INSTITUT DE LA VIE

FROM THEORETICAL PHYSICS TO BIOLOGY

Proceedings of The Second International Conference on Theoretical Physics and Biology

Palais des Congrès, Versailles 30 June-5 July 1969

Edited by

M. MAROIS

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INSTITUT DE LA VIE

DE LA
PHYSIQUE THÉORIQUE
A LA BIOLOGIE

Comptes rendus de la Seconde Conférence Internationale de Physique Théorique et Biologie

Palais des Congrès, Versailles 30 juin-5 Juillet 1969

Publiés sous la direction de

M. MAROIS

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Préface

L'objet de l'Institut de la Vie est d'engager le dialogue de la science et des hommes sur les thèmes de la vie et de l'avenir humain. L'Institut de la Vie étudie en permanence les problèmes posés au maintien et au développement de la vie par le développement de la science. Sa visée est double : fondamentale et appliquée.

La présente conférence tenue du 30 juin au 5 juillet 1969 est une manifestation de son activité fondamentale.

Dans la préface de la première réunion « Physique théorique et biologie » (Versailles, 26 au 30 juin 1967) *, nous écrivions : « Une telle conférence n'est pas une manifestation isolée. Elle marque le début d'une série dont nous n'entrevoyons pas le terme ». Il est vrai qu'un courant irréversible semble s'être créé : un troisième Congrès international « de la physique théorique à la biologie » est organisé par l'Institut de la Vie à Versailles : il aura lieu du 21 au 26 juin 1971.

Par la science, l'homme se donne les moyens de la destruction physique de l'espèce; il acquiert une maîtrise progressive du protoplasme, un pouvoir d'intervention pour le meilleur ou pour le pire. Aussi, est-il important de sensibiliser l'humanité à la valeur de la vie. C'est à cette prise de conscience qu'est voué l'Institut de la Vie. Puisse cette conférence y contribuer : elle est une réflexion sur la vie par quelques-uns des meilleurs esprits de ce temps.

M. MAROIS

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* *Theoretical Physics and Biology*, North Holland Publishing, Amsterdam-Londres, 1969, 443 pages, M. Marois éditeur.

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Monsieur le Ministre, Monsieur le Conseiller Général, Monsieur le Maire,
Monsieur le Recteur, Mes Chers Collègues,
Mesdames, Messieurs,

D'abord merci. Merci à Monsieur le Ministre de l'Education Nationale et à Monsieur le Ministre chargé de la Recherche Scientifique et des Questions Atomiques et Spatiales du précédent gouvernement, à Monsieur le Ministre de l'Education Nationale et à Monsieur le Ministre du Développement Industriel et de la Recherche Scientifique de l'actuel gouvernement pour l'honneur qu'ils ont fait à l'Institut de la Vie en accordant leur haut patronage à notre conférence.

Merci Monsieur le Ministre de l'intérêt dont vous voulez bien honorer notre congrès en ayant tenu à venir personnellement présider notre soirée officielle.

Merci à l'Union Internationale de Physique Pure et Appliquée pour son patronage renouvelé. Merci au Comité d'Organisation qui a conçu cette manifestation scientifique, en a fixé le programme, désigné les présidents, les rapporteurs et les participants et qui jour après jour, avec foi, ardeur et vigilance, a suivi la réalisation de notre grand dessein. Merci à tous les savants célèbres ici réunis, venus des points les plus divers de la planète. Ils ont franchi souvent des milliers de kilomètres pour répondre à notre invitation. Ils nous ont donné leur pensée, leur temps, leur énergie. Grâce à eux, notre congrès est une haute manifestation de l'esprit. Par leur rassemblement à Versailles ils illustrent cette ville illustre...

Merci aux organismes publics, aux sociétés privées, à tous les membres de l'Institut de la Vie dont le concours matériel et le soutien moral ont fait de cette conférence le lieu de convergence de la science, de la confiance, de l'amitié et de l'espoir.

⁂

Perdue au milieu de dizaines de milliers de systèmes planétaires, de centaines de milliers de galaxies en mouvement dans l'univers, point minuscule dans le poudroiement des astres, une planète : la terre est née il y a cinq milliards d'années. Sur cette planète, depuis deux milliards et demi d'années, la vie. Parmi les formes de vie, un dernier né il y a près d'un million d'années : l'homme, qui pour survivre, connut une longue histoire semée de vicissitudes; puis c'est la lente découverte par l'esprit humain de l'univers et de soi-même.

Alors des sentiments divers s'emparent du cœur de l'homme. Après l'illusion rassurante d'un monde fermé où l'homme se sacre roi de la création, voici l'effroi devant la petitesse, la fragilité, la solitude et l'abandon. Voici le vertige pascalien des deux infinis, l'écoute du silence éternel des espaces illimités, l'interrogation sur la pluralité des mondes habités, enfin la tristesse du néant et de l'absurde. A ces sentiments heurtés se mêle l'émerveillement, et l'homme chante un hymne à la vie, hélas rythmé par le contre chant de la mort. Dominant toutes les tentations du désespoir, un sentiment de fierté envahit l'esprit qui s'étonne de comprendre l'univers. Comment ne pas citer l'exclamation d'Einstein « La chose la plus incompréhensible dans le monde est que le monde nous soit compréhensible ». Et pour certains l'émerveillement se sublime en chant de grâce.

⁂

La science apporte à la passion interrogeante de l'homme les riches joies de la connaissance. Mais elle n'est pas seulement le savoir, elle est aussi le pouvoir : la science secrète la puissance

Elle permet désormais d'organiser rationnellement la planète. Elle ouvre à l'esprit de conquête d'immenses possibilités d'action par la pénétration dans une parcelle du cosmos, et par la maîtrise de certaines formes de vie, et bientôt du protoplasme humain.

Et l'histoire de l'homme s'accélère, menacée par des périls qui sont à la mesure des chances.

Valéry, il y a quarante ans déjà, s'interrogeait : « ce monde prodigieusement transformé mais terriblement bouleversé par tant de puissance appliquée avec tant d'imprudence, peut-il enfin recevoir un statut rationnel... arriver rapidement à un équilibre supportable ? En d'autres termes, l'esprit peut-il nous tirer de l'état où il nous a mis ? »

Le temps de l'homme adulte commence. C'est le sentiment que j'éprouve plus intensément que jamais ce soir.

Contemplons le spectacle offert par tous ces savants réunis. Leurs langues maternelles sont très diverses : elles sont celles de presque toute l'humanité. Leurs langues scientifiques sont si différentes qu'elles sont presque incommunicables aux savants de disciplines éloignées. Et pourtant, les voici rassemblés dans une manifestation de l'esprit.

Vous êtes les témoins de l'unité de la science. La république des savants est indivisible. Elle est mondiale. Son unité est définitive. L'unité de la science se forge à la poursuite d'une œuvre commune qui répond à une aspiration profonde : le besoin de connaître. Elle est scellée par une charte commune : l'objectivité, l'honnêteté intellectuelle dans la liberté de l'esprit créateur, la soumission au réel. Vous êtes la preuve que l'unité des hommes est possible, en dépit de leurs différences. Et voici qu'aujourd'hui, grâce à vous, la science franchit un nouveau pas dans sa quête d'unité de la connaissance.

En ouvrant notre premier congrès, le 26 juin 1967, le Professeur Grassé déclarait : « Notre rencontre prend, à mes yeux, une valeur particulière, en quelque sorte symbolique. Elle annonce que physique théorique et biologie contractent une union étroite et définitive. Elle marquera une date dans l'histoire des sciences ».

Oui, il est frappant de voir la diversité des disciplines confrontées pour interroger la vie : de la physique théorique à la biologie, tout l'éventail des activités de l'esprit scientifique est ici rassemblé dans un effort de recherche avancée sur la vie. Et il est frappant de constater l'ampleur du champ que notre conférence a voulu saisir : l'ordre dans les systèmes physiques, structure et maintien de la vie, génération des molécules biologiques, auto-organisation et auto-assemblage, structures dissipatives en biologie, mutation et processus de l'évolution, l'information au niveau moléculaire, le stockage de l'information dans les systèmes nerveux, les aspects théoriques.

Il est frappant, enfin, qu'à l'issue de nos confrontations, cette conférence apparaisse comme une ouverture sur l'avenir : n'a-t-elle pas tracé quelques avenues du futur dans lesquelles la science pourra s'engager ?

De l'atome à l'esprit, du passé au futur, notre congrès est une tentative d'intégrer dans une vision globale quelques processus fondamentaux de la vie. Et l'enjeu n'apparaît pas seulement scientifique. Nos travaux apportent à l'inspiration du poète, à la réflexion du philosophe, à l'interrogation de l'honnête homme, à l'inquiétude de la multitude, un aliment pour méditer sur notre destin biologique.

Mais, nos travaux laissent aussi pressentir aux moralistes, aux responsables, aux gouvernants, que le temps viendra vite où l'homme disposera du pouvoir de transformer l'homme.

Et voici que s'élève l'avertissement du Prix Nobel Nirenberg. Evoquant la possibilité d'intégrer dans le patrimoine génétique des acides nucléiques artificiels, des messages synthétiques qui s'inscriront dans le programme de la cellule, il s'inquiète : « l'humanité sera-t-elle prête ? ». Oui, le temps de l'homme adulte est venu, le temps de la responsabilité envers nous-mêmes, envers notre espèce, envers la vie. Redoutables seront nos proches options biologiques et éthiques. Il faudra bien dominer notre pouvoir nouveau. Cette tâche n'a jamais été aussi actuelle. C'est la mission que s'est donné l'Institut de la Vie.

Oui, l'humanité sera prête si elle se rend compte à temps qu'aujourd'hui la révolution c'est la science. Elle sera prête si, consciente enfin d'être entrée dans l'ère scientifique que certains penseurs appellent déjà l'ère post-industrielle, elle en mesure très vite les conséquences : modification radicale de notre condition, bouleversement des concepts traditionnels sur lesquels reposaient nos société et nos morales.

Elle le sera si, sans rien perdre des richesses et sagesses du passé, elle scrute toutes les potentialités de l'avenir, et les confronte avec les exigences permanentes de l'homme. Au sein de l'Institut de la Vie, la science est elle-même, mais elle n'a pas l'ambition d'être seule maître à penser. L'humanité sera prête si, avec la science, tous les courants constructifs de civilisations s'unissent pour ne rien négliger des besoins et des aspirations de tous les hommes et de tout l'homme.

Il est arrivé à Jean Rostand de dénoncer notre monde déshumanisant. Ce congrès s'est consacré à l'une des activités les plus humanisantes qui soient : connaître la vie. Stefan Zweig évoquait dans un livre célèbre les heures étoilées de l'humanité. Nous venons de vivre une heure étoilée.

Allocution de S. L. SOBOLEV

Monsieur le Ministre,
Mesdames, Messieurs,
Chers Collègues,

Dans les grandes universités de l'Union Soviétique, par exemple à Moscou ou à Leningrad, les étudiants chantent toujours des chansons très drôles, dans lesquelles ils essaient de prouver leur supériorité sur les autres Facultés. Je ne connais que deux chansons, l'une créée par les étudiants de la Faculté de Mathématiques de l'Université de Moscou, l'autre, par les étudiants de la Faculté de Mathématiques de l'Université de Léningrad, mais il y est absolument démontré que les mathématiques sont la meilleure des sciences.

Il existe les mêmes chansons, les mêmes hymnes, dans d'autres Facultés : les étudiants de ces Facultés y démontrent leur supériorité.

Quand nous nous sommes rassemblés à Versailles, chacun d'entre nous en apportant ses traditions, fut absolument certain que la science qui est sienne est la science supérieure. Or, grâce à l'Institut de la Vie nous sommes réunis ici et chacun de nous cache son sentiment de supériorité et contribue au travail commun, sur des problèmes communs, ce qui est extrêmement difficile, et ce qui est peut-être le plus grand problème de l'humanité d'aujourd'hui.

Je suis très heureux de participer à cette conférence pour la seconde fois.

Nous savons que la vie peut être différente. Il y a la vie douce, la vie amère, il y a peut-être la vie riche, la vie pauvre; il y a la vie ennuyeuse, la vie intéressante. Et je pense que l'Institut de la Vie a donné un très grand intérêt à notre vie, car nous sommes présents à une conférence qui a pour objet le plus important des thèmes.

Merci beaucoup, Monsieur le Ministre, merci beaucoup à l'Institut de la Vie.

*Allocution d'*I.I. RABI, *Prix Nobel*

Your Excellencies,
Ladies and Gentlemen,

I am the last to speak to you on behalf of the guests here and I am very honored and pleased when I remember that this is the 4th of July, the anniversary of the beginning of the American Republic, the United States of America.

It was a very great event in human history and as you all know, very closely connected with the history of France, which probably could not have existed without the enormous, generous aid of France. In keeping with the spirit of our meeting we remember the greatest of American scientists and perhaps the grestest of American diplomats, Benjamin Franklin, who made such an enormous success in France in a very French way, also a very American way.

This combination of France and Benjamin Franklin apparently produced a critical mass and a new world took off in new directions, the results of which anyone can read in history.

You see this is a very special occasion, and a great privilege, to be asked to speak to you today. And I will come over to our subject.

The American Republic was founded in the spirit of science. It was a new invention, it was a bold innovation. No such system had ever existed before and the people who devised it were thoroughly imbued with the spirit of science which flourished in the great age of reason, the 18th century, especially in France, In many ways not so readily acknowledged, the American Republic is one of the many great offsprings of French culture.

We are now entering a new period where science has grown immensely strong and yet differently from what it was in the 18th century. Then it was a part of the common culture of the educated classes, when nobody in government, for example, would confess that he was ignorant of science. Now it is often a matter of pride for many people who think of themselves as educated to be ignorant of science.

The establishment of the Institut de la Vie is therefore a very timely effort to bring us back to the time when science was a strong element of culture, perhaps as it once was, a ruling element of culture. A future in which the human race, our civilization, would survive without such an amalgamation of science and the general culture is hardly imaginable.

With this feeling which I am sure is in the spirit of the striving of Professor Marois to mutually fructify both physics and biology, it was for me a great joy to be here this week. Beyond this undertaking it is my dream that the spirit of science and of life will combine to give us a deeper understanding and a more profound feeling for the mystery of man and his universe.

This spirit which is emerging slowly but surely will in time affect all mankind, and lead us to a golden age when we can live in the light of knowledge and discovery.

I am sorry to have taken so long in a few introductory remarks but now I want to thank Professor Marois, and Madame Marois, who did so much to make this a very happy stay, and all those who supported this venture. And, of course, I can't stop without thanking my colleagues at this meeting, mostly on the biological side, who did so much to instruct my ignorance, and if it is slightly less, this is due to their efforts.

Thank you.

Allocution de P. DOURNES

Monsieur le Ministre,

Qu'il me soit permis de vous saluer d'abord, non seulement parce que le représentant de la ville est heureux de recevoir un membre du Gouvernement, mais aussi parce que depuis quelque vingt-cinq ans nous nous connaissons.

Mesdames, Messieurs,

Je voudrais dire au nom de la Ville de Versailles combien nous sommes heureux et fiers d'accueillir tant d'hommes de science et pour un si noble objectif.

Depuis les débuts de l'humanité, et depuis Euclide et Archimède, cinquante pour cent des savants qui ont jamais existé sont encore vivants. Or, de cette moitié, vous êtes parmi les plus éminents.

Je dois avouer que j'ai été extrêmement impressionné par une longue conversation avec le Professeur Marois sur les objectifs de l'Institut de la Vie.

L'Institut de la Vie a réuni les sciences qui pouvaient paraître les plus éloignées, la physique théorique et la biologie, disons le chiffre et la vie. A la pensée de cette confrontation à Versailles entre physiciens et biologistes sur cette question fondamentale que nous nous posons tous, savants ou profanes : « Qu'est-ce que la vie, que sommes-nous, où va la vie ? », je me suis interrogé sur la contribution de la Ville de Versailles. Hélas, pour l'instant, elle n'a pu que vous offrir, ce qui est exceptionnel, le Palais des Congrès. J'espère que nous ferons mieux la prochaine fois avec l'aide du Gouvernement représenté si brillamment par Monsieur le Ministre Lafay.

Comment parvenir à une définition de la vie qui concilie les exigences de la science la plus positive et les aspirations de l'homme le plus généreux ? Ce problème me paraît l'objectif ultime de vos recherches. Je ne fais que le poser au nom de chacun de nos concitoyens qui, confusément, en ressentent l'urgence.

En hommage à tous les savants dont la présence parmi nous honore infiniment la Ville de Versailles et en hommage particulier aux Prix Nobel, je voudrais terminer mon propos par une phrase admirable d'un autre Prix Nobel, Bergson, Il n'était ni physicien ni biologiste, bien qu'il fût d'une grande culture scientifique. Cette phrase a plus d'un tiers de siècle, mais elle n'a pas perdu de son actualté. Elle terminait cet ouvrage majeur : « Les deux sources de la morale et de la religion ». La voici : *Au corps démesurément agrandi de l'humanité, il faut un supplément d'âme.*

Ce que j'ai entendu ce soir ne contredit pas l'exigence de Bergson. Vous avez conscience du développement explosif de la science et du corps matériel de l'humanité. Il faut à ce corps un supplément d'âme : nous comptons sur vous pour le lui donner.

Allocution de BERNARD LAFAY

Monsieur le Président,
Mesdames, Messieurs,

Aux remerciements qui viennent d'être si aimablement adressés aux membres du Gouvernement, aux organismes et aux personnalités qui ont contribué ou participé aux travaux de cette conférence internationale organisée par l'Institut de la Vie, vous me permettrez d'ajouter l'expression de ma gratitude personnelle.

C'est, en effet, une grande joie pour moi que de me trouver parmi tant d'hommes de science illustres, appartenant aux disciplines les plus diverses mais communiant dans le même idéal qui est celui du respect de la vie, de son mystère et de sa lumière.

Vous me pardonnerez, Messieurs, de ne pouvoir dire nommément à chacun de vous combien le Gouvernement français apprécie votre présence et travaux, et, en particulier, Monsieur François-Xavier Ortoli, Ministre du Développement Industriel et Scientifique qui m'a demandé de présider votre soirée officielle de clôture.

Importance de la conférence

Je soulignerai seulement, en vous en félicitant et en y trouvant de hautes raisons d'espoir, le caractère largement international de votre rencontre et la qualité exceptionnelle des assistants.

Je crois qu'entre votre comité d'organisation et les participants, on dénombre 16 nations réparties sur 3 continents. Vous comptez, au sein de votre comité, 7 titulaires de Prix Nobel et dix autres ont pris part effectivement à vos travaux parmi 100 savants éminents venus de tous les points du globe.

Je crois pouvoir, Mesdames et Messieurs, être votre interprète, en remerciant chaleureusement votre Comité d'Organisation et, spécialement, M. le Professeur Marois, Président de l'Institut de la Vie, à qui nous devons le beau succès d'une organisation parfaite en tous points. Si parfaite, et si attentive, qu'aux austères travaux des spécialistes est venu s'adjoindre un programme réservé aux dames, prévu avec autant de goût que d'éclectisme par le Comité féminin d'accueil. J'espère, Mesdames, que vous y avez trouvé plaisir et intérêt, autant que nous sommes sensibles ce soir à votre présence.

Un thème de confrontation interdisciplinaire

Vous avez choisi, Messieurs, comme thème majeur de votre rencontre, l'étude de la chaîne ininterrompue qui lie l'énergie fondamentale aux phénomènes organiques les plus complexes.

« De la physique générale à la biologie », ce simple énoncé eut fait sursauter de surprise et d'incrédulité les physiologistes les plus hardis du début de notre siècle. Pourtant l'histoire des sciences a montré combien sont fécondes les incursions des chercheurs dans les domaines étrangers à leur spécialité.

N'est-ce pas un médecin, Mayer, qui, partant d'études sur le métabolisme du corps humain, établit le premier principe de thermodynamique, au milieu du XIXe siècle et n'est-ce pas un cristallographe, Pasteur, qui révolutionna la médecine quelques années plus tard ?

Mais à ces recherches tâtonnantes dont l'aboutissement était dû au hasard presque autant qu'au génie, la science actuelle substitue systématiquement l'approche multidisciplinaire du problème de la vie. Renonçant dans certains domaines à l'analyse, elle procède par synthèse, comme le remarquait M. Szent-Gyorgyi lors de votre conférence internationale de 1967. Ainsi est apparue la biologie moléculaire, née de l'intérêt que les physiciens portent aux phénomènes biologiques.

C'est le grand mérite de l'Institut de la Vie d'avoir appelé des hommes de formation et de philosophie très différentes à confronter les apports contemporains de toutes les sciences et de toutes les techniques pour répondre à l'éternelle question : *Qu'est-ce que la vie ?*

Conception actuelle de la vie

Nous savons au moins qu'elle est une énergie au service d'une organisation privilégiée, ce qui est définir plutôt qu'élucider. Mais la biologie nouvelle a franchi plusieurs étapes de la route du mystère et se fonde non plus seulement sur l'étude de la cellule, mais sur celle de l'atome régi par des lois quantiques.

Déjà, la science exprime les phénomènes vivants en langage moléculaire et efface progressivement les frontières qui séparaient les acquisitions distinctes de la biologie, de la chimie, de la physique atomique et des mathématiques.

La première moitié du XXe siècle a connu la révolution atomique. Peut-être sommes-nous à la veille de la révolution biologique, plus bouleversante encore puisque la recherche fondamentale est sur la voie de mettre la vie en équation. Perspective exaltante, certes, mais aussi perspective déroutante où les meilleurs esprits trouvent bien des motifs d'inquiétude et même d'angoisse.

L'homme se rencontre lui-même

La question se pose, dès maintenant, de savoir ce qu'il adviendra de l'homme lorsque les phénomènes vitaux, y compris ceux du domaine psychique, pourront être ramenés à des déplacements d'électrons.

Au terme du cheminement séculaire de l'esprit, après avoir franchi les barrières opposées à la connaissance par l'univers hostile, affirmant sa maîtrise sur la matière inanimée et sur le règne vivant, l'homme en vient à se rencontrer lui-même, à se confronter à sa propre nature.

La sagesse antique, par une de ses intuitions si troublantes dans l'histoire de l'intelligence humaine, avait pressenti, il y a des millénaires, le drame où nous nous engageons. Pour avoir dérobé la lumière du ciel, Prométhée fut condamné à la souffrance éternelle et à la destruction de soi-même. Ce mythe prémonitoire, qui rejoint celui de l'Arbre de la science au Paradis terrestre nous oblige à prendre conscience des écrasantes responsabilités que l'homme assume désormais.

Les sciences de la vie : redoutable danger

Demain, les biologistes auront le pouvoir d'agir sur les données fondamentales de l'existence humaine, sur les processus de l'hérédité organique et psychique. A quelles redoutables tentations la société ne sera-t-elle pas alors conduite ? Comment le savant se défendra-t-il contre l'usage monstrueux qui pourrait être fait de sa puissance ? Comment sauvera-t-il l'héritage de sagesse et de bonté, de liberté et de spiritualité accumulé par l'homme au cours de sa longue route ? Dernier né de la vie, cet homme triomphant restera-t-il capable de comprendre la simple leçon des plus humbles organismes vivants, saura-t-il maintenir et transmettre la vie ?

Ce sont les graves questions que vous posez, Monsieur le Président, dans le cadre de l'Institut de la Vie, et, c'est à vous, Messieurs, d'y apporter des réponses décisives. Ceux qui ont en charge le destin des sociétés les attendent de vous. La politique du XXI^e^ siècle sera ce que les hommes de sciences voudront qu'elle devienne : tyrannique ou fraternelle, inhumaine ou exaltante, libératrice ou inexorablement contraignante.

Je n'ai pas dissimulé nos motifs de craindre, mais les raisons d'espérer ne nous manquent pas, car l'optimisme de l'homme est invincible. Ainsi, jamais la société ne s'est penchée avec autant de sollicitude sur les déshérités de la vie, sur ces enfants victimes des fatalités tragiques de l'hérédité, fatalités que nous n'acceptons pas puisqu'elles témoignent d'un dérèglement des lois harmonieuses de la vie.

Retrouver l'âme par la science

Non seulement la science de notre temps en pénètre les fondements, mais elle entreprend d'en corriger les déviations, au nom d'un idéal d'ordre et de beauté, de bonheur et de générosité qui reste le but suprême des efforts de l'esprit.

Messieurs, on a beaucoup commenté la formule brutale de Claude Bernard, à l'aube balbutiante et géniale de la physiologie moderne. « Je n'ai jamais, disait l'illustre chercheur, trouvé l'âme sous mon scalpel ». Sans doute le scalpel était-il trop rudimentaire. Vous disposez de moyens infiniment plus subtils. Je souhaite que lorsque la vie sera mise en équation rigoureuse, l'âme y apparaisse comme une donnée essentielle de la structure de l'homme éternel.

Allocution d'ouverture de A. FESSARD

Voici donc que, dans quelques instants, va s'ouvrir la seconde conférence internationale désignée par l'expression ambitieuse que nous savons, c'est-à-dire : « De la Physique théorique à la Biologie ».

Au nom du Comité d'Organisation qui m'a fait l'honneur de me demander de prononcer les premiers mots de cette Conférence, je viens tout d'abord, et solennellement, vous souhaiter la bienvenue, solennellement comme il convient à la grandeur du thème que nous allons traiter; mais je m'empresse aussitôt de remplacer la solennité par la cordialité qui sera, je l'espère, l'atmosphère de nos entretiens.

Je remercie tout particulièrement ceux qui ont accepté les dérangements et la fatigue d'un voyage qui entraîna souvent ce décalage horaire que nous connaissons bien et qui est un premier affrontement des lois de la Physique avec celles de la Biologie.

Malheureusement nous avons à enregistrer quelques défections. Ce sont celles de MM. Bresler (Leningrad), Casimir (Eindhoven), Dalcq (Bruxelles), Dulbecco (San Diego), Katchalsky (Rehovot), Klug (Cambridge), Lifshitz (Moscou), Yang (New York), Yukawa (Kyoto).

Inutile de dire que nous regrettons vivement l'absence de ces collèges, dont plusieurs participaient à la première Conférence.

Ainsi, selon le vœu pressant formulé lors de la première Conférence, et deux ans après elle, nous pouvons nous réunir à nouveau; cela grâce à l'Institut de la Vie, à sa généreuse initiative, grâce aussi aux appuis que nous avons reçus, et tout particulièrement à celui que nous a donné l'Union Internationale de Physique Pure et Appliquée.

Ce désir que nous avions exprimé à l'issue de notre première Conférence, de nous retrouver sans trop tarder, n'était pas seulement né du succès indéniable de notre première réunion. Il n'était pas non plus né du seul désir de renouveler à Versailles, dans une certaine douceur de vivre dans cet hôtel accueillant, un séjour agréable comportant la chance de pouvoir rencontrer à nouveau des collègues venus souvent de pays fort éloignés du nôtre.

La raison principale en était, vous le savez bien, que nous avions tous eu le sentiment que nous n'avions fait qu'une attaque d'avant-garde lors de notre précédent effort pour atteindre les vérités fondamentales des phénomènes de la Vie, et qu'il fallait nous réunir à nouveau pour essayer d'aller plus loin. Sans nous bercer de l'illusion que nous pourrions cette fois épuiser le sujet, un sujet qui est vraiment inépuisable, nous espérons que vous trouverez ici, au cours de ces cinq journées de travail, ce que vous êtes venus chercher, c'est-à-dire un substantiel enrichissement de la connaissance. Sur les autres plans, nous espérons aussi que vous apprécierez notre accueil, l'accueil que vous réserve non seulement l'Institut de la Vie, mais Versailles, mais Paris.

Et maintenant, mettons nous au travail : « Je déclare ouverte la seconde Conférence internationale *De la Physique théorique à la Biologie* ».

J'ai le plaisir d'appeler à la présidence le Professeur Fröhlich, et je lui donne immédiatement la parole.

Journée du 30 juin 1969

Première séance

L'ORDRE DANS LES SYSTÈMES PHYSIQUES

PRÉSIDENT H. FRÖHLICH

H. FRÖHLICH
Introduction générale

G. CARERI
Far infrared spectra of hydrogen bonded biopolymers

Discussions

De la Physique théorique à la Biologie, C.N.R.S., 1971.

INTRODUCTION GÉNÉRALE

Chadwick Laboratory, University of Liverpool, Liverpool, England

H. FRÖHLICH

Before we actually begin I should like to say a few words on account of the International Union of Pure and Applied Physics.

The Union has sponsored this conference again as it did two years ago on the suggestion of its commission for statistical mechanics and thermodynamics. We do this because we hope that in a conference of this kind which is a very unusual one, and which is intended to be very unusual, the possibility will arise of a true interchange of ideas between physicists and biologists.

We faced two years ago considerable difficulties because the two sections in a way, speak different languages and we hope that both the physicists and the biologists will try not to speak principally to their own colleagues, but to the other side so that we might possibly hope to start an interchange on new general ideas which may lead to specific results.

Thank you.

The great success of microbiology consists in the successful correlation of biological properties with the structure of certain giant molecules. These molecules are extremely complicated compared with those one normally deals with in physics, and the opinion has been expressed that the pecularities of biology are essentially connected with this complication, and that all important biological properties will ultimately be expressed in terms of molecular properties.

This idea is reminescent of opinions in physics round the turn of the century : If the properties of the elementary particles that constitute matter were known then all properties of mater would follow in principle.

This is, manifestly, untrue for some of the most important properties of materials imply concepts which have no meaning in terms of single particles or of their pair interactions. Order in physical systems is one of these concepts. As a very simple example consider the order that refers to the arrangement of atoms in a monoatomic crystal lattice. This order can be expressed in terms of certain correlations between distances of the lattice points on which the atoms are based.

Now while the distance of two atoms is, of course, a concept referring to a pair of atoms, the particular correlation of distances is a new concept that can be expressed in terms of very many atoms only. While the particular concept has been known for very many years, another concept referring to a much less trivial order has been found rather recently only in connection with the properties of superconductors and superfluids. Subsequently the common feature of this type of order has been formulated in terms of the concept of long range phase correlations. The same type of order, it has been found later, is present in lasers; at the last conference, 2 years ago, I suggested that in a different form it is also present in biological systems.

This conjecture implies that biological systems, in spite of the complication of their molecular structures, may exhibit certain general simple features. Let me remind you in this connection of the three observations I made two years ago.

1. Biological systems have extraordinary dielectric properties. They are expected to be capable of electric vibrations in a frequency region of about 10^{11}-10^{12} sec^{-1}. These vibrations are composed of locally vibrating electric dipoles based either on certain regions of the cell membrane, or on certain groups in giant molecules. Such oscillating electric dipoles interact over large distances. In the discussion, Careri did specify relevant molecular groups.

2. With respect to some degrees of motion biological systems are expected to be far from thermal equilibrium provided that the systems are active, i.e. that energy flows through them. This same idea was presented by Prigogine who had elaborated it in more detail.

3. Biological systems should exhibit a certain order. This order should not be of a trivial spacial type but should be connected with the more subtle long range phase correlations. I then conjectured that this order should arise from strong excitations of a single mode (or a few modes) of the electric vibrations mentioned above. It was possible to show that such excitations would be stabilized by non linear effects leading to deformations and tensions in the system.

Meanwhile this conjecture has — from a theoretical point of view — received strong support from the properties of a simple model. Suppose the system of oscillating dipoles to be placed into a heat bath with which it can interact. Such a system is described in terms of its normal modes which form a relatively narrow band of frequencies. The usual interaction with the heat bath would be described in terms of emission and absorption of single vibrational quanta. The present model, however, also permits double processes which essentially implies that with the help of the heat bath a single quantum at a frequency ω' may jump to another frequency ω''. We now assume that energy is supplied to the system of oscillators at a constant rate; when a steady state has been reached then the oscillators will transmit energy at the same rate to the heat bath. This requires that their distribution differs from their thermal equilibrium distribution. When the supply of energy is small then the deviations are small. Above a critical supply, however, a qualitative change arises which is very similar to the so called Einstein condensation of a Bose gas : The mode with the lowest frequency becomes very strongly excited, so that it soon contains more energy than the rest of the system. As a consequence the system shows the long range phase correlation that had been postulated.

It will be noticed that a phenomenon comparable to a thermal phase transition has been enforced by the supply of energy in conjunction with the attempts of the system to come to terms with the heat bath into which it is placed.

This simple consideration should be used in connection with the influence of the non linear effects arising from the strong excitation of a single mode. The stabilization mentioned above implies that the frequency of the strongly excited mode is reduced. In principle such non linear effects may lead to a great complexity of behaviour.

The main consequences of the described conjecture essentially arise from the fact that the supplied energy is not entirely thermalized but placed into the storage mode from where it may be used in more efficient ways.

1. The arising tensions will lead to forces which increase as the storage is built up. This may give rise to a variety of dynamic effects e.g. connected with cell division.

2. With slight modifications a similar storage may be expected in the frequency region of visible light, and hence may occur in connection with photo synthesis. The storage mode would here (and in other cases) be able to transfer energy in units of more than one quantum.

The overall picture which thus arises makes use of possible activities that follow the molecular structure of the materials involved. This quasi static element is now, however, supplemented by dynamic features. The possible activities are supposed to supply energy into the storage mode which gradually would build up leading to various types of deformations owing to non linear effects. They will lead to new forces which ultimately may initiate processes like cell division. The long range correlations make it possible, in principle, to visualize correlations between processes which are separated in space and time, as is required e.g. in cell division.

The actual processes may, of course, be extremely complicated; their possibilities must depend on the detailed structure. The succession of actual events may, however, be regulated by the change of excitation of the storage mode. I think it should be possible to construct a dielectric model which would exhibit such features.

Application of the ideas expressed here to actual biological systems would, of course, require detailed knowledge of the dielectric properties of the constituents in a frequency region which only recently has become accessible.

De la Physique théorique à la Biologie, C.N.R.S., 1971.

FAR INFRARED SPECTRA
OF HYDROGEN BONDED BIOPOLYMERS

G. CARERI

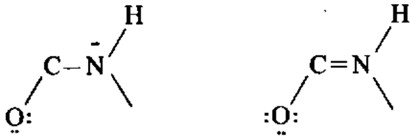
Istituto di Fisica, Università di Roma

At the first Versailles conference, in 1967, during the discussion following the Fröhlich paper [1], I suggested [2] that the hydrogen bond of some biopolymers could provide the soft modes active in the far infrared, offering a preliminary molecular background for the Fröhlich theory. This has been the motivation to carry out, in our laboratory, an experimental investigation on the absorption spectra of several biopolymers in the far infrared, in the region around 100 cm^{-1}. In this lectureI plan to introduce first the nature of the coupling due to the hydogen bonding between amide groups, then I will report our experimental results on proteins and model systems in the far infrared, and finally I will discusse their significance both for the Fröhlich theory and other speculative possibilities that can be mentioned in conference like this one.

Coupling by H-bonding between amide groups

The hydrogen bond (in the following H-bond) is present all almost in biological materials (water, proteins, nucleic acids), where it is well know to be responsible for a number of different chemical effects. Perhaps the most interesting physical manifestation of the H-bond is found in the near infrared spectra of these substances, where the absorption bands assigned to the motion of the H atom are very intense, broad and someewhat shifted in frequency. These features are reasonably well accounted for [3] in terms of a charge transfer process which occurs between the two partners when they move. Because of the partial covalent nature of the H-bond, the charge distribution on both partners is strongly dependent on their relative positions, and intense absorptions are therefore expected when their position changes.

The effect of the H-bond on the electronic structure of the bonded molecules is most important when these molecules are resonating between close electronic structures. This is precisely the case of the amide groups, where the planarity of the group and the shortening of the C-N bond to 1.32 Å are attributed to resonance between structures which are relized about 60 % and 40 % respectively. As a matter of fact, the change in the frequencies of the characteristic bands of the amide group of monosubstituted amides with the change of state [4] (e.g. as in melting), confirms that the electronic structure of an amide group is affected by the relative positions of the neighbouring groups which are H-bonded to it.



The effect of H-bonding in inducing changes in the electronic structure of the bonded will be most intense whenever these H-bonded groups are arranged in a chain (as in α or β polypeptides). No doubt, this effect would follow from a correct theoretical treatment of the H-bonded chain, including the partly covalent nature of the H-bond, but this task has never been attempted. Therefore we will follow an intuitive approach and will content ourselves with the simple consideration that the cooperative effect between the alternating H-donor and acceptor terminals will cause the amide groups to be more « polar » in the field of the two neighbur H-bonded partners, and what is most important, the converse effect whereby the more polar electronic structure once stabilized will form stronger H-bonds. Therefore in these chains one can speak of a « collective » electronic state of the chain, in the sense that there is a more than pairwise cooperative charge resonance, or charge transfer, between bonded groups. A similar cooperative effect is already known in molecular crystals [5] (e.g. benzene) when the intermolecular charge transfer in the Mulliken sense is significant, and in Rome we do find evidence for it in some model H-bonded crystals like acetoanilide. By virtue of the strong coupling between neighbours, the intermolecular « weak » stretching mode of the H-bonded "dimer" must give rise to a band of "soft" vibronic modes in the linear "polymer", in much the same way as the infrared active vibrational mode of an isolated NaCl molecule gives rise to an optical phonon band in a crystal of NaCl, as well known in solid state physics.

To conclude, we must expect some bands of soft modes, active in the far infrared, to be intense in H-bonded matter particularly when the electronic structure of the bonded group is easily influenced by the H-bond, and when the geometry allows cooperative effects among the groups. We find these conditions to be likely to be met in proteins, due to the electronic resonating structure of the amide group, to the peculiar polypeptide chain geometry, and to the presence of tightly bound water; however the proteins are far from being ordered repetitive polymers and therefore a wide distribution of soft modes involving charge displacement must occur as well, as in a "liquid" structure. We will make use of these conclusions later on, to discuss the far infrared absorption spectrum of some proteins.

The far infrared spectra of some polypeptides and proteins

Let us now consider the far infrared absorption spectra of the biological materials. Water absorbs very strongly and almost everywhere, particularly below 1 cm^{-1} (namely in the microwave region) due to Debye dissipative absorption, and above 200 cm^{-1} due to the "lattice" modes. At still higher frequencsies both water and all other biomaterials are known to absorb because of their intramolecular modes. Therefore the only region of interest for us, namely the only frequency range where some electric oscillations could be relatively undamped, must lie around 10-100 cm^{-1}.

There are very few measurements as yet on the far infrared (in the following F.I.R.) of H-bonded matter, because only in recent times some new techniques became available to investigate this part of the electromagnetic spectrum. In the past two years this author, in collaboration with U. Buontempo and P. Fasella, has made an extensive study on some synthetic polypeptides, native and denaturated proteins, in different physical conditions and over a wide range of frequencies. The instrument used for F.I.R. measurement is a Michelson interferometer (manufactured by R.I.I.C.) which can give the transmission spectrum by Fourier transform of the interferogram with the help of an electronic computer. Experimental details of this work will be given elsewhere [6]. In the following I will quote some significant results only :

a) To begin with, we have investigated the F.I.R. spectrum of a synthetic polypeptide, Poly-D-Glutamic Acid (PGA), which is known to be in the α-helix configuration in the solid state, in order to compare it with the analogous spectrum of its sodium salt (Na PGA), which is known to be in a random coil configuration in the solid state. We have selected this particular polypeptide because a similar investigation by slow neutron scattering has already been made [7] to detect a possible change in the frequency distribution of the collective modes in the order-disorder transition. While the neutron work hardly shows any difference between the frequency spectra of the two conformations, our F.I.R. spectra shows striking differences both in the band shape and in the band intensity (normalized to one amide group) : more exactly we detect the presence of strong and well defined peaks only in PGA, while in NaPGA there is a continuous band of low and decreasing intensity at the higher frequency. We believe that the above reported F.I.R. results can be explained in terms of the H-bond distribution, which is well different in the two configurations : an array of bonds of the same length and with the same angle NH ... OC (close to 180°) in the α-helix, while in the random coil configuration the bonds are distributed in a wide range of lengths and angles, and the average H-bonding is much weaker than in the α-helix.

To summarize, we have clear evidence that H-bonding is the main factor responsible for the dielectric properties of the polypeptide chains in the F.I.R.

b) Newt we consider Bovine Serum Albumin (BSA), the only protein where neutron work has been carried out [8]. Here again we find the F.I.R. spectrum completely different from the frequency distribution indicated by neutrons. In the region around 100 cm^{-1} we find a very broad and intense absorption band, which was not found in the neutron diffrection spectrum.

c) An extensive F.I.R. study has been carried out on Lysozime, a low molecular weight protein well investigated by X-rays, in different conditions of sample preparation, temperature, etc. The F.I.R. spectrum of Lysozime has been found to be always very similar to that of BSA.

The broad band around 100 cm^{-1} merits some comment. This band is mainly due to the H-bonded groups, but any other soft mode of the macromolecule, which involve a charge displacement, can contribute as well. In spite of the high resolution obtained in same runs, no fine structure has been revealed. Even the spectra recorded at liquid nitrogen temperature did not show any appreciable difference in the smothness of this band, while in other part of the spectrum they showed a better resolution. Moreover, a glance to F.I.R. spectra of BSA and Lysozime shows that these spectra cannot be due to a properly weighted superposition of the spectra of the α, β and random coil configurations.

One may wander if this broadning can merely arise from the fact that the oscillators are essentially independent, but that they have somewhat different force constants and effective masses by some trivial geometrical reasons. Then even a very modest coupling could be responsible for a small broadning of each single peak, and the observed smothness of the absorption band could result from the superposition of very many peaks. Although the above trivial explanation must be kept in mind, we believe that the main reason for the lack of fine structure in F.I.R. must be due to the strong coupling among the oscillators, so that the absorption band must be considered as one "band" in the sense of the solid state physics, namely as the one resulting from several interacting levels. We believe this to be the case because we find that in the region of very low frequencies, where the wide distribution of effective masses and force constants should be most felt, the absorption of both native and denaturated Lysozime are identical inside experimental errors. From accurate measurements of circular discroism and other structure sensitive infrared amide bands, we known that in our two samples the percentage of the α and β structures differ by a factor about two. We cannot get convinced that such a large change in the geometry could not affect the F.I.R. frequency distribution, if this distribution would be due merely to the trivial superposition of the different peaks.

To summarize, we have good reasons to believe that in a protein the lack of periodicity must be responsible for the absence of well definite absorption peaks in the F.I.R., and that the observed broadning of the intense absorption band must be due to the collective coupling of the oscillators, most of them being H-bonded amide groups. At the present time it is difficult to say more, in molecular terms, about these soft modes which are not detected in the neutron spectra.

Some speculations

In the following we will assume that a band of soft modes, which involve charge displacement, is realized in globular proteins. Then one can imagine some interesting consequences, which are briefly outlined here in order to rise a common discussion between physicist and biologist.

The proteins are macromolecules where collective charge displacements can be realized. This holds for energy changes which are of the order of magnitude of KT or less, T being the room temperature and K the Boltzman constant. Inside this energy limit, the vibronic energy is essentially delocalized, and is then easily transmitted from one place to another, because of the large coupling between the different modes. We emphasize the fact that for frequencies larger than KT/h the energy is essentially localized in the molecular modes, while for frequencies lower than $10^{-2}\,KT/h$ the dissipative Debye absorption absorption of water makes impossible any long range charge correlation. Only inside this range, from 1 to $100\,\mathrm{cm}^{-1}$, we find a band of interacting vibronic levels, already populated at room temperature, which can possibly give rise to new phenomena. The relevance of this fact is left for consideration to biochemists.

Finally we must consider the possibility suggested by Fröhlich [9], namely the presence of longitudinal electronic modes in living systems. In order to built up these modes, the biomolecular system must exhibit a strong coupling between its modes below KT, this being a preliminary requirement that our experimental work has certainly shown to be true. The presence of the sharp longitudinal mode suggested by Fröhlich remains to be detected, but the occurence of a band of soft modes in proteins *in vitro,* in a region of the electromagnetic spectrum where water is relatively transparent, is encouraging further efforts in this direction.

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DISCUSSIONS

H. Fröhlich : We shall now start with the discussion and maybe I should start it myself.

You will realise that of course I am very happy about these results because they form a confirmation of my first point which is a necessary condition for the existence of the particular modes.

I should mention two things which occured to me during your talk : the one is that these effects would be strongly non-harmonic and therefore the optical properties would yield much more information than was originally thought; but this is a matter for physicists only.

Another thing that occured to me refers to something completely different, not quite within physics. Careri told us that these modes are exited at room temperature. They form a continous background noise; so what my suggestion would do if certain of these modes are amplified by the act of biological living processes acts much in the same way as the one I heard in a very modern concert, where the background noise was amplified by the musicians with the help of certain instruments and gave a very interesting and specific noise.

But now I should like to open the discussions.

D. Hodgkin : There are very great differences between different protein molecules in peptide chain conformation and arrangement. In lysozyme some of the chain is present as α helix and as β pleated sheet, but also much is folded in other configurations; one would not expect lysozyme to show an infra red absorption spectrum similar to that of α polyglutamic acid. On the other hand, myoglobin has a high proportion of the chain folded in αhelices, though these are of varying lengths and angles to one another. Have you examined myoglobin and if so, how does the spectrum compare with that of lysozyme ?

G. Careri : Yes we have investigated five proteins for the time being. I only gave the results of two, as they look almost the same, all five; they are rather different in the near infrared and in the circular dicroism due to the presence of different amount of α and β structures.

H. Fröhlich : I wish to point out that quite apart from a molecular interpretation, far reaching conclusions might possibly be deduced from a very general semi-phenomenological investigation of the experimental results.

H. C. Longuet-Higgins : I think I understand Prof. Fröhlich's ideas, but I feel that one does want to be hard-headed about their application to biological systems. As I understand it, the essence of his idea is that if we have a system where there is a flow of energy through the boundary then we can expect to find very interesting effects; but the theory cannot help us to understand the allosteric phenomenon, for example, because this phenomenon is exhibited in thermal equilibrium. That is my first point.

The second point is a more general one. If one is thinking that the theory might apply to a particular situation, then one must somehow face the problem of applying it quantitatively to that situation. How is the energy supposed to be pumped into any particular mode ? I would be interested to know what clues you could throw out on this point.

H. FRÖHLICH : Well, that isn't known, but in protein synthesis, for instance, the sections that are deposited will not go immediately into the ground state. Being electrically dipolar they will share their energy with other dipolar oscillations, etc. The picture is very general but so far no attempts have been made to specify it because that would require some very specific knowledge, a suggestion which I hope will be taken up by chemists. What has been achieved experimentally, so far, is to show that the frequency region in question is very strongly active. A second type of experiment is in progress to decide whether in biologically active systems, some modes are very strongly excited. We hope soon to obtain an answer to this question. Perhaps Professor Palma would wish to make some remarks.

M.U. PALMA : With his theory, Fröhlich has essentially raised the general problem of the possible influence that a particular dynamical state may have on the biological properties of macromolecules (proteins, in particular). I shall comment on two aspects of this problem, while making some proposals for experiments that can hopedly yield information on the subject.

Most of these proposals involve a deuteration (at least, a partial deuteration) of the molecules to be studied. They arise from some results obtained at Palermo in the course of a study on the dynamical properties of a class of inorganic crystals which may be thought of as a simplified model of hydrogen-bonded solids. In particular, my wife and G. Aiello (to be published) have found that the height of the potential barriers, as seen by one proton, may depend remarkably upon the (mass-dependent) state of motion of the remaining protons and/or deuterons. The potential barrier heights and the state of motion of protons may depend, therefore, one from the other. This provides a feed-back that can be responsible for abrupt and collective changes in the state of motion of the system, as a function of some parameter. In particular, there may be a certain concentration of deuterium beyond which, free or very slightly hindered motion above the potential barriers (or tunneling through them) is not possible. In turn, this would cause a further and cooperative rise of the potential barriers, so that the system as a whole would switch to a completely different type of motion.

We see therefore the possibility for these remarkable changes to occur immediately above a certain critical concentration of Deuterium vs. Hydrogen. These motional changes are the analogous of a paraferromagnetic transition, at which one has the onset of a correlation — in fact, of an interlocking — among spins. Here one may have, instead, an interlocking among the hydrogen (and/or deuterium) ions, in the sense that I have just discussed. In fact, the interlocking is likely to give rise to ferroelectricity, when occurring in crystals (hydrogen-bonded ferroelectrics). And just as one has « spin waves » in interlocking spins, one may have « proton waves » in the case of protons interlocking in the sense just mentioned — that is, a completely different dynamical situation, as compared with that of the free or slightly hindered motion.

Let us venture to see now how this may be reflected in some biological experiments. We may remark that, supposing a certain dynamical state of a particular type of macromolecule has a strong influence on its biological properties, there may be a threshold percentage of deuteration, above which that dynamical state will be so modified as to give rise to a loss, or at least a strong change in that particular biological property. If the biological property is in particular due to a Fröhlich mode, the exchange of hydrogen for deuterium by the molecule may on its own right, that is, apart from the effect discussed, show a « threshold effect ». In other words, a certain minimum concentration of heavy water in normal water may be required for the molecule to exchange hydrogen with deuterium, thus causing the drastic change or, in particular, the destruction of that particular biological property. Remember, anyway, that this behaviour is expected on the basis of Fröhlich's theory, but it can also occur whenever a dynamical effect is relevant to that particular biological property.

An all-important circumstance is that cells live in aqueous media. From what I have been discussing, a deuteration will affect the dynamics of the molecules involved, as well as that of the water, as well as the dynamical interaction between molecules and water. Attention must be therefore payed and experiments of this type must be carefully planned in an attempt to discriminate among these three main effects. All of them are interesting, of course, but it is desirable to distinguish among them.

It may be thought that in any case such a major change as a deuteration (even if partial) will affect a number of relevant properties of the molecule (such as a kinetics constants, etc.), which will affect its biological functions. In point of fact, this is what one is looking for, since the difference in the H vs. D zero-point energy essentially does not affect the bond length (and therefore the molecular geometry), so that all changes observed upon deuteration must be connected with either the dynamics of hydrogen bonds between the molecule and the aqueous medium, or that of the molecule proper. The crucial point is to see whether the dependence upon deuteration is smoothly progressive, or it shows some kind of spectacularly catastrophic behaviour, such as that of Fig. 1, a. The occurrence of this case, in fact, will support (even if far from conclusively) the hypothesis of the existence of collective dynamical effects.

For the reasons that I have discussed, one should expect that, whenever a biological activity or property, or function, B depends upon the motional state of the molecule(s) involved, it may be quenched, by deuteration, in a way that is qualitatively shown in Fig. 1, b. Of course, nothing can be said if such a behaviour is not found. If it is found, however, it may suggest that either the dynamics of the molecule proper, or that of the interaction of the molecule with the aqueous medium, play a relevant rôle in the particular biological activity, or function.

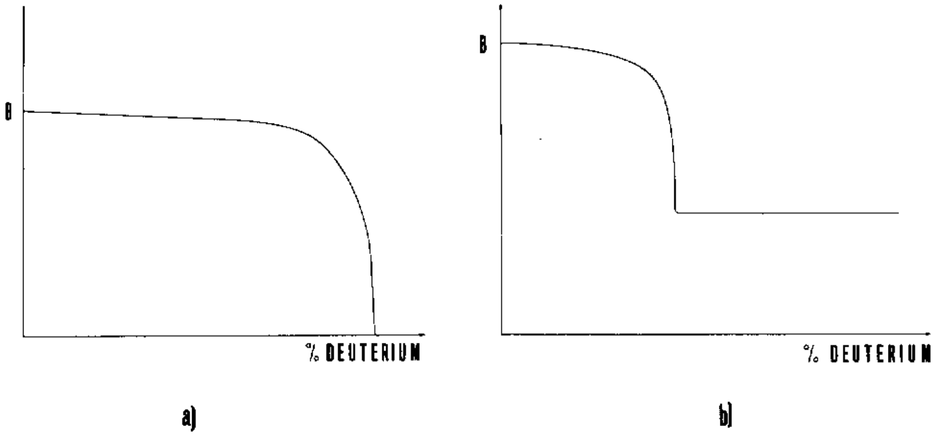

Fig. 1. Hypothetical dependence of a biological property (B) upon substitution of Hydrogen by Deuterium.

To the best of my knowledge, no biological experiment has been as yet undertaken in a search for the occurrence of a behaviour of this type. However, in a paper of MARSLAND & ZIMMERMANN (*Ex. Cell. Res.*, **30**, 23 (1963) it may be found information which, although very crude for our purposes, is exceedingly interesting.

These Authors have studied the effects of D_2O on cell division in sea urchins eggs (unfortunately, I have had no access to the papers by Gross et al. on the same subject). If we plot the few points that they give in their Table I, we find what is shown in Figs. 2 and 3, which is just the expected behaviour illustrated in Fig. 1, a.

Although, admittedly, these results do not have a univocal interpretation, they anyway point out the rôle that *collective* motional effects can have in the living state.

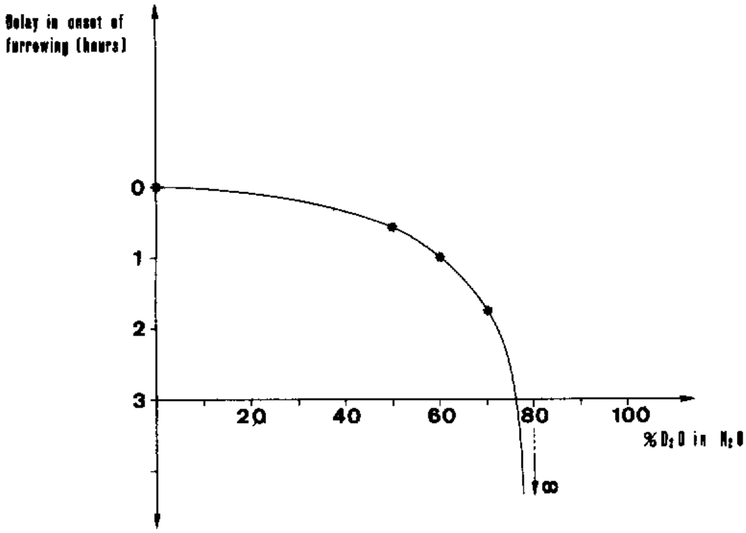

Fig. 2. Drawn from data by Marsland and Zimmermann (see text).

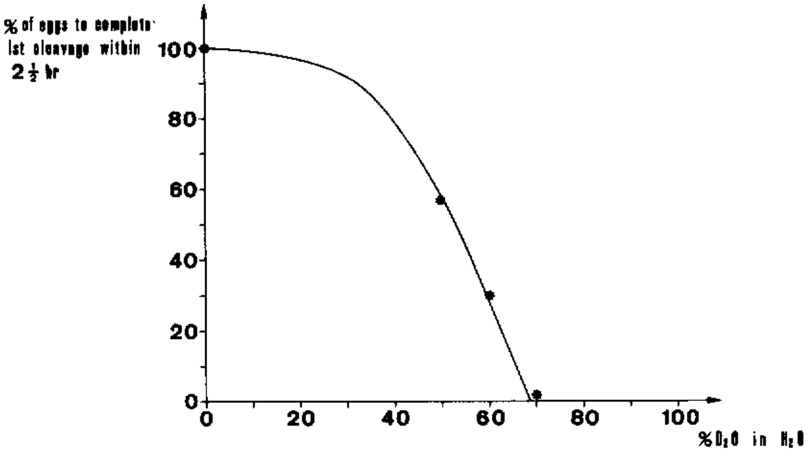

Fig. 3. Drawn from data by Marsland and Zimmermann (see text).

An extension of this type of experiments to obtain plots of the same kind for different cases and biological functions may be expected to be very rewarding. Of course, one must carefully select simple and clear-cut experiments that do not involve many biological steps, each of which may be conspicuously affected by deuteration, since this might simulate a cooperative effect that in fact might not exist.

One of the fascinating fields in which to look for a possible rôle of dynamic effects, is that of the behaviour of regulatory (allosteric) enzymes, as we have proposed elsewhere already last year. In this case, the presence of a dynamic long range correlation, in particular, if not necessarily, due to a Fröhlich mode of appropriate simmetry, might be responsible for a multimolecular kinetics — a dynamical extension of the allosteric model by Monod, Changeux, Wyman and Jacob.

Experiments along this line are under way, in a collaboration with E. Scarano's group, and the experiments to be performed in Naples concern : a) the influence of deuteration upon the activity and regulatory properties of Deoxycytidylate aminohydrolase and its Inhibitor (dTTP) and Activator (dCTP) and b) the possible influence of the (static and dynamic) Jahn-Teller effect on the specificity of this enzyme. Although this latter type of experiment presents difficulties, it has been suggested by the fact that, while for the activity of the enzyme a divalent metallic ion is requested, the Cu^{++} ion completely destroys the activity of the enzyme itself.

On the other hand, physical experiments can be done and we have indeed started them, in collaboration with Dr. Pike's group in Malvern, by the technique of Laser Raman spectroscopy and, more in general, Laser light scattering (one should remember that Fröhlich modes are not expected in first approximation to be optically active, so that they may be expected to be Raman active). These experiments will be hopedly extended to other types of enzymes.

Of course, the possible existence of dynamical effects should profitably also be studied in emoglobin, which is in fact a very convenient model of allosteric enzyme. In this particular case, in view of the Bohr effect, the dynamics of the aqueous medium may play a particularly important rôle. Emocyanin would be a particular case, suitable of being investigated by ESR techniques also with respect to the rôle of the Jahn-Teller effect, since Cu^{++} ions exhibit in a particularly clear way this effect.

Another field of interest is that of the « inner clock », that is of circadian rhythms and of the related frequency multiplication due to environmental entrainment. I have recently learned with great interest from my Colleagues biologists in Palermo that the phototactic rhythm in the single-celled alga Euglema changes from about 24 to about 28 hrs upon change from H_2O to D_2O. Again, for the reasons just discussed, it would be perhaps very rewarding to try to obtain experimental information on the possibly cathastrophic dependence of this frequency change upon D_2O concentration (although one must be aware of the several different possible ways to obtain an inner rhythm.

Finally, it may be interesting to perform the experiments with D_2O at different temperatures, in order to explore the whole temperature range which corresponds (for H_2O containing different proportions of D_2O) with a minimum in the specific heat.

In conclusion, as Figs. 2 and 3 already suggest, dynamical effects may play a central rôle in the living state. The actual problem is that of a clear-cut and thorough search, as well as that of sorting out those effects which depend on the dynamics of the particular molecules involved, from those (also interesting on their own right) which depend on the dynamical interaction of the same molecules with water, via the collective dynamical behaviour of the hydrogen bonds. It is very interesting, in this connection, to note that very recently (F.W. Cope : *Biophys. J.*, 9, 303, 1969 and C.F. Hazlewood *et al.*, *Nature,* 222, 747, 1969) an « interlock » between water molecules and muscle (or brain) molecules has been confirmed. This is likely to be the type of interlock that I have discussed here.

H. HAKEN : I'd like to make a comment on Prof. Careri's work. You concluded that you have a broad absorption band and from this that it is similar to a band in a solid. Well, I would just like to say that there's another possibility which I would like to put to discussion, namely a broad band can also be caused by dissipation or by strong damping. This strong damping would be caused by a nonlinear interaction between the normal modes. I think that just a nonlinear interaction could be a support for Fröhlich's ideas : When one has a strong electron-phonon interaction in metals one finds super-conductivity according to Fröhlich. So a strong damping can cause or can be internally connected with the strong excitation of modes. A similar thing happens in lasers if one has a strong absorption. If one feeds energy into the nonlinear system one gets a strong selection of modes. So this was just a possibility that I wanted to put forward.

A.D. MCLACHLAN : I would like to make some comments on Dr. Careri's talk. One of his main points is that there are important collective vibrations in protein molecules. A general feature of vibrations that are collective is that you must have strong resonance coupling which leads to a single sharp band. And in order to have this I think you need a highly repetitive structure. Now on the contrary, a highly disordered structure would give you a single, rather broad band in which these vibrations are irregular. One of the most striking features of the structures of those proteins which have been examined by X-ray crystallography, is that the structure is orderly but entirely irregular, and that there is much more irregularity than regularity in most of these proteins. For example, in myoglobin, although there is a high content of alpha helix, many of the helices are of different lengths and point in different directions in space. But the structure of myoglobin, which is today one of the most regular protein structures we know about, does not look at all conducive to collective vibrations. Instead it seems much more probable that the soft modes which Dr. Careri has been talking about, could be a collection of highly random vibrations in a very broad band. Now I would also doubt whether collective vibrations are important in allosteric effects, because in haemoglobin, where one knows quite a lot about the structure of the molecule, it seems probable at present that the cooperative effects are transmitted between these groups which are about 30 angströms apart by purely stereo-chemical changes; there seems to be no need to invoke any kind of charge transfer process. It would appear at present that it's quite sufficient simply to have amino acid side chains — some of them non-polar, moving past one another. Thus it seems that in small protein molecules the main importance of hydrogen bonds is as links which keep the structure in the correct, precisely formed shape. I would suggest that the evidence we have up to date from the structure of proteins would lead to the conclusion that in proteins made of a small number of subunits there are not many collective charge transfer or vibration effects.

H. Fröhlich : I would like to make some comments myself, on these latter remarks. There seems to be, of course, divergencies with respect to the interpretation of Prof. Careri's interesting results.

I would like to point out what I mentioned before in half a sentence namely that there is a completely objective way of getting some ideas of what conclusions to draw. The question of what is collective is very badly handled may I say. Every mode is collective because it's a mode; this is a tautology. I would stay on this although it's too technical. From the observations made, if they're quantitative, one can with the help of dispersion relations obtain a certain quantity which is essentially the contribution of this particular mode of motion to the static dielectic constant. There exists a very general formula which permits to tell us the following : that if we have a unit which makes a contribution to this polarization, then, this formula, together with the quantitative results, permits us to say what is the polarization owing to short range interaction and what is due to long range interaction in the whole region. That will give us an idea of how the units are coupled with each other. Now this of course has to be done and we have to see first whether the results are sufficiently quantitative and if they are not yet we will certainly have a chance of improving this.

This does not make any use of particular structures because it is a theory which is completely, completely general.

H.C. Longuet-Higgins : Could I make just a few disconnected observations ? First of all I very much like your suggestion that the idea of the energy all falling into one particular vibration is probably relevant to the initial act in photo-synthesis, where we have of course a highly non-equilibrium situation.

Now if I may comment on one of the remarks that you addressed to another speaker. I didn't quite understand what you had in mind when you distinguished between thermal free energy and configurational free energy. In a sense all free energy is thermal and all free energy, equally, has some degrees of freedom with which it is associated. So, I don't think one can make a dichotomy between thermal and configurational free energy. The next point I wanted to raise — you mentioned the dynamical Jahn-Teller effect. If one replaces one metal atom by another in an enzyme, one introduces all kinds of differences, in oxidation potential, size etc. I think to pin down a change of function to one particular, highly sophisticated spectroscopic property is jumping perhaps a bit too far. Finally, I am told that one can train algae to grow in 100 % D_2O, in which one has replaced every single hydrogen atom by a deuterium atom and that once they've got used to this they live perfectly happily. But I don't think this counts either for or against the Fröhlich theory.

H. FRÖHLICH : Every single hydrogen atom goes back to where it started from only with a different set up...

M.U. PALMA : Let me reply your last comment first. Of course, it is wrong to think that the results that I mentioned must necessarily be explained in terms of the Jahn-Teller effect. This effect, however, may have implications which go much beyond the spectroscopic grounds on which it was discovered, in account of its statical and dynamical implications. The hint is given by the fact that, apparently, whenever you substitute, let's say, magnesium with iron or copper or other Jahn-Teller ions, this seems to have a very strong influence on the way e.g. an enzyme works. Concerning your first comment, my point is discussed in a paper by Morrison (*Reviews of Modern Physics,* 36, 1964) that you have perhaps missed. Finally, concerning the effect of deuterium in circadian rhythms, you may indeed have a frequency entrainment for a circadian rhythm, but when you substitute hydrogen with deuterium, the entrainment may occur at a different frequency. You can still have a frequency duplication, but the rate, the basic rate is different. Of course this does not speak in itself for the existence of a Fröhlich mode, since the first alternative that you can think of is something which resembles a relaxation oscillation. But even if you have a relaxation type of oscillation, you have to account for the change of some particular rate which controls your relaxation time.

B. PULLMAN : I would like to make two comments on Dr Careri's paper. The hydrogen bond is of course of such a fundamental importance in biology that we have to be careful about what we say about its nature. From this point of view I must say I was somewhat embarrassed with the very strong accent that you put upon what you call a charge transfer character of the hydrogen bond which implies that between the two units linked together by the hydrogen bond there should be a rather strong electron displasement from one such unit to the other to the point that you would expect such a transfer to be important for the conformation of proteins. I must say that following rather recent and very refined calculations which exist about hydrogen bonding, such a pronounced transfer character of the hydrogen bond does not really seem to exist. If I can have my slide, please. This slide shows some recent calculation carried out by the so-called CNDO method which is a method of calculation including all valence electrons. I must say that absolutely similar results are obtained if you use a still more refined method of calculation which is the so-called ab initio method which take into account all the electrons altogether. In this slide you have numbers which indicate the variation of the sigma electrons (upper number) and of the pi electrons (lower number) population in this hydrogen bonded system with respect to the isolated molecules — two hydrogen bonded formamides. If you look carefully at these numbers you can see that there are displacements of both sigma charges and pi charges upon hydrogen bonding.

The minus sign on an electron means gain of electrons and the plus sign means loss of electrons. The numbers are in 10^{-3} electronic units. But if you look carefully you can see that essentially the displacements are inside such units, so shat if you consider the overall transfer or electrons from one unit to the other, you can find that there is practically no charge displacement or a very small one. If you count it carefully you find that there are 6 thousandth 0.006 of an electron which are transferred from one unit to the other. These are sigma electrons and not pi electrons as might have appeared from the drawing you made on the blackboard. So as a matter of fact, the charge transfer character of hydrogen bonding is exceedingly small. The second remark which I would like to make concerns to some extent the same problem. The drawing you made on the board previously and in which different units were hydrogen bonded between them, was used for another problem in biochemistry : it was used in order to try to account for semi-conductivity of biological system and energy bands have been calculated following this model. The model has been unsuccessful to account for the experimental observations, although the situation may be very complex. The model may be inappropriate — there's no reason why a band model of protein structure should be particularly acceptable. But the reason may also be different, namely that the experiments are wrong in the sense that you don't have any intrinsic electronic semi-conductivity in proteins but the semi-conductivity is extrinsic, due to impurity levels. Whatever it may be I would like just to stress the point that this model has been used in other problems in biochemistry and was unsuccessful for corelations with experimental facts. So that the idea of charge transfer in hydrogen bonding as an important contributor to the nature of the hydrogen bonds is I think questionable.

G. CARERI : In my opinion, it is not important whether there is static charge transfer or not. But what you should explain is why there is a charge flow, because only charge flow is wanted to explain the strong intensity of the near infra-red absorption, as you know. I used the concept of charge transfer because I found it very convenient and introduced by Mulliken, who is here in person; he stressed that the charge transfer model was a very useful way to answer the question of the very intense absorption in the near infra-red. So if it is not the charge transfer in the Mulliken sense, it is certainly some thing else that you certainly want to explain, and explain well, because the effect in the near infra-red is immense, and I do not see how this can appear from your calculation. This is my reply to the first question. To the second point, about the array of several hydrogen bonds and their importance for electronic conductions — since I'm talking only about the ground state and not of the excited states — (because those soft modes do not involve at all electronic excited states) I don't see this to be relevant about what I said.

Professor Prigogine, on the invitation of Professor Fröhlich gives a summary of his talk « Dissipative structures in biological systems ».

I. PRIGOGINE : The experiment shows the oxydation of malonic acid in the presence of Ce ions. This reaction has been studied extensively by Zhabotinski and al. It has been shown that the concentration of Ce^{3+} and Ce^{4+} oscillates in time. The details of the mechanism are not known. The major steps are

$$CH_2(COOH)_2 + 6\,Ce^{4+} + 2\,H_2O \longrightarrow 2\,CO_2 + HCOOH + 6\,Ce^{3+} + 6\,H^+ \quad (1)$$

$$10\,Ce^{3+} + 2\,HBrO_3 + 10\,H^+ \longrightarrow 10\,Ce^{4+} + Br_2 + 6\,H_2O \quad (2)$$

$$CH_2(COOH)_2 \xrightarrow{Br_2} CHBr\,(COOH)_2$$

There are autocatalitic and inhibitory steps in the reaction scheme.

The experimental procedure is the following. One mixes homogeneously equal amounts of Ce $(SO_4)_3$, $KBrO_3$, $CH_2(COOH)_2$, H_2SO_4, and few drops of a redox indicator.

After a while in the resting solution oscillations appear, seen by the change of color from blue (due to an excess of Ce^{4+} ions) to red (excess of Ce^{3+} ions). The oscillations start at a given point in the solution and propagate in all directions. After a few oscillations one can see horizontal blue and red lines in the test tube. This demonstrates experimentally a space organization corresponding to a *symmetry breaking dissipative structure.*

H. FRÖHLICH : I'd like to say just one word. The reason I've asked Prof. Prigogine to speak now is that he has demonstrated on quite a different subject that if energy flows through a system it can get into states of quasi-equilibrium which are completely different from the states when energy does not flow through it, which is exactly the same situation which I mentioned earlier this

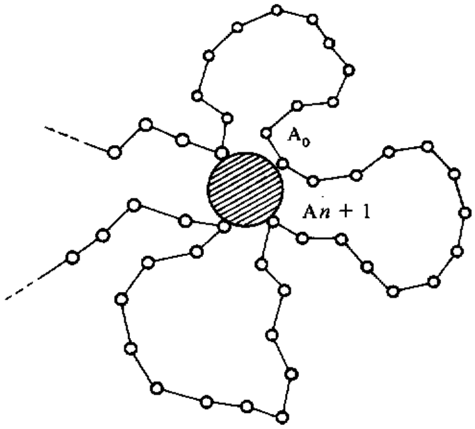

Fig. 1. A receptor site (hatched area) involving directly 4 animoacids (black dots). The connecting chain (white dots) comprises three loops and two open ends.

morning. Now we'll make an interval of exactly two minutes for the people who would like to see this experiment.

P.G. de Gennes : I wish to discuss a specific example where we can compare the physicist's notion of order with the biologist's view of order. This is related to the conformation of globular proteins.

From the point of view of polymer physics, globular proteins are reminiscent of certain polysoaps, where the hydrophobic part of the chain clusters in a central core, while the hydrophilic residues tend to lie on the outer surface [1]. This conformation provides both *stability* and *solubility*. The crucial difference with polysoaps lies of course in the presence of *specific receptors* on the protein. A schematic representation for such a receptor is shown on fig. 1. The active site directly involves a number p of aminoacids ($p \sim 5$ to 10). These are linked together by comparatively long loops of the peptidic chain. As has been emphazised by Monod [2], it is of some interest to estimate the *minimum size* required for each of these loops, when the conformation of the active site itself is prescribed. This should lead in particular to one lower bound for the molecular mass of a globular protein carrying one active site.

One possible approach to this problem would be to use Monte Carlo methods on a computer. This, however (a) is expensive and (b) gives very little insight. Here we shall restrict ourselves to a much more modest, but explicit, calculation, based on rough statistical arguments. We consider first a single loop, inserted in a dense proteic medium. We show that there does exist a well defined (and rather large) minimum size for such a loop. Then we extend the argument to a set of $p - 1$ loops converging towards the same active site, and show that the minimum size per loop remains essentially unaltered in this case.

THE ONE LOOP PROBLEM

A) *Statement of the problem and number of allowed conformations*

Consider a sequence A_0, A_1, ..., A_n, A_{n+1} of aminacids, following each other in the primary structure of the protein. We assume that A_0 and A_{n+1} belong to the active site, as shown on fig. 1. Their relative positions and orientations are fixed. The other aminoacids (A_1 ... A_n) are assumed not to play a direct rôle in the receptor activity. We postulate that for a given sequence (A_1 ... A_n) there is one (and only one) conformation of lowest energy which can be achieved. Depending on the choice of (A_1 ... A_n) we might then have 20^n conformations for our loop. However, all of them are not realisable. First, we must retain only those conformations for which the loop under study does *not* intersect other portions of the overall protein chain; we call these the allowed conformations. Counting the allowed conformations corresponds to an excluded volume problem in a dense homogeneous medium, familiar from the theory of concentrated polymer systems [3]. The result (originally derived by Flory with the help of a lattice model) is that for each unit A added to the chain, the probability of not intersecting any other part of the chain is a constant, and roughly of order e^{-1} (where e is the base of Neperian logarithms). With this estimate, the number of allowed conformations is reduced to $(20/e)^n$. Our problem is to find how many of these are compatible with the geometrical requirements imposed by the active site.

B) *Position and orientation of the end group*

To each of the $(20/e)^n$ conformations would correspond a well defined position and orientation of the end group A_{n+1} with respect to A_0. The positions may be for instance labeled by the coordinates (x, y, z) of the α carbon in A_{n+1} with respect to the α carbon in A_0. Similarly the angular properties may be specified in terms of a rotation vector (Ω_x, Ω_y, Ω_z) relating the actual orientation of, say, the amide group in A_{n+1}, to a chosen reference state. The set (x, y, z, Ω_x, Ω_y, Ω_z) definies one point M in a 6-dimensional space. There are $(20/e)^n$ such points M to consider. How are they distributed?

As soon as $n \geqslant 4$ the angular variables are spread more or less at random (each of them over an interval 2π). The spatial variables on the other hand, have a nearly gaussian distribution, even in the presence of strong excluded volume effects, provided that the medium is densely filled and homogeneous [4]. This leads us to a distribution function ρ (M) for the points M of the form:

$$\rho(\mathrm{M}) = \left(\frac{20}{\mathrm{e}}\right)^n (2\pi)^{-3}\left(\frac{2\pi}{3}\, nb^2\right)^{-3/2} \exp\left[-\frac{3}{2}\,\frac{x^2+y^2+z^2}{nb^2}\right] \qquad (1)$$

where nb^2 is the mean square elongation of a chain containing n arbitrary aminoacids. The general aspect of this distribution is shown on fig. 2.

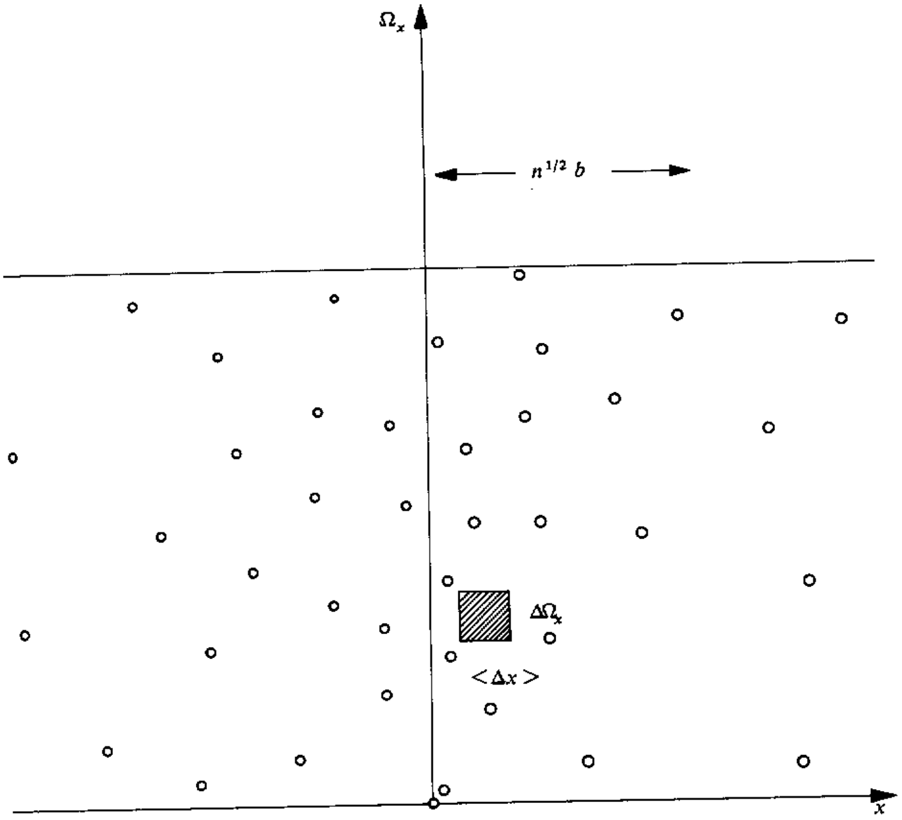

Fig. 2. Distribution of the positions (x) and rotation angles (Ω_x) of the terminal aminoacid A_{n+1} with respect to the first A_0, for various primary sequences ($A_1 \dots A_n$) of the interconnecting loop. The region which satisfies the requirements on A_{n+1} to build up the active site is Hatched.

In all what follows, we shall be interested only in *loops*, i.e. in conformations for which the end point is rather close to the starting point, or $|x_i|, |y_i|, |z| < n^{1/2}b$. In this limit ρ (M) takes the simple form :

$$\rho(M) \longrightarrow \rho_0 = C\left(\frac{20}{e}\right)^n n^{-3/2}\, b^3 \tag{2}$$

$$C = 3^{3/2}\,(2\pi)^{-9/2}$$

C) *Number of " successful " conformations*

We call successful the conformations for which A_{n+1} is indeed located as desired to build up the active site. This implies that x, for instance, be in a certain interval :

$$x_0 \leqslant x \leqslant x_0 + \Delta x$$

and that the rotations Ω_x also belong to a certain small interval :

$$\Omega_{0x} \leqslant \Omega_x \leqslant \Omega_{0x} + \Delta\Omega_x$$

(with similar inequalities for the other compoments). The amplitudes Δx, $\Delta\Omega_y$, etc., are imposed by the thermal fluctuations of the overall structure, and depend essentially on the rigidity of the protein. In what follows we shall take typically :

$$\Delta x = \Delta y = \Delta z = 0{,}2\ \text{Å}$$

$$\Delta\Omega_x = \Delta\Omega_y = \Delta\Omega_z = 1/10\ \text{rad} \cong 6^\circ$$

The points M associated with successful conformations of the loop are thus all contained in a small 6-dimensional volume w :

$$w = (0.2)\ \text{Å}^3 \times 10^{-3}$$

The probability of failure p_f is the probability that *no* point M falls into the volume w. Let us assume that, in the region of interest, the points M are distributed at random, with an average density ρ_0. (We shall discuss this assumption in more detail in section III). Then p_f is given by a standard formula for random events :

$$p_f = \exp\,[-\,\rho_0\, w] = \exp\left[-\left(\frac{20}{e}\right)^n \frac{C\,w}{n^{3/2}\, b^3}\right]$$

The probability of success is $p_s = 1 - p_f$. The dependence of p_s on n, as deduced from this equation, is shown on fig. 3. Because of the "doubly exponential" character of eq. (3), p_s is essentially 0 far all values of n below a certain threshold n_c, and $p_s \cong 1$ for $n > n_c$. Taking $b = 4$ Å, and the aforementioned value for w, we are led to $n_c \cong 13$. It turns out in fact that

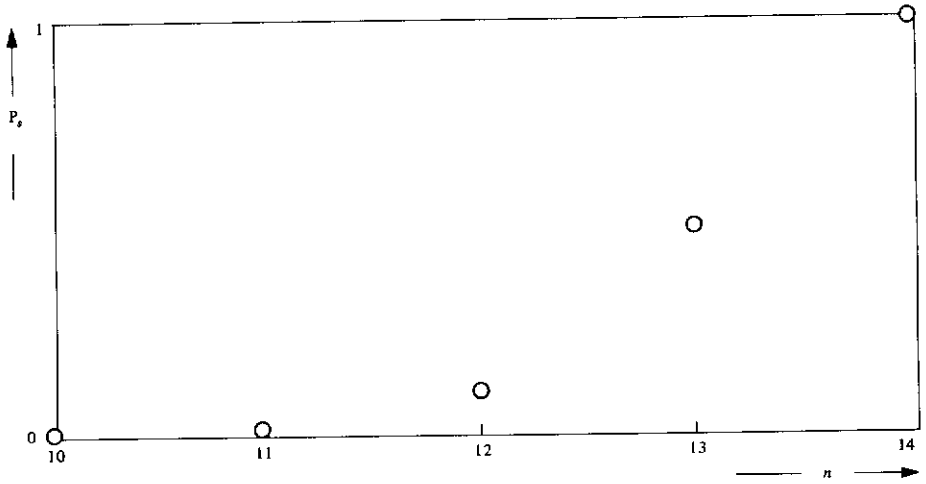

Fig. 3. Probability of success versus loop length. Note the abrupt variation near $n = 13$.

n_c is remarkably insensitive to the exact values chosen for b and w (the dependence being only logarithmic).

DISCUSSION

A) *Could the same conformation be obtained with more than one sequence?*

One basic assumption underlying equation (3) is that, in the region around w, the points M be spread out at random, without any strong correlation between them. This might be incorrect in some cases. Consider for instance the " Stereochemical code " proposed recently for aminoacid residues by Liquori [5]. In this model, the aminoacids may have only five different conformations (i.e. different sets of angles φ and ψ in the conventional notation [6]. If this rule was strictly obeyed, the number of distinct conformations would be reduced from 20^n to 5^n; the same point M_0 would correspond to a large number of primary sequences. Our calculation would then lead to a much larger value (~ 30) for n_c.

We believe, however, that our earlier estimate for n_c is the correct one, for the following reason : the angles φ and ψ for each amino acid do have some small deviations from the idealised values selected by Liquori. Thus, the many sequences, which, in a strict version of the code, would conserve to the same point M_0, will in fact lead to a *cluster* of points M centered around M_0 (fig. 4). Are these clusters well separated from each other, or do they overlap ? The calculation of section II applies only when they overlap, since then the overall density of points M becomes uniform.

To answer this question, let us first estimate the size of one cluster. A spread ε in the angles for each aminoacid implies for the orientation of the final group A_{n+1} and uncertainty :

$$\delta\Omega_x \sim \delta\Omega_y \sim \delta\Omega_z \sim \varepsilon n^{1/2}$$

As regards the position of A_{n+1}, since the overall loop size is of order $n^{1/2} b$, a change ε in the angles for *one* aminoacid will displace x, y, and z by $\sim \varepsilon n^{1/2} b$. The cumulative effects of n aminoacids will give a result $n^{1/2}$ larger, i.e. :

$$\delta x \sim \delta y \sim \delta z \sim n\varepsilon b$$

Thus the volume of one cluster is approximately :

$$\Delta = \varepsilon^6 \, n^{9/2} \, b^3$$

On the other hand, the number of cluster per unit volume in 6 dimensional space, is given by the analog of eq. (2) :

$$\tilde{\rho}_0 = \mathrm{C} \, b^{-3} \, n^{-3/2} \, \left(\frac{5}{e}\right)^n$$

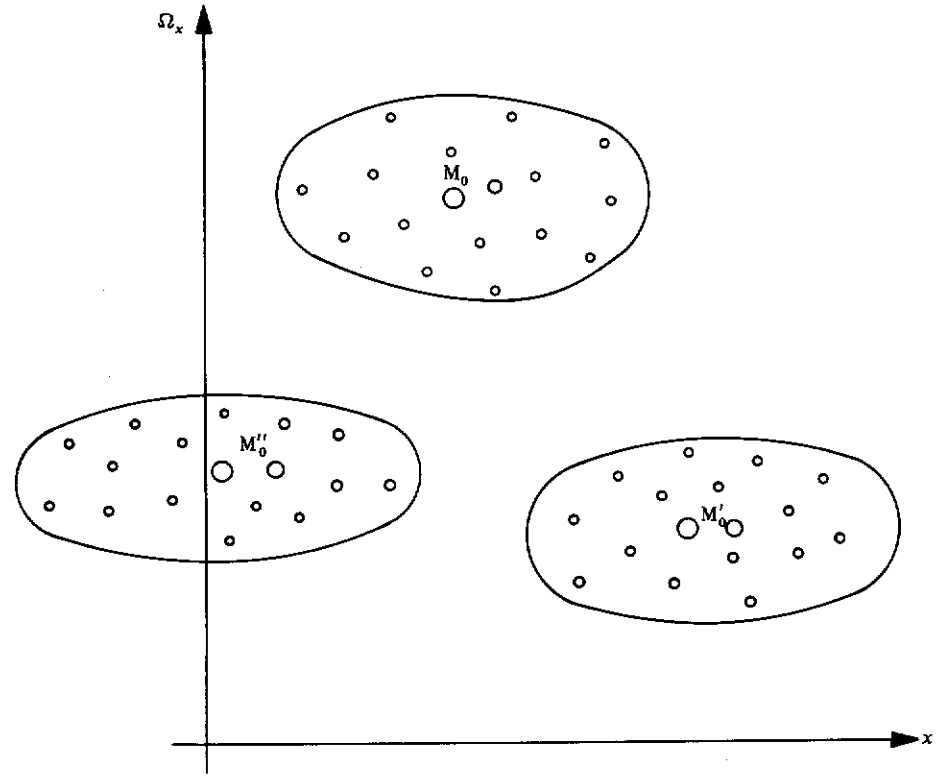

Fig. 4. Clustering effects in the (x, Ω_x) diagram. If the stereochemical code of Liquori was strictly obeyed, various primary sequences would give the same point M_0. Small deviations from the code spread these points in a cluster around M_0.

The overlap will be large if :

$$\tilde{\rho}_0 \Delta \equiv C\, \varepsilon^6 n^3 \left(\frac{5}{e}\right)^n \gtrsim 1$$

From the experimental distributions of points in the (φ, ψ) plane for proteins of known structure [7] we estimate $\varepsilon \cong 0{,}2$ rad (or 12°). Then $\rho_0\Delta$ becomes larger than 1 as soon as $n > 15$. Thus (if our guess on ε is correct), this « clustering » effect will at most increase n_c by two units, and is not too important.

B) *Effets of the protein surface*

The receptor site has to be close to the surface, and the loops must reach the surface sometimes. As a model system, we may take a planar surface, and restrict the loop paths to one half space limited by this plane (the receptor site being treated as a point on the surface). The result of such a model is simply to modify slightly the coefficient C in eq. (2), and the effect on n_c is very weak.

Of course, the surface will also influence the hydrophilic/hydrophobic balance of the loop sequence, and this should be included in a more refined calculation.

THE MANY-LOOP PROBLEM

When the receptor site under consideration involves directly a number $p > 2$ of aminoacids (as it probably does in most cases), we have $p - 1$ loops. Does the same value of n_c apply to all of them?

At first sight the answer would seem to be: no, since the first loop restricts the amount of phase space available for the second one, etc. But we believe in fact that the answer is: yes for the following reasons:

A) The Flory factor exp $(- n)$ does contain the effect on one loop, of all the material present, and in particular of the other loops.

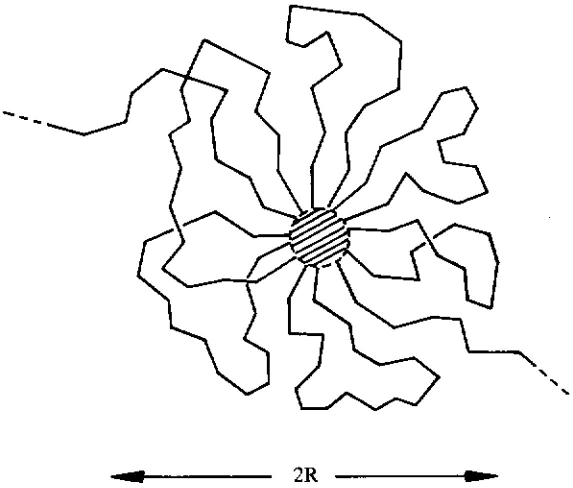

Fig. 5. When the number of loops is large, their paths near the active site area streched, with a corresponding reduction in entropy.

B) It is of course true that, if p is comparatively large, many loops will have to converge towards the same small region defined by the active site: this implies a reduction in entropy since the loops will have to stretch radially as shown on fig. 5. A reduction in entropy means an unfavorable factor. However, we shall now estimate this reduction and show that it is weak.

Assume for instance that the $p - 1$ loops fill completely a spherical region of radius R around the active site. Apart from numerical coefficients, the volume of this region will be

$$\exp\,[-\,R^2/nb^2] \sim \exp\,[-\,p^{2/3}\,n^{-1/3}]$$

where v is the average volume per aminoacid, and n the average chain length. Each loop is streched over a distance of order R and suffers a reduction in entropy $\sim R^2/nb^2$ (in dimensionless units). This corresponds to a reduction factor in the number of allowed conformations per loop, of order :

$$R^3 \sim (n + 1)\,(p - 1)\,v \sim n\,p\,v$$

(assuming $v \sim b^3$). For the values of p and n of interest here, this factor is not much smaller than unity, and it may be omitted completely.

Thus, at least to a first approximation, our estimate of n_c should apply to the many loop problem. Since there are $p - 1$ loops, each of them involving at least $n_c + 1$ aminoacids, the total number of aminoacids required to build up the receptor site may not be smaller than $(p - 1)\,(n_c + 1)$. Of course, this is only a lower limit : in particular, the requirements of stability, rigidity, and solubility, will often impose longer chains.

Acknowledgments :

We wish to thank in particular prof. J. Monod, who suggested this problem, and H. Buc, A. Krzwicki, M. Piattelli, for various related discussions.

Références :

[1] For a recent review, see : L. STRYER, *Ann. Rev. Biochemistry,* **37,** 25 (1968).

[2] J. Monod, *Cours Collège de France,* 1969.

[3] For a discussion of this concept applied to proteins, see P.J. Flory, *Journ. Pol. Science,* **XLIX,** p. 105 (1961), especially section IV.

[4] Homopeptide chains are known to become gaussian in solution only at very large n (~ 30). But here we are interested in heteropeptide chains with a statistical distribution of sequences : such systems tend to be gaussian even for rather small n (> 10).

[5] A.M. Liquori, in *The stereochemistry of macromolecules,* **3,** 287 (A.D. Ketley ed. Dekker, New York, 1968).

[6] Edsall *et al., Biopolymers,* **4,** 121 (1966).

[7] See for instance the review by G.N. Ramachandran in *Structural chemistry and molecular biology,* p. 77 (Rich and Davidson, ed.) (Freeman, London, 1968).

J. POLONSKY : En électronique nous distinguons deux types d'ordre : l'un uniforme répétitif à caractère thermodynamique et le second différencié à caractère informationnel. Ces deux catégories d'ordre s'opposent à l'entropie, c'est-à-dire au désordre dans le système et jouent ainsi le rôle d'entropie négative.

Néanmoins, il y a lieu de les traiter séparément dans l'étude d'une structure physique car leurs effets sont différents.

Le taux d'ordre global dans un système peut être exprimé par la redondance $R = (1 - H/H_{max})$.

H représentant l'entropie du système à l'état considéré;

H_{max} est l'entropie maximale que le système peut atteindre en désordre complet.

Pour $H = 0$, $R = 1$, le système est parfaitement ordonné pour $H = H_{max}$, $R = 0$, le système est totalement désordonné.

Rappelons que H/H_{max} représente le taux des structures aléatoires, tandis que la redondance R exprime le taux des contraintes ou des corrélations dans le système.

Comment distinguer dans la redondance R l'entropie négative thermodynamique de l'entropie négative d'information ?

Les contraintes réparties uniformément sur tous les états de la distribution (ces états peuvent être indiscernables) expriment la part thermodynamique de l'entropie négative, tandis que les contraintes réparties non uniformément sur les états, ayant chacun son adresse et ses probabilités propres, représentent l'entropie négative informationnelle du système. Par exemple, dans le cas de macromolécules biologiques (DNA ou protéines) :

1. – La rigidité du squelette hélicoïdal ou double hélicoïdal représente la redondance thermodynamique et son effet est de réduire globalement les phénomènes aléatoires dans la macromolécule; ceci est équivalent à une réduction de la température statistique du système macromoléculaire;
2. – Les contraintes spécifiques et différenciées dans le message informationnel contenues dans la séquence linéaire des bases ou acides aminés, représentent la redondance informationnelle.

La redondance thermodynamique peut être exprimée par un nombre entre 0 et 1, la redondance informationnelle doit par contre être représentée par des matrices des probabilités de distribution et des probabilités des transitions.

De ces matrices, on peut déduire la redondance du code. La langue anglaise par exemple possède une redondance voisine de 0,5, ce qui veut dire que dans la structure d'un message en anglais il n'y a que 50 % environ des contraintes.

La matrice des probabilités des transitions joue un rôle très important dans l'élaboration d'un système organisé. C'est à travers une telle matrice que l'on peut se rendre compte si le mode fonctionnel projeté sera univoque ou aléatoire.

Ma dernière remarque se réfère à l'intervention du Professeur Prigogine sur les conditions d'établissement d'un régime d'oscillation hydrodynamique dans un système perturbé fortement par rapport à son point d'équilibre l'amenant dans une zone de fonctionnement non linéaire.

Je voudrais mentionner que ce modèle répond également au mode fonctionnel d'oscillateurs électroniques; ceux-ci ne se mettent en régime d'oscillation qu'à la faveur d'une perturbation électrique de grande amplitude amenant le système dans une zone non linéaire.

Si des oscillations à très haute fréquence existent dans des structures biologiques dont nous a parlé ce matin le Professeur Careri, la pompe pourrait éventuellement être fournie par les molécules ATP. Il est intéressant de signaler que l'on a pu réaliser récemment des lasers chimiques où le rôle de pompe a été rempli par des phonons de la chaleur dégagée au cours de la réaction.

H. FRÖHLICH : I think there is very little time now. I would like to ask Löwdin whether he likes to make some general remarks about his impression of the situation because he is a physicist who has been involved in biology.

P. O. LÖWDIN : I really do not feel prepared to do this, but I have one general comment concerning this morning, as to the relation between theoretical physics and biology, and this is that in theoretical physics one was long ago accustomed to work with laws of a different type, namely the mechanical laws. In mechanics, one has initial conditions, one has the Newton laws, and one has final results, and with the development of thermodynamics and the treatment of the equilibrism state, one could start looking at the final results in terms of the equilibrium. Finally, there came quantum mechanics, and Niels Bohr and his stationary states, and the development of spectroscopy on the idea of the existence of the stationary states. Even today theoretical physics is very much concerned with the treatment of stationary states in atomic spectroscopy, in molecular spectroscopy, in solid state physics, and in nuclear physics, and so on. We are accustomed to making definite predictions, because today the initial conditions in physics are essentially based on the fact that one just counts the number of elementary particles in the system : the number of electrons, the number of protons, of neutrons, and so on. The initial conditions are hence given by Rutherford's discovery of the atom as a small solar system and of the concept of the atomic number.

And then, when we come to biology, we physicists certainly have a tendency to forget that biology is neither a stationary state, nor an equilibrium state, but a "steady state" which is so highly dynamical that if the initial conditions were not given by life itself, there would be no life. This is the main difficulty, I feel, in translating physics over to biology. We need dynamics, we need to know more about the initial conditions, and particularly about whether the initial conditions have "order". We have to treat the fundamental transport phenomena in biology from the point of view of dynamics, but when we go back to physics for help, we realize that physics today has not done too much about treating time-dependent phenomena. We do have a new type of spectroscopy, which is dealing with time-dependent phenomena, where for instance one has two protons hitting a molecule between two molecular collision, and so on, but, for the physicist and the chemist, it has many times been much more convenient to stay with the stationary states.

This does not mean that I don't think what Mr. Careri said this morning is highly interesting, because I believe that the hydrogen bonds are as fundamental as you think they are. However, what we need is a dynamic theory of the hydrogen bond, and when we speak about hydrogen bonds, we have to realize that they are not as easily transferable as other chemical bonds. The hydrogen bonds are highly flexible depending on the particular situation they are put in, depending on the fact that the double- and single-well potential of the protons is depending strongly on the environment, on the polarization of the π electrons, and on similar effects. This means that in order to interpret phenomena in biology from the point of view of the hydrogen bond, one may need a new nomenclature of a dynamic nature, and this is a big drawback for us just now, because such a nomenclature is simply not existing. Perhaps, I should say that I am now not referring to the work by Professor Prigogine and his associates in their study of the approach to the equilibrium, which is one of the outstanding examples of a treatment of a time-dependent phenomenon, but I am thinking essentially of the quantum mechanical aspects of such a process. This also leads to difficulties for us in understanding the operations of the enzymes and the general theory of chemical kinetics, because again such an understanting involves time-depending phenomena. In my own opinion, there will hence be a very long time before one has a good marriage between theoretical physics and biology, simply because biology is so difficult depending on the fact that the initial conditions are given by life itself.

H. FRÖHLICH : I wish to thank the speakers of this morning.

Journée du 30 juin 1969

Deuxième séance

STRUCTURES ET MAINTIEN DE LA VIE

PRÉSIDENT K. MENDELSSOHN

K. MENDELSSOHN

Structures and function of life
Introduction : application of the methods of macroscopic physics
to the problem of life

D. C. HODGKIN

Some characteristics of protein crystals

H. E. HINTON
Reversible suspension of metabolism

Discussions

De la Physique théorique à la Biologie, C.N.R.S., 1971.

STRUCTURES AND FUNCTION OF LIFE

Introduction :

Application of the methods of macroscopic physics to the problem of life

K. MENDELSSOHN

The Clarendon Laboratory, Department of Physics, University of Oxford, Oxford, England

When the present session was planned it was decided to devote it to the borderline between life and inanimate matter, a region which is remarkably ill defined in two ways. First of all, we have to decide whether there is a clear dividing line between a complex molecule which shows the features of life and another one which is lacking them. In other words, we have to formulate the criterion for the state which we call life in a meaningful manner. Secondly, we have to make up our mind at which stage and under what conditions a structure to which we have attributed the criterion of life has ceased to show them. The two main addresses which are to follow this introduction will show that these are questions which at present cannot be answered in a simple and unambigous way. It is hoped that the general discussion which is to follow may help to clarify these inssues.

The borderline between life and non-life is also the borderline between two languages, that of the biologist and that of physics. So far, practically all applications of the methods of physics to biological problems have been concerned with particular aspects of the latter. Some of these have been enormously successful, such as the interpretation of physiological observations or the X-ray investigation of molecular structure. In fact, in this last field the success has been so spectacular and far reaching that the opinion has gained ground that the ultimate goal is almost in sight. Many people think that a few steps further on the same road of structure study and stereo-chemical fitting will eventually lead to an interpretation of living processes which would then form a continuous extension of the structures of non-living matter.

This may well be so. However, there are others, and I admit that I belong to them, who suspect the existence of a well-defined gap which still has to be bridged before we can describe the phenomena of life in terms of physics. It is sometimes assumed that the language of physics is simply incapable of accomplishing this task but it seems to me that this attitude would ultimately mean a rejection of the universal validity of the laws of physics or a return to vitalism.

Assuming then that the physicists are not in ignorance of some fundamental "vital force" still awaiting discovery, it should be possible to express structure and function of life in terms which we already know. Success must then depend entirely on the choice of our approach within the conceptual framework of physics. Unless we think that this approach is already narrowed down to the concepts of stereo-chemistry or some other well defined subject, we have to look for some extremely general principle. It must be so general that it is capable of encompassing every known aspect of life, structure and function not only of the living entities themselves but also of those non-living entites which have been created by life.

The most general term which the conceptual language of physics can offer the phenomenon of life is to regard it simply as a particular form of aggregation of matter. In the parlance of modern theoretical physics we would call it a collective assembly. The next step is to discover the salient features of this particular type of physical system and we must then hope that these features turn out to be the significant ones.

Comparing life with other collective assemblies known to us, we note its enormous complexity. The constituent units of our assembly are arranged in well defined configurations in space which differ, however, from the crystal by the lack of periodicity. On the other hand, while the patterns involved are complex, they do not permit a variation in this complexity if they are to retain the aspect of life. In other words, the structures of life have a very low statistical probability, it is extremely unlikely that they will arise from random processes. In fact, randomness is excluded from the structures of life to a remarkable extent and one might, indeed, argue that this exclusion of randomness is the most significant feature of the collective assembly which we call life. As we shall see presently, this principle of the exclusion of randomness satisfies our primary condition; it is of extreme generality and it covers at the same time every aspect of life.

Whereas we can define in terms of physics the structures of life as presenting an extreme degree of orderliness, the concept of function is reserved to devices which have been created by man or other forms of life. The component units of a collective assembly may be linked by relationships that can be expressed numerically as functions of each other but the biological concept of "function", involving a purpose, does not exist. If the physicist wishes to interpret the phenomena of life in his own terms, biological function must emerge as the general "tendency" of the system to change in a certain manner under external constraint. Function which can be described as purpose may indeed appear in the complex behaviour pattern of living structures, but we cannot invoke it as a primary consequence of external constraint. In physics this reaction to constraint must be spontaneous.

Replacing the concept of function by that of spontaneous change in response to external constraint, we can make use of a well known empirical rule in physics. This is the Le Chatelier-Braun principle which states that any physical system subject to external constraint will behave in such a manner as to evade this constraint. If, as we postulate, the exclusion of randomness is the characteristic feature of our collective assembly, then the assembly will react to constraint in such a way that this aspect tends to be retained. Applying this generally valid principle to living matter, immediately relegates such features as replication, development and metabolism to the status of necessary corollaries. The basis from which all these aspects spring is simply the tendency of the central feature, exclusion of randomness, to be retained. This tendency, as we have just seen, is not limited to life. It is shared by all physical systems.

The constraints suffered by the collective assembly of life include not only the physical conditions of the inanimate world but, above all, life itself. In as much as any change of the physical and chemical environment is evaded by a consequent change in our assembly, similar evasions are forced upon it by the existence of other living structures. Thus we see that competitive development as the biologist knows it, is again nothing but the physical tendency exhibited equally rigorously by any other form of matter. One would therefore suspect that not only replication but also replication involving mutation have to be regarded as direct, if complex, consequences of a general physical rule. Summing up, we can express the application of the Le Chatelier-Braun principle on the assemblies of life simply as the tendency to survive. Surviving, of course, means nothing else but the retention of our central characteristic, the exclusion of randomness.

As I said earlier, this central feature of highly ordered structures turns out to be of extreme generality. This generality not only covers the structures themselves but also their biological function, that is, the maintenance of the central feature under constraint. It further extends to every development and all growth in complexity forced on our assemblies by this constraint. Turning from the simplest living structures as they appear in protein molecules to the other end of the scale, to man and to all of man's works, we find that the basic principle of excluding randomness, of creating order out of chaos, is apparent as the characteristic feature of all our actions. As life has transformed the barren surface of our planet by covering it with statistically improbable forms of matter, so man has made an entirely new contribution, employing the same basic physical principle, but in an entirely new form.

Orderly structures beyond the space occupied by the organism itself have been created by various species of the animal kingdom. In the case of ants, termites and bees, these structures are highly elaborate but their origin rests always on instinct, evidently due to messages laid down beforehand in the genetic material. The orderly structures created by man are infinitely more elaborate and complex, and they are not instinctive, at least not on the same plane. They are the result of reason, that is, due to a much higher combination between acquired items of knowledge. Its operation and the vast memory required for it are seated in the frontal lobes of the brain about which we know next to nothing. It seems that the exclusion of randomness, the creation of order, based on instinct, has been exhausted in its potentialities and that we are standing at the beginning of an entirely new development in which the operating mechanism has been switched to another centre.

Even so, in this most highly developed form of life the basic principle which we postulated as the characteristic feature of our collective assembly is retained as the fundamental theme determining all our actions. By and large, whatever we do, we create more ordered structures. An ocean liner, in fact, contains a greater number of separate parts which are required for its function, and which all have to be in their prescribed relation to each other, than a simple protein molecule. In addition to its structural complexity, it begins to acquire the function of living structures, it moves and it metabolizes. This work of our hands and, more important, of our brains, faithfully obeys the basic principle of counteracting the random processes. In all our actions, even those involving modern technological development, we act under compulsion like puppets, blindly obeying the one basic principle of life, creating more and more order.

Let us now turn to the persistence of life, to the retention of the ability to exclude randomness under various types of constraint. Although some forms of life disappear, the overall balance is in its favour and there are statisticians who forecast the moment in the not too distant future when there will be standing room only. It all goes to show that life, in spite of the fragility of the individual organism, is exceedingly tough and resilient. The basic physical rule which we have invoked for the interpretation of this phenomenon, the Le Chatelier-Braun principle, is an empirical law and not bound to any condition of thermodynamic equilibrium. Altogether the importance of thermodynamic equilibrium has often been overrated. It is a convenient stratagem for calculation but it does not really exist anywhere except in specially designed chemical factories and even there it is retained only for every much shorter periods of time than the steady state of the rest of the world around us. The sun, the galaxy and the universe are clearly not in thermodynamic equilibrium and neither is the interior of the earth. All these things are nevertheless pretty persistent and the lack of thermodynamic equilibrium need not be counted as a threat to the persistence of life. In fact, we are dealing with observationally fairly permanent features. The average human being presents a tolerably steady state lasting for about 70 years and the helix spelling out the code *homo sapiens* has been with us for the better part of 10^{13} seconds.

Strangely enough, life conforms in its own peculiar manner to the physical criterion for stability. This criterion says that a system is stable when after a small and temporary interference, it returns to the original condition. A sphere, resting in a concave dish will return to its centre after it has been slightly displaced. A living structure which has sustained slight damage will equally return to its original state through repair or regeneration. In our very general considerations it is quite immaterial as to how this return to the steady state is accomplished. All that matters is the unexpected but satisfying compliance of living assemblies with the general physical principle.

Using these extremely general considerations, we have seen that a great variety of the aspects of life can be interpreted in terms of physical language and on the basis of well recognized physical principles. The only postulate used has been the assumption that exclusion of randomness is the significant feature. The problem now arises as to whether this general approach can lead us to a formulation of more specific and relevant questions. The obvious thing is to look at the inanimate collective assemblies in their statistically improbable states. In this case thermodynamics is of help to us because one of its laws provides a direct relation between orderliness and temperatures. It tells us that as absolute zero is approached order must become perfect in equilibrium systems.

In quantum mechanical interpretation of physical events, conjugate quantities play an important part. The importance of one of these pairs was recognized already in classical statiscal thermodynamics; the quantities involved are position and momentum. Statistical ordering can take place with respect to either of them.

Ordering with respect to position is well known and has been thorougly investigated. This is the periodic pattern presented by the crystal lattice. It is significant that this form of an orderly assembly again shows extremely general features. The forces which hold atoms and molecules in the lattice sites can be completely different manifestations of the Coulomb interaction but the order pattern is always the same, a regular array in space, exhibiting strict periodicity. We can go even further and show that any assembly of smooth and massive spheres must form a regular crystalline pattern under its gravitational attraction. In other words, the nature of the operating interaction forces is of secondary importance in the interpretation of the solid state whereas the spatial order pattern of the periodic crystal is the basic feature.

It is only fairly recently that patterns of a high degree of orderliness with respect to momentum have been recognized. These occur in assemblies which are cooled to a low temperature where orderliness must appear but in which the high value of the kinetic zero point energy prevents condensation in the crystalline pattern. In these cases orderliness is not apparent in the spatial distribution of the constituent particles and this is the reason why their significance as ordered structures is not immediately apparent. However, they exhibit quite unusual properties in the state of motion. These are the phenomena of superfluidity in liquid helium and of electrical superconductivity. We known as yet far less about them than about the spatial order pattern of crystals. However, one aspect which is of importance to our considerations is immediately apparent. The correlation of momenta of the constituent particles is of immensely long range. The extent of such an assembly can be of macroscopic dimensions but nevertheless local interference will be sensed by the assembly as a whole.

The space order pattern of the crystalline solid provides definite form and rigidity but the effect of local interference with it will not be transmitted further than to the next or next but one atomic neighbour in the assembly. The momentum condensate, on the other hand, is never a structure in co-ordinate space but interference does not remain localized. Any change to which one part of it is submitted is transmitted throughout the whole space occupied by it.

To which extent have these two inanimate order patterns any bearing on the collective assembly of life ? As a matter of fact we find in it vestiges of both these order patterns but we would find great difficulty in describing life in terms of only one of them.

Living structures certainly exhibit the properties of shape and rigidity shown by the crystal, but the periodicity on which the crystalline order pattern is based is missing. Many years ago Schroedinger coined for it the name of "aperiodic crystal", but one wonders whether this may not be a contradiction in terms that must lead to an evasion of the true issue. In fact, you can make a crystal of protein molecules but this assembly then fails to exhibit the properties of life. These remain completely hidden by the crystalline pattern which even prevents us from finding out in this state whether the constituent units are capable of life or not.

The vestiges of the momentum condensates in the living assembly are apparent in its ability to sense local interference throughout the whole of its structure. For instance, local damage involving only a few atoms will change or completely annihilate the essential aspect of life of the total structure. In other words, chopping off the end of a protein chain will significantly alter the whole as far as a meaningful description of life is concerned whereas, chopping off the end of a single crystal of copper will not alter anything but its length.

At the beginning of my talk I mentioned the existence of a gap in our understanding, that between life and non-life, with which this session is concerned. There is no difficulty in pinpointing the position of this gap in our collective assembly. As far as the nature and distribution of elementary particles in concerned there is no difference between life and non-life. Neither is there a difference in the physical properties of a crystal of protein molecules and one of other large molecules which do not exhibit the phenomenon of life.

The gap clearly must lie between these limits, that is in the linkage of the atoms constituting the protein molecule.

Why does one complex molecule live and another not? If, as has been done so far, only chemical binding between the nearest neighbour atoms is invoked, then it is extremely difficult to see how any significant difference can arise. The elucidation of the replication process in the double helix is a magnificent achievement but somehow it is still unconvincing that this stereo-chemical fitting should be based on random processes, involving nothing more than short range forces.

Moreover, at this stage something entirely new begins to happen; unlike a non-living macro-molecule, the protein chain starts acting as a whole. It can fold up and uncoil, and it seems quite inconceivable that these concerted changes, involving hundred or a thousand atom distances should be the result of nearest neighbour forces. However, we have just seen that physics provides us with ordered assemblies of very long correlation, the momentum condensates, in which local interference is transmitted to the whole.

It is therefore tempting to try and bridge the gap by a combination of the two basic ordering processes known in physics. In such a combination the short range forces and valency angles of stereo-chemical fitting will provide the structure of life. Its function, however, will be vested in electron wave functions which extend over the whole length of the chain or at least over long distances within it. In this interpretation the basic unit of life has a dual nature, a spatial array of atoms in a well defined configuration, that is the protein molecule, to which coherence, i.e. a common "purpose", has been given by sharing the long range order of the electron fluid. Neither by itself can do the job alone. The electron fluid requires the "container" provided by the atomic structure and this structure could not acquire the specific configuration, except through resonance states of the electron fluid. Perhaps, this is, in fact, the physical meaning of life : A combination of the two basic ordering processes known to exist separately in inanimate matter.

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SOME CHARACTERISTICS OF PROTEIN CRYSTALS

DOROTHY CROWFOOT HODGKIN

Chemical Crystallography Laboratory, South Park Road
Oxford (England)

There is an early paper by A. Trécul (1858) with the title, « Note sur des cristaux organisés et vivants » which provides a background to the discussion that I wish to provoke. The crystals Trécul described were not actually " living " but were protein crystals occuring naturally as hexagonal plates in *sparganium ramosum*. They had peculiar properties — they swelled with dilute sulphuric acid and were penetrated and coloured yellow by iodine. Other naturalists in the nineteenth century made observations similar to those of Trécul — that protein crystals were soft, non-rigid to the touch when in their natural surroundings, and that they shrank on removal from their mother liquor.

Today, through X-ray crystallographic analysis, we know the detailed arrangement of the atoms in crystals containing some ten different protein molecules of weights between 12,000 and 34,000 and we have incomplete evidence of the structures of many more. The properties observed long ago are explained by the fact that the molecules are large globular bodies in contact with one another at certain points in the crystals and also having between them spaces, almost equally large, filled with solvent of crystallisation. The method used to find the detailed arrangement of the atoms within these molecules depends upon the regular relative arrangement of the molecules within the crystals and also on the possibility of introducing heavy atoms among them without noticeably disturbing their regular arrangement. X-ray diffraction spectra can be recorded from both the native and the modified isomorphous crystals; their detailed interpretation in favourable cases leads to the calculation of a map in three dimensions of the electron density in the crystals; from this the atomic arrangement may be seen. The calculation is not straightforward. It depends on the application of the mathematical theory of Fourier series. The measured intensities of the X-ray diffraction spectra provide the amplitudes of the terms of series and the study of the heavy atom derivatives, the evidence of their phase relations which is necessary to the calculation. There are two general limitations which affect the quality of the electron density map obtainable. The first is a limitation on the extent of the diffraction data obtainable which is probably a consequence of the large size of most protein molecules and the fact that they are literally floating in the crystals. This limitation is more severe with a large protein 'molecule', such as haemoglobin, than for smaller ones, such as myoglobin or ribonuclease — the reflections in the former case extend to planes of spacings of about 2.4 Å, in the latter to 1.5 Å or even less. So the

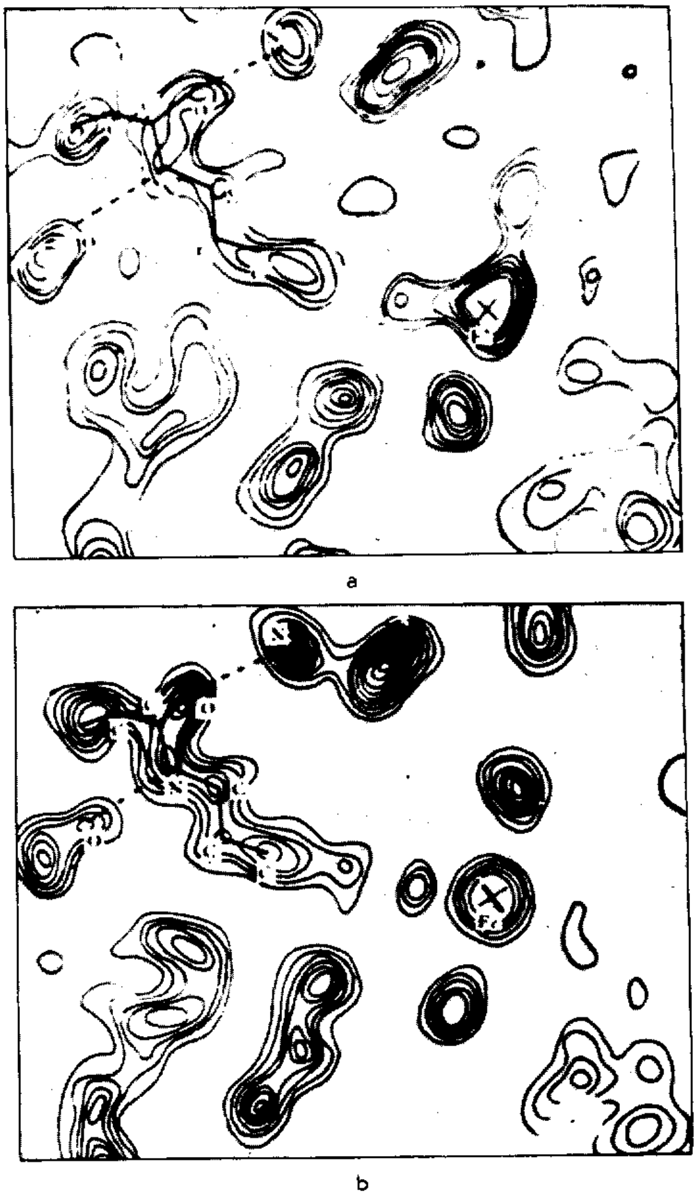
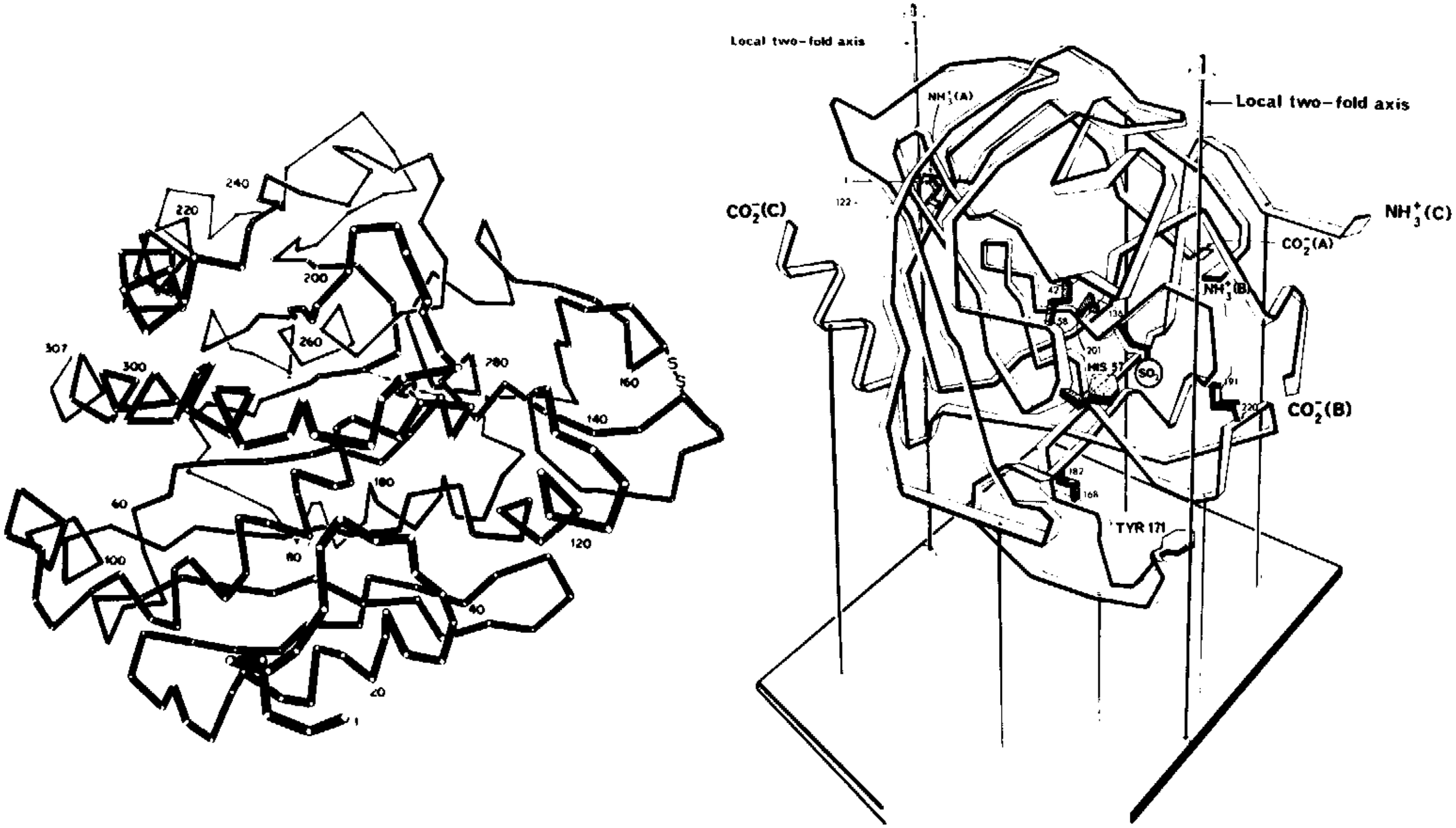


Fig. 1. Section of electron density map calculated for myoglobin: a) at 2 Å resolution; b) at 1.4 Å resolution.

(Reproduced from John Kendrew, *Le Prix Nobel,* 1962).

small section of the electron density map for myoglobin in figure 1 illustrates both the degree to which atomic positions are defined in most protein electron density maps — 1a — and the degree to which they have been refined in the best maps yet obtained — 1b. (Kendrew, 1963) The second limitation on atom definition arises from the imperfect knowledge we have of the phases appropriate for the diffraction spectra. Our evidence for these, in the first instance, is derived from the effects observed when heavy atoms are introduced into the crystals at a small number of definite sites. In the first experiments to succeed, those of Perutz on haemoglobin, the small number and definite positions were secured by covalent bonding of mercurials to the sulphydryl groups on the protein. This is still probably the best method when available; only too few proteins contain a single residue capable of attaching a heavy atom. With enzymes, as an alternative, use may often be made of inhibitors interacting specifically with the active site. Otherwise, the formation of heavy atom derivatives of proteins has to be approached by trial and error, often by soaking the crystals in heavy atom-containing solutions, as in Trécul's experiment with iodine, and testing the measured X-ray data for evidence that the heavy atoms adopt definite sites in the crystal. The whole process of trial and measurement and calculation for any one protein may take a number of research workers several years. On the other hand, the structure of one protein, elastase, was recently solved in a few months by a Ph. D. student following procedures previously found to work for the closely related protein, chymotrypsin.

Within each protein molecule studied, the atoms present can be seen to belong to amino acids linked in peptide chains as expected from chemical sequence studies. Some of the protein molecules so far surveyed in detail belong to well-defined, closely related, families of molecules within which the peptide chains adopt very similar conformations. Thus the α and β chains, which together compose half a haemoglobin 'molecule', each separately is very similar to myoglobin. (Cullis *et al.*, 1962) Elastase, (Shotton and Watson, 1969) has essentially the same chain structure as chymotrypsin (Matthews *et al.*, 1967) and this, from less complete evidence, almost certainly also appears in trypsin. S-ribonuclease is essentially similar to ribonuclease, (Kartha *et al.*, 1967). Apart from these family resemblances, the impression given by the protein molecules so far studied is one of great diversity. Only the most general principles appear to guide the chain arrangements adopted — conformational stability plus the tendency for hydrophobic groups to be inside a protein molecule and hydrophilic ones outside. The actual paths taken by the chains through the molecules of, for example, carboxypeptidase or chymotrypsin, shown in figure 2, are extremely complicated and entirely different from one another or from subtilisin (Wright *et al.*, 1969) or papain, (Drenth *et al.*, 1968), though all four molecules are proteolytic enzymes. In carboxypeptidase, (Recke *et al.*, 1967) there is a particularly complicated spiral region in the centre of the molecule,



(a)
(Reproduced from *Phil. Trans. Roy. Soc.*, 1970).

(b)
(Reproduced from *Nature*, 1967, **214,** 652; **B 25,** 75).

Fig. 2. Skeleton chain course in a) carboxypeptidase, b) chymotrypsin.

formed of extended chains running backwards and forwards, sometimes in parallel, sometimes in antiparallel β pleated sheet-like structures, linked by short stretches of α helices. In chymotrypsin, there is only a very small region of α helix, some β pleated sheet but much casually extended or folded chain. In all the enzymes so far studied there are definite pockets or grooves recognisable as the site at which the enzyme reactions occur. Adjacent to these sites are the groups such as serine and histidine known to participate in the reactions and placed so that hydrogen or charge transfer may readily occur.

In general, it is difficult to see why the peptide chains adopt the particular arrangements found within any protein molecule. Local packing conditions within the molecule, preferred bond directions and possibilities of hydrogen bonding can all be seen as important and their interrelations estimated in detailed calculations. But they appear insufficient in themselves to define the actual conformations found experimentally. There seems something very purposeful in the way in which, for example, the first fifty residues of lysozyme stretch out along two arms, beginning to define the groove in the centre of the molecule which is the active site of the enzyme (Blake *et al.*, 1967). Experiments on synthesising the proteins, insulin and ribonuclease, underline the problem. In both cases, the first products obtained showed rather low specific activities. There were evidently reasons for this, particularly in relation to the problems of the correct formation of the interchain disulphide bonds, which is interlocked with that of chain folding. The general methods used throughout involved the preparation, first, of the correct sequence of amino acids in the chains, with the potential cystine groups present as SH groups, which then interacted together on oxidation in air under controlled conditions. One might expect the correct disulphide bridges to be formed more easily within the single chain of ribonuclease than between the two chains of insulin and the first trial specific activities suggest that this is so. The two syntheses for ribonuclease reported very recently were very different in detail. That of Gutte and Merrifield (1969) was carried out stepwise in the solid phase, starting from the C terminal. That of Hirschmann *et al.* (1969), involved the more usual type of preparation and linking together serially of a number of peptides. Both gave preparations of specific activity variously estimated between 13 % and 24 % in different tests. The three different series of preparations made of synthetic insulin all gave initially lower activities of only 1-2 %, probably due mainly to the difficulties of forming the correct disulphide bridges; (Meienhofer *et al.*, 1963, Katsoyannis *et al.*, 1963, Kung *et al.*, 1965) in nature it is significant that the required disulphide bridge formation in insulin is ensured by the existence of a single chain precursor, proinsulin, (Steiner and Clark, 1968). Experimentally various conditions have been found for improving the activity of synthetic insulin preparations and these conditions also probably help to guide the insulin chains to fold correctly. That correct folding does eventually take place is shown most clearly by the work of Kung *et al.* who have, through various fractionation procedures, obtained from their synthetic insulin, crystals with an activity of more than 20 international units per mg., closely similar to commercial insulin from natural sources.

In the electron density maps calculated for protein crystals one can observe regions of very low density which correspond with the space occupied by water of crystallisation. The boundary between these regions and the protein molecules themselves is not sharp. It is often found that amino acid side chains, usually hydrophyllic such as tyrosine or lysine, projecting into the mother liquor are less well defined than those in the body of the protein molecule as if they were waving about in the water; lysine is sometimes shown by a low forked distribution corresponding to two alternative chain positions. Attached to the polar groups, often linked by hydrogen bonds between them and the peptide nitrogen or oxygen atoms, there are electron density peaks indicating water molecules which may be as well defined in position as the atoms of the protein molecule. Other water molecule positions, weaker in definition, can be traced, bonded only to one molecular polar group. Beyond these the water pattern becomes progressively more indistinct. In the maps calculated for carboxypeptidase, for example, there are many small low electron density peaks which may represent sites occasionally occupied by moving water molecules. Occasionally too, heavier peaks mark the position of some ion present; in the myoglobin maps a well defined sulphate ion appears.

It is characteristic of many protein molecules that they aggregate to form polymers — some so stable that they usurp the name of the molecule. Thus horse met haemoglobin itself is often described as of molecular weight 67,000; it actually consists chemically of four sub-molecules, each of weight about 17,000, related in pairs by exact two-fold axes and approximate two-fold axes one to one another so that the symmetry of the aggregate as a whole approximates to 222. (Cullis *et al.*, 1962). The forces producing the aggregation seem to be largely van der Waal's attraction between non polar groups only occasionally reinforced by hydrogen bonding. In haemoglobin the contact between the α_1 and β_1 chains involves 34 residues, with 110 atoms in contact while that between the α_1 and β_2 chains involves 19 residues and 80 atom contacts (Perutz *et al.*, 1968). When oxy haemoglobin is converted into deoxyhaemoglobin, relative movements of the chain occur which are more pronounced (up to 5.7 Å), as might be expected at the α_1 β_2 contact than at that involving α_1 and β_1 chains (c. 1 Å). The kind of packing of four sub units in haemoglobin seems to be a very common variety among medium sized enzymes and there are now many examples. One particularly interesting system is that of dog fish muscle lactic dehydrogenase. (Adams *et al.*, 1969) Here the enzyme oligomer, symmetry 222, molecular weight 35,000 × 4 or 140,000, has been obtained in crystals separately, as a double complex with the coenzyme, nicotinamide adenine

(a)
(Reproduced from *Proc. Roy. Soc.*, 1962, **265**, 161).

(b)
(Unpublished).

Fig. 3. a) model of haemoglobin molecule seen along two-fold axis, b) model of insulin hexamer seen along three-fold axis; both are derived from low resolution electron density maps.

dinucleotide, and as a triple complex with coenzyme and an inactive form of substrate. The three crystals are not isomorphous but are closely related; solving in detail their structures will take a tremendous amount of work but should lead straight to an understanding of the central role played by coenzymes in many enzyme reactions. In addition to tetramers there are a number of protein polymers which are hexamers of symmetry, 32. So far the molecular aggregate of this kind studied in most detail, is the quite small hexamer, molecular weight 36,000, formed by six insulin molecules in the presence of zinc. This at present, is only defined by a low resolution electron density map shown to compare with haemoglobin in figure 3. There is preliminary crystallographic evidence that the plant storage protein, excelsin has the same symmetry, 32, and is composed of six molecules of weight 50,000 giving an aggregate of weight 300,000 (Drenth and Wiebenga, 1955). Asparate trans carbamylase, from Wiley and Lipscomb's measurements (1968), seems very similar to this, both in size and also in symmetry, 32 : the subunit here consists of a regulatory subunit of weight 17,000 and a catalytic subunit of weight 33,000, giving to the aggregate molecule once again the molecular weight 300,000.

Protein molecules also often aggregate in dimers, e.g. lactoglobulin and insulin, or long polymers, compare insulin again, in acid solution, or the muscle protein, actin. The more open form of aggregation indicated by these structures may lead to open, basket-like complexes such as the fat synthesising system described by Lynen. Here the large space, surrounded by seven or more enzyme molecules seems particularly well designed to accommodate the very large coenzyme molecule, coenzyme A, necessary to promote fat synthesis.

Amongst the many different molecules which play essential roles in living matter, protein molecules hold quantitatively a rather special position; in many tissues, they compose over three quarters of the dry weight of all matter present. In the highly concentrated aqueous solution that occupies the body of most cells, they must frequently be in contact with one another as they are in protein crystals, and it is not surprising that very often actual crystals separate out in living cells. Indeed, with the coming of the electron microscope, one can see many examples of such crystals, too small to be visible in the light microscope. Thus in rat pancreas islet cells, there are patches which look extremely like small insulin crystals, within which are globular units, roughly 50 Å across, the size of the insulin hexamer. Very often, in egg yolk, platelets occur within which large periodicities can be seen; and very similar looking protein crystals frequently appear on the edge of chloroplasts. The chloroplasts themselves present similar but more complicated organisations in which the quantosomes, of the order of magnitude of large protein molecules, are arranged in very regular layers among which must be fitted many of the other molecules active in photosynthesis. Some of these, such as the carotenes, frequently also appear as single crystals in the plastids.

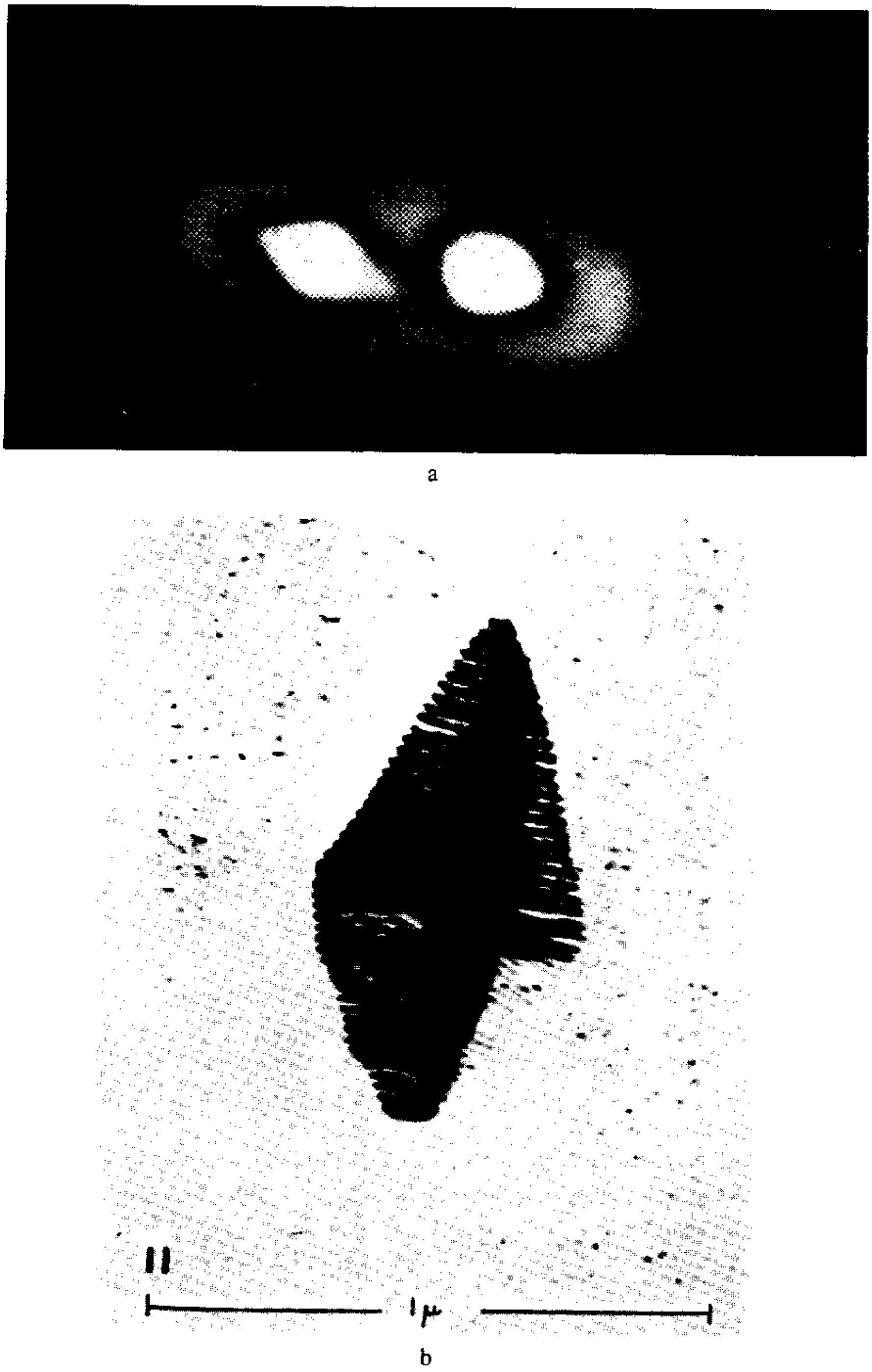


Fig. 4. *Bacillus thurigiensis :*

a) crystal and spore lying in a cell from which other constituents have been lysed;

b) shell of protein crystal produced by the intensity of electron bombardment in the electron microscope (Reproduced from *Canadian Journal of Microbiology,* 1954, **1**, 694).

The formation of protein and other crystals in living cells provides clear evidence of tendencies towards certain kinds of long range regularity. One should not, however, exaggerate their importance in relation to the properties of living creatures. There is a very remarkable small bacillus, bacillus thuringiensis *Berliner,* isolated from diseased flour moths in 1911, which is worth noticing. On the cessation of vegetative growth, a single protein crystal grows within it until it fills one half of the bacillus (Hannay and Fitzjames, 1954). This crystal appears first spherical, then rhomboid, and finally takes the form of a very regular tetragonal bipyramid, figure 4. After some exposure to the electron beams it shows in the electron microscope well defined bands about 200 Å across. However the other half of the bacillus is occupied by a single spore of more complex and less regular structure and certainly it is from the spore that life will continue to develop.

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REVERSIBLE SUSPENSION OF METABOLISM

H. E. HINTON

Department of Zoology, University of Bristol (England)

Introduction

It has long been known that many acellular organisms, such as bacteria and protozoa, could resume normal activity after a total suspension of metabolism for extended periods, but relatively little attention has been paid to the capacity of complex multicellular organisms to tolerate a similar suspension of metabolism. During the last two decades some work has been done on the capacity of whole insects and some of their tissues to tolerate a total suspension of metabolism. The relevance of this work proved to be more general than at first seemed likely. In this paper the work that has been done on the reversible suspension of metabolism in insects is briefly reviewed, and some of its wider implications are noted, particularly those that bear upon theories about the origin of life.

Definition of cryptobiosis

When the metabolism of an organism is reversibly suspended, the organism is popularly said to be in a state of suspended animation. The technical terms for suspended animation, meaning suspension of metabolism, were until recently *anabiosis* introduced by Preyer of Jena or *abiosis* introduced by the Soviet worker Schmidt. Neither of these terms is particularly suitable for the phenomenon we are now discussing, but fortunately Keilin (1959, p. 166) proposed the term *cryptobiosis* to replace *anabiosis* and *abiosis*. Keilin defined his term cryptobiosis as " ... the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable, or comes reversibly to a standstill ". This was not a very satisfactory definition because there is a clear qualitative difference between a very low level of metabolism and a total absence of metabolism. For this reason, therefore, the term cryptobiosis was restricted (Hinton, 1960 a) to describe a state when metabolic activity comes reversibly to a standstill, and the term is used in this sense throughout this paper.

The cryptobiotic state

When an organism is in a state of cryptobiosis all of the chemical reactions normally responsible for its maintenance or for both its growth and maintenance cease, and all metabolic processes come to an end. Of course in most environments some chemical reactions occur even when the organism is in a state of cryptobiosis, but these would occur if the organism were in fact dead and not in a state of cryptobiosis. Such adventitious oxidations and reductions as may occur even when the organism is in cryptobiosis have nothing to do with metabolism if by metabolism we mean the orderly chemical changes that contribute to the maintenance and growth of the organism and which, generally speaking, are under the control of the organism.

In previous epochs many were reluctant to consider even the possibility of a temporary discontinuity in metabolic processes and they therefore held the view that however slow metabolic processes might become they nevertheless proceeded continuously in order to supply the energy for the upkeep of the complex structure of the organism. However, the essential characteristic of the phenomenon that we now know as cryptobiosis was correctly described by Claude Bernard (1878) when he defined latent life as a state of absolute chemical indifference characterised by the suppression of all interrelationships between organisms and their media.

In cryptobiosis the organism is reduced to a purely morphological state, the successful maintenance of which does not require interactions between cells or between constituents of cells but only requires that certain spatial relations be preserved. No other conclusion seems possible in the light of the results of certain experiments on organisms in a state of cryptobiosis. For instance, dehydrated bacterial spores, algae, moulds, fragments of lichens, rotifers, nematodes, and tardigrades revive and grow normally after being subjected to temperatures as low as 0.008 K obtained by demagnetization of iron alum in liquid helium (Becquerel, 1951).

Decrease of viability with time during cryptobiosis

In order to understand some of the attributes of organisms in a cryptobiotic state, such as their decrease in viability with time, it is essential to appreciate the distinction already made between chemical reactions that are part of metabolism and those that can only be described as adventitious. If it is true that in a cryptobiotic state all interactions between cells and between the constituents of cells have ceased, the organism should be able to exist indefinitely in a cryptobiotic state. It is entirely because of what we have described as adventitious chemistry that in any ordinary environment there is an appreciable decrease with time of the viability of organisms in a cryptobiotic state. The decrease of viability with the passage of time can be reduced by suitable storage methods, for instance, storage at low temperatures in inert gasses. It has, for example, been calculated that if chemical reactions are possible at —200° C, they would be about 8×10^{-6} slower than at 20 °C.

The decrease of viability with time because of adventitious chemistry can of course be greatly accelerated simply by raising the temperature. It has, for instance, been found that when dehydrated cryptobiotic eggs of the brine shrimp (*Artemia salina* L.) were exposed to high temperatures in an electric oven there was relatively little decrease in the percentage that subsequently hatched until a certain critical period had been exceeded. Experiments with one lot of eggs showed that about 36 per cent of unheated eggs hatched when placed in a salt solution. When eggs of this lot were heated for as long as one hour and 30 minutes at 103-104° C, no less than 26 per cent hatched when subsequently placed in a salt solution. However, by two hours only 1.7 per cent hatched, and after an exposure of 4 hours to 103-104° C only one egg in 7,000 hatched (Hinton, 1954). Experiments with other batches of *Artemia* eggs, and with other organisms in a cryptobiotic state, show that the critical period at any particular temperature depends upon the previous history of the organism. The critical period is simply the time when the cumulative damage from adventitious chemistry begins to exceed the capacity of the organism to repair when it once more becomes a physiological entity instead of only a morphological one.

It is now known that there is no really serious practical difficulty in the way of making a fair number of plants and animals spend part of their active lives in one century and part in some other century. There would seem to be no theoretical reason why the same cannot be done with warmblooded animals, although the technical difficulties here are so great that they will not be mastered for a very long time to come. The best that has so far been done with whole warm-blooded animals is to freeze hamsters (Rodentia : *Mesocretus auratus*) at an external temperature of — 5° C for as long as 45 minutes without lasting ill effects of any kind. During this time as much as 50 to 58 per cent of the water in the brain and 60 to 90 per cent of the water in the skin was converted to ice (Farrant and Smith, 1966).

An organism in a state of cryptobiosis would theoretically survive indefinitely if it could be safeguarded against adventitious chemistry and other types of adventitious damage. The possibility of plants and animals surviving for immense periods of time has always appealed to the imagination, as witness the numerous reports that appear in the popular press and elsewhere. It may be remembered that for many years after the discovery of the tomb of Tut Ankh-Amen numerous reports appeared to the effect that wheat kernels and bacteria that had remained in a state of suspended animation since the tomb was closed about 1,500 B. C. could be got to grow. Sometimes a report that a live toad or some other animal that has been found entombed in the foundations of a building for a hundred years or so receives wide publicity. When reports like these have been carefully examined, they have proved to be untrue almost without exception : they are either pure inventions or result from accidental contamination by organisms living nearby.

Nevertheless, not all such reports are false. Bacteria have been known to grow after 46 years in alcohol in a sealed museum jar, and fungal spores were found to be viable after 35 years in a high vacuum. Until rather recently the longest authentic record of suspended animation concerned the seeds of the sacred lotus (*Nelumbium nuciferum*) found in peat deposits in northeastern China. This plant does not now grow within 1,000 miles of the peat deposits. Various analyses of the residual carbon^{14} in the seeds puts their age from 800 to 2,000 years. Seeds of *Chenopodium album* and *Spergula avensis* have been germinated in Denmark after cryptobiosis for 1,700 years (Ødum, 1965). Porsild *et al.* (1967) have grown the arctic lupin (*Lupinus arcticus*) from seeds at least 10,000 years old that had been deeply buried in permanently frozen silt of Pleistocene age in the central Yukon.

Some years ago Dombrowski showed that if salt is gradually added to a medium containing certain bacteria until the solution is supersaturated the bacteria are not killed. If now the solution is slowly dried until all the salt is crystallised, bacteria are trapped within the salt crystals. Bacteria trapped within salt crystals in the laboratory in the manner described have been revived after more than seven years. In the crystals they are largely protected from adventitious chemistry : the close packing of the chlorine ions makes the crystals virtually impermeable to oxygen. However, the claims by Dombrowski (e. g. 1963) that bacteria embedded in salt crystals of Palaeozoic age can be revived require confirmation even though it is said that the most stringent precautions were taken against contamination.

A distinction between biological age and calendar age

Experiments on the fly larva, *Polypedilum vanderplanki* Hinton, have shown in a particularly clear fashion that it is necessary to make a distinction between biological or developmental age on the one hand and calendar age on the other hand (Hinton, 1960a). The biological age of two larvae may be exactly the same, say, three weeks, whereas the calendar age of one is three weeks and that of the other four years and three weeks if it has been in cryptobiosis for four years. Although the calendar age of one larva is 70 times that of the other, there appear to be no differences between them in activity or subsequent developmental history.

When an animal is in a state of cryptobiosis, the changes that make death more likely with the passage of time do not occur. These changes only begin again when activity is restored, that is, when the animal is changed from a purely morphological entity into a physiological one. In other words, the time that the organism has spent in a state of cryptobiosis does not necessarily make it older in a biological sense : from a biological point of view time has been abolished.

A necessary consequence of a state in which, so to speak, biological time is at a standstill is that all mechanical and other injuries sustained by the organism are strictly cumulative. When the organism is in a physiological state instead of a purely morphological one, damage to it depends both upon the total amount inflicted and the rate at which it is inflicted. When an organism is in a physiological state, it has the capacity to repair damage. Because of this capacity to repair, the organism can sustain a series of injuries over a period of time that had they occurred simultaneously would have caused its immediate death. In a state of cryptobiosis only the total amount of damage is of concern and not the rate at which it is inflicted.

A consideration of some of the attributes of the cryptobiotic state mentioned in the preceding paragraphs makes it possible to consider seriously some aspects of an idea that has often been discussed in the popular press and sometimes elsewhere. That is, the idea that extended journeys into outer space may in some distant future be undergone by humans in a state of suspended animation. It is generally assumed that if humans can be put into a state of suspended animation it will be by freezing.

We know from the experiments of Hornsey (1957) on mice that when these animals are frozen to between 1° and 0° C, their resistance to radiation is increased by nearly three times. It has already been pointed out (Hinton, 1968 b) that even assuming an increase in resistance of this order, freezing humans is not likely to be a fruitful method of space travel for the simple reason that all radiation damage would be strictly cumulative.

The median lethal radiation dose for man is about 400 rads. Just outside the earth's atmosphere it would take about 36 years to accumulate this dose from galactic cosmic radiation but only about half of this time in deep space away from the shelter of the earth. We must, however, remember that high energy particle radiation from solar flares may reach a fatal level in a few hours even at 100,000 feet, as happened on February 23rd 1956 and again on November 12th and 15th 1960. Of course it is much easier to shield from this solar particle radiation than from the harder galactic cosmic rays, but it still seems evident that organisms in an active state and competent to repair at least part of the damage as it occurs are more suitable for extensive journeys into outer space.

Cryptobiosis by dehydration

It has previously been pointed out (Hinton, 1960a) that the capacity to tolerate a high degree of dehydration at developmental temperatures is usually certain evidence that cryptobiosis can be induced. Many arthropods are remarkably resistant to desiccation, but they nevertheless tolerate only a certain fall in their moisture content : sooner or later a critical level is reached below which they cannot survive. Only a few arthropods differ qualitatively in this respect : at physiological temperatures there is no apparent limit to the dehydration they will tolerate, e.g. most tardigrades, eggs of some crustacea (Branchiopoda, Copepoda, Ostracoda), and the larvae of a few flies. From a consideration of the minimum moisture content that can be tolerated by animals that do not tolerate more or less total dehydration, it may be predicted with some confidence that if an animal will tolerate a moisture content of, say, 20 per cent, it will almost certainly tolerate a more or less total dehydration and will become cryptobiotic.

In some insects dehydration of the tissues may be so slowed down by the impermeability of the embryonic membranes, chorion, or cuticle, or by the production of metabolic water, or by both, that the animal may survive desiccation for many years. Such animals are not, of course, in a state of cryptobiosis. For instance, Payne (1929) found that eggs of the moth *Notolophus leucostigmus* S. & A. would hatch after two years over calcium chloride, and Hinton (1953) found that 0.08 per cent of the eggs of the mosquito, *Aedes aegypti* L., would hatch after 114 days over calcium chloride. The egg of *Locustana pardalina* Walk. will survive over three years in relatively dry conditions. Eggs of this species have remained viable at room humidities in my laboratory for just over two years. Matthee (1951) has shown that the eggs are able to tolerate a fall in moisture content from their normal of about 85 per cent to a moisture content of about 40 per cent. Buxton (1930) found that larvae of the beetle, *Tenebrio molitor* L., kept over sulphuric acid did not begin to die until after 210 days. During this time the weight of the larvae decreased considerably, but their moisture content was more or less maintained by the water produced by metabolising their reserves. The South American coccid, *Margarodes vitium,* was found to be alive after 17 years in a museum (Ferris, 1919). The development of the larva of the North American Cerambycid *Eubria quadrigeminata* Say, has been delayed up to 40 years in dry wood (Jaques, 1918).

Although cryptobiosis by dehydration at developmental temperatures can be induced in some tissues of possibly most or all insects, cryptobiosis by dehydration of the whole animal is now known among insects only in the larvae of three species of flies : (1) an African species of Chironomidae, *Polypedilum vanderplanki* Hinton, (2) an Australian species of Chironomidae; and (3) a European species of Mycetophylidae, *Sciara medullaris* Giard. Of these, detailed experiments have so far been published only on *Polypedilum* (Hinton, 1951, 1952, 1953, 1960a, b).

The capacity of whole insects to tolerate dehydration is very uncommon indeed, but there is reason to believe that the capacity of the epidermis to tolerate total dehydration is very widespread among insects that are irreversibly damaged when they loose a large amount of their moisture. For instance, in preliminary experiments it was shown (Hinton, 1957a) that the epidermis of the larvae of four families of beetles (Gyrinidae, Haliplidae, Elminthidae, and Psephenidae) and that of one larval fly (*Chironomus,* Chironomidae) and one adult fly (*Antocha,* Tipulidae) repaired wounds to legs, or gills, or body wall with plugs of tanned cuticle after dehydration for long periods over phosphorus pentoxide. In these experiments the whole insects were dried over phosphorus pentoxide, and after 692 hours they were placed in tap water. The insects died shortly after they were placed in a desiccator, but decomposition of the tissues that did not tolerate the dehydration was prevented by lack of water. When the insects were later placed in tap water, the capacity of the epidermis to repair wounds was rather short-lived. The reason for this appeared to be that once in water the decomposition products of the tissues that did not survive the dehydration began to poison the epidermal cells that did. Cryptobiosis of the epidermal cells isolated in the spiracular gills of a number of insect pupae has recently been summarised (Hinton, 1968a). Selman (1960) has shown that both blood and epidermal cells of the larva of *Sialis lutaria* L. tolerate more or less complete dehydration. The epidermal cells of the gills would form tanned wound plugs after 17 months over phosphorus pentoxide. When placed in water after prolonged dehydration, the blood cells in the gills would clot and form pseudopodia as they did before dehydration.

Freezing experiments have shown that the capacity to tolerate a total suspension of metabolism by freezing is widespread among plants and animals. The capacity to tolerate a total suspension of metabolism by dehydration at developmental temperatures is very much less common. The capacity of acellular organisms (bacteria, protozoa) to tolerate total dehydration at developmental temperatures is a primitive feature. It has been pointed out (Hinton, 1968b) that the capacity to tolerate total dehydration is a *primitive* feature of protoplasm that has *secondarily* been lost in most highly evolved organisms although it may be retained by some of the tissues of the latter, such as the epidermis of insects.

In most of the major groups of invertebrate animals there are isolated species, or small groups of species, that will tolerate total dehydration at developmental temperatures. The sporadic distribution of this capacity makes it very evident that it has been independently evolved within each major group of invertebrates. For instance, *Polypedilum* is a very large genus of Chironomid flies. *Polypedilum vanderplanki* can tolerate total dehydration at developmental temperatures, but attempts to dehydrate the larvae of several other species of the genus were not successful, although there can be little doubt that some of the tissues, such as the epidermis, of every species will tolerate total dehydration. Thus, in this group of flies it is clear that tolerance to total dehydration is not even established at a generic level. All of this of course suggests, as previously noted (Hinton, 1968b), that we are dealing with a capacity that is not particularly difficult for invertebrates to evolve. In turn, this suggests that perhaps many tissues of invertebrates have retained the primitive capacity of protoplasm to tolerate total dehydration.

Cryptobiosis in insects by freezing

During the last 20 years much has been discovered about how to induce a cryptobiotic state in hydrated multicellular animals, or some organs of such animals, by cooling them with or without antifreeze agents.

It may be noted here that cryptobiosis can be induced in hydrated larvae of *Polypedilum vanderplanki* by placing them in a solution of dimethyl sulphoxide for an hour and then cooling them at — 40 °C for two hours before immersion in liquid air (— 190 °C). One of my students, Dr. J. P. Leader, found that up to 80 per cent of hydrated larvae that had been in liquid air subsequently metamorphosed.

Asahina and his co-workers in Japan (e.g. Asahina & Aoki, 1958) have shown that when carefully pre-cooled several kinds of lepidopterous larvae survive the temperatures of liquid gasses even in the absence of glycerol or other protective agents. Work on cooling animals has shown us that some organisms have a metabolism of maintenance and growth under conditions that induce cryptobiosis in others. For instance, the freezing point of water in Don Juan Pond in Victoria Land, Antarctica is — 40 °C, but bacteria grow in the water of this pond at temperatures of — 3° to — 24 °C, temperatures at which a mammal would almost certainly be in a state of cryptobiosis. It is of interest to note that at the other end of the temperature scale most hydrated organisms are irreversibly damaged in a few seconds at temperatures of around 50 °C. Nevertheless, a few normally grow at temperatures 20 °C or more above this, e.g. bacteria and algae in hot springs. Kempner (1963) has shown that algae living in hot springs in Wyoming will incorporate radioactive phosphorus into the nucleic acids of growing cells up to a temperature of 73 °C.

Cryptobiosis in the larva of *Polypedilum vanderplanki*

Most of the work on cryptobiosis by dehydration in insects has been done on the larva of a fly, *Polypedilum vanderplanki* Hinton (Chironomidae).

This insect breeds in small pools formed in shallow hollows on unshaded rocks in Northern Nigeria and Uganda.

At the beginning of the rainy season the small pools on the rocks may fill and dry several times, and they may very occasionally be filled with water for brief periods during the dry season. When the pools dry out the larvae dry out, but when the pools fill with water, the larvae rapidly absorb water through their skins until their normal moisture content of 85 to 90 % is restored. About an hour after the dry larva has been immersed in water it may be feeding again. The larva is thus very well adapted to an environment that is subjected to flooding alternating with periods of extreme desiccation

The moisture content of larvae exposed to less than 1 % relative humidity is below 3 %, at 60 % relative humidity the moisture content of the larva was 8 %, and at higher humidities the curve for moisture content rose more steeply. These figures for moisture content are based upon the assumption that the larva contains no water when its weight is constant at 106 °C. When dry larvae are immersed in water, the pharynx and dorsal vessel begin to beat when the moisture content rises to about 55 % or so, but they have, of course, to take up much more water before they can crawl or before they swell to their normal shape when active.

1. *Survival after prolonged dehydration*

It has been recorded (Hinton, 1953) that larvae stored for 3 years and 3 months in dry mud at room humidities (40-80 % R. H.) and temperatures produced normal adults when placed in water. Since that time normal adults have been produced from material stored in a similar way for 5 years.

The results of attempts to activate larvae that had been stored at room temperatures for 7 and 10 years respectively in the dry mud in which they were collected in the field are shown in Table 1. Some of the larvae made a temporary recovery, but none survived the prolonged dehydration. Because many movements of the body may be due entirely to the mechanical effects of absorbing water rapidly, larvae were considered to have recovered after prolonged dehydration only if after immersion in water rhythmical waves of contraction were established in the pharynx or dorsal vessel or both, or, if in the absence of either of these signs of life, the larvae responded to tactile stimulation by making distinct avoiding movements. As previously noted (Hinton, 1960a), larvae were immersed only in small groups, usually 5 or 6, because it is very difficult to observe more than this number at the same time. Many of the entries in Table 1 are therefore the sums of a number of experiments.

In the first experiments all of the larvae that had been stored for the 7- and 10-year periods failed to recover in tap water. Various saline solutions up to a pressure of 8 atm were then tried without success. Small amounts of ATP up to 25 μg/ml were added to the water or to the saline, but the larvae still did not recover. Most of the supply of the 7- and 10-year old larvae was used before it was discovered that exposure to a relative humidity of about 100 % for a few hours before immersing them directly into water or saline ensured a high percentage of recovery.

TABLE 1

Recovery of larvae stored at room temperature for periods of 7 and 10 years respectively (after Hinton)

years dry	relative humidity during storage (%)	hours at 100 % r.h. before immersion in water	number of larvae	number recovered
7	40-80	0	120	0
7	40-80	3	10	1(*)
10	40-80	0	108	2(*)
10	40-80	4	9	0
10	40-80	3	10	0
10	40-80 (7 years ca. 4 %)	0	24	0
10	40-80 (7 years ca. 4 %)	4	7	6
10	40-80 (7 years ca. 4 %)	3	11	9
10	40-80 (7 years ca. 4 %)	20-24	8	0
(*) recovery doubtful.				

As may be seen in Table 1, no less than 83 % of the larvae dried for 10 years made a temporary recovery when they were exposed to a saturated atmosphere before being immersed in liquid. Most of the larvae that recovered temporarily after a 10-year dehydration ceased to show any evident signs of life after about 2 hours in water, but the dorsal vessel of one larva was beating regularly a little more than 5 ½ hours after immersion.

The experiments (Table 1) indicate that the capacity of dry inactive larvae to survive prolonged periods of dehydration is greater the drier they are. For instance, from Table 1 it would appear that storage at relative humidities of 40 to 80 % for 7 years is more damaging to them than storage at the same humidities for 3 years followed by a further period of storage for 7 years over calcium chloride. At the end of the experiment the relative humidity in the desiccator containing the calcium chloride had risen to 4 %. But this is the kind of result that might well be expected on general grounds : the chance of damage by adventitious chemistry is reduced as the larvae become drier. It is hoped in due course to report on a large scale experiment now in progress. In this experiment larvae are stored over silica gel in an atmosphere of nitrogen in sealed glass tubes. Every few years some of the tubes are broken and the larvae fed in water for a few days. After this the larvae are dried again and then packed in sealed glass tubes containing silica gel and an atmosphere of nitrogen.

2. *Tolerance to repeated dehydration*

As was noted in an earlier paragraph, at both the beginning and end of the rainy season the pools in which the larvae live may fill with water and dry out several times within a few days. It would therefore appear to be of great selective advantage for the larvae to be able to tolerate alternate hydrations and dehydrations.

Of 12 larvae subjected to alternate hydrations and dehydrations, one survived and metamorphosed after 10 different dehydrations, each separated by an interval in water during which it fed (Hinton, 1960a). Most dehydrations were done by placing the larvae in tubes containing a column of wet mud 5-8 mm high and 5 mm wide. The larvae burrowed in the mud. When the mud appeared to be quite dry, the tube was heated to 56-57 °C for two hours in an electric oven. Survival after this heat treatment was considered to be certain evidence that the larvae were indeed dry because wet larvae are irreversibly injured at 56 °C in about a minute. The 3 mm thickness of glass and dry mud between the larvae and the ambient air in the oven will not have shielded them for more than a small fraction of the two hours to which they were exposed. After each exposure to 56-57 °C, the larvae were washed out of the mud and allowed to feed in tap water in an incubator at 25 °C. After feeding for 1 to 3 or more days, they were transferred to tubes containing wet mud, and the drying treatment was repeated.

The total number of alternate hydrations and dehydrations that the larva will tolerate may perhaps be limited only by the time taken by them to complete their development in the sense that during each hydration they complete a necessary fraction of their total growth.

Larvae will also tolerate a number of partial dehydrations. For instance, it was found in a few experiments that larvae could be got to tolerate up to 9 or 10 partial dehydrations in which their moisture content was allowed to rise in water only up to about 55 per cent before they were then thoroughly dried again (Hinton, 1960a). The selective advantage of being able to tolerate partial dehydration is clear enough when we consider the fact that in nature they will sometimes be exposed to partial and incomplete wetting, especially at the beginning and end of the rainy season.

3. *Tolerance to high temperatures*

When *Polypedilum* larvae are dry they are able to tolerate temperatures far above and far below any they are likely to encounter in their natural environment. This is of course also true of any other organisms (or tissues) that can tolerate more or less total dehydration. It thus follows that to an organism that can tolerate dehydration, which may be of great selective value, accrue capacities that may be of little or no selective value, e.g. tolerance to the temperatures of liquid gasses.

a) *Hydrated, active larvae*

It has been shown (Hinton, 1951, 1952) that active larvae tolerate water temperatures up to 41 °C for continuous periods longer than any they encounter under natural conditions. They do not survive temperatures over 43 °C for more than about 1 hour. It may be noted here that some other species of Chironomidae are more tolerant to high water temperatures than *Polypedilum vanderplanki*. For instance, the larva of *Chironomus tentans* F. has been found living in hot springs at 44.8 °C in Yellowstone National Park (Brues, 1932), and the larva of the fly *Scatella thermarium* Collin (Ephydridae) has been found living at 47.7 °C in hot springs in Iceland (Tuxen, 1944).

b) *Dry larvae*

When the shallow pools in which the larvae live become dry, the larvae remain in their tunnels in the mud and dry out as the mud dries. In the dry season the shade temperatures of some of the localities in which the larvae live may rise to a little more than 40 °C. The surface of a fine soil may be 20° to 30 °C above the shade temperature. The temperature at the surface of the dry mud may therefore be about 70 °C or a little more because of radiation from the rock sides of the pools. Most larvae were found 4 to 8 mm below the surface. There is a steep fall in temperature away from the surface. Assuming a surface temperature of 80 °C, the temperature at 4 mm is 70 °C and at 8 mm it is 60 °C. However, the larvae are probably rarely exposed to such temperatures because the surface of the dry mud is normally protected by a layer of plant debris.

When dry larvae were exposed to the kind of temperatures mentioned in the preceding paragraph, it was found (Hinton, 1951) that 93 per cent survived exposure to 61 °C for 14 hours and 26 per cent survived exposure to 68 °C for 11 hours. It thus appears that the capacity of the dry larvae to tolerate high temperatures is more than adequate to meet the exigencies of their natural habitat.

When *Polypedilum* larvae are thoroughly dried they may be exposed to remarkably high temperatures and when later placed in water will nevertheless make a temporary recovery sufficient to enable them to crawl about and feed or attempt to feed. For instance, some of the larvae exposed to 106 °C for 3 hours, 126-130 °C for 21 minutes, and 199-200 °C for 5 minutes made a temporary recovery (Hinton, 1951). In one experiment, 2 of 8 larvae exposed to 199-200 °C for 5 minutes made a temporary recovery. One of these lived for 21 hours. During this time it would respond readily to touch by making avoiding movements. It is thus clear that at least some of the sensory-motor pathways in the central nervous system remained intact, evidence that no very gross damage was done to the ganglia, nerves, or sensory end organs by exposure to 200 °C for 5 minutes.

Although all larvae were irreversibly damaged by exposure when dry to temperatures of considerably more than 100 °C, they nevertheless recovered temporarily. It seemed possible that some larvae might not be irreversibly damaged when exposed for every brief periods to temperatures of just over 100 °C. In Table 2 it may be seen that 3 out of 30 larvae exposed to 102-104 °C for 1 minute metamorphosed and produced apparently normal adults, although two of the three were unable successfully to shed their pupal cuticles.

TABLE 2

Temporary recovery and survival of larvae exposed to temperatures above 100 °C
(after Hinton)

preheating for 10 min (°C)	temperature (°C)	time (min)	number of larvae	% recovery	greatest life (h)	number metamorphosed
55-70	102-104	1	25	100	-	2
55-70	103-104	1	5	100	-	1
55-70	104	5	24	100	31	0
57	103-105	5	12	100	41	0
57	104-105	5	6	100	6	0
65	106	5	8	100	5	0
57	102-106	10	6	100	10	0
57	106	60	80	99	24	0

Temporary recovery of large fragments of larvae after exposure to temperatures of over 100 °C seems to be as good as that of whole larvae. The pieces are judged to have recovered if a regular beat is established even for a short time in the pharynx or in the dorsal vessel. In some instances responses to tactile stimuli could be elicited from the larval fragments.

In one series of experiments, pieces consisting of the head, thorax, and up to the first five abdominal segments recovered temporarily after they were heated when dry to 102-104 °C. Pieces consisting of abdominal segments 3-10 or 5-10 also recovered after prolonged cryptobiosis and heating for one minute at 102-104 °C. The fact that *Polypedilum* larvae that have been cut into half or into smaller pieces when dry and kept for a year or more will recover temporarily when placed in water is what we might well have expected from an organism in a state of cryptobiosis : in such a state mechanical and other injuries are tolerated that are absolutely incompatible with the requirements of the larva when in a hydrated state.

4. *Tolerance to low temperatures*

Dry larvae with a moisture content of as much as 8 per cent will tolerate immersion in liquid air (— 190 °C) or liquid helium (— 270 °C) and subsequently produce normal adults. A dry larva can thus be exposed to a temperature range of 372 °C and still produce an apparently normal adult. It will live for a short time but will not metamorphose after exposure to a temperature range of 470 °C.

The behaviour pattern of the larva is the same before and after a period when its metabolism has been brought reversibly to a standstill by a high degree of dehydration or by a combination of dehydration and very low (— 270 °C) or very high (+ 102-104 °C) temperatures. This can only mean that the factors that determine the responses of the active (non-cryptobiotic) animal to its environment are at some level purely structural and are not dependent for their preservation on any activity between cells or between the constituents of cells.

5. *Survival in pure alcohol and glycerol*

At one time methods of transporting dry larvae that had been picked out of the mud were investigated. Such larvae are easily abraided and otherwise injured, and because in a cryptobiotic state all injuries are strictly cumulative, a high percentage of them died when subsequently placed in water. It seemed that mechanical injuries could be reduced by transporting them in a dehydrated liquid.

The first attempt to preserve larvae in a dehydrated liquid was made with alcohol. The larvae were first dried over silica gel at 25 °C until their moisture content was reduced to 3 or 4 per cent. They were then immersed directly in ethanol. After the larvae were removed from the alcohol they were dried for several hours to as much as a day before being immersed in water. As may be seen from Table 3, dry larvae will recover and metamorphose after 24 hours in absolute ethanol, but they are irreversibly damaged if the ethanol is hydrated : none recover even temporarily after 24 hours in 90 per cent ethanol although in two experiments all larvae recovered temporarily after immersion for a week in absolute ethanol. Resistance of the dry larvae to ethanol does not depend upon the failure of the ethanol to penetrate through the body wall : there appeared to be no significant difference between the recovery rates of fragments of larvae and whole larvae (Hinton, 1960a). For instance, in one experiment a fragment of a dry larva consisting only of the head, thorax, and half of the first abdominal segment was immersed in absolute ethanol for

TABLE 3

Temporary recovery and survival of dry larvae for various periods in different concentrations of ethanol (18 to 20 °C) (after Hinton)

% ethanol	time (h)	number of larvae	% recovery	greatest life (h)	number metamorphosed
100	24	5	100	-	1
100	24	3	100	576	0
100	24	8	100	> 27	0
100	24	2	100	> 22	0
100	24	2	100	> 10	0
100	24	6	83	< 12	0
100	24	7	29	< 5	0
100	48	7	100	> 51	0
100	48	11	54	< 41	0
100	168	4	100	> 55	0
100	168	6	100	> 31	0
95	24	5	100	> 6	0
90	24	12	0	0	0

24 hours. After this treatment, the fragment was dried and then immersed in water. It responded readily to touch from the 17 th to the 30 th minute after immersion in water.

TABLE 4

Temporary recovery and survival of dry larvae immersed in pure glycerol for periods of 1 to 7 days (18 to 20 °C) (after Hinton)

time (h)	number of larvae	% recovery	greatest life (h)	number metamorphosed
24	4	100	-	2
67	6	100	-	1
168	12	100	-	1
168	11	100	> 96	0
168	10	20	> 10	0

The experiments in which pure glycerol was used as a preservative are shown in Table 4. The moisture content was reduced to 4 per cent or a little less before they were immersed in glycerol. In one experiment with 11 specimens (Table 4), however the moisture content of the larvae had not been reduced below 8 per cent before immersion in glycerol. Two of four larvae that had been immersed in pure glycerol for 24 hours eventually metamorphosed, whereas in another experiment 1 of 12 larvae kept in glycerol for 168 hours later metamorphosed.

Cryptobiosis and the origin of life

The capacity to tolerate total dehydration at developmental temperatures appears to be a primitive feature of living systems (Hinton, 1968b). This capacity appears to be retained by most if not all recent acellular plants and animals. It is also retained by some of the tissues of complex multicellular animals that as whole organisms are irreversibly damaged if they lose an appreciable part of their water.

In order clearly to understand the evolution of a particular kind of organism, it is necessary to understand the characteristic features of the environment in which it originated. This is a necessary consequence of the fact that the specific features of any organism are determined by the selective pressures to which it is subjected by the nature of its environment. In speculations about the origin of the first living systems it is no less important to define the precise nature of the environment in which they are supposed to have been evolved.

When the origin of the first living systems is considered in the light of what we now know about the cryptobiotic state, many difficulties that previously appeared to be intractable cease to be of serious concern. It is, for instance, possible for the first time to visualize an enormously wide range of terrestrial environments as possible sites for the origin of life, environments previously excluded on the ground that they were never wet for continuous periods of sufficient length. The land provides an environment of a physical and chemical diversity incomparably greater than the sea and thus one very much more likely to have provided just the right conditions for the abiogenic synthesis of the first living systems.

It is generally believed that some of the organic compounds from which the first living systems originated were formed abiogenically in the atmosphere. Such compounds would fall into the sea and on to the land. Those falling on land would be subjected to alternate wetting and drying. The frequency with which they were wetted and dried would depend upon how close they remained to the surface. For instance, compounds that came to rest a few centimetres below the surface of the soil might be in a more or less permanently saturated atmosphere in many regions of the earth. Suggestions that life could have originated on land or in small pools have been discounted by Bernal and others on the grounds that such environments are apt to dry out. But, as we have seen, alternate hydration and dehydration is not only tolerated by large numbers of primitive organisms but it is the normal way of life of many.

There is no theoretical or other reason to suppose that the complex organic compounds from which life arose were not subjected to periods of dehydration, or to suppose that such dehydrations were necessarily damaging. In short, there seems to be no reason why occasional dehydrations should adversely affect the abiogenic synthesis of the first living systems.

The land provides innumerable environmental niches, such as crevices in dust particles and rocks, that are insulated against damage by ultraviolet and high energy radiation. I have previously drawn attention to the fact that the alternate flooding and drying that is characteristic of many terrestrial niches may have provided ideal conditions for the abiogenic synthesis of organic compounds. Drying in such niches would permit high concentrations of chemicals to occur, and this would favour chemical reactions when they were wet. During evaporation the dissolved materials become progressively more concentrated and therefore chemical reactions are more likely to occur.

In contrast to terrestrial niches, chemicals in the ocean would be too dilute. The formation of coacervates from the agglomeration of materials has been invoked by Oparin (e.g. 1961) and many others to explain how large molecules could be brought together in a dilute medium, but it is not easy to visualize the formation of the large molecules themselves except in a concentrated solution.

The synthesis of proteins from amino acids in an aqueous solution is not a reasonable possibility, as has been pointed out by Dixon and Webb (1964). The amino acid units will not spontaneously polymerize in the presence of water. The initial reaction would be :

$$\text{amino acid (1)} + \text{amino acid (2)} \rightarrow H_2O + \text{peptides}$$

To produce one mole of linked amino acids requires 2000 to 4000 calories. Water in the medium tends to drive the reaction to the left, and in equilibrium the process would favour the breakdown of protein. According to the calculations of Dixon and Webb, in order to make a protein molecule of a molecular weight of 12,000 would require a volume of amino acids at 1 molar concentration equivalent to 10^{50} times the volume of the earth. Amino acids in an aqueous solution below the boiling point of water would yield very small amounts of peptides without some external source of energy. However, at temperatures above the boiling point of water, so that water is removed as the reaction continues, the formation of peptides does not present the same difficulty :

$$\text{amino acid (1)} + \text{amino acid (2)} \rightleftarrows H_2O \uparrow \text{(gas)} + 2 \text{ (linked) amino acids}$$

The higher temperature supplies the energy needed to form the chemical bond between the two amino acids, and it also removes the water as a gas and so prevents the reaction going to the left. It has been shown by others that when all types of amino acids are heated together above the boiling point of water, yields of proteinoids of up to 40 % are produced that contain all of the amino acids found in protein.

It has previously been pointed out (Hinton, 1966, 1968b; Hinton & Blum, 1965) that among the factors that would favour the synthesis of complex organic compounds in small and sheltered terrestrial niches are : (1) the highly concentrated solutions that are possible of both organic and inorganic compounds; (2) the enormous variation in the composition of the available solutions — every niche could have a solution of a different composition; and (3) the wide range of acidity or alkalinity.

If these minute collections of organic compounds subsequently dried out, they would not be lost but they would be available again when wetted. During periods when they were dry the organic compounds could be blown about and so accumulate in different combinations and proportions in the terrestrial niches. Thus a far greater variety of reacting systems could be obtained than would be possible in a continuously wet environment. Synthesis of the increasingly complex chemicals required to form the first living systems may well have occurred in a series of discontinuous steps.

Dehydration permits a qualitative break to occur in any reaction system but does not necessarily impose limits of space or time on the potential reactions of the dry particles when they are again wetted. A dry organic particle may thus undergo further chemical reactions long after it was formed and be available to react with compounds that were synthesized long after is own genesis.

Experiments with organisms in a cryptobiotic state show us that life can be reduced to a purely morphological state in which it is only necessary that certain spatial relations be preserved. This suggests the possibility of the spontaneous generation of a living system that was not necessarily evolved step by step from less complex systems. It thus becomes easier to imagine the qualitative break between living and non-living systems, and it becomes possible to visualize spontaneous generation producing a living system with some independence from its immediate environment.

My theory of the origin of life has been criticised by Bernal (1967) who says that it is most unlikely that life could have originated in "rapidly drying up cracks in rocks". However, I do not postulate "rapidly drying up" cracks. The surface of the land is so varied that almost any condition of humidity will obtain for long periods in one region or another. But even in regions that are often dry for long periods, the dryness does not necessarily extend in depth : the air spaces between the particles of earth may be more or less permanently saturated a short distance below the surface.

The first living systems formed near the top surface of the soil would be washed to lower levels in the soil where the humidity is much more constantly at a high level. Some of them would be washed into the sea. Both the soil below the surface and the sea provide less difficult and more stable environments where the further evolution of organisms produced spontaneously near or at the top surface of the soil may have taken place.

The asymmetry (L-amino acids and D-sugars) of all living organisms known today suggests a single origin. When the first living systems arose spontaneously about 3×10^9 million years ago there was but a single land mass, and the oceans were much less extensive. It is difficult to believe that the conditions necessary for the origin of the first living systems were not present for a very long period over an extensive area of the surface of the earth, at least within certain latitudes. For this reason it becomes difficult to believe that living systems were not produced independently on many occasions. In other words, it is hard to accept the proposition, which has become almost axiomatic by constant repetition, that when conditions were suitable for the abiogenetic origin of a living system, the origin of such a system was a unique event. There is no evidence that the origin of life was an improbable event: it happened and therefore the only evidence we have suggests that it was probable. The view that there was a multiple or polyphyletic origin of living systems can be held if it be assumed that those with the particular asymmetry that is now universal eliminated all other systems by competition, i.e. by eating, starving, poisoning, or crowding them out of existence.

Although competition and other density dependent factors play an important role in determining population densities at certain levels, density independent factors, such as climate, are primarily responsible for population densities except in certain special instances (Hinton, 1957 b). After all, in all parts of the world today many species that compete with each other live in the same area and have done so for millions of years. If interspecific competition is not primarily responsible for population densities and the number of species that can coexist in any region, competition cannot be evoked to account for the particular asymmetry of living systems as was done by Haldane and many others, e.g. Frank (1953). Nevertheless, the predominance of one of the two possible isomers in living systems suggests a monophyletic origin unless it can be shown that some natural event favoured the preservation of one of the two isomers.

The kinetics of the chemical reactions of laevorotary and dextrorotary amino acids, as well as racemic mixtures of amino acids, are the same from a thermodynamic point of view. Long ago Van't Hoff and others thought that circularly polarized light could explain the optical asymmetry of organisms, but the source of the circularly polarized light in nature was unknown. However, the experiments of Garay (1968) have shown that when racemic mixtures of alanine, tryptophan, and tyrosine in an alkaline solution are treated with β-particles of strontium^{90} yttrium (in equilibrium) the D isomers decompose significantly more quickly than the L isomers. When strontium^{90} decays the β-particles are polarized, even if the nuclei are unpolarized, and the beta bremsstrahlung are circularly polarized. It thus seems possible that β-particle decay from strontium^{90} or even from phosphorus^{32} or potassium^{40} may have led to the selection of L-amino acids from the abiogenetically produced racemic mixtures. The selection of L-amino acids by circularly polarized radiation might also account for the predominance of D-sugars if nucleic acids with D-sugars unite with proteins more easily than those with L-sugars.

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DISCUSSIONS

K. MENDELSSOHN : Can we start on the discussion please. Before entering the general discussion may I suggest that first of all we deal with contributions that have direct bearing on what has been said by the speakers in the earlier part of the session. I like to call on Professor PATTEE who will make a comment on the introduction.

H.H. PATTEE : There are two main physical ideas presented at this conference which are attempts to find, if you like, the " physical basis of life ". These are the ideas of *long-range quantum mechanical correlations,* and the idea of *non-equilibrium statistical processes.* If these physical concepts are to be productive in biology, then I believe that physicists must not develop them in isolation, but must take into full account all the " facts of life " as known by today's molecular biologists.

I will make two statements which I won't have time to fully justify here (See e.g., Pattee, H. The Vital Statistics of Quantum Dynamics in *Thermodynamics and the Origin of Life,* O. Reynolds, C. Tobias, and G. Oster, eds., Academic Press, New York, 1970. Also Longuet-Higgins, C. What Biology is About in *Towards a Theoretical Biology Vol. 2,* C. H. Waddington, ed., Edinburgh University Press, 1969, p. 227). First, it is my feeling that the bulk of molecular genetics and development (including the evidence for cryptobiosis that Dr. Hinton has just discussed) shows that the living system is not adequately described as a dynamical system in the sense of elementary physics in which all particles follow equations of motion in real physical time. Living systems appear to be memory-controlled growth processes, or in the language of automata theory memory controlled sequential machines which do not operate *functionally* in real physical time. That is, although living systems clearly have a detailed embodiment in real physical time, they perform all their biological *functions* in their own sequential order under the programs in the genetic memory.

The second statement is that molecular biologists have shown that the basic elements which record, transcribe, and control these sequential programs are *single* molecules — the enzymes and nucleic acids. These single molecules correspond in some ways to the switching elements in a computer. Physical theory is essential for understanding the speed and reliability of these switches, but once we assume their *function* as switches, we no longer use physical description, but speak of the logic of networks and the languages in which programs can be written.

If there are crucial quantum mechanical or statistical properties, which I believe there are (Pattee, H., *J. Theoretical Biol.,* 17, 1967), it follows that we should expect these properties in single enzyme or nucleic acid molecules. While there may be longer range quantum effects, as Pr. Fröhlich has suggested, there is at present no biological necessity for them that I know.

Now with regard to non-linear statistical processes, again there is no doubt that the cell is a highly non-linear, non-equilibrium aggregation of matter. But the program control is still executed by single molecules, and while we can often derive statistical laws without knowing the details, it is impossible to derive the motions of single molecules from their statistics. Therefore, I do not think non-linear statistical theory is as relevant to collections of molecules as to the behavior of single macro-molecules. These single molecules, such as the enzymes, which can be turned on or off by smaller molecules, or DNA which can be locally turned on or off depending on what gene the cell needs to transcribe, must in some sense be *dissipative* structures. In fact for switching processes in general, the strongest dissipation is needed for the fastest and most reliable switches. Therefore, it is in the operation of these *molecular switches* that non-linear statistics must be used for explanations.

In other words, I am saying that basic physics in the form of laws of motion and laws of statistics are most applicable to living systems, as well as computers, only at the level of the smallest control or switching elements. Once the *function* of these elements is assumed, then it is more useful to use a new level of language to describe how networks of these elements combine to perform higher level functions.

Now it is my own strategy to study these relations of physics to biology at their confluence, so to speak, that is, at the origin of life. After all, every biological object that we now study, has a programmed memory accumulated over some three or four billion years, which is very accurately recorded in the gene and which, as I said, is the essential difference between living and non-living systems. I have little doubt that the memory control systems obey physical laws in all details, though this is far from demonstrated. The point is however, that there is a clear *difference* between living and non-living matter. You can all see this, I hope very easily, since this is the fundamental empirical fact which stimulates our curiosity. And if the origin of life is to be explained, from the physical point of view, a reason for this difference must be provided in clear, physical terms.

So one way of looking at the problem of the relation of physics to biology is to try to state, in terms of physics, what the difference is between living matter and non-living matter. Unless this is stated explicitly, one does not know the physical basis of life. Once one creates a memory control system which has the feature of universality, like a big computer, it can do almost anything. This is, in one way, the property of life which is so amazing. Once it has the code, genetic recording and readout, and developmental subroutines to generate multicellular organisms, it can do virtually anything. I have suggested what the physicist has to take into account about the facts of life to usefully apply physical principles. He has to accept and explain this molecular machinery which executes autonomous control of the rates of the chemical reactions.

The problem of the origin of life also poses very serious metaphysical problems (i.e., problems of interpretation of laws). A physicist, when he looks at things in terms of first principles, sees that all detailed motions are inescapable. There is no arbitrariness about the microscopic dynamical processes which govern matter. Pr. Wigner expresses this by saying that events can happen only the way they do. There is no alternative possible because the laws of motion exclude contingencies.

Along with these laws there are what we call the *initial conditions,* which are in fact the records of measurements that we put into these laws of motion to predict what is going to happen. And because the physicist works with simple things like one and two body problems, and sometimes a few more, it is possible to use these records in dynamical equations to get answers as long as you stay simple. But most of the world is more complicated, and so there has grown up statistical laws are virtually independent of the initial conditions. And the statistical laws, whether they are equilibrium or non-equilibrium, are still basically the type of law you get when you do not allow detailed records about initial conditions.

To put it another way, in order to have statistical laws at all, you must have a very special additional principle — a " principle of ignorance " of initial conditions — otherwise you have complete dynamical order. Now this raises the basic problem I think. We speak about order in biological systems as being exceptional — a characteristic of living matter. In fact, that is how we recognize biological systems.

The order in biological systems in the course of evolution grows in complexity in a way which the order in non-living matter does not. And yet if you look at the dynamical laws of motion, the picture you get of the world is that of total, inexorable order. There is no opportunity available for electrons or atoms or cells to evade these laws of motion.

On the other hand if you use statistical description, in so far as this statistical description results from a lack of detailed records about the system, the time evolution of the statistical system results in decreasing order in the course of time (See e.g., Born, M. *J. Physique et le Radium 20,* 43, 1959).

Now the only way to make sense of the increasing complexity in living matter in the course of evolution is to say that living systems, to increase their order, must *accumulate records* in the course of time. This is precisely what they do. The biological memory is an accumulation of genetic records gained by natural selection. But the mystery of the origin of life is just this: Why should some matter begin making records ? Why should matter record its own interactions ? What laws of physics tell us that this will happen ?

I cannot answer these questions clearly, but I believe I can state somewhat imprecisely what is needed. One of the universal characteristics of life is that collections of elements generate constraints which limit the freedom of individual elements; for example, the enzyme molecule is a collection of bonds which constrain the reaction of single bonds. The individual is a collection of cells which, in development, controls the replication and genetic expression of single cells. And of course a community of individuals establishes constraints on the freedom of each individual. This is generally called *hierarchical control.* (See e.g., Pattee, H. The Physical Basis and Limits of Hierarchical Control in *Biological Hierarchies,* H. Pattee, ed., Gordon and Breach, 1970). This is not the situation in most physical systems we study. The individual molecules in a gas produce a collective pressure, but we do not speak of the pressure constraining the individual molecules. Most statistical systems in physics are chosen for simplicity, but this simplicity is not used to control individuals within the system. On the other hand, we see in all biological behavior a kind of *simple result* of *complicated dynamics*. This is what we see in all machines, particularly in switches which have very simple behavior even though their detailed dynamics is very complex. This simple behavior we call the function.

The concept of function seems to be inseparable from life — and at the same time it has no direct relation to normal physics. I believe the smallest natural functioning unit is the enzyme. Its function can be expressed quite simply, but its physical dynamics is too complex to imagine in terms of solving its equations of motion. How does such simplicity come about from dynamical complexity ? It must involve statistical or dissipative processes, but at the same time it must be exceptionally reliable in its simple function. It is in understanding the behaviour of single molecules like the enzyme that I believe both quantum and dissipative processes must play a crucial role.

To summarize : I would expect that there are crucial non-linear statistical dissipative processes in cells that function as constraints on selected microscopic degrees of freedom in single molecules like the enzyme. In other words the enzyme is the essential elementary machine of life which must be explained. The enzyme recognizes substrates and activator molecules which act as switches to turn on or off selected bond formation. I believe that the concept of a longer range order has no questions to answer once one assumes a memory controlled sequential switching system, because we know that such a system is sufficiently rich to generate or develop the kind of order that one sees in highly evolved biological systems. Similarly if there is to be any use for the idea of coherence I believe it must be in individual enzyme molecules. I would say any molecule which acts only after performing a classification process, and which must reset itself after each selective event, and does this at high speed for billions of cycles without failure is a candidate for a coherent quantum process.

K. MENDELSSOHN : Thank you very much Dr. Pattee.

Are there any other contributions to the general ideas about order and living patterns ?

G. CARERI : Dr. Pattee, could you please explain better what you mean " to understand an enzyme " ?

H.H. PATTEE : I would mean " to understand an enzyme ", first of all, to understand the dynamical and statistical events that are significant to explain specificity and speed in enzymes, as well as what we would generally call their persistent reliability, in terms of first principles. Now I certainly do not mean that you have to solve in detail the equation of motion. I only mean that one must have at least a model of a structure that size, that is, few hundred angstroms across with some 10^5-10^6 degrees of freedom, and be able to show, at least order magnitude, how the speed and specificity and the number of times that it operates without failure is consistent with basic laws. This would be one level of understanding.

Below that on a very much more mysterious level, one must explain how such molecular machines spontaneously originate. It's not enough to produce just one enzyme. The whole problem is that one enzyme, or in general, as many enzymes as you want, will not produce anything except a faster approach to equilibrium. That is not life. A genetic memory or program can only be written in a *definite language,* and that means a set of enzymes with very special interrelations. The key problem is to understand *integrated systems* of enzymes or selective catalytic molecules which function as a language or a code.

I believe this requires a threshold of complexity, or the first level of *hierarchical control,* which allows the evolution of matter in a path we recognize as living (See e.g., Pattee, H. How Does a Molecule Become a Message ? in *Dev. Biol. Suppl.,* **3**, 1, Academic Press, New York, 1969).

H. FRÖHLICH : The misunderstanding between what we have been saying this morning and what Pr. Prigogine is going to say to-morrow afternoon, and Dr. Pattee arises, because what he says physicists have not done and should do, they actually have done. You know there are a lot of physicists here who do statistical mechanics and have done it for a long time and what you call dynamics is the mechanics of the dynamics of individual particles. We have not been speaking of this. We have been speaking of many-body problems which are very complicated and we have shown, and Dr. Prigogine is going to show to you again, in considerable detail, that, in spite of all the complications, once energy streams through the system, the usual concepts of decreasing order do not hold but that a new order can arise. Tomorrow afternoon in Pr. Prigogine's talk, who I think is not here now, the same problem will again be treated from some specific point of view, different from the one I treated this morning.

Thank you.

H. H. PATTEE : All I can say is that I agree that we get order of this type. I am proposing as a hypothesis that this order must be reflected in single molecules such as the sequence of a protein or a nucleic acid before it is relevent to what we know about life.

Thank you.

S. LIFSON : It is very interesting to ponder about momentum correlations and life. But for some of the biologists, and even for some of the molecular physicists, it will need a little more than what we have learnt until now in order to stimulate further thinking. The same, I think, is true with respect to the idea of collective hydrogen-bond energy transfer. I could not get exactly the answer to the question by Professor Hodgkin, which I wish to state somewhat differently.

If it is true that the observations of infrared in the region of about one hundred wave numbers are due to collective effects in hydrogen bonding then there is a crucial test to determine it. We know that different proteins have a very different content of α-helix, therefore we should see pronounced differences between different proteins; if you say it was not observed, is it not a crucial evidence against your suggestion? On the other hand, I would say that as long as it was not proven that local or short-range interactions cannot explain the function and structure of proteins, this kind of correlation should also be followed without prejudice.

If you take twenty amino-acids and create chains of 300 units each, you get 20^{300} different possibilities; this may be, perhaps, more than the number of electrons in the universe. Therefore, when we look at real proteins we observe a very biased sample. Therefore, statistical mechanical calculations of order-disorder equilibria in proteins based on random sequencing, would make little sense. Indeed, it has been proven both theoretically and by computer calculations, that random sequences of even two different amino-acids only, have a shallow order-disorder transition as a function of temperature. Denaturation of proteins, on the other hand, is a sharp temperature transition, so it is evidently in contradiction with the concept of randomness. This is because among the practically infinite number of different sequences of amino-acids, those who became the object of our study are those who have been selected by natural selection through billions of years. Therefore, proteins are systems which obey all the laws of chemistry and physics except that they are particular, being selected by evolution in a way which made them persist and therefore become the objects of our observations. I think generalities are therefore somewhat dangerous in this field. What we have to do is to try to understand at least one single protein, and then another one, fully. We might then realise whether they do have in common a collective hydrogen bonding or not. They might; they might not. I think this is still an open question.

Now, to understand any single protein thoroughly, one has to know in addition to the nature of the hydrogen bonds also the effect of other intramolecular, or interatomic and interorbital interactions. Such a study of the relation between protein structure and intramolecular forces is now in progress in our group, and is closely linked with the study of similar interactions in simpler molecules which are composed of the same groups of atoms, like molecules with aliphatic chains, amide groups, etc. We try to find the appropriate energy functions from which we may calculate simultaneously the thermodynamics of such molecules, their molecular equilibrium conformations, their crystal structure, molecular and crystal lattice vibrations, heats of sublimation, and other observable properties. This is evidently a long-range programme, but the results hitherto obtained are quite encouraging, and it is hoped that within a few years one may have a more quantitative understanding of the relative importance of hydrogen bonding, Van-der-Vaals forces and protein-solvent interactions, which together determine the physico-chemical properties and biological functions of proteins.

K. MENDELSSOHN : Thank you very much Professor Lifson. Since some of your remarks were clearly in response to what I said, in the introduction, I hope the meeting will permit me two minutes to answer these particular points.

Life is just not random. It's just the opposite from any random process to a most remarkable degree. The question is what are the selection rules that make the rather limited number of molecules which bear the relevant features. Now first of all, I should like to say that momentum condensates, which I mentionned, are real physical system. That we know not enough about them is another matter. If we talk about long-range correlations then they are just a possible solution of this problem. We know a good deal about interatomic forces. They are features to which quantum mechanics has been applicable and you can explain for instance how atoms are bound together in the metal by exchange mechanisms. You have ionic bonding and you can have resonance forces holding atoms together. These are all different forms of the "Coulomb" interaction. However, they essentially describe effects of nearest neighbours upon each other. The question which I think is central to it all is how can we expain certain specific given configurations in large molecules without making vague assumptions. What I have done is an attempt to invoke another well-known phenomenon namely, such collective electron modes, as known in the superfluids which extend over a whole system. I would think that it's likely that either the whole protein molecule, or more likely bits of it would appear as resonant states of some particular electron wave functions. These resonances must be few and far between. Let me close with reference to work which has, in fact, been coming from a completely different field namely, the wish to produce superconductive electro-magnetic machinery at room temperature which certainly would be a highly desiderable economic proposition. For this purpose, people have been looking at long organic chains. The original idea was given by Fritz London at the same time when he investigated the interatomic forces, pointing out, the fact, that in the benzene ring where you have 6 carbon atoms, the electrons are shared completely by all 6 atoms. We now have to look for states in which these correlations extend to very much greater units. One would suspect straight away that the only possible selected configurations will be those which correspond to such correlations of sharing the electrons, probably as resonance states of electron pairs.

G. CARERI : The hydrogen bonds of the α-helics are not the only ones, there are several other groups which are bound by H-bonding as well. I suggest that the broad band arises from coupling among all the soft modes, as a result of the interaction of several levels. In this sense I speak of collective effects of the Hydrogen bonding.

K. MENDELSSOHN : I would like to make sure that the other parts of this afternoon's papers are being discussed as well. So may I ask the speakers to take up these points now.

W. FAIRBANK : There are two parts of physics, experimental and theoretical. We have been talking theoretically about long range order in momentum space with respect to Biological molecular this is a very difficult theoretical problem. The experimental question is : Do cooperative currents really flow in these molecules and how can they be observed ? One possible type of cooperative currents are represented by oscillations. This is what Careri is looking for in the for infra-red. Another type of momentum ordering would manifest itself in quantized circulating currents. This could be observed by their magnetic effects. If they are characteristic only of living systems then one is forced to look at living systems in all their magnetic complexity. One has large amounts of water and possibly small amounts of the particular molecules which exhibit the magnetic currents. Electron resonance is difficult and may not be applicable. One other possibility is to look at the D.C. magnetic susceptibility. Low temperature research has produced two important new experimental techniques which make this possible, one is perfect magnetic shelding and the other is a very sensitive magnetometer. It appears possible, for example, to measure 10^{-8} of the diamagnetism of water in a field of 50 kilograms. If one applies the time resolution techniques of Eigen with the addition of magnetic field measuring equipment it appears possible to measure 10^{-5} of the diamagnetism of water in a field of 50 kilograms in 1 micro second. Of course one can use lower magnetic fields with appropriately lower sensitivity. One can imagine a very exhaustive search to see if there are any interesting magnetic changes when a living cell is changed or destroyed or in an enzyme reaction stopped in a micro second. If one discovers long range currents which flow in living systems then one can begin to explore their significance. We are building such an apparatus. For us it is an interesting physics problem to see if the kind of long range momentum ordering we see in quantezed flux in superconductors exists anywhere else in physics, for example in a living system. It is, I am sure, a very difficult experimental problem.

H. HAKEN : I would like to comment on the collective excitation. You may have excitations which are not connected with charge transfer. There are the excitons in molecular crystals or other crystals predicted almost 40 years ago by Frenkel in Russia. When you excite atoms or molecules in a crystal, it turns out that energy is transferred all over the crystal by exchange coupling and other mechanisms. So you have even in usual physics long range phenomena connected with local excitations with no charge transfer. Perhaps this may be of interest also for big molecules.

A. FESSARD : I am a layman in molecular biochemistry and in internal molecular forces, and I would put questions that may seem naive. I think the remark made by our colleague, according to which enzymes usually exert stable and simple functions in comparison with their complicated dynamics is a very pertinent one. What makes this function stable ? Couldn't it be that the final effects of enzymes upon their environment is such that in many cases, they exert a feed-back control on the enzymes themselves, so that these would appear as having a well-regulated, stable and "purposeful" behavior ? This might be the reason why some enzymes have been selected by Nature, because after all the selection should imply usefulness and efficacy and in the case of those that had not been endowed with a specific vital function we perfectly understand that they were rejected. This selection you have mentioned should be considered not only in relation to the internal forces between the atoms inside the molecules but also to the environment, that counts above all with regard to the work an enzyme has to perform. Couldn't it be the secret of the narrowly selective action Nature seems to have had on active proteins, because these proteins must have been selected according to their usefulness and stability of behavior ?

K. MENDELSSOHN : Now perhaps I should interrupt the discussion and come back later to Professor Fessard's question because at the moment I have two requests for comment on Professor Hinton's paper.

L. F. BELANGER : Professor Mendelssohn I would like to compliment Professor Hinton on this most stimulating talk he gave us on "cryobiosis" and in this respect, I would like to add a few facts he may like to comment upon, facts which I believe bring some light on the subject. I refer to experiments that were made with tissue culture in the United States by Leonard Hayflick; you may be aware of those. Alexis Carrel many years ago, in cultivating samples from the chick-embryo heart over many years thought that life was "immortal" so to speak, in tissue culture. Dr Hayflick has recently shown that this concept was possibly erroneous, due perhaps to contamination of the culture. By carefully performing the "repiquage" he was able to establish that there was a limited time factor in the number of divisions the cells can go through in tissue culture. In the case of chick fibroblasts it is about 50 times. If in a given period of this cycle of life the investigator froze the culture, the cells once they were revived, would go only the remaining number of divisions which they had to go through. Examining this material to understand what may be responsible for this limited life span of single cells, he found that towards the end of their life, these cells actually showed chromosomic aberrations, at least morphological differences in the chromosome morphology. I am asking now : Were your larvae frozen ? If so, what happened when they were revived, especially what happened to the wounded ones ? At any rate, when Hayflick recognized these chromosomic aberrations he thought that may be the machinery of replication and coding got a little tired out at the end — and that actually at that time, the coding was less and perfect. Would you kindly comment on these observations.

M. U. PALMA : I have two questions :

1) I wonder if the following experiment can be done (or has already been done) : let us think of a suitable species of insects, exhibiting some neatly defined collective behaviour. Let us suspend the life of some larvae and then let us wait for (or provoke) some slight mutation in that species, causing a definite change in the particular collective behaviour concerned.

Let us now bring back to life the "preserved" larvae and let them grow together with the others.

I wonder if and in which way the "preserved" larvae would pick or react to the new collective behaviour. Has any experiment been done along this line ?

2) I wonder if an effect of slowing down (if not of suspending) vital processes, say metabolism, is obtained if, instead of removing the normal water,

one substitutes it with heavy water. The step (if any) could be also in this case reversible ?

P. O. LÖWDIN : If I understood your beautiful paper correctly, biological aging should go parallel with the metabolism. We have been working on the phenomena of aging in Florida and in Uppsala with a general idea that it has to do with the corrosion of DNA through the transcription process, very much in agreement with the idea exposed before by Pr. Pattee, here. Then aging of the human being would be connected with the phenomena that in a brain cell, for instance, the genetic code is slowly corroded by reading. I have been told that we have got most of our brain when we are eight years old, and then the only thing that happens to the brain cells, is that they may lose the information contained in the DNA, and die. It is remarkable that, fortunately, we do not get offsprings which are born old. For some reason, the gamates, the sperma and the ova, are set aside in a state where the metabolism is kept very low or negligible, and they are put into full metabolism only shortly before being used.

K. MENDELSSOHN : Thank you very much. Pr. Hinton, would you be kind enough to reply to this ?

H. E. HINTON : My first questioner, Professor Belanger, has pointed out that in a degenerating colony of cells in tissue culture, the processes of degeneration can be arrested by freezing. However, once freezing ends, the processes of degeneration begin again. There was, as we would have expected, no reversal of the process of degeneration but merely a temporary halt during the actual period of freezing.

In one sense my fly larva responds in the same way to dehydration. All processes that increase its biological age are arrested by dehydration. For instance, if we experiment with a larva that, let us say, will become mature in 36 days at 35 °C, this time can be interrupted as much as one likes by intercalating periods of dehydration. Nevertheless, the sum total duration of the active period of the larva will not exceed 36 days although its calendar age has been greatly extended. In other words, there seems to be a built-in clock in the larva about which one can do nothing. All you can do, and this is the answer to your question, is to stop the clock and so prevent ageing or degeneration. But you cannot increase the amount of time that will be spent in the active state. In short, when the clock is running I cannot extend the period of its running. I am unable to contribute to the interesting comments of Professor Löwdin because all of my experiments on the fly have been done on the larva, which is the only stage that can be induced to enter cryptobiosis by dehydration.

In reply to Professor Palma I should say that I do not know anything about the effects of substituting deuterium for hydrogen in my larva. I certainly have not tried it. I ought to point out that the insects that can be totally dehydrated are large complex animals. But particular tissues of much more advanced animals can sometimes be dehydrated. For instance, some years ago a Soviet scientist, Juscenko, showed that rabbit sperm treated with glycerol and dried in a high vacuum could then be stored at room temperatures for several months and still remain capable of fertilization.

Cryptobiosis can be produced in other ways besides by dehydration. For instance, the larva on which I work will tolerate liquid gasses in the fully hydrated state provided it is frozen very slowly and provided that it is protected by soaking at some stage in dimethyl sulfoxide. Dimethyl sulfoxide in some way protects it from damage when it has been frozen in the hydrated state. I want to point out that a sojourn in liquid helium has no effect on the subsequent behaviour pattern of the larva. When in a state of cryptobiosis the larva may nevertheless be affected by adventitious chemistry, that is, by non-metabolic oxidations and reductions. It has quite correctly been pointed out some time ago by Professor Maynard-Smith that such adventitious oxidations and reductions could cause mutations.

K. MENDELSSOHN : Thank you very much. Before we turn on to Professor Fessard's question, I would like to ask if there are any questions to be asked about Pr. Hodgkin's paper ?

G. CARERI (to Madame Hodgkin) : I would like to ask you about the number of protein structures that are going to be solved in a near future, and about the possibility of further refinement of their crystal structure.

D. HODGKIN : The number of protein structures solved is now increasing quite rapidly : since I wrote my paper for this meeting, two new protein crystal structures have been solved, cytochrome C and a staphyllococcal nuclease. Behind each, however, are several years' work. From the number already being investigated, one would estimate about twenty new crystal structures in five years time and the number might well be greater if more research workers come into the field.

The possibility of refinement of a protein crystal structure beyond the 2 Å limit seems at present to be limited to the rather few protein crystals which give very good X-ray reflections at high angles. Apart from myoglobin, ribonuclease, at present, appears to be the best of those known and refinement of this crystal structure by Kartha and Harker is in progress.

Question. — I would like to ask Mrs Hodgkin to which extent she is saying that the state of the molecule in the cristal state is all through for the state in solution.

D. HODGKIN : I think that protein molecules are not likely to change very extensively in solution; those already studied seem very well composed molecules, already in contact with water molecules within the liquid of crystallisation so that their condition must be largely that of molecules in solution. On the other hand, there are additional degrees of freedom in the solution which are important in their effects on enzyme activity. First the actual movement of substrate through the crystal liquid and approach to the active site may be hindered in the crystal compared with the solution. Second, quite small conformational movements in the neighbourhood of the active site, which are necessary for action, might well be hindered by intermolecular contacts in the crystal. In the ordinary crystalline modification of egg white lysozyme it is physically impossible to get the substrate into the crystal owing to these intermolecular contacts though it is quite easy to fit model substrates into the isolated molecular model.

Question. — The living cell is as near to crystal as to the solution condition so the most interesting question is : is there a difference between the protein in the living cell and in the crystal ?

D. HODGKIN : As I said in my talk, protein crystals do occur in living cells so that it is natural to deduce that protein molecules are essentially the same in the crystals and in the living cell. This is not to say that it is not generally necessary for them to be set free from the restrictions imposed by packing in the crystals and to move more freely to take part in reactions.

K. MENDELSSOHN : Thank you. Now, you remember I had to cut short Pr. Fessard's question in order to get time for the discussion of the other speakers. I wonder if I can now persuade Pr. Fessard to ask once more his question.

A. FESSARD : I would apologize for speaking about questions I don't know very well myself, but I cannot ignore that enzymes perform highly specific actions that make them appear as operating purposefully. I wonder if the narrowly selective action Nature seems to have had on active proteins in the course of evolution is not simply due to the fact that only those enzymes that were stable, and efficacious enough to contribute to the survival of a living being could themselves survive and be retained.

K. MENDELSSOHN : I think Mr. Löwdin will answer this.

P. O. LÖWDIN : No, I won't be able to answer this question directly, but perhaps I may comment on a few things. First of all, I would like to agree with Pr.

Lifson that the forces involved are simple, since the entire discussion is about Coulomb forces. Perhaps, one should also include the Fröhlich modes in the discussion, and the time dependent Coulomb forces as well as the electro magnetic forces. The basis for the intermolecular interaction is hence very simple, and one has now been studying this for about ten years in biology, and still the outcome seems to be more complicated, than anyone could anticipate.

First one may start by looking at the elementary processes involving proton transfer and electron transfer.

In the hydrogen bonds, the proton usually has two equilibriums positions, and if one brings one of the protons to jump, it changes the gross electric charge of the next molecule and may induce another proton to jump.

This leads to a collective behaviour which is well known for all hydrogen-bonded systems, and this is just a manifestation of the Coulomb forces. On the other hand, if one considers the electrons, there has been a great deal of discussion about long-range order and coherence in biology. In chemistry, coherence is essentially associated with definite phase relations; for example, in a hydrogen molecule with the atomic orbitals a and b, one may form either the bonding molecular orbital $(a + b)$ or the non-bonding orbital $(a — b)$, and the shift of sign of b is associated with an energy jump of about 8 eV. Coherence is hence a phenomenon associated also with chemical bonding, and long-range interaction in biology is often conveniently discussed in terms of molecular orbitals extending over a protein chain or over a polynucleotide.

If an electron travels alone over such an extended molecular orbital, its unpaired spin may easily interact with the environment, whereas two electrons travelling together with paired spins form an excellent transport unit in close analogy with the electron pairs in superconductivity, etc.

In biology, one often asks the question if there is any charge transfer. The life process is a burning process under the influence of oxygen, and, in this oxidation-reduction process, the electrons flow from the burning substance through a chain of enzymes to the oxygen molecules sitting in the middle of the haeme-groups of the haemoglobin. It is possible that the electron flow goes through a series of amide groups in the proteins joined by hydrogen bonds, and it seems probable that the long-range transport occurs in terms of electron-pairs. This leads to an accumulation of negative charge at the end of the haeme group leading to the reaction.

$$4\,e^- + O_2 + 2\,H_2O = 4\,OH^-$$

and the gross electric neutrality of the system is then established by a flow of OH^- ions going the other way. This leads to an electric current which one should be able to study experimentally with the refined methods available today.

In such a picture, a chain of oxidation-reduction enzymes would essentially serve as an "electron bridge". Theoretically, this is very difficult for us to explain in full detail, since we are not trained in describing the motion of electronic wave packets along such complicated bridges. However, it seems likely that long-range interactions of this type may be very essential in biology.

A. M. LIQUORI : I would like to point out that interactions which are very important in influencing the optical properties of a system, for instance, a crystal, do not necessarily have a significant bearing on its stability. This is clear from our experience on the calculation of the stability of molecular crystals carried out on the basis of calculations of non bonded interactions. These considerations certainly apply to a protein molecule. It is therefore quite possible that the kind of interactions discussed by Professor Careri for a protein molecule, are indeed responsible for the optical affects he has observed, but do not significantly contribute to the stability of the tertiary structure. I hope that this will eliminate some possible misundersandings.

K. MENDELSSOHN : Any other comment ?

Perhaps in closing this meeting I might be permitted as chairman to make just one remark. We have talked a good deal about interaction forces and the forces between atoms. It is worth remembering that the order patterns are highly insensitive to the particular forces which operate. I tried to point out before that whatever forces operate, once you get ordering in position, it is always a crystal. Equally, ordering in momentum space, irrespective of the particular interaction mechanism involved, always seems to result in a superfluid with general properties.

Life in its various form is an extremely general phenomena and it may be that order patterns are at least as important as the interaction forces we are looking for.

I would like to end this discussion by thanking you all very much for the contributions and the remarks you have made and I do feel that slowly but gradually we will arrive at a more manageable understanding between the physicists and the biologists.

Thank you very much.

Journée du 1er juillet 1969

Première séance

GÉNÉRATION DES MOLÉCULES BIOLOGIQUES

PRÉSIDENT P. AUGER

J. E. SULSTON and L. E. ORGEL

Polynucleotides and the origin of life

S. SPIEGELMAN

Extracellular evolution of a replicating RNA molecule (abstract)

W. GILBERT (*)

DNA replication

Discussions

(*) W. Gilbert's report has been presented orally. We have not received the manuscript.

De la Physique théorique à la Biologie, C.N.R.S., 1971.

INTRODUCTION

P. AUGER

J'ouvre la séance de ce matin. Le professeur Marois m'a demandé de présider alors que je n'ai aucune capacité particulière pour cela. En l'ouvrant je voudrais reprendre pour quelques minutes une partie du débat d'hier et vous dire ceci, en ce qui concerne un contraste très frappant pour beaucoup de personnes, je veux parler de celui qui sépare l'extraordinaire complexité des molécules biologiques qui nous ont été décrites hier avec un talent remarquable et qui sont le fruit de travaux dont nous admirons tous la profondeur et la précision, de la simplicité du fonctionnement de ces enzymes lorsqu'elles se trouvent effectivement dans le milieu vivant. Et si vous permettez, je ferai quelques comparaisons, disons de mathématicien ou de physicien. Si j'écris au tableau une série de chiffres comme ceux-ci par exemple 3,14159 ces chiffres vous les reconnaissez.

En principe je dois les retrouver très facilement grâce à une petite poésie française qui donne ces nombres d'après le nombre de lettres des mots :

> « que j'aime à faire apprendre un nombre utile au sage, immortel
> Archimède artiste ingénieux... »

Si vous comptez le nombre de nombre de lettres vous obtenez tous ces chiffres : eh bien ce nombre ainsi chiffré est très complexe, et on ne voit pas du tout, du point de vue arithmétique, apparaître une règle quelconque.

Il n'y a d'ailleurs pas de loi, comme vous savez, qui règle la série de ces décimales, série indéfinie et qui est exactement comparable à celle obtenue par le jeu du hasard. On a montré qu'il n'y avait absolument aucune différence entre cette série de nombres tirée au hasard. C'est donc un nombre très particulier mais excessivement compliqué si on l'exprime ainsi. Et cependant ce nombre a des fonctions, si j'ose dire, extrêmement simples lorsqu'il est mis en présence d'autres nombres qui, eux aussi, sont tout aussi complexes. Par exemple on peut écrire une équation très simple comme celle-ci :

$$e^{\pi i} = 1$$

C'est une relation très simple de ce nombre π et du nombre e qui est un nombre aussi compliqué que celui que j'ai écrit tout à l'heure si on l'exprime en décimales. C'est donc une relation simple entre deux nombres complexes. Je pense que lorsque l'on dit que les enzymes sont des complexes extrêmement compliqués, et que l'on s'étonne qu'ils aient des fonctions simples, il faut faire attention qu'ils se trouvent avoir des fonctions simples lorsqu'ils sont en rapport avec d'autres corps qui sont tout aussi complexes qu'eux.

Je crois qu'aujourd'hui nous avons à traiter justement de fonctions de corps extrêmement complexes que sont les enzymes et la première communication est celle de Monsieur Sulston sur *Polynucleotides and the origin of life.*

J'appelle la communication de Monsieur Sulston.

De la Physique théorique à la Biologie, C.N.R.S., 1971.

POLYNUCLEOTIDES AND THE ORIGIN OF LIFE

J.E. SULSTON and L.E. ORGEL

The Salk Institute, San Diego, California 92112, U.S.A.

Introduction

One approach to the problem of the origin of life is to try to picture the simplest version of an essentially modern living organism which could, in an appropriate environment, evolve by natural selection. This fictional creature must contain at least one protein molecule, for only proteins are known to possess catalytic activity; it must also contain nucleic acid, for protein is not of itself known to replicate and is synthesized only by translation under the direction of nucleic acid. The third requirement is the most severe : the organism must possess an apparatus which links amino acids together in the sequence required for protein, under the precise control of the sequence of nucleotides in the nucleic acid.

The complex contemporary translation system cannot reasonably be supposed to have arisen suddenly from an abiotic world, and our major difficulty is that we cannot see how to simplify it greatly without destroying its function entirely. Yet it seems that without nucleic acid the organism cannot replicate and without protein it can have no function, so that translation is an essential operation. We are therefore forced to simplify our picture of the proto-organism by introducing principles not found in its modern descendants.

For example, it has been suggested that primitive enzymes were formed by the specific coupling of amino acids directly on polynucleotide templates, so that a specialized translation mechanism was unnecessary. However, experiments have so far failed to demonstrate the strong, specific interactions between amino acids and short nucleotide sequences which would be required. The reverse process, specific synthesis of polynucleotides on polypeptides, seems unlikely for the same reason. (The random synthesis of polynucleotides, on the other hand, may well be catalyzed by appropriate polypeptides).

Another possibility is an all-protein system, with neither nucleic acid nor a translation apparatus. Whereas it is probably true that an abiotically synthesized mixture of polypeptides contains many molecules with particular catalytic activities, there is no known mechanism by which these molecules can replicate : therefore, so far as we know at present, there will be no natural selection in such a system.

Here we shall consider a third possibility, which has already been mentioned by one of us [1] : a system based on polynucleotides, without protein. The ability to replicate is, in principle, built-in to polynucleotides owing to the inherent pairing properties of the bases; the extent to which this is true in practice is examined in the experimental section of this paper. Although contemporary polynucleotides are not known to have catalytic activity, there seems no reason why this should always have been the case. Nucleotides possess a range of groups which can ionize, be protonated and form hydrogen bonds, and polynucleotides can form complex three-dimensional structures. Furthermore, polynucleotides might chelate small molecules which would form "active sites" and extend the range of functional groups available. (We envisage the folded polynucleotide forming a site which chelates a specific molecule; this process is very different from the one-to-one pairing between amino acids and short oligonucleotides, which would be necessary for direct translation). However, the limitations in the variety of nucleotide residues, and particularly on the range of functional groups, could mean that a polynucleotide of a certain size would always be much less efficient than an appropriate protein of that size in catalyzing a given reaction : this would account for the eventual disappearance of the majority of functional polynucleotides in living organisms. But not all have disappeared. In the ribosome and in t-RNA polynucleotides retain a functional, if not a catalytic, role; adenylic acid is an unexpected but essential component of certain coenzymes. Ribosomes, t-RNA and NAD and ATP are universal, basic, and therefore probably ancient parts of the cell machinery; the involvement of nucleotides in all three is highly suggestive.

The non-enzymic replication of polynucleotides

We have recently reviewed our experimental results concerning the template activity of polynucleotides [2]. The following summary briefly recapitulates the salient points.

Under conditions in which the triple helices A : 2 poly U* and pA : 2 poly U are known to be stable [3] poly U facilitates the condensation of A with pA in aqueous solution in the presence of a water soluble carbodiimide [4]. Under the same conditions, poly U has no effect on the condensation of G, C or U with pA, nor does poly C have any effect on the condensation of A with pA [5]. Poly U also facilitates the self-condensation of pA [4], and the condensation of pA with oligomers such as ApApApA [6]. Poly C does facilitate the self-condensation of pG and the condensation of pG with G, but not the condensation of pG with A, C or U [7]. Thus, the Watson-Crick pairing rules are

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* Abbreviations A, adenosine; G, guanosine; C, cytidine; U, uridine; poly U, polyuridylate; pA, adenosine-5′-phosphate; ApA, adenylyladenosine; etc.

obeyed, and both chain initiation and extension of oligoadenylates on a poly U template have been achieved. Chain extension of oligoguanylates on a poly C template is very inefficient because most of the internucleotide bonds formed are (5′ → 5′).

Various "prebiotic" condensing agents [8] have been investigated, and cyanogen bromide has proved quite effective for the condensation of A with pA on poly U [9]. Prior activation of pA as the imidazolide, however, gives the best results [10] : the yield of ApA is 25 times that obtained for each equivalent of carbodiimide in the earlier experiments. The imidazolide of pG, on the other hand, fails to yield oligoguanylates on a poly C template.

Carbodiimide and particularly imidazolide condensations yield principally (2′ → 5′) internucleotide links rather than the naturally occurring (3′ → 5′) links. However, Michelson and Monny [11] have shown that the helices formed by oligo-(2′ → 5′)-adenylates with poly U are only slightly less stable than those formed by oligo-(3′ → 5′)-adenylates with poly U. It is therefore possible that primitive polynucleotides contained both (3′ → 5′) and (2′ → 5′) links. We have found that the hydrolysis of poly U in a solution containing Mg^{++} is slowed five fold by the addition of poly A. Thus, the slightly greater stability of complexes of (3′ → 5′) polynucleotides and their consequent resistance to hydrolysis may have been one reason for the selection of enzymes which form exclusively (3′ → 5′) links.

The template reaction on poly U is specific for D-β-adenosine. L-β-adenosine [12], D-α-adenosine, and the 2-epimer, D-1-β-arabinosyladenine [13], do not react with the imidazolide of pA to anything like the same extent.

Poly A does not facilitate the condensation of the imidazolide of pU with U or UpUpU [9]. This result is in accord with the failure to find stable complexes between U and poly A [3]. Similarly, poly G is unlikely to facilitate condensation reactions of pC.

We conclude that base-pairing indeed provides adequate specificity for some sort of non-enzymic replication of polynucleotides, but that general replication is unlikely to proceed in dilute aqueous solution. In future experiments we shall investigate such special conditions as adsorption on minerals, complexing with polypeptides of non-biological origin, and reaction inside proteinoid microspheres.

Polynucleotides as catalysts

A second experimental approach is to look for catalytic activity in polynucleotides, or in complexes of polynucleotides with small molecules. Of course, a direct search would be very difficult on account of the large number of different nucleotide sequences to be screened. We must also bear in mind that the expected catalytic activities are much lower than those of proteins. What is required is a laboratory system for the natural selection of polynucleotides, and we are undertaking experiments using the Q_β replicase and variant RNA described by Spiegelman *et al.* [14].

The Origin of life

In conclusion, we shall outline one possible sequence of events by which biological natural selection could have begun. This is a purely fictional account, intended merely to illustrate a general principle. The reader can readily produce an alternative scenario, more to his individual taste.

Suppose that many different polynucleotides were generated on the prebiotic earth by random polymerization. Any sequences which had replicase activity, however slight, would recur more frequently than inactive sequences. Other polynucleotide molecules might show nuclease activity, and the association of such a "predator" with a resistant "replicase" would yield a much more successful unit. The polynucleotides would be expected to exploit every aspect of their environment, developing progressively more effective catalytic powers. Some sequences might specifically bind certain amino acids. If a particular dipeptide was valuable, an association of polynucleotides carrying the component amino acids would be favored; such polynucleotides could be regarded as the ancestors of contemporary t-RNA. Gradually, as longer polypeptides were synthesized and utilized, simple association would no longer be efficient. A polynucleotide framework which oriented the "t-RNA's" appropriately and a template which ensured the correct order of assembly of the amino acids would be at first valuable and later indispensable. Thus, we may imagine the ribosome and m-RNA coming into being, together with the first use of a genetic code.

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EXTRACELLULAR EVOLUTION OF A REPLICATING RNA MOLECULE (ABSTRACT)

S. SPIEGELMAN

Department of Microbiology, University of Illinois, Urbana, Illinois - 61803 U.S.A.

Proof that purified Qβ-replicase catalyzes the synthesis of both normal and mutant infectious Qβ-RNA established that the RNA is the instructive agent in the replicative process. These experiments showed that the RNA molecules satisfy the operational definition of a self- duplicating entity and thus generated the possibility of performing extracellular Darwinian experiments.

The first step in exploiting the inherent potentialities of this system was a serial transfer experiment designed to impose on the replicating molecules the condition that they multiply as fast as possible. As a result of this selection, a mutant was isolated (V-1) which replicated some 15 times faster than the original Qβ-RNA and retained only 550 of the 3600 residues originally present in the parental molecules. It would appear that in order to satisfy the imposed condition of fast growth, the mutant eliminated those parts of its genetic information which were no longer necessary in the test tube for completing the act of replication.

It was then shown purified Qβ-replicase can be initiated to synthesize copies by a *single* molecule template. The resulting *clone* of descendants possess evident advantages for sequence studies and in addition made possible the inception of an *in vitro* genetics of replicating molecules.

In performing the cloning experiments a new variant (V-2) was isolated which replicated faster than V-1. Thus measurable RNA synthesis occurred in a 15 mn reaction when initiated with as little as 0.29 μμg of V-2. However, more than 300 times as much was required for V-1. This phenotypic difference has been maintained over many transfers.

The experiments thus far noted were concerned with the isolation of mutants possessing increased growth rates under standard conditions. We then turned our attention to another question of no little theoretical as well as practical interest and inquired whether other mutant types can be isolated. In effect we attempted to answer the following question, "Can qualitatively distinguishable phenotypes be exhibited by a nucleic acid molecule under conditions in which its information is replicated but never translated ?"

Two avenues were explored in attempting to answer this question. One approach for obtaining a variety of mutants is to run the synthesis under less than optimal conditions with respect to a component or parameter of the reaction. If a variant arises which can cope with the imposed suboptimal situation, continued transfer should lead to its selection of a wild type.

The rate of synthesis as a function of the concentration of one of the required riboside triphosphates (CTP) was determined. Selections were carried out at levels which supported limited synthesis of the initiating variant. By serial transfer it was possible to isolate a series of variants which clearly had acquired a heritable feature permitting them to overcome the disadvantages imposed by the low level of CTP. Several of the properties of these variants compared to wild type will be discussed.

We then showed that it was possible to isolate a variant resistant to inhibitory analogue of one of the riboside triphosphates. Tubercidin is an analogue of adesnosine in which the nitrogen atom in position seven is replaced by a carbon atom. Tubercidin triphosphate (TuTP) is an effective inhibitor of the synthesis of Qβ-RNA *in vitro.* An attempt was then made to see whether one could derive a mutant which would show resistance to this agent by carrying out serial transfers in its presence. This was successful; variant V-9 was isolated which multiplied at twice the rate of the wild type molecules in the presence of TUTP.

Other types of resistant variants can be isolated by a similar procedure and we will describe a mutant isolated by J. Sulston and L. Orgel which is resistant to the presence of ethidium bromide.

The experiments here described show that numerous differentiable variants can in fact be isolated, the number depending on the ingenuity expended in designing the appropriate selective conditions. The data also suggest that precellular evolution could have involved selective forces of previously unsuspected diversity and subtlety through interactions between the replicating nucleic acid and primitive surface catalysts.

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De la Physique théorique à la Biologie, C.N.R.S., 1971.

DISCUSSIONS

S. Bennett : I wish to make some points which may be of general significance in relation to the reports of Dr. Sulston and Dr. Spiegelman. In earlier work, Dr. Orgel and others demonstrated that if one creates a medium containing simple ingredients, such as methane, formaldehyde, ammonia, carbon dioxide, and phosphate, and then supplies a source of energy such as ultra-violet light or an electrical discharge, one can get recombinations of the atomic ingredients and the appearance of many of the simple components of biological systems. Dr. Orgel and his group, amongst others, have demonstrated that from simple mixtures of this kind, one can detect the formation of nucleotides. This, then, is part of the background from which Dr. Sulston reported his work. Dr. Sulston emphasized that a chain of polynucleotides can provide catalytic sites for the organization of other nucleotide chains, which though not identical to those existing in biological systems are nevertheless similar. This demonstrates that proteins are not necessary for catalytic action and that a certain degree of catalysis is actuated by the nucleotide chains themselves. This, of course, is slow catalysis.

From these studies we can conclude that, given the chemical ingredients and the sources of energy which we think were present under primitive conditions on earth, the rearrangement of constituent atoms to form configurations which characterize biological molecules appears to be highly probable. Whereas it has often been stated that the configurations of atoms in biological systems are improbable, this has been an unconsidered statistical judgment based simply on the distribution and the relative numbers of the atoms, without considering their properties. If one considers their properties, it turns out that life is probable, rather than improbable.

A second point emerges, which I suggest for general discussion. Perhaps the principle basis on which competition between living forms is won or lost in the evolutionary process may be the rates of catalysis. With simple systems, such as might be derived directly from ingredients in a primitive atmosphere, we can postulate slow rates of catalysis. The invention of catalytic proteins gives advantage to systems possessing them.

P. Auger : In the problem of the probability of creation by ordinary chemical processes of complicated molecules like nucleotides, I think we should distinguish clearly two stages : the production of single nucleotides which may be very probable and the combination of these nucleotides in specific series, which then is very improbable.

K. Mendelssohn : What I really want to ask my biological colleagues is how much do you know about possible combinations of these basic bits of biological material, such as nucleotides. How many possible combination can exist which are chemically possible and which never occur ?

P. Auger : I would like to stress that you cannot calculate the probability of single events. The physicists know perfectly well and the mathematicians too, that a single event which happens only once, you cannot give any value of its probability. So if life has happened only once on this earth, then the probability of the appearing of life has no systematic value. You don't know what it means. The experiments that have been described, are experiments which are repeatable; that is you know how, what is really the probability of appearance of certain single nucleotides, you know nothing about the appearance of a chain which would be self-duplicating.

This is another problem, because this may have happened only once, and then the probability has no real value. Would anybody like to answer the question that has been just asked now, which is about the appearance of change which has no meaning, no biological meaning but is chemically possible.

J. Monod : Just to go on to Dr. Spiegelman's talk I wanted to ask him whether one could specify at what stage in the replication of his "monsters" as they are called, do the mutations affect the growth rate, because one can think of three steps at which the rate could be modified. One is of course the affinity of the RNA for the proteins which could be significantly increased. The second is the rate of elongation of the chain itself. I must say that seems less probable to me that you could modify the structure in such a way that it could be replicated faster except perhaps on the basis of secondary structure of something like that. And the third possible step is the rate at which the finished polynucleotide gets liberated from the enzyme these three are different selective pressures which could be boosted under specific conditions.

L. Rosenfeld : I should like to come back to the concept of probability. When physicists use this concept in their own problems they always take care to define the conditions under which they apply it. One can calculate the probability of some event relatively to certain conditions which have to be specified; if the conditions change even slightly, the resulting probability can be very different. They ought not to forget this simple requirement when speculating about problems with which they are not familiar. Now, I would recall the question asked yesterday by Prof. Fessard, about the interaction between an enzyme and its environment. I had the impression, listening to Dr Spiegelman, that his experiments give a clear answer to this question.

G. M. Edelman : I would like to ask Dr. Spielgelman about the mechanism of action of this enzyme. Does the enzyme molecule complete one particle before it goes on to the next ?

J. Monod : Just almost to make a philosophical point. I think Dr. Spiegelman's experiment illustrates one point which is very often not clearly understood by non biologists. Let me first make the statement that the most astonishing property of living being is not that they are able to evolve but rather that they are so fantastically stable. The fact is that species like lingula have not changed their structure for 500.000 years. This is a very astonishing situation. It is often felt or even said, that the basis of that stability is the fidelity of DNA replication. Now that's absolutely wrong as Spiegelman's experiment illustrates. If he were to multiply the RNA not in his artificial system, giving many degrees of freedom to his structure, but rather multiply the virus itself in the cell virtually all the variants he picks up in his artificial system would be needed out in the course of selection. I merely want to make the point that the fundamental reason for stability of living beings is not the stability of the replicating machine which suffers many perturbations, but rather the stability of the structure which has been built on that basis by selection : in other words, *stability is selective.*

The stability is not physical, it is purely biological. It is due to selection, it is not due to some mysterious properties of the structure.

K. Mendelssohn : Could you be kind enough to define biological stability. Is it possible to define it in terms of physics ? What is biological stability ?

J. Monod : This is a fact. It is a fact that there are species, that are still alive to-day and were alive 500.000 years ago in exactly the same structure. The point I was making was that the reason for this fantastic stability is not the fact the replicating machinery is perfect. It is not. It makes all sorts of mistakes all the time.

K. Mendelssohn : I would like to see the answer to my question. If you now have a strand of DNA, if it has been injured, if I understand Dr. Spiegelman right, repair takes place, how can repair take place in a structure that has once been injured but doesn't know anything.

M. Kotani : I am sorry to come back to the first paper. The problem of the origin of life or the origin of bio molecule, this paper is very nice in explaining a possible replication mechanism of nucleo polynucleotide without protein but, as Pr. Monod emphasized, there must be a certain stage in the evolution of nucleic acids, in which proteins come into play.

This may be the stage of oligonucleotides and oligopeptides. There must be some cooperative play between primitve nucleic acids and primitive proteins and, in this sense, I think it very important to study the very feeble weak catalytic activity of oligopeptides. My knowledge in this field is too limited, and I wonder whether there is a previous study of such very weak catalytic activities of oligo peptides; this may be too weak to measure, I don't know and I would be grateful if somebody gives some evidence on the weak activity of oligopeptides, or of primitive protein. Thank you.

J. SULSTON : It is quite true that some oligopeptides have catalytic activity; may be somebody else can give us detailed information. The point I wanted to make in my talk is that, as far as we can see, oligopeptides could not replicate without nucleic acid : that is to say, a given oligopeptide has no way of directing its own synthesis and no way of increasing its rate of appearance on the earth. This is the difference between life and death.

L. ROSENFELD : With regard to the question of stability or unstability, we have no clear-cut situation. I think that the reason why the situation appears to us strange is not that we doubt that it is ultimately a physical process, but that there is no analogy in the physical systems that we know for this kind of interaction. In physics, we have to the first approximation either completely stable systems, metals for instance, in which the interaction between the molecules dominates, or complete randomness, such as prevails in the gaseous state. In biology we have more complicated situations, in which we see the two opposite influences at work with about the same strength, and this creates this feeling of strangeness, since we have no simple analogy to guide us, but only the example of life itself. However, realizing this situation gives us some hope that we may formulate in physical terms some biological problem that we would be able to tackle.

J. BRONOWSKI : I am a mathematician who used to work with physicists and now works with biologists. I would like to speak specifically about the questions raised by Mendelssohn and Rosenfeld which concern probabilities and stabilities. I shall try to do this very simply.

It is indeed pointless to make any calculations either of the absolute or of the relative probabilities of single events, for the reason which Auger has already given. But beyond this, it is pointless to make such calculations even for whole species of events, when the time scale and the numbers involved are so large that they dwarf the individual occurrences. I mean by this remark that we should not single out the improbability of life and of the evolution of man, when evolution even in the inanimate world hinges on steps which, as individual events, are wildly improbable. For example, all life as we know it requires the presence of carbon, but it is wildly improbable that any specific carbon atom should have come into existence at all. The creation of a carbon atom requires the triple coincidence at a point in space of three separate helium atoms within a time of 10^{-13} seconds. Nobody is going to break the bank at Monte Carlo by backing that kind of odds.

So the fact that the universe has created life on earth depends on a property in the evolutionary process which is quite separate and different from the improbability of the individual steps. Evolution is made possible, in inanimate matter as well as in living matter, by the essential fact that what is created on these rare occasions is an extremely stable entity which accumulates and perpetuates itself and becomes the basic material for the next evolutionary step. For example, the carbon atom is extremely stable, and it perpetuates itself at the expense of the helium atoms which are progressively used up to make carbon. In Spiegelman's sense, it is as if the complex carbon atom were a tail growing from the simple helium atoms (as some of his nucleic acid molecules do) for the good evolutionary reason that it is more stable in its total environment.

I think this is a very important point to remember : that the evolution of the atoms is just as extraordinary a process as is the evolution of human beings. It depends on just the same evolution of more complex units from simple ones by reason of their stability. So much for that.

Secondly, then, we have to ask of these calculations that if they are relevant to life as we know it, they must in fact lead forward step by step in complexity without regression. Again, this is different from the questions that were asked yesterday, when the physical discussion was concerned with the means by which an organism is able to survive at all, to carry messages, and so on. Today we have shifted to quite a different field, namely, how a species is created and then evolves — so that we are not talking about the organism as such any more, but about the species as if it were a super-organism. Here the discussion today has been very important because, starting with the paper by Sulston and Orgel and then by Spiegelman, we have come to see that the evolutionary processes which we picture in the elaboration of the complete organism are already visibly at work in rather simple structures built up from little more than the nucleotides. Let us disregard whatever mystery there is about the formation of the nucleotides from their precursors : what we have been shown is that once the nucleotides assemble in sequences, the assemblies evolve in a straightforward way. Darwinian selection works on quite a simple nucleic acid — an absolutely bare piece of genetic message. In one set of conditions, the piece shrinks because rapidity of proliferation is the only important thing for it : just as, if a lawn is grown from mixed seeds, the seed that will conquer is the one that reproduces most quickly. But on the other hand, as Spiegelman showed, as soon as you impose a more complex condition than speed of reproduction, the molecule accomodates itself (as a species) by exploiting the evolutionary advantage of greater complexity.

This example shows that the increasing complexity of organisms may be induced by external selection, and to this extent Jacques Monod is right in saying that internal stability must not be assigned a dominant role in the evolution of complexity. I would put this differently by saying that internal stability must not be defined as if it had an absolute meaning. That is, stability is relevant to the total context, internal and external — for the organism in its environment as for the heavy atoms in the environment of a hot star. But the problem of viable complexity is peculiarly subtle for living things, which have to solve the special problem of coherent growth, and I will turn to this as my last point.

When we were talking about a single organism yesterday, we tended to argue as if it was only necessary to catch the organism at one moment of life, to show that it could pass on a message — and that was enough. But that is not enough for living organisms. The extraordinary thing about them is that they have to exist and function at every step of their lives. What is most remarkable about you and me is not that we work as we are at this instant, but that we worked as organisms at every step in our growth — as babies and children and students before we worked as adults. These stages of growth in time remind us that the processes of life are all sequential, and that each depends on the coherence and stability of its precursor.

The essential characteristic of the evolution of species is that the sub-units work and are stable within their environment, and are then able to assemble into larger units such as cells, and so on. Throughout evolution the extraordinary phenomenon is that greater complexity can only be achieved because the sub-units already work as replicating organisms (such as cells) at lower complexity. Since we have been talking a good deal about probability, I will close with an analogy from that favorite textbook source of examples, the game of cards. If you ask "What is the chance of dealing thirteen spades to one hand ? " then the answer is, infinitesimal. But suppose you make all the spades sticky, and you do not make the other cards sticky. (This is the analogue to having atomic, molecular or cellular structures which prefer to stick together in some configurations and not in others.) If you make the spades and only the spades sticky, then it will not take you many deals before you have all thirteen spades in one hand. And that is precisely the nature of the evolution of complexity : it proceeds by building one layer or stratum of stability on another, and for this reason I have named it Stratified Stability.

P. O. LÖWDIN : I would like to comment on the discussion here between Dr. Rosenfeld and Dr. Monod about the stability of the genetic code, since we have carried out some calculations on this problem. One has to understand the long-time stability of the code over millions of years, but also the stability with respect to frequent replications in such tissues as are changed after 3-4 hours.

I agree with Dr. Monod that it is likely that DNA would make all sorts of mistakes, and that the most common one would be the incorporation of tautomeric bases. The DNA-molecule of E. coli contains about 3 million base pairs, since the frequency of the tautomeric bases of cytosine is around 1 : 10^4, one can expect around 300 errors per replication. Even if most of them would be lethal and eliminated, the question is how the mutation rate can be kept below 10^{-8} per base pair and generation.

Apparently there must be some mechanism which assures that every DNA-replication can occur almost perfect. If one looks at the famous Y-model which is based essentially on the same ideas as Dr. Gilbert has discussed here, there is a definite "replication region " where the two original strands get separated and two nucleotides are added. This implies that, for a moment, there are four nucleotides in close neighbourhood of each other with their 6-positions forming a parallelogram with two temporary hydrogen bonds. In a normal replication, there are two aminogroups and two keto-oxygens in this parallelogram. In a tautomeric replication, however, one of the keto-oxygen is replaced by an amino-group or one amino-group is replaced by an enolgroup. In both cases, the energy is raised several electron volts, and, since every electronvolt corresponds to a Boltzmann factor of 10^{-16}, the tautomeric incorporations are indeed very rare. Even the incorporation of a double tautomeric error is very rare for the same reason.

This is a physicist's explanation of the enormous stability of the replication process, and I realize that it may be too simple and naive and that biological phenomena are usually much more complex and involved. However, in the long run we may have to look for physical and chemical factors of this type.

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J. Duchesne : Je voudrais revenir, à mon tour, un instant sur le problème évoqué par le Pr. Rosenfeld relativement à la notion de probabilité et, d'autre part, dire un mot sur la notion de stabilité qui a été introduite ici à différentes reprises. J'essayerai de discuter ceci non pas en fonction de concepts théoriques *a priori,* mais en cherchant dans la nature, d'après nos connaissances actuelles, quelles sont les réponses que l'on peut faire à ces questions évoquées. Dans ce but, je serai contraint d'élargir un peu le débat, dépassant la physique, d'une part, la biologie, d'autre part, et regardant quelques résultats obtenus dans le domaine de la chimie proprement dite, dans celui de la photo-chimie et de la géologie organique. Ce sont notamment les travaux de Urey et de ses associés dans le premier cas et ceux de Calvin et d'autres dans le second cas.

C'est en 1955 que commence l'étude de l'action des décharges électriques et des rayonnements sur des mélanges de gaz qui représenteraient, symboliseraient, si l'on veut, l'atmosphère primitive de la terre. On s'aperçoit que les acides aminés, fondements des protéines sont formés avec une troublante aisance, pourvu que le milieu soit réducteur, et l'on dirait, par conséquent, qu'il est infiniment probable que les protéines se manifestent avec la plus grande aisance partout dans l'univers. Lorsque l'on continue son examen et que l'on examine et regarde de près la constitution chimique des météorites carbonées, qui sont des fragments de corps célestes fournis très généreusement par l'univers, on s'attendrait, n'est-ce pas, à découvrir de hautes concentrations d'acides aminés. Eh bien, c'est le contraire qui se passe, on n'en trouve pas, ou guère, et on réalise dès lors que la notion de probabilité, par conséquent, ne s'applique pas si l'on ne tient pas compte, ou ne peut tenir compte, des conditions de formation des substances dont on veut parler, ceci en accord complet avec les généralités exposées, il y a quelques moments, par le Pr. Rosenfeld. Par contre, alors que, dans le modèle adopté, l'on ne trouve pratiquement pas de bases nucléiques, ni purines, ni pyrimidines, on trouve des concentrations significatives de ces substances dans les météorites carbonées. C'est juste, en gros, l'inverse de ce que les modèles, interprétés naïvement, auraient permis de prévoir. Cela montre à quel point nous devons être prudents dans nos conclusions lorsque nous faisons usage de la notion de probabilité. Par ailleurs, en examinant les terrains du précambrien parmi les plus anciens, on trouve, dans les sédiments vieux de quelque deux à trois milliards d'années, des molécules très élaborées, hydrocarbures saturés, caractérisés par des chaînes carbonées de l'ordre de 20 atomes de carbone, et qui se rattachent singulièrement à une chaîne de la chlorophylle que l'on appelle le phytol.

Est-ce que cela veut dire que ces substances dérivent d'une vie préexistante ou sont-elles le résultat d'une synthèse chimique élaborée suivant des mécanismes que nous ignorons encore partiellement. Là est le problème qui se pose et Calvin, lui-même, avait cru y répondre, car, à un moment donné, il déclare, et ceci pour le problème de l'origine de la vie serait d'une importance capitale, il déclare, dis-je, que ces molécules sont de véritables marqueurs biologiques qui ne pourraient pas se rattacher à autre chose qu'à une vie pré-existante. Eh bien, ceci, rapidement, s'est avéré, incertain, car des synthèses assez simples ont été réalisées au laboratoire dans des conditions de température qui ne dépassent pas quelque 100 °C. Mais il est d'autres issues bien entendu et je voudrais en signaler une ici qui n'a guère été étudiée. Il s'agit d'une suggestion de Calvin lui-même. C'est que la vie sélectionne les isotopes et que l'on constate dans les plantes des rapports isotopiques Carbone 12, Carbone 13, qui ne semblent pas correspondre au rapport correspondant caractérisant normalement l'anhydride carbonique de l'air. Il y a peut être là, dans la chimie isotopique, quelque moyen de découvrir le marqueur biologique que Calvin désirait.

En ce qui concerne les espoirs de synthèse simple en matière de nucléotide et de polynucléotide, il faut bien avouer que si les travaux exécutés jusqu'à présent constituent d'impressionnants résultats, il s'agit de possibilités en ce qui concerne les synthèses dans la nature et non pas encore de résultats dont la validité peut être appréciée en termes de probabilité. Il faut cependant bien reconnaître que les conditions de leur formation, selon nos connaissances actuelles, ne paraissent pas devoir être extrêmement répandues, ni sur la terre, ni dans l'univers, puisque aussi bien la formation de polynucléotides, autant que je me le rappelle, suppose la présence de catalyseurs comme les acides poly-phosphoriques par exemple, d'une part, et de températures au fond assez élevées de l'ordre d'environ 100° à 150° C, d'autre part. On réalise donc bien que tout cela n'est pas simple. On ne pourrait guère citer que les isomères optiques en ce qui concerne les sucres liés aux nucléotides. Ceux-ci sont lévogyres, ce qui semblerait indiquer, sur la base de la probabilité 1/2 que la vie aurait eu une origine unique !

Quant au problème de la stabilité, aurait-on pu même prévoir, que dans les terrains du précambrien, on découvrirait des molécules saturées aliphatiques à longue chaîne ? On a mesuré leur durée de vie et l'on a obtenu 10^{12} années, dans les conditions d'environnement adoptées !

Tout ceci pour indiquer que les résultats que nous pouvons espérer obtenir dans l'avenir, seront la conséquence de l'expérimentation et seront dus, non pas seulement à l'exploration physique, et à l'exploration biologique, mais aussi à l'exploration chimique, physico-chimique, biologique, terrestre et extra-terrestre. Tout ce que nous pouvons faire en ce moment, c'est dire que nous avons d'une façon très générale abouti, et c'est une conquête extraordinaire, une conquête absolument remarquable, abouti à élaborer des modèles qui, en principe, pourraient convenir aux résultats que nous contemplons, c'est-à-dire à la vie, mais au sujet desquels on ne peut encore se prononcer, ni sur la probabilité, ni sur la stabilité en général. Hors de cet évantail de possibilités et de probabilité, il y a cependant une certitude, qui émerge avec force de l'expérience. C'est que, chose que l'on n'aurait pu affirmer il y a peu, la matière première de la vie, ou en d'autres termes, les biomolécules les plus simples, comme les acides aminés, les bases d'acides nucléiques, les polypeptides et même les polynucléotides, doivent être largement répandues dans l'univers, que nous savons aujourd'hui constitué de quelque 10^{17} planètes, où une évolution chimique prébiotique a pu se dérouler. Merci !

L. ROSENFELD : To the point raised by Professor Kittel. I shall certainly not contradict his example with molecular hydrogen, since, in fact, the presence of molecules in interstellar space was first pointed out by Swings and myself; but I must emphasize in contrast to his remark (unless I have misunderstood him) that when we speak of statistics we do not necessarily think of equilibrium. In that particular example our argument was based on statistics, but it involved of course non-equilibrium considerations.

The same is done for the problem of the creation of elements he mentioned. This last problem is interesting in the context of this discussion, because here we have now a solution that we are rather confident about and that is based on a dynamical analysis, using non-equilibrium statistics. The first attempt was of course to treat the problem as one of equilibrium, and that was a complete failure. By contrast, the dynamical treatment has given us a very suggestive picture, because it illustrates how an evolutive process of an immensely simpler nature than the biological one can be analysed by physical methods. The main process for building up the stable nuclei of the elements is neutron capture, starting from an initial state which is of course hypothetical; but it is a hypothesis that is supported by rather strong evidence from the present state of the universe. This process alone, however, would soon lead to unstable nuclei; but it is compensated by another process — beta decay — leading to the stable structures. Thus, there is a "regulative principle", if we may use this terminology, leading the construction of the heavy elements along a definite path, with about as many neutrons and protons in stable nuclei.

Now, an interesting point is that there are "bottle necks" in this evolutive process, when there is no stable intermediary to go up by neutron capture from one stable nucleus to the next one; in all these cases, however, other chains of processes have been found to circumvent these obstacles and restore the main chain. This, then, is an example of how such an evolutionary problem could be treated by a method which is a combination of dynamics and statistics.

P. AUGER : Anyway the comparison between what's happening in nuclear physics and in biology is interesting but has a rather philosophical meaning : it is not directly connected with our problems now. Events concerning life do not happen outside of a large flow of negative entropy. Things happen, which would not happen if there was not that flow of negative entropy which is in fact due to the rays of the sun. And it is very difficult to go from one system to the other. It is only the general aspect which is the same but we cannot go into detailed comparison between the two phenomena.

I think it would be good for the order of this discussion to limit ourselves to a certain number of subjects and discuss them one after the other. Who wants to speak on the Spiegelman intervention or — Longuet-Higgins wants so speak — on what intervention do you want to speak ?

H. C. LONGUET-HIGGINS : In all your experiments you actually supply the replicating enzymes, do you ?

I wonder if you think this makes any difference in principle to the bearing of your observations on evolution : a complete self-replicating organism has to provide its own replicating apparatus; so is your system a guenuine paradigm of the evolutionary process ?

Could I just make one more point. I think there has been a misunderstanding. Pr. Monod was talking about the stability of the genetic code, but Pr. Löwdin's comment concerned not the stability of genetic code but the accuracy of replication, which is quite another matter. The stability of the genetic code is absolutely essential for the reasons Pr. Monod has been describing; it can't vary from time to time because this would mean immediate death to the organism.

P. AUGER: Could you dwell on the same objects for a moment or if anybody else wants to speak on that specific point ? You want to deal with this specific point ?

H. C. LONGUET-HIGGINS : I would like to make one more point on the question of stability. The old example of a flame is a good one for consideration because flames have got many of the self-replicating, stable, homeostatic properties of living organisms. The essential difference is that a living organism proceeds according to a program and that such programs can be varied within very wide limits. You could say that a flame follows a program but this would hardly be a natural way of speaking.

K. MENDELSSOHN : I would truly agree with Pr. Longuet-Higgins and possibly this is the mainly question where life and non life start or the other way round. I would like to say I cannot agree with Pr Levinthal that living systems only respond to an external constraint. In fact non-living systems do it as well. The Le Chatelier-Braun principle tells us that any non living system react to external constraint so that it evades the constraint. In other words, if you compress a gas it gets harder to compress as you go on. The system resists and retains its identity. In the same way the important thing about life is that whatever you do to it, it evades the constraint, retaining is identity.

P. AUGER : May I draw a very simple comparison. You have a valley here with water, and if the temperature is the same every where the system is stable and nothing happens. The probability that the water will climb up the hill here and form a lake there is very small. But the sun shines and produces an enormous flux of negative entropy, and then the water distills up, and comes down in rain, and so on, and some of the rain falls there, the presence of the lake is part of a new domain of stability. But, if the water goes up a little over the brink, then it goes down to the valley. So finally, inside of a flux of free energy, of negative entropy, the conditions of stability are absolutely local. They have a certain local value, and you can consider this as being the field where certain proteins or nucleotides are effectively stable; stable for certain changes, not too big ones.

J. BRONOWSKI : Since I introduced the concept of Stratified Stability, I ought to answer Auger about my usage. Taken in the simplest sense, what I am saying is that in evolution every organism along the path to man has had to be a working organism, in itself and within its environment, or it has failed. (Mendelssohn put a very good question when he asked, can we tell why one evolutionary line succeeds and another comes to a stop; and the answer is, we do not know — Neanderthal man was evolved with as large a brain as we have and yet was a failure in that he has entirely disappeared.) Even this simple point needed to be made, because the earlier discussion yesterday of the mechanism of life quite neglected the important part played by evolution. But in fact the concept of Stratified Stability goes deeper than this, and says (to use Levinthal's terminology) that a stable level of organization not only resists the normal perturbations imposed by the environment, but under the perturbation of genetic rearrangement moves up to higher complexity. The clearest illustration of that is the cellular structure of all higher organisms. There are perfectly good working organisms which consist of one cell. And without a pause at the unicellular animal on the ladder of evolution, the animal with the large number of cells that we are could not have been evolved.

Journée du 1er juillet 1969

Deuxième séance

AUTOORGANISATION ET AUTOASSEMBLAGE

PRÉSIDENT S. EBASHI

A. KLUG

Self-assembly of biological structures out of equal subunits (abstract)

E. P. KENNEDY

Molecular organization of membranes

Discussions

De la Physique théorique à la Biologie, C.N.R.S., 1971.

INTRODUCTION

S. EBASHI

The concept of "self-assembly", the idea that one kind of molecules could form an ordered and stable structure without covalent bonding, template, or other external aids, has now found concrete illustrations in various kinds of biological structures. It has been scheduled that Professor Klug, who has introduced and developed this ingenuous idea, should talk about the properties of bonding which would enable the "self-assembly" out of equal subunits. As a person working on muscle proteins, I had also been looking forward to his lecture personally, because the principles of the formation of the ordered structure of flagella or virus would also be applicable to the filament formation of muscle proteins, the filamentous structures which play the key role in the contractile process. Unfortunately, however, unavoidable circumstances obliged him to absent himself. We regret that we shall miss one of the most interesting papers of this conference.

The surface membrane of the cell is another example of the self-assembly, in which lipids interact with the protein molecules in a subtle way. The model of the surface membrane postulated by Danielli has been familiar to us for past several decades. However, a trend has recently come out to assume an entirely different model of the fine structure of the membrane. Now I should call Professor Kennedy, who is leading this new trend.

De la Physique théorique à la Biologie, C.N.R.S., 1971.

SELF-ASSEMBLY OF BIOLOGICAL STRUCTURES OUT OF EQUAL SUBUNITS

(Abstract)

A. KLUG

Cambridge (Grande-Bretagne)

Many large, organized structures are formed by making use of the specificity of the relatively weak noncovalent interactions that are possible between macromolecules, particularly between protein molecules. Moreover, the subunits of many smaller aggregates such as oligomeric enzymes can reversibly associate into definite structures held together by noncovalent bonds between the units. A very significant feature of organised structures built in this way is that their design and stability can be determined completely by the bonding properties of their constituent units. Thus, once the component parts are made they "assemble themselves" without a template or other specific external control. Structures of this kind necessarily possess point group or line group symmetry. Examples may be found in viruses, oligomeric enzymes and various fibrous proteins.

Because of the complex nature of protein molecules and the fact that the interactions between them may involve several distinct sets of combining regions, many ordered structures built of equal subunits with specific bonding properties cannot be represented by using the abstraction of strict equivalence. It is often possible to arrive at a structure of lower free energy by systematically deforming a set of bonds in a number of slightly different ways. This could come about, for instance, by the action of another component in the system which interacts with the protein molceules or more generally, by the formation of another set of bonds. To deal with such systems it is necessary to introduce the concept of quasi-equivalence which may be defined as a small nonrandom variation in a regular bonding pattern that leads to a more stable structure than does strictly equivalent bonding. Examples of this may be found in larger viruses and in bacterial flagella. The plausible minimum energy designs for these particular types of structure can be systematically described in terms of this extension of classical symmetry concepts.

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* A fuller account of this topic may be found in a paper by the author " Formation and Fate of Organelles ". *Symp. Int. Soc. Cell Biol.*, **6**, p. 1. Academic Press, New York (1967).

De la Physique théorique à la Biologie, C.N.R.S., 1971.

MOLECULAR ORGANIZATION OF MEMBRANES *

Eugene P. KENNEDY

Department of Biological Chemistry,
Harvard Medical School
Boston, Massachusetts U.S.A.

Membranes proteins

Biological membranes are made up of two fundamental kinds of structural units — proteins and lipids. Vital membrane-localized processes such as photosynthesis, oxidative phosphorylation, the transmission of the nerve impulse, and the specific transport of metabolites into and out of cells obviously require functional proteins, from which it follows that the protein composition of a given membrane must be highly specific. Indeed, the mode of organization of such functioning proteins in the membrane may be regarded as the central problem of membrane biochemistry. The study of membrane proteins while they are functioning *in situ* is beset with formidable difficulties. The conventional methods of biochemistry offer little prospect of a satisfactory solution of this problem. For this reason, there is growing interest in the application of powerful and subtle physical methods, such as nuclear magnetic resonance (Chapman, *et al.*, 1968), spin labeling (Hubbell and McConnel, 1968), and the use of fluorescent probes (Azzi, *et al.*, 1969). As this very brief review may make clear, our understanding of the molecular organization of membranes is at present only fragmentary.

Early models of membrane structure based largely on that of Danielli and Davson (1935) suggested that biomenbranes consist essentially of a bimolecular leaflet of lipid to which two layers of spread sheets of protein are bound by ionic interaction with the polar head groups of the lipids. This model appeared to be strikingly supported by studies of a large variety of membrane structures with the electron microscope which revealed triple layered structures approximately 80-100 Å in thickness, thought to represent two layers of protein separated by a layer of lipid. These findings, together with the earlier X-ray studies of myelin, gave rise to the "unit membrane" hypothesis (Robertson,

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1962) which postulated that biomembranes throughout nature are fundamentally similar, and have structures essentially like that of the Danielli-Davson model. There appears, however, to be considerable uncertainty as to whether the image found in the electron microscope accurately localizes protein and lipid in the membrane. This uncertainty arises in part, at least, from a limited knowledge as to the reactions which occur between fixatives such as potassium permanganate and osmic acid with membrane components. In a striking experiment, Fleischer, *et al.* (1967) were able to remove more than 90 % of the lipid from mitochondrial membranes and still observed a triple-layered unit membrane image in the electron microscope after fixation with osmium. Studies of biological membranes with the electron microscope at a higher resolution also appear to provide evidence for sub-structure in membranes. Since the morphology of membranes will be dealt with authoritatively by Dr. Fernandez-Moran elsewhere in this symposium, this topic will not be further pursued here, except to observe that much of the evidence based on membrane morphology which appeared to support the unit-membrane concept may require further evaluation.

Binding of proteins to membranes by hydrophobic interactions

In the simplest version, at least, of the unit membrane model, the protein is postulated to be held to the membrane by ionic interaction with the polar head groups of phospholipids. Recent biochemical investigations do not support this feature of the model. Ionic bonds undoubtedly do contribute to the binding of proteins to membranes, but hydrophobic interactions appear to be the major forces that stabilize the membrane. Lenard and Singer (1968) studied the mode of binding of proteins to erythrocyte membranes and found that treatment of membranes with phospholipase C removed a large fraction of the phosphorylcholine polar head groups of membrane-bound lecithin. This treatment resulted in only a small release of membrane protein, leading to the conclusion that the head groups of the phospholipids must be in sites on the membrane exposed to the action of phospholipase C, but not involved in the binding of significant amounts of membrane protein.

Most membranes are comparatively stable to high salt concentrations, and to reasonable variations in pH. Furthermore, the bulk of the phospholipid can usually be separated from the protein by treatment with chloroform-methanol, a solvent in which ionic interactions should be strong. In contrast, membrane proteins can generally be extracted under mild conditions with aqueous buffers containing detergents, either non-ionic or ionic. All of these facts support the view that hydrophobic forces play a large role in binding membrane proteins.

Amongst the anionic detergents, sodium dodecyl sulfate appears to be especially useful for extracting membrane proteins and dissociating them into monomeric sub-units (Jones and Kennedy, 1969). Disc electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate, developed by Shapiro, *et al.* (1967) is a very valuable procedure for analyzing membrane proteins and determining their size.

Membrane proteins extracted with sodium dodecyl sulfate can be fractionated on columns of Sephadex previously equilibrated with buffers containing sodium dodecyl sulfate. In this method, the proteins (or their monomeric subunits) are also separated on the basis of size. The Sephadex procedure can conveniently be run on a much larger scale than polyacrylamide electrophoresis. During fractionation of membrane proteins from *E. coli* on Sephadex in the presence of sodium dodecyl sulfate, the phospholipid is cleanly separated from the principal protein peaks (Jones and Kennedy, 1969). Apparently the phospholipid migrates with micelles of the detergent. The previous work of Salton and Schmitt (1967) similarly showed that phospholipid is separated from protein during electrophoresis in the presence of deoxycholate.

Despite the ample evidence that hydrophobic interactions bind membrane proteins to these structures, it appears that membrane proteins as a class do not have a higher content of hydrophobic residues than do cytoplasmic proteins (Wallach and Gordon, 1968).

Conformation of membrane proteins

Numerous studies of the conformation of membrane proteins have been reported, employing the techniques of infra-red spectroscopy, optical rotatory dispersion, and circular dichroism. This work has been reviewed by Wallach and Gordon (1968).

Membrane proteins apparently have a considerable content of α-helix. The optical rotatory dispersion and circular dichroism spectra of membrane fractions from a variety of sources are essentially similar to that of α-helical polypeptides, except that the amplitude of the peaks is smaller, and the spectrum is shifted to higher wavelengths (Wallach and Gordon, 1968). Recently, Ji and Urry (1969) have presented evidence that these anomalies may be related to light scattering and absorption by the particulate membrane fragments employed in these optical studies. In their examination of the circular dichroism of mitochondrial fragments obtained by sonic disruption, the greatest anomalies were observed with fractions containing the largest fragments.

The presence of β-conformations in proteins can be detected by infra-red spectroscopy, since the amide I band is shifted to higher wavelengths in this conformation in comparison with α-helical or random coil structures. No evidence was found for β-conformation in the native proteins of the plasma membranes of erythrocytes or of Erlich ascites carcinoma cells (Wallach, 1968. More recently, however, Wallach, *et al.* (1969) have reported that the infra-red spectrum of mitochondrial membranes suggests the presence of some proteins with β-conformation.

The studies to date of the conformation of membrane proteins have necessarily been made on total membrane protein, for the most part unfractionated. Since most membranes undoubtedly contain a very complex mixture of scores of individual proteins, the urgency of developing methods for the separation and study of specific proteins in functional form is quite obvious.

Membrane " structural " protein

The isolation of a protein claimed to represent 30-70 % of the total mitochondrial protein was reported by Green and his collaborators in 1961. The procedures used for the isolation of this protein were further developed and improved by Richardson *et al.* (1964). The method consists essentially of extraction of mitochondrial low degree of saturation with ammonium sulfate, and washing the precipitate to remove the detergent. The product so obtained is strikingly insoluble in water at neutral pH, and can be brought into solution only by treatment with detergents, alkali, or certain denaturing agents. The protein was said to be devoid of catalytic activity, but had remarkable properties of binding nucleotides and other cofactors, lipids and other proteins. The name "structural protein" was proposed for it, and the hypothesis was advanced that this protein plays a major role in organizing not only mitochondrial membranes, but biomembranes generally, by acting as kind of matrix to which other membrane constituents are bound. Reports that "structural protein" could also be isolated from *Neurospora* (Woodward and Munkres, 1966) appeared to support the idea that this protein may be widely distributed throughout nature.

Criddle, *et al.* (1966) examined the purity of the "structural protein", the molecular weight of which was reported to be 22,500, and concluded that the preparation was homogenous or nearly so by several criteria. However, the homogeneity of "structural" proteins has been the subject of controversy ever since its first description. It appeared unlikely to many workers that a pure protein could be isolated by the relatively non-selective procedures employed. A recent report by Green *et al.* (1968) now concedes that the "structural" protein prepared by the methods previously described is grossly inhomogenous and contains, amongst other proteins, a family of proteins of molecular weight 60-65,000. These results which are not easy to reconcile with the previous studies of Criddle *et al.* (1966), and from Green's own laboratory, indicate that there is no single protein which can be designated "structural" protein in the original usage of that term. This conclusion is supported by the work of Kiehn and Holland (1968), who analyzed mitochondrial proteins by disc electrophoresis in the presence of sodium dodecyl sulfate, and found that mitochondrial proteins are very numerous and cover a wide range of molecular weights. No evidence for a single predominant protein was obtained. Similar studies on the membrane proteins of *Escherichia coli* in this laboratory have also revealed a very large number of proteins in the membrane with no evidence for a single major structural protein.

In the light of these findings, it is difficult to understand the results reported by Woodward and his collaborators on the "structural" protein of *Neurospora* (reviewed by Woodward, 1968). These workers applied the same methods which had previously been used for the isolation of structural protein from beef heart mitochondria to *Neurospora* mitochondria, and isolated a protein strikingly similar in properties, molecular weight and amino acid composition to the beef heart protein. It was reported that in a mutant of *Neurospora* (Mi-l) a single amino acid replacement in the structural protein (cysteine for tryptophane) led to a profound disruption of mitochondrial function and cell physiology. If the "structural protein" is really a mixture of several proteins how could this be the case ? Perhaps an explanation may lie in the demonstrated fact that the Mi-l mutation is strikingly pleiotropic. The entire protein composition of the cell may be altered, with the mutant proteins having slightly more cysteine and slightly less tryptophane. Tryptophane and cysteine are difficult to measure with the same precision as other amino acids in proteins, and in fact, the differences reported do not precisely correspond to single residue changes (Woodward and Munkres, 1967). It is perhaps significant that when the methods for isolating "structural" protein were applied to the soluble supernatant fraction, about 40 % of the soluble protein was isolated as "structural" protein (Woodward and Munkres, 1967). This result may be interpreted either as indicating that the "structural" protein is very much more abundant than expected, or that the method of preparation leads to the aggregation of a heterogeneous mixture of previously soluble proteins. In view of the now accepted heterogeneity of "structural" protein, the latter interpretation seems more likely.

In summary, it appears that there is no compelling evidence to support the idea that a single, predominant species of protein plays a major role in the organization of membranes.

Membrane lipids

The lipid composition of membranes varies strikingly from species to species, and in different membranes from a given organism. Indeed, the bewildering variety of complex lipids encountered in membranes has been a major handicap in understanding their function. The only type of lipid found universally in biomembranes is the glycerophosphatides, which often account for 20-30 % of the dry weight of membranes. The type of glycerophosphatide, however, may vary widely from species to species (van Deenen, 1965). Thus choline-containing phosphatides are major components of mammalian membranes, but are found only rarely in bacteria.

Two kinds of choline-containing phosphatides (lecithin and sphingomyelin) are major constituents of the erythrocyte membrane of various species. The relative proportion of lecithin and sphingomyelin in ruminant and non-ruminant species is widely different, although the sum of these two lipids remains more nearly constant (van Deenen, 1965). Clearly, the function of erythrocyte membranes is not greatly affected by the substitution of lecithin for sphingomyelin.

Cholesterol is an important component of the membranes of animal tissues, but is lacking in the true bacteria. It has been suggested that cholesterol may form a complex with the choline-containing phospholipids, leading to a tighter packing of the lipid in the membrane (Finean, 1953). Polyene antibiotics such as filipin apparently exert their toxic effects by forming rather specific complexes with cholesterol. The true bacteria, which do not contain cholesterol, are relatively resistant to antibiotics of this type. However, the pleuropneumonia-like organism *Mycoplasma laidlawii,* when grown in medium containing cholesterol will incorporate the sterol into its membranes. Under these conditions, the organism becomes much more sensitive to filipin (Kinsky *et al.*, 1966).

Perhaps the most striking example of the versatility of living organisms in elaborating unusual patterns of membrane lipids is provided by the extreme halophile *Halobacterium cutirubrum*. The membrane lipid of this organism, which requires extremely high salinity for its growth, is almost entirely a unique derivative of phosphatidylglycerophosphate, in which all of the fatty acid ester bonds have been replaced by phytanyl ether linkages (Kates *et al.*, 1967).

One must conclude that certain rather general chemical features are all that are needed for lipids to function as constituents of membranes. In addition to the obvious requirement that the lipid be relatively insoluble in water, the molecule must have certain space filling properties, with an appropriate distribution of hydrophobic and hydrophilic regions. In the glycerophosphatides, the hydrophilic regions are provided by the head groups to which the phosphatidic acid moiety is linked.

There is one important element of specificity of membrane lipids, however, which must not be overlooked. Within a given membrane, the interaction between proteins and lipids may be highly specific. For example, the β-hydroxybutyrate dehydrogenase of mitochondria, after partial purification, is almost completely dependent upon phosphatidylcholine for its activity (Sekuzu, *et al.*, 1963). Other phospholipids are much less effective in activating the enzyme. Phosphatidylcholine is unlikely to participante catalytically in the dehydrogenation of β-hydroxybutyrate, and it appears that its binding is needed for the enzyme to assume its catalytically active conformation. Since mitochondrial membranes contain large amounts of phosphatidylcholine, the affinity of the enzyme for this lipid may also play a role in the intracellular localization. Similarly, an enzyme involved in the biosynthesis of the cell envelope of *E. coli* is activated by phosphatidylethanolamine (Rothfield, *et al.*, 1966). Phosphatidylethanolamine is largely concentrated in the cell membrane in this organism, and localization of this enzyme at, or in, the cell membrane appears to be highly appropriate for its function.

Since membrane lipids vary widely from species to species, during the course of evolution there must be a high degree of selection based on the membrane. Obviously, a membrane enzyme such as β-hydroxybutyrate dehydrogenase which requires phosphatidylcholine cannot function in *E. coli,* an organism that does not contain this lipid. This aspect of the evolution of proteins has perhaps not been sufficiently considered in the past.

Self-assembly and the biogenesis of membranes

The biogenesis of two types of membranes, mitochondrial membranes and the cell membrane of bacteria, will be considered briefly here.

Biogenesis of Mitochondrial Membranes.

Two distinct models of mitochondrial biogenesis may be envisaged. According to one view, the mitochondrion itself may be the site of all of the biosynthetic reactions leading to the formation of new mitochondria, that is, the mitochondrion may be a largely autonomous, self-duplicating structure. According to the second model, one may suppose that the components of the mitochondrial structure are synthesized elsewhere in the cell, and then are transferred to the growing mitochondrion in a process which might be termed self-assembly. From the fragmentary information on mitochondrial biogenesis presently available, it appears that the processes envisaged in both types of models may contribute to the biogenesis of mitochondria.

Isolated mitochondria contain DNA, ribosomes and the enzymatic apparatus needed for protein synthesis. However, studies of mitochondrial protein synthesis *in vitro* indicate that only about one-third of the mitochondrial proteins are synthesized in that structure. The other two-thirds are coded by nuclear genes and are synthesized extra-mitochondrially (Work, *et al.,* 1968). These facts argue that self-assembly may be the major but not the exclusive process involved in the biogenesis of the protein components of mitochondria.

Mitochondrial membranes, particularly the outer membrane contain some of the enzymes needed for the biosynthesis of mitochondrial phospholipids, but the bulk of phospholipid biosynthesis is catalyzed by extra-mitochondrial enzymes (Wilgram and Kennedy, 1963; Stoffel and Schiefer, 1968; McMurray and Dawson, 1969; Stein and Stein, 1969). As mentioned above, lecithin is one of the major lipids in mitochondrial membranes. The final step in its biosynthesis is the transfer of phosphorylcholine from CDP-choline to an α, β-diglyceride acceptor (Kennedy, 1961). The enzyme catalyzing this reaction is almost exclusively extra-mitochondrial. This finding led to the suggestion (Wilgram and Kennedy, 1963) that lecithin may be synthesized on the endoplasmic reticulum, and then transported to the mitochondria. Some evidence in support of this suggestion has been provided by the experiments of Wirtz and Zilversmit (1968). These workers demonstrated an exchange of phospholipids between liver mitochondria and microsomes *in vitro*. McMurray and Dawson (1969) confirmed the earlier finding that the terminal reaction of lecithin biosynthesis from choline is localized almost exclusively in the microsomal fraction of the liver cell and obtained evidence suggesting that the small activity of isolated mitochondria in catalyzing this reaction may be due to contaminating microsomes. McMurray and Dawson (1969) also showed that lecithin synthesized on the microsomes may be rapidly transferred to the mitochondria.

It seems clear that the assembly of building blocks formed elsewhere in the cell is one of the major processes involved in mitochondrial biogenesis. The question arises as to whether this assembly is entirely spontaneous and can accurately be termed « self-assembly ». The fact that the synthesis of some proteins and of some lipids takes place in mitochondria makes attractive the hypothesis that products of mitochondrial synthesis play a special role in initiating or promoting the assembly of the constituents synthesized extra-mitochondrially. The proteins synthesized by mitochondria *in vitro* are principally those not easily solubilized, i.e., of the type formerly classified as " structural " (Work, *et al.*, 1968). The enzymes catalyzing the synthesis of phosphatidylglycerol are largely mitochondrial, in contrast to the systems leading to the synthesis of lecithin and phosphatidylethanolamine, which as mentioned above, are largely extra-mitochondrial. Phosphatidylglycerol is in its turn the precursor of cardiolipin, a phospholipid principally localized in the mitochondrion. If the idea that the products of mitochondrial biosynthesis play a special role in the assembly process has any merit, then the polyglycerophosphatides such as phosphatidylglycerol and cardiolipin deserve special attention in this regard.

How are the building blocks which are synthesized extra-mitochondrially incorporated into the mitochondrial membranes ? Special factors which mediate transport within the cell may be involved here. The studies of Wirtz and Zilversmit (1968) and McMurray and Dawson (1969) have led to the finding of a soluble factor, presumably an intracellular lipoprotein, which greatly accelerates the rate of transfer of phosphatidylcholine from microsomes to mitochondria. The isolation and characterization of this factor may represent an important advance in our understanding of the biogenesis of membranes and cell organelles. That a factor or factors of this kind may quite generally be involved in intracellular lipid transfer had previously been suggested by a study of the intracellular sites for the formation and decarboxylation of phosphatidylserine in animal tissues. This sequence of reactions involves two enzyme-catalyzed steps (Kennedy, 1961) :

1. Phosphatidylethanolamine + L-serine $\rightleftharpoons$ Phosphatidylserine + Ethanolamine
2. Phosphatidylserine $\rightarrow$ Phosphatidylethanolamine + CO_2

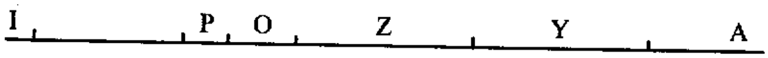
Sum : L-Serine $\rightarrow$ Ethanolamine + CO_2

The enzyme-catalyzing reaction (1) is almost entirely localized in the microsomes, while the decarboxylase is almost exclusively mitochondrial (Kennedy, unpublished results). How-ever, in whole homogenates of liver, added L-serine is rapidly decarboxylated, as is the case *in vivo*. Clearly a rapid and effective transfer of phosphatidylserine from microsomes to mitochondria must take place. The phosphatidylethanolamine formed in mitochondria by this pathway is a phospholipid which originated in the microsomes.

Bacterial Membranes.

The study of bacterial membrane systems is a relatively new but very rapidly expanding field. The advanced state of bacterial genetics, as well as many other aspects of working with micro-organisms, offer great technical advantages to those interested in probing membrane structure and function.

The brilliant contributions of workers at the Institut Pasteur have led to much of our present knowledge of the system for the utilization of lactose in *E. coli*. Thanks to their efforts, and the contributions of other workers throughout the world, the genetic regulation of the *lac* can now be depicted in some detail, as shown in fig. 1.



Gene	Protein	Sub-unit size	Number per cell
Z	β-galactosidase	130,000	72,000
Y	M protein	31,000	18,000
A	Acetylase	31,000	8,000

Fig. 1. Genetic Regulation of the *lac* System in *E. coli*.

The *lac* operon contains three structural genes. Two of these genes, the Z gene and the A gene, code for soluble enzymes, β-galactosidase and thiogalactoside transacetylase respectively. The Y gene has long been known to regulate some element needed for the transport of lactose and other β-galactosides via the *lac* permase system. Work in this laboratory has led to the finding that the product of the Y gene is a membrane-localized protein (M protein) some properties of which are briefly summarized in Table I. The function of this protein in the *lac* transport system will be reviewed elsewhere, and will not be dealt with in detail here. It may be useful to consider here some experimental facts about the *lac* system which may shed some light on membrane biogenesis in *E. coli.*

TABLE I

Properties of the membrane protein component of the lac *transport system*

1. Product of the Y gene of the *lac* operon.
2. Essential for transport in living cells.
3. Contains reactive cysteine essential for function.
4. Certain substrates protect against sulfhydryle reagents can be specifically labeled with N-ethylmaleimide.
5. Contains two sites for binding galactosides.
6. Localized in spheroplast membrane fraction.
7. Solubilized by detergents - MW of soluble protein = 30,000.

The product of the Y gene has thus far been detected only in the particulate membrane-containing fraction. [Previous claims by Kolber and Stein (1966) to have found a soluble protein which is the product of this gene have since been withdrawn]. The products of the neighboring Z and A genes, β-galactosidase and thiogalactoside acetylase, however, are almost entirely recovered in the soluble supernatant fraction. Since all three proteins are translated from the same polycistronic message, clearly the site of synthesis does not determine the eventual site of intracellular localization of a protein. Rather, that information appears to be contained in the primary sequence of amino acids in the protein.

Cells of wild-type *E. coli* are inducible for the *lac* operon. Products of the *lac* genes do not appear in the cell in significant amounts unless the cells are exposed to an inducer, such as isopropylthiogalactoside. However, very soon after induction, the M-protein is incorporated into the membrane in functional form, as revealed by the early appearance of the transport system. Since other membrane systems are also inducible, this argues that the membrane structure is rather flexible, and not highly specific. Indeed, the membranes of cells of *E. coli* may contain amounts of M protein corresponding to as much as 12 % of the total membrane protein, as will be discussed below, while the membranes of uninduced cells contain none. These results hint, but of course do not prove, that self-assembly may play a large role in the biogenesis of the membranes of *E. coli.*

New light has been shed on the biosynthesis of the functional form of the M protein by experiments in the laboratory of C. F. Fox at the University of Chicago. Fox (1969) has studied the induction of the *lac* system in certain mutants which require added oleic acid for growth. Mutants of this type which are blocked in the synthesis of unsaturated fatty acids which are required for the normal pattern of membrane phospholipids were first isolated by Vagelos, *et al.,* (1968). Fox has discovered that when induction of the *lac* operon takes place under conditions where oleic acid is withheld, preventing normal lipid synthesis, β-galactosidase immediately appears in the cell, but the transport system, which requires functional M protein, does not. Coordinate synthesis of normal lipids and M protein must take place, since if induction is carried out during starvation for oleic acid, the inducer is withdrawn, and then oleic acid is added to medium, the transport system does not appear. Thus, M protein synthesized while normal lipid synthesis is prevented cannot later be " rescued " when normal lipid synthesis is resumed.

It is clear that further work is needed before these findings can be unambiguously interpreted. One hypothesis that deserves consideration is that the newly formed M protein must immediately combine with a specific phospholipid, perhaps while the M protein is still on the ribosome. The lipoprotein, thus packaged during its synthesis, or immediately after, is then incorporated in functional state into the membrane. In the absence of the specific phospholipid, the M protein is irreversibly inactivated.

Prompted by these observations of Fox, we have examined transport function in certain mutants of *E. coli* in which phospholipid metabolism is altered unless the cells are supplemented with high levels of divalent cations. The exact nature of the lesion in these mutants is not yet known but they produce a pattern of membrane lipids in which the ration of phosphatidylethanolamine to cardiolipin is greatly reduced. The *lac* transport system is lost in the cells at a rate roughly similar to the alteration in lipid pattern, while the amount of β-galactosidase increases slightly (Lusk and Kennedy, unpublished experiments). The results suggest that even after incorporation into the membrane, the M protein requires a normal complement of lipids for its function.

Are there a limited number of highly specific sites which a given membrane protein, such as the *lac* permease protein can be incorporated, or can the protein be inserted at an indefinite number of sites ? If the sites are highly specific and limited in number, it is possible that saturation of the membrane is the limiting factor for the *lac* transport system in fully induced wild-type cells of *E. coli.* Recent work, however, argues that this is not the case.

A method has been developed for the direct measurement of the binding of radioactive thiodigalactoside to membrane fragments from cells of *E. coli.* When the cells of strain A 324-4, which contains *lac* genes on an F' *lac* episome as well as on the bacterial chromosome, were compared with the parent strain A 324, devoid of the episome, it was found to contain about twice as many binding sites. Similarly, when the rate of transport of *o*-nitrophenyl-β-galactoside into intact cells was measured, the F′ *lac* strain was found to have twice the rate of the F parent strain. In extension of these experiments, a strain was examined which is lysogenic for phage 80, and contains the *lac* operon attached to the defective phage genome, and also in the F′ *lac* episome. (This strain was kindly made available to us by Drs. Beckwith and Miller). After heat induction of the phage to obtain a maximum dosage of the associated *lac* genes, the membrane fragments were found to contain about 4 times as many sites for binding TDG as wild-type, an increase roughly comparable to the increase in β-galactosidase over the wild-type. In fully induced wild-type cells, the M protein represents about 3 % of the total membrane protein (Jones and Kennedy, 1969). The phage 80 strain, with four times higher levels, must incorporate M protein into its membranes to the extent of about 12 %. These results argue that there is no specified number of sites for the insertion of M protein into the membrane, but rather, the amount of this protein in the membrane is a function of gene dosage over a surprisingly wide range.

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DISCUSSIONS

J. Monod : Just one question about this last experiment. Was the function measured in term of accumulation or of transport ?

E.P. Kennedy : Both accumulation and the permease-mediated entry of ONPG have been measured. Neither function appears unless lipid synthesis is going on at the time of induction.

J. Monod : So you could also predict the existence of mutants where no accumulation occurred.

H. Longuet-Higgins : I wonder if anybody present would care to comment or inform us on an interesting new idea in membrane structure put forward by Changeux not very long ago. Is Changeux here ?

J.P. Changeux : What we have tried is to correlate the " apparent cooperativity " of membrane response to a cooperative membrane structure. First of all, it is clear that a biological membrane is a condensed phase; it is a " cooperative " structure simply by the fact that proteins and lipids are strongly associated one to the other. Second, we have tried to extend to the problem of membrane excitation some of the concepts proposed by Monod, Wyman and myself on allosteric proteins. Various authors have shown that, in a number of biological membranes, proteins and lipids are organised into some type of lattice structure. The question we ask is whether such cooperative lattice structure have something to do with the cooperative *response* of the membrane. In addition, one must interpret the electrical phenomena in terms of ion transport. R. Blumenthal R. Lefever and myself have recently proposed a unified theory for the action potential in terms of cooperative diffusion through the membrane.

H.C. Longuet-Higgins : If one can account for the cooperative effects which are obviously associated with, for example, the propagation of nervous impulses, in terms which relate to the cooperative effect which underlies allosteric behaviour, then we should have a very nice unifying concept.

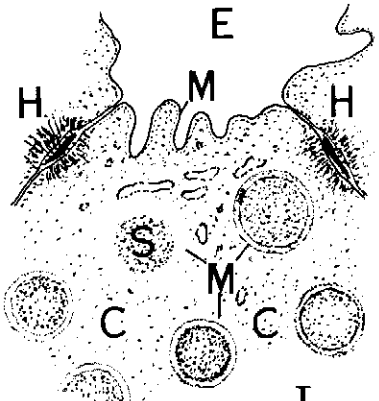
S. Bennett : Pr. Kennedy has outlined for us the manner in which the lipid and protein components can assemble themselves into single membrane units. He pointed out that under many circumstances the constituent proteins of these membranes have biological activities of a catalytic nature or of a nature related

Fig. 1. A diagram drawn from the work of Ichikawa (*J. Cell Biol.*, **24,** p. 369, 1965). The apical, or secretory pole of a pancreas acinar cell is represented at three different moments in time, representing three stages in secretion. In all three sketches, the apical portion (tip) of the cell is shown as presenting to an extracellular fluid space, E, which in this example is the lumen of a duct which carries the secretion to the intestine. The cytoplasm of the cell is indicated by light stippling (C). The letter, H, indicates cytoplasms of neighboring cells. Membrane is indicated by M. In stage I, the periplasmic or cell membrane is in black, whereas the membranes of each of the membranes of zymogen-containing vesicles (S) are shown in a distinct color. The black periplasmic membrane separates the entire cell interior from the extracellular fluid compartment (E). The colored membranes of the zymogen vesicles are internal (endoplasmic) membranes separating the aqueous cytoplasm (C) from the internal fluid compartments (S), which in turn contain concentrations of enzymes and enzyme precursors (zymogens) synthesized earlier in other parts of the same cell. In the figure there are represented only a few of the many thousands of these membrane bound vesicles, each about 0.5 μm in diameter, and each storing millions of zymogen molecules, separating them from the precious and delicate synthetic and regulating molecular machinery in the cytoplasm of the cell (C).

Stage II shows the situation in the same cell a few moments after the cell receives a stimulus to secrete, as by pancreozymin reaching the cell through the blood stream. In response to such a stimulus, the cell moves the membrane-bound vesicles containing zymogen to the vicinity of the periplasmic membrane over the apical portion of the cell. One by one but in quick succession, the zymogen vesicles approach the surface, the membrane of the vesicle makes contact with the periplasmic membrane of the cell, the two membranes fuse, the molecular components of the two membranes recombine, the membrane area initially bounding the zymogen vesicle becomes part of the surface or periplasmic membrane of the cell and the contents of the vesicle are discharged (K) into the extracellular space (E) of the duct, which is to carry the secretion so liberated to the intestine, where it exercises its function. Intermediate details of the fusion and recombination of membranes are shown in Figure 2. The portion of the periplasmic membrane now derived from membrane area originally internal and surrounding a vesicle is shown in contrasting color.

In III of Figure 1, a later stage of secretion is represented. Additional zymogen vesicles have moved to the surface and their membranes have fused and recombined with those of their predecessors, so that a considerable portion of the apical part of the periplasmic membrane is now constituted of membrane area recruited from former zymogen vesicle membrane, as is shown by the several colors now included in this membrane. K designates a cave-like invagination (caveola) of the surface membrane of the cell, in this case derived from membrane material originally bounding zymogen veiscles within the cytoplasm. Each colored area can be traced to a vesicle with the same color in stages I and II.

Some features of movement, fusion and recombination of membranes are thus illustrated in this figure. Membranes move within the cell, carrying the contents of the vesicle they enclosed. By fusion and recombination of two membranes, in the example shown here, two separate fluid compartments are fused and become confluent. In other cases, a single fluid compartment can be divided into two, a new aqueous channel can be created where there was none before, or an aqueous channel can be obliterated.



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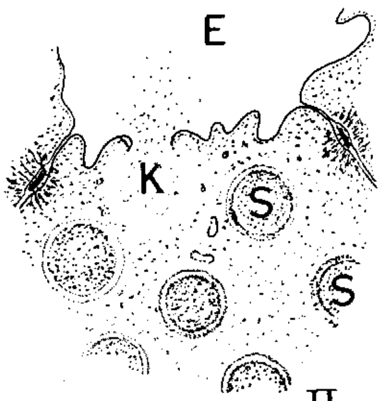

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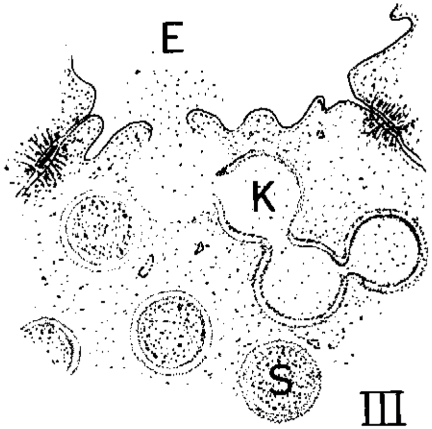

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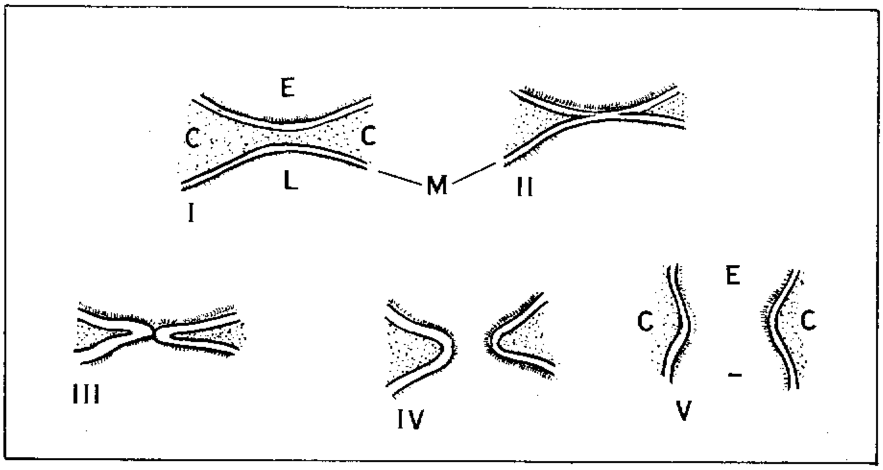


Fig. 2. A sequence of diagrams showing membrane relationship at various stages as the membrane of a secretion vesicle such as thet designated S in Figure 1, I, approaches, fuses and recombines with the surface or periplasmic membrane of the cell. In Figure 2, the cytoplasm of the cell is stippled and designated by C. The lines from M indicate the membrane of the vesicle, which is shown in red. The membrane of the cell surface is the similar blue structure above the cytoplasm, and underlying the letter E, which indicates extracellular space, in this example, the lumen of the pancreatic duct (see Figure 1, E).

In Stage I of Figure 2, the membrane of the vesicle (M) (red) is shown as having approached closely to the periplasmic or cell membrane (blue), with only a narrow bit of cytoplasm (C) intrevening. In II, the membrane of the vesicle and that of the cell surface are shown as having made contact and as beginning to fuse. The intervening cytoplasm has been moved aside. The diagram is meant to suggest a mingling of molecular components from the two membranes. An hypothesis holds that if the lipid components of the two membranes come to fuse with each other, an unstable molecular configuration may result which, in accordance with the principles of self-assembly, rapidly rearranges. The membranes after fusion as in II, recombine so that new topological relations are established. The model holds that the recombining molecular components constrain the membrane configuration to pass very rapidly through an nypothetical stage depicted in III and then quickly into stage IV, which more slowly adjusts to stage V. Stage III has never been observed, but membrane configurations as represented in I, II, IV and V have been recorded in electron micrographs.

It is to be noted that the two membrane areas represented in IV and V are each derived in part from the upper (or periplasmic) membrane of I, and in part from the lower (or secretory vesicle) membrane of I. The origins of the two portions are reflected in the assigned colors. Thus the membrane areas of III, IV and V represent recombinations of membrane areas represented in I, following the fusion represented in II. This process of membrane fusion followed by recombination is called synhymenosis.

to specific transport capabilities and hence have more than a purely structural function.

Membranes have, in addition, capabilities for moving, flowing, and otherwise changing their topographic configurations, and for fusing and recombining with each other. These phenomena comprise special examples of the principle of self assembly. By invoking participation of the surface, or periplasmic membrane of cells, in company with the vast and complicated system of intracytoplasmic or endoplasmic membranes which are found in most kinds of cells, the configurational translations, fusing and recombinations mediate important regulatory functions in cells, and manipulate, fuse and divide the several varieties of aqueous volumes which are found in cells, and which the membranes separate. The versatility and power of membrane regulatory activities is greatly extended by the capacity of two separate membrane areas to fuse and to recombine so that their topological relationships are formally changed. Let us illustrate this by a well-known example.

In the mammalian pancreas there are cells which synthesize protein enzymes and enzyme precursers which serve useful functions in digestion of the contents of the alimentary canal. Included amongst these enzymes are several proteases, nucleases, lipases and amylases which ferociously disassemble food molecules, and which are capable of equally destructive action on essential molecular components of the cell which makes them. The pancreas cells escape digestion by their own products of synthesis by depositing each dangerous enzyme molecule as soon as it is made into a fluid compartment separated by a membrane from the delicate molecular synthetic and control mechanisms of the cell. Enzyme molecules so deposited accumulate in these membrane-bound sacs, and are thus properly sequestered. They are transferred from the sacs to balloon-like membrane bound vesicles near the tip (apex) of the cell (see Fig. 1 S). Between meals, thousands of these vesicles accumulate in the apical portions (tips) of these cells. Each vesicle contains many millions of enzyme and enzyme precursor molecules. When food appears in the upper part of the intestine a chemical, pancreozymin, is released into the blood from cells lining that region. The pancreozymin is carried by the circulating blood to the pancreas, where it acts as a specific stimulus for pancreatic secretion. Some features of the response of the pancreas cell are illustrated in Figure 1. The explanation of that figure recounts the membrane movements, fusions and recombinations which participate in the discharge of secretion from the pancreas cell.

We can pause to contemplate in more detail the events that transpire between Stages I and II of Figure 1. In this sequence, the membrane bound granule or vesicle designated by the letter S in part I moves to the surface membrane, the two membranes fuse and recombine, and the vesicle S of part I is converted to the indentation or invagination called a caveola (K) of part II. Figure 2 shows some of the stages intermediate between I and II of Figure 1.

These illustrations and the explanations thereof suffice to introduce the basic concepts of membrane fusion and recombination, and the term, synhymenosis, which refers to the process. The example chosen conforms to the usual mode of secretion of protein products. Synhymenosis also occurs in the internal manipulation of protein secretory products by gland cells, as the newly-synthesized proteins are passed from vesicle to vesicle within the cell. Synhymenosis occurs at least once, and usually many times, during the course of every cell division. Synhymenosis occurs in every cell fusion, and provides the mechanism where by the separate membranes and cell volumes of the two originally separate cells become one. Cell fusion is not a rare event, and is the mechanism whereby male and female gametes fuse to form zygotes, as in fertilization of animal ova by spermatozoa, or ova of plants by pollen. In many fertilization modes, synhymenosis also serves to mediate fusion of male and female pronucleii. Synhymenosis takes place many times as each blood platelet is liberated from a megakaryocyte. The separation of the erythroblast nucleus from the maturing mammalian red blood cell involves several synhymenosis events for each of the 2×10^{11} red cells made each day in each adult human being alive on this earth.

Time does not permit one to detail here examples of the many regulatory mechanisms in cells which invoke the process of synhymenosis. But the list of examples cited earlier emphasizes its importance and calls attention to the merits of exploring further the nature of the self-assembly mechanisms which determine when this interesting event takes place. Membranes are indeed versatile, and their versatility goes far beyond the properties of selective permeability, active transport, excitability, membrane conduction, and membrane catalysis.

J. BRACHET : The electron micrographs I saw indicate that the sperm nucleus is not surrounded by a membrane; it seems that the genetical material is injected in the *egg* without membrane and that the membrane of the male pronucleus is formed by the endoplasmic reticulum of the egg. However, this is a miner point. What I wanted to say is that Dr. Bennett's pictures showed very beautifully the structure of the nuclear membrane and that I think that there is some evidence that this membrane might be very important in the regulation of protein synthesis in the cytoplasm : we know that, at least in the cells of higher organisms, there are many kinds of messenger RNA's which are formed and that many of them apparently never move into the cytoplasm and thus never become part of polysomes. So they do not play any role in the synthesis of cytoplasmic proteins. And there is some evidence coming from work on sea urchin embryos that the nuclear membrane plays a role like that of customs, this is to say that it will allow some of these messenger RNA's to go out of the nucleus, where others have to stay. I would add that I have some evidence for that view, coming out of studies on hybrids between two species of sea urchins : you can fertilize with sperm of species " A " the egg of species " B " and you will find that there is a big excess of messenger RNA of the paternal " A " species made; it apparently remains to a very large extend blocked in the nucleus of the hybrid. Perhaps the nuclear membrane, in this case, can recognize the messenger RNA's made by the maternal " B " species from the one formed by the abnormal paternal nucleus. In these hybrids and also in other cases, there are good nuclear membrane. Therefore, I would like to ask a question because we have autoradiographic indications that the synthesis of DNA starts around the the advantage of having here both Dr. Jacob and Dr. Gilbert : I would like to know whether one could put together the work suggesting the initiation of DNA synthesis and the kind of schemes which were presented by Dr. Gilbert. I don't know whether you have any comment to make.

E. ZEUTHEN : Sonneborns studies with *Paramecium* just quoted by Dr. Spiegelman deal with a system of surface membranes with associated fibres, kinetosomes and cilia, together of far higher complexity than that of the plasma membrane dealt with to-day.

The pretty colours of the plasma membranes of different cellular origin shown in Doctor Bennett's diagrams raise the question if the original differences are retained after the membranes have come to cover a common cytoplasma. In other words, are plasma membranes differentiated by their own virtue or do they just represent the cytoplasmic environment they delimit. If they can exchange macromolecular components with the underlying cytoplasma the latter possibility would seem possible.

We know from the work of Goldstein and Prescott that two nuclei in a common cytoplasma exchange a large fraction of their proteins rather quickly by migration across the separating cytoplasm, and that no proteins are conserved indefinitely in a nucleus. Williams, Michelsen and I (*Journ. Cell Science* *) have recently found evidence that a somewhat comparable situation exists for the proteins of complex cytoplasmic organenelles present in duplicate in the cytoplasm of the ciliated protozon *Tetrahymena* The structures are associated with the cell surface and they are built from great numbers of centrioles or kinetosomes held together by bundles of microtubules and other filaments, and thus show some similarity to the mitotic apparatus in other cells. Only they do not engage the chromosomes. A newly formed cell has one such structure (OA), a cell ready to divide has two. The new OA is an exact copy of the old, but it is fabricated at a distance (25 μ) of one half cell length. Each OA is carried unchanged through a number of cell generations. We have grown cells in the presence of tritiated amino acids, then removed the label and chased. OAs were isolated 0, 1, 2 and 3 cell generations (g) after label removal; there

(*) 5, 143-162 (1969) : Synthesis of cortical proteins in *Tetrahymenae*.

are the two extreme possibilities that OA proteins are tightly bound to this permanent cell structure, or that they shutle forth and back to equilibrium between old and new OA before the cell divides. The protein label scored in the OAs harvested at Og is conserved in the 1 g, 2 g, and 3 g populations, but it is not found in a steadily smaller fraction of the OAs. It is always evendely distributed over the whole OA population. Our results therefore suggest that the proteins of the OAs are migratory even though the structures are as stable as are the nuclei studied by Goldstein and Prescott. One wonders if a similar situation could exist in the case of plasma membranes. The issue is important because it deals with the question whether certain cellular differentiations shall be considered to reside in the plasma membrane or in thicker cortical layers of the cell.

Dr. Bennett : Colors were meant only to indicate the topographically distinct membrane areas and were not meant to be interpreted as indicating molecular differences. However, the phenomenon of membrane fusion and recombination offers physiological opportunity for cells to exchange information in the form of chemical components of membranes. I believe that the recognition of this phenomenon makes it appropriate to look for this kind of transfer of information. Moreover, the recognition of the phenomenon of exchange between internal membrane and the surface membrane (exchanges which go both ways), now well evidenced from many experiments, gives us opportunity to look for physiological effects of a molecular nature which might be mediated by these movements.

E. P. Kennedy : I think the comment which has just been made does indeed offer some interesting possibilities for a study of biological transport of ions.

The question arises as to whether in living cells generally there are lipid-soluble compounds which can complex ions with high specificity and which mediate the passage of these ions across membranes. We recently have done some work on magnesium transport in *Escherichia coli* and find as Silver has done in St-Louis, that there is a highly specific transport system for magnesium transport in this organism. It appears that so the *Escherichia coli* are extremely tight through passages of magnesium. They don't freely equilibrate internal and external pools of magnesium. If one grows the cells for several generations on radioactive magnesium, then the total magnesium content of the cell, of course, becomes labelled, and, if one adds energy poisons to the cell a variety of types, such as acid or carbonyl cyanide phenyl-hydrazones and then suspends the cell in cold magnesium externally, there is no exchange of magnesium inside and outside, and similarily if you suspend the cells in medium containing no added magnesium, there is no loss of the radioactive magnesium. The cells are extremely tight. This hints, at least that the membrane does not contain a large amount of lipid-soluble material which would equilibrate the internal and external pools as do these antibiotics in model systems. However, one still may argue that the carriers at this time are involved, but are regulated in relation to the cells metabolism. But the experiments rather hint that the membrane does not contain molecules which would freely exchange external and internal ions.

J. POLONSKY : What is striking in biological membranes is the association of uniformity and differentiation. This fact is a very general feature in biological structures on different scales of organization.

It is of interest to confront the general mathematical model with different physical or chemical models in biological systems, where in a uniform matrix is located an organized differentiated linear, bi- or three-dimensional pattern.

I would like to summerise some qualitative conclusions of the study :

1. Uniform redundancy in physical or chemical structures on the microscopic level links generally to specific thermodynamic active or passive functions (for instance to energy conversion), on the microscopic level by uniform cooperative phenomena of space and time correlations.

2. Differentiated redundancy in microscopic structures links to specific informational active or passive functions, on the microscopic level for instance : information transduction, filtering or catalysis of complex molecules, by cooperative phenomena of non uniform space and time correlations.

3. Association of uniform and differentiated redundancy in structures links to several number of combinations of thermodynamic and informational functions.

Three cases can be considered :

a) The two functions (termodynamic and informational) operate *independently* — the first by the uniform, the second by the differentiated structures.

b) The uniform matrix may play in respect to the informational pattern the role of a very low statistical temperature reservoir.

The signal to noise ratio of the informational message is then increased by constraints introduced by the matrix in the informational structure (the number of parasitic degrees of freedom in the informational structure is reduced).

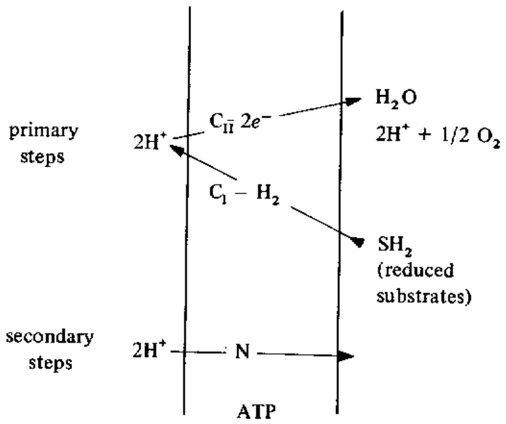
c) The differentiated pattern may play in respect to the uniform matrix the role of "impurities" in the sense used in solid state physics, these impurities can modify deeply the thermodynamic properties of the uniform structure.

C. LEVINTHAL : I want to ask Pr. Kennedy to what extent he has worked with proteins separated SDS, can one remove the SDS and do anything with the protein.

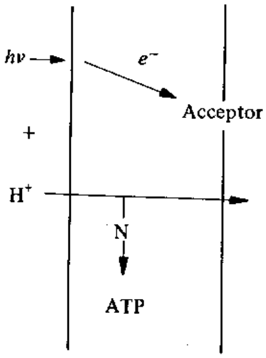
E. P. Kennedy : This particular protein is extremely sensitive to denaturation by detergents. Using the binding test which we recently developed, it appears that treatment with 0.2 milligram per ml of Triton X-100 leads to 50 % of inactivation of the binding capacity Deoxycholate also inhibits the binding function, but reversibly. One can get rid of the deoxycholate and partially restore the binding function. Sodium dodecyl sulfate is an extremely powerful denaturant and inhibitor of the binding function.

M. Klingenberg : I think a major function of membranes, which is of main interest for this mixed audience, has not yet been properly discussed, i.e. energy conservation. We know that the two main energy processes in cells, the photosynthetic primary processes and the primary energy transfer in the electron transfer of respiration, are membrane functions and, in fact, as one can assume now after many years of great efforts and failures in search of a

Biological Oxidation



Photosynthesis



primary chemical intermediate, these processes are physical elementary steps. Let me illustrate this in a scheme.

These energy transfer processes are based on the fact that the membranes are built asymetrically, containing an asymetric localization of electron transfer components. Furthermore, the membranes separate two spaces, thus making two compartments and, most important, the membrane structure itself is constructed asymetrically.

For example, in the membrane which carries the photosynthesis apparatus, light excites electrons which can be trapped by a carrier at the other side of the membrane. A charge separation ensues which is based on the vectorial arrangement of the donor and acceptor across the membrane. The membrane potential generated in this primary process may then be converted by a transport or by diffusion of cations or H^+ to ion gradients which may run the chemical machinery designed to utilize the physical gradients for the formation of chemical "energy-rich" bonds.

Similar processes take place in the biological oxidation. However, here the primary event is not the transfer of electrons, but the transfer of another "elementary" charged species, the transfer of protons. The scheme illustrates how a substrate is being oxidized at one side of the membrane. The carriers accept the hydrogen from the substrate shuttle across the membrane and are oxidized on the other side of the membrane by an electron acceptor. Then two protons are released. The electron transfer carrier then enters another transfer process across the membrane which brings electrons finally to oxygen.

In this case one has a net charge transfer of protons which corresponds to a charge separation across the membrane and forms a membrane potential. This can be utilized in a secondary process in a chemical machinery, which finally leads to the formation of the phosphate anhydride bond in ATP which is the main energy carrier in the cell.

One may suppose that the photosynthetic energy transfer process is a more basic process than the respiration, since the latter in the evolution probably has originated from the photosynthetic process. The main task of photosynthesis can be considered to be the generation of reducing equivalents and, to a lesser degree, the energy conservation. In contrast, the chemical energy formation is the major result of respiration. It may therefore be understandable that the electron transport reactions, although bound to the membrane, are not destroyed by fragmentation or breakage of the membrane. However, the energy conservation is linked to the intact membrane.

Therefore, the energy conservation and energy transfer is a consequence and function of biological membranes. It is this aspect of membrane function which should raise the primary interest of physicists, as the primary energy conservation processes are mainly physical in nature and do not involve chemical bond formation or breakage.

D. Hodgkin : I thought it might be interesting to interject here a quite specific small example of how movement through membranes of specific ions may take place. This arises from some work on various antibacterial substances and particularly on nonactin of which I have two slides.

S. Bennett : I have a question for Pr. Hodgkin which perhaps she answered in presenting the features of her interesting molecules, but if so it escaped me. Pr. Hodgkin, how well does this molecule distinguish between sodium and potassium ?

H. C. Longuet-Higgins : Let me ask a question to Dr. Hodgkin. I think she spoke of channels through which something went; I did not catch what these channels were.

D. C. Hodgkin : The molecule itself is cyclic and contains uncharged oxygen atoms belonging to furane rings and Keto groups. Professor Dunits and B. Kimball in Zurich have found that in the crystal structure of the potassium complex the molecule is folded round the ion which makes contact with eight oxygen atoms while all the nonpolar groups remain on the outside. A somewhat similar condition is found in the potassium complex of valinomycin. In both complexes it would be possible for the ions to be carried through lipid layers wrapped up in the small molecule. But in both crystal structures, it is also notable that the antibiotic molecules are stacked directly, one on top of the other, so that they compose a channel through which it would be possible to imagine ions handed on from one molecule to another.

In these molecules the framework of the oxygen atoms provides a geometrical space fitting potassium better than sodium. In most membranes it would be more likely that specific protein molecules would provide the framework, perhaps through coming together in fours or sixes. In such systems (compare haemoglobin and insulin) there are neutral central spaces, which could provide either a trap or a channel controlling the movement of ions.

M. Luzzati : These cyclic antibiotics have been used as a model for membrane function, by several workers and I am thinking of a very interesting model suggested recently by Pr. Eisenman for the transmission of nerve impulses. His idea is that some of these small compounds act as specific carriers for Na and K across lipid barrier, and that the structure of the paraffin chains of the lipids are voltage dependent. Thus when the chains stiffen, the carrier does not move any more. A simple model of that kind can take into account several events that take place in a nerve membrane. I might comment that these antibiotics have been divided into two classes : those which behave like carriers, with monomolecular kinetics; and those that behave like pores, which display multi-molecular kinetics. To some extent the pores are dependent on the structure of the membrane, while the carriers are not. These two classes are fairly sharp-cut : some antibiotics behave like carriers; other like pores.

J. MONOD : These are interesting models of how the choice could be made between sodium and potassium. But, as anyone ever observed or found antibiotics and polypeptides of that kind in membrane preparations ?

A.D. MCLACHLAN : I would like to say that as well as there being two classes — the carriers and the pores — there may also be another class of antibiotics which simply disturb the structure of the lipid, which disorder the lecithin molecules and thus make the membrane porous. I do not know if there is any proof of the idea that regular channels of antibiotic molecules do appear in a membrane. I would like to ask if anyone knows if this has been demonstrated.

M. KLINGENBERG : May I just come back to the problem of the nature of the actual carrier in the membrane. I think Dr. Kennedy would also agree that most of the carriers in biological membranes can be considered to be long chain proteins and not oligopeptides. Here I think of a well-defined biological cation carrier system such as the calcium carrier in the cytoplasmatic reticulum as well as in the Na-K-pump of plasma membranes. These cation carriers as well as other metabolite carriers are long chain peptides, like other enzymes. Therefore the low molecular peptide carriers may not be a good model for the biological carriers existing in most of the membranes.

M. LUZZATI : Pr. Monod, it occurs to me that the beginning of an answer to that may exist. Indeed several cycling antibiotics, and many of the uncouplers of oxydative phosphorylation — DNP and compounds of that kind — have been studied both in membranes and in lipid bilayers.

It has been shown that several parameters have similar effects in membranes and in bilayers. So, there are very firm reasons to believe that the action of the uncouplers is similar in bilayers and in mitochondria; the same occurs with the cyclic antibiotics. Is this a beginning of an answer ?

J. MONOD : My question was a very simple analytical one. You take a membrane preparation from a cell that you know has a sodium pump. Do you find cyclic polypeptide in that preparation ? That is my question.

M. LUZZATI : In any event the cyclic polypeptides cannot do the whole job of the pump.

J. MONOD : You will still need that for the choice.

E. P. KENNEDY : Of course, the real trouble is that there are so few examples in which anything is known about biochemistry of ion transport. It seems premature to rule out any plausible mechanism. However, if one considers ion transport systems in bacteria, such as the transport of sulfate in *Salmonella* studied by Pardee and his collaborators, the binding of ions appears to require specific proteins. In the case of the sodium and potassium pump of animal tissues, as you know the very important work of Scou strongly indicates that an ATPase, activated by sodium plus potassium, is an essential component of the pump. So, in several examples, it appears that proteins are carriers for ions. However, one cannot exclude the possibility that a compound like valinomycin could be a prosthetic group for a protein. It could seem too soon to rule out any plausible mechanism but I think in the few examples that have been studied, there is a strong implication that proteins are involved in ion transport.

M. KLINGENBERG : In this context the uncouplers of oxidative phosphorylation might be mentioned. They are quite specific carriers of protons for the membranes, and, of course, artifacts. Therefore uncouplers of artificial substance are only a functional model for biological proton carriers, but not structural models for them. This situation is analogous to the potassium transport, where the oligopeptide carrier may be a good model for some particular part of its function but not for other aspects which are linked to high molecular carriers and to the structure of the carriers.

Journée du 1er juillet 1969

Troisième séance

STRUCTURES DISSIPATIVES EN BIOLOGIE

PRESIDENT L. ROSENFELD

I. PRIGOGINE

Dissipative structures in biological systems

Discussions

De la Physique théorique à la Biologie, C.N.R.S., 1971.

INTRODUCTION

L. ROSENFELD

Unfortunately, I must open this session with a piece of had news : Professor Katchalsky who was scheduled to speak after Professor Prigogine, has been prevented by illness from coming. It is the intention of the Organizing Committee to send him a message and I may take it for granted that you all agree to join in this token of our sympathy for him. (Applause)

This morning, I had the feeling that the biologists gave us hope that we could find some simplicity in life processes. However, this afternoon they were again reminding us physicists of the terrible complexity of the biological structures. Now, if the biologists look at this blackboard they may perhaps have the impression that the physicists are going to take a revenge. I hope however that Pr. Prigogine will not lose sight of the special purpose of this conference and be as kind to the biologists as the biologists have been kind to us. Let us assume that these forbidding formulas are just there as an ornamental background for his talk.

De la Physique théorique à la Biologie, C.N.R.S., 1971.

DISSIPATIVE STRUCTURES IN BIOLOGICAL SYSTEMS *

I. PRIGOGINE

*Faculté des Sciences, Université Libre de Bruxelles **,*
Belgium

1. Introduction

As has been emphasized at this conference, many important steps in molecular biology involve only a small number of interacting molecules, but even so, these molecules have to come together at the right moment and in the right configuration. This is a " traffic problem " which involves a large number of degrees of freedom. Consideration of problems involving few molecules or many molecules are in my opinion not exclusive but complementary.

Any reference to order or to deviation from randomness implies some reference to a given set of laws. It would be very astonishing if ten molecules orbit around each other as a planetary system. References to order also involve a characteristic scale factor (for example, this scale factor is of the order of a few angstroms in classical liquids, while it is in the macroscopic range, i.e. 1 cm, for hydrodynamic problems).

The most detailed analysis of order made in physics refers to equilibrium situations. Everybody knows that the entropy reaches a maximum at equilibrium for an isolated system, the free energy a minimum for a system of given temperature and volume, and so on. (The relation between this macroscopic description and classical or quantum dynamics is far from being trivial, but will not be dealt with here [1]. Briefly, each configuration contributes to equilibrium through its number of " complexions " (or its statistical weight). One of the most interesting problems of statistical physics and thermodynamics (apart from any possible application to biology) is the extension of the concept of thermodynamic order to non equilibrium situations.

How will the processes which go on in a given system modify the concept of order ? To borrow an expression introduced by Paul Weiss [2], we must study molecular ecology, analyze the order in terms of population dynamics and compare it with the order in equilibrium systems. A first step in this direction

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** Also Center of Statistical Mechanics and Thermodynamics, University of Texas, Austin, Texas, U.S.A.

has been made through the development of non equilibrium thermodynamics of irreversible processes beyond the linear range. Since my report at the First International Conference on Theoretical Physics and Biology in 1967 [3] much progress has been achieved : the thermodynamic theory of dissipative structures which may occur in far from equilibrium conditions has been worked out in greater detail and the existence of such structures has been confirmed both by machine calculations and by laboratory experiments. Furthermore, the role of dissipative structures in specific biological problems such as metabolic processes, membranes or morphogenetic motions, is now being investigated. It seems likely that many interesting developments will occur along these lines in the near future.

I shall now present a report on the progress in this field since 1967, but in order to make this report self contained, let us begin with a few remarks.

2. Macroscopic laws and fluctuations

One generally associates classical physics with a deterministic, causal description, but even in problems involving a large number of degrees of freedom such a description may no longer be sufficient.

Consider a fluid in laminar flow. Suppose there appears a small fluctuation δE_{kin} in the kinetic energy. To this fluctuation will correspond some small " hump " in the velocity profile (see fig. 2.1)

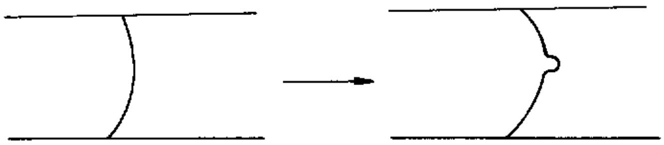

Fig. 2.1. Fluctuation in the velocity profile.

If δE_{kin} vanishes for $t \to \infty$, the flow is stable; on the contrary, if δE_{kin} increases with time, then a new state of flow will be reached. As is well known, this will be so if the Reynolds number lies beyond the critical value corresponding to turbulence.

The main problem is therefore the following : a new structure (such as that involved in the transition from the state at rest to the cellular convection in the Bénard problem) is always the result of an instability. It originates in a fluctuation. Whereas a fluctuation is normally followed by a response that brings the system back to the unperturbed state, on the contrary, at the point of formation of a new structure, fluctuations are amplified. This is of course the basis of classical stability theory derived from the normal mode analysis [4]. One considers small perturbations which satisfy linear equations of evolution.

The time dependence of each normal mode is of the form exp ωt where ω is in general a complex quantity $\omega_r + i\,\omega_i$, solution of the dispersion equation. The stability condition implies then that for each normal mode :

$$\omega_r < 0 \tag{2.1}$$

What we want is to relate the stability theory to thermodynamics of irreversible processes in order to obtain as much information as possible independently of detailed normal mode analysis.

Clearly we must then incorporate in some way in our thermodynamic description the response of the system to fluctuations, in other words we have to build a *generalized thermodynamics* which will also include a macroscopic theory of fluctuations.

It should be understood that the existence of many degrees of freedom in a molecular system implies automatically the existence of fluctuations. Therefore, we really are concerned with the response of the system to its spontaneous fluctuations.

3. Generalized thermodynamics

We first present a brief summary of this generalized thermodynamics. More details may be found elsewhere [5, 6].

Let us start with the balance equation for entropy [7, 8] :

$$dS = d_e S + d_i S \tag{3.1}$$

where d_eS denotes the contribution of exchanges with the outside world and d_iS the entropy production due to the irreversible processes inside the system. The second law of thermodynamics postulates that

$$d_i S \geqslant 0 \tag{3.2}$$

Classical thermodynamics is concerned essentially (but by no means exclusively) with equilibrium situations where entropy production vanishes. On the contrary thermodynamics of irreversible processes studies non equilibrium situations on the basis of their entropy production.

As is well known [7, 8, 9] the explicit form of entropy production can easily be derived using the balance equations for matter, momentum and energy once the assumption of " *local equilibrium* " is introduced. This means that collisional effects are sufficient to eliminate large deviations from equilibrium. One obtains in this way the basic bilinear expression for the entropy production per unit time :

$$P = \frac{d_i S}{dt} = \int_V dV\, \sigma\,[S] = \int_V dV \sum_\alpha J_\alpha X_\alpha \geqslant 0 \tag{3.3}$$

Here J_α and X_α are the flows (or rates) of the irreversible processes and the corresponding generalized forces (for more details see [7, 8]). In the case of chemical reactions we have

$$J_\alpha = v_\alpha$$

$$X_\alpha = \frac{A_\alpha}{T} = \frac{\Sigma\, \nu_{\gamma\alpha}\mu_\gamma}{T} \tag{3.4}$$

where v_α is the reaction rate and A_α the corresponding affinity. In the *linear range* of thermodynamics of irreversible processes we have (see [7, 8])

$$J_\alpha = \sum_{\alpha'} L_{\alpha\alpha'}\, X_{\alpha'} \tag{3.5}$$

This assumption of linear relation between flows and forces is specially restrictive in the following two cases :

a) *inertial effects* : for example, hydrodynamic circulation as a result of thermal gradients (the so-called Bénard instability, see [3, 4]) is not included in the linear approximation;

b) *chemical effects* : to obtain a linear law between reaction rate and affinity we need the condition (see [7])

$$\left|\frac{A}{RT}\right| < 1 \tag{3.6}$$

which is generally not satisfied except in the immediate neighborhood of equilibrium.

How can we go beyond this approximation and in particular include the effects of fluctuations ?

A precious hint as to how this can be done is provided by the basic Einstein formula for an isolated system (see [10, 11] for an excellent summary of fluctuation theory) :

$$P_r \sim \exp \frac{\Delta S}{k} \tag{3.7}$$

where ΔS is the change of entropy associated with the fluctuations. Neglecting higher order terms we may also write, for small fluctuations,

$$P_r \sim \exp \frac{1}{2k}(\delta^2 S) \tag{3.8}$$

The work of Greene and Callen (1951) [12], Tisza and Quay (1963) [13] definitely establish the validity of (3.8) for small fluctuations around equilibrium.

Does (3.8) remain valid for non equilibrium conditions ? For all systems which may be described by the local equilibrium assumption, expression (3.8) keeps its meaning and it can be shown that (see [5])

$$\delta^2 S < 0 \tag{3.9}$$

It is therefore very tempting to assume the validity of (3.8) even for fluctuations in non-equilibrium conditions [14, 15, 16].

This has been substantiated recently by detailed calculations based on stochastic models (Nicolis and Babloyantz) [17, 18]. They have shown that Einstein's formula remains valid whenever there exists a separation between the time scales of the fluctuating system and the " outside world ". As a matter of fact, such a separation is always implied in the usual macroscopic description of non equilibrium situations; at each time it is assumed that well defined boundary conditions are prescribed, independently of the actual fluctuating state of the system.

The Einstein theory thus shows that one may incorporate the effect of fluctuations by studying the time evolution of $\delta^2 S$. This is precisely the central point of our approach. *We derive a balance equation not, as in (3.1), for the total entropy S but for the quantity* $\delta^2 S$ *which measures the fluctuations.* This permits us to distinguish immediately between situations where fluctuations will regress or be amplified.

It is worth noting that in a very interesting paper, G.N. Lewis [19] has already proposed to unify fluctuation theory and thermodynamics. He was however concerned only with equilibrium situations where the effect of fluctuations is generally negligible (with the exception of critical phenomena).

Our approach to the stability problem agrees completely with the basic ideas of Liapounoff's theory [20]. We may say that we choose once for all $\delta^2 S$ as the Liapounoff function. Indeed $\delta^2 S$ not only has a well defined sign in the whole range of macroscopic physics, but also its time evolution has a deep physical meaning related to the regression or amplification of fluctuations.

We believe that our approach combines various aspects treated independently till now (classical thermodynamic stability theory, Einstein's fluctuation theory, Liapounoff stability theory, etc.), and increases substantially the power of macroscopic methods. Let us consider in more detail the case of chemical reactions in open systems.

4. Chemical reactions in open systems

We consider a sequence of reactions such as

$$A \rightleftharpoons \boxed{X, Y, Z} \rightleftharpoons F$$

where the concentration of the initial and final compounds A and F is maintained constant through continuous exchanges with the outside world.

X, Y, Z are the intermediate compounds. In each problem there exists a parameter which expresses the thermodynamic constraint : here it may for example be the ratio of the concentration of A and F. If this ratio is taken as equal to that corresponding to minimum free energy (or equivalent to the law of mass action), the system will evolve to equilibrium. In other words intermediates will also evolve to values corresponding to the law of mass action.

In general if we use a non-linear scheme of reactions, there may be different time independent solutions of the kinetic equations

$$\frac{dX}{dt} = 0 \ , \ \frac{dY}{dt} = 0 \ , \ \frac{dZ}{dt} = 0 \ , \tag{4.1}$$

all satisfying obvious physical conditions : the concentrations must be real, positive quantities, etc... For given values of A and F, one of these solutions, say X_1, Y_1, ... contains as a special case the equilibrium solution corresponding to the minimum of free energy. Will this solution be stable for any deviation from equilibrium ?

In accordance with the general theory outlined in § 3 we have to calculate the quantity :

$$\frac{d(\delta^2 S)}{dt} \tag{4.2}$$

We shall call this quantity the excess entropy production and denote it by the symbol δ_x P. If we have

$$\frac{d(\delta^2 S)}{dt} > 0 \tag{4.3}$$

the system is stable. On the contrary, if

$$\frac{d(\delta^2 S)}{dt} < 0 \tag{4.4}$$

for perturbations satisfying the kinetic equations, the system is unstable.

Therefore the transition occurs when

$$\frac{d(\delta^2 S)}{dt} = 0 \tag{4.5}$$

These conditions are derived and discussed in [5, 6]. In our earlier work we derived the thermodynamic stability condition from our " reversible " evolution criterion (see [21, 22, 23, 7, 3]). The result is of course the same for the simple cases we discuss here. For chemical reactions we obtain

$$\delta_X P = \frac{d(\delta^2 S)}{dt} = \sum_a \delta v_a \delta A_a \tag{4.6}$$

Instabilities can occur only if at least one term in this sum is negative. As we have already shown in our preceding report [3] (see also [7]) this implies that some of the steps involved must have an autocatalytic or cross catalytic character.

Later on we shall study some examples in detail. It must be emphasized that instabilities are only possible far from equilibrium, beyond the range of linear relations of the type (3.5).

We shall focus our attention on instabilities with respect to space dependent inhomogeneous perturbations that may lead to states no longer homogeneous in space. These are " symmetry breaking " instabilities [3, 24, 25, 26].

The close relation between instabilities and chemical oscillations [3, 7] must be stressed. Two remarks should be kept in mind :

1°) Since it is easily shown that oscillations around steady states can only occur if the system is far from equilibrium (outside the range of linear thermodynamics of irreversible processes [7]), it is clear that in systems where they are observed, the basic condition for the possible occurrence of symmetry breaking instabilities is fulfilled.

2°) Undamped oscillations may arise both around *stable* steady states (the real part of the frequency corresponding to small perturbations then vanishes) and around *unstable* steady states. A simple example of the first category is the well-known Lotka-Volterra mechanism (for more details see [27]), but in this case the characteristics of the oscillations depend on the perturbations and this leads to an infinite number of possible limit cycles.

On the contrary, should the oscillations proceed around an unstable steady state, the number of limit cycles can become very small, and the characteristics of the phenomenon will be independent of the initial perturbations [28].

These remarks dealing with the general theory are now followed by a detailed review of a simple example investigated by Lefever by computer calculations.

5. Example of a chemical instability

A very simple scheme of reactions which may give rise to instabilities is the following.

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X \tag{5.1}$$

$$2X + Y \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} 3X \tag{5.2}$$

$$B + X \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} Y + D \tag{5.3}$$

$$X \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E \tag{5.4}$$

As the concentrations A, B, D, E are held constant, there are only two independent variables.

This scheme is obviously not very realistic because it involves the third order step (5.2). Some results concerning this scheme (see [3]) have already been given but as it has been analyzed in greater detail by Lefever [26], it will again be used to illustrate some interesting features of chemical instabilities. If one neglects all reverse reactions, the kinetic equations (including diffusion) are

$$\frac{\partial X}{\partial t} = k_1 A + k_2 X^2 Y - k_3 BX - k_4 X + D_x \frac{\partial^2 X}{\partial t^2}$$

$$\frac{\partial Y}{\partial t} = k_3 BX - k_2 X^2 Y + D_Y \frac{\partial^2 Y}{\partial t^2} \tag{5.5}$$

It is simpler to consider one dimensional diffusion with periodic boundary conditions. It is easy to verify that (5.5) admits the homogeneous steady state solution

$$X_0 = \frac{k_1}{k_4} A \ ; \ Y_0 = \frac{k_3 k_4}{k_1 k_2} \frac{B}{A} \tag{5.6}$$

The stability of this solution is studied using normal mode perturbations of the form

$$X = X_0 + x \, e^{(\omega t + \frac{ir}{\lambda})}$$

$$Y = Y_0 + y \, e^{(\omega t + \frac{ir}{\lambda})} \tag{5.7}$$

As already indicated in our previous report there exists a critical wave length λ_c given by

$$\lambda_c^2 = \left(\frac{k_4}{k_1^2 k_2}\right)^{1/2} \frac{(D_x D_y)^{1/2}}{A} \tag{5.8}$$

As the computational problem involved in the study of the time evolution beyond the instability is rather complicated, a further simplification is introduced by considering instead of disturbances or arbitrary wavelength, a two box model. In this system the initial and final products are distributed homogeneously, whereas X and Y may diffuse freely between the two parts. Instead of (5.5) there are now four equations which are written in the form

$$\frac{dX_1}{dt} = A + X_1^2 Y_1 - BX_1 - X_1 + D_X(X_2 - X_1)$$

$$\frac{dX_2}{dt} = A + X_2^2 Y_2 - BX_2 - X_2 + D_X(X_1 - X_2)$$

$$\frac{dY_1}{dt} = BX_1 - X_1^2 Y_1 + D_Y (Y_2 - Y_1)$$

$$\frac{dY_2}{dt} = BX_2 - X_2^2 Y_2 + D_Y (Y_1 - Y_2) \tag{5.9}$$

when all the kinetic constants are put equal to one.

As in (5.6) we still have a single time-independent homogeneous solution

$$X_i = A \quad ; \quad Y_i = \frac{B}{A} \qquad (i = 1, 2) \tag{5.10}$$

We make the following choice of numerical values for the diffusion coefficient of X and for A

$$D_X = 1 \quad , \quad A = 2 \tag{5.11}$$

as a result there are *two* arbitrary parameters whose values determine the properties of the steady states. Elementary transformations on the conservation equations yield the system of equations (these equations are derived in [3]).

$$\begin{aligned} &3X_2^5 - 30\,X_2^4 + [96 + 2\,D_Y\,(B + 3)]\,X_2^3 \\ &\qquad - [96 + 12\,D_Y\,(B + 3)]\,X_2^2 + 16\,D_Y(6 + B)\,X_2 - 96\,D_Y = 0 \end{aligned} \tag{5.12}$$

$$X_1 = 4 - X_2 \tag{5.13}$$

$$\begin{aligned} Y_2 = {} & B(8X_2^2 - 4D_Y - 16\,X_2 - X_2^3)/[8X_2^3 - 3X_2^2\,(D_Y + 8) \\ & + 8\,D_Y\,(X_2 - 2) - X_2^4] \end{aligned} \tag{5.14}$$

$$Y_1 = Y_2 + (X_2^2\,Y_2 - BX_2)/D_Y \tag{5.15}$$

This system has two types of solutions :
the homogeneous steady state solution given by (5.10) and an inhomogeneous solution which, because of the symmetry of the model, may be written in two equivalent ways

$$\begin{aligned} \text{either} \quad X_1 > X_2 \quad \text{and} \quad Y_1 < Y_2 \\ \text{or} \quad X_1 < X_2 \quad \text{and} \quad Y_1 > Y_2 \end{aligned} \tag{5.16}$$

The stability analysis of the steady state solutions of (5.12)-(5.15) has been performed both for homogeneous and inhomogeneous fluctuations. One finds that the homogeneous state is unstable with respect to homogeneous perturbations when

$$B > 5 \tag{5.17}$$

and with respect to inhomogeneous perturbations when

$$B > B_C = \frac{1}{2D_Y}\,(6D_Y + 12) \tag{5.18}$$

These results combined with those obtained for the inhomogeneous steady state are shown in fig. 5.1.

Conditions (5.17)-(5.18) define a domain I where only the homogeneous steady state exists and is stable. This state becomes unstable with respect to homogeneous perturbations in II and with respect to inhomogeneous perturbations beyond the curve (b). In regions II, III, V no time independent stable state

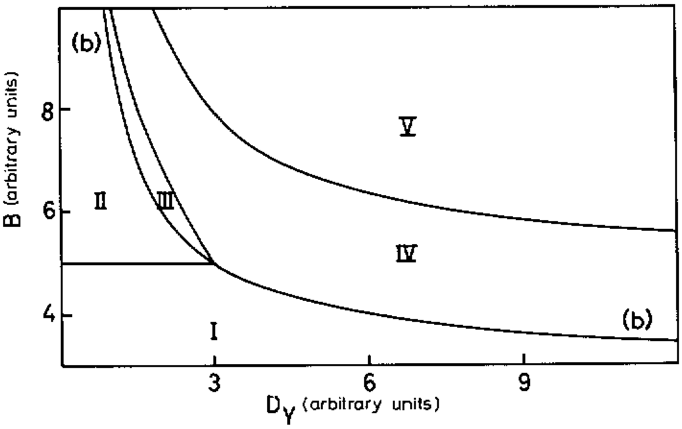

Fig. 5.1. Stability of steady states as a function of B and D_Y.

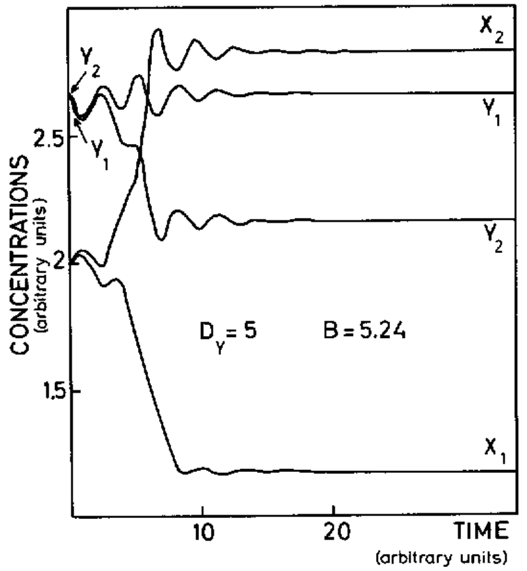

Fig. 5.2. A perturbation of the homogeneous steady state. Concentration of Y in box 2 increases the rate of production of X in that box (due to the autocatalytic step). This effect grows till the new state is reached.

exists, but in region IV the inhomogeneous steady state is stable. This region corresponds therefore to what we call a dissipative structure. It is particularly interesting to investigate how such an inhomogeneous state is reached. A typical result is reported in fig. 5.2.

The homogeneous state corresponding to $X_1 = X_2 = 2$ and $Y_1 = Y_2 = 2.62$ is destroyed by a small fluctuation $Y_2 - Y_1 = 0.04$. It is clearly seen how the initial perturbation is magnified till the inhomogeneous steady state is reached. The configuration (5.16) chosen by the system depends crucially on the nature of the initial perturbations.

In fig. 5.3 we see how a perturbation around the inhomogeneous state may lead to the exchange of the two inhomogeneous solutions corresponding to the two box problem.

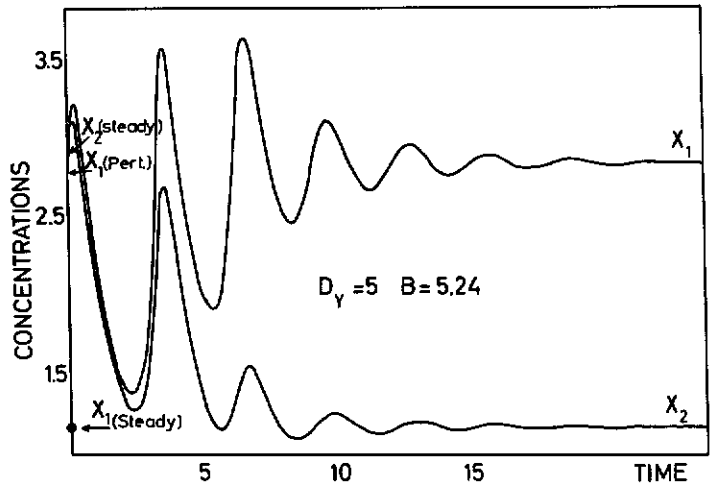

Fig. 5.3. Exchange of stability. At the initial time we have $X_1 = 2.6$ and $X_2 = 2.829$ while the steady state values are given by $X_1 = 1.170$ and $X_2 = 2.829$.

The important point which must be remembered is that *small fluctuations can no longer reverse the configurations*. This reversal only occurs if there are variations in the steady state concentrations comparable to the difference between the two boxes. Thus the possibility of an oscillatory behavior between the two accessible configurations due to random disturbances is clearly out of question.

It is interesting to note that in agreement with our remark in § 2, the description of the two box system involves a statistical element. In addition to the " causal " laws (5.9) we have to know in which box will appear the fluctuation determining the choice between the two solutions (5.16); the subsequent evolution of the system will be different accordingly. This choice appears therefore as a primitive type of " information " which has to be fed into the system and which in addition to the causal laws determines the future evolution of the system.

Fig. 5.4 shows the typical behavior of the system in the region where a limit cycle is observed. Whatever the initial conditions the system reaches the same periodic behavior. This is a " chemical clock ".

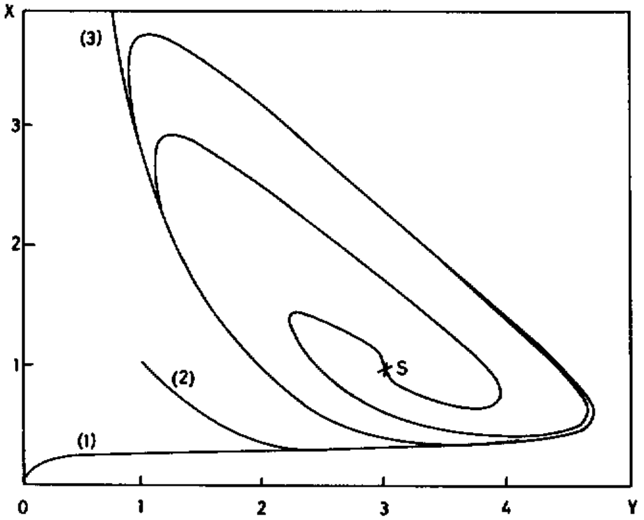

Fig. 5.4. Limit cycle corresponding to region V in Fig. 5.1.

We see that 5.1 represents a kind of non-equilibrium phase diagram. According to the regime considered specific time or space organization is found. The results obtained with the two box model remain valid when diffusion is represented in a more realistic way, considering the steady state distribution for a large number of subsystems. Results are reported in Fig. 5.5 and 5.6.

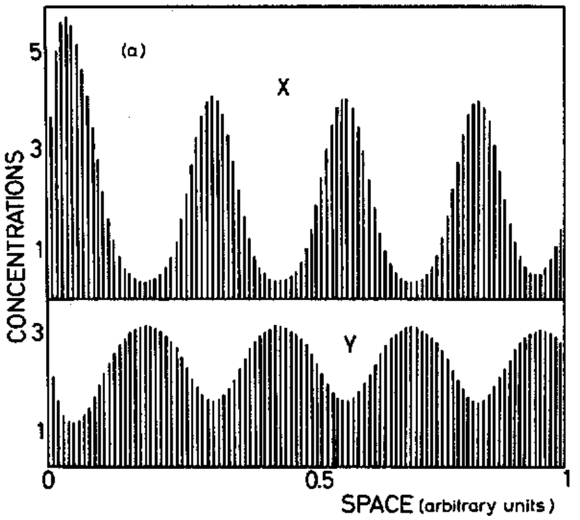

Fig. 5.5. Steady state distribution for a system with fixed X and Y concentrations at the boundaries (points 1 and 101 where the values of X and Y are maintained equal to the homogeneous steady state values X = Y and Y = 2.62.).

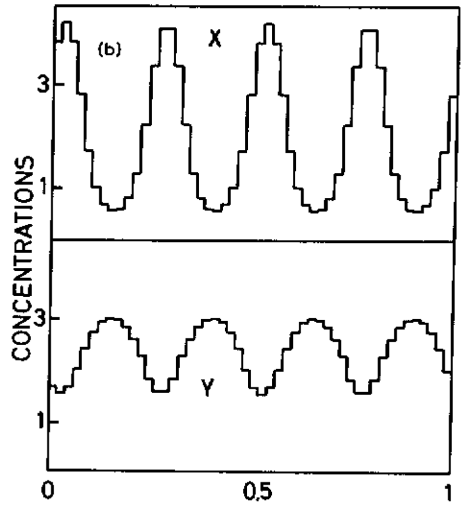

Fig. 5.6. Steady state distribution for a system of 50 boxes with periodic boundary conditions.

In both cases X and Y dominate alternatively over a number of regions yielding similar patterns. This is a striking example of dissipative structure, i.e. a structure created under the influence of far from equilibrium conditions. Seen this way this organisation can be brought of in terms of giant fluctuations which do not decay but are stabilized by the flow of energy and matter from the boundaries.

The example discussed in this section establishes the existence of chemical instabilities and of symmetry breaking dissipative structures, thus confirming the conclusions reached by Turing in his 1952 paper [30] and the discussion presented in our preceding report [3]. The next chapter is devoted to a dissipative structure that has been observed experimentally.

6. The Zhabotinsky reactions

A.M. Zhabotinsky and his coworkers have studied extensively the oxydation of malonic acid in the presence of cerium sulfate and potassium bromate. (for a detailed description see the papers of Zhabotinsky and coworkers [31]). The remarkable feature is the occurrence of reproductible time oscillations in the concentrations of Ce^{4+} and Ce^{3+} which are easily studied through spectrometric methods. Fig. 6.1 gives an example of such a behavior.

The mechanisms of the Zhabotinsky reactions are not known. One would expect three global reactions (see Degn [32])

a) oxydation of the malonic acid

$$CH_2(COOH)_2 + 6\,Ce^{4+} + 2H_2O \longrightarrow 2\,CO_2 + HCOOH + 6\,Ce^{3+} + 6H^+ \quad (6.1)$$

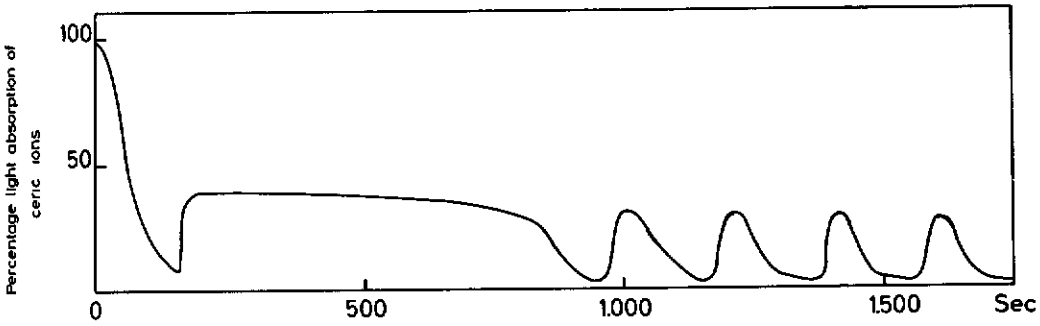

Fig. 6.1. Time oscillation of Ce^{4+} for the Zhabotinsky reactions (cfr. Degn [32]).

b) oxydation of the cerium ions

$$10\,Ce^{3+} + 2\,HBrO_3 + 10\,H^+ \longrightarrow 10\,Ce^{4+} + Br_2 + 6H_2O \qquad (6.2)$$

c) transformation of malonic acid into bromomalonic acid

$$CH_2(COOH)_2 + Br_2 \longrightarrow C_3H_3BrO_4 + Br^- \qquad (6.3)$$

The transformation Ce$^{4+} \rightarrow$ Ce^{3+} in reaction (6.1.), is compensated by the transformation Ce$^{3+} \rightarrow$ Ce^{4+} in reaction (6.2).

As these oscillations are reproductible they could correspond to a limit cycle around an *unstable* thermodynamic state (see Fig. 5.4). A comparison with the example studied in § 5 shows that for other values of the concentrations one could expect a space organization corresponding to a *symmetry breaking dissipative structure*. This has been verified very recently by Büsse [33]. His results have been repeated in our laboratory and Fig. 6.2 shows a typical space dependent structure.

As this reaction is going on in a closed system the structure can only maintain itself for a limited time (for example 30 mins.), after which the system approaches equilibrium, jumps to the thermodynamic branch and goes back to a *homogeneous* distribution of matter. As expected the transition is sharp.

This example shows that dissipative structures occur in nature : scalar free energy can give rise to an organisation in space implying an abnormally low entropy.

7. Dissipative structures in multienzymatic reactions

Recently there has been a remarkable accumulation of theoretical and experimental data on short time oscillations in biochemical reactions (see [31, 34]). As in the Zhabotinsky case, these oscillations correspond to limit cycles around unstable thermodynamic states [35, 36], and one may therefore

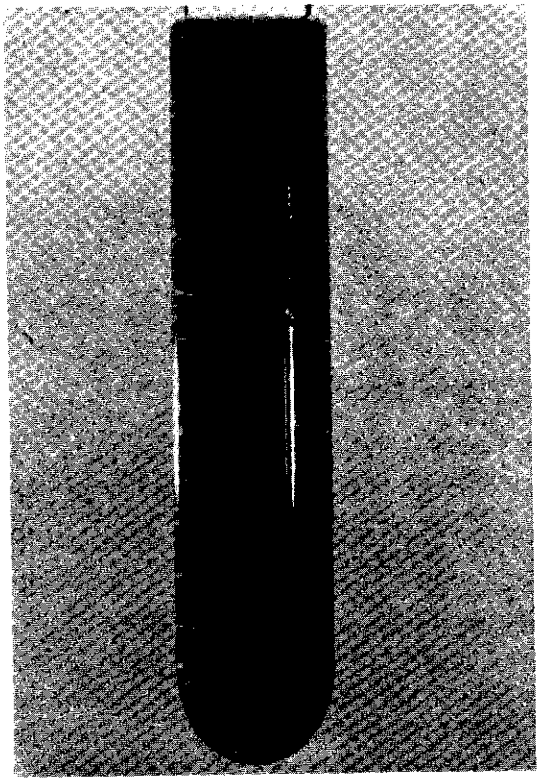

Fig. 6.2. Dissipative structure observed for the Zhabotinsky reaction.

investigate the possibility of dissipative symmetry breaking instabilities leading to a spatial organization in such systems.

Three characteristic examples typical of biochemical processes, but presenting widely different catalytic properties, have been studied recently from that point of view [37] :

a. – the model of Chernavskaia and Chernavskii [35] for the *dark part of photosynthesis*;

b. – a substrate and product inhibited enzymatic reaction (Selkov [31]);

c. – the well-known product activated enzymatic reaction of *phosphofructokinase in the glycolytic cycle* [34, 36].

Each of these processes had already been studied, but only in connection with the problem of *sustained chemical oscillations.* In each case a model yielding satisfactory results has been proposed. We do not intend to discuss possible improvments of such models, but rather *show that within the framework of the approximations leading to a good representation of their time-dependent behavior, the same biochemical systems can become unstable with respect to* *diffusion. This indicates that the existence of dissipative structures is compatible with the experimental and theoretical data presently found in the literature.* The photosynthesis case will not be discussed as it corresponds to a quite complex set of chemical reactions. Instead the other two examples will be considered.

a) *Substrate and product inhibited enzymatic reaction*

A model proposed by Sel'kov [38] for such a process is the following

$$\xrightarrow{v_i} \mathrm{S}_1 + \mathrm{E} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{S}_1\mathrm{E} \qquad (7.1)$$

$$S_1\mathrm{E} \xrightarrow{k_{+3}} \mathrm{E} + \mathrm{S}_2 \xrightarrow{v_f}$$

$$\mathrm{S}_1 + \mathrm{S}_1\mathrm{E} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{S}_1\mathrm{S}_1\mathrm{E} \qquad (7.3)$$

$$\mathrm{S}_2 + \mathrm{E} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} \mathrm{E}\mathrm{S}_2 \qquad (7.4)$$

$$\mathrm{S}_2 + \mathrm{S}_1\mathrm{E} \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} \mathrm{S}_1\mathrm{ES}_2 \qquad (7.5)$$

$$\mathrm{S}_2 + \mathrm{S}_1\mathrm{S}_1\mathrm{E} \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} \mathrm{S}_1\mathrm{S}_1\mathrm{ES}_2 \qquad (7.6)$$

where :

- S_1 represents the substrate, S_2 the product, E the enzyme and $\mathrm{S}_1\mathrm{E}$ the *active enzyme substrate complex*;
- ES_2, $\mathrm{S}_1\mathrm{S}_1\mathrm{E}$, $\mathrm{S}_1\mathrm{ES}_2$ and $\mathrm{S}_1\mathrm{S}_1\mathrm{ES}_2$ are inactive enzymatic complexes;
- v_i is the rate at which S_1 enters the system and is given by

$$v_i = V_0 - k_{-1}\, s_1$$

- v_f is the rate at which S_2 disappears. It is assumed to be of the form

$$v_f = \frac{V s_2}{K_m + s_2}\ ,$$

following an enzymatic irreversible reaction (V is the maximum rate when $s_2 \to \infty$ and K_m is the Michaelis constant of the reaction*).

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* In the derivation of (7.7) (7.8) it is assumed that

$$s_1 \approx s_2 \approx 1\ ;$$
$$k_{+4} = k_{+5} = k_{+6}\ ;$$
$$k_{-4} = k_{-5} = k_{-6}\ ;$$
$$k_{\pm 1},\ k_{\pm 3},\ k_{\pm 4},\ k_{+2} \gg 1.$$

The kinetic equations describing the system are then given by :

$$\frac{\partial s_1}{\partial t} = \mathrm{v}_i - \frac{k_{+2}\,\mathrm{e}_0 \dfrac{s_1}{\mathrm{K}_{s1}}}{\left(1 + \dfrac{s_2}{\mathrm{K}_{s2}}\right)\left[1 + \dfrac{s_1}{\mathrm{K}_{s1}} + \dfrac{\mathrm{K}_{s1}}{\mathrm{K}'_{s1}}\left(\dfrac{s_1}{\mathrm{K}_{s1}}\right)^2\right]} + \mathrm{D}_{s1}\frac{\partial^2 s_1}{\partial r^2} \tag{7.7}$$

$$\frac{\partial s_2}{\partial t} = -\,\mathrm{v}_f + \frac{k_{+2}\,\mathrm{e}_0 \dfrac{s_1}{\mathrm{K}_{s1}}}{\left(1 + \dfrac{s_2}{\mathrm{K}_{s2}}\right)\left[1 + \dfrac{s_1}{\mathrm{K}_{s1}} + \dfrac{\mathrm{K}_{s1}}{\mathrm{K}'_{s1}}\left(\dfrac{s_1}{\mathrm{K}_{s1}}\right)^2\right]} + \mathrm{D}_{s2}\frac{\partial^2 s_2}{\partial r^2} \tag{7.8}$$

where

$$\mathrm{K}_{s1} = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad ; \quad \mathrm{K}'_{s1} = \frac{k_{-3}}{k_{+3}} \quad ; \quad \mathrm{K}_{s2} = \frac{k_{-4}}{k_{+4}} \tag{7.9}$$

and e_0 is the total quantity of enzyme. Putting

$$\alpha = \frac{\mathrm{K}_{s1}}{\mathrm{K}'_{s1}} \tag{7.10}$$

the instability with respect to diffusion can be expressed in terms of this parameter. The critical value beyond which the system becomes unstable is given by :

$$\alpha_c = \frac{\mathrm{D}_{s2}\mathrm{K}_{s2}}{\mathrm{D}_{s1}\mathrm{K}_{s1}} \cdot \frac{\zeta^2(2\beta - \mathrm{V}_0)^2}{\beta^2(\mathrm{V}_0 - \beta)^2} \cdot \frac{1}{\left[1 + \sqrt{\dfrac{\mathrm{V}_0 - \beta}{\beta}}\right]\left[1 + \sqrt{\dfrac{\beta}{\mathrm{V}_0 - \beta}}\right]} \tag{7.11}$$

and corresponds to the critical wavelength

$$\lambda_c^2 = \frac{(\mathrm{V}_0 - \beta) + \sqrt{\beta(\mathrm{V}_0 - \beta)}}{\xi\,(2\beta - \mathrm{V}_0)} \cdot \frac{\mathrm{D}_{s1}\,\mathrm{K}_{s1}}{k_{+2}\,\mathrm{e}_0} \tag{7.12}$$

with

$$\xi = \frac{k_{-i}\,\mathrm{K}_{s1}}{k_{+2}\,\mathrm{e}_0} \quad ; \quad \mathrm{V}_0 = \frac{\mathrm{v}_0}{k_{+2}\,\mathrm{e}_0} \quad ; \quad \beta = \frac{\mathrm{V}}{k_{+2}\,\mathrm{e}_0} \tag{7.13}$$

(7.11) and (7.12) yield physically acceptable values when

$$\frac{\mathrm{v}_0}{2} < \beta < \mathrm{v}_0$$

In this case, for almost equal diffusion coefficient of S_1 and S_2 the instability is enhanced when product inhibition is large.

b) *Product activated enzymatic reaction*

Catalytic processes of this type are rather exceptional in biology. Nevertheless the reaction of phosphofructokinase is well known, and can be described by the following model (see [36, 39]) :

$$\xrightarrow{v_1} A_3 \tag{7.14}$$

$$A_3 + D_1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} D_3 \tag{7.15}$$

$$D_3 \xrightarrow{k_{+2}} D_1 + A_2 \tag{7.16}$$

$$\gamma A_2 + D_2 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} D_1 \tag{7.17}$$

$$A_2 \xrightarrow[k_2]{} \tag{7.18}$$

— A_3 and A_2 are ATP and ADP ;

— D_1, D_3, D_2 respectively the active form of phosphofructokinase, the complex enzyme-substrate, and the inactive form of the enzyme.

Reactions (7.14), (7.15), (7.16), (7.18) are simply the rates of income of substrate and going out of product, and coupled with a so called Michaelis-Menten process. It is not of course this part of the mechanism that is responsible for the instability. The time-independent steady states are destabilized by reaction (7.17), where activation of the enzyme is catalyzed by the product of reaction. The coefficient γ must not be considered as a stoechiometric coefficient, but rather as a parameter whose value is adjusted so as to make the time behavior of the system agree with experiments. In this way the inhibition of the enzymatic activity by the substrate is taken into account. This inhibitive effect could cause instabilities for small values of v_1 *.

Indeed when this is the case, and under quasi stationary conditions the *critical value of* v_1 below which the instability appears is :

$$v_1 = \sqrt[\gamma]{\left(\frac{k_{-1} + k_{+2}}{k_{+1} \, . \, k_{+2}}\right) \left(\frac{k_{-3}}{k_{+3}}\right) \frac{k_2^{\gamma+1}}{e_0} \cdot \frac{D_{A3}}{D_{A2}} \, . \, (\sqrt{\gamma} - 1)^2} \tag{7.19}$$

and

$$\lambda_c^2 = \frac{D_{A_2}}{(\sqrt{\gamma} - 1) \, . \, k_2} \tag{7.20}$$

Such results make one think that symmetry breaking chemical instabilities could play an *important role in biological systems*. The space differentiation produced in this way is characterized by the maintaining of the chemical potential gradients of the substances involved in the reactions. Such gradients may even in turn modify the physico-chemical state of other compounds which are not participating directly in the reactions connected with the instability.

Extrapolation of the physico-chemical state corresponding to equilibrium or near equilibrium conditions cannot explain such systems, as the instabilities introduce essential new features.

It is significant that the characteristic lengths found are of the order 10^{-2} — 10^{-4} *cm.*, i.e. large with respect to molecular dimensions, so that the macroscopic treatment used is justified. On the molecular scale we may speak of local thermodynamic equilibrium.

8. Role of chemical instabilities

The preceding paragraph showed that instability of the thermodynamic branch can lead to time or space organization. Another interesting aspect is that instabilities can lead to mechanisms of amplification and transmission. In the "phase diagram" represented in Fig. 5, region IV corresponds to a situation in which the homogeneous state is unstable. Therefore a small fluctuation occurring in the homogeneous system, such as represented in Fig. 5.2, is amplified till a new inhomogeneous state is reached.

More generally, near equilibrium the system is constrained to remain on the thermodynamic branch which is then the *only* stable one, but far from equilibrium many states may become accessible to the system. A small perturbation can then cause the system to switch from one state to another.

Delmotte [40] has recently used such a concept in developing an interesting model for the amplification of the photon absorption by a saccule of the chloroplast granule. In essence the absorption of the photon is treated as a "fluctuation" which leads to the transition from one steady state to another.

A particularly interesting problem is the amplification of information received by membranes. Changeux [41, 42] has developed a model that incorporates a possible cooperative character of the membrane action, but it does not take into account the effect of transport processes through the membrane.

We would also like to mention the work of Katchalsky and coworkers [44] on the role of dissipative structures in active transport.

All the examples we have considered so far refer to relatively simple situations where an analysis in terms of molecular concepts is possible, but more complicated and global manifestations of instability have also been studied. A striking example is provided by the recent work of E. F. Keller and L. A. Segel [45] who have shown that aggregation of slime molds may be viewed as a manifestation of instability in a uniform distribution.

There are certainly many other examples of morphogenetic motion which could be treated in a similar fashion (the origin of bilateral symmetry in the embryo, see [46] is extremely interesting).

Any theory of biological structures must retain two aspects. To quote P. Weiss [47] : " Considering the cell as a population of parts of various

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* This is in agreement with experiments. The calculations are actually made assuming that $\nu_1 \ll 1$.

magnitudes, the rule of order is objectively described by the fact that the resultant behaviour of the population as a whole is infinitely less variant from moment to moment than are the momentary activities of its parts ". This corresponds to our emphasis on stability theory, but the plasticity of biological structures, necessary to elaborate responses to changes in the environment must also be considered. One is thus forced to deal with far from equilibrium situations where a multitude of stable situations can become accessible to the system.

9. Concluding remarks.

It is very tempting to associate biological structures with chemical instabilities that lead to a spontaneous "self organisation" of the system both from the point of view of its space order and its function. It is a characteristic feature of such instabilities that their occurrence depends on a minimum level of dissipation and on specific non-linear types of kinetics that lead to negative contributions to the thermodynamic stability conditions (4.6).

To the evidence presented in §§ 6-7, it is interesting to add that the entropy production per unit mass and unit time (as measured by the metabolism) increases during the first period of oogenesis [48], then passes through a maximum and decreases to reach eventually a steady state value. To say that the first stage corresponds to the period where the treshold for instability has been reached is at least very tempting.

Correspondingly, in the prebiological stage, important steps would be associated with the occurence of dissipative structures which would permit the system to maintain far from equilibrium conditions [49].

So far this is sheer speculation. However, what is not speculation is that there indeed exists a *new state of matter* induced by a flow of free energy far from equilibrium. In this new state we have a new physical chemistry on a *supramolecular level* while all laws referring to the *molecular level remain essentially unchanged.*

This for a physicists is a quite interesting and challenging situation which requires a considerable amount of thought and study.

Acknowledgments

The thermodynamic concepts used in this paper have been developed in collaboration with Prof. P. Glansdorff. I must also express my appreciation of Mr. R. Lefever's aid in the preparation of this manuscript. Finally I thank Professors J. P. Changeux and A. Katchalsky as well as the members of our group, B. Edelstein, A. Goldbeter, M. Herschkowitz-Kaufman, B. Lavenda, G. Nicolis and A. M. Rodeyns for many interesting discussions and suggestions.

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De la Physique théorique à la Biologie, C.N.R.S., 1971.

DISCUSSIONS

J. Maynard Smith : I think dissipative systems of the kind you have described are most likely to be important in development biology. Let me give an example. Some years ago I studied the bristles on the top of the head of the fruit fly Drosophila. Along the edge of the eyes, on each side, there are four large evenly spaced bristles. How do they come to be there ? It would be nice to think that any cell on the top of the head could form a bristle, and that these is a standing wave of concentration of some chemical substance, arising in the way you have suggested, so that peaks of concentration induce particular cells to form bristles in particular places. I had this idea from the earlier work of Turing. If it is right, one should be able to alter the number of bristles by altering the chemical wave length λ. I and my colleague Dr. K. C. Sondhi tried to do this by selection, by breeding from flies with the smallest distance between two other structures on the head. Ultimately we obtained a fly with four bristles on one side and five on the other side of the head. By breeding from this fly, we finally obtained a population in which most of the flies had five bristles. The five bristles were evenly spaced in the region normally occupied by four, as if with the changed wave length five peaks could be fitted into the boundary which previously would hold only four peaks. This and other observations we made on bristle patterns could be very well explained by processes such as you have described. But of course dissipative processes may have nothing at all to do with it !

S. Lifson : If I understood correctly, the separation lengths λ are dependent on viscosity. Therefore they probably depend on thermodynamic properties like temperature. Did you study the temperature dependence to see how it fits with theory ?

I. Prigogine : Not yet.

H. H. Pattee : I just think that I should mention the well-known alternative hypothesis for this sort of developmental behaviour. Prigogine uses a continuous description. There is also a discrete description which I suppose goes back to Turing too, since he invented the Turing machine which generates a kind of discrete morphology in one dimension. It is perfectly possible to imagine a sequential switching network, controlling the expression of the genes in growing cells which would produce such a pattern, (see, e.g., Arbib, M : *J. Theoret. Biol., 14,* 131, 1967). I suppose also there is a kind of discrete-state analogue to the limit cycle in continuous non-linear systems. A recent paper by Kauffman

(*J. Molec. Biol.*, *22*, 437, 1969) shows the interesting developmental behavior of a random network of random switches, which in effect condenses out short, stable cycles. The important result is that they were *stable* cycles, so that if one switch misfired, in a short time it would usually come back into the same cycle again. Developmental biologists have many facts illustrating continuous and discrete control, but the discrete control processes appear more often at the subcellular level, while continuous processes appear in larger organisms. Developmental programs are certainly recorded in the discrete nucleotides of the gene, but the signal which results in the switching on and off these genes may originate with continuous fields, forces, or concentrations. This amounts to what computer people call "threshold logic". (See e.g., Wolpert, L.: *J. Theoret. Biol.*, *25*, 1, 1969).

Journée du 3 juillet 1969

Première séance

MUTATION ET PROCESSUS DE L'ÉVOLUTION

PRÉSIDENT D. GLASER

Ch. YANOFSKY

Protein structure and evolution

Discussions

E. MARGOLIASH and W.M. FITCH

The evolutionary information content of protein amino acid sequences

J. MAYNARD SMITH

Population genetics and molecular evolution

Discussions

S.E. BRESLER

Physical and Chemical processes leading to a mutation

De la Physique théorique à la Biologie, C.N.R.S., 1971.

INTRODUCTION

D. GLASER

My name is Glaser and I have been asked to be chairman this morning because Professor Dulbecco has not been able to come to the meeting. Also Professor Bresler of Leningrad will not be here, but we are fortunate in having a contribution from Professor Eigen, who will speak to us after the interval. All the talks before this interval will be devoted to the main topics of this mornings meeting.

For the sake of the physicists it might be useful to make a few general remarks about developments in molecular biology that have made possible a really new confrontation of the classical theory of evolution. You will learn from the 3 talks that we will hear first this morning, that it is beginning to be possible to make a really quantitative examination of the theory of evolution, because it is possible to define evolutionary events at the molecular level in a way that makes one of them strictly comparable with another.

This provides a sound basis for building a quantitative theory in which the rate of evolution would be predicted by the rate of mutation together with specification of selection pressures and such properties of the population as migration and mating patterns.

I could make one remark that the number 10^{11} agrees roughly with the results of measurements of phenotypic mutation rates in bacteria which are in the neighbourhood of 10^{-8}. That's the probability of finding a phenotypic mutant which is an auxotroph, or has acquired drug resistance, per generation per bacterium. But only a small portion of all the base changes will be seen phenotypically. That's based on the fact that a typical cistron has about 1000 nucleotides in it, let's say. Now if you say that the phenotypically detectable mutations constitute only 1 % all the base changes there's a discrepancy of a factor 100 between the estimate you quoted and the final one. Another remark is that I think the mutation rate probably is not dominated by thermal effects, but more likely by errors in the action of polymerases. These inaccuracies are the result of selection for the structure of the polymerase and one can make a qualitative argument that the mutation rate is optimized, and that it may not be the object of evolution to produce polymerases which are the most accurate possible within the limitations of quantum machanics and of kT, but rather to pick one which is a compromise between accurency and a mutation rate which allows evolution. I don't know how to estimate what the accuracy limit of a polymerase could be. The theoretical chemists have to do that for us some day.

A difficulty arises when one can't define the importance or calculate the probability of a particular step in evolution. Clearly the development of an eye is a much larger event than the change of skin pigment, for example.

The assignment of a quantitative measure to the size of an evolutionary step in gross biology is very difficult. When one can speak of a single base change at the nucleic acid level and can make the chemical statement that a large number of base changes are equally likely, perhaps all base changes under some conditions, then a single base change can be taken as a unit of evolutionary change and the number of such changes per century can be taken as an input to a quantitative theory. The papers that we will hear this morning will contain descriptions of measurements of rates of evolution defined at the molecular level together with explorations of mechanisms which can account for these evolutionary steps at the DNA level. These basic events will be correlated with phenotypic results of evolutionary events at the protein level and to some extent at the organismic level.

With that brief introduction to the physicists describing the significance of these developments in molecular kinetics and their application to evolution, I'd like to call on the first speaker, Professor Yanofsky of Stanford, who will speak on the " Protein structure and evolution ".

De la Physique théorique à la Biologie, C.N.R.S., 1971.

PROTEIN STRUCTURE AND EVOLUTION

CHARLES YANOFSKY

Department of Biological Sciences, Stanford University,
Stanford, California 94305

Present day genetic and biochemical techniques provide the means by which we can attempt to answer fundamental questions on the molecular evolution of functional proteins. The considerable knowledge acquired in recent years in studies of gene structure-protein structure relationships serves as the basis for the design of experiments which may reveal why a protein in a particular organism has a unique primary structure, and how that structure changes when the organism is subjected to the forces of evolution. In this article I would like to describe mutational studies we have performed which provide some insight into structure-function relationships in a specific protein. I will also discuss experiments which are directed towards achieving the ' evolution ' of a functional protein.

The tryptophan operon of *E. coli*

The gene cluster we have studied in our analyses of gene structure-protein structure relationships is the tryptophan operon of *Escherichia coli.* This operon

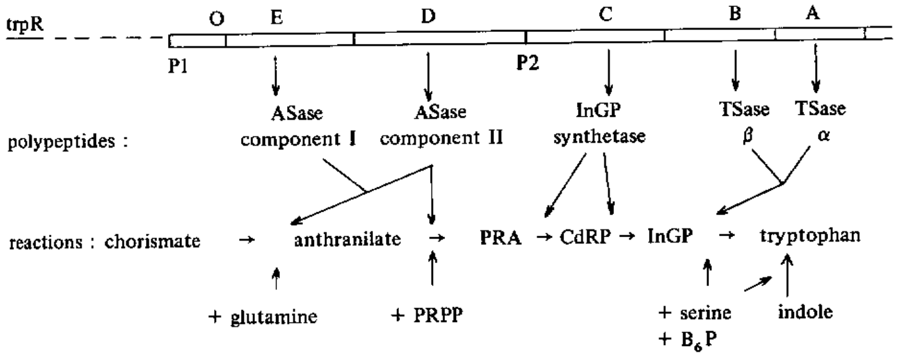


Fig. 1. The tryptophan operon of *E. coli.* The operon consists of 5 structural genes and adjacent controlling elements. TrpR is an unlinked gene specifying a protein repressor of the operon. O is the operator region and P1 and P2 are promoter regions. The various reactions in the pathway and the enzymes and enzyme complexes that serve as catalysts are indicated.

consists of five structural genes, each specifying a polypeptide which by itself or as a component of an enzyme complex catalyzes one or more of the terminal reactions in the biosynthesis of tryptophan (Fig. 1). Extensive mutational studies performed with this operon suggest that no segment of it is concerned with other essential bacterial functions. One implication of the existence of gene clusters of this type is that the component genes were derived from a common ancestral gene. To establish this point is of course one major objective of modern biology.

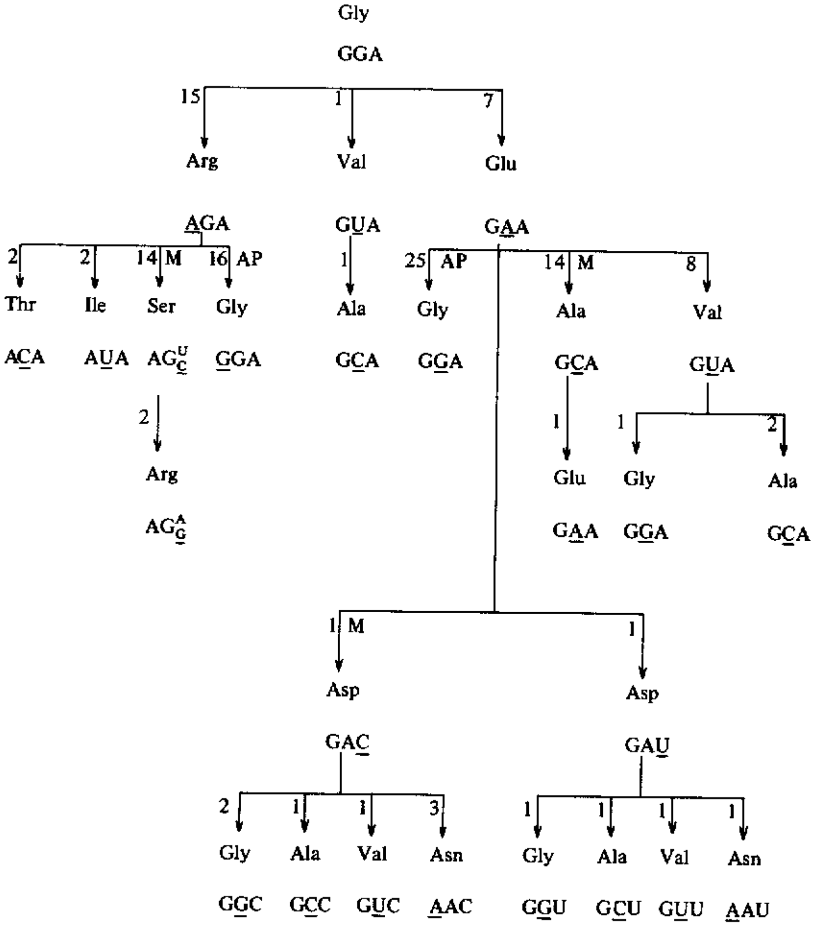


Fig. 2. Amino acid changes that have been observed at position 210 in the A protein and the probable corresponding codons [2, 3]. The number alongside each arrow indicates how many times the change was demonstrated by protein structure analyses. AP and M indicate changes favored by 2-aminopurine and the Treffers mutator gene, respectively. A bar under a codon letter identifies the nucleotide that is presumed to be introduced by the mutational change.

Tryptophan synthetase A protein alterations

Most of our gene structure-protein structure studies have been performed with the tryptophan synthetase A gene and A protein. The A protein is a single polypeptide chain 267 amino acid residues in length [1]; it has been shown to correspond linearly with the genetic map of the A gene [1]. Mutational changes in the A gene often lead to the production of altered A proteins which have single amino acid differences from the wild-type protein. At several positions in the A protein multiple amino acid changes have been detected. At position 210, for example, ten different amino acids have been inserted [2] (Fig. 2). Each of the observed amino acid substitutions is consistent with the interpretation that single mutational events involve single base-pair changes [2, 3]. Multiple amino acid substitutions have also been observed at positions 182 and 233 [3] (Fig. 3). It is clear from these cases and from comparable ones with other gene-protein systems that different amino acids can occupy a given position in a protein and permit function. We also know from amino acid sequence comparisons that enzymes isolated from related or unrelated species may have many sequence differences and nevertheless exhibit comparable enzymic activity. These observations focus on an important question: To what extent is the amino acid residue at each position in a protein essential for maximum effectiveness of that protein in its respective organism? The same question phrased in terms familiar to the evolutionary biologists is: Are neutral mutational changes preserved during evolution? In order to attack this problem experimentally we sought some means of rigorously assessing the

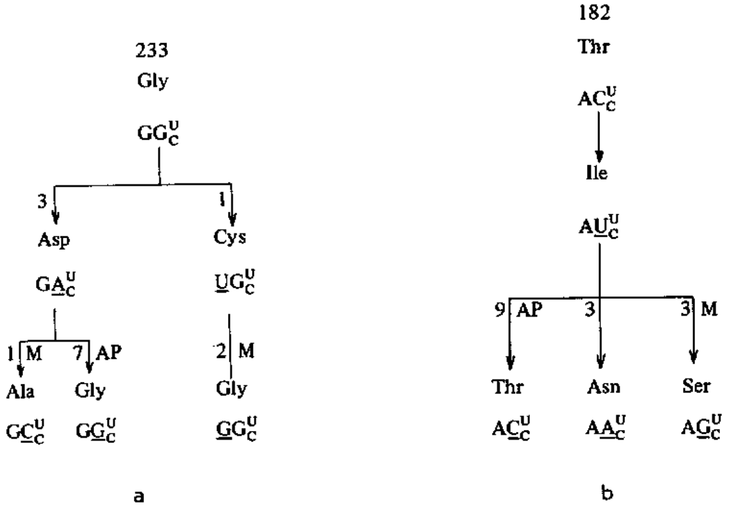


Fig. 3. Amino acid changes at position 182 (Fig. 3 A) and 233 (Fig. 3 B) and the probable corresponding codon changes [3]. See legend to Fig. 2 for other explanatory information.

functional capability of active A proteins with different amino acids at the same position. Many of the properties of the isolated altered A proteins have been examined but, since it is *in vivo* activity which is of concern to the organism, only tests performed in the growing cell could be considered relevant.

Perhaps the most sensitive means of examining the catalytic capability of an altered A protein in an organism such as *E. coli* is to determine the (tryptophan operon) enzyme levels attained when a culture is growing logarithmically in a minimal synthetic medium lacking tryptophan. Since under these conditions the organism must synthesize tryptophan to sustain its growth, a decrease in the catalytic capability of an enzyme should result in the production of elevated levels of all the enzymes specified by the operon; i.e., *E. coli* normally responds to a tryptophan deficiency by derepressing or turning on the synthesis of the enzymes of the pathway. Thus a very sensitive measure of true *in vivo* synthetic capacity is the effect on the levels of the biosynthetic enzymes. As can be seen in Table 1, when experiments of this type are per-

TABLE 1

Tryptophan synthetase B protein specific activities and generation times of strains with different amino acids at position 210 in the A protein.

Amino acid at position 210	Tryptophan synthetase B protein specific activity	Generation time (min)
Gly (wild type)	3.0 3.2 3.3	60, 60
Ala	3.2 3.3 3.5	60, 60
Ser	3.7 3.9 3.9	59, 60
Thr	7.7 8.0 8.2	58, 59
Val	30.1 31.3 31.9	69, 77
Ile	27.9 24.8 27.7	74, 74
Asn	50.9 51.4 55.1	104, 106

To ensure a constant genetic background each A gene was introduced by transduction into the same strain, a mutant with the A gene deleted. Several non-lysogenic colonies from each transduction were isolated and purified. Specific activities were determined with independent cultures harvested during log phase growth on minimal medium. Generation times were determined at 37 °C with cultures growing in minimal medium with glucose as carbon source. Estimates are based on cell population increases from 2×10^8 to 8×10^8/ml.

formed some of the proteins appear to be as active as the wild-type protein while others are probably less efficient and therefore signal the production of increased amounts of the biosynthetic enzymes. The same relationship is evident from the data presented in Table 2 for strains with altered proteins with amino acid changes at other positions in the A protein. Thus it is clear from this test that some amino acids are equally as effective as the wild-type amino acid. However, other amino acids at the same protein positions limit *in vivo* enzyme activity.

TABLE 2

Tryptophan synthetase B protein specific activities and generation times of strains with different amino acids at positions 182 and 233 in the A protein.

Amino acid at	Tryptophan synthetase B protein specific activity	Generation time (min)
position 182		
Thr (wild type)	2.6	58, 59
Ser	2.2	60, 60
Asn	9.1 9.3 10.8	58, 61
position 233		
Gly (wild type)	3.0 3.2 3.3	60, 60
Ala	11.3 12.9 13.1	61, 61

See legend to Table 1 for experimental conditions.

In view of these findings we might ask a related question: When an organism produces elevated enzyme levels does it do so at the expense of its ability to perform other metabolic reactions? For example, in the wild-type strain growing in minimal medium the tryptophan biosynthetic enzymes constitute 0.4 % of the soluble protein. If the organism were forced to increase this level to ca. 4 % to provide suffiicent tryptophan for maximal growth rates would it do so at the expense of its ability to perform other metabolic reactions, thereby limiting its growth rate? It is evident from the data in Tables 1 and 2 that significant increases in enzyme levels can be tolerated without any noticeable effect on the generation time. Thus, as can be seen in Table 1, when either serine or threonine occupies position 210 in the A protein the generation time is unaffected (Table 1). However, when the enzyme levels are increased 10-fold (valine and isoleucine proteins) a significant lengthening of the generation time is evident. When still higher levels of enzyme are produced (asparagine protein) even longer generation times are observed. On the basis of the latter finding it seems likely that the enzyme levels and generation times in the valine and isoleucine strains represent the consequence of a *balance* between the rate of tryptophan synthesis and the effect of the formation of large amounts of these proteins on the growth rate of the organism. In Table 2 we also see that significant increases in specific activity are not correlated with appreciable changes in generation time. We might have expected to see such increases when enzyme levels are increased 3- to 4-fold. It should be pointed out, however, that studies performed in the manner described in Tables 1 and 2 are incapable of detecting minor changes in generation time. Despite this, we may tentatively conclude that different amino acids are equally acceptable at certain positions in the A protein and that moderate increases in enzyme levels can be tolerated without exerting a noticeable effect on the growth rate. Thus neutral or near-neutral mutational changes probably can occur-whether they are preserved is a much more difficult question to answer.

Compensating amino acid changes

In many missense mutants reversion events occur at second sites within the A gene as well as in the codon affected by the primary mutation[4, 5]. Several cases of second-site reversion have been analyzed and the findings obtained have revealed structural relationships within the folded protein molecule. For example, the change from glycine to glutamic acid at position 210 in the A protein is reversed by a change from tyrosine to cysteine at position 174 (Fig. 4). Interestingly, only the latter change reverses the effect of the presence of glutamic acid at position 210, i.e., mutational changes in other A gene codons cannot restore functional activity and only the change from tyrosine to cysteine at position 174 is effective. Similarly, mutant A187, an auxotroph with two amino acid differences from the wild-type protein, valines instead of glycines at positions 210 and 212, reverts at three positions,

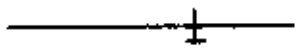
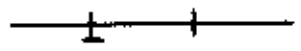
Strain	Amino acids at corresponding positions			Activity of protein	Locations of genetic changes
	174-175-176		210-211-212		
wild type	-Tyr-Leu-Leu-	33 residues	-Gly-Phe-Gly	active	
A46	-Tyr-Leu-Leu-		-Glu-Phe-Gly	inactive	
A46PR8	-Cys-Leu-Leu-		-Glu-Phe-Gly	active	

Fig. 4. Second-site reversion of mutant A46 [4]. As indicated, a Tyr → Cys change at position 174 activates the protein with Glu at position 210.

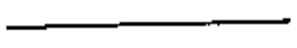
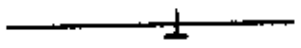
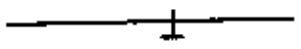
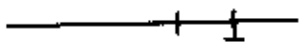
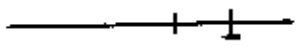
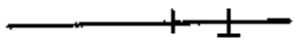
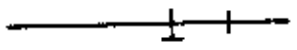
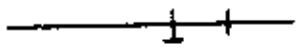
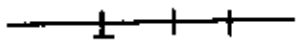
Strain	Amino acids at corresponding positions		Activity of protein	Locations of genetic changes
	174-175-176	210-211-212		
wild type	Tyr-Leu-Leu- 33 residues	-Gly-Phe-Gly	active	
A46	Tyr-Leu-Leu-	-Glu-Phe-Gly	inactive	
A46PR9	Tyr-Leu-Leu-	-Val-Phe-Val	active	
A187	Tyr-Leu-Leu-	-Val-Phe-Val	inactive	
A187SPR4	Tyr-Leu-Leu-	-Val-Phe-Gly	active	
A187SPR3	Tyr-Leu-Leu-	-Val-Phe-Ala	active	
A187SPR5	Tyr-Leu-Leu-	-Gly-Phe-Val	active	
A187SPR2	Tyr-Leu-Leu-	-Ala-Phe-Val	active	
A187SPR1	Tyr-Leu-Arg-	-Val-Phe-Val	active	

Fig. 5. Second-site reversion of mutant A 187 [5]. The A 187 protein has two changes; the Gly residues at positions 210 and 212 are replaced by Val residues. When either Val is replaced by Gly or Ala, the protein is functional. Both valines are retained in a functional protein in which the Leu residue at position 176 is replaced by Arg.

210, 212 and 176. At positions 210 and 212 the replacement of valine by either glycine or alanine restores activity, demonstrating that the A 187 protein is inactive only because both valines are present. One further point of interest is that the position of the distal reversion change, at 176, is two residues from the position of the second-site reversion in mutant A 46. These observations suggest that the two regions of the polypeptide chain indicated in Figs. 4 and 5 interact in the native molecule. We may conclude from these studies that because of the spatial relationships in the folded molecule the effects of an amino acid change in one region of the molecule can only be overcome by distal changes by specific alterations in an interacting region.

These observations raise the possibility that a neutral mutational change at one site may permit a subsequent change to confer a selective advantage. This gain in functional acceptability would then preserve what originally was a neutral event.

Attemps to "evolve" a functional A protein in strains lacking a segment of the A gene

I would like to known whether it is possible to produce a functional A protein by mutationally altering a protein fragment lacking the 20 or so amino acid residues at the carboxyl end of the molecule. To determine this, deletion mutants lacking the end of the A gene were subjected to mutagenic treatments and the treated populations were added to a medium which would only sustain the growth of cells with a functional A protein. The deletion

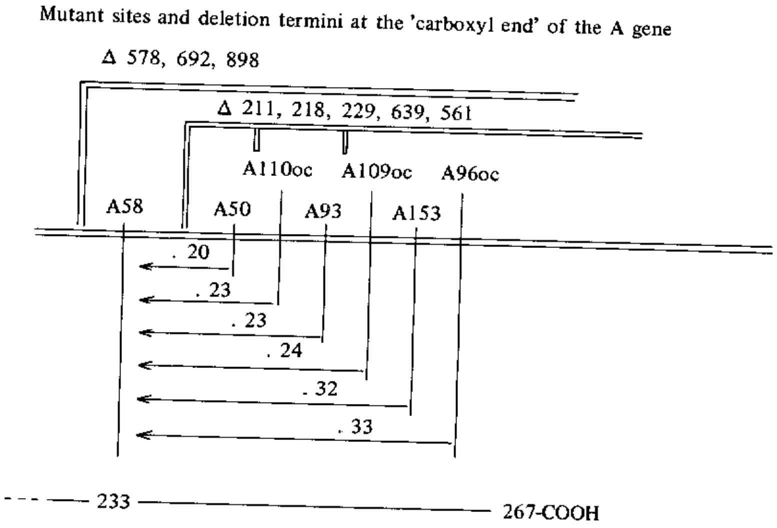


Fig. 6. Mutant sites and deletion termini in the region of the A gene specifying the carboxyl end of the A protein. Map distances are indicated above the arrows. Three of the point mutants (A 110, A 109, A 96) are ochre nonsense mutants. The precise terminus of each deletion is not known but it ends in the region indicated.

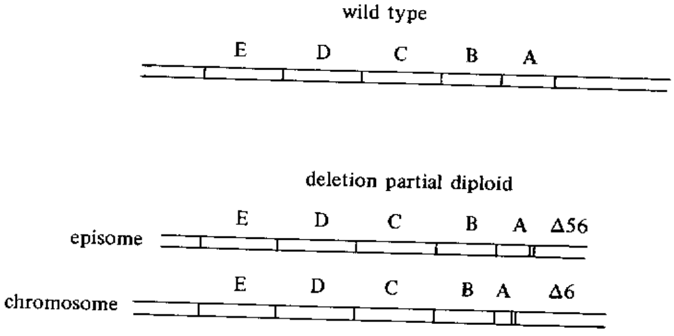


Fig. 7. A diploid strain employed in a prototrophy selection experiment. Deletion 6 removes most of the A gene while deletion 56 removes that region specifying the last thirty or so residues.

mutants examined are listed in Figs. 6 and 7. They were heavily mutagenized with nitrosoguanidine and ICR 191, powerful mutagens which produce base substitutions and base deletions and additions, respectively. Selective conditions were employed which would have permitted the outgrowth of bacteria with a functional A protein. In none of the deletions listed was a functional A protein detected — this despite the fact that large populations of bacteria were employed and the bacteria were permitted to divide several times before the tryptophan supplement in the growth medium was depleted. These negative results suggest that the carboxyl-terminal end of the A protein is essential for enzyme activity and that alterations in the remaining portion of the molecule cannot compensate for the loss. This conclusion is supported by the fact that nonsense codons in the terminal region of the A gene result in enzyme inactivity (see Fig. 6). Diploid strains with terminal deletions were also examined in these studies to eliminate the possibility that inactivating mutations occurred in other genes of the operon concomitantly with mutations in the A gene. Furthermore, episomes with A gene terminal deletions were transferred out of a heavily mutagenized population into haploid cells lacking only the A gene. In every case except one, to be described below, we did not detect a functional A protein. Despite these negative results other findings to be described subsequently suggest that the amino acid sequence at the carboxyl terminus of the wild-type A protein is not the only sequence that will permit this protein to be catalytically active. In the diploid strain described in Fig. 7, in which A gene deletions were present on both chromosome and episome, an active A protein was formed as a consequence of mutational changes in the A gene segment. The prototrophic strain obtained grows very poorly without tryptophan, however, suggesting that the functional A protein that is produced is at best inefficient. To eliminate the possibility that in the diploid strain one of the other genes of the tryptophan operon was assuming the function of the A gene, the *trp* operon of the episome was introduced by transduction into a haploid strain, replacing the operon of the recipient; i.e., the transductants had only one copy of each of the genes of the operon. These transductants were slow-growing prototrophs, suggesting that the mutation or mutations responsible for A protein activity were in or near the A gene.

In related studies we attempted to modify mutationally the E, D, C or B gene in a diploid strain so that the altered protein it produced could function as an A protein. The partial diploid prepared for the experiment had 90 % of the A gene deleted on both chromosome and episome. To date, these experiments have also given negative results, suggesting that each of the operon

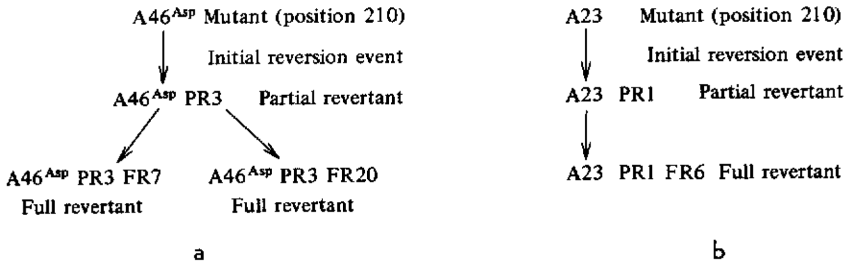


Fig. 8. Sequential reversion events [2] starting with mutant $A46^{Asp}$ (aspartic acid at position 210), A, and mutant A23 (arginine at position 210), B.

proteins is considerably different from its ancestral protein and thus many amino acid changes would be required for it to acquire A protein activity.

Frameshift mutations and protein function

Mutant A46$^{\mathrm{Asp}}$ and A23 both yield slow-growing partial revertant strains in which prototrophy is due to second-site mutations [2]. In order to analyze the effect of these mutations on the structure of the A protein, faster-growing full revertants were selected from the partial revertant strains (Fig. 8). This extra step was necessary because the partial revertant A proteins were extremely labile and could not be isolated. When the full revertant A proteins were analyzed [2] we were surprised to find that several contiguous amino acids had changed in each (Fig. 9). The amino acid differences in each revertant could be explained by assuming that the primary mutational event resulted in a single base addition and the second mutation involved a single base deletion. The greater activity of the full revertant A proteins compared to the partial revertant proteins is readily understandable since, with the exception of the residues in the vicinity of position 210, the amino acid sequences would be unaltered. The activity of the partial revertant A proteins is surprising, particularly in view of the conclusions reached in the previous section. We would expect that in these strains (A46$^{\mathrm{Asp}}$ PR3 and A23 PR1) the entire sequence of the terminal portion of the A protein would be altered as a result of the single

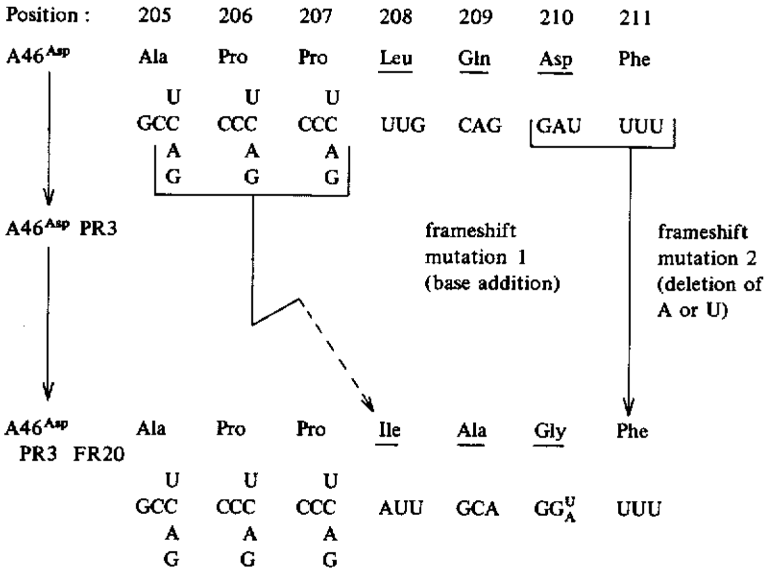


Fig. 9 a

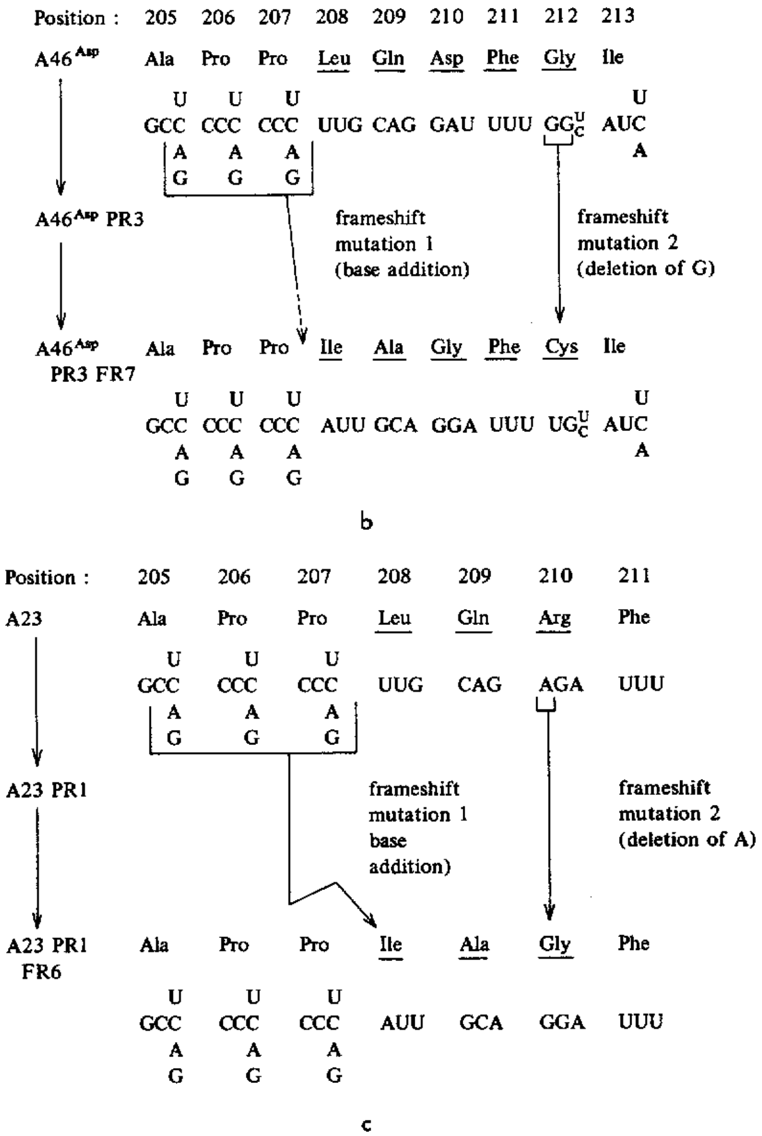


Fig. 9. Amino acid changes and probable corresponding nucleotide sequences in frameshift revertants A46^{Asp} PR3 FR20 (A), A46^{Asp} PR3 FR7 (B) and A23 PR1 FR6 (C) [2].

base addition. The new sequences that would be generated in the vicinity of position 210 are shown in Fig. 10. In strain A46^{Asp} PR3 a glycine residue would presumably replace the aspartic acid residue which is present at position 210 in A46^{Asp} and is responsible for enzyme *inactivity*. In A23 PR1, however, the arginine residue at position 210 would be replaced by a charged