SEMIPERMEABLE AQUEOUS MICROCAPSULES

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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April, 1965

ACKNOWLEDGEMENTS

I am indebted to Professor F. C. MacIntosh for directing this work and for making this study possible by encouraging me in my rather unconventional idea of making artificial red blood cells and for finding support for this project during the summer months of medical school and on a full-time basis after medical school, for his many useful suggestions, his guidance towards the best lines of approach and his interest in this work.

I appreciate Professor A. S. V. Burgen's advice and discussions at the initial part of this work and his stimulating lectures on membrane transport which started my interest in permeability phenomena.

I am grateful to Dr. S. G. Mason for suggesting to me the possibility of modifying my original microencapsulation-technique by using interfacial polymerization to prepare Nylon microcapsules, and for his allowing me to use his laboratory facility for six months to work out this possibility and for his useful advice. While in Dr. Mason's laboratory, I had the pleasure of meeting E. Anczurowski, who introduced me to the various equipment in the laboratory; F. Chan , who discussed with me his approach to sulphonating polystyrene; and Dr. D. Goring, who clarified various aspects of differential sedimentation.

Dr. M. A. Nawab had been very helpful in introducing me to n-butylbenzoate and in furnishing me with samples of it.

I thank Professor O. F. Denstedt, Professor Karl Sollner and Professor R. E. Forster for their interest and discussions. Professor Sollner

has been very helpful in suggesting possible ways for preparing negatively charged membranes and supplied me with numerous reprints of his own work on the subject of permselective membrane.

Karl Holeczek has been extremely capable in the various technical aspects of this work, especially in the preparation of most of the illustrations in this thesis. During the latter half of this work, the excellent and precise technical assistance of Mrs. Noris de Malave, especially on the very tedious analysis of blood ammonia levels, has been much appreciated. D. Cameron has been most helpful in supplying me with apparatus during the initial part of this work and in preparing illustrations and slides for other aspects of this work. I thank W. Ferch, who prepared a continuous recording device for the pH meter and the electronic component for the measurement of volume changes of microcapsules, and T. Keilpart, who prepared part of the microscope stage attachment. I appreciate O. Ransome for his help in determining the fate of intraperitoneally injected microcapsules in rats, and T. Horder for his help on the Van Slyke apparatus during one summer.

I appreciate Dr. J. Hinchey for proof-reading the manuscript of portions of the "Introductory Section"; my wife for typing the first copy of part of the manuscript and Mrs. Anna Stefani for the final typing of the thesis.

I am thankful to the members of the Department for their interest.

The support of the Faculty of Medicine, McGill University, and the Medical Research Council has been appreciated.

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INTRODUCTION

Terminology

The title of this thesis requires some explanation. The term "microcapsules" is a coinage of my own. The Concise Oxford Dictionary gives several definitions of "capsule" (from Latin capsa, a case). Two of these definitions are relevant to my usage: a capsule is a "membranous envelope (Physiol.)" or a "gelatine envelope enclosing pill (Med.)". A "semipermeable aqueous microcapsule" is therefore an envelope of semipermeable membrane enclosing a very small aqueous compartment.

At this point it may be useful to discuss also the terms "membrane" and "semipermeable". For "membrane", Sollner's definition is an excellent one: "A membrane is a phase of structure interposed between two phases or compartments which obstructs or completely prevents gross mass movement between the latter, but permits passage, with various degrees of restriction, of one or several species of particles from the one to the other or between the two adjacent phases or compartments, and which thereby acting as a physicochemical machine transforms with various degrees of efficiency according to its own nature and the nature and composition of the two adjacent phases or compartments the free energy of the adjacent phases or compartments, or energy applied from the outside to the latter into other forms of energy". (Sollner, 1945a). Very commonly both the "adjacent phases" which are separated by the membrane are aqueous; and it is only this situation that will be dealt with in this thesis. In the strictest sense, a semipermeable membrane is one which is not permeable to any

solute, but is only permeable to the solvent. However, no such a membrane has been prepared, and it can be predicted with confidence that no such membrane ever will be prepared because if the solute molecules are no larger than the solvent molecules, and do not interact chemically with the membrane, the diffusion of the solute will be no more restricted than diffusion of the solvent. Thus for convenience, we can say that a membrane which allows water to pass, but not a particular group of solutes, is semi-permeable with regard to this group of solutes. In biological research, a semipermeable membrane is usually one that is permeable to water and almost or quite impermeable to large water-soluble molecules such as those of protein or polysaccharide; the ability of small molecules other than water to pass through the membrane depends on the physicochemical characteristics of both membrane and solute, and will be considered later.

For the purpose of this thesis, the description "very small" in my definition of microcapsules will refer to membrane-bound compartments whose diameter is in the range of 1 μ to 1 mm.

Background of this research

Until the present work was begun, the class of semipermeable aqueous microcapsules was made up wholly of structures of biological origin. The modern cell theory and the identification of membrane-bound compartments as the fundamental unit of living matter goes back, beyond Schleiden and Schwann, to the French physiologist and botanist Dutrochet (1824) who said "All organic tissues are actually globular cells of exceeding smallness, which appear to be united only by simple adhesive forces". The further development of microscopy and electron

microscopy has revealed the existence of a variety of intracellular organelles that also may be classified as semipermeable aqueous microcapsules: nuclei, mitochondria, vesicles, secretory granules, lysosomes, elements of the endoplasmic reticulum, etc.: and a great wealth of information is currently being collected about their composition, their permeability, and their significance in the economy of the cell.

It is perhaps rather remarkable, considering the fundamental importance of these biological units which possess a microcapsular structure, that no artificial microcapsules of comparable dimensions have, to the best of my knowledge, ever been manufactured. One might have expected that such preparations would have many useful applications: for instance, in pharmacy for the dispensing of drugs, in colour photography for the separation of photosensitive dyes, or in the printing and duplicating industry, as vehicles for ink; in fact, each of these industries has developed materials that have some points of resemblance to microcapsules, but in every case there has been a fundamental difference, since the synthetic elements have not contained an internal aqueous phase. The familiar "capsules" of pharmacy contain oily or powdery materials enclosed within a comparatively large envelope (a few millimeters in diameter) which can disintegrate in the gastrointestinal tract. In colour photography, the light-sensitive materials have commonly been bound to microstructures such as starch granules or tanned erythrocytes, but the features of a distinct membrane and/or an aqueous interior have not been present. An important printing process (Green and Schleicher, 1957) depends on

the dispersion of ink by the rupture of microcapsules in which the pigment is held within an oily internal phase. None of these industrial processes, however, has much relevance to solving the problem in which I became interested, namely the enclosure of an aqueous protein solution within artificial membranous envelopes of small dimensions. The solution of the latter problem has turned out, as this thesis will show, to be remarkably straight-forward: indeed, two quite different solutions have been found, each of which is capable of manifold variations; and in retrospect, it seems rather strange that the solution was not sought for and found long ago. I have no way of knowing whether the attempt was ever made. In my own approach I had, as can now be seen, one distinct advantage: from the beginning I was interested in microencapsulating aqueous solutions rich in protein, and it is easier (unless the microcapsules are to have relatively large dimensions) to make satisfactory aqueous microcapsules if the aqueous phase has a significant colloidal osmotic pressure.

Arrangement of the thesis

Because my research topic is a new one, the arrangement of the thesis will be slightly unconventional. In the remainder of this introductory section, I will give a brief account of how my interest in this problem arose and of my initial approach to its solution. Following that I will give a historical account of what appears to me the most significant developments in our knowledge of the structure and permeability of artificial and biological membranes, especially those that bear directly on my own work. I will then consider briefly

the reasons why the preparation and properties of microcapsules seem to be worthy of investigation. This will complete the Introduction. It has seemed convenient to postpone an account of other aspects of the historical background to the beginning of the individual chapters of the thesis to which they relate.

HISTORY OF PRESENT WORK

Early in 1957, as a 4th-year student in Honours Physiology at McGill University, I was required to carry out a small research project, and I suggested that it would be interesting to try to prepare microcapsules similar in size to erythrocytes and containing active hemoglobin. I thought that collodion, since it was the best-studied material for semipermeable membrane, might serve as the envelope for microcapsules. My reasons for thinking that this would be worth trying were first, that I thought that these "artificial blood cells" would not become fragile after prolonged in-vitro storage in the blood bank; and secondly, that they could be transfused without cross-matching, since they would lack the ABO and other antigens that are attached to the outer surface of the erythrocytes. If this could be achieved, and if the "artificial cells" were nontoxic, impermeable to protein, and capable of circulating for a long time in the blood stream, an unlimited supply of animal hemoglobin would be available for their manufacture. Of course, even if such microcapsules could be made, it was uncertain that they would have satisfactory physico-chemical and physiological properties: their contained hemoglobin might not be sufficiently stable; their membranes might offer too great a barrier to gas exchange to make them useful for O_2 and CO_2 transport in-vivo; and they might have undesirable immunological properties related either to the membrane material or to the contained foreign protein.

A search of the literature, and informal inquiries of a number of scientists in different departments of McGill University, made it

almost certain that no method had been reported for the microencapsulation of an aqueous solution of biologically active protein inside a thin semipermeable membrane dispersed in another aqueous phase. Thus the first problem was to devise a method. My first attempts were rather unsuccessful. Dried hemoglobin powder or hemoglobin solution was sprayed at an angle of 90^{o} through a spray of collodion dissolved in an ether-alcohol mixture, in the hope that as the protein material passed through the other liquid, it might become coated with collodion solution which on drying would form a membrane. A few suggestive forms were observed under the microscope, but most of the material formed a sticky mass from which individual microcapsules could not be separated; and when I attempted to avoid this by collecting the coated protein under water, the protein separated from the collodion and went into solution. It is now apparent that this last procedure allowed no time for the newly-formed membrane to set. A variety of other techniques, with nebulizers for both types of liquids, also failed to give good results. A quite different approach was then tried, along the following lines. I had observed that at the interface between a solution of collodion ether and an erythrocyte hemolysate, a film formed slowly; it took about 15 minutes to be well developed. By naked-eye observation it appeared that the surface of the film facing the aqueous phase was well defined, while the interface on the side of the collodion solution was not well defined but showed a gradual merging of film and solvent. The practical problem of forming a stable membrane thus had two aspects: the slow deposition of the collodion membrane in contact with the aqueous phase; and the

"setting" of the membrane, with a distinct boundary, on the surface facing the organic phase. After various attempts, I found it possible to prepare relatively large membrane-bound droplets in the following way. A solution of collodion in ether was layered a few centimeters deep over an organic liquid, e.g. paraffin oil, which was immiscible with water and whose specific gravity was intermediate between that of the organic liquid and that of the hemolysate. (Though paraffin oil is not a solvent for collodion, no visible precipitation of collodion occurred at the ether-paraffin junction during the course of the experiment.) A very small drop of erythrocyte hemolysate was then let fall from about 15 cm. above the surface of the collodion solution; during the next minute or two, as the aqueous droplet slowly fell through the organic liquid, a film of collodion became attached to its surface. After traversing the collodion solution the coated droplet continued to fall, but more slowly, through the paraffin oil, in which collodion is insoluble. The inner and outer surfaces of the newly-formed membrane could now "set"; the process took an appreciable time to be completed, but after 30 minutes in the paraffin oil, the membrane was strong enough to withstand gentle handling. The capsule could then be transferred to an aqueous medium, after decanting off as much of the organic liquid as possible, by adding water and gently stirring so that the remaining paraffin floated upward as fine droplets, leaving the capsule in the aqueous phase at the bottom of the vessel. With this method, it was possible to prepare single capsules, containing undenatured hemoglobin in solution, and having a diameter of about 2 mm. I then attempted to enclose smaller droplets of hemolysate, by

shaking the aqueous solution in ether, and then adding the ethereal collodion solution to the emulsion with continued agitation. Membranes were formed as before, and many of the spherical forms produced were of microcapsular dimensions. I tried to harvest the microcapsules by centrifuging the suspension over paraffin oil, but the attempt was not very successful, for the paraffin could not then be emulsified finely enough to separate it from the microcapsules when water was added. At this point, I began to ask colleagues with greater knowledge of physical chemistry whether they could suggest a liquid that would assist in the transfer of the microcapsules from the organic medium into an aqueous medium. Such a liquid would have to be, like paraffin oil, immiscible with water and less dense than hemoglobin solution; but unlike paraffin oil, it should be capable of emulsification into very fine microdroplets which could then be separated on the centrifuge from the microcapsules. Dr. Nowab, then a graduate student in Physical Chemistry, told me that he had been using such a liquid, butyl benzoate, and was so helpful as to furnish me with samples of it. When I replaced the paraffin oil with butyl benzoate and repeated the experiment just described, I found that the newly-formed microcapsules could now be dispersed in the aqueous phase without being coated by a thick layer of organic liquid. This first successful method for preparing microcapsule suspensions thus consisted of three main steps: first, the hemolysate was emulsified in an organic liquid, ether; then, on the addition of collodion in ether to the stirred emulsion, a membrane was formed on the surface of the microdroplets and allowed to set in butyl benzoate; and finally, the microcapsules were dispersed in an aqueous phase. I now recognize that the deposition of the membrane

in the second of these steps is an example of interfacial coacervation. With various modifications, the three main steps tested above - emulsification, membrane formation and dispersion in water - have been used throughout this work for the preparation of microcapsules; but in much of the later work, the second step has involved the principle of interfacial polymerization rather than interfacial coacervation.

During the next summer (1957) and my subsequent vacation from medical school (1958-1960) I was able to follow up this work in the laboratory of Professor MacIntosh. The difficulties of stabilizing hemoglobin after microencapsulation were rather discouraging: a good deal of the enclosed hemoglobin was converted to methemoglobin or some other derivatives; and an attempt was then made to see whether enzymes could be encapsulated with retention of their activity. The idea here was that enzyme-loaded microcapsules might retain sufficient activity after injection into an animal or patient to replace an enzyme with which the body was inadequately provided. Certain molecular diseases, as is well known, are due to mutations as a result of which an enzyme is lost or is in short supply. Injection of the missing enzyme, derived from a different animal source, is unsatisfactory, because the foreign protein is either rapidly inactivated or else gives rise to antibodies, which in turn neutralize the injected enzyme or lead to allergic complications if the treatment is repeated. It would seem that if the extracellular location of the enzyme were not too critical, for instance if it were a blood or liver enzyme, and if the substrate and product of the enzyme were small molecules that could

diffuse freely through a collodion membrane, there would be some hope of achieving a prolonged in-vivo effect with the encapsulated enzyme. I did indeed find that several enzymes could be microencapsulated with retention of their catalytic activity. Among these were carbonic anhydrase and trypsin (for a dipeptide substance), and less certainly uricase. The suspension of microcapsules retained significant activity after week-long storage in the refrigerator, and there was little if any tendency for the enclosed enzyme to leak out of the microcapsules. Some of the microcapsules were injected into mice, and it was found that, though they disappeared rapidly from the circulation, their acute and chronic toxicity in doses up to 20 cc of a 10% suspension of microcapsules/kilogram was apparently negligible.

Suitable equipment for making reproducible emulsions was not available in the Physiology Department at that time, and at first success in making small microcapsules was achieved irregularly. The chief difficulty was in step (3), the transfer of the newly formed microcapsules to an aqueous medium: troublesome aggregation occurred at this stage, and yields of well-separated microcapsules were low. Many variations of the procedure were tried out, and gradually the results became better and more reproducible; but from long experience, I can testify that shaking by hand is not the ideal method for producing a standardized emulsion. The research was pursued up a number of blind alleys: for instance, most of one summer was spent in trying to load radioopaque materials into microcapsules to form a contrast medium suitable for angiography; and after four summers the basic method still appeared to be insufficiently reliable to justify publication. I was

however encouraged by the interest shown in my work by people in Physiology and other McGill Departments: in particular Dr. S. G. Mason asked me if I would spent the summer of 1961, after my graduation from medical school, in his laboratory preparing microcapsules for his experiments on the flow of suspensions, but I was unable to make use of the opportunity at that time.

After I had completed my internship, I was still interested in continuing and if possible completing the research, and in 1962 with the award of a MRC fellowship I returned to the Physiology Department as a Ph.D. candidate under the supervision of Professor F. C. MacIntosh. At this time I was fortunate enough to be allowed by Dr. S. G. Mason to work for six months in his laboratory in the Pulp and Paper Research Institute on the McGill campus. Dr. Mason's own research, as I have mentioned, gave him an interest in my problem and enabled him to make many valuable suggestions. Perhaps the most important of these was when he pointed out that Morgan and his colleagues had been able to make sheets of nylon membrane by the procedure of interfacial polycondensation. Nylon is made by condensation of a diamine (usually hexamethylenediamine) with the dihalide of a dicarboxylic acid (usually sebacoyl chloride). In the original Carothers procedure the reaction was carried out at high temperature and pressure, but Morgan (1959a) realised that, since diamine is water-soluble and the sebacoyl chloride is soluble only in organic liquid, the reaction can occur equally well at room temperature at the interface between water and an organic solvent. This process of "interfacial polycondensation" had thus a certain resemblance to the process of "interfacial coacervation"

that I was using, and Dr. Mason suggested that my basic emulsification technique might be modified to produce nylon microcapsules. At first the idea seemed rather unpromising, since one of Dr. Mason's graduate students told me that an attempt to produce large (5 mm diameter) capsules by the interfacial polymerization technique had been unsatisfactory, the membranes formed were so unstable that the idea was temporarily dropped. My first work in Dr. Mason's laboratory was therefore an attempt to improve the interfacial coacervation procedure. However, I was so fascinated by the principle of interfacial polycondensation that I read Morgan's important papers, and then started working on the problem seriously. After various tests, I found that if the reactions were not stopped within a few minutes, the membrane gradually increased in thickness and roughness, and finally disintegrated. This result was in line with Morgan's finding that as the thickness of the interfacial membrane increases, there is increased restriction to diffusion of the reactants, with the result that the subsequently formed polymers are of low molecular weight and accumulate on the organic-liquid side of the membrane, resulting in the formation of a membrane of very poor tensile strength. With this information, I continued the experiments, and found that a thin stable membrane could be made by simply decreasing the concentration of the reactants and shortening the reaction time to 3 minutes. The reaction was stopped by diluting the sebacoyl chloride with organic solvent and quickly transferring the microcapsules into an aqueous medium.

Much time was spent on the problem of transferring the newly-formed nylon microcapsules from the organic phase. The chief source

of difficulty was that the organic solvent adhering to the microcapsules caused them to stick together forming a mass that could not be dispersed in the aqueous suspension. Many procedures aimed at dispersing the microcapsules (ultrasonic vibration, freeze-drying and electrical emulsification) were tried without success. One obvious approach was to use a solvent, miscible with both the aqueous and the organic phases, in order to remove the latter: examples of such miscible solvents are ethanol, acetone and dioxane. It was indeed found possible, by repeated washing with such a solvent and then with water, to resuspend the microcapsules in an aqueous medium. This procedure, however, resulted in a further difficulty: the hemoglobin within the microcapsules was precipitated by the solvent, and did not redissolve when the solvent was removed. Thus though aqueous suspensions of microcapsules could be made by this method, the aim of preparing them with a content of undenatured protein could not be realized. The miscible-solvent procedure was therefore discarded, and another approach was tried, which finally led to a useful technique. Since the collodion microcapsules had been transferred from the organic solution to water by emulsifying the organic solvent in excess of water, allowing gravity separation of the microcapsules from the organic microdroplets, a similar attempt was made to emulsify the organic solution containing the nylon microcapsules. The microcapsules were transferred into a small volume of butyl benzoate, and this suspension was emulsified in water, resulting in a successful transfer. As mentioned in the case of the collodion microcapsules, there was aggregation of at least 50% of the nylon microcapsules, since a thin layer of butyl benzoate still adhered to them after the transfer. (Butyl benzoate is insoluble in water.) In the

hope of improving the final dispersion of both the collodion and the nylon microcapsules, I asked Dr. Mason for advice about how to make still finer emulsions of the organic liquid. He directed me to the literature on emulsifying agents, and after reading and experimenting on a variety of these, I decided that Tween 20 might be the most suitable agent for making emulsions with a continuous aqueous phase, while Span 85 might be the most suitable for making emulsions with a continuous organic phase. (Each of these emulsifying agents is a mixture of non-ionic complex esters or ester-ethers derived from the hexahydric alcohol sorbitol.) Unlike some other types of emulsifying agents, these two detergents did not appear to affect the protein which was to be enclosed; furthermore, they are known to be of very low toxicity - especially Tween 20 which has been used in the pharmaceutical industry for making emulsions for oral administration, and in the dairy industry for making ice cream. There was however still one technical problem to be solved, because Morgan had found that detergents generally interfere with the formation of nylon membranes (Morgan, 1959a). Fortunately, I found that, by using a rather small amount of Span 85 in steps (1) and (2), and a larger amount of Tween 20 in step (3), microcapsules down to 15 μ in diameter could be made to disperse completely in the aqueous phase.

With further work in both Dr. Mason's laboratory and in the Department of Physiology, where I returned at the end of 1962, I found that various polymers could be used to form microcapsule membranes, by both the coacervation and the polycondensation procedures; and with

minor changes in the emulsification technique better control was achieved over the dimensions and physicochemical properties of the product. The procedures that have been found most serviceable for the manufacture of microcapsules with different membrane materials, and the physicochemical and biological properties of such microcapsules, are described in detail in the later sections of this thesis.

During the last two years some aspects of the research have been presented verbally at scientific meetings (Chang, MacIntosh and Mason 1963; Chang and MacIntosh, 1964a, 1964b and 1964c), and a short summary of results has been published in Science (Chang, 1964).

Recently, I have discovered that I am not the only worker in the aqueous microcapsule field. Dr. Mason has drawn my attention to two Belgian patents summarized in Chemical Abstracts (1964: patents obtained in 1963), taken out by the well-known photographic firm of Gevaert. These patents are based on the principle of secondary emulsion: in this procedure, an aqueous phase is emulsified in an organic phase, and the resulting emulsion is then emulsified in another aqueous phase, resulting in the formation of a three-phase system. In the Gevaert processes, the organic phase is polystyrene in benzene, and each polystyrene microdroplet may randomly contain one or more smaller aqueous microdroplets. The organic solvent of the secondary emulsion is then evaporated by raising the temperature to 50-55^{o} C, with resulting hardening of the polystyrene. The principle of secondary emulsion used in this procedure does not result in the production of microcapsules with a true membrane, but rather of solid microspheres containing randomly one or more drops of aqueous solution. It will

also be noted that the evaporating temperature of 50-55° C is not suitable for the incorporation of biological material; and in general the Gevaert product, though it may have important applications in colour photography, would hardly be suitable for any of the possible uses of microcapsules that are envisaged in this thesis. I might perhaps note that I spent some time during the summer of 1962 attempting to use the principle of secondary emulsion for making microcapsules of collodion and other materials. The products that resulted were very similar to those described in the Belgian patents, and indeed there were remarkable points of correspondence between some of the recipes I used and those later patented by Gevaert. The procedure being so unpromising for the applications I had in mind, I discontinued work with it.

A very recent review (Chemical Week, 1965) which arrived as this thesis was approaching completion, indicates that a number of industrial firms are working on microencapsulation, besides those described for National Cash Register and Gevaert. For instance, the IIT Research Institute namufactures microcapsules by mixing two aerosols with a sonic jet diffusion charger, giving the sprays opposite electrostatic charges; the two aerosols combine, one becoming the coating, the other the core. For example, microcapsules can be made with a glycerin content and a carnauba wax wall; the wall is thick, accounting for half the weight of the microcapsules; thinner walls of acrylic resin can be made to coat barium permanganate particles. The Southwest Research Institute has been working on encapsulation procedures for the manufacture of floor cleaners and polishes, and also on aerospace

uses - encapsulation of a hydrophobic catalyst dispersed in a silicone resin; meteorite puncture of the craft would release the catalyst which would then polymerise the silicone and seal the puncture. A further process being studied by the Southwest Research Institute involves the gelation of hydrocarbon fuel to make "gasoline bricks". The Wisconsin Alumni Research Foundation and the National Research Corporation are both interested in the encapsulation of solid particles. The National Cash Register Company, too, appears to be expanding its programme of studies on microencapsulation. It will be noted that all these new processes involve the use of impermeable walls and non-biological materials, and so far as can be seen from the brief outline provided, none of them would serve for the microencapsulation of enzymes or cellular material. The last paragraph of the review refers to our own work, which is specifically mentioned as one that permits the encapsulation of enzymes and other proteins.

SEMIPERMEABLE ARTIFICIAL MEMBRANES

Many types of artificial polymer membranes have been produced, but only a few of these have been studied from the standpoint of their permeability or their usefulness in biological research. The following is a brief summary of those types which have been of particular interest to physiologists.

i. Parchment paper membranes

Graham (1861), the father of classical colloid chemistry, distinguished "crystalloids" from "colloids" on the basis of his finding that colloids diffuse more slowly in water than crystalloids. In other experiments he used a parchment paper membrane to separate a mixture of crystalloids and colloids in aqueous solution from a second aqueous phase, and he found that though the crystalloids passed through readily, the colloids (for example, gelatin and egg albumin) were unable to do so. Parchment paper, although it contributed much to the concept of colloid chemistry and was the means by which the process of dialysis was discovered, long ago fell into disfavour because it so greatly restricts the diffusion of water and permeable solutes. Other types of semipermeable membranes have taken its place.

ii. Cupric ferrocyanide membranes

Traube (1867) was the first to prepare cupric ferrocyanide membranes. The material he used was cupric ferrocyanide, formed by the interaction of potassium ferrocyanide and copper sulphate. He placed a crystal of copper sulphate in a piece of gauze and suspended this in a test-tube filled with a solution of potassium ferrocyanide: as the

copper sulphate dissolved it reacted with the ferrocyanide to form a membrane. Traube showed that this membrane did not allow the passage of cane sugar, barium chloride, calcium chloride, potassium sulphate, barium nitrate or ammonium sulphate, but was permeable to water and potassium chloride. The original membrane was very fragile, but Pfeffer in 1877 produced a stronger membrane by incorporating it in the wall of an unglazed pottery jar: potassium ferrocyanide solution was placed inside the jar and copper sulphate solution outside, and the membrane was deposited within the porous wall when an electric current was passed between the two solutions. With this system, Pfeffer made the observation that the osmotic pressure of sucrose was proportional to its concentration. His observations later became the basis of van't Hoff's classical analysis of osmotic pressure in terms of the gas-law constants. Donnan's (1911) important work on the equilibrium attained across a membrane when one of the ions involved is impermeant was carried out with cupric ferrocyanide membranes of this sort. The membrane deposited within unglazed pottery is, however, inconveniently thick, and osmotic equilibrium is reached very slowly. More useful membranes were produced by modifications of Pfeffer's procedure: for instance, Walden (1892) precipitated the cupric ferrocyanide within hardened gelatin; Tammann (1892) incorporated it into parchment paper; and Hartung (1937) deposited it on filter paper. Pfeffer (1877) assumed that the membrane was porous, and that solutes penetrated by diffusion through water-filled pores rather than by solvation in the substance of the membrane. This assumption, however, was not proved

until Walden (1892) demonstrated that though the membrane was impermeable to triethylamine, it was slightly permeable to diethylamine and readily permeable to monoethylamine, so that permeability was found to be a function of the molecular size. Later Bartell (1911) showed that the movement of water obeyed Poiseuille's Law for capillary flow under hydrostatic pressure through a membrane of cupric ferrocyanide.

iii. Cellulose ester membranes

Various cellulose esters have been used to form membranes. However, the one which has been of the greatest interest is "collodion" (this is a solution of cellulose nitrate, usually 4% w/v, in a mixture of one volume of alcohol and three volumes of ether). Bechhold (1907) discovered that membranes could be precipitated from such a solution by contact with water, a process which might now be called interfacial coacervation. He attempted to measure the porosity of such membranes by passing air through them, and by this test found that the porosity could be diminished by increasing the concentration of the dissolved ester. Later workers devised other methods for varying the porosity: thus Schoep (1911) mixed different amounts of castor oil and glycerol with the collodion solution; Farmer (1917) showed that the membranes became less permeable when they were dried for a longer time; Brown (1915) and Eggerth (1921) obtained membranes of increasing permeability by adding increasing proportions of alcohol. Other workers impregnated their collodion membranes with varous materials in an attempt to increase their selectivity, with some success: thus Meigs (1913) found that membranes impregnated with calcium phosphate were impermeable to the

chlorides of sodium, potassium and calcium and to sucrose and alanine, and somewhat permeable to glycerol and urea, though they were freely permeable to alcohol; and Philippson (1913) showed that when collodion membranes were impregnated with an ether extract of muscle, they became almost impermeable to inorganic acids, while retaining their permeability to organic acids, this permeability increasing in the series formic-acetic-lactic-butyric.

The molecular sieve action of the "dried" collodion membrane was demonstrated by Collander (1924), using solutions of non-electrolytes and weak electrolytes with substances of different molecular volume in either group. In the next decade, Elford (1937) reported his classical studies, in which he showed how collodion membranes of graded porosity could be prepared by using different solvents. He measured pore diameter by testing membrane permeability to suspended or dissolved particles of known dimensions whose size had been estimated by ultramicroscope, ultracentrifuge and diffusion techniques: for example, proteins like albumin, haemoglobin and globulin for the smaller particles, and viruses and phages for the larger particles. Using this method, he found that a standard collodion solution when allowed to set in air produced membranes with a mean pore diameter of 7000 Å (700 mμ). This pore diameter could be reduced by replacing 1/3 of the alcohol in the collodion solution by a better solvent of the collodion - for instance, ethylene glycol monoethylether reduced the mean pore size to 12 Å, acetic acid to 55 Å; whereas the addition of a poor collodion solvent increased the pore diamter - for instance, the addition of amyl alcohol increased the mean pore size to 9600 Å. More recent work

by Robbins and Mauro (1960), who measured the fluxes of water and several solutes across their membranes, showed that the porosity of collodion could be varied by the addition of different proportions of ethylene glycol to the collodion solution, the mean pore diameter being then controllable over the range 21 Å to 97 Å, with the value increasing with the proportion of ethylene glycol. Their calculation of pore size was based on the relative fluxes under the influence of diffusion alone, and diffusion plus a hydrostatic pressure gradient, the assumption being made that hydrostatic flow of water obeys Poiseuille's Law involving the fourth power of the pore radius whereas diffusion depends only on pore area, i.e. the square of the radius. Durbin (1960) in Solomon's laboratory used the Staverman principle (this will be considered later in the section under "Permeability"). He measured the net volume flow due to a difference in solute concentration across different types of cellulose-ester membranes and found that the mean pore diameters were 23 Å for "Visking", 41 Å for "Dupont 450 PD 62", and 82 Å for "Wet gel of Sylvania, 300 weight".

None of these workers were seriously concerned with the ultra-structure of collodion membranes. Sollner (1945b), summarizing in 1945 the work done up to that time, concluded that a collodion membrane could be considered as a heteroporous structure with a Gaussian distribution of pore diameters. This heteroporous character could be explained by the mode of formation of the membrane, when the nitro-cellulose molecules of varying lengths arranged themselves randomly

to form a structure similar, in Sollner's words, to "a plate of compressed straw or hay or a coconut fibre door". With this type of structure, as Sollner pointed out, it was hardly justifiable to picture semi-permeable membranes as possessing well-defined individual pores. It had repeatedly been found that although collodion membranes, prepared from various commercially available collodion solutions, behaved in the same manner towards nonelectrolytes, their behaviour towards electrolytes was very variable (Michaelis and Perlzweig, 1927; Wilbrandt, 1935; Sollner and Abrams, 1940; Sollner, Abrams and Carr, 1941). Further work (Sollner, Abrams and Carr, 1941) showed that this difference was due partly to the presence of acidic impurities derived from partial oxidation during the manufacturing process, and partly to acidic groups present in the native cellulose fibres. The impurities in either case were nitrocellulosic acids - nitrocellulose molecules of high molecular weight, carrying one or several carboxyl groups, which thus presented as fixed anions in the membrane. When collodion from different commercial sources was analyzed (Sollner, 1945b) for acid number (ml of 0.01 N KOH to titrate one gram of dry collodion) different values were obtained: and Sollner thought that the anomalous permeability to electrolytes could be explained on that basis. The anionic charge on such commercial collodion membranes was, however, extremely small when compared to that of membranes specially prepared to be selectively permeable to ions. Such membranes, following Sollner's terminology, may be referred to as permselective membranes.

iv. Permselective membranes (ion-exchange membranes)

Sollner in his 1958 review summarized much of the work done up to that time by himself and others on the electrochemistry of permselective membranes (Sollner, 1958). Such membranes characteristically have high permeability for non-electrolytes and high selectivity for ions. They may be electronegative and preferentially cation-permeable, or electropositive and preferentially anion-permeable. The fixed-charge theory, first clearly stated by Teorell, describes these membranes as having a definite number of dissociable groups firmly attached to the walls of their pores - anionic groups in the case of electronegative membranes, or cationic groups in the case of electropositive membranes. In either case, ions whose charge is opposite to the fixed charge can move freely across the membrane, whereas ions with the same charge as the membrane are repelled by the fixed charges on the pore wall, and consequently are less likely to penetrate. The narrower the pores, the more effective is the exclusion of such ions; and the greater the number of potentially dissociable fixed groups in the wall of the membrane, the more pronounced are its electrochemical properties. Polyvalent ions with the same charge as the membrane are more severely restricted than univalent ions, because of their greater hydrated volume as well as their greater charge.

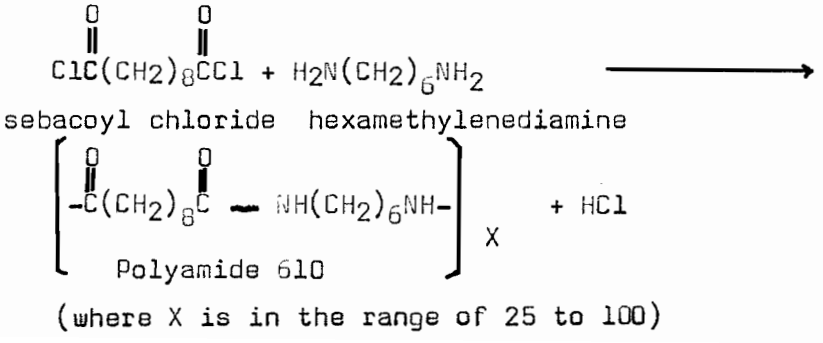
Many methods have been developed for the preparation of membranes with high electrochemical activity. Five principles have been used, each of them with variations. (1) The membrane may be activated by oxidation, resulting in the formation of dissociable groups (Carr and Sollner, 1944; Gregor and Sollner, 1946). (2) The membrane may also be activated by the absorption of polyelectrolytes (Carr, Gregor and

Sollner, 1945; Sollner and Neihof, 1951; Neihof, 1954; Gottlieb, Neihof and Sollner, 1957). (3) A polyelectrolyte may be dissolved in a high-polymer solution, which is then used for forming the membrane: the membrane so formed has polyelectrolyte molecules permanently enmeshed in its matrix (Neihof, 1954). (4) A polyelectrolyte may be polymerized on a supporting polymer membrane. (5) Polystyrene may be copolymerized on polyethylene film, followed by sulphonation of the polystyrene. Some of the more useful variants may be mentioned. Under class (1), collodion has been oxidised by NaOH in the presence of air, or by bromine, with the formation of fixed nitrocellulosic acid groups, as already mentioned in the case of the accidental impurities in some commercial membranes. Under class (2), membranes of "strong-acid" type have been made by adsorbing sulphonated polystyrene from aqueous solution on highly porous collodion membranes: when these are dried, pore diameter is reduced, and the sulphonated polystyrene is trapped in the membrane (Neihof, 1954). Correspondingly, membranes of "strong-base" type have been prepared by the absorption of poly-2-cinyl-N-methylpyridinium bromide (Gottlieb, Neihof and Sollner, 1957) followed by drying. And under class (3), membranes may be cast directly from collodion solution containing dissolved sulphonated polystyrene, again resulting in the formation of a strong-acid type of membrane (Neihof, 1954). In the examples just given of classes (2) and (3), the most highly selective membranes of strong-acid type are 1000-2000 times more permeable for univalent cations than for univalent anions, 0.1N KCl being used as the solute; with the same solute, the most highly selective strong-base membranes are 250 to 450 times more permeable to the anion than to the cation. With more dilute solutions of univalent electrolytes,

or with uni-divalent electrolytes (e.g. CaCl2 with a strong-base membrane, or K2SO4 with a strong acid membrane) the selective permeability is even higher.

v. Membranes prepared by interfacial polycondensation

The classical material here is Nylon, but the procedure is of relatively recent origin. In the pioneer work of Carothers (1931), the preparation of polyamides by condensation-polymerisation depended on reactions that require high temperature and reduced pressure. It was not until 1959 that Morgan and his colleagues first reported on their extensive studies, in which they introduced interfacial polycondensation to prepare the polymer at room temperature. Morgan (1959) described his methods for the preparation of Nylon (e.g. Polyamide 610) in great detail, and analysed the factors involved in the formation of the membrane. In this procedure, a diamine (e.g. hexamethylenediamine) in the aqueous phase reacts at the interface with a diacid halide (e.g. sebacoyl chloride) in the organic phase. Since the diamine has an appreciable partition potential towards the organic phase, whereas the acid chloride has very little solubility in water, most of the reaction takes place on the organic-solvent side of the interface. The term Polyamide 610 derives from the use of a C6 diamine and a C10 organic acid halide. The chemistry of the reaction is as shown:



(where X is in the range of 25 to 100)

The hydrogen chloride produced may be neutralized in the aqueous phase by an inorganic base. Sebacoyl chloride has very low solubility in water and thus is hydrolysed only very slowly. The rate of production of the polymer depends on the organic solvent chosen, the concentration of the reactants, the area of the interface, and the temperature. The optimum condition for the polymerization of Polyamide 610 is a molar ratio of diamine to acid chloride of about 6.5 when carbon tetrachloride is used as solvent. With higher concentrations of diamine, because of its partition potential towards the organic phase, a less compact polymer network is formed. On the other hand, increasing the concentration of acid chloride results in a thinner membrane, since this reactant, because of its very low solubility in water, can diffuse only up to the interface. Changing the organic solvent alters the optimum ratio of diamine to diacid chloride: for a solvent like chloroform, which extracts the diamine strongly, the acid chloride concentration should be higher; on the other hand, the acid chloride concentration should be lower for a solvent like cyclohexane, in which the diamine is less soluble. For similar reasons, the thickness of film depends on the solvent chosen, on the time allowed for the reaction, and on the absolute concentration of the reactants, as well as on their ratio: by appropriate variation of these parameters the thickness of the wet films can be varied from 0.1 μ to 200 μ. The density of such films is 0.10-0.25 gm/cm^3; it appears to decrease with increasing reaction time. Electron micrography shows that the film has a rough side facing the organic phase and a smooth side facing the aqueous phase. Morgan found that films of Polyamide 610 thus prepared are readily permeable to inorganic salts

and small dye molecules, but less permeable to large dye molecules.

Many other polymers can be prepared by the principle of interfacia l polycondensation (Morgan, 1959b): for example, polyamides can be prepared as the aliphatic type, the aliphatic-aromatic type, or the o-phthalamide type, depending on the choice of the basic and acid reactants. Polymers with other types of linkage can also be formed by this procedure. Examples are polyureas, polyurethanes, polysulfonamides, polyphenyl esters, etc.

vi. Silicone rubber membranes

Mullison (1964) has recently summarized the structure and properties of the relatively new group of silicone polymers. These substances are analogous to the more familiar polymers that have a skeleton composed largely or wholly of carbon molecules. In the silicon polymers molecules have silicon rather than carbon forming the chain; organic groups are added to the inorganic skeleton satisfying the two remaining valences of the silicone atoms. The silicones are available in the form of fluids, resins and rubbers. A thick liquid polymer "Silastic RTV" (Dow Corning) can be vulcanized at room temperature by the addition of catalyst (dibutyl tin dilaurate or stannous octoate) to form a rubber that has useful mechanical properties and is chemically remarkably inert (Braley, 1960). Because of these properties, it has found extensive applications in the construction of surgical prostheses of many different types: it is readily handled and produces little or no tissue reaction when implanted into the body.

The properties of silicone rubber do not permit its use as a dialyzing membrane, because the material, though it can be formed into membranes, is extremely impermeable to liquid water and aqueous solutes. It is, however, extremely permeable to oxygen and carbon dioxide: 150-300 times more permeable than polyethylene and 800-1000 times more permeable than cellulose acetate (Dow Corning Co., 1959). It is also quite permeable to water vapour. The extraordinary permeability of silicone rubber to oxygen and carbon dioxide, coupled with its extreme inertness, has suggested its use as a diffusing material in membrane oxygenators functioning as artificial lungs in surgical procedures (Mullison, 1964). Another use has been investigated by Folkman and Long (1963) who sealed various drugs into closed Silastic tubes of 3.5 mm internal diameter, and found that fat-soluble materials, which could penetrate the Silastic wall by solvation - e.g. triiodothyronine and digitoxin - were slowly released from the external surface.

vii. Lipid membranes

Artificial lipid membranes are potentially of great interest to biologists, especially in view of the widespread belief that cellular and subcellular membranes incorporate lipids as basic elements. Artificial lipid membranes show phenomena that have been thought to be related to the electrical excitability of natural membranes, but they have been little used for the study of diffusion of aqueous solutions: porous membranes, through which such solutes pass by aqueous channels, rather than lipid membranes, through which solutes must pass by solvation, have generally been used for this sort of study.

The enormous literature on lipid films, beginning with the classical studies of Robert Hooke and Sir Issac Newton on thin soap films, and continuing - especially under the leadership of Langmuir, Bernal, Schulman and other distinguished modern scientists - need scarcely be summarized here. Other workers, such as Folch and Lees (1951), have been interested in the properties of lipid membranes formed at the interface between water and an immiscible liquid. There are, however, a few studies on the behaviour of lipid membranes inserted between two aqueous phases. Mueller and his colleagues (1962 and 1963) prepared such membranes by depositing lipid (dissolved in an organic solvent) in a hole (diameter 1 mm) in the wall (0.2 mm thick) of a 5-ml polyethylene cup. They found that the membrane could be "excited", as indicated by experiments in which d.c. or a.c. pulses were applied and the potential across the membrane was recorded. Nash and Tobias (1964) prepared a model membrane by depositing a lipid solution on a Millipore filter and allowing the solvent to evaporate. They found that the electrical behaviour of such a membrane resembled that of a cation-exchange membrane, and tentatively attributed this property to the phosphatidylserine component of their lipid mixture, which might provide negatively-charged sites for cation exchange.

Since the information in the literature on artificial lipid membranes separating two aqueous phases is so scanty, further discussion of the properties of lipid membranes will deal mainly with the role of the lipid components in natural membranes, and will be deferred to the next section of the Introduction, which deals with cell membranes.

THE CELL MEMBRANE

ORIGIN OF THE CONCEPT OF THE PLASMA MEMBRANE

Pfeffer (1877) was the first to postulate an invisible "Plasma-membrane" for cells, and to suggest that this membrane might have properties like those of artificial semipermeable membranes such as those he himself had made with copper ferrocyanide. His hypothesis, however, was formed without any positive supporting evidence, and was not accepted by his contemporaries. The general concept at that time was that there were no cell membranes, and that all the phenomena of cell permeability could be readily explained on the basis that the protoplasm of cells behaves like a colloid gel. In 1899 Overton gave suggestive evidence for the existence of a lipoid membrane by his permeability studies on the unicellular plant Chara ceratophylla, in which he showed a close correlation between the permeability to a number of nonelectrolytes and their oil-water partition coefficient. It was not, however, until 1912 that Höber presented an important piece of further evidence by his demonstration of the good conductivity of the erythrocyte interior as compared to the high electrical resistance of packed erythrocytes. This evidence, apparently, was not enough to convince his contemporaries; and H. W. Smith (1962), reviewing the history of the plasma membrane, summarised their attitude by saying that "Höber believed that there was a 'plasma membrane' and thus he was in that minority who were viewed askance by dog, cat, rabbit and white rat physiologists as being a little queer." The minority opinion, however, received substantial support from the elegant experiments of Chambers. Using his newly developed microinjection technique Chambers (1922) demonstrated that water-soluble materials, which could

not penetrate the cell if placed outside it, diffuse rapidly throughout the cell when injected into its interior - a convincing demonstration, I think, of both the aqueous nature of the cell interior and the existance of a cell membrane. Extension of this work by Chambers, and his demonstration that mechanical rupture of the cell surface allowed its cytoplasm to escape, led to the general acceptance of the plasma membrane concept.

During the last 40 years, physiologists have been concerned not with the existence of the cell membrane, but rather with its structures and properties. There is still, however, an occasional lonely dissenting voice; for instance Ponder, the distinguished authority on erythrocyte permeability, in his 1961 review questioned the existence of an erythrocyte membrane, suggesting that the surface properties of these cells may be due merely to a modification of their ultrastructures brought about by forces at their interfaces. He is not convinced that the surface forms a diffusion barrier comparable to that of an artificial membrane (Ponder, 1961). Ponder's view must be treated with respect; but since he agreed that there is something special about the surface ultrastructure and was willing to have his review titled "The cell membrane and its properties," it seems legitimate in this thesis, which is not primarily concerned with biological membranes, to accept the familiar terminology and to use it in the sense by which it is used by most investigators.

STRUCTURE OF THE CELL MEMBRANE

The lipoid theory of cell membrane structure

In 1899, Overton, as already mentioned, demonstrated a clear

relationship between the partition coefficient of a substance between oil and water and its penetration into the Chara ceratophylla cell. From this he concluded that the membrane was probably composed of lipid molecules. Collander (1937) extended Overton's findings and confirmed his conclusions. Gorter and Grendel (1925) extracted lipid from blood cells and spread it into a monolayer the area of which was found to be about twice the total surface area of the extracted cells: they therefore concluded that the cell membrane consisted of a bimolecular leaflet of lipid. Schmitt and Palmer (1940) studied the birefringence of red cell ghosts with polarized light and concluded that the cell membrane was composed of radially-arranged lipoid molecules. Grendel (1929) found that the relative contributions of the surface lipids to the spread area were cholesterol 35%, cepahlin and lecithin 50% and sphingomyelin 13%, and computed that the thickness of the double layers of lipid should be 13Å ; but Schmitt and Palmer arrived at a figure of 54Å from their X-ray analysis of orientated lipids. Since Höber's earlier work, others (Fricke, 1925; McClendon, 1926; Cole, 1937) continued with the approach of measuring the electrical impedence of cell membranes and obtained results compatible with the hypothesis that the surface is composed of a thin lipoid phase. Still further support for the lipid theory of cell membrane came from the wetting properties of cells: for example, erythrocytes passed readily into the interior of an oil layer, as would be expected if the surface layer was composed of a fatty material which could coalesce with oil (Mudd and Mudd, 1931). Besides, cells are readily lysed, not only by lipid solvents but also by lecithinase which acts presumably on the lecithin of the cell membrane (Ponder, 1953).

Molecular sieve hypothesis: the protein theory of membrane structure

The concept that the cell membrane is perforated by aqueous pores that permit the passage of small water-soluble molecules was first explicitly presented by Ruhland and Hoffman (1925). They studied the relationship between molecular size and rate of penetration of solute through the cell membrane of Beggiatoa mirabilis, a large sulphur bacterium, and found, as opposed to Overton's results with Chara ceratophylla, that permeability correlated well with the molecular size and not at all with the partition coefficient. The "Molecular Sieve" theory of the cell membrane which they proposed was found attractive by many other workers, especially those who found mammalian erythrocytes convenient for the study of permeability. Mond, Hoffman and Gertz (1928, 1929), using solutes of low lipoid solubility, demonstrated for the erythrocytes a close relationship between permeability and molecular volume; and Höber and Orskov (1933) showed that this relationship held for several series of homologous organic compounds. More recently, similar findings were reported by Solomon and his colleagues (Sidel and Solomon, 1957; Goldstein and Solomon, 1960) who developed an elegant optical method for measuring rapid changes in erythrocyte volume and found likewise that permeability for substances of low lipoid solubility correlated well with molecular radii. Höber summarized in his classical monograph, "The Physical Chemistry of Cell and Tissues", a thesis that while lipid-soluble molecules could penetrate by solvation in the membrane, water-soluble molecules entered through pores in the membrane. The earlier workers believed that the sieve-like character of the membrane could be best explained by the hypothesis that it was

composed of protein rather than lipid: the protein surface layer, it was thought, formed a meshwork through whose interstices water and water-soluble materials could diffuse.

Besides the sieve-like behaviour of the membrane, other properties also appear to support the notion of its protein structure. A membrane composed entirely of lipid, it was thought, would not exhibit the elasticity and strength of the red blood cell membrane (Mitchison and Swann, 1954). The same authors suggested that the fibrous protein might bind the lipid together to produce a structure with the properties observed. Furthermore, the low surface tension exhibited by some cells, as illustrated by the centrifugal force required to break an Arbacia egg into two, suggested the presence of a protein surface (Davson and Danielli, 1943). Moreover, erythrocytes are lysed by polyhydroxyphenols, which denature proteins but do not affect carbohydrates or lipids (Ponder, 1948). Finally, agglutination of many types of cells by specific antibodies suggests the presence of protein antigens at their surfaces.

The mosaic theory of membrane structure

Many investigators have devised hypothetical models of the cell membrane which would exhibit both the lipid-solubility and molecular-sieve properties. The first of these was Nathanson (1904), who suggested that plasma membrane might be a mosaic with areas differing in structure and properties, some regions having lipid properties and some having protein and molecular-sieve properties. This suggestion was not adopted by many subsequent investigators, but a modified form of it has recently been proposed by Hoffman (1962) on the basis of electron microscopic studies

on red cell ghosts.

The Danielli and Davson model of the cell membrane

Danielli and Davson (1943), whose model has dominated text-book accounts of the cell membrane since 1943, pictures it as composed of two layers of lipid molecules arranged radially with their hydrocarbon chains facing each other, and their hydrophilic groups (hydroxyl, carboxyl and phosporic acid) directed outward and helping to form loose linkages with two layers of protein, one on the inside and one on the outside of the membrane. The arguments they bring forward, in support of this model, are too well known to need detailed recapitulation here. The evidence discussed under the lipoid theory of cell membrane structure pointed to a bimolecular leaflet of lipid arranged radially. The evidence presented under the molecular sieve theory of cell membrane structure pointed to the presence of protein in the cell membrane; the study on the tension at the surface of cells described earlier is compatible with the location of the protein on the outer surface of the lipid component of the cell membrane. Davson and Danielli also suggest that there are specialised areas on the cells responsible for active transport and that they are similar to the active centres of enzymes.

Although the Danielli and Davson model has won remarkably wide acceptance, it is by no means alone in the field today. In particular, Hillier and Hoffman have revived the mosaic theory in a modified form; and Meyer and Teorell (Teorell, 1935) have proposed a model to account for the differential permeability of many biological membranes to anions and cations.

The Meyer-Teorell Model of cell membrane

They propose that cell membrane has a sieve-like network structure with lipids and phosphatide molecules in its outer regions. In the meshes are immobile carboxyl groups or amino groups, or both. This type of model would resemble very much the charged porous artificial membrane (Teorell, 1935).

Model of cell membrane from electron microscopic studies

Hillier and Hoffman (1953) suggest that the lipid component of the red cell membrane may be situated between the meshes of a lattice of fibrous protein, since electromicrographs of erythrocyte ghosts extracted by lipid-soluble solvents show a fibrous structure with holes -- these holes are not present in those not treated with solvent. It has been suggested that the membrane may be a mosaic -- where the polar groups of lipids are attached to the polar groups of protein, water-soluble material of suitable radii can enter the cell: and where the nonpolar groups of lipids are attached to the nonpolar groups of protein, lipid-soluble material can pass through. Hoffman has recently summarised the results of electron microscopic studies of red blood cells (1962): the ghost consists of plaques 40 Å in thickness and 250 Å in diameter; the fibres, which are 20 Å in diameter and 100 Å to 4000 Å in length, lie below the plaques. These plaques and fibres are held together by ether-extractable lipid. He feels that if the lipid is radially oriented and arranged as a bimolecular leaflet (plaque-lipid-lipid-fibre) then this is consistent with the Danielli-Davson model: on the other hand, since ether only removes part of the lipid, the remaining lipid may be inside the plaques oriented tangentially; this would be consistent with the mosaic membrane model.

REASONS FOR MAKING SEMIPERMEABLE AQUEOUS MICROCAPSULES

CELL MODELS

In my account ("History of present work") of how this work began, I described my original interest in preparing "artificial erythrocytes" which, I thought, might be more stable and less likely to cause transfusion reactions than their natural equivalent. This general idea, of creating cell models containing all or part of the intracellular components of natural cells, but having their natural membrane replaced by an artificial one, still seems worthy of retention and development.

Many important problems relating to the origin of cellular responses pose the question of whether the triggering mechanism is located at the cell surface, or in intracellular organelles, or in the soluble constituents of the cytoplasm. Modern techniques of homogenation and differential centrifugation permit the isolation, in more or less intact form, of many subcellular particles; but except in special cases - e.g. microelectrode studies giving information about ion permeability - the role of the membrane as distinct from that of the cell interior cannot be evaluated. It might be theoretically possible, as an approach to this problem, to eliminate the specific functions of the natural membrane by using a sheet of artificial membrane to separate homogenised cellular materials from the plasma or from a synthetic extracellular fluid such as Krebs' solution, and then to study the changes in the "intracellular" medium produced by changing the composition of the "extracellular" medium. But unless an extremely thin layer of intracellular medium was used, the surface-volume relationship would be very unfavourable, and rapid responses

of the intracellular phase would not be expected. No doubt for this reason, experiments of this sort have not, so far as I know, been carried out. On the other hand, the microencapsulation of cell contents within artificial membranes might well provide preparations with which studies of this kind would become feasible. For instance, it might be possible to determine whether the effects of certain hormones, and of changes in extracellular electrolytes, are primarily due to an action of the substances on specific surface receptors and only indirectly affect the intracellular material, or are exerted directly upon the latter. I have not myself made studies of this sort, except for a few preliminary tests too tentative to deserve reporting in this thesis; but I hope that an opportunity may be found in the future for experiments along this line by myself or others.

MICROENCAPSULATION OF ENZYMES

Many inborn errors of metabolism are due to the deficiency of a single enzyme as a result of a mutation or some other genetic accident. Hsia in his recent monograph on "Inborn Errors of Metabolism" (1960) enumerates twenty-five diseases in which the biochemical lesion is the absence or modification of a particular enzyme; and another four diseases for which such an etiology has been suspected as a primary or predisposing cause.

An obvious approach to the therapy of such diseases would be the parental administration of the missing enzyme. As yet, however, this approach has met with little success; and indeed there are only a few cases in which it has been tried. The difficulties are obvious. The

injected enzyme would have to be obtained from a different individual, and perhaps in most cases from a non-human species. Thus, the injected enzyme would be a foreign protein, and might not survive very long in the body of the recipient. The first administration of such a foreign protein might produce undesirable reactions, such as the numerous reactions that depend on the activation of plasma proenzymes by foreign macromolecules. The treatment would be expected also to sensitize the recipient specifically to the injected protein, so that a second injection some days or weeks later might result in an anaphylactic reaction. Moreover, the production of specific antibodies to the injected protein would result in its increasingly rapid inactivation when the treatment was repeated. An example of such behaviour has been reported in the case of urease, which is not a natural tissue enzyme in vertebrates, but may be produced by the bacterial flora of the gastrointestinal tract. Visek and Thomson (Visek and Thomson, 1961; Thomson and Visek, 1963) found that by repeatedly injecting small doses of crystalline urease into human subjects they were able to raise the level of circulating antibody to urease, and they suggested that this might be a useful way of treating hepatic coma patients, in whom bacterial urease action contributes to the rising level of ammonia of the blood.

The problems just described are relatively unimportant when the desired enzyme action has to be achieved only locally in some accessible part of the body. A few examples may be cited of situations of this sort in which enzyme therapy has been found useful. Trypsin has been administered orally to patients with cystic fibrosis where there may be

a deficiency in the pancreatic exocrine secretion. Hyaluronidase, a mucolytic enzyme which depolymerizes hyaluronic acid (a polysaccharide component of intercellular substance), has been used clinically to facilitate the absorption of transudates or blood accumulated locally (Goodman and Gilman, 1955). Streptokinase and streptodornase, enzymes produced by a haemolytic streptococci, act on fibrin and desoxyribonucleoprotein respectively: a mixture of these two enzymes, available commercially as "Varidase", has been used topically for the debridement of wounds, and has been injected into body cavities to facilitate the removal of inflammatory exudates or clots. In this case, however, there may be some dangers of local allergic or generalized anaphylactoid reactions; furthermore, antibodies are formed to the enzymes, with the result that if the administration is continued for more than a week or two the dose has to be increased (Drill, 1954). In addition, various enzymes and proenzymes associated with blood coagulations (e.g. fibrinolysin (plasmin), thrombin, and thromboplastin-generating factor) have been administered intra- or extra-vascularly in various degrees of purity to retard or accelerate thrombus formation; but no attempt will be made here to review this specialized field.

In some cases the missing enzyme or protein component of an enzyme system is one that normally occurs in the plasma. The classical example is true hemophilia, in which a sex-linked deficiency of antihemophilic globulin (AHG) in the plasma globulin fraction causes impaired formation of thromboplastin and a consequent tendency to hemorrhage. The condition, as is well known, can be temporarily relived by transfusion of fresh human plasma or a human AHG concentrate. Other hemorrhagic diseases

have also been shown to be due to a deficiency of one or another of the clotting factors, and some of these can also be temporarily relieved by the administration of the factor, or plasma that contains it. Such conditions include Christmas disease, deficiency of PTA, Factor V deficiency, Factor VII deficiency, and still others that are more or less well recognised. In these cases the prospect of using microcapsules to convey the missing protein to its site of action appears to be remote, because the clotting factor in each case has to interact with other macromolecular constituents of plasma and these could not be expected to make contact with a protein sequestered within an artificial semi-permeable membrane. In a few cases, however, the missing plasma protein may be one that interacts with diffusible constituents of plasma. In this category may be placed hypophosphatasia, in which early bone lesions of the rachitic type appear to be related to a diminution of alkaline phosphatase in the serum (Hsia, 1960). Pseudocholinesterase (serum cholinesterase), an enzyme whose natural function is unknown, is necessary for the rapid inactivation of certain drugs of ester structure such as suxamethonium (succinyldicholine). If a patient who happens to have a low pseudocholinesterase level receives suxamethonium for muscular relaxation during surgery, he is likely to develop prolonged apnea. Administration of microencapsulated enzyme might be useful in these two cases, where the substrates for the deficient enzymes are diffusible and could perhaps be inactivated with sufficient rapidity by the appropriate microencapsulated enzyme, especially if the enzyme-loaded microcapsules were able to circulate like blood cells through the cardiovascular system. Mention might also be made of

ceruloplasmin, a copper-binding plasma protein whose function is poorly understood,but whose congenital absence leads to hepatolenticular degeneration (Wilson's disease) in which the tissue appears to become overloaded with copper. According to Usia, treatment by the injection of ceruloplamsin is impractical at the present time because of the very limited quantities of the protein available, and the most effective treatment at present is the administration of chelating agents for copper such as BAL (2,3 dimercaptopropanol) or DL-penicillamine. Recently Dr. A. Hoffer (1964) has informed me that he suspects that a relative deficiency of ceruloplasmin may be an etiologic factor in schizophrenia, and believes that ceruloplasmin may act as an enzyme to detoxicate a hypothetical toxic factor; as evidence for this, he reports that injected ceruloplasmin can produce a remission of the symptoms. This remission is very fleeting because the injected protein is rapidly inactivated. Dr. Hoffer has asked me about the possibility of encapsulating ceruloplasmin and testing its possible therapeutic action; but it has not so far been possible for me to give him any help along this line.

In some of these so-called "molecular diseases" the enzyme in short supply is one that is located, and carries out its metabolic function, in a particular type of cell; and the parenteral administration of the enzyme would be without value unless it could be deposited selectively in that type of cell. One example of such a hereditary enzyme deficiency is congenital methemoglobinemia type I, in which the deficient enzyme is diaphorase I which is necessary for the reduction of methemoglobin to hemoglobin with the assistance of co-enzyme I. Other examples are total

albinism, due to the absence of tyrosinase inside skin melanocytes; familial cretinism, which appears to be caused by the deficiency of an enzyme involved in the iodination of tyrosine within thyroid cells; and a common form of the adrenogenital syndrome, in which a deficiency of the specific enzyme "21-hydroxylase" for hydroxylation of the carbon at position 21, results in the excessive production of adrenal androgen. Unless some device, as yet unimagined, can be found for inducing enzyme-loaded microcapsules to settle in a particular kind of cell, the application of microcapsules to the therapy of these diseases appears quite unpromising.

In a considerable number of inborn errors of metabolism, the enzyme is one that is located and functions principally in the liver, but whose deficiency results in the under- or over-production of the substance that affects tissues elsewhere in the body. In these cases, it would seem that the hepatic location of the enzyme is not critical for its effective functioning, but is merely an example of the versatility of the liver in accomplishing a great number of metabolic transformation for the benefit of the body as a whole. Such diseases, it would appear, might be candidates for treatment by enzyme replacement with the aid of microcapsules; and in these cases the microcapsules could be deposited in any region of the body where there is a sufficient turnover of substrate in the extracellular kind. It would of course be necessary for success that certain conditions be fulfilled: (1) the enzyme should be reasonably stable in the microencapsulated form both _in vitro_ and _in vivo_; (2) the microcapsule membrane should be sufficiently permeable

to both the substrate and the product of the enzyme; and (3) either the enzyme must be one that requires no soluble coenzyme; or else the microcapsular membrane must have a high degree of selective permeability, retaining the coenzyme (which would have to be encapsulated along with the enzyme) while allowing the passage of substrate and product; or else the coenzyme should be one that can be maintained at the necessary level by its administration at intervals. A number of metabolic diseases would seem in the light of present knowledge to meet these criteria.

A typical example is Phenylketouria, a hereditary condition characterised by mental retardation and the presence of phenylpyruvic acid in the urine. This condition is due to the deficiency of an enzyme system called "Phenylalanine hydroxylase" which involves two protein fractions, a labile fraction I which is present only in the liver, and a more stable fraction II found also in kidney and heart. The enzyme system is required for the conversion of phenylpyruvic acid to tyrosine, and when it is absent, the excessive phenylpyruvic acid is transaminated to p-hydroxyphenylpyruvic acid. The disease can be treated more or less effectively in infants by administrating a diet low in phenylalanine, and it can be mimicked in animals by the oral administration of large amounts of phenylalanine (Waisman and Harlow, 1965). The phenylalanine hydroxylase system might be suitable for microencapsulation, because phenylalanine and tyrosine are both small amphoteric molecules which would be expected to diffuse rather easily through microcapsular membrane; on the other hand, the system requires a number of soluble co-factors like DPNH and iron; these might be permeant and would have to be administered separately or chemically linked to a larger molecule before encapsulation.

Alcaptonuria is a rare inborn error of amino acid metabolism characterised by the accumulation of homogentisic acid in the body fluids as a result of the deficiency of the liver enzyme homogentisic oxidase, whose product is maleyl-acetoacetate which is then transformed by other enzymes to fumarate and acetoacetate. Deposition of homogentisic acid in the large joints leads to a deforming arthritis in middle age. Homogentisic acid and its oxidation products are small water-soluble molecules that can diffuse through microcapsule membrane. Because the arthritic pathology develops so slowly, enzyme replacement therapy would have to be prolonged; but the disease may be tentatively classified with that group of diseases where the possibility of the microcapsule-technique might be investigated.

In "Maple Sugar Urine Disease" the three branched chain amino acids - valine, leucine, and isoleucine - are greatly increased in the body fluids, apparently because of the deficiency of a single enzyme necessary for the decarboxylation of the corresponding branched chain ketoacids. The disease occurs in infancy and is fatal. The enzyme involved has not been characterised; but if it is found to have the necessary stability, it might be worth while to encapsulate it, since the substrates and products of its action are all small water-soluble molecules.

The conversion of dietary galactose to glucose is accomplished by two liver enzymes: galactokinase, which phosphorylates galactose in the presence of ATP to galactose-1-phosphate, which is then transformed by phosphogalactose-isomerase in the presence of uridine diphosphate galactose to glucose-1-phosphate. In galactosemia, the absence of the second enzyme causes an accumulation of galactose during milk-feeding in

infancy, with a variety of pathological consequences. The disease, however, is so adequately treated by withholding milk and milk products that it seems unnecessary to consider more complex forms of therapy.

Final reference must be made to some pathological conditions which might respond to enzyme therapy even though there is no evidence that the condition is due to the deficiency of that particular enzyme - the most obvious example is perhaps gout, a disease which does not occur in most mammals other than man, because they possess the liver enzyme uricase, which converts uric acid into the more soluble allantoin, so that there is no opportunity for urates to crystallize in joints. In healthy human beings the uric acid derived from purine metabolism can be excreted sufficiently well, mainly by the kidney; but in gout, for reasons which are still not well understood, the excretory system is unable to handle the excessive uric acid that is produced. The enzyme uricase is available in crystalline form and the substrate uric acid is known to penetrate artificial membranes; indeed, its plasma concentration can be effectively lowered within a few hours when patients in renal failure are dialysed with an artificial kidney. The product allantoin should be still more diffusible, and the enzyme requires no co-factor. The possibility that microcapsules may eventually be useful for the treatment of this nonfatal but troublesome disease is perhaps not too remote. Other pathological conditions that might be conceivably treated by enzyme administration might include hypertension of renal origin, which is probably due at least in part to the excessive production of the pressor product angiotensin, whose destruction might be accomplished by the specific enzyme angiotensinase; and poisoning by organic phosphates,

used so extensively as insecticides and in chemical warfare, which act by inactivating cholinesterase, might be treated by injecting a large excess of the enzyme, which would then trap the poison and hydrolyse the excess of acetylcholine in the body fluid.

It will be shown in the experimental part of this thesis that microcapsules are of low intrinsic toxicity and that in favourable cases, a well-marked enzyme action can be obtained in the whole animal even with intraperitoneal injection of enzyme loaded microcapsules. The possibility is therefore reinforced that further investigation of enzyme-loaded microcapsules may lead in time to useful therapeutic preparations.

MICROENCAPSULATION OF INTACT CELLS

A further possibility that I have thought worth exploring is the microencapsulation of intact cells or tissue fragments. The idea in this case would be similar to that underlying the investigation of model cells and microencapsulated enzymes: the enclosed material might be protected from destruction and from participation in immunological processes, while the enclosing membrane would be permeable to small molecules of specific cellular products which could then enter the general extracellular compartment of the recipient. For instance, encapsulated endocrine cells might survive and maintain an effective supply of hormone after removal, or destruction by disease, of the recipient's own endocrine tissue. The situation would then be comparable to that of a graft placed in an immunologically favourable site.

Rejection by the host of heterograft or homograft tissue is well known and has been studied in enormous detail during the last decade. In the case of endocrine tissues, however, the results obtained with homografts have been rather confusing and inconclusive. There is a general impression that foreign endocrine tissue is somewhat less readily rejected than skin, which is the tissue on which by far the greatest number of investigations have been carried out. For instance, Billingham and Parkes (1955) found that ovarian tissue survived longer than did skin after homotransplantation in rats; and Russell and Gittes (1959) obtained similar results with parathyroid tissue. Some workers reported favourable metabolic effects after both the homogenic (Schatten, Bloom and Hamm, 1961) and heterogenic (Dunphy and Jacob, 1961) graft of parathyroid tissue, but they supplied no histological evidence of prolonged graft survival, and did not exclude the possibility that the beneficial effect was due to the preformed hormone present in the implanted tissue. Other workers failed to observe survival of ovarian (Krohn and Ingram, 1956), adrenal (Medawar and Russell, 1958) or thyroid (Woodruff, 1959), tissues for periods much longer than were found with skin grafts involving the same donor-recipient strains.

A new approach to the problem of endocrine transplantation was begun by Algire and his co-workers (Algire, 1954; Algire and Prehn, 1955; Algire, Weaver and Prehn, 1957) who found that such homografts enclosed in Millipore chambers survived for long periods, even when placed in a previously immunised host. The walls of these chambers were made of sheets of Millipore filter cemented on the side of lucite rings

which were 1.75 cm in diameter (Millipore filters have a matrix of cellulose esters, and can be made to have graded but remarkably uniform porosity, and have been found useful in the separation and analysis of a great variety of materials). The Millipore filters used for the chambers had an effective pore size of approximately 0.45 μ and were 100 to 150 μ thick. These Millipore chambers were permeable, at any rate initially, to the body fluids and proteins, but not to cellular elements, and were implanted intraperitoneally into animals - mice and rats. Clear evidence was obtained that adrenal glands thus homotransplanted into adrenalectomised rats significantly prolonged their survival. Histological examination after a period of up to 6 months showed that although much fibrosis was present, viable adrenal cells of relatively normal appearance were scattered throughout the tissue with no sign of a leucocytic type of rejection reaction (Brooks, Sturgis and Hill, 1960). Homografts of ovary similarly enclosed also survived for up to 6 months as judged by histological evidence and vaginal smears in rats (Brooks, Sturgis and Hill, 1960). The results with enclosed parathyroid homografts were less conclusive (Wilson, Zollinger, Mahan and Brooks, 1959): some cells survived, but much fibrosis was present in the chamber. Thyroid homografts enclosed in Millipore chambers have been implanted into both rats, dogs and man: in the rat the tissue functioned for up to four months as judged by I^{131} uptake and histologic studies (Brooks, Sturgis and Hill, 1960), after which there was a steady decline in tissue viability; in dogs the tissue survived for up to 30 weeks as judged by the same type of studies (Gough, Pugh and Brooks, 1962); in man, some thyroid follicles were still present after 10 weeks in five out of ten cases, although there was much fibrosis (Brooks, Sturgis and Hill, 1960).

The Millipore chamber technique has been extended to include heterograft transplantation, the assumption being that if cellular rather than humoral antibodies are principally involved in the rejection process, the conditions for survival might be as favourable as in the case of homografts. Stone et al. (Stone, Eyring and Kennedy, 1960) implanted Millipore chambers containing human thyroid tissue into guinea pigs, choosing the fascia above the rectus abdominal muscle for the implantation site, and found histological evidence of endocrine tissue survival for over three months in one case. They (Stone and Kennedy, 1962) also implanted enclosed dog thyroid tissue into one human patient, and found that although some tissue survived, it had lost most of its characteristic structural organisation. An interesting finding was that the human recipient showed no general or local reaction to the foreign tissue.

Transplantation of islet-cell tissue within Millipore chambers has also been attempted, but without success even though an autotransplant was used (Carnevali, ReMine, Grindlay and Harrison, 1960). In this case it appears likely that the graft was destroyed by enzymes released from contaminating pancreatic exocrine tissue.

It has been suggested that the "late rejection" of grafts in Millipore chambers is due to diffusion limitations leading to failure of nutrition of the enclosed cells (Brooks and Hill, 1960; Bassett, Andrew and Campbell, 1960; Stone and Kennedy, 1962). Restricted diffusion of nutrient may be present from the outset because of the thickness of the Millipore filter (100-150 μ) and the low surface-to-volume ratio

of the chambers; but there is also a tendency for gradual impairment of diffusion by plugging of the filter by insoluble calcium salts, and overgrowth of fibrous tissues. Stone and Kennedy (1964) had some success in overcoming this difficulty by cutting slits in the chamber wall after four to five weeks of implantation: the improved survival in this case appeared to indicate that the graft had become less susceptible to immunological rejection. It should be noted, however, that though humoral antibody and complement diffused with difficulty into implanted chambers (Wakefield and Amos, 1958), some types of enclosed homografts were destroyed when these antibodies were present in high concentration in the body fluid: observations of these sort have been made with fibroblasts (Gabourel, 1961) and leukemic cells (Russell and Monaco, 1964).

Although Algire's work with Millipore chambers has won much attention, and is being followed up by many other investigators, it does not seem that much thought has been given to the possibility of using chambers with more favourable surface-to-volume relationship, or with Millipore membranes not permeable to humoral antibodies. The enclosure of intact cells within microcapsules of suitable size would appear, if it were feasible, to offer solution to these difficulties. There would be the further advantage that implantation could be accomplished by a simple injection procedure rather than by a surgical operation. Time has not yet been found to extend my own studies to include experiments of this sort with endocrine replacement. I have found, however, that erythrocytes can be encapsulated by modifications of the procedure I have used for proteins. These tests are briefly

described and illustrated in the Experimental part of this thesis, and they encourage the hope that in-vivo experiments with endocrine tissues may yield interesting results.

DIALYSIS

Graham in 1861 discovered that he could separate colloids from a mixture containing both colloids and crystalloids in aqueous solution by using a membrane of parchment paper. He named the process "dialysis", and since then much use has been made of the principle, especially in biochemical preparative work; as already noted, other membrane materials, especially collodion, have taken the place of parchment paper. The applications of dialysis are too numerous to mention, and would be even more numerous were it not that the process, as ordinarily carried out, is intolerably slow, whether the solution under investigation is dialysed against a large volume of water or aqueous solution of crystalloids, or against air with the aid of hydrostatic pressure in the process of ultrafiltration. Many workers have used a variety of devices to speed the equilibration of diffusible substances by increasing the ratio of membrane area to fluid volume. One of the most recent and interesting applications of this sort is that of Craig (1964), who by reducing the volume of both watery phases in a concentric counter-current system with a membrane partition was able to separate dissolved crystalloids on the basis of their molecular dimensions. The kinetics of Craig's system are analogous to those of column chromatography, with the diffusibility of the solute through the membrane being substituted for its affinity for a stationary phase. It is reasonable

to suppose that the principle of differential dialysis could be applied more efficiently if the surface-volume ratio were increased by incorporating the stationary aqueous phase within microcapsules loaded into a column of suitable dimensions. A tentative exploration of this possibility is described in the experimental section, but much more work needs to be done to establish the usefulness of the technique.

The use of microcapsule columns to alter the composition of circulating blood seems at present to be a very remote possibility. It may, however, be worth while to mention, though the point is somewhat outside the scope of this thesis, that the principle of interfacial polymerization can be applied for the production of microtubules as well as spherical microcapsules, as preliminary experiments of mine have shown. If an array of microtubules, arranged in parallel and connected at either end to a larger tube, could be inserted as a shunt into a large blood vessel, with the outer surface of the microtubules in contact with flowing air or Krebs solution, one might have the basis for an artifinial lung or artificial kidney, of more convenient dimensions than the devices presently available. The technical problems of constructing such an array will require some engineering ingenuity for their solution , but it is thought that the information obtained from the preparation of microcapsules by interfacial polymerization may be quite relevant to this problem, which I would like to have an opportunity of attacking at some future time.

MISCELLANEOUS

Other possible applications of the microencapsulation-technique that have been suggested will merely be listed without any attempt to evaluate their feasibilities:

1. Microencapsulation of drugs for slow release after oral or parenteral administration (This has already been mentioned under "Silicone rubber" but other membrane materials may also be used);

2. Microencapsulation of radioactive isotopes or antimetabolites for intra-arterial injection into tumour-bearing tissue - in this case some of the microcapsules might lodge at the tumour site, while others would be carried by lymphatic channels to metastases in regional lymph nodes;

3. Microencapsulation of radioopaque material to provide a contrast medium of lower specific gravity for the radiography of the gastrointestinal tract, whose motor functions might be distorted by the heavy materials currently in use;

4. Provided microcapsules can be made that will circulate readily in the blood stream, they might be used as vehicles for contrast materials in angiography - all the presently available materials carry some hazard when injected intravascularly;

5. The microencapsulation of highly magnetic alloys might provide a useful preparation for the measurement of blood flow in unopened vessels by electromagnetic techniques - the weak ferromagnetism of

hemoglobin has been used successfully for this purpose, but instrumentation could be much simplified if microcapsules containing magnetic alloys could be made to circulate;

6. If membranes of cross-linked protein ("Preparation of cross-linked-protein-membrane microcapsules") can be made to retain the immunological characteristics of the protein, there might be a place for microcapsules in serological studies.

Other possible applications have been considered or have been suggested to me by others, but this list is perhaps rather too long already.

II. METHODS FOR THE PREPARATION OF MICROCAPSULES

PREPARATION OF SEMIPERMEABLE AQUEOUS MICROCAPSULES

GENERAL PRINCIPLE

As has already been noted, the preparation of semipermeable aqueous microcapsules involves three main steps: (1) the aqueous solution of protein is emulsified in an organic liquid, usually with the aid of a suitable surface-active agent; (2) on the addition of a suitable material to the continuous phase, a permanent membrane is formed at each interface by either coacervation or polymerization; and (3) the microcapsules so formed are transferred to an aqueous medium. These procedures are illustrated in schematic form in Fig. 1 . With both the coacervation and polymerization techniques, each of the three main steps can be modified in a variety of ways. I will not describe all of the variations in procedure that I have used, but instead will give a detailed account of one coacervation procedure and one polymerization procedure, with reasons for selecting the particular materials and manipulations prescribed. Most of the principles involved have already been mentioned in my accounts (p. 6 to p. 17) of how these techniques were developed; but a number of additional points are more conveniently considered in this experimental section. Special attention will be given to the different polymers that have been found suitable for the microencapsulation of protein solutions. In many of my trials, the encapsulated material was a red cell hemolysate, and the procedure described refers specifically to this material; but other proteins in aqueous solution can be enclosed by very similar procedures. Some attention will be given to the modifications that are indicated when material other than a red cell hemolysate is to be encapsulated.

PROCEDURE FOR MAKING MICROCAPSULES:

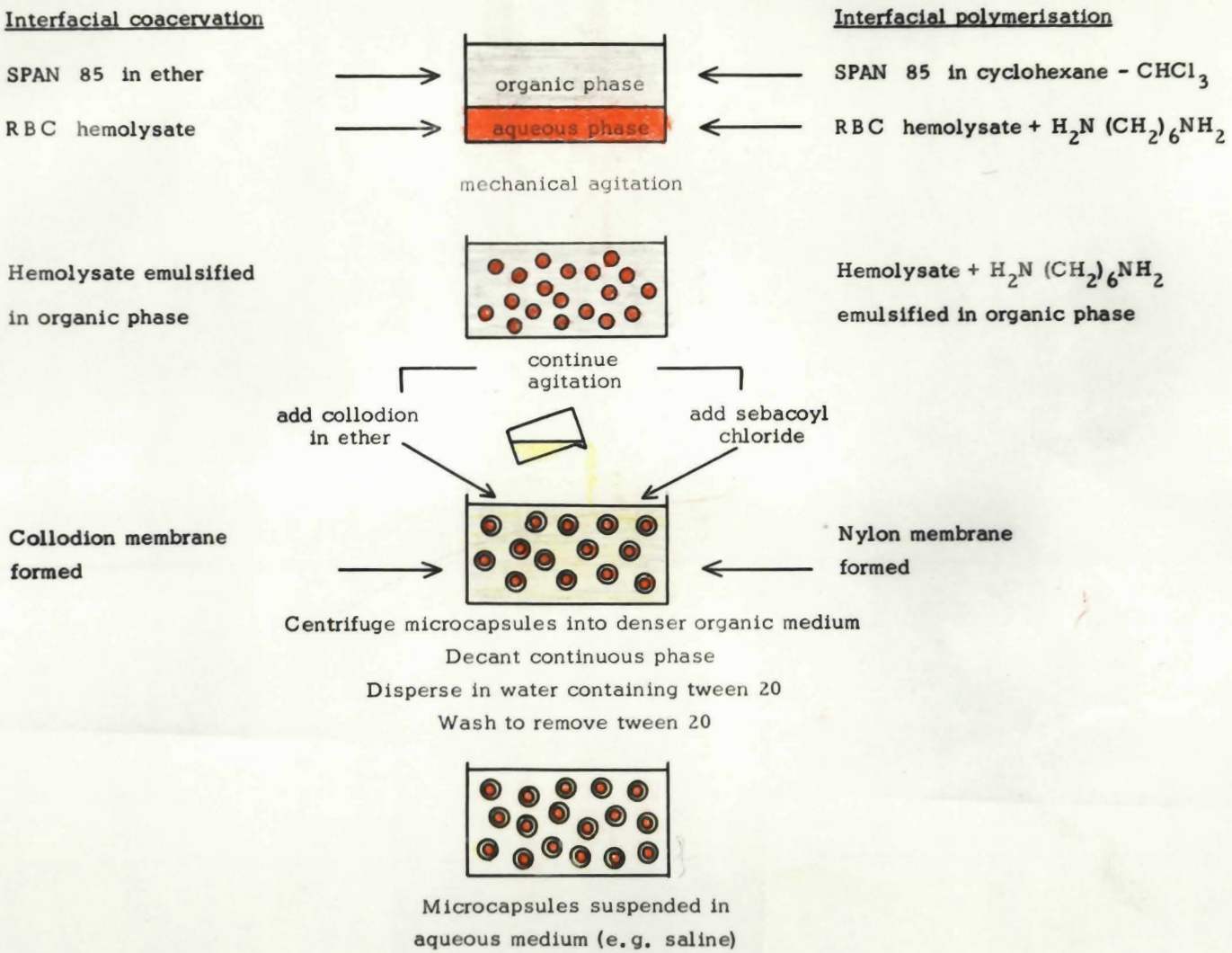


Fig. 1

Schematic representation of the two basic procedures for making microcapsules. For explanation see text.

INTERFACIAL COACERVATION

Principle

In this process the formation of the membrane depends on the lower solubility of a polymer, dissolved in a water-immiscible fluid, at the interface of each aqueous microdroplet in an emulsion. This principle is illustrated below, with collodion as the membrane material and an erythrocyte hemolysate as the internal phase.

Preparation of collodion-membrane microcapsules

The starting material is mammalian blood, heparinized or citrated. About 10 ml of the erythrocytes are spun down and washed three times with 2 volumes of saline, and after removal of the supernatant, the erythrocytes are hemolysed by shaking with water (2 volumes) containing ether (U.S.P., 0.15 volume) and left standing covered for 30 minutes. The use of ether facilitates the hemolysis of erythrocytes and the removal of stromata by centrifugation in the next step. The hemolysate is centrifuged at 1500 g for 30 minutes, and the clear hemolysate is siphoned off, avoiding contamination by the superficial layer of ether containing stromata and by any deposited red cells which might have escaped hemolysis. If the hemolysate is to be stored for an appreciable time, residual ether is removed by stirring in a ventilated hood for 30 minutes (a Jumbo magnetic stirrer in a 100-ml beaker is convenient) and the liquid is kept in the refrigerator. Just before use, the hemolysate is buffered by the addition of tris-hydroxymethyl amino-methane, unbuffered, (final concentration 0.08 M) to neutralise acidic impurities present in most cellulose nitrates of the collodion solution. 2.5 ml of the buffered hemolysate is mechanically emulsified in an

ice-cooled covered vessel with 25 ml of ether, previously saturated with water and containing 1% (v/v) "Span-85" (Atlas Powder) as the emulsifying agent.

A Jumbo magnetic stirrer (Fisher Scientific) with a 4-cm stirring bar in a covered 100-ml beaker is particularly convenient. All stirring involving electrical appliances should be done in an ice-cooled vessel in a well ventilated hood to avoid fire hazards. The mean diameter of microdroplets is determined by the speed setting of the stirrer; for example, with a speed setting of "7.5", microcapsules of mean diamter 31.9 μ are produced. Smaller microcapsules (mean diameter down to 5 μ) can be made by using a Virtis 45 homogenizer and a 30-ml cylindrical flask with diagonal fluting. With the Virtis homogenizer, a 16-gauge needle is passed through a hole in the cover of the flask so that reactants may be added without stopping the homogenizer; the edges and the tip of the homogenizer blade should be blunted to prevent destruction of the nascent membranes in the next step. A homogenizer setting of "1" yields microdroplets with mean diameter about 10 μ.

The hemolysate and other aqueous protein solutions can be emulsified without the use of Span 85 or other surfactants, but higher homogenizer speeds are then required. If Span 85 is used at a concentration much higher than 1%, results are unsatisfactory, apparently because the thicker film of detergent interferes with the formation of the membrane. As already noted ("History of present work"), Span 85 is superior to many other surfactants because it does not denature the protein in the aqueous phase.

When the emulsifier has been running for 1 minute, 25 ml of collodion solution (Collodion U.S.P., freed from alcohol by evaporation and made up to its original volume with ether) is added and stirring is continued for another 30 seconds. The cellulose ester precipitates at the interface, and the emulsion is allowed to stand covered for 45 minutes while the membranes set. During this period, the vessel should be kept covered to prevent evaporation of ether. The presence of alcohol in the collodion solution is undesirable because it causes precipitation of protein in the aqueous phase.

At the end of 45 minutes, the outer surfaces of the microcapsules are still ill-defined; to complete the formation of the membranes the microcapsules must be transferred into another medium. The microcapsules are first resuspended in n-butyl benzoate by the following procedure. First, a further 1 ml of undiluted Span 85 is added with gentle stirring: its function is to prevent microcapsules from aggregating during centrifugation. Then the microcapsule suspension is distributed into four Parafilm-covered 15-ml centrifuge tubes and spun down for 5 minutes, at 350 g for the larger microcapsules or 1500 g for the smaller microcapsules. All but about 1 ml of the supernatant is then removed; and the microcapsules are stirred with a glass rod until they are dispersed: this can be judged without too much difficulty by the naked eye. Next, 7.5 ml of n-butyl benzoate (Eastman) containing 1% v/v Span 85, is added to each tube and thoroughly mixed with the suspension, which is allowed to stand uncovered for a further 30 minutes to allow for the outer surfaces of the microcapsules to set, and then centrifuged as before. To transfer the microcapsules from the organic-liquid phase

into an aqueous phase, the supernatant is discarded except for about 0.25 ml in each tube, and, after the microcapsules have again been dispersed with a glass rod, 6.25 ml of Tween 20 solution (Atlas Powder, 50% v/v in water) is added to each tube and the microcapsules are dispersed in the aqueous medium, first by stirring with a glass rod, and then, after pooling the suspension in a 100-ml beaker, by 30 seconds agitation with the magnetic stirrer (Jumbo speed setting of "8"). At this point the microcapsules are crenated in the hypertonic Tween 20 solution, but they gradually recover their spherical form if the suspension is immediately diluted with 200 ml of water. The slightly turbid supernatant may now be removed by centrifugation, and the microcapsules are then resuspended in a 1% Tween 20 solution and stored in the refrigerator until they are to be used. Tween 20 in the higher concentration facilitates the transfer of the microcapsules from the organic medium into the aqueous medium and prevents their aggregation during the transfer; in the lower concentration, it prevents aggregation of the microcapsules during prolonged storage. When necessary, the microcapsules may be washed free of Tween 20 on a centrifuge just before use.

Collodion microcapsules as prepared are shown in Fig. 2.

Preparation of microcapsules with other than collodion membrane

Membranes can also be formed by interfacial coacervation from polymers other than collodion that are soluble in organic solvents. For example, polystyrene (20 gm% w/v) may be dissolved in benzene, and the above procedure repeated, with benzene substituted throughout for

ether, and polystyrene in benzene for collodion in ether.

Microencapsulation of other proteins

The success of the procedure described above appears to depend on the presence of protein in sufficiently high concentration in the aqueous phase. With a very dilute solution of hemolysate, protein or enzyme, microencapsulation usually is not possible;on the other hand, dilute solutions of trypsin added to the initiate hemolysate have been successfully encapsulated with retention of enzymatic activity. Presumably, the principal requirement is the existence in the aqueous phase of a sufficiently high collodial osmotic pressure to maintain the turgor of the microcapsules; but this point requires further investigation. With the interfacial polymerization technique, which has occupied most of my time during the last two years, it has been shown that proteins other than red cell hemolysate can be microencapsulated alone if they are present in high concentration.

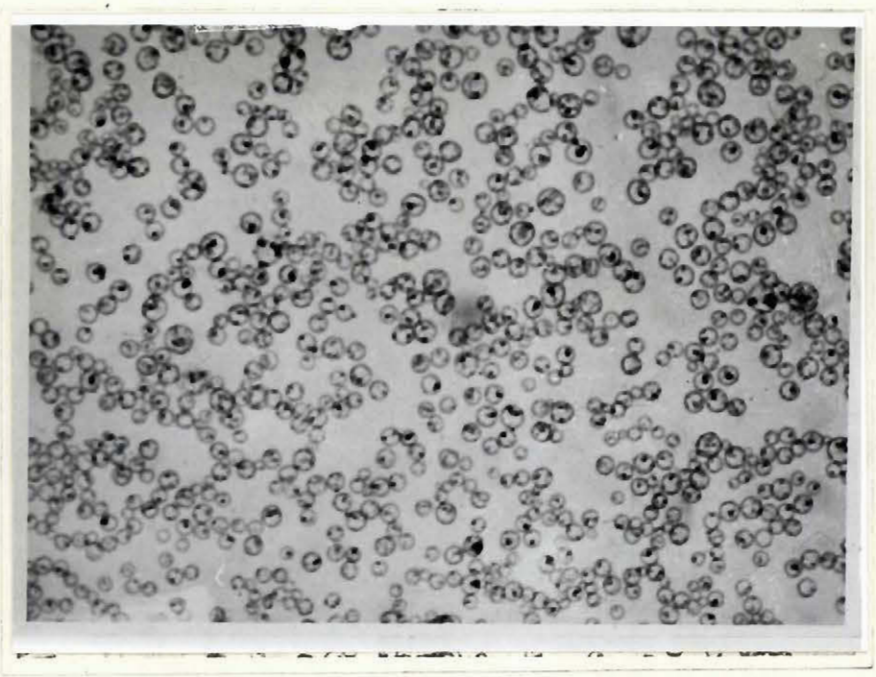


Fig. 2

Collodion-membrane microcapsules, containing hemolysate, in aqueous suspension. Mean diameter 184: magnification 60X. Note presence of precipitated protein in internal phase. The microcapsules in the original suspension had a yellowish-brown cast.

INTERFACIAL POLYMERIZATION

Principle

In this process, the formation of the membrane depends on the reaction between a water-soluble diamine and a diacid halide soluble in an organic solvent: the reaction takes place at the interface of each aqueous microdroplet in an emulsion in which the aqueous phase is discontinuous. The principle is illustrated below with Nylon (Polyamide 610) as the membrane material and an erythrocyte hemolysate as the internal phase.

Preparation of Nylon-membrane microcapsules

The formation of Nylon by the reaction of sebacoyl chloride with hexamethylenediamine is represented by the following equation:

$$\underset{\text{(hexamethylenediamine)}}{NH_2(CH_2)_6NH_2} + \underset{\text{(sebacoyl chloride)}}{ClCO(CH_2)_8COCl} \longrightarrow$$

$$HCl + \underset{\text{(Nylon - Polyamide 610)}}{NH_2(CH_2)_6NH\text{-}CO(CH_2)_8CO\text{-}NH(CH_2)_6NH\text{-}CO(CH_2)_8CO\text{-}NH(CH_2)_6NH\text{-}CO(CH_2)_8CO\text{-}} \text{ etc.}$$

As before, the starting material is an erythrocyte hemolysate, prepared as described under "Interfacial Coacervation" (P. 62). To 1.5 ml of the unbuffered hemolysate is added an equal volume of alkaline 1,6-hexamethylenediamine solution (Eastman, 0.4 M in 0.45 M $NaHCO_3$-Na_2CO_3 buffer , pH of buffer 9.8). The purpose of the buffer is to neutralize HCl formed during the polymerization step. Morgan in his pioneer studies

on the formation of Nylon membranes found that carbonate was a particularly satisfactory buffer (Morgan, 1959). On the other hand, he (Morgan, 1962) found that weaker membranes were formed with bicarbonate as the buffer, and I can confirm this; but I have found that haemoglobin is rapidly converted to alkaline hæmatin at the pH of carbonate, whereas with the mixed buffer, haemoglobin is better preserved and the microcapsule membranes have satisfactory properties.)

The buffered hemolysate, containing diamine, is mechanically emulsified at 0^{o} for 1 minute with 15 ml of a "stock organic liquid" (chloroform:cyclohexane 1:4, containing 15% v/v of Span 85 as emulsifying agent). Although many other organic liquids may be used, this combination was chosen for the following reasons. First, the liquid should be one that does not inactivate enzymes:cyclohexane, carbon-tetrachloride and chloroform are suitable (Allfray, 1959). Secondly, it is desirable to have a liquid with a specific gravity close to 1.00, because when the specific gravity is much more than 1.00, the microcapsules tend to rise and are less conveniently handled on the centrifuge; whereas if the specific gravity is much less than 1,00, the microcapsules sediment rapidly with much agglomeration. Finally, as was mentioned in the introductory section (P. 29), the partition coefficient of the organic liquid for diamine determines the properties of the membrane (Morgan, 1959): chloroform, which has a high affinity for diamine, produces strong but coarse and thick membranes, whereas cyclohexane produces smooth and very thin but weak membranes. These two solvents combined in the stated proportion give a fluid of specific gravity of 0.91,

which is very satisfactory, and also produce a thin membrane of sufficient strength. Chloroform was chosen in preference to carbon tetrachloride because I found it inconvenient to carry out all the manipulations in a fume-hood, and wished to ensure that laboratory personnel would not be exposed to the higher hepatotoxic effect of the carbon tetrachloride.

As in the procedure for collodion microcapsules, the size of the microdroplets depends on the speed of mechanical emulsification: with the Jumbo magnetic stirrer (speed "5") their mean diameter is 28 μ; with the homogenizer (speed "45") the mean diameter can be reduced to 5 μ, though the finished microcapsules then show some tendency to clump. As with the coacervation procedure, the homogenizer blade must be blunted to prevent damage to the nascent microcapsules.

The second step of the procedure occurs when the diacid halide is added to the stirred emulsion. The only diacid halide I have used is sebacoyl chloride: a satisfactory solution is made by adding 0.1 ml of pure sebacoyl chloride (Eastman) to 25 ml of the "stock organic liquid" (concentration of sebacoyl chloride, 0.019 M) immediately before the solution is added to the stirred emulsion; if the solution is allowed to stand it becomes cloudy and deteriorates. Without stopping the stirring, 15 ml of the sebacoyl chloride solution is injected into the emulsion with a syringe, the addition taking a few seconds, and the stirring is continued for a further 3 minutes at 0°. (When the Virtis homogenizer is used, the speed should be slowed to "30" for the last 2 minutes to reduce damage to the microcapsules. The formation of the

polyamide (Nylon) takes place rapidly on the outer surface of each microdroplet; the HCl set free during the reaction is buffered in the aqueous phase by the alkaline buffer.

Some comment should be made about the optimal ratio of the two reactants in the polymerization, hexamethylenediamine and sebacoyl chloride. The significant ratio here is that of the concentrations of the reactants in their respective solvents, rather than that of their equivalent weights in the system as a whole. It should be pointed out that the formation of the polymer is most rapid immediately after the addition of sebacoyl chloride to the emulsion: the rate slows greatly within a minute or two, less by exhaustion of the reactants than by the slowing of their diffusion by the newly formed membrane barrier. As was pointed out in the introductory section (P. 14), a too brief contact between the two reactants results in the formation of a leaky macroporous membrane, whereas a long prolonged contact results in a thick membrane with an excess of low molecular weight polymer on the outside. My selection of an optimal concentration ratio was based on studies of Morgan (1958), who determined the ratio that produces polymers of the highest molecular weight; Morgan found that this varies with the type of organic solvent used. Thus the diamine:acid-chloride ratio for cyclohexane was 17:1, while that for chloroform was 1.7:1 : the organic solvent with the higher partition coefficient for diamine (e.g. chloroform) requiring a higher concentration of the diacid chloride. The absolute concentration of the reactants in this connection was much less important than their ratio.

Morgan's results were obtained with an unstirred interface, where the aqueous phase contained only diamine with NaOH as the hydrogen-ion acceptor. In our case, however, the aqueous phase contained haemoglobin and bicarbonate-carbonate buffer, and the reaction was carried out at the interface of the microdroplets in a stirred emulsion. To find the optimal concentration ratio in this special case, four ratios were tested (11.1:1,22.2:1,44.4:1 and 88.8:1), the organic phase being the cyclohexane-choroform mixture already mentioned. The strength of the microcapsule membranes thus produced was tested by stirring the aqueous microcapsule suspension with the Jumbo magnetic stirrer at a speed of "2.5" for 2 minutes, and noting whether there was any leakage of haemoglobin on gross inspection or rupture of membranes on microscopic inspection. It was found that no microcapsules were formed at a concentration ratio of 88.8:1; there was gross leakage of haemoglobin when the ratio was 11.1:1 or 44.4:1; but the microcapsules prepared at a concentration ratio of 22.2:1 showed neither gross leakage of haemoglobin nor rupture of membranes. Therefore, a diamine/acid-chloride ratio of 22.2:1 was chosen for the subsequent preparations. In terms of the two reactants in the system, the excess of diamine over sebacoyl chloride was much lower, the ratio being 2.2:1; but under the reaction conditionsdescribed, the sebacoyl chloride was not used up in the time allowed, as was evident by the findings that (A) its odour was still detectable, and (B) the membranes became much thicker if the reaction time was prolonged.

To remove the microcapsules from the reacting mixture, the remaining

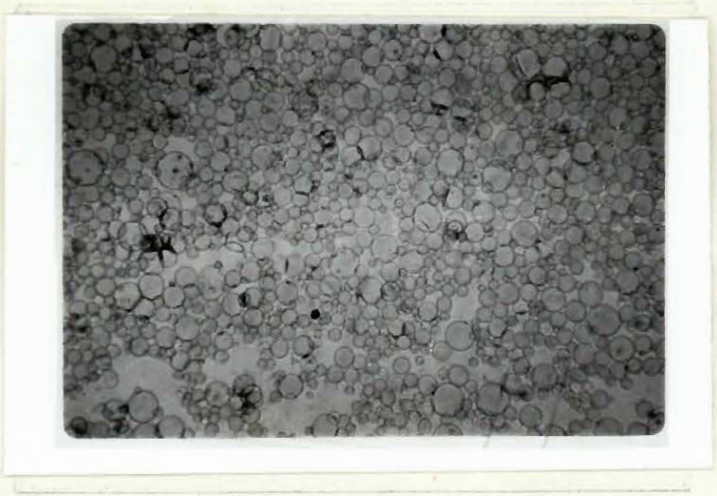
steps must be carried out quickly for the reasons just indicated. The remaining sebacoyl chloride must be separated from the newly formed microcapsules. I have found that the larger microcapsules, made with the magnetic stirrer, sediment sufficiently fast if 30 ml of stock organic liquid is added to the stirred suspension, which is then centrifuged at 350 g for 30 seconds. To separate the smaller microcapsules made with the homogenizer, the suspension is layered in 7-ml portions over 5 ml of a slightly denser organic liquid (chloroform: cyclohexane 1:3, containing 15% v/v of Span 85) in each of four 15-ml centrifuge tubes, and centrifuged for 30 seconds at 350 g. In either case, the supernatant, containing some debris, is immediately discarded; and the microcapsules are dispersed as completely as possible in 25 ml of Tween 20 solution (50% v/v in water; this concentration was chosen because at concentrations much below this, microcapsules are not completely separated from the organic liquid), first by stirring with a glass rod, and then by agitation of the suspension for 30 seconds in a 250-ml beaker on the magnetic stirrer, at a speed setting of "8" for the larger microczpsules and "10" for the smaller. The speed is then decreased to "3", and 25 ml of water is added to the stirred suspension, which is then stirred for another 30 seconds. (The speed is decreased because the addition of water greatly reduces the viscosity of the suspension: at the higher speed the microcapsules would be damaged.) The diluted suspension is then centrifuged for 1 minute, at 350 g for the larger microcapsules and 1500 g for the smaller microcapsules: to recover the smaller microcapsules which still remain in the supernatant, the latter is removed and centrifuged again. (The supernatant is

centrifuged separately in order that the microcapsules that have already sedimented should not be too tightly packed.) The microcapsules are than resuspended in 25 ml of water. At this point, they may still be crenated, because of their exposure to the hypertonic Tween 20 solution; but, as in the case of the collodion microcapsules, they regain sphericity in the hypotonic solution provided that the period of contact with the strong Tween 20 medium is kept as short as possible. After the microcapsules have recovered their sphericity, it is convenient to store the microcapsules in saline; for the smaller microcapsules the saline should contain 1% of Tween 20 to prevent agglomeration. When they are ready for use, they may be washed to remove Tween 20.

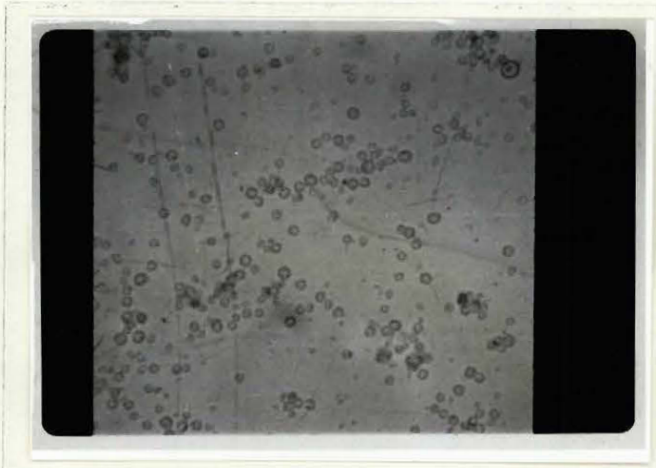
It might perhaps be emphasized that an untrained assistant can easily make microcapsules in the 10-20 μ range or larger with the aid of the Jumbo magnetic stirrer; but some practice is generally necessary for the production of satisfactory microcapsules in the 3-10 μ range. Nylon microcapsules as prepared are shown in Fig. 3 .

Preparation of microcapsules with membranes of cross-linked protein

Under the high power of the microscope the Nylon membranes of microcapsules prepared by the above procedure are clearly visible but too thin for accurate measurement: the impression gained is that their thickness is about 0.5 μ, but it is obvious that errors of reflection and refraction do not permit an accurate estimate. I calculated the theoretical thickness of the membrane on the basis of their number and surface area, making the assumption (untrue) that all of the sebacoyl



(A)



(B)

Fig. 3

(A) Nylon-membrane microcapsules, containing hemolysate, in aqueous suspension. Mean diameter 27 μ: magnification 60X. The darker patches in the microphotograph are areas where microcapsules are superimposed on one another.
(B) Smaller microcapsules of similar composition. Mean diameter 5 μ: magnification 250X.

chloride was used up and that the density of the porous membrane was 0.1 gm - 0.25 gm/ml, the range given by Morgan (1958) for Nylon films formed by interfacial polymerization. On this basis, average membrane thickness was calculated to be only 650 Å for microcapsules of 10 μ mean diameter: a surprisingly low figure in view of the mechanical strength of the microcapsules.

The possibility was then considered that since haemoglobin, like other proteins, has many free amino groups, these protein molecules might have been cross-linked with one another by sebacoyl chloride and thus incorporated into the Nylon membrane. This was readily tested by bringing a hemolysate, without added diamine, into contact with the usual sebacoyl chloride solution, with the result that a well-formed membrane was produced. Following this observation, microcapsules with membranes of cross-linked protein were manufactured according to a procedure exactly the same as that for the preparation of nylon microcapsules, except that the aqueous solution contained no diamine.

These observations make it clear that at least a part of the membrane of the "Nylon" microcapsules is formed of cross-linked protein. It is probable, however, that the greater part of the membrane is derived from the diamine rather than from the protein, because (A) the diamine, unlike the protein, can diffuse to a significant degree into the organic phase where the reaction mainly occurs, (B) microcapsules can be made in the absence of protein, though they tend to collapse, and (C) the properties of the microcapsules made,

as described in the next section, with a sulphonated diamine added to the hexamethylenediamine solution are distinctly different from those of the ordinary Nylon microcapsules. Since it is very difficult, once the microcapsules have been formed, to separate their membrane completely from their protein-rich internal phase, there seems to be no simple way to estimate chemically the proportion of protein in the membrane.

The cross-linking of haemoglobin by sebacoyl chloride may be represented schematically as shown in Fig. 4 . Nylon membranes described in the preceding section, which contained an unknown quantity of protein, can perhaps be represented as shown in Fig. 5

Preparation of microcapsules with negatively-charged membranes

Because it was thought that microcapsules with negatively charged membranes might have selective permeability for electrolytes and might be more suitable for intravascular injection, the regular procedure for preparing Nylon microcapsules was modified. An attempt was made to form membranes with a sulphonated diamine, 4,4'-diamino-2,2'-diphenyldisulphonic acid (Eastman). When an aqueous solution of this compound was brought in contact with a sebacoyl chloride solution, no well-formed membrane resulted; but when a solution containing this substance and hexamethylenediamine was used, the resulting membrane was well formed. It was assumed that such a membrane would contain free sulphonic acid groups, and microcapsules were manufactured according to the standard procedure, with the usual concentration of hexamethylenediamine, and the

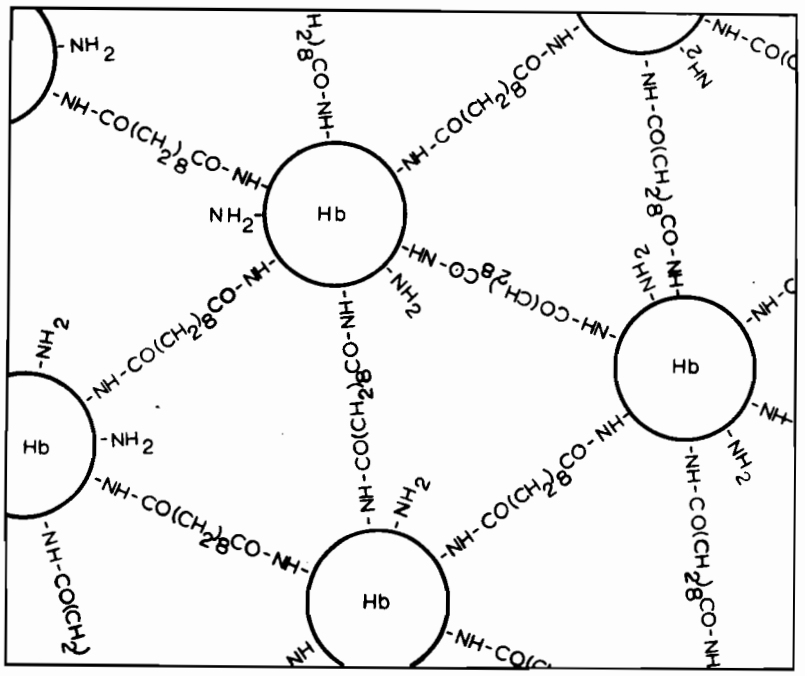


Fig. 4

Schematic representation of cross-linking of haemoglobin by sebacoyl chloride. The actual polymer would have a less regular three-dimensional structure.

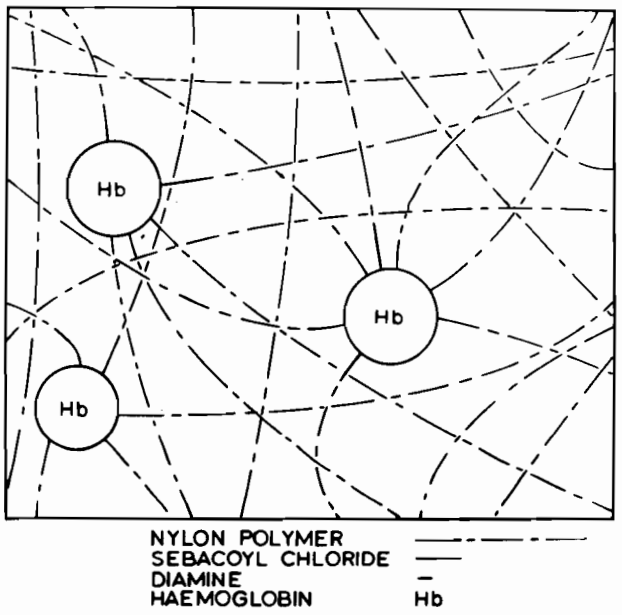


Fig. 5

Schematic representation of a Nylon membrane containing some cross-linked protein.

sulphonated compund added to the aqueous phase in 3.0 mM concentration; otherwise the concentration of the reactants and the procedure followed was the same as already described. Very satisfactory microcapsules were produced (Fig. 6) and the test described in a later section (p.107) showed that the sulphonated diamine was incorporated into the membrane, giving it a fixed negative charge. It is in fact somewhat easier to prepare microcapsules of the smallest size with the sulphonated diamine incorporated, because the negatively charged microcapsules showed less tendency to adhere to one another when they were centrifuged or stored in aqueous suspension.

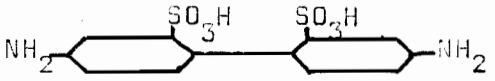
The reaction of hexamethylenediamine and 4,4'-diamino-2,2'-diphenyldisulphonic acid with sebacoyl chloride may be represented by the following equation:

$NH_2(CH_2)_6NH_2$

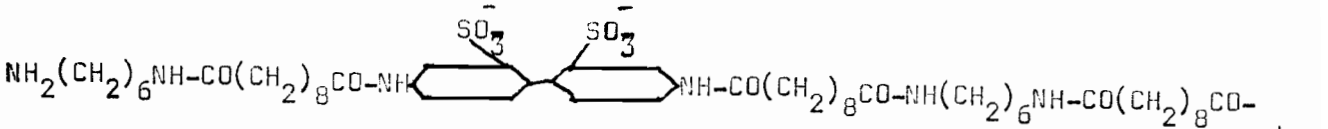
1,6,-Hexamethylenediamine

+ $ClCO(CH_2)_8COCl$

Sebacoyl Chloride $\longrightarrow$



4,4'-diamino-2,2'-biphenyldisulphonic acid



Nylon analogue membrane with strong acidic groups

+ HCl

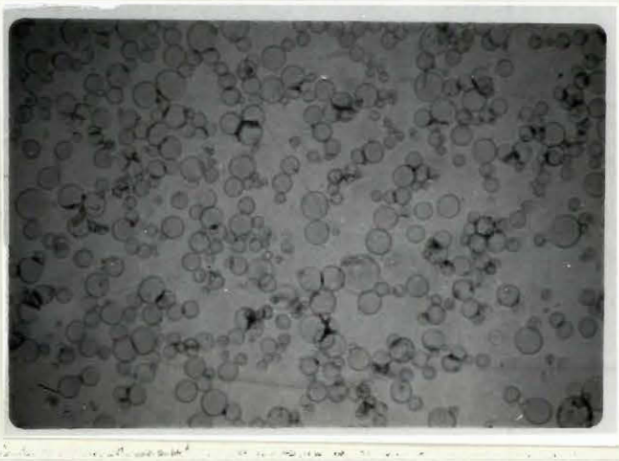


Fig. 6

Sulphonated-Nylon microcapsules containing hemolysate in aqueous suspension. Mean diameter 27 μ: magnification 60X.

Microencapsulation of enzymes and other proteins

As will be shown in a later section ("In-vitro properties of microcapsules"), microencapsulated hemolysate prepared by either the coacervation or the polymerization procedure described above retains well-marked carbonic anhydrase activity. Other enzymes may be microencapsulated by dissolving them in hemolysate and proceeding in the usual fashion. Retention of enzymatic activity after encapsulation has been demonstrated in the case of urease (see "In-vitro properties of microcapsules") and in preliminary experiments with trypsin acting on a synthetic dipeptide substrate. As has already been pointed out, the preparation of stable spherical microcapsules is facilitated by the presence of a fairly high concentration of protein in the aqueous phase. Thus dilute enzyme preparations can be more conveniently microencapsulated if they are mixed with hemolysate; on the other hand, a concentrated enzyme solution can be microencapsulated without the addition of hemolysate. For example, I was unable to encapsulate Sigma Type IV urease, which is supplied as a powder of low solubility, except by combining it with hemolysate; but Sigma Type V urease, which is much more soluble, could be encapsulated without hemolysate.

Two kinds of urease-loaded microcapsules have been used in the experiments to be described later.

(1) 200 mg of Sigma type V urease (300 Sumner units/100 mg) is dissolved in 1.5 ml of alkaline diamine solution, and this solution is emulsified with a magnetic stirrer at 0° for 1 minute with 15 ml

of stock organic liquid containing 1% v/v of Span 85. The remainder of the procedure is the same as for microencapsulation of hemolysate except that the stock solution contains 1% v/v Span 85 instead of 15% v/v.

(2) When Sigma Type V urease was not available, the preparation supplied by the Nutritional Biochemicals Corporation under the name of "NBC Soluble Urease" was used in conjunction with hemolysate. 200 mg of this preparation (150 Sumner units/100 mg) was dissolved in 3 ml of the hemolysate and diamine mixture; the rest of the procedure was as described under the microencapsulation of hemolysate, using a magnetic stirrer (Span 85 concentration in the "stock organic liquid" was 1%).

The other proteins that have been microencapsulated have been I^{131}-labelled plasma albumin (with hemolysate), hen egg albumin (without hemolysate), and Cr^{51}-labelled haemoglobin. In all these cases, encapsulation within nylon was achieved by the usual method without special difficulty.

VARIATION IN DIAMETER OF MICROCAPSULES

Principles

As already has been emphasized, diameter of the microcapsules depends on the diameter of the emulsified aqueous microdroplets. This in turn is determined mainly by three factors: (1) the speed of the mechanical emulsifier; (2) the concentration of the emulsifying agent; and (3) the viscosity of the organic liquid. After describing the methods I used for measuring microcapsule diameters, I will discuss the influence of each of these factors.

Measurement of microcapsule diameter

An aqueous suspension of microcapsules was placed in a hemocytometer chamber and photographed to include part of the hemocytometer scale. With a filar eye-piece focussed on the negative of the microphotograph, the diameters of 200 microcapsules in each preparation were measured. Means, standard deviations and standard errors were calculated in the usual manner.

Factors affecting the diameter of microcapsules

Speed of stirring:

The relationship between microcapsule diameter and stirring speed was tested for collodion and nylon microcapsules made by the standard procedure. In each case, the concentration of emulsifying agent, Span 85, was kept constant, at 15% v/v for the nylon microcapsules and 1% v/v for the collodion microcapsules; while the speed of the Jumbo magnetic stirrer was set at 1, 2.5, 5, 7.5.(Absolute speed in r.p.m.

was not measured, since it would be expected to change during the process of membrane formation). The results are shown in Table I and Figs. 7 - 8 for the collodion microcapsules, and in Table II and Figs. 9-10 for the nylon microcapsules. There is some tendency for the size-distribution curve to be skewed to the left, especially with the slower speeds and collodion microcapsules; but the size variation is similar to what is generally found in mechanically-formed emulsions. Coalescence of nascent membranes in the collodion microcapsules during the 45 minutes setting period may account for some of the "tail" of larger microcapsules, and this coalescence was actually observed under the microscope in the case of the collodion preparation at the early stage of membrane formation. Some narrowing of the size range can be obtained by differential sedimentation in saline, but this point has not been pursued in any detail.

Concentration of emulsifying agent:

The effect of this variable was tested in the same way: speed setting of Jumbo magnetic stirrer was kept constant at "5" while the concentration of Span 85 was either 1%, 5%, 10% or 15% v/v. The results for nylon microcapsules are presented in Table III and Figs. 11 - 12. The effect of detergent concentration on the diameter of collodion microcapsules was not tested because, as discussed under the section of "Preparation of collodion-membrane microcapsules", microcapsule formation in the interfacial coacervation procedure was unsatisfactory at Span 85 concentration higher than 1% v/v.

TABLE I

COLLODION MICROCAPSULES: EFFECT OF SPEED OF MECHANICAL EMULSIFIER

Rate of stirring (speed setting)	1.0	2.5	5.0	7.5
Conc. of Span 85 (% v/v)	1	1	1	1
Number of microcapsules counted	200	200	200	200
Mean diameter (μ)	77.8	48.1	45.2	31.9
Standard deviation (μ)	± 28.9	± 23.6	± 16.1	± 12.7
Standard error (μ)	± 2.0	± 1.6	± 1.1	± 0.8

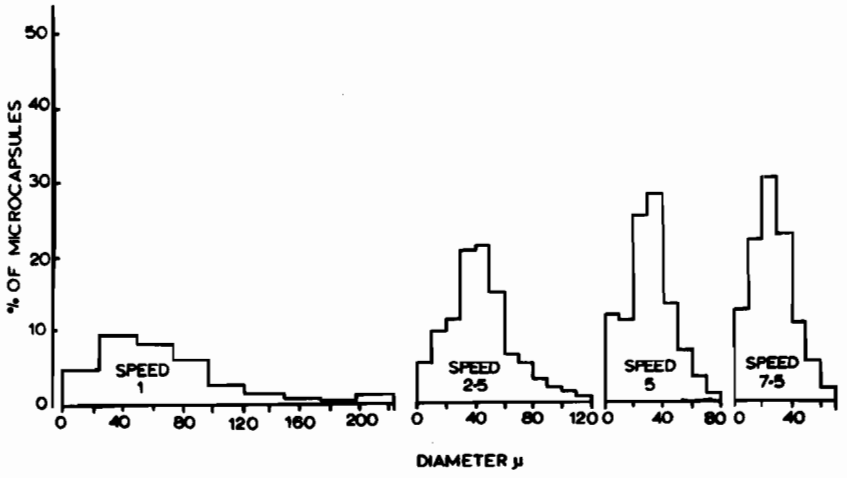


Fig. 7

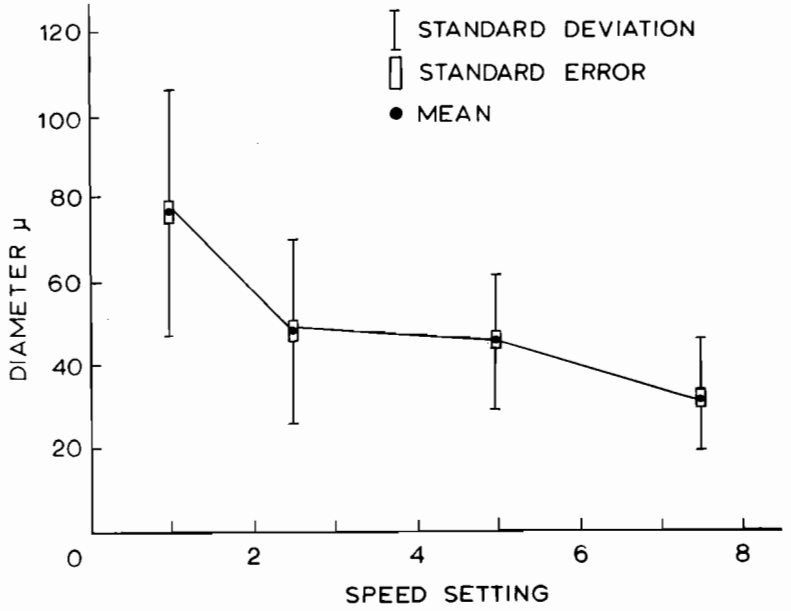


Fig. 8

Collodion microcapsules: effect of stirring speed on diameter. The same data are plotted above (Fig. 7) as histograms and below (Fig. 8) as a graph summarizing the statistical analysis.

TABLE II

NYLON MICROCAPSULES: EFFECT OF SPEED OF MECHANICAL EMULSIFIER

Rate of stirring (speed setting)	1.0	2.5	5.0	7.5
Conc. of Span 85 (% v/v)	15	15	15	15
Number of microcapsules counted	200	200	200	200
Mean diameter (μ)	79.5	45.9	28.7	13.7
Standard deviation (μ)	± 35.5	± 21.0	± 15.0	± 11.7
Standard error (μ)	± 2.5	± 1.5	± 1.0	± 0.8

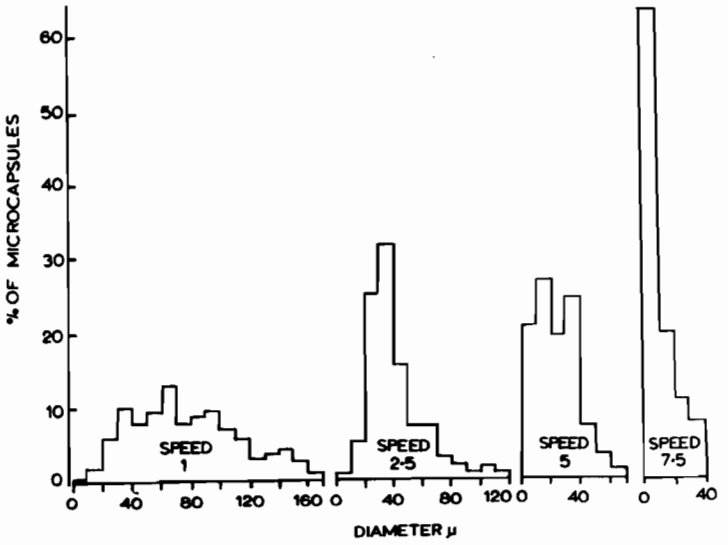


Fig. 9

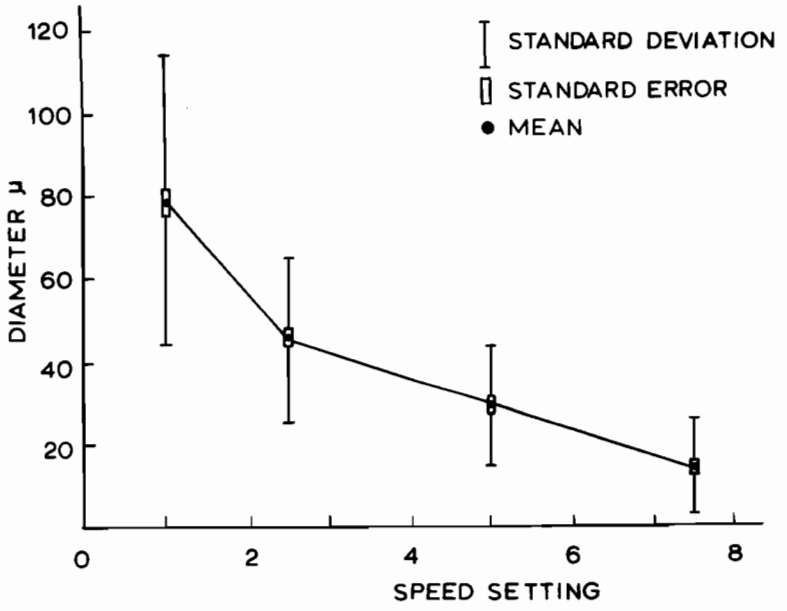


Fig. 10

Nylon microcapsules: effect of stirring speed on diameter.

TABLE III

NYLON MICROCAPSULES: EFFECT OF CONCENTRATION OF EMULSIFYING AGENT

Conc. of Span 85 (% v/v)	1	5	10	15
Rate of stirring (speed setting)	5	5	5	5
Number of microcapsules counted	200	200	200	200
Mean diameter (μ)	77.4	40.7	31.8	28.7
Standard deviation (μ)	± 21.5	± 14.7	± 17.8	± 15.0
Standard error (μ)	± 1.5	± 1.0	± 1.2	± 1.1

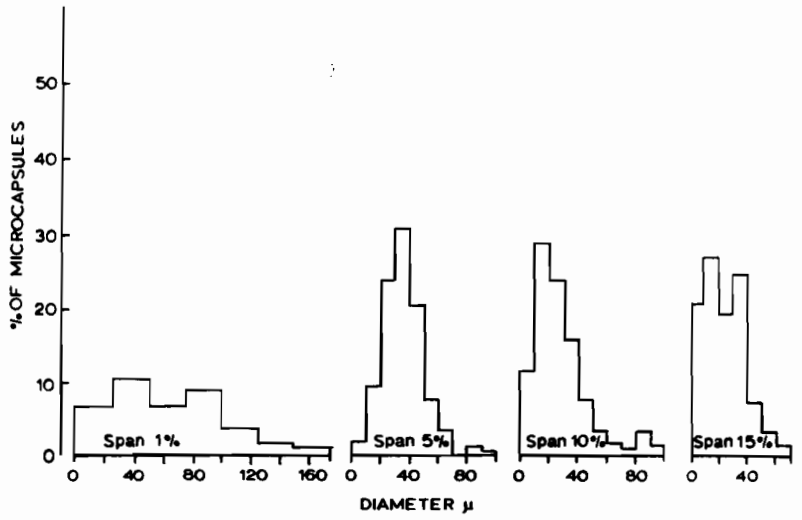


Fig. 11

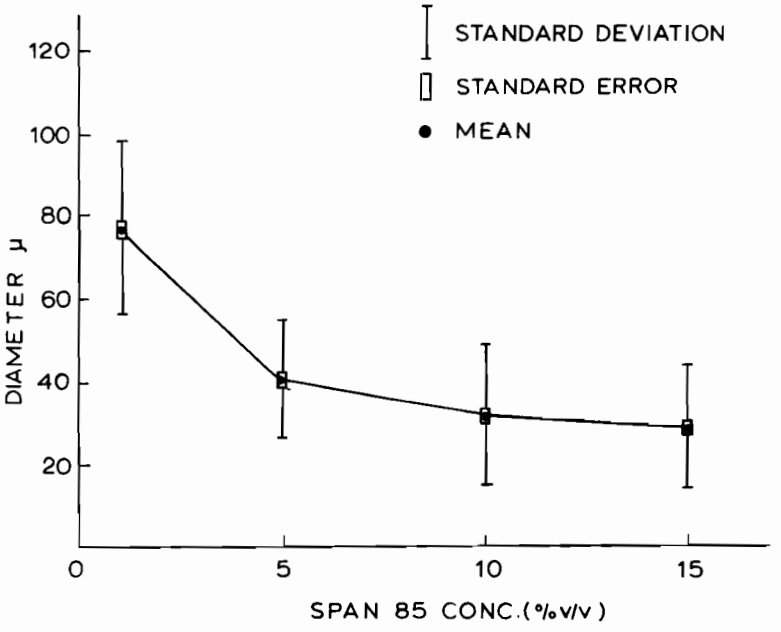


Fig. 12

Nylon microcapsules: effect of varying Span 85 concentration on diameter.

Effect of viscosity of organic liquid:

The effect of varying the viscosity of the organic fluid has not been studied quantitatively, but it is well known to all who work with emulsions that droplet size varies inversely with the viscosity of the continuous phase. Very large microcapsules may be obtained by emulsifying in a high-viscosity continuous phase, e.g. one of the silicone fluids prepared by the Dow Corning Corporation, which are available in a wide range of viscosities. Since sebacoyl chloride is insoluble in these silicone fluids, nylon microcapsules are made by adding sebacoyl chloride in a small volume of the stock organic solution. In this way, microcapsules of up to 1/2 cm diameter may be obtained with silicone fluid (Dow Corning 200 fluid) with the Jumbo magnetic stirrer set at 1, and with no Span 85 in the stock organic liquid.

Still larger microcapsules may be prepared, one at a time, by using a syringe with needles of different diameters to let drops of the alkaline diamine solution fall into the sebacoyl chloride solution in the stock organic liquid (concentration 0.018 M). For even larger microcapsules, stock organic liquid containing sebacoyl chloride (concentration 0.18 M) may be mixed with 9 volumes of the silicone fluid and drops of the alkaline diamine solution are let fall into the liquid as above. In both cases, Span 85 and stirring are omitted; the time allowed for the reaction and the transfer procedure are as usual.

MICROENCAPSULATION OF PARTICULATE MATTERS

Principle

Particulate matter may be encapsulated in aqueous suspension by adding it to the aqueous phase at the outset of the procedure. No particular difficulty was encountered, for instance, with the encapsulation of an enzyme preparation that was partly soluble in water: the membrane was formed of nylon, but presumably collodion could also have been used. Two further examples are given below, of procedures where special particulate materials are enclosed within relatively large microcapsules by interfacial polymerization.

Microcapsules within microcapsules

Nylon microcapsules containing hemolysate were prepared by the usual procedure.(Stirring speed of 5 and Span 85 concentration of 15% v/v). A 25% (v/v) suspension of these microcapsules in the hemolysate-diamine-buffer mixture was then encapsulated by the standard procedure, with a stirring speed of 1 and Span 85 concentration of 1% v/v. Such microcapsules are shown in Fig. 13: mean diameter was about 20 μ for the enclosed microcapsules and about 100 μ for the outer capsules. If the outer microcapsule is ruptured by pressing on the overlying cover-slide, the enclosed microcapsules can be observed under the microscope to flow out through the ruptured outer microcapsule.

Microencapsulation of intact cells

Hitherto, I have only attempted to microencapsulate mammalian erythrocytes using the interfacial polymerization technique. The standard technique for making large nylon-membrane microcapsules

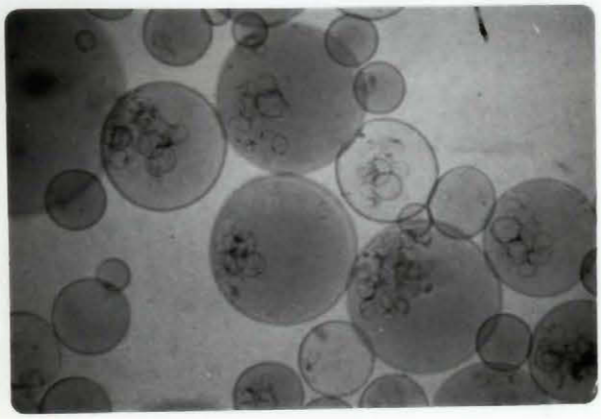
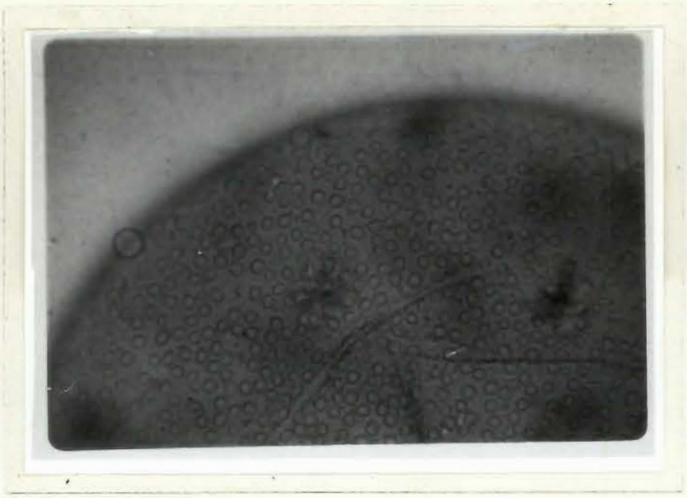


Fig. 13

Nylon microcapsules enclosing smaller Nylon microcapsules.
Magnification 60X.

cannot be used for enclosing erythrocytes for two reasons: (1) erythrocytes undergo at least partial lysis when emulsified in the stock organic liquid of chloroform and cyclohexane; and (2) erythrocytes are rapidly and completely lysed by the alkaline-diamine solution. After a number of trials, the following solution was found for these problems. The erythrocytes were suspended in hemolysate rather than in the diamine solution; and a silicone oil (Dow Corning 200 Fluid) was substituted for the stock organic liquid. The microencapsulation was then carried out by either of the two procedures involving the use of silicone fluid, as discussed under "Effect of viscosity of organic liquid" (p.92), by the principle of interfacial polymerization for membranes of cross-linked proteins. That the erythrocytes enclosed are intact is shown by the observation that when the microcapsule is mechanically ruptured in a saline medium, a stream of erythrocytes can be observed to flow out of the ruptured site, leaving the cross-linked protein membrane behind.

Fig. 14 shows a large number of human erythrocytes suspended in hemolysate within a microcapsule of about 500 μ diameter prepared by the syringe method (p.92). Because of the size of the microcapsule, many of the erythrocytes were out of focus. Fig. 15 shows erythrocytes escaping from such a microcapsule ruptured by pressure on the cover-slip.



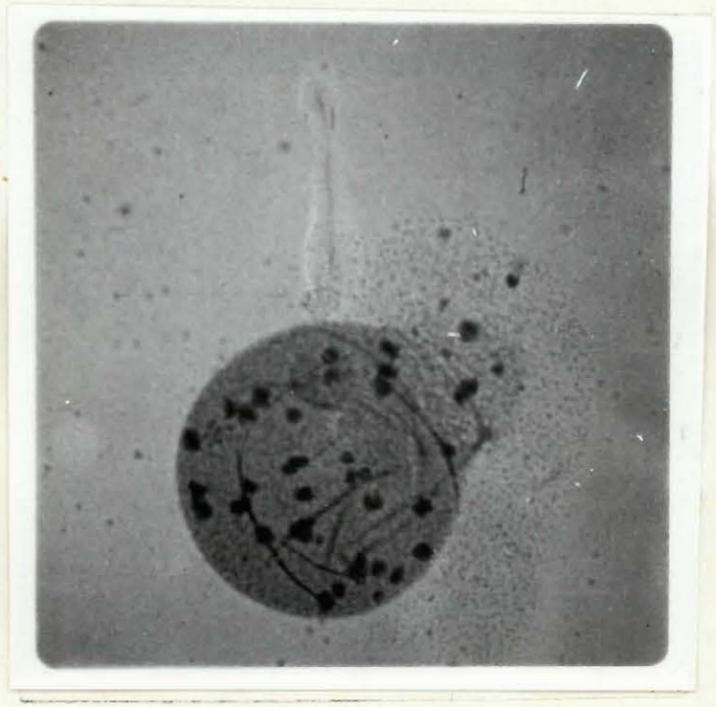


Fig. 14

Above Human erythrocytes enclosed within a large (500 μ) microcapsule with membrane of cross-linked haemoglobin. The reddish tint of the enclosed material is due partly to haemoglobin in free solution and partly to erythrocytes above or below the focal plane. There has been some folding of the microcapsule membrane under the influence of gravity. Magnification 250X.

Below Similar microcapsule in saline. The membrane has been ruptured by pressing on the coverslip and the contained erythrocytes are escaping. The dark patches are erythrocyte clumps produced by the cross-linking process. Magnification 60X.

III. IN VITRO PROPERTIES OF MICROCAPSULES

IN VITRO PROPERTIES OF MICROCAPSULES

GENERAL APPEARANCE

It can be seen from the microphotographs (Figs. 2, 3 and 6) that microcapsules prepared by interfacial coacervation are more likely to show visible precipitation of part of the enclosed protein than microcapsules prepared by interfacial polymerization. Furthermore, the membranes of microcapsules made by the latter method are much thinner, and also, as will be demonstrated later, more flexible. The polymerization procedure is less tedious, no more than 10 minutes being required to make Nylon microcapsules as against about 90 minutes for collodion microcapsules. For these reasons, although many of my preliminary studies were with collodion microcapsules, the more detailed studies reported in the remainder of this thesis were made mainly on Nylon or Nylon-analogue microcapsules.

That the Nylon microcapsule membranes are flexible may be shown by the following experiments. When one Nylon microcapsule of about 100 μ diameter is forced under hydrostatic pressure to flow through the narrow portion of a glass tube drawn out to about 50 μ minimal diameter, the microcapsule membrane is seen to fold in the same way as during crenation by a hypertonic solution (Fig. 15). After passing through the narrowed portion of the tubing, the microcapsule remains folded for a few seconds, then gradually returns to its spherical shape. The time course of the change makes it apparent that water is forced out of the microcapsule as it passes through the constriction and returns after the microcapsule has emerged. Another observation illustrating the flexibility of the Nylon membrane is shown in Fig.16 . Here two microcapsules (diameter about 1 mm) are suspended

in silicone fluid and subjected to shear stress and collision in a Couette flow situation. The apparatus used was designed by Dr. S. G. Mason for studies on the flow of suspensions, and I am indebted to him and Mr. E. Anczurowski for showing me how to use it. (In Couette flow, the fluid is placed in the annulus between two concentric cylinders rotating in opposite directions, and a stationary annular interface is thus formed between the rotating fluid layers: a particle placed at the line of zero flow is subjected to the same shear as a particle in Poiseuille flow, but with the advantage that its orientation and change of shape can be more conveniently examined.) In Fig. 16a the liquid is stationary; in Fig. 16b the two microcapsules are under shear stress applied in opposite directions, and it can be seen that the microcapsules are elongated in the line of shear stress; Fig. 16c shows their collision, and the flattening of the membranes at the point of contact can be clearly seen; in Fig. 16d the still elongated microcapsules are separating; and in Fig. 16 e the microcapsules are again at rest. The apparatus and technique developed by Dr. S. G. Mason would be very suitable for exploring the flexibility and flow characteristics of different types of microcapsules; but because my attention was focussed mainly on the biological aspects of microencapsulation, I did not carry out any quantitative anslysis of their behaviour in viscous-flow systems.

Microcapsules suspended in distilled water present a smooth, nearly spherical surface; but microcapsules placed in a hypertonic solution quickly shrink and show folding of their membranes. This is very striking in the case of Nylon microcapsules (Fig. 17), but much less obvious in the case of collodion microcapsules which have a thicker membrane. The shrinkage and folding are clearly analogous to the phenomena of crenation in erythrocytes and

plasmolysis in plant cells: as a result of the osmotic pressure gradient, water is withdrawn from the microcapsules. The shrinkage in Fig. 17 exceeds 50% of the initial volume as indicated by centrifugation of the suspension in a haematocrit tube. When microcapsules are suspended in a hypertonic solution containing a solute of large molecular or ionic radius, crenation is long-lasting, since penetration of the microcapsule by the solute is slow. But when this solute is of smaller molecular or ionic radius, penetration of the solute is faster and crenation is more quickly reversible, the microcapsules swelling to recover their original form. As would be expected, the duration of crenation is related to the microcapsule diameter, the smaller microcapsules recovering their spherical form more quickly because of the more rapid equilibration of solute across the membrane. Fig. 18 summarizes date from a series of tests in which large Nylon microcapsules from the same batch (mean diameter 88 μ) were exposed under the microscope to equimolar solutions of solutes of different molecular or ionic radius: 100 microcapsules were counted during each minute during the period of most rapid reversal of crenation, and classified as "crenated" or "uncrenated". In this way an estimate, accurate to ± 1 minute, was obtained with each solute for the mean duration of crenation (*i.e.* the time required for 50% of the microcapsules to regain sphericity). The figure shows a satisfactory correlation of crenation and molecular weight in the case of the nonelectrolytes tested, and a similar correlation with the hydrated ionic radii in the case of the three alkali metals - Li^+, K^+ and Na^+ tested as the chlorides. The values of the hydrated radii are those given by Anaki, Ito and Oscarsson (1961) and Ito, Kostyuk and Oshima (1962). Crenation on contact with non-

electrolytes of molecular weight < 100 was absent at the solute concentration used in these tests. In the next section of the thesis (p. 119 *et sequ.*) a more quantitative method, based on the threshold concentration of the solute for causing crenation, will be described for the measurement of membrane permeability and mean pore radius.

PHOTO MISSING

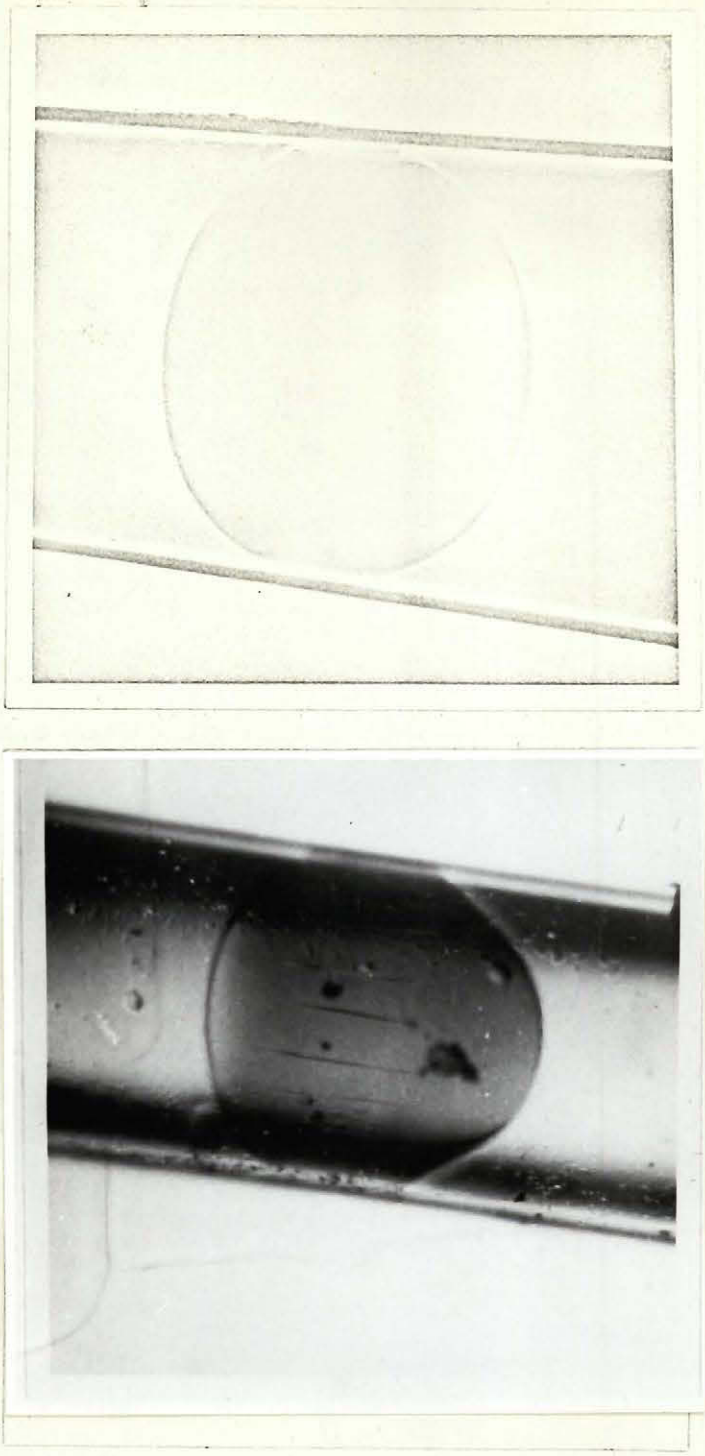


Fig. 15

Deformability of a Nylon microcapsule. The microcapsule, about 100 μ in diameter and suspended in saline, is moving from left to right along a tapering glass capillary. Above: (flow momentarily stopped) the microcapsule is just occluding the lumen of the capillary. Below: (flow again stopped) the microcapsule is in the narrowest part of the capillary and is subjected to a hydrostatic pressure gradient from left to right. Note flattening of upstream surface, bulging of downstream surface, longitudinal folding of membrane, and smaller volume of microcapsule as a result of filtration of fluid through downstream surface into capillary.

Fig. 16

Deformability of Nylon microcapsules. Two microcapsules about 1 mm in diameter, suspended in silicone oil and placed in Couette flow apparatus..

(a) Microcapsules at zero flow.

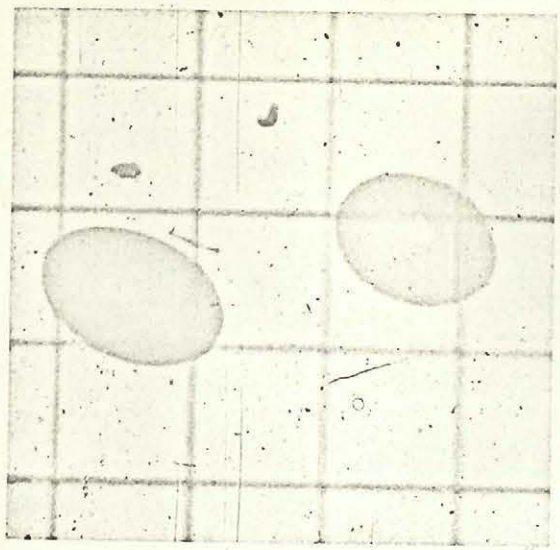
(b) About a second after onset of Couette flow: the elongated microcapsules are oriented across the zero-flow annulus and are moving towards each other.

(c) About three seconds later: the microcapsules have collided.

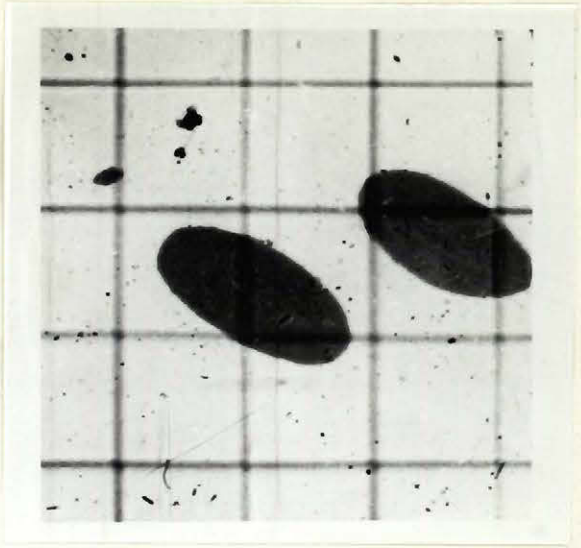
(d) Three seconds after (c): the microcapsules have separated after collision and are moving apart.

(e) After cessation of flow, the microcapsules quickly recover their original shape (Because the two microcapsules moved rapidly out of the field of observation after collision, the flow was momentarily reversed to bring them back to approximately their original position before this picture was taken).

PHOTO MISSING



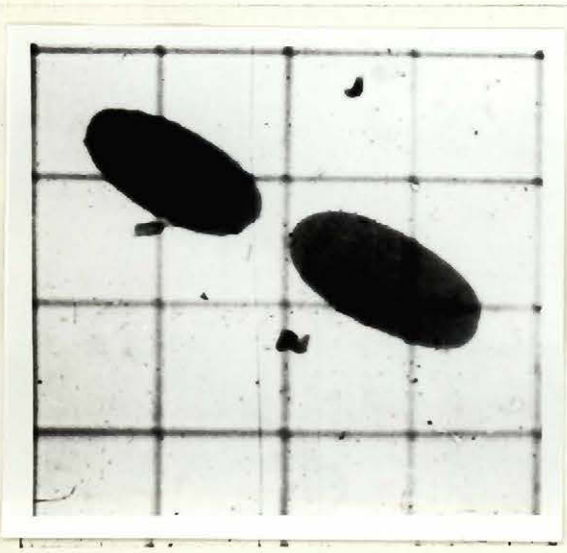
(a)



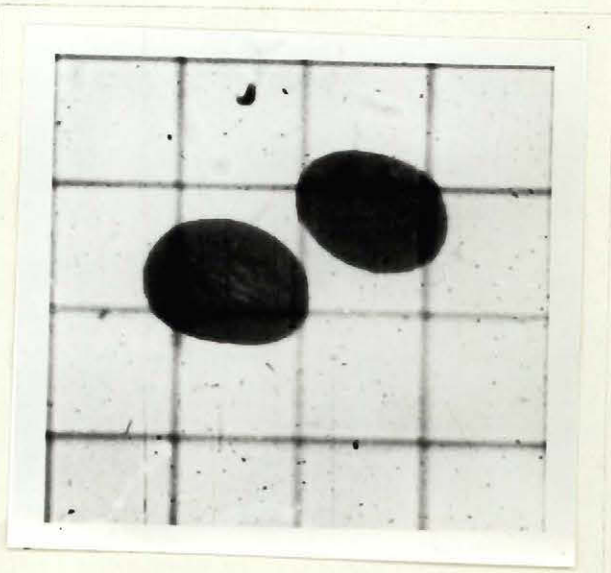
(b)

(c)

Figure 16



(d)



(e)

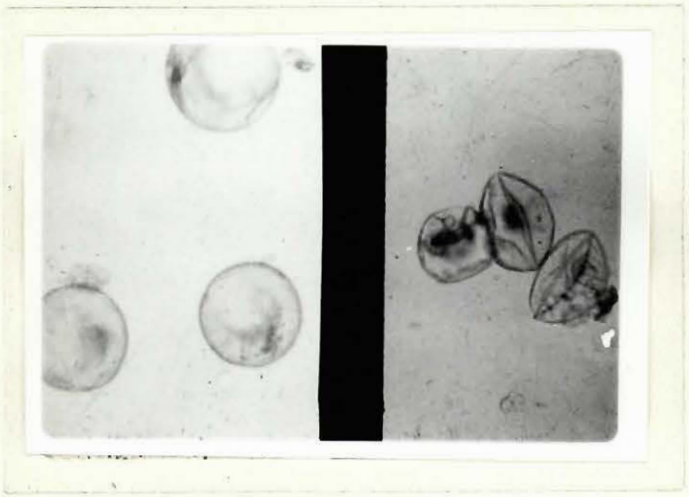


Fig. 17

Above Nylon microcapsules: mean diameter of microcapsules in batch about 90 μ, but the three shown here have diameters of about 250 μ. Left, in water; right, shortly after being placed in hypertonic saline. Magnification 60X.

Below Smaller Nylon microcapsules after crenation in hypertonic saline. Magnification 60X. (Compare with Fig. 3a, at the same magnification, which shows microcapsules of the same batch before crenation.

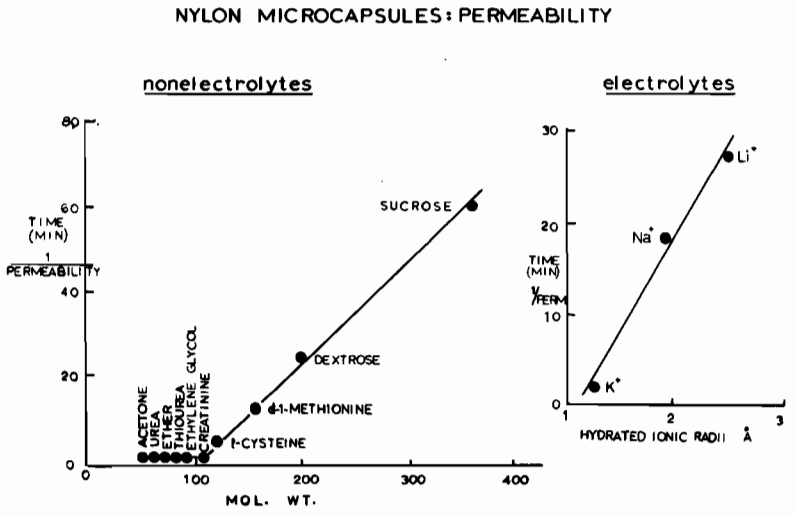


Fig. 18

Left: crenation of Nylon microcapsules (mean diameter 88 μ) in non-electrolyte solutions. Abscissae: molecular weight of solute. Ordinates: time required for 50% of crenated microcapsules to recover sphericity. All solutes 0.2 M.

Right: crenation of similar microcapsules in solutions of electrolyte: KCl, NaCl and LiCl. Abscissae: hydrated ionic radii of cations. Ordinates: time for 50% recovery from crenation.

In these tests concentration of hemolysate within microcapsules was only 1/10, as against 1/6 in the standard procedures.

SURFACE CHARGE OF MICROCAPSULES

Measurement

The surface charge of microcapsules prepared by interfacial polymerization was measured by determining their electrophoretic mobility according to the method described by Davies (1958). The electrophoresis apparatus used was loaned to me by Dr. Mason. "Migration time" was taken, following Davies, as the time taken for a particle to move a given distance (in our case 750 μ in 0.9 NaCl) at a constant current of 5 mamp. as shown by a milliammeter. The usual precautions were taken to ensure that mobility was measured at the proper distance from the capillary wall. Movement of each particle was measured twice by reversing the polarity of the cell. 10 particles were chosen at random for each suspension tested.

Cat erythrocytes were obtained from freshly drawn venous blood washed 3 times with saline and tested as a 0.5% v/v suspension. Microcapsules of about 5 μ average diameter were also tested as a 0.5% v/v suspension.

Results

Nylon microcapsules free from Tween 20 and suspended in saline had no net surface charge measurable by this method: some moved slightly towards the anode or cathode while others were nearly immobile, the migration time in all cases being too long for measurement (>1000 seconds). On the other hand, cat erythrocytes and sulphonated Nylon microcapsules invariably moved towards the anode, with the migration times shown in Table IV.

TABLE IV

Electrophoretic Mobility

Particle No.	Cat erythrocyte	Sulphonated microcapsules (5μ average diameter)	
		Sample A	Sample B
	Migration time* (seconds)	Migration time* (seconds)	Migration time* (seconds)
1	60,61	40,45	45,45
2	70,73	55,65	40,62.5
3	75,74	35,35	41,55
4	62,71	35,45	50,52
5	64,70	35,45	40,40
6	71,64	40,50	45,55
7	73,70	50,50	60,65
8	70,74	45,50	40,55
9	61,62	57,40	75,65
10	72,75	45,49	45,50
Mean	68.6	47.1	51.3
Standard deviation	± 6.4	± 8.8	± 10.2
Standard error	± 1.4	± 2.0	± 2.3

* Duplicate measurements made on each particle by reversal of polarity.

With the number of tests made, the "migration time" of the two batches of sulphonated microcapsules was not significantly different ($T = 1.38$, $p > 0.05$). On the other hand, both these batches of microcapsules had a significantly higher mobility than the cat erythrocytes tested under identical conditions ($T = 8.95$, $p < 0.001$ in one case and $T = 6.4$, $p < 0.001$ in the other)

No measurements were made of the "migration time" of erythrocytes or microcapsules in plasma or other protein solutions.

PERMEABILITY STUDIES ON MICROCAPSULES

Introduction

Membrane transport mechanisms

From the enormous literature on the mechanisms by which dissolved substances may pass through natural or artificial membranes separating two aqueous phases, one may, following Park (1961), select nine types of processes which account, separately or in combination, for the phenomena that have been investigated. These are:

(1) Simple diffusion. The substance penetrates aqueous pores in the membrane by its random molecular motion without interacting with membrane components. An example is the diffusion of water or urea across cell membranes.

(2) Solvent drag. As a result of bulk flow of water under a hydrostatic or osmotic gradient, the solute is dragged through aqueous pores. An example is the movement of thiourea in association with osmotic water flow in the skin of the toad.

(3) Diffusion restricted by membrane charge. Charged membranes allow ions of the opposite charge to pass through, but exclude or restrict diffusion of ions of the same charge. Examples are the perm-selective membranes referred to on p. 26 of this thesis.

(4) Diffusion accompanied by solvation in the membrane. The substance soluble in the membrane material may cross the membrane by diffusion which is not restricted to aqueous pores. An example is the good correlation of permeability with lipid-water partition coefficients in the case of many non-electrolytes.

(5) Facilitated diffusion (mediated transport). The solute molecule combines reversibly with a carrier in the membrane which is free to oscillate between the two surfaces to release or combine with molecules on either side. The motion of the carrier and the substrate-carrier complex could be accounted for either by thermal agitation or by molecular deformation arising from the binding and/or release of the substrate. A probable example is the rapid diffusion of oxygen across millipore filters wetted with haemoglobin solution (Scholander, 1960).

(6) Exchange diffusion. For each molecule (usually an ion) transported across the membrane in one direction a similar molecule is transported back. An example is the exchange of sodium ions across the erythrocyte membranes.

(7) Active carrier transport. The carrier is subjected to chemical modifications in the membrane as a result of energy made available by the breakdown of a "high energy component" such as ATP. As a result, the carrier exists in two forms, one which carries the molecule across the membrane as a complex and one which has a lower affinity; as a result there is a one-way transport of the substrate, which may be "uphill" against the concentration gradient. Many examples are known of this important process, e.g. the active absorption and secretion of various substances across the renal tubule, and the active transport of sodium and potassium ions across cell membranes.

(8) Pinocytosis. The cell membrane invaginates to trap substances, and is then pinched off to form intracellular vesicles, which may dissolve and release the transported material into the cell. This process permits the penetration of large molecules like proteins, and micellar

aggregates of water-insoluble materials such as lipids.

(9) Phagocytosis. This is similar to (8) but involves the engulfing of particles of much larger dimensions. Examples are the uptake of bacterial and foreign particles by the leucocytes and macrophages.

We may regard the first four of these mechanisms as transport processes of the simple passive type and the last four as representing special types. The work reported in this thesis has not been concerned with the special types of membrane transport, but only with the simple passive types, and especially with the passive diffusion of molecules across aqueous pores in artificial semipermeable membranes along an electrochemical gradient. Consideration will be given mainly to the process described in (1) above, which is the one involving crenation of microcapsules exposed to hypertonic solutions of non-electrolytes. The volume changes of microcapsules so treated can be analysed quantitatively in terms of these processes. In special cases the permeability of microcapsules also involves processes (3) and (4), but these will not be mentioned in the theoretical treatment given in the next section.

The theoretical analysis of water and solute movements across porous membranes

The literature contains an immense body of data on the movements of water and solutes across natural and artificial membranes under the influence of concentration gradients. The processes involved are those referred to as (1) and (2) in the last section, simple diffusion and solvent drag. Prior to 1951, the results obtained from permeability studies of these sort were analysed only in terms of two coefficients,

one for solute flow and one for volume flow. The equation for solute flow (in the absence of volume flow) was based on Fick's Law, which states that the rate of solute diffusion is proportional to concentration gradient and area, i.e.

$$dS/dt = Ks\ A\ (Co - Ci) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where S is the number of moles of solute entering the inner compartment, Ks is the permeability coefficient for the solute and includes the thickness of the membrane, A is the area of the membrane, and Co and Ci are the molar concentrations of solute outside and inside the membrane. Davson and Danielli (1952) modified equation (1) by substituting S/Vi for Ci, where Vi is the volume of the inner compartment, and their equation is then

$$dS/dt = Ks\ A\ (Co - S/Vi) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2).$$

Volume flow may take place either under an osmotic pressure gradient, or under a hydrostatic pressure graduent, or under these two gradients in combination. The classical equation for volume flow under an osmotic pressure gradient, where no hydrostatic pressure difference exists between the inner and outer compartments, is

$$dVi/dt = K'_w\ A\ (\pi i - \pi o) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

where dVi/dt is the rate of volume flow, K'_w is the permeability coefficient of the membrane for water, A is the membrane area and πi and πo are the osmotic pressures of the inner and outer compartments. By substituting RTC for π (where R and T have their usual meaning and C is the molar concentration of the solute) and absorbing RT into the coefficient K'_w, equation (3) may be rewritten

$$dVi/dt = Kw\ A\ (Ci - Co) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4).$$

Equation (4) was modified by Jacobs (1952) to take account of the case, common in experiments on simple biological systems, where osmotic pressure is exerted by non-permeable as well as permeable solutes,

$$dVi/dt = Kw\ A \frac{Sm - Si}{Vi} - (C_m^o + C_s^o) \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

where Sm and Si are the molar quantities of the impermeable and permeable solutes in the inner compartment, and C_m^o and C_s^o are the molar concentrations of the solutes in the outer compartment.

Where a hydrostatic pressure difference ($\Delta p = p_o - p_i$) exists between the compartments in addition to an osmotic pressure difference ($\Delta\pi = \pi i - \pi o$), the effects of the two pressure gradients summed algebraically, as discussed for instance by Pappenheimer *et al.* (1953), giving permeability equations of the type

$$dVi/dt = K_w''\ A\ (\Delta p - \Delta\pi) \quad \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

In the cases dealt with in this thesis, the effects of hydrostatic pressure differences are not significant, and the remainder of this discussion will be concerned principally with situations in which volume flow is determined by the osmotic pressure gradient. The basic equations for solute flow (1) and for volume flow (3) given above, however, do not adequately describe the movements of solute and solvent. Their short-comings were first clearly recognised by Staverman (1951) who, using the principles of irreversible thermodynamics, pointed out that, in a stationary state where volume flow is zero, the effective osmotic pressure (πe) across a membrane that is permeable to the solute will always be less than the theoretical osmotic pressure (πt) calculated from the van't Hoff equation, the ratio of these two ($\pi e / \pi t$) being the reflection coefficient (σ)

$$\pi e = \sigma\, \pi t \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (7).$$

The reflection coefficient is zero for a membrane that is non-selective as between solute and solvent, and is equal to unity for an ideal semipermeable membrane, permeable to solvent but not to solute.

The equation used by Staverman could not be directly applied to biological permeability measurements; but Kedem and Katchalsky (1958) adapted Staverman's approach and produced equations involving the reflection coefficient,

$$Jv = Lp (\Delta p - \sigma RT \Delta Cs) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (8)$$

$$\dot{n}_s = \omega RT \Delta Cs + (1 - \sigma) Cs Jv \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (9)$$

Equation (8) for volume flow corresponds to equation (6), $Jv = dVi/dt.1/A$, and Lp is the equivalent of K''_w ; but equation (8) becomes identical with (6) only if the reflection coefficient is equal to 1, i.e. if the membrane is ideally semipermeable. Equation (9) for solute flow corresponds to equation (1), but $Ks = \omega RT$ (where ω is the mobility of the solute) only when $Jv = 0$. It is thus seen that besides the coefficients for solute flow and volume flow, a third coefficient, the reflection coefficient, is required to correctly analyse water and solute movements across porous membranes.

The significance of the reflection coefficient has been widely recognised since the appearance of the paper by Staverman (1951). Kedem and Katchalsky (1958) themselves, using data from other workers, showed how the reflection coefficient could be calculated to permit the quantitative treatment of observed fluxes in artificial and biological membrane systems. This coefficient has been used by Goldstein and Solomon (1960) in the case of red blood cells, and by Durbin (1959)

in the case of collodion membrane. The analysis of the permeability data in terms of reflection coefficient has allowed a more quantitative treatment of membrane behaviour and structure. For instance, it has enabled Solomon and his colleagues (Goldstein and Solomon, 1960; Solomon, 1961) to derive expressions enabling the "equivalent pore radius" to be calculated for systems in which an uncharged solute transverses a membrane by diffusion through aqueous pores.

Solomon's derivation for equivalent pore radius

Solomon (1961) has described equivalent pore radius as being "characteristic of an idealised water-filled cylindrical pore which is presumed to transverse cellular membranes, provides a coherent concept in terms of which passive transport of nonlipid-soluble non-electrolytes may be described. Such description implies that the cellular membrane discriminates between these nonlipid-soluble non-electrolyte molecules by size alone; the equivalent pore radius is thus a measure of the relative restriction offered by the membrane to solvent and solute." The equivalent pore radius can be quantitatively related to the reflection coefficient by a series of equations. The first equation is given by Durbin, Frank and Solomon (1956) who state that

$$1 - \sigma = Asf/Awf, \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (10)$$

where σ is the reflection coefficient and Asf and Awf are the apparent pore areas available for filtration for solute and solvent. Durbin *et al.* have expanded equation (10) by incorporating the expressions for Asf and Awf obtained by Renkin (1954), who has calculated the restriction to flow of solute molecules in pores on the basis of Poiseuille flow. His equations are

$$Asf = Ao\left[2(1 - a/r)^2 - (1 - a/r)^4\right]\left[1 - 2.104(a/r) + 2.09(a/r)^3 - 0.95(a/r)^5\right] \quad \ldots\ldots\ldots (11)$$

$$Awf = Ao\left[2(1 - a_w/r)^2 - (1 - a_w/r)^4\right]\left[1 - 2.104(a_w/r) + 2.09(a_w/r)^3 - 0.95(a_w/r)^5\right] \quad \ldots\ldots\ldots (12)$$

where Ao is the total cross-sectional pore area in the membrane. and a and a_w are molecular radii for solute and solvent respectively, and r is the equivalent pore radius. Combining equations (10), (11) and (12) one obtains the equation

$$1 - \sigma = Asf/Awf = \frac{Ao\left[2(1-a/r)^2 - (1-a/r)^4\right]\left[1-2.104(a/r) + 2.09(a/r)^3 - 0.95(a/r)^5\right]}{Ao\left[2(1-aw/r)^2 - (1-aw/r)^4\right]\left[1-2.104aw/r) + 2.09(aw/r)^3 - 0.95(aw/r)^5\right]} \quad \ldots\ldots (13)$$

Using these data, Durbin, Frank and Solomon found that equation (13) provides a satisfactory fit for cellulose membranes with pore radii of 20 Å to 80 Å. Goldstein and Solomon (1960) therefore felt justified to use equation (13) and a crystallographic radius of 1.5 Å for water to derive a family of curves of $(1-\sigma)$ as a function of the molecular radius of the solute (Fig.24). The parameter of these curves is the equivalent radius. By selecting the curve which best fitted the experimental values for $(1-\sigma)$ they were able to find the equivalent pore radius for erythrocytes, and found that it agreed satisfactorily with the values obtained by a different method (Paganelli and Solomon, 1957) depending on the ratio of the osmotic to the diffusion flow of water into human red blood cells.

The method used by Goldstein and Solomon (1960) for determining the reflection coefficient experimentally was a very elegant one based on

a sensitive optical procedure, designed by Sidel and Solomon (1957), for measuring minute volume changes of erythrocytes in dilute suspension immediately after the erythrocytes had been exposed in a rapid-mixing chamber to a solution of the substance under investigation. The mixed suspension flowed at a known rate through a tube, and by altering the length of the tube the volume change could be assessed with a light-scattering device at known intervals after mixing. By adjusting the concentration of the permeant molecule, they were able to ascertain the concentration which caused neither swelling nor shrinking (isosmolar concentration). This observed concentration Ce (corresponding to an osmotic pressure of RTCe) could then be compared with the theoretical isosmolar concentration Ct (with an osmotic pressure of RTCt) according to Staverman's equation ($RTCt = \sigma RTCe$) to obtain the reflection coefficient (σ) for the particular molecular species used in the experiment. By this ingenious procedure, Goldstein and Solomon obtained a value of 4.2 Å for the equivalent pore radius in human erythrocytes. It should be emphasized that their method relates only to the permeability of nonlipid-soluble non-electrolytes: lipid-soluble materials, which enter through the fabric of the membrane, and electrolytes, whose charge can interact with fixed charges in the membrane, have their permeabilities governed by other relationships.

Permeability of Nylon microcapsules

Applicability of Staverman's and Solomon's principles to microcapsules

I have used methods analogous to those of Solomon and his colleague to determine the equivalent pore radius of microcapsule membranes for nonlipid-soluble non-electrolytes; and, like Solomon, I have derived the reflection coefficient from the ratio of the observed isosmolar concentration that causes no volume change, and the theoretical isosmolar concentration, based on Vant' Hoff's law, which would apply for an impermeant solute. In the case of the microcapsules, however, it was impracticable to use the null point of zero volume change directly, for two reasons: first, because though microcapsules shrink in a hypertonic medium, they do not swell significantly in a hypotonic medium, and secondly, because Solomon's elegant optical method for assessing volume changes could not, for technical reasons, be readily applied to microcapsule suspensions. I have, however, explored the possibility of determining the effective isosmolar concentrations for solutes of different molecular radii by measuring the % crenation of microcapsules at each of a number of solute concentrations, and extrapolating to find the threshold concentration just before any crenation can be observed (0% crenation): this threshold concentration should be equivalent to the "effective isosmolar concentration", provided that the microcapsule membrane is readily deformable in response to small changes in the internal hydrostatic pressure of the microcapsule.

The microcapsule membrane, however, is thicker than the erythrocyte membrane, and it might be though that it would offer some resistance to deformation. An attempt was therefore made to see whether any such

resistance to deformation would interfere with the value found for the reflection coefficient. The argument presented later in this section indicates that this is not the case, and therefore that the reflection coefficient, and consequently the equivalent pore radii, can be calculated by determination of the threshold crenation concentrations.

Let Ci be the concentration of impermeant substances inside the microcapsules: then the osmotic pressure calculated from van't Hoff equation will be RTCi.

Suppose the microcapsule wall has some rigidity and offers resistance to crenation, and that this can be expressed by a force equal to p: then the force opposing collapse of the wall is

$$RTCi + P \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (14)$$

Thus for crenation to occur the external force must exceed RTCi + P.

Now, for an impermeant solute m in the external phase of concentration C_o^m, the osmotic pressure will be RTC_o^m; and at its threshold concentration for crenation,

$$RTC_o^m = RTCi + P \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (15)$$

For a permeant solute s, the theoretical osmotic pressure calculated from the van't Hoff equation will be RTC_o^s, but the effective osmotic pressure according to Staverman's principle is σRTC_o^s, where σ is the reflection coefficient. Thus the threshold concentration for crenation in the case of such a solute would be given by

$$\sigma RTC_o^s = RTCi + P \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (16)$$

From equations (15 and (16)

$$RTC_m^o = \sigma RTC_s^o = RTCi + P$$

Thus

$$\sigma = \frac{RTC_m^o}{RTC_s^o} = \frac{C_m^o}{C_s^o} = \frac{\text{Threshold crenation conc. of impermeant solute}}{\text{Threshold crenation conc. of permeant solute}}$$

..... (17)

Thus the same relationship of C_m^o/C_s^o to reflection coefficient holds despite the possible rigidity of the membrane: after all, the microcapsules are serving as osmometers and allowing a comparison to be made of the molar concentrations of the different substances which will give the same osmotic pressure as given by threshold crenation concentrations.

Method of measuring threshold crenation concentrations

Measurement of permeability will here be based on the principle of Ruhland and Hoffman (1925), as the log. molar concentration of the solute which causes 50% of cells to crenate and, by extrapolating to 0% crenation, the log. threshold crenation concentration may be obtained in the following way.

Nylon microcapsules are prepared by the method described in procedure (p. 68) (interfacial polymerization of hexamethylene-diamine and sebacoyl chloride using the magnetic stirrer at a speed setting of 5, and Span 85 at 1% concentration). The newly prepared microcapsules are washed twice on the centrifuge with 10 volumes of water, then left in the refrigerator with 100 volumes of water over night to allow residual diamine and other permeant solutes to leak out. In the morning the microcapsules are again washed three times with 10 volumes of water, then suspended in 2 volumes of water and placed

in a water bath at $37^{o} \pm 0.35^{o}$ C. Solutions of the substance whose permeability is to be tested are made up to be 1.1 times the desired final concentration, and 1 ml of each concentration is placed in a test-tube which is sealed with parafilm and left in the water bath at 37^{o} C for at least one hour. At zero time, 0.15 ml of the microcapsule suspension is rapidly injected into one of the tubes, and a drop of the final suspension is immediately placed on a slide previously warmed to $37^{o} \pm 0.4^{o}$ C on the heated stage of a Biological Microscopic Stage Hot Plate (Reichert), so that examination of the microcapsules suspension can be carried out without delay and without change of its temperature. Exactly 60 seconds (stopwatch) after the addition of the microcapsules to the test solution, a microphotograph is taken at 40 X magnification. The other solutions are examined in the same way. After preliminary tests to determine the concentration causing crenation of 50% of the micro-capsules, permeability of the solute is estimated in the following way.

Seven glucose solutions (final concentrations 0.2M to 0.8M in steps of 0.1M) are used to establish a standard curve for the batch of micro-capsules under investigation. Three concentrations usually in the ratio 1, 1.5 and 2 are used for each of the other solutes. The estimation of percentage of crenation at different solute concentrations is carried out by the same individual on the negatives of the microphotographs as seen under a binocular microscope at 12.5 X magnification. All the microcapsules on each negative - more than one hundred in each micro-photograph - are classified as crenated or not crenated: crenation being defined here as the presence of any observable foldings in the membrane of the microcapsule. Well-prepared microcapsules suspended in water

are never crenated, but above the threshold concentration of each solute, a small proportion of the microcapsules are detectably wrinkled (Fig. 19a) and as the solute concentrate is progressively increased both the proportion of crenated forms and the degree of shrinking increase (Fig.19b). No attempt is made to classify the microcapsules according to the degree of crenation. Better results with less chance for subjective error are obtained when the microcapsules are recorded as "crenated" or "not crenated". I found it helpful to give my assistant negatives of microphotographs designated by numbers only, with no hint of the expected result; she was able to reproduce crenation counts on the same microphotograph presented to her at different times without her knowledge, with a standard deviation of ± 1.13% (Mean 46.65%) in five trials.

Results

With glucose as the standard solute, the percentage of crenated microcapsules was measured for each of seven molar concentrations (Table V). The binomial distribution applies to such data, which are therefore conveniently handled by probit analysis. The line of best fit was plotted as described by Emmens (1948) and the 95% binomial confidence limits were plotted for each point in the usual manner on probit paper (Fig.20). All points were well fitted by a single line, and this was considered to justify the plotting of subsequent data with other solutes for which crenation was measured at only three concentrations according to the same method. The heterogeneity factor for the standard curve was calculated as 3.4041 (Emmens, 1948) and this factor

was included in subsequent calculations based on results of solutes other than glucose. The confidence limits thus calculated make no allowance for subjective variation in counting. Repeated measurement on the same sample, however, showed that only a small additional broadening of the confidence limits would be introduced by including this sort of error. For example, repeated independent counts on individual microphotographs gave the following values - e.g. 95.88%, 95.95%, 95.95%, 97.95% and 96.00% for higher concentration, and 46.10%, 46.15%, 47.67%, 48.00% and 45.35% for lower concentration. It was calculated that the inclusion of this source of variation would in all cases change the height of the confidence limit bars in Fig.20 by no more than S.D. ± 0.45% to 1.13% of the plotted value; and this increment was considered to be negligibly small.

The results obtained from different test solutes are shown in Table VI and are plotted in Fig. 21 , using the probit plot as before to relate percentage crenation to log molar concentration. The straight line joining the three concentrations of each solute is drawn, and the intercept at the 50% crenation level is taken as the median effective concentration and the extrapolation to 0% crenation is taken as the threshold crenation concentration; standard deviation for each median effective concentration is obtained by probit analysis (Emmens, 1948), and this standard deviation is also applied to the threshold crenation concentration. Results obtained are summarized in Table VII .

In Fig. 22 the log median effective concentration for each of six non-electrolyte solutes are plotted against their molecular weights which range from 45.040 to 342.308, along with the standard deviations

for the median effective concentrations. The result fitted roughly a hyperbolic curve, showing that the effectiveness of the solutes tested in producing crenation is a steep function of their molecular weight. The values obtained for the threshold crenation (0% crenation) concentrations for the non-electrolytes will be related to their molecular radii and analysed according to Solomon's principle of equivalent pore radii in the next section.

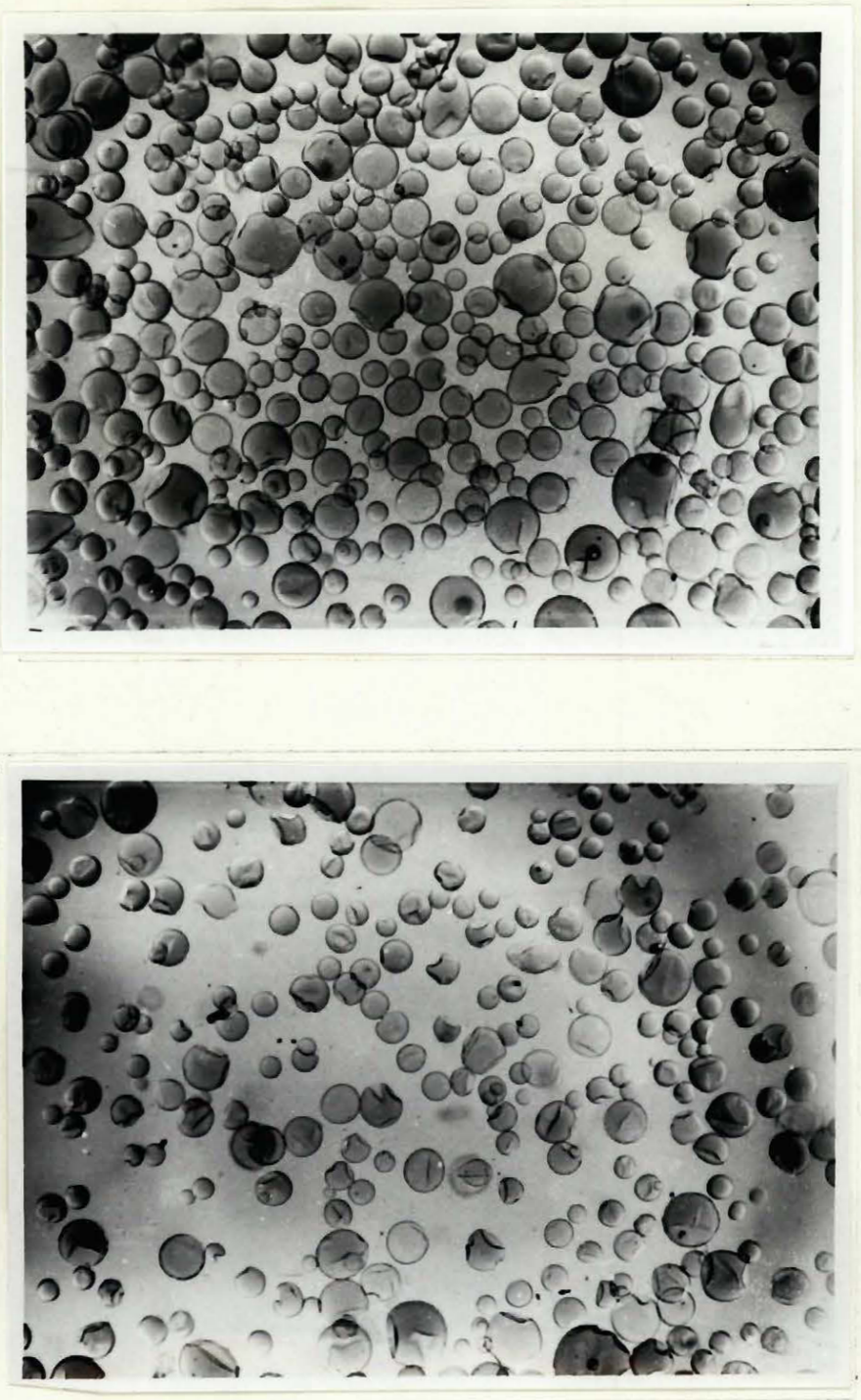


Fig. 19

Varying degrees of crenation of Nylon microcapsules (mean diameter 27 μ) 1 minute after exposure to hypertonic solutions of different concentrations.

(A) Proportion of crenated microcapsules was determined by counting to be 45%

(B) Proportion of crenated microcapsules was determined by counting to be 72 %.

TABLE V

STANDARD DOSE–RESPONSE CURVE - Glucose

Concentration (molar)	% Crenation	Number of microcapsules counted	Binomial 95% confidence limits
0.2	5.5	346	3.3 - 8.6
0.3	30.0	200	24.0 - 37.0
0.4	51.6	178	45.0 - 59.0
0.5	73.9	115	65.0 - 82.0
0.6	84.1	145	77.0 - 89.0
0.7	96.0	122	93.0 - 98.0
0.8	97.0	105	94.0 - 98.6

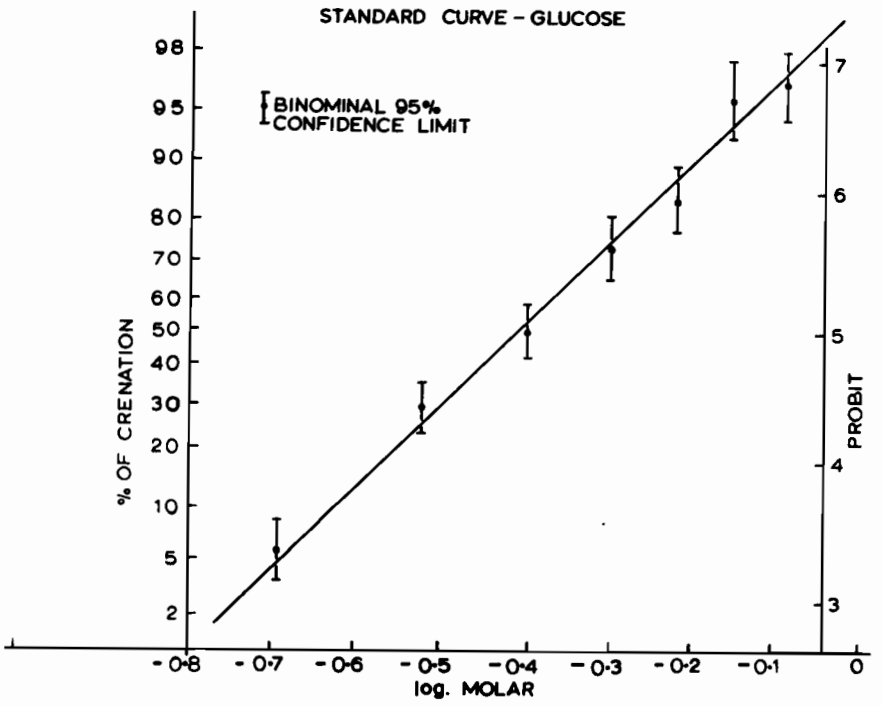


Fig. 20

Crenation of microcapsules as a function of glucose concentration in the medium. Abscissae: logarithm of glucose molarity (concentration of medium increases from left to right). Ordinates (plotted on probit scale): percentage of microcapsules found to be crenated 60 seconds after contact with the glucose medium. In this and subsequent graphs (Figs. 21-26) the data refer to Nylon microcapsules of 27 μ mean diameter containing hemolysate.

TABLE VI

EXPERIMENTAL RESULTS

Substance	Concentration (molar)	% Crenation	Number microcapsules counted	Binomial 95% confidence limits
Formamide	10.0	80.5	97	71.5 - 87.5
Formamide	7.5	68.5	212	61.5 - 74.5
Formamide	5.0	36.0	195	29.0 - 43.0
Urea	8.5	90.2	128	83.0 - 94.7
Urea	6.0	81.0	114	73.0 - 88.0
Urea	4.0	41.5	157	33.5 - 49.5
Ethylurea	2.0	86.0	187	78.6 - 90.7
Ethylurea	1.5	85.5	151	78.5 - 90.6
Ethylurea	1.0	74.5	156	66.5 - 81.5
Ethyleneglycol	2.0	73.0	157	65.0 - 77.0
Ethyleneglycol	1.5	58.0	167	50.0 - 66.0
Ethyleneglycol	1.0	14.3	315	10.3 - 18.3
Propylene glycol	2.0	84.3	101	76.0 - 90.0
Propylene glycol	1.5	79.5	161	73.0 - 89.0
Propylene glycol	1.0	40.5	146	32.5 - 48.5
Dextrose	0.6	84.1	145	77.0 - 89.0
Dextrose	0.5	73.9	115	65.0 - 82.0
Dextrose	0.4	51.6	178	45.0 - 59.0
Sucrose	0.4	76.5	111	67.5 - 83.5
Sucrose	0.3	55.5	108	46.0 - 66.0
Sucrose	0.2	16.3	141	11.0 - 23.0

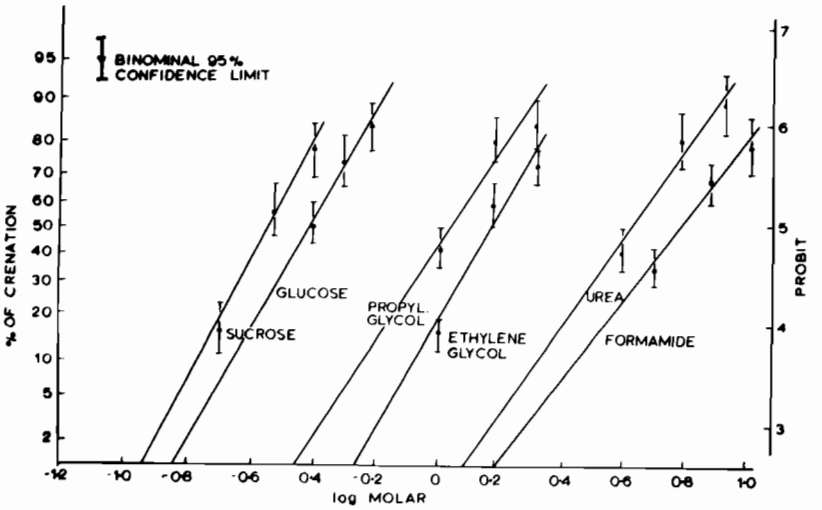


Fig. 21

Crenation of microcapsules in non-electrolyte solutions. Same experimental conditions as for Fig. 20.

TABLE VII

Solute	Median Effective Conc. (50% crenation conc.) Mean ± S. D. (Molar)	Threshold Crenation Conc. (0% crenation conc.) Mean ± S. D. (Molar)
Formamide	6.025 ± 0.375	1.641 ± 0.080
Urea	4.217 ± 0.411	1.202 ± 0.120
Ethylene Glycol	1.491 ± 0.062	0.545 ± 0.038
Propylene Glycol	1.047 ± 0.064	0.350 ± 0.035
Dextrose	0.389 ± 0.031	0.145 ± 0.009
Sucrose	0.285 ± 0.014	0.116 ± 0.006

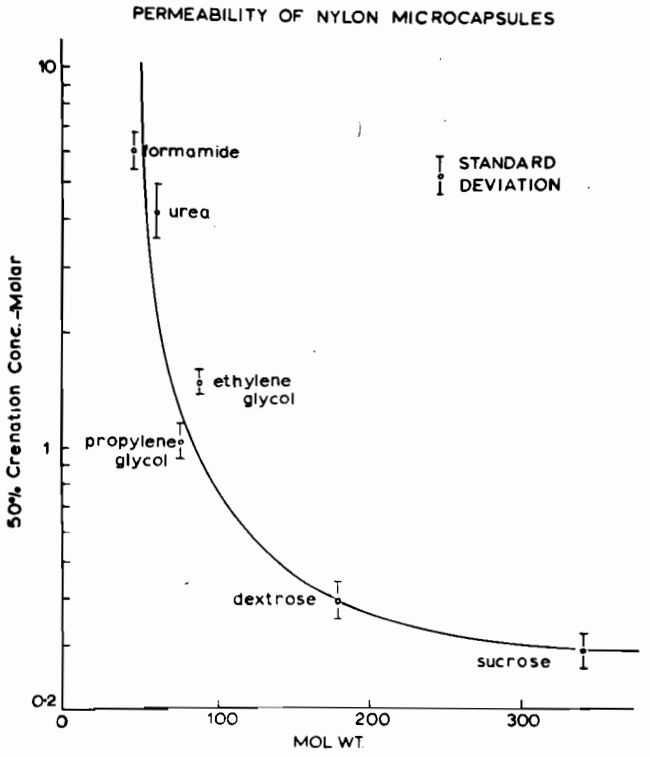


Fig. 22

Permeability of microcapsules to non-electrolytes as a function of molecular weight. Abscissae: molecular weight. Ordinates: concentration of non-electrolyte causing 50% crenation after 60 seconds. (Calculated from data of Fig. 21).

Analysis of equivalent pore radius for Nylon-microcapsules

The threshold crenation concentrations, listed in Table VII for a number of non-electrolytes, will now be used to estimate the equivalent pore radius of Nylon microcapsules prepared by the standard method. This will be done in two ways, (1) following the same approach as Solomon et al. (Goldstein and Solomon, 1960; Solomon, 1961) and (2) following a slight modification of their approach.

(1) Solomon et al.'s approach, as has already been seen, requires that the reflection coefficient should be determined experimentally for at least one solute whose molecular radius is known - preferably the data for several solutes should be available, as was the case in their investigation. In the case of their experiments, their calculation of reflection coefficient (σ) was simplified by the fact that the theoretical osmotic pressure at T = 0 was known for both the external and internal phases. In the case of the microcapsules, the reflection coefficient, defined as Cm/Cs (the ratio of threshold crenation concentration for an impermeant and a permeant solute) had to be determined by ascertaining both numerator and denumerator of this ratio, because no impermeant solute could be found which was sufficiently water-soluble to cause crenation; Cm had to be determined by extrapolation of the curve relating threshold crenation concentration to molecular radii (Fig. 23). The value for Cm thus obtained was approximately 0.08M: the reliability of this estimation will be discussed later. Values of Cs for formamide, urea, ethylene glycol, propylene glycol, dextrose and sucrose were those obtained experimentally and given in Table VIII.

Values for the molecular radii of these substances were obtained from the literature: those for urea, ethylene glycol and propylene glycol were taken from Goldstein and Solomon's paper (1960); since they did not use dextrose and sucrose in their experiments, the values for these substances, following Durbin (1960), were those given by Longsworth (1953). I have found no experimentally determined value for the molecular radius of formamide, and since the various equations used for calculating this parameter lead to widely different results, I have omitted the threshold crenation concentration value obtained with formamide from the calculation of equivalent pore radius.

Following Solomon et al., I calculated σ and $(1 - \sigma)$ for each of the test substances, using equation (13) on p.117 , and plotted a family of curves against molecular radius for theoretical pore radii ranging from 8 to 30 Å. The plotted curves are shown in Fig. 24 , on which are plotted also the experimental values of $(1 - \sigma)$ (Table VII) for each of the five molecular species used for the calculation. From Fig. 24 it is apparent that the line which best fits the experimental results is close to the lines for 15 Å and 16 Å, except in the case of urea, which lies closer to the line for 20 Å. It seems likely that the divergence in the case of urea arises from the small size of this molecule, which would penetrate the relatively large pores of the Nylon membrane so rapidly that my procedure for observing crenation (which allowed 60 seconds between exposure to the solution and taking of the microphotograph) was not rapid enough to determine the crenation produced by a truly threshold concentration of urea. Nevertheless, it

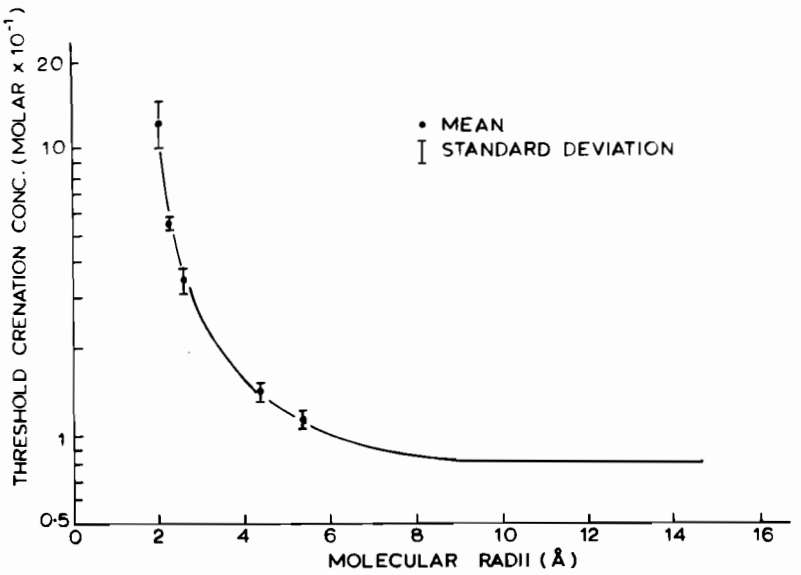


Fig. 23

Permeability of microcapsules to non-electrolytes as a function of molecular radius. Same data as Fig. 21. Abscissae: molecular radius of solute. Ordinates: threshold concentration for crenation (0% crenation) obtained by extrapolation from data of Fig. 21. (For further explanation see text).

TABLE VIII

REFLECTION COEFFICIENTS

Solute	Threshold Crenation Conc. (Molar) ± S. D.	Reflection Coefficient (σ)	1 - Reflection Coefficient ($1 - \sigma$) ± S. D.
Cm	0.080 (Extrapolated)		
Sucrose	0.116 ± 0.006	0.689	0.311 ± 0.015
Dextrose	0.145 ± 0.009	0.552	0.448 ± 0.029
Propylene Glycol	0.350 ± 0.035	0.229	0.771 ± 0.077
Ethylene Clycol	0.545 ± 0.038	0.147	0.853 ± 0.061
Urea	1.202 ± 0.120	0.067	0.933 ± 0.093

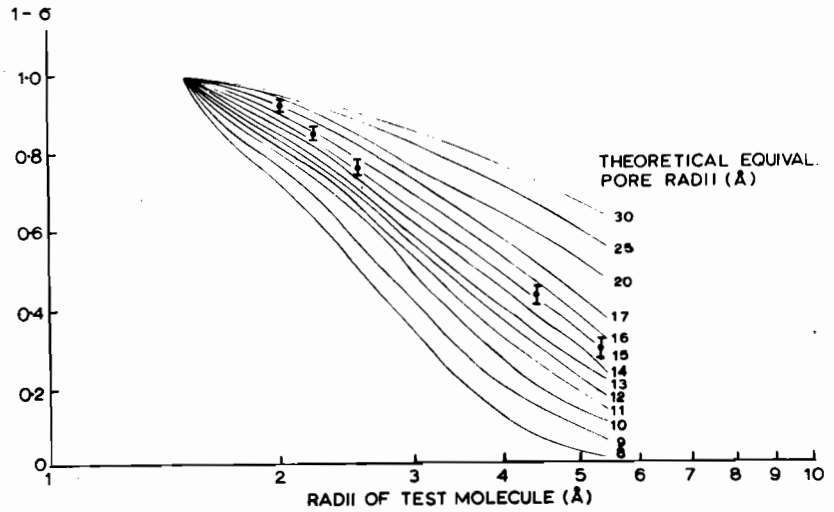


Fig. 24

Calculation of equivalent pore radius for Nylon membranes of 27 μ microcapsules. The theoretical curves for different equivalent pore radii are plotted against (1-D) according to equation (13) on p. 117 . The inserted points (mean ± S.D.) represent experimentally determined values of (1-D) plotted against values given by Solomon and Durbin for the radius of each test molecule. From above downward the points represent urea, ethylene glycol, propylene glycol, glucose and sucrose. Note that all points except the one for urea lie close to the lines for 15 and 16 Å. For further explanation see text.

can be noted that the estimates for effective pore size obtained with this relatively simple technique agreed at least as well with one another as those obtained by Solomon's elaborate procedure applied to an erythrocyte population which was presumably more homogeneous as to size and membrane thickness than the microcapsule population studied in these experiments.

(2) Though the values of effective pore radius obtained by the methods just described are in rather good agreement, they would all be subject to a systematic error if Cm, the threshold crenation concentration for an impermeant solute, had been wrongly estimated. In the calculation just presented, this value was obtained by extrapolating the data of Fig. 23 , and the question may now be raised as to the reliability of this value. Is it possible to obtain an estimate of the equivalent pore radius without knowing the threshold concentration for an impermeant solute? An attempt is made below to answer this question.

If C, as before, represents threshold crenation concentration, and m stands for an impermeant solute and s_1 and s_2 for two permeant solutes having different values for C, then

$$\sigma_{s_1} = Cm/Cs_1 , \text{ and}$$

$$\sigma_{s_2} = Cm/Cs_2 : \text{ whence}$$

$$\frac{\sigma_{s_1}}{\sigma_{s_2}} = \frac{Cm/Csl}{Cm/Cs2} = \frac{Cs2}{Csl}$$

Thus, it is seen that although σ cannot be determined without knowing Cm,

the ratio of the values of σ for any two permeant solutes can be obtained if the threshold crenation concentration is known for each solute.

The threshold crenation concentrations (C) of sucrose (s), dextrose (d), propylene glycol (p) and ethylene glycol (e) are used in the following ways. The values for threshold crenation concentrations (C) given in Table VIII for sucrose (Cs), dextrose (Cd), propylene glycol (Cp) and ethylene glycol (Ce) are paired to give six such ratios: the data for formamide and urea are omitted for the reasons already mentioned. From the remaining six ratios (Ce/Cs, Cp/Cs, Cd/Cs, Ce/Cd, Cp/Cd and Ce/Cp), the theoretical values of $Cs1/Cs2 = \sigma_{s2}/\sigma_{s1}$ are plotted against equivalent pore diameter according to equation (13) to give a family of curves, one for each solute pair. These are shown in Fig. 25 , and the experimentally determined values for four of the ratios (Ce/Cs, Cp/Cs, Ce/Cd and Cp/Cd) are tabulated in Table IX , and inserted as points on the appropriate curves. (Two pairs, Ce/Cp and Cd/Cs, give no useful information because the slopes of the line for these pairs are too flat.) For the remainding four pairs, the observed ratios fell on the plotted lines corresponding to equivalent pore radius between 17 Å and 20 Å. There appears to be a tendency for the estimated equivalent pore radius to approach 17 Å as the values for the ratio Cs1/Cs2 diminishes; in this figure, 17 Å is perhaps the best single estimate for the equivalent pore radius. The value for equivalent pore radius obtained by the second method is slightly higher than the value obtained by the first method, but in substantial agreement with it. The value obtained by method (2) may be more reliable than those by method

TABLE IX

Ratio of $\frac{\text{Threshold Crenation Conc. (Cx)}}{\text{Threshold Crenation Conc. (Cy)}} = \frac{\text{Reflection coefficient } (\sigma x)}{\text{Reflection coefficient } (\sigma x)}$

$Ce/Cs = \sigma s/\sigma e = 0.545/0.116 = 4.70$

$Ce/Cd = \sigma d/\sigma e = 0.545/0.145 = 3.76$

$Cp/Cs = \sigma s/\sigma p = 0.350/0.116 = 3.02$

$Cp/Cd = \sigma d/\sigma p = 0.350/0.145 = 2.43$

$Ce/Cp = \sigma p/\sigma e = 0.545/0.350 = 1.54$

$Cd/Cs = \sigma s/\sigma d = 0.145/0.116 = 1.25$

Where e - ethylene glycol

p - propylene glycol

d - dextrose

s - sucrose

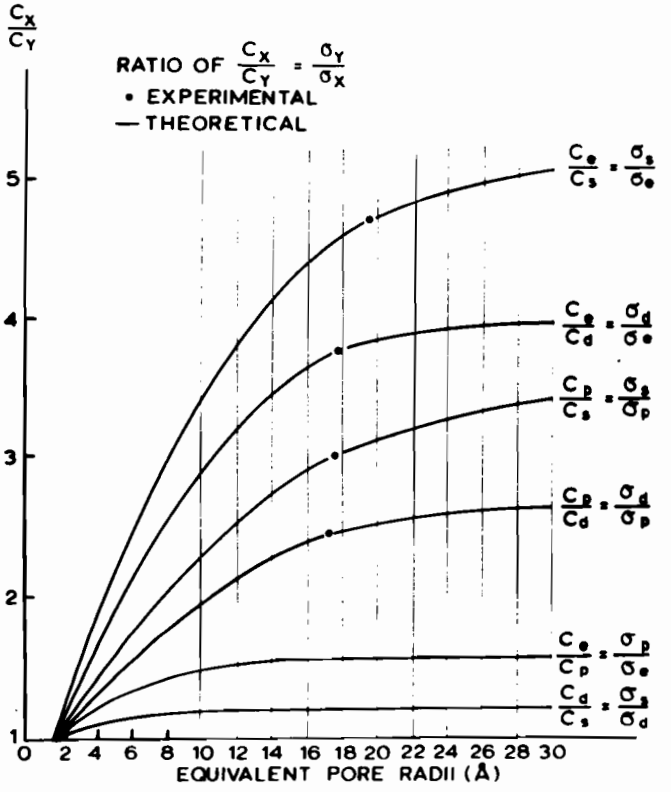


Fig. 25

Calculation of equivalent pore radius for Nylon membranes of 27 μ microcapsules. For explanation see text. The six theoretical curves, each relating pore radius to the ratio of reflection coefficients for a pair of solutes, were calculated for equivalent pore radii between 10 and 38 Å: the left-hand portion of each curve was obtained by extrapolation to the origin. The plotted points are experimentally determined values for four solute pairs.

(1) since it is not based on an extrapolated estimate of Cm.

The equivalent pore radius for the Nylon microcapsules is thus several times greater than the molecular radii of sucrose, the largest molecular species used in this experiment; nevertheless, the microcapsule membrane presents a rather effective barrier to the diffusion of this substance. It has long been recognised by workers on membrane permeability (cf. for example, the discussion by Pappenheimer (1953)) that the diffusion of a solute is greatly restricted in aqueous pores even when the pore diameter is several times the diameter of the solute molecule. The explanation of this phenomenon in terms of Poiseuille flow, steric hindrance and viscous drag has been discussed at length by the author mentioned and by others. It is consequently not surprising that molecules like those of ovalbumin(radius from diffusion coefficient 28.5 Å) and haemoglobin (radius from diffusion coefficient 32.5 Å) do not leak detectably out of microcapsules in which they have been enclosed. Even allowing for some heteroporosity of the microcapsule membrane, with the possibility that a proportion of pores may have radii greater than that of the calculated equivalent pore radius, the chance that an individual protein molecule would escape in a finite time would be negligibly small. The dimensions of Nylon microcapsule equivalent pore radius as calculated above, and of human erythrocyte membrane pores as calculated by Goldstein and Solomon (1960), are represented in Fig. 26; for comparison, the dimensions of a number of

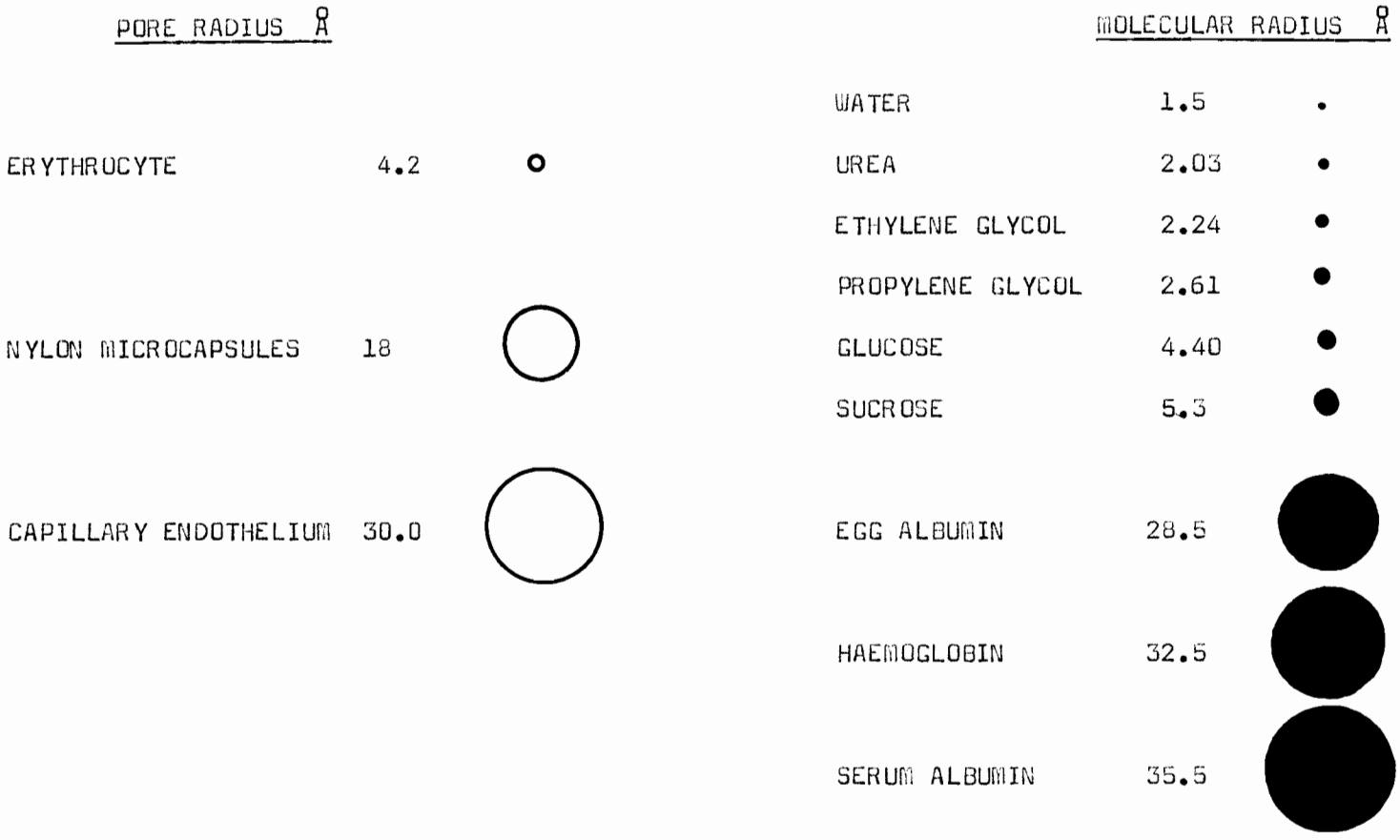


Fig. 26

The pore radius of Nylon microcapsule membranes, as calculated in the thesis, is here compared to the pore radius calculated by Goldstein and Solomon (1960) for the erythrocyte membrane and the pore radius calculated by Pappenheimer, Renkin and Borrero (1951) for the capillary wall. For comparison, the molecular radii of a number of non-electrolytes and proteins are shown on the right.

non-electrolytes and protein molecules are also indicated. It needs scarcely be pointed out that solute molecules generally depart significantly from sphericity, and that pores in membranes are unlikely to be exactly cylindrical. In the case of the Nylon membrane, and probably also in the case of the cell membrane, the membrane matrix should be thought of as a loose meshwork of asymmetrical molecules rather than as a sheet with discrete perforations; but it will be a long time before an exact description of the structure of either sort of membrane is available. Nevertheless, the data obtained from permeability studies based on the principles introduced by Staverman, Kedem, Katchalsky and Solomon provide a useful baseline for predicting the behaviour of membranes with respect to a variety of solutes other than those used to specify the characterisation of the membrane.

It should be emphasized again that the treatment of microcapsule permeability just presented refers only to non-electrolytes that would not be expected to dissolve in the membrane matrix. The permeability of lipid-soluble species would no doubt be much greater, as shown in the case of pyridine. The permeability of electrolytes as already discussed would depend not only on their hydrated-ionic radii, but also on possible interaction with fixed charges on the membrane, and has not yet been systematically investigated; though preliminary tests have suggested that the diffusion of polyvalent anions into Nylon microcapsules is slower than would be expected on the basis of their diffusion across uncharged pores.

The differential permeability of Nylon microcapsules for aqueous solutions of different molecular size is further illustrated in the next section, and has given some guidance for the studies on enzyme encapsulation reported in a later part of the thesis.

DIFFERENTIAL DIALYSIS

The different permeability of microcapsules for lipid-insoluble substances of different molecular size suggests a number of applications for analytical and preparative work. The theoretical principles involved would be similar to those employed in counter-current separation and chromatography, and need not be detailed here. One obvious analogy would be to the use of "Sephadex" granules as molecular sieves in column chromatography. This material, a cross-linked dextran, forms a hydrophilic gel whose porosity is controllable. Solutes of large molecular size that cannot penetrate the gel are excluded and emerge from the column without retardation while solutes capable of diffusing into the interior are retarded.

Fig.27 shows that a solumn of microcapsules can behave similarly, separating haemoglobin from glucose almost completely in a single passage. The characteristics of the column are given in the legend. Haemoglobin was estimated by the micro-method of King (1947) and glucose by the micromethod of Folin. In other tests partial separation was obtained of glucose and sucrose, and of sucrose and urea; but optimal conditions for the separation of low molecular weight solutes have not yet been worked out; obviously the size and permeability of the microcapsules and the flow rate will be important parameters. The theoretical advantages of differential dialysis for the separation of solutes on the basis of molecular radii has been discussed, with experimental illustrations, in a recent important paper by Craig (1964). In Craig's technique, as already noted (p.55), the necessary speed of diffusion equilibrium was secured by the use of an annular membrane separating two aqueous phases in counter-current flow, the external phase being a very thin film. Craig's procedure involves the preparation of membranes of very accurately controlled dimensions. It seems likely that equally good results could be secured more conveniently by the use of microcapsules; but further studies are necessary before this possibility can be properly assessed.

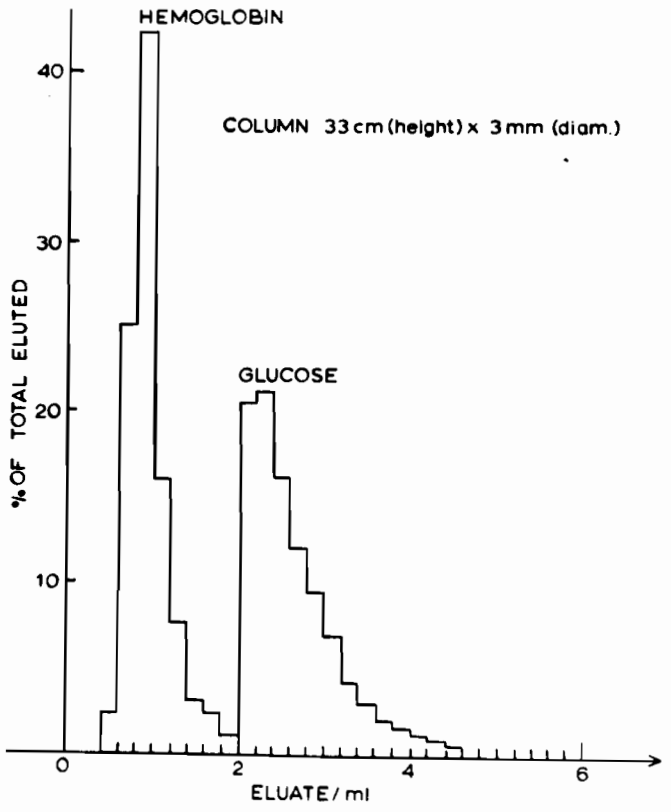


Fig. 27

Column with dimensions as shown, packed with Nylon microcapsules of mean diameter about 90 μ, prepared by the Standard method using 1% v/v Span 85 and Jumbo speed setting of 1. Flow rate 0.09 ml/min.; eluate collected in 0.2 ml fractions.

IN VITRO ENZYME ACTIVITY

General Introduction

A good deal of my time during the research reported in this thesis has been devoted to studying the possibility that enzymes enclosed within microcapsules might continue to act, more or less efficiently, on water-soluble substrates present in the external medium. This section of the thesis deals with the behaviour after encapsulation of two enzymes, carbonic anhydrase and urease. The latter enzyme, in encapsulated form, has also been used for *in vivo* experiments which will be described in the following section of the thesis. Attention has been focussed on these two enzymes because their substrates and products (CO_2 and bicarbonate in the first case; and urea, NH_4^+ and HCO_3^- in the second case) are small molecules or ions which might be expected to diffuse with relatively little hindrance across the microcapsule membrane.

The application of the encapsulation technique to certain other enzymes has been mentioned in the introduction (p.41), but the fragmentary results obtained, at an early stage of the work, have not been thought worth reporting.

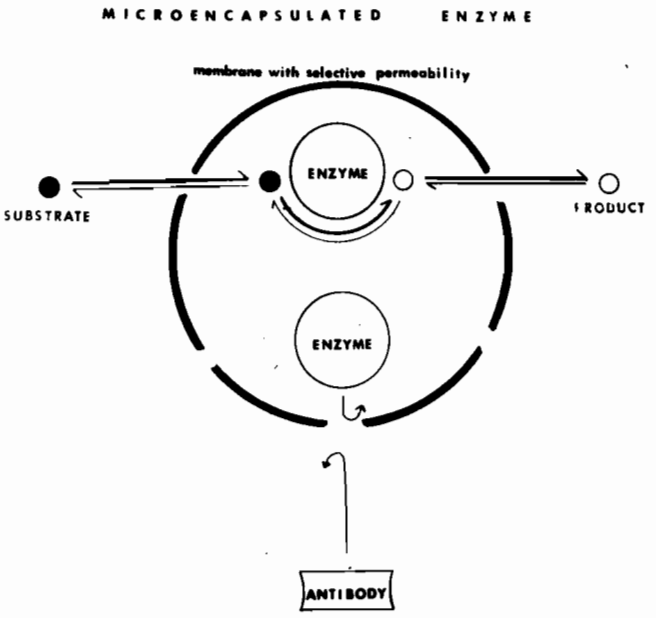


Fig. 28

Schematic representation of the behaviour of an encapsulated enzyme, acting <u>in vitro</u> on small-molecular substrate to produce a small-molecular product. Substrate and product can diffuse through membrane pores which are too small to allow passage of enzyme or antibody.

Carbonic Anhydrase

. Introduction

I found it convenient to begin my enzyme studies with carbonic anhydrase, because this enzyme is known to be present in rather high concentration in erythrocyte hemolysates such as I have used in my standard microencapsulation techniques. There is a wealth of literature on the biochemical and physiological aspects of carbonic anhydrase, but only those aspects related to the present problem will be discussed in the following brief review.

Carbonic anhydrase catalyses the first step of the reversible reaction

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

In the absence of the enzyme the first step is slow. The second step, the partial ionization of H_2CO_3, occurs to a degree that depends on the pH of the medium, but is never rate-limiting. Kinetic studies have however suggested (Davies, 1961) that the enzyme-substrate compound breaks down to yield H^+ and HCO_3^- directly, rather than H_2CO_3.

Carbonic anhydrase is a zinc-containing protein (Keilin and Mann, 1939). It is present in high concentration in mammalian erythrocytes (Keilin and Mann, 1939) and in lower concentration in renal cortex (Davenport and Wilhelmi, 1941) and gastric mucosa (Davenport and Fisher, 1938). Human carbonic anhydrase has a molecular weight of 34,000 (Dixon and Webb, 1964) and does not dialyse through an ordinary cellophane membrane (Day and Frank, 1946). It is an unusually stable enzyme unless highly purified: a concentrated solution can be stored in a refrigerator

for at least two years with no loss of activity (Davies, 1958); in the dried state it is stable indefinitely (Scott and Mendive, 1941). Meldrum and Roughton (1934), comparing the carbonic anhydrase activity of laked and unlaked blood, found that defibrinated ox blood from the abattoir had 23-35% of the activity of laked blood from the same source, whereas goat venous blood obtained by syringe had only 0.05 - 0.01% of the activity of the corresponding laked blood. They discussed the possibility that this remarkable difference might be due to species variation, or alternatively to the manner in which the blood was obtained; but they were unable to decide between the alternatives, and curiously enough later investigations appear to have paid no attention to this problem. In any case it is clear from the greater activity of the laked blood that the efficiency of the enzyme *in vivo* must be sharply reduced by diffusion limitations. The overall reaction of laked blood has a Q10 of 1.4 and is inhibited by heavy metals, sulphide and sulphonamides, especially acetazolamide (Diamox: 2-acetylamino-1, 3, 4-thiadiazole-5-sulphonamide). The efficiency of the enzyme increases over the pH range 6.0 to 9.0.

Carbonic anhydrase activity has been measured by a number of different methods. (1) Meldrum and Roughton (1933) originally used a manometric technique to measure the uptake of CO_2 by laked blood in contact with a gas mixture of known CO_2 tension. Their method has a number of limitations (*cf.* Davies, 1961) and is not now used. (2) Indicator methods were introduced by Brinkman (1934) and Philpot and Philpot (1936): they depend on measurement of the time required for

the pH of the reaction mixture containing the enzyme preparation in a bicarbonate-carbonate buffer to fall to a level indicated by the colour change of an indicator, e.g. bromothymol blue. These methods have been criticized, because both the high pH and the high concentration of indicator reduce the activity of the enzyme (Wilbur and Anderson, 1948); but Maren and his colleagues (Maren, Mayer, Wadsworth, 1954; Maren, 1963) who used a slight modification of the Philpot technique found it useful for their studies on the kinetics and inhibitors of the enzyme. (3) Wilbur and Anderson (1948) were the first to use an electrometric method to measure the pH shift: they substituted a veronal buffer for the bicarbonate and carbonate mixture, so that the pH was still high, although they dispensed with indicators. Their method was modified by Davis (1958), who recorded the pH change in a weakly buffered solution, modifying the glass electrode to reduce its impedance. In my own experiments I have retained the buffer mixture of Philpot and Philpot, but have measured the pH change electrometrically. Though a broad pH change is involved, and the technique is unsuitable for exact analysis of the kinetics of the enzyme in free solution, it is convenient and satisfactory for comparing the activity of the encapsulated and the erythrocyte-bound enzyme.

Measurement of carbonic anhydrase activity

The method of Philpot and Philpot (1936), in which the rate of hydration of CO_2 bubbled through an alkaline buffer is measured, was used with the following modifications. (1) The bromothymol blue indicator was omitted, and the pH change was recorded continuously with

a Radiometer pH meter connected to a Gilson polygraph. (2) In order to slow the reaction time, a mixture of 5% (checked by Haldane apparatus: the exact percentage was 5.02± 0.02) CO2 and 95% O2 was substituted for the 100% CO2 of the Philpot method. (3) Instead of the very dilute (0.00263M) NaHCO3 solution used to dissolve the enzyme preparation, 0.9% NaCl containing the same proportion of NaHCO3 was employed, so that the enzyme activity of intact erythrocytes could be compared with that of enzyme-loaded microcapsules. Otherwise the procedure was the same as that described by Philpot and Philpot.

In each analysis 10 ml of bicarbonate-saline solution was pipetted into a 15-ml beaker kept at 0°C. 1 ml bicarbonate-saline solution, with or without an enzyme, was added, followed by a drop of octyl alcohol to prevent foaming. The CO2 - O2 mixture was bubbled through the beaker at a constant rate as described by the Philpots, and pH was recorded continously. After exactly 2 minutes (stop-watch), 1 ml of a standard bicarbonate - carbonate buffer at 0°C was blown into the reaction mixture from a pipette, and the recording was continued until the pH reached 7.0.

In a typical experiment, the following tests were made. First, a control curve was recorded without enzyme. Then a series of curves were obtained with varying amounts of red cells: 1/64 ml, 1/32 ml, 1/16 ml and 1/8 ml of red blood cells, each volume being made up to 1 ml in bicarbonate saline; the red cells from dog carotid blood had been washed three times with 2 volumes of saline and kept overnight

suspended in the appropriate amount of saline. Nylon microcapsules were prepared by the procedure described on p. 68 , except that the hemolysate contained red cells lysed by repeated freezing and thawing and diluted with an equal volume of the alkaline diamine solution, the final dilution of the red cell content being thus 1:1. The microcapsules so prepared were washed five times with ten volumes of saline at 1/2 hour intervals to remove residual diamine and detergent, then made up as a 50% suspension in bicarbonate-saline and stored for 24 hours in the refrigerator. On the day of the test, the suspension was centrifuged and 0.5 ml of the supernatant was tested for enzyme activity, in order to detect any possible leakage of carbonic anhydrase from the microcapsules. The microcapsules were then resuspended in bicarbonate saline, and 1 ml portions of the suspension, containing 1/16 ml and 1/8 ml of microcapsules respectively, were tested for enzyme activity. Finally the enzyme activity of erythrocytes and microcapsules was tested in the presence of sodium acetazolamide: 10 mg of solid sodium acetazolamide was added to 1 ml of bicarbonate-saline suspension (containing either 1/16 ml of dog erythrocytes or 1/8 ml of microcapsules) and the mixture was left standing for 1 hour (final concentrate of acetazolamide 0.04M) before being tested for enzyme activity.

Results

The curves obtained in a typical experiment are shown in Fig. 29. In the absence of enzyme, the fall of pH from 9.5 to 7.0 at 0^{o} took about 15 minutes. In the presence of dog erythrocytes or Nylon microcapsules (mean diameter 11.3 μ) the fall was steeper, the slope of the

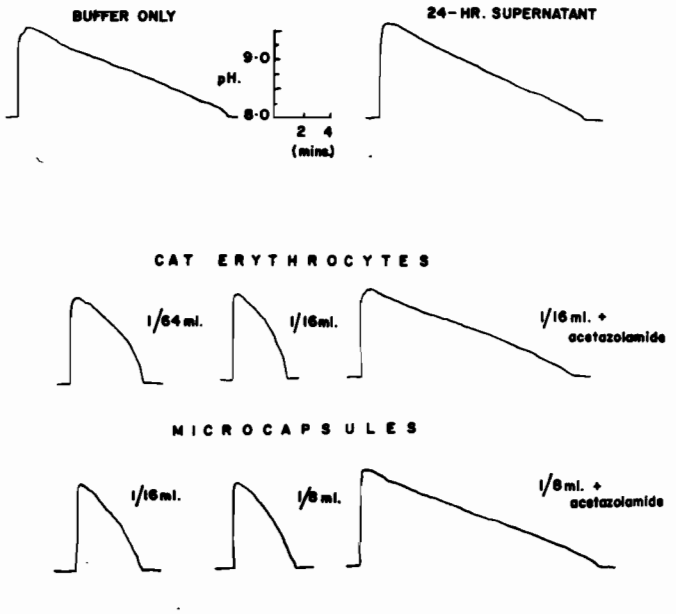


Fig. 29

Carbonic anhydrase activity, measured as the rate of fall of pH while CO_2 bubbles through 12 ml of alkaline buffer: method as in Philpot and Philpot (1936), but with 0.15 M NaCl added to the medium. Fall of pH is accelerated by the presence of erythrocytes, or of Nylon microcapsules containing erythrocyte hemolysate diluted 1:1 (1 volume of unlaked erythrocytes yields 2 volumes of microcapsules). No acceleration occurs in the presence of acetazolamide, or when the microcapsules are replaced by supernatant from a 50 percent suspension of microcapsules stored for 24 hours.

curve increasing with the quantity of added enzyme. It will be noted also that the rate of pH fall became more rapid with time as the buffer capacity of the solution decreased. The sample of buffered saline that had been in contact with its own volume of microcapsules for 24 hours showed no detectable enzyme activity: control tests with added hemolysate showed that the leakage of enzyme must have been less than 0.1%. Finally, as Fig.29 shows, acetazolamide in the concentration used completely inhibited the catalytic activity of both the erythrocytes and the Nylon microcapsules. The buffer capacity of the erythrocytes, microcapsules and acetazolamide in this system was calculated to be negligible in comparison with that of the bicarbonate-carbonate mixture; and the results of the acetazolamide test showed that this was in fact the case, the rate of pH fall in the presence of the inhibited enzyme preparation being about the same as with buffer alone. In Fig.30, erythrocyte concentration is plotted against enzyme activity, here measured as the reciprocal of the time required for pH to fall from 9.5 to 7.0. After correcting for the dilution factor (the microcapsules contained a 1:1 dilution of hemolysate), the microencapsulated enzyme in this experiment was found to have about 75% (± 5%) of the activity of the erythrocyte bound enzyme, assuming that no enzyme was destroyed by the process of encapsulation. Other experiments gave very similar results.

Preliminary tests with microcapsules having membranes of collodion or cross-linked haemoglobin have shown that carbonic anhydrase activity is also well retained in these preparations; but quantitative comparisons

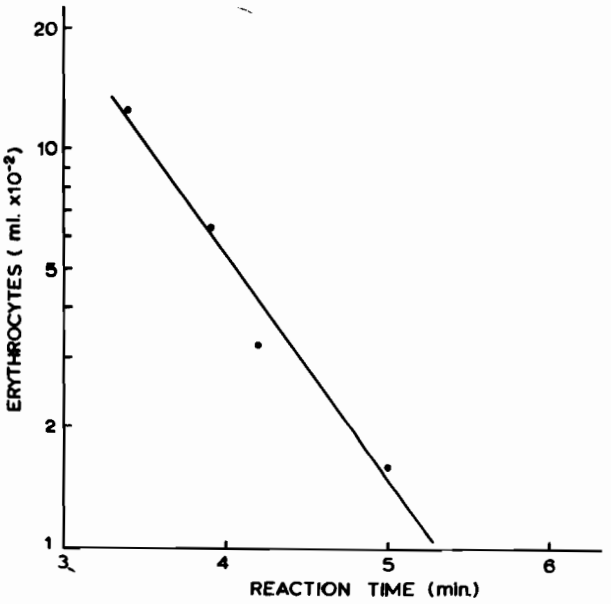


Fig. 30

Carbonic anhydrase activity of intact erythrocytes. Reaction time is the time taken for the pH of the medium to fall from 9.5 to 8.0.

with Nylon microcapsules of the same size have not yet been made, nor has carbonic anhydrase activity been determined as a function of microcapsule diameter.

Discussion

It would be of some interest to analyse the results just presented in order to determine, if possible, whether the somewhat lower activity of the microcapsule-bound enzyme, as compared to the erythrocyte-bound enzyme, could be ascribed to either the differences in geometry or the differences in membrane permeabilities between the two kinds of particles. The Nylon microcapsules with mean diameter 11.3 μ had a smaller surface-to-volume ratio than the dog erythrocyte with mean diameter 7.1 μ; moreover since the latter were biconcave, diffusion distances within the erythrocyte would be further reduced as compared with the situation in the microcapsule. A further possibility to account for the result would be the inactivation of up to 25% of the enzyme by the encapsulation procedure, for instance by cross-linking with the membrane polymers as can happen with other proteins.

Some attempts have been made to assess semiquantitatively the relative diffusion restrictions affecting the velocity of the enzyme action in erythrocytes and microcapsules respectively, but the results of the calculations could not be regarded with any confidence, since too many assumptions had to be made about the boundary conditions in the diffusion equations. The problem could be attacked experimentally by incorporating haemolysate into batches of microcapsules made to be different in mean

diameter and membrane thickness, but this has not yet been done; nor have any tests been made to determine the effect of varying the concentration of haemolysate in the enclosed phase. Lastly it may be noted that no comparison has been made of the activity of the erythrocyte-bound enzyme with that of the enzyme in free solution: as was noted earlier (p.151), the data in the literature are divergent on this point. Nevertheless, the fact that the encapsulated enzyme was not much less active than the erythrocyte-bound enzyme was rather impressive, and it encouraged me to attempt the encapsulation of an enzyme which (unlike carbonic anhydrase) could be expected to produce measurable effects when administered to an intact animal.

Urease

The second enzyme selected for encapsulation was urease, which catalyses the hydrolysis of urea :

$$(NH_2)_2.CO + 2H_2O \longrightarrow 2NH_4^+ + CO_3^{=};\ H_2O + CO_3^{=} \longrightarrow HCO_3^- + OH^-$$

No coenzyme or activator is required. In the absence of the enzyme the reaction does not occur to an appreciable degree.

Urease was the first enzyme to be crystallized (Sumner, 1926) and is available commercially in the crystalline form or in the form of a crude extract of soybean or jackbean meal. It is a globulin with a molecular weight of 480,000 (Dixon and Webb, 1964); it is moderately soluble in water at pH 6 or higher, but only slightly soluble at its isoelectric point of 5.0-5.1. It is inactivated by pepsin, reducing agents, heavy metals, oxidation products of diamines and ultraviolet light (Sumner 1951). The inactivation of urease by heavy metals can be prevented by proteins.

The purified enzyme is unstable in solution, losing 50% of its activity in 24 hours at 37°C (Sumner and Dounce, 1937), but it can be stabilised by 2% gum arabic (Sumner and Myrback, 1951) or 5% egg albumin (Van Slyke and Archibald, 1944): it is uncertain whether or not the protection depends solely on trapping of heavy metal. When protected by gum arabic and sulphite-bisulphite buffer, the crystalline enzyme is stable for 95 hours at room temperature, but then slowly deteriorates (Sumner and Dounce, 1937). Urease crystals suspended in 50% glycerol can be stored for months at 2°C (Kistiakowaky, Mangelsdorf and Rosenberg, 1952).

The occurence of urease in the animal body, except as a product of intestinal bacteria, has been a matter of controversy; in any case it is clear that tissues other than the intestinal mucosa and lumen contain at most the merest traces of the enzyme. On the other hand its substrate, urea, is the major diffusible nitrogenous constituent of the extracellular fluid. Both the high permeability of microcapsules to urea, demonstrated in the preceding section (p.123), and the interesting possibility of demonstrating an *in vivo* action of encapsulated urease, have prompted me to investigate the efficiency of this enzyme after microencapsulation.

Measurement of Urease Activity

Most of the published methods for the assay of urease depend on measuring the rate of ammonia formation under standard conditions of incubation. One of the simplest methods is that introduced by Van Slyke and Archibald (1944), who measured the time for the pH of a standard urea solution in phosphate buffer to rise from 6.7 to 7.7 under the influence of the enzyme, with phenol red as indicator. I adopted their technique with one modification; the rise of pH was recorded continuously with a pH meter and Gibson polygraph, in the same way as the fall of pH in the carbonic anhydrase assay.

The buffer-urea solution contained, in 100 ml, 3 gms of urea, 10 ml of molar NaH_2PO_4 and 10 ml of molar K_2HPO_4 (pH 6.7). To 5 ml of this solution at 20°C, 0.5 ml of a buffered urease preparation, containing a known weight of the standard urease or a known volume of microcapsules in the same

phosphate buffer (without urea), was added at zero time, with a drop of octyl alcohol added to prevent foaming, and the mixture was kept stirred until the pH rose to 7.7. The time required for the reaction was read from the record. All tests were performed in triplicate and the results were expressed in Sumner units (mean ± standard deviation). The Sumner unit was defined by Van Slyke and Archibald as the amount of urease capable of producing 1 mg of ammonium nitrogen in 5 minutes under these conditions; Van Slyke and Archibald noted further that 10 units of urease were required to raise the pH of the reaction mixture from 6.7 to 7.7 in 5 minutes at 20°C, and provided a formula for correcting readings made at other temperatures.

The urease preparation (Sigma V) used as standard had been assayed by the maker (Sigma Biochemicals) as containing 7000 Sumner units/gm. Its potency was checked by the Van Slyke and Archibald method and was found to agree approximately with the labelled potency. Thereafter it was assumed that the labelled potency was correct, and standard dilutions of the preparation were made to contain 10.0, 5.0 and 2.5 Sumner units/ml. With these concentrations the time required for the pH shift was 10 to 45 minutes, and measurements in this range appeared to give more reproducible results than when higher or lower concentrations of enzyme were used.

A standard activity curve, relating urease activity in Sumner units to reaction times in minutes, was plotted (Fig. 32) from the data in Table X , with urease added at the three levels just mentioned. From this curve the urease activity of microcapsule suspensions was read off

TABLE X

Standard Curve for Urease in Solution

Urease (Sumner units)	Time for pH change 6.7 to 7.7 (minutes)	Mean of 3 (minutes)	Standard deviation (minutes)
5.00	11.00		
5.00	10.00	11.00	± 1.00
5.00	12.00		
2.50	17.50		
2.50	18.50	17.33	± 1.25
2.50	16.00		
1.25	42.00		
1.25	41.00	42.33	± 1.52
1.25	44.00		

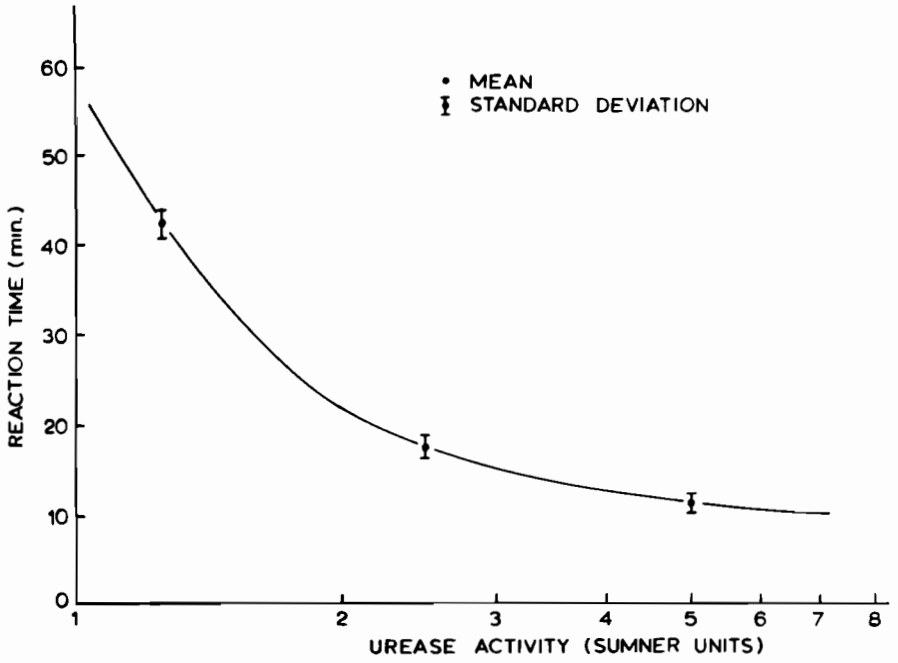


Fig. 32

Standard curve of measurement of urease activity, determined according to the method illustrated in Fig. 31. See also Table X .

and expressed as Sumner units/ml of microcapsules. The standard curve was not linear, but showed a downward concavity for reasons that are not clear; Dixon and Webb (1964) have suggested that such departures from lineality may be due to the presence of traces of inhibitors.

During the course of the tests the standard preparation of "Sigma" urease was exhausted and the only available preparation was a "soluble" one supplied by the Nutritional Biochemicals Co., whose labelled potency was 1500 Sumner units/gm. The potency of this preparation was also found to be in close agreement with the labelled potency; thereafter it was assumed that the labelled potency was correct, and the original standard curve was used with this preparation also.

Both the Sigma and the NBC urease preparations were encapsulated within Nylon as already described (p.82), the Sigma enzyme without hemolysate and the NBC enzyme in the presence of hemolysate. The NBC enzyme was also used for the preparation of two batches of microcapsules differing in mean diameter. The smaller microcapsules (27.1 ± 11.9 μ; mean diameter ± S.D.) were prepared with a Jumbo speed setting of 2.5 and the larger ones (89.8 ± 26.0 μ) with a speed setting of 1.0. The batch of smaller microcapsules was used for tests of the stability of the encapsulated enzyme.

Results

The results obtained with Sigma V urease after microencapsulation are given in Table XI , and specimen records are shown in Fig.31 .

TABLE XI

Microencapsulated Urease (Sigma V)

Vol. of microcaps. (ml)	Total urease (Sumner units)	Time of reaction (min.)	Assayed urease (Sumner units)	Mean ± Stand. Dev. (Sumner units)
1/80	5	26.5	1.75	
1/80	5	24.0	1.95	1.83 ± 0.10
1/80	5	25.5	1.80	

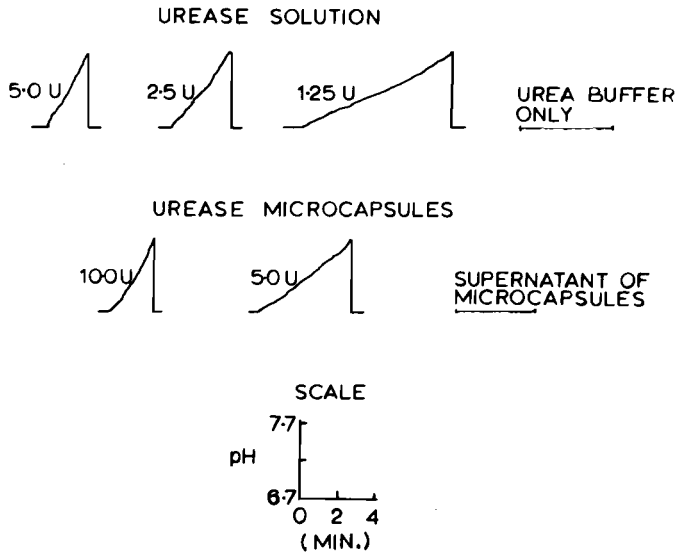


Fig. 31

Urease activity, measured as rate of rise of pH of urea-buffer medium. Rise of pH is accelerated by the presence of urease (Sigma V) in solution or enclosed in their Nylon microcapsules: further description in text. Urease in Sumner units. No pH change in the presence of supernatant from a 50% suspension of microcapsules stored for 12 hours.

From Table XI it can be seen that 5 Sumner units of the enzyme after encapsulation had an activity corresponding to that of 1.83 ± 0.10 Sumner units of enzyme in free solution. Thus the activity of the encapsulated enzyme was about 37% of the activity of the same amount of enzyme in free solution. Fig. 31 shows also that a sample of buffer in contact with its own volume of urease-loaded microcapsules for 12 hours acquires no measurable enzymatic activity. Thus urease, like carbonic anhydrase, does not leak to a significant degree out of stored Nylon microcapsules.

Very similar results were obtained with the NBC enzyme. In this case, however, the effect of varying microcapsule diameter was also observed. The results are given in Table XII. . . The data of Table XII show that the activity of the encapsulated enzyme was about 39% of the activity in free solution in the case of the smaller microcapsules, and about 21% of the activity in free solution in the case of the larger microcapsules.

The stability of the encapsulated urease preparation was not uniform. The Sigma V enzyme, encapsulated without the addition of hemolysate, formed beautiful clear microcapsules which however rapidly lost their activity: at 37° C the potency fell by more than 50% in 3 hours. In the hope of achieving a more stable preparation, later batches of urease-loaded microcapsules were made with the addition of hemolysate, using the less potent NBC enzyme which was then the only one available. With this procedure the stability of the enzyme was greatly improved, as shown

TABLE XII

Microencapsulated Urease (NBC Urease with Hemolysate)

Mean diameter (μ)	Vol. of microcap. ml	Total urease (Sumner units)	Time of reaction (min.)	Assayed urease (Sumner units)	Mean ± Standard Dev. (Sumner units)
27.1 ± 11.9	0.05	5	25.0	1.90	
27.1 ± 11.9	0.05	5	23.0	2.05	1.95 ± 0.09
27.1 ± 11.9	0.05	5	25.0	1.90	
89.8 ± 26.1	0.05	5	57.5	1.05	
89.8 ± 26.1	0.05	5	65.0	1.00	1.04 ± 0.03
89.8 ± 26.1	0.05	5	55.0	1.07	

TABLE XIII

Effect of Storage at 37° C

Time of Storage (days)	Reaction Time (minutes)	Assayed Urease (Sumner units)	Mean ± Standard Deviation (Sumner units)
Control	25.0	1.90	
Control	23.0	2.05	1.95 ± 0.08
Control	25.0	1.90	
1 day	31.0	1.58	
1 day	28.0	1.72	1.65 ± 0.007
1 day	29.5	1.65	
2 days	40.0	1.30	
2 days	31.0	1.58	1.44 ± 0.06
2 days	35.0	1.43	
3 days	37.0	1.38	
3 days	35.0	1.43	1.39 ± 0.03
3 days	37.0	1.38	
6 days	55.7	1.06	
6 days	55.0	1.07	1.10 ± 0.05
6 days	47.5	1.16	

TABLE XIV

Effect of Storage at 0°C

Time of Storage (days)	Reaction time (minutes)	Assayed urease (Sumner units)	Mean ± Standard Deviation (Sumner units)
Control	25.0	1.90	
Control	23.0	2.05	1.95 ± 0.08
Control	25.0	1.90	
7 days	36.0	1.40	
7 days	36.0	1.40	1.40 ± 0.00
7 days	36.0	1.40	
10 days	40.5	1.28	
10 days	41.0	1.25	1.24 ± 0.04
10 days	45.0	1.20	
14 days	65.0	1.00	
14 days	70.0	0.98	1.01 ± 0.04
14 days	61.0	1.05	

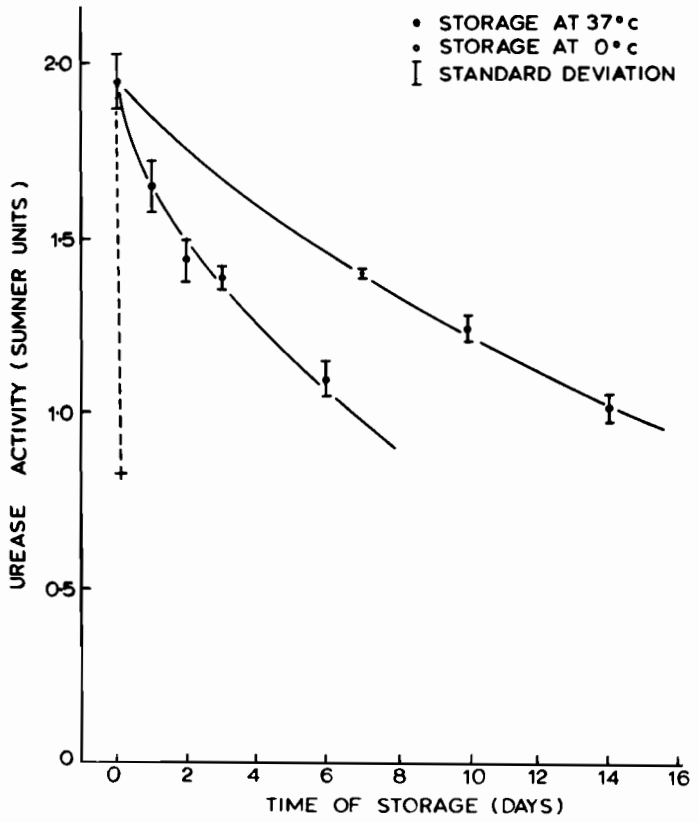


Fig. 33

Stability of urease enclosed in Nylon microcapsules, stored in phosphate buffer at pH 6.7. Continous lines: activity after storage at 0° (upper line) and at 37° (lower line) for enzyme encapsulated with hemolysate. Discontinous line: activity after storage at 37° for enzyme encapsulated without hemolysate. See also Tables XIII-XIV.

in Tables XIV and XIIIfor storage at 0^{o} and at 37^{o}. From Fig.33, which summarizes the data of all the stability tests, it can be seen that the half-life of the hemolysate-stabilized microencapsulated enzyme was about 2 weeks at the lower temperature.

Discussion

The results just presented showed that encapsulated urease can possess at least a third of the activity of the enzyme in free solution. This may be considered to be a rather satisfactory result, in view of the fact that (a) the enzyme may have been inactivated to some degree during the process of encapsulation, and (b) its action must be slowed at least to some extent by diffusion limitations. The data available do not permit an exact assessment of the contributions made by these factors to the diminution of activity. It is likely that at least a small part of the enzyme must have reacted with sebacoyl chloride and been incorporated in an inactive form into the membrane. This, however, is probably a minor cause in the diminution of activity, because, as has already been seen, the initial loss of activity was as great with the NBC urease incorporated in the presence of haemoglobin as with the Sigma V urease microcapsulated in the absence of haemoglobin. One would expect that the non-enzymatic protein present in high concentration in the internal phase would have competed for the reacting sebacoyl chloride molecules, and so protected the enzyme. Furthermore, it would be expected, if there were serious inactivation of urease at the interface,that this would be more serious in the case of the smaller microcapsules, which present the greater ratio of surface to enzyme; yet

enzyme activity was found to be significantly higher with the smaller microcapsules than with the larger one. It seems likely, therefore, that the lower activity of the encapsulated enzyme as compared with the free enzyme is to be explained on the basis of diffusion restrictions. This idea obviously fits in a general way with the higher activity of the small microcapsules which has just been referred to: other things being equal, diffusion exchange across the membrane would be facilitated by the higher surface-to-volume relationship. Unfortunately, as in the case of carbonic anhydrase, it is very difficult to develop this argument in a quantitative manner and to state exactly which molecular or ionic species involved in the enzyme action account for the diffusion limitation. There are two sets of possibilities to be considered. First, as already indicated, the enzyme reaction itself would be slowed by either a restricted access of substrate to the enzyme or a restricted diffusion of product away from the enzyme. Secondly, even though the enzyme action were efficient, the pH change by which it is revealed might occur, as a result of diffusion limitation, more slowly outside the membrane than inside it. In connection with the first possibility, it seems rather unlikely that the inward diffusion of urea could be so much slowed by the presence of the membrane as seriously to retard the formation of the substrate-enzyme complex. The urea molecule has a radius of about 2.0 Å (Goldstein and Solomon, 1960) and it is unlikely to be strongly hydrated in aqueous solution. Its cross-sectional area is thus less than 2% of the mean pore area for these microcapsules (mean pore radius of 17 - 20 Å: cf. p.139). In fact, my crenation threshold experiments have shown that urea has a reflection coefficient of 0.067

and so penetrates such a membrane almost as readily as water. The anions produced by the reaction are, however, larger, with hydrated radii of about 4.0 Å and 5.3 Å for HCO_3^- and $CO_3^=$ respectively (Anaki, Ito and Oscarsson, 1961; Ito, Kostyuk and Oshima, 1962). They interact with the still larger $H_2PO_4^-$ and $HPO_4^=$ ions of the buffer: the last of these has been observed to penetrate the microcapsular membrane with great difficulty, causing crenation that lasts for hours (p.100). Thus it seems likely that the low permeability of the membrane for these anions is the chief factor accounting for the slower action of encapsulated urease as compared with free urease; but the point cannot be regarded as settled. It may be possible to throw some light on the matter by varying the conditions of the enzyme assay: thus buffer anions of different permeance could be used, or indicators could be incorporated into the microcapsules to detect a possibly higher pH in the internal phase during the production of ammonia, or the microcapsule membrane could be made of sulphonatdd Nylon with a still lower anionic permeability.

IV. IN VIVO PROPERTIES OF MICROCAPSULES

IN VIVO PROPERTIES OF MICROCAPSULES

TOXICITY AND FATE OF INJECTED MICROCAPSULES

Introduction:

A number of possible in vivo applications of microcapsules have been discussed in the Introductory section of this thesis. None of these applications would be possible without a knowledge of the toxicity and fate of injected microcapsules. These points have been investigated and the results are reported below, with special attention to the fate of intraperitoneally and intravenously injected microcapsules. The fate of intraperitoneally injected microcapsules was studied because in the experiments on the in vivo action of microencapsulated urease, the microcapsules were to be injected intraperitoneally, and a knowledge of their fate would assist in the interpretation of the results. The fate of intravenously injected microcapsules was studied because originally it was hoped that microcapsules might survive in the circulation: when this hope was disappointed, in the case of Nylon microcapsules, an attempt was made to find what happend to intravenously injected microcapsules, and what factors determined their survival in the circulation. In relation to the last point, special attention was paid to their surface charge.

Toxicity and tissue reactions:

Introduction

Suspensions of washed microcapsules are likely to be contaminated by traces of residual hexamethylenediamine and Tween 20 in the case of Nylon microcapsules, or by traces of Tween 20 in the case of collodion microcapsules. In addition, the microcapsules themselves might embolize small blood or lymph vessels or might produce a foreign-body reaction on tissues close to the site of injection. Finally, any outward leakage of protein from the microcapsule interior might sensitize the animal with untoward results if the injection was repeated.

As to the possible contaminating materials, both hexamethylenediamine and Tween 20 are rather nontoxic materials: Tween 20 indeed has been added in considerable quantities to ice-cream and other foods for many years without giving rise to any difficulty (Krantz, 1948), and hexamethylenediamine has not been a source of trouble to workers in the manufacture of Nylon. Both substances are weak histamine liberators when injected intravenously (MacIntosh and Paton, 1947; Krantz, 1948), but from the data in the literature, no significant effect of this sort would be expected from the traces likely to be present in the suspensions of washed microcapsules.

The reaction of tissues to implanted solid polymers varies with the material. Teflon and Silicone rubber (Mullison, 1964) are extremely inert even in the form of sheets and blocks, whereas Nylon in these forms causes some inflammation followed by fibrosis. The physical form of the polymer is important since, curiously enough, many plastics that produce

a tissue reaction when implanted as sheets or blocks do not do so when implanted as granules or powder (Oppenheimer, Oppenheimer, Danishefsky, Stout and Eirlick, 1955): Nylon, for instance, though it causes some tissue reactions in the larger forms, is nearly inert when used as suture material (Nichols, 1940; Aries, 1941). Teflon and silicone rubber though extremely inert are not semipermeable to water or aqueous solutes and, in addition, Teflon polymerizes only with the aid of high temperature and pressure which would denature protein and other biological material. Collodion and other cellulose derivatives notoriously produce a strong tissue reaction on implantation. For these reasons, Nylon, which is semipermeable and evokes little tissue reaction, has been used for my *in vivo* studies with microcapsules.

Results

A 10% suspension of Nylon microcapsules (mean diameter about 20 μ) was injected intraperitoneally into three mice weighing about 20 grams, the dosage being 6.3, 12.5, 25.0 ml/kg. Exactly similar tests were carried out with suspensions of collodion microcapsules and cross-linked haemoglobin microcapsules, and, as a control, saline on three other mice. No adverse reaction was observed in any of the mice immediately or later. A week after the injections, the animals were killed, their peritoneal cavities were washed out with saline, and the washings were examined microscopically. A strong leucocytic reaction was found in each of the mice that had received collodion microcapsules, but only a minimal reaction in the mice that had received Nylon or cross-linked protein microcapsules. Subcutaneous (2.5 ml/kg) and intramuscular (2.5 ml/kg)

injection of the Nylon microcapsule suspension produced no adverse effect, and no gross tissue reaction at the injection site could be detected a month later. Intravenous injection of up to 7.5 ml/kg of a 10% suspension of collodion microcapsules also had no adverse effect; but this volume of a more concentrated or a larger volume of the same suspension was quickly lethal: the exact cause of death was not determined. With Nylon microcapsules, up to 7.5 ml/kg of a 10% suspension have been given by vein to mice without perceptible ill-effect.

In addition to these toxicity tests on mice, incidental observations have been made also on rats, cats and dogs, as will be noted later. In these species also, the tissue reaction to intra-peritoneally injected microcapsules was minimal, but in anaesthetized cats and dogs the arterial pressure was lowered by even quite small amounts (< 0.5 ml/kg) of a 10% suspension of Nylon microcapsules (mean diameter 5 μ); with this smaller doses, the depressor effect was transient, but with larger doses (e.g. > 5 ml/kg) the blood pressure fell rapidly to zero and the animals died - presumably of pulmonary embolism.

Intraperitoneal injection:

Introduction

Von Recklinghausen (1862) was the first to study the absorption of particulate material from the peritoneal cavity. On the basis of his microscopic observations of the structure of diaphragm and the removal of particles by the intact diaphragm, he presented the concept of preformed openings between the peritoneal cavity and the diaphragmatic lymphatics. By 1926, when Cunningham reviewed the physiology of serous membranes, Von Recklinghausen's concept had been replaced by MacCallum 's (1903) concept that the openings between the endothelial cells are only potential openings: MacCallum suggested that the pumping action of the diaphragm causes the cells lining the peritoneal surface of the diaphragm to separate, allowing particles to pass easily between them. Cunningham suggested a third possibility, on the basis of his own experiments in which he found that some of the injected material had entered the endothelial cells; he thought that particulate matter absorbed from the abdominal cavity might have to pass through the cytoplasm of these cells.

The next review on the physiology of serous membranes was not published until thirty years later. The authors, Courtice and Simmonds (1954), discussed the absorption of particles from the peritoneal cavity under four headings.

(1) Site of absorption. It was generally agreed that a variety of intraperitoneally injected materials - graphite, colloidal silver, frog and chicken red cells, mammalian red cells and radioactive glass spheres

and thorotrast - are absorbed mainly through the peritoneal surface of the diaphragm (especially its right half), and that absorption by the rest of the parietal peritoneum and the mesenteric fold is much less significant. The omentum appears to affect absorption mainly by elaborating a coagulating fluid which tends to fix the particles, after which, if they are not removed through the diaphragmatic lymphatics, they are ingested or surrounded by phagocytes, and may then persist in this state for many months, especially in the greater omentum.

(2) Transfer of material to the site of absorption. It is remarkable that the sticky particulate matters fixed by the omental fluid drift to the under surface of the diaphragm and become absorbed there, even when they are denser than the lymph. The mechanism underlying this directional drift is not known, though it has been suggested that the movements of diaphragm and intestine may be involved in it. The removal of particles is slower in the upright posture, or in paralysis of the diaphragm.

(3) Passage of material into the lymphatics. Cunningham's idea of absorption through the cytoplasm of endothelial cells has not been substantiated: the evidence is in favour of penetration through gaps in the endothelial and connective tissue layers and through fenestrations of the basement membrane over these gaps. As the diaphragm is stretched during inspiration, reflux from the diaphragmatic lymphatics is prevented by valves but outflow of lymph towards the venous system is increased

during expiration; the diminished stretch of the diaphragm results in a decreased pressure within the lacunae, and particles are aspirated through the openings.

(4) Effect of particle size. Red blood cells of different mammals (diameter 7-8 μ) are readily reabsorbed and collected from the right lymph duct (little or none from the thoracic duct) in rats, guinea-pigs and rabbits, but frog erythrocytes (diameter 11-12 μ) treated with fixative do not enter the lymphatic system.

Stern (1963), using Cr^{51}-labelled sheep erythrocytes injected intraperitoneally into mice, found that their uptake was retarded by the addition of a polyanionic substance, polyglutamic acid, but not by a polybasic substance, polylysine. He suggested that surface charge might be a factor affecting the rate of removal of particulate matter. In Stern's and other workers' experiments, the absorption of Cr^{51}-labelled erythrocytes was conveniently followed by measuring the accumulation of the labelled isotope in liver and spleen, I used a similar technique to investigate the fate of injected Nylon microcapsules. These were prepared with a content of Cr^{51}-labelled hemolysate from human erythrocytes as described on p. 183 . This series of intraperitoneal injection of microcapsules into 18 rats was carried out with the assistance of a fourth year honours physiology student, Mr. Oscar Ransome.

Method

The microcapsules were prepared as described on p. 68 (with Span 85 at 1% v/v concentration and Jumbo speed setting of 2.5) to have

a mean diameter of about 29 μ. After washing to remove Twen 20, they were immersed in a saline solution containing 1 μc/ml of $Na_2Cr^{51}O_4$ at 37 C for 3 hours, washed three times to remove excess label, and kept in the refrigerator as a 25% suspension in saline. The supernatant from this suspension was found on the next day to have negligible radioacticity, whereas the microcapsules themselves were strongly labelled. This technique was adopted in preference to labelling of the haemoglobin before encapsulation, in order to minimize the risk of contaminating apparatus and vessels with the isotope. The CrO_4 anion, with a hydrated ionic radius of about 3.5 Å, appeared to have penetrated the microcapsules, since the degree of labelling was adequate for the experiment to be performed (it may be noted that erythrocytes, with an effective pore radius of only 4.2 Å, can be tagged by Cr^{51} by contact with $NaCr^{51}O_4$ solution for one hour).

For the experiments, 18 healthy male rats of average weight 130 grams were divided into six groups of three rats each. The animals of five groups were each given 1 ml intraperitoneally of the 25% suspension of labelled microcapsules: as a control, each rat of the 6th group received 1 ml of the supernatant from the suspension. Before the injection, the animals had been anaesthetized with intraperitoneal sodium pentobarbital (Abbott, 25 mg/kg): this was done because preliminary tests showed that in the absence of anaesthesia there was a risk that part of the injection in the struggling animals might enter the anterior or posterior abdominal wall or even the spleen. At weekly intervals, the animals of one group were examined and weighed and anaesthetized as before with sodium pento-

barbital. A 1 ml blood sample was obtained by cardiac puncture; the chest was then opened and both lungs were removed; 2 ml of saline was injected intraperitoneally; the abdomen was massaged gently and then opened; and 1 ml of the peritoneal fluid was removed. The abdomen was inspected for gross evidence of inflammation or fibrosis; all visible microcapsules or aggregates of microcapsules adhering to the omentum or abdominal wall were picked up with forceps and placed in a separate test tube; lastly, the liver, any visible lymph nodes and the spleen were removed and placed in separate tubes. The radioactivity of weighed aliquots of blood sample, lung tissue, peritoneal washing fluid, microcapsules removed, the liver, spleen and lymph nodes were estimated.

Results

All the animals injected intraperitoneally with Nylon microcapsules or control supernatant remained active and healthy, without significant change of weight, abdominal tenderness or rigidity, or alteration of bowel movements. On opening the abdomen, there was no sign of inflammation in any abdominal structure, except for one case in which by accident all the microcapsules had been injected into a small pocket of omentum: there was some local inflammation and fibrosis around this collection of undispersed microcapsules. In all the animals except the one just mentioned, larger individual Nylon microcapsules and small aggregates of Nylon microcapsules could be seen sticking to the abdominal wall or omentum: they were not attached to these structures, since they could easily be picked up with fine forceps, leaving no signs of fibrosis or inflammation at the sites.

After the first week, the microcapsules were well dispersed over the whole peritoneal cavity, but at the second week and thereafter there was some tendency for them to be found in larger numbers in the upper part of the peritoneal cavity, where many of them adhered to the upper surface of the liver and spleen just below the diaphragm; none were observed on the peritoneal surface of the diaphragm. No significant radioactivity was detected in lung, liver, spleen, lymph nodes, blood or saline wash fluid. Most of the recovered radioactivity was associated with the microcapsules or small aggregates of microcapsules which adhered loosely to the abdominal structures.

Intravenous injection:

Introduction

Much work has been done on the fate of intravenously injected foreign particles since Wyssokowitsch's first studies on the removal of micro-organisms from the circulation in 1886. Halpern and his colleagues, whose work contributed importantly in this field, have summarized the results they obtained up to 1958 in their "Physiopathology of the Reticulo-endothelial System". They injected many kinds of insoluble foreign particles - e.g. carbon (250 Å), saccharated iron oxide (25 Å), chromium phosphate ($\overline{P^{32}}$), pigeon erythrocytes, and heat-coagulated plasma proteins (I^{131}) - into different species of animals (rabbit, guinea-pig, rat, mouse). They found that particles of all these types were removed from the circulation at a rate that declined exponentially with time, and defined a clearance constant K as $C = Co\ 10^{-Kt}$, where C was the concentration at time t after injection and Co was the initial concentration. The value of K, in the experiments of the Halpern group, depended on a number of factors. It varied in different animals, decreasing in the order mouse > rat > guinea-pig > rabbit. K decreased also in the following conditions: increased dosage of injected particles, reduction of portal blood flow (as after total removal of intestine or partial hepatectomy), administration of cortisone or adrenaline, or when the particles were administered in gelatin solution. In the latter condition it was found that the clearance of chromium phosphate suspended in gelatin solution diminished with increasing gelatin concentration over the range of 1% to 5% w/v, and the possibility was suggested that this might have been related to an effect of gelatin on the surface charge

of the particles. Clearance was also shown to depend on the age of the animal, increasing in rats from 3 weeks and then gradually decreasing: this was ascribed to changes in the activity rather than merely the size of the reticuloendothelial system. Clearance was not affected by thyroidectomy, castration, adrenalectomy, hypophysectomy or administration of testosterone.

Robson (1958), using $CrP^{32}O_4$, studied other factors that affected clearance: on some points his results were different from those of Halpern et al. Thus he found that in different animals, K decreased in the series mouse > chicken > rabbit > rat > dog > man; and he related this experimentally to the liver: body-weight ratio, which diminished in the same order in these species. Robson also found that there were two phases of disappearance, both exponential, an early rapid phase and a slower tail, and he attributed the first phase to uptake of the larger particles and the second phase to uptake of the smaller particles. Most particles smaller than 0.03 μ, according to Robson, were removed by the liver and spleen: but particles larger than 0.3 μ were temporarily retained to a great extent by the lungs, through a process other than phagocytosis.

In the above experiments, there was a great size dispersion of the injected particles, and this made the interpretation of the results much more difficult. A later group of workers (Schoenberg, Gilman, Mumaw and Moore, 1961) overcame this difficulty by injecting polystyrene latex particles (PLP), which were very uniform in diameter in any one batch, but were available in batches of different diameters

(0.514, 0.557 and 1.171 μ). Their experiments were made with albino rabbits, and they found no significant difference in clearance associated with either diameter or dosage. They found that most of the particles were inside the reticuloendothelial cells of the liver and spleen; some were present in the lung, and these were in the capillary spaces rather than in the cytoplasm of cells; occasionally polystyrene latex beads were observed in the circulating leucocytes. Even with this homodisperse material, the disappearance curves showed a distinct tail, which was thought to be due to temporary trapping of polystyrene latex by lungs or to prolonged circulation of polystyrene latex captured by leucocytes.

Ring, Blum and Kurbatov (1961) also used uniform polystyrene particles in their experiments; but they extended the investigation to polystyrene latex beads of greater diameter, injecting them into the pulmonary artery and collecting them from the pulmonary vein after one passage. The recovery of the microspheres under these conditions was as follows: 6% for 8 μ diameter particles, 50% for 2.8 - 4 μ diameter particles, 94 - 100% (expiratory phase) and 72 - 83% (inspiratory phase) for 1.4 - 2.0 μ diameter particles.

Other workers have studied the removal of intravenously injected lipid emulsions from the blood stream. Byers (1960) reviewed the subject and described the removal of chylomicrons derived from the diet as being very similar to the removal of foreign particles. Again the blood levels fell exponentially and most of the lipids were taken up by the phagocytic cells of the liver. Waddell, Geyer, Saslaw and Stare (1953) used emulsions of less than 1 μ diameter prepared from different types of

lipids (phosphatide, cerebroside, coconut oil, olive oil, cottonseed oil, cocoa butter, linseed oil and mineral oil) and found that the half-time of disappearance after intravenous injection ranged from 120 — 200 minutes, and that the pH of the various preparations, ranging between 4.35 and 7.02, did not correlate significantly with the clearance. When, however, they used Triton, an anionic emulsifying agent, to disperse the emulsion, the half-time was extended to more than 500 minutes, and they suggested that the surface charge might have something to do with survival of the lipid particles in circulation. It may be pointed out that Seaman and Swank (1963) found that the electrophoretic mobilities of lipid emulsions suspended in saline were related to their composition; but that if such emulsions of different pH were suspended in plasma, their electrophoretic mobilities were similar in all cases. Seaman et al. offered the very reasonable explanation that the surface charge, and therefore the electrophoretic mobilities, of these particles were determined by their coating of absorbed protein rather than by the charged groups present in the original lipid emulsion.

From all these results, and from others that might have been quoted, a few conclusions can be drawn with at least moderate confidence. The ability of foreign particles to remain in the circulation depends in the first place on their diameter (and presumably also on their deformability); but below a certain range of diameter - roughly 1 to 2 μ - the effect of diameter is not the significant factor. Particles larger than the critical size pass with difficulty through some capillaries, especially the pulmonary capillaries, in which they may be retained for a greater or lesser period. Smaller particles, and also larger particles

that have escaped capillary trapping, are liable to be captured by the cells of the reticuloendothelial system, especially those in the liver and spleen, and to a minor extent by circulating leucocytes. The likelihood of capture appears to depend on the effective surface charge of the particle, and this in turn is determined largely by the properties of plasma protein (or of such adjuvants as gelatin or Triton added as emulsifiers) which may be present in high concentration at the particle surface.

At this point it may be appropriate to refer to a familiar fact which in this context has been curiously disregarded, namely that the formed elements of blood circulate for a very long time. The question may be asked how far their ability to do so can be accounted for by their size, deformability and effective surface charge.

Erythrocyte diameter in most mammals is in the range of 6 - 8 μ, much larger than the upper limit for the free passage of foreign particles through the pulmonary circulation: as has just been noted, 50% of polystyrene microspheres of 2.8 - 4 μ diameter were retained in the pulmonary capillaries during a single passage of blood through the lung of dog (Ring et al., 1961). In this regard it is interesting to note that erythrocytes have membranes which, though not elastic, are plastic (Harris, 1964) and very deformable, as Rand and Burton (1964) have demonstrated by very elegant microtechniques. Rand and Burton were able to measure the "stiffness" of biconcave human erythrocytes, and to assign a value for this quantity of only 0.019 ± 0.002 dynes/cm, and their micromanipulation tests indicated that the membrane could be

grossly bent without rupture, though it had little resistance to stretching. Because of this high degree of flexibility, the erythrocytes can pass through Millipore filters with a pore diameter of 5 μ (Jandl, Simons and Castle, 1961); and correspondingly, erythrocytes passing through the microcirculation can be observed to undergo deformation in the form of "a flow parabola (parachute head) ..... plug flow, resembling the fit of a dart in a blowgun" (Wells, 1964). That this deformability is important for the free passage of erythrocytes through capillaries is illustrated by the behaviour of sickle-cell erythrocytes, which, when the oxygen tension is low, contain crystalline haemoglobin, and consequently have an irregular spiculated shape and a high internal viscosity. Such rigid erythrocytes cannot pass through 5 μ Millipore filter (Jandl *et al.*, 1961), and although they can pass through the microcirculation when not in the sickled form they are notorious for blocking the capillaries when in the sickled state.

The other formed elements of the blood also have characteristics that enable them to squeeze easily through fine capillaries. The ability of leucocytes to change their shape, even passing by diapedesis through the interstices between closely-packed cells of the capillary endothelium, has been known for generations. The platelets, with a diameter of 2 - 4 μ, are smaller than some capillaries, and presumably can pass through finer vessels because of their discoid shape and deformability.

Another physical factor which may well be related to the ability of the blood cells to survive in the circulation is their negative surface charge. In 1941, Kozawa, by adding acid to mammalian erythrocyte

suspension, observed that the cells of each species had a characteristic isoelectric point within the range pH 3.5 to 4.7. The surface charge of blood cells can, however, be more conveniently studied on the basis of their electrophoretic mobility. The first quantitative measurements of the electrophoretic mobility of erythrocytes were made by Abramson (1934), with the following results: man, 1.31 μ/second; dog, 1.65 μ/second; cat, 1,39 μ/second; rat, 1.45 μ/second; mouse, 1.40 μ/second; monkey, 1.33 μ/second and rabbit, 0.55 μ/second.* Abramson found that the other formed elements of blood were also negatively charged: in the horse, the electrophoretic mobility of both platelets and polymorphonuclear leucocytes was 0.49 μ/second. Abramson suggested that the negative charge of the leucocytes might explain their migration into injured tissue, but he did not consider a possible relationship between surface charge and survival in the circulation. Ponder and Furchgott (1941) repeated the electrophoretic studies, and suggested that the erythrocyte surface is dominated by strongly acidic groups such as those of phosphoric acid.

The situation was greatly clarified by Gottschalk's (1957) finding that the combination of certain viruses with the erythrocyte depends on the presence in the membrane of a strongly negatively-charged mucopolysaccharide, the terminal grouping of which he was able to isolate and characterize as N-acetylneuraminic acid. Later Gottschalk (1960) found that the oxo-group of N-acetylneuraminic acid forms a glycosidic link with D-galactosamine, which in turn is linked to a trypsin-susceptible part of a mucoprotein. An enzyme, neuraminidase, found in

* Above values refer to a voltage gradient of 1 volt/cm.

viruses and bacteria, could split neuraminic acid off from the red cell membrane and so remove the "virus receptors". Cook, Heard and Seaman (1961) demonstrated a relationship between N-acetylneuraminic acid and erythrocyte surface charge: they found that most of the surface charge was lost after treatment with neuraminidase; that the effect was not due merely to absorption of the enzyme; and that the change in electrophoretic mobility could be related to the amount of N-acetylneuraminic acid released by the enzyme. The identity of the groups responsible for the negative surface charge of platelets and leucocytes has not been established, although platelets are known to contain a sulphated polysaccharide (cf. Wintrobe, 1961). Very recently Rambourg, Neutra and Leblond (1965), in still unpublished work at McGill University, have demonstrated histochemically the presence of a well-marked carbohydrate coating on mammalian erythrocytes and leucocytes.

An observation possibly relevant to the physiological significance of the surface charge of the blood formed elements was made by Hirschboeck (1940), who found delayed coagulation and absence of clot retraction in blood placed in a collodion-lined container, although this surface is not much less wettable by water or by blood than a glass surface. (Collodion, as has been noted in the section on "Artificial semipermeable membranes" (p. 25), contains acidic groups and is thus negatively charged.) It is interesting in this regard that clot retraction is a function of the platelets (cf. Wintrobe, 1961), which are also negatively charged. Lovelock and Porterfield (1951) prepared tubes lined with sulphonated polystyrene, and found that though they

had a water-wettable surface, they delayed the coagulation of blood even more effectively than tubes of unsulphonated polystyrene; and they attributed the superiority of the sulphonated surface to its negative charge. Sawyer and Pate (1953) showed that if the normal surface negativity of the arterial intima was reversed by the passage of an electric current, blood cells and platelets adhered to the positively-charged area. Since then a considerable literature has grown up relating the surface charge of blood cells and vessels to various physiological and pathological phenomena. For instance, Sachtleben and Ruhenstroth-Bauer (1961) found that erythrocytes and bacteria showed a reduction in surface charge after treatment with antibodies and other agglutinating agents; and Davies and his colleagues (Davies, 1958; Davies and Clark, 1961) reported that the electrophoretic mobility of erythrocytes was diminished in myocardial infarction, and suggested that such a reduction of surface charge would favour their adhesion to one another or to blood-vessel walls.

The fragments of information discussed above, when placed together, seem to suggest that the surface negativity of the formed elements of the blood may be important for their survival in the circulation.

The Nylon microcapsules whose fate after intraperitoneal injection has already been discussed were also injected intravenously; and as has been pointed out, they were not well tolerated unless given in low dosage; and, as will be recorded later (p.210), they were also found to be rapidly removed from the circulation. The possibility was considered that their lack of surface negative charge might have been

one of the factors that prevented them from remaining for more than a short time in the blood stream. This idea suggested that it would be useful to test microcapsules whose membranes carried a negative charge, in order to determine whether or not they would remain longer in the circulation than ordinary Nylon microcapsules. Before attempting to prepare negatively charged microcapsules, I thought it would be of interest to test the survival of more readily available particles of varying surface charge.

The first possibility that suggested itself was to compare the survival in circulation of uncharged polystyrene microspheres, which as already noted (p.188), are available in very uniform diameter in the range 1 - 8 μ, with that of negatively charged microspheres of the same diameter. Sollner and other workers had already shown that membranes could be given a strong negative charge by coating them with sulphonated polystyrene, and it was anticipated - correctly, as it turned out - that uniform particles of polystyrene latex might be sulphonated to give them a negative surface charge.

The second possibility is one that must be described in greater detail. This is that neuraminidase-treated erythrocytes, which have lost most of their surface negativity, would behave like foreign particles in the circulation, and would be rapidly removed, especially by the reticuloendothylial system. The only report that could be found with a bearing on this point was one by French and Ada (1953). These workers infused guinea-pigs with homologous neuraminidase-treated erythrocytes, and found that whereas half-an-hour after the transfusion

13% of the circulating erythrocytes were "slow cells" with low electrophoretic mobility, 24 hours later the figure had dropped to 8%, and 72 hours later only cells of normal mobility could be found. They observed that neuraminidase-treated erythrocytes showed increased osmotic fragility in vitro; but since urine urobilinogen was not increased, they concluded that the enzyme-treated cells were not destroyed more rapidly than normal cells, but rather that the disappearance of the "slow cells" was due to the repair of their surfaces by "some constituents of the mammalian red cells undergoing rapid metabolism". The findings that have already been presented suggest, however, another interpretation of the results of French and Ada: namely that the "slow cells" disappeared because their reduced surface negativity permitted them to be removed from the circulation. Since the technique of Cr^{51}-labelling is now well established as a means for studying erythrocyte life-time, it was decided to use this technique to reinvestigate the fate of intravenously injected neuraminidase-treated erythrocytes. The hypothesis was even entertained that the normal lifetime of circulating erythrocytes might be a function of their ability to retain their original surface charge. In this connection, the report of Danon and Marikovsky (1961) was thought to be significant. These workers, taking account of the well-established fact that young erythrocytes, being lipid-rich, have a lower density than aged erythrocytes, determined the surface charge of erythrocytes separated by centrifugation into younger and older fractions, and found that the younger cells were about 30% more strongly charged than the older ones. On this basis it seemed possible that aging erythrocytes gradually lose their neuraminic acid, until the

time is reached when their surface negativity is too low to prevent their removal from the circulation. It therefore seemed appropriate to use the Cr^{51} method to determine whether neuraminidase-treated erythrocytes, reinjected into the donor animal, would disappear more rapidly than erythrocytes similarly handled, but without exposure to the enzyme.

Methods

(1) Fate of intravenously injected polystyrene microspheres : The experiments were carried out on cats weighing about 1.5 kg. They were first anaesthetized with ethyl chloride followed by ether; and as soon as possible, chloralose (80 mg/kg) was given intravenously and the volatile anaesthetic was withdrawn. One femoral vein was cannulated for infusion, and the opposite femoral artery was used for the collection of blood samples. Polystyrene latex (Dow Chemical Co.) containing particles of 2.05 ± 0.018 μ (mean diameter and standard deviation) was diluted 1:100 in saline, and the particle count of the dilute suspension was determined by hemocytometry. A similar suspension of sulphonated polystyrene particles was prepared by a method similar to that described by Neihof (1954) for the preparation of soluble sulphonated polystyrene, involving treatment with Ag_2SO_4 and H_2SO_4; but in the case of the latex the treatment was done in a Virtis homogeniser at a speed of "1"; and instead of being continued for 1-3 hours was stopped after 30 seconds by quenching the stirred suspension with water. Then the sulphonated microspheres were quickly collected on a Millipore filter, washed free of acid, resuspended in saline, and counted as before with a hemocytometer. (It should be noted

that the reaction time must be brief, so that only the surface of the microspheres is sulphonated; prolonged reaction would result in the sulphonation of much of the polystyrene, which would then become soluble in water.) The blood volume of the cats was estimated as 70 ml/kg of body weight, and the volume of suspension injected was calculated to give a particle count of about 250/mm^3 after mixing in the circulation, provided uptake and trapping were absent. Arterial samples were collected at half-minute intervals for the first 2 1/2 minutes and thereafter at intervals of 1 and 5 minutes. In another set of experiments, the animals were eviscerated to cut down the portal circulation, and simultaneous blood samples were collected from the right and left ventricles in order to determine whether the microspheres could be trapped in the pulmonary circulation. In still another experiment, the uncharged polystyrene latex was suspended in an 0.25% v/v solution of the anionic emsulsifying agent "Tergitol" (Union Carbide Chemical Co.), and arterial samples were collected at intervals after the intravenous injection. As a control, a similar suspension of microspheres was administered in 1% Tween 20: this, as has already been noted, is an non-ionic detergent.

(2) Intravenously injected neuraminidase-treated and untreated erythrocytes: These experiments were carried out on unanaesthetized dogs weighing about 10 kg. 3-ml samples of washed erythrocytes from heparinized venous blood of the animal were treated with neuraminidase (L. Light & Co. Ltd.) by the procedure of Cook et al. (1961), washed once with saline, tagged with Cr^{51} according to the method described by Chien and Gregersen (1962), and washed 3 times with saline. They

were injected intravenously as a 50% suspension in saline. Control samples of erythrocytes were treated in the same way, except that the neuraminidase solution was replaced by the solvent alone. The electrophoretic mobility of both erythrocyte samples was measured as described in p. 107. Alternate injections were made, at appropriate intervals, of the control and enzyme-treated erythrocytes, the dose in each case being 2.5 ml of the 50% suspension. At intervals after the injection, venous samples were obtained from the opposite leg, and their radioactivity was measured.

(3) Intravenously injected Nylon and sulphonated-Nylon microcapsules: These experiments were carried out on cats, anaesthetized with chloralose as already described: the arrangements for injecting the suspension and obtaining the arterial samples were the same as before. The preparation of the Nylon and sulphonated-Nylon microcapsules has already been described (p. 68): mean diameter in both cases was close to 5 μ. The survival of the injected microcapsules was determined either (1) by direct counting of blood samples hemolyzed with dilute acetic acid and stained with Wright's stain (the stained leucocytes in the hemolyzed suspension were readily distinguished from the unstained microcapsules); or (2) by incorporating a small amount of I^{131} -labelled albumin into the hemolysate before microencapsulation; or (3) by labelling the hemolysate with Cr^{51} after encapsulation by the method already described (p.184). Method (2) was used for the microcapsules with uncharged Nylon membranes, and method (3) for the microcapsules with sulphonated Nylon membranes, (It would have been preferable to have used only one of these two

methods, but permission to use the more convenient Cr^{51} method was only obtained after the control tests with the uncharged microcapsules had been carried out. The quantity of I^{131}-labelled protein was, however, very small; and leakage of the label from the microcapsules was insignificant in both cases, so it was difficult to see how the use of the two methods could have invalidated the result.) A separate cat had to be used for these acute experiments, and the possibility of seasonal variations was not ruled out; moreover some of the experiments were incomplete, because of toxic effects produced by the microcapsule suspension. For these reasons, the results obtained in the comparative tests have to be treated with reserve, but it is thought that they are of sufficient interest to be worth reporting. At the end of each experiment, the lungs, liver, spleen and one kidney were removed and their radioactivity was determined.

Results

The arterial blood levels of the uncharged and charged microspheres are shown, plotted against time after injection, in Fig. 34 . With each type of microsphere, the results of the two tests were in good agreement. The theoretical blood levels after mixing are shown by the heavy horizontal line at the top of the graph: this was calculated on the assumption that if the microspheres circulated without restriction, they would be suspended in a blood volume corresponding to 70 ml/kg. On this basis, the expected microsphere count in all experiments was $240 \pm 25/mm^3$. With the negatively charged microspheres, the initial counts, at a time when mixing in the blood stream was probably not complete, were quite close to this figure; thereafter the number of

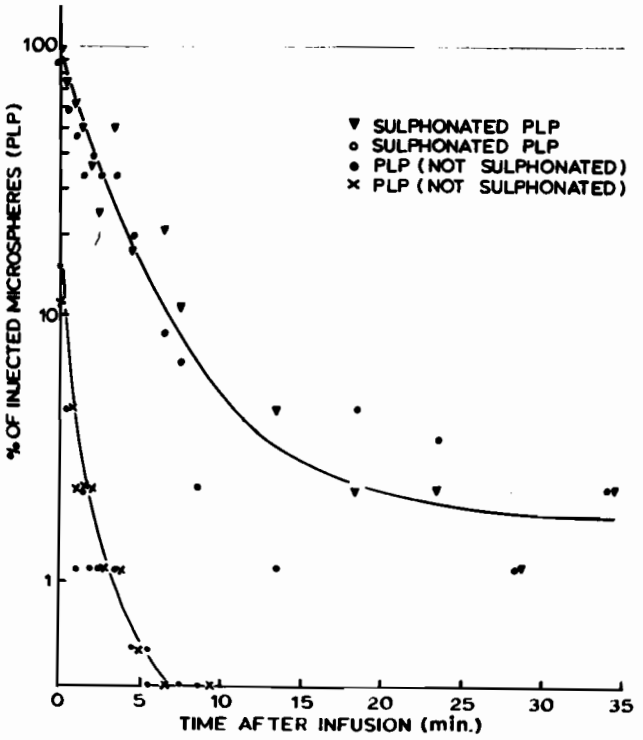


Fig. 34

Survival of polystyrene microspheres infused into cats. (Infusion took 30 seconds ending at zero time). Data from four experiments, two with untreated and two with sulphonated microspheres. Ordinates give arterial counts of microcapsules, as percentage of the count expected on the assumption that all injected microcapsules would be uniformly distributed in a blood volume of 70 ml/kg. (Note that ordinates in Figs. 34, 35, 36, 38 and 39 are plotted logarithmically.

microspheres declined, roughly exponentially at first, with a t 1/2 of about 1.6 minutes, and then apparently more slowly; but the later counts were subject to large errors because so few microspheres were left in the circulation. The uncharged microspheres behaved quite differently. Even in the first sample collected, half a minute after the injection, the arterial blood contained only 10 - 15% of the theoretical number of microspheres; and in the subsequent samples, the microsphere count fell with a much steeper slope than in the experiments with charged microspheres, the t 1/2 being no more than 15 - 30 seconds. No uncharged microspheres were found in the arterial blood later than 6 minutes after the injection.

The fate of these unlabelled microspheres could not be followed by tissue analysis, but some evidence was obtained to suggest that trapping occurred mainly in the abdominal viscera. In two experiments, the stomach, intestine and spleen were removed, occluding both arterial and portal blood supply to the liver; and serial blood samples were taken simultaneously from left and right ventricles after the intravenous injection of the microspheres. One cat received an injection of the charged microspheres, and one the uncharged microspheres. The results of these tests are shown in Fig. 35. While caution is appropriate in interpreting these limited data, some points seem to be well established. (1) Since the curves for right and left ventricles practically coincide in the case of the charged microspheres, these particles must have passed through the lung without significant trapping; but since the right ventricular curve fell consistently below

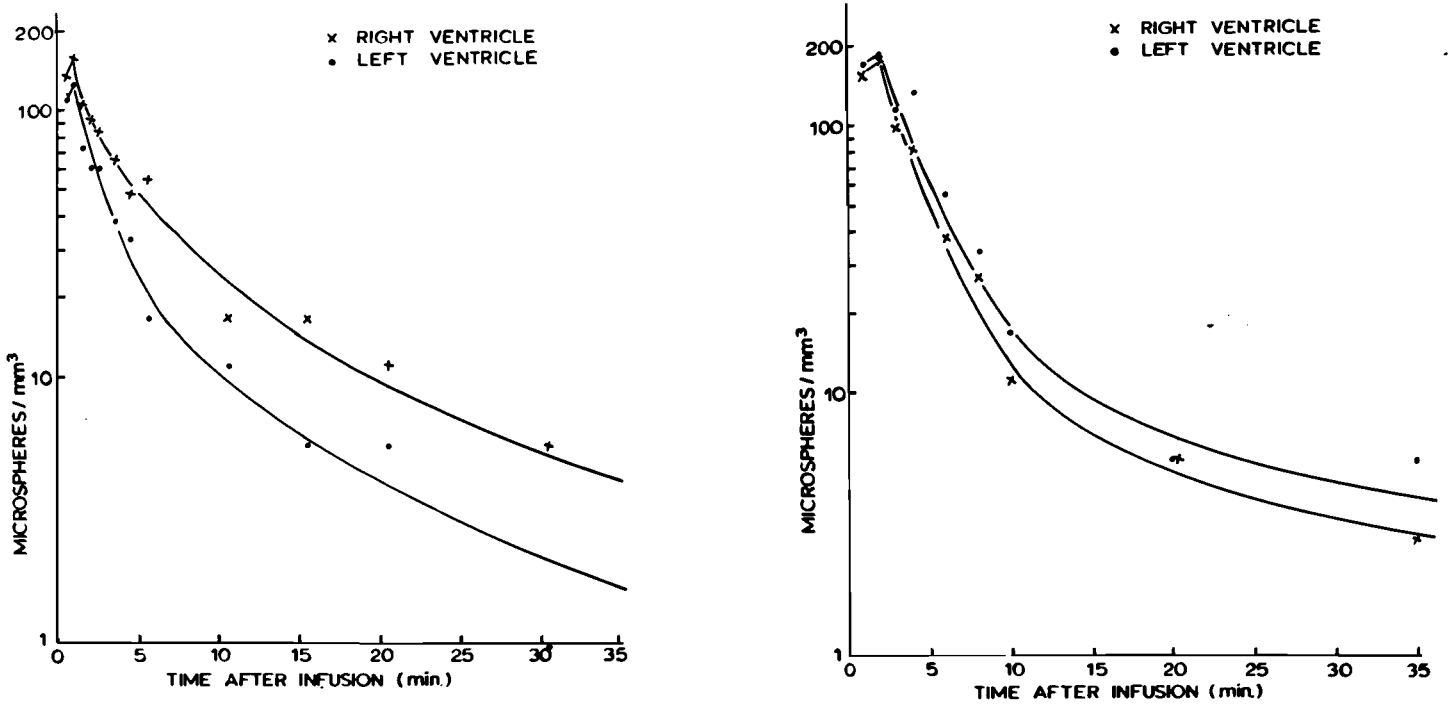


Fig. 35

Polystyrene microsphere counts in simultaneous blood samples from right and left ventricles after intravenous infusion in an eviscerated cat. Left: untreated microspheres; right: sulphonated microspheres.

the left ventricular curve in the case of the uncharged microspheres, these particles must have been trapped or delayed in the pulmonary vessels. (2) Since the slope of the left-ventricular (arterial) curve for the charged microspheres was only a little less steep (t 1/2 = 2.5 minutes) in the eviscerated animal than in the previous tests on the intact animal (t 1/2 = 1.6 minutes), the portal circulation can hardly account for the major part of the removal of these particles from the circulation. And (3) since the uncharged microspheres were removed much more slowly in the eviscerated animal (t 1/2 = 1.75 minutes) than in the intact animals (t 1/2 < 0.5 minutes), the portal circulation must have played a major role in trapping them. Thus it seems likely that uncharged particles in the 2 μ diameter range are trapped fairly efficiently (though perhaps only temporarily) by the lungs, and very efficiently (and permanently) by the extrapulmonary tissues, especially the portal circulation; while negatively charged particles of the same diameter are relatively immune from trapping by the lungs, and are trapped at a slower rate, but still quite efficiently, by the extrapulmonary tissues, including tissues other than the abdominal viscera.

The single experiment in which a comparison was made of the survival of microspheres suspended in an anionic versus a nonionic detergent gave similar results. These are shown in Fig. 36 , from which it may be seen that the anionic detergent, though added in lower concentration, was more successful in prolonging the circulation time (t 1/2 = 12 minutes) than the nonionic detergent (t 1/2 = 5.5 minutes). (The absolute half-time values should be disregarded, since the injected

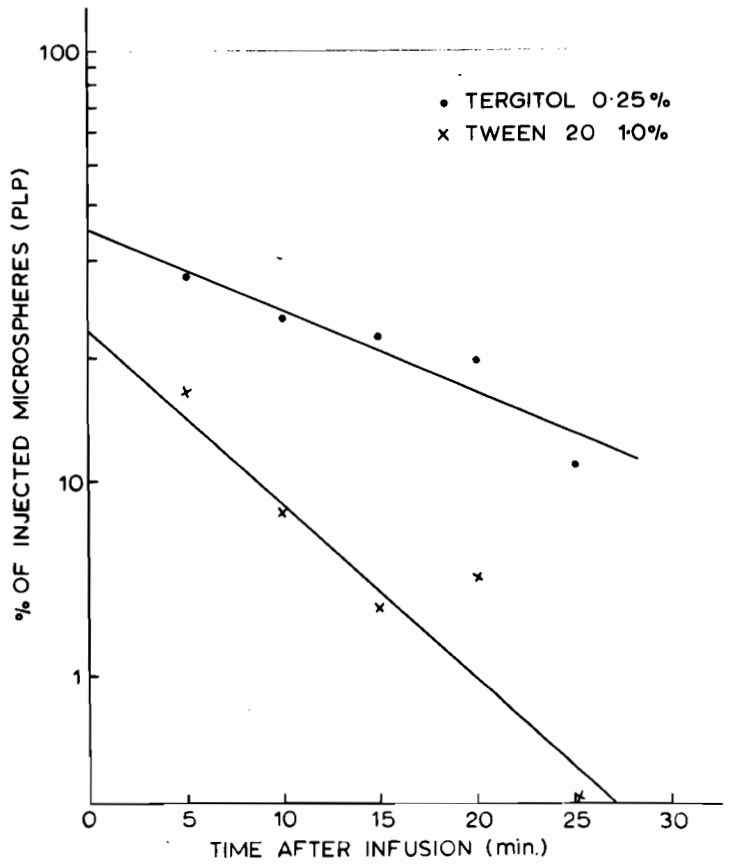


Fig. 36

Survival of polystyrene microspheres infused into a cat. Data from two tests: first with microspheres suspended in 1.0% Tween 20, and then with microspheres suspended in 0.25% Tergitol. Procedure as in Fig. 34, but dose of microcapsules was about 10 times larger.

volume of microspheres was not the same as in the preceding experiments.)

(2) Intravenously injected neuraminidase-treated and untreated erythrocytes: Experiments on the survival of labelled erythrocytes were carried out on four dogs according to the plan shown in Fig. 37 . The animals' own erythrocytes were injected, after tagging, on three occasions. On the first and last occasions, the erythrocytes were untreated except for the labelling procedure, while on the second occasion, they were exposed to neuraminidase as described under Methods. The arrangements for injecting the erythrocytes and obtaining and counting the blood samples were the same throughout, except that after the injection of the neuraminidase-treated cells, blood samples were withdrawn more frequently. The proportion of injected erythrocytes surviving at the time of sampling was recorded in each case as a percentage of the radioactivity of the first blood sample taken 15 minutes after the first injection of control (no enzyme treatment) erythrocytes.

All four experiments gave very similar results, as Fig. 37 shows. The control cells disappeared very slowly from the circulation: the duration of the experiment was too short to permit an accurate estimate of their survival, but the results were compatible with the usually stated lifetime of about 3 - 4 months for dog red cells (cf. Berlin, Waldman and Weismann, 1959). The neuraminidase-treated erythrocytes, on the other hand, behaved quite differently. The initial rise in blood radioactivity after they were injected was significantly less than in the case of control erythrocytes (although the first sample in this case was taken only 5 minutes after the injection), and thereafter the values

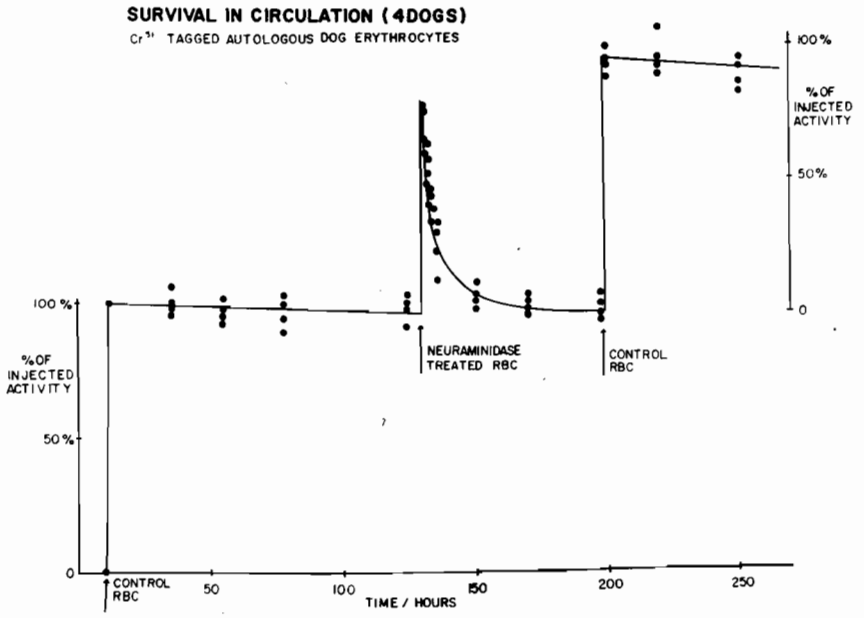


Fig. 37

Survival of reinfused autologous dog erythrocytes, tagged with Cr^{51}. Data from four dogs: each point represents the radioactivity of one blood sample from a limb vein. In each case the first and third tests were with normal erythrocytes and the second test was with neuraminidase treated erythrocytes. Ordinates give percent of injected label in circulation at time of sampling, assuming that blood volume, calculated as the dilution volume of the injected label in the first test, was the same throughout. Note rapid disappearance of neuraminidase-treated erythrocytes.

fell exponentially with a half-time of only about 2 hours. The response of each animal to the final injection of control erythrocytes was not significantly different from its response to the first injection.

It would have been of interest to learn the fate of the labelled enzyme-treated erythrocytes which disappeared so rapidly from the blood, but in these tests on conscious animals, it was not feasible to obtain tissue samples. It was noted, however, that whereas after injection of the control erythrocytes, all of the radioactivity was found in the cells and none in the plasma, after the injection of the enzyme-treated erythrocytes the plasma contained visible haemoglobin and had some radioactivity. Thus the enzyme-treated erythrocytes, besides being removed from the blood stream, must also have undergone some lysis; but whether this lysis occurred in the circulation as the result of mechanical stresses, or in the tissues after uptake of the cells, could not be determined from these experiments.

(3) Intravenously injected Nylon and sulphonated-Nylon microcapsules: The limited information I have been able to obtain at the time of writing shows that Nylon microcapsules are also removed very rapidly from the blood stream. Sulphonated-Nylon microcapsules are also removed rapidly, but they appear to survive somewhat longer than uncharged microcapsules of the same diameter, and to be trapped in a different way. Experiments on the fate of intravenously administered labelled microcapsules present the dilemma that large doses are toxic, obstructing the pulmonary circulation, whereas with small doses, the

diminishing radioactivity of the blood soon approaches background. Small numbers of microcapsules can be detected by hemocytometry of laked blood samples, but not by microscopic examination of tissues by the methods available at present. Of the considerable number of experiments undertaken to determine the fate of Nylon microcapsules in the circulation, the results of three seemed to be worth reporting. In these three experiments, the condition of the experimental animals remained satisfactory, and blood levels of the microcapsules could be followed by hemocytometry or by radioactivity measurements. In two of the experiments, the radioactivity of tissue samples provided some information about the tissue sites where microcapsules were removed from the circulation.

In the first such experiment, a suspension of uncharged microcapsules (mean diameter 5 μ) was infused by vein over 2 1/2 minutes, and arterial samples were obtained from the unanaesthetized cat during and following the infusion. At the end of the infusion, the arterial microcapsule count (Fig. 38) reached 20% of the theoretical level, calculated on the assumption that the microcapsules would be uniformly distributed in a blood volume of 70 ml/kg. Upon discontinuing the infusion, the arterial level fell rapidly, at first with a half-time of about 1.6 minutes and thereafter somewhat more slowly. This result made it obvious that though some of the microcapsules successfully completed one or more circulations, the majority were removed from the blood stream within a single circulation. The size spectrum of the surviving microcapsules was not determined, but the microscopic inspection showed, as might have been expected, that the smaller ones (diameter less

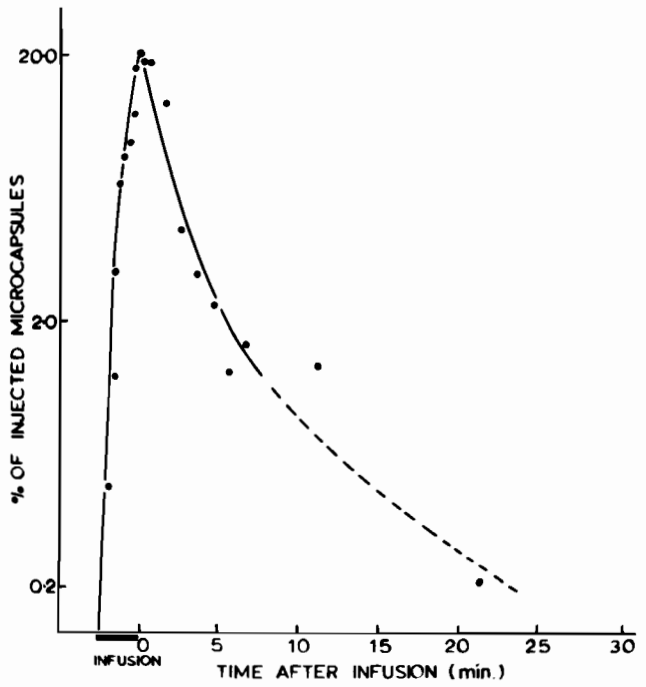


Fig. 38

Survival of unlabelled Nylon microcapsules (mean diameter 5 μ) after intravenous infusion into a cat, arterial sampling. Survival estimated as percentage of expected microcapsule count on the basis of uniform distribution of microcapsules in a blood volume of 70 ml/kg.

than 5 μ) survived longer than the larger ones.

Fig. 39 gives the result of two experiments with labelled microcapsules of the same mean diameter, one with Nylon and one with sulphonated Nylon as the membrane material. The ordinates give the radioactivity of the successive arterial samples, again calculated as a percentage of the theoretical level on the basis of uniform distribution in a blood volume of 70 ml/kg. Removal of the uncharged microcapsules was very rapid. At the end of the infusion, the peak level of radioactivity was only 8% of the theoretical value; thereafter the level declined approximately exponentially with a half-time of 36 seconds. This was much shorter than the half-time calculated for the previous experiment, in which the surviving microcapsules were counted in a hemocytometer; but the difference may not have been a real one, because, as already noted, a given number of smaller microcapsules (which have a longer survival) account for a much smaller amount of radioactivity than the same number of larger microcapsules: for this reason, the half-time obtained by hemocytometry of a size-disperse population of microcapsules cannot be fairly compared with the half-times obtained by radioactivity measurements. On the other hand, it seems fair to compare the two curves of Fig. [39], since both were obtained by radioactivity measurements on microcapsules that had a similar mean diameter and size distribution. The upper curve of Fig. 39 shows the disappearance of the sulphonated-Nylon microcapsules. Here, the peak value at the end of the infusion was 36% of theoretical, and the half-time was about 90 seconds, or 2.5 times that for the uncharged microcapsules. While only limited confidence can be placed

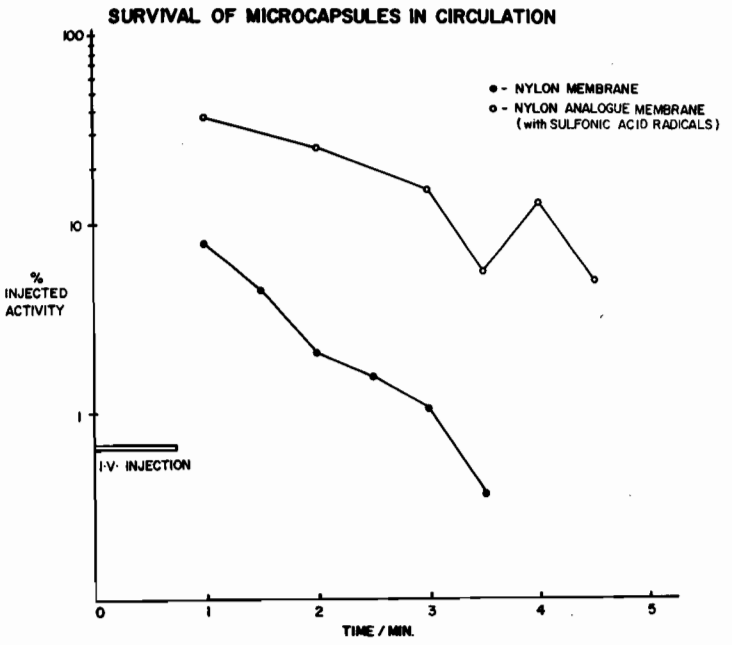


Fig. 39

Survival of labelled Nylon microcapsules (mean diameter 5 μ) after intravenous infusion into cats: arterial sampling. Note logarithmic ordinates. Above: sulphonated Nylon membrane microcapsules. Below: ordinary Nylon membrane microcapsules. Survival estimated as percentage of radioactivity expected on the basis of uniform distribution of the label in a blood volume of 70 ml/kg.

TABLE XV

Uptake of Intravenously Injected Microcapsules by Tissues

Organ	Percentage of injected label	
	Nylon microcapsules	Sulphonated-Nylon microcapsules
Lungs	65	6
Liver	21	11
Spleen	0.5	0.6
Kidney	1.0	0.9

in the result of a single comparison, it does seem highly probable that the negative charge of the sulphonated-Nylon microcapsules enabled them to circulate longer than the uncharged microcapsules.

At the end of the experiments of Fig. 39 the cats were killed by opening the chests, and aliquot samples of lung, liver, kidney and spleen were taken for radioactivity measurements. The results, given in Table XV , showed that in the case of the uncharged microcapsules about 2/3 of the label was trapped in the lung, with the liver as the next most important site; whereas in the case of the charged microcapsules there was little trapping in the lung, and though about 1/10 of the label was found in the liver, most of the microcapsules must have been removed by the carcass tissues which were not analysed.

Discussion

The rapid disappearance of both charged and uncharged microcapsules from the circulation was rather disappointing. The reasons why they were removed have not been completely analysed, but no doubt some importance should be attached to their relatively large size (in all the microcapsule suspensions the greater part of the bulk was accounted for by particles $> 3\,\mu$ in diameter), and probably also by the lower deformability of microcapsules as compared with the normal biconcave red cells. Nevertheless, it seems clear, both from the present results and from those quoted in the literature, that factors other than simple mechanical occlusion of the microcirculation must pay an important role in the removal of foreign particles from the blood stream. I have attempted to test the hypothesis that a negative surface charge, such as

that normally carried by the formed elements of the blood, may be one of the unidentified factors. This does indeed seem to be the case; but it also seems that surface negativity *per se* is not the only factor, and that its effect is primarily to protect circulating particles from trapping in the pulmonary circulation. I have found, in confirmation of Ring *et al.* (1961), that 2 μ polystyrene microspheres are mostly removed from the blood during a single passage through the lungs; but I can add the further finding that such microspheres when negatively charged are hardly trapped at all in the lung. An even more striking difference was seen with Nylon microcapsules: here again, the negatively charged ones were much less likely to be trapped in the lungs.

Nevertheless, both kinds of artificial particles - microspheres as well as microcapsules - had only a brief survival in the circulation, even when they possessed a surface negativity. The tissues responsible for both their removal are still largely unknown, though as expected, the data indicate that the liver is involved to some degree. The failure of the negatively charged microcapsules to survive cannot be attributed merely to a deficiency of fixed anionic groups, since the electrophoretic mobility of these microcapsules when suspended in saline suspnsion exceeded that of erythrocytes suspended in saline (see p.107). Nevertheless, it must be pointed out that the *effective* surface charge of a suspended particle depends very much on the nature of the fluid in which it is suspended, and in particular, that contact with protein can greatly modify the effective surface charge. For example, Seaman *et al.* observed that though lipid emulsions of varying pH had electrophoretic

mobilities in saline that corresponded to their pH, the same lipids emulsified in plasma had identical and much lower mobilities. Likewise, Mirkovitch and his colleagues (1964), measuring the surface charge (Zeta potential) of various solid surfaces, including polystyrene and sulphonated polystyrene, found that these surfaces had well-defined and individually different values of Zeta potential when tested against Ringer's solution, but had the same value, close to zero, when tested against blood. Both groups of workers suggested that the absorption of plasma protein at the interface was responsible for lowering the effective surface charge. Knisely's (1964) visual observations may also be cited: he found that soon after a foreign particle was injected into the blood stream, it acquired a visible gelatinous coating, and was then rapidly removed from the circulation by the phagocytic cells of the liver. All these findings suggest that the surface of blood cells differs from the surface of injected foreign particles in having the ability to retain an effective negative surface charge even in the presence of plasma protein. There is direct evidence that this is the case. Davies (1958) found that the electrophoretic mobility of human erythrocytes in their own plasma was nearly equal to that of the same erythrocytes in saline, except in the case of older or atherosclerotic subjects. Davies did not relate his findings to the ability of blood cells to survive in the circulation; but his data, in combination with those that have already been quoted, certainly make it an attractive hypothesis that the effective surface charge of particles immersed in plasma is a major factor determining the survival of these particles when introduced into the circulation.

The failure of neuraminidase-treated erythrocytes to survive after being reinjected suggests that neuraminic acid may be the constituent of the erythrocyte membrane that is chiefly responsible for maintaining its Zeta potential in the presence of plasma. Unfortunately, no reference has been found to the electrophoretic mobility of neuraminidase-treated erythrocyte in plasma, nor have I determined this quantity myself; but it can hardly be higher than their mobility in saline, and it might even be much lower. The possibility has not yet been excluded that the brief survival of erythrocytes that have lost their neuraminic acid may be due to increased fragility: French and Ada (1953) have found that such erythrocytes showed increased osmotic fragility as compared to normal ones; and I have observed that they tend to be weakly agglutinated when suspended in their own plasma, although the clumps are readily dispersed by gentle stirring. If this tendency to agglutinate is also present in vivo, there might well be increased sequestration and perhaps hemolysis of the neuraminidase-treated cells. Such a process would certainly help to explain their rapid disappearance from the major vessels, and might also operate in the case of injected microcapsules. The fascinating and voluminous literature that has accumulated around the subject of "sludged blood" (cf. Wells, 1964) may be referred to here, though neuraminic acid, so far as I know, has not hitherto been implicated in this phenomenon.

At present, then, one can only state as an attractive hypothesis that one of the reasons blood cells survive in the circulation is because they have surfaces rich in neuraminic acid, or a similar acidic carbohydrate, and so maintain an effective negative charge even in the presence

of plasma protein; and one can add the corollary hypothesis that artificial particles provided with such a surface and sufficiently small or flexible, would have a better chance to survive in the circulation. On this hypothesis the negative charge of the vascular endothelium with respect to the plasma would be a principal factor opposing the adhesion and uptake of circulating cells and particles. The presence of a carbohydrate-rich coat on the vascular side of the endothelium has been observed (Rambourg et al., 1965); but whether it contains material like neuraminic acid is not known. The hypothesis may be extended to include, for example, erythrocyte-agglutination phenomena. On this basis the clumping of erythrocytes by group-specific isoagglutinins would depend on the specific masking of acidic carbohydrate moieties on the cell surface by the antibody; in support of this idea one may cite the observation (Sachtleben et al., 1961) that the electrophoretic mobility of erythrocytes is reduced by contact with group-specific serum. But it must be stressed that the hypothesis is, as yet, only a hypothesis. If it becomes possible to manufacture microcapsules with membranes that incorporate neuraminic acid or a similar material, the hypothesis might be tested by measuring their ability to survive in the circulation.

ACTION OF MICROENCAPSULATED UREASE IN VIVO

Introduction:

1. Exchange of material across the peritoneal membrane

Starling and his co-workers (Starling and Tubby, 1894; Leathes and Starling, 1895) found that intraperitoneally injected dyes appeared in the urine before they appeared in the lymph, and that little dye was removed by lymphatic channels. On the basis of these findings they suggested that small molecules enter and leave the intraperitoneal cavity mainly by blood vessels, the lymphatics playing only a minor role. Kjollerfeldt (1917), who studied the absorption of albumins, proteoses, peptones, and amino acids from the peritoneal cavity, found that amino acids were absorbed much more rapidly than the more complex bodies, and concluded that the cells lining the peritoneum and/or the endothelium of the blood vessels possess a differential permeability with regard to these substances. Cunningham (1926) found that when the ommentum was exteriorized and immersed in a solution of ferric ammonium citrate or potassium ferrocyanide, the solute was excreted by the kidney: since there was no change in the rate of excretion after ligation of the thoracic duct, he suggested that these substances passed into the blood vessels directly, and not through the lymphatic system. Maurer, Warren and Drinker (1940) analyzed peritoneal fluid from rabbits and dogs, and concluded that it is an ultrafiltrate of blood plasma with some leakage of protein. Courtice and Simmonds (1954), who summarized the available data, including their own, were satisfied that small molecules are exchanged directly between the peritoneal blood vessels and the cavity, whereas colloids, small

particles and cells are removed solely by the lymphatic system. The dynamics of exchange for the pleural cavity, according to Courtice and Simmonds, is similar to that for the peritoneal cavity. Stewart and Burgen (1958), who studied quantitatively the turnover of fluid and solutes in the dog's pleural cavity, found that small molecules like Na^{22} and p-aminohippurate were removed very rapidly by direct exchange with the blood while protein was reabsorbed mainly by the lymphatics. In their experiments the transfer of water-soluble crystalloids between pleural fluid and blood could be fitted to a simple diffusion scheme: for molecules of intermediate size (e.g. p-aminohippurate) the exchange was diffusion-limited, but for small molecules (e.g. Na^{22} with a clearance of 80 ml/hr = pleural blood flow) it was blood-flow-limited. Corresponding data for the peritoneal cavity have not been reported, but the situation there is no doubt similar.

The rapid diffusion of small-molecular substances across the peritoneal lining has been made use of clinically. One or two of the more recent reports may be mentioned. Schupak, Hampers, Prager, Mathy, Poulos and Kushner (1964) were able to reduce the blood urea and creatinine by more than 50% through peritoneal dialysis, and calculated the net peritoneal urea clearance to be 24 ml/min. with a dialysing volume of about 2 litres/hour. Barry, Sherman, Schwartz and Davis (1965) recently reviewed their results of peritoneal dialysis in more than 100 patients with renal failure, intoxication, or fluid and electrolyte problems. For such patients they found the procedures more convenient than the use of the artificial kidney: they were able to

lower the blood urea nitrogen of a uraemic patient from 200-230 to 60 mg/100 ml with a half-time of 30 hours, and also to lower the plasma potassium level in hyperkalemia and the blood levels of barbiturate, glutethimide (Doriden) and salicylate in cases of intoxication by these drugs.

The potentially rapid exchange of solutes across the peritoneal membrane, and the low toxicity and long survival of intraperitoneally injected microcapsules, suggested the use of the intraperitoneal route for testing the in vivo action of enzyme-loaded microcapsules. Urease was chosen as the enzyme to be tested, for several reasons: its ready availability; the small molecular size of its substrate; the ease with which its in vivo activity could be assessed by following the blood ammonia levels; and the fact that its activity after microencapsulation had already been demonstrated by in vitro tests. The results with urea, it was hoped, would serve as a basis for a theoretical test of the applicability of the principle of administering enzyme-loaded microcapsules.

The principle is shown diagrammatically in Fig. 40. For the microencapsulated urease to have significant in vivo activity, its substrate, urea, would have to diffuse across the peritoneal membrane, and then across the microcapsule membrane, to be acted on by the enzyme; then the product, ammonium carbonate, would have to diffuse in the reverse direction to raise the blood ammonia level. It would be expected that the diffusion of urea and NH_4^+ across the peritoneal membrane would not be too greatly restricted, on the basis of Stewart and Burgen's studies

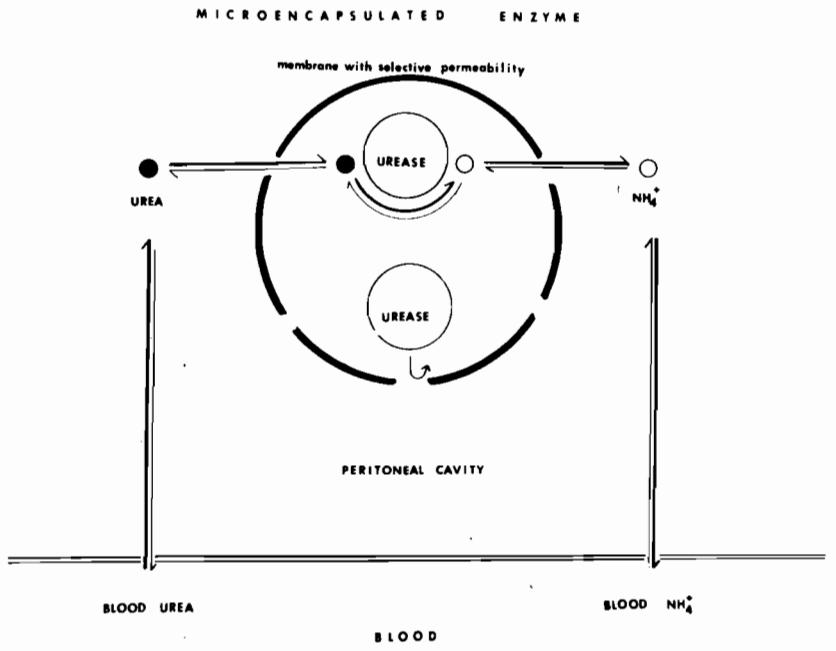


Fig. 40

Schematic representation of the action of intraperitoneally-injected microcapsules loaded with urease.

on Na^{22} (whose ion is larger than NH_4^+) and the peritoneal dialysis studies on the removal of urea. Efficient diffusion of substrate and product across the second barrier - the microcapsule membrane - could also be expected, on the basis of the *in vitro* tests with the enclosed enzyme, unless the microcapsules became less permeable as a result of their introduction into the peritoneal cavity.

2. Blood ammonia, urease, hepatic coma

It may be appropriate at this stage to summarize briefly our current knowledge of ammonia metabolism. According to Sherlock (1960), the blood ammonia level is the resultant of several processes: ammonia production by the intestine, ammonia uptake by the liver, ammonia uptake by and release from other tissues, and ammonia production by the kidney. Intestinal ammonia production depends on the bacterial activity on protein in the intestine, as is shown by two findings: the rise of blood ammonia after hepatectomy is prevented by evisceration; and the oral administration of antibiotics (*e.g.* neomycin) decreases the blood ammonia level. Bacterial urease is very similar to urease of plant origin: no other enzyme is known that will hydrolyze urea, and urease has no other known substrate (Kornberg and Davies, 1955).

Many investigators have studied the toxicity of parenterally injected urease. Tauber and Kleiner (1931) and Kirk and Sumner (1931) were the first to demonstrate that death in animals injected with urease occurs when the blood ammonium reaches a critical level; and Tauber and Kleiner were able by injecting ammonium carbonate into rabbits to reproduce the signs of intoxication by crystalline jack-bean

urease. Dang and Visek (1964) determined the acute LD50 of urease, in Sumner units per kg of body weight, to be 25-50 subcutaneously or intraperitoneally in mice, rats, guinea-pigs and rabbits, and 6-25 intravenously in rabbits, mice and rats. In rabbits, following an intravenous lethal dose of urease, depression appeared in 30-60 minutes, followed by excitation and laboured breathing which persisted for 5-15 minutes, after which the animal convulsed and died. With intraperitoneal injection in the same species, the time of onset of symptoms varied from 1/2 hour to several hours after the injection: there was successively lachrimation, urination, weakness, scratching and loss of the righting reflex, followed by a hypnotic state lasting from 1 to 4 hours with laboured breathing; death was preceded by a violent convulsion. Animals that recovered from the hypnotic state survived with no ill effects. Dang and Visek found that crystalline jack-bean urease is a potent antigen, repeated injection of sublethal doses resulting in the appearance of titratable antiurease in the serum. These authors concluded cautiously that their experiments did not prove that urease toxicity is due to ammonia intoxication, but they added that their findings did not in any way detract from this hypothesis. The only alternative hypotheses would be that the observed toxicity was due to an impurity in the enzyme preparation, or to some effect of urease unrelated to its enzymatic activity.

Hepatic coma is accompanied by, and has often been attributed to, an elevation of blood ammonia; but whether or not the cerebral intoxication can be wholly accounted for by the raised ammonia level is still uncertain. Sherlock (1960), who has admirably reviewed the copious literature on this subject, points out that the blood ammonia rises because the ammonia present in high concentration in the portal vein

fails to be converted to urea by the liver. Theoretically an elevated blood ammonia could interfere with cerebral metabolism by two possible mechanisms: excessive production of glutamine from ammonia and glutamate, depriving the brain of essential glutamate; and excessive reductive amination of d-ketoglutarate, removing the later substance from the Krebs cycle and so reducing oxidative phosphorylation in the brain. Sherlock reports a good general correlation between the blood ammonia level and the degree of neurological involvement in patients with hepatic insufficiency. She distinguished three grades of the condition: in grade 1, there was minor disorder of consciousness and the motor system: and in a group of such patients the mean blood ammonia level was 1.35 µg/ml, twice the mean level for normal subject; in grade 2, there was gross disorder of consciousness, and the mean blood ammonia was 2.5 µg/ml; in grade 3 there was full coma, and the mean blood ammonia was 3.5 µg/ml. Sherlock notes, however, that 10% of hepatic coma patients had blood ammonia levels in the normal range, regardless of the degree of neurological disturbance, while a few patients with very high blood ammonia levels had little CNS symptomatology; and she points out, further, that it is difficult to compare the effects of infused ammonium salts with the effects observed in hepatic coma patients having similar blood ammonia levels, because pH and electrolyte balance is likely to be different in the patients and the experimental animals.

Whether or not blood ammonia is a major factor in the pathogenesis of hepatic coma, it is clear that when blood ammonia is elevated in this condition, the ammonia is produced by urease in the intestine; and it is therefore of interest to learn how far an *in vivo* action of urease can reproduce

the signs of hepatic coma that frequently accompany a given elevation in the blood ammonia level. The administration of encapsulated urease would have a possible advantage over the administration of urease in free solution, since any non-specific effects of the foreign protein would be eliminated, and the effect of the preparation could be attributed wholly to its enzymatic action.

The experiments reported below show that microencapsulated urease on intraperitoneal injection in the dog acts effectively to raise the blood ammonia level, and to produce neurological disturbances.

Methods:

Seven experiments were carried out, six on anaesthetized dogs and one on an unanaesthetized dog. The acute experiments were on male dogs, weighing about 10 kg and anaesthetized with intravenous sodium pentobartital (25 mg/kg). Carotid blood pressure, rate and depth of respiration and electrocardiograph were recorded continuously on a Gilson polygraph. Estimates of ammonia in blood taken from a leg vein were performed by the method of Hutchison and Labby (1962).

Two sets of acute experiments were carried out, each on a group of three dogs. The animals of the first group were given 1 ml/kg of a 10% suspension of Nylon microcapsules (mean diameter 29 μ) containing a solution of Sigma V crystalline urease (400 Sumner units/ml): the dose of urease was therefore 40 Sumner units/kg. The animals of the second group were similarly treated, except that the dose was 2.5 ml (100 Sumner units)/kg. One or more control samples of blood were taken before the encapsulated

enzyme was injected; thereafter blood samples were taken at hourly intervals. All the animals, except one dog of the first group, received as a further control an equal volume of a suspension of Nylon microcapsules made up without included enzyme, but otherwise identical with the enzyme-loaded ones, which were given later.

As a test for leakage of protein from the injected microcapsules, Nylon microcapsules containing Cr^{51}-tagged haemoglobin were injected intraperitoneally at a dosage of 2.5 ml/kg of a 10% suspension. Four venous samples were taken at hourly intervals and tested for radioactivity; and at the end of the experiment, the liver, spleen and kidney were removed for counting. At the same time the abdominal cavity was irrigated with saline, and the recovered irrigation fluid was also tested for radioactivity.

The one chronic experiment was carried out on a small male dog weighing 6 kg. In order to avoid gross fluctuations of the blood ammonia level, the dog was kept throughout the experimental period on a low-protein diet. Blood samples for ammonia estimation were taken from a leg vein before and after the intraperitoneal injection of 5 mg/kg of a 10% suspension of Nylon microcapsules containing hemolysate only, and further blood samples were taken twice a day for two days. The test was repeated a week later, but on this occasion the administered microcapsules contained NBC soluble urease (100 Sumner units/ml of microcapsules) in addition to haemoglobin; the dose was again 5 ml/kg of a 10% suspension corresponding to an enzyme dosage of 50 Sumner units/kg. The blood ammonia level was followed for six days after the injection and the behaviour of the animal was closely observed.

Results

The results of the first group of acute experiments are shown individually in Fig. 41 , and the pooled results of the second group of three experiments in Fig. 42 . It is obvious that the dummy microcapsules produced no significant effect, while in every case the blood ammonia level rose steeply after injection of the urease-loaded microcapsules. At the end of the experiments, 3-4 hours after the injection of the encapsulated enzyme, the blood ammonia level of each animal was still rising. No significant change was observed in blood pressure, electrocardiogram or respiration, except ... that the breathing of the animals of the second group tended to become laboured towards the end of the experiment.

The changes in blood ammonia obtained in the unanaesthetized dog are shown in Fig. 43 . Again the intraperitoneal injection of the dummy microcapsules had no significant effect on either the blood ammonia level or the general state of the dog, which continued to be active, gained weight, and showed no sign of abdominal tenderness or rigidity. The enzyme-loaded microcapsules likewise produced no immediate effect on the behaviour of the animal. During the first hour or two it remained active and playful; but after 2.5 hours, when the first postinjection blood samples were collected, it appeared to be more sedate. Half-an-hour later, it preferred to lie down, but became active when approached by the experimenter. Thereafter it became more torpid; and four hours after the injection it no longer responded actively when approached, but when led walked with an unsteady gait. Its legs were observed to twitch occasionally, but there was no change in respiration. The dog's condition remained the same until

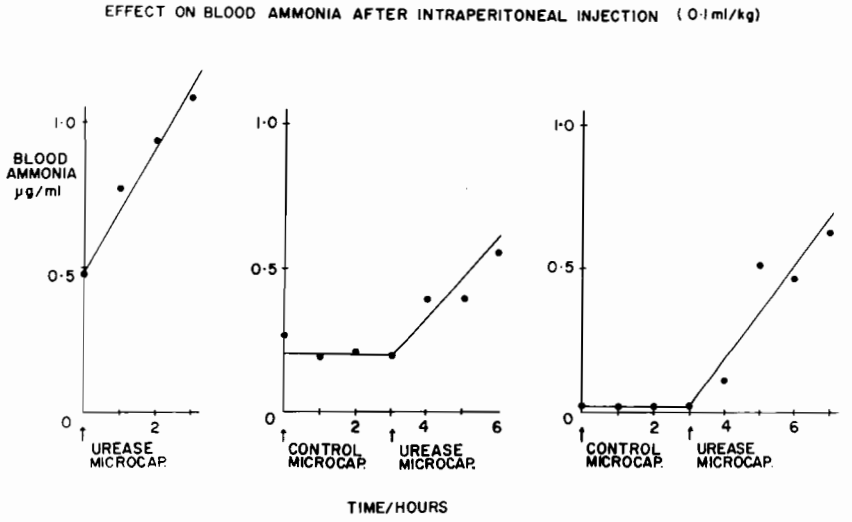


Fig. 41

Effect of microcapsules on blood ammonia in dogs anaesthetized with Nembutal: each graph represents an experiment on one animal. All three animals were given Nylon microcapsules (diameter 27 µ) containing urease (40 Sumner units/kg). The second and third animals were previously given similar microcapsules containing no urease. Dosage of microcapsules at each injection was 0.1 ml/kg.

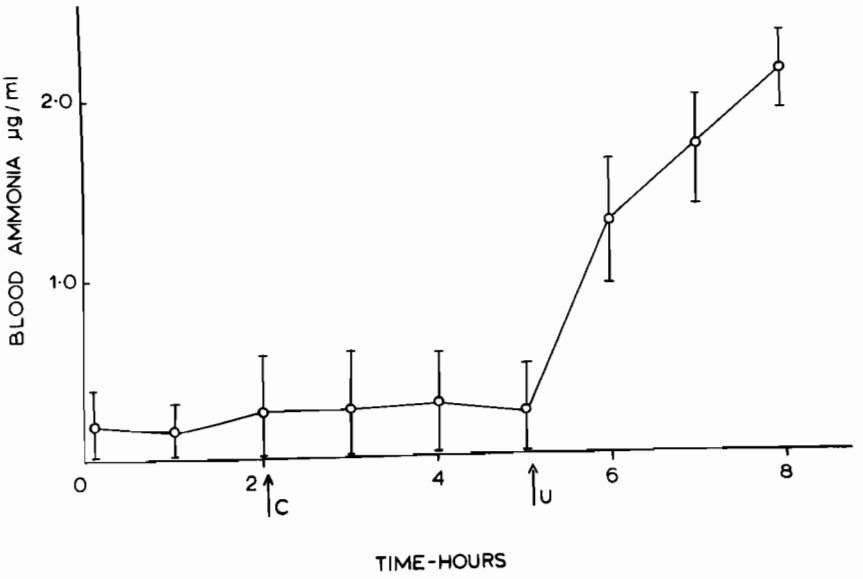


Fig. 42

Effect of microcapsules on blood ammonia in dogs anaesthetized with Nembutal: the graph summarizes the data from three experiments (Mean ± S.D.). C: injection of control microcapsules (no urease, 0.25 ml/kg). U: injection of urease loaded microcapsules (0.25 ml and 100 Sumner units/kg).

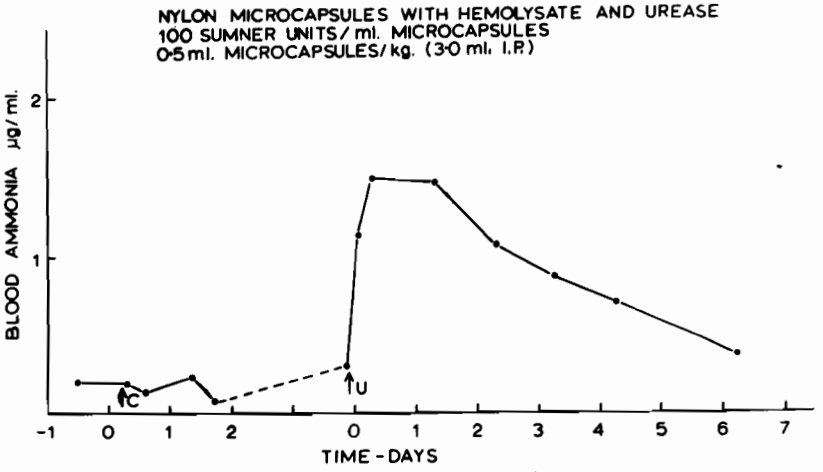


Fig. 43

Effect of microcapsules on blood ammonia in an unanaesthetized dog. C: control microcapsules (0.5 ml/kg). U: urease-loaded microcapsules (0.5 ml and 50 Sumner units/kg). In this test, the urease preparation (NBC) was stabilized by the addition of hemolysate.

late in the evening, when the observations were interrupted. Next morning it was better, though still sedate; but by afternoon it seemed to be perfectly normal, and has remained so up to the time of writing (about 6 months after the injection).

The blood ammonia concentration correlated only imperfectly with the signs of intoxication. The peak level of 1.6 μg/ml, found in the second and third postinjection samples, would be slightly above Sherlock's mean level for grade I intoxication. Clinically, the dog's status was between grade I and grade II at the first and second samplings, but it was not even grade I at the third sampling. The animal's apparent clinical recovery during the period when its blood ammonia was still at its peak was quite striking, and strongly suggests that its brain had some capacity to adapt itself to a high level of blood ammonia. From this elevated level the blood ammonia declined towards normal with a half time of 2-3 days.

Discussion

The results of these experiments show conclusively that micro-encapsulated urease when injected intraperitoneally can act efficiently on endogenous urea, converting it into ammonia. Only a small proportion of the body's urea was changed to ammonia in these tests, but it must be remembered that the intact liver was simultaneously reconverting ammonia to urea. While due caution is indicated in comparing the results of the different experiments, attention may be drawn to the fact that the *in vivo* action of the encapsulated urease appeared to rise steeply with enzyme dosage. This is illustrated in Fig. 44 in which the rate

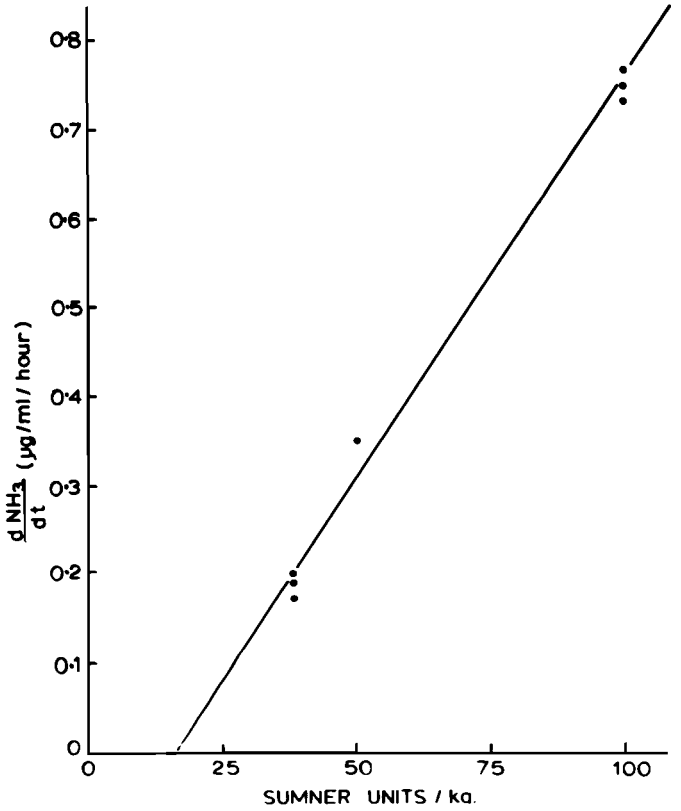


Fig. 44

Summary of experiments with encapsulated urease on dogs. Abscissae: dosage of urease (Sumner units). Ordinates: rate of rise of blood ammonia during first 3 hours after injection. Data from experiments shown in Figs. 41-43.

of increase in blood ammonia during the first 2-3 hours of each of the 7 tests has been plotted against the enzyme dosage in Sumner units/kg. The greater effectiveness of the higher dosage indicates that the rate-limiting factor for ammonia formation under the conditions of these tests was the amount of enzyme present in the peritoneal cavity rather than the rate of transfer of urea or ammonia across the peritoneal membrane. Extrapolation of the curve back to the abscissa (Fig. 44) suggests that the livers of these dogs were removing ammonia from the blood at about the rate that it would be formed by 20 Sumner units/kg of encapsulated urease.

V. GENERAL DISCUSSION

DISCUSSION

Most of the experimental results have been fully discussed at the end of each section. Consequently this final discussion will be brief, and will deal with some more general aspects of this research.

Some apology is perhaps called for by the fact that this thesis for a Ph.D. in Physiology contains so much material that is not primarily physiological in nature. It had been my intention, after I worked out the procedures for preparing microcapsules of reproducible specifications, to try to use such microcapsules to solve various biological problems. This attempt was delayed because it was felt that a more detailed study of the physical properties of microcapsules would provide a better basis for the *in vivo* enzymatic studies. The general principle and feasibility of studies of the latter sort has been illustrated by the experiments on the *in vivo* action of microencapsulated urease. Further work along this line is contemplated. For instance, the *in vivo* urease experiments will be continued in order to determine whether there is a genuine adaptation of the central nervous system to a high level of blood ammonia, as is suggested by clinical data from hepatic coma patients and by my one test on a conscious dog. This point cannot readily be settled by either repeated injections of free urease or infusion of ammonium salts, since the response of the animal would be complicated in the first case by foreign protein reactions, and in the second case by acid-base or electrolyte disturbances from the accompanying anions. The groundwork for such a study is now complete and the experiments should be straightforward, except that more refined methods will have to be developed for

assessing the degree of central depression in the experimental animals.

Various other problems that might be investigated with encapsulated enzymes *in vivo* have occurred to me or have been suggested to me. Some of these might be mentioned here, though most of them would be expected to involve greater experimental difficulties than the urease study just outlined. It would, for example, be of interest to see whether changes in the normal levels of various plasma constituents, other than NH_3, could be produced by means of encapsulated enzymes, and if so, what alterations of function would result. Examples of substrate-enzyme pairs that might be studied in this way would be uric acid and uricase, choline and choline oxidase, dopa (diphydroxyphenylalanine) and dopa decarboxylase, histidine and its decarboxylase, histamine and diamine oxidase, glucose and glucose oxidase, angiotensin and angiotensinase (in experimental hypertension). Each of these investigations might throw light on some hitherto obscure points; but in each case a good deal of preliminary work (and in some cases, the development of microcapsule membranes of higher selectivity) might be necessary before critical animal experiments could be begun. Finally, it would be of interest to test the possibility of using encapsulated enzymes to combat experimental intoxications: for instance phenylalanine "hydrolase" against dietary excess of phenylalanine (experimental phenylketonuria: Weisman & Harlow, 1965), acetylcholinesterase against organic phosphate insecticides, or aldehyde dehydrogenases against methanol poisoning. In these cases also a good deal of preliminary work would be needed.

An ultimate aim of these *in vivo* studies with encapsulted enzymes would

be, as mentioned in the Introduction, to explore the feasibility of applying the technique to the treatment of "molecular diseases". But many problems have yet to be solved before this possibility can be put to a practical test. The three problems that loom largest in this connection have been referred to earlier, but may be mentioned again.

First, there is the limited effective lifetime of most enzymes *in vivo*, or in solution at 37^{o}. No comprehensive treatment of this subject occurs in the literature, although there is a great deal of scattered data on the deleterious effect in certain cases of elevated temperature, atmospheric O_2, heavy metals, etc. and on the protective action of substrate and proteins. In the case of encapsulated urease the stabilizing effect of a high concentration of haemoglobin was a striking one, and further exploration of the manner in which this protein protects the purified enzyme might be rewarding. Even crystalline enzymes are not perfectly pure, and a trace of contamination with, for example, heavy metals or proteases might seriously curtail the life of some enzymes at body temperature. The value of therapy with an encapsulated enzyme would obviously depend largely on how long the enzyme remained active after the injection. Repeated injections would eventually encumber the body with a load of membranous material, which could not easily be got rid of, and would be undesirably bulky even if not actually toxic.

The second problem facing an attempt to develop an effective enzyme therapy would be that of controlling the permeability of the microcapsule membrane. This can be divided into three subsidiary problems. First, the initial permeability of the membrane for substrate and product of the enzyme

must be high, while permeability to the enzyme itself must be negligible. This problem has been solved more or less successfully for enzymes like carbonic anhydrase and urease, which have small-molecular substrates and products. Secondly there is the problem, in the case of some enzymes, of preventing the outward leakage of the necessary co-factors from the microcapsules. This might be solved by better adjustment of pore size or charge, or by inclusion in the microcapsule (or in smaller encapsulated microcapsules) of other enzyme systems which would generate the co-factor. Thirdly there is the possibility that the microcapsule membrane might become less permeable in vivo, through being coated or having its pores plugged by protein or lipids. My in vivo experiments with intraperitoneal urease-loaded microcapsules suggest that this process, if it occurs at all, is slow; but I have not yet used the crenation method to measure the permeability of microcapsules recovered from body cavities or tissues a long time after injection.

The third major problem is that of locating the enzyme-loaded microcapsules in a site where they will be sufficiently accessible to the substrate. In the case of urease the peritoneal cavity proved to be such a site, but this may not be the case for enzymes acting on a less diffusible substrate. For some enzymes the preferred arrangement would be to have the microcapsules circulating freely in the blood stream; and if this could be achieved many interesting experimental uses - for instance in studies of the circulation - could also be opened for microcapsules. I have made only very limited progress toward solving this problem. Batches of microcapsules prepared by the procedures I have described, even

with optimal stirring speed and detergent concentration, have always contained a proportion of forms too large to pass freely through capillaries. My attempts to screen microcapsules for size by differential centrifugation alone have not had much success, apparently because microcapsules of different size have about the same specific gravity. The principle of counterstream centrifugation may yield better results. When I attempted to use the electrical dispersion method of Nowab and Mason (1958) to prepare starting emulsions more uniform in droplet diameter, the high voltage required caused much denaturation of protein. Some modification of this technique may later be found serviceable, for making uniform emulsions, but the chemical and engineering problems involved have discouraged me from pursuing the matter.

The finding of a solution to the problem of making microcapsules uniformly smaller than capillaries, however, would still leave the problem of eliminating those properties of the membrane surface that are responsible for the trapping of the microcapsules in the microcirculation. From my experiments with both microcapsules and polystyrene microspheres, as well as with neuraminidase-treated red cells, it appears that the pulmonary circulation is no longer a major site of trapping for particles that have a negatively charged surface. But such particles are still rapidly removed from the blood stream by the liver and the tissues of the carcass, and in this respect they are far inferior to natural blood cells, which, though they have no stronger intrinsic surface negativity, can circulate for many days or weeks. I have suggested the hypothesis that a principal reason why blood cells can remain "afloat" in the circulation is because, in contradiction to my artificial particles, they can maintain

an effective surface negativity when in contact with plasma; and I have suggested further that their carbohydrate-rich surface is important in this connection. It should eventually be possible to produce a microcapsule membrane that mimics this feature of the blood-cell surface, and some testable ideas along this line have been envisaged. The simplest of these ideas is to determine the survival of labelled collodion-membrane microcapsules; others will involve attempts to prepare membranes of cross-linkdd polysaccharides, mucopolysaccharides or mucoproteins. An investigation of this sort is likely to be tedious and is more suitable for a team than for a single worker. It is however possible that more detailed knowledge of the chemical composition and steric structure of natural cell membranes will speed progress in this direction.

The possibility of a quite different solution to these problems should also be mentioned. Most of the difficulties considered above would be circumvented by placing the microcapsules in a replaceable container bathed by a stream of blood. A recent surgical advance is the development of vascular shunts which can be permanently installed in a limb artery, and "plugged in" when required to an artificial kidney. Blood clotting could be controlled with systemic anticoagulants or by selecting membrane materials that do not promote clotting.

While my <u>in vivo</u> experiments with microcapsules have so far been concerned only with their toxicity, fate and activity as enzyme carriers, I have referred also to other <u>in vivo</u> applications whose feasibility deserve to be explored: for instance their possible use as vehicles for drugs, radio-opaque materials or radioactive substances. The most engaging

possibility of this sort, however, seems to be that of using them to hold cell hemogenates or intact cells, thus providing the equivalent of a tissue or organ which could be administered by injection to replace or supplement one that had been damaged by disease. My finding that erythrocytes can be encapsulated without apparent injury, by a modification of the interfacial polymerization technique, seems to encourage the hope that progress can be made in this direction, especially when taken in conjunction with the partial success obtained by Algire and others with implanted Millipore chambers containing fragments of endocrine tissue. As I have pointed out, microcapsules should serve at least as effectively as Millipore chambers to limit the exchange of immunologically active materials; and they should provide a more favourable surface-to-volume ratio for the exchange of nutrients, wastes, metabolites and hormones. The peritoneal cavity would probably be the best site of implantation. Besides the obvious case of encodrine tissues, it may be of interest to try also the encapsulation of liver tissue, since hepatic cells can readily be separated with the aid of enzymes and suspended in a nutrient medium, with preservation of at least some of their metabolic capacities. (The biliary system would of course still be missing.)

Lastly reference should be made to various <u>in vitro</u> applications of microcapsules, some of which I have explored in a preliminary way. The experimental material presented in the thesis shows that microcapsules behave, as expected, like tiny dialysis bags, whose small dimensions permit the rapid equilibration of diffusible solutes across semi-permeable membranes. They should thus be useful without special difficulty for some of the analyses usually accomplished by dialysis or ultrafiltration; for example,

determining how much of a plasma constituent is bound to protein. In this case a small volume of microcapsules, after equilibration with the plasma, would be filtered off and leached with a protein-free fluid, which would then be analyzed after removal of the microcapsules. Another possible application, briefly illustrated in the thesis, is the use of microcapsule columns to separate solutes of different molecular dimensions: this procedure could probably be made much more efficient by varying the dimensions of column and microcapsules and the permeability characteristics of the membrane, and perhaps also by using the counter-current principle. Lastly the use of microencapsulated enzymes *in vitro* might be convenient for analytical or preparative studies, since an enzyme could be separated from a reaction mixture by merely centrifuging or filtering, and used again. A variant of this technique, suggested to me by Professor Avram Goldstein, would be to add substrate to the top of a column of encapaulated enzyme and recover the reaction products continuously from the eluate. Such procddures as these might be particularly convenient where different enzyme reactions are to be performed in sequence. The use of encapsulated whole cells or subcellular organelles, rather than of enzymes, might also be considered in some cases.

Lastly, though perhaps with the feeling that I have already speculated enough along these lines, two somewhat different lines might be mentioned as deserving further research. One would be to attempt the formation, especially by interfacial coacervation, of microcapsular membranes out of natural lipids and associated materials, and to explore the possibility that such membranes might retain some of the special permeability features associated with cell membranes. The second, which I have found to be

easily realizable on the small scale, is to use the interfacial polymerization technique to produce microtubules: for example, by continuously extruding an aqueous diamine solution from an orifice into an organic liquid containing sebacoyl chloride. If one could solve the technical problem of connecting many such tubules in parallel to a larger tube at either end, one could expect to obtain s amall rapid dialysis unit which might provide a basis for miniaturizing the artificial kidney (or, with silicone-rubber tubules, the membrane oxygenators used as artificial lungs in cardiac bypass surgery).

VI. GENERAL SUMMARY

AND STATEMENT OF CLAIMS TO ORIGINAL RESEARCH

GENERAL SUMMARY

(* Statement of claims of original work marked by asterisk)

* 1. Methods have been devised , for the first time, for the preparation of small hollow spheroidal membranous structures containing an aqueous phase.

* 2. The limiting membranes of these "microcapsules" have been formed by a physical process, interfacial coacervation, or by a chemical process, interfacial polymerization.

* 3. With the interfacial coacervation process, membranes have been formed from collodion, polystyrene or cellulose acetate.

* 4. With the interfacial polymerization process, membranes have been formed from Nylon (Polyamide 610), from a sulphonated analogue of Nylon, or from cross-linked haemoglobin. The two last named polymers have not been made previously.

* 5. Various proteins, including haemoglobin and the enzymes carbonic anhydrase and urease, have been encapsulated by these procedures with retention of their biological activity.

* 6. By these procedures, microcapsules have been made that are impermeable to protein but permeable to small water-soluble molecules and ions.

* 7. Methods have been devised for controlling the mean diameter of the microcapsules over the range 5 μ to 1 mm or more.

* 8. Methods have been devised for controlling the thickness, surface charge and porosity of the membranes.

* 9. Methods have been devised for encapsulating smaller microcapsules within larger ones.

* 10. Methods have been devised for encapsulating intact cells (erythrocytes) within large microcapsules.

* 11. The crenation of microcapsules in an aqueous solution of non-electrolytes has been shown to depend on the concentration and molecular radius of the solute.

* 12. Two methods have been devised for the estimation of the equivalent pore radius of microcapsule membranes, based on the application of Staverman's reflection coefficient to crenation data. These methods resemble those used by Solomon to calculate the equivalent pore radius of the erythrocyte membrane.

* 13. It has been demonstrated experimentally that a column packed with microcapsules can be used to separate water-soluble materials of different molecular weights.

* 14. It has been shown that carbonic anhydrase can catalyse the hydration of CO_2 almost as efficiently when encapsulated as when bound within erythrocytes.

* 15. It has been shown that, under favourable circumstances, urease when encapsulated can catalyse the hydrolysis of urea with more than one-third the reaction velocity obtained when the enzyme is present in free solution.

* 16. The survival of microspheres and microcapsules in the circulation after intravascular injection has been shown to be affected by the surface charge of the particles as well as by their size. Negatively charged particles survived longer than uncharged ones.

* 17. Uncharged small particles after intravenous injection have been found to be trapped efficiently in the pulmonary circulation, whereas negatively charged particles are much less likely to be trapped in this way.

* 18. It has been found that erythrocytes which have lost their surface charge through treatment with neuraminidase do not survive long in the circulation when they are reinfused.

* 19. Nevertheless, it has been demonstrated that some surface property other than charge is related to the survival of the particles in the blood stream.

* 20. The toxicity of subcutaneously, intramuscularly, or intraperitoneally injected microcapsules is low, and they elicit little local tissue reaction.

* 21. Microencapsulated urease injected into the peritoneal cavity in moderate dosage (less than 1 ml/kg) can raise the blood ammonia concentration within a few hours to levels similar to those found in hepatic coma in man.

* 22. The stability of microencapsulated urease after injection is similar to that of the enzyme <u>in vitro</u>, in aqueous solution at body temperature.

* 23. The stability of microencapsulated urease is improved if it is accompanied by a high concentration of haemoglobin.

* 24. In a dog intoxicated by encapsulated urease, recovery from central depression was more rapid than the return of the blood ammonia level to normal.

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