REVIEW ARTICLE

BEHAVIOUR OF PROTEINS AT INTERFACES

By M. J. Fraser*, B.Sc., M.Sc., Ph.D.

National Institute for Research in Dairying, Shinfield, Reading

Speculation concerning the biological importance of thin films of proteins began as early as 1870 when Ascherson[1] noted that a tough skin of protein formed spontaneously around oil droplets suspended in egg albumin solutions and suggested such droplets as models for living cells. Devaux[2], in 1903, was the first to spread proteins at the air : water interface. He observed that such films could be collapsed to form artificial insoluble fibres, the "Devaux effect". About the same time Ramsden[3] noted that egg albumin is precipitated from solution after being present at an air : water interface. Thus it was early recognised that a considerable change in the properties of protein molecules occurs on their adsorption at interfaces. Wu and Ling[4] later studied this phenomenon of "surface denaturation", finding that shaking solutions of egg albumin, oxyhaemoglobin and methaemoglobin in air leads to an irreversible loss of solubility of these proteins. Langmuir and Waugh[5] calculated that an increase of film pressure of 15 dynes/cm., i.e., great enough to collapse the protein films, should increase the solubility of the film by a factor of 10^{95}, if the adsorption of the protein on to the water surface followed a simple Gibb's isotherm. Apparently then, the loss of solubility of proteins on adsorption is due to some drastic structural change in the molecules.

This review undertakes to consider what is known of the nature of these structural changes that occur on adsorption of proteins at air : water, oil : water, solid : water and the perhaps biologically more important lipid : water interfaces. Secondly the effects of adsorption on the activity of enzymes and other biologically active proteins will be discussed. Finally these physico-chemical studies will be related to the problems involved in the study of the activity of intracellular enzymes, especially those "surface enzymes" associated with the membrane, nucleus, mitochondria, endoplasmic reticulum (microsomes) and other morphological entities of the living cell. Several excellent reviews[6–9] of the physical chemistry of protein monolayers, both of the older and more recent literature, are extant. Among those, Bull[7] has discussed many of the surface chemical techniques employed in the study of spread monolayers of protein which are not elaborated here. The papers of Rothen[8] and Cheesman and Davies[9] discuss the effects of spreading at interfaces on biological activity and the latter in particular dwells on the structure of protein monolayers. Now attempts at building up more complex systems, chiefly studies on enzyme-lipid interactions, can be discussed, and with the advent of the technique of differential centrifugation, these systems can be compared with the simpler ones isolated from living cells.

* Holder of a National Research Council of Canada Special Overseas Scholarship.

Adsorption of Proteins at Interfaces

Protein Structure and Denaturation

Before attempting to discuss the development of the topic of the adsorption of proteins at interfaces it is perhaps best to prejudice the reader with a simple picture of the modern concepts of protein structure and "denaturation", so that the development may be seen in a better light. One of the most useful of such pictures for our purposes is that of Kauzmann[10,11] on protein denaturation, following the studies of Pauling and others[12] on the structure of "native" proteins.

Briefly, the structure of a native globular protein is considered under three aspects: (1) the "primary" structure or linkage of amino acids (for example, 51 in insulin, 288 in ovalbumin) through peptide bonds to give a long polypeptide chain; (2) the "secondary" or helical structure of this long chain stabilised by numerous hydrogen bonds between the amide H-atoms of peptide bonds and O-atoms of the peptide carbonyl groups; (3) the "tertiary" structure or folding of the helix into a globular form, a structure stabilised partly by hydrogen bonding between the folds of the helix and by van der Waal's forces between the non-polar groups. These hydrophobic bonds are the result of the tendency of the non-polar side-chains such as the benzyl groups of the phenylalanines, the butyls of the leucines, propyls of the valines and indoles of the tryptophanes, to adhere to one another because of their low affinity for water. Salt linkages between positively charged lysine and arginine residues and negatively charged glutamic and aspartic acid residues may also play some role in holding the molecule in a specific "tertiary" configuration, though, according to Jacobsen and Linderstrøm-Lang[13] they are not numerous in most proteins. More important are probably disulphide links both in the secondary (between the coils of the helix) and tertiary (between folds of the helix) structures[14,15].

The tertiary structure is the most labile, and probably for many proteins changes in it are reversible under mild conditions. Such things as hydrogen-bonding agents, organic solvents and detergents (though their primary binding on to proteins is probably through polar groups of the protein) can alter the tertiary structure. Hydrogen-bonding agents such as urea and guanidine, by competition for the intramolecular H-bonds, serve to modify both the secondary and tertiary structures considerably, but in some examples reversibly, if conditions are not too extreme. In all but a few instances heat effectively and irreversibly destroys the specific secondary and tertiary configurations of the protein molecules. Any process that succeeds in doing this without proteolysis or damage to the primary structure is generally called a "denaturation". The course of the action of denaturing agents on proteins may usually be followed by measuring either the loss of solubility at the isoelectric point, the loss of biological activity of the protein, or both. Loss of the primary structure or proteolysis can be induced, for example, enzymatically or by digestion with acid or alkali. With this simple picture of protein structure and

denaturation in mind we can go on to consider the changes in structure that occur when proteins are adsorbed at various interfaces.

Films of Protein Spread at the Air: Water Interface

One of the first steps in the study of spread monolayers of proteins was to measure the thickness of the films. It was soon discovered by optical methods and direct measurement[6] of the thickness of the layers deposited on top of one another on conditioned metal slides by the Langmuir-Blodgett technique[7] that the films were only 8 to 10 Å thick, the average length of an amino acid residue. These layers were prepared from films spread at zero surface pressure to allow maximum structural rearrangement. Since the globular molecules in solution were about 40 to 80 Å in diameter and no dissociation had occurred, it was clear that a considerable intramolecular unfolding had taken place, a "surface denaturation". What is more, the amino acid residues were oriented in the monolayer, for if the film was deposited on the plate such that the top side of the film was exposed, it was found to be hydrophobic (not wetted by water). If the deposit was made such that the bottom side of the film was exposed it was found to be wettable or hydrophilic. Thus the orientation of the residues in the film appeared to be similar to that in fatty acid films, the polar groups anchored in the aqueous phase, the non-polar groups in the air. These conclusions were supported by surface potential measurements and interaction studies[16]. The former showed that the orientation could be changed by compressing the films. At low pressure the amino acids lay nearly flat in the interface; in highly compressed films their angle of tilt reached 90°.

In the region of low compression, 0 to 1 dynes/cm., the film molecules act like a 2-dimensional gas and a molecular weight can be obtained from the force-area curves. In general, where there is no dissociation of the molecule on spreading, these molecular weights agree closely with those obtained by other physical methods[7]. Above 1 dyne/cm. a coherent film is obtained whose surface viscosity and elasticity increase sharply as the film is compressed. Above 15 dynes/cm. a time-dependent collapse of the films occurs. With a dark field ultramicroscope long faint striations or folds parallel to the compressing slide[17] are seen at the onset of collapse. Finally, at complete collapse visible fibrils appear and these can be collected in the form of an artificial fibre—the "Devaux effect". Not all proteins exhibit this effect. Most "gelled" films below the collapse pressure generally show marked hysteresis effects on compression, decompression and recompression of the films[7,18].

Several lines of evidence suggest that surface denaturation, once thought to be the most drastic form of denaturation[6], does not always succeed in destroying the tertiary and secondary structures entirely, leaving only a fully extended two-dimensional primary structure. For example, some proteins first heat denatured or treated with the Ställberg-Teorell spreading solution exhibit larger limiting areas than films spread from native proteins[9,19]. Also ultra-violet light has been shown to expand fully spread ovalbumin films[44] and it has been found that the Devaux

fibres prepared from collapsed films of proteins denatured by different agents before spreading possess widely varying morphological properties and tensile strengths[19]. Thus it is likely that these denaturing agents succeed in attacking bonds not broken when the native molecules unfold at the air:water interface[9,19]. The visco-elastic properties of various proteins, and of the same protein treated by different denaturing agents, are seen to vary considerably by the different expansion patterns they give with indicator oils also[7]. Thus, despite the great similarities in the force area curves for proteins other properties of the films may vary considerably[9].

Most of the topics mentioned in this section have been more elaborately reviewed by Bull[7] and Cheesman and Davies[9].

Interactions of Protein Monolayers with Soluble Substances in the Sub-phase

The study of fully spread monolayers of protein, though of great interest in structural studies, has not much direct bearing on the behaviour of proteins at interfaces of biological interest. Of closer relevance are the studies of interactions of proteins at interfaces: (1) of protein monolayers with soluble substances in the sub-phase and (2) of soluble proteins in the sub-phase with insoluble monolayers of other substances, the lipids being probably of most interest. This section deals with the former topic and the succeeding sections with the latter.

Schulman and Hughes[20] early reported that spread monolayers of casein could be digested by both chymotrypsin and pepsin under appropriate pH conditions. The thickness or extent of spreading of the casein films is not known, and these experiments are complicated by the fact that the enzymes are film-forming also. Schulman[21] later studied the interaction of amphipathic substances with protein monolayers coming to the general conclusions that if the molecules in the sub-phase have hydrophilic "heads" and hydrophobic "tails", i.e., are surface-active, three situations could arise: (1) if there is no association between the polar heads of the injected molecules and polar groups in the film there will be no alteration in the film's characteristics, e.g., no change in either film pressure or in surface potential; (2) if there is association between these polar groups but no association between the hydrophobic tails of the molecules in the sub-phase and the non-polar groups of the protein then there is an adsorption of the substance on to the protein with a consequent change in surface potential; (3) if there is association of both polar and non-polar groups of both molecular species then the molecules in the sub-phase penetrate the protein film giving rise not only to changes in surface potential but also to increases in surface pressure. Penetration of the hydrophobic tails can be prevented if the film molecules are tightly packed by compression before injection of the amphipathic substances into the sub-phase, or, in the case of a mixed film, compression may result in the squeezing out of the component with the lowest collapse pressure as the surface pressure increases. These systems thus act as models for the action of haemolytic and agglutinating agents on red blood cells. Lytic agents were found to penetrate either cholesterol films or disperse

protein (gliadin) films and are capillary or surface-active. Agglutinating or sensitising agents adsorb only on protein films, exhibiting no penetrating properties and no appreciable surface activity. Thus the red blood cell membrane, a lipoprotein complex probably containing cholesterol, is likely to be structurally altered by penetrating lytic agents, perhaps even partially dispersed, while the agglutinating agents merely adsorb on the membrane.

With mixed monolayers of two species of surface-active substance one of two phenomena can occur on compression of the films: (1) one component may displace the other from the interface usually at the collapse point of the displaced material or (2) both components may remain as a stable mixed film collapsing as a complex, at pressures above the collapse points of either component. More stable complexes are found with penetrating substances which show association between both polar and non-polar portions of the film molecules. Thus penetrating molecules cannot always be displaced from the interface but remain in strong association with the film molecules.

There are several other instances in which there is found a correlation of the association (adsorption with or without penetration) or non-association of various structurally similar substances in the sub-phase with monolayers of proteins and the biological activity of these compounds. For example, Rideal and Schulman[22] studied the adsorption of a series of compounds related to stilboestrol on monolayers of gliadin. They found a good correlation of the rates of adsorption on the protein, as measured by changes in surface potential, and oestrogenic activity, suggesting that the event of oestrus is initiated by the adsorption of the hormone at a protein surface. This and other examples have been reviewed by Cheesman and Davies[9], and the general interactions of protein films with substances dissolved in the sub-phase have been reviewed by Schulman[23].

Adsorption of Proteins at Clean and Lipid-Stabilised Oil: Water Interfaces

Studies of spread monolayers of proteins at the oil: water interface are considerably less numerous than those at the air: water interface because of the technical difficulties encountered. Alexander and Teorell[24] and Cumper and Alexander[25] have made force-area measurements on proteins spread at non-polar oil: water interfaces, finding the major difference from films at the air: water interface to be a larger specific area. The hydrophobic tails of the amino acid residues no longer cohere at low pressure; the chief van der Waal's forces to be considered are between these groups and the oil. Davies[26] has used a vibrating plate technique to measure film surface potentials at the light petroleum: water interface. Cheesman and Davies[9] have discussed the orientation of the amino acid residues at the oil: water interface and the visco-elastic properties of protein films in some detail, basing their discussion chiefly on data obtained for films of poly-α-amino acids and their copolymers.

An interesting, if somewhat neglected, method employed in the study of the adsorption of proteins to oil droplets in aqueous suspension is the

microscopic electrophoretic mobility technique developed by Abramson, Moyer, Gorin[27] and others. These workers followed the changes in electrophoretic mobility with pH of proteins adsorbed on minute particles of paraffin wax, pyrex glass, carbon, collodion, quartz, and kieselguhr, by microscopic observation, using reflected light, of the rate of movement of the particles across a measured field. Most studies were concerned with adsorption of protein on "inert" particles where the surface protein is electrokinetically equivalent to the bulk globular protein under the same environmental conditions of pH, ionic strength and buffer type. Apparently adsorption on these solid surfaces took place without unfolding of the protein. This type of study was thus chiefly concerned with the elucidation of the properties of the adsorbed protein, the effects of the underlying surface tending to be neglected. In some examples where deviation from the bulk behaviour was noted the results were explained by a partial unfolding or structural rearrangement of the protein which presents a different array of polar groups to the shear boundary layers that determined the mobility[27]. Cumper and Alexander[28] applied the method to a study of the adsorption of proteins (like bovine γ-globulin, haemoglobin, and insulin) on to suspended droplets of non-polar and polar hydrocarbon derivatives like mineral oil, oleyl and cetyl alcohols. In a number of instances the surface isoelectric point for the protein-coated particles coincided with the bulk isoelectric point of the protein, suggesting that the protein in the shear boundary layer was in the globular form. Since this behaviour was closely observed only in protein solutions of higher concentration it is quite likely that a layer of native protein was adsorbed on a layer of unfolded protein. Cumper and Alexander have elsewhere[29] presented a dynamic picture of proteins adsorbing, unfolding and spinning off oil : water interfaces in a denatured form. With some particles, especially cetyl alcohol, a marked shift in isoelectric point was observed, and on either side of this the mobility of the particles depended to a certain extent upon the nature of the particles. Seaman and Fraser[30] have observed differences in apparent isoelectric point for trypsin, even on the non-polar paraffin wax particles in the presence of excess protein, of as much as 6 pH units. Cumper and Alexander[28] have explained such observations as being due either to a specific chemical reaction between the proteins and particles, or as the result of incomplete coverage of the surface. There is thus considerable difficulty in interpreting the results of these mobility measurements, though no extensive study of any one system has been made. Among some of the possible factors that would have to be considered are the variation in the amount of protein adsorbed with changing conditions of pH, salt concentration, or buffers, and the adsorption or loss of trace ions as conditions are altered. The method is a very sensitive one and worthy of fuller exploration. It has found more successful use, in combination with other methods, in the study of the adsorption of the enzyme trypsin at various lipid-stabilised oil : water interfaces[31], as will be discussed later.

The first extensive studies of the interaction of soluble proteins with lipid interfaces were made by Elkes, Frazer, Schulman and Stewart[32],

using what we shall call the "emulsion technique". These authors studied the adsorption of oxyhaemoglobin (and albumin) on emulsified mineral oil droplets stabilised with the cationic detergent, hexadecyl trimethyl ammonium bromide (C_{16}TAB) and the anionic detergent sodium hexadecyl sulphate ($SC_{16}S$) over a wide range of pH at constant ionic strength. They found that adsorption of the protein resulted in flocculation of the droplets and occurred when the protein and interface were oppositely charged, e.g., on the acid side of the isoelectric point with the negatively charged interface ($SC_{16}S$). The amount of protein required to cause maximum clarification of the subnatant fluid, that is flocculation of all the droplets, corresponded to that needed to give a firmly gelled monolayer at the air : water interface. This was 2·5 mg. per sq. metre surface area. With less protein than this the droplets remained discrete because the surface charge had not been neutralised, and with more protein (25 mg./M^2) in the sulphate system, the droplets did not flocculate either, because of further adsorption of protein in the form of multilayers reversing the sign of the charge on the droplets. Further, this surface haemoglobin could be desorbed by altering the pH of the medium so that the protein and interface came to have the same sign of electrical charge. The protein was thus repelled from the surface. This reversible adsorption-desorption resulted in the denaturation of the haemoglobin to parahaematin which was shown by changes in the absorption spectrum and by solubility tests. Ultracentrifugal analysis of the desorbed protein showed it had the same molecular weight as oxyhaemoglobin, indicating that no association or dissociation had occurred.

In these experiments some of the denaturation could have taken place through the bulk interaction of the partially soluble detergents and haemoglobin. Nevertheless, that surface denaturation does occur has been confirmed[31,33], using a water-insoluble anthracene long-chain sulphonate as stabiliser. Here it required 10 mg./M^2 of proteins (haemoglobin, catalase, trypsin) to neutralise the surface charge and amounts above 15 to 20 mg./M^2 could be desorbed in the native forms, the absorption spectrum of oxyhaemoglobin being still present, and the enzyme activities still considerable. The outer multilayers of proteins were firmly bound and could not be washed off with washes of the same medium as that used in adsorption. The formation of these multilayers without loss of the globular structure, or at most only slight reversible changes in the molecules, presents an interesting physico-chemical problem, for it is difficult to account for such strong adsorption when the surface charge has been neutralised. Possibly the layers of proteins are strongly polarised, the polarisation being induced by the specially oriented lipoprotein layers underneath. Such forces have been suggested by Rothen[8] to account for the build-up of multilayers of insulin 450 Å thick on top of a layer of protamine 30 to 50 Å thick, and the fact that slides covered with barium stearate and "conditioned" with uranyl ions can hold large numbers of layers of protein transferred to them by the Langmuir-Blodgett technique[7]. Without being conditioned they can apparently hold only one layer of protein[8].

It is to be noted that in the adsorption of proteins on the anthracene long-chain sulphonate stabilised oil droplets[31,33] considerably more protein was required to neutralise the surface charge to give flocculation than previously reported for the sulphate system[32]—10 mg./M^2 as opposed to 2·5 mg./M^2. This amount of protein is equivalent to ten fully spread layers of protein, and must be thought of as being in the form of multilayers. These inner layers of protein are irreversibly denatured. It is not likely that all of the sulphonate stabiliser remains at the oil:water interface but probable that layers of protein-detergent-like complex are formed. It is possible too, that some of the adsorbed protein is actually solubilised in the oil phase through this complex formation, for it was not possible to desorb the protein entirely. Such oil-soluble complexes have been reported previously by Pankhurst[34]. Putnam[35] has reviewed the bulk interaction of proteins with soluble ionogenic detergents. The amount of detergent bound increases with the concentration of detergent present, at first approximately linearly as a few ions are bound on the surface of the protein, then logarithmically as the molecules are "wedged open" and unfolded, exposing many more binding sites, until all the sites are saturated. Friend, Harrap and Schulman[36] have followed the unfolding by the light-scattering technique, studying bovine serum albumin-sodium dodecyl sulphate (SDS) interaction. Few, Ottewill and Parriera[37] have found that dodecyl trimethyl ammonium bromide (DTAB) binds to this protein at relatively high protein and detergent concentrations giving a sudden increase in molecular asymmetry (as measured by viscosity increases) after six molecules of DTAB per molecule of protein have been bound. These complexes dissociate on dilution and are affected by strong salt concentrations, whereas the formation of the protein-SDS complexes, though affected by salts[36], is virtually irreversible[35]. These observations were paralleled at the oil:water interface[31,33] where interactions with quaternary ammonium ions were reversible, but not so interactions with long-chain sulphates and sulphonates. The former are thought to bind through the carboxyl groups of the protein[37], the latter through the free amino groups, but these may not be the only binding sites[35].

Interaction of Proteins with Lipid Monolayers

Doty and Schulman[38] and Matalon and Schulman[39] studied the interaction of lipid monolayers with soluble proteins injected into the subphase. The lipid films were held at 14 dynes/cm. pressure, the collapse pressure of protein films, to prevent spontaneous spreading of the protein. The interactions of serum albumin, haemoglobin and globulins with monolayers of cephalin, cholesterol, cardiolipin, stearyl choline and a long-chain C_{22}-sulphate were studied. The experiments were carried out over a wide pH range using a Langmuir surface balance; (1) allowing the film pressure to increase from 14 dynes/cm. at constant film area and (2) allowing the area of the film to increase at constant pressures above 14 dynes/cm. Phenomena similar to those encountered at the oil:water interface were observed. For example, the interaction of the proteins on the acid side of their isoelectric points was strong with negatively

charged films. If the pH of the sub-phase was then made alkaline the protein, now having the same charge as the lipid, could be nearly all ejected from the film. Some "bound protein" remained where the penetration of the protein into the lipid had been strong. With the non-ionogenic cholesterol there is only weak association. In mixed films compression above the collapse pressure of the protein results in ejection of the protein[40]. The pH range where strong interaction took place at the air : water interface was found to coincide with the range for flocculation of oil : water emulsions stabilised with the same, or similar, lipids[38,39].

One of the lipids studied by this method, cardiolipin, is the main component, with the sensitising lipids lecithin and cholesterol, of the specific antigen used in the Kahn, Kline, Eagle, Mazzini and Wasserman diagnostic tests for syphilis. In all but the Wasserman test a positive test is given by a flocculation, visible to the eye or microscopically, of the antigenic lipid particles in the presence of syphilitic serum due to the adsorption on them of the specific antibody protein whose production in the human body is induced by the presence of the spirocaete *Treponema pallidum*. Normal human serum under the conditions of the tests does not give the flocculation. Doty and Schulman and others[38] have tried to demonstrate this specific interaction by the monolayer penetration technique without success. They injected small quantities of normal and sylphilitic sera underneath films of cardiolipin and mixtures of this lipid with cholesterol and lecithin. The same penetration was observed by both types of sera, however, perhaps because of the very great dilution of the sera. The specific adsorption has been followed under conditions more nearly approximating those of the clinical test by Eagle[41] using the microscopic electrophoretic mobility technique described above.

Unfortunately very little work has been done to develop further the use of the monolayer penetration technique in the study of lipoprotein interactions at interfaces. There is especially a need for a method to quantitate the results, being able to measure exactly how much protein adsorbs on to the lipid film. A step in this direction has recently been taken by Eley and Hedge[42,43] who have adopted the technique of injecting the protein solutions at the lipid interface 1 mm. or so beneath the film. They claim that the proteins spread rapidly beneath the film without appreciable loss of protein to the bulk phase. However, they have made the injections of protein under lipid films held well below the collapse pressure of proteins (2 and 10 dynes/cm.) so that spontaneous spreading of the protein took place. The final pressures of the "penetrated films" rarely rose above 14 to 16 dynes/cm. Thus their reports of demonstration of interactions between lecithin, cholesterol and uncharged stearic acid (on acid substrate) with bovine plasma albumin, fibrinogen, lysozyme and insulin must be interpreted with caution. Many of these reported interactions do not occur when the lipid films are held above 14 dynes/cm.[38,39]. The fact that the proteins still unfold at an interface covered with a lipid film at 2 to 10 dynes/cm. pressure does not prove there is interaction between the lipid and protein. The unfolding process does however, present ample opportunity for association between the polar

and non-polar groups to occur and one could wish to know more about the properties of these mixed films. In any case these authors have not claimed that the interaction energies are strong.

As these interesting systems warrant further investigation and development so do the effects of ultra-violet light on lipoprotein films. Kaplan and Fraser[44] have observed that strong doses of ultra-violet light first expand albumin films before causing them to dissolve in the substrate at constant film pressure, presumably due to proteolysis. As noted above this expansion is due to a rupture of bonds not broken in the unfolding processes on adsorption. A dose of ultra-violet light that is strong enough to solubilise the protein film was found to have no effect on a film of "cephalin" (chiefly phosphatidyl ethanolamine) but when a lipoprotein film was formed by allowing catalase to penetrate the cephalin at 20 dynes/cm. the entire film was solubilised by the same dose of ultra-violet light[45]. The complex formation between the lipid and protein must have been strong indeed.

In summary of the interactions of proteins with lipid covered interfaces, either oil : water or air : water, where the interfacial tension has already been considerably reduced by the presence of the lipid, one has to consider chiefly the association of the polar and non-polar groups of both molecular species. Many factors which affect these associations can be elucidated by bulk studies, for example, effects of pH, salt concentration, dielectric constant, specific interaction between polar groups, which alter the hydrogen bonding, ion-dipole attractions and van der Waal's forces between the lipid and protein. In other words, one must recognise all the interactions that occur in the bulk phase together with the special steric restrictions imposed by the alignment of the lipid molecules at the air : water or oil : water interface. The orientation and packing of the lipid molecules can be varied considerably, from the two-dimensional gaseous to the solid states, by compression of the films or addition of lipids of other types which associate with that already present, changing the properties of the film. Thus with highly compressed or tightly packed lipid monolayers the penetration of the non-polar groups of the protein may be prevented, and the unfolding of the protein that usually occurs thereby greatly limited, unless possibly there is a high density of polar groups which strongly react with numerous polar groups on the protein. These films would resemble the immobile solid : liquid interfaces where the solid surface greatly restricts movement in the adsorbed film.

Effect of Adsorption on Biological Activity of Proteins

Biological Activity at Air : Water and Oil : Water Interfaces

Surface denaturation, or the non-proteolytic structural modification that occurs on adsorption at air : water or oil : water interfaces, affords excellent conditions for determining the contributions of the secondary and tertiary structures of the intact protein molecule to its biological activity. This is an important point in the light of some recent experiments on the controlled digestion of large portions of the amino-acid residues of certain proteins with proteolytic enzymes in bulk solution.

Rogers and Kalnitsky[46] for example, report that 17 amino acid residues of ribonuclease can be removed without loss of its enzymatic activity. And, most striking of all, Hill and Smith[47] have shown that no less than 120 of the original 180 amino acid residues of the enzyme papain can be removed without loss in activity, though a considerable loss of stability of the protein was noted. Such findings would seem to indicate that only a relatively small portion of the molecule near the active centre need remain structurally intact for the protein to exhibit its enzymatic activity. Also a considerable portion of the amino acid residues are not essential in preserving this specific structure but probably do act to stabilise the entire molecule. On the other hand, adsorption of the protein at air : water and oil : water interfaces, accompanied by drastic overall structural unfolding will be seen to have profound effects on the biological activity, showing that the "native" secondary and tertiary structures of at least part of the molecule are essential for its activity. This fact is also shown by the effects of other denaturing agents in bulk solution, such as heat, ultra-violet light, and urea. Thus catalase and other large enzymes are inactivated by urea[48] and heat[49] while the smaller, more stable enzymes like ribonuclease and trypsin can lose their activities reversibly in the presence of heat[50,51] or urea[52,53].

The effects of spreading proteins at air : water and oil : water interfaces on their biological activity have been previously reviewed by Rothen[8] and Cheesman and Davies[9]. From the outset it was noted that adsorption of enzymes on to these mobile interfaces generally led to a great reduction in their activities. Thus Gorter[54] found that films of pepsin spread at pH = 2·85, and of trypsin spread at pH = 7·00, then removed from the surface with a silk net, still retained as much as 80 per cent activity when tested under appropriate conditions. Langmuir and Schaefer[55] deposited films of pepsin, urease and catalase on metal slides, finding that they retained only about 5 per cent activity. The catalase films were 23 Å thick, and thus not completely unfolded. A few years later Sobotka and Block[56] reported that films of saccharase deposited on slides retained full activity. Here again the protein was not unfolded, the films being 45 Å in thickness. More recently, Hayashi and Edison[57], Hayashi[58] and Kaplan[59] have reported that spread monolayers of pepsin-albumin complexes, actomyosin and catalase respectively, retain a certain fraction of their enzymatic activity. In each case however, closer investigations[60,9,61] have subsequently revealed that if films of these enzymes were spread carefully under no compression for long times to obtain homogeneous films 8 to 10 Å in thickness, no activity could be recovered. The same has been found for pepsin[62], and trypsin too, spread at air : water or oil : water interfaces, loses its activity completely and irreversibly[8,31]. It is now generally agreed that this is the case for most enzymes spread at these interfaces, so that we may now say that at least part or, in some cases, possibly all, of the secondary and tertiary structure of the enzyme molecule is essential for its function.

One difficulty attending measurements of enzyme activities of spread monolayers is that the protein must be collected for assay, either by

deposition on slides, draining the substrate away leaving the film spread on filter paper, collapsing the films to form insoluble fibres and testing their activity or redissolving the fibres in other media, etc. In all these procedures it is tacitly assumed that processes subsequent to the spreading of the enzyme film do not lead to renaturation. There is thus a need for assaying the enzyme *in situ*, but the dangers of contaminating the film with unspread globular protein from the sub-solution are great.

Harkins and colleagues[63] have deposited films of catalase 55 Å thick on conditioned metal slides which exhibited only 5 to 10 per cent activity. They reported that this layer adsorbed first, a layer of antibody protein which gave no further decrease of activity and second, another layer of enzymes only 10 Å thick possessing no activity. The first layer, though it had lost much of its enzymatic activity had apparently retained high immunological specificity. Danielli and others[64], on the other hand, failed to demonstrate the reaction of pneumococcus type II polysaccharides with spread films of the specific antibody proteins. Bateman and others[65] reacted spread egg albumin films transferred to slides with rabbit antisera, but the films were 14 to 20 Å thick, suggesting incomplete spreading. Rothen and Landsteiner[66] however, claim to have obtained immunological specificity with spread films of egg albumin, heat denatured egg albumin, human and horse serum albumins, horse serum globulins, etc., where all the films were carefully spread at the air : water interface to 8 to 10 Å thickness. The globulins lost their ability to react with antibody rapidly on standing while the egg and horse serum albumins retained this property even if left for sixteen hours under no compression. This certainly suggests that at least a few immunological reactions are probably dependent only on the primary structure of the protein or on a very stable or reversibly lost secondary structure.

Rothen and colleagues (see [8])* have also tested the effects of surface denaturation on various protein hormones, being careful to spread the films to 7 to 9 Å thickness. They found that films of *metakentrin*, a gonadotrophic hormone of the anterior lobe of the pituitary gland, and the oxytocic hormone of the posterior lobe of the pituitary, lost 95 to 100 per cent activity on spreading, redissolving and testing for hormonal activity. Insulin, however, retained full activity after having been spread at the air : water interface. Possibly this small molecule can undergo reversible unfolding. It would be interesting to see this observation repeated. Rothen[8] reports also that all these hormones retain their ability to react with specific antisera when spread at the air : water interface and deposited on conditioned plates. In these transfer experiments it is important that the layers recovered should contain only protein unfolded to a uniform thickness, and not be contaminated with unspread globular material. On such considerations Dean, Gatty and Stenhagen[67] have called into question the techniques employed in obtaining these results. However, a generalisation of the usually accepted view that unfolding on adsorption at the air : water and oil : water interfaces leads to a complete

* This review contains a description of the technique of measuring thicknesses of deposited films using elliptically polarized light.

and irreversible loss of biological activities of proteins awaits further experimentation.

Enzyme-Lipid Interactions at Interfaces

In view of the findings that many intracellular enzymes are associated with lipids and nucleic acid in various particulate fractions like the membrane, nucleus, mitochondria, or microsomes, the study of enzyme-lipid interactions at interfaces is likely to yield more practical results from a biological point of view than are studies at the clean air : water and oil : water interfaces. Some early studies at lipid covered interfaces have shown that the packing of the lipid molecules in the film is important in enzyme-lipid interactions. Rideal[68] showed that the rate of digestion of lecithin monolayers by black tiger snake venom in the sub-phase changes on compression of the films. Similarly Schulman[69] showed that the orientation and packing of the long-chain esters at the oil : water interface is a prime factor in the rates of their hydrolysis by pancreatin. However, little is known of the nature of these enzyme surface lipid interactions, for example, to what extent the enzyme unfolds on adsorption at the substrate-lipid surfaces.

Elkes and Frazer[70,71] found that a lecithinase of *Clostridium welchii* type A toxin caused flocculation of the chylomicrons in lipaemic sera due to interference with the stabilising phospholipid film. This enzyme also creamed lecithin stabilised emulsions, but had no effect on intestinal emulsified fat, likely stabilised by a fatty acid-glyceride-soap film. The study of the adsorption of enzymes on lipid stabilised oil droplets by the "emulsion technique" has been extended considerably by Fraser, Kaplan and Schulman[33] and Fraser and Schulman[31]. They have followed the adsorption of the enzymes catalase and trypsin at oil : water interfaces stabilised with water-insoluble lipids so as to minimise the bulk interaction. Changes in the activity of the large (M.W. = 225,000) haem-enzyme catalase in the decomposition of hydrogen peroxide, and of the small (M.W. = 17,000) basic protein trypsin in the hydrolysis of benzoyl arginine ethyl ester were followed. Catalase is reported to have an isoelectric point of 5·7[72], while that for trypsin is nearly pH = 11·00[73]. Trypsin lacks a prosthetic group also, so that the two enzymes are of vastly different natures. The water insoluble stabilisers used were an anthracene-C_{22}-long-chain sulphonate, cephalin (chiefly phosphatidyl ethanolamine) mixed sodium lauryl phosphates, oleic acid-glyceryl mono-oleate, and mixed films of octadecyl trimethyl ammonium bromide (C_{18}TAB) with various long-chain and cyclic alcohols. The adsorption was followed over a wide range of conditions.

Briefly, the technique was to add appropriate amounts of buffered emulsion to dilute solutions of the enzymes, separate the oil droplets with their adsorbed protein by centrifugation, wash them in buffer, resuspend and assay them (also the subnatant fluid and washes) for enzymatic activity. The washed globules could be resuspended in medium in which the protein was desorbed from the interface, the enzyme being released into the bulk phase, then assayed for activity. Thus measures were had

of the amount of protein that adsorbed per unit of surface area of emulsion, the reduction of activity on adsorption, and the reversibility on desorption.

By the appropriate choice of stabilisers it was found possible to obtain these two enzymes adsorbed on emulsion droplets in the form of monolayers and multilayers varying in state from fully active to completely and irreversibly inactive enzyme. It was found that the loss of activity of the enzyme on adsorption could be related to the degree of unfolding of the protein which was dependent on the charge on the protein and interface, pH, polar group specificity and steric factors. Provided that the unfolding process was not allowed to go to completion, desorption of the enzyme from the interface, by charge reversal or displacement with a surface-active agent, resulted in partial to complete restoration of activity. In the extreme cases, catalase could be adsorbed on the positively charged $C_{18}TAB$ + *n*-lauryl alcohol-stabilised interface at pH = 8·3 without loss in activity, while both enzymes adsorbed on the sulphonate-stabilised interface at pH = 4 to 5 were in the form of completely inactive monolayers. Desorption of the protein in the former case occurred with full retention of activity, while none could be regained in the latter. However, in the sulphonate system if excess protein were present it adsorbed strongly in the form of multilayers (over 100 mg./M^2) which possessed considerable activity and yielded active enzyme on desorption. The more protein that adsorbed the greater the activity recovered on desorption. A great number of intermediate forms between these extreme cases were demonstrated. Both enzymes lose their activities irreversibly at the clean air : water and oil : water interfaces as mentioned above.

These surface interactions were compared to the bulk interactions of the enzymes with the soluble lipid analogs SDS and DTAB. As in the bulk phase, the action of the quaternary ammonium compounds was much milder than the action of long-chain sulphates and sulphonates. Wills[74] has recently studied the latter for a wide range of enzymes, suggesting that they are inactivated by adsorption on to micelles. However, several instances in the above papers seemed to indicate that the unfolding of the proteins through the binding of detergent could take place well below the micelle point, a picture more in line with the mechanisms of Putnam[35].

In the case of the adsorption of trypsin on the ionogenic (charged) lipid interfaces observations by the "emulsion technique" were paralleled with the microscopic electrophoretic mobility technique. For example, in the presence of increasing amounts of trypsin at pH = 4·0, the high negative mobility of the long-chain sulphonate-stabilised droplets decreased rapidly to zero when 10 mg. protein/M^2 surface area had been added. Then the mobility slowly reversed in sign taking a constant small positive value above 15 to 20 mg./M^2, approaching that for trypsin itself at this pH. All of the protein added, at least up to 25 mg./M^2, resided at the interface. Another use of this technique was to follow the pH mobility curves for the droplets in the presence of just enough protein to

neutralise the surface charge at pH = 4·0. The mobility of the sulphonate-stabilised droplets is almost constant over the pH range 3 to 12. In the presence of trypsin it decreases by an amount which is proportional to the bulk mobility of the protein, i.e., the amount of protein that adsorbed was proportional to the net charge on the protein. The same was found to hold roughly for adsorption on the lauryl phosphate interface, but quite a different effect was observed at the oleic acid-glyceryl mono-oleate-stabilised oil : water interface. Here as the pH of the medium was lowered the carboxyl groups of the fatty acid were titrated so that at pH = 4·0 the mobility was nearly zero. As expected, there was no measurable adsorption of trypsin at this pH. However, at hydrogen-ion concentrations above pH 8·0 where the droplets had a constant high negative mobility (all the carboxyls were ionised) the positively charged trypsin did not adsorb either. Only in the narrow pH range 5 to 8 did appreciable adsorption occur. These results were confirmed by direct measurement of adsorption using the "emulsion technique". Apparently there must be both charged and uncharged carboxyl groups in the interface to react with the polar groups on the protein. Perhaps special steric requirements are thus fulfilled.

Enzymes at Solid : Water Interfaces

Mention should be made at this point of the adsorption of enzymes at solid : water interfaces, though more work has been done in this connection in the field of chromatography of proteins[75] than with a view to testing the effects of adsorption on enzymatic activity. Talibudeen[76] has studied the adsorption of several proteins and amino acids on montmorillonoid clays by X-ray diffraction methods. The information obtained was used to calculate the thickness of one and two layers of protein which were shown to correspond to the thickness of one and two close-packed polypeptide chains of the β-keratin type, that is, spread monolayers 6 to 9 Å thick. It is doubtful whether enzymes could exhibit activity under these circumstances. Adsorption on these negative surfaces was stronger when attempted at acid pH values where the proteins bore strong positive charges. More than three layers of spread protein could be adsorbed on these clays.

McLaren[77–79] has studied the adsorption of enzymes on negatively charged kaolinite particles, finding that the adsorption led to a reduction or loss in activity of lysozyme, pepsin, trypsin, or chymotrypsin. The adsorption apparently involved both an ion exchange with adsorbed metal cations, and simple adsorption on the external surface of the particles. Some activity of lysozyme and trypsin could be regained on elution (desorption) of these enzymes with ethylamine. Chymotrypsin was still capable of digesting adsorbed heat-denatured lysozyme, but not adsorbed native lysozyme. Apparently, then, the adsorption of these enzymes to kaolin, under the conditions employed, does not radically modify their structure. This adsorption can thus be compared with that to the milder-acting lipid-stabilised oil : water interfaces. The dense packing and immobility of the "adsorbent" molecules probably prevents

extensive penetration and unfolding from occurring. Thus solid surfaces are of more use in the chromatographic separation of enzymes.

Partially Active Enzymes

In any study of the action of denaturing agents in reducing the activity of enzymes one of the problems that arises is whether or not the effect of the agents is "all-or-none" or whether there are intermediate stages in the action giving rise to partially active enzymes. Thus, though the loss in activity of an enzyme parallels its general structural unfolding as measured by changes in viscosity, solubility, birefringence of flow, exposure of –SH and phenolic –OH groups, etc., the activity measurements do not reveal whether one is following displacement of an equilibrium between active and inactive forms of an enzyme, or of an equilibrium between intermediate forms of the enzyme having only partial activity. For example, Harris[53] has found that the inactivation of trypsin in solution by high concentrations of urea is paralleled by a great increase in viscosity. This he states is due to unfolding of the molecules, and he postulates a reversible equilibrium between active and inactive forms.

A virtually unexplored approach to the problem of the action of denaturing or "unfolding" agents, as opposed to specific inhibitors, may be found in the study of the kinetics of partially denatured enzymes. The inhibition by sodium dodecyl sulphate (SDS) of many enzyme activities, though it takes place at fairly low concentrations[74], is accompanied by unfolding of the protein molecules[36]. It may be easily seen from the inhibition curves (conc. SDS vs. enzyme activity) in the literature[31] that a normal type of competitive or non-competitive inhibition does not take place. The usual linear plot of reciprocal of activity vs. concentration of inhibitor is found in these examples to give a hyperbolic curve[31]. This suggests that the effect of SDS is not through an action at the active centre, and it is logical to associate it with the unfolding that occurs.

If strained configurations of enzyme molecules can work at lower efficiency, exhibiting partial activity, then it is possible that kinetic and thermodynamic studies will enable one to differentiate between losses in activity by a drastic (all-or-none) or a slight modification of the structure of the active centre. For example, one might expect the combination of the substrate with the enzyme to be much more difficult and hence would predict an increase in K_m for this reaction. No such measurements have been made, either in bulk or for enzymes adsorbed at lipid-stabilised or solid interfaces. Fraser, Kaplan and Schulman[33] have observed that the activation energy for the catalase-H_2O_2 reaction (the overall reaction, followed by the manometric method) increased by 10 Kcal./mole on adsorption of the enzyme at a cephalin-stabilised olive oil : water interface. They took this as an indication of the action of a partially active form of catalase. The surface enzyme had only one-eighteenth the activity of the bulk enzyme. Desorption of the enzyme, though it did not restore the catalase to full activity, did result in a return of the activation energy to its original level. This system was thus a good model for the intracellular yeast catalase studied by Fraser and Kaplan[49]. The yeast enzyme

possesses a low activity and high activation energy compared with crystalline catalase in bulk. Treatment of the yeast cells with various physical and chemical agents or extraction from the cells converts or alters the enzyme to the bulk state. This was considered to come about through a desorption of the partially unfolded normal enzyme from some interface within the cell accompanied by its renaturation. The argument that an unfolded enzyme is expected to exhibit a high activation energy for substrate decomposition[49] was based on Eyring's picture of protein denaturation and is too involved to be reproduced here. However, it is well to point out that there are many possible causes for a change in activation energy for an enzyme-substrate reaction on adsorption of the enzyme at an interface, for example, the interfacial pH might be considerably different from the bulk pH, the active centres of the enzymes may become partially buried on adsorption so that the substrate no longer has free access to them, and, if water takes part in the reaction, changes in hydration of the protein or of dielectric constant may have occurred. McLaren[80] reports, for example, that the optimum pH, and the pH for half-maximal activity are 2 pH units higher for chymotrypsin adsorbed on kaolinite particles than in bulk solution, indicating that the H^+ concentration at the interface is 100 times that of the bulk concentration. Thus a method of dealing with the problems of partially active enzymes by kinetic and thermodynamic measurements, though fascinating and very worthwhile, must be approached with caution.

There are indications that partially active forms of enzymes can exist in relatively simple systems. Hammond[81] has found that the loss of activity of ficin in hydrolysing benzoyl arginine ethyl ester at alkaline pH is due to the titration of a positive group. On shifting the pH back to the optimum full activity is regained. However, if dimercaprol (BAL) is present only 70 per cent of the activity is recovered at any time. Hammond suggests that this is due to a structural change in the ficin molecules probably resulting from the scission of –S–S– links (with unfolding?). Alkaline pH is known to accelerate the breakage of these bonds by thiol compounds[82]. Citri[83] has found that adsorption of the purified α-penicillinase from *B. cereus* on pyrex glass results in its conversion to the γ form, resembling the bound intracellular enzyme in its antigenic properties and sensitivity to inactivation by iodine[84]. Though little decrease in activity is observed on adsorption to the glass, an increase of activity is observed on desorption in the presence of gelatin.

The State of Intracellular Enzymes

Interfaces in Biological Systems

Cytologists and cell physiologists have long recognised the importance of interfaces in biological systems. Danielli[85] has pointed out that many reactions involving the major components of living tissues, fats, proteins, nucleic acids and carbohydrates, must take place at interfaces. The great majority of neutral fats, for example, are so insoluble in water that the final steps in their synthesis and initial steps in their utilisation must

occur in the interior of the lipoid phase or at the lipid: water interface. That *only* water soluble enzymes can act at the interface, however, is now less likely with the discovery that some lecithinases can operate in organic solvents[86], for example, 80 to 90 per cent ethanol or "wet" ether. The very special orientation of proteins in combination with lipids in the plasma membrane, and membranes of the nucleus, mitochondria and cytoplasm (endoplasmic reticulum) gives rise to unique properties concerned with the transfer of materials and substrates to the proper localities of the cell where they are stored or utilised.

Membranous and other interfacial regions of living cells differ from homogeneous phases with which they are in contact. The pH, ionic strength, oxidation-reduction potential, ratio of univalent to polyvalent ions all may be considerably different from the neighbouring bulk phase. Special forces due to the orientation of the molecules at these interfaces must be considered. Changes in orientation through the penetration of other substances into these layers can alter the rates, activation energies and entropies of chemical or enzymic reactions taking place there[87]. With the great variety of substances to be found in a living cell there are multitudes of possible "micro-environments" in which enzymes can act. To speak of an "intracellular pH" or the "concentration of ions" in a cell in the same manner as in homogeneous solution is not only misleading but quite ridiculous.

Nor must one fall into the danger of thinking of the cell as a static system. New functions come into the fore as the cell grows, divides, differentiates, encapsulates, or reacts to changes in its environment in any way. In view of this constant change we might expect the phenomena of reversible denaturation and "unmasking" of enzyme activities along with the synthesis of "new" enzymes to play an important role in the life of the cell. Meyer, Mark, Goldacre and Lorch[88] have suggested that the reversible folding and unfolding of protein molecules plays an important role in amoeboid movement, cytoplasmic streaming and muscular contraction. Local variations in intracellular pH may help to bring about these changes. It is possible that combination of already synthesised enzymes with macromolecules and interfaces, like ribonucleic acid (RNA) and mixed lipid complexes results in many cases in the temporary "masking" of enzymes. Thus RNA[89] or polysaccharides[90] can act as enzyme inhibitors in certain cases. Subsequent changes in physiological state may serve to unveil their activities, giving rise to different pathways of metabolism of certain materials. It is the hope of the surface chemist to be of some aid to the cell physiologist in elucidating these states and changes in states of intracellular enzymes. Perhaps what is needed at this stage of our knowledge is a Frey-Wyssling[91] with an enzymological bias.

Enzymes Associated with Cell Structures

Since the classical work of Claude[92], Schneider and Hogeboom[93] and others on the isolation of particulate cell components by the technique of differential centrifugation of homogenates, it has become increasingly clear that a large number of enzymes are not haphazardly distributed

in the cytoplasm. They are to be found localised in association with lipids and nucleic acids on such morphologically distinct structures as the plasma membrane, nucleus, mitochondria, microsomes, Golgi apparatus, zymogen and pigment granules, and chloroplasts. It has become clear also that the processes of metabolism are controlled in part by the spatial relations between the enzymes and their substrates, the metabolites. A brief survey will be made of the various enzymes known to be associated with cell particulates, and the effect this binding has on their activities. A review by Schneider[94] will be found to be more comprehensive on the former topic.

(a) The Cell Membrane

There are many aspects of the study of the plasma membrane of the cell, all of which must be related ultimately to the structure of this complex of lipids and proteins and its function in controlling the passage of materials in and out of the cell. Only the slightest of references to these can be made here. By direct chemical analyses of isolated cell membranes[95], studies of the effects of lytic agents, digestive enzymes[96], detergents and other agents, the use of the microscopic electrophoretic mobility technique in determining the ion-spectra for surface charge reversal by adsorption of polyvalent ions[97], the plasma membranes of red blood cells and bacterial protoplasts have been characterised. Though much work has been done along these lines there is a long way to go towards working out the exceedingly complicated structure of a "simple" cell membrane and to relate this with its functions in "facilitated"[98] or "activated"[99] transport of metabolites.

Sizer[100] studied the properties of yeast invertase which is associated with the cell surface. He found that the activity was the same whether or not the enzyme was associated with the cell. The activation energy for inversion of sucrose was identical for both states of enzyme also. Later Wilkes and Palmer[101] found that the pH activity curves were identical, so that the enzyme is probably directly exposed to the extracellular pH. McLaren[80] however, has recently claimed that these data show a slight surface pH effect due to the negative charge on the yeast cell. Apparently the association of invertase with the cell surface does not affect its activity. We are unfortunately ignorant of the structure of this surface enzyme, but the above data suggest that it is probably in the globular form. Rothstein[102] has reviewed the surface enzymology of the yeast cell (containing invertase, lactase, maltase and phosphatases) suggesting that it is a multimembranous, compartmentalised system. This is a much more complicated picture than that presented for the membranes of red blood cells and bacteria. Even the latter contain adaptively formed "permeases"[103] which concentrate certain substrates rapidly in the cells.

(b) Nucleus and Mitochondria

Both these elements of the cell are structurally very complex, containing their own enzymic environments within limiting membranes. Little is

known of the states of nuclear enzymes. Much more work has been done on the mitochondria. Half their total mass appears to consist of soluble proteins found in the interlamellar spaces, among which are the enzymes glutamic dehydrogenase, fumarase, ribonuclease, deoxyribonuclease, while the cytochrome oxidase, cytochrome c, DPN-cytochrome c reductase and succinic dehydrogenase are bound to the lipid double membranes. Contrary to previous notions, all of the Krebs tricarboxylic acid cycle enzymes do not reside in the mitochondria, some being found in the soluble cell fraction[94]. Some enzymes concerned in fatty acid oxidation are also localised on the mitochondria[94]. Meyers and Slater[104] have recently examined isolated mitochondria for ATP-ase activity and have come to the conclusion that four of these enzymes are present, with different pH optima. As with many other mitochondrial enzymes, physical disruption, in this case freezing and thawing or ageing, results in an activation of some of the forms. Surface-active agents also "unmask" latent ATP-ase activity of mitochondria[105]. One can attempt to generalise that where these enzymes are bound to lipoprotein or to the strongly acidic RNA they probably exhibit only part of their potential activity when supplied with excess substrate. Many agents can act to release these bound enzymes, leading to a "renaturation" with increases in activity in some cases. Loss of co-factors or if the activity depended strongly upon the structural intactness of the lamellae, would result in the loss of certain activities on disruption of the mitochondria.

(*c*) *Microsomes*

Recent electron microscopic studies[106,107], have revealed that the smallest sedimentable cell particles, the microsomes, are comprised of electron dense spheres of nucleoprotein 20 to 150 mμ in diameter, adsorbed on lipoprotein double membranes found in the cytoplasm, the "endoplasmic reticulum". These membranes carry the enzyme activities found to be associated with this fraction. The lipids comprise 45 per cent of the fraction and include lecithin, cephalin, inositol phospholipids and cholesterol-like steroids[108]. Treatment of suspensions of microsomes with strong solutions of sodium deoxycholate solubilises the lipoprotein membranes and allows the small nucleoprotein particles to be sedimented[109]. These consist of as much as 60 per cent RNA. The high interest in the microsome fraction lies in the fact that it is probably chiefly responsible for the synthesis of the cytoplasmic proteins. The nucleoprotein particles are likely concerned in the actual stages of peptide bond formation linking up the "activated amino acids" from the soluble fraction of the cytoplasm[110]. The most mysterious stage in the synthesis at the moment is how the polypeptide chains fold up to produce globular proteins with specific functions, superficially an opposite process to surface denaturation. One may speculate that the lipoprotein membranes, through some adsorption-desorption mechanism, possibly with the help of a substrate-like substance (the "inducer" in the case of adaptive enzyme formation), are functional in the folding-up process. It is interesting to note in this connection that several adaptively formed enzymes are found

associated with the microsome fraction from bacterial cells[111]. With the adaptive formation of β-galactosidase in *E. coli* and of catalase in yeast Bonner[112] and Bonner and Kaplan[113] have suggested that these enzymes are piled up in layers at their sites of synthesis. Their activities can be unmasked by suitable treatment of the cells.

Many cases of increases of microsomal enzyme activities on solubilisation or release from the sedimentable components have been reported. Rat liver aryl sulphatase, for example, is released by cationic and non-ionic surface-active agents with increased activity[114]. The microsomal alkaline phosphatase and xanthine oxidase of cow's milk are released with considerable activity increases by various physical and chemical agents from the surface of the milk fat globules[115,116]. What role reversible folding and unfolding of the protein molecules has to play in these phenomena has yet to be shown. The observations are highly suggestive in the light of the studies of adsorption of enzymes at lipid-stabilised oil : water interfaces discussed above[31,33].

Changes in Physiological States of Cells

It is evident from the discussions on partially active enzymes and the various enzymes associated with cellular particulate fractions that many intracellular enzymes, by virtue of being combined with lipids, nucleic acids, polysaccharides or other components, perhaps at some interface, could be expressing only part, or possibly none, of their potential activity. Splitting, or changes of physical state of these complexes by some environmental change can lead to expression of increased or full activity. Such processes would thus partake in, if not "cause", a change in physiological state of the cell. In these we have possible mechanisms for the evocation or "unmasking" of intracellular enzyme activities alternative to "de novo" synthesis of enzymes. To what extent such mechanisms play a part in the life of the cell remains to be seen. Cheesman and Davies[9] have called attention to the findings of the cytologists Runnström[117] and Holtfreter[118] in this connection. The former demonstrated the release at the cell surface of a powerful protease immediately after fertilisation of sea-urchin eggs. He suggested that it was held in the inactive form in a complex with a heparin-like polysaccharide such as that demonstrated in the jelly coat of these eggs by Immers and Vasseur[90]. The protease could release other enzymes, triggering a new chain of metabolic pathways. Holtfreter[118] found that interaction must occur between yolk platelets and the lipochondria of the amphibian egg before development can proceed. He suggested that the membranes of the lipochondria contain proteases in the inactive form which became activated by this interaction, initiating the new chain of processes required for development.

A number of other instances of unmasking of intracellular enzyme activities by treatment of intact cells with various physical and chemical agents are known. Reference has already been made[49] to the case of yeast catalase studied by Kaplan[119]. The hypothesis was proposed that the normal enzyme exists adsorbed at some intracellular interface in a partially unfolded state where it exhibits only one-eighteenth of its potential

activity, a high activation energy for substrate decomposition and resistance to inactivation by heat and ultra-violet light. Treatment of the cells with ultra-violet light, grinding for extraction, by toluene, chloroform or the homologous series of aliphatic alcohols, aldehydes and ketones, etc., "alters" the catalase to the bulk state. Even if the enzyme remains *in situ* it has all the properties of crystalline catalase in solution. This "alteration" was thought to be due to a desorption of the enzyme from the interface with a consequent folding up of the molecules into the more active globular form. Such phenomena have also been observed in the case of β-galactosidase of *E. coli*[112], and other instances may occur[119].

The difficulty in the interpretation of such observations comes when it is realised that all these treatments of cells result in the breakdown of the specific permeability properties of the cell membrane, permitting equilibration of the cell contents with the external suspension medium and resulting in the "death" of the cell as measured by loss of viability. Thus Few, Fraser and Gilby[120] have proposed that the observed 4-fold increase in catalase activity and changes in activation energy for H_2O_2 decomposition of *Micrococcus lysodeikticus* on treatment with "altering agents" could be accounted for if the intracellular enzyme were acting at an effective pH of 4·6, lower than the pH of the external medium by two units. Chance[121], using spectrophotometric techniques on intact cells, previously concluded that the physical state of this bacterial catalase was essentially identical with that of the purified enzyme in solution. In the case of the yeast catalase however, it is unlikely that pH changes or increases in the permeability of the cells to hydrogen peroxide can account for all the observed changes in the properties of the enzyme, either qualitatively or quantitatively[119,49]. The interfacial hypothesis remains the most likely explanation. The effects of ultra-violet light[122] and ionising radiations[123] in the alteration of yeast catalase suggest that the enzyme is in close association with RNA, which is in line with the suggestion of Bonner and Kaplan[113] that the adaptively formed catalase of anaerobic cells is "piled up" at its site of synthesis. One can thus speculate that the "interface" involved in alteration is provided by the microsomes.

Finally, attention should be drawn to the effect of homologous series of alcohols, aldehydes and ketones in altering yeast catalase activity[124]. The effectiveness of these agents in "altering" the enzyme was found to correspond to their ability to lower the surface tension at the air : water interface, increasing approximately three-fold for each $-CH_2$ group added on the chain, thus following Traube's rule. This relation between alteration and surface activity was taken as further indication that the phenomenon of alteration involved desorption of the enzyme from some intracellular interface through the action of these agents[124]. Another possibility is that this type of alteration occurs by indirect action on the cell membrane, breaking down its permeability properties allowing equilibration of the cell contents with the external buffer medium. Not only would changes in pH and ion concentrations shift the observed enzyme activities and activation energies for substrate decomposition but also they would allow the loosening or splitting of various complexes of proteins

with lipids and nucleic acids, further enhancing the alteration of the properties of the enzymes. Thus "alteration" may be another of the numerous examples of the result of the collapse of lipoprotein cell membranes by surface-active agents[125]. The phenomenon is also related to the augmented action of narcotics with increasing chain length. Whether the mechanism of action of such compounds involves a change in surface activity, according to Traube, Warburg and Clark, or in fat solubility, according to Meyer and Overton, remains unsettled[126]. Dethier, reviewing this topic[127], points out that it is fundamentally a change in thermodynamic activity which changes with chain length.

References

1. Ascherson, *Arch. Anat. Physiol.*, 1840, **44**.
2. Devaux, *Prob. verb. soc. des sci. phys. et nat. Bordeaux*, Nov. 1903.
3. Ramsden, *Proc. Roy. Soc., Lond.*, 1903, **B72**, 156.
4. Wu and Ling, *Chinese J. Physiol.*, 1927, **1**, 407.
5. Langmuir and Waugh, *J. gen. Physiol.*, 1938, **21**, 745.
6. Neurath and Bull, *Chem. Revs.*, 1938, **23**, 391.
7. Bull, *Adv. in Prot. Chem.*, 1947, **3**, 95.
8. Rothen, *ibid.*, 1947, **3**, 123.
9. Cheesman and Davies, *ibid.*, 1954, **9**, 439.
10. Kauzmann, *J. cell. and comp. Physiol.*, 1956, **47**, Supp. 1, 113.
11. Kauzmann, *The Mechanism of Enzyme Action*, ed. McElroy and Glass, Johns Hopkins Press, Baltimore, U.S.A., 1954, p. 70.
12. Edsall, *J. cell. and comp. Physiol.*, 1956, **47**, Supp. 1.
13. Jacobsen and Linderstrøm-Lang, *Nature, Lond.*, 1949, **164**, 411.
14. Haurowitz, *J. cell. and comp. Physiol.*, 1956, **47**, Supp. 1, 1.
15. Kauzmann and Douglas, *Arch. Biochem. Biophys.*, 1956, **65**, 106.
16. Cockbain and Schulman, *Trans. Faraday Soc.*, 1939, **35**, 1266.
17. Hughes and Rideal, *Proc. Roy. Soc., Lond.*, 1932, **A137**, 62.
18. Kaplan and Fraser, *Proc. N.S. Inst. Sci.*, 1950–1, **23**, 91.
19. Kaplan and Fraser, *Nature, Lond.*, 1953, **171**, 559.
20. Schulman and Hughes, *Biochem. J.*, 1935, **29**, 1236.
21. Schulman, *Trans. Faraday Soc.*, 1937, **33**, 1116.
22. Rideal and Schulman, *Nature, Lond.*, 1939, **144**, 100.
23. Schulman, *Cytology and Cell Physiology*, ed. Bourne, 2nd Ed., Oxford, 1951, p. 119.
24. Alexander and Teorell, *Trans. Faraday Soc.*, 1939, **35**, 727.
25. Cumper and Alexander, *ibid.*, 1950, **46**, 235.
26. Davies, *Nature, Lond.*, 1951, **167**, 193.
27. Abramson, Moyer and Gorin, *Electrophoresis of Proteins*, Reinhold Publ. Corp., N.Y., U.S.A., 1942.
28. Cumper and Alexander, *Austral. J. sci. Res.*, 1951, **A4**, 372.
29. Cumper and Alexander, *Revs. pure and appl. Chem.*, 1951, **1**, 121.
30. Seaman and Fraser, unpublished results.
31. Fraser and Schulman, *J. Colloid Sci.*, 1956, **11**, 451.
32. Elkes, Frazer, Schulman and Stewart, *Proc. Roy. Soc., Lond.*, 1945, **A184**, 102.
33. Fraser, Kaplan and Schulman, *Faraday Soc., Discussions*, 1955, **20**, 44.
34. Pankhurst, *ibid., Surface Chemistry*, Butterworth, London, 1949, p. 109.
35. Putnam, *Adv. in Prot. Chem.*, 1948, **4**, 79.
36. Friend, Harrap and Schulman, *Nature, Lond.*, 1951, **168**, 910.
37. Few, Ottewill and Parriera, *Biochim. Biophys. Acta.*, 1955, **18**, 136.
38. Doty and Schulman, *Faraday Soc., Discussions*, 1949, **6**, 21.
39. Matalon and Schulman, *ibid.*, 1949, **6**, 27.
40. Schulman and Rideal, *Proc. Roy. Soc., Lond.*, 1937, **B122**, 29.
41. Eagle, *J. Immunol.*, 1935, **29**, 467.
42. Eley and Hedge, *Faraday Soc., Discussions*, 1956, **21**, 221.
43. Eley and Hedge, *J. Colloid Sci.*, 1956, **11**, 445.
44. Kaplan and Fraser, *Biochim. Biophys. Acta*, 1952, 9, 585.
45. Fraser, unpublished results.
46. Rogers and Kalnitsky, *Biochim. Biophys. Acta*, 1957, **23**, 525.

47. Hill and Smith, *ibid.*, 1956, **19**, 376.
48. Nagahisha and Takamiya, *Symp. on Enz. Chem. (Japan)*, 1950, **5**, 46.
49. Fraser and Kaplan, *J. gen. Physiol.*, 1955, **38**, 515.
50. Sela and Anfinsen, *Biochim. Biophys. Acta*, 1957, **24**, 229.
51. Anson and Mirsky, *J. gen. Physiol.*, 1933–4, **17**, 151.
52. Anfinsen, Harrington, Hvidt, Linderstrøm-Lang, Ottesen and Schellman, *Biochim. Biophys. Acta*, 1955, **17**, 141.
53. Harris, *Nature, Lond.*, 1956, **177**, 471.
54. Gorter, *Proc. Roy. Soc., Lond.*, 1936, A**155**, 706.
55. Langmuir and Schaefer, *J. Amer. chem. Soc.*, 1938, **60**, 1351.
56. Sobotka and Bloch, *J. phys. Chem.*, 1941, **45**, 9.
57. Hayashi and Edison, *J. Colloid Sci.*, 1951, **5**, 1437.
58. Hayashi, *J. gen. Physiol.*, 1952, **36**, 139.
59. Kaplan, *J. Colloid Sci.*, 1952, **7**, 382.
60. Mazia and Hayashi, *Arch. Biochem. Biophys.*, 1953, **43**, 424.
61. Kaplan, personal communication.
62. Cheesman and Schuller, *J. Colloid Sci.*, 1954, **9**, 113.
63. Harkins, Fourt and Fourt, *J. biol. Chem.*, 1940, **132**, 111.
64. Danielli, Danielli and Marrack, *Brit. J. exp. Path.*, 1938, **19**, 393.
65. Bateman, Calkins and Chambers, *J. Immunol.*, 1941, **41**, 321.
66. Rothen and Landsteiner, *J. exp. Med.*, 1942, **76**, 437.
67. Dean, Gatty and Stenhagen, *Nature, Lond.*, 1939, **143**, 721.
68. Rideal, *Science*, 1939, **90**, 217.
69. Schulman, *Trans. Faraday Soc.*, 1941, **37**, 134.
70. Elkes and Frazer, *J. Physiol.*, 1943, **102**, 24P.
71. Elkes and Frazer, *ibid.*, 1945, **104**, 3P.
72. Sumner and Dounce, *J. biol. Chem.*, 1937, **121**, 417.
73. Bier and Nord, *Arch. Biochem. Biophys.*, 1951, **33**, 320.
74. Wills, *Biochem. J.*, 1954, **57**, 109.
75. Zittle, *Adv. in Enzymol.*, 1953, **14**, 319.
76. Talibudeen, *Trans. Faraday Soc.*, 1955, **51**, 582.
77. McLaren, *J. phys. Chem.*, 1954, **58**, 129.
78. McLaren, *Proc. Soil Sci. Soc. of America*, 1954, **18**, 170.
79. McLaren, *Arch. Biochem. Biophys.*, 1956, **61**, 158.
80. McLaren, *Science*, 1957, **125**, 697.
81. Hammond, personal communication, to be published.
82. Barltrop, Hayes and Calvin, *J. Amer. chem. Soc.*, 1954, **76**, 4348.
83. Citri, communicated and confirmed by Kaplan.
84. Pollock, *J. gen. Microbiol.*, 1956, **15**, 154.
85. Danielli, *Nature, Lond.*, 1945, **156**, 468.
86. Hanahan and Vercamer, *J. Amer. chem. Soc.*, 1954, 76, 1804.
87. Rideal, *J. chem. Soc.*, 1945, 423.
88. Meyer, Mark, Goldacre and Lorch, *Nature, Lond.*, 1951, **167**, 736.
89. Swartz, Kaplan and Frech, *Science*, 1956, **123**, 50.
90. Immers and Vasseur, *Experientia*, 1949, **5**, 124.
91. Frey-Wyssling, *Submicroscopic Morphology of Protoplasm*, 2nd ed., Elsevier Pub. Co., London, 1953.
92. Claude, *Adv. in Prot. Chem.*, 1949, **5**, 423.
93. Schneider and Hogeboom, *Ann. Rev. Biochem.*, 1956, 201.
94. Schneider, *3rd Internat. Cong. Biochem., Brussels, rapport*, 1955, 143.
95. Gilby and Few, *Proc. 2nd Internat. Cong. Surface Activity*, Butterworth, London, 1957.
96. Salton, *ibid.*, 1957.
97. Seaman and Pethica, *ibid.*, 1957.
98. Stein and Danielli, *Faraday Soc., Discussions*, 1956, **21**, 238.
99. Davson and Danielli, *The Permeability of Natural Membranes*, Cambridge, 1943.
100. Sizer, *J. gen. Physiol.*, 1938, **21**, 695.
101. Wilkes and Palmer, *ibid.*, 1932, **16**, 233.
102. Rothstein, *The Enzymology of the Cell Surface, Protoplasmologia*, II E4, Springer Verlag, Vienna, 1954; *Science*, 1951, **114**, 683.
103. Monod, *Henry Ford Hospital Internat. Symp.: "Enzymes: Units of Biological Structure and Function"*, ed. Gaebler, Academic Press, N.Y., U.S.A., 1956, p. 7.
104. Meyers and Slater, *Nature, Lond.*, 1957, **179**, 363.
105. Pressman and Lardy, *Biochim. Biophys. Acta*, 1956, **21**, 459.

106. Palade, *Anat. Rec.*, 1952, **114**, 427.
107. Sjöstrand, *Nature, London*, 1953, **171**, 30.
108. Claude, *Harvey Lectures, Ser.* **48**, Jan. 1948.
109. Palade, *Henry Ford Hospital Internat. Symp.*: "*Enzymes: Units of Biological Structure and Function*", ed. Gaebler, Academic Press, N.Y., U.S.A., 1956, p. 185.
110. Hoagland, Keller and Zamecnik, *J. biol. Chem.*, 1956, **218**, 345; Littlefield and Keller, *ibid.*, 1957, **224**, 13.
111. Bowen and Dagley, *Biochem. J.*, 1955, **59**, xxiv.
112. Bonner, *Amino Acid Metabolism*, ed. McElroy and Glass, The Johns Hopkins Press, Baltimore, U.S.A., 1955, p. 193.
113. Bonner and Kaplan, personal communication, to be published.
114. Spencer, Dodgson, Rose and Thomas, *3rd Internat. Cong. Biochem.*, Brussels, 1955, 4–20.
115. Morton, *Biochem. J.*, 1954, **57**, 231.
116. Robert and Polonovski, *Faraday Soc., Discussions*, 1955, **20**, 54.
117. Runnström, *Adv. in Enzymol.*, 1949, **9**, 312.
118. Holtfreter, *J. exp. Zool.*, 1946, **101**, 355.
119. Kaplan, *Exptl. Cell. Res.*, 1955, **8**, 305.
120. Few, Fraser and Gilby, *Biochim. Biophys. Acta*, 1957, **24**, 306.
121. Chance, *Science*, 1952, **116**, 202.
122. Kaplan and Paik, *J. gen. Physiol.*, 1956, **40**, 147.
123. Aronson, Fraser and Smith, *Radiation Res.*, 1956, **5**, 225.
124. Kaplan, *J. gen. Physiol.*, 1954, **38**, 197.
125. Pethica, *Faraday Soc. Discussions*, 1956, **21**, 286.
126. Danielli, *Cell Physiology and Pharmacology*, Elsevier Pub. Co., Inc., Amsterdam, 1950.
127. Dethier, *Ann. N.Y. Acad. Sci.*, 1954, **58**, 139.

RESEARCH PAPERS

CELLULAR CONSTITUENTS. MAJOR AND MINOR METALS IN NORMAL AND ABNORMAL TISSUES

PART I. ANALYSIS OF WISTAR RAT LIVERS FOR COPPER, IRON, MAGNESIUM, MANGANESE, MOLYBDENUM AND ZINC

BY F. BERGEL, J. L. EVERETT, J. B. MARTIN AND J. S. WEBB

From the Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, and the Department of Geology, Royal School of Mines, London

Received March 25, 1957

Assessment of the usefulness of emission spectrography for comparative analyses of six elements, Cu, Fe, Mg, Mn, Mo and Zn, in rat liver has been made. It has uncovered the presence or absence of differences in the concentrations of these metals in livers between age groups, animals on different diets, pregnant animals and male animals with normal, regenerating and tumourous organs. A discernible trend in concentrations of certain elements from the embryonic tissue to that of the adult animal emerged. In some instances the abnormal and regenerating materials appeared to carry lower or higher amounts of the element compared with normal livers. With molybdenum, the livers of embryonic, newborn and young animals had low concentrations, slowly increasing; the results obtained with older animals formed a higher level rugged plateau.

COMPARATIVE analyses of normal and abnormal tissues or cells for organic constituents and for levels of enzyme activities have been carried out over a number of years in many laboratories[1]. Estimation of nucleic acids, proteins, amino acids, lipids, carbohydrates, vitamins, coenzymes, hormones, etc. have been undertaken in embryonic, regenerating, adult and corresponding tumourous material. While so far usually only quantitative and not qualitative differences between the contents of various comparable tissues have been found, these observations are helping towards a greater understanding of the biochemistry of growth, development and cancer.

More recent work has attempted to find more experimental support for the hypothesis[2] that induction of malignancy is due, at least in part to loss of, or change in essential proteins. Apart from the studies of the Millers[3] with azobenzenes, Weiler[4] from an immuno-chemical viewpoint established that the feeding to animals of butter yellow (4-dimethylamino-azobenzene) which leads to the development of hepatomas, causes also a loss of a liver antigen. He demonstrated later[5] a similar antigen loss in the kidney of the male hamster after insertion of stilboestrol pellets in the flank, producing over a period tumours[6] in that organ. Bhargava and Heidelberger[7] showed that 1:2:5:6-dibenzanthracene, a carcinogenic polycyclic hydrocarbon, in form of an oxidation product combined to a certain extent with the skin-proteins of mice which had been treated with the agent.

The chemical nature of the changed or partly deleted proteins has not been established up to now. But estimations of enzyme activities have shown that some of these levels are lower in tumours than in normal tissues, although they fall rarely to zero. To quote one example, the livers of animals treated with butter yellow according to Westerfeld, Richert and Hilfinger[8] have a diminished xanthine oxidase activity compared with those of untreated animals. This enzyme, which contains flavin adenine nucleotide, molybdenum and iron, is being submitted to a chemical study by Bergel, Bray and colleagues[9,10] and has been the subject of analytical investigations by Lewin[11]. He estimated the level of xanthine oxidase activity in the breast tissue of mice belonging to low (C−) and high (C+) tumour strains, and found that it fell per cell from that in the C− mice, via that in non-tumourous tissue of the C+ mice, to a low value in the breast carcinoma itself. Another aspect of changes of proteins and related compounds, this time in the cell membrane, was uncovered by Ambrose, James and Lowick[12] who found that the surface charge of tumour cells was of a more negative character compared with normal cells. Before then DeLong, Coman and Zeidman[13] had drawn attention to the loss of mutual adhesiveness between malignant cells and put forward the suggestion that this was due to a deficiency in calcium in the cell membrane.

These few examples give additional impetus to the idea that tissue or cell analysis in the field of cancer research ought to include estimation of major and minor mineral constituents. Many investigations have been made to correlate the content of trace elements in water, soil, plants and animals[14–16] and, to establish their general role in nutrition (cf. Underwood[17]). Lately, Tipton and colleagues[18,19], Koch, Smith, Shimp and Connor[20] and Stitch, and Sowden and Stitch[21,22] have commenced studies on the concentration of certain metals in human organs. Their exploratory researches leading to semiquantitative data should be contrasted with work aiming at the purification and characterisation of metal-carrying cellular constituents. One aspect of recent investigations on metalloflavoproteins has been mentioned before[9,10,23]. Systematic studies by Vallee and colleagues[24] resulted in the discovery that zinc formed an integral part of a number of dehydrogenases.

In other instances, such as the pyridoxal-pyridoxamine enzymes, and many more, the role of metals as activators through chelation of coenzyme, substrate and, maybe, apoenzyme has been established. Not yet fully confirmed is the possibility, as proposed by Kirby[26,27] and followed up by us in collaboration with him, that metals may participate in the bonding of deoxyribonucleic acid to proteins.

For our work, as presented in this paper, we have chosen an experimental approach which amounts to a restriction of the analytical procedures, in this instance emission spectrography, to one organ of a defined animal species of different ages and of known nutritional status, and to a small number of elements, namely Cu, Fe, Mg, Mn, Mo and Zn. In this way we were hoping to compensate for the numerous technical difficulties in obtaining significant results. Apart from the problem of selecting

normal and abnormal tissues for reliable comparison, a problem which we temporarily solved by concentrating on livers, there is the complication through variations of tissues in contents of fat, connective material, residual blood, necrotic or inflamed parts. Moreover, there remains the decision of chosing a practical base line, may it be wet or dry weight of the organ, its total nitrogen or phosphorus content or its cellularity, on which one can calculate the analytical data. Thus we were less expecting "absolute" values but more hoping for disclosure of certain trends in the changes of metal levels from one age group to the next, and in animals fed with carcinogenic agents or having a regenerating organ. Consequently, leads might emerge which could be followed by assessment of coenzyme or enzyme contents, or of metal-containing cellular components of a still undetermined functional character.

Animal Material

The livers of Wistar rats, bred at the Chester Beatty Research Institute and killed by breaking the neck, were removed immediately after death and pooled, so that sufficient material for analysis was available and the effect of variations between individual livers was diminished. Throughout, metal tools were avoided and glass knives and rods substituted. The following groups of livers (not perfused, except one tumourous liver) were used.

(1) Normal livers from male rats at different age levels and fed on a 14 per cent special cake nuts diet (North-Eastern Agricultural Co-operative Society, Ltd., Aberdeen). (*a*) 127 foetal livers for four samples; (*b*) 50 new-born for two samples; (*c*) 30 one-week old; (*d*) 30 two-weeks old; (*e*) 25 three-weeks old; (*f*) 21 four-weeks old; (*g*) 24 five-weeks old; (*h*) 30 six-weeks old; (*i*) 24 seven-weeks old; (*j*) 12 eight-weeks old; (*k*) 12 sixteen-weeks old; (*c*) to (*k*) one sample each.

(2) Normal livers (20 for three samples) from pregnant rats, 16 to 20 weeks old, fed on rat cakes and kitchen scraps; these animals were used also as a source of the embryos whose foetal livers were mentioned under (1*a*).

(3) Normal livers from rats fed on rat cake and kitchen scraps (*a*) 198 male for seven samples, 4 to 8 weeks old; (*b*) 100 for four samples 8 weeks old.

(4) Regenerating livers (8 for two samples) from rats, 8 weeks old, previously operated on for partial hepatectomy (removal of one lobe of the liver), the organ then being allowed to regenerate for 5 and 8 days respectively. The operations were carried out by Dr. Sheila Doak, to whom we are greatly indebted.

(5) Tumour tissue, excised from livers (7 for 7 samples, one perfused) of rats fed for thirteen to fourteen months on a diet with 20 per cent protein and 0·42 per cent of methyl butter yellow (4-dimethylamino-3′-methyl-azobenzene). The types of tumour present could not be accurately distinguished; they consisted mostly of hepatomas but also some cholangiomas and adenocarcinomas. As Price, Harman, Miller and Miller[28] have expressed the opinion that usually there is a gradual transition

from one type of abnormal tissue to the other, we refrained from classifying histologically the malignant liver-parts, and shall refer to them as "liver tumours".

Analytical Methods

(*a*) *Ashing of biological material.* Each group of pooled livers was weighed ("wet weight") and then dried in an electric oven at 110° for 24 hours. This material was powdered by grinding it in a glass mortar with glass pestle and dried at 110° to constant weight ("dry weight"). The powder was subsequently defatted by extraction in a soxhlet with absolute ether. The extracted residue was freed from solvent *in vacuo* with warming up to 50°. The product, weighed for "dry-defatted weight" ("W_{DD}") was incinerated in a "Vitreosil" crucible, placed in an electrically-heated muffle furnace at temperatures reaching 435° ± 1° inside 3 hours[15], when the heating was continued for another 7 hours. After cooling, the crucible was removed and its contents ground with an agate pestle. A few crystals of spectroscopically pure ammonium nitrate were mixed with the powder and the heating in the furnace continued at 435 ± 1° for 24 hours. The resulting colourless ash was weighed ("ash weight", "W_A") and stored in polythene specimen tubes until submitted to emission spectrographic analysis.

(*b*) *Emission spectrography.* The ash (*ca.* 20 mg.) was mixed with an equal weight of pure carbon powder and the mixture compressed into the bore of a Jelfke electrode made from spectrographically pure carbon rod to the following standard specification: external diameter of electrode, 3·0 mm.; internal diameter and depth of longitudinal bore, 0·8 mm. and 8·0 mm., respectively.

Four replicate spectrograms of each sample were obtained by the cathode layer arc technique (cf. Mitchell[14]) using a Hilger Large Quartz Spectrograph (E.492) and a 9 amp. D.C. arc. The external optical train consisted of a condensing lens and step filter (ratio 1 : 1·7). The lens focussed the cathode layer on the collimator of the spectrograph, and the spectrograms were recorded on Kodak Photoscript B.10 plates over the range 2700 to 4800 Å. The samples were burned to completion in the arc; this required a 200 second exposure.

The wavelengths of the lines used for the estimation of each element are included in Table I. The density of the spectral lines on the sample plates were measured using a Hilger microphotometer, correction being made for "background" in the normal manner. The concentration of each element sought was estimated in a manner similar to that described by Mitchell[14], with the exception that Seidl density values[29] were used instead of normal density values, the concentration being read graphically from a working curve correlating log concentration in parts per million of ash (log W_{E1}) and log per cent relative density of the spectral line.

The mean working curves were established from the line densities on replicate spectrograms given by a series of synthetically prepared standards containing known quantities of the elements sought. Each standard was spectrographed four times.

TABLE I

WAVELENGTHS OF LINES USED AND REPRODUCIBILITY OF RESULTS IN EMISSION SPECTROGRAPHY

Element and wavelength (A)	A		B	
	Known conc. in standard (p.p.m.)	Rel. standard deviation ± per cent	Mean conc. in sample (p.p.m.)	Rel. standard deviation ± per cent
Cu 3274	100	9·5	235	8·4
	316	8·4	386	6·6
	1000	2·8	509	7·8
	3162	6·5	1390	8·3
Fe 3059	1000	7·35	2560	21·1
	3162	7·98	6270	9·73
	10,000	6·54	7810	8·96
	31,620	7·05	20,200	10·89
	100,000	13·06	45,200	8·63
Mg 2783	1000	10·6	1040	15·1
	31,620	17·4	13,100	11·8
			16,000	9·9
Mn 2801	31·6	5·4	38	2·9
	100	4·5	55	5·8
	316	6·0	80	6·6
Mo 3170	3·2	15·8	7	13·8
	10·0	22·2	24	15·6
	31·6	11·4	34	18·8
	100	10·1	45	9·0
Zn 3345	3162	17·8	2450	12·1
	10,000	10·6	3250	10·9
			4830	12·7

The synthetic base, to which the elements were added in appropriate logarithmic proportions, was prepared by Messrs. Johnson and Matthey, London, by fusing the following mixture: KH_2PO_4 (21·0 g.); NaH_2PO_4 (4·96 g.); $CaCO_3$ (0·37 g.); Na_2CO_3 (0·42 g.); $NaNO_3$ (0·16 g.); NaCl (1·64 g.) and $(NH_4)_2SO_4$ (0·28 g.). The composition of this mixture was based on results of a bulk analysis of ashed rat organs, kindly undertaken by J. F. Harringshaw and L. S. Theobald of Imperial College, London, who found:—K_2O, 29·93; Na_2O, 8·19; CaO, 0·82; MgO, 2·15; Fe_2O_3, 0·04; SiO_2, 0·11; P_2O_5, 48·21; SO_3, 0·69; CO_2, 0·44; Al_2O_3, 0·02; N_2O_5, 0·4; NaCl, 2·97; H_2O, 1·02 per cent. Organic matter (containing N and/or O, 5·13 per cent); total, 100·12 per cent.

(*c*) *The reproducibility of the estimations.* As previously mentioned, the present examination has been concerned only with the estimation of copper, iron, magnesium, manganese, molybdenum and zinc. Figures for the reproducibility of the results for those elements over the range of concentrations encountered are given in Table I.

Reproducibilities were estimated using both synthetically prepared standards (A) and the results obtained on selected samples (B). For the former, the reproducibility is given as the relative standard deviation obtained by expressing the standard deviation of four estimations as per cent of the known content of the standard. With the samples (B), the standard deviations are expressed as per cent of the mean of four replicate estimations.

The significance of these figures is that statistically the limits of the relative standard deviation should be exceeded only 1 in 3 times by

chance. Data A and B show relatively good agreement, and in view of the fact that the samples represented in B are those which showed maximum variation, it is considered that a satisfactory degree of reproducibility has been achieved with the spectrographic method employed.

Results and Discussion

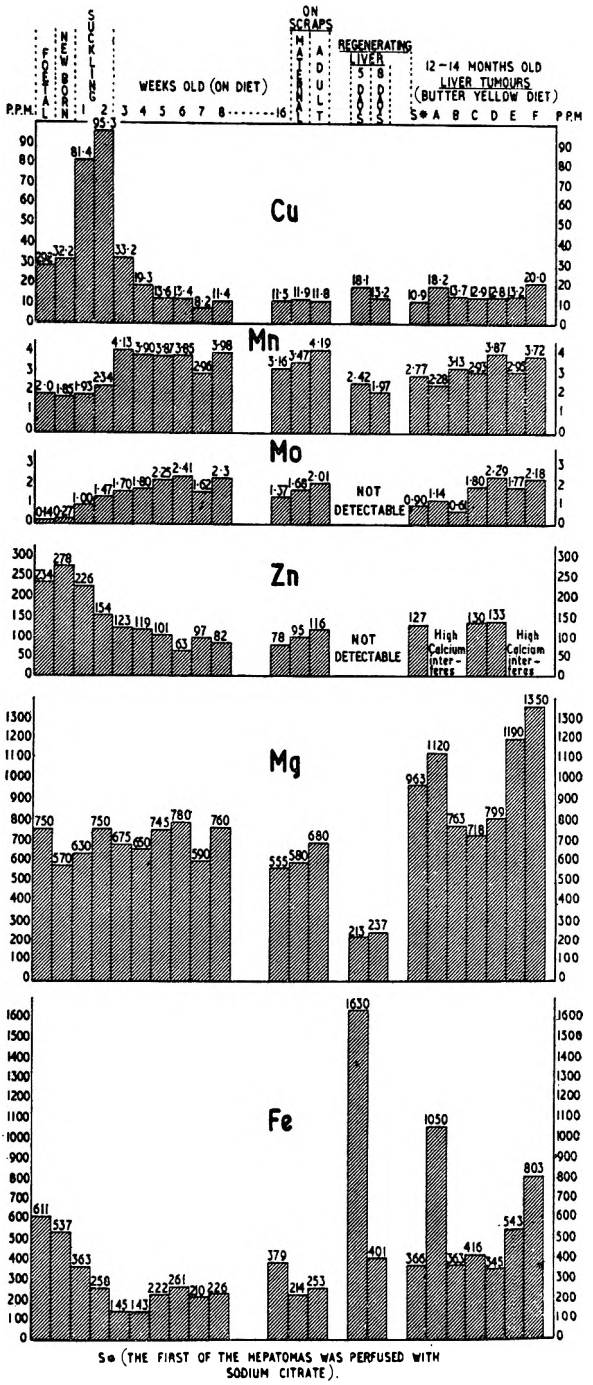
In a preliminary spectrographic survey of 30 elements the following were not detected under our experimental conditions: Be, Bi, Cd, Co, Cs, Ga, Ge, Hg, In, Li, Sb and Tl. While Rb was a contaminant of the synthetic base (cf. p. 526), Ag, Ni, Sn and Zr were found to be present in small amounts in certain samples only, and Ba, Cr and Pb seemed to form genuine constituents of some of the livers. Our more extensive studies were restricted to six elements, namely, Cu, Fe, Mg, Mn, Mo and Zn, which are known to play an important role as part of enzymes or as their activators.

The results are reported in Table II and Histogram I, where by using different scales for the various metals and averaged values for some groups, the changes are visually summarised. The data are expressed as μg. of element per g. of dried defatted tissue (W_{DD}) or p.p.m., calculated from the ratio W_A/W_{DD} (as given in Table II, column 1) and multiplied by W_{E1} where $W_{E1} = \mu$g. of element per g. of ash as obtained from the photometric values by the method mentioned above.

Copper (*cf.* Underwood[17], pp. 63–74). In normal livers the content increases suddenly from that of the embryos and newborn animals (*cf.* Lorenzen and Smith[30]) to about treble their amount in the one and two weeks old rats which are suckling at the time. The reason for this rise is rather obscure, particularly if one considers the low copper content of milk reported in the literature[31]. Our observation confirms that of Brückmann and Zondek[32], who have found a peak in copper concentrations in rat livers between 10 to 15 days after birth. Like these authors we established that in our animals, fed on a controlled diet, the copper content declined during the period from the 3rd to 16th week. Animals, 4 to 8 weeks old and pregnant rats 16 to 20 weeks old, both groups fed on kitchen scraps, did not differ very much from the former (*cf.* Ashikawa, Smith and Helwig[33]). The "liver tumours" from rats over 12 months old and the regenerating livers, after 5 and 8 days regeneration show only a slight increase compared with the organs of all adult animals mentioned above.

Iron (*cf.* Underwood[17], pp. 26–33). The level in tissues of this major element is, for obvious reasons, subject to fluctuations by the presence or absence of residual blood. Attention is therefore drawn only to the higher values noted in foetal livers of embryos and newborn animals, in liver tumours and in the regenerating organ. The average iron content of all other livers (21 groups) given in Table II is 236 p.p.m.

Magnesium. Figures for this other major element, so far obtained, are of restricted significance without the corresponding data for calcium. However, the values for the regenerating organ seem to be genuinely lower and the average for the liver tumours (986 p.p.m. from 7 samples) appear to be higher than the average of all other livers, namely 627 p.p.m.



HISTOGRAM 1. Individual analytical results of samples.

TABLE II

INDIVIDUAL ANALYTICAL RESULTS OF ALL SAMPLES

Age	Ratio	Copper		Iron		Magnesium		Manganese		Molybdenum		Zinc	
	W_A/W_{DD}	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample
Foetal	0·056	509	28·8	16,400	928	13,700	776	32	1·81	3	0·17	4260	241
	0·077	524	33·1	8540	658	13,400	847	32	2·02	2	0·13	2760	174
	0·063	470	29·7	6600	417	11,300	715	33	2·08	2	0·13	3370	213
	0·072	351	25·2	6150	441	9380	673	30	2·15	2	0·14	4300	308
New born	0·047	676	31·9	12,300	581	12,600	595	38	1·79	7	0·33	6510	308
	0·055	599	32·9	10,150	557	9830	540	35	1·92	4	0·22	5510	302
1 week	0·059	1390	81·3	6210	363	10,800	632	33	1·93	17	1·00	4350	255
2 weeks	0·055	1750	95·3	4730	258	13,800	752	43	2·34	27	1·47	4150	228
3 weeks*	0·057	588	33·2	2560	145	12,000	679	73	4·13	30	1·70	2820	159
4 weeks*	0·050	386	19·3	2850	143	13,000	650	78	3·90	36	1·80	2470	123
5 weeks*	0·049	278	13·6	4520	222	15,200	745	79	3·87	46	2·25	2430	119
6 weeks*	0·051	262	13·4	5080	261	15,200	780	75	3·85	47	2·41	1980	101
7 weeks*	0·041	205	8·3	5170	210	14,600	591	73	2·96	40	1·62	1560	63
8 weeks*	0·051	224	11·4	4430	226	14,900	760	78	3·98	45	2·30	1890	97
16 weeks*	0·045	252	11·5	8390	379	12,100	553	69	3·16	30	1·37	1790	82
4 weeks†	0·052	236	12·2	2450	126	10,700	551	67	3·45	34	1·75	1930	100
5 weeks†	0·043	241	10·2	3340	142	11,100	472	67	2·84	34	1·44	2240	95
6 weeks†	0·038	228	8·68	4580	175	13,000	495	77	2·93	34	1·29	2100	80
7 weeks†	0·046	215	9·95	5190	240	13,100	605	79	3·65	35	1·62	2170	100
8 weeks†	0·043	204	8·8	9800	422	12,700	547	67	2·88	28	1·23	1510	65
8 weeks‡	0·049	207	10·0	5530	268	14,400	699	82	3·98	42	2·04	2130	103
8 weeks‡	0·053	193	10·2	3750	199	12,000	635	82	4·35	32	1·70	2000	106
8 weeks‡	0·051	237	12·0	4700	238	11,800	596	86	4·35	39	1·97	2260	114
8 weeks‡	0·048	268	13·0	6340	307	14,600	705	84	4·06	43	2·08	2970	143
16–20 weeks§	0·046	212	9·7	4050	186	11,300	520	68	3·13	34	1·56	2110	97
	0·046	235	10·8	4230	195	12,400	571	75	3·45	34	1·56	2060	95
	0·051	301	15·2	5110	259	12,600	639	76	3·85	38	1·92	1820	92
5 days¶	0·055	329	18·1	29,600	1630	3870	213	44	2·42	Nd	Nd	Nd	Nd
8 days¶	0·052	229	13·2	6940	401	4100	237	34	1·97	Nd	Nd	Nd	Nd
12–14 months**	0·096††	157	10·9	3800	366	13,900	963	40	2·77	13	0·90	1840	127
	0·076	240	18·2	13,800	1050	14,700	1120	30	2·28	15	1·14	Ca	High
	0·055	250	13·7	6620	363	13,900	763	57	3·13	11	0·61		
	0·047	273	12·9	8810	416	15,200	718	62	2·93	38	1·80	2750	130
	0·051	251	12·8	6770	345	15,700	799	76	3·87	45	2·29	2620	133
	0·074	179	13·2	7370	543	16,200	1190	40	2·95	24	1·77	Ca	High
	0·091	220	20·0	8880	803	14,900	1350	41	3·72	24	2·18	Ca	High

* Rats were fed on a controlled diet.
† Rats were fed on a normal diet plus kitchen scraps.
‡ Rats were fed on a normal diet plus kitchen scraps and the livers were pooled (four).
§ Maternal livers from pregnant rats.
¶ Regenerating livers.
** Tumours arising from rats fed on a diet containing a carcinogenic azo-dye.
†† Perfused with sodium citrate.

Manganese (see Underwood[17], pp. 235–240). An increase in content of this metal can be discerned (see Histogram I) from the embryo via the newborn and one to two weeks old animals to the three to 16 weeks old rats. It may be significant that the values for the regenerating livers are more like those of the earlier age groups, while those of the liver tumours are similar to the results with the later age groups.

Molybdenum (see Underwood[17], pp. 125–129). This trace element is of special interest to us in view of the work in these laboratories[9] on xanthine oxidase, a molybdeno-flavoprotein. The low levels of foetal and newborn livers rise over the next five weeks and arrive at an average value for the 16 weeks old, the pregnant females and the rats fed on kitchen scraps of 1·7 p.p.m. This should be contrasted with the content of the liver tumours of which three showed a low molybdenum concentration and four an average of 2·0 p.p.m. It has been mentioned before that the histological nature of the tumours was somewhat uncertain. This might explain the differences between the low and higher figures. The low values could be quoted in support of a report by Westerfeld and colleagues[8] on the diminished level of xanthine oxidase in livers of animals fed with butter yellow. Our figures obtained with foetal livers invite a similar comparison with the low level of xanthine oxidase activity in embryonic organs[34]. No molybdenum could be detected in our regenerating livers. If this is an observation reproducible with other material, it should be followed up by estimation of molybdeno-flavoproteins in regenerating organs.

Zinc (*cf.* Underwood[17], pp. 208–216). Our figures suggest a higher zinc content in the foetal, newborn and one week-old livers. Then they fall to an average of 102 p.p.m. in the organs of animals between 2 and 16-weeks old, organs with the data for all the other normal livers being about the same[35]. We cannot offer, at present, any explanation for the apparent absence of zinc in the regenerating livers. Some of the tumour livers did not yield results, because the presence of surprisingly high amounts of calcium made the spectrographic estimation of zinc impossible. This observation makes it very desirable to analyse at an early date for calcium. The remainder of the tumour-livers showed slightly higher metal contents, average 130 p.p.m. The other point which should be mentioned here, is that the keeping of rats in zinc cages has seemingly very little effect on the content of zinc in the livers.

There is no doubt that further work has to aim at the inclusion of other elements including major ones, such as sodium, potassium and calcium and to be backed up with coenzyme: enzyme determinations or assessment of other metal-bearing cellular constituents. Future studies should be extended to cellular particles and to other organs in the same species and to organs of other species, finishing in the end with comparable human material.

Acknowledgements. We wish to thank Messrs. J. F. Harringshaw and L. S. Theobald for the bulk analysis, Dr. L. A. Elson for the supply of liver-tumours, Mr. C. Smith for the supply of all the animals. We

appreciate the constant interest of Professor A. Haddow and the initial suggestions by Mr. A. Chester Beatty, Jr.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service. Acknowledgement is also made for the use of spectrographic equipment purchased with the aid of a special grant to the Royal School of Mines from the Central Research Fund of London University.

REFERENCES

1. Greenstein, *Biochemistry of Cancer*, 2nd Ed., Acad. Press Inc., New York, 1954. Winzler, *The Chemistry of Cancer; Tissue Physiopathology of Cancer*, Hoeber, N.Y., 1953, p. 552.
2. Haddow, *Biochemistry of Cancer, Ann. Rev. Biochem.*, 1955, **24**, 699.
3. Miller and Miller, *Adv. Canc. Res.*, 1953, **1**, 339. Acad. Press Inc., New York.
4. Weiler, *Z., Naturforsch.*, 1952, 7*b*, 324.
5. Weiler, *Brit. J. Cancer*, 1956, **10**, 553, 560.
6. Horning and Whittick, *ibid.*, 1954, **8**, 451.
7. Bhargava and Heidelberger, *J. Amer. chem. Soc.*, 1956, **78**, 3671.
8. Westerfeld, Richert and Hilfinger, *Cancer Res.*, 1950, **10**, 486.
9. Avis, Bergel and Bray, *J. chem. Soc.*, 1955, 1100.
10. Avis, Bergel, Bray, James and Shooter, *ibid.*, 1956, 1212, 1219.
11. Lewin, Lewin and Bray, *Nature, Lond.*, 1957, in the press; cf. also Bergel, Bray, Haddow and Lewin, *The Chemistry and Biology of Purines, Ciba Foundation Symposium*, 1957, 256, J. and A. Churchill, London.
12. Ambrose, James and Lowick, *Nature, Lond.*, 1956, **177**, 576.
13. DeLong, Coman and Zeidman, *Cancer*, 1950, **3**, 718.
14. Mitchell, Commonwealth Bureau of Soil Science, Techn. Communication No. 44, 1948; *Chemistry of Soil*, A.C.S. Monograph No. 126, Reinhold Publ. Corp., New York, 1955, p. 253.
15. Webb and Millman, *Trans. Inst. Min. Metall. (Lond.)*, 1950–51, **60**, 473.
16. Cuthbertson and Allcroft, *The Advancement of Science*, 1956, **12**, 485.
17. Underwood, *Trace Elements in Human and Animal Nutrition*, Acad. Press Inc., New York, 1956.
18. Tipton, Steiner, Foland, Mueller and Stanley, *Progress Report Oak Ridge Nat. Laboratory*, 1954, 54-12-66.
19. Tipton, Cook, Steiner, Foland, Bowman and McDaniel, *ibid.*, 1956, 56-3-60.
20. Koch, Smith, Shimp and Connor, *Cancer*, 1956, **9**, 499.
21. Stitch, A.E.R.E., M.R.C./R 1952, Part I, 1956.
22. Sowden and Stitch, A.E.R.E., M.R.C./R 2030, Part II, 1956.
23. Mahler and Green, *Science*, 1954, **120**, 7; cf. also, The role of metals in oxidations catalysed by xanthine oxidase and other metallo-flavoproteins, *Biochem. J.*, 1956, **64**, 34P.
24. Vallee, *Adv. Protein Chemistry*, 1955, **10**, 318, Acad. Press Inc., New York.
25. Metzler and Snell, *J. Amer. chem. Soc.*, 1952, **74**, 979.
26. Kirby, *Biochem. J.*, 1956, **62**, 31P.
27. Kirby, *ibid.*, 1957, in press.
28. Price, Harman, Miller and Miller, *Cancer Res.*, 1952, **12**, 192.
29. Black, *Spectrochimica Acta*, 1952, **4**, 519–524.
30. Lorenzen and Smith, *J. Nutrit.*, 1947, **33**, 143.
31. Cox and Mueller, *ibid.*, 1937, **13**, 249.
32. Brückmann and Zondek, *Nature, Lond.*, 1940, **14***b*, 30.
33. Ashikawa, Smith and Helwig, UCRL-3530, *Health and Biology Distribution*, U.S. Atomic Energy Commission.
34. Greenstein and Thompson, *J. Nat. Cancer Inst.*, 1943, **4**, 271.
35. Gilbert and Taylor, *Biochim. Biophys. Acta*, 1956, **21**, 545.

SEPARATION OF THE HORMONES OF THE POSTERIOR PITUITARY FROM A CRUDE EXTRACT BY ELECTRO-CHEMICAL MEANS

By G. W. L. James and D. B. Garraway

From the Department of Pharmacology, Welsh National School of Medicine, The Parade, Cardiff

Received February 15, 1957

Pressor and oxytocic activities were separated from a crude extract of pig posterior pituitary tissue using a continuous electrophoretic technique. Both voltage and rate of application were kept constant. The material obtained during the first 24 hours was discarded; collections could then be made for up to 7 days. With a Whatman No. 2 filter paper, a rate of feed of 3·5 ml. of crude extract in 24 hours and 400 V., pooled activities had about 5 per cent of contamination.

In 1928 Kamm, Aldrich, Grote, Rowe and Bugber[1] separated by means of a long and tedious chemical process two of the active substances of the posterior lobe of the pituitary. These active substances were isolated and a structure suggested for them by du Vigneaud and his colleagues[2,3] and by Fromageot, Acher, Clauser and Maier-Huser[4]. Taylor, du Vigneaud and Kunkel[5] found that a mixture of purified oxytocin and vasopressin could be separated electrophoretically using glass beads as supporting medium. Since it may well be many years before the pure polypeptides are commercially available some attempt was made to separate these hormones from a crude extract by an economical and reasonably quick method on filter paper.

Materials and Method

The apparatus used was one for "Continuous Electrophoresis on Paper" built upon the specifications published by Holdsworth[6], except that a pump was not necessary, the buffer being fed into the apparatus from a 20 litre aspirator at a rate of 250 ml./hour and the effluent allowed to flow down the sink. The aspirator was refilled daily. The crude extract is applied to the top corner of a sheet of filter paper supported vertically and the various components move in a direction which is the resultant of two forces. The two forces are: (1) a flow of material down the paper due to gravity, and (2) an electrical pull across the paper due to an applied voltage. The supported filter paper was serrated at its lower vertical end. Under each serration was positioned a collecting tube. The buffer solution plus separated activity having vertically traversed the paper would then drip from a serration into a collecting tube, of which there were 29 in number.

The effect of temperature on separation was not analysed and for the purpose of these experiments was kept approximately constant at 20°, no cooling device being necessary in the apparatus.

Pig posterior pituitary powder, kindly supplied by Dr. Tindall of Organon Limited, was the raw material. The potency of these powders

varied between—vasopressin, 1080 and 1500 I.U./g. and oxytocin 1680 and 2180 I.U./g. A crude extract was quickly made by boiling one g. of the initial powder with 10 ml. of 0·3 per cent acetic acid for 3 minutes, cooling rapidly and filtering. The material remaining on the filter paper was then washed with a further 5 ml. of 0·3 per cent acetic acid, and the two filtrates mixed. The buffer used in the apparatus was also 0·3 per cent acetic acid.

The assay for oxytocin was that specified by the B.P. 1953 for the "rat uterus method" with the following alterations. Magnesium chloride was omitted from the saline, which was aerated and kept at a temperature of 30°. The female rat taken was in early dioestrus.

The assay of vasopressin was as specified by the B.P. 1953 for the "pressor activity method", but an anaesthetised rat of minimum weight of 300 g. was used.

Results

The crude extract was fed to the top corner of the filter paper on the anode side so that the polypeptides would be drawn across the paper towards the cathode, the hormones being positively charged. Alteration of the voltage applied alters the resultant paths of polypeptides. By increasing the voltage, whilst maintaining all other factors constant, the position of collection of both hormones was moved nearer to the cathode. An increase of about 10 volts was found to move the peak of any one activity one tube nearer the cathode. For this reason, it was found necessary to stabilise the voltage, the most suitable for separation being 400 volts.

Alteration of the rate of application of the crude extract also considerably altered the position of recovery of the hormones. By increasing the rate of application, the position of recovery of the activities was found to move towards the anode. Thus it was also necessary to stabilise the rate of application by a synchronously driven constant infusion syringe. Attached to the end of this was a capillary polythene tube which led to the top corner of the filter paper.

Differing kinds of filter paper also greatly affected the position of recovery of the activities. For example with Whatman No. 1 filter paper, and a voltage of 500, current 2·2 milliamps and a rate of application of 4 ml. crude extract in 24 hours, and numbering the collection tubes from 1 to 29 in ascending order from the anode, a peak value for oxytocin activity was found in tube No. 11. But with Whatman No. 2 filter paper under the same conditions, the peak value was found in tube No. 23. For the purposes of these experiments, the filter papers were termed "fast" or "slow", depending on the rate at which the materials seeped through them. For a given set of conditions, the "slower" the filter paper, the nearer to the cathode the activities are found.

All these variables affect the positions of recovery of the activities, but not the amount and concentrations recovered. There are two factors which affect the latter, namely, the length of time of running the apparatus and the quality of the initial powder.

The apparatus was normally run for 72 hours. For the first 24 hours of collection, the concentrations of activities collected continually varied. No significant difference was then found for collection made between 24–48 hours and 48–72 hours. It was found that the better the initial sample of pituitary powder the more the concentration of any one activity at its peak, and the less the spread of the activity around the peak.

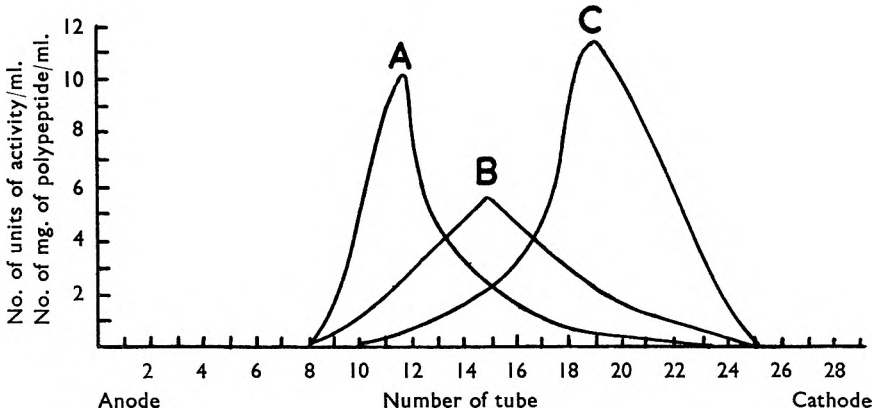


FIG. 1. Showing the distribution of activities of a typical run. A represents the distribution of oxytocic activity, C that for pressor activity and B shows the distribution of proteins and polypeptides.

Having found the most suitable conditions for separation (in this particular instance a Whatman No. 2 filter paper, 400 V., rate of application of 3·5 ml. crude extract/24 hours) the peaks of oxytocic activity and pressor activity are found. The activities in the neighbourhood of the peaks are then pooled so as to give a resulting solution of fairly concentrated activity, but lying within the B.P. limits of contamination, one with the other. For example, from the experiment recorded in Figure 1, tubes 9–12 would be taken in the collection of oxytocin. The two separate "pools" are then freeze dried. In the results obtained by us, from 2·5 ml. of such a "pool" of oxytocin, the activity recovered was about 25 I.U./mg. of freeze dried product.

DISCUSSION

At the "peak" tubes the concentration of either activity is about 10 I.U./ml.

To be economical the rate of application of extract, and hence the rate of collection of the individual activities, must be high. However, increasing the rate of application moves the separated activities towards the anode, whilst maintaining the same distance between the peaks. Thus there must be a compensatory increase of voltage to return the peaks to their original positions. But, now the two peaks move nearer to one another and the contamination of pooled activities increases as curves A and C of Figure 1 overlap. Thus there is an upper limit to the rate of application.

The overlapping of the activities can be limited in two ways. The first is by increasing the vertical length of the filter paper. Then the two directional forces mentioned above will act for a longer time on the polypeptides and hence their separation will be greater. The second is the "spread" of the activity along the tubes. This "spread" can be reduced by careful stabilisation of the voltage and rate of application.

From Figure 1 it can be seen that most of the protein and polypeptides were collected between the two activities. This suggests that the third hormone of the posterior pituitary, the melanophore-expanding hormone, might be found between the other two activities. This aspect of the work was not pursued.

It was found that after running the apparatus with the same paper for a long time, separation was adversely affected. This was probably due to the accumulation of inert material around the point of application of the crude extract.

REFERENCES

1. Kamm, Aldrich, Grote, Rowe and Bugbee, *J. Amer. chem. Soc.*, 1928, **50**, 573.
2. du Vigneaud, Lawler and Popenoe, *ibid.*, 1953, **75**, 4880.
3. du Vigneaud, Ressler and Trippett, *J. biol. Chem.*, 1953, **205**, 949.
4. Fromageot, Acher, Clauser and Maier-Huser, *Biochim. Biophys. Acta*, 1953, **12**, 424.
5. Taylor, du Vigneaud and Kunkel, *J. biol. Chem.*, 1953, **205**, 45.
6. Holdsworth, *Biochem. J.*, 1955, **59**, 340.

THE ASSAY OF ACETYLCHOLINE ON THE SUPERFUSED FROG RECTUS MUSCLE

By A. Ahmed* and N. R. W. Taylor

From the Department of Pharmacology and Therapeutics, University of St. Andrews, Queen's College, Dundee

Received March 15, 1957

A method of superfusion for assaying acetylcholine on the eserinised frog rectus muscle applying each dose in a volume of 0·2 ml. is described. The sensitivity of the superfused preparation is compared with that of the preparation in a 5 ml. bath and is 10 to 20 times more sensitive than the bath preparation. Results of 10 parallel assays (2 + 2) suggest that the method of superfusion gives a reliable and accurate assay of acetylcholine.

The estimation of acetylcholine in biological materials requires a method of high sensitivity when the volume and concentration likely to be found is very small. In recent years the technique of superfusion has been increasingly used for assaying minute quantities of active substances such as histamine and 5-hydroxytryptamine on isolated preparations. The present investigation was undertaken with the object of applying the method of superfusion to the assay of acetylcholine on the frog rectus muscle. In order to assess the sensitivity, reliability and the accuracy of the superfusion method, parallel assays by the usual bath method were also made using muscles from the same frogs. The results show that the superfusion method is highly sensitive and that the assay can be made using only 1/20 to 1/10th of the dose required for the bath method with a dose volume of only 0·2 ml.

Methods

One rectus abdominis muscle of the pithed frog was suspended in a 5 ml. bath and the other was suspended in air enclosed in a wide glass tube. Frog Ringer solution from a reservoir placed about 30 inches above the bench was used to supply both preparations. Each litre of frog Ringer solution contained NaCl 6·5 g.; KCl 0·14 g., $CaCl_2$ 0·12 g.; $NaHCO_3$ 0·2 g.; NaH_2PO_4 0·01 g. and glucose 2 g. The fluid in the bath was continuously aerated. Eserine salicylate was added to the Ringer solution to give a concentration of 10^{-5} (as salicylate).

The rate of flow for superfusing the muscle was controlled by a screw clip on the rubber tube and the flow interrupted or continued by turning the stopcock in the glass tube. A flow rate of 80 drops per minute was employed.

Frontal writing levers were used and adjusted to give a 10 fold magnification and a tension on the muscle equivalent to 2·0 g.

The procedure of administration of drug solution to the superfused preparation was similar to that of Gaddum[1]. Superfusion was stopped

* Assam Government Scholar.

10 seconds before the application of a dose. The doses were administered in a volume of 0·2 ml. from a 0·2 ml. blood pipette. The pipette was rested near the capillary end of the glass tube touching the thread and the drug solution was allowed to fall dropwise down the thread and flow over the surface of the muscle. The drug solution was left in contact with the muscle for 90 seconds, after which the superfusion was restarted.

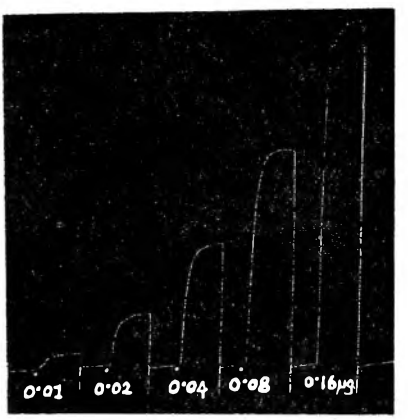


FIG. 1. Frog rectus muscle superfused with Ringer's solution (without eserine). Records of contractions due to different doses of acetylcholine solution (as acetylcholine chloride). Muscle allowed to contract for 90 seconds.

Assay Procedure

The muscles were prepared and left for half to one hour, then doses of acetylcholine were applied regularly at 6 to 10 minute intervals. The acetylcholine was dissolved in frog Ringer solution and added to both preparations in a constant volume of 0·2 ml. When the response had become regular the assay was made by Schild's method[2]. The approximate concentration of the unknown solution was estimated by the bath method, diluted if necessary and four responses obtained at each of two doses, the ratio of the high to low dose being 2 : 1. In assaying by the superfusion method these concentrations had to be further diluted 10 to 20 times. Four responses were obtained at each dose level for standard and unknown solutions in each assay, the doses being randomly distributed in a 4 × 4 Latin square design.

RESULTS

Response of Superfused Frog Rectus Preparation to Acetylcholine Solution

Figure 1 shows the type of contracture produced by different doses of acetylcholine in superfused frog rectus muscle not sensitised by eserine. The contracture starts as soon as the drug solution comes in contact with the surface of the muscle, reaches a maximum within 20–30 seconds and remains at this level until the superfusion is restarted. Relaxation commences soon afterwards and is complete within 3–6 minutes. In the eserinised preparation, the contracture does not reach the maximum even in 90 seconds (see Fig. 3).

Sensitivity of Superfused Preparations

The sensitivity of the preparations to acetylcholine varied moderately with different frogs. The smallest effective dose for the superfused preparation was 0·0025 to 0·005 μg. of acetylcholine per 0·2 ml. dose; that for the 5 ml. bath was 0·05 to 0·1 μg.

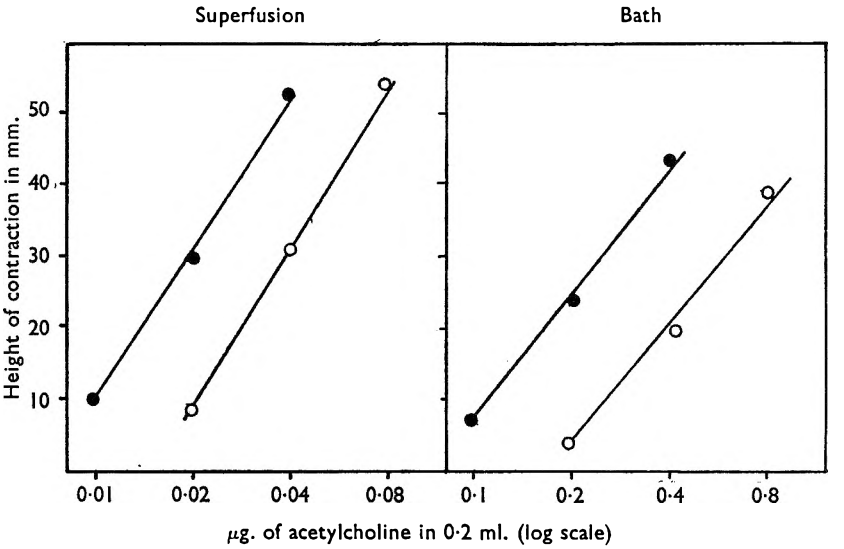


FIG. 2. Log dose response curve of frog rectus muscle obtained by superfusion and bath method. Closed circle—preparations sensitized with eserine; open circle—not sensitised with eserine. Each point represents the mean value obtained from six muscles.

TABLE I

SUMMARY OF ACETYLCHOLINE ASSAY RESULTS BY BATH (5 ML.) AND SUPERFUSION METHODS

No. of Assay	Method	Amount of acetylcholine μg./ml. Actual	Amount of acetylcholine μg./ml. Found	Limits of error P = 0·05	Standard Deviation (s)	Slope (b)	λ (s/b)
1	Bath	4	4·16	92·6–107·9	2·34	78·45	0·029
	Superfusion		4·4	86·8–115·1	1·86	47·36	0·039
2	Bath	3	3·3	93·9–106·4	3·61	120	0·0308
	Superfusion		3·25	94·2–105·9	3·4	119·1	0·0286
3	Bath	8	9·3	76·4–130	6·13	71·84	0·086
	Superfusion		7·6	92·3–108·3	1·76	73·92	0·024
4	Bath	3	3·08	86·6–115·6	2·83	67·27	0·043
	Superfusion		3·15	77·6–125	3·22	42·85	0·075
5	Bath	2·5	2·55	88·9–112·4	2	64·78	0·0308
	Superfusion		2·60	95·5–104·7	1·27	41·52	0·0306
6	Bath	0·725	0·72	87·1–114·4	2·48	41·5	0·060
	Superfusion		0·69	87·8–113·9	4·72	69·35	0·068
7	Bath	2·5	2·65	82·2–121·1	3·23	63·5	0·0508
	Superfusion		2·32	86·0–116·3	3·64	79·3	0·046
8	Bath	2·75	2·78	85·3–117·8	3·39	84·7	0·046
	Superfusion		3·0	86·2–116·1	5·8	83·8	0·070
9	Bath	0·77	0·863	89·0–111·7	1·63	52·45	0·031
	Superfusion		0·813	81·4–122·7	3·35	52·82	0·063
10	Bath	2·75	2·8	95·9–104·7	1·83	75·58	0·024
	Superfusion		2·81	82·6–121·1	4·93	80·56	0·061

Figure 2 shows the dose response curves of eserinised and noneserinised preparations in the superfusion and bath methods. The dose response was found to be linear within the range of 0·01 to 0·04 μg. (eserinised) and 0·02 to 0·08 μg. (noneserinised). The bath method gave linearity of response within the range of 0·1 to 0·8 μg. (eserinised). The assays (2 + 2) were carried out using these dose ranges.

2 + 2 *Assays*

Assays were made simultaneously on superfused and bath preparations and the results of ten such parallel assays are given in Table I. The same standard and unknown solutions were used for both methods except that in the case of the superfusion method, they were diluted 10 to 20 times. The first five assays were made on noneserinised preparations and the last five assays were made one hour after eserinisation.

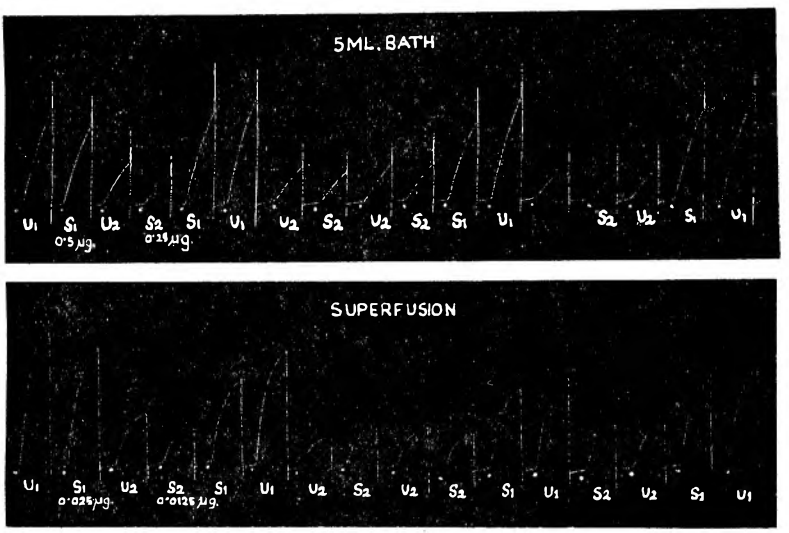


FIG. 3. Record of a parallel assay (2 + 2) with acetylcholine solution on frog rectus eserinised muscles by bath and superfusion methods (assay No. 10, Table I). In bath method—$S_1 = 0{\cdot}5$ μg.; $S_2 = 0{\cdot}25$ μg.; $U_1 = 0{\cdot}55$ μg.; $U_2 = 0{\cdot}275$ μg. In superfusion method—$S_1 = 0{\cdot}025$ μg.; $S_2 = 0{\cdot}0125$ μg.; $U_1 = 0{\cdot}0275$ μg.; $U_2 = 0{\cdot}01375$ μg.

A typical parallel assay on eserinised preparations is shown in Figure 3. The actual concentrations of the test solutions were unknown to the operator until the assay had been completed.

It will be seen from the Table that the accuracy of the assay and the limits of error were much the same for both bath and superfusion methods, though the sensitivity of the superfused preparation was 10 to 20 times greater than the bath preparation. The index of precision (λ) was found to be less than 0·05 in five assays by the method of superfusion. The estimates of the assays and their errors were calculated by the method of analysis of variance. In all the assays the quantity "t" for parellelism

was less than 2·18 for 12 degrees of freedom thus indicating no significant deviation from parallelism.

Discussion

Kwiatkowski[3] used the superfused frog rectus for assaying acetylcholine by injecting acetylcholine into the superfusing fluid. In these circumstances the dose of acetylcholine is immediately diluted so that the senstitivity is greatly reduced. Cambridge and Holgate[4] found that a graded response to acetylcholine could be obtained from a superfused frog rectus if the solution containing it were applied directly to the muscle. They found the optimum dose was 2·5 ml. of solution containing 0·3 to 0·8 μg./ml. at a rate of 1 drop per second for 50 seconds. The present investigation shows that the concentration of the acetylcholine solution and the dose volume may be reduced. The concentration of acetylcholine in the solutions assayed in our superfusion experiments ranged from 0·1 to 0·4 μg./ml. in noneserinised preparations and from 0·05 to 0·2 μg./ml. in eserinised preparations. The volume of the dose was also lower—0·2 ml.

Statistical evidence is adduced to show that the method of superfusion and bath method are of equal accuracy when used under comparable conditions. In the case of the superfusion method assayable responses could be obtained with 1/10 to 1/20th of the dose required in the bath method.

References

1. Gaddum, *Brit. J. Pharmacol.*, 1953, **8**, 321.
2. Schild, *J. Physiol.*, 1942, **101**, 115.
3. Kwiatkowski, *ibid.*, 1941, **100**, 147.
4. Cambridge and Holgate, *Brit. J. Pharmacol.*, 1954, **9**, 370.

A PHARMACOGNOSTICAL STUDY OF THE SEEDS OF A SPECIES OF THE GENUS *PAPAVER* GROWN IN EGYPT

By I. R. Fahmy, M. A. El-Keiy and F. M. Hashim

From the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Egypt.

Received September 23, 1955

The cultivation and possession of plants or seeds of certain *Papaver* species is strictly forbidden in Egypt, and to aid identification a comparison of the seeds of some species of *Papaver* was made. Winton[1] reported on the histology of seeds of *Papaver somniferum* var. *nigrum*. Fedde[2] discussed the taxonomy and morphology of the flowering and fruiting plants of the genus *Papaver*. Muschler[3] similarly studied *Papaver* species which grow in Egypt, and Aleksandrov and Vislaukh[4] reported on the histology of the flowering and fruiting plants of *P. somniferum*.

Material

The seeds were obtained from the following: Chelsea Physic Garden, London; Kew Gardens, London; The Medicinal Plant Garden, University of Washington, Seattle, U.S.A., The Pharmacognosy Department, University of Southern California, U.S.A.; The Botanical Garden, Graz University, Austria; The Pharmacognosy Department, Faculty of Pharmacy, Cairo University and Zuhria Garden, Gizerah, Cairo.

The seeds of each species were separately cultivated in the Medicinal Plants Garden, Faculty of Pharmacy, Cairo University, as an additional means of authentication.

Seeds: Morphology

The seeds of the different *Papaver* species (Fig. 1) are minute, varying in colour and size, being somewhat laterally compressed, more or less reniform in shape and with a narrow, pointed end. They are thicker towards the convex edge which is mostly rounded, while the concave edge shows in the centre a pale depression, the hilum. The microphyle is hardly seen. The seeds are hard, but can be easily crushed. They are odourless with an oily taste when crushed.

Externally, the seeds are reticulated, the reticulations being polygonal, isodiametric or somewhat elongated with nearly straight or wavy sides, surrounding shallow depressions. The number of the reticulations lengthwise and across the flat lateral side of the seed varies in the different species.

Internally, the seeds consist of a thin testa, enclosing a fleshy endosperm in which is embedded a curved embryo; the cotyledons being slightly longer than the radicle which is pointed towards the hilum.

The seeds as well as an alcoholic extract (1 in 10) when examined in filtered ultra-violet light, emit a bluish fluorescence.

The morphology of the seeds is given in Table I.

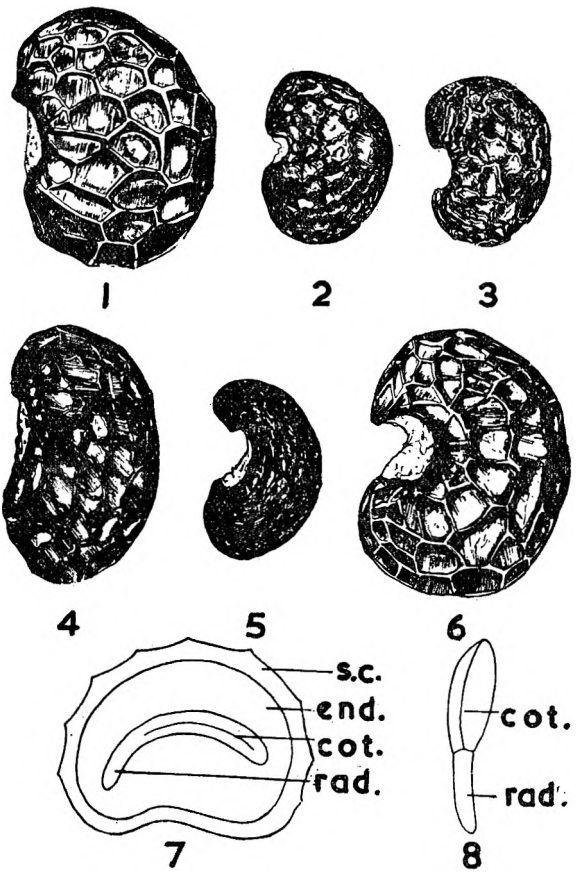


FIG. 1. Seeds (all ×25); 1, *Papaver somniferum* var. *nigrum*; 2, *P. rhoeas*; 3, *P. dubium*; 4, *P. argemone*; 5, *P. nudicaule*; 6, *P. orientale*; 7, longitudinal cut surface in the seed of *P. somniferum* (var. *nigrum*); 8, embryo; *cot.*, cotyledons; *end.*, endosperm; *rad.*, radicle, *sc.*, seed-coat.

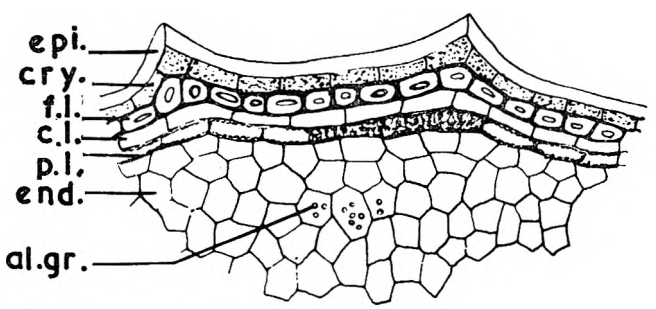


FIG. 2. T.S. in the seed of *P. somniferum* var. *nigrum* (by 160); *epi.*, epidermis; *cry.*, crystal layer; *f.l.*, fibrous layer; *c.l.*, cross layer; *p.l.*, pigment layer; *end.*, endosperm; *al.gr.*, aleurone grains.

HISTOLOGY

The seed-coat (Fig. 2, 3, 4) consists of five layers, the epidermis, the crystal layer, the fibrous layer, the cross layer and the pigment layer. The first three layers correspond to the outer integument, while the inner two correspond to the inner integument. Each layer of the seed-coat consists of a single row of cells.

The epidermal cells appear tabular and tangentially elongated in transverse section and usually polygonal, isodiametric or somewhat elongated in surface view with slightly thickened almost straight or wavy anticlinal walls. The epidermal cells on the convex edge towards the

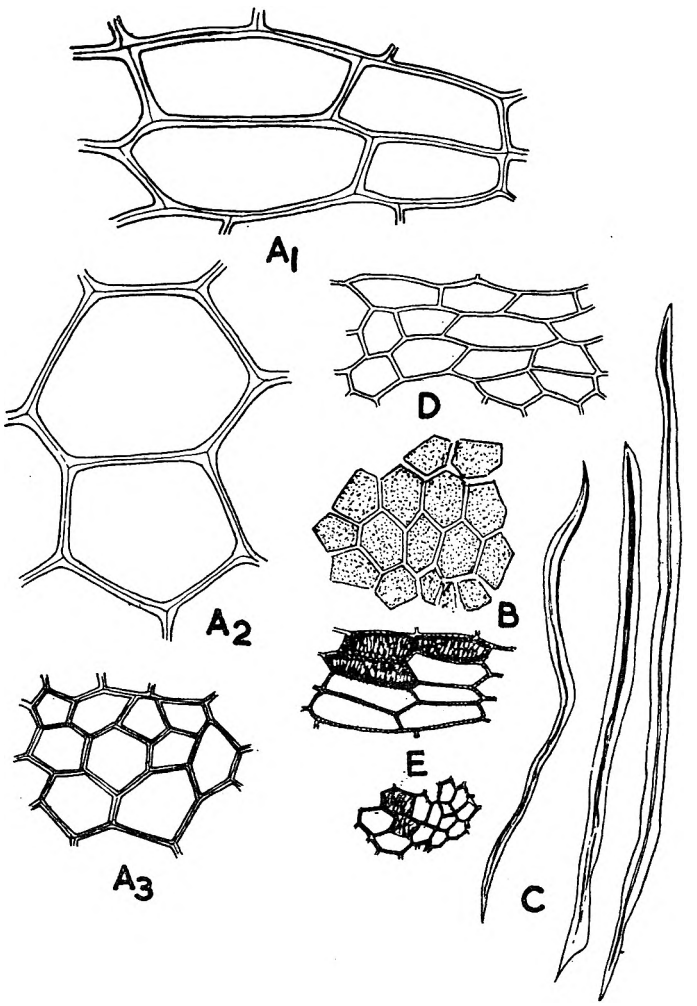


FIG. 3. Elements of the seed-coat of *P. somniferum* var. *nigrum* (× 120); A_1, epidermal cells on the convex edge towards the narrow end; A_2, epidermal cells; A_3, epidermal cells in the neighbourhood of hilum; B, cells of crystal layer; C, cells of fibrous layer; D, cells of cross layer; E, cells of pigment layer.

TABLE I

MORPHOLOGY OF SEEDS OF *Papaver* SPECIES

Name	Shape	Colour	Size	Weight of 100 seeds	Outline of reticulations	Number of reticulations
1a. *Papaver somniferum* var. *nigrum* var. *glacum*	Somewhat laterally compressed, reniform with a narrow and pointed end; convex edge mostly rounded; concave edge, narrow containing the hilum and micropyle.	Greyish to greyish-brown to dark brown or even blackish.	1 to 1·2 mm. long 0·7 to 1·0 mm. wide 0·6 to 0·8 mm. thick	35 to 40 mg.	Polygonal, mostly isodiametric, with almost straight sides.	8 to 11 lengthwise, 6 to 8 across the flat lateral side.
1b. var. *album*	As for 1a	Whitish to pale yellowish white.	1 to 1·6 mm. long 0·7 to 1·2 mm. wide 0·6 to 0·8 mm. thick	40 to 50 mg.	As for 1a	As for 1a
2. *Papaver rhoeas*	As for 1	As for 1a	0·6 to 0·8 mm. long 0·5 to 0·6 mm. wide 0·4 to 0·5 mm. thick	12 to 17 mg.	Polygonal, mostly isodiametric with wavy sides.	8 to 11 lengthwise, 5 to 7 across, the flat lateral side.
3. *Papaver dubium*	As for 1	As for 1a	0·6 to 0·7 mm. long 0·4 to 0·5 mm. wide 0·4 to 0·5 mm. thick	10 to 15 mg.	As for 2	As for 2
4. *Papaver argemone*	As for 1, but usually narrow and elongated; the length about twice the width.	Dark reddish-brown to dark brown.	1 to 1·2 mm. long 0·5 to 0·6 mm. wide 0·4 to 0·5 mm. thick	17 to 20 mg.	Polygonal, somewhat elongated with slight wavy sides.	10 to 15 lengthwise, 5 to 7 across the flat lateral side.
5. *Papaver nudicaule*	As for 4	Pale brown to dark brown	0·7 to 0·9 mm. long 0·3 to 0·4 mm. wide 0·3 to 0·4 mm. thick	8 to 10 mg.	As for 4	10 to 15 lengthwise, 8 to 10 across, the flat lateral side.
6. *Papaver orientale*	As for 1, but the narrow ends are more acute and more elongated.	As for 1a	As for 1a	As for 1a	As for 1	As for 1

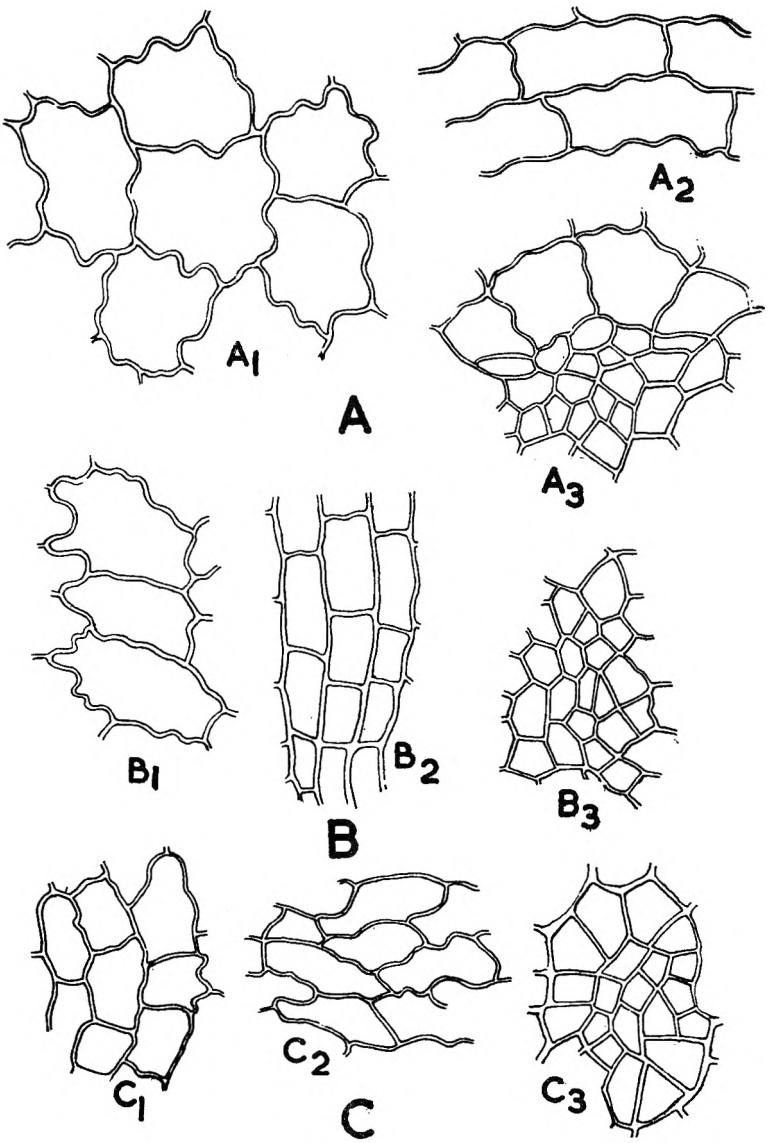


FIG. 4. Surface view of epidermal cells of seed coat (× 140); A, *P. rhoeas*; A_1, epidermal cells in flat sides; A_2, epidermal cells on the convex edge; A_3, epidermal cells in the neighbourhood of hilum; B, *P. argemone*; B_1, epidermal cells on flat sides; B_2, epidermal cells on the convex edge; B_3, epidermal cells in the neighbourhood of hilum; C, *P. nudicaule*; C_1, epidermal cells on flat sides; C_2, epidermal cells on the convex edge; C_3, epidermal cells in the neighbourhood of hilum.

narrow end of the seed are usually narrow, elongated and nearly rectangular; while those in the neighbourhood of the hilum are usually smaller in size than those of other regions. In the crystal layer the cells are tabular and somewhat tangentially elongated in transverse section

TABLE II

HISTOLOGY OF SEEDS OF *Papaver* SPECIES

Name	Epidermis	Crystal Layer	Fibrous layer	Cross layer	Pigment layer	Endosperm and embryo
Papaver somniferum 1a. var. *nigrum* var. *glaucum*	Epidermal cells, tabular, polygonal, isodiametric and slightly elongated, with straight walls; 18 to 337 μ long and 225 μ wide.	Cells tabular polygonal, isodiametric or somewhat elongated with nearly straight walls; contain minute shining microcrystals of calcium oxalate; 30 to 75 μ long and 20 to 60 μ wide.	Cells rounded or laterally compressed, elongated, somewhat twisted with pointed ends and thickened, non-lignified walls; 15 to 45 μ in diameter; and 500 to 750 μ long.	Cells tabular, polygonal, elongated with straight fairly thickened brownish walls; 30 to 150 μ long; 20 to 60 μ wide.	Cells tabular, polygonal, isodiametric to elongated with straight reticulately thickened walls; contain amorphous dark brownish pigment; 15 to 130 μ long; 10 to 75 μ wide.	Endosperm cells polygonal, usually isodiametric with thin walls; embryo cells smaller, rounded, thin-walled. Endosperm and embryo cells contain fixed oil globules and aleurone grains; aleurone grains minute, rounded, containing one or more globoid and one crystalloid; 4 to 7 μ in diameter.
1b. var. *album*	As for 1a, but bigger; 18 to 375 μ long; 15 to 280 μ wide.	As for 1a	As for 1a	As for 1a, but the walls colourless.	As for 1a, but contain no pigment.	As for 1a
2. *Papaver rhoeas*	Epidermal cells, tabular, polygonal, isodiametric or slightly elongated, with wavy walls; 15 to 225 μ long; 15 to 150 μ wide.	As for 1a, but usually smaller; 15 to 37 μ long; 12 to 30 μ wide.	As for 1a in shape, but smaller; 10 to 30 μ in diameter; 300 to 600 μ long.	As for 1a, but usually smaller; 30 to 90 μ long; 10 to 45 μ wide.	As for 1a, but usually smaller; 15 to 110 μ long; 10 to 60 μ wide.	As for 1a, but aleurone grains, smaller; 2 to 5 μ in diameter.
3. *Papaver dubium*	As for 2	As for 2	As for 2	As for 2	As for 2	As for 2
4. *Papaver argemone*	Epidermal cells, tabular, elongated, polygonal with slightly wavy walls; 15 to 200 μ long; 15 to 95 μ wide.	As for 1, but smaller; 30 to 45 μ long; 20 to 37 μ wide.	As for 2	As for 2	As for 2	As for 2
5. *Papaver nudicaule*	Epidermal cells, smallest of species examined; resemble in shape those of 4. 15 to 150 μ long; 15 to 75 μ wide.	As for 1, but are the smallest of species examined; 15 to 30 μ long; 12 to 26 μ wide.	As for 1 in shape, but are the narrowest of species examined; 7 to 20 μ in diameter; 300 to 600 μ long.	As for 1 in shape, but are the smallest of species examined; 30 to 60 μ long; 10 to 30 μ wide.	As for 1 in shape, but are the smallest of species examined; 15 to 60 μ long; 10 to 37 μ wide.	As for 1
6. *Papaver orientale*	As for 1a	As for 1	As for 1	As for 1a	As for 1a	As for 2

and polygonal, nearly isodiametric or slightly elongated and with nearly straight, anticlinal walls in surface view. They contain minute, shining microcrystals of calcium oxalate.

The fibrous layer consists of fibrous cells which, in transverse section, appear nearly rounded or somewhat laterally compressed and in surface view are elongated, and twisted with fairly thickened non-lignified walls and acute apices.

The cells of the cross layer are tabular, almost rectangular in transverse section and polygonal, somewhat elongated in surface view with nearly straight, fairly thickened and brownish anticlinal walls excepting those of *P. somniferum* var. *album*, which are colourless.

In transverse section, the cells of the pigment layer appear tabular and nearly rectangular and in surface view, they are polygonal, either isodiametric or elongated, with usually straight and reticulately thickened walls. These cells contain amorphous, dark brownish pigment except those of *P. somniferum* var. *album* which do not contain pigment.

The endosperm is composed of numerous rows of polygonal, usually isodiametric, thin-walled cells, containing fixed oil globules and small aleurone grains. The radicle consists of small rounded, thin-walled cells and the cotyledons of a single row of short palisade-like cells and of several rows of small rounded, thin-walled cells. The cells of the radicle and cotyledons contain also fixed oil globules and small aleurone grains. The comparative histology is given in Table II.

DIFFERENTIATION

The seeds are differentiated by the following:

1. The seeds of the species examined are dark in colour except those of *P. somniferum* var. *album* which are whitish to pale yellowish-white.
2. The seeds of the three varieties of *P. somniferum* and of *P. orientale* are nearly equal in size, being larger than those of the other species. The seeds of *P. orientale* differ from those of *P. somniferum* by having pointed and narrower ends.
3. The reticulations of the three varieties of *P. somniferum* seeds and those of *P. orientale* seeds have almost straight walls, while those of the other seeds have wavy walls. In *P. rhoeas* and *P. dubium* seeds the reticulations have more wavy walls than those of *P. argemone* and *P. nudicaule*.
4. The epidermal cells of the three varieties of *P. somniferum* and those of *P. orientale* are larger than those of the other seeds. They have almost straight anticlinal walls, differing from those of the other seeds which have wavy walls. The epidermal cells of *P. rhoeas* and *P. dubium* seeds are mostly isodiametric, while those of *P. argemone* and *P. nudicaule* are usually elongated and less wavy.
5. The cells of the other layers of the seed-coat of the three varieties of *P. somniferum* and of *P. orientale* seeds are larger than those of the other seeds, while those of *P. nudicaule* are the smallest in size. In *P. somniferum* var. *album* the cells of the cross layer and pigment layer differ from the other species in being colourless.

6. The aleurone grains of the three varieties of *P. somniferum* seeds are larger than those of the other seeds.

References

1. Winton, *Agr. Exp. Sta. Rep.*, 1903, p. 195.
2. Fedde, *Pflanzenreich*, 1909, IV, 104.
3. Muschler, *A Manual Flora of Egypt*, Vol. 1, 1912, p. 376.
4. Aleksandrov and Visloukh, *Botanicheskii Zhurnal S.S.S.R.*, 1934, **19** (2), 141.

SYNTHETIC ANALOGUES OF ADRENAL CORTICAL HORMONES: SOME 6:7:8:9-TETRAHYDRO-4:5-BENZINDANES

By D. B. Cowell and D. W. Mathieson

From the Department of Pharmaceutical Chemistry, School of Pharmacy, University of London

Received March 8, 1957

6:7:8:9-Tetrahydro-1-oxo-4:5-benzindane and its 3′-methoxy analogue have been synthesised from 1-oxotetralin and 1-oxo-6-methoxytetralin. Side chains typical of cortical hormones have been built up by known methods via the intermediate ethynyl alcohols: 1-acetoxy-1-glycollyl-6:7:8:9-tetrahydro-4:5-benzindane has thus been produced. In the case of the corresponding methoxy analogue, ring expansion probably occurs to furnish the corresponding "homo" compound—a substituted hydrophenanthrene.

The action of basic reagents on 2-cyano-6-methoxy-2-methyl-1-oxotetralin leads to ring fission and the formation of γ(2-carboxy-5-methoxyphenyl) α-methylbutyronitrile.

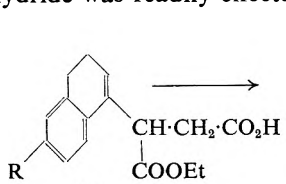
For the purpose of biological evaluation as adrenal cortical analogues, the attempted synthesis of two 4:5-benzindan derivatives I, (R = H, OMe) is described. This may be regarded as an extension of published work[1] on bicyclic steroid analogues.

Synthesis of the tricyclic ketones

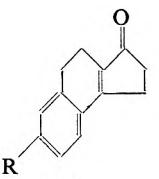
Construction of the carbon skeleton of such compounds started most conveniently by attaching a five membered ring on to the readily available 1-oxo tetralin and 1-oxo-6-methoxytetralin, the method used being that described by Johnson[2] and outlined overleaf (formulae II → IV). Thus, starting from 1-oxo-tetralin, condensation with diethylsuccinate in presence of potassium *tert.*-butoxide gave the intermediate half ester II (R = H) which was smoothly cyclised with zinc chloride:acetic anhydride to yield 6:7-dihydro-1-oxo-4:5-benzindane (III, R = H)[2]. With 6-methoxy-1-oxo-tetralin, slight modification of the published details[3] led to considerable improvement in yield. Thus as a first step the half ester II (R = OMe) was obtained in 75 per cent yield from the corresponding tetralone. Ring closure of such half esters may conceivably take two courses, either on to the benzene ring to form a perinaphthane derivative or on to the double bond, forming the required benzindan structure. Thus Johnson and colleagues[2] found with II (R = H), that with zinc chloride, III (R = H) was obtained, whilst with anhydrous hydrogen fluoride, V resulted, accompanied by disproportionation products. Consideration, however, of the structure of II (R = OMe) led to the conclusion that position 8 would be deactivated towards attack by an electrophilic reagent and that during ring closure (attack by an acyl carbonium ion) only one product would obtain viz. III (R = OMe). This proved to be so and III (R = OMe) resulted with either anhydrous hydrogen fluoride or zinc chloride:acetic anhydride. The structure of the

product was confirmed by dehydrogenation to 1-oxo-4:5-(3′-methoxy) benzindane identical with a specimen previously described by Billeter and Miescher[4].

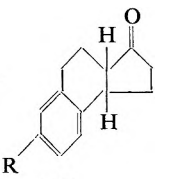
Hydrogenation of the unsaturated ketones III (R = H, OMe) was easily accomplished[5] to give the requisite saturated compounds IV (R = H, OMe). Although concomitant reduction of the ketone group to secondary alcohol took place to some extent, reoxidation with chromic anhydride was readily effected.



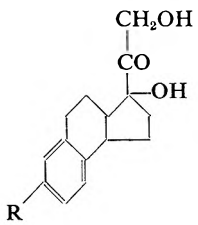
(II)



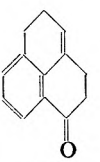
(III)



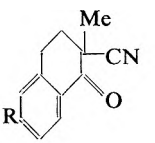
(IV)



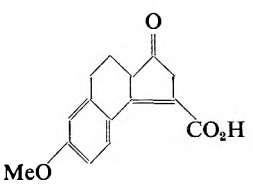
(I)



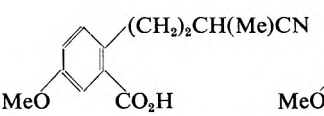
(V)



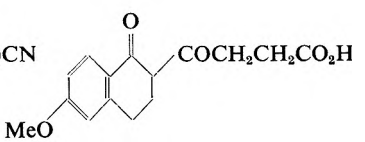
(VI)



(IIIA)



(VII)



(VIII)

Several alternative routes to these and similar tricyclic ketones were explored and one unsuccessful attempt has already been described elsewhere[6]. Analogously with that report it has now been found that treatment of VI (R = OMe) with potassium *tert.*-butoxide (and other basic reagents) leads to ring fission and the production of the acid VII. For this reason we were unable to effect a Stobbe condensation between VI (R = OMe) and diethylsuccinate.

In place of potassium *tert.*-butoxide, the use of sodium hydride as a condensing agent[7,8] has been explored. With 1-oxo-tetralin, the dicarboxylic acid corresponding to II (R = H) resulted, that is the normal "Stobbe" type of condensation. With 6-methoxy-1-oxo-tetralin, however, nearly half of the product consisted of a monocarboxylic acid which displayed anomalous properties. It was assigned the structure VIII on the following evidence and would arise by the alternative "Claisen" type of condensation: (cf. Daub and Johnson[8]). (i) It gave a green colour

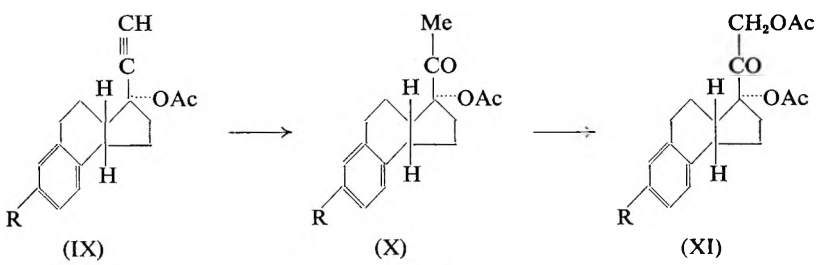
with ferric chloride, (ii) it proved stable to acid and was degraded by alkali to 6-methoxy-1-oxotetralin (iii), its spectroscopic properties are λmax 350 mμ ε 16,980 (ethanol): λmax 360 mμ ε 21,300 (0·1N NaOH). These spectral characteristics are in accord with the analogous case of benzoyl acetone, the bathochromic shift in 0·1N alkali being attributed by Morton, Hassan and Calloway[9] to the enolate ion.

Whilst 6:7:8:9:-tetrahydro-1-oxo benzindane IV (R = H) was a colourless oil and therefore difficult to assess from a stereochemical viewpoint, the methoxy analogue IV (R = OMe) proved to be a crystalline substance of sharp melting point and was deemed stereochemically homogenous. Comparison with other hexahydroindanes suggests that the more stable form of this substance would be *cis* about the C(8)–C(9) bond (cf. the classical work of Hückel and also references cited by Dreiding[10]).

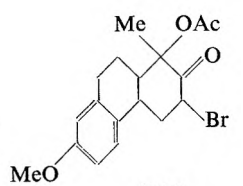
In addition, and more directly analogous, there remains the change from equilin to 14-*epi*-$\Delta^{8:9}$ equilin[11] and of 3-desoxyequilenin to 3-desoxy-14-*epi*-equilenin[12]: both isomerisations represent a change from a *trans* fusion of rings C and D to a *cis*. For these reasons the tentative assignment of the 6–5 membered ring junction in IV (R = OMe) is *cis*. Such is probably the case where R = H likewise.

Attachment of the Side Chain

By reacting the saturated ketones IV (R = H, OMe) with potassium acetylide in ether the corresponding ethynyl alcohols were obtained and these were converted to their acetates, IX (R = H, OMe).



(IX) (X) (XI)



(XII)

Addition of aniline to the triple bond by the method of Stavely[13] gave an anil which was readily hydrolysed to the ketone X. With X (R = H) the resulting ketone was brominated then treated with potassium acetate in acetone[14]: 1-glycollyl-1-hydroxy-6:7:8:9-tetrahydro-4:5-benzindan diacetate XI (R = H) thus resulted. With the ethynyl acetate IX (R = OMe), on the other hand, addition of aniline

undoubtedly gave a ketone and this in turn furnished a bromo ketone. Using a large variety of conditions, however, we were unable to effect replacement of this bromine atom either by the acetoxy or hydroxyl grouping. Under many conditions of hydroxylation of the triple bond ethynyl alcohols of the steroid series undergo at the same time a rearrangement similar to the pinacol rearrangement thus causing ring expansion to the so-called D-homo steroids[15]. With IX (R = OMe) the corresponding homo compound would probably have the structure shown in XII without the bromine atom. Although the conditions of Stavely[13] used here were specifically designed to avoid this rearrangement, such may in fact have occurred. Bromination of the "homo compound" would then furnish a substituted bromo*cyclo*hexanone XII where the bromine atom would be most likely to occupy an axial position[16] and thus be relatively inert to the action of alkali.

The ethynyl acetates IX (R = H, OMe) isolated in crystalline form would appear to be stereochemically homogenous and a tentative assignment of configuration is shown in IX (cf. reference 1). The same configuration applies to X and XI (R = H).

Experimental

β-Carbethoxy-β [1-(3 : 4-*dihydro*-6-*methoxy*) *naphthyl*] *propionic acid* (II, R = OMe). (i) *Stobbe condensation with potassium* tert.*-butoxide.* 6-Methoxy-1-oxotetralin (50 g.) and diethyl succinate (75 g.) were refluxed for 7 hours under nitrogen with potassium *tert.*-butoxide (from potassium (15 g.) and *tert.*-butanol (600 ml.)). After cooling, the *tert.*-butanol was removed *in vacuo*, water was added to the residue and this was extracted with ether. The acidic fraction was then removed therefrom with 10 per cent sodium carbonate solution, reprecipitated with hydrochloric acid and extracted again with ether. Evaporation gave a yellow gum, 66 g. (75 per cent) b.p. 190° at 0·5 mm.[3] Found: C, 66·9; H, 6·4; titration equivalent 300. Calc. for $C_{17}H_{20}O_5$, C, 67·1; H, 6·6 per cent. Titration equivalent 304. Light absorption in ethanol λmax 273 mμ (ε 10,000).

(ii) *With sodium hydride.* 6-Methoxy-1-oxotetralin (17·6 g.) and diethyl succinate (52 g.) were dissolved in benzene (300 ml.) and the solution dried azeotropically by distilling a 50 ml. portion of solvent. To the residual solution at 20° under nitrogen and with stirring, sodium hydride (4·8 g.) was quickly added followed by ethanol (1 drop). Evolution of hydrogen, negligible at first, markedly increased after 2 hours and ceased after 4–5 hours when the mixture was gelatinous and deep red. After pouring the mixture into water, the required compound was extracted therefrom with dilute sodium hydroxide. Acidification yielded a reddish-brown oil from which a crystalline material separated. On dissolving the oil in ether remained behind and was filtered off (2 g.). The ether layer was then re-extracted with 10 per cent sodium carbonate solution and this was reacidified. Ether extraction gave a solution of the required compound which was boiled with charcoal and on evaporation gave 22 g. (72·5 per cent) of a light brown oil from which crystals separated after a few days. This crystalline substance was separated with ether as described

above (3 g. thus resulted and the oily portion (II, R = OMe) distilled (b.p. 190° at 0·5 mm.) as a yellow gum. Found: C, 66·2; H, 6·35; titration equivalent, 300. Calculated for $C_{17}H_{20}O_5$; C, 67·1; H, 6·6 per cent. Titration equivalent 304.

The bulked crystalline material (5 g.) crystallised from ethanol in pale yellow prisms m.p. 166–167°. Found: C, 65·4; H, 5·6; OCH_3, 11·4. Molecular weight (ebullioscopic in acetone), 250; titration equivalent 280; $C_{15}H_{16}O_5$ requires C, 65·2; H, 5·8; OCH_3, 11·2 per cent; titration equivalent 276. Light absorption in ethanol λmax 241 mμ (ε 4,900); 272 mμ (ε 5,300); 350 mμ (ε 16,900). By adding sodium hydride to the reactants at 60° the induction period was reduced from 2 hours to several minutes and the yield of crystalline VIII (R = OMe) was increased to about 45 per cent of the total yield.

β-Carboxy-β [1-(3:4-*dihydro*)*naphthyl*] *propionic acid.* 1-Oxotetralin (14·6 g.) and diethylsuccinate (26 g.) in dry benzene (100 ml.) were treated as described above with sodium hydride (3·6 g.). There resulted a brown oil (14 g.) which partially solidified and on crystallisation from aqueous ethanol gave m.p. 184–186° (lit.[2] value m.p. 182–182·7°) (decomp.). Found: C, 68·1; H, 5·5; titration equivalent 123. Calculated for $C_{14}H_{14}O_4$, C, 68·3; H, 5·7 per cent, titration equivalent 123. Light absorption in ethanol, λmax 261 mμ (ε 8,900).

1-*Oxo*-6:7-*dihydro*-4:5-(3′-*methoxy*)*benzindane* (III, R = OMe). To redistilled half ester II, (R = OMe) (30 g.) in a polythene bottle, anhydrous hydrogen fluoride (250 ml.) was added and allowed to stand for two days. Evaporation of the reagent left a dark brown residue which was dissolved in a mixture of glacial acetic acid and concentrated hydrochloric acid (1:1) (200 ml.) and refluxed. When evolution of carbon dioxide had ceased (1 hour), acetic acid was removed under reduced pressure and the residual acid liquors neutralised and then extracted with ether. After washing with 10 per cent sodium hydroxide, then water, the solution was treated with charcoal, dried over sodium sulphate and evaporated. A mass of yellow crystals resulted whence crystallisation from ether gave the required ketone in colourless needles m.p. 96° in agreement with the literature value.[3]

The 2:4 *dinitrophenylhydrazone* crystallised from dioxan-ethanol in dark red needles charring at 255° (Birch, Quartey and Smith[3] cite m.p. 275°). Found: C, 61·3; H, 4·8; N, 14·6. $C_{20}H_{18}O_5N_4$ requires C, 60·9; H, 4·6; N, 14·2 per cent. Light absorption, (main bands in chloroform) λmax 423 mμ (ε 37,460) 326 mμ (ε 15,580).

The *semicarbazone* crystallised from glacial acetic acid in yellow micro needles m.p. 252° (decomp.). Found: C, 66·0; H, 6·0; N, 15·9; OCH_3, 11·31. $C_{15}H_{17}O_2N_3$ requires C, 66·4; H, 6·3; N, 15·5; OCH_3, 11·4 per cent.

From the residue left on evaporation of hydrogen floride, trituration with ether gave some crystalline material m.p. 141° (with gas evolution). The crystals were soluble in sodium carbonate and on attempted crystallisation from ethanol gave only the tricyclic ketone III (R = OMe). This was probably the intermediate keto acid IIIA.

1-*Oxo*-4:5-(3'-*methoxy*) *benzindane*. The above dihydro ketone (500 mg.) was heated with sublimed sulphur (100 mg.) at 220° (1½ hr.) when hydrogen sulphide evolution had almost ceased. The tarry residue was then extracted with boiling ether, the extract decolourised with charcoal and evaporated to yield a residue 211 mg. (42 per cent). Crystallisation from ether gave the required ketone in yellow needles m.p. 135°. The semicarbazone had m.p. 284° (decomp.) (lit. values[4] are m.p. 133° and 280–284° respectively).

1-*Oxo*-6:7:8:9-*tetrahydro*-4:5-(3'-*methoxy*) *benzindane*. The dihydro ketone (22·5 g.) in *iso*propanol (700 ml.) was hydrogenated (2 hr.) at a 5 per cent palladium:barium sulphate catalyst at 80 ats. and 80°. The filtered solution yielded a light yellow oil (23 g.) which contained 56 per cent ketone on assay with 2:4-dinitrophenylhydrazine. It was therefore dissolved in glacial acetic acid (25 ml.) and to it was slowly added with stirring a mixture of sodium dichromate (15 g.), conc. sulphuric acid (10 ml.) and water (50 ml.). The temperature was kept below 60°. After addition, the whole was kept at 55° for 1 hour, then sufficient ethanol added to destroy excess dichromate. Acetic acid was then removed and the whole extracted with ether. Evaporation gave an oil 18·3 g. (80 per cent) which had b.p. 145–150° at 0·4 mm. (16 g.). The distillate partly crystallised yielding thus the required *ketone* (8 g.) m.p. 63–64° on recrystallisation from ether. A further quantity of crystalline *ketone* (4 g.) was obtained from the oily residue by formation and hydrolysis of the semicarbazone. Found: C, 78·3; H, 7·0; OCH_3, 14·2. $C_{14}H_{16}O_2$ requires C, 77·8; H, 7·4; CH_3O, 14·35 per cent.

The *semicarbazone* crystallised from glacial acetic acid in small yellow needles m.p. 245°. Found: C, 66·2; H, 7·1; N, 15·5. $C_{15}H_{19}O_2N_3$ requires C, 66·0; H, 7·0; N, 15·4 per cent.

The 2:4-*dinitrophenylhydrazone* crystallised from ethanol-dioxan in red needles m.p. 220°. Found: C, 60·7; H, 4·8; N, 14·4. $C_{20}H_{20}O_5N_4$ requires C, 60·7; H, 5·1; N, 14·14 per cent. Light absorption (main band in chloroform) λmax 367·5 mμ (ϵ 24,000).

2-*Hydroxymethylene*-6-*methoxy*-1-*oxo tetralin*. Ethyl formate (5 g.) in dry benzene (40 ml.) was added at 0° over 30 minutes to sodium methoxide (from sodium 1·6 g.) under nitrogen. 6-methoxy-1-oxotetralin (5 g.) in dry benzene (40 ml.) was then added under the same conditions. After stirring (1 hr.) the mixture was allowed to stand at room temperature overnight. Water was added and the benzene layer extracted with 5 per cent sodium hydroxide. Acidification and ether extraction yielded a brown oil on evaporation of solvent. This crystallised on standing and was carried forward thus to the next stage. A sample distilled (b.p. 140–142° at 0·5 mm.) and crystallised from ether in pale greenish-yellow plates m.p. 67–68°, had the following analysis: found: C, 70·6; H, 5·5. $C_{12}H_{12}O_3$ requires C, 70·5; H, 5·9 per cent.

2-*Cyano*-6-*methoxy*-1-*oxo tetralin*. The above substance (25 g.) and hydroxylamine hydrochloride (17·5 g.) in glacial acetic acid (200 ml.) were stirred at 75° for 8 hours. Most of the acetic acid was removed, water added and the whole extracted with ether. After washing this with

sodium bicarbonate, evaporation gave the crude isoxazole (25 g.). To this was slowly added a 10 per cent solution of sodium methoxide in methanol and after standing 2 hours, the whole was diluted with water and impurities extracted with ether. Acidifying the aqueous phase followed by ether extraction, furnished 22·8 g. (90 per cent) of the required *cyano ketone* which crystallised from ether in colourless needles m.p. 98–99°. Found: C, 71·9; H, 5·7; N, 7·3. $C_{12}H_{11}O_2N$ requires C, 71·6; H, 5·5; N, 7·0 per cent.

2-*Cyano*-6-*methoxy*-2-*methyl*-1-*oxotetralin*. The above ketone (14 g.) with sodium ethoxide (from sodium 2·3 g.) in ethanol (120 ml.) was refluxed for 8 hours with methyl iodide (14 g.). There resulted 12 g. (80 per cent) crude product which crystallised from ether in colourless plates m.p. 70–70·5°. Found: C, 73·05; H, 6·0; N, 6·7. $C_{13}H_{13}O_2N$ requires C, 72·5; H, 6·1; N, 6·5 per cent.

γ-(2-*Carboxy*-5-*methoxy phenyl*) α-*methylbutyronitrile* (VII, R = OMe). The above ketone (1·05 g.) was stirred (5 hr. at 55°) with potassium *tert.*-butoxide (from potassium 1 g. in *tert.*-butanol 30 ml.). The acidic fraction of the resulting mixture weighed 1·21 g. and crystallised from ether in colourless prisms m.p. 108–110°. Found: C, 66·5; H, 6·4; N, 6·1; Titration equivalent, 228. $C_{13}H_{15}O_3N$ requires C, 66·9; H, 6·5; N, 6·0; titration equivalent 233.

Hydrolysis with ethanolic potassium hydroxide gave γ(2-*carboxy*-5-*methoxy phenyl*)α-*methylbutyramide* m.p. 181–183° from ethanol. Found: C, 61·9; H, 6·5; N, 5·5; titration equivalent 246. $C_{13}H_{17}O_4N$ requires C, 62·2; H, 6·7; N, 5·6 per cent. Titration equivalent 252.

1-*Ethynyl*-6:7:8:9-*tetrahydro*-1-*hydroxy*-4:5-*benzindane*. Dry ether (1000 ml.) was saturated with acetylene at 20° by bubbling the gas through for 1 hour. To this was then added over 30 minutes with stirring a mixture of 6:7:8:9-tetrahydro-1-oxo-4:5-benzidane (9·3 g.) in dry ether (100 ml.) and potassium *tert.*-amylate (from potassium 13 g. and dry *tert.*-amyl alcohol 300 ml.). After 8 hours stirring and passage of acetylene, water was added, the aqeous layer made faintly acid to litmus and saturated with sodium chloride. The organic layer was removed, the aqueous portion extracted with ether and the combined extracts evaporated to give the *acetylenic alcohol* (7·25 g.) b.p. 112–114° at 0·1 mm. Found: C, 84·7; H, 7·6. $C_{15}H_{16}O$ requires C, 84·9; H, 7·6 per cent.

1-*Acetoxy*-1-*ethynyl*-6:7:8:9-*tetrahydro*-4:5-*benzindane* (IX, R = H). The above ethynyl carbinol (7 g.) was refluxed under nitrogen for 20 hours with freshly distilled pyridine (40 ml.) and acetic anhydride (40 ml.). After pouring into water and extracting with ether, there resulted a mass of yellow crystals 7 g. (84 per cent). Crystallisation from light petroleum (b.p. 40–60°) gave colourless prisms m.p. 92–93°. Found: C, 79·9; H, 7·3; $COCH_3$ 16·7; absorbed 2·07 H_2. $C_{17}H_{18}O_2$ requires C, 80·3; H, 7·14; $COCH_3$, 16·9 per cent.

1-*Acetoxy*-1-*acetyl*-6:7:8:9-*tetrahydro*-4:5-*benzindane* (X). The above ethynyl acetate (3 g.) and redistilled aniline (1·4 g.) were dissolved in benzene (160 ml.) at 60°. To this was added mercuric chloride (8 g.) in boiling distilled water (40 ml.) and the two-phase system

vigorously stirred for 8 hours at 60°. After standing a further 16 hours at 25°, benzene was removed by steam distillation and in the aqueous residue there remained an insoluble brown gum. Water was decanted and the gum dissolved in acetone. Hydrogen sulphide was bubbled through until precipitation of mercury was complete. The filtered solution was then evaporated to give a brown oil, and treated with charcoal in ether to leave on evaporation, a crystalline mass 2·3 g. (70 per cent). Crystallisation from ether : light petroleum (b.p. 40–60°) (1 : 1) gave colourless needles m.p. 74°. Found : C, 74·7; H, 7·4. $C_{17}H_{20}O_3$ requires C, 75·0; H, 7·4 per cent. Infra-red absorption in carbon tetrachloride (NaCl prism) 1743 cm.$^{-1}$ (carbonyl) 1370 cm.$^{-1}$ (acetate methyl) 1419 cm.$^{-1}$ (active methylene).

This substance failed to form a semicarbazone and on prolonged boiling with 2 : 4-*dinitrophenylhydrazine* in 10 per cent ethanol : sulphuric acid gave the 2 : 4-dinitrophenylhydrazone of an $\alpha\beta$ unsaturated ketone—probably formed by elimination of acetic acid and consequent formation of a double bond in the 1 : 8 position (cf. ref. 1). It crystallised from ethanol-dioxan in dark red needles m.p. 187–188°. Found : C, 64·7; H, 5·6; N, 14·3. $C_{21}H_{20}O_4N_4$ requires C, 64·3; H, 5·1; N, 14·3 per cent. Light absorption (main band in chloroform) λmax 386 mμ (ϵ 27,300).

1-*Acetoxy*-1-*bromoacetyl*-6 : 7 : 8 : 9-*tetrahydro*-4 : 5-*benzindane*. The above ketone (1·36 g.) in glacial acetic acid (10 ml.) was treated with bromine (0·8 g.) also in acetic acid; on warming to 50–60° the bromine colour was discharged. Acetic acid was then removed. The residue was treated with charcoal in either to yield the *bromoketone* (1·2 g.) which crystallised from light petroleum (b.p. 60–80°) in colourless prisms. m.p. 106–109° (softens at 100°). Found : C, 58·8; H, 5·3; Br, 20·9. $C_{17}H_{19}O_3Br$ requires C, 58·1; H, 5·5; Br, 22·7 per cent.

1-*Acetoxy*-1-*glycollyl*-6 : 7 : 8 : 9-*tetrahydro*-4 : 5-*benzindane* (XI, R = H). The bromoketone (2 g.) in acetone (100 ml.) was refluxed for 30 minutes with potassium iodide (2 g.). Potassium acetate (6 g.) was then added and the whole refluxed 15 hours. Working up in the usual way gave 1·77 g. (94 per cent) of crystalline material which crystallised from ether in colourless needles m.p. 135–137°. Found : C, 69·0; H, 6·9; CH_3CO, 26·5. $C_{19}H_{22}O_5$ requires C, 69·1; H, 6·7; CH_3CO, 26·1 per cent.

Infra-red absorption in carbon tetrachloride (NaCl prism) 1743 cm.$^{-1}$, 1758 cm.$^{-1}$ (carbonyl), 1372 cm.$^{-1}$ (acetate methyl), 1418 cm.$^{-1}$ (methylene in $COCH_2OAc$).

Attempts to form the free glycollyl side chain either by direct replacement of Br by OH in the bromoketone, or by hydrolysis of the diacetate, gave oily mixtures which could not be evaluated.

1 - *Ethynyl* - 6 : 7 : 8 : 9 - *tetrahydro* - 1 - *hydroxy* - 4 : 5 - (3′ - *methoxy*) *benzindane*. Starting from 6 : 7 : 8 : 9-tetrahydro-1-oxo-4 : 5-(3′-methoxy) benzindane (5 g.) and proceeding in an identical manner to that described above for the desmethoxy compound, the required *acetylenic alcohol* was obtained as a yellow oil b.p. 150–152° at 0·4 mm. 3·4 g. (60 per cent). It was carried on thus to the next stage.

1 - *Acetoxy* - 1 - *ethynyl* - 6:7:8:9 - *tetrahydro* - 4:5 - (3' - *methoxy*) *benzindane* (IX, R = OMe). The above ethynyl alcohol (2·1 g.) was acetylated as described for the desmethoxy compound to yield 2·36 g. (95 per cent) of the required *ethynyl carbinol acetate* which crystallised from light petroleum (b.p. 60–80°) in colourless prisms. m.p. 98–99°. Found: C, 76·7; H, 7·2; OCH_3, 10·4; absorbed 2·04 moles H_2. $C_{18}H_{20}O_3$ requires C, 76·0; H, 7·1; OCH_3, 10·5 per cent.

1-*Acetoxy*-1-*acetyl*-6:7:8:9-*tetrahydro*-4:5-(3'-*methoxy*) *benzindane?* Treatment of the above ethynyl acetate in the same manner as described above for the desmethoxy compound gave a *compound* crystallising in colourless plates m.p. 93–94° from ether-light petroleum (b.p. 40–60°). Found: C, 71·6; H, 7·4; OCH_3, 10·1. $C_{18}H_{22}O_4$ requires C, 71·5; H, 7·3; OCH_3, 10·3 per cent. Infra-red absorption in carbon tetrachloride (NaCl prism) 1743 $cm.^{-1}$ (carbonyl), 1372 $cm.^{-1}$ (acetate methyl).

1 - *Acetoxy* - 1 - *bromoacetyl* - 6:7:8:9 - *tetrahydro* - 4:5 (3' *methoxy*) *benzindane?* (XIII). Bromination of the above compound in the usual manner gave a *bromoketone* crystallising from ether in colourless prisms m.p. 189–190°. Found: C, 57·1; H, 5·6; Br, 20·8; CH_3CO, 11·4. $C_{18}H_{21}O_4Br$ requires C, 56·7; H, 5·6; Br, 20·9; CH_3CO, 11·3 per cent.

Acknowledgement. We thank Professor W. H. Linnell for his advice and interest during the course of this work.

References

1. Coles, Linnell, Mathieson and Shoukri, *J. chem. Soc.*, 1954, 2617.
2. Johnson, Johnson and Petersen, *J. Amer. chem. Soc.*, 1945, **67**, 1360.
3. Birch, Quartey and Smith, *J. chem. Soc.*, 1952, 1768.
4. Billeter and Miescher, *Helv. chim. Acta*, 1946, **29**, 859.
5. Mathieson, *J. chem. Soc.*, 1953, 3248.
6. Cowell, Linnell and Mathieson, *ibid.*, 1953, 3723.
7. Daub and Johnson, *J. Amer. chem. Soc.*, 1948, **70**, 418.
8. Daub and Johnson, *ibid.*, 1950, **72**, 501.
9. Morton, Hassan and Galloway, *J. chem. Soc.*, 1934, 883.
10. Dreiding, *Chem. and Ind.*, 1954, **73**, 992.
11. Hirschmann and Wintersteiner, *J. biol. Chem.*, 1938, 126, 737.
12. Bachmann and Dreiding, *J. Amer. chem. Soc.*, 1950, **72**, 1323.
13. Stavely, *ibid.*, 1941, **63**, 3127.
14. Romo Rosenkranz Djerassi and Sondheimer, *ibid.*, 1953, **75**, 1277.
15. Shoppee and Prins, *Helv. chim. Acta*, 1943, **26**, 185.
16. Corey, *J. Amer. chem. Soc.*, 1953, **75**, 2301, 3297, 4832.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

***Veratrum album* L., A New Alkaloid from.** H. R. Hegi and H. Flück. (*Pharm. Acta Helvet.*, 1957, **32**, 57.) This new alkaloid was isolated from the leaf and could not be demonstrated in the subterranean organs. It was separated from the other alkaloids by partition chromatography, and its homogeneity was established by paper chromatography. The melting point, specific rotation and ultra-violet absorbtion characteristics were determined. These latter showed no absorption above 270 mμ, and an ascending curve between 270 mμ and 212 mμ without any maximum, indicating the absence carbonyl groups, $\alpha\beta$-unsaturated carbonyl groups, conjugated double bonds, aromatic rings in an alkamine structure, and unsaturated and aromatic acid components. The reaction with concentrated sulphuric acid was different from that of other veratrum alkaloids. It was concluded to be an ester alkaloid of proposed empirical formula $C_{25}H_{39}O_6N$ (unconfirmed). D. B. C.

ANALYTICAL

Adrenaline, Chormatographic Separation of, from Local Anaesthetics. K. Zachau-Christiansen and J. B. Jensen. (*Dansk Tidsskr. Farm.*, 1957, **31**, 1.) Separation of adrenaline from local anaesthetics (procaine and lignocaine) by the method of Björling is not always satisfactory. The cause has been traced to the use of buffer solutions, which are able to displace adrenaline from alumina. A satisfactory method is as follows: 10 to 20 ml. of a solution containing 50 to 200 μg. of adrenaline plus one drop of methyl red solution is titrated to the neutral point with 0·1N sodium hydroxide. This solution is passed through a column of 2 g. of alumina (Brockmann) in a tube having a diameter of 0·5 cm. After washing with 20 ml. of water, the column is eluted with 30 ml. of 0·1N hydrochloric acid. The eluate is treated with 2·0 ml. of N sodium hydroxide, 2·00 ml. of Folin and Ciocalteau reagent and 8·0 ml. of a 20 per cent aqueous solution of anhydrous sodium carbonate. After making up to 50 ml., and standing for at least 30 minutes, the blue colour is measured at 570 mμ in a 1 cm. cell, after centrifuging if necessary. The solutions used for calibration should contain procaine (or lignocaine) and bisulphite as in the solution to be analysed. G. M.

Aneurine, Determination of, with 6-Aminothymol. K. J. Hayden. (*Analyst*, 1957, **82**, 61.) The rapid photometric method described is suitable for certain pharmaceutical and cereal products and depends upon the intense yellow colour produced on treatment of the vitamin with diazotized 6-aminothymol. The reagent is shown to be more specific than the more widely exploited reagent *p*-amino-acetophenone, and the method is suitable for materials containing upwards of 0·1 mg. of aneurine per g., and the maximum experimental error of a single determination is 1·5 per cent (P = 0·95). D. B. C.

Prednisolone and Prednisone, Determination of. J. Buur Jensen. (*Dansk Tidsskr. Farm.*, 1956, **30**, 293.) A solution containing about 0·3 to 1·0 mg. of the compound in absolute ethanol is treated with 0·5 ml. of semicarbazide acetate reagent, refluxed for 2 hours, and made up to 100 ml. The absorption is then determined at 292 mμ. In the case of tablets the active substance is first extracted by shaking with cold absolute ethanol. The presence of cortisone or hydrocortisone in prednisolone can also be detected by comparison with standards, since the addition of these shifts the maximum absorption from 292 mμ towards lower wavelengths, while the maximum at 242 μ disappears and is substituted by a point of inflection at about 250 mμ. The method has an error which is estimated at about 3 per cent, but it cannot distinguish between prednisolone and prednisone. These two compounds themselves may be distinguished by their melting points and by the solution in sulphuric acid, in which prednisolone gives a wine red colour and prednisone a yellowish green fluorescent solution. G. M.

Rauwolfia Alkaloids, Colorimetric Determination of. H. Wunderlich. (*Pharm. Zentralh.*, 1957, **96**, 68.) After extraction of the medicament with a methanol-acetic acid-water mixture, the solution was cooled to 0° and the alkaloids precipitated by the addition of excess ammonium reineckate after the addition of ether to reduce the solubility of the complex. After filtration, the precipitate was dissolved in methanol and the absorption measured at 427 mμ. Concentrations of about 1 mg./ml. were suitable for constructing a calibration curve, showing that the method is more sensitive than many other methods The author maintains that only by the use of the above solvent in the extraction process, will the alkaloids obtained be fully pharmacologically active. D. B. C.

Sulphonamides, Identification of. H. Baggesgaard Rasmussen, J. Berger and G. Espersen. (*Dansk Tidsskr. Farm.*, 1957, **31**, 66). Sulphonamides were acetylated at the *p*-amino group by boiling the sulphonamide with glacial acetic acid and acetic anhydride for 3 minutes, precipitating by the addition of water and recrystallising from alcohol or glacial acetic acid, followed by drying at 105° for 3 hours. The equivalent weights were determined by titration of the acetyl derivatives with 0·1N lithium methoxide in dimethylformamide, using thymol blue as indicator. The acetyl derivatives did not exhibit sharp melting points, droplets being formed in the capillary tube 10 to 15° below the temperature at which the substance melted completely. The temperature of complete melting was, however, a well-defined character, varying by at most 1 or 2° between determinations. The hydrolysis of sulphonamides was carried out by heating with sulphuric acid or pyrolysis. Pyrolysis required a careful technique and was applicable to only 4 of the sulphonamides investigated. Hydrolysis was best carried out by heating with sulphuric acid (40 per cent) until a precipitate of sulphanilic acid appeared. The liquid was cooled and an excess of sodium hydroxide solution added to dissolve the sulphanilic acid. The amine was extracted with ether, and the extract filtered through exsiccated sodium sulphate, evaporated and dried over silica gel for 24 hours. While the acetyl derivatives were satisfactory for the identification of most of the sulphonamides, the melting points were rather high and in some cases did not provide complete proof of identity. The amines obtained by hydrolysis with sulphuric acid provided a ready means for the identification of all the sulphonamides examined. G. B.

Sympathomimetic Amines, Determination of, by Ion Exchange. M. C. Vincent, E. Krupski and L. Fischer. (*J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 85). Assays of sympathomimetic amines were carried out by adsorbing the amines on columns of ion exchange resins and subsequently eluting them and titrating with acid. For this purpose a strongly basic anion exchange resin (Amberlite IRA–400) gave satisfactory results except with amines containing phenolic groups; these became firmly bound to the resin and could not be recovered under the usual assay conditions. A carboxylic cation exchange resin (Amberlite IRC–50) was satisfactory for the assay of pure amine salts, but not for preparations containing sodium and other ions which were eluted with the amine and interfered in the estimation. This was avoided by the use of a weakly basic anion exchange resin (Amberlite IR–45), which binds weakly both phenolic and non-phenolic amines. Sodium ions and other interfering substances from pharmaceutical preparations can be removed by washing the column with water prior to removal of the amine with ethanol. Quantitative results were obtained by this method using a number of amines. In assaying amines such as adrenaline, which are sensitive to alkali and light it was necessary to protect the column from light and to collect the eluate in 0·1 N hydrochloric acid. The method was successfully applied to a range of pharmaceutical preparations, including capsules, tablets, jellies, inhalers, emulsions and oily solutions. G. B.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Fluorocarbon, Removal of Anticomplementary Activity and Host Antigens from Viral Preparations by. K. Hummeler and V. Hamparian. (*Science*, 1957, **125**, 547.) Fluorocarbon Freon 112 with *n*-heptane to give a solution of s.g. 1·30 (1 part) was mixed with poliomyelitis culture fluid, still containing cell debris (10 parts) and homogenised in a Servall Omnimixer at 14,500 r.p.m. for 3 to 4 minutes, whilst being cooled to 0°. The homogenate was centrifuged at 1000 r.p.m. for 5 to 10 minutes, to separate the aqueous and fluorocarbon layers. The aqueous phase revealed little loss of infectivity, but was free of anticomplementary effects, and specific reactions, not previously evident, were readily apparent. Exposure to fluorocarbons of crude antigens, which were not anticomplementary, caused no decrease of specific reactions. Other tissue-culture antigens (adeno group and Coxsackie B) and antigens derived from chick chorio-allantoic membranes (herpes simplex, mumps soluble antigen) or allantoic fluids (mumps and influenza virus antigens) were similarly treated with fluorocarbon, without loss of antigen titre. J. B. S.

Lysergic Acid Diethylamide, Metabolism of. E. Rothlin. (*Nature, Lond.*, 1956, **178**, 1400.) The report (Axelrod, Brady, Witkop and Evarts (*Nature, Lond.*, 1956, **178**, 143) that little is known of the biological fate of lysergic acid diethylamide, is refuted. It is neither bound nor destroyed in rat blood after incubation for 6 hours at 38°. In the presence of rat tissue homogenates activity decreases within a few minutes, but there is little further decrease thereafter. With liver and muscle homogenates a 50 per cent decrease occurs almost immediately, but there is no further decrease thereafter. With liver and muscle homogenates a 50 per cent decrease occurs rapidly but no further reduction in 17 hours; brain homogenates show 58 per cent reduction in 10 minutes and 79 per cent in 17 hours. Experiments with carbon-14 labelled

lysergic acid diethylamide showed that most organs reached the highest level after 10–15 minutes, but lost it gradually in the course of a few hours, the highest concentration being found in the liver after 30 minutes. Other experiments showed that 7–8 per cent of the activity was excreted within 12 hours, of which about half was in the expired air and the rest in the urine and faeces; 70 per cent was found in the intestinal contents. Labelled diethylamide injected into rats with bile fistula was found in the bile to the extent of 70 per cent within 2 hours. Lysergic acid diethylamide is excreted as water-soluble decomposition products; three substances R_F 0, 0·13 and 0·18 have been separated on paper but not identified. J. B. S.

Purines, Transformation of, into Pteridines. A. Albert. (*Biochem. J.*, 1957, **65**, 124.) The conversion of purines into pteridines has been studied since it is considered that it may shed light on the biological origin of natural pteridines. 2-Hydroxypurine gives 4-amino-5-formamido-2-hydroxy pyrimidine at 20° and pH 5, and 2-mercaptopurine behaves similarly. The formyl group is lost more and more rapidly as it becomes autocatalysed by H^+ ions from the liberated formic acid, giving 4:5-diamino-2-hydroxypyrimidine. The latter combines with glyoxal to give 2-hydroxypteridine in 93 per cent yield at 37° and pH 7. This is typical of a general reaction which proceeds under physiological conditions regardless of substituents in the pyrimidine and requires no catalyst. 1:2-Diketones, aldehydo acids and keto acids react similarly to glyoxal. These reactions are illustrated by a number of examples in which the yields vary from 16 to 85 per cent. Guanine and hypoxanthine undergo similar transformations but less readily. Experiments by Weygand and Waldschmidt (*Angew. Chem.*, 1955, **67**, 328), in which [2-^{14}C]2:4:5-triamino-6-hydroxypyrimidine was fed to pierid caterpillars and [2-^{14}C]xanthopterin was isolated from the wings, support the view that the above reactions are of biological significance. Ziegler and Günder and others (*Z. Naturf.* B., 1956, **11**, 82) found that larvae of the amphibian *Xenopus* similarly were able to convert [2-^{14}C]guanine in labelled pteridine. It is suggested that this reaction may regulate growth in certain circumstances. J. B. S.

CHEMOTHERAPY

Ethyl Mercaptan and Related Compounds. G. E. Davies, G. W. Driver, E. Hoggarth, A. R. Martin, M. F. C. Paige, F. L. Rose and B. R. Wilson. (*Brit. J. Pharmacol.*, 1956, **11**, 351.) In mice infected with *Mycobacterium tuberculosis*, human type strain 905, ethyl mercaptan showed a high activity. Examination of a large number of related compounds has shown that only derivatives that can be metabolized to ethyl mercaptan are active. The ethyl thiol-esters, particularly ethyl thiobenzoate, were most active and found to be superior to other potential sources of ethyl mercaptan. G. F. S.

***Trypanosoma congolense* and *T. vivax*, New Compound Active Against.** W. C. Austin, H. O. J. Collier, M. D. Potter, G. K. A. Smith and E. P. Taylor. (*Nature, Lond.*, 1957, **179**, 143.) Decamethylene bis *iso*quinolinium bromide) (I) and a number of analogues have been prepared and examined for activity in trypanosomiasis. (I) possessed only slight activity against *T. congolense*; a similar order of activity was shown by a series of related polymethylene compounds, activity being at a maximum in this series with hexamethylene bis(*iso* quinolinium bromide). Decamethylene bis(4-aminoquinaldinium iodide) (Dequadin) was appreciably active, and in near lethal doses cured *T. rhodesiense* infections in mice, and also showed prophylactic properties. Dequadin also

possesses high antibacterial and antifungal activity. The primary product of the reaction between 4-aminoquinaldine and hexamethylene di-iodide showed good activity against *T. congolense*, but this was later resolved by fractional crystallisation into three fractions. The most active of these, substance II, was identified by an alternative and unambiguous synthesis as 6′(4-quinaldylamino)hexyl-4-aminoquinaldinium iodide hydrodide. Both the symmetrical isomers hexamethylene bis(4-aminoquinaldinium iodide) (III) and *NN*-bis(4′quinaldyl)-1:6-hexamethylenediamine dihydroiodide (IV) were less active against *T. congolense*, although (III) was slightly more active against *T. rhodesiense*. It is suggested that in general unsymmetrical compounds are more active against *T. congolense*, whilst symmetrical structures have the greater activity against *T. rhodesiense*. Substance II was more active and less toxic than antrycide against *T. congolense* and *T. vivax*. The corresponding chloride to substance II, and its sparingly soluble suramin salt show low toxicity and useful prophylactic activity against *T. congolense*. J. B. S.

PHARMACY

Agar Clarification. J. G. Feinberg. (*Nature, Lond.*, 1956, **178**, 1406.) A simple procedure for agar clarification, adapted from the method Viswanatha and Liener for the purification of a proteinase, is described. New Zealand agar (1 per cent in distilled water) was filtered twice through well rinsed glass wool to remove grossly insoluble particles. At this stage 1 per cent w/v sodium azide may be added as a preservative. A mixture of equal parts of powdered bentonite and 'Hyflo Super Cel' is added (1–2 per cent by volume) and the whole shaken vigorously by hand to disperse the clarifying agents. The suspension is stored at 56° for several days, the clarifying agents being resuspended daily by gentle inversion of the bottles. The clarified agar is decanted and filtered through No. 5 Whatman paper in a hot funnel, the first few ml. being returned for refiltering. The product has the colourless clarity of water in the molten state, and only a faint, clean, unclouded opalescence when set in plates.

J. B. S.

Decanol-1, Solubility of, in Solutions of Sodium Caprate, Laurate and Myristate above the CMC. K. Passinen and P. Ekwall. (*Acta chem. scand.*, 1956, **10**, 1215.) The solubility of decanol in solutions of sodium caprate, laurate and myristate above the critical micelle concentration has been determined, the point of maximum solubility being that at which the solution becomes turbid, due to the separation of a mesomorphic phase composed of decanol, soap and water. The solubility curves are linear over a wide range of concentration above the CMC, before first sloping upwards in a narrow concentration range, and then continuing again as a straight line. The position of the break point in the curves shifts to lower soap concentrations as the molecular weight of the soap increases. The decanol solubility of the mixed micelles decreases with increasing molecular weight of the soap; it is also dependent on the nature of the ionic group in the association colloid and on the gegenions. The meaning of 2nd CMC, and factors responsible for the separation of the mesomorphic phase are discussed. J. B. S.

Decanol-1, Solubility of, in Sodium Oleate Solutions Containing Sodium Chloride. K. Passinen and P. Ekwall. (*Acta chem. scand.*, 1956, **10**, 1228.) The effect of sodium chloride on the solubility of decanol in sodium oleate solutions has been studied, and it has been shown that decanol solubility decreases rapidly with increase of sodium chloride concentration, though the

general shape of the solubility curve is unaltered by sodium chloride concentrations up to 0·05 M. In the presence of sodium chloride the break point in the solubility curve occurs at lower oleate concentrations (0·08 M oleate) than in the absence of salt (at about 0·12 M oleate), though the break is less marked. The solubility of decanol could not be measured when the sodium chloride concentration exceeded about 0·15 M, because solutions become viscous and gel-like. Since the break-point occurs at the so-called 2nd CMC, this must be a function not only of the concentration of the micelle forming hydrocarbon chain ions, but also of the concentration of the gegenions. Factors are discussed, which are responsible for the decrease in the solubility of decanol. J. B. S.

Ferric Saccharate for Injection. J. Büchi and R. Zoppi-Hug. (*Pharm. Acta Helvet.*, 1956, **31**, 497.) The use of colloidal ferric saccharate solution allows the intravenous administration of large quantities of iron and is well tolerated by the patient. A suitable preparation may be made as follows: 18 g. of crystalline sodium carbonate dissolved in 250 ml. of cold water is added in small portions to a solution of 10 g. of ferric chloride in 100 ml. of cold water. The temperature must not exceed 15°. The precipitate is washed free from chloride, and the excess of water is sucked off on a vacuum filter. The fresh ferric hydroxide is rubbed down with 14·5 g. of sucrose plus 0·5 g. of glucose, and a solution of 0·85 g. of sodium hydroxide in 10 ml. of water is added. The mixture is warmed on the water bath until on dilution with 10 parts of water a clear solution is obtained. The liquid is evaporated to dryness on the water bath, and dried at 105°. The solution for injection is prepared by dissolving the preparation in water and adjusting to a content of 2 g. of Fe/100 ml., sterilising through a filter. This preparation does not contain any ions, provided that the pH is not below 7. Its toxicity is low, the LD50 for mice being 150 mg. Fe/kg. G. M.

PHARMACOGNOSY

***Atropa belladonna*, Adulteration of.** M. Wellendorf. (*Dansk Tidsskr. Farm.*, 1956, **30**, 281.) Adulteration of belladonna leaf with *Scopolia carniolica* is difficult to detect in the powdered drug. A useful indication is given by counting the number of idioblasts, containing sandy crystals, per sq. mm. For scopolia this value ranges from about 5 to 8, for belladonna it is about 20. If a value of below 10 is found, then the palisade ratio should be determined as confirmation. This method applies only to the leaf drug; for the herb the results are quite different. G. M.

Saponin in Drugs, Determination of, by Haemolysis. J. Petričić and V. Petričić. (*Acta pharm. Jug.*, 1956, **6**, 95). Extracts were prepared from samples of various saponin-containing drugs grown in Jugoslavia or imported. Although aqueous ethanol was a better solvent than water in some cases, the authors preferred to use aqueous decoctions for the determinations, since the drugs are generally used in the form of aqueous extracts. The addition of sodium bicarbonate did not increase the efficiency of extraction. The saponin content of the extracts was assessed by determination of the degree of haemolysis of citrated cattle blood caused by dilutions prepared in arithmetical progression. A sample of pure saponin was the standard of comparison. On the basis of the results obtained the following are proposed as minimum standards for the Jugoslavian Pharmacopoeia: herniary herb 30, primula root 120, quillaia bark 75, saponaria root 50 and senega root 75 units per g. The Ph. Jug. unit is equivalent to 1 mg. of the standard saponin sample. G. B.

PHARMACOLOGY AND THERAPEUTICS

Antifoam Agents in Pulmonary Oedema. R. C. Balagot, R. M. Reyes and M. S. Sadove. (*J. Amer. med. Ass.*, 1957, **163**, 630.) A new compound, No. 5507, which consisted of silicone 0·01 per cent, Superinone (a polyhydric alcohol) 0·75 per cent, glycerol 1 per cent, and potassium bicarbonate 1 per cent, was tested for its ability to suppress foam in adrenaline-induced pulmonary oedema in rabbits. It was compared with octyl alcohol, and 10 and 20 per cent ethanol and was found much superior to any of these, giving a survival rate (in 24 rabbits) of 53 per cent; it also compared favourably with results previously obtained (Luisada and Cardi) with 95 per cent ethanol. Animal studies showed the compound to be non-toxic and to have no depressant effects on the central nervous system. Clinically, excellent results were obtained in 8 patients with pulmonary oedema, there was almost immediate suppression of foam, the oedema quickly cleared, and the cyanotic condition of the patients improved. S. L. W.

β-Diethylaminoethylphenothiazine-10-carboxylate Hydrochloride (Transergan), Studies on. S. Wiedling. (*Acta. pharm. tox. Kbh.*, 1957, **13**, 59.) This is a new phenothiazine derivative which has been found to be valuable clinically as a non-specific spasmolytic and antiparkinsonism. It has a strong spasmolytic action against spasms induced in isolated tissues by acetylcholine, histamine, 5-HT and barium, but against adrenaline and noradrenaline its action is weaker. It has a strong local anaesthetic action on the eye, being about five times as active as lignocaine, but it also has a local irritant action. Intravenously it does not potentiate the local anaesthetic action of lignocaine and it is considered therefore to have no central analgesic action. Doses of 1 mg./kg. or over reduce the rabbits blood pressure, but it does not antagonise the actions of acetylcholine, histamine, adrenaline or noradrenaline. Only with toxic doses (10 mg./kg.) is there a noticeable reduction of the body temperature of the rabbit. The acute LD50's to mice were oral 0·44 g./kg., subcutaneous 0·62 g./kg., intraperitoneal 0·14 g./kg. and intravenous 0·027 g./kg. Toxic doses cause tonic and clonic convulsions, while lower doses have a sedative effect preceded by a stage of excitation, irregular respiration and Straub phenomena. The chronic administration of doses up to 30 mg./kg. daily over one month does not reduce the growth of rats and produces no pathological effects. Against a haemolytic strain of *Staphyloccus aureus* a concentration of 4×10^{-4} has a bacteriostatic effect and 8×10^{-4} a bactericidal effect.

G. F. S.

Ganglion-blocking Activity of a Series of 4-Aminoethylpiperidine Derivatives. K. I. Colville and R. V. Fanelli. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 727.) A series of derivatives of 4-β-aminoethylpiperidine of the general formula $XRR'N{\cdot}C_5{\cdot}H_9{\cdot}CH_2{\cdot}CH_2{\cdot}NR''R'''RX$ was examined. The compounds were tested for hypotensive activity in anaesthetised cats and for ganglion-blocking action using the nictitating membrane of the anaesthetised cat. The LD50 was determined in mice. The most potent compound examined was R′=Me,R″R‴N=pyrrolidino,R=Et,X=I, and the analogous compound where R=Me was slightly less potent. The corresponding tertiary amines were found to be much less active than their quaternary derivatives. The hypotensive action appeared to be almost entirely due to the ganglion-blocking activity of the compounds. The effect of varying the substituent groups is discussed.

G. B.

Glutethimide in Labour. T. M. Abbas. (*Brit. med. J.*, 1957, **1**, 563.) This study is based on a survey of 100 parturient patients, consisting of equal numbers of primiparae and multiparae, given two tablets of glutethimide (each containing 250 mg.) early in the first stage of labour. For control purposes a second series of 100 cases of normal parturient patients were treated with 30 grains of chloral hydrate as a syrup. In the glutethimide group good relief of pain was obtained in 70 of the patients, fair relief in 12, and poor relief in 18. Mental and physical relaxation, with ability to sleep between contractions, was good in 72, fair in 10, and poor in 18. In the glutethimide group the average length of labour was 12 hours for primiparae and 7½ hours for multiparae; in the control series the corresponding figures were 13½ and 8 hours. There were no maternal or foetal deaths and no significant untoward effects attributable to the drug. Four patients had nausea and 5 vomited within 3 hours of administration of glutethimide. In the chloral hydrate group vomiting occurred in 77 patients. The total amount of pethidine needed for additional sedation and analgesia in the glutethimide group was substantially less than that required in the control series. Glutethimide was well tolerated by all patients, and none showed any allergic reaction. There was no significant difference in the incidence of post-partum haemorrhage in the two groups. All patients who initially were apprehensive lost their fear and became fully cooperative shortly after administration of the drug, greatly facilitating the general management of the first stage. S. L. W.

Lead Poisoning, Treatment of, with Calcium Edetate. O. Wegelius and A. Harjanne. (*Scand. J. clin. lab. Invest.*, 1956, **8**, 335.) A series of 5 patients, 3 with acute lead poisoning and 2 with mild subjective symptoms and definite signs of lead poisoning were given daily intravenous infusions of 2·5 g. of calcium edetate in 1000 ml. of 5 per cent dextrose solution for a period of 10 days. For those patients with the more severe symptoms, the treatment was repeated after an interval of one week. Urinary excretion of lead increased greatly during the treatment, and in all cases nausea, cramping abdominal pains, fatigue and any anaemia present disappeared, and no basophilic stippling of the red cells was visible. The urinary coproporphyrin returned to the normal level. No untoward effects, such as renal damage were observed at the dosage level employed, although they have been reported when larger doses of calcium edetate were given. G. B.

Methylpentynol; Toxic Effects and Side-Effects. E. Marley and J. S. W. Chambers. (*Brit. med. J.*, 1956, **2**, 1467.) Eight case histories of toxic reactions to methylpentynol given in therapeutic dosage are described. The physical signs included pupillary abnormalities, nystagmus, diplopia, ptosis, loss of facial muscle tone, dysarthria, tremor of the protruded tongue, and cerebellar ataxy in the limbs or admixtures of this with posterior column ataxia. Muscular tone was usually diminished. Plantar reflexes were flexor and sensation remained unaffected. Mood change, particularly depression, dominated the accompanying mental state in these patients. Disorientation was not observed, but nominal dysphasia, paraphasia, distortion of subjective time experience and body image were noted. Illusions or hallucinations were common, as was impairment of memory for the toxic episode. Withdrawal symptoms were seen in one patient who had been taking from 1 to 1·5 g. of methylpentynol daily for 10 months. In addition to the foregoing, side-effects (as distinct from toxic phenomena) were noted in two groups, each of 15 patients, the one given therapeutic doses (0·5 to 2 g. a day) for 1 to 6 weeks, and the other

receiving larger doses (about 5 g.) during labour. Side-effects on these two groups were confined to minor alterations in the mental state; dizziness and light-headedness were the most frequent side-effects noted. The authors emphasise the resemblance to barbiturate or alcoholic intoxications, and also the small doses and brief duration of action which may precipitate toxic reactions, especially in patients with psychiatric disorders. S. L. W.

Methyprylone, Clinical Trial of. J. S. Stewart. (*Brit. med. J.*, 1956, **2**, 1465.) A controlled trial was carried out on 100 patients in the ear, nose and throat wards of a London hospital to compare the hypnotic effect of methyprylone with amylobarbitone sodium and butobarbitone. Four sets of tablets identical in appearance and closely similar in taste were prepared: amylobarbitone sodium 100 mg., butobarbitone 100 mg., methyprylone 200 mg., and a control (lactose, starch, and a trace of infusion of quassia). One of the tablets was given on each of four consecutive nights, and the results assessed objectively by the nursing staff (speed of onset and duration of action) and subjectively by the patients (speed of onset, quality of sleep, and nature of after-effects). The results showed no significant difference between the mean scores for the three drugs. The score for the control was not greatly lower but was statistically significant. The only side-effect recorded was headache which occurred twice after each drug and once after the control. The author concludes that methyprylone is a reliable non-barbiturate hypnotic of comparable effect to amylobarbitone sodium and butobarbitone. S. L. W.

Noradrenaline in Shock Due to Visceral Perforation. D. D. Davies. (*Brit. med. J.*, 1957, **1**, 261.) Three cases are described in which noradrenaline had to be used to maintain adequate blood pressure after traditional methods of resuscitation had failed. Two patients with perforation of the large bowel recovered completely; the third patient, with peptic ulcer perforation, showed a good immediate recovery but died 12 hours later from a pulmonary embolus. The noradrenaline was given by intravenous infusion, commencing with a strength of 8 mg. of noradrenaline per 1000 ml. of dextrose-saline, the strength being increased to 16 mg. and later to 32 mg. per 1000 ml., and the drip-rate varying between 25 and 80 drops per minute. The strength of the solution and the rate of drip were adjusted throughout to raise and maintain the systolic pressure at about 90 mm. Hg or above. In the treatment of shock due to visceral perforation simple infusions of blood or dextran alone are usually effective in permanently maintaining an adequate blood pressure level provided that the factors causing the shock are effectively treated. In these cases infusions of blood or dextran should therefore first be instituted, but if these measures fail then the early use of continuous noradrenaline infusion holds out the best chance of recovery. Its early use in the shocked state probably produces an increase in blood flow to vital organs. This and the correction of the myocardial ischaemia, following the increase in coronary blood flow, may be important factors in helping to prevent the development of an irreversible phase in which noradrenaline therapy is of no avail and may do more harm than good. The author stresses the paramount importance of adequate oxygenation in the shocked patient. S. L. W.

Nystatin in Moniliasis. E. T. Wright, J. H. Graham and T. H. Sternberg. (*J. Amer. med. Ass.*, 1957, **163**, 92.) Nystatin was used topically in the treatment of 122 patients with infections due to *Candida albicans*. There were 42 patients with oral moniliasis, 17 with vaginal moniliasis, and 63 with such cutaneous

involvement as paronychia, intertrigo, and perlèche. Nystatin was applied in the form of ointments, solutions, powders, troches, capsules, suppositories and jelly for vaginal use. The ointment and gel consisted of a plasticised petrolatum base containing 5,000 to 200,000 units/g. of Nystatin. Two types of solution, containing 5,000 and 100,000 units/ml. were used; one was propylene glycol solution, and the other contained 2 per cent procaine hydrochloride and 0·25 per cent polysorbate 80. The latter solution was prepared both with and without 2·5 mg./ml. of hydrocortisone. The powder contained 175,000 units of nystatin per teaspoon. The troches contained 200,000 units, and in addition some contained 2·5 mg. of neomycin sulphate and 0·25 mg. of gramicidin. The capsules and tablets used as troches and suppositories contained 125,000 to 500,000 units of Nystatin. The suppositories contained 10,000 to 100,000 units. The cutaneous infections were treated with ointments and solutions 4 times daily. Oral infections were treated with solutions, troches, capsules and tablets used as troches 4 times daily. In addition the powder was used orally 4 times daily (one teaspoonful in glass of milk or water as a mouthwash). Vaginal infections were treated once daily with the use of disposable applicators, suppositories, and tablets and capsules used as suppositories. Treatment periods varied from 3 days to 1 month. In all but 5 cases the response to treatment was good to excellent. The lesions ordinarily disappeared in 2 or 3 days in thrush or perlèche, though some patients required 2 or 3 weeks. Clinical relapses were infrequent, though in 43 per cent of the patients repeat cultures revealed the presence of *C. albicans* even though the lesions had disappeared. This was especially true in patients with severe systemic diseases, especially diabetes. The type of vehicle in which the Nystatin was placed influenced the clinical effectiveness. Solutions were more effective in intertriginous areas. The addition of hydrocortisone to the solution shortened the course of therapy.

S. L. W.

APPLIED BACTERIOLOGY

Lactobacilli and Streptococci, Differentiation of, by Paper Partition Chromatography. A. T. R. Mattick, G. C. Cheeseman, N. J. Berridge and V. Bottazzi. (*J. appl. Bact.*, 1956, **19**, 310.) This is a report of the application of 2-dimensional paper partition chromatography of extracts of washed bacterial cells to the differentiation of species of *Lactobacillus* and *Streptococcus*. Preliminary experiments revealed that application of bacterial cells directly to the paper yielded chromatograms full of faint streaks. Delayed extraction of soluble material was held to be responsible for this streaking. The method afterwards adopted consisted of preparing a suspension from a 24 hour culture of the organisms, adjusting the suspension to a definite optical density, and washing the cells twice by centrifuging and resuspending in standardised volumes of water. Finally the cells were suspended in 10 per cent v/v acetic acid. The suspension was allowed to stand for 4 hours, centrifuged and drops of the supernatant spotted on to the chromatogram paper. The chromatogram was developed first with butanol-acetic acid and then with *m*-cresol and phenol for the second dimension. Results were recorded as widths and lengths of spots and as R_F values relative to the position of alanine. The positions were recorded with the aid of an "adjustable shadowgraph" which is described in detail. The results indicated that the patterns of amino acids and peptides revealed by ninhydrin were generally constant for closely related strains within a species. Variations were found with different species, some being otherwise difficult to

BOOK REVIEW

COLORIMETRIC ANALYSIS. Second Edition. Vol. I. Determinations of Clinical and Biochemical Significance. By Noel L. Allport and J. W. Keyser. Pp. xi + 424 (including Index). Chapman and Hall, London. 1957. 50s.

The first edition of Mr. Allport's "Colorimetric Analysis," in one volume, was published in 1945 and was an immediate success. A second impression was taken in 1947 and a third in 1951. Since the original edition, colorimetric analysis has been developed considerably and many refinements in technique have established its use in clinical and biochemical analysis.

In approaching a second edition the specialisation which had occurred in analytical methods indicated to the author that he could no longer deal single handed with such an extended field. In addition the increase in colorimetry made it impossible to compress the subject within the limits of one volume. It was therefore decided to devote the whole of the first volume of the second edition to clinical and biochemical analysis under the joint authorship of Mr. Allport and Dr. Keyser. As in the original edition all theoretical considerations of instrumental techniques have been excluded and only the analytical descriptions of chemical technique are given.

The widened scope of the new edition compared with the old is indicated by the increase in the number of pages (almost double) and by the inclusion of 98 monographs compared with about 50 in the first edition. Monographs have been much revised and extended—as an example 17-ketosteroid estimations now occupy 8 pages with 23 references instead of one and a half pages.

Although the book deals solely with colorimetric methods a general approach has been maintained and the limitations of the methods are clearly given. Thus under barbiturates it is stated that methods based on the measurement of ultra-violet absorption will generally be found to be more sensitive than existing colorimetric methods. It was just this approach which made the first edition so valuable and the retention of this critical evaluation makes it a worthy successor. The authors are to be congratulated on their work and one will await with interest the publication of the second volume.

R. E. STUCKEY.

(ABSTRACTS *continued from page* 567)

differentiate. Thus the 4 strains of *Lactobacillus casei* examined were distinguished by absence of detectable amounts of ornithine, serine and lysine. Distinctions could readily be made between this species and *L. plantarum*, *L. acidophilus*, *L. helveticus*, *L. arabinosus*, *L. brevis*, and *L. fermenti*. The distinction between the last two of these species was regarded as useful because they are not otherwise well defined; the well marked differences found between *Streptococcus lactis* and *S. cremoris* were considered of value for the same reason. Other organisms examined included streptococci of Lancefield groups B, C, and D, micrococci and *Leuconostoc* species. Differences between species were always definite. However, age of culture and type of medium appeared to affect the chromatogram patterns and this constituted an obvious disadvantage of the method. B. A. W.