GENERAL DISCUSSION

Dr. D. B. Wetlaufer (*Copenhagen*) (*communicated*): In reference to the paper by Massey, Harrington and Hartley, since they find that chymotrypsinogen labelled with the fluorescent dye can be activated to chymotryptic activity, and the reaction between chymotrypsin and the dye chloride leads to an inactivation, it would appear that a re-location of the dye molecule occurs in the activation of dye-chymotrypsinogen. This raises two questions that can be answered experimentally: (i) does activated dye-chymotrypsinogen have the same activity (as chymotrypsin) toward substrates that are strongly bound to native chymotrypsin; and (ii) does the function (ρ_{h20}/τ_0) change as a result of the activation of dye-chymotrypsinogen. These experiments should help show whether there actually has been a re-location of the dye during the enzymatic activation of this dye-labelled zymogen.

I should also like to comment on the low ionic-strength polymerization of chymotrypsin (fig. 3 and 4 of their paper). The persistence of about 10 % monomer

even at the highest protein concentrations seems quite inconsistent in a system undergoing polymerization to a tetramer on higher polymer. These results might be explained if one postulated the presence of two species of chymotrypsin, one of which does not polymerize under their conditions. Do they have any information on the homogeneity of their chymotrypsin preparations which bears on this point ?

Prof. H. Neurath (*University of Washington*) said: While it is interesting to note that under the special conditions of the experiments, Dr. Massey and co-workers were able to demonstrate what appears to be a polymerization of chymotrypsinogen, I should like to raise the question whether in solutions of 0·03 ionic strength primary charge effects have been sufficiently suppressed to render the numerical values of the sedimentation constants meaningful. One might expect a retardation of sedimentation rates, and hence, sedimentation constants lower than those observed in the absence of charge effects. Actually, the authors report a sedimentation constant of 2·9 S for the monomer of chymotrypsin, as compared to 2·5-2·6 found by others at 0·1 ionic strength. The question arises, therefore, whether what the present authors consider to be the monomer is actually a dimer and whether the component with a sedimentation constant of 8·2 S does not correspond to a higher degree of polymerization than has been suggested in the paper.

Dr. Hartley (*Cambridge*) said: In reply to Dr. Neurath's suggestion that inhibition of enzymic activity in fluorescent conjugates of ChTr might be due to denaturation of the enzyme, we think that this does not occur for the following reasons. In the first place the mild conditions used in preparing the conjugates cause no inactivation of the enzyme. The conjugates have exactly the same sedimentation properties as the native enzyme. Dye-labelled chymotrypsin can be reactivated by incubation at pH 9·7, which causes the dye to hydrolyze from the protein, and finally a fully-active dye-labelled chymotrypsin can be prepared by trypsin activation of labelled chymotrypsinogen.

Dr. V. Massey (*Cambridge*) said: In connection with the suggestion by Dr. Neurath and Dr. Dreyer that the N-terminal *iso*lencine might be important in the catalytic activity of chymotrypsin, I would like to point out that we have found that *iso*lencine does not react with FDNB when the enzyme is in the native state. This presumably means that the N-terminal *iso*lencine is sterically blocked in native chymotrypsin, and it is therefore unlikely that it can be concerned in the catalytic activity of the enzyme.

Concerning the argument presented in the Discussion on our paper that chymotrypsinogen and DIP-chymotrypsin contain a chemically reactive grouping which is identical with part of the active centre of chymotrypsin, fig. 10, which shows the degree of inhibition against the extent of reaction of the dye, clearly shows that the first molecule in the enzyme with which the dye reacts also destroys the enzymic activity. Fig. 8 shows that the ratio $\rho h_{20}/\tau_0$ increases markedly when more than one molecule of dye has reacted per molecule of enzyme. As no changes in molecular size of the enzyme could be detected over the degrees of labelling studied, we have interpreted this change to a decrease in τ_0 when more than one molecule of dye has reacted per molecule of enzyme. A change in ρ_0 would be expected if the first group labelled had different fluorescent characteristics from the groups subsequently labelled. Now it will be noticed from fig. 8 that the same changes of $\rho h_{20}/\tau_0$ also occur with chymotrypsinogen and DIP-chymotrypsin. Hence it seems very likely that the first group to react with the dye is the same in these three proteins, and as it has been found that the first group to react with the dye in chymotrypsin is a group necessary for activity, then it would follow that this active-centre group is also free to react in chymotrypsinogen and DIP-chymotrypsin. Now this argument rests upon the assumption that the low ρ_{h20}/τ_0 values found at low degrees of labelling is due to reaction of the dye with the active centre. That this is in fact so has been

shown by reacting with dye in the presence of the competitive inhibitor, sodium β-phenylpropionate. Under these conditions labelled enzyme is obtained which has very much greater activity than a similar preparation labelled in the absence of any inhibitor. Such labelled chymotrypsin derivatives have been prepared at various degrees of labelling below 1 mole per mole, and found to have ρ_{h20}/τ_0 values of 14-15, compared to the normal value of 7. Thus there is little doubt that the specific reaction of the dye with part of the active centre is responsible for the low ρ_{h20}/τ_0 values found at low degrees of labelling, and that this active centre grouping is also free to react in chymotrypsinogen and DIP-chymotrypsin.

As Vaslow and Doherty have shown that chymotrypsinogen will also bind one molecule of a substrate with much the same affinity as it is bound by chymotrypsin, it is quite possible that this group reacting specifically with the dye is the substrate-combining site. From a comparison of the stability properties of the labelled chymotrypsin with various model systems we believe that this site is probably an imidazole group, although we have as yet no conclusive evidence that this is the case. However, in support of this possibility we find that one, and only one, of the two histidine residues in these three proteins is free to react with fluorodinitrobenzene.

Against this concept of a common active centre site in the active and inactive proteins is the following work. Labelled chymotrypsinogen, when treated with trypsin, gives active labelled chymotrypsin. This evidence seems to be in direct contradiction to that presented above, as the most obvious interpretation is that the dye was not on a group in chymotrypsinogen which is essential to enzymic activity. We do not want to minimize the seriousness of this contradiction, but as usual several explanations are possible, of which we would like to mention two.

(i) During the activation process the dye may have been transferred from the histidine (or whatever the group may be) to some other group not essential for catalytic activity.

(ii) Chymotrypsin and chymotrypsinogen each possess one free imidazole group, but it is possible that it is not the same one in each protein. In other words, while chymotrypsinogen might possess a free imidazole group which would react preferentially with the dye and confer special fluorescent characteristics to it, it may not be the same imidazole residue which is necessary for catalytic activity in chymotrypsin.

Further information about the substrate-combining site has been obtained by reaction with fluorodinitrobenzene under mild conditions. This work was reported at the Third International Congress of Biochemistry, but we would just like to emphasize one point here, as it is undoubtedly important in our understanding of the mode of action of this enzyme. When chymotrypsin was reacted with fluorodinitrobenzene under mild conditions it was shown that only the K_m increased with increasing time of reaction but that the maximum velocity remained unchanged. Thus one can say that none of the FDNB-reacting groups (imidazole, phenolic, ϵ-amino groups and α-amino groups) contribute to the activation process, but that one or more of them is concerned in the formation of the enzyme-substrate complex. If the group concerned here is an imidazole group, as we believe from our fluorescent studies, one might expect an effect of pH on K_m in the pH range 5-8. According to Gutfreund and to Laidler, K_m is independent of pH in this range. Although we have made no detailed study we must mention in this connection that we have found the K of β-phenyl propionate to be very different at pH 7·2 and pH 7·9.

Dr. Hartley (*Cambridge*) said: I should like to confirm Dr. Jandorff's observation that imidazole rings catalyze the hydrolysis of *p*-nitrophenyl-acetate, which is a substrate for chymotrypsin. We have demonstrated that α-benzoyl histidine methyl ester—which is a reasonable analogue of a histidine residue in a peptide chain—will catalyze the hydrolysis of this compound quite appreciably. We believe that some earlier observations on the catalytic properties of other proteins

such as insulin for this compound are due to the histidine residues which they contain.

Dr. G. A. Gilbert (*Birmingham*) said: The papers by Massey, Harrington and Hartley,[1] and Neurath and Dreyer [2] provide further data on the sedimentation of associating molecules, and also draw attention to the absence of a quantitative theory for the sedimentation of complexes in a state of reversible equilibrium. I should like to make some suggestions towards the development of such a theory, based on an analogy between the sedimentation (or electrophoresis) of associating molecules and chromatography. I pointed out this analogy in a previous *Discussion of the Faraday Society*,[3] without being aware that the theory of chromatography predicts that when, as happens only rarely, the adsorption of a substance depends to a higher power than the first on its concentration in solution, a diffuse instead of a sharp leading boundary is formed. To make the analogy clear, we may consider the sedimentation of a monomer, M, in *instantaneous* equilibrium with a polymer, P, composed of n molecules of M. If the centrifuge cell is imagined to be given a velocity equal and opposite to the sedimentation velocity of P, P is brought to rest and is analogous to the adsorbed species in chromatography, while M streams past P and takes the place of the free solute carried along by the developing solvent. Since P is proportional to M^n, with $n > 1$, the boundary is diffuse. Its shape and mobility are given (in the *idealized* case *when diffusion is neglected* and no account is taken of other factors such as cell shape, etc., discussed in the paper by Baldwin, Gosting, Williams and Alberty [4]), by the equations for chromatography deduced by De Vault [5] (see also Wilson [6] and Weiss [7]).

A brief indication of the method of treatment is given below for the general case of a polymer P sedimenting in the presence of its monomer M. Comparison is then made with the experimental results in ref. (1) and (2) for chymotrypsin obtained under two sets of conditions, (i) when P is a hexamer, and (ii) when P is a dimer. No new principle is involved when a group of complexes (each of different n) is present, but the algebra is much more complicated.

It is convenient to imagine M halted by giving the system an equal and opposite velocity to M. Let P then sediment with a velocity v relative to the stationary monomer and consider a point at a distance x from the initial sharp boundary, after a time of sedimentation t. Then, within the diffuse boundary, the discussion of De Vault [5] shows that the following partial differential equation holds

$$\mathrm{d}M + \mathrm{d}P + v\left(\frac{\partial P}{\partial x}\right)_t \mathrm{d}t = 0, \tag{1}$$

where the concentrations *by weight* of monomer and polymer are expressed by M and P respectively.

If
$$M^n = KP, \tag{2}$$

eqn. (1) can be integrated within the region of the boundary to give for boundary conditions corresponding to an initially sharp boundary at the meniscus at time $t = 0$,

$$\frac{nvtM^{(n-1)}}{K} - \left(1 + \frac{nM^{(n-1)}}{K}\right)x = 0. \tag{3}$$

If x/vt is replaced by the parameter δ we obtain

$$M = \left(\frac{K}{n}\,\frac{\delta}{1-\delta}\right)^{\frac{1}{n-1}}. \tag{4}$$

[1] Massey, Harrington and Hartley, this Discussion.
[2] Neurath and Dreyer, this Discussion.
[3] Gilbert, *Discussions Faraday Soc.*, 1953, **13**, 159.
[4] Baldwin, Gosting, Williams and Alberty, this Discussion.
[5] De Vault, *J. Amer. Chem. Soc.*, 1943, **65**, 532.
[6] Wilson, *J. Amer. Chem. Soc.*, 1940, **62**, 1583. [7] Weiss, *J. Chem. Soc.*, 1943, 297.

On substituting for M from (2) it also follows that

$$P = \frac{K^{\frac{1}{n-1}}}{n^{\frac{n}{n-1}}}\left(\frac{\delta}{1-\delta}\right)^{\frac{n}{n-1}} \tag{5}$$

giving by addition

$$(P + M) = \left(\frac{K}{n}\frac{\delta}{1-\delta}\right)^{\frac{1}{n-1}}\left\{1 + \frac{1}{n}\frac{\delta}{1-\delta}\right\}. \tag{6}$$

This equation describes the variation of the concentration by weight $(P + M)$ of the substance through the boundary. However, the observed *Schlieren* pattern in the cell is a measure of the gradient of $(P + M)$ with respect to x. This is obtained by differentiating (6) to give

$$\frac{\partial(P+M)}{\partial x} = \frac{1}{vt}\frac{1}{(n-1)}\left(\frac{K}{n}\right)^{\frac{1}{n-1}}\delta^{\frac{2-n}{n-1}}\left(\frac{1}{1-\delta}\right)^{\frac{2n-1}{n-1}}. \tag{7}$$

Thus, if diffusion could be neglected, and the individual sedimentation constants of the polymer and monomer were independent of concentration, etc., the *Schlieren* pattern would have the shape given by this equation. Although these ideal conditions are *very* far from being realized, it is interesting to draw the patterns for different values of n and compare them with actual cases. Since the above treatment was the result of an attempt to explain fig. 4 in the paper of Massey, Harrington and Hartley,[1] I will take as the first case polymer P of sedimentation constant 8·2 Svedberg units in equilibrium with a monomer M of sedimentation constant 2·5 units corresponding to their values for α-chymotrypsin in 0·01 M phosphate, pH 7·9. A reasonable value for n is then 6.

Inspection and differentiation of eqn. (7) shows that if n is 2, the *Schlieren* pattern consists of a *single* peak. For values of n greater than 2, it is a *double* peak with the valley lying at $\delta = \frac{n-2}{3(n-1)}$. For $n = 6$ the minimum will occur at $\delta = 0{\cdot}267$. Only one further item of data is needed to solve eqn. (6) and (7). It is provided by the observation in fig. 3 of ref. (1) that the two peaks of the pattern are equal in area when $(P + M)$ is 3·5 mg/ml (corresponding to 50% polymer according to ref. (1)). The area of the first, slower peak, which terminates at $\delta = 0{\cdot}267$, is therefore equivalent to 1·75 mg/ml, and this fact enables K to be calculated from eqn. (6), leaving no further unknown quantities. Using equation (7) and this data I have drawn in fig. 1*a* the "ideal" *Schlieren* pattern for a protein concentration $(P + M)$ of 3·5 mg/ml. The sharp peaks in the pattern would, of course, be rounded off in practice by diffusion,

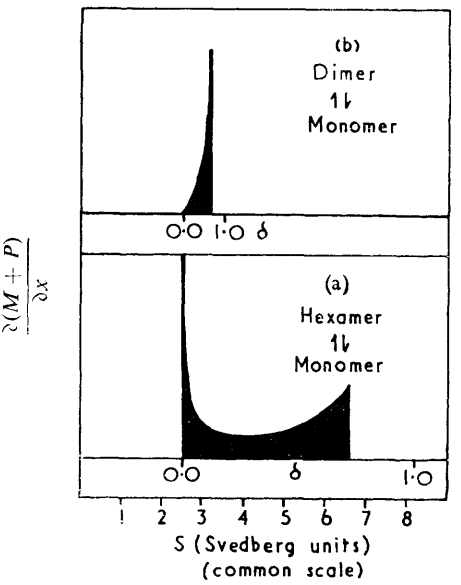

FIG. 1.—Idealized *Schlieren* patterns for sedimenting complexes.

[1] Massey, Harrington and Hartley, this Discussion.

and the pattern would then be more comparable to the experimental pattern in fig. 2*a* of ref. (1).

It is a simple matter to transform the abscissa of the pattern in fig. 1 from δ into equivalent values of sedimentation constant to give the pattern that would result from unit field acting for unit time. An alternative scale in Svedberg units is therefore added.

The slow peak ($\delta = 0$ to 0·267) has a *constant area* for all protein concentrations above 1·75 mg/ml, and it is only for concentrations above this value that the fast

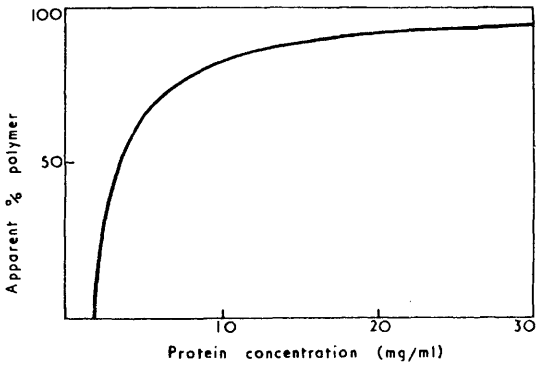

FIG. 2.—Sedimentation of hexamer-monomer equilibrium mixture. Theoretical ratio of area of fast peak to total area.

peak is seen at all. Thus the ratio of the area of the fast peak to the total area is given by $\{(P + M) - 1{\cdot}75\}/(P + M)$. This ratio is plotted as a function of protein concentration in fig. 2. Comparison should be made with fig. 3 of ref. (1), which gives the experimentally found ratio, expressed as " % polymer ".

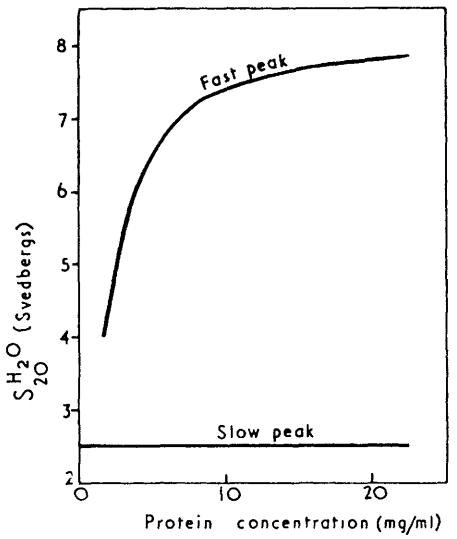

FIG. 3.—Theoretical variation of sedimentation velocity with concentration for hexamer-monomer equilibrium mixture.

If the sedimentation velocity of a peak is taken as measured by the velocity of the vertical bisector of the area of the peak, the variation of this velocity with concentration can be calculated using eqn. (6). In this way the velocity of the slow peak is found to be practically independent of concentration, and approximately equal to that of monomer, whereas the velocity of the fast peak rises very steeply with concentration. The calculated sedimentation constants are shown in fig. 3, which can be seen to resemble fig. 4 of ref. (1) even to the extent of close numerical agreement. The somewhat higher experimental value $S = 2{\cdot}9$ for the slow peak may be the result of the simultaneous presence of dimer.

The ratio P/M can be calculated and it is of interest that whereas the immediate intuitive interpretation of the *Schlieren* pattern when it has two peaks of equal area is that about equal weights of polymer and monomer are present in solution,

calculation shows that there is then actually 2·3 times as much monomer by weight as hexamer. Equal weights are not present until the fast peak has 2·3 times the area of the slow peak.

As a second example, I will consider the dimerization of chymotrypsin,[1] accepting values of 2·5 and 3·5 Svedberg units for the respective sedimentation constants, S_M and S_D, of monomer and dimer. Putting $n = 2$ in eqn. (7), I have drawn in fig. 1*b* the idealized *Schlieren* pattern for a solution containing equal weights of monomer and dimer. The peak is *asymmetrical* with a trailing edge towards the meniscus (see ref. (2) and (8)), and this asymmetry would only be greatly reduced, not eliminated, by taking into account diffusion.

The peak for this pattern is bisected by the vertical line that corresponds to a sedimentation constant of 3·05 units. Schwert [1] has shown that the equilibrium constant of dimerization depends upon pH and ionic strength. In the specific case of DIP + δ-chymotrypsin at pH 7·5, ionic strength 0·1, it appears from fig. 7 of ref. (2) that the sedimentation constant has this particular value of 3·05 units for a protein concentration of 3·5 mg/ml. K in eqn. (2) is therefore $\frac{1}{2}$ (3·5) mg/ml. Using this value in equation (6) I have calculated the theoretical variation of the sedimentation constant with protein concentration and plotted the result in fig. 4. A close resemblance to the corresponding experimental curve in fig. 7 of ref. (2) will be noted. Similar experimental curves in fig. 5 and 7 of ref. (1) show an initial rise of the expected kind, but a subsequent decrease, due presumably to a dependence of S_D on concentration not taken into account in this treatment.

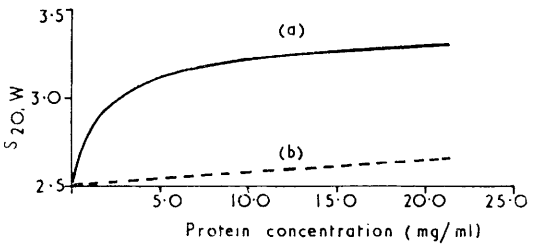

FIG. 4.—Theoretical variation of sedimentation constant with concentration for dimer-monomer equilibrium mixture. Curve (*a*) $K = 1{\cdot}75$ mg/ml, curve (*b*) $K = 100$ mg/ml.

Even a very weak tendency to dimerize may have a measurable effect on the sedimentation constant. To illustrate this, I have drawn as a dotted line in fig. 4 the variation of S with concentration for a protein of dimerization constant 100 mg/ml and S_M and S_D as for chymotrypsin. Thus in many cases where S falls less rapidly with concentration than the increase in viscosity would lead one to expect, dimerization may be accelerating the sedimentation.

To conclude, it is obvious that the above treatment remains unreal as long as diffusion is neglected, but it still seems to account for the main features of the sedimentation of complexes.

Prof. H. Neurath (*University of Washington*) said: Let me state at the outset that I strongly believe in experimental facts; and since there is no adequate theory for the sedimentation behaviour of macro-molecules at finite concentrations, I believe that we should be guided primarily by our experimental observations.

The sedimentation patterns shown in the accompanying slide [2] indicate that the sedimenting peaks are skewed whenever the sedimentation rate-concentration relation suggests reversible polymerization; and this skewness is absent whenever we are dealing with what appears to be a single molecular species. Thus, it may be noted that with DIP-δ-chymotrypsin, at pH 3·86, where it does not dimerize, the sedimenting boundary is symmetrical, whereas at pH 7·5 where dimerization

[1] Schwert, *J. Biol. Chem.*, 1949, **179**, 655.
[2] Dreyer, Wade and Neurath, *Arch. Biochim. Biophys.*, 1955, **59**, 145.

occurs, the boundary is skewed. When sedimentation at pH 7·5 is carried out in a synthetic boundary cell, no asymmetry is discernible in the synthetic boundary where protein concentration is relatively high on both sides of the boundary, and is in a region where the slope of the concentration dependent curve is relatively small; in contrast, the boundary formed between the protein solution and the pure buffer is slightly asymmetric and would probably have been more so had the experiment been extended for a longer period of time.

I was most interested to hear from Dr. Hartley that under mild conditions, the α-amino group of *iso*leucine in α-chymotrypsin does not react with FDNB. However, this does not preclude the involvement of the hydrocarbon side-chains of the *iso*leucyl-valine sequence in the configuration of the active centre; and I believe that more direct chemical evidence is required before the idea of an involvement of this sequence in the configuration of the active site can be discounted. As indicated in the introduction to our paper, I am inclined to ascribe more significance to the structural change in the molecule which occurs upon activation of chymotrypsinogen, as evidenced by the decrease in laevo-rotation and by the fact that the rate of change of optical rotation goes parallel to the rate of appearance of enzymatic activity. Since no significant change in either rotation or activity occurs during the conversion of π-chymotrypsin to the δ form, it is clear that the structural changes towards a more nearly helical configuration must have occurred incidental to the cleavage of the first peptide bond.[1]

Referring to Dr. Hartley's comments on his own paper I am glad to note that, following removal of the dye, the enzyme regained activity; I am sure that he, too, would feel happier about this evidence if reactivation were complete or nearly so.

Prof. Felix Haurowitz (*Indiana University*) said: Is the protein component of catalase essential in Schulman's experiments? Would not protein-free haemin, which has weak catalase activity, give the same results? It is well known that the lipoxidase activity of haemin is the same as that of haemoglobin and that both haemin and haemoglobin act as lipoxidases in heterogeneous emulsions of linseed oil or of linoleic acid, but not in the clear solutions of these lipids in organic solvents or in ox bile.[2] If such clear solutions are converted into emulsions by the addition of water, haemin begins to act again as a lipoxidase.

Prof. J. G. Kaplan (*Halifax N.S.*) (*communicated*): The biological implications of our paper have been made somewhat less clear-cut as a result of some of our recent work on the induced biosynthesis of yeast catalase (a phenomenon first reported by Chantrenne). Our data now show that this enzyme can exist in no less than 3 discrete "states"', the first 2 of which having been previously described by Fraser and myself. These "states" are:

(i) that of the enzyme in the normal, aerobic cell, in which there is a high catalase content but in which one can demonstrate in the intact cell only a small fraction of the total activity, the latter being characterized by a comparatively high activation energy (approx. 8 kcal/mole);

(ii) that of the enzyme within the lysed cell, or in aqueous solution, in which the enzyme has undergone *alteration*, shown by an increase in activity of approx. 17-fold, and a decrease in activation energy (to approx. 3 kcal/mole), as well as by change in other enzymatic properties;

(iii) that of the enzyme within the anaerobic cell, exhibiting a low, basal level of catalase activity *which cannot be increased by lysis of the cell*, the enzyme nevertheless being characterized by the same high μ as the catalase in the aerobic cell.

State (iii) in the "unadapted" cell, is intermediate between states (i) and (ii), since the enzyme possesses the high μ and other enzymatic properties of state (i), and the full expression of activity of state (ii); although lysis changes but little

[1] Rupley, Dreyer and Neurath, *Biochim. Biophys. Acta*, 1955, **18**, 162.
[2] Haurowitz and Schwerin, *Enzymologia*, 1940, **9**, 193.

the specific activity of the suspension, it nevertheless causes enzyme alteration, shown by the drop in μ values to the level characteristic of state (ii). Work done at Yale in collaboration with Dr. D. M. Bonner shows that these same 3 states are demonstrable in the case of the β-galactosidase of *E. coli*. Here, state (i) is that of the enzyme in the fully adapted cell (i.e., grown in the presence of lactose) in which only a fraction of total activity is expressed, state (ii) is that following lysis of the cell or extraction of the enzyme with full expression of activity, and state (iii) is that in the unadapted cell, where the basal level of activity per cell is but little affected by lysis or extraction (Rickenberg and Bonner) but in which these procedures cause the μ to change from the high level of state (i) (approx. 16 kcal/mole) to the low level of state (ii) (approx. 9 kcal/mole).

These findings suggest, first, that the interface at which catalase is adsorbed within the cell may be the surface at which it is synthesized, since the activation energy is high (and unchanged) before, during, and after the process of enzyme biosynthesis; additional weight is lent this hypothesis by the evidence from our ultra-violet studies (Kaplan and Paik) that catalase within adapted and unadapted yeast exists in a complex with ribonucleic acid. They suggest further that the most satisfactory type of model interfacial system may be the C_{18}TAB *n*-lauryl alcohol-stabilized emulsion studied at Cambridge since it is the only one at which the crystalline catalase may be stably adsorbed with some increase in activation energy (from 4·1 to 6·9 kcal after adsorption) but without loss of activity (see table 4), thus constituting a reasonably accurate model of state (iii). (It might be noted that the magnitude of the ratio of activity of desorbed (or altered) enzyme to activity of adsorbed (or unaltered) enzyme—the " factor " of Rickenberg and Bonner and " activation coefficient " of Robert and Polonovski—will change somewhat depending on the temperature of assay, since the μ of catalase in the 2 states differs; hence a factor of 1 (table 4) would be valid only at a single temperature of assay and would increase slightly at lower temperatures and decrease to a fractional value at higher temperatures.) If to this model interfacial system we could add some hypothetical agent which would block reversibly the activity of a high proportion of the adsorbed catalase molecules without affecting the temperature characteristics of the remaining active enzyme, we would have a working model of state (i). Finally, if desorption of the enzyme were accompanied by release of the inhibition we would have a model of state (ii), and would thus be able to manipulate the model interfacial system as one can the intracellular enzyme. However, even if we never arrive at a perfect model, it is clear that many of the interfacial systems presented in our paper are nonetheless better imitations of the cell enzyme than is a simple aqueous solution of the extracted enzyme.

Dr. L. Robert (*Paris*) (*communicated*): The ingenious model experiments of Dr. Frazer, Kaplan and Schulman prove the importance of adsorption-desorption equilibria in organized enzyme systems and parallels our findings on milk xanthine oxidase. It would be tempting to admit that a similar mechanism as proposed by the authors is responsible for the structural localization of x.o. in milk globules (adsorption to the lipid particles, microsomes, in multilayer, in the mammery gland) as well as its " activation " by physicochemical means (desorption of enzyme from the lipid surface). To be able to accept this mechanism, it would be necessary to prove that x.o. in the mammary gland is in " solution " and is incorporated in microsomes during fat globule synthesis, and that multilayer adsorption takes place. Another important feature of this mechanism would be, according to the comment on our paper by Dr. Frazer, a great difference between enthalpies and entropies of activation of the " free " and " adsorbed " enzyme. The only data available are due to Prof. Sizer,[1] who found the same activation energy for the purified enzyme as for milk. (As milk is a mixture of " soluble " and " adsorbed " enzyme, this measurement needs to be repeated,

[1] *Enzymologia*, 1940, **8**, 75.

with exhaustively washed fat globules, containing only the non-desorbable enzyme, with liposomes and with purified enzyme.) But this interpretation does not explain the irreversible structural changes occurring on " activation ": a certain number of fat globules is destroyed, the size distribution is changed, the average diameter being higher after activation (see ref. (15) of our paper), non-saturated fatty acids appear (" oiling off "), etc. So it seems more probable that the " activation " of x.o. is of a double nature: breakdown of the equilibrium in a binary or more complicated isomorphous mixture with liberation of x.o. from a lipoprotein complex, as was proposed by Neuzil *et al.* (see ref. in our paper), and perhaps an adsorption-desorption mechanism concerning the enzyme bound to microsomes.

We agree entirely with Dr. Fraser, Kaplan and Schulman as to the interpretation of the activation energy of the thermal activation process of x.o. ($\sim$ 20 kcal, see our paper); it corresponds probably to the rupture of a lipoprotein complex with the transfer of the equivalent of about 280 sq. Å hydrocarbon-like chains to the oil/water interface.[1]

It is interesting to note the relative resistance of x.o. in milk to denaturation by urea, which is probable due to complex formation between fatty acids and urea, diminishing greatly its effective concentration. Resistance to urea denaturation diminishes with purification of the enzyme.

Dr. R. K. Morton (*University of Melbourne*) (*communicated*): There are several points in the interesting paper by Dr. Robert and Dr. Polonovski on which I would like to comment.

The origin of xanthine oxidase of milk and comparative studies of the physical state of the enzyme in mammary gland and milk, have been investigated in my laboratory.[2] Comparison was made of the chemical composition and enzymic (xanthine oxidase and alkaline phosphatase) activities of fractions isolated from the mammary gland of the cow and from the milk of the same cow (obtained immediately before death). The results confirm my earlier suggestion that " milk microsomes "—the lipoprotein particles in cows' milk—are largely derived from microsomes from the secretory epithelian cells of the mammary gland. During incubation in the collecting ducts and cistern of the cow, however, the mammary gland microsomes undergo certain changes, notably: (i) a small but significant loss of lipid, (ii) a considerable loss of nucleic acid, and (iii) a marked increase in xanthine dehydrogenase activity. However, there is little or no change in the alkaline phosphatase activity. Hence, in answer to the question as to whether microsomes (" liposomes ") are performed elements of milk, the answer is certainly " yes ".

Whereas the xanthine oxidase of fresh milk is almost entirely associated with the fat fraction as a microsome complex, in the mammary gland the enzyme appears to be distributed between the cytoplasmic particles and the supernatant remaining after centrifuging the mammary gland homogenates (in 0·25 M sucrose) at approximately 50,000 *g* for 2 h. Incubation of mammary gland microsomes at 37° for 10 h, either in milk serum or in phosphate buffer (pH 7·4) leads to an appreciable increase (two to four times) in xanthine oxidase activity. Such " activated " gland microscomes still have considerably less xanthine oxidase activity than do milk microsomes. The xanthine dehydrogenase activity of freshly-isolated milk microsomes may be equal to 5-15 % of that of the most highly purified xanthine oxidase preparations.

In contrast to xanthine oxidase, alkaline phosphatase in the mammary gland is almost wholly associated with the microsome fraction, as it is in milk. As yet, no activation of this enzyme by physical means has been observed.

[1] see Eyring, Lumry and Spikes in McElroy and Glass, *Mechanism of Enzyme Action* (Johns Hopkins Press, Baltimore, 1954), p. 129.

[2] Baillie, *M.Sc. Thesis* (University of Melbourne, 1955).

Dr. Tsoo E. King and **Dr. V. H. Cheldelin** (*Oregon State Coll.*) said: In connection with the report by Robert and Polonoski on the thermal activation and inactivation of xanthine oxidase, we would like to raise a question whether the inactivation or denaturation of enzymes follows a simple order of reaction. This question has been debated for a long time, cf., for example, a review by Putman.[1] From the practical view-point of enzymologists, this type of information is very useful.

Recently we have studied properties of a new TPN-linked acetaldehyde dehydrogenase from *Acetobacter suboxydans*. This enzyme catalyzes the following reaction: [2]

$$CH_3CHO + TPN^+ + H_2O \rightarrow CH_3COOH + TPNH + H^+.$$

The inactivation or denaturation rate of the enzyme was determined at temperatures between 300 to 310° K. It was found that the rate constants changed with the time at any temperature determined. No simple order of reaction could be observed. The enzyme used in the investigation was a purified preparation with a k_3 of 10 mmoles acetaldehyde/min per g protein. Determinations were performed at an enzyme concentration of 0·0005 % in 0·1 % solution of crystalline bovine serum albumin.

On the other hand, the activation energy of the oxidation was successfully determined by measuring initial zero order rates (with respect to either acetaldehyde or TPN) at temperatures between 273 to 310° K. The activation energy was found to be 14·28 kcal/mole by the conventional Arrhenius plot. The standard error was 0·29 kcal and the confidence limit at a confidence coefficient of 0·95 was 13·53 to 14·96. This result indicates that our methodology was precise enough for such kind of work. However, the fact that rate constants of the thermal inactivation of this enzyme *changed* with time, suggests the very complex nature of its denaturation.

Prof. J. G. Kaplan (*Halifax, N.S.*) (*communicated*): There are certain other parallels between the interesting work of Roberts *et al.* and our own, in addition to the increase in both X.O. and catalase activities following release of these enzymes from the surface of oil droplets. Pre-treatment with heat had been found by von Euler [3] to cause increased catalase activity of the yeast cell, a finding which we were able to confirm [4] but which Fraser and I have found to be a quite variable effect; [5] the "maximal activation coefficient" was rarely more than one-fifth that obtained after treatment of cells with $CHCl_3$, surface-active agents, u.-v., etc., and heat does not alter the enzymatic properties the way these other agents do. I have found that freezing the yeast also caused the increased catalase activity which we have called the Euler effect. Treatment of cells with anionic detergents also caused the Euler effect. We believe the Euler effect follows, directly or indirectly, from desorption of the catalase from some intracellular interface of the oil/water type; [6] the change in various enzymatic properties which accompanies the Euler effect we have called enzyme alteration.

The activation energy for heat "activation" of X.O. (or what we should call heat-induced alteration, if change in enzyme properties could be demonstrated) was 21 kcal. Fraser and I [5] on the basis of the observed 21 kcal difference between the μ values for heat inactivation of altered and of unaltered intracellular yeast catalases, predicted that the process of alteration itself would have an energy of activation of about, but no less than, 21 kcal. The subsequent kinetic study of catalase alteration [7] closely confirmed this prediction, yielding values for butanol-,

[1] in Neurath and Bailey, *The Proteins*, vol. 1, part B (Academic Press, N.Y., 1955) p. 807.
[2] King and Cheldelin, unpublished.
[3] von Euler, *Z. physiol. Chem.*, 1919, **105**, 83.
[4] *Expt. Cell. Res.*, 1955, **8**, 305.
[5] *J. Gen. Physiol.*, 1955, **38**, 515.
[6] *Physiol. Zool.*, 1952, **25**, 123.
[7] Kaplan and Paik, *Can. J. Biochem. Physiol.*, 1956, in press.

$CHCl_3$- and u.-v.-induced alteration within the narrow range of 20-24 kcal. Evidence that an interfacial process is involved in catalase alteration has been presented.[1] The rather striking similarity between our values for catalase alteration and that of Robert and Polonovski for X.O. " activation " might indicate a common, or similar, rate-limiting step, especially since they conceive this to be a splitting of a lipo-protein complex, a process which might well be only *semantically* different from a desorption of protein from a lipid/water interface. In this connection, I should like to ask Dr. Robert whether his distinction between " a physical or chemical nature " of " activation " is a meaningful one, since the " fission of the lipoprotein complex " must represent rupture of some chemical bond, regardless of whatever agent is used to cause it (see also Langmuir's classical paper in this regard).[2]

I should like also to ask Dr. Robert whether he has studied the effect of temperature on the rate of the reaction of X.O. with its various substrates in order to obtain an activation energy for this process, and if so, whether this constant changes following desorption of the enzyme into the aqeuous phase? We should predict on the basis of our work on catalase and β-galactosidase that the μ would decrease significantly upon desorption from the oil droplets.

May I ask further whether the low " activation coefficient " observed with the aliphatic aldehydes could not possibly be due to a desorption of the X.O. in the fresh, uncooled milk during assay of these surface-active substrates? We have shown that the aliphatic aldehydes cause alteration of yeast catalase;[3] this was seen to be related to their surface-activity, and the homologous alcohols of about equal surface-activity, caused desorption of crystalline catalase from the olive oil/cephalin/water interface.

Finally, I think that the term " activation ", used in the sense of increased enzyme activity following treatments of various kinds, is rather confusing. I would propose that terms such as " activation coefficient ", " crypticity ", etc., be replaced by the term " factor ", introduced by Bonner [4] and defined as the ratio of activities of maximally active enzyme to that of the enzyme in its original, or less active, state, whatever this may be.

Dr. L. Robert (*Paris*) (*communicated*): To the interesting comment by Prof. Kaplan I should like to reply that the distinction between activation of a " physical " or " chemical " nature concerns the activating agent rather than the process of activation itself. It would be tempting to admit that the nature of " activation " or " alteration " is the same with physical means (high pressure, heat, ultrasonic vibrations), as with chemical means (surface-active agents, etc.). On the other hand, I do not think that the " fission " of a lipoprotein complex must represent a rupture of some chemical bond; such complexes may well be held together by van der Waals' forces.[5] The interesting comment by Dr. Morton confirms the hypothesis that formation and disintegration of milk fat globules and of liposomes is very similar in nature to the adsorption-desorption phenomena observed by Prof. Kaplan, Prof. Fraser and Dr. Schulman at oil-water interfaces.

We are now studying the activation energy of desorbed and of the highly purified enzyme (kindly provided by Dr. Bray, Prof. Bergel and Dr. Avis) so I cannot yet answer the second question of Prof. Kaplan concerning the experiments of Prof. Seizer. The low " activation coefficient " observed with aliphatic aldehydes could well be due to desorption of the enzyme by the aldehyde. Direct experimental proof is difficult to obtain, because these aldehydes inactivate the enzyme rather rapidly.

[1] *J. Gen. Physiol.*, 1954, **38**, 197; also Kaplan and Paik.[5]
[2] *J. Amer. Chem. Soc.*, 1917, **39**, 1848.
[3] *J. Gen. Physiol.*, 1954, **38**, 197.
[4] *Amino Acid Symposium* (Johns Hopkins U. Press, 1955).
[5] see, for instance, the classical papers of Macheboeuf, in Hughes, *Blood Cells and Plasma Proteins*.

The term activation is in itself perhaps confusing, but it indicates at least that the alteration produced in the enzyme system resulted in a higher activity. Alteration is perhaps a more general term, but I think it is too vague. Mr. Polonowski called the phenomenon in French " revelation " which is as acceptable as the " Euler effect ", if a good definition is given of the reaction which is understood by this phrase. Adsorption and desorption is not acceptable, because for X.O. at least much more happens than is described by these terms. The " activation coefficient " is by definition the same as Dr. Bonner's " factor ".

Prof. D. D. Eley (*Nottingham*) said: Dr. King has mentioned the change in reaction order observed during enzyme inactivation Coleman, Davies and I have examined this problem for acetylcholinesterase A plot of log activity against time gives two lines connected by an " elbow ", corresponding to a rapid initial reaction followed by a slower final process The initial reaction has a high entropy of activation which suggests that the decreasing activity at the active site is due to structural changes in the neighbouring parts of the protein molecule. The final slow process may well concern destruction of the active site itself, and in support of this view we have some evidence that this final process is slowed down in the presence of butyryl choline, which is known to be adsorbed on the active site from its behaviour as an inhibitor in the hydrolysis of acetyl choline by the enzyme. We have also evidence that the initial rapid phase is associated with a decrease in both enzyme-substrate affinity, $1/K_m$, and a somewhat less marked decrease in $v_{\max.}$. It seems clear that this inactivation process is not a simple decrease in enzyme concentration with time, but involves different levels of enzyme activity in one and the same molecule.