THE LABELLING OF HUMAN SERUM BY ^{32}P-DI*ISO*PROPYLPHOSPHOROFLUORIDATE (DF ^{32}P)

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In previous communications[1,2,3] methods were described for estimating the life span of red cells and thrombocytes in humans after injection of DF^{32}P. In the same way the turnover of serum protein could be studied[2] by estimating the radioactivity of the serum from blood samples drawn at intervals after an intramuscular injection of DF^{32}P. A plot of the logarithm of the radioactivity of serum against time elapsed after injection revealed a linear relationship. From this, after correction for physical decay, the half life of the labelled component could be calculated. It was assumed that the labelling would be limited to a particular component of the serum, the pseudo-cholinesterase. Furthermore it seemed probable that this combination would, like other known reactions between DF^{32}P and esterases, lead to an irreversibly labelled product. It would follow that the rate of disappearance of the serum radioactivity would reflect that of the normal breakdown of the pseudo-cholinesterase, assuming that the breakdown of the unlabelled protein would be reflected by that of the DP*-enzyme. Since in the normal state of dynamic equilibrium breakdown equals synthesis, the calculated value for the half life for the disappearance of the serum-bound radioactivity would also apply to the turnover of the pseudo-cholinesterase. This value was formerly found to be 12–14 days. From the preceeding argument it follows that in order to arrive at an exact interpretation it remained to be proven that in fact only one component was labelled using the technique described and that this component was indeed pseudo-cholinesterase. The linearity of the log. radioactivity-time plots was rather suggestive of the labelling of one component only; heterogeneity would probably have led to a more complicated relationship. However the possibility of the presence of several components of similar half life or of heterogeneity masked by the preponderance of one of several components could not be excluded. Moreover, results obtained in this laboratory by GOUTIER[4] showed that caution should be exercised with regard to the conclusion that pseudo-cholinesterase is exclusively involved. He found that after injection of DF^{32}P into rabbits and guinea pigs the radioactivity of the serum was chiefly bound to the B-ali-esterase, whereas the contribution of the pseudo-cholinesterase fraction to the total radioactivity was insignificant. On the other hand our assumption that the method, when applied to humans, was indeed indicative of the turnover of pseudo-cholinesterase found strong support in the results reported by MOUNTER AND WHITTAKER[5]. These authors investigated human sera and could establish the presence

* DP signifies di*iso*propylphosphoryl.

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of only two esterases, *viz.* pseudo-cholinesterase which could, and A-ali-esterase which could not be inhibited by DFP. Obviously only the former enzyme may reasonably be expected to be labelled by $DF^{32}P$ under our circumstances. On the other hand it should be remembered that this purely enzymic study did not exclude the possibility that other protein components, *e.g.* esterases of substrate specificity not revealed by the substrates used or proteins with little or no enzyme action, could conceivably react with $DF^{32}P$.

These considerations led us to make the present study, which consisted in the comparison of the exact localisation of the cholinesterase activity in the electrophoretic pattern of human serum and that of the radioactivity of this serum after it had been in contact with $DF^{32}P$.

EXPERIMENTAL METHODS

1. *Zone electrophoresis* on starch columns was carried out according to FLODIN AND PORATH[6] in 0.1 *M* veronal buffer at pH 8.9 in the cold room. The column was further cooled by ice-cold water circulated through a surrounding glass compartment. For every experiment 2 ml of serum was dialysed during one hour against the buffer, stained by the addition of bromophenol blue and applied onto a 40 cm column of 5.41 cm^2 diameter (retention volume approx. 76 ml). The serum was allowed to travel a distance of 5 cm before the current was switched on (600 V – 20 mA) for approx. 30–40 h; during that time the stained albumin fraction had covered a distance of 30 cm towards the anode. Elution followed using the same buffer in 1 ml fractions separated by a fraction collector. In these fractions cholinesterase was estimated manometrically (AMMON[7]) in 0.5 ml, protein colorimetrically (LOWRY *et al.*[8]) in 0.02 ml and radioactivity by counting the plated and dried remainder of each fraction.

2. *Paper electrophoresis* and localisation of protein and enzyme activity on the strips was performed as previously described (GOUTIER[4]). The composition of the agar plates was 500 ml 1 % agar, 10 ml 0.1 % (in 10 % ethanol) phenol red and 10 ml 10 % (w/v) acetylcholine. Cholinesterase activity caused marked yellow decoloration of the agar after 18 h. It was photographed at intervals using a lightbox and filter to follow the course of the enzymic reaction.

EXPERIMENTAL RESULTS

I. Localisation of cholinesterase activity by means of paper electrophoresis

Our first aim was to localize the cholinesterase-containing fraction after paper electrophoresis of human serum. If this should prove feasible, an attempt was to be made to relate this fraction to the one that contained radioactivity after paper electrophoresis of serum of $DF^{32}P$ injected human subjects. Sera were subjected to electrophoresis and the strips were analysed for cholinesterase activity as described. Representative experiments are illustrated by Fig. 1. Fig. 1a indicates that the centre of enzyme activity is localised between the α-2 and the β-peak. This localisation was much more circumscript when the enzyme activity just started to show up on the plate; it was then, however, too weak to give a satisfactory photograph. Later it spread out on both sides to give the picture shown. Fig. 1b gives the results of the electrophoresis of serum that had been previously exposed to $DF^{32}P$ ($2 \cdot 10^{-7}$ g/ml); no cholinesterase activity was found. Some enzyme activity (again between the α-2 and the β-peak) is found after electrophoresis of serum of a patient injected 24 h previously with 0.921 g oil containing 0.7 mg $DF^{32}P$ per g (Fig. 1e). This activity could no longer be demonstrated if the same serum was dialysed before the electrophoresis (Fig. 1f). In the same way dialysis of the control serum taken before the DFP injection caused considerable loss of enzyme activity (Fig. 1c compared with 1a).

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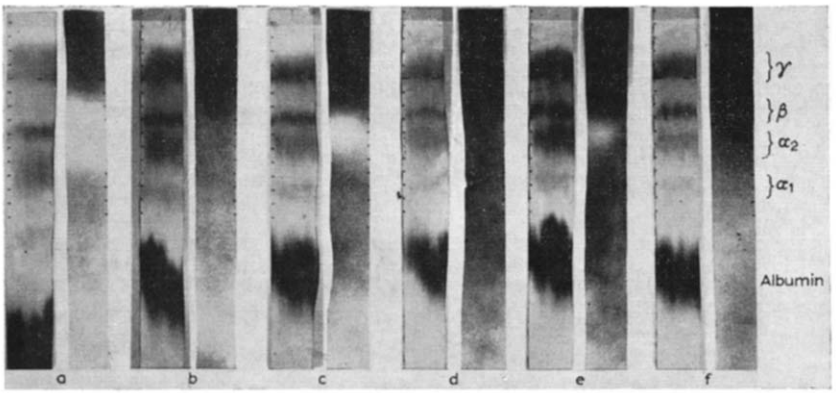

Fig. 1. Localisation of cholinesterase activity after paper electrophoresis of human serum. Veronal buffer. The left strip of each pair is stained for protein, the right one indicates the localisation of cholinesterase activity on the agar plate. For detailed explanation see text.

Fig. 1d represents the results obtained on dialysis of $DF^{32}P$ pretreated serum of the same patient before injection; of course no activity was found. Dialysis was carried out overnight against 1% NaCl in the cold room. While it was possible to assess cholinesterase activity in fractions separated by paper electrophoresis, all subsequent attempts to demonstrate radioactivity in the electrophoresis strips from sera that had been treated in some way by $DF^{32}P$ failed. None of the strips of Fig. 1 produced any blackening of an X-ray film (Gevaert Curix) after 5 weeks of exposure. It was concluded that for this purpose the method was too insensitive. An obvious way to overcome this difficulty would be the adoption of a method enabling us to obtain larger quantities of the various fractions. Zone electrophoresis on starch allowing the processing of batches of several ml seemed to be the method of choice. Since it was our intention to relate results obtained by this method to those described in the previous section, it was important to know whether the electrophoretic patterns obtained by each method were mutually comparable. It was of particular significance to know whether cholinesterase would again be found between the α-2 and the β-peak when the column method is used, and whether these peaks would be comparable to those found on the paper. Two ml of serum were subjected to column electrophoresis. The result is illustrated by Fig. 2. It will be seen from Fig. 2 that at least five different fractions are separated. They were tentatively called albumin, α-1, α-2, β en γ. The fractions belonging to each peak were pooled, dialysed and concentrated by evaporation; the pooling was as follows: α-1 (tubes 18–26), α-2 (tubes 29–39), β (tubes 40–48) and γ (tubes 56–64). The evaporated residues were dissolved in 40 μl of water and half of this volume was mixed with 20 μl of the original serum and subjected to paper electrophoresis. The results are given in Fig. 3. Fig. 3f represents the results of paper electrophoresis of the original serum in the absence of additions; a, b, c, d and e are obtained by mixing it as described with the fractions α-1, α-2, β, γ and α-2 + β respectively. It is evident that the α-1, α-2 and β-fractions as separated by column electrophoresis correspond closely to those as obtained after paper electrophoresis. Only for γ-globulin is the outcome more doubtful, probably on account of the strong dilution of this fraction occurring in the course of the column electrophoresis.

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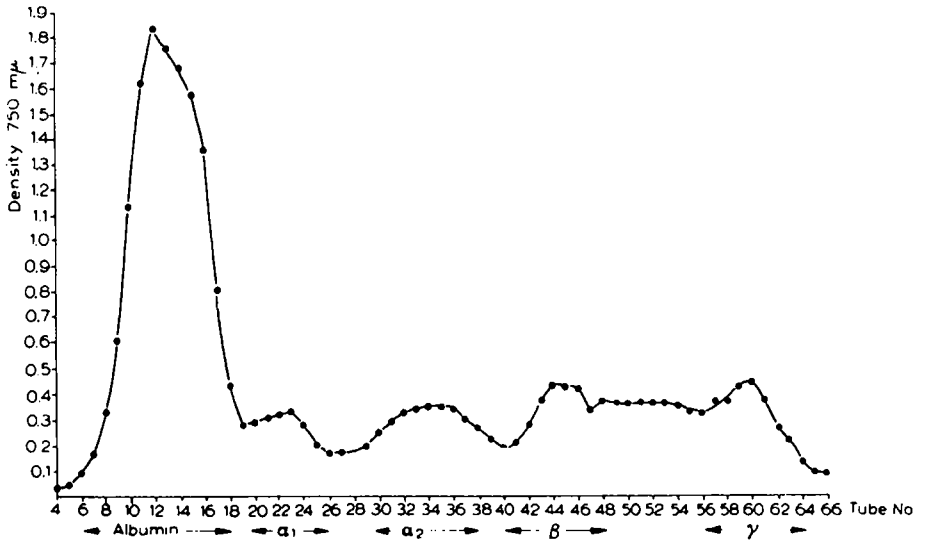

Fig. 2. Zone electrophoresis on starch columns of normal human serum. Volume of fractions 1 ml; the numbers of the eluted fractions are plotted against their absorption at 750 mμ when analysed by the Lowry method.

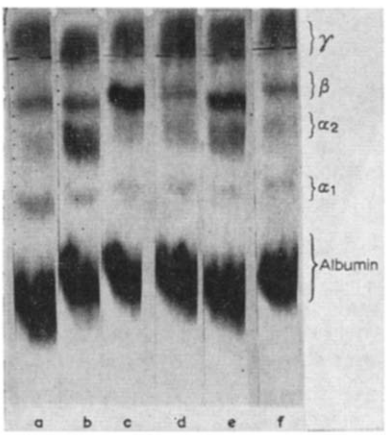

Fig. 3. Localisation after paper electrophoresis of serum components separated by preceding column electrophoresis. For further explanation see text.

II. Localisation of cholinesterase activity and DF^{32}P combining power of serum fractions separated by zone electrophoresis on starch columns

Serum was subjected to zone electrophoresis on starch in the manner described before. All eluted fractions were analysed for cholinesterase activity. The results are summarized in Fig. 4. The values for the cholinesterase activity are inserted into the normal electrophoresis diagram. This activity is exclusively located between the α-2 and the β-peak.

2.5 ml of serum were incubated with 0.25 ml of DF^{32}P $5 \cdot 10^{-6}$ (w/v) during 40 minutes at room temperature. 0.5 ml of a non-radioactive carrier solution containing 3.5 mg of DFP and 3.5 mg of di*iso*propylphosphate (DIP) were then added.

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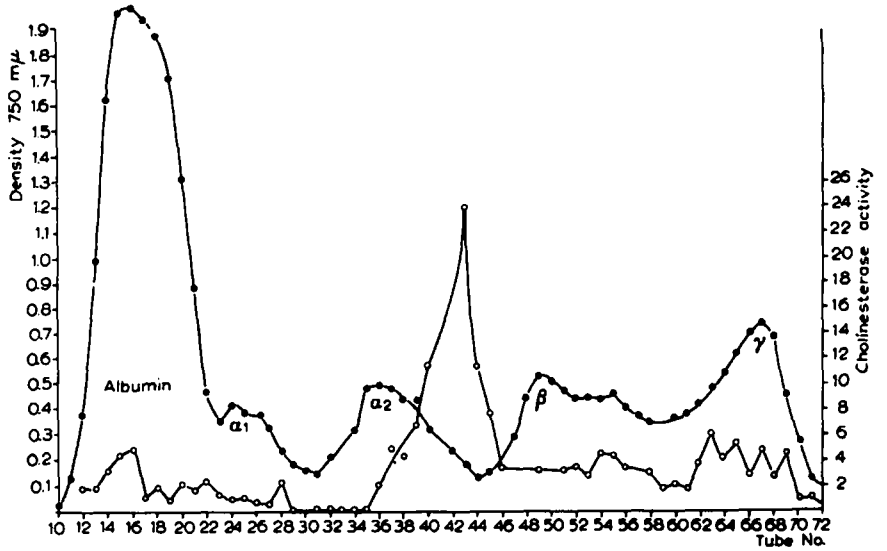

Fig. 4. Localisation of cholinesterase activity after zone electrophoresis on starch column. ●—● protein content (absorption at 750 mμ; Lowry method); ○—○ cholinesterase activity in μl CO_2/15 min/ml. For further explanation see Fig. 2 and text.

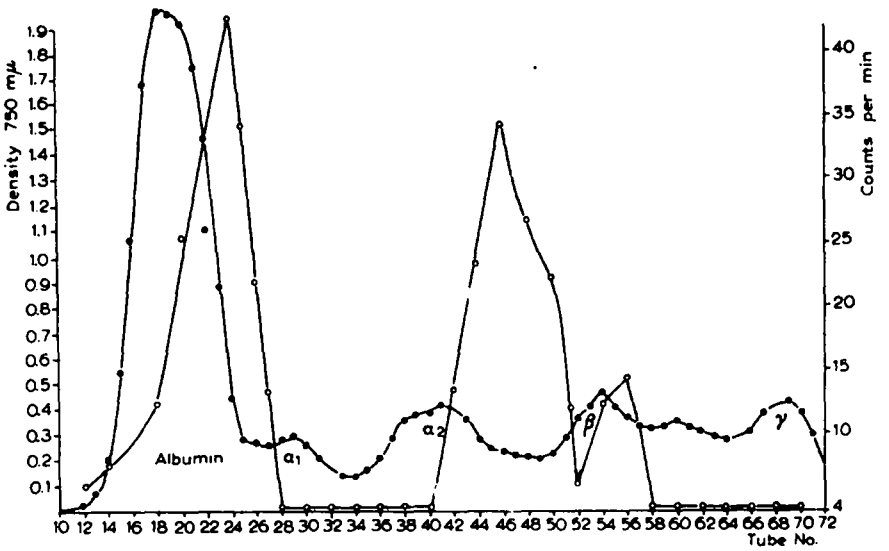

Fig. 5. Localisation of radioactivity after short dialysis and column electrophoresis of DF^{32}P treated serum. ●—● protein content; ○—○ radioactivity in counts/min/fraction. For explanation see previous figures and text.

The product was dialysed overnight in the cold against distilled water and subsequently during one hour against veronal buffer. The pattern obtained on column electrophoresis of 2 ml of the serum is represented in Fig. 5; the values for the radioactivity are inserted into the diagram. The radioactivity is obviously mainly localised in a fast-running component coinciding partly with the albumin fraction and in a slow one between the α-2 and the β peak. The fast component probably represents

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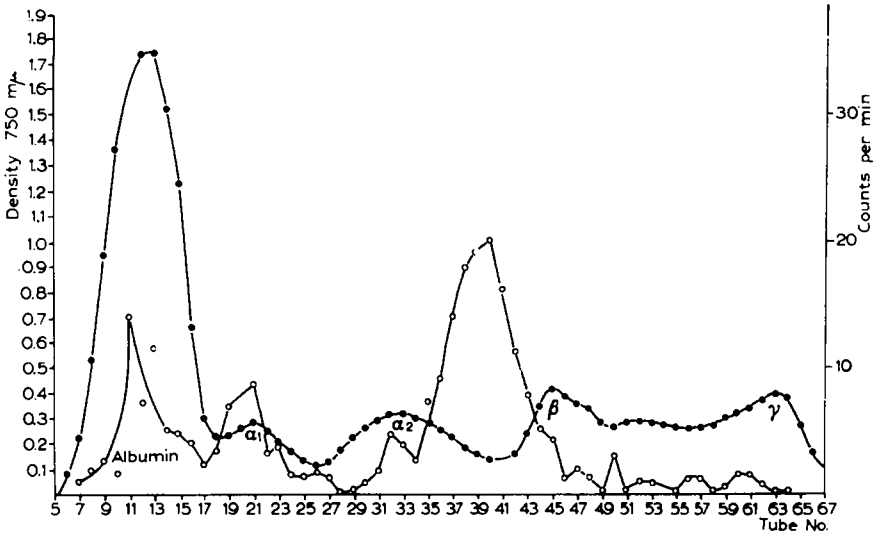

Fig. 6. Localisation of radioactivity after prolonged dialysis and column electrophoresis of DF^{32}P treated serum. ●—● protein content; ○—○ radioactivity in counts/min/fraction. For further explanation see previous figures and text.

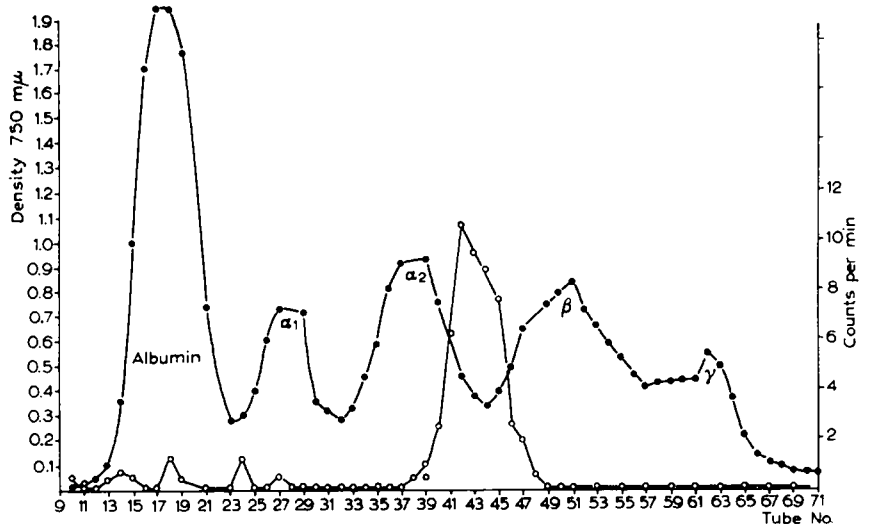

Fig. 7. Localisation of radioactivity after column electrophoresis of serum from a DF^{32}P injected person. ●—● protein content; ○—○ radioactivity in counts/min/fraction. For explanation see previous figures and text.

a reversible and non-specific combination of serum protein with DF^{32}P, DIP or radioactive impurities of the DF^{32}P preparation. The slow component was considered to reflect the specific irreversible reaction between DF^{32}P and pseudo-cholinesterase. If this interpretation were true, then prolonged, intensive dialysis should result in a decrease in the radioactivity associated with the fast component leaving the slow one essentially unaltered. Moreover, the radioactivity in serum of subjects injected not too recently with DF^{32}P would be expected to be mainly localised between the α-2 and β peaks, that is at the site of cholinesterase activity.

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The following experiments prove the validity of the proposed interpretation.

a. When the duration of the dialysis was extended to 3 × 24 h (Fig. 6) the radioactivity was indeed redistributed in the predicted sense, *viz.* a large shift of the distribution of the total activity in favour of the cholinesterase peak and at the expense of the fast peak.

b. Serum of a patient, collected 3.5 days after the i.m. injection of 1.26 mg of $DF^{32}P$ in 1 g of arachis oil, was dialysed during one hour against veronal buffer before it was subjected to column electrophoresis. Fig. 7 shows that the radioactivity is almost exclusively limited to the cholinesterase region between the α-2 and the β peaks.

DISCUSSION

On zone electrophoresis of human serum on starch columns the fraction containing the cholinesterase activity invariably travelled at a speed intermediate between that of the α-2 and the β peaks. No radioactivity could be detected on paper strips resulting from electrophoresis of $DF^{32}P$ treated serum. The reason is that probably the 20–40 μl of serum applied do not harbour sufficient radioactivity to give rise to an unambiguous autoradiogram. However, radioactivity is demonstrable in the eluates of starch columns after electrophoresis of $DF^{32}P$ treated serum or serum drawn from $DF^{32}P$ injected people. Sera from human subjects injected more than 2 days previously with $DF^{32}P$ as well as thoroughly dialysed $DF^{32}P$ treated human sera showed a sharp peak of radioactivity between the α-2 and the β peaks of the electrophoresis diagram, that is at exactly the same location as that of cholinesterase. These results strongly suggest that in human serum only a single protein, cholinesterase, reacts with $DF^{32}P$. There can be no doubt that this is pseudo-cholinesterase because this is the only cholinesterase known to occur in human plasma. The possibility that two different proteins of similar electrophoretic properties could be responsible for cholinesterase activity and combination with $DF^{32}P$ respectively cannot be altogether excluded. It is even possible that the serum component, which reacts with DFP is originally localized elsewhere and acquires its cholinesterase-like electrophoretic properties only after its combination with $DF^{32}P$. However it is important to realize that serum does contain pseudo-cholinesterase and that this protein will be labelled by $DF^{32}P$. Therefore on electrophoresis a peak representing $D^{32}P$-cholinesterase must be present. The fact that in the relevant experiments, after sufficient washing out, only one peak is found, which is moreover localized at the site of the cholinesterase renders it difficult and artificial to avoid the conclusion that the non-dialysable radioactivity of the sera concerned is due to irreversible combination with pseudo-cholinesterase. It follows that under the conditions of our method of estimation of the turnover of proteins[1], the protein to be labelled will be the pseudo-cholinesterase. The half-life value of 12–14 days found for the disappearance of the serum-bound radioactivity after injection with $DF^{32}P$ must therefore represent the turnover of serum pseudo-cholinesterase.

SUMMARY

1. On paper electrophoresis the pseudo-cholinesterase activity of human serum is localised between the α-2 and the β-peak.

2. The same localisation is found after electrophoresis on starch columns.

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3. Thoroughly dialysed DF^{32}P treated human sera as well as sera obtained from humans a few days after the injection of DF^{32}P were submitted to zone electrophoresis. No radioactivity could be detected on the paper electrophoresis strips, but after column electrophoresis it was possible to localise the radioactivity between the α-2 and the β-peaks.

4. The conclusion is reached that in human sera, which have been in contact with DF^{32}P, only one component, the pseudo-cholinesterase, is irreversibly labelled by ^{32}P. The values obtained for the turnover of serum proteins by means of DF^{32}P therefore clearly reflect the turnover of the pseudo-cholinesterase component.

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THE EFFECT OF SUBSTRATE SIZE UPON HYALURONIDASE ACTION*

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WEISSMANN[1] has shown that the activity of hyaluronidase increased with substrate size. This paper describes in quantitative terms the effect of substrate size upon the thermodynamics of hyaluronidase action, as well as providing a measure of the size of the active center of the enzyme.

MATERIAL AND METHOD

Partially degraded substrate

250 mg of human umbilical cord hyaluronate and bovine tracheal chondroitin sulphate A were dissolved separately in 50 ml of 0.10 *M* acetate buffer (pH 5.0) containing 0.15 *M* sodium chloride. To five 10 ml portions of each solution 0.10 ml of buffer containing 40 TRU of a preparation of hyaluronidase, assaying 6500 TRU/mg N was added. The enzymic reaction was stopped after 0, 4, 8, 12 and 15 minutes incubation at 36° C by thermal inactivation, and the solutions were pooled. This pooled material contained a mixture of degraded substrate of greatly varying chain length. Ethanol was then added until the first traces of a precipitate occurred. The solution was then stored at 4° C for 30 minutes. After storage the precipitate was collected by centrifugation and more ethanol was added to the clear supernatant. This procedure was repeated until no more centrifugable material formed. The precipitates were washed in ethanol, dried, and redis-

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