The Identification and Estimation of Elastase in Serum and Plasma

By DAVID A. HALL
Department of Medicine, University of Leeds

1. Electrophoretic separation of partially purified elastase preparations from pancreas followed by incubation of the electrophoretogram in contact with an agar gel containing 4% of either Congo Red-stained or unstained elastin demonstrated that the enzyme which dissolves elastin can be identified with that which releases dye from the stained preparation. 2. A method for the estimation of elastase based on the release of dye from Congo Red-stained elastin is described. It is 23 times as sensitive as methods employing protein determination. 3. With the method, elastase activity can be identified in plasma and in a partially fractionated plasma protein preparation. 4. Lineweaver–Burk plots of these estimates of activity at a variety of substrate concentrations indicate that the reaction between elastase and the dyed elastin is more closely similar to that between elastase and the soluble substrate elastin rather than to that between elastase and the solid substrate. 5. Values for K_m and $V_{max.}$ calculated for the enzyme present in plasma present further evidence for its identity with the pancreatic enzyme. 6. By calculation of the slopes of the Lineweaver–Burk plots for various enzyme concentrations it has proved possible to demonstrate that the inhibitor which is also present in the plasma is without effect on the enzyme when it acts on the dyed substrate.

Since the discovery of the enzyme elastase (endopeptidase, EC 3.4.4.7) by Balo & Banga (1949*a*), the tacit assumption has been made that, at some stage after its secretion by cells of the pancreas, elastase can appear in the circulation and perform a systemic function (Hall, 1961, 1964). This is not in agreement with the numerous observations which ascribe a purely digestive role to the enzyme (Cohen, Megel & Kleinberg, 1958; Kokas, Foldes & Banga, 1951; Grant & Robbins, 1955; Bertelheimer, Kulpe & Somerkamp, 1958; Campagnari & Greggia, 1959; McIvor & Moon, 1959; Moon & McIvor, 1960; Lamy & Lansing, 1961), but two groups of workers (Cohen *et al.* 1958; Tolnay, Solyom & Borsy, 1962) have reported elastolytic activity in urine, and others (Gore & Larkey, 1960) have reported this in an extract from arterial wall. Further, Lansing (1954, 1959) has reported systemic activity after the administration of elastase by mouth. It has become increasingly essential therefore that this controversy be settled, since all theories about the possible intervention of elastase and other pancreatic enzymes in the onset and/or control of arterial lesions (Hall, 1961) depend on the assumption that they pass into the vascular tissues via the circulation.

Hitherto all attempts to characterize elastase in plasma have failed (Graham, 1958; Balo, 1963; J. W. Czerkawski, unpublished work) and this may be assumed to be due to the additive effect of two factors. First, the amount of circulating elastase may be less than the concentration measurable by the methods available and, secondly, plasma is known to contain appreciable quantities of inhibitor (Balo & Banga, 1949*b*; Graham, 1958; Loeven 1962; Hall, 1963), which will suppress the activity of any elastolytic enzyme. During his studies on the elastase inhibitor, Loeven identified in the plasma the associated enzyme E_1 (Hall, 1957), which has the effect of increasing the activity of pure elastase when tested against solid elastin as substrate. Hall (1957, 1961) has suggested that this enzyme acts synergistically by liberating sites on the substrate to which elastase can become attached, thus effectively increasing rates of elastolysis by increasing the effective substrate concentration.

To identify elastase, should it be present in plasma, a method of estimation is required which is more sensitive than those already in use, namely the gravimetric (Balo & Banga, 1950) or interferometric (Hall & Czerkawski, 1959) assessment of tissue loss, or the biuret (Hall, 1955) or nephelometric (Banga, Balo & Horvath, 1959) assessment

of protein passing into solution. Such a method has been devised by Sachar, Winter, Sicher & Frankel (1955), Naughton & Sanger (1961) and Banga (1963). By using dyed elastin preparations these groups of workers have estimated elastolytic activity by a spectrophotometric assessment of the orcein or Congo Red released by the enzyme as the dyed protein passes into solution. Chao, Sciarra & Vosburgh (1962) have further developed the method by extracting the liberated dye with butan-1-ol and recently Sciarra, Chao & Mandl (1963) have employed it for the estimation of the inhibitor content of serum during pregnancy. Cacciola, Cristaldi & Giustolisi (1961) have also employed the release of dye for the estimation of elastase in plasma. They did not, however, advance any rational explanation for their ability to estimate the enzyme in the presence of its inhibitor.

Findlay (1954) observed that stained sections of elastic tissue were completely resistant to elastase attack whereas stained powdered tissue was susceptible. Apparently the reduction to a powdered form and the removal of the associated proteins and mucopolysaccharides provides a number of sites for enzyme attachment which are not blocked by stain. It has been shown (Hall, 1962) by the use of kinetic studies that elastase apparently has two active and independent centres associated respectively with the elastolytic and general proteolytic activities of the enzyme. The present paper proves, by the application of similar techniques, together with electrophoretic studies, that the use of dyed substrate is justifiable for the estimation of elastase and moreover that it can be employed for the characterization of the enzyme in plasma even in the presence of excessive amounts of inhibitor.

MATERIALS AND METHODS

Materials

Elastin. Powdered elastin was prepared from ox ligamentum nuchae by the method described by Hall (1955). After the removal of the collagen, the powder was milled in a hammer mill until the whole passed a 100 mesh/in. sieve. It was then subjected to further treatment with 2% acetic acid in the autoclave and thoroughly washed with distilled water. Part of the powder was retained as a suitable substrate in the undyed state and the rest dyed with Congo Red (Naughton & Sanger, 1961). Elastin powder was suspended in 100 vol. of saturated aqueous Congo Red solution and agitated gently for 3 hr. Excess of dye was removed by repeated washing on a Buchner funnel, first with distilled water and then with acetone. In each case, washing was continued until no further dye could be removed. The dyed mass was finally dried by washing with ether and exposing to the atmosphere.

Elastase. The enzyme preparations employed were obtained from pig pancreas (Viobin Corp., Monticello, Ill.,

U.S.A.) by the method of Hall & Czerkawski (1959). Crude preparations (AEI) were used for some of the electrophoretic experiments and highly purified material (E$_2$I), which runs as a single band on a cellulose acetate electrophoretogram at a number of pH values (Hall & Czerkawski, 1959), for the kinetic studies.

Plasma and serum. Whole fresh citrated plasma from young adults was employed for the experiments aimed at the characterization of the enzyme, and also a protein fraction prepared from ox plasma by precipitation between 33% and 40% saturation of ammonium sulphate. This latter preparation contained α_2-, α_1- and β-globulins. Fresh citrated plasma or serum from patients of various ages was used for the estimation of enzyme and inhibitor content.

Methods

Measurement of elastolysis. Elastolysis with undyed substrate was measured by methods described by Hall & Czerkawski (1959). Activity is expressed in terms of the amount of protein taken into solution (measured by its biuret colour at a wavelength of 520 mμ)/mg. of enzyme under the specified conditions of the experiment, namely 3 hr. incubation at 37° in 10 ml. of borate buffer (pH 8·7, I 0·364). The normal amount of substrate present at the commencement of the reaction is 50 mg., but in the kinetic studies this was varied appropriately.

The measurement of activity with dyed substrate was carried out as follows. Various amounts of dyed elastin (between 0·2 and 10 mg.) were distributed in 15 ml. stoppered tubes. If the original elastin preparation was sufficiently fine a bulk preparation of dyed elastin (ECR) could be prepared by suspension in borate buffer and the amounts required delivered from a pipette. Enough buffer was added to make the total volume, together with subsequently added plasma or enzyme solution, 10 ml. The mixtures were incubated for 30 min. at 37° with inversion every 5 min. to ensure adequate mixing and undissolved substrate was removed by filtration through Whatman no. 541 paper. E_{485} of the clear-red solution was measured in a Unicam SP. 600 spectrophotometer in a 1 cm. cell. Similar measurements on blanks to which no enzyme had been added demonstrated that no protein was dissolved nor any dye released into solution by the buffer alone.

The dye present in the filtrate obeys Beer and Lambert's Law up to a concentration of 0·5 mg./ml. (Fig. 1) even in the presence of the dissolved protein. The estimation is 23 times more sensitive than the biuret methods as used for the undyed substrate, as can be seen by comparison of the two lines in Fig. 1.

Electrophoresis. Partially purified preparations of elastase were fractionated on cellulose acetate strips (Oxo Ltd., London, E.C. 4) in veronal buffer (pH 8·6, I 0·15, with a potential gradient of 12 v/cm. for 90 min.) and compared with samples of elastase and chymotrypsin treated in the same way. The electrophoretograms were cut into two strips longitudinally and one half was stained with Solway Purple (Hall, 1957), ninhydrin (Matheson, Tigane & Hanes, 1961) or azocarmine (Turba & Enenkel, 1950). The other strip was pressed on to the surface of a 2% agar gel set in the bottom of a shallow trough formed on a glass sheet with vertical walls of plasticine. The agar gel contained 4% of either dyed or undyed elastin in suspension. Evaporation was prevented by pressing another glass plate

on to the top of the plasticine walls to cover the electrophoretogram without touching it. The 'sandwich' was incubated for periods varying from 30 min. to 3 hr.

With the gel containing the dyed elastin, removal of the acetate strip showed it to be stained red in the region identified with the elastolytic enzyme on the azocarmine- or ninhydrin-stained portion of the strip, the dye having diffused out of the gel after being released by the enzyme which had diffused in. The choice of incubation time represented a compromise. Conditions had to be chosen which permitted adequate diffusion to occur at right angles to the plane of the acetate sheet while preventing excessive diffusion in the plane of the sheet.

Sbarra, Gilfillan & Bardawil (1960) and Oakley & Banerjee (1963) have employed gels containing unstained elastin for the estimation of elastin activity, measuring the areas cleared by the enzyme as it diffuses outward from wells cut in the gel. If the 'sandwich' with gel containing undyed enzyme was incubated for a considerable period of time (16–24 hr.) clear bands could be detected in the agar layer. Such prolonged incubation, however, resulted in so much lateral diffusion that it was impossible to ascribe these areas to individual components of the mixture as identified on the stained half of the electrophoretogram. Better separation could be obtained by the transfer of soluble protein and peptides from the gel to a superimposed strip of filter paper pressed firmly on to the upper surface of the 'sandwich'. In this case the gel was poured and set on top of the electrophoretogram, which was placed in the bottom of the trough. The proteins migrating from the cellulose acetate did not penetrate through the agar, only the peptides originating in the elastin within the gel. This could be proved by carefully removing the gel and preparing a print of the lower surface. Here all the components of the crude elastase preparation could be identified with the active band showing a marked intensification due to the products of elastolysis. The print from the upper surface on the other hand only demonstrated these products of the reaction. Owing to their polypeptide and peptide nature

spraying with ninhydrin solution proved the most suitable means for their identification.

RESULTS

Proof that the liberation of the dye is due to elastolysis. Fig. 2 demonstrates the distribution of elastolytic fractions among the stainable components of crude elastase preparations. It can be seen that there are two components in these crude elastase preparations which attack both the dyed and the undyed substrates. Similar records, on the other hand, which demonstrate the effects of purified elastase and chymotrypsin, indicate that the slower moving of the active components is lost during the final stages of purification and that the release of dye from ECR cannot be ascribed to chymotrypsin since this enzyme does not attack either the dyed or the undyed substrate.

Kinetic studies with dyed substrate. The activity of pure elastase against ECR was determined at a variety of enzyme and substrate concentrations. Values for the reaction rate were obtained by
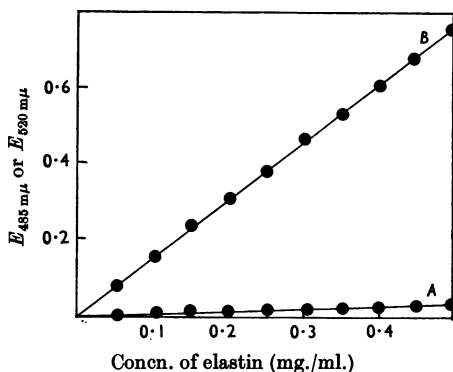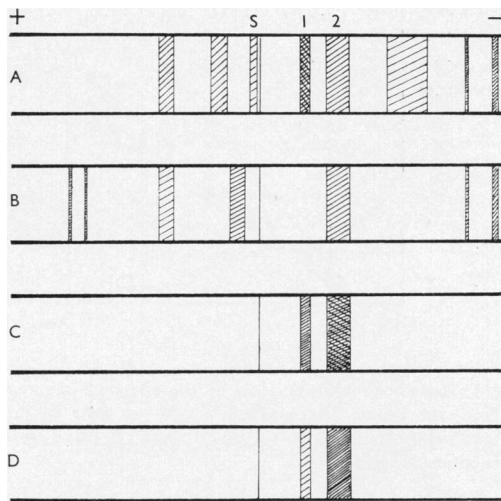

Fig. 2. Diagrammatic representation of cellulose acetate electrophoretograms of crude elastase preparations. The intensity of hatching is proportional to the degree of staining of the different bands. A, Stained with azocarmine. The bands labelled 1 and 2 are the two having elastolytic function. B, The same preparation stained with ninhydrin, showing the differing amounts of free amino groups in the various fractions. C, The Congo Red which has diffused back from the gel in the region of the two bands 1 and 2 after the elastolytic enzymes in these fractions have diffused into the agar containing the Congo Red-dyed substrate. D, The ninhydrin-positive material transferred from the surface of the agar gel containing unstained substrate after the migration of the same two bands (1 and 2).
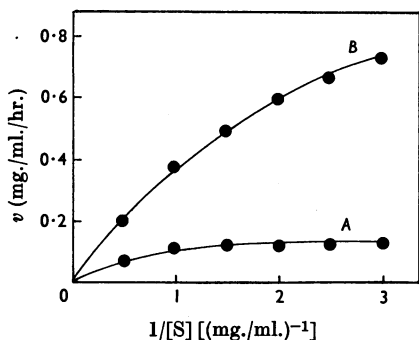

Fig. 1. A comparison of the extinction of the biuret colour of the dissolved protein (520 mμ) (*A*) and the released Congo Red (485 mμ) (*B*) obtained by total dissolution of unstained and stained substrates respectively at a variety of concentrations.

Fig. 3. Effect of changes in substrate concentration on the release of dye by (A) 5 μl. of pooled human citrated plasma and (B) 10 μg. of purified pancreatic elastase (E₂I) incubated with 5–30 mg. of Congo Red-dyed elastin in 10 ml. of borate buffer (pH 8·7, I 0·364) for 30 min. at 37°. Values of v are calculated from the curve in Fig. 1 and converted into an hourly base.

Fig. 5. Lineweaver–Burk plots for the action of fresh pooled human citrated plasma on Congo Red-dyed elastin: (A) 2; (B) 4; (C) 6 μl./ml. final concentration.
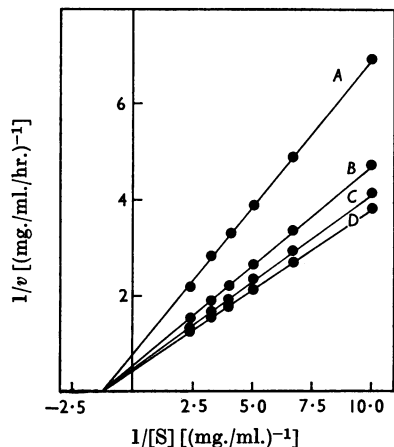

Fig. 4. Lineweaver–Burk plots of the effect of pancreatic elastase on Congo Red-stained elastin at four different concentrations of enzyme: (A) 2; (B) 4; (C) 6; (D) 8 μg./ml. final concentration.

Fig. 6. Lineweaver–Burk plots for the action of an ox-plasma fraction obtained by precipitation between 33% and 40% ammonium sulphate concentration: (A) 20; (B) 40; (C) 60; (D) 80; (E) 100 μg./ml. final concentration.

assessing the dye released during the first half-hour of the reaction in tubes incubated without agitation. The results obtained with systems containing enzymes from two different sources over a sixfold range of substrate concentrations are given in Fig. 3, where the rate has been expressed in terms of mg. of protein released/ml./hr. calculated from the graph in Fig. 1. To facilitate comparison of the reaction with those between the enzyme and undyed solid and soluble elastins (Hall, 1962) the results were plotted as reciprocals of the rate against the reciprocal of the substrate concentration (Fig. 4).
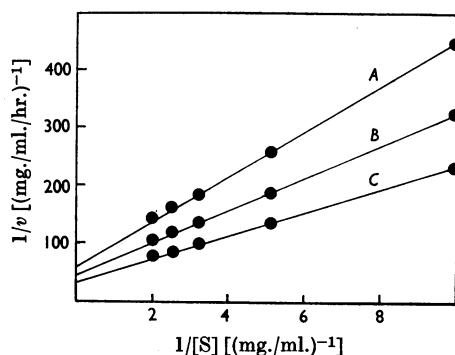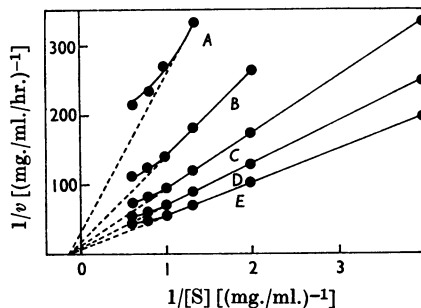
Similar Lineweaver–Burk plots are recorded in Figs. 5 and 6 for the effect of fresh plasma and the plasma protein fraction when acting alone on ECR. Here the values for enzyme concentration are given in terms of μl. of plasma/ml. or mg. of plasma protein.

Time-course of the reaction with the plasma enzyme. When samples of ECR were incubated with a given volume of plasma for various lengths of time an exponential curve which changed after between ½ and 1 hr. to a linear relationship was obtained (Fig. 7). That this was truly exponential could be shown by the logarithmic plot of Fig. 8. Although there appears to be constant falling off of rate from the beginning of the reaction, the linearity of the log plot up to 1 hr. indicates that over this period of time a measurement of the total dye release in a given time is a justifiable estimate of the reaction rate, since the rate calculated in this way bears a mathematical relationship to the true
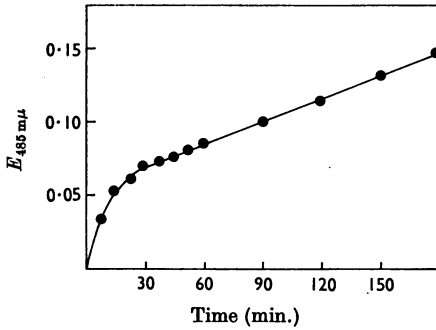
Fig. 7. Time-course of the reaction between human plasma elastase and Congo Red-dyed elastin expressed in terms of the extinction of the released dye solution at $485\,m\mu$.
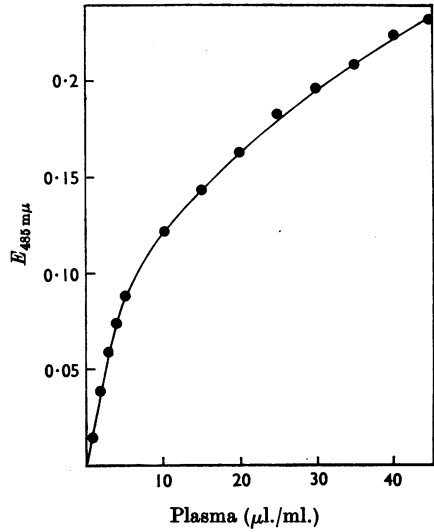

Fig. 9. Effect of variations in concentration on the response of the system human plasma elastase–Congo Red-dyed elastin. The response is linear up to concentrations of $5\,\mu l.$ of plasma/ml.
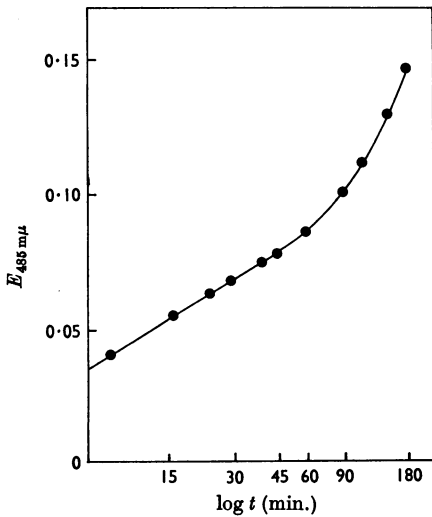

Fig. 8. Logarithmic plot of the time-course of the reaction between human plasma elastase and Congo Red-dyed elastin, demonstrating the justification for the use of incubation periods of up to 1 hr. in view of the simple mathematical relationship existing between time and response over this period.
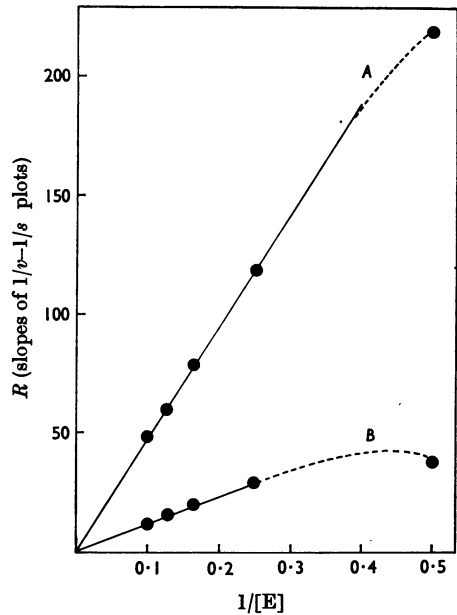

Fig. 10. Slopes of Lineweaver–Burk plots for systems containing Congo Red-dyed elastin together with (A) human plasma elastase and (B) pancreatic elastase plotted against the reciprocal of the enzyme concentration. Units of the ordinate are $\mu l./ml.$ for the plasma enzyme and $\mu g./ml.$ for the pancreatic enzyme.

initial rate. After incubation for 1 hr., the extinction of the released dye was 0·085, whereas after $\frac{1}{2}$ hr. it had reached a value of 0·07. It does not appear therefore that doubling the incubation time is worth while, except in cases where initial experiments show that the amount of enzyme present is too low to be accurately computed from the reading obtained after $\frac{1}{2}$ hr.

Effect of plasma concentration on activity determinations. The response of activity to increased enzyme concentration as demonstrated in Fig. 9 is

linear up to 5 μl./ml. The addition of larger amounts of plasma to the system as recorded in Fig. 10 demonstrates that at these higher concentrations the effect of the inhibitor becomes apparent. Plasma concentrations up to 5 μl./ml. should therefore be used for the determination of the enzyme in plasma.

DISCUSSION

Previous workers who have employed dye release as an estimate of elastolytic activity have assumed that the enzymic action which they were recording was in fact that of elastase, and of elastase alone. Without strict proof this would not appear to be a justifiable assumption. The lack of activity of chymotrypsin on both dyed and undyed elastin as indicated by 'sandwich' incubation after electrophoresis showed that at least this enzyme, which on the basis of certain similarities in specificity towards synthetic substrates has been claimed to resemble elastase, was no more capable of acting on the dyed substrate than it is on the native elastin. The identification of the activity with the same band on the electrophoresis strip, and of both these two with elastase itself, depends on the one hand on the definition of elastase and on the other on the criteria of purity which are acceptable for this enzyme. Hall & Czerkawski (1959) have demonstrated that the pure enzyme fraction E$_2$I which is used here moves as a single entity at a variety of pH values and has the solubility properties of a pure substance. It may be that at a later date such a preparation will be shown to be complex, but at the present time the enzyme which dissolves undyed elastin can be identified with the enzyme which brings about the liberation of dye when ECR is employed as substrate.

The appearance of two bands in the crude elastase preparations which have elastolytic activity is not surprising in view of the observations of the present author and others on the dual nature of elastase. Dvonch & Alburn (1959), by separating elastase from pancreas on ion-exchange resins, demonstrated two active bands in the eluate. Two fractions were also mentioned by Grant & Robbins (1955). Hall (1957) and later Loeven (1963) suggested that this division of activity might be due to the presence of the synergistic factor E$_1$, which, together with a small amount of elastase present in these fractions either due to tailing in Dvonch & Alburn's experiment or partial solubility in Grant & Robbins's preparations, produces an effective peak of activity. More recent studies (Hall, 1964, 1965), however, have demonstrated that elastase can in fact exist in two forms which appear to bear the relationship of monomer and dimer to one another. They act respectively on elastin molecules which are cross-linked by calcium and on free carboxyl groups in the elastin molecules. The reaction with dyed elastin is not dependent on the calcium content of the substrate and both enzymes liberate the dye (Fig. 2).

The family of curves in Fig. 5 represent typical Lineweaver–Burk plots of $1/v$ against $1/[S]$ for systems differing with respect to their enzyme concentrations [E], demonstrating the expected geometric relationship between slope and [E] in view of the linear relationship which exists between V and [E]. Similar curves can be obtained over various ranges of substrate concentrations and from the intercepts of these lines on the $1/[S]$ and $1/v$ axes, values for the constant K_m and V_m can be calculated. The former is characteristic of the enzyme and is independent of enzyme concentration, whereas the latter can be determined for a number of enzyme concentrations. The figures in Table 1 are the values of these constants, calculated for systems covering various ranges of substrate concentration; those for V_m are recorded for an enzyme concentration of 5 μg./ml. Also in Table 1 are the values for the same constants determined for the reaction between elastase and solid undyed substrate and the soluble substrate, α-elastin (Hall, 1962). The values for K_m and V_m for the reaction between elastase and ECR show a more marked similarity to the values obtained when soluble

Table 1. *Values of constants for the interaction between elastase and Congo Red-dyed elastin (ECR) compared with similar figures for the interaction of elastase with undyed solid elastin and soluble elastin*

Substrate	Range of substrate concn. (mg./ml.)	K_m (mg./ml.)	V_m (mg./ml./hr.)
ECR	0·2 – 1·0	6·4	—
	0·5 – 5·0	7·0	
	1·0 – 4·0	6·8	
	1·25– 8·0	6·25	28·6
Solid elastin	2·5 –10·0	51·3	3·0
Soluble elastin	2·0 –10·0	3·9	25·0

α-elastin is used as substrate than for the reaction between the enzyme and its natural undyed substrate. It would appear that the attachment of the dye molecule results in the conversion of the substrate into a form in which it resembles, as far as its reaction with the enzyme is concerned, the soluble substrate rather than the native solid one. Congo Red contains both amino and sulphonic groups by which it may attach itself to elastin, and it is not known as yet how the attachment occurs. Since the rate of reaction is increased it may be assumed that the effect is one of increasing the net negative charge on the elastin molecule. Hall & Czerkawski (1961) have demonstrated that a number of processes which introduce extra negatively charged centres into the elastin molecule result in greater susceptibility to the enzyme. Similar increases in free carboxyl groups may be expected to occur when the solid substrate is converted into α-elastin by the fission of peptide bonds. From the practical point of view, the dyeing of the molecule with Congo Red results in the greater reactivity associated with the soluble substrate being attained without loss of the ease of manipulation associated with the use of a solid substrate.

Inhibition by substrate is apparent at high substrate concentrations in the curves relating to the effect of the plasma protein fraction (Fig. 6). Here again a value for K_m can be calculated and this is recorded in Table 2. There is good agreement between the value obtained for the pure enzyme and that determined for the enzyme present in plasma.

At first sight it might appear impossible to determine the absolute enzyme concentration in plasma since the simultaneous presence of the inhibitor might be expected to lower the effective value of V. That this is not the case can be seen from the lines in Fig. 10. These represent plots of the slopes of the lines in Figs. 5 and 6 (i.e. the values $K_m/k_2[E](1 + [I]/K_i)$ obtained by plotting $1/V$ against $1/[S]$ for systems containing plasma and the plasma protein fraction) against $1/[E]$. In these cases 'enzyme concentrations' are calculated in terms of μl./ml. and μg./ml. for plasma and plasma protein fraction respectively. As can be seen both lines are linear over a considerable range of enzyme concentration, and both pass through the origin. Proof that the inhibitor which is also present in the added plasma and to a lesser extent

in the plasma fraction (cf. Graham, 1958) has no effect is contained in the following.

The equations for uninhibited and inhibited enzyme reactions can be written:

$$1/V = 1/k_2[E] + K_s/k_2[E][S] \tag{1}$$

and

$$1/V_i = 1/k_2[E] + K_s/k_2[E][S].(1 + [I]/K_i) \tag{2}$$

respectively.

Hence the slopes of these two lines (R and R_i) may be expressed as:

$$K_s/[E]k_2 \text{ and } K_s/[E]k_2.(1 + [I]/K_i) \tag{3a \& b}$$

Plotting these slopes against $1/[E]$ should therefore give a straight line through the origin for the uninhibited reaction and a line of different slope and displaced from the origin by a distance proportional to the inhibitor concentration for the inhibited reaction. In the present instance, however, simplification of equation (3b) is possible. Since both enzyme and inhibitor are present in any given amount of plasma in a constant relationship to one another, I can be written as rE where r represents this constant relationship. Hence the appropriate form of equation (3b) may be written:

$$R_i = K_s/[E]k_2.(1 + r[E]/K_i) \tag{4}$$

which will reduce to

$$R_1 = K_s/[E]k_2 = rK_s/K_i k_2 \tag{5}$$

This represents a line parallel to that of the uninhibited reaction but displaced upwards by an amount such that its intercept on the $1/[E]$ axis is $rK_s/K_i k_2$. The two lines in Fig. 10, however, both pass through the origin and only at higher values of $1/[E]$ and hence at correspondingly lower plasma concentrations is there deviation from linearity such as to indicate that the lines might cross the R axis above the origin due to activity of the inhibitor. The grossly different slopes are due to the fact that the scales of the $1/[E]$ axis are in the one case μg. of a rather inactive plasma protein preparation/ml. and in the other μl. of active plasma/ml. It is therefore apparent that conditions can be determined under which the inhibitor, although simultaneously present in the plasma, is without effect on the reaction and hence valid values for the enzyme content of the plasma can be obtained. The method is of considerable simplicity and lends itself to a study of large numbers of samples. Typical figures for the elastase content of a series of plasma samples are given in Table 3.

If the elastin powder from which the dyed substrate is prepared has a particle size of less than 0.2 mm. it remains suspended between inversions of the reaction tubes. With larger particle size, not

Table 2. *Values for the kinetic constants for plasma elastase*

Source of enzyme	K_m (mg./ml.)
Pancreas (av. value)	6·5
Whole plasma (human)	6·3
Plasma fraction (ox)	6·35

Table 3. *Elastase content of the plasma of representative young adults*

A significant difference (P 0·05) between the values for the sexes is shown.

Sex	E_{485}	Units of elastase/ml. of plasma	
Male	0·075	102·6	
	0·120	164·0	
	0·115	157·0	
	0·095	129·7	
	0·087	118·8	Mean value 130·1 ± 7·5
	0·092	125·6	
	0·080	109·0	
	0·103	140·6	
Female	0·083	113·0	
	0·085	116·0	
	0·093	127·1	
	0·076	103·7	Mean value 115·9 ± 9·8
	0·086	117·2	
	0·087	118·7	

only is it difficult to measure accurate amounts by volume, but the rate of dissolution is fractionally decreased.

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