of Smith (1959) suggest, however, that the combination velocity rates may be determined chiefly by the chemical configuration of the haems, which apparently all combine with the same site on the protein surface (Rossi-Fanelli & Antonini, 1959b), though not necessarily forming identical linkages. Determinations have shown that the ultracentrifugal molecular weight is the same in all three cases, so that the lower degree of haem–haem interaction shown by mesohaemoglobin and deuterohaemoglobin cannot be attributed to splitting to half-molecules. The results reported here suggest that experiments with more widely different haems may help in understanding the mechanism of haemoglobin function.

SUMMARY

1. The rates of combination of natural and reconstituted protohaemoglobin with carbon monoxide have been compared both for the first and fourth molecules of carbon monoxide to combine. The natural and reconstituted proteins give rates agreeing with one another.

2. The rates of dissociation of the first molecule of oxygen and of carbon monoxide from fully saturated natural and reconstituted protohaemoglobin have been similarly compared and found to agree.

3. The corresponding reactions of mesohaemoglobin and deuterohaemoglobin have been examined. The rate of combination of the first molecule of carbon monoxide is greater than for protohaemoglobin. The rate of dissociation of the first molecule of oxygen from saturated haemoglobin is slow for mesohaemoglobin (k_{43} 8 sec.$^{-1}$) and rapid for deuterohaemoglobin (k_{43} 60 sec.$^{-1}$).

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Study of a Proteolytic Enzyme from Rabbit Spleen

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It has long been known that aqueous extracts of spleen exhibit proteinase activity (Hedin, 1903). The intracellular enzymes responsible for this activity and a similar activity found in other tissues have been named cathepsins (Willstätter & Bamann, 1929). The cathepsins of ox spleen have been studied in some detail and classified according to their specificity toward synthetic substrates (Tallan, Jones & Fruton, 1952).

In order to study the catabolism of a protein

antigen one of us undertook the investigation of the degradation of human-serum albumin by aqueous extracts of rabbit spleen. It was found that the extracts were capable of causing the degradation of human-serum albumin at pH 3·0–4·0 (Lapresle, 1955a). The immunochemical study of the products of this degradation showed that some of these fragments precipitated independently with distinct antibodies present in sera of rabbits previously immunized with undegraded human-serum albumin (Lapresle, 1955b). It was concluded that the albumin molecule had different antigenic groups which were part of different split products.

A comparative study of the degradation products obtained by the action of several proteolytic enzymes on human-serum albumin has shown that spleen extract was a convenient tool for studying the antigenic structure of the albumin molecule (Lapresle, Kaminski & Tanner, 1959). Moreover, an enzyme or enzymes of spleen extracts capable of degrading human-serum albumin in this manner could have an important role in the catabolism of proteins. For these two reasons we have undertaken the study of this activity and the partial purification of the enzyme responsible. Preliminary results of these studies have been reported elsewhere (Lapresle & Webb, 1960).

MATERIALS

Rabbit spleens. Rabbit spleens were obtained from exsanguinated rabbits and frozen for storage.

Human-serum albumin. Squibb fraction V was prepared by ethanol precipitation and was kindly given by the American Red Cross.

Diethylaminoethylcellulose and carboxymethylcellulose. These were prepared according to the methods described by Peterson & Sober (1956) and were obtained from Brown and Co., 500 Fifth Avenue, New York, 36, U.S.A.

Antisera. Rabbit sera were prepared by intravenous injection of alum-precipitated human-serum albumin as described by Lapresle (1955b).

METHODS

Protein determination. The amount of protein in the enzyme preparations was determined, with the biuret reagent prepared according to Weichselbaum (1946), by reference to a standard curve prepared with various weights of a freeze-dried crude spleen extract.

Enzyme assay. A solution of human-serum albumin, 10% in water containing 2 mM-CaCl₂, 0·2 mM-MgCl₂ and 8 mM-cysteine, was adjusted to pH 3·5 with HCl. Albumin solution (0·5 ml.) was added to 0·5 ml. of enzyme preparation (in 0·15M-NaCl) and the mixture was incubated for 30 min. at 45°. Then 1 ml. of 4% trichloroacetic acid was added and the suspension was incubated for 10 min. at 45° and centrifuged. A portion (1 ml.) of the clear supernatant was added to 1·5 ml. of biuret reagent prepared according to Weichselbaum (1946) but containing twice the usual quantity of NaOH in order to neutralize the trichloro-

acetic acid. After 30 min. of incubation at 37° the extinction of the colour developed by the biuret reagent was read at 555 mμ. Enzyme–substrate blanks were prepared by incubating the substrate solution for 30 min. at 45° and then adding the enzyme and trichloroacetic acid simultaneously. A curve was drawn relating extinction of the supernatant to milligrams of crude extract used. An arbitrary unit of proteolytic activity was defined from the tangent to the initial part of the curve as the activity which would cause an increase of 0·001 unit of extinction/min. of digestion. A further standard curve (Fig. 1) was then constructed relating the increases in extinction of the supernatant to the number of units employed.

Determination of optimum pH. The same conditions were used as those for the measurement of enzyme activity except that albumin solutions were prepared with the pH adjusted with HCl to 2·5, 3·0, 3·5, 4·0, 4·5 and 5·0 as measured by a Radiometer pH meter.

Determination of the Michaelis constant. The Michaelis constant was determined under the following conditions: pH 3·5, temp. 45°, reaction time 30 min. The velocity of the reaction was measured by the quantity of material formed which was no longer precipitated with 2% trichloroacetic acid. To increasing amounts of human-serum albumin a constant quantity of 'crude' preparation (3·3 units) of 'purified' preparation (3·0 units) was used. The Michaelis constant was calculated by the graphic method of Lineweaver & Burk (1934) relating the reciprocal of the initial velocity of the reaction to the reciprocal of the substrate concentration.

Preparation of the enzyme

Step 1. The frozen rabbit spleens were broken in a small volume of water with a Waring Blendor for 1 min. and dialysed with agitation against distilled water for 1 hr. The suspension was centrifuged at 15 000g for 1 hr. and the clear supernatant was decanted.
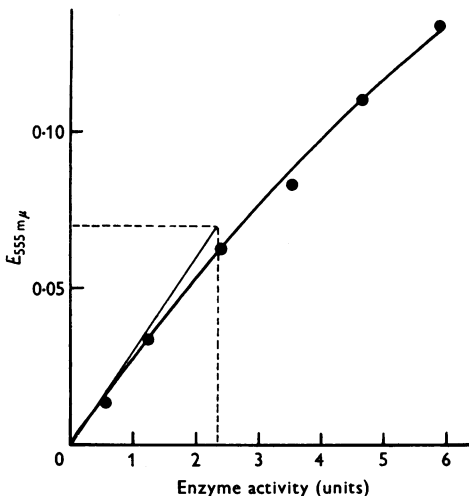

Fig. 1. Standard curve for the digestion of human-serum albumin by rabbit-spleen extract. The extinction figures refer to changes/30 min.

Step II. Sufficient NaCl was added to the aqueous extract to give a concentration of 0·15M and HCl was added to give pH 5·0. The suspension thus formed was centrifuged and the precipitate was discarded.

Step III. The clear supernatant was precipitated by the addition of 2 vol. of saturated $(NH_4)_2SO_4$ solution, pH 7. After 30 min. the suspension was centrifuged and the supernatant discarded. The precipitate was dissolved in a small volume of water, dialysed free of $(NH_4)_2SO_4$ and frozen for storage. This preparation is referred to as the 'crude' preparation.

Step IV. Further purification was achieved by adsorbing a portion of the inactive material on to diethylaminoethyl-cellulose equilibrated with a 0·03M-potassium phosphate buffer, pH 8·0. This adsorption was carried out by mixing the cellulose into a dialysed solution of the enzyme preparation (3 g. of cellulose/g. of protein). After 30 min. of contact the reddish supernatant was filtered from the cellulose and the cellulose washed with 0·5 vol. of phosphate buffer.

Step V. The eluate thus obtained was dialysed against a 15 mM-potassium phosphate buffer, pH 6·75, and mixed with carboxymethylcellulose equilibrated with the same buffer (15 g. of cellulose/g. of protein). After 30 min. of contact the supernatant was again filtered from the cellulose and the cellulose washed with 0·5 vol. of phosphate buffer.

Step VI. The eluate was dialysed against 5 mM-potassium phosphate buffer, pH 8, and put on a column of diethylaminoethylcellulose equilibrated with the same buffer. Some of the inactive material was eliminated by washing the column with the same buffer. The active material was eluted with a 0·1M-potassium phosphate buffer, pH 7. This preparation is referred to as the 'purified' enzyme.

Chromatographic analysis. The chromatographic analysis was carried out with a preparation obtained at step V. The preparation was dialysed against a 5 mM-potassium phosphate buffer, pH 8, and put on a column of diethylaminoethylcellulose equilibrated with the same buffer. After the passage of a 'break through' the elution of the enzyme was obtained with a concave gradient of potassium phosphate buffer from 5 mM, pH 8, to 0·2M, pH 7, realized with the cone–sphere technique of Sober & Peterson (1958).

Immunoelectrophoresis. For these experiments the albumin was degraded under the same conditions as for the

measurement of proteolytic activity except that the final albumin concentration was 2% and the time allowed 1 hr. The degradation was stopped by neutralizing the reaction medium with one drop of NaOH solution at a suitable concentration. Immunoelectrophoresis of human-serum albumin degraded by the 'crude' and by the 'purified' enzyme was done according to the technique of Grabar & Williams (1955) under the following conditions: veronal–HCl buffer 0·05M, pH 8·2, duration 4 hr. A rabbit serum anti-human-serum albumin was used to develop the immunoelectrophoresis pattern.

RESULTS

Preparation of the enzyme. Table 1 gives the results of the different steps in the purification of the proteolytic enzyme. With this procedure, where the preparation is made with the double purpose of purifying the activity and recovering as much activity as possible, the final preparation contains about ten times as much activity per milligram as the original preparation and the overall recovery is about 50%. It should be noted that a much better purification can be obtained if one takes at each step the most active portion, and by this means we have obtained a preparation having about 200 units/mg., i.e. 40 times the specific activity of the original preparation.

Chromatographic analysis. Chromatographic analysis of an enzymic preparation obtained at step V shows (Fig. 2) that the proteolytic activity was eluted as a single peak. All the activity was eluted by phosphate concentration of between 0·01 and 0·04M, pH 8·2–7·0. In other experiments where the pH of the eluting buffer was kept constant at pH 8·0 the activity was also eluted as a single peak between the same concentrations in phosphate (0·01–0·04M).

The proteins in the preparation were eluted as several peaks, none of which seemed to be enzyme protein. The peak of activity in Fig. 2 and in other experiments under the same conditions appears

Table 1. *Preparation of a proteolytic enzyme from rabbit-spleen extracts*

For details see Methods section.

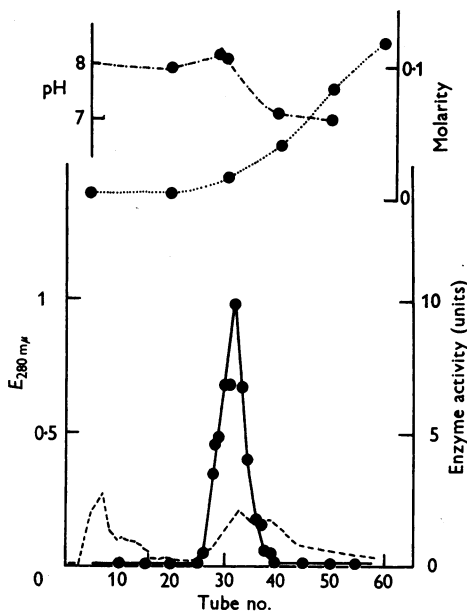| Step no.	Procedure	Enzyme activity	
		Total units	Units/mg. of protein
I	Aqueous extract	10 000	5·3
II and III	Removal of inactive material precipitated at pH 5 and subsequent precipitation of active material at 66% saturation by $(NH_4)_2SO_4$	6 600	16·5
IV	Adsorption of inactive material on diethyl-aminoethylcellulose at pH 8	4 950	22·4
V	Adsorption of inactive material on carboxymethylcellulose at pH 6·75	4 800	27·2
VI	Elution from a column of diethylamino-ethylcellulose at pH 7	4 800	55

Fig. 2. Chromatography of a purified spleen extract. Enzyme solution (step V; 23 ml.) containing 1·23 mg. of protein/ml. and 21·5 units/ml. was put on a column of 1·5 g. of diethylaminoethylcellulose. The gradient was started at tube no. 19. Each tube contained 5 ml. of eluate. The proteolytic activity of each eluate was measured in duplicate. —, Activity; - - -, extinction.
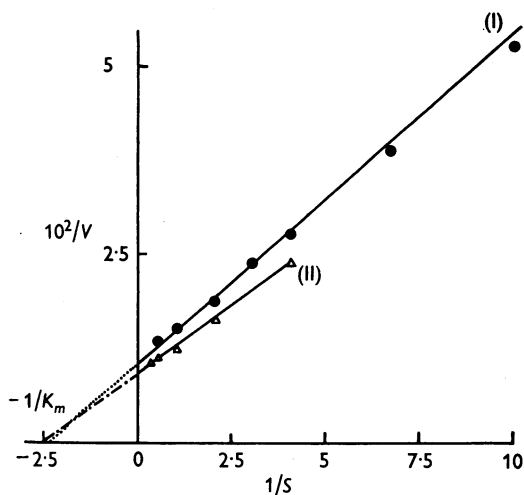

Fig. 3. Graphic representation according to Lineweaver & Burk (1934) for the determination of the Michaelis constant of rabbit-spleen extracts. (I) 'Crude' extract; (II) 'purified' extract. S is the concentration (g./100 ml.) of human-serum albumin used as substrate; V is the extinction at 555 mμ of the colour given by 1 ml. of the trichloroacetic acid supernatants when treated with the biuret reagent.

slightly in advance of the first protein peak eluted by the gradient.

Optimum pH. It was found for both the 'crude' enzyme and the 'purified' enzyme that the activity was optimum over the range pH 3·0–4·5.

Michaelis constant. The study of the enzymic reaction with a constant amount of 'crude' or 'purified' enzyme and increasing amounts of human-serum albumin showed that it obeyed the Michaelis–Menten law. The experimental points thus obtained when plotted according to Lineweaver & Burk corresponded in each case to a straight line (Fig. 3). The Michaelis constant derived from the intercept of these lines with the abscissa was almost the same for the 'crude' enzyme (0·423 g./100 ml.) as for the 'purified' enzyme (0·404 g./100 ml.).

Immunoelectrophoresis. Fig. 4 shows the immunoelectrophoresis of degraded human-serum albumin revealed by an anti-human-serum albumin serum. The split products of human-serum albumin give three different precipitin lines as was described by Lapresle (1955b). These lines have the same shape, situation and density whether the albumin has been degraded by a 'crude' or a 'purified' preparation provided that the same number of proteolytic units are used. An example of this pattern is given in Fig. 4 by using human-serum albumin degraded by 10 units/ml. of 'crude' (4 units/mg.) or 'purified' (55 units/mg.) preparation.

DISCUSSION

As we were interested in the enzyme or enzymes of rabbit-spleen extracts which can degrade human-serum albumin, this protein was used as
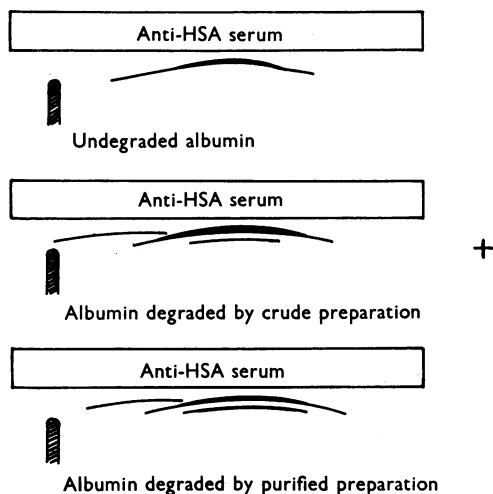

Fig. 4. Schematic representation of the immunoelectrophoresis pattern given by undegraded and degraded human-serum albumin (HSA).

substrate. To measure the activity we arbitrarily used the amount of product formed which is not precipitated in 2 % trichloroacetic acid (the minimum trichloroacetic acid concentration required for complete precipitation of the undegraded human-serum albumin). This non-precipitable material was not measured by its absorption at 280 mμ as is usually done, because the enzyme blanks with the 'crude' preparation were found to contain considerable material which absorbed at this wavelength. The non-precipitable material in the 'crude' enzyme preparation did not react with the biuret reagent. This reagent was therefore used to measure the quantity of the degradation product formed. This procedure is justified by the finding (Lapresle, 1955a) that the degraded albumin retains, almost completely, its capacity to react with the biuret reagent. The activity was increased by the presence of cysteine, Ca^{2+} and Mg^{2+} ions, and these substances were added to the reaction medium to avoid the effect of their possible elimination during the course of the purification of the enzyme.

Steps I–IV in the procedure for the preparation of the enzyme are based on preliminary experiments reported by Lapresle & Webb (1960). The main purpose of step V is to remove from the preparation a contaminating reddish pigment which cannot be separated from the activity by chromatography on diethylaminoethylcellulose columns. Step VI eliminates a portion of inactive material as a 'break through' and concentrates the active material.

Chromatographic analysis of the 'crude' preparation carried out by Lapresle & Webb (1960) did not permit a definite conclusion about the homogeneity of the proteolytic enzyme. With the 'stepwise'-elution procedure several peaks of activity were obtained but with the gradient technique one large peak of activity was obtained with an extended and irregular trailing. The analysis of the 'purified' preparation reported herein and several other experiments carried out under similar conditions showed that the protein responsible for the enzymic activity was eluted as a single substance. It would seem that one of the three reasons suggested in our earlier publication (i.e. the existence of a single enzyme in the form of complexes with various other components) was responsible for the apparent existence of several active substances in the 'crude' extracts.

We were interested in the spleen proteases capable of degrading human-serum albumin in such a way as to split the antigenic structure of the albumin molecule in the manner described by Lapresle (1955b). Therefore a method has been developed for measuring the proteolytic activity with albumin as substrate, immunoelectrophoresis being used to check the immunochemical properties of the degradation products of albumin. It is most probable that the same activity was being studied by these two procedures because the same immuno-electrophoretic patterns were obtained only when the same number of proteolytic units of each enzyme preparation (steps I–IV) was used.

Under these conditions we have found only one enzyme in the 'purified' preparation (step V). This enzyme is most probably the one which is responsible for the proteolytic activity of the 'crude' preparation since the pH optimum and the Michaelis constant are the same. This conclusion is supported by the similarity of the immunoelectrophoretic patterns given by human-serum albumin degraded with crude and purified preparations, which suggests that in both cases the bonds split were in the same situation relative to the antigenic groups of the albumin molecule. As pointed out by Lapresle *et al.* (1959), human-serum albumin, degraded by pepsin, gives the same immunoelectrophoretic pattern as that given by human-serum albumin degraded by the rabbit-spleen extract, which suggests that the specificity of the rabbit-spleen protease resembles that of pepsin.

The existence of several cathepsins in ox-spleen extracts has been demonstrated by the use of synthetic substrates. The latest classification by this method has permitted the identification of at least three cathepsins: A, B and C (Tallan *et al.* 1952). Lapresle *et al.* (1959) have shown that the enzyme responsible for the degradation of human-serum albumin at pH 3·5 cannot be analogous to either cathepsin B or C. The enzyme which we have described in this work could be analogous to cathepsin A or another intracellular proteinase. Further clarification of this point will await studies on the specificity of this enzyme.

After this paper had been submitted for publication, Press, Porter & Cebra (1960) described the isolation from ox spleen of an intracellular enzyme with properties similar in many respects to those of the enzyme from rabbit spleen that we have been studying.

These two enzymes have the same acid pH optimum, are heat-labile, are eluted from diethylaminoethyl- and carboxymethyl-cellulose under similar conditions and both seem to have specificities resembling that of pepsin.

SUMMARY

1. The proteolytic activity of rabbit-spleen extracts towards human-serum albumin has been studied. With serum albumin as substrate a method of measuring the proteolytic activity of spleen extracts has been developed, based on the determination with the biuret reagent of the

amount of products which are not precipitable by trichloroacetic acid.

2. The enzyme responsible for this proteolytic activity has been purified by adsorption of inactive material on diethylaminoethylcellulose and carboxymethylcellulose.

3. Chromatography on diethylaminoethylcellulose has demonstrated the existence of only one enzyme in the purified preparation.

4. The optimum pH and the Michaelis constant of this enzyme are the same as those of the proteolytic activity in the crude preparation. It was therefore concluded that this enzyme is responsible for the proteolytic activity of the crude spleen extracts.

5. A comparative study of the immunoelectrophoresis patterns given by human-serum albumin, degraded with the crude and purified spleen extracts, has shown that it is this enzyme which is responsible in both cases for the splitting of the antigenic structure of human-serum albumin.

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Effects of the Insecticides DDT and Dieldrin on Phosphorus Metabolism of the Adult Housefly *Musca domestica* L.

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DDT [1:1:1-trichloro-2:2-di-(p-chlorophenyl)ethane] and dieldrin (1:2:3:4:10:10-hexachloro-6:7-epoxy - 1:4:4a:5:6:7:8:8a - octahydro - exo - 1:4 - endo - 5:8-dimethanonaphthalene) respectively represent two important classes of insecticides. Both compounds are toxic to mammals as well as to insects at comparable tissue concentrations, but comparatively little is known of their mode of action in terms of one or more specific biochemical lesions. There is some evidence that DDT may affect oxidative metabolism in a way that is not explained by the exhaustion of endogenous reserves (see review by Winteringham & Lewis, 1959).

The application of a ³²P-labelled-pool technique for studying changes in the relative concentrations of the soluble phosphorus compounds of intact adult houseflies as a result of flight, starvation, etc., has been described (Winteringham, 1960). The use of the same technique for studying the effects of DDT and dieldrin is now reported.

EXPERIMENTAL

Treatment and analysis of insect material. Female adult houseflies (*Musca domestica* L.) not less than 3 days old at the time of extraction were used throughout. The strain used was susceptible to the action of DDT or dieldrin. The principal soluble phosphorus compounds were uniformly labelled with ³²P *in vivo*, extracted and assayed exactly as explained by Winteringham (1960). Any special treatment of the insects before extraction, such as exposure to insecticides, is indicated in the tables. Insecticides were applied topically to the dorsal surface of the thorax in acetone solution, while the insect was under mild *cyclo*propane anaesthesia. Aqueous glucose was injected intrathoracically by means of an Agla micrometer syringe (Burroughs Wellcome and Co., London) fitted with a 27 s.w.g. needle. Treated insects were always kept at 27–30° in aerated glass chambers without food or water in artificial light. The injection of water alone or the topical application of acetone alone was without detectable effect on phosphorus distribution (Winteringham, 1960).