

ISOLATION AND PARTIAL CHARACTERIZATION OF AN ANTIGEN FROM THE CATTLE TICK, *BOOPHILUS MICROPLUS*

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Abstract—The isolation is reported of a component of *B. microplus* larvae which causes an immediate hypersensitivity reaction in cattle which have been exposed to the tick. It has been purified and shown to be a protein with a mol. wt of approx 60,000. Evidence is also given that the component is an esterase whose irreversible inhibition by the organophosphate reagent, DFP, shows the presence of a serine residue essential for enzymatic activity.

INTRODUCTION

At the present time, the immunology of a number of host-parasite relationships is being increasingly investigated. The association between the cattle tick and its host has, however, despite its interesting features, received little attention. Although cattle develop an immunologically-mediated resistance to infestation by the cattle tick, *B. microplus* (Roberts, 1968a), the nature of the antigens and the types of immunological response involved are unknown. The resistance mechanism is likely to be complex. Complete immunity to infestation is rarely achieved and cattle display a range of fairly stable resistance levels (Roberts, 1968a). The cellular responses in cattle skin to the attachment of larvae and their relationship to host resistance have been described (Schleger *et al.*, 1975). Further, it has been suggested that many of the events observed histologically may be due to the host's reaction to tick antigens (Tatchell, 1969). It seemed likely to us that skin reactions of cattle to tick components would provide a suitable way of investigating those antigens which the tick has injected into the host in the course of natural infestation. Riek (1956, 1962) has reported that animals which have been exposed to ticks show an immediate hypersensitivity to tick extracts which is small or absent in unexposed animals and may increase in intensity with increasing exposure.

All stages of the life-cycle of *B. microplus* could be sources of antigenic material but a major expression of resistance is the rejection of larvae during the first 24 hr of the life cycle (Roberts, 1968b). This indicates that the critical antigens must be present at that stage. In this paper therefore, we report the isolation of a component of *B. microplus* larvae which produces an immediate hypersensitivity reaction in hosts which have been naturally infected with ticks.

It is possible that in a parasitic infection a protein which is isolated as an antigen could have additional and possibly non-antigenic sites with some other physiological function. One can only guess at possibilities for such a function, but a likely one is that of a hydrolytic enzyme. We have therefore tried to see if the

antigen being purified had an identifiable enzymatic activity.

MATERIALS AND METHODS

Sources of materials

Fluorescein dibutyrate was obtained from ICN Pharmaceuticals and tritium-labelled di-isopropyl phosphorofluoridate, DFP, 3.9 Ci/m-mole, from the Radiochemical Centre, Amersham. Starch for gel electrophoresis, Lot 166-1, was from Connaught Medical Research Laboratories and Cyanogum-41 from BDH. The DEAE-cellulose used was a medium mesh product from Sigma and the Sephadex G-200 was of normal grade. *B. microplus* larvae were obtained as described by Roberts (1971) and frozen at -20°C , 7-14 days after hatching.

Immediate hypersensitivity tests

The animals used were *Bos taurus* cattle which had received substantial natural exposure to ticks. Materials to be tested were diluted in phosphate-buffered saline containing 1 mg/ml bovine serum albumin and 0.1 ml was injected intradermally. The oedematous reaction reached its maximum size between 20 and 30 min after injection and did not alter in size for another 30 min. The diameter was measured in two directions at right angles and the product of these two measurements taken as the result. It was found that, within the useful range of response (a 10-30 mm dia swelling), the size of the reaction was proportional to the logarithm of the amount of material injected. During purification procedures, a standard line was obtained from dilutions of the initial crude extract. Using this, the concentration of antigen in any fraction was related to that in the crude extract, and expressed as the number of μl of crude extract which would give the same sized reaction as one μl of the fraction. The response of different animals varied and the standardization with crude antigen was repeated with each animal and usually each series of determinations. Tests for non-specific activity were done in the same way, using cattle which had not been exposed to ticks.

Esterase assay

Enzyme activity was measured in 0.05 M phosphate buffer pH 7.5 containing 1 mM 2-mercaptoethanol and 0.5 mM EDTA (EDTA and mercaptoethanol were added

routinely to all buffers at these concentrations and henceforth, in this report, buffers will be identified only by the buffering component). Fluorescein dibutyrate was used as substrate at a final concentration of $8.1 \times 10^{-6} M$. An aliquot of the solution to be assayed was added to 3 ml of the substrate solution and incubated at $37^\circ C$. Incubation times were 6, 12 or 60 min, depending on the activity of the sample. The reaction was terminated by addition of 1 ml 10% TCA, the solution centrifuged if there was a significant precipitate and the fluorescence was measured in a Perkin-Elmer Model 203 Fluorescence Spectrophotometer using excitation and emission wavelengths of 440 and 515 nm respectively. Not more than 0.2% of the substrate was hydrolyzed in any assay. The fluorescence change was proportional to the duration of the incubation and the amount of enzyme added.

To convert the enzyme activity from the arbitrary fluorescence units into the normal units of μmoles of substrate hydrolyzed per min, it was necessary to prepare a fluorescence standard. For this, a $0.959 \mu M$ solution of fluorescein dibutyrate in the assay buffer was hydrolyzed enzymically, until absence of a change on addition of more enzyme or prolongation of the reaction time showed the hydrolysis to be complete. The fluorescence of this solution, when measured as described for the normal assay procedure, was taken as the standard.

Gel electrophoresis

Starch gel electrophoreses were run in a 12.2% gel, 22 cm long, set as a thin layer in an LKB2117 Multiphor apparatus. Small wells were cut in the gel, $50 \mu\text{l}$ samples were pipetted in and, after electrophoresis for 5 hr at 400 V, the gel was cut into 5 mm sections. These were then eluted overnight into 1.5 ml electrophoresis buffer, prior to testing for antigenic and esterase activities.

Polyacrylamide gels were prepared from 7% Cyanogum in 0.02 M Tris, 0.04 M glycine buffer, and set as 15 cm gels in narrow bore glass tubes. After pre-electrophoresis for 3 hr at 400 V in the same buffer, to which had been added 1 mM mercaptoethanol, samples were loaded and run for 2 hr at 400 V. The gels were stained for protein

with Coomassie blue (Chrambach *et al.*, 1967). To localize radioactive components, the gels were cut into 2 mm sections which were solubilized in 0.5 ml 30% hydrogen peroxide (Tishler & Epstein, 1968) and counted, after addition of 6 ml Packard Insta-gel, in a Packard Tri-Carb liquid scintillation counter.

Protein determinations

The method of Warburg & Christian (1941) and Kalckar (1947) was used.

RESULTS

Purification of antigenic activity

Tick larvae were homogenized, with cooling, in a Braun Cell Homogenizer MSK for 2 min, then extracted for 30 min in 0.05 M phosphate buffer pH 7.5 using 2.5 ml buffer/g of larvae. This, and all subsequent steps of the purification, were carried out at $4^\circ C$. The extract was then centrifuged for 30 min at 10,000 *g* followed by a further centrifugation for 30 min at 25,000 *g*. The supernatant was filtered and the filtrate centrifuged once more at 25,000 *g* for 30 min. As a preliminary experiment intended to show the number of antigens present, 2 ml of the supernatant was chromatographed in 0.10 M phosphate buffer pH 7.5 on a 2.5×62 cm column of Sephadex G-200 and the fractions were tested for antigenic and esterase activity. The results are shown in Fig. 1. The Figure shows the results when 2.8 void volumes had been collected, representing molecules with apparent mol. wts of about 20,000 or larger. There were no additional peaks of antigenic activity in the lower mol. wt range. The breadth of the peak of hypersensitivity activity strongly suggests that more than one antigen is involved. The work to be described concerns the purification of the material shown at the

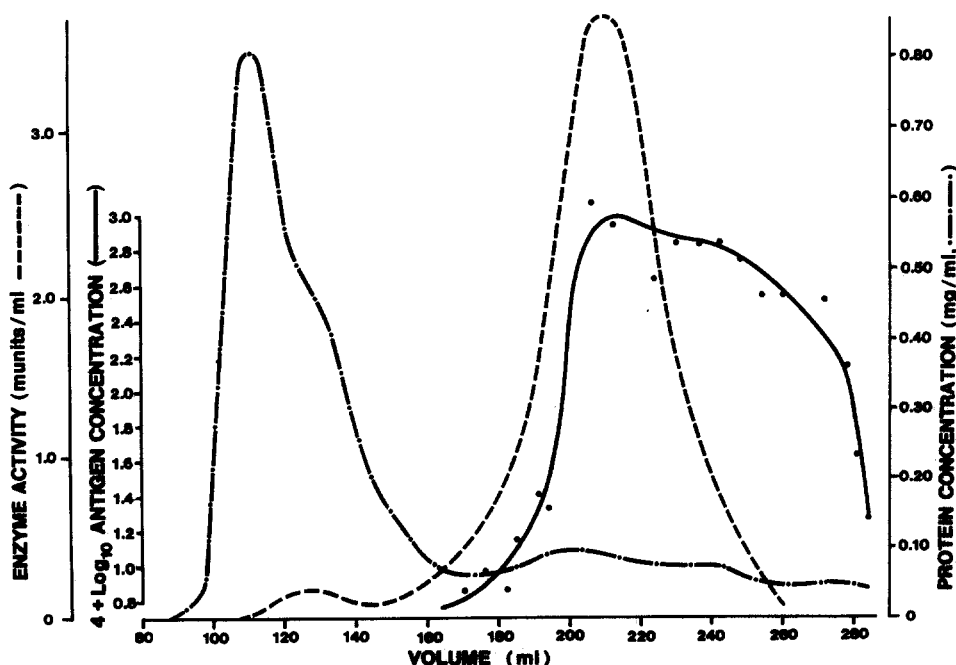


Fig. 1. Chromatography of crude extract on G-200. Experimental points for antigen concentration are shown as dots.

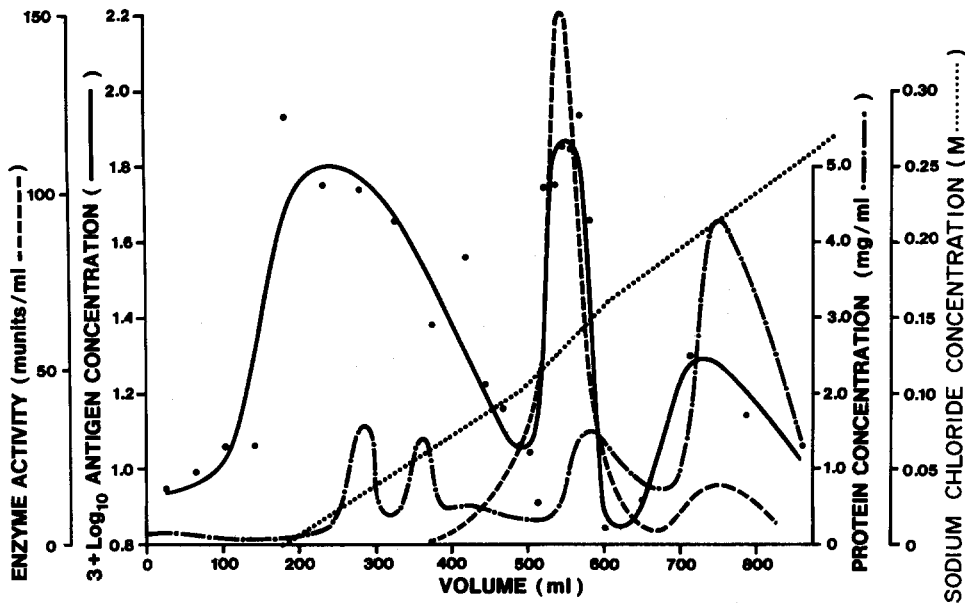


Fig. 2. Chromatography of salt-fractionated extract on DEAE-cellulose. Experimental points for antigen concentration as in Fig. 1. The preliminary washing of the column with starting buffer has been omitted from the diagram.

leading edge in this chromatography and the co-purification of an esterase activity.

A typical purification will be described. The supernatant from the extraction of 100 g larvae, prepared as before, was fractionated between 40% and 55% saturation ammonium sulphate at pH 7.5. The precipitate was dissolved in approx 40 ml of 0.05 M phosphate buffer pH 7.5 and dialyzed overnight against two changes of a 20-fold excess of 0.05 M Tris buffer pH 8.7. Approximately half of the protein precipitated during dialysis. After centrifugation, the supernatant was loaded on a 2.5 × 27 cm column of DEAE-cellulose equilibrated in the same Tris buffer. After washing with 230 ml of buffer, the protein concentration of the eluate fell to 0.1 mg/ml and a 1 l

linear gradient 0–0.3 M in sodium chloride was applied. The results in Fig. 2 show that one of the two major antigenic components co-chromatographed with the esterase activity. This material was pooled and brought to 60% ammonium sulphate saturation at pH 8.2, centrifuged and the precipitate dissolved in, and dialyzed for 3 hr against, 0.1 M phosphate buffer pH 7.5. The product, in 2.7 ml solution, was then chromatographed on a 2.6 × 62 cm column of Sephadex G-200 in phosphate buffer. The result, Fig. 3, shows that the antigen and esterase activities also co-chromatograph on the Sephadex column. The active material was finally concentrated on an Amicon UM-10 membrane. The purification procedure is summarized in Table 1.

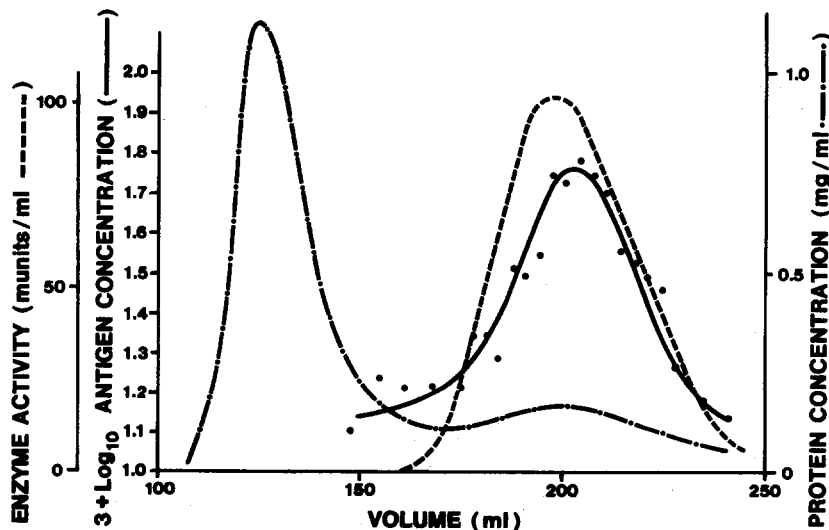


Fig. 3. Chromatography of the product of ion-exchange chromatography on G-200. Experimental points for antigen concentration as in Fig. 1.

Table 1. Purification of larval antigen and esterase activities

Fraction	Total protein	Sp. act esterase $\times 10$	Esterase (% yield)	Purification factor of esterase	Antigen (% yield)	Purification factor of antigen
Extract	7970mg	0.036	100	1	100	1
40–55% Satd. (NH ₄) ₂ SO ₄ , before dialysis	1900	0.077	52	2.1	48	2
40–55% Satd. (NH ₄) ₂ SO ₄ , after dialysis	960	0.15	52	4.2	54	5
DEAE chromatography product	41	0.85	12	23.9	4	9
Sephadex chromatography	4.1	6.18	9	173	2	40

Starch gel electrophoresis of the product of this preparation was carried out using two buffers—a 0.05 M Tris buffer pH 8.60 and an 0.02 M HEPES buffer pH 7.31. After electrophoresis and elution, the samples were tested for antigenic and esterase activity. The results are shown in Fig. 4 where, for ease of comparison, the antigen concentrations are shown on a linear scale and not a logarithmic one as in previous diagrams. In this experiment, antigen concentrations were standardized using dilutions of the material applied to the gel.

The purified antigen produced a dermal reaction only in animals which had been exposed to ticks. Injection of 100 times as much as the maximum amount

normally used in antigen quantitation into an unexposed animal produced no significant swelling.

Determination of apparent mol. wt of the esterase

The apparent mol. wt of the esterase was determined from its elution volume in gel filtration on Sephadex G-200, under the conditions used in the final step of the purification. Ovalbumin, bovine serum albumin, rabbit muscle lactate dehydrogenase and yeast alcohol dehydrogenase were used as standards with the mol. wts assigned to them by Andrews (1970). The value thus obtained for the esterase was 60,000. The chromatographic behaviour of the enzyme was not significantly dependent on its concen-

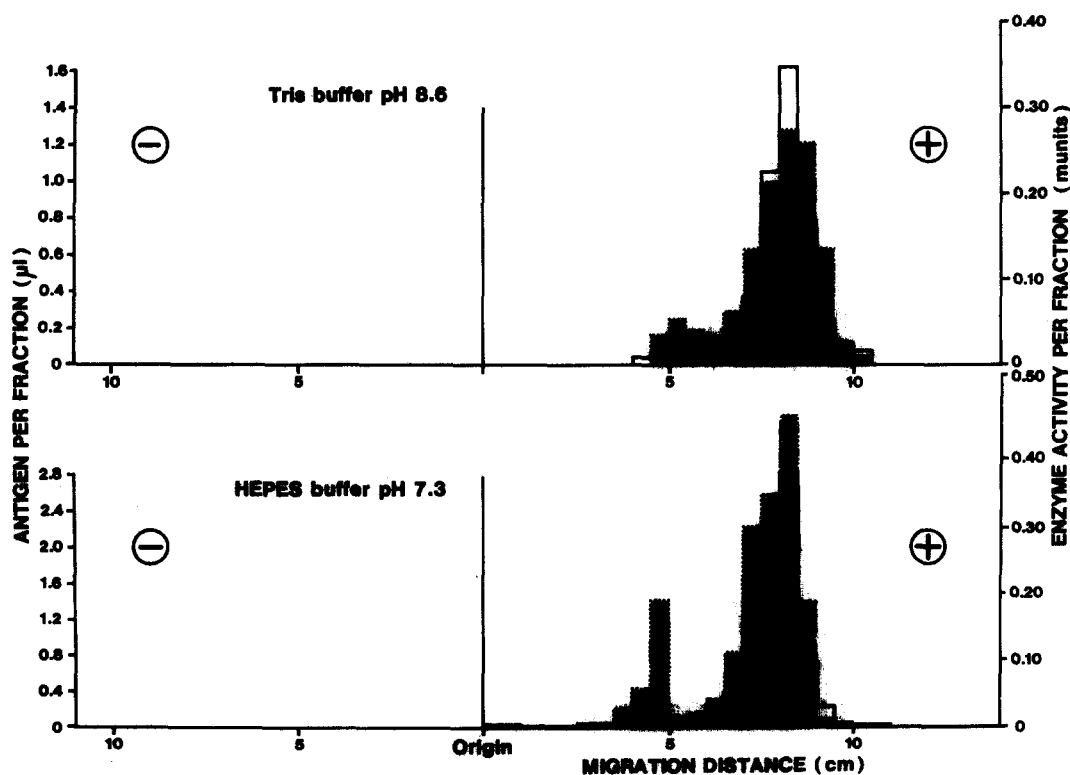


Fig. 4. Electrophoresis of purified antigen on starch gel. The shaded area shows the esterase activity, the thick line the antigen activity.

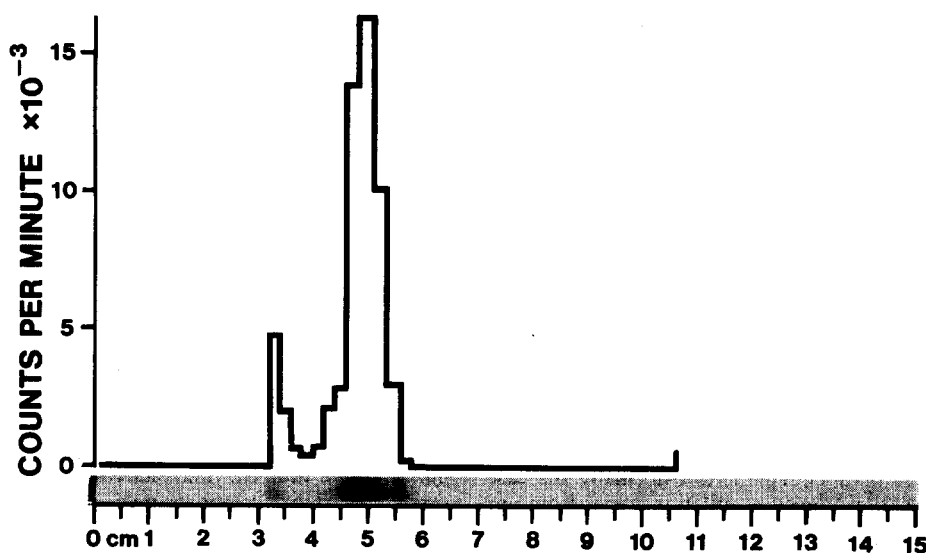


Fig. 5. Electrophoresis of tritium-labelled esterase. The gel is stained for protein and the bars show distribution of tritium.

tration, within the range of concentrations used. During the final step of the purification, where a total of 4 mg esterase was present in the sample loaded, the elution volume was 1.90 void volumes. Rechromatography of 20 μ g gave a ratio of 1.91.

Inhibition of esterase activity

Despite the fact that mercaptoethanol was necessary for the stability of the esterase, particularly of purified material, the esterase was not sensitive to the normal sulphydryl reagents. Incubation at 37°C in a Tris buffer pH 8.6 with 0.7 mM iodoacetamide for 1 hr, 2.5 mM iodoacetic acid for 40 min or 0.9 mM *p*-mercuribenzoate for 30 min failed to cause any inhibition. For these experiments, although the EDTA concentration was maintained at 0.5 mM, the concentration of mercaptoethanol was 0.1 mM or less, so as not to interfere with possible enzyme inhibition.

However, the esterase was completely and irreversibly inhibited by DFP in a very rapid reaction. The effect of DFP on esterase activity was measured by adding an aliquot of DFP in ethanol to a solution of enzyme in 0.05 M phosphate buffer at 37°C, then withdrawing 200 μ l samples at 10–15 sec intervals and adding these directly to an assay mixture, the assay reaction being stopped after 10 min. For a second order inhibition reaction, this dilution will result in a decrease in the reaction rate of more than 250-fold and hence the duration of the inhibition was taken as the time up to dilution of the 200 μ l samples. The inhibition was difficult to follow accurately because of the low levels of activity which had to be used. At the lowest concentration of DFP, 1.8×10^{-8} M and with an enzyme concentration of 1.9 μ g/ml, the loss of activity still appeared to be first order for almost two half-lives. Further dilution of the DFP led to incomplete inhibition, presumably because the concentration of enzyme became greater than that of the inhibitor. Using DFP concentrations in the range 1.8 – 5.2×10^{-8} M, it was found that the reaction was first order in DFP concentration, giving an ap-

proximate second order rate constant for the reaction of 1.7×10^7 M⁻¹ min⁻¹ (range 1.5 – 2.2×10^7).

Antigenic activity of DFP-inhibited esterase

Inhibited esterase was prepared by reacting duplicate samples of the enzyme in phosphate buffer with 0.11 mM DFP at 37°C for 10 min. After this time, there was no residual enzymic activity. Overnight dialysis was carried out to remove DFP, without any activity being recovered in the inhibited esterase. A control sample which was treated identically except for the addition of DFP remained fully active. Tests for antigenic activity showed no measurable difference between inhibited and uninhibited esterase samples.

Electrophoresis of tritium-labelled esterase

Purified antigen, 1.8×10^{-2} units, in 0.8 ml 0.10 M phosphate buffer pH 7.5 was mixed with 10 μ l of [³H]DFP at 37°C. After 4 min, the enzyme was more than 99% inhibited. After 7.5 min, the solution was dialysed against cold buffer. Overnight dialysis giving a total dilution factor of more than 10^4 was carried out without recovery of enzyme activity. A 100 μ l aliquot of the product was run on polyacrylamide gel electrophoresis, stained for protein and then sectioned for counting of radioactivity. The correlation between protein bands and the radioactive label is shown in Fig. 5. Four bands of protein are visible. The two bands between 4.4 and 5.2 cm were barely separated and contain almost all of both the protein and tritium label. They are presumably very similar forms of the same enzyme. Two minor protein components are visible. One, at 3.2 cm, is also tritium labelled and can reasonably be attributed to the small amount of slowly-migrating esterase activity observed on starch gel electrophoresis (Fig. 4). This component was not an antigen. It is uncertain from Fig. 5 whether or not the fourth component, at 5.5 cm, is radioactive or not.

DISCUSSION

The initial objective of this work was to purify, from tick larvae, components which were potentially relevant to the immunologically-based resistance of cattle to the tick. The isolation and characterization of antigenic material which has been described depends on the accuracy with which antigen concentrations can be determined from the immediate hypersensitivity reaction. This accuracy is limited by two factors. Firstly, any single estimate may be in error by a factor of two. That is, the deviation of points from the regression line obtained in standardizing an antigen-containing solution is commonly 0.3 log units on the concentration scale. Secondly, although it is necessary to estimate the antigen content of different fractions during a purification, this can only be done rigorously if the dose response lines for all antigen fractions are parallel. This is not normally the case, but the deviation from this situation varies from animal to animal. The results which have been given for antigen purification were obtained with animals in which all fractions did give parallel lines, at least within experimental error. The results thus give a semi-quantitative idea of the progress of purification, but do not imply that identical figures would be obtained in all animals.

Despite the limitations of the skin test, it has been shown that one of the major antigens giving an immediate hypersensitivity reaction in exposed animals can be successfully purified. At the maximum concentration used in skin testing, there was no detectable delayed reaction. Moreover, tests for a reaction to the antigen with unexposed cattle were negative, eliminating the possibility that the material isolated was a toxic or pharmacologically active agent.

However, the most interesting conclusion from the work reported here is that there is evidence that the antigen isolated is a hydrolytic enzyme. This is suggested by the fact that the two activities co-chromatograph on both DEAE-cellulose and Sephadex G-200. It is strongly supported by the fact that in the purified product, the two activities are not separated by electrophoresis on starch gel in two buffer systems. As an additional similarity, mercaptoethanol was found to be necessary for the purification and electrophoresis of both the esterase and antigen. In the absence of mercaptoethanol, very little esterase was recovered after electrophoresis and the only antigenic activity detectable was concentrated at the origin and trailed a short distance towards the anode. It would be desirable as well to have some idea of the purity of final product in terms of presence or absence of other protein species. This was the purpose of the electrophoresis on polyacrylamide gel. Esterase activity could not be visualized directly since we have so far been unable to stain a gel using fluorescein dibutyrate as substrate. The result was obtained indirectly, after it had been shown that the esterase was very sensitive to inhibition by DFP, by using tritiated DFP to prepare a radioactively-labelled enzyme and correlating protein and radioactive bands. The result shown in Fig. 5 demonstrates that the DFP-sensitive esterase is the major protein component present. Thus, if the antigen is not the esterase, it must either co-electrophorese again in this, the third electrophoretic system, or be a relatively minor protein com-

ponent. The possibility that the esterase and antigen are different proteins with very similar physico-chemical characteristics cannot be eliminated. However, it seems reasonable on the accumulated evidence to suggest that they are the same material.

There are two pieces of evidence that the dermal reactions observed are due to antigenic activity and not just to the enzymic activity of the protein. Firstly, as mentioned before, such reactions do not occur in animals which have not been exposed to the cattle tick. Secondly, if the enzyme is inhibited by DFP, the inhibited material is still antigenic. This second observation does not, however, comment on the identity or non-identity of the esterase and antigen proteins. Inhibition of an enzyme by DFP should cause very little alteration of the tertiary structure. As well, antibodies to enzymes frequently cause only partial inhibition of the enzymic activity, particularly for small substrates, demonstrating that antigenic and enzyme active sites are separate parts of the molecule. These two facts together mean that an antigenic site on the esterase protein would probably be unaffected by inhibition of the enzyme.

The rapid, irreversible inhibition by DFP is evidence that a serine residue is essential for the activity of the enzyme (Vallee & Riordan, 1969). The natural substrate of the esterase is unknown. Fluorescein dibutyrate has been used for the assay of lipase, chymotrypsin and acylase (Guilbault & Kramer, 1964) and so nothing can be said about the nature of the enzyme from this non-specific substrate. The excretion of a variety of hydrolytic enzymes by larvae into the host has been demonstrated histochemically (Schleger & Lincoln, 1975) and it is likely that one of these enzymes is the antigenically active material which has been isolated. Here again, however, the histochemical reactions have been carried out with substrates which could be non-specifically hydrolyzed by a variety of enzymes. For this reason, the enzyme has been referred to throughout this paper as an esterase, but it is not implied that its function is to hydrolyse simple aliphatic esters. This is the second time that a material which naturally produces an immediate hypersensitivity reaction of clinical importance has been identified with a hydrolytic enzyme. Sobotka *et al.* (1974) have reported that phospholipase A is the major allergen of honeybee venom.

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