The Purification of the Enzyme Hydrolysing Diethyl *p*-Nitrophenyl Phosphate (Paraoxon) in Sheep Serum

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Aldridge (1953*b*) reported an enzyme in mammalian sera which hydrolysed the acetylcholinesterase inhibitor, diethyl *p*-nitrophenyl phosphate (paraoxon) to diethylphosphoric acid and *p*-nitrophenol. No serious attempts have previously been made to purify this enzyme either from serum or from other tissues. Aldridge (1953*b*) fractionated rabbit serum with ammonium sulphate, but none of the fractions showed significant purification. In addition he showed that the Cohn *et al.* (1950) method 10 precipitated the enzyme in the (I+II+III) fraction. The yield was 85% and purification 2·8-fold.

An enzyme hydrolysing a related substrate, di*iso*propylphosphorofluoridate, was purified 13-fold from rabbit kidney by Mazur (1946). Using a similar procedure, Mounter, Floyd & Chanutin (1953) purified hog-kidney di*iso*propyl phosphorofluoridatase 65–100 times. The most pure (A-2) fraction was shown by Cohen & Warringa (1957) to contain two and possibly three additional enzymes. Bergmann, Segal & Rimon (1957) have reported a 'C'-esterase from the A-2 fraction. 'C'-Esterase hydrolysed *p*-nitrophenyl acetate under essentially the same conditions as A-esterase, as defined by Aldridge (1953*a*). Aldridge had concluded that the enzyme hydrolysing paraoxon in serum was identical with A-esterase, which hydrolysed *p*-nitrophenyl acetate and was not inhibited by paraoxon. However, since an enzyme exists which does not hydrolyse paraoxon but does hydrolyse *p*-nitrophenyl acetate under the conditions defining an A-esterase, this identity may not hold in all cases. Evidence supporting the view that more than one paraoxon-resistant esterase hydrolyses *p*-nitrophenyl acetate in serum will be presented in a later paper dealing with the properties of sheep-serum paraoxonase. The activity hydrolysing paraoxon has been called paraoxonase to distinguish it from other A-esterases.

The relative contributions of these enzymes towards the hydrolysis of various substrates is difficult to assess in impure tissue homogenates. The situation is made more complex by the variable effects of metal ions with different substrates and tissues (Mounter & Chanutin, 1953, 1954) and by the possible presence of naturally occurring activators and inhibitors (Cohen & Warringa, 1957). The use of partially purified preparations has also given rise to uncertainties (Cohen & Warringa, 1957). The purpose of the present work was therefore to obtain a homogeneous preparation both to differentiate paraoxonase from enzymes with similar substrate specificities and, if possible, to gain some knowledge about the chemical nature and possible physiological function of this enzyme.

Since paraoxonase was a relatively labile enzyme, a purification procedure with mild fractionating conditions was desirable. The Cohn low-temperature ethanol-fractionation approach was satisfactory in this respect, but the procedures were not followed in detail. In the present work conditions were determined which simplified the Cohn *et al.* (1946) method 6 in the initial stages and at the same time increased both the yield and purification. In addition, it was desired to keep the dilution of the serum to a minimum to utilize the capacity of the refrigerated equipment to best advantage. In the later stages an independent study of the effect of ethanol, ionic strength and pH at low temperatures proved necessary.

EXPERIMENTAL

Colorimetric determination of paraoxonase activity. *p*-Nitrophenol in the paraoxon was removed by repeatedly shaking a 20% solution in $CHCl_3$ with a 1% $NaHCO_3$ soln. according to the method of Aldridge (1953*b*). A stock solution (0·8 M) was prepared by diluting 11·008 g. of *p*-nitrophenol-free paraoxon to 50 ml. with absolute methanol.

A buffered paraoxon substrate (8 mM) solution was prepared by blowing 1 ml. of 0·8 M-paraoxon stock solution into 100 ml. of M/15-sodium phosphate buffer, pH 7·6, containing 0·1% of gum acacia and by shaking vigorously. This reagent could be used for several days if stored in the cold.

A sample of buffered substrate (5 ml.) was pipetted into each of three or more 10 ml. volumetric flasks. If required, one flask was used for a reagent blank. The second served to correct for non-enzymic hydrolysis and the third flask was used to measure the *p*-nitrophenol liberated during incubation. After the solution in the second and third flasks had reached temperature equilibrium (37°), 0·02 ml. of the

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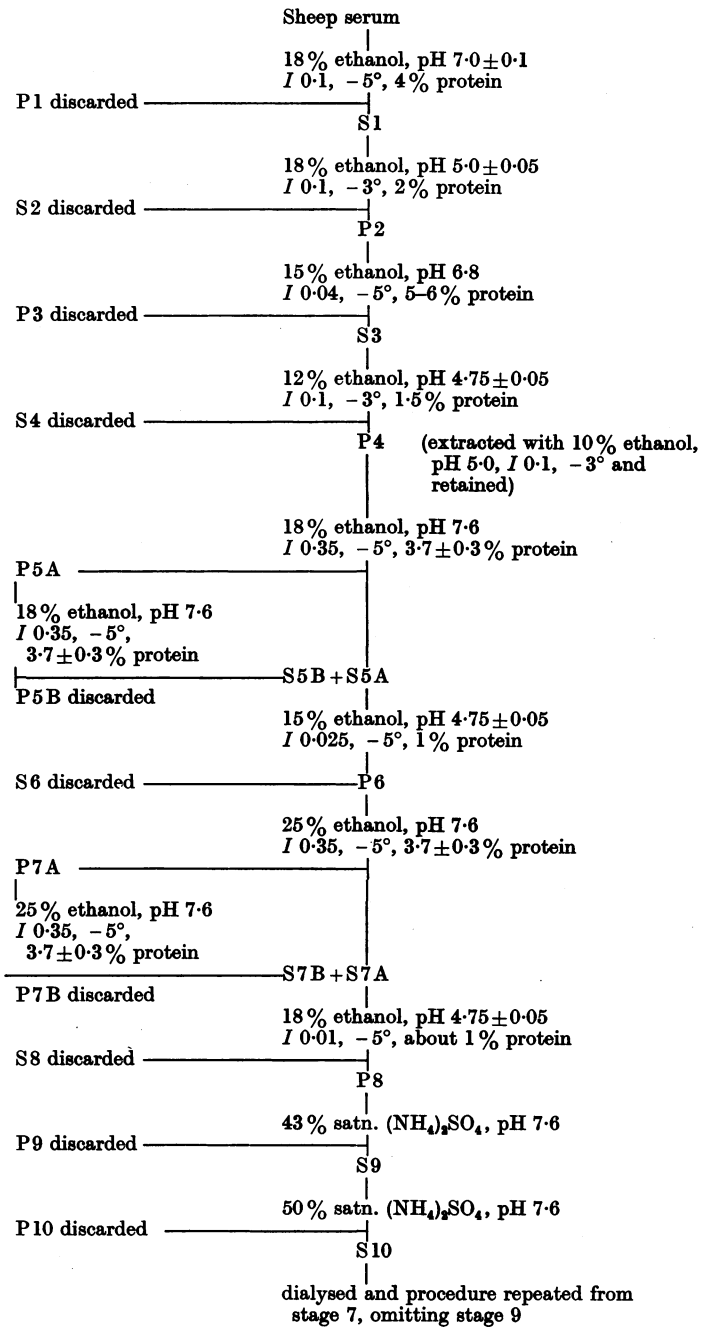
enzyme preparation was added to the third flask and the time was noted. After incubation for a suitable length of time, as judged visually by the colour in the flask, the third flask was removed from the bath and immediately made to volume with ethanol reagent (400 ml. of 95% ethanol and 6·25 ml. of aq. 2N-NH_3 soln., diluted to 500 ml. with water) to terminate enzymic hydrolysis and to bring the pH to 9·2. The time was again noted.

The pH was adjusted to 9·2 to prevent small pH fluctuations from affecting the extinction and to maintain the stability of paraoxon. Spontaneous paraoxon hydrolysis became rapid at pH 10·3, but was negligible in the region of

Flow scheme 1. Purification of serum paraoxonase. S, Supernatant; P, precipitate.



pH 9·2. The 400 mμ light absorption by *p*-nitrophenol increased from pH 6·0 to 8·5, but then became almost constant at higher pH.

The extinction was read at 400 mμ in cuvettes with a 1 cm. light path at any convenient time after the addition of ethanol reagent. The precision of the method was ±2·5%, with Sahli pipettes 'calibrated to contain' 0·02 ml. This volume of most enzyme preparations liberated a convenient amount of *p*-nitrophenol in 1–30 min. and thus eliminated the need for more than one dilution.

The *p*-nitrophenol calibration curve was made in the presence of 40% ethanol, since ethanol significantly altered the light absorption.

Manometric determination of paraoxonase. A variation of the manometric technique described by Aldridge (1953*a*) was also used to determine the activity against paraoxon and *p*-nitrophenyl acetate. Generally, 0·5 ml. of enzyme preparation was placed in the side arm of the manometer flask and 2·5 ml. of buffered substrate in the centre well. The final concentrations of the components in the digestion medium, exclusive of substrate and enzyme, were 25 mM-$NaHCO_3$, 0·162 M-NaCl and 0·2% of gelatin. The activities were determined at 37° after gassing for about 15 min. with CO_2+N_2 (5:95) at room temperature.

Determination of protein. Routine protein determinations were made by measuring the light absorption at 280 mμ with a quartz u.v. spectrophotometer. In general a dilution of 0·02 ml. of sample in 4 ml. of 50 mM-$NaHCO_3$ soln. gave a convenient reading. The biuret method of Robinson & Hogden (1940) was also used to determine protein. Both methods were calibrated against the Kjeldahl method of Chibnall, Rees & Williams (1943). The spectrophotometric method at 280 mμ could be used for the earlier fractions from the procedure, but gave readings which were two to three times that of the Kjeldahl and biuret methods with the fractions including and following S7 (see flow scheme 1). The protein in these samples was routinely determined by the biuret method.

Phosphorus determination. Phosphorus was determined by the amidol method of Allen (1940).

Enzyme purification. Each stage of the purification procedure was associated with definite final precipitating conditions as given in the flow scheme. These conditions were determined by investigating the effect on the yield and purification of such variables as pH, alcohol concentration, ionic strength, time of standing after precipitation and rate and order of addition of reagents. Three examples illustrating the scope and nature of these investigations are given below. These examples also indicate the permissible limits of the specified variable necessary to obtain satisfactory purification and yields.

Sheep serum was selected as a source of paraoxonase since it was reasonably rich in enzyme and was readily available.

Stage 1. Defibrinated sheep blood (13 l.) was obtained from an abattoir on the day of slaughter and was used immediately. The fresh blood was centrifuged at 2000 *g* for 45 min. at room temperature. The serum was removed by suction and the volume measured. A volume convenient to the apparatus available was poured into a stainless-steel bucket and cooled in a bath at −5° until freezing just began. The remaining serum was stored at 0°.

To each litre of serum was added 500 ml. of reagent 1 (567 ml. of 95% ethanol and 17 ml. of 2 M-acetic acid mixed and diluted to 1 l. at room temperature) at the rate of 25 ml./min., with vigorous mechanical stirring. After standing overnight the mixture was centrifuged at 1800 *g* for 45 min. at −6°. The supernatant S1 was retained and the precipitate P1 was discarded. The supernatant volume was about 1380 ml./l. of serum.

Stage 2. S1 was adjusted to −3° and 25 ml. of 2 N-acetic acid/l. of serum was added, at the rate of 1·5 ml./min., with vigorous stirring. The pH was determined and, if necessary, adjusted to 5·0±0·05. After standing for at least 12 hr. the solution was centrifuged at 1500 *g* for 30 min. at −3°. The supernatant S2 was discarded. The precipitate P2 was dissolved in 33 ml. of 0·1 M-$NaHCO_3$ soln./l. of serum and transferred with sufficient rinse water to make the final volume 125 ml./l. of serum.

The permissible limits of pH variation to obtain the highest yield and purification are shown in Fig. 1. In this example the limits were narrow, requiring careful pH control.

Table 1 illustrates the effect of standing time.

Stage 3. The pH of the dissolved precipitate P2 was adjusted to 6·8 with 2 N-acetic acid or aq. NH_3 soln. and cooled in a bath at −5° until ice crystals just appeared. Pre-cooled 95% ethanol (23 ml./125 ml. of P2) was added slowly with stirring. After standing overnight the solution

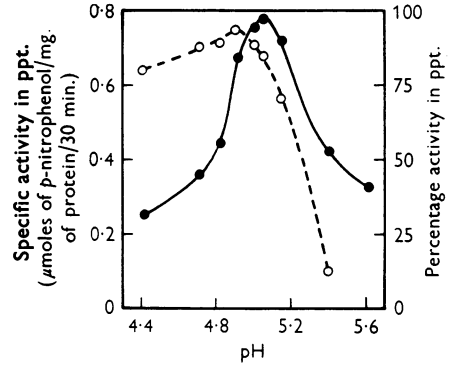


Fig. 1. Stage 2, pH fractionation. Effect of pH on the specific activity and yield of paraoxonase in the precipitate. ●, Specific activity; ○, percentage yield. Starting material S1 of specific activity 0·18 mg. of *p*-nitrophenol liberated/mg. of protein/30 min. Precipitating conditions: 18% (v/v) of ethanol; *I* 0·10; −3°; standing time for precipitate formation, 15 hr.

Table 1. *Rate of precipitation of paraoxonase from S1*

Starting material: S1; pH 5·0, *I* 0·1; ethanol 18%; temperature −3°.

Time of standing (hr.)	Activity in precipitate (%)	Activity in supernatant (%)
1	39	57
3	71	26
6	77	16
12	85	11
16	87	7

was centrifuged at 2000 *g* for 60 min. at −5°. The precipitate P3 was discarded. The supernatant S3, which was usually cloudy, was retained.

Stage 4. To each 100 ml. of S3 at −3° to −5° was added 8 ml. of 2M-NaCl solution and 87 ml. of water at room temperature. The diffuse precipitate cleared. The solution was placed in a cold bath at −3°.

A sample of convenient volume, usually 25 ml., was removed and titrated to pH 4·75 with 2N-acetic acid. Immediately after titration the sample was returned to the solution. The total volume of acid required to adjust the pH of S3 to 4·75 was calculated and was added with vigorous mechanical stirring at the rate of 2 ml./min. (about 2 ml. of acid/100 ml. of S3 was necessary).

To each initial 100 ml. of S3, now at pH 4·75, was added 9·5 ml. of pre-cooled 95% ethanol at the rate of 10 ml./min. After standing for 3 hr. at −3°, the solution was centrifuged at 1500 *g* for 30 min. at −3°. To collect the precipitate centrifuging was done with one 500 ml. centrifuge bottle and as many runs as necessary. The supernatant S4 was discarded.

The precipitate P4 was extracted at −3° with about 25 ml. of reagent 2 (105 ml. of 95% ethanol, 50 ml. of 2M-sodium acetate and 21 ml. of 2M-acetic acid, mixed and diluted to 1 l. pH 5·0)/100 ml. of S3. The extracting solution was stirred mechanically with the precipitate for about 30 min. at −3° and the mixture was centrifuged at 1500 *g* for 1 hr. at −3°. The supernatant was discarded.

The precipitate P4 was dissolved in 5 ml. of 0·1M-$NaHCO_3$ soln./100 ml. of S3 and rinsed into a measuring cylinder with an equal volume of water. Solubilization was aided by the use of a Potter homogenizer. The determination of the optimum ethanol concentration for the highest yield and purification in stage 4 is shown in Fig. 2.

Stage 5. The volume of the dissolved P4 was adjusted with water to 25 ml./l. of starting serum. A few drops of aq. 2N-NH_3 soln were added to adjust the pH to 7·6. To every 100 ml. of dissolved P4, 28 ml. of 2M-sodium acetate was added and the solution was warmed for 10 min. at 35°. After cooling the solution to 0°, 30 ml. of pre-cooled 95% ethanol/100 ml. of dissolved P4 was added slowly with stirring. During addition, the temperature was lowered to −5°. After standing overnight, the solution was centrifuged at 15 000 *g* for 60 min. at −5°.

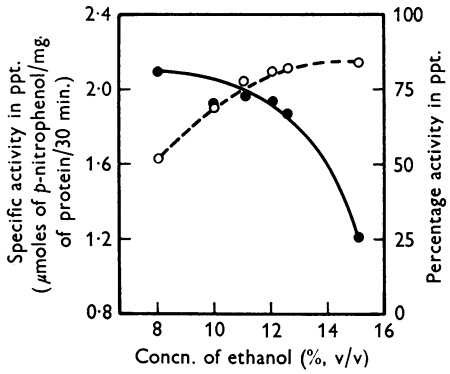


Fig. 2. Stage 4, ethanol fractionation. Effect of ethanol on the yield and purification of paraoxonase in the precipitate. ●, Specific activity; ○, percentage yield. Starting material, S3 of specific activity 0·91. Precipitating conditions: pH 4·75±0·05; *I* 0·10; temperature −5°; standing time for precipitate formation, 3 hr.

The supernatant, S5A, was dialysed overnight against 35 times its volume of cold mM-$NaHCO_3$ soln. and was then stored in the cold until needed. The precipitate P5A was dissolved in 12·5 ml. of water/l. of starting serum with the aid of a Potter homogenizer. The volume was adjusted to 20 ml./l. of serum. To every 100 ml. of dissolved P5A, 20 ml. of 2M-sodium acetate soln. was added. The solution was warmed at 35° for 10 min. After cooling the solution to 0°, 24 ml. of pre-cooled 95% ethanol was added slowly with stirring. During addition the temperature was lowered to −5°. After standing for 4 hr. the solution was centrifuged at 15 000 *g* for 60 min. at −5°. The precipitate P5B was discarded.

The supernatant S5B was dialysed overnight against 35 times its volume of cold mM-$NaHCO_3$ soln. The dialysed S5B was combined with the previous diffusate, S5A.

Stage 6. The volume of the combined diffusate, S5A + S5B, was measured. A 25 ml. sample was removed and titrated to pH 4·75 with 2N-acetic acid. The volume of acid required to adjust the pH of the diffusate to 4·75 was calculated. Usually about 1·4 ml. of acid/100 ml. of diffusate was necessary.

The diffusate was cooled to 0°, and 19 ml. of pre-cooled 95% ethanol and 1 ml. of 2M-NaCl soln./100 ml. of combined diffusate were added slowly with stirring. As the temperature of the solution was lowered to −5°, the volume of 2N-acetic acid calculated to bring the pH to 4·75 was added at the rate of 6 ml./min. After standing for 3 hr. the solution was centrifuged at 2000 *g* for 45 min. at −5°. The supernatant S6 was discarded.

The precipitate P6 was dissolved in 3 ml. of 0·1M-$NaHCO_3$ soln./100 ml. of combined diffusate. The dissolved P6 was transferred with a minimum of rinse water to a measuring cylinder.

Stage 7. The dissolved P6 was diluted with water to 12·5 ml./l. of starting serum and the pH was adjusted to 7·6 with a few drops of aq. NH_3 soln. Sodium acetate trihydrate (8 g./100 ml. of dissolved P6) was dissolved as the solution was warmed at 35° for 10 min. The solution was then cooled until it began to freeze and 33 ml. of 95% ethanol/100 ml. of P6 was added slowly, with stirring, as the temperature was lowered to −5°. After standing overnight the solution was centrifuged at 15 000 *g* for 60 min. at −5°.

The supernatant S7A was dialysed overnight against 50 times its volume of cold mM-$NaHCO_3$ soln. and then stored until needed. The precipitate P7A was dissolved in water and diluted to about 10 ml./l. of starting serum. To every 100 ml. of dissolved P7A was added 8 g. of sodium acetate trihydrate. The salt dissolved as the solution was warmed for 10 min. at 35°. The solution was cooled to 0°. As the temperature was lowered to −5°, 35 ml. of pre-cooled 95% ethanol/100 ml. of P7A was added slowly with stirring. After standing for 4 hr., the solution was centrifuged at 15 000 *g* for 60 min. at −5°. The precipitate P7B was discarded. The supernatant S7B was dialysed overnight against 50 times its volume of cold mM-$NaHCO_3$ soln. The diffusate S7B was combined with S7A.

Stage 8. The volume of the combined diffusates, S7A + S7B, was measured. A sample (25 ml.) was removed and

titrated to pH 4·75. The volume of acid required to adjust the pH of the diffusate to 4·75 was calculated. About 0·8 ml. of 2N-acetic acid/100 ml. of combined diffusate was usually required.

The diffusate was cooled to 0° and 24 ml. of pre-cooled 95% ethanol/100 ml. of combined diffusate was added slowly with stirring as the temperature was lowered to −5°. The calculated volume of 2N-acetic acid was then added at the rate of 3 ml./min. After standing for 1 hr., the solution was centrifuged at 12 000 *g* for 20 min. at −6°. The supernatant S8 was discarded. The precipitate P8 was dissolved in 1 ml. of 0·1M-$NaHCO_3$ soln./100 ml. of combined diffusate, and transferred with a minimum of rinsing water to a dialysis bag. The dissolved precipitate P8 was dialysed against 1 l. of cold mM-$NaHCO_3$ soln. for at least 6 hr.

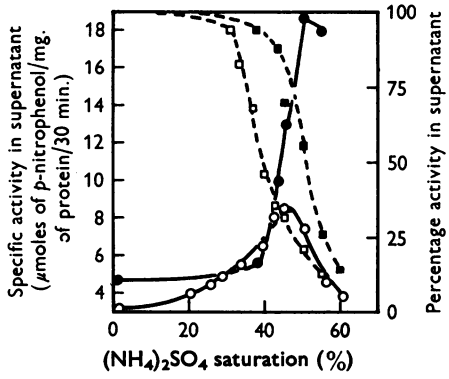


Fig. 3. Stages 9 and 10, ammonium sulphate fractionation. Effect of ammonium sulphate on the yield and purification of paraoxonase in the supernatant. ●, Specific activity, starting material, P6S; ○, specific activity, starting material, P4S; ■, percentage yield, starting material, P6S; □, percentage yield, starting material, P4S. Starting materials: P6S of specific activity 4·60 and P4S of specific activity 3·02 mg. of *p*-nitrophenol liberated/mg. of protein/30 min. Precipitating conditions: pH 7·6, adjusted with sodium phosphate buffer; 4°; standing time for precipitate formation, 1 hr.

Stage 9. The volume of dialysed P8 was measured and adjusted to 2–3 ml./l. of starting serum to keep the protein concentration at about 6%. Sufficient saturated $(NH_4)_2SO_4$ solution (7·5 ml./10 ml. of P8) was added with stirring at room temperature to make the solution 43% saturated. After standing for 4 hr. at about 4°, P8 was centrifuged at 15 000 *g* for 60 min. at 2°. The supernatant S9 was retained and the precipitate P9 was discarded (see Fig. 3).

Stage 10. The volume of S9 was measured and 1·5 ml. of saturated $(NH_4)_2SO_4$ soln./10 ml. of S9 was added to make the solution 50·5% saturated. After standing for 4 hr. at 4°, S9 was centrifuged at about 100 000 *g* for 30 min. at 4°. If a high-speed centrifuge was not available, centrifuging was done at 15 000 *g* for 2 hr. The precipitate P10, which was usually lighter than the supernatant S10, was discarded. When the volume of the precipitate was more than 20% of the total volume, the precipitate was washed with an equal volume of 50% saturated $(NH_4)_2SO_4$ soln. and recentrifuged. The supernatant of the wash was added to S10.

The supernatant S10 was retained and dialysed against cold mM-$NaHCO_3$ soln. for at least 12 hr. The dialysing solution was renewed once.

The term 'S10 repeated' in the text refers to a repetition of stage 10 on S10 after dialysis and re-concentration by stage 11.

Stage 11. After dialysis, S10 was treated as the combined diffusates, S7A+S7B, of stage 8. The supernatant S11 was discarded and the precipitate P11 was dissolved in 0·1M-$NaHCO_3$ soln. The solution was dialysed as in stage 8. If P11 was not clear or straw-coloured, stage 10 and stage 11 were repeated as noted above.

Efficiency of procedure. The results of each stage of the purification procedure are summarized in Table 2, which gives the results of run 31 for comparison with a composite run. Only those stages which resulted in significant purification are included.

The stages which are omitted served either to remove small amounts of interfering protein or to concentrate the enzyme. Of these, stage 3 precipitated 14·4±3·7% of the protein and 6·1±2·2% of the activity in P2. Only a small purification, of about 1·1 times, resulted in stage 3 but the purification obtained by stage 4 was much improved. Stages 6 and 8 concentrated the enzyme but did not result

Table 2. *Summary of the enzyme purification obtained in run* 31 *and in a composite run*

The composite run was constructed by averaging the results for each stage of a number of runs. Activities were determined by the colorimetric method. The specific activity is given in terms of μmoles of *p*-nitrophenol liberated/mg. of protein/30 min. at $V_{max.}$ and the absolute activity in terms of mg. of *p*-nitrophenol liberated/30 min. at $V_{max.}$.

	Run 31				Composite run			
Stage no.	Total activity	Specific activity	Yield (%)	Purification	No. of runs averaged	Specific activity	Yield (%)	Purification
Serum	11 000	0·093	100	1	16	0·095	100	1
1	9 650	0·166	88	1·8	9	0·176	86	1·85
2	9 140	0·78	83	8·4	15	0·76	81	8·0
4	7 060	2·81	64	30	8	2·70	63	27·5
5	5 470	5·25	50	58	9	4·75	48	50
7	3 780	11·8	34	127	5	10·8	32	114
10*	1 400	28·8	12·7	310	4	30·1	15	317

* Stages 9 and 10 in run 31 were repeated. The yield was lowered because of the incomplete removal of ethanol during dialysis.

in significant purification. The same precipitating conditions were used in stages 8 and 11 to concentrate the enzyme.

This procedure resulted in about 315-fold purification with a 15% yield up to stage 10. This material was usually slightly reddish but this colour could be removed by repeating stage 10. The purification then increased slightly to approx. 350±25 times and the yield decreased by about 20%.

The performance of the individual stages is given in Table 3.

RESULTS AND DISCUSSION

Electrophoresis. Samples were subjected to electrophoresis in the analytical cell of the Perkin–Elmer model of the Tiselius boundary-electrophoresis apparatus. The schlieren diagrams of the purest preparations from seven runs showed the presence of two components. The leading component (Fig. 4*a*) was present in a higher concentration than the rear one. The leading boundary of the material shown in Fig. 5 was permitted to pass out of the descending limb, leaving only material represented by the rear boundary. The cell components were then isolated and the contents analysed. The material in the descending limb contained no activity towards paraoxon. It was water-clear and showed no fluorescence, whereas the starting material had been slightly cloudy and highly fluorescent. The material in the descending limb was about 0·04% of protein in a volume of 2·2 ml. This represented about 0·9 mg. of protein and is approximately the amount expected, considering the relative areas under the two peaks, the percentage of the material which remained in the descending limb after electrophoresis for 150 min. and the initial protein concentration. Analysis of the material in the bottom limb and in the ascending limb showed a specific activity, recalculated to $V_{max.}$, of 23·6 and 27·0 μmoles of *p*-nitrophenol liberated/mg. of protein/30 min. respectively. This

Table 3. *Summary of the yield of enzyme and purification obtained for each stage from a number of large-scale preparations*

Stage no.	No. of runs averaged	Purification from stage	Yield on stage (%)
1	9	1·9±0·2	86±2
2	16	4·3±0·5	94±3
4	8	3·4±0·4	79±4
5	9	1·8±0·2	76±2
7	5	2·4±0·2	67±10
9, 10	4	2·8±0·2	47±9

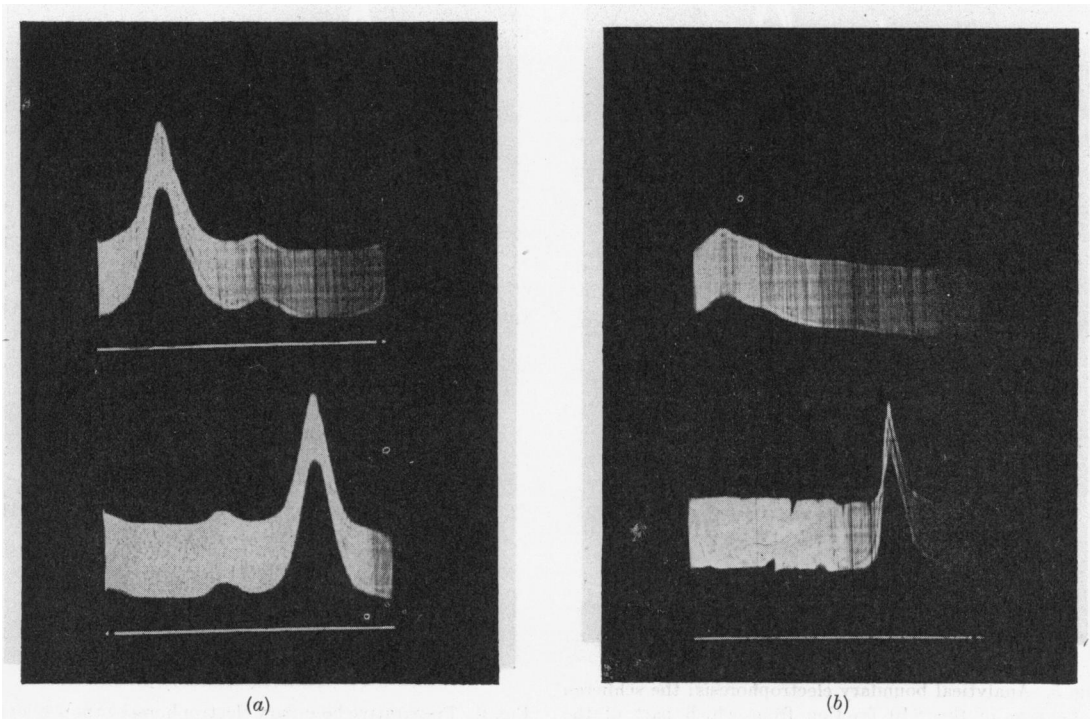


Fig. 4. Analytical boundary electrophoresis; schlieren diagrams of 365-fold purified product at two pH values. (*a*) pH 8·5; top, ascending limb; bottom, descending limb; 0·1 M-veronal buffer; 105 V, 5·5 mA; time, 120 min. (*b*) pH 5·4; descending limb, top 45 min., bottom 165 min.; 0·01 M-sodium acetate, 0·09 M-NaCl; 100 V, 10 mA.

represented a purification of 307 times in the ascending limb. The contents of these two limbs were highly fluorescent and slightly cloudy. It was concluded that the material represented by the leading boundary contained the activity.

A repeated S10 fraction was subjected to electrophoresis at pH 8·5 in veronal buffer and then at pH 5·4 in sodium acetate–acetic acid buffer supported with NaCl. At pH 8·5 the schlieren diagram of this preparation showed two peaks (Fig. 4*a*). The ratio of the area under the peaks suggested that the symmetrical leading peak was 95% pure. When the same preparation was subjected to electrophoresis at pH 5·4 only one asymmetric peak developed (Fig. 4*b*). This evidence suggested that this preparation consisted of only two components, the predominant one being paraoxonase.

Preparative boundary electrophoresis. Attempts were then made to separate the leading components by taking advantage of the different migration velocities in veronal buffer, pH 8·5. The large preparative cell with one closed electrode of the Tiselius apparatus (Perkin–Elmer model) was employed. The ascending limb communicated with the closed electrode, which had a gassing device permitting the boundary to be displaced backwards.

Preparative electrophoresis was successful with run 31. The bottom and descending limbs of the cell were filled first with a 1% protein solution in 0·05 M-veronal buffer, pH 8·5. The electrophoresis was run at 130 V and 7 mA and the progress of the boundaries was observed at intervals. The relative positions of the two boundaries just before the leading boundary disappeared from the ascending limb (135 min.) is shown in Fig. 6. Electrophoresis was continued for a total of 195 min., when the leading boundary disappeared entirely from the ascending limb. The boundaries were then displaced back with a gassing apparatus until the rear boundary had disappeared into the bottom limb (24 min.). The limbs were then isolated.

Electrophoresis of the material obtained from the ascending limb showed one component (Fig. 7). A small distortion appeared at the rear shoulder of the boundary in the descending limb, but since it did not occur in the ascending boundary or change

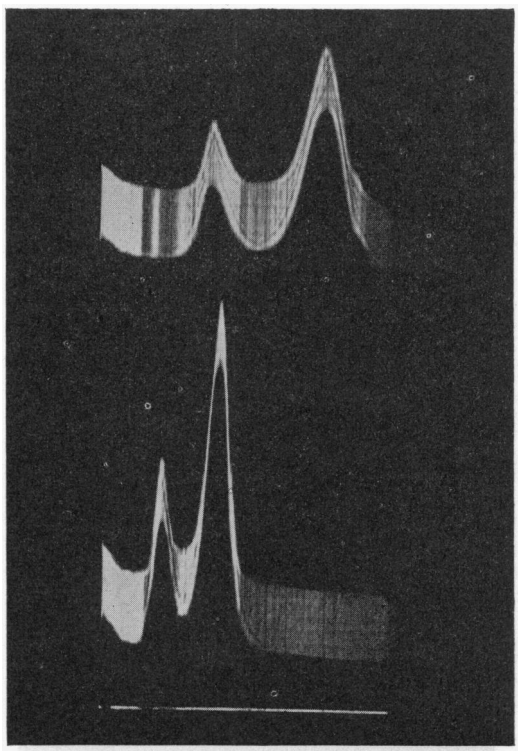


Fig. 5. Analytical boundary electrophoresis; the schlieren diagram of the S10 fraction from which part of the material represented by the rear boundary was separated and analysed. Descending limb; pH 8·2, 0·1 M-veronal buffer; 108 V, 5·5 mA; top, 120 min.; bottom, 58 min.

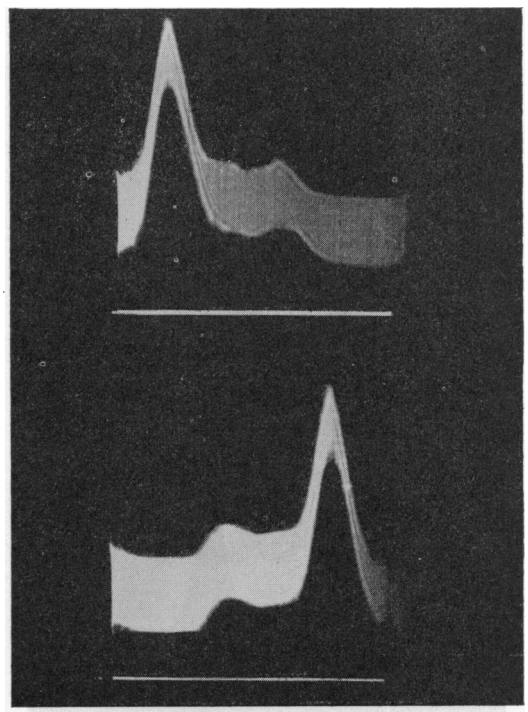


Fig. 6. Preparative boundary electrophoresis; the schlieren diagram of the S10 repeated fraction just before the main peak left the ascending limb. Top, ascending limb; bottom, descending limb.

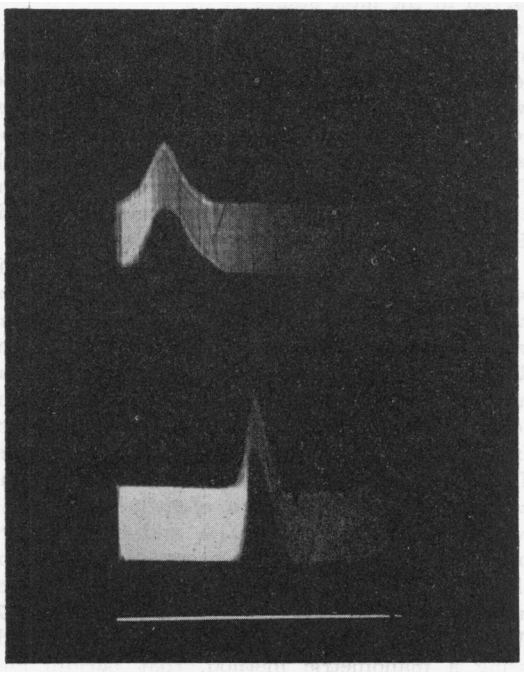


Fig. 7. Analytical boundary electrophoresis; the schlieren diagram of the component isolated by preparative electrophoresis. Ascending limb: top, 51 min.; bottom, 85 min.; pH 8·4; 0·05M-veronal buffer; 140v, 3·8 mA.

Table 4. *Specific activity of the material from run* 31 *in the limbs of the preparative electrophoresis cell*

The activities were determined after electrophoresis and boundary displacement. The specific activity is in terms of μmoles of *p*-nitrophenol liberated/mg. of protein/30 min. at $V_{max.}$. The paraoxonase activities are as percentages of the activity recovered finally.

Limb	4 mM-Paraoxon specific activity	Paraoxonase activity (%)
Ascending	4·76	36
Bottom	3·70	27
Descending	3·26	37

its position relative to the main peak during the electrophoresis, it is probable that it did not represent another component. It seemed justifiable to conclude that the material shown in Fig. 7 was electrophoretically pure paraoxonase with a mobility of $0{\cdot}88 \pm 0{\cdot}01 \times 10^{-4}$ cm.^{2}/v/sec.

Repeated electrophoresis resulted in a continuous loss of activity. In run 31 about 80 % of the activity was lost after the final electrophoresis. Part of the loss was caused by repeated electrophoresis due to gassing of the electrodes. Analysis

Table 5. *Paraoxon K_m of the fractions in run* 31

K_m was determined from colorimetric activity determinations, which were plotted according to the Dixon (1953) modification of the Lineweaver & Burk plot (1934).

Fraction	Purification	K_m (mM)
Serum	1	0·29
P2	84	0·38
P4	30	0·58
S5	56	1·0
S7	127	1·7
S10	310	4·4

of the contents of the three limbs confirmed that the activity was in the ascending limb (Table 4).

Paper electrophoresis. Various fractions from the purification procedure were examined by paper electrophoresis at pH 8·5 in 0·1M- and 0·05M-veronal buffer. The protein in fraction S9 resolved into three zones, with the activity in the leading zone. The papers were examined under ultraviolet light and the position of the fluorescence in the leading zone was marked. The papers were then dipped in paraoxon-buffered substrate and the position of the activity was marked by the appearance of yellow *p*-nitrophenol. The position of the protein was determined either with ninhydrin or Amido schwarz 10B dye (1-amino-8-hydroxy-3:6-disulphonic acid-2-diazo(*p*-nitrophenyl)-7-diazophenyl naphthalene). In every instance the position of the activity and fluorescence coincided with the protein in the leading zone. More purified fractions (S 10 etc.) tended to remain at the origin, but when the paper was overloaded a crescent-shaped zone moved down the paper. The coincidence of the fluorescence, activity and all the protein in these patterns was taken to confirm the essential homogeneity of these preparations.

The protein in the active zone tended to stain more lightly with Amido schwarz 10B dye and more brilliantly with ninhydrin than the protein in the other zones, suggesting a relatively small molecule.

Ultracentrifuging. On the basis of a single determination the sedimentation coefficient was 3·69s (uncorrected). This suggested a molecular weight of 35 000 to 50 000 according to Svedberg & Pedersen (1940). The schlieren diagram of the material used is shown in Fig. 4. The ultracentrifuge picture showed one symmetrical peak.

Increase in the paraoxon K_m. The K_m of successive fractions from the purification procedure progressively increased. The K_m doubled during the first 30-fold purification and increased seven times with the final ten-fold purification (Table 5).

The increased K_m suggested the presence of an activator in sheep serum which was lost during purification. Repeated dialysis of serum and other

fractions did not result in a loss of activity, suggesting that the activator, if present, was a relatively large molecule.

The K_m did not increase significantly beyond 4·4 mM in S10 despite further purification, suggesting that the activator had been removed by this stage (Table 6). Partial irreversible deactivation of an electrophoretically homogeneous sample (Fig. 4) did not alter the K_m.

If the increase in the K_m of retained fractions reflected the removal of an activator from the bulk of the enzyme, then the K_m of the discarded fractions containing the activator would be significantly lower than the K_m of either the retained fraction or the starting material. The K_m of the retained fraction S7 and the starting material P6 was 0·17 mM and 0·10 mM respectively, whereas that of the discarded fraction P7 was 0·72 mM. Similar results were found for the fractions of stage 5. These results also suggest that the activation was reversible.

Recovery of activity. The recovery of a stage was the sum of the activity in the precipitate and supernatant, expressed as the percentage of the activity in the starting material. Low recoveries were observed in the later stages of the purification procedure when the activity determinations were made with 4 mM-paraoxon. Recalculation of the recovery with the maximum velocity associated with the K_m of each fraction increased the recovery, suggesting that the apparent loss could be largely accounted for by the change in K_m (Table 7). The overall recovery to stage 10 increased from an apparent 1060 units to 2260. The calculated purification increased by a comparable ratio.

Inhibition and inactivation. Both ethylenediaminetetra-acetic acid (EDTA) and barium inhibited paraoxonase, as shown in Table 8. The inhibitions were reversible as judged by dilution, dialysis, the effect of time on the progress of inhibition and, with barium, by the addition of sodium sulphate to precipitate the barium. Inhibition by barium and EDTA was additive. Thus 0·132 mM-EDTA alone inhibited 33 % of the activity and 0·18 mM-barium alone inhibited 35 % of the activity. When EDTA and barium were present together at these concentrations, 67 % of the activity was inhibited. The pI_{50} of barium inhibition was 3·2 and of EDTA inhibition 3·7. Augustinsson & Heimburger (1955) observed, using a manometric method, that barium and strontium activated serum tabunase but inhibited kidney tabunase. Barium inhibition of paraoxonase was stronger than the inhibition of tabunase. However, in the present work it has been observed that the apparent barium inhibition depends on the method of analysis. The manometric method gave lower results than the colorimetric method of analysis. The reason for this may be that in the manometric method most of the barium in excess of 0·1 mM was precipitated as barium bicarbonate.

The pH stability of paraoxonase was examined over a range of conditions particularly related to purification. In sheep serum, paraoxonase retained its full activity between pH 5·0 and 9·5 for at least 30 min. at room temperature. The stability decreased on the acid side as the enzyme became more

Table 6. *Paraoxon K_m of highly purified fractions from various runs*

K_m was determined from colorimetric activity determinations, which were plotted according to the Dixon (1953) modification of the Lineweaver & Burk plot (1934).

Run no.	Sample	Purification	K_m (mM)
31	Electrophoresis product	400*	4·5
31	Electrophoresis starting material (S10 repeated)	328	3·1
31	S10	310	4·4
30	S10	264	3·3
29	Electrophoresis product	385	4·2†
26	S10	340	4·5

* Estimated; the sample was partially inactivated during electrophoresis.
† K_m was determined manometrically.

Table 7. *A comparison of activity recoveries calculated from two reaction velocities*

Activity recoveries were calculated from the reaction velocity at 4 mM-paraoxon and from the $V_{max.}$; activity is expressed as mg. of *p*-nitrophenol liberated/30 min.

		4 mM Velocity			Maximum velocity		
Stage no.	Starting fraction	Starting activity	Activity in supernatant + ppt.	Activity recovered (%)	Starting activity	Activity in supernatant + ppt.	Activity recovered (%)
1	Serum	11 000	10 800	98	11 800	11 600	98
2	S1	9 650	9 680	100	10 550	10 400	100
4	S3	9 000	8 120	95	9 480	9 280	98·5
5	P4	6 210	5 550	89	7 060	6 920	98
7	P6	4 300	3 140	73	4 970	4 450	90
10	P8	1 720	1 060	62	2 450	2 260	92

pure, as shown in Fig. 8 where serum is compared with an S10 fraction of about 250-fold purity. Over long periods of time, such as those necessary for storage, the optimum stability range was between pH 6·5 and 8·5.

The temperature stability of a S10 fraction, purified 250-fold, is shown in Fig. 8. Above 42° the activity was rapidly destroyed. At 37° the enzyme was stable for 3 hr. and for longer periods at room temperature.

When stored at 2° purified preparations lost activity at the rate of about 7 % per day at pH 7·6. When a purified preparation was frozen from a weak bicarbonate solution, 10 % of the activity was lost but the remainder of the activity was retained for at least 10 weeks. The paraoxonase activity of frozen serum remained intact over a period of months.

Paraoxonase activity in sheep serum (3·5 % of protein) tolerated 15 % (v/v) of ethanol for 1 hr. at room temperature. Higher concentrations or longer periods of contact resulted in progressive irreversible inactivation. At the more dilute protein concentrations necessary for activity determinations (0·03 % of protein) ethanol inactivation was much more rapid. At −5° the enzyme in a 2·34 % serum-protein solution was stable in 35 % ethanol for at least 24 hr. and in 42 % (v/v) ethanol for about 4 hr. Complete deactivation occurred in 1 hr. in 50 % (v/v) ethanol (2 % serum-protein solution).

At −5° saturated butanol solutions (about 12 % of butanol in a 4·1 % serum-protein solution) resulted in an 84 % loss in 2 hr., but 5 % of butanol was tolerated with a 10 % loss of activity in 3 hr. At room temperature 5 % of butanol resulted in rapid irreversible inactivation of the activity in serum.

Prolonged and repeated dialysis against water at pH 7·0 did not deactivate the enzyme.

Nature of the pure enzyme. The absorption spectrum of electrophoretically homogeneous paraoxonase in mM-$NaHCO_3$ soln. is shown in Fig. 9. Since the normal protein peak at 280 mμ is absent, the spectrum suggests that two or more components of the molecule are absorbing in the

Table 8. *Effect of ethylenediaminetetra-acetic acid alone and in the presence of barium on the paraoxonase activity of serum*

Paraoxonase activity was determined colorimetrically.

Barium (mM)	EDTA (mM)	Paraoxonase activity: mg. of *p*-nitrophenol/ml./30 min.	Paraoxonase activity: % inhibition
—	—	1·32	0
5	—	0·05	96
1	—	0·53	60
0·5	—	0·79	40
0·18	—	0·86	35
0·09	—	1·12	13
—	1·0	0·10	93
—	0·132	0·88	33
—	0·066	0·96	27
0·18	0·132	0·44	67
0·07	0·066	0·84	37
0·5	1·0	0·06	95

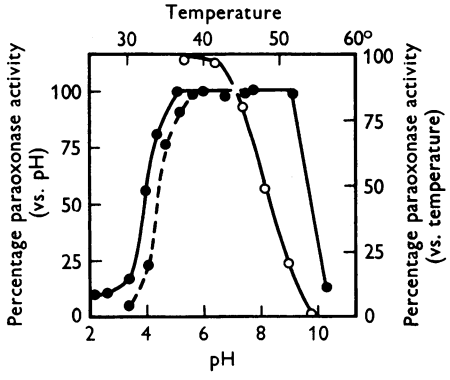


Fig. 8. Thermal and pH stability of paraoxonase. The pH stability of sheep-serum paraoxonase (●—●) and of a 250-fold purified fraction (●- - -●) is shown after 30 min. at each pH at 20°. The thermal stability of a S10 paraoxonase sample (○—○) is shown after 5 min. at each temperature, pH 7·6.

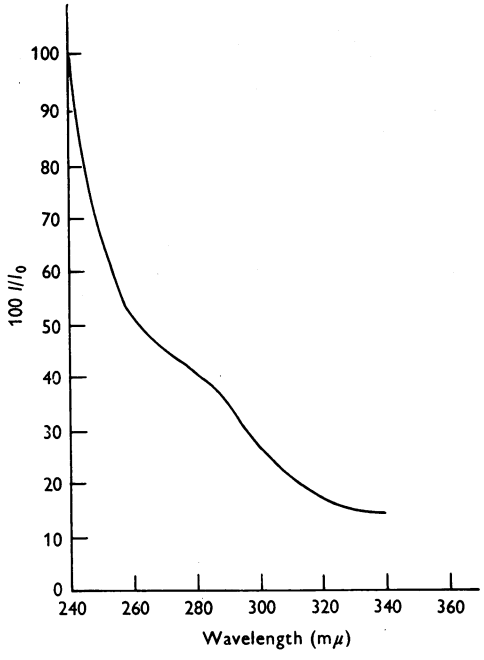


Fig. 9. Absorption spectrum of electrophoretically pure paraoxonase in mM-$NaHCO_3$ solution.

ultraviolet region and that their absorption peaks overlap. The ratio of the absorptions at 260 mμ compared with that at 280 mμ was 0·85 for purified material. The ratio of the combined proteins of the serum was 1·63.

Phosphorus analysis indicated the presence of 1·5% of phosphorus in the paraoxonase, on the assumption that paraoxonase protein was 16% nitrogen. The phosphorus to nitrogen ratio of S10 fractions was about 10 times that of the average for serum protein. When a repeated S10 fraction was run chromatographically on silica-impregnated paper, according to the method of Lea, Rhodes & Stoll (1955), choline-containing spots were detected at the origin and near the solvent front parallel to lecithin. The reaction for choline was strong as judged by the method of Chargaff, Levine & Green (1948). These results suggested that paraoxonase contained phospholipid, part of which was loosely bound and came off readily in the chloroform solvent. The absence of streaking suggested that the choline-containing component remaining at the origin was tightly bound.

SUMMARY

1. The enzyme in sheep serum which hydrolyses diethyl *p*-nitrophenyl phosphate (paraoxon) has been purified 330- to 385-fold. Electrophoretic evidence suggests that the product was 80–95% pure and contained only one other, non-active, component.

2. Low-temperature ethanol, pH and ionic-strength fractionation resulted in a 114-fold purification with a 32% yield. Ammonium sulphate fractionation increased the purification to 300-fold and lowered the yield to about 15%. Repeated ammonium sulphate fractionation brought the purification to 330- to 385-fold.

3. The paraoxon K_m of purified fractions (4·2 mM) was 15 times that of sheep serum (0·29 mM). The characteristics of the increase in K_m suggests the presence of a paraoxonase activator in sheep serum which was removed during purification.

4. Paraoxonase solutions fluoresced blue when exposed to ultraviolet radiation.

5. Analysis of the enzyme indicated the presence of about 1·5% of phosphorus and chromatographic experiments indicated the presence of choline-containing phospholipids.

6. The sedimentation constant and other observations suggest a molecular weight of 35 000–50 000.

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