Biochemical Pharmacology, 1963, Vol. 12, pp. 981–988. Pergamon Press Ltd., Printed in Great Britain.

ESTERASES OF RAT-LIVER CELL FRACTIONS

CORRELATION OF DF^{32}P-BINDING CAPACITY TO ESTERASE ACTIVITY

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(*Received* 14 *February* 1963; *accepted* 1 *May* 1963)

Abstract—The soluble proteins of the particulate and the supernatant fractions of the rat-liver cells have been separated into a number of enzyme peaks by DEAE-cellulose chromatography. By a technique using Sephadex G-50, the capacity of each chromatographic fraction to bind radioactive di*iso*propyl phosphorofluoridate (DF^{32}P) has been determined and correlated to its esterase activity towards some phenyl, ethyl and glyceryl esters. It is found that fractions having the maximal esterase activity exactly coincide with those with the highest DF^{32}P-binding capacity. Except for one peak in the supernatant fraction, all other esterase peaks take up the radioactive label and there are no radioactive peaks without esterase activity. The esterase peaks show some broad differences in their relative capacity to hydrolyze simple esters. The microsomal fraction has the highest esterase and DF^{32}P-binding activity. Though the unfractionated cell components exhibit some activity towards choline esters, this is not found to be localized in any of the esterase peaks obtained by DEAE-cellulose chromatography.

IT WAS demonstrated in a previous paper[1] that the subcellular proteins of the rat-liver cells, after incubation with radioactive di*iso*propyl phosphorofluoridate (DF^{32}P) could be separated into a number of distinct radioactive peaks by DEAE-cellulose chromatography. Since DF^{32}P completely inhibited all esterase activity, a parallel series of experiments were run without the inhibitor, wherein a pattern of esterase peaks was obtained which seemed to agree in number and positions with the radioactive peaks. However, for the actual proof of the identity of the two sets of peaks, as also to locate any radioactive peak without concomitant esterase activity and vice versa, it would be necessary to carry out the determinations of the two activities in the same chromatographic sample.

The present paper describes the details of experiments in which the above has been accomplished. It is found that the esterase peaks exactly coincide with the radioactive peaks. All the particulate fractions, of which the microsomal portion contains the highest activity, have been separated into four distinct enzyme peaks which show broad differences in substrate specificity.

MATERIALS AND METHODS

These were the same as described in the previous paper.[1] In addition, the following materials were obtained from the sources indicated: phenyl acetate, phenyl propionate, ethyl-n-butyrate, tri-n-butyrin (Eastman Organic Chemicals); phenyl-n-butyrate and triacetin (B.D.H.); acetylcholine chloride (Merck). The samples of butyrylcholine iodide and acetyl-β-methylcholine iodide used were obtained from local sources.

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The four cell fractions were obtained as described previously and after passing through a column of Sephadex G-25 to remove deoxycholate, they were fractionated on DEAE-cellulose without incubation with $DF^{32}P$. Aliquots of the chromatographic fractions were used for enzyme determinations and $DF^{32}P$-incubations. Esterase activity was determined with *p*-nitrophenyl acetate according to the method of Huggins and Lapides.[2] With the other esters the colorimetric method of Hestrin[3] was used. The substrates were prepared by dissolving about 0·5 g of the accurately weighed ester in 5 ml methanol and shaking up 1 ml of the methanolic solution with 100 ml 0·067 M phosphate buffer, pH 7·2, which also contained 0·1% Triton X-100. In the case of tributyrin and phenyl butyrate, uniform suspensions were obtained by homogenizing the methanolic solution of the ester with the buffer in a Potter–Elvehjem type homogenizer. All the substrates were prepared immediately before use. The final concentrations of the esters ranged from 2 to 8 $\times$ 10^{-3} M. In the case of choline esters the final concentration was 4 $\times$ 10^{-3} M. Standard curves were prepared for each ester and controls were run with each determination to correct for non-enzymatic hydrolysis and volatilization. As losses due to these sources were considerable at 37°, all determinations were carried out at room temperature between 20–22°.

Routine determinations of esterase activity for the location of enzyme peaks were carried out on all chromatographic fractions by incubating suitable aliquots with

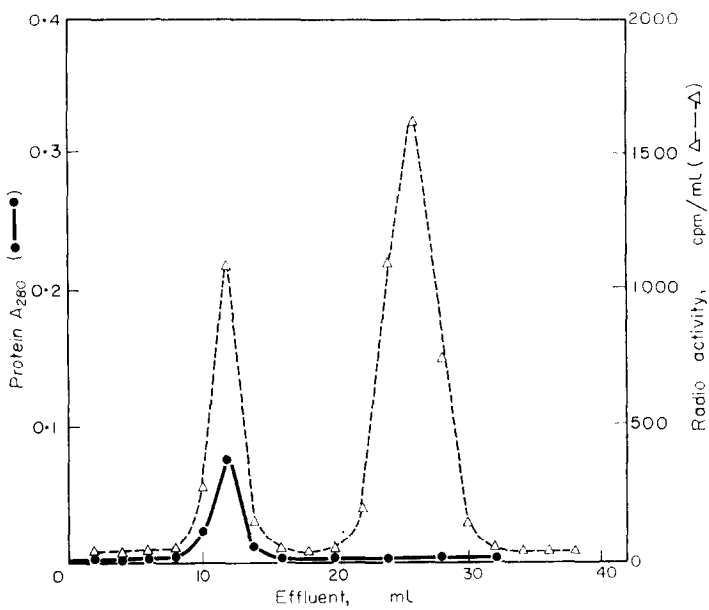

FIG. 1. Standardized procedure for the determination of $DF^{32}P$-binding capacity of esterase fractions derived from rat-liver intracellular proteins. In the typical experiment above, 2 ml of a microsomal fraction obtained by DEAE-cellulose chromatography was incubated with 0·05 ml 4 $\times$ 10^{-5} M $DF^{32}P$ in propylene glycol for 30 min at 0°. One ml was applied to a column of Sephadex G-50 (1·3 $\times$ 25 cm; capacity 37 ml) and filtration was carried out with 0·01 M Tris–acetic acid buffer, pH 7·4. Fractions of 2 ml were collected manually for protein and radioactivity determinations. In the routine analysis of the chromatographic fractions, a series of 50 columns of the same dimensions and capacity were used. The first 8 ml were rejected. Aliquots from the next 10 ml which were collected were used for radioactivity determinations.

0·5 ml of the substrate, diluting to a final volume of 1 ml with buffer and developing the colour after 1 hr. For the determination of the relative activity of the enzyme fractions on various substrates, the tubes corresponding to each esterase peak, as determined by the *p*-nitrophenyl acetate method, were pooled and aliquots were used for esterase determinations. In these cases the enzyme fraction was incubated with the substrate in stoppered tubes and 1 ml portions were withdrawn at various intervals for colour development. The initial linear part of the curve was extrapolated for calculations of esterase activity. In many cases the determinations were checked at two different enzyme concentrations. In the case of cholinesterase determinations of the pooled enzyme peaks both phosphate[3] and bicarbonate[4] buffers were used.

For the determination of the $DF^{32}P$-binding capacity 2 ml of each chromatographic fraction was incubated at 0° for 30 min with 0·05 ml 4×10^{-5} M $DF^{32}P$ (specific activity about 300 mc/g, Radiochemical Centre, Amersham) in propylene glycol. By trial experiments it was found that a good separation of the $DF^{32}P$-bound protein from an excess of the reagent could be achieved by using Sephadex G-50, Medium

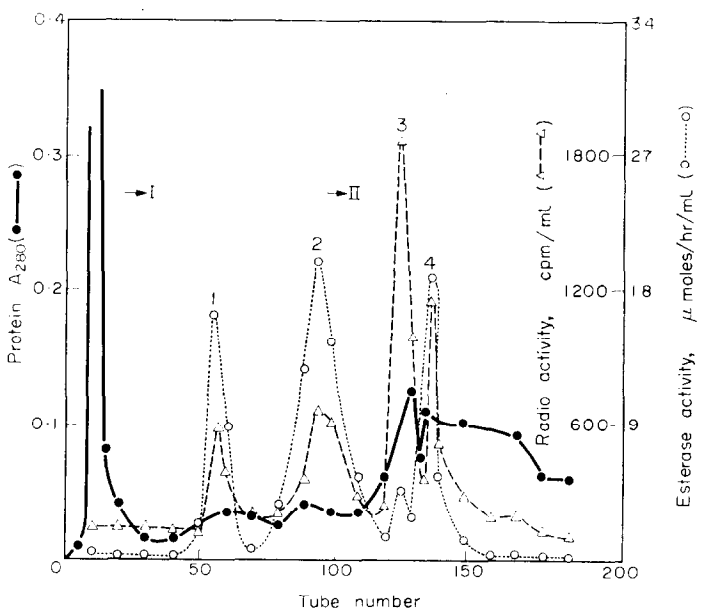

FIG. 2. Esterase and $DF^{32}P$-binding activity of the nuclear fraction of rat-liver cells. The protein derived from one rat liver was solubilized with 0·5% sodium deoxycholate in 0·01 M Tris–acetic acid buffer, pH 7·4. After passing through Sephadex G-25 to remove excess deoxycholate, the material (90 ml) was applied to a column of DEAE-cellulose (capacity 350 ml; dimensions 2·3 × 84 cm). Solvent system: 0·01 M Tris–acetic acid buffer, pH 7·4. Gradient elution started at (I) with the above buffer in the mixing chamber and the same buffer containing 0·1 M sodium acetate in the reservoir. At (II) the mixing chamber contained the buffer with 0·1 M sodium acetate and the reservoir the same buffer with 1·0 M sodium acetate. Fractions of 10–12 ml were collected at intervals of 15 min. All operations were carried out between 0–4°.

$DF^{32}P$-binding activity was determined according to procedure described under Fig. 1. Esterase determinations were carried out with various substrates according to Hestrin.[3] With *p*-nitrophenyl-acetate the method of Huggins and Lapides[2] was used. Only the curve obtained with *p*-nitrophenyl acetate is shown in the figure above. The others were of the same pattern, each peak coinciding exactly with the radioactivity peak, with differences only in the magnitude of esterase activity.

(Pharmacia, Uppsala) for filtration under standardized conditions as shown in Fig. 1, with a column of dimensions 1·3 × 25 cm and capacity 37 ml. For routine analysis a battery of 50 columns was used. One millilitre of the DF^{32}P-incubated mixture was applied to the column and filtration was carried out using 0·01 M Tris–acetic acid buffer, pH 7·4. The first 8 ml were rejected and from the next 10 ml which were collected, aliquots were taken for radioactivity determinations. The column was washed with 30 ml more of the buffer for regeneration. Sephadex G-75 was found to give equally good separations under the above conditions.

RESULTS

Figures 2–5 represent the radioactivity and esterase activity curves obtained respectively with the nuclear, mitochondrial, microsomal and the supernatant fractions. Only the esterase activity curves obtained with *p*-nitrophenyl acetate are shown in the diagrams. The patterns obtained were the same with the other substrates, viz., phenyl acetate, phenyl propionate, phenyl butyrate, ethyl butyrate and tributyrin, with differences only in the magnitude of esterase activity. In every case the fractions having highest DF^{32}P-binding activity were found to exhibit the highest esterase activity. It is also seen that there are no radioactive peaks without corresponding esterase activity. All the tubes were also analyzed with ethyl acetate, triacetin, acetylcholine, butyrylcholine and acetyl-β-methylcholine but only with negative results.

The first protein peak in the case of the three particulate fractions (Figs. 2–4) does not have any esterase or radioactivity. This is followed by four distinct DF^{32}P-binding esterase peaks, numbered 1 to 4 in the figures, in approximately the same positions in the chromatograms. There are distinct differences in their capacity to

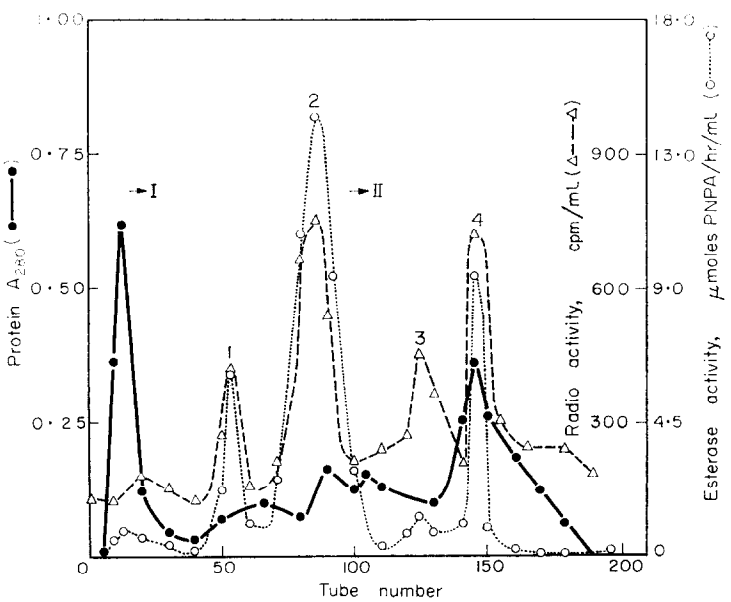

FIG. 3. Esterase and DF^{32}P-binding activity of the mitochondrial enzymes. Material derived from four rat livers and solubilized with deoxycholate was used, after removal of excess of deoxycholate by Sephadex G-25 filtration. Volume applied to DEAE-cellulose column was 60 ml. Column dimensions 2·5 × 67 cm; capacity 350 ml. Solvent system and other particulars as described under Fig. 2.

bind DF^{32}P; for example, peak 3 (Fig. 4) which has a comparatively low activity on *p*-nitrophenyl acetate has a very high radioactivity. It is also clear that the microsomal fraction has the highest esterase and DF^{32}P-binding activity.

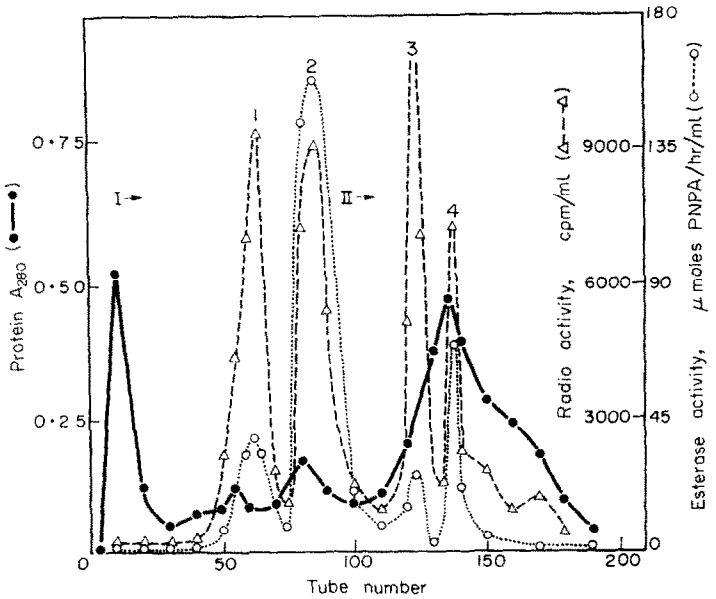

FIG. 4. Esterase and DF^{32}P-binding activity of the microsomal enzymes of the rat liver. The proteins were derived from four rat livers by deoxycholate treatment as usual. Volume of solution applied 60 ml. DEAE-cellulose column dimensions 2·3 × 82 cm. Capacity 350 ml. Other details as under Fig. 2.

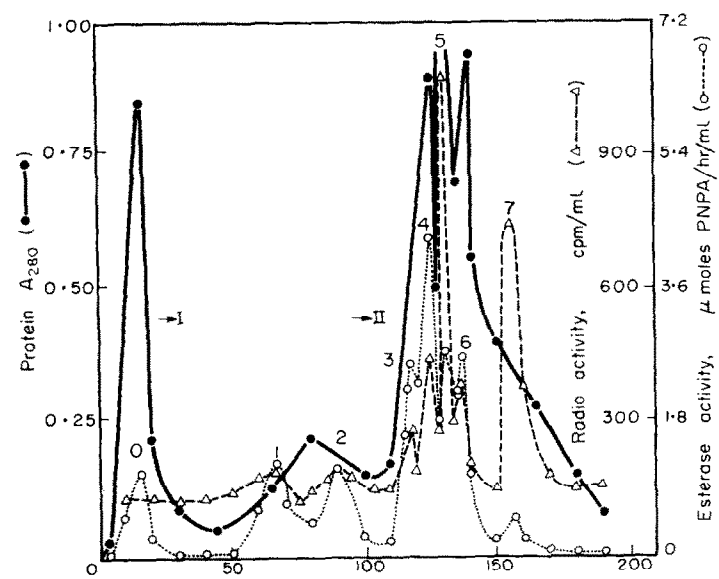

FIG. 5. Esterase and DF^{32}P-binding capacity of the supernatant fraction from rat-liver cells. The material was obtained from one rat liver. Volume after Sephadex filtration 117 ml. Capacity of DEAE-cellulose column 350 ml; dimensions 2·3 × 82 cm. Other details as under Fig. 2.

The pattern obtained with the supernatant fraction (Fig. 5) is different from those of the particulate fractions. The first esterase peak (No. 0) in the region of tubes 10–20 does not take up any $DF^{32}P$. It is also not completely suppressed by 10^{-6} M $DF^{32}P$ at which concentration all the other esterases in the particulate as well as in the supernatant fractions are completely inhibited. This peak probably contains one of the DFP-resistant enzymes.[5, 6] This is followed by two small peaks (1 and 2 in Fig. 5) which are approximately in the same positions as the first two major peaks in the particulate fractions. The region of tubes 110–160 in the chromatogram has as many as five closely associated esterase peaks each with an exactly coinciding radioactive peak. Both in the case of the particulate fractions as well as in the supernatant, all $DF^{32}P$-labelled peaks also contain esterase activity.

To obtain a clearer picture of the substrate specificity pattern of the various esterase peaks, enzyme activity was determined in the pooled fractions. The results are given in Table 1, together with the ultra-violet absorbancy values/cm at 280 mμ. There is a

TABLE 1. ESTERASE ACTIVITY OF ENZYME PEAKS OBTAINED FROM RAT-LIVER SUBCELLULAR FRACTIONS BY DEAE-CELLULOSE CHROMATOGRAPHY

Enzyme peak		Protein A_{280}/cm	PA	PP	PB	EB	PNPA	TB
Nuclear	1	0·14	4·7	10·1	8·5	1·2	1·1	0·0
	2	0·09	1·6	4·3	6·8	4·0	2·0	0·1
	3	0·19	3·2	7·0	4·8	0·8	0·7	2·9
	4	0·18	4·4	9·2	3·7	0·6	1·1	1·6
Mitochondrial	1	0·17	4·0	6·8	7·6	1·5	0·5	0·0
	2	0·25	1·5	0·9	3·8	1·6	2·5	0·0
	3	0·24	0·8	0·0	0·0	0·0	0·1	0·0
	4	0·34	1·3	1·9	2·3	0·0	0·2	0·0
Microsomal	1	0·23	52·9	86·7	81·5	25·6	19·8	0·8
	2	0·32	27·8	36·0	46·0	42·8	78·0	0·8
	3	0·35	13·0	34·0	40·0	1·9	6·2	4·3
	4	0·50	26·5	37·5	46·4	0·9	14·4	4·1
Supernatant	0	1·10	0·0	0·0	0·0	0·0	0·1	0·0
	1	0·19	0·8	0·5	1·0	0·0	0·1	0·0
	2	0·10	0·0	0·0	0·3	0·0	0·2	0·0
	3	0·35	3·8	1·2	0·2	0·0	0·6	0·0
	4	0·85	8·1	4·9	2·5	0·0	0·8	0·0
	5	1·00	3·6	3·2	1·1	0·0	0·5	0·0
	6	0·80	2·2	1·9	0·5	0·0	0·3	0·0
	7	0·44	1·1	1·7	0·7	0·0	0·1	0·0

Numbers of the enzyme peaks refer to the numbers in Figs. 2–5. Aliquots from the above pooled fractions (1–3 ml) were incubated with ester substrates (4–10 ml) in 0·067 M phosphate buffer, pH 7·2 at 20–22°, 1 ml fractions being withdrawn at various time intervals for determination of ester remaining unhydrolyzed.[3] In the case of *p*-nitrophenyl acetate the determination was carried out in a cuvette in the Beckman model DU spectrophotometer at a final volume of 2·5 ml. All values are expressed as μmole of substrate hydrolyzed by 1 ml of the fraction in 1 hr. The material was derived from four rats in the case of mitochondrial and microsomal, and one rat in the case of the nuclear and supernatant fractions. The approximate weight of a rat liver was 8 g. Abbreviations: PA, phenyl acetate; PP, phenyl propionate; PB, phenyl-n-butyrate; EB, ethyl-n-butyrate; PNPA, *p*-nitrophenyl acetate; TB, tri-n-butyrin. The enzyme fractions had no measurable activity on triacetin, ethyl acetate, acetylcholine, butyrylcholine and acetyl-β-methylcholine.

considerable overlapping of specificities of the esterases, but the following points are noteworthy: In the particulate fractions peak 1 has a higher activity towards phenyl esters than toward *p*-nitrophenyl acetate which is acted upon best by peak 2 in every case. In these two peaks ethyl butyrate follows also the same pattern as *p*-nitrophenyl acetate but is hardly acted upon by peaks 3 and 4. These peaks, however, have a higher activity towards tributyrin than peaks 1 and 2. In the case of the supernatant fractions the activity of peaks 0,1 and 2 are too low for enzyme determinations. Peaks 3 to 7 also have a general reactivity towards phenyl esters. The esterases of the particulate fractions show a progressively increasing activity from the acetate to the butyrate in the series while in the supernatant fractions the reverse is the case. None of the pooled fractions was found to have any measurable activity towards acetylcholine, butyrylcholine or acetyl-β-methylcholine whether phosphate buffer or bicarbonate buffer containing Mg^{2+} ions[4] was used.

To further investigate the absence of cholinesterase activity, the nuclear, mitochondrial, microsomal and the supernatant fractions were isolated from two rat livers and the soluble proteins were prepared from the particulate fractions by deoxycholate-treatment. After removal of the latter by Sephadex filtration, all the four fractions were analyzed for total acetyl- and pseudo-cholinesterase activity. The residues from the deoxycholate-treatment of the particulate fractions were also analyzed. The results are given in Table 2 from which it will be seen that the unfractionated cell components

TABLE 2. CHOLINESTERASE ACTIVITY OF UNFRACTIONATED RAT-LIVER CELL FRACTIONS

Cell fraction	Vol. ml	N/mg/ml	Acetyl-choline	Butyryl-choline	Acetyl-β-methyl-choline
Nuclear (DOC soluble)	96	0·90	2·5	2·9	3·0
Nuclear (DOC insoluble)	30	0·95	1·1	1·4	2·3
Mitochondrial (DOC sol)	35	1·25	0·5	1·2	1·2
Mitochondrial (DOC insol)	25	0·60	0·0	0·0	0·0
Microsomal (DOC soluble)	46	1·80	23·2	34·4	10·1
Microsomal (DOC insol)	20	0·40	5·5	3·7	2·9
Supernatant	217	0·65	0·8	1·0	0·8

The cell components were obtained from two rat livers (20 g) by the differential centrifugation technique and the particulate fractions were solubilized with 0·5% sodium deoxycholate (DOC) in 0·01 M Tris–acetic acid buffer, pH 7·4. Deoxycholate was removed by Sephadex G-25 filtration. The insoluble residues were suspended in 0·067 M phosphate buffer, pH 7·2. All determinations were carried out by incubating aliquots of the fractions with choline esters (final concentration 4×10^{-3} M) at 20–22° and withdrawing 1 ml samples at various intervals for colorimetric estimation.[3] All values are expressed as μmole of choline ester hydrolyzed by 1 ml of the fraction in 1 hr. Nitrogen was determined by the micro-Kjeldahl method. All cholinesterase activity was suppressed by 10^{-5} M eserine sulphate.

have some cholinesterase activity, the major portion of which is in the soluble portion of microsomes. It was also found that this activity towards choline esters could be completely suppressed by 10^{-5} M eserine sulphate while in the presence of this inhibitor the enzymes were still active towards *p*-nitrophenyl acetate. At present it is not known whether this cholinesterase activity, which cannot be localized in any of the esterase peaks separated in the DEAE-cellulose fractionation is merely due to a low unspecific activity which hydrolytic enzymes in general can exhibit towards various substrates.

DISCUSSION

In spite of the mass of favourable data, doubts have repeatedly been raised whether the toxic effects of DFP and similar compounds are entirely attributable to their inhibitory effect on acetylcholinesterase activity.[8, 9] This question which is complicated by the general overlapping specificities of the esterases can be elucidated by obtaining more data with regard to the penetration of DFP into the cell and the specificities of intracellular hydrolytic enzymes other than acetylcholinesterase which take up DFP. The present series of investigations were started with this object in mind and were initially concentrated on the fractionation of the enzymes at the subcellular level, to localize, if possible, the intracellular components which are attacked by DFP. From the evidence obtained in this investigation it is clear that the microsomal portion is the major site of DFP attack, and from the very similar pattern of the DEAE-cellulose chromatograms (Figs. 2–4) and the general substrate specificities the possibility cannot be excluded that the enzyme activities found in the nuclear and the mitchondrial portions may be merely those derived from microsomes contaminating them. At least, in the case of mitochondria, this has been suggested by Underhay *et al.*[10] on evidence based on glucose-6-phosphatase activity. The supernatant fraction contains comparatively little esterase activity.

The microsomal portion offers a rich source of DFP-sensitive esterases, which can be separated into four distinct peaks by DEAE-cellulose chromatography. All these peaks take up high amounts of $DF^{32}P$ and yield phosphorylserine on acid hydrolysis. These enzyme peaks do not have any acetylcholinesterase activity. The exact superimposability of the radioactive and esterase peaks and the absence of any $DF^{32}P$-binding peak without concomitant esterase activity are findings of considerable interest in the study of intracellular esterases with selective inhibitors and reactivators. They can be followed more easily by this method as their normal physiological substrates are still not known.

Acknowledgements—This work was supported by grants from the Committee for Medical Defence of the Swedish Medical Research Council. The authors are grateful to Mr. Roman Bijan and Mrs. Inga Hägglöv for meticulous technical assistance.

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