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FRACTIONATION OF DF^{32}P-BINDING PROTEINS OF RAT-LIVER CELL FRACTIONS BY DEAE-CELLULOSE CHROMATOGRAPHY

DISTRIBUTION OF ESTERASE ACTIVITY

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Abstract—The proteins of the desoxycholate-treated nuclear, mitochondrial, microsomal and supernatant fractions of rat liver have been incubated with radioactive di*iso*propyl phosphorofluoridate (DF^{32}P), and fractionated by DEAE-cellulose chromatography. More than twenty labelled peaks have been obtained and radioactive phosphorylserine could be isolated from the acid hydrolysates in every case. The reacting serine residues are most probably active sites of enzymes. Esterase determinations in parallel experiments without DF^{32}P, using *p*-nitrophenyl acetate as substrate, suggest that most of the labelled proteins have esterase activity. DF^{32}P completely inhibited this activity. The esterase activity of some of the peaks was found to be eserine-sensitive.

INTRODUCTION

IT WAS reported in a preliminary communication that radioactive phosphorylserine (Ser^{32}P) could be isolated from different rat-liver cell fractions incubated with ^{32}P-labelled di*iso*propyl phosphorofluridate (DF^{32}P).[1] The results indicated that all fractions, especially the microsomal one, contained relatively large amounts of DF^{32}P-inhibited enzymes with serine at their active sites. It was, therefore, of interest to achieve further separation of the DFP-reacting proteins.

In the experiments described in the present paper, the proteins of the desoxycholate-treated cell fractions were incubated with DF^{32}P, and subsequently separated by DEAE-cellulose chromatography. In parallel experiments without DF^{32}P the esterase activity of the chromatographic fractions was determined and correlated to the radioactivity.

MATERIAL AND METHODS

The livers were taken from Wistar rats of the departmental colony. The animals weighed about 300 g and were 10–12 months old. The nuclear, mitochondrial, microsomal and supernatant fractions were prepared as described by Hogeboom,[2] with some minor modifications.[3] The particulate fractions were homogenized with 0·5% sodium desoxycholate (Merck) in 0·01 M Tris–acetic acid buffer, pH 7·4. After 15 min at 0 °C, the homogenized fractions were centrifuged at 81,000 × *g* for 60 min. The volume obtained from each rat liver of approximately 8 g was as follows: nuclear fraction

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20–35 ml, mitochondrial 10–12 ml and microsomal fraction 15–25 ml. The corresponding volume of the supernatant fraction was 50–75 ml. Each cell fraction was incubated with 10^{-6} M $DF^{32}P$ for 30 min at 0–4 °C. The incubation medium also contained 0·01 M sodium phosphate, pH 6·8, and 2·5% propylene glycol (to dilute the inhibitor). $DF^{32}P$ with a specific activity of about 300 mc/g was obtained from the Radiochemical Centre, Amersham, England. The residues from centrifugation of the desoxycholate-treated particulate fractions were also incubated under similar conditions.

To remove the surplus of $DF^{32}P$, the incubated fractions were run through columns of Sephadex G-25 Medium (Pharmacia, Sweden). Elution was performed with 0·01 M Tris–acetic acid buffer, pH 7·4, in less than 1 hr at about 4 °C. The volumes of the Sephadex columns were at least four times the volume of the samples applied.

The protein fractions from Sephadex filtration were chromatographed on 200–350 ml columns of DEAE-cellulose (Eastman Organic Chemicals). The starting buffer was 0·01 M Tris–acetic acid, pH 7·4. After applying the protein solution, the column was first washed with 150 ml of this buffer. Elution was then continued with an increase in sodium acetate concentration. A concave gradient was used.[4] A 1000 ml round-bottomed flask, containing 520 ml of starting buffer, served as a mixing chamber. The 1000 ml conical reservoir flask was filled with 580 ml of 0·01 M Tris–acetic acid buffer, also 0·1 M with regard to sodium acetate. About 100 fractions of 10–12 ml each were then collected at 15-min intervals. A second gradient, ranging from 0·1 M to 1·0 M sodium acetate in 0·01 M Tris–acetic acid buffer, pH 7·4, was then used in the same way.

In some experiments, the protein of all labelled peaks was precipitated by trichloroacetic acid to the final concentration of 10%, after which it was hydrolysed with 2 N HCl for 20 hr at 100 °C. Two milligrams of unlabelled SerP were added to the dried hydrolysates, which were then chromatographed on 50 ml Dowex 50 columns. $Ser^{32}P$ was isolated according to Ågren *et al.*,[5] and further identified by rechromatography on Dowex 1 and paper electrophoresis.[5]

The radioactivity of each fraction from the DEAE-cellulose and Dowex columns was measured in glass cups, using a Robot Scaling Equipment with an end-window Geiber tube (LKB-produkter, Stockholm, Sweden).

The protein concentration of each fraction from the DEAE-cellulose columns was estimated by measuring the ultra-violet absorbancy/cm at 280 mμ in a Beckman model DU spectrophotometer.

Esterase activity was estimated by measuring the cleavage of *p*-nitrophenyl acetate (Mann Research Laboratories) according to Huggins and Lapides.[6] The esterase activity was also measured after preincubation with 10^{-6} M $DF^{32}P$ for 30 min at 0 °C. In addition, the enzyme activity was tested in the presence of 10^{-5} M eserine sulphate (Mann Research Laboratories) which, at this concentration, inhibits cholinesterases.[7]

RESULTS

Fig. 1 (a) shows the chromatogram of the solubilized proteins from the nuclear fraction incubated with $DF^{32}P$, and Fig. 1 (b) the corresponding control experiment without $DF^{32}P$. The material in this experiment was derived from two rat livers, one

half having been used for $DF^{32}P$ incubation and the other half for the parallel experiment (esterase activity determinations). The first high protein peak was of little interest, since it was unlabelled and contained only small amounts of esterase activity. This peak was followed by a series of small ones. At the beginning of the second gradient, a high, labelled protein peak appeared (Fig. 1 (a)), also showing esterase activity (Fig. 1 (b)). The peak with the highest labelling was found in about tube 70. A large esterase peak appeared in the same region in the control. In addition, a few smaller radioactivity peaks were obtained, as well as at least three esterase peaks. Slight, uniform inhibition of esterase activity was observed in all fractions in the presence of eserine. The esterase activity was completely inhibited by $DF^{32}P$.

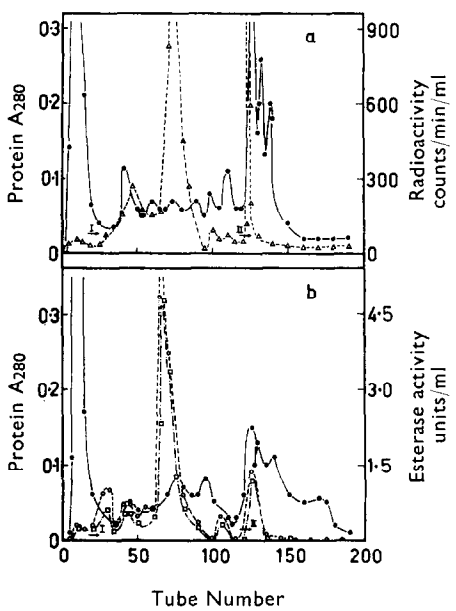

FIG. 1. Chromatography of nuclear proteins of rat-liver cells on DEAE-cellulose. The material from two livers was solubilized with 0·5% sodium desoxycholate in 0·01 M Tris–acetic acid buffer, pH 7·4. After centrifugation, half portions (30 ml each) were used for experiments (a) and (b), run in parallel. In (a), the material was incubated with 10^{-6} M $DF^{32}P$ and passed through Sephadex G 25, before being applied to DEAE-cellulose. In (b), the fractions were used for esterase activity determinations. Column dimensions: 2 × 56 cm. Fractions: 10–12 ml each at 15-min intervals. Solvent system: 0·01 M Tris–acetic acid buffer, pH 7·4, initially; gradient elution started at (I) with the above buffer in the mixing chamber, and the buffer containing 0·1 M sodium acetate in the reservoir. At (II), the two chambers contained 0·01 M Tris-acetic acid buffer, pH 7·4, with 0·1 M sodium acetate and 1·0 M sodium acetate, respectively. ●, Absorbancy at 280 mμ; △, radioactivity; ○, esterase activity; □, esterase activity in the presence of eserine.

The chromatogram of the solubilized proteins of the mitochondrial fraction from two rat livers incubated with $DF^{32}P$ is seen in Fig. 2 (a), and the corresponding control experiment without $DF^{32}P$ in Fig. 2 (b). The material used for the latter experiment was also obtained from two livers, but the experiments were not run in parallel.

The first high protein peak was slightly labelled, and contained some esterase activity. It was followed by three highly labelled regions (Fig. 2 (a)) which seemed on

the whole, to coincide with the position of three esterase peaks (cf. Fig. 2 (b)). The esterase activity was insensitive to eserine, but was completely inhibited by $DF^{32}P$.

Fig. 3 (a) represents the chromatogram of the solubilized proteins of the microsomal fraction from two rat livers, one half of the material being incubated with $DF^{32}P$. Fig. 3 (b) shows the corresponding chromatogram of the control experiment without $DF^{32}P$, using the other half of the material.

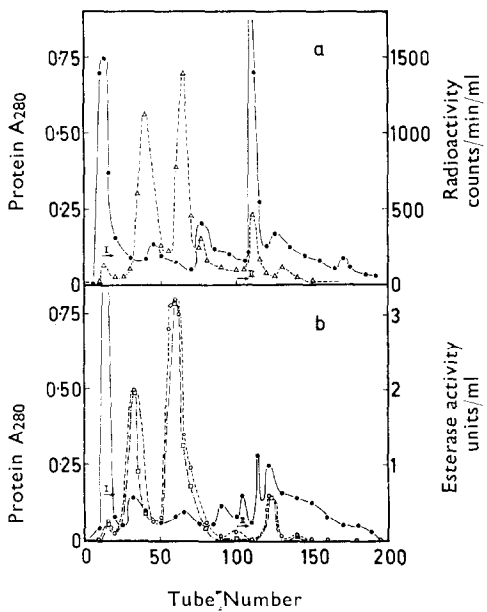

FIG. 2. Chromatography of mitochondrial proteins of rat-liver cells on DEAE-cellulose. The mitochondria used for each experiment were derived from two livers and solubilized with desoxycholate. The volume applied was 50 ml in experiment (a) and 40 ml in (b), the former after incubation with 10^{-6} M $DF^{32}P$ and the latter without $DF^{32}P$. The experiments were not run in parallel. Column dimensions: 2 × 60 cm and 2 × 80 cm. Other details and symbols as in Fig. 1.

The first protein peak was slightly labelled, and also contained a small amount of esterase activity. It was followed by two large esterase peaks in about the same position as the two radioactive peaks in Fig. 3 (a). An interesting feature is that the first esterase activity peak in particular was inhibited to a considerable extent by eserine. Although this peak seemed to contain less esterase activity than the following one, it was much more highly labelled.

After changing to the second gradient, a radioactive protein peak appeared (Fig. 3 (a)), also with esterase activity (Fig. 3 (b)). The largest amount of esterase activity was present in the microsomal fraction.

Fig. 4 (a) is the chromatogram of the proteins of the supernatant fraction from two rat livers; one half of the material was used in incubation with $DF^{32}P$. Fig. 4 (b) shows the corresponding chromatogram of the control experiment without $DF^{32}P$, using the other half of the material.

The first high protein peak contained relatively high esterase activity, whereas the corresponding region in Fig. 4 (a) showed only very small amount of radioactivity.

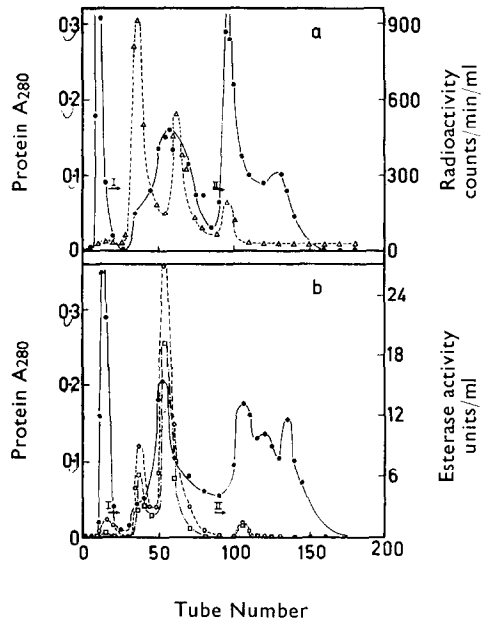

FIG. 3. Chromatography of microsomal proteins of rat-liver cells on DEAE-cellulose. Microsomes from two livers were solubilized with desoxycholate. Half portions (20 ml each) were used for experiments (a) and (b), run in parallel, the former after incubation with DF^{32}P. Column dimensions: 2 × 56 cm. Other details and symbols as in Fig. 1.

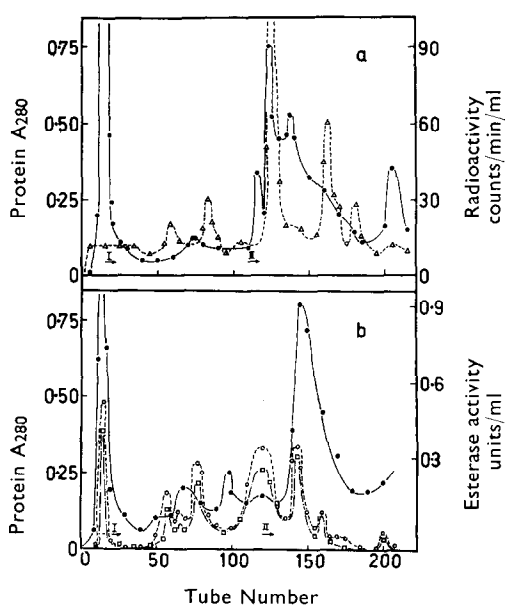

FIG. 4. Chromatography of supernatant fraction of rat-liver cells on DEAE-cellulose. Material from two livers was used in half portions (50 ml each) for experiments (a) and (b), run in parallel. Column dimensions: 2·2 × 80 cm. Other details and symbols as in Fig. 1.

This was followed by no less than six peaks of esterase activity, the first three of which coincided with the labelled peaks in Fig. 4 (a). There was slight, uniform suppression of esterase activity in the presence of eserine. As in the case of the three previous cell fractions, all esterase activity was inhibited by $DF^{32}P$.

The following procedure was used to investigate the linking of $DF^{32}P$ to the proteins of the labelled fractions. The protein of the pooled fractions corresponding to each radioactive peak was precipitated with trichloroacetic acid, to a final concentration of 10%. After centrifugation of the precipitated material, not more than 5 per cent of the total activity remained in solution. $Ser^{32}P$ was isolated from the hydrolysates of all the dried precipitates in an amount representing at least 15 per cent of their total activity. Since, in our experience, only about 20–25 per cent of the acid-stable phosphoprotein phosphorus can be isolated as SerP, these results indicate that all the $DF^{32}P$ was covalently bound to serine molecules in the proteins.

The residues left after desoxycholate treatment and centrifugation of the nuclear, mitochondrial and microsomal fractions were incubated with $DF^{32}P$, as previously described, in a volume of about 20 ml $Ser^{32}P$ was isolated in every case, but amounted to only a few per cent of its total quantity from the soluble proteins of the corresponding particulate fractions.

DISCUSSION

This seems to be the first attempt to fractionate the soluble proteins of the different cell fractions after incubation with $DF^{32}P$. A finding of considerable interest is that this inhibitor reacted with so many proteins in all four cell fractions. Since $Ser^{32}P$ could be isolated from all labelled peaks, it is reasonable to infer that they contained $DF^{32}P$-inhibited enzymes with a serine residue at their active sites. From this point of view, it is noteworthy that the esterase activity peaks seemed to coincide with a similar series of labelled peaks, suggesting that all $DF^{32}P$-inhibited enzymes may possess esterase activity.

The use of substrates other than *p*-nitrophenyl acetate may reveal specific functions of the many active fractions. Our observation that the microsomal fraction contained the largest amount of esterase activity is in accordance with the results of Underhay *et al.*[8] Since the whole of the liver tissue has been accounted for in these studies, and since large amounts of $DF^{32}P$ are taken up in this organ,[9] it is reasonable to believe that the toxicity of the inhibitor is to be ascribed not only to its action on acetylcholinesterase, but also to its inhibitory effects on other enzymes.

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