Biochemical Pharmacology, 1964, Vol. 13, pp. 849–854. Pergamon Press Ltd., Printed in Great Britain.

DETERMINATION OF DFPase IN RABBIT AND RAT TISSUES USING DF^{32}P

B. V. RAMACHANDRAN* and G. ÅGREN

Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden

(*Received* 22 *December* 1963; *accepted* 26 *February* 1964)

Abstract—The subcellular esterases of rabbit liver cells have been fractionated by DEAE-cellulose chromatography and the esterase activity of the fractions correlated with their capacity to bind radioactive di*iso*propyl phosphorofluoridate (DF^{32}P). As in the case of the rat liver enzymes the two activities are found to coincide with each other. The di*iso*propyl phosphorofluoridate-hydrolysing activity (DFPase) has been determined in rabbit and rat tissues using DF^{32}P as the substrate. The values obtained are much lower than those reported in literature. The enzyme is found to be localized in the supernatant fraction and is identifiable with one of the major enzyme peaks obtained in DEAE-cellulose chromatography.

WHEN radioactive di*iso*propyl phosphorofluoridate (DF^{32}P) is injected to rats large amounts of the label are found to be incorporated in the liver.[1] A similar effect has been observed in the rabbit.[2] The liver tissue of these species is also known to contain an active enzyme, DFPase, which hydrolyses di*iso*propyl phosphorofluoridate (DFP) to di*iso*propyl phosphate.[3, 4] This enzyme, in analogy with A-esterases[5] would be expected to act on simple substrates like *p*-nitrophenyl acetate (PNPA)[6] and not to bind DF^{32}P since it would hydrolyse it to an inactive form.[2] However, in our previous study on rat liver fractions wherein the esterase activity was correlated with DF^{32}P-binding capacity we failed to detect any enzyme peak which could be identified as DFPase.[7] We have now extended similar studies to rabbit liver esterases, the original source of DFPase.[3] Using a micro-method for the determination of DFPase activity it has been found that the enzyme is present only in small amounts in rat and rabbit tissues. In the liver it is found to be localized in the supernatant fraction.

MATERIALS AND METHODS

The materials used and the fractionation procedure adopted were the same as described in the previous papers.[7, 8] The subcellular fractions of the rabbit liver were isolated from 32 g of the liver tissue.

DFPase was determined as follows: 1 to 5 ml of the enzyme fraction was diluted to 50 ml with 0·01 M Tris-acetic acid buffer, pH 7·4, and incubated with or without 10^{-3} M Mn^{2+} for 20 min at 37°. A solution of 0·01 M DF^{32}P in propylene glycol (50 μl) was then added to the enzyme–buffer mixture to give a final DF^{32}P concentration of 10^{-5} M and the incubation continued in the thermostat. The total radioactivity in 1 ml of the digest ranged from 10 to 25 $\times$ 10^3 cpm. Aliquots of 5 ml were withdrawn every 5 min and extracted thrice with 10-ml portions of ether. This was found to

* Visiting Lecturer from the National Chemical Laboratory, Poona, India.

remove the undecomposed $DF^{32}P$ completely. The aqueous phase which contained the protein-bound $DF^{32}P$ and the hydrolytic products was precipitated with an equal volume of 5% trichloracetic acid (TCA) to remove the former and 1-ml aliquots of the filtrate were plated in glass cups with 3 drops of 5 N NaOH for radioactivity determinations.

Corrections were applied for the spontaneous hydrolysis of $DF^{32}P$ which under the above conditions amounted to 2·7 and 3·5% respectively in 1 hr without and with Mn^{2+} ions. The $DF^{32}P$ stock solution also contained 8·8% of unextractable radioactive material for which a correction was applied. DFPase activity was calculated from a straight-line curve obtained by plotting the radioactivity released in TCA-filtrates against time. It was expressed in terms of μmoles of $DF^{32}P$ hydrolysed per hr, the standardization being carried out by determining the specific activity of a known weight of the stock solution of $DF^{32}P$ in propylene glycol. The digest contained 0·001% of propylene glycol but the solvent even at 2·5% concentration was found not to materially affect the results. The sensitivity of the method is approximately 0·001 μmole of $DF^{32}P$ hydrolysed in 1 hr. Radioactivity was measured either in a Robot Scaling Equipment (LKB-produkter, Stockholm) or in a Tracerlab Superscaler 18 fitted with an end-window TGC-2 Geiger tube.

RESULTS

Figure 1 gives the pattern obtained in the DEAE-cellulose chromatography of rabbit liver subcellular enzymes. The esterase activity as determined by the PNPA-method[9] has been correlated with the capacity of each fraction to bind $DF^{32}P$ and the curves indicate that as in the case of the rat enzymes the two activities run parallel to each other. The nuclear fraction has two major peaks and the mitochondria are found to contain numerous enzyme peaks of low esterase activity. It is likely that some of these are derived as impurities from other fractions. The microsomal fraction has the maximum esterase and $DF^{32}P$-binding activity. The esterase activity of the particulate fractions towards PNPA is completely inhibited by 10^{-6} M $DF^{32}P$.

The supernatant fraction has 7 esterase peaks and all of them are found to incorporate $DF^{32}P$. However, the esterase activity of peaks 3 and 4 (Fig. 1) is not completely suppressed by 10^{-6} M $DF^{32}P$, about 32 and 14% respectively of the original activity being found to be DFP-resistant. The amount of DFP-insensitive esterase in other enzyme peaks is too small to be determined accurately. The esterase peaks of the particulate as well as the supernatant fraction are in general more active towards phenyl butyrate and propionate than the acetate.

Since in these studies there is no esterase peak which can be identified as DFPase, this activity was determined in tissue homogenates of the rabbit and the rat to obtain an idea of the approximate amounts present. The results are given in Table 1. The values are expressed as μmoles of $DF^{32}P$ hydrolysed by 1 g of tissue, 1 ml of plasma or subcellular material derived from 1 g of liver tissue. Nitrogen values to which the DFPase activity refers are also given. The results are only indicative and not absolute since they are based only on a few experiments and it is well known that individual animals may show large variations.[11] It is observed that the DFPase activity as determined by this method is very low in tissues, only of the order of a fraction of a μmole $DF^{32}P$ hydrolysed in 1 hr. The liver and kidney DFPases are activated by Mn^{2+} while the plasma enzyme is inhibited. The results are in broad qualitative agreement

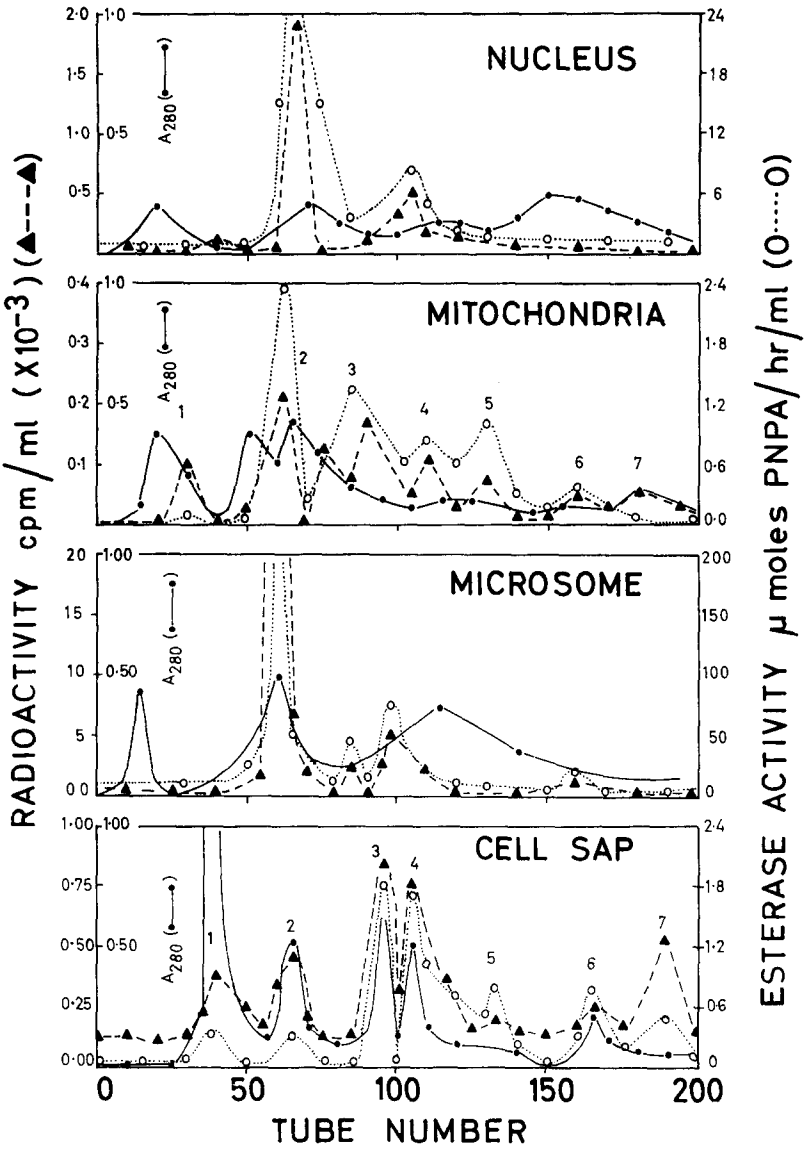

FIG. 1. Esterase and DF^{32}P-binding activity of subcellular enzymes of rabbit liver cells. The microsomes and mitochondria were derived from 32 g and the nuclear and supernatant fractions from 8 g of liver tissue. The particulate fractions were solubilized with 0·5% sodium deoxycholate in 0·01 M Tris-acetic acid buffer, pH 7·4. After filtering through Sephadex G-25 the material was applied to a column of DEAE-cellulose (volume 350 ml; dimensions 2·3 × 84 cm). Solvent system: 0·01 M Tris-acetic acid buffer, pH 7·4 up to 10 tubes after which gradient elution was started with the above buffer in the mixing chamber and 0·1 M sodium acetate in buffer in the reservoir. At about tube No. 100 the mixing chamber contained 0·1 M sodium acetate in buffer and the reservoir the same buffer containing 1·0 M sodium acetate. Fractions of 10–12 ml each were collected at 15-min intervals. All operations were carried out between 0–4°.

Esterase activity was determined according to Huggins and Lapides[9] and DF^{32}P incorporation by the procedure described previously.[7] Optical readings were taken in a Zeiss Model PMQ II spectrophotometer.

with those of Mounter *et al.*[10] In no case was histidine found to potentiate the activation by Mn^{2+}. With the exception of the heterogenous rat nuclear material, both in the rabbit and in the rat liver, DFPase is concentrated in the supernatant fraction. The degree of activation of rabbit liver supernatant by Mn^{2+} is much higher than that of the rat liver supernatant. The localization of DFPase in the supernatant fraction indicates that the hydrolysis of DFP is brought about by a distinct enzyme and is not due to the unspecific hydrolytic activity exhibited by esterases in general towards various substrates since the microsomes which have the highest esterase activity towards PNPA are found to exhibit a comparatively low DFPase activity. Adie and Tuba[11] found that sarinase is localized in the supernatant fraction. In our own

TABLE 1. DFPASE CONTENT OF RABBIT AND RAT TISSUE MATERIAL

Material	N (mg)	DFPase activity in μmoles of $DF^{32}P$ hydrolysed in 1 hr		
		Without activator	With Mn^{2+} (10^{-3} M)	Values from literature*
Rabbit				
Kidney	18·2	0·958	5·124	167·0
Plasma	11·9	0·443	0·125	113·4
Liver	17·5	0·261	1·617	244·6
Liver Nucleus	5·5	0·021	0·041	
Liver Mitochondria	0·4	0·008	0·004	
Liver Microsomes	2·2	0·028	0·040	
Liver Supernatant	6·9	0·072	0·656	
Rat				
Kidney	24·3	0·315	2·175	113·9 (489·6)
Plasma	5·8	0·125	0·049	
Liver	19·0	0·483	1·620	187·5 (356·3)
Liver Nucleus	7·2	0·146	0·328	
Liver Mitochondria	2·3	0·025	0·089	
Liver Microsomes	4·5	0·091	0·070	
Liver Supernatant	8·7	0·177	0·332	

The values are expressed as μmoles of $DF^{32}P$ hydrolysed by 1 g of tissue, 1 ml of plasma or subcellular material derived from 1 g of liver tissue in 1 hr at 37° and at pH 7·4 (0·01 M Tris-acetic acid buffer). Nitrogen values (micro-Kjeldahl) given refer to this quantity of material. Tissue homogenates and liver fractions were dispersed in Tris-acetic buffer in a dilution of 1 : 10 or 1 : 5 and suitable aliquots were used for determinations. The procedure used is given under the methods. The sensitivity of the method is approximately 0·001 μmole $DF^{32}P$ hydrolysed in 1 hr.

* The rabbit and rat values were calculated from figures given by Mazur[3] and Mounter[13] respectively.

in-vivo studies[1] with rats it was observed that that the $DF^{32}P$ content of the supernatant fraction decreased progressively with time probably due to the DFPase activity of this fraction.

As DFPase seems to be concentrated mainly in the supernatant, the chromatographic fractions obtained by DEAE-cellulose chromatography (Fig. 1) were analysed for this activity by the method described. One ml of each fraction was incubated with Mn^{2+} for 20 min at 37° and then with 10^{-5} M $DF^{32}P$ for 1 hr. The radioactivity

released in the TCA-filtrate was determined for each fraction and corrected for hydrolysis. Figure 2 gives the DFPase activity curve of the supernatant fraction superimposed on the curve for esterase activity towards PNPA. It is observed that DFPase is concentrated in peak 3 which, incidentally, is also not completely inhibited by $DF^{32}P$. The amount of DFPase in other enzyme peaks of the supernatant as well as of the particulate fractions is too small for accurate assay.

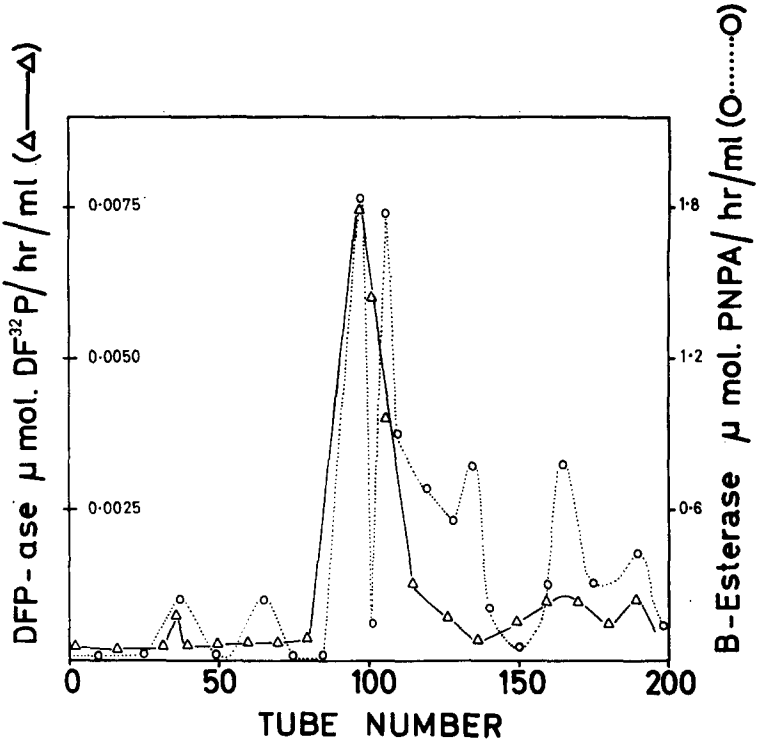

FIG. 2. Correlation of DFPase and B-esterase in the supernatant fraction of rabbit liver cells. The fractions used were the same as indicated in Fig. 1 (cell-sap). For DFPase, 1 ml of each fraction was incubated with 0·05 ml of 2×10^{-3} M Mn^{2+} for 20 min at 37°. One ml of 2×10^{-5} M $DF^{32}P$ in 0·01 M Tris-acetic acid buffer, pH 7·4, was then added and tne incubation continued for 1 hr. The digest was extracted thrice with 5-ml portions of ether and 1 ml of the aqueous phase was precipitated with an equal volume of 5% TCA. Other details are given under Methods.

DISCUSSION

Interest in enzymes which hydrolyse organophosphorus anticholinesterases centres on their possible role in the detoxicating mechanism of the animal and the evaluation of their use in the therapy of organophosphate poisoning.[12] However, little is known about the identity of the various enzymes hydrolysing organophosphates or their amounts present in tissues to assess the extent to which they may counter toxicity. The method used by most workers for the assay of these enzymes is the manometric technique in which the acid liberated is taken as a measure of the hydrolysis of the organophosphate. In the case of DFP, 2 molecules of acid will be generated for each molecule of DFP hydrolysed. It is also known that when DFP acts as an inhibitor

one molecule of HF is liberated. The error from this source in the assay of DFPase is assumed to be negligible but it may be significant if the amount of DFP-sensitive enzymes is very large compared to DFPase. From the present series of studies[7, 8] it appears likely that the B-esterases (DFP-sensitive esterases) of the microsomal fraction may affect the determination of DFPase in unfractionated tissues by the manometric method.

The values obtained by the present method are much lower than those reported by Mazur[3] and Mounter *et al.*[4] for rabbit and rat tissues The reason may be the one stated above or due to the nature of the substrate used by us whose purity cannot be so effectively controlled as that of un-labelled DFP.

It is observed that DFPase occurs in the same enzyme peak which also incorporates radioactivity (Fig. 2). This may mean that the enzyme peak is still heterogenous or that the enzyme has more than one active centre, one inhibited by $DF^{32}P$ (B-esterase)[5] and therefore incorporating radioactivity and another concerned with the hydrolysis of DFP. Jandorf and McNamara[2] drew attention to the fact that the tissues which are known to be rich in DFPase are also those which actively incorporate radioactivity from $DF^{32}P$. Another possibility is that the DFP-DFPase intermediate which may be formed is hydrolysed so slowly that the radioactive label can be detected as protein-bound in the present technique. Mn^{2+} ions may play a role in accelerating this rate-determining second step. The finding that DFP-sensitive and insensitive esterases are localized in the microsomes and supernatant respectively is of interest in the purification and further study of these enzymes.

Acknowledgements—The authors are thankful to the Medical Defence Committee of the Swedish Medical Research Council for generous financial support. The skilful technical assistance rendered by Miss Gunilla Bengtsson, Mr. Roman Bijan and Mrs. Inga Hägglöv is gratefully acknowledged.

REFERENCES

1. G. ÅGREN and B. V. RAMACHANDRAN, *Acta physiol. scand.* (in press).
2. B. J. JANDORF and P. D. MCNAMARA, *J. Pharmacol.* **98**, 77 (1950).
3. A. MAZUR, *J. biol. Chem.* **164**, 271 (1946).
4. L. A. MOUNTER, L. T. H. DIEN and A. CHANUTIN, *J. biol. Chem.* **215**, 691 (1955).
5. W. N. ALDRIDGE, *Biochem. J.* **53**, 110, 117 (1953).
6. L. A. MOUNTER, *J. biol. Chem.* **209**, 813 (1954).
7. B. V. RAMACHANDRAN and G. ÅGREN, *Biochem. Pharmacol.* **12**, 981 (1963).
8. B. V. RAMACHANDRAN, L. ENGSTRÖM and G. ÅGREN, *Biochem. Pharmacol.* **12**, 167 (1963).
9. C. HUGGINS and J. LAPIDES, *J. biol. Chem.* **170**, 467 (1947).
10. L. A. MOUNTER, *J. biol. Chem.* **215**, 705 (1955).
11. P. A. ADIE and J. TUBA, *Canad. J. Biochem. Physiol.* **36**, 21 (1958).
12. J. A. COHEN and M. G. P. J. WARRINGA, *Biochim. Biophys. Acta*, **26**, 29 (1957).
13. L. A. MOUNTER in *Handbuch der Experimentellen Pharmakologie, Ergänzungswerk*, XV, p. 486. Springer-Verlag, Berlin (1963).