ISOLATION OF RADIOACTIVE PHOSPHORYLSERINE FROM DIFFERENT RAT-LIVER CELL FRACTIONS INCUBATED WITH ^{32}P-LABELLED DI-ISOPROPYL PHOSPHOROFLUORIDATE

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DI-ISOPROPYL phosphorofluoridate (DFP) is known to inactivate several hydrolytic enzymes by reacting with their active sites[1]. Phosphorylserine has been isolated in several cases after acid hydrolysis of the inactivated enzymes, showing that a particular serine molecule, probably essential for the enzyme activity, is phosphorylated by the inhibitor. In addition, two transphosphorylating enzymes, phosphoglucomutase[2] and alkaline phosphatase[3] — although not inhibited by DFP—seem to contain a serine molecule at their active sites. These enzymes undergo intermediary phosphorylation by their substrates, with the phosphoryl groups bound to a serine molecule after acid hydrolysis. Serine thus appears to be important in many enzyme mechanisms.

To obtain some information about the amount and intracellular localization of the rat-liver enzymes which react with DFP, we studied the incorporation of acid-stable, radioactive phosphate from DF^{32}P into the proteins of rat-liver cell fractions, prepared according to Hogeboom[4]. Liver was chosen, because it has been shown by Jandorf and McNamara[5] that the incorporation *in vivo* of DF^{32}P in rabbits is particularly high into the liver, kidneys and lungs.

Each cell fraction was incubated with different concentrations of DF^{32}P for 30 min at 0° and pH 6·8. The specific radioactivity of the DF^{32}P used was about 300 mC/g. The medium contained 0·25 M sucrose, 0·01 M sodium phosphate, pH 6·8, and 2·5% propylene glycol, with the inhibitor added to the final concentration of 10^{-8}–10^{-2} M. The total volume of the nuclear, mitochondrial, microsomal and supernatant fractions was 140, 60, 90 and 130 ml., respectively. The incubation mixtures were inactivated by precipitation with trichloroacetic acid to a final concentration of 10%. The precipitates were washed with 10% trichloroacetic acid, dried with ethanol and ether and then hydrolysed by 2 N HCl at 100°C for 20 hr. The hydrolysates were chromatographed on

Abbreviations: DFP, di-isopropyl phosphorofluridate; SerP, phosphorylserine.

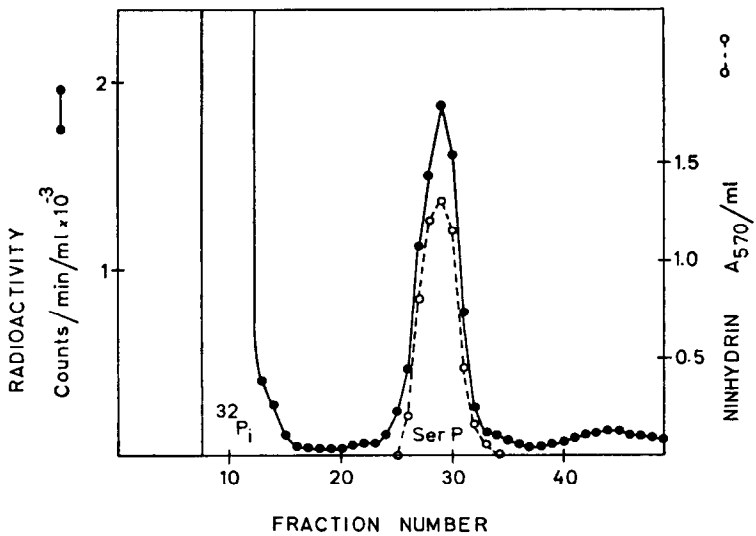

FIG. 1. Dowex 50 W × 8 chromatogram: hydrolysate of a supernatant fraction incubated with 10^{-7} M DF^{32}P. Column dimensions: 1·2 × 45 cm. Elution with 0·01 N HCl. Fraction volume: 3 ml. ●—●, radioactivity; ○- - -○ ninhydrin colour.

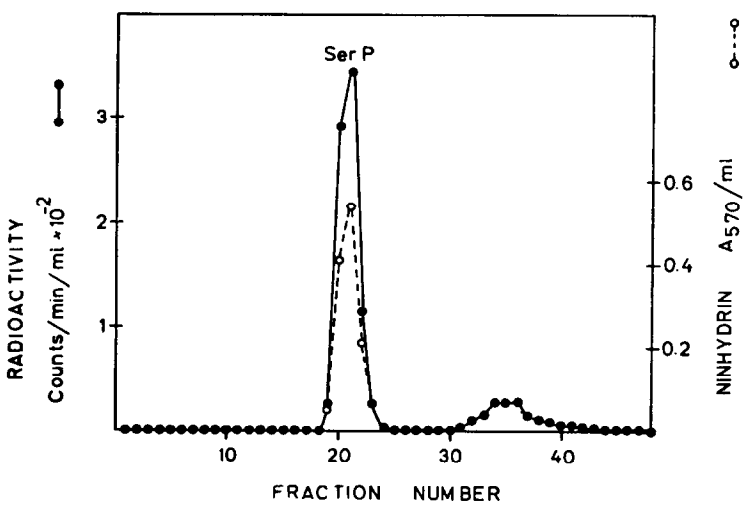

FIG. 2. Rechromatography on Dowex 1 × 10: SerP fraction from Dowex 50 W chromatography of a hydrolysate of a microsomal fraction incubated with 10^{-6} M DF^{32}P. Column dimensions: 1·2 × 23 cm. Gradient elution with a closed mixing chamber containing 100 ml. of water, refilled with 0·025 N HCl. Fraction volume: 6 ml. ●—● radioactivity; ○- - -○ ninhydrin colour.

Dowex 50 W × 8 columns, with the addition of 1 mg of unlabelled phosphate and 2 mg of SerP as carriers. The inorganic phosphate was added to achieve good separation, and the SerP to indicate the position of the labelled Ser^{32}P. Elution was performed with 0·01 N HCl. The fractions were analysed for radioactivity and ninhydrin-reacting substances[6].

Fig. 1 shows a typical chromatogram of a hydrolysate of a supernatant fraction incubated with 10^{-7} M DF^{32}P. After the inorganic phosphate, some radioactive material appeared in front of the activity peak, which was eluted together with the carrier SerP. By rechromatography of the SerP peak on Dowex 1 × 10, most of the radioactivity was again eluted with the carrier SerP (Fig. 2). The labelled material was further identified as Ser^{32}P by paper electrophoresis and paper chromatography[7]. The amount of Ser^{32}P isolated was estimated from its total radioactivity divided by the specific activity of the DF^{32}P used. The total radioactivity was obtained by summation of the activities of the Ser^{32}P-containing fractions from the Dowex 50 W columns, and correction for the samples withdrawn for the ninhydrin analyses, as well as for decay of the isotope. After the Ser^{32}P, some labelled substances were eluted from the Dowex 50 W columns in a position usually occupied by phosphopeptides[7].

TABLE I

Amount of Ser^{32}P *isolated from different rat-liver cell fractions after incubation with* DF^{32}P

(Calculated as μmoles/g. dry weight)

Fraction	Molar concentration of DF^{32}P						
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Nuclear	0·08	0·02	0·05	0·04	0·04	0·008	0·002
Mitochondrial	0·04	0·02	0·06	0·02	0·01	0·006	0·002
Microsomal	0·21	0·16	0·15	0·13	0·08	0·012	0·001
Supernatant	0·03	0·07	0·01	0·01	0·02	0·004	0·001

The results of incubation of the cell fractions with various concentrations of DF^{32}P are listed in Table I. It is seen that labelled phosphate was incorporated into all the fractions. In every series of experiments, the incorporation was greatest into the microsomal fraction. With the two lowest DF^{32}P concentrations used, the small amount of inhibitor added was probably consumed, the low values being due to a large excess of incorporating groups.

In our experience[3,8], only about 20–25% of acid-stable phosphoprotein phosphorus can be isolated as SerP, since most of the phosphorus

is split off as inorganic phosphate during hydrolysis. Using this approximation, the incorporation into the microsomal fraction corresponds to about one μmole per gramme dry weight, or one mole per 10^6 g. Moreover, it must be borne in mind that the dried precipitates contained nucleic acids and fats, in addition to proteins.

With respect to the fairly low $DF^{32}P$ concentrations used in some of the experiments, it seems probable that most of the $Ser^{32}P$ are derived from enzymatically active proteins. Consequently, all the rat-liver cell fractions appear to contain relatively large amounts of DFP-inhibited enzymes with serine at their active sites.

REFERENCES

1. D. R. DAVIES and A. L. GREEN; *Advanc. Enzymol.* **20** 283 (1958).
2. L. ANDERSON and G. R. JOLLÈS; *Arch. Biochem. Biophys.* **70** 121 (1957).
3. L. ENGSTRÖM; *Biochim. Biophys. Acta* **52** 49 (1961).
4. G. H. HOGEBOOM; In *Methods in Enzymology*, Vol. 1, p. 16, (Edited by S. P. COLOWICK and N. O. KAPLAN) Academic Press New York, 1955.
5. B. J. JANDORF and P. D. MCNAMARA; *J. Pharmacol. Exptl. Therap.* **98** 77 (1950).
6. S. MOORE and W. H. STEIN; *J. Biol. Chem.* **211** 907 (1954).
7. G. ÅGREN, C.-H. DE VERDIER and J. A. GLOMSET; *Acta Chem. Scand.* **8** 1570 (1954).
8. C.-H. DE VERDIER; *Acta Soc. Med. Upsaliensis* **60** 48 (1955).