JCI The Journal of Clinical Investigation

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J Clin Invest. 1954;33(3):459-467. https://doi.org/10.1172/JCI102917.

Research Article

THE FATE OF P^{32} LABELLED DIISOPROPYLFLUOROPHOSPHONATE IN THE HUMAN BODY AND ITS USE AS A LABELLING AGENT IN THE STUDY OF THE TURNOVER OF BLOOD PLASMA AND RED CELLS

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(Submitted for publication May 12, 1953; accepted October 28, 1953)

In recent years diisopropylfluorophosphonate (DFP) has established itself as a useful drug in such conditions as intestinal and vesical atonia, glaucoma and myasthenia gravis. With the consolidation of its place in therapy the desirability of more precise knowledge of the fate of this compound in the human organism became obvious. In the present study the absorption, the distribution in the blood and the excretion of DFP have been studied after intramuscular injection of the radioactive labelled substance (DFP^{32}) into three patients suffering from myasthenia gravis (two cases) and arteriosclerosis cerebri, respectively, and into two normal persons. There are no theoretical reasons why the metabolic fate of DFP should be different from normal in the patients studied. Indeed, no essential differences were observed between results obtained from the three patients and those from the normal persons. Therefore, all results to be described should be considered as applicable to the metabolism of DFP in the normal human organism.

In the course of this work it became obvious that the phosphorus of DFP^{32} was irreversibly bound to the proteins of blood plasma and red cells and that DFP was exclusively broken down to diisopropylphosphate, a substance which is not further metabolised nor taken up into any normal metabolic pool. Such properties suggested that DFP^{32} might prove to be a useful tool in the study of the turnover of blood plasma proteins and red cells. Preliminary results of the use of DFP^{32} in the determination of the life span of red cells and the half life of a plasma protein will be included in this study.

EXPERIMENTAL METHODS

Synthesis of DFP^{32}. DFP^{32} was prepared from $H_3P^{32}O_4$ purchased from the Atomic Energy Research Establishment at Harwell by a method to be described elsewhere. It was injected intramuscularly as a solute in 1 to 2 ml. of peanut oil.

Radioactivity measurements. In the absorption experiments, samples of 0.1 ml. blood obtained from the fingertip were pipetted into flat counting dishes, which were provided with a snugly fitting piece of filter paper in the bottom. After drying, the samples were counted with a conventional Geiger Müller tube. All other radioactivity measurements were carried out with a G.M. liquid counter (M_6 of 20th Century Electronics). All measurements were related to standards consisting of diisopropylphosphate obtained by alkaline hydrolysis of a sample of DFP^{32} of known quantity and radioactivity.

Treatment of blood, urine, and feces for radioactivity measurements. Ten milliliters of blood obtained by venepuncture were collected in sodium citrate (final concentration 0.76 per cent). Red cells were separated from the plasma and washed thrice in normal saline. Counting was carried out on the hemolysate in distilled water. Samples, diluted if necessary, from 24-hour portions of urine were counted directly in the liquid counter. Feces were collected in aliquots over a period of 24 hours and mixed. Samples of 2 Gm. were suspended in a small volume of water and hydrolyzed during 15 minutes at 100° C. in molar NaOH.

Cholinesterase activity. Cholinesterase activity was measured manometrically at 37° C. according to Ammon (1) using acetylcholine as a substrate.

Paper chromatography. Paper chromatography was carried out on samples of 40 µl. of urine using an ascending method with butanol-acetic acid-water mixtures as solvent. The development period lasted 16 hours at a temperature of 23° C.

Organic phosphates were made visible on the paper by a method which was essentially that of Hanes and Isherwood (2) with slight modifications.

Radioactivity on the paper was assessed by exposing x-ray films in direct contact with the chromatogram in the dark room for 72 hours.

RESULTS

The first series of experiments were performed on three young women. Two of these were treated in the Neurological Clinic of the Leyden Uni-

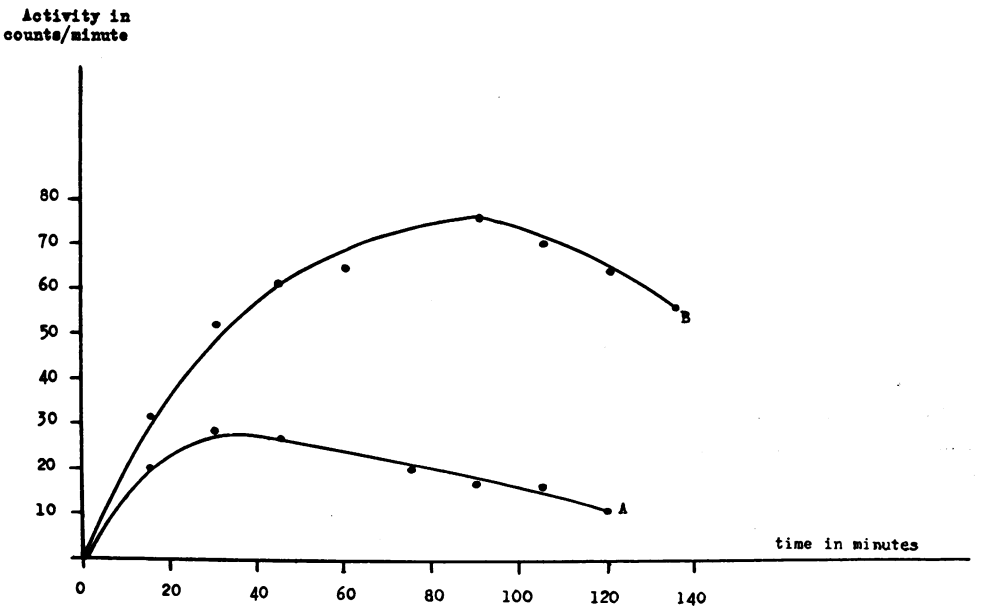


FIG. 1. RADIOACTIVITY IN WHOLE BLOOD AFTER INJECTION OF DFP^{32}
Activity expressed in counts per minute per 0.1 ml. sample of blood of patients A and B.

versity Hospital for myasthenia gravis (A and B), the third one (C) was a healthy volunteer. A, B, and C received dosages of 1.990, 0.240, and 0.433 mg. of DFP^{32}, respectively. The specific activity of the preparation used was approximately 40 μc. per mg. DFP. One of the patients (A) had been treated with OMPA (octamethylpyrophosphoramide) prior to the experiment. At the start of the experiment practically no cholinesterase activity was present in this case in blood plasma and red cells.

After completion of this series it became de-

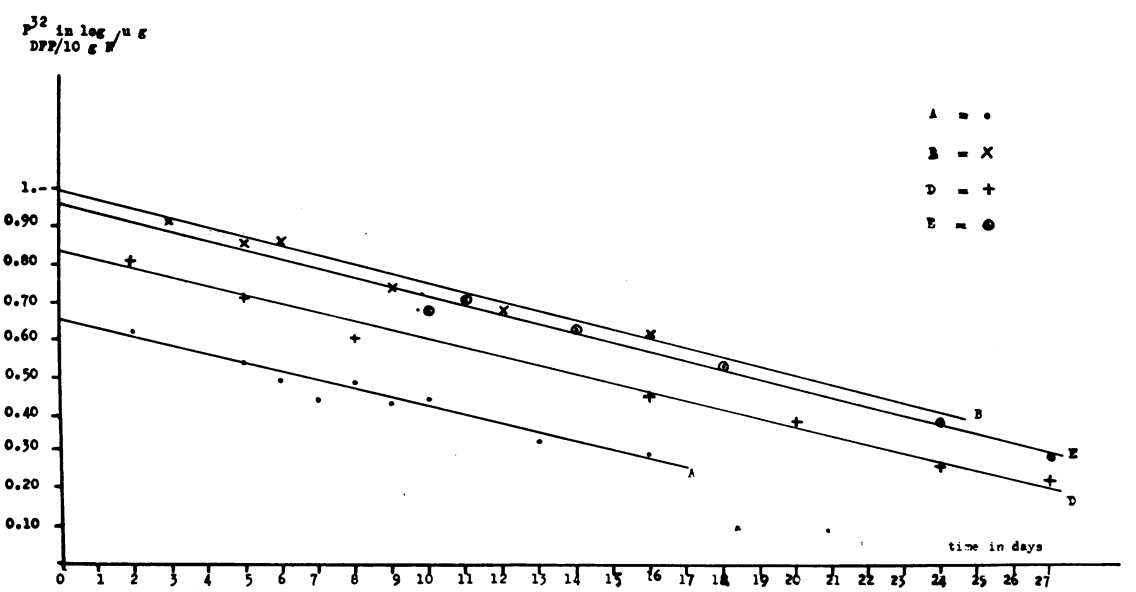


FIG. 2. PLASMA BOUND RADIOACTIVITY AFTER INJECTION OF DFP^{32}
Activity expressed as the logarithm of the weight in μg. of DFP bound to 10 Gm. of plasma nitrogen of Subjects A, B, D, and E. Curves drawn by method of least squares.

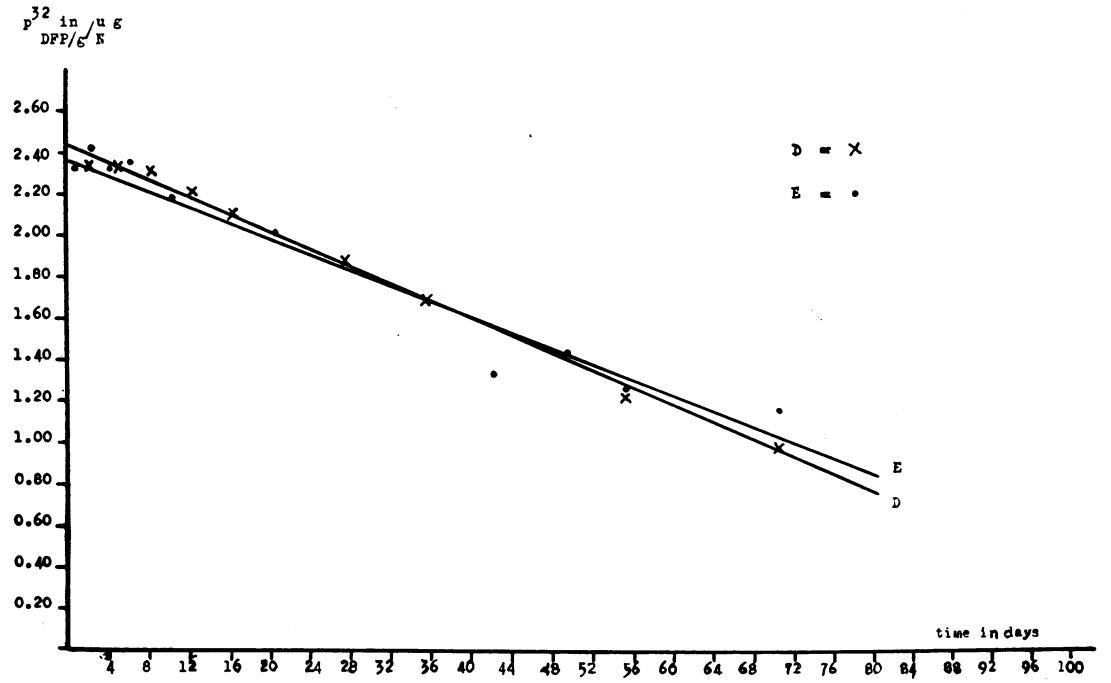


FIG. 3. RED CELL BOUND RADIOACTIVITY AFTER INJECTION OF DFP^{32}

Activity expressed in μg. of DFP reacting per Gm. of erythrocyte nitrogen in the blood of Subjects D and E. Curves drawn by method of least squares.

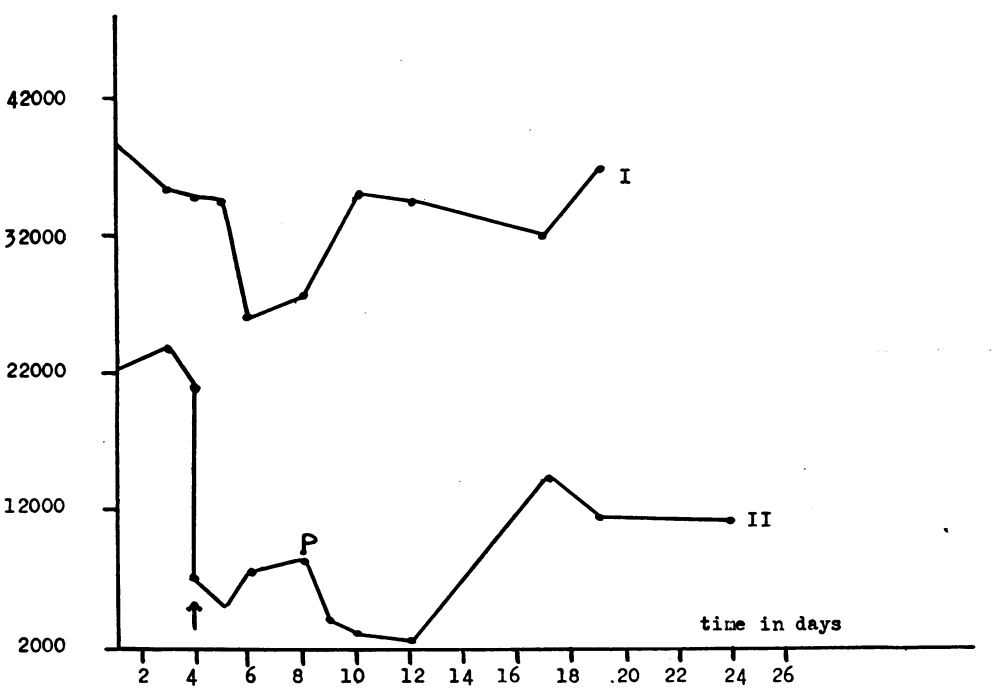


FIG. 4. PLASMA AND RED CELL CHOLINESTERASE

I, Red cell cholinesterase of Case B expressed in μl. CO_2 per hour per 100 Gm. Hb. II, Plasma cholinesterase of same person in μl. CO_2 per hour per 100 Gm. N. The arrow marks the moment of injection of 240 μg. DFP^{32}. P indicates the moment of neostigmine injection.

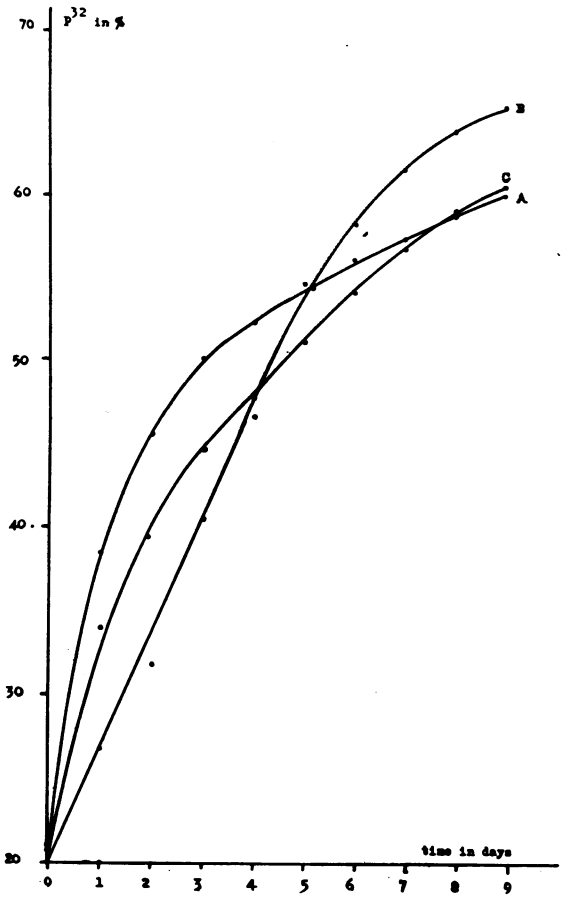


FIG. 5. EXCRETION OF P^{32} IN URINE
The excretion of radioactivity is expressed as percentage of the dose injected.

sirable to repeat in particular the experiments on plasma and red cell turnover using longer periods of observation. For this purpose a higher initial radioactivity of the labelled material was required. This could be achieved by injecting a preparation of a specific activity of approximately 200 μc. per mg. DFP. Two patients of the City Hospital of the Hague were thus treated. One was an 80-year-old man suffering from arteriosclerosis cerebri (D); the other (E) was a 63-year-old woman who, at the time the experiment was started, had completely recovered from an attack of apoplexia and was considered normal. They received dosages of 0.44 and 0.51 mg. of DFP^{32}, respectively.

Resorption of DFP

Blood samples from the fingertip from A and B were examined for radioactivity at 15-minute intervals. Figure 1 shows the course of the radioactivity of the whole blood in counts per minute during an observation period of 130 minutes. There are two main differences between the curves obtained from A and B. Firstly, the maximal activity is reached after 30 minutes in Case A and only after 90 minutes in Case B. Secondly, the level of radioactivity is much higher in the latter case in spite of the fact that only one-eighth of the amount of radioactivity had been injected. These differences are readily explained as the result of the pretreatment with OMPA to which A had been subjected. This treatment has certainly resulted in blocking of the majority of the sites present on proteins of blood plasma and cells which are capable of reacting with DFP. These sites are readily accessible in Case B where they react with DFP to form the irreversible protein-DFP-reaction products well known from cholinesterase inhibition studies. The unbound DFP is assumed to be rapidly removed by the liver, the kidney, and other organs and the difference in level of curves A and B is thus explained on account of differences in bound DFP content of the blood. If the supposition is right that the protein-DFP linkages are irreversible, radioactivity will disappear at the rate of replacement of those proteins by newly formed molecules.

Binding of P^{32} to elements of the blood

For this experiment blood samples from A, B, D, and E were drawn at intervals. After separation of red cells and plasma the amount of DFP in micrograms that had reacted with plasma and cells, respectively, was calculated from the observed radioactivity.

Results are expressed in Figures 2 and 3. It is apparent that, in spite of the larger amount of DFP injected into A, the plasma and red cells harbour considerably less radioactivity than those of B, D, and E, as would be expected from the results presented in the previous passage.

Red cell and plasma cholinesterase activity was measured in the blood of B (Figure 4). A marked inhibition of plasma cholinesterase is observed down to approximately 10 per cent of the initial value. Low values are still observed on the eighth day. Unfortunately at that time it was necessary to start neostigmine treatment so that the further

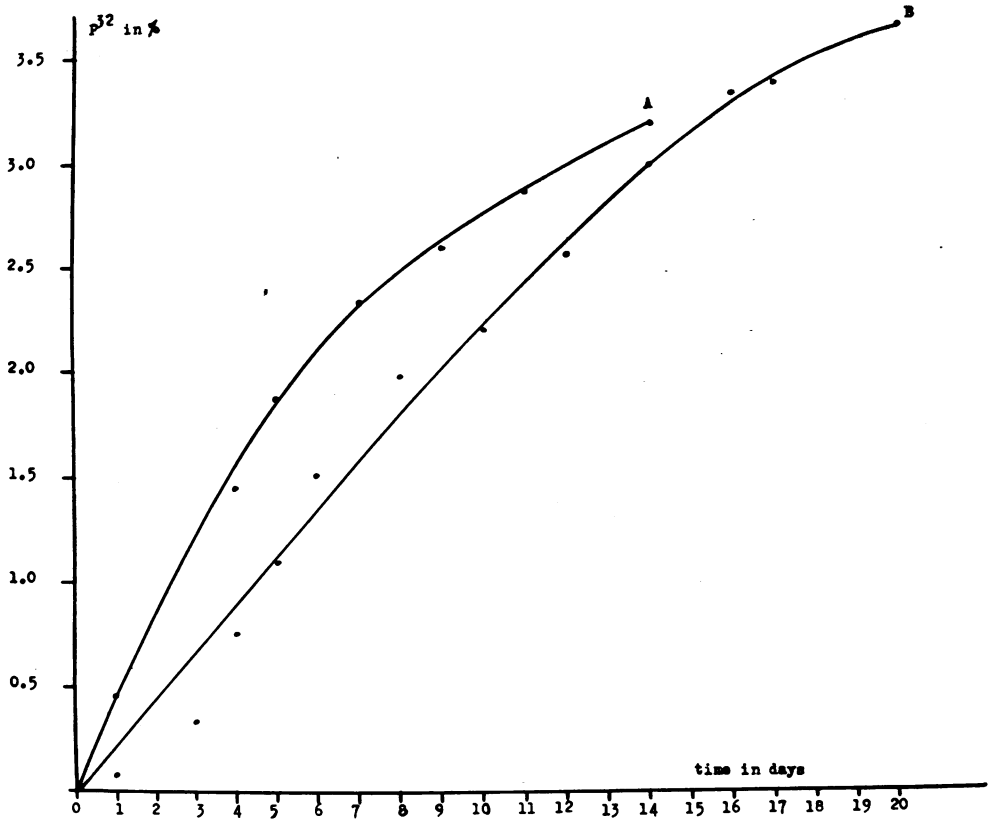


FIG. 6. EXCRETION OF P^{32} IN FECES
The excretion of radioactivity is expressed as percentage of the dose injected.

course of the curve is not fit for detailed analysis. Slow restoration of activity seems however apparent after the twelfth day. The curve for the red cell cholinesterase, which is known to be less sensitive to DFP than the plasma enzyme, shows only a shallow and transitory dip. Moreover it occurred at least 24 hours after the injection. It seems highly unlikely that this transitory dip, occurring after a latent period on a dose of DFP of only 240 micrograms should be attributed to a true DFP inhibition of red cell cholinesterase.

Excretion of DFP^{32}

Radioactivity was measured in the urine of A, B, and C and also in the feces of A and B. In all cases, 60 to 70 per cent of the P^{32} injected as DFP appeared in the urine during the first 10 days (Figure 5). One-third was found in the urine in the first 24 hours. The amount of P^{32} excreted in the urine of A was slightly greater in the first few days and slightly less after the first three to four days compared with B and C. This difference may be due to the OMPA treatment in case A, resulting in a modification of the ratio protein-bound/free P^{32}.

During the observation period less than 5 per cent of the injected radioactivity was recovered in the feces (Figure 6).

Paper chromatography of urine

The results so far reported provide only information on the distribution of the P^{32} incorporated in the injected DFP molecules. They convey nothing concerning the chemical alterations of the DFP molecule between its injection and its excretion. Therefore, the urine was further analysed by paper chromatography to elucidate the chemical character of the radioactive compounds it contained. Portions of urine of B and C collected during the first days after the injection were analysed. Portions of the same urine mixed with diisopropylphosphate (DIP) monoisopropylphos-

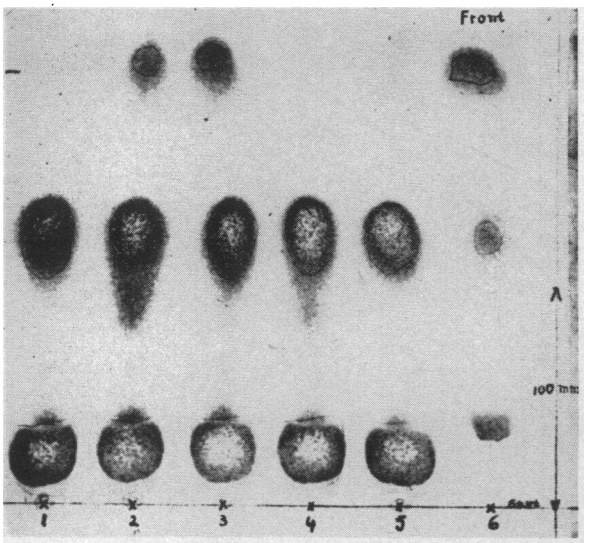


FIG. 7a. PAPER CHROMATOGRAM OF THE URINE OF C

Solvent: butanol-acetic acid-water.

1 urine
2 urine + DIP (diisopropylphosphate) + MIP (monoisopropylphosphate) + AP (anorganic phosphate)
3 urine + DIP

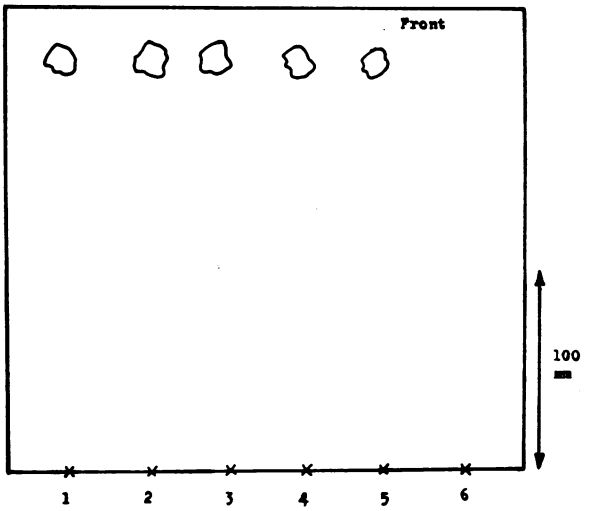


FIG. 7b. RADIOGRAM OF THE CHROMATOGRAM OF FIGURE 7a

4 urine + MIP
5 urine + AP
6 water + DIP + MIP + AP

phate (MIP) and inorganic phosphate were run simultaneously. These substances were considered to be the most likely phosphates to occur in the urine as a result of DFP break-down in the body.

In Figure 7 a chromatogram is presented of five samples of urine of C with additions as indicated. Number 6 is a control in water.

Unmixed urine (No. 1 in Figure 7) shows two large unidentified spots. Comparison with numbers 2, 5, and 6 suggests that the upper part of the lower spot contains probably inorganic phosphate. MIP is located immediately under the upper spot (2, 4, and 6) whereas DIP travels in the front (2, 3, and 6). The original urine (1) cannot be expected to give readily demonstrable spots resulting from DFP or its break-down products because the technique used is not sensitive enough to give a colour with the traces of phosphate^{32} excreted in 40 μl. of urine (see Figure 5).

If, however, an x-ray film is exposed to the same chromatogram, an autoradiogram is obtained. A drawing of this radiogram is presented in Figure 7b. It proves conclusively that the injected DFP appears in the urine in only one form, DIP. It is concluded that at least the main part of the injected DFP is transformed into DIP and excreted as such. It should be noted that these results were obtained on urine of the first days following injection of DFP^{32}. Later, radioactivity becomes too slight to measure dependably. The possibility that in later periods other products appear can, therefore, not be entirely excluded. It could be argued that some of the injected DFP^{32} might be eliminated as such. This product would not appear on the chromatogram because of its volatility. A watery solution of DFP^{32} brought on filter paper rapidly loses its radioactivity due to this volatility. When a drop of fresh urine of C was treated in this way, no radioactivity disappeared on drying, showing that no free DFP was present nor any other volatile P^{32} containing compound.

DISCUSSION

The results obtained on the normal individuals C and E did not differ essentially from those observed in the patients A, B, and D. Such differences as were found between A on the one hand and B, C, D, and E on the other could be easily explained by the OMPA treatment of A prior to the administration of DFP.

The following picture of the fate of DFP may be tentatively suggested as a result of the present

experience with humans and by earlier experiments on animals by other authors (Figure 8). DFP is rapidly absorbed from the intramuscular depot. The presence of radioactive material in the blood can be demonstrated a few minutes after the injection. At the dosage used, maximal radioactivity in the blood is observed after 90 minutes, after which a slow decline occurs, indicating that removal now is predominant over resorption. Free DFP disappears rapidly from the circulation into such organs as kidney, liver, and lung which have been shown to contain radioactivity in similar experiments on rabbits described by Jandorf and McNamarra (3). A large fraction is probably hydrolyzed in the tissues to DIP and excreted into the urine and to a far less extent into the feces. The hydrolysis may be spontaneous but is more likely to be essentially enzymatic. The enzyme system responsible is probably the fluorophosphatase, present in liver and kidney, as described by Mazur (4).

A certain amount of DFP^{32} reacts with the proteins of red cells, blood plasma and tissues. It is implied in the scheme of Figure 8 that this reaction is irreversible and that the reaction products are stable. This assumption is based on many studies reported in the literature, where it has been shown that a number of animal proteins, such as chymotrypsin, trypsin, cholinesterases, aliesterases etc. react with DFP. In all these instances the reaction was irreversible and the reaction product stable.

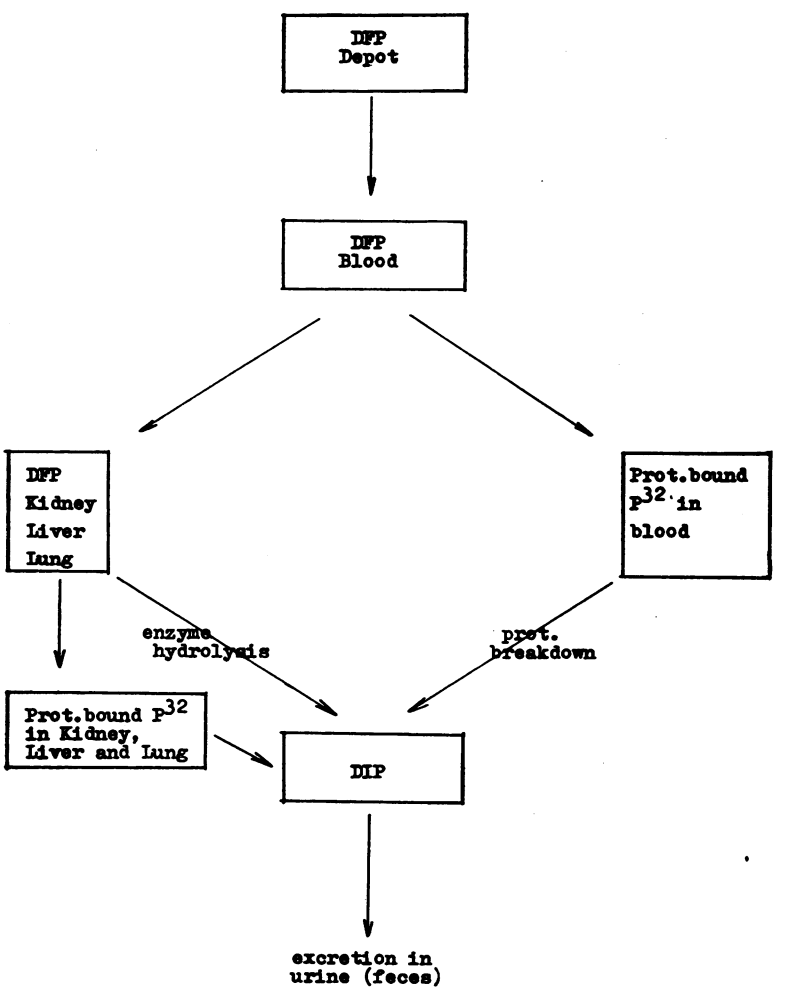
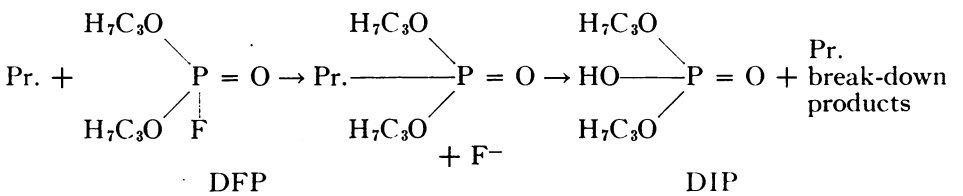


FIG. 8. TENTATIVE DIAGRAM OF THE METABOLIC FATE OF DFP IN THE HUMAN BODY

It seems likely that this protein-DFP interaction bears the same character as the reaction between DFP and chymotrypsin described by Jansen, Nutting, Jang, and Balls (5, 6). It was found that in this reaction DFP lost its fluorine atom whereas the diisopropylphosphate moiety became firmly attached to the protein. As a result of the irreversible character of this linkage further metabolism of the protein bound P^{32} containing moiety is henceforth dependent on the break-down of the protein molecule to which it is attached. When this break-down occurs, again DIP is the product formed.



Pr. = protein

All processes involved result, therefore, in a single final product, the DIP, which is excreted essentially in the urine. DIP, once formed, is not to any extent bound or stored in the body, as has been demonstrated by Jandorf and McNamarra (3). It is not further metabolised and quickly disposed of. It does not give rise to any P^{32} containing compound which is actively involved in body metabolism.

The rapid irreversible reaction of DFP^{32} with certain body proteins suggests its use for the labelling of these proteins. Since, for instance, the radioactivity of labelled red cells can only be reversed by the ultimate destruction of these cells, simple estimations, during a certain period, of red cell linked radioactivity after injection of DFP^{32} should provide information on the rate of break-down of these cells and their life span. In Figure 3 the concentration of red cell linked P^{32} in the subjects D and E is plotted against the time. It will be seen that these two cases have been studied for a period of as long as three months. The P^{32} content of the red cells had reached half its initial value after 58.1 and 64.4 days in D and E, respectively, corresponding to a mean life span of 116 and 129 days. These values compare satisfactorily with those reported in the literature (mean life span 120 days [7]). From the curves of Figure 3 it would seem that during the first 7 to 10 days after the injection of DFP^{32} the red cell bound radioactivity remains constant. After that period radioactivity falls at a steady rate which may be measured and used for calculations of life span. The reason for this initial constancy has not been studied. Similarly irreversible combination of injected DFP^{32} with blood plasma can be used for the calculation of plasma protein turnover (Figure 2). In this case, the curves depicting the elimination of the labelled plasma protein fraction were exponential reflecting random destruction. They were plotted logarithmically to produce straight lines from which the half life time could be calculated. Values of 12 to 14 days were found for the half life of this blood plasma component in the patients A, B, D, and E (13.7, 13.7, 13.1, and 12.5 days, respectively).

It should be kept in mind that the method outlined above can only provide information on the turnover of that plasma component which reacts with DFP. It is probably mainly or exclusively the pseudo-cholinesterase component of human plasma. The half life of 12 to 14 days found by us for this plasma fraction in adult humans is comparable with the figures given by Dixon, Talmage, Maurer, and Deichmiller (8) for gamma globulin in human adults (13 days) and by London (9) for serum proteins (albumin 20 days, β and γ globulin 12 days, γ globulin 19 days). The most important advantage, next to its simplicity, of the present procedure over methods so far described is probably the fact that only one metabolite, the DIP, results from the impact of DFP on the organism, and that this DIP never takes part in any form in further metabolic processes but is rapidly eliminated. Therefore, no active metabolic pool can get polluted with P^{32} and resynthesis of red cells and plasma involving radioactive material is not likely to interfere with the turnover estimations. It might be advantageous to tag plasma or red cells *in vitro* by DFP^{32} and then inject the reaction

products. This possibility has not been explored but seems worth considering. This procedure might also be useful for the determination of red cell and plasma volume, particularly because of the irreversibility of the P^{32} label.

The possibility has not been ruled out as yet that the reaction-products of DFP and plasma or red cells do not behave entirely like the unlabelled structures. More evidence is required before the method can be confidently recommended as a reliable general technique. The values obtained seem reasonable compared with those described in the literature and the regular pattern of the curves provide some confidence that the methods might be useful.

With the dosages used no symptoms of toxicity have been observed.

SUMMARY

1. Five human subjects were injected intramuscularly with varying non-toxic doses of DFP^{32} (diisopropylfluorophosphonate labelled with P^{32}).

2. Under normal conditions radioactivity originating from DFP could be demonstrated in the peripheral blood within a few minutes; maximum radioactivity was reached in 90 minutes.

3. A small portion of the injected P^{32} was irreversibly bound to red cells and plasma. This fraction disappeared from the blood at a rate corresponding to the normal replacement of these elements.

4. This relationship could be exploited to calculate the rate of replacement of a plasma protein and the mean life span of red cells.

Values of 12 to 14 days were found for the half life of this plasma component in four persons.

A mean red cell life span of 116 and 129 days, respectively, was found in two subjects.

5. Injected DFP is excreted in the urine as diisopropylphosphate (60 to 70 per cent in the first ten days); less than 5 per cent of the P^{32} injected as DFP is recovered in the feces during that period.

6. In one person DFP receptors were blocked by treatment with OMPA (octamethylpyrophosphoramide) prior to the injection of DFP^{32}. In this case less radioactivity was bound to plasma and red cells and maximal radioactivity in the blood was reached as soon as 30 minutes after the injection of DFP. Excretion was slightly accelerated. These data are understandable on account of the relative decrease of firmly bound and increase of free DFP^{32} as a result of the OMPA pretreatment. The free DFP is more readily removed from the blood than the bound fraction.

7. It is suggested that labelling by means of DFP^{32} may also be used for the evaluation of red cell and plasma volume.

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