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A Rapid Method for the Determination of Radioactive Diisopropyl Phosphate ($DI^{32}P$), Monoisopropyl Phosphate ($MI^{32}P$) and Inorganic Phosphate (^{32}Pi) in Biological Material

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$DI^{32}P$ is completely recoverable from acidified trichloroacetic acid filtrates by two extractions with a (1:1) mixture of isobutanol and benzene. $MI^{32}P$ is extractable to the extent of about 50 %. On addition of molybdate and further extraction with the solvent mixture only the $^{32}P_i$ is extracted quantitatively, the $MI^{32}P$ remaining in the aqueous phase under the conditions employed. By applying appropriate corrections the true values for $DI^{32}P$, $MI^{32}P$, and $^{32}P_i$ can be calculated. The method has the advantage of being simple, rapid and capable of being used for the routine analysis of a large number of samples in combination with the available methods for the determination of $DF^{32}P$ and $^{32}P_i$.

The biological degradation of diisopropyl phosphorofluoridate (DFP) has been extensively studied,[1–4] but interest has mainly centred on the hydrolysis of the acid anhydride (P—F) bond which is concerned with the toxic and enzyme-inhibitory effects. Very little is known about the further degradation of diisopropyl phosphate (DIP) to monoisopropyl phosphate (MIP) and inorganic phosphate (P_i). The study of the secondary hydrolysis of DFP in the animal system is of importance, since it is known that acetylcholinesterase which has been inactivated by DFP changes quickly from the oxime-reactivable to the non-reactivable form, a process known as 'aging',[5,6] and this conversion is accompanied by the loss of one of the isopropyl groups from the phosphorylated enzyme.[7] The biochemical mechanism by which this is brought about is not understood. To investigate this and similar problems it is necessary to have a convenient and rapid method for the estimation of DIP, MIP and P_i in animal tissues. The available methods based on ion-exchange and paper chromatography [7,8] are time-consuming and not suited for application when large numbers of samples have to be analysed. The present communication

describes a method for the determination of $DI^{32}P$, $MI^{32}P$, and $^{32}P_i$ when $DF^{32}P$ is the starting material. It is similar to the solvent extraction procedure used by Harris *et al.*[9,10] for the determination of the metabolites of Soman.

MATERIALS AND METHODS

$DF^{32}P$ (300 mC/g) was obtained from Radiochemical Centre, Amersham, England. Stock solutions were prepared in propylene glycol to contain 1.85 mg (10 μmoles)/g and preserved at −16° and quantities were weighed out before experiments. $^{32}P_i$ was also obtained from the same source.

Preparation of $DI^{32}P$. $DF^{32}P$ is very rapidly hydrolysed to $DI^{32}P$ by dilute alkali at room temperature. The isopropyl groups are resistant to alkaline hydrolysis but are slowly hydrolysed by boiling with 1 N H_2SO_4.

About 1 g of the stock solution of $DF^{32}P$ was dissolved in 10 ml of 0.2 N NaOH and left at room temperature for 1 h. It was diluted to about 25 ml, acidified with 3 ml of 10 N H_2SO_4 and extracted twice with 30 ml portions of a (1:1) mixture of isobutanol and benzene (BB). The solvent layer was extracted thrice respectively with 10, 5, and 5 ml of 0.1 N NaOH in which the $DI^{32}P$ was completely recovered. This $DI^{32}P$ was found to be completely free from $MI^{32}P$ and $^{32}P_i$ as determined by paper chromatography [8] followed by autoradiography and ion-exchange column chromatography on Dowex-1 (Ref. 8). In case it was found to be impure a repetition of the cycle by acidification and re-extraction with BB gave a pure product. The solution was adjusted to pH 9 at which it was stable. Aliquots were suitably diluted in the following experiments.

Preparation of $MI^{32}P$. When $DI^{32}P$ was boiled under reflux with 1 N H_2SO_4 and aliquots were withdrawn at intervals and diluted with 1 N H_2SO_4 and extracted with BB, the extractable radioactivity was found to decrease progressively with a corresponding increase of $^{32}P_i$ and the un-extractable radioactivity (Table 1, second and third columns, respectively). Since at 6 h the amount of activity in the acidic aqueous phase (corresponding to $MI^{32}P$) seemed to decrease, a sample of $DI^{32}P$ was hydrolysed for this period and repeatedly extracted with an equal volume of BB each time. Extracts 1 and 2, extract 3 and extracts 4—10 were pooled and the radioactivity recovered by re-extraction with 0.1 N NaOH. The pooled extracts 1 and 2 consisted of a mixture of $DI^{32}P$

Table 1. Acid hydrolysis of $DI^{32}P$.

Time (h)	Radioactivity as per cent of the total in Solvent ext. of hydrolysate	Solvent ext. after addition of molybdate	Acidic aqueous phase
0	95.7	3.0	1.3
2	78.9	3.9	17.2
4	63.7	10.0	26.3
6	50.8	19.2	30.0
8	41.5	26.4	32.1
12	25.4	46.8	27.8
24	17.0	60.6	22.4

14 ml of a solution of $DI^{32}P$ (containing approximately 80 000 cpm/ml of radioactivity) was diluted to 140 ml after the addition of 14 ml of 10 N H_2SO_4 and boiled under reflux. 6 ml aliquots were withdrawn at intervals and extracted once with 6 ml of a (1:1) mixture of isobutanol-benzene. Of the aqueous phase, 3 ml were mixed with 0.5 ml of a 10 % solution of ammonium molybdate and extracted with 3 ml of the solvent mixture. Radioactivity was determined in the solvent layers and the acidic aqueous phase as described under Methods.

and $MI^{32}P$ as determined by paper chromatography and autoradiography. Extract 3 showed predominantly $MI^{32}P$ with a faint spot of $DI^{32}P$. The pooled extracts 4—10 consisted only of $MI^{32}P$.

If the logarithm of the radioactivity in each extract was plotted against the number of extractions a straight line graph was obtained after the third extract, the slope of which was 0.7. This showed that about 30 % of the $MI^{32}P$ in the aqueous phase was extracted each time.

The pH of the $MI^{32}P$ solution was adjusted to 9 by the addition of dilute H_2SO_4 and aliquots were suitably diluted for further experiments.

Radioactivity measurements. These were done by pipetting out 0.5 ml aliquots in glass cups in triplicate, drying them in an air-oven at 80—110° and counting in a Rotatory Automatic Fraction Changer (LKB-Produkter AB, Stockholm) fitted with an end-window GM tube. Solvents and aqueous solutions which did not already contain H_2SO_4 were evaporated in the presence of 2 drops of 1 N H_2SO_4 as otherwise low values were obtained especially for $DI^{32}P$.

RESULTS AND DISCUSSION

Extractability of $DI^{32}P$ and $MI^{32}P$ from various media. Table 2 gives the results. It is seen that from acidic solutions of pH 1.0 and below $DI^{32}P$ was extractable to the extent of more than 90 %, which would give an almost complete recovery in 2 extractions. The extractability decreased progressively

Table 2. Extractability of $DI^{32}P$ and $MI^{32}P$ from various media.

No.	Medium	Percent radioactivity extracted (with S.D. and No. of trials) $DI^{32}P$	$MI^{32}P$
1.	1.0 N H_2SO_4	93.1 ± 0.9 (46)	29.8 ± 2.9 (4)
2.	0.5 »	91.8 ± 0.4 (18)	29.2 (2)
3.	0.1 »	90.3 ± 0.5 (21)	22.5 (2)
4.	20 % TCA	93.2 ± 0.6 (18)	20.3 ± 0.9 (8)
5.	10 % »	90.8 ± 0.2 (9)	20.1 ± 0.4 (8)
6.	5 % »	87.5 ± 1.7 (9)	19.5 ± 0.4 (8)
7.	2.5 % »	85.3 ± 0.7 (9)	18.7 ± 0.3 (8)
8.	KCl—HCl buffer, 0.05 M, pH 1.0	92.3 ± 0.4 (9)	29.0
9.	» » » 2.0	58.5 ± 2.0 (9)	5.6
10.	Glycine-HCl buffer » 3.0	17.3 ± 1.7 (9)	0.8
11.	Acetate buffer » 4.0	4.2 ± 0.2 (9)	0.3
12.	» » » 5.0	0.6 ± 0.1 (9)	0.0
13.	1.0 N N_2SO_4 + molybdate	92.2 ± 0.2 (4)	23.4 ± 2.3 (4)
14.	0.5 » »	89.7 ± 0.5 (6)	3.4 ± 1.4 (3)
15.	0.1 » »	83.4 ± 0.6 (6)	0.0 (3)

10 ml of $DI^{32}P$ solution in the appropriate medium and containing 4000—8000 counts/min/ml of radioactivity was extracted once with 10 ml of a (1:1) mixture of isobutanol and benzene. 0.5 ml aliquots of the solvent and the aqueous phase were plated with 2 drops of 1 N H_2SO_4 for radioactivity determinations. The results are expressed as percentage of radioactivity in the solvent layer in terms of the total in the two phases. In the case of $MI^{32}P$, the experiments with TCA were carried out as above. With others 10 ml of $MI^{32}P$ solution containing 10^5—10^6 counts/min/ml in the appropriate medium were extracted 10 times with 10 ml portions of the solvent and the logarithm of the radioactivity extracted each time was plotted against the number of extractions. From the slope of the straight line part of the graph the extractability was calculated.

as the acidity was decreased. It was unextractable from media of pH 5 and above. $MI^{32}P$ was extractable to a much less extent than $DI^{32}P$. It was extractable from media containing 0.5 to 1.0 N H_2SO_4 about 29 % each time. This showed that almost exactly 50 % of the total $MI^{32}P$ would be extracted in two extractions. Not included in the table are figures for the extractability of $DI^{32}P$ and $MI^{32}P$ from solutions which contained both H_2SO_4 and 10 to 20 % trichloroacetic acid (TCA). The values were found to be almost the same as with H_2SO_4 alone.

Separation of $MI^{32}P$ and $^{32}P_i$. In the presence of ammonium molybdate $MI^{32}P$ was extractable to the extent of 23.4 % from solutions which contained 1.0 N H_2SO_4 (item 13, Table 2). The extractability steeply decreased (2 to 5 %) as the acidity was lowered to 0.5 N and it was completely un-extractable at 0.1 N (items 14 and 15, Table 2). By trial experiments it was found that $^{32}P_i$ was only partially (87 %) extracted as the phosphomolybdate from acid of 0.1 normality but that it was quantitatively extracted (99—101 %) from 0.5 N H_2SO_4. Thus, by adding ammonium molybdate and extracting with BB from media containing 0.5 N H_2SO_4 it will be possible to effect an almost complete separation of $MI^{32}P$ and $^{32}P_i$.

Suggested procedure for a mixture of $DI^{32}P$, $MI^{32}P$ and $^{32}P_i$. Trial experiments were carried out with 1:10 aqueous homogenates of mouse liver, kidney, lungs, and brain to which known amounts of $DI^{32}P$, $MI^{32}P$ and $^{32}P_i$ were added either individually or as a mixture. TCA (100 % w/v) was added immediately to a final concentration of 16 % and the mixture was filtered. Radioactivity determinations in the TCA-filtrates showed that recoveries were quantitative. There was no degradation of $DI^{32}P$ or $MI^{32}P$ on standing at room temperature with TCA in concentrations up to 20 % for 48 h.

For the estimation of $DI^{32}P$, $MI^{32}P$, and $^{32}P_i$, a known volume of the TCA-filtrate (9.5 ml) was acidified with 10 N H_2SO_4 (0.5 ml) to a final acid concentration of 0.5 N and extracted twice with an equal volume each time of BB. Radioactivity was determined in 0.5 ml aliquots of the solvent extract (S). An aliquot (5 ml) of the aqueous phase was treated with 0.5 ml of a 10 % solution of ammonium molybdate in water and extracted once with 5 ml of BB. The radioactivity in the solvent phase (MS) and the aqueous phase (MW) was determined. The activity in MW represented 50 % of the total $MI^{32}P$ (29.2 % + 20.8 % in the two extractions respectively to obtain S) if the small amount of $MI^{32}P$ carried over with the $^{32}P_i$ (MS fraction) was ignored. The true value for $MI^{32}P$ originally present was thus taken as equal to twice the amount of radioactivity in the MW fraction. The activity in fraction MS was taken to represent that of $^{32}P_i$. The true value of $DI^{32}P$ was equal to the activity in S minus that in MW.

Table 3 gives the results of some experiments. Larger volumes of TCA-filtrates (50—100 ml) which contained lower radioactivity such as those obtained from brain tissue have been worked up as above. In these cases the activity in the solvent phases were concentrated by rendering them alkaline with 4 N NaOH in which all the activity was extractable. These as well as the MW fractions were then further concentrated, if necessary, by evaporation before plating.

Table 3. Recovery of DI^{32}P, MI^{32}P, and $^{32}P_i$ added to mouse liver homogenates.

Expt. No.	Radioactivity in counts/min				
	Fraction S	Fraction MS (=$^{32}P_i$)	Fraction MW	DI^{32}P (S minus MW)	MI^{32}P (MW × 2)
1	74 280	83 700	19 932	54 348	39 864
		111 %		101 %	108 %
2	69 280	80 000	19 206	50 074	38 412
		106 %		93 %	104 %
3	68 120	79 400	19 426	48 694	38 852
		105 %		90 %	105 %
4	69 320	71 880	18 458	50 862	36 916
		102 %		94 %	100 %
5	69 720	76 620	17 270	52 450	34 540
		102 %		97 %	93 %
6	70 040	76 560	18 502	51 538	37 004
		101 %		95 %	100 %

To 10 ml of a mixture which contained 2 ml each of DI^{32}P, MI^{32}P, $^{32}P_i$ and 100 % (w/v) TCA, 1 ml of 10 N H_2SO_4 and 1 ml water, 10 ml of a (1:10) mouse liver homogenate in water was added. The mixture was filtered and 10 ml aliquots were analysed as described. (S = solvent extract, MS = solvent extract on addition of molybdate and further extraction, MW = the acidic aqueous phase after extraction of $^{32}P_i$ as phosphomolybdate. The values are expressed as CPM in 10 ml of the TCA filtrate which contained DI^{32}P (54 000), MI^{32}P (36 980) and $^{32}P_i$ (75 360) counts/min. The percent recoveries refer to these figures.

For the determination of DI^{32}P and MI^{32}P in DF^{32}P-inhibited enzyme material, the phosphoprotein was obtained by any one [11] of the well-known methods. The precipitate was then treated with 1 N NaOH to release the organophosphate moieties and the solution treated with TCA. The filtrate was then acidified and analysed as described above.

The suggested procedure is only approximate but it has the advantage of being simple, rapid and capable of being combined with the well-known Martin and Doty procedure for the determination of $^{32}P_i$ (Refs. 12, 13). The foregoing results also indicate the possible sources of error in the determination of $^{32}P_i$ by the above method where DF^{32}P has been used such as in enzyme-inhibition studies.

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