Phosphorus Compounds in the Cell

1. PROTEIN-BOUND PHOSPHORUS FRACTIONS STUDIED WITH THE AID OF RADIOACTIVE PHOSPHORUS

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It has previously been shown (Brues, Tracy & Cohn, 1944; Hammarsten & Hevesy, 1946; Davidson, 1947) that when nucleic acids are isolated from the tissues of animals to which radioactive phosphorus (^{32}P) has been administered, the specific activity of the ribonucleic acid phosphorus (RNAP) is much greater than that of the deoxyribonucleic acid phosphorus (DNAP). These measurements have been carried out on ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) which have been isolated in a purified state from animal tissues (mainly the liver), but such a procedure requires a larger amount of tissue than is commonly available from small laboratory animals. It was therefore thought desirable to follow the distribution of radioactive phosphorus in the fractions separated by some such scheme as that of Schmidt & Thannhauser (1945), to determine whether isolation of nucleic acids in a pure state was in fact necessary, and to investigate phosphorus compounds other than the nucleic acids. Preliminary accounts of this work have already appeared (Davidson, Gardner, Hutchison, McIndoe, Raymond & Shaw, 1949; Davidson, Gardner, Hutchison, McIndoe & Shaw, 1949).

METHODS

Each animal (rabbit or rat) received radioactive P (^{32}P as inorganic phosphate; $100\text{ }\mu\text{c./kg.}$ body weight) by subcutaneous injection between the shoulder blades. After an interval of usually 2 hr. the animals were killed either by cervical dislocation or by exsanguination under ether anaesthesia. The abdomen was quickly opened and the liver perfused with warm 0.9% NaCl solution through the portal vein. The organs and tissues required were rapidly removed, weighed, and chilled in ice. They were then homogenized in a Waring blender with an ice jacket, or in a Nelco blender, in 4 (or 9) vol. of ice-cold distilled water or 0.9% NaCl solution. For small organs, such as the spleen, the entire homogenate was taken for analysis. With larger organs, e.g. the liver, an appropriate portion such as 20 ml. corresponding to 4 (or 2) g. tissue was employed. The homogenate was then submitted to the slight modification of the Schmidt-Thannhauser (1945) method of fractionation shown in Table 1. One half vol. of 30% (w/v) A.R. trichloroacetic acid (TCA) was added to the homogenate which was then allowed to stand for at least 30 min. in ice. In some of the later experiments ^{32}P in 30% TCA was added to homogenate of tissues from animals which had received ^{32}P 2 hr. previously or from control animals which had received no ^{32}P . The precipitated material was then centrifuged down and washed with ice-cold 10% (w/v) TCA. In the initial experiments two washings with TCA were

Table 1. *Scheme of separation of phosphorus compounds in tissues*

(Slightly modified from Schmidt & Thannhauser, 1945.)

employed; subsequently as many as seventy washings were employed using a refrigerated centrifuge. The supernatant fluid and washings were set aside on ice for further fractionation as the acid-soluble P fraction (A_1S). The precipitate was extracted successively with acetone, ethanol, CHCl_3 , ethanol-ether (3:1) (twice), and ether, and the extracts combined. These extracts were then taken to dryness on the water bath and the dry residue extracted with CHCl_3 to provide the lipid fraction (lipid P). A small amount of material in the lipid extract remained insoluble in CHCl_3 , but contained a small amount of P.

The dry residue which remained was treated with a volume of N-NaOH equal to that of the original homogenate and was then incubated overnight at 37° . It was invariably found, contrary to the experience of Schmidt & Thannhauser (1945), but in agreement with observations of Mann & Gruschow (1949), who ignored the phenomenon, that the alkaline digest was very slightly turbid owing to the presence of a minute amount of insoluble material. This was usually filtered off through a hardened paper (Whatman no. 50), the process of filtration which took up to 24 hr. being accelerated by warming to 37° . The small precipitate was washed with distilled water and set aside for analysis (insoluble P). A 30 ml. portion of the alkaline digest plus the washings (alkali-soluble P) (total vol. 40 ml.) was then treated with 1.5 ml. of 10 $\text{N-H}_2\text{SO}_4$ and 20 ml. ice-cold 30% (w/v) TCA. The precipitate containing proteins and DNA was centrifuged down and washed twice with small quantities of 10% TCA. The final residue was dissolved in N-NaOH to yield fraction R. The supernatant fluid and washings, containing, among other things, the ribonucleotides formed by the breakdown of RNA, were combined and set aside as the second acid-soluble P fraction (A_2S).

Both acid-soluble fractions A_1S and A_2S contained inorganic phosphate, derived in the former from the inorganic phosphate of the tissue, and in the latter presumably from phosphoprotein (Plimmer & Bayliss, 1906). A portion of each extract was therefore taken for precipitation of inorganic phosphate as $\text{Mg}(\text{NH}_4)\text{PO}_4$. To such portions Mathison's (1909) reagent was added (1 ml. to 10 ml. extract) and the mixture made alkaline with NH_4OH . After standing overnight in the refrigerator the precipitate (which was minute and almost invisible in the case of the material from A_2S) was filtered off through a Whatman no. 42 paper, washed with dilute NH_4OH and dissolved in N-HCl . This provided the inorganic phosphate fractions P_1 and P_2 respectively. The mother liquors were taken as the organic phosphate fractions S_1 and S_2 respectively. An outline of the scheme of separation is shown in Table 1. Suitable portions of all fractions (in some cases the entire fractions) were taken for the determination of P by the method of Allen (1940) suitably modified for small quantities where necessary.

In some instances the separation procedure of Schneider (1945) was also used. The homogenate (20 ml.), corresponding to 4 g. of tissue, was submitted to extraction with TCA and lipid solvents as already described. The dry and finely powdered residue was then extracted for 15 min. with 5% TCA at 90° to remove nucleic acids, leaving a P-containing protein residue. The precipitate was washed twice with 5% TCA, supernatant fluid and washings being combined as the 'Schneider extract' and the final residue being dissolved in N-NaOH as the 'Schneider residue'.

For the determination of radioactivity, portions of each solution were pipetted into a liquid counter (Type M6

manufactured by 20th Century Electronics) attached to a conventional probe unit and scaling unit (Type 200; manufactured by Dynatron Radio Ltd.). All specific activities were calculated as counts/min./100 μg . P.

Isolation of nucleic acids

Since RNA is mainly cytoplasmic in origin while DNA is wholly nuclear, the isolation of each nucleic acid free from contamination by the other is clearly facilitated by a preliminary separation of cytoplasmic from nuclear material. Accordingly, a portion of liver homogenate in 0.9% NaCl was centrifuged at low speed for 5 min. The turbid supernatant fluid was treated with 2 vol. of acetone and the precipitate centrifuged down, washed with ethanol and ether and dried. It was used for preparation of RNA.

The precipitate of nuclei and cell debris was washed twice with ice-cold 0.9% NaCl and was then treated with acetone, ethanol and ether and dried. It was used for isolation of DNA.

RNA. The dried cytoplasmic material was extracted three times for 1 hr. each with 40 ml. of 10% (w/v) NaCl solution at 100° . The combined extracts were treated with 2 vol. of ethanol and the precipitate of sodium nucleate was centrifuged down and washed with ethanol and ether. It was then dissolved in 10 ml. of water and the small insoluble residue was discarded. The supernatant fluid was treated with 2 vol. of glacial acetic acid and the precipitate so obtained was collected, washed with ethanol and ether and dried. The dry powder was dissolved in a small volume of ice-cold water with the cautious addition of 0.1 N-NaOH in the cold. The solution was made just alkaline to phenol red; a small insoluble residue was centrifuged down and discarded. Traces of protein were removed from the supernatant fluid by shaking with a small amount of CHCl_3 -octanol mixture (4:1) and centrifuging. The aqueous supernatant layer was poured into 10 vol. of glacial acetic acid, the precipitated nucleic acid being centrifuged down, washed with ethanol and ether and dried.

DNA. The dried nuclear material was extracted for three successive 1 hr. periods in the boiling-water bath with 10 ml. portions of 10% NaCl. The combined extracts were treated with 2 vol. of ethanol and the precipitate so obtained was centrifuged down, washed with ethanol and ether and dissolved in 10 ml. water. A small insoluble residue was centrifuged down and discarded. The supernatant fluid was then treated with 2 vol. of ethanol and the precipitate collected and dissolved in 2 ml. of N-NaOH . The solution was incubated overnight at 37° to hydrolyse any RNA present and was then treated with 2 ml. of 2 N-HCl and 2 vol. of ethanol. The precipitate was collected, washed with ethanol and ether, dried and dissolved in about 5 ml. of water with the cautious addition of 0.1 N-NaOH . CHCl_3 -octanol (4:1) (0.5 ml.) was added and the mixture shaken and centrifuged. The supernatant fluid was poured from the protein gel into 5 vol. of a mixture of 2 ml. of conc. HCl and 10 ml. of ethanol, and the precipitate so obtained was washed with ethanol and ether and dried.

RESULTS

A large number of different organs and tissues from different species has been examined, and the results have been found to follow the same general pattern. The results of typical experiments on rabbit liver and spleen are shown in Tables 2 and 3 respectively.

Table 2. *Distribution of phosphorus and radioactivity in rabbit liver 2 hr. after injection of ^{32}P*

(Fractionation by modified Schmidt-Thannhauser procedure. Rabbit wt., 2200 g.; liver wt., 59 g.; dose of ^{32}P , 220 μc . by subcutaneous injection.)

Fraction	Phosphorus content (mg./100 g. fresh tissue)	Specific activity (counts/min./100 μg . P)
A_1S	142.7	2470
S_1	109.4	1572
P_1	32.0	4933
Lipid P	116.1	127
Alkali-soluble P	88.2	214
Insoluble P	2.5	331
A_2S	66.4	252
S_2	65.7	225
P_2	0.89	818
R	20.4	24

Table 3. *Distribution of phosphorus and radioactivity in rabbit spleen 2 hr. after injection of ^{32}P*

(Fractionation by modified Schmidt-Thannhauser procedure. Rabbit wt., 2480 g.; spleen wt., 1.6 g.; ^{32}P dose, 250 μc . by subcutaneous injection.)

Fraction	Phosphorus content (mg./100 g. fresh tissue)	Specific activity (counts/min./100 μg . P)
A_1S	76.9	2539
S_1	52.5	1564
P_1	22.3	3754
Lipid P	33.1	44
Alkali-soluble P	105.0	284
Insoluble P	2.7	321
A_2S	52.5	456
S_2	49.0	457
P_2	1.04	1101
R	51.2	53

It is seen that after 2 hr. the inorganic phosphate P_1 shows the highest activity of all, and contributes to the high activity of the A_1S fraction, although the organic phosphorus (S_1), which makes up the rest of this fraction and consists chiefly of phosphate esters, is also highly active. Of the remaining fractions the

P_2 fraction (the 'phosphoprotein phosphorus'), although present in amounts of the order of only 1 mg./100 g. of liver, has an activity which is much higher than those of fractions S_2 and R in which the phosphorus is predominantly derived from the nucleic acids. The activity of the P_2 fraction is responsible for the high activity of fraction A_2S which is almost invariably higher than that of either of the nucleic acid fractions.

The insoluble P, i.e. the phosphorus contained in the insoluble material present in the alkaline digest, is also low in amount, but its activity is usually high, though not so high as that of the P_2 fraction.

Table 4 shows a comparison for rabbit liver of the activities of fractions S_2 and R obtained by the Schmidt-Thannhauser separation procedure with those of nucleic acids isolated from the same samples of tissue. The results show a wide scatter, but the activity of fraction S_2 , which contains the ribonucleotides derived from RNA, is invariably higher than that of isolated RNA, while that of R is higher than that of isolated DNA. The discrepancy in the latter case is not so great as in the former, so that the activities ratio S_2/R is in most cases higher than the ratio RNAP/DNAP.

In some of our experiments an additional portion of the original tissue homogenate was subjected to the separation procedure of Schneider (1945). Table 5 shows a comparison of the results obtained for rabbit liver by this method with those obtained by the method of Schmidt & Thannhauser (1945). The specific activity of the phosphorus in the Schneider extract, which should contain both types of nucleic acid or their hydrolysis products, should give a value intermediate between those found for the separate S_2 and R fractions in the Schmidt-Thannhauser method. Though this is generally approximately true, the phosphorus content is always less than that of S_2 plus R . The Schneider residue, on the other hand, shows a much higher phosphorus content than would be expected on the basis of 'phosphoprotein' alone. Since the activity of this fraction, though high, is invariably lower than

Table 4. *Specific activities of phosphorus fractions obtained from rabbit-liver homogenates by the Schmidt-Thannhauser method compared with the activities of nucleic acids isolated from the same material*

(Dose of ^{32}P , 100 μc ./kg. body wt., 2 hr. before killing. Activities are expressed as counts/min./100 μg . P.)

Rabbit no.	Activities of fractions		Ratio of activities S_2/R	Activities of isolated nucleic acids		Ratio of activities RNAP/DNAP
	S_2	R		RNAP	DNAP	
26	281	15	18.7	57	7	8.1
29	173	32	5.4	54	3	18.0
32	487	61	8.0	105	11	9.5
33*	311	16	19.4	45	5	9.0
46	118	30	3.9	52	15	3.5
47	322	21	15.3	59	13	4.5
49	237	16	14.8	91	12	7.6

* Pregnant.

Table 5. *Comparison of distribution of phosphorus and radioactivity in fractions isolated from rabbit-liver homogenates by the Schmidt-Thannhauser and by the Schneider procedures*

(Dose of ^{32}P , 100 $\mu\text{c.}$ /kg. body wt., 2 hr. before killing. P , amount of phosphorus in mg./100 g. fresh tissue; C , specific activity of phosphorus in counts/min./100 $\mu\text{g. P.}$)

Method	Schmidt-Thannhauser						Schneider			
Fraction	S_2		P_2		R		Extract		Residue	
Rabbit no.	P	C	P	C	P	C	P	C	P	C
26	44.1	281	1.0	428	13.9	15	52.3	82	22.0	133
29	77.7	173	1.4	1336	12.1	32	65.9	127	24.3	423
32	82.6	487	1.0	566	14.3	61	40.5	281	80.5	393
33	74.2	311	0.8	2585	17.9	16	60.0	203	38.5	525
46	75.8	118	0.2	2008	14.3	30	68.1	108	68.5	306
49	71.4	237	3.3	1208	22.4	16	70.9	131	23.2	137
50	65.7	225	0.9	818	20.4	24	60.6	153	24.0	416

that of P_2 it would appear that the phosphoprotein phosphorus in the Schneider residue is diluted by phosphorus of lower activity. The amount of phosphorus in the Schneider residue, which is often casually labelled 'phosphoprotein phosphorus', was so much greater than the amount of phosphorus in the P_2 fraction that it was thought desirable to carry out a Schmidt-Thannhauser separation on the Schneider residue. Table 6 shows the results of an

organic phosphorus fraction S_3 and an inorganic phosphorus fraction P_3 . All these fractions had widely differing specific activities, but fraction P_3 , the true inorganic phosphate, was present in the same order of quantity as P_2 and had a similar specific activity. It seems probable therefore that in the Schneider residue only fraction P_3 corresponds to fraction P_2 in the Schmidt-Thannhauser procedure, i.e. protein-bound phosphorus which is liberated in the inorganic state on incubation with alkali.

Table 6. *Distribution of phosphorus and radioactivity in fractions obtained from rabbit-liver homogenates by application of the Schmidt-Thannhauser separation alone and in combination with the Schneider procedure*

Fraction	Phosphorus content (mg./100 g. fresh tissue)	Specific activity (counts/min./ 100 $\mu\text{g. P.}$)
Schmidt-Thannhauser method		
Alkali-soluble P	59.4	79
Insoluble P	0.3	105
A_2S	47.7	170
S_2	47.0	156
P_2	0.4	1641
R	11.7	19
Schneider method		
Schneider extract	44.5	52
Schneider residue dissolved in alkali and fractionated by the Schmidt-Thannhauser procedure, to yield:		
Alkali-soluble P	11.6	359
Insoluble P	0.1	15
Acid-soluble fraction (A_3S)	10.8	404
S_3	9.9	369
P_3	0.2	1850
R_3	1.5	235

experiment in which the Schneider residue was dissolved in N -sodium hydroxide and incubated in the usual Schmidt-Thannhauser manner, thereafter yielding on acidification an acid-soluble fraction A_3S and a residue R_3 . Fraction A_3S was divided by precipitation with Mathison's reagent into an

In our usual separation procedure the amount of fraction P_2 actually handled is invariably very small (of the order of 10 or 20 $\mu\text{g.}$) and yields a precipitate of magnesium ammonium phosphate which is scarcely visible. Since the error in estimating such minute amounts of phosphorus tends to be high, we have carried out one or two experiments in which the amount of liver tissue extracted was of the order of 100 g. instead of the usual 4 g. In such experiments the amount of P_2 obtained was about 1 mg. and could easily be estimated with accuracy. Its specific activity was as high as in experiments conducted on a much smaller scale.

In one set of experiments an attempt was made to follow the variations in the activity of fraction P_2 with time. Female albino rats weighing about 150 g. each received a dose of 10 $\mu\text{c.}$ of radioactive phosphorus per 100 g. body weight intramuscularly into the right thigh. Groups of six animals each were killed 2, 4, 24 and 48 hr. after injection and the livers were submitted to the fractionation scheme shown in Table 1. The results of such an experiment are summarized in Fig. 1 in which the mean specific activities of the fractions A_2S , S_2 , P_2 and R relative to that of fraction P_1 as unity are plotted against time. There is a very considerable scatter in the results obtained from different animals, but it is clear from the graph that there is no direct correlation between the specific activity of fractions P_1 and P_2 . The graph also shows that there is a steady rise in the specific activity of fractions A_2S and S_2 relative

to that of fraction P_1 , and that in the later stages of this experiment these activities actually exceeded that of fraction P_2 .

Fig. 1. Variations in the specific activities of phosphorus-containing fractions of rat-liver homogenates (relative to the activity of liver inorganic phosphate as unity) up to 48 hr. after intramuscular injection of ^{32}P (as inorganic phosphate). Schmidt-Thannhauser procedure. Each point represents mean value from six animals. (For the significance of P_1 , P_2 , A_2S , S_2 and R , see text, p. 312.)

It is generally considered that three or four extractions with TCA are sufficient to remove all acid-soluble material from a tissue homogenate, but we have thought it necessary to investigate this point in some detail. Table 7 shows the results of an experiment in which three equal portions of liver homogenate were extracted three, six and twenty times respectively with ice-cold 10% TCA. There is little difference between the results following three and

six extractions, and in both cases the amounts and activities of fraction P_1 are of the same order. Twenty extractions, however, result in the removal of rather more phosphorus from the tissue homogenate, but from the eleventh to the twentieth extraction the specific activity of the phosphorus removed steadily falls to a value far below that of the main bulk of the acid-soluble fraction and indeed below that of most of the fractions containing phosphorus originally protein-bound. This suggests that the phosphorus removed in the later washings is not merely acid-soluble phosphorus which has failed to be extracted in the earlier washings, but that it is more probably derived from the breakdown of protein-bound phosphoric esters. It is important to note that fraction P_2 in all three cases is of the same order of amount and activity. Indeed the activity of the P_2 fraction from the tissue which has been extracted twenty times is not in any way diminished and is greater than that of the phosphorus extracted in the sixteenth to twentieth washings. These results suggest that fraction P_2 must contain inorganic phosphate other than that which has failed to be removed owing to inadequate extraction with TCA. Indeed, an experiment recorded in Table 10 shows that the amount of fraction P_2 is in no way reduced even after fifty-seven extractions with TCA.

While fraction P_2 appears to be bound to protein in such a way that it is released only on incubation with alkali, it is nevertheless possible that the specific activity of this fraction, and indeed of all the protein-bound fractions, may be unduly high owing to contamination with traces of inorganic phosphate derived from fraction P_1 , and, as has already been mentioned, the results recorded in Table 4 strongly suggest this possibility. It should be emphasized that the specific activity of the inorganic phosphate

Table 7. Comparison of the effects of extracting rat-liver tissue three, six and twenty times with 10% TCA

(Dose of ^{32}P , 100 μC /kg., 2 hr. before killing. Schmidt-Thannhauser procedure. P , amount of phosphorus in mg./100 g. fresh tissue; C , specific activity in counts/min./100 μC P.)

Fraction	Number of extractions with TCA					
	3		6		20	
	P	C	P	C	P	C
A_1S	107.5	5380	111.6	5120	111.4	5410
A_1S , 11th-15th extractions	—	—	—	—	2.9	1348
A_1S , 16th extraction	—	—	—	—	2.2	710
A_1S , 17th-19th extractions	—	—	—	—	5.3	481
A_1S , 20th extraction	—	—	—	—	1.0	189
S_1	66.8	3415	59.7	3490	64.9*	3910*
P_1	40.4	7450	41.3	7635	43.7*	7820*
Lipid P	108.1	871	106.0	891	117.0	874
Alkali-soluble P	125.5	372	129.7	348	128.2	334
Insoluble P	1.53	347	0.89	283	0.61	396
A_2S	97.7	444	100.1	404	97.5	399
S_2	93.3	470	97.5	403	95.4	383
P_2	0.12	1340	0.17	1195	0.20	1221
R	25.5	32	26.9	30	26.1	28

* Figures from 1st to 10th TCA extracts only.

fraction P_1 is so much greater than that of any of the protein-bound phosphorus fractions from the alkali-soluble P onwards, that contamination of any of these fractions with even a minute trace of fraction P_1 would result in a very considerable raising of their specific activities. In an attempt to achieve a more adequate removal of possible contaminating P_1 we have adopted a somewhat similar expedient to that suggested by Jeener (1949*b*) and by Friedkin & Lehninger (1949), of adding non-radioactive inorganic phosphate to minimize the activity of any inorganic phosphate still present after TCA extraction (Table 8). This procedure causes no appreciable alteration in the specific activities of the protein-bound phosphorus fractions.

In Table 8 also is shown the result of an experiment in which ^{32}P was added along with TCA to the homogenate of the liver tissue of animals which had received radioactive phosphorus by injection 2 hr. previously. Such artificially added inorganic radioactive phosphate might be expected to be completely removed by the extraction procedure with TCA so that the protein-bound phosphorus fractions should show the same radioactivity as is obtained from control tissues without such supplement. Table 8, however, reveals that this is by no means the case, and all the protein-bound phosphorus fractions, notably P_2 and R , show distinctly elevated specific activities. Similar results are shown in the experiment recorded in Table 9, in which even twenty

Table 8. *Effect of adding $\text{NaH}_2^{31}\text{PO}_4$ and ^{32}P as inorganic phosphate to homogenates of liver tissue from rats which had received ^{32}P 2 hr. previously*

(P , amount of phosphorus in mg./100 g. fresh tissue; C , specific activity in counts/min./100 μg . P. Schmidt-Thannhauser procedure.)

Fraction	No addition		3 mg. ^{31}P added as $\text{NaH}_2^{31}\text{PO}_4$ to 4 g. tissue		0.5 μc . ^{32}P added to 4 g. tissue	
	P	C	P	C	P	C
A_1S	73.8	6725	145.4	3950	77.2	10420
S_1	28.9	5320	33.4	5110	31.5	5990
P_1	40.2	7860	117.1	4070	42.3	9690
Lipid P	76.8	743	75.1	928	84.8	782
Alkali-soluble P	102.6	452	111.5	433	108.7	486
Insoluble P	0.33	356	0.26	320	0.26	1915
A_2S	77.3	695	85.4	666	84.6	815
S_2	77.6	576	85.5	674	82.7	792
P_2	0.38	1465	0.54	1632	0.38	4410
R	24.3	41	24.3	45	24.1	215

Table 9. *Effect of adding ^{32}P as inorganic phosphate to homogenates of liver tissue from rabbits which had and which had not received ^{32}P by injection 2 hr. previously*

(P , amount of phosphorus in mg./100 g. fresh tissue; C , specific activity in counts/min./100 μg . P.)

Additions to homogenate	Injected animal* No addition				Injected animal* ^{32}P , 0.5 μc . / 4 g. tissue				Uninjected animal† ^{32}P , 0.5 μc . / 4 g. tissue			
	6		20		6		20		6		20	
	P	C	P	C	P	C	P	C	P	C	P	C
Fraction	Schmidt-Thannhauser procedure											
A_1S	145.2	4279	146.4	4625	142.7	6017	141.4	5942	128.3	2070	133.7	2048
S_1	107.3	3035	112.5	2962	107.3	3155	116.4	2926	102.2	120	109.2	142
P_1	25.4	8572	20.1	9190	26.8	17365	21.3	16600	21.1	9264	19.5	7525
Lipid P	61.0	165	76.9	161	66.3	194	80.6	238	50.1	45	47.8	35
Alkali-soluble P	73.6	407	72.1	521	78.4	1133	73.4	1238	45.8	1172	44.8	1113
Insoluble P	3.99	354	3.86	366	3.13	1553	2.65	1349	0.95	1103	1.94	1451
A_2S	57.2	522	57.9	482	63.9	1259	59.1	1295	39.2	1320	37.2	1286
S_2	56.5	462	56.2	404	58.4	945	56.2	1206	37.3	1238	35.5	1399
P_2	0.55	2191	0.57	1304	0.62	5069	0.48	2825	0.25	1458	0.25	856
R	12.0	139	12.8	122	13.0	667	13.2	649	6.1	768	6.1	592
Schneider procedure												
Schneider extract	—	—	40.8	276	39.5	996	41.2	981	25.3	1030	24.8	1236
Schneider residue	—	—	30.7	589	37.5	1286	28.3	1580	16.1	1334	15.5	1587

* Female rabbit, wt. 2277 g., liver wt. 51.5 g.

† Male rabbit, wt. 2576 g., liver wt. 125.5 g. The exceptionally high weight of the liver of this animal may be related to the unusually low concentrations of P in all fractions from lipid P onwards.

extractions with TCA failed to remove inorganic radioactive phosphate added to a tissue homogenate along with the TCA. These results indicated such extreme difficulty in removing a serious contamination that we investigated the effect of adding ^{32}P as a minute amount of inorganic phosphate along with the TCA to the homogenate of liver tissue from an animal which had not received radioactive phosphorus. ^{32}P was mixed with TCA before addition to the homogenate in order to preclude the possibility of its enzymic incorporation into organic derivatives. The amount of ^{32}P added was such as was found to give a specific activity in fraction P_1 of the same order as that found 2 hr. after the injection of our customary dose of ^{32}P . It might reasonably be expected that if the extraction procedure were adequate, such contaminating inorganic radioactive phosphate would be completely removed during the extraction with TCA, but Table 9 shows that this is not so. As might be expected, the bulk of the radioactivity appears in fraction P_1 and only a little in fraction S_1 and in the lipid-P fraction. But whereas the protein-bound fractions from alkali-soluble phosphorus onwards should be free from radioactivity, most are in fact significantly radioactive. This contaminating radioactivity is not reduced when the number of extractions with TCA is increased from six to twenty (Table 9). It has been our experience that the activities of the protein-bound phosphorus fractions obtained in experiments of this type show much greater variation than those from animals which had received the ^{32}P by injection. When the Schneider procedure is adopted, similar contamination is also revealed; both the Schneider extract and the Schneider residue, which should not be radioactive, show considerable activity.

Table 10 shows the results of an experiment in which radioactive phosphorus was added along with

TCA to a homogenate of liver tissue from an un-injected rabbit. The precipitate was subsequently extracted either with ice-cold 10% TCA or with 10% TCA containing 15% (w/v) of non-radioactive sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$). The extractions were carried out on a refrigerated centrifuge and were continued for several days in an attempt to obtain an extract with a count as low as that of the background. After forty-three extractions with TCA containing phosphate, fourteen further washings were carried out with 10% TCA to remove the added carrier phosphate. Even after this very exhaustive extraction the protein-bound phosphorus fractions still showed appreciable radioactivity, and it is clear from Table 10 that TCA containing phosphate is not much more effective in removing contaminating radioactive phosphorus than is TCA alone. Reprecipitation of the R fraction, however, has the effect of reducing both the amount of phosphorus, and the specific activity of that which remains after reprecipitation. From this it seems clear that the R fraction is not homogeneous as regards the phosphorus compounds present, even after more than fifty extractions of the protein material with 10% TCA.

We have also considered the possibility that addition of ^{32}P to the homogenate along with the first addition of TCA might produce an unusually persistent contamination of the protein precipitate, so giving rise to an exaggerated impression of the difficulty of freeing such precipitates from unwanted inorganic phosphate. In one group of experiments, therefore, we delayed the addition of ^{32}P until the first washing with TCA, i.e. until after the protein was fully precipitated. Table 10 indicates that while the resulting contamination of the protein fractions is slightly less than when the same amount of ^{32}P is added with the first quantity of TCA, it is, nevertheless, both extensive and persistent.

Table 10. *Effects of extraction with 10% TCA, and with 10% TCA containing 15% $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, until extracts were free from radioactivity, on rabbit-liver tissue to which ^{32}P had been added along with TCA after homogenizing*

(P , amount of phosphorus in mg./100 g. fresh tissue; C , specific activity in counts/min./100 μg . P .)

Fraction	^{32}P added with 30% TCA				^{32}P added with first washing			
	57 washings with 10% TCA		43 washings with 10% TCA + 15% NaH_2PO_4 , followed by 14 washings with 10% TCA		57 washings with 10% TCA		43 washings with 10% TCA + 15% NaH_2PO_4 , followed by 14 washings with 10% TCA	
	P	C	P	C	P	C	P	C
Alkali-soluble P	57.0	57	59.6	51	57.2	44	56.8	39
A_2S	46.3	42	49.6	49	45.1	25	45.1	30
S_2	45.3	34	48.0	42	42.7	18	42.9	11
P_2	0.9	122	0.8	310	0.9	176	1.4	269
R	9.3	82	10.3	67	11.4	76	11.7	76
R^*	7.3	52	7.4	49	10.2	58	10.5	64

* Reprecipitated.

DISCUSSION

In the Schmidt-Thannhauser separation procedure the fraction which we have labelled A_2S contains acid-soluble ribonucleotides derived from the breakdown of RNA under the influence of warm alkali. Such compounds contain organically bound phosphorus, but the fraction also contains a small amount of inorganic phosphate. This is usually termed 'phosphoprotein phosphorus', since it is known from the work of Plimmer & Bayliss (1906) that the phosphorus of phosphoproteins is liberated in inorganic form by the action of alkali. 'Phosphoprotein phosphorus' is present in very minute amounts in most animal tissues, but usually tends to be completely ignored except in egg yolk and milk where it is abundant. Any objection that this fraction might consist merely of inorganic phosphate which has failed to be extracted during the preliminary washings with TCA can be dismissed in view of our observations that it persists in undiminished amount in tissues which have been extracted as many as fifty-seven times with 10% TCA.

In the Schneider (1945) procedure the tissue residue from which all acid-soluble phosphorus and lipid phosphorus have been removed is treated with hot 5% TCA to split off the nucleic acids as acid-soluble fragments, leaving a residue of protein-bound phosphorus which is usually assumed to be entirely derived from phosphoproteins. Schneider (1946) observed that this 'phosphoprotein phosphorus', as determined by his extraction procedure, was very much greater in amount than that which he found by the procedure of Schmidt & Thannhauser (1945), although he did not comment on the discrepancy. Our own observations amply confirm the fact that the Schneider 'phosphoprotein phosphorus' is many times greater than the Schmidt & Thannhauser 'phosphoprotein phosphorus'. That the Schneider residue contains other phosphorus compounds is shown by the fact that it has a very much lower specific activity than that of the 'phosphoprotein' fraction obtained by the Schmidt-Thannhauser method. This is supported by the results of our experiments in which the Schneider residue is dissolved in alkali and submitted to a Schmidt-Thannhauser separation. The inorganic phosphate fraction P_3 of Table 6 corresponds both in amount and in specific activity to the 'phosphoprotein' fraction P_3 found by the Schmidt-Thannhauser method. It is therefore clear that in any experiments on the protein-bound phosphorus fractions of tissues it cannot be assumed that the phosphorus of the Schneider residue represents only 'phosphoprotein', and it would appear that in the Schneider procedure the treatment with hot 5% TCA does not split off all the nucleic acid phosphorus.

In the Schmidt-Thannhauser separation, fraction A_2S contains ribonucleotides plus a small amount of inorganic phosphate from 'phosphoprotein'. Its pentose content, therefore, or better still the pentose content of fraction S_2 (on which pentose is more difficult to determine), should correspond in terms of phosphorus to that of a digest of RNA. This, however, is not the case. We have generally found that the pentose content of fraction A_2S corresponds to about 85–90% of the theoretical value for a digest of yeast RNA as calculated from phosphorus determinations. Similar figures have been found by Campbell & Kosterlitz (1949). It would appear probable, therefore, that fraction A_2S contains phosphorus compounds other than the acid-soluble ribonucleotides and the inorganic phosphate derived from 'phosphoprotein'.

It is clear from our results that protein-bound phosphorus fractions have a specific activity which is in general very much lower than that of acid-soluble phosphorus derivatives and is in consequence more liable to be greatly raised by traces of contaminating inorganic phosphate. Of the protein-bound phosphorus fractions, R has by far the lowest activity, but since even this low value is considerably higher than that of isolated DNA, contamination of the DNA in the R fraction with small amounts of phosphorus compounds of higher activity obviously occurs. As might be expected from results of early experiments on isolated nucleic acids, the specific activity of fraction S_2 is considerably higher than that of R . It is, however, clear that the S_2 fraction is also contaminated by phosphorus compounds of higher activity than that of isolated RNA.

The specific activity of fraction A_2S is in general slightly higher than that of fraction S_2 owing to the presence of the small amount of highly active phosphorus which later appears in the P_2 fraction. We have already commented on the particularly high activity of this fraction (Davidson *et al.* 1949) which has also been noted by Euler, Hevesy & Solodkowska (1948), by Friedkin & Lehninger (1949), and by Marshak & Calvet (1949). Juni, Kamen & Spiegelman (1948) have observed a high specific activity in the corresponding fraction from yeast, but in this case the fraction is very much more abundant than in animal tissues and consists essentially of metaphosphate. In confirmation of the work of Ebel (1949) we have failed to find any trace of protein-bound metaphosphate in animal tissues.

It might be considered that the so-called phosphoprotein fraction of the tissues, i.e. our fraction P_2 , might arise not so much from true phosphoprotein, in which phosphate is esterified with hydroxy amino-acid residues, as from inorganic phosphate which had failed to be removed during the preliminary extraction with TCA. Such a possi-

bility has been kept in mind by Friedkin & Lehninger (1949), by Euler *et al.* (1948), and by Jeener (1949*b*), and in this connexion it is important to consider the number of extractions with TCA which is required to remove all traces of acid-soluble phosphorus. It appears to be the general practice to extract some three or four times with TCA and to assume that further washing will remove only negligible amounts of acid-soluble material. For example, McCarter & Steljes (1948) found that the last of four successive TCA extracts of 0.5 g. of rat-liver tissue contained only 0.006–0.008 mg. of phosphorus and concluded that further extraction was unnecessary. Our own extraction process has in many cases been much more exhaustive, and has revealed that even after fifty-seven extractions, using a refrigerated centrifuge, a small amount of phosphorus remains attached to protein and is released as inorganic phosphate only after incubation of the tissue residue with alkali. It appears probable, therefore, that our P_2 fraction does in fact arise mainly from true protein-bound phosphorus. Nevertheless, the possibility remains that the high specific activity of this fraction might be due to contamination with traces of inorganic phosphate which should have appeared in fraction P_1 . This danger has been recognized by Friedkin & Lehninger (1949), by Euler *et al.* (1948) and by others. For example, McCarter & Steljes (1948) added radioactive phosphorus to a homogenate of rat-liver tissue and found that 96.5 % of the added ^{32}P could be recovered in the acid-soluble extract. Since only minute amounts of the order of 0.005 % were found in the nucleic acid fractions, they concluded that valid specific activity figures for these fractions were obtainable by the method employed (the Schmidt-Thannhauser procedure). This, however, is not necessarily the case since even minute contamination of the nucleic acid fractions with traces of inorganic radioactive phosphate might make very significant alterations to their normally low specific activities.

Dziewiatkowski & Bodian (1950), using mouse-brain tissue, found that four washings of their homogenate with 5 % TCA were more than sufficient to remove all acid-soluble radioactive phosphorus, and that further extracts gave counts as low as that of the background. This has not been our experience with liver tissue, as can be seen in Table 7, from which it is evident that even the twentieth extraction with TCA is removing small amounts of phosphorus with appreciable radioactivity. It is evident from Table 7, however, that whether the number of extractions with TCA be three, six or twenty the overall picture of the specific activity of the protein-bound fractions is unchanged, and the activity of fraction P_2 is as high after twenty extractions with TCA as after 3. If the high activity of P_2 is in fact due to contaminating inorganic phosphate the contaminant

is certainly not easily removed by further washing with TCA.

The extreme difficulty of removing the last traces of inorganic phosphate is abundantly clear from the results in Tables 9 and 10, where ^{32}P was added to the tissue homogenates along with the TCA. Not only does it appear that more than forty washings with TCA are required to obtain an extract with a count as low as that of the background, but even then the protein-bound fractions contain a significant amount of radioactive phosphorus which has failed to be removed by the previous exhaustive washing. Whether this phosphate exists in chemical combination with the proteins, or whether, as is more probable, it is physically adsorbed has not been determined, but it is clear from our results that the extent of the contamination is sufficiently serious to make it difficult to place any reliance on specific activity figures of fractions derived from protein-bound phosphorus.

It has been considered possible that the removal of contaminating inorganic radioactive phosphorus might be facilitated by repeated washing with non-radioactive phosphate. Friedkin & Lehninger (1949) employed three extractions with 10 % TCA followed by three washings with 0.04M- $\text{NaH}_2^{31}\text{PO}_4$ in TCA before applying the Schmidt-Thannhauser and Schneider procedures. Jeener (1949*a, b*), while investigating the fraction corresponding to our A_2S , recognized that the precipitation of the small amount of inorganic phosphate derived from 'phosphoprotein' might be incomplete, and could therefore give rise to unduly high specific activities in the S_2 fraction. He accordingly carried out repeated dilutions of the A_2S fraction with carrier inorganic phosphate and repeated precipitations with magnesia mixture. He found, however, that even the sixth washing with inorganic phosphate was still removing appreciable amounts of radioactive phosphorus.

Our own results shown in Table 10 reveal that repeated washing with inorganic carrier phosphate in TCA does not usefully aid in the removal of radioactive phosphorus.

In view of the difficulty of removing the last traces of inorganic phosphorus from precipitated tissue proteins it is not surprising to find (Table 4) that the specific activity of our fraction S_2 is invariably much greater than that of the phosphorus in isolated RNA and that the activity of our fraction R is greater than that of the phosphorus in isolated DNA.

There would therefore appear to be little justification for the assumption made by a number of authors, e.g. Campbell & Kosterlitz (1949), Holmes (1949*a, b*) and Hull & Kirk (1950*a, b, c*), that in order to obtain the specific activity of the nucleic acids in a sample of tissue from an animal to which ^{32}P has been given it is sufficient to carry out a Schmidt-

Thannhauser separation and to assume that the specific activity of the phosphorus in the A_2S fraction represents that of RNA while that of the phosphorus in the R fraction represents that of DNA. Not only does this assumption ignore the existence of the highly active P_2 fraction, which is of course included in A_2S , but it overlooks the complex nature of the fractions obtained in a Schmidt-Thannhauser separation which, in general, do not represent pure substances. As we have shown, the specific activities of the nucleic acids are invariably considerably lower than the specific activities of the fractions which contain them. The dangers of contamination have been recognized and stressed by Barnum, Nash, Jennings, Nygaard & Vermund (1950) and by Friedkin & Lehninger (1949) and especially by Jeener (1949*a, b*) and Jeener & Szafarz (1950*a, b*). The last named conclude that no reliance can be placed on the specific activities of nucleic acid fractions obtained by the Schmidt-Thannhauser method, and that the only way to determine the true specific activity is to isolate the nucleic acids in a rigorously purified form. Our own results lend ample support to this view, but we are by no means certain that any method of isolation so far described yields nucleic acids of the required standard of purity.

SUMMARY

1. The Schmidt-Thannhauser and the Schneider separation procedures have been applied to tissues, chiefly liver, from rats and rabbits injected with radioactive phosphorus (^{32}P), and the specific activities of the various phosphate fractions have been determined. The specific activities of the fractions containing the nucleic acids are invariably considerably higher than those of the nucleic acids isolated from the same tissues.

2. The 'phosphoprotein phosphorus', i.e. phosphorus set free from protein by incubation with alkali, invariably has a high specific activity. The 'phosphoprotein phosphorus' determined by the Schneider procedure is always much larger in amount but lower in activity than that obtained by the Schmidt-Thannhauser procedure owing to the presence of other phosphorus compounds in the Schneider residue.

3. Exhaustive extraction with ice-cold 10% TCA fails to remove 'phosphoprotein phosphorus' from liver tissue or to reduce its high specific activity. On the other hand, the high activity of this fraction may be due partly to persistent traces of acid-soluble phosphate, since ^{32}P added as inorganic phosphate to a tissue homogenate is not completely removed even by numerous washings with 10% TCA. Washing with TCA containing carrier phosphate is also inadequate.

4. It is concluded that it is unjustifiable to assume that the specific activities of the nucleic acid fractions obtained by the Schmidt-Thannhauser procedure represent the true activities of the nucleic acids. In all experiments on protein-bound phosphorus fractions involving the use of ^{32}P it is essential to eliminate the possibility of contaminating inorganic phosphate. To do this it is desirable to isolate the compounds under investigation in the pure state.

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Note added 2 March 1951. The experiments described in this paper were carried out with ^{32}P supplied by Atomic Energy Research Establishment, Harwell, which issued a statement in January 1951 that 'a number of solutions of phosphorus 32 dispatched from Harwell before the end of November 1950 have contained variable amounts of particulate matter... In some solutions the activity is partially concentrated on these particles.' We have therefore repeated experiments of the general type shown in Tables 2-7 and 9 with two different batches of ^{32}P stated to be free from this defect and have found that the general pattern of activity in injected animals is as before. When ^{32}P was added with TCA to homogenates of liver tissue as in Table 9 we again found persistent radioactive contamination of the protein-bound phosphorus fractions. The conclusions arrived at do not therefore appear to be affected.

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The Hexokinase Activity of Animal Tumours

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Slices of normal and tumour tissue which convert glucose to lactic acid rapidly (Warburg, 1930) have but little action on hexosediphosphate (Boyland & Boyland, 1938). This difference is probably due to the fact that hexosediphosphate cannot diffuse into the tissue cells, but it was interpreted as an indication of non-phosphorylating glycolysis in tissues (Needham & Lehmann, 1937). The demonstration of glycolysis of glucose, fructose and hexosediphosphate by fortified tumour extracts at the same rate as glycolysis in tumour slices (Boyland, Boyland & Greville, 1937) indicated that glycolysis in tumours involved phosphorylation. No positive evidence of a glycolytic system in tumour tissue differing from that of other tissues has been put forward and enzymes of the normal glycolytic cycle have been found in tumour tissue. Meyerhof & Wilson (1949) showed that glycolysis in tissue extracts is dependent on the ratio of phosphorylating and dephosphorylating enzymes present, and that tumour extracts contain excessive amounts of adenosinetriphosphatase (ATP-ase) relative to the hexokinase content.

The facts presented in this paper show that the hexokinase activity of certain rat tumours is sufficiently great to allow all the glucose used in anaerobic glycolysis of the tumour slices to pass through the stage of the hexokinase reaction. Some properties of the hexokinase of various rat and mouse tumours have also been investigated.

METHODS

Preparation of homogenates. The animals were killed by a blow on the head, the tumour removed and necrotic material dissected away as quickly as possible. The tissue was then

allowed to cool on ice for 10 min. A portion (2–3 g.) was chopped with scissors, weighed and immediately added to an all glass homogenizer (Potter & Elvehjem, 1936) and homogenized with 7 vol. of ice-cold 0.002 % glucose. The resulting homogenate was strained through a wisp of glass-wool to remove fibrous strands.

Preparation of acetone powders. The tumour was removed immediately after the animal was killed, dissected free of necrotic material and allowed to cool on ice for 10 min. The tissue was then homogenized in a Waring blender with 5 vol. cold acetone (–5°) for 2–3 min. at 2°. The solid material was filtered off on a Büchner funnel and washed at least five times with cold acetone without allowing the filter cake to crack. It was then dried in a vacuum desiccator over CaCl₂. The dry powder, if kept *in vacuo* at 2°, retained its hexokinase activity for at least 1 month. Aqueous extracts of the acetone powders were prepared by triturating with 10 vol. of ice-cold glass-distilled water and then centrifuging at approx. 2000 g for 15 min. at 2°. The resulting supernatant fluid, after being passed through a wisp of glass-wool, was used for determination of the hexokinase activity.

Estimation of hexokinase activity. (a) *In homogenates.* The reaction was carried out in open test tubes at 38°. To each tube was added 0.50 ml. buffer; 0.10 ml. 0.2 M-MgCl₂; 0.10 ml. 1.5 % glucose and 0.50 ml. water and/or additions. Freshly prepared homogenate (1.00 ml.) was then added followed by 0.30 ml. M-KF (the fluoride was added after the homogenate had been added to the Mg-containing buffer mixture in order to minimize precipitation of MgF₂). The tubes were then incubated at 38° for 5 min. and the reaction started by the addition of 0.50 ml. adenosinetriphosphate (ATP) solution (containing approx. 2 mg. acid-labile P). Duplicate tubes were removed immediately after the addition of the ATP in order to give values for the initial glucose concentration. The other tubes were incubated at 38°, while being shaken at the rate of 120 cyc./min., for 10–20 min. The reaction mixtures were deproteinized by the addition of successive equal volumes of 0.3 N-Ba(OH)₂ and 5 % ZnSO₄;