- Consden, R., Gordon, A. H. & Martin, A. J. P. (1946). *Biochem. J.* **40**, 33.
- Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
- Davidson, J. N., Frazer, S. C. & Hutchison, W. C. (1951). *Biochem. J.* **49**, 311.
- Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
- Gordon, A. H., Keil, B., Sebesta, K., Knessl, O. & Sorm, F. (1950). *Czechoslovak Chemical Communications*, **15**, nos. 1 and 2.
- Holiday, E. R. & Johnson, E. A. (1949). *Nature, Lond.*, **163**, 216.
- Magasanik, B., Vischer, E., Doniger, R., Elson, D. & Chargaff, E. (1950). *J. biol. Chem.* **186**, 37.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Markham, R. & Smith, J. D. (1951a). *Biochem. J.* **49**, 401.
- Markham, R. & Smith, J. D. (1951b). *Nature, Lond.*, **168**, 406.
- Marrian, D. H., Spicer, V. L., Balis, M. E. & Brown, G. B. (1951). *J. biol. Chem.* **189**, 533.
- Marshak, A. & Vogel, H. J. (1950). *Fed. Proc.* **9**, 85.
- Marshak, A. & Vogel, H. J. (1951). *J. biol. Chem.* **189**, 597.
- Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
- Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
- Smellie, R. M. S. & Davidson, J. N. (1951). *Biochem. J.* **49**, xv.
- Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.
- Volkin, E. & Carter, C. E. (1951). *J. Amer. chem. Soc.* **73**, 1516.
- Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

Phosphorus Compounds in the Cell

3. THE INCORPORATION OF RADIOACTIVE PHOSPHORUS INTO THE RIBONUCLEOTIDE FRACTION OF LIVER TISSUE

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In previous work on the distribution of radioactive phosphorus among the protein-bound phosphorus fractions of animal tissues (Davidson, Frazer & Hutchison, 1951) it has been pointed out that the removal of the last traces of contaminating acid-soluble phosphate from such fractions is an extremely difficult process, and the conclusion has been reached that it is unjustifiable to assume that the specific activities of the nucleic acid fractions obtained by the separation procedure of Schmidt & Thannhauser (1945) represent the true activities of the nucleic acids. In such experiments it is essential to eliminate the possibility of the presence of contaminating inorganic phosphate by a rigorous purification of each fraction. In the Schmidt-Thannhauser procedure the fraction most susceptible to interference is that containing the acid-soluble nucleotides formed from ribonucleic acid (RNA) by alkaline hydrolysis and subsequent acidification. A method for the separation and purification of these nucleotides in small quantities would therefore be of great value. The method of ionophoresis described by Smellie & Davidson (1951) and Davidson & Smellie (1952) has proved satisfactory for the purpose and its application will now be described.

METHODS

The technique of ionophoresis was employed as described in the previous paper (Davidson & Smellie, 1952).

Two separate methods were adopted for preparing material for ionophoresis.

Procedure 1. A modification of the Schmidt & Thannhauser (1945) method for the separation and estimation of nucleic acids was used. The tissue homogenate was extracted with trichloroacetic acid (TCA) and with lipid solvents to remove acid-soluble P and lipid P as described by Davidson *et al.* (1951). Water was then added in the proportion of 1 ml. to 100 mg. of the dry lipid-free residue and 5N-KOH to give a final concentration of 0.3N. The mixture was then incubated at 37° for 18 hr. The resulting digest was brought to pH 1 with 60% (w/w) HClO₄ and the precipitate of protein, deoxyribonucleic acid (DNA) and KClO₄ centrifuged down, the supernatant fluid plus washings being used for ionophoresis as the fraction A₂S of Davidson *et al.* (1951) after adjusting the pH to 4.

Procedure 2. Since the isolation of RNA from animal tissues by the method of Davidson *et al.* (1951) is tedious and time-consuming, the following modification was adopted. The tissue homogenate was extracted with TCA and lipid solvents as described in procedure 1. The residue was extracted three times for 1 hr. each at 100° with 10% (w/v) NaCl. The extracts were pooled and treated with 2 vol. ethanol. The precipitate so formed was washed with ethanol and ether and dried. It was hydrolysed to mononucleotides by digestion with 0.3N-KOH for 18 hr., using 1 ml. alkali/20 mg. powder. The alkaline digest was brought to pH 1 by adding 60% HClO₄. DNA, protein and KClO₄ were centrifuged out and washed, and the supernatant fluid and washings adjusted to pH 4 with N-KOH.

Radioactive phosphate supplied by the Atomic Energy Research Establishment, Harwell, as H₃PO₄ was submitted to 10 min. hydrolysis with N-HCl before use to destroy any traces of pyro- or poly-phosphates.

The general procedure with radioactive material was that of Davidson *et al.* (1951). ^{32}P was administered subcutaneously to rats in doses of $50\text{ }\mu\text{c.}/100\text{ g.}$ body weight, and the animals killed 2 or 24 hr. later. The livers were perfused with saline, excised, homogenized in saline in an M.S.E. Nelco Blender and submitted to procedures 1 and 2 above. Papers for ionophoresis were usually set up in duplicate or triplicate. One set was photographed in ultraviolet light according to the method of Markham & Smith (1949) and then left in contact with Kodak Industrex Type D X-ray film for 2 weeks for the preparation of autoradiographs. A second set of papers was cut up and eluted, the eluates being used for the determination of P and radioactivity.

RESULTS

Several authors (Marshak & Vogel, 1950; Jeener & Szarfarsz, 1950) have recently stressed the difficulties of removing traces of contaminating ^{32}P from the nucleotide-containing fraction of tissues, and Davidson *et al.* (1951) have demonstrated that, if a small amount of inorganic radioactive phosphate is added to a homogenate of non-radioactive rat liver along with TCA, there is still considerable activity in the ribonucleotide fraction obtained by the method of Schmidt & Thannhauser (1945) even after exceedingly exhaustive washing of the tissue with TCA and with TCA containing phosphate.

Experiments have therefore been carried out to investigate the efficiency of ionophoresis in removing such contaminating ^{32}P . Small amounts of inorganic radioactive phosphate were added to a spot of mixed nucleotides on paper and the mixture submitted to ionophoresis. The progress of the inorganic phosphate band was followed by means of a 'Panax' monitor set, and ionophoresis was generally continued until this band was approaching the end of the paper. The position of the band was obtained, after drying the paper, by moving the monitoring counter slowly over its surface. It was found that all the activity was located in one well defined area, and this was marked in pencil. On subsequent examination of the paper in ultraviolet light, the radioactive region proved to be well clear of the four nucleotides. When the separate nucleotides were eluted the amount of contaminating ^{32}P as determined in a 20th Century Electronics Type M6 liquid counter was found to be negligible.

In another experiment, a small amount of radioactive phosphorus was added to the paper at the starting point along with a volume (containing about $80\text{ }\mu\text{g.}$ phosphorus) of a Schmidt-Thannhauser nucleotide fraction prepared from non-radioactive rat liver. After ionophoresis for 6 hr. at 14 V./cm. , the paper was dried and the ultraviolet-absorbing areas marked in pencil. When the 'Panax' monitor was moved slowly over the paper, the only area which exhibited radioactivity was found to lie well ahead of the nucleotides, and to

coincide with component *A* (see below). Determination of the activity of the nucleotides indicated negligible contamination from added ^{32}P .

In a third experiment $0.88\text{ }\mu\text{c.}$ ^{32}P was added to a homogenate of 4 g. rat liver along with TCA as had been done by Davidson *et al.* (1951). The precipitated material was washed four times with ice-cold 10% (w/v) TCA and subjected to the usual modified Schmidt-Thannhauser procedure (procedure 1), the alkaline digest being precipitated with perchloric acid. The ribonucleotides separated by ionophoresis showed negligible radioactivity on elution.

It is clear therefore that the method of ionophoresis on paper can yield nucleotides free from all traces of contaminating inorganic phosphate which might distort activity measurements. The method was therefore applied to the nucleotide fraction obtained by the Schmidt-Thannhauser (1945) method as described in procedure 1 above, using the liver tissue of rats which had received radioactive inorganic phosphate by subcutaneous injection 2 or 24 hr. before killing.

The result of ionophoresis as shown in an ultraviolet light print is given in Fig. 1 which reveals that the four nucleotide spots are preceded by a fast-moving crescentic spot which absorbs ultraviolet light faintly.

To demonstrate the presence of this unknown component which we have called *A*, a short run on a 57 cm. paper strip at 14 V./cm. for 6 hr. sufficed, but this was inadequate for the complete separation of the four nucleotides. In the normal procedure for good nucleotide separation on a longer paper the fast-moving components run off the end. For each solution two runs were therefore usually employed—a short one for the separation of the fast-moving component (Fig. 1) and a conventional longer run on a 72 cm. paper for nucleotide separation (Fig. 2). The fast-moving component *A* was present both in solutions acidified with perchloric acid and in solutions prepared by acidification of the alkaline digest with TCA and subsequent removal of TCA with ether. It is discussed in greater detail below.

An ultraviolet photograph and an autoradiograph from a short run are shown side by side in Fig. 1. The four nucleotides are not adequately separated in the ultraviolet photograph, although they appear as discrete spots in the autoradiograph. The characteristically crescent-shaped spot *A* is clearly visible in both photograph and autoradiograph, but is more pronounced in the latter. Between *A* and uridylic acid lies a spot *C*, clearly visible in the autoradiograph, while uridylic acid itself is accompanied by two more spots *D* and *E* lying immediately in front of and behind it respectively. An additional spot *F* which appears in the autoradiograph lies behind cytidylic acid.

It is clear, therefore, that the RNA fraction obtained in the Schmidt-Thannhauser separation contains, in addition to the expected four nucleotides, at least five other compounds containing phosphorus, one of which can be shown up by ultraviolet absorption.

The results of a long ionophoresis run are shown in Fig. 2 in which components *A* and *C* have passed beyond the end of the paper. Here the nucleotide separation is adequate, but uridylic acid is still accompanied by components *D* and *E*, which show up on the autoradiograph but not in the ultraviolet photograph. These two components remain very close to uridylic acid and do not separate as discrete spots. Since their specific activity is much higher than that of uridylic acid, the effect of their presence is to make uridylic acid appear unduly active.

strips. When the ultraviolet-absorbing area *A* was eluted it was found possible to separate the eluate into an inorganic and organic constituent, both of which contained phosphorus. The inorganic phosphate content was determined by adding sulphuric acid, ammonium molybdate and 2:4-diaminophenol hydrochloride to a portion of the eluate without digestion. The intensity of the blue colour was measured giving the concentration of inorganic phosphorus, and the blue phosphomolybdate complex was then extracted with *isobutanol* for radioactive assay. Radioactive measurements were also carried out on the aqueous layer. A second portion of the eluate was used for determination of total phosphorus. Organic phosphorus was determined by difference.

The inorganic portion was designated *B* while the organic portion, which must be responsible for the

Table 1. Separation by ionophoresis of the components of the ribonucleotide fraction of rat-liver tissue submitted to the modified Schmidt & Thannhauser method (procedure 1)

(For an explanation of *A*, *B*, *C*, *D* and *F*, see Fig. 1 and text. Results are expressed in $\mu\text{g. P}/100\mu\text{g. P}$ in the whole fraction.)

Exp. no.	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>F</i>	Recovery (%)
1	14.2	24.2	20.6	16.7	4.3	3.8	6.1	6.5	—	89.1
2	14.6	25.0	24.0	16.4	1.9	2.2	8.1	8.0	—	96.2
3	13.3	23.5	20.8	15.3	1.4	1.5	9.7	11.0	—	96.5
4	13.2	25.4	21.5	15.9	3.5	2.0	6.9	3.0	—	91.2
5	14.0	26.4	20.6	15.9	3.8	2.5	3.4	6.2	—	92.8
6	12.8	25.8	19.9	18.2	4.6	5.6	8.7	4.6	—	100.2
7*	12.3	28.4	19.7	15.4	6.0	1.5	4.5	4.2	1.1	93.1

* Liver cytoplasm only.

The relative amounts of phosphorus in the component parts of the ribonucleotide fraction obtained in procedure 1 above (the A_2S fraction in the modified Schmidt & Thannhauser procedure used by Davidson *et al.* 1951) are shown in Table 1. The figures for nucleotides were obtained from long ionophoretic runs and those for the concomitants *A–F* by the corresponding short runs. The total recovery of phosphorus from all components is usually less than 100%, but it is clear that the nucleotides together comprise some 75% of the total phosphorus in the fraction. The relative amounts of the nucleotides are of the same order as those obtained from isolated liver RNA (see previous paper). The amounts of each concomitant vary quite widely from experiment to experiment. This may be due in part to the arbitrary way in which the paper was cut, since considerations of time made it impossible to prepare an autograph from each strip before it was cut up, eluted and counted, and in part to the fact that the amount of phosphorus in each eluate was at the lower limit of accuracy of the method of estimation. This precluded the use of more numerous but narrower

ultraviolet absorption, retained the name *A*. The presence of the inorganic component *B* accounts for the fact that the width of the band on the autoradiograph is considerably greater than that on the ultraviolet photograph. The other components *C*, *D* and *F* contained no inorganic phosphate, nor were they visible on examination of the paper in ultraviolet light. The relative positions occupied by all these components and by the nucleotides on the paper is apparent from the photographs in Fig. 1.

The specific activities of the various components listed in Table 1 are shown in Table 2. It will be seen that the activity of the whole fraction is much higher than that of any of the nucleotides. This is due to the high activity of the concomitants and particularly of the inorganic component *B* which corresponds to the 'phosphoprotein' phosphorus or P_2 fraction of Davidson *et al.* (1951) which likewise had a very high activity. The discrepancy between the activities of the nucleotides and of the whole fraction is most noticeable 2 hr. after injection. After 24 hr. the activity of the nucleotides has increased while that of the concomitants has decreased.

Fig. 1.

Fig. 2.

Fig. 3.

Of the three nucleotides adenylic acid, guanylic acid and cytidylic acid, the first was generally found to have the highest activity although the differences between them were not large. Uridylic acid, on the other hand, showed an activity so much higher than any of the other nucleotides that it was clear that contamination by components *D* and *E* was occurring. Accordingly, the areas corresponding to *D* and *E* were cut out and eluted along with the uridylic acid itself as obtained from a long run. Although uridylic acid showed a higher activity than the other three nucleotides, the concomitants *D* and *E* showed still higher values (Table 3). It would appear probable therefore that values obtained for uridylic acid in ionophoretic runs of this fraction would be liable to exhibit excessively high

activities owing to the likelihood of contamination by closely associated highly active concomitants. The presence of *D* and *E* is clearly shown in the autoradiograph in Fig. 2.

It was thought that the presence of concomitants *A-F* might be due in part at least to incomplete removal of acid-soluble or lipid phosphorus during the preliminary extraction procedure. Experiments were therefore carried out in which the number of extractions with 10 % TCA was increased to five and the lipid extraction was supplemented by two extractions of 30 min. with a boiling ethanol-chloroform (3:1) mixture. Subsequent analysis revealed that neither procedure had reduced the concomitants in number or amount. Of these concomitants the most troublesome are *D* and *E* which lie in close

Table 2. *Specific activities of the phosphorus compounds separated by ionophoresis of the ribonucleotide fraction obtained by the modified Schmidt & Thannhauser method (procedure 1)*

(The results were obtained from the livers of rats which had received 50 μ c. ³²P/100 g. body weight at different intervals before killing. For component *E* see Table 3.)

Time after injection (hr.)	Specific activity (counts/min./100 μ g. P)									Whole fraction
	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>F</i>	
2	485	184	344	660	5075		1350	1590	—	—
2	723	431	490	990	1500	7800	2000	2600	—	2050
2	715	390	565	920	500	19800	2350	2800	—	1780
2	975	570	810	870	4160	21300	6700	2300	—	2560
2	293	278	275	702	1820	3120	1180	775	—	1280
2	300	255	215	588	2470	4270	1120	3020	—	1190
2	287	249	234	525	2000	3320	1000	1720	—	1050
2	1240	785	1165	2325	487	60500	3960	3400	6750	3500
24	2150	2090	2040	2290	950	2550	3600	2650	—	2850
24	2000	1960	1840	2340	5700		4180	3050	—	2780

Table 3. *Specific activities of the four nucleotides and of the eluates from D and E (Fig. 2), obtained in a long ionophoresis run of the ribonucleotide fraction obtained by the modified Schmidt & Thannhauser method (procedure 1)*

(Rat liver, 50 μ c. ³²P/100 g. body weight administered 2 hr. before killing.)

Exp. no.	Specific activity (counts/min./100 μ g. P)							Whole fraction
	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	<i>D</i>	<i>E</i>		
1	559	323	469	749	2660	1595		2360
2	978	434	838	1110	2805	2030		4280

Ultraviolet prints (above) and autoradiographs (below) of the corresponding ionophoretic runs of the nucleotide fractions of liver tissue. Cy., cytidylic acid; Ad., adenylic acid; Gu., guanylic acid; Ur., uridylic acid.

Fig. 1. Short ionophoretic run of the nucleotide fraction prepared by procedure 1. Components *A-F* are visible in the autoradiograph, but only *A* shows up in the ultraviolet print. The nucleotides have not yet separated from each other.

Fig. 2. Long ionophoretic run of the ribonucleotide fraction prepared by procedure 1. Separation of the nucleotides is adequate, but uridylic acid is accompanied by components *D* and *E* which appear in the autograph but not in the ultraviolet print.

Fig. 3. Long ionophoretic run of the ribonucleotide fraction prepared by procedure 2. The nucleotides are adequately separated and no concomitants are visible. The tendency for the isomeric guanylic acids to separate is seen on the autoradiograph.

association with uridylic acid. Attempts to separate them by extending the length of the run and altering the pH were unsuccessful.

In several cases, ionophoresis of a radioactive ribonucleotide fraction (procedure 1) was carried out down one edge of a wide strip of paper, the paper dried in the usual fashion, and then subjected to chromatography in the form of an ascending chromatogram at right angles to the direction of ionophoresis. The solvents used were the *isobutyric acid/ammonia*, *isopropanol/hydrochloric acid* and *methanol/formic acid* systems of Magasanik, Vischer, Doniger, Elson & Chargaff (1950), Wyatt (1951) and Bandurski & Axelrod (1951), respectively. The uridylic acid spot obtained in this way showed a reduced activity comparable with that of the other nucleotides, but this combined ionophoresis and chromatography was cumbersome, and at the best semi-quantitative.

Table 4. *Specific activities of the nucleotides separated by ionophoresis of the nucleotide fractions, obtained by procedure 1 (modified Schmidt & Thannhauser method), and procedure 2 (partial isolation method)*

(Rat liver, 50 μ c. 32 P/100 g. body weight administered 2 hr. before killing.)

Nucleotide	Specific activity (counts/min./100 μ g. P)	
	Procedure 1	Procedure 2
Adenylic acid	482	565
Guanylic acid	412	370
Cytidylic acid	430	455
Uridylic acid	1335	562
Whole fraction	1355	540

Accordingly, an attempt was made to obtain nucleotide material free from the concomitants by carrying out a partial isolation of RNA by sodium chloride extraction as described in procedure 2 above. The results of such an experiment are shown in Table 4 in which the specific activities of the nucleotides obtained by ionophoresis of the ribonucleotide fraction from a Schmidt & Thannhauser separation (procedure 1) are compared with those obtained by ionophoresis of the material extracted in procedure 2. An ultraviolet print and an autoradiograph are shown in Fig. 3. Only the nucleotides and no concomitants are visible.

It is clear that procedure 2 yields a fraction containing no large proportion of highly radioactive contaminants since the specific activities of the nucleotides and of the whole fraction are comparable. Procedure 2, moreover, yields uridylic acid with an activity much lower than that found in procedure 1 and comparable with that found for the other nucleotides. The activities of the other three nucleotides obtained by the two methods are of the same order.

DISCUSSION

A useful application of the method of ionophoresis lies in the separation of the components of the ribonucleotide fraction of a tissue from an animal which has received a tracer dose of radioactive phosphorus. Davidson *et al.* (1951) have shown that when liver tissue from such an animal is submitted to the conventional separation procedure of Schmidt & Thannhauser (1945), the specific activity of the phosphorus in the ribonucleotide fraction (A_2S) is much greater than that in the RNA isolated from the same tissue. This difference could in part be accounted for by the presence in the ribonucleotide fraction of a small amount of highly active inorganic phosphate derived from 'phosphoprotein', but even after its removal, the activity of the residue (fraction S_2 of Davidson *et al.* (1951)) is still greater than can be explained by the presence of ribonucleotides alone. Similar drawbacks to the use of the Schmidt-Thannhauser procedure in experiments with 32 P have also been recorded by Marshak & Vogel (1950), by Jeener & Szarfaz (1950), and by Szarfaz & Paternotte (1951), who used paper chromatography in an attempt to free the ribonucleotides from radioactive contaminants such as inorganic phosphate.

By the use of the ionophoresis procedure to separate the components of the ribonucleotide fraction these difficulties can be resolved. The experiments with 32 P added to nucleotide mixture make it clear that ionophoresis can remove all traces of contaminating inorganic phosphate from the nucleotides. Such purification is virtually impossible by chemical methods alone, and ionophoresis therefore provides a satisfactory solution to the problem of determining true specific activities of the individual nucleotides.

The method of ionophoresis used in conjunction with autoradiography has revealed that of the ribonucleotide fraction obtained in the conventional Schmidt & Thannhauser separation, only some 75 % of the phosphorus is in fact derived from the ribonucleotides. The remaining 25 % consists of at least six protein-bound phosphorus derivatives which are released as acid-soluble compounds on incubation with alkali. Of these six, five are organic phosphates of unknown composition while the sixth (component B) is inorganic phosphate presumably derived from 'phosphoprotein' and corresponding to the fraction P_2 of Davidson *et al.* (1951). These authors pointed out that on the basis of pentose estimations nucleotide phosphorus accounted for not more than 85 % of the total phosphorus in the ribonucleotide fraction obtained by the Schmidt & Thannhauser method. Of the remainder only a small proportion could be determined as inorganic phosphate, and it is now clear that this is due to the

presence of the other concomitants. The Schmidt & Thannhauser (1945) method, as usually employed, tends therefore to give unduly high results for the RNA concentration in a tissue.

The chemical nature of the concomitants is at present under investigation. Only one of them (component *A*) exhibits any appreciable absorption in ultraviolet light. It has the unusual property of migrating as rapidly as inorganic phosphate. All the concomitants are present in all three cytoplasmic fractions corresponding to mitochondria, microsomes and cell sap (Smellie & Davidson, 1952). Similar concomitants, not necessarily identical with those found in the cell cytoplasm, are found in the cell nucleus (McIndoe & Davidson, 1952).

It is quite clear from our results that the nucleotide fraction obtained by the Schmidt & Thannhauser (1945) procedure is in itself useless as an indication of the specific activities of the nucleotides which it contains as has already been pointed out by Davidson *et al.* (1951). Separation of its component parts by ionophoresis yields reliable values for the specific activities of adenylic, cytidylic and guanylic acids. Uridylic acid, on the other hand, is so closely accompanied by components *D* and *E* that reliable specific activity figures cannot be obtained, and this accounts for the high activities for uridylic acid recorded by Davidson, McIndoe & Smellie (1951) and Davidson (1951). Moreover, the presence of these concomitants leads to high values for the phosphorus content of the uridylic acid fraction (Table 1).

The most satisfactory solution to the problem of obtaining the true specific activities of the nucleotides is to carry out a partial isolation of the RNA by our procedure 2, and to submit the material so obtained to ionophoresis.

This method yields material free from all concomitants including *D* and *E* which, especially the latter, cling so obstinately to uridylic acid. It is moreover much simpler and quicker than the exhaustive purification previously described (Davidson *et al.* 1951), but it should be noted that even this material contains traces of component *C* as does highly purified RNA.

The extraction of nucleic acid in procedure 2 is not quantitative, yields of about 70 % being usual. The material obtained, however, is satisfactory for determining the relative molar proportions of bases or the specific activities of the nucleotides although not for estimating the RNA content of a sample of tissue. The chief danger in its use is the possible preferential extraction of one RNA from material not homogeneous with respect to nucleic acid content.

Procedure 2, then, gives material which on ionophoresis provides a true measure of the specific activities of all four nucleotides (Table 4). Only by such separation of the component nucleotides can the true specific activity of an RNA be determined. The differences between the nucleotides are not great, but adenylic acid is in general the most active. Volkin & Carter (1951), who carried out similar experiments involving the separation of the constituent ribonucleotides of liver RNA by ion-exchange chromatography after the administration of much larger doses of ^{32}P 20 min. before killing, found the highest activity in adenylic acid and the lowest in guanylic acid.

SUMMARY

1. In experiments with ^{32}P the use of the procedure of ionophoresis on paper makes it possible to obtain nucleotides free from all traces of contaminating inorganic phosphate.

2. When the ribonucleotide fraction obtained by the procedure of Schmidt & Thannhauser (1945) from the tissues of an animal receiving ^{32}P is submitted to ionophoresis, at least six non-nucleotide phosphate derivatives can be detected as well as the four ribonucleotides. Their presence can conveniently be demonstrated by making autoradiographs of the ionophoretic run.

3. One of these six concomitants is inorganic phosphate derived from phosphoprotein. The other five are organic phosphates.

4. Two hours after administration of ^{32}P to rats and rabbits the concomitants all have higher specific activities than the nucleotides. This accounts for the abnormally high specific activity of the Schmidt-Thannhauser fraction.

5. A modified Schmidt-Thannhauser procedure involving partial isolation of the ribonucleic acid is described which, in combination with ionophoresis, gives a true picture of the specific activities of the ribonucleotides.

6. Ribonucleotides comprise only about 75 % of the total phosphorus in the ribonucleotide fraction obtained from tissues by the Schmidt-Thannhauser procedure.

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REFERENCES

- Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.
- Davidson, J. N. (1951). *Ciba Conference on Isotopes in Biochemistry*, p. 175. London: Churchill.
- Davidson, J. N., Frazer, S. C. & Hutchison, W. C. (1951). *Biochem. J.* **49**, 311.
- Davidson, J. N., McIndoe, W. M. & Smellie, R. M. S. (1951). *Biochem. J.* **49**, xxxvi.
- Davidson, J. N. & Smellie, R. M. S. (1952). *Biochem. J.* **52**, 594.
- Jeener, R. & Szarfarsz, D. (1950). *Arch. Biochem.* **26**, 54.
- McIndoe, W. M. & Davidson, J. N. (1952). *Brit. J. Cancer*. (In the Press).
- Magasanik, B., Vischer, E., Doniger, R., Elson, D. & Chargaff, E. (1950). *J. biol. Chem.* **186**, 37.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Marshak, A. & Vogel, H. J. (1950). *J. cell comp. Physiol.* **36**, 97.
- Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
- Smellie, R. M. S. & Davidson, J. N. (1951). *Biochem. J.* **49**, xv.
- Smellie, R. M. S. & Davidson, J. N. (1952). Unpublished results.
- Szarfarsz, D. & Paternotte, C. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1518.
- Volkin, E. & Carter, C. E. (1951). *J. Amer. chem. Soc.* **73**, 1519.
- Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

Studies on the Synthesis of Lactose by the Mammary Gland

2. THE SUGAR-PHOSPHATE ESTERS OF MILK

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In the course of investigations on the intermediary metabolism involved in the synthesis of lactose in the mammary gland, we have made repeated attempts to detect phosphate esters of galactose and lactose, both in extracts of actively secreting guinea pig glands and in media containing similar tissue metabolizing glucose *in vitro* (Malpress & Morrison, unpublished; Malpress, 1950). The results have been disappointing. In isolated cases faint positive tests were obtained for both these sugars in chromatographic studies after acid hydrolysis or after the action of alkaline phosphatase preparations on solutions obtained by barium fractionation of the initial extracts, but the indications were too weak and inconsistent to allow any conclusion regarding the normal occurrence of such esters in the active gland synthesizing lactose.

It seemed possible, however, in view of reports by Graham & Kay (1933) and by others of the presence of acid-soluble phosphate esters in milk, that an analysis of milk by a barium fractionation procedure might provide a new approach and one that might well yield results of interest in connexion with the problems of lactose synthesis. This was the more likely, since theories of milk secretion (Richardson, 1947) recognize both the diffusion and the extrusion of milk constituents from the alveolar cells, and in the latter case the rupture or 'decapita-

tion' of the cells might be expected to lead to the presence of intermediates of mammary cell metabolism in the milk.

This paper reports the results of such an investigation in which minute amounts of α -galactose-1-phosphate, glucose-1-phosphate, a second glucose phosphate (probably glucose-6-phosphate) and a lactosephosphate have been detected in normal milk; phosphopyruvic acid has also been found.

Hitherto, apart from the production of α -galactose-1-phosphate by lactose-fermenting or galactose-adapted yeasts (Trucco, Caputto, Leloir & Mittelman, 1948; Wilkinson, 1949), the only known formation of galactose esters in living organisms is that, again of galactose-1-phosphate, reported by Kosterlitz (1943) in the livers of rats fed on galactose, though Laszt & Sullmann (1935) and Verzár & Sullmann (1937) have adduced circumstantial evidence for the formation of these compounds in the intestine during the absorption of galactose. The esterification of galactose in the carbon-1 position depends, in yeasts, upon the presence of a galactokinase, and the work of Bacila (Trucco *et al.* 1948) suggests that the same mechanism operates in the liver.

We know of no reported occurrence of any lactose phosphate in animal tissue.