Acid-Soluble Phosphate Metabolism in Nuclei from Rat Thymus Gland

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The discovery of nuclear phosphorylation and of the ability of isolated nuclei to incorporate protein and nucleic acid precursors (Allfrey, Mirsky & Osawa, 1955, 1957a) focused attention on the part that might be played by the nucleus in the metabolism of the whole cell. Anderson (1953) from his work on the permeability of isolated calf-thymus nuclei to proteins concluded that quite large molecules could enter the nucleus. On the other hand, thymus nuclei isolated in an aqueous medium retained nucleotides (Allfrey *et al.* 1955) so that some process might be envisaged by which precursor molecules are held in the nucleus before the formation there of new molecules. We therefore attempted to define more precisely the relationship between acid-soluble phosphate metabolism in the nucleus and that in the whole cell and this led to some observations on uptake of inorganic phosphate and arginine by nuclei from rat-thymus glands which may be relevant in other types of animal cells.

MATERIALS AND METHODS

Animals weighing between 80 and 130 g. were taken from the laboratory stock of Wistar rats. Most of the experiments were made with thymus glands from females, but no obvious difference was found between preparations from either sex. Unless otherwise stated, the animals were injected intramuscularly with 25–50 μc of ^{32}P/100 g. body wt. 5–60 min. before death.

Isolation of nuclei. The sucrose medium A of Creasey & Stocken (1959) and their ionic medium C were used, except that 5 mM-tris replaced the 5 mM-triethanolamine hydrochloride in medium A. Medium C: 45 mM-glucose, 85 mM-KCl, 8·5 mM-NaCl, 2·5 mM-CaCl₂, 2·5 mM-MgCl₂, 5 mM-triethanolamine hydrochloride adjusted to pH 7·2 with KOH. Neither medium contained acid-soluble phosphate. The homogenization was performed in a tube of 0·5 in. diameter with the homogenizer described by Philpot & Stanier (1956) and all operations were carried out at ±2°. The nuclei were washed twice before final resuspension. The method of removal of the thymus and the time that this took were critical, as was the concentration of the thymus during the first homogenization. These details are described in the text.

Cell sap. The supernatant from the first sedimentation of the nuclei was centrifuged for a further 60 min. in the Spinco centrifuge model L (rotor 30, 30 000 rev./min.; rotor 40, 40 000 rev./min.), and the supernatant from this

was used for the determination of the specific activity of the inorganic phosphate of the cell sap.

Isolated thymocytes. These were obtained as described earlier (Ord & Stocken, 1958a). They were suspended in ionic medium C in which the Na⁺ and K⁺ ion concentrations were reversed.

Acid-soluble phosphate analysis. In some experiments the reactions were stopped by the addition of HClO₄ to give a solution N with respect to HClO₄. The precipitate was separated by centrifuging and the supernatant fluid filtered before analysis. Usually the nuclear suspension was centrifuged before acidification, and the supernatant separated, made N with respect to HClO₄ and then filtered. The phosphate compounds present in this fraction are referred to as the soluble or supernatant fraction of the nuclei. The residue from the nuclear suspension was redispersed in medium and acidified to give the acid-extractable or bound-phosphate fraction.

Inorganic phosphate was estimated by the method of Berenblum & Chain (1938). Acid-labile phosphate was that liberated by heating for 15 min. in N-HCl at 100°. When the sucrose medium was used a yellow colour appeared on hydrolysis, but this did not interfere with the measurement of the inorganic phosphate.

In all experiments the samples were brought to a final volume of 10 ml. with *iso*butanol and filtered before estimation of the inorganic [^{31}P]phosphate and ^{32}P. When inorganic phosphate of the acid-extractable nuclear fraction was assayed a precipitate was produced in the presence of the phosphomolybdic acid. This was retained in the *iso*butanol extract which was assayed for ^{32}P before and after filtration. The ^{32}P content of the precipitate could then be obtained by difference. Direct measurement of its ^{31}P content was not always performed since experiments showed that it contributed only a small amount to the ^{31}P in the acid-extractable, acid-labile fraction.

Arginine. This was estimated by the method of Rosenberg, Ennor & Morrison (1956).

Hexokinase. The enzyme was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. (yeast enzyme, crude type II), and used to determine the specific activity of the γ-phosphate group of ATP.

Separation of ribonucleotides. This was achieved chromatographically on Dowex 1 (formate form; X10) columns (Hurlbert, Schmitz, Brumm & Potter, 1954).

Estimation of deoxyribonucleic acid. The method of Burton (1956) was used. A sample from the HClO₄ extract was taken for colorimetric assay and the remainder was made up to 10 ml. so that the ^{32}P activity in the DNA could be obtained.

Measurement of radioactivity. ^{32}P activity was determined in a Geiger–Müller M 6 liquid counter. ^{14}C activity was measured in a Nuclear-Chicago Corp. counter. Sufficient counts were recorded to give an error of less than 3%.

RESULTS

Isolation of nuclei. Considerable care is required to obtain reproducible conditions for studying nuclear behaviour (Allfrey, Mirsky & Osawa, 1957b). It was therefore necessary to define as closely as possible the conditions under which the nuclei used in this work were prepared. We found that factors leading to a good yield of nuclei, containing a reproducible amount of acid-soluble phosphate, appeared to be similar for both the sucrose and the ionic media. With sucrose it was essential to increase the $CaCl_2$ concentration above 1·8 mM used by Hogeboom, Schneider & Striebich (1952) for liver nuclei; 3·3 mM-$CaCl_2$ (Allfrey et al. 1955) gave good yields of nuclei which were very little contaminated by whole cells. The pH of both media at room temperature was between 7·15 and 7·25 (glass electrode); no pH outside this range was used.

If the thymus was not removed rapidly from the animal at death, a considerable increase in nuclear inorganic phosphate occurred mainly at the expense of acid-labile phosphate (Table 1). Even the 30 sec. necessary for exsanguination decreased the nuclear acid-labile phosphate. The properties of the nuclei were found to be very dependent on the relative proportions of inorganic and acid-labile phosphate which they contained when first isolated.

Attempts were made to increase the yield of nuclei by taking a greater number of thymus glands in the initial homogenization. This proved to be unsatisfactory, especially with the sucrose medium, when the homogenization was always less easy than with the ionic medium. Examination of the sucrose nuclei isolated from a concentrated homogenate showed a considerable leakage of the DNA and a reduced yield owing to poor sedimentation from the more concentrated homogenates.

Unless otherwise stated the final conditions chosen for the preparation of the nuclei were the rapid removal of the thymus from rats killed by cervical fracture and the homogenization of not more than six glands at a time. Usually the twice-washed nuclei from two such operations were pooled to give the final nuclear suspension. Eight consecutive experiments in ionic medium gave: inorganic phosphate, 1·82 ± 0·46 μg./mg. of DNA P; acid-labile phosphate, 9·86 ± 2·32 μg./mg. of DNA P.

Properties of isolated nuclei. The retention of nucleotides by thymus nuclei has been discussed by Allfrey et al. (1955). In our experiments the final nuclear suspension, when recentrifuged, gave a supernatant containing inorganic but not acid-labile phosphate. This inorganic phosphate was about 50 % of the total inorganic phosphate of the suspension (Table 2). Treatment of the nuclei with acid was required to extract the remaining inorganic phosphate and the acid-labile phosphates. In nuclei from thymus glands that had aged within the rats after death the increased inorganic phosphate was distributed between both the soluble and acid-extractable fractions. From an approximately 1:1 ratio in nuclei from thymus glands removed immediately after death, the distribution shifted towards a bound:soluble ratio of 1·5:1 (Table 2).

Chromatographic separation of the nucleotides in the acid-extracted fraction of the nuclei, from thymus glands which had been rapidly removed, confirmed the earlier findings of Allfrey et al. (1955) that adenine nucleotides predominated. In our

Table 1. *Effect on the nuclear acid-soluble phosphates of the time after death when the thymus was removed*

| Time (min.) after death (and no. of experiments) | Acid-soluble phosphates (μg. of P/mg. of DNA P) | | | |
| | Ionic medium | | Sucrose medium | |
	Inorganic	Acid-labile	Inorganic	Acid-labile
< 0·3 (5)	1·7	10·9	1·8	10·1
0·5 (3)	4·3	8·9	—	—
5 (2)	5·6	7·7	3·6	6·9
10 (3)	7·1	4·8	—	—
20 (1)	13·4	4·1	7·5	3·8

Table 2. *Distribution of acid-soluble phosphates in isolated rat-thymus nuclei*

Time (min.) of thymus removal after death (and no. of experiments)	Acid-soluble phosphate					
	Total (μg. of P/mg. of DNA P)		% present in			
			Soluble fraction		Bound fraction	
	Inorganic	Acid-labile	Inorganic	Acid-labile	Inorganic	Acid-labile
< 0·5 (3)	2·8	7·8	54	0	46	100
10–15 (4)	6·0	5·8	40	8	60	92

Table 3. *Specific activities of acid-soluble phosphates in nuclei from rat thymus after injection of 50 μc of ^{32}P/100 g. body weight*

Time (min.) after injection (and no. of experiments)	Mean specific activities (counts/min./μg. of P)			
	DNA	Soluble inorganic	Bound inorganic	Acid-labile
5 (1)	0·4	97	158	—
10 (2)	0·8	187	341	133
20 (5)	1·6	310	431	259
30 (2)	3·4	280	425	260
60 (4)	6·2	125	276	222
120 (1)	—	—	218	213
240 (1)	—	—	190	158

Table 4. *Specific activities of inorganic phosphate and adenosine triphosphate from whole rat thymus and from cell fractions of the gland*

The rats received 50 μc of ^{32}P/100 g. body weight except †, where approx. 40 μc were used. The nuclear inorganic phosphate was the 'bound' fraction.

Time after injection (min.)		Specific activity of phosphate (counts/min./μg. of P) in					
		Cell fraction			Whole thymus		
		Inorganic	Acid-labile	γ-P of ATP	Inorganic	Acid-labile	γ-P of ATP
5	Cell sap*	146	99	105	210	75	104
5	Cell sap	118	—	—	224	91	96
6	Nuclei	175	39	73	232	114	171
10		—	—	—	258	175	208
10†	Nuclei	185	77	79	212	116	102
10†	Nuclei	169	70	80	230	107	102
20	Cell sap	288	207	—	335	262	299

* In this experiment a Ca^{2+}-free medium was used (see text).

extracts ATP and AMP were the main components, very little ADP being present. A small amount of guanosine triphosphate and uridine triphosphate was found, but it was evident that about 70 % of the acid-labile phosphate was ATP. This was confirmed in the course of hexokinase assays of the specific activity of the γ-phosphate group of ATP (see below). In these rat nuclei, in contrast with those from calves but in agreement with the findings from whole rat thymus (Ord & Stocken, 1958b), cytidylic acid was only slightly lower in concentration than AMP. In extracts from rats given ^{32}P 30 min. before death, the activity of ATP was greater than in ADP, and AMP had insignificant counts.

Specific activity of inorganic phosphate in different cell fractions. It was observed in these experiments, in which the rats had received ^{32}P at various times before death, that the specific activity of the acid-extractable inorganic phosphate of the nucleus was higher than either the soluble inorganic phosphate or the acid-labile phosphate of the nucleus (Table 3). Determination of the specific activities of inorganic and acid-labile phosphates in the cell sap apparently indicated that the specific activity of bound inorganic phosphate in the nucleus was higher than the inorganic phosphate in the cell sap at times between 5 and 60 min. after injection. It was, however, noticed that the amount of acid-

labile phosphate in the cell sap was appreciably lower than that found earlier (Ord & Stocken, 1958b) when whole rat thymus was analysed. This suggested that, in the course of fractionation at 0°, breakdown of less highly labelled compounds had taken place, so diluting the specific activity of the inorganic phosphate. When the activity was determined on extracts from the whole gland, frozen in liquid air immediately after removal, much higher specific activities for the inorganic phosphate were found (Table 4).

Assays of the γ-phosphate group of ATP indicated that ATP in the nucleus was not identical with that from the whole extract. The activity of the γ-phosphate group in the whole extract was initially lower than that of the inorganic phosphate; dilution from the nucleus could have occurred although the amount of ATP in the nucleus is less than in the cytoplasm. It did not prove possible to isolate mitochondria containing ATP from the thymus although a Ca^{2+}-free medium was used (Bellamy & Bartley, 1960; 0·15M-KCl–1 mM-tris–1 mM-EDTA), which had previously given good results for oxidative phosphorylation with thymus mitochondria. In cell sap obtained after sedimentation of the mitochondria in the Ca^{2+}-free medium the specific activity of the γ-phosphate group was identical with that from the whole thymus.

To find out why the nuclei retained their highly active inorganic phosphate during fractionation and to explore further the interactions between nuclear and cytoplasmic ATP and P, uptake of ^{32}P was studied *in vitro* with thymus nuclei and thymocytes.

Nature of the inorganic phosphate binding by the nucleus

'*Soluble*' *fraction of the nucleus.* The reality of the division of this phosphate into at least two components was based mainly on the physical separation of two inorganic phosphate fractions of different specific activities (Table 3). Since the soluble, low-specific-activity inorganic phosphate was obtained after acidification of the supernatant from the centrifuged nuclear suspension, and since this acidification produced a fairly copious white precipitate, it was possible that the inorganic phosphate had been split off from some substance(s) in the supernatant. This was tested by dialysing the supernatant from nuclei isolated in ionic medium against 2 or 3 vol. of medium at 0° (Table 5).

It was clear from determination of the total counts in the soluble fraction at the start and from the counts attributable to inorganic phosphate, that

Table 5. *Effect of dialysis against ionic medium on the 'soluble' phosphate component of nuclei from rat thymus*

Specific activities are given as counts/min./μg. of P. Expt. 1: the mean of 2 experiments with rats given 100 μc of ^{32}P/100 g. body weight 1 hr. before death. Expt. 2: rats given 50 μc of ^{32}P/100 g. body weight 20 min. before death.

	Expt. no.	
	1	2
At start		
Total counts in sac	3754	718
Counts due to inorganic phosphate	2020	423
Total inorganic [^{31}P]phosphate (μg.)	4·03	1·46
Specific activity of inorganic phosphate	502	289
Specific activity of bound inorganic phosphate	653	373
Ratio volume of sac:volume of diffusate	1:2·5	1:3
After dialysis for 2 hr. at 0°		
Counts in sac	2426	406
Specific activity of dialysable inorganic phosphate	663	359
Amount of dialysable inorganic phosphate (μg.)	2·01	0·78
Specific activity of non-dialysable material giving inorganic phosphate on treatment with N-HClO₄	424	214

not all the ^{32}P activity was due to material giving inorganic phosphate after treatment with cold N-HClO$_4$. ^{32}P could be extracted from the white precipitate by 0·5 N-HClO$_4$ at 70° and the extract showed traces of deoxyribose. This suggested that some DNA nucleoprotein was present in the soluble fraction, either as a result of leakage from the nuclei or because of poor sedimentation.

It was also evident from the specific activity of the dialysable inorganic phosphate that this inorganic phosphate was identical with the inorganic phosphate bound to the nucleus. Since the specific activity of the material within the sac fell as a result of dialysis, it seemed that the inorganic phosphate of the 'soluble' fraction was made up of two components: a dialysable fraction and a fraction of comparatively low specific activity which gave inorganic phosphate after treatment with acid. The nature of this non-dialysable component has not been studied further, although it was noted that incorporation of ^{32}P into the soluble fraction was inhibited by iodoacetate (Table 10).

The dialysable fraction apparently represented inorganic phosphate of high specific activity, which was either dissociated from its nuclear binding in the last stages of the isolation procedure or was normally trapped within the nucleus.

'*Bound*' *inorganic phosphate of the nucleus.* This fraction had been recognized because of its high specific activity when nuclei were isolated from animals given ^{32}P 5–60 min. previously. It was of interest to determine whether the nuclear suspension, after the 'soluble fraction' had been separated by centrifuging, would take up the isotope when shaken gently in ionic medium containing ^{32}P. Preliminary experiments established that resuspending the nuclei three times in unlabelled medium reduced the ^{32}P of the supernatant to a low level. Further washing appeared to cause breakdown of the nuclei, as shown by the appearance in the supernatant of acid-labile phosphate. It is clear that (Table 6) the specific activity of the bound inorganic phosphate after incubation of the nuclei with ^{32}P *in vitro* was much greater than that of the other two fractions examined and that there was no difference between the uptake at 0° and 37°, although it is possible that the nuclei were only able to tolerate rather short incubations at 37°. Additional experiments at 0° indicated that the uptake of ^{32}P into the bound inorganic phosphate was linear from 0 to 30 min. If nuclei from animals that had been given ^{32}P were incubated similarly in ionic medium containing 1 μmole of inorganic [^{31}P]phosphate/ml. (Table 7) and the soluble and bound fractions separated, there was a fall in the specific activity of the bound inorganic phosphate fraction. The change in specific activity was not as marked as with the uptake experiments but the specific

Table 6. *Specific activities of acid-soluble phosphates from rat-thymus nuclei labelled* in vitro *in ionic medium*

Specific activities are given as counts/min./μg. of P.

Time of incubation (min.)	Incubation at 0°			Incubation at 37°		
	Last wash	Bound inorganic phosphate	Acid-labile phosphate	Last wash	Bound inorganic phosphate	Acid-labile phosphate
30	760	2 055	66	—	—	—
30	240	1 580	120	264	1 357	91
10*	5 510	28 420	—	4530	29 950	—
30*	19 710	55 000	2740	3158	23 250	1131

* 1 μc of ^{32}P present/ml.

Table 7. *Specific activities of inorganic phosphate from rat-thymus nuclei labelled* in vivo *and incubated* in vitro *in ionic medium at 0° with inorganic* [^{31}P]*phosphate* (1 μmole/ml.)

Time (min.)	Specific activities of phosphate (counts/min./μg. of P)	
	Soluble inorganic	Bound inorganic
0	6·44	191
30	8·44	161
0	2·57	72·1
10	2·96	71·5

Table 8. *Effect of extraction with* M-*sodium chloride on the 'bound' inorganic phosphate fraction from rat thymus gland*

Specific activities are given as counts/min./μg. of P. In Expts. 1 and 2 the M-NaCl extracts were dialysed for 2 hr. at 0° against 2 vol. of M-NaCl; in Expt. 3 11 vol. of M-NaCl was used.

	Expt. no.		
	1	2	3
Specific activity of bound inorganic phosphate	650	655	—
Specific activity of inorganic phosphate after acidification of M-NaCl extract	671	500	—
Specific activity in DNA of M-NaCl extract	—	9·6	—
Total inorganic phosphate in M-NaCl extract (μg.)	2·54	1·6	4·32
Inorganic phosphate dialysable (%)	41	44	66·6
Specific activity of inorganic phosphate in diffusate	—	532	—

activity of the inorganic phosphate of the nucleus was about 30 times that of the supernatant as compared with an extremely high activity in the supernatant during incorporation *in vitro*.

The fact that the bound inorganic phosphate fraction was removed after treatment with acid suggested that an ionic linkage might be involved and this is supported by the high rate of exchange

of the phosphate at 0°. The possibility that the inorganic phosphate might be associated with a lipid component was investigated by using the $CHCl_3$–methanol procedure described by Getz & Bartley (1961). Shaking the lipid solution with N-HClO$_4$ did not liberate appreciable inorganic [^{31}P]- or [^{32}P]-phosphate into the aqueous phase.

It was therefore decided to investigate extraction procedures that had been applied to nuclei in other work. Allfrey *et al.* (1957*a*) have described the sensitivity of nucleotide retention by calf-thymus nuclei to extraction with 0·02M-acetate buffer, pH 4·5–5·0. This was confirmed in our experiments but short-time treatment with acetate buffer, comparable with that with N-HClO$_4$, was less effective in dissociating the phosphate and nucleotides than acid extraction.

Extraction of nuclei, from which the soluble fraction had been removed, by M-NaCl at 0° (Allfrey *et al.* 1957*a*) led to the isolation of a clear, viscous fraction, which on treatment with N-HClO$_4$ gave inorganic phosphate of the same specific activity as in the original nuclei, in about 70 % yield (Table 8). Although there was a considerable purification on a DNA basis the extraction was by no means complete and there were indications that inorganic phosphate could be obtained from the residue after centrifuging off the viscous supernatant. The specific activity of the inorganic phosphate obtained by acidification of the M-NaCl extract was enormously greater than that of the DNA present in the same fraction. Dialysis of the non-acidified material for 2 hr. against M-NaCl showed that 40 % of the inorganic phosphate bound in the sol was dialysable. When dialysed against a larger volume of M-NaCl a greater proportion of inorganic phosphate passed through the membrane, and it would appear therefore that the complex with inorganic phosphate was dissociable by solutions of high ionic strength.

Arginine uptake by nuclei

In some experiments analyses were made for components other than phosphates in the acid extracts from the nuclei or nucleoprotein. Chro-

Table 9. *Uptake of ^{32}P by thymocyte suspensions* in vitro

Control and experimental incubations were for 15 min. at 37° unless otherwise stated. In all the experiments 0·3 μc of ^{32}P/ml. was present. The concentration of iodoacetate was 1 mM.

		Free nuclei (%)	Specific activities of phosphate (counts/min./μg. of P)				
	10^{-8} × Cells/ml.		Nuclei			Cell sap	
			Total inorganic	Soluble inorganic	Bound inorganic	Inorganic	Acid-labile
Control	26·9	19	575	—	—	224	103
15 min. at 0°			229	—	—	7	3
Control	51·2	6	256	—	—	183	105
Iodoacetate–O₂			242	—	—	198	211
Control	48·6	0	—	100	259	256	135
Iodoacetate–N₂			—	34	93	98	41
Control	53·7	20	—	483	601	490	297
Iodoacetate–N₂			—	143	661	69	45
Control*	23	36	—	595	772	502	253
Iodoacetate–N₂			—	500	900	222	104

* Rabbit thymocytes used.

matographic evidence indicated the presence of arginine, so that the possibility of nuclear binding of arginine was also investigated. Allfrey & Mirsky (1959) have provided evidence that amino acid incorporation into protein in calf-thymus nuclei is likely to be through an activated amino acid–RNA complex similar to that in microsomal protein synthesis. Hoagland, Zamecnik & Stephenson (1957) established that amino acid–soluble RNA complexes were not broken down by cold 0·4N-HClO₄. It therefore seemed probable that the arginine found in the extracts was not derived from breakdown of an arginine–RNA complex. Estimation of the arginine:inorganic + acid-labile phosphate ratios in the 'bound' fraction from isolated nuclei showed the presence of about 0·5 mole of arginine/mole of total inorganic + acid-labile phosphate. Incubation of the nuclei in ionic medium at 0° with [^{14}C]arginine showed a linear uptake. [^{14}C]Arginine (50 000 counts/min.) was added to nuclei from 12 thymus glands suspended in 14 ml. of ionic medium. At 0, 5 and 15 min. incubation at 0° the specific activities of the bound arginine were 1100, 1745 and 2880 counts/min./μmole. The rate of uptake appeared to be similar to that for inorganic phosphate.

Uptake of ^{32}P by thymocyte suspensions

Suspensions of whole cells are comparatively easy to obtain from rat thymus (Ord & Stocken, 1958a). The thymocytes can be distinguished from naked nuclei by examination with phase-contrast microscopy in albumin solution (Barer, Joseph & Esnouf, 1956). After incubation with ^{32}P the suspensions were immediately diluted with 20 vol. of medium at 0° and washed three times. The cells were suspended in a small volume of medium C and homogenized with a plunger of 0·002 in. clearance.

The nuclei were then washed in the usual way. The first supernatant obtained after sedimentation of the nuclei was centrifuged at 10 000g and the specific activities of inorganic and acid-labile phosphate were determined.

Uptake of ^{32}P into the cell sap was negligible at 0° although the nuclear inorganic phosphate became labelled. In all the experiments the specific activity of the nuclear inorganic phosphate exceeded that of the cell sap by an amount that seemed to be proportional to the number of free nuclei present in the original suspension (Table 9). This was confirmed by incubating in the presence of 1 mM-iodoacetate. Preliminary experiments showed that 1 mM-iodoacetate had no effect on ^{32}P uptake into the bound inorganic phosphate of isolated nuclei; with thymocyte suspensions iodoacetate under anaerobic conditions reduced the uptake into the cell sap by about 70 % but where free nuclei were present their uptake was unaffected or slightly increased. In the experiment with iodoacetate under aerobic conditions ^{32}P uptake into the cell sap was not decreased.

DISCUSSION

The interpretation of the results presented here rests on the purity of the nuclear fraction and on the accuracy of the phosphate determinations. Preparations of nuclei were examined by phase-contrast microscopy and after staining. Very few whole cells were found and there was very little contamination from cytoplasmic debris or red cells. The majority of the nuclei were small and round but about 10 % were larger and less regular in shape. Some of the nuclei, especially larger ones, had tags of cytoplasm attached. Classical radioautography does not enable the presence of water-soluble compounds in nuclei to be detected; a technique has,

however, been devised permitting the retention of these compounds (Fitzgerald, Ord & Stocken, 1961). By this means it has been possible to show, in nuclei from thymus glands of rats given the isotope *in vivo*, ^{32}P that was not present in nuclei that had been washed with N-perchloric acid or fixed by the usual methods. A detailed examination of the nuclei has not so far been made so that it is not possible to say whether the uptake is a feature of all types of nuclei at all stages of their development.

Ionic medium was used for the isolation of nuclei in most of the experiments with thymus, and no difference in properties has been found between nuclei prepared in this medium and those in sucrose. We have found that nuclei in ionic medium were easier to prepare and handle and appeared to tolerate the final resuspension to a small volume better than in sucrose.

The amount of inorganic phosphate in the nuclear suspensions was very low so that quantities of $0.5 \mu g$. were sometimes estimated. It was difficult to increase the amount assayed because of the limit to the number of thymus glands that could be used in the preparation of the nuclei. In nuclei from thymus glands that had been removed immediately after death, where the total inorganic phosphate was particularly low, the amount of inorganic phosphate in the 'soluble' and 'bound' fractions was approximately the same but the difference in ^{32}P activity was very marked. The accuracy of the ^{32}P determinations was $\pm 3 \%$.

Much of the pioneering work on uptake of ^{32}P by tissues was carried out by Hevesy (1948) and his colleagues and he and Marshak (1941) were the first to study incorporation into the nuclear fraction of cells. The isolation procedure entailed a low pH and little activity was detected in the residual acid-soluble fraction. Recent work on yeast (Jennings, Hooper & Rothstein, 1958) and erythrocytes (Glynn, 1957) has shown that the entry of inorganic phosphate into these cells is markedly dependent on glycolysis and that initially glycolytic intermediates and ATP have higher specific activities than intracellular inorganic phosphate. Sacks & Altshuler (1942) studied the uptake of phosphate by cardiac and striated muscle and concluded that it was first converted into organophosphate. In Ehrlich ascites-tumour cells Wu & Racker (1959) found that, whereas addition of glucose stimulated the entry of inorganic phosphate, in the absence of glucose the entry appeared to be by an exchange diffusion that was unaffected by iodoacetate. Our experiments on the entry of inorganic phosphate into the cytoplasm of thymocytes lead to a similar interpretation, and it seems that in these cells uptake of inorganic phosphate may be linked to glucose utilization but does not occur exclusively through glycolytic intermediates.

The reversible uptake of inorganic phosphate by nuclei at 0°, the absence of a temperature effect on the reaction, its insensitivity to 1 mM-iodoacetate and the removal of the inorganic phosphate by treatment with cold, dilute acid suggest that the inorganic phosphate is bound to the nucleus in an ionic linkage. The site of the binding remains obscure. Association with histones seems probable and is consistent with the extraction of the bound material by M-sodium chloride. Arginine binding might be expected since Mirsky & Ris (1951) and Davison & Butler (1956) have reported the presence of free negative charges on DNA–nucleoprotein. Kuzin & Shabadash (1959) have proposed that excessive negative charges are especially evident in nuclei from lymphoid tissues. It is not yet known whether other basic amino acids are similarly bound. Na^+ ion binding in calf-thymus nuclei is indicated from the work of Itoh & Schwartz (1957), who found a reversal of the Na:K ratio from that in the cytoplasm. Preliminary experiments have shown that ^{32}P uptake by isolated rat-thymus nuclei was unaffected by the addition of arginine to the medium, suggesting either that the inorganic phosphate and arginine are taken up independently of one another or that the system is already saturated with arginine.

The relationship of the nucleoprotein described here to chromosomes or the nucleolus is unknown but the results suggest that the charged macromolecules have associated with them in the intact nucleus an ordered and reproducible array of charged, low-molecular-weight compounds. It may not be coincidence that nucleotide binding by rat liver is very low or absent (Rees & Rowland, 1961) and that so far nuclear phosphorylation, which presumes the presence of bound nucleotide acceptors, has been described only in nuclei from tissues containing dividing cells (Creasey & Stocken, 1959). It seems possible that the binding of low-molecular-weight compounds in the nucleus may be relevant to the incorporation of those same molecules into the macromolecular structure of the nucleus. However, Allfrey *et al.* (1955) found no significant inhibition of amino acid incorporation into the proteins of isolated nuclei when free phosphate groups in the DNA were blocked by various reagents.

SUMMARY

1. Some of the conditions have been defined for the isolation of nuclei from rat thymus gland containing reproducible amounts of acid-soluble phosphates. The nuclei contained 1–2 μg. of inorganic phosphate and about 10 μg. of acid-labile phosphate/mg. of deoxyribonucleic acid phosphorus.

2. ^{32}P was taken up readily by nuclei *in vivo* and *in vitro*. The process occurred rapidly at 0°, was not

accelerated by increasing the temperature to 37° and was not inhibited by 1 mM-iodoacetate.

3. The binding of inorganic phosphate by thymus nuclei was investigated. Linkage to nucleoprotein is probable but the exact nature of the complex is not yet certain.

4. Entry of inorganic phosphate to the nucleus did not require prior uptake into adenosine triphosphate. Entry into the cytoplasm was possibly linked with glucose utilization; its dependence on high-energy phosphate compounds is discussed.

5. Arginine also could be extracted from the nuclei with 0·5–1·0N-perchloric acid at 0°. The molar ratio of total inorganic and labile phosphates: arginine was approximately 2:1.

6. The arginine in the nuclei exchanged rapidly with [^{14}C]arginine at 0°.

REFERENCES

Allfrey, V. G. & Mirsky, A. E. (1959). Proc. nat. Acad. Sci., Wash., 45, 1325.

Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1955). Nature, Lond., 176, 1042.

Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1957a). J. gen. Physiol. 40, 451, 491.

Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1957b). In Chemical Basis of Heredity. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Anderson, N. G. (1953). Science, 117, 517.

Barer, R., Joseph, S. & Esnouf, M. P. (1956). Science, 123, 24.

Bellamy, D. & Bartley, W. (1960). Biochem. J. 76, 78.

Berenblum, I. & Chain, E. (1938). Biochem. J. 32, 295.

Burton, K. (1956). Biochem. J. 62, 315.

Creasey, W. A. & Stocken, L. A. (1959). Biochem. J. 72, 519.

Davison, P. F. & Butler, J. A. V. (1956). Biochim. biophys. acta, 21, 568.

Fitzgerald, P. J., Ord, M. G. & Stocken, L. A. (1961). Nature, Lond., 189, 55.

Getz, G. S. & Bartley, W. (1961). Biochem. J. 78, 307.

Glynn, I. M. (1957). Progr. Biophys. 8, 241.

Hevesy, G. (1948). Radioactive Indicators. New York: Interscience Publishers Inc.

Hoagland, M. B., Zamecnik, P. C. & Stephenson, M. L. (1957). Biochim. biophys. acta, 24, 215.

Hogeboom, G. H., Schneider, W. C. & Striebich, M. J. (1952). J. biol. Chem. 196, 111.

Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). J. biol. Chem. 209, 23.

Itoh, S. & Schwartz, I. L. (1957). Amer. J. Physiol. 188, 490.

Jennings, D. H., Hooper, D. C. & Rothstein, A. (1958). J. gen. Physiol. 41, 1019.

Kuzin, A. M. & Shabadash, A. L. (1959). Progr. Nuclear Energy, series VI, 2, 364.

Marshak, A. (1941). J. gen. Physiol. 25, 275.

Mirsky, A. E. & Ris, H. (1951). J. gen. Physiol. 34, 475.

Ord, M. G. & Stocken, L. A. (1958a). Biochem. J. 68, 410.

Ord, M. G. & Stocken, L. A. (1958b). Biochim. biophys. acta, 29, 201.

Philpot, J. St L. & Stanier, J. E. (1956). Biochem. J. 63, 214.

Rees, K. R. & Rowland, G. F. (1961). Biochem. J. 78, 89.

Rosenberg, H., Ennor, A. H. & Morrison, J. F. (1956). Biochem. J. 63, 153.

Sacks, J. & Altshuler, C. H. (1942). Amer. J. Physiol. 137, 750.

Wu, R. & Racker, E. (1959). J. biol. Chem. 234, 1029.

Phospholipid Metabolism in Nervous Tissue

4. INCORPORATION OF ^{32}P INTO THE LIPIDS OF SUBCELLULAR FRACTIONS OF THE BRAIN*

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In earlier investigations (Davison & Dobbing, 1960a, b; Davison, Morgan, Wajda & Payling Wright, 1959) it was shown, by introduction of radioactive precursors into developing animals,

* Part 3: Davison & Dobbing (1960b).

that part of the brain lipids remained metabolically stable during growth and subsequently in the adult animal. It was suggested that this metabolic stability was associated with the myelin sheath and possibly other anatomical structures in the brain. This concept was supported by the demonstration