Phosphate-Transfer Reactions by Nuclei from Rat Thymus Gland

BY MARGERY G. ORD AND L. A. STOCKEN
Department of Biochemistry, University of Oxford

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The ability of isolated calf-thymus nuclei to convert nucleoside monophosphates into triphosphates was discovered by Allfrey, Mirsky & Osawa (1955). A production of acid-labile phosphate compounds has also been found to occur in nuclei from tissues containing a relatively high proportion of dividing cells (Creasey & Stocken, 1959). The demonstration that nuclei from rat thymus reversibly exchanged inorganic phosphate and arginine (Ord & Stocken, 1961*a*) led to an investigation of the conditions under which the exchanges took place and in particular to the effect of nucleoside triphosphates on phosphorylation and phosphorolysis catalysed by isolated nuclei. Two types of reaction have been found depending mainly on the extent of phosphorylation of the nucleosides in the initial nuclear suspension. In nuclei in which triphosphates predominated, acid-labile phosphate disappeared from the acid-extractable fraction of the nuclei during incubation at 0°; if the amount of triphosphate was low, nuclear phosphorylation could be shown or, if the nuclei were incubated in the presence of ATP, uptake of ATP was found.

MATERIALS AND METHODS

The preparation of the nuclei and the determination of the inorganic phosphate and acid-labile phosphate were as described by Ord & Stocken (1961*a*). The 'ionic medium C' of Creasey & Stocken (1959) was used for all the experiments. Usually the nuclear reactions were stopped by centrifuging the preparation, separating the supernatant and resuspending the sedimented nuclei in the original volume of medium. The supernatant and resuspended nuclei were then acidified separately and the precipitates discarded, leaving respectively the 'soluble' and 'bound' acid-extractable fractions of the nuclei.

Enzyme assays on isolated nuclei

All enzyme activities were expressed as μm -moles of substrate transferred/min./mg. of DNA phosphorus.

Hexokinase. This was measured by the disappearance of labile phosphate after incubating 2 ml. of the standard preparation of nuclei in ionic medium (which contains 100 mg. of glucose/100 ml.) for 25 min. at 0° with a final concentration of 1.45 mM-ATP. Because of the presence in the nuclei of a very active adenosine triphosphatase the fall in total inorganic phosphate plus acid-labile phosphate was followed.

'Glucose 6-phosphate dehydrogenase'. This was estimated by incubating 2 ml. of nuclei with glucose 6-phosphate

(final concn. 1 mM) and NADP⁺ (final concn. 0.25 mM) for 25 min. at 0°. The suspensions were centrifuged and the NADPH in the clear supernatant was measured at 340 $\mu\mu$ at room temperature. Controls were run without the addition of glucose 6-phosphate. No attempt was made to separate the different NADP⁺-dependent reactions.

'Glucose 1-phosphate isomerase'. This was assayed as for the 'glucose 6-phosphate dehydrogenase' except that glucose 1-phosphate replaced glucose 6-phosphate in the incubation medium.

Adenosine-triphosphatase activity. This was followed by determining the inorganic phosphate liberated after incubating 2 ml. of nuclei with 1.45 mM-ATP at 0°.

Substrate assays

Sugar phosphates. These were analysed chromatographically by the method of Khym & Cohn (1953). Rats were given 100 μC of ³²P-labelled phosphate/100 g. body wt. 1 hr. before death and the nuclei prepared from 24 thymus glands. Then 1 μmole each of AMP, ADP, ATP, glucose 1-phosphate, glucose 6-phosphate, ribose 5-phosphate, fructose 1,6-diphosphate, NAD and NADP was added to the extract before conversion into the free acids by passage through a cation-exchange resin (Amberlite IR-100H). The extract was then applied to an anion-exchange column (Dowex 1 X 10; 200–400 mesh; Cl[−] form) after adjusting to pH 8.0 with aq. NH₃. The ³²P activity of the eluate was followed continuously with an automatic scanner.

Firefly determination of adenosine triphosphate. In later experiments ATP was measured by the method of Holton (1959). This necessitated precipitation by trichloroacetic acid (final concn. 6%, w/v); the acid was then removed by extraction with ether. Because of inhibition by Cl[−] ions present in the medium, the method was calibrated with 10 μM -ATP in the presence of ionic medium.

Pyrophosphate. This was determined with yeast pyrophosphatase (Bailey & Webb, 1944).

Glycogen. This was precipitated from acid extracts of nuclei by the addition of 1 vol. of ethanol. After hydrolysis for 2 hr. at 100° with N-H₂SO₄ and neutralization the glucose was determined either by the method of Hagedorn & Jensen (1923) or Nelson (1944), or with glucose oxidase (Huggett & Nixon, 1957).

Histone protein. This was estimated by the biuret method (Gornall, Bardawill & David, 1949).

RESULTS

Phosphate-transfer reactions

Ord & Stocken (1961*a*) demonstrated that one of the most critical factors in the isolation of nuclei was the time at which the thymus was removed

from the rat after death. When the thymus was removed within 20 sec. the nucleosides were present mainly as triphosphates of which about 70% was found to be ATP by chromatographic analysis or with hexokinase ('fresh' nuclei). When these nuclei were incubated at 0° with gentle shaking nuclear phosphorylation could not be detected. Though this might be expected if only small amounts of acceptor were present for phosphorylation, it was surprising to find that acid-labile phosphate disappeared from the acid-extractable fraction during the incubation without the concomitant appearance of inorganic phosphate (Table 1). If nuclei were prepared from rats given ³²P-labelled phosphate before death the disappearance of ATP could be confirmed. Incubations were usually carried out for 15–25 min. but the reaction was rapid and was often complete within 5 min. The average (acid-labile phosphate):(inorganic phosphate) ratio of the nuclei showing this reaction was 2.3.

In the belief that phosphorylation would require the presence of acceptors, nuclei were isolated from thymus glands which were not removed from the animal until 10 min. after death, so as to promote ATP breakdown. The (acid-labile phosphate):(inorganic phosphate) ratio of these nuclei was 0.91 ('aged' nuclei). During incubation for 15–25 min. at 0° phosphorylation then took place (Table 1), but not invariably. An attempt was therefore made to investigate the mechanisms of the transfer of labile phosphate by nuclei in more detail so that reproducibility might be attained.

Table 1. *Phosphate-transfer reactions by nuclei isolated from rat thymus gland*

The concentrations are expressed as μ m-moles of phosphate/mg. of DNA phosphorus. The nuclei were incubated for 25 min. at 0° with gentle shaking. The reaction was stopped by the addition of perchloric acid and the inorganic phosphate (P_i) and acid-labile phosphate (P_{15}) were determined on the deproteinized extract.

	Initial		Change after incubation at 0°	
	P_i	P_{15}	P_i	P_{15}
Rapid removal of thymus	60	120	+40	-50
	20	180	+10	-60
	100	200	0	-60
	100	360	+10	-70
	210	310	0	-80
	100	200	0	-40
Mean	100	230	+10	-60
Delayed removal of thymus	80	60	+10	+40
	130	100	-20	+50
	80	90	+10	+50
	130	160	-20	+50
	220	160	-50	+60
Mean	110	100	-10	+50

Nuclear enzymes and substrates

Insight into the mechanism whereby ATP disappeared from nuclei having an (acid-labile phosphate):(inorganic phosphate) ratio > 2 came from the observation that nuclei isolated in sucrose-calcium chloride solution did not show the reaction but would do so if supplemented with sodium chloride, magnesium chloride and glucose (Logan, Errera & Ficq, 1959). This suggested that a Mg^{2+} ion-dependent kinase might be involved. Direct assay of hexokinase in nuclei isolated in ionic medium, which contains 100 mg. of glucose/100 ml., showed that the enzyme was present, 10 μ m-moles of the γ -phosphate of ATP being transferred/min./mg. of DNA phosphorus at 0°. This rate would be sufficient to account for the loss of labile phosphate found on incubation of nuclei with an (acid-labile phosphate):(inorganic phosphate) ratio > 2, and the presence of the enzyme would also reduce the amount of ATP apparently formed during the phosphorylation reaction.

After the finding of hexokinase in the nuclear preparation other enzymes were investigated which might be concerned in carbohydrate metabolism. Glucose 6-phosphate dehydrogenase had already been described in thymus, kidney and liver nuclei (Stern & Mirsky, 1953; Siebert, 1960). Nuclear suspensions obtained after a single wash reduced about 3 μ m-moles of $NADP^+$ /min./mg. of DNA phosphorus at room temperature. Chromatographic evidence (see below) indicated the presence of glucose 1-phosphate, and assays with glucose 1-phosphate and $NADP^+$ gave a rate of reduction of 0.8 μ m-mole of $NADP^+$ /min./mg. of DNA phosphorus at room temperature, which suggested that phosphoglucumutase was present.

Adenosine triphosphatase was also detected in the nuclei, W. A. Creasey's (unpublished work) and our findings showing the liberation of about 90 μ m-moles of inorganic phosphate/min./mg. of DNA phosphorus at 0°. It was noticed that incubation of nuclei at 0° without additional ATP did not necessarily lead to a breakdown of the ATP present at the start of the incubation (Table 1). This is in contrast with the utilization of nuclear ATP by the hexokinase present in the preparation, and suggests that for steric or other reasons nuclear ATP is not susceptible to nuclear adenosine triphosphatase, although conditions can easily be envisaged in which the barrier to its hydrolysis is removed. A decreased concentration of nuclear ATP was also found after the unfractionated homogenate had been kept at 0°, which suggested that the triphosphate was, or became, freely accessible to cytoplasmic adenosine triphosphatase in the ionic medium containing Ca^{2+} (and Mg^{2+}) ions. The high activity of these cytoplasmic adenosine triphos-

Table 2. *Effect of washing on certain nuclear enzymes and substrates*

'Fresh' nuclei were prepared as described in the text. After the first centrifuging, the nuclei were suspended in a measured volume of medium. One portion was diluted, resedimented and resuspended in a final volume equivalent to one thymus/ml. With a second portion this process was repeated twice.

	After first wash	After second wash
Phosphorylation ($\mu\text{m-moles}$ of phosphate/25 min./mg. of DNA phosphorus)	+ 54	+ 7
Glucose 1-phosphate isomerase ($\mu\text{m-moles}$ of NADPH formed/min./mg. of DNA phosphorus)	0.6	0.8
Adenosine triphosphatase ($\mu\text{m-moles}$ of inorganic phosphate liberated/min./mg. of DNA phosphorus)	35	38
Glycogen ($\mu\text{m-moles}$ of glucose/mg. of DNA phosphorus)	7.2	6.5

phatases in ionic medium had been noticed by Ord & Stocken (1961a).

When once- and twice-washed nuclei were prepared from the same homogenate (Table 2), washing did not alter the activity of either the adenosine triphosphatase or the enzyme complex linking glucose 1-phosphate with NADP^+ reduction.

With the finding of the firm attachment of the enzymes of the Embden-Meyerhof and hexose monophosphate pathways to the nuclei, a search was made for substrates which might be utilized by these enzymes for phosphorylation. Rats were given ^{32}P -labelled phosphate 1 hr. before death and nuclei were prepared in ionic medium not supplemented by glucose. Chromatographic analyses were made for sugar phosphates present in the perchloric acid extract from the nuclei. Trace amounts of possible phosphates were added and the ^{32}P activity in the eluate was followed. A peak was found in ^{32}P activity immediately preceding that due to labelled inorganic phosphate and in the position expected for glucose 1-phosphate. No other ^{32}P -labelled peaks were obtained except those of the nucleoside phosphates described by Ord & Stocken (1961a). A second experiment under identical conditions, but with only glucose 1-phosphate added, again indicated a peak in ^{32}P activity corresponding to glucose 1-phosphate. Unfortunately the amount of material available for analysis from rat thymus is very small so that identification of the sugar phosphate must be regarded as provisional, but confirmation of the presence of endogenous substrate which could be linked to NADP^+ reduction came from the enzyme assays performed with the addition of NADP^+ but without either glucose 1-phosphate or glucose 6-phosphate. These controls always showed an increase in extinction at $340\text{ m}\mu$ during incubation.

The possible presence of sugar phosphates suggested that glycogen might also be found in nuclei. Nuclei were therefore isolated in ionic medium not supplemented with glucose and a concentrated extract (equivalent to 12 thymus glands in 5 ml. of

6% trichloroacetic acid) was obtained from which any glycogen could be precipitated (Table 2). Small amounts of the polysaccharide were found and the concentration was not appreciably diminished by further washing of the nuclei before acidification.

Adenosine triphosphate exchange by isolated nuclei

The presence of glycolytic enzymes and substrates firmly attached to the nuclei and the strong binding of the ATP raised the question of the reversibility of these attachments and of the utilization by nuclei of cytoplasmic ATP. Allfrey *et al.* (1955) reported that added AMP was not used for nuclear phosphorylation but as far as we are aware no results on ATP exchange between nuclei and their medium are available.

Two types of experiment were carried out: in the first, ^{32}P -labelled phosphate was administered to the rats 30 min. before death and the nuclei were isolated and incubated for 30 min. at 0° in ionic medium containing unlabelled ATP, but no glucose. Alternatively, unlabelled nuclei were incubated in medium with the same final concentration of ATP ($1\text{ }\mu\text{mole}$ of acid-labile phosphate/ml.) to which ^{32}P -labelled ATP (contaminated with labelled inorganic phosphate) had been added. The excess of unlabelled ATP in the medium was required because of the rapid breakdown of exogenous ATP by nuclei. The ^{32}P was taken up by or released from the nuclei (Table 3) during the incubation, without change in their total bound acid-labile phosphate. The nuclear adenosine triphosphatase was active during the experiment, and as to be expected from previous work on the exchange reactions shown by bound inorganic phosphate in the nucleus (Ord & Stocken, 1961a) ^{32}P was taken up by, or released from, this fraction also.

These experiments coincided with the introduction of the firefly method for the determination of ATP, and an unexpected feature of the results was the fall in concentration of ATP in the nuclei during the incubation, although the total chemically-estimated acid-labile phosphate remained con-

stant. Inhibition of the firefly determination of ATP by histones liberated by the acid used to stop the reaction (see below) failed to account for the loss of triphosphate and it was concluded that the fall in the concentration of ATP from an initial level of 60–100 % of the total acid-labile phosphate might reflect either the action of adenylate kinase, which has been implicated in much recent work on thymus nuclei and its presence confirmed by Miller & Goldfeder (1961), or utilization of ATP for the activation of amino acids or other substrates retained by the nuclei. The presence of pyrophosphate in nuclei is described below.

Experiments in which the incubation with ^{32}P -

labelled ATP was performed in the presence of 0.2 mM-dinitrophenol showed very little difference in uptake into bound acid-labile phosphate from control incubations; the adenosine-triphosphatase activity of the nuclei was not significantly affected.

In these experiments, with nuclei isolated rapidly ('fresh'), no net uptake of ATP occurred. If nuclei were isolated from thymus gland which had not been removed until 10 min. after the death of the rat ('aged'), actual uptake of ATP could be shown (Table 4). In the second of the two experiments the nuclei incubated without additional ATP showed an increase in chemically-determined acid-labile phosphate which was not accounted for

Table 3. *Adenosine triphosphate exchange reactions in isolated nuclei*

The nuclei were shaken in ionic medium, without glucose, for 30 min. at 0°. Unlabelled ATP was added (1 $\mu\text{mole/ml.}$) in both types of experiment and sufficient ^{32}P -labelled ATP (also containing labelled inorganic phosphate) to give about 3000 counts/min./ml. The nuclear preparation was centrifuged and the supernatant (1) separated. The sedimented nuclei were washed and resuspended in the original volume of ionic medium. This final suspension and the supernatant (1) were acidified with trichloroacetic acid to give, after further centrifuging, the 'bound' and 'soluble' fractions respectively. Inorganic phosphate (P_i) and acid-labile phosphate (P_{15}) were measured in the acid extracts and ATP was assayed in the two fractions after removal of the trichloroacetic acid.

Time of incubation (min.)	^{31}P ($\mu\text{g./ml.}$)						^{32}P (counts/min./ml.)			
	Soluble			Bound			Soluble		Bound	
	P_i	P_{15}	ATP	P_i	P_{15}	ATP	P_i	P_{15}	P_i	P_{15}
Unlabelled nuclei										
0	3.9	80.7	58.3	0.51	0.38	0.37	2024	1626	20	38
30	15.5	68.7	52.1	0.42	0.34	0.2	2315	1185	23	72
0*	4.9	87.3	—	0.58	1.32	0.81	3553	2342	37	19
30*	22.8	69.0	—	0.90	0.98	0.16	3453	1923	119	65
30*	22.5	65.0	—	—	1.12	0.18	3720	2243	—	104
^{32}P -labelled nuclei										
0	6.1	—	—	1.73	1.75	—	47	18	87	32
30	18.6	—	—	1.85	1.91	—	54	23	81	26

* Dinitrophenol added to give a final concn. of 0.2 mM.

Table 4. *Concentrations of labile phosphate in nuclei incubated for 30 min. at 0° in the presence and absence of adenosine triphosphate*

The concentrations are expressed as $\mu\text{g.}$ of phosphorus/ml. Nuclei were isolated from 'aged thymus gland' (see text) in ionic medium without glucose. The nuclear suspensions were centrifuged and washed once before extraction to give the 'bound' fraction. In Expts. 1–3, ATP was added to give a final concn. of 1 $\mu\text{mole/ml.}$; in Expt. 4 the final concn. of ATP was 0.25 $\mu\text{mole/ml.}$ P_i , inorganic phosphate; P_{15} , acid-labile phosphate.

Expt.	Phosphate	Initial		Change after 30 min.	
		Without ATP	With ATP	Without ATP	With ATP
1	P_i	0.06	0.10	+0.12	+0.52
	ATP (as P_{15})	0.071	0.071	–0.018	+0.010
2	P_i	0.34	0.62	–0.28	–0.16
	P_{15}	0.18	0.34	+0.34	+0.86
	ATP	0.029	0.029	–0.010	+0.086
3	P_i	0.14	0.31	–0.08	+0.15
	P_{15}	0.47	0.43	–0.21	–0.07
	ATP	0.10	0.03	–0.10	+0.016
4	P_i	0*	0.04	+0.10*	+0.10
	P_{15}	0.62*	0.22	–0.10*	+0.20
	ATP	0.067*	0.032	–0.035*	+0.021

* ADP (final concn. 0.5 $\mu\text{mole/ml.}$) added.

Table 5. *Amino acid activation and pyrophosphate formed by thymus nuclei at 0°*

Concentrations are expressed as $\mu\text{m-moles/mg.}$ of DNA phosphorus. The reaction was stopped by the addition of trichloroacetic acid, and the ATP, pyrophosphate and arginine were determined after the removal of the trichloroacetic acid from the deproteinized extracts. Arginine was determined by the method of Rosenberg, Ennor & Morrison (1956). In the first two experiments 'fresh' nuclei were used. Phosphate concentrations only were determined on 2 ml. portions of the nuclear preparation stopped at 0 min., and on other 2 ml. portions stopped after incubation. In the second series of experiments, only acid-extractable arginine was measured. P_i , inorganic phosphate; PP, pyrophosphate.

	Initial concn. of phosphate		Change after 30 min.		
	ATP	PP	P_i	ATP	PP
	3.9	0	+5.3	-2.2	+16.9
	4.6	0	+14.0	-1.4	+40.9
	Initial concn. of arginine		Change after 30 min.		
'Fresh' nuclei	117			-73.5	
	155			-41	
'Aged' nuclei	62			-10	
	81			0	
	88			-5	

Table 6. *Labile-phosphate content of nuclei isolated from rat thymus gland*

The results are expressed as $\mu\text{m-moles}$ of pyrophosphate/mg. of DNA phosphorus. 'Fresh': nuclei prepared after rapid removal of the thymus gland; 'aged': nuclei prepared from thymus gland removed from rats 10 min. after death. P_{15} , acid-labile phosphate.

	'Fresh' nuclei	'Aged' nuclei
Chemically determined P_{15}	60-180	30-90
Pyrophosphate	0-10	8-33
ATP	30-180	0-7

by ATP. When the effects of ADP and ATP in the medium were compared ATP uptake was found only in the presence of ATP, indicating that ADP was not taken up and phosphorylated to ATP. When ADP was added to the incubation medium breakdown to inorganic phosphate occurred at nearly the same rate as with ATP.

Pyrophosphate formation

The loss of ATP and constancy of total acid-labile phosphate found when 'fresh' nuclei were shaken at 0° suggested that pyrophosphate might be produced. This could be demonstrated even in preparations with low ATP contents (Table 5), and there was also a fall in the concentration of arginine bound on the nuclei (Ord & Stocken, 1961*a*) and in total ninhydrin-reacting material under the same conditions of incubation. In 'aged' nuclei the concentration of bound arginine was diminished and little disappeared on subsequent shaking. These nuclei (Table 6) normally contain pyrophosphate, in contrast with those prepared after rapid removal of the gland. Activation of amino acids in

nuclei proceeds by mechanisms essentially similar to those in the cytoplasm (Hopkins, 1959), but it was unexpected to find the reactions occurring so rapidly at 0°.

Factors concerned in nuclear phosphorylation

All attempts at phosphorylation were made with 'aged' nuclei. These nuclei have very little bound ATP; in some cases virtually all the acid-labile phosphate may be due to pyrophosphate (Table 6), but usually there is an excess of acid-labile phosphate over that accounted for as ATP plus pyrophosphate. The concentration of ADP is believed to be very low (Allfrey *et al.* 1955), and it is suggested that the excess of acid-labile phosphate is due to glycogenolysis occurring in the nuclei in the 10 min. in which the thymus remains in the animal after death, giving rise to such acid-labile compounds as glucose 1-phosphate and fructose 1,6-diphosphate. The decreased concentrations of bound arginine (Table 5) and ATP, and the presence of the pyrophosphate, are ascribed to activation of bound amino acids on the nuclei during the 10 min. period.

In two consecutive years positive results were obtained in the mornings from March to May inclusive but in June and July no phosphorylation occurred (Table 7). Since the reaction might be required for cell division rats were kept under controlled lighting conditions for later experiments. The animals were put from the time of weaning into a room with a 12 hr. light-period, from 8 a.m. to 8 p.m. Food and water were available *ad lib.*, and from their liver-glycogen concentrations the animals probably ate when the light went off at night and just before it went on in the morning.

Table 7. *Analysis of variables affecting nuclear phosphorylation*

The number of experiments in which phosphorylation occurred is given. The month refers to the month through which the experiments were carried out. Controlled daylight was with light in the animal house from 8 a.m. to 8 p.m. When present the glucose concentration was 100 mg./100 ml. of ionic medium.

First variant	Invariant	Second variant. No. of washes	
March-May	Glucose present	1 5/7	2 2/7
		Daylight	
July	Glucose present, one wash	Controlled 3/3	Uncontrolled 1/5
		Glucose	
March-May and controlled daylight	One wash	Present 5/7	Absent 3/3

This regimen increased the number of positive results but still phosphorylation could not be shown when the rats were used in the afternoons. Constancy of nuclear glycogen between the morning and the afternoon and the lack of effect of glucose in the medium on the phosphorylation (Table 7) suggested that the availability of glycolytic intermediates was unlikely to be the limiting factor in the reaction. This is consistent with phosphorylation in sucrose-calcium chloride solution as described by Allfrey *et al.* (1955) and Creasey & Stocken (1959).

One further difference from the procedure of Creasey & Stocken (1959) was that the nuclei were usually washed twice; when only one wash was employed phosphorylation was more usually observed (Tables 2 and 7) but the washing out of the substrates and enzymes shown in Table 2 did not seem probable.

In most of the experiments (Table 1) phosphorylation was followed by the increase in acid-labile phosphate after shaking at 0°. In one experiment in which ATP was determined directly (second, Table 4) the increase in acid-labile phosphate was not accompanied by a gain in ATP. This is in contrast with the results of Allfrey *et al.* (1955) where chromatographic evidence showed a real formation of ATP at the expense of AMP, and is probably due to the very active utilization of ATP in these rat nuclei for reactions producing pyrophosphate (Table 5). Neither in the experiments of Allfrey *et al.* nor in ours was the change in inorganic phosphate sufficient to account for the acid-labile phosphate formed.

Adenosine triphosphate binding by thymus nuclei

The evidence of a dinitrophenol-insensitive exchange of nucleotide into acid-labile phosphate suggested that neither nuclear nor mitochondrial phosphorylation was involved. The nucleotides might, however, be bound to some nuclear com-

Table 8. *Effect of histone on the firefly determination of adenosine triphosphate*

The histone solution was the supernatant obtained after acidification of the nuclear preparation with trichloroacetic acid and centrifuging. The solution contained 0.4–0.5 mg. of protein/ml. The final volume of ATP, histone, enzyme and buffer used in the assay of ATP was 1 ml.

Concn. of ATP (ml. of 10 μ M soln. added/ml.)	Concn. of histone (ml. added/ml.)	Inhibition by histone (%)
0.02	0.2	53
0.03	0.2	45
0.04	0.2	42
0.02	0.05	0
0.02	0.1	25, 22
0.02	0.2	50, 56
0.02	0.4	63

ponent through an ionic linkage, possibly involving Mg^{2+} ions (Mirsky & Osawa, 1961). The acidification used to release the bound nucleotides caused also a release of histone (Daly & Mirsky, 1955). The presence of the histone interfered with the estimation of ATP by the firefly method (Table 8). Additional experiments showed that, under special conditions in which the amount of enzyme rather than ATP was the limiting factor, added histone did not have any effect. This indicated that the protein was probably competing for ATP but calculations showed that the amount of nucleotide bound by the histone would only be of the order of 1–2 μ m-moles of ATP/mg. of histone.

Equilibrium dialysis of arginine-rich and lysine-rich histones (Daly & Mirsky, 1955) against ionic medium containing 1 μ mole of ATP/ml. did not show retention of ATP by either type of histone, nor were the proteins active as adenosine triphosphatases, in contrast with nucleoprotein isolated from the same nuclei (Ord & Stocken, 1957; Siebert, 1960). Nucleoprotein prepared in potassium dihydrogen phosphate buffer, pH 6.8, by the method of Zubay & Doty (1959) was also unable to

bind ATP, and neither this nucleoprotein nor its gel form had adenosine-triphosphatase activity even in the presence of Ca^{2+} or Mg^{2+} ions. Crude nucleoprotein, from nuclei isolated in ionic medium and washed with water, though retaining activity as an adenosine triphosphatase, again failed to bind ATP.

DISCUSSION

It will be convenient to consider the properties of nuclei from rat thymus gland under two heads: those shown by nuclei after rapid removal of the gland ('fresh') and those shown by preparations obtained from glands which were not taken out until 10 min. after the death of the rat ('aged').

The reactions shown by the nuclei seem to depend critically on the initial concentration of ATP. This was shown with the hexokinase reaction in medium containing glucose and with the activation of amino acids, both of which required 'fresh' nuclei. Net formation of acid-labile phosphate, on the other hand, required 'aged' nuclei. The mechanism of the phosphorylation is still obscure. The presence of pyrophosphate in 'aged' nuclei would possibly permit a reversal of either amino acid or nicotinamide (Hogeboom & Schneider, 1952) activation, and in the chromatograms shown by Allfrey *et al.* (1955) a fall in NAD^+ , coincident with the conversion of AMP into ATP, can be discerned. These reactions, however, are not oxidative, nor do they account for a net formation of acid-labile phosphate, but the presence of glycolytic enzymes in nuclei (Dounce & Beyer, 1948; Stern & Mirsky, 1953; Siebert, 1960, 1961; Siebert, Bässler, Hannover, Adloff & Beyer, 1961), and the possibility of substrate-level phosphorylation utilizing glycogen or glucose 1-phosphate, cannot be dismissed. The glucose 6-phosphate-dehydrogenase pathway might certainly be concerned, and the necessity for isolating nuclei in a bivalent-ion-rich medium, with dangers of concomitant alterations in mitochondrial function, leaves open the possibility of leakage of flavoprotein or haem components from the mitochondria and their subsequent isolation in the nuclear fraction.

This type of mechanism would not, however, account for the inability to obtain the reaction in conditions which are probably associated with the time of feeding, but not with glycogen availability. If glycolytic reactions should be involved, NAD^+ might be important and it is possible that supplies of NMN for NAD^+ synthesis by the nuclei could be limiting and linked directly to the intake of food. None of these mechanisms by which NAD^+ might be concerned in the phosphorylation has yet been investigated.

The binding of exogenous ATP by isolated nuclei suggests that in the intact cell use of cytoplasmic

ATP for nuclear syntheses is a normal procedure. Experiments in which the γ -phosphate groups of cytoplasmic and nuclear ATP could be distinguished for up to 10 min. after giving ^{32}P -labelled phosphate *in vivo* (Ord & Stocken, 1961*a*) indicate that the rate of binding of the ATP on the nuclear site may be comparatively slow, but from the speed of arginine disappearance at 0° it is likely that the activation reactions proceed very rapidly in the intact cell at 37° . The uptake and binding of low-molecular-weight compounds indicates that such binding may be a necessary preliminary to their utilization. This is especially the case with ATP, where all the triphosphate is present in the 'bound' rather than the 'soluble' fraction (Ord & Stocken, 1961*a*) and where the binding appears to be preferential to ADP. The deoxyribonucleoproteins isolated did not show binding ability and ribonucleoproteins have not been explored. The capacity for polyionic macromolecules to attract charged precursors for their own syntheses has also been considered by others (for discussion see Mirsky & Osawa, 1961).

Should the nuclei normally use cytoplasmic ATP to an appreciable extent the interpretation of the sensitivity of nuclear phosphorylation to X-radiation is re-opened. If the binding of charged low-molecular-weight compounds and the absorption of extranuclear components are significant in the phosphorylation reaction, the physicochemical changes caused by X-radiation may be sufficient to alter the properties of nuclei concerned in their binding abilities and so prevent the phosphorylation (Ord & Stocken, 1961*b*).

SUMMARY

1. Nuclei were isolated from rat thymus gland in an aqueous ionic medium in two conditions: in 'fresh' nuclei nucleosides were retained in relatively high concentration, predominantly as triphosphates; in 'aged' nuclei the amount of acid-extractable triphosphate was very much less but the amount of pyrophosphate was increased.

2. 'Fresh' nuclei were able to exchange their ATP with that in the medium at 0° . The reaction was insensitive to 0.2 mM-dinitrophenol. Net uptake of ATP was not found.

3. 'Aged' nuclei showed a net uptake of ATP from the medium at 0° but added ADP was not bound as ATP.

4. 'Fresh' nuclei had a rapid uptake of acid-extractable arginine and converted bound ATP into pyrophosphate at 0° . If glucose was present in the medium bound ATP was used for the formation of glucose 6-phosphate.

5. The presence was detected of a number of enzymes of the Embden-Meyerhof and hexose

monophosphate pathways which could lead to the observed formation or disappearance of acid-labile phosphate from the nuclei. The possible function of these enzymes in nuclear phosphorylation is discussed.

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REFERENCES

- Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1955). *Nature, Lond.*, **176**, 1042.
- Bailey, K. & Webb, E. C. (1944). *Biochem. J.* **38**, 394.
- Creasey, W. A. & Stocken, L. A. (1959). *Biochem. J.* **72**, 519.
- Daly, M. M. & Mirsky, A. E. (1954-55). *Amer. J. Physiol.* **38**, 405.
- Dounce, A. L. & Beyer, G. T. (1948). *J. biol. Chem.* **173**, 159.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
- Hogeboom, G. H. & Schneider, W. C. (1952). *J. biol. Chem.* **197**, 611.
- Holton, P. W. (1959). *J. Physiol.* **145**, 494.
- Hopkins, J. W. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1461.
- Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, ii, 368.
- Khym, J. & Cohn, W. E. (1953). *J. Amer. chem. Soc.* **75**, 1153.
- Logan, R., Errera, M. & Ficq, A. (1959). *Biochim. biophys. Acta*, **32**, 147.
- Miller, L. A. & Goldfeder, A. (1961). *Exp. Cell Res.* **23**, 311.
- Mirsky, A. E. & Osawa, S. (1961). In *The Cell*, vol. 2, p. 677. Ed. by Brachet, J. & Mirsky, A. E. New York: Academic Press Inc.
- Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
- Ord, M. G. & Stocken, L. A. (1957). In *Advances in Radiobiology*, p. 65. Ed. by de Heversy, G., Forseberg, A. & Abbatt, J. D. Edinburgh: Oliver and Boyd Ltd.
- Ord, M. G. & Stocken, L. A. (1961a). *Biochem. J.* **81**, 1.
- Ord, M. G. & Stocken, L. A. (1961b). In *Mechanisms in Radiobiology*, p. 320. Ed. by Errera, M. & Forseberg, A. New York: Academic Press Inc.
- Rosenberg, H., Ennor, A. H. & Morrison, J. F. (1956). *Biochem. J.* **63**, 153.
- Siebert, G. (1960). In *The Cell Nucleus*, p. 176. Ed. by Mitchell, J. S. London: Butterworth and Co. Ltd.
- Siebert, G. (1961). *Biochem. Z.* **334**, 369.
- Siebert, G., Bässler, K. H., Hannover, R., Adloff, A. E. & Beyer, R. (1961). *Biochem. Z.* **334**, 388.
- Stern, H. & Mirsky, A. E. (1953). *J. gen. Physiol.* **37**, 177.
- Zubay, G. & Doty, P. (1959). *J. molec. Biol.* **1**, 1.

Biochem. J. (1962) **84**, 600

The Effects of 200 r. of X-radiation *in vivo* on Phosphate-Transfer Reactions in Nuclei from Rat Thymus Gland

By MARGERY G. ORD AND L. A. STOCKEN

Department of Biochemistry, University of Oxford

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Creasey & Stocken (1959) showed that phosphorylation by nuclei from thymus and various other tissues of the rat was completely inhibited by exposures of 100 r. given either *in vivo* or *in vitro*. With the elucidation of some of the factors involved in the phosphorylation (Ord & Stocken, 1962) it was decided to re-examine the radiosensitivity in an attempt to pin-point the lesion more precisely.

MATERIALS AND METHODS

These were as described by Ord & Stocken (1962).

X-Radiation. This was done either at the Medical Research Council Radiobiological Research Unit, Harwell, or at the Department of Radiotherapy, the Churchill Hospital, Oxford. The characteristics were respectively: half-value layer 2.5 mm. Cu, peak voltage 250 kv at 14 mA; or half-value layer 1.1 mm. Cu, peak voltage 220 kv at 15 mA. The animals were killed 15-50 min. after exposure.

RESULTS

When nuclei were prepared from thymus glands of normal rats that had been removed from the animals as rapidly as possible after death ('fresh' nuclei) and were then shaken for 30 min. at 0°, pyrophosphate formation occurred to a greater extent than accounted for by the apparent disappearance of ATP. It was considered that this gave evidence for phosphorylation by the isolated nuclei. When rats were exposed to 200 r., and the nuclei isolated in a similar way, no increase in pyrophosphate was found (Table 1), although the initial preparation contained more pyrophosphate than did 'fresh' nuclei from control rats (Ord & Stocken, 1962). The concentration of arginine also resembled that in 'aged' nuclei from normal rats.

When 'aged' nuclei from irradiated rats were