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Studies of Phosphorus Metabolism by Isolated Nuclei. II. Investigation of Optimal Conditions for Its Demonstration*

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Rat liver nuclei isolated by sedimentation in dense sucrose have been found capable of converting inorganic orthophosphate to forms not extractable by organic solvents such as phosphomolybdate. The phosphorylation proceeds linearly with time and is maximal at *p*H 7.0 and 30°. The process is aerobic; is relatively insensitive to dinitrophenol, sodium cyanide, or antimycin A; requires Mg^{2+}; and is stimulated by fluoride. Label is incorporated into both the acid-soluble and acid-insoluble fractions produced by perchloric acid treatment of the nuclei. Mitochondria isolated in similar fashion in dense sucrose retain their normal phosphorylative activity. The evidence indicates that liver nuclei possess inherent phosphorylative activity which is unique to the nucleus itself.

The use of labeled precursors has amply shown that nuclei can synthesize proteins and nucleic acids (Allfrey, 1954; Allfrey *et al.*, 1957). Other studies have shown that isolated nuclei of calf and rat thymus can convert endogenous nucleoside mono- and diphosphates to nucleoside triphosphates upon aerobic incubation (Osawa *et al.*, 1957; Creasey and Stocken, 1959). Such observations indicate that nuclei possess an active phosphorylation system, akin in many ways to that of mitochondria. Recently it has become increasingly clear that cell nuclei have extensive enzyme complements for possible energy transduction (McEwen *et al.*, 1963; Siebert, 1963). The systems responsible for phosphorylation by calf thymus nuclei have been studied in detail (McEwen *et al.*, 1963). Phosphorylation by thymus nuclei is principally aerobic and may be inhibited by DNP, NaCN, and the like, but it is unaffected by 95% CO–5% O_2, 3 mM Ca^{2+}, or methylene blue. The latter properties are felt to indicate that the phosphorylation is truly a nuclear process.

While the properties of thymus nuclear phosphorylation have been clarified extensively, the possibility that nuclei of radioinsensitive tissues such as liver or kidney possess such an activity is not so clear-cut. Isolated nuclei of rat liver and kidney can incorporate amino acids into protein and convert P_i to covalent form (Rees and Rowland, 1961; Rees *et al.*, 1962); and it has been postulated that such nuclei possess a phosphorylation system. However, the difficulty in studying nuclei of such tissues lies in that, upon exposure to aqueous-sucrose media, the nuclei tend to lose all or most of their endogenous complement of nucleotides and some protein (McEwen *et al.*, 1963). In addition, it is impossible to isolate liver nuclei in pure state by straightforward repetitive sedimentation in 0.25 M sucrose systems (Roodyn, 1962; Rees and Rowland, 1961). This difficulty may be obviated if a sedimentation in dense sucrose is employed to separate by flotation possible subcellular contaminants of lower density than the nuclei. Liver nuclei so isolated have been found capable of incorporating amino acids into protein (Rendi, 1960).

In early experiments on rat spleen and liver nuclei (Penniall *et al.*, 1962) in which we sought to extend the work of Creasey and Stocken (1959), we found that liver nuclei isolated by means of 2.2 M sucrose have a capacity to convert $^{32}P_i$ to covalent form. Our results verified reports that liver nuclei lost most of their complement of nucleotides upon isolation.[1] However, despite this objectionable aspect to the isolation of liver nuclei in aqueous media, it was felt significant that they retained some phosphorylative activity. Therefore we have explored the problem further, since such a system affords a chance to study liver nuclei under conditions wherein extranuclear contamination is truly minimal or absent. In this paper we wish to present the results of such studies. The first paper in this series has appeared in print (Penniall *et al.*, 1963).

Experimental Procedures

Isolation of Nuclei.—In this work mature male rats of Sprague-Dawley descent were used. Upon ether anesthesia of the rats the livers were perfused with cold isotonic saline prior to excision. In all manipulations after perfusion the tissues or extracts therefrom were kept at 0–3°. Upon excision each liver was homogenized singly in a medium containing 0.25 M sucrose: 3mM $MgCl_2$–5 mM triethanolamine, *p*H 7.0, in a Teflon-glass Potter-Elvehjem apparatus, and the pooled homogenates were well mixed with further medium to give a final ratio of 60 ml of medium/liver. This homogenate was filtered with gentle suction through two layers of fine-weave nylon cloth and centrifuged 15 minutes at 900 × *g* in the International

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[1] R. Penniall, unpublished data.

centrifuge. The sediment so obtained was resuspended by manual homogenization in a similar amount of medium and resedimented at 900 × *g*. This sediment was then homogenized into uniform suspension in a total of 80 ml of 2.2 M sucrose/liver in a large Potter-Elvehjem apparatus fitted with a loose Lucite pestle. The suspension was centrifuged for 30 minutes at 53,000 × *g* in a Spinco No. 21 rotor. The nuclei separated as a clear almost invisible sediment, and were freed of the bulk of the dense sucrose by decantation and draining in an inverted position for 20–30 minutes. The nuclei so isolated were suspended for final use by hand homogenization in further 0.25 M sucrose–3 mM $MgCl_2$–5 mM triethanolamine medium. The ratio of ml of medium/original liver used for final suspension of the nuclei has been varied from 6 to 10. No differences in activity directly attributable to this variable have been noted.

Mitochondria have been isolated from the pad derived by flotation in the dense sucrose sedimentation of the nuclei. This was accomplished by lifting the pads from the tops of several tubes with a spatula prior to decantation of the sucrose. The combined pads were homogenized in 50 ml of 0.25 M sucrose and centrifuged at 6500 × *g* for 10 minutes. The sediment so obtained was resuspended in 50 ml 0.25 M sucrose and centrifuged at 1000 × *g* for 10 minutes. The supernatant was carefully decanted at this point and again centrifuged at 6500 × *g* for 10 minutes. The mitochondria so obtained were homogenized into suspension in an appropriate volume of 0.25 M sucrose–3 mM $MgCl_2$–5 mM triethanolamine, *p*H 7.0.

Incubation of Nuclei.—Throughout much of this work the conversion of $^{32}P_i$ to organic form has been measured in an incubation system containing the following: 250 μmoles sucrose; 50 μmoles DL-histidine, *p*H 7.0; 5–10 μmoles KF; added ribonucleosides; $^{32}P_i$; and 1.0 ml of nuclear suspension in a final total volume of 3.2 ml. Frequently the system was scaled down by a factor of 0.5. Incubations were run in 15 × 150-mm test tubes or small beakers in a shaking water bath and were terminated by the addition of cold $HClO_4$ to a final concentration of 0.5 M. At this point 2–4 μmoles of P_i were added and equilibrated for 10 minutes, and the insoluble material was sedimented by low-speed centrifugation. The supernatant was decanted to a graduated test tube and the acid-soluble nuclear sediment was then washed at the centrifuge with two successive 2-ml volumes of 0.5 M $HClO_4$. The combined supernatants were diluted to 10 ml, mixed, and analyzed for total, acid-stable, and acid-labile covalent ^{32}P.

Determination of Labeled Covalent Phosphate.—The presence of labeled phosphate in covalent form was determined by the Martin and Doty procedure essentially as described previously (Penniall *et al.*, 1962). Acid-labile ^{32}P is that part of the [^{32}P]RP rendered extractable as $^{32}P_i$ by 10 minutes in 1 N HCl at 100°.[2]

Presence of label in RNA was determined after acid-insoluble fractions had been depleted of their lipids by extraction with the following: 5 ml 95% ethanol (2 X); 5 ml ethanol-ether (3:1) (2 X) for 20 minutes at 50°; 5 ml ether (1 X). Such preparations were then extracted twice with 5-ml volumes of 1.7 M NaCl–0.4 M sodium acetate, *p*H 7.0, at 100° for intervals of 60 and 30 minutes. The combined extracts were treated with 2.5 volumes of cold 95% ethanol and the nucleates were allowed to precipitate at 5° overnight. The sodium nucleates were collected by centrifugation and washed twice with cold 95% ethanol. The nucleates were dissolved in two 1-ml samples of water, transferred to planchets, and dried, and radioactivity was counted. Results obtained by this procedure agree very well with simultaneous determinations run by another procedure (Ives and Barnum, 1962).

[2] Abbreviations used are as follows: [^{32}P]RP, labeled phosphorus covalently bound in forms nonextractable as P_i; [^{32}P]ASP, acid-stable covalent phosphorus; [^{32}P]ALP, acid-labile covalent phosphorus.

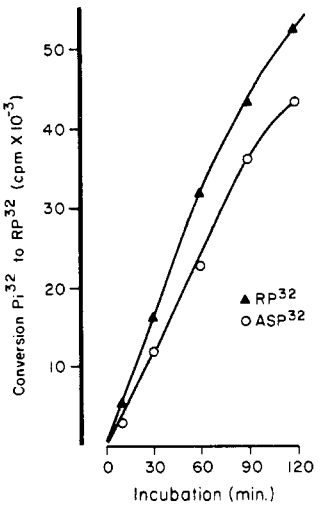

FIG. 1.—Time course of phosphorylation by rat liver nuclei. Conditions: 5.4 mg nuclear protein; 38 mμmoles each of A, U, G, and C; 5 μmoles Mg^{2+}; and 0.2 μcurie $^{32}P_i$ in 3.2 ml total volume. Incubated at 30°. P_i^{32} = $^{32}P_i$; RP^{32} = [^{32}P]RP; ASP^{32} = [^{32}P]ASP; see text, footnote 2.

Nucleic Acid Determinations.—DNA and RNA were determined by the procedure of Tsanev and Markov (1960).

Protein Determination.—The protein content of nuclei was determined by the procedure of Gornall *et al.* (1949) following a pretreatment with 1 N $HClO_4$ at 80° for 30 minutes. The insoluble material was packed by centrifugation, solubilized with alkali, and analyzed in the usual manner. This procedure removes only a small percentage of the arginine-rich histone of nuclei, but it eliminates a nonlinearity in the development of the biuret color that occurs when nuclei are assayed directly.

Materials.—$^{32}P_i$ was purchased from the Oak Ridge National Laboratory. Prior to use, the $^{32}P_i$ was made 1 N with HCl and subjected to 3-hour hydrolysis at 100°. The preparation was then brought to neutrality with NaOH and diluted with distilled water for ultimate use. More recently, the $^{32}P_i$ has been purified by chromatography on Amberlite CG-4B (Boyer *et al.*, 1962) or on Dowex-1 8X, using either a NH_4HCO_3 gradient or 0.05 M KCl–0.01 M HCl for elution of the $^{32}P_i$. Results with such preparations are indistinguishable from early results with unchromatographed $^{32}P_i$.

RESULTS

Under the conditions outlined we have found that liver nuclei will convert $^{32}P_i$ to covalent forms in linear fashion with time (Table I). There is an active incorporation of $^{32}P_i$ into RNA which parallels closely the appearance of label in acid-soluble forms. This conversion will on occasion proceed linearly for as long as 2 hours, sometimes for only a shorter period (Fig. 1), a variation we attribute to unknown differences between nuclear preparations.

In addition to showing a linear time course of incorporation, the nuclear phosphorylation is also temperature and *p*H dependent. Initial experiments had

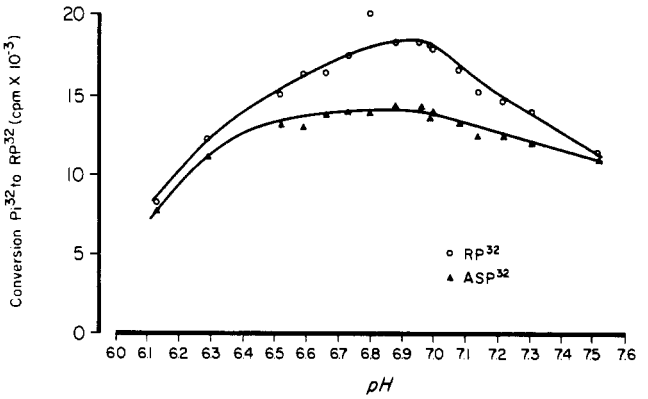

FIG. 2.—Effect of variation of *p*H on phosphorylation by rat liver nuclei. Conditions: 2.25 mg nuclear protein; 16 μmoles each of DL-histidine and glycylglycine; 19 mμmoles each of A, U, G, and C; 0.3 μcurie $^{32}P_i$ in 1.6 ml total volume. Incubated 45 minutes at 30°. Abbreviations as in Fig. 1.

TABLE I

TIME COURSE OF PHOSPHORYLATION BY ISOLATED RAT LIVER NUCLEI[a]

Incubation (min)	[^{32}P]RP	[^{32}P]ASP (cpm × 10^{-3})	RNA
20	37.5	29.5	12.9
40	75.2	66.0	21.6

[a] Conditions: 4.2 mg nuclear protein; 19 mμmoles of each ribonucleoside (A, U, G, and C); and 0.82 μcurie $^{32}P_i$ in 1.6 ml total volume. Incubated at 30°.

shown that the optima for these parameters were approximately 30° and *p*H 6.9–7.0, respectively. More definitive studies using a histidine-glycylglycine buffer indicate that nuclear phosphorylation exhibits a broad response to *p*H variation with an optimum at *p*H 6.9–7.0 (Fig. 2). This has been verified with imidazole as the buffer, and other experiments indicate a steady decline in phosphorylative activity beyond *p*H 7.6.

Figure 3 shows that formation of [^{32}P]ALP ([^{32}P]RP minus [^{32}P]ASP) is maximal at 30°. We have found that phosphorylation will proceed at temperatures as high as 40–50°, although such activity is variable and the products formed usually are of acid-stable nature only. Thus, we have chosen to investigate the nuclear phosphorylation at 30°.

Exogenous Na^+ has been reported as required for amino acid transport and as presumably innocuous to phosphorylation by nuclei (Allfrey *et al.*, 1955; Rendi, (1960). Initially, we added Na^+ and Mg^{2+} to the system, following the aforementioned lead and the results of others regarding Mg^{2+} (Naora *et al.*, 1961). We have recently evaluated the ionic requirements of liver nuclei (Table II) and find they need no added Mg^{2+} other than that in the suspension medium. Added levels of 10 or more μmoles/1.6 ml usually inhibit covalent ^{32}P formation (expts 1 and 2), although 5 μmoles of added Mg^{2+} may in part restore a failing capacity for formation of acid-labile ^{32}P (expt 2). It is most likely that the nuclei acquire all the Mg^{2+} for which they have a need in the course of their isolation and resuspension. Fluoride has a stimulatory effect on the phosphorylation (expts 2 and 3). This stimulation will occur in the presence or absence of added Mg^{2+}, but added Mg^{2+} is antagonistic to the action of fluoride. On occasion, the effect of fluoride on covalent ^{32}P formation is quite dramatic (expt 3); it has an apparent optimum at 10 μmoles/3.2 ml. NaCl at levels of 20 and 40 μmoles/

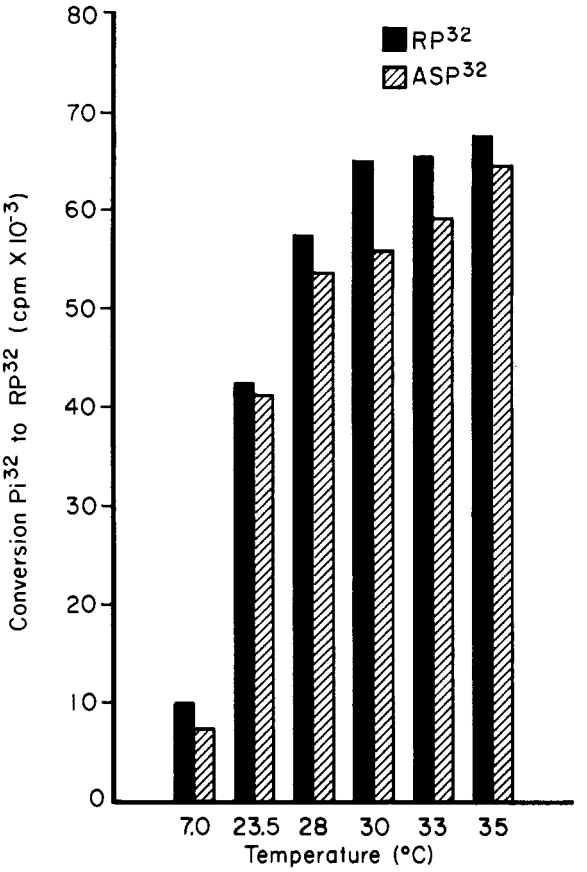

FIG. 3.—Effect of incubation temperature on phosphorylation by rat liver nuclei. Conditions: 3.3 mg nuclear protein; 19 mμmoles each of A, U, G, and C; and 0.36 μcurie $^{32}P_i$ in 1.6 ml total volume. Incubated 30 minutes at indicated temperatures. Abbreviations as in Fig. 1.

TABLE II

INFLUENCE OF VARIOUS IONS ON PHOSPHORYLATION BY RAT LIVER NUCLEI[a]

Expt	Addition	[^{32}P]RP	[^{32}P]-ASP	[^{32}P]-ALP
		(cpm × 10^{-3})		
1		34.3	23.3	11.0
	20 μmoles $MgCl_2$	28.6	22.7	5.9
	40 μmoles $MgCl_2$	22.2	17.7	4.5
2		36.6	37.3	
	5 μmoles $MgCl_2$	33.2	32.0	1.2
	10 μmoles KF	49.1	40.8	8.3
	5 μmoles $MgCl_2$ + 10 μmoles KF	36.0	31.5	4.5
	20 μmoles NaCl	30.9	29.3	1.6
	20 μmoles NaCl + 10 μmoles KF	34.8	30.9	3.9
	40 μmoles NaCl	27.3	23.1	4.2
	40 μmoles NaCl + 10 μmoles KF	31.9	28.3	3.6
3		42.4	40.8	1.6
	5 μmoles KF	68.6	55.9	12.7
	10 μmoles KF	75.0	60.9	14.1
	20 μmoles KF	65.7	55.6	10.1

[a] Conditions: Expt 1, 15.6 mg nuclear protein; 76 mμmoles each of A, U, G, and C; and 0.8 μcurie $^{32}P_i$ in 6.5 ml total volume. Incubated 60 minutes at 26°; expt 2, 2.8 mg nuclear protein; 19 mμmoles each of A, U, G, and C; and 0.34 μcurie $^{32}P_i$ in 1.6 ml total volume. Incubated 45 minutes at 30°; expt 3, 3.3 mg nuclear protein; 19 mμmoles each of A, U, G, and C; and 0.36 μcurie $^{32}P_i$ in 1.6 ml total volume. Incubated 30 minutes at 30°.

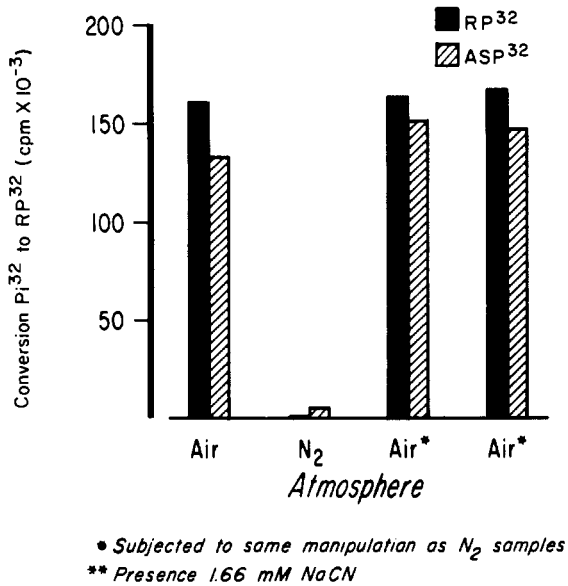

FIG. 4.—The aerobic nature of phosphorylation by rat liver nuclei. Conditions: 10.3 mg nuclear protein; 38 mμmoles each of A, U, G, and C; 5 μmoles Mg^{2+}; and 1.4 μcuries $^{32}P_i$ in 3.2 ml total volume. Incubated 60 minutes at 30°. Samples were made anerobic by repeatedly (5 ×) evacuating the tubes to the point of incipient boiling and thereupon filling them with N_2. Abbreviations as in Fig. 1.

tube is also stimulatory to the phosphorylation. However, as with Mg^{2+}, the action of NaCl is not additive with, but rather is antagonistic to, that of KF. Similar actions have been found for KCl; and the sulfate salts of both sodium and potassium are even more inhibitory than the chlorides. Thus, with few exceptions, the only salt routinely added to the incubation mixture, in addition to P_i, has been KF.

Although Table II indicates that it is possible to add too much Mg^{2+} in the nuclear incubation system, Table III shows the importance of the presence of

TABLE III

ABILITY OF Mg^{2+} TO PROTECT THE PHOSPHORYLATION SYSTEM DURING ISOLATION OF NUCLEI[a]

Preparation	Additions	[32P]RP	[32P]-ASP	[32P]-ALP
		(cpm × 10⁻³)		
A		65.2	22.8	42.4
B		4.2	3.1	1.1
	1.5 μmoles Mg^+	6.1	2.8	3.3

[a] Conditions: 3 mg nuclear protein (preparation A) and 2.1 mg nuclear protein (preparation B); 1 μmole 2-deoxyguanosine; and 50 mμmoles $^{32}P_i$ (0.6 μcurie) in 1.6 ml total volume. Incubated 30 minutes at 30°. Preparations A and B were isolated simultaneously. Media for isolation and final suspension of A and B contained 3 mM and 0.3 mM Mg^{2+}, respectively.

Mg^{2+} during the isolation of liver nuclei. Preparations A and B were isolated simultaneously and suspended in media which differed only in Mg^{2+} content. Preparation A was far more active in phosphorylation than was B, and, indeed, preparation B showed little response to further added Mg^{2+}. No obvious physical differences between preparations A and B were observed either during or after their isolation.

The phosphorylation has proved in repeated experiments to be strictly aerobic in nature. Figure 4 represents the result of an experiment accomplished in Thunberg tubes made anaerobic by evacuation and filling with N_2. It can be seen that when the tubes are made anaerobic the phosphorylation is abolished, although appropriate controls eliminate manipulative procedures as the cause. It is interesting that the phosphorylation is not affected by 1.7 mM NaCN.

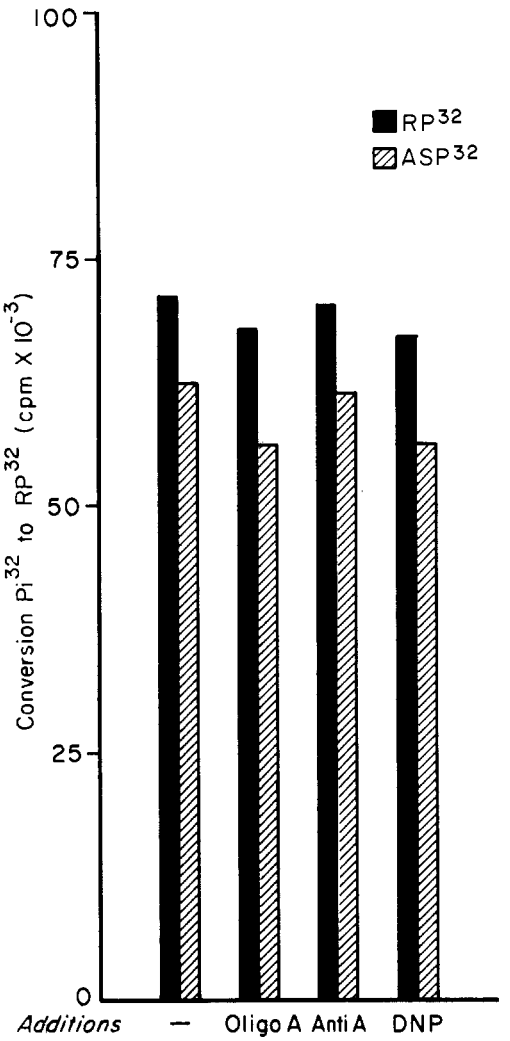

FIG. 5.—Effects of inhibitors of mitochondrial oxidative phosphorylation on nuclear phosphorylation. Conditions: 3.4 mg nuclear protein; 19 mμmoles each of A, U, G, and C; and 0.8 mμmole $^{32}P_i$ (0.4 μcurie) in 1.65 ml total volume, 2 μg of oligomycin A or antimycin A both kindly provided by National Cancer Institute Chemotherapy Testing Service and 0.38 mM DNP where indicated. Incubated 30 minutes at 30°. Abbreviations as in Fig. 1.

The synthetic as well as phosphorylative activities of thymus nuclei have been found quite susceptible to inhibitors such as DNP, NaCN, azide, antimycin A, and amytal (McEwen *et al.*, 1963). We have indicated previously that phosphorylation by rat liver nuclei is relatively insensitive to inhibitors such as DNP and NaCN (Penniall *et al.*, 1963). This is strikingly apparent in the results presented in Figures 5 and 6. In Figure 5 it can be seen that neither oligomycin A, antimycin A, nor DNP has any distinct effect on nuclear formation of [32P]RP.

The effects of DNP are detailed in Figure 6. It can be seen that DNP does affect total [32P]RP formation, but only as the concentrations approach 3–4 mM DNP does the degree of inhibition reach 45%. In contrast, 0.2 mM DNP completely obliterates phosphorylation by calf thymus nuclei (McEwen *et al.*, 1963).

To further affirm that the phosphorylative activity

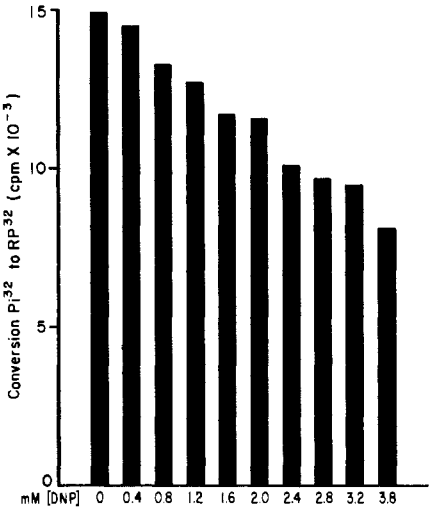

FIG. 6.—Effects of DNP on formation of [^{32}P]RP by liver nuclei. Conditions: 3 mg nuclear protein; 19 mμmoles each of A, U, G, and C; and 0.25 mμmole $^{32}P_i$ (0.1 μcurie) in 1.6 ml total volume. Incubated 45 minutes at 30°. Abbreviations as in Fig. 1.

is nuclear in origin, we have isolated mitochondria exposed to dense sucrose in the same fashion as the nuclei (Fig. 7). Mitochondria when so isolated can be seen to retain a potent capacity to form [^{32}P]ALP, and although this activity was measured without added substrate, the mitochondria are capable of a considerable response to hexokinase and glucose. In this experiment, in the presence of 1 mg of hexokinase and 25 μmoles of glucose, formation of [^{32}P]RP increased 5.5-fold and [^{32}P]ASP formation simultaneously increased almost 110-fold. However the activity of these mitochondria was still responsive to several classic inhibitors. Mitochondrial formation of both [^{32}P]RP and [^{32}P]ALP was most decidedly affected by 1 mM NaCN, 2 μg of antimycin A, or 3.8 mM DNP (Fig. 7). The degree of inhibition ranges from 78 to 85%. Other experiments show that mitochondrial phosphorylation is inhibited at least 85% by 2 μg of oligomycin; it is also stimulated by added substrate such as succinate. Thus, upon isolation by conventional procedures, rat liver mitochondria exposed to dense sucrose retain many if not all their usual properties.

Discussion

One of our concerns in this study has been whether the phosphorylation is truly enzymic and not owing to a binding process, inasmuch as the presence of a protein, or proteins, with such a capacity has been discerned in nuclei.[3] The evidence indicates, however, that binding can play no part in our results: (1) The phosphorylative activities expressed herein represent net increments in covalent ^{32}P upon incubation (2). Covalent ^{32}P formation is linear with time and exhibits a decided response to variation of incubation temperature. (3) The phosphorylation is eliminated by heating nuclei suspensions for 2 minutes at 100° at *p*H 7.0, or by 6 mM iodoacetamide (Penniall *et al.*, 1963), or by N_2. Perhaps the most conclusive evidence for an enzymic process is provided by Table IV. In this experiment the addition of unlabeled P_i after 30 minutes' incubation in no way altered, during the course of a subsequent 30-minute incubation, the level of covalent ^{32}P formed prior to that P_i addition. However, specific activity data indicate that the P_i added at 30 minutes increased the P_i concentration by at least two orders of magnitude—surely a change sufficient to reverse a binding phenomenon.

[3] B. S. McEwen, personal communication.

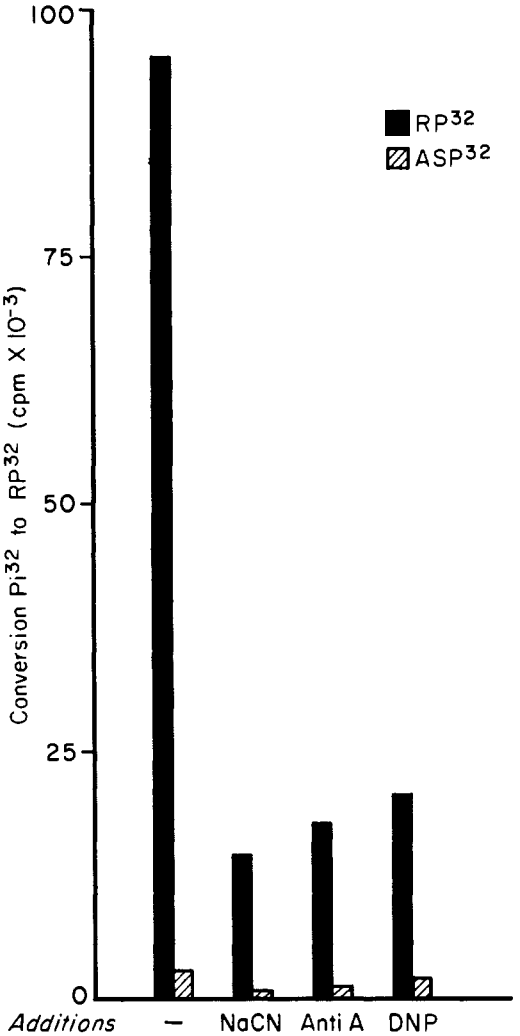

FIG. 7.—Effects of various inhibitors on liver mitochondria isolated from dense sucrose. Conditions: 2.8 mg mitochondrial protein; 19 mμmoles each of A, U, G, and C; and 0.4 μcurie $^{32}P_i$ in 1.6 ml total volume; 2 μg antimycin A; 3.8 mM DNP and 1 mM NaCN where indicated. Incubated 30 minutes at 30°. Abbreviations as in Fig. 1.

TABLE IV

EFFECTS OF CARRIER P_i ON PHOSPHORYLATION BY RAT LIVER NUCLEI[a]

Incubation (min)	[^{32}P]RP	[^{32}P]ASP (cpm × 10^{-3})	[^{32}P]ALP
30	16.2	11.8	4.4
60	31.8	23.3	8.5
60[b]	21.4	16.5	4.9

[a] Conditions: 5.4 mg nuclear protein; 38 mμmoles each of A, U, G, and C; 5 μmoles of Mg^{2+}; and 0.2 μcurie $^{32}P_i$ in 3.2 ml total volume. Incubated at 30° as indicated. [b] Denotes samples to which 0.4 μmole P_i was added after 30-minute incubation which were subsequently incubated a further 30 minutes.

It is of interest that phosphorylation by rat liver nuclei is aerobic in nature. Liver nuclei isolated by nonaqueous procedures have been shown (Siebert, 1963) to contain enzymes and intermediates of glycolysis and, according to McEwen *et al.* (1963), this system is operative in thymus nuclei. The evidence indicates that

liver nuclei, as isolated in this work, do not have a functioning glycolytic system because (1) the phosphorylation is aerobic; (2) fluoride gives no inhibition but rather is stimulatory; and (3) experiments with glucose provide no evidence that this substrate, to which isolated nuclei are permeable, in any way enhances the phosphorylation. Such results are not unexpected in view of the aforementioned effects of aqueous sucrose upon liver nuclei.

The phosphorylative activity of rat liver nuclei resembles that of nuclei of other tissues with respect to optimum temperature and *p*H, a need for O_2, the necessity for a divalent cation in media for isolation of nuclei, and in that the activity proceeds on substrates endogenous to the nuclei (McEwen *et al.*, 1963). Liver nuclei differ from other nuclei in that upon isolation they lose their glycolytic system, and in that fluoride has a capacity to stimulate phosphorylation. The mechanism of the fluoride action is unknown but, since its activity is antagonized by Mg^{2+}, it appears that it does not involve fluorophosphate formation. The most striking properties of the phosphorylation lie in its insensitivity to inhibitors such as oligomycin A, antimycin A, NaCN, and DNP. At present it is not known whether this insensitivity is due to the nature of the process, to membrane-permeability properties of liver nuclei, or to a combination of both. In any event these properties, in conjunction with the others presented herein, leave no doubt that mitochondria play no part in the observed activity. However these results show that isolated rat liver nuclei do possess a phosphorylative capacity. Although we are aware that the magnitude of the reported activity is small, we hope that further knowledge of the process will allow a closer approach to optimum conditions for the activity. At that time we hope to study the character of the process in more intimate detail.

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Studies of Phosphorus Metabolism by Isolated Nuclei. III. Some Fundamental Properties of the System.*

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The aerobic phosphorylation catalyzed by liver nuclei isolated in dense sucrose has been found insensitive to a number of potential substrates, cofactors, inhibitors, other conditions that influence the phosphorylation catalyzed by mitochondria, or nuclei from other tissues. The activity is inhibited by artificial electron acceptors, sulfhydryl compounds, and flavoprotein antagonists; amytal exerts a biphasic action, being stimulatory at low concentrations and inhibitory at high concentrations. The phosphorylation is presumably supported by endogenous substrate(s) and is vastly stimulated by added ribo- or 2-deoxyribonucleosides. With both types of nucleoside those containing guanine are the most active in fostering phosphorylation. The presence of either purine-2-deoxyribonucleoside leads to formation of more acid labile ^{32}P (covalently bound in forms not extractable as P_i) than its counterpart ribonucleoside. It is concluded that the phosphorylation is not catalyzed by enzymes of glycolysis, nucleoside or polynucleotide phosphorylases, or possible exchange enzymes.

We have previously reported the presence of a phosphorylative system in isolated rat liver nuclei and detailed some of its properties (Penniall *et al.*, 1963, 1964). These experiments provide the first conclusive demonstration of such activity in nuclei from other than a radiosensitive tissue. Phosphorylative activity has previously been reported in nuclei from calf thymus (Osawa *et al.*, 1957) and rat thymus and spleen (Creasey and Stocken, 1959). In certain respects the phosphorylation of rat liver nuclei is like that of thymus nuclei and that of mitochondria; but in other respects it is unique. In this paper we present additional data in support of our contention that liver nuclei possess their own energy-transduction system.

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