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.

References

Allfrey, V. G. (1954), *Proc. Natl. Acad. Sci. U. S. 40*, 881.

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

Allfrey, V. G., Mirsky, A. E., and Osawa, S. (1957), *J. Gen. Physiol. 40*, 451.

Boyer, P. D., DeLuca, M., Ebner, K. E., Hultquist, D. E., and Peter, J. B. (1962), *J. Biol. Chem. 237*, PC 3306.

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

Gornall, A. G., Bardawill, C. S., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Ives, D. H., and Barnum, C. P. (1962), *J. Biol. Chem. 237*, 2599.

McEwen, B. S., Allfrey, V. G., and Mirsky, A. E. (1963), *J. Biol. Chem. 238*, 758, 2571, 2579.

Naora, H., Naora, H., Mirsky, A. E., and Allfrey, V. G. (1961), *J. Gen. Physiol. 44*, 713.

Osawa, S., Allfrey, V. G., and Mirsky, A. E. (1957), *J. Gen. Physiol. 40*, 491.

Penniall, R., Hall, A. H., Griffin, J. B., and Irvin, J. L (1962), *Arch. Biochem. Biophys. 98*, 128.

Penniall, R., Liu, S. M., and Saunders, J. P. (1963), *Biochim. Biophys. Acta 76*, 170.

Rees, K. R., Ross, H. F., and Rowland, G. F. (1962), *Biochem. J. 83*, 523.

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

Rendi, R. (1960), *Exptl. Cell Res. 19*, 489.

Roodyn, D. B. (1962), *Biochem. J. 84*, 3P.

Siebert, G. (1963), *Proc. Intern. Congr. Biochem. 5th, Moscow, 1961, 2*, 93.

Tsanev, R., and Markov, G. G. (1960), *Biochem. Biophys. Acta 42*, 442.

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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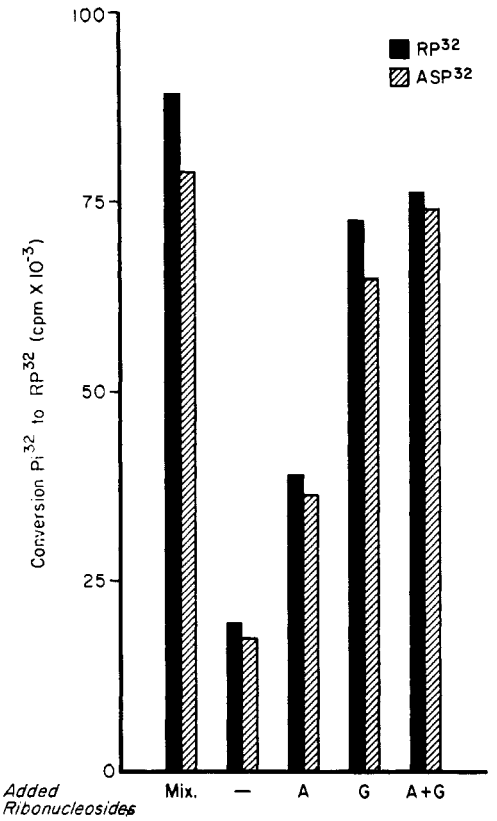

FIG. 1.—Effects of exogenous ribonucleosides on nuclear phosphorylation. Conditions: 3.33 mg nuclear protein, 18 mμmoles of added adenosine and guanosine where indicated, and 0.25 mμmole $^{32}P_i$ (0.84 μcurie) in 1.6 ml total volume. Incubated 30 minutes at 30° Pi^{32} = $^{32}P_i$; RP^{32} = [^{32}P] RP; ASP^{32} = [^{32}P] ASP; see text, footnote 1.

EXPERIMENTAL

The procedures of nuclei isolation, measurement of phosphorylation, incubation conditions, and the like, used in this work have been detailed previously (Penniall *et al.*, 1962, 1964).

RESULTS

In our work we have recognized that liver nuclei isolated in sucrose media lose most or all of their nucleotide complement (McEwen *et al.*, 1963) and that they are permeable to free purine and pyrimidine bases or nucleosides (Rees and Rowland, 1961). Accordingly, we have always included exogenous nucleosides in the incubation system. It was hoped that the nucleosides would undergo phosphorylation in appropriate kinase reactions and thereby foster $^{32}P_i$ uptake, or that the bases derived by hydrolysis of the nucleosides would be caught up in ATP-requiring reactions and thus stimulate phosphorylation. We began this work by employing an equimolar mixture of all four ribonucleosides: adenosine, uridine, guanosine, and cytidine. The importance of exogenous nucleosides to phosphorylation by liver nuclei can be seen in Figure 1. When the ribonucleoside mixture is added to the system, $^{32}P_i$ conversion is increased almost 4-fold over the endogenous activity. By itself, adenosine doubles the endogenous rate; but guanosine is twice as potent as adenosine, fostering almost 82% of the activity found with the mixture of A, U, G, and C. It is interesting that adenosine plus guanosine yield 85% of the activity observed with the ribonucleoside mixture. Obviously, the activities of adenosine and guanosine are not additive and the pyrimidines foster only a minor portion of the total activity. Separate experiments verify this figure, and also indicate that pyrimidine activity is due to uridine, since cytidine is essentially inactive in fostering phosphorylation.

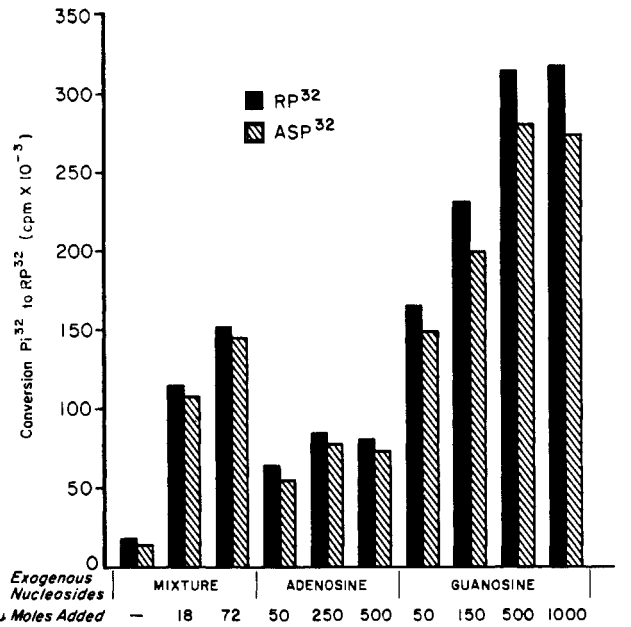

FIG. 2.—Optimal levels of exogenous ribonucleosides for nuclear phosphorylation. Conditions: 2.6 mg nuclear protein and 0.25 mμmole $^{32}P_i$ (0.68 μcurie) in 1.6 ml total volume. Incubated 30 minutes at 30°. Abbreviations as in Fig. 1.

Figure 2 shows that the ribonucleoside concentrations originally employed (19 mμmoles/1.6 ml reaction volume) were too low and that they do not foster the maximum phosphorylative activity. Increasing the nucleoside concentration increases the phosphorylation appreciably. The effects of guanosine are maximal at 500 mμmoles/1.6 ml, at which it increases formation of [^{32}P]RP and [^{32}P]ALP over the endogenous rate some 17-fold and 10-fold, respectively.[1] The effects of adenosine are maximal at 250 mμmoles/1.6 ml, although at this concentration the activity of adenosine was less than that of the ribonucleoside mixture at concentrations less than 0.1 that of the adenosine. It is important to note that guanosine fostered considerable formation of [^{32}P]ALP, a result not obtained with adenosine at any concentration. In this same experiment it was found that, at concentrations of guanosine exhibiting maximal phosphorylation, the addition of equimolar amounts of adenosine and/or uridine give no increased phosphorylation. Such additions merely decrease [^{32}P]ALP formation, in both cases, to the level exhibited by the system containing all four ribonucleosides. Similar results can be seen with the guanosine-plus-adenosine system in Figure 1.

It has also been found that 2-deoxyribonucleosides can foster phosphorylation by liver nuclei (Table I). Again, as with the ribonucleosides, the purine-2-deoxyribonucleosides are more efficient in promoting nuclear phosphorylation than their pyrimidine counterparts. What is most important, however, is that there is a fundamental difference in the manner in which

[1] Abbreviations used are as follows: [^{32}P]RP, total radioactive phosphorus covalently bound in forms not extractable as P_i; [^{32}P]ASP, acid-stable [^{32}P]RP; [^{32}P]ALP, acid-labile [^{32}P]RP; A, U, G, and C are adenosine, uridine, guanosine, and cytidine, respectively; 2-deA, 2-deG, 2-deC, and T are 2-deoxyadenosine, 2-deoxyguanosine, 2-deoxycytidine, and thymidine, respectively.

TABLE I

COMPARATIVE ABILITIES OF PURINE 2-DEOXYRIBONUCLEOSIDES AND RIBONUCLEOSIDES TO FOSTER NUCLEAR PHOSPHORYLATION[a]

Addition	[^{32}P]RP	[^{32}P]-ASP	[^{32}P]-ALP	[^{32}P]ALP Formation ($\mu\mu$moles/45 min)
	(cpm × 10^{-3})			
	6.9	6.9	0	
A, 0.5 μmole	27.6	25.1	2.5	152
0.5 μmole 2-deA	14.5	5.5	9.0	549
1.0 μmole 2-deA	13.0	6.0	7.0	427
G, 2.0 μmoles	57.4	53.5	3.9	238
2.0 μmoles 2-deG	24.7	7.8	16.9	1030

[a] Conditions: 3.3 mg nuclear protein; and 50 mμmoles $^{32}P_i$ (0.37 μcurie) in 1.6 ml total volume. Incubated 45 minutes at 30°.

purine-ribo- and 2-deoxyribonucleosides affect nuclear phosphorylation. In each instance in Table I, the 2-deoxyribonucleoside fosters formation of less [^{32}P]RP than its counterpart ribonucleoside; but, of that [^{32}P]RP which is formed in the presence of the 2-deoxyribonucleoside, far more is [^{32}P]ALP. For example, with equimolar amounts of nucleoside, formation of [^{32}P]RP in the presence of 2-deoxyadenosine is only 53% of that formed in the presence of adenosine. However, 2-deoxyadenosine leads to formation of 3.5 times more [^{32}P]ALP than does adenosine. Similar results obtain with guanosine and 2-deoxyguanosine.

Phosphorylation with 2-deoxyadenosine is maximal at 500 mμmoles/1.6 ml (Table I). The optimal concentration of 2-deoxyguanosine is approximately 1–2 μmoles/1.6 ml (Table II). Although the results are not quite so striking as in Table I, 2-deoxyguanosine can again be seen (Table II) to foster increased [^{32}P]ALP

TABLE II

OPTIMAL CONCENTRATION OF 2-DEOXYGUANOSINE FOR NUCLEAR PHOSPHORYLATION[a]

Addition	[^{32}P]RP	[^{32}P]ASP	[^{32}P]ALP
	(cpm × 10^{-3})		
Guanosine			
2 μmoles	47.3	40.6	6.7
2-Deoxyguanosine			
0.5 μmole	17.3	7.7	9.6
1.0 μmole	16.9	7.3	9.6
2.0 μmoles	18.0	7.2	10.8
4.0 μmoles	16.6	6.6	10.0

[a] Conditions: 2.9 mg nuclear protein and 50 mμmoles $^{32}P_i$ (0.4 μcurie) in 1.6 ml total volume. Incubated 45 minutes at 30°.

formation as compared to guanosine.

The relative efficiency with which the 2-deoxyribonucleosides foster nuclear phosphorylation is shown in Table III. As with the ribonucleosides, the order of efficiency is the same, with 2-deoxycytidine being relatively inactive. However, thymidine is almost as active as adenosine—a situation that does not obtain with its counterpart, uridine. Again as with the ribonucleosides, there is no apparent additive action of a 2-deoxynucleoside mixture as opposed to the activity of each of the 2-deoxynucleosides alone.

We have reported the insensitivity of nuclear phosphorylation fostered by exogenous ribonucleosides to various inhibitors of mitochondrial oxidative phosphorylation (Penniall, *et al.*, 1963, 1964). It can be seen in Table IV that with both 2-deoxyguanosine and 2-deoxyadenosine the same situation obtains. The

TABLE III

RELATIVE ABILITY OF 2-DEOXYRIBONUCLEOSIDES TO FOSTER NUCLEAR PHOSPHORYLATION[a]

Addition	[^{32}P]RP	[^{32}P]ASP	[^{32}P]ALP
	(cpm × 10^{-3})		
	2.6	2.4	0.2
1 μmole 2-deA	8.0	2.5	5.5
2 μmoles 2-deG	21.6	5.2	16.4
1 μmole 2-deC	1.9	1.3	0.6
1 μmole T	6.5	2.4	4.1
Nucleoside mixture	19.7	3.3	16.4

[a] Conditions: 1.8 mg nuclear protein and 100 mμmoles $^{32}P_i$ (1.36 μcuries) in 1.6 ml total volume. Incubated 30 minutes at 30°. Nucleoside mixture contained indicated amounts of each of the nucleosides.

TABLE IV

EFFECT OF INHIBITORS OF MITOCHONDRIAL PHOSPHORYLATION ON NUCLEAR PHOSPHORYLATION FOSTERED BY 2-DEOXYRIBONUCLEOSIDES[a]

Expt	Addition	[^{32}P]RP	[^{32}P]ASP	[^{32}P]ALP
		(cpm × 10^{-3})		
1		17.0	5.5	11.5
	Oligomycin A (1 μg)	12.0	5.0	7.0
	Ethanol	10.8	4.2	6.6
	DNP, 0.8 mM	13.2	5.2	8.0
	NaCN, 1 mM	15.5	8.0	7.5
2		8.8	4.6	4.2
	DNP, 0.8 mM	7.9	3.9	4.0
	NaCN, 1 mM	7.9	3.8	4.1

[a] Conditions: 2.6 mg nuclear protein and 50 mμmoles $^{32}P_i$ (0.4 μcurie). Incubated 60 minutes at 30°. Expt 1, 1 μmole 2-deoxyguanosine and 1.7 mmoles ethanol ± oligomycin A; expt 2, 1 μmole 2-deoxyadenosine.

phosphorylative activity with 2-deoxyadenosine (expt 2) is not affected by either DNP or NaCN. In the presence of 2-deoxyguanosine (expt 1), [^{32}P]RP formation is inhibited 22% and 9% by 0.8 mM DNP and 1 mM NaCN, respectively. Oligomycin A exhibits no inhibition over an ethanol control. As with the ribonucleosides, the properties of this system are not consistent with phosphorylative systems described heretofore.

Thus far no stimulation of nuclear phosphorylation has been observed for a number of potential substrates. Tested and found to be without effect were various glycolytic intermediates; pyruvic, citric, α-ketoglutaric, succinic, glutamic, and β-hydroxybutyric acids; NADH and NADPH, and D- and L-amino acids. Figure 3 shows that both succinate and β-hydroxybutyrate are in fact inhibitory to formation of [^{32}P]ASP. In Table V (expt 1) glutamate can also be seen to inhibit phosphorylation, and in both experiments (Fig. 3 and Table V) hexokinase has an apparent inhibitory action. At this point the reason for such inhibitions is uncertain. However, we know (Penniall *et al.*, 1964) that phosphorylation by liver nuclei is inhibited by sodium and ammonium salts. The presence of sodium ion in each of the substrates and the possible presence of ammonium ion in the hexokinase constitute a common point in each of these experiments. It is possible that the presence of such ions underlies the observed inhibitions, although this point has not been fully investigated. Table V shows that NAD^+, $NADP^+$, and ATP are all strongly inhibitory to phosphorylation. Isolated liver nuclei possess considerable nucleotidase activity (Rees and Rowland, 1961; Siebert, 1963). We have also found that liver nuclei will rapidly release P_i from ribonucleoside mono-, di-, and triphosphates.[2]

[2] R. Penniall, unpublished experiments.

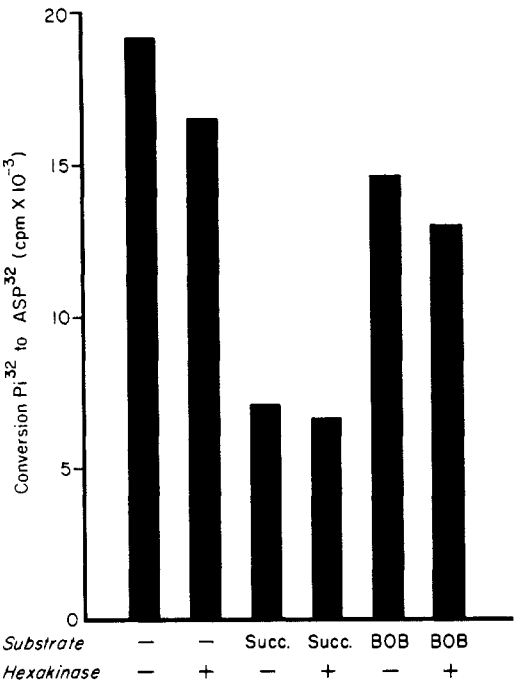

FIG. 3.—Effects of mitochondrial substrates and hexokinase on formation of [^{32}P] ASP by liver nuclei. Conditions: 3.8 mg nuclear protein, 18 mμmoles each of A, U, G, and C, 10 μmoles of succinate (Succ.) or β-hydroxybutyrate (BOB) and 1 mg hexokinase plus 25 μmoles glucose where indicated, and 0.43 μcurie $^{32}P_i$ in 1.6 ml total volume. Incubated 30 minutes at 30°. Abbreviations as in Fig. 1.

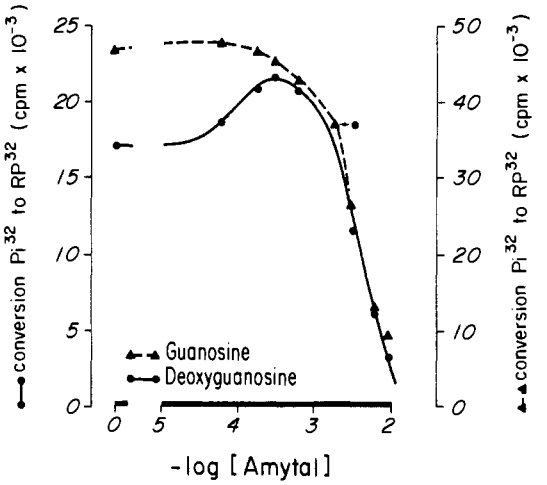

FIG. 4.—Effects of amytal on nuclear phosphorylation. Conditions: 1.8 mg nuclear protein, 2 μmoles of guanosine or 2-deoxyguanosine where indicated, and 0.1 μmole $^{32}P_i$ (0.74 μcurie) in 1.6 ml total volume. Incubated 60 minutes at 30°. Abbreviations as in Fig. 1.

TABLE V
EFFECTS OF VARIOUS ADDITIVES ON NUCLEAR PHOSPHORYLATION[a]

Expt	Addition	[^{32}P]RP	[^{32}P]-ASP	[^{32}P]-ALP
		(cpm × 10^{-3})		
1		24.4	19.5	4.9
	Hexokinase	22.0	16.3	5.7
	Hexokinase + 10 μmoles glutamate	19.3	14.6	4.7
2		89.3	79.0	10.3
	NAD^+, 1 μmole	68.3	66.0	2.3
	$NADP^+$, 1 μmole	39.4	29.0	10.4
3		22.0	21.3	0.7
	NAD^+, 0.7 μmole	15.6	16.0	
	NAD^+, 0.7 μmole + 15 μmoles nicotinamide	14.4	14.6	
4		62.3	36.3	26.0
	ATP, 0.6 μmole	2.8	2.5	

[a] Conditions: Expts 1, 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° (0.5 mg hexokinase and 25 μmoles glucose where indicated). Expt 2, 3.3 mg nuclear protein; 19 mμmoles each of A, U, G, and C; and 0.25 mμmole $^{32}P_i$ (0.8 μcurie) in 1.6 ml total volume. Incubated 30 minutes at 30°. Expt 3, 2.9 mg nuclear protein; 38 mμmoles each of A, U, G, and C; and 50 mμmoles $^{32}P_i$ (0.4 μcurie) in 1.6 ml total volume. Incubated 45 minutes at 30°. Expt 4, 17 mg nuclear protein; 50 μmoles DL-histidine, *p*H 7.0; 75 mμmoles each of A, U, G, and C; 40 μmoles $MgCl_2$; 10 μmoles KF; and 1.1 μcuries $^{32}P_i$ in 6.6 ml total volume. Incubated 60 minutes at 24°.

It is reasonably certain this is the manner of action of ATP. The pyridine-nucleotide inhibition is possibly due in part to hydrolysis, although expt. 3, Table V shows that nicotinamide has no effect on the action of NAD^+. However, there is a considerable distinction between the actions of NAD^+ and $NADP^+$ in that the latter inhibits the formation of [^{32}P]ASP only. The complement of nuclear components that acquires label in acid-labile form continues to do so, even though the formation of [^{32}P]ASP is considerably depressed. These results imply a fundamental difference in the effects of the two pyridine nucleotides on phosphorylation which will require further work for clarification.

We have found that DNAase treatment completely eliminates phosphorylation by liver nuclei (Penniall *et al.*, 1963), and that nuclei so treated show no response to polyanions such as polyethylene sulfonate. In susceptibility to DNAase, liver nuclei are like those of calf thymus (McEwen *et al.*, 1963). However, thymus nuclear phosphorylation and energy-dependent syntheses are restored after DNAase treatment by polyethylene sulfonate, DNA, RNA, or the like (Allfrey, 1963). In this connection, basic proteins such as nuclear histones inhibit phosphorylation by both thymus nuclei and mitochondria (McEwen *et al.*, 1963). With liver nuclei we are measuring an admittedly small phosphorylative activity and, accordingly, have sought ways to restore any activity lost or masked for any reason. Since we isolate nuclei at a *p*H close to optimal for DNAase activity, and our nuclei could therefore be inhibited at least in part by excess histones, we have tested polyanions for their effect on nuclear phosphorylation. Table VI shows that neither polyethylenesulfonate, RNA, nor DNA has any stimulatory effect on phosphorylation. Polyethylenesulfonate is actually inhibitory to the phosphorylation, and similar effects have been noted for the homolog of average molecular weight of 12,900. It can also be seen in Table VI that nuclear histones have no effect on phosphorylation. Again, liver nuclei differ from nuclei of other tissues as well as from mitochondria.

The energy metabolism of both thymus nuclei (McEwen *et al.*, 1963) and liver nuclei (Rees and Rowland, 1961; Penniall *et al.*, 1963) is inhibited by flavoprotein antagonists. Figure 4 shows that nuclear phosphorylation is also inhibited by amytal; 3 mM amytal causes 50% inhibition of activity. It is most interesting that amytal's action is biphasic; although a stimulatory phase is not truly apparent in Figure 4

TABLE VI

EFFECTS OF POLYANIONS AND NUCLEAR HISTONES ON NUCLEAR PHOSPHORYLATION[a]

Expt	Addition	[^{32}P]RP	[^{32}P]-ASP	[^{32}P]-ALP
		(cpm × 10^{-3})		
1		89.3	79.0	10.3
	0.2 mg Polyethylene sulfonate (mw 5900)[b]	25.7	24.4	1.3
	0.4 mg Polyethylene sulfonate	18.8	16.9	1.9
	DNA (Worthington), 7.0 μg	86.3	75.6	10.7
	RNA (Worthington), 12.0 μg	85.6	74.9	10.7
2		13.4	4.3	9.1
	Nuclear histone, 0.1 mg[c]	13.8	4.2	9.6

[a] Conditions: Expt 1, 3.3 mg nuclear protein; 19 mμmoles each of A, U, G, and C; and 0.25 mμmole $^{32}P_i$ (0.8 μcurie) in 1.6 ml total volume. Incubated 30 minutes at 30°. Expt 2, 1.7 mg nuclear protein; 1 μmole 2-deoxyguanosine; and 100 mμmoles $^{32}P_i$ (0.7 μcurie) in 1.6 ml total volume. Incubated 30 minutes at 30°. [b] Kindly provided by the Upjohn Co., Kalamazoo, Mich. [c] Unfractionated nuclear histone kindly provided by Dr. J. Logan Irvin of this department.

in the presence of guanosine, it is unmistakable with 2-deoxyguanosine. Other experiments have also shown a biphasic effect of amytal on phosphorylation fostered by ribonucleosides. In addition to the effects of flavoprotein antagonists, nuclear phosphorylation is also inhibited by artificial electron acceptors such as neotetrazolium and $K_3Fe(CN)_6$ and by sulfhydryl compounds such as thioglycolate (Table VII). Similar

TABLE VII

EFFECT OF VARIOUS ADDITIVES ON NUCLEAR PHOSPHORYLATION[a]

Expt	Addition	[^{32}P]RP	[^{32}P]-ASP	[^{32}P]-ALP
		(cpm × 10^{-3})		
1		14.9	14.3	0.6
	Neotetrazolium, 0.25 mg	9.9	9.0	0.9
2		80.8	65.2	15.6
	$K_3Fe(CN)_6$, 1 μmole	10.5	4.9	5.6
	Thioglycolate, 1.6 μmoles	61.7	52.0	9.7

[a] Conditions: Expt 1, 3.0 mg nuclear protein; 19 mμmoles each of A, U, G, and C; and 0.25 mμmole $^{32}P_i$ (0.12 μcurie) in 1.6 ml total volume. Incubated 45 minutes at 30°. Expt 2, 2.3 mg nuclear protein; 18 mμmoles each of A, U, G, and C; and 0.77 μcurie $^{32}P_i$ in 1.6 ml total volume. Incubated 30 minutes at 30°.

inhibitions have been found for 1,3-dimercaptopropanol and reduced glutathione.

In much of the work with liver nuclei we have used preparations of nuclei over a considerable period of time (Table VIII). If liver nuclei are frozen as a pellet after being drained of 2.2 M sucrose, they retain their phosphorylative activity. A month is the longest interval we have tested, but many preparations retain most or all of their activity for that time. In this property liver nuclei are again quite unlike liver mitochondria (Weinbach, 1959).

We have long known that the nuclear phosphorylation system has a high affinity for P_i and that the net

TABLE VIII

STABILITY OF NUCLEAR PHOSPHORYLATION TO STORAGE AT −15°[a]

Expt	[^{32}P]RP	[^{32}P]ASP	[^{32}P]ALP	[^{32}P]RP Formation (mμmoles/30 min)
	(cpm × 10^{-3})			
1	65.2	22.8	42.4	2.6
2	35.5	11.7	23.8	2.88

[a] Conditions: Upon isolation the preparation of nuclei was split and one part was assayed immediately (expt 1); the second part was stored in pellet form at −15° for 28 days prior to final suspension and assay (expt 2). Expt 1, 2.8 mg nuclear protein; and 20 mμmoles $^{32}P_i$ (0.57 μcurie); expt 2, 2.7 mg nuclear protein; and 100 mμmoles $^{32}P_i$ (0.6 μcurie). Incubations: 30 minutes at 30°.

phosphorylation rate is low. However, the rate is reproducible. In eight consecutive experiments, the average incorporation of $^{32}P_i$ in acid-soluble [^{32}P]RP was 1.2 (0.7–1.7) mμmoles/mg protein/60 min, in the presence of 2 μmoles of guanosine. We have made reciprocal plots of phosphorylative activity in the presence of each of the purine nucleosides (Fig. 5). Values for the apparent K_M and V_{max} obtained from these plots are given in Table IX. The results show

TABLE IX

KINETIC CONSTANTS FOR NUCLEAR PHOSPHORYLATION WITH PURINE NUCLEOSIDES[a]

Nucleoside	V_{max}	K_M
Guanosine	5.6×10^{-3}	2.8×10^{-5}
2-Deoxyguanosine	1.6×10^{-3}	1.5×10^{-5}
Adenosine	2.6×10^{-3}	3.8×10^{-5}
2-Deoxyadenosine	5.9×10^{-4}	8.1×10^{-6}

[a] Conditions: 3.2 mg nuclear protein; 0.6 μcurie $^{32}P_i$ with varying concentrations of $^{32}P_i$; 2 μmoles of guanosine nucleosides, 1 μmole adenosine, and 0.5 μmole 2-deoxyadenosine where indicated. Incubated 45 minutes at 30°.

quite strikingly the high affinity of this system for P_i, and its low rate of activity. In view of the similar magnitude of the apparent K_M values for P_i in the presence of each of the nucleosides, we have sought evidence of competition between nucleosides so far as is presently possible. One approach we have tried is to determine the effects of adenosine, presence of which leads to far less nuclear formation of [^{32}P]RP and little or no [^{32}P]ALP, compared to phosphorylation in the presence of guanosine. Experiments such as that of Table X show no clear-cut evidence of adenosine inhibition of [^{32}P]RP formation fostered by guanosine. The presence of as much as a 15-fold excess of adenosine with 2

TABLE X

INABILITY OF ADENOSINE TO INHIBIT PHOSPHORYLATION FOSTERED BY GUANOSINE[a]

Nucleoside	[^{32}P]RP	[^{32}P]ASP	[^{32}P]ALP
	(cpm × 10^{-3})		
Adenosine, 1 μmole	13.3	11.6	1.7
G, 2 μmoles	39.0	33.3	5.7
G, 2 μmoles, + 1 μmole A	40.8	33.6	7.2
G, 2 μmoles, + 30 μmoles A	37.2	35.0	2.2

[a] Conditions: 1.7 mg nuclear protein and 100 mμmoles $^{32}P_i$ (0.77 μcurie) in 1.6 ml total volume. Incubated 30 minutes at 30°.

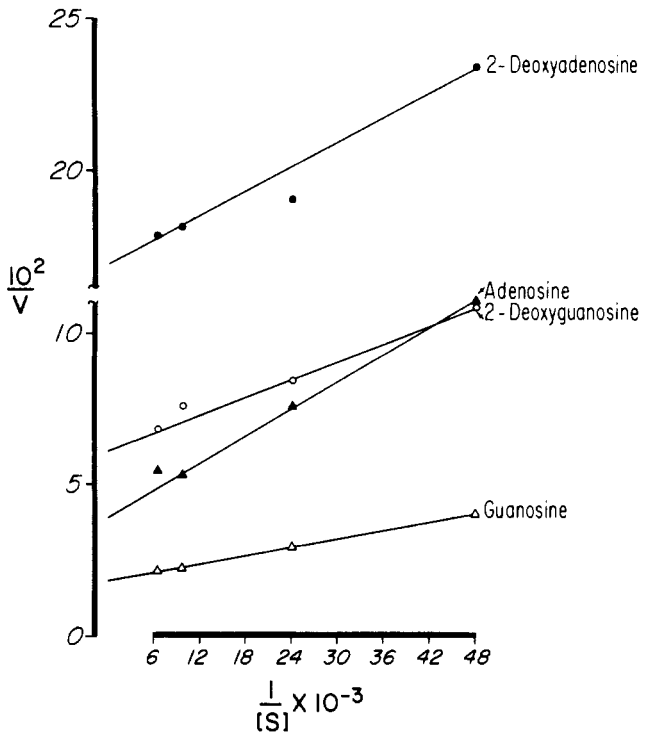

FIG. 5.—Reciprocal plots of nuclear phosphorylation with different purine nucleosides. Conditions: 3.2 mg nuclear protein; 0.5, 1, 2, and 2 μmoles of 2-deoxyadenosine, adenosine, 2-deoxyguanosine, and guanosine, respectively; and 0.6 μcurie $^{32}P_i$ plus varying levels of carrier P_i in 1.6 ml total volume. Incubated 45 minutes at 30°.

μmoles of guanosine causes no real change in the [^{32}P]RP formation from that with 2 μmoles of guanosine alone. However, this high concentration of adenosine does diminish the level of [^{32}P]ALP formation that occurs with guanosine alone. These results we interpret as indicative that there is most likely no true competition between nucleosides at the level of the phosphorylation system itself. Instead, variations in ability of nucleosides to foster nuclear phosphorylation rests in the amounts and characteristics of transphosphorylation enzymes affected by the nucleosides. A conclusive interpretation of the results will have to await a more intimate knowledge of the phosphorylation system.

DISCUSSION

In this paper, and preceding ones of this series, we have reported some of the properties of the phosphorylation catalyzed by rat liver nuclei. The system is unique and can be distinguished from mitochondria by: its sensitivity to various inhibitors, potential substrates, or cofactors; its stability to storage; and its stability to isolation in either Ca^{2+} or Mg^{2+} media. Liver mitochondria similarly exposed to dense sucrose retain their normal response to inhibitors, substrates, and the like (Penniall *et al.*, 1964). The data presented have been supplemented in many experiments by phase and/or electron-microscopic examination of the nuclei. Most preparations contain no mitochondria, but contamination when present has always been minute and estimated at 1 to 2 mitochondria per 200 nuclei. Examinations of smears show only an occasional whole cell or nucleus with cytoplasmic tabs.

In their susceptibility to flavoprotein antagonists, liver nuclei resemble mitochondria as well as nuclei from other tissues. Rees and Rowland (1961) found synthetic activities of liver nuclei to be practically eliminated by 0.15–0.30 mM chlorpromazine. However, our preparations of nuclei do not display this degree of sensitivity to such inhibitors. The biphasic action of amytal on nuclear phosphorylation was unexpected, although this type of response is not unprecedented. For example, Löw (1959) found that mitochondrial ATPase activity was affected in biphasic fashion by promazines. From the effects of amytal and the like, we infer that a flavoprotein is involved in some fashion in the observed phosphorylation, or in providing the energy for it. Actually, a number of the properties of nuclear phosphorylation, when considered together, provide some insight into the nature of the process. These are: its aerobic nature and insensitivity to cyanide, DNP, oligomycin and antimycin; its inhibition by chlorpromazine, amytal, and artificial electron acceptors; its apparent lack of a requirement for exogenous substrate; Rees and Rowland's (1961) observation of NADH–cytochrome c reductase in liver nuclei; and Siebert's (1963) demonstration of various dehydrogenases within liver nuclei. These properties seem to indicate an inherent system for substrate oxidation within the nucleus which could be coupled to the observed phosphorylation. Any condition or material which is capable of diminishing electron flow through the system thereby diminishes phosphorylation. The system can be presumed to contain a flavoprotein; and, assuming nuclei to be permeable to cyanide or azide, it can be presumed to not contain a terminal component such as the cytochrome oxidase of mitochondria.

The means whereby added nucleosides foster phosphorylation by liver nuclei is a matter of conjecture. The guanine nucleosides are the most active of those tested. A consideration of the manner of action of nucleosides on nuclear phosphorylation must include the following: the phosphorylation is aerobic; free bases such as adenine or guanine[2] have little or no capacity to foster phosphorylation, although purines can penetrate and be utilized *in vitro* RNA synthesis by liver nuclei isolated in 0.25 M sucrose (Rees and Rowland, 1961); the qualitative and quantitative differences in activity between ribo- and 2-deoxyribonucleosides; and the lack of any additive or clearly discernible inhibitory action of one nucleoside upon the activity of another. These properties make it unlikely that intranuclear phosphorolysis of nucleosides is the basis of the phosphorylation. Such an activity would not be aerobic and, furthermore, the acid stability of the labeled materials formed in largest amount in the acid-soluble fraction is inconsistent with the acid-lability of the α-D-ribose-1-phosphates which would result from nucleoside phosphorylase activity. In addition, the aerobic nature of the phosphorylation eliminates polynucleotide phosphorylase and possible exchange activities as bases for the phosphorylation.

Although labeled nucleosides have long been used to label liver nuclear nucleic acids, the source of the energy involved has been left open to question. The distribution and intracellular localization of kinases capable of phosphorylating nucleosides in the presence of ATP is an ill-defined area (Caputto, 1962). Although the bulk of nucleoside phosphate synthesis in tissues is thought to proceed by *de novo* pathways, it is possible that nuclei contain one or more kinases capable of directly phosphorylating nucleosides. From our data we presume at present that such is the case and that one or more substrates and a minute complement of bound nucleotides endogenous to the nuclei support the phosphorylation. An alternative possibility is that nucleosides act by altering penetration of nuclei by another material, possibly P_i. For P_i, this is a remote possibility because

all evidence indicates that isolated nuclei are very permeable to P_i.

It must be noted that Rees and Rowland (1961) and Rees *et al.* (1962) previously postulated that liver and kidney nuclei possess a phosphorylative system. The significance of their data was obscured in part however because they used preparations of nuclei containing demonstrable levels of mitochondrial contamination. It is impossible to draw any concrete conclusions at this time concerning the nature of the phosphorylation catalyzed by liver nuclei, but our results verify that they do possess an energy-transduction system. The results provide substance to the postulations by the English investigators. Work is in progress on the nature of the phosphorylation products formed in the presence of the various nucleosides.

References

Allfrey, V. G. (1963), *Proc. Intern. Congr. Biochem. 5th, Moscow, 1961, 2,* 127.

Caputto, R. (1962), *Enzymes 6,* 133.

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

Löw, H. (1959), *Biochim. Biophys. Acta 32,* 1, 11.

McEwen, B. S., Allfrey, V. G., and Mirsky, A. E. (1963), *J. Biol. Chem. 238,* 758, 2571, 2579.

Osawa, S., Allfrey, V. G., and Mirsky, A. E. (1957), *J. Gen. Physiol. 40,* 491.

Penniall, R., Hall, A. H., Griffin, J. B., and Irvin, J. L. (1962), *Arch. Biochem. Biophys. 98,* 128.

Penniall, R., Liu, S. M., and Saunders, J. P. (1963), *Biochim. Biophys. Acta 76,* 170.

Penniall, R., Saunders, J. P., and Liu, S. M. (1964), *Biochemistry 3,* 1454 (this issue; preceding paper, paper II of this series).

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

Rees, K. R., Ross, H. F., and Rowland, G. F. (1962), *Biochem. J. 83,* 523.

Siebert, G. (1963), *Proc. Intern. Congr. Biochem. 5th, Moscow, 1961, 2,* 93.

Weinbach, E. C. (1959), *J. Biol. Chem. 234,* 1580.

A Monodehydro Form of Ascorbic Acid in the Autoxidation of Ascorbic Acid to Dehydroascorbic Acid

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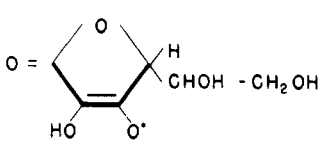
The existence of monodehydroascorbic acid in the oxidative pathway of L-ascorbic acid to dehydroascorbic acid was suggested by available literature. Experiments performed in this laboratory have now led to the isolation and characterization of this intermediate compound. Aqueous solutions of L-ascorbic acid and L-[1-^{14}C]ascorbic acid were chromatographed using descending chromatography. Kinetic data obtained using the ^{14}C-labeled ascorbate places the intermediate in the pathway as well as establishing the rate of intermediate formation. These studies indicate that this intermediate is monodehydroascorbic acid and may be complexed with ascorbic acid.

The existence of ascorbic acid in a monodehydro form as a free radical (I) is assumed in order to support the theoretical role of L-ascorbic acid in intermediary metabolism such as hydroxylation reactions. This



Structural Formula I

postulate necessitates a one-electron-transport system present in the oxidative pathway of L-ascorbic acid to dehydroascorbic acid. Recent work (Finholt *et al.*, 1963) has supported the existence of a monodehydro form of the acid, based on the maximum in the *p*H-rate profile at $pH = pK_a$ of ascorbic acid and on the observed first-order characteristics of the reaction. Other work (Barr and King, 1956) on the γ-ray-induced oxidation of ascorbic acid was interpreted in terms of radical mechanisms. The absence of a chain utilization of oxygen by ascorbic acid is consistent with the known protective effect on biological systems (Géro and Le Gallic, 1952). The compounds that exert this effect are characterized by their ease of oxidation and by their formation of stable one-electron oxidation products. The remainder of the oxidative pathway, the decomposition of dehydroascorbic acid to 2,3-diketogulonic acid and the elucidation of the Browning-reaction products, has recently been reported (Kamiya, 1960a).

Experiments (Baker *et al.*, 1963) dealing with the catabolism of L-[1-^{14}C]ascorbic acid in man have shown that changes in $^{14}CO_2$ expiration occurred with degradative changes of the acid. Work was initiated to identify the intermediates in the pathway responsible for these changes (Levandoski *et al.*, 1963). This paper centers on the identification and characterization of a proposed monodehydroascorbic acid–ascorbic acid complex as one of the intermediates. Work with other organic acids has shown that the acid molecule can combine with its own ion to form a charge-transfer complex. This acid-anion complex is seen in adipic acid, *p*-hydroxybenzoic acid, dihydroxybenzoic acid, and phenylacetic acid (Finholt *et al.*, 1963). Data from this laboratory suggest that a radical transfer complex may exist with monodehydroascorbic acid–ascorbic acid.

Materials and Methods

The three sources of L-ascorbic acid used in this study were: L-[1-^{14}C]ascorbic acid with a specific activity of 1.65 mc/mM obtained from California Corp. for

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