quick technic for separate expired air samples. Both CO_2 and O_2 analysis may be completed within 5 minutes as compared to 15-20 minutes by Haldane. Ninety-five per cent of the duplicate CO_2 analyses as determined with the Beckman C model (110-160 mm Hg range) will agree within 0.10% with duplicate Haldane data. A Beckman instrument of a narrower range and/or greater sensitivity would improve this accuracy. In our experience the Beckman C model (110-160 mm Hg range) allows better results than does the more elaborate and expensive E-2 model.

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Activation of Pyruvate Oxidation in Tumor Mitochondria by Diphosphopyridine Nucleotide.* (19089)

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(Introduced by S. P. Reimann.)

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Previous studies from our laboratory have disclosed that intact tumor cells (as exemplified by slices of various transplanted mouse neoplasia) are able to oxidize glucose and fatty acids to completion via the citric acid cycle(1,2). It therefore appeared that previous failures to observe oxidation of pyruvate or oxalacetate in homogenates of tumors(2-6) were due to loss of some labile factor in homogenization rather than to lack of oxidative capacity of the tumor cell.§ In an investigation of this matter it was found that good oxygen uptake by whole homogenates of tumors could be achieved in the presence or absence of substrates if rather high concentrations of diphosphopyridine nucleotide (DPN) were added; some preliminary results of these experiments have already been reported(1). The necessity for DPN in oxidation processes of tumors has been more strikingly demonstrated in experiments with isolated mitochondria, and these are reported in the present communication.

Methods and materials. The tumors used in this study are maintained by subcutaneous implantation in adult mice of the A or C3H strains. When tumors attained a size of 1 cm the animals were sacrificed by decapitation. Mitochondria of normal and neoplastic tissues were obtained by following exactly the procedure of Schneider(7).‡ The washed mitochondria were suspended in isotonic sucrose and kept refrigerated until used. All operations were carried out at $<5°C$. The tissue suspension was added to chilled Warburg vessels containing the ions, factors and substrates designated in Table I. The pyruvate and malate were commercial products. Diphosphopyridine nucleotide was obtained from various commercial sources, and assayed about 40% by the enzymatic procedure of Racker

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† This work will constitute part of a thesis to be presented by C. E. Wenner to the Graduate School of Temple University in partial fulfillment of the requirements for the Ph.D. degree.

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§ Potter and Lyle(6) have recently reported that pyruvate oxidation will occur in tumor homogenates in the presence of fluoride.

7. Schneider, W. C., *J. Biol. Chem.,* 1948, v176, 259.

TABLE I. Oxidation of Pyruvate by Mitochondria of Mouse Tumors. Experiments run 1 hr in air at 38°. The basic system contained the following substances in the designated final concentrations: $MgSO_4$, 3×10^{-3} M.; fumarate, 7×10^{-5} M. (as a primer); adenosine triphosphate (ATP) 2×10^{-3} M.; cytochrome C. 4×10^{-6} M.; phosphate buffer, 7.4 pH, 6×10^{-3} M.; and .6 ml of the mitochondria suspension representing 300 mg of original tissue. To this there was added diphosphopyridine nucleotide (DPN) 1.9×10^{-3} M. or sodium pyruvate, 8×10^{-3} M., or both; and the final volume made to 1.6 ml. Values are in μl per hr.

Tissue	Additions to basic system: None	DPN	Pyruvate	DPN + pyruvate
C3H hepatoma	23	62	22	187
A hepatoma	13	28	97	176
Mammary carcinoma	2	21	10	154
Sarcoma 37	22	43	21	77
Rhabdomyosarcoma	—	22	10	66

(8). Cytochrome C and ATP were commercial products. After an initial 7-minute equilibration, the flasks were shaken one hour at 38° with air as the gas phase, and oxygen uptake measurements made by the usual methods. Nitrogen was determined by a micro-Kjeldahl procedure(9).

Results. The effect of DPN in activating pyruvate oxidation by tumor mitochondria is demonstrated in Table I, in which data are given for 5 neoplastic tissues. In a system containing all of the known cofactors required for oxidation of pyruvate, oxalacetate, other citric acid cycle components, and fatty acids by mitochondria of normal tissues(3,7,10,11) oxygen consumption was very low, and was not stimulated appreciably by the addition of pyruvate. This observation is essentially the same as reported by Potter *et al.*, for pyruvate and oxalacetate oxidation in homogenates and in mitochondria of several transplanted rat tumors(3,4). Only one apparent exception was noted in the case of the A strain hepatoma, which displayed appreciable oxygen uptake on the addition of pyruvate alone. When DPN in a final concentration of 2 x 10^{-3} M was added to the fortified mitochondria, small increments in oxygen consumption occurred, which could be attributed to DPN activation of endogenous metabolites; however, when both pyruvate and DPN were present together high levels of oxygen consumption were observed, which were of similar magnitude to those obtained with mitochondria of normal liver(5,11).

The rigid requirement of added DPN for oxidation of pyruvate by mitochondria of C3H mouse hepatoma is displayed by Table II, in which there is recorded a study of the effects of omission of various factors from the system. Diphosphopyridine nucleotide was the only substance whose omission lowered oxygen consumption to an almost negligible value. The approximately 30% decrease in oxygen consumption on omission of fumarate is a reflection of the well-established priming effect of small amounts of Krebs cycle components on oxidation in mitochondria(11) and can be taken as further evidence for occurrence of the citric acid cycle in mitochondria of neoplastic tissues. The drop in activity on omission of ATP is in harmony with

TABLE II. Effects of Omissions on Pyruvate Oxidation by Mitochondria of Mouse Hepatoma. The complete system was that of Table I with DPN and pyruvate present. Values are in μl per hr.

Conditions	O_2 consumption
Complete system	187
DPN and pyruvate omitted	23
DPN "	22
Pyruvate "	62
Fumarate "	112
ATP "	117
Cytochrome C "	133

‡ Owing to a defective transformer, which was not discovered until most of our experiments were completed, the centrifugations of mitochondria were conducted at 5000-6000 g instead of the recommended 8000 g. This resulted in recoveries of mitochondria which were about 60% of the amounts obtained at the higher speed. This fraction contained no nuclei or intact cells which could be seen microscopically in stained preparations. Had all of the mitochondria been sedimented our observed oxygen uptakes, recorded in Tables I and II, would have been considerably higher, but the figures in Table III, which are on a per mg nitrogen basis would not have been changed.

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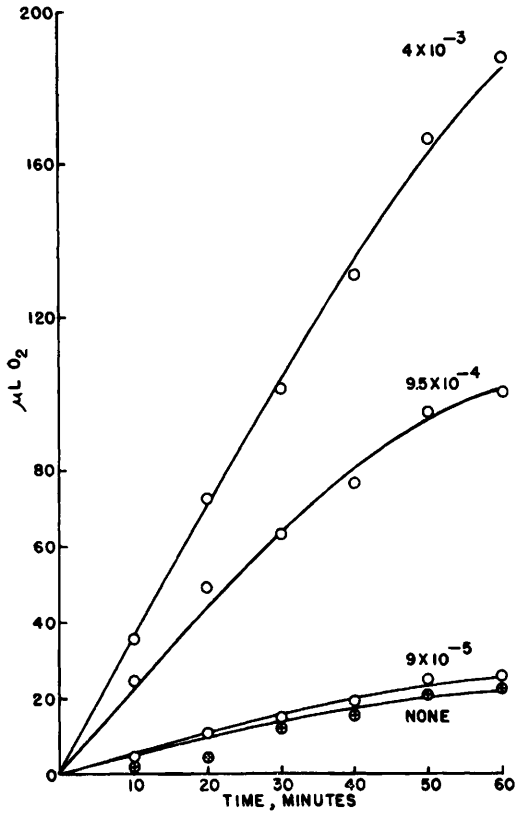

FIG. 1. DPN levels and oxygen consumption by suspensions of hepatoma mitochondria. Conditions are as given in Table I.

previous findings concerning its stimulating effect on oxidation in liver mitochondria (5,11). Apparently the ATP requirement in tumor mitochondria is not so high in the presence of DPN, since omission of the former resulted in only a 30% drop in activity. This is also in accord with previous observations of Lehninger and Kennedy(10), and Potter *et al.*(4) who found that DPN could at least partially replace the ATP requirement for oxidations in normal cell particles. The loss of some activity on omission of cytochrome C may be considered as a result of a deficiency in tumor mitochondria of this electron transport factor(12), however, here again a similar reduction in octanoate oxidation has been reported with respect to omission of cytochrome C in liver mitochrondria(11).

12. Dubois, K. P., and Potter, V. R., *Cancer Research,* 1942, v2, 290.

The relationships between oxygen uptake and DPN level in the oxidation of pyruvate by mitochondria from the C3H hepatoma is revealed in Fig. 1. Relatively high concentrations of DPN of the order of 0.002 M are required for maximal activation; further increase in DPN concentration did not raise oxygen consumption above the level shown in this figure. In the experiments of Potter *et al.*(3,4) on oxalacetate and pyruvate oxidation in homogenates of rat tumors, no effect of DPN was observed; however, the highest concentrations used were only .0003 M. It is evident from Fig. 1 that this concentration was too low for appreciable activation in our experiments. It may be presumed, since the two sets of experiments were carried out under almost identical conditions except for DPN concentration, that Potter *et al.* would also have been able to activate oxalacetate oxidation if their DPN concentration had been higher. The use of 40% DPN in our experiments raises the question whether its effects are actually due to some contaminant. This seems unlikely for two reasons. First, the effects obtained with different samples were proportional to the DPN content as determined enzymatically(8); and, second, occasional checks with 90% DPN (obtained from General Biochemicals Inc.) gave the same results as the less pure specimens.

When these studies disclosed the capacity of tumor mitochondria for pyruvate oxidation, it was of interest to make comparisons with mitochondria of normal tissues. It was already evident from Warburg's experiments (13) that the respiratory activity of tumor slices was somewhat low when compared with metabolically active normal tissues such as liver and kidney; and essentially the same finding was reported in a recent isotopic tracer study(2). Inasmuch as it is well established that at least a major portion of the oxidative activity of cells is concentrated in the mitochondria(14) it appeared that such differences as exist between normal and neo-

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14. Schneider, W. C., and Hogeboom, G. H., *Cancer Research,* 1951, v11, 1.

TABLE III. Pyruvic and Malic Oxidase Activities of Mitochondria of Normal and Neoplastic Tissues.

Tissue	O_2 uptake, μl/hr/mg N Pyruvate	Malate
Neoplastic		
A-strain hepatoma	315 318 246	314
C3H strain hepatoma	312	—
Rhabdomyosarcoma	150	159
Sarcoma 37	292	230
Normal		
Kidney (C3H mouse)	292	278
Liver (A mouse)	252 224 169	145
Kidney, rat	380* 642†	
Liver, "	220* 394†	
" "	141‡	

* Data of Potter, *et al.*(16). Substrate, oxalacetate and pyruvate.

† Data of Schneider and Potter(5). Substrate, oxalacetate and pyruvate.

‡ Data of Kennedy and Lehninger(11). Substrate, pyruvate plus oxalacetate.

plastic tissue in oxidative capacity could be due to the lower content of mitochondria in tumors(14). Some support for this view is found in recent data of Schneider and Hogeboom(15) who reported that although the total nitrogen content of a mouse hepatoma was three-fourths that of mouse liver, the tumor mitochondrial nitrogen constituted only 13% of the total cell nitrogen, whereas liver mitochondrial nitrogen was 23% of the total. Thus, if a particular enzymatic activity is found only in mitochondria and is proportional to the nitrogen content thereof, the apparent enzymatic activity of the normal tissue will be $\frac{23}{13} \times \frac{100}{75} = 2.4$ times that of the tumor.

To compare the pyruvic (and malic) oxidative activities of tumor mitochondria with those of normal tissues, separate aliquots of mitochondria were simultaneously tested under the conditions outlined in Table I, and assayed for nitrogen; and oxidative activity was calculated on the basis of the nitrogen content. Although liver mitochondria generally do not need added DPN for maximal activity, it was added to all flasks in order to make the experiments comparable. The data are given in Table III. Three of the four tumors gave essentially equal pyruvic oxidative activities per mg of mitochondrial nitrogen, and these were fully as high as those displayed by mouse liver and kidney, the only exception being rhabdomyosarcoma. Whether this tumor is actually different in this respect from the others is now under study. Malic oxidative activities were, on the whole, in the same range as those of pyruvate.

In several instances it was possible to compare these values with data from the literature. Potter, *et al.*(16) reported graphic data (Fig. 1) for oxalacetate oxidation by mitochondria of rat liver and kidney, and similar data were reported by Schneider and Potter(5) (Tables I and II). These were recalculated on a per mg nitrogen basis by assuming, from Schneider and Hogeboom's data(15), that normal tissues contain 3% of nitrogen and that the mitochondria contain 25% of the total cell nitrogen. A similar calculation was made for a single experiment reported by Kennedy and Lehninger(11) (Table IV). These values are included in Table III. When one considers all the differences in techniques and conditions between these various experiments the agreement is surprisingly good.

We conclude from these results that mitochondria of neoplastic cells have the same capacity to oxidize pyruvate and malate as those of normal tissues. It does not necessarily follow that all of the enzymes concerned with the reactions of the citric acid cycle or those involved in electron transport through the pyridine nucleotides, flavoproteins and cytochromes occur in the same amounts in both types of mitochondria. Indeed, according to experiments of Schneider and Hogeboom(15) mitochondria from mouse hepatoma have less than half as much succinic oxidase and cytochrome oxidase activity as mitochondria from normal mouse liver. It does appear, however, that mitochondria of normal and neoplastic tissues do have essentially the same makeup of oxidative enzymes and also it would appear that in the

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16. Potter, V. R., Lyle, G. G., and Schneider, W. C., *J. Biol. Chem.*, 1951, v190, 293.

complex system of reactions resulting in pyruvate oxidation the same step is rate-controlling in both types of mitochondria.

Discussion. The fact that DPN must be added to tumor mitochondria to bring about oxidation of pyruvate is not surprising. It is more surprising, in fact, that it is apparently not necessary for oxidations by mitochondria of normal tissues, when one considers the important role of this soluble coenzyme in at least one step of the citric acid cycle, *viz.* malic oxidation, and probably also in pyruvate and α-ketoglutarate oxidation(17). However, a DPN requirement can be demonstrated under certain conditions. Lehninger (18) has shown that washed sedimentable liver particles which lost their activity on "ageing" could be reactivated by addition of DPN; and Potter, *et al.* and Kennedy and Lehninger(4,11) have pointed out that DPN can partially replace the adenine nucleotide requirement for mitochondrial oxidations. Further evidence of a DPN requirement in oxidations by kidney homogenates has been provided by Pardee and Potter(19), who found that loss in oxidative activity is paralleled by a decrease in particle-bound DPN; and that further addition of DPN would partially restore oxidative activity.

In an extensive study of the "cyclophorase" system of rabbit liver and kidney, the active component of which is the mitochondrial fraction, it was found by Green and members of his laboratory that mild physical or chemical treatment resulted among other changes in a loss of oxidative activity which could be regained by addition of DPN(20-22). It was concluded that the pyridinoproteins occur normally in a conjugated form in which the nucleotide is tightly bound to its apoenzyme; and that the effect of the various treatments was to dissociate the pyridine nucleotide. Whether or not one accepts this explanation, these studies have indicated that the nucleotide is present in normal mitochondria in a form which is not freely dissociable, and that alterations can easily be produced which create a DPN requirement but which do not otherwise impair the integrity of the particles. On this basis it might be assumed that the tumor mitochondrion differs from its normal counterpart by an excessive "fragility," so that the process of homogenization is sufficient to bring about changes resulting in the DPN requirement. The ease with which tumor mitochondria lose DPN may be related to the relative content of oxidized and reduced forms of the nucleotide(1). This point is now under investigation.

Another possibility is that tumors may be extremely active in splitting DPN. Although direct studies of this point have not been carried out, to our knowledge, Potter and Lyle(6) have recently shown that in comparison with normal tissues several rat tumors displayed an excessive rate of organic phosphate breakdown and have concluded that the primary reason for the loss in oxidative activity in tumor homogenates is an unusually high rate of destruction of high energy phosphate bonds, thus preventing maintenance of a sufficiently high level for oxidative activity. In support, these authors cited experiments in which oxidation was maintained in tumor homogenates if fluoride was added to minimize splitting of organic phosphate linkages. It is interesting in this connection that fluoride has been reported by Huennekens and Green (20) to stabilize the kidney cyclophorase system so as to make DPN addition unnecessary for oxidative activity.

Further work is obviously needed to clarify the nature of the DPN requirement for oxidation of pyruvate by tumor mitochondria; it seems apparent now, however, from the present study and from recent results of Potter and Lyle(6) that tumor mitochondria do not differ fundamentally from their normal counterparts in the enzymatic mechanisms of

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pyruvate oxidation.

Summary. Suspensions of mitochondria of various transplanted mouse tumors are able to oxidize pyruvic acid if the system, containing Mg ions, ATP, cytochrome C, and priming concentrations of fumarate, is fortified with diphosphopyridine nucleotide. The concentration of the nucleotide required for maximal activation is 0.002M. When mitochondria of tumors were compared with those of normal tissues it was found that on a per mg nitrogen basis pyruvate oxidation was similar in magnitude in both types of mitochondria.

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Metabolism of Plasma Substitutes II. Oxypolygelatin (OPG) (19090)

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The use of gelatin solutions as substitutes for plasma have long been investigated(1,2). Although gelatin appears to fill the requirements for a practical plasma expander, the main difficulty lies in its high gel point. Recently, Campbell and co-workers(3) have developed a modified gelatin, oxypolygelatin, (OPG) which appears to overcome this particular fault.

This paper is a report of the studies on the metabolism of this new plasma expander. Changes in the urinary dextrose to non-protein nitrogen ratio (D/N) of the phlorizinized, starved dog were used as an index of metabolism of OPG. The details of these experiments have been reported in the first paper of this series(4). The results are summarized in Table I. In each case, during the 24-hour period following the infusion of 200 ml OPG in physiologic saline, we have a marked increase in the D/N ratio, well beyond the limits of the pre-infusion spread.

TABLE I. Changes in D/N Ratio After Infusion of 200 ml of 5% Solution of OPG in Physiological Saline.

Dog No.	Pre-infusion avg	Range	0-24 hr post-infusion D/N
1	1.39	1.23-1.69	2.4
3	2.76	2.17-3.75	4.42
6	2.27	2.17-2.30	3.34
48	3.47	3.30-3.91	5.29

Since the phlorizinized starved dog stabilizes at a constant D/N ratio, a change in this value reflects changes in the glucose or nitrogen metabolism. Because phlorizin acts by preventing the reabsorption of glucose by the tubules, an increase in D/N ratio indicates an increase in the excreted glucose relative to the excreted nitrogen. As was pointed out in the previous discussion(4), it is felt that glycogen can be ruled out as a source of the increased glucose as can the plasma proteins. Therefore, the highly probable possibility left which might cause this rise in the D/N ratio is the metabolism of OPG to glucose.

Summary. Evidence is presented to show the metabolism of OPG to glucose by the phlorizinized starved dog.

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