Action of Fluoride and Other Reagents on Phosphorylation in Malignant and Certain Normal Tissues. (19876)

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The suggestion of Warburg(1,2) that tumors derive a large proportion of their energy for growth from anaerobic processes has stimulated inquiry into the mechanism of energy liberation and transfer in both tumor and normal tissues. It has recently been shown that enzymes of the tricarboxylic acid cycle occur in tumors (see the articles by Potter(3), Weinhouse and collaborators(4,5), and Olson(6), including reviews of literature. See also Schneider and Hogeboom(7) for literature regarding normal and cancer tissue mitochondria). Potter and Lyle(8), Clowes and Keltch(9), Williams-Ashman and Kennedy(10), and Kit and Greenberg(11) found that oxidations carried on by such enzymes from tumors can generate high-energy phosphate bonds which can be used for synthesis of cellular structural elements(10,11). It has also been shown by LePage(12,13), by LePage and Schneider(14), and by Clowes and Keltch(9,15) that the supernatant fraction remaining after the particulate elements of tumor and normal tissue have been centrifuged off can utilize the energy from glycolysis to synthesize organic phosphate compounds. The nature of the glycolytic reactions involved is now under study by Groth, LePage, Heidelberger, and Stoesz(16).

The object of the present investigation is to form a more quantitative estimate than has hitherto been made of the relative importance of anaerobic and aerobic processes in energy liberation in one tumor, the Walker 256 rat carcinoma, as compared to normal rat liver and brain.

Results. The methods used were those of Clowes and Keltch(9) as adapted from those of LePage and of Potter, except for the order in which the reaction components were mixed. In the present experiments the inorganic phosphate was a part of the mixture placed in the main compartment of the Warburg vessel and the reaction was started by addition of the tissue fractions from the side arm after equilibration with the gas phase; in the previous experiments(9) the tissue fractions were put into the main compartment and the reaction started by addition of phosphate from the side arm. Tissues were obtained and homogenized as described before(9). Particles were obtained by centrifuging at 20,000 g for one-half hour at 4°C, washing, and resuspending, as previously described. The reaction mixture contained the same components in the same concentration as before, unless otherwise stated. The substrate in all experiments was hexose diphosphate (HDP) and pyruvate.

Effect of fluoride concentration on phosphorylation by particulate and supernatant fractions. One of the principal difficulties in measurements of phosphorylation in cell-free fractions is the high phosphatase activity which appears as cells are broken up; this activity can be to some degree suppressed by the inclusion of fluoride in the incubation medium(3,17,18). The effects of fluoride upon aerobic and anaerobic phosphorylation by homogenates, particles, and supernate fractions were found to depend very sharply upon its concentration (Table I). The point to be stressed is that phosphorylation by the particles *increased* with addition of fluoride up to the optimum concentration of 0.0033 to 0.015 M. Optimum phosphorylation by the glycolytic system of the supernatant fractions was obtained when fluoride was omitted entirely. When the optimum rates of phosphorylation obtainable with homogenates or particles by adjustment of fluoride concentration were compared, it was found that the ratio of aerobic to anaerobic phosphorylation was greatest in liver and least in tumor, with brain intermediate between the other two. The phosphorus uptake of a given supernate was approximately the same in air or nitrogen and was lowered to a corresponding extent by any given concentration of fluoride. These results confirm and extend the observations

TABLE I. Effect of Fluoride on Phosphorus Uptake by Homogenates of Rat Liver, Brain, and Tumor and by the Particulate and Supernate Fractions Derived Therefrom by Centrifuging at 20000 g for 30 Min. Particles were washed once with buffered KCl, recentrifuged, and resuspended in buffered KCl. Incubation time, 40 min at 25°C. Substrate for both acrobic and anaerobic experiments was hexose diphosphate and pyruvate. Gas phase in aerobic experiments was air, in anaerobic experiments nitrogen. Each experiment based on material derived from 125 mg original tissue, except in the case of tumor particles, where material from 500 mg original tissue was used. All results expressed in μM.

Final molar conc. fluoride	Liver		Brain		Tumor	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
			Homogenate			
.0	14.7	−1.2	15.9	6.9	4.3	3.2
.0017	16.6	.8	16.5	5	8.8	7.2
.0033	17.1	2.1	17	7.3	13.3	10.6
.0067	17.5	3.3	17.3	8.7	14.2	10.8
.013	17.5	3.4	17.5	8.5	13.6	10.7
.02	17.3	3.1	17.3	8.1	12.8	10.5
.026	16.6	3.6	16.9	7.9	13.2	10.8
.033	16	2.6	15.9	7.8	12.8	10.7
.04	15	3.4	15.5	7.8	12.1	10.5
			Particles			
.0	4.6	−2	0	−1.1	−2.8	−1.2
.0017	10.5	−2.5	2.3	−1.6	−2.4	−1.1
.0033	14.1	−1.2	5.1	−1.2	− .8	− .5
.0067	13	−1.3	6.3	.5	0	.3
.013	12.4	− .5	8.4	.8	3.3	2.8
.02	10.8	− .6	7.5	.7	2.9	2.3
.026	8.5	− .4	7.6	.7	2.5	2.5
.033	7.7	−1.3	6.9	1	1.9	2.1
.04	6.1	0	6.3	.9	2.1	2
			Supernate			
.0	8.9	11.1	18	17.1	17.3	17.9
.0017	5.7	7.6	17.2	14.7	17.3	16.8
.0033	5.7	7.5	16.6	14.4	17.3	16.9
.0067	5.1	6.7	16.9	13.5	13.6	14.6
.013	5.1	6.2	13.5	11.7	8.3	10.5
.02	5.4	6.1	13.1	10.5	8.5	11
.026	5.6	6.4	13.1	9.7	8	11.5
.033	5.4	6.7	11.8	9.7	8.5	11
.04	5.3	6.2	12.2	9.3	7.6	11

previously reported(9,15).

Under carefully controlled conditions phosphatase activity of particulate elements from a mouse tumor is smaller when the particles are prepared in 0.25 M sucrose than in 0.15 M KCl(19). In the present experiments, brain or tumor particles prepared and tested in 0.25 M sucrose did not take up more phosphate than those prepared and tested in KCl; P uptakes in micromoles were: brain particles in sucrose, 10.8; in KCl, 14.6; tumor particles in sucrose, 5.7; in KCl, 5.0.

Effect of anaerobiosis on phosphorylation by homogenates and by particulate and supernatant fractions. As another attempt to assess the relative importance of anaerobic and aerobic processes in the 3 types of tissues, phosphate uptake and lactate production by homogenates, by particles, and by supernatant fractions of liver, brain, and tumor were measured (Table II).

The anaerobic and aerobic uptakes of inorganic phosphate by liver, brain, and tumor homogenates from 125 mg tissue were respectively, in micromoles, as follows: liver: anaerobic 2.3, aerobic 16.6; brain: anaerobic 9.0, aerobic 17.2; tumor; anaerobic 10.2, aerobic 13.1. Thus, phosphate uptake by the Walker 256 tumor homogenates under these experimental conditions was chiefly based on anaerobic reactions, that by liver chiefly on aerobic reactions, confirming the conclusion reached on the basis of measurements on the particulate and supernatant fractions(9).

The question arises whether competition may exist for phosphate between the anaerobic

TABLE II. Phosphorus uptake (P) and lactate production (L) by homogenate, particulate, and supernate fractions of liver, brain, and tumor as a function of amount of original tissue from which each fraction per vessel is derived, as a function of presence or absence of DPN, and as a function of presence or absence of DNC. Fractions were prepared and measurements made as described in Table I and text. Incubation time 40 min. at 25°C. Results in micromoles.

	Tissue equiv. per vessel, mg	Conc. DPN, moles/l	Conc. DNC, moles/l	Liver				Brain				Tumor			
				Homog.		Part.	Super.	Homog.		Part.	Super.	Homog.		Part.	Super.
				P	L	P L	P L	P	L	P L	P L	P	L	P L	P L
In air	250	.0002	0	17.5	4.6	16.7 1.2	9.2 12.6	19.2	7.6	9.8 2	18.4 10.8	18.2	14.2	2.2 2.8	19 16
		0	0	17.3	.7	16.2 .9	1.9 2.8	18.8	4.4	.3 1.1	— —	5.5	3.4	-.1 1.1	— —
		.0002	.0005	.8	7.7	— —	— —	8.9	9.8	-.1 1.8	— —	14.1	15.1	1.4 2.7	— —
	125	.0002	0	16.6	4.1	10.2 .5	4.7 7.2	17.2	6.5	5.8 1.5	10 7.8	13.1	11.2	1.2 1.8	8.5 8.7
		0	0	15.5	.9	9.1 .6	1.2 1.4	2.4	2.2	.5 .5	1.7 2.1	2.9	1.4	-.2 .3	-.8 1.1
		.0002	.0005	2	.5	-.5 .6	5.2 7.1	8.9	8.8	.7 1.4	12.3 9.9	9.9	9.4	— —	10.5 9.4
	62.5	.0002	0	12.1	3.8	4.7 .6	— —	16.6	4.7	3.2 1.6	5.8 4.8	6.6	5.5	— —	— —
		0	0	6.7	.8	3.9 .5	— —	.7	1.1	-.2 1.2	— —	.4	1.1	— —	— —
		.0002	.0005	1.9	3.8	0 .5	— —	5.1	5.9	— —	— —	5.6	5.5	— —	— —
In nitrogen	250	.0002	0	2.1	9.1	-.2 1.5	8 12.1	10	9.9	-.7 2.7	18.2 11.7	14.6	18.9	1.6 3.4	17.4 16.9
		0	0	-4.8	.9	-1.1 1.4	.9 3.4	1.6	6.2	-1.9 1.2	— —	-1.2	3.4	-1.3 1.6	— —
		.0002	.0005	1	7.7	— —	— —	8.9	9.8	-1 2.3	— —	14	17.1	1.5 3.1	— —
	125	.0002	0	2.3	6.2	0 .3	5.6 8.1	9	8.6	.9 1.8	9.8 9.8	10.2	11.2	— —	8.7 10
		0	0	-4.3	.6	-.5 .4	2.1 2.8	1.4	3.4	-.1 1.4	2.4 3.7	.5	2.6	— —	-1.3 1.5
		.0002	.0005	1.5	5.9	-.8 .6	5.7 7.1	8.8	9.6	.8 2.2	12 9.7	9.9	11.5	— —	9.9 9
	62.5	.0002	0	3.2	4.3	.5 1.1	— —	6.2	5.4	.5 1.3	6.1 4.7	5.4	5.7	— —	6 6.1
		0	0	-1.2	.8	.1 .6	— —	.3	3.2	— —	— —	.8	2.2	— —	— —
		.0002	.0005	1.6	3.5	0 .9	— —	6.1	5.2	— —	— —	6.3	5.8	— —	— —

and aerobic systems, such that the actual phosphate uptake in oxygen occurring via glycolysis may be much less than the anaerobic values of 2.3, 9.0, and 10.2 for liver, brain, and tumor, respectively; *i.e.*, that there is a pronounced Pasteur effect. The anaerobic and aerobic lactate productions for the same series from 125 mg liver, brain, and tumor homogenates were, respectively, in micromoles, as follows: liver: anaerobic 6.2, aerobic 4.1; brain: anaerobic 8.6, aerobic, 6.5; tumor: anaerobic 11.2, aerobic 11.2, thus indicating a moderate Pasteur effect for liver and brain but not for tumor. If the lactate figures for 250 mg of original tissue are compared, liver, brain, and tumor all show a moderate Pasteur effect, that of the liver being greatest. In a previous communication (9) it was noted that with brain and liver but not with tumor the combined lactate production of the individual particle and supernate fractions was considerably larger than that of the homogenate. This was interpreted as a Pasteur effect. In the experiments reported in Table II the lactate production of particles plus supernate greatly exceeded that of homogenate in the experiments with liver and brain and also in the experiment in which 250 mg of tumor tissue was employed, but with 125 mg of tumor tissue the combined lactate production of particles and supernate was less than that of homogenate. It would appear from the above results that a moderate Pasteur effect is observed in the case of all 3 tissues, greatest in liver, intermediate in brain, and least or absent in tumor tissues.

One question which was not completely settled in previous publications(9,15) was whether or not the particulate elements could effect phosphorylation under anaerobic conditions with HDP and pyruvate as substrate. It has now been found that when the particles are washed virtually free of phosphate and kept separate from the glycolytic substrates until the equilibration with nitrogen has been completed, there is no significant phosphorus uptake by liver and brain particles and sometimes no significant uptake by tumor particles under anaerobic conditions. For example, the phosphorus uptake under aerobic and anaerobic conditions of liver particles was 16.4 and

TABLE III. Phosphorus Uptake, Lactic Acid Production, and Oxygen Consumption by Particulate and Supernate Fractions of Liver, Brain, and Tumor in Air and in Nitrogen. Each experiment is based on material derived from 250 mg original tissue, except in the case of the tumor particles, where material from 500 mg original tissue was used. For other experimental details see Table I and text. All results expressed in μM.

	Liver			Brain			Tumor		
Fraction	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed	O_2 uptake
				In air					
Particles	16.4	.6	6	11.3	2.5	3.6	3.4	1.5	2.3
Supernate	9.2	12.6	1.1	18.4	10.8	1	19	16	—
				In nitrogen					
Particles	−.2	1.5	—	.3	2.4	—	.7	5.8	—
Supernate	8	12.1	—	18.2	11.7	—	17.4	16.9	—

—0.2, of brain particles 11.3 and 0.3, and of tumor particles 3.4 and 0.7 (Table III). The phosphorus uptake of the supernates was, as previously observed, the same under aerobic and anaerobic conditions: in the case of liver 9.2 and 8.0, in the case of brain 18.4 and 18.2, and in the case of tumor 19.0 and 17.4. Furthermore, the lactate production in all supernates was virtually the same under aerobic and anaerobic conditions. It appears that the phosphorylation by tumor particles under anaerobic conditions which was previously mentioned may have arisen, at least in part, from traces of oxygen which were present during the 10-minute equilibration period with nitrogen, particularly at the beginning. It is possible that the particles in the intact cell may have a system capable of glycolysis, in addition to the nonparticulate glycolytic system dealt with here.

On account of the relatively high fragility and low content of particles in the tumor tissues, larger amounts have frequently been employed in the hope of obtaining significant results. This makes it more difficult to wash the particles free from supernate. It is possible, therefore, that the small phosphorylating effect sometimes observed when tumor particles are used under anaerobic conditions may be due to occlusion of small amounts of the water-soluble, anaerobic, glycolytic, phosphorylating mechanism. This question is being further investigated with other tumors.

The absolute rates of phosphorus uptake by the homogenates were found to depend upon the amount of tissue used and upon addition of diphosphopyridine nucleotide (DPN) to the medium: with the homogenates under aerobic conditions great dependence upon DPN addition was found with tumor and brain and relatively little with liver. The same was true with particles, confirming other investigators(10,19). Omission of DPN led to a marked depression of phosphate uptake by the supernatant fractions of all 3 tissues, being about the same under both aerobic and anaerobic conditions. For example, the anaerobic P uptakes in micromoles per 40 minutes with and without addition of 0.0002 M DPN were (all for 125 mg tissue): liver supernate, 5.6 and 2.1; brain, 9.8 and 2.4; tumor, 8.7 and —1.3 (Table II).

Dinitrocresol (DNC), 5×10^{-4} M, did not significantly inhibit phosphorylation by homogenates of brain and tumor under anaerobic conditions (Table II) and may therefore be used as a reagent to test for the fraction of the phosphate uptake under aerobic conditions (*i.e.*, in an atmosphere of air or O_2) which is dependent on glycolytic reactions. In the case of liver homogenates, the anaerobic phosphate uptake of which is relatively low, the addition of dinitrocresol under anaerobic conditions sometimes causes further lowering of phosphorus uptake.

Efficiency of phosphorylation under anaerobic conditions. Formation of one mole of lactic acid from hexose diphosphate under the experimental conditions employed should lead to the uptake of one atom of inorganic phosphorus. To determine the actual ratio, lactate formation and phosphorus uptake were measured for the supernate fractions as a function of the incubation time, fluoride concentration, and presence or absence of added hexokinase (Table IV). The optimum values of the

TABLE IV. Phosphorus Uptake and Lactic Acid Production by Supernate Fractions of Liver, Brain, and Tumor, in Relation to Incubation Time, Addition of Excess Hexokinase, and Presence or Absence of Fluoride. Gas phase, air. Each experiment based on material derived from 250 mg original tissue. For other experimental details see Table I and text. All results expressed in μM.

	No yeast hexokinase added				Yeast hexokinase added			
	With fluoride		Without fluoride		With fluoride		Without fluoride	
Incubation time (min)	P uptake	Lactate formed	P uptake	Lactate formed	P uptake	Lactate formed	P uptake	Lactate formed
				Liver				
5	2.4	3.3	−.2	3.1				
10	3.3	4.9	1	5.8				
20	5.1	7	−.5	7.8				
40	7.3	11.4	.5	14				
				Brain				
5	3.6	1.8	3	1.6	10.7	7	14.9	12
10	5.3	3.3	4.9	4.2	14.8	9.7	19.7	14.4
20	8.8	4.5	6.6	5.5	—	—	—	—
40	10.2	6.5	9.1	8.4	14.3	11.1	20.4	23.1
80	16.6	11.8	—	—	13.6	11.8	20.4	24.2
				Walker 256 tumor				
5	2.8	2.8	2.1	1.8	6.4	6.1	12.2	12.2
10	3.2	4.2	2.8	3.6	6.9	6	16.5	16.5
20	5.8	6.4	3.2	6.5	—	—	—	—
40	7.9	9.4	4.8	8.8	6.7	8	17.3	18.3
80	12.7	10.8	7.2	14.6	8.2	10.9	17.3	20.7

ratio of phosphorus uptake to lactate formation were found to be as follows: liver 0.7, 20 minutes incubation, fluoride present, hexokinase absent; brain 1.5, 10 minutes incubation, fluoride present, hexokinase either present or absent; tumor 1.2, 10 minutes incubation, fluoride present, hexokinase present. In the absence of added yeast hexokinase, lactate formation and phosphorus uptake follow an approximately linear course for the first 40 minutes of incubation. With added hexokinase the reaction under the experimental conditions employed was virtually complete after 10 minutes. The limiting factor in the overall phosphorus uptake was, therefore, the rate at which the high-energy phosphate could be transferred to a stable form. The reactions responsible for the phosphate uptake in excess of that accounted for by lactate production have not been identified; they may be the same as those recently described by Groth, LePage, Heidelberger, and Stoesz(16).

Iodoacetate and phosphate uptake. It was mentioned above that only aerobic phosphorylation is suppressed by dinitrocresol, that associated with glycolysis being left unaffected (Table II). The effects of various concentrations of iodoacetate, which can combine with sulfhydryl groups(20) of enzymes such as phosphoglyceraldehyde dehydrogenase (21) and those of the tricarboxylic acid cycle (22), were determined upon both particulate and supernatant fractions (Fig. 1). Both aerobic and anaerobic phosphorylations were suppressed by iodoacetate.

Discussion. It now appears that the particulate elements isolated from tumors can generate high-energy phosphate bonds by oxidation of substrates of the tricarboxylic acid cycle(3-10,19). However, since the tumor materials employed have invariably contained a certain amount of normal tissue, the possibility remains that the small oxidative phosphorylating effect observed may be derived from that source. There is no doubt that the supernate fractions can generate such high-energy phosphate bonds by glycolytic reactions(9,12-14). From the published results now available it appears that generation of high-energy phosphate bonds in the Walker 256 tumor takes place largely at the expense of glycolytic rather than oxidative reactions, whereas generation of such bonds in liver is largely based on aerobic reactions. Particulate elements of other tumors may have a relatively greater capacity for aerobic phosphorylation than the Walker 256 tumor (10,19).

In this and preceding papers(9,15) the

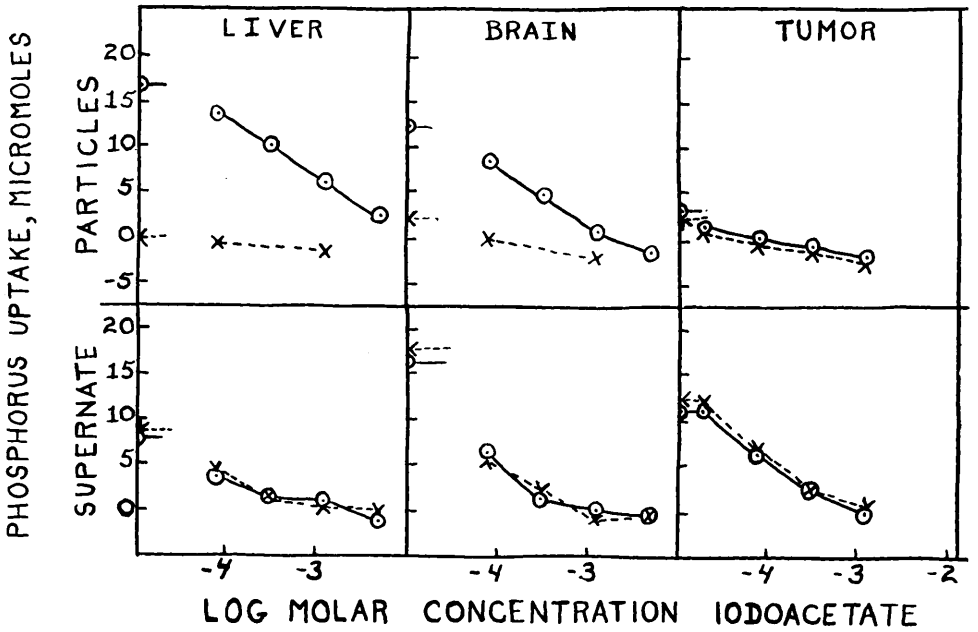

FIG. 1. Effect of iodoacetate on phosphorus uptake by particulate and supernate fractions of liver, brain, and tumor: O———O, in air; X- - - -X, in nitrogen. Respective control values in air and in nitrogen designated by horizontal lines perpendicular to the ordinates. For other experimental details see Table I and text.

sensitivity to inhibitors of the particulate and supernatant fractions of tumor has been measured and compared with that of the same fractions from normal tissues. It was found that aerobic phosphorylation by particles of liver and brain was inhibited by dinitrocresol or by calcium ion and that phosphorylation in the supernate fractions of liver, brain, and tumor was relatively insensitive to these reagents. The only difference between the normal and tumor fractions with respect to the action of these agents was that the tumor particles exhibited a slight residual phosphorylation even in the presence of DNC or calcium chloride. These observations have been confirmed in the present investigation. However, as it is difficult to obtain enough tumor particles to permit the extensive washing employed with the liver and brain particles, it is possible that the DNC-resistant phosphorylation of the tumor particles may arise from occlusion of supernate in the tumor particulate fraction. This possibility requires further investigation.

In the present paper the effects of fluoride and iodoacetate on homogenates, particulate fractions, and supernate fractions from the same tissues have been determined. While aerobic phosphorylation is increased and anaerobic phosphorylation is decreased by addition of fluoride, no significant difference between the sensitivity of the tumor and the normal fractions has been found.

Although the phosphorylation by the cell-free fractions from the tumor drops markedly when DPN is omitted from the medium, this dependence on added DPN is not unique to the tumor; similar decreases in phosphorylation by fractions from brain and to a lesser degree from liver were observed upon omission of DPN.

Since, with the exceptions noted, the tumor tissue appears to differ only quantitatively and not qualitatively from the 2 normal tissues, the high anaerobic phosphorylation cannot, at least for the present, afford an explanation of the rapid growth of tumor relative to normal tissues.

Summary. The effects of fluoride, of anaerobiosis, and of iodoacetate upon esterification of phosphate by homogenates, by particles, and by supernatant fractions of liver, brain, and tumor have been determined. The requirement for DPN by the various fractions has also been investigated.

1. Phosphorylation by the homogenates or particles increases with addition of fluoride up to an optimum concentration of about 0.01 M; optimum phosphorylation by the supernatant fractions is obtained when fluoride is omitted entirely. By this adjustment of fluoride concentration the preponderance of glycolytic over oxidative phosphorylation in the Walker 256 tumor is even more marked than has been previously recognized. 2. Under the present experimental conditions phosphorylation by the particulate fractions of liver and brain and of tumor within the range of experimental error was found to be dependent on aerobic processes, while that in the supernatant fractions was completely dependent on anaerobic reactions. The maximal values obtained with the supernate fractions under anaerobic conditions for the ratio of phosphorus uptake to lactate formation were: liver, 0.7; brain, 1.5; tumor, 1.2. 3. Phosphorylation by the particulate and supernate fractions was markedly inhibited by iodoacetate; no significant differences between the sensitivity of the tumor fractions and that of the liver or brain fractions was observed. 4. Phosphorylation in the Walker 256 tumor appears to differ from that in normal tissues principally in quantitative rather than in qualitative respects.

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