

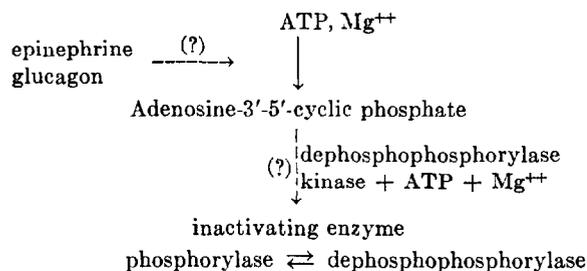
A Biochemical Characteristic of Ascites Tumor Cells

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Glycogen and glycogen phosphorylase are almost ubiquitously distributed among mammalian tissues. The enzymatic activation of phosphorylase is hormonally regulated, and the following scheme summarizes the major findings with liver phosphorylase (1, 2).



It seemed reasonable to suppose that a study of phosphorylase and its activating enzymes in tumor tissues might yield some information bearing upon (a) the characteristically high rate of glucose utilization by tumors (3), and (b) the degree of control exerted by certain hormones over these enzymes in neoplastic cells.

In previous studies (4-6) various aspects of carbohydrate metabolism and its hormonal control were investigated in several strains of ascites tumors and an absence of glycogen was noted in one of the tumors. In further preliminary reports many different ascites tumors were shown to possess little or no glycogen and glycogen phosphorylase (7, 8).

It seemed striking that cells with such large capacities to utilize glucose should be unable to degrade glycogen appreciably and should therefore be free from this hormonally controlled regulatory mechanism. The present report contains more complete information concerning these findings.

EXPERIMENTAL

The tumor strains and the strains of host mice used in this study are listed in Table I. The majority are ascites tumors; exceptions are the HeLa carcinoma grown in tissue culture and the Rous sarcoma, a solid tumor. The method of harvesting the ascites tumors has been presented previously (5). HeLa cells grown both on glass and in suspension were the generous gift of Dr. Harry Eagle. The HeLa cells were harvested by centrifuging the cells at $200 \times g$ for 5 minutes. The cells were washed with Earle's solution and were recentrifuged.

Cells were homogenized at 0-5° by the following techniques. Sonic disintegration for 5 to 10 minutes with a Raytheon 10 kc. sonic oscillator; cell shearing in a motor-driven, all-glass Potter-Elvehjem homogenizer (Kontes Company); the application and

rapid release of a pressure greater than 1500 lb. per sq. in. of N₂ in a small stainless steel tank at room temperature (9); hand homogenization in an all-glass Tenbroeck homogenizer (Kontes Company); and, agitation in a Nossal shaker (10) for 90 seconds. The degree of homogenization was followed microscopically in all experiments.

Glycogen (Nutritional Biochemicals Company) was precipitated three times from ethanol before use. Dipotassium glucose 1-phosphate was obtained from Schwarz Laboratories. Glucose 1,6-diphosphate was a gift from Dr. Victor Ginsburg, crystalline glucagon from Dr. O. Behrens. Glucose 6-phosphate dehydrogenase was prepared from yeast (11). DL-Epinephrine bitartrate was obtained from Winthrop Laboratories; some experiments were performed with epinephrine chloride solution (1:1000) obtained from Parke, Davis and Company. Caffeine was obtained from the Eastman Chemical Company.

Glycogen was determined by the method of Stadie, *et al.* (12) and by the anthrone method (13). Phosphorylase was assayed routinely by the method of Sutherland and Wosilait (14). The Cori *et al.* phosphorylase assay (15) was used where stated. The method of Rall *et al.* (16) was slightly modified for dephosphophosphorylase activation experiments and details are presented with the experimental data. Phosphorylase inactivating enzyme was assayed by the technique of Wosilait and Sutherland (17). Protein was determined by the method of Bucher (18) with crystalline bovine albumin used as the standard. Purified preparations of dog liver phosphorylase, dephosphophosphorylase and dephosphophosphorylase kinase were the generous gift of Dr. Earl Sutherland. Histochemical assays were very kindly performed by Dr. Samuel Spicer.

RESULTS

Polysaccharide Content of Tumor Cells

The total polysaccharide content of freshly harvested tumors is given in Table II. The average concentration of polysaccharide in each tumor was approximately 5 μ moles of glucose equivalents per g of protein. This may be compared with normal rat liver containing approximately 1400 μ moles of glucose equivalents per g of protein. No glycogen was found in tumor cells even after incubating cells aerobically for one hour in Krebs-Ringer-bicarbonate buffer containing 10% glucose.¹

Since 3 to 8% of ascites tumor cell populations consisted of normal erythrocytes and leukocytes, it was of interest to determine whether polysaccharides were present in the normal cells, in the tumor cells, or distributed among both. Fresh suspensions of the Ehrlich carcinoma, hepatoma, and the Krebs-

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¹ J. F. Hogg and M. W. Nirenberg, unpublished results.

2 carcinoma were stained with the periodic acid-Schiff reagent (19). Glycogen granules were visible in the normal polymorphonuclear leukocytes contaminating the ascites tumor suspensions, and the glycogen could be removed by treatment with diastase. No glycogen could be demonstrated in the tumor cells. The tumor cells exhibited a light reddish, diffuse color after staining which did not disappear after diastase digestion. It is likely that the tumor cells contain very low levels of unidentified polysaccharides, probably mucopolysaccharides.

The chemical analyses for polysaccharide contained by the mast cell tumor revealed somewhat higher levels than the other tumor cells. Bright red granules were found in the mast cell tumor after periodic acid-Schiff staining. The granules did not disappear after diastase digestion; hence the polysaccharide was not glycogen. Since this tumor strain is known to synthesize heparin (20) it seemed likely that the granules were aggregates of heparin. This assumption was tested by the use of the azure A metachromatic stain, specific for acidic polysaccharides. The granules gave a positive reaction. Therefore, a close correlation was obtained between the chemical and the histochemical assays.

Phosphorylase Contents of Tumor Cells

The phosphorylase contents of the tumor cells are presented in Table III. Normal mouse liver contains an active phosphorylase, whereas the phosphorylase activity of the hepatoma, taken from the same animal was one-tenth to one-twentieth that of liver. Addition of 5'-AMP did not result in an increased activity. Similar results were obtained with all of the tumors. Five homogenization techniques were applied in the hope that an increased activity could be obtained. In each case whole homogenates were used. In additional experiments, whole homogenates were centrifuged at $100 \times g$ for 2 minutes to remove debris, and the supernatant suspensions were used. No significant changes in phosphorylase activity resulting from different methods of homogenization could be found.

Incubation of the reaction mixture for various intervals of time, up to 2 hours, had no effect upon phosphorylase activity. Assaying phosphorylase by the method of Cori *et al.* (15) did not result in an increased activity. Addition of $MnCl_2$ (10^{-3} M), ATP (10^{-3} M), $MgCl_2$ (2×10^{-3} M), and UTP (10^{-3} M) to separate reaction vessels also did not increase phosphorylase activity.

The pH optima of both liver and muscle phosphorylase lie between pH 6 and 7 (14, 21). The pH optimum of Ehrlich ascites tumor phosphorylase was 6.4 and was therefore similar to that of normal liver and muscle.

The phosphorylase assay was validated by demonstrating the stoichiometry of the reaction (Table IV). The appearance of a large amount of inorganic phosphate release from glucose 1-phosphate paralleled the net synthesis of glycogen in mouse liver and muscle. With Ehrlich ascites tumor homogenates, however, a small amount of phosphate was released from glucose 1-phosphate, but no synthesis of glycogen could be detected.

The phosphorylase reaction also was measured in the reverse direction, *i. e.*, from glycogen to glucose 1-phosphate by incubating tumor homogenates with glycogen and determining the disappearance of the glycogen (Table V). Mouse muscle and liver homogenates catalyzed the rapid disappearance of glycogen; the rate of the reaction in hepatoma homogenates was approximately one-tenth to one-twentieth that of liver. Essentially similar results were obtained with all tumor homogenates tested.

TABLE I
Tumor Strains

Tumor	Type	Host	Source
Ehrlich carcinoma.	Ascites	Swiss mouse	Dr. Arthur Schade
Krebs-2 carcinoma	Ascites	Swiss mouse	Dr. Mark Woods
Hepatoma-129-F (Reference 36) ..	Ascites	C ₃ H mouse	Dr. Morris Belkin
Lymphocytic leukemia-388-S.....	Ascites	BALB/c x dba mouse	Dr. Michael Potter
Plasma cell-70429 (Reference 37) ..	Ascites	C ₃ H mouse	Dr. Michael Potter
Mast cell-815 (Reference 38) ..	Ascites	BALB/c x dba mouse	Dr. Michael Potter
Sarcoma-37.....	Ascites	CFW mouse	Drs. Peter Eck, Margaret Ogara Dr. Harry Eagle
HeLa carcinoma..	Tissue culture		Dr. Harry Eagle
Rous sarcoma.....	Solid	Chicken	Dr. W. Bryan

TABLE II
Total Polysaccharide Content of Tumor Cells

Tissue	Total polysaccharide μ moles glucose equivalents/g protein
Hepatoma ascites.....	4.1
Ehrlich carcinoma ascites.....	0.48
Lymphocytic leukemia ascites.....	6.1
Plasma cell ascites.....	5.2
Mast cell ascites.....	13.0
Krebs-2 carcinoma ascites.....	1.2
Sarcoma-37 ascites.....	1.2
HeLa carcinoma (tissue culture).....	5.3

TABLE III

Phosphorylase activity of tumor and normal tissue homogenates

Tumor cells and liver slices were washed once with 0.9% NaCl and were homogenized at 5° in an all-glass Potter-Elvehjem homogenizer (Kontes Glass Company). The concentration of 5'-AMP was 2 μ moles/ml reaction mixture, when present. Phosphorylase was assayed by the method of Sutherland and Wosilait (14). In some cases the results were checked by the assay of Cori *et al.* (15).

Tissue	$\Delta\mu$ moles P _i /10 min./mg protein	
	-AMP	+AMP
Mouse liver (normal).....	1.57	1.56
Hepatoma ascites.....	0.125	0.0990
Ehrlich carcinoma ascites.....	0.081	0.0845
Lymphocytic leukemia ascites....	0.114	0.116
Plasma cell ascites.....		0.0993
Mast cell ascites.....	0.0613	0.0674
Krebs-2 carcinoma ascites.....	0.144	0.135
Sarcoma-37 ascites.....	0.140	0.134
HeLa carcinoma (tissue culture)..	0.116	0.129
Chicken muscle (normal).....	1.63	7.23
Rous sarcoma.....	0.165	0.204

glycogen, and 0.081 μ mole P_i released from glucose 1-phosphate. Separate Ehrlich ascites tumor homogenates were prepared for each analysis, yet the results of the three different types of assays essentially agree with each other. It seems reasonable to conclude that Ehrlich ascites tumor homogenates have 3 to 10% of the phosphorylase activity of normal liver or muscle and that most or all of the observed activity is derived from normal leukocytes present in the ascites suspensions.

Ehrlich ascites tumor homogenates can utilize glucose 1-phosphate. Supplementation with glucose 1,6-diphosphate and cysteine was necessary for optimal phosphoglucomutase activity; both were routinely added for phosphorylase assays. The results of Fig. 1 demonstrate that phosphoglucomutase is present and is not rate limiting in glycogen degradation by Ehrlich ascites tumor homogenates. Phosphoglucomutase has also been demonstrated in the Novikoff hepatoma (23).

Phosphorylase Activity of Tumor of Viral Origin

The phosphorylase content of a virus-induced tumor, the Rous sarcoma, and normal chicken muscle are presented in Table III. Both samples of tissue were removed from the same animal. The Rous sarcoma was a solid tumor and was not studied as completely as the other tumors, but nonetheless low phosphorylase levels, comparable to the ascites tumors and the HeLa carcinoma, were found.

Activation of Phosphorylase by Epinephrine and Glucagon

Since epinephrine and glucagon are involved in the activation of phosphorylase, it seemed logical to determine whether either hormone could facilitate the activation of phosphorylase in hepatoma and Ehrlich ascites tumor cells. The addition of 50 μ g/ml of epinephrine and 25 μ g/ml of glucagon to mouse liver slices resulted in a marked and rapid reactivation of phosphorylase. Additions of epinephrine and glucagon to hepatoma and epinephrine to Ehrlich ascites tumor cells had no effect upon phosphorylase activation. These data demonstrate the absence of this hormonally controlled enzymatic response in these tumors.

Studies with Phosphorylase Activating System

Since the tumor cells had negligible phosphorylase activities and were not responsive to epinephrine and glucagon, it was of interest to determine whether the phosphorylase activating enzymes were present. The data of Table VI demonstrate that HeLa carcinoma, hepatoma, and Ehrlich carcinoma homogenates can convert dephosphophosphorylase to phosphorylase. Addition of dephosphophosphorylase kinase had no effect upon phosphorylase activation in HeLa and Ehrlich carcinoma homogenates, but increased the phosphorylase activity of the hepatoma homogenate. The conversion of dephosphophosphorylase to phosphorylase was not proportional to the amount of homogenate added, possibly due to the involvement of adenosine-3'-5'-cyclic phosphate in the over-all reaction. The activation of phosphorylase was dependent upon the presence of ATP. The results of Table VI demonstrate that the tumors possess a vigorous dephosphophosphorylase-activating system and a relative absence of dephosphophosphorylase.

Phosphorylase Inactivating Enzyme

The question may be asked, "Does the Ehrlich ascites tumor have a high phosphorylase inactivating enzyme activity?" The

TABLE VI

Activation of Dephosphophosphorylase by HeLa, Hepatoma and Ehrlich Homogenates

Reaction mixtures contained 4 μ moles of tris(hydroxymethyl)aminomethane, pH 7.4, 0.34 μ mole of ATP, 0.5 μ mole of $MgSO_4$, 0.039 μ mole of epinephrine Cl_2 , whole homogenate, and where indicated, dog liver dephosphophosphorylase and dog liver dephosphophosphorylase kinase. 0.05 ml of the HeLa and hepatoma homogenates contained 2.37 and 1.90 mg of protein respectively. 0.15 ml of Ehrlich homogenate contained 9.04 mg of protein. Total volume was 0.2 ml. Reaction mixtures were incubated at 30° for 5 minutes. 1 ml of the phosphorylase reagent (14) containing 2.0 μ moles 5'-AMP was then added, and the tubes were incubated at 37° for 20 minutes. Samples were deproteinized by trichloroacetic acid precipitation at 0 and 20 minutes.

No.	Homogenate	Addition	HeLa carcinoma	Hepatoma	Ehrlich carcinoma
			$\Delta \mu$ moles P_i /20 min.		
1	0.05	None	0.190	0.033	
2	0.05	+ Dephosphophosphorylase	6.48	0.850	
3	0.10	None	0.346	0.549	
4	0.10	+ Dephosphophosphorylase	15.7	11.7	
5	0.15	None	0.768	1.19	1.68
6	0.15	+ Dephosphophosphorylase	19.7	15.8	13.2
7	0.15	+ Dephosphophosphorylase + Dephosphophosphorylase kinase	20.9	28.0	12.3
8	0.15	+ Dephosphophosphorylase - ATP	3.96	2.01	
9	0.15	- Dephosphophosphorylase + Dephosphophosphorylase kinase			1.90
10	0.15	- Homogenate + dephosphophosphorylase + dephosphophosphorylase kinase			0

conditions for assaying liver phosphorylase inactivating enzyme have been described (17). Purified preparations of dog liver phosphorylase were added to homogenates of normal mouse liver and Ehrlich tumor cells and the disappearance of phosphorylase activity was measured at 10 and 20 minutes. The specific activity of the phosphorylase inactivating enzyme in homogenates of dog liver is reported to be 1.2 to 1.6 (17). Under identical conditions the specific activity of this enzyme in mouse liver was 0.04 and in Ehrlich ascites tumor, 0.02. The low value obtained in mouse liver, as compared to dog liver possibly may be a species difference. These experiments demonstrate that mouse liver can inactivate phosphorylase at approximately twice the rate of Ehrlich ascites tumor. The possibility that the tumor extract contains a powerful inhibitor of phosphorylase can therefore be excluded.

DISCUSSION

Tumors exhibit such diversity in form and type that it would seem highly unlikely to expect a relative absence of phosphorylase in all tumors. The results obtained with ascites tumors should not be extrapolated to other types of tumors. Phosphorylase has been demonstrated in two types of solid tumors (24); however, considerably decreased phosphorylase levels have also been attributed to a solid hepatoma (25). Since solid tumors contain

variable numbers of normal cells such as connective tissue, ascites tumors were used primarily in this study. One advantage of ascites tumors is the relative purity of cell type which can be obtained.

Glycogen synthesis has been shown to proceed in a variety of tissues by Leloir and others (26-29) by an irreversible UDP glucose transferase reaction. This, rather than phosphorylase, may be the main route of glycogen synthesis. Dr. R. Wu² has found UDP-glucose transferase in HeLa cells. Under certain growth conditions the cells can accumulate glycogen; then low levels of phosphorylase, about 1% that of an equivalent amount of muscle, can be demonstrated. Since HeLa carcinoma homogenates can rapidly reactivate added dephosphophosphorylase (Table VI), the rate-limiting factor appears to be the availability of dephosphophosphorylase. UDP-glucose transferase has not been looked for in ascites tumors. Although no stored glycogen can be found in these tumor cells, the absence of glycogen need not always go hand in hand with the absence of phosphorylase. The possibility exists that some cells contain UDP-glucose transferase but lack phosphorylase. This situation might result in a marked accumulation of glycogen.

Rat muscle and brain have little phosphorylase at birth, and after approximately 10 days the phosphorylase activities rise to adult levels (30). The phosphorylase content of rat liver 1 day after term also is greatly reduced.³ Fetal guinea pig liver, however, contains adult quantities of phosphorylase (31). Some, but not all embryonic tissues, therefore, have greatly reduced phosphorylase levels when compared to the corresponding adult tissues.

Although both glycogen and phosphorylase are present in almost all adult mammalian tissues, every cell type need not contain these substances. Histochemical studies have demonstrated, for example, the uneven distribution of phosphorylase activity among different cell types of a given tissue (22). Preliminary work with a virus-induced tumor, the Rous sarcoma, has revealed remarkably low phosphorylase levels when compared with normal chicken muscle taken from the same animal. Although this tumor is a sarcoma, chicken muscle may not be an adequate control, for the Rous sarcoma can arise from infected avian fibroblasts (32). Although no answer is available, the question should be raised, "Do certain types of normal cells such as fibroblasts also have low phosphorylase levels, and, if so, are ascites tumors derived primarily from these cell types?"

The breakdown of glycogen in both liver and muscle is clearly regulated by a complex hormonal mechanism. The extremely rapid interconversion of dephosphophosphorylase and phosphorylase in resting *versus* contracting muscle has been emphasized (33), and it is possible that the activation and deactivation of phosphorylase controls the release of distinct waves of glucose-1-phosphate which can be converted quickly to lactate either with the concomitant production of pulses of ATP, if it is metabolized via the Embden-Meyerhof pathway, or with the production of waves of TPNH if it is metabolized via the hexose monophosphate shunt. Although ascites tumor cells have exceedingly high rates of carbohydrate metabolism, they lack to a large extent this hormonal regulatory mechanism. The suggestion has been made (34) that some tumors become insensitive to certain controlling forces, such as hormonal regulation, through loss or inhibition of particular enzyme pathways. Transhydro-

genase is present in normal liver but has not been found in a number of ascites tumors (35), including a hepatoma. Addition of epinephrine and glucagon to hepatoma cells in this study did not result in an increased level of phosphorylase, possibly because of the low amount of dephosphophosphorylase available.

Since it is always difficult to validate a negative finding, such as the absence of an enzyme, an attempt has been made to investigate thoroughly the parameters of the phosphorylase assay. It seems reasonable to conclude that the phosphorylase contents of the ascites tumors studied, such as the hepatoma, are very low when compared with normal liver or muscle. It is not possible with the methods available to ascribe a total absence of phosphorylase to ascites tumors. It should be noted that the phosphorylase levels herein ascribed to ascites tumors undoubtedly represent maximal values, for histological examination revealed high phosphorylase activity in normal leukocytes also present in ascites suspensions. No phosphorylase whatsoever could be detected histologically in the tumor cells.

It seems striking that these tumors, utilizing monosaccharides at such rapid rates, should be relatively unable to degrade glycogen. In normal cells glycogen appears to serve the cell as a hormonally controlled reservoir, or buffer, for "energy" and substrates. Clearly, the tumor cells studied neither possess a carbohydrate reserve nor have, to any appreciable extent, the control mechanism which may release pulses of intracellular glucose 1-phosphate. It is not known what effect this may have upon the metabolism and economy of these neoplastic cells.

SUMMARY

The glycogen phosphorylase activities of seven types of ascites tumors, a tumor grown in tissue culture, and a solid virus-induced tumor were determined by chemical and histochemical techniques. Negligible phosphorylase activities were found compared to normal control tissues. Although glycogen is present in some neoplastic cells, little or no glycogen could be found in the ascites tumors. The phosphorylase activating enzymes of three tumors were studied. All contained ATP-dependent phosphorylase activating enzymes but had negligible amounts of dephosphophosphorylase. No epinephrine or glucagon-induced activation of phosphorylase was observed.

It is not known whether this enzymatic defect in ascites tumors can be extrapolated to other types of tumors. The relative absence of a hormonal mechanism regulating stored carbohydrate utilization in ascites tumors was discussed and was held in contrast to the rapid degradation of monosaccharides by these tumors.

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