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Hexose Transport in Ascites Tumor Cells[1]

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RECEIVED APRIL 3, 1958

The competitive inhibition of hexose utilization in ascites tumor cells by the non-utilizable hexose, galactose, has been ascribed to competition for a binding site which appears to be a functional part of the transport mechanism. Galactose inhibited neither hexose utilization by homogenates nor hexokinase action in extracts of the ascites tumors. In Gardner cells where hexose transport was shown to limit the rate of glycolysis, kinetic analysis served to identify the galactose-sensitive reaction with a step by which the sugar gains access to the glycolytic enzymes. The relative affinities for the binding site are in the order: glucose > galactose = fructose. Several steroid compounds also were found to inhibit hexose utilization in a manner resembling in several respects the galactose inhibition.

Although chemically mediated transport of monosaccharides is shown more easily for complex systems such as the intestinal and renal tubular walls, sugars apparently enter many cells by processes which are at least in some respects similar. The studies of LeFevre, Wilbrandt and others[5-8] have shown that sugar entry into human erythrocytes apparently involves a reversible combination of the sugar with a membrane constituent. This conclusion has been extended to foetal erythrocytes of some other mammals[8] and to yeast[9,10] and bacteria.[11] Evidence for a comparable mode of hexose entry into Ehrlich ascites tumor cells was presented in a preliminary communication.[3] More recently, Crane, *et al.*,[12] have verified and extended these results by means of a tracer technique.

Although sugar transfer often has been supposed to depend upon phosphorylation, the insulin-stimulated step in sugar uptake appears to precede such reaction.[13-17] Other reasons for doubting that the transfer reaction is a phosphorylation are a poor correlation for the intestine between the transfer rates and phosphorylation rates of sugars[18] and the concentrative transfer of 1-deoxy- and 6-deoxy-D-glucose from an intestinal pouch.[19]

(1) Portions of this work have been the subject of a preliminary communication[3] and have been presented before the meeting of the American Society of Biological Chemists, Chicago, April, 1957.[4] This material was taken in part from a doctoral dissertation submitted to the Horace H. Rackham School of Graduate Studies by Marshall W. Nirenberg in 1957. The investigation was supported in part by American Cancer Society Institutional Grants Nos. 22C and 22D and by the Michigan Memorial-Phoenix Proejct No. 45.

(2) To whom inquiries should be directed.

(3) M. W. Nirenberg and J. F. Hogg, THIS JOURNAL, **78**, 6210 (1956).

(4) M. W. Nirenberg and J. F. Hogg, *Federation Proc.*, **16**, 227 (1957).

(5) P. G. LeFevre, *Symp. Soc. Expt. Biol.*, **8**, 118 (1954).

(6) W. Wilbrandt, *ibid.*, 136 (1954).

(7) D. Reinwein, C. F. Kalman and C. R. Park, *Federation Proc.*, **16**, 237 (1957).

(8) F. Bowyer and W. F. Widdas, *Disc. Faraday Soc.*, **21**, 251 (1956).

(9) A. Rothstein, *Symp. Soc. Expt. Biol.*, **8**, 165 (1954).

(10) E. R. Blakely and P. D. Boyer, *Biochem. Biophys. Acta*, **16**, 576 (1955).

(11) J. Monod, in "Enzymes: Units of Biological Structure and Function," Academic Press, Inc., New York, N. Y., 1956, p. 7.

(12) R. K. Crane, R. A. Field and C. F. Cori, *J. Biol. Chem.*, **224**, 649 (1957).

(13) R. Levine and M. S. Goldstein, *Rec. Prog. Hormone Res.*, **11**, 343 (1955).

(14) A. E. Renold, A. B. Hastings, F. B. Nesbett and J. Ashmore, *J. Biol. Chem.*, **213**, 135 (1955).

(15) C. R. Park, *J. Clin. Invest.*, **32**, 593 (1953).

(16) C. R. Park and L. H. Johnson, *Am. J. Physiology*, **182**, 17 (1955).

(17) C. R. Park, J. Bornstein and R. L. Post, *ibid.*, **182**, 12 (1955).

(18) A. Sols, *Rev. espan. fisiol.*, **11**, 277 (1955).

(19) R. K. Crane and S. M. Krane, *Biochem. Biophys. Acta*, **20**, 568 (1956).

Our investigation[3] on the mode of hexose uptake in the Ehrlich ascites tumor has been augmented and comparable results have been obtained with the Gardner tumor which, however, showed a specific dependence of glycolytic rate on hexose concentration for a wide range of values. Furthermore, certain steroids have been found to exert a selective inhibitory action upon fructolysis.

Experimental

Materials and Procedures.—Both the Ehrlich carcinoma and Gardner lymphosarcoma ascites tumors[20] were obtained from Dr. T. Hauschka.[21] The Ehrlich tumor (clone 2) was carried in unselected white Swiss mice; the Gardner (6C3HED) in C3H mice obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine. At 7 to 9 days after the intraperitoneal injection of 0.2 ml. of ascites tumor, the ascites tumors were aspirated into a heparinized syringe, centrifuged 5 min. at 400 × *g*, the fluid decanted and the contaminating erythrocytes removed by aspiration with a capillary pipet. The cells were then washed twice with Krebs–Ringer bicarbonate solution,[22] erythrocytes being removed after each centrifugation.

Quantitative homogenization was obtained by subjecting a 33% suspension of cells in isotonic KCl[23] to the application and rapid release of a pressure of greater than 1500 lb./sq. in. of N_2 in a small stainless steel tank at room temperature.[24] Initially, for comparison, homogenates also were prepared in a Brendler homogenizer[25] at 5°. Soluble tumor hexokinase preparations were obtained by centrifuging the homogenates at 20,000 × *g* for 30 min. at 5°. Hexokinase was found in the aqueous supernatant fluid; the precipitate was almost devoid of hexokinase activity.

Anaerobic glycolysis was measured in a Warburg apparatus shaken at 37° with an atmosphere of 95% N_2–5% CO_2.[22] Tumor cells were suspended in Krebs–Ringer bicarbonate buffer[22] at *p*H 7.45; tumor homogenates in a medium, modified from that of LePage[23] (Table I), which held the *p*H at 7.4 under an atmosphere of 95% N_2–5% CO_2. Intact cells maintained linear rates for at least 90 min.; homogenates for 40 min.

Glucose[26] and fructose were Pfanstiehl reagents. Glucose-free galactose was kindly furnished by Dr. A. Campbell. The galactose had been freed of glucose by allowing two successive lots of washed cells of baker's yeast to ferment any glucose present. Additional purification was accomplished by twice-repeated treatment of a 10% solution with 30 g./l. of Norite. Using the hexokinase assay described below with yeast hexokinase, the purified galactose contained less than 3 μg. of glucose in 80 mg. The steroids tested were commercial preparations (Ciba or Schering) kindly supplied

(20) G. Klein, *Expt. Cell Res.*, **2**, 518 (1951).

(21) T. S. Hauschka, *Trans. N. Y. Acad. Sci.*, **16**, 64 (1953).

(22) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques and Tissue Metabolism," Burgess Publishing Co., Minneapolis, Minn., 1949.

(23) G. A. LePage, *J. Biol. Chem.*, **176**, 1009 (1948).

(24) C. S. French and H. Milner, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," New York, N. Y., **1**, 64 (1955).

(25) H. Brendler, *Science*, **114**, 61 (1951).

(26) All carbohydrates and their derivatives had the D-configuration unless otherwise stated.

by Dr. Burton L. Baker; nystatin (Squibb Mycostatin) by Dr. J. O. Lampen.

Analytical Methods.—Sugars were measured in samples deproteinized with $Ba(OH)_2$ and $ZnSO_4$.[27] Reducing sugar was determined by the colorimetric method of Somogyi[28] and fructose by the method of Roe.[29] A correction proportional to the galactose level was applied for the latter method. Hexokinase activity was measured by a direct spectrophotometric assay[30,31] with a reaction mixture containing in a total volume of 3.0 ml. the following: hexose substrate, 100 μmoles of tris-(hydroxymethyl)-aminomethane buffer (*p*H 8.0), 5 μmoles of disodium adenosine triphosphate (ATP) (Schwarz), 10 μmoles of $MgCl_2$, 1.2 μmoles of triphosphopyridine nucleotide (TPN) (Sigma), 5 μmoles of KF, 0.2 K units of glucose 6-phosphate dehydrogenase (Sigma), 2.5 mg. of crude phosphohexoisomerase (Sigma) (omitted for tumor extracts), and sufficient tumor hexokinase preparation (0.05–0.10 ml.) to give the required reaction rate. The formation of reduced TPN (TPNH) was measured by the absorption at 340 mμ in a model DU Beckman spectrophotometer using a photomultiplier attachment. Both the glucose 6-phosphate dehydrogenase and the phosphohexoisomerase were found to be free of hexokinase. Of the reactants, only hexokinase was at a rate-limiting level. When the concentration of fructose was made limiting (0.1–1.0 μmoles per 3 ml.), the rate of TPNH formation was proportional to the concentration of fructose. The tumor extracts apparently contained phosphogluconate dehydrogenase since the ratio, TPNH production/fructose consumption, was approximately 2.

Results and Conclusions

Ehrlich Carcinoma Ascites Tumor.—In *intact* cells glucolysis and fructolysis proceeded at approximately equal rates (Table I). Raising the amount of glucose or fructose in the Warburg vessel from 11 to 278 μmoles did not increase significantly the rate of glycolysis.[32] Lesser amounts precluded accurate manometric measurements of rate. Galactose was not metabolized, since it did not serve as a glycolytic substrate and, when incubated with the tumor cells, did not disappear progressively from the medium.[3] Although galactose had no effect upon glucolysis (Table I), it markedly inhibited fructolysis in intact cells. The inhibition of fructolysis was completely reversed by additional fructose. Plotting results for fructolysis according to Lineweaver and Burk[33] (Fig. 1) showed that the galactose inhibition was competitive, thus indicating a common binding site for galactose and fructose.

Such a common binding site was indetectible in homogenates; here galactose failed to inhibit fructolysis. Glucolysis and fructolysis proceeded at about equal rates, these being equally high in homogenates and intact cells (Table I). The manometric results were confirmed by simultaneously determining the disappearance of fructose from the medium (Table II). Both methods of analysis demonstrated clearly that galactose inhibited fructolysis in intact cells but not in homogenates. The hexokinase step was examined more critically by another technique, namely, a spectrophotometric assay of hexokinase activity. Both glucose and fructose, at the same concentrations used for measurements of glycolysis were found to be phosphorylated at equal rates and levels of galactose high enough to inhibit fructolysis by 80% or more in a cellular system did not affect the hexokinase action even when the fructose concentration was rate-limiting (Fig. 2). Therefore galactose did not inhibit fructolysis by competing for hexokinase.

TABLE I

EFFECT OF SUBSTRATE, CONCENTRATION AND GALACTOSE UPON ANAEROBIC GLYCOLYSIS IN EHRLICH ASCITES TUMOR

Additions per vessel. μmoles	CO_2/mg. dry wt./hr. μl.
Intact cells	
13.9 glucose	24.5
13.9 fructose	26.7
13.9 galactose	0
444.0 glucose	29.0
8.7 glucose + 444.0 galactose	29.1
16.7 fructose	25.3
16.7 fructose + 222.0 galactose	8.7
16.7 fructose + 444.0 galactose	4.8
27.8 fructose + 220.0 galactose	15.3
55.6 fructose + 220.0 galactose	26.7
Homogenates[a]	
16.7 glucose	24.7
16.7 fructose	24.6
16.7 fructose + 444 galactose	24.0
444.0 galactose	1.5

[a] The medium consisted of: 0.0024 *M* K_2HPO_4, 0.025 *M* $KHCO_3$, 0.04 *M* nicotinamide (Merck), 0.002 *M* disodium adenosine triphosphate (Schwarz), 0.004 *M* diphosphopyridine nucleotide (Pabst), 0.0005 *M* potassium fructose 1,6-diphosphate (Schwarz), 0.0005 *M* sodium pyruvate (Schwarz) and 0.01 *M* $MgCl_2$. Rates of glycolysis in the homogenates were corrected for a blank glycolysis of approximately 10 μl. CO_2/mg. dry wt. homogenate/hr. due to the fructose 1,6-diphosphate in the medium. Therefore the figures given represent the complete Embden–Meyerhof pathway of glycolysis starting from glucose or fructose.

TABLE II

COMPARISON BETWEEN MANOMETRIC DETERMINATIONS OF ANAEROBIC FRUCTOLYSIS AND MEASUREMENTS OF FRUCTOSE DISAPPEARANCE FROM THE MEDIUM IN EHRLICH ASCITES TUMOR CELLS AND HOMOGENATES

Substrate, μmoles	Fructose disappearance, μmoles	Extra CO_2 produced, μl.	Theoretical fructose disappearance,[a] μmoles
Intact cells			
16.7 fructose	7.2	296	6.6
16.7 fructose + 139 galactose	4.3	135	3.0
Homogenates			
11.1 fructose	5.6	204	4.6
11.1 fructose + 220 galactose	5.6	190	4.3

[a] The theoretical fructose disappearance was calculated from the manometric data by assuming that one μmole of fructose yields 2 μmoles of lactic acid and therefore should produce 44.8 μl. of CO_2.

An alternative to concluding that galactose and fructose compete for a common site is the possibility that *intact tumor cells* but not *homogenates*

(27) M. Somogyi, *J. Biol. Chem.*, **160**, 62 (1945).
(28) M. Somogyi, *ibid.*, **195**, 19 (1952).
(29) J. H. Roe, *ibid.*, **107**, 15 (1934).
(30) M. W. Slein, G. T. Cori and C. F. Cori, *ibid.*, **186**, 763 (1950).
(31) R. K. Crane and A. Sols, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," New York, N. Y., **1**, 277 (1955).
(32) In two experiments, the rate of *fructolysis* increased approximately 25% between 10 and 40 μmoles. Apparently a concentration of 10 μmoles per vessel just saturates the Ehrlich cells and a small alteration of cellular condition may result in a very limited concentration dependency.
(33) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

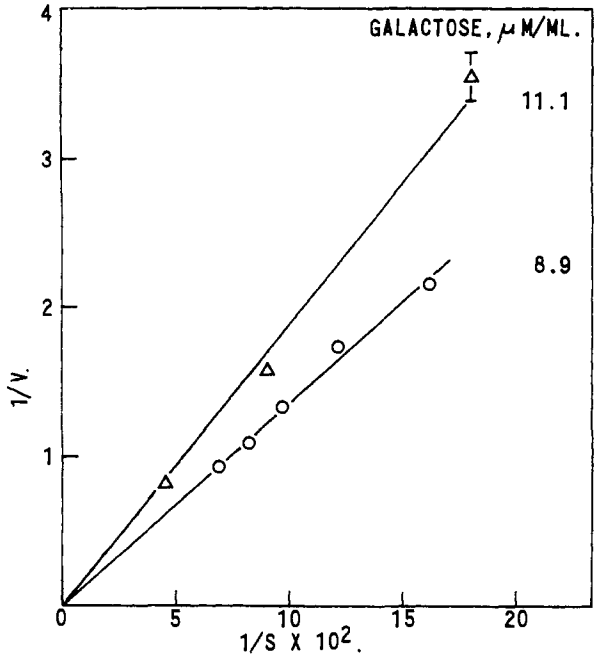

Fig. 1.—A Lineweaver–Burk plot of galactose inhibition of fructolysis in Ehrlich ascites tumor cells. The substrate concentration is expressed as μmoles per ml. and the velocity is expressed as μmoles CO_2 produced per mg. dry weight of tumor cells per hr.

produce a glycolytic inhibitor in the presence of galactose. This was tested by incubating cells for 45 min. with a mixture of galactose and fructose (ratio of 300/1). The cells should then have contained the supposed inhibitor. After centrifugation the cells were homogenized and lyophilized. A suspension of tumor cells incubated without galactose was treated in the same way. The rate of fructolysis by a fresh homogenate was then determined after adding either the galactose-treated or the control homogenate. Since the experiments yielded 31 and 30 μl. CO_2/mg./30 min., respectively, the galactose-treated cells did not contain a glycolytic inhibitor. Therefore we must return to the concept of a common binding site for the two sugars. Although one would anticipate this site to be on an enzyme, galactose inhibited neither fructolysis in homogenates nor phosphorylation in extracts of the cells. These results then suggest that in cell suspensions an unknown initial reaction must occur before the sugars can enter glycolysis. Plausibly this reaction may participate in the transport of fructose into the cell.

Gardner Lymphosarcoma Ascites Tumor.—Here again galactose was not glycolyzed whereas both fructose and glucose served as glycolytic substrates (Table III). In these cells, as in the Ehrlich cells, galactose inhibited fructolysis and again galactose was inhibiting competitively (Fig. 3). At very high galactose/glucose ratios (200/1), however, an inhibition of *glucolysis* also was observed with these cells, the inhibition being fully reversed by additional glucose. Although glucolysis inhibition was not obtained in Ehrlich cells at the highest ratios experimentally feasible, competition between glucose and galactose for entry has been shown re-

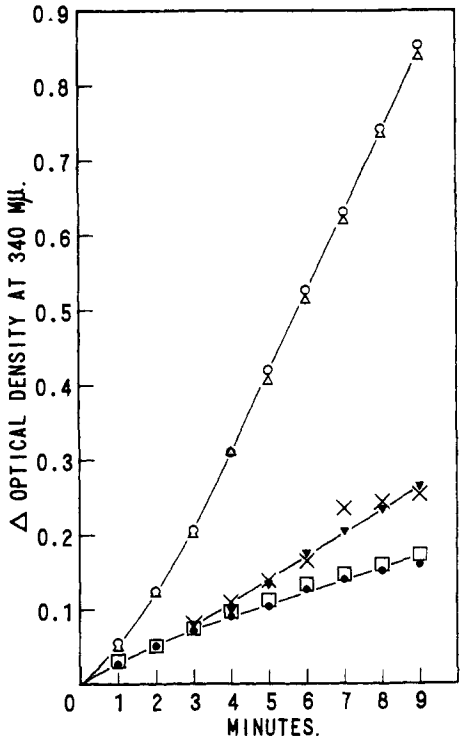

Fig. 2.—The effect of galactose upon phosphorylation of fructose by Ehrlich ascites tumor hexokinase: ⊡, no substrate; ●, 355 μmoles galactose; ▼, 1 μmole fructose; ×, 1 μmole fructose + 125 μmoles galactose; ◬, 11 μmoles fructose or glucose; ⊙, 11 μmoles fructose + 355 μmoles galactose.

cently by means of a different technique.[13] With Gardner cell *homogenates*, the results agreed in all respects with the results for Ehrlich cell homogenates, including Fig. 2. Therefore the Gardner cells seem also to contain the reaction preceding glycolysis.

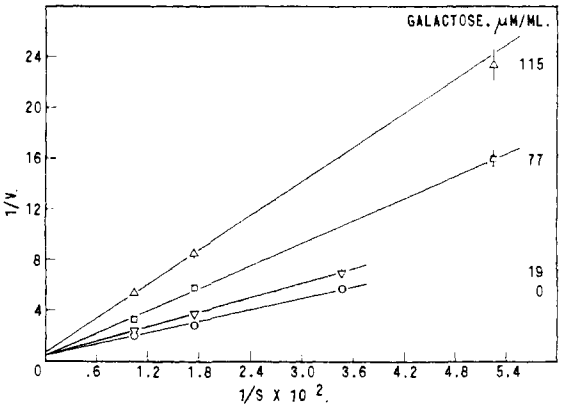

Fig. 3.—Lineweaver-Burk plot of galactose inhibition of fructolysis in Gardner lymphosarcoma ascites tumor cells. The substrate concentration is expressed as μmoles per ml. and the velocity as μmoles CO_2 produced per mg. dry weight of tumor cells per hr.

A distinct and significant feature in the Gardner cell, however, is the dependence of the rate of CO_2-production upon the level of fructose or glucose

taken, although glycolysis was always more rapid with the latter sugar (Table III). This concentration dependency, as well as the difference in rate with the two sugars, disappeared upon homogenization, both rates being substantially increased (Table III). The lack of concentration dependency in tumor homogenates has been observed previously by LePage.[34] Although the acceleration of glucolysis upon homogenization possibly could be explained by an increased availability of coenzymes or phosphate,[35] this can hardly be true for the much greater acceleration (30-fold) of fructolysis. This acceleration upon homogenization means that a barrier has been removed. The barrier, however, is the necessity that the sugar pass through a reaction, one in which galactose competes and for which the sugars have affinities in the order: glucose > galactose = fructose. The disappearance of the substrate specificity and concentration dependency upon homogenization means that this otherwise necessary initial reaction is associated with cellular integrity and is rate-limiting to sugar utilization by the intact cell. These features of the initial reaction appear to describe a transport reaction.

The same conclusion may be drawn independently from the results of kinetic studies with the intact cells alone (Figs. 3 and 4). The line de-

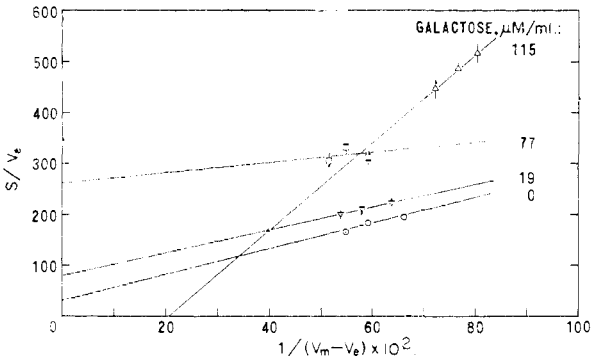

Fig. 4.—A Blum–Jenden plot of galactose inhibition of fructolysis in Gardner lymphosarcoma ascites tumor cells. Values of V_m were taken from Fig. 3.

scribing increasing fructose catabolism with the rising fructose level (Fig. 3, lowest line) has the same intercept on the reciprocal velocity axis as do the three lines measuring catabolic rate at three levels of the competitive inhibitor, galactose. This identifies the rate-limiting step in fructose utilization with the galactose-inhibited step.

Furthermore, with the determination of reciprocal maximal velocity by these plots, the equation of Blum and Jenden[36] for rate behavior in geometrically constrained enzyme systems may now be applied. The form of the equation for this case is

$$S/V_e = 1/\bar{K}(V_m - V_e) + r_0/3H$$

TABLE III

EFFECT OF SUBSTRATE, CONCENTRATION AND OF GALACTOSE UPON ANAEROBIC GLYCOLYSIS IN THE GARDNER ASCITES TUMOR

Additions per vessel, μmoles	CO_2/mg. dry wt./hr. μl.
Intact cells	
16.7 glucose	14.0
16.7 fructose	0.75
16.7 galactose	0
5.5 glucose	9.32
55.5 glucose	15.2
278 glucose	21.3
5.5 glucose + 1100 galactose	5.28
55.5 glucose + 1100 galactose	14.6
55.5 fructose	3.30
167 fructose	8.20
278 fructose	10.5
55.5 fructose + 55.5 galactose	1.60
Homogenates (see Table I for medium used)	
16.7 glucose	21.6
16.7 fructose	22.8
333 galactose	0
55.5 glucose	20.9
278 glucose	17.7
55.5 fructose	25.5
278 fructose	24.1
11.1 fructose	27.1
11.1 fructose + 333 galactose	25.8

where S is the concentration of substrate; V_e, the experimental velocity at S; V_m, the maximal experimental velocity for the system; $\bar{K}$, the reciprocal Michaelis–Menten constant for the one intracellular enzyme which is responsible for the observed rate (presumably hexokinase in this case); r_0, the cell radius and H is the permeability constant of the cell. In this form of the equation the diffusion constant within the cells is assumed to be comparable to that for the substrate in aqueous solution. Plotting S/V_e *vs.* $1/(V_m - V_e)$, the intercept on the S/V_e axis (Fig. 4) where $1/(V_m - V_e)$ equals 0 gives the value of $r_0/3H$. Therefore the increasing value of this intercept with rising galactose level supports the conclusion that galactose reduced the permeability of the cell to fructose (see Addendum).

Other Inhibitors of Tumor Glycolysis.—The inhibitory action of 2-deoxy-D-glucose is considered elsewhere.[37] In contrast to yeast,[38] the tumor glycolysis was unchanged in the presence of the antibiotic, nystatin. As with erythrocytes,[39] insulin had no measurable effect upon hexose uptake in the Gardner cell or, in agreement with Crane, *et al.*,[12] in the Ehrlich cell. Certain steroid compounds, however, were effective inhibitors of fructolysis, but not of glucolysis, in the ascites tumor cells (Table IV).[40] Although the substrate speci-

(34) G. A. LePage, *Cancer Res.*, **10**, 77 (1950).

(35) Other measurements have shown that the amount of homogenate is the rate-limiting factor in our medium. Enzyme losses in homogenization, however, will minimize the acceleration from removal of a barrier to entry.

(36) J. J. Blum and D. J. Jenden, *Arch. Biochem. Biophys.*, **66**, 316 (1957).

(37) M. Nirenberg and J. F. Hogg, *Cancer Res.*, in press.

(38) J. O. Lampen, E. R. Morgan and P. C. Slocum, *Federation Proc.*, **15**, No. 1, 963 (1956).

(39) W. Wilbrandt, E. Guensberg and H. Lauener, *Helv. Physiol. et pharmacol. Acta*, **5**, C20 (1947).

(40) The selective inhibition of fructolysis by steroids has been verified for the melanoma S-91 (solid) and Krebs-2 (ascites) tumor by Wood and Burk ("Pigment Cell Biology," Academic Press, Inc., New York, N. Y., 1958. (Report of the Pigment Cell Conference,

ficity, the reversal of inhibition by increased fructose concentration and the loss of inhibition on homogenization resemble closely the galactose inhibition, the equal effectiveness of testosterone (and deoxycorticosterone) with either tumor is a marked contrast to the results for galactose. This difference means either that steroids and galactose act at different sites on the transport mechanism or, less probably, that steroids do not affect the cellular permeability. Steroids of quite diverse hormonal activities were found to inhibit tumor fructolysis. This finding, however, does not rule out the possibility that the testosterone inhibition of fructolysis is the mode of control of fructose secretion in the male accessory glands by this hormone.[41]

TABLE IV

EFFECT OF STEROIDS AND OTHER COMPOUNDS ON ANAEROBIC GLYCOLYSIS IN ASCITES TUMORS[a]

Test compound[b]	μg./ml.	Substrate	μM/ml.	CO_2/mg. dry wt./hr. μl.
Gardner cells				
None		Glucose	4.4	25
Testosterone	15	Glucose	4.4	22
None		Fructose	44.0	7.7
Testosterone	15	Fructose	44.0	4.3
Testosterone	1.5	Fructose	44.0	6.7
Deoxycorticosterone	15	Fructose	44.0	4.5
Ehrlich cells				
None		Glucose	6.7	33
Testosterone[c]	20	Glucose	6.7	32
None		Fructose	6.7	33
Testosterone[d]	20	Fructose	6.7	9.5
Testosterone	20	Fructose	67.0	32
Testosterone	5	Fructose	6.7	25
$\Delta^{1,4}$-Androstene-3,17-dione	20	Fructose	6.7	22
Δ^{4}-Androstene-3,17-dione	20	Fructose	6.7	16.0
Diethylstilbestrol	25	Fructose	6.7	12.0
Progesterone	25	Fructose	6.7	22
Deoxycorticosterone	20	Fructose	6.7	8.2
Podophyllin	25	Fructose	6.7	20
Gardner cell homogenate[e]				
None		Fructose	3.7	27
Testosterone	20	Fructose	3.7	28
Deoxycorticosterone	20	Fructose	3.7	27

[a] In comparable experiments, estradiol, cortisone, cortisol, insulin, nystatin and epinephrine were without effect on either tumor. [b] Alcoholic solutions of the steroids were diluted 20-fold in Krebs–Ringer bicarbonate buffer immediately before use. Each Warburg vessel received 0.5 ml. of steroid dispersion or of the alcoholic buffer. [c] In comparable experiments, diethylstilbestrol, deoxycorticosterone, progesterone and podophyllin also had no significant effect on glucolysis. [d] Three different preparations (Ciba or Schering) gave the same result; one of these contained an extraneous 10-fold quantity of cholesterol as diluent. All compounds listed in this table were found to be inactive also in *Ehrlich* cell homogenates at the concentrations shown.

Discussion

Three assumptions necessary to our conclusions are that anaerobic CO_2-production is a specific index of hexose uptake, that no important galactose-sensitive unknown routes of glucose and fructose catabolism occur in these cells, and that the substrate-binding characteristics of hexokinase do not change upon homogenization. That the first assumption is substantially correct is demonstrated by the parallel analytical results of Table II. Furthermore hexoses cannot be converted to glycogen or arise from endogenous glycogen in these tumors,[42] possibly because phosphorylase is lacking.[43] Although the hexose monophosphate shunt does occur at low level in these tumors,[44] calculations based upon the distribution of carbon-14 from 1-, 2-, or 6-labeled glucose into CO_2 and lactic acid in Ehrlich cells,[45] or into ribose phosphate in Gardner cells,[46] show that 90% or more of the glucose catabolism follows the Embden–Meyerhof pathway. In homogenates TPN addition is necessary to elicit catabolism *via* the shunt.[47]

The second assumption is more difficult to support directly because the process of homogenization conceivably could destroy an unknown catabolic pathway of hexoses. Glycolytic rates in tumor homogenates, however, have always equaled or exceeded the rates in the cells (Tables I, III); therefore no loss of catabolic activity upon homogenization is apparent. Furthermore galactose has been shown to compete with ribose for *uptake* into Ehrlich ascites tumor cells.[13] Since neither ribose[48] nor galactose is metabolized by the Ehrlich cell, they could scarcely compete in a catabolic reaction.

The third assumption is necessary because a hexokinase with lower and differential hexose-affinities in the intact cell, which are increased upon homogenization, could represent the obligatory transport reaction.[49] Two factors make hexokinase unsuitable for this role: sugars which are not phosphorylated by hexokinase pass through the transport-reaction,[12] and erythrocytes transport sugars well despite very slow phosphorylation.[5,7] Also the failure of cell fructolysis to equal homogenate fructolysis even at 0.1 *M* fructose argues against this possibility.[49]

The structural specificity both of passage and of inhibition of passage eliminates diffusion as the rate-limiting step in hexose transport, as does also the non-linear relationship between hexose concentration and utilization in the Gardner cell. Other results[12,50] also have excluded this possibility in ascites tumors.

Houston, Texas, November 14, 1957)). In addition, inhibition of glucolysis at higher levels of steroids was found.

(41) T. Mann, "The Biochemistry of Semen," Methuen, London, 1954.

(42) M. W. Nirenberg and J. F. Hogg, unpublished.

(43) L. Slechta, A. Jakubovic and F. Sorm, *Coll. Czechoslovak Chem. Comm.*, **21**, 24 (1956).

(44) E. Racker, *Ann. N. Y. Acad. Sci.*, **63**, 1017 (1956).

(45) C. E. Wenner and S. Weinhouse, *J. Biol. Chem.*, **222**, 399 (1956).

(46) C. E. Wenner, J. Hackney and J. Herbert, *Proc. Amer. Assoc. Cancer Res.*, 259 (1957).

(47) E. Kravitz and A. J. Guarino, unpublished.

(48) E. Kun, P. Talalay and H. G. Williams-Ashman, *Cancer Res.* **11**, 855 (1951).

(49) J. G. Kaplan, *Expt. Cell Res.*, **8**, 305 (1955).

(50) W. D. Yushok and W. G. Batt, Abstracts, 130th Meeting, American Chemical Society, p. 22-D, Atlantic City, September 16–21, 1956.

A selective inhibition of fructose permeability by a steroid, cortisone, was reported for the foetal erythrocytes of sheep.[51] Bacila and Guzman-Barron[52] previously found no effect of cortical steroids on Ehrlich tumor glucolysis, but glucolysis in the mouse diaphragm was inhibited at low substrate concentration, the inhibitory action being attributed to a reversible ketosteroid reaction with sulfhydryl groups. Since various sulfhydryl reagents inhibit sugar transport in erythrocytes,[8] a possible mode of steroid inhibition is suggested. In agreement with this suggestion, the ketoses react much more poorly with cysteine than do the aldoses.[53]

Addendum

By J. J. BLUM[54]

The purpose of this addendum is to discuss briefly the ambiguities inherent in the kinetic analysis of constrained enzyme systems when inhibitors are present.

Consider an enzyme, E, uniformly distributed in a system bounded by a membrane of permeability H and with an internal diffusion constant D for the substrate whose (maintained) external concentration is S. The enzyme is supposed to catalyze the following reaction in the presence of an inhibitor I.

$$\begin{array}{ccc} E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} E + P & & V_m = k_2 E_0 \\ + & + & \bar{K} = \dfrac{k_1}{k_{-1} + k_2} \\ I & I & \\ \downarrow\uparrow K_i & \downarrow\uparrow K_i' & K_i = \dfrac{[E\cdot I]}{[E][I]} \\ E\cdot I & E\cdot S\cdot I & K_i' = \dfrac{[E\cdot S\cdot I]}{[E\cdot S][I]} \end{array}$$

In the previously published method[36] for obtaining $\bar{K}$, V_m, D and H from experimental data, the presence of inhibitors was not considered. It can be shown that in the presence of an inhibitor the appropriate equation is

$$\frac{S}{V_e} = \frac{1 + K_I I}{\bar{K}[V_m - V_e(1 + k_i' I)]} + \frac{r_0^2}{(p+2)(p+4)D} + \frac{r_0}{(p+2)H}$$

where $p = -1, 0, +1$ for a tissue slice, cylindrical cell and spherical cell, respectively. For the Gardner tumor cell $p = 1$, and it may be assumed that the term containing D is negligible compared to the permeability term. There are two opposite ways in which the inhibitor (galactose) may act. Possibly galactose acts as a competitive inhibitor ($K_i' = 0$) of the intracellular enzyme responsible for the rate of fructolysis (*cf.* Fig. 3). Alternatively galactose may act to decrease the permeability, as is indicated by the lack of inhibition and loss of concentration dependency in homogenates.

If one first assumes that the permeability term is of negligible importance ($H \to \infty$), then the above equation is a rearranged form of the Michaelis-Menten equation with competitive inhibition, and the data of Fig. 4 give a value of $\bar{K} = 4.0$ liters/mole (computed from the slope of the line with O galactose). The values of K_i computed from the slopes of the lines for 19, 77 and 115 μmoles/ml. galactose are, respectively, (negative), (negative) and 20 liters/mole. Thus this assumption does not yield consistent values for K_i, even though consistent values are obtainable from the Lineweaver-Burk plot (Fig. 3). The Lineweaver-Burk plot used on whole cells, however, simply indicates a competitive action of galactose, and offers no possibility of deciding whether the inhibitor affects permeability or an intracellular enzyme, or both.

If one assumes that the galactose affects only the permeability (*i.e.*, $k_i - k_i' = 0$), the slopes and the extrapolated intercepts of the lines in Fig. 4 yield the following values of the parameters, taking the radius of the cells as 5.2×10^{-4} cm.[55] and the cellular volume as 4.5×10^{-8} cc. per mg. dry weight.

Galactose, μmoles/ml.	K_i, l./mole	H (cm./sec.) $\times 10^{+7}$
0	4.0	3.9
19	4.6	1.4
77	(3.0-6.7)	0.45
115	1.2	(Negative)

Except for the highest value of galactose, where the data do not fit either assumption—presumably because some other phenomenon is occurring—the values of $\bar{K}$ are reasonably constant and the permeability decreases with increasing galactose concentration.

We have previously[34] emphasized the need for a large number of accurate determinations of the steady-state rate if one is to get reliable estimates of the parameters. Furthermore the equation required to get $\bar{K}$, V_m and H from kinetic data on *whole* cells is necessarily more sensitive to experimental variation than the Lineweaver-Burk equation. The presence of an inhibitor in the system further increases the requirement for many accurate measurements if ambiguity is to be avoided. Nevertheless the present relatively limited data tend to support the conclusion that galactose decreases the permeability of the Gardner ascites tumor cell to fructose and provides an order of magnitude estimate of the permeability.

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(51) A. Pleischer, P. Van Planta and W. A. Hunzinger, *Helv. physiol. et pharmacol. Acta*, **13**, 18 (1955).

(52) M. Bacila and E. S. Guzman-Barron, *Endocrinology*, **54**, 591 (1954).

(53) M. P. Schubert, *J. Biol. Chem.*, **130**, 601 (1939).

(54) Dept. of Biological Chemistry and Mental Health Institute.

(55) L. Revesz and G. Klein, *J. Natl. Cancer Inst.*, **15**, 253 (1954).