Factors Affecting Anaerobic Glycolysis in Mouse and Rat Liver and in Morris Rat Hepatomas [1,2]

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SUMMARY—Anaerobic glycolysis, in cells of mouse or rat liver and the Morris series of rat hepatomas, consists of a glucolytic component and a glycogenolytic component. Under anaerobic conditions both components become strongly inhibited as a result of $DPNH_2$ (reduced form of DPN) accumulation from nonglycolytic reduction of diphosphopyridine nucleotide (DPN) in hepatic tissues. Thus the real glycolytic potential of the cells becomes rapidly masked. Additions of DPN, triphosphopyridine nucleotide (TPN), or pyruvate temporarily relieve the glycolytic inhibition by increasing the DPN/$DPNH_2$ ratio. This paper presents detailed information concerning differential responses of glucolysis and glycogenolysis to additions of DPN[4], TPN, pyruvate, 2-deoxyglucose, Δ^1-testololactone, and insulin in mouse or rat livers, in a spectrum of rat hepatomas, and in certain nonhepatic tumors for comparison. In the liver-hepatoma spectrum glucolysis increases and glycogenolysis decreases, with increasing malignant growth capacity. The data reported have a bearing on the problem of localization of glycolytic activities within intact cells, and further support the Warburg concept that an increased glucolytic capacity and enhanced capacity for anaerobiosis are basic to malignant transformation. The *in vitro* results show that cells of liver and of hepatomas of very diverse growth rates follow the same phenomena of insulin : anti-insulin behavior, as do other tested mammalian tissues and malignant spectra derived therefrom.—J Nat Cancer Inst 41: 267–286, 1968.

OUR EARLIER STUDIES (*1–9*) of many types of normal and malignant mammalian cells have

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[2] Abbreviations: DPN, diphosphopyridine nucleotide; $DPNH_2$, reduced form of DPN; TPN, triphosphopyridine nucleotide; $TPNH_2$, reduced form of TPN; TPD, triosephosphate dehydrogenase; LDH, lactic dehydrogenase; 2-DG, 2-deoxy-D-glucose; Δ^1-TL, Δ^1-testololactone (NSC-23759); K-R-B, Krebs-Ringer-bicarbonate medium (unless otherwise specified, with 0.2% $NaHCO_3$).

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developed extensive evidence that hormonal control of glucose metabolism in mammalian cells is critically related to the regulation of cell growth. These investigations indicate that, among other essential factors, small increases in anaerobic glucolytic potential are involved in the initiation of a low order of malignancy, and that further increases in malignant growth capacity involve further increases in anaerobic glucolysis and decreases in susceptibility of glucolysis to insulin: steroid ("insulin:anti-insulin") regulation. From preliminary studies with rat liver and hepatomas, we reported in 1961 [(*6*) p 258] that ". . . in these rat hepatomas, as in sarcoma-producing tissue cultures, a range of tumors forms a glycolytic spectrum." Although it has been reported (*10–15*) that the slow-growing Morris rat hepatomas do not have more glycolytic activities than those in the normal tissue of origin, liver, we have been able to show (*7–9, 16, 17*) that under proper conditions of *in vitro* incubation even the slowest growing rat hepatomas display anaerobic glucolytic capacities greater than those in normal or host livers. The data support the Warburg concept (*18–21*) that an increased glucolytic capacity and adaptation to a more anaerobic life are basic to malignant transformation.

Proper measurements of the anaerobic glycolytic capacities of hepatic tissues, especially in liver and the slow-growing ("minimal deviation") hepatomas, depend on the use of agents (*e.g.*, DPN, TPN, or pyruvate) to combat the excessive non-glycolytic reduction of DPN that leads to profound inhibition of glycolysis as a result of too low a DPN/$DPNH_2$ ratio at the TPD reaction. This paper presents detailed information concerning differential responses of glucolysis and glycogenolysis to DPN, TPN, pyruvate, 2-DG, Δ^1-TL, and insulin in normal mouse or rat livers, in a spectrum of rat hepatomas and host livers, and in certain nonhepatic tumors for comparison.

MATERIALS AND METHODS

All normal rats and tumor-bearing rats were maintained in the National Cancer Institute laboratories of Dr. Harold P. Morris (normal and Morris hepatoma rats) or Dr. Miloslav Rechcigl (normal, HC, and LC hepatoma rats) on standard diets supplied *ad libitum*. Normal mice were similarly maintained in our laboratory. Methods of tissue collection, preparation, and dry weight determination were as previously described (*9*), as were the chemical, manometric, or other procedures and reagents used. Insulin was kindly supplied by Dr. Otto Behrens of the Lilly Research Laboratories, Indianapolis, Indiana, and was amorphous lot 192-235B-188 from trypsin-treated crystalline insulin. It was essentially devoid of glucagon. In some experiments, crystalline low HGF zinc insulin (Lilly 795 372) or amorphous lot W-1302 (Lilly) was used. All these insulins produced the same type of effects (stimulation of glucolysis but *not* glycogenolysis), so that any contamination by hyperglycemic factor (HGF) present in W-1302 insulin had little or no effect on either glycogenolysis or glucolysis.

Δ^1-TL (NSC-23759) was obtained through the kindness of Dr. Erwin P. Vollmer of this Institute. We prepared the solutions by dissolving the micronized crystals in a minimal amount of absolute ethanol (*e.g.*, 18 mg/cc) and, immediately before use, rapidly adding a large volume (*e.g.*, 15 cc) of warm K-R-B to yield a perfectly clear solution that showed no precipitation for 15 minutes or more. The solution of Δ^1TL thus prepared was promptly added to the manometric flasks that already contained tissue suspended in K-R-B. The temperature of the tissue when the steroid was added (or K-R-B alcohol in controls) was 10–15°C.

In most instances glycolytic activities are expressed as $Q_A^{N_2}$ values (mm^3 acid produced per mg dry weight per hour, measured as CO_2), but in some instances the acid produced per hour per total weight of tissue is given. The actual weights (wet and dry) of tissue slices added per respirometer vessel are given in the table footnotes and text-figure legends. The supernatant (SN) fluids of *in vitro* hepatic slice preparations contained soluble LDH (as does centrifuged Ehrlich ascitic serum). However, the overall metabolic performance of the preparations as a function of time showed that the cells were responsible for most of the manometric changes. The centrifuged SN from our hepatic preparations did not glucolyze anaerobically, nor in the absence of pyruvate oxidize $DPNH_2$. There were no notable gas changes on addition of pyruvate in the absence of $DPNH_2$.

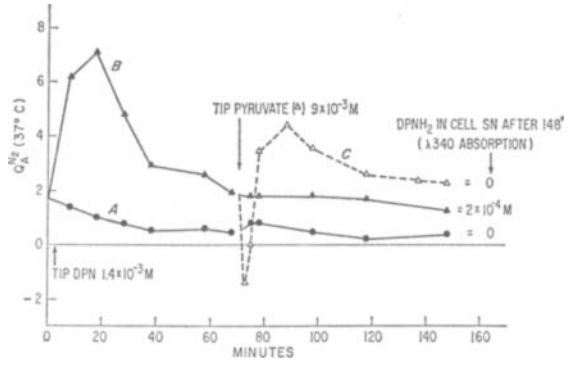
RESULTS

Effects of Exogenously Added DPN, TPN, $DPNH_2$, $TPNH_2$, and Pyruvate on Total Glycolysis

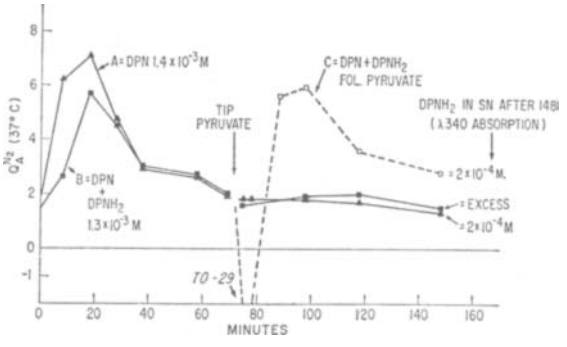
Ascites tumor cells: In previous work (*22*) with Ehrlich ascites tumor cells we found that under anaerobic conditions very small amounts of added $DPNH_2$ (*e.g.*, 20 μg/cc) strongly inhibited glucolysis, up to 75%. The added $DPNH_2$ was remarkably stable in the cell suspension. But in K-2 ascites tumor cell suspensions under anaerobic conditions, added $DPNH_2$ was rapidly destroyed, and very high levels of $DPNH_2$ (several mg/cc) were required to produce and maintain inhibition of anaerobic glycolysis (*22*). $DPNH_2$-induced inhibition of glucolysis could be reversed by addition of sufficient DPN.

Mouse liver: Text-figures 1 and 2 illustrate the effects of added DPN and $DPNH_2$ on total glycolysis in mouse liver. Text-figure 1, curve A, shows the autoinhibited anaerobic glycolytic rate in the control tissue. When DPN (1.4×10^{-3} M) was added at $t = 3$ minutes (curve B), glycolysis was temporarily activated to a $Q_A^{N_2}$ of *ca.* 7, but fell after 1 hour to a value of *ca.* 2. Spectrophotometric analysis of the SN at 340 mμ showed after 148 minutes about 14% of the initially added DPN was present in the reduced form. On the other hand, when a large excess of pyruvate was added to the DPN-treated cells at $t = 70$ minutes (curve C), a short period of negative manometric readings corresponded to OH^- formation (*22*) from reoxidation of accumulated $DPNH_2$. This was followed immediately by marked reactivation of glycolysis. Spectrophotometric analysis of the cell SN after 148 minutes showed that all $DPNH_2$ had been oxidized by the added pyruvate. Apparently, intracellular reduction of DPN continued to compete with its reoxidation so that the glycolytic rate again fell (curve C), but remained poised at a higher level than where pyruvate had not been added (curve B). Similar experiments showed that a second addition of DPN could again temporarily reactivate the inhibited glycolysis.

Text-figure 2 shows the effect of addition of $DPNH_2$ simultaneously with DPN. Obviously (text-fig. 2, B vs. A), addition of approximately a molar equivalent of $DPNH_2$ significantly lowered the initial (first 20 min) glycolytic increase due to DPN. Addition of excess pyruvate at $t = 70$ minutes to cells previously treated with DPN and $DPNH_2$ (curve B) produced (curve C) a rapid reoxidation of $DPNH_2$, as indicated by the negative phase of the curve. This was followed by a nearly complete recovery in glycolytic rate for a short period. Here again partial inhibition reappeared, apparently as a result of the continued excessive reduction of DPN. At $t = 150$ minutes the SN contained 2×10^{-4} M $DPNH_2$ as determined from the absorbance at 340 mμ. The



TEXT-FIGURE 1.—Stimulation of total anaerobic glycolysis (= glycogenolysis plus glucolysis) in mouse liver by added DPN, followed by secondary inhibition with $DPNH_2$ accumulation. A, control tissue without DPN addition; B, DPN added; C, as in B but following addition of pyruvate. One hundred mg wet weight (34 mg dry wt) of tissue per vessel. (C × DBA)F_1 male mice, 8 months old.



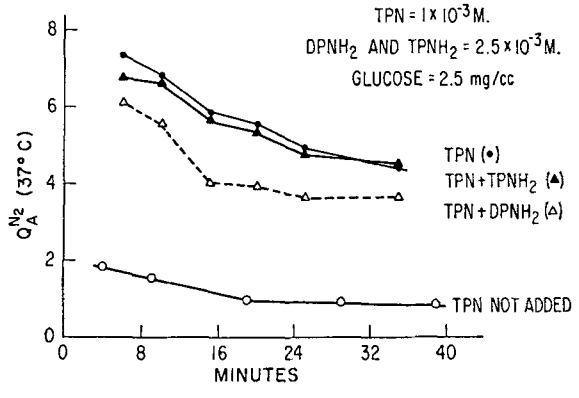
TEXT-FIGURE 2.—$DPNH_2$ inhibition of DPN-stimulated total glycolysis and reversal by pyruvate. A, DPN only added; B, DPN plus $DPNH_2$ added; C, as in B, but following pyruvate addition. Run simultaneously with curves shown in text-figure 1, with additional fractions of the same livers.

glycolytic rate in curve C remained poised above curves A or B, presumably in part because C contained a larger amount of DPN due to the oxidation by pyruvate of over 85% of the added $DPNH_2$.

Addition of substrate amounts of TPN (*e.g.*, 10^{-3} M) markedly reactivated autoinhibited anaerobic glycolysis in mouse (and rat) liver. This apparently resulted in part from a sparing effect on nonglycolytic reduction of endogenous DPN; *i.e.*, TPN was rapidly reduced by the nonglycolytic process, but since the $TPNH_2$ formed did not inhibit glycolysis, the net effect was a counteraction of glycolytic inhibition. Text-figure 3 shows that the simultaneous addition of $DPNH_2$ with TPN significantly inhibited the glycolytic increase due to TPN, whereas the simultaneous addition of $TPNH_2$ with TPN did not inhibit. Addition of excess pyruvate reactivated glycolysis wherever DPN or $DPNH_2$ had originally been added. Furthermore, where TPN only had been previously added, pyruvate did not appreciably reactivate glycolysis. Reoxidation of $TPNH_2$ by pyruvate and LDH is much slower than with $DPNH_2$.

Effects of Pyruvate on Glucolysis and Glycogenolysis

Normal liver: Table 1 shows some of the effects of added pyruvate on glycogenolysis and glucolysis



TEXT-FIGURE 3.—Inhibition of TPN-stimulated glycolysis by $DPNH_2$, but not by $TPNH_2$. One hundred mg wet weight (33 mg dry) of tissue per vessel. (C × DBA)F_1 male mouse, 8 months old.

in normal rat liver. The rate of glucolysis at a physiological level of added glucose (1 mg/cc) was not significantly increased by any level of added pyruvate and decayed rapidly to a very low value. However, with an unphysiologically high level (1%) of added glucose, pyruvate markedly increased both the rate and the stability of liver glucolysis. Increasing the level of pyruvate above 1 mg/cc in other experiments did not yield higher rates, and therefore in most cases this level was used. While glycogenolysis was increased by only 0.1 mg/cc of pyruvate the rate of decay was rapid and the maximum rates attained were considerably below a $Q_A^{N_2}$ of 8, such as can easily be obtained with DPN addition. Maximal Q values for glucolysis with added DPN were not higher than those which could be obtained with added pyruvate alone. For measurement of the glucolytic potential alone, it is preferable to add pyruvate without DPN because of the rapid secondary inhibition caused by nonglycolytic reduction of the nucleotide.

Hepatomas: In slow-growing hepatomas much lower levels of both pyruvate and glucose were required for maximal glucolytic stimulation than was true in liver (normal or host). Table 2 shows that 0.2 mg/cc of pyruvate gave maximal stimulation of glucolysis in slow-growing hepatoma 5123C. Significant glucolytic stimulation occurred with as little as 0.016 mg/cc of pyruvate. In this particular experiment, 1 and 2 mg/cc of pyruvate actually gave somewhat lower rates than did 0.2 mg/cc, instead of usually the same rate. This and other experiments make clear that much higher levels of both glucose and pyruvate are required in liver for maximal rates of glucolysis than are needed for even the slowest-growing hepatomas.

Lactic Acid Formation

A number of chemical determinations of lactic acid in liver and hepatomas by the well-known Barker-Summerson method confirmed that the manometrically indicated acid production obtained from glycolytically activated liver and hepatomas was largely, even if not entirely, lactic acid. Thus table 3 shows that glucolysis in intermediate hepatoma 7288C activated by DPN plus pyruvate was strongly inhibited by 2-DG, but glycogenolysis was

TABLE 1.—Effect of added pyruvate on glycogenolysis and glucolysis in normal rat liver*

Minutes at 37°C	$Q_A^{N_2}$ glycogenolysis †			$Q_A^{N_2}$ glucolysis ‡					
	+2-DG 0.5%			+ Glucose 0.1%			+ Glucose 1%		
	mg Na pyruvate/cc								
	0	0.1	1.0	0	0.1	1.0	0	0.1	1.0
0–36	0.79	1.47	3.65	0.45	0.55	0.57	0.60	0.95	1.07
36–66	0.54	1.09	2.15	0.27	0.21	0.16	0.27	0.63	1.51
66–96	0.60	0.69	1.03	0.03	0.09	0	0	0.34	1.33

*Female Buffalo rat age 4 months, 200 mg wet weight (64 mg dry) of tissue per vessel in 4.5 cc K-R-B with 0.2% $NaHCO_3$, gas phase 5% CO_2/N_2.

† Rate of acid production in the presence of 0.5% 2-DG in the absence of added glucose.

‡ Rate of acid production with glucose minus rate with added 2-DG but no added glucose; thus, in column 5, the value 0.45 ($Q_A^{N_2}$ in the absence of added pyruvate, 0–36 min) was derived by subtracting the measured rate of acid production in the presence of 0.5% 2-DG but no added glucose ($=Q_A^{N_2}$ glycogenolysis = 0.79, col 1) from the measured $Q_A^{N_2}$ total glycolysis of 1.24 (not included in the table). Thus $Q_A^{N_2}$ total glycolysis minus $Q_A^{N_2}$ glycogenolysis = $Q_A^{N_2}$ glucolysis (1.24 − 0.79 = 0.45).

TABLE 2.—Effects of varying levels of added pyruvate on rate of anaerobic glucolysis in hepatoma 5123C*

Minutes at 37°C	$Q_A^{N_2}$ glucolysis†						
	mg Na pyruvate/cc						
	0	0.016	0.050	0.10	0.20	1.0	2.0
0–23	0.70	1.04	1.22	1.30	2.18	1.74	1.55
23–83	0.59	1.26	1.33	1.63	2.04	1.78	1.59
83–113	0.48	0.82	1.22	1.44	2.03	1.26	1.49
Average 23–113	0.56	1.11	1.29	1.57	2.04	1.61	1.56

*One hundred and fifty mg wet weight (30 mg dry) of tissue per vessel in 4.5 cc of K-R-B with 0.2% $NaHCO_3$ and 0.5% 2-DG or 0.3% added glucose.

†Rate with added glucose minus rate with 2-DG but no added glucose.

TABLE 3.—Effect of 2-DG on anaerobic glycolysis and lactic acid formation in intermediate hepatoma 7288C

Measurements	$Q_A^{N_2}$*					
	Minus added glucose			Plus added glucose†		
	−2-DG	+2-DG‡	(% inhib.)	−2-DG	+2-DG	(% inhib.)
Manometric CO_2	4.46	4.24	(5)	8.92	6.46	.....
Chemically determined lactic acid§	3.52	3.24	(8)	8.44	5.77	.....
Glucolysis (manometric)				4.45	2.22	(50)
Glucolysis (lactic)				4.92	2.53	(49)
Lactic as % of total manometric CO_2	79	76	.....	95	89	.....

*Two hundred mg wet weight of tissue (41 mg dry) per vessel in 4.5 cc K-R-B plus 1.4×10^{-3} M DPN and 9×10^{-3} M pyruvate. Incubation time 145 minutes.

† Glucose, 5 mg/cc (2.8×10^{-2} M).

‡ 2-DG, 5 mg/cc (3×10^{-2} M).

§ Expressed as mm^3 CO_2 (4 μg lactic acid = *ca.* 1 mm^3 CO_2).

not significantly inhibited. In the absence of added glucose, 76–79% of the manometrically indicated acid could be accounted for as lactic, and with glucose over 90% was lactic acid. Similar agreement between manometry and chemical determinations of lactic acid in slow-growing and intermediate hepatomas (5123D and 7288C), together with the respective host livers, is shown in table 4. In the absence of added glucose the average rate of acid production ($Q_A^{N_2}$) in the 5123D hepatoma was lower than in the host liver. This was to be expected because of the very fast decay in rate,

TABLE 4.—Total acid production (CO_2) and lactic acid formation in 2 hepatomas and respective host livers

Tumors* and additions	Hepatoma							Host liver						
	Total acid production			$Q_X^{N_2}$§				Total acid production			$Q_X^{N_2}$§			
	CO_2†	Lactic‡ acid	Lactic as % of CO_2	Total acid		Glucolysis		CO_2†	Lactic‡ acid	Lactic as % of CO_2	Total acid		Glucolysis	
				CO_2	Lactic	CO_2	Lactic				CO_2	Lactic	CO_2	Lactic
5123D‖														
−glucose	279	146	52	3. 4	1. 8	—	—	575	569	98	4. 6	4. 5	—	—
+glucose	317	227	71	3. 9	2. 8	0. 46	0. 98	605	562	93	4. 8	4. 5	0. 24	0. 02
7288C¶														
−glucose	447	352	79	4. 4	3. 4	—	—	626	468	75	3. 9	2. 9	—	—
+glucose	892	844	95	8. 7	8. 2	4. 3	4. 8	636	534	84	4. 0	3. 3	0. 06	0. 42

*All vessels contained 1.4×10^{-3} M DPN, and 9×10^{-3} M pyruvate. Glucose where added was 0.5% (2.8×10^{-2}M).

†Total mm^3 CO_2 produced during time periods indicated in ‖ or ¶ footnotes per 200 mg wet weight of tissue per vessel. Gas phase, 5% CO_2/N_2, 37°C.

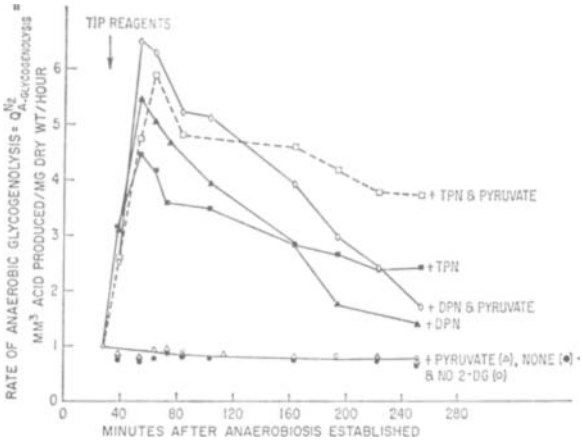
‡Total lactic acid produced during indicated time in ‖ or ¶ footnotes, expressed as equivalent mm^3 CO_2 (4 mm^3 CO_2 = *ca.*1 μg lactic acid).

§Activities expressed as mm^3 CO_2 per mg dry weight per hour (total acid = glycogenolysis glucolysis, glucolysis = Q Δ glucose).

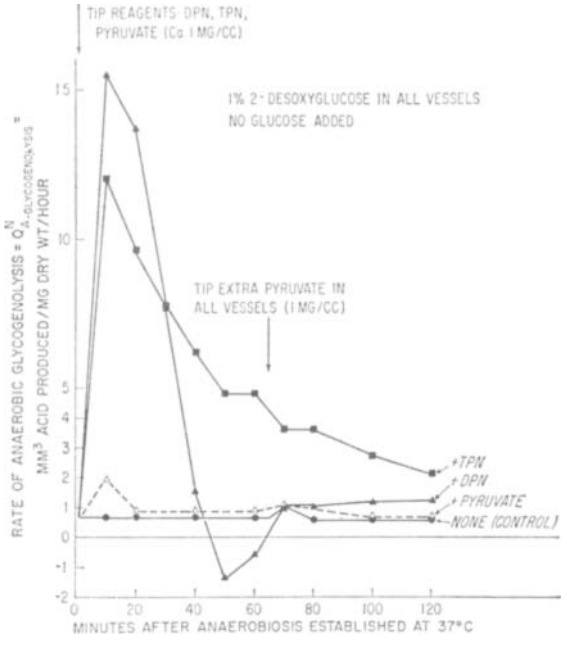
‖Two hundred mg wet weight of tissue (hepatoma 42 mg dry, liver 64 mg dry weight) per vessel, 117 minutes' incubation).

¶Two hundred mg wet weight of tissue (hepatoma 42 mg dry, liver 66 mg dry weight) per vessel, 145 minutes' incubation).

which characterizes DPN-stimulated glycogenolysis in 5123 tumors relative to liver (*e.g.*, *see* text-figs. 4 and 5). However, the Q values for glucolysis (indicated by the differences in rates of acid production plus and minus glucose) were significantly higher in the hepatoma than in the liver. In the faster growing hepatoma 7288C, DPN-stimulated glycogenolysis decayed less rapidly than in hepatoma 5123D, and glucolysis was considerably higher. Clearly in both tumor and host liver tissues, in the presence of added glucose, most of the acid production was accounted for as lactic acid. Table 5 shows determinations of manometric CO_2, enzymatically indicated glucose (Glucostat), and chemically determined lactic acid in fast-growing hepatoma 3924A and host liver. Again, the manometrically indicated amounts of acid production



TEXT-FIGURE 4.—Effects of added DPN, TPN, and/or pyruvate on anaerobic glycogenolysis in normal rat liver: 0.5% 2-DG added at $t = -20$ minutes. One hundred twenty-five mg wet weight (40 mg dry) of tissue per vessel. Female Buffalo rat 4 months old. DPN, 2.2×10^{-3} M; TPN, 2.4×10^{-3} M; pyruvate, 7×10^{-3} M.



TEXT-FIGURE 5.—Effects of added DPN, TPN, and pyruvate on anaerobic glycogenolysis in slow-growing hepatoma 5123D. Two hundred mg wet weight of tissue (45 mg dry) per vessel. DPN 1.4×10^{-3} M, TPN 1.3×10^{-3} M, pyruvate 9×10^{-3} M. K-R-B, 4.5 cc per vessel with 1% (6×10^{-2} M) 2-DG; no added glucose.

TABLE 5.—Total acid production, lactic acid formation, and glucose uptake or production in fast-growing hepatoma 3924A and host liver

Measurements*	Hepatoma†		Host liver†	
	− Glucose	+ Glucose‡	− Glucose	+ Glucose‡
Total acid (manometric CO_2)	275	626	709	710
Δ glucose		351		1
Total glucose (glucostat)	0	−336	1135§	336§
Δ glucose		−336		−799
Total lactic acid (chemical)	113	625	738	688
Δ glucose		512		−50
Lactic as % of total CO_2	41	100	104	96

* Expressed as equivalent mm^3 CO_2 (4 μg lactic acid = *ca.* 1 mm^3 CO_2). One mg glucose = 249 mm^3 CO_2 as lactic equivalent. Total incubation = 128 minutes.

† Two-hundred mg wet weight of tissue (hepatoma 32 mg dry, liver 68 mg dry) per vessel in 4.5 cc K-R-B.

‡ Glucose, 0.5% (2.8×10^{-2} M), added.

§ Glucose produced (less in presence of added glucose).

are accounted for mainly as lactic acid, and, in glucolysis in the hepatoma, by net disappearance of glucose.

Differential Effects of DPN, TPN, and Pyruvate on Glycogenolysis and Glucolysis

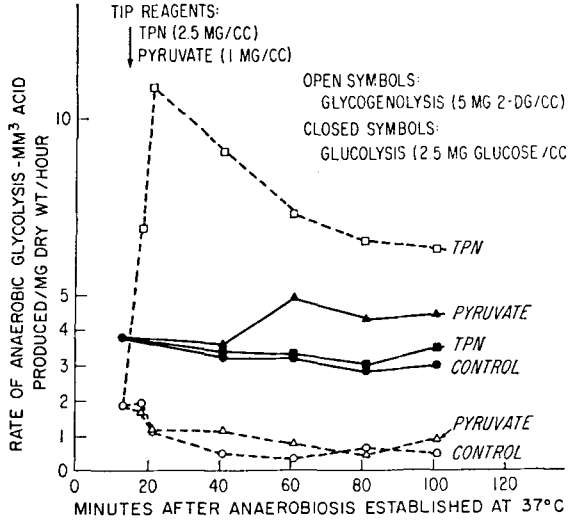
Liver: Text-figure 4 shows the effects of DPN, TPN, plus and minus pyruvate on glycogenolysis (with 0.5% 2-DG) in normal rat liver. The addition of DPN alone gave an initial glycogenolytic rate higher than with TPN alone, but the rate of decay was then considerably higher with DPN. Pyruvate alone had no significant effect on acid production but did increase the rates of acid production with DPN or TPN. Many other experiments with both normal and tumor host liver showed essentially the same patterns of response of glycogenolysis to these three activating agents except that pyruvate sometimes gave a small and rapidly decaying stimulation.

Hepatomas: Text-figure 5 shows the pattern of response characteristic for glycogenolysis by slow-growing hepatoma 5123. DPN gave a greater initial stimulation than did TPN, but the rate of decay was extraordinarily fast. This was consistently observed in all 5123 tumors tested. Apparently these hepatomas reduce added DPN more rapidly than does liver. A net negative manometric reading in due course (here at 40–50 min) was observed in almost every experiment with hepatoma 5123 involving the single addition of DPN, but never occurred in liver of hepatoma-bearing or normal rats. It presumably resulted from an initial excessively rapid reduction of added DPN to $DPNH_2$ followed by a limited reoxidation by pyruvate and LDH of the reduced coenzyme to yield an excess of OH^- ions resulting in CO_2 uptake. In any case there is ordinarily a period during which there is a net production of anions, involving net gas consumption. Significantly such negative manometric readings never occurred with TPN addition.

In progressing from normal liver, or slow-growing hepatomas (*e.g.*, 5123), through intermediate 7288C, to fast-growing hepatoma 3683, glycogenolysis became progressively diminished but showed remarkably uniform and characteristic response patterns to added DPN, TPN, or pyruvate.

Intermediate hepatoma 7288C is specially suited to comparative studies of glycogenolysis and glucolysis, since both are quite active in this tumor. Table 6 shows anaerobic responses of both processes in 7288C to added DPN and/or pyruvate. Pyruvate had little effect on glycogenolysis, but stimulated glucolysis up to 40%. DPN greatly increased glycogenolysis (*ca.* 16-fold) and initially increased glucolysis (nearly 40%). However, in time, the glucolytic rate with added DPN fell to a level even lower than in the controls. The simultaneous addition of pyruvate with DPN retarded the fall in rates of both glycogenolysis and glucolysis. Text-figure 6 shows that TPN greatly stimulated glycogenolysis in hepatoma 7288C, but as in liver and the other hepatomas, had little or no effect on glucolysis.

In fast-growing hepatomas (3683, H-35 tc_2, 3924A) the glucolytic capacity exceeded the glycogenolytic capacity. Table 7 shows the response of anaerobic glycogenolysis and glucolysis in hepatoma 3683 to the addition of DPN, TPN, and/or pyruvate. While glycogenolytic activity was much lower than in liver or slow-growing (5123), or intermediate (7288C), hepatomas, the pattern of response to pyridine nucleotides and to pyruvate



TEXT-FIGURE 6.—Effects of TPN or pyruvate on anaerobic glycogenolysis and glucolysis in intermediate hepatoma 7288C; 125 mg wet (23 mg dry) weight of tissue per vessel; 2-DG 3×10^{-2} M, glucose 1.4×10^{-2} M, TPN 2.5×10^{-3} M, pyruvate 9×10^{-3}.

TABLE 6.—Effects of added DPN and/or pyruvate on rates of anaerobic glycogenolysis and glucolysis in intermediate hepatoma 7288C

Minutes* at 37°C	$Q_A^{N_2}$†								
	No glucose or 2-DG added	Glycogenolysis				Glucolysis			
Tip at 110 from side arms:	K-R-B	K-R-B	Pyruvate	DPN	DPN+ pyruvate	K-R-B	Pyruvate	DPN	DPN+ pyruvate
60–108.....	0. 6	0. 4	0. 4	0. 6	0. 7	1. 7	1. 4	1. 4	1. 2
	At 110′ tip all side arms								
118–148.....	0. 5	0. 6	0. 7	7. 9	8. 0	1. 9	2. 7	2. 6	3. 0
148–193.....	0. 6	0. 4	0. 7	5. 9	6. 5	1. 8	2. 4	1. 8	2. 7
193–253.....	0. 4	0. 4	0. 4	2. 9	3. 8	2. 0	2. 4	1. 4	2. 3

* t = 0 was time of transfer to thermostat at 37°C, anaerobic gassing began at t = 8′.

† One hundred and twenty-five mg wet weight of tissue (25 mg dry wt) per vessel in 4.5 cc K-R-B. Glycogenolysis=rate with 0.5% (3×10^{-2} M) 2-DG; glucolysis=rate with 0.25% (1.4×10^{-2} M) glucose minus rate with 2-DG only. DPN, 2.2×10^{-3} M; pyruvate, 7×10^{-3} M.

was similar in all 3 tissues. In hepatoma 3683, pyruvate increased the rate of glycogenolysis 77%, but the absolute change was small ($Q_A^{N_2}$ of 0.35 up to 0.62). On the other hand, DPN increased the rate of glycogenolysis from 0.35 to 3.4 (871%). Glucolysis, which was initially very high, was increased only 4% ($Q_A^{N_2}$ of 15.8 up to 16.4), and DPN gave no significant increase in glucolysis unless possibly when combined with pyruvate (15.8 up to 16.4). Pyruvate had similar effects in fast-growing hepatoma 3924A in that the rate of glycogenolysis was increased 63% (Q of 0.40 up to 0.65), and glucolysis 34% (Q of 4.4 up to 5.9) during the last 125 minutes of a 170-minute run.

Separate and Combined Effects of 2-DG and Δ^1-Testololactone

Nonhepatic tumors: In certain highly anaplastic nonhepatic cancers, such as the Ehrlich ascites tumor, anaerobic fermentation depends entirely on exogenously supplied glucose, there being essentially neither (endogenous) glycogenolysis nor glucogenesis (table 8). The addition of 2-DG to such cells anaerobically in the absence of glucose did not lead to any detectable acid formation. However, fermentation with 0.2% added glucose was strongly (82–84%) inhibited by 0.5% 2-DG. Since glucolysis in the Ehrlich ascites tumor has only a very low susceptibility to insulin : anti-insulin control (*1, 9, 23*), the addition of Δ^1-TL gave only slight inhibition (6 and 20%, without and with 2-DG).

The S91 melanoma (table 9), on the other hand, had a small but definite endogenous glycolysis that was not significantly altered in rate by 2-DG or Δ^1-TL. However, the much greater glucolysis with 0.2% added glucose was completely eliminated by 0.5% 2-DG and was inhibited 33% by Δ^1-TL. In this tumor anaerobic glycolysis was not markedly altered by the addition of sodium pyruvate to the incubation medium. There appears to be little or no significant production of glucose by S91 tumor tissue. The addition of DPN and/or pyruvate did not activate glycolysis in either the Ehrlich or S91 tumors except in ascites cells where glucolysis had been previously inhibited by added $DPNH_2$ (*22*).

Hepatic tissues: Determination of the true capacities for anaerobic glycogenolysis and glucolysis in liver and the Morris hepatomas requires discrimination between acid production from endogenous substrate other than glucose (glycogenolysis), and from added glucose (glucolysis). Since the amounts of glucose formed endogenously may be enough to support a significant rate of acid production, the endogenous glucolysis must be suppressed to determine the true nonglucolytic baseline of acid production for a given tissue. This is of relatively more importance in liver and the slow-growing hepatomas than in the more strongly dedifferentiated tumors.

TABLE 7.—Effects of DPN, TPN, and/or pyruvate on anaerobic glycolysis in fast-growing hepatoma 3683*

Minutes at 37°C	$Q_A^{N_2}$												
	Endogenous†	Glycogenolysis‡						Glucolysis‡					
Tip at§ t = 43	K-R-B	K-R-B	Pyruvate	DPN	TPN	DPN + pyruvate	TPN + pyruvate	K-R-B	Pyruvate	DPN	TPN	DPN + pyruvate	TPN + pyruvate
29–40	0. 8	Average pre-tip Q = 0. 9						Average pre-tip Q = 18. 3					
Tip at t = 43													
40–70	0. 1	0. 3	0. 6	5. 6	4. 5	5. 1	3. 9	16. 4	16. 8	14. 7	15. 8	17. 4	18. 2
70–110	0. 3	0. 5	0. 8	3. 1	3. 9	3. 5	3. 8	16. 3	16. 9	15. 8	16. 4	16. 5	16. 5
110–140	0. 4	0. 2	0. 4	1. 6	3. 1	1. 8	3. 3	14. 8	15. 5	15. 2	15. 0	15. 2	14. 3
Average 40–140	0. 3	0. 4	0. 6	3. 4	3. 8	3. 4	3. 7	15. 8	16. 4	15. 2	15. 5	16. 4	16. 4

*One hundred and fifty mg wet weight (24 mg dry) of tissue per vessel in 4.5 cc K-R-B.
†Without 2-DG or added glucose.
‡Glycogenolysis = rate with 0.5% 2-DG (3×10^{-2} M); glucolysis = rate with 0.25% (1.4×10^{-2} M) glucose minus rate with 2-DG only.
§DPN, 1.1×10^{-3} M; TPN, 1.2×10^{-3} M; pyruvate, 7.2×10^{-3} M.

TABLE 8.—Effects of 2-DG and/or Δ^1-TL on anaerobic glycolysis in Ehrlich ascites tumor cells*

Measurements	Endogenous	0.5% 2-DG	Δ^1-TL†	0.2% glucose	Δ^1-TL†	0.5% 2-DG + 0.2% glucose	Δ^1-TL†
Total acid in 120 minutes....	3, 3	0, 2	0, 2	187, 188	176, 178	29, 38	28, 29
Average mm³ CO_2.....	3	1	0	188	177	34	29
Δ glucolysis (mm³ CO_2).....	3	1	—‡	188	177	34	29
$Q_A^{N_2}$ glucolysis.....	0. 6	0. 2	—	37. 0	35. 0	7. 0	5. 6
% inhibition of glucolysis by Δ^1-TL	.....	.....	.....	.....	6	.....	20
% inhibition of glucolysis by 2-DG	.....	.....	.....	.....		82	84

*Sixteen mm³ cells (2.56 mg dry wt) per vessel in 4.5 cc K-R-B with 0.2% $NaHCO_3$ plus 0.2% sodium pyruvate (0.018 M).

†Δ^1-TL, 190 μg/cc.

‡ Rate of glucolysis with 2-DG + Δ^1-TL = 0.

TABLE 9.—Effects of 2-DG and/or Δ^1-TL on anaerobic glycolysis in S91 melanoma cells*

Measurements	Endogenous	Δ^1-TL	0.5% 2-DG	Δ^1-TL	0.2% Glucose	Δ^1-TL	0.5% 2-DG + 0.2% glucose	Δ^1-TL
Total acid in 90 minutes.....	18, 19	17, 15	20, 21	20, 18	332, 288	217, 206	16, 18	15, 18
Average mm³ CO_2.....	19	16	21	19	310	212	17	17
Δ glucolysis (mm³ CO_2)	0	−3	2	—†	291	193	−2	−2
$Q_A^{N_2}$ glucolysis.....	0	0	0. 07	—	10. 8	7. 2	0	0
% inhibition of gluclolysis by Δ^1-TL.....	.....	.....	.....	.....	.....	33	.....	.....
% inhibition of glucolysis by 2-DG.....	.....	.....	.....	.....	.....		100	100

*One hundred mg wet weight (18 mg dry) of tissue per vessel, otherwise same as for table 8 except 8 μg/cc of low zinc insulin (Lilly W-1302) added to all vessels.

†Glucolysis zero.

Fast-growing hepatomas: Table 10 shows the glycolytic responses obtained with highly anaplastic fast-growing hepatoma 3683 under the same experimental conditions used in the study of the Ehrlich ascites tumor (table 8), and S91 melanoma (table 9). Endogenous glucolysis was either absent or very small. The addition of 0.2% glucose activated a high rate of glucolysis which was inhibited 89% by 0.5% 2-DG. In line with the previously observed relative insensitivity of glucolysis in hepatoma 3683 to inhibition by diethylstilbestrol (*9*), Δ^1-TL inhibited the rate of glucolysis only 10%.

Slow-growing hepatomas: Table 11 shows the glycolytic response pattern to 2-DG and Δ^1-TL in very slow-growing hepatoma 7794B which lies close to the liver end of the glucolytic spectrum. In the absence of added glucose there appeared to be a small endogenous glucolysis ($Q_A^{N_2}=0.16$) which was inhibited appreciably by Δ^1-TL and 2-DG. However, in such cases we have found little or no actual accumulation of free glucose in the medium. The $Q_A^{N_2}$ with 0.2% added glucose, 0.48, was inhibited 21% by Δ^1-TL and 74% by 2-DG. As previously described, addition of DPN or TPN to such minimal deviation tumors greatly activated the rate of glycogenolysis (to $Q=10$). The maximum rate of glucolysis in DPN-activated cells was usually not higher than with pyruvate alone, and in fact decayed more rapidly, presumably due to $DPNH_2$ accumulation.

TABLE 10.—Effects of 2-DG and/or Δ^1-TL on anaerobic glycolysis in fast-growing hepatoma 3683 (generation 355)*

Measurements	Endogenous	Δ^1-TL	0.5% 2-DG	Δ^1-TL	0.2% glucose	Δ^1-TL	0.5% 2-DG + 0.2% glucose	Δ^1-TL
Total acid in 60 minutes.....	22, 26	18, 21	20, 20	20, 17	273, 276	262, 239	43, 50	46, 49
Average mm³ CO_2.....	24	20	20	19	275	250	47	48
Δ glucolysis (mm³ CO_2).....	5	1	1	—†	256	231	28	29
$Q_A^{N_2}$ glucolysis (mm³ CO_2).....	0. 31	0. 06	0. 06	—	14. 2	12. 8	1. 6	1. 6
% inhibition of glucolysis by Δ^1-TL.....	.....	80	.....	.....	.....	10	.....	0
% inhibition of glucolysis by 2-DG.....	.....	.....	80	.....	.....	.....	89	89

*One hundred mg wet weight (18 mg dry) of tissue per vessel, same conditions as in table 8.

†Glucolysis zero.

TABLE 11.—Effects of 2-DG and/or Δ^1-TL on anaerobic glycolysis in very slow-growing hepatoma 7794B (generation 6)*

Measurements	Endogenous	Δ^1-TL	0.5% 2-DG	Δ^1-TL	0.2% glucose	Δ^1-TL	0.5% 2-DG + 0.2% glucose	Δ^1-TL
Total acid in 120 minutes....	59, 54	48, 48	49, 51	45, 43	78, 85	78, 69	58, 50	59, 54
Average mm³ CO_2.....	57	48	50	44	82	74	54	57
Δ glucolysis (mm³ CO_2).....	13	4	6	—†	38	30	10	13
$Q_A^{N_2}$ glucolysis (mm³ CO_2).....	0. 16	0. 05	0. 08	—†	0. 48	0. 38	0. 13	0. 16
% inhibition of glucolysis by Δ^1-TL.....	.....	70	.....	.....	.....	21	.....	.....
% inhibition of glucolysis by 2-DG.....	.....	.....	54	.....	.....	.....	74	57

*One hundred and fifty mg wet (40 mg dry) weight of tissue per vessel; same conditions as in table 8.

†Glucolysis zero.

Normal rat liver: Table 12 shows a glycolytic response pattern characteristic for normal rat liver. The endogenous glucolysis of $Q_A^{N_2} = 0.16$ was apparently completely inhibited by Δ^1-TL and markedly so by 2-DG. The rate of endogenous glycolysis with both 2-DG and Δ^1-TL was the same as with Δ^1-TL alone. The percent inhibition of glucolysis by Δ^1-TL decreased with increasing glucose concentration, but was very marked (42%) at 2% added glucose. The data are in harmony with the previously established high susceptibility of liver glucolysis to anti-insulin inhibition (*8, 9*).

Comparative Glycolytic Rates in Liver and Slow-Growing Hepatomas

Table 13 compares the effects of 0.5% 2-DG on glycolysis in hepatoma 5123C and host liver with and without 0.1% added glucose. In these cases apparently 0.5% 2-DG alone could abolish both the endogenous glucolysis as well as that occurring with 0.1% added glucose. However, in some instances (*e.g.*, table 12) 2-DG apparently failed to inhibit the endogenous glucolysis as effectively as Δ^1-TL, and especially Δ^1-TL plus 2-DG.

Further investigation of the effects of Δ^1-TL on anaerobic glucolysis in the liver-Morris hepatoma

TABLE 12.—Effects of 2-DG and/or Δ^1-TL on anaerobic glycolysis in normal rat liver*

Measurements	Endogenous		0.5% 2-DG		0.2% glucose		0.5% 2-DG + 0.2% glucose		2% glucose	
		Δ^1-TL		Δ^1-TL		Δ^1-TL		Δ^1-TL		Δ^1-TL
Total acid in 225 minutes	98, 97	62, 73	80, 86	64, 74	114, 102	76, 79	88, 91	77, 73	118, 124	100, 98
Average mm³ CO_2	98	68	83	69	108	78	90	75	121	99
Δ glucolysis (mm³ CO_2)	29	−1	14	—†	39	9	21	6	52	30
$Q_A^{N_2}$ glucolysis (mm³ CO_2)	0. 16	0	0. 08	—	0. 22	0. 05	0. 12	0. 03	0. 30	0. 17
% inhibition of glucolysis by Δ^1-TL		100				77		71		42
% inhibition of glucolysis by 2-DG			52				46			

*Osborne-Mendel male rat *ca.* 3 months old; 150 mg wet (47 mg dry) weight of tissue per vessel. Same conditions as in table 8.

†Glucolysis zero.

TABLE 13.—Effects of 2-DG on anaerobic glycolysis in slow-growing hepatoma 5123C and host liver*

Measurements	Hepatoma 5123C (gen. 48)				Host liver			
	Minus added glucose		0.1% added glucose		Minus added glucose		0.1% added glucose	
		0.5% 2-DG		0.5% 2-DG		0.5% 2-DG		0.5% 2-DG
Total acid in 65 minutes	50, 58	50, 44	77, 78	46, 45	102, 90	97, 91	99, 118	98, 87
Average mm³ CO_2	54	47	78	46	96	94	108	93
Δ glucolysis (mm³ CO_2)	7	—†	31	−1	2	—†	14	−1
$Q_A^{N_2}$ glucolysis (mm³ CO_2)	0. 16	—	0. 72	0	0. 02	—	0. 17	0

*Two hundred mg wet (40 mg dry in hepatoma, 74 mg dry in liver) weight of tissue per vessel in 4.5 cc K-R-B + 5% polyvinylpyrrolidone with 0.2% $NaHCO_3$ and 0.1% pyruvate.

†Glucolysis zero.

spectrum indicated that the combined use of 2-DG and Δ^1-TL provides a slightly better baseline, at least in some instances, for estimation of the full glucolytic capacity of liver and slow-growing hepatomas than the use of 2-DG alone. We therefore set up a series of new experiments in which determinations of the glucolytic activities were based on both the glycogenolytic baseline with 2-DG only and with 2-DG plus Δ^1-TL (for detailed examples *see* tables 8–12). Table 14 summarizes these results.

The use of Δ^1-TL together with 2-DG in determining the nonglucolytic baselines increased the calculated average $Q_A^{N_2}$ of glucolysis 19% in the 11 liver experiments, and 0–6% in the tumor experiments. Even though the combined use of 2-DG plus Δ^1-TL as suppressor of endogenous glucolysis yielded a 19% higher glucolytic quotient for liver than did calculations based on the use of 2-DG alone (Q = 0.31 and 0.26, respectively) obviously the new determinations calculated in either way confirm the essential conclusion previously reached, *i.e.*, even the very slow-growing hepatomas have significantly higher anaerobic glucolytic capacities than do normal or host livers. On the whole there is a remarkable fit to the similar data previously published [(*9*) table 2].

TABLE 14.—Anaerobic glucolytic activities in a rat liver-hepatoma spectrum, corrected to baseline glycogenolysis as determined or affected by either 2-DG or 2-DG + Δ^1-TL; two mouse tumors are presented for comparison

Tissues*	$\frac{\text{Average}}{\text{Range}}$ $Q_A^{N_2}$ (number determinations)		Average % change with Δ^1-TL
	2-DG only for baseline†	2-DG + Δ^1-TL for baseline‡	
Liver (normal and host)	$\frac{0.26}{0.08–0.51}$ (11)	$\frac{0.31}{0.17–0.53}$ (11)	+19
Very slow-growing hepatomas (7787, 7795, 7794B, R-1)	$\frac{0.48}{0.42–0.61}$ (7)	$\frac{0.50}{0.32–0.58}$ (7)	+4
Slow hepatomas (5123 B,C,D; 7794A)	$\frac{1.25}{0.7–1.81}$ (4)	$\frac{1.32}{0.8–1.77}$ (4)	+5
Intermediate hepatomas			
HC (Rechcigl)	$\frac{3.6}{3.2–4.3}$ (3)	$\frac{3.8}{3.4–4.6}$ (3)	+5
LC (Rechcigl)	$\frac{6.5}{5.0–8.9}$ (3)	$\frac{6.8}{5.0–9.2}$ (3)	+5
7288C (ip)	$\frac{7.4}{7.1–7.7}$ (2)	$\frac{7.9}{7.4–8.3}$ (2)	+6
Advanced hepatoma 3683	14.2 (1)	14.2 (1)	0
S91 mouse melanoma	10.7 (1)	10.8 (1)	+1
Ehrlich ascites tumor	37.0 (1)	37.0 (1)	0

*Tissue slices (or cell suspensions in the ascites tumor) suspended in K-R-B with 0.2–0.25% $NaHCO_3$, glucose levels in all cases 0.2–0.5%.

†2-DG, 0.5%.

‡Δ^1-TL, 190 μg/cc.

Insulin : Anti-Insulin Activity in Liver-Hepatoma Spectrum

Δ^1-TL as an anti-insulin: Previous work (*9*, *23*) has shown that Δ^1-TL acts as a typical anti-insulin inhibitor of glucolysis in a variety of mammalian tissues. Because of its demonstrated anti-tumor activity *in vivo*, together with the demonstrated lack of endocrinological activities (*24*), it was interesting to compare the anti-glucolytic effects of Δ^1-TL in the liver-hepatoma spectrum as was previously done for diethylstilbestrol (*9*). Table 15 shows the percent inhibitions caused by 190 μg/cc of Δ^1-TL in the identical series of tissue preparations shown in table 14 (calculated to baselines with 2-DG + Δ^1-TL). Clearly the percent inhibitions of glucolysis caused by Δ^1-TL form a spectrum that roughly parallels the glucolytic activities and established growth rates. Thus liver glucolysis was inhibited an average of 70%, very slow and slow-growing hepatomas 23–32%, intermediate hepatomas 13–20%, and the fast-growing 3683 hepatoma only 10%.

Insulin action: From the glucolytic inhibitions obtained with diethylstilbestrol and Δ^1-TL in the liver-hepatoma spectrum [(*9*) fig. 13; present MS table 15] it appeared likely that direct glucolytic stimulation by exogenously supplied insulin should be obtainable throughout the spectrum. While it has not been possible to demonstrate insulin-induced stimulation of glucolysis in every individual liver or tumor studied, direct glucolytic stimulation by insulin can often be demonstrated well beyond the experimental error involved. The patterns of response obtained indicate that in liver and hepatomas the insulin : anti-insulin control mechanism is of the same nature as that found in nonhepatic

TABLE 15.—Percent inhibitions of anaerobic glucolysis by Δ^1-TL in the rat liver-hepatoma spectrum shown in table 18*

Tissues	Percent inhibition of glucolysis by Δ^1-TL (190 μg/cc)			Number of experiments
	Minimum	Average	Maximum	
Liver (normal and host)	47	70	100	11
Very slow-growing hepatomas (7787, 7794B, 7795, R-1)	12	23	35	7
Slow hepatomas (5123B, C, D; 7794A)	27	32	40	4
Intermediate hepatomas				
HC (Rechcigl)	3	15	28	5
LC (Rechcigl)	10	20	27	5
7288C (ip)	.....	13	.....	2
Advanced hepatoma 3683	.....	10	.....	1

*Data from same experiments as in table 14 (0.2–0.5% glucose).

normal mammalian tissues and cancers (*1*, *4–6*, *9*, *25*, *26*). Table 16 shows stimulatory effects of insulin in two different livers of (C × DBA)F_1 mice that had been exposed for 20 hours to an environmental temperature of 37°C just before being killed. During exposure to 37°C they were given 5% glucose in the drinking water. Anti-insulin restraint of glucolysis appeared to be very strong, since large and significant insulin effects occurred only at the highest glucose levels (2%). A similar relationship between degree of insulin : anti-insulin action and glucose (or fructose) concentration has been described for nonhepatic tissues [(*5*) p 471–479] and for hepatic tissues [(*9*) p 855–856].

Table 17 further illustrates the relationship between substrate concentration and the effect of insulin in hepatoma 5123C which has a lower degree of susceptibility to anti-insulin regulation of glucolysis than does liver. In this instance exogenous insulin did not increase the rate of glucolysis until a concentration of 0.025% glucose was added to the incubation medium. Thus the approximate maximum rate of glucolysis occurred at 0.025% glucose, only in the presence of added insulin. At the highest glucose level (0.1%) the endogenous level of insulin was already rate saturating.

Table 18 shows the pattern of response to insulin in slow-growing hepatoma 7800 where, in the

TABLE 16.—Stimulation of anaerobic glucolysis in mouse liver by insulin*

Measurements	Mouse #1					Mouse #2				
	1% 2-DG	1% glucose Insulin		2% glucose Insulin		1% 2-DG	1% glucose Insulin		2% glucose Insulin	
Total acid in 100 minutes	53, 50	89, 76	63, 88	76, 73	104, 104	61, 71	70, 76	74, 77	78, 77	94, 92
Average mm³ CO_2	52	83	76	75	104	66	73	76	78	93
Δ glucolysis (mm³ CO_2)	—†	31	24	23	52	—†	7	10	12	27
$Q^{N_2}_A$ glucolysis	.....	0. 56	0. 44	0. 42	0. 94	.....	0. 13	0. 18	0. 22	0. 49
Insulin effect										
CO_2	.....	.....	−7	.....	29	.....	.....	3	.....	15
Percent	.....	.....	−20	.....	126	.....	.....	43	.....	125

*(C × DBA)F_1 male mice *ca.* 11 months old, held 20 hours at 37 °C air temperature, with 5% glucose in the drinking water; 100 mg wet (33 dry) weight of slices per vessel in 4.5 cc of K-R-B with 0.25% $NaHCO_3$ + Na pyruvate.

†Glucolysis zero.

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TABLE 17.—Stimulation of anaerobic glucolysis in slow-growing hepatoma 5123C (generation 46) by insulin*

Measurements	0.5% 2-DG		Endogenous		0.025% glucose		0.1% glucose	
	Insulin		Insulin		Insulin		Insulin	
Total acid in 190 minutes	41, 46	42, 41	58, 55	57, 58	84, 77	104, 100	108, 110	106, 92
Average mm³ CO_2	44	42	57	58	81	102	109	99
Δ glucolysis (mm³ CO_2)	—†	−2	13	14	37	58	65	55
$Q_A^{N_2}$ glucolysis (mm³ CO_2)		0	0. 14	0. 15	0. 39	0. 61	0. 68	0. 58
% insulin effect		0		8		57		−15

*One hundred and fifty wet (30 mg dry) weight of tissue per vessel in 4.5 cc K-R-B with 0.25% $NaHCO_3$ and 0.1% pyruvate. Insulin, HGF-free 192-235 B, Lilly 8 μg/cc.
†Glucolysis zero.

presence of 0.2% added glucose, a small (11%) stimulation of glucolysis by insulin was increased to 57% in the presence of Δ^1-TL. Such counteractions by insulin of steroid-induced inhibitions of glucolysis are characteristic also of nonhepatic tissues in which the insulin:anti-insulin system is operative. Table 19 summarizes a series of experiments in which insulin stimulated glucolysis in mouse livers and in the rat liver-hepatoma spectrum. Two experiments with S91 mouse melanoma are included for comparison. Clearly, insulin-induced stimulations of glucolysis can be demonstrated throughout the liver-hepatoma spectrum except that in fast-growing hepatoma 3683 the use of fructose in place of glucose was necessary to obtain an anti-insulin action sufficient to require exogenous insulin [*cf.* also (*9*) text-fig. 14]. Similar results with fructose have been obtained in other tissue systems (*5, 25*).

DISCUSSION

Nonglycolytic Reduction of DPN or TPN and Action of Pyruvate

Oxidation of a mole of either $DPNH_2$ or $TPNH_2$ yields one mole of OH^- ions, and the reduction of a mole of either nucleotide produces one mole of H^+ ions. Therefore, any nonglycolytic reduction of DPN or TPN could account for some of the increased acid formation after addition of these substances. Such acid formation, even though occurring at the expense of endogenous substrate,

TABLE 18.—Effects of insulin on anaerobic glycolysis in slow-growing hepatoma 7800 (generation 29) in the presence of 2-DG or glucose plus and minus Δ^1-TL*

Measurements	0.5% 2-DG				0.5% Glucose			
	Insulin		Δ^1-TL Insulin		Insulin		Δ^1-TL Insulin	
Total acid in 120 minutes	72, 78	80, 80	70, 70	66, 70	118, 114	120, 121	90, 92	107, 98
Average mm³ CO_2	75	80	70	68	116	121	91	103
Δ glucolysis (mm³ CO_2)	5	10	—†	−2	46	51	21	33
$Q_A^{N_2}$ glucolysis (mm³ CO_2)	0. 07	0. 14	—	—0. 03	0. 67	0. 74	0. 30	0. 48
% inhibition of glucolysis by Δ^1-TL							54	35
% stimulation of glucolysis by insulin						11		57

*One hundred and fifty mg wet (34.5 mg dry) weight of tissue per vessel in 4.5 cc K-R-B with 0.2% $NaHCO_3$ and 0.2% pyruvate, Δ^1-TL 190 μg/cc, insulin (W-1302 Lilly) 8 μg/cc.
†Rate with 2-DG + Δ^1-TL taken as baseline (= zero glucolysis).

TABLE 19.—Hepatic tissues in which insulin stimulated anaerobic glucolysis or fructolysis, with S91 melanoma for comparison*

Tissues and date of experiment	mg glucose /cc	μg Δ¹-TL /cc	Minus Δ¹-TL			Plus Δ¹-TL		
			$Q_{A}^{N_2}$ glucolysis		% insulin effect	$Q_{A}^{N_2}$ glucolysis		% insulin effect
				+ insulin			+ insulin	
Mouse liver								
2/25/65	20	—	0. 42	0. 94	126	—	—	—
2/25/65	20	—	0. 22	0. 49	125	—	—	—
2/4/65	10	—	0. 47	0. 71	52	—	—	—
Normal rat liver								
1/7/65	10	—	0. 83	1. 31	58	—	—	—
2/1/65	5	—	0. 32	0. 50	56	—	—	—
5/10/67	5	98	0. 23	0. 30	26	0. 14	0. 26	86
5/12/67	5	98	0. 37	0. 53	42	0. 28	0. 42	49
Host rat (5123C) liver								
12/28/64	5	—	0. 33	0. 73	122	—	—	—
Hepatoma 7794B (gen. 6)								
2/3/67	2	—	0. 38	0. 59	58	—	—	—
Hepatoma 7800 (gen. 29)								
5/9/67	2	190	0. 67	0. 74	11	0. 30	0. 48	57
Hepatoma 5123C (gen. 46)								
2/9/65	0. 25	—	0. 39	0. 61	75	—	—	—
Hepatoma 7288C (gen. 34)								—
2/15/65	1	—	2. 05	2. 50	31	—	—	
Hepatoma 3683 (gen. 303)								
2/11/65	1	—	13. 7	13. 4	−2	—	—	—
	16. 6 (fructose)	—	7. 2	9. 4	30	—	—	—
S91 melanoma								
9/9/66	2	74	12. 3	15. 4	25	10. 3	14. 6	42
9/12/66	2	172	9. 8	13. 2	35	7. 7	10. 2	32

*Tissue suspended in K-R-B with 0.2–0.25% $NaHCO_3$ and 0.1–0.2% pyruvate. Baseline glycogenolysis calculated from rate minus added glucose but plus 2-D G (0.5%) or 2-D G plus Δ¹-TL. Insulin 8 μg/cc. Q values are averages of duplicate determinations.

would not be a true glycolysis (conversion of carbohydrate to lactic acid via the Emden-Meyerhof pathway).

That nonglycolytic reduction of added DPN or TPN did not account for more than a minor part of the total acid formed in pyridine nucleotide-activated glycolysis in liver or hepatoma slices was indicated in several ways. First, the amount of increased acid formation, after the addition of DPN or TPN in many experiments involving all types of hepatomas and livers studied, far exceeded the total amount of nucleotide given, even assuming that all the DPN or TPN was reduced. An accelerated rate of acid production was often still proceeding after the increase in CO_2 production was already three times the amount equivalent to all the nucleotide added. Second, the simultaneous or prior addition of sodium pyruvate in a large molar excess (relative to the DPN or TPN added) did not quench the rate of acid production, but actually increased it. If the accelerated rate of acid production had been due primarily to a mere reduction of the added DPN, excess pyruvate and LDH present would have resulted in a rapid reoxidation of the reduced nucleotide with concomitant OH^- formation canceling the increase in CO_2 production due to the reductive process.

Activation of anaerobic glycolysis by pyruvate was reported for Jensen rat sarcoma by Dickens and Greville (*27*) and Rosenthal (*28*), and for

liver by Rosenthal (*29–31*). Yushok (*32*) found an initial lag period in anaerobic glycolysis in washed K-2 ascites tumor cells that could be eliminated by 0.001 M pyruvate, or by prior treatment with oxygen. Most significant, Yushok noted [(*32*) p. 109] that ". . . it is possible that the presence of oxygen or of pyruvate may counteract the lag period by shifting the ratio of reduced to oxidized diphosphopyridine nucleotide in favor of the oxidized form." As described in preceding sections of this paper and in earlier publications (*7*, *8*, *16*, and *17*), our data support the view that pyruvate activates anaerobic glycolysis in liver and hepatomas by helping to maintain an adequate DPN/$DPNH_2$ ratio.

Inhibition of Glycolysis by 2-DG

Since liver slices produce considerable amounts of glucose during *in vitro* anaerobic incubation, often up to 0.2% in the surrounding medium, determinations of the full extent of the glucolytic capacity requires a correction for endogenous glucolysis as well as for endogenous glycogenolysis (*5*, *7–9*, *16*, *17*). In the present paper we have presented further data on the use of 2-DG ($\pm$ Δ^1-TL) as a means of quenching endogenous glucolysis in liver or in some hepatomas which may also produce very low levels of endogenous glucose. Under certain conditions, such as aerobic incubation of cells (*33–35*) or in homogenates supplied with ATP (*36*), considerable phosphorylation of 2-DG takes place, and this could conceivably lead to generation of enough acid, or sequestration of enough inorganic phosphate (Pi), to vitiate the use of 2-DG as a blocking agent in measuring endogenous glucolysis. However, from a consideration of the literature (*35*, *37–40*) as well as the data from our own experiments, clearly with intact cells under *anaerobic* conditions these counterindications to such use of 2-DG are either trivial or absent.

Action of Insulin

Our studies over the last 15 years have made it evident that a decrease in the level of insulin:anti-insulin control of the rate of glucolysis is one of the most fundamental and universal changes accompanying the malignant transformation of normal cells into cancer cells, or in the progressive increase in malignant growth capacities of already existing cancer cells. The data obtained in these and other experiments make it clear that liver and hepatomas show the same phenomena of insulin:anti-insulin behavior as do other mammalian tissues. The activation of glucokinase (hexokinase) by insulin, or increasing the availability of glucose by decreasing anti-insulin (steroid) inhibition of the enzyme, may well represent a master control reaction that sets in motion a series of anabolic reactions leading to increased nucleic acid synthesis, increased protein synthesis (including more glucokinase), and finally cell growth and multiplication. The "glucolytic potential" as measured in terms of the anaerobic glucolytic capacity correlates well with growth in melanomas (*1*, *3*, *6*), tissue culture fibrosarcomas (*4*), and hepatomas (*7–9*). The key factor may be the critical balance in glucose-6-phosphate formation in growth-controlling regions of the cell (*e.g.*, mitochondria and/or ribosomal regions of the cytoplasm).

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