On the Significance of Glucolysis for Cancer Growth, With Special Reference to Morris Rat Hepatomas [1,2]

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SUMMARY—Anaerobic fermentation (glycolysis of glucose) was measured in a progressive growth spectrum of normal and host rat livers (zero net growth) through very slowly growing, slowly growing, intermediately growing, and rapidly growing lines of Morris rat hepatomas, maintained as slices in Krebs-Ringer bicarbonate-glucose-pyruvate medium at 37 C with 5% CO_2 in N_2 as gas phase. All hepatoma lines, including even the most slowly growing, showed glucose fermentation values in excess of those of normal or host rat livers. A continuous, positive correlation was observed between *in vivo* growth rate and *in vitro* anaerobic glucolytic potential; also, of equal importance, $K_{m\text{-}glucose}$ (concentration of glucose required for half-maximum rate of fermentation) and degree of inhibition by the anti-insulin stilbestrol decreased with increasing growth rate. Recent data of Gullino are cited which provide overwhelming evidence as to the actuality of *in vivo* fermentation of glucose to lactate by slow-growing hepatomas of the Morris series, compared to *in vivo consumption* of lactate normally observed with livers. The results reported make clear that a dominant biochemical difference between a normal metabolism and one characteristic of even the lowest degree of malignancy studied involves a critical, however small, increase in glucolytic capacity over that in the normal tissue of origin, in this instance, liver. Progression in malignant growth capacity is associated with increasing fermentation capacity. These results and conclusions confirm and extend the well-known findings of Otto Warburg over the past 40 years and our similar findings made with mouse melanoma spectra and Sanford-Earle tissue

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[2] The 1965 Gerhard Domagk Prize for Cancer Research was awarded for work detailed in this paper demonstrating for the first time the connection between cellular glucose fermentation and growth rate in a continuous, positive spectrum of Morris rat hepatomas compared with normal or host liver.

[3] It is a pleasure to acknowledge the aid and interest of Harold Morris, Billie Wagner, and Pietro Gullino, of the National Cancer Institute, in generously supplying the hepatomas used, for suggesting many types of experiments, and for providing important correlative data. In this paper the terms "submininal, minimal, intermediate, or advanced" hepatomas refer to the cellular parameters of growth rate, glucolysis, or stilbestrol inhibition that we have found to form experimentally demonstrable, continuously increasing spectra, and do not necessarily refer to various subcellular or enzymic parameters unless they, too, show such spectra. We use the term "glycolysis" as originally defined by Warburg (*1*, p 199) as ". . . the splitting up of carbohydrate into lactic acid," or such acid as may be measured manometrically. When the carbohydrate is glucose, one has, more specifically, *glucolysis*, when fructose *fructolysis*, when glycogen *glycogenolysis*, etc.

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culture spectra, and, as reported here, also in the Hilf rat mammary tumor spectrum. Implications of these findings for chemotherapy and hyperthermy therapy of cancers are briefly indicated.—J Nat Cancer Inst 38: 839–863, 1967.

THE FUNDAMENTAL metabolic property of all cancer cells so far tested under suitable conditions was first discovered over 40 years ago by Warburg (*1*), namely, their fermentation of glucose to lactic acid. This glucolytic fermentation in various living forms was well known to Berzelius, Liebig, Pasteur, and Claude Bernard, as was alcoholic fermentation. As Pasteur showed over 100 years ago, such types of fermentation were commonly prevented by the presence of oxygen gas. The profound significance of this effect was elaborated on and named the Pasteur Effect by Warburg in 1926 (*1*, p 246). He reported then that all cancer cells he studied *in vitro* and *in vivo* produced lactic acid under aerobic conditions, albeit to a still greater extent under anaerobic conditions *in vitro*. A relatively few normal and other noncancer cells produced some lactic acid from glucose aerobically under *in vitro* conditions arranged to simulate *in vivo* conditions as far as possible, though by no means perfectly. Nevertheless, no *growing* normal tissue could be demonstrated to do so under *in vivo* conditions in the animal body, which is the ultimate criterion of significance.

Warburg's cancer studies since 1950 (*2–4*) on the highly anaplastic, rapidly growing ascites cancer cells led him to the more general conclusion that the shifting of the metabolism to the anaerobic (fermentative) state is the main biochemical difference between the tumor and normal cell. The best *in vitro* indicator of this shift is the increase in anaerobic glucolytic potential, much more critically so than aerobic glucolysis, which *in vitro* is " . . . too labile and too dependent on external conditions" (*2*, p 309). Warburg has increasingly emphasized the facultative fermentative way of life of cancer cells, involving a permanent adaptation to potential survival and growth under anaerobic or near anaerobic conditions (*2–4*): "Die Krebszellen fakultative Anaerobier sind, im Gegensatz zu den normalen Zellen, die ohne Ausnahme nur aerob wachsen kann (obligate Aerobier sind)." [5] The extreme forms of rapidly growing ascites cancer cells can produce lactic acid from glucose anaerobically at a sustained rate probably faster than any other living mammalian tissue—up to half the tissue dry weight per hour. Even a hummingbird, whose wings may beat up to at least one hundred times a second, consumes at best only half its dry weight of glucose equivalent per day (*5*).

In the past decade, Morris, Wagner, and coworkers at the National Cancer Institute (*6–9*) have created a graded series of rat hepatoma tumor lines that extend far down into the slow-growing end of the cancer growth-rate spectrum. These workers have produced a large number of relatively stable lines of liver tumors that vary from a few weeks up to over a year for attainment of full size (25 g or more). Such lines of hepatomas, derived from parenchymal rat liver cells on treatment with various chemical carcinogens, offer special promise for study in their so-called "minimal deviation" forms, namely, those lines with the slowest growth rates and the least biochemical or other deviations from the normal host tissue of origin, liver. Such minimal deviation hepatomas should in principle permit a closer distinction between essential and incidental differences between a cancer cell and its cell of origin than heretofore possible. Whereas several series of tumor spectra have been available in recent years, the Morris hepatomas currently offer the widest range of comparable growth rates as one proceeds from adult liver (with no net growth) through minimal deviation, intermediate deviation, and advanced deviation hepatomas. Before any such tumor spectra were available, one was limited to grosser comparisons between all cancers as a whole and the bulk of normal tissues as a whole.

Many biochemical studies on the Morris hep-

[5] Personal communication, April 2, 1966.

atoma spectrum have already been carried out, but most of these have involved preparations of nonliving broken-up cells (homogenates, particulates, supernatants), with results whose applicability to the living or *in vivo* state often remains highly questionable.[6] Many individual enzyme or composition differences have occurred among subcellular preparations of normal liver and the various hepatomas, as might be expected from the enzymic complexity of liver. However, no quantitatively fundamental and universal enzyme or composition distinction between liver preparations and hepatoma preparations, such as might have been hoped for, has as yet been established.[7] A reason for this state of affairs need not be far-sought, when it is realized that nothing in the past extensive history of cancer biochemistry investigations, whether aimed, unaimed, or aimless, has encouraged much expectancy or even hope in this direction, except for the fermentation component, enhanced (*1–4*) or in the other ways altered [8] (*11–27*) in so many thousands of living cancers. This definitive component may not be the only definitive component, but it is the only one found with great regularity so far, and its present uniqueness in this respect is, peculiarly, shied away from by many workers in the Morris hepatoma field (*28–45*) with, if ever Isaiah was right, eyes that have seen not, ears that have heard not (*46*), and therefore pens that have cited not. As Pasteur wrote for the motto on the cover and title page of his book (*47*), "Le plus grand dérèglement de l'esprit est de croire les choses parce qu'on veut qu'elles soient."

Studies on the biochemistry of living liver and Morris hepatoma cells and tissues, *in vitro* or *in vivo*, have been far fewer than those on subcellular preparations thereof; on the comparative glucolysis in these living tissues there are but three or four independent contributions (*33–36*) of very limited experimental scope. Curiously, however, these brief contributions, methodologically inadequate in many respects of commission as well as omission,[9] have led to widely echoed conclusions or implications to the effect that minimal deviation hepatomas have no significant glucolysis above that in their normal tissue of origin (liver), and that the generality set forth long ago by Warburg for cancers he then studied is now faced with a notable, undermining, and far-reaching exception. An example of an extreme, recently published expression of this inadequately supported and much-bandied outlook is: "The minimal deviation hepatomas fail to glycolyze glucose aerobically or anaerobically, . . . resemble normal liver as closely as one sample of liver resembles another, . . . do not remotely resemble the tumor tissue studied by Warburg"

[6] *See* table 3 and discussion thereof. Carefully prepared and maintained *in vitro* cellular tissues (slices, cell suspensions) have long been generally regarded as "living," though outside the body. They have demonstrable ability to grow on transplantation into appropriate host or tissue culture and metabolic behaviors often closely resembling such cells *in vivo* where tested, in ways not exhibited by tissue homogenates or fractions thereof. It is incorrect to require, as has occasionally been suggested, a restriction of the term "living" to cells *in vivo*.

[7] An exception to this statement might be the reported absence of thymidylate synthetase in adult normal rat liver (*10*), were it not for the fact that regenerating adult and embryonic normal rat livers contained large amounts of this enzyme, in the range of the rat hepatomas studied.

[8] The alterations in glucolytic capacity that distinguish the cancer cell from its corresponding normal cell of origin include a chronically lowered susceptibility of glucolysis to anti-insulin steroid regulation of the glucokinase step, increased affinity for glucose, and increased rate of lactic (or equivalent) acid production anaerobically at physiological glucose concentrations. The critical biochemical lesion is the loss in susceptibility to anti-insulin steroid regulation. Various lines of evidence indicate that the altered glucokinase activity is a *primary* lesion underlying uncontrolled growth or degree of escape from host suppression of growth (*11–27*).

[9] Thus in (*33–35*) no distinction was made between anaerobic glucolysis and (endogenous) "glycogenolysis"; no data were reported on the differential effects on either of these types of glycolysis by inhibitors such as 2-desoxyglucose, *N*-acetylglucosamine, or anti-insulin steroids, with either liver or minimal deviation hepatomas; glucose concentration functions ($K_{m\text{-}glucose}$) for glucolysis were not reported, nor in (*33, 34*) was use of any glucose at all in the glycolysis measurements specified; no account was given of time course decays due to accumulation of $DPNH_2$ that inhibits glycolysis at the triosephosphate dehydrogenase level, somewhat differently for glucolysis than glycogenolysis; fortifying oxidants suitable for eliminating $DPNH_2$ and yielding maximum potential glycolysis rates were not used; and the liver glycolysis values reported differed by as much as an order of magnitude from values reported in the classic series of papers by Rosenthal (*48–53*) and much confirmatory work since, including that of Orr and Stickland (*54*), Negelein and Noll (*55*), and Dickens and Weil-Malherbe (*56*), all of whom were well aware of the important effect of liver glycogen content on liver glycolysis rate.

(Potter, *45*, p 193). The numerical data accompanying and used to support these conclusions are reproduced in table 1 taken from the same page of (*45*). With only this minnow of data at hand, William of Occam himself might conceivably have come to the same conclusions, but assuredly with a Triton of suspended judgment.

TABLE 1.—Data of Aisenberg and Morris (*33*, *34*) cited by Potter (*45* p 193) re glucolysis of liver and minimal deviation hepatomas

	$Q_{lactic\ acid\ (glucose)}$	
	Anaerobic	Aerobic
Normal liver	1.0	0.6
Hepatoma 5123	1.0	0.5
Hepatoma 7800	1.0	1.0
Hepatoma H-35	1.0	0.9

The just-quoted conclusions involve the heart of the question of whether cancer cells generally do or do not possess a common, definitive, metabolic alteration, for obviously, in the Morris series of parenchymal cell hepatomas, we have the best possibility as yet for making a critical comparison of such cancers with their tissue of origin: parenchymal cells of normal adult liver. If the quoted conclusions of Potter are correct, then the world certainly loses the one and only broad definitive biochemical characteristic of all [10] cancers that was, until recently, generally regarded as established.

We present in this paper a part of a wealth of experimental facts we have gathered on the *in vitro* metabolism—mainly in terms of anaerobic glucolysis—found in studies on the spectrum of Morris hepatomas at the cellular level. In our opinion, the results presented invalidate the conclusions of Potter. To this we add foreground and background data on the nature and mechanism of glucolytic control by insulin and anti-insulins (*e.g.*, steroids) that greatly extend the certainty of a definitive cancer glucolysis and thereby the value of a rational cancer therapy based on such a generality.

MATERIALS AND METHODS

All rats and tumors were maintained by Dr. Harold P. Morris and co-workers on standard diets supplied *ad libitum*, although in certain instances animals were subjected to periods of starvation or other special treatments for comparative purposes (*e.g.*, wire bottoms vs. sawdust bedding to vary degree of stress; composition of the food pellets) with results of no evident significance for the present study. Hepatomas H-35, H-35TC$_2$, 3924A, and 3683 were grown on ACI/N rats, and all other hepatomas on Buffalo rats. When the tumors had reached a sufficient size, the host animals were sacrificed, and livers and tumors rapidly removed to petri dishes placed on crushed ice. The tumors were carefully dissected free of hemorrhagic, necrotic, and nontumorous material and then "sliced" with a sharp safety razor blade and forceps to pieces 1 mm thick or less. Certain hepatomas (*e.g.*, 5123 series; 3683) consisted of loosely adherent cells and required little or no sectioning. After the separated tissue was thoroughly mixed, representative portions of usually 125–200 mg fresh weight, as determined rapidly with a Roller-Smith torsion balance, were transferred to manometric vessels (total volume 15–20 ml) immersed in cracked ice and containing normally 4.5 ml of Krebs-Ringer 0.25% $NaHCO_3$ medium, with glucose, 0.01–0.1% sodium pyruvate and 2-desoxyglucose (2-DG), or other additions as indicated in the text, tables, or text-figures. Separate fractions of tissue were dried in a hot-air oven (*ca.* 50 C) to constant weight for determination of initial dry weights. Dry weight ran close to 33% of the wet weight of liver, 28% in very slow-growing hepatomas (*e.g.*, 7787), 20% in intermediate (7288c) and slow-growing (5123) hepatomas, and 16% in fast-growing (3683) hepatomas. These dry weights have been remarkably constant and characteristic for each type of tissue. With all hepatic tissues it is most important to employ "initial" dry weights rather than "final" dry weights obtainable at the ends of experimental runs, where, in long experiments, up to 50% of the tissue may not be readily recoverable. This could lead to Q values up to 50% higher than those based on initial dry weights, and values reported in the literature must always be recon-

[10] Morris (*6*) lists some 10 purported biochemical alterations in various hepatomas, but these are not characteristic of all cancers.

sidered in this light, especially determinations made decades ago.

After the vessels were filled, they were attached to the manometers, rapidly transferred to the thermostat at 37 C, and gassed for 10–15 minutes with the appropriate gas mixture. For anaerobic studies, 5% CO_2/N_2 was used, being first passed over hot copper with a little H_2 to remove traces of O_2. Aerobic incubations were carried out in an atmosphere of 5% CO_2/O_2. After the vessels were gassed, a few minutes were allowed for equilibration, and manometric readings were commenced.

In certain experiments trichloroacetic acid to 5% was added to the tissue suspensions after incubation for varying lengths of time and the centrifuged supernatant used for determination of lactic acid by the Barker-Summerson procedure (*57*), and/or glucose measurements by the Glucostat method (*58*). Similar replicate vessels were treated at time zero for base-line comparisons. Such experimentation will be detailed elsewhere (*27*).

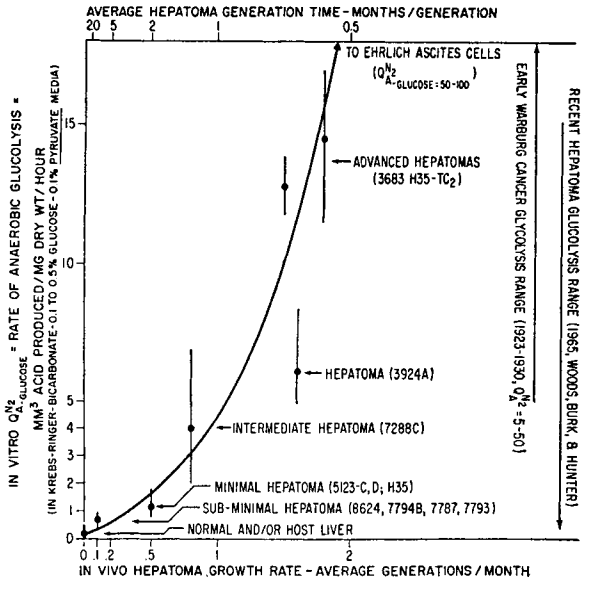
All preparations of diphosphopyridine nucleotide (DPN) were Sigma DPN grade II from yeast containing 0.3% sodium and were dissolved in Krebs-Ringer medium; the *p*H was adjusted to 7.2–7.6 with NaOH. Triphosphopyridine nucleotide (TPN) was Sigma, monosodium salt (reported purity 98%), and was similarly dissolved and the *p*H adjusted to 7.2–7.6. Reduced pyridine nucleotides were Sigma $DPNH_2$ disodium salt type I, prepared enzymatically from yeast DPN by alcohol dehydrogenase. Solutions of $DPNH_2$, prepared freshly each day in Krebs-Ringer medium (*p*H 7.6 without adjustment), were stable for several hours at 37 C. $TPNH_2$ (Sigma type II) was the tetrasodium salt having a reported purity of 98% and was prepared immediately before use. The amounts of $DPNH_2$ listed in the tables or figures are based on spectrophotometric assay at 340 μ or on the amounts of actual nucleotide as measured by a manometric assay (*59*) involving pyruvate and lactate dehydrogenase (LDH). The glucose used was Merck anhydrous reagent dextrose; 2-DG was Mann chromatographically pure (sp. rot. +45 in water, C = 2); *N*-acetylglucosamine (NAGA) was supplied by K and K Laboratories, Plain View, New York, and pyruvate was the Sigma sodium salt, type II. The insulin was kindly supplied by Dr. Otto Behrens of the Lilly Research Laboratories, Indianapolis, Indiana, and was amorphous lot 192-235 B-188 from trypsin-treated crystalline insulin. It was essentially devoid of glucagon.

RESULTS

Anaerobic Glucolysis as a Function of Growth Rate in the Morris Hepatic Spectrum

Text-figure 1 and table 2 present a summary of typical results obtained in *in vitro* manometric measurements of anaerobic glucolysis of normal adult liver compared with that of increasingly rapidly growing hepatoma lines. Aside from our preliminary reports (*4*, *23–26*), no such spectrum based on living Morris hepatoma cells is available in the literature. It is evident in text-figure 1 that there is a *progressively increasing*[11] glucolysis as one proceeds up the spectrum of increasing growth rate, and that at the upper and extrapolated range beyond the indicated ordinate scale the values fall in line with the range heretofore obtained with thousands of more rapidly growing tumors, as first reported by Warburg and innumerable workers since. In short, there is a quantitative as well as qualitative fit of agreement between the older and the new hepatoma data.

[11] Sweeney *et al.* (*36*), working with Morris hepatomas in cellular form *in vitro*, under *aerobic* conditions, refer to a "spectrum," but state that minimum deviation hepatomas produce lactate at rates no higher than and, on the average a little lower than, normal liver. Clearly this "spectrum" bears no resemblance to the anaerobic spectrum of text-figure 1 with respect to the critical region in question, namely, the slowly growing hepatomas. The same lack of positive continuity is also found with respect to subcellular ATP-glucose-phosphotransferase (hexokinase-glucokinase) activity, as will be shown in table 3. When Weber and Lea (*32*, vol IV, p 123 and, similarly, *29*) write in 1966 that "The correlation of glycolysis with growth rate was recently also recognized by Burk and associates, yet their report in form of abstracts at a cancer meeting was presented two years after publication of our papers having established this relationship (*3*, *4*) and subsequent to a number of our publications in which this concept was outlined (*5–10*)" they overlooked our earlier experimental paper (*20*) submitted for publication in 1961, and, as just indicated, that our reported hepatoma "relationship" (spectrum) is *continuously positive* with respect to liver, including glucolytic differentiation between the latter and the slowly growing hepatomas.



TEXT-FIGURE 1.—*In vitro* anaerobic glucolysis versus *in vivo* growth rate of liver-Morris hepatoma spectrum. Points represent averages of available comparable determinations in Krebs-Ringer 0.25% $NaHCO_3$, 0.1–0.5% glucose, 0.1% pyruvate medium, initial *p*H 7.4, with 5% CO_2/N_2 in the gas phase. *Vertical lines* through the points indicate range of ordinate determinations. The points are based on averages of several to many determinations made on each class of hepatoma or liver (table 2). Average hepatoma generation time refers to average number of months required for growth from an inoculum of *ca.* 0.1 cm³ tumor tissue to an average size of about 25 cm³ at the next transplant, not to average cell generation time (average cell doubling time). Average cell generation time=$g_{cell} = 1/t\ (ln)\ m/m_o$, where t is time, m is final tumor mass, and m_o is initial tumor mass. Then when average hepatoma generations/month=1, this 250-fold increase in tumor mass means, in integrated form, about 8 cell generations a month ($25/0.1 = 250 = 2^8$), and, in differential aspect, g cell = (1/month) $ln\ 250$ = 5.5/month = 1.4/week = 0.18/day. Manometric determinations refer to experimental periods of 1 ± ½ hour, with media containing 0.1–0.5% glucose and (to minimize $DPNH_2$ accumulation and secondary glucolytic inhibition therefrom, *see later*) 0.1% sodium pyruvate. As determined in our experimentation, Q values in the lower growth-rate range, for single determinations on samples of a given lot of tissue, are accurate to within about 0.1 mm³ acid produced/mg initial dry weight/hour (*ca.* 4 mm³ acid produced/vessel) and more accurate for replicate determinations. The glucolysis values are calculated against baseline control vessels containing no added glucose, also often run with addition of inhibitors such as 2-DG or NAGA that decreases or eliminates any endogenous glucose utilization (NAGA only with liver, not hepatomas), as will be described in text-figures 5 through12 or text.

TABLE 2.—Number of specimens of each Morris hepatoma type and liver type reported in text-figure 1, with numerical average, minimum, and maximum glucolysis values*

Tissue	$Q^{N_2}_{CO_2}$-glucolysis (0.1–0.5% glucose in medium)			Number of individual specimens studied
	Minimum	Maximum	Average	
Normal liver.....	0.18	0.50	0.32	6
Host liver.....	0	0.68	0.33	26
Slowest growing hepatomas (subminimal deviation) 8624, 7794B, 7787, 7793...	0.40	1.00	0.69	9
Slow-growing hepatomas (minimal deviation) 5123B-D; H-35.....	0.58	1.80	1.03	13
Hepatomas (7288C) intermediate deviation.....	2.00	6.40	4.10	15
Fast-growing hepatomas (advanced) 3924A.....	4.5	8.0	6.7	3
H-35TC_2, 3683.....	11.8	17.5	13.7	6

*The same conclusions are reached whether one uses the average, minimum, or maximum values: Glucolysis is a continuous, positive function of increasing growth rate. In no single instance, for a liver and slow-growing hepatoma from the same host rat, was the liver glucolysis ever as high as the hepatoma glucolysis. The absolute Q^{N2}_{Co2} value of a liver or slow-growing hepatoma depends to some extent on the degree of stress of the particular host rat, and hence there is some overlap in range values at low Q^{N2}_{Co2} values. This table includes an occasional experiment performed after text-figure 1 was prepared, but without alteration of significance. The transplant generations studied were: (slowest growing) 2, 4, 5–6 and 8–10 for lines 8624, 7794B, 7787, and 7793, respectively; (slow-growing) 31, 30–51, 41–42, and 40–55 for lines H-35, 5123C, 5123D, and 5123B, respectively; (intermediate-growing) 28–34 for line 7288C; and (fast-growing) 8, 192–287, and 297–303 for lines H-35TC_2, 3924A, and 3683, respectively.

One could scarcely expect all points in text-figure 1 to fall exactly on the smoothed curve drawn, since various factors in addition to growth rate can affect glucolysis, but the dominating trend in relation to growth rate is unmistakably demonstrated under the carefully chosen comparable conditions of experimentation, even down to the most slowly growing hepatomas, with liver falling below them. The ordinate differences between points at the lower end of text-figure 1 may appear small on an absolute basis, but this is because the scale is chosen to show the whole

hepatoma spectrum range. The *relative* differences between liver and slowly growing hepatomas are large, and represent large differences in terms of actual manometric readings involved (*cf.* table 2, and text-fig. 3). We have seldom, if ever, found a slow-growing hepatoma whose glucolysis was not unmistakably, well beyond the limit of experimental error, *greater than the glucolysis of the liver from the same host rat measured simultaneously under otherwise identical experimental conditions*, including the same prior treatment of the mutual host and therefore identical degree of host stress. Since the hepatomas themselves, like most cancers, produce some host stress, conceivably a large number of determinations on livers from hepatoma-bearing and hepatoma-free animals might show a small, statistically significant difference between such livers, but with physiological concentrations of glucose supplied (0.5–2 mg/cc), we have not as yet detected such a difference.

Looking at the entire spectrum (text-fig. 1 and table 2), one sees that a surprisingly small increase in glucolytic fermentation potential suffices, as a seemingly essential factor, to permit liver cells to dedifferentiate (or redifferentiate), from whatever additional "causes" that may be involved, into the slowly growing hepatomas. A causal connection between fermentation and malignancy, such as long strongly indicated for rapidly growing cancers, is now extended down to the very slow-growing hepatomas, whose full growth requires nearly the full lifetime of the host rat, and would probably require the entire lifetime if smaller tumor inocula were used. In short, the ultimate in "slow-growingness" has virtually been reached, and with it, the ultimate in connection between fermentation and "minimal" malignancy.

In Vivo Glucolysis by Minimal Deviation Hepatomas Growing on Host Rats Under Gullino Methodology

The experimentation of text-figure 1 was carried out *in vitro* under conditions which decades of experience have indicated to be a close, though not perfect, approach to *in vivo* conditions, but within the framework of the bulk of conventional glucolytic determinations, short of the use of sera as suspending media.[12] In the last few years Gullino and co-workers have developed at the National Cancer Institute exact, highly controlled methods for studying, over periods of days, not only hours, the metabolic exchanges of glucose, lactic acid, O_2, and many other compounds in a variety of cancers, including the Morris hepatomas, growing *in vivo* in host rats (*60*). They have been able to measure rates of flow of blood into and out of a tumor, together with determinations of the concentrations of various compounds in the afferent and efferent blood, as well as in the interstitial fluid within the tumor, namely, the liquid phase interposed between the newly formed vascular walls of the tumor and the plasma membrane of the neoplastic cells. In all cancers studied they have observed high concentrations of lactic acid and low concentrations of glucose in the interstitial fluids, much higher and lower, respectively, than in the afferent or efferent blood. The lactate content of the interstitial fluid of the slow-growing minimal deviation Hepatoma 5123 was roughly comparable with that of Fibrosarcoma 4956, but considerably lower than that of the highly anaplastic Walker carcinoma 256, whereas the glucose levels were near zero in all three tumors.

Recently Gullino (*61*) measured afferent and efferent blood levels without removal of interstitial fluid and again with all cancers tested, including minimal deviation hepatomas, found a marked increase in lactate and decrease in glucose concentrations on passage of blood through the tumor. Small quantities of Hepatoma 5123 were inoculated into rat ovaries and when they had grown to 4–6 g were studied metabolically. The $Q_{lactate}$ found was 1.7 mm^3 lactate produced/mg final dry weight/hour, within a 95% confidence limit of ± 0.7 (9 degrees of freedom), whereas the respiration was $Q_{O_2} = 5.3$ mm^3 O_2 consumed/mg final dry weight/hour. In confirmation of the techniques yielding these values, the more rapidly growing Walker carcinoma in a series of 19 tumors

[12] We have found high potassium levels to retard the rate of anaerobic glucolysis in intact cells (melanoma, brain, ascites carcinomas), though such levels may be stimulating in subcellular glycolyzing preparations. Since we have been concerned here with maximum potential anaerobic glucolysis in intact hepatic cells, we have avoided high potassium levels.

(range 3–10 g) gave a $Q_{lactate}$ value of 5.2 ± 1.0, and, in remarkable confirmation of Warburg's observations (*1*, *3*) of lowered respiratory values in highly developed cancers, a Q_{O_2} value of 3.3, clearly lower than that of the slow-growing hepatomas: The ratio of $Q_{lactate}/Q_{O_2}$ in the latter of 1.7/5.3 = 0.36 had increased to 1.6 in the rapidly growing Walker carcinoma. In both tumors the concentration of glucose was of the order of 50% lower in the efferent blood compared to the afferent blood, and the lactate was of the order of 10% higher. These values indicate fairly good circulation through the tumors, possibly a little better in the (on the average) smaller hepatomas, and the probability (but not certainty) that most of the lactate production by the cells was aerobic rather than purely anaerobic; efferent blood hemoglobin was of the order of 50% saturated with O_2.

Glucose consumption in the tumors was of the order of 2–3 times the lactate production in equivalent units, which indicated that the observed lactate formation represented true glucolysis accompanied by, under aerobic conditions, conversion of a reasonable fraction of the glucose also to CO_2, H_2O, other excretion products, and cellular constituents. With well-developed tumors, the blood lactate level in the plasma of the host was gradually raised from a normal level of about 30 mg% – 45 mg%, with additionally the indicated added difference between efferent and afferent blood. Tumors grown under the Gullino methodology represent a high percentage of cancer cells, with little or no necrosis or fibrocytic infiltration or capsule (the latter being replaced by the artificial parafilm capsule supplied in the technique).

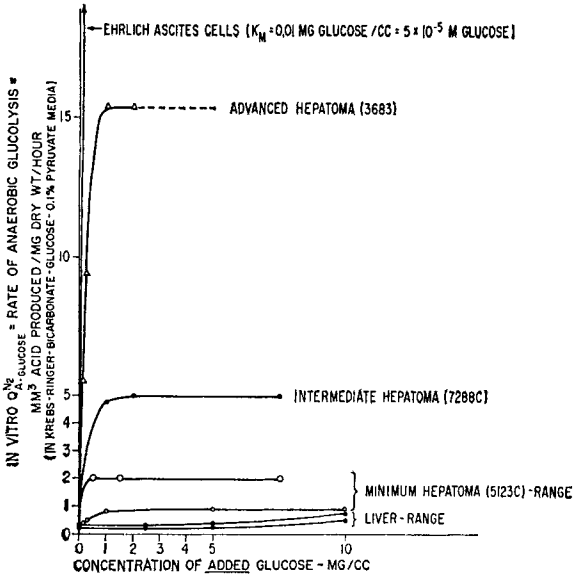
The results of Gullino (*61*) are in harmony with those of Warburg and the Cori's (*1*, p 254) obtained with spontaneous cancers 40 years ago by more casually selected and less reproducibly obtained tumor specimens *in vivo*, and is overwhelming evidence as to the actuality of the *in vivo* glucolytic potential in the slow-growing Morris hepatomas compared to similar experimentation with perfused liver *in vivo*, where ordinarily lactic acid is not produced but *consumed* in passage through this organ. The *order of magnitude* of aerobic $Q_{lactate}$ in the minimal deviation hepatomas *in vivo* is that of values we observe *in vitro* anaerobically for short time periods under our most optimum conditions in media fortified with pyruvate and DPN (text-figs. 5 through 12) and not greatly higher than the longer time averages in text-figure 1.

Effect of Added Glucose Concentration on *In Vitro* Anaerobic Glucolysis

Having established an absolute difference in cellular glucolytic potential between liver and the slow-growing hepatomas, we further found a variety of other glucolytic differences of equal or greater significance. One of the easiest of these to measure is the effect of added glucose concentration (text-figs. 2 and 3). Whereas the effect of added glucose concentrations on cellular liver glucolysis is seldom detectable until "unphysiological" concentrations of at least 0.5–0.75% are supplied, with full rate-saturation requiring at least 1–2% glucose, half-saturation rates among the hepatomas are usually attained by 0.05% glucose, and near saturation by 0.1% glucose, except for the twofold to threefold higher concentrations required for the very slow-growing Hepatoma 7787, which also has a very high glycogen content compared with any of the other Morris hepatomas studied so far. Our impression, not yet supported by sufficiently detailed data, is that there is an inverse functional relationship between *in vivo* growth rate and glucose concentration required to attain any given percentage of maximum *in vitro* glucolytic rate. In any event, it is quite evident from studies of glucolysis in intact cells that the development of the malignant state in any of the Morris hepatomas has involved a marked lowering in $K_{m\text{-}glucose}$[13] of the order of tenfold and more, though not to so low an extent as in Ehrlich ascites cancer cells, where, in our measurements, half-maximum rate is attained at about 0.001% glucose, and is still lower in the experiments of von Ardenne *et al.* (*65*).

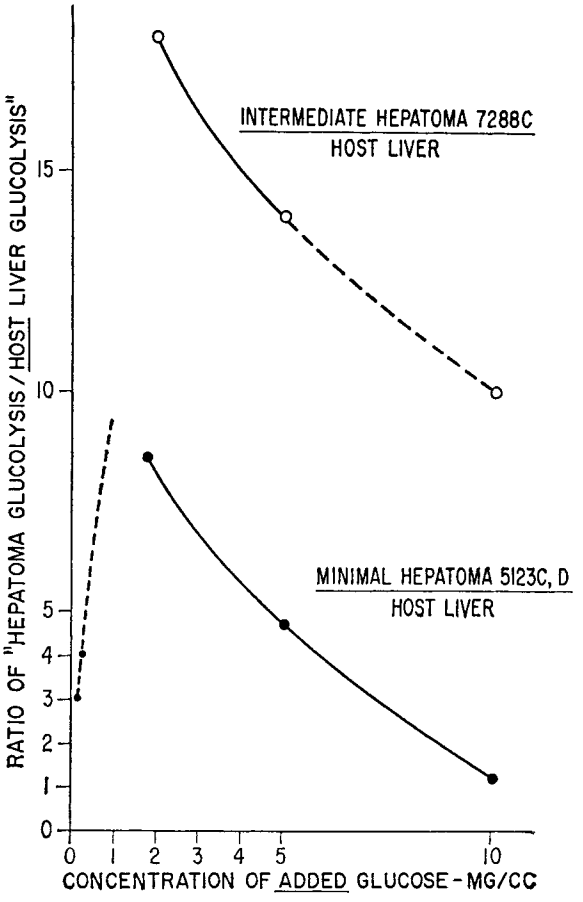
Other investigators (*37–39*, *43*, *44*) have reported markedly lower $K_{m\text{-}glucose}$ values for glucose phosphorylation in *subcellular* extracts of Morris 5123 and other hepatomas as compared with liver extracts. In the very slow-growing, relatively glycogen-rich Hepatoma 7787, Sharma *et al.* (*43*)

[13] Km of Michaelis and Menten (*62*), Haldane (*63*), and Lineweaver and Burk (*64*).



TEXT-FIGURE 2.—Glucolysis as a function of added glucose concentration in the Morris hepatoma spectrum.

found both a low $K_{m\text{-}glucose}$ hexokinase and a moderate-to-high $K_{m\text{-}glucose}$ glucokinase (as they called it), which more nearly resembled the liver pattern for this particular hepatoma. However, as we have already pointed out (*24*) and indicate in table 3, the glucose-ATP-phosphotransferase activities of all such solubilized preparations are much too high to account for the regulated rate of glucolysis in *intact living cells*, and therefore cannot be used to establish a rate limitation for the cellular process. Attempted correlations between *in vivo* growth rate and extractable glucophosphotransferase activity fail of explanation not only on an absolute rate basis but also in continuity, in that normal liver (with zero net growth rate) had a notably higher extractable glucophosphotransferase activity than did slow-growing minimal deviation hepatomas. Quantitative correlations between extractable glucophosphotransferase activity and glucolysis by hepatic *homogenates* (*38*, *39*) are more nearly possible, but are not the point of consideration here for living cell glucolysis. As long as the very great "discrepancy factor" shown in table 3 exists, it is impossible to conclude from the extract data that glucokinase or hexokinase activity *per se* limits cellular glucolysis, just because such activity is smaller than that of other extracted, *comparably measured* glucolytic enzymes: a line of



TEXT-FIGURE 3.—Ratio of "hepatoma glucolysis/host liver glucolysis" as a function of added glucose concentration. *Points* on *solid lines* refer to particular individual experiments, and *dotted lines* to approximations from several experiments. Only at very high, unphysiological glucose concentrations does the ratio approach 1 for the minimal deviation hepatoma, and never for faster growing hepatomas.

reasoning (or lack of reasoning) that is nevertheless a widely prevalent, popular fallacy. The main reason for differential glucolytic limitation in living cancer cells (and even more so in most normal cells) is to be found in the further endogenous (or exogenous) cellular inhibitors (*e.g.*, antiinsulins, *see later*) that specifically inhibit cellular glucophosphotransferase (hexokinase, glucokinase) activity; these inhibitors and their decreasing activity with increasing tumor anaplasticity and growth rate appear to be the underlying basis for the glucolytic differences between liver and Morris hepatoma cells, as we have found with other tumor spectra (*11–22*).

TABLE 3.—Comparison of "approximate order" of rates of *in vivo* growth, cellular glucolysis, and solubilized ATP-glucose-phosphotransferase (GPT) in Morris liver-hepatoma spectrum, with estimated "discrepancy ratio" between last 2 rates

Column parameter	A Average % dry weight of fresh tissue	B Average rate of growth (gen./mo.)	C Average 37 C observed (text-fig. 1)	D $Q^{N_2}_{Glucolysis}$ 24 C* calculated	E Average total (hexokinase + glucokinase) (GPT) of cell extract supernatant equivalent to $Q^{N_2}_{Glucolysis}$ at 24 C†	F Average "discrepancy ratio"‡ $= \frac{Q^{N_2}_{Glucolysis}(GPT)}{Q^{N_2}_{GPT}\ (cells)} = \frac{col.\ E}{col.\ D}$
Normal liver.....	33	0	0.3	0.1	8	80
Morris hepatoma submininal 7787, 7793.....	27	0.15	0.6	0.2	8.5	42
Minimal 5123A–D, H-35, 5123TC....	20	0.6	1.3	0.43	5.5	13
Intermediate 7288C.	20	1.4	4.1	1.4	17	12
Advanced 3683.....	16	2.0	14.5	4.8	48	10

*Calculated with an average reversible temperature coefficient for glucolysis of 3/13° between 24 and 37 C (as we have found in Hepatoma 5123TC for both glucolysis and glycogenolysis).

†Based on "average" of available recent data of Elwood *et al.* (*39*), Sharma *et al.* (*45*), and Shonk *et al.* (*44*), where reported as units of GPT per g wet weight of tissue, catalyzing the reduction of 1 μmole of TPN per minute at (in these experiments) 24 ± 2 C. Such units were converted to *equivalent* $Q^{N_2}_{GPT}$ = mm³ acid formed/mg dry wt/hr by multiplying by 60 (min/hr) × 2 (moles lactic acid/mole glucose) × 22.4 (mm³ acid formed/μmole), all divided by the mg dry wt per g fresh tissue. The data of (*39*) and (*45*) were further divided by 1.7 (moles of $TPNH_2$ formed per mole glucose consumed), whereas the value of 1 was used for (*44*), as per indications of these various authors.

‡The "discrepancy ratios" here given refer to *average*, not highest individual, anaerobic glucolytic and GBT rates of the various tissues assayed under the specified but carefully chosen conditions of experimentation. Various arbitrary but reasonable assumptions have had to be made to bring the data together, but we are not aware of any reasonable alterations in the assumptions that would change the estimated "discrepancy ratios" of 10–80 down to near unity (no discrepancy); and the same would be true for insertion of several-fold higher values of $Q^{N_2}_{GPT}$ for liver or slow-growing hepatomas. In col. E the GPT value of minimal hepatomas is lower than in liver (5.5 vs. 8), and in the earlier reports of Weinhouse *et al.* (*38*) was even lower (about one third as great), yielding even poorer correlation of extracted GPT with *in vivo* growth rate when liver is included in the spectrum, as it should be.

Text-figure 3 expresses the lowered glucose concentration function in Morris hepatomas in another way, by showing that the ratio "hepatoma glucolysis/host liver glucolysis" may in instances reach a value of eight or more with slow-growing hepatomas when a near-saturating, physiological concentration of 0.1% glucose is reached, and then falls to near unity only as the glucose concentration attains 1–2% where the liver rate attains saturation and becomes about equal to that of the slow-growing hepatomas. The absolute values of the ratio for intermediate deviation hepatomas are very much higher, as would be expected from text-figures 1 or 2. The Morris hepatomas (except possibly the very slow-growing 7787) produce little or no glucose during experimental runs, whereas liver samples commonly produce up to several tenths percent glucose in their circumambient medium. It might be thought that this latter phenomenon could explain why liver glucolysis does not ordinarily respond to additions of 0.1–0.2% glucose, but use of specific glucolytic inhibitors such as 2-DG or NAGA shows this is not true. They produce little or no inhibition of total glycolysis by liver samples to which no glucose is added and have no effect on glycogenolysis, which accounts for the endogenous glycolysis shown by liver samples in the absence of added glucose (or in its presence up to several tenths percent), as we shall now consider in some detail.

Glucolysis and Glycogenolysis as Affected by Pyruvate, DPN, TPN, 2-DG, and NAGA

Any extensive study of liver and Morris hepatoma glucolysis with added oxidants can readily become complicated and confused by a frequently concurrent process, endogenous glycolysis, which, together with glucolysis, then makes up the total observable *glycolysis*, often largely glycogenolysis. By the use of inhibitors and certain coenzymes and oxidant substrates, we have found ways of maximalizing or minimalizing one or the other form of glycolysis to make a feasible quantitation of each, but in particular to arrive at relatively unambiguous values for glucolysis, since except for hepatic cancers, liver, and a few other tissues, glycogenolysis is rarely met with *in vitro*, and probably still less so *in vivo*, at least glycogenolysis that, by definition, leads to lactic acid formation.

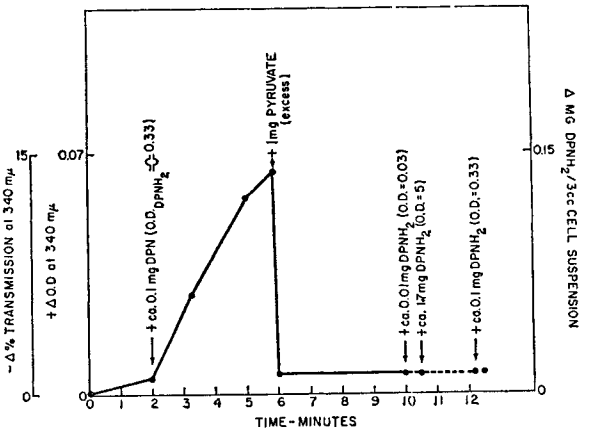
The history of the subject goes back at least 35 years to the classic experimental papers of Otto Rosenthal (*48–53*), who showed that either brief (*e.g.*, 15 min) preincubation of liver slices with O_2 at 37 C or simple addition of other oxidant hydrogen acceptors (pyruvate, acetaldehdye, methylene blue) could raise the commonly observed values of total anaerobic liver glycolysis of $Q_A^{N_2} = Q_{lactate}^{N_2} = 1$–3 (based upon final, not initial, dry weights) up to values 5–10 times as great. In 1963 Negelein and Noll (*55*) confirmed, without prior knowledge thereof, Rosenthal's finding with respect to O_2 incubation. In 1941 Orr and Stickland (*54*) did not observe this phenomenon as such, but reported values for liver glycolysis that varied from $Q_A^{N_2} = 2$–16 that they could roughly correlate with increasing liver glycogen content, and in 1943 Dickens and Weil-Malherbe (*56*) published much work in harmony with the foregoing considerations. Parenthetically, many papers on liver and hepatoma glycolysis have been written over the past 35 years with total neglect or unawareness of Rosenthal's basic findings.

Rosenthal recognized the existence of, but could not identify, some catalytic, internal cellular component immediately affected by his added oxidants, because his work was done before the identification and isolation of the coenzyme pyridine nucleotides. In 1963 we found (*19*, *59*, *66*) that the normally very high anaerobic glycolysis (glucolysis) of our Ehrlich and K_2 ascites cancer cells could be promptly and greatly inhibited (60–75%) by low concentrations of externally added $DPNH_2$, and that such inhibition could likewise be promptly and completely overcome by addition of sufficient DPN, pyruvate, or methylene blue, or by brief exposure to O_2, all of which agents or treatments can convert $DPNH_2$ to DPN (or yield a lower $DPNH_2$/DPN ratio) in our ascites cancer cell suspensions, and can do so in net effect both inside and outside the cells, since DPN and $DPNH_2$ were shown to readily enter and leave such cells. We have further found (*cf.* text-figs. 5 through 11) that consistently high values of total anaerobic glycolysis by rat liver and all hepatoma cells studied can be ob-

tained on addition of DPN to the suspending medium, even without prior O_2 incubation, and that marked secondary inhibition of such high fermentation values occurred on accumulation of $DPNH_2$ as the result of extra, *nonglycolytic* reduction of DPN that occurs in all liver and hepatoma tissues we have studied [though, in ascites cells, only in the presence of light and certain acridines (*67*, *68*) or thiazines (*69*)].

We conclude that a major factor controlling the attainment af maximum rates of *total* glycolysis (glycogenolysis plus glucolysis) in these cellular[14] hepatic tissues *in vitro*, in addition to insulin: anti-insulin regulation and hexokinase activity, is the DPN/$DPNH_2$ ratio prevailing at, and controlling, the rate of triosephosphate-dehydrogenase activity, just as in ascites cancer cell glycolysis. Addition of pyruvate, together with DPN, yields more prolonged high anaerobic fermentation, since the pyruvate tends to reduce and maintain the concentration of $DPNH_2$ at a low level, via pyruvic reductase (LDH), and thus yield a more favorable DPN/$DPNH_2$ ratio, as well as more DPN. Unfortunately, in due course, in hepatic tissues, the nonglycolytic reduction of the added DPN becomes overpowering, and $DPNH_2$ inhibition of glycolysis (both glucolysis and glycogenolysis) sets in, so that the beneficial effect of the added DPN on glycolysis is soon overcome, even within 15–30 minutes (depending on the state of the cells), by the $DPNH_2$ formed. Under such conditions, $DPNH_2$ inhibition of triosephosphate dehydrogenase masks any limitation at the hexokinase step. Large amounts of $DPNH_2$, derived from the DPN added exogenously, may be found in the medium surrounding the cells. The $DPNH_2$ so produced may readily be measured spectrophotometrically at 340 mμ. Because of the phenomenon of DPN reduction to $DPNH_2$ yielding secondary inhibition, the results of text-figures 1, 2, and 3 were obtained with media containing added pyruvate but seldom added DPN, at least over the lower ranges of the curves; ordinate points would be a little higher for the short periods of DPN stimulation if DPN were added (text-figs. 5 through 12). Pyruvate increases mainly glucolysis, DPN mainly glycogenolysis (*27*). If an Aminco-Chance dual wavelength spectrophotometer is used to measure $DPNH_2$ formation, cell suspensions may be studied without prior centrifugation, as shown in text-figure 4.

Cells of the minimal deviation Hepatoma 5123D could usually be readily teased into a uniform cell suspension, and in the experiment of text-figure 4 were then washed twice, and taken up, in standard Krebs-Ringer bicarbonate-glucose medium without initially added pyruvate or DPN, but containing



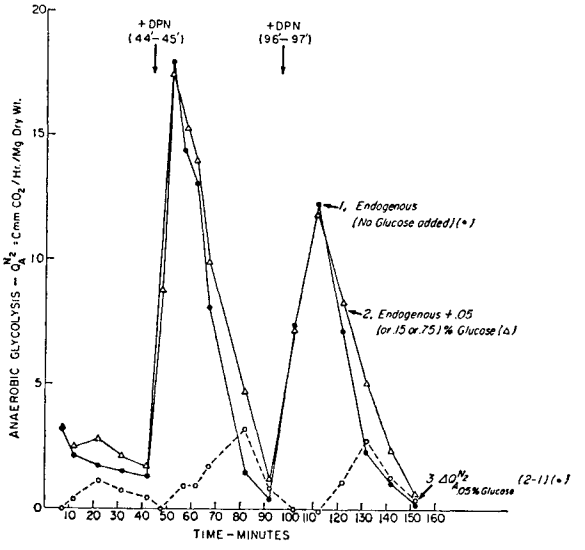
TEXT-FIGURE 4.—Reduction of DPN to $DPNH_2$ by cell suspension of minimal deviation Hepatoma 5123D in Aminco-Chance dual wavelength spectrophotometer and reoxidation by added pyruvate; 12 mm^3 cells (=2.4 mg dry weight)/3 cc/cuvette. Curve corrected for dilutions by, and nonspecific optical density of, reagents added during the experimentation.

10% polyvinylpyrrollidone for maintenance of prolonged stability. After a preliminary observation period of the first 2 minutes of text-figure 4, during which there was apparently a small reduction of endogenous DPN to $DPNH_2$ (that did not occur in later experiments with samples of the same suspension), DPN equivalent to a potential yield of $DPNH_2$ of optical density (OD) = circa 0.33 was added, and by 4 minutes later $DPNH_2$ equivalent to OD = 0.06 had been formed, when excess pyruvate was added. Within 15 seconds of mixing and equilibration, virtually all the $DPNH_2$ had been oxidized back to DPN by the pyruvate added, catalyzed by pyruvate reductase (LDH) present endogenously in the system. Later additions of

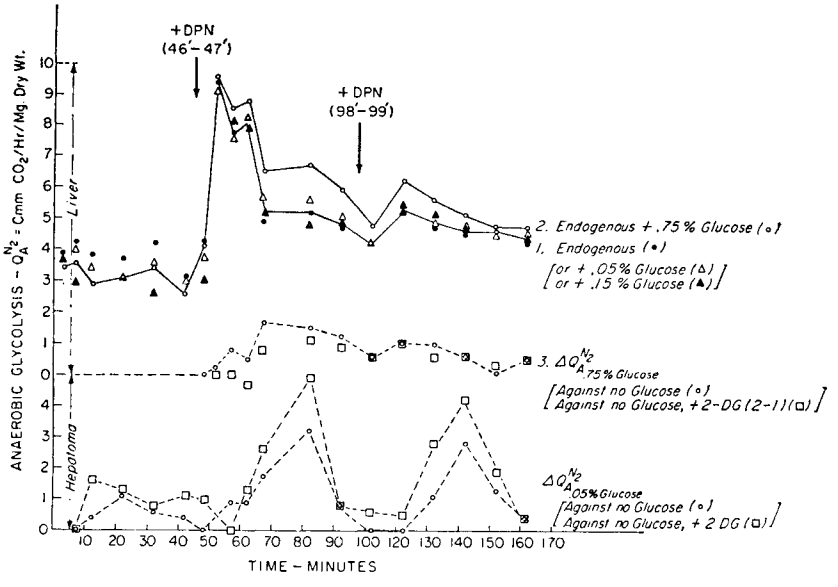
[14] Claims that phosphofructokinase may be a rate-limiting step in liver and hepatoma glycolysis are largely based on studies of subcellular preparations and do not appear to us to have been shown to be very relevant to glycolysis in intact cells.

varying amounts of $DPNH_2$ (one quite large, 1.7 mg) were completely oxidized similarly within no more than half a minute. Similar reduction results were obtained if TPN were substituted for DPN, but reoxidation by added pyruvate was considerably slower and far from complete.

In text-figure 5 the *solid lines* show the effect on manometric total anaerobic glycolysis (glycogenolysis plus glucolysis) of minimal deviation Hepatoma 5123D, in curve 1 for no glucose addition, and in curve 2 for addition of glucose at either 0.05, 0.15, or 0.75%. Curve 2 was unaffected by glucose concentration over this range (rate-saturation by 0.05%), in harmony with similar indication in text-figure 2. The *dotted curve* 3 in text-figure 5 represents the difference between curves 1 and 2, *i.e.*, the minimum rate of glucolysis with the 0.05, 0.15, or 0.75% glucose added. The actual rate of glucolysis was a little higher, because the "endogenous base line" of curve 1 involved a small amount of endogenous glucose, as shown by the fact that when the corresponding endogenous curve was simultaneously performed with addition of the specific glucolytic inhibitor, 2-DG, the Q values were slightly reduced, and hence the difference between the curve with added glucose, and the curve without glucose but with added 2-DG, slightly increased. This minor distinction is shown in text-figure 6, where the *bottom curve* is reproduced from text-figure 5, and the curve above it is for a simultaneous run with added 2-DG. Under the indicated conditions, 0.5% 2-DG will inhibit any endogenous glucolysis completely and will do so to the extent of 90–95% in the presence of 0.1% glucose; at a competitive ratio of 2:1 (*e.g.*, 1% 2-DG:0.5% glucose) the inhibition is usually about 80% (*see* text-figs. 8 and 10).



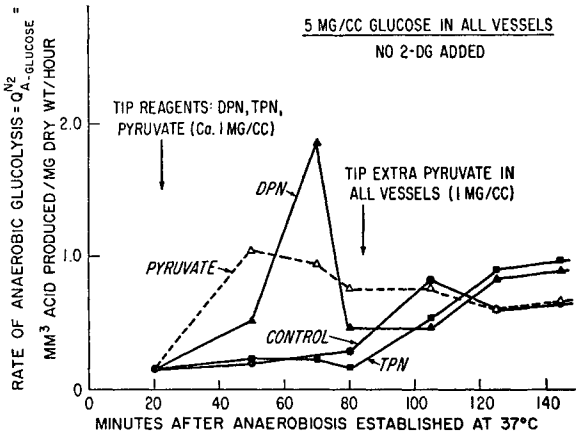
TEXT-FIGURE 5.—Anaerobic glycolysis of minimal deviation Hepatoma 5123D as a function of time, in the presence and absence of glucose, and later additions of DPN, to initial Krebs-Ringer, 0.2% $NaHCO_3$, 0.1% pyruvate medium. *Curve 1* is approximate glycogenolysis (*see* text), *curve 2* is glycogenolysis plus glucolysis, and *curve 3* is glucolysis of added glucose, uncorrected for small endogenous glucose (*see* text and text-fig. 6).



TEXT-FIGURE 6.—Anaerobic glycolysis, glucolysis, or glycogenolysis of minimal deviation Hepatoma 5123D compared to liver of same host rat, as a function of time, presence and absence of glucose and 2-DG, and later additions of DPN. Simultaneous experiment with that of text-figure 5 (*see* text).

The three upper curves in text-figure 6 show equivalent and simultaneously obtained data with the liver of the same hepatoma-bearing host rat used in text-figure 5. With the liver, no effect of added glucose was here noticeable until the concentration as high as 0.75% was employed, in harmony with indications in text-figure 2 and with earlier statements as to the great difference between glucose saturation curves for liver and for Morris hepatomas, slow-growing as well as fast-growing. The data for the lower ordinate values in text-figures 1, 2, and 3 were based on "no added glucose controls" that were usually run simultaneously in both the presence and absence of added 0.5% 2-DG values, where indicated, in calculation of the glucolysis. For relatively high glucolytic values, this procedure was scarcely necessary.

Text-figures 5 and 6 show not only our procedure for obtaining total glucolytic values, but the tremendous fold increase in total glycolysis (mainly glycogenolysis) on addition of DPN (at 44–45 minutes), and the equally tremendous and rather prompt onset of subsequent, secondary inhibitions (by $DPNH_2$ formed), and similarly with a second DPN addition (at 98–99 min), though in lesser degree. Our general experience is that the secondary inhibition occurs even more promptly in the minimal hepatoma than in the host liver, *i.e.*, this hepatoma has an even greater capacity for non-glycolytic reduction of DPN to $DPNH_2$, although this is not true for the more rapidly growing hepatomas. TPN addition produces a variety of effects similar and dissimilar to those of DPN, as will be considered in detail elsewhere (*27*). As indicated in text-figure 7, added TPN has little or no effect on minimal deviation hepatoma glucolysis in the absence of added pyruvate, but may do so in the presence of added pyruvate (TPN curve from 84 min on). In text-figure 7 are also illustrated, in a very typical experiment, the initially increased glucolysis, the rapidly following secondary glucolytic inhibition, and finally (after 84 min) partial recovery therefrom on addition of pyruvate. The effect of pyruvate in increasing glucolysis, for a prolonged period in the absence of any added DPN, is also clearly shown; it does so by preventing accumulation of inhibitory $DPNH_2$ formed from the endogenous DPN to yield an unfavorable $DPNH_2$/DPN ratio. The glucolysis curves in text-figure 7 were obtained by the subtraction method illustrated in text-figures 5 and 6.



TEXT-FIGURE 7.—Anaerobic glucolysis of minimal deviation Hepatoma 5123D in Krebs-Ringer $NaHCO_3$ glucose medium as affected by later individual additions of pyruvate, DPN, and TPN. Glucolysis values represent differences from simultaneously obtained values in the absence of added glucose.

NAGA inhibited liver glucolysis (as many have shown for homogenate preparations) at high, non-physiological glucose concentrations, but did not inhibit glycogenolysis; indeed we have occasionally observed stimulation thereof. NAGA inhibited neither glucolysis nor glycogenolysis by any of the Morris hepatomas, including (relatively glycogen-rich) Hepatoma 7787, at physiological glucose concentrations.

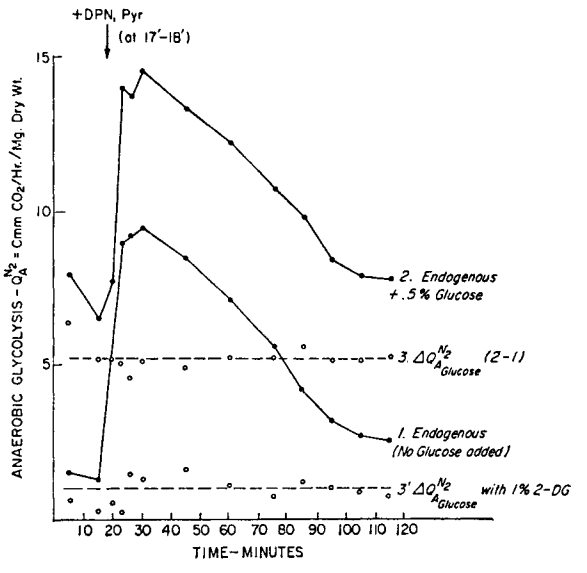
Text-figures 8, 9, and 10 show typical, corresponding, simultaneous results obtained with a specimen of intermediate-growing Morris Hepatoma 7288C, and text-figures 11 and 12 with a specimen of fast-growing Morris Hepatoma H-35TC_2 and 3683, instead of minimal, slow-growing Morris Hepatoma 5123D shown in text-figures 5 and 6. The faster the growth rate, the greater the glucolysis and, to a lesser degree, the smaller the glycogenolysis, and various effects of added glucose, 2-DG, pyruvate, DPN, and TPN on glucolysis or glycogenolysis, as earlier described, are further illustrated and extended. Curve 2 in text-figure 8 is reproduced as curve 2 in text-figure 9 for ready comparison of results obtained with liver of the host rat (which may be compared with the host liver reported on in text-fig. 6). The rate of anaerobic glucolysis by

this specimen of intermediate Hepatoma 7288C is here about $Q_{CO_2}^{N_2} = 5$, whether obtained as in text-figure 8 by curve 1 without glucose subtracted from curve 2 with added glucose, or obtained as in text-figure 9 by curve 1 for liver (with or without added glucose) subtracted from curve 2 for Hepatoma 7288C with added glucose. Looked at another way, maximum endogenous glycogenolysis in the intermediate hepatoma (curve 1, text-fig. 8) has been reduced to about the maximum level of glycogenolysis in liver (curve 1, text-fig. 9)—provided DPN has been added. The glycogenolysis component of both hepatic tissues falls with time not long after the added DPN has established maximum rate of glycogenolysis (as in text-figs. 5 and 6), whereas, by comparison, the considerable glucolysis (occurring with the intermediate hepatoma, not with the liver) is relatively stable with time and no longer requires DPN or pyruvate to establish near maximum glucolytic rate (curve 3 values before 17–18 min compared to values after 17–18 min). Curve 3′ in text-figure 8 (reproduced in text-fig. 10) shows that, as already pointed out, 1% 2-DG will not completely inhibit (here only *ca.* 80%) glucolysis at the high glucose concentration of 0.5%.

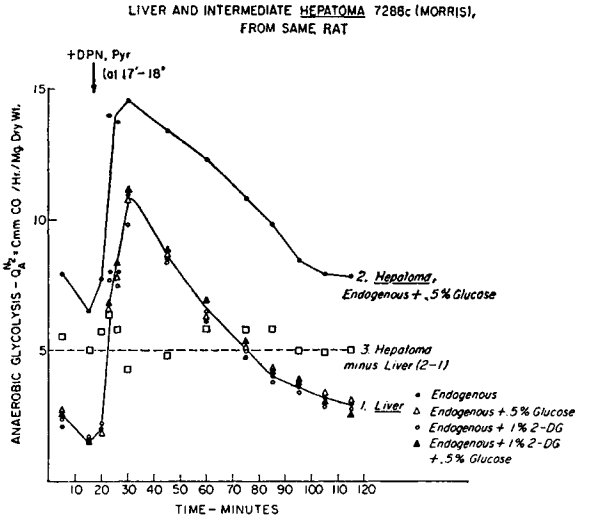
Text-figures 11 and 12 show with specimens of fast-growing Morris Hepatomas H-35TC$_2$ and 3683 still greater glucolysis rates ($Q_{CO_2,\ max}^{N_2}$ = *ca* 12 and 17), again relatively independent of addition of DPN and pyruvate, but with here some falloff in rate over 140–160 minutes; maximum glycogenolysis with DPN and pyruvate (*see* especially text-fig. 12) is, respectively, $Q_{CO_2}^{N_2} = 10$ and 5, again falling notably with time to values of *circa 2* by 140–160 minutes.

Insulin : Anti-Insulin (Steroid) Control of Glucolysis at the Hexokinase (Glucokinase) Level

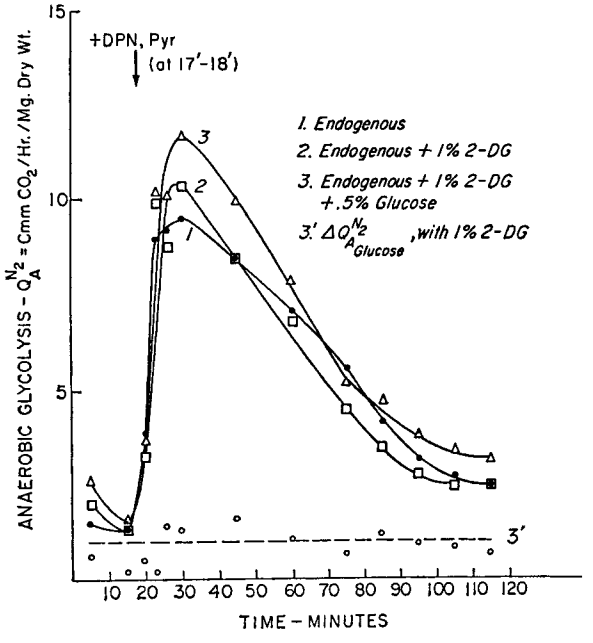
Our work over the past 10 years (*11–27*) has not only confirmed the Warburg concept of over-all cancer metabolism, but has greatly extended it by indicating the enzymatic site of the primary biochemical lesion involved in altered glucolysis in cancer cells, *relative to their respective tissues of origin.* Insulin : anti-insulin (steroid) regulation of the initial step of glucose utilization, namely, glucose phosphorylation in the hexokinase (glu-



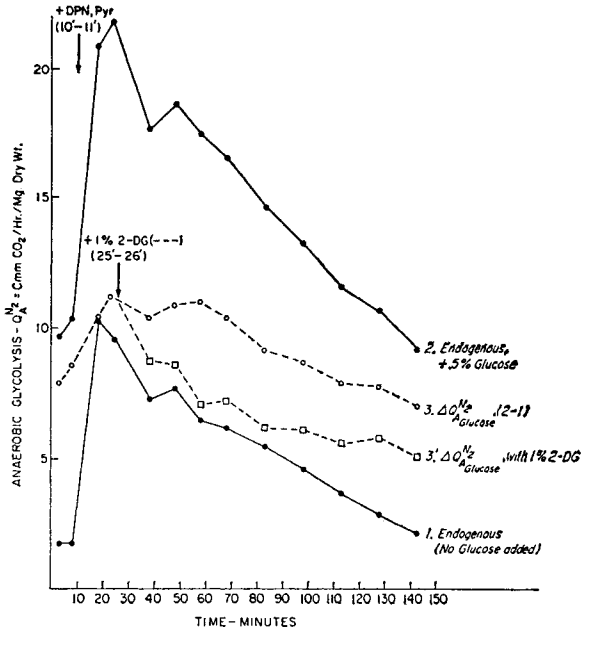
TEXT-FIGURE 8.—Anaerobic glycolysis, glucolysis, and glycogenolysis of Morris intermediate Hepatoma 7288C as a function of time, in the presence and absence of glucose and 2-DG, and of DPN plus pyruvate. *Curve 1* is approximately glycogenolysis; *curve 2*, glycogenolysis plus glucolysis; *curve 3*, glucolysis; and *curve 3′*, glucolysis 80% inhibited by 2-DG at twice the concentration of glucose.



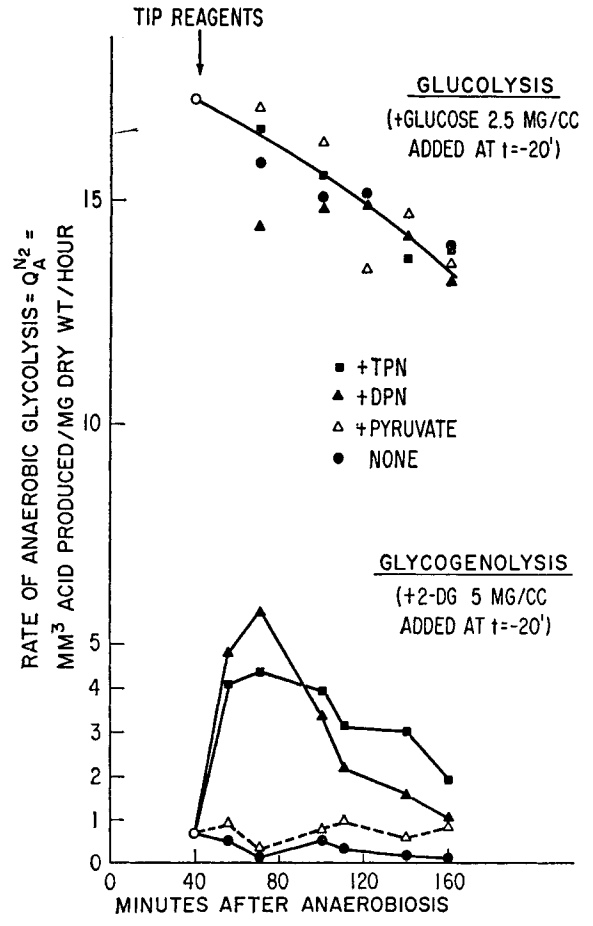
TEXT-FIGURE 9.—Anaerobic glycolysis, glucolysis, and glycogenolysis of Morris intermediate Hepatoma 7288C compared to liver of same host rat, as a function of time, presence or absence of glucose, 2-DG, and later additions of DPN and pyruvate.



TEXT-FIGURE 10.—Endogenous anaerobic glycogenolysis of Morris intermediate hepatoma as a function of time and additions of 2-DG and/or glucose.



TEXT-FIGURE 11.—Anaerobic glycolysis, glucolysis, and glycogenolysis of advanced Morris Hepatoma H-35TC$_2$ as a function of time, in the presence and absence of glucose, and 2-DG, and later additions of DPN and pyruvate.



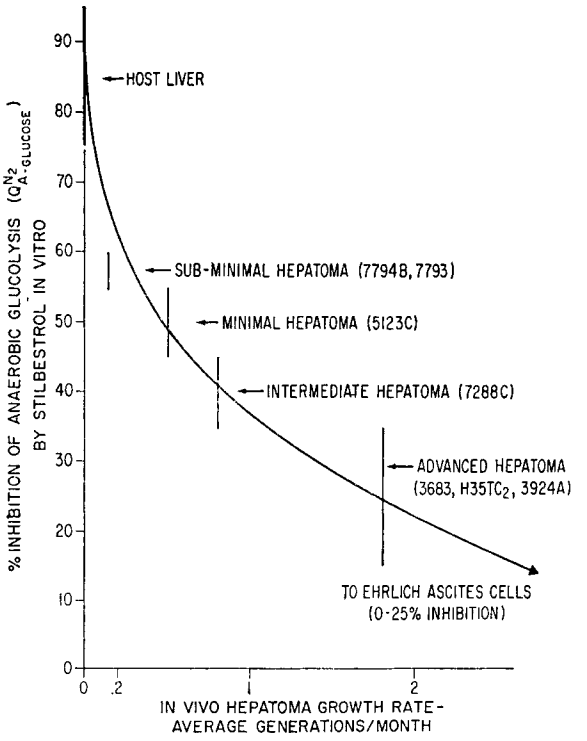
TEXT-FIGURE 12.—Anaerobic glucolysis and glycogenolysis of rapidly growing Morris Hepatoma 3683, as a function of time, in the presence and absence of TPN, DPN, or pyruvate.

cokinase [15]) step, plays a dominating role in the control of innumerable subsequent cellular reactions in both cancer and normal cells, as our

[15] In this paper we use the word "glucokinase" in the classical sense of the enzyme or enzymes that phosphorylate glucose, without regard to different isolated (but possibly not cellular) isoglucokinases of different K_m values; as with hexokinase, whether glucokinase acts on fructose also is immaterial here, inasmuch as all indications so far obtained suggest that they do so act. Many workers have repeatedly shown that solubilization of glucokinase in subcellular preparations of various tissues results in a reduction of the hexokinase (glucokinase) response to steroid or insulin regulation (*16*). It is probable that in hepatic, as in other tissues, subcellular preparations of hexokinases (glucokinases) may be obtained with varying K_m-glucose values (*i.e.*, isolated *iso*glucokinases), depending on the degree of disruption of the phosphotransferase from the cell structures, but perhaps scarcely existing in the cell as such.

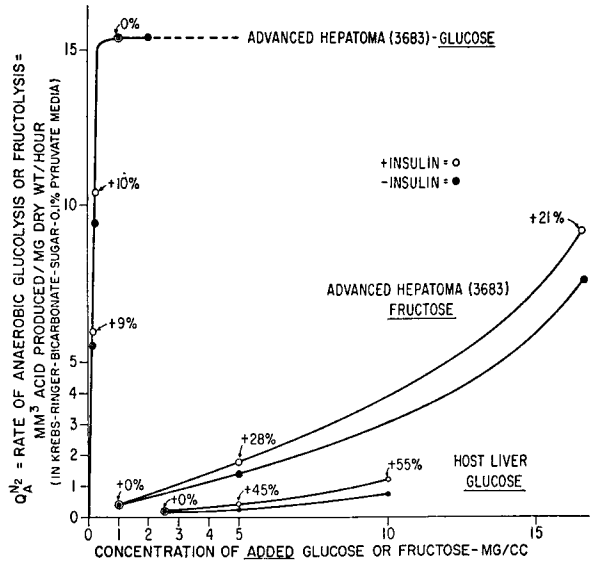
previous work has demonstrated in melanomas, mammary cancers, ascites cancer cells (weakest control), human myeloid and lymphocytic leukemias, peritoneal macrophages and spleen, tissue-culture fibroblast cell lines and sarcomas derived therefrom, mouse kidney and brain, and now liver and Morris hepatomas. The difference between normal maintenance-growth metabolism in adult noncancer tissues showing virtually no net growth, on the one hand, and "uncontrolled" malignant-growth metabolism on the other, depends on critical alterations in the indicated insulin: anti-insulin control mechanism. A reliable index of the susceptibility of glucolysis to anti-insulin regulation is the percentage inhibition of glucolysis given by a steroid, or steroid-like material such as the synthetic estrogen stilbestrol. Our previous work with spectra of melanomas and tissue-culture cell lines has clearly shown that the more progressive the change from normalcy through increasing degrees of malignancy, the greater the loss in restraint of cellular glucolysis induced by anti-insulin hormone steroids, or steroid-like compounds acting at the hexokinase reaction (*11–27*). The same relationships appear to hold for the Morris liver-hepatoma spectrum, as indicated in text-figures 13 and 14.

Text-figure 13 shows an inverse relationship between percentage inhibition of glucolysis by stilbestrol *in vitro* and the *in vivo* growth rate, and therefore, from text-figure 1 also, the glucolytic rate itself: the higher the growth rate and glucolytic rate, the lower the percentage inhibition by stilbestrol, a "synthetic estrogen." Most significant is that even in the most slow-growing hepatomas the glucolytic component shows a considerably lowered sensitivity to restraint by the anti-insulin inhibitor stilbestrol compared to nonmalignant host liver, just as the $K_{m\text{-glucose}}$ is similarly lowered (text-fig. 2). In our experience with liver and the hepatomas, as with other tumors, anti-insulin action tends to increase $K_{m\text{-glucose}}$, but less so in the hepatomas than in liver because the hepatomas have progressively lost sensitivity to such restraint or regulation of glucolysis. There is a close, experimentally demonstrable connection between lowered $K_{m\text{-glucose}}$ and lowered sensitivity to restraint wherever we have obtained correlative data in a variety of tumors and normal tissues. At one extreme stand the ascites cancer cells, with very low $K_{m\text{-glucose}}$ and near-vanishing sensitivity to restraint; at the other extreme stands liver, with perhaps the highest $K_{m\text{-glucose}}$ and near the highest sensitivity to restraint by anti-insulins.



TEXT-FIGURE 13.—*In vitro* inhibition of glucolysis by stilbestrol as a function of *in vivo* liver-hepatoma growth rate spectrum.

Any discussion of anti-insulin action in a tissue presupposes, of course, an action of insulin, though not necessarily always equal and opposite, any more than an equal and opposite action between an enzyme substrate and an enzyme inhibitor (*i.e.*, K_m rarely equals K_I). Numerous reports in the literature deny a direct action of insulin *in vitro* in liver (and, similarly, in brain tissue). In our studies on melanomas and certain other tissues we have shown clear-cut *in vitro* effects of insulin on glucolysis, *provided such factors as prior host stress substrate concentration, and incubation temperature were duly considered and controlled*, and this is also true of liver and Morris hepatomas, as indicated in text-figure 14.



TEXT-FIGURE 14.—Effect of glucagon-free insulin on glucolysis or fructolysis by advanced Hepatoma 3683 or host liver; 0.2 unit insulin/cc.

As might be expected under a variety of *in vitro* conditions, effects of insulin on glucolysis can vary from zero to high values, depending on many experimental factors, three of the most important of which have just been delineated. Liver is a tissue where endogenous anti-insulin restraint is so great that greatest effort and understanding in varying factors are required to obtain at least unmistakable if not regularly controllable insulin effects. Brain is a tissue which one can readily arrange *in vitro* conditions to obtain insulin effects on glucolysis with reasonable regularity. At the other extreme, Ehrlich ascites cancer cells have lost so much insulin:anti-insulin sensitivity to control of glucolysis that effects of insulin and anti-insulins are usually small but nevertheless the rule: In at least 90% of hundreds of determinations we have made, insulin increased the rate of glucolysis by 5–15% with a maximum error of 1–2%; this in spite of the concluding statement of Crane, Field, and Cori (*70*) that ". . . every effort to show an effect of insulin on the sugar uptake of these cells (Ehrlich ascites tumor cells) failed." In our experiments, often performed with the sensitive and accurate Mechrolab self-recording automanometer system, the effect of added insulin on the glucolytic rate was evident within no more than a few minutes and persisted until the added glucose was virtually gone, as confirmed by glucose oxidase analyses. Here again, as so often in the history of biochemistry, the manometric method (always to be confirmed by spot-check chemical analysis) is *the method of discovery and of choice*, being commonly far more sensitive, especially in time-rate studies, than methods of chemical analysis.

Even more important than methodological sensitivity and accuracy, however, is an appreciation of the biological and other factors involved in insulin:anti-insulin sensitivity, above all the effect of prior stress of the host animal because stress tends to enhance the secretion of endogenous anti-insulin steroids. We have no doubt that the general uncertainty and difficulty of reproducing the work of the Cori school of nearly 20 years ago, reporting an interaction of insulin with adrenal cortex and anterior pituitary extracts, is to be laid to an inadequate understanding and handling of factors whose nature we have been delineating for many years (*11*–27) in detail for a large number of tissues.

In our hands, insulin acts not only against the hormonal materials implicated by the original Cori school, but also against sex steroids and steroid-like substances, podophyllins, etc., all of which we class as operational anti-insulins; the quantitative interactions vary greatly from system to system, however disturbing this may be to "nonbiological biochemists," but grist for the mill of "biological biochemists." Thus, for example, $K_{m\text{-}glucose}$ (likewise the entire glucose concentration-rate saturation curve) will depend not only on the particular tissue, but also on the level of stress existing in the host animal at the time of collecting the tissue; increased levels of stress increase the $K_{m\text{-}glucose}$ (and very much so $K_{m\text{-}fructose}$), especially in tissues with high susceptibility to anti-insulin steroid regulation, such as mouse Melanoma S91.

The experiments in text-figure 14 illustrate greater insulin effects obtained with fructose than glucose in advanced Hepatoma 3683. This may well reflect an atavism reminiscent of the hepatic parenchymal tissue of origin. Text-figure 14 also shows the importance of an *optimum* glucose concentration for maximum percentage insulin stimulation of glucolysis, realized here with Hepatoma 3683, but not yet by liver at 1% glucose. It is up to both the nonbiological as well as biological

biochemist to vary all known established factors before concluding that an insulin effect *in vitro* is not to be obtained; otherwise such a negative conclusion can scarcely invalidate positive insulin effects.[16] Small but unmistakable insulin and anti-insulin effects can be quite important as indicators of the degree of insulin : anti-insulin control of glucolysis at the hexokinase reaction in the system under investigation. Parenthetically, the action of insulin is remarkably simulated by low concentrations of purified bacterial endotoxins, and the quantitative and qualitative actions of these widely different biochemicals generally run parallel in any given experimental system under investigation (*71*, *72*).

Other Alleged Exceptions to Cancer Glucolysis and Steroid Control

Scientific human nature being what it is, and in view of the observation of Warburg, "In science one cannot prove that there are no ghosts" (*76*, p 320), we may look forward with confidence to the appearance of future reports of alleged exceptions to cancer glucolysis and insulin:anti-insulin control thereof. The latest of such reports to come to our attention is that of Hilf *et al.* (*77*, *78*), who describe a "lack of anaerobic utilization of glucose substrate by the R323OAC tumor," a slow-growing, transplantable mammary tumor maintained in Fischer rats that arose spontaneously from a faster growing, lactating tumor R323OAB which itself utilized glucose anaerobically, as measured by CO_2 or lactic acid production. Estrogen treatments given *in vivo* were stated not to alter the utilization of glucose substrate by R323OAC tumor *in vitro*. Since the experimentation was reported (*78*, p 287 and table 7) to have been carried out with tissue homogenates (*i.e.*, at the "hammered-up watch level") and since "exceptions" must always be referred back to living cells to be within the framework of the question at issue, Dr. Hilf at our request kindly supplied us with several R323OAC tumor-bearing Fischer rats, which, in view of the importance of the question, we preferred to use instead of R323OAC tumors carried on another strain of rat by Gullino. Our results with *living* R323OAC tumor tissue are reported in table 4, based on standard methods within the aforementioned "classical" framework of experimentation for living cells.

The data of table 4 show that living cells of the relatively slow-growing rat mammary Carcinoma R323OAC do indeed utilize glucose and form lactic acid therefrom anaerobically at a rate of $Q^{N_2}_{glucolysis} = 4$–6, well within a cancer range consonant with the rate of growth, and that this glucolysis is almost completely inhibited by 0.5% 2-DG, whereas endogenous "glycolysis" is scarcely affected by this inhibitor. In the determinations without added inhibitors, the agreement between manometric and chemically measured lactic acid formation is excellent, but with somewhat higher values for glucose consumption that probably represent a small glucose consumption occurring in addition to production of lactic acid. The endogenous nonglucolytic "glycolysis" is of the order of $Q^{N_2}_{endogenous} = 1.5$, much smaller than the $Q^{N_2}_{glucolysis} = 4$–6.

The data indicate that by 50 parts per million (ppm) (mg/liter) testosterone is more inhibitory than stilbestrol *in vitro* in this sex-steroid-linked mammary tumor, whereas in the cases of all non-sex-oriented tumors we have otherwise tested, *e.g.*, melanoma as shown in table 4, stilbestrol is considerably more inhibitory than testosterone, and we believe this contrast is of significance in connection with possible chemotherapeutic applications. The agreement between manometric, lactic, and glucose measurements is not as close in the presence of these two inhibitors (as in their

[16] Weinhouse *et al.* (*38*, vol I, p 366) reported that glucokinase activity of rat liver is markedly lowered in alloxan diabetes, but is restored by insulin treatment *in vivo*. However, they reported insulin to be inactive *in vitro*. Sharma *et al.* (*73*, vol II, p 189) further noted that insulin is necessary for substrate induction of glucokinase and that the effect is on the synthesis of the enzyme rather than a direct stimulation. Sols *et al.* (*74*, vol II, p 186) also reported their results " . . . suggest that liver glucokinase is an inducible enzyme whose synthesis is induced directly, or indirectly, by insulin." Since a direct stimulatory effect of insulin on hepatic hexokinase has been reported by Ilyin (*75*, vol II, p 151), claims that insulin does not act directly on liver glucokinase must be considered with great reservation. Moreover, our own results show that *in vitro* administration of insulin to liver slices (as well as to certain rat hepatomas) can stimulate anaerobic glucolysis. Such stimulation occurring *in vivo* might indirectly lead to enhanced protein synthesis, including an increase in the amount of glucokinase itself.

TABLE 4.—Anaerobic glucolysis by Hilf slow-growing, rat mammary tumor R3230AC (or S91 mouse melanoma) in the absence or presence of stilbestrol, testosterone, 2-desoxyglucose, Δ^1 testololactone, or *N*-acetylglucosamine*

Expt.	Tumor	Addition other than glucose	$Q^{N_2}_{glycolysis}$ or $Q^{N_2}_{glucolysis}$ = mm³ acid equivalent/mg dry wt/hr											
			No glucose added† (endogenous)			+ Glucose			Δ Glucose (glucolysis)			% Inhibition of Δ glucose		
			Manometric	Lactic	Glucose	Manometric	Lactic	Glucose	Manometric	Lactic	Glucose	Manometric	Lactic	Glucose
2	Rat mammary R3230AC	None	1.71	1.49	0	6.22	6.20	6.05	4.51	4.71	6.05			
		50 ppm stilbestrol	1.80	1.75	0	5.03	6.01	6.05	3.23	4.26	6.05	28	10	0
		50 ppm testosterone	1.37	0.08	0	4.05	3.88	5.29	2.68	3.80	5.29	41	19	13
		0.5% 2-desoxyglucose	1.62	1.60	0	2.10	0.91	1.07	0.48	−0.69‡	1.07	89	100	82
		50 ppm Δ^1 testololactone	1.62	1.07	0	6.64	7.04	8.57	5.02	5.97	8.57	−11‡	−27‡	−42‡
3	Rat mammary R3230AC	None	1.65			7.65			6.00					
		25 ppm stilbestrol	1.60			5.70			4.10			32		
		25 ppm testosterone	1.65			5.85			4.20			30		
		140 ppm Δ^1 testololactone	1.45			6.00			4.55			24		
3a	S91 mouse melanoma (3a simultaneous with 3)	None							9.94					
		25 ppm stilbestrol							4.56			54§		
		25 ppm testosterone							7.28			27§		
		140 ppm Δ^1 testosterone							7.22			27§		

*1% *N*-acetylglucosamine in another experiment inhibited the manometric glucolysis of 4.06 by 13%.

†No glucose detectable by glucostat method at beginning and end of experiment (*i.e.*, at 75 min).

‡−=stimulation;

§Correction to endogenous "no-glucose control" negligible (per indication from other experiments); 4.5 cc Krebs-Ringer-0.2% $NaHCO_3$-0.1% sodium pyruvate medium in manometric vessels of *ca.* 17 cc total volume; gas phase 5% CO_2/N_2 (purified of O_2 with hot copper and trace of H_2); temperature, 37 C; final *p*H values, 7.6-7.25; Q values based on averages of duplicates or more; glucose determined by the glucostat method, lactate by the Barker-Summerson method; expts. 3 and 3a run simultaneously to insure identical stilbestrol and testosterone concentrations for both tumor types. The two compounds were taken up in alcohol and then diluted with larger volume of medium to yield a uniform suspension that was added to the manometric vessels immediately; equivalent alcohol-water solution without compounds being added to controls in same small volume; in expts. 2, 3, and 3a, respectively: 125, 100, 100 mg wet wt of tissue, of 21, 20, 18% dry weight, per vessel; 75, 110, 110 minutes experimental period; uniformly 0.15% glucose where added. Rat mammary tumor-bearing animals obtained through courtesy of Dr. Russell Hilf, Squibb Institute for Medical Research, of New York, New York.

absence), and is suggestive of differential influences on the path of glucolysis by these inhibitors, but this point needs further confirmation and extension to a wider range of concentrations than studied. Testosterone at 25 ppm did not affect endogenous glycolysis, but did produce inhibition at 50 ppm.

Δ^1 Testololactone (which, like podophyllins, shows little if any endocrinological activity), although inhibiting glucolysis at 140 ppm, appeared to stimulate glucolysis at the lower concentration of 50 ppm. One percent NAGA, which, as already indicated, is an effective inhibitor of liver glucolysis at high glucose concentrations, but ineffective against Morris hepatoma glucolysis at physiological glucose concentrations, likewise produced little inhibition of R323OAC tumor glucolysis, as we have also found with our Ehrlich ascites cancer cells and mouse Melanoma S91. Addition of 1 mg DPN/cc increased the rate of endogenous glycolysis over 200%, as in liver and Morris Hepatoma 7288C, but had little effect on glucolysis [as likewise in Hepatoma 7288C (*27*)]. Whether the endogenous "glycolysis" of 323OAC tumor is truly glycogenolysis or based on some other substrate remains to be determined.

So much for the latest alleged "exception"; in future, *caveat emptor et caveat emptor*.

Chemotherapeutic and Hyperthermic Implications

We have already pointed out the central importance of chemical, physiological (stress), or genetic inhibitions or modifications of glucolysis at the hexokinase level for rational cancer chemotherapy or extreme hyperthermy (*11–14*, *19*, *79–104*) that need not be recapitulated. Suffice to say, most types of cancer chemotherapeutic agents and extreme hyperthermy employed clinically today, regardless of original rationale or lack of rationale in their derivation or discovery, have been found *in vitro* to have powerful and immediate effects on cancer glucolysis and/or respiration that can result in many secondary or subsequent chemical and biological effects, including derangement of coenzyme syntheses, general cell breakdown, and death of the cancer cell. The new data and conclusions in this paper regarding the slow-growing Morris hepatomas and the Hilf (sex-hormone oriented) mammary carcinomas again emphasize the potential importance of the antiglucolytic activity of chemotherapeutic agents possessing anti-insulin action and the great need for initiating studies with the former and continuing studies with the latter cancers, but with full appreciation of the glucolytic, anti-insulin component.

We have noted in previous publications a parallelism between *in vivo* responsiveness of various tissues to growth inhibitory action of anti-insulins and their *in vitro* action, most notably and in greatest detail with the mouse melanoma spectrum (*81*, *82*, *85*, *86*), and in mouse and human leukemias (*87–91*). *Knowledgeable* exploitation of agents which potentiate or supplement the anti-glucolytic action of steroids, or agents with similar activity, can depend on precise determination of the degree of insulin:anti-insulin regulation characteristic for specific tumors and normal tissues. Anti-insulins, such as estrogens (including stilbestrol), testosterone, decadron, prednisolone, 9-α-fluoroprednisolone, Δ^1 testololactone, and even podophyllins, if not all-sufficient in themselves, can be powerful concomitant or sequential adjuncts in multiple chemotherapy by increasing susceptibility to other agents (*81*, *82*, *85*, *86*), and would be particularly valuable in studies of the Morris hepatoma series. In an *in vitro* model system, and with melanomas growing *in vivo*, we have indicated the combined actions of prednisolone and 5-fluorouracil, or dinitrophenol (or pentachlorophenol), 5-fluorouracil, and *in vitro* elevated oxygen pressure. Even the simple presence of glucose in a cellular milieu, with concomitant glucolysis, may be chemotherapeutically essential in various ways (*19*), *e.g.*, as necessary for the entrance of a chemotherapeutic agent into a cell, as with 5-fluorouracil and related derivatives thereof (*89*); or as a protective agent in the detoxification of H_2O_2 by glutathione peroxidase (*19*, *105*). Chemotherapeutic applications to human cancers can be facilitated by use of the new metabolic and histologic evaluation procedures recently developed with great skill by Gold (*106*, *107*), who with this technique has found markedly differential stimulation by insulin of anaerobic glucolysis of human colon carcinomas and malignant colon polyps as compared with benign polyps or normal mucosa.

We conclude that there is more reason than ever to seek to capitalize on the now still more

firmly established central biochemical lesion and target *common to cancer cells generally*, namely, their altered glucolysis resulting from a less restrained and increased hexokinase reaction with respect to their tissues of origin. Such emphasis on this potential Achilles heel of cancers is not meant to preclude chemotherapeutic attacks at other enzyme sites such as triosephosphate dehydrogenase (reviewed *19*) or LDH (*108*).

Finally, some forty papers by von Ardenne, Kirsch, and co-workers (*e.g.*, *94–98*) during the past 3 years have greatly stimulated renewed interest in extreme hyperthermy, total-body therapy of cancers, following earlier work of Crile (*99–101*), Lampert (*102*), and hosts of others still earlier, back to Hippocrates. The technical advances of von Ardenne and Kirsch appear to have opened the way to clinical treatments (*94–98*) of patients at body temperatures of 42–44 C, and to have raised many interesting questions regarding the biochemical mechanism of selective killing of cancer cells at such temperatures in a way specifically involving glucose, glucolysis, respiration, and the Pasteur Effect (*93*, *96–98*, *103*, *104*). Whereas cancer chemotherapeutic methods of recent years appear to have settled down to relatively stabilized channels of procedure and outlook—streamlined canal and river dredging, extreme hyperthermy may offer, both on its own and also in conjunction with chemotherapy, the equivalent of a "new route to India" or a Northwest passage.

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