value, while it was normal in regenerating and newborn liver. The authors feel that the absence of fructose-1,6-diphosphatase in the liver tumor, like the absence of glucose-6-phosphatase, favors the channeling of glucose into the Embden-Meyerhof glycolytic pathway. A later study (489) demonstrated that the enzyme level was also low in a number of normal tissues (brain, lung, and muscle).

6. The Requirement for DPN and Other Nucleotide Additions in the Oxidative Metabolism of Tumors

Boyland (54), in his studies on glycolysis, recognized that tumor extracts rapidly destroyed DPN and ATP, and that to obtain continued activity of the extracts these nucleotides had to be added. Later, Schlenk (406) determined the DPN and DPNH content of a group of tumors. Working with animal and human tissues he first reported that, whereas in most normal tissues the oxidized form was present in excess, in some tumors, particularly the Jensen rat sarcoma, the reduced form predominated. However, in a later study extended to other tumors (AH rat sarcoma, rat methylcholanthrene tumor, and a C3H mouse breast carcinoma), the finding of an excess of reduced DPN in tumors could not be confirmed (168). Recently, detailed studies on the levels of reduced and oxidized pyridine nucleotides have been carried out by Jedeikin and Weinhouse (231) and Glock and McLean (180, 181). The studies of Glock and McLean include measurements of reduced and oxidized TPN, as well as DPN, and are therefore presented (Table 38). A very sensitive method was used, involving coupling with the specific cytochrome *c* reductase, and measuring spectrophotometrically the cytochrome *c* formed. In all the normal tissues studied DPN was chiefly in the oxidized form, and TPN chiefly in the reduced form. Normal tissues with high levels of DPN and TPN included liver, adrenal, kidney, and lactating gland. With the solid neoplastic tissues studied, the DPN content was in general lower than in most normal tissues, while the level of TPN was negligible. The form of DPN and TPN was the same in tumor as in normal tissue, i.e., in both tissues the bulk of DPN was oxidized and the bulk of the TPN was reduced.

Carruthers and Suntzeff studied the distribution of pyridine nucleotides (81) in the various cell fractions of liver, kidney, spleen, brain, and a group of neoplastic tissues (Table 39). In liver, kidney,

TABLE 38

THE LEVELS OF OXIDIZED AND REDUCED PYRIDINE NUCLEOTIDES[a] IN NORMAL AND NEOPLASTIC TISSUES (180, 181)

Tissue	DPN+	DPNH	DPN+ + DPNH	TPN+	TPNH	TPN+ + TPNH
Normal rat tissues						
Liver	370	204	574	6	205	211
Adrenal	315	154	469	17	116	133
Diaphragm	287	138	427	22	13	13
Cardiac muscle	299	184	483	4	33	36
Kidney	223	212	435	3	54	57
Lactating mammary gland	227	83	310	22	51	51
Ovary (rabbit)	181	34	215	2	42	42
Brain	155	67	222	8	16	24
Spleen	116	35	151	2	12	12
Thymus	108	52	160	9	18	27
Lung	115	78	193	2	12	12
Pancreas	126	30	156	2	2	2
Seminal vesicles	128	11	139	2	12	12
Ventral prostate	80	17	97	2	11	11
Testis	80	71	151	2	6	6
Placenta	90	11	101	2	3	7
Blood	55	36	91	5	3	8
Rat and mouse tumors						
Azo dye hepatic tumors						
Mixed tumor (rats)	226	83	309	3	27	30
Hepatoma	255	25	280	3	28	28
Normal liver of tumor-bearing rat	441	151	592	4	92	96
Jensen rat sarcoma	118	41	159	3	4	4
Crocker mouse sarcoma	112	61	174	5	9	14
Sarcoma 37	111	22	133	3	3	3
Carr lymphosarcoma	134	37	171	3	3	3
EL4 mouse leukemia	101	33	134	3	3	3
Walker 256 carcinoma (rat)	94	50	145	3	3	3
MC/63 mouse carcinoma	131	40	172	3	3	3
Krebs ascites tumor	132	36	168	3	3	3

[a] Expressed in micrograms per gram fresh tissue.

and spleen the largest amount of pyridine nucleotide was in the soluble supernatant fraction; the smallest amount, in the microsomes. In the tumors examined the levels of pyridine nucleotides were decreased in all fractions, but most markedly in mitochondria and microsomes. Indeed, in the tumor microsomes the level of

pyridine nucleotide was so low that it could not be detected by the sensitive methods used.

TABLE 39

THE DISTRIBUTION OF TOTAL PYRIDINE NUCLEOTIDE[a] IN THE CELL FRACTIONS OF NORMAL TISSUES AND TRANSPLANTABLE TUMORS OF THE MOUSE (81)

Tissue	Homogenate	Nuclei	Mitochondria	Microsomes	Soluble fraction
Normal tissues					
Liver	438	59	49	9.0	275
Kidney	406	72	78	14	195
Spleen	179	80	21	7.2	58
Brain	—	—	21	8.0	—
Mouse tumors					
Rhabdomyosarcoma	102	10.8	5.3	—[b]	70
Mammary carcinoma	136	17.8	3.2	—[b]	96
Squamous cell carcinoma	147	29.0	4.0	—[b]	86
Hepatoma	106	19.4	5.1	—[b]	64

[a] Expressed in milligrams per gram fresh tissue.
[b] No detectable pyridine nucleotide.

Several laboratories have independently described the need for the addition of high levels of DPN to tumor mitochondria in order to obtain adequate oxidation of a number of oxidative substrates (241, 501, 509, 510). Wenner and Weinhouse have studied this DPN requirement in detail (501). The effect of DPN addition on the oxidation of pyruvate, citrate, α-ketoglutarate, succinate, fumarate, and malate by mitochondria from normal and tumor tissues of the rat and mouse are presented in Table 23. Tumor mitochondria showed a requirement for added DPN for the oxidation of pyruvate, citrate, α-ketoglutarate, fumarate, and malate, but not for succinate oxidation. Brain displayed a similar requirement, but liver and kidney did not. The oxidation of pyruvate in the presence of DPN (2×10^{-3} M) was proportional to the mitochondrial content of the various normal and tumor tissues (500, 501), from which the authors inferred a similarity in normal and tumor mitochondria in the presence of DPN (Table 40).

The reason why tumor mitochondria require DPN for adequate oxidation of these substrates is unclear. It may be due in part to the lower initial level of pyridine nucleotide of the tumor. Another possibility is that the DPN is destroyed by the active DPNase

known to be present in tumors (54, 263, 330, 493). Quastel and Zatman (387) have assayed the DPNase activity of various normal and tumor tissues in an ingenious way. Acetone powders prepared from various tissues were found to inhibit the glucose fermentation of an acetone powder of yeast extract. This inhibition was shown to be due to DPNase, and the DPNase was assayed quantitatively from its ability to inhibit the glycolysis of the yeast system. Of the normal tissues, spleen and lung showed the highest DPNase, and kidney and liver showed the lowest activity. The levels of DPNase of tumors were spread over a wider range than those of normal tissues, but the enzyme levels in the neoplastic tissues were not consistently higher than in the non-neoplastic tissues.

TABLE 40
OXIDATION[a] OF PYRUVATE AND MALATE BY NORMAL AND NEOPLASTIC MITOCHONDRIA IN THE PRESENCE OF 0.002 *M* DPN (500)

Tissue	Pyruvate	Malate
A-hepatoma	270	314
C3H hepatoma	312	—
Rhabdomyosarcoma	150	159
Sarcoma 37	292	230
Kidney	292	278

[a] Oxygen uptake expressed in microliters per hour per milligram mitochondrial nitrogen.

Emmelot *et al.* have studied the ATPase and DPNase activities of mitochondria from a number of animal tumors (156, 157). They found that with regard to these activities tumors fell into two groups. The mitochondria with high ATPase activity (as determined by release of inorganic phosphate from ATP) and high DPNase activity (as determined enzymatically with alcohol and alcohol dehydrogenase by disappearance of DPN) were from a transplantable mouse sarcoma, an adrenal cortical carcinoma, a mouse ovarian sarcomatoid tumor, and an aminoazo dye rat hepatoma. Mitochondria from these tumors were unable to oxidize either octanoate or *d*- or *l*-β-hydroxybutyrate and they inhibited the oxidation of these substrates by normal liver mitochondria when the tumor mitochondria were added to the liver particles. The addition of DPN or nicotinamide to the mixed liver-tumor mitochondria system restored the ability to oxidize the *d*-β-hydroxybutyrate but not the *l*-isomer or octanoate. The authors attributed failure to obtain oxidation of the last two compounds by the combined mitochondria

to the ATPase activity of the tumor mitochondria. A second group of tumors possessed low ATPase and DPNase activities, did not inhibit oxidations of liver mitochondria, and were themselves able to oxidize β-hydroxybutyrate with a P:O ratio of 2–3. The same authors found (157) that dinitrophenol (DNP) did not enhance the mitochondrial swelling of those tumor mitochondria with high ATPase and DPNase activities as it did enhance the swelling of liver mitochondria. They concluded that these tumor mitochondria were already damaged with activated ATPase and DPNase before DNP addition, and were thus comparable to liver mitochondria after the compound had been added. Unfortunately, a later study from the same laboratory (154) indicated that the entire picture of high mitochondrial ATPase and DPNase of certain tumors was due, in all probability, to admixture of the mitochondrial fraction from these tumors with microsomes. The microsome fraction is known to be rich in these enzymes. [In normal rat liver 83% of the total DPNase is located in the microsomes, while in Novikoff hepatoma the microsomes contain 88% of the enzyme of the whole homogenate (48).]

An alternate explanation of the DPN requirement of *in vitro* tumor oxidations is that DPN synthesis is faulty rather than that there is excess destruction of the coenzyme. There is a limited amount of experimental evidence to support this explanation. For example, it has been reported that DPN synthesis in liver homogenates of CAF mice bearing sarcoma 37 was decreased (464). A subsequent report (463) has indicated that both in the sarcoma and in L-1210 leukemia there was a reduction in the DPN-synthesizing capacity of the blood and spleen as well as liver of the tumor-bearing animal. In addition, Branster and Morton have found that the DPN-pyrophosphorylase activity (the enzyme that synthesizes DPN from ATP and nicotinamide mononucleotide) of mouse mammary gland carcinomas of C3H mice is only 22% of the enzyme level of normal lactating mouse mammary tissue (55). In later work (330) it was found that the level of this DPN synthesizing enzyme (which is localized in the cell nucleus) was also very low in hepatoma (20% of the value of normal liver), in Ehrlich ascites cells, in samples of human neoplasms obtained at operation, and in fetal mouse liver. Other workers (501) have suggested that the DPN requirement of tumor mitochondria may be caused by less efficient binding of DPN in tumors than in their

non-neoplastic counterpart. Such a rapid dissociation of the bound DPN from the mitochondria could be associated with a subsequent hydrolysis of the compound (160).

Certainly, the cause of the well-defined DPN requirement for oxidations by cell-free tumor systems is unclear. It could be the result of a low DPN content of intact tumor cells, or a loose conjugation of the nucleotide with mitochondrial apo-enzymes, or an increased mitochondrial permeability, or a high DPNase activity, or an impaired ability of the tumor cell to synthesize pyridine nucleotides (181). Further work on this point is desirable. A phenomenon similar to the DPN requirement for the oxidation of Krebs cycle intermediate is to be found in the report that homogenates of Walker 256 carcinosarcoma and Flexner-Jobling carcinoma show a 20- to 40-fold increase in acetate activation when CoA is added to the medium (6).

7. Oxidative Phosphorylation in Tumor Mitochondria

The difficulties of early authors in obtaining oxidative phosphorylation in tumor homogenates has been reviewed in a previous section. With the adoption of cell fractionation methods employing sucrose, and with fortification of the medium with DPN, active oxidative phosphorylation by preparations of tumor mitochondria has been obtained in both the presence and the absence of fluoride. Keilly studied oxidative phosphorylation in sucrose mitochondria (241) isolated from transplantable mouse hepatomas and from normal liver. With such preparations, good oxidative phosphorylation with a variety of substrates (succinate, α-ketoglutarate, and glutamate) was obtained in the absence of fluoride. P:O ratios of 2.22 with α-ketoglutarate and of 1.55 with succinate were obtained with tumor mitochondria; these compared well with P:O ratios of 2.42 and 1.58 respectively with the two substrates in normal mouse liver. Keilly found that tumor mitochondria upon aging showed a marked loss of ability to carry on oxidative phosphorylation. Thus, tumor mitochondria lost 30–50% of their activity after 2–3 hours at 0°, and 100% was lost after 24 hours at 0°, or after 25 minutes at 28°. In contrast, liver mitochondria lost only 30–40% of their ability to carry on oxidative phosphorylation after 24 hours at 0°. Part of this lost activity of tumor mitochondria with α-ketoglutarate and glutamate as substrate could be restored by the addition of 0.001 *M* DPN.

Williams-Ashman and Lehninger (510) and Williams-Ashman and Kennedy (509) studied oxidative phosphorylation in sucrose mitochondria of two rat tumors, the Jensen sarcoma, and the Walker 256 carcinosarcoma, and in a mouse amelanotic melanoma. They were able to demonstrate good oxidative phosphorylation of succinate by these particles in the presence of magnesium, inorganic phosphate, ATP, and fluoride; this phosphorylation was dependent on substrate and was sensitive to DNP. The P:O ratios with succinate as substrate and with fructose and hexokinase as phosphate acceptor were 1.24 for the melanoma, 0.32 for the Walker 256 tumor, and 0.61 for the Jensen sarcoma. With ADP as phosphate acceptor, the P:O ratio was only one-third of that with fructose and hexokinase, while fluoride was found to be an obligatory component for oxidative phosphorylation with either phosphate acceptor. The addition of 0.001 *M* DPN was demonstrated to permit the oxidation of glutamate but not of fumarate, α-ketoglutarate, or citrate. Cytochrome *c* caused a 2- to 10-fold increase in the rate of oxidation of succinate by mitochondria from Flexner-Jobling carcinoma, but oxidation of the other substrates could not be obtained even with cytochrome *c* addition.

The first studies on oxidative phosphorylation in Ehrlich ascites cell mitochondria were performed by Kun *et al.* (263), who reported P:O ratios of 1.15–1.18 for succinate in the absence of fluoride. Later, Lindberg *et al.* (288) studied oxidative phosphorylation by ascites cell mitochondria, using a variety of substrates in the presence of glucose, hexokinase, and adenosine-5′-phosphate (AMP), but again in the absence of fluoride. They found that the over-all rate of respiration and phosphorylation, on a per milligram mitochondrial nitrogen basis, was low (uptake of phosphorus of 0.05–0.3 μ*M* per milligram of nitrogen per minute, compared with values of 2.0 and 4.0 for liver and heart respectively). The P:O ratios were also somewhat low except those for succinate (Table 41). Thus, malate and pyruvate gave P:O ratios of 2.0 and 1.2 respectively (compared with the theoretical value of 3.0); α-ketoglutarate, a ratio of 1.8 (compared with a theoretical value of 4.0); and succinate, a ratio of 1.4 (comparing well with the theoretical value of 2.0). DPN was not added to the medium in these experiments, which may explain the rather poor oxidative phosphorylation with substrates other than succinate (succinate is the only

substrate that does not require pyridine nucleotide for its oxidation).

TABLE 41
OXIDATIVE PHOSPHORYLATION IN ASCITES CELLS (288)

Substrate	Oxygen consumption (μatoms/mg. protein N)	Phosphate esterified (μ*M*/mg. protein N)	P:O ratio
None	0.0	0.0	—
Pyruvate	1.4	1.7	1.2
Pyruvate + succinate	4.8	9.7	2.0
Citrate	0.3	1.3	—
α-Ketoglutarate	5.3	9.6	1.8
Succinate	8.6	11.8	1.4
Malate	3.9	7.8	2.0

With succinate as the substrate, P:O ratios as high as 1.8 have been reported in ascites cells by other investigators (96). Indeed, Ács *et al.* (2.3) have obtained a P:O ratio of 3.0 for Ehrlich ascites cell mitochondria oxidizing glucose and endogenous substrate in the absence of added fluoride or hexokinase. This last result agrees well with Quastel and Bickis (385) who, using indirect methods, calculated that the P:O ratios of intact Ehrlich ascites cells and of slices of Novikoff hepatoma and Walker 256 carcinosarcoma were between 2.9 and 3.2. Emmelot *et al.* (156) have also reported high P:O ratios (2–3) for a group of transplanted testicular and ovarian tumors oxidizing β-hydroxybutyrate.

Indirect studies on oxidative phosphorylation have been performed by Siekevitz *et al.* (422), and Siekevitz and Potter (420), through study of the effect of fluoride and DNP on the oxidative rate of tumor homogenates. Whereas fluoride tends to maintain the high energy phosphate pool (inhibits ATPase), DNP uncouples oxidation from phosphorylation and leads to a running down of the high energy phosphate reservoir (ATP). These authors postulated that the most active respiration will be produced when the balance between high energy phosphate (ATP) and phosphate acceptor (ADP) is optimal. Thus, in the Flexner-Jobling rat carcinoma dephosphorylation outstripped the phosphorylative processes until fluoride was added. With the addition of this compound, active oxidation of pyruvate and fumarate was obtained with this tumor, while addition of DNP led to a further deterioration of the already

low oxidative rate. However, when the rate of oxidation of a number of other tumors was studied with DNP and fluoride, no consistent pattern emerged. Thus, a human fibrosarcoma was markedly stimulated by fluoride and slightly stimulated by DNP, both a mouse and a rat hepatoma were inhibited by fluoride and unaffected by DNP, an adrenal tumor was inhibited by both compounds, and a mouse mammary tumor was stimulated by fluoride but not affected by DNP. It thus appears that factors other than those considered by the authors are involved in the effects of fluoride and DNP on tumor oxidations. The authors found that DPN (in low concentrations) would not substitute for fluoride in the stimulation of oxygen uptake by the Flexner-Jobling carcinoma (420).

Wenner and Weinhouse (502) studied the effect of DNP and fluoride on the glucose-C^{14} oxidation (conversion to $C^{14}O_2$) of homogenates and slices of tumors and normal tissues. These authors found (Table 42) that fluoride inhibited glucose oxidation in homogenates of neoplastic and nonneoplastic tissues, while dinitrophenol stimulated glucose oxidation at low concentrations and inhibited it at high concentrations. Studies with slices differed from the homogenate studies in that low concentrations of DNP stimulated glucose oxidation of slices and the compound inhibited only at higher concentrations.

In a recent review, Potter and Siekevitz have summarized their results and those of others in studying oxidative phosphorylation in tumors (378). There can be little doubt that cell-free preparations of certain tumors (Flexner-Jobling carcinoma and Walker 256 carcinosarcoma, for example) fail to perform active oxidative phosphorylation under conditions which allow good oxidative phosphorylation in normal tissues such as liver, kidney, brain, and heart (378). This may be in part a reflection of the fact that these tumors have a limited capacity for oxidative phosphorylation (limited mitochondrial content) while the ATPase activity is in the range of the active normal tissues (normal content of microsomes and nuclei). Supporting this hypothesis is the beneficial effect on oxidative phosphorylation of the addition of fluoride, and the greater ease of obtaining oxidative phosphorylation in tumor mitochondria isolated free of other cell particles. Another factor contributing to the difficulty of obtaining oxidative phosphorylation in these tumors is the DPN requirement discussed in the preceding section. This require-

TABLE 42

EFFECT OF FLUORIDE AND DINITROPHENOL ON $C^{14}O_2$ FORMATION[a] FROM LABELLED GLUCOSE IN HOMOGENATES OF NORMAL AND NEOPLASTIC TISSUES (502)

Tissue	No addition	NAF (0.0075 *M*)	DPN (0.002 *M*)	NAF (0.0075 *M*) + DPN (0.002 *M*)	DPN (0.002 *M*)	DPN (0.002 *M*) + DNP (1.2×10^{-5} *M*)	DPN (0.002 *M*) + DNP (6×10^{-5} *M*)	DPN (0.002 *M*) + DNP (2.5×10^{-4} *M*)
Heart	0.2	0.1	11.2	0.1	17.8	17.6	0.3	0.3
Kidney	0.1	0.1	7.2	0.1	3.4	1.5	0.5	0.5
Brain	0.4	0.3	1.6	0.2	2.1	—	4.3	3.7
Liver	0.5	0.0	0.6	0.1	0.7	0.6	0.23	0.20
Hepatoma	0.1	0.0	1.8	0.1	2.0	1.3	0.1	0.2
Rhabdomyosarcoma	0.1	0.1	1.2	0.2	3.5	2.0	1.55	0.16
Sarcoma 37	0.1	0.0	0.4	0.1	0.59	0.56	0.24	0.19
Mammary adenocarcinoma	0.1	0.0	0.8	0.2	0.37	0.30	0.07	—
Ehrlich ascites	0.5	0.4	0.7	0.7	—	—	—	—

[a] Expressed in μatoms of carbon.

TABLE 43
CELL FRACTION STUDIES OF NORMAL LIVER AND AZO DYE HEPATOMA IN THE RAT (410)

Tissue	Succinoxidase (μl. O_2 per 10 min. per 100 mg. fresh tissue)	Cytochrome oxidase (μl. O_2 per 10 min. per 100 mg. fresh tissue)	ATPase (μg. phosphorus liberated per 15 min. per 100 mg. tissue)	RNA (μg. per 100 mg. fresh tissue)	"Protein" nitrogen (μg. per 100 mg. fresh tissue)	Dry material (μg. per 100 mg. fresh tissue)
Normal rat liver						
Homogenate	383	1012	865	65.2	1970	31.9
Nuclear fraction	25.4	54.6	231	4.9	197	3.3
Mitochondria	289	748	416	11.4	434	5.7
S_1 (mitochondrial supernatant)	45.5	147	257	47.8	1250	23.3
Hepatoma						
Homogenate	86.7	322	802	66.1	1093	21.3
Nuclear fraction	10.0	29.4	99.5	7.4	265	5.3
Mitochondria	48.5	203	98	6.6	94	1.7
S_1 (mitochondrial supernatant)	20.4	101	594	50.1	206	13.8

ment is evidenced by the greater ease in obtaining good oxidative phosphorylation with succinate (which does not involve a pyridine-linked dehydrogenase) than in obtaining it with other oxidative substrates. A second group of tumors, including the mouse hepatoma and the Ehrlich ascites tumor, appears to be similar to normal tissue since good oxidative phosphorylation can be obtained without the many considerations discussed above. Whether the difference in behavior between the two groups of tumors is caused by a different content of mitochondria, by a difference in ATPase or DPNase activity, or other factors such as differences in mitochondrial permeability, is not clear at the present time.

8. Number and Nitrogen Content of Tumor Mitochondria

With regard to tumor mitochondria, it would be desirable that the following two questions were answered: (1) What differences are there between the amount (nitrogen content) of mitochondria in the normal and in the neoplastic cell? (2) In what ways are the mitochondria of the neoplastic cell similar to and different from the mitochondria of the normal cell? Unfortunately, only very incomplete answers to these questions are available. The best studies that bear on this question have been comparative studies of hepatoma and liver in rats and mice.

Schneider studied the mitochondrial content of normal rat liver and of hepatoma produced by the administration of the hepatic carcinogen dimethylaminoazobenzene, measuring the ribonucleic acid (RNA), protein content, and dry weight of the mitochondrial fraction (obtained by centrifugal fractionation). As indicated in Tables 43 and 44, the mitochondrial content of the hepatoma was

TABLE 44
INTRACELLULAR COMPOSITION OF LIVER AND TUMOR OF RATS FED 4'-DIMETHYLAMINOAZOBENZENE (383)

	Protein (mg. protein/gm. wet weight)		Ribonucleic acid (mg. RNA/gm. wet weight)		Riboflavin (μg./gm. wet weight)	
Cell fraction	Liver	Tumor	Liver	Tumor	Liver	Tumor
Homogenate	131	122	5.22	6.88	5.5	4.0
Nuclei	27	42	0.82	1.74	0.6	0.8
Mitochondria	37	13	1.54	0.69	2.6	1.2
Microsomes	14	13	1.20	1.24	0.9	0.6
S_2 (soluble fraction)	52	54	1.47	3.32	1.5	1.7

only 22% that of the normal liver on a "protein" nitrogen basis, or 33% of normal on a dry weight basis. The investigator felt that the tumor mitochondria were similar to normal mitochondria because of the similar content of succinoxidase, cytochrome oxidase, and ATPase of the two mitochondria on a per milligram protein nitrogen basis (410). For normal liver the activities were 665 for succinoxidase, 1720 for cytochrome oxidase, and 960 for ATPase, while for rat hepatoma the respective figures are 516, 2160, and 1040 (enzyme activities being expressed in the same units. However, it should be noted that the tumor mitochondria contained about twice the RNA per milligram nitrogen of liver mitochondria. A later study by Price *et al.* (383) confirmed the results on mitochondrial content of hepatoma as measured by protein and RNA content of the mitochondrial fraction, and extended the study to show that the riboflavin content of the mitochondrial fraction was also markedly reduced (2.6 μg. per gram in liver and 1.2 μg. per gram in hepatoma). It has also been found that the preneoplastic liver of rats fed dimethylaminoazobenzene showed a diminished mitochondrial content (35% decrease of mitochondrial protein, 40% decrease of mitochondrial RNA, and 45% decrease of mitochondrial riboflavin) (381, 382). It was even possible to show (380) that the decrease in mitochondrial content was proportional to the carcinogenicity of all but one of a group of aminoazobenzene dyes (Fig. 7). The exception was 4′-fluoro-4-dimethylaminoazobenzene which, though a very potent carcinogen, caused only a modest decrease in mitochondrial content. Extending these studies, Potter *et al.* (376) showed that the level of succinoxidase and oxaloacetic oxidase fell in a manner parallel to the fall of mitochondrial content (as judged by protein, riboflavin, and nitrogen). It is known that these oxidase activities are localized in the mitochondria (411). It was also possible to show that the fall of mitochondrial content did not take place in hamsters, which are not sensitive to the carcinogenic activity of this group of dyes, while it did take place in mice, which are slightly sensitive (384). Laird and Miller have shown that feeding 2-acetylaminofluorine, a hepatic carcinogen belonging to a different chemical group from the aminoazo dyes, also brings about a fall in the level of liver mitochondria (Fig. 8) (265).

Similar cell fractionation studies have been done on the mouse hepatoma 98/15, a well differentiated hepatoma that histologically resembles adult liver and is carried by subcutaneous transplanta-

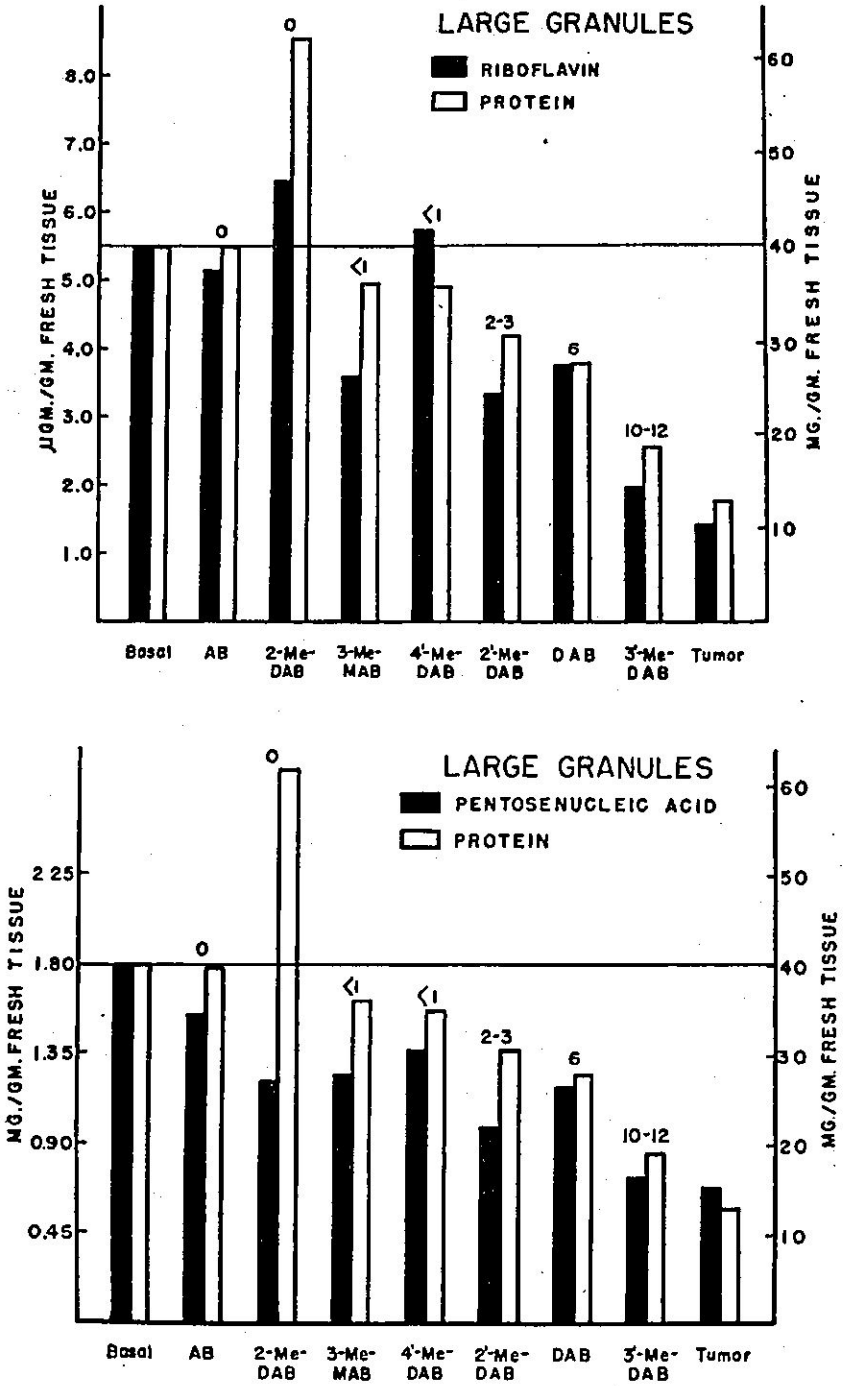


FIG. 7. Comparison of the levels of riboflavin, protein, and pentosenucleic acid in the mitochondria from livers of rats fed various azo dyes, and in mitochondria of tumors induced with 4-dimethylazoaminobenzene. From Price *et al.* (380).

tion. The results (412), reported in Table 45, indicate that the liver has 2.4 times as much mitochondrial nitrogen (on a per gram wet tissue basis) as does the hepatoma. In this tumor the cytochrome oxidase and succinoxidase of the mitochondria are decreased more than the mitochondrial nitrogen, with the result that the enzyme activities of the mitochondria, on a per milligram nitrogen basis, are only 35–50% that of the liver (Table 46).

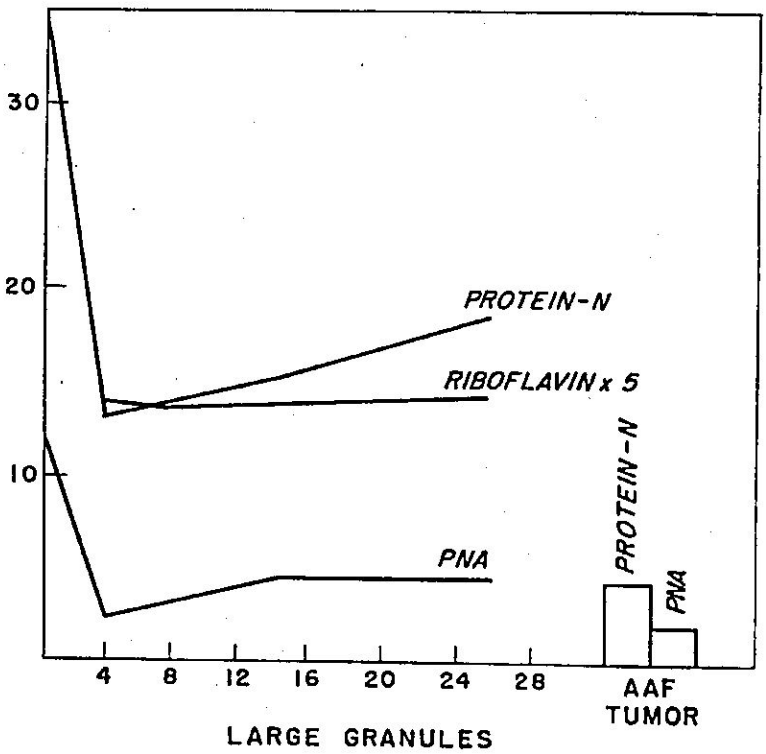


FIG. 8. Changes in the levels of riboflavin, protein, and pentosenucleic acid of rat liver mitochondria during the course of carcinogenesis with acetylaminofluorene (AAF). The ordinate is expressed in grams $\times 10^{-12}$ per average liver cell and the abscissa in weeks. From Laird and Miller (265).

More recent studies have been done on direct counting of mitochondria of normal and neoplastic hepatic tissue. It was found (424, 444) that in mice the number of mitochondria per gram of tissue was markedly reduced in the hepatoma from the level of normal liver, but the number of mitochondria per nucleus were about equal in normal mouse liver and mouse liver tumor. In more refined studies, Hogeboom and Schneider (218) studied the individual mitochondrial proteins of mouse hepatoma and C3H mouse liver. The mitochondria were disrupted, either by sonic vibration or from being passed under great pressure through a small orifice,

TABLE 45

PROTEIN AND RIBONUCLEIC ACID CONTENT OF CELL FRACTIONS OF C3H MOUSE LIVER AND HEPATOMA 98/15 (412)

	Nitrogen				Ribonucleic acid			
	C3H liver		Hepatoma 98/15		C3H liver		Hepatoma 98/15	
Cell fraction	Total N (mg./ 100 mg. fresh tissue)	Per cent of total homogenate N	Total N (mg./ 100 mg. fresh tissue)	Per cent of total homogenate N	Total μg./ 100 mg. fresh tissue	Per mg. N	Total μg./ 100 mg. fresh tissue	Per mg. N
Homogenate	3.19	100	2.30	100	92.9	28.1	97.5	42.4
Nuclei	0.57	17.9	0.52	22.8	10.2	19.2	16.0	32.2
Mitochondria	0.75	23.5	0.30	13.1	15.6	18.4	10.3	30.9
Microsomes	0.73	23.1	0.45	19.6	48.7	64.2	38.8	84.0
Soluble fraction	1.20	376	0.04	46.1	15.3	12.0	25.7	24.5

TABLE 46

SUCCINOXIDASE, CYTOCHROME OXIDASE, ADENOSINE TRIPHOSPHATASE, AND DIPHOSPHOPYRIDINE NUCLEOTIDE (DPN)-CYTOCHROME *c* REDUCTASE IN FRACTIONS OF C3H MOUSE LIVER AND HEPATOMA 98/15 (217, 412, 413)

	C3H mouse liver			Hepatoma 98/15		
	Per 100 mg. fresh tissue	Per cent of total homogenate activity	Per mg. N	Per 100 mg. fresh tissue	Per cent of total homogenate activity	Per mg. N
Succinoxidase (mm.^{3} O_2/hr.)						
Homogenate	4,250	(100)	1,340	755	(100)	325
Nuclei	842	19.8	1,650	128	17.0	239
Mitochondria	2,400	56.5	3,180	445	58.9	1,220
S_1 (mitochondrial supernatant)	184	4.3	91	58	7.7	37
Cytochrome oxidase (mm.^{3} O_2/hr.)						
Homogenate	6,860	(100)	2,060	1,520	(100)	633
Nuclei	1,360	19.8	2,440	195	12.8	379
Mitochondria	5,390	78.6	6,460	964	63.4	3,300
Microsomes	292	4.1	351	247	16.3	624
Soluble fraction	0	0	0	0	0	0
Adenosine triphosphatase (μg. inorganic phosphate from ATP/ 15 min.)						
Homogenate	1,580	(100)	485	907	(100)	379
Nuclei	495	31.3	822	342	37.8	606
Mitochondria	790	50.0	1,050	117	12.9	392
Microsomes	240	15.2	316	318	35.1	690
Soluble fraction	80	5.17	63	125	13.8	122

TABLE 46 (*Continued*)

	C3H mouse liver			Hepatoma 98/15		
	Per 100 mg. fresh tissue	Per cent of total homogenate activity	Per mg. N	Per 100 mg. fresh tissue	Per cent of total homogenate activity	Per mg. N
DPN-cytochrome *c* reductase (μ *M* cytochrome *c* reduced/min.)						
Homogenate	6.95	(100)	2.14	8.80	(100)	3.82
Nuclei	0.63	9.1	1.10	1.30	14.8	2.54
Mitochondria	1.97	28.3	2.56	2.40	27.3	7.48
Microsomes	4.12	59.3	5.49	4.56	51.8	9.22
Soluble fraction	0.24	3.5	.19	.69	7.8	.65

and 1576 per nucleus. Because the increase of liver mitochondria in rats fed the non-carcinogenic dye was not associated with a proportional increase in mitochondrial nitrogen and mitochondrial enzymes (cytochrome, uricase, octanoxidase, and nucleases), the authors felt that this increase was not an increase in mitochondria biochemically similar to those of normal liver. On the other hand, the remaining mitochondria of the preneoplastic liver (after the feeding of carcinogenic dye) appeared by these criteria to be identical with normal mitochondria. Howatson and Ham (223) have used the electron microscope to compare the number of mitochondria of the Novikoff hepatoma and the LT-124 liver carcinoma with normal rat liver. They found that the neoplastic tissue had only one-fourth the number of mitochondria of the normal cell, and that the neoplastic mitochondria exhibited a greater variation of size than did their normal counterparts.

There have been few other systematic studies on the mitochondrial content of tumors. Figure 9, from Wenner and Weinhouse (501), indicates that the mitochondrial content, on a per milligram nitrogen per wet weight of tissue basis, is lower in a miscellaneous group of tumors than it is in normal tissue. These authors also found that in terms of pyruvate oxidation the activities of tumor and normal mitochondria are similar, the differences in pyruvate oxidation between normal and tumor tissue being mainly a reflection of the higher mitochondrial content of normal tissues. Table 49 indicates that the mitochondria of ascites cells accounts for less than 4% of the total nitrogen of this tumor cell. Laird (264a) determined the nitrogen content of the combined mitochondria and fluffy layer of a number of normal and neoplastic tissues, and found that on a per cell basis tumors were uniformly low in this combined fraction but not lower than several of the normal tissues examined. (Since the fluffy layer contains microsomes and, probably, other particles, as well as mitochondria, such measurements of combined mitochondria and fluffy layer are difficult to interpret.) The following data come from the paper of Schneider and Hogeboom (411) and are expressed as the per cent of the total homogenate nitrogen contained in the mitochondrial fraction: rat liver, 23–26%; mouse liver, 24%; rat kidney (Table 10), 17%; Flexner-Jobling carcinoma (Table 10), 8%; Novikoff hepatoma, 4%; spleen (normal), 2.5%; spleen (leukemic), 2.2%. There is a manifest need for more data on the amount of mitochondria in

tumor tissue compared with that of normal tissue and their enzyme complement, at least with regard to a group of key enzymes.

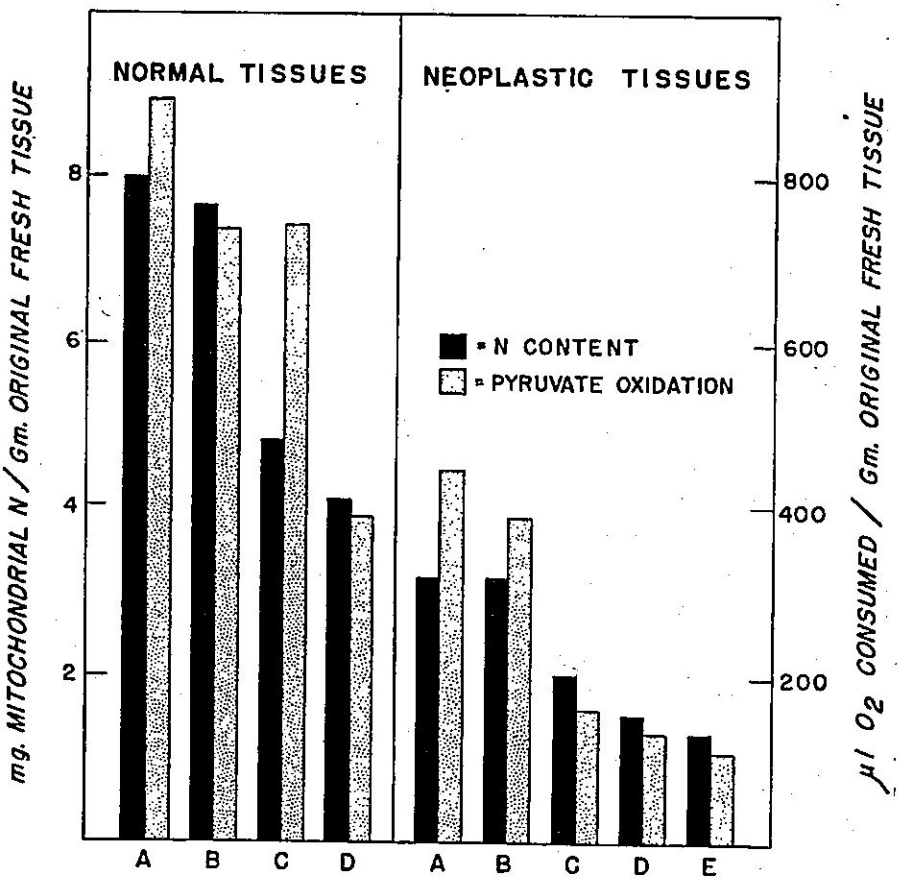


FIG. 9. Pyruvate oxidation versus nitrogen content of mitochondria of normal and neoplastic tissues. The unshaded bars on the left of each column represent the milligrams mitochondrial nitrogen per gram of original whole tissue. The shaded bars on the right side of each set of columns represent the net respiratory activity in microliters of O_2 consumed per gram of fresh tissue per 30 minutes. Normal tissues: column A, rat liver; column B, mouse liver; column C, mouse kidney; D, rat brain. Neoplastic tissues: column A, mouse hepatoma (strain A); B, mouse hepatoma (strain C3H); C, sarcoma 37; D, rhabdomyosarcoma; E, rat hepatoma. From Wenner and Weinhouse (501).

9. Miscellaneous Studies on Tumor Mitochondria

A group of miscellaneous studies with tumor mitochondria, which do not rightfully belong in any of the previous sections are included here. Graffi and Schneider (189, 190) and Graffi *et al.* (191, 192) studied the effect of the addition of various concentrations of mitochondria from liver and from various tumor tissues on the glycolysis of combined glucose and hexose diphosphate by the

TABLE 49

NITROGEN CONTENT, CYTOCHROME OXIDASE ACTIVITY, AND GLUTAMIC ACID DEHYDROGENASE ACTIVITY OF CELL FRACTIONS OF EHRLICH ASCITES TUMOR AND MOUSE LIVER (82)

	Ehrlich ascites cells					Mouse liver				
		Cytochrome oxidase		Glutamic acid dehydrogenase			Cytochrome oxidase		Glutamic acid dehydrogenase	
Cell Fraction	Nitrogen (mg.)	Total activity (Δ O.D.)[a]	Specific activity (Δ O.D. mg. N)	Total activity (Δ O.D.)	Specific activity (Δ O.D. mg. N)	Nitrogen (mg.)	Total activity (Δ O.D.)	Specific activity (Δ O.D. mg. N)	Total activity (Δ O.D.)	Specific activity (Δ O.D. mg. N)
Homogenate	54.1	258	4.7	1.32	0.02	89	3480	39.9	22.2	0.26
Nuclei	21.4	146	6.8	0.42	0.02	15.0	173.7	11.6	0.8	0.48
Mitochondria	2.1	95	45	0.22	0.11	25.4	2476	97.4	9.6	0.22
Microsomes	1.5	5.8	3.8	0.33	0.05	20.2	381	18.8	5.9	0.06
Supernatant	22.2	—	—	0.25	0.01	32.0	—	—	1.7	0.26

[a] O.D. = optical density.

glycolytic system derived from the soluble particle-free supernatant fraction of liver and tumor. While liver mitochondria added to liver soluble fraction produced a maximal stimulation of glycolysis of only 158% of the control without mitochondria, Ehrlich tumor mitochondria produced a 272% stimulation. Liver mitochondria produced a 260% stimulation of the glycolysis of sarcoma 37 glycolytic system; Jensen sarcoma mitochondria, a 288% stimulation; and Ehrlich tumor mitochondria, a 461% stimulation. The authors attributed the stimulatory effect of the added mitochondria to the increased availability of phosphate acceptor in the presence of the added mitochondria. A similar observation has also been reported with mitochondria from Walker 256 carcinosarcoma and Flexner-Jobling carcinoma (8).

In a preceding section on tumor glycolysis the view has been presented that glycolysis takes place primarily in the particle-free soluble fraction of the cell. Although this view is generally accepted and is consistent with a considerable body of experimental work, a few authors hold a different view. Dubuy and Hesselbach (141, 142, 207) feel that the entire energy-producing mechanism of the cell (respiratory and glycolytic) is located in the mitochondria, the supernatant glycolytic activity representing artifact due to the leakage of mitochondrial glycolytic enzymes. Their data in Table 50 demonstrate that mitochondria from brain, S-91 melanoma, a hepatoma, and Krebs-2 tumor display significant glycolysis, with glucose or hexose diphosphate as the substrate. However, it is not clear to what extent this mitochondrial glycolytic activity represents contaminating supernatant enzymes, since the authors report that washing brain mitochondria once reduced the glycolysis from a $Q_{La}^{N_2}$ of 13.6 to the value of 3.5 reported in the table, and reduced the glycolysis of the S-91 melanoma from 2.5 to 0. Hochstein (215, 216) has also reported glycolytic activity by melanoma mitochondria which required boiled supernatant fraction (later found to be ATP). One other report mentions glycolytic activity of tumor mitochondria (300), while several observers have reported glycolysis by brain mitochondria (19, 176). Only one investigator (176) has attempted to determine the percentage of the original glycolytic activity found in the carefully washed mitochondrial preparation. He found that about 10% of the glycolytic activity of the original brain homogenate was present in the mitochondria. At the present

TABLE 50

UTILIZATION OF GLUCOSE, HEXOSE DIPHOSPHATE, PYRUVATE, AND α-KETOGLUTARATE BY WASHED MITOCHONDRIA AND BY MITOCHONDRIAL SUPERNATANT (S_2) OF NORMAL AND NEOPLASTIC TISSUES (142)

	Glucose		Hexose diphosphate		Pyruvate		α-Ketoglutarate		Endogenous	
Tissue	Q_{O_2}	Q_{La}	Q_{O_2}	Q_{La}	Q_{O_2}	Q_{La}	Q_{O_2}	Q_{La}	Q_{O_2}	Q_{La}
Brain										
Mitoch.	4.7	3.5	4.8	5.3	9.6	1.3	9.0	0	1.1	0.6
S_2	0.2	22.8	0	13.4	0	—0.1	— 0.1	0.5	0.9	0.7
Testis										
Mitoch.	1.6	0.3	1.9	1.8	3.0	1.4	—	—	1.2	0.2
S_2	0.3	1.9	0.5	10.5	0.4	0.4	—	—	0.2	0.8
S-91										
Mitoch.	3.5	1.6	6.2	5.9	—	—	12.5	0.5	1.3	1.4
S_2	—0.1	29.4	—0.1	16.7	—	—	0	0.3	0.4	0.7
Krebs-2										
Mitoch.	0.8	0.9	3.6	3.0	4.3	—	8.0	—	0.6	0.5
S_2	0.1	19.6	0.1	7.9	—0.2	—	—	—	0.3	0.6
Hepatoma										
Mitoch.	0.5	0.4	3.7	3.9	0.9	1.0	—	—	6.5	0.3
S_2	0.3	35.2	—0.3	12.2	—0.4	5.2	—	—	0.5	1.8

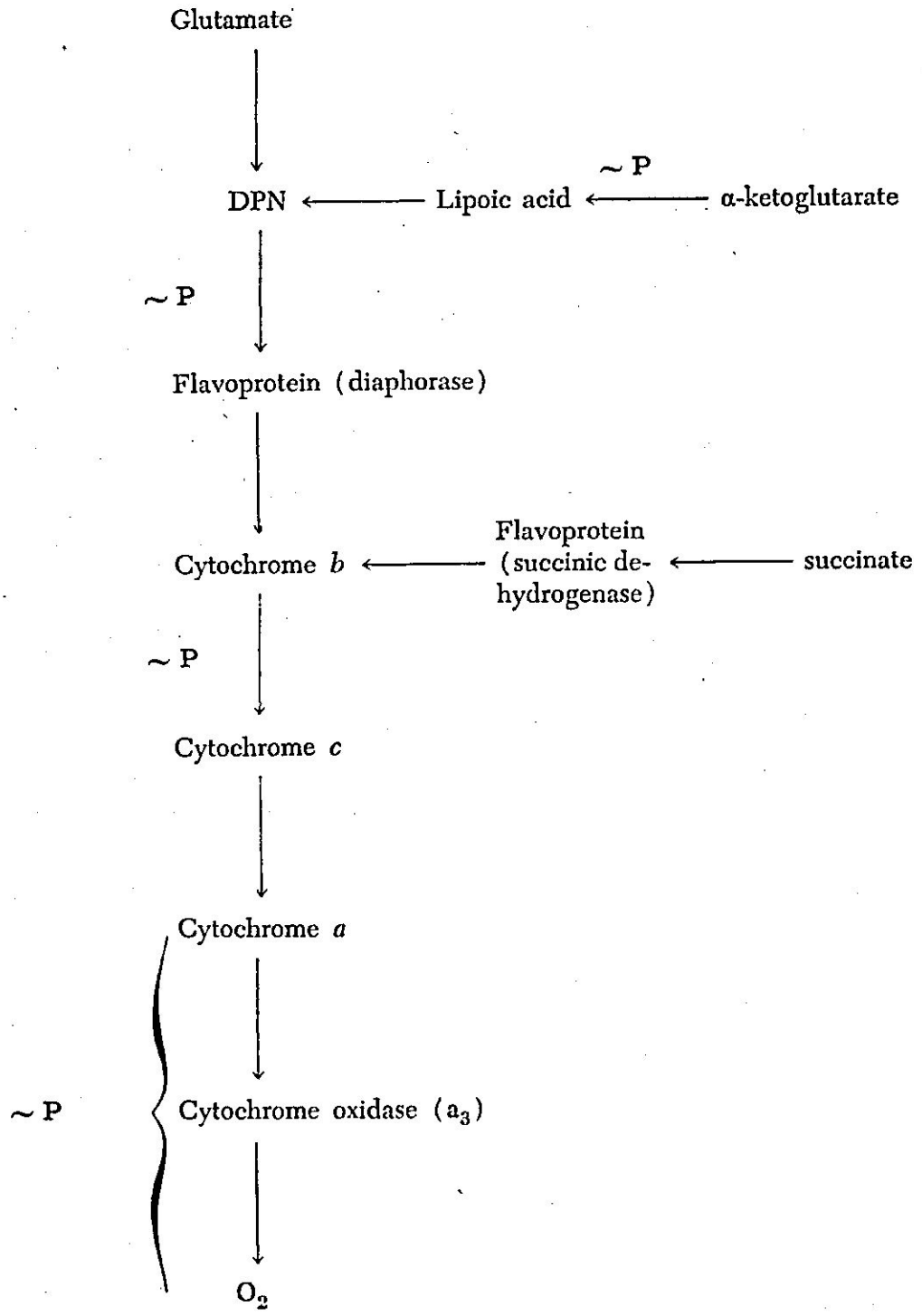
time (until mitochondrial contamination with soluble fraction is more carefully controlled), it seems unwise to assume that the mitochondria from normal or tumor tissue possess quantitatively important glycolytic activity (391, 409). A number of earlier studies have suggested that the glycolytic system is located in the soluble fraction of the cell (178, 247, 285), and the tumor work reported above requires careful evaluation while taking into account contamination of the mitochondria with soluble fraction. On the other hand, it is quite possible that mitochondria added to a competent glycolytic system derived from the soluble cytoplasmic fraction of the cell can enhance glycolysis, either through an effect on adenine nucleotide balance or by supplying a single limiting glycolytic enzyme such as hexokinase.

Mutolo and Abrignani (332) have found that tumor mitochondria differ from the mitochondria of normal tissues in their swelling response to hypotonicity, detergents, and trypsin. Similarly, it has been found that DNP does not enhance the swelling of tumor mitochondria as it does normal mitochondria (157). Arcos *et al.* (13) have studied alterations in the swelling of mitochondria isolated from the livers of rats fed carcinogenic amino azo dyes. They found, in the mitochondria from the liver of rats fed the carcinogenic dyes, a decrease in mitochondrial swelling compared with that of normal liver mitochondria, and found that swelling was minimal at the time of maximal tumor incidence. No change in swelling occurred after administration of a non-carcinogenic azo dye. They interpret this decrease in mitochondrial swelling as reflecting an increase in mitochondrial rigidity which, they feel, is in turn due to alterations in the mitochondrial membrane. Obviously, much further work must be done before the significance of these studies is evident.

10. The Level of Specific Oxidative Components in Tumors

A. Introduction

The elucidation of the specific pathways of the transport of substrate hydrogen and electrons to molecular oxygen is one of the most impressive achievements of modern biochemistry (174, 267). This pathway, as generally conceived today (433), is presented on the following page:



Briefly, for most substrates hydrogen and the electron pair are first transferred to pyridine nucleotide with the aid of a specific dehydrogenase. The next step in the chain is the transfer of the hydrogen and electron pair to the flavin moiety of a flavin enzyme. The electron pair is then passed along a group of porphyrin en-

zymes (the cytochromes). These have been principally studied by their spectrophotometric characteristics, and the order of transfer is believed to be *b*, *c*, *a*. Finally, the electron pair is transferred to molecular oxygen through cytochrome a_3 (cytochrome oxidase). As a substrate, succinate is unusual in that it bypasses the DPN step and is directly oxidized by a flavin enzyme, succinic dehydrogenase. Associated with the transfer of electrons from substrate to oxygen there is esterification of inorganic phosphate to yield ATP from ADP. In the case of most substrates, for each electron pair transferred to oxygen three molecules of ATP are realized. For succinate, which bypasses the pyridine nucleotide step, the yield appears to be only two. The notation $\sim$ P represents the currently conceived sites of the three respiratory chain phosphorylations. In addition, there is, for certain substrates such as α-ketoglutarate, a substrate level phosphorylation.

B. Succinic Dehydrogenase

Succinic dehydrogenase is generally measured by the rate of oxygen consumption in a tissue preparation (slice or homogenate of appropriate concentration) in a system that is usually fortified with cytochrome *c*. Since measurement has shown that in a large variety of tissues the cytochrome oxidase is present in excess of the succinic dehydrogenase, it is assumed that under the condition of added cytochrome *c* the succinic dehydrogenase is the limiting component. Under these conditions the rate of oxygen uptake is a measure of activity of the dehydrogenase. The data in Table 51 indicate that, in general, the succinic dehydrogenase activity of tumors is lower than that of active normal tissues (414), but that the activity of neoplastic tissues is in the range of the least active normal tissues (spleen, lung, and skeletal muscle). Earlier measurements by Elliott and Grieg (148) of the level of this enzyme yielded similar results. Rat and mouse hepatomas (410, 412, 413) display a succinic dehydrogenase activity (Tables 43 and 46) that is one-fourth that of normal liver. Succinoxidase (the entire succinic acid-oxidizing respiratory complex) is contained entirely in the mitochondrial fraction of the cell (411), and it has been found (380) that in azo dye feeding the decrease of mitochondrial protein that occurs in the neoplastic and preneoplastic liver is accompanied by a parallel decrease in the succinoxidase (Figs. 7 and 8).

Reif and Potter (394) extended the studies on succinoxidase in

TABLE 51

LEVELS OF SUCCINIC DEHYDROGENASE, CYTOCHROME *c*, AND CYTOCHROME OXIDASE IN FRESH NORMAL AND NEOPLASTIC TISSUES

	Succinic dehydrogenase	Cytochrome *c*			Cytochrome oxidase			
	Ref. (414)[a] (μl. O_2/ mg. dry tissue/hr.)	Ref. (139)[b] (μg./ gram wet weight)	Ref. (441) (mg./gm. dry weight)	Ref. (400) (mg./gm. dry weight)	Ref. (414)[c] (μl. O_2/ mg. dry tissue/hr.)	Ref. (420)[d] (μl. O_2/ mg. wet weight)	Ref. (441) (μl. O_2/ mg. wet weight)	Ref. (195)[e] (μl. O_2/ mg. wet weight)
Normal rat tissues								
Heart	219	371	2.34	—	974	68.7	97	40.2
Kidney	195	247	1.36	1.43	549	—	47	14.0
Skeletal muscle	36	97	0.68	—	180	8.2	23	6.3
Brain	49	50	0.35	0.38	420	—	35	13.0
Adult liver	88	90	0.24	0.61	281	30.9	17	11.8
Fetal liver	—	—	—	—	—	12.3	—	—
Regenerating liver	—	—	—	—	—	30.1	—	4.3
Spleen	23	43	0.21	—	195	9.5	16	—
Lung	18	21	0.11	0.02	92	—	13	—
Neoplastic tissue								
Rat								
Primary hepatoma (DAB)	26	20	—	—	101	6.8	—	3.1
Transplanted hepatoma	18	—	—	—	124	—	—	—

[a] In homogenate with succinate and excess cytochrome *c*.
[b] In homogenate.
[c] In homogenate with ascorbate and excess cytochrome *c*.
[d] In presence of *p*-phenylenediamine and excess cytochrome *c*.
[e] In presence of *p*-phenylenediamine.

TABLE 51 (*Continued*)

	Succinic dehydrogenase Ref. (414)[a] (μl. O_2/mg. dry tissue/hr.)	Cytochrome *c*			Cytochrome oxidase			
		Ref. (139)[b] (μg./gram wet weight)	Ref. (441) (mg./gm. dry weight)	Ref. (400) (mg./gm. dry weight)	Ref. (414)[c] (μl. O_2/mg. dry tissue/hr.)	Ref. (420)[d] (μl. O_2/mg. wet weight)	Ref. (441) (μl. O_2/mg. wet weight)	Ref. (195)[e] (μl. O_2/mg. wet weight)
Neoplastic tissue								
Rat (*Continued*)								
Walker 256 carcinosarcoma	11	9	—	0.07	70	—	—	—
Flexner-Jobling carcinoma	15	12	—	—	84	—	—	—
Jensen rat sarcoma	18	12	—	—	129	2.6	—	—
Tumor R-256	—	—	0.02	—	—	—	29	1.6
Spontaneous tumor	—	—	0.01	0.05	—	—	24	—
Mouse								
Spontaneous hepatoma	—	—	—	—	—	5.1	—	—
Hepatoma (AAT)	—	—	—	—	—	6.3	—	—
Hepatoma (CCl_4)	—	—	—	—	—	6.3	—	—
Adenocarcinoma (stomach)	—	—	—	—	—	3.0	—	—
Adenocarcinoma (intestine)	—	—	—	—	—	4.4	—	—
Adenocarcinoma (breast)	28	14	—	—	88	6.1	—	—
Yale 1 tumor	20	16	—	—	97	—	—	—
Leukosis	—	—	—	—	—	4.4	—	—
Malignant melanoma	—	—	—	—	—	6.5	—	—
Ear tumor (u.v.)	19	11	—	—	64	—	—	—
Sarcoma 37	—	—	—	—	—	6.5	—	—
Sarcoma 180	—	—	—	—	—	5.5	—	—
Rous chicken sarcoma	11	12	—	—	44	—	—	—

TABLE 52
OXIDATIVE RESPONSE OF NORMAL AND NEOPLASTIC HUMAN TISSUE SLICES TO ADDED SUCCINATE AND *p*-PHENYLENEDIAMINE (402)

Tissue	$-Q_{O_2}$	Succinate (% increase in oxidative rate)	*p*-Phenylenediamine (% increase in oxidative rate)
Normal			
Kidney	9.2	+127	+118
Skin	4.0	+158	+118
Thyroid	5.6	+134	+118
Prostate	6.5	+176	+173
Testicle	6.4	+107	+167
Rectal mucosa	4.2	+100	+ 93
Tongue (leukoplakia)	3.1	+152	+213
Neoplastic			
Rectosigmoid adenocarcinoma	4.9	0	0
Rectosigmoid adenocarcinoma	9.0	+ 20	+ 8
Rectosigmoid adenocarcinoma	8.9	+ 16	+ 18
Breast papilloma	8.7	+ 41	− 7
Rectosigmoid adenocarcinoma	8.0	+ 23	+ 16
Chronic cystic mastitis	4.8	− 21	− 10
Fibroxanthoma	10.8	+ 47	+ 39
Synovial sarcoma	7.4	+ 11	+ 35
Verrucous tumor of neck	3.0	+ 23	+ 20
Sympathicoblastoma (kidney)	13.3	− 10	+ 7
Cancer of stomach	9.4	+ 1	+ 30
Fibroxanthoma of leg	10.1	+ 34	− 28

TABLE 52 (*Continued*)

Tissue	$-Q_{O_2}$	Succinate (% increase in oxidative rate)	*p*-Phenylenediamine (% increase in oxidative rate)
Neoplastic (*Continued*)			
Brain tumor	8.9	+ 27	− 20
Lymph node metastasis (cancer of rectum)	5.3	+ 36	+ 36
Hypernephroma (kidney)	4.5	+ 2	+ 18
Splenic flexure adenocarcinoma	10.8	+ 51	+ 27
Basal cell carcinoma	7.3	− 29	− 6
Lymphoblastoma (inguinal node)	7.7	− 16	+ 38
Cancer of stomach (lymph node)	11.2	+ 27	—
Adenocarcinoma (kidney)	9.7	+ 21	+ 97
Sigmoid tumor	6.6	+ 24	+ 30
Testicular tumor	7.8	+ 76	− 15
Breast carcinoma	15.3	—	+830
Breast carcinoma	—	−	+ 87
Breast carcinoma	2.0	+ 52	0
Mycosis fungoides	−	−	+ 29

measuring the inhibition of succinoxidase by antimycin A, a compound that inhibits electron transport in the region of cytochrome *b*. They found that the antimycin A titer (amount of antimycin A required to cause a 50% inhibition of succinoxidase *in vitro*) is roughly parallel to the succinoxidase activity *in vitro*. With the idea of possible chemotherapeutic applications, the studies were extended to *in vivo* inhibition of succinoxidase by antimycin A. It was hoped that neoplastic tissues that possess very low succinoxidase levels might be most sensitive to the agent. Unfortunately, a good correlation between *in vivo* inhibition of succinoxidase and *in vitro* inhibition (or antimycin titer) was not obtained. Thus, of six tissues with low *in vitro* succinoxidase levels (spleen, lung, thymus, Flexner-Jobling carcinoma, Walker 256 tumor, and Jensen rat sarcoma) only three (lung, spleen, and the Jensen tumor) showed the expected *in vivo* sensitivity.

In a preceding section, the early work on the response of the oxidative rate to added substrate was considered. For convenience, later studies on the response of the oxidative rate of slices of tumor and normal tissues to added succinate and *p*-phenylenediamine are considered in this section (109, 402, 403). As discussed previously, the oxidative rate in the presence of succinate can be taken as a measure of succinoxidase. *p*-Phenylenediamine acts as a substrate in a manner similar to that of succinate, but rather than requiring reaction with a flavin enzyme such as succinic dehydrogenase, it reacts directly with the cytochrome chain. Thus, assuming that cytochrome oxidase is the limiting member of the cytochrome chain, the rate of oxidation of *p*-phenylenediamine may be assumed to be a measure of the cytochrome oxidase activity of a tissue. In general, the studies to be considered here are developments of the earlier work of Elliott *et al.* (146, 149, 150) and have been reported in terms of percentage increase in oxidative rate over the endogenous rate after the addition of substrate. Glucose has usually been present in this medium.

The basic conclusion to be drawn from the data of Tables 52 and 53 is that tumor tissues display a low activity of succinoxidase and cytochrome oxidase (measured by the percentage increase in oxidative rate upon addition of succinate and *p*-phenylenediamine respectively) compared with normal tissues in general and, more specifically, with the normal tissue of origin. Thus, while normal liver, diaphragm, and granulation tissue show responses of the

TABLE 53

RESPONSE OF OXIDATIVE RATE TO ADDED SUCCINATE AND *p*-PHENYLENEDIAMINE IN NORMAL AND NEOPLASTIC TISSUES OF THE MOUSE (109)

	$-Q_{O_2}$	Succinate (% increase in oxidative rate)	*p*-Phenylenediamine (% increase in oxidative rate)
Normal liver	10.6	+137	—
Hepatoma	10.5	+ 17	—
Diaphragm	7.0	+242	—
Rhabdomyosarcoma	8.7	+ 41	—
Granulation tissue	1.5	+192	—
Sarcoma 180	8.2	+ 27	—
Benign sarcoma 180	6.4	+ 37	—
Normal lactating breast	—	+ 37	+12 to +362 (5 samples)
Mammary adenocarcinoma (transplanted)	—	−10 to +62 (128 samples)	−12 to +75 (11 samples)
Mammary adenocarcinoma (spontaneous)	—	—	+15 to +37 (7 samples)
Normal lung	10.1	+ 66	+98
Yale 1 tumor	6.6	+ 10	+24
Lymphosarcoma	8.8	+ 3	−13
Mouse "chest tumor"	7.6	− 8	+16

oxidative rate to added succinate in excess of 100% stimulation, the response of the homologous tumors (hepatoma, rhabdomyosarcoma, and sarcoma 180) were all less than 50%. With the two other sets of homologous tissues studied, the results were much less satisfactory. Normal lactating mouse mammary gland displayed a small response to succinate, but a variable response to *p*-phenylenediamine. Of the mammary neoplasms, the mouse tumors showed a low response to both substrates, while one of the human carcinomas displayed an increase of 830% in oxidative rate upon *p*-phenylenediamine addition. Both normal and leukemic leukocytes showed no response of oxidative rate to succinate addition, but a good response to *p*-phenylenediamine. Later studies (402) on the response of liver to succinate and *p*-phenylenediamine during the course of azo dye carcinogenesis (Table 54) revealed a normal response until

TABLE 54
RESPONSE OF OXIDATIVE RATE TO ADDED SUCCINATE AND *p*-PHENYLENEDIAMINE DURING HEPATOCARCINOGENESIS WITH BUTTER YELLOW (402)

Days on butter yellow diet	Succinate (% increase in oxidative rate)	*p*-Phenylenediamine (% increase in oxidative rate)
21	+118	+147
32	+140	+101
44	+164	+103
49	+150	+106
62	+279	+279
76	+ 69	+ 12
88	+ 71	+ 82
95	+ 89	+102
114	+ 67	+ 95
127	+ 31	+ 73
136	+ 22	+ 28
150	+ 23	+ 44
163	− 29	− 15
186	+ 10	+ 20

about the 50th day, with the loss of response developing at about the 70th day of dye feeding (tumors first developed on day 163). Similarly, experiments (Table 55) with the Shope papilloma indicated a loss of the high percentage oxidative response of normal skin to addition of the two substrates at about the 10th day after virus inoculation. (No carcinoma had developed at the time this experiment was terminated.) The responses of a large group of

normal tissues and tumors to added succinate and *p*-phenylenediamine are included in Table 52. A large oxidative response of normal tissue and a low response of neoplastic tissue was consistently noted. A later investigation by Woods (515) indicated that the percentage response of tissue slices to succinate addition was dependent, to a considerable extent, on variables such as medium and DPN addition. However, the above author also found the percentage response of liver, brain, and kidney much greater than that of S-91 melanoma and Krebs-2 tumor, although chorion and embryo responded as tumor did.

TABLE 55

RESPONSE OF OXIDATIVE RATE OF RABBIT SKIN TO ADDED SUCCINATE AND *p*-PHENYLENEDIAMINE DURING EPIDERMAL CARCINOGENESIS WITH THE SHOPE PAPILLOMA VIRUS (402)

Time after inoculation (wk.)	Succinate (% increase in oxidative rate)	*p*-Phenylenediamine (% increase in oxidative rate)
6	+222	+316
7	+382	+440
10	+ 28	+ 1
14	+ 4	+ 14
46	− 8	+ 40
55	+ 14	+ 6
79	+ 19	+ 60

Two groups of investigators have extended these studies on the responses of normal and neoplastic tissue to succinate and *p*-phenylenediamine addition. Kidd *et al.* (251) studied the normal and neoplastic tissues of the rabbit skin in the presence of glucose. While with normal tissues increases in Q_{O_2} following succinate addition varied from 59 to 9000% and, following *p*-phenylenediamine, from 12 to 7400%, with neoplastic tissues the increase following addition of substrate did not exceed 40% for succinate and 120% for *p*-phenylenediamine. The benign tumor of the rabbit skin resembled the malignant tumor more closely than it did normal skin, a result in keeping with the result of Salter *et al.* (402). Rosenthal and Drabkin (399), in studies differing from the preceding only in the omission of glucose from the medium, arrived at the following conclusions. They found that normal tissues fell into two groups with regard to their responses to added succinate and *p*-phenylenediamine. The first group displayed a high oxidative response to the added substrate and included liver (3000–6000), kidney cortex

(450–550), and brain and muscle (250–550). The second group of normal tissues gave a poor oxidative response (gastrointestinal mucous membrane and lung (50–150) and, to a lesser degree, skin, mammary gland, and lymphatic tissues). The response of neoplastic tissues resembled this second group of normal tissues having a low percentile response (120–300) to the added substrate.

C. Cytochrome *c* and Cytochrome Oxidase

These two components represent the most studied members of the cytochrome chain. Cytochrome oxidase is localized almost entirely in the mitochondria, while cytochrome *c* is equally divided between the mitochondria and the soluble portion of the cytoplasm (411). These compounds have been examined in neoplastic tissue by a number of investigators. They have been assayed in a homogenate system by Potter and his group. Schneider and Potter (414) studied cytochrome oxidase by measuring the rate of oxidation of reduced cytochrome *c* (generated by the addition of ascorbic acid), and DuBois and Potter (139, 371) studied the level of cytochrome *c* by a method involving the specific enzymatic reduction of cytochrome *c*. The results of these studies on cytochrome *c* as well as of those of earlier investigators are included in Table 53. The level of cytochrome *c* in neoplastic tissues is quite low in comparison to normal tissues, although one normal tissue, lung, is in the same range. The levels of cytochrome oxidase are also low in neoplastic tissue but, again, the lowest normal tissues are in the same range. In the transition of normal liver to hepatoma, the cytochrome *c* content is reduced to one-quarter and the cytochrome oxidase to one-third. Similar conclusions on the levels of cytochrome *c* and cytochrome oxidase in normal and tumor tissues had been arrived at earlier by Stotz (441), employing different methods. Rosenthal and Drabkin (400) found that the cytochrome *c* content of normal epithelial tissues falls into a high and a low group. The epithelial neoplastic tissues studied were in the same range as the normal tissues of the low cytochrome *c* group (lung and mammary gland). Shack (420), on the basis of his studies on cytochrome oxidase (which are included in Table 53), has suggested that the level of the cytochrome components is so low in neoplastic tissue that the respiratory rate is limited by them. He feels that the cytochromes are not limiting in normal tissues.

The levels of the oxidative enzymes, cytochrome *c*, cytochrome

oxidase, and succinoxidase have been studied by Carruthers and Suntzeff during the course of methylcholanthrene epidermal carcinogenesis in mice (80, 83). Figure 10 indicates that there is a slight fall in the cytochrome *c* level of the epidermal neoplasm as compared with normal or preneoplastic skin, while the level of succinic dehydrogenase has more than doubled in the neoplasm. Cytochrome oxidase is slightly higher in the neoplasm and in the

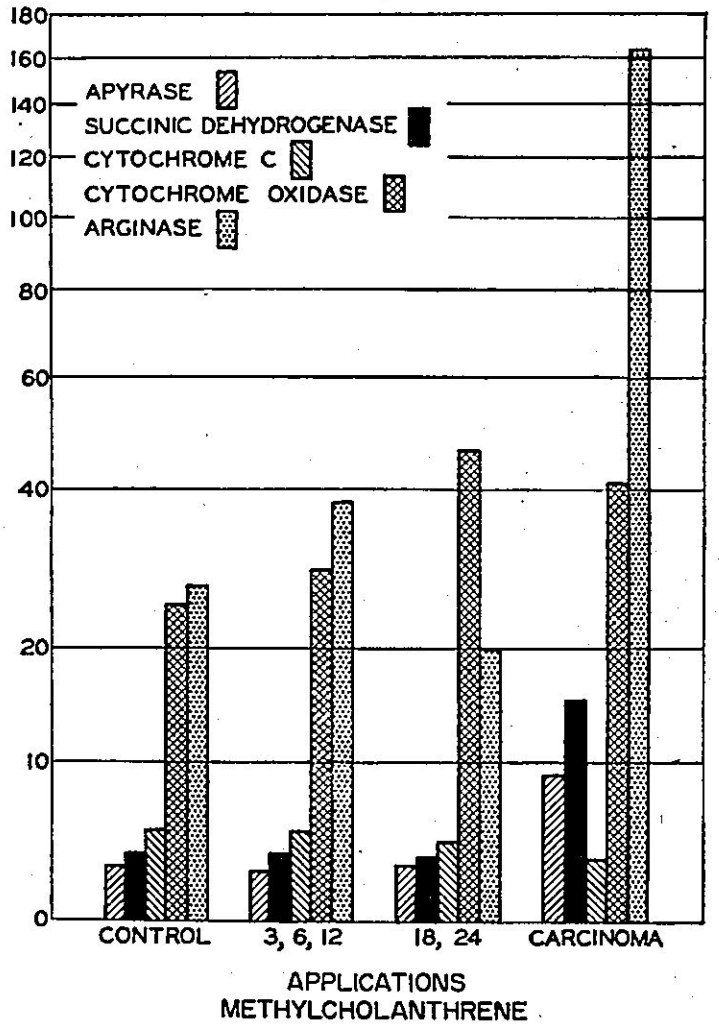


FIG. 10. The activities of adenosine triphosphatase (Apyrase) succinic dehyrogenase, cytochrome oxidase, and arginase, and the level of cytochrome *c* during epidermal carcinogenesis with methylcholanthrene (M.C.) in the mouse. The activities of succinoxidase and cytochrome oxidase are expressed in the conventional manner as Q_{O_2}; cytochrome *c*, as micrograms per 100 mg fresh tissue; adenosine triphosphatase, as micrograms of phosphorus liberated per milligram of fresh tissue in 15 minutes; and the arginase, as micrograms urea per milligram fresh tissue. From Carruthers (79).

late preneoplastic period as compared to the early preneoplastic and control periods. In a later study (79) the measurements of cytochrome oxidase levels during epidermal carcinogenesis were repeated with similar findings, and the work was extended to a determination of DPNH- and TPNH-cytochrome *c* reductases and diaphorase.* TPNH-cytochrome *c* reductase and diaphorase were found to be present in the same low or trace levels in normal, preneoplastic hyperplastic, and neoplastic epidermis. DPNH-cytochrome *c* reductase in normal epidermis and in three-fourths of the squamous cell carcinomas examined was found to be twice as high as in the preneoplastic hyperplastic skin and in papillomas. In interpreting these observations that there is no fall in the level of the respiratory enzymes during the course of epidermal carcinogenesis it should be remembered that the level of these enzymes in normal skin is very low, in the range of that seen in most neoplasms.

Cytochrome oxidase and cytochrome *c* have also been studied in acetone powder preparations of normal and neoplastic tissues by Greenstein *et al.* (195). The over-all cytochrome oxidase-cytochrome *c* activity of the system was studied by determining the rate of oxygen uptake in the presence of *p*-phenylenediamine. When cytochrome *c* was added to the system, the rate of oxygen uptake was then felt to be a measure of the cytochrome oxidase activity of the system. In all cases an increased rate of oxygen uptake was obtained when cytochrome *c* was added, suggesting that the oxidase actually was limiting. As indicated in Table 56, cytochrome oxidase is high in heart, brain, kidney, and liver, and low in prostate, bladder, uterus, spleen, lung, and neoplasms. The distribution of cytochrome *c* was similar to that of cytochrome oxidase, being high in heart muscle, liver, kidney, and brain, and low in lung, uterus, spleen, and neoplasms. In both these and the previous studies the levels of cytochrome *c* appear to be disproportionately low relative to the levels of cytochrome oxidase in neoplastic tissues, as compared to these levels in normal tissues.

Greenstein (194) classified tissues according to their relative lack of cytochrome *c* compared to their levels of cytochrome oxidase, dividing them into four groups by the per cent response of oxidative

* Diaphorase is a flavoprotein respiratory component that reacts with DPNH, but only very sluggishly with cytochrome *c*. Its activity was therefore measured as the rate of oxidation of an oxidation-reduction dye (2,6-dichlorobenzenoneindophenol) in the presence of cyanide-blocked cytochrome system.

rate to cytochrome *c* addition in the presence of *p*-phenylenediamine. Group 1 displayed a percentage response to added cytochrome *c* of 100–400, and included only normal tissues (heart, skeletal muscle, liver, kidney, and brain). Groups 2 and 3 displayed an intermediate response of 600–1200 and included both normal tissues (thyroid, spleen, pancreas, adrenal, intestine, stomach, bladder, uterus, prostate, and lung) and neoplastic tissues (thyroid adenomas, hyperthyroid prostate, gastric carcinoma, granulosa cell tumor, uterine fibroid, early prostatic carcinoma, chondrosarcoma, mixed parotid tumor, synovioma, desmoid tumor, and a rectal adenoma). Group 4 responded with an increase of oxidative rate of 1200–6000 (the lowest levels of cytochrome *c* as compared to cytochrome oxidase), and included only neoplastic tissue (spindle cell sarcoma, malignant melanoma, epidermoid carcinoma, malignant meningioma, lymphosarcoma, bronchogenic carcinoma, and gastric, mammary, rectal, and colonic carcinomas).

Waravdekar, Paradis, and Leiter (276, 462) have investigated the effect of a number of tumor-damaging agents (podophyllotoxin, α- and β-peltatin, two arsenicals, three antimonials, two phenazines, one quinoxaline, and four colchicines) on the *in vitro* cytochrome oxidase activity of a number of mouse tumors (sarcoma 37, lymphomas 1 and 2, leukemic 1210, mammary adenocarcinoma C3HBA, and melanoma S-91). The drug was injected *in vivo* into the tumor-bearing animal, and the enzyme activity of the tumor homogenate determined *in vitro*. In each case there was a drop in the cytochrome oxidase activity of the tumor at 2 hours after injection, with the most marked rate of drop at 4–8 hours. There was also a drop in the activity of cytochrome oxidase activity of the spleen and lymph node homogenates from the animals bearing lymphomas, which was attributed to the effects on the tumor infiltration of these tissues. Homogenates of liver and other non-neoplastic tissues displayed much smaller drops following drug injection than did the tumor homogenates. Later studies with podophyllotoxin and α- and β-peltatin (462) indicated that these drugs, in addition to causing a marked decrease in the cytochrome oxidase activity of sarcoma 37 homogenates, decreased the cytochrome *c*, the succinoxidase activity, and the over-all respiratory rate of the tumor. Furthermore, it was found that there was also a decrease of the anaerobic glycolysis of this tumor homogenate, which could be overcome by increasing the DPN concentration of the medium and which was

TABLE 56
CYTOCHROME OXIDASE AND CYTOCHROME *c* IN NORMAL AND NEOPLASTIC HUMAN TISSUES (195)

Tissue	Cytochrome oxidase ($V_{max.}$)[a]	Cytochrome *c*[b]	$v_{obs.}$[c]	$v_{calc.}$[d]	Response[e]
Normal					
Heart muscle	8.2	10.2	4.0	4.0	100
Skeletal muscle	2.4	2.4	0.7	0.5	375
Liver	2.8	2.3	0.8	0.6	400
Kidney	3.6	2.4	1.0	0.8	375
Brain	3.9	2.3	0.9	0.8	400
Thyroid	0.6	1.0	0.1	0.08	750
Spleen	0.4	0.8	0	0.03	1000
Pancreas	0.5	0.8	0.1	0.05	1000
Adrenal	0.7	0.8	—	0.06	1000
Intestine	0.5	0.9	0.1	0.06	857
Stomach	0.5	0.9	0.1	0.06	857
Uterus	0.4	0.7	0	0.03	1200
Bladder	0.4	0.7	0	0.03	1200

[a] Expressed in cubic millimeters oxygen taken up per hour per milligram wet tissue. Cytochrome *c* added in excess (1×10^{-4} *M*) plus *p*-phenylenediamine. Activity designated $V_{max.}$

[b] Concentration in milligrams per 100 gm. wet tissue.

[c] $v_{obs.}$ refers to cubic millimeters oxygen taken up in presence of *p*-phenylenediamine but not added cytochrome *c*.

[d] $v_{calc.}$ refers to cubic millimeter oxygen per milligram wet tissue calculated from the experimental quantities of cytochrome oxidase ($V_{max.}$), cytochrome *c*, and the Michaelis-Menten equation.

[e] The response (calc.) is given as % by the expression:

$$\frac{V_{max.} - v_{calc.}}{v_{calc.}} \times 100 = \frac{K_M}{\text{cytochrome } c} \times 100.$$

TABLE 56 (*Continued*)

Tissue	Cytochrome oxidase ($V_{max.}$)[a]	Cytochrome *c*[b]	$v_{obs.}$[c]	$v_{calc.}$[d]	Response[e]
Normal (*Continued*)					
Prostate	0.2	0.8	0	0.02	1000
Lung	0.4	0.7	0	0.03	1200
Neoplastic					
Thyroid adenoma	0.38–1.42	0.9–1.6	0–0.22	0.04–0.20	600–1000
Dermatofibrosarcoma	0.68	0.6	0	0.04	1500
Hypertrophied prostate	0.40–0.42	0.9–1.4	0	0.03–0.05	644–1000
Early prostatic carcinoma	0.32	0.8	0	0.03	1200
Granulosa cell tumor (ovary)	0.90	1.3	0.12	0.11	750
Uterine fibromyoma	0.34–0.48	0.7–0.7	0	0.02	1200–1500
Giant cell tumor	0.54–0.68	1.3–1.6	0.12	0.07–0.10	600–750
Chondrosarcoma	0.68	1.0	0.12	0.07	1000
Mixed parotid tumor	0.70	1.4	0.12	0.08	750
Synovioma	0.54	0.8	0	0.04	1200
Spindle cell sarcoma	0.48	0.7	0	0.02	1500
Connective tissue tumor	0.42	0.6	0	0.02	1500
Melanoma	0.30–0.35	0.7–0.8	—	0.02	200–1500
Epidermoid carcinoma	0.34–0.44	0.3–0.4	0	0.01–0.02	2000–3000
Meningioma	0.32–0.34	0.3–0.4	0	0.01	2000–3000
Bronchogenic carcinoma	0.42	0.3	0	0.01	3000
Lymphosarcoma	0.22	0.3	0	0.01	3000
Early gastric carcinoma	0.22	0.9	0	0.01	1200
Gastric carcinoma	0.28–0.38	0.4–0.6	0	0.01	2000–3000
Colonic adenocarcinoma	0.38–0.50	0.4–0.5	0	0.01–0.02	2000–3000
Rectal adenoma	0.82	0.9	0.12	0.08	1000
Rectal adenocarcinoma	0.36–0.50	0.4–0.7	0	0.01–0.03	1500–3000
Mammary carcinoma	0.24–0.48	0.2–0.4	0	0.00–0.02	3000–6000

not accompanied by changes in ATPase, aldolase, or hexokinase activity.

Before concluding this section it is most important to discuss briefly the validity of the above measurements. Each of the methods of enzymatic assay used above involves an arbitrary and often quite destructive tissue treatment (homogenization or acetone powder preparation) followed by enzymatic assay, under conditions which, on the basis of existing knowledge and materials, are considered to be limiting for the particular enzyme being studied. Thus, the assay may reveal only a part of the true cytochrome present if a significant amount of enzyme has been destroyed in the experimental procedure; this is an important problem, particularly in the case of the acetone powder procedures. These assays, therefore, measure enzyme activity, not enzyme amount. Another objection, of less importance, concerns the possibility that in the artificial conditions of the assay all the integrating and controlling mechanisms of the intact cell are removed. To circumvent these difficulties Chance and coworkers (85, 90, 92, 96–98) have developed methods for the measurement of the cytochromes which involve microspectrophotometric determination of the enzymes in intact cells or whole mitochondria. Specific inhibitors are used to obtain the various components of the electron transport chain in the proper state of oxidation or reduction for spectrophotometric measurement (carbon monoxide for the terminal oxidase, antimycin A for the contribution of the b_5 pathway, and amytal for the relative importance of DPN- and succinate-linked pathways). Chance and Hess find for example, that amytal (Fig. 11) blocks the respiratory pathway of the ascites cell between pyridine nucleotide and flavoprotein, as evidenced by the presence in the ascites cell inhibited by this agent of the reduced form of pyridine nucleotide and the oxidized form of flavoprotein and cytochrome *b*. Amytal acts at the same site in the liver mitochondria. Furthermore, the nearly complete inhibition of respiration in the ascites cell, which is observed in the presence of either antimycin A or amytal, suggested to the authors that the electron transport pathways of the ascites cell are similar to those of liver mitochondria. They further found, from spectroscopic study, that the cytochrome content of the ascites cell is largely contained within the mitochondria, an observation which they feel eliminates significant respiratory function by cytoplasmic cytochromes.

The results of these spectroscopic measurements with intact

ascites cells and mitochondria derived from them are at great variance with the enzyme data mentioned earlier. Chance and Hess (92, 96) found the levels (Table 57) of all the cytochromes of the ascites cell and ascites mitochondria to be comparable with the levels found in the other actively respiring tissues that they had studied by this method (rat liver mitochondria, muscle cells, and yeast cells). In particular, cytochrome *c* was not deficient compared with the other cytochrome components, as had been suggested by other workers. Studies on the turnover number of cyto-

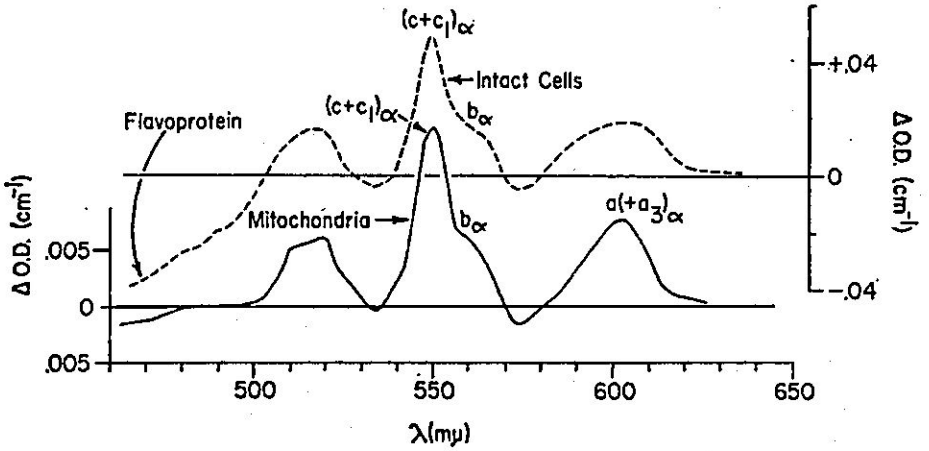


FIG. 11. Spectrum corresponding to absorbency differences between the anaerobic ascites tumor cells and the aerobic cells treated with amytal in order to cause oxidation of the cytochrome components. For comparison, the difference spectrum of cytochrome components of the mitochondria isolated from the tumor cell is included. From Chance and Hess (96).

chrome *a* of the intact ascites cell revealed a value one-third that of ascites mitochondria or mitochondria from other sources. This led the authors to suggest that the cytochrome of the intact ascites cell is under physiological control, a control which they thought to be due most likely to a lack of phosphate acceptor (ADP). Upon addition of glucose to resting ascites cells (94, 96) there was a brief period of marked respiratory stimulation (2–6 times the control rate) followed by respiratory inhibition (Crabtree effect, see Part III). The authors point out that this clearly indicates that the cytochrome chain is capable of much greater activity than that found in the endogenous respiratory state, a conclusion which is in disagreement with the previously held view that the cytochromes (particularly cytochrome *c*) of the tumor cell are functioning

microsomal fractions. In a subsequent study, Reinafarje and Potter (395) compared normal rat liver and Novikoff hepatoma with regard to the activities of DPNH-cytochrome *c* reductase, TPNH-cytochrome *c* reductase, and transhydrogenase (the enzyme that transfers hydrogen between TPN and DPN). The mitochondria and microsomes of both liver and hepatoma were found to contain DPNH-cytochrome *c* reductase, but the transhydrogenase was limited to the mitochondria of liver and was not present at all in hepatoma. Although the TPNH-cytochrome *c* reductase was also absent from the tumor, it was contained in the normal liver mitochondria and microsomes. The latter mitochondrial enzyme could be dissociated from the particle in non-sedimentable form.

Lenta and Riehl (279) have studied the rate of oxidation of DPNH by acetate buffer extracts of normal and neoplastic tissue. They found that total DPNH oxidase activity* (based on the ability of the extract to decolorize the oxidation-reduction dye 2,6-dichlorophenolindophenol) to be the following: heart, 230; kidney, 112; liver, 60; brain, 40; and muscle, 8. On the other hand, a group of tumors, including two sarcomas, two adenocarcinomas, and a hepatoma, displayed values of 6–8. These workers then went on to study the specific DPNH-cytochrome *c* reductase activity, the cytochrome oxidase activity, and the diaphorase activity (DPNH reductase activity in the presence of cyanide-blocked cytochrome chain) of these tissues. Table 58 contains the summarized results which indicate that the levels of diaphorase and DPNH-cytochrome *c* reductase in the sarcoma and the adenocarcinoma were below those of the normal tissues studied, while the level of these enzymes in the hepatoma were similar to the levels of normal liver. The levels of the cytochrome *c* and cytochrome oxidase were lower in the three tumors than in any of the five normal tissues studied, which is consistent with the studies of these enzymes reported in an earlier section.

E. Other Dehydrogenases

Meister (311) has made extensive measurements of the level of lactic dehydrogenase in normal and tumor tissues. By employing a spectrophotometric method, the levels were determined in a water homogenate. Table 59 indicates that the lactic dehydrogenase

* Decrease in optical density at 340 mμ × 1000.

TABLE 58

SUMMARY OF STUDIES ON THE DPNH OXIDASE SYSTEM AND ITS COMPONENTS IN NORMAL AND TUMOR TISSUE EXTRACTS OF THE MOUSE (279)

Tissue	DPNH oxidase	Diaphorase	Cytochrome *c* reductase	Cytochrome *c*	Cytochrome oxidase
Liver	+++	++++	+++++	++	+++
Kidney	++++	++++	++++	+++	++++
Heart	+++++	++++	++++	++++	++++
Brain	++	++	+++	++?	+++
Muscle	+	+	+	++?	+++
Sarcoma S-37	+	+	++	+	+
Adenocarcinoma	+	+	++	+	+
Hepatoma 98/15	+	+++	++++	+	++

activity of tumors was in about the middle range of the activities of normal tissues. Furthermore, it was not possible to correlate the glycolytic rate of normal or tumor tissues with the level of lactic dehydrogenase. A group of dehydrogenases studied by Weinhouse *et al.* (Table 26) displayed a low activity in tumors as compared to normal tissues.

TABLE 59
LACTIC DEHYDROGENASE ACTIVITY OF NORMAL AND NEOPLASTIC TISSUE (311)

Tissue	Lactic dehydrogenase activity[a]	Tissue	Lactic dehydrogenase activity[a]
Normal mouse tissues		Neoplastic mouse tissues	
Liver	420–455	Hepatomas (T)	381, 669
Kidney	354–377	Lymphomas (T)	292, 335
Skeletal muscle	948–1010	Lung tumors (I)	466, 561
Heart	292	Thymoma (T)	316
Lung	83	Sarcoma 37 (T)	419
Intestine	470	Gastric carcinoma (T)	336
Stomach	261	Melanomas (T)	163, 367
Pancreas	155	Mammary carcinomas (T)	299, 389
Spleen	140	Granulosa cell tumor (T)	257
Submaxillary gland	136	Myoepithelioma (T)	492
Brain	226	Rhabdomyosarcoma (T)	250
Thymus	189		
Lymph nodes	206		
Uterus	140		
Ovary	116		
Lactating breast	373		
Testis	188		
		Rat tissues	
		Adult liver	419
		Fetal liver	640
		Hepatoma (azo dye)	495
		Fibrosarcoma (T)	414

[a] Activity in moles $\times 10^{-8}$ pyruvate per milligram total nitrogen per minute.

(T) = transplanted tumor.

(I) = induced tumor.

Lenta and Riehl (278) employed a Thunberg technique (in which the rate of decolorization of methylene blue by an acetone powder of tissue in the presence of appropriate substrate is determined), to measure the levels of lactic and malic dehydrogenases. They found the levels of both dehydrogenases were lower in a mouse adenocarcinoma and in sarcoma 180 than in any of the normal tissues studied (muscle, spleen, kidney, liver, and brain).

F. Riboflavin and Other B Vitamins

Riboflavin is a component of the known enzymes that catalyze the transfer of hydrogen and electrons from reduced pyridine nucleotide to the cytochrome chain, and is also a component of the oxidases that short-circuit the cytochrome chain, transferring hydrogen and electrons directly to molecular oxygen (amino acid oxidase, etc.). Since sensitive and specific methods for the determination of riboflavin are available, it has been measured in many normal and neoplastic tissues (249, 250, 262, 367, 397). As indicated in Tables 60 and 61, the level in tumors is lower than in the more active normal tissues such as liver, kidney, and brain, but the tumor level is no lower than that of a large group of non-neoplastic tissues including lung, skeletal muscle, and spleen. As previously discussed, the level of hepatic riboflavin falls in the preneoplastic and neoplastic rat liver after azo dye feeding in a manner that parallels the fall in mitochondrial protein (383).

The other B vitamins fulfill roles, understood to varying degrees today, in the oxidative metabolism of the cell. For the sake of completeness, studies on their levels in tumors are included (362–366) in Tables 61 and 62. As with riboflavin, the values of tumors fall in the range of the low normal tissues. The level of the pyridine nucleotides, of which nicotinic acid is a component, has been discussed in an earlier section.

G. Adenosine Triphosphatase

At the present time adenosine triphosphatase (ATPase) is an enzyme whose activity is easily measured by the release of inorganic phosphate from ATP, but whose biochemical significance is poorly understood. It occurs principally in the particles (nuclei, microsomes, and mitochondria), and has been most carefully studied in mitochondria. Carefully prepared sucrose mitochondria show little ATPase activity unless activated (244, 245, 377) in various ways (preincubation at 30° C. without substrate, calcium ions, dinitrophenol, etc.). It would seem reasonable to suggest that ATPase activity represents a group of ATP donor reactions in which the physiological recipient of the high energy phosphate is replaced by water. This is consistent with localization of the activity in the most actively synthetic particles, the microsomes and nuclei (410, 413).

TABLE 60
RIBOFLAVIN CONTENT OF NORMAL AND TUMOR TISSUES

Tissue	Content[a]	Ref.
Normal rat tissues		
Liver (adult)	29.4	(397)
Liver (fetal)	5.5	(397)
Liver (regenerating)	24.7	(397)
Heart	12.0	(362)
Brain	3.1	(362)
Lung	4.4	(362)
Spleen	3.3	(362)
Kidney	28.0	(362)
Skeletal muscle	1.9	(362)
Neoplastic rat tissues		
Hepatoma	2.6–5.7	(397, 362)
Walker 256 tumor	3.2	(362)
Jensen sarcoma	3.6	(397)
Normal mouse tissues		
Liver	25–51	(362)
Heart	20–36	(362)
Brain	2.4–4.3	(362)
Neoplastic mouse tissues		
Sarcoma	6	(362)
Adenocarcinoma	2.1–3.2	(362)
Lymphosarcoma	3.7	(397)
Intestinal carcinoma	3.9	(397)
Salivary gland tumor	3.5	(397)
Ear tumor (ultraviolet)	4.4	(397)
Malignant melanoma S-91	5.6	(397)
Sarcoma 37	3.7	(397)
Crocker sarcoma 180	4.1	(397)
Brain tumor	2.4	(397)
Mammary adenocarcinoma	5.5	(397)
Adenocarcinoma (stomach)	4.3	(397)
Squamous carcinoma (stomach)	3.3	(397)
Hepatoma (azo toluene)	8.4	(397)
Spontaneous hepatoma	15.3	(397)
Hepatoma (CCl_4)	15.2	(397)

[a] Micrograms per gram wet weight.

The data of Potter and Liebl (374) and DuBois and Potter (140) on the ATPase activity of a number of normal and tumor tissues are included in Table 63. It is clear that tumors have a

TABLE 61
B VITAMIN CONTENT OF NORMAL AND NEOPLASTIC HUMAN TISSUES (362–365)

Tissue	Ribo-flavin[a]	Nicotinic acid[a]	Biotin[a]	Pantothenic acid[a]
Normal tissues				
Liver	16	54	620	31
Heart	7.8	41	170	16
Cerebrum	2.1	20	31	13
Lung	1.6	18	19	5.0
Spleen	3.0	30	40	5.4
Kidney	25	33	580	16
Skeletal muscle	2.0	50	21	10
Neoplastic tissues				
Breast carcinoma (6)	1.1–5.7	13–29	36–97	3–11
Breast carcinoma (metastasis) (2)	2.0, 2.8	15, 22	86, 87	5.5, 7.9
Spindle cell sarcoma (4)	2.1–2.8	21–28	42–86	4.2–9.5
Neurogenic sarcoma	2.1	24	19	2.8
Lymphosarcoma	2.4	26	24	5.4
Melanoma (2)	1.1, 2.3	11, 23	27, 30	3.7, 4.5
Ovarian adenocarcinoma (2)	2.2	15, 27	5, 13	4.5, 5.0
Renal adenocarcinoma	2.0	18	8	4.4
Sigmoid adenocarcinoma	2.0	24	51	73
Rectal adenocarcinoma	2.5	30	71	—
Epidermoid carcinoma	1.6	18	20	4.0

[a] Measured in micrograms per gram of fresh tissue.

TABLE 62
B VITAMINS IN HUMAN AND ANIMAL NEOPLASMS (366)

Vitamin	Human normal tissues (μg./gm. wet wt.)	Human cancer tissues (μg./gm. wet wt.)	Cancer/ normal (%)	Rat normal tissues (μg./gm. wet wt.)	Rat cancer tissues (μg./gm. wet wt.)	Cancer/ normal (%)
Thiamine	1.80	1.28	70	3.7	1.54	42
Riboflavin	8.10	2.35	29	9.6	3.4	35
Nicotinic acid	31.2	23.5	75	87.0	23.6	27
Pantothenic acid	10.3	5.54	54	20.4	7.7	32
Pyridoxine	0.52	0.11	21	0.87	0.196	22
Biotin	0.18	0.04	21	0.22	0.05	23
Inositol	632	877	138	924	516	56
Folic acid	1.4	2.86	200	3.7	3.51	100

rather high ATPase activity, in the same range as active normal tissues. The data of Schneider (410) on the intracellular distribution of ATPase in normal rat liver and hepatoma (butter yellow) are included in Table 43. It will be noted that the ATPase activity of the whole homogenate is similar for the two tissues but the intracellular distribution is quite different. Thus, while in liver

TABLE 63
ADENOSINE TRIPHOSPHATASE ACTIVITY[a] OF NORMAL AND NEOPLASTIC ANIMAL TISSUES (140, 374)

Tissue	Without calcium	With calcium
Normal rat tissues		
Heart muscle	4.8	27.3
Skeletal muscle	12.5	23.3
Lung	3.0	21.8
Kidney	3.5	20.3
Submaxillary gland	3.6	16.4
Spleen	1.4	13.0
Liver	3.9	12.9
Pancreas	1.5	11.5
Smooth muscle	1.9	8.2
Brain	2.4	7.0
Neoplastic tissues		
Jensen rat sarcoma	2.0	20.2
Ultraviolet tumor (mouse)	3.5	19.3
Spontaneous mammary tumor	2.9	13.3
Rat hepatoma (AAB)	1.9	11.8
Walker 256 carcinosarcoma	1.6	11.5
Flexner-Jobling carcinoma	1.3	8.0
Yale 1 mouse tumor	1.6	5.6

[a] Activity of homogenate in micrograms inorganic phosphate per milligram fresh tissue in 15 minutes.

50% of the ATPase activity is in the mitochondrial fraction, in the hepatoma the mitochondria contain only 12% of the activity. On the other hand, the mitochondrial supernatant fraction (microsomes + soluble cytoplasmic fraction) contains only 30% of the activity of the rat liver, but this fraction contains 75% of the activity of the tumor homogenate. Schneider *et al.* (413) have extended these experiments on the intracellular distribution of ATPase to mouse liver and mouse hepatoma (Table 46). The cell fractionation in this later study was carried a step further to include the separation of the microsomes from the soluble supernatant cytoplasmic fraction.

Although ATPase activity of the mouse hepatoma was only about 60% that of liver, the intracellular enzyme distribution resembled that of the rat tissues. In mouse liver the major part of the ATPase activity was in the nuclear and mitochondrial fractions; in the mouse tumor the major part of the activity was in the nuclear and submicroscopic fractions. This finding of the high ATPase activity of mouse and rat hepatoma microsomes compared with the normal liver is of interest, and further study in a variety of tumors would appear worthwhile. Reid and O'Neal have also reported on the levels of mitochondrial ATPase during azo dye carcinogenesis in the mouse (393).

Ács *et al.* have investigated the ATPase of the Ehrlich ascites cell and found that it is largely located in the cell membrane (3). They found (2) that the mitochondria of this tumor cell differed from the mitochondria of brain and liver in several important respects. Thus, the ATPase of the Ehrlich ascites cell mitochondria was only one twenty-fifth that of the two normal tissues, while the mitochondrial hexokinase activity of the tumor was six times that of brain, and twenty times that of liver. Because of these properties of the tumor mitochondria, the authors were able to obtain an interesting system by centrifuging the homogenate of the Ehrlich ascites cell at low speeds to free it of nuclei and cellular debris. This preparation showed no Pasteur effect (aerobic and anaerobic lactate formation were equal), yielded P:O ratios in the neighborhood of 3.0 without added fluoride or hexokinase, and aerobically esterified phosphate with hexose diphosphate accumulation (but not anaerobically). These properties were attributed by the authors to the low ATPase and high hexokinase activity of the tumor mitochondria.

Though clearly much more data is needed on ATPase levels both in normal tissue and tumor, studies to date suggest that the mitochondrial level of ATPase is decreased, at least in certain tumors. On the other hand, the total ATPase levels of the tumor homogenate appear to be on the high side in comparison with normal tissues. The low ATPase activity of tumor mitochondria may only be a reflection of the low mitochondrial content of neoplastic tissue.

11. Warburg's Theory of a Damaged Tumor Respiration

No aspect of cancer research has been the subject of such acrimonious debate (35, 50, 51, 65, 119, 474, 493, 495) as has centered about Warburg's view (473, 478) that the crux of the cancer problem is a damaged tumor respiration. It is the present author's feeling that any evaluation of this issue must be separated from the undeniably important contributions of Otto Warburg to our understanding of tissue metabolism in general and of tumor metabolism in particular. It is the purpose of this section to present Warburg's position, including certain data that have not been presented in the earlier sections of this monograph, and also to consider the views of other authors who are both in favor of and in opposition to the Warburg position. In so far as possible the author will attempt to reserve his own views for the summary section constituting the final section of this monograph.

As should be clear from the preceding sections, Warburg's main experimental approach to tumor metabolism has been the measurement of the respiration and glycolysis of tumor slices in oxygen and in nitrogen. No argument exists among investigators about the consistently high anaerobic glycolysis of tumors or the equally impressive though less consistent aerobic glycolysis. It is in respect to the explanation of the cause of this glycolysis, its relation to the etiology of the neoplastic process, and the qualitative nature of tumor respiration that agreement is lacking.

Warburg's first suggestion, that the high tumor glycolysis resulted from quantitatively inadequate respiration of the tumor cell, was based on initial studies in a group of tumors with atypically low respiratory rates (144). He recognized this himself, and the view of a quantitatively inadequate tumor respiration does not deserve further consideration. A view which has a number of points in common with his presently held view appears in his preface of the English edition of "The Metabolism of Tumors" (471):

"Aerobic glycolysis results if the respiration of growing cells is injured, whether by diminishing its extent or by interfering with the relationship which holds between respiration and fermentation (glycolysis) . . . . Interference with the respiration of growing cells is, from the standpoint of the physiology of metabolism, the cause of tumors. If the respiration is disturbed, as a rule, the cell dies. If the cell does not die, a tumor cell results. This is no theory, but

a comprehensive summary of all the measurements at present available."

It should be pointed out that Warburg, in referring to the relationship between respiration and fermentation, is not referring to the Pasteur effect in terms of the inhibition of glycolysis per unit of respiration, but rather to the over-all quantitative relationship between amount of respiration and amount of glycolysis of the tumor cell. To quote a later section of the same work:

"We determined the Meyerhof quotient for carcinoma tissue, lactic acid bacteria, embryonic tissue and a number of glycolyzing tissues, and as a rule obtained the same mean values as Meyerhof. As a rule 1 mole of breathed oxygen, just as in muscle, caused the disappearance of 1–2 moles of lactic acid. This result . . . proves that the influence of the respiration on the cleavage metabolism of the carcinoma cell is normal . . . Although in the tumor every molecule of oxygen breathed is just as effective as in muscle—the Meyerhof Quotient is equal in the two cases—yet the respiration does not cause the glycolysis to disappear. The respiration of the carcinoma cell is too small in comparison with its glycolytic power."

Finally, the Warburg view may be presented by quoting from the rather long summary in a recent review by Warburg (473):

"Cancer cells originate from normal body cells in two phases. The first stage is the irreversible injury of respiration. Just as there are many remote causes of plague—heat, insects, rats—but only one common cause, the plague bacillus, there are a great many remote causes of cancer—tars, rays, arsenic, pressure, urethane—but only one common cause into which all the other causes of cancer merge, the irreversible injuring of respiration.

"The irreversible injury of respiration is followed, as the second phase of cancer formation, by a long struggle for existence by the injured cells to maintain their structure, in which a part of the cells perish for lack of energy, while another part succeeds in replacing the irretrievably lost respiration energy by fermentation energy. Because of the morphological inferiority of fermentation energy, the highly differentiated body cells are converted by this into undifferentiated cells that grow wildly—the cancer cells.

"To the thousands of quantitative experiments on which these results are based, I would like to add, as a further argument, the

fact that there is no alternative today. If the explanation of a vital process is its reduction to physics and chemistry, there is no other explanation today for the origin of cancer cells, either specific or general. From this point of view mutation and carcinogenic agent are not alternatives, but empty words unless metabolically specified. Even more harmful in the struggle against cancer can be the continual discovery of miscellaneous cancer agents and cancer viruses, which by obscuring the underlying phenomena, may hinder necessary preventive measures and thereby become responsible for cancer cases."

At this point some of the evidence cited by Warburg to substantiate these statements may be presented. First, the statement that carcinogenesis is due to injury to the respiratory apparatus will be considered. Warburg points out that a number of chemical carcinogens, arsenous acid (475), thiourea and thioacetamide (198), and urethane (269, 448) are known respiratory poisons. However, the large variety of known chemical carcinogens (hydrocarbons, azo dyes, etc.) are not respiratory poisons in the usual sense, nor are the respiratory poisons as a group notably carcinogenic. Both chemical carcinogens and respiratory poisons include a formidable list of member compounds, so that it is not surprising that occasional substances are members of both groups. Though no systematic study on this last point has been made—to be completely objective—on the whole, the evidence is not in favor of a widespread overlapping of the two activities.

R. K. Keilley has attempted to determine the effect of a number of carcinogens on isolated liver mitochondria, in order to evaluate Warburg's hypothesis that carcinogens act through injury to the respiratory apparatus of the cell. She found that the carcinogen *N*-2-fluoreneyldiacetamide (239, 240, 242) inhibited glutamate oxidation by mitochondria. Other pyridine nucleotide-linked phosphorylations were less inhibited (α-ketoglutarate, isocitrate, and β-hydroxybutyrate) and succinate was not inhibited at all. In a group of dyes of this series (aminofluorene) there was a rough correlation between the potency as a carcinogen and the degree of inhibition; the mechanism appeared to be a competition between the carcinogen and DPN. The inhibition was released by the uncoupling agent dinitrophenol. Emmelot and Bos (155) confirmed these findings, and found that the inhibition could be overcome by

DPN addition. Keilley then went on to study the effect of the aminoazo dyes on glutamate oxidation, and she found (243) that both carcinogenic and non-carcinogenic aminoazo dyes were able to inhibit glutamate oxidation by mitochondria from the livers of riboflavin-deficient rats, but not from non-deficient rats. The inhibition ranged from 50 to 60% of the controls and was similar in extent to the inhibition achieved with carbon tetrachloride. In all these studies the P:O ratios of the inhibited mitochondria were unchanged, suggesting that the primary effect is on oxidation itself rather than on the phosphorylation process.

A number of authors have studied the effect of decreased and increased oxygen tension on carcinogenesis. An early study showed that increased oxygen tension (exposure to compressed air) reduced the incidence in mice of spontaneous mammary gland adenocarcinomas (305). It has been found that fowl kept at high altitudes (in the Peruvian mountains) display a lower incidence of spontaneous leukemia but a higher incidence of lung tumors than animals kept at sea level (328, 329). An increased incidence of lung tumors in mice injected with dibenzanthracene (208) and urethane (134) has also been demonstrated in animals exposed to 100% O_2. Hypoxia during gestation inhibited the development of spontaneous leukemia in the offspring of AKR mice (12). An extensive series of experiments concerning the influence of prolonged oxygen change upon the formation of spontaneous and induced mouse cancer has been performed by DiPaolo and Moore (134). They found that the effect of altered oxygen tension on the production of the tumors studied was neither quantitatively important nor general. Thus, the incidence of spontaneous leukemia was the same in AKR mice raised in 10% oxygen, room air, and 70% oxygen. There was also no difference in the incidence of lymphomas in C-57 mice exposed to 700 r raised either in room air or 10% oxygen. DBA mice painted with methylcholanthrene developed a higher incidence of skin carcinomas when exposed to 70% oxygen than when exposed to 10% oxygen or room air, while Swiss mice painted with the same chemical showed the highest incidence of skin tumors in the group of animals exposed to compressed air (lower and equal incidence in room air, 10%, and 70% oxygen). There would thus appear to be no evidence that hypoxia increases the incidence of either spontaneous or induced neoplasms, as might be expected from the Warburg hypothesis.

Graffi observed that mitochondria of many tissues absorb carcinogenic dyes *in vitro* (187, 188), and has also reported that particulate components are involved in the absorption of hydrocarbons painted on the skin of mice. On the other hand, detailed *in vivo* studies by the Millers of hepatic azo dye carcinogenesis in the rat (379–381) suggest that the soluble particle-free cytoplasm of the cell is the site of action of the azo dye carcinogens, since the highest concentration of the protein-bound azo dye (50% of total bound dye) is located in this fraction. (However, some dye is bound to the other cell fractions.) The Millers favor a mechanism of azo dye carcinogenesis that involves the deletion of a growth-controlling cytoplasmic protein (324). They conceive of the primary carcinogen as a metabolite of the azo dye which combines with certain soluble cytoplasmic proteins of the liver. These cytoplasmic proteins are considered to be the primary site of action of the carcinogen, and are thought to play a key role in the response to growth controls exercised by the rest of the organism (competitive reactions, hormonal, etc.). They suggest that the binding of the dye to the proteins reduces or prevents further synthesis of these proteins and that eventually, in subsequent generations, cells arise with less and finally none of the proteins originally bound by the dye. It is hypothesized that such cells could only respond to nutrition by continued growth and would be tumor cells.

Potter *et al.* (376) have independently proposed an enzyme deletion theory, but the detailed mechanism is somewhat different from that of the Millers. He feels that the specific enzyme deletion may involve the respiratory complex (368, 376). He suggests that the rate of reproduction of the respiratory complex (mitochondria) may be unable to keep up with the rate of division of the whole cell (376), eventually resulting in a stabilized strain of tumor cells lacking completely the respiratory complex. Potter visualizes alternate pathways existing in the cell for many metabolites, of which one leads to their combustion with energy formation and the other to their utilization for synthetic processes. Thus the cancer cell, as a cell lacking the alternate respiratory pathway, is obliged to use these building blocks or metabolites for synthetic processes and therefore for cell growth and division. Potter has more precisely defined Warburg's concept of a damaged respiration, but clearly recognizes that although this is an attractive unifying concept it remains unproven (368).

Warburg has reported that X-radiation in high dosage will destroy the respiratory activity of mitochondria (478), and he relates the carcinogenic activity of X-radiation to this effect. In view of the variable results of X-radiation on tissue respiration (25), and the well-known effects of X-radiation on nuclear morphology and DNA synthesis (219), Warburg's conclusion as to the mode of carcinogenic action of X-rays does not appear to be justified at the present time. In a recent paper Warburg *et al.* have suggested that the therapeutic effect of X-radiation on neoplastic disease is due to the glycolysis-inhibiting action of the H_2O_2 formed (478). They attribute the particular sensitivity of tumor tissue to a relative deficiency of catalase in neoplastic tissue. Burk *et al.* (64) believe that the primary position of the glycolysis of neoplastic tissue in the cancer problem is indicated by observations made in their laboratory, that all the agents of value in the chemotherapy of malignant disease in man have some *in vitro* effect on tumor glycolysis. Thus, Methotrexate, 6-mercaptopurine, and hydrocortisone were found to inhibit the glycolysis of acute lymphatic leukemia cells (60, 275), while Myeleran, 8-azaguanine, and certain bacterial polysaccharides stimulated glycolysis (64, 520). Furthermore, these authors felt that they could demonstrate (516, 519) with a variety of agents (8-azaguanine, Methotrexate, 6-mercaptopurine, hydrocortisone, and 5-fluorouracil) that metabolic effects could only be produced in cell lines which could be expected to show an *in vivo* susceptibility to the drug. It was felt that all effective chemotherapeutic agents against cancer have an ability to alter significantly the energy metabolism of the cancer cell, regardless of the direction of the alteration. In the present author's opinion, it is difficult to reconcile that both a stimulation and an inhibition of glycolytic activity *in vitro* can be related to the antineoplastic activity of chemotherapeutic agents. Furthermore, there is a large and convincing body of evidence that a number of the more effective human antineoplastic agents owe their effectiveness to an ability to interfere with the synthesis of deoxyribonucleic acid (DNA).

Goldblatt and Cameron (183) have reported on the development of neoplasia in fibroblasts grown in tissue culture under conditions of intermittent anaerobiosis. They found, upon transplantation, that one strain of fibroblasts originating in the myocardium and grown under intermittent anaerobiosis (15 minutes in N_2 every

12 hours for several years) showed neoplastic behavior, whereas the control grown without this treatment did not. However, it is open to question whether the exposure to anaerobiosis played a critical role in this development of malignant characteristics. The development of such characteristics in tissue culture has been repeatedly observed without employing conditions of anaerobiosis, and most observers feel that anaerobiosis cannot be incriminated as the cause of this *in vitro* neoplastic transformation (404).

Warburg has raised the point that energy derived from fermentation is different from energy derived from respiration. As yet there is little evidence to bear out this statement. In regard to the incorporation of labelled amino acid into protein, the studies of Quastel and Bickis (385) appear to give the clear answer that glycolytic and respiratory energy can be used with equal efficiency. These studies do suggest, however, that tumor tissues are able to utilize glycolytic energy for protein synthesis more efficiently than can normal tissues.

In concluding this section the situation may be summarized in the following way: the high anaerobic glycolysis of tumors is a well-established phenomenon which, combined with the normal inhibitory effect of oxygen on glycolysis and a moderate respiratory rate, results in a high anaerobic glycolysis. The result is that even in oxygen, tumors derive an appreciable part of their energy from glycolysis. (Although even for ascites cells, which display the most marked glycolysis, less than 40% of the aerobic energy comes from glycolysis, while for solid tumors it is only one-third to one-half that of ascites neoplasms.) However, the objective data on oxidation of labelled substrate, oxidative phosphorylation, and efficiency of utilization of glycolytic and respiratory energy for amino acid incorporation [as summarized in Section II and stressed by others (407, 493)] have not revealed any important quantitative or qualitative differences between the respiration of tumors and normal tissues. This does not mean that as more is learned of the energy metabolism of normal tissues and tumors that a qualitative or major quantitative difference may not emerge. However, at the present time such differences and Warburg's entire concept of an injury to respiration must remain hypotheses. It remains to be proven that carcinogenic agents as a group act by damaging the respiratory apparatus of the cell. The concept that glycolytic energy is morphologically inferior to oxidative energy and consequently

leads to the neoplastic state is also vague and without experimental basis. It has been the attempt to ascribe to these hypotheses the status of established scientific fact that has led to the intense disputes which have arisen. As hypotheses they merit further study, although clearly certain facets have not been adequately defined. However, it would be a mistake to mould prematurely the existing body of scientific data on tumor respiration to fit a hypothesis. Although at the present time a body of significant scientific data in support of Warburg's views does not exist, it must not be forgotten that the intuition of an outstanding mind may grasp a phenomenon beyond the reach of experimental verification and beyond the general understanding of the time. Eventually, such intuition is translated into scientific proof. The same point of view is expressed more concisely by R. K. Keilley (243): "Although one may agree that there is still considerable evidence that does not support the view, the basic concept has not been disproved, and continues to challenge the imagination and resources of those engaged in the problem of carcinogenesis."

PART III: Regulatory Mechanisms in Respiration and Glycolysis

This section contains a discussion of two fascinating problems in present-day intermediary metabolism. The two are the important interrelationships of the glycolytic and oxidative pathways. The Pasteur effect, the inhibition of glycolysis by oxygen, is a biochemical phenomenon of almost universal occurrence and of great subtlety. Its intriguing simplicity has defied solution since Pasteur's original description, despite the immense progress that has been made in understanding the many individual steps of both the aerobic and anaerobic pathways of glucose breakdown. The Crabtree effect, the inhibition of oxygen uptake by glycolysis, is a less widespread phenomenon, appearing in its most impressive form in the rapidly glycolyzing ascites cell. Although the Crabtree effect is far from explained, a solution appears to be closer at hand than in the case of the Pasteur effect.

I. The Pasteur Effect

A. Introduction

Pasteur's masterly studies on yeast fermentation (356) described in simple terms a phenomenon so complex that its understanding still eludes biochemists. Pasteur's original observation concerned yeast capable of both aerobic and anaerobic existence. He found that in the presence of oxygen the yeast consumed sugar much more slowly than under anaerobic conditions, but that the sugar was utilized more effectively, judging by the higher aerobic ratio of weight of yeast formed to weight of sugar consumed. Numerous subsequent studies have established the constant occurrence of respiratory inhibition of fermentation among organisms capable of energy production by the two pathways (yeast, ascites cells, slices of normal, and neoplastic tissues, etc.). In terms of the modern understanding of the energy relations of fermentation and respiration, the significance of the Pasteur effect as a mechanism for the conservation of substrate is evident (120, 289). Only 2 moles of high energy phosphate (ATP) are realized per mole of glucose fermented, while the complete oxidation of 1 mole of glucose to

CO_2 and water yields 38 moles. These figures represent some 40–70% of the 700 kcal. of energy (calculated from thermodynamic data) that is available from the oxidation of glucose, or of the 56 kcal. of energy available when a mole of glucose is fermented.

B. Theoretical Considerations

Experimentally, the Pasteur effect can be defined in either of two ways (135): either as the oxidative inhibition of glucose disappearance, or as the oxidative inhibition of split product formation (lactate in mammalian systems). It is important to recognize clearly the distinction between the two definitions.

Mechanisms of the Pasteur effect may be divided into two groups. The first group, referred to as "equilibrium" mechanisms by Lipmann (292), imply a stoichiometric coupling of respiration to fermentation through some common metabolic intermediate (DPN, pyruvate, ADP, or inorganic phosphate). The oxidation of the fermented pyruvate (360) was suggested at an early date as a mechanism of the Pasteur effect but, as will be discussed below, the oxygen uptake was found to be insufficient to account for the inhibited fermentation. More recently, Ball (22) suggested on purely theoretical considerations that the intermediate involved was DPN. This mechanism has received little in the way of experimental confirmation and, like a pyruvate mechanism, it is hard to explain an inhibition of glucose utilization on the basis of oxidative removal of the glycolytically generated DPNH. The "equilibrium" Pasteur mechanism, which has received by far the most favorable consideration by most biochemists, involves a competition for inorganic phosphate or phosphate acceptor between the oxidative and glycolytic systems. This mechanism was proposed at about the same time by Lynen (295) and Johnson (232). Johnson formulated the view that a competition for inorganic phosphate or phosphate acceptor might be responsible, and indicated that such a theory had several critical theoretical requirements:

(1) "The glycolytic reactions must be readily reversible.

(2) "Oxidative phosphorylation must be capable of reducing inorganic phosphate and phosphate acceptor to a lower level than that obtained at glycolytic equilibrium, i.e., oxidative phosphorylation must be possible at concentrations too low to permit glycolytic phosphorylation.

(3) "The same reserves of phosphate ester, inorganic phosphate

and phosphate acceptor must be available to both the glycolytic and oxidative systems."

Several important considerations preclude the ready acceptance of phosphate acceptor or inorganic phosphate competition as mediating the Pasteur effect. The first is that from a practical point of view the hexokinase and phosphohexokinase reactions are not freely reversible (389). However, at least for the hexokinase reaction, this may not stand in the way of a phosphate competition Pasteur effect, since glucose-6-phosphate has been shown to inhibit brain hexokinase (111) although it does not inhibit the hexokinase of yeast (297). Second, it should be recalled that phosphate acceptor and inorganic phosphate are not required for the early reactions of glycolysis but only for steps beyond the cleavage of hexose (glyceraldehyde phosphate dehydrogenase, phosphoglycerokinase, and phosphopyruvic kinase reactions). Thus lack of inorganic phosphate or phosphate acceptor would be expected to lead to a Harden-Young-like effect with hexose diphosphate accumulation (200), a finding which is not characteristic of the Pasteur effect. To circumvent this, several authors have recently suggested a Pasteur effect based on the shuttling of adenine nucleotides or inorganic phosphates between the oxidative and glycolytic systems (91, 390).

Opposed to the equilibrium mechanisms of the Pasteur effect, certain investigators have favored non-equilibrium mechanisms (282). They view the connection between glycolysis and respiration as a nonstoichiometric one (catalytic). Thus the presence of oxygen or respiration, rather than the amount of respiration, is viewed as responsible for the Pasteur effect. Engelhardt and Sakov (163) have made this mechanism more explicit with the suggestion that the inhibition by respiration takes place at the phosphohexokinase reaction. Their view was that in the presence of oxygen the phosphohexokinase reaction is inhibited and tissue respiration takes place through the hexose monophosphate shunt.

The Pasteur effect differs from the Crabtree effect (the inhibition of respiration by glycolysis) in one significant regard. The Crabtree effect appears to be mediated through a tightly coupled phenomenon (see Section 2, Part III). That is to say, assuming 1 mole of ATP is formed per mole of lactate, and 6 moles of ATP per mole of oxygen consumed (P:O ratio of 3.0), it can be calculated that the energy lost from inhibited respiration is just replaced by

that derived from respiration (385). This situation does not obtain in the Pasteur effect. Thus, whereas the ATP derived from 1 mole of oxygen respired equals the energy from 6 moles of lactate formed, 1 mole of oxygen respired actually inhibits only 1 to 2 moles of lactate formation (see Tables 1, 3, 4). Much more energy is available aerobically than anaerobically in both normal and tumor tissues. Any mechanism of the Pasteur effect must take into consideration the finding that respiration replaces by 3- to 6-fold the energy lost from inhibited glycolysis, as well as the observation that for different tissues the actual amount replaced per mole of oxygen varies.

C. The Pasteur Effect in the Slice

The simplest explanation of the Pasteur effect is that under oxidative conditions the fermentation products (e.g., lactic acid) are completely oxidized (360). This was ruled out by Meyerhof's studies, which indicated that in muscle and in other cells and tissues (312) the oxygen uptake was sufficient to account for the disappearance by oxidation of only one-third or one-sixth of the lactate accumulation that was actually inhibited. Meyerhof defined a quantity, the Meyerhof oxidation quotient (M.O.Q.):

$$\text{M.O.Q.} = \frac{Q_{CO_2}^{N_2} - Q_{CO_2}^{O_2}}{1/3\ Q_{O_2}}$$

which expresses the theoretical requirement of 3 moles of oxygen for the oxidation of 1 mole of lactate. Experimentally, this quantity was found to be 3–6 (Tables 1, 3, 4). Meyerhof himself proposed that the Pasteur effect was mediated through the oxidative resynthesis of a glycolytic intermediate, but experimental evidence for such a theory has not been found, and the theory has found little acceptance (120).

Many substances will increase the aerobic glycolysis of the surviving tissue slice and other tissue preparations to the anaerobic level. However, to be a specific inhibitor of the Pasteur effect in any sense, a compound must be able to increase aerobic glycolysis without inhibiting tissue respiration (442). If this is borne in mind, fewer compounds deserve consideration. One of the first of these selective compounds found was ethyl carbylamine. The effects of this compound, the ethyl ester of hydrocyanic acid, was described by Warburg (469) and, unlike cyanide itself, the ester releases aero-

bic glycolysis without any inhibition of primary respiration. Dinitrophenol and dinitrocresol (137) both release aerobic glycolysis of the tissue slice (as well as a number of other tissue preparations) without a corresponding inhibition of respiration. Great interest has been aroused by the action of these compounds on the Pasteur effect because of the later findings of another of their rather selective actions, the uncoupling of phosphorylation from oxygen uptake (293). Several authors (84, 117, 234) have studied the effect of various dyes on the Pasteur effect and a number of these compounds, including phenosafranine, pyocyanine, neutral red, ethyl red, and guanidine, have been found to be selective inhibitors. Of these, only the first three also uncouple oxidative phosphorylation, while the two last mentioned do not appear to inhibit the phosphorylating mechanism.

Seits and Engelhardt (418) studied the effect of a number of agents (DNP, azide, ethyl carbylamine, and arsenate) on glycolysis and oxidative phosphorylation of bird erythrocytes, baker's yeast, and tumor tissue. Oxidative phosphorylation was studied by measuring oxygen and inorganic phosphate uptake and ATP level and, in some cases, by P^{32} experiments. Although DNP and azide were the strongest inhibitors of aerobic phosphorylation and the Pasteur effect, all four compounds were able to relieve the Pasteur effect and check the aerobic synthesis of ATP without inhibiting oxygen uptake. These authors felt (418) that the first action of these respiratory poisons was toward the process of respiratory phosphorylation, as a consequence of which the Pasteur effect was removed.

Although Warburg observed in yeast a differential sensitivity of the Pasteur effect to carbon monoxide inhibition (472), later studies by Laser (271) indicated that selective inhibition of the Pasteur effect by carbon monoxide was most marked in animal tissues. Stern and Melnick (439) determined the absorption spectrum of a so-called Pasteur enzyme (the component involved in the carbon monoxide inhibition of the Pasteur effect), employing the method used by Warburg to determine the spectrum of the respiratory pigment (cytochrome oxidase). The latter had been measured by Warburg by taking advantage of the photochemical dissociation of the carbon monoxide-respiratory pigment complex. The spectrum of the "Pasteur enzyme" was similar to the respiratory pigment although there were some minor differences. It has recently been pointed out that these differences between the "Pasteur enzyme"

and the respiratory pigment may involve differences in the distribution of the two compounds within the cell, rather than intrinsic differences of chemical structure (238).

D. The Pasteur Effect in Extracts

Since at the present time it is not possible to separate the respiratory mechanism of the cell from the cell particles (whole or disrupted mitochondria), it is questionable that Pasteur effects in particle-free extracts have more than the crudest analogy to Pasteur effects in more intact systems. However, several of these extract systems have considerable historical interest. Lipmann demonstrated (290, 291) that it was possible to obtain reversible inhibition of glycolyzing extracts of yeast and muscle with weak oxidizing agents such as iodine and quinone. Thus, by adding indophenols as oxidants, inhibition of glycolysis in oxygen was produced, which then disappeared in nitrogen when the oxidized dye was reduced by enzymatic activity of constituents of the extract. In such a system, inhibition of glycolysis could be demonstrated with negligible respiratory activity.

Important work along similar lines was done by Engelhardt and Sakov (163). They demonstrated that purified phosphohexokinase was very sensitive to mild oxidizing agents, being inhibited by oxidation-reduction indicators with E_0 greater than 0.05 volts. On the basis of this finding these investigators suggested that the Pasteur effect operated through the phosphohexokinase reaction, with tissue respiration taking place via the hexose monophosphate shunt in the presence of oxidatively inhibited phosphohexokinase.

E. The Pasteur Effect in Yeast

Yeast has always been a favored material for the study of the Pasteur effect. The very early studies of Meyerhof (312) on the respiration and glycolysis of various forms of yeast are included in Table 64. It will be noted that from the almost completely anaerobic, glycolytic brewer's yeast, through the partially aerobic, partially glycolytic baker's yeast, to the almost completely aerobic wild yeast the Meyerhof oxidation quotient remains remarkably constant.

More recently, Lynen has extensively studied the Pasteur effect in yeast (296, 297). It will be recalled that Lynen was one of the first to suggest a Pasteur effect mediated through inorganic phosphate or phosphate acceptor (295), and it was in part on the basis

of studies with yeast that this hypothesis was formulated. He showed that the rate of oxidative phosphorylation exceeded the rate of glycolytic phosphorylation, and that in yeast the level of inorganic phosphate was lower and the levels of triose phosphate and hexose diphosphate were higher under aerobic than under anaerobic conditions (220, 299). At that time Lynen suggested that

TABLE 64
THE METABOLISM OF VARIOUS KINDS OF YEAST (312)

Type	Q_{O_2}	$Q_F^{O_2}$	$Q_F^{N_2}$	$\frac{Q_F^{N_2} - Q_F^{O_2}}{1/3\ Q_{O_2}}$	Inhibition (%)
Wild yeast	—180	18	260	4	93
Baker's yeast	— 87	95	274	6.2	65
Brewer's yeast	— 8	213	233	7.5	8
Brewer's yeast after 15 hours' aeration	— 73	113	193	3.3	42

the low level of inorganic phosphate was responsible for the inhibition of triose phosphate dehydrogenase. [Warburg had shown that crystalline preparations of this enzyme require inorganic phosphate for activity (427).] However, other workers have pointed out (348) that the differences in inorganic phosphate levels found in the steady states of aerobic and anaerobic yeast were too small to account for the different rates of sugar breakdown. For example, Strickland has found that the inorganic phosphate level was only 20% higher aerobically than anaerobically (443).

We now turn to the question of phosphate acceptor competition in yeast. It was found that in oxygen the level of ATP was higher and the level of ADP lower than when the yeast were in nitrogen (297). Lynen has also found that DNP releases the Pasteur effect in yeast, an observation made earlier by Meyerhof in quick-dried yeast preparations (316). Recognizing the existence of certain incompatibilities with his previous theory of inorganic phosphate and phosphate acceptor competition (particularly the contradiction of decreased hexose diphosphate and triose phosphate in the presence of increased ATP), Lynen more recently suggested (297) that the Pasteur effect may be mediated through an intracellular localization or compartmentation of inorganic phosphate or phosphate acceptor. Some evidence in favor of this has been the finding of a higher level of inorganic phosphate in the particulate fraction of

disrupted respiring yeast as compared with this fraction of fermenting yeast (221).

Strickland has studied specific inhibitors of the Pasteur effect in yeast in considerable detail (443). He found two compounds of particular interest. One was DNP, which he found inhibited aerobic phosphorylation (i.e., inorganic phosphate uptake) but not anaerobic phosphorylation. DNP, both aerobically and anaerobically, inhibited polysaccharide synthesis and raised the level of inorganic phosphate of the yeast cell. However, of equal interest was propri-onitrile (ethyl cyanide), a compound closely related to ethyl carbylamine (ethyl isocyanide). This nitrile also specifically inhibited the Pasteur effect, but had no effect either on respiration or on phosphorylation.

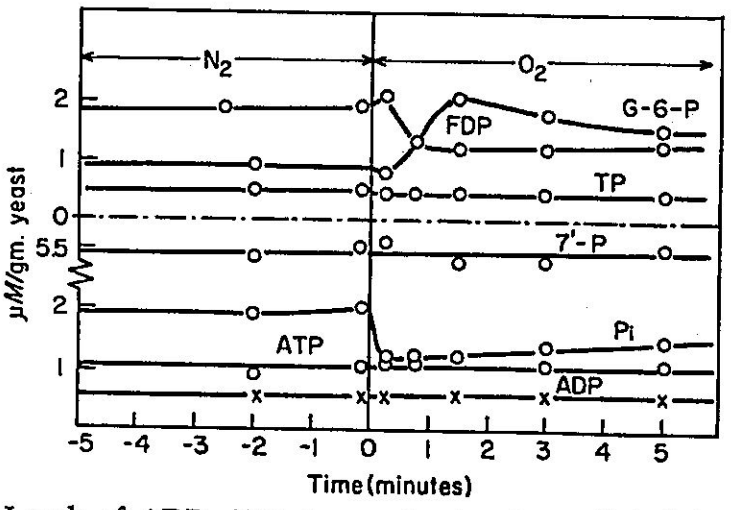


FIG. 12. Levels of ADP, ATP, inorganic phosphate (Pi), labile phosphate (7'-P), total phosphate (TP), hexose diphosphate (FDP), and glucose-6-phosphate (G-6-P) in yeast under aerobic and anaerobic conditions. From Lynen (297).

Lynen's latest studies with yeast have been summarized in two recent articles (297, 298). Figure 12 reproduces the time curves for levels of sugar phosphates and adenine nucleotides of yeast transferred from nitrogen to oxygen. It will be noted that, while the levels of ADP and ATP are essentially unchanged in the transition from anaerobic to aerobic conditions, there is a sharp rise in glucose-6-phosphate, and a corresponding fall in hexose diphosphate. On the basis of these studies Lynen at present believes (298) that several mechanisms are involved in the Pasteur effect in yeast. The phosphorylation reactions that parallel the respiratory process

exhaust the inorganic phosphate pool available for the glyceraldehyde phosphate dehydrogenase reaction, thereby inhibiting the fermentative breakdown of glucose. He interprets the reduced glucose uptake by the respiring cells, in comparison with the fermenting cells, as indicating an impairment of the hexokinase reaction due to lack of ATP at the site of glucose phosphorylation. Finally, to explain the higher aerobic level of G-6-P and the lower aerobic level of hexose diphosphate, he feels that the unavailability of ATP for the phosphohexokinase reaction must be greater than its unavailability for the hexokinase reaction (under aerobic conditions). Lynen reconciles the actual levels of ATP and ADP found (the levels are the same in aerobic and anaerobic yeast) with this theory of the unavailability of ATP for hexose phosphorylation under aerobic conditions in terms of localization of the enzymes in question in cell compartments. Thus, while the glycolytic enzymes are located in the soluble fraction of the cytoplasm, the oxidative enzymes are found in the mitochondria. He postulates that the ATP formed during respiration is accumulated in the mitochondria and so is not readily available for extramitochondrial glucose phosphorylation. He further suggests that knowledge of enzyme localization in terms of cellular ultrafine structure may be necessary to fully understand the Pasteur effect. Dinitrophenol could be visualized as acting by releasing the ATP from its sequestered mitochondrial site so that it can function in glycolytic phosphorylation.

F. The Pasteur Effect in Ascites Cells

Extensive studies of the Pasteur effect in ascites cells have been carried out by Racker and Wu (391, 529). They first studied the distribution of the enzymes of glycolysis among the soluble cytoplasmic and mitochondrial fractions of the cell. The only glycolytic enzyme present in large amounts in ascites cell mitochondria was hexokinase; 60% of the total enzyme was located in the mitochondria. All the glycolytic enzymes were present in the soluble cytoplasmic fraction, with hexokinase, phosphohexokinase, and glyceraldehyde phosphate dehydrogenase found to be limiting compounds. The extract contained a 10- to 40-fold excess of ADP-transphosphorylating enzymes (pyruvate kinase and 3-phosphoglyceric kinase) over hexokinase and phosphofructokinase. However, the rate of glycolysis of extracts and homogenates fortified with cofactors greatly exceeded the rate of glucose utilization in the intact

cell. Wu and Racker concluded from this last observation that none of the enzymes limited glycolysis in the intact cell, the critical factor being either a cofactor or an intermediate. They measured the intracellular concentration of inorganic phosphate and the adenine nucleotides under various conditions, and found that only the level of inorganic orthophosphate appeared to fluctuate parallel to the rate of glycolysis. Inorganic phosphate also appeared to be a major limiting factor in glycolysis, since high concentrations of external orthophosphate markedly stimulated lactic acid production in intact cells (rates of glycolysis approaching the homogenate rate). Other experiments indicated that the ability of ascites tumor cells to phosphorylate glucose was not related to the intracellular level of ATP. By preincubation of the cells with DNP, ATP could be depleted to a fraction of its maximal levels while glycolysis proceeded maximally. Thus, Wu and Racker concluded that glycolytically produced ATP could be utilized more efficiently for the phosphorylation of glucose than could ATP produced by oxidative phosphorylation. Since a high extracellular level of inorganic phosphate could diminish the Pasteur effect (both lactate production and glucose uptake), these authors felt that the Pasteur effect could be visualized as due to a low availability of intracellular inorganic phosphate for glyceraldehyde phosphate dehydrogenase under aerobic conditions, with consequent limitation of glycolytic synthesis of ATP and phosphorylation of glucose. However, the actual measurements of total inorganic phosphate and ATP were not consistent with this formulation. Wu and Racker felt that if compartmentation of inorganic phosphate and adenine nucleotides within the cell were postulated, a satisfactory mechanism of the Pasteur effect would emerge which would be consistent with the observed experimental data. The authors further suggest that this compartmentation may be less rigid anaerobically, and that it may be less rigid in tumor cells than in normal cells. The diminished rigidity of the compartmentation in tumor cells may find expression in an increased penetration of extracellular phosphate and other compounds which enter with difficulty, and may explain the high aerobic glycolysis of the tumor cell. Kvamme (264) has performed similar experiments with ascites cells and has, in general, arrived at similar conclusions.

G. The Pasteur Effect in Particulate Systems

The development of cell-free systems in which an active Pasteur effect is displayed has in the past caused considerable difficulty. One such system has recently been described (5, 7, 8), in which the glycolysis of the soluble supernatant fraction of brain or tumor (Flexner-Jobling or Walker 256) is inhibited by the addition of varying amounts of liver mitochondria. The inhibition involves both glucose utilization and lactate formation. Direct measurement of the levels of adenine nucleotides and inorganic phosphate in this system reveals values not consistent with this model Pasteur effect operating through a competition for inorganic phosphate, for phosphate acceptor, or for ATP. The inhibited (by addition of mitochondria) glycolytic system was characterized by a higher level of glucose-6-phosphate and fructose-6-phosphate and a lower level of hexose diphosphate and glyceraldehyde-3-phosphate than was the uninhibited system. The levels of AMP, ADP, and ATP were similar in the inhibited and uninhibited systems. Furthermore, the system in which glucose utilization was inhibited by mitochondrial addition was still able to glycolyze hexose diphosphate freely. These findings suggested that the glycolytic system was being inhibited at the phosphohexokinase step and, possibly, that the hexokinase reaction was also being inhibited. Studies with inhibitors (cyanide, antimycin A) indicated that the mitochondrial cytochrome chain was necessary for the inhibition. The authors suggested that the inhibition might be mediated through some intermediate of the electron transport chain interposed between electron transport and ADP phosphorylation, such as Hunter's hypothetical Y~phosphate (227).

Gatt and Racker have described a similar model Pasteur effect (177, 178) in which the glycolytic system was prepared by adding each of the purified glycolytic enzymes in proper amount. Mitochondria were again used to supply the oxidative component. In the presence of limiting adenine nucleotide concentrations and excess inorganic phosphate, addition of mitochondria resulted in a pronounced inhibition of lactate production. Under these conditions there was no inhibition of glucose utilization, while hexose diphosphate accumulated. This inhibition therefore resembled a Harden-Young effect rather than a true Pasteur effect. However, by limiting inorganic phosphate in the presence of added ATP (to maintain respiration and glycolysis), it was possible to obtain

mitochondrial inhibition of both lactate production and glucose consumption. Under these conditions mitochondrial phosphorylation led to a depletion of inorganic phosphate with a cessation of glycolysis when a critical inorganic phosphate concentration was reached. Gatt and Racker concluded that, although a true Pasteur effect could be demonstrated in this reconstructed system at limiting concentrations of inorganic phosphate, since such low inorganic phosphate levels were not observed in the intact cell it was unlikely that such a mechanism was operating in the whole cell.

Chance (96) has pointed out a serious drawback of experiments with a Pasteur effect in reconstructed systems. It is a very real possibility that, through manipulation of the enzyme, nucleotide, and inorganic phosphate concentration, Pasteur effects are achieved which have no relation to the mechanism of the Pasteur effect in the intact cell. Nevertheless, in the present author's opinion there is reason to hope that results with purified systems, cautiously interpreted, will be as valuable in the study of the Pasteur effect as they have been in other areas of biochemistry.

H. Summary of Mechanism

Despite the present uncertain state of the situation, it seems clear that real progress is being made in understanding the mechanism of the Pasteur effect. A simple competition for inorganic phosphate or phosphate acceptor between oxidizing mitochondria and the glycolytic system does not seem to explain adequately the aerobic inhibition of glucose utilization, an essential feature of the Pasteur effect. Such a competition would be expected to cause a Harden-Young type of phenomenon, with hexose diphosphate accumulation. It is generally believed that an adequate explanation of the Pasteur effect must involve inhibition of either the hexokinase or the phosphofructokinase reactions, or both. Several observers feel, because of the measured levels of hexose phosphates in the inhibited system (high glucose-6-phosphate and low fructose diphosphate), that a component of phosphofructokinase inhibition must be involved. At the present time two mechanisms seem plausible for this kinase inhibition: either the ATP concentration in the immediate vicinity of the glycolytic enzyme is decreased, or the reaction is being inhibited by some other factor. The most acceptable explanation of the Pasteur effect remains that there is some interaction between respiration and glycolysis involving the

adenine nucleotide-inorganic phosphate system. Direct measurement of the over-all ATP concentration in several systems would seem to make it clear that the kinase inhibition cannot be explained in terms of the average intracellular concentration of ATP. Racker, Lynen, and Chance have all suggested that oxidatively generated ATP is somehow unavailable for glucose phosphorylation. At the same time it is suggested that under aerobic conditions inorganic phosphate and/or phosphate acceptor is utilized for oxidative phosphorylation in the mitochondria and is therefore unavailable for the glyceraldehyde-3-phosphate dehydrogenase reaction in the soluble fraction of the cytoplasm. This leads to an unavailability of glycolytically generated ATP. In the absence of ATP in the soluble fraction of the cytoplasm, glucose phosphorylation ceases. This formulation requires a compartmentation, either structurally or functionally, of inorganic phosphate or adenine nucleotide within the cell. A second, less likely, possibility exists concerning the mechanism of the oxidative inhibition of glucose phosphorylation. It remains possible that the Pasteur effect is mediated by an intermediate, as yet undefined and other than ATP, which in some way inhibits the hexokinase reaction and/or phosphohexokinase reactions. A satisfactory explanation of the Pasteur effect must explain the release of the Pasteur effect by agents, such as proprionitrile, which have no known effect on the phosphorylation mechanism, as well as such agents as DNP, whose uncoupling action is well established. There is every reason to expect considerable progress in our understanding of the Pasteur effect in the next decade.

I. The Pasteur Effect in Tumors

Consideration will next be given to the Pasteur effect in tumors. Column 4 of Tables 1, 3, and 4 gives the absolute Pasteur effect, which varies from about 5 to about 15 in tumors and in normal tissues. The anaerobic glycolysis of a number of normal tissues is so low that appreciable inhibition is impossible. If the Pasteur effect is expressed as a function of oxygen uptake, as it is in the Meyerhof oxidation quotient of Column 5, it is seen that tumors and normal tissue also behave in a similar manner. Meyerhof oxidation quotients vary from 3 to 6, indicating that roughly 1 mole of oxygen taken up inhibits the appearance of 1–2 moles of lactate. Thus, it would seem well established that the Pasteur effect is func-

tioning in tumors in the same qualitative and quantitative manner as in normal tissue, and that the high aerobic glycolysis of the tumor cell is a manifestation of the high anaerobic glycolysis in the presence of a modest respiratory rate.

2. The Crabtree Effect

The phenomenon of respiratory inhibition by glycolysis is generally known in the English literature as the Crabtree effect, although some investigators prefer the term, reversed Pasteur effect. Crabtree observed (108) that the addition of glucose, but not of nonmetabolizable xylose, to slices of Jensen rat sarcoma, Crocker mouse sarcoma, and a mouse tar sarcoma, resulted in inhibition of the oxygen uptake. The inhibition was quantitatively not large (10–12%), but it was consistent, and not shown by the normal tissues studied (liver and kidney). Probably because of the relatively small inhibition observed in slices, a few observers have had difficulty demonstrating the Crabtree effect (122). However, most careful experimenters have been able to obtain the phenomenon in slices of transplantable tumors (145), and it has also been observed in slices of leukemic lymph nodes (457).

Recently, the Crabtree effect has been demonstrated in a number of normal tissues. Slices of renal medulla display a 20% inhibition of respiration on the addition of glucose, while bovine articular cartilage is 57% inhibited (401). Normal leukocytes (164, 294) have been found in several laboratories to display a Crabtree effect, although one observer (416) disputes this point. It is of interest that postnatal retina displays a Crabtree effect (up to 40% inhibition of respiration), while adult retina does not (102). In the retina the loss of the Crabtree effect is associated with the development of the sensory elements of this tissue, at a time when there is a 4- to 6-fold increase in respiration but no change in glycolysis. It will be noted that all the tissues which display a Crabtree effect share in common a high aerobic glycolysis.

It has only been with the development of the ascites cell that detailed study of the Crabtree effect has been possible. This is due in part to the ease with which this experimental tumor tissue is handled, but principally to the more marked Crabtree effect displayed by this tumor tissue. The Crabtree effect in ascites tumor cells was described at about the same time, in this country by Kun

et al. (263) and in the Soviet Union by El'tsina and Seits (151). It was found (263) that Ehrlich ascites cells showed a considerable oxygen uptake ($Q_{O_2} = 8.1$) in the absence of added glycolytic substrate, and possessed a respiratory quotient of 0.86. The addition of glucose resulted in a 50% inhibition of oxygen uptake and an elevation of the R.Q. to 1.16. Neither the inhibition of oxygen uptake nor the elevation of the R.Q. occurred upon the addition of lactate. The elevation of the R.Q. upon glucose addition had been previously described in tumor slices (145), and the effect in ascites cells has been repeatedly confirmed (43, 161, 228). Subsequent studies on Ehrlich ascites tumor cells (58, 390) has shown that sugars which are substrates for hexokinase (glucose, fructose, and mannose) exert a Crabtree effect, while nonfermentable sugars do not (sucrose, ribose, galactose). Of great interest is the observation that 2-deoxyglucose, a sugar which is phosphorylated by hexokinase but not further glycolyzed, also inhibits oxygen uptake by tumor cells (228, 535). With glucose the respiration returns to its unstimulated rate after all the sugar is glycolyzed, while with 2-deoxyglucose the respiratory inhibition persists (228). It has also been noted that very low concentrations of glucose, instead of inhibiting respiration in the usual manner, produce some stimulation of oxygen uptake (301).

The inhibition of respiration by glucose addition (Crabtree effect) has been demonstrated to involve inhibition of both glucose oxidation and endogenous oxidation of fatty acids (309). There is evidence that the inhibition of glucose oxidation is mediated through an inhibition of the oxidative decarboxylation of pyruvate and lactate at the step preceding the entry of the latter two compounds into the citric acid cycle (42). Glucose does not significantly inhibit the oxidation of labelled acetate (156, 309), suggesting that the inhibition of endogenous fatty acid oxidation does not involve depression of oxidation of two-carbon units.

Compounds that uncouple oxidation from phosphorylation (dinitrophenol, dicumerol) also relieve the inhibition produced by glucose on the oxygen uptake of ascites cells. It is also of great significance that there is no relationship between the actual rate of glycolysis and the Crabtree effect. In addition to the fact that 2-deoxyglucose produces inhibition of respiration, many observers have found that glycolysis can be blocked with iodoacetate (228, 264, 529) or bromoacetate (151) without interference with the

Crabtree effect. The observation that actual glycolysis is not necessary for the Crabtree effect also rules out a change in intracellular pH (417) as a cause of the glucose-induced respiratory inhibition. Parenthetically, it should be noted that oxamic acid, a compound which inhibits lactic dehydrogenase, inhibits anaerobic glycolysis and abolishes the Crabtree effect (354). Methylene blue, ethylurethane, and sulfhydryl compounds also counteract the Crabtree effect in ascites cells (165).

The precise mechanism of the Crabtree effect remains uncertain, though it is thought at present that very likely this phenomenon, like the Pasteur effect, may result from a competition between the oxidative and glycolytic systems for inorganic phosphate or phosphate acceptor. Since the work of Engelhardt in 1932, it has been known that inorganic phosphate is required for maximal respiration. More recently, it has been conclusively demonstrated with mitochondria from a variety of normal tissues (liver, kidney, brain, etc.) (233, 267, 268, 431) that the rate of oxygen uptake by these respiratory particles is also dependent on the presence of phosphate acceptor. Thus, in the absence of phosphate acceptor the rate of mitochondrial oxidation of a variety of substrates is quite low. Upon addition of phosphate acceptor, usually glucose plus hexokinase, there is marked acceleration of oxygen uptake, the extent of which varies with the substrate. Stimulation up to 4-fold is seen with succinate and caprylate, 5- to 15-fold with glutamate, while with β-hydroxybutyrate the uptake of oxygen is often completely dependent on phosphate acceptor (267). On the basis of this known dependence of the rate of oxidation of isolated mitochondria on phosphate acceptor, and of certain original work to be discussed, Racker (390) and Chance (91) have suggested a mechanism for the Crabtree effect which involves a shuttling of the adenine nucleotides or inorganic phosphate out of the mitochondria into the soluble fraction of the cell with the onset of the glycolysis that follows glucose addition. The mitochondria are thus deprived of phosphate acceptor, with a consequent decline in oxidative rate. The localization of the oxidative system to the mitochondria, and the glycolytic system to the soluble particle-free fraction of the cell cytoplasm, appears to be well established experimentally (115, 411).

Gatt and Racker (177, 178) studied the Crabtree effect in a model system of isolated mitochondria and a glycolytic system re-

constructed by the combination of the individual isolated glycolytic enzymes. It was found that in this system, at low ADP concentrations (3×10^{-4} M), there was inhibition of mitochondrial oxygen uptake by the addition of glucose. At high ADP concentrations (2×10^{-3} M) there was no inhibition, and omission of a single glycolytic enzyme resulted in a failure to obtain inhibition. The inhibition was dinitrophenol-sensitive. There was also no inhibition when the glycolytic transphosphorylating enzymes (pyruvate kinase and 3-phosphoglycerate kinase) in the synthetic glycolytic system were reduced to limiting activity. Presumably, when these enzymes were limiting they could no longer compete with mitochondria for phosphate acceptor. It was also possible in this reconstructed system to obtain conditions in which inorganic phosphate appeared to be limiting. This was done by having the level of ADP relatively high and the level of inorganic phosphate low, and by adding ATPase. Thus Gatt and Racker were able to set up a Crabtree effect in which the mechanism appeared to be either ADP competition or inorganic phosphate competition, depending on the conditions. Although these experiments have established that in a reconstructed system the Crabtree effect may be mediated by either inorganic phosphate or ADP competition, they do not indicate how the Crabtree effect is mediated in the intact cell.

Chance and Hess (91, 94–96), on the basis of studies with intact ascites cells and ascites mitochondria, favor a mechanism of the Crabtree effect involving a competition for phosphate acceptor (ADP) between the oxidative and glycolytic systems. They have reported that mitochondria from ascites cells resemble mitochondria from normal tissue in their response to added phosphate acceptor. Thus, succinate oxidation by ascites mitochondria is accelerated 3- to 6-fold by the addition of phosphate acceptor (93). Spectrophotometric studies indicate that ascites mitochondria also resemble liver mitochondria in the response of the substrate-treated mitochondria to added ADP, Chance's "state 3–4 transition." This addition causes a disappearance of the absorption bands (oxidation) of reduced pyridine nucleotide, flavoprotein, cytochrome *b*, and cytochrome *c*. On the other hand, exhaustion of ADP, "state 4–3 transition," is marked by the appearance of bands corresponding to the reduction of pyridine nucleotides, flavoprotein, and cytochromes *b* and *c*. When glucose is added to respiring ascites cells or mitochondria, there is a transient 2- to 6-fold stimulation of respiration,

which is then followed by a marked inhibition of respiration, the Crabtree effect. If a sensitive measuring device, such as the oxygen electrode used by Chance, is not used, the preliminary period of stimulation is missed. The glucose-activated phase of respiration is characterized spectrophotometrically by the appearance of bands corresponding to reduced pyridine nucleotide, reduced flavoprotein, and reduced cytochromes *b* and *c*. This pattern is consistent with the glucose-activated respiration being caused by the transient accumulation of ADP resulting from the initial phosphorylation of glucose. The Crabtree effect, the inhibitory period that follows the initial period of oxidation, is characterized by the disappearance of these bands (oxidation of the respiratory components) and is consonant with the picture of ADP exhaustion. Thus Chance and Hess feel the Crabtree effect is caused by glycolytic depletion of phosphate acceptor. If glucose utilization is measured at the same time as oxygen uptake, it is found that the onset of respiratory inhibition following glucose addition is associated with a marked fall in the rate of glucose utilization. This signifies, to the authors, that the ATP oxidatively generated is not available for glucose phosphorylation. They feel that the entire picture of respiratory and glycolytic regulation in the ascites cell might be explained by the following three hypotheses:

"(1) An overriding ADP affinity for mitochondria (*as opposed to the glycolytic enzymes*) which are under control of the ADP level.

"(2) A compartmentalization of newly phosphorylated (*oxidatively*) ATP which can be released by uncoupling agents and anaerobiosis.

"(3) A sensitivity of ATP utilization to the intracellular ATP level."

They feel, therefore, that the Crabtree effect is caused by the glycolytic system's depriving the respiring mitochondria of ADP. Chance (86) believes that it is unlikely that inorganic phosphate is a respiratory control chemical, because mitochondria have a very low affinity for the compound and their respiratory activity cannot be triggered by low concentrations (measured by the respiratory response of mitochondria to phosphate in a phosphate-depleted medium). On the other hand, ADP has the characteristics of a desirable respiratory control chemical because mitochondrial respira-

tion is triggered by low concentrations of this compound, and maximal activity is obtained in response to a small amount.

Although such a shuttle of adenine nucleotides is an attractive hypothesis for explaining the Crabtree effect, as yet it is not backed by a substantial amount of direct experimental evidence. Direct determination of the levels of AMP, ADP, and ATP in the resting ascites cell, before and after the addition of glucose, has been done by Ibsen *et al.* (228). In the resting ascites cell, before the addition of glucose, about two-thirds to three-quarters of the total adenine nucleotide is found as ATP, the remainder being ADP, and almost no AMP is found. Upon addition of glucose, there is a rapid fall in ATP and a rise in ADP and AMP, but within 2 to 3 minutes the levels of all three compounds return almost to those found before glucose addition. (The final levels of AMP and ADP are both slightly higher than before glucose addition.) Under the conditions of these experiments the inhibition begins within 30 seconds of glucose addition and lasts for 20–30 minutes. However, these data, as the authors point out, do not bear on whether or not a localized mitochondrial deficiency of adenine nucleotides, particularly of ADP, is produced by glucose addition.

Wu and Racker (528) have also measured the intracellular concentration of adenine nucleotides, inorganic phosphate, and hexose phosphates in Ehrlich ascites cells. In the absence of iodoacetate, the levels of adenine nucleotides were similar in the absence and in the presence (Crabtree effect) of glucose. With the addition of both iodoacetate and glucose, a good Crabtree effect was obtained in the presence of marked depletion of cellular adenine nucleotides. The Crabtree effect both in the presence and absence of iodoacetate was characterized by a lower level of cellular inorganic phosphate than in the absence of glucose-inhibited respiration. Wu and Racker also observed that there was a partial inhibition of the Crabtree effect in the presence of high levels of inorganic phosphate in the medium. They concluded that in the intact ascites cell a competition for inorganic phosphate (and, under some conditions, probably also for adenine nucleotides) was the most likely cause of the Crabtree effect.

Although other investigators (56) have reported that the Crabtree effect can be partially overcome by raising the inorganic phosphate level of the medium, several careful reports indicate that the level of inorganic phosphate of the medium is not an important

TABLE 65

RELATION BETWEEN AEROBIC GLYCOLYSIS AND THE CHANGE OF RESPIRATORY RATE DUE TO GLUCOSE ADDITION (385)

Tissue	Q_{O_2} (no glucose present)	$Q_{O_2}(G)$ (with glucose present)	$Q_{O_2} - Q_{O_2}(G)$	$Q_L^{O_2}$	$\frac{Q_L^{O_2}}{Q_{O_2} - Q_{O_2}(G)}$
Ehrlich ascites					
(1)	14.0	8.0	6.0	33	5.5
(2)	12.3	7.1	5.2	30	5.7
(3)	12.1	8.1	4.0	29	7.2
(4)	11.3	6.9	4.4	26	5.9
(5)	11.8	6.3	5.5	26	4.7
					Average value = 5.8
Sarcoma 37	9.8	6.6	3.2	20	6.2
Novikoff hepatoma	9.7	7.0	2.7	162	6.0
Walker 256 carcinosarcoma	6.8	5.4	1.4	9.0	6.4

variable in the Crabtree effect (228, 264). Kvamme (264) and Ibsen *et al.* (228) have concluded on the basis of their experiments that the total intracellular level of inorganic phosphate is not the critical factor in the Crabtree effect. Clearly, for the evaluation of the role of inorganic phosphate depletion in the respiratory inhibition of the Crabtree effect, the significant experimental quantity needed is the concentration (activity) of inorganic phosphate at the intramitochondrial site of oxidative phosphorylation, a measurement that is difficult to make.

Unlike the Pasteur effect, the Crabtree effect appears to be a tightly coupled phenomenon in which the high energy phosphate from the inhibited respiration is exactly replaced by glycolytically generated ATP. Thus, the data of Quastel and Bickis (385) in Table 65 indicate that, assuming the reasonable figure of a P:O ratio of 3.0 for tumor respiration, the energy lost by glucose-suppressed respiration is exactly replaced by glycolytically formed high energy phosphate.

The mechanism of the Crabtree effect remains unclear. This phenomenon is displayed by tumor slices and slices of normal tissues that possess a high aerobic glycolysis; the most marked Crabtree effect is displayed by ascites tumor cells. As it has been studied in ascites tumor cells, it appears to require a sugar that is a hexokinase substrate, but not to require active glycolysis. (2-Deoxyglucose inhibits respiration, and the Crabtree effect persists in the presence of glycolysis blocked by iodoacetate.) Uncoupling the oxidative system from phosphorylation releases the Crabtree effect. Indirect evidence suggests that a competition either for ADP or inorganic phosphate between the glycolytic system and the oxidizing mitochondria is responsible for the Crabtree effect. However, the crucial experimental data, i.e., the concentration of the intermediate (inorganic phosphate or ADP) at the site of oxidative phosphorylation in the mitochondria, must be known before it will be possible to decide which, if either, of these compounds is responsible for the respiratory inhibition of glucose. Compartmentation of inorganic phosphate and the adenine nucleotides between the soluble and particulate cytoplasmic fractions, a phenomenon which is a necessary condition for the above Crabtree effect mechanism, is a problem of considerable biochemical interest about which little is known at present.

PART IV: The Dependence of Tumors on Oxidative and Glycolytic Energy

1. The Dependence of Synthetic Processes on Oxidative and Glycolytic Energy

The high aerobic and anaerobic glycolysis of tumors has been the subject of Part I of this monograph, while Part II has been devoted to a detailed study of tumor respiration. It is the purpose of the present section to relate the energy metabolism of tumors to synthetic processes. Relatively little work has been done on this aspect of tumor metabolism; the studies to be presented mainly represent comparisons of the abilities of tumor and normal tissues to incorporate radioactive precursors into protein and nucleic acid under aerobic and anaerobic conditions in the presence and absence of glucose. It is beyond the purpose of the present book to review the field of protein synthesis. A number of excellent reviews are available (214), one of which considers the problem from the point of view of growth processes (77).

Tumors are very active in the incorporation of labelled amino acids into protein (536, 538). In Table 66, for example, in which the rates of incorporation of labelled alanine into liver and into

TABLE 66

RATE OF INCORPORATION OF RADIOACTIVE ALANINE INTO PROTEIN OF VARIOUS TYPES OF RAT HEPATIC SLICES (170, 536)

Tissue	Incorporation (c.p.m./mg. protein)	Oxygen uptake (μl.)
Normal control liver	38	—
Normal liver from hepatoma	91	—
Hepatoma	255	—
Regenerating liver	91	—
Fetal liver	179	—
Normal liver (0 DNP)	31	54
Normal liver (5×10^{-5} *M* DNP)	17	75
Normal liver (5×10^{-4} *M* DNP)	0.9	54
Hepatoma (0 DNP)	255	80
Hepatoma (5×10^{-5} *M* DNP)	171	99
Hepatoma (5×10^{-4} *M* DNP)	1.2	26

hepatoma slices are compared, it will be noted that over 6 times as much radioactivity is incorporated into the liver tumor as into normal rat liver. An energy source is necessary for amino acid incorporation. Thus, there is no incorporation into protein in the absence of oxygen, and uncoupling the phosphorylation of ATP from oxygen with DNP (Table 66) also eliminates amino acid incorporation (170). Incorporation studies with particulate systems have revealed similar energy requirements. Thus, Zamecnik *et al.* have shown that, although the amino acid-incorporating system is contained in the microsomes and the soluble supernatant fraction, a source of energy must also be supplied for incorporation, either mitochondria and oxidizable substrate (429) or a phosphate donor such as creatine phosphate or phosphoenolpyruvate with the appropriate kinase enzyme (537).

Farber *et al.* (166) and Kit and Greenberg (254) have studied the incorporation of radioactive amino acids into minces of Gardner lymphosarcoma. They found that the addition of glucose stimulated the incorporation of glycine-2-C^{14} into the proteins of this tumor both under aerobic and anaerobic conditions. In oxygen the incorporation was 35 counts per minute (c.p.m.) with glucose present and 15 c.p.m. without glucose, while in nitrogen the radioactivity of the protein was 7 c.p.m. with glucose and no incorporation when glucose was omitted. The incorporation in oxygen was inhibited 94% by 0.005 *M* cyanide, 98% by 0.005 *M* azide, and 99% by 0.005 *M* dinitrophenol. The stimulating effect of glucose was studied further, and it was found, with a variety of amino acids, that aerobic incorporation was stimulated from 50 to 300% in the tumor mince by glucose addition. In the aerobic medium fortified with glucose, glycolytic inhibitors (0.001 *M* fluoride, 0.01 *M* arsenate, and 0.001 *M* iodoacetate) all markedly inhibited the amino acid incorporation, although at these concentrations a rather marked inhibition of respiration was also produced in this system (Table 67). Oxidative substrates such as citrate, pyruvate, lactate, malate, and α-ketoglutarate could not be substituted for glucose in stimulating amino acid incorporation. As indicated in Table 67, arsenite, fluoroacetate, and malonate (inhibitors of oxidation and the citric acid cycle) all inhibited the uptake of labelled amino acid. Thus these studies indicate that in the Gardner lymphosarcoma optimal incorporation of radioactive amino acids into protein requires both respiration and glycolysis. More recently, Blecher

and White have performed experiments with thymic lymphocytes and Murphy-Sturm lymphosarcoma cells and have obtained results that differ somewhat from the results of Farber *et al.* (166, 254). They found (41) that the incorporation of glycine-2-C^{14} into protein and mixed nucleic acids of the lymphosarcoma cell was low in the absence of glucose, regardless of whether the conditions were

TABLE 67

EFFECT OF INHIBITORS OF GLYCOLYSIS AND OF THE TRICARBOXYLIC ACID CYCLE ON RADIOACTIVE GLYCINE INCORPORATION BY CELL SUSPENSIONS OF THE GARDNER LYMPHOSARCOMA (254)

Exp. no.	Addition (concentration, mmoles/l.)	Incorporation (μg. C^{14}/gm. protein)	Oxygen uptake (μl.)
1	None (control)	17.4	220
	Arsenate (10)	3.7	62
	Fluoride (1)	0.8	108
2	None (control)	69.0	—
	Iodoacetate (1)	5.1	—
	Iodoacetate (0.25)	12.6	—
3	None (control)	16.0	110
	Glucose (20) + fluoroacetate (10)	7.8	42
4	None (control)	17.4	220
	Glucose (20)	30.3	146
	Glucose (20) + fluoroacetate (15)	6.5	75
	Glucose (20) + fluoroacetate (15)	6.2	62
5	Glucose (25)	144	—
	Glucose (25) + arsenite (5)	4.8	—
6	α-Ketoglutarate (20), control	9.4	102
	α-Ketoglutarate (20) + arsenite (2.5)	0.6	21
7	None (control)	18.3	98
	Malonate (10) + succinate (10)	11.6	152
	Malonate (10)	4.9	80

aerobic or anaerobic. Maximal incorporation was obtained anaerobically with glucose present; the aerobic incorporation with glucose was only about one-third of this maximal value. However, with thymic lymphocytes results similar to those of earlier workers (166, 254) were obtained. Aerobic incorporation both in the presence and absence of glucose was higher than anaerobic incorporation, and maximal incorporation was obtained aerobically in the presence of glucose.

Rabinovitz *et al.* (388) have found that the incorporation of radioactive amino acids into the Ehrlich ascites tumor proceeds anaerobically if active glycolysis is maintained. Their data in Table 68 indicate that the anaerobic incorporation in the presence of glucose is about two-thirds of the aerobic incorporation (for a variety of labelled amino acids). Without glucose, there is no

TABLE 68
OXIDATION-LINKED AND ANAEROBIC GLYCOLYSIS-LINKED INCORPORATION OF AMINO ACIDS INTO PROTEIN OF EHRLICH ASCITES CARCINOMA (388)

	Aerobic		Anaerobic			
			Without glucose		With glucose (0.015 *M*)	
Amino acid	O_2 uptake (μl.)	Incorporation (μ*M*/gm. protein/hr.)	CO_2 evolved (μl.)	Incorporation (μ*M*/gm. protein/hr.)	CO_2 evolved (μl.)	Incorporation (μ*M*/gm. protein/hr.)
Leucine	142	6.8	33	0.0	659	4.9
Valine	207	6.2	37	0.0	682	4.5
Lysine	167	5.1	33	0.0	651	3.4
Phenylalanine	170	2.7	35	0.0	644	1.8
Methionine	193	2.0	39	0.0	690	1.2

anaerobic incorporation. For a reason which is not clear, the glycolysis-linked incorporation shows an induction period, although the aerobic incorporation does not. When this induction period is taken into account, the rate of incorporation anaerobically in the presence of glucose is the same as the aerobic rate. Dinitrophenol inhibits the aerobic incorporation at a concentration which stimulates oxygen uptake, while the compound does not affect the glycolysis-linked incorporation. Unfortunately, both this and the preceding study fail to compare tumor tissue with normal tissue, a comparison that is necessary before any conclusion can be made concerning the relation of these phenomena to tumor glycolysis.

A very important and extensive study of the relation of energy metabolism to protein synthesis has been done by Quastel and Bickis (385). They measured incorporation of amino acid into protein under aerobic conditions (with glucose) and anaerobic conditions (with glucose), and related it to the energy available under the two conditions. Thus, they obtained an aerobic efficiency which is the incorporation efficiency of aerobically generated ATP

(aerobic ATP $= 6 \times Q_{O_2}$, assuming a P:O ratio of 3.0) and an anaerobic efficiency which is the incorporation efficiency of glycolytically generated ATP (anaerobic ATP $= Q_{La}$, assuming 1 mole of ATP per mole of lactate). The figures for a variety of normal and neoplastic tissues are given in Table 69. It should be stated here that only a very small amount of the total energy available is used for amino acid incorporation (assuming that one ATP molecule is needed for each molecule of amino acid incorporated, only 1 of 500 molecules of ATP formed is used for protein synthesis). In contrast to the studies presented previously, Quastel and Bickis found that in ascites cells under aerobic conditions the presence or absence of glucose makes no difference in the amino acid incorporation, and the efficiency of incorporation is the same under the two conditions. Thus, glycolytic and oxidative energy are used with equal efficiency by the tumor cell for protein synthesis. Without glucose there is no anaerobic incorporation. The highest efficiency in both aerobic and anaerobic incorporation occurred in growing tissue, i.e., tumor and embryo. The ratio of anaerobic to aerobic efficiency approximates unity for tumors, the anaerobic efficiency being slightly higher, if anything. However, with adult rat tissues and with chick and rat embryos the aerobic efficiency usually exceeds the anaerobic. Furthermore, a number of normal tissues (for example, renal medulla and retina), though possessing a relatively high rate of anaerobic glycolysis, have little or no anaerobic efficiency for the incorporation of labelled glycine into protein. The authors suggest that such tissues are unable to use extramitochondrial ATP for protein synthesis. Many adult tissues also display relatively low aerobic efficiencies compared with those of tumor and embryo. Although tumor and embryo both display a high aerobic and anaerobic efficiency, they differ in several respects. Embryo, in contrast to tumor, usually displays an aerobic efficiency which exceeds the anaerobic value. Furthermore, while malonate inhibits the amino acid incorporation into the protein of both Ehrlich ascites cells and chick embroys, the addition of glucose overcomes this inhibition in the tumor cell but has no effect on the embryo incorporation. Thus tumor appears to differ from normal tissue in the greater efficiency with which it can use glycolytic energy for protein synthesis.

Concerning these highly significant investigations of Quastel and Bickis, the present author believes that too literal an interpreta-

TABLE 69

EFFICIENCIES OF AEROBIC AND ANAEROBIC INCORPORATION OF GLYCINE-1-C^{14} INTO TISSUE PROTEINS (385)

Tissue	Oxygen uptake, Q_{O_2} (G)[a]	Aerobic amino acid incorporation, A_{O_2} (G) (c.p.m./mg.)	Aerobic efficiency,[b] E_{O_2} (G)	Anaerobic glycolysis, $Q^{N_2}_{CO_2}$	Anaerobic amino acid incorporation, A_{N_2} (G) (c.p.m./mg.)	Anaerobic efficiency, E_{N_2} (G)
Ehrlich ascites carcinoma	7.1	137	1.9	62	138	2.2
Sarcoma 37 (s.c. in mice)	5.0	60	1.5	19.7	24	1.2
Sarcoma 37 (fertile eggs)	6.6	105	1.8	40.1	89	22
Sarcoma 37 ascites (mice)	4.5	97	20	44	102	2.3
Novikoff hepatoma	7.0	80	1.5	33.6	68	2.0
Walker 256 carcinosarcoma	5.4	70	1.6	27.2	55	2.0
Melanoma S-91 (DBA mice)	4.6	45	1.3	16.7	25	1.5
Embryo (rat)	10.9	121	1.7	21.8	29	1.3
Embryo (chick)	14.8	221	2.5	30.6	45	1.6
Rat liver	6.5	33	0.8	2.2	0.0	0.0
Rat "regenerating" liver	10.0	85	1.4	3.2	0.0	0.0
Rat kidney cortex	22.2	9	0.07	2.8	0.0	0.0
Rat kidney medulla	19.9	20	0.17	11.5	1	0.09
Rat spleen	6.3	48	1.2	4.6	2	0.4
Rat thymus	8.8	81	1.5	127	9	0.7
Rat lung	4.7	31	1.1	5.7	2	0.3
Pigeon pancreas	4.8	42	1.4	4.6	1.0	0.2
Rat brain	12.8	2	0.03	9.4	0.0	0.0
Cattle retina	10.2	4	0.07	32.4	3	0.1

[a] See Table 65.

[b] Corrected for aerobic glycolysis.

tion of the word efficiency should be avoided. Certainly, one cannot make an analogy of incorporation efficiency, in which a multitude of equally significant pathways are utilizing energy, with mechanical efficiency, which applies to a much simpler problem. Bickis *et al.* (37) have also performed interesting experiments on the effect of Ehrlich ascites antisera on the *in vitro* biochemical activities of the Ehrlich ascites cell. Earlier studies from other laboratories had shown that in the presence of specific antisera ascites cells lost their ability to respire in the presence of glucose but retained succinate respiration (169), and that antisera against whole cells and ribonucleoprotein fractions could inhibit amino acid incorporation (104). Bickis *et al.* (37) confirmed that antisera could inhibit respiration, glycolysis, and amino acid incorporation of the ascites cell. Of these the amino acid incorporation was the most sensitive, occurring at antiserum concentrations that effected neither respiration nor glycolysis. Succinate respiration, while insensitive to the antiserum, was unable to support amino acid incorporation. The authors felt their experiments indicated that the inhibition of amino acid incorporation was unrelated to either inhibition of respiration or glycolysis.

An earlier study by Mann and Gruschow (303) dealt with the incorporation of radioactive phosphorus into slices of tumor, kidney, and liver, in oxygen and in nitrogen, in the presence and in the absence of glucose. The P^{32} incorporation represents a measure of synthesis of combined phosphoprotein, phospholipid, and nucleic acid. The authors found that when glucose was absent the incorporation of P^{32} into tumor slices was inhibited by anaerobiosis by 80% of the aerobic value, but was inhibited only 15% when glucose was present. With kidney and liver the presence or absence of glucose made little difference in the per cent of inhibition; thus, for kidney in nitrogen there was a 69% inhibition with glucose and a 59% inhibition without glucose, while for liver the respective figures were 51% and 69%. Kennedy and Williams-Ashman (248) studied the incorporation of P^{32} into the particles of the tumor cytoplasm (combined phospholipid, phosphoprotein, and nucleic acid) under conditions of oxidative phosphorylation. They found that the incorporation was substrate-sensitive and DNP-sensitive. For good incorporation, it was found necessary to add fluoride to prevent the intense dephosphorylation of the adenine nucleotides which occurs in the tumor cytoplasm. Several laboratories have

confirmed the glucose requirement for anaerobic incorporation of P^{32} into the combined phospholipid, phosphoprotein, and nucleic acid of ascites tumor cells (113, 326). One group of investigators (113) has obtained evidence that the P^{32} uptake in the absence of ascitic fluid was exchange reaction, while in the presence of ascitic fluid the uptake represented largely *de novo* synthesis—an observation which requires further study. As in the case of amino acid incorporation into ascites cells, uncoupling agents [DNP, Sarkomycin (38), and decanoic acid] led to a marked inhibition of P^{32} incorporation in the absence of glucose but not in its presence (112). These uncoupling agents led to a rapid loss of ATP and ADP from the cell but little alteration of the respiratory rate (113).

Shacter has studied the effects of the uncoupling agent DNP (421), the glycolytic inhibitors *p*-chloromercuribenzoic acid and iodosobenzoic acid (432), and stilbestrol (423) on the incorporation of P^{32} into cellular and medium inorganic phosphate and cellular labile phosphate, ester phosphate, and DNA of the Ehrlich ascites tumor cell. The studies with DNP are presented in Fig. 13. It will be noted that at DNP concentrations of 2×10^{-5} *M*, while respiration and P^{32} incorporation into DNA were stimulated, P^{32} incorporation into labile phosphate was unchanged, and the actual level of labile phosphate was decreased. At somewhat higher concentrations of DNP, stimulation of respiration remained, but the level and radioactivity of the labile phosphate decreased, as did the P^{32} incorporation into DNA. The author concluded from these studies that the mitotic rate (P^{32} incorporation into DNA) was independently related to both respiration and high energy phosphate. Stilbestrol was felt to act in a fashion similar to that of DNP. In a later study (349) *p*-chloromercuribenzoate and *o*-iodosobenzoic acid were found to inhibit respiration, decrease the concentration and specific activity of the labile phosphate, and lower the incorporation of P^{32} into DNA at high concentrations; at low concentrations these glycolytic inhibitors neither inhibited respiration nor incorporation into DNA. Nitrogen mustard had an effect, similar to that of the glycolytic inhibitors, on respiration and incorporation, though less marked. It thus appears necessary to inhibit severely respiration in order to inhibit the incorporation of P^{32} into DNA.

Obviously, further studies on the relationship of synthetic processes of the tumor cell to its glycolytic and oxidative metabolism are needed. For these studies to be of value, they must be controlled

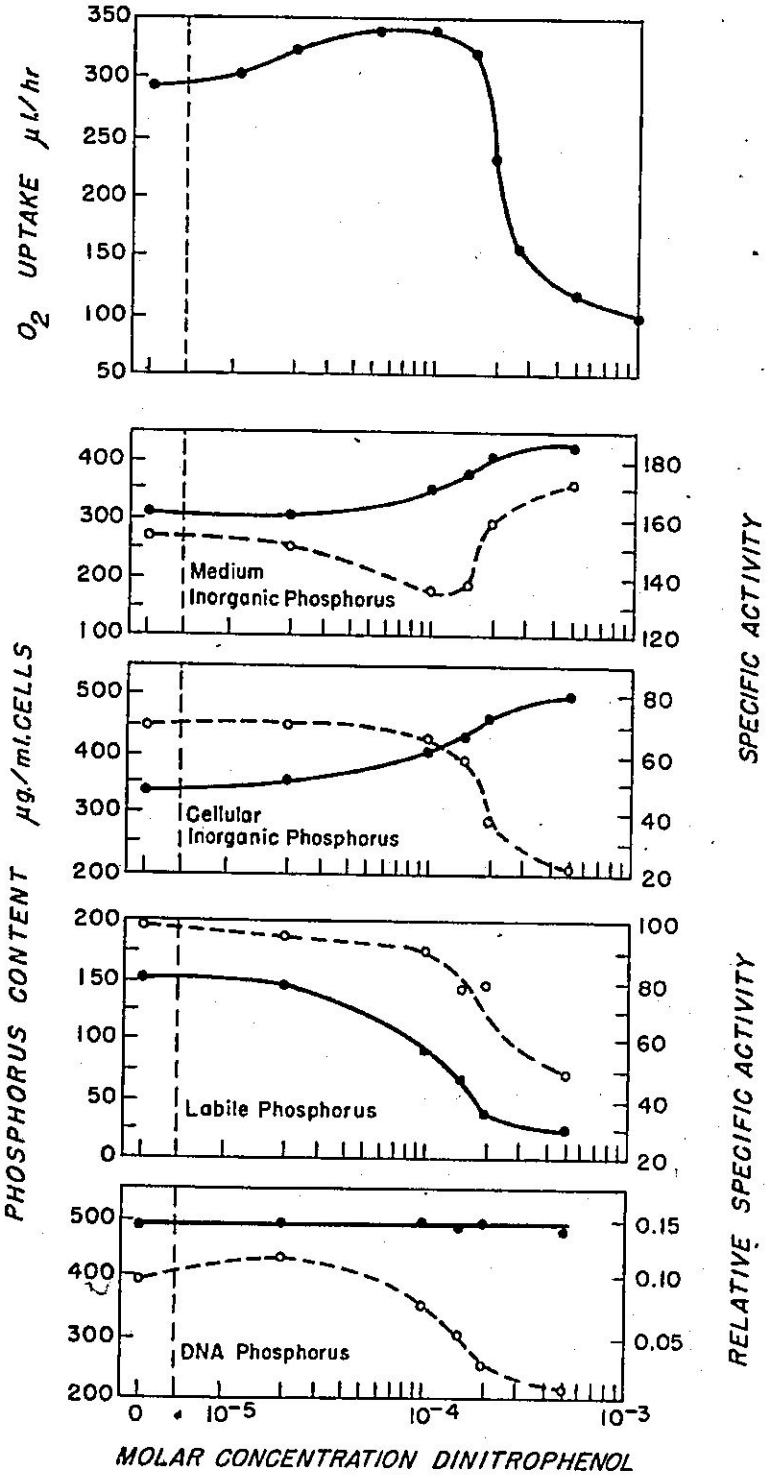


FIG. 13. Effect of graded concentrations of dinitrophenol on oxygen uptake, inorganic phosphate of the medium, and inorganic, labile and deoxyribonucleic acid phosphate of the ascites cell. The solid lines are phosphorus content, and the broken lines relative specific activity. The cells were incubated in a medium containing radioactive phosphate. From Shacter (421).

by similar studies done in normal tissues, particularly tissues with a high rate of aerobic and anaerobic glycolysis, such as renal medulla, embryo, and retina. Little is known about the energy dependence of nucleic acid synthesis in tumor as opposed to normal tissue. With protein synthesis, the available evidence suggests that tumor has a double energy source as compared with that of most adult tissues. While normal adult tissues, in general, do not seem to be able to effectively incorporate amino acids with glycolytic energy alone, tumor tissue seems to be able to make equally effective use of aerobically and anaerobically generated ATP.

2. The Nature of the Energy Supply for Cell Division

The qualitative and quantitative aspects of the energy source for cell division are difficult and unsettled subjects whose detailed treatment is beyond the scope of the present monograph. Two recent general references are available (54a, 444a). For the most part, experimental work in this field has been confined to lower animals because of the technical difficulties involved in the study of mammalian tissues. The actual process of cell division (not considering the preceding synthetic processes) appears to be independent of a direct energy supply from either respiration or glycolysis, the required energy being stored in advance in some chemical form. The amount of energy needed for cell division itself is not accurately known but, in cells other than yolky eggs, it is thought to account for only a negligible fraction of the total respiration. On the whole, the evidence favors the view that the energy for mitosis is derived from oxidative phosphorylation and carbohydrate metabolism. However, it is not clear whether any particular glycolytic or respiratory pathway is involved. Certain experimental evidence favors the position that glycolysis is particularly linked to cell division, while other evidence suggests that it is only net total energy production which is necessary for mitosis.

3. The Relationship of Tumor Survival to Glycolysis and Respiration

The ability of tumor tissue to survive on either glycolysis or respiration alone was studied in Warburg's laboratory in the late 1920's. Okamoto (349) found that even after incubation for 72 hours in nitrogen there were 100% takes on subsequent transplantation of slices of Flexner-Jobling carcinoma if adequate glucose were

supplied in the medium (Table 70). With the Jensen rat sarcoma there were 30% takes on transplantation after 120 hours in an anaerobic glucose containing medium (Table 70). On the other hand, when oxygen was supplied, the tumor slice survived for con-

TABLE 70

SURVIVAL OF SLICES OF FLEXNER-JOBLING CARCINOMA (F-J) AND JENSEN RAT SARCOMA (J) UNDER ANAEROBIC CONDITIONS IN THE PRESENCE AND ABSENCE OF GLUCOSE (TRANSPLANTATION STUDIES) (349)

Tumor	Gas	Medium	Glucose	Duration of incubation (hr.)	No. of animals inoculated	Tumor takes (%)
F-J	N_2	Ringer's	No	24	10	0
F-J	N_2	Ringer's	No	24	3	0
F-J	N_2	Ringer's	Yes	24	11	30
F-J	N_2	Ringer's	Yes	24	4	100
F-J	N_2	Ringer's (0.001 M C≡N)	Yes	24	20	40
F-J	N_2	Ringer's	Yes	24	10	80
F-J	N_2	Ringer's	Yes	72	5	100
J	N_2	Serum	Yes	24	10	80
J	N_2	Serum	Yes	24	6	33
J	N_2	Serum	Yes	48	7	100
J	N_2	Serum	Yes	48	6	100
J	N_2	Serum	Yes	72	10	80
J	N_2	Serum	Yes	72	9	78
J	N_2	Serum	Yes	120	10	30

siderable periods of time in the absence of glucose. (Table 71 indicates that the Flexner-Jobling carcinoma would still actively glycolyze when placed into glucose after being maintained aerobically for 72 hours in the absence of glucose.) Warburg reports that to destroy the cells of the tumor slice a period of 4 hours was necessary, in which the tumor had neither oxygen nor glucose (349). More recently, Warburg *et al.* found (478) that to kill the ascites cancer cell (at 38° C.) it is necessary to deprive it of oxidative and glycolytic energy for at least 24 hours, and that only one-fifth of this energy was sufficient to preserve the transplantability of this neoplastic cell.

Studies with tissue culture (513) were less conclusive. It appeared that, while life in tissue culture was maintained by either

TABLE 71

SURVIVAL OF SLICES OF FLEXNER-JOBLING CARCINOMA (F-J) AND JENSEN RAT SARCOMA (J) UNDER AEROBIC AND ANAEROBIC CONDITIONS IN THE PRESENCE AND ABSENCE OF GLUCOSE (METABOLISM STUDIES) (349)

Tumor	Gas	Medium	Glucose	Metabolism estimated in Ringer's solution (0.2% sugar) after 0 Hr.		24 Hr.		48 Hr.		72 Hr.	
				$-Q_{O_2}$	$Q_{CO_2}^{N_2}$	$-Q_{O_2}$	$Q_{CO_2}^{N_2}$	$-Q_{O_2}$	$Q_{CO_2}^{N_2}$	$-Q_{O_2}$	$Q_{CO_2}^{N_2}$
F-J	N_2	Ringer's	No	—	28	—	0	—	—	—	—
F-J	O_2	Ringer's	No	—	34	—	—	—	—	—	13
F-J	N_2	Ringer's	Yes	—	31	—	—	—	—	—	8
F-J	N_2	Ringer's (N/1000 HCN)	Yes	—	28	—	16	—	—	—	—
F-J	N_2	Serum	Yes	—	21	—	16	—	17	—	15
F-J	N_2	Serum	Yes	—	20	—	—	—	15	—	6
F-J	O_2	Serum	Yes	—	21	—	23	—	23	—	—
J	N_2	Serum	Yes	9	42	1	26	—	—	—	—
J	N_2	Serum	Yes	13	35	—	—	2	22	1	25
J	N_2	Serum	Yes	6	30	—	—	2	20	2	21
J	N_2	Serum	Yes	6	29	—	—	—	—	—	—
J	N_2	Serum	Yes	7	35	—	—	—	—	2	20
J	O_2	Serum	Yes	13	35	—	—	2	21	0	15
J	O_2	Serum	Yes	6	30	—	—	—	—	5	15
J	O_2	Serum	Yes	7	35	—	—	—	—	2	19

95% oxygen or 0.2% glucose, for optimal growth both were needed. The absence of control studies with explants of normal tissues makes interpretation of these results difficult.

A number of investigators (24, 76, 361, 482) have examined the effect of hypoxia on tumor growth. It has been possible to obtain tumor weight loss with or without the loss of host body weight after exposure to 5–11.5% oxygen for varying periods of time.

PART V: Summary and Conclusions

This concluding part of the present review of the energy metabolism of tumors is divided into two sections. The first is a summary of the more important conclusions that have emerged from the extensive experimental work presented in the earlier sections. The second section of this part will be even more speculative, and will consider the relationship of tumor energy metabolism to the fundamental nature of the neoplastic process, with a brief consideration of chemotherapeutic implications.

1. Summary

The most striking property of neoplastic energy metabolism remains the high glycolytic rate of the slice of tumor tissue. The anaerobic glycolysis of the tumor slice is quantitatively great and experimentally more constant than the aerobic rate, while the aerobic glycolysis, though smaller in absolute terms and experimentally more variable, is more impressive when the rarity of significant aerobic glycolysis among normal tissues is considered. Having emphasized the remarkable constancy of the glycolysis of the tumor slice, it is unfortunately necessary to add a number of qualifying remarks that detract considerably from the significance of the finding. The most important qualification is that a high rate of glycolysis is not uniquely restricted to tumor tissue. Many normal adult tissues have a high rate of anaerobic glycolysis, and a few (retina, myeloid bone marrow, renal medulla, and jejunal mucosa) have a significant and sustained aerobic glycolysis as well. In addition, embryonic tissues and certain non-neoplastic lesions of the skin (viral) and lymph nodes appear to display aerobic glycolysis. Furthermore, in tumor tissue the metabolic controls between glycolysis and respiration appear to be functioning in the same manner as in normal tissues. Thus, the influence of the introduction of aerobic conditions on glycolysis (Pasteur effect) is the same in neoplastic as in non-neoplastic tissue. Each mole of oxygen consumed by the tumor slice results in the inhibition of the accumulation of 1–2 moles of lactate, a figure in the same range as that found for normal tissues. It also appears well established, from studies

with extracts and homogenates, that the details of glycolysis (reactions and enzymes), at least as understood today, are the same in tumor as in normal tissue. In this regard, tumor glycolysis is phosphorylating in character.

Another point which must be made is that the constancy of the glycolysis of the tumor slice does not imply that the tumor slice derives most of its energy from fermentation. Even the ascites cell, which displays the most extreme energy metabolism of the type we associate with tumor cells (i.e., high aerobic and anaerobic glycolysis) derives only about one-half of its energy from glycolysis, while for most solid tumors only 15–40% of the total aerobic energy available comes from glycolysis. Furthermore, the prominent Crabtree effect of the tumor slice indicates that, if glycolytic substrate is not available, the tumor slice is able to extend its respiratory rate to produce oxidatively the energy that would ordinarily be produced by glycolysis. Although these many qualifying points in no way negate the uniformity with which a high rate of glycolysis appears in tumor tissue, or the rarity of the finding in normal tissues of the adult, they do lead to caution in accepting the finding as being specific to the tumor cell.

The respiration of the tumor slice is not quantitatively deficient. If one excludes the normal tissues with a particularly high oxidative rate, such as liver and kidney (which for reasons of convenience have been the tissues most extensively studied by the biochemist), the bulk of cellular neoplastic tissues displays an over-all oxidative rate similar to that of normal tissues. However, it appears to be clearly established that the oxidative rate of tumors is fixed, and quite refractory to the stimulation seen when excess carbohydrate or fatty acid substrate is added to most normal tissues. This fixed over-all oxidative rate of neoplastic tissues does not mean that tumors do not oxidize these same substrates—such an interpretation was at one time given to this observation. Studies with radioactive carbohydrate and fatty acid substrates and studies of the levels of the enzymes involved indicate that the oxidative pathways are present and that the added materials are oxidized but, apparently, the added substrate replaces the endogenous substrate and the total oxidative rate remains unchanged. Unlike most normal tissues which, when they display a high rate of glycolysis, also display a respiratory quotient of unity (pure carbohydrate oxidation), neo-

plastic tissues possess an intermediate respiratory quotient, in the 0.75–0.90 range, indicating a mixed fat-carbohydrate respiration. Isotope studies have confirmed this conclusion that fat forms a considerable part of the endogenous substrate for tumor respiration. Thus, neoplastic tissues possess the distinctly unusual combination of a high aerobic and anaerobic glycolysis with an endogenous respiration which is not purely carbohydrate but, on the contrary, is largely dependent on fat for endogenous oxidative substrate. However, when glucose is added to neoplastic tissue, coincident with an inhibition of respiration (Crabtree effect) there is a slight rise in the respiratory quotient of the neoplastic slice and a marked rise in the quotient of the ascites cell,* indicating a shift to carbohydrate respiration. Isotope studies have confirmed the shift from fat to carbohydrate respiration in this situation.

The specific oxidative pathways of the tumor slice, Krebs cycle, hexose monophosphate shunt, and acetate activation, at least as far as they have been investigated, all appear to function actively in tumor tissue, much as they do in their normal counterparts.

The Crabtree effect, together with the Pasteur effect, describes the interesting relationships whereby the energy requirements are apportioned between the oxidative and glycolytic processes. The tumor slice and, particularly, the ascites cell display an active Crabtree effect (inhibition of respiration by glucose addition). Indeed, the Crabtree effect is most impressive in neoplastic tissue with its high aerobic glycolysis, though it is seen in some normal tissues which display active glycolysis.

As would be expected, from the relatively recent development of methods of cell fractionation, the properties of tumor mitochondria have been incompletely studied. Certainly in mouse and rat hepatoma, the neoplastic tissue has a much smaller mitochondria content than its homologous normal counterpart. The mitochondria content of other tumors also appears to be low, but adequate studies on either tumors or normal tissues are at present not available. Of

* A point may be raised with regard to the tendency in recent years of restricting studies on neoplastic tissues to the ascites cell for reasons of its convenience as an experimental tool. The free-living existence of the ascites cell may alter its metabolic characteristics in ways which are not necessarily part of the neoplastic process per se. It would therefore seem wise not to abandon studies with solid tumors.

the oxidative components, cytochrome oxidase and succinoxidase are localized chiefly in the mitochondria, while cytochrome *c* is equally divided between the mitochondria and the soluble part of the cytoplasm. These components are low in tumor, not lower than in the lowest normal tissue but clearly lower than in the majority of non-neoplastic tissues. Of these respiratory components, perhaps cytochrome *c* is disproportionately low in tumor tissue compared with the other respiratory components. However, it should be noted that measurements of respiratory components are measurements of enzyme activity under particular circumstances, and not determinations of the amount of enzyme present in a chemical sense. Essentially, these assays for enzyme activity are based on oxidative response to added substrate under particular conditions and signify something that is already clear, i.e., that the oxidative response of tumor tissue to added substrate is small. Furthermore, direct spectrophotometric measurement of the cytochrome system in intact ascites cells suggests that the absolute amount of these components may not be decreased, at least in this neoplasm.

There are at least two alternate explanations for the fixed respiratory rate of the tumor slice. First, there may be a smaller than normal number of mitochondria present in the neoplastic cell, equipped with a normal complement of respiratory enzymes, which are functioning at their maximal rate on endogenous substrate alone and cannot be further stimulated by the addition of exogenous substrate. In the intact slice, tumor mitochondria may be in the same environment as isolated normal mitochondria in the presence of excess oxidative substrate and phosphate acceptor; i.e., incapable of further increase in respiratory rate. The data on the mitochondrial content and enzymes of rat and mouse liver and hepatoma support the idea that the neoplastic tissue has fewer mitochondria than the normal homologous tissue and that these mitochondria are normal in enzymatic composition (410, 412). Information available about other tumors is insufficient for drawing any conclusion in this regard. The idea of tumor as a tissue having fewer mitochondria than non-neoplastic tissue, which are normal in composition, and are functioning at maximal rate on endogenous substrate alone, is a unifying hypothesis worth further study.

The alternate explanation for the fixed respiratory rate of the tumor cell is that proposed by Chance and Hess (96). On the basis

of spectrophotometric studies, they feel that the respiratory apparatus of the neoplastic cell (mitochondrial content and enzymes) is present in amount similar to that of normal cells, but is under some restraining influence.

There are other features of tumor mitochondria which, though at present only vaguely defined quantitatively and qualitatively, are worthy of note. The requirement for pyridine nucleotide addition for adequate oxidation of many substrates by tumor mitochondria appears well documented, as is the fluoride requirement of certain tumors for adequate oxidative phosphorylation. These requirements, though not as yet well understood, suggest that tumor mitochondria have more exacting requirements as to their intracellular environments for obtaining active oxidative phosphorylation than do the mitochondria of the normal tissues which have been widely studied. To what extent, and in what way, this phenomenon is related to the ATPase and DPNase of the tumor and to the mitochondrial permeability are interesting questions meriting further study. Indeed the physiological significance of these functions in tissues in general requires investigation.

Thus, tumors possess the full complement of glycolytic enzymes as well as a balanced activity of hexokinase and ATPase, and so maintain a high level of steady glycolysis which is coupled to phosphorylation. Neoplastic tissue has a limited respiratory mechanism (mitochondria) which maintains a normal over-all respiratory rate of the tissue and appears to function at a maximal rate on endogenous substrate alone (fat and probably protein being important components of the endogenous metabolism). Added substrate is unable to stimulate respiration beyond its already maximal rate, although a shift in substrate takes place if glucose is added. Tumor mitochondria appear to utilize the same substrates in the same reactions (Krebs' cycle, hexose monophosphate shunt) with the same phosphorylation mechanism as does normal tissue. Furthermore, the interrelations of glycolysis and respiration (the Pasteur and Crabtree effects) appear to be in no way different from those of normal tissues. Because of a high anaerobic glycolysis and a limited oxidative apparatus, a high level of residual glycolysis is found in oxygen. With regard to their energy metabolism, tumors tend to converge, enzymatically, to a common type of tissue (193).

2. Relationship of the Energy Metabolism of Tumors to the Fundamental Nature of the Neoplastic Process

The most important question of the relationship of tumor metabolism (in the restricted sense used in this monograph, of an energy metabolism deriving a disproportionate amount of its high energy phosphate from glycolysis rather than from respiration) to the fundamental nature of the neoplastic process remains to be considered. Clearly, at the present time a definite answer to this question cannot be given, and the following is in large measure the author's personal opinion.

In the light of the existing knowledge of tumor biology, pathology, and biochemistry, it would seem unwarranted to give the tumor energy metabolism the central role in the fundamental nature of the neoplastic process. It seems most reasonable to the present author to consider growth and cell division, whether normal or neoplastic, as similar phenomena. To obtain growth and cell division, more than high energy phosphate and building blocks is necessary in the living organism. Presumably, more than proteins or enzymes are needed, and more than the ribonucleoprotein which is currently believed to be essential for protein synthesis. Cell growth and cell division are functions of the integrated organizational apparatus of the cell, presumably the deoxyribonucleoprotein of the cell nucleus. This organizational apparatus channels building blocks and high energy phosphate through the activity of proteins, enzymes, and ribonucleic acid into the complex phenomenon which constitutes cell division, and it is abnormal cell growth and division that best characterizes the neoplastic process. Thus it is difficult to accept the view that the neoplastic process is in essence tumor energy metabolism. This view, which attributes overwhelming significance to the energy metabolism of cancer, is clearly expressed in Warburg's theory, that damage to the respiratory apparatus of the cell leads to the energy pattern we have been describing and to the unfavorable biological and pathological process known as cancer.

At the opposite extreme is the view that the energy metabolism of tumor is merely a coincidental finding, rather common, to be sure, but in no way related to the fundamental nature of the neoplastic process and in no way related to the survival of the neoplasm.

The present author inclines to a view between the previously mentioned extremes, namely, that the carcinogenic stimulus brings about the nuclear transformation which is the crux of the neoplastic process and that a subsidiary feature of this neoplastic transformation is a particular type of energy metabolism. Clearly, two critical aspects of this formulation are whether the carcinogenic stimulus can give rise to cancer (as defined biologically and pathologically) without the neoplastic energy metabolism and, even more important, whether this particular energy metabolism is essential for the survival of the neoplastic cell. The energy metabolism of tumors remains such a striking metabolic property of tumors that it deserves to be exploited chemotherapeutically in the hope that it may indicate a specific requirement of the neoplastic cell. Certainly, less rational chemotherapeutic attacks have been pursued most vigorously, in contrast with the relative lack of interest in the development of chemotherapeutic agents based on the specific energy metabolism of tumors.

The question of the susceptibility of the tumor cell to chemotherapeutic attack through its energy metabolism is open to two a priori views. It is possible to reason that the tumor cell, with both a glycolytic and an oxidative energy pathway available, is particularly resistant to restriction of its energy supply. The previously reported studies, which indicate that the incorporation of labelled precursors into protein and nucleic acid takes place with either high energy phosphate derived from oxidative phosphorylation or from glycolysis, support this first hypothesis, as do the early studies, which indicate that the tumor slice could survive either aerobically in the absence of glucose or anaerobically when glucose was present. However, this evidence is too indirect to be considered conclusive. A second a priori view, equally tenable at the present time, is that the metabolic pattern of neoplastic tissue may indicate an unusual susceptibility to chemotherapeutic agents. This chemotherapeutic attack could be either through an agent which attacked the tumor cells' respiratory mechanism (assuming, for example, that the very limited respiratory enzymes of the neoplastic cell are just sufficient to maintain it, and agents further damaging these enzymes would lead to death of the neoplastic cell before its normal counterparts) or through an agent which attacked the glycolytic mechanism (perhaps on the assumption that the high rate of glycolysis was uniquely necessary for the neoplastic process).

At the present time, it is not possible to say with any assurance that the cancer cell can or cannot be controlled by a selective chemotherapeutic attack on its energy metabolism. However, in view of the urgency of the problem it is most important to avoid the premature relinquishment of any possibly fruitful therapeutic approach.

Appendix

Chart 1

GLYCOLYSIS

Historical Review (120, 174, 200, 341)

1861—Louis Pasteur recognized the fundamentally anaerobic nature of the alcoholic fermentation of glucose by yeast. "Fermentation is life without air."

1877—Claude Bernard established the importance of the fermentation of glucose to lactate as an energy-yielding process in animal tissues.

1905 to 1908—Harden and Young, studying yeast fermentation:

(1) Showed that inorganic phosphate was required for rapid glycolysis by yeast, and that in the course of alcoholic fermentation this inorganic phosphate was bound in organic form as fructose-1,6-diphosphate (HDP). A 1:1 stoichiometric relationship was found between CO_2 production, alcohol production, and inorganic phosphate taken up.

(2) Established that a heat-stable dialyzable cofactor was present in fresh yeast juice and was essential for alcoholic fermentation. Later studies by Warburg and von Euler showed this cofactor to be DPN.

1914—Embden demonstrated that muscle press juice formed inorganic phosphate on incubation; HDP, unlike many other materials studied, gave rise to inorganic phosphate and lactate when added to muscle juice. Furthermore, HDP could be isolated from fermenting muscle.

1914—Harden and Robison were able to isolate from fermenting yeast (in addition to HDP) a hexose monophosphate. This was later shown to be a mixture of glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P). In 1922 Robison studied G-6-P more carefully and suggested that it was involved in yeast fermentation.

1926—Meyerhof working with muscle extract demonstrated that:

(1) Inorganic phosphate was required for glycolysis.

(2) Glycogen of muscle was converted to lactic acid.

(3) A heat-labile factor was present in yeast maceration juice (in addition to DPN) which, when added to muscle extracts, gave them the ability to attack fermentable hexoses. Factor called "hexokinase."

1927—Embden and Zimmermann isolated, from glycolyzing muscle, hexose monophosphate, which was found to resemble the hexose monophosphate from yeast fermentation.

1927 to 1929—Embden and Parnas isolated adenylic acid from muscle.

1929 to 1931—Lohmann, and Fiske and Subbarow reported that adenylic acid is actually the breakdown product of ATP, the naturally occurring compound which is an essential cofactor for muscle glycolysis.

1930 to 1931—Lundsgaard studied muscle glycolysis with iodoacetate, a compound which blocks lactate formation by muscle. Hexose monophosphate and hexosediphosphate were found to accumulate in iodoacetate-blocked muscle. The role of phosphocreatine and phosphoarginine as energy stores was clarified.

1933—Lohmann studied the reversible conversion of glucose-6-phosphate to fructose-6-phosphate by an enzyme present in muscle and yeast.

1933—Embden discovered that muscle extract acts on 3-phosphoglyceric acid to give pyruvic acid and inorganic phosphate. Later, in 1934, Lohmann and Meyerhof isolated phosphoenolpyruvate as an intermediate in this reaction. Then, in 1935, Meyerhof and Kiessling postulated a second intermediate, 2-phosphoglyceric acid, which they were able to isolate.

1934—Meyerhof and Lohmann demonstrated the conversion of HDP to 2 moles of triose phosphate by an enzyme termed aldolase.

1934 to 1936—Parnas clarified the transfer of inorganic phosphate from phosphoenolpyruvic acid to ADP which was associated with the conversion of phosphoenolpyruvate to pyruvate.

1937—Needham and Pillai found that when triose phosphate was oxidized to phosphoglyceric acid inorganic phosphate disappeared and ATP was formed. Arsenate replaces phosphate but the ester hydrolyzed spontaneously. In 1939 this reaction was further clarified by Negelein and Bromel, and Warburg and Christian as follows:

(1) 3-phosphoglyceric acid + IP + DPN → 1,3-diphosphoglyceric acid + DPNH

(2) 1,3-diphosphoglyceric acid + ADP → 3-phosphoglyceric acid + ATP

1941—Lipmann and Kalckar clarified the significance of high phosphate "energy-rich" and "energy-poor" bonds.

1942—Warburg showed that the inhibition of glycolysis by sodium fluoride involved the enolase reaction with the subsequent accumulation of 2-phosphoglyceric acid.

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