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Comparative oxidative enzyme activity of normal and tumor tissues

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THE COMPARATIVE OXIDATIVE ENZYME ACTIVITY OF NORMAL AND TUMOR TISSUES

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INTRODUCTION

Investigation of the oxidative enzymes of tumor tissues is a comparatively recent development in the field of scientific research, and is the result of the application of the expanding field of enzyme chemistry to the perplexing problems of tumor metabolism. Tumor growth is unique among pathological conditions (Boyd, 1938). It is not contagious, and there is every reason to beleive that it is not due to a specific infectious agent (Voegtlin, 1942). Clinical experience, and animal experimentation have clearly shown that tumors are the result of the transformation of normal body cells into malignant cells. As normal cells undergo transformation, there is an alteration of the normal cellular metabolism (Orr and Stickland, 1941). There may be a change in one or more of the enzymes or enzyme systems of the cell. Normal emzymic catalysis follows definite patterns. We wish to discover whether there is alteration of these patterns in neoplasia.

As early as 1903, Rogers suggested that malignancy was due to a derangement of carbohydrate metabolism. Of all the foodstuffs, it is carbohydrate which is used the most for the energetics of cell activities. Not only does this system provide energy, but pyruvate, an intermediary substance produced during the meta-

bolism of food, is the hub toward which converge carbohydrate, fats, and proteins in their catabolic and anabolic reactions. (Barron, 1943) Carbohydrate metabolism is accomplished by means of a series of oxidation-reduction systems interlinked between the carbohydrate and molecular oxygen. Some of these systems are electroactive, some sluggish, and some enzymatic-sluggish, but almost all depend upon one or more enzymes or coenzymes for the activity of the system. Intensive work has been done on some of these enzymes, and recent investigations of their role in tumor respiration and glycolysis have made them suitable for such a study as this.

In 1923, Warburg announced his epic discovery of a qualitative difference in metabolism between normal and tumor tissues. His results showed,in part, that tumor metabolism was was characterized by a high rate of glycolysis which was only partially supressed by oxygen. He believed that tumor tissue had suffered some damage to respiration, and he inspired a number of studies of individual respiration mechanisms in tumor tissue. These studies, although benefitting by Warburg's great technical contributions, encountered tremendous difficulties in their efforts to obtain valid results. One special difficulty almost halted investigation of tumor enzymes; this was the lack of suitable material. (Voegtlin, 1937)

A new era in tumor study was initiated with the development of inbred strains of rats and mice, in which tumors, either spontaneous or induced, could be transplanted and propagated from one generation to the next. This made available much more tumor tissue than heretofore, of fairly constant character, and frequently composed almost entirely of tumor tissue in contrast to the primary tumor. Several types of tumors have been developed, and of these, the hepatomas, either spontaneous or induced, have proved admirably suited to enzyme investigations. (Greenstein, 1942) Among tissues, the liver is richly endowed with enzymes. It rapidly regenerated when partially resected, thus furnishing a rapidly growing, homologous tissue for comparison with the hepatomas. It is also available in adequate quantities from the fetus. All of these qualities make the hepatoma and its homologues excellent material for investigation.

There are many valuable comparisons to be drawn between tumor materials and adult tissues widely separated from them embryologically. (Burk, 1942) However, suitable criteria for ascertaining significant differences between normal and tumor metabolism are lacking, and until they are developed, the studies of most value will be those which furnish comparisons between tumors and tissues as closely homologous as possible. (Berenblum, Chain and Heatley, 1940) In tumors which develop in laboratory animals, the primary tumor, as well as tumors derived from it, is

available for histologic study to determine the tissue and site of origin. (White, Dalton and Edwards, 1942) Frequently, genetically identical material is available for study as a control tissue, which is, actually, the tissue of origin. (Edwards, Dalton and Andervont, 1942) .

Tumor cells have not infrequently been compared to embryonic cells, primarily because both show rapid growth characteristics. Enzymatic study may further elucidate this comparison. Yet it must be noted that enzymes in the embryo play a double role. Not only do they participate in the elaboration of growth substances and energy production, but these catalysts also cause the differentiation of cells during embryological development (Mathews, 1936). Fetal growth depends upon the appearance of a series of catalysts at particular points in the body of the organism, and at a particular time in relation to development. These catalysts may either accelerate or retard certain chemical processes, and so promote regional differentiation and progressive ontogenetic growth. Thus, enzymes, under the influence of the genes, become the real agents of heredity. This embryologic role of enzymes is suggestive of the existence of an enzyme-altering mechanism which might accidentally be thrown into play to produce neoplasia.

Such a suggestion is pure speculation, as are perhaps

all attempts to reconcile and integrate the diverse factors in the etiology of tumors (see Eggers, 1932). Yet one is tempted to speculate that when the pathological physiology of neoplasia is known, it will be found to be an altered enzyme system (see Potter, 1943). It is not difficult to imagine the alteration of a delicately balanced enzyme system by the assults of radiation (Crabtree, 1932), irritation, and trauma. The metabolized breakdown products of some carcinogenic chemicals have been shown to have an inhibitory action on certain enzymatic reactions (Kensler, 1942; Potter, 1942; Kensler and Rhoads, 1943). And perhaps a breakdown or weakening of the growth-controlling hormone system may allow the escape of cells into neoplasia. However, the fact remains that no enzyme system peculiar to tumor has thus far been demonstrated (Hogeboom and Adams, 1942), nor has any definite and peculiar characteristic of tumor metabolism been proved (Elliott, 1942).

With this pessimistic outlook, we set out to report the alterations of some oxidative enzymes of tumor tissues as compared to their tissues of origin, and from these comparisons we will draw such conclusions as are possible concerning tumor metabolism.

HISTORICAL REVIEW

Van den Corput in 1883 made the suggestion that cancer might be the result of impairment of the oxidative metabolism. In 1897, Buchner published his classical study of alcoholic fermentation by cell-free yeast juice, and thus initiated the modern study of the respiratory enzymes. Rogers, in 1903, suggested that malignancy was due to a derangement of carbohydrate metabolism rather than of the protein metabolism being stressed at that time.

Buxton, in 1903, set out to test systematically the enzymes of tumor tissues. Enzyme activity was ingeniously demonstrated by the preparation of agar plates with indicators and reagents on which were placed small portions of ground, glycerinated tumors. The size of the ring of enzymatic activity afforded a crude estimation of the quantitative result. Of thirty malignant tumors tested, he found that most contained an amylase, and a lipase-like enzyme (butyrase) which converted butyne into butyric acid and glycerine. In a considerable number, proteolytic enzymes were found. In many, there was a milk-coagulating enzyme, and catalase and peroxidase were invariably present.

Harden and MacFadyen were spurred, evidently by Buxton's report, to publish in 1903 a preliminary report of their investi-

gations of tumor enzyme activity. Without giving details of their methods, they reported that they had found in tumor tissues, (chiefly cancers of the breast), "an invertase, a maltase, an amylase, proteolytic enzymes acting in acid and alkaline solutions, traces of a lipase, and possibly a peroxidase." They were unable to detect a lactase.

Buxton and Schaffer made a second report in 1905, enlarging upon the report of Buxton's earlier work. They had made further studies of tumors, and had attempted to study normal and embryonic tissues, but were handicapped by the lack of adequate material. They reported that tumors contained enzymes identical in action with those found in normal adult and embryonic tissues. They noted wide variation in the amount of enzyme activity of various tissues, but this did not seem to be related to the character or malignancy of the tissue. In the course of their investigation, they observed the fact that tumor cells often assume, at least partially, the function of the cells which they replace.

Cramer (1908) studied the gaseous exchange of tumor-bearing rats, and reported that he was unable to detect any essential difference between the tumor-bearing rats and the control rats, either as regards the absolute amounts of oxygen and carbon dioxide involved, or the respiratory quotient.

Chisholm (1910) made similar studies on mice, and came to the same conclusion regarding tumor-bearing mice that Cramer had made on rats.

Blumenthal and Brahn (1910) found less catalase in cancerous livers than normal, especially in the cancerous nodules.

Drew in 1920 studied the power of normal and tumor tissue to reduce a dilute solution of methylene blue. There were large differences in reducing power between the different tissues, and lesser differences between the various tumor tissues, yet all the tumor tissues were much less powerful in reducing methylene blue solution.

Russell and Gye (1920) measured the oxygen consumption of normal and cancerous tissues of the mouse <u>in vitro</u>. They found that most tumor tissues consumed less oxygen than normal mouse tissues, but considerably more than embryonic tissue. They concluded that more oxygen is used by tumors which are more highly differentiated histologically. Russell and Woglom (1920) continued these experiments, and determined in addition the rate of production of carbon dioxide, which allowed them to compute respiratory quotients for normal mouse tissues and tumor tissue.

They drew the tentative conclusion that the more rapidly growing, undifferentiated tumors drew their energy from carbohydrates, while the more slowly growing differentiated tumors consumed fats. They pointed out as the exception to this statement a glycogen-rich, slowly-growing tumor, which gave the highest average respiratory quotient. This seemed to be due to the excessive amount of carbohydrate present.

Braunstein, in 1921, reported that he had autolyzed tumor tissue with sugar solution in an incubator, and found thirty to forty per cent decrease in the sugar content. He ran control experiments on normal muscle, heart, liver, and a non-malignant fibromyoma and observed no change in the sugar content. Mauriac, Bonnard, and Servantie in 1923 described experiments in which they placed tumor and normal tissues in a solution containing sugar and salt for twenty-four hours, and estimated the sugar consumption titrimetrically. They concluded that tumors rich in cells consumed more sugar than those poor in cells, and that tumors in general did not consume more sugar than normal tissues.

In 1923, Professor Otto Warburg and his co-workers first announced their discovery that whereas slices of a number of normal tissues produced lactic acid from glucose or glycogen rapidly in the absence of oxygen, (anaerobic glycolysis), cancer

tissue slides showed an unusual ability to continue production of lactic acid from glucose in the presence of oxygen (aerobic glycolysis). This change occured without the interaction of oxygen, and resulted in the liberation of an appreciable amount of energy. It was therefore probable that this was not a chance property of the cancer cells, but that it had some real significance in relation to the life and growth of the tumor. In his early studies, Warburg happened to study tumors which showed very low oxygen up-take rates; he therefore concluded that in cancer tissue, the respiratory mechanism was impaired, and that glycolysis took its place as a means for producing energy. This gave it the property of growing under conditions where normal cells, not posessing this extra function, would die. Bierich, (Bierich and Rosenbohm, 1924) taking a different view of the process, held that the production of lactic acid enabled tumor tissue to attack and invade normal connective tissue, and that the resulting breakdown products of these tissues supply the material for the growth of the cancer cells. Later, Warburg 1927 observed that the oxygen up-take rate of most tumor tissue under good conditions is not usually lower than that of many normal tissues.

Warburg concluded that since the respiration rate of cancer tissue might be normal, while rapid aerobic glycolysis continued, there must be something wrong with the type of res-

piration in the tumors. Some sort of damage to the respiratory mechanism must have occured in the production of tumor cells which caused a loss of efficiency of respiration in supressing glycolysis. High anaerobic glycolysis appeared to be a general property of growing or multiplying tissues, since it was found in embryonic tissue and testis, but in these normal tissues, glycolysis was largely abolished when respiration occured, that is, in the presence of oxygen (Pasteur effect). Warburg therefore concluded that interference with the respiration of growing cells was, from the standpoint of the physiology of metabolism, the cause of tumors. When the respiration of a growing cell was disturbed, either the cell died, or a tumor cell resulted.

To Warburg goes much of the credit for the development of a systematic attack on the problem of tumor metabolism. He found appropriate experimental methods for his studies, then systematically utilized a variety of tissues, reporting data on many samples of each tissue under different conditions. These self-evident approaches and methods were lacking in most of the previous work in this field. Warburg pioneered the field of tumor metabolism in the measurement of quantities. He developed sensitive monometric methods for the measurement of respiration and glycolysis in small pieces of isolated tissues which are today valuable additions to the technical resources of the cancer

laboratory. He was the first to show a qualitative difference in metabolism between normal and tumor tissues (Orr and Stickland, 1941). Warburg's interpretations and conclusions have been modified by later workers, but his approach to the subject may serve as a model, because of his intelligent use of the best available material, and the employment of appropriate experimental conditions.

Fleish in 1924 showed tumors to be lacking in succinic oxidase.

Voegtlin, Johnson and Dyer (1925) used the Clark series of oxidation-reduction indicators to compare the reducing power of slices of normal rat tissues with those from some well-known transplantable rat tumors. They concluded that these tumors do not exhibit a diminished reducing power.

Bièrich, Rosenbohm and Kalle (1927) found that cytochrome apparently occured in malignant tissues in proportion to cell density.

Lewis and Cossman (1928-1929) found low tumor catalase activity.

Dickens and Simers (1930) advanced the idea that whereas under aerobic conditions, the oxidation of the trioses in normal tissues proceeds by way of pyruvic acid to carbon dioxide and water, the malignant tumors produce lactic instead of pyruvic acid. They also suggested that in place of Warburg's descriptive phrase, "damage to respiration", the expression, "inability (of tumors) to bring about normal oxidation of carbohydrates" be used.

Lewis, Barron and Gardner (1931) found undiminished reducing power in rat tumors and rabbit carcinoma; however, the Rous sarcoma, rabbit myxoma, and some non-malignant growths produced by viruses showed a low reducing power.

Cori and Warburg (Cori, 1931) confirmed Warburg's observation of increased lactic acid production by tumor tissue by demonstrating high aerobic lactic acid production by tumors in situ.

Vietorisz (1932) found rat, mouse and fowl tumors to be deficient in succinoxidase as compared with other tissues.

Barron (1932) found succinoxidase in rat tumors, but not in Rous sarcoma or the infective myxoma of the rabbit.

Elliott and Baker (1935) reported on the influence of oxidation-reduction indicators on the respiration and glycolysis of normal and tumor tissue. They observed that the effect varies with the indicator concentration, high concentrations causing respiratory inhibition, lower concentrations, stimulation. This confirmed results obtained with pyocyanine by Friedheim (1934) and with dinitrocresol by Dodds and Greville (1934).

Voegtlin, Fitch, Kahler, Johnson and Thompson (1935) reported a striking decrease in the pH of the tumor as measured by the capillary glass electrode, when fasting, tumor-bearing animals were given intraperitoneal injections of glucose, fructose and maltose. The drop is due to the production of lactic acid. This indicates that the buffer capacity of these tumors can be overcome by glycolysis, even in vivo.

Boyland and Boyland (1935) discovered that although the Jensen sarcoma contained large amounts of coenzymes I and II, these were rapidly inactivated after excision or destruction of the tissue.

Scharles, Baker and Salter (1935) reported lactic acid production from hexose-diphosphate in extracts of sarcoma 180.

Elliott, Benoy and Baker (1935) found the succinic oxidase system inactive in slices of certain tumors. Euler, Adler and Günther (1937) reported that succinic dehydrogenase in tumor tissue is variable, but on the whole, its activity is less than that of normal tissue. They stated that the amounts of cozymase and flavin enzymes in the Jensen sarcoma are insufficient for full activation.

Banga (1936) found that certain tumors were scarcely able to reduce added oxalacetate; this, and the lack of succinic dehydrogenase, indicated impaired catalysis by the mechanism postulated by Szent-Györgyi (1937).

Elliott and Grieg (1938) described the succinic dehydrogenase and cytochrome oxidase activities of various tissues. They showed that most tumor suspensions tested were low in succinic dehydrogenase and cytochrome cytochrome-oxidase systems.

Masayana and Yokoyama (1939) found the cocarboxylase in rat tumors to be decreased.

Stotz (1939) observed little difference between the cytochrome oxidase activities of rat tumor R-256 and of spontaneous rat tumor, and the cytochrome oxidase activity of normal

tissues that he studied.

Fujita, Hata, Numata and Ajusaka (1939) reported a low tumor cytochrome content. Stotz (1939) and DuBois and Potter (1942) found low cytochrome c content in a number of cancer tissues.

Breusch (1939) found that tumors were low in succinic dehydrogenase activity.

Craig, Bassett and Salter (1941) reported succinoxidase and cytochrome oxidase determinations of slices of homologous normal and tumor tissues. Both groups demonstrated diminished activities of these enzymes in tumor tissues.

Orr and Stickland (1941), by the use of chemically induced liver tumors, showed that liver cells manifested a sudden change in the type of their metabolism when they became neoplastic.

Kensler, Dexter, and Rhoads (1942) reported that induced rat hepatomas were very low in coenzyme I.

Albaum and Potter (1943) found the inhibiting effects of necrotic tissue on succinoxidase and cytochrome oxidase acti-

vities to be marked, but to be absent from healthy tumor tissue.

Schneider and Potter (1943) suggest that the weakest link in the oxidative mechanism of tumor tissue lies somewhere between pyruvic acid and succinic acid. The succinoxidase system is not the limiting factor in the oxidative mechanism of tumors.

To Greenstein and co-workers (1941-1942-1943) we are indebted for extensive research into the enzymes and other components of tumor tissues. From this laboratory has come the most extensive information to date concerning many phases of enzyme activity, not only of tumors, but of tumor-bearing animals and normal control animals.

II TECHNICAL PROBLEMS

Two types of difficulties have plagued those who have studied the relative enzymatic activity of normal and tumor tissues. The first of these is the lack of adequate material suitable for study. Not only has it been difficult to get enough tumor tissue which is in a healthy, growing, and uncontaminated condition, but also there has been a lack of comparable normal control tissues for the tumors studied (Greenstein, Jenrette, Mider and White, 1941). In order to obtain good results, both tumors and control tissues must be found in histological units large enough for relatively pure slices and emulsions to be made. They must be free from extraneous material such as stroma, leukocytic infiltration, and islands of foreign tissue. They should not be damaged by necrosis or post-mortem degeneration.

The transplantation of growing neoplastic tissue in strains of inbred rats and mice now affords an excellent method of obtaining satisfactory material under controlled conditions. The rat strains were developed in Japan, and Iikubo was the first to successfully accomplish transfer of a rat hepatoma (Shear, 1937). The mouse strains were developed in this country, and tumors have been subjected to intensive study, especially by

workers under Voegtlin at the National Institute for Cancer. Because of the two species of animals studied, we are enabled to compare the tissues and tumors by species, as well as by the different strains of animals.

Inbred strains of laboratory animals have been developed by brother-and-sister matings through at least twenty generations. This prolonged inbreeding has produced strains in which all animals may be regarded as identical twins. The establishment and use of such strains, besides making available large amounts of healthy tumor tissue, has markedly reduced the number of variables encountered in the study of experimental cancer (Andervont, 1942).

Each tumor is derived from normal tissues, and the behavior of the tumor is mainly determined by the properties of the tissue of origin(Ewing, 1940). It becomes essential that the histogenesis of the tumor be known, if possible. Unfortunately, some of the most-studied tumors have a doubtful or unknown histogenesis. Biologists have now made available tumors in which the primary tumor was studied clinically, histologically, metabolically, and in situ at post mortem. When this is accomplished by thorough histologic and metabolic studies of the subsequent generations of transplanted tumor, and similar studies

of the tissue from which the tumor originated, we have a very complete picture of that tumor.

The selection of tumors of the liver for experimental study has proved to be a fortunate choice. While primary hepatomas are rare in man, they are not uncommon in some strains of laboratory animals. They not only arise spontaneously, but also can be induced by a variety of carcinogenic chemicals administered at distant sites. As has already been pointed out, liver tissue is plentiful in both adult and fetus, although fetal liver contains large amounts of hematopoetic elements which may alter metabolism. Furthermore, the liver is rich in enzymes, especially those engaged in carbohydrate metabolism.

The second major difficulty of the study lies in the complicated problems which arise in the development of an adequate technique for enzyme investigations. The study of individual enzyme systems necessitates, in many cases, disruption of the cell structure, which, of itself, introduces a number of complications. The living cell is a heterogeneous system in which the various proteins taking part in enzymatic reactions are separated from each other by semi-permeable membranes, and the whole separated from the environment by another semi-permeable membrane (Barron, 1943). This organization is responsible for the stabi-

lity of certain enzyme systems, including that which accomplishes tumor glycolysis. Any destruction of tissue by freezing, grinding, etc., rapidly abolishes tumor glycolysis, apparently by the destruction of coenzymes I and II (Euler and Schlenk, 1938). Activity similar to that of tumors can be restored by addition of considerable quantities of adenylic acid and cozymase (Boyland and Boyland, 1935).

Alterations are not limited to destruction of adenine compounds and nucleotides, but with the archetecture ruined, proteins may be unfolded or precipitated. Some reactions possible only at the surface of the membranes may be diminished, or may disappear altogether. Others, which do not normally take place because of interposition of these membranes or other steric hindrances, will appear, especially those coupled oxidation-reductions which might take place in carbohydrate breakdown. In general, respiratory enzymes are inactivated,while cathepsins become very active when cell structure is disrupted.

The rate of reaction of isolated enzyme systems is extremely high when compared to the rate of respiration in living cells. In the higher animals, these reactions are not only governed by the usual physio-chemical factors controlling the rate of reaction, the distribution of enzymes in heterogeneous systems,

and the interposition of interfaces, but also are controlled by a specific regulating device, the hormonal system. This system appears to exert its influence by enzymatic inhibition, which is regulated by the interaction of the hormones. For example, insulin, which has a marked effect on the blood sugar level and on the combustion of carbohydrate, does not appear in the series of reactions from carbohydrate to molecular oxygen. It may act through modification of the rate at which glucose penetrates the cell, but this is, as yet, unproved. Adrenalin increases the the rate of phosphorolysis of glycogen in muscle, but in the isolated enzyme system, it has no effect (Cori, 1940). The corticosterones of the adrenal cortex, and the hormones of the hypophysis are also parts of the regulating mechanism. Together, they tend to maintain a regulated flow of energy, thus increasing the efficiency of the body chemical activities, but they add to the problems of the investigator. For-example, it has been shown that diabetic tissue in vitro gradually regains its ability to oxidize carbohydrate (Shorr, 1942). A complete restoration takes place in four hours at 41 degrees Centigrade, in ten hours at 37.5 degrees Centigrade. This change, which appears to be a release phenomenon, can be checked by the use of other than inorganic phosphate buffers, one of which is beta-glycerophosphate. As a result of this release, metabolism toward the end of an experimental run may be very different in type from that at the

start of the experiment.

The ionic composition and concentration of the medium in which tissue respires are known to exert profount effects upon the extent of respiration. For example, when 20 micrograms of calcium, as calcium chloride, were added to 20 milligrams of homogenized heart tissue in the presence of 3×10^{-8} mole of cytochrome c, an increase of two hundred per cent in the activity of the succinoxidase activity was observed (Axelrod, 1942). Axelrod believes the presence of a dissociable complex involving calcium is indicated. The addition of aluminum ions overcomes the effect of dilution on tissues (Potter, 1942). Chlorides inhibit the succinoxidase system, so these are omitted from Potter's media. When studying intracellular components, buffers based on extracellular fluids such as Ringer's or serum may not give optimum results.

Many investigations have been made using Warburg's tissue-slice technique. However, where Warburg sliced his tissues by hand with a wet razor blade, investigators now use expensive microtomes. Warburg, (1923) devised a formula based on the laws of diffusion of gases to give the limiting thickness of tissue slices. The general tendency, since then, has been to make the slices as thin as possible. Histologic and metabolic

studies have revealed this to be poor technique. The thicker the slice, the smaller is the ratio of damaged cells to undamaged cells. Thicker slices frequently have a higher rate of respiration than thin ones (Shorr, 1942), and it may be that there are mechanisms for maintaining oxygen pressure other than the gradient st up by the tension in the solution, such as oxygen-carrying, iron-containing compounds of the tissues. However, it is not the unrespiring cells but the damaged cells which are more likely to invalidate results.

At times, muscle fibers can be teased out intact for metabolic studies. This is an excellent technique. The other method is that utilizing minced tissue. Some of the sources of error in this method have been previously mentioned. In addition, in machines such as the Latapie mincer, there is incomplete disintegration of some cells so that the investigators are working with with a mixture of disintegrated and intact cells. The homogenizing apparatus described by Potter (1942) accomplishes a complete and uniform disintegration of the cells. The latter shows a lower rate of respiration indicating the absence of intact cells.

Further complicating factors arise between the time of removal of the tissue and the time the test is run. Lactic acid

accumulates in the tissue. This can be minimized by vigorous oxygenation of the solution containing the tissue until such a time as the test starts. Also, the phosphorylating mechanism is destroyed, and must be replaced before tests can be made.

Investigators are justified in working with broken cell suspensions and cell extracts because it is the only way that intracellular enzymes can be studied. They must be isolated, their equilibrium constants and kinetics determined, their distribution in tissues established, their interactions studied, and the interaction of the intracellular and extracellular fluids determined. But finally, the worker must turn to the intact animal with the task of verifying the fact that these enzymatic reactions <u>do</u> occur in living cells as they do in solutions.

III CARBOHYDRATE OXIDATION

A complete discussion of the oxidation of carbohydrate in tissue is beyond the scope of this paper. However, it does not seem amiss to sketch the primary characteristics of this oxidation, in order to provide a common basis for discussion of enzymatic activity. There are many theories and schemes of carbohydrate oxidation. These schemes are made up of a series of intermediate compounds and may undertake to show the enzyme involved in the specific reaction and type of reaction. Schemes of reactions receive credence when it can be demonstrated that a cell or tissue has the ability to perform certain of those reactions, even though under experimental, and, therefore, unphysiological conditions. This is following Hopkins' (1913) contention that, in general, a tissue is able to deal only with what is customary to it. To go a step farther, we may adduce that the greater the ability of a tissue to perform a reaction, the greater is the probability that that reaction lies on the main pathway of tissue metabolism. It is well to remember that this flimsy formulation is as near as we can come to "proof" of any particular scheme in this very controversial field.

The first phase of carbohydrate metabolism is the conversion of glucose (or glycogen or starch) to pyruvate. This

proceeds through a series of phosphorylations, either with or without the presence of oxygen. This chain of reactions has been unraveled by the combined work of many investigators, especially those in the laboratories of Meyerhof, Embden, Parnas, and Cori. There are eight steps in the cycle from glucose to pyruvate, all of which are reversible but the last, which is the dephosphorylation of phosphopyruvic acid by adenylic acid to pyruvate. The enzymes of this system are specific metallo-(usually magnesium) proteins, (Barron, 1943), and most are combined with adenylic acid or its polyphosphates. This phase involves the anaerobic or fermentative phase of carbohydrate breakdown, and is not our primary concern.

In tissue in which oxygen is present, pyruvate undergoes oxidation. It is, therefore, considered the intermediary which divides the anaerobic phase of carbohydrate breakdown from the aerobic phase. This is a remarkable substance, for no intermediary product of the metabolism of foodstuff posesses the reactivity of pyruvate. Seventeen different pathways of its metabolism are known to exist in living cells (Barron, 1942). In the absence of oxygen, part of the pyruvate formed by the breakdown of carbohydrate is reduced by dihydrophosphopyridine neucleotide to lactate, part to alcohol or alanine, and part may be used in transaminations or dismutations. The interconversion of protein

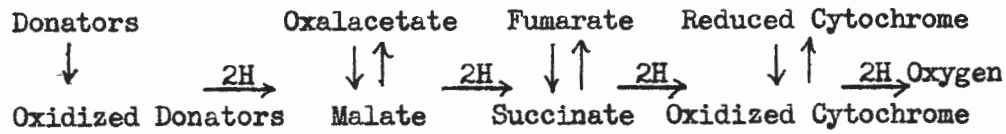
and carbohydrate may occur through the link of pyruvate and the simple amino acids. Fats seem to be related through the aceto-acetic acid link to pyruvate. The synthesis of carbohydrate from fats seems not improbable, since Weil-Malherbe (1938) reported the synthesis of carbohydrate by the kidney incubated with aceto-acetate.

Buchanan, Hastings, and Nesbett (1942), through the use of radioactive carbon, showed that glycogen synthesis in the liver occured by way of pyruvate condensation. While it is not improbable that other mechanisms may exist, it is interesting to note that by this means, the organism circumvents the irreversible dephosphorylation of phosphopyruvic acid to accomplish synthesis of glycogen.

While stressing the importance of pyruvate, we must not fail to notice that oxidative pathways exist which do not include pyruvate. Barron (1943) comments that there is now incontrovertible evidence against the view that carbohydrate utilization starts only after its anaerobic breakdown to pyruvate. Harrison (1932) found an enzyme system for the direct oxidation of glucose in liver tissue. Dickens (1938) reported on the direct oxidation of hexose monophosphate, an intermediate product.

The aerobic phase of carbohydrate metabolism embraces

the series of reactions from pyruvate to molecular oxygen. The first attempt to formulate a scheme of carbohydrate oxidation was drawn up by Thunberg (1920) and Knoop (1923). Toeniessen and Brinkmann (1930) found this scheme unsatisfactory, and offered their modification of the scheme. Szent-Györgyi and his team (1936-1937) offered an entirely new explanation for the presence in muscle of powerful enzymes catalyzing the oxidation of the four-carbon dicarboxylic acids. He suggested that the main function of malate, fumarate, and oxaloacetate in muscle was as catalytic hydrogen carriers between carbohydrate and cytochrome. This is represented by the following highly simplified diagram (after Elliott, 1942) in which heavy arrows indicate transfers of hydrogen atoms (or electrons) from one system to the next:



According to this theory, hydrogen from tissue donators reduces oxalacetate to malate; the malate is reoxidized to oxalacetate, and the hydrogen is transfered to fumarate, which repeats the process. This theory has proved to be very attractive, and has been incorporated in some way into nearly all of the succeeding schemes. Krebs (Krebs and Johnson, 1937) presented the "citric acid cycle", which has been the center of much controversy.

Later, a tricarboxylic acid cycle was presented (Krebs, 1943). No attempt will be made to examine critically these schemes.

According to the Szent-Györgyi theory, pyruvate is oxidized through a series of sluggish oxidation-reduction systems. These are systems depending upon collision, or upon more active intermediary catalysts for electron transfer. Systems of negative oxidation-reduction potential are oxidized here by systems of more positive potential through the mediation of flavoproteins. To this, Krebs' addition of citric acid provided a path for the gradual withdrawl of electrons and of carbon dioxide molecules for the complete breakdown of pyruvate.

From this sketch it will be seen that a series of oxidation-reduction systems, some of them sluggish or enzymatic-sluggish, and some electroactive, are interspersed between the carbohydrate and molecular oxygen, releasing energy not in a rush, but by a smooth and gradual flow of electrons. The pathways which the metabolism can take are numerous, but are designed to meet a variety of conditions within the cell. For example, the fate of pyruvate depends upon the oxygen tension, the concentration of the reacting substances, the concentrations of phosphates and other electrolytes, the prosthetic groups of the activating proteins, and the presence of regulating hormones which control the

speed of reactions, - leaving aside the influence of foreign agents. All these determine the orientation and the rate of metabolism of pyruvate and hence, of carbohydrate.

IV OXIDATIVE ENZYMES

We have selected oxidative enzymes from the literature which have been the object of controlled study. A brief note is made of the composition, if known, the type of reaction of which it is the catalyst, the discoverer of the enzyme, outstanding investigators, and findings as to activity in tumor tissue.

Succinic dehydrogenase is a flavin enzyme, and along with cytochrome oxidase and cytochrome c, catalyzes the succinooxidase system. Succinic acid is oxidized to fumaric acid by the catalytic removal of two atoms of hydrogen from each mole of succinate. The electrons from this hydrogen are transferred to oxidized cytochrome c via succinic dehydrogenase, and from the reduced cytochrome c to molecular oxygen (Schneider and Potter, 1943). Elliott and Grieg, (1937) made an extensive survey of the distribution of the enzyme, which is widely scattered through many tissues. Breusch (1937) and Craig, Bassett and Salter(1941) have reported that its activity in tumor tissues is invariably low. Schneider and Potter (1943), as a result of their investigations, concluded that liver tumors had only about one-fourth the succinic dehydrogenase activity of normal liver.

Cytochrome oxidase has been identified by its spectrum as a hemin-containing compound. It seems to be related to a number of enzymes, none of which have been isolated. It may be a copper protein (Keilin and Mann, 1938). Shack (1943) found the ratio of cytochrome oxidase of normal liver to hepatoma was about four or five; to regenerating liver, one; to fetal liver, two and five tenths, and to liver of tumor bearing animals, one and one tenth. A variety of other tumors posessed low cytochrome oxidase activities of the same order of magnitude as hepatoma.

Cytochrome c appears to be quantitavely the most important of the iron-containing respiration catalysts. It was first isolated by Theorell, in 1936. Theorell postulated thioether bonds between hemin and the amino acids of the protein, but the actual structure is yet to be worked out. DuBois and Potter, in 1942, reported that liver tumors produced by feeding butter yellow contained about one-fifth as much cytochrome c as normal liver.

Catalase is a widely-distributed, iron-containing enzyme, whose function in the cell is not known. It is identified by its rapid action in releasing oxygen from hydrogen peroxide. The prosthetic group of the enzyme is protoferriheme IX (Stern, 1936). Catalase activity in rat hepatomas is tremendously

reduced from that of normal liver. In mouse hepatomas, it is also much less than normal (Greenstein, 1942). Dialysis of the extracts of liver tissues showed absence of a readily dissociable inhibitor (Greenstein, 1943). Activity was shown to vary somewhat with the strain of animals studied. Lymphoma showed half the activity of normal lymph nodes.

Xanthine oxidase , which was isolated in a purified state by Ball, (1939) catalyzes the oxidation of the purines, particularly xanthine, the aldehydes, and diphosphopyridine neucleotide (Ball and Ramsdell, 1939) by oxygen. Its prosthetic group is alloxazine adenine dinucleotide, with perhaps other groups as yet unknown. It was once known as Schardinger's enzyme, and was first identified in milk by Morgan, Stewart and Hopkins, in 1922. Xanthine oxidase showed greatly diminished activity in all hepatomas studied, with one exception, which showed normal activity. This drop may be partially correlated with a lowered flavin content of tumor tissue, (Kahler and Davis, 1940,and Robertson and Kahler, 1942), but the drop is greater than can be accounted for by that reasoning. The activity in spontaneous mammary tumors in mice is greater than that in stilbesterol-induced, hyperplastic breast (Greenstein, Jenrette and White, 1941).

d- Amino acid oxidase is another flavin enzyme. Krebs in 1935 identified both d-and l-amino acid oxidases. Warburg and Christian (1938) isolated the d-amino acid oxidase and found that its prosthetic group was a dinucleotide made up of riboflavin phosphate and adenylic acid. It oxidizes most of the d-amino acids, except d-glutamic acid (Krebs, 1935), splitting them into alpha-keto acids and ammonia. It is highly reactive with molecular oxygen. This enzyme is also reduced in activity in hepatomas, and to a greater extent than xanthine oxidase. Shack (1943) suggested that this may be due to a deficiency in the protein component as well as the flavin prosthetic group. Shack (1943) reported the ratio of d-amino acid oxidase activity of rat liver to hepatoma 31 as about ten, to regenerating liver as one and two-tenths, to fetal liver as seven, and to liver of rats bearing tumor as about one and five-tenths. Neither mouse liver nor mouse hepatomas exhibited measurable d-amino acid oxidase activity.

The comparitive activity of the enzymes is presented in chart form following Greenstein (1943) with additions from subsequent literature. In the chart, the value for the activity of each enzyme in normal adult liver, irrespective of species or strain, is arbitraily set at 100. The corresponding values of the enzymes in other control tissues and hepatomas are then given on the comparitive basis, and will be less or greater than 100, depending on the activity of the enzyme.

RELATIVE ACTIVITY OF OXIDATIVE ENZYMES
IN HEPATOMAS, FETAL AND REGENERATING LIVER

	Rat Hepatomas induced with		Mouse Hepatomas						Fetal Liver		Regenerating
	p-dimethylamino-azobenzene		Aminoazotoluene induced in			Spontaneous		Carbon tetra Chloride induced	Rabbit	Rat	Rat Liver
	Transplanted	Primary	I Mice	C Mice	C3H Mice	C3H Mice	A Mice	A Mice			
Catalase	0.1		18		3	4	11	36	10		100
Xanthine dehydrogenase	50		100		26	28	50	60	0		50
d-Amino acid oxidase	12									12	91
Cytochrome oxidase	32 28	25 33	33		33	33	33	33		33	100
Cytochrome c		22									
Succinic dehydrogenase	24	28		14							

LYMPHOMA AND NORMAL LYMPH NODES	
ENZYME	LYMPHOMA
Catalase	50
Xanthine dehydrogenase	960

MAMMARY TUMORS AND BREAST TISSUE	
ENZYME	SPONTANEOUS MAMMARY TUMORS
Catalase	100
Xanthine dehydrogenase	150
(Control tissue: Hyperplastic breast induced by stibestrol)	

RHABDOMYOSARCOMA	
ENZYME	RHABDOMYOSARCOMA (transplanted tumor)
Succinic oxidase	17

DISCUSSION

It is apparent from the chart that, with the exception of xanthine oxidase, all oxidative enzymes are equal or reduced in amount in tumor tissue. Many show only one-third to one-fourth the activity in tumor tissue as in normal tissue, and in one case, catalase activity is reduced to one-thousandth of its activity in normal tissue. Such low values suggest a greatly decreased activity of the oxidative mechanism. But most malignant tumors show a moderately high respiration rate (Elliott, 1942), an intermediate respiratory quotient (Burk, 1942), and a high sustained aerobic and anaerobic glycolysis. Perhaps the most striking characteristic of tumor metabolism is the accumulation of lactic acid in the presence of oxygen, with the implied defect in the mechanism which controls glycolysis and which gives us the Pasteur effect. The demonstration of the deficiency of succinic dehydrogenase, cytochrome oxidase, and cytochrome c provides a basis for lactate accumulation, since the succinoxidase system is an integral part of the normal oxidative mechanism. A decrease in the activity of this system would necessarily result in a decreased activity of the whole cycle, since the reaction rate of the cycle is governed by the slowest system in the cycle. If the tissue oxidation is restricted, then the tumor would show low respiratory quotients.

Schneider and Potter (1943) found that these values were low, but no lower than some normal tissues such as lung, spleen, and skeletal muscle.

Deutsch and Raper (1938) suggested that certain active tissues such as heart, liver and kidney, possess large amounts of oxidative enzymes to meet the variation in the activity of the tissue in vivo. Tumors, on the other hand, have been considered as having but one function, that of steady, continuous growth. For that reason, tumors have no need for the amounts of oxidative enzymes found in normal tissues. This is in contrast to the surmise by Euler, Malmberg, Günther and Nystrom (1936) that a deficiency in cytochrome oxidase, cytochrome c, and possibly catalase was the cause of the abnormal metabolism in tumor tissue.

Potter (1942) suggested that the growth process outpaces the synthesis of the oxidative enzymes so that the latter become diluted as compared with their concentration in normal tissues. He points out the importance of the dilution effect on diffusible substances such as cytochrome oxidase. These depend both on the density of reactants in the tissue, and upon the concentration of the enzyme itself. Robertson and Kahler (1942) reported a marked decrease of total solids in all tumors studied, with a corresponding increase in the water content of the tumors, which may

throw some light on this theory.

Schneider and Potter (1943) concluded that deficiencies in the components of the succinoxidase system were not sufficient to account for the low respiratory quotient in tumors. Further evidence is furnished by Elliott and Grieg (1937), who found that succinic acid did not accumulate in tumor slices that were oxidizing pyruvates. It seems probable that the weakest link in the oxidative chain lies somewhere between succinic acid and pyruvic acid. Additional investigations are being made of this system, and especially of certain flavin enzymes which may act as intermediary carriers (Bernheim, 1942).

The meaning of the decreased amounts of oxidative enzymes which have not as yet been placed in the respiratory scheme is no longer more clear than those of the succinoxidase enzymes. Respiration through d-amino acid oxidase is quantitatively not very important for either rat liver or hepatoma, representing only about one-thirtieth of the total respiration of the liver. As to its qualitative significance, very little can be said, since its substrates, the d-amino acids, do not appear in the diet. Its absence in mouse liver and hepatoma is puzzling. Catalase presents another problem, since hydrogen peroxide is lacking from many tissues in which this enzyme is found.

The increased activity of xanthine dehydrogenase in some instances, which is contrary to the behavior of the other oxidative enzymes, may in some way be linked to the behavior of the proteolytic enzymes. When a tissue is subjected to autolysis, the respiratory enzymes are soon inactivated, while the hydrolytic agents attacking carbohydrates and proteins continue undiminished in their activity, and may even be activated (Bodanski, 1938). This characteristic is heightened in the necrotic portions of tumors. There is increasing evidence that it is also a characteristic of healthy tumor tissue. Albaum and Potter (1943) found that oxidative enzymes in extracts of healthy tissues were inhibited by extracts of tumor tissues. Maver, Mider, Johnson, and Thompson (1941) found increased catheptic activity in tumor tissue. Euler and Schlenk (1938) found that there was rapid destruction or at least, inactivation, of phosphorylating enzymes in tumor tissue.

This rapid destruction of respiratory enzymes by the proteolytic enzymes in tumor tissue has been ignored by many investigators. For this reason, many of the results, as published, are to be received with much scepticism. For example, Greenstein (1941), one of the foremost workers in the field, has allowed his suspensions of tumor tissue to stand for eighteen hours at five degrees Centigrade to achieve equilibrium. While acti-

vity is certainly low at this temperature, the combined assaults of grinding, chilling, and heightened catheptic activity of tumor tissue may give results far below those of normal tissues subjected to similar assaults. This is a difficult criticism to evaluate, but it is certain that more valid results will be obtained by replacement of the enzymes which can at times be demonstrated in the tissues, but which are lacking, or greatly reduced, in tissue preparations (Boyland and Boyland, 1935 , Schneider and Potter, 1943).

Maver, Mider, Johnson and Thompson (1941) suggest two possibilities to account for the increased activity of proteolytic enzymes. One is that other altered metabolic systems, such as the carbohydrate mechanism, may provide a tissue medium which stimulates more rapid synthesis. The other possibility is that a definite qualitative change occurs in the specific activity of the cathepsin, with the formation of chemically-different proteins.

If there is a real deficiency in oxidative enzymes in tumor tissues, and, as a consequence, there is an increase in glycolysis, with the formation of lactic acid, it still remains to be determined whether this is an inherent characteristic of tumor metabolism, or merely a manifestation of extensive and unusually rapid growth. Burk (1942) suggests that if it could be found that certain growing tissues do not exhibit marked glycolysis,

then it might well be said that the glycolysis of tumors has a more specific and characteristic significance for tumor metabolism. He points out that in neither embryonic liver , regenerating liver, (in which growth may increase fifty to one hundred per cent in twenty-four hours) nor in adult liver, is there a noteworthy glycolysis, in contrast to the various malignant hepatomas. He concludes that the production of lactic acid in hepatic tumors, or tumors generally, is not necessarily an expression of the requirements of growth. He suggests that malignant tumors possess glycolysis not because of growth, but because glycolysis is better correlated with the more primitive organization and lesser differentiation involved.

Recent observations show that the pronounced anaerobic metabolism of malignant growths is not an isolated phenomenon. Many tissues, through environmental conditions such as insufficient circulation, are forced to rely in part on an anaerobic source of energy. A study of the early blastomere reveals that it may be in a state of partial anaerobiosis (Philips, 1941). This condition is probably associated with the rapidly increasing size of the embryo during early periods of growth before the circulatory system can function as an adequate oxygenating mechanism. The relatively poor vascularization of many tumors is evidence that this may apply to malignant growths. After consideration of the evidence, Lipmann

(1942) concludes that the high capacity for anaerobic metabolism in malignant, growing tissues should be attributed to their partly anaerobic state of life rather than to an unlikely special growth function of glycolysis. From this we can infer that lack of development of oxidative enzymes is to be attributed not to any derangement of functioning systems, but simply to a shortage of oxygen.

Many explanations have been offered for phenomena related to the oxidative enzymes of tumors. Only time and continued investigation will show which of the observations and conclusions are correct. For the present, we are like the blind men of India who disagreed as to whether the elephant was snake-like, tree-like, wall-like, or rope-like, and were able to cite "experiments" to prove their contentions. Our conclusions may prove to be as far removed as theirs from the real nature of things, but one thing we know: "There is little doubt that we are faced not with one elephant, but with the jungle itself." (Schneider, 1939).

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