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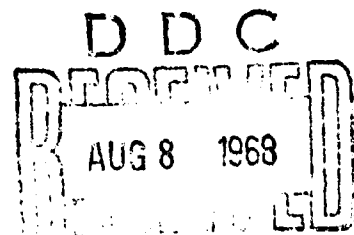
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Fort Detrick, Maryland

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On the distribution patterns of enzymes of the energizing metabolism in the flight muscle, leaping muscle and fatty substance of *Locusta migratoria*, and their cytological coordination.

by A. Delbrueck, E. Zebe and Th. Buecher.

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The majority of known principal chain enzymes in the flight muscles of insects has been demonstrated in the past few years (2, 11-14, 33, 42, 50-52). It may be assumed, therefore, that the energizing metabolism of this highly specialized tissue proceeds in the customary manner. A number of peculiarities were discovered, however: relatively low lactic dehydrogenase and relatively high glycerol-1-P-oxidase (P = esteric phosphate) (10, 53-55, 66, 67, 69). These have occupied us in past studies (8, 9, 67-69). Further attempts to classify and understand these deviations lead almost inescapably to the plotting of enzyme distribution patterns. By this we mean the systematic coordination of the quantitative factors connected with enzymatic activities that are related functionally.

The analytical evaluation of such distribution patterns in the sense of enzymological characterization leads into the realm of comparative method. As phenomena, they may be compared to morphological patterns. Greenstein (27) was the first to describe this aspect in its essential consequences in connection with the problems of malignant growth. We became aware of it during studies of different types of muscle in the migratory locust (Fig. 1). Our example indicates further that the comparative method permits a coordination of enzyme patterns and tissue functions.

The evaluation of enzyme distribution patterns in the cytophysiological sense may be seen as the first step from analysis to reconstruction. The patterns convey a picture, although a somewhat distorted one, of the relations between rates of intracellular reactions. They make it possible to estimate the locations at which defiles and concomitant flow equilibria may be found in the metabolic chain, and the ratios in which the flow of metabolites branches out at the metabolic distribution

points. (*)

Cytological coordination.

The indicated possibilities are available only when the enzyme patterns are based on a realistic basis of cytological coordination. Some of the enzymes relevant to this study are encumbered with doubtful data in the literature (22, 26, 29, 56). We therefore took up methods of cytological fractionation. These investigations will be described first.

Results listed in Fig. 2 demonstrate the method used by us. It is a modification of Hogeboom, Schneider and Pallade's (31) sucrose technique. The same tissue specimen was subjected to six successive extractions. The extraction medium and the mode of mechanical treatment were changed in line with ascending osmotic (16) and mechanical aggressivity.

Only the water-soluble portion of the protein was separated from the various extracts (centrifugation at 100,000 g), while the entire sediment was subjected to renewed treatment and extraction. Action in the first step produces fully functional mitochondria, while treatment in the last step leads to complete liberation of enzymatic activities in mitochondrial solution. Finally, isolated mitochondria were destroyed by the sixth step of extraction for comparison purposes and were tested for specific activity of enzymes studied in the remaining extracts.

It is important that both the absolute and specific activity of enzymes (applied to the extractible total protein) were determined for each extract. We thus start from the premise that the presence of enzymes in different conditions of extractibility is indicated by changes in specific activity upon repeated extraction. The more the intracellular status of two enzymes approaches identity, the more similar will be this activity. It will start at a maximum in the case of enzyme activities in a state of good extractibility. It will fall when the percentual share of extracted protein is dominated by difficultly extractible, and consequently continually extractible, protein fractions.

As reflected in Fig. 2, three different types of enzymes or, more precisely, of enzymatic activities, may be differentiated by their behavior: Type I: Specific activity is highest in the first (most gently liberated) tissue extract and falls rapidly from step to step (for example, glycerol-1-P dehydrogenase). Type II: Activity is minimal in the first extract and rises from fraction to fraction, reaching its peak with the most thorough destruction of cellular components (example: glutamic dehydrogenase). Type III: Activity decreases initially and

(*) In a preceding report (68) we listed a few factors by way of an example using competition between the glycerol-1-P cycle and direct DPNH oxidation, which must be considered in any improved method of reconstruction.

risers subsequently (for example: malic dehydrogenase).

The relations of extractible activities of Type I during the first three extractions indicate conditions that would result upon washing of a matrix saturated with solution, if its capsule had been torn. These enzymes are linked very loosely in the hyaloplasm of the tissue, if not nearly free in solution (C-space)(8). The enzymes of the Embden-Meyerhof route, to the extent tested, belong to this type (Table 1).

The assignment of Type II to the mitochondrial cell space (M-space), i.e., to its water-soluble fraction, follows from the comparison of specific activities of the last extract with those from isolated mitochondria. The higher specific activity of the latter suggest a constant contamination of the extracts from the total homogenate by quantities of relatively poor and, therefore, constant extractibility.

The conditions with respect to enzymes of Type I and II agree with data obtained by our precursors. The important fact is that their differentiability in extraction tests enables us to decide on the status of enzymatic activities of Type III. Apparently these enzymes of Type III are in two distinctly different states of extractibility, and this not only after the dissolution of cellular structures, but even prior to this treatment: If these enzymes possessed uniform, but peculiar properties of solubility which would be partially favored by the first extraction, then their specific activity would increase already in the second step (an identical repetition of the first). On one hand, a large portion of cellular protein (enzymes of Type I) would have been extracted at this time, on the other, repetition of the homogenization process would liberate additional quantities from even larger reserves. If the biphasic behavior is to be explained by adsorption of the original cytoplasmic enzyme on certain structures, an explanation must be offered about the manner in which enzyme is set free during the last steps by purely mechanical means.

These considerations make it very probable that the descending branch of Type III activity indicates a hyaloplasmic enzyme and the ascending branch reflects a mitochondrial one.

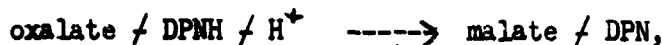
It would be premature to assume an identity of these two enzymes before positive proof can be offered. DeDuve and Berthet (22) have recently presented this aspect in a critique of the differential centrifugation method. The findings described in the following chapter offer an example of its cytological importance.

C-enzymes and M-enzymes. The occurrence of principal chain enzymes for the same reaction in different states of extractibility has been studied in the last few years in the case of various promising enzymes, although not systematically. Hogeboom et al. (31) and Shepherd et al. (58) have investigated this aspect in isocitric dehydrogenase, Christie

et al. (15) in malic dehydrogenase and Dickman et al. (21) as well as Shepherd et al. (59) in aconitase.

The existence of such "Type III" enzymes, which undoubtedly creates special interest, has caused uncertainties (26), since it seems to contradict the essential discovery of cell fractionation, the principle of the unequivocalness of cytological localization of biochemical properties.

When Hogeboom and Schneider (30) first met with a Type III enzyme in isocitric dehydrogenase, they postulated three criteria for unequivocal cytological coordination. The indicated contradiction probably is based on inadequate consideration of these criteria, which have been developed further in the meantime (22, 57). Fig. 3a and 3b show this in tests with malic dehydrogenase. Here we compare the first (hyaloplasmic) and last (mitochondrial) extracts of fractionation done by the method described in the previous chapter. It appears that both extracts catalyze the same reaction:



but differ in the kinetic details.

Since both enzymes are in a clear, aqueous solution, these differences cannot be ascribed to environmental influences, but are based on different properties of enzymatic proteins. It follows that we are dealing not with one, but with two different enzymes. We wish to designate these as C-malic dehydrogenase and M-malic dehydrogenase.

The substratal saturation of oxaloacetate for C-enzyme lies at the tenfold concentration (200 micromoles) of that for M-enzyme (20 micromoles). In addition, an effective inhibition by the native substrate sets in when the optimum is exceeded, as already demonstrated by Davies and Kun (18) in the case of malic dehydrogenase isolated from heart muscle mitochondria.

The Lineweaver-Burk plot of initial rates of M-enzyme from locust mitochondria (Fig. 5) shows the same characteristic picture as the corresponding tests of Davies and Kun (cf. the experimental part). A possible objection blaming the inhibition on substratal contamination is contradicted by the kinetics in Fig. 3b. It is evident that the reaction accelerates steadily with utilization of substrate and reaches a maximum shortly before total uptake of substrate. The difference between the two enzymes is not removed by dialysis of the extracts.

The two enzymatic activities differ also with respect to inhibition by sulfite-DPN adduction, as discovered by Pfeleiderer, Jeckel and Wieland (48) (Fig. 6; cf. experimental part).

Apposition of kinetic action shown in Fig. 3b reveals the difficulties inherent in determinations of activity of both malic dehydrogenases in the same test system, and in a comparative evaluation of results. It is evident that we were able to define Type III in the preceding chapter only because we neglected Hogeboom and Schneider's demand for adequate specific characterization of the biochemical phenomenon. This is valid also for the experiments of our precursors. Dickman and Speyer (21), while pointing to the kinetic differences between cytoplasmic and mitochondrial enzyme in the case of aconitase, did not arrive at the indicated conclusions.

Distribution patterns.

In the plotting of distribution patterns, the extraction method discussed above was usually carried to the second extraction; the extracts were then combined. Our results are given in Fig. 1 and 4 as well as in Table 2. The figures contain conversion values obtained predominantly at pH 7.6, the substratal optimum, and applies to fresh weight (micromoles/g fresh tissue/hours). The scale is logarithmic; relations between the activities of the various enzymes may thus be read from the reciprocal distances on the scale. In addition, the activities of the different enzymes were related to the activity of glyceraldehyde-3-P-dehydrogenase, the hydrogen-supplying enzyme of Embden-Meyerhof's chain (numbers prefixed to the enzyme nomenclature). This is designed to facilitate comparison of different tissues. Arrows stress the relation lactic dehydrogenase/glycerol-1-P-dehydrogenase, which is of particular interest here.

In the distribution pattern of enzymes from the flight muscle, the low activity of lactic dehydrogenase and the high activity of glycerol-1-P-dehydrogenase are especially conspicuous. The dispersion of lactic dehydrogenase is relatively great (Tab. 2). Possible causes are discussed in the descriptive part. In no case did we measure a relation glycerol-1-P-dehydrogenase/lactic dehydrogenase below 50. In some cases lactic dehydrogenase could not be demonstrated, suggesting that indirect flight musculature completely free of foreign tissue may contain no lactic dehydrogenase at all. In comparison to the skeletal muscle of warm-blooded animals, the two dehydrogenases of oxidative carbohydrate degradation and malic dehydrogenase are strongly represented. In spite of these peculiarities, the type of voluntary musculature may be derived from this distribution pattern: The activity of aldolase, enolase, pyruvic kinase and glycerol-1-P-dehydrogenase amounts to more than 1000 enzymatic units (for definition, see experimental part) per gram of fresh tissue and exceeds by 1/10 the activity of glyceraldehyde-3-P-dehydrogenase.

In contrast to the flight muscles, the leaping musculature of the locust contains copious activity of lactic dehydrogenase, while the activity of glycerol-1-P-dehydrogenase is relatively low. The leaping muscle resembles the skeletal muscle of the rat in this respect (20) (Fig. 4), as is true also of the relations of Embden-Meyerhof enzymes as such. Absolute values of enzymatic activities in the insect's muscle are lower by one-half compared to the muscle of the mammal. This finds partial explanation in the higher share of mitochondria in certain areas of the former tissue. Analogous to the flight musculature, including directly and indirectly operating parts, the leaping musculature is similarly inhomogeneous. It encompasses two morphologically and enzymologically different types of muscle which will be discussed elsewhere.

The fatty substance was included in our tests, since its involvement in the energizing metabolism during flight is considerable. This tissue again failed to reveal appreciable activity of lactic dehydrogenase. The activities of glycerol-1-P-dehydrogenase, glyceraldehyde-3-P-dehydrogenase and malic dehydrogenase, on the other hand, retain the same magnitude. In this sense the fatty substance resembles the mammalian liver, to which it has been related at times due to its high content of glycogen and the presence of enzymes typical of the liver, e.g., arginase and a series of transaminases (36), and due to its supplying function as such. The same is true of the extremely low relation of typical Embden-Meyerhof enzymes to the indicated dehydrogenases. On the other hand, the mammalian liver's content of lactic dehydrogenase exceeds that of the fatty substance. It contains the highest relative activity of lactic dehydrogenase of all tissues examined by us to date.

It is significant for the evaluation of the absolute enzymatic activities of the fatty substance that this tissue contains only about one-fifth as much water-soluble protein as the musculature (68).

Discussion.

C-space. The space into which we project our distribution patterns is limited experimentally by the extractibility of enzymes. Within the group, the differences in extractibility are defined by the method's limits of dispersion, while the group as such is differentiated from the action of other enzymes and from other cellular proteins. The facility and completeness with which these enzymatic proteins are obtained after elementary mechanical destruction points to their operation within a space in which structural hindrance (22) is insignificant for the interaction of metabolites between the various enzymes. It seems necessary, therefore, with regard to cytophysiological relations, to define a space in which the catalytic effects of this group operate.

Since this space encloses major portions of the cytoplasm, we have designated it (8) as C-space (cytoplasmic space). The location of its limits within the cell has not been established, nor do we know whether they can be clearly defined topographically. The scarcity of enzymes in the extracellular space (E-space) on one hand and the distinct delimitation from M-enzymes on the other, point to an area within the cell membrane and outside of the mitochondrial double-membrane. Both membranes may be assumed to constitute specific barriers of permeability for certain metabolites, especially for transport metabolites. The question, whether the area on the other side of the nuclear membrane should be excluded, cannot be answered at the present stage of knowledge (cf. 60a and the pertinent discussion). It is not significant for the present study of muscular problems due to the scant involvement of nuclei.

The area defined here "per exclusionem" is nearly identical with the cytological concept of hyaloplasm. It also contains a large number of electron-microscopically demonstrable structures, especially myofilaments, the apparatus of "Z-bands" and an extensive endoplasmic reticulum which was recently shown by Porter and Pallade (48a) in excellent pictures of insect muscles. Whether these structures are merely inserted in the C-space or whether they enclose distinct spaces delimited by permeability barriers, -- this poses an important question. Its treatment will disclose information on the localization of C-enzymes.

The following may be said about the uniformity of tissues studied: The flight muscles consist predominantly of indirectly operating musculature. The involvement of connective tissue and other cell types may be ignored. The leaping musculature is composed of two functionally and morphologically different parts which differ noticeably, although not principally, in their enzymatic distribution patterns. We shall discuss this aspect in a subsequent paper. Part of the leaping musculature contains fatty tissue; in other respects the homogeneity resembles that of the flight muscles. The fatty substance consists essentially of two fundamentally different cell types, the fat cells themselves and so-called enocytes. In the case of migratory locusts, it does not contain bacterial symbions (36).

Comparative enzymology. The comparative biochemistry of the same insect's musculature was started by Gilmour and Calaby (24, 25). They demonstrated several differences between flight and leaping muscles in their supply of proteins with specific operations. In addition to deviations in the properties of water-soluble, Mg-activated apyrases, they investigated the differing action of actomyosines. The general viewpoint from which Gilmour and Calaby evaluated their results, "that these differences... are related in some way to the different physiological roles of the two types of muscle seems obvious..." may similarly be the guiding principle for the discussion of our results. The viewpoint according to which enzymatic organization and structure of a tissue reflect not only general cytophysiological situations, but also

specific performances and conditions of performance, receives relatively clear support from the enzyme distribution patterns described here. We shall try below to replace the "some way" of the Australian authors with concrete speculations pertaining to three enzymes studied by us.

Lactic dehydrogenase. The fact that *Locusta*'s flight muscle contains a low activity of lactic dehydrogenase practically indicates absence of glycolysis in the proper sense, a circumstance that invites general interest, since it represents the first instance of an energy-transforming tissue in which the last metabolic function of the Embden-Meyerhof chain (anaerobiosis and carbohydrate digestion) is performed. In some respects, this offers a subject for comparative studies of malignant tumors.

Davis and Slater (19) as well as Blanchard and Dimulescu (4), in their initial investigations of the lactate level in anaerobiosis of insects, discovered and discussed peculiarities in contrast to the action of vertebrate muscles described by Fletscher and Hopkins (23a). Barron and Tahmisian (2), upon incubation of cockroach leg muscles under exclusion of oxygen, found only a low increase in lactate. Humphrey and Siggins (34) discovered formation of important amounts of pyruvate and relatively little lactate in grasshopper flight muscles. Scant, but distinct amplification of cellular respiration due to pyruvate and other Embden-Meyerhof metabolites shown by Sacktor (52) in tests with homogenates of flight muscles of the fly could not be demonstrated with lactate. Only McGinnis, Cheldelin and Newburgh (42) recently reported on high production of lactate in tests in which homogenates of flies' flight muscles were incubated with pyruvate and fructose-di-P in an atmosphere consisting of CO_2 and N_2 . Oddly enough they did not find an increase in lactic acid when they used extracts of their homogenate, although this was to be expected. In similar studies of the same material, Zebe and McShan (69) found only scant production of lactate, which was steeply increased by addition of LDH. Chefurka (11, 14) worked with a closely related species of fly. He determined that the lactate production of the extract was practically equal to the whole homogenate. His values of lactic acid production and LDH activity are in the same order of magnitude as ours. They do not quite support his assertion of an effective system of glycolysis in the insects examined by him.

It is obvious that anaerobiosis of a tissue is feasible only if the level of end products within the organism is held below the toxic margin by removal, excretion or regeneration. The danger of autointoxication is as great as the potential energy development of an organ and the duration of its work. The flight muscle represents an extreme in both respects. It constitutes one-fifth of the body by weight; its productive respiration amounts to ten to one-hundred times that of the remaining organism. If only a fraction of the converted energy equivalents were supplied by glycolysis, extraordinary quantities of lactic acid would be produced. Inability to fly would be the result within a short period of time.

Consequently there is a characteristic difference between the flight muscle and other voluntary muscles in their oxygen supply. In the vertebrate, this supply is limited by the performance potential of the circulation and the capacity of the intracellular oxygen-storing agent, myoglobin. The latter is absent from the flight muscle. Oxygen is supplied very effectively either directly or nearly so through the system of tracheae and tracheoli which penetrates into the cellular realm to each mitochondrion through ultra-fine branches. It is linked to the flight movements, and there is no flight and no work performance without the inexhaustible presence of air. Oxygen supply through the blood seems to have less significance.

The morphological pattern of the flight muscle cell corresponds to what may be expected of an extremely aerobic tissue. About one-half the cell volume is taken up by mitochondria arranged in columns parallel to the relatively thin myofibrils. The tracheoli which enmesh the mitochondria have already been discussed. Everything has been adjusted to the shortest routes of diffusion.

Metabolite levels during rest and flight (8, 9) indicate that the muscle shows no signs of oxygen depletion, even during prolonged periods of heavy muscular work.

These arguments suggest that the function, structure and metabolic activity of this highly specialized tissue are in agreement with the peculiarities of the enzymatic distribution pattern. Their impact is heightened by a comparison of the flight muscle with the leaping musculature.

The sustained performance of flight is contrasted with the instantaneous action of the leap. The latter requires extreme muscular exertion which may exceed the oxygen supply quite considerably due to its short duration. The presence of lactic dehydrogenase in the enzyme distribution pattern of this organ and its similarity to the mammalian voluntary musculature agrees with the function in this respect. The morphological appearance of a considerable portion of the leaping musculature reveals the dearth of mitochondria known from the study of mammalian muscles, and instead shows thick bundles of fibrils in the cell.

It is noteworthy that there are instances in the insect kingdom of fully glycolysing muscles with a high activity of lactic dehydrogenase. Comparison of different species has shown (69) their presence wherever increased performance under conditions of inadequate oxygen supply is indicated, whereas the leg muscles of slowly running insects, e.g., the kitchen cockroach, reveal relatively low values of lactic dehydrogenase.

Glycerol-1-P-dehydrogenase. During the past few years, glycerol-1-P-dehydrogenase and glycerol-1-P have usually been discussed from the aspect of lipid exchange. In fact, the flight muscles do contain considerable amounts of lipids, especially phosphatides. It is unlikely, however, that these play an essential role in catabolism. Rather, it indicates the high fraction of mitochondria in the composition of the flight muscle.

Additional correlations between lipid exchange and the relatively high activity of glycerol-1-P-dehydrogenase could be derived from the locust's ability to utilize fats as an energy source for muscular work in flight. Krogh and Weis-Fogh (38a) demonstrated the latter in measurements of the respiratory quotient. A short computation shows, however, that only a few percent of oxygen necessary for the combustion of a lipid molecule are reduced via dehydration of glycerol-1-P. In this case, again, a larger involvement of glycerol-1-P-dehydrogenase in energy production is improbable, especially since the flight muscle also contains an extremely active, structurally linked glycerol-1-P-oxidase. Even during protracted flight, there is neither an accumulation of inorganic phosphate nor of glycerol-1-P, nor of dihydroxyacetone-P, as could be expected from combustion of phosphatides for purposes of energy procurement. The remaining possibility involves the participation of glycerol-1-P-dehydrogenase in the synthesis of lipids. But even here its activity seems excessively high, particularly in a functionally and structurally so extensively specialized tissue as the flight muscle. Finally, the assumption that the function of glycerol-1-P-dehydrogenase in total metabolism must be other than participation in synthesis and digestion of fats, is supported by comparative observations: insects that are extensively specialized for the utilization of carbohydrates as energy source for flight show the same extreme values of glycerol-1-P-dehydrogenase in the flight muscles as the locust (53).

In the light of these arguments, a question is raised about the general functions of glycerol-1-P-dehydrogenase in the network of redox systems which carry the energy-supplying metabolism.

The similarity between redox potentials of the systems glycerol-1-P/dihydroxyacetate-P and the system lactate/pyruvate suggests study of these functions in the sense of an acceptor system for hydrogen (8,39,67). In fact, the relation of glycerol-1-P-dehydrogenase to glyceraldehyde-3-P-dehydrogenase with respect to its activity in the flight muscle approaches that of lactic dehydrogenase in other muscles. However, there are important differences between the substrates of the lactate and glycerol-1-P systems. For one, pyruvate and lactate are exchanged with relative ease between the intracellular and extracellular spaces, while the glycerol-1-P system remains restricted to the intracellular space. Secondly, glycerol-1-P has a corresponding reaction site in the respiratory chain, whereas this is not always valid for lactate.

Dihydroxyacetate-P may, to a certain extent, act as hydrogen acceptor in the flight muscle under conditions of anaerobiosis. This mechanism has been demonstrated recently in tests measuring the content of metabolites in the flight muscle under conditions of experimental oxygen depletion (glycerol-1-P-pyruvate dismutation)(8, 39, 68). Its operation is limited, however, by the amount of inorganic phosphate available in the cell. The hydrogen deposited on glycerol-1-P is retained by the cell, since glycerol-1-P does not permeate.

Perhaps there are situations in which the flight muscle assumes a temporary oxygen debt in spite of its extraordinary respiratory capacity, because the energy requirement exceeds the respiratory capacity. One could assume, for example, that the myofibrils of a muscular bundle do not contract at the same time, but alternately (multiple beat) during the rhythmic motions of flight and thus engage the hydrogen buffer rhythmically at minimal intervals during continued cellular respiration.

In our opinion, however, the most important function of the glycerol-1-P system is its dual role of acceptor system for hydrogen in the C-space and donor system for the respiratory chain (M-space) (7, 23, 67). The first report of this series (68) attempted to establish the flow rates in this glycerol-1-P cycle.

Malic dehydrogenase. The same conditions as described in connection with the flight muscle (nearly equal distribution of activity on mitochondria and supernate, as well as masking of mitochondrial enzyme by an accessibility barrier) were discovered by Christie and Judah (15) in studies of rat livers. The resultant differences in the quality of malic dehydrogenase in both fractions remove the last doubts about localization of two malic dehydrogenases in two distinct metabolic spaces. We have demonstrated analogous conditions in tumor cells (20).

Malic dehydrogenase from mitochondria of insect flight muscles has been studied by Sacktor (51, 52). However, the stress of this study rests on factors quite different from ours. Valuable information can be obtained from a comparison of our kinetic investigations of M-enzyme with the corresponding data of Davis and Kun pertaining to purified M-malic dehydrogenase from liver mitochondria. Both enzymes show the same characteristic properties (cf. Fig. 5 and the experimental part). Parenthetically, kinetic analysis of malic dehydrogenase isolated from heart muscle according to Straub and of the commercial preparation of the Boehringer Works identifies them as M-enzyme.

The identity of conditions in phylogenetically quite different tissues indicates that the duplicity of malic dehydrogenase activity is a general cytological principle. The great effectiveness of the apparatus in the flight muscles shows it as an essential property of the energy-supplying metabolism. Its meaning is an important problem, for hardly another metabolic group has as many relations to the network of

metabolism as the system malic oxaloacetate. Its most important compounds lead 1. to the condensing reaction which, in turn, influences the direction and rate of conversion in the acetyl-CoA pool, 2. to carboxylating reactions of pyruvate or phosphoenolpyruvate and 3. to transamination in reciprocal action with the redox system glutamate-alpha-ketoglutarate.

The structural hindrance which prevents the testing of M-enzyme in gently prepared sucrose homogenates is based primarily on the cytoplasmic-mitochondrial barrier to permeation of DPNH discovered by Lehninger (40a). On both sides of this barrier, malic oxaloacetate is related to the DPN system through the C and M-enzyme. We may assume that a uniform DPN pool exists in the C-space, while several of them may coexist in the M-space. The relatively high activity of dehydrogenase suggests that the redox potentials (actual potential, 8) of the malate system and the DPN system are closely allied. Metabolites connected to the malate system through transamination, carboxylation and condensing reaction have multiform relations to the DPN system. However, they are invariably localized on but one side of the barrier: glutamic dehydrogenase and the enzymes of oxidative decarboxylation of alpha-ketoglutarate and pyruvate in the M-space, lactic dehydrogenase in the C-space. Assuming that the cytoplasmic-mitochondrial barrier is sufficiently permeable for malate and oxaloacetate, the indicated relations offer numerous possibilities for coupling of redox systems across the barrier by way of hydrogen transport utilizing these systems.

It is noteworthy, on the other hand, that a relation to the system TPN-TPNH of the C-space is given via the malic enzyme (3a) localized in considerable activity in the C-space of muscles. Detailed discussion of these possibilities would be premature. It is gainful, however, to stress the difference between the system of corresponding malic dehydrogenases and the enzymatic apparatus based on the glycerol-1-P cycle: glycerol-1-P reacts on both sides of the barrier with partners (DPN and flavoprotein) whose normal potential in the opposite direction is quite different from that of the transporting system. The normal potential of DPN in the M and C-spaces, on the other hand, even if modified by specific linkages, is considerably less negative in this sense than the normal potential of the malate system. If malate and oxaloacetate are able to permeate through the cytoplasmic-mitochondrial barrier in the same manner, then a state of adjusting fluctuation is more likely than independent hydrogen transport. We have indicated elsewhere how such a mechanism could affect the stabilization of the oxaloacetate level.

Description of tests.

Preliminary remarks (*). The maintenance of test animals was described briefly in a preceding paper. Adults between the 10th and 30th day after last dehiscence were employed. No consideration was given to sex. If there are sex-specific differences, they do not exceed the limits of standard deviation in Table 2, since male and female individuals were used in nearly equal proportions. The test reflected in Fig. 2 utilized 15 males. In addition to possible sexual differences, the standard deviations which, in some parts, are quite considerable, are due to variable content of moisture and hemolymph, to uncertainties in the unit of measure, and to such technical circumstances as the method's development in the course of experimentation. In addition, special factors must be considered, examples of which are discussed below in connection with the enzyme lactic dehydrogenase.

Enzymes were measured in optical tests according to O. Warburg. Detailed instructions published in a previous paper (3) were followed. The enzymatic unit defined therein for technical reasons corresponds to a conversion of 1.09 micromole/hour, nearly equal to the scale of distribution patterns (micromole/hour).

Fractional extraction of flight muscles (cf. Fig. 2). The grasshoppers were decapitated, the thorax was opened ventrally and the exposed musculature was drawn out after separation of the digestive tract and careful absorption of the fatty substance. The tissue was suspended in a ten-fold amount of 0.25 M sucrose solution adjusted to pH 7.3 with 10 mM triethanolamine buffer. Homogenization was carried out for 10 sec. in a Potter-Elvehjem homogenizer with Teflon template (A.H. Thomas Co., Philadelphia). This was followed by centrifugation at 100,000 g (Spinco centrifuge) for 10 min. The supernate was used for protein analysis and enzyme tests. The sediment was homogenized with fresh sucrose solution (15 sec.) and centrifuged once more. This process was repeated a third time; the three clear supernates contained the major portion of "soluble" cell proteins. Next the sediment was suspended in 0.1 M phosphate buffer at pH 7.3 and homogenized for 1 min. in a glass-in-glass Potter-Elvehjem homogenizer. Centrifugation for 10 min. at 100,000 g produced extract 4. Extract 5 is produced identically by homogenization for 2 min. Finally, upon renewed centrifugation and

(*) Abbreviations: TRAP = triethanolamine buffer produced from triethanolamine-hydrochloride (C.F. Boehringer & Sons, Mannheim) and NaOH, EDTA = triplex III = ethylene-diaminotetraacetic acid-disodium salt (E. Merck, Darmstadt). Phosphate buffers were prepared routinely from secondary sodium salt and primary potassium salt. Oxaloacetic acid, TPN and glucose-6-P-dehydrogenase were supplied by Sigma Chemical Co, St. Louis. The remaining biochemicals came from C.F. Boehringer & Sons, Mannheim. Triose-isomerase was a preparation crystallized from rabbit muscle in our laboratory.

suspension in phosphate buffer, the sediment is treated for 2 min. in an Ultra-Turrax homogenizer (Model TP 18/2, Janke and Kunkel KG. Staufen i. Br.) and again centrifuged. The result is extract 6 and sediment. All operations were conducted at 0°C, occasionally under refrigeration.

The sediment of homogenate extract 1 yields mitochondria by the customary process (centrifugation at 600 g and 10,000 g) whose biochemical (content of cytochrome c and pyridine nucleotides, respiratory activity) and morphological (electron-microscopic appearance) criteria show them to be essentially intact. In some cases these mitochondria were obtained and disintegrated for 2 min. in the Ultra-Turrax homogenizer, as described in connection with extract 6. Enzymatic distribution of these mitochondria, shown in the last column of Fig. 2, corresponds qualitatively to that of the sediment of extract 5; however, the specific activity of enzymes in solution after disintegration is approximately twice as high as that of the homogenate's sediment, suggesting the possibility that disintegration of homogenate sediment opens additional sources of soluble protein, other than those of the mitochondria.

Reversed test for glyceraldehyde-3-P-dehydrogenase (3). Test solution: DPNH 0.15 mM, glycerate-3-P 7 mM, ATP 0.35 mM, Mg^{2+} 3.3 mM, glutathion red 1.2 mM, glycerate-3-P-kinase 200 units/ml = 120 μ /ml, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

Rates of reaction achieved in this test solution were 3 to 4 times higher than in the previous one (3). The concentration of magnesium or its ratio to concentrations of EDTA and substrates is critical, as also pointed out by Trautschold (63). Magnesium must not be excluded, however, since the activation of glycerate-3-P-kinase would otherwise depend on magnesium impurities in the substrates and the reproducibility of the test would be impaired. The latter is true also of the addition of glutathion and EDTA, which prevent inactivation of the enzyme.

If, in addition to glyceraldehyde-3-P-dehydrogenase, the test material contains triose-isomerase and glycerol-1-P-dehydrogenase, it is possible that further reduction of resultant triose simulates exaggerated activity. This source of error, which is easily neutralized by addition of hydrazine sulfate (1.2 mM), has no practical effect on tissues tested by us to date: For one, the activity of glycerol-1-P-dehydrogenase is usually lower than that of the tested enzyme, and secondly, the resultant quantity of triose phosphate is so small that its concentration remains far below the saturation point of interfering enzymes.

The concentration of sulfate should not exceed 5 mM. This must be watched particularly during addition of the coenzyme. Test values obtained by us initially in the above-mentioned reversed test (3) were multiplied by the factor 2.9 which we obtained from locust extracts by comparison of the two tests. The corresponding error for crystallized glyceraldehyde-3-P-dehydrogenase from rabbit muscle is 3.8. The rate of the "reversed" test therefore corresponds to that of the "forward" test with arseniate.

Test for glycerol-1-P-dehydrogenase (1a). Test solution: DPNH 0.15 mM, dihydroxyacetone-P 1 mM, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

We used dihydroxyacetone-P, the preparation of which was described in the preceding report (68). The concentration was adjusted to the substratal optimum. This removes two disadvantages of the previous test (3): competitive inhibition by fructose-1,6-di-P and inadequate saturation of the enzyme. Test values are higher than earlier results by the factor 2.4.

Young and Pace (65) recently discussed the errors inherent in the above test system (due to presence of glyceraldehyde-3-P-dehydrogenase and triose-isomerase) in connection with determination of glycerol-1-P-dehydrogenase in organ extracts. Theoretically, a certain amount of DPN formed in the test is transferred to DPNH through oxidation of glyceraldehyde-3-P, thus masking part of the conversion. This cycle has no practical impact, provided the test system contains no more phosphate than is present in the substrates (as impurities) and in the strongly diluted tissue specimens. In this case the potential concentration of glyceraldehyde-3-P is far below the substratal optimum, and the state of equilibrium does not induce a noticeable reaction, since no phosphate acceptor system is available. In order to avoid the ostensible error, Young and Pace added 5 mM iodoacetate to their tests. This probably is the reason why they were unable to demonstrate the relatively high activity of glycerol-1-P-dehydrogenase in the brain (8, 20).

It must be pointed out that the preceding test is impaired by a non-specific principle of DPNH oxidation in some mammalian tissues, especially those containing copious blood. We shall describe this complication in a forthcoming publication (20).

The pH of our test solution is within the pH optimum of the enzyme. Upon variation of pH by 0.1 units up or down, the test value falls by about 10%.

Malic dehydrogenase. Test solution: DPNH 0.15 mM, oxaloacetate 0.2 mM, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

The pyruvate formed by degradation of oxaloacetate amounts to no more than 2% of the initial admixture at the end of testing, provided the parent solution (10 mM) of oxaloacetic acid (Sigma Chemical Co.) is not neutralized and is less than 2 hours old (0°C).

The concentration of oxaloacetate in this test, based on the principle of Mehler et al. (43), was adjusted to furnish the substratal optimum for C-malic dehydrogenase. M-malic dehydrogenase was thus inhibited by about 30% of optimal activity. At the concentration listed in the original instructions (43), activity is about 30% of optimum.

The measurement of this enzyme's optimal activity with the customary commercial photometers is not feasible due to the extraordinarily low substratal concentration. Above-mentioned relations were measured with the Chance photometer (37).

This technique also produced the data contained in Fig. 5. The Michaelis constants of the curves' principal branches (pH 7.6) amount to 40 micromoles for C-enzyme and 5 micromoles for M-enzyme. The substratal optimum of the latter enzyme is near 20 micromoles. Davies and Kun (18) have measured the following parameters for M-enzyme from rat liver mitochondria at pH 6.7, i.e., in a range about 1 unit away from the pH optimum: $K_M = 18$ micromoles, substratal optimum near 100 micromoles. The kinetic analysis of oxaloacetate inhibition encounters relatively complicated conditions, since the competition between DPNH and DPN-sulfite adductor, together with the inhibition due to oxaloacetate, points to a ternary complex in the active group of the enzyme.

In order to compare inhibitor affinities (Fig. 6) in inhibition due to DPN adductor (hereafter called I), Pfeleiderer's et al. method (48) was used in adding sodium sulfite in concentrations of 10^{-5} to $2 \cdot 10^{-4}$ to the test solution. The enzyme was first incubated for 10 min. with the inhibitor without oxaloacetate; the reaction was then elicited by addition of the substrate. The supernatant of the first sucrose homogenate (C-enzyme) and extract from isolated mitochondria (M-enzyme) were employed.

The DPN content of the utilized DPNH preparation is near 10 micromoles. The dissociation constant of sulfite-DPN adductor

$$D_I = \frac{(DPN)(SO_3^{2-})}{(I)}$$

at the pH of our test solution is near 10^{-2} m. Both malic dehydrogenases are saturated with DPNH under the test conditions. If $\lg V/(V_0 - V)$ is entered against $\lg (SO_3^{2-})$, (V_0 = test value without inhibitor, V = test value with inhibitor), the results (Fig. 6) are located roughly on a line with 45° declension. In the simplest case, this indicates the competition of two substances for the same active group of the enzyme in the sense of Michaelis' theory (cf. 5 and 1):

$$\frac{V}{V_0 - V} = \frac{K^*}{(SO_3^{2-})}$$

In the above relation, K^* is a function involving partly experimental and partly system-specific conditions (*) (p. 293). Since the first factor was constant in all tests, differences in values of K^* would indicate differences in the active groups of C and M-enzyme. First considerations should be given to the relation of affinities of the competing substances DPNH and sulfite-DPN adductor.

Test for lactic dehydrogenase (40). Test solution: DPNH 0.15 mM, pyruvate 2.4 mM, EDTA 5 mM, TRAP 50 mM, pH 7.6, 25°C.

The concentration of pyruvate was lower than in previous tests (3). It corresponds to the substratal optimum for enzymes from rat liver, rabbit muscles and locust muscle. These enzymes are not inhibited by EDTA within the scope of this test.

The relatively wide range of fluctuation in flight muscle LDH may be explained partly by the fact that the leaping muscles and their insertions, relatively rich in LDH, continue into the thorax, which makes it difficult to separate them completely. Perhaps this enzyme is more prevalent in underdeveloped muscles shortly after dehiscence than at the time of most efficient flight. These circumstances require more thorough study. It is known that enzymatically prepared DPNH is important for the reproducibility of lactic dehydrogenase activities (48).

Test for glucose-6-P-dehydrogenase (64). Test solution: TPN 0.15 mM, glucose-6-P 1.8 mM, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

The same activities are tested in glycyl-glycine buffer or upon addition of magnesium. The concentration (38) does not correspond to the substratal optimum which is 1.8 mM for locust enzyme.

(*) We designate the Michaelis constants for DPNH and sulfite-DPN adductor as K_{DPNH} and K_I , respectively. Provided $(DPNH) \gg K_{DPNH}$,

$$\frac{V}{V_0 - V} = \frac{K_{DPNH} (I)}{K_I (DPNH)} .$$

Moreover, since $D_I \gg (SO_3^{2-})$ and $D_I \gg (DPN)$, we can substitute

$$(I) = \frac{D_I}{(SO_3^{2-}) (DPN)}$$

for the concentration of sulfite-DPN adductor. In this manner we get:

$$K^* = \frac{K_{DPNH} D_I}{K_I (DPNH) (DPN)} .$$

Test for isocitric dehydrogenase (46). Test solution: TPN 0.15 mM, D-isocitrate 1.3 mM, Mg^{2+} 12 mM, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

If manganese (46, 60) is substituted for magnesium, activities tested over a very narrow range will be twice as high. Reproducibility is better with magnesium, however, due to the wider maximum, especially if different tissues are compared.

Glutamic dehydrogenase (47). Test solution: DPNH 0.15 mM, alpha-ketoglutarate 3 mM, ammonium acetate 40 mM, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

The ketoglutarate concentration of this batch corresponds to the substratal optimum.

Fructose 1,6-di-P-aldolase (49). Test solution: DPNH 0.15 mM, FDP 4 mM, triose-isomerase 40 units/ml, glycerol-1-P-dehydrogenase 40 units/ml, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

Due to the high activity of triose-isomerase in all tissue extracts tested to date, the solution contains a relatively large amount of crystallized triose-isomerase. The measured units of activity thus refer to conversion of the trioses. They were included in the tables in this form. A number of commercial fructose-diphosphate preparations contain slight impurities of pyruvate which may cause considerable interference in tests with tissue extracts of high lactic dehydrogenase content.

Hexose-P-isomerase (61). Test solution: TPN 0.3 mM, fructose-6-P 0.6 mM, Mg^{2+} 50 mM, glucose-6-P-dehydrogenase 40 units/ml, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

Glucose-6-P-dehydrogenase, which was added as coenzyme, also contains hexose-P-isomerase. For this reason the listed values were determined by differential analysis. No particular care was taken in the development of this test.

Triose-isomerase (44). Test solution: DPNH 0.15 mM, D,L-glycer-aldehyde-3-P 1 mM, glycerol-1-P-dehydrogenase 40 units/ml, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

Pyruvate-kinase (cf. 3).

Enolase (cf. 6).

Gluconate-6-P-dehydrogenase (38). Test solution: TPN 0.15 mM, gluconate-6-P 0.7 mM, Mg^{2+} 10 mM, TRAP 50 mM, EDTA 5 mM, pH 7.6, 25°C.

Glutamate-oxaloacetate-transaminase and glutamate-pyruvate-transaminase (35). Test solution: DPNH 0.15 mM, D,L-aspartate or D,L-alanine 20 mM, alpha-ketoglutarate 10 mM, malic dehydrogenase or lactic dehydrogenase 20 units/ml, phosphate buffer 100 mM, pH 7.4, 25°C.

This solution is suitable for testing of transaminases only if glutamic dehydrogenase activity is low. Tests are triggered by addition of amino acids and the slow initial rate is subtracted from the test results.

Illustrations.

Fig. 1. Enzyme distribution pattern (C-space) in the flight muscle, leaping muscle and fatty substance of *Locusta migratoria*. Optical tests at pH 7.6 in substratal optimum, applied to fresh weight. (Abbreviations in Table 2).

Fig. 2. Fractionated extraction of locust flight muscle. Left scale (dark columns) enzymatic units/g fresh tissue. Right scale (light columns) enzymatic units/mg extract protein.

Fig. 3. Malic dehydrogenases of locust flight muscle. a. Dependence of enzymatic activity upon oxaloacetate concentration. b. Tests with a registering spectrophotometer (Beckman DK 1). DPNH 0.4 mM, triethanolamine buffer 0.05 M, pH 7.6, irradiation 375 millimicrons (not 340 millimicrons, as shown erroneously in the graph).

Fig. 4. Comparison of enzyme distribution patterns of the leaping muscle of *Locusta migratoria* (left) and the hind leg muscle of the rat (right). (Abbreviations in Table 2).

Fig. 5. Lineweaver-Burk plot of malic dehydrogenases. I M-MDH from locust flight muscle (extract from isolated mitochondria). II C-MDH from locust flight muscle (supernate I of the test shown in Fig. 2). III MDH from liver mitochondria after Davies and Kun (18).

Fig. 6. Sulfite inhibition of malic dehydrogenases. Explanation in the text.

Tables.

Table 1. Fractional extraction of C-enzymes from locust flight muscles. (In brackets: control number; P = peristone extraction, R = sucrose extraction). Supernatant enzymatic units per gram of fresh weight. U = supernate.

Table 2. Activities of C-enzymes in locust flight muscle, including abbreviations.