or no effect on the sedimentation behaviour of synovial fluid (Platt, Pigman & Holley, 1959) and the hyaluronic acid complex (Fessler, 1960).

SUMMARY

1. Human synovial fluid was analysed electrophoretically before and after treatment with testicular hyaluronidase.

2. After hyaluronidase treatment, the electrophoretic mobilities of the synovial proteins decreased markedly and were similar to the values obtained for blood serum proteins.

3. The π -component, a complex of hyaluronic acid and albumin, could not be detected after hyaluronidase treatment.

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The Control of some Oxidative Pathways in Guinea-Pig Mammary-Gland Mitochondria

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The control of metabolic pathways can occur on three different time scales. First, the steady-state levels of metabolite concentrations can be changed within seconds by the activation or inhibition of enzymes due to the introduction of metabolites, hormones or other modifiers. These rapid changes can involve the release of preformed enzymes but are not likely to involve synthesis of enzymes. The second time scale covers a long range from minutes to days, which are the periods required for adaptive enzyme formation. In this paper we are concerned with a third and very much longer time scale required for tissue to adapt itself to a special purpose during the evolution of the organism. For the biosynthesis of proteins and fatty acids, the mammary gland is the specialized organ *par excellence*. Turner (1951) has already shown that the tricarboxylic acid cycle functions in mammary tissue. The present work was undertaken as part of a study of the biochemistry of the mammary gland. We are also interested in examining mito-

chondria, from specialized tissue, for quantitative differences in enzyme activities of steps of the citric acid cycle, and of steps involved in the supply and utilization respectively of substrates and products of the cycle. A study of a wide range of oxidative and other metabolic reactions can be shown to reflect the predominantly anabolic activity of the mammary gland.

Many different criteria have been formulated to define intact mitochondria. We have relied upon the evaluation of the P/O ratio (phosphorylation of adenosine diphosphate to adenosine triphosphate/oxygen uptake) in the presence of different substrates and of respiratory control (respiration in the presence and absence of the phosphate acceptor, adenosine diphosphate) (Lardy, 1956) as a measure of the biochemical integrity of our mitochondrial preparations. By far the largest number of investigations on mitochondria has been carried out with preparations from liver. From this source good preparations can be obtained with relative ease by

a variety of methods. The mammary gland, like some other specialized tissues, has, however, presented greater difficulties and the isolation of intact phosphorylating mitochondria from such sources has only been reported recently when Ciaccio & Nelson (1958) and Jones & Gutfreund (1959) were successful with such preparations. Guinea-pig mammary tissue is still the only one which can be homogenized sufficiently gently so that mitochondria are not damaged. The choice of glands from guinea pigs together with some care in the removal of milk constituents, especially of calcium, are the two factors mainly responsible for the success in obtaining satisfactory mammary-gland mitochondria. As will be seen, the yields and reproducibility of mammary-gland mitochondria are still not up to the level now universally achieved from liver preparations, but the results of our studies show that the mitochondria of a tissue with such specialized synthetic activity have features which make them a most useful system for the study of the enzymic control of its anabolic processes.

EXPERIMENTAL

Preparation of mitochondria

Liver mitochondria were prepared from young male guinea pigs by the procedure of Schneider (1948). Mammary-gland mitochondria were prepared from guinea pigs 2-6 days after parturition. The animals were stunned and bled by cutting their throats, and the glands excised. All subsequent operations were carried out at 0-5°. The glands were passed through a Latapie mincer into approximately twice their weight of cold medium (0.25 M-sucrose, 5 mM-ethylenediaminetetra-acetate, adjusted to pH 7.4 with KOH). The mince was squeezed dry in a muslin bag and homogenized with fresh medium in a Potter homogenizer with a nylon pestle to give an approximately 20% homogenate (g. of fresh gland tissue per volume of homogenate). The homogenate was first centrifuged for 5 min. at 600g to remove debris and then for 15 min. at 12 000g to deposit the mitochondria. The mitochondria were resuspended in half the original volume of medium and again centrifuged for 15 min. at 12 000g. All centrifugings were carried out in a refrigerated Spinco preparative ultracentrifuge. The final mitochondrial pellet was again resuspended in the above medium and stored at 2-4°; experiments were carried out within 3 hr. of completing the preparation. Kjeldahl N determinations were carried out in most cases on these mitochondrial suspensions, the N content of the suspension medium was subtracted from the blank. In some experiments only relative rates are recorded; this was done when only small samples were available and we did not reserve any material for N estimations.

Determination of oxygen uptake and phosphorylation

Two methods were used for the measurement of the rate of O₂ uptake by mitochondria, first the conventional Warburg technique and secondly the polarographic technique. Experiments were carried out at 25° or 30°.

The polarographic apparatus consisted of a pair of electrodes, one a gold wire, the other a silver wire, vibrating at 50 cyc.; they were insulated by polythene tubing except at the tips. The gold electrode was put at a negative potential of 0.8 v with respect to the silver electrode and the flow of current was recorded on a polarograph (Evershed and Vignoles Ltd., London, W. 4). Each experiment is self-calibrating on the basis that the O₂ concentration is linearly related to the polarographic current and that the start of the experiment gives the current for a 250 μM-solution of O₂, whereas the final anaerobic solution gives the residual current at zero O₂ concentration.

The electrodes are immersed in 5 ml. cuvettes containing 3 ml. of reaction mixture; the cuvette is immersed in a thermostat. The incubation medium for polarographic measurements (medium A) of the rate of O₂ uptake consisted of a solution: 8 mM-KH₂PO₄, 0.02 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), 8 mM-MgSO₄, 0.1 M-sucrose (pH 7.4); substrates, inhibitors and ADP were added to give the concentrations stated for each experiment; mitochondrial suspensions (0.5 ml.) were added to start the experiments. In experiments proceeding at such a rate that all the O₂ of the solution was used up in about 10 min., no correction was required for diffusion or stirring in of O₂.

For some of the measurements of O₂ uptake and of oxidative phosphorylation the Warburg technique was used with the following medium (medium B): 8 mM-KH₂PO₄, 0.02 M-tris, 8 mM-MgSO₄, 8 mM-glucose, 4 mM-adenosine triphosphate (ATP), 2 mM-substrate, 0.5 mg. of hexokinase (Pabst Laboratories, Milwaukee, Wisc., U.S.A.) (pH 7.4). Consumption of O₂ during the equilibration period of 10 min. was assumed to be equal to that during the first equivalent measured period. Phosphorylation was measured by trapping phosphate as glucose 6-phosphate formed from the generated ATP and glucose in the presence of hexokinase; the disappearance of inorganic phosphate from the medium was determined by application of the method of Fiske & Subbarow (1925) to a sample of the trichloroacetic acid filtrate. The reaction was terminated by tipping 0.3 ml. of 40% (w/v) trichloroacetic acid from the side arm of the Warburg flask.

All the substrates as well as adenosine diphosphate (ADP) and ATP were made up as potassium salts, pH 7.4.

A number of experiments were carried out to test the agreement between measurements of O₂ consumption as recorded by the polarographic technique and by the Warburg method at 25°. Data obtained from succinate oxidation by heart-muscle preparation (Keilin & Hartree, 1940) and from the respiration of liver and mammary-gland mitochondria in the presence of α-oxoglutarate showed that the two methods gave results which agreed within 5%.

RESULTS

Yields and integrity of mammary-gland mitochondria

A portion (60 ml.) of a 20% (w/v) mammary-gland homogenate yielded mitochondria and debris, which were made up to volumes of 9 and 13.5 ml. respectively. From Warburg experiments with samples of the two fractions it was calculated that at 30° the whole of the debris fraction oxidized

140 μ moles of citrate/hr. and 232 μ moles of α -oxoglutarate/hr. The oxygen consumption was 452 μ g.atoms/hr. and 712 μ g.atoms/hr. in the presence of added citrate and α -oxoglutarate respectively. The whole of the mitochondrial fraction with added citrate oxidized 104 μ moles of citrate/hr., with an oxygen uptake of 304 μ g.atoms/hr.; with added α -oxoglutarate it oxidized 164 μ moles of α -oxoglutarate and the oxygen consumption was 392 μ g.atoms/hr. Re-homogenization of the debris yielded more respiratory activity in a second mitochondrial fraction, but this was not carried out as a routine. In a comparison with the yield of guinea-pig-liver mitochondria, it was found that the mitochondria obtained from a gram of liver mince had an oxygen uptake of 96 μ g.atoms/hr. with added α -oxoglutarate, whereas mitochondria from mammary gland consumed 21 μ g.atoms of oxygen/hr. in the presence of α -oxoglutarate.

Fig. 1. Polarographic record of oxygen uptake at 25°. *A*, By guinea-pig mammary-gland mitochondria on addition of glutamate and ADP. Reaction mixture: 1.08 mg. of mitochondrial N in 3 ml. of medium A; 1.7 mm-glutamate; 0.67 mm-ADP. *B*, By guinea-pig-liver mitochondria on addition of α -oxoglutarate and ADP. Reaction mixture: 1.2 mg. of mitochondrial N in 3 ml. of medium A; 1.7 mm- α -oxoglutarate; 0.67 mm-ADP.

Re-extraction of the debris from mammary-gland homogenate yielded an equal amount of activity.

It has been stated that respiratory control and P/O ratios, as defined above, have been taken as criteria for the integrity of our mitochondrial preparations. It would be useless to quote mean values for these ratios since the mammary-gland preparations show considerable variability. We suggest that since we have used only such preparations for critical experiments which gave respiratory control of 3 or more it is sufficient to say that values between 3 and 6 can be, but are not always, obtained. Figs. 1*A* and *B* show the response of good preparations of mammary gland and liver mitochondria to the addition of ADP. Similarly, it is justifiable to quote the highest

Fig. 2. Polarographic record of oxygen uptake at 25° by guinea-pig mammary-gland mitochondria in the presence of malate and ADP and on subsequent addition of malonate and glutamate. Reaction mixture: 0.72 mg. of mitochondrial N in 3 ml. of medium A; 1.7 mm-malate; 3.3 mm-malonate; 1.7 mm-glutamate; 1 mm-ADP.

Fig. 3. Polarographic record of oxygen uptake at 25° by guinea-pig mammary-gland mitochondria (*A*) in the presence of pyruvate, and ADP on subsequent addition of malate, (*B*) in the presence of oxaloacetate and ADP and on subsequent addition of pyruvate, and (*C*) in the presence of oxaloacetate and ADP and on subsequent addition of alanine. The reaction mixtures, which were in each case made up to 3 ml. with medium A, contained 0.67 mm-ADP, and were 1.7 mm, with respect to each of the other substrates added.

values obtainable for P/O ratios from our preparations of mammary-gland mitochondria: on addition of succinate, 1.4; on addition of α -oxoglutarate in the presence of malonate, 3.8; on addition of citrate, 2.5.

Rate of oxidation of citric acid cycle intermediates

The q_{O_2} (μ g.atoms/mg. of mitochondrial nitrogen) values at 25° for both liver and mammary-gland mitochondria in the presence of citrate, α -oxoglutarate, succinate and malate were all indistinguishable and within the range 13–15. In the presence of oxaloacetate, liver mitochondria gave a q_{O_2} of 9, whereas mammary-gland mitochondria produced no significant oxygen uptake (see Fig. 3B). Another important difference between the mitochondria from the two tissues lies in the failure of mammary-gland mitochondria to oxidize malate after the period of equilibration in Warburg experiments. Fig. 2 shows the polarographic record of the oxygen uptake by mammary-gland mitochondria in the presence of malate. When the oxygen concentration of the solution is lowered from 250 to 115 μ M respiration stops. On the assumption that all the oxygen has been used for the conversion of malate into oxaloacetate one can calculate that the respective concentrations are 1.73 and 0.27 mM when the oxidation of malate stops. In the Warburg method malate oxidation will cease during equilibration, before the first reading is taken. No significant uptake of oxygen is observed by either method in the presence of oxaloacetate. Fig. 3A and B give the records of the relative rates of oxygen uptake by mammary-gland mitochondria in the presence of pyruvate alone, oxaloacetate alone, pyruvate plus oxaloacetate and pyruvate plus malate and illustrate that both malate and oxaloacetate oxidation are stimulated by the addition of pyruvate. Fig. 3C illustrates the stimulation of oxaloacetate oxidation by alanine, and Fig. 2 that of malate by glutamate in the presence of malonate, which inhibits glutamate oxidation (Fig. 5B) in mammary-gland mitochondria.

The steady-state rate of respiration in the presence of malate and pyruvate is 1.5 times as fast as in the presence of oxaloacetate and pyruvate. This illustrates a difference in rate control of the citric acid cycle to be discussed below together with the usefulness of mitochondria with a block in oxaloacetate oxidation in the absence of added pyruvate as a tool for the study of a variety of phenomena.

Metabolism of glutamate

An assay method was developed which allows one to determine the rate of reduction of diphosphopyridine nucleotide (DPN) by various dehydro-

genases in the presence of negligible amounts of reduced diphosphopyridine nucleotide (DPNH) (H. Gutfreund & E. A. Jones, in preparation); this is based on the procedure suggested by Ells (1959) and involves the reduction of 2:6-dichlorophenol-indophenol by DPNH in the presence of catalytic concentrations of phenazine methosulphate. We used these conditions for the determination of the activity of glutamic dehydrogenase of mitochondria which had been stored frozen. These preparations did not oxidize α -oxoglutarate and the rate of appearance of DPNH in the presence of glutamate (10 mM), DPN (0.1 mM) and phenazine methosulphate (0.05 mM) could, therefore, be attributed entirely to the reaction:

Preliminary studies indicate that the glutamic dehydrogenase of guinea-pig-liver mitochondria can under these conditions reduce 0.1 m-mole of DPN/hr./mg. of N at 20° in the presence of 10 mM-glutamate. In contrast with this very high activity of glutamic dehydrogenase in liver mitochondria, no activity could be detected in mammary-gland mitochondria.

Two experiments were carried out with guinea-pig-liver mitochondria to gain some information about the inhibition of the glutamic-dehydrogenase reaction by the addition of α -oxoglutarate under the assay conditions described above. With 9 mM-glutamate and 1 mM- α -oxoglutarate the rate of reduction of DPN was lowered by a factor of 3 over the rate obtained in the presence of 10 mM-glutamate alone; with substrate and product present at equal concentrations (5 mM each) no reduction of DPN could be detected.

Figs. 4A and B and 5A and B illustrate the rate of oxygen uptake by liver and mammary-gland mitochondria in the presence of glutamate or α -oxoglutarate and the inhibition of these reactions by malonate. The q_{O_2} at 25° for the two experiments in the presence of glutamate alone is 13 for liver and 12 for mammary gland. The apparent rate of glutamate oxidation for the two different mitochondria is remarkably similar, considering the great difference observed for the activity of glutamic dehydrogenase.

Fig. 5B illustrates the inhibition of glutamate oxidation by malonate and the revival of respiration on addition of malate.

DISCUSSION

The experiments described in this paper were designed to investigate a number of metabolic properties of guinea-pig mammary-gland mitochondria and to compare these with properties of

liver mitochondria. Such differences as have been established can be usefully analysed from the point of view of their contribution to the control of the synthetic activity of such a very highly specialized tissue as the mammary gland.

First of all it has to be established that mammary-gland mitochondria can be prepared which have a sufficient number of critical properties in common with liver mitochondria so that one can be satisfied about their integrity; only then is it justifiable to interpret any differences as due to different properties of specialized intact mitochondria. It is for instance of fundamental importance that experiments on the control of the citric acid cycle by substrates and products are carried out with mitochondria which do not only maintain their *in vivo* complement of citric acid cycle enzymes during isolation but which also preserve the close coupling between the dehydrogenases and electron transport to oxygen and phosphorylation of ADP to ATP. Any disruption of these naturally interlinked processes would change the energy balance and could give misleading results by an alteration of the steady-state control of dehydrogenase reactions.

Respiratory control in living tissue may be defined as the ability to accelerate its rate of respiration during activity and to slow down its rate of respiration at rest when the expended energy has been restored. For isolated mitochondria respiratory control can be studied as illustrated in Fig. 1. The mitochondria show a clear response to the addition of a phosphate acceptor (ADP).

The fact that the q_{O_2} values for liver and mammary-gland mitochondria were approximately the same for most substrates of the citric acid cycle does not mean that the concentrations of the individual enzymes are necessarily the same in the two tissues. This point will have to be investigated separately. It does mean, however, that the rate of control of overall coupled dehydrogenation and electron transport for the DPN-linked steps of the cycle is the same for the two tissues. The two mitochondrial enzyme reactions which have been found to be markedly different in liver and mammary gland (glutamic dehydrogenase and oxaloacetate decarboxylase) are concerned with the supply or disposal of citric acid cycle intermediates. This may well be a more general phenomenon since no marked differences in enzyme activities of the citric acid cycle have been reported, whereas the supply lines to the cycle have been shown to exhibit marked variations; Zebe (1960), for instance, showed that in locust flight-muscle the enzyme reaction involved in the supply of citric acid cycle intermediates from fatty acids can be between 10 and 100 times as fast as in tissues from other animals. The rate of respiration of

Fig. 4. Polarographic record of oxygen uptake at 25° by guinea-pig liver mitochondria (A) in the presence of α -oxoglutarate and on subsequent addition of malonate, and (B) in the presence of glutamate and on subsequent addition of malonate. Each reaction mixture contained, in 3 ml. of medium A, 1.82 mg. of mitochondrial N, and was 1.3 mM with respect to ADP; the other substrates were added to give final concentrations of 1.7 mM.

Fig. 5. Polarographic record of the rate of oxygen uptake at 25° by guinea-pig mammary-gland mitochondria (A) in the presence of α -oxoglutarate and on subsequent addition of malonate, (B) in the presence of glutamate and on subsequent successive addition of malonate and malate. Each reaction mixture contained, in 3 ml. of medium A, 0.72 mg. of mitochondrial N, and was 1 mM with respect to ADP; the substrates were added to give a final concentration of 1.7 mM and malonate to give a final concentration of 3.3 mM.

mammary-gland mitochondria in the presence of malate and pyruvate or of citrate is 1.5 times as fast as in the presence of oxaloacetate and pyruvate. We interpret this difference as an indication that the oxidation of malate or of citrate saturates the capacity of the system to reoxidize reduced DPN, whereas the rate of respiration in the presence of oxaloacetate and pyruvate is determined by the rate of formation of citrate. Krebs & Kornberg (1957) have postulated that the rates of many metabolic sequences are determined by the low intermediate concentrations and not by the enzyme concentrations. This is applicable to the constitutive enzyme reactions of the citric acid cycle itself, but many of the supply lines in specialized tissues are likely to be controlled by enzyme concentrations.

Krebs & Bellamy (1960) have shown that the major pathway for glutamic acid utilization in a number of respiring tissues goes via transamination to oxaloacetate to form α -oxoglutarate and aspartate and concomitant oxidation of α -oxoglutarate to oxaloacetate.

The metabolism of glutamate by mammary-gland mitochondria appears to be straightforward. Respiration proceeds at about the same rate in the presence of glutamate or α -oxoglutarate, whereas the activity of glutamic dehydrogenase is negligible. It is therefore evident that transamination produces α -oxoglutarate from glutamate very rapidly. Since there is a block in the disposal of oxaloacetate, conditions should be favourable for transamination. This sequence of events should be most useful for the synthetic activity of the mammary gland as nitrogen is preserved for anabolic activities instead of being lost via an energy-requiring process.

In liver mitochondria, however, we have shown that the level of enzymic activity for glutamic dehydrogenase under optimum conditions is between 5 and 10 times that required to account for the oxygen consumption on addition of glutamate. The control between transamination and oxidation must rest with the level of α -oxoglutarate concentration and the ratios of DPN/DPNH.

Using the data tabulated by Burton (1957) one can calculate the following equilibrium at pH 7 and 25°:

$$\frac{[\alpha\text{-Oxoglutarate}][\text{NH}_4^+][\text{DPNH}]}{[\text{Glutamate}][\text{DPN}]} = 10^{-6.28} \text{ M.}$$

According to the data for the tissue concentration levels of the components of this reaction system (Krebs & Bellamy, 1960) no direct oxidation of glutamic acid is likely to occur *in vivo* under normal circumstances. In isolated and washed mitochondria a variety of situations can develop which depend on the endogenous concentrations and the

accumulation of the various reactants during subsequent processes. On addition of glutamate to liver mitochondria the action of dehydrogenase and transaminase should rapidly produce enough α -oxoglutarate to inhibit the former enzyme.

The effect of malonate on the oxidation of glutamate by heart-muscle sarcosomes has been studied by Borst & Slater (1960), who find that no respiration occurs if the supply of oxaloacetate is cut off by the inhibition of succinate oxidation. In the presence of glutamate the oxygen uptake by guinea-pig-liver mitochondria was reduced to about a third on addition of malonate, whereas in mammary-gland mitochondria malonate inhibited respiration completely (Fig. 5). This indicates that some direct glutamate oxidation occurs in liver mitochondria. Mammary-gland mitochondria, with their block in malate oxidation due to accumulation of oxaloacetate, enable one to demonstrate the Borst & Slater effect in another way. Fig. 2 gives the record of oxygen uptake during an experiment in which malate is oxidized in the presence of malonate until the accumulation of oxaloacetate inhibits malate oxidation, at which point glutamate is added. Though it has been shown that glutamate is not oxidized in the presence of malonate (Fig. 5B), when oxaloacetate is present oxidation occurs. The lag in reaching optimum respiration rate on addition of glutamate is probably due to the inhibition of both succinate and malate oxidation by oxaloacetate and respiration is stimulated as oxaloacetate is converted into aspartate.

Liver mitochondria could oxidize glutamate directly, but it is not known under which physiological conditions they will do so. Mammary-gland mitochondria, on the other hand, are safeguarded from this wasteful disposal of a useful metabolite. The primitive control of metabolic pathways relying on the concentrations of various reactants for the choice of alternate metabolic routes may be of greater importance in the liver, where both anabolic and catabolic reactions are important and have to be balanced. The mammary gland on the other hand should have adapted its complement of enzymes for maximum efficiency of synthetic pathways.

SUMMARY

1. Methods for the preparation and criteria for the integrity of guinea-pig mammary-gland mitochondria are described, and the oxidation of a number of substrates is compared with that of liver mitochondria.

2. Oxidations of the citric acid cycle proceed at approximately the same rate in liver and mammary-gland mitochondria. However, whereas liver mitochondria respire on addition of oxaloacetate,

mammary-gland mitochondria do not; they are apparently unable to decarboxylate oxaloacetate at a sufficient rate. The glutamic-dehydrogenase activity of mammary-gland mitochondria is also negligible compared with the high activity of liver mitochondria.

3. The control of the metabolism of glutamic acid by liver and mammary-gland mitochondria presents a number of special and interesting features. The mammary gland appears to have control mechanisms which favour its synthetic activity by preserving metabolites and energy. These are discussed in the light of current ideas on the primitive control of metabolism.

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Biosynthesis of C₄ Acids in *Pseudomonas fluorescens* KB1

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In recent years much has been learned about the mechanisms for the biosynthesis of C₄ acids in micro-organisms. The occurrence of fixation of carbon dioxide of the C₃ + C₁ type has been demonstrated in baker's yeast (Davis, Cheldelin, Christensen & Wang, 1956; Stoppani, Conches, de Favelukes & Sacerdote, 1958), *Escherichia coli* (Abelson, Bolton & Aldous, 1952), and others. Fixation of carbon dioxide of the C₁ + C₂ + C₁ type was detected in *Rhodospirillum rubrum* (Cutinelli, Ehrensvar, Reio, Saluste & Sternholm, 1951), *Clostridium kluyveri* (Tomlinson, 1954) and *Penicillium digitatum* (Noble, Reed & Wang, 1958). Recently the operation of the malate-synthetase reaction (Wong & Ajl, 1956), a key step in the glyoxylate cycle, has been reported as playing an important role in acetate-grown *Pseudomonas fluorescens* KB1 and *E. coli* cells (Kornberg & Madsen, 1958). In the former organism, the fixation of carbon dioxide was also considered as an essential process in cellular biosynthesis (Kornberg & Quayle, 1958). More recently, the adaptive formation of both malate synthetase and isocitratase has been

demonstrated by Reeves & Ajl (1960) in *E. coli* induced to utilize acetate as the sole carbon source.

In the present work the nature and the participation of major catabolic pathways functioning in proliferating cells of *P. fluorescens* KB1 were examined by an analysis of the rate and extent of ¹⁴CO₂ production from cells metabolizing specifically ¹⁴C-labelled glucose samples. The fixation of carbon dioxide known to occur in this organism has been identified as the C₃ + C₁ type. The C₃ component in the carbon dioxide-fixation process is believed to be the pyruvate derived from the degradation of glucose via the Entner-Doudoroff pathway, a predominant pathway for glucose catabolism in this organism. An estimation has been made of the relative contributions of fixation of carbon dioxide and of the malate-synthetase reaction towards the overall biosynthesis of C₄ acids in this organism.

EXPERIMENTAL

Culture and incubation procedures. The culture of *P. fluorescens* KB1 was kindly furnished by Dr H. L. Kornberg and has been kept on agar slants and in broth cultures.

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