SYNTHESIS OF CELL CONSTITUENTS FROM C_2-UNITS BY A MODIFIED TRICARBOXYLIC ACID CYCLE

By Dr. H. L. KORNBERG and Prof. H. A. KREBS, F.R.S.

Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

MAJOR advances have recently been made towards the closure of one of the outstanding gaps in the knowledge of intermediary metabolism. This gap concerns the metabolic processes by which two-carbon compounds, such as acetate or ethanol, can be converted to cell constituents in those organisms which can meet all their carbon requirements from these compounds. Bacteria of the genus *Pseudomonas*, many strains of *Escherichia coli* and many moulds belong to this group. The occurrence of a cyclic process, representing a modification of the tricarboxylic acid cycle, has been newly established in these organisms.

Experiments on Acetate-grown Cells of *Pseudomonas*

To find the first, or an early, metabolic product of acetate, acetate labelled with carbon-14 was added to cells of *Pseudomonas KB* 1[1] growing on ammonium acetate as sole carbon source, and the radioactive compounds formed after very short periods of incubation were located by autoradiography and analysed[2]. In the hands of Calvin and his collaborators, this procedure had led to the identification of phosphoglyceric acid as the first organic compound containing fixed carbon dioxide in photosynthesizing cells[3]. However, analogous experiments with acetate on whole cells of *Pseudomonas*[2,4] remained inconclusive. This organism oxidizes acetate by the reactions of the tricarboxylic acid cycle[1,2], and the intermediates of this cycle therefore became rapidly labelled. Even after only 3 sec., citrate, malate, fumarate and succinate, and some amino-acids directly derived from the cycle (glutamate and aspartate), contained radioactive carbon. It did not prove possible in these experiments to limit the radioactivity to virtually one compound, as in the case of photosynthesis. The distribution of the radioactivity among the labelled compounds formed from labelled acetate, and the change of labelling with time, suggested that acetate entered the tricarboxylic acid cycle at two points, to form citrate at one and a C_4-dicarboxylic acid at another[2]. Subsequent work on extracts[5,6] confirmed this conclusion.

Malate Synthetase

A decisive step was the observation made on extracts of *Pseudomonas* that the removal of acetate can be promoted in two ways. The first, established in 1951 by Ochoa, Stern and Schneider[7], consists of the addition of oxaloacetate, in the presence of coenzyme *A* and adenosine triphosphate. The reactions occurring under these conditions are the well-established stages of the tricarboxylic acid cycle leading to citrate:

Acetate + coenzyme *A* + ATP →
acetyl coenzyme *A* (+ AMP + PP)*
Acetyl coenzyme *A* + oxaloacetate →
citrate (+ coenzyme *A*)*

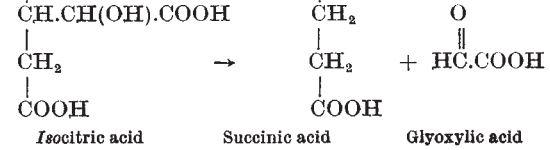
The second way of promoting acetate utilization consists of the addition of glyoxylate, in the presence of coenzyme *A* and adenosine triphosphate. The metabolic process responsible for this removal of acetate proved to be identical with the malate synthetase reaction discovered by Wong and Ajl[8] in extracts of *E. coli*:

Acetate + coenzyme *A* + ATP + glyoxylate →
malate (+ coenzyme *A* + AMP + PP)*

The action of malate synthetase is closely analogous to that of the citrate-forming condensing enzyme[7]. In both cases, the methyl group of acetyl coenzyme *A* condenses with a —CO.COOH grouping.

*Iso*citritase

Pseudomonas was already known to possess an enzyme which can supply glyoxylate. This is the '*iso*citritase' which splits *iso*citrate to glyoxylate and succinate[9–12]:



Isocitric acid Succinic acid Glyoxylic acid

This enzyme, which belongs to the class of aldolases, has also been found in *Penicillium chrysogenum*[13] and *E. coli*[14]. The reaction is reversible. Saz and Hillary[12] suggested that its function might be to provide glyoxylate for the biosynthesis of glycine and, in reverse, to provide a cyclic mechanism for the oxidation of compounds more highly oxidized than acetate, such as glycine and glycollate. It has also been suggested[14] that the glyoxylate may be oxidized to carbon dioxide and water, possibly via formate, and that this therefore represents an alternative mechanism for the complete oxidation of acetate.

The 'Glyoxylate Bypass'

The following results[5,6] show that the glyoxylate, formed by the action of *iso*citritase, can react with acetyl coenzyme *A* to form malate:

(*a*) Cell-free extracts of *Pseudomonas*, in the presence of glutathione, adenosine triphosphate and coenzyme *A*, catalyse the condensation of labelled

* These compounds (in brackets) have not been identified in the present work, but are presumed to arise, by analogy with previous work.

acetate and glyoxylate to form malate as the only labelled compound.

(*b*) When *iso*citrate is used instead of glyoxylate in the above system, malate is again the only labelled compound formed in short-term experiments.

(*c*) Incubation of extracts with *iso*citrate leads to the production of equal amounts of glyoxylate and succinate. In the presence of acetate, coenzyme *A* and adenosine · triphosphate, the glyoxylate disappears and malate is formed. The amounts of *iso*citrate utilized are approximately equal to the amount of succinate, and the amounts of malate + glyoxylate, formed.

(*d*) No malate is formed when boiled cell extracts are used for the above experiments, nor when acetate, coenzyme *A* or adenosine triphosphate is omitted.

It is evident that the combined action of *iso*citritase and malate synthetase can replace the steps of the tricarboxylic acid cycle leading from *iso*citrate to malate. In the tricarboxylic acid cycle, these steps are oxidative:

$$Isocitrate + 1\tfrac{1}{2}\ O_2 \rightarrow malate + 2\ CO_2$$

whereas the combined action of *iso*citritase and malate synthetase is an anaerobic process:

$$Isocitrate + acetate \rightarrow succinate + malate$$

The existence of this 'glyoxylate bypass'[5] implies that there are two variants of the tricarboxylic acid cycle in *Pseudomonas*. One is the well-established terminal pathway of acetate oxidation (Fig. 1). The effect of one turn of this cycle is the complete oxidation of acetate:

$$CH_3COOH + 2\ O_2 \rightarrow 2\ CO_2 + 2\ H_2O$$

The second variant employs most of the reactions of the tricarboxylic acid cycle but substitutes the 'glyoxylate bypass' for the degradative reactions between *iso*citrate and malate. The net effect of one turn of this cycle, henceforth referred to as the 'glyoxylate cycle' (Fig. 2), is the synthesis of one molecule of succinate from two molecules of acetate:

$$2\ CH_3COOH + \tfrac{1}{2}\ O_2 \rightarrow HOOC.CH_2.CH_2.COOH + H_2O$$

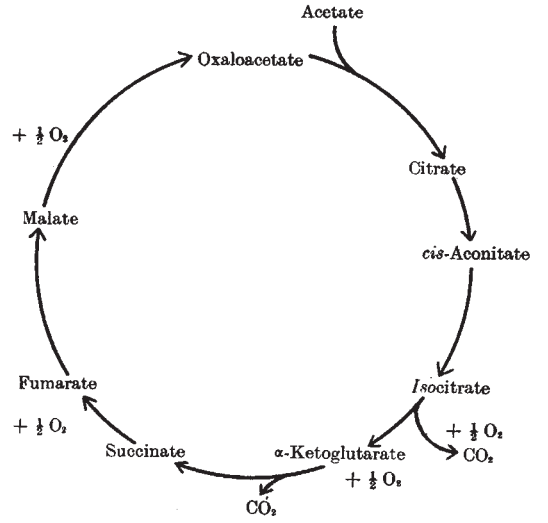


Fig. 1. The main stages of the tricarboxylic acid cycle

Acetate reacts in the form of acetyl coenzyme *A*. The net effect of one turn is: acetate + 2 O_2 → 2 CO_2

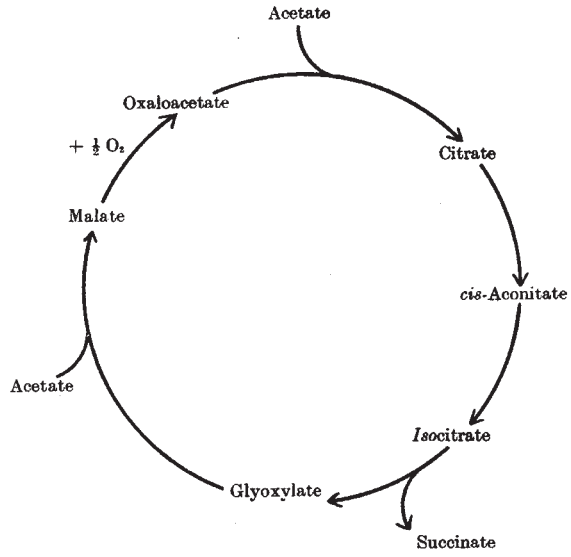


Fig. 2. The main stages of the 'glyoxylate cycle', which is a variant of the tricarboxylic acid cycle

Acetate reacts in the form of acetyl coenzyme *A*. The net effect of one turn of the cycle is 2 acetate + $\frac{1}{2}O_2$ → succinate. If the succinate formed is further metabolized, the cycle, together with the subsequent reactions of succinate, can lead to the synthesis of other metabolites

The occurrence of a reaction leading from two molecules of acetate to one molecule of succinate was first postulated by Thunberg[15] in 1920, and later by Knoop[16]. These authors assumed a rather direct condensation of two molecules of acetate ('Thunberg condensation'); but it is now apparent that the same effect can be achieved by an entirely different mechanism, involving as intermediate stages the reactions of the glyoxylate cycle:

Acetate + oxaloacetate	→ citrate
Citrate	→ *iso*citrate
*Iso*citrate	→ succinate + glyoxylate
Acetate + glyoxylate	→ malate
Malate + $\frac{1}{2}\ O_2$	→ oxaloacetate.
Sum: 2 acetate + $\frac{1}{2}\ O_2$	→ succinate.

Biological Role of the Glyoxylate Cycle

The glyoxylate cycle accounts for the net synthesis of C_4-dicarboxylic acids from acetate when this is the sole source of carbon, and it therefore provides the oxaloacetate required for the continued operation of the tricarboxylic acid cycle. It is also a key step in the synthesis of many cell constituents. Extracts of *Pseudomonas*, in the presence of pyridine nucleotides, can convert malate to phosphopyruvate, which can yield hexoses by the reversal of the reactions of glycolysis[17,18]. Hexoses in turn can supply pentoses through the pentose phosphate cycle, as well as the carbon skeletons of several amino-acids. The intermediates of the tricarboxylic acid cycle together with acetyl coenzyme *A* are already known to supply the carbon skeletons of most amino-acids. Such intermediates, drained from the tricarboxylic acid cycle, can be readily regenerated from acetate by the glyoxylate cycle.

Conversion of Fat to Carbohydrate

In what has already been discussed, it is implied that the glyoxylate cycle could also account for the conversion of fat to carbohydrate, the stages being:

fatty acid
↓
acetyl coenzyme *A*
↓ (glyoxylate cycle)
succinate
↓
oxaloacetate
↓ (Utter–Kurahashi reaction, ref. 18)
phosphopyruvate
↓
carbohydrate

It is uncertain to what extent a net conversion of fat to carbohydrate takes place in higher animals, but this conversion is known to occur in seedlings rich in oil, such as those of the castor bean (*Ricinus*). Cell-free extracts of castor bean seedlings contain *iso*citritase and malate synthetase, and are able to catalyse the formation of malate from acetate and *iso*citrate via the glyoxylate bypass[19]. In conjunction with the reactions whereby fatty acids give rise to acetyl coenzyme *A*, and those whereby malate produces phosphopyruvate[18], the bypass thus provides a route for the net conversion of fat to carbohydrate.

Tissues of higher animals are known to be capable of incorporating labelled acetate (and therefore the carbons of fatty acids) into carbohydrates; but this does not imply a net conversion of fat to carbohydrate. When labelled acetate enters the tricarboxylic acid cycle, the citrate formed is labelled in such a manner as to lead, on completion of one turn of the cycle, to the formation of C_4-dicarboxylic acids still retaining all the isotope of the added acetate: the two molecules of carbon dioxide evolved are not directly derived from the added acetate. Hence, although the entry of two carbon atoms (as acetate) has been followed by the liberation of two carbon atoms (as carbon dioxide), and no net synthesis has occurred, the C_4-dicarboxylic acids of the tricarboxylic acid cycle have nevertheless become labelled. Since these acids can readily give rise to phosphopyruvate[17], isotope will also appear in carbohydrates, but will not be accompanied by any net formation of carbohydrate. The glyoxylate cycle, by supplying extra C_4-dicarboxylic acids from acetate, could, however, account for a net synthesis of carbohydrate from acetate and hence from fat. It remains to be investigated whether higher animals can under special conditions—such as when fat is the main source of energy—convert fat into carbohydrate, and whether this involves the glyoxylate cycle.

Synthesis of Citric and Fumaric Acid in Moulds

The conversion of sugars to citric acid by *Aspergillus niger* is taken to involve a fission of C_6-sugars to triose phosphates, oxidation to pyruvate, the fixation of carbon dioxide to form oxaloacetate, and a condensation of this with a molecule of acetyl coenzyme *A* derived from another molecule of pyruvate (see Chain[20]).

This pathway cannot account for the synthesis of citric acid from acetate or from other two-carbon compounds. The occurrence of *iso*citritase in at least one mould[13] suggests that the accumulation of citric acid involves the glyoxylate cycle, the intermediate stages being:

Acetate + *iso*citrate	→ succinate + malate
Malate + $\frac{1}{2}$ O_2	→ oxaloacetate
Succinate + O_2	→ oxaloacetate
2 Acetate + 2 oxaloacetate	→ 2 citrate
Citrate	→ *iso*citrate
Sum: 3 Acetate + $1\frac{1}{2}$ O_2	→ citrate

Citrate, besides being the end-product of acetate utilization under these conditions, also acts in the manner of a catalyst, in that it initiates the sequence of reactions and is regenerated by them.

Foster and his co-workers[21,22] concluded from isotopic data that the synthesis of fumaric acid from acetate or ethanol in the mould *Rhizopus nigricans* involved a 'dicarboxylic acid cycle', in which the two methyl groups of acetate or ethanol, via the Thunberg condensation, give rise to the two methylene carbons of the C_4-compound. The experimental results are, however, in even better agreement with the assumption that fumarate is formed by the reactions of the glyoxylate cycle, the steps being:

*Iso*citrate	→ succinate + glyoxylate
Acetate + glyoxylate	→ malate
Malate + $\frac{1}{2}$ O_2	→ oxaloacetate
Acetate + oxaloacetate	→ citrate
Citrate	→ *iso*citrate
Succinate + $\frac{1}{2}$ O_2	→ fumarate
Sum: 2 Acetate + O_2	→ fumarate

The accumulation of fumarate in this organism could be ascribed to the absence or low activity of fumarase. As in the case of the citric acid-forming moulds, citrate and *iso*citrate act like catalysts.

Conclusion

Recent work, carried out in several laboratories, has established the occurrence of a metabolic cycle in micro-organisms which can derive all their carbon requirements from two-carbon compounds. The cycle (see Fig. 2) represents a variant of the tricarboxylic acid cycle. The stages between *iso*citrate and malate are replaced by reactions in which glyoxylate is a key metabolite. The cycle is therefore referred to as the 'glyoxylate cycle'.

The main discoveries which led to the elaboration of the cycle were: (1) the finding that *iso*citrate, apart from undergoing dehydrogenation, is split enzymically to form succinate and glyoxylate[9–14,23]; (2) the recognition by Wong and Ajl[8] of an enzyme system bringing about the synthesis of malate from glyoxylate and acetyl coenzyme *A*; (3) the demonstration of the ready occurrence of the combined action of the two enzyme systems in cell-free extracts[5,6,19].

The overall effect of one turn of the glyoxylate cycle is the formation, from two molecules of acetate, of one molecule of C_4-dicarboxylic acid. This, together with acetate, can serve as a precursor of many cell constituents. The cycle is therefore a stage in the

synthesis of cell material from acetate. It can also account for the net formation from acetate of citric, fumaric and other organic acids in moulds[20].

The key reactions of the glyoxylate cycle have further been demonstrated in *Ricinus* seedlings[19]. In these seedlings, it can account for the conversion of fat to carbohydrate.

[1] Kogut, M., and Podoski, E. P., *Biochem. J.*, **55**, 800 (1953).
[2] Kornberg, H. L., *Biochem. J.* (in the press).
[3] Calvin, M., "The Harvey Lectures", **46**, 218 (1950–1951). (C. C. Thomas Publ. Co., Springfield, Ill.)
[4] Kornberg, H. L., *Biochim. Biophys. Acta*, **22**, 208 (1956).
[5] Kornberg, H. L., and Madsen, N. B., *Biochim. Biophys. Acta* (in the press).
[6] Kornberg, H. L., and Madsen, N. B., *Biochem. J.* (in the press).
[7] Ochoa, S., Stern, J. R., and Schneider, M. C., *J. Biol. Chem.*, **193**, 691 (1951).
[8] Wong, D. T. O., and Ajl, S. J., *J. Amer. Chem. Soc.*, **78**, 3230 (1956).
[9] Saz, H. J., *Biochem. J.*, **58**, xx (1954).
[10] Smith, R. A., and Gunsalus, I. C., *J. Amer. Chem. Soc.*, **76**, 5002 (1954).
[11] Smith, R. A., and Gunsalus, I. C., *Nature*, **175**, 774 (1955).
[12] Saz, H. J., and Hillary, E. P., *Biochem. J.*, **62**, 563 (1956).
[13] Olson, J. A., *Nature*, **174**, 695 (1954).
[14] Wong, D. T. O., and Ajl, S. J., *Nature*, **176**, 970 (1955).
[15] Thunberg, T., *Skand. Arch. Physiol.*, **40**, 1 (1920).
[16] Knoop, F., *Klin. Wochschr.*, **2**, 60 (1923).
[17] Krebs, H. A., *Bull. Johns Hopkins Hosp.*, **95**, 19 (1954). Bartley, W., *Biochem. J.*, **56**, 387 (1954). Bartley, W., and Avi-Dor, Y., *Biochem. J.*, **59**, 194 (1955).
[18] Utter, M. F., and Kurahashi, K., *J. Biol. Chem.*, **188**, 847 (1953).
[19] Kornberg, H. L., and Beevers, H., *Nature* (in the press).
[20] Chain, E. B., Proc. 3rd Int. Congr. of Biochemistry, Brussels 1955, p. 523 (Academic Press, Inc., New York, 1956).
[21] Foster, J. W., Carson, S. F., Anthony, D. S., Davis, J. B., Jefferson, W. E., and Long, M. V., *Proc. U.S. Nat. Acad. Sci.*, **35**, 663 (1949).
[22] Foster, J. W., and Carson, S. F., *Proc. U.S. Nat. Acad. Sci.*, **36**, 219 (1950).
[23] Campbell, J. J. R., Smith, R. A., and Eagles, B. A., *Biochim. Biophys. Acta*, **11**, 544 (1953).

RADIOBIOLOGY AND CANCER

By Dr. L. H. GRAY

Director of the Research Unit in Radiobiology, Mount Vernon Hospital

THE opening of the British Empire Cancer Campaign Research Unit in Radiobiology by His Grace the Duke of Devonshire on May 20 marks a new phase in the research into the nature and treatment of cancer which the Cancer Campaign has supported at the Mount Vernon Hospital for more than twenty-five years. It is also an expression of the belief that the study of fundamental problems in radiobiology by a group of scientists can make a significant contribution to our understanding of cancer, if the group is consciously working to this end and is located at a hospital in close association with medical and scientific colleagues whose efforts are similarly directed. Experimental research interests at the Mount Vernon Hospital include the etiology of cancer, chemical carcinogenesis, chemotherapy and radiobiology. In close association with those conducting clinical research in radiotherapy, a group is studying the cytological changes induced in human tumours by a course of X-ray treatment. The activities of all the groups are co-ordinated and controlled through the Cancer Research Committee of the Hospital.

Radiobiological research was at first particularly concerned with the measurement of the relative biological effectiveness of different types of radiation, and the first biological experiments with neutron radiation to be made in Britain were carried out at this Hospital by Mottram and by Spear, using neutrons from a 380 kV. (D-D) generator built for this purpose some twenty years ago by Gray, Read and Wyatt[1].

The new Research Unit in Radiobiology was created nearly three years ago when plans were initiated for the programme outlined below. In the meantime, the Unit has been growing, and has been working in temporary quarters. The theme of most of this work is the role of oxygen in radiobiology and radiotherapy. In this, as in other respects, the research work in progress to-day at Mount Vernon Hospital owes much to J. C. Mottram, who was appointed pathologist when the Hospital became entirely devoted to cancer treatment and research in 1931. Mottram was a keen observer—indeed, he was a naturalist at heart—and carried out many experiments with the view of discovering the factors which influence the regression of an irradiated tumour. He observed that when the blood-flow in the tail of a mouse was stopped, either by compression or by ligature, radiation damage to the skin was much reduced. He also observed that the regression of a rat tumour, following a given dose of radiation, was much reduced when the rat was rendered anæmic by bleeding just before irradiation. These experiments have a very modern flavour, but it is interesting to note that this approach to the study of the influence of oxygen on the response of cells to radiation was in origin through an exploration of the role of the vascular tumour bed. It was only later, in the light of the experiments of Crabtree and Cramer[2], that Mottram studied the influence of anaerobiosis on the radiosensitivity of roots, and came to identify oxygen transport as a key function of the vascular bed. Read pursued this theme, and there resulted his classical experiments with Thoday[3,4], which established (1) that anaerobiosis may reduce the radiosensitivity of a tissue by a factor of 3, (2) the fact that oxygen has little influence on the damage induced by α-radiation, (3) the speed with which the radiosensitivity of a cell changes in response to changes in oxygen tension, and (4) the quantitative relationship which exists between the radiosensitivity of a tissue and the oxygen tension in its immediate environment.

Why is it that X-radiation is several times more damaging if oxygen is freely available to cells at the time of irradiation ? Is the role of oxygen metabolic ? Is oxygen unique, or can its place be taken by another molecule ? Or again, why is it that structural damage to chromosomes, which is almost invariably dependent upon oxygen when induced by X-radiation, is independent of oxygen tension when induced by nitrogen mustard and many other of the so-called radiomimetic chemicals ? One may also ask why the induction of chromosome structural damage by radiation is sensitive to oxygen, while the induction of some kinds of gene mutation, as well as the inactivation of pneumococcal 'Transforming Principle' which is practically pure deoxyribonucleic acid, is apparently not sensitive to oxygen. These are a few