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Metabolic Conversions during Pupation of the Cecropia Silkworm

2. TESTS FOR THE OPERATION OF THE GLYOXYLATE CYCLE

By MARIA L. BADE*

Department of Biochemistry, Yale University, New Haven, Conn., U.S.A.

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It has been widely maintained that insects may carry out net conversion of fat into carbohydrates. Although quantitative analyses of cecropia silkworms taken during the non-feeding prepupal stage led to an alternative explanation of the source of glycogen synthesized at the end of this time (Bade & Wyatt, 1962), the possibility that some of this glycogen might be derived from fat could not be ruled out. Tissues of these insects have therefore been examined for activity of the glyoxylate cycle, an enzymic pathway by which fat is utilized for carbohydrate synthesis in micro-organisms and plants (Kornberg & Collins, 1957; Kornberg, Phizackerley & Sadler, 1960; Canvin & Beevers, 1961).

MATERIALS AND METHODS

Chemicals. All chemicals and solvents were of reagent grade, except that the diethyl ether used for chromatography was anaesthetic grade. CoA was obtained from the Pabst Brewing Company, Milwaukee, Wis., U.S.A., and oxaloacetic acid, glutathione (reduced), glyoxylic acid (sodium salt hydrate) and ATP (disodium salt) from Sigma Chemical Co., St Louis, Mo., U.S.A. They were stored in a desiccator at -10° . The glyoxylate gave a 2,4-dinitrophenylhydrazine (prepared in 65% yield by Dr Anne

Sayigh) which had m.p. $194-195^{\circ}$ and gave only two spots on chromatography in a basic solvent system. Crystalline bovine serum albumin was a product of Pentex Inc., Kankakee, Ill., U.S.A. New England Nuclear Corp., Boston, Mass., U.S.A., supplied sodium $[2-^{14}\text{D}]$ acetate which was stated to have a specific activity of 2.00 mc/m-mole. Ion-exchange resins were as described by Bade & Wyatt (1962).

Biological material. Specimens of *Hyalophora cecropia* were reared as described by Bade & Wyatt (1962). Tissues utilized for enzyme assays were obtained from prepupae 5 or 6 days after the beginning of spinning. For comparison, assays were also carried out with tissue homogenates from feeding larvae in instar V, and from early pupae (30 days after spinning).

Determination of protein. Protein was assayed by the biuret method (Layne, 1957). Triplicate samples of each homogenate were prepared by precipitating the proteins in 0.1 ml. with 75% (v/v) ethanol, washing with ether and drying at 110° . For the assay, they were dissolved in 1 ml. of hot N-NaOH.

Preparation of tissues and assay conditions. Preliminary experiments on incorporation of $[2-^{14}\text{C}]$ acetate into total ethanol-soluble non-volatile components of fat-body homogenate established that a good rate of incorporation could be obtained in presence of histidine-HCl buffer (0.05M) with little difference over the pH range 6.5-7.5. Exogenous phosphate was not required. Keeping the enzyme preparation at 0° for 40 min. reduced incorporation only slightly, freezing and thawing somewhat more so. Addition of CoA and ATP was necessary for maximal incorporation.

Fat body and midgut from one individual were used as enzyme sources for each assay. Tissues were dissected out

* Present address: Conant Chemical Laboratory, Harvard University, Cambridge, Mass., U.S.A.

under ice-cold 'insect Ringer' solution (Ephrussi & Beadle, 1936). Each tissue was macerated by hand in a chilled glass homogenizer with the addition of 1-2 vol. of ice-cold KCl (0.2M). Homogenates were then kept at 0°. Assays were begun within 20 min. from the beginning of dissection.

For the assays, tubes were set up with all components except enzyme and substrate or inhibitor in a final volume of 0.5 ml. Where necessary, solutions were adjusted with dilute KOH to pH 7 before use, except that glutathione was kept at pH 4.5. Reactions were initiated by adding enzyme and stopped with 1.5 ml. of hot ethanol. Incubation was at 30°. For the time curves, no other addition was made. Experiments on incorporation in the presence of substrate or inhibitor were run for 30 min. each. Oxaloacetate and glyoxylate solutions were freshly prepared immediately before use. These, as well as malonate, were pipetted into their respective tubes in two portions: 2.5 μ moles into the initial mixture (immediately preceding enzyme addition), and 2.5 μ moles after 15 min. of incubation. Water was added to a fourth set of tubes in two 0.05 ml. portions. All tubes were shaken once/min. during the incubation.

Chromatographic procedures. Tubes were kept at 70° for 30 min. after the reactions were stopped with ethanol. Coagulated proteins were then spun down, washed once with 75% (v/v) ethanol and discarded. To convert the acetyl-CoA present into its hydroxamate, 0.1 ml. of neutralized 2M-hydroxylamine was added to the combined extract and washings. The volume was reduced to 0.2-0.5 ml. under a stream of air at 60°. Acidic compounds were isolated by adsorption on a column (6 mm. $\times$ 20 mm.) of Dowex 1 X10 (formate form). After washing neutral and basic compounds through the column with water (2 ml.), acidic compounds were eluted with 5 bed vol. (6 ml.) of 6N-formic acid. The eluate was evaporated to dryness at room temperature *in vacuo* in a rotary evaporator with the condenser cooled in ethanol-solid CO₂. The residue was dissolved in 0.1 ml. of 30% (v/v) ethanol, and 0.02 ml. of solution containing 2 μ equiv. each of citric acid, malic acid and succinic acid was added. A portion (0.06 ml.) of the resultant solution was spotted on Whatman no. 3MM filter paper (29 cm. $\times$ 38 cm.). In a number of experiments 1 μ equiv. of acetohydroxamate was also spotted on each origin. The filter paper was developed for 7 hr. as an ascending chromatogram in diethyl ether-formic acid-water [5:2:1 (v/v) (Palmer, 1955); thorough equilibration of the vapour phase with the solvent was essential for success with this system]. The chromatogram was air-dried for 12 hr. and sprayed with 0.04% bromophenol blue in ethanol adjusted to pH 8 with KOH.

Radioautography and sample counting. The sprayed chromatograms were placed in contact with sheets of No-screen Safety X-ray film (Eastman Kodak Co., Rochester, N.Y., U.S.A.). The films were developed after 1-2 weeks of exposure. The chromatograms were then kept in envelopes at room temperature until further processing.

Aluminium planchets were prepared by grinding the surface with carborundum powder in a circle of area 1.54 cm.²; grinding facilitated even spreading of the samples. The limits of radioactivity of each chromatogram spot were traced from the radioautograph; then the spot was cut out and eluted with water directly on to the planchet (Dent, 1947). Samples were dried, respread over the ground planchet area with a small amount of water and

redried under an infrared lamp. They were counted in a windowless gas-flow counter (Packard Instrument Co., La Grange, Ill., U.S.A.) operated in the proportional range at 50% counting efficiency. No correction for self-absorption was made since sample weights were generally much below 1 mg.

Confirmation of identities. The marker acids separated cleanly on chromatograms by Palmer's method (R_F values: acetohydroxamic acid, 0.34; citric acid, 0.49; malic acid, 0.57; succinic acid, 0.82). No separation occurred during rechromatography of the radioactive malic acid spot in a basic solvent system [ethanol-water-ammonia (sp.gr. 0.88) (8:1:1, by vol.) (Jones, Dowling & Skraba, 1953)] or during high-voltage electrophoresis at pH 2 (Gross, 1956).

Acetyl-CoA was converted into acetohydroxamate because the former did not separate completely from citric acid in Palmer's solvent system. About 15% of radioactivity was lost in the conversion. The derivative, which gave distorted spots of characteristic R_F in the Palmer system, moved with an R_F identical with that of an authentic sample of acetohydroxamate after being twice rechromatographed in ammoniacal ethanol.

In the absence of added hydroxamate marker, citric acid moved as a single round spot in Palmer's solvent system and showed no separation during 3 hr. of high-voltage electrophoresis at pH 2. The high salt concentration in added hydroxamate marker caused citric acid to trail somewhat or separate into two spots connected by heavy trails; even in such cases, however, separation of citrate from acetohydroxamate was clean. Single radioactive spots were obtained when double spots were rechromatographed in Palmer's solvent system or subjected to high-voltage electrophoresis.

Rechromatography in ammoniacal ethanol of the radioactive spot with R_F of succinate resolved it into three radioactive components, one of which corresponded to succinate; the other two were not identified. The 'succinate' spot was, therefore, not counted (except as mentioned below).

Reproducibility. Duplicate 0.5 ml. portions of reaction mixture (as used for the construction of time curves) were taken at 10, 15 and 30 min. from a single tube. All duplicates were inactivated in ethanol within 5 sec. of each other. After processing, the malate and 'succinate' spots were counted; differences from the means varied between 4.0 and 4.8% for malate, and between 5.1 and 6.6% for 'succinate'.

RESULTS

Time curves were constructed for the incorporation of [2-¹⁴C]acetate into citrate, malate, and acetyl-CoA (in the form of the hydroxamate) by fat-body homogenates from larva, prepupa and pupa. Results with pupal fat body are shown in Fig. 1. Similarly shaped curves were obtained with tissue from prepupa and larva; activities for all acids were higher for the prepupa than for the pupa, and were highest for the larva. Since these activities are expressed as total counts in each acid/min./mg. of protein in the reaction mixture, and not as specific activities, no deductions may be made from the shape of these curves as to any precursor-product relationships.

Table 1 shows the influence of added substrates and of malonate upon incorporation of radioactive acetate into metabolic intermediates by fat-body and gut enzymes. Malate, citrate, and aceto-hydroxamate accounted for most of the radioactivity on the chromatograms. The impure 'succinate' spot generally showed the most activity among components that were not counted. Another radioactive spot, commonly appearing after incubation with added oxaloacetate, had R_f 0.90 corresponding to that of fumarate or pyruvate. The only other spot of notable radioactivity appeared after incubation with glyoxylate; it had R_f 0.74.

Fig. 1. Incorporation of radioactivity into components of the citric acid cycle during incubation of pupal-fat-body homogenate with $[2-^{14}\text{C}]$ acetate at 30° . The reaction mixture contained (μ moles): histidine-HCl buffer (pH 7.0), 20; $[2-^{14}\text{C}]$ acetate, 0.5 ($1.0 \mu\text{C}$); MgCl_2 , 5; reduced glutathione, 4; CoA, 0.05; ATP, 5; total vol., 0.5 ml. $\circ$, Citrate; $\triangle$, aceto-hydroxamate; $\square$, malate.

DISCUSSION

The assay system used here to test for the operation of the citric acid cycle follows in many particulars the work of Kornberg (Kornberg & Beevers, 1957; Kornberg & Madsen, 1958). In the glyoxylate cycle that he formulated, isocitrate (resulting from the action of aconitase on citrate) is split by isocitratase into succinate and glyoxylate. Malate synthetase condenses the latter with a molecule of acetyl-CoA to form malate, which is transformed into oxaloacetate by malate dehydrogenase. The cycle is completed by condensation of oxaloacetate with a further molecule of acetyl-CoA to regenerate citrate.

Were this cycle operating, a great increase in ^{14}C incorporated into malate should be observed when glyoxylate is added. In marked contrast with organisms which do possess the glyoxylate cycle, however (cf. Madsen & Hochster, 1959), no such increase is observed here. Instead, ^{14}C incorporated into malate in the presence of added glyoxylate is either lower or (as with larval gut) essentially unchanged when compared with that entering malate in the presence of endogenous substrate only. Inhibition of tissue metabolism by glyoxylate has been observed in rat liver and ascribed to the formation of an inhibitor for aconitase (Ruffo, Romano & Adinolfi, 1959). This would be expected to cause accumulation of citrate. Since the effect of added glyoxylate on accumulation of radioactive citrate varied in the present experiments with different stages and tissues, however, one cannot decide whether the same mechanism may be operative in insect material.

The curve for citrate (Fig. 1) reveals that incorporation occurs in two phases, which may be interpreted as a rapid condensation of acetyl-CoA with preformed endogenous oxaloacetate until the latter is exhausted, followed by a slower phase

Table 1. *Radioactivity in citric acid-cycle components after incubation of fat-body and gut homogenates from three developmental stages for 30 min. with $[2-^{14}\text{C}]$ acetate*

Reaction mixtures were as for Fig. 1 with the following additions (5μ moles each): C, control (no addition); O, oxaloacetate; G, glyoxylate; M, malonate. Results are expressed as $10^{-3} \times$ counts/min./mg. of protein and are means of two experiments.

Cycle component	Instar V				Prepupa				Pupa			
	C	O	G	M	C	O	G	M	C	O	G	M
In fat body												
Aceto-hydroxamate	2.1	0.23	1.1	0.7	1.0	0.19	0.59	0.32	0.24	0.07	0.31	0.07
Citrate	4.1	26	7.4	3.3	2.1	8.4	0.93	0.77	1.2	3.9	1.3	0.58
Malate	0.8	0.28	0.5	0.10	1.1	0.12	0.39	0.05	0.09	0.04	0.07	0.02
In gut												
Aceto-hydroxamate	8.0	1.1	8.1	3.2	1.3	0.30	1.2	0.26	0.76	0.21	1.1	0.15
Citrate	36	72	25	6.4	5.6	8.2	4.4	2.2	1.8	3.5	2.2	0.86
Malate	17	10	17	0.19	2.8	0.85	1.5	0.08	1.4	0.18	1.0	0.08

dependent on the rate of endogenous oxaloacetate production. This explanation is supported by the observations that, during incubation for 30 min., added oxaloacetate invariably both increases incorporation into citrate and lowers accumulation of ^{14}C in acetyl-CoA (Table 1). If malate were being produced by condensation of acetyl-CoA with glyoxylate, one would expect a biphasic curve for malate also, but this is not observed. This could mean either that the malate-synthetase reaction is not involved in malate production in cecropia tissue, or that the endogenous concentration of glyoxylate in insect tissues is too low to permit observation of the rapid condensation stage under the experimental conditions employed. The latter possibility seems to be ruled out by the observation that added glyoxylate did not act efficiently either to increase synthesis of malate or to remove acetyl-CoA. It therefore appears that the main route of malate production is not via malate synthetase.

It is also significant that malonate causes almost complete blocking of incorporation of ^{14}C from acetate into malate. This confirms that little or no activity is shown by enzymes which would operate to bypass the succinate-oxidation step in the citric acid cycle.

The consistently lowered levels of radioactivity of malate when oxaloacetate was added may reasonably be ascribed to the operation of the citric acid cycle in reverse. The equilibrium constant of malate dehydrogenase greatly favours malate (Euler, Adler & Günther, 1937; Straub, 1942); an excess of unlabelled oxaloacetate will thus tend to produce much unlabelled malate. This, in turn, would suppress the production of (labelled) malate from fumarate since the equilibrium constant of fumarase favours malate production only slightly (Bock & Alberty, 1953). A radioactive spot with R_f corresponding to that of fumarate appears on the chromatograms after incubation with added oxaloacetate.

It may be concluded that malate synthetase, a key enzyme of the glyoxylate cycle, is not active in the pupating cecropia silkworm. The results for all developmental stages studied are consistent with the operation of the citric acid cycle as a major pathway for metabolism of acetate. In agreement with this finding, Levenbook (1961) has demonstrated the presence of all enzymes of the citric acid cycle in homogenates of whole instar V larvae of the Southern army-worm, *Prodenia eridania*, though assays for isocitratase and malate synthetase on the same material were negative. Further, Fukuda & Hayashi (1960) found that, when [^{14}C]glucose is fed to silkworms, the label is not incorporated appreciably into glyoxylate, whereas α -oxoglutarate and oxaloacetate acquire considerable activity.

The lowering of radioactivity in acetyl-CoA in the presence of malonate is of interest. Zebe & McShan (1957) showed that malonate is required for maximal incorporation of acetate into long-chain fatty acids by fat-body homogenates of the lepidopteran *Prodenia*. Malonate is known to enhance fatty acid synthesis, e.g. in rat mammary gland (Popják & Tietz, 1955) and pigeon liver (Brady, 1958), and a malonate-activating system is present in pig heart and rat brain (Brady, 1958). It is thus possible that addition of malonate stimulated fatty acid synthesis in cecropia homogenates.

The chief change in enzymic activity in passing from larva to pupa appears to be quantitative. From the similar patterns shown by the several stages (Table 1), one may infer that all enzymes diminish in activity, the incorporation rates in the early pupa being roughly one-tenth of those in the mature larva. It is probable that this is representative of losses in activity undergone by many enzymes during this phase of metamorphosis. The cytochromes, for example, show even greater losses during pupation of cecropia silkworms (Shappirio & Williams, 1957).

SUMMARY

1. The possible operation of the glyoxylate cycle in tissues of the pupating cecropia silkworm has been assayed by incubating the tissues with [^{14}C]acetate and comparing the incorporation of radioactivity in the presence of various substrates and of malonate into component acids of the citric acid cycle.
2. There is a decrease of radioactivity in malate when glyoxylate is added; also, unlike oxaloacetate, glyoxylate is not an efficient remover of active acetate. Both observations suggest that malate synthetase is not active.
3. Very little ^{14}C is incorporated into malate in the presence of malonate, suggesting that the major route of malate synthesis is via the citric acid cycle.
4. Tissues obtained from feeding larvae and early pupae show qualitatively the same incorporation pattern; enzymic activity/mg. of protein diminishes greatly in the order: larva, prepupa, pupa.
5. These results are consistent with the operation of the citric acid cycle as a major pathway of acetate metabolism in the stages of silkworm development investigated and fail to support appreciable activity by the glyoxylate-cycle enzymes in any of them.

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Oxidative Metabolism of Protocatechuic Acid by Certain Soil Pseudomonads: A New Ring-Fission Mechanism

BY D. W. RIBBONS* AND W. C. EVANS

*Department of Biochemistry and Soil Science, School of Agriculture,
University College of North Wales, Bangor*

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Dagley, Evans & Ribbons (1960) have recently drawn attention to the diversity of the intermediary pathways by which micro-organisms metabolize aromatic compounds. These divergences may occur at three different stages:

(1) Before ring fission; benzenoid structures are modified in different ways, depending on the micro-organism and cultural conditions, before the cleavage step. Substituent groups may be added or eliminated, e.g. by hydroxylation or decarboxylation or both (Ribbons & Evans, 1960).

(2) After ring fission; e.g. protocatechuic acid (I) gives rise to *cis-cis*- β -carboxymuconate (*cis-cis*-3-carboxyhexa-2,4-dienedioic acid) (II) by the action of protocatechuate oxidase (protocatechuate oxygenase, EC 1.99.2.3) present in a variety of micro-organisms; its further conversion into β -oxoadipate (III), however, may occur via at least three alternative routes. Examples of these pathways may be found among the following organisms: *Neurospora crassa* [β -carboxymuconolactone intermediate (Gross, Gafford & Tatum, 1956)]; *Pseudo-*

monas fluorescens A3.12 [unidentified intermediate (Macdonald, Stanier & Ingraham, 1954; Cain, Ribbons & Evans, 1961)]; *Vibrio* O1 [muconolactone intermediate (Cain, 1961)]; and *Nocardia erythropolis* [unidentified lactone intermediate (Cain & Cartwright, 1960)].

(3) During ring fission. Cleavage of catechol to *cis-cis*-muconic acid (*cis-cis*-hexa-2,4-dienedioic acid), by rupture of the C-C bond between the hydroxyl groups, is effected by pyrocatechase (catechol oxygenase, EC 1.99.2.2) (Hayaishi & Hashimoto, 1950; Evans, Smith, Linstead & Elvidge, 1951).

Until recently this last reaction was the only known mechanism by which catechol could be enzymically transformed into aliphatic compounds. Dagley & Stopher (1959), however, discovered that some *Pseudomonas* strains contained an enzyme which oxidatively opened the catechol nucleus between C-2 and C-3, giving α -hydroxymuconic semialdehyde (2-hydroxyhexa-2,4-dienedioic 6-aldehyde). It transpired that these organisms were not unique, and a soil pseudomonad with the new ring-fission mechanism for the cleavage of protocate-

* Present address: Department of Biochemistry, University of Glasgow.