

## Citrate and the Conversion of Carbohydrate into Fat

### THE ACTIVITIES OF CITRATE-CLEAVAGE ENZYME AND ACETATE THIOKINASE IN LIVERS OF STARVED AND RE-FED RATS

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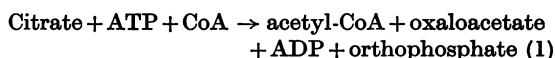
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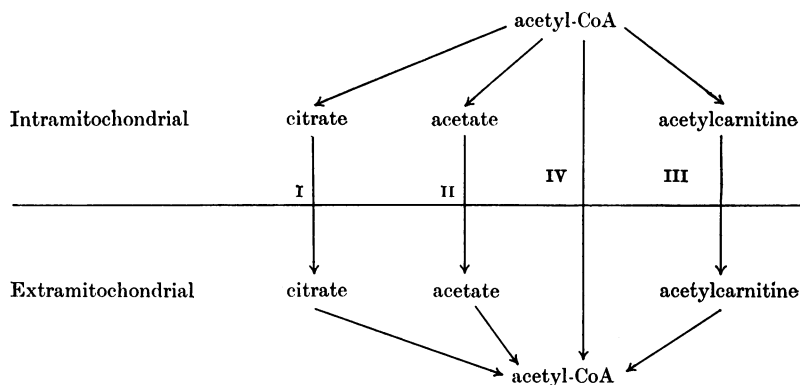
1. The activity of citrate-cleavage enzyme varies in accordance with the nutritional state of the animal. It is suppressed on starvation and restored on re-feeding after starvation. 2. The increase in enzyme activity that occurs on re-feeding starved animals depends on the diet. It is largest on diets high in carbohydrate and low in fat, and smallest on diets high in fat. Intermediate increases are obtained with balanced diets. 3. The ratio of activities of citrate-cleavage enzyme to acetate thiokinase varies from 2.5 for animals maintained on a balanced diet to 20 for animals re-fed with a diet high in carbohydrate. 4. The changes in activity of citrate-cleavage enzyme correlate with changes in the rate of fatty acid synthesis and provide evidence for the involvement of the citrate-cleavage reaction in fatty acid synthesis.

The synthesis of fatty acids in animals is predominantly an extramitochondrial process (Popják & Tietz, 1955; Brady, Mamoon & Stadtman, 1956; Langdon, 1957; Matthes, Abraham & Chaikoff, 1960; Masoro, Korchak & Porter, 1962; Dils & Popják, 1962; Abraham, Matthes & Chaikoff, 1963; Harlan & Wakil, 1963; Spencer, Corman & Lowenstein, 1964). Pyruvate derived from the breakdown of carbohydrate, and fatty acids liberated from fats, are oxidized to acetyl-CoA in the mitochondria. If sufficient energy is available much of the acetyl-CoA so formed will be converted into fatty acids. Four possible pathways are considered in Scheme 1 for

the transport of the acetyl group of intramitochondrial acetyl-CoA into the extramitochondrial space of the cell. The first pathway (I) involves the conversion of intramitochondrial acetyl-CoA into citrate, the diffusion of citrate from the mitochondria, and the cleavage of extramitochondrial citrate to acetyl-CoA. The cleavage of citrate occurs

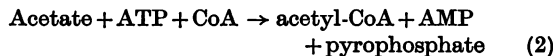


according to eqn. (1) (Srere & Lipmann, 1953; Srere, 1959). The second pathway (II) involves the hydrolysis of intramitochondrial acetyl-CoA to free



Scheme 1.

acetate (Gergely, 1955), the diffusion of acetate from the mitochondria, and the activation of extra-mitochondrial acetate to acetyl-CoA. Acetate can also be formed from ethanol. In ruminants, acetate is made in large quantities by the rumen. The activation of acetate by the extramitochondrial acetate thiokinase occurs according to eqn. (2).



The present paper gives an evaluation of pathways I and II in terms of the activities of citrate-cleavage enzyme and acetate thiokinase in various nutritional states. A preliminary account has appeared (Kornacker & Lowenstein, 1963).

The third pathway (III) shown in Scheme 1 involves carnitine as carrier of the acetyl group (Bremer, 1962*a,b*; Fritz, 1963; Fritz, Schultz & Srere, 1963; Fritz & Yue, 1964; Norum, 1963). The fourth pathway (IV) involves the direct diffusion of acetyl-CoA across the mitochondrial pathway (Lowenstein, 1963). A quantitative evaluation of pathways III and IV will be presented subsequently.

## EXPERIMENTAL

**Animals and diets.** All rats used were males of the Sprague-Dawley strain. They were supplied by Charles River Breeding Laboratories, Brookline, Mass., U.S.A., and by Holtzman Rat Co., Madison, Wis., U.S.A., and weighed between 250 and 500 g. at the time of use. Unless otherwise indicated, the rats were maintained on a balanced diet of Wayne Lab-Blox (Allied Mills Inc., Chicago, U.S.A.). This diet contained 24% of protein, 4% of fat, not more than 4.5% of fibre, 8.6% of ash and, by difference, 59% of carbohydrate. A complete analysis of vitamins and trace elements is available from the manufacturer. The high-fat diet (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) contained 45% of vegetable oil, 29% of sucrose, 18% of casein, 4% of brewer's yeast powder, 4% of salt mixture no. 2 (U.S.P.) and a vitamin fortification mixture. White bread was a locally available type. It contained approx. 2.8% of fat, 8.4% of protein, 47.3% of carbohydrate and 37.6% of water. The high-glucose diet was essentially that described by Baker, Chaikoff & Schusdek (1952). It contained, per 100 g. of diet: 58 g. of glucose, 22 g. of vitamin-free casein, 6 g. of salt mixture W, 11.8 g. of powdered cellulose, 2.0 g. of liver extract concentrate and 0.2 g. of vitamin and mineral supplement (Pervinal). Each 100 g. of diet mixture was suspended in 40 ml. of 0.5% non-nutrient agar that had been liquefied by warming. On setting, the suspension formed a cake. All animals were given water *ad libitum*.

The animals lost 12.1% of their body weight after 2 days of starvation (average of 45 values). The weight gained by starved animals on re-feeding is shown in Table 1.

**Preparation of tissue extract.** Animals were decapitated and bled. Livers were excised quickly and placed in ice-cold 0.25 M-sucrose. The cold livers were passed through a Fischer mincer constructed of stainless steel. The mince was suspended in 3 vol. (by wt.) of 0.25 M-sucrose, and

Table 1. *Weight gained by starved animals on re-feeding*

Animals were starved for 2 days and re-fed with one of the following diets: *a*, balanced; *b*, high glucose; *c*, white bread; *d*, high fat. The gain in weight is shown as percentage of the body wt. before starvation.

Period of re-feeding (days)	Diet ...	Percentage weight gain			
		<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
1		9.0	6.0	5.5	6.0
2		10.1	9.6	7.3	6.6
3		18.5	7.9	9.3	7.0
4		15.6	9.2	9.6	—
5		19.6	—	12.6	20.1
6		—	12.1	—	—
7		19.1	—	12.2	—

homogenized in a Philpot homogenizer with an internal diameter of 2.5 cm. and a stroke length of 20 cm. (Philpot & Stanier, 1956). The homogenate was centrifuged at 4500 *g*<sub>av</sub> for 10 min. at 0° in the International centrifuge. The supernatant was decanted and centrifuged at 59 000 *g*<sub>av</sub> for 30 min. at 0° in the Spinco model L centrifuge. The clear supernatant fluid was carefully withdrawn with a drawn-out dropper.

**Analytical methods.** Assays of the activities of citrate-cleavage enzyme and acetate thiokinase were carried out by the methods of Srere & Lipmann (1953) and Lipmann & Tuttle (1945) respectively. The reaction mixture contained ATP (sodium salt) (5.0 mM), MgCl<sub>2</sub> (5.0 mM), CoA (about 0.56 mM), reduced glutathione (10 mM), hydroxylamine hydrochloride (500 mM) neutralized with NaOH, and sodium acetate (5 mM) or sodium citrate (5 mM). Protein concentrations were in the range 0.2–6 mg. per assay, the final volume was 1.0 ml., the pH was 7.5 and the incubation was carried out at 37° for 40 min. The reaction was stopped by the addition of 3 ml. of a solution containing: HCl (0.8 N), FeCl<sub>3</sub>·6H<sub>2</sub>O (2%, w/v) and trichloroacetic acid (6.7%, w/v). The precipitated protein was removed by filtration and the extinction was read at 540 mμ. Authentic acetohydroxamic acid, a gift from Dr W. P. Jencks, was used as standard. Each assay of citrate-cleavage enzyme was performed at three or four different protein concentrations to ensure proportionality between enzyme activity and protein concentration. The values cited are the results of at least two points in the linear range. This proportionality failed at high protein concentrations, confirming the observation of Srere (1959). Because of frequent reports in the literature that hydroxylamine inhibits various enzymes, the hydroxamate method for determining citrate-cleavage activity was checked as follows. In one set of tubes the assay was run as described above. In a duplicate set of tubes the hydroxylamine buffer was replaced by 0.1 M-tris buffer, pH 7.5. In this set the reaction was stopped by rapidly chilling the reaction mixtures to 0°. The oxaloacetate formed was then assayed spectrophotometrically with an excess of malate dehydrogenase and NADH (Ochoa, 1955). Both methods gave the same results (Table 2). Srere (1959) has used the malate-dehydrogenase method to follow the citrate-cleavage reaction continuously. The discontinuous

Table 2. Comparison of hydroxamate and malate-dehydrogenase assays for citrate-cleavage enzyme

Details are given in the Analytical methods section.

High-speed-supernatant protein (mg.)	Citrate cleavage ( $\mu$ moles/40 min.)	
	Hydroxamate assay	Malate-dehydrogenase assay
0.25	0.30	0.29
0.51	0.58	0.58
1.02	0.98	0.99
2.03	1.60	1.33

method was preferred in the experiment described above since it is more directly comparable with the hydroxamate method.

Protein was measured by the Folin method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard.

*Sources of materials.* Compounds were obtained as follows: malate dehydrogenase, CoA, ATP, NADH, methionine and ethionine from the Sigma Chemical Co., St Louis, Mo., U.S.A.; salt mixture W and vitamin-free casein from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; Celufi cellulose powder and liver extract concentrate from Mann Research Laboratories Inc., New York, U.S.A.; Pervinal from United States Vitamin Corp., New York, U.S.A. All other chemicals were of reagent grade.

## RESULTS

*Effect of starvation on the activities of citrate-cleavage enzyme and acetate thiokinase.* The ratio of the rate of citrate cleavage over the rate of acetate activation (referred to below as the C/A ratio) is 2.5 in high-speed supernatants prepared from animals fed with the balanced diet. Fig. 1 shows that the scatter of points is relatively large for animals fed with this diet. However, Fig. 1 does not show that the ratio of the two rates remains relatively constant. When rats are starved there is little or no change in the activity of the citrate-cleavage enzyme for 24 hr., but it falls to one-third of the normal value after 2 days, and one-fifth of the normal value after 6 days of starvation. Except for the first day of starvation the activity of acetate thiokinase falls at approximately the same rate (Fig. 1). Fatty acid synthesis decreases on starvation. The decrease in activity that occurs on starvation is consistent with a role of citrate-cleavage enzyme in fatty acid synthesis.

*Effect of re-feeding starved animals with a balanced diet.* When animals that have been starved for 2 days are re-fed with the balanced diet there is a rapid increase in the activity of citrate-cleavage enzyme (Fig. 2). After re-feeding for 1, 2 and 3 days the activity of the enzyme increased 2.3-, 3.3- and

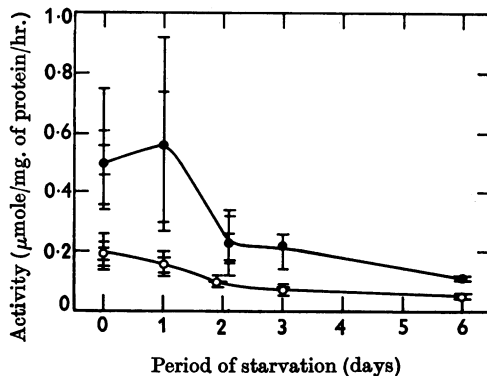


Fig. 1. Effect of starvation on the activities of citrate-cleavage enzyme (●) and acetate thiokinase (○).

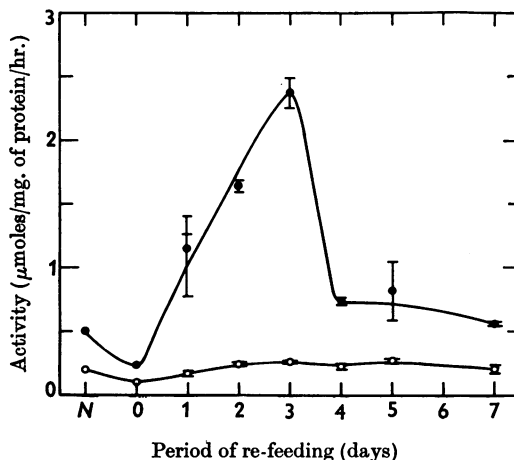


Fig. 2. Effect of re-feeding animals starved for 2 days with a balanced diet on the activities of citrate-cleavage enzyme (●) and acetate thiokinase (○). Normal values (N) are shown for comparison.

4.7-fold respectively over the activity found in unstarved animals on a balanced diet. The activity then falls so that after re-feeding the diet for 4, 5 and 7 days it approaches normal levels. The activity of acetate thiokinase changes much less under the same treatment. After re-feeding for 1, 2, 3, 4 and 5 days the activity of the enzyme increased only 0.85-, 1.2-, 1.3-, 1.1- and 1.3-fold respectively over the activity found in animals on a balanced diet. After 7 days of re-feeding the activity is back to normal. That is to say, the enzyme activity increases by a maximum of only about 30% above normal after 2 and 3 days of re-feeding. This is not a significant increase. Fig. 2 also shows that the

activity of citrate-cleavage enzyme increases well above that of acetate thiokinase during the period of re-feeding. In preparations from normal animals, or from animals that have been starved for 2 days, the C/A ratio is 2.5. After re-feeding for 1, 2, 3, 4, 5 and 7 days, the C/A ratios are 6.7, 6.7, 9.3, 3.3, 3.0 and 2.8 respectively.

Animals were also starved for 6 days and then re-fed with the balanced diet. Under these conditions the activities of the citrate-cleavage enzyme were 0.11, 1.38 and 1.75  $\mu$ moles/mg. of protein/hr. after 0, 1 and 2 days of re-feeding respectively. The increase in the activity of citrate-cleavage enzyme on re-feeding starved animals parallels the increases observed in fatty acid synthesis under similar conditions.

*Effect of re-feeding starved animals with a diet high in glucose.* When animals that have been starved for 2 days are re-fed with the high-glucose diet there is an even more rapid increase in the activity of the citrate-cleavage enzyme than that in animals re-fed with the balanced diet. After re-feeding for 1, 2, 3, 4 and 6 days, the enzyme

activity is 6.8-, 12-, 13-, 8- and 5.5-fold greater respectively than that in animals maintained on a balanced diet (Fig. 3). Again the activity of acetate thiokinase changes much less under these conditions. After re-feeding for 1, 2, 3, 4 and 6 days, the enzyme activity is 1.3-, 3.1-, 2.1-, 2.2- and 1.4-fold greater respectively than that found in animals on a balanced diet. Examination of the C/A ratio also shows that the activity of citrate-cleavage enzyme changes much more in response to the high-carbohydrate diet than does the activity of acetate thiokinase. After re-feeding for 1, 2, 3, 4 and 6 days the C/A ratios are 14, 9.4, 15, 9.2 and 9.7 respectively, compared with the normal ratio, 2.5.

Similar results are obtained when animals that have been starved for 2 days are re-fed with a high-carbohydrate diet consisting of white bread. After re-feeding with white bread for 1, 2, 3, 4, 5 and 7 days, citrate-cleavage activity is 1.5-, 6-, 10-, 6-, 9- and 4-fold greater respectively than that in animals on the balanced diet (Fig. 4). If animals that have been re-fed with white bread for 3 days are put back on the balanced diet, citrate-cleavage activity continues to rise for 1 day and then declines. Again the

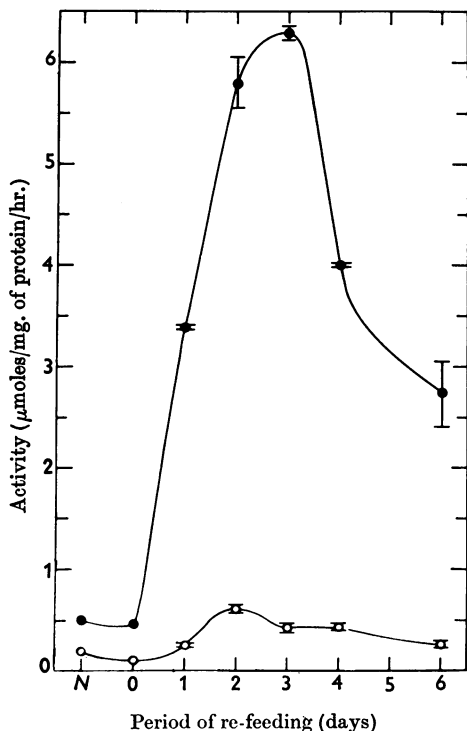


Fig. 3. Effect of re-feeding animals starved for 2 days with a diet high in carbohydrate on the activities of citrate-cleavage enzyme (●) and acetate thiokinase (○). Normal values (N) are shown for comparison.

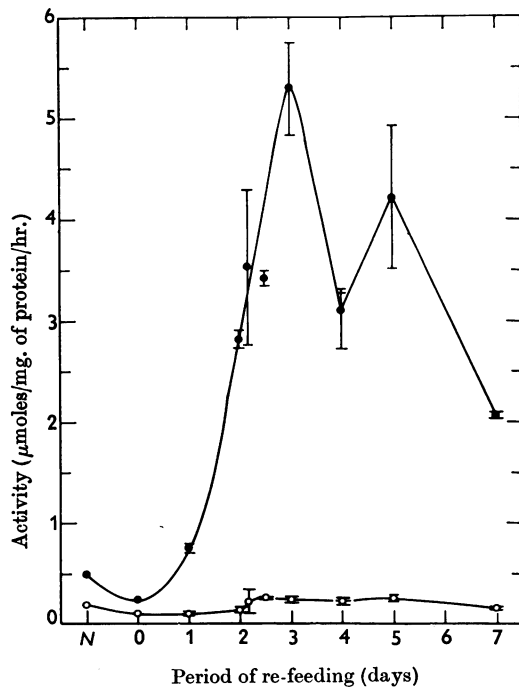


Fig. 4. Effect of re-feeding animals starved for 2 days with white bread on the activities of citrate-cleavage enzyme (●) and acetate thiokinase (○). Normal values (N) are shown for comparison.

activity of acetate thiokinase changes much less under these conditions. After re-feeding for 1, 2, 3, 4, 5 and 7 days the enzyme activity is 0.5-, 0.7-, 1.2-, 1.1-, 1.2- and 0.8-fold greater respectively than that found in animals on a balanced diet. In other words, the activity returns to normal within a few days (Fig. 4). There is a small but definite peak of activity between the second and third day of re-feeding, during which the acetate-thiokinase activity is 1.3-fold greater than that found in animals on a balanced diet. After re-feeding for 1, 2, 3, 4, 5 and 7 days the C/A ratios are 7.6, 20, 22, 14, 17 and 14 respectively, compared with the normal ratio, 2.5.

The effect of re-feeding starved animals with a diet high in carbohydrate is to elevate the rate of fatty acid synthesis far above normal. The much greater increase in the activity of citrate-cleavage enzyme when starved rats are re-fed with the high-carbohydrate low-fat diet instead of the balanced diet is consistent with the much greater increase in the rate of fatty acid synthesis that is observed under these conditions.

The changes in C/A ratio rule out the possibility that changes in the activity of citrate-cleavage enzyme are simply due to changes in the number of cells or the amount of protein in the liver. Moreover, Weber, Banerjee & Bronstein (1962) and Weber & McDonald (1961) have shown that the total number of cells in the liver does not undergo large changes when starved rats are re-fed. These workers also found that the homogenate and high-speed-super-natant nitrogen values differ only slightly in the livers of starved as compared with the livers of starved and re-fed animals.

*Effect of re-feeding starved animals with a diet high in fat.* When animals that have been starved for 2 days are re-fed with a diet high in fat there is an

increase in the activity of citrate-cleavage enzyme (Fig. 5). However, this increase is much less than the increases shown in Figs. 3 and 4. After re-feeding for 1, 2, 3 and 5 days the enzyme activity is 0.57-, 1.8-, 1.7- and 1.5-fold greater respectively than that found in animals maintained on a balanced diet. The activity of acetate thiokinase returns to normal within 2 days after putting the animals on the high-fat diet. The effect of re-feeding starved animals with a diet high in fat is to cause little change in the rate of fatty acid synthesis. The observation that there is little change in the activity of citrate-cleavage enzyme under these conditions is consistent with the role of this enzyme in fatty acid synthesis.

*Effect of ethionine on the increase in activity of citrate-cleavage enzyme.* In view of the large increases in the activity of citrate-cleavage enzyme that can be elicited by re-feeding starved animals, it was decided to test whether these changes are due to the synthesis of new enzyme. Ethionine was used for this purpose. An attempt was made to administer the antimetabolite by intraperitoneal injection. Sufficiently large doses could not be given this way without the animal becoming visibly sick, and this method was abandoned. The administration of ethionine in the diet was therefore adopted. The increase in the activity of citrate-cleavage enzyme that occurs when starved animals are re-fed with the high-glucose diet is decreased by 0.2% of ethionine, and is suppressed almost completely by 0.5% of ethionine in the diet (Table 3). The addition of 0.5% of methionine to the diet containing 0.5% of ethionine restores the increase observed in the absence of the antimetabolite to the extent of about 57%. The results suggest that the increase in citrate-cleavage activity observed on re-feeding is due to the synthesis of enzyme protein. However, Table 3 shows that, on the first day of re-feeding, animals receiving ethionine gained less weight than those not receiving ethionine. On the second day of re-feeding, animals receiving ethionine actually lost weight. This indicates that ethionine exerts a general effect on the metabolic state of the animals, and suggests that its effect on the activity of citrate-cleavage enzyme may be secondary. It has been shown by Bartels & Hohorst (1963) that ethionine exerts a general inhibition on the energy metabolism of rat liver.

## DISCUSSION

Citrate serves as a precursor for the extramitochondrial synthesis of fatty acids (Kallen & Lowenstein, 1962; Spencer & Lowenstein, 1962; Srere & Bhaduri, 1962; Formica, 1962; Bhaduri & Srere, 1963; Spencer *et al.* 1964). Experiments with stereospecifically labelled citrate provide evidence

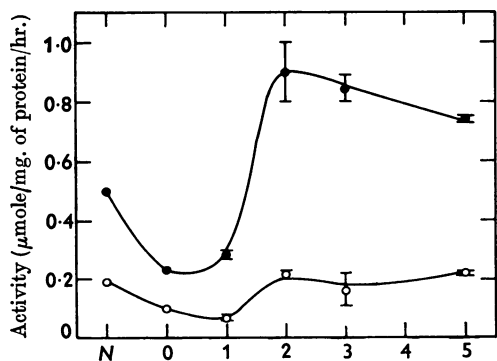


Fig. 5. Effect of re-feeding animals starved for 2 days with a diet high in fat on the activities of citrate-cleavage enzyme (●) and acetate thiokinase (○). Normal values (N) are shown for comparison.

Table 3. *Effect of ethionine on the increase in activity of citrate-cleavage enzyme*

Male rats that had been kept on the balanced diet were starved for 2 days and then re-fed with the high-glucose diet containing the following additions: *A*, none; *B*, 0.2% of ethionine; *C*, 0.5% of ethionine; *D*, 0.5% of ethionine + 0.5% of methionine. Citrate-cleavage activity of high-speed supernatant prepared from liver was assayed after the animals had been re-fed for 48 hr. The results are for two animals, (i) and (ii), on each diet. The enzyme activities of two control animals that were starved 48 hr. but not re-fed were 0.2 and 0.2  $\mu$ mole/mg. of protein/hr. respectively.

Nutrition	Wt. of animals (g.)							
	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)
Before starvation	343	309	350	324	353	328	315	321
Starved for 48 hr.	299	257	308	289	302	282	290	289
Re-fed diet ...	<i>A</i>		<i>B</i>		<i>C</i>		<i>D</i>	
for 24 hr.	322	277	313	297	309	291	298	300
for 48 hr.	333	288	299	279	285	268	287	283
	Citrate cleavage ( $\mu$ mole/mg. of protein/hr.)							
	5.5	6.0	1.6	2.8	0.2	0.6	3.3	3.3

that the first step of the incorporation of citrate into fatty acids is catalysed by citrate-cleavage enzyme (Spencer & Lowenstein, 1962). It was also shown that, on re-feeding starved animals with a diet high in carbohydrate, the rate of citrate incorporation into fatty acids increases to much higher levels than does the rate of acetate incorporation (Spencer *et al.* 1964).

The activity of citrate-cleavage enzyme declines on starvation (Fig. 1). When starved animals are re-fed with a balanced diet (Fig. 2) or a high-carbohydrate diet (Figs. 3 and 4), the activities of citrate-cleavage enzyme and acetate thiokinase rise considerably above normal levels. The overshoot in the activity of the citrate-cleavage enzyme is much greater than that observed for the acetate thiokinase. These changes in enzyme activity parallel the inhibition of fatty acid synthesis that occurs on starvation, and the exceptionally large increases in fatty acid synthesis that occur when starved animals are re-fed with diets high in carbohydrate and low in fat (Boxer & Stetten, 1944; Bernhard & Steinhauser, 1944; Bloch, 1948; Shapiro & Wertheimer, 1948; Masoro, Chaikoff, Chernick & Felts, 1950; Medes, Thomas & Weinhause, 1952; Hutchens, Van Bruggen, Cockburn & West, 1954; Miller, Cooper & Freeman, 1957; Emerson, Bernards & Van Bruggen, 1959; Tepperman & Tepperman, 1958*a,b*, 1961; Cockburn & Van Bruggen, 1959; Masoro & Porter, 1960; Langdon, 1960; Fritz, 1961; Masoro, 1962; Masoro *et al.* 1962; Spencer *et al.* 1964). Acetyl-CoA carboxylase and the enzymes of the fatty acid-synthetase complex show similar changes in activity (Gibson & Hubbard, 1960; Numa, Matsuhashi & Lynen, 1961; Korchak & Masoro, 1962).

When starved animals are re-fed with a diet high in fat (Fig. 5), the activities of citrate-cleavage enzyme and acetate thiokinase return to normal after 2 days, and there is little or no overshoot during the re-feeding. The diet used in these experiments contained 45% of fat, but it also contained 29% of carbohydrate. The small increases in the activities of the two enzymes shown in Fig. 5 may be due to the presence of this carbohydrate. The rate of fatty acid synthesis is regulated by both the fat and the carbohydrate content of the food. The higher the fat content, and the lower the carbohydrate content, the lower the rate of fatty acid synthesis (Masoro *et al.* 1950; Hausberger & Milstein, 1955).

In each of the cases discussed the activity of citrate-cleavage enzyme changes in the manner expected of an enzyme involved in fatty acid synthesis. Acetate thiokinase shows similar but smaller changes at a much lower level of activity. This suggests that the pathway involving citrate is quantitatively much more important than the pathway involving free acetate (Scheme 1).

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