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INTERRELATIONSHIPS BETWEEN LIVER COMPOSITION, PLASMA GLUCOSE AND KETONES, AND HEPATIC ACETYL-CoA AND CITRIC ACID DURING PROLONGED STARVATION IN THE MALE RAT

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SUMMARY

1. Male rats were starved 0, 6, 24, 48, 72 and 120 h to compare the fed state with phases of starvation ranging from the early mobilization of fat to the depletion of peripheral depots. Hepatic levels of acetyl-CoA and citric acid at all time points were correlated with liver composition, and plasma glucose and ketones. Animals were sacrificed by cervical fracture since elevations of liver acetyl-CoA were observed following ether or Nembutal anesthesia.

2. Analyses disclosed constant values for hepatic DNA throughout starvation whereas liver water, protein, glycogen, phospholipids and esterified fatty acids diminished. Glycogen displayed the earliest reductions: Hepatic glycogen was significantly lowered after 6 h, fell to lowest levels after 48 h, and subsequently increased again.

3. Values for acetyl-CoA and citric acid were derived for the whole liver to compensate for the preservation of liver cell numbers despite the contraction of cell mass. By this criterion, the total hepatic content of acetyl-CoA was increased significantly after 6 h of starvation, restored to "fed" values after 24 and 48 h, and reduced significantly thereafter. Total liver citric acid declined to nadir levels after 48 h and displayed a secondary rise to achieve one-half the values observed in fed animals by 120 h.

4. Plasma ketones exhibited reciprocal changes: Ketonemia was maximal after 48 h and fell again as fasting was prolonged. Acute administration of insulin (with sufficient glucose to prevent hypoglycemia) to 48-h-starved rats abolished the ketonemia and restored liver citrate without altering hepatic acetyl-CoA.

5. The findings have been discussed with regard to the regulatory effects of acetyl-CoA and citric acid upon intrahepatic gluconeogenesis, lipogenesis and ketogenesis during starvation.

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INTRODUCTION

Hepatic gluconeogenesis, ketogenesis and lipogenesis during starvation may be influenced by the regulatory effects which the content of acetyl-CoA and citric acid within liver cells can exert upon certain enzyme activities[1,2] (*e.g.* pyruvate carboxylase[3] and pyruvate dehydrogenase[4], in the case of acetyl-CoA; acetyl-CoA carboxylase[5] and phosphofructokinase[6,7], in the case of citric acid). However, published figures for the changes in hepatic acetyl-CoA and citric acid during starvation have varied qualitatively as well as quantitatively. For example, estimates for acetyl-CoA in male rat liver after 24-h starvation have ranged from 39.2 to 100 nmoles/g wet wt.[8–13], and citric acid per g liver in starved rats has been described as increased[8,9], unchanged[14,15] or diminished[12,16,17]. Indeed, inconsistencies in the direction of citrate changes have been reported under identical conditions even from the same laboratory[18,19]. None of the observations have been extended beyond 48–72 h of starvation, and corrections for the shrinkage of hepatocytes that occurs when food is withheld[20] have not been instituted previously.

MATERIALS

Reagents

Acetyl-CoA was purchased from Calbiochem, Calif. Enzyme preparations were obtained from Boehringer-Mannheim, N.Y. Adenine and pyridine nucleotides were purchased from Sigma, Mo. Reagents for determination of glucose (Glucostat) were obtained from Worthington Biochemical, N.J. Insulin (glucagon-free insulin U-40) was obtained from Lilly, Indianapolis, through the courtesy of Dr. WILLIAM KIRTLEY. All organic chemicals were of Reagent Grade. Organic solvents were 'spectranalyzed' grade. [1-^{14}C]Palmitic acid and [*carboxy*-^{14}C]tripalmitin were purchased from New England Nuclear, Mass. Silicic acid for lipid chromatography (*minus* 325 mesh) was obtained from Bio-Rad Laboratories, Calif., and Hyflo-Super-Cel (Celite Diatomite Filter Aid) from Johns-Manville, N.Y.

Animals

Male albino rats (Caeserian-derived; Charles River Laboratories, Wilmington, Mass.) weighing 240–280 g at the onset of starvation were employed. The animals had been maintained on Purina pellets and were housed in individual cages in windowless air-conditioned animal quarters at 22–24°. Lights in the animal quarters were turned off at 6 p.m. and on again at 8 a.m. Animals were sacrificed between 9 and 10 a.m., and starvation was timed on the basis of the prior period of food deprivation (*i.e.* food was withdrawn at 3–4 a.m. from rats which were to be starved for 6 h *etc.*). At the beginning of starvation, the individual animals were transferred to clean, wire-bottomed cages which provided unrestricted access to drinking water.

EXPERIMENTAL

Sacrifice of animals

Animals were stunned by a blow on the head. Less than 20 sec thereafter, livers were excised and frozen in liquid N_2 or segments were frozen *in situ* with aluminum

tongs which had been chilled with liquid N_2: Significant differences between the two techniques could not be demonstrated: Acetyl-CoA and citric acid averaged 53.2 $\pm$ 2.9 nmoles and 0.449 $\pm$ 0.013 μmoles per g wet wt., respectively, in five livers which had been excised and frozen, and 45.4 $\pm$ 2.4 nmoles and 0.442 $\pm$ 0.029 μmoles per g in five other "freeze-clamped" livers.

Following removal of the liver, both epididymal fat pads were excised immediately distal to the entrance of the epididymal blood vessels and weighed.

Preparation of tissue extracts for estimation of citric acid and acetyl-CoA

Frozen liver was powdered in a porcelain mortar that was continuously chilled with liquid N_2. Aliquots of the powder were weighed by introducing them into tared beakers containing chilled 6% (w/v) $HClO_4$. Preliminary extraction was performed in the beakers by stirring on ice for 15 sec with 2 vol. 6% $HClO_4$. The mixtures were then introduced into loosely-fitting chilled glass homogenizers for 1 min of homogenization–extraction. Mortar, pestles and homogenizer were rinsed with 1 vol. of 6% $HClO_4$, and the washings were added to the original extracts. Precipitated proteins were removed by centrifugation for 20 min at 4° (100000 $\times$ g; Beckman Spinco model L-2). Aliquots of supernatant were neutralized in an ice-bath to pH 6.8 with 1.6 M KOH and sufficient K_2HPO_4–KH_2PO_4 buffer (pH 6.8) to yield 0.05 M buffer in the final mixture. The solutions were allowed to settle on ice for 30 min prior to removal of precipitated $KClO_4$ by centrifugation for 10 min at 4° (800 $\times$ g; International centrifuge PR-2). Buffer was omitted in the neutralization of the supernatant when extracts were to be analyzed for citric acid with citrate lyase. The supernatants were analyzed for acetyl-CoA immediately; other aliquots were stored at —18° to —70° for subsequent citric acid assays.

Acetyl-CoA was estimated with an Eppendorf recording fluorimeter by the method of HERRERA AND FREINKEL[10]. This procedure utilizes internal standards and eliminates the need for the correction factors which PEARSON[21], and BUCKEL AND EGGERER[22] have recommended. Concentrations of acetyl-CoA in the internal standards were verified each day by spectrophotometric assay with phosphotransacetylase.

Citric acid was estimated by the pentabromacetone method as described by STERN[23] and based on the method of NATELSON, PINCUS AND LUGOVOY[24]. Evaluation for specificity disclosed no interference by the addition of 0.150 to 0.300 μmole of oxaloacetic acid, 2-ketoglutaric acid, isocitric acid, cisaconitic acid or pyruvic acid. Recovery of citric acid added to powdered frozen livers from fed and fasted rats ranged from 94 to 105%. As previously reported for acetyl-CoA[10], approx. 60% of the endogenous citric acid was lost when frozen pieces of livers were homogenized and extracted with $HClO_4$ without prior pulverization.

To validate the pentabromacetone method, liver extracts from fed (n = 4) and 24-h fasted (n = 4) rats were also analyzed by the citrate lyase method, as described by MOELLERING AND GRUBER[25]. Significant differences were not observed. Values derived by pentabromacetone *vs.* citrate lyase method were 0.384 $\pm$ 0.037 *vs.* 0.407 $\pm$ 0.036 μmole per g fresh wt. of liver in the fed and 0.157 $\pm$ 0.012 *vs.* 0.180 $\pm$ 0.009 μmole per g in the 24 h starved animals, respectively.

Extraction and fractionation of tissue lipids

Another portion of frozen liver was extracted with 20 vol. of chloroform–methanol (2:1, v/v). The extracts were purified by the method of FOLCH, LEES AND

STANLEY[26]. One aliquot was used to estimate lipid phosphorus[27] after digestion with 72% $HClO_4$ as described elsewhere[28]. A second aliquot was employed for the gravimetric estimation of total fat. A third aliquot was reduced to dryness under N_2 at 50° and resuspended in chloroform prior to passage through 2.5 cm × 1.0 cm columns containing 1:1 mixtures of activated silicic acid and Hyflo-Super-Cel. Neutral lipids were eluted from the columns with chloroform.

The chloroform eluates were reduced to dryness under N_2 at 50° and resuspended in heptane. Esterified fatty acids of the neutral lipids were separated from free fatty acids by the differential solubilization technique of KERPEL, SHAFRIR AND SHAPIRO[29]. Recovery experiments with [^{14}C]tripalmitin or [^{14}C]palmitic acid added to lipid extracts prior to passage over silicic acid columns indicated that less than 3% of the labeled free fatty acid and more than 95% of the labeled triglyceride are recovered in the esterified fatty acid fraction by this method. The fractions containing the esterified fatty acids were saponified with 1 M KOH in 95% ethanol for 1 h at 100°, and the fatty acids were re-extracted into heptane by acidification. Final heptane extracts were evaporated to dryness under N_2 at 50° and resuspended in chloroform for estimation of fatty acids by the method of DUNCOMBE[30].

Other measurements of liver composition

Water content was estimated by repetitive weighings to constant weight of a portion of frozen liver which had been dehydrated in a tared vessel at 110°. DNA-phosphorus was isolated from the residual pellet after lipid extraction by the method of SCHMIDT AND THANNHAUSER[31]; inorganic phosphorus was estimated[27] after digestion with 72% $HClO_4$. Total hepatic protein was estimated by the procedure of LOWRY *et al.*[32] following digestion of an aliquot of frozen liver. Separately weighed portions of frozen liver were digested with KOH for precipitation of glycogen by the method of GOOD, KRAMER AND SOMOGYI[33]. The precipitates were solubilized in water and reprecipitated twice with ethanol for purification prior to acid hydrolysis (2.5 M H_2SO_4; 2 h; 100°) and enzymatic assay with glucose oxidase.

Analyses of plasma

The necessity for immediate freezing and processing of livers made it impossible to sample blood from the same animals. Therefore, blood specimens were secured from other rats maintained under identical conditions and stunned by a blow on the head as described above. Immediately after opening the abdomen, a nick was made in the aorta, and blood was collected in a tube containing dried heparin. 2–3 ml of blood could be obtained from each animal in this fashion. Plasmas were separated by centrifugation at 4° and stored at −18°. Protein-free filtrates were prepared with $Ba(OH)_2$–$ZnSO_4$ (ref. 34) and analyzed for glucose[35] and total ketones[36].

RESULTS

Changes in plasma glucose and ketones; and weight of whole body, liver and epididymal fat pads during starvation

Results are summarized in Table I (A and B). Body weight diminished slowly and progressively; liver weight fell more precipitously. The most marked reductions occurred in the weights of the epididymal fat pads which were monitored as an index

TABLE I

EFFECT OF STARVATION IN THE MALE RAT

Mean ± S.E. values for fed and starved animals are shown below; values in parentheses are the number of rats in each group. All animals were sacrificed by a blow on the head between 9:00–11:00 a.m. Experiments involving anesthesia prior to stunning have been designated: ether–air or Nembutal. Duration of starvation denotes period of time during which food was withheld before sacrifice. Asterisks denote significance (P) of deviations from values observed in fed animals (*i.e.* 0 starved) in the experiments without anesthesia. Values for liver acetyl-CoA and citric acid have been expressed per g fresh liver wt. (μmoles/g) and on basis of total liver content (μmoles/g × fresh liver wt.). Significant effects of anesthesia with ether–air or Nembutal on liver acetyl-CoA and citric acid in fed and 48 h starved rats have been designated by §'s.

Duration of starvation (h):	*0*	*6*	*24*
A. Weight			
Total body (g)	248 ± 10 (12)	238 ± 11 (5)	217 ± 2 (12)***
Liver (g)	10.7 ± 0.2 (11)	10.4 ± 0.1 (5)	7.5 ± 0.3 (9)***
Epididymal fat pads (g)	1.15 ± 0.05 (12)	0.99 ± 0.08 (5)	0.98 ± 0.06 (12)*
B. Plasma			
Glucose (mg/100 ml)	128 ± 5 (7)	106 ± 6 (5)*	94 ± 7 (7)***
Ketones (μmoles/l)	289 ± 76 (6)	318 ± 45 (9)	1191 ± 99 (5)
C. Liver acetyl-CoA			
nmoles/g	35.8 ± 3.8 (6)	61.4 ± 4.6 (5)**	48.5 ± 2.0 (6)*
nmoles/liver	400 ± 41	644 ± 49**	378 ± 16
nmoles/g (ether–air)	66.3 ± 6.8 (5)§§		
nmoles/(g) (Nembutal)	65.7 ± 5.9 (5)§§		
D. Liver citric acid			
μmoles/g	0.379 ± 0.047 (6)	0.331 ± 0.057 (5)	0.146 ± 0.013 (6)***
μmoles/liver	4.05 ± 0.050	3.44 ± 0.61	1.10 ± 0.11***
μmoles/g (ether–air)	0.203 ± 0.034 (5)§		
μmoles/g (Nembutal)	0.353 ± 0.085 (5)		

Duration of starvation (h):	*48*	*72*	*120*
A. Weight			
Total body (g)	205 ± 4 (12)***	193 ± 2 (12)***	167 ± 3 (12)***
Liver (g)	6.8 ± 0.3 (8)***	6.5 ± 0.2 (9)***	5.8 ± 0.1 (7)***
Epididymal fat pads (g)	0.93 ± 0.04 (12)***	0.81 ± 0.09 (12)**	0.16 ± 0.04 (12)***
B. Plasma			
Glucose (mg/100 ml)	89 ± 4 (10)***	96 ± 5 (10)***	91 ± 7 (7)**
Ketones (μmoles/l)	1319 ± 150 (5)***	1162 ± 177 (5)***	589 ± 145 (5)*
C. Liver acetyl-CoA			
nmoles/g	55.0 ± 4.2 (6)**	42.2 ± 1.9 (6)	27.0 ± 3.2 (6)**
nmoles/liver	374 ± 29	274 ± 12*	157 ± 19***
nmoles/g (ether–air)	70.1 ± 2.4 (5)§		
nmoles/(g) (Nembutal)	78.1 ± 8.2 (6)§		
D. Liver citric acid			
μmoles/g	0.109 ± 0.008 (6)***	0.202 ± 0.026 (6)*	0.309 ± 0.036 (6)
μmoles/liver	0.74 ± 0.05***	1.31 ± 0.17***	1.79 ± 0.21**
μmoles/g (ether–air)	0.099 ± 0.022 (5)		
μmoles/g (Nembutal)	0.168 ± 0.038 (6)		

* $P < 0.05$. § $P < 0.05$.
** $P < 0.01$. §§ $P < 0.01$.
*** $P < 0.01$.

of peripheral fat stores. After 120 h of starvation, less than 15 % of their initial mass was still present (Table I, A), thus suggesting that mobilizable depot fat had been virtually exhausted.

Plasma glucose declined from an initial value of 129 mg/100 ml to nadir values of 89 mg/100 ml after 48 h (Table I, B). Plasma ketones exhibited a more biphasic

TABLE II

EFFECT OF STARVATION ON LIVER COMPOSITION IN THE MALE RAT

Mean ± S.E. values for fed and starved animals are shown below. Conditions of sacrifice were as described in Table I. Asterisks denote significance (P) of deviation from o time. Values are expressed on basis of wet wt. per g of liver (Section A) and on basis of total liver content (Section B). Values in parentheses are number of animals in each group.

Duration of starvation (h):	*o*	*6*	*24*
A. Concn. per g liver (wet wt.)			
DNA-phosphorus (μmoles)	6.69 ± 0.27 (6)	7.12 ± 0.56 (5)	9.95 ± 0.28 (5)***
Water (g)	0.706 ± 0.067 (5)	0.716 ± 0.019 (5)	0.707 ± 0.012 (5)
Protein (g)	0.146 ± 0.008 (5)	0.164 ± 0.006 (5)	0.155 ± 0.008 (5)
Glycogen (mg)	40.8 ± 2.1 (5)	29.1 ± 3.2 (5)**	1.4 ± 0.2 (5)***
Phospholipid-phosphorus (μmoles)	42.6 ± 1.1 (12)	42.4 ± 1.2 (5)	47.9 ± 1.1 (12)***
Esterified fatty acids (neutral lipid) (μmoles)	17.1 ± 1.4 (12)	18.9 ± 0.58 (5)	16.9 ± 1.0 (12)
B. Amount per whole liver			
DNA-phosphorus (μmoles)	71.9 ± 2.9 (6)	76.2 ± 6.0 (5)	74.2 ± 1.9 (5)
Water (g)	7.55 ± 0.08 (5)	7.45 ± 0.02 (5)	5.30 ± 0.09 (5)***
Protein (g)	1.56 ± 0.09 (5)	1.71 ± 0.06 (5)	1.16 ± 0.06 (5)*
Glycogen (mg)	432 ± 22 (5)	302 ± 33 (5)**	11 ± 2 (5)***
Phospholipid-phosphorus (μmoles)	453 ± 12 (12)	441 ± 12 (5)	359 ± 8 (12)***
Esterified fatty acids (neutral lipid) (μmoles)	183 ± 15 (12)	196 ± 6 (5)	128 ± 7 (12)***

Duration of starvation (h):	*48*	*72*	*120*
A. Concn. per g liver (wet wt.)			
DNA-phosphorus (μmoles)	9.72 ± 0.06 (5)***	10.34 ± 0.48 (5)***	11.27 ± 0.42 (5)***
Water (g)	0.710 ± 0.010 (5)	0.711 ± 0.006 (5)	0.716 ± 0.001 (5)
Protein (g)	0.176 ± 0.012 (5)	0.171 ± 0.011 (5)	0.178 ± 0.018 (5)
Glycogen (mg)	3.5 ± 0.8 (5)***	8.5 ± 1.3 (5)***	6.1 ± 3.2 (5)***
Phospholipid-phosphorus (μmoles)	47.5 ± 2.1 (12)**	46.3 ± 1.4 (12)**	40.5 ± 2.1 (12)
Esterified fatty acids (neutral lipid) (μmoles)	16.5 ± 1.7 (12)	16.8 ± 1.8 (12)	4.9 ± 0.8 (12)***
B. Amount per whole liver			
DNA-phosphorus (μmoles)	67.7 ± 3.1 (5)	68.6 ± 3.1 (5)	66.6 ± 2.8 (5)
Water (g)	4.83 ± 0.07 (5)***	4.62 ± 0.04 (5)***	4.15 ± 0.04 (5)***
Protein (g)	1.19 ± 0.08 (5)*	1.11 ± 0.07 (5)*	1.03 ± 0.06 (5)***
Glycogen (mg)	24 ± 5 (5)***	55 ± 8 (5)***	35 ± 19 (5)***
Phospholipid-phosphorus (μmoles)	323 ± 14 (12)***	301 ± 9 (12)***	235 ± 12 (12)***
Esterified fatty acids (neutral lipid) (μmoles)	112 ± 12 (12)***	109 ± 11 (12)***	29 ± 5 (12)***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

pattern (Table I, B): In confirmation of AMATRUDA AND ENGEL[37], maximal increases were achieved after 48 h of starvation. Thereafter, plasma ketones fell coincident with the depletion of peripheral fat.

Effect of starvation on liver composition

Analyses are expressed on the basis of concentration per g fresh liver weight in Table II (A), and per whole liver (*i.e.* concn. per g × fresh liver wt.) in Table II (B). HARRISON[20] has previously reported that the mass of the liver-cell nucleus is not affected by 6 days of starvation in the rat and that the DNA per diploid liver cell is unaltered. In confirmation of HARRISON[20], DNA-phosphorus per whole liver did not change during 5 days of starvation (Table II, B). Presumably, therefore, the number of liver cells remained constant, and the reduction of liver weight resulted from a loss of cytoplasmic constituents. Total liver water, protein, glycogen, phospholipid phosphorus, and esterified fatty acids were significantly diminished after 24-h starvation. On a percentage basis, the reductions were greatest for glycogen; indeed, glycogen was the only constituent affected significantly after only 6 h. Glycogen stores were replenished during more prolonged starvation, as others have shown[20,38], and total glycogen increased five-fold between 24 and 72 h. On the other hand, when starvation was prolonged beyond 24 h, the reductions of liver water and phospholipid phosphorus continued but at a much slower rate, whereas total protein remained relatively constant. Like peripheral fat, the neutral lipid fatty acids exhibited a precipitous decline between 72 and 120 h.

Effects of starvation on liver acetyl-CoA and citric acid

Values for acetyl-CoA (Table I, C) and citric acid (Table I, D) have also been expressed as concentration per g fresh wt. (μmoles/g) and on the basis of total liver content (*i.e.* μmoles/g × fresh liver wt.). The latter may reflect regulatory content within individual hepatocytes more accurately in view of the shrinkage of cytoplasm and preservation of cell numbers during starvation.

After 6 h of starvation, before detectable changes in liver weight (Table I, A), acetyl-CoA increased more than 60% (Table I, C) whereas citric acid fell slightly but not significantly (Table I, D). The total liver content of acetyl-CoA returned to control levels after 24 and 48 h (although acetyl-CoA per g liver was still increased significantly). Thereafter, total acetyl-CoA was depressed below control values (Table I, C).

Citric acid was reduced significantly at 24 h (Table I, D). Nadir values for total liver citric acid (*i.e.* 18.3% of the content in fed animals) occurred at 48 h coincident with 4–5-fold increases in plasma ketones (Table I, B). Thereafter, citric acid increased. Thus, at 120 h, when plasma ketones were only twice as great as in fed animals (Table I, B), the total liver content of citric acid averaged 44.2% of that which was present in the fed state (Table I, D).

The seemingly inverse correlations between liver citric acid and plasma ketones prompted more acute studies. Five male 48-h-starved rats were injected intraperitoneally with 0.1 unit insulin (dissolved in 0.5 ml 50% glucose (w/v) to prevent hypoglycemia), and six others were given equivalent volumes of saline. All animals were sacrificed by a blow on the head 30 min later. FOSTER[11] has demonstrated that acute reductions of plasma ketones by insulin under such circumstances are due to an abrupt cessation of ketone body production by the liver. In confirmation of

FOSTER[11], in the insulin–glucose *vs.* saline injected 48 h starved rats, plasma glucose was higher (176.8 ± 18.2 *vs.* 84.6 ± 0.8 mg/100 ml; $P < 0.001$) and plasma ketones were markedly lower (334 ± 32 *vs.* 1331 ± 134 μmoles/l; $P < 0.001$), although the liver content of acetyl-CoA was not significantly different (340.1 ± 13.7 *vs.* 373 ± 21.5 nmoles/liver). However, the liver content of citric acid was strikingly increased in the insulin–glucose-treated animals (3.08 ± 0.18 *vs.* 1.19 ± 0.09 μmoles/liver; $P < 0.001$)—a phenomenon which has not been reported previously.

Effects of anesthesia on liver acetyl-CoA and citric acid

Since most previous estimates have been secured in anesthesized animals[9,11,12,16], separate experiments were performed to examine the effects of anesthesia in fed and 48 h starved rats. Animals were exposed to ether–air mixture in large, closed, glass jars into which ether-impregnated balls of gauze had been suspended. Nembutal (40 mg/kg body wt.) was administered intraperitoneally. Animals were sacrificed by a blow on the head after 5 min of exposure to ether–air mixtures, or 20 min after the injection of Nembutal. Significantly higher concentrations of acetyl-CoA per g liver were observed in fed and 48 h starved animals following Nembutal or ether–air anesthesia (Table I, C). Citric acid was unaffected except in fed animals, anesthetized with ether–air (Table I, D). START AND NEWSHOLME[12] have recently reported that the method of anesthesia may influence the content of acetyl-CoA in freeze-clamped livers[12]. Our independent experiences reinforce this observation.

DISCUSSION

In the present studies, as in earlier reports[8–13], the concentrations of acetyl-CoA per g of liver increased during 48 h of starvation. However, our concurrent measurements of total liver weight and composition indicated that cell size must be considered in estimates of regulatory content. In confirmation of older observations[20], we found that total hepatic DNA (and presumably the total number of liver cells) remained constant during 120 h of starvation whereas total liver weight, water and protein diminished coincident with a contraction of the cyloplasmic mass of individual hepatocytes. When appropriate corrections for this shrinkage were instituted, by relating acetyl-CoA to total liver weight (and by inference, to individual liver cells), we found that the values after 24 and 48 h of starvation did not differ significantly from the fed state, and that they were actually reduced after 72 and 120 h. Thus, although elevations in the concentration of acetyl-CoA within liver cells have been implicated in the activation of pyruvate carboxylase for gluconeogenesis[39–41], our measurements are not consistent with such a function from 24 h of starvation onward.

However, an earlier contribution cannot be excluded. We observed highly significant elevations of liver acetyl-CoA in 6-h-starved rats. Plasma ketones were unchanged at that time, liver citrate was beginning to decline, and glycogen had fallen to levels which occur whenever the delivery of fatty acids to the fed liver is increased[42]. The elevations of acetyl-CoA could reflect a transient "pile-up" within the mitochondria because fat oxidation was increasing, whereas removal of acetyl-CoA *via* the formation of citrate was becoming attenuated, and ketogenesis had not yet been established fully. During such a brief disequilibrium, the accumulated acetyl-CoA might be meaningful for the activation and/or induction of intramitochondrial pyru-

vate carboxylase* (refs. 39–41). The failure of liver slices from fasted rats to form more glucose from lactate or pyruvate until after 8–9 h of starvation[44], and the increase of pyruvate carboxylase in hepatic mitochondria which has been seen after 24 h of starvation[40] could be compatible with the above sequence. It need not be excluded by the finding of unaltered concentrations of acetyl-CoA after gluconeogenesis and ketogenesis have been increased[13] and new "steady-states" established.

During the preparation of this manuscript, START AND NEWSHOLME[12] reported that the citric acid per g liver is significantly reduced in rats starved for 12–48 h. Reduction of total liver content of citric acid during 24–72 h starvation may also be derived from the data of SPENCER AND LOWENSTEIN[14] when corrections for shrinkage of liver cells are applied. Our data confirm these observations. In the present studies, observations were extended beyond 72 h for the first time, and it was demonstrated that the total content of citric acid in liver doubles between 48 and 120 h of starvation.

The factors influencing tissue content of citric acid have been considered in several recent reviews[1,45]; the available data preclude an explanation for the unique, early lowering in liver during starvation. The implications of this fall in citric acid have been discussed by START AND NEWSHOLME[12]. As they suggested, the lowered content, in contrast to previous reports of increased citric acid[8,9], negates the suggestion[19,46] that the heightened hepatic gluconeogenesis of early starvation is facilitated by an inhibition of phosphofructokinase by citric acid. However, the reduction of liver citric acid may be linked to the impairment of lipogenesis at this time[12]. Our finding of a rise in total liver citric acid later in starvation raises the question of concomitant readjustments in lipogenesis and glycolysis as peripheral fat becomes depleted. In this regard, the apparently reciprocal changes in plasma ketones and liver citric acid, which we observed throughout prolonged starvation as well as in acute studies, prompts a search for some direct interactions in the formation of citrate and ketogenesis.

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* Since the concentration of acetyl-CoA which we encountered in livers of fed animals (*i.e.* 35.8 nmoles/g wet wt.) already approximates the K_a of acetyl-CoA for pyruvate carboxylase at pH 7.8 (*i.e.* 33 μM)[43], compartmentation must be invoked to support any postulate concerning the regulatory effects of acetyl-CoA upon mitochondrial pyruvate carboxylase.

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