The method used here to assess the contribution of liver to plasma acylcarnitine is based on the idea that in rat, shortly after administration of [3H]butyrobetaine the [3H]carnitine appearing in the plasma derives from the liver and so does the acyl moiety of [acyl-3H] carnitine. In the perchloric acid extracts of plasma and liver, the ester fraction of total carnitine was determined by high performance liquid chromatography. The ester fraction of total carnitine in the plasma of fed rats was 32.6% while that of [3H]carnitines was 67.9%. 1 h following injection of [3H]butyrobetaine. For 48 h starved rats the equivalent values were 54.2 and 84.0%, respectively, 24 h after the administration of [3H]butyrobetaine, the ester content became the same in the total and [3H]carnitines. That the newly synthesized carnitine was more acylated (67.9 versus 32.6%, fed) indicates that liver exports acyl groups with carnitine as carrier.

The observation that the ester fraction in the newly synthesized plasma carnitine increased with fasting (84.0 versus 67.9%) indicates that the surplus plasma acylcarnitine in fasting ketosis derives from the liver. Perfused livers, however, released carnitine with the same ester content (60–61%) whether they were from fed or fasted animals. Probably, the increased plasma [acylcarnitine] in fasting develops not by an increased ester output from the liver but by an altered handling in extrahepatic tissues.

In ketotic states, whether in starvation or diabetes, the contribution of short-chain acylcarnitines to the total plasma carnitine markedly increases at the expense of free carnitine. This fact has been established in animal (1–3) as well as in human (4–7) studies. The mechanism of the development of increased plasma [acylcarnitine], however, has not quite been revealed, and the tissue of origin is also obscure. The candidate with the most potential as the source of extra acylcarnitines was the liver since it responds uniquely by enhancing its capacity to oxidize fatty acids in ketotic states. Really, the participation of acylcarnitines in the total carnitine released by the liver cells (8) or perfused liver (3, 9, 10) is relatively high, about 50–60%. However, we also observed that the percentage of acylcarnitines in the perfusate of liver did not increase when the liver was from fasted animals (3, 9). These latter observations seem to challenge the view that liver supplies the surplus plasma acylcarnitines in ketosis. Alternatively, muscle or kidney may be the source.

**Liver Perfusion**—The perfusions were performed in situ in outflow fashion as previously described (9). The perfusion medium was composed of human erythrocytes suspended to a hematocrit of 15% in Krebs bicarbonate buffer, pH 7.4, containing 4% bovine albumin. Animals were injected with [3H]butyrobetaine 1 h prior to perfusion.

**Sample Preparation and Analyses—**Neutralized perchloric acid extract of 0.5 g of liver was purified on 0.5-cm columns of Dowex 50-W-X8 (200–400 mesh, Cl- form) and Dowex 50-W-X8 (200–400 mesh, H+ form) resins and N-ethylmaleimide were purchased from Serva Heidelberg. [1-14C]Acetyl coenzyme A was from Amersham Corp. L-Carnitine for standard was a gift from Sigma Tau Rome. [3H]Butyrobetaine was prepared as described previously for labeled carnitine (12) using 4-(dimethylamino)butyric acid purchased from Aldrich. Acetyl coenzyme A was prepared by the method of Stadtman (13).

**Treatment of Animals—**Male Wistar rats weighing 190–200 g were injected intraperitoneally with 15 × 10^6 cpm of [3H]butyrobetaine in 1.0 ml of 0.9% saline 1, 2, or 24 h prior to being killed. For fed animals, food was available until they were killed, while the starved animals were deprived of food 48 h before. (In the 24-h experiment, starved animals were injected in the 24th h of starvation.) The animals were killed by decapitation between 8:00 and 10:00 a.m., blood was collected into heparinized tubes, and livers were quickly removed and frozen in liquid N2. Neutralized perchloric acid extracts were prepared on the day of experiment, and the extracts were stored at −20 °C until analysis.

EXPERIMENTAL PROCEDURES

**Materials—**Carnitine acetyltransferase and coenzyme A were from Boehringer Mannheim. Dowex 1-X8 (300–400 mesh, Cl– form) and Dowex 50W-X8 (200–400 mesh, H+ form) resins were prepared by the method of Stadtman (13).

**Sample Preparation and Analyses—**Neutralized perchloric acid extract of 0.5 g of liver was purified on 0.5 × 8-cm columns of Dowex 50W-X8, NH4+, and Dowex 1-X8, F– resins as previously described (14). The final methanol extract was evaporated, reconstituted in 1.0 ml of water, neutralized to pH 7.0, and subjected to a second purification on resins. In the latter procedure, one-half amounts of resin (0.5 × 4-cm columns) were used. The extract of 1.0 ml of plasma was purified in the same way, but in the second purification on resins, Dowex 1 was employed in acetate– form (instead of F– form). The final evaporated samples were reconstituted in 400 μl of water, of which 200 μl was used for high performance liquid chromatography (HPLC). The remaining 200 μl was made up to half of the original volume of the extract and used for enzymatic carnitine analysis (15).

To analyze the perfusate, the red blood cells were first spun down at 10,000 × g for 20 min (less than 5% of radioactivity was found in the pellet). The resulting supernatant was treated as plasma, but 10 ml was processed. The extract of 10 ml of supernatant was purified first on 1.0 × 8-cm columns of resins, and in the second step of purification the size of resins was reduced to 0.5 × 4 cm. The final sample was reconstituted in 400 μl of water, of which 200 μl was used for HPLC and the other half was made up to 1.0 ml with water and used for carnitine assay. A portion of perfusion fluid, which was not...
passed through liver, was also purified in the same way and involved in carnitine assay as blank.

Carnitine was determined by enzymatic analysis as described previously (15). The difference between free and total (obtained after alkaline hydrolysis) gave short-chain acylcarnitines. Note that in this work total carnitine means acid-soluble total carnitine determined by enzymatic analysis. For perfusate, the values were corrected for the nonused perfusion fluid, the carnitine content of which was less than 10% of total carnitine found in the perfusate passed through liver. The recovery after sample processing (usually above 80%) was also measured by following the tritium content of samples and was taken into correction.

HPLC Conditions—Radioactive carnitine and its esters were separated by reversed phase ion-pairing chromatography using a step gradient of methanol as previously described (14). Peaks of carnitine, butyrobetaine (if present), acetylcarnitine, and propionylcarnitine were identified.

Radioactive Measurements—Radioactivity was measured in 10.0 ml of a toluene/Triton X-100 (2:1, v/v) mixture containing 2,5-diphenyloxazole (5 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (200 mg/liter) scintillants with a Beckman LS 200 spectrometer. When 14C radioactivity was to be measured (carnitine determination), the tritium content was excluded by narrow window setting.

RESULTS AND DISCUSSION

The ester portion of total carnitine and [3H]carnitine were determined in the acid-soluble fractions of liver (Table I) and plasma (Table II) of fed and fasted rats. In liver (Table I), the total carnitine level increased with fasting in accord with earlier observations (16), while the percent contribution of esters did not change (1, 2, 16). It is also seen that the ester content of [3H]carnitines (obtained by HPLC separation) was very close to that of total carnitine (obtained by enzymatic analysis). (In the 1-h experiment, however, the ester [3H]carnitine fraction tended to be higher, indicating that the newly formed carnitine did not quite equilibrate with the total pool in this time.) The contribution of acid-insoluble carnitine to total increased from 6.45 to 12.0% with fasting, and the same percentages of [3H]carnitine were found in this fraction (not shown).

Data obtained in the plasma are shown in Table II. As appears from the enzymatic analysis, the percentage of carnitine esters markedly increased with fasting. An essential question for this work is the organ origin of this surplus acylcarnitine. An analysis of the conversion of [3H]butyrobetaine into [3H]carnitine and [acyl-3H]carnitine may elucidate this question. 1 or 2 h following [3H]butyrobetaine injection, the newly synthesized [3H]carnitine in the liver cannot equilibrate with the carnitine pool of extrahepatic tissues. Therefore, both [acyl-3H]carnitine and [3H]carnitine are derived from the liver.

The results of Table II can be analyzed first by comparing the changes in course of time (1, 2, and 24 h) regardless of nutritional state. As Table II shows, the relative contribution of esters within total carnitine was highest after 1 h and significantly higher than the ester fraction within total carnitine (e.g. 67.9 versus 32.5% in fed). This difference decreased by the 2nd h, and after 24 h the ester fractions in the [3H]carnitine and total carnitine were almost the same (23.2 and 29.3%, fed). The same trend was valid for the fasted groups. A plausible explanation is that extrahepatic tissues took up [acyl-3H]carnitines at a faster rate than [3H]carnitine. This led us to the conclusion that in rat, liver delivers acyl groups

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### Table I

| Time (h) | Animal Condition | Carnitine | [3H]Carnitine |
|---------|-----------------|-----------|---------------|
|         |                 | Total (nmol/g) | Ester (%) | Total (10^3 cpm/g) | Ester (%) |
| 1       | Fed             | 194 ± 10.1 | 32.6 ± 1.20 | 423 ± 40.5 | 41.6 ± 2.11 |
| 2       | Fed             | 378 ± 11.2 | 33.1 ± 1.12 | 661 ± 41.1 | 39.6 ± 1.18 |
|         | Starved         | 191 ± 9.80 | 30.4 ± 0.21 | 155 ± 14.2 | 31.5 ± 0.80 |
| 24      | Fed             | 243 ± 11.1 | 39.8 ± 1.28 | 566 ± 4.01 | 43.3 ± 2.81 |
|         | Starved         | 359 ± 21.2 | 40.1 ± 1.30 | 104 ± 6.50 | 40.4 ± 2.01 |

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### Table II

| Time (h) | Animal Condition | Carnitine | [3H]Carnitine |
|---------|-----------------|-----------|---------------|
|         |                 | Total (nmol/ml) | Ester (%) | Total (10^3 cpm/ml) | Ester (%) |
| 1       | Fed             | 36.5 ± 1.20 | 32.6 ± 1.11 | 35.5 ± 3.20 | 67.9 ± 3.10 |
|         | Starved         | 52.1 ± 1.18 | 54.2 ± 2.31 | 40.5 ± 2.15 | 84.0 ± 4.20 |
| 2       | Fed             | 37.8 ± 1.20 | 33.2 ± 1.88 | 19.3 ± 1.05 | 61.5 ± 3.08 |
|         | Starved         | 33.7 ± 2.11 | 55.7 ± 2.78 | 22.1 ± 1.52 | 77.9 ± 1.06 |
| 24      | Fed             | 40.1 ± 2.21 | 28.3 ± 1.00 | 9.12 ± 0.66 | 32.2 ± 2.10 |
|         | Starved         | 34.4 ± 1.28 | 57.0 ± 3.11 | 10.2 ± 0.61 | 55.1 ± 2.85 |

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a Determined by enzymatic analysis.
b Determined by HPLC separation and includes counts/min in the acetylcarnitine plus propionylcarnitine peak.

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Origin of Surplus Plasma Acylcarnitines in Ketotic State
to extrahepatic tissues via carnitine as carrier, in accord with recent observations by Snoswell and co-workers (17) on sheep.

The second question addressed is how the increment in plasma acylcarnitines develops with fasting. As shown in Table II, the acylation of the newly synthesized [3H]carnitine also markedly increased with fasting (84.0 versus 67.9% in the 1-h experiment and 77.9 versus 61.5% in the 2-h experiment). This observation, together with the fact that [3H]carnitine was acylated in the liver, proves' that the surplus plasma acylcarnitines derive from the liver.

The contribution of newly synthesized carnitine from the liver to circulating carnitine can be calculated by dividing the plasma carnitine counts/min by the apparent specific activity in the corresponding liver. Calculating with data from the 2-h experiment (Table II), the ester content did not change either when the perfusates of livers from fed and fasted rats were perfused in outflowing fashion at the rate of 10 ml/min as previously described (9). The perchloric acid extracts of liver tissue and perfusate were analyzed for acid-soluble total and ester carnitine as specified under "Experimental Procedures." Values are means ± S.E. for three animals.

**TABLE III**

| Animal condition | Liver tissue | Perfusion | Ester fraction of carnitine and [3H]carnitine in the tissue and perfusate of livers from fed and fasted rats |
|------------------|--------------|-----------|---------------------------------------------------------------|
| Origin          | Carnitine<sup>a</sup> | [3H]Carnitine | Carnitine<sup>b</sup> | [3H]Carnitine<sup>b</sup> |
|                  | Total | Ester | Total | Ester | Total | Ester | Total | Ester |
| Fed             | 158 ± 8.30 | 40.1 ± 2.03 | 555 ± 60.1 | 46.5 ± 1.90 | 1.74 ± 0.11 | 67.3 ± 2.10 | 11.9 ± 0.51 | 80.6 ± 2.22 |
| Starved         | 257 ± 10.2 | 35.1 ± 1.82 | 714 ± 30.1 | 38.3 ± 2.08 | 1.76 ± 0.15 | 67.4 ± 3.01 | 10.0 ± 0.50 | 82.5 ± 2.35 |

<sup>a</sup> Obtained by enzymatic analysis.

<sup>b</sup> Obtained by HPLC separation and includes counts/min in acetylcarnitine plus propionylcarnitine peak.

**TABLE IV**

| Animal condition | Additions | Liver tissue | Perfusion | Total | Ester | Total | Ester |
|------------------|-----------|--------------|-----------|-------|-------|-------|-------|
|                  |           |              |           |       |       |       |       |
| Experiment I     |           |              |           |       |       |       |       |
| Fed             | 5 mM glucose plus 0.3 mM oleate | 114 ± 12.7 | 38.4 ± 2.43 | 1.02 ± 0.25 | 60.8 ± 3.84 |
| Starved         | 4 mM glucose plus 0.7 mM oleate | 227 ± 9.77 | 40.7 ± 3.95 | 0.75 ± 0.12 | 60.3 ± 8.20 |
| Experiment II    |           |              |           |       |       |       |       |
| Starved         | None | 205 ± 11.2 | 39.2 ± 2.11 | 0.79 ± 0.10 | 63.7 ± 5.10 |
| Starved         | 2.1 mM oleate | 199 ± 15.1 | 43.2 ± 2.05 | 0.60 ± 0.07 | 69.0 ± 5.50 |
| Starved         | 3.5 mM oleate | 217 ± 10.4 | 46.2 ± 3.55 | 0.54 ± 0.05 | 75.0 ± 7.20 |

Rats were injected with 15 × 10⁶ cpm [3H]butyrobetaine 1 h before the experiment. Livers from fed and 48-h starved rats were perfused in outflowing fashion at the rate of 10 ml/min as previously described (9). The perchloric acid extract of liver tissue and perfusate were analyzed for acid-soluble total and ester carnitine as specified under "Experimental Procedures." Values are means ± S.E. for four animals.

Livers from fed and 48-h starved rats were perfused in outflowing fashion in the presence of the substrates indicated. The perchloric acid extracts of liver tissue and perfusate were analyzed for acid-soluble total and ester carnitine as specified under "Experimental Procedures." Values are means ± S.E. for four animals.

A further question posed in this work is what mechanism leads to the increased plasma [acylcarnitine] in fasting? Since the surplus plasma acylcarnitines derives from the liver, the most plausible mechanism would be that liver in fasting released carnitine with a higher ester content. To answer this we perfused livers from fed and fasted rats and measured the ester content in total carnitine and [3H]carnitines. The perfusions in this case were performed in outflowing fashion to prevent reuptake, unlike former recirculating perfusions (3, 9). From the results of Table III, it can be seen that in the absence of added substrates the ester content of released carnitine did not change when the livers were either from fed or fasted rats. In another experiment (Table IV, Experiment I), the ester content did not change either when the perfusates were supplemented with substrates at physiological concentrations (18). An increased release of carnitine esters could be forced only with 3.5 mM oleate (Table IV, Experiment II) which was 5 times physiological (and caused hemolysis of red blood cells). The results show that livers even from fed rats release carnitine and [3H]carnitine (Table III) with ester content as high as (or a little higher) that found in the plasma of fasted rats (Table II). Not surprisingly, this value did not increase further in the perfusate when livers were from fasted animals, suggesting that liver does not supply extra acylcarnitines into the bloodstream in the fasting state. This result could also be predicted from the fact that the ester fraction did not increase in the liver tissue either with fasting (Tables I, III, and IV).

To account for these observations, we propose that the increase of [acylcarnitines] in the plasma of fasted rats is caused by an altered handling in extrahepatic tissues. This may be either a decreased uptake and utilization of acylcarnitines or an increased uptake of free carnitine by extrahepatic tissues in the fasted state. The lowered total plasma carnitine in fasting (Table II) suggests an increased uptake of free carnitine. To investigate this possibility, we would also need to know how carnitine turnover changes in fasting, which requires further studies. An altered renal handling cannot play a significant role because carnitine excretion is markedly
reduced by the 2nd day of starvation in rats and the ester fraction of excreted carnitine does not change (1, 19).

The results of Tables III and IV are also interesting in terms of carnitine transport. While the relative contribution of carnitine esters to the total acid-soluble carnitine in the liver tissue ranged between 30 and 40%, the liver released carnitine containing 60–67% ester. This is in accord with the report that rat liver releases acylcarnitines at twice the rate of free carnitine release (20). It seems that the transport protein promoting the release of carnitines (9) prefers acylcarnitines or that, in its microenvironment, substrate channeling takes place.

The present experiments also revealed that the newly synthesized [3H]carnitine had not quite equilibrated with the liver carnitine pool 1 h after administration of [3H]butyrobetaine. The percentage of esters based on label distribution was higher than that measured enzymatically both in the tissue (Tables I and III) and in the effluent of perfused liver (Table III). Also, as shown in Table III, the specific activity of total carnitine was twice as high in the perfusate (6839 cpm/nmol) as in the tissue (3512 cpm/nmol) for fed animals. Obviously, the apparent tissue specific activity of carnitine used above to estimate the hepatic contribution to the circulating carnitine pool was an underestimate. (In another experiment, not shown, the perfusion was done 2–2.5 h following administration of [3H]butyrobetaine, and the specific activities in the tissue and effluent were almost the same.) The reason for these observations may be that butyrobetaine hydroxylase (the enzyme responsible for making carnitine from butyrobetaine) is involved in some protein-protein interactions that result in a substrate channeling in the microenvironment of the enzyme.

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