Fatty Acid and 3-\(\beta\)-Hydroxysterol Synthesis in the Perfused Rat Liver

INCLUDING MEASUREMENTS ON THE PRODUCTION OF LACTATE, PYRUVATE, \(\beta\)-HYDROXYBUTYRATE, AND ACETOACETATE BY THE FED LIVER*

(Received for publication, October 5, 1972)

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SUMMARY

Conditions are described for perfusing rat livers in vitro which result in rates of fatty acid synthesis which equal the rates observed in vivo. Rates of 3-\(\beta\)-hydroxysterol synthesis by the perfused liver are equal to or greater than those observed in vivo.

Livers from starved animals perfused with low concentrations of glucose show low rates of fatty acid and 3-\(\beta\)-hydroxysterol synthesis. When such livers are perfused with high concentrations of glucose they show a substantial increase in the rate of fatty acid and 3-\(\beta\)-hydroxysterol synthesis. These increases are compared with similar increases in vivo, which are observed when starved animals are refed.

Relationships are examined between rates of fatty acid synthesis and the production of lactate, pyruvate, \(\beta\)-hydroxybutyrate, and acetoacetate, as well as the oxygen consumption. Metabolite contents are reported for perfused livers that exhibit high and low rates of fatty acid synthesis.

The rate of fatty acid synthesis is controlled by two different mechanisms which can be distinguished on the basis of the time which they take to come into operation. One is a short term mechanism; in the rat it comes into play in as little as 30 min, although it may take as long as 2 hours or more to come into full effect. The other is a long term mechanism; it comes into play in 6 to 8 hours or more, and it takes 2 to 4 days to come into full effect (1).

Experimental evidence suggests that the short term control of fatty acid synthesis does not involve changes in the total amounts of the enzymes involved in fatty acid synthesis. It has been inferred that short term control is exerted by changes in the intracellular concentrations of metabolites which act as activators or inhibitors. However, so far it has proven difficult to identify the modifiers which control the rate of fatty acid synthesis in the intact cell (1-4). Long term effects involve changes in the total amounts of the enzymes involved in fatty acid synthesis, in particular of citrate cleavage enzyme, acetyl-CoA carboxylase and fatty acid synthase (1, 4, 5).

The liver of the rat exhibits very high rates of fatty acid synthesis. In vivo rates of animals fed a diet which is high in glucose and which lacks fat, fall in the range of 1 to 2.5 pmoles of acetyl group incorporated into long chain fatty acid per g dry weight of liver per min (6). Such high rates have not been reported for liver slices. The present paper describes conditions for perfusing rat liver which result in rates of fatty acid and 3-\(\beta\)-hydroxysterol synthesis that equal those observed in vivo. The total rate of fatty acid synthesis, which was obtained by measuring the incorporation of \(^{14}\)C from \(^{14}\)C glucose incorporation into fatty acids and with the rates of formation of lactate plus pyruvate, \(\beta\)-hydroxybutyrate plus acetoacetate, as well as with the rate of operation of the citric acid cycle.

EXPERIMENTAL PROCEDURE

Methods—Rats of the Sprague-Dawley strain were obtained from the Charles River Breeding Laboratories, North Wilminton, Mass. Their weight on arrival was 100 to 110 g. The animals were placed on a scheduled diet high in glucose (7) given to one animal per cage from 9 am. to 12 noon (6). Water was made available ad libitum. The animals were weighed every day before the meal. After an initial loss, the weight of the rats remained almost constant for about 6 days and then increased steadily (Fig. 1). The animals were used when they were in the weight range of 145 to 155 g, during the 4th week of the feeding schedule. In each group of 15 rats, the two fastest and the two slowest weight gainers were discarded. Experiments on fed rats were started between 12 noon and 1 p.m., that is to say 3 to 4 hours after the animals started their last meal. This period corresponds to the maximum rate of fatty acid synthesis in vivo (6).
Livers were perfused with Krebs-Ringer bicarbonate buffer (8) containing 4% bovine serum albumin (Fraction V, fatty acid poor, Miles Biochemicals, Kankakee, Ill.) and glucose as indicated. Analyses by the method of Novák (9) showed that the batches of bovine serum albumin used by us contained less than 0.1 mole of free fatty acid per mole of albumin. Before use, a 10% solution of albumin was dialyzed against 30 volumes of Krebs-Ringer bicarbonate buffer (10) at 5°C with six changes of buffer for 48 hours. Enzymatic analyses (11) showed that the albumin contained 0.83 mole of citrate per mole of albumin before dialysis and 0.016 mole of citrate per mole of albumin after dialysis. Measurements of citrate in the perfusate showed that it did not increase during 2 hours of perfusion. The initial concentration of free fatty acid, which was introduced with the albumin, was always less than 0.06 mM. Where indicated the perfusate also contained the amino acid mixture recommended by Mallette et al. (12).

Metabolite contents were determined fluorometrically by enzymatic methods according to the procedures described by Williamson and Corkey (11), except that oxaloacetate was determined by the method of Löfler and Wieland (13).

**Materials**—Pyridine nucleotides were purchased from P-L Biochemicals. The enzymes used in the assays were purchased from Boehringer and Worthington. An exception was α-ketoglutarate dehydrogenase, which was prepared according to the procedure of Sanadi (14). Contaminating glutamate dehydrogenase was largely removed by centrifuging the preparation at 100,000 × g for 2 hours and resuspending the sediment in 20 mM phosphate buffer, pH 7.0 (15). The centrifugation step was repeated once more. Radioactive compounds were obtained from New England Nuclear. All other chemicals were of reagent grade or the purest grade available commercially.

**Perfusion Apparatus**—The apparatus, which is shown in Scheme 1, combines various features of the designs described by Hems et al. (16), Scholz (17), and Staib et al. (18), plus modifications of our own, which we have found to possess advantages over other designs. The details of the apparatus were evolved in a series of 100 perfusions; it has been used in its present form for 300 perfusions.

The whole apparatus is set up in an illuminated cabinet which is kept at 38°C by means of a heater, fan, and thermostat. The air circulation is designed to be gentle and uniform. This arrangement is inadequate for keeping the apparatus at a constant temperature, its purpose is to bring the apparatus near the correct temperature. The perfusate is brought to exactly 38°C by the water-jacketed oxygenator just up-stream of the liver.

Most of the perfusate is contained in a flat-bottomed glass reservoir of 125-ml capacity (H. Vincent, 14 Lyndworth Close, Headington, Oxford OX3 9ER, England), with five top inlets and a bottom outlet. A peristaltic pump (model 1203, Harvard
Apparatus Co., Millis, Mass. 02054) sends the perfusate from the reservoir through a Swinnex-47 filter holder containing a glass fiber prefilter and a Millipore filter with a pore size of 1.2 μm, 47 mm in diameter (Millipore Corporation, Bedford, Mass. 01730). The perfusate then passes to a water-jacketed oxygenator maintained at 38° (also supplied by H. Vincent). The water jacket eliminates fluctuations of the order of 1 or 2° in the temperature of the perfusate entering the liver. The oxygenator is gassed with 95% O₂-5% CO₂ at a rate of 1 liter per min. From the oxygenator the perfusate is sent to the liver by a low pulsing pump (Polyostaltic pump, Buchler Instruments, Fort Lee, N. J. 07024). An overflow at the bottom of the oxygenator returns excess perfusate to the reservoir. The liver is held in a Lucite block by an adjustable clamp in such a manner that it is in part suspended from the inferior vena cava cannula by a residual fragment of the pericaval diaphragm, and in part resting on the perforated platform in the center of the block. The perfusate is passed into a bubble trap which is located in a corner of the block. The bubble trap is connected via a short piece of tubing to the portal vein cannula, which rests in a horizontal slot cut into the block. The perfusate leaves the liver through the inferior vena cava cannula and most of it is drained into the reservoir.

A portion of the effluent is diverted immediately after the cannula and is pumped at a constant rate, say 3 ml per min, past an oxygen electrode and then into the reservoir. The tubing leading from the cannula to the oxygen electrode is made of glass, except for the portion that passes through the peristaltic pump, which is made of thick-walled, narrow internal diameter Tygon tubing (3/16 inch outer diameter, 3/32 inch inner diameter). Oxygen consumption is measured by modifying the apparatus to permit the measurement of both influent and effluent PO₂. A portion of the influent is diverted immediately after the bubble trap and is pumped at a constant rate, say 3 ml per min, past an oxygen electrode and then into the reservoir. The electrode (type E5046) is mounted in Chamber D616 and is connected to blood analyzer PHM71 (all obtained from Radiometer, Copenhagen).

The bottom drain in the perfusion block serves to drain the perfusate before the inferior vena cava cannula is connected to the outflow tubing. After the circuit has been closed, liquid oozing from the liver passes from the bottom of the drain in the Lucite block to the reservoir, but livers which ooze are rejected. Two three-way stopcocks are located between the oxygenator outlet and Pump 2. They connect the main circuit with two reservoirs containing preperfusion fluids. One reservoir (connected via Stopcock B) contains about 500 ml of Krebs-Ringer bicarbonate buffer with or without albumin; it is used for perfusing the liver during surgery. The second reservoir (connected via Stopcock A) contains 50 ml of Krebs-Ringer bicarbonate buffer with albumin; it is used when the operation is completed, just before switching the liver to the main perfusate. Both perfusion fluids are equilibrated with 95% O₂-5% CO₂ and pass through small auxiliary preheating coils kept at 38°. (Small coil condensers are suitable for this purpose.) During the preperfusion period, Stopcock A is used to shut off the oxygenator outlet, and the perfusate running through the oxygenator is returned to the reservoir via the oxygenator overflow.

The contents of the reservoir are mixed with a magnetic stirrer. The reservoir is fitted with a glass electrode connected to a pH meter (Radiometer model 51). The pH meter is linked to a titrator (Radiometer model 11), which operates a magnetic valve (Radiometer, type MNV 1c). The latter operates a burette containing 0.284 N NaOH plus 0.006 N KOH which is connected to the reservoir via polyethylene tubing. The titrator is set to maintain the pH at 7.40.

The effluent gas of the oxygenator is passed through a drying tube containing anhydrous CaCl₂, followed by a drying tube containing magnesium perchlorate. These serve to trap H₂O. The gas is then passed through two gas washing bottles containing 5 N NaOH, followed by one containing 0.1 N Ba(OH)₂. A build up of gas pressure in the apparatus is avoided by sucking the gas through the train of drying tubes and washing bottles by means of a water pump. A drop in pressure below atmospheric is avoided by means of an oil valve between the oxygenator outlet and the first drying tube. Dilution of the gassing mixture by air is prevented by extending the perfusate overflow line from the oxygenator below the perfusate level in the reservoir.

The apparatus offers the following advantages. The bulk of the perfusate is contained in an easily accessible reservoir. Additions to the reservoir are mixed rapidly and completely. A constant pH is maintained automatically, and the rate of alkaline consumption can be recorded as a function of time. The technique permits a constant rate of perfusion to be chosen so that a given effluent PO₂ is maintained.

Perfusion Technique—The method described below avoids anesthesia and anaerobic conditions. It yields rates of fatty acid synthesis comparable to rates observed in vivo.

The rat is killed by a blow on the head (but not on the neck or the back). Immediately thereafter an assistant holds the animal firmly flat on its back, grasping the four limbs to oppose contractions. A large V-shaped incision is made into the abdomen from the pubis toward the flanks with a sharp pair of scissors, grasping through fur and muscle. The portal vein is located, grasped with forceps, an entrance hole is knicked with micro-scissors, and an 18-gauge plastic cannula is inserted through which oxygenated Krebs-Ringer bicarbonate with or without 4% bovine serum albumin, kept at 38°, is perfused at a rate of 30 ml per min throughout the entire operation. The Krebs-Ringer solution contains glucose at the same concentration as that used during the experimental period. The time elapsed between the blow on the head and the portal vein cannulation should not exceed 20 to 30 s. The liver should be completely cleared of blood in 30 to 45 s after cannulation. The portal vein and the cannula are held gently but firmly in the correctly aligned positions by the operator until the rat has stopped moving. (The portal vein is held with forceps in one hand, and the cannula is held in the other hand.) As soon as possible the inferior caval vein is cut below the kidneys to prevent the liver from swelling and to facilitate the washing out of blood. Two ligatures are then made and tightened around the portal vein cannula, one between the entry of the duodenal vein and the hilum of the liver, and the second on the mesenteric side of the duodenal vein. The liver is separated from the stomach, the spleen, and the intestine; and the bile duct is cut off at the hilum of the liver. A loose ligature is put around the inferior caval vein (Ligature a) just above the right renal vein. The thorax is opened, and a loose ligature is put around the inferior caval vein between the heart and the diaphragm (Ligature b). A 10-gauge plastic cannula is pushed through the right auricle into the atrium and thence into the inferior caval vein until it almost reaches the liver. (If desired, an entrance hole for the cannula can be prepared by knicking the right auricle with small scissors.) Ligature b is tightened; then a piece of diaphragm is tied with the same thread in a second knot. Ligature
a is tightened, and the inferior caval vein is cut caudally, close to the ligature. The assistant now raises the rat by the neck. The operator lifts the heart cannula and cuts away the heart, the esophagus, and the posterior part of the diaphragm. (Both ligatures remain with the preparation.) The liver preparation is put into the Lucite perfusion block; the latter is drained by suction. The diaphragm is trimmed except for the tendinous portion, and the esophagus is dissected out. The liver is rinsed with Krebs-Ringer bicarbonate at 38°. A thermocouple (Yellow Springs Instruments) is slipped between two lobes, and a Lucite cover is put on the perfusion block.

The perfusate is now changed to 50 ml of Krebs-Ringer bicarbonate containing 4% bovine serum albumin, and the effluent perfusate is discarded. The recirculating perfusion is now started by switching over to the main circuit. This is done by connecting the heart cannula to the line which returns most of the effluent to the reservoir. The bottom drain of the Lucite block is also connected to the reservoir. The closing of the main circuit is taken as zero time; usually 8 to 10 min elapse between starting the operation and zero time. The recirculating perfusate has a volume of 150 ml. A portion of the effluent is pumped at a constant rate (say 3 ml per min) past an oxygen electrode and thence back into the reservoir. A pCO₂ electrode can be mounted in series if desired.

The bile duct is cut purposely to prevent complete loss of the compounds reabsorbed by the gut in vivo, in the so-called enterohepatic cycle. These substances are thus recycled, although they become diluted in the perfusate. If desired, the bile duct can be cannulated just after the portal vein ligatures are tied.

During the perfusion the gross appearance of the liver is watched, and oozing of liquid from the surface of the organ is assessed. In a successful perfusion the liver retains an even and undamaged appearance, and oozing is minimal. The effluent pO₂ is monitored to check on the adequacy of the oxygen supply. Samples of perfusate are taken at 5-, 10-, or 15-min intervals and the concentrations of pyruvate, lactate, β-hydroxybutyrate, acetoacetate, and glucose are determined (19-29). The ratios of [lactate] to [pyruvate] and [β-hydroxybutyrate] to [acetoacetate] are used to monitor the oxidation-reduction status of the extra- and intramitochondrial compartments, respectively (17, 23). Less than 1 liver in 20 exhibited unusual ratios; these were rejected. Acceptable ratios are given under “Results.”

At the end of the experiment the liver is lifted out of the perfusion block by the heart cannula with the perfusate still running. The liver is clamped rapidly between aluminum blocks which are precooled in liquid nitrogen (24). The livers are stored in a liquid nitrogen freezer.

The average dry weight of rat livers after 2 hours of perfusion with the Krebs-Ringer bicarbonate medium containing albumin was 23% of the wet weight for 150 perfusions. The average dry weight determined on rat livers taken from animals without perfusion was 31%. Both groups of animals received the scheduled diet high in glucose which lacked fat. The determinations of dry weight to wet weight ratios of livers which were not perfused were carried out 5 to 6 hours after the animals received their last scheduled meal. The difference in dry weight of 8% can be accounted for in part by the absence of blood from the perfused livers as compared to the in vivo livers. Calculations show that the absence of blood lowers the dry weight by about 3%. Our wet weight to dry weight ratios are in keeping with those reported for fed rat livers by Woods and Krebs (25), who found 29% dry weight for livers not subjected to perfusion, and 26% dry weight for livers perfused with red cells. Unless otherwise indicated, labeled compounds were added to the reservoir 58 min after starting the perfusion.

Maintenance of Glucose Concentrations during Measurements of Rates of Lipid Synthesis—The effect of varying the initial glucose concentration in the perfusate upon the glucose concentration of the perfusate after equilibrium had been attained was investigated in a series of preliminary experiments. In the case of animals which were fed their last meal 3 ½ hours before the start of the perfusion the results were as follows. When glucose was omitted from the perfusate, the livers released glucose until the concentration in the perfusate was 4 mm. This level was reached about 45 min after starting the perfusion. Thereafter the concentration of glucose in the perfusate remained approximately constant. As 4 mm glucose was the lowest steady state level that we were able to attain when perfusing livers from schedule-fed animals, this is the lowest reported under “Results.” When the perfusion was started with 5 mm glucose in the perfusate, the livers released glucose until a steady state perfusate level of 8 mm was reached after about 45 min. When the perfusion was started with 15 mm glucose in the perfusate, the level remained constant throughout the perfusion. Lastly, when the perfusion was started with 25 mm glucose in the perfusate, the level remained at 23 mm or declined slightly; the maximum decline observed was to a level of 22 mm.

In the course of animals which missed their last meal before the perfusion, a steady state perfusate level of 4 mm glucose was achieved by starting the perfusion with 1 mm glucose. In the case of animals which were starved for 2 days before the perfusion, a steady state perfusate level of 4 mm glucose was achieved by starting the perfusion with 4 mm glucose. Lastly in the case of animals which were starved for 1 or 2 days, steady state levels in the range of 23 to 25 mm glucose were achieved by starting the perfusions with 25 mm glucose. The actual concentrations of glucose in the perfusate were measured at 10- or 15-min intervals during each perfusion as described under “Methods.” The autoregulation of glucose uptake and output in perfused liver from starved rats was studied by McCreaw et al. (26), who found a balance point between uptake and output of glucose to occur at 12 mm. These authors also reviewed earlier work on this subject.

Specific Activity of H₂O—An aliquot of perfusate is withdrawn at 90 min, that is in the middle of the period during which H₂O is present in the perfusate, and is diluted and counted in a liquid scintillation counter using the fluid recommended by Bray (27). A water concentration of 52.8 m is used to calculate the specific radioactivity of the water. This concentration can be calculated from the specific gravity and the dry weight to wet weight ratio of the perfusate.

Specific Activity of [¹⁴C]Glucose—Samples (0.2 ml) of perfusate are deproteinized according to Nelson (28). An aliquot of the supernatant is treated with a mixture of cationic and anionic resins. Exton and Park (29) have shown that this procedure removes practically all ionized compounds derived from [¹⁴C]-glucose metabolism. An aliquot of the resin-treated supernatant is counted, and another aliquot is assayed enzymatically for glucose (22). Radioactive Precursor Incorporation into 3-β-Hydroxyysteroids—The procedure which follows is based on the method of Sperry and Webb (30). The amounts of reagents are quoted for 1 g fresh weight of liver. The liver is homogenized with 19 ml of chloroform-methanol (2:1) in a Sorvall Omni-Mixer. The stainless steel cup of the mixer has a capacity of about 60 ml. The extract is filtered, evaporated under nitrogen, and saponified.
with 2 ml of 5 x NaOH at 80° for 3 hours. The turbid, alkaline solution is diluted with 5 ml of water and is extracted three times with 8 ml of petroleum ether. The pooled extract is evaporated to dryness and is dissolved in 5 ml of acetone-ethanol (1:1), acidified with 1 drop of 10% acetic acid, and precipitated with 2 ml of 0.5% digitonin in 50% ethanol (30). The digitonide precipitate is washed with 6 ml of acetone-ether (1:2) followed by 6 ml of ether. It is dissolved in 1 ml of methanol and counted in toluene scintillation fluid.

Contamination of the digitonide fraction by radioactive fatty acids was checked as follows. The petroleum ether extract containing the total nonsaponifiable fraction is extracted with 5 ml of 0.1 x NaOH. The aqueous phase is separated, acidified, and extracted with petroleum ether. The petroleum ether extract is evaporated and counted. In 13 samples examined, an average of 1.0 ± 0.09% of the total radioactivity of the nonsaponifiable fraction could be extracted in this manner.

Radioactive Precursor Incorporation into Long Chain Fatty Acids.—The aqueous residue of the alkaline extraction is acidified with 1.25 ml of 10 N HSO₄ and is extracted three times with 8 ml of petroleum ether. The extract is evaporated and counted in toluene scintillation fluid.

Counting efficiencies of all samples are determined by recounting each sample in the presence of internal standards of either [1H]toluene or [14C]toluene or both. Rates of fatty acid and 3-β-hydroxysterol synthesis are expressed as micromoles of hydrogen from H₂O incorporated per g dry weight per hour. These values are converted to micromoles of acetyl group incorporated into fatty acids by multiplying by 1.15 (31) or to micromoles of acetyl group incorporated into 3-β-hydroxysterols by multiplying by 1.31 (32).

Analyses for radioactive fatty acids and 3-β-hydroxysterols released into the perfusate were performed as follows. A sample of each perfusate (25 ml) is freeze-dried. The residue is extracted with 25 ml of a mixture containing 1.25 ml of water, 15.8 ml of chloroform, and 7.9 ml of methanol for 12 hours. The resulting extract is filtered, evaporated, and saponified with 9 ml of 5 N NaOH. The nonsaponifiable and saponifiable fractions are separated and counted.

RESULTS

Rates of Fatty Acid and 3-β-Hydroxysterol Synthesis in Perfused Liver.—Fig. 2 shows rates of fatty acid synthesis obtained in livers from schedule-fed rats which had been starved for 1 or 2 days. In the presence of 25 mM glucose, livers from animals which received the last schedule-fed meal just before the perfusion was started showed a rate of 170 pmol of acetyl group incorporated into fatty acids per g dry weight per hour. In contrast to this, livers from animals which missed the last one or two of the scheduled meals showed rates of 57 and 29 pmol of acetyl group incorporated per g dry weight per hour. In the presence of 4 mM glucose the rates of fatty acid synthesis were reduced to 72 pmol of acetyl group incorporated per g dry weight per hour in the case of livers from fed animals; and to 25 and 31 pmol of acetyl group incorporated in the case of livers from animals starved 1 and 2 days, respectively.

The rate of fatty acid synthesis reaches one-half of maximum when the glucose concentration in the perfusate is about 5 mM, 90% of maximum when it is 8 mM, and maximum when it is 15 mM. The rate of fatty acid synthesis at 25 mM glucose is essentially the same as that observed at 15 mM glucose. Two perfusions were run in the presence of 50 mM glucose, these yielded the same rates of fatty acid synthesis as were obtained with 15 and 25 mM glucose (not shown in Fig. 2).

Two types of perfusate were used, one lacked added amino acids, the other contained a mixture of amino acids similar to that found in rat plasma (12). No statistically significant differences in the rates of fatty acid synthesis were observed in the presence and absence of the amino acid mixture. As is shown later, significant differences in acid production were observed between perfusions containing and lacking the amino acid mixture.

Newly synthesized fatty acids which were released into the perfusate were 8.7 ± 1.1% (S.E.) (n = 17) of the amounts found in the livers. This percentage was independent of the glucose concentration in the perfusate. The form in which the fatty acids were released was not studied. Unless otherwise indicated, the amounts released are not included in the graphs and tables.

Unlike fatty acid synthesis, the rate of 3-β-hydroxysterol synthesis in livers of fed rats does not change as the glucose concentration of the perfusate is increased from 4 to 25 mM. In livers of rats which have been starved 2 days, the rate is greatly diminished in the presence of 4 mM glucose, but it is diminished to a much smaller extent in the presence of 25 mM glucose (Fig. 3).

Comparison of Rates Observed in Perfused Livers with Rates Observed in Vivo.—In order to obtain a valid comparison of the rates of fatty acid and 3-β-hydroxysterol synthesis obtained in perfused livers, a series of similar measurements was made on rat livers in vivo. The animals used in these experiments were treated identically in every respect to those used for the perfusions, and the in vivo measurements were conducted at times corresponding to the perfusion measurements. Results are
Fig. 1. Rates of fatty acid synthesis in perfused livers and in vivo

The results of Figs. 1 and 2 and Table I have been combined in simplified form. Rates obtained with livers from fed rats perfused with 25 mM glucose are compared with rates obtained in vivo in livers of fed rats. Results obtained with livers from starved rats perfused with 4 mM glucose are compared with rates obtained in vivo in livers of starved rats. Results obtained with livers from starved rats perfused with 25 mM glucose are compared with rates obtained in vivo with livers of starved rats which were reared for 1 hour. Note that the rates were measured during the 2nd hour of the perfusions. All rates are expressed as micromoles of acetyl group incorporated per g dry weight per hour.

![Graph](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Nutritional status</th>
<th>(Glucose) in perfusate</th>
<th>Rate of fatty acid synthesis</th>
<th>Rate of 3-β-hydroxysterol synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>25</td>
<td>170</td>
<td>4.1</td>
</tr>
<tr>
<td>Starved 1 day</td>
<td>4</td>
<td>25</td>
<td>3.8</td>
</tr>
<tr>
<td>Starved 2 days</td>
<td>4</td>
<td>3.2</td>
<td>0.65</td>
</tr>
<tr>
<td>Starved 1 day, a or starved 1 hr, b</td>
<td>25</td>
<td>73</td>
<td>3.8</td>
</tr>
<tr>
<td>Starved 2 days, a or starved 2 days and reared 1 hr, b</td>
<td>25</td>
<td>12</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*In the case of perfusions.

†In the case of in vivo experiments.

Nutritional status

<table>
<thead>
<tr>
<th>Fed</th>
<th>Starved 1 day</th>
<th>Starved 2 days</th>
<th>Starved 1 day, a or starved 1 hr, b</th>
<th>Starved 2 days, a or starved 2 days and reared 1 hr, b</th>
</tr>
</thead>
<tbody>
<tr>
<td>154.3 ± 11.6 g</td>
<td>16.9 ± 3.2 g</td>
<td>4.8 ± 1.0 g</td>
<td>154.6 ± 19.1 g</td>
<td>58.9 ± 3.0 g</td>
</tr>
<tr>
<td>178 g</td>
<td>19 g</td>
<td>5.5 g</td>
<td>178 g</td>
<td>66 g</td>
</tr>
<tr>
<td>3.35 ± 0.52 μmol</td>
<td>0.55 ± 0.08 μmol</td>
<td>0.22 ± 0.05 μmol</td>
<td>0.80 ± 0.23 μmol</td>
<td>0.21 ± 0.03 μmol</td>
</tr>
<tr>
<td>g dry wt liver/hr</td>
<td>g dry wt liver/hr</td>
<td>g dry wt liver/hr</td>
<td>g dry wt liver/hr</td>
<td>g dry wt liver/hr</td>
</tr>
</tbody>
</table>

shown in Table I. In order to facilitate comparison of the results obtained in the perfusions with those obtained in the in vivo experiments, some of the data shown in Figs. 2 and 3 and in Table I have been combined to give Table II.

The rate of fatty acid synthesis in livers of rats fed the scheduled diet is the same in perfused livers as in livers in vivo, namely 170 versus 178 μmoles of acetyl group incorporated per g dry weight per hour. The rates are also approximately the same after 21 hours of starvation, namely 25 versus 19 μmoles. The perfused livers exhibit lower rates than livers in vivo in the case of animals which were starved for 45 hours.

It was thought that the perfusion of livers from starved animals with 25 mM glucose would mimiick refeeding of starved animals. Accordingly livers were perfused with 25 mM glucose for 1 hour prior to measuring the rate of fatty acid synthesis. This treatment resulted in a large increase in rate compared to livers from similar animals which were perfused with only 4 mM glucose. In the case of perfused livers obtained from rats starved for 21 hours the change was from 25 to 73 μmoles. With perfused livers from rats starved 45 hours the change was from 9.2 to 12 μmoles. These increases in the rate of fatty acid synthesis suggest that the perfusion of starved livers with high concentrations of glucose resembles the refeeding of starved animals.

The change in the rate of fatty acid synthesis in vivo observed when rats starved for 21 hours were reared for 1 hour was from 19 to 178 μmoles. When rats starved for 45 hours were reared for 1 hour, the change was from 3.5 to 66 μmoles. This comparison shows that the changes in the rate of fatty acid synthesis which occur in vivo after refeeding are greater and more rapid than those observed by us in perfused livers. Additional factors must come into play in vivo which are lacking or which are suppressed in our perfusion experiments.

Concerning 3-β-hydroxysters synthesis, the rates observed with the perfused livers were all somewhat higher than those observed in vivo (Table II). As has already been mentioned, in the perfused liver, the rate of 3-β-hydroxysters synthesis does not respond to increasing glucose concentrations in the same manner as does the rate of fatty acid synthesis. Taken together, these observations suggest strongly that there are factors which
regulate 3-β-hydroxy-sterol synthesis independently of the regulation of fatty acid synthesis. This statement does not rule out factors which are common to both pathways.

Lactate and Pyruvate Production and β-Hydroxybutyrate and Acetoacetate Production by Perfused Liver from Schedule-fed Animals—Pyruvate is the immediate precursor of acetyl-CoA during the conversion of carbohydrate into fatty acids. The major rates of the acetyl group of acetyl-CoA are conversion into long chain fatty acids, β-hydroxybutyrate, acetoacetate, and CO₂. In addition to measuring the rate of fatty acid synthesis, we also measured the rates of formation of pyruvate and lactate, and of β-hydroxybutyrate and acetoacetate as a function of the glucose concentration in the perfusate. Results are shown in Figs. 4 and 5. Combustion of acetyl groups to CO₂ via the citric acid cycle was calculated from the oxygen consumption.

The initial rates of lactate plus pyruvate output by the perfused liver are 10.9, 11.3, 13.3, and 14.5 μmoles per g dry weight of liver per min at perfusate glucose concentrations of 4, 8, 15, and 25 mM, respectively (Fig. 4). The initial rates are 11.43, 5.1, and 4.8 times greater than the rates of fatty acid synthesis observed during the 2nd hour of the perfusion (Fig. 2). In other words, the maximum capacity of the glycolytic pathway is four to five times greater than the maximum rates of fatty acid synthesis observed by us. It should be stressed that such high rates of glycolysis are observed only during the first 15 or 30 min of the perfusion (Fig. 4). There is a particularly sharp reduction in the rate of lactate plus pyruvate output between 30 and 45 min after starting the perfusion; this coincides with the attainment of the balance point in the glucose concentration in the perfusate which has already been described above. In the case of livers perfused with 4 mM glucose, the pyruvate plus lactate concentration remains constant between 45 and 60 min; subsequently, the livers show a slow but significant uptake of lactate and pyruvate. After the initial rapid release of lactate and pyruvate is over, livers perfused with 8 mM glucose show a slow release of lactate and pyruvate which almost stops by 120 min, while livers perfused with 25 mM glucose continue to show an appreciable rate of lactate and pyruvate release during the period between 45 and 120 min. As the glucose concentration in the perfusate is increased, there is a barely perceptible decrease in the oxidation-reduction potential of the lactate-pyruvate couple, from -236 to -241 mv (Fig. 6).

In the absence of fatty acids, livers from fed rats produce β-hydroxybutyrate and acetoacetate at a steady rate which is almost independent of the concentration of glucose in the perfusate. The rates of β-hydroxybutyrate plus acetoacetate output by the perfused liver are 0.71, 0.69, 0.62, and 0.51 μmole per g dry weight per min at glucose concentrations in the perfusate of 4, 8, 15, and 25 mM, respectively, during the 1st hour (Fig. 5). These differences are probably not significant. There is little or no change in the rate of ketone body production during the 2nd hour. Moreover, the oxidation-reduction potential of the β-hydroxybutyrate-acetoacetate couple decreases only slightly with increasing glucose concentrations from -236 to -301 mv (Fig. 6).

The rates of ketone synthesis are equivalent to 1.42, 1.32, 1.24, and 1.02 μmoles of acetyl group incorporated per g dry weight per min at 4, 8, 15, and 25 mM glucose, respectively. The corresponding rates of fatty acid synthesis are 1.2, 2.6, 2.9, and 2.8 μmoles of acetyl group incorporated per g dry weight per min, respectively (Fig. 2). The ratios of the rates of fatty acid synthesis to ketone body production, both expressed as micromoles of acetyl group incorporated, are 0.84, 1.95, 2.32,
and 2.68, at perfusate glucose concentrations of 4, 8, 15, and 25 mm glucose.

The midpoint potentials used in calculating the oxidation-reduction potentials shown in Fig. 6 were: [lactate] to [pyruvate] = -215 mv (33), and [β-hydroxybutyrate] to [acetoacetate] = -297 mv (34). In most perfusions the [lactate] to [pyruvate] ratio fell between 6 and 8 at all times, corresponding to oxidation-reduction potentials of -238 to -242 mv. Occasionally the ratio was as low as 5 or as high as 9 (336 to 214 mv). The [β-hydroxybutyrate] to [acetoacetate] ratio varied over a somewhat wider range; it usually rose from 0.3 to 0.4 at 15 min to 0.8 to 1.2 at 60 min after starting the perfusion. Ratios of 0.8 to 1.2 correspond to -294 to -299 mv. The ratios usually remained constant or fell by about 20% between 60 and 120 min. The ratios and oxidation-reduction potentials quoted above were obtained with livers from rats fed the scheduled diet high in carbohydrate.

The two oxidation-reduction couples exhibit a constant difference of about 57 mv (maximum range 54 to 61 mv), in harmony with the concept that there is a constant difference in the oxidation-reduction balance of the extra- and intramitochondrial [DPNII] to [DPN] ratios, at least under a given set of steady state conditions (17, 34). The constant difference reported by Scholz (17) for livers from starved rats is 40 mv. These authors used the same midpoint potentials as were used in our calculations. The disagreement between our value and theirs can be traced to the [β-hydroxybutyrate] to [acetoacetate] ratios obtained by them.

Acid Production—The automatic titrator was placed so that the effluent from the liver was neutralized before it passed through the oxygenator, where gaseous exchange takes place. This resulted in the titration of both nonvolatile and volatile acids produced by the liver. Acid production, as measured by the amount of alkali added to maintain the pH at 7.40, is shown in Fig. 7. As is to be expected, the total acid production was less in the presence than in the absence of the α-keto acid mixture in the perfusate.

Acid production in the presence of 25 mm glucose and in the absence of the α-keto acid mixture was 11.67 and 6.76 μeq during the 1st and 2nd hours of the perfusion, respectively. Multiplied by 150/1.67 this yields 994 and 607 microequivalents of acid neutralized per g dry weight per hour during the 1st and 2nd hours of the perfusion, respectively (Table III). When the major metabolic processes that produce acid are subtracted from these values, as is shown in Table III, the amount of acid unaccounted for is 73 and 46 microequivalents of acid per g dry weight per hour, or 7.3% and 7.6% of the total acid produced during the 1st and 2nd hour of the perfusion, respectively. This agreement is excellent considering the many processes that are added together in Table III, and considering that minor sources of acid production, such as free fatty acids, were not taken into account.

The calculations in Table III were made on the assumption that the citric acid cycle contributes none and the pentose phosphate pathway contributes 46% of the TPNH, then in the 2nd hour 12% of the acid production would be unaccounted for.
TABLE III
Comparison of total acid production with various metabolic processes

Acid production in the presence of 25 mm glucose and in the absence of the amino acid mixture (Fig. 7) is used as an example.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>1st hour</th>
<th>2nd hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/g dw hr</td>
<td>pmol/g dw hr</td>
</tr>
<tr>
<td>a. Acid neutralized.</td>
<td>994</td>
<td>607</td>
</tr>
<tr>
<td>b. Lactate plus pyruvate.</td>
<td>475</td>
<td>115</td>
</tr>
<tr>
<td>c. a - b.</td>
<td>519</td>
<td>492</td>
</tr>
<tr>
<td>d. d.</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>e. c - d.</td>
<td>488</td>
<td>461</td>
</tr>
<tr>
<td>f. CO₂ produced in citric acid cycle.</td>
<td>292</td>
<td>292</td>
</tr>
<tr>
<td>g. CO₂ produced in pentose phosphate pathway.</td>
<td>161</td>
<td>161</td>
</tr>
<tr>
<td>h. Fraction of h that is bicarbonate at pH 7.40 (h x 11/12).</td>
<td>453</td>
<td>453</td>
</tr>
<tr>
<td>i. CO₂ produced in pentose phosphate pathway.</td>
<td>453</td>
<td>453</td>
</tr>
</tbody>
</table>

* Table V, Line p, Case A x 2.

Metabolite Contents—The livers used in the perfusion experiments described in Fig. 2 were analyzed for the content of certain metabolites at the end of the 2nd hour of perfusion, that is at the end of the hour during which the rate of fatty acid synthesis was measured. The results, which are shown in Table IV, are followed by three sets of comparisons between values obtained with livers in Group A, which showed the highest rate of fatty acid synthesis, and other groups, which showed lower rates.

The levels of all metabolites in the segment of the glycolytic pathway between glucose 6-phosphate and glyceraldehyde 3-phosphate increase very considerably in going from fasted livers perfused with 4 mM glucose to fed livers perfused with 25 mM glucose. One can describe this as a “filling up” of the glycolytic pathway with metabolites. Fructose 6-phosphate and fructose diphosphate are both activators of phosphofructokinase (36-38). The high concentrations of these metabolites in the livers of Group A indicate that the activity of phosphofructokinase is much higher in this group than in the livers of the other groups. In relative terms, the increase in dihydroxyacetone phosphate is associated with an equal increase in α-glycerophosphate; but in absolute quantities the increase in the content of α-glycerophosphate is much larger. This increase may play a role in the regulation of fatty acid synthesis, since it has been shown that α-glycerophosphate can accelerate fatty

TABLE IV
Metabolite contents of perfused livers

Results are expressed as micromoles of metabolite per g dry weight. For details see “Methods.” The symbol < means that the values were considerably lower than the corresponding values shown in the previous column.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose 6-phosphate</td>
<td>0.56 ± 0.056</td>
<td>0.16 ± 0.015</td>
<td>0.11 ± 0.017</td>
<td>0.679 ± 0.057</td>
</tr>
<tr>
<td>fructose 6-phosphate</td>
<td>0.17 ± 0.0016</td>
<td>0.065 ± 0.019</td>
<td>0.048 ± 0.016</td>
<td>0.031 ± 0.016</td>
</tr>
<tr>
<td>fructose diphosphate</td>
<td>0.18 ± 0.014</td>
<td>0.070 ± 0.013</td>
<td>0.027 ± 0.002</td>
<td>0.015 ± 0.015</td>
</tr>
<tr>
<td>dihydroxyacetone phosphate</td>
<td>0.58 ± 0.083</td>
<td>0.11 ± 0.032</td>
<td>0.067 ± 0.013</td>
<td>&lt;</td>
</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>2.33 ± 0.25</td>
<td>0.55 ± 0.069</td>
<td>0.34 ± 0.028</td>
<td>0.01 ± 0.018</td>
</tr>
<tr>
<td>glyceraldehyde 3-phosphate</td>
<td>0.060 ± 0.0039</td>
<td>0.040 ± 0.009</td>
<td>0.024 ± 0.002</td>
<td>&lt; 1.7 ± 0.018</td>
</tr>
<tr>
<td>phosphoenolpyruvate</td>
<td>4.8 ± 0.029</td>
<td>0.55 ± 0.009</td>
<td>1.19 ± 0.067</td>
<td>1.55 ± 0.069</td>
</tr>
<tr>
<td>pyruvate</td>
<td>2.38 ± 0.22</td>
<td>0.93 ± 0.14</td>
<td>0.90 ± 0.29</td>
<td>0.66 ± 0.26</td>
</tr>
<tr>
<td>lactate</td>
<td>28.1 ± 3.0</td>
<td>5.82 ± 1.15</td>
<td>2.20 ± 0.75</td>
<td>0.84 ± 0.48</td>
</tr>
<tr>
<td>oxaloacetate</td>
<td>0.11 ± 0.021</td>
<td>0.057 ± 0.010</td>
<td>0.019 ± 0.006</td>
<td>0.017 ± 0.019</td>
</tr>
<tr>
<td>malate</td>
<td>2.66 ± 0.98</td>
<td>1.17 ± 0.23</td>
<td>0.50 ± 0.093</td>
<td>0.15 ± 0.21</td>
</tr>
<tr>
<td>citrate</td>
<td>2.11 ± 0.44</td>
<td>1.59 ± 0.10</td>
<td>0.74 ± 0.19</td>
<td>0.47 ± 0.14</td>
</tr>
<tr>
<td>CoA</td>
<td>0.25 ± 0.012</td>
<td>0.34 ± 0.041</td>
<td>0.37 ± 0.042</td>
<td>0.44 ± 0.074</td>
</tr>
<tr>
<td>acetyl-CoA</td>
<td>0.15 ± 0.090</td>
<td>0.18 ± 0.019</td>
<td>0.20 ± 0.026</td>
<td>0.16 ± 0.083</td>
</tr>
<tr>
<td>long chain fatty acyl-CoA</td>
<td>0.17 ± 0.018</td>
<td>0.15 ± 0.021</td>
<td>0.16 ± 0.027</td>
<td>0.098 ± 0.018</td>
</tr>
<tr>
<td>AMP</td>
<td>1.10 ± 0.21</td>
<td>0.58 ± 0.15</td>
<td>0.40 ± 0.050</td>
<td>0.42 ± 0.19</td>
</tr>
<tr>
<td>ADP</td>
<td>1.80 ± 0.15</td>
<td>3.07 ± 0.12</td>
<td>2.14 ± 0.47</td>
<td>3.63 ± 0.59</td>
</tr>
<tr>
<td>ATP</td>
<td>0.80 ± 0.57</td>
<td>7.57 ± 0.13</td>
<td>7.8 ± 0.36</td>
<td>7.33 ± 0.90</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>3.8 ± 0.43</td>
<td>2.4 ± 0.45</td>
<td>3.3 ± 0.6</td>
<td>2.0</td>
</tr>
<tr>
<td>ATP/AMP</td>
<td>10 ± 4</td>
<td>21 ± 8</td>
<td>21 ± 3</td>
<td>17</td>
</tr>
<tr>
<td>β-hydroxybutyrate</td>
<td>1.73 ± 0.24</td>
<td>1.17 ± 0.29</td>
<td>0.89 ± 0.19</td>
<td>0.63 ± 1.48</td>
</tr>
<tr>
<td>α-glycerophosphate/dihydroxyacetone phosphate</td>
<td>4.7 ± 1.0</td>
<td>2.4 ± 0.43</td>
<td>4.4 ± 0.4</td>
<td>1.97 ± 0.97</td>
</tr>
<tr>
<td>malate/oxaloacetate</td>
<td>24 ± 4</td>
<td>22 ± 3.9</td>
<td>35 ± 3.0</td>
<td>12.09 ± 0.69</td>
</tr>
</tbody>
</table>

* Table V, Line p, Case A x 2.

* Assuming that 100% of the TPNH required for fatty acid synthesis is supplied by the pentose phosphate pathway. The generation of 2 moles of TPNH leads to the production of 1 mole of CO₂: 321 (Table V, Line q) + 2 = 161.
acid synthesis in vivo, probably by facilitating the removal of long chain acyl-CoA (39), which is an inhibitor of acetyl-CoA carboxylase (40).

In contrast to the above changes, the phosphoenolpyruvate content is lowest in the livers of Group A (Table IV), indicating an increase in the rate of operation of the pyruvate kinase reaction. The total amount of pyruvate kinase present in the liver can increase as much as 10-fold when starved animals are fed a diet high in carbohydrate (41-43). A difference in the total activity of pyruvate kinase may account for the differences in phosphoenolpyruvate content between the livers from the 2-day-starved animals and the fed animals. It probably does not account for the difference between Groups A and B. Perhaps the higher levels of phosphoenolpyruvate in Group B are the result of a reduced activation of pyruvate kinase by fructose diphosphate (42, 44). Table IV shows that fructose diphosphate is reduced 2.6-fold in Group B as compared to Group A.

The lactate content of the livers in Group A is 4.8 times larger than that in Group B, in good agreement with the greater steady state levels of lactate found in the corresponding perfusate (Fig. 4). The oxidation-reduction potentials of lactate and pyruvate found in the livers of Groups A and B are 6 and 2 mv less than the oxidation-reduction potentials in the corresponding perfusates. In other words, they are a little more reduced in the livers than in the perfusates. This may be due to a systematic error in the analyses, but we have not traced its origin.

Malate and oxaloacetate levels are elevated in Group A as compared to the other groups. Oxaloacetate is particularly high in fed livers perfused with 25 mM glucose. Perhaps this is the result of a very low rate of removal of oxaloacetate in the phosphoenolpyruvate carboxykinase reaction, and a very high rate of extramitochondrial oxaloacetate production by the citrate cleavage reaction. Both would be expected under conditions when lipogenesis is high.

Citrate levels are highest in the livers showing the highest rates of fatty acid synthesis. This is consistent with a role of citrate in regulating the activity of acetyl-CoA carboxylase. It is not consistent with the higher rates of glycolysis which are observed in these livers, since citrate inhibits phosphofructokinase. However, the inhibitory action of citrate is probably overcome by the powerful activating effect of fructose diphosphate on phosphofructokinase. Differences in citrate content of the type shown in Table IV were not found in previous measurements made on rat liver in vivo (45, 46), although some diurnal variations were reported (47). The animals in the in vivo experiments were not scheduled-fed and were anesthetized. These differences may in part explain the differences between the perfusion and in vivo results.

Citrate levels of 3.4 and 3.6 μmoles per g dry weight have been reported to occur in livers from rats starved 1 day, when they were perfused with lactate or pyruvate, respectively (35, 48). Such livers would be expected to give low rates of fatty acid synthesis. Addition of 0.5 mM oxaloacetate to the perfusate, which would be expected to result in even lower rates of fatty acid synthesis, increased the citrate levels to 10.0 μmoles per g dry weight (35). The oxaloacetate content of these livers were very similar to those shown in Table IV, Columns C and D. Such extraordinarily high levels of citrate (48), under conditions when fatty acid synthesis is low, are inconsistent with a role of citrate in regulating the activity of acetyl-CoA carboxylase.

The changes in ATP, ADP, and AMP contents are not significant, except that livers in Group A have double the amount of AMP found in the other livers. The higher level of AMP is consistent with the higher rate of glycolysis observed in Group A, since AMP is an activator of phosphofructokinase (38, 49, 50).

CoA levels are lowest in livers which show the highest rates of fatty acid synthesis. The changes in acetyl-CoA and long chain acyl-CoA content are not significant. In other words, a decrease in the rate of fatty acid synthesis does not appear to be accompanied by an increase in the level of long chain acyl-CoA under our conditions. There is a slight decline in the sum of the three CoA species reported in Table IV in going from C to B to A. This may reflect the accumulation of an additional CoA species not measured by us, possibly malonyl-CoA, under conditions when fatty acid synthesis is high.

If it is assumed that glucose can freely enter the parenchymal cells of the liver via a carrier mechanism that does not require insulin as an activator (51-53), then the substrates and products of the gluconeogenesis and hexokinase reactions are far from mass action equilibrium. Either the total activities of the two enzymes in rat liver are rate-limiting or their activity is modified by inhibition. The substrates and products of phosphofructokinase are also far from mass action equilibrium, both at low and high concentrations of perfusate glucose.

**Contribution of Carbon from Perfusate Glucose to Fatty Acid Synthesis**—At a perfusate concentration of 25 mM glucose, only 31.5% of the total carbon of perfusate glucose is incorporated into fatty acids (Fig. 8). Most of the carbon used for fatty acid synthesis must therefore be derived from endogenous sources, the most likely source being glycogen. The percentage of exogenous glucose carbon incorporated into fatty acids increases almost but not quite in parallel with the increase in the total rate of fatty acid synthesis between 8 and 25 mM glucose (Fig. 8).

The ratio of [1-14C]carbon to [6-14C]carbon incorporation into fatty acids makes possible a tentative calculation of the contribution of the pentose phosphate pathway to TPNH production (54-56). According to Katz et al. (56) the fraction contributed by the pentose phosphate cycle is equal to (1 - γ)/(1 + 2γ), where γ is the ratio of [1-14C]glucose to [6-14C]glucose incorporation into fatty acids. Applying this equation to the results shown in Fig. 8, the contribution of the pentose phosphate cycle is 38, 27, and 17% at 8, 15, and 25 mM glucose, respectively. The use of this equation requires complete tricose phosphate isomerization or at least a relatively low amount of glycerol (and, by implication, glycerophosphate) synthesis (56). In the present experiments the formation of free glycerol was negligible. As is calculated under "Discussion," the amount of glycerophosphate formed was approximately 65 μmoles out of a total of over 1000 μmoles of C3 units generated per g dry weight per hour. Thus the requirement for the validity of the calculations of a relatively low glycerol and glycerophosphate production seems to be met. Katz et al. (56) found that in rat epididymal fat pad the pentose phosphate pathway can provide 50 to 88% of the TPNH required for fatty acid synthesis.

**Discussion**

The relation between the concentration of glucose in the perfusate and the rate of fatty acid synthesis which is shown in Fig. 2 suggests that the saturation of gluconeogenesis with glucose is one of the factors involved in determining the rate of fatty acid synthesis by the liver. The Kₘ for glucose in the glucohexokinase reaction is 10 mM (57). The peripheral blood level of glucose in fasted rats is 3.5 to 4 mM (58); it is 10.1 ± 0.25 mM...
2 days and then fed a fat-free, high carbohydrate diet for 4 days. Animals perfused with 4 mM glucose, can account for the de novo synthesis, namely 5.2 pmoles of acetyl incorporated per g and 63% less in the presence of 4 mM glucose. Fatty acid synthesis is 12% less in the presence of 8 mM glucose, specific radioactivities of perfusate glucose so determined. A, incorporation of [1-14C]glucose; V, incorporation of [6-14C]glucose; this is equivalent to a micromole of acetyl group incorporated. The broken lines show the percentage contribution. The latter was calculated from the incorporation of 3H from 3H2O. The percentage scale was adjusted so that it coincides with the microatom scale at 25 mM glucose. Each point represents four to seven perfusions.

(n = 10) 1 hour after the end of the 3-hour feeding schedule. Considerably higher levels of glucose may be reached in the portal vein after a schedule-fed meal.

When the glucose concentration in the perfusate is 15 and 25 mM, the rate of fatty acid synthesis is 170 pmol of acetyl group incorporated per g dry weight per hour. When divided by 8, this yields 21.3 pmol of palmitate synthesized per g dry weight per hour. This is equivalent to a de novo synthesis of 4.6% of the total fatty acid of rat liver per hour. The rate of fatty acid synthesis is 12% less in the presence of 8 mM glucose, and 63% less in the presence of 4 mM glucose.

Similar calculations show that the highest rate of 3-β-hydroxy-steroid synthesis, namely 5.2 pmol of acetyl incorporated per g dry weight per hour which was observed in livers from fed animals perfused with 4 mM glucose, can account for the de novo synthesis of 0.62% of the total cholesterol of rat liver per hour.

The rate of fatty synthesis obtained in the perfusion experiments agrees well with the rates observed in vivo (Table II). They are much higher than rates reported previously, which were obtained with slices or cell-free extracts (60, 61). In this respect fatty acid synthesis resembles gluconeogenesis (62) and ketogenesis (10). All three pathways appear to operate at relatively low rates in slices and in cell-free extracts prepared and used according to current practices. The amounts of newly synthesized fatty acids that were released by the perfused liver and by the liver in vivo are not taken into account in these comparisons. The perfused liver released an average of 8% of the newly synthesized fatty acids; comparable data for in vivo conditions are not available. Considerably higher rates of release of fatty acids were observed by Windmueller and Spaeth (59, 63), who studied some of the factors in the perfuse that might affect the rate of release.

The rates of cholesterol synthesis observed in our perfusion experiments are much higher than those obtained by others. Typical rates reported with slices are 0.29 and 0.026 pmol of acetyl group incorporated per g dry weight of liver per hour for fed animals and for animals which had been starved for 48 hours, respectively (64). Corresponding rates obtained by us in perfused livers are 6.1 and 0.65 pmol per g dry weight per hour. The 21- to 25-fold difference in rates may be due to the use by previous workers of acetate as labeled precursor; in most circumstances acetate incorporation accounts for only a small fraction of the total rate of cholesterol synthesis. As has already been pointed out, H incorporation from 3H2O does not suffer from this disadvantage.

At a concentration of 25 mM, the glucose in the perfusate contributes only 32% of the total carbon used in fatty acid synthesis (Fig. 8). At lower glucose concentrations the contribution is much less still, being 20% at 10 mM, 9.5% at 8 mM, and 1.6% at 4 mM perfusate glucose. Although the rate of fatty acid synthesis increases by only 12% between 8 and 15 mM glucose, and there is no increase in the rate between 15 and 25 mM glucose, the percentage of carbon from added glucose which goes into fatty acids changes almost as a linear function of the glucose concentration (Fig. 8). This result would not be expected simply on the basis that glucokinase exhibits Michaelis-Menten kinetics with a k m value for glucose of 10 mM. Some possible explanations for this discrepancy follow. (a) Glucokinase in vivo has a k m for glucose greater than 10 mM. (b) Glucokinase may exhibit sigmoidal saturation kinetics with respect to glucose, or the enzyme is inhibited by an unidentified metabolite, or a combination of both, which is to say that an inhibitor imposes sigmoidal kinetics on the reaction. (These are hypothetical properties which have not previously been reported.) (c) As the concentration of glucose is decreased there is an increase in glycolysis which is proportionately greater than the reduction in glucose utilization via glucokinase. This might occur, for example, if glycolysis is inhibited by high concentrations of glucose (65-67). (d) As the glucose concentration is decreased, additional carbon flows into the acetyl pool from some source other than carbohydrate, such as fatty acid oxidation.

The high initial rate of lactate plus pyruvate production, namely 14.5 × 60 = 870 pmol per g dry weight of liver per hour, is probably a consequence of a sudden switching on of glycolysis during the operative procedure. It compares to a rate of citric acid cycle operation during the 2nd hour of 172.3
Table V
Comparison of rate of fatty acid synthesis with rate of operation of citric acid cycle and other pathways

| a. Average O₂ uptake (60 to 120 min) | 864 |
| b. O₂ uptake in presence of cyanide | 100 |
| c. Cyanide sensitive O₂ uptake | 674 |
| d. β-Hydroxybutyrate formation | 15.3 |
| e. Acetoacetate formation | 15.3 |
| f. O₂ used in β-hydroxybutyrate formation assuming its carbon comes from | 19.1 |
| i. Palmitate | 19.1 |
| ii. Lactate | 22.9 |
| g. O₂ used in acetoacetate formation assuming its carbon comes from | 26.8 |
| i. Palmitate | 26.8 |
| ii. Lactate | 30.6 |
| h. Total O₂ used for ketogenesis assuming carbon comes from | 45.9 |
| i. Palmitate | 45.9 |
| ii. Lactate | 53.5 |
| i. Fatty acid synthesis (corrected for 8% loss into perfusate) | 183.6 |
| k. Sterol synthesis. | 6.1 |
| l. O₂ consumed to oxidize lactate to acetyl-CoA that is subsequently used for fatty acid and sterol synthesis | 189.7 |
| m. O₂ consumed in oxidation of lactate to CO₂ (e - h - i) | 430.8 |
| n. O₂ consumed to oxidize lactate → acetyl-CoA that is subsequently oxidized via citric acid cycle: | 143.6 |
| Case A (see below) | 172.3 |
| Case B (see below) | 287.2 |
| Case A, is oxidized (m - n) | 258.5 |
| Case B, is used for reductive synthesis of fatty acids (m - n) | 321 |
| q. TPNH required for fatty acid synthesis (i × 2 × j)/8 | 128% |
| Case A | 107% |
| Case B | 4.2% |
| t. 100 × sterol synthesis/citric acid cycle (100 × j/p) | 3.5% |

*a* Palmitate + 5 O₂ → 4 β-hydroxybutyrate.
*b* 2 lactate + 2 O₂ → β-hydroxybutyrate + 2 CO₂ + 2 H₂O.
*c* Palmitate + 7 O₂ → 4 acetoacetate + 4 H₂O.
*d* 2 lactate + 2 O₂ → acetoacetate + 2 CO₂ + 3 H₂O.
*e* Lactate + O₂ → acetate + CO₂ + H₂O.

No allowance was made for the following metabolic processes:

(i) *Urea Synthesis*. This was probably slight, because the per- fusions considered in the table lacked the amino acid mixture. Amino acid analyses of some of the perfusates as a function of time showed that the livers actually put out a mixture of amino acids.
(ii) *Pyruvate Secreted into Perfusate*.—This amounted to about 21 μmoles per g dry weight per hour during the 2nd hour (Fig. 4 shows that 1.1 mm × 150 ml = 165 μmoles of lactate plus pyruvate were liberated during the 2nd hour of the perfusion. The average [lactate] to [pyruvate] ratio was 7, and 165/8 = 21 μmoles of pyruvate.) The average dry weight per liver was 1.67 g, thus the average amount of pyruvate secreted into the perfusate was 21 × 1.67 = 34.6 μmoles per g dry weight, during the 2nd hour. The conversion of 12.6 μmoles of lactate to pyruvate requires 6.3 μmoles of O₂ or about 1% of the cyanide-sensitive oxygen consumption. (ii) *TPNH Used in Sterol Synthesis*.—The maximum amount of sterol synthesis was 6.1 μmoles of acetyl group incorporated per g dry weight per hour. Less than 1 μmol of TPNH is required to convert 1 μmol of acetyl group into cholesterol. This is less than 2% of the amount required for fatty acid synthesis.
triglycerides, this would add 183.6 (Table IV, Line i) + 61.2 μmoles of glycerophosphate generated for a total of 1293 μmoles of C-3 units generated per g dry weight per hour.

The rates of lactate plus pyruvate production observed by us during the first 15 to 30 min of the perfusion (Fig. 4) are somewhat higher than the rates of lactate production reported by Woods and Krebs (25) for perfuse glucose concentrations of 20 and 40 mM, and much higher than the 13% reported by these authors for perfuse glucose concentrations of 5 and 10 mM.

Table V provides comparisons of the rate of fatty acid synthesis with the rate of operation of the citric acid cycle and certain other metabolic processes at perfuse glucose concentrations of 25 mM. Fatty acid synthesis consumes somewhere between 7 and 28% more acetyl groups than the citric acid cycle (Table V, Line s), a remarkable result considering that fatty acid synthesis by the liver has hitherto been considered a "slow" process, and the citric acid cycle vs a "rapid" process.

Ketogenesis operates at 17% of the rate of fatty acid synthesis (Table V, compare Lines d and e with Line i). Our results show that ketogenesis is not entirely switched off, even when conditions for fatty acid synthesis are most favored.

Cholesterol synthesis operates at 3.3% of the rate of fatty acid synthesis (Table V, compare Line j with Line i). Although slower than the other processes mentioned so far, this is still an appreciable fraction of the total acetyl group utilization.

The maximum amount of TPNH that the citric acid cycle might supply for the reductive steps of fatty acid synthesis through the operation of extramitochondrial, TPN-specific isocitrate dehydrogenase is 54% of the total (Table V, Line v). The remainder (46 × 321/100 = 147.7 μmoles of TPNH) must be supplied by glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme. If all of the remaining TPNH is supplied by the pentose phosphate pathway dehydrogenases, the amount of glucose metabolized through this pathway would be 147.7 + 2 = 149 μmoles. If extramitochondrial, TPN-specific isocitrate dehydrogenase does not contribute any TPNH for the reductive steps of fatty acid synthesis, and all TPNH is generated through the pentose phosphate pathway, the amount of glucose metabolized through this pathway would have to be 321 (Table V, Line q) + 2 = 169.5 μmoles per g dry weight per hour.

The maximum rate of glycolysis, observed during the initial stage of the perfusion in the presence of 25 mM glucose, was 870 / 2 = 435 μmoles of hexose utilized per g dry weight per hour (Fig. 4). As has already been mentioned, the maximum initial rate may have been higher still. Thus the pentose phosphate pathway would have to operate at about 100 × 180/435 = 37% of the maximum observed rate of glycolysis in order to account for all of the TPNH requirement of fatty acid synthesis, or at about 100 × 74/435 = 17% of the maximum rate of glycolysis in order to account for the TPNH requirement if all of the TPNH generated in the citric acid cycle is utilized for fatty acid synthesis. It should be stressed that the rate of glycolysis is probably much less during the 2nd hour of the perfusion than during the first 15 or 30 min. In other words the percentage contribution of the pentose phosphate pathway would have to be much higher if it were to account for all of the TPNH needed for fatty acid synthesis.

The data presented under "Results" show that the pentose phosphate pathway can account for 17% (Fig. 5), and the citric acid cycle can account for a maximum of 54% (Table V), for a total of 71% of the TPNH required at the maximum rate of fatty acid synthesis. The malic enzyme reaction may contribute some or all of the TPNH not accounted for, but our results throw no light on this possibility. At low rates of fatty acid synthesis the pentose phosphate pathway and the citric acid cycle can account for all of the TPNH required for fatty acid synthesis.

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