

The absolute rate of fatty acid synthesis by mammary gland slices from lactating rats

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Abstract Mammary gland utilizes a combination of substrates in vivo. To determine if and how the substrates presented to the gland alter milk fat synthesis, slices of mammary gland of lactating rats were incubated with combinations of glucose, L-lactate, and pyruvate. Uptakes and conversions of uniformly and specifically labeled substrates to CO₂ and fatty acid were measured. The absolute rate of fatty acid synthesis was measured by incorporation of tritium from tritiated water into fatty acid. The extent, but not the type, of fatty acids synthesized was affected by the substrates utilized. Glucose stimulated uptake and conversion to fatty acid of L-lactate and, to a smaller extent, of pyruvate. Analysis of CO₂ and fatty acid yields revealed that (a) the major stimulatory effect of glucose was on the conversion of acetyl coenzyme A from other substrates to fatty acid; (b) the rate of fatty acid synthesis paralleled the activity of the hexose monophosphate pathway up to a point. A likely source of the NADPH required beyond this point is provided by oxidation of malate to pyruvate. Reduction by NADH of oxaloacetate, produced during citrate cleavage, would yield malate. Hence, maximal synthesis would require production of NADPH and NADH, a condition met when glucose and L-lactate were substrates. Decreased fatty acid synthesis in pyruvate's presence supports this suggested requirement for cytosolic NADH in rat mammary gland.

Supplementary key words substrate interactions · glucose stimulation · regulation of lipogenesis · milk fat synthesis · mitochondrial-cytosolic interactions

Mammary gland in vivo does not utilize solely glucose, but a combination of substrates depending upon the state of the animal (1, 2). The concentrations of the major nonlipid substrates in arterial blood perfusing the mammary gland of the lactating rat have been reported to be 5–6 mM glucose, 1–2 mM L-lactate, and 0.03 mM ketone bodies (1, 2). A 16-hr starvation tends to decrease the levels and the net uptakes of glucose and L-lactate and to increase those of β -hydroxybutyrate and acetoacetate (1). If alterations in the rate of fatty acid synthesis are brought about by changes in the combination of substrates presented to the gland, the substrate composition of the blood could have a significant influence on the extent of milk fat synthesis.

In mammary glands from lactating rats, synthesis of fatty acids from acetate, propionate, short chain fatty

acids (C-4 to C-8), lactate, pyruvate, and various amino acids is markedly stimulated by the concomitant utilization of glucose (3–15). However, the absolute rate of fatty acid synthesis from combinations of substrates has not been reported. Such knowledge is essential to evaluate the physiological significance of this stimulatory effect. For example, fatty acid synthesis from glucose is decreased by the addition of pyruvate (4, 12–14), so that the total quantity of fatty acids formed by the gland actually decreases (4, 13, 14). The effect on total lipogenesis of the observed stimulation by glucose on the conversion of another substrate to fatty acid would depend, then, on the effect of the other substrate on glucose conversion to fatty acid.

Even though the stimulatory effects of glucose (3–15) and, to a lesser extent, of pyruvate (4, 12) on the conversion of other substrates to fatty acids were first observed several years ago, the mechanism of stimulation is not completely clear. Several possibilities have been suggested: provision of another carbon source (16), formation of glyceride-glycerol (5, 15, 16), increased rate of production of NADPH (7, 17, 18) and of ATP (12, 16), among others.

To clarify both the physiological significance and the biochemical mechanism of this stimulatory action, we conducted experiments with combinations of glucose, lactate, and pyruvate and analyzed their effect on (a) the absolute rate of fatty acid synthesis, (b) the conversions of exogenous substrates to fatty acid, and (c) the activity of related pathways.

EXPERIMENTAL PROCEDURE

Tissue preparation

Lactating rats of the Long-Evans strain, which were raised and maintained on an adequate stock diet (FP special rat breeder food) and had each suckled at least

Abbreviations: HMP, hexose monophosphate; GLC, gas-liquid chromatography; acetyl-CoA, acetyl coenzyme A; acetyl-CoA carboxylase, acetyl coenzyme A:CO₂ ligase.

six pups, were killed 10–16 days postpartum by a blow on the neck. All mammary glands were quickly excised, trimmed of connective tissue and muscle, and placed in an ice-cold, oxygenated Krebs–Henseleit bicarbonate buffer (19), pH 7.35–7.45.

Slices of the mammary glands (0.4 mm thick) were prepared with the tissue chopper described by McIlwain and Buddle (20). The slices were washed repeatedly with the bicarbonate buffer until milk was no longer observed in the wash and were then gently blotted dry with coarse filter paper. A precisely weighed portion (80–105 mg) of slices was transferred to the main compartment of a flask, provided with a center well (21), that contained the labeled and unlabeled substrates as indicated in the tables. The concentration of all added substrates was 10 mM. The flasks were gassed with a mixture consisting of 95% O₂ and 5% CO₂, closed with a self-sealing rubber serum cap, and incubated for 30 min with shaking in a water bath maintained at 37°C. At the end of that time, the tissue was inactivated and the CO₂ was released by injecting 0.5 ml of 1 N H₂SO₄ into the outer well with a syringe and needle.

Analytical procedures

Carbon dioxide was collected into 0.3 ml of a solution of ethanolamine added to the center well of the incubation flask prior to acidification as described previously (22). The contents of the incubation flasks were transferred to conical centrifuge tubes. The slices were separated from the medium by centrifugation in the cold. The supernatant fraction was saved for assay of glucose, L-lactate, and pyruvate. The slices were washed twice with copious amounts of water and reisolated by centrifugation. Fatty acids in the washed tissue were saponified and extracted into hexane (23).

The methyl esters of the isolated fatty acids, prepared as described previously (24), were separated by gas–liquid chromatography (GLC). A flame ionization detector was used to determine the mass and the radioactivity of each fatty acid fraction was measured with a modified Nuclear-Chicago (Des Plaines, Ill.) radioactive monitor. The percentage distribution was determined by integrating the area under each peak by triangulation. Appropriate factors to correct for the effect of retention time on peak area were developed from determinations with standard fatty acid methyl esters corresponding to each major sample peak.

The fatty acids in a portion of some of the hexane extracts were converted to their sodium salts by extraction into an excess of aqueous NaOH. After the hexane was evaporated under a stream of nitrogen gas, a portion of each solution of sodium salts was assayed in the scintillation spectrometer. Another

portion was decarboxylated by a modification of the Schmidt reaction (11, 24, 25). The ¹⁴CO₂ released during this reaction was trapped in 0.5 N NaOH and assayed in the liquid scintillation spectrometer under conditions identical to those used for the sodium salts.

All measurements of radioactivity were done in a liquid scintillation spectrometer (Nuclear Chicago). The liquid scintillation medium was 10 ml of a 2:1 (v/v) mixture of toluene–2-ethoxyethanol containing 98 mg of 2,5-diphenyloxazol (PPO) and 2 mg of *p*-bis-(*o*-methylstyryl)-benzene (Bis-MSB). All samples were adjusted to the same counting efficiency by the use of quench factors derived from the external standard ratio. In samples containing both isotopes, the net counts per minute of ¹⁴C and ³H were determined by the isotope screening method (26).

Glucose was determined by a glucose oxidase method (Glucostat, Worthington Biochemical, Freehold, N. J.) in which 0.1 M phosphate buffer pH 7.2, was substituted for water in making up the reaction mixture. L-Lactate was determined with lactate dehydrogenase in hydrazine–glycine buffer, pH 9.5 (27). Pyruvate was also determined with lactate dehydrogenase (28).

Significance was calculated on the basis of Student's *t* test (29). A random probability of less than one percent was considered statistically significant.

Materials

Uniformly ¹⁴C-labeled glucose ([U-¹⁴C]glucose) was prepared photosynthetically from *Canna indica* (30, 31). All other labeled compounds were purchased from the New England Nuclear Corporation, Boston, Mass. Labeled pyruvate was purified and stored as recommended by Von Korff (32). Solutions of carrier pyruvate were made up the morning of the experiment.

RESULTS

Rate of substrate utilization

In our initial studies, we found that with substrate concentrations of 10 mM, a majority of the substrates had been utilized during the 3 hr incubation period used previously (6–11, 22, 24). This situation was particularly disturbing in the case of pyruvate as over 90% of the original substrate was taken up by the end of the 3 hr period. Rather than increase the substrate concentration beyond physiological levels, we resolved this problem by reducing the incubation time to a period, namely 30 min, in which less than 40% of each exogenous substrate had been utilized.

Using these conditions, the rate of glucose uptake

was 8 μ moles per hour per 100 mg fresh tissue (**Table 1**). The rate of glucose utilization was significantly decreased by the addition of equimolar quantities of pyruvate. A similar decrease in glucose uptake due to the addition of pyruvate, both in the presence and the absence of acetate, has been reported by others (12, 13). L-Lactate as an additional substrate also significantly decreased the rate of glucose disappearance but not to the extent observed with pyruvate (**Table 1**).

The uptake of pyruvate in the medium was stimulated slightly ($P < 0.05$) by the addition of glucose. In contrast, the rate of utilization of L-lactate in the presence of glucose was more than doubled. Pyruvate or L-lactate as the second substrate did not significantly alter the rate of uptake of the other.

Oxidation of exogenous substrates

The conversion of glucose to CO_2 was decreased by the presence of added pyruvate regardless of the site of the label (C-1, C-6, or uniformly labeled glucose) (**Table 2**). This effect was observed many years ago with slices (4, 12) and more recently with cells (13, 14) from glands of lactating rats. The percentage decrease was similar to that observed on the rate of glucose utilization due to the addition of pyruvate. Although the presence of equimolar quantities of L-lactate had no effect on the $^{14}\text{CO}_2$ yields from glucose labeled in carbon-1, it decreased this yield from carbon-6 and from uniformly labeled glucose. This observation confirms that made by Abraham, Hirsch, and Chaikoff (6) several years ago. We have shown, in addition, that the percentage decrease in CO_2 production from uniformly labeled glucose was of the same magnitude

TABLE 1. Uptake of glucose, pyruvate, and lactate by slices of mammary gland from lactating rats

Substrate(s)	N ^b	Disappearance from Media of: ^a		
		Glucose	Pyruvate	L-Lactate
Glucose	7	4040 \pm 303 ^c		
Glucose + pyruvate	11	2370 \pm 273	2400 \pm 180	
Glucose + L-lactate	14	3310 \pm 161		3400 \pm 300
Pyruvate	10		1800 \pm 220	
L-Lactate	10			1600 \pm 360
L-Lactate + pyruvate	9		2000 \pm 260	1000 \pm 110

A precisely weighed portion (80–105 mg) of washed tissue slices was incubated at 37°C for 30 min in 1 ml of Krebs–Henseleit bicarbonate buffer (19), pH 7.3–7.4, with 95% O_2 and 5% CO_2 as the gas phase. The concentration of each substrate was 10 mM. Other experimental details are given in the text.

^a Results are presented as nmoles/100 mg fresh tissue/30 min.

^b Number of incubations.

^c Mean \pm SE.

TABLE 2. Effect of pyruvate and lactate on the oxidation of labeled glucoses to CO_2 by slices of mammary gland from lactating rats

Substrate(s)	Incorporation into $^{14}\text{CO}_2$ from: ^a		
	[1- ^{14}C]Glucose	[6- ^{14}C]Glucose	[U- ^{14}C]Glucose
Glucose	763 \pm 56.4 ^b (6) ^c	43 \pm 1.7 (3)	483 \pm 20.0 (4)
Glucose + pyruvate	383 \pm 23.6 (6)	20 \pm 3.6 (3)	117 \pm 4.7 (3)
Glucose + L-lactate	722 \pm 56.7 (6)	11 \pm 1.5 (3)	352 \pm 27.8 (4)

Slices were prepared and incubated as described in the text and Table 1. The concentration of each substrate listed was 10 mM. Other experimental details are given in the text.

^a Results are presented as nmoles/100 mg fresh tissue/30 min.

^b Mean \pm SE.

^c Number of incubations.

as that in glucose uptake due to the presence of L-lactate.

While the CO_2 yield from C-1 of pyruvate was unaffected by the presence of glucose, that from the comparable carbon of L-lactate was increased almost 5-fold (**Table 3**). The yield of $^{14}\text{CO}_2$ from pyruvate labeled in its methyl carbon was decreased in the presence of glucose. It is notable that this effect was the opposite of that observed due to glucose on the rate of pyruvate utilization. Similarly, equimolar quantities of glucose along with L-lactate reduced the conversion of C-3 of lactate to CO_2 by 2-fold. In this case, the result was particularly striking since the addition of glucose markedly increased the CO_2 yield from C-1 of L-lactate. As in the case of uptake, pyruvate had no effect on the conversion of L-lactate to CO_2 , and vice versa.

Conversion of exogenous substrate to fatty acid

Of the compounds tested as the sole substrate, glucose supported the highest rate of fatty acid synthesis (**Table 4**). Pyruvate alone acted as a much better exogenous substrate for fatty acid synthesis than did L-lactate, but the incorporation from pyruvate did not compare with that from glucose. L-Lactate as the only added substrate was the poorest substrate for fatty acid synthesis of those used in these experiments. This general relationship of lipogenesis from these substrates has been reported by others (4, 6, 9, 13, 33).

As was observed before (4, 13, 14), pyruvate in combination with glucose markedly reduced the conversion of glucose carbon, either from C-6 or uniformly labeled glucose, to fatty acid (**Table 4**). This inhibitory effect of pyruvate was much greater than its inhibition on glucose uptake (**Table 1**) or conversion to CO_2 (**Table 2**). Conversion of C-1 of glucose to fatty acid was also reduced by the presence of exogenous pyruvate, but to a lesser extent than the other carbons of

TABLE 3. Effect of glucose, pyruvate, or lactate on the incorporation of specifically labeled pyruvates and lactates into CO₂ by slices of mammary gland from lactating rats

Substrate(s)	Incorporation into ¹⁴ CO ₂ from: ^a			
	[1- ¹⁴ C]Pyruvate	[3- ¹⁴ C]Pyruvate	[1- ¹⁴ C]L-Lactate	[3- ¹⁴ C]L-Lactate
Pyruvate	683 ± 26.2 ^b (6) ^c	218 ± 20.0 (10)		
L-Lactate			447 ± 14.3 (6)	101 ± 5.9 (10)
Glucose + pyruvate	691 ± 28.3 (6)	133 ± 7.4 (4)		
Glucose + L-lactate			2204 ± 276.5 (5)	51 ± 2.5 (7)
Pyruvate + L-lactate		179 ± 15.9 (3)		97 ± 12.2 (6)

Slices were prepared and incubated as described in the text and Table 1. The concentration of each substrate listed was 10 mM. Other experimental details are given in the text.

^a Results are presented as the nmoles of labeled substrate converted/100 mg fresh tissue/30 min.

^b Mean ± SE.

^c Number of incubations.

glucose. L-Lactate also decreased the conversion of C-6 of glucose and the carbons of uniformly labeled glucose to fatty acid, but the magnitude of the decrease was similar to that observed on glucose uptake (Table 1) and conversion to CO₂ (Table 2). In contrast to the effect of pyruvate on the conversion of C-1 of glucose to fatty acid, the decrease induced by L-lactate was greater on C-1 than on C-6 or on carbon from uniformly labeled glucose.

Conversion of methyl carbon of pyruvate to fatty acid was doubled by the addition of glucose but was unaffected by equimolar additions of L-lactate as a second substrate (Table 4). The rate of incorporation of the methyl carbon of L-lactate into fatty acid was stimulated both by the addition of pyruvate and of glucose. Glucose, however, was a 10-fold more effective stimulator than pyruvate. Furthermore, with glucose the stimulatory effect on the conversion of L-lactate to fatty acid was directly related to an increase in L-lactate uptake, but this was not true with pyruvate (Table 1). The highest rate of conversion of exogenous substrates to fatty acid was observed with glucose and L-lactate. This maximal conversion was solely due to the tremendous stimulatory effect of glucose on the incor-

poration of carbon from L-lactate as the incorporation of glucose was actually decreased under these circumstances. While the fatty acid synthesis from glucose and lactate and from glucose and pyruvate has been studied (4, 6, 9, 13, 14), this investigation is the first in which it can be shown that the substrate concentrations were adequate in all cases to sustain fatty acid synthesis throughout the entire incubation period.

Pattern of fatty acids formed

Decarboxylation of fatty acids formed from uniformly labeled glucose revealed that 6.6% of the total activity resided in the carboxyl carbon. The presence of pyruvate or L-lactate during the incubation period had no effect on this pattern. Similarly, when [2-¹⁴C] pyruvate was the labeled substrate, the proportion of total radioactivity in the carboxyl carbon was unchanged by the addition of either glucose or L-lactate to the incubation medium. In the experiments with labeled pyruvate, the percentage of the total radioactivity in the carboxyl carbon was 2-fold that from the labeled glucose experiments. If the acetyl units formed from [2-¹⁴C]pyruvate were incorporated directly into fatty acids without randomization, such a difference

TABLE 4. Incorporation of specifically labeled glucoses, pyruvates and lactates into fatty acid by slices of mammary gland from lactating rats

Substrate(s)	Incorporation of ¹⁴ C into Fatty Acids from: ^a				
	[1- ¹⁴ C]Glucose	[6- ¹⁴ C]Glucose	[U- ¹⁴ C]Glucose	[3- ¹⁴ C]Pyruvate	[3- ¹⁴ C]L-Lactate
Glucose	455 ± 48.5 ^b (6) ^c	1404 ± 186.0 (3)	360 ± 42.9 (4)		
Glucose + pyruvate	42 ± 5.2 (6)	44 ± 3.5 (3)	34 ± 15.3 (3)	420 ± 59.2 (4)	
Glucose + L-lactate	126 ± 14.7 (6)	627 ± 103.7 (3)	260 ± 29.2 (4)		1859 ± 61.2 (7)
Pyruvate				221 ± 15.3 (10)	
L-Lactate					55 ± 5.5 (10)
L-Lactate + pyruvate				262 ± 16.8 (3)	155 ± 23.3 (5)

Slices were prepared and incubated as described in the text and Table 1. The concentration of each substrate listed was 10 mM. Other experimental details are given in the text.

^a Results are presented as nmoles/100 mg fresh tissue/30 min.

^b Mean ± SE.

^c Number of incubations.

in carboxyl labeling would be expected since only every other carbon in the acetyl units would be labeled.

In the case of [U-¹⁴C]L-lactate, the incorporation into the carboxyl carbon with lactate as the sole exogenous substrate was twice that when either glucose or pyruvate was present in the incubation medium. It should be noted that the rate of fatty acid synthesis from L-lactate alone was very low (Table 4). In the presence of either glucose or pyruvate, the extent of labeling was the same as that from uniformly labeled glucose. If the labeled carbons were evenly distributed along the carbon chain of the synthesized fatty acid, the results indicate that the length of the chain was 13–16 carbons in all cases except from L-lactate alone.

When the fatty acids formed from each substrate and each combination of substrates were separated by GLC, the complement of labeled fatty acids was similar in all cases. The mean carbon number of fatty acids synthesized was slightly longer than that reported previously for lactating rat mammary gland by Abraham, Matthes, and Chaikoff (23). The mean carbon number of the fatty acids fell between 13–15 in all cases.

This agreement between the mean length of the carbon chain as calculated from the incorporation of label into individual fatty acids and from the results of the Schmidt degradation demonstrates that the acetyl units derived from all substrates, except from L-lactate alone, were incorporated into fatty acids via synthesis de novo as defined by Smith and Abraham (34).

Conversion to fatty acid of tritium from tritiated water

In the absence of exogenous substrates, mammary gland slices incorporated minute quantities of tritium, derived from labeled water, into fatty acid (Table 5).

TABLE 5. Effect of glucose, pyruvate, and lactate on the incorporation of tritium from ³H₂O into fatty acid by slices of mammary gland from lactating rats

Substrate(s)	N ^a	Incorporation of ³ H into Fatty Acids ^b
None	6	43 ± 9.7 ^c
Glucose	4	1716 ± 97.7
Glucose + pyruvate	5	791 ± 55.2
Glucose + L-lactate	3	2593 ± 102.9
Pyruvate	3	296 ± 23.5
L-Lactate	3	65 ± 13.1
Pyruvate + L-lactate	3	389 ± 40.8

Slices were prepared and incubated as described in the text and Table 1. Substrates were added at a concentration of 10 mM each. The media were labeled with ³H₂O. Other experimental details are given in the text.

^a Number of incubations.

^b Results are presented as nmoles of ³H₂O converted to fatty acid/100 mg fresh tissue/30 min.

^c Mean ± SE.

TABLE 6. Formation of lactate from glucose and pyruvate by slices of mammary gland of lactating rats

Substrate(s)	L-Lactate Formed ^a
Glucose	380 ± 32 ^b (11) ^c
Pyruvate	660 ± 62 (19)
Glucose + pyruvate	600 ± 51 (15)

Slices were prepared and incubated as described in the text and Table 1. The concentration of each substrate listed was 10 mM.

^a Results are presented as the nmoles of L-lactate formed/100 mg fresh tissue/30 min.

^b Mean ± SE.

^c Number of incubations.

In conformance with the experiments in which the exogenous substrates were labeled (Table 4), the results in Table 5 indicate that L-lactate as the sole substrate provided the poorest support for fatty acid synthesis, and that glucose plus L-lactate was the most productive of the combinations tested in these studies. This level of synthesis was followed in increasing order by glucose alone, glucose plus pyruvate, and either pyruvate alone or pyruvate plus L-lactate, both of which supported similar rates of fatty acid synthesis. The ratio between the incorporation of nmoles of water into fatty acid and the incorporation of acetyl units, as calculated from the ¹⁴C-labeled substrates, approached unity for all substrate combinations studied (Tables 4 and 5). Such a relationship has been observed before in mammary gland preparations (13, 35).

L-Lactate production

When glucose was the sole substrate, less than 10% of the glucose taken up was converted to L-lactate (Table 6). The extent of L-lactate accumulation was identical to that observed at 5 mM glucose and a lower concentration of mammary gland slices (36). The addition of pyruvate to glucose doubled the production of L-lactate. On the basis of the total substrate utilization, however, this still represented only a small fraction of the total potential L-lactate production (Tables 1 and 6). Even though the L-lactate production was the same from pyruvate or pyruvate together with glucose, in the case of pyruvate alone the production of L-lactate accounted for over one-third of the pyruvate consumed (Table 6).

DISCUSSION

Validity of experimental conditions

The superiority of isolated cells over slices for studies in vitro has been repeatedly emphasized (13, 37, 38). Recently, however, Elkin and Kuhn (2) have shown that, based on glucose utilization and lactate

production, slices of rat mammary gland are better indicators of intact gland metabolism than are cells. Abraham and co-workers (37, 39) could find few metabolic superiorities of cells over slices of murine mammary gland. While the rates of glucose uptake and of fatty acid and lactose synthesis were greater in cells, the production of lactate was increased to an even greater extent in the cells, and the utilization of NADPH from the hexose monophosphate (HMP) pathway for fatty acid synthesis was reduced in cells compared to that in slices (39). To us, these results, in conjunction with those of Elkin and Kuhn (2), indicate that the metabolism of the intact mammary gland is more accurately portrayed by slices.

When slices of mammary gland are incubated as they were here, their rates of glucose uptake and lactate production approach those of Elkin and Kuhn for intact gland (2). This rate is compatible with the hexokinase activity measured in mammary gland from comparable rats (40), but is 3-fold higher than that obtained with a 3 hr incubation period (22), and twice that observed during 1 hr incubation with 5 mM glucose (36). Assuming a dry weight of 10 percent of the wet weight for isolated acini, this rate of uptake is identical to that reported for the dispersed gland preparation (13). We propose that, during the longer incubation periods, the concentration of glucose decreases to the point at which glucose uptake is restricted. Since this concentration is above the K_m of the hexokinases for glucose (41), such an explanation implies that membrane permeability, rather than the affinity of the enzyme, regulates glucose uptake; this explanation is in harmony with current concepts of glucose uptake (42).

The inability of Haut, London, and Garfinkel (43) to construct a model in a steady state with respect to all metabolic pathways may be related to this changing rate of glucose uptake. According to their model, the HMP activity of mammary gland does not reach a steady state until after 2 hr of *in vitro* incubation. Compared with the results of Elkin and Kuhn (2), the metabolic state at 2 hr is remote from that in the intact gland. One reason for this may be related to the concentration of glucose. The computer model is based on experiments with tissue slices in which the glucose concentration decreased with time (7) and the rate of this decrease would, by necessity, also be decreasing. The slower rate of change would enhance the establishment of seemingly steady state conditions.

If the glucose concentration were kept constant, as it is in intact gland, the decline in uptake, in HMP activity, and in fatty acid synthesis might not occur and the metabolic activity might approach that observed with slices in the first 30 min. The metabolic similarity

between the intact gland (2) and slices in the present study and the fact that the glucose concentration never fell below 6 mM (ca. 100 mg/100 ml) led us to the conclusion that the short term incubation more accurately mimics the situation in the intact gland.

Absolute rate of fatty acid synthesis

The total extent of fatty acid synthesis from both exogenous and endogenous substrates can be gauged by the incorporation of labeled hydrogen from water (44, 45). Jungus (44) has shown that in adipose tissue the absolute rate of fatty acid synthesis in terms of acetyl units is 1.15 times the rate of incorporation of tritiated water. Since synthesis in the absence of exogenous substrate was not examined (44), the extent of tritium incorporation along with unlabeled carbon is unknown. Because of large endogenous sources of carbon in liver, significant incorporation of tritium from tritiated water is observed in the absence of added substrates (46, 47). Hence, the ratio in fatty acid of ^3H from tritiated water and of ^{14}C from labeled substrates can vary widely in this tissue depending upon the experimental conditions (46). Katz and co-workers have demonstrated a one-to-one relationship in both mammary gland slices (35) and cells (13) with varying rates of synthesis. The discrepancy between adipose tissue and mammary gland could be due to differences in the contribution of chain elongation to total fatty acid synthesis (48), to the extent of synthesis from endogenous substrates, and to the origin of NADPH utilized for fatty acid synthesis (44).

This discrepancy does not alter the interpretation of the results of our experiments. The major pathway of synthesis was the same from all substrates, with the noted exception of lactate alone, and synthesis of fatty acids was dependent upon the addition of exogenous substrates (Table 5, Ref. 13). Thus, the incorporation of tritium from tritiated water represents an absolute rate of synthesis for this tissue with these substrate combinations.

Physiological role of substrates in regulating lipogenesis

Our results demonstrate that alterations in the rate of fatty acid synthesis can be brought about by the combination of substrates presented to the gland. That this observation has physiological significance is supported by the fact that mammary gland *in vivo* does not utilize only one substrate, but a combination. The major substrates for the mammary gland of the rat are glucose, lactate, ketone bodies, volatile fatty acids, and other lipids (1, 2). We included pyruvate in this study, not as a physiological substrate, but as a biochemical probe to examine a

specific stage of glycolysis. The concentrations of these compounds in the blood perfusing the gland fluctuate depending upon the dietary and physiological state of the animal (1). We have shown that altering the proportions of two of these major substrates, glucose and lactate, can have a modulating effect on the extent of fatty acids synthesized by mammary gland. Recently, similar manipulations with acetoacetate and glucose have been shown to alter the extent of fatty acid synthesis in mammary gland slices from lactating rats (36).

Except for acetyl-CoA carboxylase, the activities of the mammary gland enzymes (49–57) involved in the conversion of the substrates used to fatty acid exceed the absolute rate of fatty acid synthesis, as gauged by the incorporation of hydrogen from water. The reported activity of acetyl-CoA carboxylase in lactating gland is slightly less (52) or equivalent to (50, 53, 56) the maximal rate of fatty acid synthesis listed in Table 5. This activity, however, may be higher in vivo since the maximal activity of this enzyme is difficult to determine in vitro (58). The total activity of pyruvate dehydrogenase (56, 57) exceeds the rate of synthesis observed here by a small amount, but only about one-half of this enzyme is in the activated form at mid-lactation (57). Therefore, either pyruvate dehydrogenase or acetyl-CoA carboxylase or both could act as the rate limiting enzymes in the formation of fatty acids by mammary gland from lactating rats. It is interesting to note that the activity of both enzymes can be modulated by metabolite levels in the tissue (57–61). Whether the modulating effects of exogenous substrates are mediated by this means will have to await measurement of intracellular metabolites in the presence of the various substrate combinations.

Relationship between uptake of substrate and fatty acid synthesis

A portion of the stimulatory action of glucose on lipogenesis from other substrates can be related to their increased uptake. The presence of glucose increased the uptake of lactate by 1.8 μ moles, which is the magnitude of the increase in lipogenesis from lactate. On the other hand, the addition of glucose increased pyruvate uptake by 0.6 μ moles, but at the same time, fatty acid synthesis from pyruvate was increased by only 0.2 μ moles. Addition of lactate, and to an even greater extent of pyruvate, decreased the uptake of glucose disproportionately more than the decrease in fatty acid synthesis from glucose. Hence, changes in lipogenesis are not proportionately related to changes in uptake. This

TABLE 7. Effect of glucose on the formation and conversion of acetyl-CoA to fatty acid by slices of mammary gland of lactating rats

Substrate		AcCoA from Substrate 1 (A)	Conversion of AcCoA from Substrate 1 to Fatty Acid (B)	Percent of AcCoA Converted to Fatty Acid (B/A \times 100)
1	2			
<i>nmoles/100 mg/30 min</i>				
Pyruvate	None	683	221	32
Pyruvate	Glucose	691	420	61
L-Lactate	None	447	55	12
L-Lactate	Glucose	2204	1859	91

Slices were prepared and incubated as described in the text and Table 1. The concentration of each substrate listed was 10 mM. The acetyl-CoA formed was taken as equivalent to the $^{14}\text{CO}_2$ production from the carboxyl labeled organic acids (Table 3). The conversion of acetyl-CoA formed from pyruvate and L-lactate was taken as the conversion of the methyl-carbon labeled acids to fatty acid (Table 4). See text and Tables 3 and 4 for other experimental details.

conclusion is confirmed by the experiments with tritiated water. There is a direct relationship between the total uptake of exogenous substrates and the absolute extent of lipogenesis but it is not a linear one. Factors other than simply increased uptake can evidently modify the metabolic fate of exogenous substrates.

Effect of substrates on the utilization of acetyl-CoA

The relationship between uptake and lipogenesis may not be a simple one, because the addition of a second substrate can alter the route of utilization of metabolites at branch points. The rate of formation of acetyl-CoA from pyruvate or lactate, and its utilization for fatty acid synthesis, can be calculated from the results presented here. Acetyl-CoA and CO_2 from pyruvate are produced in equimolar quantities in the reaction catalyzed by pyruvate dehydrogenase. Therefore, the moles of acetyl-CoA formed from pyruvate or lactate are equivalent to the $^{14}\text{CO}_2$ yields from [1- ^{14}C]pyruvate or [1- ^{14}C]lactate. The incorporation into fatty acid of label from C-3 of pyruvate or lactate represents the portion of the acetyl-CoA formed from these compounds that is utilized for fatty acid synthesis.

Glucose as an additional substrate had no effect on the extent of acetyl-CoA formation from exogenous pyruvate (Table 7). On the other hand, glucose addition almost doubled the extent to which the acetyl-CoA formed from pyruvate was utilized for fatty acid synthesis. In the presence of glucose, 61% of the acetyl-CoA derived from pyruvate ended up in fatty acid compared to 32% in the absence of glucose (Table 7). In incubations containing L-lactate, the in-

crease in acetyl-CoA formation due to the presence of glucose (1.8 μ moles) was almost identical to that in fatty acid synthesis. These conversions were the same as the increase in lactate uptake due to addition of glucose (Table 1). It should be noted, however, that in the absence of glucose, only 12% of the acetyl-CoA derived from lactate was converted to fatty acid; addition of glucose increased the percentage by almost 8-fold (Table 7). Clearly, the effect of glucose on fatty acid synthesis from exogenous lactate was on both the formation and the conversion of acetyl-CoA to fatty acid. The major site of glucose stimulation of fatty acid synthesis from both pyruvate and lactate was, however, on the diversion of acetyl-CoA toward fatty acid synthesis and away from oxidation.

Relationship between hexose monophosphate pathway (HMP) and fatty acid synthesis

Enzymatic studies (49, 52, 55) and measurements of carbon flux (6, 13, 35, 43, 62, 63) have clearly established that the HMP pathway is the major source of NADPH in the rat mammary cell. The direct relationship between the respiratory quotient and the extent of fatty acid synthesis, observed many years ago in mammary gland studies *in vitro* (3, 16, 64), is based on this relationship between lipogenesis and HMP activity.

The low conversion of C-6 of glucose to CO₂, in combination with the high CO₂ yield from C-1 of glucose, provides an easy means of calculating the NADPH produced in the HMP pathway in lactating mammary gland (63). The glucose flux through the HMP pathway must be between the yield of CO₂ from C-1 of glucose minus that from C-6 and the yield from C-1 alone (65). For each glucose molecule utilized in this pathway, two moles of NADPH are produced. Calculating the glucose flux through the HMP pathway in this manner reveals that 15–21% of the glucose taken up from the medium was utilized in this pathway regardless of the substrates provided. This relationship between the HMP flux and glucose uptake is rather constant in face of a glucose uptake that was significantly altered by the addition of the other exogenous substrates (Table 1). Hence, in rat mammary gland, it would appear that either glucose uptake and the HMP pathway are coupled or the activities are regulated by a common mechanism.

When the activity of the HMP pathway is compared to the capacity for fatty acid synthesis from exogenous substrates, the carbon flux through the HMP pathway increases linearly with the rate of fatty acid synthesis up to 1700 nm/min. Obviously, below this level the availability of glucose could stimulate fatty acid synthesis by providing the necessary substrate

for the production of reductive hydrogen in the HMP pathway. However, fatty acid synthesis can be increased without a concomitant increase in HMP activity. Therefore, the extent of fatty acid synthesis in the gland is not necessarily coupled to the activity of the HMP pathway; other factors are involved.

Relationship between mitochondrial activity and fatty acid synthesis

Maximal synthesis was observed with glucose in the presence of L-lactate, but not pyruvate, and this maximal rate was not completely coupled to HMP pathway activity. The specificity of the fatty acid synthetase multi-enzyme complex for NADPH (54) and the lack of significant incorporation of carbon from lactate (Table 4) and, more specifically, of tritium from [2-³H]lactate into fatty acids¹, all argue against NADH as a direct source of reductive power in mammary gland. Further analysis of the results presented here provide circumstantial evidence that the source of this reductive power is a series of reactions that provide a means of converting NADH to NADPH in the cytosol.

We have shown directly (66) and there is supportive, circumstantial evidence (11, 50, 67) that at least a portion of the acetyl-CoA for fatty acid synthesis is transported out of the mitochondria of rat mammary gland in the form of citrate. Subsequent cleavage of the citrate yields acetyl-CoA, which can be converted to fatty acids, and oxalacetate. Conversion of oxalacetate to malate would promote fatty acid synthesis by two possible mechanisms: (a) production of NADPH during oxidation of malate to pyruvate and (b) providing the anion required for the facilitated transport of citrate across the mitochondrial membrane (68, 69). The conversion of oxalacetate to malate requires a source of NADH in the cytosol. Lactate, by supplying such a source of NADH, would provide an indirect means of stimulating fatty acid synthesis. Furthermore, utilizing lactate as the major carbon source for fatty acid synthesis in the presence of glucose would decrease the amount of ATP produced during the conversion of exogenous substrates to acetyl-CoA. In mammary gland, ATP is produced from glucose in excess of that required for fatty acid synthesis (13), a situation proposed as limiting lipogenesis in other tissues (70).

The explanation for the inhibitory effect of pyruvate on incorporation of glucose into fatty acid may also be related, in part, to a requirement of NADH in the cytosol. The formation of lactate from exog-

¹J. C. Bartley and S. Abraham, unpublished observations.

enous substrates (Table 6) would lessen the availability of NADH and, thereby, hinder the conversion of oxalacetate to malate in the cytosol. In this situation, malate may not be required as a source of NADPH, but its lack could limit fatty acid synthesis due to the lack of malate-facilitated transport of citrate to the cytosol (68, 69, 71).

The inability of the gland to form fatty acids with lactate as the sole substrate is due in part to the lack of mechanism for converting cytosolically generated NADH to NADPH (13, 33). Providing glucose or pyruvate as an additional substrate could overcome this block by generating mitochondrial citrate which, upon transfer to the cytosol, would yield oxalacetate. The NADH would then be utilized in converting oxalacetate to malate. Of course, pyruvate with lactate does not provide NADPH via the HMP pathway and, hence, cannot support the same extent of fatty acid synthesis as does glucose. ■■

Ms. Shirley Barber is gratefully acknowledged for her expert technical assistance. These investigations were supported by USPHS Grants GM-19837 from the National Institutes of General Medical Sciences and RR-05467 for General Research Support.

Manuscript received 20 November 1975 and in revised form 17 February 1976; accepted 10 May 1976.

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