FIG. 4. Effects of cardiotonic agents on the isolated spontaneously beating frog heart perfused with EDTA. Time scale = 5 seconds. 1. At A, the Ca-Ringer's solution was replaced by a Ca-free Ringer's solution containing 1.34 mM EDTA-Na_2. At B, 10 min after A, a Sr-Ringer's solution containing 6.7 $\times$ 10^{-7} M ouabain replaced the EDTA solution. At C, 120 min after B, no cardiotonic response was observed. 2. At A, the Ca-Ringer's solution was replaced by a Ca-free Ringer's solution containing 1.34 mM EDTA-Na_2. At B, 10 min after A, a Ca-Ringer's solution containing 6.7 $\times$ 10^{-7} M ouabain replaced the EDTA solution. At C, about 15 min after B, weak contractions were recordable. At D, about 30 min after C, maximal positive inotropic action was elicited. 3. At A, the Ca-Ringer's solution was replaced by a Sr-Ringer's solution containing 1.7 $\times$ 10^{-6} M rhodochlorin. At B, 60 min after A, 0.14 mM $CaCl_2$ was added to the perfusion solution. At C, about 60 min after B, a positive inotropic response was elicited.

effect when present in the same solution. Following perfusion with Ca-free Ringer's solution containing EDTA, contractility may be partially restored by perfusing the heart with Ca-Ringer's solution with or without ouabain, but contractility was not restored by supra-normal Sr-Ringer's solution with or without ouabain. Sub-effective concentration of calcium when added to Sr-Ringer's solution containing rhodochlorin also elicited a weak cardiotonic action on the heart. It is suggested that strontium has little or no cardiotonic action of itself but depends upon calcium for its positive inotropic action, and that factors in addition to the facilitation of transmembrane flux of calcium or strontium ions may be important for the cardiotonic action of ouabain.

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Effects of Glucose, Pyruvate and α-Ketoglutarate on Acetate Metabolism In Rat Mammary Gland.* (32529)

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It is well known that lactating mammary gland slices have a high capacity for fatty acid synthesis and that the synthesis of fatty acids from C^{14} labelled substrates such as acetate, pyruvate, lactate, propionate and a number of amino acids is markedly stimulated by addition of glucose to the incubation medium(1-6). Abraham and Chaikoff(7) showed that this pronounced effect of glucose on the fatty acid synthesis of mammary gland slices is limited to the period of lactation. In mammary glands from pregnant or postlactating animals a much smaller effect of glucose on fatty acid synthesis could be detected.

In experiments on lactating rat mammary gland slices Hirsch *et al*(1) found a moderate stimulation of fatty acid synthesis from labelled acetate, when pyruvate was added to the incubation medium. If pyruvate were added in the presence of glucose, the stimulating effect of glucose was reduced.

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From the works mentioned above it seems as if the stimulation of fatty acid synthesis by glucose has two components: A major related to glucose metabolism above the pyruvate level and a minor related to carbohydrate metabolism at or below the pyruvate level. The purpose of the present work was to compare the effects of glucose and pyruvate on fatty acid synthesis in pregnant, lactating and postlactating animals in order to see whether both components of the glucose effect depend on the lactating state of the animal.

Observations were made on the interactions of pyruvate and glucose metabolism, and the effect of α-ketoglutarate on the incorporation of acetate-C^{14} into fatty acids was investigated.

Experimental. Animals and preparation of tissue. Mammary glands were obtained from 3 groups of Wistar rats that had been fed a stock diet *ad libitum*.† One group consisted of pregnant rats 0-3 days before parturition. The second group consisted of lactating rats which had suckled 6-8 pups for 10-14 days. The third group consisted of rats that had suckled for about 20 days. One or two days after weaning they were killed by cranial fracture, decapitation and destruction of the spinal cord, and the mammary glands were quickly excised and placed in ice-cold Krebs-Henseleit bicarbonate buffer of pH 7.3 to 7.4 (8). The tissue was sliced in 0.3 mm slices with a McIlwain-Buddle tissue chopper and washed 3 times with the ice-cold buffer. The slices were blotted on filter paper and 250 mg portions were incubated in 2.5 ml medium.

Substrates. Acetate-2-C^{14}, D-glucose-1-C^{14} and D-glucose-6-C^{14}, were obtained from New England Nuclear Corp., Boston. The labelled substrates were shown to be chromatographically pure. Acetate-2-C^{14}, unlabelled pyruvate and α-ketoglutarate were used as sodium salts.

Incubation conditions. The incubation media were made from Krebs-Henseleit bicarbonate buffer of pH 7.3 to 7.4. Labelled and unlabelled substrates were added as shown in the tables.‡ All incubations were carried out in duplicate or triplicate as indicated in the Tables. The results reported are averages of these.

The incubations were undertaken in 25 ml Erlenmeyer flasks provided with a 3 ml detachable vessel hung under the rubber serum caps. After 90 minutes' incubation at 37°C the tissue was inactivated by addition of 0.4 ml 5 N H_2SO_4. The addition was made through the serum stopper with a syringe mounted with a long needle. In the same way 1 ml of hyamine was added to the small vessel. The system was shaken for 1 hour at room temperature to allow complete absorption of CO_2 in the hyamine.

Analytical procedures. Carbon dioxide. The smaller vessel with $C^{14}O_2$-hyamine was placed in a liquid scintillation vial with 15 ml of scintillation mixture containing 5 g of 2,5 Diphenyloxazole and 0.3 g of 1,4-bis-2-(4-Methyl-5-Phenyloxazolyl)-Benzene per 1000 ml of toluene. After mixing, the C^{14}-activity was assayed in a Packard Tri-Carb liquid scintillation spectrometer. The presence of the glass vessel in the scintillation system was without any effect on the counting efficiency.

Fatty acids were isolated from the tissue by KOH saponification followed by n-hexan extraction, as described by Abraham *et al*(9). The extract was assayed for C^{14}-activity in the liquid scintillation spectrometer.

Glucose was determined by the glucose oxidase method(10). Glucose oxidase GOD III 15421 from C.F. Boehringer and Soehne GmbH, Mannheim was used. In order to neutralize the acidified media, the procedure was carried out in a phosphate buffer (pH 7).

Assay of C^{14}-activity in aqueous solutions was done by mixing 20 μl of the sample with 2 ml methanol and adding 10 ml scintillation mixture.

Results. Incorporation of acetate into fatty acids by mammary glands. From Table I it appears that acetate-C^{14} was incorporated into fatty acids by pregnant, postlactating as well as by lactating mammary gland slices. The incorporation in the glands from pregnant animals was higher than in the postlactating

† Karensmølle rat pellets.

‡ Pyruvate in the concentration used (5 μmol per ml) was shown to have maximal effect on fatty acid synthesis, *i.e.,* an increase in pyruvate concentration was without further effect on the incorporation of acetate-2-C^{14} into fatty acids whether or not glucose was present.

TABLE I. Incorporation of Acetate-2-C^{14} into Fatty Acids by Rat Mammary Gland Slices in Presence of Different Unlabelled Substrates. Acetate-2-C^{14} was added to the medium at a concentration of 4 µmoles per ml, 2 × 10^5 dpm/ml. Unlabelled substrates were added at a concentration of 5 µmoles per ml. See Methods for other details on incubation conditions. Results are given as % of added C^{14} activity recovered in fatty acids ± SEM. All incubations were carried out in duplicate.

		Unlabelled additions to incubation media					
Type of tissue	No. of animals	No addition	Glucose	Glucose and pyruvate	Pyruvate	Glucose and α-KG	α-KG
		%					
Lactating	7	.14 ± .039	17.40 ±1.12	6.51 ± .636	1.95 ± .252	17.81 ±1.94	.81 ± .244
1 day postlactating	5	.07 ± .023	.26 ± .052	.58 ± .088	.35 ± .065	.27 ± .069	.11 ± .032
2 day postlactating	3	.05 ± .012	.18 ± .082	.33 ±1.152	.18 ± .078	.17 ± .060	.06 ± .012
Pregnant*	5	.18 ± .035	1.26 ± .258	.50 ± .038	.32 ± .042	1.31 ± .254	.31 ± .080

* 0-3 days before term.

animals, but lower than in the lactating ones.

Effect of glucose on fatty acid synthesis from acetate-2-C^{14}. When glucose was added to the incubation medium containing lactating mammary gland slices the increase in acetate-C^{14} incorporation into fatty acids was more than 100-fold on the average. In accordance with the findings of Abraham and Chaikoff (7) a small increase (3-4 fold) was observed in postlactating tissue. A somewhat greater effect of the glucose addition was found with mammary tissue from pregnant rats (Table I).

Effect of pyruvate on fatty acid synthesis from acetate-2-C^{14}. Addition of pyruvate to the incubation medium caused an increase in the acetate-C^{14} incorporation into fatty acids not only in lactating mammary gland, but in pregnant and postlactating tissue as well. The effect was most pronounced in the lactating tissue.

If pyruvate was added to incubation media already containing glucose in addition to labelled acetate, the pyruvate inhibited acetate incorporation into fatty acids in lactating and pregnant tissue, *i.e.*, pyruvate counteracted the stimulating effect of glucose (Table I). In the postlactating tissue, however, pyruvate stimulated in the presence of glucose, *i.e.*, the effects of glucose and pyruvate were additive.

Effect of α-ketoglutarate on fatty acid synthesis from acetate-2-C^{14}. In lactating mammary gland slices fatty acid synthesis from acetate-2-C^{14} was increased by 5-6 times after the addition of α-ketoglutarate to the incubation medium (Table I). A slighter effect of α-ketoglutarate was seen in tissue from pregnant animals. If α-ketoglutarate was added to incubation media already containing glucose, no effect of α-ketoglutarate could be detected.

$C^{14}O_2$-recoveries. The recoveries of $C^{14}O_2$ are listed in Table II. It is evident that the oxidation of acetate was much more vigorous

TABLE II. Incorporation of Acetate-2-C^{14} into CO_2 by Rat Mammary Gland Slices in Presence of Different Unlabelled Substrates. See Table I and Methods section for incubation conditions and compositions of media. Results are given as % of added C^{14}-activity recovered in CO_2 ± SEM. All incubations were carried out in duplicate.

		Unlabelled additions to incubation media					
Type of tissue	No. of animals	No addition	Glucose	Glucose and pyruvate	Pyruvate	Glucose and α-KG	α-KG
		%					
Lactating	7	.44 ± .132	.55 ± .148	.35 ± .060	.68 ± .180	.61 ± .163	.78 ± .177
1 day postlactating	5	.36 ± .246	.32 ± .061	.18 ± .037	.22 ± .045	.37 ± .070	.48 ± .083
2 day postlactating	3	.52 ± .054	.42 ± .063	.24 ± .061	.27 ± .073	.46 ± .113	.48 ± .146
Pregnant	5	3.34 ± .356	3.28 ± .455	1.38 ± .161	1.46 ± .157	3.10 ± .402	3.65 ± .364

in tissue from pregnant animals than in the lactating or postlactating state.

Pyruvate consistently exerted an inhibition of the oxidation of acetate-2-C^{14} in mammary gland of pregnant animals. A less pronounced inhibition was seen in the postlactating tissue.

Addition of α-ketoglutarate increased the $C^{14}O_2$ yields from acetate-C^{14} in all experiments with slices from lactating and 1-day postlactating animals. In tissue from pregnant and 2-day postlactating animals the effect was variable.

Glucose uptake. The greatest glucose uptake was seen in tissue from lactating animals (Table III). The uptake in the postlactating glands was lowest, with a somewhat higher uptake in glands from pregnant animals. Pyruvate inhibited glucose uptake in lactating glands and in glands of pregnant animals. No such effect was seen in the postlactating gland. α-ketoglutarate had no significant effect on glucose uptake in any of the 3 types of glands.

Experiments with 1- and 6-labelled C^{14}-glucose. Using tissue from 3 pregnant rats experiments were performed with glucose-1-C^{14} and glucose-6-C^{14} in the absence and presence of pyruvate (Table IV). In all experiments the incorporation of C^{14} from the 6-position into fatty acids was found higher than that from the 1-position, while the $C^{14}O_2$ production from C-1 exceeded that from C-6. These findings indicate that the pentose phosphate shunt is operating in the tissue. When pyruvate was present in the incubation medium, the absolute amounts of glucose-C^{14} recovered as $C^{14}O_2$ and C^{14}-fatty acids decreased. $C^{14}O_2$-recovery from glucose-6-C^{14} was reduced by a greater factor than $C^{14}O_2$-recovery from glucose-1-C^{14}. Conversely the recovery of C^{14} in fatty acids was reduced by a greater factor when glucose was labelled in the 1-position, than when the label was in the 6-position.

Discussion. It is generally agreed that glucose stimulates fatty acid synthesis in the lactating mammary gland by the generation of TPNH in the pentose-phosphate shunt (7,11,12). No mechanism has been proposed to explain the stimulation of fatty acid synthesis that is caused by addition of pyruvate to the system.

Pyruvate probably exerts its stimulating effect on fatty acid synthesis at the Krebs cycle level, since α-ketoglutarate causes a similar stimulation. It seems unlikely that α-ketoglutarate stimulates *via* a conversion to pyruvate, since α-ketoglutarate does not interfere with glucose metabolism as pyruvate does. Pyruvate may stimulate fatty acid synthesis after conversion to oxaloacetate by increasing the production of ATP and reductive power in the Krebs cycle. In this respect it is of interest that Matthes *et al*(13) found that not only TPNH, but also DPNH may act as hydrogen donor in fatty acid synthesis in mammary gland homogenates, though TPNH is the more effective.

It was found for pyruvate as well as for glucose that their stimulating effect on fatty acid synthesis depends on the functional state of the gland. This result could be expected, as Howanitz and Levy(14) showed that, when lactation is terminated, a precipitous fall occurs in the activity of acetyl-CoA carboxylase and the citric acid cleavage enzyme. These are the enzymes that catalyze the initial steps in fatty acid synthesis from acetyl-CoA and citrate, respectively.

The finding of similar effects of glucose and pyruvate on fatty acid synthesis in the

TABLE III. Uptake of Glucose by Rat Mammary Gland Slices Incubated with Acetate and Other Substrates. See Table I and Methods section for incubation conditions and composition of media. Results are given as % of added glucose utilized during the incubation ± SEM. All incubations were carried out in duplicate.

Type of tissue	No. of animals	Unlabelled additions to incubation media		
		Glucose	Glucose and pyruvate	Glucose and α-KG
		%		
Lactating	7	50 ± 2.99	32 ± 3.23	49 ± 2.43
1 day postlactating	5	16.7 ± 2.60	18.0 ± 2.36	16.6 ± 2.56
2 days "	3	10.0 ± 3.55	9.8 ± 3.84	7.9 ± 3.37
Pregnant	5	18.7 ± 1.94	11.8 ± 2.05	16.8 ± 1.86

TABLE IV. Metabolism of Acetate-2-C^{14}, Glucose-1-C^{14} and Glucose-6-C^{14} in Pregnant Rat Mammary Gland Slices. Effect of Pyruvate. Composition of media and incubation conditions as given in Table I and in Methods section. Activity of glucose-1-C^{14} was 4×10^5 dpm/ml; of glucose-6-C^{14} 2×10^5 dpm/ml. All recovery figures are averages of triple incubations.

Additions to media	% of added C^{14}-activity recovered in: Fatty acids, No addition	Fatty acids, Glucose	Fatty acids, Glucose and pyruvate	Fatty acids, Pyruvate	CO_2, No addition	CO_2, Glucose	CO_2, Glucose and pyruvate	CO_2, Pyruvate	% of added glucose utilized: Glucose	% of added glucose utilized: Glucose and pyruvate
	%									
Rat No. XXXII:										
Acetate-2-C^{14}	.23	2.07	.82	.42	4.61	4.09	1.36	1.52		
Glucose-1-C^{14}		.23	.03			3.47	2.20		27.8	17.8
Glucose-6-C^{14}		.26	.06			1.39	.27			
Rat No. XXXIII:										
Acetate-2-C^{14}	.64	3.83	1.23	.93	7.93	6.73	1.83	1.56		
Glucose-1-C^{14}		.29	.02			3.63	1.94		24.7	19.3
Glucose-6-C^{14}		.47	.07			2.13	.24			
Rat No. XXXIV:										
Acetate-2-C^{14}	.23	3.98	.79	.59	5.49	6.30	1.74	1.87		
Glucose-1-C^{14}		.33	.03			3.94	1.73		30.1	19.0
Glucose-6-C^{14}		.59	.06			2.73	.30			

postlactating gland suggests that glucose exerts its effect at the same level as pyruvate in this type of tissue. This is consistent with the finding of Abraham and Chaikoff(7) that the pentose phosphate shunt activity subsides rapidly after weaning.

The demonstration of Hirsch, Baruch and Chaikoff(1) that pyruvate antagonizes the stimulating effect of glucose has been confirmed. That this effect is not simply due to isotope dilution is evident from their figures, which allow a calculation of the total fatty acid synthesis from acetate, glucose and pyruvate. It can also be seen from our experiments XXXII and XXXIII (Table IV), where C^{14}-fatty acid synthesis from glucose-1-C^{14} and from glucose-6-C^{14} are reduced by different factors in the presence of pyruvate.

The antagonism can not be caused by a mere shift of glucose metabolism from pathways involving stimulation of fatty acid synthesis to pathways not involving such stimulation. It was not possible to calculate the participation of the pentose phosphate shunt and the Embden-Meyerhof pathway in glucose metabolism, since only part of the utilized glucose is metabolized *via* these pathways. However, the inhibition must mainly involve the Embden-Meyerhof pathway, since $C^{14}O_2$-recovery from glucose-6-C^{14} was reduced by a greater factor than $C^{14}O_2$-recovery from glucose-1-C^{14}. The same conclusion can be drawn from the finding that the recovery of C^{14} in fatty acids was reduced by a greater factor when glucose was labelled in the 1-position, than when the label was in the 6-position.

Duncombe and Glascock(15) have shown that pyruvate inhibits the $C^{14}O_2$ formation from glucose-1-C^{14} as well as from uniformly labelled glucose. They suggested that this effect of pyruvate might be explained by gluconeogenesis causing isotope dilution at the glucose-6-phosphate level. This possibility is excluded by our finding that the $C^{14}O_2$-recovery from glucose-6-C^{14} is reduced by a 4-fold greater factor than is the $C^{14}O_2$-recovery from glucose 1-C^{14}. It may be added that although gluconeogenesis occurs in the bovine udder(16,17), there is no indication of gluconeogenesis in the rat mammary gland(18).

Uyeda and Racker(19) found that phos-

phoenolpyruvate inhibits phosphofructokinase activity in rabbit muscle preparations. A similar situation may exist in the mammary gland and might account for the inhibition of the Embden-Meyerhof reactions. An inhibition of phosphofructokinase would lead to accumulation of fructose-6-phosphate and possibly of glucose-6-phosphate, which is known to inhibit hexokinase and thus glucose-uptake. It is however, difficult to understand how glucose stimulation of fatty acid synthesis *via* the TPNH production can decrease in the presence of an accumulation of glucose-6-phosphate. It is conceivable that some other metabolic product of pyruvate may inhibit hexokinase. The formation of such an intermediate would most likely depend on the functional state of the gland.

With respect to fatty acid synthesis, stimulation of fatty acid synthesis by glucose or pyruvate, and to the interaction of pyruvate and glucose metabolism, tissue from pregnant animals close to parturition occupies an intermediary position between the lactating and the postlactating tissues. Glucose is found to stimulate fatty acid synthesis somewhat more than pyruvate, which suggests that a certain pentose-phosphate shunt activity is present at this time of pregnancy. In this respect our Wistar rats differ from the Long-Evans rats used by Abraham and Chaikoff(7), which showed no pentose-phosphate activity before the onset of lactation.

Summary. Glucose and pyruvate stimulate the incorporation of acetate-2-C^{14} into fatty acids in mammary gland slices from pregnant, lactating and postlactating rats. α-ketoglutarate did so only in tissue from pregnant and lactating animals. The effect of the 3 added substrates varied with the functional state of of the gland, being most pronounced in lactating tissue. In mammary glands from lactating and pregnant rats pyruvate inhibited glucose uptake by the tissue and the glucose stimulation of fatty acid synthesis from acetate-2-C^{14}. In postlactating tissue pyruvate neither inhibited glucose uptake nor antagonized the glucose effect on fatty acid synthesis. On the contrary, the effect of glucose and pyruvate was additive. α-ketoglutarate did not interfere with glucose metabolism.

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