Relative Utilization of Glucose and Acetate Carbon for Lipogenesis by Mammary-gland Slices, Studied with Tritium, ^{13}C and ^{14}C

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Respiration and isotope-incorporation results, previously obtained in our laboratory, indicate that lactating mammary-gland slices actively effect net synthesis of fatty acids from small molecules *in vitro*, though there are marked species differences. Thus, udder slices from lactating ruminants actively respire with R.Q. > 1 in a medium containing acetate as sole substrate (Folley & French, 1950), and under these conditions will incorporate carbon from the carboxyl group of acetate into fatty acids, though the rate of this incorporation of acetate is considerably increased in the presence of glucose (Balmain, Folley & Glascock, 1952). On the other hand, mammary slices from lactating rats, in common with mammary tissue from other non-ruminants (mouse, rabbit, guinea pig), will utilize glucose as sole substrate with R.Q. > 1 (Folley & French, 1949), but will only incorporate carboxyl carbon of acetate into the fatty acids in the presence of glucose (Balmain *et al.* 1952).

Mammary tissue from the rat and sheep also differ as regards the effects of insulin and glycerol on lipogenesis from small molecules *in vitro*. The R.Q. of rat-mammary slices in a solution of glucose plus acetate is significantly increased by insulin or glycerol added *in vitro* (Balmain & Folley, 1951) and these agents also increase the incorporation of the carboxyl carbon of acetate into the fatty acids (Balmain *et al.* 1952). The respiration of sheep-udder slices, on the other hand, is unaffected by either agent in the presence of acetate plus glucose, though glycerol (but not insulin) increases both the R.Q. (Balmain & Folley, 1951) and the incorporation of acetate (carboxyl) carbon into the fatty acids in presence of acetate alone (Balmain *et al.* 1952).

The main object of the present work was to determine the relative values of acetate and glucose as sources of carbon for fatty acid synthesis by mammary tissue when both substrates are present in the incubation medium. In view of the abovementioned species differences, it seemed likely that mammary tissue from rat and sheep might differ in the proportions of acetate and glucose carbon used for lipogenesis under these conditions. The simultaneous incorporation of both carbon atoms of acetate and of glucose carbon into fatty acids by the same sample of mammary tissue has therefore been studied in the rat and sheep by means of a triple-labelling technique in which the methyl carbon of acetate was labelled with tritium, the carboxyl carbon with ^{13}C, and all the carbon atoms of glucose with ^{14}C. Studies of the effects of insulin and glycerol on the simultaneous incorporation of glucose carbon and both acetate carbon atoms into fatty acids by mammary slices have also been included in these experiments in the hope of gaining further insight into the mechanism of the stimulatory effect of insulin upon fat synthesis. A preliminary account of part of this work has already appeared (Balmain, Folley & Glascock, 1953).

METHODS

The experimental procedure was in general similar to that described previously (Balmain *et al.* 1952), sliced mammary tissue from primiparous lactating rats and ewes being obtained as before. Separate experiments were set up with tissue from each udder half from three lactating ewes, while for each of the six experiments with rats a pool of abdominal mammary slices from two or more animals was used. Each incubation flask contained 2 g. of slices which were incubated with constant shaking for 3 hr. at 37° in 20 ml. of medium. The medium was Krebs bicarbonate-saline, the gas phase 95% O_2 and 5% CO_2 (v/v) and the substrates 0·02 M sodium [*carboxy*-^{13}C-*Me*-^{3}H]acetate and 0·3% (w/v) [^{14}C]glucose. The specific activity of ^{3}H in the experiments on rat, which were carried out before the experiments on sheep, was 6·5 μc/g. acetate, but because the amounts of ^{3}H found in the fatty acids were rather lower than expected, the specific activity of the substrate was doubled in the experiments on sheep, unnecessarily as it afterwards proved. In the tables the specific activities of ^{3}H in the fatty acids isolated from sheep tissue have therefore been corrected to a substrate specific activity of 6·5 μc/g. The ^{13}C abundance in the whole acetate molecule was 5 atoms per cent excess and the specific activity of ^{14}C was 3·5 μc/g. glucose. In each experiment four flasks were set up with substrates (concentrations as above) as follows: (1), ^{14}C-glucose, (2), (3), (4), [^{14}C]glucose + [*carboxy*-^{13}C-*Me*-^{3}H]acetate; insulin (1 i.u./ml.) was added to (3) and glycerol (0·2%, v/v) to (4). This design was replicated six times for each species. The mixed fatty acids were isolated from the slices as Ca salts as described previously (Balmain *et al.* 1952). The three isotopes were determined after combustion of the fatty acid salts as described by Glascock (1952).

^3H-labelled sodium acetate was prepared by the reaction used by Halford & Anderson (1936) for the preparation of [^2H]acetate. Tritio-malonic acid was prepared by dissolving 0·62 g. of unlabelled acid in an equal weight of radioactive water and evaporating to dryness *in vacuo*. This yielded malonic acid labelled in all H atoms. The dry acid was then decarboxylated by being heated to 180° in a sealed, evacuated tube large enough to accommodate all the CO_2 produced and the resultant acetic acid was condensed into a small side arm. The side arm was cut off and the acetic acid was exactly neutralized with N-NaOH to displace the labile hydrogen.

RESULTS

Acetate and glucose as sources of carbon for lipogenesis

The yields of mixed fatty acids from 2 g. batches of slices were, on the average, about the same for both rat and sheep, thus indicating that the amounts of pre-formed tissue fat diluting the fat newly formed during the experiment from labelled substrates was also about the same for each species. Comparison of the abundance of any of the three isotopes in the fatty acids isolated from the mammary tissue of the two species therefore gave an approximate measure of the relative substrate incorporation during the experiment. The values for the incorporation of all three isotopes into the fatty acids of the mammary slices are given for both species in Table 1. No ^{13}C values are given for two of the rat experiments because the abundances were too low to be measured accurately in the spectrometer. It will be seen that the fatty acids of rat mammary tissue contained, on the average, about eight times more labelled glucose carbon than did those of sheep slices, whereas the fatty acids of sheep mammary tissue contained about six times as much of either of the two acetate carbon atoms as did rat slices. The agreement between the results obtained for the two acetate carbons may be noted.

From the ^{13}C and ^{14}C incorporation data, taken in conjunction with the known abundances of the two isotopes in the substrates, the respective contributions of glucose carbon and carbon of the acetate carboxyl group to the newly synthesized fatty acids can be calculated. Values calculated in this way from the mean isotopic abundances shown in Table 1 are given in Table 2, the values for acetate referring to both carbon atoms. This table shows that, allowing for the experimental error inevitable in this kind of experiment, rat mammary gland utilizes about equal amounts of acetate and glucose carbon for fatty acid synthesis, with perhaps a slight preference for glucose carbon. Sheep mammary tissue, by contrast, uses some 30 times more acetate carbon than glucose carbon for fatty acid synthesis. It will also be noted that the fatty acids present in the sheep-udder slices contained about twice as much newly synthesized fatty acid carbon per 100 mg. carbon as did those present in the rat mammary tissue (cf. the sums of columns 2 and 4 of Table 2). Since the tissues from both species gave about the same yields of mixed fatty acids, this indicates that sheep udder tissue synthesizes fatty acids *in vitro* at about twice the rate of rat mammary tissue. These calculations are made on the assumption that the glucose molecule is uniformly labelled. If the specific activities of carbon

Table 1. *Relative incorporation of glucose and acetate carbon into the fatty acids of mammary-gland slices incubated with* [^{14}C]*glucose* (0·3 %, *w/v*) *plus* [*carboxy*-^{13}C-*Me*-^3H]*acetate* (0·02 M)

Species							Mean	Ratio (*a*/*b*)
	^{14}C(glucose carbon) counts/min./mg. C							
Rat	307	381	124	245	110	170	223 (*a*)	7·9
Sheep	38·4	17·4	22·5	24·7	35·8	31·6	28 (*b*)	
	^3H (acetate; methyl carbon) counts/min./mg. combustion water							
Rat	84·5	94·8	41·7	109·5	43·9	71·2	73·3 (*b*)	6·3
Sheep	421	405	501	502	451	502	464 (*a*)	
	^{13}C(acetate; carboxyl carbon) atom per cent excess							
Rat	0·07	0·05	—	0·06	—	0·04	0·055 (*b*)	5·9
Sheep	0·252	0·249	0·373	0·378	0·370	0·320	0·324 (*a*)	

Table 2. *Incorporation of substrate carbon into fatty acids by mammary-gland slices utilizing acetate plus glucose*

	Acetate (^{13}C)		Glucose (^{14}C)	
	mg. C/100 mg. fatty acid* C	% of total C of newly formed fatty acids	mg. C/100 mg. fatty acid C	% of total C of newly formed fatty acids
Rat	1·1	38	1·8	62
Sheep	6·5	97	0·2	3

* Calculated for both carbon atoms of acetate.

atoms (1), (2), (5) and (6) were less than the average specific activity for the whole molecule, then the amount of glucose carbon incorporated would be greater than that shown in Table 2.

Effect of acetate on the incorporation of glucose carbon into fatty acids of rat-mammary tissue

The experimental design permitted the comparison of the incorporation of glucose carbon into the mixed fatty acids of mammary slices incubated in glucose alone and in glucose plus acetate. The results for the rat are given in Table 3. In all experiments the incorporation of glucose carbon was decreased when acetate was present in the medium, and on the average it appeared that the addition of acetate roughly halved the utilization of glucose carbon. This may perhaps be due to the dilution of ^{14}C-labelled two-carbon units derived from glucose by two-carbon units derived from acetate or their diversion to oxidation (see later). The results for the experiments on sheep (Table 4) show that the relatively trivial incorporation of the carbon of glucose when the latter is present in the medium by itself was not materially altered by the addition of acetate.

Relative utilization of the two acetate carbons

These experiments provided results bearing upon the question of whether the two carbon atoms of acetate were utilized to an equal extent by the slices for lipogenesis. If both carbon atoms were used equally for fatty acid synthesis and no loss of tritium occurred, the ratio of tritium to ^{13}C would be the same in the fatty acids as in the substrate acetate. According to the mechanism of the reaction postulated by Popják, French, Hunter & Martin (1951), however, tritium would be lost both by the process of condensation of two-carbon units and by exchange owing to an activation of the methyl group. If the ratio of tritium to ^{13}C in the substrate acetate is defined as unity, that in the fatty acids must therefore be less than one. It would thus be possible to compare the utilization of the acetate carbon atoms only if the tritium to ^{13}C ratio actually found in the fatty acids could be compared with that found when they had been formed from acetate doubly labelled in the methyl group. Fortunately these data were available for comparison, having been previously obtained in some separate experiments designed to test the usefulness of tritium as a label for alkyl groups (Glascock, unpublished). In those experiments, mammary tissue from both the rat and the sheep was incubated in the usual medium containing glucose, together with acetate doubly labelled in the methyl group ($^{14}C^{3}H_3COONa$).

In the rat experiments three animals were used, three determinations being carried out on tissue from the first and two determinations on tissue from each of the others. In the sheep experiments, one determination was made on tissue from each of seven udder halves. The ratio of tritium to labelled carbon in the substrate acetate molecules again being defined as unity, the value of the ratio in the fatty acids extracted from the tissue was calculated. Statistical analysis of the results obtained (Table 5) shows that for both the rat and the sheep the value of the ratio of tritium to labelled carbon does not differ significantly when the carboxyl carbon atom is labelled (with ^{13}C) from that obtained when the methyl carbon atom is labelled (with ^{14}C), thus providing a formal proof that both acetate carbon atoms are used equally for fatty acid synthesis.

These results therefore provide evidence that the process of fatty acid synthesis does not involve fission of the two-carbon chain of acetate and also show that about two-thirds of the hydrogen atoms

Table 3. *Effect of acetate on utilization of* ^{14}C*-labelled glucose carbon for fatty acid synthesis by rat-mammary slices* in vitro

(Results are expressed as counts/min./mg. fatty acid C.)

Substrate	Expt. no. 245	246	247	248	249	250	Mean	Ratio
Glucose (0·3%, w/v)	357	566	210	510	282	530	409	1·83
Glucose (0·3%, w/v) + acetate (0·02 M)	307	381	123	245	110	169	223	

Table 4. *Effect of acetate on the utilization of* ^{14}C*-labelled glucose carbon for fatty acid synthesis by sheep-udder slices* in vitro

(Results are expressed as counts/min./mg. fatty acid C.)

Substrate	Expt. no. S4L	S4R	S5L	S5R	S6L	S6R
Glucose (0·3%, w/v)	26	41	46	35	21	23
Glucose (0·3%, w/v) + acetate (0·02 M)	38	17	23	25	36	32

Table 5. *Isotopic ratios in mixed fatty acids of mammary-gland slices incubated with glucose plus [carboxy-^{13}C-Me-^{3}H]acetate or glucose plus [Me-^{14}C-Me-^{3}H]acetate*

(In calculating the tritium to labelled-carbon ratios in the fatty acids, that in the substrate was defined as unity. Degrees of freedom given after standard error in parentheses.)

	^{3}H/^{13}C			^{3}H/^{14}C	Differences		
	No addition *a*	Insulin *b*	Glycerol *c*	No addition *d*	*a*−*b*	*a*−*c*	*a*−*d*
Rat							
No. of observations	4	4	4	7	—	—	—
Mean	0·378	0·300	0·293	0·291	$0·05 > P > 0·02$	$0·05 > P > 0·02$	$P > 0·05$
S.E.	0·0208 (6)*	0·0208 (6)	0·0208 (6)	0·0191 (2)	—	—	—
Sheep							
No. of observations	6	6	6	7	—	—	—
Mean	0·336	0·396	0·365	0·361	$P > 0·05$	$P > 0·05$	$P > 0·05$
S.E.	0·0225 (10)*	0·0225 (10)	0·0225 (10)	0·0123 (3)	—	—	—
Difference (sheep − rat)	—	—	—	$P > 0·05$	—	—	—

* Standard error for use in comparisons with treatment (*d*): rat, 0·0336 (3); sheep, 0·0176 (5).

Table 6. *Effects of insulin and glycerol on the incorporation of glucose and acetate carbon into the fatty acids of slices of rat mammary gland incubated with* [^{14}C]*glucose* (0·3 %, *w/v*) *plus* [*carboxy*-^{13}C-*Me*-^{3}H]*acetate* (0·02 M)

	Expt. no. 245	246	247	248	249	250
	^{14}C(glucose C): counts/min./mg. fatty acid C					
No addition	307	381	124	245	110	170
Insulin	768	805	488	630	435	663
Glycerol	508	563	263	435	359	332
	^{3}H(acetate; methyl C): counts/min./mg. combustion water					
No addition	85	95	42	110	44	71
Insulin	176	202	138	203	131	193
Glycerol	301	301	143	359	332	286
	^{13}C(acetate; carboxyl C): atom per cent excess					
No addition	0·07	0·05	0·01	0·06	0·01	0·04
Insulin	0·13	0·14	0·07	0·16	0·08	0·17
Glycerol	0·26	0·23	0·07	0·26	0·16	0·24

Table 7. *Effects of insulin and glycerol on the incorporation of glucose and acetate carbon into the fatty acids of sheep-udder slices incubated with* [^{14}C]*glucose* (0·3 %, *w/v*) *plus* [*carboxy*-^{13}C-*Me*-^{3}H]*acetate* (0·02 M)

	Expt. no. S4L	S4R	S5L	S5R	S6L	S6R
	^{14}C(glucose C): counts/min./mg. fatty acid C					
No addition	38·4	17·4	22·5	24·7	35·8	31·6
Insulin	43·7	10·0	27·6	20·6	27·0	23·7
Glycerol	9·1	14·1	12·1	13·4	13·2	12·8
	^{3}H(acetate; methyl C): counts/min./mg. combustion water					
No addition	843	810	1002	1004	903	1004
Insulin	710	820	970	928	1045	1235
Glycerol	815	751	1006	1115	1130	790
	^{13}C(acetate; carboxyl C): atom per cent excess					
No addition	0·25	0·25	0·37	0·38	0·37	0·32
Insulin	0·20	0·25	0·33	0·31	0·28	0·30
Glycerol	0·26	0·28	0·35	0·34	0·27	0·28

of the acetate methyl group are lost or exchanged. A mechanism such as that postulated by Popják *et al.* (1951), involving the stepwise elongation of the fatty acid chain by successive addition of two-carbon units, is therefore in accord with our findings.

Table 5 shows that in the sheep experiments the tritium to ^{13}C ratios obtained in the presence of insulin or glycerol did not differ significantly from the mean ratio obtained in their absence. However, this was not so in the case of the rat, the difference in both cases just reaching the accepted level of significance. The reason for this is unknown.

Effects of insulin and glycerol on the utilization of acetate and glucose carbon

The effects of insulin and glycerol on the incorporation into the fatty acids of all three isotopes are shown for the rat in Table 6. The results confirm previous findings (Balmain *et al.* 1952) that insulin and glycerol increase the rate of incorporation of the carboxyl carbon of acetate into the fatty acids of slices of rat mammary gland and show further that the same is true for the methyl carbon of acetate and for glucose carbon.

The results for sheep-udder slices, given in Table 7, show that insulin and glycerol have no stimulatory effect on fatty acid synthesis from either acetate or glucose by sheep tissue incubated with acetate plus glucose confirming our previous findings for this species in respect of the carboxyl carbon of acetate and extending them to the methyl carbon of acetate and to glucose carbon. In fact there is an indication that the small uptake of glucose carbon (^{14}C) into the fatty acids was slightly inhibited by glycerol.

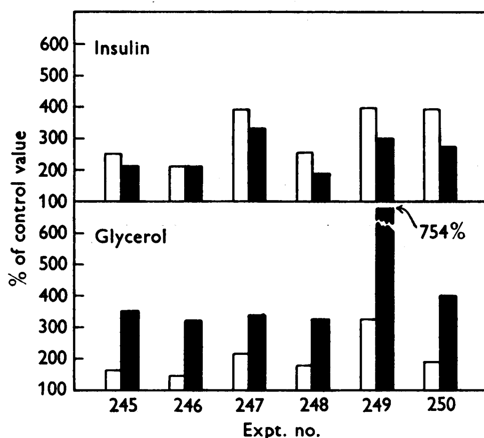

Fig. 1. The effects of insulin and glycerol, added *in vitro*, on the incorporation of acetate and glucose carbon into the fatty acids of slices of rat mammary gland incubated with [^{14}C]glucose plus [*carboxy*-^{13}C-*Me*-^{3}H]acetate; □, ^{14}C-(glucose C); ■, ^{3}H (acetate; methyl C). Drawn from the results of Table 6.

This is in keeping with our previous results (Balmain & Folley, 1951; Balmain *et al.* 1952) suggesting that glycerol slightly inhibits lipogenesis in sheep-udder slices incubated in acetate plus glucose.

Examination of the results for the rat (Table 6) shows that the effect produced by insulin differs markedly from that produced by glycerol. The difference between the effects of the two agents is more clearly shown in Fig. 1 in which, for each of the six experiments, the isotope abundances of tritium and ^{13}C in the fatty acids found in presence of insulin or glycerol are expressed as percentages of the corresponding control values. Fig. 1 shows that whereas in most experiments insulin increased the incorporation of glucose carbon to a slightly greater extent than it increased that of acetate carbon, glycerol increased the utilization of acetate carbon significantly more than it did that of glucose carbon.

DISCUSSION

Our results indicate that while mammary slices from lactating rats utilize acetate and glucose carbon for fatty acid synthesis to an approximately equal extent (with perhaps a slight preference for glucose), slices of lactating sheep udder utilize glucose carbon much more slowly than acetate carbon for this purpose, if at all. Our results for rat mammary tissue *in vitro* are thus in general agreement with those of Popják, Hunter & French (1953) for the lactating mammary gland of the living rabbit.

The present work indicates that in the mammary gland of the ruminant, as exemplified by the sheep, the mechanism which breaks down glucose to an 'active' two-carbon unit, in the form of acetyl coenzyme A, must be almost completely absent. If this is so it would be expected that sheep-udder slices would be unable to oxidize glucose by the accepted pathway leading through the glycolytic system to the formation from pyruvate of a two-carbon unit which enters the Krebs cycle as acetyl coenzyme A. However, recent isotope experiments in this laboratory by Duncombe & Glascock(1953), show that, contrary to what Folley & French (1950) had concluded from respiratory measurements, sheep-udder slices can oxidize appreciable amounts of glucose either alone or in the presence of acetate. It may therefore be that, in the ruminant mammary gland, pyruvate or some other three-carbon unit formed from glucose can enter the Krebs cycle as such, or else that glucose can be oxidized in this tissue by a pathway not involving the glycolytic system (see Dickens, 1953).

The respiratory measurements of Folley & French (1949) indicate that rat mammary tissue can form acetyl coenzyme A from glucose, and though it cannot 'activate' acetate alone it can do so if

glucose is also present (Folley & French, 1950; Balmain *et al.* 1952). The present work shows that so long as glucose is present, acetate seems to be as good a source of carbon for lipogenesis as glucose itself, just as in the mammary gland of the living rabbit. The explanation of how glucose permits the activation of acetate in rat mammary tissue must await further work.

While the metabolic difference between ruminant (sheep) mammary tissue and non-ruminant (rat) mammary tissue, apparent from previous work in this laboratory, has been further emphasized and to some extent clarified by the present experiments, further work will be needed before these interesting species differences can be fully explained. It may not be out of place, however, to suggest that they may have some connexion with the fact that the blood of ruminants normally contains appreciable levels of acetate, while the level of sugar is rather low as compared with non-ruminants.

The present experiments have provided further insight into the nature of the stimulatory effects of insulin and glycerol respectively upon fatty acid synthesis from acetate plus glucose in rat-mammary slices. Glycerol increases the incorporation of acetate carbon into the fatty acids much more than it increases that of glucose, while insulin stimulates the utilization of acetate and glucose more or less to the same extent. At first sight this might be regarded as evidence against our suggestion (Balmain & Folley, 1951; Balmain *et al.* 1952) that the stimulating effect of insulin upon lipogenesis might be exerted through the breakdown of glucose to glycerol. However, if the effect of insulin is exerted at more than one point in the chain of reactions leading from carbohydrate to fat, this need not necessarily be the case. It is admittedly difficult to see why, if the effect of glycerol in stimulating fatty acid synthesis is merely a 'mass action' effect as suggested previously (Balmain *et al.* 1952), the utilization of both substrates should not be increased by glycerol to an equal extent. One possibility that needs consideration is that the slices convert glycerol into glucose itself, or into a three-carbon compound intermediate in glucose breakdown, thus diluting the ^{14}C-labelled glucose carbon. This, however, seems unlikely, since previous work has shown that glycerol, by contrast with glucose, does not appreciably increase the utilization of acetate carbon by rat-mammary slices (Balmain *et al.* 1952).

SUMMARY

1. When incubated in a medium containing [^{14}C]glucose plus [*carboxy*-^{13}C-*Me*-^{3}H]acetate, mammary-gland slices from lactating rats incorporate into the fatty acids about eight times more glucose carbon and about six times less acetate carbon than do slices from lactating ewes.

2. Mammary tissue from the rat utilizes carbon from acetate or glucose for fatty acid synthesis at approximately equal rates, while udder slices from the ewe utilize for this purpose in a given time about 30 times more acetate carbon than glucose carbon.

3. In presence of acetate plus glucose, rat-mammary slices utilize less glucose carbon for fatty acid synthesis than in presence of glucose alone. Acetate has no effect on the trivial utilization for fatty acid synthesis of glucose carbon by slices of sheep udder.

4. Insulin, added *in vitro*, increases the incorporation of glucose and acetate carbon into the fatty acids of rat-mammary slices to about the same extent; glycerol, however, increases the utilization of acetate carbon significantly more than that of glucose carbon.

5. Both rat and sheep mammary tissue utilize both carbon atoms of acetate for lipogenesis to an equal extent. There is no evidence that the two-carbon chain is split in the course of lipogenesis. About two-thirds of the hydrogen atoms of acetate are lost or exchanged during chain elongation.

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