Further Observations on the *in vitro* Stimulation by Insulin of Fat Synthesis by Lactating Mammary Gland Slices

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(*Received* 26 *February* 1951)

The incorporation of [*carboxy*-^{14}C] acetate into fatty acids by rat-liver slices *in vitro* was studied by Bloch & Kramer (1948), who showed that the rate of incorporation was increased by pyruvate and still further enhanced if insulin was present as well. The insulin effect was, however, not seen if glucose replaced pyruvate, nor did it occur in acetate alone. Later, Bloch (1948) reported that these results could not be reproduced regularly with other strains of rats, and he concluded that further work was necessary before the general significance of this insulin effect, believed to be particularly related to the utilization of pyruvate for fatty acid synthesis, could be evaluated.

Following upon this work we were able, by using lactating mammary gland tissue of the rat, which is an eminently favourable medium for the study of *in vitro* fat synthesis (Folley & French, 1949*a*, 1950), to demonstrate a marked stimulating effect of insulin upon *in vitro* fat synthesis in this tissue in mixtures of acetate and glucose, as judged by respiratory data and acetate uptake (Balmain, French & Folley, 1950). A definite but less marked effect was observed in glucose alone. Our results thus differ in certain respects from those of Bloch & Kramer (1948) as do the recent results of Brady & Gurin (1950), who have reported that insulin increases the incorporation of ^{14}C into long-chain fatty acids by rat-liver slices in presence of methyl- or carboxyl-labelled acetate alone, but not in acetate plus pyruvate.

In this paper we report further observations upon the stimulating action of insulin *in vitro* on fat synthesis by lactating mammary gland slices. The phenomenon has now been studied in mammary tissue from the mouse, rabbit and ewe, as well as more extensively in tissue from the rat, especially in relation to the functional state of the mammary gland. The important question of the mechanism of this remarkable *in vitro* insulin effect has also been considered in a preliminary way. The active mammary gland normally secretes large quantities of neutral fat in the form of glycerides, and it would therefore appear that the problem of the net fat synthesis which mammary gland slices are clearly capable of effecting *in vitro* under suitable conditions (Folley & French, 1950) is essentially a problem of glyceride formation rather than merely of fatty acid synthesis. That being so, the question of the origin of the glycerol must be taken into consideration, and the possibility of the availability of glycerol being critical for the synthesis of neutral fat, and at one remove of fatty acids, in mammary tissue, naturally arises. In view of the possibility that the stimulating action of insulin on fat synthesis in this tissue, demonstrated by the present work, might, partly at any rate, be connected with the regulation of the supply of glycerol, we have studied the effects of the addition of glycerol on the respiratory metabolism and acetate uptake of lactating mammary tissue. Some of this work has already been communicated in preliminary form (Balmain & Folley, 1951).

EXPERIMENTAL

The experiments were carried out on hooded Norway rats, fawn mice (Glaxo FF strain), Dutch rabbits and sheep, only animals which were lactating satisfactorily being used, except when tissue taken at the end of lactation was required. As regards laboratory species, abdominal mammary glands were always used for slicing, which was carried out as before (Folley & French, 1949*a*). Respiratory measurements were made by the method of Dickens & Šimer (1931), most experiments lasting 2 hr., but some 3 hr. Quotients ($-Q_{O_2}$, Q_{acid}, $Q_{glucose}$) are expressed throughout as μl./mg. final dry wt./hr. Experimental details were as described previously (Folley & French, 1950), $-Q_{acid}$ being taken as an approximate measure of acetate uptake, since acetate was the only consumable anion used as substrate. In all cases, respiratory measurements in the presence and absence of insulin were made on tissue from the same gland. In one series of experiments residual glucose was determined in the medium at the end of the 2 hr. manometric run by the method of Nelson (1944).

'Regular' insulin was used in the experiments on rabbits, but for all the others a preparation of crystalline insulin (Novo), assaying approximately 20 i.u./mg. and free from glycogenolytic factor was used. The insulin was dissolved in the water to a concentration of 0·5 mg./ml. using the least possible amount of NaOH. The final pH of the solution was 7·4 (glass electrode).

RESULTS

In vitro *effect of insulin on fully lactating mammary tissue*

Rat. Results for four series of experiments in glucose plus acetate on tissue from rats in full lactation, thirty-seven animals in all, are summarized in Table 1. In all these experiments the substrate

concentrations were: sodium acetate 0·02 M; glucose, 0·3 %. This concentration of glucose was used because it had previously been found, by measurement of the time curve of total gas exchange, that while the pressure increase, due to excess production of carbon dioxide over oxygen uptake, soon fell off in 0·1 % glucose, it was approximately linear over at least 3 hr. when the initial glucose concentration was 0·3 % (Balmain *et al.* 1950).

It will be seen that in all four series $-Q_{O_2}$, $-Q_{acid}$ (≏acetate utilization) and R.Q. were significantly increased by addition of 1 i.u. insulin/ml. In the last column but one are given the values for 'extra CO_2', i.e. the CO_2 produced in excess of that required for an R.Q. of unity (the algebraic sum of Q_{CO_2} and $-Q_{O_2}$), the value for complete oxidation of either substrate. In this system this quantity may be taken as a rough indication of the extent of the occurrence of synthetic reactions leading to the formation of oxygen-poor substances (e.g. fatty acids) from oxygen-rich materials (carbohydrate, acetate). From the figures in the last column it will be seen that in all four series insulin increased the 'extra CO_2' production 2·5–3·0 times. Not only was the increase in $-Q_{O_2}$ and R.Q. accompanied by an increase in acetate utilization as indicated by a two- to three-fold increase in $-Q_{acid}$ but, as Table 2 shows, insulin also nearly doubled the glucose utilization.

Table 1. *Effect of insulin on the respiration and acetate utilization of lactating rat mammary gland slices in glucose* (0·3 %) *plus sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml. In this and succeeding tables the values given are means ± S.E.M.)

Experimental series	No. of rats	Stage of lactation at autopsy (days)	Duration of respiration experiments (hr.)	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)	Increase in 'extra CO_2' due to insulin (%)
1	10	12–18	3	Insulin	13·4 ± 0·9	−4·7 ± 0·6	2·03 ± 0·02	13·7 ± 1·0	149
				None	10·5 ± 0·9	−2·1 ± 0·3	1·53 ± 0·03	5·5 ± 0·6	
2	11	14–15	2	Insulin	14·7 ± 0·8	−4·8 ± 0·5	2·15 ± 0·02	16·8 ± 0·7	195
				None	10·1 ± 0·5	−1·4 ± 0·2	1·57 ± 0·04	5·7 ± 0·5	
3	10	13–15	2	Insulin	11·7 ± 0·6	−2·5 ± 0·2	2·07 ± 0·03	12·5 ± 0·8	178
				None	8·6 ± 0·6	−0·7 ± 0·2	1·53 ± 0·05	4·5 ± 0·5	
4	6	14–16	2	Insulin	9·7 ± 0·9	−1·7 ± 0·5	1·89 ± 0·03	8·6 ± 0·7	161
				None	8·6 ± 0·5	−0·5 ± 0·2	1·40 ± 0·04	3·3 ± 0·4	

Table 2. *Effect of insulin on the glucose uptake of lactating rat mammary gland slices in glucose* (0·3 %) *plus sodium acetate* (0·02 M)

(Residual glucose in medium determined by the method of Nelson (1944) at end of respiration experiment.)

Experimental series	No. of rats	Stage of lactation at autopsy (days)	Duration of experiment (hr.)	Addition	$-Q_{glucose}$
3	7	13–15	2	Insulin	9·2 ± 0·9
				None	4·7 ± 0·8

Table 3. *Effect of insulin on the respiration and glycolysis of lactating rat mammary gland slices in glucose* (0·3 %)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.)

Experimental series	No. of rats	Stage of lactation at autopsy (days)	Duration of respiration experiments (hr.)	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)	Increase in 'extra CO_2' due to insulin (%)
1	10	12–18	3	Insulin	10·5* ± 0·7	1·7 ± 0·3	1·80 ± 0·02	8·2 ± 0·5	52
				None	9·2* ± 0·7	1·7 ± 0·4	1·57 ± 0·03	5·4 ± 0·6	
2	11	14–15	2	Insulin	11·0† ± 0·7	3·2 ± 0·2	1·87 ± 0·04	9·3 ± 0·7	79
				None	9·4† ± 0·6	3·3 ± 0·3	1·55 ± 0·04	5·2 ± 0·4	

* Statistical analysis of these 10 pairs of differences gave $0·1 > P > 0·05$.
† Statistical analysis of these 11 pairs of differences gave $0·05 > P > 0·04$.

The results of two series of experiments carried out with 0·3 % glucose alone as substrate are given in Table 3. Here again R.Q. was significantly increased in the presence of insulin, but not to the same extent as when acetate was also present. The mean values of $-Q_{O_2}$ were greater in presence of insulin than in its absence, the difference in series 1 being significant at a level of probability between 5 and 10 % and in series 2 at a level just below 5 %. In glucose alone the 'extra CO_2' increased by 60–85 % as compared with 146 and 191 % in the same two series of experiments with glucose plus acetate as substrate.

In the presence of acetate alone, which is hardly utilized by rat mammary gland slices (Folley & French, 1950), insulin had no effect on the respiration of the tissue under the conditions of these experiments (Table 4).

Rabbit. Results for seven experiments on mammary gland slices from lactating rabbits (3–5 weeks) are given in Table 5. These were early experiments in which 'regular' insulin was used and a few of them have been previously reported (Balmain *et al.* 1950). They were carried out before it was found that a glucose concentration of 0·3 % was necessary for the full expression of the insulin effect, at any rate in experiments with rat mammary tissue. The substrate concentrations used in these experiments were: glucose, 0·1 %; acetate, 0·02 M, and the duration of all but one experiment was 3 hr. Even under these possibly suboptimal conditions insulin in all cases caused marked increases in R.Q., oxygen uptake and acetate utilization. The 'extra CO_2' production was substantially raised by insulin in all experiments.

Mouse. The mean results for an experiment on a group of five lactating mice (14 days in lactation) are given in Table 6. In glucose alone insulin had no appreciable effect on the respiration of mouse mammary gland slices, the R.Q. being of the same order as found previously for mouse tissue (Folley & French, 1949*a*), irrespective of whether or not insulin was present. In the absence of insulin, addition of acetate to the medium appreciably

Table 4. *Effect of insulin on the respiration and acetate utilization of lactating rat mammary gland slices in sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.)

Experimental series	No. of rats	Stage of lactation at autopsy (days)	Duration of respiration experiments (hr.)	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.
1	10	12–18	3	Insulin	4·6±0·4	−0·5±0·2	0·82±0·04
				None	4·6±0·3	−0·2±0·1	0·76±0·03
2	3	14–15	2	Insulin	4·6	−0·2	0·82
				None	5·1	+0·8	0·75

Table 5. *Effect of insulin on respiration and acetate utilization of lactating rabbit mammary gland slices in glucose* (0·1 %) *plus sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; 'regular' insulin (Wellcome) added to final concentration of 1 i.u./ml.)

Rabbit no.	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)	$\Delta -Q_{O_2}$	ΔQ_{acid}	Δ R.Q.	Δ 'Extra CO_2'	Increase in 'extra CO_2' due to insulin (%)
101	Insulin	11·7	− 9·1	1·66	7·7	1·1	−2·8	0·46	5·6	267
	None	10·6	− 6·3	1·20	2·1					
C4	Insulin	12·3	− 9·2	1·63	7·8	2·2	−3·2	0·35	5·0	179
	None	10·1	− 6·0	1·28	2·8					
C5	Insulin	9·1	− 7·3	1·72	6·6	0·8	−2·9	0·49	4·7	247
	None	8·3	− 4·4	1·23	1·9					
108	Insulin	12·3	− 9·4	1·52	6·4	1·1	−3·6	0·31	4·0	167
	None	11·2	− 5·8	1·21	2·4					
114	Insulin	9·8	− 6·8	1·47	4·6	1·6	−2·3	0·30	3·2	229
	None	8·2	− 4·5	1·17	1·4					
116*	Insulin	19·6	−17·6	1·56	11·0	6·5	−8·3	0·28	7·3	197
	None	13·1	− 9·3	1·28	3·7					

* 1 hr. experiment; all others 3 hr.

lowered the R.Q. as compared with the results obtained in glucose, and Q_{acid} was still positive though somewhat smaller than in glucose alone, indicating possibly a slight utilization of acetate. The effect of glucose on acetate utilization by mouse mammary tissue is thus much less marked than that found previously in the rat and rabbit (Folley & French, 1950). Under these conditions, however, the mouse tissue was now responsive to insulin which restored Q_{O_2} and R.Q. to levels similar to those obtained in glucose alone and definitely increased the acetate uptake. In fact there is an indication that in glucose plus acetate the 'extra CO_2' production in presence of insulin was somewhat above the level found in glucose alone.

Sheep. Three lactating ewes were used for this study, biopsy specimens being taken from their udders, under *cyclo*propane anaesthesia, at various times from days 11–48 of lactation. Because of this it was considered advisable to subject the results to an analysis of variance which, however, revealed no regular trends with advance of lactation. Mean values in acetate are therefore given in Table 11 and in acetate plus glucose in Table 12. It is clear that in neither substrate had insulin any significant effect on the metabolism of the slices. The stimulatory effect of glucose on acetate utilization by udder slices from lactating ewes, previously reported (Folley & French, 1950), is confirmed by the present results (cf. Tables 11 and 12).

Insulin sensitivity of rat mammary tissue at different functional stages

The effect of insulin on the respiration of mammary tissue was studied on tissue from groups of rats killed at the end of pregnancy (19–20 days), at the height of lactation (14–15 days) and 2 days after the removal of the young at the 20th–21st day of lactation. The results obtained in glucose + acetate are given in Table 7. As found previously (Folley & French, 1949*b*), for tissue from rats of the same colony metabolizing glucose, the respiration of tissue taken at the end of pregnancy was relatively low, and the R.Q. below unity. Addition of insulin had no effect at this stage in contrast with the usual marked effect observed with fully lactating tissue (14–15 days). Similarly, after 2 days' weaning, low values for Q_{O_2} and R.Q. were found as before, and the slices no longer responded to insulin. Essentially similar changes in insulin sensitivity at the three stages studied were found in glucose alone (Table 8).

Comparison of the effects of glycerol and insulin on the respiration of lactating mammary gland slices

The results of 2 hr. determinations in glucose plus acetate on tissue from ten lactating rats, killed at days 13–15, are summarized in Table 9. It will be seen that glycerol in 0·2% concentration, like insulin, increases R.Q., $-Q_{O_2}$ and acetate utilization,

Table 6. *Effect of insulin on the respiration and acid utilization or production of lactating mouse mammary gland slices in glucose* (0·3%) *plus sodium acetate* (0·02 M) *or in glucose* (0·3%) *respectively*

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium, Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; duration of experiments: 2 hr.; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml. Five mice at the 14th day of lactation were used.)

	Glucose				Glucose + acetate			
Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)
Insulin	9·8±1·1	3·1±0·4	2·14±0·07	10·7±0·9	12·1±0·4	−1·0±0·7	2·14±0·08	13·7±1·3
None	10·6±0·8	2·4±0·2	1·90±0·07	9·5±0·9	10·1±0·3	0·1±0·3	1·46±0·05	4·4±0·2

Table 7. *Insulin sensitivity in glucose* (0·3%) *plus sodium acetate* (0·02 M) *of mammary gland slices from rats at the end of pregnancy, mid-lactation and after weaning at 20–21 days*

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.)

Stage	No. of rats	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)
Pregnancy (19–20 days)	11	Insulin	1·4±0·1	0·1±0·04	0·90±0·03	—
		None	1·4±0·1	0·2±0·03	0·90±0·05	—
			Δ 0·0	Δ −0·1	Δ 0·00	—
Lactation (14–15 days)	11	Insulin	14·7±0·8	−4·8±0·5	2·15±0·02	16·8±0·7
		None	10·1±0·5	−1·4±0·2	1·57±0·04	5·7±0·5
			Δ 4·6	Δ −3·4	Δ 0·58	Δ 11·1
Post-weaning (2 days)	10	Insulin	4·5±0·2	2·7±0·2	0·81±0·04	—
		None	4·6±0·4	2·8±0·2	0·81±0·04	—
			Δ −0·1	Δ −0·1	Δ 0·00	—

Table 8. *Insulin sensitivity in glucose* (0·3 %) *of mammary gland slices from rats at the end of pregnancy, mid-lactation and after weaning at 20–21 days*

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.)

Stage	No. of rats	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)
Pregnancy	11	Insulin	1·3±0·1	0·8±0·1	0·85±0·03	—
(19–20 days)		None	1·1±0·1	0·9±0·04	0·74±0·04	—
			Δ 0·2	Δ −0·1	Δ 0·11	—
Lactation	11	Insulin	11·0±0·7	3·2±0·2	1·87±0·04	9·3±0·7
(14–15 days)		None	9·4±0·6	3·3±0·3	1·55±0·04	5·2±0·4
			Δ 1·6	Δ −0·1	Δ 0·32	Δ 4·1
Post-weaning	10	Insulin	4·5±0·2	3·3±0·1	0·80±0·03	—
(2 days)		None	3·4±0·3	2·9±0·2	0·78±0·05	—
			Δ 1·1	Δ 0·4	Δ 0·02	—

Table 9. *Comparison of the effects of insulin and glycerol on the respiration and glucose and acetate utilization of lactating rat mammary gland slices in glucose* (0·3 %) *plus sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; duration of experiments, 2 hr.; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.; final concentration of glycerol, 0·2 %. Ten rats (13–15 days of lactation) used for respiration measurements; glucose determinations carried out on seven of these.)

Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)	$-Q_{glucose}$
None	8·6±0·6	−0·7±0·2	1·53±0·05	4·5±0·5	4·7±0·8
Insulin	11·7±0·6	−2·5±0·2	2·07±0·03	12·5±0·8	9·1±0·9
Glycerol	10·9±0·7	−3·0±0·5	1·70±0·03	7·8±0·7	4·2±0·8

though under the conditions used the increase in R.Q. and 'extra CO_2' was less with glycerol than with insulin. It is noteworthy that glycerol differed from insulin in that it evoked no increase in glucose utilization. Table 10 shows that the effects of insulin and glycerol were not additive. Control experiments showed that glycerol alone was not utilized by rat mammary gland slices.

Table 10. *Lack of additive effect of insulin and glycerol on the respiration and acid utilization of lactating rat mammary gland slices in glucose* (0·3 %) *plus sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.; final concentration of glycerol, 0·2 %. The values given are means of three experiments carried out on rats at the 14th–15th day of lactation.)

Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)
None	6·9	−0·3	1·63	4·3
Insulin	10·0	−2·5	2·19	11·9
Glycerol	9·7	−2·5	1·76	7·5
Insulin + glycerol	11·4	−4·0	2·07	12·5

Since mammary gland slices from ruminants, in contrast with tissue from non-ruminants such as the rat, will utilize acetate alone with R.Q. > 1 (Folley & French, 1950), it was felt that a comparison of the *in vitro* effects of glycerol and insulin on mammary tissue from lactating ruminants would be of interest. The studies on lactating udder tissue from ewes, referred to above, therefore included an investigation of the effects of glycerol. The results (Table 11) indicate that while lactating sheep udder tissue is inert to insulin in acetate, the slices respond to glycerol by noticeable increases in $-Q_{O_2}$, R.Q. and acetate utilization which, though not dramatic, are nevertheless highly significant statistically. In glucose plus acetate (Table 12), on the other hand, glycerol appeared to cause a slight inhibition of fat synthesis as manifested by small but significant decreases in $-Q_{O_2}$, R.Q. and acetate utilization.

DISCUSSION

There is little doubt that the utilization of acetate or glucose with R.Q. > 1 by lactating mammary gland slices signifies the net synthesis of fat in the tissue. This conclusion, reached by Folley & French (1950), has been reinforced as far as the incorporation of acetate carbon into fatty acids is concerned by the

Table 11. *Comparison of the effects of insulin and glycerol on the respiration and acetate utilization of udder slices from lactating ewes in sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; duration of experiments, 2 hr.; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.; final concentration of glycerol, 0·2%. Fourteen biopsy samples were taken from three sheep at various times between days 11–48 of lactation.)

Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2'* ($Q_{CO_2}+Q_{O_2}$)
None	5·1 ± 0·3	−3·1 ± 0·4	1·09 ± 0·04	0·4
Insulin	5·4 ± 0·3	−3·4 ± 0·4	1·09 ± 0·04	0·5
Glycerol	6·7 ± 0·3	−5·6 ± 0·4	1·27 ± 0·04	1·8
	Significance of treatment comparisons			
Insulin—no addition	$P > 0·05$	$P > 0·05$	$P > 0·05$	—
Glycerol—no addition	$0·01 > P > 0·001$	$P < 0·001$	$P < 0·001$	—

* Calculated from the mean values of $-Q_{O_2}$ and R.Q.

Table 12. *Comparison of the effects of insulin and glycerol on the respiration and acetate utilization of udder slices from lactating ewes in sodium acetate* (0·02 M) *plus glucose* (0·3 %)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; duration of experiments, 2 hr; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.; final concentration of glycerol, 0·2%. Fourteen biopsy samples were taken from three sheep at various times between days 11–48 of lactation.)

Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2'* ($Q_{CO_2}+Q_{O_2}$)
None	7·6 ± 0·3	−6·4 ± 0·4	1·52 ± 0·04	4·0
Insulin	8·0 ± 0·3	−6·5 ± 0·4	1·50 ± 0·04	4·0
Glycerol	6·4 ± 0·3	−4·3 ± 0·4	1·42 ± 0·04	2·7
	Significance of treatment comparisons			
Insulin—no addition	$P > 0·05$	$P > 0·05$	$P > 0·05$	—
No addition—glycerol	$0·02 > P > 0·01$	$0·01 > P > 0·001$	$0·05 > P > 0·02$	—

* Calculated from the mean values of $-Q_{O_2}$ and R.Q.

demonstration that the mammary gland of the pregnant rabbit (Popják & Beeckmans, 1949, 1950), lactating goat (Popják, French & Folley, 1950, 1951) and lactating cow (Kleiber, Smith & Tolbert, 1950) actively incorporates $CH_3{}^{14}COOH$ into fatty acids of milk glycerides. It follows, therefore, that the *in vitro* insulin effect on the metabolism of lactating mammary gland slices, described in this and the preceding paper (Balmain *et al.* 1950), is an expression of a stimulation of net fat synthesis by the tissue. While an insulin concentration of 0·05 mg. (1 i.u.)/ml. has been uniformly used in the present work, in unpublished experiments we have been able to detect, by measurement of overall pressure changes (see Balmain *et al.* 1950), definite *in vitro* effects of insulin on lactating rat mammary tissue at concentrations as low as 0·0001 mg. (0·002 i.u.)/ml.

The fact that the effect we have studied is of such a magnitude as to be regularly demonstrable by respiratory measurements is no doubt due to the fortunate circumstance that in mammary tissue we have at our disposal a particularly favourable medium for the study of fat synthesis *in vitro*. This may be largely because this tissue is capable of effecting the synthesis and secretion of glycerides, a process which by 'fixing' fatty acids and removing them from the site of synthesis and breakdown would tend to favour their synthesis. Though the liver can synthesize fatty acids, it may be that their assembly into glycerides is not so important a feature of the fat metabolism of isolated liver tissue. If so, and if the tentative suggestion (see below) that the action of insulin on fat formation might be partly concerned with the regulation of the supply of glycerol for glyceride synthesis should prove correct, it would be understandable that *in vitro* insulin effects on fat synthesis from acetate in liver slices have so far only been demonstrated by increased incorporation of labelled carbon into fatty acids, which may signify merely an increased rate of fatty acid turnover rather than net synthesis.

The general significance of our results as regards the role of insulin as a regulator of fat synthesis in the mammary gland is emphasized by the fact that the insulin effect has been observed, under suitable conditions, in tissue from the rabbit and mouse as well as from the rat. Mammary tissue from lactating ewes was, however, inert to insulin both in acetate alone and in glucose plus acetate. This may be another facet of the metabolic difference between mammary tissue from ruminants and non-ruminants described by Folley & French (1950). With rat

mammary tissue the same insulin effect, but of lesser magnitude, was obtained as before (Balmain *et al.* 1950) in glucose alone, while mouse mammary gland was unresponsive to insulin with this substrate. Insulin had no effect on the metabolism of rat (or sheep) mammary tissue in presence of acetate alone, a result which differs from those of Brady & Gurin (1950), who studied the incorporation of ^{14}C-labelled acetate into fatty acids of rat-liver slices. Though the insulin effect studied by us is manifest in glucose alone (rat) but not in acetate alone (sheep and rat), it nevertheless seems particularly related to acetate utilization for fat synthesis. This is illustrated in Table 13, which shows that the enhancing effect of addition of acetate on fat synthesis by rat mammary gland slices metabolizing glucose is greatly increased in presence of insulin.

Table 13. *Acetate metabolism of lactating rat mammary gland slices in relation to insulin*

(The Δ values given are differences between mean values in glucose (0·3%) plus acetate (0·02 M) and in glucose (0·3%).)

Experimental series	Addition	(Glucose + acetate − glucose) $\Delta -Q_{O_2}$	ΔQ_{acid}	Δ R.Q.	Δ 'Extra CO_2'
1	Insulin	2·9	−6·4	0·23	5·5
	None	1·3	−3·8	−0·04	0·1
2	Insulin	3·7	−8·0	0·28	7·5
	None	0·7	−4·7	0·02	0·5

deposition by rat diaphragm muscle (Gemmill & Hamman, 1941), an effect which is antagonized by the anterior pituitary, as indicated by the results of Stadie, Haugaard & Marsh (1951), who found rat diaphragm to be more sensitive to insulin after hypophysectomy of the donor.

The important question of the mechanism of the insulin effect on fat synthesis must be largely a matter of speculation at the present time, but the fact that glycerol is capable of similarly stimulating fat synthesis by mammary gland slices may be significant in this connexion. It was previously suggested (Folley & French, 1949*c*, 1950) that the stimulating effect of glucose on fat synthesis from acetate by mammary gland slices might be explained, at least partly, on the assumption that glucose provides glycerol necessary for the formation of glycerides which, as pointed out above, might well indirectly promote fatty acid synthesis in a tissue capable of synthesizing and secreting glycerides. This suggestion has received support from the recent demonstration by French & Popják (1951) that radioactive carbon is incorporated into glycerol as well as fatty acids isolated from milk glycerides secreted by rabbits receiving ^{14}C-labelled glucose, which led them to conclude that glycerol might be a limiting factor in fatty acid synthesis. Our results with glycerol thus open up the possibility that insulin, acting at some point in the metabolic pathway of glucose breakdown, might promote the formation of glycerol from glucose. Two of our findings are in keeping with this hypothesis: (*a*) though R.Q. and acetate utilization of rat mammary gland slices is increased by glycerol in presence of glucose plus acetate, the glucose uptake does not increase as it does in presence of insulin; (*b*) sheep mammary gland slices metabolizing acetate alone, while unresponsive to insulin, gave evidence of increased fat synthesis in presence of glycerol. Other results, such as the unresponsiveness of sheep udder tissue to insulin in glucose + acetate and the slight inhibitory effect of glycerol in this substrate, are, however, less easy to explain in these terms.

The inertness of rat mammary tissue to insulin at the end of pregnancy or after weaning parallels the low oxygen uptake and R.Q. at these stages previously observed in glucose (Folley & French, 1949*b*), and now confirmed both in glucose and glucose plus acetate. It would seem that insulin by itself cannot stimulate fat synthesis by fully developed but essentially non-functional mammary tissue, though this may appear surprising in view of recent evidence that the mammary gland of the rabbit in late pregnancy is capable of the synthesis of short-chain fatty acids characteristically found in milk fat (Popják & Beeckmans, 1949, 1950; Popják, Folley & French, 1949). The effect of insulin on the fat metabolism of mammary tissue seems to require for its expression the existence of an active secretory condition, manifested by the active respiration with high R.Q. exhibited by post-partum tissue (Folley & French, 1949*b*) and presumably related to the action of the complex of anterior-pituitary hormones concerned in lactogenesis and galactopoiesis (Folley & Young, 1941). The loss of insulin sensitivity following weaning emphasizes the probable importance of the regularly applied suckling stimulus, acting on the anterior pituitary (Selye, 1934), in maintaining the conditions essential for responsiveness to insulin. In respect of this evident necessity for anterior-pituitary participation the insulin effect studied by us differs from the well known stimulating action of insulin on glucose uptake and glycogen

It must be emphasized, however, that this interpretation is at present purely hypothetical. The alternative possibility that the insulin effect is related to the utilization of glucose as a source of

energy for driving the fat synthesis must not be overlooked. If this were the explanation the inertness of ruminant mammary tissue to insulin would be understandable in view of the fact that in ruminants the mammary gland appears to use acetate rather than carbohydrate as an energy source. The whole question clearly needs further investigation.

SUMMARY

1. Insulin added *in vitro* markedly increased $-Q_{O_2}$, acetate and glucose utilization, and especially R.Q. and 'extra CO_2' (carbon dioxide produced in excess of that required for R.Q. = 1) of lactating rat mammary gland slices in glucose plus acetate. This is interpreted as signifying a stimulatory action of insulin on net fat synthesis. Similar but less marked effects were obtained in glucose, but in acetate the tissue was inert to insulin.

2. Rat mammary gland slices were unresponsive to insulin, both in glucose plus acetate and in glucose, at the end of pregnancy (19–20 days) and after 2 days' weaning at days 20–21 of lactation.

3. Lactating rabbit mammary tissue was similarly responsive to insulin in glucose plus acetate.

4. Insulin had no effect on the metabolism of lactating mouse mammary gland in glucose alone. In the absence of insulin, the R.Q. of this tissue was lower in glucose plus acetate than in glucose, but the acid production was somewhat depressed, indicating a slight utilization of acetate. In glucose plus acetate the tissue responded to insulin by moderate increases in $-Q_{O_2}$ and acetate uptake and marked increases in R.Q. and 'extra CO_2'.

5. Udder slices from lactating ewes were inert to insulin both in glucose plus acetate and in acetate.

6. Glycerol exerted a qualitatively similar effect to insulin, though less marked as regards the increases in R.Q. and 'extra CO_2', on the metabolism of lactating rat mammary tissue, except that the glucose uptake was not increased by glycerol. The effects of glycerol and insulin were not additive. Udder slices from lactating ewes responded to glycerol in acetate alone, but in glucose plus acetate glycerol exerted a slight inhibitory effect.

7. It is suggested that the stimulating effect of insulin on fat synthesis by mammary tissue may be due, partly at any rate, to stimulation of the formation of glycerol from glucose. Since mammary tissue can synthesize and secrete glycerides, the supply of glycerol might well be a critical, rate-limiting factor in fat synthesis by this tissue. It is, however, equally possible that the insulin effect is related to the utilization of glucose as an energy source for fat synthesis.

We are indebted to Dr T. H. French for many helpful discussions and for allowing us to include the rabbit data which were obtained by him; to Mr C. P. Cox for statistical analysis of the data and advice on their interpretation; to Dr A. T. Cowie for performing mammary gland biopsies on sheep; and to Dr S. K. Kon for extending to us the facilities of his rat colony. Our best thanks are also due to Dr K. Hallas Møller, of Novo Terapeutisk Laboratorium, Copenhagen, for generous supplies of crystalline insulin devoid of glycogenolytic factor.

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