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Increase in Activity of Glucose 6-Phosphate Dehydrogenase in Mouse Mammary Tissue Cultured with Insulin

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1. In organ cultures of mammary tissue from C3H mice we observed increases in the activity of glucose 6-phosphate dehydrogenase similar to that occurring at parturition. 2. In 22hr. cultures of tissue from late-pregnant mice insulin was required for the increases, but the further addition of prolactin, corticosterone and certain other hormones had no effect. The rise in activity occurred over the second half of the culture period. 3. Results from culture of adipose tissue, and mammary tissue rich in adipose tissue, strongly suggest that the rise in activity occurs in mammary parenchymal rather than adipose cells. 4. In 45hr. cultures prolactin prevented a fall in enzyme activity between 22hr. and 45hr. If the medium contained serum the activity at 22hr. was unaffected, but it continued to rise up to 45hr., and prolactin then had no effect. 5. The enzyme also increased in activity in cultures of mammary tissue from mid-pregnant mice. Insulin was again required, the activity was higher at 45hr. than at 24hr. and prolactin increased the activities at both these times. 6. Actinomycin D, cycloheximide and puromycin at low concentration in the media of 22hr. cultures all prevented increases in enzyme activity. Hydroxyurea at a concentration that inhibited the incorporation of [^{3}H]thymidine into DNA by 92% had little effect. 7. Actinomycin D and cycloheximide largely failed to prevent the rise in enzyme activity if added after 3·5hr. and 12hr. respectively. Hence all essential RNA and protein synthesis appears to be finished by 3·5hr. and 12hr., although most of the increase in enzyme activity occurs gradually between 12hr. and 22hr. 8. We suggest that the increases in enzyme activity, both in culture and in the living animal at parturition, are induced by an influx of glucose that is restrained during pregnancy by the growth-hormone-like action of placental lactogen.

During pregnancy and lactation marked biochemical changes occur in the mammary gland under the influence of hormones. The study of these changes has been greatly facilitated by organ culture of the mammary gland (Elias, 1959). Mammary tissue from mice in mid-pregnancy can be maintained for several days if the culture medium contains insulin. If it also contains prolactin and an adrenal corticosteroid the parenchymal cells enlarge, and the alveoli that these cells surround become filled with secretion that contains casein (Rivera & Bern, 1961; Juergens, Stockdale, Topper & Elias, 1965).

Our previous experiments have shown effects of hormones on RNA and protein formation in cultures of mammary tissue from C3H mice (Mayne, Barry & Rivera, 1966; Mayne & Barry, 1967; Mayne, Forsyth & Barry, 1968). We now present results of a study in mammary-organ cultures of glucose 6-phosphate dehydrogenase, which, in the gland of the living animal, increases sharply in activity at parturition (Glock & McLean, 1954). We have also included some measurements of 6-phosphogluconate dehydrogenase activity. Some of these results have been briefly reported (Leader & Barry, 1968).

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MATERIALS AND METHODS

Materials. L-[U-^{14}C]Lysine, [2-^{14}C]uridine and [6-^{3}H]-thymidine were bought from The Radiochemical Centre, Amersham, Bucks. Tissue-culture medium 199 was bought from Glaxo Laboratories Ltd., Greenford, Middlesex. Before use an extra 132mg. of $NaHCO_3$/100ml. was added to give pH7·4 during incubation. The composition of medium 199 is given by Morgan, Morton & Parker (1950). Glucagon-free insulin was a gift from Dr Otto Behrens, Eli Lilly Research Laboratories, Indianapolis, Ind., U.S.A. Bovine prolactin (NIH-P-B1) and bovine growth hormone (NIH-GH-B10) were gifts from the Endocrinology Study Section, National Institutes of Health, Bethesda, Md., U.S.A. Corticosterone

and 3,3',5-tri-iodothyronine were bought from Sigma (London) Chemical Co. Ltd., London, S.W.6 and oestradiol and progesterone from British Drug Houses Ltd., Poole, Dorset. Insulin was freshly dissolved before use in 3mM-HCl and prolactin and growth hormone were dissolved in medium 199. The other hormones were dissolved in ethanol, the concentration of which in the culture medium never exceeded 0·25%. Oxytocin (Syntocin; 1unit/ml. of ethanol) was bought from Sandoz Ltd., Basle, Switzerland.

Actinomycin D was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A., and a stock solution (1mg./ml.) was stored in the dark at −40°. Cycloheximide and hydroxyurea were bought from Sigma (London) Chemical Co. Ltd. and puromycin from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Aqueous solutions of these inhibitors were prepared for each experiment. Bovine serum albumin (fraction V) was bought from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. Other materials were bought from Sigma (London) Chemical Co. Ltd. or British Drug Houses Ltd. Plastic Petri dishes (5cm. diam.; Sterilin Ltd., Richmond, Surrey) had lids designed to allow gas to enter freely.

Mice. These were C3H strain and derived from the M.R.C. Laboratory Animal Centre, Carshalton, Surrey. They were fed on diet FFG(M) (Dixon and Sons Ltd., Ware, Herts.). Virgin females about 3 months old were put in separate cages, each with a male. The morning on which a vaginal plug was detected was called day 1 of pregnancy. Young were born between midnight on day 19 and midday on day 20. 'Mid-pregnant' and 'late-pregnant' refer to mice killed on the mornings of day 12 and day 19 respectively.

Organ culture. The watch-glass organ-culture procedure (Elias & Rivera, 1959) was modified to allow the culture of enough tissue for enzyme assays. In each experiment a mouse was killed by cervical dislocation, immersed in 70% (v/v) ethanol and dried, and the inguinal mammary glands were removed and put in medium 199 in a Petri dish. Explants of about 1·5mm.^{3} were cut with iridectomy scissors and batches of 20 explants, selected at random, were put into Petri dishes containing medium 199 (5ml.) and the necessary hormones. The explants floated on the surface of the medium. In each experiment two batches of 20 explants were stored at −40° for later enzyme assay, one batch having first been blotted and weighed. Enzyme activities, both before and after culture, were calculated per unit weight of tissue at the start of culture. There were negligible differences in weight and enzyme activity between replicate batches of 20 explants from each mouse. The Petri dishes were incubated at 37° under O_2+CO_2 (95:5). All the above operations were done under sterile conditions.

Enzyme assays. Twenty explants were ground in the cold in a glass homogenizer with a mechanical drive in a solution (0·75ml.) of 0·15M-KCl in 10mM-tris–HCl buffer, pH7·6. The homogenate was centrifuged at 100000*g* for 45min. and the supernatant drawn off from beneath the pad of fat. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were determined by the method of Glock & McLean (1953). Storage of the explants at −40° did not affect their enzyme activities.

DNA and RNA. These were determined by procedures developed by Dr R. Mayne in our Laboratory. Tissue was ground with acetone (3ml.) in a glass homogenizer, and after 20min. the residue was washed three times with acetone on the centrifuge, and then twice with 0·2M-$HClO_4$ (3ml.), all at 2°. It was then incubated with 0·3M-KOH (2ml.) for 2hr. at 37° and, after cooling, 1·2M-$HClO_4$ was added to give pH2. After 10min. the residue and supernatant were separated by centrifugation. Ribonucleotides in the supernatant were determined by the orcinol method (Mejbaum, 1939). The residue was washed twice with 0·2M-$HClO_4$ and then heated with 0·5M-$HClO_4$ (4ml.) at 70° for 25min. and DNA in the supernatant was determined (Burton, 1956).

Mouse serum. Mice were anaesthetized with ether and blood was collected by cutting the carotid artery and then the heart. Blood was left to clot for 30min. at 37°, and the serum drawn off after gentle centrifugation.

Inhibitors. The incorporation of [^{14}C]uridine into the RNA of explants, of [^{3}H]thymidine into the DNA and of [^{14}C]lysine into the proteins, with and without inhibitors, was determined by the procedure of R. Mayne & J. M. Barry (unpublished work).

Statistical treatment of results. Each condition of organ culture was carried out in duplicate, and experimental values reported are means from these duplicates, which seldom differed by more than 5%. To determine the significance of a difference between two conditions of culture each duplicate value was used in an analysis of variance, and values of P were obtained from Snedecor's F (variance ratio) distribution.

RESULTS

Table 1 shows that the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase/g. of inguinal mammary

Table 1. *Changes in mammary glands of mice during pregnancy and lactation*

Values are the means ± S.E.M. of separate determinations on tissue from six mice.

	Wt. of inguinal glands (g.)	Glucose 6-phosphate dehydrogenase (units/g.)	6-Phosphogluconate dehydrogenase (units/g.)	RNA (mg./g.)	DNA (mg./g.)
Virgin	0·214±0·018	3·37±0·30	0·75±0·08	1·11±0·13	1·12±0·10
Day 12 pregnancy	0·362±0·018	2·49±0·13	0·61±0·09	2·01±0·28	1·71±0·16
Day 19 pregnancy	0·974±0·114	2·50±0·18	0·61±0·07	2·92±0·15	2·49±0·18
Day 1 lactation	1·12±0·13	2·96±0·28	0·76±0·12	3·57±0·27	2·54±0·22
Day 2 lactation	0·784±0·036	4·49±0·40	1·10±0·19	5·12±0·20	3·20±0·08
Day 8 lactation	1·64±0·13	10·4±0·4	1·55±0·34	7·07±0·18	2·31±0·09

Table 2. *Increase in enzyme activities of mammary tissue from pregnant mice after 22 hr. culture with insulin*

In each experiment four batches of 20 explants were prepared. Two batches were frozen and the other two were each incubated for 22 hr. in medium 199 (5 ml.) containing insulin (5 μg./ml.). Enzyme activities were then determined in all batches of tissue. Values are means (± S.E.M.) of 19 experiments on mice at day 19 of pregnancy and seven experiments on mice at day 12. Changes in weight and total protein content of the explants during culture were negligible.

	Glucose 6-phosphate dehydrogenase (units/g.)		6-Phosphogluconate dehydrogenase (unit/g.)	
	Day 19	Day 12	Day 19	Day 12
Initial value	1·83 ± 0·21	1·50 ± 0·11	0·56 ± 0·04	0·54 ± 0·05
Increase over 22 hr.	1·53 ± 0·13	1·03 ± 0·12	0·36 ± 0·06	0·29 ± 0·12
Increase (%)	84	68	64	54
P	< 0·001	< 0·001	< 0·001	< 0·001

Table 3. *Effect of insulin compared with that of insulin plus prolactin and corticosterone in 22 hr. and 45 hr. cultures of tissue from pregnant mice*

In each experiment six batches of 20 explants were prepared. Two batches were frozen, and the remainder was incubated in medium 199 (5 ml.) containing insulin alone (I) (5 μg./ml.), or insulin (5 μg./ml.) + prolactin (P) (10 μg./ml.) + corticosterone (C) (1 μg./ml.). Enzyme activities were then determined. Values are the means of duplicate determinations made in the numbers of experiments shown in parentheses.

	Glucose 6-phosphate dehydrogenase (units/g.)			
Incubation time (hr.)	22		45	
	Day 19	Day 12	Day 19	Day 12
Start	2·37 (4)	1·50 (7)	1·80 (8)	1·56 (4)
Cultured + I (*a*)	3·73 (4)	2·54 (7)	2·74 (8)	3·83 (4)
Cultured + I + C + P (*b*)	3·98 (4)	3·17 (7)	3·52 (8)	4·70 (2)
$\frac{b}{a} \times 100$ (± S.E.M.)	106 ± 4·4	124 ± 5·6	129 ± 2·4	138 ± 12·2
P	> 0·05	< 0·01	< 0·001	< 0·01

tissue of our mice change little during pregnancy, but increase over the first 8 days of lactation, as they do in rats (Glock & McLean, 1954). The RNA concentration increases during both pregnancy and early lactation, whereas the DNA concentration mainly increases during pregnancy. It was, however, calculated from the weights of the glands that there is some increase in total DNA after parturition. The fall in weight at day 2 of lactation could be due to removal of milk. These results are similar to those found by others (Munford, 1964; Baldwin & Milligan, 1966).

Tissue from late-pregnant mice in 22 hr. cultures. Since the enzyme activities begin to rise only at parturition, we initially used tissue from mice at the end of pregnancy in our attempts to obtain increases in activities in culture. Table 2 shows that in tissue from mice at day 19 of pregnancy cultured for 22 hr. with insulin the activities of both enzymes increased by amounts roughly equal to their average daily increases in the living animal over the first 8 days of lactation (see Table 1). Why the average initial activities were somewhat lower than those in Table 1 is unclear, but the two series of measurements were made over different periods of time. Without insulin the activities of both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase fell by an average, in three experiments, of 20% and 15% respectively. [When a supernatant from tissue at the start of culture was incubated with insulin (5 μg./ml.) for 1 hr. at 0° or 37° the enzyme activities did not rise.]

Because of the smaller rise in activity of 6-phosphogluconate dehydrogenase, it was possible to study precisely only changes in the activity of glucose 6-phosphate dehydrogenase. Fig. 1 shows that the activity of this enzyme begins to rise only after about 12 hr. of culture.

In cultures of mammary tissue from mid-pregnant mice the presence of prolactin and an adrenal corticoid, in addition to insulin, causes marked effects on RNA and protein formation (Stockdale, Juergens & Topper, 1966; Mayne *et al.* 1968). Table 3 shows that with tissue from

late-pregnant mice (day 19) these hormones did not affect the increase in activity of glucose 6-phosphate dehydrogenase during the first 22hr. of culture although, as discussed below, they did cause a difference by 45hr. Prolactin or corticosterone added separately with insulin also had no effect, nor did they affect the fall in activity without insulin.

It has been suggested that the initiation of milk secretion before parturition is, in the living animal, restrained by high concentrations of oestrogens and progesterone (see Cowie, 1961). The addition to our cultures with insulin of oestradiol (1μg./ml.), progesterone (1μg./ml.) or oestradiol (0·01μg./ml.)+progesterone (5μg./ml.) did not affect the rise in enzyme activity.

Experiments were carried out to determine the location of the increase in enzyme activity. Explants from mice in late pregnancy could be seen under the microscope to contain parenchymal (secretory) cells and a smaller proportion of adipose cells. Table 4 shows a typical experiment that compares the change in enzyme activity in normal explants, in explants of abdominal adipose tissue, and in explants taken from the edge of the mammary gland ('peripheral' mammary tissue), which contained a higher proportion of adipose cells. The enzyme activity in the abdominal adipose tissue was lower than in the mammary tissue and it decreased during culture. The activity in the peripheral tissue lay between that in normal mammary tissue and in abdominal adipose tissue. Although the activity in this tissue rose during culture by about the usual percentage, the absolute rise was smaller. These facts strongly suggest that the increase in enzyme activity in mammary explants during culture occurs in the parenchymal and not in the adipose cells. This is to be expected, since the proportion of adipose cells in the mammary gland continuously falls during pregnancy and lactation (Nicoll & Tucker, 1965). There is also strong evidence that in the living mouse the rise in glucose 6-phosphate dehydrogenase activity in the mammary gland at parturition occurs in parenchymal cells rather than in adipose cells (Hershey, Lewis, Johnston & Mason, 1963; Bartley, Abraham & Chaikoff, 1966).

Tissue from late-pregnant mice in 45hr. cultures. It has been shown (Table 3) that prolactin and corticosterone, when present in addition to insulin, did not affect the enzyme activity after 22hr. of culture. Table 3 shows, however, that in 45hr. cultures these two hormones did increase the final activity. Table 5 shows the results of experiments in each of which the enzyme activity was measured at both 22hr. and 45hr. in cultures from a single mouse at day 19. The difference at 45hr. is due to the activity falling after 22hr. in the absence of prolactin and corticosterone; activity is maintained in the presence of the hormones. Further experiments showed that the difference was solely due to prolactin, corticosterone having no effect on cultures either with insulin or with insulin and prolactin.

That the enzyme activity did not increase during the second day of culture (Table 5) suggests that the conditions of culture are not optimum. Renewal of the medium at 22hr. did not affect the activity at 45hr.; nor did the addition at the start of culture of

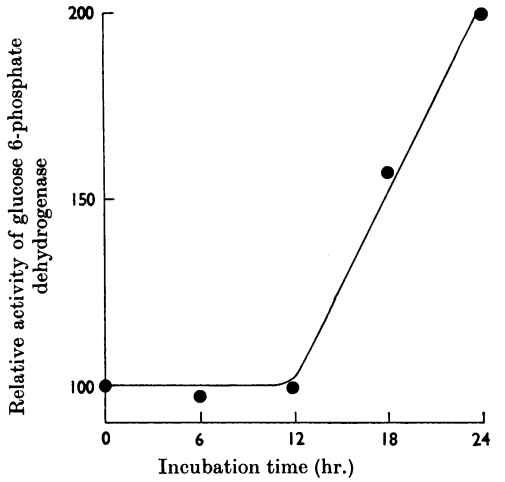

Fig. 1. Glucose 6-phosphate dehydrogenase activities of mammary tissue from mice in late pregnancy during culture with insulin. The points are means from two identical experiments in each of which batches of 20 explants from a single mouse were incubated in duplicate for the times shown. In each experiment the activities have been adjusted to give 100 at 0hr. and 200 at 24hr. The actual initial and final activities averaged 1·97 and 3·45 units/g.

Table 4. *Change in enzyme activity of mammary tissue, 'peripheral' mammary tissue and abdominal adipose tissue after culture for 22hr. with insulin*

Explants of mammary tissue and abdominal adipose tissue were taken from the same mouse. Conditions otherwise were as described for Table 2.

	Glucose 6-phosphate dehydrogenase (units/g.)		
	Mammary tissue	'Peripheral' mammary tissue	Abdominal adipose tissue
Start	1·05	0·56	0·23
Finish	2·82	1·42	0·14
Change	1·77	0·86	−0·09
Change (%)	169	154	−39

Table 5. *Glucose 6-phosphate dehydrogenase activities of mammary tissue from pregnant mice during culture with insulin or insulin plus corticosterone and prolactin*

Each set of values are means from three experiments, in each of which batches of 20 explants from a single mouse were incubated in duplicate for the times shown. In each experiment the activities were adjusted to give 100 at 0 hr. and 200 at 45 hr. with three hormones: insulin (I), 5 μg./ml.; corticosterone (C), 1 μg./ml.; prolactin (P), 10 μg./ml.

	Incubation time (hr.)	Relative activities 0	22	45
Day 19:	I	100	183	157
	I+C+P	100	198	200
Day 12:	I	100	138	162
	I+C+P	100	171	200

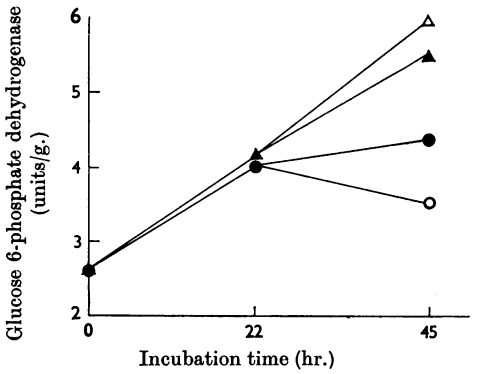

Fig. 2. Glucose 6-phosphate dehydrogenase activities of mammary tissue from mice in late pregnancy during culture with and without serum. Batches of 20 explants from a single mouse were incubated in duplicate for the times shown. ○, Insulin (5 μg./ml.); ●, insulin (5 μg./ml.)+prolactin (10 μg./ml.)+corticosterone (1 μg./ml.); △, insulin (5 μg./ml.)+serum from lactating mouse (to give 10%, v/v); ▲, insulin (5 μg./ml.)+prolactin (10 μg./ml.)+corticosterone (1 μg./ml.)+serum from lactating mouse (to give 10%, v/v).

growth hormone (10 μg./ml.), 3,3′,5-tri-iodothyronine (5 μg./ml.), oestradiol (0·05 μg./ml.), oxytocin (10^{-5} unit/ml.) or extra prolactin (to give 100 μg./ml.).

Serum is required in many organ cultures, and Fig. 2 shows the effect of serum from lactating mice on our cultures. It did not affect the activity at 22 hr., but allowed the rise in activity to continue over the second day. When serum was present prolactin and corticosterone caused no further increase in activity at 45 hr. Serum from mice in the second half of pregnancy, or from virgin mice, gave the same increment in enzyme activity at 45 hr., whereas foetal calf serum gave 90% of the increment, horse serum 50% and calf serum 35%. Bovine serum albumin (4%, w/v) had no effect.

Tissue from mid-pregnant mice. Table 2 shows that both enzymes again increased their activity in cultures with insulin of tissue from mid-pregnant (day 12) mice. Without insulin the activities fell. Table 3 shows that prolactin and corticosterone caused the activity of glucose 6-phosphate dehydrogenase to increase further in both 22 hr. and 45 hr. cultures. Table 5 shows the results of experiments in each of which the enzyme activity was measured both at 22 hr. and 45 hr. in cultures from a single mouse at day 12. With insulin alone, or with the three hormones, the activity continued to rise over the second day of culture. Further experiments showed once more that the stimulation caused by prolactin plus corticosterone was solely due to prolactin.

These results contrast with those obtained on tissue from late-pregnant mice in that prolactin stimulated the activity at 22 hr. as well as at 45 hr., and even with insulin alone the activity continued to rise over the second day.

Experiments with inhibitors. The effect of inhibitors of RNA, DNA and protein synthesis on the enzyme activities in 22 hr. cultures of tissue from late-pregnant mice was tested. The inhibitors were used at the lowest concentrations that would give almost complete inhibition. These were: actinomycin D (0·5 μg./ml.), which inhibited the incorporation of [^{14}C]uridine into the RNA of the explants by 87%; hydroxyurea (50 μg./ml.), which inhibited the incorporation of [^{3}H]thymidine into the DNA by 92%; cycloheximide (1 μg./ml.) and puromycin (10 μg./ml.), which inhibited the incorporation of [^{14}C]lysine into the proteins by 93% and 90% respectively. The above concentration of actinomycin D inhibited the incorporation of [^{3}H]thymidine into DNA by 16% and of [^{14}C]lysine into proteins by 17%. The above concentration of hydroxyurea inhibited the incorporation of [^{14}C]-uridine into RNA by 8%.

Table 6 gives typical experiments showing the effect on the activities of both enzymes of having any one of these inhibitors in the culture medium. Actinomycin D, puromycin and cycloheximide all prevented any increase in enzyme activity whereas hydroxyurea had little effect. In experiments with actinomycin the activity of each enzyme was about the same at 22 hr. as at 0 hr. In four experiments with cycloheximide the average activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase at 22 hr. were 67% and 66% of those at 0 hr., possibly as a result of turnover of the enzymes. With puromycin the decreases were greater.

Table 6. *Effect of inhibitors on increases in enzyme activities in 22 hr. cultures of mammary tissue from mice in late pregnancy*

Values are means of duplicate determinations. Percentages of initial values are given in parentheses. Inhibitors were present at the start of culture. Conditions otherwise were as described for Table 2.

	Glucose 6-phosphate dehydrogenase (units/g.)		6-Phosphogluconate dehydrogenase (units/g.)	
Expt. no.	1	2	1	2
Start	2·02 (100)	1·53 (100)	0·60 (100)	0·46 (100)
22 hr.	4·20 (208)	2·91 (190)	1·20 (200)	1·06 (230)
22 hr. + puromycin (10 μg./ml.)	0·16 (8)	—	0·16 (27)	—
22 hr. + cycloheximide (1 μg./ml.)	1·50 (74)	—	0·30 (50)	—
22 hr. + hydroxyurea (50 μg./ml.)	—	2·80 (183)	—	0·82 (178)
22 hr. + actinomycin D (0·5 μg./ml.)	—	1·10 (72)	—	0·46 (100)

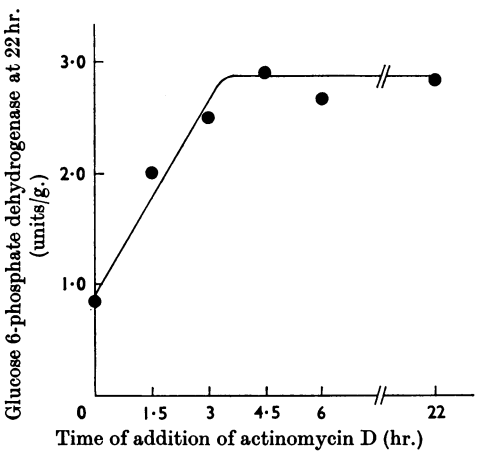

Fig. 3. Effect of delayed addition of actinomycin D on glucose 6-phosphate dehydrogenase activity after 22 hr. of culture. Batches of 20 explants from a single mouse at day 19 of pregnancy were cultured with insulin (5 μg./ml.) for 22 hr. Actinomycin D was added to duplicate cultures at each of the times shown to give 0·5 μg./ml.

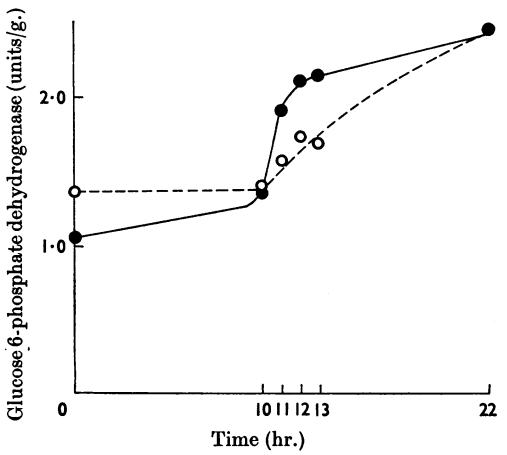

Fig. 4. Effect of delayed addition of cycloheximide on glucose 6-phosphate dehydrogenase activity after 22 hr. of culture. Experimental details were as described for Fig. 3, cycloheximide being added to give 1 μg./ml. ●, Enzyme activities at 22 hr., cycloheximide being added at times shown. Also shown are glucose 6-phosphate dehydrogenase activities at intervals during culture of tissue from the same mouse (experimental details were as described for Fig. 1). ○, Enzyme activities at times shown.

We next studied the effects of actinomycin D and cycloheximide when added at various intervals after the start of culture. Fig. 3 shows that actinomycin D failed to prevent the rise in enzyme activity if added after about 3·5 hr. (Its inhibition of the incorporation of [^{14}C]uridine into RNA was the same at 3·5 hr. as at 0 hr.)

In two preliminary experiments we found that cycloheximide, if added at 12 hr. when the enzyme activity was still almost at the starting value, largely failed to prevent the rise in activity. It therefore appeared that the rise in activity could occur in the absence of protein synthesis, and the experiment in Fig. 4 was designed to elucidate this point. When cycloheximide was added at 10 hr. it still prevented the rise in enzyme activity, but when added at 12 hr. or later it largely failed to do so. The increase in enzyme activity in cultures of tissue from the same mouse largely occurred over 12–22 hr. (see also Fig. 1). Within 5 min. of its addition cycloheximide inhibited the incorporation of [^{14}C]lysine into protein when added at 12 hr., by over 90%, as it did when added at 0 hr. Hence, although protein synthesis is required before 12 hr., the enzyme activity can in fact rise in the absence of protein synthesis.

Preliminary attempts to detect an inactive precursor of glucose 6-phosphate dehydrogenase in tissue cultured for 12 hr. were unsuccessful. Thus

the enzyme activity in a mixture of two supernatants that had been cultured for 12hr. and 22hr. was the average of the two. The enzyme activity in supernatants from tissue that had been cultured for 12hr. was not increased by incubation with $NADP^+$, glucose 6-phosphate and β-mercaptoethanol (Nevaldine & Levy, 1965).

DISCUSSION

In mammary tissue from mid-pregnant mice the secretory cells are small and surround small alveolar lumina, which contain no secretion. Culture of this tissue with insulin produces little change in structure, but with the further addition of prolactin and an adrenal corticosteroid the alveoli after 48hr. resemble those in a late-pregnant mouse: they have become swollen with secretion and the parenchymal cells are enlarged and contain secretory vacuoles.

It has been shown that culture with insulin of tissue from mid-pregnant, as well as late-pregnant, mice produces marked increases in glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities, which are little affected by the further addition of prolactin and corticosterone (Table 3). Hence these increases in enzyme activity will occur in parenchymal cells whether or not they are undergoing or have undergone developmental changes similar to those that occur in the second half of pregnancy. In this respect they contrast with changes in formation of milk protein, which require prolactin (Stockdale *et al.* 1966; R. Mayne & J. M. Barry, unpublished work; Turkington, Brew, Vanaman & Hill, 1968), and resemble the insulin-stimulated uptake and oxidation of glucose, which is only slightly affected by prolactin and corticosterone (R. Mayne & J. M. Barry, unpublished work). The small effect of prolactin on the enzyme activities during the second day of culture of tissue from late-pregnant mice (Tables 3 and 5) could be due to prolactin being needed to maintain the structure of the fully developed mammary gland (Cowie, 1961).

Our experiments do not enable us to decide whether insulin induces the synthesis of enzymes directly (Weber, Singhal, Stamm, Lea & Fisher, 1966), or through some metabolic intermediate whose concentration is raised by the stimulated glucose uptake and metabolism. Nevertheless the experiments with inhibitors do give a few hints about how the inducer might act. That hydroxyurea did not prevent the rise in enzyme activity suggests that prior DNA synthesis and cell division are not required as they appear to be for stimulated casein synthesis (Stockdale & Topper, 1966). That actinomycin D and cycloheximide completely inhibit the increase in enzyme activity when added at the start of culture suggests that both RNA and protein synthesis are involved. The inhibitory effect of actinomycin D fell off steadily between 0hr. and 3·5hr., whereas that of cycloheximide fell off between 10hr. and 12hr. (Figs. 3 and 4). Hence all RNA on which the increase in enzyme activity depends must be synthesized over 0–3·5hr.; at least one protein on which it depends must be absent at 10hr., and all essential protein synthesis must be complete at 12hr.

These facts strongly suggest that the increase in enzyme activity is initiated by an inducing compound stimulating the synthesis of specific RNA within the nucleus. The simplest mechanism would be that over the first 3·5hr. the inducer stimulates the synthesis of RNA, which includes template RNA that is translated between 10hr. and 12hr. into a protein precursor of the enzyme (or conceivably into active enzyme bound to cell particles). Active enzyme then appears in the cytoplasm between 10hr. and 22hr. The delay between transcription and translation could be the time taken for organization of the RNA on to new endoplasmic reticulum (Tata, 1967). It is possible, however, that the template synthesized over the first 3·5hr. is almost immediately translated into a protein that, by a chain of metabolic changes, stimulates the synthesis of the enzyme precursor between 10hr. and 12hr. by a mechanism that does not immediately depend on RNA synthesis. Alternatively it is possible that the protein formed between 10hr. and 12hr. is not the enzyme precursor, but is merely involved in the activation of enzyme protein that was present in the tissue from the start of culture. These possibilities will, we hope, be clarified by experiments with an antibody to the enzyme.

It appears unlikely that the inducer acts in the cytoplasm rather than the nucleus, either by initiating the translation of pre-formed template RNA or by stabilizing the protein formed on it. These mechanisms would require that the inducer is not present until after 10hr. Dependency on RNA that is synthesized some hours earlier could result from the template RNA being unstable and being continually replaced by new RNA that takes some hours to travel from the nucleus to the site of protein synthesis. However, the fact that inhibition by actinomycin D is complete at 0hr., but falls off steadily between 0hr. and 3·5hr., suggests that the essential RNA is absent at 0hr. and present soon after, and hence that its synthesis is induced by entry of the tissue into the culture medium.

Why do these enzyme activities increase in the mammary gland of the living mouse at parturition? This is not due to a rise in plasma insulin at parturition, since the average insulin concentration is elevated in late pregnancy and gradually returns to normal after parturition (Spellacy & Goetz, 1963).

A possible factor limiting the uptake and use of glucose by the gland during pregnancy could be placental lactogen, which occurs in C3H mice (Cerruti & Lyons, 1960), and which, at least in man, has an anti-insulin action resembling that of growth hormone (Grumbach, Kaplan, Sciarra & Burr, 1968). With the disappearance of placental lactogen at parturition the elevated concentration of plasma insulin could well cause an increased uptake of glucose by the mammary gland and induction of the enzymes. A similar induction would occur when the tissue is removed from the pregnant animal and placed in culture.

That the loss of placental lactogen may cause the induction of these enzymes at parturition does not appear to have been considered before. It is possible that the loss of placental lactogen, rather than an increased secretion of pituitary hormones, may also stimulate other biosynthetic processes at parturition.

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