substrate at different fixed concentrations of the second substrate gave convergent lines intersecting to the left of the ordinate and below the abscissa. These results are characteristic of a sequential mechanism in which the Michaelis constants for both the substrates are greater than the respective dissociation constants. However, this procedure does not distinguish between the various possible sequential mechanisms, which include (Cleland, 1963) Ordered Bi Bi, Theorell-Chance, Random Bi Bi and Rapid-Equilibrium Random Bi Bi. Product-inhibition studies have now served to distinguish between these possibilities.

Inhibition by CoA was competitive with respect to acetyl-CoA and non-competitive with respect to choline; these results eliminate both the random mechanisms and suggest that an ordered addition of substrates is required. Inhibition by acetylcholine was competitive with respect to choline at saturating acetyl-CoA concentrations. In the presence of a saturating concentration of choline, acetylcholine produced no inhibition with respect to acetyl-CoA. These findings are consistent with the Theorell-Chance mechanism and inconsistent with the Ordered Bi Bi mechanism, which would require acetylcholine to produce non-competitive and uncompetitive inhibition respectively under the above conditions. Considerable problems were encountered in the experiments with acetylcholine owing to the small amount of choline that it contained; this difficulty was overcome by assaying the choline content and making the necessary allowance in the kinetic experiments. A further problem was that eserine, a competitive inhibitor of cholinesterase present in the enzyme preparation, was not totally effective in the presence of the high concentrations of acetylcholine used in the inhibition experiments, resulting in a considerable release of choline. This problem was overcome by pre-incubating the enzyme with 0.1 mM-di-isopropyl phosphorofluoridate, an irreversible inhibitor of the esterase.

The Theorell-Chance mechanism involves an ordered addition of substrates and the results described above are consistent with acetyl-CoA being the leading substrate. Enzyme binding studies with radioactive acetyl-CoA and choline are now being undertaken.

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A Quantitative Investigation of the Phospholipid-Dependence of Rat Liver Microsomal Glucose 6-Phosphohydrolase

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Since the classical studies of Beaufay & de Duve (1954), much work has been done on the phospholipid dependence of glucose 6-phosphohydrolase (glucose 6-phosphatase). However, the specificity of this dependence is still unknown. We have initiated a quantitative investigation of glucose 6-phosphatase dependence on phosphatidylcholine and phosphatidylethanolamine.

Rat liver microsomal phospholipids were hydrolysed with phospholipase C (*Clostridium welchii*; Sigma) at 5 and 37°C. Glucose 6-phosphatase was assayed after hydrolysis had been stopped with EGTA,* which removes calcium essential for this phospholipase. Hydrolysis of phospholipids was measured quantitatively by using a sensitive radiochemical method involving direct counting of radioactive phospholipids in acid-washed microsomal precipitates (Hallinan, Nagley, Murty, Bennett & Grant, 1969). This simple radioassay was possible because conditions had been devised giving virtually specific labelling of phosphatidylcholine by radioactive choline *in vivo*, over 97% of the incorporated radioactivity entering phosphatidylcholine (Nagley & Hallinan, 1968). Similar specific labelling of phosphatidylethanolamine by radioactive ethanolamine has now been developed (B. R. Cater, unpublished work).

With EGTA present, phospholipase C caused no phospholipid hydrolysis and no inhibition of glucose 6-phosphatase. With calcium present, phospholipase C hydrolysed 90–100% of the microsomal phosphatidylcholine and 50–70% of the phosphatidylethanolamine at 37°C. Under these conditions glucose 6-phosphatase activity decreased approximately in parallel to phosphatidylcholine, approaching zero at 90–100% hydrolysis of the latter. However, at 5°C the kinetics were completely different. Though the same degree of phospholipid hydrolysis occurred, the enzyme activity no longer decreased in parallel with the phospholipid. Rather, enzyme activity decreased only 40–60% and then reached a plateau, following somewhat phosphatidylethanolamine, which reached a plateau at 50–70% hydrolysis.

Removal of exactly the same proportion of phospholipids has different effects at 5°C compared with 20 or 37°C, because when some phospholipid is hydrolysed glucose 6-phosphatase is

* Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetic acid.

rendered heat-labile. This was demonstrated by post-incubation at 20 or 37°C of microsomes hydrolysed by phospholipase at 5°C, EGTA being added before post-incubation. Heat-inactivated enzyme could not be reactivated by sonicated phosphatidylcholine, though this completely restored both activity and heat-stability to glucose 6-phosphatase from microsomes with 95% of the phosphatidylcholine and 70% of the phosphatidylethanolamine hydrolysed at 5°C. Lecithin probably failed to reactivate glucose 6-phosphatase for Duttera, Byrne & Ganoza (1967) because they treated with phospholipase at 20°C.

Preliminary conclusions are that glucose 6-phosphatase is not wholly lecithin-dependent, since 40–60% of the activity remains when 90–100% of the lipid is hydrolysed. Superimposition of heat-inactivation prevents quantitative definition of the phospholipid-dependence of glucose 6-phosphatase, so this must be investigated by low-temperature techniques.

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Effect of Vitamin A Status of the Content of some Steroids in the Ovarian-Vein Blood of Pregnant Rats

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Female rats given a vitamin A-deficient diet with retinoic acid maintain normal oestrous cycles and conceive when mated with normal males, but pregnancy is terminated by resorption at about day 14 (Thompson, Howell & Pitt, 1964; Juneja, Murthy & Ganguly, 1964). Juneja, Moudgal & Ganguly (1969) showed that the resorption is prevented by injections of pregnenolone or oestradiol-17 β , whereas progesterone is only partially effective. Further, the specific activity of 3 β -hydroxy Δ^5 -steroid dehydrogenase is less in the ovaries of retinoate-fed rats during pregnancy.

Groups of rats maintained on a vitamin A-deficient diet supplemented with either retinyl acetate or retinoic acid grew and conceived normally,

but after day 12 of pregnancy those receiving retinoate ceased to increase in body weight and no pups were born; pregnancy in the other group was normal.

To investigate steroid secretion rates, ovarian-vein blood was taken at days 9 and 15 of pregnancy. Tracers of ^3H -labelled steroids were added to pooled extracts of blood each obtained by the addition of acetone to the sample. Extracts were fractionated by partition between NaHCO_3 and ether, column chromatography (silica-gel adsorbent) and partition between toluene–light petroleum and 0.4M-NaOH, giving fractions containing neutral and phenolic steroids. Oestradiol-17 β and oestrone, separated by t.l.c. on silica gel, were finally purified by paper chromatography. Each oestrogen was dissolved in propane-1,2-diol–water (1:10, v/v), for bioassay by the intravaginal tetrazolium-reduction method of Martin (1960, 1964). Progesterone and 20 α -hydroxy-4-pregnen-3-one were determined by g.l.c. after further purification by solvent partitioning and t.l.c.

With both supplements there were marked increases in the secretion rates of progesterone and 20 α -hydroxypregnenone as pregnancy advanced, as has been reported for normal rats by Eto, Masuda, Suzuki & Hosi (1962), Fajer & Barraclough (1967) and Hashimoto, Hendricks, Anderson & Melampy (1968). However, the secretion rates of these steroids were about one-third less in the retinoate-fed rats. No differences in the secretion rates of oestradiol-17 β and oestrone were found. It is suggested that the decreased secretion of progesterone and 20 α -hydroxypregnenone reflected the lowered specific activity of 3 β -hydroxy Δ^5 -steroid dehydrogenase in the ovaries of the retinoate-fed rats and may have been a cause of the pregnancy disorder.

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