2. The inhibition by the last-named three substances potentiates the inhibition by oxaloacetate of succinate oxidation.

3. The role of oxaloacetate as a rate-regulator of oxidative metabolism through the citric acid cycle is discussed.

This work was supported in part by research grants from the Life Insurance Medical Research Fund and from the National Science Foundation, Division of Molecular Biology.

REFERENCES

- Avron, M. & Biale, J. (1957). *J. biol. Chem.* **225**, 699.
 Brummond, D. O. & Burris, R. H. (1953). *Proc. nat. Acad. Sci., Wash.*, **39**, 754.
 Corwin, L. M. & Schwarz, K. (1959). *J. biol. Chem.* **234**, 191.
 Davies, D. D. & Kun, E. (1957). *Biochem. J.* **66**, 307.
 Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
 Pardee, A. B. & Potter, V. R. (1948). *J. biol. Chem.* **176**, 1085.
 Rosenthal, O. (1937). *Biochem. J.* **31**, 1710.
 Tyler, D. B. (1954). *J. Pharmacol.* **110**, 49.
 Tyler, D. B. (1955a). *Proc. Soc. exp. Biol., N.Y.*, **88**, 333.
 Tyler, D. B. (1955b). *J. biol. Chem.* **216**, 395.
 Tyler, D. B. (1957). *Fed. Proc.* **16**, 130.
 Wilson, P. W. (1949). In *Respiratory Enzymes*. Ed. by Lardy, H. A. Minneapolis: Burgess Publishing Co.
 Wolff, E. C. & Ball, E. G. (1957). *J. biol. Chem.* **224**, 1083.

Biochem. J. (1960) **76**, 297

Stimulation of Fatty Acid Synthesis by Oestradiol *in vitro*

By N. HOSOYA,* D. HAGERMAN AND C. VILLEE

Department of Biological Chemistry, Harvard Medical School, and Research Laboratories of the Boston Lying-in Hospital, Boston, Massachusetts, U.S.A.

(Received 23 November 1959)

Previous experiments in this Laboratory demonstrated the presence in human placenta of an enzyme system which responds to oestradiol (Vilee & Hagerman, 1953). This system, originally thought to be an *isocitric* dehydrogenase which required diphosphopyridine nucleotide as hydrogen acceptor (Vilee, 1955), was subsequently found to require minute amounts of triphosphopyridine nucleotide and was shown to be a transhydrogenase (Talalay & Williams-Ashman, 1958; Vilee & Hagerman, 1958). The transhydrogenation reaction, $\text{TPNH} + \text{DPN} \rightarrow \text{DPNH} + \text{TPN}$, can be coupled to glucose 6-phosphate dehydrogenase as well as to *isocitric* dehydrogenase, and presumably can be linked to any dehydrogenase requiring triphosphopyridine nucleotide (Vilee & Hagerman, 1958).

Talalay & Williams-Ashman (1958) suggested that this system is mediated by a 17β -oestradiol dehydrogenase with dual pyridine nucleotide specificity which alternately oxidizes and reduces oestradiol:

Sum: $\text{DPN} + \text{TPNH} \rightleftharpoons \text{DPNH} + \text{TPN}.$

Recent experiments in this Laboratory (Hagerman & Vilee, 1959) have shown that the trans-

hydrogenase stimulated by oestrogen and the oestradiol dehydrogenases are separate and distinct enzymes. They differ markedly in their response to thyroxine, to adenosine 2'-monophosphate and to sulphhydryl inhibitors; they have different rates of thermal inactivation; and they may be separated physically by electrophoresis on a starch block or on a filter-paper curtain or by adsorption on to and elution from diethylaminoethylcellulose columns. Talalay & Williams-Ashman (1958) had postulated that all steroid hormones produce their effects by way of hydroxysteroid dehydrogenases with dual pyridine nucleotide specificity that rapidly and reversibly oxidize and reduce the steroid. Our recent evidence renders it unlikely that this is the mechanism of action of oestrogens. Other evidence against this theory has been presented by Vilee & Spencer (1959) with respect to the mode of action of testosterone and by Bush & Mahesh (1959) with respect to the mode of action of 11β -hydroxy-steroids.

The reaction mediated by the transhydrogenase stimulated by oestrogen should be reversible. In experiments reported earlier (Vilee & Hagerman, 1958) we were unable to couple the transhydrogenation reaction with a system in which a diphosphopyridine nucleotide-linked dehydrogenase was presented with substrate amounts of triphosphopyridine nucleotide and catalytic amounts of diphosphopyridine nucleotide. However, the trans-

* Present address: Department of Biochemistry, Tokyo Women's Medical College, Tokyo, Japan.

hydrogenase is inhibited by oxidized triphosphopyridine nucleotide. The present paper reports experiments which demonstrate a coupling of the transhydrogenase stimulated by oestrogen with a fatty acid-synthesizing system which requires reduced triphosphopyridine nucleotide. In this reaction, under the special conditions of the system *in vitro*, hydrogen is transferred from reduced diphosphopyridine nucleotide to triphosphopyridine nucleotide and the synthesis of fatty acids is stimulated. These experiments also indicate that the enzymic synthesis of fatty acids in the placenta occurs by way of the biotin-requiring-enzyme system described by Wakil (1958) and by Brady (1958).

MATERIALS AND METHODS

The diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), adenosine triphosphate (ATP) and coenzyme A (CoA) used in these experiments were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) or from Difco Laboratories (Detroit, Mich., U.S.A.) and were 95% pure.

Preparation of fatty acid-synthesizing enzyme

Human placentae (12–20 weeks gestational age) were obtained at vaginal hysterotomy for therapeutic interruption of pregnancy and were used within 5 min. of their removal from the patient's body. A sample (10–20 g.) of placenta was washed twice in 10 vol. of ice-cold 0.15M-NaCl. The yolk sac was removed, the placenta was cut into small pieces with scissors and the pieces were homogenized in 4 vol. of 0.25M-sucrose or 4 vol. of potassium phosphate-potassium bicarbonate buffer, pH 9 (0.1M- K_2HPO_4 –0.07M- $KHCO_3$). The homogenization was carried out in a glass vessel with a Teflon pestle or in a Waring Blendor for 30 sec. The homogenate was centrifuged at 2000 *g* for 10 min. at 5° to remove cellular debris. The supernatant fluid was centrifuged in a Spinco preparative ultracentrifuge for 60 min. at 60 000 *g*. The resulting supernatant fluid was recovered and fractionated by the addition of solid ammonium sulphate. The protein which precipitated when the solution was made 60% saturated with respect to ammonium sulphate was recovered by centrifuging at 10 000 *g* for 15 min. in the cold. The precipitate was dissolved in 5 ml. of 0.04M- $KHCO_3$ buffer, pH 8.5, and glutathione was added to a final concentration of 1 mM. This solution was dialysed for 2 hr. in the cold against 1 l. of 0.04M- $KHCO_3$. The protein solution after dialysis was frozen and stored overnight at –20°. It was then thawed and centrifuged at 1000 *g* for 10 min. The resulting supernatant solution was used in these studies.

Enzymic synthesis of malonyl-coenzyme A

An enzyme system for the activation of malonic acid has been found in acetone-dried preparations of pig heart (Beinert & Stanly, 1953), in rat tissues (Nakada, Wolfe & Wick, 1957) and in human placenta (Hosoya & Kawada, 1958). An acetone-dried powder of pig-heart or human placenta was dissolved in 10 vol. of a solution containing

0.02M- $NaHCO_3$, 1 mM-sodium ethylenediaminetetraacetate, 1 mM-glutathione and 1 mM-potassium malonate. The fraction precipitating between 40 and 60% saturation with ammonium sulphate was prepared according to the directions of Green, Goldman, Mii & Beinert (1953). This preparation contains a malonate-activating enzyme which requires ATP, CoA, Mg^{2+} ions and malonate. The enzyme preparation was incubated at 37° for 30 min. in a mixture containing 100 μ moles of 2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer, pH 8.2, 10 μ moles of $MgCl_2$, 100 μ moles of potassium malonate, 0.2 μ mole of CoA, 10 μ moles of ATP, 10 μ moles of glutathione, 500 μ moles of hydroxylamine and 1.5 mg. of purified pig-heart or placenta enzyme. The product of the reaction was extracted and chromatographed according to the directions of Stadtman & Barker (1950) and Hayaishi (1955). A single hydroxamic acid derivative with R_f 0.33 was detected when the chromatograms were developed with water-saturated butanol. The mobility of this derivative corresponded exactly with that of authentic monomalonyl hydroxamic acid chromatographed simultaneously.

Large-scale incubations were performed as described by Brady (1958), with 10 times the quantity of the materials used in the assay system described above but containing a total of 10 μ moles of CoA. The reaction was terminated by the addition of 2 m-equiv. of acetic acid and the acidified mixture was heated at 100° for 1 min. The resulting suspension was centrifuged and the supernatant solution was recovered and freeze-dried. The dried residue was dissolved in a minimal volume of water and applied to a sheet of Whatman no. 3 filter paper. The chromatogram was developed with a solution of equal volumes of ethanol and 0.1M-acetic acid-potassium acetate buffer, pH 4.5. After the paper had been dried a small strip was cut out and treated with nitroprusside reagent (dilute ammoniacal solution of sodium nitroprusside) to detect the CoA derivative. The material was eluted with water from the remainder of the chromatogram, and concentrated by freeze-drying and taken up in a small volume of water. A total of 2.2 μ moles of malonyl-CoA was recovered, measured by the method of Lipmann & Tuttle (1945).

RESULTS

Stimulation by oestradiol of the synthesis of long-chain fatty acids

The synthesis of fatty acids by the enzyme system prepared from the placenta was measured by the incorporation of [^{14}C]acetate into the lipid fraction. The following components were placed in a small glass-stoppered test tube: coenzyme A, 0.02 μ mole; ATP, 2.5 μ moles; Mn^{2+} ion, 0.02 μ mole; isocitrate, 6.0 μ moles; TPN, 0.2 μ mole; DPN, 0.2 μ mole; glucose 1-phosphate, 15.0 μ moles; lipoic acid, 0.15 μ mole; glutathione, 8.0 μ moles; [^{14}C]acetate, 4 μ moles; purified human placental enzyme, 2.0 mg.; potassium phosphate buffer, pH 6.5, 30 μ moles. The final volume of the incubation mixture was 0.5 ml. To one of each pair of vessels an aqueous ethanol solution of oestradiol was added to a final concentration of 4 μ M.

The tubes were flushed gently with nitrogen for 30 sec., stoppered and incubated in a water bath at 38° for 2 hr. The reaction was then stopped by the addition of 0.5 ml. of ethanolic 10% KOH and the tube was placed in a boiling-water bath for 30 min. When saponification was complete, 0.5 ml. of 2N-HCl was added to bring the pH to between 2 and 3. The free fatty acids were extracted by adding 4 ml. of *n*-pentane and shaking the tightly stoppered tube for 15 min. After the two phases had separated, the pentane layer was transferred to a 15 ml. graduated centrifuge tube with a medicine dropper. The extraction was repeated with two additional 4 ml. portions of pentane. The pooled pentane extracts were concentrated to 1 ml. in a stream of warm air. Samples (50 μ l.) of the pentane solution were plated on aluminium planchets and dried. The radioactivity on the planchet was then measured in a gas-flow counter. The measured radioactivity represents the amount of acetate incorporated into long-chain fatty acids, since only long-chain fatty acids are extracted into the pentane under these conditions. From the specific activity of the substrate acetate, and the total counts/min. in the long-chain fatty acids isolated at the end of the incubation, it was calculated that, without added oestradiol, 0.42 μ -mole, and in the presence of oestradiol (4 μ M) 0.51 μ -mole, of acetate was incorporated into fatty acid during the incubation for 2 hr.

A sample of the pentane extract was transferred to filter paper and developed with ethylene glycol-diethyl ether-propionic acid-water solvent by the method of Benson *et al.* (1950). The radioactivity was located by prolonged exposure of the chromatogram to X-ray film. The spot had an R_f identical with that of palmitate.

These experiments indicated that oestradiol may accelerate the incorporation of [1-¹⁴C]acetate into long-chain fatty acids. The first step in the synthesis of fatty acids is the carboxylation of acetyl-CoA to yield malonyl-CoA, catalysed by a biotin-containing enzyme which requires ATP, Mn²⁺ and HCO₃⁻ ions. The enzyme fraction (containing 1 mg. of protein) prepared from pre-term human placenta was incubated at 37° in an atmosphere of nitrogen in a system containing 0.5 μ -mole of acetyl-CoA, 5 μ -moles of phosphate buffer, pH 7.4, 5 μ -moles of ATP, 5 μ -moles of KH¹⁴CO₃ (10⁵ counts/min.), 1 μ -mole of MnCl₂, 0.05 μ -mole of biotin, with and without added oestradiol (final concn. 4 μ M). After incubation for 30 min. 300 μ -moles of hydroxamic acid were added and the system was incubated for 10 min. Ethanol (5 vol.) was added, the mixtures were centrifuged and the supernatant fluid was evaporated to dryness. The dry residue was extracted with 2 ml. of acetone, the acetone extract was concentrated under nitrogen to 0.1 ml.,

and this was applied to paper for chromatography. By the method of Stadtman & Barker (1950) malonyl monohydroxamate was identified by its R_f (0.35). The radioactivity of the malonyl monohydroxamate was the same whether or not oestradiol was added, which indicates that the hormone has no effect on the enzymic synthesis of malonyl-CoA.

The second step in fatty acid synthesis is a condensation of malonyl-CoA with acetaldehyde to yield β -hydroxybutyryl-CoA (Brady, 1958) or of malonyl-CoA with acetyl-CoA (Wakil, 1958) to yield butyryl-CoA. Reaction mixtures were prepared containing 25 μ -moles of phosphate buffer, pH 7.4, 5 μ -moles of KF, 1 μ -mole of MnCl₂, 5 μ -moles of ATP, 0.5 μ -mole of CoA, 5 μ -moles of glutathione, 15 μ -moles of substrate and 1 mg. of placental enzyme, in a total volume of 1 ml. The mixtures were incubated for 30 min. at 37°, and then 300 μ -moles of hydroxylamine hydrochloride were added to terminate the reaction. The mixture was kept at room temperature for 10 min. and then 5 vol. of ethanol was added; the mixtures were centrifuged and each supernatant solution was evaporated to dryness. The residue was extracted with 2 ml. of acetone, the solution was concentrated to 0.1 ml. under nitrogen, transferred to filter paper and developed with butanol-propionic acid-water solvent (Benson *et al.* 1950). In this system, the R_f values of the hydroxamic acid derivatives of malonic acid, acetic acid, β -hydroxybutyric acid and butyric acid are 0.30, 0.51, 0.74 and 0.81 respectively.

When the incubation was carried out with malonate alone or with malonate plus acetate as substrates, β -hydroxybutyrate was formed, as indicated by the production of a hydroxamic acid derivative with R_f 0.75. With malonate and acetaldehyde as substrate no such derivative was formed. With malonate and acetate as substrate an additional hydroxamic acid derivative, with R_f 0.66, was obtained. This was not identified but is probably some intermediate in the reaction system. With malonate alone as substrate, both acetate and β -hydroxybutyrate were formed. Thus the protein prepared from placenta contains enzymes for the decarboxylation of malonate to form acetate as well as ones for the condensation of malonyl-CoA and acetyl-CoA to yield β -hydroxybutyrate.

The condensation step is followed by a reductive step which requires reduced triphosphopyridine nucleotide (TPNH; Wakil, 1958). Since oestradiol has been shown to stimulate a specific pyridine nucleotide transhydrogenase (Hagerman & Villee, 1959), it seemed possible that the stimulation with oestradiol observed in the synthesis of long-chain fatty acids from [1-¹⁴C]acetate resulted from a

Fig. 1. Spectrophotometric measurement of the effect of oestradiol on synthesis of β -hydroxybutyryl-CoA. The reaction mixture contained 150 μ moles of potassium phosphate buffer or tris buffer (pH 7.0), 0.25 μ mole of DPNH, 0.5 μ mole of MnCl_2 , 0.2 μ mole of acetyl-CoA and malonyl-CoA. One cuvette (line B) contained oestradiol, final concn. 4 μM . Line A gives extinction of control cuvette to which no oestradiol was added. After incubation for 10 min. (at arrow), 0.01 μ mole of TPN was added. Total volume in each cuvette was 3 ml. Temperature was 22°.

stimulation of transhydrogenase coupled with the TPNH-linked reductive step. To test this possibility, a reaction mixture was set up in which fatty acid synthesis was followed spectrophotometrically by the oxidation of reduced diphosphopyridine nucleotide (DPNH) in the presence of the transhydrogenase and catalytic amounts of TPN. Under these conditions the reaction was accelerated by the presence of oestradiol in a final concentration of 4 μM (Fig. 1). This indicates that the pyridine nucleotide transhydrogenase stimulated by oestrogen catalysed the transfer of hydrogen from DPNH to TPN and that the TPNH formed was then reoxidized in the course of the synthesis of β -hydroxybutyrate from acetyl-CoA and malonyl-CoA.

DISCUSSION

The evidence presented here shows that the placenta contains enzymes for the synthesis of fatty acids by the condensation of acetyl-coenzyme A and malonyl-coenzyme A, by reactions similar to those described by Wakil (1958). A partially purified enzyme preparation from human pre-term placenta contains both the enzymes for fatty acid synthesis and the transhydrogenase stimulated by

oestrogen. Under conditions in which the supply of TPNH is made rate-limiting but in which there is an adequate supply of DPNH, the synthesis of fatty acids can be stimulated by the addition of oestradiol to the system. The present experiments indicate that this results from a coupling of the TPNH-requiring reductive step which follows the condensation of malonyl-coenzyme A with acetyl-coenzyme A and the pyridine nucleotide transhydrogenase stimulated by oestrogen which in this instance catalyses the transfer of hydrogen from DPNH to TPN. Previous attempts in this Laboratory to demonstrate the transfer of hydrogen in this direction had been unsuccessful (Villee & Hagerman, 1958). However, in the former experiments, substrate amounts of oxidized TPN were supplied to a system containing a DPN-specific dehydrogenase, the transhydrogenase stimulated by oestrogen and catalytic amounts of DPN. Under these conditions the transhydrogenase is inhibited by the oxidized TPN. A TPN-linked glutathione reductase can be coupled with the oestrogen-sensitive transhydrogenase and under these conditions the latter transfers hydrogen from DPNH to TPN (Talalay, Hurlock & Williams-Ashman, 1958). Again, this could be demonstrated only when the concentration of TPN was kept extremely low.

In other experiments (Villee, Hagerman & Joel, 1960) with placenta slices incubated in a medium containing ^{14}C -labelled acetate or glycine, the rate of incorporation of the ^{14}C into fatty acids as well as into proteins and purines was stimulated by the addition of oestradiol. In the intact cell, with most of the TPN in the reduced state and most of the DPN in the oxidized state (Glock & McLean, 1955), it seems unlikely that oestradiol could increase the rate of fatty acid synthesis by increasing the concentration of reduced TPN. Instead, the evidence suggests that the stimulation of the transhydrogenase by oestrogen leads to an increase in the amount of biologically useful energy available to the cell and that this secondarily leads to an increase in the rates of endergonic reactions such as the synthesis of fatty acids, proteins and purines. The present experiments are of interest in showing that under certain conditions *in vitro* the synthesis of fatty acids can be coupled to transhydrogenase which provides the requisite TPNH. They should not be taken as evidence that the oestrogen stimulation of fatty acid synthesis in the intact placental cell necessarily occurs by way of the same mechanism.

SUMMARY

1. Evidence is presented that the human placenta contains enzymes for the carboxylation of acetyl-coenzyme A to yield monomalonyl-coenzyme

A and for the condensation and reduction of these two to yield β -hydroxybutyric acid.

2. These enzymes, which are located in the non-particulate fraction of the cell, were partially purified by ammonium sulphate fractionation.

3. The coupling of the fatty acid-synthesizing system to the transhydrogenase stimulated by oestrogen was demonstrated by the increased rate of conversion of [^{14}C]acetate into labelled fatty acid and by the increased rate of oxidation of reduced pyridine nucleotide when oestradiol was added to the system.

REFERENCES

- Beinert, H. & Stansly, P. G. (1953). *J. biol. Chem.* **204**, 67.
 Benson, A. A., Bassham, J. A., Calvin, M., Groodale, T. C., Haas, V. A. & Stepka, W. (1950). *J. Amer. chem. Soc.* **72**, 1710.
 Brady, R. O. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 993.
 Bush, I. E. & Mahesh, V. B. (1959). *Biochem. J.* **71**, 705.
 Glock, G. E. & McLean, P. (1955). *Biochem. J.* **61**, 381.
 Green, D. E., Goldman, D. S., Mii, S. & Beinert, H. (1953). *J. biol. Chem.* **202**, 137.
 Hagerman, D. D. & Vilee, C. A. (1959). *J. biol. Chem.* **234**, 2031.
 Hayaishi, O. (1955). *J. biol. Chem.* **215**, 125.
 Hosoya, N. & Kawada, N. (1958). *J. Biochem., Tokyo*, **45**, 363.
 Lipmann, F. & Tuttle, R. (1945). *J. biol. Chem.* **158**, 565.
 Nakada, H. I., Wolfe, J. B. & Wick, A. N. (1957). *J. biol. Chem.* **226**, 145.
 Stadtman, E. R. & Barker, H. A. (1950). *J. biol. Chem.* **184**, 769.
 Talalay, P., Hurlock, B. & Williams-Ashman, H. G. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 862.
 Talalay, P. & Williams-Ashman, H. G. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 15.
 Vilee, C. A. (1955). *J. biol. Chem.* **215**, 171.
 Vilee, C. A. & Hagerman, D. D. (1953). *J. biol. Chem.* **205**, 873.
 Vilee, C. A. & Hagerman, D. D. (1958). *J. biol. Chem.* **233**, 42.
 Vilee, C. A., Hagerman, D. D. & Joel, P. A. (1960). *Recent Progr. Hormone Res.* (in the Press).
 Vilee, C. A. & Spencer, J. M. (1959). *Abstr. 135th Meet. Amer. chem. Soc.*, p. 4C.
 Wakil, S. J. (1958). *J. Amer. chem. Soc.* **80**, 6465.

Biochem. J. (1960) **76**, 301

The Yellow Pigmentation of Bull Semen and its Content of Riboflavin, Niacin, Thiamine and Related Compounds

BY I. G. WHITE AND G. J. LINCOLN

Department of Veterinary Physiology, The University of Sydney, Sydney, and M.B.T. Research Laboratories Pty. Ltd., Bourke Street, Darlinghurst, N.S.W., Australia

(Received 23 November 1959)

Semen is usually white or slightly creamy in colour, but during the course of unrelated studies on the physiology of spermatozoa (White, 1954) the semen from one particular Friesian bull was found to be quite yellow. The colour was confined to the plasma fraction and u.v. light produced a green fluorescence similar to that emitted by riboflavin. By visual examination, the colour of the specimen was comparable with that of an aqueous solution containing at least 10 μg . of riboflavin/ml.

Much lower values for the riboflavin content of bull semen were quoted by VanDemark & Salisbury (1944), who were presumably dealing with unpigmented specimens. The reports of Brochart (1952), Kaemmerer & Krampitz (1955) and the analyses of Mann (1954) suggest that the yellow pigmentation of bull semen may be attributed largely to riboflavin.

Recently it has been possible to obtain several samples of very yellow bull semen, and these have

provided material for more extensive investigations into the nature of the pigmentation and its effect on spermatozoa (see White & Lincoln, 1958).

EXPERIMENTAL

Semen and blood. Semen was collected from 13 bulls by the use of an artificial vagina; the semen from five was yellow and the remainder white. Frequently, two ejaculates were obtained from each animal within a few minutes. The samples were protected from light during and after collection, and precautions were taken to prevent any contact with cork, plastic or rubber. The semen was either cooled slowly to 10° or frozen to -79° before transport to the laboratory. Seminal plasma was separated from the spermatozoa by centrifuging. Spermatozoa counts, made with a haemocytometer under high power, were done in duplicate on semen samples diluted with 3% (w/v) NaCl in blood-cell pipettes.

Blood was collected from the jugular vein and the serum that separated from the clot after standing in the dark was used.