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Esterification of Glycerol 3-Phosphate in Lactating Guinea-Pig Mammary Gland

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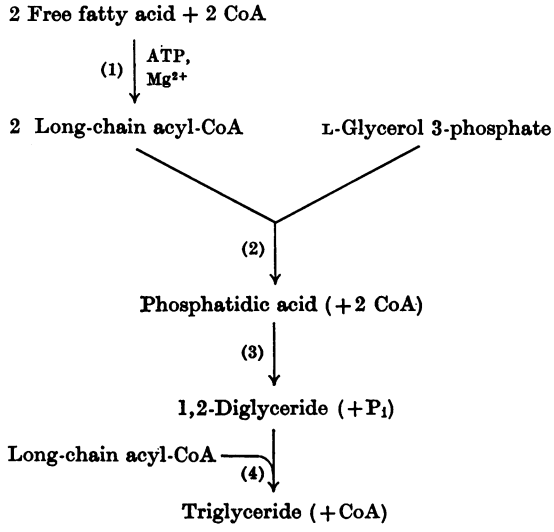
1. The presence of palmitoyl-CoA–L-glycerol 3-phosphate palmitoyltransferase (EC 2.3.1.15) has been demonstrated in a particulate fraction of mammary tissue from lactating guinea pigs. 2. Cell-free preparations also catalysed the activation of palmitate and oleate, and the conversion of enzymically formed phosphatidic acid into glycerides, in accord with the Kennedy pathway of glyceride formation. 3. The properties of the system that esterifies L-glycerol 3-phosphate were studied with respect to substrates and cofactors, and the reaction product was shown to be phosphatidic acid (1,2-diacyl glycerol 3-phosphate). 4. The extent to which newly formed phosphatidic acid was converted into glyceride in a cell-free system was dependent on the nature of the acyl donor, the concentration of subcellular particles, the time of incubation and the concentration of Mg^{2+}.

Current understanding of the biosynthesis of triglyceride in mammals is derived largely from studies on liver and intestine, where experiments with isotopically labelled fatty acids, phosphate and glycerol 3-phosphate, together with the examination of partially purified enzymes, have led to the accepted pathway (Kennedy, 1961) shown in Scheme 1.

Sufficient work has been done with adipose tissue to show that the same overall pathway functions in this tissue (Shapiro, Statter & Rose, 1960; Steinberg, Vaughan & Margolis, 1961). On the other hand, continuing studies have revealed that with intestine this is not the only, or perhaps even major, pathway. In this tissue an alternative route for the formation of 1,2-diglyceride has been demonstrated in the direct acylation of 2-monoglyceride derived from triglyceride lipolysis in the gut lumen (see review by Senior, 1964).

The fourth mammalian tissue in which triglyceride synthesis occurs on a large scale, namely mammary gland, has received very little detailed study at the enzymic level. Dils & Clark (1962) first showed that the incorporation of fatty acid into complex lipid by homogenates of rat mammary gland required ATP, Mg^{2+} and CoA, and that it was stimulated by glycerol 3-phosphate. Since then, Pynadeth & Kumar (1964) have reported that particulate fractions of goat mammary gland catalyse the esterification of both glycerol 3-phosphate and diglyceride. These studies constitute evidence for the operation of the Kennedy pathway in this tissue. On the other hand, the possibility of an alternative route, such as the 'monoglyceride' pathway established for intestine, cannot yet be ruled out. In fact, McBride & Korn (1964) have described the enzymic esterification of mono-



Scheme 1. Pathway for the biosynthesis of triglyceride (Kennedy, 1961). (1) Acid–CoA ligase (AMP) (EC 6.2.1.3); (2) acyl-CoA–L-glycerol 3-phosphate *O*-acyltransferase (EC 2.3.1.15); (3) L-α-phosphatidate phosphohydrolase (EC 3.1.3.4); (4) acyl-CoA–1,2-diglyceride *O*-acyltransferase.

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glyceride in guinea-pig mammary tissue. Since, however, ethanol was esterified under the same conditions, such reactions may be due to a non-specific transferase whose relation to fat synthesis remains to be demonstrated. In contrast, Dils & Clark (1962) and Pynadeth & Kumar (1964) have reported the inability of monoglyceride to act as acyl acceptor in the presence of mammary-gland preparations of the rat and goat respectively.

In the present paper evidence is provided for the importance of the Kennedy pathway in the lactating mammary gland of the guinea pig. The results of studies *in vitro*, in which [^{14}C]glycerol 3-phosphate is esterified by palmitoyl radicals, demonstrate the presence of the enzyme palmitoyl-CoA–L-glycerol 3-phosphate palmitoyltransferase (EC 2.3.1.15), subsequently referred to as 'transferase', in the gland. They also define the nature of the products by means of chromatography and by measurements of reaction stoicheiometry, and demonstrate how the nature of these products is dependent on the incubation conditions.

METHODS

Animals. Normal female guinea pigs were used within the first week of lactation while suckling their young. Animals were exsanguinated under light ether anaesthesia, and the mammary tissue was rapidly dissected out and placed in ice-cold 0·25 M-sucrose–0·1 M-sodium phosphate buffer, pH 6·5 (henceforth referred to as 'sucrose buffer').

Enzyme preparations. Most of the reported experiments were carried out with an acetone-dried powder of whole tissue, or with freshly prepared subcellular particles.

(1) Fresh preparations. The chilled tissue was coarsely snipped with scissors and homogenized in 9 vol. of ice-cold sucrose buffer in a glass homogenizer fitted with a loose-fitting Teflon pestle. The homogenate was strained through two layers of cheesecloth and centrifuged at 2° for 20 min. at approx. 5000*g* in an MSE bench centrifuge. The supernatant fraction was withdrawn from the floating fat and from the sedimented tissue debris. From this preparation a particulate fraction was obtained by centrifugation at 0° for 30 min. at 100000*g* in the Spinco model L preparative ultracentrifuge, the pellet being resuspended in a volume of sucrose buffer equal to that of the tissue from which the pellet was derived. This fraction, which is presumed to contain most of the tissue mitochondria and microsomes, is referred to as 'MM fraction'. In a few experiments the low-speed supernatant fraction was centrifuged first at 0° for 10 min. at about 8000*g* in the Spinco model L preparative ultracentrifuge. The pellet was washed by resuspension and resedimentation under the same conditions and finally resuspended in a volume of sucrose buffer equal to that of the tissue from which the pellet was derived. This fraction is referred to as 'mitochondrial fraction'. The supernatant fraction from the centrifugation at 8000*g* was then re-centrifuged at 0° at 100000*g* and the pellet resuspended to yield what is referred to as the 'microsomal fraction'.

(2) Acetone-dried powders. These were prepared by homogenizing 9 g. of fresh tissue in 12 ml. of ice-cold water and pouring the homogenate slowly into 500 ml. of stirred acetone previously chilled to −15°. The suspension was filtered on a Buchner funnel, and the powder washed successively with 1 l. of acetone and 1 l. of ether at 0°. The cake was sucked dry on the funnel, powdered in a mortar and stored in a desiccator over P_2O_5 at 0°. This yielded 0·93 g. of dry powder. Portions of powder were resuspended in 0·1 M-sodium phosphate buffer, pH 6·5, just before use, at a concentration of 33 mg./ml.

Transferase assays. Transferase activity was generally measured by the incorporation of [^{14}C]glycerol 3-phosphate into an acid-insoluble ethanol-soluble form, as described by Kornberg & Pricer (1953). Where specified, however, lipids were extracted from the reaction mixture by the milder procedure of Folch, Lees & Sloane-Stanley (1957) before the radioactivity was measured. Assays by the two methods agreed to within 10%. Where lipids were extracted by the method of Folch *et al.* (1957), they were subsequently separated into glyceride and phosphatide fractions by chromatography on columns of silicic acid. The lipid from each reaction mixture was dissolved in chloroform and placed on a silicic acid column, approx. 8 cm. internal diam. (375 mg. of Mallinckrodt silicic acid, activated overnight at 110°, mixed with 185 mg. of Celite). Successive elution with 10 ml. portions of chloroform and methanol separated the glyceride and phosphatide fractions respectively. Each was reduced in volume under a current of air and transferred with 1 mg. of carrier triglyceride to an aluminium planchet for counting in a Geiger–Müller gas-flow counter.

Transferase activity was also determined on occasions by the release of CoA from palmitoyl-CoA by the method of Stansly (1955), by utilizing the different acid solubilities of these two substances.

Thin-layer chromatography. The general procedures used have been described (Kuhn & Lynen, 1965). Plates were developed with the following solvents: (1) *n*-hexane–ether–acetic acid–methanol (90:20:2:3, by vol.) (Brown & Johnston, 1962); (2) chloroform–methanol–aq. 30% (v/v) methylamine (65:25:8, by vol.) (Kuhn & Lynen, 1965); (3) di-isobutyl ketone–formic acid–pentan-1-ol (8:3:1, by vol.). A solution of 40 mg. of bromothymol blue in 100 ml. of 0·01 N-KOH was used as a spray to locate phosphatides. All solvents were of reagent grade.

Radioactivity counting techniques. (*a*) Lipids obtained from the radioactive transferase assay were quantitatively transferred to aluminium planchets, and the solvent was removed by heating under a lamp. Each planchet was counted twice for not less than 1000 counts each time in a Nuclear–Chicago gas-flow counter and the mean values were recorded. No corrections for self-absorption were made, owing to the small amounts of lipid involved.

(*b*) Radioactive scanning of thin-layer plates was performed by transferring sequential segments of thin layer to glass vials. After these had been dried at 40° on top of a warm oven, 4 ml. of scintillation fluid (0·3%, w/v, of solid 2,5-diphenyloxazole in toluene) was added to each and the vials were counted for 400 sec. in an IDL liquid-scintillation counter. Background values of about 100 counts/400 sec. were obtained by using a blank area of thin layer, and have been subtracted from the values given in this paper.

Preparation of chemicals. (*a*) L-[^{14}C]Glycerol 3-phosphate was prepared by phosphorylation of glycerol. A 0·05 mc sample of [^{14}C]glycerol (1·57 mc/m-mole), obtained from The Radiochemical Centre (Amersham, Bucks.), was dis-

solved in 0·5ml. of water together with 32μmoles of unlabelled glycerol, 40μmoles of ATP and 1·0ml. of glycine–hydrazine–Mg^{2+} buffer (Wieland, 1963) in a final volume of 2·5ml. at room temperature. The reaction was started by adding 0·05mg. of glycerokinase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany), and the reaction was followed by pipetting 0·02ml. portions of the reaction mixture into 0·06ml. of 0·1M-EDTA solution and assaying for glycerol 3-phosphate (Hohorst, 1963). Phosphorylation was complete in about 2hr. Then 0·5m-mole of carrier DL-glycerol 3-phosphate was added and, by following the procedure described for the preparation of L-glycerol 3[^{32}P]-phosphate (Kuhn & Lynen, 1965), was isolated as the barium salt and converted into the sodium salt. Radioactive purity was confirmed by paper electrophoresis in the presence of carrier glycerol and glycerol 3-phosphate. A migration time of 2hr. and a voltage gradient of 30v/cm. were used, the electrolyte being 0·05M-borax, pH9·2. After the paper was dry, glycerol was located by spraying with a solution containing 33ml. of 0·1M-boric acid, 26·7ml. of 0·1N-NaOH and 40ml. of 0·02% methyl red in 60% (v/v) ethanol, and drying at 90°. The reagent of Hanes & Isherwood (1949) served to locate the glycerol 3-phosphate. The paper was scanned for radioactivity in an end-window strip counter (IDL Tritomat 2029) and the radioactivity was found to coincide solely with the glycerol 3-phosphate.

(*b*) Egg lecithin was prepared by the method of Pangborn (1951).

(*c*) Phosphatidic acid was prepared from egg lecithin as described by Kates (1954) with a commercial preparation of phospholipase D (C. F. Boehringer und Soehne G.m.b.H.) instead of carrot chromoplasts.

(*d*) Diglyceride was obtained by incubating 20mg. of crude *Clostridium welchii* toxin (Wellcome Research Laboratories, Beckenham, Kent) with 60μmoles of egg lecithin dispersed in 30ml. of 0·33M-sodium borate buffer, pH7·6, at 30° overnight. The diglyceride, which was quantitatively released, was extracted several times into light petroleum (b.p. 60–80°), the combined extracts being washed three times with water and evaporated to dryness in a rotary-film evaporator. Analysis of the product gave an ester value corresponding to 98% of the theoretical value, and a glycerol content corresponding to 91% of the theoretical value, taking 830 as the molecular weight of the lecithin used. Thin-layer chromatography in solvent (1) showed the two spots expected of a mixture of 1,2- and 1,3-diglyceride isomers (Brown & Johnston, 1962), with the 1,2-isomer predominating.

(*e*) Monoglyceride was purified from grade C 1-monostearin (Calbiochem, Los Angeles, Calif., U.S.A.) by washing a chloroform solution successively with 2vol. of saturated $KHCO_3$ solution and, after acidification, with water. It was then precipitated by cooling, rapidly filtered off, dried over P_2O_5 and twice recrystallized from ether containing a little ethanol. The product melted at 73–74°, was homogeneous by thin-layer chromatography in solvent (1), and had 100% of the theoretical ester content and 103% *cis*-diol content (as shown by oxidation with lead tetraacetate).

(*f*) Palmitoyl-CoA was prepared by the method of Seubert (1959). An analysis of one such preparation was made by treatment with 0·8M-hydroxylamine, pH7, for 30min. at room temperature. An amount of solution that contained 100mμmoles of total CoA, as determined from the absorption of the adenine ring at 260mμ, released 96mμmoles of free thiol (estimated by the method of Ellman, 1959), 95mμmoles of acid-soluble CoA (estimated from the absorption at 260mμ after treatment with 5%, w/v, $HClO_4$ in the presence of 5mg. of bovine serum albumin) and 129mμmoles of hydroxamate (estimated from the absorption of the ferric chloride complex at 530mμ in 95% ethanol, compared with standard palmitoylhydroxamate). In this paper, stated concentrations of palmitoyl-CoA are based on the total CoA content.

Analysis. Esters were determined by the method of Stern & Shapiro (1953), glycerol was determined by the method of Wieland (1963) and L-glycerol 3-phosphate was determined by the method of Hohorst (1963).

RESULTS

Experiments with palmitoyl-CoA generated endogenously. Whole-tissue homogenates, acetone-dried powders and subcellular particulate fractions have all been found to catalyse the esterification of glycerol 3-phosphate when fortified with fatty acid, ATP, Mg^{2+} and CoA. The results of an experiment with an acetone-dried powder are shown in Fig. 1 and indicate that incorporation of [^{14}C]glycerol 3-phosphate into lipid proceeds linearly for over 1hr. and then ceases. The reason for the cessation of reaction at this point has not been investigated.

With acetone-dried powder as the source of enzyme, the effect of varying the concentrations of the reaction components was investigated.

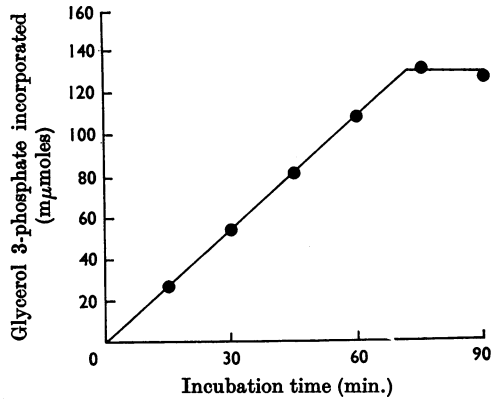

Fig. 1. Time-course of [^{14}C]glycerol 3-phosphate incorporation by a homogenate of acetone-dried powder of mammary gland, when palmitoyl-CoA is generated *in situ*. Reaction mixtures were incubated at 30°, and contained: acetone-dried powder (3·3mg.); sodium phosphate buffer, pH6·5 (50μmoles); serum albumin (5mg.); cysteine, pH7 (10μmoles); L-glycerol 3-phosphate (4μmoles, $8{\cdot}4\times10^4$ counts/min.); sodium palmitate (400mμmoles); ATP (2μmoles); CoA (100mμmoles); $MgCl_2$ (2μmoles). The final volume was 0·51ml. and the assay method that of Kornberg & Pricer (1953).

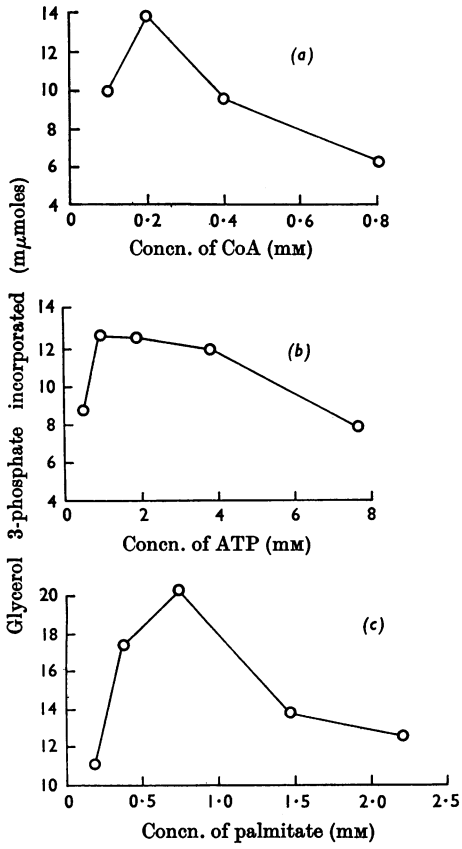

Fig. 2. (*a*) Activity of coupled thiokinase–transferase system as a function of CoA concentration. Reaction mixtures were incubated at 30° for 20min. and contained: acetone-dried powder (3·3mg.); serum albumin (5mg.); sodium phosphate buffer, pH6·5 (50μmoles); cysteine, pH7 (10μmoles); L-glycerol 3-phosphate (2μmoles, $8{\cdot}4 \times 10^4$counts/min.); sodium palmitate (500mμmoles); ATP (1μmole); CoA (as indicated); $MgCl_2$ (3μmoles). The final volume was 0·5ml. (*b*) Activity of coupled thiokinase–transferase system as a function of ATP concentration. Reagents were as for (*a*) except for: sodium palmitate (400mμmoles); CoA (100mμmoles); ATP (as indicated). The final volume was 0·525ml. Reaction mixtures were incubated for 15min. at 30°. (*c*) Activity of coupled thiokinase–transferase system as a function of palmitate concentration. Reagents were as for (*a*) except for: CoA (100mμmoles); sodium palmitate (as indicated). The final volume was 0·545ml. Reaction mixtures were incubated at 30° for 15min. All assays were by the method of Kornberg & Pricer (1953).

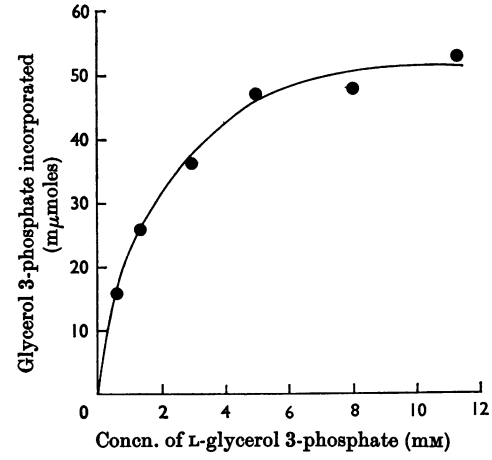

Fig. 3. Variation of transferase activity with concentration of L-glycerol 3-phosphate. Reaction mixtures were incubated at 30° for 20min. and contained: acetone-dried powder (3·3mg.); sodium phosphate buffer, pH6·5 (50μmoles); serum albumin (5mg.); cysteine, pH7 (10μmoles); L-glycerol 3-phosphate (as indicated); sodium palmitate (400mμmoles); ATP (1·5μmoles); CoA (100mμmoles); $MgCl_2$ (2μmoles). The final volume was 0·5ml. The assay method of Kornberg & Pricer (1953) was used.

Figs. 2(*a*), 2(*b*) and 2(*c*) show that there are optimum concentrations for CoA, ATP and palmitate, with higher concentrations leading to inhibitory effects. CoA at 0·25M also gave a 40% inhibition when added palmitoyl-CoA was used as substrate (results not shown). Magnesium chloride gave maximum rates at 3mM, and did not show inhibition at concentrations up to 8mM.

The concentration curve for glycerol 3-phosphate followed the classical hyperbolic form (Fig. 3), giving a straight-line reciprocal plot (Lineweaver & Burk, 1934) from which the K_m was determined to be 2·7mM.

Experiments with added palmitoyl-CoA. As shown by Kornberg & Pricer (1953) in liver and by Kuhn & Lynen (1965) in yeast no added cofactors are required for the direct esterification of glycerol 3-phosphate by palmitoyl-CoA (Fig. 4) other than the protective thiol shown to be required by Stansly (1955).

However, the experiment in Fig. 4 shows that linear incorporation of glycerol 3-phosphate does not proceed for more than 5–10min., and the reaction virtually ceases by 50min. Although at this time less than half the added palmitoyl-CoA can have been used to esterify glycerol 3-phosphate, the concentration of palmitoyl-CoA was probably limiting since, when this substrate is preincubated with the enzyme in the absence of glycerol 3-phosphate, and a 30min. reaction is then initiated by the addition of glycerol 3-phosphate, there is only 56% of the incorporation that is achieved in the absence of a preincubation. Moreover, in an experiment with a preparation of fresh tissue, the

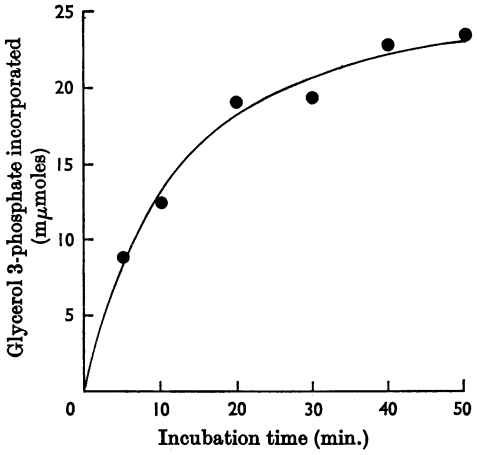

Fig. 4. Time-course of [^{14}C]glycerol 3-phosphate incorporation by homogenate of acetone-dried powder. Reaction mixtures were incubated at 30° and contained: acetone-dried powder (1·7mg.); sodium phosphate buffer, pH 6·5 (40μmoles); serum albumin (5mg.); cysteine, pH 7 (10μmoles); L-glycerol 3-phosphate (2μmoles, $8{\cdot}4\times10^4$ counts/min.); palmitoyl-CoA (130mμmoles). The final volume was 0·4ml. The assay method of Kornberg & Pricer (1953) was used.

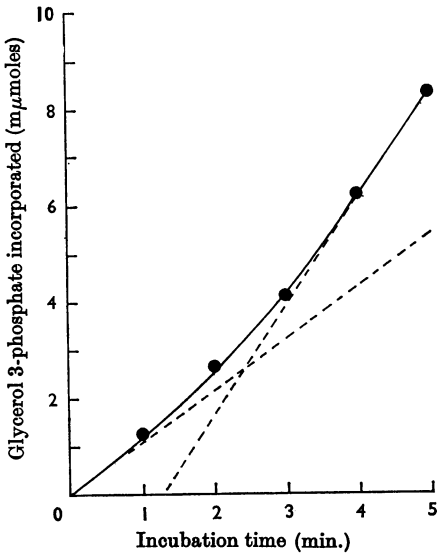

Fig. 5. Incorporation of [^{14}C]glycerol 3-phosphate over 5min. by a homogenate of acetone-dried powder. Incubation conditions were as described for Fig. 4.

incorporation of glycerol 3-phosphate during a 10min. incubation was nearly doubled by the addition of a second amount of palmitoyl-CoA followed by a further 10min. incubation. These results, together with the ability of a palmitoyl-CoA-regenerating system to cause linear incorporation for over 1hr., indicate the presence of an active deacylase in mammary-gland preparations. Such an enzyme has already been demonstrated in other tissues (Vignais & Zabin, 1958; Srere, Seubert & Lynen, 1959; Lands & Merkl, 1963).

Closer examination of the rate of incorporation of glycerol 3-phosphate during the first 5min. of incubation, under the conditions given in Fig. 4, revealed a slight lag (Fig. 5). Tangents drawn to the curve at 0 and 5min. showed a twofold increase of rate over this period. It was found that the lag occurred only when the reaction was initiated with palmitoyl-CoA, and that initiation with enzyme caused linear incorporation from the very start. The same phenomenon was revealed when Stansly's (1955) transferase assay was used, the amount of CoA released in 5min. being 50% greater when enzyme was used to start the reaction than when palmitoyl-CoA was used. This phenomenon, which may be due to a change in the micellar state of palmitoyl-CoA on dilution, was not further studied. In subsequent work, reactions were therefore started by addition of enzyme.

Fig. 6 shows that the incorporation of glycerol 3-phosphate in a short incubation period is proportional to the amount of enzyme added. When the rate of incorporation of glycerol 3-phosphate was studied as a function of palmitoyl-CoA concentration, an increase up to about 0·3mM-palmitoyl-CoA was found, but above this concentration severe inhibition occurred (Fig. 7). This property resembles that of the same enzyme in brain microsomes (Sanchez & Cleland, 1962) and in yeast particulate preparations (Kuhn & Lynen, 1965).

Intracellular distribution of transferase. Enzymes concerned in the synthesis of complex lipids have been found to occur largely or exclusively in the particulate fractions of tissue homogenates (Sedgwick & Hübscher, 1964). It was noted in early experiments that high-speed supernatant fluids prepared from guinea-pig mammary-gland homogenates by centrifugation in 0·25M-sucrose buffer for 30min. at 105000*g* were completely inactive in the assay method of Kornberg & Pricer (1953). Since both mitochondria and microsomes have been found to be important sites of complex lipid synthesis in other tissues, the distribution of transferase among these subcellular particles of mammary gland was studied by the fractionation procedure of Berthet & de Duve (1951). The mitochondrial and microsomal fractions obtained resembled corresponding fractions from liver in their visual appearance, but were not otherwise characterized. (The microsomal fraction contained less than 0·3% of the glucose 6-phosphatase activity

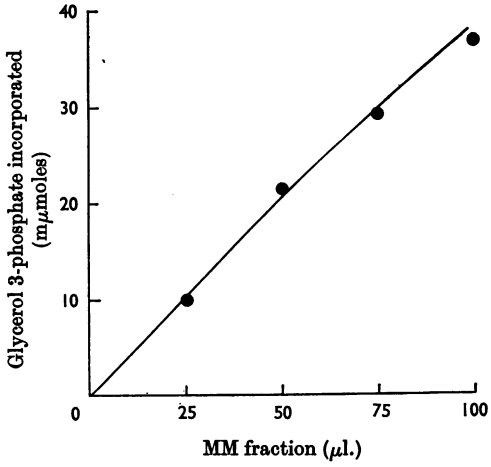

Fig. 6. Proportionality of [^{14}C]glycerol 3-phosphate incorporation to amount of enzyme. Reaction mixtures contained: MM preparation (as shown; 1ml. is equivalent to 0·33g. of fresh tissue); sodium phosphate buffer, pH 6·5 (40μmoles); serum albumin (5mg.); cysteine, pH7 (8μmoles); L-glycerol 3-phosphate (3μmoles, $8{\cdot}4\times10^4$ counts/min.); palmitoyl-CoA (156mμmoles). The final volume was 0·4ml. and incubations were performed at 30° for 6min. The assay method of Kornberg & Pricer (1953) was used.

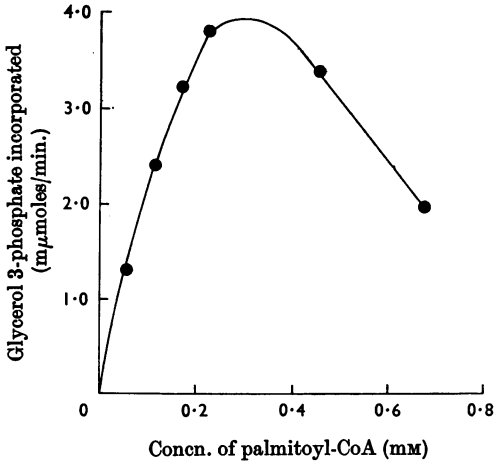

Fig. 7. Variation of transferase activity with palmitoyl-CoA concentration. Reaction mixtures were incubated at 37° for 5min. and contained: MM preparation (equivalent to 0·02g. of fresh tissue); sodium phosphate buffer, pH6·5 (50μmoles); serum albumin (5mg.); cysteine, pH7 (10μmoles); L-glycerol 3-phosphate ($3{\cdot}04$μmoles, $1{\cdot}31\times10^5$ counts/min.); palmitoyl-CoA (as indicated). The final volume was 0·5ml. The assay method of Kornberg & Pricer (1953) was used.

Table 1. *Transferase activity in subcellular particulate fractions of mammary tissue*

Values are expressed as μmoles of glycerol 3-phosphate esterified/min./subcellular fraction derived from 1g. of tissue.

Fraction	Activity	% of combined activity
Nuclei + cell debris	0·142	17
Mitochondria	0·055	7
Microsomes	0·622	76
Mitochondria + microsomes	0·675	83

of liver.) With the assay method of Kornberg & Pricer (1953), the results given in Table 1 were obtained. The bulk of the transferase activity occurred in the microsomal fraction, whereas the mitochondrial fraction contained a very little activity, which could represent microsomal contamination. About 17% of the combined activities was present in the nuclear + cell-debris fraction, but it is not known whether this represents true nuclear activity or the presence of grosser fragments of endothelial reticulum.

Characterization of reaction products. (*a*) Stoicheiometry of esterification reaction. In the experiment illustrated in Fig. 8, the time-course of the transferase reaction was followed by measuring, simultaneously in each reaction mixture, the release of CoA from palmitoyl-CoA (□) and the incorporation of [^{14}C]glycerol 3-phosphate into lipid (○) at different periods, with the assay methods of Stansly (1955) and of Kornberg & Pricer (1953) respectively. A control curve (■) shows the release of CoA due to deacylation of palmitoyl-CoA in the absence of glycerol 3-phosphate. The remaining curve (●) is the calculated difference between the curves for CoA, and is taken to represent CoA released through transferase action. From these results the ratios of CoA released, due to transferase action, to glycerol 3-phosphate incorporated are: 3min., 2·07; 6min., 2·10; 12min., 1·98; 20min., 1·75. The fall in the calculated curve after 12min. is presumably due to the invalidity of this subtraction at a time when palmitoyl-CoA has been used up in the presence of glycerol 3-phosphate but is still present in its absence. The possibility that the rate of hydrolysis of palmitoyl-CoA is itself affected by glycerol 3-phosphate has not been investigated, but is unlikely in view of the work of Stansly (1955).

The results therefore suggest that two palmitoyl radicals are esterified for each molecule of glycerol 3-phosphate incorporated, and there is no indication that monoacylated glycerol 3-phosphate accumulates as an intermediate at an early time in the

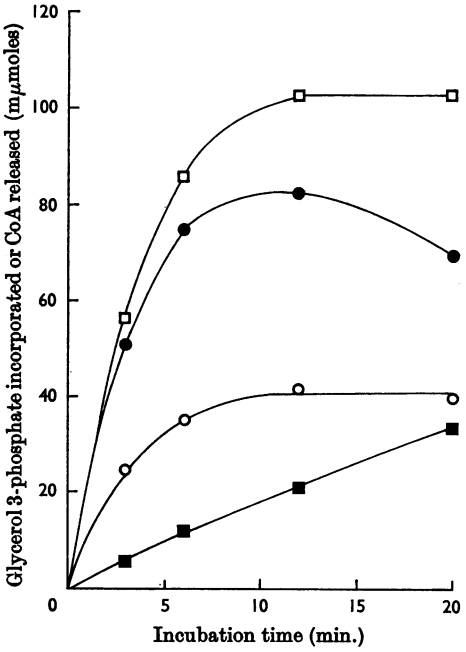

Fig. 8. Simultaneous time-course of incorporation of [^{14}C]glycerol 3-phosphate and release of CoA from palmitoyl-CoA. Reaction mixtures were incubated at 37° and contained: MM preparation (equivalent to 0·017g. of fresh tissue); sodium phosphate buffer, pH 6·5 (40μmoles); serum albumin (5mg.); cysteine, pH 7 (8μmoles); L-glycerol 3-phosphate (3·07μmoles, $6{\cdot}96\times10^4$ counts/min.); palmitoyl-CoA (156mμmoles). The final volume was 0·4ml. The assay methods of Kornberg & Pricer (1953) and of Stansly (1955) were used. Curves represent CoA released in the presence (□) and absence (■) of glycerol 3-phosphate, and the difference between these two (●), as well as the glycerol 3-phosphate incorporated into lipid in the presence of palmitoyl-CoA (○).

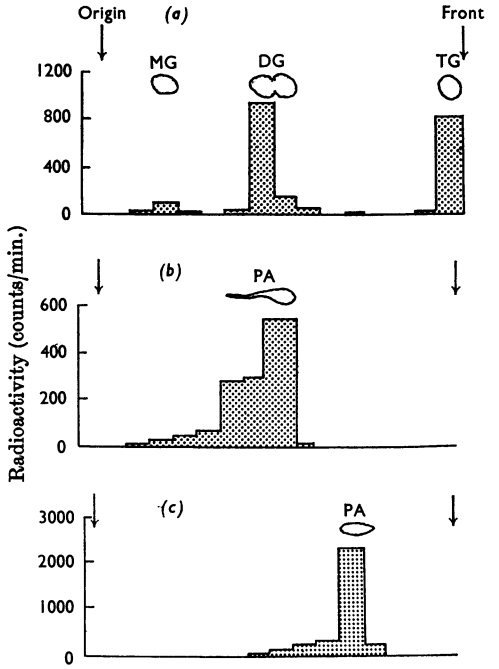

Fig. 9. Coincidence of radioactivity with position of known carrier lipid on thin-layer chromatograms. Arrows indicate the origin and front of each chromatogram, and radioactivity is shown in stippled areas. Known carrier lipids are indicated by the abbreviations: MG, monoglyceride; DG, diglyceride; TG, triglyceride; PA, phosphatidic acid. Chromatograms are as follows: (*a*) glycerides run on silica gel G in solvent (1); (*b*) phosphatides run on silica gel G in solvent (2); (c) phosphatides run on kieselguhr G in solvent (3).

reaction. The ratio is consistent with the formation of phosphatidic acid or diglyceride and inconsistent with the formation of triglyceride.

Fig. 8 shows that CoA release stops before all the added palmitoyl-CoA has been used up. The unused palmitoyl-CoA can be accounted for as the impurity in the preparation, which may be a diacyl-CoA (see the Methods section).

(*b*) Chromatographic properties of the reaction product. A reaction mixture containing MM fraction (derived from 0·04g. of tissue), sodium phosphate buffer, pH 6·5 (40μmoles), magnesium chloride (0·8μmole), serum albumin (5mg.), cysteine (4μmoles), [^{14}C]glycerol 3-phosphate (2·8μmoles, $1{\cdot}86\times10^5$ counts/min.), ATP (3μmoles) and palmitoyl-CoA (total of 0·437μmole) in a final volume of 0·5ml. was incubated at 30° for 45min., the palmitoyl-CoA being added in three equal portions at 0, 15 and 30min. to minimize the hydrolysis of this substrate. The magnesium chloride and ATP were intended to provide a regenerating system for palmitoyl-CoA. At the end of the incubation the total lipids, extracted by the method of Folch *et al.* (1957), had an activity of 9260counts/min., equivalent to the esterification of 142mμmoles of glycerol 3-phosphate. The lipids were dissolved in chloroform and separated into glyceride and phosphatide fractions on a small column of silicic acid (see the Methods section). Of the applied radioactivity 71% was recovered in the eluates; of this, 14% appeared as neutral lipid and 86% as phosphatide. In two other experiments, where an acetone-dried powder of the MM fraction was used as the enzyme source, the radioactivity recoveries from the column were 87% and 94%, and in each case only 6% of the eluted radioactivity appeared in the neutral fraction.

These radioactive fractions were further characterized by thin-layer chromatography. The

glyceride fraction was mixed with carrier monoglyceride, diglyceride and triglyceride and chromatographed on a layer of silica gel G, impregnated with 2′,7′-dichlorofluorescein (Brown & Johnston, 1962), in solvent (1). The finished plate was scanned for radioactivity as described in the Methods section. Fig. 9(*a*) shows that essentially all the radioactivity coincided with the di- and tri-glyceride markers, most of the 'diglyceride' radioactivity being in the position of the slower-running isomer (presumably the 1,2-isomer). Similarly, portions of the eluted phosphatide fractions were chromatographed with carrier phosphatidic acid on silica gel G in solvent (2) and on kieselguhr G in solvent (3). The carrier phosphatidic acid was located by treating with bromothymol blue and iodine vapour respectively, and the distribution of radioactivity was scanned as before. Figs. 9(*b*) and 9(*c*) show that the radioactivity coincided with phosphatidic acid in both systems. The 'tailing' seen in Fig. 9(*b*) is not a normal phenomenon with phosphatidic acid in this system, and in this instance only appeared when the reference phosphatide was mixed with the methanol eluate of the column. In other experiments a cleaner peak was obtained.

These experiments were twice repeated with acetone-dried powder of MM fraction as the enzyme. The results were very similar although with somewhat lower radioactivity in the glyceride fraction.

Factors affecting the nature of the reaction product. Since the major part of the reaction product was shown above to be phosphatidic acid, it was necessary to demonstrate that this enzymically formed intermediate could be further metabolized by mammary-gland preparations to give glycerides according to the scheme of Kennedy (1961). Reaction mixtures, containing [^{14}C]glycerol 3-phosphate as the one substrate and either palmitoyl-CoA or palmitate + ATP + CoA + magnesium chloride as the other, were therefore extracted at the end of the reaction by the method of Folch *et al.* (1957) and the lipids separated into phosphatide and glyceride fractions as described in the Methods section.

(*a*) Comparison of palmitate and oleate. Table 2 shows the results of an experiment in which sodium palmitate and oleate were the substrates; the total incorporation of glycerol 3-phosphate is given,

Table 2. *Effect of palmitate and oleate on the incorporation of* [^{14}C]*glycerol 3-phosphate into lipid fractions*

Each reaction mixture was incubated at 37° for 1 hr. and contained: microsomes (equivalent to 0·25 g. of tissue); ATP (2 μmoles); sodium phosphate buffer, pH 6·5 (50 μmoles); $MgCl_2$ (1 μmole); CoA (100 mμmoles); cysteine (8 μmoles); fatty acid (0·4 μmole of each when present singly, and 0·2 μmole of each when present together); serum albumin (3 mg.); L-glycerol 3-phosphate (0·833 μmole, $8·1 \times 10^4$ counts/min.). The final volume was 0·525 ml.

Incubation mixture...	1	2	3	4	5	6
Palmitate	+	+	−	−	+	+
Oleate	−	−	+	+	+	+
Total incorporation (mμmoles)	8	7	16	18	12	12
Incorporation into glycerides (%)	66	69	84	86	78	78
Incorporation into phosphatides (%)	34	31	16	15	22	23

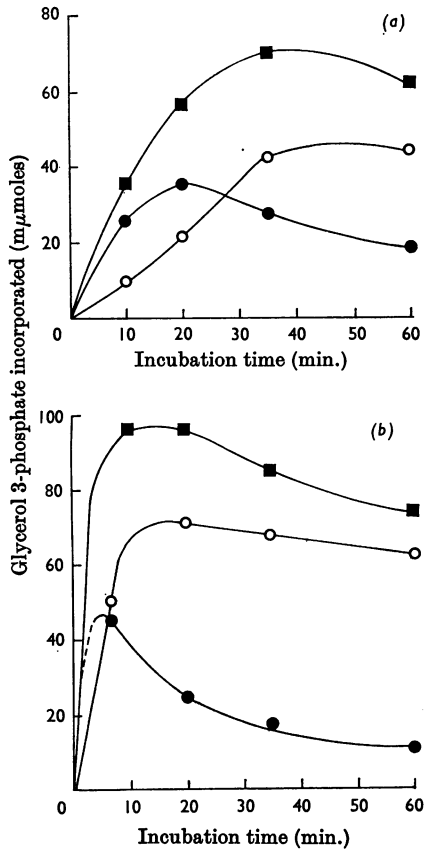

Fig. 10. Effect of incubation time and of concentration of MM fraction on incorporation of [^{14}C]glycerol 3-phosphate into lipid fractions, palmitoyl-CoA being generated *in situ*. Reaction mixtures were incubated at 37° for up to 1 hr. and contained: MM fraction [equivalent to 0·04 g. (*a*) or 0·24 g. (*b*) of tissue]; sodium phosphate buffer, pH 6·5 (50 μmoles); serum albumin (5 mg.); cysteine (8 μmoles); sodium palmitate (0·4 μmole); CoA (100 mμmoles); ATP (2 μmoles); L-glycerol 3-phosphate (1·7 μmoles, $1·08 \times 10^5$ counts/min.). The final volume was 0·525 ml. ■, Total lipid; ●, phosphatide; ○, glyceride.

together with its percentage distribution between phosphatides and glycerides. In contradistinction to the results given above of the analysis of reaction products, nearly 70% of the glycerol 3-phosphate esterified by palmitate was found in the glyceride fraction after 1 hr. incubation. In the presence of oleate a greater total esterification occurred, as well as a larger percentage conversion into glyceride. It is clear that, under these conditions, there is active dephosphorylation of phosphatidic acid formed enzymically.

(*b*) Variation of glycerol 3-phosphate concentration. An increase from 1·7 mM- to 6·3 mM-glycerol 3-phosphate gave a 40% increase in total incorporation, but did not change the distribution of radioactivity between phosphatides and glycerides. At lower concentrations, down to 0·4 mM, there was a slight rise in the percentage of label in the phosphatide fraction.

(*c*) Time-course of incorporation of glycerol 3-phosphate. Fig. 10 shows the time-course obtained at two different concentrations of enzyme. High enzyme concentration and increased incubation times promote transfer of label from phosphatide to glyceride. The decline of total incorporation towards the end of the incubation must be due to enzymic deacylation.

(*d*) Added palmitoyl-CoA as substrate. A completely different pattern of reaction products is obtained when added palmitoyl-CoA is used in place of that generated endogenously. Fig. 11 shows that most of the label now remains in the phosphatide fraction, especially at 'low' microsome concentrations.

(*e*) Effect of Mg^{2+}. Fig. 12 shows that, when palmitoyl-CoA is used as the fatty acyl substrate, the presence of magnesium chloride radically alters the distribution of radioactivity between phosphatide and glyceride fractions at the end of 1 hr. incubation. The magnitude of the effect is greatest at about 1 mM-magnesium chloride and decreases at higher concentrations. The variation in the total incorporated radioactivity could be explained by a partial lipolysis of the glyceride that has been formed, the rate increasing with the amount of newly formed glyceride.

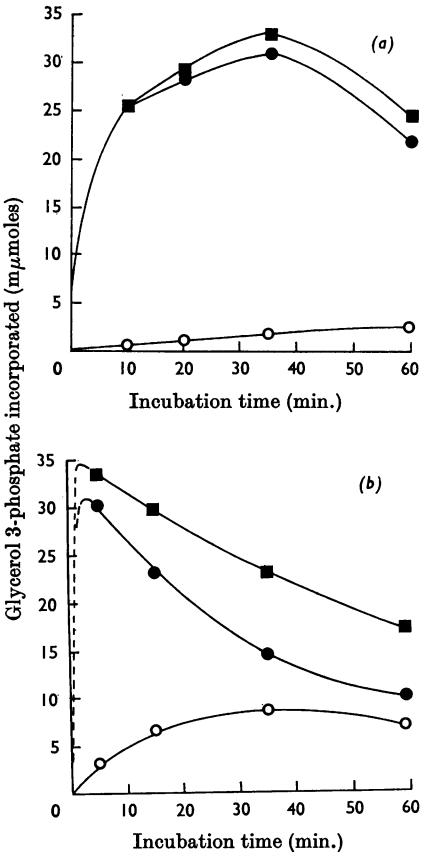

Fig. 11. Effect of microsome concentration on [^{14}C]-glycerol 3-phosphate incorporation into lipid fractions in the presence of added palmitoyl-CoA. Reaction mixtures were incubated at 37° for up to 1 hr. and contained: microsomes [equivalent to 0·04 g. (*a*) or 0·24 g. (*b*) of tissue]; sodium phosphate buffer, pH 6·5 (50 μmoles); serum albumin (3 mg.); cysteine (8 μmoles); palmitoyl-CoA (173 mμmoles); L-glycerol 3-phosphate (1·7 μmoles, 1·08 × 10^5 counts/min.). The final volume was 0·525 ml. ■, Total lipid; ●, phosphatide; ○, glyceride.

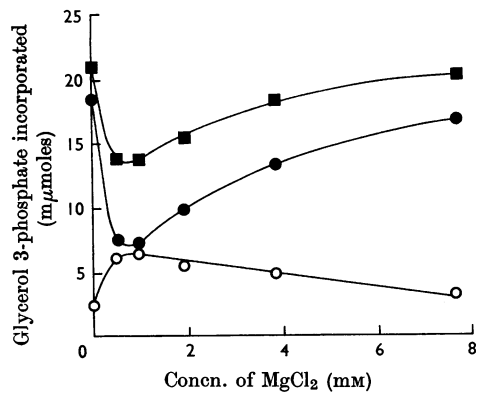

Fig. 12. Effect of Mg^{2+} concentration on the incorporation of [^{14}C]glycerol 3-phosphate into lipid fractions. Reaction mixtures were incubated for 1 hr. at 37° and contained: microsomes (equivalent to 0·04 g. of tissue); sodium phosphate buffer, pH 6·5 (50 μmoles); serum albumin (3 mg.); cysteine (8 μmoles); palmitoyl-CoA (216 mμmoles); $MgCl_2$ (as indicated); L-glycerol 3-phosphate (1·7 μmoles, 1·08 × 10^5 counts/min.). The final volume was 0·525 ml. ■, Total lipid; ●, phosphatide; ○, glyceride.

DISCUSSION

The results presented above demonstrate the presence, in lactating guinea-pig mammary gland, of fatty acid thiokinase, transferase and phosphatidate phosphatase activities. Thus three of the four enzymes on the Kennedy pathway of glyceride formation are present in this organ.

The nature of the reaction product of transferase activity has been shown to be diacyl glycerol 3-phosphate, as for the enzyme of liver microsomes (Kornberg & Pricer, 1953). This differs from findings in yeast, where neither stoicheiometry nor chromatographic evidence indicated a diacyl derivative as product (Kuhn & Lynen, 1965). Since it appears unlikely that the same enzyme catalytic site transfers both acyl radicals, it may be supposed either that the transferase has two functional sites, or else that two enzymes exist. In all tissues where the enzyme has been examined it has proved to be particulate, and solubilization will be required before the presence of two enzymes can be shown.

Since palmitoyl-CoA stands at a branch point in metabolism, and since the esterification of glycerol 3-phosphate represents the interaction of two major areas of metabolism, the transferase would seem to be a suitable point at which the pathway of fat synthesis might be regulated. The present work has shown that CoA, palmitoyl-CoA, palmitate and ATP can all inhibit the esterification of palmitate when used at high concentrations. Moreover, high concentrations of CoA and palmitoyl-CoA both inhibit the transferase directly. Since CoA is a product of the reaction, it might compete with palmitoyl-CoA for a common site on the enzyme surface. The inhibition by excess of palmitoyl-CoA might be a non-specific effect due to the detergent-like properties of this substance, and this interpretation is supported by the finding (Taketa & Pogell, 1966) that a variety of other enzymes are inhibited at low palmitoyl-CoA concentrations. On the other hand, there is some evidence that long-chain acyl radicals can be transferred from CoA to thiol groups on the yeast transferase before their further transfer to glycerol 3-phosphate. Palmitoyl-CoA might compete with 'palmitoyltransferase' as the immediate acyl donor in the second step of the acyl transfer. However, these substances are inhibitory only at concentrations greater than their measured tissue concentrations (Kuhn, 1967), so that their physiological role in this capacity remains doubtful.

The studies on the nature of the reaction products have shown that these depend greatly on the exact conditions of incubation. The results are best interpreted as being due to a varying activity of phosphatidate phosphatase. An increase in microsome concentration and in the duration of incubation naturally promote conversion of phosphatidic acid into glyceride. This was not significantly altered by the addition of mitochondria (results not shown). Oleate radicals appear to favour the dephosphorylation as compared with palmitate radicals. Unexpected was the finding that endogenous generation of palmitoyl-CoA permits greater conversion of phosphatide into glyceride than when synthetic palmitoyl-CoA is added. It is possible that phosphatidate phosphatase is irreversibly inhibited by the high initial concentration of palmitoyl-CoA.

The dephosphorylation of phosphatidic acid is promoted by magnesium chloride, though there is no absolute requirement for this salt. Several authors have reported Mg^{2+} effects on phosphatidate phosphatase activity, though the direction of the effect varies according to the source of enzyme (Smith, Weiss, & Kennedy, 1957; Hokin & Hokin, 1959; Agranoff, 1962; Coleman & Hübscher, 1962).

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