The Biosynthesis of Glycerides in Homogenates of Normal Lactating Mammary Gland and of Mammary Tumor Tissue of the Mouse*

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SUMMARY

A study of the biosynthesis of glycerides in lactating mammary gland and in a spontaneous tumor (DBA/2JAX) of the mouse is reported. Homogenates of both tissues, supplemented with yeast hexokinase to ensure maximum rates of glycolysis, were incubated under aerobic conditions with glucose-\textsuperscript{14}C (randomly labeled). Diglycerides and triglycerides labeled exclusively in the glycerol portion were formed at more rapid rates in tumor than in normal tissue. In view of the limited capacity of neoplastic tissue to synthesize fatty acids from precursors such as acetate (17), its high capacity to esterify preformed fatty acids may be an important factor in its economy. On incubation with mevalonate-\textsuperscript{2-14}C certain fractions of the fatty acids of tumor, but not of normal mammary tissue, were found to be labeled; cholesterol was, however, not radioactive in either tissue preparation.

MATERIALS AND METHODS

Mice bearing spontaneous tumors, mammary type (DBA/2JAX), were supplied by the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine; normal lactating mice with litters were obtained from a local breeder. The tumor-bearing mice were sacrificed in batches of ten to twenty when the size of the tumors approached 1 inch in diameter, the normal mice between the 10th and 19th day of lactation. The mammary glands and the tumors (free from necrotic tissue) were treated identically throughout the subsequent experimental procedures. The tissues were passed through a Latapie tissue mincer and washed with cold 0.154 M KCl to remove as much milk as possible from the normal tissue and blood and tissue debris from the tumors. The mince was then ground in a stainless steel homogenizer of the Potter type in a medium of 0.154 M KCl containing 0.024 M KHCO\textsubscript{3} and 0.02 M nicotinamide. The homogenate was squeezed through muslin, and its volume was adjusted by adding 0.154 M KCl to result in a tissue-to-volume ratio of 1 in 4 or 1 in 5 (dry wt., 50–60 mg/ml).

Reaction mixture.—The basal medium contained in addition to 0.154 M KCl: MgCl\textsubscript{2}, 0.01 M; nico-
tinamide, 0.02 M; ATP, 10⁻³ M; NAD, 10⁻⁴ M; NADP, 3 × 10⁻⁴ M; phosphate buffer, pH 7.4, 0.01 M; Tris(hydroxymethyl)aminomethane buffer, pH 7.4, 0.01 M; KHCO₃, 0.02 M; fumarate, 0.002 M. Further additions were made as stated in the text and in the tables. The volume per flask of the complete reaction mixture including 10–15 ml. of homogenate and substrates was 50 ml. The C¹⁴-labeled substances were prepared as 0.1 M solutions containing 100 μc/mmole and were added to the reaction mixture in a final concentration of 0.008 M for glucose-C¹⁴ (randomly labeled) and 1.67 × 10⁻⁶ M for mevalonate-2-C¹⁴. When the latter was the radioactive substrate, nonradioactive glucose (0.003 M) was also included. Yeast hexokinase (6 units/ml of reaction mixture) was added at the start of the incubation period. (For definition of hexokinase units see [24].) The flasks were flushed with a gas mixture of 5 per cent CO₂ and 95 per cent oxygen and were shaken for 60 min. in a water bath at 37°C.

Extraction of lipides.—At the end of the incubation period the flasks were chilled in iced water, their contents transferred to centrifuge tubes, and the particulate material was sedimented by high-speed centrifugation at 4°C. The sediment was extracted by homogenization in a mixture of chloroform and methanol (1:1 v/v); the supernatant aqueous solution was shaken with ethyl acetate. After evaporation of the ethyl acetate, the residue was taken up in chloroform-methanol mixture and added to the extract of the sediment material. The combined extracts were washed according to the method of Folch et al. (8) with precautions to minimize loss of material as outlined previously (25). The lipide extracts of tissue from at least 100 mice were accumulated for each set of experimental conditions. On the average, 100 tumor-bearing mice yielded 146 gm. of tumor tissue; the total dry weight of the homogenates prepared from the tumors was 4.6 gm.; the weight of the washed lipide extract was 8.82 gm. One hundred lactating mice yielded 235 gm. of mammary tissue; the dry weight of the homogenates was 14.8 gm., and 4.03 gm. of lipide material was recovered.

Solvent partition and column chromatography.—The washed lipide extracts were partitioned in separating funnels between hexane and 70 per cent methanol with seven transfers. The hexane fraction (60–70 per cent of the total) was taken to dryness under nitrogen, dissolved in chloroform, and passed through a silicic acid column to remove the phosphatides (4). The eluate was taken to dryness, redissolved in hexane and fractionated on a silicic acid column according to the scheme of Fillerup and Mead (6). On rechromatography of individual fractions on silicic acid columns, the sequence of solvent mixtures recommended by Barron and Hanahan (1) was employed.

Separation of glycerides and cholesterol.—The fractions eluted from the silicic acid columns by mixtures of ethyl ether and hexane (1:9 and 1:3 v/v) contained cholesterol, as indicated by the Liebermann-Burchard reaction. The cholesterol was separated by precipitation with digitonin, and the glycerides were recovered as previously described (23). Unesterified fatty acids were recovered mainly from the fractions eluted from the silicic acid columns by ethyl ether-hexane (1:9 v/v) by washing with dilute aqueous NaOH. The cholesterol-free glyceride fractions were rechromatographed on silicic acid columns until analysis by paper chromatography (16) and thin-layer chromatography (15) indicated that the diglycerides and triglycerides were free from contaminant material.

Purification of cholesterol.—The digitonin-insoluble material was cleaved by heating with pyridine (3). The sterol was passed through a column of aluminum oxide and was then brominated and regenerated according to Schwenk and Werthessen (20).

Analysis of fatty acids.—Methyl esters of the fatty acids were prepared by two methods: (a) transesterification by refluxing the glycerides for 2 hours in methanol containing 1 per cent sulfuric acid; (b) saponification of the glycerides in 0.5 N-ethanolic KOH, followed by esterification of the isolated fatty acids according to Radin et al. (19). Radin’s method was also employed in the preparation of methyl esters of the unesterified fatty acid fractions.

The methyl esters were separated by chromatography on silicic acid columns (10). Three fractions were obtained by elution with 3 per cent and 20 per cent ethyl ether in pentane and finally with 100 per cent methanol. Each fraction was then treated with mercuric acetate and further separated by thin-layer chromatography (15). The zones corresponding to the saturated, mono-, di-, and polyethenoic esters were recovered and eluted. After removal of the mercury from the addition compounds the esters were analyzed by gas chromatography. The gas chromatograph was equipped with a 6-foot column packed with 15 per cent ethylene glycol succinate on chromosorb W and an argon ionization detector. The argon flow rate was 60 ml/min.; the oven temperature was 175°C. Standard mixtures of methyl esters of fatty acids were obtained from the Metabolism Study Section, National Institutes of Health;
individual methyl esters were purchased from the Applied Science Laboratories, State College, Pa., or prepared in the laboratory.

**Determination of glycerol.**—The aqueous residue remaining after the extraction by ethyl ether of the transesterification or saponification mixture was deionized by passing through a mixed-bed ion exchange resin and concentrated by lyophilization. Portions of the solution were taken for the estimation of the glycerol content by the periodate chromotropic acid method (14) and for the determination of its radioactivity.

**TABLE 1**

<table>
<thead>
<tr>
<th>Hexokinase (units)</th>
<th>(-Q_{02})</th>
<th>Labeled glucose (mumoles/hr) appearing in respiratory CO(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.4</td>
<td>84</td>
</tr>
<tr>
<td>18</td>
<td>7.2</td>
<td>200</td>
</tr>
<tr>
<td>36</td>
<td>7.8</td>
<td>615</td>
</tr>
<tr>
<td>72</td>
<td>8.7</td>
<td>280</td>
</tr>
</tbody>
</table>

**RESULTS**

**Hexokinase and respiratory activity of homogenates.**—It was previously reported that, in order to achieve maximal rates of glycolysis, yeast hexokinase had to be added to homogenates of the lactating mammary gland of the rat, rabbit, and guinea pig (21, 22). In the present study as well, the addition of hexokinase accelerated the rate of glycolysis of homogenates of lactating mammary tissue and of tumor tissue of the mouse. Under aerobic conditions supplementation with hexokinase resulted in an increase of the rate of oxygen consumption and an acceleration of the appearance of glucose-carbon in the respiratory CO\(_2\) (Table 1). Under the experimental conditions chosen for substrate incorporation studies (see “Methods”), the addition of 6 units of hexokinase per ml. of reaction mixture was found to be adequate. No measurable radioactivity appeared in the respiratory CO\(_2\) when mevalonate-2-C\(_{14}\) was the radioactive substrate.

**C\(_{14}\) labeling of glycerides in normal and tumor tissue.**—The pattern of labeling of the lipide fractions of tissues incubated with glucose-C\(_{14}\) (randomly labeled) showed that the fractions recovered from silicic acid columns expected to contain glycerides were the most intensely labeled (Table 2). Removal of the accompanying cholesterol and repeated chromatographic purification of the remaining material (see “Methods”) yielded pure diglyceride and triglyceride fractions. The specific activity of the glycerides of tumor tissue greatly exceeded that of the glycerides of normal mammary glands; in normal mammary tissue the specific activity of the diglycerides was predominant, whereas in tumor tissue the specific activities of both di- and triglycerides were of a comparably high level. On saponification and isolation of the fatty acids and the glycerol portions the radioactivity was found to reside exclusively in the glycerol (Table 3); the fatty acids were not radioactive. The purified diglyceride and triglyceride fractions of normal tissue which had been incubated with mevalonate-2-C\(_{14}\) were not radioactive; the corresponding fractions from tumor tissue contained a small amount of radioactivity which was found to be associated with their fatty acids.
(Table 4). The unesterified fatty acids of the tissues incubated with glucose-C\(^{14}\) were also devoid of measurable radioactivity, whereas those from tumor tissue, but not normal tissue, which had been incubated with mevalonate-\(^{2-14}\)C were slightly radioactive.

**Fractionation of radioactive fatty acid mixtures.**—Because of the complexity of the fatty acid mixtures shown by gas chromatography, no attempt was made at this stage to search for qualitative or quantitative differences in the fatty acid composition of the nonradioactive glycerides and unesterified fatty acids. Efforts were directed at localizing the radioactive components of the fatty acid mixtures derived from tumor tissue incubated with mevalonate-\(^{2-14}\)C. On chromatography of the methyl esters on silicic acid columns (10) radioactive material was eluted by 20 per cent ethyl ether in pentane and by 100 per cent methanol (Table 5). On further separation by thin-layer chromatography (15) each of these fractions into saturated and unsaturated groups of fatty acids, the radioactive material was located in the fractions designated as “monoethenoic” fatty acids but was absent from the saturated and polyethenoic subgroups. Gas chromatographic analysis showed that, although the saturated fatty acid fraction was free from unsaturated fatty acids, the allegedly unsaturated groups contained both saturated and unsaturated fatty acids—e.g., fraction 2 (Table 5) contained: myristic acid, 1.8 per cent; palmitic acid, 27.4 per cent; palmitoleic acid, 9.2 per cent; stearic acid, 18.8 per cent; oleic acid, 23.8 per cent; linoleic acid, 16.1 per cent; unidentified, 2.9 per cent. Since the saturated fractions, containing myristic, palmitic and stearic acids, were not radioactive, it may be assumed that either the unsaturated fatty acids or unidentified minor components, possibly of short chain length, contained the C\(^{14}\) label. The analysis by identical procedures of the fatty acids of normal lactating mammary tissue incubated with mevalonate-\(^{2-14}\)C did not uncover radioactive fractions.

**Cholesterol.**—After rigid purification (see “Methods”), cholesterol (m.p., 149°) isolated from both normal and tumor tissue, whether incubated with glucose-C\(^{14}\) or mevalonate-\(^{2-14}\)C, did not contain measurable radioactivity.

**DISCUSSION**

**Hexokinase activity in homogenates of normal and tumor tissues.**—It has long been known that, in contrast to the high glycolytic capacity of tumor tissues, hexokinase activity in homogenates of normal and tumor tissues is deficient and, as shown by Meyerhof, may be restored by the addition of ATPase inhibitors or of yeast hexokinase. Meyerhof and Wilson (18) attributed the decline of hexokinase activity in tumor extracts either to an instability inherent in the various species of hexokinase in different tissues or to its separation from structural elements and subsequent dilution in the process of preparation of the homogenate. Terner (21) compared the rates of anaerobic glycolysis of brain and mammary gland homogenates from the same rabbit. Where-
as the brain homogenate showed full glycolytic activity and did not respond to added yeast hexokinase, the rate of glycolysis in the mammary homogenate could be increased by as much as 100 per cent and at the same time stabilized by the addition of the enzyme in optimal amounts. Although the beneficial effect of supplementing the homogenates of both lactating mammary gland and of mammary tumor of the mouse might be considered the result of the restoration of an enzymatic activity which may have been present in vivo, but diminished during the preparative procedure, it would not be justifiable to draw conclusions as to the level of hexokinase activity in the original normal and tumor tissues employed in the present study. The purpose of the addition of the hexokinase was to raise its activity to a nearly optimal level in an attempt to eliminate from both tissue preparations a rate-limiting factor, the possible variability of which might make it impossible to make a comparison of the rates of the subsequent reactions. That caution has to be exercised in adding hexokinase is shown by the inhibitory effect of larger than optimal amounts of the added enzyme (Table 1).

Glyceride synthesis in normal and tumor tissues. —The observation that, under optimum conditions of glycolysis, glyceride-glycerol was produced from glucose more rapidly in tumor than in normal tissue homogenates suggests a higher activity in tumor tissue of a-glycerophosphate dehydrogenase corresponding to its high glycolytic activity. The labeling of glycerol also provides a measure of the rate of turnover of glycerides shown by the liberation and re-esterification of endogenous glyceride-fatty acids. There may be some uncertainty in comparing the rates of reactions calculated, based on measurement of rates of isotope incorporation in different tissues, in that the specific activity of a labeled product depends not only upon enzymic activities but also upon the relative amounts in the tissues of preformed materials which may result in isotope dilution in varying degrees. However, since the lipide content of lactating mammary tissue was 21.8 per cent of the total dry weight and that of tumor tissue was 15.2 per cent, the lipide pool of normal tissue exceeded that of tumor tissue by not more than 50 per cent. Dilution by the larger lipide pool of lactating mammary tissue will, therefore, not account for the very large differences in specific activity of the di- and triglycerides of the two tissues, and the application of a corresponding correction factor (ca. 1.5) will not materially affect the data reported in terms of specific activities actually measured. Also, the observation that, under similar experimental conditions, the maximum rate of labeling of di- and triglycerides had been attained in tumor tissue, whereas in normal tissue the specific activity of the triglycerides was still behind that of their diglyceride-precursors (see Table 3), supports the conclusion that the rate of glyceride formation in mammary tumor tissue exceeded that of the normal mammary gland.

The presence of unesterified fatty acids in the lipide extracts of all tissue preparations suggests that the rate of breakdown of the glycerides may have exceeded the rate of their biosynthesis. Thus, the rate of labeled glyceride formation was probably not limited by the amount of free fatty acids present in the homogenates.

Fatty acid synthesis in normal and tumor tissue. —The relative inability of tumor tissue preparations to synthesize fatty acids from acetate-1-C\(^ {14} \) in vitro was reported by Medes et al. (17) and confirmed by Gore and Popjak (13). Lactating mammary tissue, on the other hand, affords an excellent preparation for the study of fatty acid synthesis in vitro (9). In the present experiments acetate was not employed as a substrate, glucose-C\(^ {14} \) being preferred, since it labeled the glycerides of both tissues without being incorporated into their fatty acids. Mevalonate-carbon, however, appeared in the fatty acids of tumor tissue. Other workers who have found mevalonate to be a precursor of fatty acids (5, 11, 12), assumed that the labeled products were higher acids of the type of farnesoic acid. Although further work is required to identify the labeled components, it is of interest that in the present study tumor tissue, but not normal mammary tissue, utilized mevalonate-carbon in this way, whereas its incorporation into cholesterol was not observed in either tissue. According to a recent report by Fluck and Pritham (7), the presence of fatty acids inhibited the conversion of mevalonate-2-C\(^ {14} \) to cholesterol in rat liver homogenates. It is possible that this pathway was also inhibited by the fatty acids present in the mammary and tumor tissues.

Synthesis of glycerol in mammary tissue. —Folley and McNaught (9) discussed the still unresolved question of the site of origin of the glycerol of milk fat. Although in intact animals milk glycerol had been shown to contain the label derived from injected glucose-C\(^ {14} \), the perfused cow udder and slices of rat and sheep mammary tissue had so far failed to incorporate glucose-carbon into glyceride-glycerol. The present experiments appear to fill a gap of information by providing clear evidence of the formation of C\(^ {14} \)-labeled glyceride-glycerol in homogenates of normal and neoplastic mammary tissue incubated with glucose-C\(^ {14} \).
Glycolysis and lipide metabolism of normal mammary gland and tumor tissue.—It appears that a powerful glycolytic system in the tumor tissue may confer upon it some metabolic advantage over normal tissue, not only by generating metabolic energy but also by favoring the deposition of glyceride reserves. If, as found by Medes et al. (17), tumor tissue is limited in its capacity to synthesize fatty acids and must obtain them from other organs via the circulatory system, the capacity of tumor tissue to hold those fatty acids in the form of glycerides may enable the tumor to maintain its lipide reserves while preserving an amount of energy equivalent to that expended by other tissues in the biosynthesis of their fatty acids. That energy may be available to support other reactions which may contribute to the unusual properties of tumor tissue.

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