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The Effect of Pituitary Growth Hormone on Phospholipid Synthesis

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Amongst the many metabolic effects described in rats treated with growth hormone are the marked modifications in fat metabolism such as an increased rate of fatty acid oxidation (Greenbaum & McLean, 1953*a*) and a decreased rate of fatty acid synthesis (Greenbaum & Glascock, 1957). Phospholipid synthesis is also affected, although previous studies on the effect of the hormone on the rate of phospholipid synthesis in the livers of rats have led to some discordant results. Greenbaum, Graymore & Slater (1957) have reported a substantial increase in the rate of incorporation of injected [^{32}P]orthophosphate into the liver phospholipids of growth-hormone-treated animals, whereas Greenbaum & Glascock (1957) found a threefold decrease in the rate of incorporation of ^{14}C from [1-^{14}C]acetate into phospholipids of similarly treated animals. It appeared desirable to investigate this discrepancy more closely and to attempt to obtain further information on the nature of the action of the hormone which results in the lower incorporation rate of ^{14}C.

To this end the decreased rate of ^{14}C incorporation into phospholipid has been examined in more detail in an attempt to establish at which steps in the pathway of biosynthesis of phospholipids this decrease occurred and how far it is explicable in terms of isotope dilution caused by the appearance of larger quantities of intermediates of the synthetic pathway in the livers of growth-hormone-treated animals. By use of [^{14}C]acetate and [^{14}C]-palmitate as precursors it has been established that one of the spans affected by the hormone is acetate → long-chain acyl-coenzyme A. A second factor leading to the decrease in radioactive labelling is tentatively identified as a dilution at the level of diglyceride, which appears to be present in greater quantities in the livers of growth-hormone-treated rats than in control livers.

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METHODS

Animals. Adult female rats of the hooded Norwegian strain aged about 3–4 months and weighing 170–200 g. were fed *ad lib.* on diet 41 of Bruce & Parkes (1946).

Growth hormone. A twice-recrystallized preparation of anterior-pituitary growth hormone prepared from ox pituitaries by the method of Wilhelmi (1955) was used. Rats were injected with 1 mg. of the hormone (in 1 ml. of water) and killed 6 hr. later. This time was chosen as it had been shown by Greenbaum & Glascock (1957) that there is a considerable inhibition of lipogenesis in the livers of growth-hormone-treated rats at this time-interval after the injection. Controls were injected with 1 ml. of 0·9% sodium chloride soln. After killing, the livers were rapidly removed, rinsed and placed in ice-cold Ringer bicarbonate (Umbreit, Burris & Stauffer, 1945). In all experiments in which incorporation of the two precursors has been compared, the liver slices used were always taken from the same liver.

Incubation and isolation procedures. For some of the experiments on the incorporation of acetate and palmitate into fatty acids, phospholipids and neutral fats, 400 mg. of slices were cut from the livers with a Stadie–Riggs (1944) microtome and incubated in 5 ml. of Krebs–Ringer bicarbonate in the presence of either [1-^{14}C]acetate or [1-^{14}C]palmitate at a final concentration of 0·1 μc/ml. The [1-^{14}C]acetate had a specific activity of 4 mc/m-mole and the [1-^{14}C]palmitate 2 mc/m-mole. Both substances were obtained from The Radiochemical Centre, Amersham, Bucks. No carrier was added. The flasks were gassed with $CO_2 + O_2$ (5:95) and incubated at 37° for 3 hr. At the end of this time the slices were collected by centrifuging, washed twice with ice-cold 0·9% sodium chloride soln. and then suspended and homogenized in 5 ml. of 5% trichloroacetic acid in an all-glass Potter–Elvehjem (1936) homogenizer. After centrifuging, the residue was again suspended in 5 ml. of 5% trichloroacetic acid and the extracts were discarded.

The residue was then suspended in 1 ml. of water, 5 ml. of absolute ethanol was added and the whole stood overnight at room temperature. The extract was then separated by centrifuging and the residue extracted twice with ethanol–ether (3:1) and once with ether. The solvents were removed by evaporation and the residue was extracted with light petroleum (4 × 3 ml.). From the combined extracts the phospholipids were separated as their magnesium complexes and purified by redissolving in 0·6 ml. of chloroform and precipitating again with acetone (Greenbaum & Glascock, 1957). The phospholipid-free extract was used as a source of the neutral lipids, the only purification procedure being the removal of the labelled palmitic acid in the experiments on the incorporation of palmitate into neutral lipids. For this purification the solution of neutral lipids in acetone was evaporated to dryness, the residue dissolved in light petroleum (b.p. 40–60°) and shaken in a separating funnel with cold aqueous 0·02 N-potassium hydroxide solution. This procedure was repeated twice and appeared to remove adequately any free palmitic acid.

For the experiments on the conversion of palmitic acid into palmitoyl-coenzyme A (CoA), liver homogenates (1 g. of liver with 4 ml. of 0·9% sodium chloride soln.) were used. A portion (1 ml.) of the homogenate was incubated in 4 ml. of water containing 7·5 m-moles of hydroxylamine hydrochloride (freshly neutralized to pH 7·4 by the addition of ammonia); 154 μmoles of sodium chloride and 0·025 μmole of [1-^{14}C]palmitate ($1{\cdot}23 \times 10^6$ counts/min.). After incubating for 2 hr. at 37°, 5 ml. of 10% perchloric acid was added to each flask, the whole then centrifuged, and the liquid phase removed with a capillary pipette. The residue was washed twice with portions (5 ml.) of 5% perchloric acid and extracted three times with portions (3 ml.) of the reagent A of Hill (1947) diluted 1:100 with methanol. Unchanged palmitic acid was separated from the ferric hydroxamate by repeatedly shaking the methanolic solution with heptane according to the method of Kornberg & Pricer (1953). Samples of the methanolic solution were plated and counted as described below.

For the experiments on the conversion of phosphatidic acid into diglyceride, 1:20 liver homogenates in 0·05 M-sodium maleate buffer, pH 6·4 (Smith, Weiss & Kennedy, 1957), were prepared. The reaction flasks contained 200 μmoles of sodium maleate buffer, pH 6·4, 14 μmoles of phosphatidic acid and homogenate equivalent to 200 mg. of rat liver. Incubation was for 1 hr. at 37° and the reaction was then stopped by the addition of 4 ml. of 10% perchloric acid. Inorganic phosphate was determined on samples of the supernatant after centrifuging and the figures so obtained were corrected for the blank values obtained in the flasks incubated without substrate (Smith *et al.* 1957).

For measurement of the rate of incorporation of [^{32}P]-orthophosphate into phosphatidic acids, liver mitochondria (in 0·25 M-sucrose) prepared by the procedure of Hogeboom, Schneider & Pallade (1948) were used. The mitochondria were incubated at 37° for 1 hr. in a medium identical with that described by Kennedy (1953) except that adenosine diphosphate (ADP) was used in place of adenosine monophosphate (AMP) and no glycerol was added. After incubation the phosphatides were extracted and purified as described by Kennedy (1953) and plated and counted as described below.

In the experiments on the incorporation of phosphorylcholine into phospholipids, the liver preparation was a particulate fraction of a sucrose homogenate prepared according to Kennedy & Weiss (1956). This fraction includes particles smaller than mitochondria. Each flask contained 100 μmoles of sodium phosphate buffer, pH 7·4, 10 μmoles of adenosine triphosphate (ATP), 1·5 μmoles of cytidine triphosphate, 20 μmoles of magnesium chloride, [^{32}P]phosphorylcholine in amounts between 4 and 20 μmoles and 'liver particles' derived from 0·4 g. of liver. ATP (1·5 μmoles) and 0·03 μmole of cytidine triphosphate were added every 12 min. during the course of the experiment. Incubation was for 1 hr. at 37°. After incubation the phospholipids were extracted and purified according to Kennedy & Weiss (1956) and counted as described below.

For the experiment on the labelling and chromatographic separation of the neutral lipids, the combined livers of four experimental or four control rats were used. The livers were rapidly weighed, chopped finely with scissors and finally reduced to a brei by pressing through the nozzle of a plastic syringe. The brei thus obtained was incubated for 3 hr. at 37° as described above for slices except that all components were increased in proportion to the weight of the liver. The substrates used were [1-^{14}C]acetate and [^{32}P]orthophosphate, present together. After incubation the lipids were extracted as before. The phospholipids were separated by precipitation with cold acetone and dried under vacuum. They were then extracted with ethanol to yield two fractions: the ethanol-soluble (lecithins) and ethanol-insoluble (kephalins). The acetone solution containing neutral lipids was evaporated to dryness at room temperature and the residue extracted with *n*-hexane (b.p. 67–70°). The residue from this extraction was set aside. The clear hexane solution was applied to a silicic acid column (11 cm. × 3·7 cm.) and the separation of neutral lipids carried out as described by Barron & Hanahan (1958). Fractions (15 ml.) were collected, evaporated to dryness and weighed and counted; the volume plated was adjusted so that the weight of lipid on the planchet did not exceed 1·5 mg. The fractions containing the triglycerides and free fatty acids were combined and dissolved in light petroleum (b.p. 40–60°), and the triglycerides separated from the free fatty acids by the method of Borgström (1952).

Chemical methods

Preparation of phosphatidic acids. The starting material for this preparation was lecithin prepared by the method of Hanahan, Turner & Jayko (1951) with alumina columns. Each 50 ml. fraction was analysed for phosphorus and choline (Glick, 1944) and only those fractions with a phosphorus to choline ratio of 1 were used for the next step. The purified lecithin was incubated for 2 hr. with carrot chloroplasts at 25°, in the presence of ether, as described by Kates (1955). The sodium salts of the phosphatidic acids were purified by repeated precipitation from ether solution by the addition of acetone and finally dried under vacuum. An aqueous suspension was prepared by the addition of water to the sodium salts of the phosphatidic acids and the suspension homogenized in a tight-fitting Potter–Elvehjem (1936) homogenizer until a very fine suspension was obtained.

Preparation of [^{32}P]*phosphorylcholine.* [^{32}P]Phosphorylcholine was prepared according to the method of Riley (1944) and purified as described by Plimmer & Burch (1937). Calcium ions were removed by passing the solution

through an IR-120 (K^+ cycle) column. The material prepared in this fashion had a specific activity of 25–45 $\times 10^3$ counts/min./μmole.

Phosphorus was estimated by the method of Allen (1940).

Esterified fatty acids were estimated by the method of Stern & Shapiro (1953).

Unesterified fatty acids were determined as described by Entenman (1957) after purification (Fairbairn, 1945).

Counting of radioactive samples. All samples were plated at infinite thinness on nickel planchet either 1·6 or 2·5 cm. in diam. The planchets were counted in a Tracerlab SC16 windowless counter with a conventional scaler. When both ^{32}P and ^{14}C were present simultaneously, these were differentially counted by interposing a thin aluminium disk, which effectively absorbed all the ^{14}C counts. The ^{32}P counts were corrected for absorption in the aluminium disk.

Calculations. The results given in the tables for experiments in liver slices always refer to the total counts present in 400 mg. of slices. The presence of variable quantities of fat mobilized from the depots in the livers of the treated animals makes this figure more useful than the more usual 'specific activities'.

RESULTS

Incorporation of [1-^{14}C]acetate and [1-^{14}C]palmitate into liver neutral lipids and phospholipids. The effect of pituitary growth hormone on the rate of incorporation of [1-^{14}C]acetate into the neutral lipids and phospholipids of rat-liver slices is shown in Table 1. In all six experiments shown in Table 1, the rate of incorporation of the precursor into phospholipids is decreased, the factor varying between three- and ten-fold. An inhibition of the rate of incorporation into the neutral lipids of the same slices is also observed although this is not so great as that observed for the phospholipids.

Table 2 shows similar experiments in which the rates of incorporation of [1-^{14}C]acetate and [1-^{14}C]-palmitate into neutral lipids and phospholipids are compared with slices from the same liver for both substrates. It will be seen that the hormone also inhibits the incorporation of [1-^{14}C]palmitate into the lipid fractions although it should be noted that the effect on the incorporation of the labelled palmitate is less than that observed with acetate.

Activation of palmitic acid. Since palmitic acid has to be activated before it enters the pathway of phospholipid or neutral-lipid synthesis, the effect of growth hormone on the rate of the activation process was measured. The results of these experiments are shown in Table 3. It is apparent that there is no difference in the rate at which the

Table 1. *Effect of pituitary growth hormone on the incorporation of acetate [1-^{14}C]into the 'neutral fats' and phospholipids of rat-liver slices*

Each flask contained 400 mg. of slices in 5 ml. of Krebs–Ringer bicarbonate. Sodium [1-^{14}C]acetate was added to a final concentration of 0·1 μc/ml. Gas phase, $CO_2 + O_2$ (5:95). Temp. 37°. Time 3 hr.

	Phospholipids (counts*/100 sec./flask)			Neutral fats (counts*/100 sec./flask)		
Expt.	Control	Hormone-treated	Treated / Control	Control	Hormone-treated	Treated / Control
1	3 600	912	0·25	6 178	5 061	0·82
2	7 338	2 748	0·37	15 988	10 710	0·67
3	7 716	2 148	0·28	7 492	5 445	0·73
4	4 608	1 524	0·33	7 944	5 639	0·71
5	8 435	1 765	0·21	15 472	4 420	0·29
6	7 644	849	0·11	15 200	3 193	0·21

* Total counts present in 400 mg. of slices.

Table 2. *Effect of pituitary growth hormone on the incorporation of [1-^{14}C]acetate and [1-^{14}C]palmitate into phospholipids and 'neutral fats' of rat-liver slices*

Each flask contained 400 mg. of slices in 5 ml. of Krebs–Ringer bicarbonate. [1-^{14}C]Acetate or [1-^{14}C]palmitate was added to a final concentration of 0·1 μc/ml. Gas phase, $CO_2 + O_2$ (5:95). Temp. 37°. Time 3 hr.

		Phospholipids (counts*/100 sec./flask)			Neutral fats (counts*/100 sec./flask)		
Expt.	Precursor	Control	Hormone-treated	Treated / Control	Control	Hormone-treated	Treated / Control
1	[1-^{14}C]Acetate	19 720	2 683	0·14	69 520	38 083	0·55
	[1-^{14}C]Palmitate	9 360	3 654	0·39	85 420	29 250	0·34
2	[1-^{14}C]Acetate	12 800	3 728	0·29	58 630	47 490	0·81
	[1-^{14}C]Palmitate	11 784	5 470	0·46	79 520	56 460	0·71

* Total counts present in 400 mg. of slices.

Table 3. *Effect of pituitary growth hormone on the formation of palmitoyl-coenzyme A*

Each flask contained 7·5 m-moles of hydroxylamine hydrochloride adjusted to pH 7·4, 154 μmoles of NaCl, 0·025 μmole of sodium [1-^{14}C]palmitate ($1{\cdot}23 \times 10^6$ counts/min./μmole) and rat-liver homogenate equivalent to 0·2 g. of liver, in a total volume of 5 ml. Gas phase, air. Temp. 37°. Time 2 hr.

	Radioactivity of ferric hydroxamate complex (counts/100 sec./flask)		
Expt.	Control	Hormone-treated	Treated / Control
1	13 690	13 070	0·95
2	13 006	9 750	0·75
3	10 840	10 470	0·93

Table 4. *Effect of pituitary growth hormone on the incorporation of [^{32}P]orthophosphate into the phosphatidic acids of rat-liver mitochondria*

Each flask contained 250 μmoles of sucrose, 15 μmoles of $MgCl_2$, 3 μmoles of ADP, 50 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol buffer, pH 7·4, 100 μmoles of sodium succinate, 0·3 μc of [^{32}P]orthophosphate and mitochondria from 0·7 g. of liver. Final volume 3 ml. Gas phase, air. Temp. 37°. Time 1 hr.

	Radioactivity of the phosphatidic acids (counts/100 sec./flask)		
Expt.	Control	Hormone-treated	Treated / Control
1	823	939	1·1
2	1080	960	0·9
3	941	997	1·1

Table 5. *Effect of pituitary growth hormone on the dephosphorylation of the phosphatidic acids*

Each flask contained 200 μmoles of maleate buffer, pH 6·4, 14 μmoles of phosphatidic acid and rat-liver homogenate equivalent to 0·2 g. of liver in a final volume of 5 ml. Temp. 37°. Time 1 hr. Figures shown are corrected for blank values obtained in flasks incubated without substrate.

	Inorganic phosphate released (μmoles/flask)		
Expt.	Control	Hormone-treated	Treated / Control
1	2·74	3·26	1·2
2	2·30	2·11	0·9
3	2·46	2·75	1·1
4	1·91	2·11	1·1
5	2·24	2·28	1·0

labelled palmitate was incorporated into the ferric hydroxamate complex in hormone-treated animals and in controls. It is evident therefore that the lowered rate of palmitate incorporation into phospholipid in growth-hormone-treated rats cannot be accounted for in terms of a decreased rate of entry into the biosynthetic pathway.

Incorporation of [^{32}P]orthophosphate into phosphatides. The rate of incorporation of phosphate into phosphatides was measured by using an ATP-generating system in the presence of ADP and inorganic [^{32}P]phosphate. The ^{32}P-labelled ATP so formed was used to phosphorylate glycerol and hence the phosphatides (Kennedy, 1953). The results of these experiments are shown in Table 4. Once again it will be observed that there is no difference between the experimental and control animals under the conditions used.

Rate of conversion of phosphatidic acids into diglycerides. The next step in the pathway of biosynthesis of phospholipids investigated was that in which the phosphatidic acids are dephosphorylated to give the diglycerides, the immediate precursors of both phospholipids and neutral triglycerides. The results of these measurements are shown in Table 5. Again, this step appears to be unaffected by previous treatment of the rat with growth hormone since in both groups of animals the rate of release of inorganic phosphate was virtually identical.

Incorporation of [^{32}P]phosphorylcholine into phospholipids. The last step investigated in phospholipid biosynthesis concerned the rate of incorporation of phosphorylcholine. A comparison of the rates of incorporation of [^{32}P]phosphorylcholine into the phospholipids of normal and growth-hormone-treated animals is shown in Table 6. Although the rate of incorporation of the phosphorylcholine increases more or less in proportion to the level of substrate present, the hormone-treated animals consistently incorporate the phosphorylcholine faster and, indeed, over the range used, the ratio treated : control remained reasonably constant at about 1·4.

Table 6. *Effect of pituitary growth hormone on the incorporation of [^{32}P]phosphorylcholine into liver phospholipid*

Each flask contained 100 μmoles of phosphate buffer, pH 7·4, 20 μmoles of $MgCl_2$, 1·5 μmoles of cytidine triphosphate, 10 μmoles of ATP, [^{32}P]phosphorylcholine (33 700 counts/min./μmole) as indicated above and particles derived from 0·4 g. of liver. Further additions of 1·5 μmoles of ATP and 0·03 μmole of cytidine triphosphate were made every 12 min. during the course of the experiment. Final volume 3 ml. Temp. 37°. Time 1 hr.

		[^{32}P]Phosphorylcholine incorporated (μm-moles/flask)	
[^{32}P]Phosphorylcholine added (μmoles/flask)	Control	Hormone-treated	Treated / Control
4	4·6	6·3	1·36
10	6·9	9·8	1·42
18	9·5	12·3	1·29
20	14·1	21·6	1·53

Effect of growth hormone on the level of the lipids in the liver and the rates of incorporation of [1-^{14}C]*acetate and* [^{32}P]*orthophosphate into these lipids.* The results shown in the foregoing tables only partially account for the degree of inhibition of acetate incorporation into phospholipids and they direct attention to the possibility that the sevenfold reduction found may have been due, in part, to a dilution of the labelled precursors by one or more of the lipids mobilized to the liver under the influence of growth hormone (Szego & White, 1949; Greenbaum & McLean, 1953*b*). In order to evaluate the degree of dilution the levels of the di- and triglycerides, phospholipids, cholesterol and cholesterol esters have been measured in the livers of normal and growth-hormone-treated rats and the incorporation of [1-^{14}C]acetate and [^{32}P]orthophosphate into these fractions has been determined. The results are shown in Table 7. The level of free fatty acids is not included in the table since it was shown by Fairbairn (1945) that hydrolysis of phospholipids to free fatty acids occurs rapidly on incubation of rat livers *in vitro*. For this reason a separate determination was made of free fatty acids in livers freshly removed from normal and hormone-treated animals. Titration of the fatty acids showed no difference between the experimental and control groups.

Table 7 shows the scale of the mobilization of triglycerides to the livers of the treated animals. Ratios as high as six have been obtained for the comparison of triglycerides between hormone-treated and control rats. It also shows that, amongst the other lipids, only diglycerides are significantly changed; the increase, by about 40%, is reproducible. Cholesterol, cholesterol esters and phospholipids remain relatively unchanged in amount.

The most outstanding difference to be observed in the values obtained in the incorporation studies is the sixfold decrease in the specific activity of the triglyceride fraction (Table 7, columns 4 and 5). This very large reduction may be accounted for by the combined action of two factors. First there is a definite decrease in the total amount of triglyceride synthesized in the livers of the growth-hormone-treated rats (columns 7 and 8), and accompanying this there is a marked mobilization of neutral fat to the livers of the treated animals (columns 1 and 2). Each of these factors lowers the specific activity about 2·5-fold. Similarly, lowered specific activity of the diglycerides in the hormone-treated rats can be accounted for in terms of a lowered rate of synthesis and a dilution; both of these factors are, however, less than with triglycerides.

With ^{32}P as a precursor there is little difference in incorporation into either lecithins or kephalins

Table 7. *Effect of pituitary growth hormone on the levels and on the incorporation of* [1-^{14}C]*acetate and* [^{32}P]*orthophosphate into the lipids of the liver*

Results are derived from the combined livers of four rats in each group.

	Total wt. (mg.)			Specific activity (counts/100 sec./mg.)			Total incorporation (counts/100 sec.)		
	Control	Hormone-treated	Treated / Control	Control	Hormone-treated	Treated / Control	Control	Hormone-treated	Treated / Control
Diglycerides	70·4	99	1·4	1 361	575	0·42	95 810	56 900	0·59
Triglycerides	287	667	2·3	1 851	280	0·15	531 000	186 700	0·35
Lecithins (^{14}C)	425	510	1·2	627	235	0·37	266 500	119 800	0·45
Lecithins (^{32}P)				469	429	0·91	199 300	218 800	1·1
Kephalins (^{14}C)	126	151	1·2	815	279	0·34	102 700	42 100	0·41
Kephalins (^{32}P)				539	543	1·0	67 900	82 000	1·2
Sterols*	51	54	1·1	4 064	6 157	1·5	207 300	332 500	1·6
Sterol esters	32·2	39·4	1·2	670	833	1·2	21 570	32 840	1·5
Pigments etc.	14·5	6·8	0·5	633	5 376	8·5	9 190	36 720	4·0

* The fraction called 'sterols' in this table is composed of up to 98–99% of cholesterol (Barron & Hanahan, 1958) and has been called cholesterol in this paper.

after treatment with growth hormone. The slightly higher phospholipid content of the livers of the treated animals (column 1 and 2) results, however, in a small rise in the total counts measured in these two groups. When [1-^{14}C]acetate is used the specific activity of these two fractions is reduced by about 60–65 % in the hormone-treated animals and the total counts by 55–60 %.

The specific activity of the cholesterol [the fraction called the sterols in the table is composed of up to 98–99 % of cholesterol (Barron & Hanahan, 1958) and will be called cholesterol in this paper] is raised in the hormone-treated animals as are the total counts. This is in agreement with previous observations of an increased rate of cholesterol synthesis accompanying a decreased rate of fatty acid synthesis. An increase is also found, both in specific activity and in the total counts incorporated, in the sterol esters. In the group 'pigments etc.' of the table, Barron & Hanahan (1958) included hydrocarbons. On examination of the values for the specific activity of the fractions as they emerged from the column it is clear that this heterogeneous group of compounds contains some substances of extremely high specific activity (up to 16 000 counts/100 sec./mg.), and in view of the fact that squalene is eluted with hexane, as are these pigments, it would appear that these high counts may be ascribed, amongst other things, to cholesterol precursors.

DISCUSSION

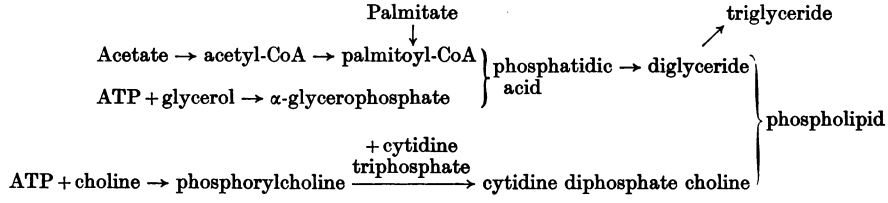
The results of the present series of experiments confirm and extend the previous results of Greenbaum & Glascock (1957) in that growth hormone is shown to cause a marked inhibition of the incorporation of acetate into phospholipids and neutral lipids. The degree of decrease of incorporation into phospholipids varies somewhat from animal to animal, but is generally about fivefold. Although the incorporation of acetate into the neutral fats does not appear to be affected to the same degree (Table 1) there is, in fact, a strong inhibition. The 'neutral-lipid' fraction is of a composite nature and includes triglycerides, diglycerides, cholesterol and cholesterol esters. When this group is fractionated to obtain pure di- and tri-glyceride fractions then it is found that growth hormone greatly decreases the incorporation of acetate into these substances also (see Table 7).

To try to find where growth hormone affects the rate of phospholipid synthesis measurements have been made of some of the component reactions of the pathway of phospholipid biosynthesis in normal and growth-hormone-treated rats. This pathway may be indicated as in Scheme 1.

The first comparisons were made of the spans acetate to phospholipid and palmitate to phospholipid (Tables 1 and 2). It was found that whereas the rate of incorporation of acetate was usually decreased fivefold (and sometimes eightfold) the decrease of the rate of incorporation of palmitate never exceeded threefold and was more usually between two- and three-fold. In this context Jedeikin & Weinhouse (1954) found that acetate incorporation into phospholipid was more affected by starvation than was the incorporation of palmitate.

This discrepancy between the decrease in rates of incorporation of acetate and palmitate could arise either from a direct effect of the hormone on the long-chain fatty acid-activating systems or on the enzymic steps which convert acetate into a long-chain acyl-CoA. A comparison was therefore made of the rate of activation of palmitate by unfortified homogenates of liver taken from normal and growth-hormone-treated animals. As will be seen from Table 3, there was an active incorporation of labelled carbon into the ferric hydroxamate complex in both sets of animals and, indeed, no difference could be detected in the rate of formation of palmitoyl-CoA in the two groups. It is thus possible to locate one of the sites of growth-hormone activity as lying on the pathway between acetate and long-chain acyl-CoA.

Another site of growth-hormone activity appears to exist in the second portion of the chain, i.e. the part leading from palmitoyl-CoA to phospholipid. In an attempt to localize this site further, comparisons were made of the steps involved, namely (*a*) the formation of phosphatidic acids as measured by the rate of incorporation of ^{32}P via the intermediate formation of labelled ATP and α-glycerophosphate, (*b*) the dephosphorylation of phosphatidic acid to diglyceride, and (*c*) the conversion of



Scheme 1

phosphorylcholine into phospholipid. In the first of these steps it has been shown that the radioactivity of the extracted phosphatidic acids is a measure of their rate of formation (Kennedy, 1953; Marinetti, Erbland & Albrecht, 1957). Table 4 shows that the radioactivities of the phosphatidic acids extracted from the livers of normal and growth-hormone-treated rats are virtually identical and it may therefore be concluded that the esterification of α-glycerophosphate by palmitoyl-CoA is unimpaired in treated rats. This is also true of the second reaction studied, the dephosphorylation of phosphatidic acids to diglycerides (Table 5). In the third reaction measured, the incorporation of phosphorylcholine into phospholipid, a small but definite increase in the incorporation rate in treated animals was recorded, i.e. this reaction would not account for the decreased rate of palmitate incorporation which had been found in growth-hormone-treated animals.

The lack of any enzymic differences between the two groups of rats studied directed attention to the possibility that the lowered rate of palmitate incorporation into phospholipid in the livers of growth-hormone-treated rats could be due to a dilution of the label by unlabelled precursor at one of the steps shown on the Scheme. For this reason the liver lipids of normal and control rats were chromatographed on silicic acid columns and the levels of the different lipids measured (Table 7).

While almost all the fractions were present in slightly greater quantity in the hormone-treated animals, only two of the fractions, the di- and triglycerides, were significantly raised. Of these two, only the diglyceride could act to dilute the isotope incorporated into the phospholipid. The 40% increase in the level of this fraction in the livers of the hormone-treated rats could account for most of the decreased incorporation of palmitate into phospholipid, both on a basis of specific activity or as total counts incorporated. It should be noted, however, that the diglyceride fraction is again a complex mixture. It is composed of diglycerides that give rise to triglycerides but not phospholipids, diglycerides that give rise to phospholipids and not triglycerides, and diglycerides that give rise to both. The present data do not show whether all these different fractions are affected by growth hormone, or only some of them. A more detailed fractionation of this large group would help to throw some light on this point.

Thus the results point to two possible regions at which growth hormone could produce an effect which would result in the great decrease in the incorporation of acetate. The first of these is in the enzymic span acetate → palmitoyl-CoA; the second is probably a dilution of the isotopically labelled intermediates at the level of the diglycerides.

SUMMARY

1. The incorporation of [1-^{14}C]acetate and [1-^{14}C]palmitate into liver phospholipids has been studied in normal and growth-hormone-treated rats. The hormone treatment greatly reduces the rate of incorporation of these substances, acetate more so than palmitate.

2. Measurements have been made of (*a*) the rate of the activation of palmitate to palmitoyl-coenzyme A, (*b*) the incorporation of ^{32}P-labelled adenosine triphosphate into phosphatidic acid, (*c*) the dephosphorylation of phosphatidic acid and (*d*) the incorporation of phosphorylcholine into phospholipid in normal and treated animals. The first three reactions proceeded at essentially the same rate in both groups of rats whereas the fourth reaction, the incorporation of phosphorylcholine, proceeded faster in the treated animals.

3. Measurements have been made of the levels of the various lipid fractions in the livers of normal and treated rats and the incorporation of [1-^{14}C]-acetate into these fractions. There is a significant increase in the levels of the di- and tri-glycerides.

4. It is suggested that the reduced rate of incorporation of acetate into phospholipids is caused partly by a slowing of the enzymic span acetate → palmitoyl-coenzyme A, and partly by an increased dilution of the labelled diglyceride by the greater amounts of unlabelled diglyceride present in the livers of the treated rats.

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The Demethylation of Griseofulvin by Fungi

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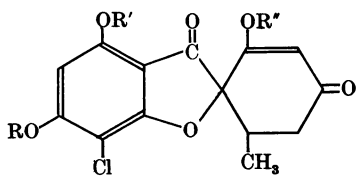
As one method of obtaining strains of *Microsporum canis* resistant to griseofulvin [7-chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione (I: R, R′, R″, Me)] the organism was grown on agar containing griseofulvin at a low concentration. At first the hyphae exhibited typical curling and



(I)

distortion (Brian, 1949), but after several days the edges of the giant colony showed normal hyphal growth, owing to the destruction of griseofulvin by the fungus (Aytoun, Campbell, Napier & Seiler, 1960). Biological assay showed that griseofulvin had disappeared from the substrate, and paper chromatography detected the presence of another substance, a new compound that has been characterized as 7-chloro-4-hydroxy-6:2′-dimethoxy-6′-methylgris-2′-en-3:4′-dione (I: R, R″, Me; R′, H) (4-demethylgriseofulvin).

In continuation of this work, many other fungi have been tested for their ability to metabolize griseofulvin. Some rapidly destroyed the griseofulvin; from two of them other griseofulvin metabolites were recovered and characterized. *Botrytis allii* and *Cercospora melonis* produced griseofulvic acid (I: R, R′, Me; R″, H) (Grove, MacMillan Mulholland & Rogers, 1952), and 6-demethylgriseofulvin (I: R, H; R′, R″, Me) (Barnes & Boothroyd, 1961) respectively.

EXPERIMENTAL

Paper chromatography. The solvent system and methods used have been reported by Barnes & Boothroyd (1961). All the reported compounds gave blue fluorescent spots when paper chromatograms of these compounds were examined under ultraviolet light; R_F values are: griseofulvin, 0·90; 4-demethylgriseofulvin, 0·70; griseofulvic acid, 0·20; 6-demethylgriseofulvin, 0·15.

Biological assay. The griseofulvin content of the various substrates was determined by a serial dilution method with *Alternaria solani* as test organism. Griseofulvin solutions absorbed on filter paper were allowed to diffuse through a cellophan disk placed on the damp filter paper so as to come in contact with germinating *A. solani* spores. End-point concentrations caused the hyphae to exhibit regular curling without stunting.

Spectrophotometry. Ultraviolet spectra were determined in ethanol containing 1% (v/v) of either 2N-HCl or aq. 2N-NH_3 soln. Infrared spectra were determined in $CHBr_3$ unless otherwise stated, in a Perkin–Elmer model 21 instrument with a NaCl prism.

Materials. The [^{36}Cl]griseofulvin was obtained by fermentation as described in a previous paper (Barnes & Boothroyd, 1961), in which are also described methods of determining the amount of radioactive material. The [^{36}Cl]griseofulvin used had a specific activity of 1·48 μc/m-mole.