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Studies on the Metabolism of Adipose Tissue. XI. Activation of Phosphorylase by Agents Which Stimulate Lipolysis*

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A regime of fasting and refeeding of rats has been employed to produce adipose tissue with a high glycogen content. Incubation of this tissue in a bicarbonate-buffered medium under anaerobic conditions, 5% CO₂-95% N₂, results in the formation of lactic acid which can be quantitatively accounted for by glycogen disappearance. The formation of acid results in the liberation of CO₂ from bicarbonate of the medium and permits the time course of the reaction to be followed manometrically. The rate of the reaction decreases with time but is promptly increased by the addition of epinephrine, serotonin, ACTH,¹ glucagon, fat-mobilizing substance, thyrotropic hormone, or β -mercaptoethylamine. Assay of the tissue for phosphorylase indicates that such agents may produce this effect by increasing the activity of this enzyme. The aerobic esterification of fatty acids to triglycerides in adipose tissue from normally fed rats is inhibited by β -mercaptoethylamine. This substance has no effect on esterification by glycogen-rich tissue, incubated under either aerobic or anaerobic conditions, from fasted and re-fed rats. The results are discussed from the standpoint of energy requirements for the esterification process and the ability of certain hormones to stimulate simultaneously fatty acid release, oxygen consumption, and phosphorylase activity in adipose tissue.

The phosphorylase of many mammalian tissues shows some specificity with regard to the hormone (or hormones) which initiates its activation. For example, in the adrenal cortex, ACTH plays this role: in the liver, glucagon and epinephrine; and in muscle it is the catecholamines (*cf.* Sutherland and Rall, 1960). On the other hand the phosphorylase of white adipose tissue appears to be unique in that it may be activated by all of the mentioned substances (Vaughan, 1960a; Hagen, 1961). In addition these same substances have been shown to be capable of stimulating the release of fatty acids from adipose tissue (Engel and White, 1960; Vaughan, 1960b; Hagen, 1961). Thus in adipose tissue a dual mobilization of the two chief storage forms of foodstuffs, glycogen and fat, may be brought about simultaneously by any one of these substances. The question therefore arises whether any substance which stimulates fatty acid release from adipose tissue will also activate the phos-

phorylase of this tissue. We describe here a procedure whereby the time course of the activation of phosphorylase in intact adipose tissue may be followed and present data obtained thereby bearing upon this question.

METHODS

The epididymal fat body of rats purchased from Holtzman Company has been used exclusively for these studies. The general care and precautions employed in handling of the animals as well as the procedure for removal of adipose tissue have been described previously (Ball and Merrill, 1961). In order to obtain tissue with a high glycogen content, rats which had been fed *ad libitum* for 1 week on Purina laboratory chow were fasted for 3 days and then re-fed *ad libitum* for 2 days. During the refeeding period the rats were given the so-called "fat-free" test diet supplied by Nutritional Biochemicals Corporation containing 21% casein, 58% sucrose, 16% cellulose, vitamins, and a salt mixture. Animals had access to water at all times. Such rats will be referred to as fasted and re-fed. The tissue obtained from them was found to contain 1.5-3.9 mg of glycogen per 100 mg wet weight. Rats of the same age which were maintained continuously on Purina chow were employed as controls. Adipose tissue

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¹ The following abbreviations are used: ACTH, adrenocorticotropin; ATP, adenosine triphosphate; G-1-P, glucose-1-phosphate; AMP, adenosine-5'-phosphate; CoA, coenzyme A.

from such animals contained 0.02–0.04 mg of glycogen per 100 mg wet weight.

In order to bring about glycogen breakdown *in vitro* in intact tissue we have used the device of placing the tissue under anaerobic conditions in a glucose-free bicarbonate medium. Under such conditions glycogen is converted to lactic acid. The time course of the reaction at 37.2° was followed by measuring the CO₂ released by the reaction of the lactic acid with the bicarbonate of the medium. The conventional Warburg apparatus was used for this purpose with a shaking rate of 120 cycles per minute. The medium employed was a Krebs-Ringer bicarbonate buffer containing half the recommended calcium (Krebs and Henseleit, 1932), and the gas phase was 5% CO₂-95% N₂. The buffer was well equilibrated with the gas mixture before use and had a pH of 7.6 at 37.2°. Conventional flasks of approximately 15-ml capacity with center well and a side-arm with a vented stopper were employed. The center well was not used. The main compartment contained 3.0 ml of bicarbonate buffer and the tissue, and the side-arm contained 0.1 ml of a solution of the substance whose action was to be tested. The compounds employed were dissolved either in the incubation medium or in 0.9% NaCl except in the case of epinephrine where the free base was dissolved in 0.1 N HCl and diluted with water to the desired concentration. This procedure was followed to avoid auto-oxidation of the epinephrine prior to its addition. After the flasks were mounted on the manometers and inserted in the water bath they were gassed for 5 minutes with 5% CO₂-95% N₂, equilibrated for a further 5 minutes, and readings begun. The time interval from decapitation of the animal to the first manometric reading was never more than 15 minutes.

Phosphorylase activity of tissue homogenates was determined by the procedure first described by Sutherland and Wosilait (1956). The successful application of this procedure to adipose tissue homogenates has already been described by Vaughan (1960a). We have prepared homogenates from 100–150 mg of the distal part of the epididymal fat body in 2.0 ml of 0.1 M NaF with the aid of an Elvehjem-Potter type homogenizer. The homogenization was carried out rapidly by hand at room temperature and the homogenate immediately transferred to a prechilled tube in an ice bath. Homogenization of adipose tissue at ice temperature was found to be unsatisfactory. The homogenate was centrifuged for 5 minutes at 1500 × *g* at 4° and the upper fat layer removed. The pellet was resuspended in the remaining fluid and rehomogenized in the cold. For the assay 0.5 ml of this homogenate was added to 0.8 ml of a basic reagent mixture so that the final volume of 1.3 ml contained 4.0 mg of glycogen, 31.5 μmoles of G-1-P, 1.4 μmoles of AMP, and 85 μmoles of NaF. The pH of the final mixture was 6.30. Determination of the optimum pH for the phosphorylase activity of adipose tissue showed it

to be between pH 6.2 and 6.4, with a sharp decline in activity on either side. In all cases the reaction was run at 37° and started by addition of the homogenate. It was stopped in one vessel at zero time and in an identically prepared vessel after 30 minutes by addition of 0.25 ml of 0.3 N HClO₄. After about 1 minute, 4.0 ml of 0.2 M Na acetate, pH 4.0, was added and the mixture was cooled in ice and centrifuged at 4° for 30 minutes at 30,000 × *g*. The inorganic phosphate content of the supernatant was determined by the method of Lowry and Lopez (1946). The difference in inorganic phosphate between zero time and 30 minutes was taken as a measure of the G-1-P units transferred to the primer glycogen, and the phosphorylase activity was expressed as μmoles/100 mg/hour. We ascertained that the formation of inorganic phosphate was linear with time during the incubation period. This is not the case if adenylic acid is omitted.

The glycogen content of the tissue was determined as follows: the weighed tissue was cut into small pieces and extracted twice with a mixture of methanol and chloroform (1:2 v/v) to remove most of the lipids. The mixture was centrifuged after each extraction and the solvent carefully drawn off. The residue was digested for 2 hours at 100° in 2–3 ml of 20% KOH and cooled, and 2 volumes of 95% ethanol were added. The precipitate which formed overnight was collected by centrifugation for 2 hours at 2000 × *g* and hydrolyzed in 1 N HCl. The glucose content of the hydrolysate was determined by the glucose oxidase method with reagents supplied by Worthington. In order to avoid losses of the small amounts of glycogen handled, all procedures except the glucose assay were carried out in the same container and no attempt was made to purify the glycogen. Known amounts of glycogen were carried through the entire procedure with a recovery of 90–98%.

The release or uptake of free fatty acids by adipose tissue was determined by incubation of tissue in Krebs-Ringer bicarbonate buffer to which bovine serum albumin had been added to yield a concentration of 50 mg per ml. Medium enriched in free fatty acid was prepared as follows (*cf.* Bragdon and Gordon, 1958): a known amount of recrystallized palmitic acid was dissolved in 100% ethanol, KOH added in slight excess of the amount calculated to neutralize the fatty acid, and the solution evaporated to dryness. The residue was dissolved in a small amount of water and the desired amount added with constant stirring to the albumin-bicarbonate medium. Any precipitate that formed on standing was centrifuged off and the free fatty acid content determined by the method of Dole and Meinertz (1960). Experiments were performed in Warburg vessels with 5% CO₂-95% air or 5% CO₂-95% N₂ as the gas phase; the temperature was 37.2° and the incubation period 3 hours. In all cases the free fatty acid content of both medium and tissue were determined initially and at the

end of the incubation. A paired piece of tissue served to give the initial tissue value. The tissue sample from the end of the incubation was immediately plunged into ice cold 0.9% NaCl, washed lightly, blotted on filter paper, and promptly homogenized in 5.0 ml of the acid isopropanol-heptane extraction reagent. Water and heptane were then added and the heptane phase promptly separated and its fatty acid content determined. Thus values presented represent the free fatty acid appearing in or disappearing from the total system, tissue plus medium.

Nitrogen content of tissue or tissue homogenates was determined by digestion with concentrated H_2SO_4 in the presence of selenized Hengar granules. If digestion was not complete in 2 hours, 30% H_2O_2 was added dropwise with continued heating until clearing occurred. The resulting ammonia was distilled into 2% boric acid and titrated with 0.01 N HCl.

Water content of tissue was measured by the loss in weight that occurred on drying to a constant weight over P_2O_5 *in vacuo*.

Lactate was determined enzymatically by the procedure introduced by Scholz *et al.* (1959) with reagents supplied by C. F. Boehringer und Soehne.

Crystalline zinc insulin (lot #466368) and crystalline glucagon (lot #758-234B 54-2) were kindly supplied by Eli Lilly and Company. L-Epinephrine was generously supplied as the free base by Burroughs Wellcome and Company. The sheep thyrotropic hormone preparation assayed 4-6 units per mg (lot #XIX 21-2) and was provided through the courtesy of Dr. A. E. Wilhelm. The sample of fat-mobilizing substance was a gift from Dr. T. M. Chalmers. The ACTH preparation was kindly supplied by Dr. J. D. Fisher of Armour Pharmaceutical Co., who reported it assayed 99.9 ± 10.2 U.S.P. units (subcutaneous) per mg. All other chemicals and reagents were the purest obtainable commercially. Serotonin was used in the form of the creatinine sulfate complex.

RESULTS

The release of CO_2 by adipose tissue from fasted and re-fed rats when incubated anaerobically in a bicarbonate buffer is shown by the experiment portrayed in Figure 1. In this experiment four vessels were run concomitantly. Each vessel received tissue obtained in the following manner: the distal portions of the left and right fat body of two rats were removed and each of these four portions cut (longitudinally) into four more pieces; each vessel then received one piece of tissue from each fat body of each rat, four pieces in all. This procedure for randomization of tissue samples was followed since it ensured more uniform initial rates in each vessel and thus facilitated comparisons of the relative action of compounds to be tested. It can be seen that in the experiment shown in Figure 1 the rate of CO_2 release initially in all four vessels was a little over

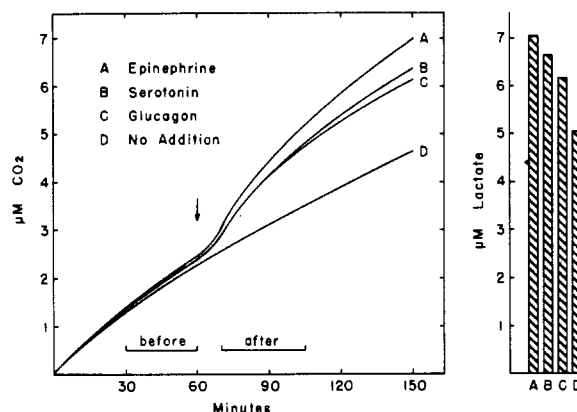


FIG. 1.—The effect of hormones on the release of CO_2 and lactic acid production by adipose tissue of fasted and re-fed rats incubated anaerobically in a bicarbonate medium. At 60 minutes the contents of the side-arm (0.1 ml) were added to give a final concentration in the flask as follows: epinephrine, 0.1 $\mu\text{g}/\text{ml}$; serotonin, 200 $\mu\text{g}/\text{ml}$; glucagon, 1.0 $\mu\text{g}/\text{ml}$. In flask D, 0.1 ml of incubation medium was added from the side-arm as a control. The lactate values were determined on the contents of the flasks at the end of the incubation period of 150 minutes. Further details are given in the text.

2 μmoles per hour per 100 mg of tissue. After 60 minutes the contents of the side-arm was tipped into each vessel. In the control vessel, where the side-arm contained 0.1 ml of the incubation medium, the rate of CO_2 release was not affected. In the other three vessels, where glucagon, serotonin, or epinephrine were present in the side-arm, the rate of CO_2 release increased markedly. This increase in rate was evident within 5 minutes, and the maximum rate (4.8 μmoles per hour per 100 mg in the case of epinephrine) was reached 10-20 minutes after the addition. The rate then gradually declined with time and approached the initial rate. At the end of 150 minutes the experiment was terminated and the media in all four vessels removed for lactic acid analysis. The results of these analyses are shown also in Figure 1. In all cases the μmoles of lactic acid formed slightly exceeded the μmoles of total CO_2 released. This excess of lactic acid can be accounted for largely by the fact that lactic acid production was undoubtedly proceeding during the 10-minute equilibration period before CO_2 measurements were begun (see Methods). Hence the release of CO_2 would appear to be quantitatively accounted for by lactic acid production. In other experiments not shown the CO_2 production has been followed for as long as 7½ hours. In a typical experiment in which serotonin was present from the start at a concentration of 200 $\mu\text{g}/\text{ml}$ the rate of CO_2 evolution during the first half hour was 4.0 μmoles per hour per 100 mg of tissue. The rate fell off gradually with time, and during the last half hour of the 7½-hour incubation the rate was 0.15 μmole per hour per 100 mg of tissue. The total CO_2 evolution during the entire

incubation was 21.35 μ moles per 100 mg and the total lactate production as determined at the end of the incubation was 22.62 μ moles per 100 mg. If this amount of lactate is assumed to be derived from the glycogen of the tissue, then it may be calculated to represent 1.83 mg of glycogen per 100 mg of tissue. As shown in Table I, direct analysis of adipose tissue from fasted and re-fed rats shows it to contain between 1.5 and 3.3 mg/100 mg tissue.

TABLE I
COMPARISON OF GLYCOGEN DISAPPEARANCE WITH CO₂ PRODUCTION DURING ANAEROBIC INCUBATION OF ADIPOSE TISSUE FROM FASTED AND RE-FED RATS

Exper.	Glycogen Content (mg per 100 mg)			Total CO ₂ Re- leased (μ mole per 100 mg)	
	Before	After	Differ- ence	Calcu- lated ^a	Ob- served
1	3.30	2.28	1.02	12.6	11.4
2	1.87	1.18	0.69	8.5	7.8
3	2.61	1.79	0.82	10.1	7.5
4	1.52	1.09	0.43	5.3	5.5
	Av.			9.1	8.1

^a Calculated from the glycogen disappearance assuming that each glucose residue (m. w. 162) gives rise to two molecules of lactic acid and two of CO₂. Total incubation time in all experiments 2 hours. In experiment 1, serotonin (400 μ g/ml) was present throughout the incubation, in experiment 2 serotonin (400 μ g/ml) was added after 60 minutes. In experiments 3 and 4 the tissue was incubated without a phosphorylase stimulator.

The fact that the rate of CO₂ production progressively diminishes as the experiment proceeds raises the question whether a pH change due to lactic acid production is responsible. Measurements show that changes in the pH of the medium are of the order of 0.1 unit during experiments of the type shown in Figure 1. In this regard it is of interest to note that if one adds both glucose (0.9 mg/ml) and insulin (0.1 U/ml) from the side-arm in place of one of the substances shown in Figure 1, CO₂ release and lactate production are accelerated and at a rate which corresponds to about the maximum achieved for example with epinephrine. There is however in this case no falling off in the rate of CO₂ production, which remains constant for several hours. The addition of glucose or insulin alone is without effect upon the rate. Data to be presented below suggest that the alteration in rate such as is seen in Figure 1 is due to changes in the phosphorylase activity.

The source of the lactic acid produced is the glycogen of the tissue, as indicated by the fact that when adipose tissue obtained from rats fed normally, in which the tissue glycogen is low, is incubated under similar conditions, the total CO₂ release and lactate production are about 0.2–0.3

μ moles. Addition of hormone has no appreciable effect upon this release. Further evidence that the tissue glycogen is the source of the lactate is provided in Table I. These data were obtained in experiments performed in a manner similar to those shown in Figure 1. In each experiment the tissue samples were taken from two rats and randomized as described but were distributed into two portions instead of four. One portion was taken for the analysis of the tissue's initial glycogen content and the other incubated anaerobically. The total CO₂ release was measured during the incubation, and at the end of 2 hours this portion of tissue was removed for glycogen analysis. As described in the legend to Table I, the total CO₂ release to be expected from the conversion to lactic acid of the glycogen which disappeared was calculated and compared with that actually measured. It can be seen from Table I that in three out of the four experiments the glycogen disappearance was more than enough to account for the measured CO₂ production. The fact that glycogen disappearance is on the average 10% higher than the measured CO₂ release is again, as in the case of lactic acid production, due in part to glycogen breakdown during the 10-minute equilibration period preceding the measurement of CO₂ release.

The same experimental procedure employed for the data of Figure 1 has been used to compare the ability of a variety of compounds to accelerate the conversion of glycogen to lactic acid. The results are given in Table II. An hourly initial rate is calculated from the rate observed during the interval of 30–60 minutes, labeled "before" in Figure 1. The interval 70–105 minutes, labeled "after" in Figure 1, has been used to calculate an hourly rate due to stimulation. These rates have then been divided by two in order to express the results in μ moles of glucose equivalents released from glycogen per hour per 100 mg of tissue. The concentration of the substance employed in each case is that found by preliminary trials to be about the lowest needed to ensure a maximum response. As can be seen from the results both ACTH and epinephrine about doubled the rate while the other materials gave a response somewhat less than this. On a weight basis epinephrine was most effective, but on a molar basis epinephrine, ACTH (mol. wt. 3500, Li [1956]) and glucagon (mol. wt. 3550, Bromer *et al.* [1957]) were about equally effective at concentrations of the order of $3-5 \times 10^{-7}$ M. If a value of 25,000 is accepted for the molecular weight of a thyrotropic hormone (Bates and Condliffe, 1960), then even on the basis of the impure sample used here (4–6 units/mg) it is effective at concentrations of at least 4×10^{-8} M. Statistical evaluation of the data showed in all experiments except those with 2,2'-dithiobis-(ethylamine) that the probability of the difference between the activity before and after stimulation being due to chance is less than 1%. Ethanolamine, mercaptoethanol, cysteine, and gluta-

thione, all employed at a concentration of 1 mg/ml, were found to be without effect.

The acceleration of the conversion of glycogen to lactic acid by all these substances would most logically be ascribed to their ability to activate phosphorylase. Vaughan (1960a) has already shown that the phosphorylase of adipose tissue is enhanced by epinephrine, ACTH, glucagon, and serotonin. Hagen (1961) has also shown that glucagon and epinephrine activate phosphorylase in this tissue. However, in view of the fact that these other workers incubated tissue under aerobic conditions it seemed worthwhile to test this premise under our conditions. For this purpose we selected thyrotropic hormone and β -mercaptoethylamine, two compounds not studied by Vaughan or Hagen. The results are presented in Table III. In these experiments tissue from two fasted and re-fed rats was randomly distributed into three portions. One portion was immediately taken for determination of its phosphorylase activity, and the other two portions were incubated in the manner described for the experiments portrayed in Figure 1 for a total of 100 minutes. One vessel received the addition of the compound under test at the end of 70 minutes. At the end of the period the tissue

from both flasks was removed for determination of the tissue phosphorylase activity. As can be seen from the data in Table III the incubation of tissue for 100 minutes results in a lowering of phosphorylase activity to about 70% of its initial value. A similar decline in activity of about this magnitude is seen in the rate of CO_2 release during incubation (*cf.* Fig. 1). The addition of either thyrotropic hormone or β -mercaptoethylamine at 70 minutes followed by a further 30 minutes' incubation results in the tissue having a phosphorylase activity higher than that of the control incubated tissue but about equal in value to that of the initial tissue sample. The percentage change in phosphorylase activity produced by these agents agrees well with the percentage change as measured from the CO_2 release (*cf.* Table II).

In view of the difference in glycogen content of adipose tissue from normally fed and from fasted and re-fed rats it seemed of interest to compare the phosphorylase activity of the two types of tissue. In a series of six rats fed normally (average weight of 180 g) the average phosphorylase activity was 8.5 μmoles per 100 mg per hour for tissue freshly excised. This value is half that for tissue obtained from fasted and re-fed rats (*cf.* Table III). However, we have found the nitrogen and water

TABLE II
THE EFFECT OF VARIOUS SUBSTANCES ON THE RATE OF GLYCOGEN BREAKDOWN IN ADIPOSE TISSUE FROM FASTED AND RE-FED RATS UNDER ANAEROBIC CONDITIONS

Substance	Conc. ($\mu\text{g}/\text{ml}$)	No. of Expts.	Rate of Glycogenolysis ^a ($\mu\text{mole}/\text{hr.}/100\text{ mg}$) (Mean $\pm$ S.E.M.)		P
			Initial	Stimulated	
ACTH	1.0	6	1.68 $\pm$ 0.19	3.30 $\pm$ 0.40	0.01
Epinephrine	0.1	6	1.32 $\pm$ 0.17	2.70 $\pm$ 0.29	0.01
Glucagon	1.0	6	1.50 $\pm$ 0.19	2.58 $\pm$ 0.27	0.01
Thyrotropic hormone	1.0	5	1.38 $\pm$ 0.13	2.34 $\pm$ 0.24	0.01
Fat-mobilizing substance	5.0	5	1.32 $\pm$ 0.06	2.10 $\pm$ 0.19	0.01
Serotonin	200.0	11	1.50 $\pm$ 0.18	2.52 $\pm$ 0.32	0.01
β -Mercaptoethylamine	500.0	11	1.56 $\pm$ 0.12	2.46 $\pm$ 0.26	0.001
2,2'-Dithiobis(ethylamine)	1000.0	5	1.68 $\pm$ 0.36	1.98 $\pm$ 0.25	0.1

^a The rate is expressed in terms of glucose formed from glycogen as calculated from the rate of CO_2 production assuming 2 μmoles of CO_2 are derived from 1 μmole of glucose. The average weight of the rats employed in this experiment was 186 g before fasting, 143 g after 3 days' fasting, and 169 g after 2 days' refeeding.

TABLE III
CHANGES IN PHOSPHORYLASE ACTIVITY OF ADIPOSE TISSUE FROM FASTED AND RE-FED RATS DURING ANAEROBIC INCUBATION

Substance	Conc. ($\mu\text{g}/\text{ml}$)	No. of Expts.	Phosphorylase Activity ^a ($\mu\text{mole}/\text{hr.}/100\text{ mg}$) (Mean $\pm$ S.E.M.)		
			Initial	Incubated; No Addition	Incubated with Addition
Thyrotropic hormone	1.0	5	18.0 $\pm$ 1.2	12.2 $\pm$ 1.2	18.7 $\pm$ 1.6
β -Mercaptoethylamine	500.0	5	22.2 $\pm$ 0.8	15.6 $\pm$ 0.4	21.3 $\pm$ 1.0

^a Activity expressed in terms of μmoles of inorganic phosphate liberated. The total incubation time was 100 minutes; when the substance was present it was added after 70 minutes of incubation. The average weight of the rats used in the thyrotropic hormone experiment was 187 g before fasting, 146 g after fasting, and 174 g after refeeding, and in the β -mercaptoethylamine experiment 175, 133, and 164 g respectively.

content of tissue from fasted and re-fed rats to be about double that of tissue from normally fed rats. Hence when the results are expressed in terms of tissue nitrogen content, this difference disappears. The value for normally fed rats then becomes 30 μ moles per mg N per hour, as opposed to an average value of 31 μ moles per mg N per hour for fasted and re-fed animals.

Several experiments have also been performed in which the effect of incubation and addition of thyrotropic hormone on the phosphorylase activity of tissue from normally fed rats were followed. These experiments were performed in the same manner as those described in Table III for tissue from fasted and re-fed rats. It was observed that on anaerobic incubation for 100 minutes the phosphorylase activity of tissue from normally fed rats also decreased and by about the same percentage as that of tissue from the fasted and re-fed rats. However, unlike tissue from fasted and re-fed rats, tissue from normally fed rats showed no appreciable change in phosphorylase activity when thyrotropic hormone (1 μ g/ml) was added after 70 minutes of anaerobic incubation and the incubation was continued for a further 30 minutes.

Many of the substances listed in Table II have been shown to increase the release of fatty acids from adipose tissue of normally fed rats when incubated *in vitro* under aerobic conditions (*cf.* Ball and Jungas, 1961). It was of importance therefore to ascertain whether these substances would have this same action not only upon tissue obtained from fasted and re-fed rats but when such tissue was incubated anaerobically. We found that ACTH, epinephrine, thyrotropic hormone, and fat-mobilizing substance do cause the release of fatty acids from this tissue when it is incubated anaerobically in a bicarbonate medium containing albumin (30 mg/ml). When these substances were used at the concentrations shown in Table II the release of fatty acid into the medium ranged from 0.34 to 0.78 μ eq per 100 mg of tissue per hour. Serotonin (400 μ g/ml) did not stimulate the release of fatty acids under comparable conditions. Thus the action of these substances under the conditions described here resembles their action upon tissue from normally fed rats when it is incubated aerobically. Engel and White (1960) in correction of an earlier report (White and Engel, 1958) have shown that ACTH releases fatty acids from adipose tissue of normally fed rats when it is incubated under anaerobic conditions. Epinephrine was also found by Engel and White (1960) to possess this property. Vaughan (1960a) has previously shown that serotonin does not release fatty acids from adipose tissue of normally fed rats when it is incubated aerobically. In this connection it may be pointed out that the release of fatty acids would appear to have no influence on the time course of the reaction in experiments of the type shown in Figure 1. If it did one would expect serotonin which releases no fatty acids to show a

response different from that of the other agents.

At this point it may help the reader to understand the results which follow if we pause for a word of explanation concerning our thought processes. Our examination of the action of β -mercaptoethylamine on the phosphorylase activity of adipose tissue was prompted by the report of Raben and Hollenberg (1959) that this compound not only stimulates the release of fatty acids from rat adipose tissue *in vitro* but also blocks the uptake of fatty acids which occurs when this tissue is incubated aerobically in a medium which contains fatty acids, glucose, and insulin. These workers suggested that β -mercaptoethylamine might exert such an action by competing with CoA in the process of reesterification of fatty acids by the tissue. Now Ball and Jungas (1961) have suggested that the ability of the hormones tested here to stimulate the phosphorylase activity of adipose tissue might be due to the production of cyclic AMP as a by-product in the reesterification process that is initiated when these hormones release fatty acids. Thus we argued that if β -mercaptoethylamine blocked the reesterification process it might not affect the phosphorylase activity of the tissue even though fatty acids were released. As shown above, β -mercaptoethylamine does stimulate the phosphorylase activity of adipose tissue incubated anaerobically. This finding made us question certain of the premises just stated, and we therefore decided it worthwhile to investigate the action of β -mercaptoethylamine in greater detail.

As shown in Figure 2, we have been able to confirm the findings of Raben and Hollenberg (1959). Under aerobic conditions and in the presence of glucose and insulin, adipose tissue from normally fed rats removes fatty acid from the incubation medium at a rate which is proportional to the concentration of fatty acid in the medium. β -Mercaptoethylamine inhibits this process but does not block it completely. Under anaerobic conditions but with all other details unaltered, uptake of fatty acid does not occur or is extremely low. In the presence of β -mercaptoethylamine a release of fatty acids may be observed.

The picture is quite different, however, if tissue from fasted and re-fed rats is employed. This is best seen by a comparison of results with the two types of tissue at a fixed concentration of fatty acid in the medium. In Table IV the results of a series of experiments are shown where the initial fatty acid concentration in the medium was in the range 5.4–6.9 μ eq per ml. First it may be seen that under aerobic conditions there is on the average a slightly greater uptake of fatty acids by the tissue from fasted and re-fed rats than by tissue from normally fed rats. More important is the fact that β -mercaptoethylamine does not block this uptake in tissue from fasted and re-fed rats. Even more striking is the difference in behavior of the two types of tissue under anaerobic conditions. Uptake of fatty acids by tissue from fasted and re-fed rats pro-

ceeds almost as rapidly under anaerobic conditions as under aerobic, and again β -mercaptoethylamine has no effect upon the process. This same behavior of tissue from fasted and re-fed rats is also seen when the fatty acid concentrations in the medium are varied over the range shown in Figure 2. It should be noted that the values under anaerobic conditions for the uptake of fatty acids by tissue from fasted and re-fed rats given in Table IV may be roughly 10% lower than the true ones. This is due to the high production of lactic acid by this tissue under these conditions, which leads to the extraction of a small amount of the lactic acid along with fatty acids from the medium. We have satisfied ourselves that for the purposes at hand the magnitude of the error involved did not warrant the application of the additional steps necessary to eliminate it.

DISCUSSION

Von Gierke (1906), in studies on guinea pigs, appears to have been the first to describe an increase in the glycogen content of the adipose tissue of animals after fasting and refeeding. Since then many workers have confirmed this finding on a variety of species. The values to be found in the literature for the glycogen content of the epididymal fat body of the rat average about 0.04 mg per 100 mg for normally fed animals, while values for tissue from fasted and re-fed animals lie between 0.3 and 0.5 mg per 100 mg wet weight. In our studies tissue from fasted and re-fed animals has yielded values as high as 3.9 mg per 100 mg. The reason for the higher values obtained by us is not clear, though they may in part reflect the fact that, unlike most previous workers, we have chosen to carefully extract the fat from the tissue before undertaking the alkaline digestion. We have found that when this is not done the resulting soapy mixtures are most difficult to handle. When one considers the fact that the water content of the epididymal fat body from fasted and re-fed rats runs around 0% of the weight of fresh tissue, then a glycogen content of 3.9 mg per 100 mg corresponds to some 13% of the nonlipid portion of the cell. The

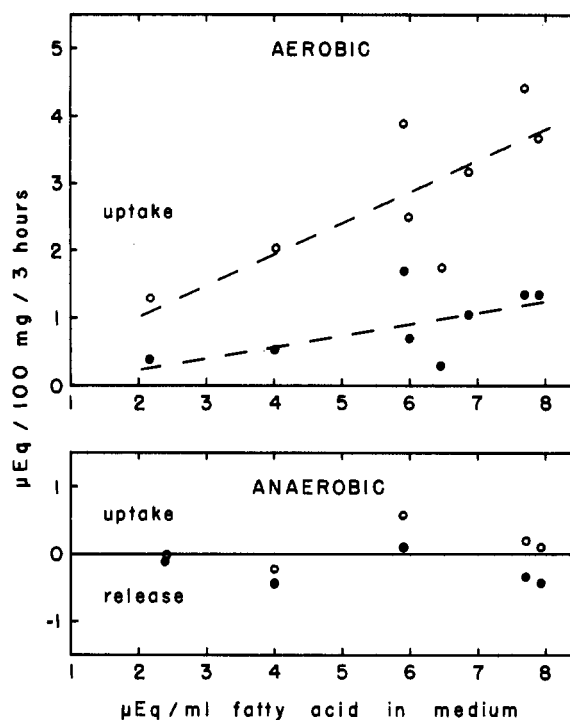


FIG. 2.—The effect of β -mercaptoethylamine on the uptake or release of fatty acids by adipose tissue from normally fed rats. Incubations were carried out for 3 hours at 37° in a bicarbonate medium fortified with fatty acids (*cf.* Methods) containing 50 mg of bovine albumin, 3.6 mg of glucose, and 0.1 unit of insulin per ml. The gas phase was 5% CO_2 -95% air for the aerobic experiments and 5% CO_2 -95% nitrogen for the anaerobic experiments. Paired tissue from rats fed normally and weighing approximately 180 g were compared in the presence and absence of β -mercaptoethylamine. The open circles represent values for control flasks, the filled circles the values obtained in the presence of 500 μg of β -mercaptoethylamine per ml.

highest value for the glycogen content of adipose tissue we have encountered in the literature is 7.4 mg per 100 mg, reported by Arndt (1928) for the subcutaneous adipose tissue from dogs.

Mirski (1942) was apparently the first to study the phosphorylase activity of adipose tissue, using

TABLE IV
A COMPARISON OF THE EFFECT OF β -MERCAPTOETHYLAMINE ON FREE FATTY ACID UPTAKE BY ADIPOSE TISSUE FROM RATS FED NORMALLY OR FASTED AND RE-FED^a

Metabolic State	No. of Expts.	Aerobic		Anaerobic	
		No Addition	β -Mercaptoethylamine	No Addition	β -Mercaptoethylamine
Fed normally	4	2.83 $\pm$ 0.46	0.92 $\pm$ 0.29	-0.07 $\pm$ 0.24	-0.33 $\pm$ 0.23
Fasted and re-fed	4	3.07 $\pm$ 0.13	3.37 $\pm$ 0.42	2.64 $\pm$ 0.26	2.62 $\pm$ 0.15

^a Uptake is expressed as μeq per 100 mg fresh weight for 3 hours; a minus sign indicates release. Conditions as described for the experiments in Figure 2 except the initial fatty acid concentration in the medium ranged from 5.4 to 6.9 μeq per ml. In each experiment adipose tissue from two rats was randomly distributed among four vessels in which the experimental conditions were varied as indicated by the column headings. The average weight of the rats fed normally was 179 g. The fasted and re-fed rats weighed 182 g before fasting, 138 g after 3 days' fasting, and 167 g after 2 days' refeeding.

partially purified extracts. More recently Vaughan (1960a) and Hagen (1961) have reported studies on the phosphorylase activity of homogenates of the epididymal fat body of normally fed rats. Both these workers used the phosphorylase assay method of Sutherland and Wosilait (1956), which follows the reaction in the direction of glucose-1-phosphate to glycogen. Though Vaughan reports no specific values for phosphorylase activity of freshly excised tissue, it may be calculated from the results given that these would lie between 6 and 9 μ moles of inorganic phosphate released per 100 mg of tissue per hour. When tissue is incubated for an hour the values are reported by Vaughan to decline to 65% of the initial value. If the incubation is carried out in the presence of epinephrine, glucagon, ACTH, or serotonin then the values at the end of an hour's incubation approach but never exceed those found for freshly excised tissue. Hagen (1961) reports only values for the increase over the control tissue produced after a 30-minute incubation in the presence of epinephrine or glucagon. These increases correspond on the average to about 2.5 μ moles of inorganic phosphate released per 100 mg of tissue per hour. We have observed values for tissue freshly excised from normally fed animals that average 8.5 μ moles of inorganic phosphate released per 100 mg per hour, and this value decreased on incubation of the tissue. Freshly excised tissue from fasted and re-fed rats gives values which average 20 μ moles per 100 mg per hour, and on incubation of the tissue for 70 minutes anaerobically this value reaches the neighborhood of 14 μ moles. The value may be restored to approximately that for freshly excised tissue by incubation with thyrotropic hormone or β -mercaptoethylamine. The higher values found for tissue from fasted and re-fed rats reflects its greater water and nitrogen contents. In spite of its much higher glycogen content, the phosphorylase activity of tissue from fasted and re-fed rats is not greatly different from tissue obtained from normally fed rats, if the results are expressed on a nitrogen basis. It is of interest to note that the phosphorylase activity of tissue from normally fed rats, in contrast to glycogen-rich tissue, is not activated by thyrotropic hormone under anaerobic conditions. This may reflect a paucity of ATP, without which activation of phosphorylase cannot take place.

It is of interest to compare the values for phosphorylase activity discussed in the preceding paragraph with the rates at which glycogen is converted to lactic acid in the intact tissue under comparable experimental conditions. Expressed in terms of glucose phosphate formed, the rate of glycogen breakdown in intact tissue is on the average 1.5 μ mole per hour per 100 mg for unstimulated tissue and 2.5–3.0 μ moles after the addition of hormones (*cf.* Table II). Thus these values are at best no more than 15% of the maximum rates to be observed for the conversion of glucose-1-phosphate to glycogen in homogenates of the tissue.

At first glance such a comparison suggests that the activity of phosphorylase might not be the rate-limiting step in the breakdown of glycogen to lactic acid in intact tissue. If this is the case then the acceleration of this process by hormones must be attributed to their effect on some reaction other than that catalyzed by phosphorylase. This is possible, though we do not favor such an interpretation of the data. We believe that the reason for the difference in the values obtained by the two procedures is to be sought among such factors as the great difference in substrate concentrations, changes in the physical state of the enzyme, and the difference in the direction in which the reaction is measured. The data presented here do not in our opinion warrant a further discussion of such possibilities at this time. However it may be noted in this connection that Villar-Palasi and Lerner (1960) have used the measurement of TPN reduction to follow the rate of glycogen breakdown in homogenates of rat adipose tissue fortified with glycogen, inorganic phosphate, phosphoglucomutase, glucose-6-phosphate dehydrogenase, and TPN. They report phosphorylase activities of 0.08 μ moles per hour per 100 mg of tissue.

As pointed out by Ball and Jungas (1961), all hormones which stimulate the release of fatty acids from rat adipose tissue also markedly accelerate the oxygen consumption of this tissue. These authors suggested that the increase in oxygen consumption reflected the utilization of energy for the purpose of reesterification of the released fatty acids to triglycerides within the tissue. They further postulated that the conversion of acyl AMP to acyl CoA in this process might result in the production of cyclic adenylic acid as a by-product and that this in turn might bring about activation of phosphorylase in the tissue. Thus all substances which stimulate fatty acid release could be expected also to increase oxygen consumption and to activate phosphorylase. The results presented here, along with those of Vaughan (1960) and Hagen (1961), support this generalization. However there are no data available as yet to support the postulated production of cyclic adenylic acid in this action of the hormones. It should be noted that the converse is not true, namely, that a substance (*e.g.* serotonin) which activates adipose tissue phosphorylase does not stimulate fatty acid release or oxygen consumption by adipose tissue.

The reasons for our investigation of the action of β -mercaptoethylamine on phosphorylase activity have been stated in the presentation of the results. As shown in Figure 2, we were able to confirm the statement of Raben and Hollenberg (1959) that the uptake of fatty acids by adipose tissue from normally fed rats incubated aerobically in the presence of glucose and insulin is inhibited by β -mercaptoethylamine. In addition we observed that if experiments with such tissue were carried out anaerobically very little reesterification occurred and the addition of β -

mercaptoethylamine resulted in either a diminution of this uptake or a slight release of fatty acids. The failure of reesterification with adipose tissue from normally fed rats under anaerobic conditions in the presence of insulin and glucose may reflect the inability of the tissue to produce sufficient high-energy phosphate for this process. We have found that insulin stimulates glucose uptake by this type of tissue under anaerobic conditions, with the production of lactic acid. The amount of this metabolite formed, however, averages only 1 μ mole per 100 mg of tissue per hour (unpublished results). This would yield about 1 μ mole of high-energy phosphate, which if entirely used for reesterification would permit at best the uptake of 0.5 μ eq of fatty acid per 100 mg per hour. In marked contrast is the behavior of tissue from fasted and re-fed rats when incubated under anaerobic conditions in the presence of glucose and insulin. Uptakes of fatty acid of the order of 1 μ eq per 100 mg per hour are observed when the fatty acid concentration in the incubation medium lies within the range 5.4–6.9 μ eq per ml. A production of 2 μ moles of high-energy phosphate per 100 mg per hour would be needed to support this rate of reesterification. This is well within the capabilities of the tissue, since under these conditions it produces lactic acid at a rate of 6–7 μ moles per 100 mg per hour. This would correspond to 6–7 μ moles of high-energy phosphate if glucose was the sole source of the lactic acid and to a still larger amount if conversion of glycogen to lactic acid was occurring. Most striking is the fact that β -mercaptoethylamine does not inhibit the reesterification process under either aerobic or anaerobic conditions. This fact suggests that β -mercaptoethylamine does not act directly upon the esterification process itself. Its inhibitory action upon this process in tissue from normally fed animals incubated aerobically may reflect its interference with the production of ATP by oxidative processes. We have observed that β -mercaptoethylamine inhibits the oxygen consumption of adipose tissue.

It is of interest to note that β -mercaptoethylamine has been investigated as an agent to counteract radiation damage. Bacq *et al.* (1953) in their studies of this substance observed that when administered *in vivo* it had a glycogenolytic effect on the liver of mice and rats. Sokal *et al.* (1959) confirmed this finding and showed that the disulfide form, 2,2'-dithiobis(ethylamine), also possesses this property. They concluded from their studies that the glycogenolytic action on

liver was direct and not mediated by the release of epinephrine or glucagon. A glycogenolytic action on muscle was, however, seen only in animals with an intact adrenal medulla. Karnovsky (1961) employed β -mercaptoethylamine as a lathyrogenic agent, and noted that it produced a marked depletion of glycogen in the epiphyseal cartilage of young rats. The action of β -mercaptoethylamine and 2,2'-dithiobis(ethylamine) as activators of phosphorylase may thus not be limited to adipose tissue.

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