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Studies on the Metabolism of Adipose Tissue. XX. The Effect of Thyroid Status upon Oxygen Consumption and Lipolysis*

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ABSTRACT: Oxygen consumption (Q_{O_2}) and glycerol and free fatty acid (FFA) formation have been measured *in vitro* utilizing adipose tissue from hypo-, eu-, or hyperthyroid rats. Spontaneous lipolysis and Q_{O_2} were enhanced in the hyperthyroid and reduced in the hypothyroid tissues compared with the normal, even when corrections were applied for the initial tissue content of glycerol and FFA and the data were expressed on a tissue nitrogen basis. Insulin alone inhibited spontaneous lipolysis irrespective of thyroid status, but tissue from hyperthyroid rats was inhibited less than that from normal or hypothyroid animals. A comparison of the dose response of the three types of tissue to epinephrine was made in the presence of insulin (1000 μunits/ml) and glucose (3 mg/ml), conditions under which release of FFA and its reesterification are favored. Sensitivity to the lipolytic action of epinephrine increased progressively as one proceeded from the hypo- to the hyperthyroid state. Sensitivity of hypo- and euthyroid tissue to the lipolytic action of epinephrine was markedly enhanced by aminophylline (10^{-4} M), the greatest lipolytic rate observed in any of these experiments being measured in hypothyroid tissue under these circumstances. A comparison of oxygen consumption with the calculated amount of FFA reesterified in these experiments indicates that the efficiency of oxidative phosphorylation in adipose tissue is not significantly altered by the thyroid status of the animal. The increased basal oxygen consumption of hyperthyroid tissue can be accounted for by the increased rate of reesterification of FFA. The conclusion is drawn that thyroid status alters the lipolytic response not by a change in the tissue content of lipase but by an alteration in the mechanism, presumably involving cyclic 3′,5′-adenosine monophosphate, by which the lipase is activated.

The magnitude of the lipolytic response of rat adipose tissue *in vitro* to the addition of epinephrine is markedly dependent upon the thyroid status of the animal. Debons and Schwartz (1961) showed that a concentration of epinephrine (2.5 μg/ml) which elicited a maximum release of FFA[1] from normal tissue produced a 250% greater response from hyperthyroid tissue. Conversely, they found little or no response by hypothyroid tissue to this same concentration of epinephrine. Similar results were reported by Deykin and Vaughan (1963) using a lower concentration of epinephrine (0.1 μg/ml). In addition, they showed that concomitant increases in glycerol production accompanied the changes seen in FFA release in hyperthyroid tissue. With these studies as a base line we began in 1964 the investigations to be described here in an effort to obtain further insight into the manner by which thyroid hormones brought about these changes in the lipolytic response of adipose tissue to

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[1] Abbreviations used: FFA, free fatty acid; AMP, adenosine monophosphate; ACTH, adrenocorticotrophin.

epinephrine.

In planning our experiments we sought to obtain certain objectives. First, we wished to avoid as far as possible any accumulation of FFA within the tissue in order to prevent inhibitory effects of FFA upon metabolic processes (Hagen and Ball, 1961). We have, therefore, performed experiments under conditions where glucose and insulin are present so that rapid reesterification of released FFA occurs (Ball and Jungas, 1961). We have chosen phosphate buffer because under otherwise identical conditions lipolysis proceeds faster in phosphate than in bicarbonate buffer (Flatt and Ball, 1963), and the use of phosphate permits the manometric measurement of O_2 consumption. This measurement affords an index to the linearity of the over-all reactions throughout the course of the incubation period and coupled with determination of FFA and glycerol production permits one to calculate the efficiency of the reesterification process as it may be affected by the thyroid status of the animal. Second, in making comparisons of adipose tissue from hypo-, eu-, and hyperthyroid animals we wished to exclude from the metabolic data any influence caused by differences in the tissues' FFA and glycerol content prior to incubation and their fat-free dry weight. We have, therefore, in all experiments corrected our data for initial tissue FFA and glycerol values and expressed metabolic rates in terms of tissue nitrogen.

Under these experimental conditions we have measured the response of adipose tissue from hypo-, eu-, and hyperthyroid animals over a range of epinephrine concentrations (0.01–10 μg/ml) and the effect of aminophylline upon the log dose–response curves so obtained. The main conclusions we have reached from these studies are two. First, the effect of thyroid hormones upon lipolytic rates in adipose tissue is not achieved by alterations in the total amount of lipase in the tissue which is capable of undergoing activation. Rather the level of cyclic AMP which is responsible for the activation would seem to be involved. Second, there is no evidence of uncoupling of oxidative phosphorylation in hyperthyroid adipose tissue as measured by the efficiency of FFA reesterification.

Materials and Methods

Male Holtzman rats were employed in these experiments. The average weights at the time of sacrifice for the normal, hyper-, and hypothyroid groups, respectively, were 190, 180, and 198 g. All animals had free access to food and water until approximately 0.5 hr before sacrifice. The normal or "euthyroid" group was fed a diet of Purina chow pellets. The group designated as "hyperthyroid" was fed in the same fashion until 4 days before sacrifice, when a diet of Purina chow meal, containing 1% desiccated thyroid, USP, was substituted. The "hypothyroid" group were, on the average, 1 week younger than the other animals at the time of arrival from the supplier. From then, until sacrifice 3 weeks later, they were fed a diet of Purina chow meal, containing 0.15% propylthiouracil. The necessary precautions observed in animal care and the procedures for obtaining the epididymal fat body have been described (Ball and Merrill, 1961). Incubations were performed for 1 hr at 37.2° in a Warburg respirometer, with a shaking rate of 120 cycles/min. The medium utilized was Krebs–Ringer phosphate buffer modified to contain only one-half the recommended amount of calcium (Umbright *et al.*, 1957). Substrate was added as described in the text, but no albumin was present in any of the experiments. The gas phase was air.

In each experiment adipose tissue from two rats was distributed into six relatively uniform groups, as previously described (Jungas and Ball, 1963). Immediately after weighing, four of these were placed into Warburg vessels containing 2.8 ml of buffer at room temperature. The fifth sample was frozen for later analysis of nitrogen content. The sixth was placed into a small stoppered erlenmeyer flask containing 1.2 ml of buffer at room temperature for determination of the "initial" FFA and glycerol content. During the time that the other tissue samples were equilibrating in the Warburg respirometer, this latter flask was also placed into a shaking water bath at 37.2°. At the moment that shaking was resumed following the initial manometer readings and tipping of the side-arm contents into the Warburg vessels (the zero time), the tissue in the erlenmeyer flask was removed and immediately homogenized in acid isopropyl alcohol–heptane extraction reagent. At the same time, an aliquot of the medium was prepared for analysis of glycerol. Previous publications have described the procedures for determination of tissue FFA and nitrogen content (Frerichs and Ball, 1962) and medium glycerol (Jungas and Ball, 1963). Stock solutions of epinephrine (free base, lot no. 38626, Burroughs-Wellcome and Co.) and crystalline zinc insulin (lot no. 466368, Eli Lilly and Co.) were prepared as previously described (Hagen and Ball, 1960; Ball and Merrill, 1961). Immediately prior to use, appropriate dilutions were made with 0.15 M NaCl.

In all experiments the epinephrine was added from the side arms immediately after the flasks were equilibrated and the first manometer readings made. Glucose and insulin, however, were present in the incubation medium from the beginning in the experiments depicted in Figures 5 and 6, both in the manometric flasks and in the flasks containing tissues for "initial" glycerol and FFA analysis. In all other experiments, insulin, when present, was added from the side arm, and neither insulin nor glucose was present in the medium in which the "initial" tissues were incubated. The total volume after all additions was 3.0 ml.

Determination of the incorporation of radioactivity from glucose-U-^{14}C into total lipids, glyceride fatty acids, and glyceride glycerol followed the procedures of Flatt and Ball (1964) with minor modifications. Glucose-U-^{14}C was purchased from the New England Nuclear Corp. Aminophylline, a compound containing 2 moles of theophylline with 1 mole of ethylenediamine,

TABLE I: Nitrogen and Initial Glycerol and FFA Content of Adipose Tissue from Rats in Different Thyroid Status.[a]

Tissue	No. of Expt	N Content (mg/100 mg wet wt)	Initial Values (μmoles/mg of tissue N) Glycerol	FFA
Hypothyroid	8	0.371 ± 0.012	0.17 ± 0.009	0.49 ± 0.024
Euthyroid	10	0.394 ± 0.012	0.23 ± 0.018	0.59 ± 0.035
Hyperthyroid	10	0.570 ± 0.034	0.41 ± 0.023	1.73 ± 0.127

[a] All data given as mean plus or minus standard error of the mean.

was used as a source of methylxanthine because its solubility properties permitted the addition of small amounts of a solution from the side arm of flasks. Experiments in which aminophylline and theophylline were compared revealed no difference in their action.

Results

In Table I are listed the average nitrogen contents and the initial values of glycerol and FFA for adipose tissues from three sets of animals, grouped according to their thyroid status. While there are only small differences to be seen between the hypo- and euthyroid groups, it is apparent that all measurements are significantly higher in the hyperthyroid tissues. The effect of subtracting initial values of glycerol and FFA from the values obtained at the end of an incubation and of expressing the data on a tissue nitrogen basis are shown by the following experiments.

Comparison of Spontaneous and Epinephrine-Stimulated Lipolysis. In Figure 1 are represented the unstimulated rates of lipolysis and oxygen consumption of adipose tissues from hypothyroid, euthyroid, and hyperthyroid rats. On the left-hand portion of the

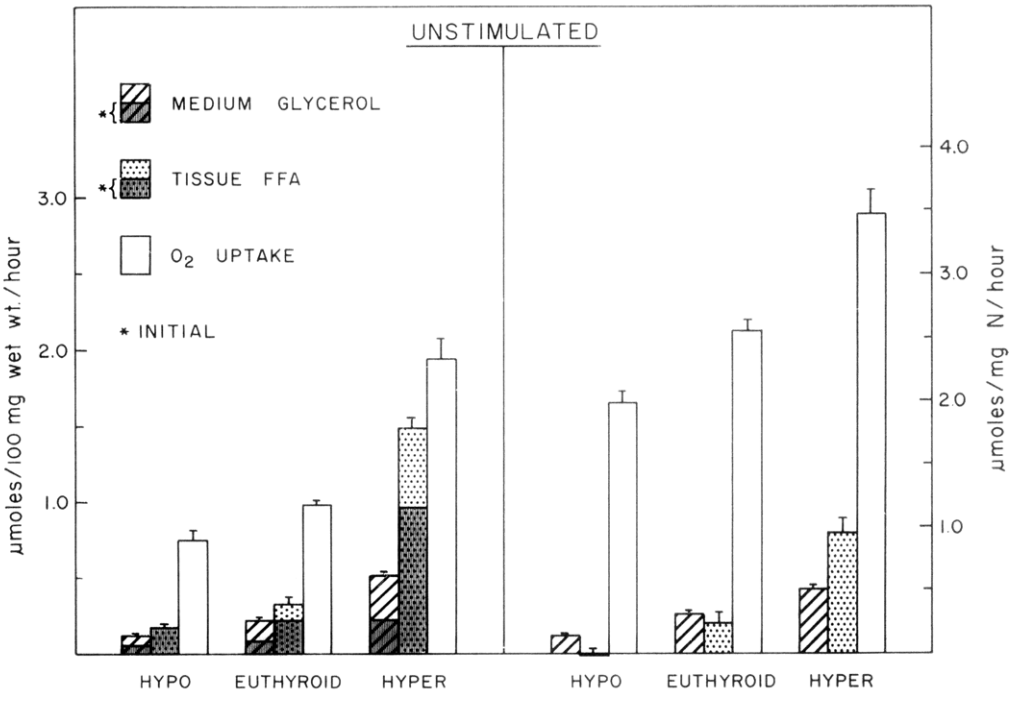


FIGURE 1: Spontaneous lipolytic and oxygen consumption rates of hypo-, eu-, and hyperthyroid rats. The data on the left half of the figure are expressed per 100 mg of tissue wet wt per hr. The shaded portions of the bars which represent glycerol and FFA indicate the μmoles/100 mg wet wt of these moieties which were already present in the tissue at the beginning of the incubation (the "initial" value). The same data are presented on the right half of the figure expressed as micromoles per milligram of nitrogen per hour after the initial values have been subtracted. The incubation medium was Krebs–Ringer phosphate buffer without added glucose or hormones. The vertical lines indicate one standard error of the mean. Each bar represents the average of seven observations for the hypothyroid, and 12 observations for both the eu- and hyperthyroid.

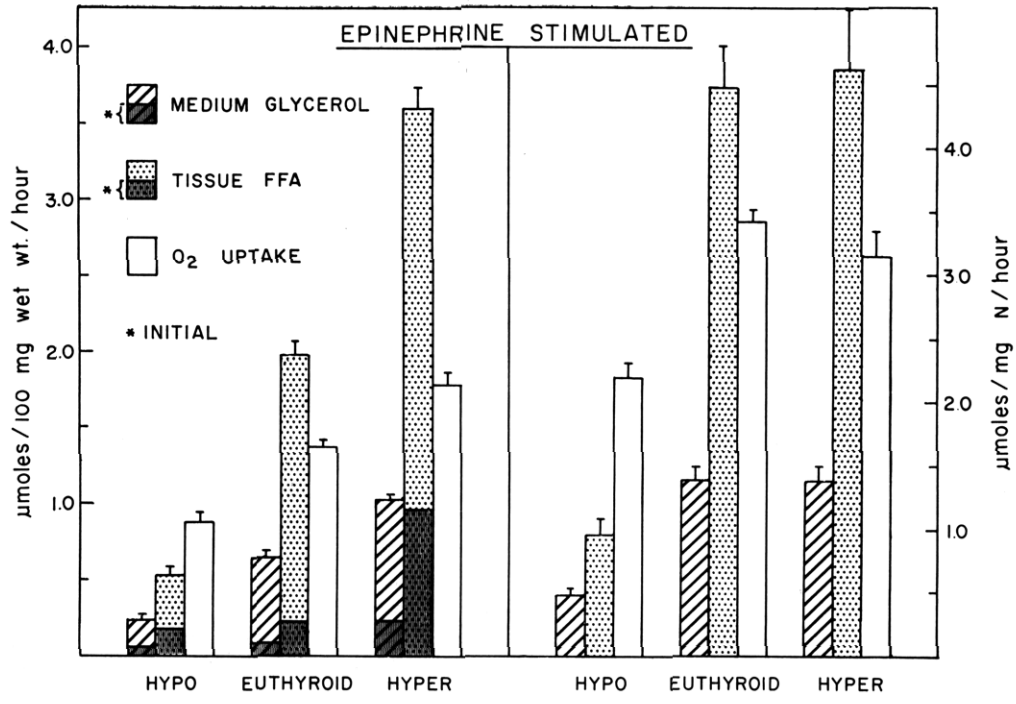


FIGURE 2: Epinephrine-stimulated lipolytic and oxygen consumption rates of adipose tissue from hypo-, eu-, and hyperthyroid rats. Experimental procedure and presentation of data are the same as in Figure 1 except that epinephrine in a final concentration of 0.1 mg/ml was added to the medium at the beginning of the incubation. Each bar represents the average of seven observations and the vertical line above each bar indicates one standard error of the mean.

figure, the results are expressed in terms of μmoles/100 mg wet weight of tissue per hr. The darker areas of the bars, representing glycerol and FFA, indicate that part of the respective moiety which was present in the tissue at the beginning of the incubation (the "initial" value). On the right-hand portion of the figure, the same data are presented in terms of micromoles released per milligram of tissue nitrogen per hour, the "initial" values having been subtracted. In Figure 2, data are presented which show the effect of addition of epinephrine (0.1 μg/ml) to the incubation medium.

In the case of the unstimulated tissue, it is apparent that there is a progressive increase in the activity of the adipose samples from the hypo- to the hyperthyroid, with respect to both lipolytic rates and oxygen consumption. This observation holds true whether the data are presented in relation to wet weight or tissue nitrogen. Similarly, there seems to be a stepwise progression in the epinephrine-stimulated group (Figure 2) when the data are related to the wet weight of the adipose samples. When the data for the epinephrine experiments are expressed in terms of the tissue nitrogen content, however, the response of the hyperthyroid tissue appears no greater than that of the euthyroid. As will be evident from the data to follow, this latter observation is probably an artifact of the experimental system employed, related perhaps to the accumulation of high levels of FFA within the tissue.

Effect of a System Permitting Rapid Reesterification of Fatty Acids. The experiments depicted in the first two figures were repeated in the same fashion except that in addition to epinephrine (0.1 μg/ml), insulin (1000 μunits/ml) and glucose (3 mg/ml) were present (Figure 3). Now striking differences are apparent in the data when adipose tissues from animals with dissimilar thyroid function are compared even when the initial values are subtracted and the data expressed on a nitrogen basis. The glycerol released from the hyperthyroid group is 180% that of the normal, whereas the hypothyroid is only 18% of that seen in the normal. In like manner, the oxygen uptake of the hyperthyroid group is 129% that of the euthyroid, while the hypothyroid group is but 51% of the control. As indicated by the values for FFA, which project beneath the base line, not only that FFA released during the incubation, but a portion of that present initially, has been reesterified. As will be shown subsequently, the increased oxygen uptake of the hyperthyroid compared to that of the normal tissue appears to be entirely due to the quantitatively greater reesterification rate of the former. The lower oxygen uptake of the hypothyroid samples similarly reflects the lower oxygen requirements for reesterification of those tissues, although it may be explained in part by a lower "basal" oxygen uptake for the hypothyroid group.

Inhibition of Spontaneous Lipolysis by Insulin. In

Figure 4 are seen the effects of incubation in the presence of 1000 μunits of insulin/ml on spontaneous lipolysis and oxygen consumption. For contrast, the previously cited control data (*cf.* Figure 1) has been replotted next to that of the insulin data. It is clear from these data that insulin is capable of inhibiting lipolysis irrespective of the thyroid status of the donor animals. As judged by the glycerol release values, the hypothyroid and normal tissues were inhibited 50 and 59%, respectively, while the hyperthyroid was inhibited only 22%. Oxygen uptake was no different in the control and insulin experiments for the hypo- and euthyroid groups. This is probably explained by the fact that, while lipolysis was decreased the by presence of insulin, the proportion of FFA reesterified was increased and the absolute amount of reesterification remained approximately the same. The reason for the apparent rise in oxygen consumption seen with insulin in the hyperthyroid group is not entirely clear. Both the percentage and absolute amount of FFA reesterified was higher in the presence of insulin. The change in total amount reesterified was, however, not great enough to account for the increase seen in the O_2 consumption. It is possible in the control experiments that a greater accumulation of FFA within the tissue results in an inhibition of O_2 consumption (Hagen and Ball, 1961). The rate of oxygen consumption in these experiments was, however, linear with time.

Response to Epinephrine over a Range of Concentrations. As demonstrated earlier (*cf.* Figure 3), when incubations are performed in the presence of excess glucose and insulin, the fatty acids released by lipolysis are quickly reesterified. So long as the rate of esterification equals or exceeds the rate of lipolysis, accumulation of FFA in the adipose samples is prevented. This system has been utilized to study the effect of thyroid status on the response to epinephrine over a wide range of concentrations. In Figure 5 glycerol release is plotted linearly on the ordinate against logarithmic increases in epinephrine concentration on the abscissa. The number in parentheses next to each point is the average net change in tissue FFA during the incubation (FFA concentration at the end of the incubation minus the initial concentration).

Without addition of epinephrine, the glycerol release under these circumstances was virtually identical for the hypothyroid and normal tissues and only slightly higher for the hyperthyroid group. A concentration of 0.01 μg/ml of epinephrine had no significant effect on lipolysis in either the normal or hyperthyroid groups and, for that reason, no experiments were performed at that concentration of epinephrine with hypothyroid tissue. Over the concentration range of 0.01–1.0 μg/ml, the normal tissue exhibited a linear increase in glycerol release (when plotted in this semilogarithmic fashion). A tenfold increase beyond that, however, produced no further increase in lipolysis, but a fall. The response of hypothyroid tissue paralleled that of normal tissue between epinephrine concentrations of 0.1 and 1.0 μg/ml, but required approximately eight times as much epinephrine for a comparable glycerol liberation. As with the normal tissue, the response of the hypothyroid declined when the epinephrine concentration was increased to 10.0 μg/ml. At an epinephrine concentration of 0.1 μg/ml, the lipolytic response of the hyperthyroid tissues was significantly higher than that of the normal, but an increase to 1.0 μg/ml produced only a small additional increment in average glycerol release.

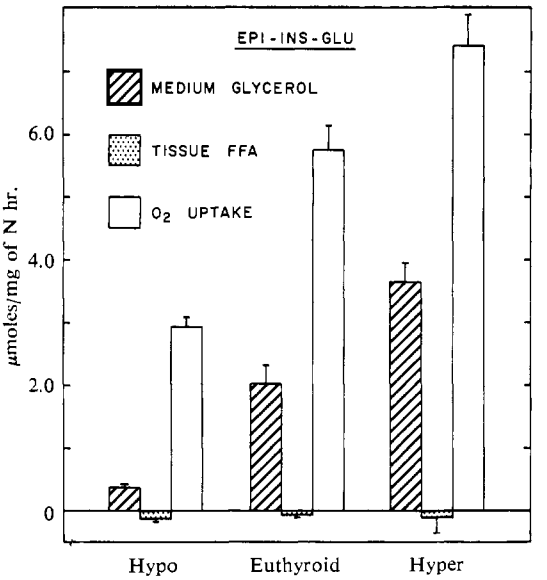


FIGURE 3: Epinephrine-stimulated lipolytic and oxygen consumption rates of adipose tissue from hypo-, eu-, and hyperthyroid rats in a system permitting reesterification of liberated FFA. The data are presented as in the right half of Figure 1. Insulin (1000 μunits/ml) and glucose (3 mg/ml) were present in the medium at the beginning of the incubation, in addition to epinephrine (0.1 μg/ml). Each bar represents the average of seven observations and the vertical line above each bar indicates one standard error of the mean.

Potentiation of Lipolysis with Aminophylline. Also plotted in Figure 5 are experiments which demonstrate the ability of aminophylline to greatly potentiate the effect of epinephrine on lipolysis of adipose tissue from hypo- and euthyroid rats. In the experiments depicted, in addition to epinephrine at the indicated concentrations, aminophylline was added from the side arms of the vessels at the beginning of the incubations for a final concentration of 10^{-4} M. It is of interest to note that a large accumulation of FFA took place in the experiment with normal tissue in which aminophylline and epinephrine (1.0 μg/ml) were present. Under the same conditions the FFA accumulation in hypothyroid tissue was only 10% of that seen in the normal even though a greater release of glycerol occurred. In experiments not shown here it was found that the effect of 10^{-4} M aminophylline alone on hypothyroid tissue was at best no more than that produced by 0.1 μg/ml of epinephrine alone. No

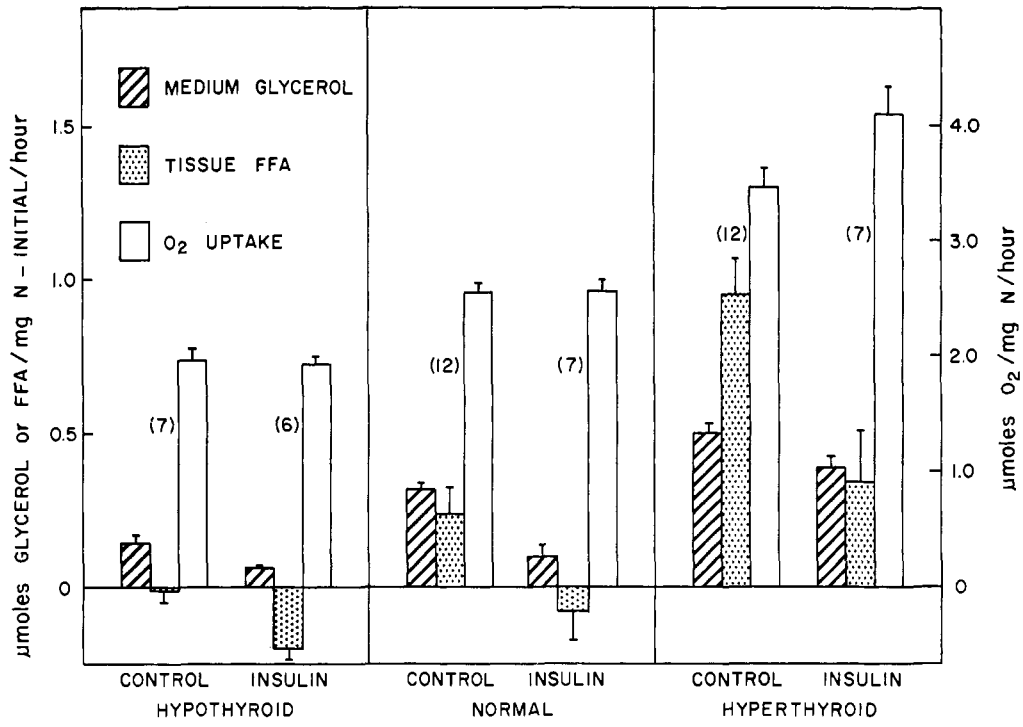


FIGURE 4: Inhibition by insulin of spontaneous lipolysis of adipose tissue from hypo-, eu-, and hyperthyroid rats. The "control" data in the left-hand portion of each set are identical with those presented in the right half of Figure 1. The "insulin" experiments represent the effect on lipolysis and oxygen consumption when insulin (1000 μunits/ml) was added at the beginning of the incubation. The vertical line above each bar represents one standard error of the mean. The number of determinations in each group of experiments is represented by the number in parentheses.

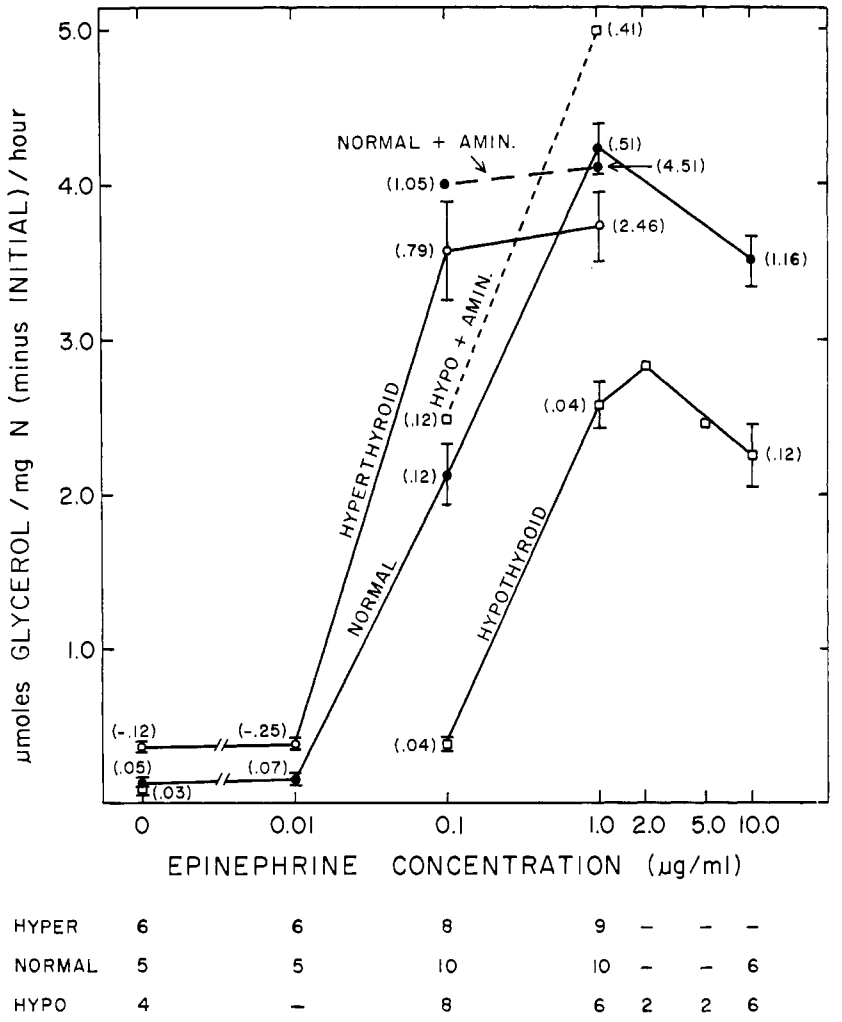
experiments were performed to assess the effect of aminophylline on adipose tissue from hyperthyroid animals.

Relation of Oxygen Consumption to FFA Reesterification. In Figure 6, the micromoles of oxygen consumed per hour are plotted on the ordinate *vs.* the micromoles of FFA reesterified per hour on the abscissa. The latter data were calculated as follows. It was assumed that for each micromole of glycerol released, 3 μmoles of FFA had been liberated. From that value was subtracted the measured net change in tissue FFA. For example, the glycerol release from the hyperthyroid tissue at an epinephrine concentration of 0.1 μg/ml was 3.58, and the net change in tissue FFA was +0.79 μmole/mg of tissue nitrogen per hr. Therefore, 3.58 × 3 = 10.74 − 0.79 = 9.95 μmoles of FFA reesterified/mg of adipose tissue nitrogen per hr.

For the purposes of discussion a line has been arbitrarily drawn on this figure which has been fitted by eye to the data calculated for normal and hyperthyroid tissue. Less weight has been given to values calculated for those situations in which considerable accumulation of FFA occurred in the tissue (*cf.* Figure 5). The slope of this line can be considered as a measure of the efficiency of the use of oxygen for reesterification of FFA: the greater the slope, the lower the efficiency. Inspection of the data in relation to this line clearly indicates that the efficiency of this process in the hyperthyroid tissue does not differ significantly from that for normal tissue. Thus, as measured in this manner, there is no indication that uncoupling of oxidative phosphorylation occurs in hyperthyroid adipose tissue.

The slope of the line drawn in Figure 6 corresponds to a process in which 1.04 μmoles of FFA is esterified for each atom of oxygen consumed. Assuming that virtually all of the FFA was esterified back to triglyceride, seven high-energy phosphate bonds would be required to form each molecule of triglyceride, six for the production of three acyl-CoA molecules and one for the production of glycerol phosphate. This is equivalent to 2.33 high-energy phosphate bonds/molecule of FFA esterified. Using these values a P:O ratio of 2.42 (1.04 × 2.33) may be calculated. As indicated in Figure 6, if the tissue were utilizing glucose exclusively as an oxidative substrate, an ideal P:O ratio of 3.17 might be expected. On the other hand, if a fatty acid were the sole substrate (and here palmitic acid is the example in the illustration), a somewhat lower ideal P:O ratio would ensue. In isolated mitochondria P:O values in the range 2.5–2.7 have been reported (Koivusalo and Slater, 1965; Van Dam, 1964).

The values for the hypothyroid tissues all fall below that for the normal and hyperthyroid tissues and along a line roughly parallel to that drawn in Figure 6. If the values are extrapolated back to the ordinate,



HYPER	6	6	8	9	–	–	–
NORMAL	5	5	10	10	–	–	6
HYPO	4	–	8	6	2	2	6

FIGURE 5: Response of adipose tissue over a range of concentrations of epinephrine. Insulin (1000 μunits/ml) and glucose (3 mg/ml) were present in all experiments. The ordinate gives the lipolytic rate in terms of glycerol released per milligram of tissue nitrogen per hour after initial values have been subtracted. The number in parentheses by each point is the average net FFA value (final minus initial concentration). The vertical lines designate the standard errors of the means. The number of observations represented by each point is indicated in the legend just below the abscissa except for the experiments where 10^{-4} M aminophylline (amin) was added where each point represents the average of four observations. At a concentration of 0.1 μg/ml of epinephrine, the average glycerol release ± one standard error of the mean was 2.45 ± 0.07 and 4.00 ± 0.22, for the hypothyroid plus aminophylline and normal plus aminophylline, respectively. At a concentration of 1.0 μg/ml of epinephrine these values are 4.98 ± 0.27 and 4.11 ± 0.29.

figures for oxygen consumption are obtained which might be considered the "basal" requirement of the tissues at a point where no reesterification is occurring. Thus, this "basal oxygen consumption" for the hypothyroid tissue would appear to be distinctly lower than that of the normal, while that of the hyperthyroid would seem to be about the same as the normal.

In constructing Figure 6 the assumption was made that the amount of FFA reesterified may be estimated by subtracting tissue FFA from three times the value of the medium glycerol and that the glycerol incorporated into the triglyceride so formed is derived from tissue glycogen or medium glucose. In order to obtain a check upon the validity of this assumption we have performed experiments in the presence of glucose-U-^{14}C and measured the radioactivity of tissue glyceride glycerol. The results of these experiments in which hyperthyroid tissue was employed are shown in Table II. Measurements of glycerol release and tissue FFA were performed in the usual manner, and the amount of FFA reesterified as calculated from these data is given in the last column. Radioactivity of glyceride glycerol was determined in two ways. First, the difference between the radioactivity of total lipids as extracted with chloroform–methanol (3:1) and the radioactivity of the fatty acids released therefrom by saponification

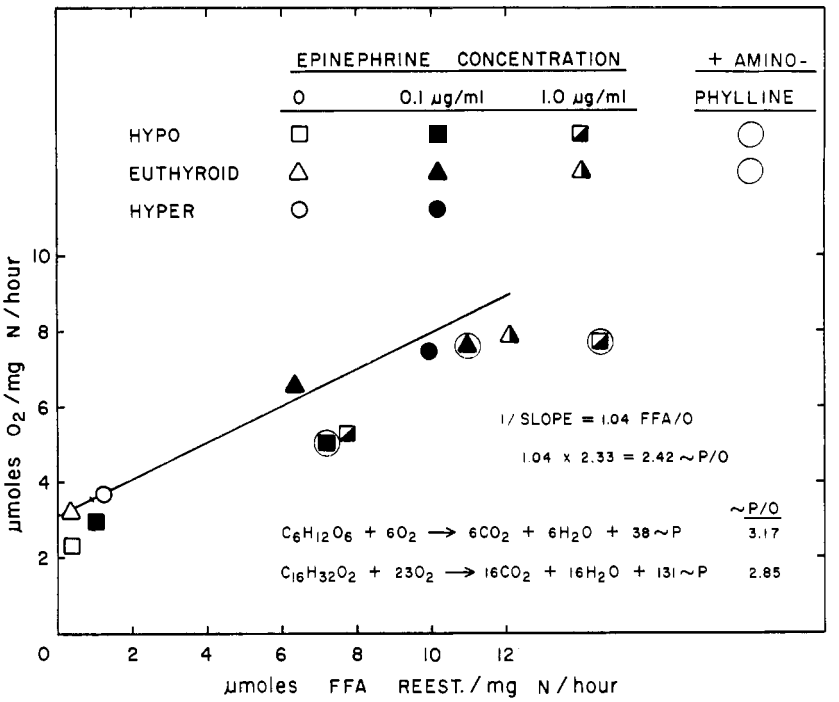


FIGURE 6: Relation of oxygen consumption to FFA reesterification. Oxygen consumption in micromoles per milligram of tissue nitrogen per hour was measured in the experiments depicted in Figure 5. Explanation of the method for calculating FFA reesterification is given in the text.

was measured. Second, the glycerol released in this saponification was treated with periodate and the formaldehyde formed was precipitated as the dimedone derivative. It should be noted that *de novo* synthesis of fatty acids from glucose under the experimental conditions employed was small. The radioactivity found in the fatty acid fraction averaged about 10% of that for the total lipid extract. Thus, if a fatty acid chain length of 17 carbons is assumed, only about 0.6% of the total lipid counts represents glycerol utilized to esterify newly synthesized fatty acids. We have, therefore, made no attempt to correct the data for this amount. The glyceride glycerol values have been multiplied by three in order to obtain the amount of FFA reesterified, and there is reasonably good agreement between the values obtained by the two procedures. The glyceride glycerol values are, however, at best only 73% of those calculated by the indirect method. The reason for this difference is not clear. Utilization of tissue glycogen for formation of glycerol-phosphate is a possibility, though the results given in Figure 2 do not favor such an interpretation in the case of hyperthyroid tissue. It is evident, nevertheless, that the results support the conclusion that reesterification of FFA is actively proceeding under the conditions of our experiments.

Discussion

The results presented here indicate that the diminished response to the lipolytic action of epinephrine seen in adipose tissue from hypothyroid rats is not due to a deficiency in the amount of tissue lipase capable of being activated. In the presence of aminophylline and epinephrine, hypothyroid tissue displayed rates of glycerol release that equaled or exceeded those shown by normal or hyperthyroid tissue under any experimental conditions. Therefore, it would appear that the influence of thyroid hormones on the lipolytic process is not due to any significant effect upon the lipase content of the tissue *per se* but must be sought in the process by which the lipase is activated. One possibility is that the rate at which epinephrine is inactivated in the tissue is under the control of thyroid hormones. This seems unlikely in view of the fact that the subnormal response of hypothyroid tissue is not increased by raising the epinephrine concentration to levels above those considered to be physiological. Moreover, it may be noted that the rate of oxygen consumption remained essentially linear throughout the experiments with hypothyroid tissue even at the highest epinephrine concentrations. This indicates that lipolysis rates were also linear throughout the experiment. If excessive destruction of epinephrine or the accumulation of inhibitory degradative products of the hormone (*cf.* Ho *et al.*, 1965) were occurring, then a progressive decline might be expected. The mechanism by which the lipase is activated thus deserves consideration. In this process it would appear that cyclic AMP is a secondary mediator of the action of epinephrine (Butcher *et al.*, 1965). Thyroid hormones might, therefore, influence the level of cyclic AMP that can be attained within

TABLE II: Estimation of Fatty Acid Esterification.[a]

Determination	Initial Value (μmoles/mg of N)	Exptl Value (μmoles/mg of N/ hr)	Exptl − Initial Value	FFA Reesterified
Medium	0.23 (3) (0.21–0.25)	1.90 ± 0.16 (6)	1.67	
Glycerol				
Tissue FFA	0.43 (3) (0.38–0.48)	0.93 ± 0.30 (6)	0.50	4.41
Glyceride glycerol				
By difference	0.20 (3) (0.15–0.21)	1.27 ± 0.13 (6)	1.07	3.21
Dimedone	0.19 (3) (0.14–0.23)	1.17 ± 0.11 (6)	0.98	2.94

[a] Experiments were performed on hyperthyroid tissue in the same manner as those given in Figure 5 and with epinephrine at a concentration of 0.1 μg/ml. Each flask contained approximately 1.0 μc of glucose-U-^{14}C; total glucose concentration 3.0 mg/ml. Three sets of experiments were run in each of which matched tissue was distributed between two experimental flasks and a flask for initial values. The values in parentheses following the average values represent the total number of determinations. In the case of the initial values the range is given in the second set of parentheses while the experimental values are given with one standard error. See text for further details.

adipose tissue, the amount increasing as the thyroid hormone level rises. Such a control could be accomplished by an alteration either in the rate of formation or rate of destruction of cyclic AMP. For example, the destruction of cyclic AMP is governed by the activity of a specific phosphodiesterase which is inhibited by the methylxanthines (Butcher and Sutherland, 1959). Thus, the ability of aminophylline to restore normal lipolytic activity to hypothyroid tissue could be interpreted as due to its action in suppressing a hyperactive phosphodiesterase. On the other hand the phosphodiesterase activity in this tissue may be normal and its suppression may merely compensate for a subnormal activity of the cyclase which forms cyclic AMP. The results presented here do not permit a firm choice between these two possibilities. The fact that insulin displays less antilipolytic action on hyperthyroid tissue than on normal tissue (*cf.* Figure 4) could be interpreted as an indication that there is an increased activity of the cyclase in the hyperthyroid tissue. This would follow from the fact that in normal tissue the antilipolytic action of insulin diminishes as the epinephrine concentration and, hence, presumably the cyclase activity increases (Jungas and Ball, 1963).

Brodie *et al.* (1966) in a recent review article report data from experiments cited as unpublished from which they have reached conclusions similar to ours. In addition these investigators have measured the adenylcyclase activity of hyperthyroid adipose tissue and found that it is markedly increased over that of the normal. If the action of thyroid hormones is upon adenylcyclase activity then it would appear to be a delayed one. This is because the addition of thyroid hormones to rat adipose tissue *in vitro* produces no immediate effects upon lipolysis rates, as shown by Debons and Schwartz (1961). We have confirmed this observation, but in preliminary studies we have found that when incubation with triiodothyronine of adipose tissue from hypothyroid rats is extended to 14 hr in the presence of an amino acid mixture, some enhancement of lipolytic rates may be detected. The action of thyroid hormones on the lipolytic process may thus be by way of a primary action in regulating protein synthesis (in this case the enzyme adenylcyclase), as suggested by the studies of Tata (1963) and Weiss and Sokoloff (1963).

A function for the thyroid hormones in regulating the cyclic AMP level in adipose tissue would add another facet to the central role that cyclic AMP appears to play in governing metabolic rates in this tissue. The key to the level of cyclic AMP in this tissue would appear to be adenylcyclase, the activity of which may be influenced by a number of hormones. Thus, the absolute amount of the inactive form of this enzyme in the tissue may be under the control of the thyroid hormones. The amount of enzyme present in an active form would depend upon the levels of those hormones (catecholamines, glucagon, ACTH, etc.) which activate it in relation to the level of insulin which counteracts their action. In turn the level of cyclic AMP within the tissues that results from the interplay of the action of these hormones would govern the activity of such diverse enzymes in the tissue as lipase, phosphorylase, glycogen synthetase, and phosphofructokinase (*cf.* Jungas, 1966).

The maximum rate of lipolysis as measured by glycerol production in these studies is 5.0 μmoles/mg of tissue nitrogen per hr obtained with hypothyroid tissue in the presence of epinephrine and aminophylline (*cf.* Figure 5). Somewhat lower rates were observed with normal and hyperthyroid tissue. It should be noted that when rates of this magnitude are approached they appear to have reached a plateau value, at least in the case of the normal and hyperthyroid tissue. At the same time FFA begin to accumulate in the tissue. Since FFA may inhibit the lipolytic process it is thus possible that the maximum lipolytic rates to be measured by our procedure are rates limited by

the tissue's ability to reesterify FFA. The value of 4 μmoles of glycerol released/mg of tissue nitrogen per hr observed by us for hyperthyroid tissue corresponds to about 23 μmoles of glycerol or 69 μequiv of FFA/g wet wt per hr (*cf.* Table I). This value may be compared with the maximum rates of around 6 μequiv of FFA/g wet wt per hr observed by Debons and Schwartz (1961) and 19 μequiv by Deykin and Vaughan (1963). These investigators carried out their experiments in bicarbonate buffer containing albumin, conditions under which the released FFA concomitantly accumulates in both the tissue and medium, as the experiment progresses. Rosenfeld and Rosenberg (1965) have reported a maximum average value of 9.3 μequiv of FFA released/g wet wt per hr for hyperthyroid tissue. These investigators employed Krebs–Ringer phosphate buffer containing 5% albumin and 10 μg of epinephrine/ml. Goodman and Bray (1966) found tissue from both normal and thyroidectomized rats to release 10–11 μmoles of glycerol or the equivalent of 30–33 μequiv of FFA/hr per g wet wt. These investigators employed Krebs bicarbonate buffer containing 4% serum albumin and 1 mg/ml of glucose, conditions under which some reesterification of FFA is favored. It thus is evident that a wide range of maximum values has been reported for lipolytic rates. A part of this variation in results may be in the difference in age of the animals used by various investigators (*cf.* Benjamin *et al.*, 1961), but the major cause seems most likely to reflect the experimental conditions, especially as they affect the fate of the released FFA. The data presented here in Figures 2 and 3 would support this conclusion.

There is a wide divergence in the results that various investigators have reported on the response of adipose tissue from hypothyroid rats to the lipolytic action of epinephrine. Using rats treated with propylthiouracil, little or no response as measured by glycerol release was found by Deykin and Vaughan (1963) at an epinephrine concentration of 0.1 μg/ml or by Debons and Schwartz (1961) at 2.5 μg/ml. We found only a slight response of hypothyroid tissue to 0.1 μg/ml of epinephrine but a definite and maximum response was obtained at 1.0 μg/ml. Rosenfeld and Rosenberg (1965) using tissue from thyroidectomized rats found an increase in FFA release in response to 0.005 μg/ml of epinephrine that was percentagewise greater than the response shown by normal tissue. Goodman and Bray (1966) also reported a good response of tissue from thyroidectomized rats to epinephrine. The results of these investigators are unusual in that as measured by both glycerol and FFA release they found that both normal and hypothyroid tissue gave responses that continuously increased as the concentration of epinephrine was raised stepwise from 0.05 μg to 50 μg/ml. At the latter concentration the response of tissue from normal and thyroidectomized animals was practically identical at about 8.0 μmoles of glycerol/g per hr. These authors reported that the feeding of propylthiouracil to thyroidectomized rats did not prevent a lipolytic response to epinephrine. A careful comparison of the response to epinephrine of tissue from propylthiouracil-treated and thyroidectomized rats, however, would be desirable.

The degree of reesterification of FFA that is seen in adipose tissue incubated in buffer without added insulin and glucose appears to vary with the thyroid status of the animal. As may be seen in Figure 1, when calculations are made on a nitrogen basis after initial values are subtracted, 100% of the FFA released by hypothyroid tissue is reesterified, as compared with 75 and 37% for the eu- and hyperthyroid tissue, respectively. Even with the addition of 0.1 μg/ml of epinephrine (Figure 2) the hypothyroid tissues showed 34% reesterification, whereas the other tissues showed none. This difference would not appear to be due to any changes in the tissue content of enzymes concerned in the reesterification process since this difference is not seen when insulin and glucose are present. Goodman and Bray (1966) reported greater rates of reesterification in tissue from thyroidectomized than from normal rats when the tissue was incubated in the presence of epinephrine and glucose. Such differences in results may reflect the relative glycogen content of the tissues employed, which in turn would govern the supply of glycerophosphate needed for the reesterification process.

A number of workers have previously reported a lowered efficiency of oxidative phosphorylation either in mitochondria isolated from hyperthyroid rats or in normal mitochondria to which thyroid hormones have been added *in vitro* (*cf.* Hoch, 1962). More recently, Tata *et al.* (1963) in a careful study reported no difference in the P:O ratios obtained with liver or muscle mitochondria isolated from normal or hyperthyroid rats. Our results indicate that no lowering of the efficiency of oxidative phosphorylation is demonstrable in adipose tissue from the hyperthyroid rat. To the best of our knowledge this is the first time that such a study has been made with intact tissue. Our results may be interpreted to discredit the long-held notion that impairment of phosphorylation mechanisms in the hyperthyroid state gives rise to increased rates of basal oxygen consumption. We believe such an interpretation should be made, however, with some caution. This is due to the fact that in the hyperthyroid state as demonstrated here and by others there is increased mobilization of fatty acids as well as higher FFA serum levels (Rich *et al.*, 1959). Fatty acids are known to be potent uncouplers of oxidative phosphorylation (Pressman and Lardy, 1956). Thus, it is not impossible that in the hyperthyroid animal some uncoupling of oxidative phosphorylation occurs by reason of the elevated FFA levels that result from the primary action of thyroxine on lipolytic processes. Such an uncoupling effect of FFA is not likely to be seen in our experiments since they were designed purposely to permit rapid reesterification of the released FFA.

One other point should perhaps be made in relation to enhanced FFA mobilization and increased basal oxygen consumption displayed by the hyperthyroid

animal. This is the possibility that an increased oxygen consumption under such conditions results from an increase in the quantity of FFA undergoing esterification to triglyceride. As discussed in more detail elsewhere (Ball and Jungas, 1961, 1964; Ball, 1965), a cyclic process of triglyceride breakdown and resynthesis is not unlike the effect to be achieved by an uncoupling of oxidative phosphorylation. The energy utilized in this process must largely appear as heat since no net energy gain has resulted. In the studies reported here it is of interest to note that the unstimulated (no added epinephrine) rate of oxygen consumption of hyperthyroid adipose tissue is about 15% greater than that of euthyroid tissue (*cf.* Figure 6). This increased rate appears to be entirely due to an enhanced rate of FFA reesterification and not to any change in the basal rate of hyperthyroid tissue relative to the normal. If a similar situation exists in the whole animal and tissues other than adipose also display an enhanced rate of FFA reesterification, then such a process could contribute to the well-known increase in basal metabolism inherent to the hyperthyroid state.

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