Studies on the Metabolism of Adipose Tissue. XVII. *In vitro* Effects of Insulin upon the Metabolism of the Carbohydrate and Triglyceride Stores of Adipose Tissue from Fasted-refed Rats*

ROBERT L. JUNGAS AND ERIC G. BALL

From the Department of Biological Chemistry, Harvard Medical School, Boston, Mass.

Received July 30, 1964

Adipose tissue from fasted-refed rats exhibits a high rate of glycerol release and oxygen consumption when incubated *in vitro* in the absence of added substrate. The free fatty acid content of the tissue remains low throughout the incubation. The addition of insulin, 1000 μunits/ml, inhibits glycerol production 85% and oxygen consumption 30% while free fatty acid values are unaffected. These results are interpreted to indicate a high rate of spontaneous hydrolysis of triglyceride to fatty acids and glycerol in this tissue and the inhibition of the process by insulin. The low fatty acid values indicate that rapid re-esterification of this product to triglyceride must occur with glycerophosphate furnished by the abundant glycogen stores found in this tissue. The decrease in oxygen consumption produced by insulin is to be accounted for by the diminished demand for high-energy phosphate needed for re-esterification purposes attendant upon the curtailed lipolytic process. Lactate production in this tissue is also high and this process is likewise inhibited by the presence of insulin. Concomitant with the decreased lactate and glycerophosphate production which results from insulin addition, a diminished disappearance of glycogen from the tissue during the incubation period can be demonstrated. Under anaerobic conditions the formation of lactic acid from glycogen stores proceeds even more rapidly and the presence of insulin slightly enhances this rate. The acceleration in lactic acid formation produced by the addition of epinephrine under anaerobic conditions is less marked if insulin is present. Measurements of oxygen consumption and net gas exchange corrected for lactic acid formation permit the calculation of total CO_2 production and RQ of the tissue during its incubation in bicarbonate-buffered medium. The corrected net-gas-exchange value is doubled and the RQ rises from 1.37 to 1.87 in the presence of insulin. These results are interpreted to indicate that the carbohydrate stores of the tissues are converted to fatty acids during the incubation. The data suggest an acceleration of fatty acid synthesis upon the addition of insulin but additional data are necessary to establish this point. Adipose tissue from fasted-refed rats thus provides a useful means for the study of certain actions of insulin which are independent of the passage of glucose across the cell membrane.

Insulin has been shown under certain circumstances to inhibit the production of glycerol by rat epididymal adipose tissue incubated *in vitro* (Jungas and Ball, 1962). Since there is good evidence that glycerol is produced in adipose tissue largely if not entirely by the process of triglyceride hydrolysis, this effect of insulin has been interpreted to indicate that insulin can reduce the rate of lipolysis in this tissue (Jungas and Ball, 1963). This antilipolytic action of insulin was seen most dramatically upon the addition of insulin to adipose tissue incubated in a medium containing epinephrine but no glucose. In order to investigate further this property of insulin, we have chosen to study adipose tissue removed from rats which have been fasted and then refed. Such fasted-refed tissue exhibits a high rate of spontaneous lipolysis (Ball and Jungas, 1963), and the antilipolytic action of insulin therefore can be studied conveniently without the concomitant addition of a lipolytic hormone to the media. These studies have led to the findings to be described here, which show that the addition of insulin *in vitro* to adipose tissue from fasted-refed rats markedly alters the metabolism of both the triglyceride and carbohydrate stores of this tissue.

METHODS

The rats employed were obtained from the Holtzman Co., Madison, Wis. After arrival they were maintained on Purina laboratory chow ad libitum for 1–2 weeks before use. Rats described as fasted-refed were fasted for 3 days and refed 2 days with the fat-free test diet of Wooley and Sebrell (1945). All rats weighed from 150 to 200 g at the time of use. The epididymal fat bodies of 2 rats were used for each experiment and randomized among the vessels used in a manner similar to that previously described (Jungas and Ball, 1963). The tissue samples in all vessels were thus as nearly alike as possible with regard to their composition and metabolic rates. Approximately 100 mg of tissue was used in each vessel.

Incubations were carried out under three different conditions. Most measurements of oxygen uptake reported here were made according to the method of Pardee (Umbreit *et al.*, 1957a), using a bicarbonate buffer of the following composition: 9.6 mM $NaHCO_3$, 135.5 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4. To distinguish this medium from the more common Krebs-Henseleit (1932) bicarbonate medium containing 25 mM bicarbonate, it will be referred to as the "Pardee bicarbonate" medium. This buffer was gassed with a mixture of 1.9% CO_2–98.1% air to yield a *p*H of 7.4. Incubation was performed in Warburg vessels of approximately 7 ml total volume. The vessels contained 1.00 ml of liquid in the main chamber plus side arm and 0.30 ml of CO_2 absorbent in the center well. This absorbent consisted of 4 M diethanolamine containing 0.1% thiourea as an antioxidant. The absorbent and incubation medium were well gassed with the 1.9% CO_2 mixture immediately before use. After the vessels were placed in the

* This work was supported in part by funds received from the Eugene Higgins Trust through Harvard University, the Life Insurance Medical Research Fund, and grants (A-3132) and (AM-08076) from the U. S. Public Health Service.

TABLE I

EFFECT OF INSULIN ON GLYCEROL PRODUCTION AND OXYGEN CONSUMPTION OF ADIPOSE TISSUE[a]

Experimental Conditions				Glycerol Release (μmoles/100 mg/2 hr)			Oxygen Consumption (μmoles/100 mg/2 hr)		
Dietary Regime	Buffer	Added Substrate	Number of Expts	Control	Insulin	Decrease (%)	Control	Insulin	Decrease (%)
Fasted-refed	Pardee-HCO_3	None	7	2.98 ± 0.22	0.46 ± 0.06	85	8.85 ± 0.33	6.10 ± 0.23	31
Same	Same	Glucose	2	3.82	0.45	88	11.0	7.0	36
Same	Krebs-PO_4	None	4	2.18	0.52	76	8.64	5.30	39
Normally fed	Same	None	5	0.24	0.10	58			
Fasted overnight	Same	None	5	0.28	0.30	0			

[a] Results are the average plus or minus the standard error of the number of experiments listed. When present the concentration of glucose was 3 mg/ml and insulin 1000 μunits/ml. Food was removed from the cages of the fasted rats at 9 P.M. and the animals were sacrificed at 10 A.M. the next morning. The lights in the animal room are automatically controlled so as to be on from 6 A.M. to 9:30 P.M.

bath they were gassed for 15 minutes and allowed to equilibrate for an additional 5 minutes. Zero-time readings were then taken and the incubation was allowed to proceed for another 2 hours. The oxygen-uptake measurements during the first 20 minutes of the incubations were found to be unreliable owing to the slow equilibration of the CO_2 absorbent with the gas phase. The rates given for the 2-hour period were obtained, therefore, by multiplying the values obtained for the period 20–120 minutes by the factor 1.2. It was also necessary to apply a small correction to the oxygen-uptake values to compensate for the slow rate of autoxidation of the CO_2 absorbent and for incomplete retention of CO_2 by the absorbent. This correction amounted to 4.8 ± 0.3% for control tissues and 5.3 ± 0.3% for tissues incubated with insulin.

When it was desired to incubate the tissue under anaerobic conditions the Krebs-Henseleit bicarbonate medium (Krebs and Henseleit, 1932), containing half the amount of calcium employed by these workers, was used. The gas phase was 5% CO_2–95% nitrogen and Warburg vessels were used in the manner described for oxygen-uptake measurements.

In a few experiments the tissue was incubated in Krebs-Ringer phosphate medium (Umbreit *et al.*, 1957b), again using half the recommended calcium. In these experiments the gas phase was air and 0.20 ml of 10% KOH was placed in the flask center wells.

Assays for tissue free fatty acids and medium glycerol and lactate were performed as previously described (Ball and Jungas, 1963). In some cases lactate and glycerol production were measured by assay of the medium at the end of the experiment and represent the amount formed during both the 20-minute equilibration and gassing period and the 2 hours during which manometric readings were made. In these cases the values obtained have been multiplied by 0.857 to reduce them to a 2-hour basis and thus make them comparable to the manometric data. Tissue "glycogen" was measured in the following way. The tissue was extracted with 5 ml of chilled $CHCl_3$–CH_3OH (2:1, v/v) in a centrifuge tube. Following centrifugation the solvent was carefully removed with a capillary pipet and the tissue residue was digested with 2 ml of 2 N HCl for 1 hour at 100°. The digest was transferred quantitatively to volumetric flasks and diluted to the mark, and suitable aliquots were assayed for glucose by the use of the glucose oxidase reagents supplied by Worthington. Since the tissue glycogen was not purified prior to hydrolysis the observed glucose values may include a contribution from tissue sources other than glycogen. Epinephrine and insulin stock solutions were prepared and diluted as previously described (Ball and Jungas, 1963).

RESULTS

The effects of insulin on the oxygen consumption and glycerol production of tissue from fasted-refed rats are illustrated by the results given in Table I. When this type of tissue is incubated in the Pardee bicarbonate medium with or without glucose, a very high rate of both oxygen consumption and glycerol release is observed. The addition of insulin (1000 μunits/ml) results in an 85% inhibition of the amount of glycerol released into the medium and reduces oxygen consumption by about 30%. Similar results are seen when a Krebs-Ringer phosphate medium is used. Measurements of free fatty acids in the tissue at the end of these experiments have yielded values which average 0.18 μmole/100 mg regardless of the presence or absence of insulin.

The spontaneous release of glycerol by adipose tissue from normally fed rats incubated in a Krebs-Ringer phosphate buffer is less than 10% of that seen with tissue from fasted-refed rats, though its production is likewise inhibited by insulin (see Table I). When rats from the same batch as those used to obtain the data given for normally fed rats were fasted overnight, the data of Table I show that glycerol release is not increased but the ability of insulin to suppress this release is lost. As shown previously by Jungas and Ball (1963), a 2- or 3-fold increase in the spontaneous glycerol release occurs from tissue taken from rats fasted for longer periods (3 days), and this release is not inhibited by insulin.

In Table II data are presented from experiments in which the lactate release, oxygen consumption, and net gas exchange of adipose tissue from fasted-refed rats was measured in the presence and absence of insulin. Also given in this table are the production of total and metabolic CO_2 and RQ values which have been calculated as described in the legend to Table II. Four vessels were employed in each experiment, two for monitoring net gas exchange and two for measuring oxygen uptake. One vessel of each pair served as control, while the other contained insulin (1000 μunits/ml) which was added to the medium prior to the start of the experiment. The Pardee bicarbonate medium was employed in all vessels.

The data of Table II show that spontaneous lactic acid production by tissue from fasted-refed rats in the

TABLE II

EFFECT OF INSULIN ON LACTATE AND RESPIRATORY GASES OF ADIPOSE TISSUE FROM FASTED-REFED RATS[a]

Experiment	Lactate (μmoles)	O_2 Uptake (μl) Observed (O_2)	O_2 Uptake (μl) Corrected (X_{O_2})	Net Gas Exchange (μl) Observed (NGE)	Net Gas Exchange (μl) Corrected	Total CO_2 Production (μl) (X_{CO_2})	Metabolic CO_2 (μl)	RQ	HCO_3^- Excess (μmoles)	Lactate Deficit (μmoles)
(1) Control	1.32	214	224	81	52	332	303	1.35		
Insulin	0.24	132	139	115	110	271	266	1.91		
(2) Control	2.53	185	195	99	43	317	261	1.34		
Insulin	1.92	150	159	121	78	299	256	1.61		
(3) Control	0.74	174	181	60	43	263	246	1.36		
Insulin	0.12	119	125	104	101	245	242	1.94		
(4) Control	1.29	154	161	73	54	253	224	1.39		
Insulin	0.21	132	138	94	89	249	244	1.77	1.29	1.26
(5) Control	2.28	175	184	95	44	301	250	1.36		
Insulin	0.44	126	133	120	110	269	259	1.95	2.08	2.15
(6) Control	1.24	194	204	89	61	318	290	1.42		
Insulin	0.24	113	119	110	105	244	239	2.01	1.17	1.17
Average										
Control	1.57		192	83	49	297	262	1.37		
Insulin	0.53		136	111	99	263	251	1.87		

[a] All values given are for 100 mg wet wt of tissue. The values have been adjusted to a 2-hour basis as explained in the text, except for the data given under the headings HCO_3^- Excess and Lactate Deficit. These data represent the actual values observed for the total experimental period of 140 minutes. The values given for lactate were obtained on the medium in the vessels used for the measurement of net gas exchange.

The equations used for obtaining the calculated values shown in the table were derived as follows. We may write for any vessel, $\Delta h = \Delta h_{CO_2} - \Delta h_{O_2}$. This equation states that the net change in gas pressure, Δh, may be considered the resultant of a positive term representing CO_2 released into the vessel atmosphere and a negative term due to O_2 consumption. Writing X_{CO_2} for the total CO_2 production in microliters, X_{O_2} for the oxygen consumption, and k for the flask constants, we have for the vessels containing no CO_2 absorbent

$$\Delta h = \frac{X_{CO_2}}{k_{CO_2}} - \frac{X_{O_2}}{k_{O_2}}$$

The measured net gas exchange, NGE, is given by $\Delta h \cdot k_{CO_2}$ and thus

$$\text{NGE} = X_{CO_2} - \frac{k_{CO_2}}{k_{O_2}} \cdot X_O \tag{1}$$

In the case of vessels containing a CO_2 absorbent we have

$$\Delta h = \frac{X_{CO_2} \cdot F}{k_{CO_2}} - \frac{(X_{O_2} + A)}{k_{O_2}}$$

where F is the fraction of CO_2 produced by the tissue which is not absorbed in the center well and A is the rate of autoxidation of the CO_2 absorbent. The observed oxygen uptake, O_2, is equal to $-\Delta h \cdot k_{O_2}$ and hence

$$O_2 = -\frac{k_{O_2}}{k_{CO_2}} \cdot X_{CO_2} \cdot F + X_{O_2} + A \tag{2}$$

Equations (1) and (2) may be solved for X_{CO_2} and X_{O_2} as follows

$$X_{CO} = \frac{\text{NGE} + \frac{k_{CO_2}}{k_{O_2}} \cdot (O_2 - A)}{(1 - F)}$$

$$X_{O_2} = \frac{F \cdot \frac{k_{O_2}}{k_{CO_2}} \cdot \text{NGE} + (O_2 - A)}{(1 - F)}$$

The corrected net gas exchange is the observed value minus the amount of CO_2 calculated to be generated by the reaction of lactic acid with the bicarbonate of the medium. Similarly, metabolic CO_2 is obtained from X_{CO_2} by subtracting the CO_2 due to lactic acid production. RQ is the metabolic CO_2 divided by X_{O_2}:

$$\text{RQ} = \frac{X_{CO_2} - \text{lactate}}{X_{O_2}}$$

Under the experimental conditions employed here $k_{CO_2}/k_{O_2} = 1.12$, $F = 0.055$, and $A = 6$ μl per 2 hours.

Pardee bicarbonate medium averages 1.57 μmoles/100 mg per 2 hours. This value is eight times greater than that seen in tissue from normally fed rats studied under somewhat similar experimental conditions (Flatt and Ball, 1964). It may be seen from Table II that insulin pronouncedly diminishes the high spontaneous lactic acid production of this tissue in addition to its effect on glycerol release and oxygen consumption. The other point of interest revealed by the data of Table II is that this tissue exhibits a high positive net gas exchange. A portion of this observed positive net gas exchange is due to the release of CO_2 from the bicarbonate of the

medium by lactic acid formation. However, there still remains a significant positive net gas exchange when the data are corrected for this factor. The average corrected value for the control tissue amounts to 49 μl/100 mg of tissue for a 2-hour incubation period. The addition of insulin increases the observed net gas exchange in spite of the simultaneous reduction that occurs in lactic acid formation. Thus the corrected net gas exchange is doubled by the presence of insulin. It will be noted in this connection that the total metabolic CO_2 production as calculated from the data is on the average only slightly decreased by the addition of insulin in spite of the diminution seen in the O_2 uptake. As a result of these changes the average RQ rises from 1.37 to 1.87 upon the addition of insulin.

Now the calculations of respiratory processes made in these experiments are based upon the assumption that no fixed acid other than lactic acid is produced. If insulin did stimulate the production of some acid which would release CO_2 from the bicarbonate of the medium then the interpretation of the results would be drastically altered. It was therefore important to check this point by comparing the bicarbonate content of the control vessel with that of the vessel containing insulin at the termination of the measurements of net gas exchange. This was accomplished by attaching the pair of vessels used for net-gas-exchange measurements to Summerson differential manometers. The pair of vessels used were chosen so as to have identical flask constants and were carefully prepared to contain the same amount of bicarbonate at the start of the incubation period. Net gas exchange was measured in the usual way and after the final reading at 120 minutes the open arms of the two manometers were closed to the atmosphere and brought into intercommunication. Sulfuric acid, which was present in the side arms of both vessels, was then tipped in and the difference in CO_2 evolution in the two vessels was obtained directly from the manometer readings. Lactate in the medium was then measured as usual. In Table II experiments 4, 5, and 6 were performed in this manner. In each case the vessels containing insulin had more bicarbonate at the end of the incubation than did the controls. The amount so found is given in Table II under the column headed "HCO_3^- Excess." Correspondingly, the vessels with insulin contained less lactate than the controls. The difference in lactate content of the two vessels is given under the column of Table II headed "Lactate Deficit." In all three cases it will be seen that the excess bicarbonate corresponded exactly with the lactate deficit. These data thus provide strong evidence that no fixed acid other than lactic was produced in significant amounts during the incubation and that the calculated values for the respiratory quotient are valid.

The experiments which have been described have been performed in media containing no added glucose. The lactate produced must therefore have been derived from the tissue's own stores of carbohydrate. It was therefore of interest to study the effect of insulin on lactate production when glucose was present. In three experiments performed in the presence of 3 mg/ml of glucose the lactate production averaged 2.9 μmoles/100 mg per 2 hours, a value nearly double that observed for the six experiments of Table II, which were carried out in the absence of glucose. In flasks containing paired tissue and glucose but with 1000 μunits/ml of insulin present the same amount of lactate was formed. Thus the inhibitory action of insulin on lactate production appears to make itself manifest only when the tissue's own carbohydrate stores are the source of the lactate. This finding prompted an investigation of the effect of insulin upon glycogen disappearance in tissue of fasted-refed rats when incubated *in vitro*.

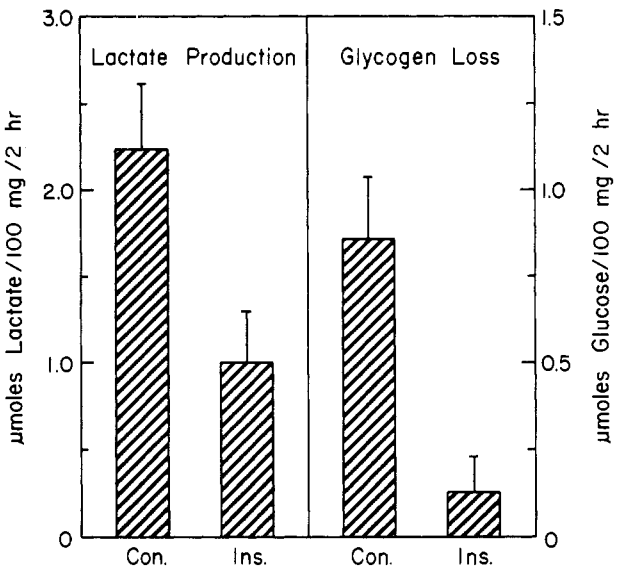

FIG. 1.—The effect of insulin on lactate production and glycogen disappearance. The results are the average of seven experiments and the standard error of the means is indicated by the vertical line at the top of each bar. The insulin concentration was 1000 μunits/ml.

In Figure 1 are shown the results of seven experiments in which the effect of insulin upon lactate production and glycogen disappearance was measured. In these experiments tissue from several rats was randomized into three portions; one portion was used to obtain the initial glycogen content and the other two were incubated with and without insulin. The unincubated tissue contained on the average about 1.1% glycogen or 6.8 μmoles of glycogen-glucose/100 mg fresh tissue. The glycogen content of the tissue incubated without insulin decreased by about 15% or 1.0 μmole of glycogen-glucose. When insulin was present this loss was reduced to only 0.15 μmole of glycogen-glucose. According to Student's *t*-test this effect of insulin on glycogen was significant at a level of 0.025. Similar results were seen in four experiments using Krebs-Ringer phosphate medium. It may be mentioned that glycogen does not give rise to any detectable amounts of glucose in the medium during incubation of the tissue. To check this possibility, 380 mg of fasted-refed tissue was incubated in 3 ml of buffer with and without insulin for 20 minutes at 37°. Glucose in the media was then assayed by the use of glucose oxidase and found to be below the limits of detection or less than 0.02 μmole/100 mg tissue.

These effects of insulin on glycogen metabolism prompted an investigation of the effect of insulin on adipose tissue phosphorylase by the procedure developed by Frerichs and Ball (1962). In this procedure intact tissue from fasted-refed rats is incubated under anaerobic conditions (5% CO_2–95%$N)_2$ in a Krebs-bicarbonate medium. The time course of the breakdown of glycogen to lactic acid may be followed by the rate of CO_2 evolution from the bicarbonate of the medium. The rate-limiting step in this process occurs in the breakdown of glycogen to glucose-6-phosphate and is presumably the phosphorylase step (Frerichs and Ball, 1962). In these experiments, portions of adipose tissue from two rats were randomized among six vessels, three of which contained insulin in the incubation medium from the start of the experiment. At zero time two vessels, one with and one without

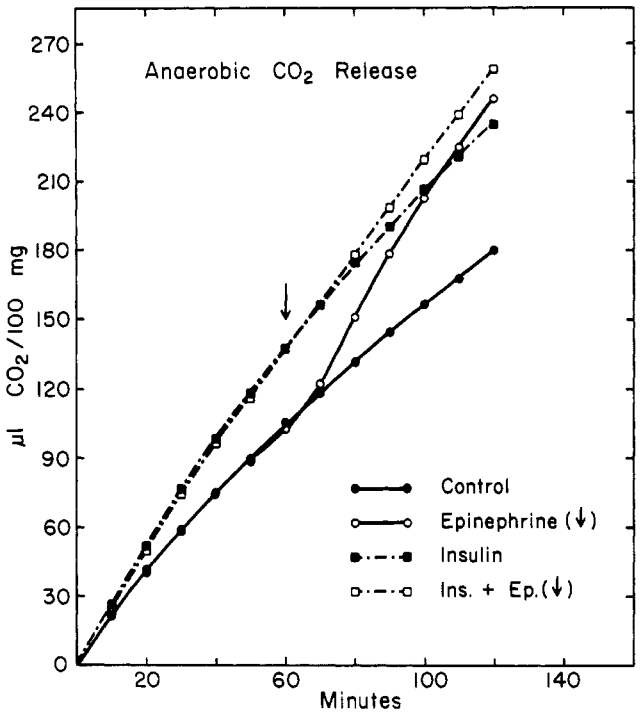

FIG. 2.—The effect of insulin and epinephrine upon the release of CO_2 by tissue incubated anaerobically in a bicarbonate medium. This graph represents the results of one experiment typical of a total of five performed. The insulin concentration was 1000 μunits/ml. Epinephrine was added at 60 minutes as indicated by the arrows to yield a final concentration in the medium of 0.1 μg/ml.

insulin, were removed from the bath and the medium was analyzed for lactate. Incubation of the other four vessels was continued for a period of 120 minutes. The results of a typical experiment are shown in Figure 2.

The amount of CO_2 released during the first 60 minutes of the incubation was greater in the vessels containing insulin than in the control vessels. In five experiments run in this manner, the amount of CO_2 production during the period 40–60 minutes averaged 28 ± 9% higher in the flasks with insulin. There is a suggestion in the data that this increase is owing to the reaction's following a more linear rate in the presence of insulin. At the end of 60 minutes epinephrine was added from the side arms of one of the vessel containing insulin and of one without. In the vessel without insulin the addition of epinephrine produced a prompt increase in the rate of CO_2 evolution. In five experiments performed in this manner the amount of CO_2 production during the period 70–90 minutes increased on the average 110 ± 10%. In the vessel containing insulin the response to epinephrine was less marked, and during the period 70–90 minutes the average increase observed in five experiments was only 16 ± 3%. At the end of the incubation period the lactate content of the vessels was determined and the appropriate zero-time value was deducted. On the average, the amount of lactate produced during the incubation period was found to equal 70–75% of that to be expected from the CO_2 evolution in all cases. These results thus indicate that under anaerobic conditions insulin increases the spontaneous rate of lactic acid formation and markedly diminishes the activation by epinephrine of the tissue's phosphorylase.

DISCUSSION

The results presented here emphasize the complex nature of the metabolic responses to be elicited by the *in vitro* addition of insulin to adipose tissue from fasted-refed rats. The fact that these changes in metabolic pattern occur in tissue incubated in the absence of glucose suggests a locus of action of insulin which lies within the cell rather than at the cell membrane. In considering stepwise the various metabolic changes induced by insulin we may conveniently begin with those involving glycerol. The high rate of glycerol release seen in tissue from fasted-refed rats indicates that triglyceride breakdown to fatty acids and glycerol is proceeding at a rapid pace. The fact that fatty acids do not accumulate during this process indicates that they are undergoing re-esterification to triglyceride and at a rate equal to their production by lipolysis. The glycerophosphate needed for this re-esterification must be furnished by the carbohydrate stores of the tissue. The marked diminution (85%) in glycerol release caused by the addition of insulin indicates that an inhibition of the lipolytic process has occurred. It seems unlikely that this effect can be attributed to an increased utilization of glycerol since there is no evidence that glycerol can be actively metabolized by white adipose tissue. Though the nature of this action of insulin upon lipolysis remains nuclear (see Jungas and Ball, 1963), one may assume that there occurs a fall in free fatty acid production of about the same magnitude (in percentage) as is seen for glycerol. Moreover, since free fatty acid accumulation remains negligible in the presence of insulin, there will be a like diminution in the amount of free fatty acid undergoing re-esterification and in the amount of glycerophosphate used for this process. This decrease in re-esterification rate is reflected in the fall in oxygen consumption which stems from a diminished demand for high-energy phosphate needed for the re-esterification process (see Ball and Jungas, 1961). On the average, when the medium is Pardee bicarbonate, each fall of 1 μmole in glycerol release (or 3.0 μmoles of fatty acid) produced by the presence of insulin is accompanied by a fall of 1.09 μmoles in oxygen consumption (Table I). Now when glycogen serves as the source of glycerophosphate it may be calculated that 6.5 μmoles of high-energy phosphate will be required to form triglyceride from 3.0 μmoles of fatty acids if the process follows that outlined by Ball and Jungas (1961). Thus it can be argued that 6.5 μmoles of high-energy phosphate are generated by the consumption of 1.09 μmoles of oxygen or that a P/O ratio of $6.5/(2 \times 1.09) = 3.0$ is exhibited by intact adipose tissue in this process. Similar calculations for the data given in Table I for the case where the buffer was Krebs phosphate yield a P/O ratio of 1.6. It must be emphasized of course that such calculations ignore other metabolic changes induced by the addition of insulin and assume that high-energy phosphate produced at the substrate level is not utilized for re-esterification.

The ability of insulin to diminish the production of lactate in adipose tissue from fasted-refed rats is less readily explainable than is its action on glycerol production and oxygen consumption. The action of insulin on lactate production does not seem to be due to any direct action of insulin upon lactate dehydrogenase. This is indicated by the fact that when glucose is added to the medium, lactate production is increased and the addition of insulin under these circumstances does not depress the rate of its formation. This premise is further supported by the effect of insulin upon lactic acid production under anaerobic conditions. Concomitantly with the decrease in lactate formation, one can show that less glycogen disappears from the tissue in the presence of insulin. These facts may indicate that insulin somehow acts

to inhibit a process in the conversion of glycogen to glucose-6-phosphate. Ball and Jungas (1961) have suggested that cyclic adenylic acid may be produced as a by-product of the re-esterification process. Thus the fall in rate of re-esterification produced by insulin could result in a lowered production of cyclic adenylic acid which in turn might be reflected in a diminution of phosphorylase activity and glycogen breakdown. On the other hand, a diminished glycogen breakdown may merely reflect the fact that less lactate and glycerophosphate are formed in the presence of insulin. It may be calculated from the data of Tables I and II and the foregoing reasoning that the formation of glycerophosphate and lactate in the control tissue require the utilization of the equivalence of 2.27 μmoles of glucose from carbohydrate stores per 100 mg per 2 hours. In the presence of insulin this is cut to the equivalence of 0.50 μmole of glucose. Thus there is spared the equivalence of 1.79 μmoles of glucose. The data of Figure 2 indicate that the glycogen spared by the addition of insulin is equivalent on the same basis to 0.73 μmole of glucose. Though the accuracy of the glycogen measurements may be open to question it is clear that the curtailment of lactate and glycerophosphate production by insulin is reflected in the amount of glycogen that disappears from the tissue. The question that remains unanswered is where in the metabolic sequence of events between glycogen and lactic acid does insulin produce this effect.

This effect of insulin becomes all the more puzzling when one considers the results obtained under anaerobic conditions. First, the measured lactate production is of the order of 6 μmoles/100 mg per 2 hours as determined by direct assay, and somewhat higher as measured by the CO_2 release from the medium. This rate is 4–5 times that seen under aerobic conditions. It is thus obvious that under aerobic conditions the limiting factor to lactic acid production is not the potential lactate dehydrogenase activity of the tissue. Moreover, the addition of insulin under anaerobic conditions increases lactate formation during a 2-hour incubation period by 29% on the average. Thus insulin inhibits lactate production from glycogen under aerobic conditions but accelerates it under anaerobic. Another point of interest is that the stimulating effect of epinephrine upon the phosphorylase system seen under anaerobic conditions is nearly wiped out by the presence of insulin. This would suggest that under anaerobic conditions, at least, insulin can somehow affect the reactions which govern the interconversion of glycogen and glucose-1-phosphate. This ability of insulin to counteract the stimulating action of epinephrine upon glycogen breakdown is thus similar to its ability to prevent the lipolytic activity of epinephrine in this tissue (Jungas and Ball, 1963).

The data presented here furnish indirect evidence that, in addition to glycerophosphate and lactate, fatty acids are formed from tissue-carbohydrate stores when adipose tissue from fasted-refed rat is incubated in a bicarbonate medium *in vitro*. The average positive-net-gas-exchange values corrected for lactic acid formation amount to 49 μl/100 mg per 2 hours in the absence of insulin and 99 μl in its presence. This positive net gas exchange does not appear to be owing to the formation of fixed acids other than lactic acid and hence is best interpreted as caused by fatty acid formation. The increase in the net-gas-exchange value in the presence of insulin is not easily interpreted with the data in hand. It could represent an increase in the rate of formation of fatty acids from carbohydrate stores. On the other hand, a shift from fatty acid to glycogen as the source of acetyl CoA burned to CO_2 and H_2O in the citric acid cycle could also contribute to an increase in the net gas exchange. The RQ values clearly support the conclusion that fatty acids are being formed. The RQ value of 1.87 observed in the presence of insulin agrees closely with the values found by Flatt and Ball (1963, 1964) for tissue from normally fed rats when synthesizing fatty acids *in vitro* from glucose in the presence of insulin. Quagliariello and Scoz (1930) observed an RQ above 2 for mesenteric adipose tissue from fasted-refed rats when incubated in a Ringer bicarbonate medium. These authors concluded that fat is synthesized from tissue carbohydrate stores under these conditions.

The oxygen consumption data also support the conclusion that fatty acid synthesis from tissue-carbohydrate stores is taking place. Assuming a nitrogen content of 0.58 mg/100 mg (Jungas and Ball, 1963), the data of Table I yield rates of O_2 consumption of 15–19.5 μmoles/mg N per 2 hours. As discussed previously, part of this high rate is due to the re-esterification of fatty acids. Yet when this process is repressed by the presence of insulin, the rate still remains high (9–13 μmoles). Tissue from normally fed rats exhibits a basic rate of 6–9 μmoles/mg N per 2 hours (Jungas and Ball, 1963). This rate is increased about 80% when the process of fatty acid synthesis is initiated (Jungas and Ball, 1961; Flatt and Ball, 1963), and so reaches values of 11–16.

An estimate of the amount of carbohydrate being converted to fatty acids in our experiments may be made by recourse to the equation of Flatt and Ball (1964) relating the corrected net gas exchange to fatty acid formation from [U-^{14}C]glucose, namely, fatty acid = 0.59 + 2.44 net gas exchange. In the experiments reported here, the corrected net gas exchange in the presence of insulin was 4.44 μmoles; hence 0.59 + (2.44 × 4.44) or 11.45 μatoms of fatty acid carbon are formed per 100 mg per 2 hours in this tissue. The comparable rate for tissue from normally fed rats synthesizing fatty acids from medium glucose in the presence of insulin is 8.9 μatoms (Flatt and Ball, 1964). However the nitrogen content of tissue from fasted-refed rats is higher than that from normally fed rats (Jungas and Ball, 1963). Calculated in terms of mg N per 2 hours, the rate for tissue from fasted-refed rats is 19.8 μatoms of fatty acid carbon while that of tissue from normally fed rats in the same weight range would be 25.4. Thus on a nitrogen basis tissue from fasted-refed rats forms fatty acid from carbohydrate stores at about 80% of the rate seen in tissue from normally fed rats incubated in the presence of 1.8 mg of glucose and 1000 μunits of insulin/ml.

A calculation of the total flow of carbohydrate through the major metabolic pathways in adipose tissue from fasted-refed rats in these experiments may be made. In terms of glucose equivalents the amount flowing from pyruvate to fatty acids amounts to (11.45/6) × (3/2) or 2.86 μmoles/100 mg per 2 hours. The data of Flatt and Ball (1964) permit one to calculate that for each μatom of fatty acid carbon formed, 0.235 μatom of carbon is released as CO_2 in the pentose cycle. Thus 11.45 × 0.235 or 2.69 μatoms of carbon or 0.45 μmole of glucose are consumed in the pentose cycle. As calculated earlier, the equivalence of at least 0.50 μmole of glucose is converted to glycerophosphate and lactate. The sum of all these pathways equals 3.81 μmoles of glucose or the equivalence of 0.62 mg of glycogen. As shown in Figure 1, the measured glycogen loss under these conditions is only equivalent to about 0.13 μmole of glucose or some 3% of that calculated. Though several explanations could be offered at this time for such a discrepancy, discussion of this

matter had best await the outcome of further investigations. It is of interest to note that under anaerobic conditions the equivalent of 4–5 μmoles of glucose is metabolized during a comparable period.

ACKNOWLEDGMENT

The authors are indebted to Miss Dorothy Harris and Miss Margery Merrill for their capable assistance in these studies.

REFERENCES

Ball, E. G., and Jungas, R. L. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 932.
Ball, E. G., and Jungas, R. L. (1963), *Biochemistry 2*, 586.
Flatt, J. P., and Ball, E. G. (1963), *Biochem. Z. 338*, 73.
Flatt, J. P., and Ball, E. G. (1964), *J. Biol. Chem. 239*, 675.
Frerichs, H., and Ball, E. G. (1962), *Biochemistry 1*, 501.
Jungas, R. L., and Ball, E. G. (1961), *Biochim. Biophys. Acta 54*, 304.
Jungas, R. L., and Ball, E. G. (1962), *Federation Proc. 21*, 202.
Jungas, R. L., and Ball, E. G. (1963), *Biochemistry 2*, 383.
Krebs, H. A., and Henseleit, K. (1932), *Z. Physiol. Chem. 210*, 33.
Quagliariello, G., and Scoz, G. (1930), *Boll. Soc. Ital. Biol. Sper. 5*, 1117.
Umbreit, W. W., Burris, R. H., and Stauffer, S. F. (1957a), Manometric Techniques, 3rd ed., Minneapolis, Burgess, p. 43.
Umbreit, W. W., Burris, R. H., and Stauffer, S. F. (1957b), Manometric Techniques, 3rd ed., Minneapolis, Burgess, p. 149.
Wooley, J. G., and Sebrell, W. H. (1945), *J. Nutr. 29*, 191.

Deoxyribonucleic Acid in Mitochondria and Its Role in Protein Synthesis*

George F. Kalf†

From the Department of Biochemistry,

Seton Hall College of Medicine and Dentistry, Jersey City, N. J.

Received May 29, 1964

This paper reports the detection of small amounts of DNA ranging between 340 and 560 μg/100 mg protein in lamb heart mitochondria. This material, which appears to be highly polymerized, was identified as DNA by its deoxyribose content, acid-precipitability, and selective sensitivity to pancreatic DNAase. Experimental evidence indicates that the DNA is not merely a contaminant arising from the breakage of nuclei during the preparation of the mitochondria. A biological role for DNA in heart mitochondria was established when the presence of a DNA-dependent RNA polymerase was observed. The ability of intact mitochondria to effect the incorporation of labeled amino acids was found to be sensitive to actinomycin D, but not to DNAase or RNAase. Inhibition by the antibiotic was partially reversed by the addition of excess DNA. The incorporating activity of a supernatant system derived from sonically disrupted mitochondria by centrifugation at 105,000 × *g* for 2–4 hours is reduced by DNAase or RNAase. In this case the addition of excess DNA reverses the inhibition of actinomycin D. Furthermore, actinomycin D is capable of inhibiting the incorporation of [8-^{14}C]-ATP by intact mitochondria.

The nucleus has been considered to be the exclusive site of DNA and hence of genetic information (Allfrey, 1959). However, reports from a number of laboratories on the presence of fibers or barlike structures with characteristics of bacterial nucleoplasm and of chemical analyses of DNA in chloroplasts (Ris and Plaut, 1962; Iwamura, 1962; Gibor and Izawa, 1963), kinetoplasts (Pitelka, 1961; Steinert, 1960; Steinert *et al.*, 1958), and the kappa particles of paramecia (Dippel, 1958; Hamilton and Gettner, 1958; Ris, 1961) speak in favor of the view that cytoplasmic particles which have been presumed or suggested to be "self-duplicating" contain DNA. This view is further strengthened by the demonstration (Nass and Nass, 1963a,b) that chick embryo mitochondria studied with the electron microscope show christae-free areas of low electron opacity which contain rodlike fibrous components with characteristic Feulgen-staining properties. These mitochondrial fibers were shown to be specifically digested with DNAase. In control incubations, RNAase and pepsin did not act upon the structures, although RNAase digested the cytoplasmic ribosomes and some nucleolar material and pepsin digested all of the mitochondrial structure except the fibers. These mitochondrial fibers appear to contain DNA.

While our studies were in progress, Schatz *et al.* (1964) observed that preparations of mitochondria from bakers' yeast which were purified by flotation in density gradients contain a significant quantity of DNA.

This report is concerned with experiments which indicate the presence of DNA in heart mitochondria and presents evidence that this DNA is not merely a contaminant but fulfills a biologically active role in mitochondrial protein synthesis.

EXPERIMENTAL PROCEDURE

Biological Material.—Lamb hearts weighing approximately 100 g were obtained from a local abattoir immediately after the animals were killed.[1] The hearts were cut into small pieces and packed in ice for the trip back to the laboratory.

* This investigation was supported by a grant (HE 06009-03) from the National Institutes of Health, U. S. Public Health Service. A preliminary report of this work was presented at the Meeting of the Federation of American Societies for Experimental Biology at Chicago, Ill., April, 1964.

† This work was carried out during the tenure of an Established Investigatorship of the American Heart Association and supported by the Essex County (New Jersey) Heart Association.

[1] We wish to thank the Charles Miller Company for their kindness in providing us with the fresh hearts.