CXXII. THE EFFECT OF INSULIN ON OXIDATIONS IN ISOLATED MUSCLE TISSUE

BY HANS ADOLF KREBS AND LEONARD VICTOR EGGLESTON

From the Department of Biochemistry, University of Sheffield

(*Received 21 March 1938*)

MUSCLE, unlike other tissues, loses many of its metabolic activities if sliced and suspended in Ringer solution or similar saline media, and the "tissue slice technique" in its usual form is therefore not applicable to muscle. Nor are Meyerhof's muscle extracts suitable if oxidations are to be studied since they lack the capacity of utilizing molecular O_2.

In order to find the optimal conditions for the study of oxidations in muscle tissue, we examined the rate of oxidation systematically in finely minced muscle under various conditions. The following factors were found to affect the rate of oxidation:

(1) The ionic composition of the medium.
(2) The proportion of tissue to medium ("dilution" of the tissue).
(3) The addition of boiled muscle or tissue extracts.
(4) The addition of oxidizable substances.
(5) The addition of insulin.

The importance of the first four factors is well known from the work of Thunberg [1909; 1910], Batelli & Stern [1911] and Meyerhof [1919], and our own experiments only elaborate some details of the role of these factors. The effect of insulin, though postulated from work on the intact diabetic organism, has not been observed before in *in vitro* experiments. The effect appears of special interest as it affords a new opportunity of studying the mechanism of the action of insulin.

Methods

Tissue. Pigeon breast muscle was used for most experiments. The animal was killed by guillotining, the breast was plucked and cooled on ice, and the cold muscle removed and finely minced in the "Latapie" instrument. The removal of the outer disk of the mincer, suggested by Szent-Györgyi [1934], caused no significant difference in the rate of metabolism and we have preferred to mince the muscle finely since it allows us to pipette the suspension with greater accuracy. The mince was weighed and thoroughly mixed with the ice-cooled medium. The tissue suspension was stirred since it settles rapidly and measured quickly into the manometric cup by means of a wide-mouthed bulb pipette.

Media. The composition of the media used is given later. It should be pointed out that the *p*H of the final suspension is generally lower than that of the medium owing to acid production by the tissue. All acidic substrates were added as neutral Na salts.

Manometric procedure. The O_2 absorption was measured manometrically in conical flasks with a centre cup containing 0·2 ml. 2*M* NaOH and absorbing filter paper. The gas space was filled with O_2. Since the rate of respiration was often very high, shaking was fast, 120–140 swings per min., and the stops for the

reading were as short as possible. Readings began after 10 min. equilibration; for calculation of the total O_2 uptake the curves obtained were extrapolated for the period of equilibration. The change of pressure often exceeded the length of the manometer scale (300 mm.). In these cases, when the end of the scale was reached, readings were suspended for a short defined period, generally 5 min., and air was admitted into the flasks to replace the O_2 used. The O_2 uptake during these periods was determined by interpolation.

Insulin. Unless otherwise stated freshly dissolved insulin hydrochloride of the British Drug Houses containing between 17 and 20 units per mg. was used. Crystalline insulin which contains much zinc [Scott, 1934] proved less active per unit (see later). Ampoules of insulin containing antiseptics were also less active.

Boiled muscle extract. One part of fresh, finely minced muscle was thoroughly mixed with one part of water, placed in a boiling water bath for 10 min. and filtered.

Bacterial infections. A number of experiments had to be continued for more than 3 hr., and in these infections occasionally occurred. The presence of bacteria was indicated by a gradual rise of the respiratory activity of the suspension and such experiments were discarded. Infection occurs so infrequently that it appears unnecessary to work under strictly sterile conditions.

Ionic composition of the medium

Confirming the older work of Thunberg [1909] and Meyerhof [1919; 1930] we find the highest rate of respiration of minced muscle in phosphate buffer. Table I

Table I. *Rate of respiration of pigeon breast muscle in various saline media*

Effect of Ca^{++} and *p*H. 1 part muscle (wet) in 10 parts of medium.

Medium 1: 8·9 g. Na_2HPO_4, $2H_2O$, 25 ml. *N* HCl in 500 ml.
2: 8·7 g. K_2HPO_4, 25 ml. *N* HCl in 500 ml.
3: 30 ml. medium (1) + 1 ml. 0·11 *M* $CaCl_2$
4: 30 ml. medium (1) + 1 ml. 0·155 *M* $MgSO_4$
5: 30 ml. medium (2) + 1 ml. 0·11 *M* $CaCl_2$
6: 4·45 g. $Na_2HPO_4.2H_2O$; 3·40 g. KH_2PO_4 in 500 ml.
7: 8·9 g. $Na_2HPO_4.2H_2O$; 10 ml. *N* HCl in 500 ml.
8: 8·9 g. $Na_2HPO_4.2H_2O$; 40 ml. *N* HCl in 500 ml.
9: phosphate saline *p*H 6·8 [Krebs, 1933]

Medium no.	1	2	3	4	5	6	7	8	9
Mol. concentration of Ca^{++}	—	—	0·0035	—	0·0035	—	—	—	0·0025
" Na^+	0·2	—	0·19	0·19	—	0·1	0·2	0·2	0·14
" K^+	—	0·2	—	—	0·19	0·05	—	—	0·0055
" Cl^-	0·05	0·05	0·05	0·05	0·05	—	0·02	0·08	0·13
" Mg^{++}	—	—	—	0·0035	—	—	—	—	0·001
*p*H	6·8	6·8	6·8	6·8	6·8	6·8	7·4	6·2	6·8
μl. O_2 absorbed by 3 ml. suspension after:									
20 min.	695	538	111	636	120	760	780	270	530
50 min.	1450	1105	190	1210	207	1640	1680	523	780
95 min.	1920	1485	239	1555	243	1960	1975	636	830
Q_{O_2} (first 20 min.)	38·6	29·6	6·1	35·0	6·6	42·1	41·5	14·8	29·4

shows the rates of oxidation in 9 different media. It will be seen that media which contain Ca^{++} in about physiological concentrations inhibit the respiration [Thunberg, 1909]. Mg^{++} also inhibits, but less than Ca^{++}. Na phosphate buffer gives slightly higher values than K phosphate buffer. It follows from these

experiments that Ringer solution and similar media are not suitable for work on minced muscle if the optimal rates of respiration are desired.

The inhibitory effect of Ca [see Thunberg, 1937; Holck, 1934] is not confined to muscle tissues as will be seen from Table II. Liver and kidney, *if minced*, show the same effect, whilst with slices of these tissues, on the contrary, the optimal rate of metabolism is obtained when the concentration of Ca in the medium is the same as in blood serum [Lasnitzki & Rosenthal, 1929; Kisch, 1934].

The *p*H of the medium has little effect between *p*H 6·8 and 7·4; at *p*H 6·2 respiration is considerably diminished.

Pyrophosphate is a very potent inhibitor in minced tissues [Dixon & Elliott, 1929; Dixon & Leloir, 1937], but not in slices (Table II).

Table II. *Comparison of the rates of respiration in minced and sliced tissues*

Tissue	Medium	Q_{O_2} (for first 30 min.) Mince	Slices
Kidney cortex, sheep	(1) Phosphate buffer 0·1 *M*; *p*H 7·1	20·6	12·5
	(2) As (1) + $CaCl_2$ 0·0035 *M*	7·1	12·5
	(3) As (1) + pyrophosphate 0·0082 *M*	10·8	11·9
	(4) Phosphate saline	—	13·6
	(5) Phosphate saline + pyrophosphate 0·0082 *M*	—	13·8
Liver, guinea-pig	(1) Phosphate buffer 0·1 *M*; *p*H 7·1	12·9	6·15
	(2) As (1) + $CaCl_2$ 0·0035 *M*	4·3	6·12
	(3) As (1) + pyrophosphate 0·015 *M*	9·65	6·02
	(4) Phosphate saline	—	7·15
Kidney cortex, guinea-pig,	(1) Phosphate buffer 0·1 *M* *p*H 7·1	17·8	12·0*
	(2) Phosphate buffer 0·1 *M* + 0·02 *M* *l* (+) glutamate	19·6	24·4*
	(3) Phosphate buffer 0·1 *M* + 0·02 *M* α-ketoglutarate	18·4	17·8*
	(4) Phosphate buffer 0·1 *M* + 0·02 *M* pyruvate	16·7	17·0*

* Phosphate saline used.

As it is not possible with muscle to compare sliced and minced material we used other tissues. Experiments carried out on liver and kidney (Table II) show that the rate of oxidation in minced tissue is, under optimal conditions, of the same order as in slices. Sometimes the respiration is even higher in the mince, but whilst the addition of suitable substrates to mince has no significant effect on the respiration it raises the respiration of slices to or above that of mince. The rate of respiration in mince thus appears to have already reached its maximum.

Although minced tissues can still respire at the same rate as slices and may be useful where the permeability of the intact cell interferes, it should be made clear that minced tissue cannot always replace slices, since synthetic processes, such as urea synthesis or the "Pasteur effect", are completely or partially destroyed by mincing.

Proportion of tissue to medium

Meyerhof [1919] has pointed out the importance of "dilution" for studying muscle oxidations *in vitro*. We have shown previously that in minced liver and kidney the rate of oxidation (per unit wt.) decreases about proportionally to the extent of dilution [Krebs, 1935]. In pigeon muscle, we find no falling off as long as the ratio medium/tissue remains below 10. On further dilution the rate of oxidation decreases rapidly as shown in Table III.

Some experiments recorded in this table were carried out at 17° in order to make certain that the O_2 supply is not the limiting factor. This may be so at 40° with the rapidly respiring, more concentrated suspensions (10–15 μl. O_2 per ml.

Table III. *Effect of "dilution" on respiration in pigeon muscle*

Muscle suspended in 0·1 *M* phosphate buffer *p*H 6·8 in Exps. 1 and 2; phosphate buffer extract in 3 and 4 (1 part muscle and 6 parts phosphate buffer (*p*H 6·8), heated in water bath for 5 min., filtered); phosphate buffer containing 0·02 *M* Na_3-citrate in 5.

1. 40°	Parts of medium / Parts of muscle	10	20	40	80	—
	Q_{O_2} (first 20 min.)	25·2	6·7	3·6	1·5	—
2. 17°	Parts of medium / Parts of muscle	5	10	20	40	—
	Q_{O_2} (first 20 min.)	5·25	5·65	1·08	0·80	—
3. 17°	Parts of medium / Parts of muscle	5	10	20	40	80
	Q_{O_2} (first 20 min.)	6·5	7·2	8·3	8·2	9·6
4. 40°	Parts of medium / Parts of muscle	10	20	40	80	160
	Q_{O_2} (first 20 min.)	46·4	51·5	46·8	50·0	56·5
5. 40°	Parts of medium / Parts of muscle	12	24	48	96	—
	Q_{O_2} (first 20 min.)	21·8	20·4	12·5	6·2	—

per min.), but experiments carried out at low temperatures are clear-cut and here it is certain that the O_2 supply does not limit the rate of respiration.

The effect of dilution naturally depends on the composition of the diluting medium. If the medium contains boiled muscle extract (Exps. 2 and 4) or certain oxidizable substrates (citrate, fumarate, succinate) Q_{O_2} remains constant up to a dilution of about 1:30.

Effect of boiled muscle extract

The stimulating effect of boiled tissue extract on respiration was discovered by Meyerhof [1919] and more recently studied by Rydin [1935], Stare & Baumann [1936] and Greville [1937]. If fairly concentrated muscle suspensions are used (1:10), boiled extracts maintain or occasionally increase the initial rate of respiration. We find that the effect is more pronounced at *p*H 7·4 than at *p*H 6·8 (Table IV). The more extract is added the longer the respiration remains at a high

Table IV. *Effect of boiled muscle extract on the respiration of muscle tissue*

1 part muscle suspended in 10 parts 0·1 *M* phosphate buffer; 40°.

	*p*H 6·8			*p*H 7·4		
ml. extract added to 3 ml. suspension ...	—	1·0	2·0	—	1·0	2·0
μl O_2 absorbed after: 30 min.	748	905	975	838	1048	1162
75 min.	1242	1750	1915	1230	1866	2255
185 min.	1470	2230	2505	1368	2200	3025

level. In more dilute tissue suspensions extracts increase the rate of respiration very considerably and prevent the falling off of respiration on dilution (Table III). If the dilution is for instance 1:80, the extract increases the rate about 3000%.

The effect of the extract is probably due chiefly to the presence of catalysts (coenzymes), but partly also to the presence of oxidizable substrates [see Meyerhof, 1919; Greville, 1937].

Addition of oxidizable substances

Although muscle is capable of oxidizing a great number of substances there are only a very few which increase the rate of O_2 uptake when added to fresh, unwashed suspensions of muscle tissue. Substances which can be oxidized in pigeon muscle but which have no effect on addition are: (*a*) glycogen, glucose, hexosephosphate, lactic acid, pyruvic acid; (*b*) α-hydroxyglutaric acid, alanine, β-hydroxybutyric acid. The absence of any effect of class (*a*) is because the enzymes are saturated with them, and of class (*b*) because the activity of the enzymes concerned is low enough to be insignificant compared with that of other enzymes. The following groups of substances on the other hand greatly increase respiration under suitable conditions: (*c*) citric acid, *cis*-aconitic acid, α-keto-glutaric acid, succinic acid, fumaric acid, malic acid, oxaloacetic acid; (*d*) *l* (+) glutamic acid, *l* (−) aspartic acid, α-glycerophosphate.

In this paper we shall only consider in detail the substances of group (*c*). These substances form a series of intermediates which arise in the breakdown of citric acid [Martius & Knoop, 1937; Krebs & Johnson, 1937]. The rapid oxidation of most of these substances in muscle tissue has long been known [Thunberg, 1909; Batelli & Stern, 1911; Meyerhof, 1919]. If they are present in smaller concentrations they act as catalysts [Stare & Baumann, 1936; Innes, 1936; Szent-Györgyi, 1936; Krebs & Johnson, 1937]; if present in high concentration, like all catalysts [see Warburg, 1937], they can serve as substrates.

The magnitude of the latter effect is shown in Table V. For this experiment muscle suspensions in 10 or 30 parts of phosphate buffer were used and it will be

Table V. *Effect of citrate, α-ketoglutarate, succinate, fumarate, oxaloacetate and* cis*-aconitate on the O_2 uptake of muscle*

Minced muscle suspended in 0·1 *M* phosphate buffer *p*H 6·8.

Substrate added (final concentration 0·02 *M*)	—	Citrate	α-Keto-glutarate	Succinate	Fumarate	Oxalo-acetate	*cis*-Aconitate
μl. O_2 absorbed by 3 ml. suspension:							
1 part minced muscle; 10 parts phosphate buffer							
After 30 min.	848	1002	1090	1205	970	928	990
70 min.	1760	2080	2205	2320	2050	2050	2010
120 min.	2355	2985	3355	3320	3045	3240	2850
185 min.	2525	3760	4160	4140	3830	4140	3710
1 part minced muscle; 30 parts phosphate buffer							
20 min.	32	208	212	—	210	96	—
30 min.	47	297	298	—	314	146	—
85 min.	106	501	711	—	782	228	—
130 min.	133	522	747	—	850	258	—

seen that with the more concentrated extract the added substances act chiefly by stabilizing the initial rate of respiration. It is noteworthy that all the substances are about equally effective. With the more dilute suspension the substances increase respiration very considerably. The effect is about the same in each case except with oxaloacetate.

It should be specially noted that the effects of citrate and α-ketoglutarate begin at the same time as that of fumarate (not after a period of induction [see Breusch, 1937]) and that they are of the same order as the effect of fumarate. These facts are relevant to the theory of the "citric acid cycle".

Catalytic effects. The catalytic effects of fumarate, malate, succinate and oxaloacetate have been demonstrated by Stare & Baumann [1936] and that of

citrate by Krebs & Johnson [1937]. Our further experiments show that these effects are not always found at *p*H 7·4, but they occur regularly if the tissue is suspended in phosphate buffer of *p*H 6·8. This influence of *p*H may explain negative results obtained by Breusch [1937].

The criterion of the catalytic effect is the ratio:

$$\frac{\text{Extra } O_2 \text{ absorption induced by catalyst } [\mu l.]}{\text{Quantity of catalyst added } [\mu l.]}. \qquad \ldots\ldots(1)$$

In the following we report a series of experiments carried out with citrate as a catalyst which are typical for the whole series of substances. α-Ketoglutarate and fumarate for instance showed the same effect.

Since 1 mol. citrate requires 4·5 mol. O_2 for complete oxidation, a catalytic effect is indicated when the ratio (1) is > 4·5. It is to be expected that the magnitude of (1) will increase with the duration of the experiment and it is

Table VI. *Catalytic effect of citrate on oxidations in muscle*

The brackets indicate parallel experiments carried out on the same tissue.

Quantity of citrate added	ml. muscle suspension	Strength of muscle suspension [ratio muscle: medium]	Duration of Exp. (min.)	*p*H	μl. O_2 absorbed: Without citrate	μl. O_2 absorbed: With citrate	Ratio (1)	Substances added to muscle suspension (final concentration)
0·15 ml. 0·02 *M*	3	1:7·5	160	6·5	2150	2836	10·2	—
0·1 ml. 0·02 *M*	2	1:7·5	200	6·8	2215	2790	12·8	—
0·1 ml. 0·02 *M*	2	1:7·5	190	6·8	2500	3030	11·8	—
0·1 ml. 0·02 *M*	3	1:11	260	7·4	2930	3720	17·5	Boiled muscle extract (30%)
0·1 ml. 0·1 *M*	3	1:7·5	190	6·8	2450	4080	7·3	—
0·1 ml. 0·02 *M*	3	1:11	225	6·8	2150	2470	7·1	Boiled muscle extract (30%)
0·1 ml. 0·02 *M*	3	1:11	225	6·8	2410	3760	30·0	Boiled muscle extract (30%) +4 units insulin
0·15 ml. 0·02 *M*	3	1:11	165	6·8	815	2095	19·0	—
0·15 ml. 0·02 *M*	3	1:11	165	6·8	1585	3170	23·5	Boiled muscle extract (25%)
0·15 ml. 0·02 *M*	3	1:11	210	6·8	1645	2150	7·5	—
0·15 ml. 0·02 *M*	3	1:11	210	6·8	1980	2350	5·5	Boiled muscle extract (25%)
0·15 ml. 0·02 *M*	3	1:11	210	6·8	2900	3240	5·2	Boiled muscle extract (25%) +4 units insulin

therefore desirable for the purpose of demonstrating the catalytic effect to follow the respiration over a long period. The time is however limited by the gradual falling off of respiration *in vitro* and conditions which prevent or retard the falling off will therefore increase the ratio (1). We find (see Table VI), as expected, larger values for (1) if the respiration is "stabilized" by tissue extract or by insulin (see later).

The highest value for (1) was 30, thus 6·7 times more O_2 was absorbed than was required for the complete oxidation of the citrate added. The time course of an experiment is given in Fig. 1, and it will be seen that small quantities of citrate, like tissue extract, have little effect on the initial rate, but they retard the falling off of respiration.

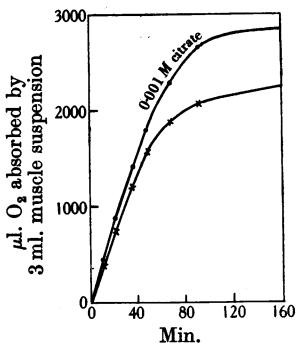

Fig. 1. Catalytic effect of citrate in respiration in pigeon muscle.

If the total respiration of the muscle passes through the citric acid cycle, the figure 30 for the ratio (1) indicates that the cycle occurs 10 times after the addition of 1 mol. citric acid (1 complete cycle involving the consumption of $3O_2$). The failure to find higher values for (1) may be ascribed to side

reactions by which the catalytic faculty of the carbon skeleton is irreversibly lost. The decarboxylation of oxaloacetic acid which is known to occur readily in the presence of muscle is such a side reaction. The stages between oxaloacetic and citric acids appear to be the most labile part of the citric acid cycle and are the first to be damaged by the artificial conditions of the experiment; the catalytic effects of added citrate (or its derivatives) may be due to a partial repair of such damage.

Insulin

Experiments on the intact organism [reviewed by Geiling *et al.* 1937] have shown that one unit of insulin, i.e. 0·045 mg. crystalline insulin, may bring about the oxidation of 1–2 g. carbohydrate and it is thus clear that insulin acts in a catalytic manner upon the breakdown of carbohydrate. Many attempts have been made to obtain catalytic effects of insulin in isolated tissues *in vitro*, but so far the only effect found is an inhibition of gluconeogenesis and of urea formation from amino-acids in liver [Bach & Holmes, 1937]. Since these effects are not concerned with the main action of insulin in the intact body, i.e. the oxidation of carbohydrate, they must be considered as side reactions or secondary effects of insulin. Certain effects on the respiration in muscle tissue described by Ahlgren [1925] could not be reproduced by other workers and have been ascribed to technical errors [Rothschild, 1930].

Negative experiments on isolated tissue would indicate that insulin is not the limiting factor for the oxidation of carbohydrate under the conditions existing in the experiments but they do not show that insulin does not act *in vitro*. We have examined the action of insulin on oxidations in minced muscle under varying conditions in an attempt to change the factors which limit the rate of oxidation in the tissue. We find as a rule, as previous workers have found, no effect when insulin is added to plain suspensions of muscle; but very great effects are observed when insulin is added together with boiled muscle extract and certain readily oxidizable substances such as citrate or α-ketoglutarate. A typical experiment is recorded in Table VII. In this experiment insulin has no

Table VII. *Effect of insulin on oxidations in pigeon breast muscle*

1 part of minced muscle suspended in 10 parts phosphate buffer (0·1 *M*; *p*H 6·8); 3 ml. suspension in each cup. "Extract" = 1 part of minced sheep heart + 1 part H_2O placed 10 min. in boiling water, filtered. "Citrate" = 0·2 *M* Na_3-citrate. "Insulin" = 0·2 ml. 0·1% insulin hydrochloride B.D.H. = 3·4 units.

	1	2	3	4	5	6	7	8
Added to suspension		Insulin	1 ml. extract	1 ml. extract; insulin	0·3 ml. citrate	0·3 ml. citrate; insulin	1 ml. extract; 0·3 ml. citrate	1 ml. extract; 0·3 ml. citrate; insulin
μl. O_2 absorbed:								
After 20 min.	656	642	604	636	600	628	562	678
40 min.	1118	1075	1160	1240	1065	1155	1090	1280
100 min.	1765	1730	2040	2225	1600	2065	1900	2650
165 min.	1970	1980	2285	2715	1645	2210	2155	3565
245 min.	2065	2085	2360	2965	1660	2245	2185	4130
Extra O_2 due to insulin (μl.)	—	+20	—	+605	—	+585	—	+1945

effect at all in the plain suspension; there is a certain effect in the presence of citrate and of boiled extract, but a very pronounced effect if both citrate and

extract are present. The total O_2 uptake during 4 hr. is increased by insulin from 2185 to 4130 μl. Most of the additional oxidation occurs during the later periods of the experiment and within the last period (165–245 min.) the O_2 uptake was only 30 μl. without insulin but 565 μl. with insulin (1800 % increase). In other words insulin acts chiefly by stabilizing the respiration over a longer period; this will be seen clearly from the graph in Fig. 2. In other experiments effects of insulin were also found in plain suspensions, but the effect was always more pronounced if both extract and an oxidizable substrate had been added. Variations of this kind may be expected since the effect of added extract or substrates will depend on the quantities of heat-stable catalysts and of substrate already present in the plain suspension. The concentration of these "preformed" substances will naturally vary from experiment to experiment.

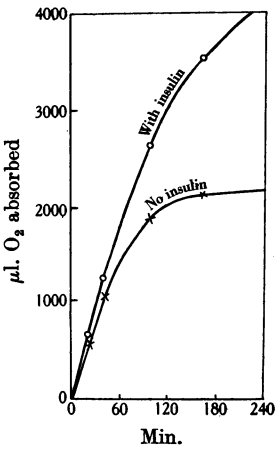

Fig. 2. Effect of insulin on respiration in pigeon muscle (in the presence of boiled muscle extract and of citrate). For data see Table VII, columns 7 and 8.

The effect of different quantities of insulin is shown in Table VIII; 0·01 unit per ml. has a slight, but definite effect, 0·1 unit shows a pronounced effect, 1 unit per ml. shows at the beginning almost the same effect but it stabilizes respiration over a much longer period than the smaller quantities.

Table VIII. *Effect of varying quantities of insulin on the O_2 uptake of pigeon breast muscle*

3 g. minced muscle suspended in 15 ml. 0·1 *M* phosphate buffer, *p*H 6·8 + 21 ml. boiled sheep's heart extract (see Table IX) + 2 ml. 0·2 *M* Na_3-citrate.

	Quantity of insulin added (units per ml.)			
	0	0·01	0·1	1·0
μl. O_2 absorbed by 3 ml. suspension after:				
20 min.	333	340	347	370
50 min.	1500	1605	1625	1730
135 min.	3265	3495	3650	3975
270 min.	3845	4155	4540	5785
μl. O_2 absorbed due to insulin		+310	+695	+1940

If citrate is replaced by other oxidizable substances similar effects of insulin are observed (Table IX). In the example given, however, the largest effect of insulin is observed in the presence of citrate.

Table IX. *Effect of insulin in the presence of various substrates*

Muscle suspension: 4 g. pigeon breast muscle + 40 ml. 0·1 *M* phosphate buffer, *p*H 6·8 + 10 ml. boiled sheep's heart extract.

	Substrates added (final concentration 0·01 *M*)		
	Citrate	α-Ketoglutarate	Fumarate
μl. O_2 absorbed by 3 ml. suspension in 275 min.:			
No insulin	2605	2950	3025
Insulin	4150	3815	3510
Effect of insulin (μl. O_2)	1545	885	485

Insulin and zinc

Scott [1934] and Scott & Fisher [1935] have shown that insulin forms compounds with zinc, nickel, cadmium or cobalt which readily crystallize. This discovery is now widely used for the preparation of the marketed insulin which is commonly a solution of "zinc insulinate".

We have compared the effect on the rate of oxidation of amorphous insulin, almost free from zinc, with the crystalline zinc insulinate using the following preparations:

(*a*) Crystalline zinc insulinate of Messrs Burroughs, Wellcome and of the British Drug Houses. Zinc insulinate contains 0·52% zinc [Scott & Fisher, 1935].

(*b*) Insulin hydrochloride of the British Drug Houses.

(*c*) A specially purified preparation of insulin containing 0·018% zinc kindly supplied to us by Boots Pure Drug Co., Ltd., Nottingham, through the courtesy of Mr W. A. Broom.

The preparations (*b*) and (*c*) showed almost identical effects whilst the preparations of zinc insulinate had practically no effect (Table X). In order to

Table X. *Effects of amorphous insulin and crystalline zinc insulinate*

1 part of pigeon breast muscle + 10 parts of 0·1 *M* phosphate buffer, *p*H 6·8 + 3·5 parts of boiled muscle extract + 0·4 parts 0·2 *M* citrate; 40°.

	No insulin added	Insulin hydrochloride; 1 unit per ml.	Crystalline zinc insulinate; 1 unit per ml.
μl. O_2 absorbed by 4 ml. muscle suspension after:			
20 min.	690	771	626
65 min.	1995	2230	1840
150 min.	3070	3520	2945
305 min.	3260	4455	3210

elucidate these differences we examined the effect of Zn^{++} on the respiration of muscle (Table XI) and it will be seen that even the minute quantity of zinc contained in zinc insulinate may inhibit respiration, especially during the later periods of the experiment. One unit of zinc insulinate per ml. corresponds to a zinc concentration of $3{\cdot}6 \times 10^{-6}$ *M* (calculated on the basis of the figure of Scott & Fisher). The inhibition caused by this concentration is sufficient to counteract the stimulating effect of insulin and to explain the negative effect of zinc insulinate.

The inactivity of zinc insulinate in our experiments suggests that zinc insulinate is not the active form of insulin in the body. This conclusion it should be made clear does not imply that zinc insulinate is of no physiological importance; it may serve for instance as an inactive storage form of insulin. The experiments of Scott & Fisher [1936] on insulin zinc protamine are of interest in connexion with this question.

The inhibitory effect of Zn^{++} on respiration can only be explained as an anticatalytic effect and the question thus arises as to which catalyst is inactivated by zinc. It is possible that the inhibition is due to a conversion of the insulin present in the tissue into the inactive zinc insulinate and if this be true, an excess of insulin should abolish the inhibition by zinc. The experiments in Table XI show that insulin abolishes the effect of zinc partly but not completely and we must therefore conclude that zinc reacts not only with insulin but also with other catalysts. It is of interest to note that Thunberg [1934] found zinc to be an inhibitor of various plant dehydrogenases.

Table XI. *Effect of Zn^{++} on respiration of pigeon breast muscle*

Each cup contained 3 ml. muscle suspension: 2·5 g. muscle + 20 ml. 0·1 *M* phosphate buffer, *p*H 6·8 + 10 ml. boiled muscle extract + 1·5 ml. 0·2 *M* citrate; 40°.

Concentration of Zn (*M*) ...	0	10^{-4}	10^{-5}	10^{-6}	0	10^{-4}	10^{-5}	10^{-6}
Concentration of amorphous insulin (units per ml.) ...	—	—	—	—	2	2	2	2
μl. O_2 absorbed after:								
25 min.	683	660	597	640	692	602	617	586
80 min.	1865	1320	1580	1725	1980	1475	1555	1580
155 min.	2665	1455	2015	2430	3135	1870	2320	2530
245 min.	2925	1475	2055	2615	3895	1915	2570	3200
310 min.	2930	1480	2060	2620	4195	1920	2585	3280

Discussion

Minced and sliced tissues. The fact that slices of muscle tissue, unlike other tissue slices, lose many of their metabolic activities if suspended in balanced salt solutions, is probably related to the specific cellular structure of muscle. Owing to the large size of the smallest cellular unit it is inevitable that every unit is injured on slicing, whereas in other tissues, such as liver, kidney or spleen, only a few % of the cells are damaged in slices of 0·3–0·5 mm. thickness. Cell lesions would upset the permeability; consequently, catalysts may diffuse out of the cell, and Ca^{++} may reach intracellular enzymes to which they have normally no access. This may explain why Ca^{++} or dilution inhibit oxidations in minced, but not in sliced tissues.

Citric acid cycle. Some observations recorded in this paper are of interest in connexion with a scheme of intermediate carbohydrate oxidation put forward recently [Krebs & Johnson, 1937]. According to this scheme citric acid and its biological breakdown products are intermediates in the oxidation of carbohydrate and it is to be postulated, therefore, that they are rapidly oxidized in muscle. It will be seen from the Table V that the rate of their oxidation is indeed very rapid and about the same for the different members of the "citric acid cycle". The great velocity of the oxidation of citric acid should be specially mentioned since other workers failed to observe the great activity of citric dehydrogenase in muscle [Langecker, 1934]. Such negative results are easily explained on account of the instability of this enzyme: confirming the older observations of Batelli & Stern [1911] we find it to be one of the most labile enzymes in animal tissues. It is completely inactivated by drying minced muscle in a desiccator over P_2O_5 or by precipitating muscle with acetone. It disappears within a few hr. from aqueous muscle extracts. The enzymic system concerned with α-ketoglutaric acid in muscle is also very labile. In liver preparations, on the other hand, the citric dehydrogenase seems to be less fragile and Langecker [1934] was thus led to believe that the liver is the chief site of citric acid breakdown in the body. Under optimal conditions, however, muscle removes citric acid about three times as rapidly as the liver.

Localization of the insulin effect. Since the effect of insulin is enhanced by the addition of substances belonging to the citric acid series, it appears probable that insulin is a catalyst concerned with one of the stages of the citric acid cycle. Since the effect of insulin is often found to be greatest with citrate itself (see Table IX) it is likely that insulin enters at a stage in the cycle near to citric acid. A difficulty in locating the effect of insulin lies in the nature of the cycle which results in the interconversion into each other of all the intermediate stages. Addition of

one member of the cycle therefore is followed by an increased formation of all the later stages.

A relation of insulin to the "citric acid cycle" is also suggested by a certain parallelism which exists between the occurrence of insulin and the citric acid cycle in different organisms. The cycle does not occur in yeasts or in bacteria (*B. coli*, *B. lactis aerogenes*, *B. pyocyaneus*, *Staphylococcus albus* or *aureus*) which do not contain insulin, but it occurs, as far as we at present know, in vertebrates which require insulin. The parallelism does not apparently, however, extend over the plant kingdom.

Quantity of insulin required. The quantities of insulin which show effects in our experiments are much above the amounts required in the intact organism. The smallest active concentration in our experiments was 0·01 unit (or 0·0005 mg.) per ml. In the intact rabbit 0·5 unit per kg. body weight has a considerable effect. The average concentration in the body after an effective dose is thus only 0·0005 unit per ml. or 20 times less than in our experiments. Another calculation shows that 0·01 unit causes an additional O_2 uptake of 375 μl. in our experiment, equivalent to about 0·5 mg. glucose; i.e. 1 unit promotes the oxidation of 50 mg. of glucose, whilst *in vivo* it promotes the oxidation of at least 1000 mg. Quantitative differences of this kind may however be expected in view of the different conditions *in vitro* and they do not contradict the view that the effect of insulin observed in our experiments is identical with the main action of insulin in the body. It should be noted that the effective concentrations are, in absolute terms, extremely low in our experiments. If the mol. wt. of insulin be 37600 [Crowfoot, 1938; Svedberg, 1937] our smallest effective concentration is 5×10^{-8} M and the optimal effect is obtained with 10 or 100 times higher concentrations. As far as we are aware no more active catalyst has ever been found to act in isolated tissues. For comparison it may be mentioned that aneurin (vitamin B_1) shows measurable effects *in vitro* at a concentration of 10^{-6} M and optimal effects at a concentration of 10^{-5} M [Peters, 1936].

Other effects of insulin. We do not yet know whether all the effects of insulin in the body are due to *one* primary effect on the oxidation of carbohydrate and we must therefore leave the question open whether there are other primary effects apart from the effect described in this paper.

The effect of Bach & Holmes. Bach & Holmes [1937] found that liver slices contain less carbohydrate, and produce less urea, after the addition of insulin. They concluded that insulin in their experiments prevented the deamination of amino-acids and thus acted by inhibiting the synthesis of carbohydrate from amino-acids. Their experimental facts can also be explained on the assumption that insulin directly promotes the oxidation of carbohydrate in liver. This would account for the relative decrease in carbohydrate and for the diminished deamination as the latter process is generally inhibited by readily oxidizable substances [Krebs, 1935]. This view has the advantage of explaining the effects of insulin in muscle and in liver in the same manner. The hypothesis of Bach & Holmes would not explain the insulin effect in muscle since amino-acids do not play a significant part as substrates for oxidations in muscle.

Limiting factors. Three different groups of naturally occurring substances are now known to stabilize respiration in minced muscle:

(*a*) Boiled muscle extract (chief active principle = coenzymes).

(*b*) Substances which are intermediates in the citric acid cycle.

(*c*) Insulin.

The different groups of substances act only under special, well defined conditions. None of the substances for instance has a pronounced effect on the

respiration of a more concentrated suspension (1:5) within the first 30 min. The substances of the group (*b*) frequently act only if muscle extract is present [see Greville, 1937]. Insulin on the other hand shows the greatest effects if both extract and citrate are present. These facts are easily understood on the grounds of the following considerations. A positive effect can only be expected if the concentration of the added substance is one of the factors limiting the rate of oxidation under the conditions of the experiment. A negative effect therefore only shows that the added substances are not limiting factors under the experimental conditions. It follows that it is only the positive experiment which is significant in so complicated a system as respiring muscle.

"Non-utilization theory" versus "overproduction theory" of diabetes. The effects of insulin described prove that insulin brings about the oxidation of carbohydrate in the peripheral tissues and they thus support the theory which explains diabetes as a condition in which carbohydrate cannot be oxidized in the tissues. (For a discussion of this subject see Geiling *et al.* [1937] and Young [1936]).

Decomposition of enzymes in minced tissue. Whilst the metabolism of sliced tissue can be maintained *in vitro* for several days [Okamoto, 1925], it is not yet possible to preserve the respiration of minced tissue for long periods. The respiratory activity of minced muscle gradually diminishes, even under optimal conditions, and usually after 4 or 5 hr. respiration ceases. The cessation is not due to the lack of substrate since the medium still contains much lactate at the end; it must therefore be ascribed to an inactivation of enzymes.

Summary

1. The optimal conditions for the respiration of minced pigeon breast muscle have been investigated. The best suitable saline medium was found to be 0·1 *M* phosphate buffer, *p*H 6·8–7·4. Ringer's solution and similar "balanced" solutions inhibit oxidations owing to the presence of Ca^{++}. If the ratio medium/tissue is above 10 the respiratory activity of the muscle is diminished.

2. Minced liver or kidney respire (for 1 or 2 hr.) at about the same rate as sliced tissue if suspended in 5 volumes of phosphate buffer. The respiration of minced tissue, unlike that of sliced tissue, is inhibited by physiological concentrations of Ca.

3. The respiration of minced muscle begins to fall off after 30–60 min. The falling off is retarded by the addition of (*a*) boiled muscle extract, (*b*) citrate or substances derived from it (α-ketoglutarate, succinate, fumarate, oxaloacetate), (*c*) insulin. The conditions under which these factors are effective have been investigated.

4. Insulin has the greatest effect if added together with citrate and muscle extract. This indicates that insulin becomes a limiting factor in oxidations only when heat-stable coenzymes (muscle extract) and a source for the carbon skeleton required for the "citric acid cycle" are present in excess.

5. Observations are discussed which suggest that insulin acts as a catalyst in the "citric acid cycle".

6. Zinc insulinate has practically no effect on the respiration of minced muscle. Traces of zinc (10^{-6} *M*) inhibit respiration. It is concluded that zinc insulinate is not the active form of insulin in metabolism.

We wish to thank the Rockefeller Foundation and the Medical Research Council for grants and Prof. E. J. Wayne for help.

REFERENCES

Ahlgren (1925). *Skand. Arch. Physiol.* **47**, 271, 275 and Supplement.
Bach & Holmes (1937). *Biochem. J.* **31**, 89.
Batelli & Stern (1911). *Biochem. Z.* **31**, 478.
Breusch (1937). *Hoppe-Seyl. Z.* **250**, 262.
Crowfoot (1938). *Proc. roy. Soc.* **164**, A, 580.
Dixon & Elliott (1929). *Biochem. J.* **23**, 812.
—— & Leloir (1937). *Enzymologia*, **2**, 81.
Geiling, Jensen & Farrar (1937). *Handbuch Exp. Pharmak., Ergänzungswerk.* **5**, 197.
Greville (1937). *Biochem. J.* **31**, 2274.
Holck (1934). *Skand. Arch. Physiol.* **70**, 273.
Innes (1936). *Biochem. J.* **30**, 2040.
Kisch (1934). *Biochem. Z.* **273**, 338.
Krebs (1933). *Hoppe-Seyl. Z.* **217**, 191.
—— (1935). *Biochem. J.* **29**, 1620.
—— & Johnson (1937). *Enzymologia*, **4**, 148.
Langecker (1934). *Biochem. Z.* **273**, 43.
Lasnitzki & Rosenthal (1929). *Biochem. Z.* **207**, 120.
Martius & Knoop (1937). *Hoppe-Seyl. Z.* **246**, 1; **247**, 104.
Meyerhof (1919). *Pflüg. Arch. ges. Physiol.* **175**, 20.
—— (1930). Die chemischen Vorgänge im Muskel. (Berlin.)
Okamoto (1925). *Biochem. Z.* **160**, 52.
Peters (1936). *Lancet*, i, 1161.
Rothschild (1930). *Biochem. Z.* **217**, 491.
Rydin (1935). Dissertation. Uppsala.
Scott (1934). *Biochem. J.* **28**, 1592.
—— & Fisher (1935). *Biochem. J.* **29**, 1048.
—— —— (1936). *J. Pharmacol.* **58**, 78.
Stare & Baumann (1936). *Proc. roy. Soc.* B, **121**, 338.
Svedberg (1937). *Nature, Lond.*, **139**, 1060.
Szent-Györgyi (1934). *Hoppe-Seyl. Z.* **224**, 1.
—— (1936). *Hoppe-Seyl. Z.* **236**, 1.
Thunberg (1909). *Skand. Arch. Physiol.* **22**, 406.
—— (1910). *Skand. Arch. Physiol.* **24**, 73.
—— (1934). *Skand. Arch. Physiol.* **69**, 247.
—— (1937). *Skand. Arch. Physiol.* **75**, 279.
Warburg (1937). *Ergebn. Enzymforsch.* **7**, 242.
Young (1936). *Lancet*, ii, 237 and 297.