CCXXIII. *IN VITRO* ACTION OF INSULIN ON MINCED AVIAN AND MAMMALIAN MUSCLE¹

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IN 1938 Krebs & Eggleston reported the interesting observation of an *in vitro* insulin effect upon oxidative metabolism. The experimental conditions which they employed to demonstrate this phenomenon consisted in the use of minced pigeon muscle as against the tissue slice technique which has been uniformly unsuccessful for the demonstration of insulin effects on respiration. A *M*/10 phosphate (*pH* 6.8) buffer containing the minced tissue in at most a one to ten dilution, the addition of citrate, and finally of boiled muscle juice resulted in a system which gave maximum results. With this system, increases of respiration of as much as 90% were noted in the presence of insulin. These effects were obtained with an amorphous insulin hydrochloride preparation (B.D.H.), but were absent when crystalline insulin was employed. This failure with crystalline insulin was attributed to its zinc content, an interpretation based on the inhibiting effects of zinc on the respiration of pigeon mince. The stimulation of respiration was roughly proportional to the insulin concentration between 0.05 and 5.0 mg./100 ml. Instead of citrate, other members of the postulated citric acid cycle could be substituted with similar effects. The only tissue employed in these experiments was the breast muscle of the pigeon, which was minced in a Latapie mincer.

From these experiments, the authors drew several conclusions. From the catalytic effects of certain dicarboxylic acids on the respiration of minced pigeon breast, Krebs & Johnson had previously proposed [1937] that they constituted a "citric acid cycle" which was responsible for the maintenance of carbohydrate metabolism. Since the citrate effect on respiration was now found to be increased in the presence of insulin, they concluded that the hormone influenced the metabolism of carbohydrate by acting catalytically on the citric acid cycle. They regarded the crystalline zinc insulin as either an unnatural form of the hormone or as an inactive depot form.

Because of the potential importance of these observations in elucidating the relationship of insulin to sugar metabolism, it was thought worth while to examine a variety of experimental animals to determine how general this phenomenon was. In order to insure the reliability of the procedures employed in this study, they were controlled by a repetition of much of the work reported by Krebs & Eggleston with pigeon breast muscle. In general, their results were confirmed. Thereafter, comparable studies were extended to the minced skeletal muscles of the chicken, dog, cat and rabbit, and the cardiac muscle of the dog. With none of these tissues was a similar insulin effect noted.

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METHODS

The pigeons were guillotined, the breast muscle rapidly plucked and immediately chilled. The same technique and the same muscle was employed with the chicken. Skeletal muscle was obtained from cats and dogs from the hamstrings with the animals under nembutal anaesthesia. Cardiac muscle was excised under the same conditions. Rabbit muscle was obtained after the animal was stunned by a blow. All tissue was rapidly chilled on ice and ground in a cold Latapie mincer. The mince obtained was thoroughly mixed, portions weighed out, suspended in the chilled media and pipetted into the conventional Warburg vessel with alkali in the wells for O_2 consumption measurements. All determinations were made in triplicate in pure O_2 at a temperature of 40° for the pigeon and 37.5° for the mammalian muscle.

The solutions used duplicated those of Krebs & Eggleston:

- (1) $M/10$ sodium phosphate, pH 6.8.
- (2) Boiled muscle extract prepared by extracting finely minced fresh beef heart with an equal volume of water for 10 min. in a boiling water bath and filtering.
- (3) $0.2 M$ trisodium citrate.
- (4) Insulin solutions containing, per 0.2 ml., 0.001–5.00 mg. of B.D.H. insulin hydrochloride or Lilly amorphous insulin. The Lilly insulin, which was furnished through the kindness of Dr G. H. A. Clowes, had an activity of approximately 22 units per mg. The final concentration of insulin in the suspensions ranged from 0.05 to 75 mg./100 ml.

The phosphate solution was used alone, and in combination with muscle extract and citrate in the proportions of 30:10:3 respectively. In some of the mammalian experiments the phosphate concentration used to make up the combinations was increased to $M/7$ in order to yield a final phosphate concentration of $M/10$.

In some experiments comparison of the respiratory metabolism of the mince was made with unminced dog skeletal muscle. For this purpose fibre bundles of suitable diameter were prepared from dog skeletal muscle by careful dissection, as previously described [Richardson *et al.* 1930]. This method yields a uniform preparation of intact fibres. Pigeon muscle was found too delicate to permit a satisfactory dissection of muscle bundles in this manner; therefore, slicing was resorted to. Cellular destruction was minimized in this case by cutting the sections parallel to the direction of the fibres after first dissecting out a layer of muscle with a uniform arrangement of fibres. By this means a muscle strip was obtained with a high percentage of relatively intact muscle fibres, as judged from histological sections. While not quite as satisfactory from the standpoint of lack of damage as the dog muscle strips, these slices respire for long periods of time at a very uniform rate. With these two unminced tissue preparations, the phosphate content of the medium was reduced to $M/75$, the conventional Ringer solution employed, and the pH raised to 7.4 in accordance with the experience of this laboratory as to conditions for optimal respiration of muscle.

The duration of the experiments and the amounts of tissue employed were varied in an effort to obtain the maximum effects of the components used. For convenience in comparing results, the respiration in all experiments shown in the Tables has been calculated on the basis of $\mu l. O_2$ per mg. wet weight of tissue per hr. In most of the pigeon experiments, 200–300 mg. of the mince were used per vessel, and the duration of the experiments was from 3 to 5 hr. Approximately the same amounts of tissue were employed in the mammalian series, but here respiration usually fell off more rapidly and the experiments were terminated in 2 to 4 hr.

The in vitro effects of insulin on the respiration of minced pigeon muscle

In the main, the results obtained by Krebs & Eggleston were confirmed. Citrate, muscle juice and insulin each exerted an additional stimulation of the respiration of the minced muscle in phosphate alone. Fig. 1, a typical experiment, shows a definite superiority of citrate plus phosphate over phosphate alone, and of muscle juice plus citrate plus phosphate over citrate plus phosphate. Finally, the addition of insulin caused a further increase in O_2 consumption amounting in this instance to 27 %. The improvement in respiration produced by

Fig. 1. Effects of citrate, muscle extract, and insulin on O_2 consumption of minced pigeon breast muscle.

Table I. *Effect of insulin on respiration of minced pigeon breast muscle*

Condition of animal	Duration of exp. hr.	Q_{O_2}		Increase due to insulin %	Amount and kind of insulin mg./ml.
		No insulin $\mu\text{l.}/\text{mg.}/\text{hr.}$	Insulin $\mu\text{l.}/\text{mg.}/\text{hr.}$		
Fed	3.7	2.99	3.81	27	5, B.D.H.
"	3.5	2.76	3.40	23	5, Lilly
			3.27	19	5, B.D.H.
Fasted	4.5	2.88	3.27	14	5, B.D.H.
Fed	3.0	5.45	5.87	8	5, B.D.H.
"	3.2	2.47	3.12	26	4, B.D.H.
			4.32	18	6, Lilly
			3.25	8	10, B.D.H.
"	3.6	3.41	4.09	20	7.5, B.D.H.
			4.40	20	7.5, B.D.H.
			4.54	22	7.5, B.D.H.
"	3.0	3.71	4.59	24	7.5, B.D.H.
Fasted	2.7	2.76	3.18	15	7.5, B.D.H.
Fed	3.4	1.40	1.78	27	1.5, B.D.H.
			2.14	53	6, B.D.H.
			2.10	50	30, B.D.H.
			2.09	49	60, B.D.H.
Fed	5.5	2.26	2.28	1	0.05, B.D.H.
			2.34	3	0.5, B.D.H.
			2.79	23	5.0, B.D.H.
"	4.3	2.97	3.52	19	5.0, Lilly
			4.21	19	5.0, Lilly
			3.14	26	5.0, Lilly
"	3.3	2.59	3.23	25	5.0, Lilly

each additional component was manifested both by an increase and better maintenance of O_2 consumption.

Table I is a summary of the experiments with pigeon breast mince. They show, uniformly, a better respiration when insulin was added to the phosphate-muscle extract-citrate-mince system. B.D.H. insulin hydrochloride and Lilly amorphous insulin were equally effective. The average improvement in respiration was about 25%, considerably less than the values of 40–90% shown by Krebs & Eggleston. The stimulation was minimal with less than about 1 mg./100 ml. of insulin, but, above this level, did not seem any better at 4, or 75 mg./100 ml. In the experiment showing the most pronounced insulin effect, a 50% increase in oxygen consumption was obtained with 6 mg./100 ml. Raising the concentration to 30 and 60 mg./100 ml. had no further effect. Fig. 2 details an experiment with two insulin concentrations below 1 mg./100 ml. and one above.

Fig. 2. Effect of increasing quantities of insulin on O_2 uptake of minced pigeon breast muscle.

Fig. 3. Increments of respiration of minced pigeon breast muscle.

The insulin effect has been described as a better maintenance of respiration. An inspection of a curve of successive increments in a typical experiment (Fig. 3), makes somewhat clearer the nature of the increased respiration in the presence of insulin. It is evident that whether or not insulin is added, the O_2 consumption of the mince falls off very rapidly. By 100 min., the rate of O_2 consumption is about half of the initial. From then on, the effect of insulin is apparent in delaying the fall of respiration in a system whose rate of O_2 consumption is diminishing rapidly. It would seem more accurate, therefore, to regard an increased O_2 consumption in the presence of insulin neither as a stimulation nor as a maintenance of respiration, but rather as due to a lag in the rate at which it is falling off.

An explanation for the effect of insulin in a mince system, in contrast to the absence of similar effect in slices, might be that destruction of the cell eliminated

the factor of impermeability to the large insulin molecule, and brought about conditions under which it had easy access to the reacting systems. Even with pigeon breast muscle, disintegration of the tissue is essential for the production of the insulin effect. This can be seen from experiments with sliced pigeon muscle (Table II), in which no insulin effect is shown.

Table II. *Effect of insulin on respiration of sliced pigeon muscle*

Ringer + M/75 phosphate + citrate	Q_{O_2}
" " " + insulin, 6 mg./100 ml.	0.59
" " " + insulin, 6 mg./100 ml.	0.51
Ringer + phosphate + glucose	0.29
" " " + insulin, 6 mg./100 ml.	0.29

Table III. *Effect of insulin on respiration of minced chicken muscle*

Insulin mg./100 ml. ...	Q_{O_2} Phosphate + muscle extract + citrate			
	0	0.5	5.0	25.0
1.11	0.59	0.99	1.07	1.06
		0.57	0.59	0.55

The experiments reported with pigeon breast muscle are in general agreement with those of Krebs & Eggleston, differing chiefly in the extent of the insulin effect. The extension of the study to a variety of other tissues, using the same technique, showed a uniform absence of this phenomenon.

Insulin effect on chicken breast mince. Entirely negative results were obtained with the minced breast muscle of the chicken. Table III summarizes the data in two such experiments. This result was surprising in view of the close relationship of these two experimental animals.

Fig. 4. Effects of citrate, muscle extract, and insulin on O_2 consumption of minced mammalian skeletal muscle (dog).

In vitro effects of insulin on the respiration of minced mammalian muscle

Addition of citrate and muscle juice to the basic phosphate suspension of minced skeletal muscle from the dog, cat and rabbit, and dog cardiac tissue, produced increases in respiration comparable with those obtained with the pigeon mince. When insulin was added no further rise was obtained. Fig. 4 illustrates

such an experiment. The addition of muscle juice to the phosphate solution increased respiration by 10 %. The further addition of citrate to the phosphate and muscle juice raised respiration by 50 % over phosphate alone, while insulin added to the combination actually caused a fall of 10 %.

Table IV. *Effect of insulin on respiration of minced mammalian muscle*

Muscle	Duration of exp. hr.	Q_{O_2}		Amount and kind of insulin mg./100 ml.
		No insulin μ l./mg./hr.	Insulin μ l./mg./hr.	
Dog skeletal:				
Post-absorptive	3.3	0.71	0.69 0.67 0.56 0.53	1.5, B.D.H. 6, B.D.H. 30, B.D.H. 60, B.D.H.
	2.1	0.89	0.83	7, B.D.H.
Phlorhizinized-fasted	3.3	0.81	0.74	6.5, B.D.H.
Post-absorptive	3.5	0.60	0.60 0.58 0.56	0.4, B.D.H. 2, B.D.H. 8, B.D.H.
Depancreatized-fasted	2.3	1.01	0.95	3, B.D.H.
Cat skeletal:				
Post-absorptive	3.8	1.45	1.42	6, Lilly
	3.2	1.03	0.82	6, Lilly
	3.9	0.95	0.80 0.91 0.88 0.83	1.5, B.D.H. 6, B.D.H. 30, B.D.H. 60, B.D.H.
	3.7	0.90	0.94 0.84 0.83 0.86	1.5, B.D.H. 6, B.D.H. 30, B.D.H. 60, B.D.H.
	3.7	1.17	1.17 1.13 1.20 1.12	1.5, Lilly 6, Lilly 30, Lilly 60, Lilly
Rabbit skeletal:				
Post-absorptive	2.3	0.60	0.56 0.59 0.58 0.59	1.5, Lilly 6, Lilly 30, Lilly 60, Lilly
Dog cardiac:				
Phlorhizinized-fasted	2.6	1.35	1.16	8, B.D.H.
	3.5	1.23	1.28 1.22 1.16	0.3, B.D.H. 1.5, B.D.H. 6, B.D.H.
Post-absorptive	3.4	1.10	1.08 0.94 0.93	0.4, B.D.H. 2, B.D.H. 8, B.D.H.
Fasted	4.2	0.91	0.92 0.87 0.94	0.5, B.D.H. 2.5, B.D.H. 10, B.D.H.
	2.6	1.00	1.07 1.03 1.17	0.05, B.D.H. 0.5, B.D.H. 5, B.D.H.
Depancreatized-fasted	2.5	1.21	1.14 1.13 1.09	0.05, B.D.H. 0.5, B.D.H. 5.0, B.D.H.

The experiments performed on this group of mammalian tissues are summarized in Table IV. No stimulating effect on respiration was noted from insulin, in concentrations ranging from 0.5 to 60 mg./100 ml. In fact, depressions of considerable magnitude were often encountered, both with B.D.H. insulin hydrochloride, as well as the Lilly amorphous insulin. It was thought that this depressant effect might be due to the zinc present in the insulin preparations. Krebs & Eggleston found respiration lowered by 22% when sufficient zinc was added to the pigeon muscle mince-insulin system to make a final concentration of 10^{-6} *M*. According to information from Dr Clowes, the Lilly insulin furnished us contained less than 0.025% of zinc. Since our decreased Q_{O_2} were often obtained with amounts of insulin less than 5 mg./100 ml. the effective zinc concentration required to depress respiration in mammalian muscles would be less than 2×10^{-7} *M*. Actual testing of the effect of added zinc shows that mammalian muscle mince is even more resistant to this metal than pigeon mince (Table V). Thus, the decreases in oxygen consumption were more likely due to the insulin itself.

Table V. *Effect of zinc upon respiration of minced cat skeletal muscle*

Zinc concentration, <i>M</i>	0	10^{-5}	10^{-6}	10^{-7}
Q_{O_2}	0.33	0.32	0.33	0.32
Inhibition Q_{O_2} , no insulin	—	30	11 [Krebs & Eggleston, 1938]	—
Inhibition Q_{O_2} , insulin	—	38	22	—

Table VI. *Effects of zinc, amorphous insulin and crystalline zinc insulin on minced pigeon muscle*

Exp. no.	No additions	Q_{O_2}			
		Zinc 5×10^{-6} <i>M</i>	Amorphous insulin 5 mg./100 ml.	Amorphous insulin + zinc	Crystalline zinc insulin 5 mg./100 ml.
1	2.97	—	3.52	—	3.47
2	3.55	—	4.21	—	4.04
3	2.49	1.98	3.14	2.42	3.53
4	2.59	2.26	3.23	2.71	3.18

Because of the discrepancy, noted above in the effects of zinc on O_2 consumption, experiments were performed on minced pigeon muscle with added zinc, zinc plus amorphous insulin, and crystalline zinc insulin. The amount of zinc added was about the same as that actually present in the crystalline insulin. The results, in Table VI, reveal that added zinc depresses respiration of pigeon muscle, in confirmation of Krebs & Eggleston, and that insulin is able to offset this effect, at least to some extent. The most interesting fact, however, is that when the zinc is actually in the insulin molecule (crystalline insulin), the usual insulin effect is seen on O_2 consumption. Krebs & Eggleston found no effect of zinc insulin on respiration with pigeon mince.

It will be noted from Table IV that animals were used in various nutritional states—post-absorptive, fasted for several days, phlorhizinized-fasted and completely depancreatized—in order to study the *in vitro* effect under all possible conditions of insulin deprivation and of lowered carbohydrate utilization. The phlorhizinized-fasted and completely depancreatized dogs show *in vivo* a marked response to the injection of insulin, and one would expect to find this hormone as a limiting factor in tissues excised from these animals.

The failure to obtain insulin effects with other than pigeon breast muscle might have been due to experimental conditions not being optimal. Therefore,

several modifications were made in an effort to obtain more favourable conditions. Since the previous experiments were done at pH 6.8, a trial was performed with cat skeletal muscle at pH 7.2 and 7.8, by varying the phosphate buffer only. Table VII shows that at pH 7.2 respiration was well maintained for a period of 3.5 hr. without any insulin effect. At pH 7.8 respiration fell off more rapidly, and stopped completely by 2.5 hr. During this period there was no difference between the O_2 consumption in the presence of insulin (5 mg./100 ml.) and that in the controls.

Table VII. *Effect of pH on metabolism of minced cat skeletal muscle*

pH	Duration hr.	Q_{O_2} Phosphate + muscle extract + citrate	
		No insulin	Insulin 5 mg./100 ml.
7.2	3.5	0.90	0.82
7.8	2.5	0.82	0.81

Stare & Baumann [1939] have recently reported that greater insulin effects are obtained with pigeon breast mince if a NaCl-KCl-MgSO₄ solution, a weaker phosphate buffer ($M/60$) and smaller amounts of citrate and muscle juice are used than those employed by Krebs & Eggleston and by ourselves. This type of solution was, therefore, tested with dog skeletal and cardiac muscles (Table VIII) with no evidence of any insulin effect, although the level of respiration of skeletal muscle was higher than with the customary solutions.

Table VIII. *Comparison of respirations (Q_{O_2}) of minced tissues in different solutions*

	No insulin	Insulin 5 mg./100 ml.	Duration of exp. hr.
Skeletal muscle: Krebs-Eggleston solution	0.57	0.54	2.0
Stare-Baumann solution	0.62	0.61	
Cardiac muscle: Krebs-Eggleston solution	1.24	1.15	2.2
Stare-Baumann solution	1.20	1.16	

From the data reported, it can be seen that mammalian muscle has a lower metabolic rate, rarely reaching values more than one-third of those of the pigeon mince. In addition, the mammalian experiments were carried out at 37.5°, whereas the pigeon experiments were done at 40°, their normal body temperature. An attempt was made to study both of these factors by carrying out an insulin experiment on cat skeletal muscle at 40°. The insulin did not produce any change (Table IX), but it should be pointed out that the higher temperature did not produce the expected increase in O_2 consumption.

Table IX. *Effect of insulin on respiration (Q_{O_2}) at 40° of minced cat skeletal muscle*

Insulin mg./100 ml.	Q_{O_2} Phosphate + muscle juice + citrate
0	0.90
0.4	0.89
4.0	0.90
20.0	0.88

Another possible limiting factor was the low cytochrome content of mammalian skeletal muscle, as compared with pigeon breast. Junowicz-Kocholaty & Hogness have reported [1939] that the concentration of cytochrome *c* in pigeon breast muscle is unusually high, about 50 μ g. per 100 g. That of mammalian cardiac muscle is also quite high, 20 μ g. per 100 g., in contrast with the very low concentration of mammalian skeletal muscle (5 to 10 μ g. per 100 g.). While it was not very likely that the lack of insulin effects on cardiac muscle could be due to cytochrome lack because of its high concentration in this tissue, cytochrome might have been the limiting factor with mammalian skeletal muscle. Accordingly, the two experiments summarized in Table X were performed to test the effect of two levels of added cytochrome *c* on the respiration of minced cat skeletal muscle. In one series, a slight rise in O_2 consumption was noted in all the solutions containing cytochrome *c*, but in no case was there an insulin effect.

Table X. *Effects of cytochrome c and insulin upon respiration of minced cat skeletal muscle*

Solution	Q_{O_2}
Exp. 1:	
Phosphate + muscle juice + citrate	0.70
Phosphate + muscle juice + citrate + 5 mg./100 ml. Lilly insulin	0.70
Phosphate + muscle juice + citrate + 0.1 mg./100 ml. cytochrome <i>c</i>	0.76
Phosphate + muscle juice + citrate + 0.1 mg./100 ml. cytochrome <i>c</i>	0.73
+ 5 mg./100 ml. Lilly insulin	
Phosphate + muscle juice + citrate + 0.1 mg./100 ml. cytochrome <i>c</i>	0.73
+ 0.5 mg./100 ml. Lilly insulin	
Exp. 2:	
Phosphate + muscle juice + citrate	0.79
Phosphate + muscle juice + citrate + 5 mg./100 ml. Lilly insulin	0.72
Phosphate + muscle juice + citrate + 50 mg./100 ml. cytochrome <i>c</i>	0.70
Phosphate + muscle juice + citrate + 50 mg./100 ml. cytochrome <i>c</i>	0.68
+ 5 mg./100 ml. Lilly insulin	

One is tempted to consider another possible explanation for the insulin effect on the basis of a non-specific change. The reason for the sharp falling off of respiration in minced tissue preparations is obscure. In many instances it does not appear to be due to the lack of substrate. Its explanation might lie in the destruction of protein carriers by proteolytic enzymes. Should this prove to be the case, the delay in falling off of respiration achieved by insulin with pigeon breast mince might be due to a temporary interference with proteolysis by the hormone. From the work of Scott on the binding of trypsin by insulin with subsequent inactivation of the hormone, such a mechanism might exist, the insulin binding part of the proteolytic enzyme system of the cell and hence delaying the disintegrating action. For such a mechanism to explain the insulin effect would necessitate a difference of some kind in the proteolytic enzymes of the pigeon muscle as compared with the other tissues studied. It is a concept, however, susceptible of direct study and may throw some light on the changes produced by insulin, as well as on the difference in survival between minced tissue and the tissue slice or strip.

Comparison of the R.Q. of muscle mince and muscle strips or slices

Since the insulin effect was so sharply limited to the pigeon of all the animals studied, it was of interest to examine the qualitative character of the respiratory metabolism of this tissue for a possible explanation of the difference in behaviour. Considerable weight would be lent to the insulin effect in pigeon muscle as a

specific phenomenon if not only the rate of respiration were accelerated by this hormone, but the character of the foodstuffs oxidized changed from a non-carbohydrate to a carbohydrate type. It was also felt that R.Q. studies on minced mammalian tissues would be necessary to rule out the possibility that, even in the absence of respiratory stimulation by insulin, isocaloric shifts in the character of the foodstuffs oxidized might have taken place. R.Q. determinations, using the method of Dickens and Šimer, were made under a variety of experimental conditions.

Table XI. *Respiratory quotients of minced pigeon breast muscle*

	Condition of animal	Solution	R.Q.	Q _{O₂}
1	Fed	Phosphate	0.89	3.82
		Phos. + muscle juice + citrate	0.92	4.96
	Fed	Phosphate	0.95	4.63
		Phos. + muscle juice + citrate	0.91	5.12
3	Fasted 3 days	Phosphate	1.01	3.87
4	"	"	0.98	4.40
5	"	"	0.96	4.03
6	Fasted 3.5 days	"	0.96	2.21
7	Fasted 4 days	"	0.88	2.04
8	Fasted 4.5 days	"	0.93	4.45

It soon became apparent that all minced muscle preparations, even in the absence of added insulin, had high R.Q., indicating almost exclusive oxidation of carbohydrate. Table XI shows the high R.Q. obtained even in the case of fasted animals which would be expected to be burning fat preponderantly. This high oxidation of carbohydrate during a fast is counter to previous experience in this laboratory with the R.Q. of excised muscle strips and cardiac slices from fasted animals. When comparisons of simultaneous R.Q. were made with minced muscle and muscle strips or slices, there was apparent in each instance a significant elevation in the R.Q. of the mince. These results are summarized in Table XII. Muscles both from pigeons and from fasted and depancreatized dogs behaved similarly. The high R.Q. of the fasted pigeon mince is particularly striking in view of the high metabolic rate of these birds, and the anticipated greater depletion of carbohydrate stores. Blood sugar determinations on the pigeon after three- and four-day fasts were surprisingly high: 229, 206, 197, 233 mg./100 ml. in four such animals.

Table XII. *Effect of mincing on muscle metabolism*

Animal	Exp. condition	Tissue		R.Q.	Q _{O₂}
Pigeon	Fasted 3 days	Breast muscle	Slices	0.79	0.81
			Mince	0.96	4.03
Pigeon	Fasted 3.5 days	Breast muscle	Slices	0.87	0.58
			Mince	0.96	2.21
Pigeon	Fasted 4.5 days	Breast muscle	Slices	0.85	0.73
			Mince	0.93	4.45
Dog	Fasted 6 days	Skeletal muscle	Strips	0.76	0.34
			Mince	0.91	1.27
Dog	Fasted 7 days	Skeletal muscle	Strips	0.69	0.33
			Mince	0.91	1.18
Dog	Fasted 10 days	Skeletal muscle	Strips	0.77	0.35
			Mince	0.86	0.76
Dog	Depancreatized 5 days	Skeletal muscle	Strips	0.73	0.43
			Mince	0.85	0.89

Preliminary experiments by Stare & Baumann indicate that, like the duck [Koppanyi *et al.* 1926], the pigeon tolerates pancreatectomy extremely well with no permanent change in the already high blood sugar. Both these birds would thus seem to be much less dependent on insulin than mammals. Some fundamental qualitative difference in the nature of certain metabolic processes might produce the difference in behaviour of the pigeon mince to insulin as compared with tissues from the other animals studied. The evidence, however, suggests that the pigeon is less dependent on insulin than the mammal, and by that token the *in vitro* effect of insulin in the pigeon is difficult to understand.

The high R.Q. of the minced tissue suggests that the effect of insulin is not in varying the nature of the substrate oxidized, but rather in prolonging the O₂ consumption of a tissue already burning carbohydrate almost exclusively. It therefore fails to provide what would be highly desirable evidence of a specific insulin phenomenon.

DISCUSSION

The interesting effect of insulin on the respiration of pigeon breast muscle described by Krebs & Eggleston has been confirmed. The chief divergence in the data obtained by these workers and by ourselves lies in the smaller increase found, which averaged 25 % as against 50 %. We are inclined to interpret the effect on respiration produced by insulin as not a true "stimulation" of respiration, but rather as a lag in the falling off of respiration which takes place at a time when the O₂ consumption has fallen markedly from its original value. These results were obtained with three different samples of insulin: B.D.H. insulin hydrochloride, an amorphous insulin preparation (Lilly), and crystalline zinc insulin (Lilly). The positive results with the crystalline insulin were unexpected since Krebs & Eggleston were unable to obtain any effect with this substance. We were also unable to find any significant proportionality between the stimulation of respiration and the concentration of insulin above about 1 mg./100 ml.

The insulin effect is, however, absent in skeletal muscle from the chicken, dog, cat and rabbit, and in dog cardiac muscle. Indeed, it is very common to find a depression of O₂ consumption with insulin with these tissues. This depression was not found to be due to the presence of zinc in the insulin used. It is of interest in this connexion that mammalian tissue seems much less sensitive to zinc than pigeon mince. This, as well as the positive results obtained in the pigeon with crystalline zinc insulin, tend to invalidate Krebs & Eggleston's suggestion that the active form of insulin in the body is probably not similar to the zinc compound because of the depressant action of the metal.

Attempts were made to set up conditions which might be more favourable to the manifestation of the insulin effect. These consisted in raising the pH, altering the electrolyte content of the solutions, elevating the experimental temperature from 37.5 to 40° and adding cytochrome c. In addition, animals were fasted and depancreatized in order to bring about conditions in which insulin was a definite limiting factor. All of these modifications were unsuccessful. The absence of the insulin effect in the mammalian tissues and in the chicken does not rule out the possibility that it might occur under proper conditions. However, it casts doubt on any generalized significance of the effect as related to the postulated citric acid cycle.

The criterion for establishing specific insulin action is, in this instance, the level of O₂ consumption. Much stronger evidence would be furnished by a situation in which insulin actually changed the nature of the foodstuffs burned from a non-carbohydrate to a carbohydrate type. It will be recalled that in the

whole animal the effects of insulin are not shown by an increased metabolic rate, but rather by a change in foodstuff oxidized. Examination of the R.Q. obtained by mincing pigeon breast, as well as mammalian skeletal muscle, brings out the interesting fact that this treatment serves to elevate the R.Q. immediately to levels approaching exclusive carbohydrate oxidation. This change took place in all tissues studied, even in the fasted and depancreatized animal where the results were not to be expected from the usual effect of these procedures on the character of the foodstuffs oxidized. The additional effect of insulin on the respiration of minced pigeon tissue would appear to be merely a continuation of the carbohydrate type of oxidation which existed in the non-insulin control.

A comparison of the R.Q. of muscle strips or slices with that of the mince is of considerable interest for workers employing any procedure such as mincing or homogenization. Obviously, by the disruption of the cell structure, more than a quantitative change in the metabolic processes is brought about. The R.Q. of the excised strip or slice parallels that obtained with the whole animal, whereas the R.Q. of the mince is strikingly elevated. Disruption of cell structure may result in the liberation of highly reactive carbohydrate systems no longer under control by the intact cellular organization. This fact should be borne in mind when interpreting data derived from tissue mince and extending them by analogy to the living cell.

A possible explanation for the limitation of this phenomenon to pigeon breast muscle may reside in some peculiar metabolic characteristic of the pigeon. Some evidence for this has been offered in the unusual reaction to fasting as evidenced by the high blood sugar and by the apparent resistance of the pigeon to pancreatectomy, as reported by Stare & Baumann. This difference in behaviour as against that of the mammals would have a parallel in the resistance of the duck to pancreatectomy. It is surprising to find a specific insulin effect only in animals so little dependent upon its action, whereas animals for which this hormone is essential should fail to be influenced.

SUMMARY

1. The insulin effect described by Krebs & Eggleston, consisting of the better maintenance of O_2 consumption in minced pigeon breast muscle, is verified.
2. No comparable effect of insulin is manifested on the respiration of minced chicken breast muscle, minced skeletal muscle of the cat, dog and rabbit, or minced dog cardiac muscle.
3. Therefore, it is doubtful if the insulin phenomenon in pigeon breast muscle exemplifies the general mechanism of insulin action on carbohydrate oxidation.
4. The result of disruption of the intercellular organization of muscle by mincing is to stimulate the oxidation of carbohydrate, even in the absence of added insulin.

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