FUMARATE IN BIOLOGICAL OXIDATIONS*

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The living cell is an extremely dynamic entity, its constituents being in a constant state of flux—constantly in the process of synthesis and degradation. The cell lives by virtue of a highly complex equilibrium between a large number of reactive chemical compounds. One of these compounds is fumarate. A discussion of fumarate in cellular respiration, therefore, involves a consideration of many substances other than fumarate, since fumarate is biologically interesting only because it is readily transformed into other substances, and because other substances are readily transformed into it.

The complexity of the respiratory process has led to much speculation as to the chemical mechanism involved, and the theories which have been formulated are both numerous and contradictory. Unfortunately, the theories have sometimes received more attention than the facts on which they were based. Theories have, however, served as convenient pegs on which to hang an assortment of isolated facts. Two distinct theories have arisen about the action of fumarate and related substances; the Szent-Györgyi theory concerning hydrogen transport, and the Krebs citric acid cycle dealing with the breakdown of carbohydrate fragments.

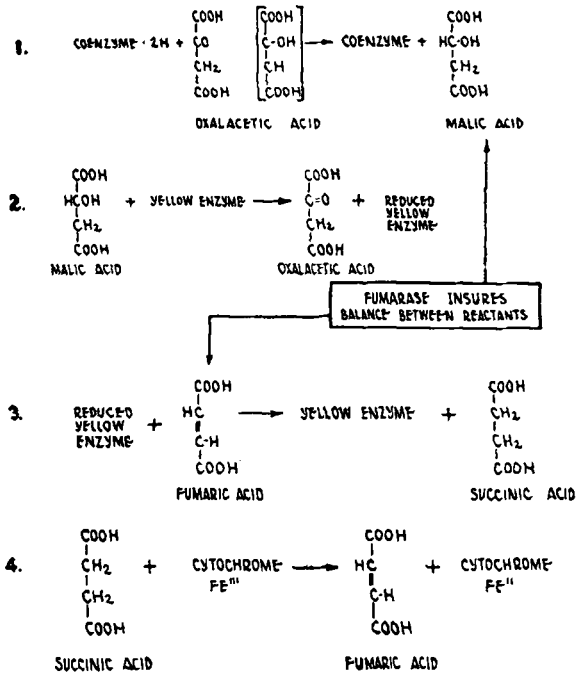

Fig. 1. Hydrogen transport according to Szent-Györgyi.

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Szent-Györgyi's theory has undergone several modifications. The present version (1) is as follows: Substrate hydrogen is transported from reduced coenzyme to oxalacetic acid which is thereby reduced to malate. The malate reacts with yellow enzyme, reducing it, and the reduced yellow enzyme then passes the hydrogen to fumarate, reducing it to succinate. According to Szent-Györgyi it is succinate, and succinate only, which reacts with cytochrome. Schematically the theory can be represented as shown in Fig. 1. The real function of fumarate and its relatives, malate, succinate, and oxalacetate, Szent-Györgyi's "C_4", is that of hydrogen transport. The compounds are readily and rapidly transformed into one another, and hence only a small amount of "C_4" is needed to transport hydrogen from reduced coenzyme to cytochrome. Szent-Györgyi actually considers "C_4" to be the active prosthetic group of the hydrogen-carrying enzymes, the "transportases."

In the Krebs citric acid cycle (2) the "C_4 acids" are assumed to function in the degradation of carbohydrate. The cycle can be pictured as shown in Fig. 2. Oxalacetate condenses with a

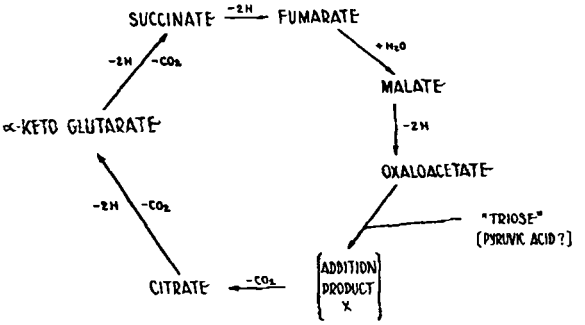

Fig. 2. The citrate cycle of Krebs.

"triose" or its oxidation product to form an intermediate which loses CO_2 and hydrogen, forming citric acid. The citric acid rearranges, loses CO_2 and hydrogen and eventually forms α keto-glutaric acid, which also loses CO_2 and hydrogen, yielding succinic acid which is transformed successively to fumaric acid, malic acid, and finally oxalacetic acid completing the cycle. As a picture the Krebs cycle has the virtue that it offers a comprehensible pathway by which CO_2 is formed by the cell, and furthermore, the step-wise formation of CO_2 harmonizes with the step-wise removal and transport of hydrogen.

The purpose of the present discussion is to consider some of the available experimental evidence dealing with fumarate and related sub-

stances, which has given rise to the above theories.

The first evidence to be cited in connection with the above theories concerns succinodehydrogenase, the enzyme which converts succinate to fumarate. This enzyme is extremely widespread and extremely active (3, 4). It has been found in all animal tissues as well as in bacteria and yeasts. This enzyme differs from most dehydrogenases in several important particulars. It not only is one of the most active respiratory enzymes known, but the enzyme belongs to the "zero order," that is, maximum activity is observed with very small concentrations of substrate (5). This property enables tissues to convert very small amounts of succinate very rapidly.

When moderate amounts of succinate[1] are added to minced tissue the rate of oxygen uptake is increased enormously. The rate of respiration, however, in contrast to oxygen consumption, is not correspondingly increased. Effectively, what happens is that the dehydrogenation of succinate is superimposed on normal respiration. This can readily be demonstrated by considering the respiratory quotient, which is lowered markedly by succinate. If the volume of oxygen consumed in normal respiration be designated as X, the CO_2 output will also be X, since the R.Q. of normal muscle tissue is approximately 1.0. On the addition of succinate the oxygen consumption may be doubled or more. Assuming it to be doubled, the extra oxygen consumption due to succinate will also be equal to X. There will, however, be no additional production of CO_2, since the enzyme merely transfers hydrogen from the substrate. The hydrogen ultimately appears as water; the end-product of the carbonaceous residue is fumarate. The calculated R.Q. will be $X/(X + X)$ or 0.5. In actual experiment, amounts of succinate have been added such that the oxygen uptake was more than doubled. Under these conditions R.Q. values of 0.43 have been observed (6). These experiments are important because they show that normal tissue contains a mechanism which can mobilize much larger amounts of hydrogen from succinate than the amounts of hydrogen ordinarily mobilized from the natural substrates in normal respiration. Kinetic studies likewise emphasize the enormous hydrogen-carrying capacity of succinate. Szent-Györgyi and co-workers have shown that the small amounts of succinate present in normal tissue are oxidized at a sufficiently rapid rate to account for all of the hydrogen transported to cytochrome in normal respiration (7, 8).

A second peculiarity of the succinate-succino-dehydrogenase system is its unique ability to react with cytochrome. In Keilin's experiments on the hydrogen-carrying function of cytochrome (9), succinate was employed as the substrate, which was able to reduce cytochrome in the presence of succino-dehydrogenase. A survey of other dehydrogenase systems, however, indicated that they do not possess this property. Ogston and Green (10, 11) examined 11 different dehydrogenase systems, and observed reduction of cytochrome only in the presence of succino-dehydrogenase. Szent-Györgyi and co-workers (1) likewise emphasize the specificity of the reaction between succinate and cytochrome.

The salient facts about succino-dehydrogenase are the following: the system is extremely widespread in animal tissues, the system is extremely active, and the reaction with cytochrome is specific. It is therefore highly probable that the system is biologically important, despite the fact that the amounts of succinate formed in metabolic processes are relatively small, or despite the fact that no one has succeeded in demonstrating that succinate is a metabolite in higher animals. Szent-Györgyi provisionally assumed that the function of the succinate system was to transport hydrogen to cytochrome (7).

Certain observations with inhibitors, however, tended to shift the emphasis from succinate to fumarate. Thus malonate inhibited the action of succino-dehydrogenase (12), and malonate also inhibited respiration (7, 8). The addition of succinate to a system poisoned by malonate did not appreciably increase respiration. However, the addition of fumarate completely compensated for malonate inhibition (6, 8). Not only that, but fumarate actually increased respiration in the presence of malonate, the increase being for all practical purposes the same as when malonate was absent (Fig. 3).

The increase in respiration due to fumarate was in reality a preservation of the initial rate, rather than a true increase. Banga (13) reported that when fumarate was added to pigeon breast muscle suspended in M/15 phosphate at pH 7.4, the initial rate of respiration was maintained for 30 minutes or more, whereas similar tissues showed a rapidly decreasing rate of respiration in the absence of added fumarate. This observation has been confirmed repeatedly (6, 14, 15). Since the function of the added fumarate appeared to be to conserve the respiration rather than to increase it, the assumption was made that necessary fumarate diffused out of the cells into the surrounding medium, decreasing the concentration within the cell until fumarate became the limiting factor in respiration. As the amount of fumarate which preserved respiration, 0.0066 M, roughly approximated the amount present in the tissue, there

[1] When very small amounts of succinate are added to respiring muscle, a true catalytic effect is observed. See below.

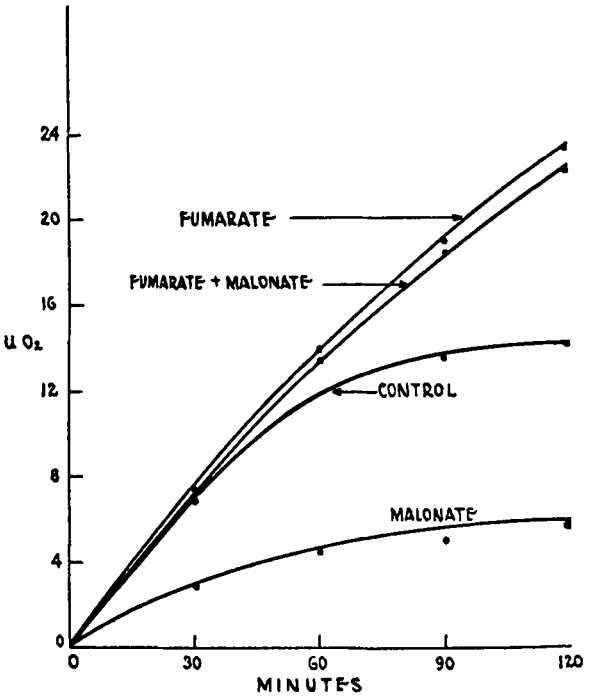

Fig. 3. The effect of malonate on the respiration of pigeon breast muscle stimulated by fumarate. 0.005 M fumarate and malonate. The results are expressed in terms of oxygen uptake (U_{O_2}), defined as the mm.[3] of oxygen absorbed per mg. dry weight of tissue in the time t (6).

appeared to be some validity to the assumption. Szent-Györgyi therefore postulated that it was not the succinate-fumarate which functioned in hydrogen transport, but rather fumarate-oxalacetate (8). Unfortunately the "preserving" effect of fumarate is not generally observed under the experimental conditions originally outlined by Szent-Györgyi. Of 24 experiments with pigeon muscle in phosphate, we observed a true conserving effect only 7 times. With 21 pig hearts, we never observed a true conserving effect, although fumarate almost always increased respiration somewhat (6). Furthermore, the amount of fumarate necessary to increase respiration in this medium was so great that the increased oxygen consumption could have been due entirely to the oxidation of the fumarate—that is, the fumarate might have been acting only as a substrate. As most tissues contain an extremely active fumarase which converts fumarate to malate, and as they also contain a malico-dehydrogenase, the substrate action of fumarate might be explained on this basis. However, as far as could be determined by questionable chemical methods of analysis, the amount of fumarate plus malate did not decrease during respiration (8, 16).

A study of the respiratory quotient indicated that the respiration of tissue fortified or preserved with fumarate was practically normal—in other words, that the measured oxygen uptake represented true respiration. We have observed R.Q. values of approximately 1.0 for fumarate respiration with both pig heart and pigeon breast muscle, using several media: M/15 phosphate, Ringer-phosphate, or the two media fortified with muscle juice (6). Szent-Györgyi reports that the respiratory quotient falls from 1.0 to approximately 0.8 as the respiration of muscle decreases (1, 17). When respiration is maintained by means of fumarate, the value of the respiratory quotient is maintained at the normal value, 1.0.

Proof of the catalytic action of fumarate came when very small amounts of fumarate were added to minced pigeon breast muscle suspended in Ringer-phosphate solution (6). In this medium much smaller amounts of fumarate conserved respiration than in plain phosphate buffer, 0.0002 M being approximately equivalent to 0.0066 M in the latter medium. A calculation of the added oxygen consumed in the presence of fumarate revealed that much more extra oxygen was consumed than could be accounted for by the complete oxidation of the fumarate to CO_2 and water. For example, in 20 experiments with concentrations of fumarate from 0.0001 to 0.0008 M, all showed increases in oxygen consumption much greater than could have been consumed by the fumarate. The discrepancy between the theoretical and the observed increases in oxygen consumption was sometimes quite spectacular. For example, when sufficient fumarate was added to form a 0.0002 M solution, the amount of oxygen sufficient to completely oxidize this fumarate in 3.00 cc. was 40 mm.[3] The increase in oxygen consumption actually observed with this amount of fumarate was 255 mm.[3], an increase over the theoretical of 6-fold.

More recent experiments indicate that the discrepancy may persist, that is, fumarate may act catalytically in concentrations up to 0.002 M. There is, no doubt, some catalytic action at even higher concentrations, but at these higher concentrations a substrate effect is also operative. Not only fumarate, but substances which yield fumarate in the presence of respiring tissue increase the respiration of pigeon breast muscle in an analogous manner. Low concentrations of succinate, malate, oxalacetate, or citrate all produced increments in oxygen consumption greater than could be accounted for by the complete oxidation of the material added. Table I presents recent data illustrating the catalytic activity.[2]

[2] Unless otherwise stated all of our experimental work discussed in this paper has been done using Ringer-phosphate buffer (6) (PO_4 = M/60, pH = 7.4, 0.2 p.c. glucose).

TABLE I

The increase in the respiration of pigeon breast muscle stimulated by small amounts of fumarate and allied substances.

Concentrations	Oxygen uptake in mm.³	
	Observed increase*	Theoretical increase from complete oxidation (6)
0.0002 M fumarate	197	26.8
0.0004 M "	169	53.6
0.001 M "	298	134.0
0.002 M "	308	269.0
0.0004 M succinate	137	62.8
0.0004 M malate	134	53.6
0.0002 M citrate	69	40.3
0.0004 M "	126	80.6
0.001 M "	211	202.0
0.002 M "	115	403.0

* Increase calculated as follows: $U_{O_2}^{120''}$ of experimental sample *minus* $U_{O_2}^{120''}$ of control sample *times* dry weight of experimental tissue.

The final evidence of catalytic action was a demonstration of catalytic potency of the medium in which tissues had respired (6). The experiments were performed as follows: small amounts of fumarate, 0.0004 - 0.0008 M, were added to pigeon breast muscle and the muscle allowed to respire for 2 hours, during which time an increased oxygen uptake was observed with respect to control muscle. At the end of the period, the tissue was filtered off and aliquots of the fluid or "used juice" were added to fresh respiring pigeon breast muscle. "Used juice" from control experiments, unsupplemented with fumarate, showed a slight inhibitory effect on the respiration of fresh tissue. "Used juice" from experiments in which the fumarate has been added originally, still stimulated respiration somewhat; it showed a marked stimulation when compared to the "used juice" from the control samples. The catalytic potency of the fluid containing small amounts of fumarate had therefore been retained, and this in spite of the fact that the respiration in the original flask had been increased to a much greater extent than would have been sufficient to destroy all of the added fumarate.

It was thus established that fumarate can act catalytically *in vitro* under certain special circumstances. Although the critical experiments had been performed with pigeon breast muscle, other tissues were subsequently shown to behave in a similar manner, usually qualitatively and sometimes also quantitatively. The advantages of pigeon breast muscle are its comparatively high rate of respiration, the ease and speed with which large amounts of tissue can be obtained from a single animal, and the low cost. It is highly desirable that the experiments begin as soon after removal from the animal as possible. The pigeon is beheaded, the breast muscle removed and placed on ice for 2 or 3 minutes, and then minced in a chilled Latapie mincer, the muscle being collected in a chilled petri dish containing filter paper moistened with saline solution. For accurate work the tissue samples must be weighed separately. Attempts at pipetting suspensions of Latapie mince, even with the use of a wide-mouthed pipette, gave variable results. We weigh our tissue samples on a torsion balance, on small pieces of cellophane, which are added to the reaction flask with the tissue, and the tissue dispersed by means of a wire. Twenty to twenty-five minutes elapse from the time the bird is beheaded until the zero reading.

With some tissues it is important that a fairly constant amount of tissue be used for a given volume of medium, since the rate of respiration decreases if the amount of tissue is too low—Krebs' "dilution effect" (18). For pigeon breast muscle in Ringer-phosphate medium this decrease is usually observed when the amount of tissue in 2 cc. of medium is 100 mg. fresh weight or less. In most of our experiments we used approximately 150 mg. of tissue. Supplements such as *koch-saft* (deproteinized muscle juice) or the "C_4 — C_6" acids abolish the dilution effect. In lower respiring tissues, such as rat skeletal muscle, the

TABLE II

The effect of dilution on the respiration of rat skeletal muscle.

Supplements	Oxygen uptake in U_{O_2}			
	Tissue = 100 mg.*		Tissue = 300 mg.*	
	1st hr.	2nd hr.	1st hr.	2nd hr.
0—Control	1.7	1.8	3.6	4.0
0.0004 M fumarate	2.4	2.9	3.4	3.7
0.0004 M citrate	1.7	1.9	3.0	3.4
0.0004 M citrate + 0.2 cc. *kochsaft*	5.3	8.0	5.4	7.4

* Wet weight.

dilution effect is more pronounced (Table II).

The difference between results obtained with minced tissue as compared to tissue slices might also be interpreted as a variation of the dilution effect, if one considers that there will be a greater diffusion of necessary substances into the medium when there is a greater degree of tissue destruction. However, cellular integrity is not essential for a demonstration of the fumarate effect. Tissues ground with sand do not respond to fumarate, but when *kochsaft* is added, the response is equal to that of intact tissue (6). Banga and Szent-Györgyi (19, 20) and Greville (21) have studied the respiration of tissue dispersions—particles which pass through muslin but which are removed by centrifugation—and find that they also respond to fumarate when suitable supplements are added.

Greville (15) working with muscle, and Elliot and Elliot (22) working with liver dispersions, have both emphasized the importance of the tonicity of the solution in demonstrating "C_4 effects." Using plain phosphate buffer M/15, they report increases in respiration when NaCl is added to the medium. In effect, this amounts to conversion of the medium from plain phosphate to the Ringer-phosphate type of medium, in which both the rate of respiration, and the sensitivity to fumarate are known to be greater (6). Elliot reports that action of malate, oxalacetate, citrate, α-keto-glutarate, and pyruvate is completely dependent upon the chloride ion.

A number of muscles other than pigeon breast, have been studied for their response to fumarate. Muscle from pig heart gave essentially the same results as muscle from pigeon breast, although the response to fumarate was quantitatively somewhat less (6). Minced rabbit heart is sensitive to both fumarate and citrate in Ringer-phosphate buffer. Using concentrations of 0.0004 M we have observed catalytic increases in respiration with these materials both in the presence and absence of *kochsaft*. The response to fumarate was usually somewhat greater than the response to citrate, and with some rabbit hearts the response to fumarate was also more consistent: that is, fumarate not only stimulated the respiration of the unsupplemented tissue, but also of tissue supplemented with *kochsaft* or insulin or mixtures of the two. Citrate did not always stimulate the supplemented tissues. The Q_{O_2} of our control rabbit hearts averaged 12.0. The protocols of a typical experiment are given in Table III.

Rabbit skeletal muscle had a lower rate of respiration than rabbit heart ($Q_{O_2} = 5.0$) and the tissue was also less sensitive to fumarate. Fumarate increased respiration in 2 out of 3 samples of muscle, but in only one case was the magnitude of the increase sufficient to indicate catalysis. A catalytic increase in respiration due to citrate was never observed, although respiration was increased somewhat in 2 out of 3 cases.

Rat skeletal muscle had an even lower rate of respiration (average $Q_{O_2} = 2.0$) and it was less responsive to fumarate and citrate than rabbit muscle. This tissue showed a pronounced dilution effect; that is, respiration decreased as the amount of tissue was "diluted" with medium (Table III). When the tissue was sufficiently dilute, 100 mg. per 2 cc., respiration was increased by 0.0004 M or 0.02 M fumarate, though not catalytically. 0.0004 M citrate was inactive; 0.02 M citrate actually decreased respiration. In contrast to pigeon breast muscle, rat skeletal muscle needed *kochsaft* as well as citrate or fumarate to overcome the dilution effect. In general, *kochsaft* was the most effective single agent in abolishing the dilution effect, particularly with low respiring tissues.

Rat diaphragm has been studied by Greville (15) who noted that fumarate increased respiration while malonate inhibited it. Sensitivity to

TABLE III

The effect of fumarate and citrate on the respiration of rabbit heart muscle.

Supplements	Oxygen uptake in U_{O_2}		
	1st hr.	2nd hr.	3rd hr.
0—Control	12.9	17.3	18.6
0.0004 M fumarate	16.2	22.4	24.3
0.0004 M citrate	14.7	20.9	22.0
0.2 cc. *kochsaft*	13.4	20.3	22.8
Fumarate + *kochsaft*	17.3	25.8	27.4
Citrate + *kochsaft*	14.4	26.0	32.2

fumarate was increased appreciably by mincing the tissue with scissors. The conclusion appeared to be that a certain amount of cellular damage was necessary before a fumarate effect could be demonstrated (even our Ringer-phosphate solution is not strictly physiological) although damage such as that produced by Ca ions rendered the tissue insensitive.

Chicken breast muscle is another tissue with a relatively low rate of respiration in Ringer-phosphate solution ($Q_{O_2} = 2.7$). Fumarate, citrate, malate, and succinate in concentrations of 0.001 M all stimulated respiration somewhat, but the increase was not sufficiently great to indicate catalysis. With 0.0004 M fumarate, however, catalytic action was indicated. With this tissue also, high concentrations of citrate, 0.01 M, inhibited respiration, although similar amounts of fumarate, malate, and succinate stimulated it. The inhibition due to high citrate could be corrected by the addition of *kochsaft*. It is of interest that a decrease in respiration due to high citrate has been recorded by Krebs and Eggleston (18), who, however, did not stress the point. They used pigeon breast muscle in M/15 phosphate.

Normal human skeletal muscle has a low rate of respiration ($Q_{O_2} = 3.8$). It shows a pronounced dilution effect. It also responds favorably to succinate, and occasionally to citrate, although the latter may inhibit respiration if present in higher amounts. Failure of succinate oxidation by human muscle tissue has been found in various myopathies (23).

Several tissues other than muscle have been studied for fumarate, "C_4", catalysis. Szent-Györgyi and co-workers (8) report that fumarate stimulates and malonate inhibits the respiration of rabbit kidney mince. Stare (24) has shown that fumarate preserves the respiration of rabbit kidney and liver mince in a manner similar to that observed in pigeon breast muscle. Fumarate compensated for malonate in this tissue, the response depending upon both the absolute and the relative amounts of the substances present.

It has been possible to demonstrate the catalytic action of a number of substances with rat kidney. Both slices and mince were used. In the presence of glucose, catalytic action was observed in 2 out of 4 samples of mince containing 0.0004 M fumarate, 1 out of 3 samples containing 0.0004 M malate, and 1 out of 1 sample containing 0.0004 M succinate. In no case (4 experiments) was catalysis observed with 0.0004 M citrate. Increased respiration was observed with all 4 acids when the concentration was increased to the point where a substrate effect was operative. With kidney slices, catalytic increases in respiration were observed when low concentrations of citrate, fumarate, or malate were added to Ringer-phosphate solution containing glucose. In the absence of added glucose, there was very little stimulation of respiration with either citrate, fumarate, or malate. This was true for both sliced kidney and kidney mince, and appeared to indicate that the acids in question increased respiration by stimulating the oxidation of carbohydrate. The demonstration of catalysis might, however, also have been somewhat dependent on the rate of respiration of the tissue. The Q_{O_2} of minced kidney was 8.0 in the absence of glucose, and 9.1 in its presence; for kidney slices, the Q_{O_2} averaged 11.0 in the absence of glucose, and 15.3 in its presence. In all cases kidney exhibited a more constant rate of respiration than muscle. As with muscle, respiration was usually somewhat better in the presence of fumarate than in the presence of citrate. The results of an experiment with kidney tissue are given in Table IV.

Rat liver, like rat kidney, was usually sensitive to the "C_4 acids." Concentrations of fumarate

TABLE IV

The effect of fumarate and citrate on the respiration of rat kidney mince and slices.

Supplements	Oxygen uptake in U_{O_2}					
	No glucose in buffer			0.2 p.c. glucose in buffer		
	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.
Mince						
Control	8.0	13.3	18.6	8.3	15.8	20.4
0.0004 M fumarate	8.2	13.5	17.7	9.6	19.0	25.1
0.0004 M citrate	7.5	12.9	17.7	8.8	15.9	21.3
Slices						
Control	11.0	18.6	26.1	13.6	27.2	39.3
0.0004 M fumarate	8.2	14.1	19.6	20.6	43.0	62.7
0.0004 M citrate	5.8	9.8	13.3	17.8	37.7	54.3

and citrate of 0.01 M and 0.001 M, increased respiration in 2 out of 3 samples of minced liver, and in 1 out of 3 samples of slices. Low concentrations, 0.0004 M, gave a catalytic response in 1 out of 2 samples of minced liver, but not with liver slices. The average Q_{O_2} for the minced liver was 3.6; for corresponding liver slices it was 5.9. The response of minced liver to fumarate and citrate was generally the same, but liver slices invariably showed greater increases in respiration with fumarate. Table V gives data from one of the liver experiments.

TABLE V

The effect of fumarate and citrate on the respiration of rat liver slices and mince.

Supplements	Oxygen uptake in U_{O_2}			
	Slices		Mince	
	1st hr.	2nd hr.	1st hr.	2nd hr.
0—Control	5.2	9.2	3.6	4.3
0.001 M fumarate	8.8	15.5	6.5	7.3
0.001 M citrate	7.6	11.7	4.5	5.4

Tumor tissue (Flexner-Jobling rat carcinoma) was less sensitive than most of the normal tissues studied. Tumor mince in Ringer-phosphate solution (containing glucose) had a Q_{O_2} of only 2.3. This material did not respond to fumarate, citrate, malate, succinate, *kochsaft,* cytochrome-c, or various combinations. Tumor slices, however, had a higher rate of respiration, $Q_{O_2} = 7.3$. They did not respond to the "C_4 acids" alone, but in the presence of *kochsaft,* a 30 percent increase in respiration was observed when 0.001 M fumarate, malate, or citrate were added. Boyland and Boyland have reported that fumarate increases the respiration of the Jensen rat sarcoma, and of the Crocker rat sarcoma 180 (14). An interesting peculiarity of the tumor slices was the persistence of the respiration. Even after 4 hours the slices were respiring at their initial rate (Fig. 4).

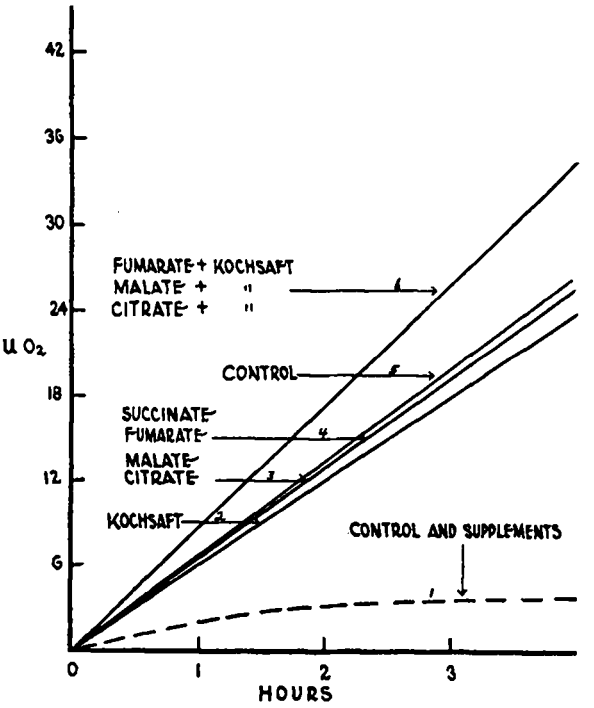

Fig. 4. The effect of fumarate and allied substances on the respiration of slices and of mince of Flexner-Jobling rat carcinoma. 0.001 M succinate, fumarate, malate, and citrate; 0.2 cc. *kochsaft.* Broken line indicates minced tissue.

Summarizing, there appears to be abundant evidence that the "four carbon acids" are active in a wide variety of tissues. It may be that they function in all tissues, for the failure to demonstrate increased respiration on the addition of more acid obviously does not mean that the acid already present is inactive. On the other hand, an extreme opposite view probably should be stated, namely, that the increases in oxygen uptake observed *in vitro* may be merely artifacts of the laboratory, without any significance in the living animal, in as much as the demonstration of "catalysis" invariably depends on a certain amount of tissue damage. Corroborative evidence, however, suggests that the truth lies somewhat nearer the former view than the latter.

The various forms of evidence leading to the revised concept of hydrogen transport are discussed in detail in Szent-Györgyi's book "Studies on biological oxidation and some of its catalysts". Briefly the evidence is as follows: Tissues contain a mechanism for the very rapid reduction of oxalacetic acid, the end product being malic acid (8, 16). The kinetics of the reaction are such that all of the hydrogen mobilized in respiration could be carried by this means. Having shown that the succinate-fumarate system reacts with cytochrome, whereas the malate-oxalacetate system acts with the substrate, it remained to link the two systems (25). Green (26) showed that the two systems could be linked by means of suitable electroactive hydrogen transmitters, dyes, the end products being succinate and oxalacetic acid. The transfer of hydrogen from malate to fumarate could be shown with dyes and also with cytochrome (27). Finally Banga demonstrated the transfer of hydrogen from donator to cytochrome by means of the double system: oxalacetate-malate and fumarate-succinate (28). The natural intermediate appeared to be a flavoprotein (29). The "old yellow enzyme" from yeast has a potential midway between that of the two "C_4 systems" (30), and Laki (31) has shown that the yellow enzyme can be reduced by malico-dehydrogenase and that it can transfer hydrogen to the succino-dehydrogenase system. However, in view of the many flavoproteins recently isolated (32) it is most unlikely that the "old yellow enzyme" of yeast is the active flavin in the mammalian cell. More likely prospects would be either the adenine-alloxazine proteid (M.B.—red. coenzyme) of Haas (33) which reacts very rapidly with methylene blue though not with molecular oxygen, or the heart flavoprotein of Straub (34) which is believed to be identical with the coenzyme factor (35).

One of the weaknesses of Szent Györgyi's scheme of hydrogen transport is that it does not explain the catalysis due to citric acid. Using suspensions of pigeon breast muscle in phosphate buffer, Krebs and Johnson (2) showed that the extra oxygen consumed in the presence of citrate was much greater than could have been used up by this citrate itself. We have repeatedly confirmed this observation using Ringer-phosphate medium (see above) and furthermore, have observed increases in respiration due to citrate in a wide variety of tissues although mol for mol citrate was usually less effective than fumarate. With pigeon muscle in phosphate Krebs and Johnson observed that citrate catalysis was much more pronounced in the presence of carbohydrates; hexose phosphate, glycogen, or α-glycero phosphate. In our experiments with kidney slices, glucose itself made possible the demonstration of a catalytic effect. The assumption, therefore, is that citrate catalyzes carbohydrate oxidation. Krebs and Johnson added citrate to respiring tissues and observed that it rapidly disappeared (2). In the presence of arsenite, which inhibits the oxidation of α-keto acids, α-keto glutaric acid accumulated. In the presence of malonate, succinic acid accumulated. In each case the "derivative" accumulated in amounts comparable to the citric acid which has disappeared. It was therefore postulated that citric acid was converted first to α-keto glutarate, then to succinate. The first step had already been accomplished *in vitro* by Martius and Knoop (36, 37).

With the demonstration that citrate acts catalytically, and the further demonstration that respiring tissue destroys citrate, it followed that a mechanism must be present for the regeneration of citrate. This was shown by incubating oxalacetic acid with muscle mince anaerobically. Citric acid was formed in large amounts. On this basis the citric acid "cycle" was formulated (Fig. 2). Szent-Györgyi and co-workers had shown that oxalacetate could be reduced directly to form malate and eventually succinate (8, 16); Krebs and Johnson showed that oxalacetate could be oxidized, presumably after condensation with a triose, to form citrate and then by further "oxidation" (dehydrogenation plus decarboxylation) form succinate. Experiments with malonate seemed to indicate that both pathways are possible (2). Oxalacetate, plus malonate, incubated with tissue aerobically yielded more succinate than when oxalacetate alone was used, the assumption being that the malonate, by checking succino-dehydrogenase, prevented further oxidation of the succinate previously formed by oxidation. Under anaerobic conditions a different phenomenon was observed. Actually more succinate was formed from oxalacetate than under aerobic conditions, presumably by reduction over malate and fumarate. In the presence of malonate, however, anaerobic succinate production was reduced, since

the inhibited succino-dehydrase lay in the direct path of succinate formation.

Krebs' citric acid cycle has been questioned by Breusch (38) who was unable to duplicate either an enhancement of citrate catalysis in the presence of glycerophosphate, or the formation of citrate from oxalacetate on incubation with muscle or liver. The first observation had been used by Krebs and Johnson (2) to link citrate catalysis with carbohydrate metabolism, the second, to indicate the mechanism of citrate regeneration. Breusch (38) contends that the citrate formed in Krebs' experiments arose by an unphysiological route, namely the formation of a condensation product *in vitro* when oxalacetic acid was neutralized, which condensation product was subsequently decarboxylated on incubation to form citric acid. In the absence of this pre-formed condensate, Breusch observed no citric acid formation from oxalacetic acid. On the contrary, malic acid was formed. Breusch further points out that citrate catalysis is usually somewhat delayed, as if the citrate had first to be converted to some other compound before it became active. Part of the discrepancy may have been due to differences in pH and in medium (plain phosphate by Krebs and Johnson, Ringer-phosphate by Breusch). It is noteworthy, however, that the catalytic activity of citric acid, iso-citric, *cis*aconitic, and α-keto glutaric acid were confirmed by Breusch, who was unsuccessful in establishing either of two possible explanations of his own for their activity. Thus, the Krebs cycle, though open to question in certain details has nevertheless served to focus attention on a new group of catalytic substances, the "C_6 acids."

Recent experiments of our own throw some doubt upon the specificity of malonate as an inhibitor for succino-dehydrogenase, since the effect of citrate on normal muscle was more effectively nullified by malonate than the effect of succinate itself. A few typical experiments will serve to illustrate the point. Using 0.001 M fumarate, succinate, and citrate, a marked stimulation of respiration was observed (Fig. 5). 0.001 M malonate inhibited respiration 31 percent; 0.01 M inhibited it 71 percent. When 0.001 M malonate was added along with 0.001 M fumarate, a 17 percent decrease in respiration was observed over the respiration of fumarate alone (U_{O_2} at 2 hours = 19.0 as compared to 23.0). The effect of 0.001 M succinate was reduced 16 percent by malonate (U_{O_2} at 2 hours = 16.0 as compared to 19.0). The effect of citrate, however, was reduced 42 percent (U_{O_2} at 2 hours with citrate = 24.0; citrate + malonate = 14.0). With excess malonate (0.01 M) approximately 70 percent inhibition was observed in the presence of 0.001 M

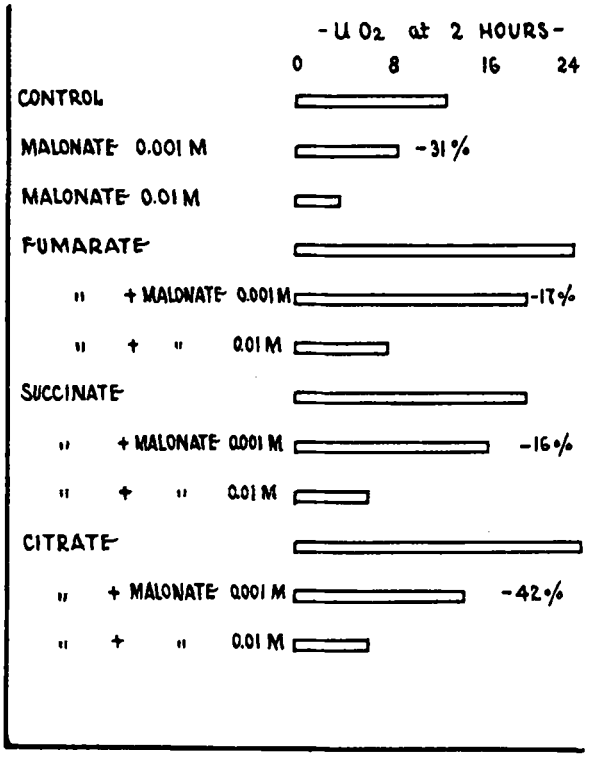

Fig. 5. The inhibiting effect of malonate on the respiration of pigeon breast muscle. 0.001 M fumarate, succinate and citrate; malonate in concentrations indicated.

succinate, fumarate, or citrate. The abnormal sensitivity of citrate was revealed even more clearly when higher concentrations of the salts were used. 0.005 M malonate inhibited respiration 60 percent; (U_{O_2} at 2 hours = 5.7 as compared to 14.2 for the control). Low concentrations, 0.001 M, of fumarate, succinate, or citrate stimulated respiration as before. When equal amounts of fumarate and malonate, 0.005 M, were added to muscle, respiration was practically the same as that observed in the presence of 0.001 M fumarate (U_{O_2} at 2 hours = 22.5 and 23.3). When equal amounts of succinate and malonate were added, respiration was equal to control respiration, but not equal to the oxygen consumption observed in the presence of low succinate only. Control, 14.2; 0.005 M succinate + malonate, 15.0; 0.001 M succinate, 23.1; 0.005 M malonate, 5.7 U_{O_2} for two hours. Increasing the amount of succinate to 0.01 M in the presence of 0.005 M malonate increased the U_{O_2} to 19.7.

In the presence of citrate, malonate inhibition was much stronger than in the presence of succinate. When equal amounts of citrate and malonate were added, respiration was not even restored to normal. Control, 14.2; 0.005 M citrate + malonate, 9.3; low citrate 0.001 M, 16.6 U_{O_2} for

2 hours. Increasing the amount of citrate to 0.01 M in the presence of 0.005 M malonate failed to increase the respiration; in fact, higher concentration of citrate actually depressed respiration somewhat (U_{O_2} for 2 hours = 7.8). It thus appears that malonate is not as specific in its action as hitherto supposed, a conclusion also reached by Weil-Malherbe (39).

A consideration of the curves of oxygen uptake indicated that the inhibiting effect of malonate was delayed for at least 20 minutes in the presence of succinate or fumarate, but in the presence of citrate the inhibiting effect of malonate manifested itself at once. This may have been due to purely physical phenomena, such as the relative rates of penetration of the substances into the cell. Some such explanation may also be involved in the frequent observations that citrate is not quite as effective in stimulating respiration as the other salts of the series. A study of the action of malonate on purified enzyme systems, as well as a study of accumulated end products, would appear highly desirable.

An interesting application of "C_4" and "C_6" catalysis has been the use of these substances in demonstrating an action of insulin *in vitro*. Krebs and Eggleston (18) proceeded from the premise that many factors are necessary in respiration, which may fail *in vitro* from a lack of any number of them. Hence they attempted to supply as many factors as possible. When enough other materials were added, insulin became the limiting factor, and insulin added to the mixture increased respiration.

A suspension of pigeon breast muscle in M/10 phosphate, pH 6.8, was used in their demonstration. It was supplemented with citrate, and with deproteinized muscle juice (*kochsaft*) which contained substrates, coenzymes, and inorganic ions. The juice stimulated respiration slightly, the citrate inhibited it. Juice and citrate appeared to counteract each other, since the addition of the two together gave a respiration approximately equal to that of the unsupplemented control. When insulin was added to such a "balanced" system, respiration was increased 90 percent. Insulin also increased respiration somewhat in the presence of citrate alone or of *kochsaft* alone. Both amorphous insulin and crystalline insulin hydrochloride were effective; but preparations containing zinc sometimes actually inhibited respiration. The experiments were continued for 4 hours.

Shorr and Barker (40) studied this effect on rabbit, cat, and dog muscle, and failed to observe any response to insulin. In pigeon muscle they observed a 20 percent increase in contrast to the 90 percent increase reported by Krebs and Eggleston. Banga, Ochoa, and Peters (41) observed stimulation due to insulin in certain brain preparations fortified with fumarate, adenylic acid, and pyruvate.

Our own experiments support the general conclusion that insulin may stimulate respiration *in vitro*. At first we attempted to repeat exactly the experimental conditions of Krebs and Eggleston (Table VI). Difficulties with their pipetting technique, however, forced us into weighing our tissue samples separately. In contrast to their results we observed a marked stimulation in respiration with citrate or *kochsaft* alone, and an even greater

TABLE VI

The effect of insulin and supplements on the respiration of pigeon breast muscle in M/10 phosphate buffer, pH 6.8.*

Supplements	Oxygen uptake				
	Total U_{O_2} values				4th hr. Q_{O_2}
	1st hr.	2nd hr.	3rd hr.	4th hr.	
0—Control	6.8	9.1	9.9	9.4	0
Citrate	13.5	19.8	22.1	23.5	1.4
Kochsaft	11.4	18.9	22.7	24.5	1.8
Citrate + *kochsaft*	14.1	22.9	27.1	29.1	2.0
Insulin + citrate + *kochsaft*	13.6	23.6	30.6	35.6	5.0
Fumarate + *kochsaft*	15.5	26.6	32.9	35.8	2.9
Insulin + fumarate + *kochsaft*	13.0	22.2	29.3	34.3	5.0

* 0.0134 M citrate and fumarate; 0.45 cc. *kochsaft*; 1.5 units amorphous insulin.

response when the two were added together (control U_{O_2} for 4 hours = 9.4, citrate plus *kochsaft* 29.1). Insulin plus citrate plus *kochsaft* increased the respiration 22 percent, the U_{O_2} being 35.6, which appeared to be about the maximum respiration for this tissue, since the same figure was reached by adding fumarate plus *kochsaft*, or fumarate plus *kochsaft* plus insulin. This effect was observed repeatedly.

Experimental conditions were therefore adopted which more nearly approached the physiological: Latapie mince, Ringer-phosphate pH 7.4, 0.001 M supplements of the "C_4 acids", 0.2 cc. *kochsaft* per 2 cc. of medium. Under these conditions *kochsaft*, fumarate, or 20 units of insulin[3] alone stimulated respiration about 20 per cent (Fig. 6). The respiration was "preserved" rather than accelerated. Any two of the supplements were more effective than one alone, in other words insulin increased respiration in the presence of fumarate or of *kochsaft*. Similarly, the three components were better than any two; insulin increased respiration in the presence of fumarate and *kochsaft*. However, insulin added only a 15 percent increment to a respiration which had already been stimulated 100 percent by fumarate and *kochsaft*. The effect was most pronounced during the last two hours of the experiment. Varying the amounts of insulin added between 2 and 40 units per flask, maximum stimulation was observed when 4 units were added to 200 mg. of tissue in 2 cc. of medium. Higher amounts decreased this effect, probably due to the preservative present in the insulin preparation. Essentially similar results were obtained when citrate (Table VII), succinate, or malate (Table VIII) was used in place

[3] Unless otherwise mentioned the insulin was the Eli Lilly preparation known as Iletin.

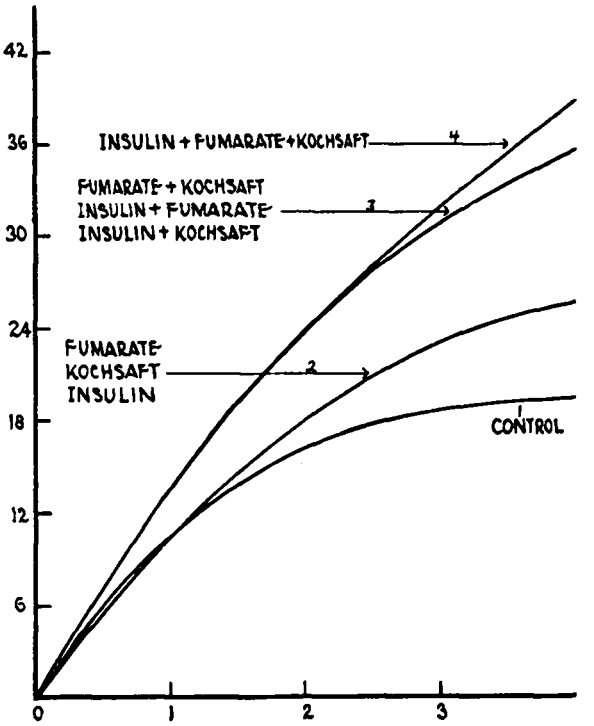

Fig. 6. The conserving action of insulin and fumarate on the respiration of pigeon breast muscle. 0.001 M fumarate; 0.2 cc. *kochsaft*; 20 units of insulin.

TABLE VII

The effect of insulin and of citrate on the respiration of pigeon breast muscle.*

Supplements	Oxygen uptake				
	Total U_{O_2} values				4th hr. Q_{O_2}
	1st hr.	2nd hr.	3rd hr.	4th hr.	
0—Control	12.6	20.7	23.3	26.2	2.9
Insulin, 4 units	15.4	26.0	33.6	39.5	5.9
" 8 units	14.9	24.7	32.5	38.4	5.9
" 20 units	12.9	23.2	29.7	34.4	4.7
" 40 units	11.8	20.2	26.6	32.0	5.4
Citrate	12.8	20.8	25.1	26.5	1.4
Kochsaft	12.8	23.3	29.2	30.6	0.4
Citrate + *kochsaft*	14.4	26.7	35.4	37.6	2.2
Insulin, 4 units + citrate	14.4	24.5	32.6	39.0	6.4
" + *kochsaft*	15.3	27.6	37.7	45.5	7.8
" + citrate + *kochsaft*	14.7	27.5	38.0	49.2	11.2

* 0.001 M citrate; 0.3 cc. *kochsaft*; 4 to 40 units of insulin as indicated.

TABLE VIII

The effect of insulin (Iletin and amorphous) and supplements on the respiration of pigeon breast muscle.*

Supplements	Oxygen uptake in U_{O_2}			
	1st hr.	2nd hr.	3rd hr.	4th hr.
0—Control	8.7	11.6	12.0	12.2
Insulin (Iletin)	11.8	13.8	14.1	14.3
" (amorphous)	10.3	13.0	13.3	13.5
Fumarate + *kochsaft*	13.9	23.5	30.3	32.8
Insulin (Iletin) + fumarate + *kochsaft*	13.9	24.2	33.4	35.2
" (amorphous) + " + "	14.6	24.0	31.5	37.2
Succinate + *kochsaft*	13.6	22.4	28.9	32.1
Insulin (Iletin) + succinate + *kochsaft*	13.3	22.6	30.3	35.2
" (amorphous) + " + "	13.4	23.6	31.1	36.0
Malate + *kochsaft*	12.8	22.0	28.6	31.3
Insulin (Iletin) + malate + *kochsaft*	15.8	27.3	36.7	39.7
" (amorphous) + " + "	12.9	22.3	30.0	35.7

* 0.001 M fumarate, succinate and malate; 0.2 cc. *kochsaft*; 4 units of insulin.

of the fumarate. Amorphous insulin[4] reacted similarly to Iletin.

We have repeated these insulin experiments on rabbit heart and skeletal muscle, and chicken breast muscle, in every case using Ringer-phosphate buffer. The effect was less striking with these lower respiring muscles but qualitatively the results were the same as those reported for pigeon breast muscle.

Summarizing, in experiments with insulin on surviving muscle, a slight stimulation of respiration was nearly always observed when the tissue was suitably supplemented. Sometimes the stimulation was quite appreciable. Insulin alone, however, was highly variable in its effect. Approximately one-third of the tissues showed some stimulation, one-third showed no effect, and one-third showed a slightly decreased respiration in the presence of insulin. A possible explanation of the variation may have been the variable amounts of insulin already present in the tissue. Hence, the effect of insulin was studied on the respiration of muscle from depancreatized pigeons. The general experimental procedure was the same as before: 150 to 200 mg. of Latapie mince, Ringer-phosphate buffer pH 7.4, 0.001 M "C_4 acids," 1 to 4 units of amorphous insulin or Iletin per 2 cc. fluid. The experiments were performed one week to 10 days after pancreatectomy.[5]

Insulin alone markedly stimulated the respiration of muscle from depancreatized birds. In the first experiment the U_{O_2} at 4 hours was 19.0 for the control, and 38.0 in the presence of insulin, an increase of 100 per cent. As with normal muscle, fumarate plus *kochsaft* increased respiration markedly, and insulin in addition added another increment to the respiration. Since the rate of respiration was "preserved", the effect of insulin was most marked during the third and fourth hours of the experiment. In fact, during the latter half of this experiment insulin alone gave an increase of over 500 per cent as compared with the control (Fig. 7).

With a second depancreatized pigeon, insulin alone stimulated respiration 50 per cent, but insulin plus citrate plus *kochsaft* gave a 100 per cent increase over the effect of citrate plus *kochsaft* (Table IX). 0.001 M malonate markedly inhibited respiration stimulated by insulin. Again the effect was most marked in the third and fourth hours.

With a third depancreatized pigeon, 1 unit of amorphous insulin stimulated respiration 30 per cent; in the presence of *kochsaft* and "C_4", it stimulated respiration 50 per cent, in contrast to

[4] The preparation assayed 20 units per milligram. We are indebted to Dr. G. H. A. Clowes of Eli Lilly Co. for kindly supplying us with this sample.

[5] We are indebted to Dr. H. P. Rusch for aid in the pancreatectomies.

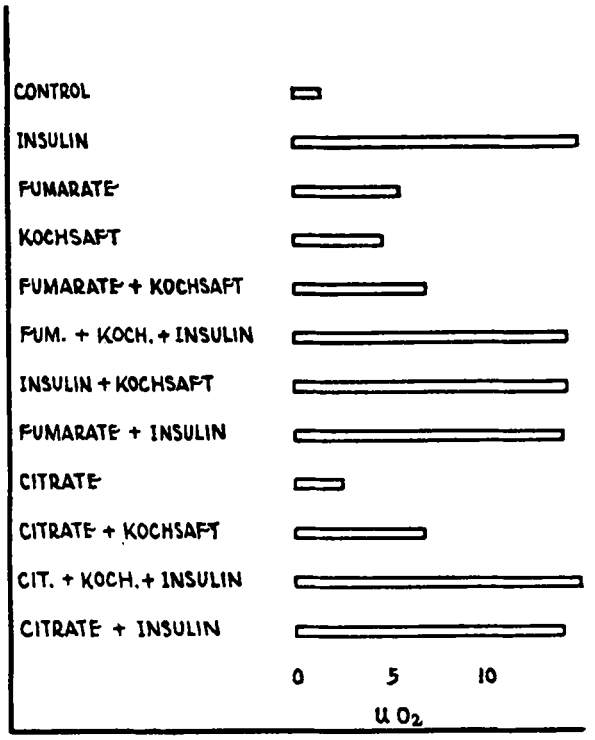

Fig. 7. The effect of insulin and supplements on the oxygen consumption of breast muscle from a depancreatinized pigeon, during the third and fourth hours of the experiment. 0.001 M fumarate and citrate; 0.2 cc. *kochsaft*; 4 units of insulin. Blood sugar before pancreatectomy was 184 mg. per cent; 9 days after, it was 210 mg. per cent.

the 20 per cent increase usually observed with normal muscle. "C_4", fumarate, citrate, malate, and succinate were interchangeable, and again the effect was most marked in the third and fourth hours.

The unexpected action of malonate in inhibiting citrate catalysis even more than succinate catalysis, has already been referred to. This effect is particularly striking in muscle fortified with insulin. For example, with our usual experimental conditions, the following 4 hour U_{O_2} values were observed: control, 28.2; Iletin (4 units), 26.7; Iletin + 0.005 M malonate, 5.1; 0.005 M citrate + *kochsaft*, 27.9; Iletin + citrate + *kochsaft*, 34.2; malonate + Iletin + citrate + *kochsaft*, 8.9 (Table X). The malonate apparently interfered with some system through which the insulin effect had been expressed. When fumarate was substituted for citrate, however, the inhibition due to malonate was usually negligible, as shown by the following 4th hour U_{O_2} values: control, 20.0; 0.005 M fumarate + *kochsaft*, 28.7; Iletin (4 units) + fumarate + *kochsaft*, 41.0; 0.005 M malonate + Iletin (4 units) + fumarate + *kochsaft*, 37.0 (Table XI).

The malonate, therefore, obviously had not inhibited the insulin directly. The results emphasize the extreme sensitivity of citrate to malonate, as well as the fact that a least a part of the "citric acid cycle" must be intact for insulin to exert an *in vitro* effect.

TABLE IX

The effect of insulin and supplements on the oxygen consumption of breast muscle from a depancreatized pigeon.*

Supplements	Oxygen uptake							
	Hourly Q_{O_2} values				Total U_{O_2} values			
	1st hr.	2nd hr.	3rd hr.	4th hr.	1st hr.	2nd hr.	3rd hr.	4th hr.
0—Control	14.7	8.9	3.5	1.0	14.7	23.6	27.1	28.1
Insulin, 1 unit	17.2	11.6	8.7	3.0	17.2	28.8	37.5	40.5
Amorphous insulin, 1 unit	17.8	12.5	7.2	2.7	17.8	30.3	37.5	40.2
Insulin, 4 units	19.2	12.8	6.0	3.4	19.2	32.0	38.0	41.4
Amorphous insulin, 4 units	17.3	10.4	6.9	4.4	17.3	27.7	34.6	39.0
Insulin, 4 units + malonate	4.8	1.7	0.3	0.2	4.8	6.5	6.9	7.1
Citrate + *kochsaft*	16.2	11.8	5.8	1.1	16.2	28.0	33.8	34.9
Insulin, 4 units + citrate + *kochsaft*	19.5	15.7	12.8	11.0	19.5	35.2	48.0	59.0
Insulin, 4 units + citrate + *kochsaft* + malonate	8.1	3.4	1.5	0.4	8.1	11.5	13.0	13.4
Fumarate + *kochsaft*	18.8	14.6	3.0	1.2	18.8	33.4	36.4	37.6
Insulin, 4 units + fumarate + *kochsaft*	17.8	15.2	11.0	10.0	17.8	33.0	46.0	56.0

* 0.001 M fumarate and citrate; 0.005 M malonate; 0.2 cc. *kochsaft*; 1 and 4 units of insulin and amorphous insulin as indicated. Blood sugar before pancreatectomy was 164 mg. per cent; 11 days after it was 216 mg. per cent.

TABLE X

The effect of malonate on the respiration of pigeon breast muscle stimulated by small amounts of insulin and citrate.*

Supplements	Oxygen uptake				
	Total U_{O_2} values				4th hr. Q_{O_2}
	1st hr.	2nd hr.	3rd hr.	4th hr.	
0—Control	12.1	20.7	26.6	28.2	1.6
Citrate + *kochsaft*	11.8	19.7	25.2	27.8	2.6
Insulin + citrate + *kochsaft*	12.0	20.6	28.0	34.2	6.2
Insulin + citrate + *kochsaft* + malonate	5.4	7.8	8.5	8.9	0.4
	(Malonate inhibition at 4 hrs. = 74 p.c.)				
Insulin	10.7	17.4	22.6	26.7	4.1
Insulin + malonate	3.5	4.4	4.7	5.1	0.4

* 0.005 M citrate and malonate; 0.2 cc. *kochsaft*; 4 units of insulin.

The inhibition of the insulin effect *in vitro*, whether directly or indirectly through the citrate cycle, raised the question whether malonate might not also hinder the action of insulin in the living animal. Accordingly, a 15 per cent solution of neutralized malonate was injected subcutaneously into rabbits, either before, or simultaneously with, a solution of insulin. The sugar content of the blood was then determined at intervals using the Shaffer-Hartman method. The exact dosages and the results of a preliminary experiment are given in Table XII. In rabbits No. 1 and 4, which received 4 units of insulin, the blood sugar fell rapidly and the animals went into convulsions. Rabbit No. 2 received the same amount of insulin, but in 2 cc. of molar malonate solution, and the blood sugar failed to decrease. Rabbits No. 3 and 5, receiving similar supplements, showed some decrease in blood sugar, though the decrease was much greater when insulin alone was given. No. 6 received malonate 15 minutes before the insulin, and here again the blood sugar fell only slightly.

Table XIII gives the results of a more complete experiment. A study of this table shows that malonate retarded the action of insulin in lowering blood sugar for about an hour (rabbits No. 6 through 9, as compared with the first 5), but as time went on the malonate effect wore off and the blood sugar decreased. With the rabbits receiving malonate without added insulin (rabbits No. 10 through 14) there was a tendency for the blood sugar to rise slightly. No. 12 and 13, which received twice as much malonate as No. 10 and 11 showed a definite rise. No. 14 which received the same total amount of malonate as No. 12 and 13, but given at hourly intervals rather than in one dose at the beginning of the experiment, showed a marked temporary rise. The temporary effect of malonate in retarding the

TABLE XI

The effect of malonate on the respiration of pigeon breast muscle stimulated by small amounts of insulin and fumarate.*

Supplements	Oxygen uptake				
	Total U_{O_2} values				4th hr. Q_{O_2}
	1st hr.	2nd hr.	3rd hr.	4th hr.	
0—Control	13.6	18.5	19.5	20.0	0.5
Fumarate + *kochsaft*	13.4	21.9	28.4	28.7	0.3
Insulin + fumarate + *kochsaft*	14.7	24.3	33.9	41.0	7.1
Insulin + fumarate + *kochsaft* + malonate	15.0	23.6	31.4	37.0	5.6
	(Malonate inhibition at 4 hrs. = 10 p.c.)				

* 0.005 M fumarate and malonate; 0.2 cc. *kochsaft*; 4 units of insulin.

TABLE XII

The effect of insulin and malonate on the blood sugar of the rabbit.*

		Blood sugar in mg. p.c.			
		0 Hour		4 Hours	
Rabbit No. 1	4 units insulin	104 mg. p.c.		39 mg. p.c.	(convulsion)
" " 2	4 units insulin in 2 cc. of M/1 malonate	116 "		113 "	
" " 3	4 units insulin in 2 cc. of M/1 malonate	142 "		111 "	
		0 Hour	1 Hour	2 Hours	
Rabbit No. 4	4 units insulin	101	80	56	(convulsion at 5 hours)
" " 5	4 units insulin in 2 cc. M/1 malonate	96	75	75	
" " 6	10 cc. M/1 malonate, 15 minutes later given 4 units insulin	103	85	90	

* Normal fed rabbits, 2 to 2.5 kg. in weight.

fall in blood sugar following insulin administration agrees with the results of Huszak (8) who observed that injection of malonate increases the excretion of acetone bodies for a period of about 2 hours.

These experiments are only preliminary. They are mentioned in the present form because they give some indication that reactions studied in the manometric vessel also proceed in the intact animal. Furthermore, they suggest another explanation for the great variability in insulin action, since insulin is only one of the factors involved in carbohydrate oxidation; others are fumarate, citrate, and the enzymes which activate them.

TABLE XIII

The effect of insulin and malonate on the blood sugar of the rabbit.*

		Blood sugar in mg. p.c. Hours				
		0	1	2	4½	
Rabbit No. 1	4 units insulin	86	26	(convulsions)		
" " 2	" " "	93	46	47	54	
" " 3	" " "	82	58	39	(convulsions)	
" " 4	" " "	98	52	45	39	(convulsions)
" " 5	" " "	83	51	42	35	"
" " 6	10 cc. M/1 malonate—15 min. later given 4 units insulin	96	83	55	37	(convulsions)
" " 7	" " " "	100	104	61	64	
" " 8	20 cc. M/1 malonate—15 min. later given 4 units insulin	83	70	66	36	(convulsions)
" " 9	" " " "	77	85	(died—no convulsion)		
" "10	10 cc. M/1 malonate	93	108	97	96	
" "11	" " "	96	114	114	115	
" "12	20 cc. " "	106	110	121	123	
" "13	" " "	84	109	102	135	
" "14	10 cc. M/1 malonate at beginning, repeated at end of 1st hour	110	99	212	107	

* Starved (24 hours) rabbits, 2 - 2.5 kg. in weight.

There is ample evidence that fumarate, malate, oxalacetate, citrate, and succinate function catalytically in the biological oxidations of isolated tissues. Insulin is somehow connected with this catalytic cycle. The cycle catalyses the oxidation of carbohydrate. Malonate, under appropriate conditions, inhibits the cycle. There is some evidence that these *in vitro* experiments are representative of reactions in the intact mammalian organism.

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DISCUSSION

Dr. Burk: There is one idea which I think was not brought out that lends much support to Szent-Györgyi's original mechanism. The idea has been somewhat expressed already by Elliot, namely, that citrate should be regarded as an incidental, perhaps unnecessary, source of the malate and fumarate, and that in this sense the Krebs cycle is incidental to Szent-Györgyi's cycle which may be regarded as a fundamental cycle. It seems to me that this view is supported throughout by the new data just presented, especially in regard to the new point that malonate inhibits citrate action more easily and more quickly than it does fumarate or succinate action. This would be necessarily true, if citrate were a source of fumarate and succinate, under the conditions when the latter are limiting. One would get a greater and quicker inhibition in the case of citrate, because the succinate-fumarate system would, for a while at least, be more limiting than if the succinate and/or fumarate were added directly and adequately. The same logic holds with regard to the insulin results, all indications being that both citrate and insulin enter somewhat incidentally (even if not wholly) into respiration and its catalysis. The malonate acts solely and directly at the fumarate-succinate position when it does act.

Dr. Stare: Our data suggest that citrate is not a necessary part of the 4 carbon dicarboxylic acid system, but that it probably serves as a source of catalyst, presumably of succinate. We have not stressed this point, but may I refer again to data we have just given which indicate that citrate is not an essential component of the so-called Szent-Györgyi "cycle". The inhibition caused by 0.005 M malonate can be completely overcome by addition of succinate or fumarate in suitable concentration, but not by citrate. If succinate and fumarate functioned in a "cycle" of which citrate was an integral component, they could not overcome malonate inhibition, because malonate effectively blocks out citrate. This then indicates that citrate is not a necessary link in succinate-fumarate catalysis, but the fact that it acts catalytically in the absence of malonate suggests that it may serve as a source of catalyst.

With regard to insulin, it may not enter directly into fumarate catalysis, but our data suggest that it certainly has something to do with cellular respiration. Its action is emphasized in the experiments with breast muscle from depancreatized pigeons (see Table X).

Our data do not permit us to determine whether malonate acts solely at the fumarate-succinate position, although that is one possibility. It appears equally possible, however, that malonate is a more general inhibitor, affecting other systems, as for example, citrate.

Dr. Shorr: As Stare pointed out, Barker and I looked for the Krebs insulin effect in minced muscle of a number of mammals, including the dog, cat, and rabbit, and failed to find it. It is even absent in the breast muscle of the chicken. In view of Stare's observation that the insulin effect with pigeon breast mince is increased by using a Ringer-phosphate solution instead of water-phosphate, it may be profitable for us to repeat our experiments with this variation in the medium. I wonder whether Stare has any data as to the concentration of chlorides in the system as employed by Krebs, in comparison with the medium used by him. One would expect that the boiled muscle juice would contain all the chlorides present in the tissue used to prepare it. Furthermore, the mince used in the experiment would contribute its salts readily to the medium. The question of tonicity *per se* can hardly be expected to be significant when preparations are used whose cellular structure is completely destroyed. In surviving tissue slices proper tonicity becomes important. Slices of cardiac tissue show an enormous stimulation of respiration in boiled muscle juice uncorrected for tonicity, but this is soon followed by a sharp fall.

The fact that we obtained the insulin effect only with the pigeon, of all the species used, made us wonder whether there might be some peculiarities in the character of the metabolic processes in this bird. We were surprised to find that the respiratory quotient of the mince was in the neighborhood of unity no matter what the nutritional state. Even after a four day fast, which is extremely rigorous for a pigeon whose metabolism is so high, such respiratory quotients were also obtained. Blood sugar levels, which are ordinarily higher in pigeons than in mammals, persisted at these levels after fasts of three and four days. We then compared the respiratory quotient of the pigeon mince with that of muscle slices prepared simultaneously. Such slices, if carefully made, contain a high percentage of relatively intact muscle bundles and maintain their respiration very well. The respiratory quotients of the slices were at the low levels to be expected after a fast, while the quotients of the mince prepared from the same muscle were around 1.0. Mincing, therefore, immediately changes the nature of the foodstuffs being oxidized, bringing about an almost exclusive oxidation of carbohydrates, to judge from the respiratory quotient. Under such conditions, the effect of insulin on the respiratory rate must be considered as a maintenance of a type of metabolism which is also going on in its absence, rather than a change in the character of the metabolites oxidized. A demonstration of a transition from fat to carbohydrate metabolism would be more convincing.

I am glad that Stare has shown that the insulin effect is far from being a maintenance of the original level of respiration. If the curves are plotted to show the respiration for each period studied, the marked falling off with time with the pigeon as well as all the mammalian tissues which we have studied, becomes apparent. The insulin effect is generally observed only at low levels of respiration, and is most striking towards the end of the experiment, when the total oxygen consumption in both the insulin vessels and the controls is small.

Dr. Stare: We have no data on the relative chloride contents of our system and that used by Krebs. There are, no doubt, chlorides present in *kochsaft,* but the amount of *kochsaft* used by us was comparatively small. We tried to add just enough to increase the sensitivity of the tissue to the "C_4" acids without increasing the oxygen uptake materially in the absence of "C_4" acids. This amounted to 0.1 to 0.2 cc. per 2 cc. of medium. Krebs used nearly 5 times that amount. Of course, if one adds more *kochsaft,* the respiration is increased enormously.

In all of our experiments with muscle we have used a "Latapie mince," and I should like to mention that mincing with the Latapie does not begin to completely destroy the cells. Microscopic examination of the mince shows a large number of intact cells.

In comparing our results with those of others, we should like to emphasize the "time factor" as well as the use of the Ringer-phosphate type of medium. We feel it is very important to work at the utmost speed from the time the tissue is excised until the cocks of the manometric vessels are closed. With all of our experiments this has been less than 25 minutes.

As for the peculiarity of the pigeon, pigeons seem to get along all right without a pancreas, at least for two weeks. They do not lose weight, nor does the blood sugar go up as much as one might expect (from 180 to 200-210 mg. p.c.). They also seem to stand a lot of insulin. We have given pigeons as much as 150 units of insulin within 24 hours; the blood sugar was reduced to about half of the normal level.

Dr. Shorr: Some of the experimental variations employed by Barker and myself in attempting to repeat Krebs' work were fasting and pancreatic diabetes in the dog. Under these conditions, as well as with skeletal muscle from cats, rabbits, and the chicken, both fed and fasted, we failed to observe any stimulation of respiration with insulin. I should like to ask another question about the blood sugar changes noticed after the injections of malonate in the rabbit. Were the rabbits fasted, and if so, for how long?

Dr. Stare: Twenty-four hours.

Dr. Shorr: What would be the respiratory quotient of the rabbit after a twenty-four hour fast?

Dr. Barker: Fasting a rabbit for that short length of time probably would not have any effect on the R.Q.

Dr. Shorr: I brought up the point by way of suggestion that you might choose experimental conditions for your malonate experiments which might be more decisive in determining their significance for carbohydrate metabolism. If malonate were given to animals whose carbohydrate metabolism was reduced to a minimum, as in pancreatic diabetes, it might be possible to ascertain whether the effects were widespread or localized, for example, in the liver.

Dr. Barker: Continuing the discussion of the effect of malonate on the whole animal, I should like to consider the significance of this phenomenon in relation to the effect of insulin on the oxygen consumption of the excised tissue. The preponderant *in vivo* effect of insulin is on storage rather than on oxidation, and any effect on oxidation of carbohydrate probably extends over a longer period of time. The effect of insulin *in vitro,* in the case of pigeon muscle, is to give a pick-up in oxygen. I wonder if you have been able to reconcile these two aspects. The situation in the whole organism may be confused by the glycogenolytic effect of malonate, which your data show. The result of this process would be to obscure the usual hypoglycemic effect of insulin. Your data, I believe, show a considerable increase in blood sugar after the injection of malonate alone, and variable effect after insulin plus malonate. This is just what one would expect if the delicately adjusted mechanisms for blood sugar regulation were disturbed by agents affecting both sides of the equilibrium.

Shorr and I always found marked stimulations with citrate, often amounting to 25 to 50 per cent of the initial value. The effect was duplicated in all of the animals we tried, including mammals, and yet the mammalian tissue did not give us any insulin stimulation, whereas the pigeon tissue did. Actually, in the case of the mammals, insulin decreased the oxygen consumption of the minced-phosphate-*kochsaft*-citrate system. In view of Krebs' experiments we thought that this depression might be due to the zinc content of insulin. The Lilly amorphous preparation we used contained less than 0.025 per cent zinc, and we have successfully ruled that out by experiments with zinc alone. Our conclusion is that the depression may well be an effect of the insulin.

Dr. Stare: The interpretation of our results with malonate *in vivo* must, of course, depend upon what insulin actually does in the living animal. If the decrease in blood sugar in insulinized rabbits is entirely due to a withdrawal of sugar into storage, this phenomenon would necessarily concern an aspect of insulin activity different from its effect in increasing the oxygen consumption of tissues *in vitro*. But whatever the interpretation, the observations are as reported.

Mr. MacLeod: There has been some discussion in this paper about the effect of a lack of and an excess of certain ions on tissue metabolism. Then, too, we are made aware of differences obtained in the use of phosphate alone and phosphate plus NaCl. In other words, in many cases the medium employed has a determining effect on the amount or type of metabolism which we may expect from certain tissues. It is obvious, I think, even under the best of *in vitro* conditions where various forms of Ringer media are used, that one is left with a doubt as to the validity of certain results. I have wondered why the workers in the field do not take the best step towards the use of a physiological medium and employ serum. True, there are certain technical difficulties in the use of serum which require careful work, but they are not so difficult nor time consuming that they render the use of serum impossible.

Serum increases the respiration of exudate leucocytes about 70 per cent relative to the respiration of the same cells in Ringer-phosphate. The same is true of bone marrow and several other tissues.

Since isolated cells and tissue slices behave differently, depending on the fluid medium employed, it is still to be shown that their response to certain substances may not depend also on the nature of the medium.

Dr. Baumann: Certainly the respiration of isolated cells depends on the nature of the medium in which they are placed. But in the study of the "C_4" acids, there is some question whether a "perfect physiological medium" would be desirable. The experimental conditions which we try to attain are those in which all factors necessary for respiration are present except the C_4 acids. This means that the medium must be sufficiently unphysiological to permit these substances to diffuse away, or to be destroyed, so that the tissue will respond when normal concentrations are restored.

An analogy might be made with the study of vitamins, in which the ideal ration is one containing all necessary factors except the one being studied, so that the animal can respond when that factor is added.

Dr. Barker: I was not quite clear whether you said you always needed glucose present in order to get your citrate effects.

Dr. Stare: With kidney, yes; with the other tissues, no. Occasionally when we have done experiments with muscle without glucose, we did not observe the usual effect with C_4 acids. So as a matter of precaution we put it in. With kidney we found it necessary.

Dr. Barker: Of course we have used a much higher citrate content than you have; however, with slices of heart muscle we have been able to get marked citrate stimulation of respiration in Ringer-phosphate.

Dr. Stare: We have never tried a heart slice.

Dr. Burk: What is the relative concentration of insulin added compared with serum protein which exists in serum? I wonder whether there is any correlation between mere protein concentration, of insulin, or serum protein, and the insulin effect. If you were to add other non-specific proteins, what would happen?

Dr. Stare: I don't know. In our insulin experiments we have used amounts varying from one unit to forty units per vessel (total volume = 2 cc.)

Dr. Warren: There are two points about which I wish to comment. One is about the use of serum. It is interesting that you can use neutralized serum in tissue work and obtain the same stimulating effect you do with untreated serum. This can be done in an ordinary Warburg apparatus, and in the case of glycolysis too there is about a 70 per cent increase in the neutralized serum compared with Ringer's solution. I would like to inquire whether you have any information about the action of these C_4 substances on glycolysis? When you increase respiration do you decrease aerobic glycolysis accordingly?

Dr. Stare: I don't know and we have not any information of our own.

Dr. Müller: There has been a good deal of discussion about damaged cells and I should like to know what we mean by a damaged cell.

Dr. Warren: Whereas we obviously cannot answer this question fully, it is pertinent to realize, as MacLeod has mentioned, that in serum the rate of respiration (of bone marrow at least) does not fall off markedly as time goes on (as in Stare's experiments) but remained absolutely constant for at least five hours. Part of the damage to the cells, then, must be due to the absence in salt solution of something that is present in serum. Three groups of workers have tried to identify this substance (or substances) but each used a different tissue and each obtained a different result. Fat soluble substances, diffusible substances and proteins have accordingly been proposed; our own experiments on bone marrow are in progress and we do not have an answer yet.

Dr. Shorr: What is the significance of the R.Q. in the presence of succinate? When succinate produces a marked stimulation of respiration, you are inclined to interpret the low R.Q. as due to surplus dehydrogenation exceeding the oxidative capacities of the system. It would seem to me that such an interpretation of the low R.Q. in the presence of a substrate would only be justified if there were no change in oxygen consumption. Whereas, in this instance, oxygen consumption is greatly increased. There is no direct evidence that the rate of dehydrogenation is actually exceeding the oxygen-transporting capacity of the cell.

Dr. Baumann: Perhaps more direct evidence would be an analysis of the system for incompletely oxidized fragments of succinate Such fragments (fumarate, malate) are said to accumulate when fairly large amounts of succinate are added to respiring tissue. The extra oxygen consumption (in addition to respiration) could then be regarded as combining with hydrogen from the succinate to form water. If this view be correct, a lowering of the R.Q. must necessarily follow, and that, in fact, is what one observes. What we really tried to indicate was that the agents transporting hydrogen from succinate to oxygen are extremely active, capable apparently of transporting amounts of hydrogen from succinate equivalent to all of the hydrogen mobilized in normal respiration. This would furnish some justification for including the succinate-fumarate transformation as one step in the various "cycles."

Dr. Ochoa: Stare and Baumann's extensive data (from experiments performed on suspensions of tissue containing preformed substrate) on the activation of the respiration of various tissues by C_4 and C_6 acids do not inform us as to the nature of the carbohydrate (H_2 donor), the oxidation of which is being catalytically stimulated by those acids. Banga and Szent-Györgyi (1) had already given evidence to show that a donator, in muscle respiration, is triosephosphate. Annau (2) obtained evidence that pyruvate can be another such donator in muscle. Our own experiments on brain dispersions, in which most of the preformed substrate and coenzymes are removed by dialysis, supply new as well as complementary information for this discussion. These experiments clearly show that the C_4 dicarboxylic acids (succinate, fumarate, malate) are also concerned with the oxidation of pyruvate. When brain dispersions (pigeon, rabbit) in 0.9 p.c. KCl

are dialysed for a short time (against 0.4 p.c. KCl) their power to oxidise added pyruvate is almost completely lost, but can be fully restored by addition of inorganic phosphate + a C_4 dicarboxylic acid + "adenine nucleotide" (3). In order to restore the oxidation of pyruvate to the level before dialysis, the presence of a C_4 dicarboxylic acid is essential, as indeed is that of any other of the substances mentioned above. The effect of the C_4 dicarboxylic acids is catalytic, their addition increases the net O_2 uptake of pyruvate from over 100 to 200 p.c. The following typical figures to illustrate this point are taken from a paper recently submitted for publication (4).

converted by transamination (6) into glutamic acid. Krebs and Cohen (7) have recently presented evidence to show that the system α-ketoglutaric acid ⇌ glutamic acid can act catalytically as a hydrogen carrier.*

Dr. Stare: The experiments reported by Ochoa add more weight to the importance of fumarate in tissue respiration. Since the oxidation-reduction system succinate ⇌ fumarate has a potential of E'_o 0.00 volt at pH 7.0, 38° C., it is thermodynamically possible that it may act as a mediator for the electron transfer in biological oxidations of systems with a lower oxidation-reduction potential, such as the oxidation of pyruvate where

Enzyme = pigeon brain dispersion (dialysed 2.5 hours) + phosphate + adenylic acid + Mg^{++}. (μl oxygen uptake in 20 min. at 38° C.)

	No addition	0.005 M fumarate	0.005 M citrate
Enzyme	36	81	40
Enzyme + pyruvate (0.009 M)	146	322	186
Net O_2 uptake of pyruvate	110	241	146

The effect of citrate. The above table raises another point. It may be seen that the catalytic effect of citrate on the oxidation of pyruvate by the brain dispersions is much smaller than that of fumarate, and also than that of other C_4 dicarboxylic acids (4); in some experiments it has been *nil,* and it does not replace the C_4 dicarboxylic acid, *i.e.,* with citrate, the original level of respiration before dialysis is not restored. In view of this it has already been pointed out (3) that oxidation of pyruvate in brain cannot be catalysed by a citric acid cycle. If such were the case, the effect of citrate should have had to be identical to that of C_4 dicarboxylic acids. Also, if a citric acid cycle were operative, any other intermediate of the cycle should have had to be as active as a C_4 acid; this did not prove to be the case with α-ketoglutarate (4).

Now Stare and Baumann also find in most cases, and with various tissues, smaller effects of citrate mol for mol. Their evidence thus reinforces our own (*cf.* also Breusch, 5) and extends it to the case of muscle tissue oxidising its own preformed (or added) carbohydrate. The obvious inference, that the citric acid cycle is unlikely to be operating in a main pathway of oxidative breakdown, is not drawn by the authors. Tentative explanations for the effect of citric acid (when present) might, however, be (*a*) supplying of C_4 acids by oxidative breakdown, citric → *cis*-aconitic → *iso*-citric → α-ketoglutaric → succinic acid, or (*b*) α-ketoglutaric acid may be partially

the E'_o value is about −0.180 volt at pH 7.0, 38° C. This apparently happens in the experiment reported by Ochoa, and future research will probably show other systems where the electron transfer goes *via* fumarate.

The poor effect of citrate in the experiment reported by Ochoa may have been due to the absence of triphosphopyridine nucleotide, as Adler and Euler have recently shown that the dehydrogenation of *iso*-citric acid requires this coenzyme.

Dr. Peters: Ochoa's remarks cover most of the points in relation to the C_4 and C_6 acid cycle raised by work in this laboratory. It may be added that ever since the start of work upon the effect of vitamin B_1 *in vitro,* we have always been aware that the oxidation of succinate appeared to be entirely distinct from that of lactate-pyruvate (1). This gave no justification for the belief that succinate was part of the oxidation system of the latter. This view was especially reinforced by the observation that with a brain *brei* oxidising succinate, the extra oxygen uptake produced by addition of pyruvate was an additive quantity (2). Now that the C_4 acids have been proven to be catalytic and therefore to have some relation

* References: (1) Banga and Szent-Györgyi, Hoppe-Seyler Z. *252,* 275 (1938). (2) Annau and Erdös, Hoppe-Seyler Z. *257,* 111, (1939). (3) Banga, Ochoa and Peters, Nature, *144,* 74 (1939). (4) Banga, Ochoa and Peters, Biochem. J. (in press). (5) Breusch. Hoppe-Seyler Z. *250,* 262 (1937). (6) Braunstein and Kritzmann, Enzymologia *2,* 129 (1937). (7) Krebs and Cohen, Nature, *144* (1939).

to the oxidase system, this point needs further discussion because in theory there is a possible path for conversion of succinate to pyruvate *via* oxaloacetic acid; it is quite clear that any such conversion is much too slow to be a main path for the oxidation of pyruvate. It is a fact that avitaminous pigeon brain tissue shaken with succinate or fumarate (3) shows evidence, after a time, of the formation of traces of pyruvate (as judged by the nitroprusside reaction). This must be formed *via* the path fumarate → malate → oxaloacetate. In the presence of sufficient pyruvate, however, this reaction is unlikely to occur to any extent. The question has not been worked out for small concentrations of pyruvate under these conditions, but Green (4) found that the activity of muscle malate dehydrogenase was inhibited 76 p.c. by 0.06 M pyruvate. It is therefore likely that in the presence of pyruvate, conversion of fumarate to pyruvate *via* oxaloacetate tends to be inhibited. There seems to be here a beautiful example of biological adaptation; fumarate (or succinate) can be slowly formed either *via* pyruvate (5) or possibly α-ketoglutarate, because the C_4 system is needed for proper functioning of the total pyruvate oxidase system. Excess fumarate so formed can escape back to pyruvate again.**

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FUMARATE IN BIOLOGICAL OXIDATIONS

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