OXIDATIVE METABOLISM OF WASHED RESIDUES OF RAT SMALL INTESTINAL EPITHELIUM†

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Few studies have been made of the energy-producing oxidation reactions catalyzed by small intestinal epithelium. Studies made with slices have shown that this tissue exhibits a high level of oxidative activity and a high rate of aerobic glycolysis (Dickens and Weil-Malherbe, 1941). Homogenates of the epithelium catalyze the oxidation of α-ketoglutarate (Moinuddin and Cushing, 1957). Isocitric dehydrogenase (Adler et al., 1939) and aconitase (Johnson, 1939) are present in intestinal extracts, and respiration of intestine was stimulated markedly by glucose and slightly by glutamic acid (Rose and Archdeacon, 1953), but the distribution of these activities in various layers of the gut was not described. Intestinal xanthine oxidase is thought to be localized in the mucosa (Westerfeld and Richert, 1954). The accumulation of citrate in the small intestine after the injection of fluoroacetate into animals indicated that the citric acid cycle probably is a major oxidative pathway in the small gut as in other animal organs (Buffa and Peters, 1949) but to what extent this is true for the different gut layers was not determined. Thus, there appears to be little known regarding specific oxidations in gut epithelium by itself. In view of the importance of oxidation reactions in the absorption of certain sugars (Darlington and Quastel, 1953; Ponz and Lluch, 1955) and in other functions of this tissue, it appeared worthwhile to study the oxidative metabolism in some detail.

It will be shown herein that washed residues prepared from gut epithelium catalyze (a) the oxidation of substrates of the citric acid cycle and of various other substances, and (b) the formation of citrate from oxalacetate or from pyruvate plus oxalacetate. Requirements for cofactor fortification of the preparations are partially delineated.

EXPERIMENTAL METHODS

Mature, male Sprague-Dawley rats were allowed access to feed (Rockland Rat Pellets, complete) until they were killed, since previous work had shown that the oxidation of added α-ketoglutarate by whole homogenates of small gut epithelium was markedly decreased in fasted rats (Moinuddin and Cushing, 1957). Homogenates of essentially the entire epithelium were prepared in ice-cold 0.25 M mannitol solution by the method of Hele (1953) with modifications (Moinuddin and Cushing, 1957). After straining out the nonepithelial residues, the homogenates were further briefly homogenized in a Potter-Elvehjem apparatus with a tight-fitting, motor-driven pestle having teeth; some columnar cells remained unbroken even after this treatment. Washed residues were then prepared by centrifuging the homogenate in Lusteroid tubes at 6900 x *g* (calculated to the ends of the tubes) for 10 min at 0 to 4° C. The supernatant fluid was poured off, carrying with it some white, solid material which floated on the surface. The residue consisted of three layers: (a) an upper, thin, white layer; (b) the main, bulky, viscous, tannish layer; and (c) at the bottom, a very small, tan disk which usually contained some red blood cells. A volume of mannitol solution equal to one-half the volume of the original strained homogenate was added to the residue and the tube was swirled, thus dislodging the upper two layers which were then poured off with

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and homogenized into the solution. The small tan disk, which adhered to the tube wall, was discarded. The homogenized upper layers were recentrifuged in clean tubes as before. The supernatant fluid was discarded, and the residue was resuspended as before except that the small tan disk frequently adhered to the main pellet rather than to the tube and could not be removed. The final suspensions contained 1.2 mg of nitrogen per milliliter on the average; they usually still contained some recognizable unbroken principal cells of the epithelium.

In the respiration studies, standard Warburg manometric procedures were employed. Each reaction component was added to the flasks in approximately isosmolar solution. Each Warburg flask contained the following standard components (amounts in micromoles): 50 of tris(hydroxymethyl)aminomethane which had been brought to pH 8.0 with hydrochloric acid, 40 of potassium phosophate at pH 8.0, 20 of magnesium chloride, 0.03 of cytochrome *c*, and 6 of adenosine triphosphate (ATP) which had been neutralized with potassium hydroxide. The high pH of the buffer was needed to give the desired pH of the mixtures (after adding the acidic residue) of 7.3 to 7.6, while still keeping the osmolarity and volume of the buffer solution within useful limits. The total volume, allowing for addition of substrates, other cofactors, and 1.0 ml of the washed residue suspension, was made up to 3.0 ml with 0.154 M potassium chloride solution. The substrates were added as potassium salts except for pyruvate, β-hydroxybutyrate, isocitrate and acetoacetate, which were added as sodium salts. The flasks containing the reaction mixtures were chilled to 0° C. before adding chilled solutions of the less stable components and the chilled, freshly-prepared residue preparation. Sodium hydroxide and a fluted filter paper were used in the center well. The gas phase was air, the temperature of the bath 35° C, and the equilibration period 10 min. The comparative results with different substrates are calculated on the basis of the first 10 min after equilibration, since the uptakes with some of the substrates decreased considerably or ceased within 10 to 20 min. Oxidation due to microorganisms is not a problem during this early period in slices (Dickens and Weil-Malherbe, 1941) or homogenates (Moinuddin and Cushing, 1957) of epithelium; probably it is not significant in the residues either since the cold temperatures at which they were prepared do not favor proliferation of most intestinal organisms and since, also, the falling oxidation observed is not characteristic of microbial metabolism.

In all but a few instances, duplicate flasks were run with each substrate and endogenous variation, and the average of closely agreeing duplicates is given.

Citrate was estimated in trichloracetic acid filtrates of some of the reaction mixtures by the method of Natelson et al. (1948), and acetoacetate was estimated in a few filtrates by the method of Barkulis and Lehninger (1951). The nitrogen content of each residue preparation was determined by a micro-Kjeldahl procedure.

Materials were obtained as follows: dipotassium ATP (non-crystalline) and coenzyme A (CoA) (75%) from Pabst Laboratories; cytochrome *c*, CoA (75%), disphosphopyridine nucleotide (DPN) (90% or better,) triphosphopyridine nucleotide (TPN) (96.5%) from Sigma Chemical Co.; oxalacetic acid and *cis*-aconitic acid from H. M. Chemical Co.; L-glutamic acid from Merck & Co.; α-ketoglutaric acid, sodium pyruvate, sodium DL-β-hydroxybutyrate, and trisodium isocitrate (mixture of *dl* and allo forms) from Nutritional Biochemicals Corp. Sodium acetoacetate was synthesized according to Krebs and Eggleston (1945). Other substrates and mannitol usually were Eastman products.

RESULTS AND DISCUSSION

Many experiments established that the standard system used herein usually would support the oxidation of a number of physiologically important compounds by the washed residues (table 1). A few preliminary studies on the standard components were conducted. These showed that when cytochrome *c* was omitted,

the oxidation of citrate in the presence of DPN failed, and the oxidation of succinate was decreased; therefore, cytochrome *c* was included routinely. Results with heart mitochondria in the presence of DPN (Plaut and Plaut, 1952) and certain results with liver mitochondria in the absence of added DPN (Lardy and Wellman, 1952) indicate that the oxidation of citrate may be depressed by adding ATP at about the same level (0.002 M) as used herein. However, oxidation of citrate by our intestinal residues in the presence of DPN was not depressed very much by ATP; and so in view of its usefulness in promoting oxidation of many substrates by certain preparations from other tissues, ATP was used in all experiments. The omission of magnesium from the system was detrimental to the oxidation of many substances, so magnesium was always included. Malonate was found to inhibit the oxidation of succinate.

TABLE 1

Rates of oxidation by washed residues of rat small intestinal epithelium

Substrate*	Oxygen uptake, μl/mg N/hr†			
	All experiments‡		Individual experiments§	
	No DPN added	3 μmole DPN added	No DPN added	3 μmole DPN added
No substrate	27± 4 (24)	51± 4 (32)		
Citrate	13± 7 (4)	98±19 (3)	30	99
			0	64
cis-Aconitate	0± 0 (2)	124±59 (2)	0	65
			0	183
Isocitrate	32± 2 (2)	118±30 (3)	34	128
			31	77
Glutamate	38±14 (5)	90±24 (2)	58	115
			13	66
α-Ketoglutarate	83±24 (9)	104±25 (8)	106	125 n.s.
			245	237 n.s.
			126	137 n.s.
Succinate	343±55 (12)	171±59 (3)	457	266
			362	185
Fumarate	57±19 (7)	80±20 (5)	7	82
			0	52
Malate	58±27 (4)	126±25 (4)	37	168
			7	77
Oxalacetate	52± 5 (2)	45± 2 (2)	47	46 n.s.
Oxalacetate, 10 μmole	81±47 (3)	67±33 (3)		
Pyruvate	96±33 (3)	68±35 (4)	53	41 n.s.
β-Hydroxybutyrate	59± 2 (2)	87±12 (5)	57	76
			60	84
Acetate		23±10 (7)		
Acetoacetate		30± 7 (4)		
Crotonate		15± 2 (2)		
Butyrate		26± 3 (6)		
Octanoate, 18 μmole		15± 5 (4)		

*30 μmole/flask, except where otherwise noted.

†Values for substrates have been appropriately corrected for the uptake with no substrate in each respective experiment, and calculated for the first 10 min after equilibration.

‡Mean ± standard error of the mean (S. E.), for the number of observations given in parentheses. S. E. $= \pm\sqrt{\frac{\Sigma(d^2)}{n(n-1)}}$, where d = the deviation of individual values from the mean, and n = number of observations.

§The effect of DPN is marked not significant (n. s.) in the individual experiments in which there was any overlap of values for flasks with DPN and flasks without DPN. (DPN increased the uptake with no substrate in all 15 experiments in which it was specifically tested; these numerous individual values are not given.)

Effect of DPN.—Most of the individual experiments were set up to test the oxidation of several compounds simultaneously under one set of conditions (i.e., either in the presence or absence of DPN). The values obtained in this way with many of the substrates were so variable under either set of conditions (table 1, all experiments) that the effect of adding DPN was specifically tested in a few experiments (last 2 columns of table 1).

DPN depressed the oxidation of succinate. This is similar to the findings with the Keilin and Hartree (1940) heart muscle preparation and also to the more recent findings of Tyler (1955a) with homogenates of heart, kidney, and brain. This depression is due to DPN increasing the formation of the actual inhibitor, oxalacetate, by stimulating the oxidation of fumarate and malate in the sequence: succinate to fumarate to malate to oxalacetate (Keilin and Hartree, 1940). Tyler (1955a) found that 0.003 M magnesium relieved to some extent the DPN inhibition of succinate oxidation in kidney and heart. In our experiments with gut epithelial residues, while the final DPN concentration was 0.001 M as in Tyler's work, the concentration of *added* magnesium was much higher (0.0067 M) and yet DPN had a considerable inhibitory effect. This different result could easily be due to other differences in the composition of the tissue preparations and reaction media, since not only formation but also metabolism and metal ion complexing of the inhibitor (oxalacetate) may influence the results obtained with DPN (Keilin and Hartree, 1940; Tyler, 1955a, 1955b).

TABLE 2

Failure of oxidation of malate to improve the oxidation of acetoacetate and certain fatty acids

Substrate*	Oxygen uptake, μl/mg N/hr†			
	Oxidized separately			Substrate and malate oxidized in same flask
	Substrate	Malate‡	Sum	
Acetate	15	52	67	62
Acetoacetate	48	67	115	110
Butyrate	24	67	91	57
Octonoate, 18 μmole	20	67	87	24
Crotonate	13	24	37	22

*30 μmole per flask except where otherwise noted.
†Corrected and calculated as in table 1.
‡10 μmole per flask except that 3 μmole were used in the acetate experiment.
Each line is for one experiment typical of the results for each substrate (except that the crotonate experiment is the only one done with this substrate), in each of which the oxidation of substrate alone was significant. The results shown for acetoacetate, butyrate and octanoate all were obtained in the same run. Three μmole DPN in each flask; other conditions as in text.

DPN enabled the fairly vigorous oxidation of all three tricarboxylic acids (citrate, *cis*-aconitate, isocitrate) to proceed. It stimulated the oxidation of glutamate, fumarate, malate and β-hydroxybutyrate in all experiments in which it was tested. DPN had little or no effect on the initial rate of oxidation of α-ketoglutarate but helped a little to prolong oxidation (figure 1).

Effect of TPN.—In the experiment in table 1 in which the rate of oxidation of citrate was 0 without added pyridine nucleotide and 64 with 3 μmole of DPN added, a rate of 83 was obtained with 3 μmole of TPN added, and a rate of 114 with 3 μmole each of DPN and TPN. Whether the effect of the combination was due to a summation of different effects of the two cofactors or merely to the

higher total level of pyridine nucleotide is not known. Perhaps the residues contain both a TPN-linked and a DPN-linked isocitric dehydrogenase, as in heart (Plaut and Sung, 1954, 1955) and liver (Ernster and Navazio, 1956).

Oxidation of acetoacetate and fatty acids.—The oxidation of the last five compounds listed in table 1 was tested only in the presence of DPN. A slow but significant oxidation of acetoacetate was observed. The oxidation of acetate, crotonate, butyrate, or octanoate sometimes was insignificant, but in other experiments proceeded very slowly for about 10 min before failing completely. Lowering the level of acetoacetate to 0.003 M or of butyrate or octanoate to 0.001 M was tried since high levels of octanoate had been shown to be deterimental to washed residues of liver (Lehninger, 1945), but this only resulted in failure of the little oxidation which did occur with the higher levels of these compounds. Attempts to improve the oxidation by addition of up to 10 μmole per flask of cosubstrates such as malate (table 2), oxalacetate, or ketoglutarate generally were unsuccessful, since the uptakes during simultaneous oxidation of substrate and cosubstrate did not exceed the sum of the uptakes in the separate oxidations. Octanoate (0.003 M) depressed the oxidations of added cosubstrates markedly, and butyrate (0.01 M) usually depressed them slightly; this is shown for malate as cosubstrate in table 2.

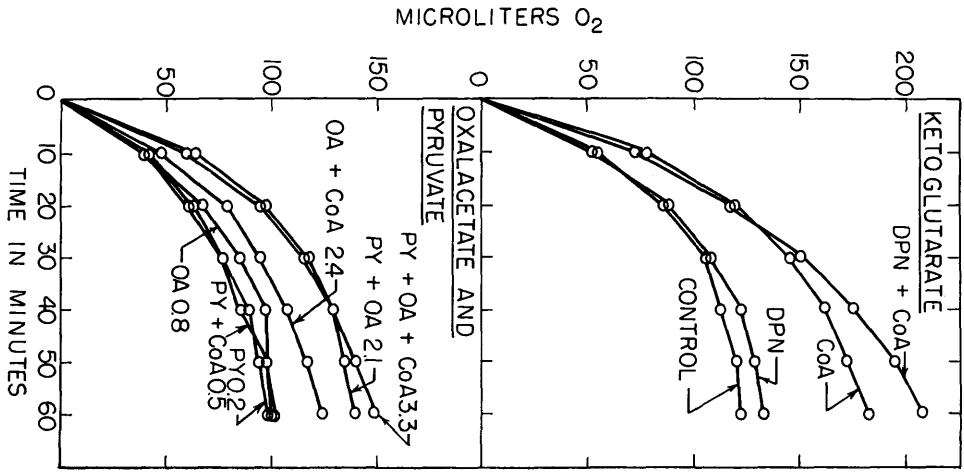

FIGURE 1. Effects of additions of certain cofactors on oxidation of certain substrates and on citrate formation by washed residues of rat small gut epithelium. The experiments graphed are those in which the most active respiration occurred with the respective substrates in the standard system, with 30 μmole of α-ketoglutarate or of pyruvate (PY) or 10 μmole of oxalacetate (OA) used per flask, and 3 μmole DPN or 1 μmole CoA added as indicated. The quantity of residue added per flask contained 1.3 mg of nitrogen in the ketoglutarate experiment and 1.5 mg of nitrogen in the oxalacetate and pyruvate experiment. All oxygen uptake values have been appropriately corrected for endogenous uptake. The numbers on the curves in the oxalacetate and pyruvate experiment refer to the μmoles of citrate formed during 70 min (i. e., 10 min equilibration plus 60 min of oxygen uptake measurements); they have been corrected for endogenous citrate formation of 0.1 and 0.2 μmole in the absence and presence of added CoA, respectively.

Oxidation of β-hydroxybutyrate.—During the oxidation of DL-β-hydroxbutyrate in the presence of DPN, acetoacetate accumulated in the reaction vessel; e.g., during the 10-min thermal equilibration period in one experiment, during which substrate was present in the flasks but oxygen uptake was not measured, about 1.4 μmole of acetoacetate accumulated. In the next 10-min period, 0.8 μmole of acetoacetate accumulated while 2.6 μatoms of oxygen were consumed. Thus, the amount of acetoacetate which accumulated during the 10-min period after

equilibration was not sufficient to account for all of the oxygen consumed during the same time period, if one assumes that the one-step oxidation of D-β-hydroxybutyrate to acetoacetate was the only reaction occurring. However, it was shown recently that tissues vary considerably in the ability to oxidize the stereoisomers of β-hydroxybutyrate (McCann, 1957). From our work with only the DL mixture, it is impossible to decide whether both or only one of the isomers is being oxidized and whether different pathways for the oxidation of the D and L isomers may be operating in our epithelial residues as in liver mitochondria (Lehninger and Greville, 1953).

Effect of CoA on oxidation of α-ketoglutarate.—The occasional failure of α-ketoglutarate to be oxidized even in the presence of added DPN appeared sometimes to be due, at least partly, to a low level or a rapid inactivation of reduced CoA in the preparations. For example, in the one experiment in which no uptake of oxygen was found with ketoglutarate in the presence of DPN, it was found that CoA (1 μmole) raised the uptake slightly to 18 and glutathione (30 μmole) had no effect, but CoA plus glutathione raised the uptake to 95, after the appropriate endogenous uptake for each cofactor combination was subtracted. However, in another similar experiment in which low uptakes were observed, neither CoA nor CoA plus glutathione was helpful; so, it appears that other limitations on ketoglutarate oxidation may be present occasionally. The effect of adding CoA was studied also in two preparations which were active to start with; the experiment in which the greatest uptakes were observed for ketoglutarate in the standard system is graphed in figure 1. In this case, CoA stimulated the oxidation initially and also prolonged the oxidation, whereas DPN had no effect initially but later helped to prolong oxidation especially in the presence of CoA; the CoA effect was repeatable with another good but somewhat less active preparation.

Formation of citrate.—The finding that *added* pyridine nucleotide was required for appreciable oxidation of added citrate but not for oxidation of pyruvate or oxalacetate by the residues, permitted a study of citrate formation under conditions which were expected to favor maximum accumulation of this metabolite. This was done in one experiment carried out on the preparation which oxidized pyruvate most rapidly, and the results are shown in figure 1. The beneficial effects of CoA on oxalacetate oxidation and on formation of citrate particularly from oxalacetate or from pyruvate plus oxalacetate are evident. These results support the impression gained from the ketoglutarate oxidation studies that even the oxidatively most active residues may be somewhat low in CoA or may inactivate this cofactor rapidly.

General discussion.—Under appropriate conditions, rat small gut epithelial residues catalyzed the oxidation of the various substrates of the Krebs cycle and of some other related compounds and catalyzed also the synthesis of citrate starting from oxalacetate or from oxalacetate plus pyruvate. These findings are consistent with the idea that the Krebs cycle with certain contributing reactions may operate in gut epithelium as in many other tissues which have been studied. On the other hand, any appreciable oxidation of several fatty acids by this tissue was not found using our standard system supplemented with DPN, even when a substrate of the Krebs cycle was cooxidized with the fatty acid. It is uncertain whether the epithelium is intrinsically low in one or more of the enzymes needed for fatty acid catabolism or whether there is some other limitation on fatty acid oxidation (perhaps insufficient CoA) in the residue preparations under our conditions.

SUMMARY

The preparation of washed residues of rat small intestinal epithelium in 0.25 M mannitol is described. These preparations exhibited no or low endogenous oxidative activity in our standard system containing buffer, phosphate, magnesium,

ATP and cytochrome *c*. They showed a somewhat higher endogenous activity when DPN was added.

The residues oxidized succinate, fumarate, malate, oxalacetate, pyruvate, glutamate, α-ketoglutarate, β-hydroxybutyrate and isocitrate in most or all experiments, and also oxidized citrate in some experiments to a slight extent.

The effect of adding DPN or CoA was tested for some of the substrates with a few of the residue preparations. The addition of DPN depressed the oxidation of succinate markedly. DPN enabled the oxidation of *cis*-aconitate to proceed, and considerably stimulated the oxidation of citrate, isocitrate, glutamate, fumarate, malate, and β-hydroxybutyrate. DPN stimulated the oxidation of α-ketoglutarate to a lesser extent and the effect was not always demonstrable. CoA addition stimulated both the oxidation of α-ketoglutarate in several experiments and the formation of citrate from added precursor substances in one experiment.

In the presence of DPN, acetoacetate was oxidized slowly but definitely. Attempts to oxidize acetate, crotonate, butyrate and octanoate in the presence of DPN resulted in failure or else in low but definite oxygen uptakes in different experiments. Reduction in the levels of certain of the acids or cooxidation of acids of the Krebs cycle did not improve the oxidation of these substances.

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