The Coupled Oxidation of Pyruvate with Glutathione and Cysteine

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Among the numerous reactions that pyruvate undergoes in biological systems its oxidation to acetic acid and carbon dioxide is at present little understood. A pyruvate dehydrogenase has been reported in animal tissues, but has not been isolated. As regards oxidative decarboxylation we know that acetate is formed from pyruvate by a number of tissues and bacteria (Lipmann, 1937; Barron & Lyman, 1939; Long & Peters, 1939), and that acetate represents an important stage in the biological degradation of pyruvate (Bartlett & Barron, 1947; Kalnitsky & Barron, 1947).

Krebs & Johnson (1937) postulated a dismutation in which one molecule is oxidatively decarboxylated and the other reduced, giving rise to the formation of lactic acid, acetic acid and carbon dioxide. Banga, Ochoa & Peters (1939) found that brain dispersions can oxidize pyruvate provided that cocarboxylase, a C_4 dicarboxylic acid, inorganic phosphate, adenylic acid and magnesium or manganese ions are present. Under these conditions, as Long (1938) showed, 20 % of the pyruvate is recoverable as acetic acid. Weil-Malherbe (1940) and Martius (1943) suggested that a reaction between the pyruvic acid and the amino group of the cocarboxylase is necessary both for oxidative decarboxylation and for anaerobic fission. Lipmann (1937, 1939, 1941), working with bacteria, suggested that the pyruvic dehydrogenase oxidizes a pyruvate-phosphate complex formed in the presence of inorganic phosphate, producing carbon dioxide and an energy-rich acetyl phosphate; in the presence of adenosinediphosphate the latter then forms adenosinetriphosphate and acetic acid.

Cavallini (1946) reported an oxidation of pyruvate to acetate during the oxidation of ascorbic acid with a suitable catalyst. This 'induced oxidation' of pyruvate by ascorbic acid may be of biological importance, since according to Cavallini & Bonetti (1946) ascorbic acid is involved in the degradation of pyruvate *in vivo*.

This paper describes experiments on the oxidation of pyruvate with glutathione and cysteine which may be of significance in connexion with the obscure problem of pyruvate breakdown *in vivo*.

EXPERIMENTAL

Materials

Glutathione and cysteine (Hoffman La Roche) were used without further purification.

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Sodium pyruvate was prepared according to Robertson (1942).

Cytochrome *c* was prepared according to Keilin & Hartree (1937).

Cytochrome oxidase was prepared from calf heart muscle by the method of Stotz & Hastings (1937). The extract was reprecipitated with acetate buffer (pH 4.5) and resuspended in the original volume of 0.067 M- Na_2HPO_4 .

Methods

O_2 uptake and CO_2 evolution were measured by the Warburg technique; conical vessels with single or double side arms were used throughout.

CO_2 evolution was determined by the use of paired vessels containing the same reactants, except that in one of the flasks the NaOH in the centre well was replaced by water. The CO_2 output was then given by the relation

$$x_{CO_2} = \left\{ h'' - \frac{h' k'_{O_2}}{k''_{O_2}} \right\} k''_{CO_2},$$

where h' and h'' represent the change in pressure at a given time in the flasks with and without NaOH; k'_{O_2} is the manometric constant for O_2 for the first flask, and k''_{O_2} and k''_{CO_2} the constants for O_2 and CO_2 of the second manometer. Bound CO_2 was estimated by tipping in tartaric acid from a side arm into those flasks which did not contain NaOH in the centre well.

Unless otherwise stated, the main compartment of the flasks contained 1 ml. of 0.067 M-phosphate buffer, pH 7.4; 0.1 ml. of 0.1 N-NaOH (for the neutralization of the cysteine hydrochloride and glutathione solutions); 0.0002 mmol. $CuCl_2$ in 0.3 ml. of water, or 1.72 mg. cytochrome *c* (0.0001 mmol.) in 0.1 ml. of water + 0.2 ml. cytochrome oxidase preparation; 0.02 mmol. sodium pyruvate in 0.2 ml. of water; water to a total volume of 3 ml. Glutathione or cysteine, 0.02 mmol. in 0.3 ml. of water, was placed in the side arm, while the second side arm (when used) contained about 0.1 g. crystalline tartaric acid. The flasks contained air and the measurements made at 38°; pH values were determined electrometrically.

RESULTS

When glutathione is oxidized either with $CuCl_2$ or with cytochrome *c* and cytochrome oxidase, an uptake of O_2 occurs corresponding to the reaction $2GSH + O \rightarrow GSSG + H_2O$. Under the conditions used in the present work the observed O_2 uptake is very close to the theoretical value of 112 $\mu l.$ /0.02 mmol. glutathione.

If, however, this reaction is allowed to proceed in the presence of sodium pyruvate the O_2 consumption is increased and CO_2 is evolved in excess of the small amount produced during the oxidation of glutathione

Fig. 1. Oxidation of glutathione. $\square$, O_2 uptake; $\blacksquare$, CO_2 output during the oxidation of 0.02 mmol. glutathione; $\circ$, O_2 uptake; $\bullet$, CO_2 output during the oxidation of the same amount of glutathione in the presence of 0.02 mmol. sodium pyruvate. A, with 0.0002 mmol. $CuCl_2$ as catalyst; B, with 0.0001 mmol. cytochrome c + cytochrome oxidase as catalyst.

Fig. 2. Oxidation of cysteine. $\square$, O_2 uptake; $\blacksquare$, CO_2 output during the oxidation of 0.02 mmol. cysteine. $\circ$, O_2 uptake; $\bullet$, CO_2 output during the oxidation of the same amount of cysteine in the presence of 0.02 mmol. sodium pyruvate. A, with 0.0002 mmol. $CuCl_2$ as catalyst. B, with 0.0001 mmol. cytochrome c + cytochrome oxidase as catalyst.

alone (Fig. 1). (As shown in Figs. 1 and 2 a small CO_2 evolution occurs during the oxidation of both glutathione and cysteine. The origin of this CO_2 is not understood.) Since the method of estimating CO_2 output is not accurate in the initial stages of the reaction, the experimental curve for CO_2 is drawn partly as an arbitrary broken line in both Figs. 1 and 2. Fig. 2 shows that the same result is obtained when cysteine is substituted for glutathione.

In the absence of glutathione or cysteine no oxidation of pyruvate was observed under these conditions; furthermore, oxidized glutathione and cystine are without effect. No oxidation occurs when the cupric chloride or cytochrome *c* and cytochrome oxidase are absent, or when potassium cyanide is present.

conditions used here an oxidative decarboxylation to acetic acid and CO_2 occurs.

In the experiments described in Table 2 cysteine was used instead of glutathione, and under these conditions the CO_2/O_2 ratio is different. This difference might be explained by assuming either a different mode of oxidation of the pyruvate or by a partial removal of cysteine by pyruvate in a form which is not catalytically oxidizable by O_2 .

Schubert (1935, 1936, 1939) has studied the compounds formed by interaction of thiols with aldehydes, pyruvic acid and sugars. He found that in the case of glutathione, thioglycolic acid and its derivatives, addition compounds (I) are formed. In the case of cysteine and thiourea, in which an amino group is adjacent to the thiol group, a condensation

Table 1. *Effect of increasing concentration of pyruvate on the oxidation of pyruvate by glutathione*

(Glutathione (0.02 mmol.) dissolved in 0.3 ml. water in side arm. In the main flask: 1 ml. phosphate buffer; 0.4 ml. 0.1N-NaOH; CuCl_2 , 0.0002 mmol. in 0.3 ml.; sodium pyruvate in different quantities. Centre well: 0.2 ml. 10% (w/v) NaOH, or 0.2 ml. water (see Experimental section). Total vol. 3 ml. Temp. 38°. Gas space filled with air.)

Pyruvate added (mmol.)	pH	Time of oxidation (hr.)	O_2 ($\mu\text{l.}$)	CO_2 ($\mu\text{l.}$)	Extra O_2 ($\mu\text{l.}$)	Extra CO_2 ($\mu\text{l.}$)	Extra CO_2/O_2
0	7.70	3.5	112.0	28.4	—	—	—
0.005	8.20	4	123.0	48.3	11.0	19.9	1.81
0.01	7.70	4	145.5	100.8	33.5	72.4	2.16
0.1	8.05	5	183.0	159.0	71.0	130.6	1.84

From these results it would appear that a secondary oxidation of the added pyruvate takes place during the oxidation of either glutathione or cysteine.

product (II) is formed by the elimination of water and the closure of the thiazolidine ring (see Sullivan & Hess, 1937).

Table 2. *Effect of increasing concentration of pyruvate on the oxidation of pyruvate by cysteine*

(Cysteine (0.02 mmol.) dissolved in 0.3 ml. water in side arm. In the main flask: 1 ml. phosphate buffer; 0.1 ml. 0.1N-NaOH; CuCl_2 , 0.0002 mmol. in 0.3 ml.; sodium pyruvate in different quantities. Centre well: 0.2 ml. 10% (w/v) NaOH, or 0.2 ml. water (see Experimental section). Total vol. 3 ml. Final pH 7.3. Time of oxidation 1 hr. Temp. 38°. Gas space filled with air.)

Pyruvate added (mmol.)	O_2 ($\mu\text{l.}$)	CO_2 ($\mu\text{l.}$)	Extra O_2 ($\mu\text{l.}$)	Extra CO_2 ($\mu\text{l.}$)	Extra CO_2/O_2
0	123.0	23.1	—	—	—
0.005	129.8	78.4	6.8	55.3	8.15
0.01	132.0	91.4	9.0	68.3	7.60
0.02	148.1	119.2	25.1	96.1	3.82
0.1	106.0	178.4	—	153.3	—

Table 1 shows the results of experiments in which the reaction between glutathione and different amounts of pyruvate was allowed to go to completion. The CO_2 production and O_2 uptake in excess of the amounts required for the oxidation of the glutathione alone were assumed to be concerned with the oxidation of the added pyruvate. The CO_2/O_2 ratio is close to 2, over the range of pyruvate used. This result suggests, therefore, that under the

This difference in the behaviour of the two thiol compounds when reacting with carbonyl groups would explain the different results in the oxidation of pyruvate. Since Schubert showed that both addition and condensation products are, in solution, in equilibrium with their components, an increase in pyruvate would displace the equilibrium towards the right in reactions (I) and (II). With glutathione, increase in pyruvate leads to a greater concentration of the addition compound, but the oxidation of pyruvate is more complete and the CO_2/O_2 ratio remains constant, suggesting that formation of an addition complex represents a stage in the coupled oxidation of pyruvate by glutathione. With cysteine, increase in pyruvate favours not only the formation of the addition compound (which we may presume

to occur as an intermediate product), but also the condensation of the two substances; the depression of the O_2 uptake observed when the concentration of pyruvate is increased, and the variable CO_2/O_2 ratios could be explained by the partial exclusion of cysteine from oxidation owing to formation of the condensation product. Since the latter, like cysteine, reduces iodine (Schubert, 1936) it is possible to estimate it in solution at the end of the catalytic oxidation.

The data in Table 3 support this explanation. Pyruvate and cysteine were incubated in the side arm for different periods in the absence of the catalyst in order to facilitate the formation of the condensation product; the contents of the side arm were then tipped into the main flask containing the catalyst and the oxidation allowed to proceed to

Table 3. *Effect of the incubation of cysteine with pyruvate for different times before contact with the catalyst*

(In the main flask: 1 ml. phosphate buffer; 0.1 ml. 0.1N-NaOH; cytochrome *c*, 0.0001 mmol. (1.72 mg.) in 0.1 ml.; 0.2 ml. cytochrome oxidase preparation. Centre well: 0.2 ml. 10% (w/v) NaOH, or 0.2 ml. water (see Experimental section). Total vol. 3 ml. Final pH 7.3. Temp. 38°. Gas space filled with air. Cysteine, 0.02 mmol. in 0.3 ml. water, and pyruvate, 0.02 mmol. in 0.2 ml. water, were placed together in the side arm just before immersion of the vessels in the water bath. After the desired time, the contents of the side arm were tipped into the main space and the oxidation left to proceed completely (1 hr.). For the recovery of non-oxidized cysteine and calculation see text, p. 4.)

Time of incubation of cysteine with pyruvate before tipping (min.)	O_2 (μ l.)	CO_2 (μ l.)	Cysteine recovered at the end of oxidation (%)	
			By I_2 titration	Calculated
0	130.0	97.4	10.0	18.4
15	98.0	80.2	48.4	47.0
30	80.0	77.4	51.2	54.0
60	58.8	61.6	74.0	74.8

Under the same conditions cysteine alone consumed 112 μ l. O_2 .

completion. At the end of the experiment the contents of the vessels were deproteinized by the addition of 2 ml. of 10% (w/v) trichloroacetic acid and centrifuged. The supernatant was neutralized with solid Na_2CO_3 and titrated with I_2 , and the results were compared with the amount of I_2 utilized by

0.02 mmol. cysteine treated in the same way. It was found that as the preliminary incubation period was increased the O_2 uptake decreased and a greater quantity of cysteine was estimated iodometrically.

If the oxidation of pyruvate proceeds as suggested above, it is possible to estimate from the amount of CO_2 evolved the amount of cysteine excluded from oxidation. Since the amount of O_2 needed for the decarboxylation of the pyruvate is equal to half the CO_2 evolved, the O_2 needed for the oxidation of cysteine is $O_2 - \frac{1}{2}CO_2$, while $112 - (O_2 - \frac{1}{2}CO_2)$ represents the cysteine excluded from oxidation in the form of a condensation compound. (The CO_2 evolved during the oxidation of the cysteine alone, 26.6 μ l., has been neglected. The slight correction involved does not affect the results.) As is shown in the last two columns of Table 3, this manometric calculation is in reasonable agreement with the iodometric titration; it further shows that with cysteine also the CO_2/O_2 ratio for the coupled oxidation of pyruvate is approximately 2. That the condensation product of cysteine with pyruvic acid is not catalytically oxidized by molecular oxygen has been confirmed by preparing it according to Schubert (1936) and subjecting it to the action of the catalyst; under the same conditions as those described in Table 3 this compound gave only a low rate of oxidation (11.5 μ l. O_2 /hr.), this low uptake being probably due to a low hydrolysis of the condensation compound to the addition compound.

DISCUSSION

From the above experiments it may be concluded that if pyruvic acid is present in the reaction mixture a secondary oxidation may occur in the course of the oxidation of glutathione or cysteine. From the extra carbon dioxide/oxygen ratios obtained in the presence of pyruvic acid it may be argued that this oxidation of pyruvate proceeds through an oxidative decarboxylation to acetate and carbon dioxide. Since glutathione, which is known to form only an addition compound, can perform the coupled oxidation of pyruvate, and since cysteine is not oxidizable when it is forced to form a condensation product, it is suggested that the reaction proceeds by means of the addition, and not the condensation, of pyruvic acid with the thiol group. Either of the following reactions might be postulated:

Free sulphhydryl groups are known to be a limiting factor in the metabolism of pyruvate; evidence for this was provided by Peters & Wakelin (1946) using maleate, and Peters, Sinclair & Thompson (1946) using arsenical vesicants.

Aneurin, one of the main factors concerned with the catabolism of pyruvate, was shown by Zima & Williams (1940) and Zima, Ritsert & Moll (1941) to be capable of undergoing reversible oxidation and reduction, $2\text{RSH} \rightleftharpoons \text{RSSR}$, both the reduced and oxidized forms having equal biological activity. The question arises, therefore, whether the catalytic activity of aneurin is due to oxidation of an addition compound formed between pyruvate and the reduced form of the vitamin, in a manner similar to

the coupled oxidation of pyruvate with glutathione described above.

SUMMARY

1. When glutathione or cysteine is oxidized either with copper ions or with the cytochrome c-cytochrome oxidase system, a secondary oxidation occurs when pyruvate is added.

2. The ratio of the extra carbon dioxide output to the extra oxygen uptake due to the added pyruvate is approximately 2, suggesting an oxidative decarboxylation to acetic acid and carbon dioxide.

3. It is suggested that this coupled oxidation of pyruvate with glutathione may throw light on the biological oxidation of pyruvate.

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The Effect of Amino-acids on the Growth of *Lactobacillus casei* A.T.C. 7469 in Pteroyl Glutamic Acid-Free Media

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Most media suggested for the estimation of pteroyl glutamic acid (PGA), using *Lactobacillus casei* as a test organism, contain hydrolysed casein plus tryptophan and cystine as a nitrogen source. Snell (1944) found that the growth response of this organism to PGA was increased by supplementing such media with DL- α -alanine (hereafter referred to as alanine). Dolby & Waters (1944) reported that leucine, isoleucine and threonine behaved in a similar manner. Subsequently a number of workers have recommended the inclusion of alanine (1–2 mg./

10 ml.) in such media (Tepley & Elvehjem, 1945; Kreuger & Peterson, 1945, and Kodicek & Mistry, 1949).

During experiments on the formulation of a medium for the estimation of PGA with *Lb. casei*, it was found that addition of alanine to a medium containing a commercial brand of 'vitamin-free' casein hydrolysate, resulted in good growth in the absence of added PGA. When alanine was omitted no growth was obtained unless PGA was added. This unexpected behaviour on the part of the test