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THE INTERACTION OF 6,6′-DITHIODINICOTINIC ACID WITH THIOLS AND WITH EHRLICH ASCITES TUMOR CELLS*

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Abstract—6,6′-Dithiodinicotinic acid reacts with thiols with formation of a disulfide and 6-mercaptonicotinic acid; the reaction can be followed spectrophotometrically. Homogenates of Ehrlich ascites cells react with 6,6′-dithiodinicotinic acid to an extent approximately 4-fold that of intact cells. This suggests that the compound has difficulty in penetrating the cell. 6,6′-Dithiodinicotinic acid can cause the indirect, enzyme-mediated oxidation of glucose 6-phosphate by providing oxidized NADP. When acting on Ehrlich ascites cells, 6,6′-dithiodinicotinic acid causes a moderate stimulation of the hexose monophosphate pathway, presumably due to the reoxidation of NADPH.

WE HAVE recently studied the interaction of 2,2′-dithiodipyridine† with thiols[2] and with Ehrlich ascites tumor cells.[3] This compound is a strong inhibitor of respiration and glycolysis of Ehrlich ascites cells;[4] the inhibitory action was found to be related to its reaction with cellular components.[3]

A study of the effect of several disulfides on the respiration and glycolysis of Ehrlich ascites cells[5] has shown that, within the group of compounds studied, two types of action can be distinguished. Certain disulfides (type A) inhibit strongly both respiration and glycolysis, whereas others (type B) have a milder inhibitory effect, which appears to act selectively on the Krebs cycle rather than the glycolytic pathway. This is suggested by the fact that in the presence of these type-B disulfides under aerobic conditions an accumulation of lactic acid takes place. The structural characteristics correlated with these two types of action have been discussed.[5]

The present communication reports a study of the interaction of 6,6′-dithiodinicotinic acid, a representative of type-B disulfides, with thiols and with Ehrlich ascites cells. The findings are compared with those obtained with 2,2′-dithiodipyridine, which is a type-A disulfide.

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† Abbreviations used in this paper: I = 6,6′-dithiodinicotinic acid; II = 6-mercaptonicotinic acid (6-carboxy-2-thiopyridone); III = 2,2′-dithiodipyridine; IV = 2-thiopyridone; NADP, NADPH = oxidized and reduced forms of nicotine-adenine dinucleotide phosphate; GSH, GSSG = reduced and oxidized forms of glutathione; GR = glutathione reductase (NADPH(NADH): GSSG oxidoreductase, EC 1.6.4.2); KRP buffer = Krebs–Ringer phosphate buffer, pH 7·2;[1] G6P-DH = glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP oxidoreductase, EC 1.1.1.49); HMP = hexose monophosphate.

EXPERIMENTAL

Materials. 6,6'-Dithiodinicotinic acid was prepared as reported previously.[5] 6-Mercaptonicotinic acid was synthesized by the procedure of Räth.[6] 2,2'-Dithiodipyridine was prepared according to Marckwald *et al.*[7] GSSG, NADP, glucose 6-phosphate, G6P-DH, GR and aldolase (fructose-1,6-diphosphate: D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.b) were purchased from Sigma Chemical Co. Bovine serum albumin was purchased from Calbiochem. (1-^{14}C)-glucose and (6-^{14}C)-glucose were purchased from New England Nuclear Corp.

Ehrlich ascites tumor cells were harvested from 7- to 10-day transplants in female Swiss mice (18–20 g) and washed free of blood as reported previously.[3] Ehrlich ascites homogenates were obtained as follows: the final suspension of washed cells was centrifuged at 15 *g* for 5 min at 2–4° and the pellet was suspended in an equal volume of KRP buffer, pH 7·2, in a Potter–Elvehjem homogenizer. The suspension was frozen by immersion in dry ice–propanol for 15 min, partially thawed by immersion in water at room temperature, then homogenized for 2 min with the homogenizer immersed in water at 5–10°, using a Telflon plunger. The mixture thus obtained was frozen again for 5 min and again homogenized for 2 min. Microscopic examination showed that this procedure caused essentially complete cell breakage.

Thiol determinations. A suitable sample of the protein or thiol (0·02 to 0·2 μmole) was added to 3·0 μmoles of I or of III, in 3·0 ml of 0·1 M phosphate buffer, pH 7·5. The absorbance at 344 mμ (for I) or at 343 mμ (for III) was determined immediately upon mixing for simple thiols or after 5 min for bovine serum albumin. With aldolase, the reaction proceeded slowly and was measured over a 3-hr period in air at room temperature (about 25°). For washed ascites cells or homogenates, a sample of 7–8 mg (dry wt.) was incubated with 5 μmole of I or of III in KRP buffer, pH 7·2 (total volume 5·0 ml), for 1 hr at 37° in air. The flask contents were then filtered through Whatman glass fiber filter paper GF/C. The amount of II or of IV formed was determined in the filtrate by its absorption at 344 mμ (II) or 343 mμ (IV). Blanks containing the disulfide and blanks containing the tissue were carried through the procedure.

Protein determinations. The 280 mμ/260 mμ method was used, with Layne's coefficients.[8]

Isotopic experiments. The procedures described earlier[9] were followed.

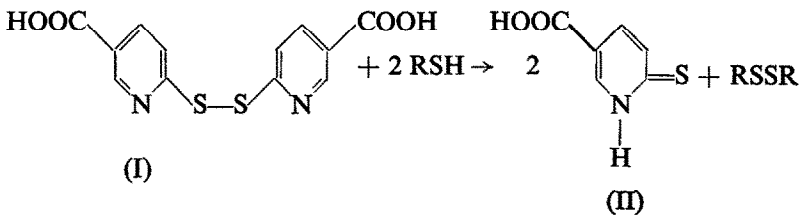
Purity of glutathione reductase. GR (0·44 mg) was dissolved in 2·0 ml of 0·1 M phosphate buffer, pH 7·5. The enzyme was denatured by heating in boiling water for 2 min and the denatured protein was removed by centrifugation at room temperature. An aliquot (0·5 ml) of the clear supernatant was assayed for glutathione as previously described.[10] The total glutathione found (expressed as GSSG/mg GR) was 0·9 mμmole. Since the recovery of added GSSG was only 50 per cent, we have concluded that the maximum amount of total glutathione present in this enzyme preparation was 1·8 mμmoles/mg GR or 0·036 mμmole/cuvette (see Fig. 3).

Dialysis of a solution of 0·44 mg GR in 2·0 ml of 0·1 M phosphate buffer, pH 7·5, was carried out against the same buffer for 70 hr at 2–4°. Solutions of GSSG and of GR + NADPH were dialyzed simultaneously. It was found that the GSSG had completely diffused out of the dialysis sack and that the GR in the other two sacks had suffered no loss of activity with I as the substrate.

RESULTS

The u.v. spectrum of I is given in Fig. 1 and that of the corresponding thiol, II, in Fig. 2. The similarity of the spectrum of this thiol with that of 2-thiopyridone[2] indicates that II is also predominantly in the thiopyridone form.*

In aqueous solution at pH 7·2, I reacts with thiols such as cysteine and glutathione in a rapid and complete manner to give II, as follows:



(I) (II)

It was found that I reacts also with sulfhydryl groups of proteins, such as bovine serum albumin or aldolase, and of Ehrlich ascites cell homogenates. The extent of

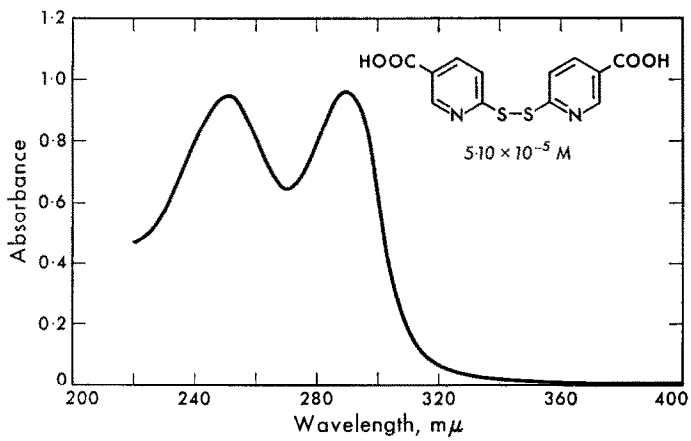


FIG. 1. The u.v. spectrum of I in KRP buffer, pH 7·2.

reaction of I and of III with sulfhydryl groups of these materials is reported in Table 1, and is seen to be comparable; however, with the intact cells, III reacts to a much larger extent than I.

The results reported in Table 1 also permit a comparison of the extent of reaction of each of these disulfides with the intact cells and with the homogenates. We find that III produces larger amounts of IV with the intact cells than with the homogenate. This difference has been at least partially explained[3, 9] as due to the enzyme-mediated oxidation of nonsulfhydryl metabolites occurring in the intact cell. On the other hand, it is found that with I the homogenates cause the production of about four times as much II as the intact cells.

We have previously found that III is capable of oxidizing glucose 6-phosphate to 6-phosphogluconate in (*a*) aqueous solution containing the appropriate reactants,

* The molar extinctions of these compounds at their absorption maxima are: I: $E_{251} = 1{\cdot}85 \times 10^4$, $E_{290} = 1{\cdot}88 \times 10^4$; II: $E_{295} = 1{\cdot}91 \times 10^4$, $E_{344} = 1{\cdot}00 \times 10.^4$

enzymes and cofactors;[3, 10] and in (*b*) Ehrlich ascites cells suspended in an appropriate buffer.[3] To establish whether I is capable of the same activity, we have studied the following reactions:

$$\text{6,6'-dithiodinicotinic acid} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} \text{NADP}^+ + 2\ \text{(6-mercaptonicotinic acid)} \quad (1)$$

$$\text{Glucose 6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+ \quad (2)$$

The net result of reactions 1 and 2 if the oxidation of glucose 6-phosphate by I, with stoichiometric formation of II; the course of this reaction can be followed spectrophotometrically, as indicated in Fig. 3. The reactions proceed smoothly at a

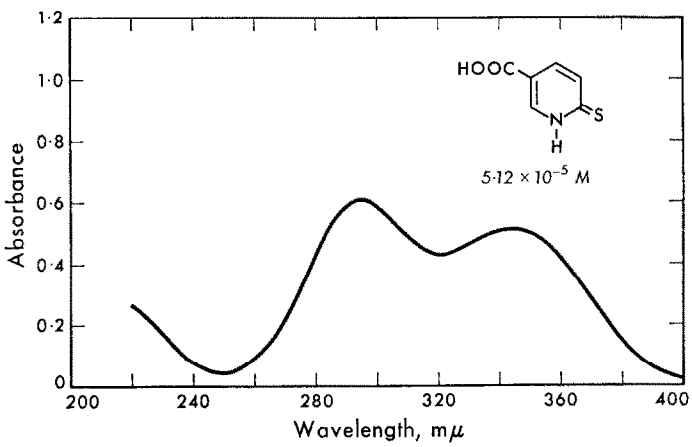


FIG. 2. The u.v. spectrum of II in KRP buffer, pH 7·2.

TABLE 1. REACTION OF DISULFIDES WITH THIOLS AND ASCITES TUMOR

Material	Units	Values obtained using*	
		6,6'-Dithio-dinicotinic acid (10^{-3} M)	2,2'-Dithio-dipyridine (10^{-3} M)
Cysteine	SH/mole	1·00	1·00
Glutathione	SH/mole	1·00	1·00
Serum albumin (bovine)	SH/mole	0·65	0·67
Aldolase	SH/mole	12·7	13·1
Ehrlich ascites cells, washed	SH/100 mg dry weight	1·56 ± 0·20 (1·21–1·95)	9·14 ± 0·12 (8·94–9·38)
Ehrlich ascites cells, homogenate	SH/100 mg dry weight	6·81 ± 0·05 (6·73–6·97)	6·32 ± 0·13 (6·06–6·62)

* The values given are the average of 4 determinations; in the case of tissues, the standard error and range are given. For the procedures used, see Experimental.

rate comparable to that obtained in the case of 2,2′-dithiodipyridine.[3] It is seen that I is a substrate for glutathione reductase.*

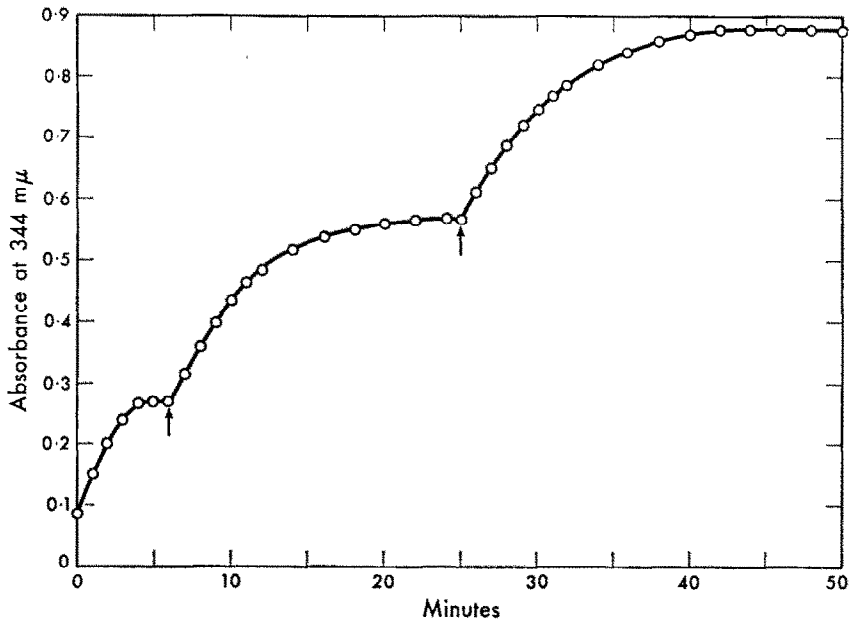


FIG. 3. The oxidation of glucose 6-phosphate in the presence of 6,6′-dithiodinicotinic acid. (Reactions 1 and 2 in text.) The spectrophotometer cuvette contained 3·0 μmoles 6,6′-dithiodinicotinic acid and 0·04 μmole NADPH in 3·00 ml of 0·1 M phosphate buffer, pH 7·5. Glutathione reductase (3·0 i.u. in 0·01 ml) was added to initiate the reaction. Glucose 6-phosphate dehydrogenase (2·8 i.u. in 0·01 ml) was added at 5·5 min. Aliquots of glucose 6-phosphate (0·041 μmole in 0·005 ml) were added at 6 min and again at 25 min. A cuvette containing 3·0 μmole of I in 3·00 ml of 0·1 M phosphate buffer, pH 7·5, was used as the blank.

Reactions 3 and 4 were also studied (Fig. 4).

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\ \text{GSH} + \text{NADP}^+ \qquad (3)$$

$$6,6'\text{-dithiodinicotinic acid} + 2\ \text{GSH} \longrightarrow$$
$$2\ (6\text{-mercaptonicotinic acid}) + \text{GSSG} \qquad (4)$$

Their rates are comparable to those occurring with 2,2′-dithiodipyridine,[3] the net result being the oxidation of NADPH to NADP (and restoration of the initial GSSG) at the expense of I.

When reactions 2, 3 and 4 are combined (Fig. 5), the overall result is the oxidation of glucose 6-phosphate to 6-phosphogluconate at the expense of I, with a corresponding linear formation of II (Fig. 6).

* Two methods have been used to demonstrate that the amount of glutathione present in the GR preparations cannot account for the rate of NADPH disappearance with I as substrate. These methods are: a) direct determination of glutathione after denaturation of the enzyme, and b) exhaustive dialysis of GR under conditions which we have shown to remove glutathione. The amount of glutathione found (0·036 mμmole GSSG/cuvette) would produce an absorbance change correspondence to 1/40 of the rate found when I is used as the substrate. The dialysis results also confirmed our conclusion that I is a substrate for GR (see Experimental).

In order to establish whether I is capable of oxidizing glucose 6-phosphate in the intact Ehrlich ascites cells, experiments with (^{14}C)-labelled glucose as the substrate were carried out. It was found that I (5×10^{-3} M) stimulates the production of $^{14}CO_2$ by Ehrlich ascites cells from (1-^{14}C)-glucose. This stimulation amounts to about

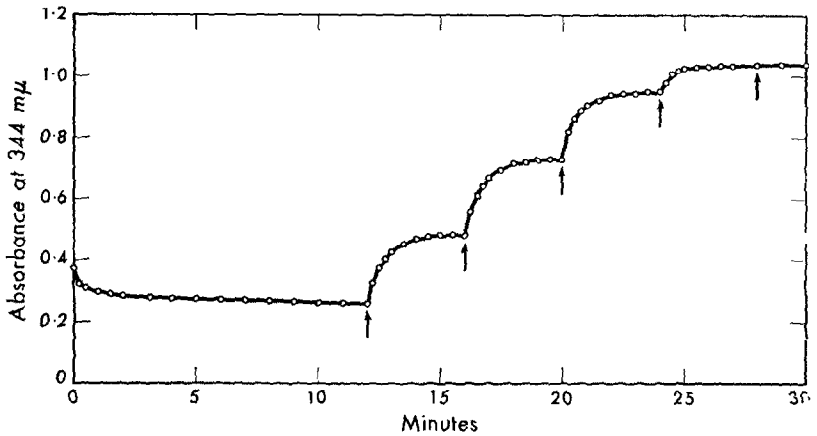


FIG. 4. Effect of 6,6′-dithiodinicotinic acid on the oxidation of NADPH by GSSG. (Reactions 3 and 4 in text.) The spectrophotometer cuvette contained 0·053 μmole of oxidized glutathione and 0·16 μmole NADPH in 3·00 ml of 0·1 M phosphate buffer, pH 7·5. Glutathione reductase (3·0 i.u. in 0·01 ml) was added to initiate the reaction. Aliquots of 6,6′-dithiodinicotinic acid (0·05 μmole in 0·005 ml) were added at the times indicated by the arrows. The blank was 0·1 M phosphate buffer, pH 7·5.

30–40 per cent (Fig. 7A and 7B). The production of $^{14}CO_2$ from (6-^{14}C)-glucose (Fig. 7C) was inhibited about 30 per cent after 60 min of incubation in the presence of added glucose. This is in agreement with our previous manometric experiments,[5] which showed that I causes a 35 per cent inhibition of oxygen uptake by Ehrlich

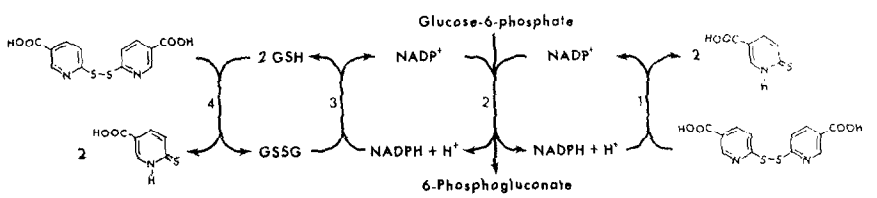


FIG. 5. Reactions involved in the oxidation of glucose 6-phosphate by 6,6′-dithiodinicotinic acid. Reactions 1 and 3 are catalyzed by glutathione reductase; reaction 2 is catalyzed by glucose 6-phosphate dehydrogenase; reaction 4 occurs spontaneously.

ascites cells in the presence of added glucose. Under similar conditions, type-A compounds caused almost complete inhibition, both of oxygen uptake and of glycolysis. Compounds of type B, such as I, on the other hand, caused a stimulation of aerobic glycolysis, and had a strong inhibitory effect on oxygen uptake in the absence of added glucose. The aerobic accumulation of lactate was taken as an indication that, although the formation of pyruvate via glycolysis is not prevented by I, its further oxidation via the Krebs cycle is inhibited.

DISCUSSION

Jacob and Jandl,[11] working with human erythrocytes, found that an increase of the ratio of oxidized to reduced glutathione causes an increase of the rate of glucose utilization via the HMP pathway. As the oxidizing agent, these investigators used hydrogen peroxide produced by appropriate enzyme systems. Our findings, using I as the oxidizing agent with Ehrlich ascites cells, are in line with those of the above authors with human erythrocytes. It would thus appear that the oxidation of NADPH stimulates the HMP pathway in Ehrlich ascites cells as well as in erythrocytes. Concurrent with the stimulation of $^{14}CO_2$ production from the C_1 of glucose, we found an increase in Ehrlich ascites cells in the amount of II formed from I.*

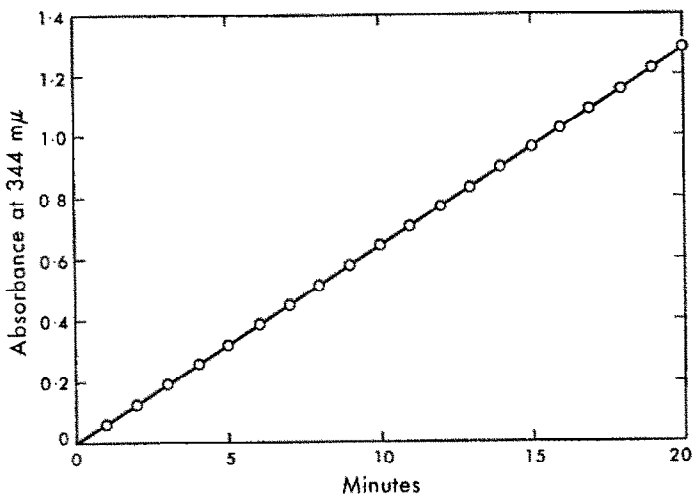


FIG. 6. Oxidation of glucose 6-phosphate by 6,6′-dithiodinicotinic acid. (Reactions 1, 2, 3 and 4 in text.) The spectrophotometer cuvette contained 1·5 μmoles of I, 1·5 μmole GSSG, 2·0 μmole glucose 6-phosphate, 1·5 i.u. of glutathione reductase and 1·4 i.u. of glucose 6-phosphate dehydrogenase. NADP (5·0 mμmole) was added to initiate the reaction. The total volume was 3·0 ml of 0·1 M phosphate buffer, pH 7·5. The blank contained 1·5 μmole of I, 1·5 μmole GSSG and 2·0 μmole glucose 6-phosphate in a total volume of 3·0 ml of 0·1 M phosphate buffer, pH 7·5.

In solution, both I and III react completely and rapidly with soluble thiols, giving a product that can easily be determined by its u.v. spectrum. It is apparent from the data presented in this paper that I would be suitable as a reagent for the determination of sulfhydryl groups, in a manner similar to that desdribed for III.[2] An advantage might be its higher solubility in aqueous buffer. I would also be suitable for the determination of NADP and of glutathione in solution, by enzymatic cycling, utilizing the reactions outlined in Fig. 5, in a manner similar to that described for III.[10]

In solution, both I and III can cause the enzyme-mediated oxidation of glucose 6-phosphate to 6-phosphogluconate. In the living cells, both compounds stimulate the HMP pathway. In the case of III, the stimulation consists of a 5-min "burst" of

* The amount of II formed in these experiments was only about one-half that expected, judged from the amount of $^{14}CO_2$ produced from the C_1 of glucose (unpublished). The cause of this discrepancy is currently under investigation.

$^{14}CO_2$ evolution from (1-^{14}C)-glucose[9] followed by essentially complete inhibition. With I, the stimulation is gradual and continues at least 3 hr. The extent of the interaction of I with intact Ehrlich ascites cells is considerably lower than that of III. In view of our finding that I and III react to a similar extent with Ehrlich ascites cell homogenates and with thiols and proteins, we suggest that I penetrates the cells less easily than III.

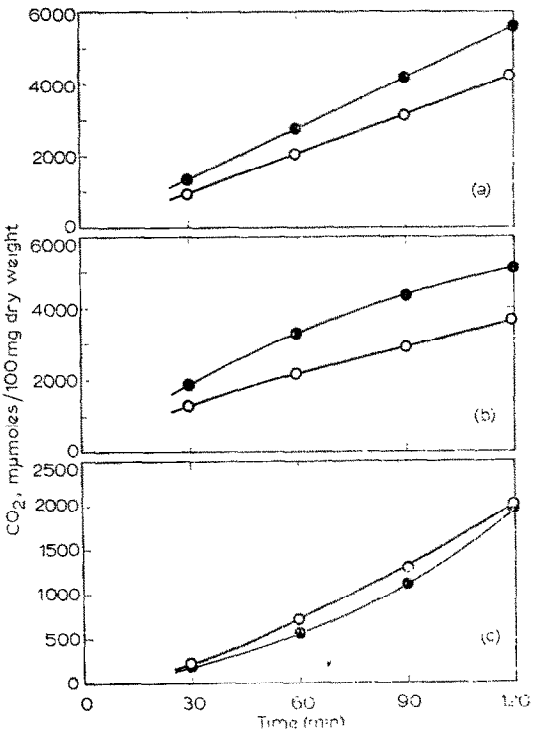


FIG. 7. Effect of 6,6'-dithiodinicotinic acid on the oxidation of glucose by Ehrlich ascites cells.

a. Anaerobic evolution of $^{14}CO_2$ from (1-^{14}C)-glucose (N_2–CO_2, 95:5).

b. Aerobic evolution of $^{14}CO_2$ from (1-^{14}C)-glucose (O_2–CO_2, 95:5); $C_1 - C_6$ is plotted.

c. Aerobic evolution of $^{14}CO_2$ from (6-^{14}C)-glucose (O_2–CO_2, 95:5).

Each flask contained 30 μmole of (^{14}C)-glucose (1 μc) and 28–45 × 10^6 washed Ehrlich ascites cells (13–15 mg dry wt.) in KRP buffer, pH 7·2. Total volume per flask was 3·0 ml. Incubations were carried out at 37°. $^{14}CO_2$ was collected and counted as reported previously.[9] ○ = $^{14}CO_2$ production by washed Ehrlich ascites cells; ● = $^{14}CO_2$ production by washed Ehrlich ascites cells in the presence of 15 μmole of I.

It may be of interest to point out the difference between I and the known artificial electron acceptors such as methylene blue and phenazine methosulfate. These latter compounds are able to oxidize NADPH nonenzymatically,[12] whereas I requires the mediation of an enzyme to carry out this oxidation.

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