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EFFECTS OF CYSTAMINE ON THE METABOLISM OF YOSHIDA HEPATOMA ASCITES CELLS *IN VITRO*

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Abstract—The effects of cystamine on some metabolic features of Yoshida hepatoma ascites cells are studied.

It is shown that cystamine inhibits the oxygen uptake; lactic acid production under anaerobic conditions is also inhibited after an initial enhancement. The Crabtree effect is partially removed by cystamine, if it is added at the same time as glucose; pre-incubation with glucose inhibits the appearance of the effect by cystamine on oxygen uptake, but under these conditions, no removal of the Crabtree effect is observed.

The enhancement of lactic acid production is correlated with the production of thiols by cystamine and the subsequent inhibition is demonstrated to occur at the hexokinase level. The action of glucose in removing the oxygen uptake inhibition is interpreted as due to the ability of this substance to re-establish the capacity of cells to reduce disulphides.

IT IS known that cystamine causes a lowering of body temperature in the mouse.[1] This substance, as well as cyanide, enhances the blood content of lactic and pyruvic acid in the rabbit.[2] It was also shown that cystamine can give origin to mixed disulphides with thiol-groups of proteins.[3] The possibility that some cellular enzymes could be inhibited through this mechanism was studied by Eldjarn and coll. using erythrocytes. Human erythrocytes are capable of reducing some disulphides to sulphydryls, but this reduction is small and temporary in the absence of substrates. When glucose is oxidized in the presence of methylene blue, the oxygen uptake is inhibited by the presence of cystamine at high concentration, and, at the same time, there is an inhibition of the reduction of disulphides.[4] The "disulphides poisoning" in the erythrocytes occurs at the hexokinase level and the presence of cystamine diverts the carbohydrates metabolism from the Embden-Meyerhof glycolytic pathway to the oxidative pentose shunt.[5]

The metabolism of erythrocytes is based on a limited number of reactions, and the purpose of the present paper is to extend the observations on the effects of cystamine to a different type of cells, namely Yoshida hepatoma ascites cells. Results presented in this paper confirm an inhibition at the hexokinase step; furthermore an inhibition of the oxygen uptake is found.

The appearance of these inhibitions is presumably due to a shortage of $NADPH_2$† in the cells; the ability of the cells to reduce disulphides is ascribed to the concentration of this coenzyme.

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† NADP nicotinamide-adenine dinucleotide phosphate; $NADPH_2$ reduced form of this dinucleotide.

While this paper was in preparation, a work by Nesbakken and Eldjarn[6] appeared which demonstrated that disulphides poisoning of glucose metabolism occurs also in other mammalian tissues.

MATERIAL AND METHODS

The strain of Ascites Hepatoma AH 130 (Yoshida) was supplied by the Medical Institute of the Sasaky Foundation (Tokyo, Japan) to the Farmitalia Inc. (Milano, Italy). It was kept in our Institute by weekly transplantations in non-imbred adult Wister rats of either sex since 1958. The animals transplanted 6–7 days previously with ascites hepatoma were sacrificed by decapitation and the ascitic fluid was removed by means of a syringe through a small opening in the peritoneal wall. The tumour cells were collected by centrifugation at 800 × *g* for 5 min at 0–4°C; the supernatants were discarded and the cells were resuspended in ice-cold medium (NaCl 130 mM; KCl 12·8 mM; $MgSO_4$, 0·65 mM; Phosphate Buffer, at pH 7·4,5 mM) and centrifuged again. This washing procedure was repeated two to three times until contamination with red blood cells, which tend to pack at the bottom of centrifuge tubes, was negligible. The final well packed, blood-free, ascites tumour cells were then resuspended 1:30 (v/v) in ice-cold medium of different composition according to the different types of experiments. For endogenous oxygen uptake measurements, the cells were suspended in the above mentioned medium and when a substrate was supplied, the NaCl concentration was modified in order to equilibrate the addition of substrate. When anaerobic glycolysis was measured, the medium employed for resuspending the cells contained NaCl 114 mM; KCl 4·7 mM; $MgSO_4$ 1·20 mM; KH_2PO_4 1·20 mM; $NaHCO_3$ 24·9 mM; glucose 15 mM. The solution was saturated with N_2–5% CO_2; final pH 7·4. Manometric measurements of oxygen uptake, anaerobic glycolysis and incubation in the presence of cystamine were performed according to conventional Warburg techniques. Cystamine was dissolved in the same Ringer medium used for cell suspensions, and the pH was accurately controlled at 7·4; the cystamine solutions were placed in the side arms of the flasks. All the experiments were carried out at 38°C.

Lactate and pyruvate were determined in TCA final 6 per cent filtrates of the cell system respectively according to the methods of Barker and Summerson[7] and Friedmann and Haugen[8] modified by Rindi and Ferrari.[9]

Ammonia content was determined on a 2 ml sample of TCA final 6 per cent filtrate; the sample was treated with about 2 g of Na_2CO_3 and the solution was fluxed with N_2 to carry ammonia in a H_2SO_4 N solution. Ammonia was determined in this solution according to the Nessler colorimetric method.

RESULTS

The action of various concentrations of cystamine on the oxygen uptake of hepatoma ascites cells is shown in Fig. 1. Inhibition starts at about 20 min whatever concentrations of cystamine are used. For each concentration tested the curves tend to asymptotic values, which are reached more rapidly at higher concentrations. Fig. 2 shows the dependence of oxygen uptake upon cystamine concentration.

It has been shown on rat liver and kidney homogenates that the oxygen consumption is inhibited by cystamine and this effect has been correlated with the formation of

deamination products of this substance by the action of diamminoxidase.[10] On this ground the presence of diamminoxidase activity has been investigated in these cells. Ammonia determinations have been carried out using 20 mM cystamine or cadaverine as substrates. All experiments at different incubation times suggest the absence of any diamminoxidase activity.

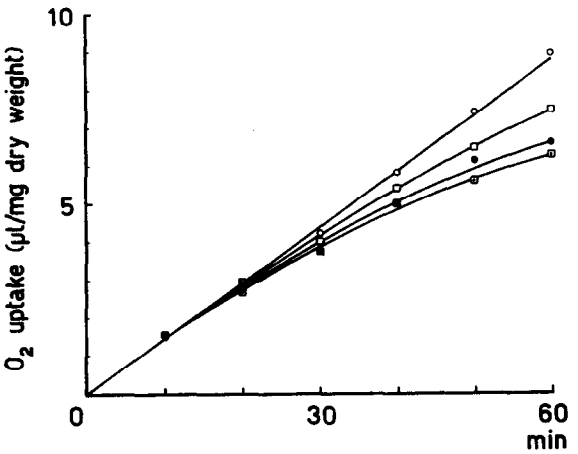

FIG. 1. Action of cystamine on oxygen uptake of Yoshida hepatoma ascites cells.
Experimental procedure: 2·8 ml of cell suspension in Warburg flasks; 0·2 ml KOH 30% in the center well; the side arms contained 0·2 ml of ringer (controls) or 0·2 ml of cystamine solution at various concentrations. Cystamine was added at 0 time.
○—○ controls; □—□ cystamine 2 mM; ⊙—⊙ cystamine 4 mM; ⊡—⊡ cystamine 8 mM.

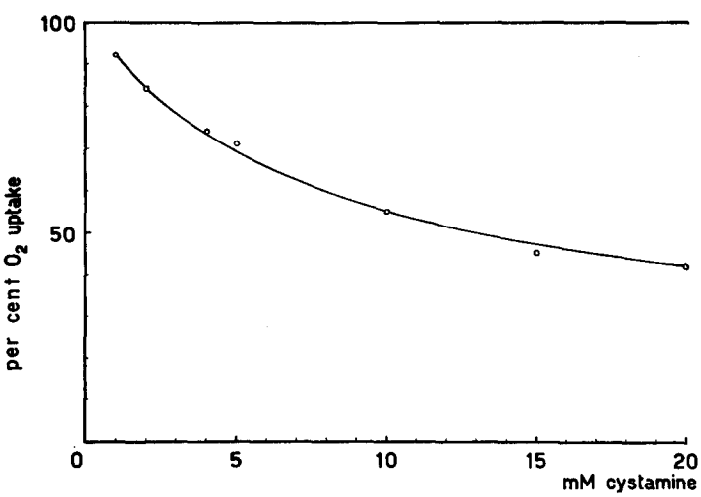

FIG. 2. Dependence of oxygen uptake of hepatoma cells on cystamine concentrations.
Procedure as in Fig. 1.
Ordinate: QO_2, per cent of control values;
Abscissa: cystamine concentration.

In order to establish whether the inhibition of oxygen uptake could be due to a blocking of one step of the oxidative metabolism, the cells were supplied with 30 mM citrate or succinate as substrates. In all tests the inhibition by 10 mM cystamine was not modified by the presence of citrate or succinate.

It was recently shown that "disulphide poisoning" is related to $NADPH_2$ concentration in the cells,[5] and it is well known that in tumour cells the NADP is present in very small amounts; most of it is found in the reduced form.[11] Studies with labelled glucose demonstrated that hexose monophosphate shunt is operating in hepatoma;[12] in ascites tumour cells the reoxidation of $NADPH_2$ generated in the monophosphate shunt is utilized for reductive syntheses.[13] For these reasons the behaviour of the oxygen uptake inhibition by cystamine in the presence of glucose has been studied. Cells incubated with 15 mM glucose for 20 min and added with 10 mM cystamine do not show any inhibition of the oxygen uptake due to cystamine. Fig. 3 shows that the

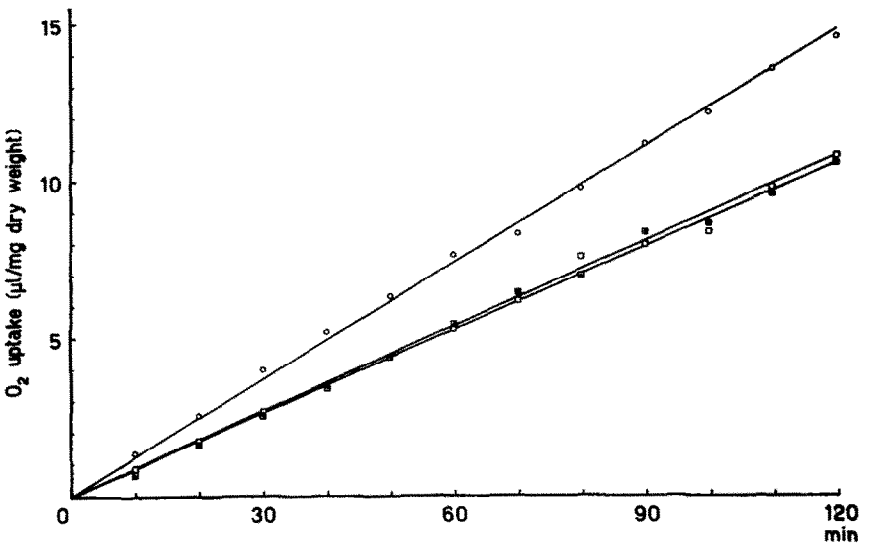

FIG. 3. Effect of glucose pre-incubation on oxygen uptake in the presence of cystamine.
○—○ controls; □—□ glucose 15 mM at 0 time;
⊡—□ glucose 15 mM at 0 time and cystamine 10 mM at 20 min.

presence of glucose inhibits the oxygen consumption (Crabtree effect), but this inhibition is not further increased by the addition of cystamine. If, however, glucose is added when the inhibition by cystamine is already operating, such inhibition is not modified (Fig. 4). When glucose and cystamine are added at the same time, the Crabtree effect is partially removed and inhibition by cystamine does not appear (Fig. 5).

In order to elucidate the mechanism of this effect, anaerobic glycolysis in the presence of various amounts of cystamine has been tested.

When anaerobic glycolysis is measured with the Warburg technique, the addition of cystamine induces an increased production of CO_2, which is followed by an inhibition.

The increase takes place during the first 5 min immediately following the addition of the disulphide and it is proportional to its concentration in the medium. After about 10 min at the high concentrations, or after about 20 min at the low ones, an inhibition appears which is also dependent upon the concentration of cystamine in the medium.

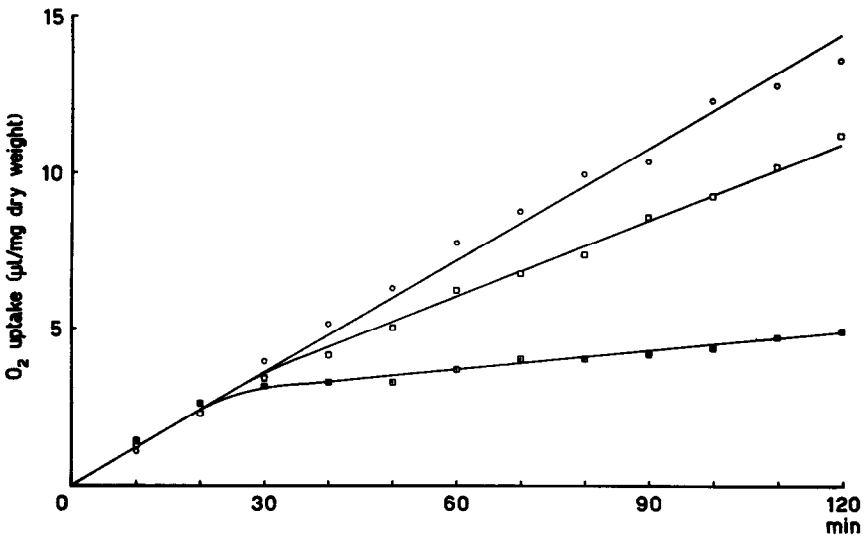

FIG. 4. Effects of glucose added when cystamine inhibition is operating.
○—○ controls.
□—□ glucose 15 mM at 30 min;
⊡—⊡ cystamine 10 mM at 0 time and glucose 15 mM added at 30 min.

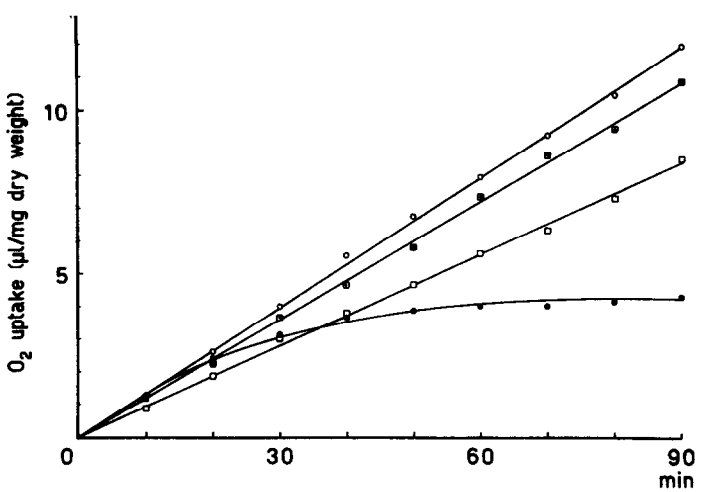

FIG. 5. Effect of glucose on oxygen uptake inhibition by cystamine when the two substances are added at the same time.
○—○ controls; □—□ glucose 15 mM at 0 time;
⊡—⊡ cystamine 10 mM and glucose 15 mM at 0 time;
⊙—⊙ cystamine 10 mM at 0 time.

This is shown in Fig. 6, where the glycolysis rate of the controls is normalized to 100 for all incubation times and the rate of glycolysis in the presence of cystamine is expressed as per cent of the controls at each time.

In order to confirm the results of manometric measurements lactic acid production has been investigated. At the same time the pyruvic acid concentrations have been

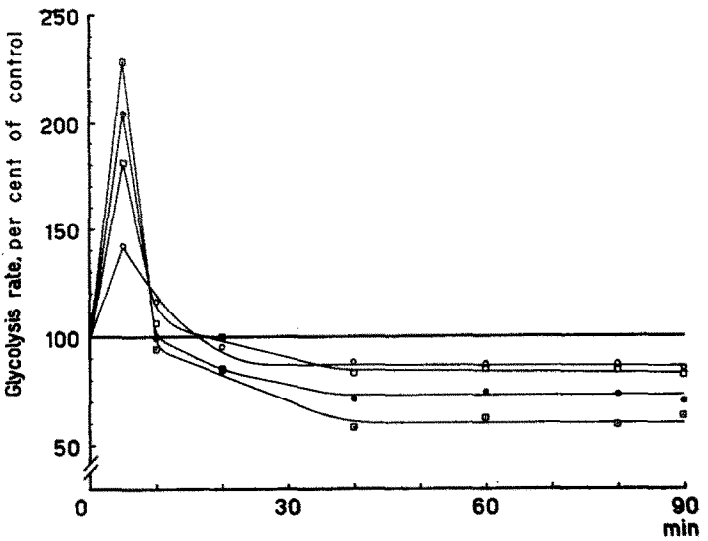

FIG. 6. Effect of different concentrations of cystamine on the rate of glycolysis. Control rate is normalized to 100 at all times and the glycolysis rate in the presence of cystamine is expressed as per cent of controls at each time. Cystamine was added at 0 time in all experiments.
○—○ cystamine 5 mM; □—□ cystamine 15 mM;
○—○ cystamine 20 mM; ⊡—⊡ cystamine 30 mM.

measured to establish whether a blocking at the lacticodehydrogenase level was induced by the presence of cystamine. The results are reported in Table 1; lactic acid determinations are in good agreement with the manometric data. Pyruvic acid concentrations demonstrate that no blocking of lacticodehydrogenase is present.

TABLE 1. INFLUENCE OF CYSTAMINE ON LACTIC AND PYRUVIC ACID PRODUCTION BY YOSHIDA HEPATOMA ASCITES CELLS

	μmoles of CO_2/ 100 mg dry weight			μmoles of pyruvic acid/100 mg dry weight			μmoles lactic acid/ 100 mg dry weight		
Incubation time (min)	5	10	90	5	10	90	5	10	90
Controls	22·5	33	265·5	0·89	0·89	1·46	19·7	28·8	250·7
Cystamine 30 mM added at 0 time	45·8	58·5	220·5	0·91	0·85	0·62	41·0	46·35	186·0

Immediately after manometric reading, 2 ml TCA 15% is added for each determination (final TCA concentration 6%); final volume 5 ml.
Lactic acid is determined on 1 ml of 1:25 diluted solution.
Pyruvic acid is directly determined on 2 ml of the sample.

DISCUSSION

It was reported that cystamine can give origin to mixed disulphides with the thiol-groups of proteins[3] and with reduced glutathione.[14] Recently Nesbakken and Eldjarn[6] have proposed the following scheme for disulphide reduction within the cells:

$$GSH + XSSH \longrightarrow GSSX + XSH \quad (1)$$
$$GSH + GSSX \longrightarrow GSSG + XSH \quad (2)$$
$$GSSG + NADPH_2 \longrightarrow 2GSH + NADP \quad (3)$$

Reactions 1 and 2 are depending upon the availability of reduced glutathion. A constant level of reduced glutathion is maintained by reaction 3, which is catalysed by glutathione-reductase; this enzyme is not active on mixed disulphides.[15] Reaction 3 is depending upon the presence of $NADPH_2$. Thiols produced by reactions 1 and 2 are metabolized or reoxidized to disulphides by molecular oxygen. Under anaerobic conditions the lack of reoxidations leads to a decreased concentration of disulphides within the cell. Disulphide poisoning occurs when reaction 3 is blocked by shortage of $NADPH_2$.

When hepatoma ascites cells are treated with cystamine, this substance diffuses rapidly into the cells and according to the observations of Eldjarn on erythrocytes,[4] it can be assumed that the intracellular concentration is dependent upon the concentration in the medium. The presence of cystamine within the cells gives origin to an increased production of lactic acid (Table 1); the origin of lactic acid is endogenous and it is not due to a greater utilization of glucose added.

This conclusion is supported by the observations on the Crabtree effect, which is dependent upon hexokinase[16–18]: in our experiments this effect is partially removed by the addition of cystamine and glucose at the same time (Fig. 5).

The endogenous origin of lactic acid is also supported by the experiments of Nesbakken and Eldjarn,[6] who found and increased production of lactic acid from endogenous substrates in rat kidney and diaphragm homogenates after 30 min incubation with cystamine. McIlwain[19] has demonstrated that the presence of thiols enhances the rate of glycolysis in brain slices. Rapid production of mixed disulphides by cystamine gives origin to cysteamine and it is suggested that cysteamine is responsible for the increased production of lactic acid, although the mechanism of this action is still unknown.

After the initial increase of glycolysis just discussed, a subsequent inhibition has been observed. However, the utilization of the glucose present in the medium is not completely inhibited even at the highest concentration of cystamine used. This is in agreement with the observation that the Crabtree effect is still present, although to a smaller degree in experiments with cystamine and glucose added together. The final values of pyruvic acid in the presence of cystamine are lower than the control levels and it is suggested that the glycolysis blocking takes place at some metabolic steps preceding the formation of pyruvic acid. From the partial removal of the Crabtree effect it can be inferred that this step may be at the hexokinase level, in agreement with observations on erythrocytes[5] and rat tissue homogenates.[6]

* GSH glutathione reduced; GSSG glutathione oxidized.

The inhibition of the oxygen consumption due to the presence of cystamine is not affected by citrate or succinate; this effect is not present if the cells are pre-incubated with glucose or if cystamine and glucose are added together. The low concentration of NADP in tumour cells has been previously pointed out.[11] The prevalence of this coenzyme in the reduced form facilitates the reduction of disulphides in ascites tumour cells, according to the Nesbakken scheme. However, this reduction is limited to a short time, because of the $NADPH_2$ shortage. The addition of glucose allows the re-synthesis of $NADPH_2$ through hexose-monophosphate shunt which is operating in hepatoma cells[12] and reduction of disulphides can be resumed. This is in agreement with the fact that $NADPH_2$ generated in the monophosphate shunt is utilized for reductive syntheses by ascites tumour cells.[13] The hypothesis that glucose is acting by re-establishing the ability of the cells to reduce disulphides, is also supported by the observation that its addition after the establishment of oxygen uptake inhibition by cystamine does not remove such inhibition (Fig. 4).

At the present time it is impossible to formulate any hypothesis about the level at which inhibition of oxygen uptake by cystamine takes place.

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