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The Inhibition of Carbohydrate Metabolism in Ascites-Tumour Cells by Ethyleneimines

By I. M. ROITT
Courtauld Institute of Biochemistry, Middlesex Hospital, W. 1

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The influence of cytotoxic agents upon carbohydrate metabolism has been the subject of several investigations. For example, mustard gas was shown to inhibit glycolysis in Ehrlich rat sarcoma (Jany & Sellei, 1935) and in Jensen sarcoma (Berenblum, Kendal & Orr, 1936). Barron, Bartlett, Miller, Meyer & Seegmiller (1948) found that the respiration of a number of rat tissues was inhibited by methyldi-(β-chloroethyl)amine hydrochloride. Suspensions of Sarcoma 37 obtained from mice after administration of α-peltatin had a decreased ability to utilize glucose anaerobically (Waravdekar, Paradis & Leiter, 1955).

The potent growth-inhibitory properties of the ethyleneimines demonstrated by Hendry, Homer, Rose & Walpole (1951) suggested that a study of the effect of these compounds on carbohydrate metabolism in normal and malignant mammalian cells would be of interest. Cardinali (1954) has already shown that aerobic glycolysis in Rous sarcoma and benzopyrene sarcoma is inhibited by ethyleneimine picrate. In the present work, attention was concentrated mainly on the effect of 2:4:6-triethyleneimino-1:3:5-triazine (TEM) on anaerobic glycolysis in ascites-tumour cells, since this material is convenient for following changes in the concentration of cellular constituents in response to the action of metabolic inhibitors.

A preliminary account of this work has been reported (Roitt, 1955).

EXPERIMENTAL

Ascites-tumour cells. In the majority of experiments, the Krebs II carcinoma in ascites form was used and was maintained in heterozygous albino mice. The animals were killed 7–9 days after inoculation with ascites tumour and the cells were washed with isotonic saline into tubes containing heparin. The ascitic fluid was removed after centrifuging for 5 min. at 2000 *g* and the cells were resuspended in saline, spun at 100 *g* for 3 min. and the bulk of the red cells, still in suspension, removed. The resuspension and low-speed spinning were repeated until contamination with red cells was negligible. The cells were finally suspended in Krebs–Ringer phosphate solution containing 0·1% glucose for aerobic studies, and in Krebs–Ringer bicarbonate containing 0·4% glucose for experiments under anaerobic conditions. The packed-cell volume was determined by centrifuging in a Wintrobe tube for 30 min. at 800 *g*. These preparations contained approximately 5% by number of inflammatory cells, representing less than 1% of the total cell mass.

Respiration and anaerobic glycolysis. The conventional Warburg techniques were used. The media used for studying the metabolism of ascites-tumour cells were also employed for parallel studies on the other mammalian tissues. Substrates were dissolved in isotonic NaCl and the pH was adjusted to 7·4. In a few cases anaerobic lactic acid production was measured (Barker & Summerson, 1941) and shown to be paralleled by the CO_2 evolved. The buffering capacity of TEM was negligible at pH 7·4, so that no correction for retained CO_2 was necessary.

Enzyme preparations

Hexokinase. The activity of a deoxycholate-treated particulate fraction of bullock brain was followed by measuring glucose disappearance (Crane & Sols, 1953; preparation *CD*). Glucose was estimated by the anthrone method (Fairbairn, 1953) after precipitation of phosphates and protein with $Ba(OH)_2$ and $ZnSO_4$.

Triose phosphate dehydrogenase. A dialysed extract of rabbit-muscle acetone powder was used with fructose diphosphate as substrate (Green, Needham & Dewan, 1937). The evolution of CO_2 from a bicarbonate medium

was followed. The enzyme was incubated with the ethyleneimines *in vacuo* to reduce loss of activity by autoxidation.

Phosphofructokinase. An acetone powder of rat brain was extracted with 0·002 M-Na_2HPO_4, pH 7·4. The extract was spun at 18 000 *g* to minimize contamination with particles having high ATPase activity (Muntz & Hurwitz, 1951). The reaction between fructose monophosphate and adenosine triphosphate (ATP) was followed by measurement of acid production manometrically. The enzyme was incubated with fructose monophosphate and the ethyleneimine in Warburg vessels gassed with N_2+CO_2 (95:5); the reaction was started by adding ATP from the side arm. The formation of fructose diphosphate in the reaction was demonstrated enzymically by using the rabbit-muscle preparation of Green *et al.* (1937).

Ascites-cell DPNase. Ascites cells were ground in a Potter-type homogenizer with powdered glass in 0·13% $NaHCO_3$. The resulting suspension was centrifuged at 2000 *g* for 15 min. and the supernatant fluid dialysed against 0·13% $NaHCO_3$ at 0° for 12 hr. The rate of disappearance of added diphosphopyridine nucleotide (DPN) was followed by means of the alcohol dehydrogenase (Racker, 1950) or cyanide (Colowick, Kaplan & Ciotti, 1951) method.

Analytical methods

Ethyleneimine grouping. The reaction of the ethyleneimine ring with $S_2O_3^{2-}$ which results in the equivalent liberation of OH^- may be used as a quantitative method (Ross, 1950). Since many of the solutions to be estimated contained phosphate, a screened indicator consisting of 0·1% aqueous solutions of thymol blue and cresol red (6:1) was used. A solution of 0·4 M-$Na_2S_2O_3$ was boiled to expel dissolved CO_2 and made just alkaline to the screened indicator; 3·0 ml. of this solution was added to the TEM (quantities of the order of 2·0 ml. of 0·01 M), which had also been made alkaline to the indicator, in a small conical flask, and the mixture was placed so that the tip of a Conway microburette dipped below the liquid surface. The mixture was gently heated near to boiling point and 0·2 N acetic acid was run in from the burette to keep the pH slightly on the acid side as the OH^- was generated in the reaction. When the reaction was complete, 0·2 N-NaOH was added until the indicator changed colour. The estimation was accurate to within 3%.

Fructose phosphates. The ascites cells were rapidly spun free of medium and homogenized with cold 10% (w/v) trichloroacetic acid; after centrifuging, the supernatant fluid was neutralized and then frozen and thawed. On spinning down the precipitate a clear supernatant liquid was obtained. The fructose esters were estimated by the method of Roe & Papadopoulos (1954), in which the quantitative separation of the Ba salts is enhanced by the addition of inorganic phosphate. With a synthetic mixture of fructose mono- and di-phosphates, the most consistent results were obtained when the fraction precipitated by Ba from aqueous solution at pH 8·2 (containing the diphosphate probably contaminated with the monophosphate) was redissolved in dilute HCl, excess of Ba acetate added and the pH again adjusted to 8·2. The precipitate was spun down and the supernatant fluids (containing the monophosphate) were combined. This procedure (recommended generally by LePage, 1949) was applied to the cell extracts. The results were confirmed in one case by paper electrophoresis of the extract (see under fructose diphosphate in the Materials section) and estimation of the fructose in successive strips of the paper: the strips were eluted with water, glucose 6-phosphate was oxidized with Br_2 and the fructose determined by heating with the Roe & Papadopoulos reagent.

Other phosphorylated intermediates. The trichloroacetic or perchloric acid cell extracts were neutralized and the Ba salts fractionated as above. P was measured by a micromodification of the Fiske & Subbarow (1925) method. Glucose 6-phosphate was estimated by a modified Hagedorn–Jensen method (Robison & King, 1931), allowance being made for the presence of fructose phosphates. Triose phosphate was estimated as the P liberated in 20 min. at room temp. by N-KOH, and phosphopyruvic acid was estimated as the P liberated by dilute KOH and I_2 relative to the control without I_2. 3-Phosphoglyceric acid (together with 2-phospho- and 2:3-diphospho-glyceric acids) was measured as 'difficultly hydrolysable P'; after 3 hr. hydrolysis with N-HCl at 100° in stoppered tubes, the bulk of the inorganic P was precipitated in 5 min. with magnesia mixture and the organically bound P in the supernatant fluid determined. ATP, adenosine diphosphate and adenylic acid were calculated from the acid-labile P and absorption at 260 mμ. (adenine) of the fractions forming soluble and insoluble Ba salts. Ribose 5-phosphate was estimated as (total ribose of fraction forming soluble Ba salts) – (ribose equivalent of adenylic acid).

Diphosphopyridine nucleotide. The method of Jedeikin & Weinhouse (1955) was followed. The concentration of reduced diphosphopyridine nucleotide was shown to be negligible and therefore only DPN was estimated. The cells (1·0 g.) were rapidly spun down in the cold. 3·0 ml. of boiling 0·04 M-KH_2PO_4, pH 5·4, was added, the cells were rapidly dispersed with a glass rod and immediately added to a Potter-type homogenizer, containing 4·0 ml. of the phosphate buffer and 0·5 g. of powdered glass, which had been heated in a boiling-water bath for at least 4 min. previously. After 60 sec., the contents were homogenized for 30 sec. and cooled in ice; when cold they were homogenized for a further 2 min., made up to a given volume, frozen, thawed and finally spun at 18 000 *g* for 15 min. The DPN content of the clear supernatant fluid was estimated by the method of Racker (1950).

Assay of triose phosphate dehydrogenase in crude cell extracts

The enzyme cannot be assayed in crude extracts by spectrophotometric estimation of the reduction of DPN with triose phosphate, since the glycerol phosphate dehydrogenase present effects the reoxidation of DPNH (Racker, 1947). The basis of the present method is as follows: in the presence of arsenate, triose phosphate formed by the action of aldolase on fructose diphosphate is oxidized by DPN and triose phosphate dehydrogenase to 1-arseno-3-phosphoglyceric acid, which spontaneously undergoes hydrolysis to 3-phosphoglyceric acid. The acid formed liberates CO_2 from the bicarbonate medium. DPNH is reoxidized by pyruvate in the presence of lactic acid dehydrogenase. The triose phosphate oxidation is made rate-limiting by addition of excess of fructose diphosphate, arsenate, DPN, pyruvate, aldolase and lactic acid dehydrogenase.

The ascites-tumour cells (0·5 g.) were dispersed in the incubation medium by homogenizing with powdered glass

for 3 min. The suspension (about 8 ml.) was centrifuged at 18 000 *g* for 35 min. at 0° and the supernatant fluid collected. For the assay, the following were added to each Warburg flask: 1·5 ml. of extract, 0·1 ml. each of solutions of aldolase and lactic acid dehydrogenase (1 part of commercial preparation diluted with 9 parts of water), 0·25 ml. of 0·1 M fructose diphosphate, 0·1 ml. of 1 M sodium pyruvate, 0·1 ml. of 0·3 M-Na_2HAsO_4, pH 7·4, and 0·4 ml. of Krebs–Ringer bicarbonate; the side arms contained 0·3 ml. of 0·17 mM DPN and 0·3 ml. of Krebs–Ringer bicarbonate. The flasks were gassed with $N_2 + CO_2$ (95:5) and allowed to equilibrate at 38°; CO_2 evolution was followed after tipping in. If either DPN or arsenate was omitted from the assay system, CO_2 evolution was negligible, indicating that acid production truly reflected the triose phosphate oxidation. Since relative and not absolute enzymic activities were required, the buffering effects of protein and arsenate could be ignored, as they were the same in both control and TEM-inhibited cells. For absolute determinations it would be necessary to measure lactic acid formation.

The DPNase activity of the cell extracts was low, presumably owing to aggregation of the cytoplasmic particles in a medium containing potassium and their subsequent removal by centrifuging. Where DPNase activity is appreciable, the assay system should include nicotinamide. The rate of acid production was linear over the first 15 min. and, in a typical assay, 100 µl. of CO_2 was evolved during this period. The concentration of TEM was maintained at 0·01 M in the assay system in case dilution affected some reversible TEM-complex.

Materials

Ethyleneimine, 2:4:6-triethyleneimino-1:3:5-triazine, ethyleneiminopropionitrile and *N*-acetylethyleneimine were kindly given by Dr A. L. Walpole and Dr J. A. Hendry (I.C.I. Dyestuffs Division).

Adenosine triphosphate was obtained from Boots Pure Drug Co. as the barium salt, and freed from heavy metals by ion exchange (Polis & Meyerhof, 1947). Calcium fructose diphosphate (L. Light and Co.) was converted into the sodium salt by treatment with sodium oxalate. The solution was analysed by electrophoresis on paper in veronal buffer pH 8·6 (Flynn & de Mayo, 1951), and the spots were localized by the method of Bandurski & Axelrod (1951). The sample was free of monophosphate. Fructose monophosphate was prepared by hydrolysis of the diphosphate (Neuberg, Lustig & Rothenberg, 1943). The product was free of the diphosphate as judged by paper electrophoresis.

*iso*Nicotinamide was prepared from *iso*nicotinic acid via the ethyl ester (Camps, 1902). DPN, aldolase, and lactic and alcohol dehydrogenases were obtained from C. F. Boehringer & Soehne, G.m.b.H. Coramine (*NN*-diethylnicotinamide) was kindly given by Ciba Laboratories, pyridine-3-nitrile by Professor H. McIlwain and nicotinylhydrazide by Dr D. E. Hughes.

The yeast used in these experiments was from The Distillers Co., Ltd.

RESULTS

Effect of TEM on anaerobic glycolysis

A delay in the onset of inhibition of anaerobic glycolysis in Krebs II carcinoma ascites cells by various concentrations of TEM is evident from Fig. 1. This delayed inhibition characterized the effect of TEM on the anaerobic glycolysis of the other tissues studied. The results obtained with Sarcoma 37 cells in the ascites form, slices of rat-brain cortex, liver, hepatoma, kidney cortex and spleen, and yeast cells, are presented in Table 1. With the exception of yeast, brain was the least sensitive to the action of 0·01 M TEM, there being

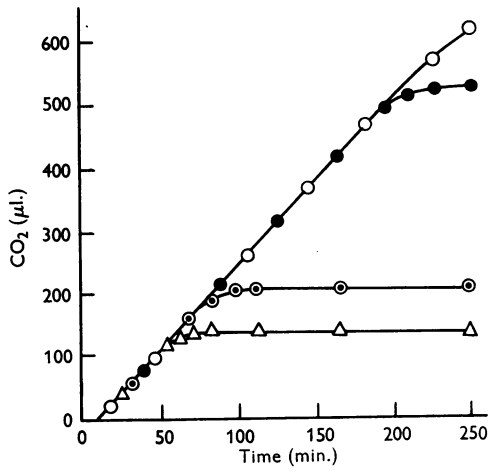

Fig. 1. Effect of TEM on anaerobic glycolysis of Krebs II carcinoma ascites cells. Each flask contained 0·04 ml. of packed cells suspended in 2 ml. of bicarbonate medium. ●, 0·001 M TEM; ⊙, 0·0067 M TEM; △, 0·01 M TEM; ○, control.

Table 1. *Inhibition of anaerobic glycolysis by* 0·01 M *TEM*

Results are the means of duplicates, and are expressed as per cent inhibition compared with control cells without TEM. Slices of rat tissues were used; each Warburg vessel contained between 60 and 100 mg. of fresh tissue suspended in 3 ml. of Krebs–Ringer bicarbonate. In experiments with ascites-tumour cells, 40 mg. of cells was suspended in 3 ml. of Krebs–Ringer bicarbonate, and with yeast, 3 mg. of cells was suspended in a medium containing 0·2% (w/v) $NaHCO_3$ and 0·002 M-NaH_2PO_4. The substrate was 0·4% (w/v) glucose. The gas phase was $N_2 + CO_2$ (95:5). Experiments with mammalian tissues were conducted at 38° and those with yeast at 30°. The reaction was started by tipping 0·2 M TEM (in 0·9% NaCl) from the side arm to give a final concentration of 0·01 M.

	Time (min.)					
Tissue	0 (%)	60 (%)	90 (%)	120 (%)	270 (%)	300 (%)
S37 Ascites cells	0	50	71	72	96	—
Krebs II ascites cells	0	30	100	—	—	—
Rat-brain cortex	0	0	14	18	62	97
Normal rat liver	0	0	100	—	—	—
Rat hepatoma	0	33	80	82	89	—
Rat-kidney cortex	0	33	52	56	73	81
Rat spleen	0	0	59	72	73	88
Yeast	0	0	0	0	43	43

only very slight inhibition after 2 hr. In contrast, inhibition was virtually complete by 90 min. in Krebs II carcinoma ascites cells (hereafter referred to as ascites-tumour cells), liver and hepatoma.

Ethyleneimine and ethyleneiminopropionitrile resemble TEM in their effects on anaerobic glycolysis. In ascites-tumour cells, anaerobic glycolysis is completely inhibited by 0·023 M ethyleneiminopropionitrile 55 min. after addition of the drug, and by 0·088 M ethyleneiminopropionitrile 120 min. after the addition. *N*-Acetylethyleneimine appears to be less potent.

The anaerobic glycolysis of ascites-tumour cells was studied in mice injected twice daily with 80 μg. of TEM/100 g. of body wt. intraperitoneally, starting 4 days after inoculation with tumour cells. Despite the almost complete suppression of mitosis (one abnormal metaphase was the only mitotic figure seen in 1000 cells) the anaerobic glycolytic rates of cells from animals treated with TEM for 24 and 48 hr. did not differ significantly from the rates of cells from control animals injected with isotonic saline.

Effect of TEM on respiration

Yeast is again insensitive, but in the other cells studied respiration appears to be more susceptible to the action of TEM than is anaerobic glycolysis. The results are summarized in Table 2.

Study of possible factors causing the delay in inhibition

The permeability of ascites-tumour cells to TEM was studied. Within 4 min. of the addition of a comparable volume of cells to medium containing TEM, the concentration of drug in the medium had fallen to a value which accorded with redistribution over the combined volume of cells plus medium. Although rapid penetration of the cells is suggested, adsorption of TEM on the cell surface cannot be excluded. The possibility that TEM reacted with the Krebs–Ringer medium to give a substance with inhibitory properties was studied by incubating TEM with the medium for 5 hr. before addition of the ascites cells. The course of the inhibition was unaltered by this procedure.

It is unlikely that TEM reacts with some cellular constituent to form an inhibitor since addition of intact or homogenized TEM-inhibited ascites cells plus their incubation medium fails to affect the time course of the TEM-inhibition of anaerobic glycolysis in fresh cells.

Table 2. *Inhibition of respiration by* 0·01 M *TEM*

Results are the means of duplicates and are expressed as per cent inhibition compared with control cells without TEM. The experimental details are the same as for Table 1 except for the following: mammalian tissues were suspended in 2 ml. of Krebs–Ringer phosphate; in the experiments with yeast, 30 mg. of cells was suspended in 3·0 ml. of 0·07 M-NaH_2PO_4 buffer, pH 6·8; the substrate was 0·1% (w/v) glucose; the gas phase was O_2.

Tissue	Time (min.) 60 (%)	120 (%)	180 (%)	240 (%)
Sarcoma 37 in ascites form	44	86	100	—
Rat-brain cortex	0	16	93	100
Rat-kidney cortex	0	32	68	77
Yeast	0	0	0	—

Effect of TEM on isolated enzyme preparations

In an attempt to elucidate the mechanism of glycolytic inhibition, the action of TEM on certain enzymes concerned in carbohydrate metabolism was studied *in vitro*.

Hexokinase. After incubation for 5 hr. with 0·01 M TEM at 38° the activity of the brain enzyme was slightly higher than that of the control. Similar results were obtained with 0·01 M ethyleneimine.

Triose phosphate dehydrogenase. The activity of a triose phosphate dehydrogenase preparation was inhibited by 20% after incubation for 4 hr. with 0·01 M TEM at 38° and 43% after incubation for 6 hr.

Phosphofructokinase. The effect of incubation with 0·01 M TEM at 38° for different times is shown in Fig. 2. This enzyme is far more sensitive to TEM than either of the other enzymes studied.

Phosphorylated intermediates in TEM-inhibited ascites-tumour cells

Since phosphofructokinase was inhibited *in vitro*, the concentrations of fructose phosphates in TEM-inhibited ascites-tumour cells were estimated to see whether there was an accumulation of the monophosphate as might be expected if phosphofructokinase were blocked. On finding that there was a greater accumulation of fructose diphosphate than of monophosphate in the inhibited cells, the analysis was extended to include other phosphorylated intermediates (Table 3). The ratio of the concentration of fructose monophosphate in TEM-inhibited slices of brain cortex to that in control slices was 2·68; the corresponding ratio for fructose diphosphate was 2·98.

Assay of triose phosphate dehydrogenase in TEM-inhibited ascites-tumour cells

Extracts of cells in which anaerobic glycolysis had been blocked by TEM were able to oxidize triose phosphate at the same rate as the controls without TEM. Further, if the aldolase and lactic acid dehydrogenase additions are omitted from the assay system (see Experimental section), the rates

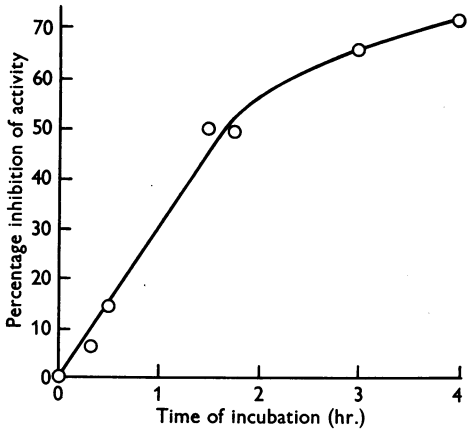

Fig. 2. Effect of incubation with 0·01 M TEM on the activity of a phosphofructokinase preparation from rat brain.

Table 3. *Acid-soluble phosphates in ascites-tumour cells immediately following complete inhibition of anaerobic glycolysis by* 0·01 M *TEM*

Conditions as for Table 1. Results are expressed as the ratio of concentration in inhibited cells to concentration in control cells without TEM.

Phosphate	Ratio
Acid-soluble P	1·0
Inorganic P	1·4
Glucose 6-phosphate	1·3
Fructose 6-phosphate	1·6
Fructose 1:6-diphosphate	3·0
Triose phosphates	1·8
Phosphoglycerate	1·0
Phosphoenolpyruvate	1·0
Adenosine triphosphate	0·17
Adenosine diphosphate	0·39
Adenosine 5′-phosphate	1·5
Ribose 5-phosphate	1·8

of evolution of CO_2 with extracts from TEM-inhibited and control cells are the same. Thus the activities of aldolase, triose phosphate and lactic acid dehydrogenases would appear to be unimpaired by the presence of TEM.

Attempts to reverse the inhibition of anaerobic glycolysis by TEM

The inhibited ascites-tumour cells were washed twice with Krebs–Ringer bicarbonate solution containing 0·4% glucose, and resuspended in this medium. Under anaerobic conditions there was now a low evolution of CO_2; that this was not due to glycolysis was shown by lactic acid estimations.

Addition of pyruvate, DPN or arsenate either at the beginning of the experiment or when inhibition was complete had no effect. There was a rapid evolution of CO_2 on addition of ATP to the inhibited cells which was unaffected by the presence of 4×10^{-3} M DL-glyceraldehyde (previously shown to inhibit anaerobic glycolysis in the ascites cells by 90%); the evolution of CO_2 was probably due to the presence of an active ATPase.

DPN concentrations in TEM-inhibited cells

The inability of pyruvate to reverse the inhibition despite the presence of excess triose phosphate and active triose phosphate and lactic acid dehydrogenases suggested that the cells were deficient in DPN. The DPN content of the cells was estimated just before the onset of inhibition, during increasing inhibition and just after the completion of inhibition by 0·01 M TEM in presence of 0·4% glucose. There was a rapid fall in DPN concentration from 83% of the control value at 70 min. to 14% at 90 min. after addition of TEM; inhibition of glycolysis was complete at approximately 80 min.

No DPN could be detected in the medium after inhibition by TEM.

These results may be compared with the rate of fall of DPN content of cells incubated at 38° under anaerobic conditions in the absence of glucose. The negligible evolution of CO_2 indicated that, as expected, glycolysis could not proceed under these conditions. The rate of fall of DPN concentration was considerably lower than that observed in the TEM-inhibited cells; after 80 min., the concentration had fallen only to 59% of the initial value.

Stability of DPN to the action of TEM

A solution of DPN (0·25 mg./ml.) was incubated at 38° with 0·01 M TEM in 0·01-Na_2HPO_4 buffer, pH 7·2, for 16 hr. The concentration of DPN as measured by the alcohol dehydrogenase method did not change over this period.

Effect of nicotinamide and related substances on the inhibition of anaerobic glycolysis by TEM

The evidence indicating that the inhibition was mediated by a fall in DPN concentration suggested that a study of the effect of nicotinamide on this system might be of value. The results obtained on adding nicotinamide (final concentration 0·01 M) at various times after the addition of TEM are shown in Fig. 3. Nicotinamide protects the system against the action of TEM and, further, it partially restores glycolysis even when added after inhibition is complete. This restoration could be demonstrated by addition of nicotinamide 78 min., but not 95 min., after complete inhibition. The protective effect falls off with time; in the presence of 0·001 M nicotinamide, added at the start of the experiment, the glycolytic rate fell gradually to 10% of the control value after 3 hr. As shown by titration, TEM did not react with nicotinamide when the two were incubated together at 38°.

A number of substances structurally related to nicotinamide were examined for their protective action against the inhibitory effect of TEM. Of these, none approached the efficiency of nicotinamide in protecting against TEM-inhibition, while only *iso*nicotinamide was able to restore glycolysis in inhibited cells, and that to a minor degree (Table 4). Typical curves are shown in Fig. 4.

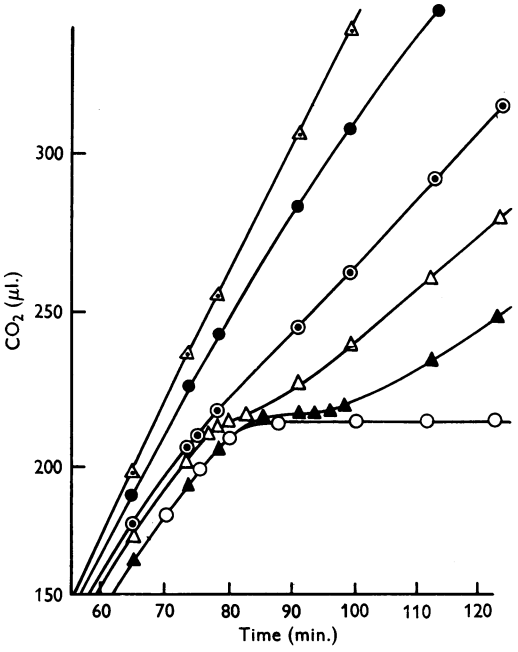

Fig. 3. Effect of 0·01 M nicotinamide on inhibition of anaerobic glycolysis of ascites-tumour cells by 0·01 M TEM. Each flask contained 0·04 ml. of packed cells suspended in 2 ml. of bicarbonate medium; TEM was present in all flasks other than the control. Nicotinamide was added at ●, 0 min.; ⊙, 75 min.; △, 80 min.; ▲, 94 min.; ○, no nicotinamide added; △, control.

TEM and the breakdown of DPN by DPNase

The presence of 0·01 M TEM did not affect the rate of breakdown of DPN by ascites-cell DPNase. When the breakdown was nearly complete, nicotinamide was added (final concentration 0·167 M). Further destruction of DPN was arrested but the concentration of DPN was not increased as might have been expected had a DPN analogue containing TEM been reversibly formed.

DISCUSSION

TEM resembles many of the related cytotoxic agents in its inhibitory action on respiration and anaerobic glycolysis in the tissues studied. Attempts

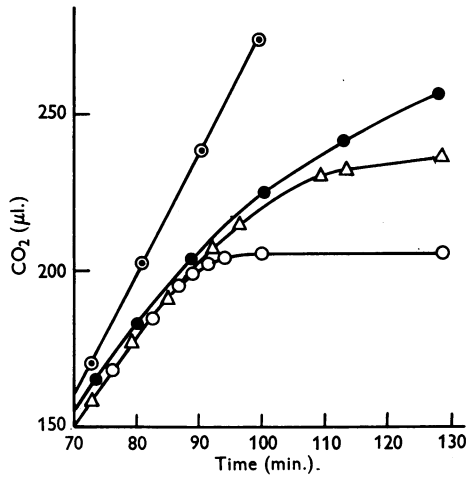

Fig. 4. Effect of *iso*nicotinamide and *iso*nicotinylhydrazide on inhibition of anaerobic glycolysis of ascites-tumour cells by TEM. Each flask contained 0·04 ml. of packed cells suspended in 2 ml. of bicarbonate medium. ●, 0·01 M TEM and 0·01 M *iso*nicotinamide; △, 0·01 M TEM and 0·01 M *iso*nicotinylhydrazide; ○, 0·01 M TEM; ⊙, control, without additions.

Table 4. *Effect of nicotinamide and related substances on the inhibition of anaerobic glycolysis in ascites-tumour cells by* 0·01 M *TEM*

Substance (0·01 M)	Protection against inhibition	Restoration of glycolysis	Reaction with DPN/DPNase*
Nicotinamide	+ + + +	+ + +	+
*iso*Nicotinamide	+ +	+	+
Nicotinylhydrazide	+	−	+
*iso*Nicotinylhydrazide	+	−	+
3-Acetylpyridine	+	−	+
Pyridine-3-nitrile	−	−	−
Nicotinic acid	−	−	−
Nicotinamide methiodide	−	−	−
Coramine	−	−	−
Nicotine	−	−	−
Pyridine	−	−	−

* + signifies ability to exchange with the nicotinamide moiety of DPN in the presence of DPNase. Data taken from Zatman, Kaplan, Colowick & Ciotti (1954).

to localize the site of inhibition by experiments with isolated enzyme preparations revealed that triose phosphate dehydrogenase was relatively stable to the action of TEM. This was unexpected in view of the finding that TEM reacts with the SH-groups of cysteine, glutathione and 2-aminoethanethiol (Roitt, 1954); presumably the SH in the glutathione prosthetic group was partially protected.

In contrast, phosphofructokinase activity was strongly inhibited by incubation with TEM, but analysis of the concentrations of phosphorylated intermediates present in ascites-tumour cells in which anaerobic glycolysis had been completely inhibited by TEM indicated that this enzyme was not blocked. These opposing results cannot be ascribed to the difference in origin of the enzymes since similar changes were observed in TEM-inhibited slices of rat-brain cortex, the tissue from which the isolated preparation was obtained.

The accumulation of both fructose diphosphate and triose phosphate suggests that the reaction affected by TEM is subsequent to the formation of triose phosphate in the breakdown of glucose. The greater accumulation of fructose diphosphate relative to triose phosphate may be connected with the equilibrium in an isolated aldolase system which favours the hexose ester. Similar concentration changes were reported by Holtzer, Haar & Schneider (1955) on inhibition of anaerobic glycolysis in ascites-tumour cells by either iodoacetate or fluoride.

Inhibition by TEM did not alter the concentration of the phosphoglycerate fraction or of phosphoenolpyruvate, suggesting that the reactions by which diphosphoglyceric acid is converted into pyruvate are unaffected. The analytical methods might not detect an accumulation of 1:3-diphosphoglyceric acid occurring at the expense of 2- and 3-phosphoglyceric acids, but the possibility that phosphoglycerokinase was blocked is unlikely since TEM inhibition was not influenced by the presence of arsenate, which permits glycolysis without participation of this enzyme.

Despite the unimpaired activity of triose phosphate and lactic acid dehydrogenases, and the presence of excess triose phosphate in the inhibited cells, the addition of pyruvate failed to restore glycolysis. It was concluded that the concentration of DPN must be inadequate to support glycolysis, and this was confirmed by analysis.

Concurrently with the inhibition of glycolysis the concentration of DPN fell very sharply. There remains the problem of whether the fall in concentration of DPN is the primary cause of glycolytic inhibition or whether some other factor is responsible for blocking glycolysis with consequent lowering in ATP concentration, and hence decreased synthesis of DPN. It was found that when glycolysis was arrested by incubating the cells in a medium devoid of glucose under anaerobic conditions, the rate of fall in concentration of DPN at no time approached that observed in the TEM-inhibited cells, and it must be concluded that the reduction in DPN concentration is probably the primary cause of glycolytic inhibition by TEM. The inhibition of respiration in ascites-tumour cells by TEM accords with this view; the endogenous substrate for the respiration of these cells is probably fat (Slechta, Jakubovic & Sorm, 1955), and DPN provides the common link between anaerobic glycolysis and aerobic catabolism of fat. Instances in which the DPN levels in certain tissues are decreased after the administration of growth inhibitors have been reported. Aminopterin treatment lowered the DPN concentration in rat liver (Strength & Mondy, 1953), while α-peltatin was shown to reduce the DPN concentration in the mouse Sarcoma 37 (Waravdekar *et al.* 1955).

That the inhibitory effect of TEM was mediated via DPNase was suggested by the findings that nicotinamide, 3-acetylpyridine and *iso*nicotinylhydrazide afforded protection against this inhibition in direct relation to their efficiency as DPNase inhibitors. Zatman *et al.* (1954) have shown that many DPNase inhibitors are able to exchange with the nicotinamide moiety of DPN in the presence of DPNase. The DPN analogues so formed usually inhibit the breakdown of DPN; the enzymes from some tissues, although catalysing the exchange, are not themselves inhibited by the analogues. Of the five substances studied which had been shown by Zatman *et al.* (1954) to interact with the DPN–DPNase system, all protected glycolysis against the action of TEM to some degree. Other pyridine derivatives having no effect on the breakdown of DPN by DPNase gave no protection against the action of TEM.

The mechanism by which TEM induces a much greater rate of breakdown of DPN than occurs by the action of DPNase in uninhibited cells remains obscure. There is little or no direct information on the activity of DPNase in intact cells, but it appears to be far less than that observed in disrupted cell preparations. Thus in the above experiments the rate of cleavage of DPN by the ascites-tumour-cell DPNase preparation was at least 10 times the rate observed in intact cells in which DPN synthesis was suppressed by failure of the exergonic reactions. A similar phenomenon was observed in brain slices and dispersions (Gore, Ibbot & McIlwain, 1950). The lower activity of the enzyme in intact cells may be due to (*a*) its localization in a relatively impermeable subcellular structure, (*b*) a low concentration of freely diffusible DPN, or (*c*) the presence of an inhibitor. TEM might act by increasing the accessibility of the enzyme to its substrate, by

increasing the concentration of DPN owing to displacement from combination with apoenzymes, or by reacting with the supposed inhibitor. It is of interest that DPNase activation has been invoked to account for the effect of *Veratrum* alkaloids on cerebral carbohydrate metabolism (Wollenberger, 1955); again, nicotinamide afforded protection against this effect.

Another possibility would be the interaction of TEM and DPN in the presence of DPNase to form a DPN analogue in which TEM replaced nicotinamide. The partial restoration of glycolysis by nicotinamide would then be understandable in terms of resynthesis of DPN by exchange with the TEM bound into the DPN analogue. If such an analogue is formed, the ribose must probably be bound to the nitrogen atom of an ethyleneimine rather than the triazine ring since ethyleneiminopropionitrile inhibits glycolysis in ascites-tumour cells in the same manner as TEM does, and here also the effect is reversed by nicotinamide.

No support for the hypothesis that a DPN analogue was formed could be adduced from experiments in which TEM was incubated with DPN in the presence of ascites-cell DPNase. The breakdown of DPN was unaffected by TEM and further, when the reaction was nearly complete, addition of nicotinamide did not alter the concentration of DPN; the presence of an analogue would have resulted in the resynthesis of DPN by reaction with nicotinamide.

Thus although TEM appears to inhibit glycolysis by increasing the rate at which DPN is broken down, the mechanism by which it does so is not yet known. The role of DPNase in cell metabolism is not fully understood, but the exchange reactions catalysed by the enzyme suggest that it might be implicated in the synthesis of nucleic acids. This would be of considerable interest in view of evidence for the blocking of nucleic acid synthesis by nitrogen mustards and certain other cytotoxic agents (Bodenstein, 1954; Skipper *et al.* 1951).

SUMMARY

1. 2:4:6-Triethyleneimino-1:3:5-triazine (TEM) inhibits respiration and glycolysis in mouse ascites-tumour cells and in a number of rat tissues.

2. Isolated preparations of hexokinase and triose phosphate dehydrogenase are only slightly affected by TEM, but phosphofructokinase is readily inhibited.

3. Fructose diphosphate and triose phosphate accumulate, while the DPN concentration falls sharply, concurrently with the onset of inhibition of anaerobic glycolysis in ascites-tumour cells by TEM.

4. Nicotinamide and a number of DPNase inhibitors protect against this inhibiting effect of TEM.

5. The inhibition is considered to be due to a rapid breakdown of DPN induced by TEM.

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