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BRAIN CARBOHYDRATE METABOLISM DURING HYDRAZINE TOXICITY

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Abstract—These studies have attempted to define the influences of hydrazine, 1-methylhydrazine (MMH), 1,2-dimethylhydrazine (SDMH), and 1,1-dimethylhydrazine (UDMH) on brain carbohydrate metabolism and to relate these effects to the hydrazines' toxicity. A positive correlation was found between the effects of these hydrazines on respiration in rat brain homogenates and their *in vivo* toxicity. Experiments with labeled glucose showed that MMH and SDMH, in 10-min incubations, increased the amount of $^{14}CO_2$ production from 1-^{14}C-glucose, presumably by stimulation of the pentose phosphate pathway. Production of $^{14}CO_2$ from 6-^{14}C-glucose was inhibited greatly by MMH. Excess lactate accumulated during MMH inhibition indicated an interference with pyruvate metabolism. Oxygen uptake could be restored by succinate and α-ketoglutarate but not by pyruvate. The effects of UDMH appear to be similar to those of MMH, whereas hydrazine causes little accumulation of lactate but appears to form a relatively stable complex with glucose, probably a hydrazone. These data indicate that part of the toxic effects of MMH and possibly UDMH may be related to their ability to inhibit pyruvate dehydrogenase (EC 1.2.4.1).

THE TOXIC effects of hydrazine and certain of its methyl derivatives are of great interest in modern technology because of their large-scale use as high-energy fuels. These hydrazines produce depressions, convulsions, and death by any mode of administration.[1, 2]

The convulsion that results from hydrazine toxicity can be prevented or counteracted by injections of pyridoxine,[3, 4] indicating an interference with pyridoxal-requiring enzyme systems; in fact, pyridoxal hydrazones do inhibit pyridoxal phosphokinase.[5]

Effects on central nervous system after sublethal doses of hydrazines persist for days. Both stimulation[1, 6] and depression[2] have been reported, which last for days, resulting in death in some cases as long as two weeks after exposure. The prolonged effects suggest that part of the toxicity of the hydrazines may be due to alterations in other metabolic pathways. Therefore, the purpose of the work presented here was to investigate the effects of hydrazines on brain carbohydrate metabolism. The studies are limited to consideration of rat brain carbohydrate metabolism *in vitro* and, for the most part, used homogenates.

MATERIALS AND METHODS

Sprague-Dawley female rats, weighing 200–250 g, were decapitated; their brains were removed and chilled immediately by submersion in ice-cold homogenizing fluid. In some experiments, the tissue was homogenized in 5 volumes of 0·25 M sucrose. In others, particularly those in which isotopes were used, the tissue was homogenized

in 2 volumes of Krebs–Ringer–glucose (1 mM) solution. Each flask contained from 100 to 250 mg tissue. Other constituents of the incubation mixture were: KCl, 0·1 M; $MgCl_2$, 0·0138 M; potassium phosphate, 0·05 M; EDTA, 0·001 M; potassium fumarate, 0·001 M; and glycylglycine, 0·0167 M. These components were mixed and their concentrations adjusted such that 2 ml of the mixture would give the indicated final concentration of each component in a total final volume of 3·0 ml. All incubations were carried out in a Warburg apparatus. The center well contained 0·1 ml of 20% KOH impregnated on a strip of filter paper. The flasks were equilibrated at 37° for 10 min, and the reaction was followed after the addition of homogenate from the side arm. The gas phase was air for all aerobic experiments, helium for the anaerobic isotope experiments, and a 95:5 mixture of nitrogen and carbon dioxide for the manometric anaerobic experiments.

In those experiments in which $^{14}CO_2$ production was determined, a volume of 0·3 ml of 6 N H_2SO_4 was added from a second side arm, and the flasks were shaken for 1 hr. This procedure forced the diffusion of any residual $^{14}CO_2$ trapped in the incubation mixtures to the KOH in the center wells. The KOH was transferred from the center wells of these flasks to the main compartments of other flasks which contained in their center wells 0·1 ml of an approximate 1 M solution of Hyamine in methanol and 0·5 ml of 6 N H_2SO_4 in their side arms. The Hyamine was adsorbed on a filter-paper strip to increase the absorption area and to facilitate subsequent transfers. After the flasks were capped, H_2SO_4 was added from the side arm, and the mixture was placed on a rotary shaker for 60 to 90 min. The Hyamine strips, containing 90–95 per cent of the $^{14}CO_2$, were transferred to counting vials. Ten ml of a scintillation fluid was added, which contained 0·4% 2,5-diphenyloxazole (PPO) and 0·01% 1,4-*bis*-2-(5-phenyloxazolyl)-benzene (POPOP) in toluene. Each sample was counted for 10 min in a Packard Tri-carb liquid scintillation counter and had an average statistical variation of 5 per cent or less. In short-term incubations, Hyamine-impregnated (instead of KOH) filter-paper strips were added to the center wells of the initial incubation flasks, thereby eliminating the necessity for subsequent transfer steps. Background radiation was determined for each experiment as described except that the initial incubation did not contain glucose-^{14}C.

Aliquots of the reaction mixtures were neutralized with KOH and subjected to descending paper chromatography on strips of Whatman 1 filter paper in a phenol:water:formic acid system (76:24:1). After about 20 hr, chromatograms were removed from the tanks and dried in a stream of air. Positions of radioactive spots were determined with a Vanguard Autoscanner 880 and were compared with those of unlabeled citrate, α-ketoglutarate, pyruvate, and oxalacetate. These unlabeled standards appeared as yellow spots on a blue background after the strips were sprayed with a 0·1% solution of bromophenol blue in 95% ethanol.

The 2,4-dinitrophenylhydrazone of pyruvate was prepared by standard procedure[7] and subjected to paper chromatography in *n*-butanol:ethanol:0·5 N NH_4OH (70:10:20).

Lactic acid was determined as described by Umbreit *et al.*[8]

Radioactive glucose was obtained from New England Nuclear Corp. The specific radioactivities expressed as mc/mmole were 3·05, 1·58, 1·93, and 1·57 for UL-^{14}C-glucose, 1-^{14}C-glucose, 2-^{14}C-glucose, and 6-^{14}C-glucose respectively. The hydrazines and other materials were obtained commercially also and were of high purity.

RESULTS

The compounds used in these studies were hydrazine, 1-methylhydrazine (MMH), 1,1-dimethylhydrazine (UDMH), and 1,2-dimethylhydrazine (SDMH). The first three are toxic with the LD_{50} expressed as moles per kilogram body weight (2×10^{-3}, 7×10^{-4}, and 2×10^{-3} respectively).[1] SDMH does not appear to be toxic in rats when given in amounts up to five times that shown here for UDMH. Because of this observation the data reported are based on control values obtained in the absence of any hydrazines to determine overall effects of the various hydrazines on carbohydrate metabolism. On the other hand, only those values that are significantly different for hydrazine, MMH, and UDMH as compared with the apparently nontoxic SDMH are considered in terms of defining the causes for toxicity of hydrazines. This assumption ignores the possibility that the *in vivo* difference could be a function of penetration, rapid metabolism by tissues other than brain, or excretion of SDMH.

Anaerobic glycolysis

When rat brain homogenates are incubated with glucose and each of the hydrazines, both oxygen uptake and lactate production are inhibited in an analogous manner. Hydrazine, MMH, SDMH, and UDMH inhibit CO_2 production by 67, 65, 78, and 54 per cent respectively. Similarly, lactate production is inhibited by 67, 75, 78 ,and 32 per cent respectively. Of particular interest is the observation that SDMH, which is the least toxic hydrazine, inhibits glycolysis most. These data indicate that the observed effects on this system probably are not related to toxicity of the hydrazines.

Aerobic catabolism

Table 1 shows the effects of the hydrazines on oxygen uptake: the overall inhibition of oxygen uptake roughly parallels the relative toxicity of the hydrazines; i.e. SDMH shows no inhibition, MMH shows most inhibition, and hydrazine and UDMH show inhibition between these values. These data also indicate that the observed effects on aerobic metabolism might be related to the hydrazines' toxicity.

TABLE 1. INHIBITION OF OXYGEN UPTAKE BY HYDRAZINES*

	Oxygen uptake (μl/hr)	Inhibition (%)
Control	64 ± 4·5 (54–68)	0
Hydrazine	27 ± 4·7 (22–34)	58
MMH	18 ± 4·0 (13–25)	72
UDMH	38 ± 11·8 (31–58)	41
SDMH	68 ± 15·3 (57–83)	−6

* Each flask contained 143 mg of brain tissue that had been homogenized in 0·25 M sucrose containing 0·001 M ethylenediamine tetraacetate, 54 mM glucose, and, where indicated, 30 mM hydrazines were included in the incubation mixture. All other components are as outlined under Materials and Methods. These data are an average of 16 values ± S.D.; ranges in parentheses.

Studies with radiolabeled glucose

Brain tissue respires almost exclusively at the expense of glucose, and under *in vitro* and aerobic conditions, glucose disappearance approximately equals that expected from oxygen consumption and lactic acid formation.[9, 10] It appeared that

information could be obtained relative to which pathway was most affected by the hydrazines by measuring $^{14}CO_2$ production from specifically labeled glucose. Because the extent of MMH inhibition is progressive with time and concentration (Fig. 1), 10 or 30 mM concentrations were mixed with the homogenate in the side arm. This allowed a preincubation period of 10 to 15 min before addition to the main compartment. Data obtained with the labeled glucose substrates and homogenates treated as

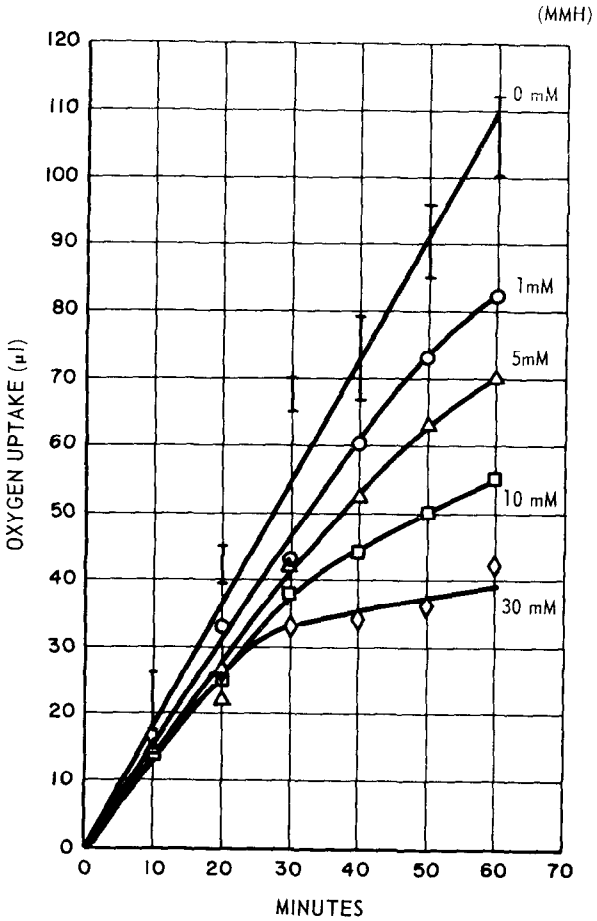

FIG. 1. Inhibition of oxygen uptake in brain homogenates by increasing concentrations of MMH. Variations in the range of values are indicated on the graph for the experiment containing no MMH. The average of the mean deviation in all other cases was ± 5·7 per cent with an average range of 3·7–8·2 per cent. The points on the curves for each concentration of MMH represent the average of duplicates. Other experiments have shown the same qualitative picture.

just described with MMH and SDMH are shown in Table 2. When these data are compared with those obtained for oxygen uptake (Table 1), it can be seen that: (1) inhibition of $^{14}CO_2$ production from 6-^{14}C-glucose in the presence of the hydrazines roughly parallels that seen with overall oxygen uptake; (2) less inhibition is observed with 1-^{14}C-glucose than with 6-^{14}C-glucose, indicating that the predominant effects of these hydrazines on $^{14}CO_2$ production were on the citric acid (TCA) cycle.

The observation that relatively strong inhibition of $^{14}CO_2$ production from 2-^{14}C-glucose occurred is consistent with this interpretation.

To gain more information about the nature of the observed inhibition and to determine which pathway was affected initially and most under the influence of the hydrazines, experiments similar to those just described were carried out for 10 min, with only the more toxic MMH and the apparently nontoxic SDMH. Results are

TABLE 2. EFFECTS OF HYDRAZINES ON $^{14}CO_2$ PRODUCTION FROM LABELED GLUCOSE*

	UL-^{14}C-Glucose	1-^{14}C-Glucose	2-^{14}C-Glucose	6-^{14}C-Glucose
Control	224 ± 47 (157–281)	110 ± 4 (107–114)	64 ± 17 (43–82)	54 ± 19 (38–86)
Hydrazine	103 ± 38 (75–155)	60 ± 12 (48–74)	34 ± 8 (22–41)	12 ± 7 (2–21)
MMH	94 ± 15 (74–116)	71 ± 9 (59–82)	10 ± 7 (2–17)	9 ± 7 (3–19)
UDMH	258 ± 7 (251–267)	103 ± 9 (95–115)	13 ± 10 (2–28)	21 ± 10 (10–34)
SDMH	251 ± 28 (211–272)	146 ± 23 (122–178)	76 ± 17 (55–97)	63 ± 18 (39–82)

* Conditions are the same as for Table 1 except that 275,000 counts/min of labeled glucose was added. Incubation time was 1 hr. Results are expressed as counts/min ± S.D.; range in parentheses.

shown in Table 3. All values are corrected to an equivalent initial specific radioactivity for glucose. These data confirm the observation above that MMH inhibits markedly $^{14}CO_2$ production from 6-^{14}C-glucose. In contrast to the above data, however, is the observation that $^{14}CO_2$ production from 2-^{14}C-glucose is inhibited to a lesser extent, whereas that from 1-^{14}C-glucose appears to be stimulated. SDMH inhibitory effects on $^{14}CO_2$ from 6-^{14}C-glucose are similar but not as marked as those of MMH. On the other hand, it appears to stimulate C-1 oxidation more than MMH. Apparently there is no direct oxidation of C-6 of glucose as compared to C-1 (Table 4). Therefore, it is valid to assume that all $^{14}CO_2$ appearing from the C-6 position of glucose comes by way of the TCA cycle.

The data accumulated so far indicate that there may be some relationship between the *in vivo* toxicity of these hydrazines and their effects on *in vitro* carbohydrate catabolism. These data with respect to oxygen uptake and CO_2 production from the specifically labeled glucoses indicate that the unfavorable hydrazine effects on carbohydrate catabolism may be on the TCA cycle.

Localization of the sites of MMH action

Aliquants of the incubation mixture were subjected to paper chromatography, based on the assumption that an accumulation of labeled intermediates indicative of specific enzyme(s) inhibition would be revealed. However, it was important to show first that the observed effects were not due to a simple removal of glucose by hydrazone formation. To do this, labeled glucose and an equivalent amount of each of the four hydrazines, after a 10-min incubation, were subjected to paper chromatography in the phenol system described above. Autoradiographic scanning of the paper strips showed no apparent reaction between any of the hydrazines and glucose except hydrazine itself. In the case of hydrazine there was an additional radioactive area

TABLE 3. PRODUCTION OF $^{14}CO_2$ FROM SPECIFICALLY LABELED GLUCOSE IN THE PRESENCE OF SDMH AND MMH*

	UL-^{14}C-Glucose		1-^{14}C-Glucose		2-^{14}C-Glucose		6-^{14}C-Glucose	
	(cpm)	(% control)	(cpm)	(% control)	(cpm)	(% control)	(cpm)	(% control)
Control	37,200 ± 9·8%	100	8,670 ± 4·7%	100	7,360 ± 8·0%	100	4,125 ± 4·1%	100
SDMH	39,750 ± 0·8%	107	30,400 ± 4·7%	351	6,340 ± 2·1%	86	2,310 ± 4·8%	56
MMH	26,750 ± 4·3%	72	11,860 ± 4·7%	137	4,940 ± 3·2%	67	1,525 ± 3·8%	37

* Conditions were as outlined under Materials and Methods. Total glucose concentration was 1 mM including 50 μg labeled glucose. The incubation time was 10 min. A total of 200 mg tissue homogenized in Krebs–Ringer–Tris buffer was used. Each value represents a minimum of four separate determinations; the mean per cent deviation is shown for each of these.

with an R_f of approximately 0·3, which contained about 40% of the label. The R_f of glucose in this system is 0·42.

When incubation mixtures similar to those of Table 3 were subjected to paper chromatography as outlined, two major radioactive components were observed in the flask containing MMH. One had an R_f of 0·42, corresponding to that of glucose in this

TABLE 4. ANAEROBIC $^{14}CO_2$ PRODUCTION FROM C-1 AND C-6 OF GLUCOSE: EFFECT OF PYRIDINE NUCLEOTIDE COENZYMES*

System	(cpm)
1-^{14}C-Glucose	
Control	282 ± 55 (5)
Control + NADP	3,042 ± 606 (6)
6-^{14}C-Glucose	
Control	94 ± 26 (6)
Control + NAD + NADPH	75 ± 42 (7)

* Experimental conditions are the same as Table 3 with the exception that helium was the gas phase. Where indicated, 1 mM pyridine nucleotide was added. The figures in parentheses represent the number of determinations.

system or to α-ketoglutarate, which has approximately the same R_f value. The second major peak had an R_f of 0·73, which is approximately that of lactate and pyruvate. Only one of the labeled spots was observed in the control flask and in those treated with SDMH. This material had an R_f of 0·42 and undoubtedly was unmetabolized glucose. It should be pointed out here that the concentration of glucose in the system was of the order of 10^{-3} M, and that at this concentration oxygen uptake is the same as with higher glucose concentrations.[11]

To determine whether the second radioactive spot could be lactate or pyruvate, the following experiment was done: 1 mg lactate was added to one half of a deproteinized incubation mixture, and 1 mg pyruvate was added to the other half. The 2,4-dinitrophenylhydrazone of pyruvate was prepared from the mixture to which pyruvate was added. Chromatography of this phenylhydrazine (R_f = 0·46 in a butanol:ethanol-ammonium hydroxide system) showed it to be completely devoid of radioactivity, indicating that pyruvate was not the material accumulating under MMH inhibition. NAD and lactic dehydrogenase were added to the half containing lactate. After a few minutes' incubation, 2,4-dinitrophenylhydrazone was added to this fraction also. The resulting 2,4-dinitrophenylhydrazone contained considerable radioactivity, thus showing that MMH inhibition of oxygen uptake is accompanied by an increase in lactic acid. Had labeled α-ketoglutarate or oxalacetate been present, they too would have been detected by this procedure. Their phenylhydrazones in this solvent system have R_f values, of 0·12 and 0·24 respectively.

The data presented indicate an inhibition of glucose metabolism by MMH, possibly at the level of pyruvate oxidation. To determine if the other hydrazines caused a similar increase in lactic acid, experiments similar to those described above for MMH and SDMH were done with UL-^{14}C-glucose as substrate. Table 5 shows the relative $^{14}CO_2$ production in the presence of the various hydrazines. Autoradiographic patterns for UDMH and hydrazine indicate that to some small extent UDMH and hydrazine

were capable of producing the same effects as MMH but that the predominant effect of hydrazine was effective removal of glucose as a substrate.

If the predominant effect of MMH on oxygen uptake is to prevent pyruvate oxidation by the TCA cycle at the pyruvic dehydrogenase step, then the addition of substrates of the TCA cycle after MMH inhibition should restore oxygen uptake to its

TABLE 5. EFFECTS OF HYDRAZINES ON $^{14}CO_2$ PRODUCTION FROM UL-^{14}C-GLUCOSE

	(cpm)	Inhibition (%)
Control	17,945 ± 1·5%	0
SDMH	17,792 ± 2·4%	1·0
UDMH	17,654 ± 11·3%	1·7
MMH	8,789 ± 7·8%	51·1
Hydrazine	6,712 ± 1·6%	62·6

* See Table 3 for conditions. The hydrazine concentration was 60 mM.

TABLE 6. REVERSAL OF MMH INHIBITION BY TCA CYCLE INTERMEDIATES*

Substrate	Additions	
	None	MMH
Glucose	63·0 ± 8·9%	29·4 ± 7·7%
Glucose + pyruvate	74·0 ± 13·8%	34·0 ± 10·3%
Glucose + α-ketoglutarate	79·0 ± 2·5%	60·6 ± 8·3%
Glucose + succinate	130·8 ± 0·9%	104·0 ± 10·6%

* Oxygen uptake was observed for 1 hr (expressed as μliters) in the presence of the indicated substrates under control conditions (column 2) and after inhibition by 10 mM MMH (column 3).

original rate. That this is true may be seen from the data of Table 6. Succinate restores oxygen uptake to about 80% and α-ketoglutarate to about 72 per cent of its control value. As expected, pyruvate was completely ineffective.

DISCUSSION

Although the pentose phosphate pathway is not believed to play a major role in brain carbohydrate catabolism, drugs and other foreign materials have shown that it has an important role in brain metabolism.[12, 13] This has been shown to be true here when brain tissue is exposed to some hydrazines.

It has been assumed that virtually all $^{14}CO_2$ produced by brain tissue from 1-^{14}C and 6-^{14}C-glucose represents oxidation via the Embden-Meyerhof and TCA-cycles.[11, 12, 14–17] This assumption was based on C-1/C-6 ratios close to 1 for $^{14}CO_2$ production from these two compounds. The fact that the ratios observed here are significantly different from 1 indicates that there must be some direct oxidation of C-1 of glucose by the pentose phosphate pathway even under controlled conditions and that the relative yield of $^{14}CO_2$ from C-1 glucose is influenced by the hydrazines. If only the Embden-Meyerhof and pentose phosphate pathways operate in brain, and if $^{14}CO_2$ from UL-^{14}C-glucose is used as a basis for calculating CO_2 yields from C-1 and C-6 positions of glucose, it can be estimated that 5–12 per cent of the $^{14}CO_2$

produced in controls come from operation of the pentose phosphate pathway. In the 10-min experiments, up to 58 per cent appears to come from the pentose phosphate pathway under the influence of SDMH and 12 per cent under the influence of MMH. These data have been calculated using formula 3 of Katz and Wood.[18] The assumption that $^{14}CO_2$ yields can be calculated by the procedure used here as opposed to that used by Katz and Wood[18] is not necessarily valid since glucose may be utilized in brain homogenates by pathways other than those considered and since the total amount of glucose carbons appearing as CO_2 probably does not reflect total glucose utilization. Moreover, none of the data accumulated would allow an estimation of either the rate of hexose isomerization or the distribution of label in glycogen or glycerol.[19] Therefore, these numbers are overestimations[18] of the per cent pentose phosphate pathway. Nevertheless, the conclusion drawn here, viz., that the hydrazines do affect differently the pathways of glucose metabolism, is valid. Although the calculated changes are not quantitative they are of value in accessing relative effects of these agents in this system.

At high concentrations, SDMH is a relatively good inhibitor of anaerobic glycolysis. Yet, under aerobic conditions total CO_2 formation is not appreciably altered. The fact that this hydrazine stimulates the pentose phosphate pathway aerobically indicates that NADP is not limiting, possibly because of its reoxidation by glutathione reductase[20] or through transhydrogenases.[21] The marked stimulation of this pathway would yield more triose phosphate for oxidation through the TCA cycle. Therefore, the reduced glycolysis caused by SDMH could be overcome, and the overall energy yield might not be affected significantly.

MMH, in contrast to SDMH, has an influence on oxygen uptake, Therefore, its net effects would be quite different. Short-term experiments indicate some stimulation of the pentose phosphate pathway. This stimulation does not persist, and after 1-hr incubation, no net stimulation could be demonstrated. According to the data shown in Table 6, accumulation of triose phosphates by some other pathway would be of no consequence in restoring oxidative metabolism, since the TCA cycle is blocked at the initial step, presumably pyruvate dehydrogenase. Succinate and α-ketoglutarate are capable of restoring oxygen uptake to nearly its normal rate. On the other hand, pyruvate is completely without effect on MMH-inhibited oxygen uptake. The observation that neither succinate nor α-ketoglutarate will completely restore the rate of oxygen utilization to that seen with these substrates in the complete absence of MMH is probably significant and may represent some inhibition of other enzymes within the TCA cycle.

It is interesting to note, however, that α-ketoglutarate dehydrogenase (EC 1.2.4.2) catalyzes a reaction very similar to that of pyruvate dehydrogenase and these two enzyme complexes have the same general properties,[22] but the α-ketoglutarate dehydrogenase did not appear to be affected appreciably by the hydrazines. Also, there was no evidence to indicate that the hydrazines had any significant effects on transketolase (EC 2.2.1.1). The one thing that all of these enzymes have in common is thiamine pyrophosphate as a prosthetic group.[23, 24] Although such be the case, one appears to be affected markedly by MMH and the others apparently are not. Thus, the possibility exists that MMH binds some group on the enzyme and interferes with pyruvate oxidation directly or causes a dissociation of the thiamine pyrophosphate prosthetic group.

Hydrazine appears to inhibit glucose utilization by complex formation with glucose itself, as contrasted with both MMH and UDMH. It is possible, however, that hydrazine too inhibits the same reactions as MMH but to a lesser extent and after a longer incubation time. In this regard MMH requires about 25 min before marked effects on oxygen uptake can be demonstrated (Fig. 1), but because of the relative potency of its action some of these products accumulate within the first few minutes.

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