NONENZYMIC FORMATION OF TOXIC LEVELS OF METHYLGLYOXAL FROM GLYCEROL AND DIHYDROXYACETONE IN RINGER'S PHOSPHATE SUSPENSIONS OF AVIAN SPERMATOZOA

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Summary: Several recent papers have reported evidence of methylglyoxal as an authentic intermediate of the metabolism of glycerol via a glycolytic bypass mechanism operable in a variety of bacteria. Certain mutant strains of E. coli produced bacteriocidal levels of this α-ketoaldehyde. Similar observations are now reported of lethal accumulations of methylglyoxal in Ringer's phosphate suspensions of avian spermatozoa in the presence of glycerol and/or dihydroxyacetone. Glycerol is metabolically converted to dihydroxyacetone and this triose is nonenzymically converted to methylglyoxal, the reaction being catalyzed principally by inorganic phosphate. Whether or not lethal levels of methylglyoxal accumulate in such a system depends upon a delicate dynamic balance of interacting factors involved in the formation and further conversion of this α-ketoaldehyde. The existence of two established mechanisms for conversions of glycerol to methylglyoxal in biological systems must be considered when methylglyoxal appears as a product.

Interest in methylglyoxal as a possible intermediate of glucose catabolism developed about 1913 with the discovery of the glyoxalase enzyme system which converts this α-ketoaldehyde to D-lactate (1,2). During the ensuing 50 years, methylglyoxal was finally concluded to be a nonenzymic artifact of glycolysis rather than an authentic intermediate (3). Recently, however, several investigators (4,5,6) have provided quite convincing evidence that this compound may indeed be an intermediate in a glycolytic bypass mechanism active in a variety of bacteria. Part of the emphasis for this renewed interest in methylglyoxal arose from observations of the production of a bacteriocidal product elaborated by certain mutant strains of E. coli when exposed to glycerol (6,7).

Without discounting the validity of these findings, but to stress the general need for caution in interpreting observations of glycerol-induced

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lethal effects associated with the appearance of methylglyoxal in biological systems, we report examples of similar phenomena observed in incubations of avian spermatozoa in the presence of phosphate ion and glycerol or dihydroxyacetone. The mechanism of methylglyoxal formation in these systems based on prima facie evidence (8) is one of a nonenzymic, phosphate-catalyzed conversion of exogenous triose or of endogenously-produced dihydroxyacetone to methylglyoxal.

MATERIALS AND METHODS. Fowl semen from white leghorns was pooled and prepared for use within one hour of collection. Storage during the interim period was at 15°C. After diluting 4-fold with warm Ringer's phosphate diluent (8), the suspension was centrifuged for 10 minutes at 1500 g. The supernatant plasma was decanted off and the cells resuspended in diluent. Sperm cell concentrations were estimated by the method of Carson, et al. (9).

Manometric measurements were made with an Aminco Warburg apparatus, Model 5-133, using 15 ml single arm flasks. Substrates were added directly to the contents of the main well or were present initially in the side arms. In all cases the final volume of incubation mixtures was 2.0 ml, with center wells containing 0.1 ml of 25% KOH. Incubations were at 39°, with air as the gaseous phase.

Following manometric measurements, mixtures were prepared for assay by denaturing 1.0 ml portions with 1.5 ml each of 2% $ZnSO_4$ and 0.4% NaOH, centrifuging and removing the supernatant solutions.

Dihydroxyacetone was measured by the Nelson method for total reducing sugars (10) or by conversion to methylglyoxal (8). Methylglyoxal was measured by a modification of the method of Vogt (11), see (8).

RESULTS.

1. Inhibition of respiration in the presence of glycerol.

Incubation mixtures contained 2.4×10^9 washed cells. Substrates when present were glucose, 2.8 mM or glycerol, 2.8, 14, or 28 mM. Respiration rates per se are influenced by the nature of substrate. In the absence

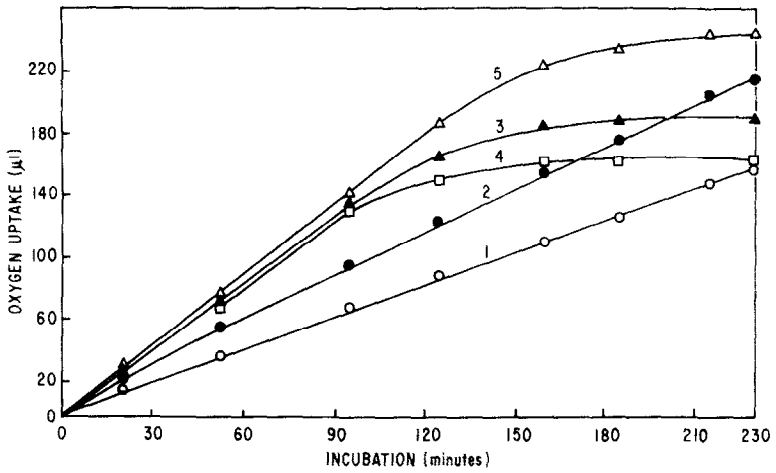

Figure 1. Inhibition of respiration in the presence of glycerol.

Incubation mixtures contained 2.4 x 10^9 washed cells in 2.0 ml final volume. Incubation was at 39°. Substrates were present as follows: (1), none; (2), glucose, 2.8 mM; (3), glycerol, 2.8 mM; (4), glycerol 14 mM; (5), glycerol, 28 mM.

See text for discussion.

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of added substrate, endogenous energy sources are metabolized by the spermatozoa. Glucose enhances the basal respiration rate, while glycerol, due to its more reduced state, induces a yet higher rate of respiration. Therefore inhibition of respiration is indicated by changes in slopes of plotted curves, Figure 1, rather than by relative oxygen consumption at any given time. With no substrate or with glucose, oxygen consumption was linear with time throughout the incubation period. With the three levels of glycerol, the initial respiration rates were linear and essentially independent of concentration, with inhibition becoming apparent after about 90 minutes. In this and all related experiments, the observed inhibition of respiration was directly associated with an irreversible loss of cell viability. Methylglyoxal was not measured following this run, as this compound was not recognized at the time as a factor associated with the inhibition. The incubations with glycerol accumulated 0.7 to 1.6 mM dihydroxyacetone by the end of the run.

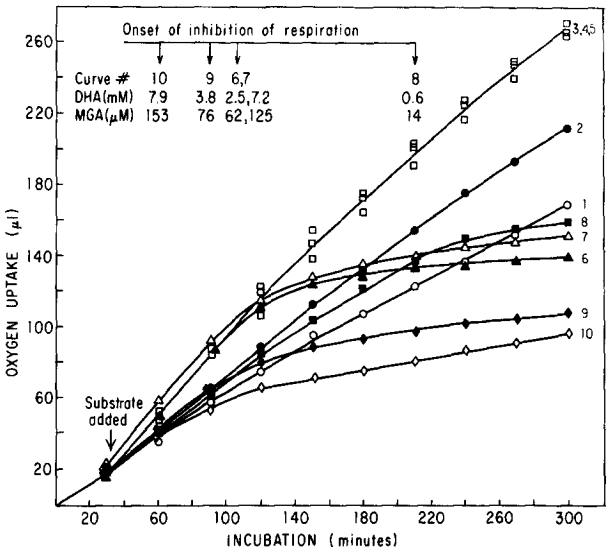

Figure 2. Inhibition of respiration in the presence of glycerol and dihydroxyacetone.

Details of preparation and incubation are provided in Table 1. Curves correspond to flask numbers, with a mean curve drawn for glycerol-containing flasks 3, 4 and 5. Arrows indicate approximate times of onset of inhibition of respiration and the relationship to final concentrations of dihydroxyacetone and methylglyoxal. See Table 1 and text for discussion.

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2. Methylglyoxal formation and inhibition of respiration.

Incubations were prepared as described in Table 1, and final assay results are given. Figure 2 plots oxygen consumption during the incubation. Essentially linear respiration rates were obtained without added substrate or with glucose present. In this run, glycerol alone did not result in detectable inhibition of respiration, although the onset of inhibition at the low level of dihydroxyacetone was markedly enhanced by the presence of glycerol (cf. curves 6 and 8). At the higher level of dihydroxyacetone, added glycerol had little effect (curves 7 and 9) on time of onset of inhibition, undoubtedly because the already high levels of added dihydroxyacetone masked any contribution from conversion of glycerol to this triose and thence to methylglyoxal.

Methylglyoxal was found in the incubated mixtures. For those containing dihydroxyacetone alone, there was a rough correlation between final

Table 1

Metabolism of glycerol and dihydroxyacetone by spermatozoa

Incubation mixtures contained 1.8×10^9 washed spermatozoa. Substrates were present in side arms of Warburg flasks and were added following 30 minutes of pre-incubation at 39°. Total incubation time was 5 hours. No inhibition of respiration was evident when glycerol was the sole substrate. All levels of dihydroxyacetone lead to inhibition with the time of its expression roughly related to the initial and final levels of dihydroxyacetone and to methylglyoxal present at the end of the incubation period. Glycerol accentuated the onset of inhibition produced in the presence of dihydroxyacetone (cf. curves 6 and 8, Figure 2).

	Substrates, mM			Results					
Flask	Glucose	Glycerol	DHA	Final pH	Oxygen uptake (ul)	DHA[a] present mM	Methylglyoxal formed uM	Onset of inhibition, minutes	Motility[b] %
1				7.30	170	0	0	none	5
2	5.5			6.90	213	-	0	none	10
3		7.0		6.80	266	(0.7)	21	none	5
4		14.0		6.75	265	(1.1)	28	none	5
5		21.0		6.75	273	(1.1)	35	none	5
6		14.0	2.8	7.05	143	2.5	62	110	0
7		14.0	8.4	7.05	155	7.2	125	110	0
8			2.8	7.20	161	0.6	14	210	0
9			8.4	7.00	109	3.8	76	90	0
10			14.0	7.00	99	7.9	153	60	0

[a] parentheses indicate net DHA formed from glycerol

[b] % of cells displaying active motility

methylglyoxal concentrations and extent of inhibition, which in turn correlated with both initial and final dihydroxyacetone concentrations. The action of glycerol in enhancing the inhibitory effects observed when dihydroxyacetone is initially present appears due to the greater accumulation of methylglyoxal when both substrates are present.

DISCUSSION.

It could be argued from the data presented that the correlation between onset of inhibition of respiration and dihydroxyacetone concentra-

tion indicates that dihydroxyacetone as much as methylglyoxal may be the inhibitory agent. The arguments opposing this view are several-fold. One is the fact that even with high levels of dihydroxyacetone, inhibition does not become apparent for one-half hour or longer, suggesting that dihydroxyacetone is not the inhibitor per se but rather that it is a precursor of an inhibitory substance. Also the inhibition observed in the presence of glycerol is not a regularly occurring event, yet dihydroxyacetone routinely appears as a metabolite of glycerol utilization. Dihydroxyacetone, present as a substrate, leads more frequently than glycerol to cell toxicity, yet dihydroxyacetone is readily metabolized and inhibition by this triose is unpredictable. Whenever glycerol or dihydroxyacetone induced toxic effects in sperm cell incubations, methylglyoxal, when sought, was found to be present. Conversely, little or no methylglyoxal has been found in the absence of observable inhibition of respiration. Methylglyoxal is known to be a potent metabolic inhibitor, whereas dihydroxyacetone is not. Phosphate-catalyzed conversion of trioses to methylglyoxal has been demonstrated (8).

A postulated dynamic chemical-biochemical mechanism consistent with established metabolic pathways of carbohydrate metabolism in spermatozoa (12) which accounts for the reported phenomena is diagrammed in Figure 3. Glycerol enters the metabolic pathway as shown and is converted to dihydroxyacetone phosphate. Dihydroxyacetone is also readily metabolized via phosphorylation. Accumulation of dihydroxyacetone in the medium, when glycerol is the substrate, is undoubtedly due to the action of phosphatases on the phosphorylated triose (13). When glycerol phosphate is oxidized to dihydroxyacetone phosphate, NAD levels within the cells may be reduced with resulting decreased activity of the 3-carbon pathway to L-lactate. Under such conditions, dihydroxyacetone phosphate and resulting dihydroxyacetone may accumulate to a greater extent. The additive inhibitory effects when both glycerol and dihydroxyacetone are present is due to the maintenance of a higher level of dihydroxyacetone than when either substrate is added

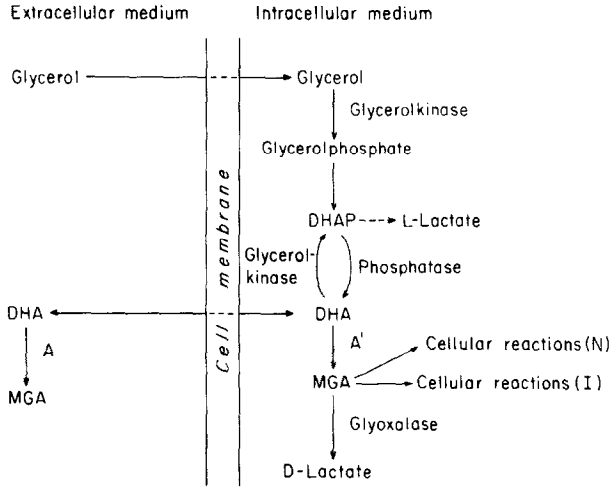

Figure 3. Chemical-biochemical interactions at the 3-carbon level of glycolysis.

Chemical and biochemical interactions are shown involving metabolism of glycerol and dihydroxyacetone, metabolic formation of dihydroxyacetone from glycerol, chemically-catalyzed formation of methylglyoxal from dihydroxyacetone, enzymic conversion of methylglyoxal to D-lactate, and cellular reactions involving accumulated methylglyoxal. Whether or not metabolic inhibition occurs when dihydroxyacetone or glycerol are substrates is dependent upon dynamic interactions of all of the factors involved in methylglyoxal formation and subsequent reactions. Abbreviations are: A, extracellular catalytic anions; A', intracellular catalytic anions; DHA, dihydroxyacetone; MGA, methylglyoxal; DHAP, dihydroxyacetone phosphate; (N), non-inhibitory; (I), inhibitory.

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alone. Methylglyoxal, nonenzymically produced from dihydroxyacetone, is converted in part to D-lactate (3), reacts with nonmetabolically active sulfhydryl groups (14) of the cells, and reacts with sulfhydryl-containing enzymes (15). The extent to which methylglyoxal accumulates and acts as a lethal metabolite of glycerol or dihydroxyacetone depends upon a delicate dynamic balance existing among these interacting metabolic and chemical factors involved in its formation and subsequent reactions.

REFERENCES

1. Dakin, H. D., and Dudley, H. W., J. Biol. Chem. 14, 423 (1913).
2. Neuberg, C., Biochem. Z. , 49, 502 (1913).
3. Racker, E., in S. Colowick, A. Lazarow, E. Racker, D. R. Schwartz, E. Stadtman and H. Waelch (Editors), Glutathione, Academic Press, New York, 171 (1954).
4. Cooper, R. A., and Anderson, A., FEBS Letters, 11, 273 (1970).

5. Hopper, D. J., and Cooper, R. A., FEBS Letters, 13, 213 (1971).
6. Freedberg, W. B., Kistler, W. S., and Lin, E. C. C., J. Bacteriol., 108, 137 (1971).
7. Zwaig, N., and Dieguez, E., Biochem. Biophys. Res. Comm., 40, 1415 (1970).
8. Riddle, V., and Lorenz, F. W., J. Biol. Chem., 243, 2718 (1968).
9. Carson, J. D., Lorenz, F. W., and Asmundson, V. S., Poult. Sci., 34, 336 (1955).
10. Nelson, N., J. Biol. Chem., 153, 375 (1944).
11. Vogt, M., Biochem. Z., 211, 17 (1929).
12. Mann, T., The Biochemistry of Semen and of the Male Reproductive Tract, John Wiley and Sons, New York (1954).
13. Wilcox, F. H., J. Reprod. Fertil., 2, 148 (1961).
14. Schubert, M. P., J. Biol. Chem., 111, 671 (1935).
15. Kun, E., J. Biol. Chem., 187, 289 (1950).