*CELL DIVISION, SH, KETOALDEHYDES, AND CANCER**

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Communicated December 17, 1965

When single cells joined to form multicellular organisms, they had to resign their unlimited proliferation. This they had to do reversibly since, in regeneration and wound healing, they might be called upon to resume proliferation. Cell division involves a great number of single processes which all have to be stopped or started in these changes simultaneously. This seems possible if these reactions had some common denominator.

It is known that SH groups are involved in cell division.[1] If these are blocked, no cell division can take place. Thio groups participate in a great number of different enzymic reactions which opens the possibility that they may be the postulated common denominator.

The literature contains various, more or less, accidental observations on acceleration or inhibition of cell division by tissue extracts. Such reactions were observed also in this laboratory.[2] The substance responsible for the retardation was tentatively called "retine," its antagonist, "promine." Such observations can become valuable through the isolation and identification of the active agent or a better understanding of the underlying mechanisms. Thus, the final isolation of retine is strived for.

Experience with partially purified preparations led to the conclusion that the substance responsible for the inhibition is a methylglyoxal derivative.[3] Methylglyoxal, as all α-ketoaldehydes, readily interacts with SH[4] and NH_2 groups forming addition products. While its reaction with SH may account for the inhibitory effect of retine on cell division, its general reactivity may account for the difficulties of its isolation. Moreover, most cells contain a very active enzymic system, consisting of glutathione, glyoxalase I and II, which system readily transforms methylglyoxal into lactic acid and methylglyoxal derivatives into the corresponding α-hydroxy acid. This enzymic system could be involved in the regulation of cell division releasing the cell from the inhibitory action of retine. At the same time, it may also be responsible for part of the difficulties of its isolation.

Until retine is isolated and its constitution is determined, the conclusion of its being a methylglyoxal derivative cannot be accepted as final. This made us collect data about the biological reactions of α-ketoaldehydes, which could serve as circumstantial evidence for or against our assumption and may facilitate isolation.

If α-ketoaldehydes interact with SH, and SH groups are involved in cell division, then we can expect to find cell division to be inhibited by relatively low concentrations of these substances. For this reason we studied the action of methylglyoxal and some of its derivatives on the division of bacteria, fertilized sea urchin eggs, seeds, flagellates, and cells of tissue cultures. We found in all cases cell division inhibited by similar, relatively low concentrations in a more or less specific manner. This paper deals with the proliferation of bacteria.

Sulphur is one of the most versatile atoms, and SH groups linked to different molecules or macromolecules may have very different reactivities even within one and the same molecule. Muscle contraction, which also depends on SH, is not inhibited by concentrations of methylglyoxal ten times higher than those needed to stop cell proliferation. We also found some specificity in the reactions of the various methylglyoxal derivatives, methylglyoxal acting somewhat stronger on bacteria than on cells of tissue cultures, while the reverse was true of Kethoxal[R]. Normal cells seemed to be less sensitive than cancer cells (HeLa, KB), so ways might be found which would allow inactivating SH-s involved in cell division in a specific way, perhaps opening the way to a specific cancer therapy. It may be more than a coincidence that several antibacterial, antiviral, and antitumor agents have been found among 1,2-dicarbonyl compounds and their derivatives.[5] Upjohn's Kethoxal (β-ethoxy α-ketobutyraldehyde) is one of them. French and Freedlander prepared several derivatives and found carcinostatic activities.[6] When we look at their structural formula, their derivatives show little resemblance to ketoaldehydes, but it seems possible that in the body the α-ketoaldehydes are liberated again and the substitute merely serves to protect them from inactivation before reaching their target. Preparing and testing various other derivatives may be rewarding.

The fact that most agents known to initiate or retard cancer react with SH also pleads for some intimate relation between cancer and SH. Mustards or other alkylating agents, radiation, and arsenic may be mentioned. The protective action of thiol against radiation damage is generally known.

Experiments.—Methylglyoxal was purchased from the Aldrich Chemical Co. as a 45% aqueous solution. The acidic contaminants were eliminated by fractional distillation or by passing a diluted solution through a Dowex-1-bicarbonate column. β-Ethoxy α-ketobutyraldehyde (Kethoxal[R]) was kindly given by Upjohn Pharmaceutical Co., Kalamazoo, Michigan, as a 56% aqueous solution. Hydroxymethylglyoxal (as a trimer alcoholate) was kindly synthesized for us by Merck, Sharpe & Dohme Co., Rahway, N. J.

The concentration of the 1,2-dicarbonyls for biological assays was estimated by oxidation with hydrogen peroxide in the presence of alkali[7] and was confirmed by calculation from the increase of absorbancy at 240 mμ as formation of thiolester with glyoxalase I.[8]

Escherichia coli[9] was kindly given by Dr. Martha Baylor (Woods Hole). A heavy inoculum of organism was allowed to grow in basic M-9 media,[10] supplemented with glucose (4%) as a sole carbon source overnight at 38°C under aerobic conditions. During this period the cell population increased to about 2–3 $\times$ 10^9/ml. From this stock suspension, the test media were inoculated with sufficient bacteria to achieve an optical density of 20 Klett units. The amount of cells in the test solution at the beginning and at the end of the experiments was estimated by cell counts after dilution with 4% formalin using a Leavy chamber and by viable count on nutrient agar containing 1% glucose.

The effect of α-ketoaldehydes on the proliferation of the microorganism was assayed in the test media containing M-9 glucose medium (4.25 ml), bacterial suspension (0.25–0.3 ml), ketoaldehyde (0.5 ml) in various concentrations, and water to bring up the final volume to 5.5 ml. The control contained the same amount of medium and bacterial suspension but the inhibitor was omitted. The reaction was followed by the increase of turbidity (Klett colorimeter 1.2-cm lightpath, red filter, maximum transmittancy 640–700 mμ) estimated against water and corrected for zero time reading. The assay mixtures were kept in a 38°C water bath and aerated under sterile conditions. The ketoaldehyde solutions were sterilized through a Millipore filter having a pore size of 0.45 μ.

Cysteine stock solution (1 mole) was made up from the hydrochloride and neutralized to pH 6.5. It was then sterilized through a Millipore filter and stored in a frozen state in small aliquots.

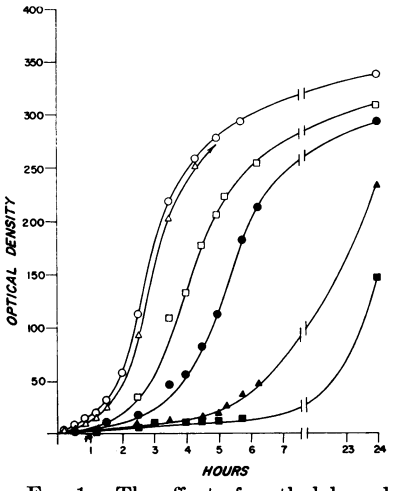

FIG. 1.—The effect of methylglyoxal on the proliferation of *E. coli*. The assay contained: 4.25 ml M-9-glucose medium, 0.25 ml bacterial suspension, 0.5 ml inhibitor in various concentrations, and 0.5 ml water. The control contained the same amounts but the bacterial suspension was replaced with water. Temperature 38°C, aerobic conditions. O—O, Control; △—△, 2×10^{-4} *M*; □—□, 4×10^{-4} *M*; ●—●, 6×10^{-4} *M*; ▲—▲, 8×10^{-4} *M*; ■—■, 1×10^{-3} *M*.

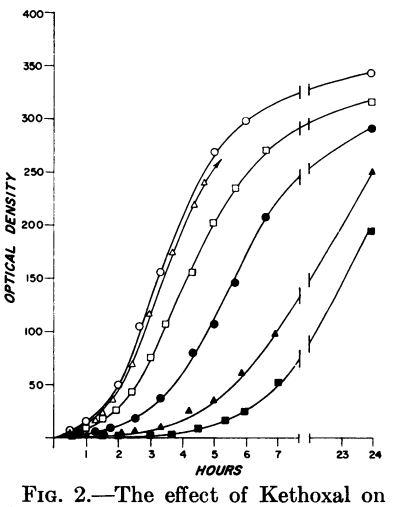

FIG. 2.—The effect of Kethoxal on the proliferation of *E. coli*. The assay contained: 4.25 ml M-9-glucose medium, 0.3 ml bacterial suspension, 0.5 ml inhibitor in various concentrations, and 0.45 ml water. The control contained the same amounts but the bacterial suspension was replaced with water. Temperature 38°C, aerobic conditions. O—O, Control; △—△, 2×10^{-4} *M*; □—□, 4×10^{-4} *M*; ●—●, 6×10^{-4} *M*; ▲—▲, 8×10^{-4} *M*; ■—■, 1×10^{-3} *M*.

Discussion.—*E. coli* was incubated with methylglyoxal of 1×10^{-3} to 2×10^{-4} mole concentrations at 38°C under aerobic conditions and the increment of optical density was measured. The family of curves (Fig. 1) obtained showed that the highest (10^{-3} *M*) concentration inhibited cell division for over 6 hr. Lower concentrations showed a gradually decreasing inhibition. The 2×10^{-4} mole had but slight effect. Kethoxal of 1×10^{-3} to 2×10^{-4} mole (Fig. 2) gave similar results.[11] The hydroxymethylglyoxal (Fig. 3) showed the least effect on cell division. In order to achieve the same length of inhibition as obtained with Kethoxal a 1×10^{-2} mole concentration had to be applied. This indicates that rendering the methyl end of the molecule more hydrophylous makes the substance less active.

As turbidity records only increase in protoplasma size, which does not necessarily correspond to increase in the number of cells, the incubation mixtures were diluted and plated out for colony count at various points and at the end of the assay (24 hr). The results indicated that the increment in turbidity was due to the increase in number of the bacteria.

The lowest curves in Figures 1, 2, and 3 indicate that after a longer incubation the cells break through their inhibition. This could be expected because the cells contain glutathione and methylglyoxalase, which transforms the added α-ketoaldehydes to α-hydroxy acids. In agreement with this it was found that the length of the lag period depended both on concentration of the inhibitor and the number of bacteria present in the assay. More bacteria contain more glyoxalase.

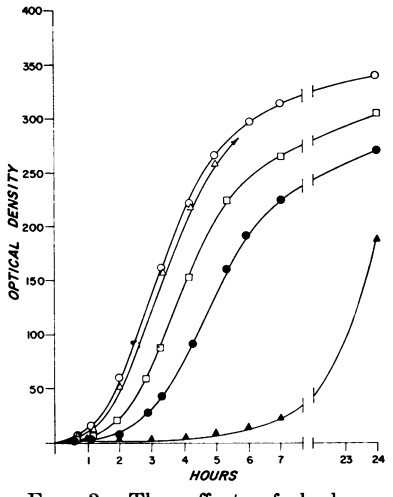

FIG. 3.—The effect of hydroxymethylglyoxal on the proliferation of *E. coli*. The assay contained: 4.25 ml of M-9-glucose medium, 0.25 ml bacterial suspension, 0.5 ml inhibitor in various concentrations, and 0.5 ml water. The control contained the same amounts but the bacterial suspension was replaced with water. Temperature 38°C, aerobic conditions. O—O, Control; △—△, 5×10^{-4} *M*; □—□, 1×10^{-3} *M*; ●—●, 5×10^{-3} *M*; ▲—▲, 1×10^{-2} *M*.

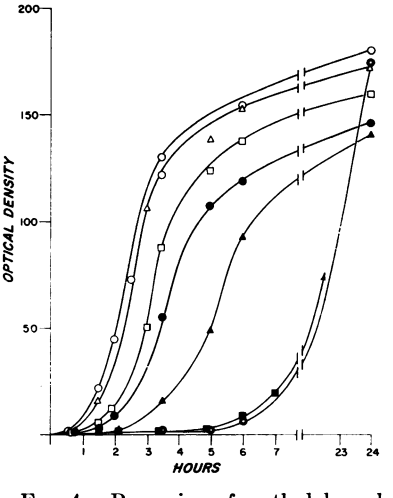

FIG. 4.—Reversion of methylglyoxal inhibition of *E. coli* by cysteine. The assay contained: 4.25 ml M-9-glucose media, 0.25 ml bacterial suspension, 0.5 ml methylglyoxal (1×10^{-3} *M*). In the control, the methylglyoxal was replaced with water. To the test, 0.1 ml cysteine (1×10^{-3} *M*) was added at various time intervals. Temperature 38°C, aerobic conditions. O—O, Control; **O—O**, no cysteine added. Cysteine added at zero time (△—△), 10 min (□—□), 30 min (●—●), 60 min (▲—▲), and 120 min (■—■) after the onset of reaction.

The breakthrough could also be explained by the development of an insensitive mutant which can tolerate the ketoaldehyde. That this was not the case was shown by transferring the bacteria at the end of the experiment to plates containing the methylglyoxals in their inhibiting concentration. No growth was obtained while the bacteria grew normally in absence of an inhibitor. The high affinity of thiols to methylglyoxal declares itself also in the fact that cysteine, added to our cultures simultaneously with methylglyoxal in isomolar (10^{-3} *M*) concentration, completely abolishes inhibition, as shown by Figure 4. If cysteine was added at various periods after the addition of the glyoxal, the inhibition showed a corresponding delay.

Bacteria in the log phase of their growth can break through the inhibition faster than bacteria taken from resting cultures (Fig. 5).

It may be asked whether the observed inhibition of growth was due to a specific inhibition of the machinery of cell division or was merely a secondary consequence of the inhibition of metabolism. As Figure 6 shows, concentration of methylglyoxal, which inhibits cell division completely, has no marked inhibiting effect on the oxidative metabolism. Similar results were obtained with Kethoxal and hydroxymethylglyoxal, indicating that the effect on growth was not a secondary consequence of cessation or respiration. Ten times higher concentrations of the inhibitor showed a reversible inhibition of respiration.

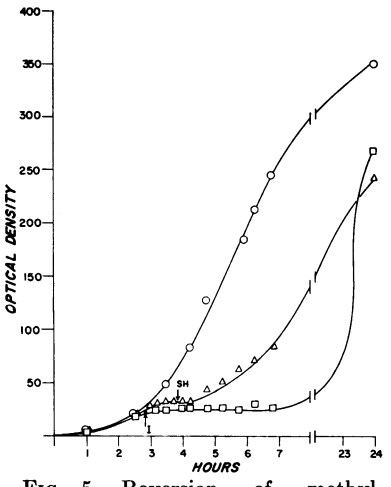

FIG. 5.—Reversion of methylglyoxal inhibition in log phase growing *E. coli* by cysteine. The assay contained: 4.25 ml M-9-glucose media, 0.25 ml bacterial suspension, 0.5 ml water at zero time. To the control (○—○), 0.5 ml water and to the tests, 0.5 ml of methylglyoxal (1×10^{-3} *M*) were added at time indicated by *I*. To one of the tests (△—△), 0.1 ml cysteine (1×10^{-3} *M*) was added at time indicated by *SH*. The other test (□—□) did not receive cysteine. Temperature 38°C, aerobic conditions.

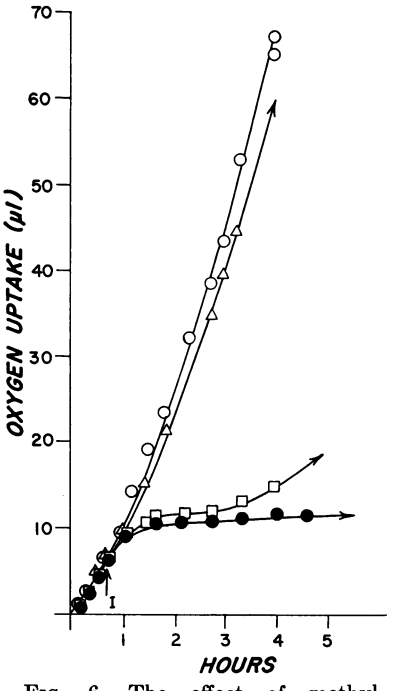

FIG. 6.—The effect of methylglyoxal on respiration of *E. coli*. Assay contained: 3.1 ml M-9-medium, 0.5 ml bacterial suspension. The center well contained 0.2 ml 20% KOH. The side arm contained 0.5 ml methylglyoxal (△—△, 1×10^{-3} *M*; □—□, 1×10^{-2} *M*; ●—●, 1×10^{-1} *M*), or water (○—○, control). The latter was added to the main compartment at time indicated by *I*. Temperature 38°C.

Part of this observed inhibitory reaction might be elicited also by aldehydes activated by other than by α-keto groups.

Recently, saturated (C_3-C_{13}) and α-β unsaturated ketoaldehydes have been synthesized chemically in this laboratory.[12] Preliminary experiments on the proliferation of *E. coli* showed a similar inhibition. This and the previously described experiments indicate that, in general, compounds having the α-ketoaldehyde groups can be effective inhibitory agents.

Summary.—α-Ketoaldehydes inhibit cell division in concentrations which do not affect respiration or motility. The inhibition is due to its reaction with SH groups, essential for cell division. Implications were discussed.

The authors thank Miss T. E. Felker for her technical assistance.

* This research was supported by the National Institutes of Health grant GM-10383.

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*HYDROXYLYSINE FORMATION FROM LYSINE DURING COLLAGEN BIOSYNTHESIS**

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Communicated December 22, 1965

Hydroxylysine as a constituent amino acid of a protein has been found only in collagen.[1] The obligatory source of collagen hydroxylysine is lysine.[2-4] Hydroxylysine itself, either fed or injected intraperitoneally, is not incorporated into collagen by rats,[5] or presumably by other animals. Hydroxylation of lysine occurs in the early stages of collagen synthesis[4, 6]; it may, as found for hydroxylation of proline, occur in the precollagen peptide chain on the ribosomes.[7]

The present paper reports a continuation of a study of the biochemical mechanism by which a hydroxyl group is attached to the number 5 carbon of lysine. Conceivably the hydroxylation could proceed by (*a*) direct uptake of an atom of oxygen on carbon 5; (*b*) oxidation of carbon 5 to a ketone —CO— group followed by reduction to —CH(OH)—; (*c*) dehydrogenation to form a 4–5 double bond followed by addition of the elements of water; (*d*) dehydrogenation to form a 5–6 double bond followed by addition of the elements of water.

We have previously reported the results of studies using lysine-4-5-H^3 as a precursor of collagen hydroxylysine.[8] In these studies we found that only one of the four labeled H atoms of lysine-4-5-H^3 was lost in the conversion to hydroxylysine. One hydrogen atom on carbon 5 was replaced by —OH; therefore none of the other three labeled H atoms could be involved in the hydroxylation in such a way that they became equilibrated with the H of the medium. The results eliminated 5-keto-lysine and 4,5-dehydrolysine (mechanisms *b* and *c*) as possible intermediates in the hydroxylation of lysine.