Glyoxal and Related Compounds as Potential Blood Sterilizing Agents. (22776)

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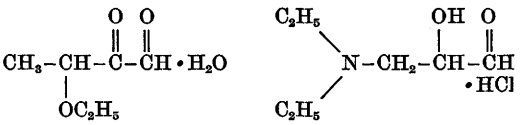
A major problem in the use of human blood and plasma for transfusions is the danger of transmitting the virus of serum hepatitis. No completely satisfactory method is available for inactivation of the virus. Ultraviolet irradiation(1), β-propiolactone (BPL)(2) and a combination of these(3,4) have been studied. Since preliminary studies in our laboratory indicated that glyoxal and many related compounds were potent virucidal agents, experiments were undertaken to determine the potential value of these compounds as blood sterilizing agents by comparing their *in vitro* virucidal activity and their toxicities with other known virucidal agents. Two of these compounds, glyoxal and methyl glyoxal, were previously tested but apparently discarded because when "added to human blood plasma, they caused an increase in the electrophoretic mobilities at pH 8.6 of the plasma proteins."

Materials and methods. Viruses: (a) influenza A, PR-8 strain, supplied by Dr. Thomas Francis, Jr., University of Michigan. It was an egg-adapted strain, lethal for 10-day embryos within 60-80 hrs; it reached a titer in 10-day eggs of about 10^9 LD_{50} per 0.1 ml when injected into the allantoic cavity. (b) Newcastle disease virus, NJ-KD strain, secured from Dr. E. S. Pomeroy, University of Minnesota. It was an egg-adapted strain, lethal for 10-day embryos within 50-70 hrs; it reached a titer in 10-day eggs of about 10^8 LD_{50} per 0.1 ml when injected into the allantoic cavity. (c) Mouse hepatitis virus, received from Dr. J. B. Nelson(5). A stock was prepared by injecting 0.1 ml volumes of a 10^{-2} dilution of the virus by the intraperitoneal route into a group of Princeton weanling mice (Millerton Research Farm, Millerton, N. Y.). Fifty hours after infection, the livers of the mice were harvested and a 10% suspension in beef infusion broth was prepared. This material had a titer of approximately 10^7 LD_{50} per 0.1 ml in Princeton weanling mice when injected intraperitoneally.

Chemicals:* (a) *Glyoxal*—A specially purified aqueous solution was obtained from Carbide and Carbon, Inc. The colorless solution was passed through Amberlite IR-45 resin to remove any acid present. The glyoxal content of the resulting solution was 36.8% by weight. (b) Pyruvaldehyde (methyl glyoxal)—This was obtained from the same source and purified in the same way as the glyoxal. The methyl glyoxal content of the aqueous solution was 33.4% by weight. (c) Formaldehyde—A fresh commercial sample (37% aqueous solution) was filtered through paper and used directly. (d) β-propiolactone—A redistilled commercial sample (B. F. Goodrich Co.) was used. To minimize hydrolysis, the BPL dilutions were made immediately before using. Water was used as the solvent except when the lysis of erythrocytes was being studied; then saline was used. (e) Kethoxal (β-ethoxy-α-ketobutyraldehyde hydrate); U-1671 (β-diethylaminolactaldehyde hydrochloride); and Kethoxal bisulfite—A description of the synthesis of these compounds will be published elsewhere. The structures of Kethoxal and U-1671 are shown below:



Kethoxal

U-1671 (Upjohn code No.)

Test for virucidal activity. One of 3 media was used: (a) A mixture of equal volumes of beef infusion broth and physiological saline, (b) whole blood, (c) plasma. Virus was incubated for 2½ hrs at 37°C with equimolar quantities of the various test chemicals. The

* The chemical synthesis and purification procedures were conducted by Dr. R. H. Levin and his associates in the Chemistry Department of The Upjohn Co.

amount of infective virus remaining in each sample after incubation was determined by making serial 10-fold dilutions in 1:1 beef infusion broth:saline and titrating in groups of 10 eggs or mice, depending on the virus used. Deaths were recorded for a period of 10 days, and the titers were calculated by the method of Reed and Muench(6).

Test for Lysis of Erythrocytes. Equimolar quantities of the test chemicals were mixed with 10 ml of 1% human red blood cells suspended in physiological saline. Five tubes for each chemical and 5 control tubes were prepared. The tubes were continuously shaken on a mechanical shaker at 25°C. The amount of lysis was determined at intervals by centrifuging one tube from each set for 5 min at 2000 rpm in a Model V International Centrifuge and determining the optical density of the supernatant at a wavelength of 590 mμ in a Beckman Model B Spectrophotometer. A standard curve was previously drawn in which optical density was plotted against known concentrations (0.1-1.0%) of red blood cells which were completely lysed by placing them in distilled water for 10 min. The % lysis of the test samples was determined by referring optical density measurements to this standard curve. A control tube which had been shaken for the same length of time served as the blank.

Electrophoresis studies. Equimolar amounts of the test chemicals were added to human plasma. After incubation under varying conditions, each sample was diluted with an equal volume of veronal buffer, pH 8.6 and ionic strength 0.1 and dialysed against 250 ml of the same buffer for 24 hrs. The samples were then diluted to approximately 1.5% total protein concentration and dialysed for another 24 hr against 2 L of fresh veronal buffer. The dialysed samples were then subjected to electrophoresis for a period of 3 hr.†

Results. Comparative in vitro virucidal activity of various chemicals. Comparative virucidal activities of several of the test chemicals were determined (Table I). Kethoxal was a more potent virucidal agent in broth, plasma or whole blood medium than were equimolar amounts of BPL, formaldehyde or U-1671. Experiment 3 (Table I) further indicated that Kethoxal and glyoxal were more potent virucidal agents for PR-8 virus in whole blood medium than were any of the other chemicals tested; that a molar concentration of approximately 6.75×10^{-3}

† The electrophoresis determinations were conducted by G. C. Colovos of The Upjohn Company, Physics Department.

TABLE I. Comparative Virucidal Activities of Various Chemicals vs. Influenza A (PR-8) and Newcastle Disease (NJ-KD) Viruses.

Equimolar quantities of the test chemicals, each in a vol of 0.1 ml, were incubated with 0.1 ml of 10-fold diluted (Exp. 1) or non-diluted (Exp. 2 and 3) infective allantoic fluid in 4.8 ml of the indicated medium. Each sample was incubated for 2½ hr at 37°C, and the amount of residual infective virus was then determined by making serial 10-fold dilutions and titrating in groups of 10 eggs. Each dilution was inj. intra-allantoically at a level of 0.5 ml/egg. Results are expressed as the $\log_{10}$ titer of 1 LD_{50}.

Exp. No.*	Chemical	Molar conc.	Final titer
1 a	BPL	1.35×10^{-4}	8.7
	Formaldehyde	"	8.9
	Kethoxal	"	6.5
	U-1671	"	8.4
	Control	—	9.0
1 b	BPL	1.35×10^{-4}	6.9
	Formaldehyde	"	7.4
	Kethoxal	"	5.6
	U-1671	"	7.0
	Control	—	7.5
2	BPL	1.35×10^{-4}	8.7
	Formaldehyde	"	8.9
	Kethoxal	"	7.5
	U-1671	"	8.5
	Control	—	9.1
3 a	Kethoxal	2.70×10^{-3}	6.7
	"	4.05×10^{-3}	5.1
	"	5.40×10^{-3}	2.8
	"	6.75×10^{-3}	<2.0
	BPL	"	6.0
	Formaldehyde	"	5.3
	U-1671	"	8.1
	Kethoxal bisulfite	"	6.2
	Glyoxal	"	<2.0
	Methyl glyoxal	"	7.4
	Control	—	8.1
3 b	Kethoxal	2.70×10^{-3}	5.1
	Glyoxal	"	4.2
	Control	—	6.4

* The medium used in Exp. 1 was 50% beef broth, in Exp. 2 was chicken plasma, and in Exp. 3 was chicken blood. The PR-8 virus was used in Exp. 1 a, 2 and 3 a; NJ-KD was used in Exp. 1 b and 3 b. The initial titer of the viruses in each exp. was as follows: 1 a = 9.4; 1 b = 7.9; 2 = 9.2; 3 a = 9.3; 3 b = 7.6.

TABLE II. Comparative Virucidal Activities vs. Mouse Hepatitis Virus.

Equimolar quantities of the test chemicals, each in a vol of 0.1 ml, were incubated with 0.5 ml of a 10% liver suspension of mouse hepatitis virus in 4.4 ml of whole chicken blood. Each sample was incubated for 2½ hr at 37°C, and the amount of residual infective virus was then determined by making serial 10-fold dilutions and titrating in Princeton weanling mice. Each dilution was inj. intraper. at a level of 0.1 ml/mouse. Deaths were recorded for a period of 10 days. Results are expressed as the reciprocal of the $\log_{10}$ titer of 1 LD_{50}.

Exp. No.	Chemical	Molar conc.	Final titer
1	Kethoxal	6.75×10^{-3}	2.1
	BPL	"	3.4
	Glyoxal	"	<2.0
	Control	—	6.8
2	Kethoxal	3.38×10^{-3}	4.0
	BPL	"	6.1
	Glyoxal	"	3.6
	Control	—	7.0

(1000 mg/L) of Kethoxal was required to inactivate all PR-8 virus in whole blood under the stated incubation conditions; and that glyoxal was slightly more potent than Kethoxal against NJ-KD virus in whole blood. The comparative virucidal activities of Kethoxal, BPL and glyoxal against the virus of mouse hepatitis were studied. This virus was chosen because of its possible closer relationship to the virus of serum hepatitis. The results (Table II) indicated that Kethoxal and glyoxal were more active than BPL against the virus of mouse hepatitis.

Comparative effect on lysis of erythrocytes. An experiment was designed to test the comparative effects of Kethoxal, BPL and glyoxal on a 1% suspension of human red blood cells. All 3 chemicals were used at a molar concentration of 6.75×10^{-3}. The results (Table III) indicated that BPL lysed the red cells more rapidly than did Kethoxal or glyoxal. When whole blood was used, the results were different. Equimolar amounts of the chemicals were mixed with whole human blood and stored for 3 weeks at 4°C, similar to storage conditions used in hospital blood banks. Under these conditions, it was found that a molar concentration of 4.05×10^{-2} (6000 mg/L) of Kethoxal caused 2.3% lysis of the erythrocytes of whole blood, an equimolar amount of BPL caused 2.0% lysis, and an equimolar amount of glyoxal caused 1.4% lysis. A control sample of human blood stored for the same period of time was found to have undergone 1.1% lysis of the red cells, indicating that the net lytic effect of the glyoxal (0.3%) was very low. Spectrophotometer readings on the supernatants from these whole blood samples were made at a wavelength of 490 mμ. The lower wavelength was used with the whole blood samples because at 490 mμ there was less interference from the yellow color apparently formed by reaction of the carbonyl compounds with blood proteins. No attempt was made in these experiments to correct the values for possible formation of methemoglobin.

TABLE III. Comparative Effect of Equimolar Amounts of Kethoxal, β-Propiolactone and Glyoxal on Lysis of a 1% Suspension of Human Red Blood Cells.

Kethoxal was used at a molar conc. of 6.75×10^{-3} (1000 mg/L). The figures in the table express % of lysis.

Time after mixing (hr)	Kethoxal	β-Propiolactone	Glyoxal
1	0	41	0
2	0	57	0
4	0	85	0
6	23	92	0
7	31	95	0

Comparative effect on plasma proteins. In order to determine the effect of Kethoxal on plasma proteins, a fresh sample of human plasma was obtained and treated with Kethoxal at a molar concentration of 6.75×10^{-3} (1000 mg/L); this was the minimum concentration previously found to destroy all detectable Newcastle and influenza viruses in artificially infected whole blood. A second portion of the plasma was treated with an equimolar amount of glyoxal, a third portion with an equimolar amount of BPL, and a fourth portion served as a control. Each sample was incubated for 93 hr at room temperature, then for an additional 48-72 hr at 4°C. In a similar second experiment, one group of samples received the 3 chemicals at a molar concentration of 6.75×10^{-3}, as above; another group of plasma samples received double this amount of each chemical.

This group of 8 samples, 6 treated and 2 controls, was incubated for 21 days at 4°C, then subjected to electrophoresis. It was found that the presence of the various chemicals under the stated incubation conditions caused no significant changes in the electrophoretic pattern of the human plasma.

Discussion. The results of these experiments indicate that glyoxal and Kethoxal may have value as virucidal agents. Both chemicals were more potent in destroying the infectivity of influenza A, Newcastle disease and mouse hepatitis viruses than was an equimolar amount of BPL. Glyoxal appeared to be less lytic than BPL or Kethoxal, and none of the chemicals caused significant changes in human plasma proteins at concentrations which were effective in sterilizing artificially-infected blood. These results suggest that glyoxal and Kethoxal hold some promise as blood-sterilizing agents. Glyoxal gave results as good or better than Kethoxal. Since thorough studies have not been made on the toxicity of glyoxal for mammalian hosts(7,8), further work is required before it can be applied to the large-scale sterilization of blood and plasma. The marked superiority of glyoxal over formaldehyde as a virus inactivating agent suggests also the possible application of glyoxal in the preparation of "killed" vaccines or as a general viral disinfectant.

Summary. Both glyoxal and β-ethoxy-α-ketobutyraldehyde (Kethoxal) were more potent virucidal agents than β-propiolactone (BPL). Glyoxal was less lytic for human erythrocytes than was Kethoxal or BPL. None of the chemicals showed significant toxicity for plasma proteins as measured by electrophoresis. These experiments indicate that glyoxal may be of potential value in the sterilization of human blood and plasma.

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Increased Erythropoietic Stimulant in Plasma of Pregnant Rats.* (22777)

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The existence of an erythropoietic substance in the blood stream was first suggested by Carnot and Deflandre(1) who showed that plasma from rabbits which had been bled repeatedly when injected into other rabbits resulted in hyperplastic bone marrow and increased reticulocyte and erythrocyte counts. Several investigators have since(2-19) confirmed the presence of erythropoietic activity in plasma of animals which had been bled. Plasma from animals exposed to low oxygen tensions has similarly been found to stimulate erythropoiesis in recipients (6b,9,20-22, 24b). Human umbilical cord blood, also fetal sheep and dog blood, have been found to increase red blood cell count in recipient animals(22-26). As these reports of an erythropoietic substance in blood were based either

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