Binding of an Antiviral Agent (Kethoxal) by Various Metabolites. (24611)

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β - Ethoxy - α - ketobutyraldehyde hydrate (Kethoxal) exhibits marked activity against several viruses in embryonated eggs(1,2). A logical sequence to demonstration of its antiviral activity in eggs was its evaluation in animal-virus systems. Kethoxal was tested against viruses of Newcastle disease in chickens, distemper in ferrets and influenza in mice. Although there was some indication of activity occasionally, it was of low level and inconsistent. The experiments reported here were designed to determine the reason for this lack of antiviral activity in animals.

Materials and methods. Kethoxal. Synthesis and some reactions of this compound have been reported(3). *Viruses.* Description of viruses used and egg technics involved in testing for antiviral activity have been described(2). *Microcolorimetric assay.* This was adapted from the procedure of Barrenscheen and Dreguss(4) for methylglyoxal. A standard curve was established by using weighed samples of Kethoxal. Kethoxal was precipitated as the 2,4-dinitrophenylosazone, which was washed successively with N hydrochloric acid and with increasing concentrations of ethanol in N HCl. The washed osazone was dissolved in 5% alcoholic potassium hydroxide and adjusted to volume. Model B Beckman spectrophotometer was balanced to zero optical density with alcoholic potassium hydroxide solution at wavelength of 565 mμ. The optical density of sample was then read at same wavelength. Average value obtained from triplicate samples represented one point on standard curve. The general procedure to assay for Kethoxal by this method in the presence of proteins, etc., can be illustrated by the reaction mixture of Kethoxal and normal chicken serum. One ml of aqueous solution of known Kethoxal content was mixed with 1 ml of normal chicken serum and 6 ml of 10% aqueous trichloroacetic acid solution was added. After centrifuging, the supernatant was transferred quantitatively to 10 ml volumetric flask. The protein precipitate was washed thoroughly with 2 ml of 10% trichloroacetic acid solution, recentrifuged, the supernatant added to the original and the whole made up to volume with 10% trichloroacetic acid solution. For analysis, a 5 ml aliquot was treated as described previously. Appropriate controls were made by diluting the serum with 1 ml of deionized water, deproteinizing, making up to volume as above and using 5 ml aliquot for analysis. The assay was accurate within $\pm$ 25% in the 5-30 μg range, within $\pm$ 10% in the 30-100 μg range and within $\pm$ 3% in the 100-200 μg range. *Alkaline peroxide assay.* This method was used for determination of Kethoxal in mg quantities. It was originally developed by Friedemann(5) and the application of the method to Kethoxal and its analogues has been described (3).

Results. Use of microcolorimetric assay to study Kethoxal binding. Since Kethoxal was active as an antiviral agent when injected into the allantois of chick embryos, a detailed study was made of its reaction with allantoic fluid, both *in vitro* and *in vivo*. Under conditions simulating those employed in routine administration of Kethoxal to infected embryonic eggs, Kethoxal at concentration of 0.5 mg/ml was added *in vitro* to normal allantoic fluid, harvested from 10 day eggs. For the *in vivo* study, a single dose of 2.5 mg of Kethoxal was injected into the allantoic cavity of the 10 day embryonic eggs and samples withdrawn at intervals and assayed for Kethoxal content. Rapid disappearance of Kethoxal from allantoic fluid *in vivo* is evident (Fig. 1). Other experiments(1,2) have indicated that there is marked antiviral activity when the same amount of Kethoxal is injected into eggs as much as 24 hours before the virus is injected. Thus, at time of infection there would be no detectable free Kethoxal present in the allantoic fluid, indicating that some bound form or some conversion product of

Kethoxal also showed antiviral activity. To learn more about the fate of Kethoxal when injected into various hosts, experiments were undertaken on recovery of Kethoxal added to body fluids and other substances *in vitro*. The general procedure in these recovery studies consisted of addition of a known quantity of aqueous solution of Kethoxal to a specified volume of serum, whole blood, etc. The mixture was deproteinized with aqueous solution of trichloroacetic acid, and an aliquot of the supernatant was analyzed essentially according to procedure described for chicken serum. It was found that Kethoxal was bound rapidly by blood, serum, urine, saliva, gelatin and other materials containing protein or protein-degradation products. Gelatin was used as a relatively simple model for more detailed studies of the reactions of Kethoxal with proteins. Two samples of sterile 10% gelatin, containing 1 mg of Kethoxal/ml, were prepared. One sample remained at room temperature throughout experiment; the other was kept at room temperature for 4 hours, then stored at –60°C. Aliquots of these samples were taken at intervals, assayed microcolorimetrically for Kethoxal content, and tested in eggs for antiviral activity. Similar experiments were also run using a synthetic gelatin hydrolysate consisting of a mixture of average quantities of amino acids reported to be present in gelatin. A 10% solution of this amino acid mixture was prepared, Kethoxal was added at a level of 1 mg/ml and disappearance of free Kethoxal and loss of antiviral activity were followed as described above. Results indicated (Table I) that Kethoxal was bound both by intact gelatin and by the synthetic gelatin hydrolysate, that rate of binding was approximately the same in the 2 cases, and that the bound Kethoxal was still active as an antiviral agent in eggs.

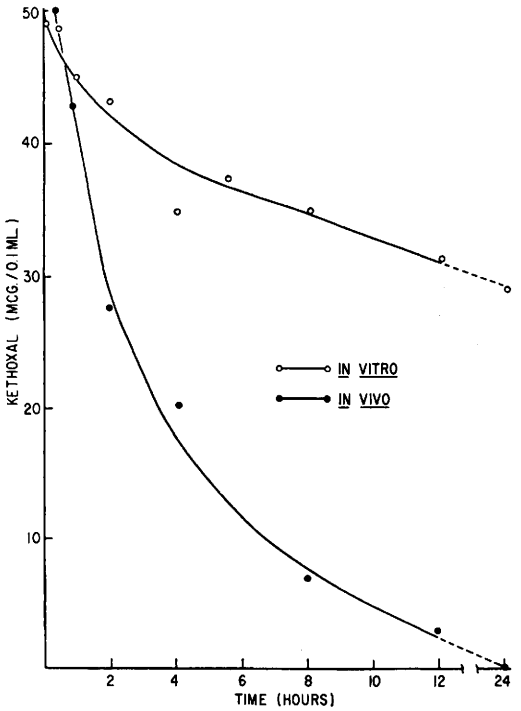

FIG. 1. Disappearance of Kethoxal from allantoic fluid.

TABLE I. Antiviral Activity of Kethoxal-Gelatin Mixtures.

Storage temp. (C)	Time after mixing (hr)	Free Kethoxal (μg/0.1 ml)	Egg activity† (% survivors)
		Mixture A*	
25°	0	100	94
	4	7	50
	8	0	10
	12	0	0
	24	0	6
25°/4 hr; –60°	8	0	47
	12	0	55
	24	0	35
		Mixture B*	
25°	0	100	100
	4	21	60
	8	1	12
	12	0	15
	24	0	6
25°/4 hr; –60°	8	20	50
	12	13	54
	24	16	36
Control		0	0

* Mixture A: 1 mg/ml of Kethoxal in 10% gelatin; Mixture B: 1 mg/ml of Kethoxal in 10% synthetic gelatin hydrolysate.

† Inj. 0.1 ml of test solution/egg into allantois of 10-day embryos; these eggs were infected 15 min. later with 25 lethal doses of Newcastle disease virus by the same route. Per cent survivors were recorded 10 days after infection. All tests for antiviral activity were carried out by our Virus Unit.

Reaction of Kethoxal with individual amino acids. Having established that Kethoxal reacted with the mixture of amino acids comprising the synthetic gelatin hydrolysate to yield biologically active products, it was of

TABLE II. Antiviral Activity of Kethoxal-Amino Acid Mixtures.

Amino acid*	Reaction time†	Free Kethoxal (μg/0.1 ml)	Egg activity‡ (% survivors)
Cysteine	5 min.	0	0
Arginine	5	0	5
Tryptophane	16 hr	28	0
Glycine	80	32	18
Lysine	63	23	16
Proline	140	32	76
Hydroxy-proline	140	36	71
Histidine	20 min.	9	40
Glutamic acid	110 hr	18	79
Serine	25 min.	7	89
Threonine	25	7	100
Control		0	0

* Kethoxal (5 mg/ml) reacted with 10 molar equivalents of the amino acid at pH 7.4.

† Time required for Kethoxal concentration to drop to 2 mg/ml or less.

‡ Inj. 0.1 ml of the test solution/egg into allantois of 10-day embryos; these eggs were infected 15 min. later with 25 lethal doses of Newcastle disease virus by the same route. Per cent survivors were recorded 10 days after infection.

interest to examine the behavior of Kethoxal with individual amino acids present in this hydrolysate. An aqueous solution of Kethoxal at initial concentration of 5 mg/ml was treated with 10 molar equivalents of a given amino acid at pH 7.4 in 0.1 *M* phosphate buffer under sterile conditions. At intervals, aliquots of the reaction mixture were assayed for their Kethoxal content by the alkaline peroxide method. When the free Kethoxal content had fallen to less than 2 mg/ml, the remainder of the reaction mixture was diluted with sufficient sterile distilled water so that the quantity of free Kethoxal would be sub-effective in virus-infected eggs, but the total Kethoxal (free plus combined) would be sufficient to afford protection, providing the combined form retained antiviral activity. Such solutions, when assayed for antiviral activity in eggs, gave results which provided several points of interest (Table II). Kethoxal was inactivated very rapidly by cysteine and by arginine. With tryptophane the binding of Kethoxal took place more slowly, but biological inactivation occurred distinctly. Kethoxal bound to glycine and to lysine displayed antiviral activity initially, but when these samples were tested after 3 additional days at room temperature, they were inactive; this phenomenon is similar to that observed with Kethoxal bound to gelatin and to the synthetic gelatin hydrolysate. The rapid reaction of Kethoxal with serine and with threonine to yield products of marked antiviral activity was of particular interest. From the reaction mixture of Kethoxal with serine a crude solid was isolated. This material showed significant antiviral activity in eggs after being kept for a month at room temperature. Repeated attempts to obtain a homogeneous, crystalline product were unsuccessful. The antiviral activity of the Kethoxal-serine complex was inhibited completely by cysteine. Other hydroxyamino compounds, including serine methyl ester, homoserine and ethanolamine, also reacted with Kethoxal to give products showing high levels of antiviral activity in eggs; however, these, too, were inactive in animals.

Discussion. While gelatin-bound Kethoxal possessed antiviral activity in the embryonic egg system after free Kethoxal was no longer detectable (as was true of allantoic fluid-bound Kethoxal), the activity declined and finally disappeared after the reaction mixture stood at room temperature for a few additional hours (Table I). One interpretation of this behavior is that the initial reaction of Kethoxal with gelatin (or with allantoic fluid) effectively bound the ketoaldehyde with retention of antiviral activity; on continued standing, however, further reactions occurred yielding biologically inactive material. Such an interpretation is sustained by data shown in Table I when Kethoxal was allowed to react with gelatin at room temperature until Kethoxal disappeared (4 hours), and the reaction mixture was then held at −60°C during the next 20 hours. In this case, the antiviral activity of the conjugate was maintained for considerably longer time. This prolongation of biological activity is thought to be due to a retardation of the secondary reactions at low temperature. The rate of disappearance of Kethoxal in the synthetic gelatin hydrolysate closely simulated that observed for native gelatin; antiviral activity was likewise of the same order of magnitude. The Kethoxal-synthetic hydrolysate conjugate further paralleled

the Kethoxal-gelatin conjugate in that bioactivity diminished after keeping the reaction mixture at room temperature for a relatively short time; however, antiviral activity could be prolonged by holding the reaction mixture at −60°C. Concerning the particular groups present in amino acids, peptides and proteins which are responsible for conjugation of these substances with Kethoxal, some concrete indications were obtained. Participation of primary amino groups seems clear from the observations that (a) Kethoxal reacted rapidly with simple primary amines; (b) Kethoxal reacted rather slowly with neutral α-amino acids, but with the corresponding α-amino acid esters rate of reaction was significantly increased; (c) in a given series of α-amino acids, rate of reaction with Kethoxal was retarded by loading the α-carbon atom with methyl groups; (d) no detectable reaction occurred between Kethoxal and N-acylated α-amino acids or N-acylated α-amino acid esters. The extremely rapid reaction of Kethoxal with arginine (and with guanidine) suggests the guanidino grouping as another site of reaction. Rapidity of reaction of Kethoxal with serine, threonine, cysteine and 2-aminobutanol-1, as compared to alanine, for instance, implies that a free primary amino group and an adjacent active group comprises another reactive area. In view of ability of Kethoxal to react rapidly with such a wide variety of chemical groupings, it was not surprising that it was rapidly bound and inactivated in the complex animal system. The fact that antiviral activity was retained in eggs was undoubtedly due to the much simpler chemical environment and low protein content of the allantoic fluid. It was thus concluded that the main reason for lack of antiviral activity of Kethoxal in animals was due to its participation in a variety of chemical reactions. To facilitate studies on fate of Kethoxal or its conversion products in animals, radioactive carbon-labeled Kethoxal was synthesized. A brief summary of results obtained with this material has been presented (1). A more detailed description, including methods of synthesis and its distribution, as well as excretion in mice, will be published.

Summary. Kethoxal (β-ethoxy-α-ketobutyraldehyde hydrate) reacted chemically with a variety of normal metabolites. Some of these reactions (*e.g.*, those with cysteine and arginine) resulted in rapid loss of antiviral activity; others (*e.g.*, with glycine and intact gelatin) in a more gradual loss of activity, while still others, particularly with serine and threonine, gave products possessing apparently undiminished activity. It was concluded that lack of significant antiviral activity by Kethoxal in animals could be attributed principally to its rapid binding and inactivation by proteins, amino acids and other metabolites.

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