Biochemical Pharmacology, Vol. 19, pp. 687–695. Pergamon Press. 1970. Printed in Great Britain

POTENTIAL ALLOXAN-DIABETIC COMPOUNDS FROM THE REACTION OF ALLOXAN, GLUTATHIONE AND NITROGEN BASES

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(*Received* 10 *March* 1969; *accepted* 11 *July* 1969)

Abstract—Alloxan and glutathione in glycine buffers formed a stable product that absorbs at 280 mμ. Glycine analogues having an amino group reacted similarly with alloxan and glutathione, whereas glycine analogues having a carboxyl group reacted with alloxan and glutathione to yield a product that absorbs at 305 mμ. The 280-mμ-absorbing compound after reacting alloxan, glutathione and ethyl aminoacetate was electrically neutral, was stable in basic solutions, and was not absorbed to either anion or cation exchange resins. Mild acid treatment produced a new compound having a cationic group. These data and other pertinent observations were used in proposing a structure for this 280-mμ-absorbing compound and in evaluating the probability that such compounds might produce experimental diabetes.

EXPLANATIONS of alloxan's action in producing a type of diabetes emphasize the ability of alloxan to react with sulfhydryl groups.[1] Several investigators[2–5] have observed that alloxan reacts with glutathione or protein sulfhydryl groups in phosphate buffer to form an unstable product that has an ultraviolet absorption maximum of 305 mμ.

When glycine was substituted for phosphate in the reaction of alloxan with glutathione, a new absorption maximum was observed at 280 mμ† and the reaction product(s) was stable. The studies now reported were designed to investigate this chemical reaction of alloxan and glutathione in the presence of glycine and similar compounds, to correlate these findings with the properties of the compound absorbing at 305 mμ, and to evaluate the probability that such compounds might produce experimental diabetes.

EXPERIMENTS

All inorganic reagents were analytical grade. Alloxan monohydrate was obtained from L. Light and Co. Ltd., England; and glutathione, amino acids and glycine analogues from British Drug Houses, Ltd., United Kingdom. ZeoKarb 225 (SRC-8) was a product of Permutit Co., Ltd., London. The anion exchange resin was AG 2-X10, 200–400 mesh, obtained from BIO-RAD Laboratories, Calif.

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† Not all of the absorbing compounds have an absorption maximum exactly at 280 mμ. However, for differentiating between the two types of products, these two wavelengths, 280 mμ and 305 mμ, were used.

Changes in ultraviolet absorption were followed by quartz cuvettes of 1-cm light path with a Unicam DU spectrophotometer, model 500. Absorption spectra, which were measured every 2 mμ, were determined against blanks that contained the buffers used for the individual experiments.

In most cases, 0·25 ml of glutathione solution (1 μmole) was added to 8·5 ml of 0·2 M buffer solution. Water was added to give, with the alloxan solution (1–2 μmoles), a total volume of 10 ml. The solution then stood at room temperature (19–22°). The reaction time in minutes represents the interval between adding the alloxan and attaining the maximal optical density. All reagents were prepared daily and glutathione and alloxan solutions were used within 1 hr of preparation. The glass-distilled water was deionized and had a resistance greater than 4 million ohm-cm.

Paper electrophoresis of the reaction mixture was carried out using the hanging-strip-type apparatus. The buffer system was 0·02 M potassium hydrogen phthalate adjusted to pH 5·9 with sodium hydroxide.[6] Samples were applied to Whatman No. 1 filter paper (15½ × 18 in.), with 3 in. between each sample. The separation was carried out for 2½ hr at a constant current of 16 mA. The chromatogram was dried at 100° for 1 hr and sprayed with ninhydrin in acetone.

Amino acids were chromatographed by the method described by Block and Weiss.[7] Sulfhydryl groups were estimated by the nitroprusside reaction.[8]

RESULTS

The importance of a third component in the reaction between alloxan and glutathione. The reaction of alloxan and glutathione in the presence of phosphate buffer yielded a

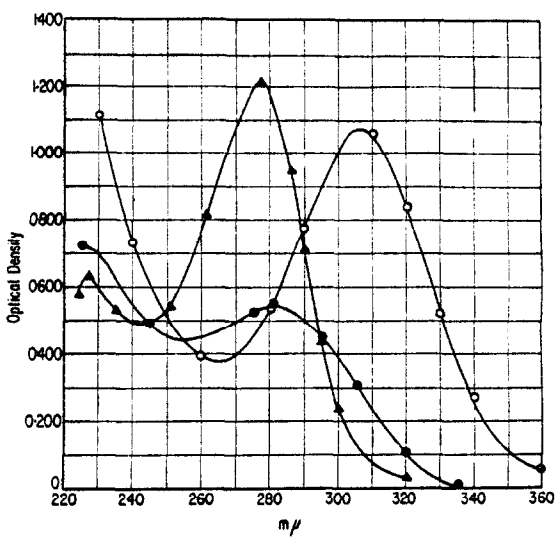

FIG. 1. The absorption spectra of the 280-mμ- and 305-mμ-absorbing compounds using either ethyl aminoacetate or phosphate in the reaction of alloxan and glutathione. The reaction mixtures contained for the 277-mμ-absorbing compound (▲—▲) 1 μmole of alloxan and glutathione in 1·7 m-moles of ethyl aminoacetate, pH 7·2; for the 305-mμ-absorbing compound (○—○) 1·3 μmoles of alloxan, 2·7 μmoles of phosphate buffer, pH 7·72. There was a total volume of 10 ml in each case. The curve (●—●) is the identical solution yielding the 305-mμ-absorbing compound (○—○) having stood at room temperature for 18 hr.

305-mμ absorption maximum,[3] whereas a 280-mμ absorption maximum was observed after reacting alloxan and glutathione in buffered glycine (Fig. 1 and Table 1). Although absorption was apparent almost instantaneously, the time for reaching maximal absorption was 60 min in phosphate buffer and 120 min in glycine. If alloxan and glutathione were reacted in the presence of both glycine and phosphate, both absorption maxima (305 and 280 mμ) were seen. The 305-mμ absorption maximum in phosphate disappeared on standing at room temperature, whereas the 280-mμ

TABLE 1. EFFECT OF VARIOUS REAGENTS ON THE REACTION OF ALLOXAN AND GLUTATHIONE*

Name of compound	Chemical structure	Absorption maximum (mμ)	pH range† (maximum)	Maximal optical density	Reaction time (min)
	ORGANIC				
Glycine	$HOOCCH_2NH_2$	278–280	7·1–9·9 (7·6)	1·207	120
Alanine (DL)	$HOOCCH(CH_3)NH_2$	278–280	6·7–8·5 (7·9)	0·960	280
2-Amino butyrate (DL)	$HCOOCCH(CH_2CH_3)NH_2$	282	6·5–8·5 (7·5)	1·048	225
Glycylglycine	$HOOCCH_2NHCOCH_2NH_2$	276	7·2–8·0 (7·2)	1·202	75
Ethyl aminoacetate	$CH_3CH_2OOCCH_2NH_2$	276	5·7–8·0 (7·0–7·2)	1·340	50
4-Amino-*n*-butyric	$NH_2CH_2CH_2CH_2COOH$	303	6·2–8·1 (6·7)	0·890	60
Acetyl glycine	$CH_3CONHCH_2COOH$	305	7·3–8·2 (7·8)	0·632	45
N:*N*'dimethyl glycine	$(CH_3)_2NCH_2COOH$	305–310	7·3–8·0 (7·7)	0·960	60
Acetic acid	CH_3COOH	311	7·4–7·6 (7·5)	0·700	30
"Tris"	$(CH_2OH)_3CNH_2$	305	7·5–8·5 (7·5)	0·708	150
	INORGANIC				
Phosphate		305	7·4	0·740	60
Arsenate		305	7·5	0·860	10
Borate			7·6–8·9		

* One μmole of glutathione was added to 8·5 ml of 0·2 M of each compound. One to 2 μmoles of alloxan was added with water to make a total volume of 10 ml. The reaction time in minutes represents the interval between adding the alloxan and attaining the maximal optical density.

† Many of the compounds studied have very little buffering capacity below pH 8·0. Therefore, the range of pH at the end of the reaction is given, with the pH yielding the maximal optical density in brackets.

absorption maximum in glycine was stable. The 305-mμ-absorbing compound—alloxan and glutathione were reacted in phosphate buffer at pH 7·7—could not be transformed to a 280-mμ-absorbing compound by the addition of glycine and vice versa.

Other compounds were evaluated as substitutes for glycine and phosphate in this reaction in terms of absorption maximum, pH, maximal yield of absorbance and time to attain maximal absorbance (Table 1). Alanine and 2-amino butyrate, both of which have the α-carboxyl and α-amino groups, yielded the 280-mμ absorption maximum. Glycylglycine and 4-amino-*n*-butyric acid have both functional groups but different p*K*'s. Glycylglycine yielded a 280-mμ absorption maximum, 4-amino-*n*-

butyric acid a 305-mμ absorption maximum. Ethyl aminoacetate, an analogue of glycine in which the α-carboxyl group is masked, led to the formation of a compound absorbing near 280 mμ (Table 1). However, *N*:*N'* dimethylglycine and acetylglycine, in which the α-amino group of glycine is masked, formed a product absorbing at 305 mμ (Table 1). Acetic acid produced a 311-mμ-absorbing compound in this reaction and Tris buffer [2-amino-2-(hydroxymethyl 1:3 propanediol)] reacted to yield a 305-mμ compound.

Two different inorganic anions (arsenate and borate) were substituted for phosphate in this reaction. The characteristic 305-mμ-absorption maximum was obtained only with arsenate. The rate of formation with arsenate was considerably faster than in phosphate buffer, and the resulting product was considerably more labile. Since no absorption peaks were observed in borate buffers over a pH range of 7·6 to 8·9, it was shown that 90 per cent of the original sulfhydryl group remained in the reduced state, whereas in those reactions yielding either a 305-mμ- or a 280-mμ-absorption maximum, less than 5 per cent of the original sulfhydryl was detected.

Effect of pH on the formation and stability of the 280-*mμ and* 305-*mμ compounds.* Maximal optical density was attained within the pH range of 7·0 to 7·6 (Fig. 2) in the

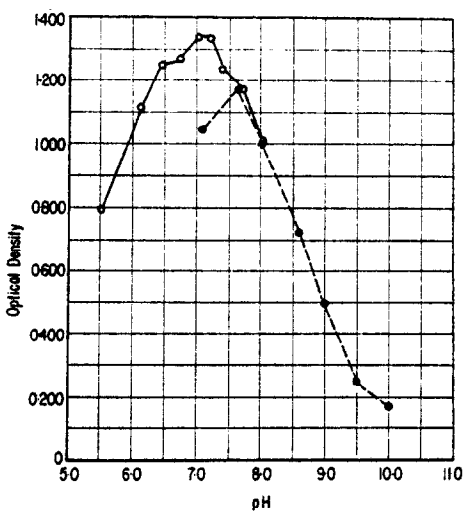

FIG. 2. The effect of pH on the production of the 276-mμ or 278-mμ absorption maxima using ethyl aminoacetate (○—○) or glycine solutions (●—●). The reaction mixture was 2 μmoles of glutathione added to 1·7 m-moles of either ethyl aminoacetate or glycine solutions of known pH. Two μmoles of alloxan solution was added to start the reaction and the total volume was 10 ml.

reaction of alloxan with glutathione plus glycine or ethyl aminoacetate. The 280-mμ compound in ethyl aminoacetate was stable at the pH maximum for at least 3 days at room temperature (20°) or at refrigerator temperatures. The product was stable for 19 hr at 30° and 37° and one-third of the original absorbing compound remained after 72 hr at 30°. This 280-mμ-absorbing compound was slightly less stable in more alkaline solution (pH 9–10) under similar experimental conditions. If the original eaction mixture were made acid (pH 1), the absorption maximum disappeared

immediately at room temperature. The lability of the 280-mμ compounds in acidic solutions is shown in Fig. 3. The pH corresponding to one-half of the initial 280-mμ absorption was 5·67 ± 0·04 irrespective of the molar ratio of alloxan and glutathione or the initial pH of the glycine buffer used. The final reaction product after acid (pH 2·6) treatment was neutralized to pH 7·2 and the resultant absorption was nearly identical to the initial reaction product. The 276-mμ compound produced after acid treatment was not stable in alkaline pH and was retained by a cationic exchanger unlike the original 276-mμ-absorbing compound. This suggests that the loss of the

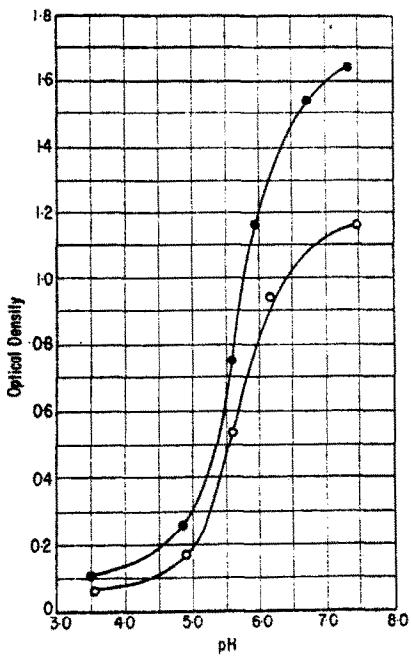

FIG. 3. The disappearance of the 280-mμ-absorbing compound on lowering the pH of the reaction mixture. The reaction mixture was 20 μmoles of glutathione added to 1·7 m-moles of glycine, pH 7·76. In each case the alloxan (○—○, 20 μmoles; ●—●, 40 μmoles) was added to start the reaction and the total volume was 100 ml. After 2 hr of standing at room temperature, the pH was adjusted with 0·25 N HCl, and after every decrease of approximately 0·2-pH unit, a 5-ml aliquot was taken for optical density measurements at 280 mμ.

280-mμ absorption in acid was not related to the titration of an ionizable functional group(s).

All of the 305-mμ-absorbing compounds were labile at room temperature as defined by disappearance of the ultraviolet absorption at the pH for maximal formation of the compound.

Ion-exchange chromatography and electrophoresis of the 280-mμ-absorbing compound. The 280-mμ-absorbing species were stable and attempts were made to isolate the absorbing products from the reactants by ion-exchange chromatography. Glutathione, alloxan and ethyl aminoacetate were reacted at pH 7·2, aliquots of the reaction mixture were adjusted to pH 5·5, 7·5 and 10·0 and these solutions were chromato-

graphed on Dowex-2 columns (11 × 1·2 cm) equilibrated with ethyl aminoacetate of the same pH. Approximately 90–95 per cent of the absorbing compound was recovered in the eluate. Thus, the original compound was not adsorbed, implying that no free carboxyl group was present. After treating the original product with acid, readjusting the pH to 7·2, and chromatographing it on Dowex-2 columns at pH 7·2, at least 80 per cent of the original absorbing compound was recovered in this eluate. This finding probably means that no free anionic group was liberated by acid treatment; also, acid treatment did not liberate any (<5 per cent) sulfhydryl groups as measured by the nitroprusside method.[8]

Experiments with a strong cationic-exchange resin (ZeoKarb 225) employed citrate buffers to maintain the desired pH. At a pH of 6·60 approximately 98 per cent of the 276-mμ-absorbing compound was recovered in the eluate. After adjusting the pH of the reaction mixture to 5·7, which lowers the absorption at 276 mμ approximately 50 per cent, about 60 per cent of the absorption was recovered in the eluate. After chromatography at pH 3·7, none of the absorbing compound was observed in the eluate even after adjusting the pH of the eluate to 7·2. The pH of the 276-mμ-absorbing solutions was first lowered to pH 3 and then readjusted to pH 6·5 with recovery of most of the absorbance. This reaction mixture was chromatographed on

TABLE 2. PAPER ELECTROPHORESIS OF PRODUCTS FROM THE REACTION OF ALLOXAN AND GLUTATHIONE IN ETHYL AMINOACETATE BUFFER*

	Distance migrated towards anode or cathode using glycine as reference	
Compounds	Anode (mm)	Cathode (mm)
Controls		
Glycine	0	0 brown†
Ethyl aminoacetate		75 yellow
Glutathione	38 purple	
Glutamic acid	52 purple	
Reaction mixture		
1. Acid-treated 276-mμ-absorbing compound		85 purple
2. Ethyl aminoacetate		75 yellow
3. 276-mμ-Absorbing compound	0	0 purple-brown
4. Tripeptide	40 purple	
5. Dipeptide	61 purple	

* After the reaction attained maximal optical density, the water was removed *in vacuo* and 20–40 μl of a concentrate was applied to the paper 1·5–2·0 cm from the center towards the anode. Electrophoresis was carried out as described in the experimental procedure. Glycine migrated towards the cathode approximately 1·5–2·0 cm and was considered electrically neutral at pH 5·9.

† Indicates the color of the ninhydrin-positive spots.

ZeoKarb 225 at pH 6·6 and none of the absorbing compound was observed in the eluate. From these data, it was apparent that acid treatment resulted in the formation of a cationic group that was not present in the original product.

Concentrates of the reaction mixture (glutathione, alloxan and ethyl aminoacetate) were subjected to paper electrophoresis and several ninhydrin-positive spots were found (Table 2). The ethyl aminoacetate reacted with ninhydrin to yield a yellow spot. A ninhydrin-positive component, purple in color, migrated in front of the ethyl aminoacetate and was apparent at greater concentrations after acid treatment. The neutral compound was absent if the reaction product had been previously treated with

acid. This neutral compound gave a positive hydroxylamine reaction for esters (ethyl aminoacetate moiety). The other two compounds that migrated towards the anode were minor constituents. These two minor constituents were isolated by chromatography on Dowex-2 columns, pH 6. The fractions were hydrolyzed in 6 N HCl for 18 hr and the amino acid identified by paper chromatography. Fraction 5 contained glutamic acid and glycine, and Fraction 4 contained glutamic acid, glycine and an unknown substance having at R_f of 0·33 in a phenol–water solvent system.

DISCUSSION

Alloxan reacts with glutathione or protein in phosphate buffer to yield a 305-mμ-absorbing product,[2–5] and several investigators[2, 3] have shown that a reduced sulfhydryl group is required for the reaction. In our studies we have demonstrated that phosphate, other oxygen anions or amine bases with a lone pair of electrons are also an integral part of the reaction between alloxan and glutathione. The nature of this third component is important in producing either a stable product having an absorption maximum around 280 mμ or an unstable product having an absorption maximum around 305 mμ.

Alloxan and glutathione reacted with amines to yield a 280-mμ-absorbing product. Ethyl aminoacetate and glycylglycine substituted for glycine in forming a compound absorbing at 280 mμ. The pH maxima of these reactions with these three compounds are 7·1, 7·2 and 7·7 respectively. The pK values for ethyl aminoacetate, glycylglycine and glycine are 7·7, 8·2 and 9·6 respectively. In 4-amino-*n*-butyric acid the amino group has a pK of 10·6. This compound yielded only the 305-mμ compound over the pH range of 6·2 to 8·1 (Table 1). Under these experimental conditions the amino group of 4-amino-*n*-butyric acid was completely ionized (more than 99·7 per cent). This suggested that the amino group as a salt does not react and that the reacting substance was R-NH_2, having a lone pair of electrons. In all probability, R-NH_2 reacted with a carbonyl of alloxan.

Alloxan and glutathione in Tris buffer reacted unexpectedly and yielded a 305-mμ compound (Table 1). The hydroxyls of 2-amino-2-(hydroxymethyl)-1,3-propanediol do not ionize and the pK value for the amino group is 8·08.[9] Tris reacts with carbonyls and in the reaction of Tris with pyruvic acid, two products (Fig. 4, I and II) have been

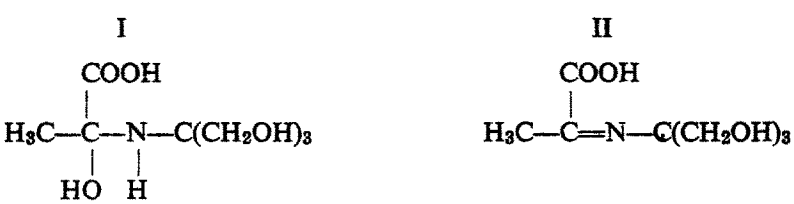

FIG. 4. Compounds I and II.

formed with a predominance of compound I.[10] Tris might react similarly with alloxan in this reaction between alloxan and glutathione, and it is probable that the 305-mμ-absorbing product may have a grouping ($>C<^{OH}_{X}$) similar to compound I. Likewise, a similar hemiketal structure may be produced if oxygen anions reacted with the carbonyls of alloxan.

Amines might react with a carbonyl of alloxan and produce a grouping (Schiff

bases) similar to compound II. The characteristics of Schiff bases ($R_2C = N—R'$) agree with the data on the formation and stability of the 280-mμ-absorbing compound: maximal formation in neutral solution, stability in alkaline solutions and lability in acid to produce a cationic group. Mild acid treatment did produce a new compound, which was adsorbed to a cation exchange resin and which migrated towards the cathode at pH 5·9.

The 276-mμ-absorbing product (alloxan, glutathione and ethyl aminoacetate) was electrically neutral and was not adsorbed to ion-exchange resins, which suggests that other functional groups of glutathione, i.e. the carboxyl groups of glycine and the amino and carboxyl groups of glutamic acid, have reacted in addition to the sulfhydryl group of cysteine. The available data[11–14] indicate that relatively mild conditions can degrade glutathione to cysteinylglycine and glutamic acid. The carbonyl group of glycine in glutathione might preferentially cyclize with the amino group of cysteine with the removal of the glutamyl moiety of glutathione. Thus, the following structure (Fig. 5) is proposed for the reaction product of glutathione, alloxan and ethyl aminoacetate.

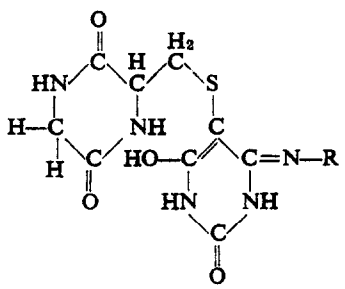

FIG. 5. Structure III. This type of structure would be electrically neutral and would contain a conjugated unsaturation that would absorb ultraviolet light.

The stability of the reaction product of alloxan, glutathione, and a third component is believed to have relevancy to the action of alloxan in producing a type of diabetes. Glutathione levels in the blood were lowered by the alloxan treatment.[15] The injection of borate simultaneously with alloxan minimized the effects of alloxan in producing diabetes.[16, 17] We have demonstrated that borate ions prevented the reaction between alloxan and glutathione. Simultaneous injection of ammonium salts with alloxan potentiates the diabetogenic action of alloxan.[18] This fact and our observation that alloxan and glutathione react in the presence of a nitrogen base to yield a stable product at physiological conditions create incentives to investigate further the product of such reactions in producing a type of diabetes.

Acknowledgement—The author is grateful to Dr. S. K. Kon for sponsoring the National Science Foundation Fellowship, and to Dr. John Moore for his helpful advice.

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