[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AND THE CLAYTON FOUNDATION FOR RESEARCH]

Benzene Analogs of Pyridoxal. The Reactions of 4-Nitrosalicylaldehyde with Amino Acids

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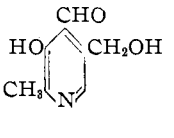
4-Nitrosalicylaldehyde simulates pyridoxal in its reactions with several amino acids. Like pyridoxal it catalyzes the dehydration of serine, the desulfhydration of cysteine and the splitting of threonine to glycine. Unlike pyridoxal, which undergoes transamination with glutamic acid to yield α-ketoglutaric acid and pyridoxamine, 4-nitrosalicylaldehyde oxidatively deaminates this amino acid to yield α-ketoglutaric acid, ammonia and an aromatic amine. Like the reactions of pyridoxal, each of these reactions is metal-ion catalyzed. Of several other substituted salicylaldehydes tested, only 6-nitrosalicylaldehyde catalyzed these reactions; salicylaldehyde itself and *p*-nitrobenzaldehyde were also ineffective. Thus the *o*-hydroxy group and a strong electron-attracting group in the 4- or 6-position of salicylaldehyde are essential for these reactions, facts that emphasize the role and significance of the corresponding groupings in pyridoxal.

In the presence of ions of aluminum, iron, or copper, pyridoxal (I) catalyzes a number of reactions with



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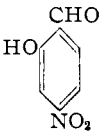

II

various amino acids that are models for those catalyzed by enzymes containing vitamin B_6, *e.g.*, amino acid transamination[1] and racemization,[2] serine dehydration,[3] and cysteine desulfhydration.[3] To determine the requirements in chemical structure needed to carry out these reactions and to gain an insight into their mechanism the possibility of finding pyridoxal-like compounds in the benzene series was considered.

In a number of reactions the behavior of the pyridine ring parallels that of nitrobenzene.[4] 4-Nitrosalicylaldehyde (II) in which the position of the nitro group with respect to the formyl and phenolic groups corresponds to that of the heterocyclic nitrogen atom in pyridoxal, was prepared and found to resemble pyridoxal closely in several of its reactions. Thus, it reacts with glutamic acid to form α-ketoglutaric acid (Tables I–III), with serine and cysteine to form pyruvic acid (Tables II and III), and catalyzes the splitting of threonine to yield glycine[5] (see Experimental). Like the analogous reactions in which pyridoxal participates, these reactions were catalyzed by aluminum ions at *p*H 4–5 but also took place at *p*H 12 or above without the mediation of aluminum. The importance of the phenolic hydroxyl group for the catalysis of these reactions was shown by the fact that *p*-nitrobenzaldehyde failed to form significant amounts of the corresponding keto acids from either glutamic acid or serine (Table I). The importance of the position of the nitro group was shown by the failure of 5-nitro- and 3,5-dinitrosalicylaldehydes to carry out any of these reactions (Table I). 6-Nitrosalicylaldehyde, however, in which the nitro group occupies an electronically equivalent position with regard to both the formyl and phenolic groups as in 4-nitrosalicylaldehyde, behaved like the latter in its reaction with glutamic acid.

TABLE I

KETOGLUTARATE FORMATION FROM REACTION BETWEEN L-GLUTAMIC ACID AND VARIOUS ALDEHYDES[a]

Aldehyde	Ketoglutarate formation[b] *p*H 4–5 With alum	*p*H 4–5 Without alum	*p*H 11.7–12.3 With alum	*p*H 11.7–12.3 Without alum
Salicylaldehyde	−		−	−
4-Nitrosalicylaldehyde	+++	+	+++	+++
6-Nitrosalicylaldehyde	+++			
5-Nitrosalicylaldehyde	−		−	−
3,5-Dinitrosalicylaldehyde	−			
4-Chlorosalicylaldehyde	−		−	−
4-Carboxysalicylaldehyde	−		−	−
4-Hydroxysalicylaldehyde	−		−	−
2-Hydroxy-1-naphthaldehyde	−			
4-Nitrobenzaldehyde	±	±		

[a] Reaction mixtures were 0.01 *M* in L-glutamic acid, 0.01 *M* in aldehyde (added either in aqueous solution or in solid form), 0.001 *M* in potassium aluminum sulfate, and contained either 2 ml. of 0.5 *M* acetate buffer, *p*H 5.0, or 2 ml. of 0.5 *N* sodium hydroxide per 10 ml. of solution. These were sealed in tubes and heated in a boiling water-bath for 30 minutes, cooled, and opened for analysis (see Experimental). [b] A strongly positive test for ketoglutarate (see text) is indicated by +++; +, ± and − indicate, respectively, a weakly positive, a very weakly positive, and a negative test for this substance.

That the presence of a powerful electron-attracting group in the 4- or 6-position of salicylaldehyde is a prerequisite for these reactions was shown by the failure of salicylaldehyde itself, of 2-hydroxy-1-naphthaldehyde, and of 4-hydroxy-, 4-chloro- and 4-carboxysalicylaldehydes to bring about the reactions. Although the carboxyl group is meta directing, it apparently is not a sufficiently powerful electron-attracting group to effect deamination (Table I). The significance of such an electron-attracting group for the reactions of 4-nitrosalicylaldehyde and of pyridoxal is discussed in an accompanying paper.[6]

The reactions of serine and cysteine with 4-nitrosalicylaldehyde appear to proceed similarly as with

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(3) D. E. Metzler and E. E. Snell, *ibid.*, **198**, 353 (1952).

(4) H. S. Mosher, "Heterocyclic Compounds," Vol. I, ed. by R. C. Elderfield, John Wiley and Sons, Inc., New York, N. Y., 1950, pp. 408–409.

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TABLE II

REACTION OF 4-NITROSALICYLALDEHYDE WITH GLUTAMIC ACID, SERINE AND CYSTEINE AT 100°[a]

Reactants	Product measured	Approx. pH of reacn.	Yield of keto acid in 30 minutes, % With alum	Yield of keto acid in 30 minutes, % Without alum
4-Nitrosalicylaldehyde + L-glutamic acid	α-Keto-glutaric acid	1.9	0.0	0.0
		4.0	9.3	.2
		5.6	2.9	.7
		8.2	1.0	.8
		9.6	0.8	.7
		11.8	2.3	1.9
4-Nitrosalicylaldehyde + DL-serine	Pyruvic acid	1.9	0.0	0.0
		4.1	7.2	.1
		5.8	1.7	.1
		8.6	0.6	.2
		9.0–9.7	0.6	.9
		11.8	7.1	7.6
4-Nitrosalicylaldehyde + L-cysteine	Pyruvic acid	1.9	0.0	0.3
		4.0	29.1	1.2
		5.6	43.5	13.9
		8.3	36.1	31.5
		8.6–9.1	40.1	25.7
		11.8	11.7	11.7
4-Nitrobenzaldehyde + DL-serine	Pyruvic acid	4.0	0.25	0.0
		11.8	0.4	0.25

[a] Reaction mixtures were 0.001 *M* in L-glutamic acid, DL-serine or L-cysteine, 0.001 *M* in 4-nitrosalicylaldehyde and 0.0001 *M* in potassium alum, and were heated in sealed tubes in a boiling water-bath.

phenyl)-4-thiazolidinecarboxylic acid (III), the product analogous to that from pyridoxal and cysteine.[7]

Pyridoxal reacts with glutamic acid by transamination to form equimolar amounts of pyridoxamine and α-ketoglutaric acid. The reaction of glutamic acid with 4-nitrosalicylaldehyde differs from that with pyridoxal in that for each mole of keto acid observed slightly more than one mole of ammonia appeared (Table III). Thus instead of a transamination reaction, an oxidative deamination of glutamic acid appears to have taken place. That oxygen is not the oxidizing agent was shown by the formation of nearly the same amount of ammonia when the reaction and subsequent operations were conducted in an atmosphere of nitrogen. The reaction mixture after hydrolysis with hydrochloric acid gave a positive Bratton–Marshall test for diazotizable aromatic amine which strongly suggests that the nitro group of the aldehyde is acting as the oxidizing agent. Estimation of the maximum recoverable 4-nitrosalicylaldehyde showed approximately 60% recovery after one hour at 100° whereas in its reaction with serine the figure was 86%, giving further evidence that the reagent was being altered in the reaction with glutamic acid. The reduction of the nitro group may involve the addition of a proton to the oxidizing agent prior to the hydrolysis of the amino acid–aldehyde–metal complex.

Limited tests of the action of 4-nitrosalicylaldehyde on microörganisms have shown it to be a potent inhibitor of growth. Its inhibitory properties have not been counteracted by vitamin B_6 in those instances tested.

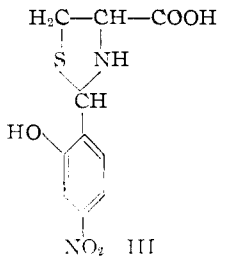
TABLE III

STOICHIOMETRY OF REACTION OF 4-NITROSALICYLALDEHYDE WITH AMINO ACIDS[a]

Reaction of 4-nitrosalicylaldehyde with	Reaction atmosphere	Hr. at 100°	μM. keto acid per 10 ml. With alum	μM. keto acid per 10 ml. Without alum	μM. NH_3 per 10 ml. With alum	μM. NH_3 per 10 ml. Without alum	Aldehyde recovered, % With alum Direct	Aldehyde recovered, % With alum After hydrol.	Aldehyde recovered, % Without alum
No amino acid	Air	1	0.00		0.04		96		
	Air	2	.00		0.04			94	
L-Glutamic acid	Air	1	.94	0.05	1.1	0.06		60	93
	O_2	1	1.02		1.2			60	
	N_2	1	0.94		1.1			58	
	N_2	2	1.25		1.4				
DL-Serine	Air	1	0.55	.01	0.7	.07	51	86	92
L-Cysteine	Air	1	0.25	.02	1.0	.20	27	41	49

[a] Reaction mixtures 0.001 *M* in 4-nitrosalicylaldehyde, 0.005 *M* in the indicated amino acid, and 0.001 *M* in potassium alum and containing 1 ml. of 0.5 *M* acetate buffer (pH 4.0) per 10 ml. were heated in sealed tubes for the indicated reaction time before analysis.

pyridoxal[3] in that both pyruvic acid and ammonia are liberated, and also hydrogen sulfide in the case of cysteine. With serine slightly more than one mole of ammonia was observed per mole of keto acid determined whereas with cysteine considerably more ammonia was observed. The reaction between cysteine and 4-nitrosalicylaldehyde is complicated by the formation of 2-(2-hydroxy-4-nitro-



III

Experimental

Materials.—Salicylaldehyde, 5-nitrosalicylaldehyde, 4-hydroxysalicylaldehyde and 4-nitrobenzaldehyde were Eastman Kodak Co. products; 2-hydroxy-1-naphthaldehyde was obtained from Abbott Laboratories. 4-Carboxysalicylaldehyde,[8] 4-chlorosalicylaldehyde,[9] 6-nitrosalicylaldehyde,[10] 3,5-dinitrosalicylaldehyde[11] and 4-nitrosalicylaldehyde[12] were prepared according to the references cited.

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Reaction between Glutamic Acid and Various Aldehydes.—The composition of the reaction mixtures is given in Table I. For the qualitative demonstration of α-ketoglutaric acid formation 2 ml. of a 0.1% solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid was added to 0.5-ml. aliquots of the reaction mixtures, the solutions allowed to stand for 30 minutes, then extracted with ethyl acetate, and suitable portions of the ethyl acetate extracts chromatographed on paper as described previously.[3,13] The low R_f value of the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid corresponded with that of an authentic sample and allowed it to be readily distinguished from the aldehyde dinitrophenylhydrazones in every case. The results are given in Table I.

Quantitative Keto Acid Determinations in the Reaction of 4-Nitrosalicylaldehyde with Glutamic Acid, Serine and Cysteine.—Composition of the reaction mixtures is given in Table II. The *p*H values were measured after the reaction, and were maintained by use of 0.02–0.04 *M* acetate or bicarbonate buffers in the central *p*H range, and the addition of appropriate amounts of HCl or NaOH at low and high *p*H values, respectively. Quantitative keto acid determinations on 3-ml. aliquots of the above reaction mixtures were carried out according to Metzler and Snell[1]; the results are given in Table II. The keto acid formed in the reactions with serine and cysteine was shown to be pyruvic acid by chromatography and spectral analysis of its 2,4-dinitrophenylhydrazone.[3,13]

Reaction of 4-Nitrosalicylaldehyde with Excess Glutamic Acid, Serine and Cysteine.—Keto acid and ammonia determinations[3] were made on aliquots of the reaction mixtures specified in Table III. Recorded values for recoverable aldehyde were estimated directly by adding 2 ml. of 6 *N* hydrochloric acid to a 5-ml. aliquot of the reaction mixture, extracting with 10.0 ml. of toluene, pipetting off a 5.0-ml. aliquot of the toluene extract, extracting the latter with 15.0 ml. of 1 *N* sodium hydroxide, filtering 10 ml. of the sodium hydroxide extract, and comparing the color intensity of the filtrate with that of a similarly treated standard in an Evelyn colorimeter using a 490 filter. With reaction mixtures containing aldehyde, amino acid and alum, the acidified solution remained yellow-orange in color even after extraction with toluene, apparently due to presence of a relatively stable complex (Schiff base?) between the aldehyde and amino acid. On heating these acidified solutions on a steam-bath for 15 minutes the glutamic acid and serine reaction mixtures became almost colorless whereas the cysteine reaction mixture retained its yellow-orange color. Aldehyde determinations were also run on these "hydrolyzed" solutions (Table III).

The Bratton–Marshall determination[14] for diazotizable aromatic amines was negative when applied directly to the glutamic acid reaction mixtures, but after hydrolysis showed a 9% conversion of 4-nitrosalicylaldehyde to aromatic amine. *m*-Aminophenol was used as the standard. The amount of aromatic amine formed in oxygen and nitrogen atmospheres was identical. A blank containing all reagents except glutamic acid gave a value of 0.5%.

Reaction between Various Amines and Nitro Compounds.—Reaction mixtures similar to those used earlier with glutamic acid, but containing benzylamine or pyridoxamine as the amino compound and *m*-nitrophenol or 4-nitrosalicylaldehyde as the nitrophenol were heated with alum at *p*H 4.0 and 100° for 1–2 hours. Negligible amounts of ammonia were formed.

Reaction between Threonine and 4-Nitrosalicylaldehyde.—This reaction was carried out at *p*H 5 as described in the section on reaction between glutamic acid and various aldehydes. Aliquots of the reaction mixtures were spotted on paper, chromatographed with 77% ethanol as the developing agent, and sprayed with ninhydrin. In the mixture in which alum had been omitted only the threonine spot appeared whereas in the mixture containing alum both threonine and glycine were observed. It has been shown previously[5] that no cleavage of threonine to glycine occurred in the absence of pyridoxal; 4-nitrosalicylaldehyde is an effective substitute for the latter.

2-(2-Hydroxy-4-nitrophenyl)-4-thiazolidinecarboxylic Acid (III).—To 25 ml. of 0.5 *M* acetate buffer (*p*H 4.0) were added 84 mg. (0.5 mmole) of 4-nitrosalicylaldehyde and 160 mg. (1 mmole) of L-cysteine hydrochloride. The mixture was heated to boiling, water added until all the solid had dissolved, and the solution heated on a steam-bath for one hour. On cooling, 98 mg. of fine silky needles was obtained. Recrystallization from water yielded 68 mg. of the dihydrate, m.p. 160° dec. (cor.).

Anal. Calcd. for $C_{10}H_{10}O_5N_2S\cdot 2H_2O$: C, 39.2; H, 4.6; N, 9.15. Found: C, 39.4; H, 4.6; N, 9.1.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY, AND THE DEPARTMENT OF SURGERY, BETH-ISRAEL HOSPITAL, AND HARVARD MEDICAL SCHOOL]

Preparation of N-Phosphorylated Derivatives of Bis-β-chloroethylamine[1a]

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In order to re-examine the distribution of phosphamidase in normal and malignant tissues with substrates of a type that could be used as possible chemotherapeutic agents, a number of N-phosphorylated derivatives of bis-(β-chloroethyl)-amine were prepared. Treatment of the amine hydrochloride with phosphorus oxychloride afforded the dichlorophosphamide III from which the triamidophosphonate IV was obtained with ammonia. Reaction of the dichlorophosphamide III with one molar equivalent of phenol gave the corresponding phenylchlorophosphamide (V). When V was treated successively with ammonia, ethanol, *t*-butyl alcohol and neopentyl alcohol intermediates were obtained from which the corresponding amido and alkyl ester monobasic acids IX, XIII, XIV and XV were prepared by replacement of the phenyl group by hydrogenolysis over platinum. When V was treated with benzyl alcohol followed by hydrogenolysis over palladium the phenyl ester monobasic acid VIII was obtained. By deamidation of the corresponding phenylamidophosphamide VI with nitrous acid VIII was also obtained.

Phosphamidase activity has been ascribed to various preparations from plant and animal sources,[2] although the natural substrate is unknown. Based on an earlier claim for the synthesis of *p*-chloroanilinophosphoric acid[3] methods have been developed for estimation of phosphamidase activity quantitatively by the titration of phosphoric acid liberated enzymatically[3]; and histochemically at *p*H 5.4–5.8 by the formation of lead phosphate at the site of enzymatic action in tissue sections.[4] From histochemical studies with this substrate

(1) (a) This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Department of Health, Education and Welfare, and in part by a research grant from Mrs. Albert B. Lasker. (b) School of Science, Brandeis University, Waltham, Mass.

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