Anal. Calcd. for $C_9H_7N_3S_2$: C, 48.84; H, 3.19; N, 18.99. Found: C, 48.51; H, 2.92; N, 18.53.

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Nitrous Acid Oxidation of Triacyl Pyridoxamine[1]

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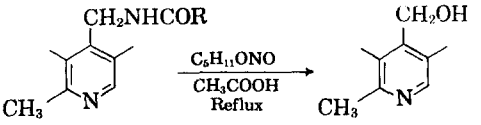
Based on elemental analysis, it appeared probable that the nitrous acid oxidation product (MDP) of *N,O,O*-tripalmitoylpyridoxamine was a mixture of mono- and di-palmitoylpyridoxine.[2] Since this reaction represented direct conversion of the acylated amino to a hydroxyl group, further studies were carried out with various acyl derivatives of pyridoxamine.

Several workers have synthesized *N*-nitroso derivatives of acylated primary amines and their reactions have been investigated.[3] Heyns *et al.* observed that the nitroso derivatives of *N*-*n*-propylacetamide, *N*-*n*-butylacetamide, and *N*-benzylacetamide could be thermally decomposed to form acetates of the corresponding alcohols.[4]

The transformation of free pyridoxamine to pyridoxine under mild treatment with nitrous acid has been reported.[5,6] The test compounds used were triacetyl-, tridecanoyl, tripalmitoyl-, tribenzoyl- and tri-*p*-nitrobenzoyl-pyridoxamine. When the acyl pyridoxamine was refluxed in a mixture of isoamyl nitrite and glacial acetic acid (1:2 v/v) for 30–60 *min.*, complete replacement of the acylated amino group with a hydroxyl group took place. Evidence for this conversion has been obtained by paper chromatography and microbiological assay using a minute quantity of the test compound.

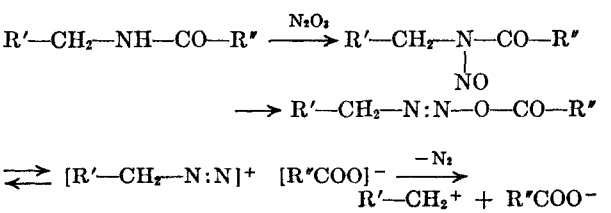
The triacylpyridoxamine was hydrolyzed with 2*N* ethanolic potassium hydroxide by refluxing for 30 min. The resulting hydrolysate contained two



fractions detectable on the papergram with *N*, 2,6-trichloro-*p*-quinoneimine. One was free pyridoxamine and the other was a high R_f fraction, which was probably the corresponding *N*-monoacylpyridoxamine (Table 1). The ester linkages at the 3- and 5-positions must have been cleaved during the alkali treatment, since complete hydrolysis of various *O,O,O*-triacylpyridoxines resulted under identical conditions. If the triacylpyridoxamine was treated with nitrous acid prior to alkali hydrolysis, the only vitamin B_6 component present in the final hydrolysate was found to be pyridoxine (Table 1). Oxidation with nitrous acid under milder conditions gave a mixture of pyridoxamine, pyridoxine, and a high R_f fraction (Table 1). In all cases, pyridoxal was found to be absent.

The theoretical amount of pyridoxine moiety in MDP was calculated from the nitrogen analysis. A portion of MDP was also subjected to microbiological assay. The two values thus obtained were in complete agreement indicating that the nitrogen present in MDP accounted for all of the nitrogen in the pyridoxine molecule. By paper chromatography, it also became clear that MDP contained pyridoxine free from pyridoxamine. However, free pyridoxine, pyridoxal, pyridoxamine as well as the pyridoxamine moiety present in the alkali hydrolysate of tridecanoylpyridoxamine[7] was totally destroyed by refluxing in a mixture of isoamyl nitrite and acetic acid for one hour. This was shown by color test with *N*, 2,6-trichloro-*p*-quinoneimine and by microbiological assay. Protection of one or both of the 3-hydroxyl and 5-hydroxymethyl groups in vitamin B_6, therefore, appeared to be essential in order to prevent destruction of the vitamin fragment. When 3-monopalmitoylpyridoxal[2] or 5-monopalmitoylpyridoxine[2] was similarly treated, the vitamin B_6 moiety withstood the nitrous acid treatment.

The following equations may be of value to explain the conversion of the acylated amino group to



(1) This work was supported by research grant A-257 from the National Institutes of Health, Department of Health, Education, and Welfare. The pyridoxamine used in this study was kindly supplied by Merck and Co. through the courtesy of Dr. Max Tishler.

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(3) (a) H. v. Pechmann and L. Frobenius, *Ber.*, **27**, 651 (1894); (b) E. Bamberger, *Ber.*, **27**, 914 (1894); (c) G. F. D'Alelio and E. E. Reid, *J. Am. Chem. Soc.*, **59**, 109 (1937); (d) Von R. Husigen and J. Reinertshofer, *Z. Naturforsch.*, **6b**, 395 (1951); (e) K. Heyns and O. Woyrsch, *Ber.*, **86**, 76 (1953); (f) Von R. Husigen and H. Nakaten, *Ann.*, **586**, 84 (1954).

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(7) Tridecanoyl pyridoxamine was refluxed in 2*N* ethanolic potassium hydroxide for 30 min. After neutralization with ethanolic hydrogen chloride, the precipitate of potassium chloride was removed by filtration and the solvent was evaporated until dryness *in vacuo*. The residue was then used for the nitrous acid oxidation.

TABLE I

PAPER CHROMATOGRAM OF ALKALI HYDROLYSATE OF ACYLPYRIDOXAMINE TREATED WITH NITROUS ACID UNDER VARIOUS CONDITIONS

Test Compound	Treatment[a]	Spots Observed[b] (Solvent: 1-Butanol Saturated with Water) Pyridoxamine (R_f 0.06)	Pyridoxine (R_f 0.24)	High R_f fraction[c]
Triacetylpyridoxamine	A	+	−	− (R_f 0.28)
	E	−	+	−
Tribenzoylpyridoxamine	A	+	−	+ (R_f 0.82)
	E	−	+	−
Tri-*p*-nitrobenzoylpyridoxamine	A	+	−	+ (diffused)
	E	−	+	−
Tridecanoylpyridoxamine	A	+	−	+ (R_f 0.86)
	B	+	−	+
	C	+	+	+
	D	+	+	+
	E	−	+	−
	F	+	−	+
Tripalmitoylpyridoxamine	A	+	−	+ (R_f 0.88)
	E	−	+	−

[a] A few mg. of the test compound was treated as follows: A—Refluxed for 30 min. in 0.5–1.0 ml. of 2*N* ethanolic potassium hydroxide solution. Each of the treatments from B to F was followed by treatment A after thorough removal of the solvent *in vacuo*. B—Dissolved in a mixture of 0.5 ml. of ethanol, 0.5 ml. of isoamyl nitrite, and 2 drops of concentrated hydrochloric acid. Allowed to stand at room temperature for 12 hr. C—Dissolved in a mixture of 0.5 ml. of isoamyl nitrite and 1 ml. of glacial acetic acid. Allowed to stand at room temperature for 120 hr. D—Same as C, but refluxed for 15 min. E—Same as C, but refluxed for 45 min. F—Same as E, but 1 ml. of dioxane was used instead of 1 ml. of glacial acetic acid. [b] Chromogenic reagent was *N*,2,6-trichloro-*p*-quinoneimine. + indicates presence; − indicates absence. [c] Probably the corresponding *N*-monoacyl pyridoxamine.

a hydroxyl group, although no attempt has been made to isolate a nitroso compound as a possible intermediate.

If the chemical conversion observed in the present study was identical with the one reported by Heyns *et al.*,[4] the immediate product from the possible intermediate, *N*-nitroso- *N,O,O*-triacylpyridoxamine, would be the corresponding triacylpyridoxine. However, when *O,O,O*-tripalmitoylpyridoxine[2] was refluxed in a mixture of isoamyl nitrite and glacial acetic acid, no chemical change took place and unchanged starting material was recovered. This indicated that MDP could not be derived from tripalmitoylpyridoxine and was probably a product formed directly from the *N*-nitroso intermediate or through some other steps.

EXPERIMENTAL

Microbiological assay of various acyl derivatives of vitamin B_6. Approximately 0.005 to 10 mg. of the test compound was hydrolyzed with a sufficient amount (0.5–10 ml.) of 2*N* ethanolic potassium hydroxide by refluxing for 30 min. This condition was mild enough to prevent alkali destruction of pyridoxine and pyridoxamine,[8,9] but caused about 10–15% destruction of pyridoxal. After cooling, the solution was neutralized to phenolphthalein with alcoholic hydrogen chloride and the solvent was removed thoroughly *in vacuo* using a rotatory evaporator. Complete removal of the alcohol was essential, since inclusion of ethanol in the assay medium caused *drift* or lower readings. The residue was then extracted with hot water and made up to volume for microbiological assay as reported by Atkin *et al.*[10] using *Saccharomyces carlsbergensis* (ATCC 4228). The recovery of vitamin B_6 from triacetyl,- tridecanoyl-, tripalmitoyl-, and tribenzoylpyridoxine was quantitative ranging between 97–104%. The recovery of pyridoxamine from tridecanoyl- and tripalmitoylpyridoxamine varied from 14 to 28% indicating resistance of the amide linkage to hydrolysis. When tridecanoylpyridoxamine was refluxed for 1 hr. in a mixture of isoamyl nitrite and glacial acetic acid (1:2 v/v) prior to alkali hydrolysis, the recovery was increased to 70%. The microbiological assay of MDP was similarly carried out with 7.7 mg. of the sample, which was hydrolyzed with 10 ml. of 2*N* ethanolic potassium hydroxide solution.

Paper chromatography. To separate vitamin B_6 and its derivatives, the ethanolic solution of the sample was acidified to Congo Red with hydrochloric acid and applied to Whatman No. 1 filter paper on a descending system. The solvent used was 1-butanol saturated with water and developed at 30°. To detect the spots, a 1% solution of *N*,2,6-trichloro-*p*-quinonimine in benzene was sprayed on the paper and the paper exposed to ammonia vapor. This solvent system was not satisfactory for the separation of pyridoxine from pyridoxal.[11] Among the three forms of vitamin B_6, however, only pyridoxal revealed a yellow spot with phenylhydrazine in acetic acid. With this chromogenic reagent, absence of pyridoxal in the oxidized triacyl pyridoxamine was confirmed.

Paper chromatography for the fatty acid component in MDP was carried out by the hydroxamate method as reported by Inouye and Noda.[12] The preparation of MDP gave a strong spot of palmitic acid and an extremely faint spot corresponding to acetic acid. The chromogenic reagent, ferric chloride in ethanol, also revealed the spot of pyridoxine.

Test compounds. *N,O,O*-Tripalmitoylpyridoxamine was

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synthesized as reported previously.[2] The preparation of MDP (C, 72.97; H, 10.19; N, 2.23) was the oxidation product of tripalmitoylpyridoxamine with nitrous acid.[2] Triacetylpyridoxamine was prepared as follows: Approximately 50 mg. of pyridoxamine dihydrochloride was refluxed in a mixture of glacial acetic acid (2.5 ml.) and acetic anhydride (2.5 ml.) for 1.5 hr. The solvent was then removed as much as possible *in vacuo*, and the residue was dissolved in a few ml. of absolute methanol. Upon addition of absolute ether containing dry hydrogen chloride, a precipitate was obtained. M.p. 129.0–130.0°.

Anal. Calcd. for $C_{14}H_{18}N_2O_5 \cdot HCl$: N, 8.47. Found: N, 8.67.

Other triacylpyridoxamines were prepared from pyridoxamine dihydrochloride and the respective acid chlorides in a manner similar to the one described for the synthesis of the tripalmitoyl derivative. *N,O,O*-Tridecanoylpyridoxamine was recrystallized from absolute methanol. M.p. 86.5–87.5°.

Anal. Calcd. for $C_{38}H_{66}N_2O_5$: C, 72.33; H, 10.54; N, 4.44. Found: C, 72.62; H, 10.33; N, 4.54.

N,O,O-Tribenzoylpyridoxamine was recrystallized from 60% ethanol. M.p. 131.0–133.0°.

Anal. Calcd. for $C_{29}H_{24}N_2O_5$: N, 5.83. Found: N, 5.98.

N,O,O-Tri-*p*-nitrobenzoyl pyridoxamine was recrystallized from pyridine-methanol. M.p. 202.0–203.0°.

Anal. Calcd. for $C_{29}H_{21}N_5O_{11}$: N, 11.38. Found: N, 11.34.

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p-Phenylazobenzoyl Chloride for Identification and Chromatographic Separation of Colorless Compounds. III. Phenols

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The value of *p*-phenylazobenzoyl chloride as a reagent for identification and chromatographic separation of alcohols and amines has been shown in previous communications.[2,3] Since the reactions of alcohols, amines, and phenols are similar in many respects, it seemed desirable to study the usefulness of *p*-phenylazobenzoyl chloride as a reagent for phenols.

Whereas alcohols and amines reacted readily with the reagent to give good yields of esters and amides respectively, in general, the phenols reacted with difficulty and gave small yields of esters. The procedure used in the preparation of the derivatives was very simple. It consisted of heating for 4 hours a solution of the phenol and acid chloride in pyridine. Longer periods of reaction time did not increase the yield of derivatives. With the exception of the derivatives of 2-methylphenol, 3,4-dimethylphenol, 2,4-dimethylphenol, 2,6-dimethylphenol, and 3-ethyl-5-methylphenol all were precipitated from the reaction mixture as solids. Although the yields were low they were adequate for easy identification. The *p*-phenylazobenzoates are highly crystalline derivatives which are easily purified. The melting points of the derivatives are high enough so that only the derivative of 2,6-dimethylphenol was obtained as an oil, and they are separated widely enough to ensure identification. In those several instances where the melting points of derivatives were similar, mixture melting points showed considerable depression and wide spreads.

The aryl-*p*-phenylazobenzoates that have been characterized are recorded in Table I.

The derivatives of phenols which are commonly used for identification are neither colored nor fluorescent. Hence they are not suitable to applications of chromatographic adsorption for the separation of phenol derivatives. The brilliantly colored aryl-*p*-phenylazobenzoates have been found to be suitable derivatives for separation of mixtures by chromatographic adsorption techniques. The adsorbents used in preparing preliminary columns for the separation of the derivatives were as follows: alumina, alumina-celite mixture, silicic acid, and silicic acid–celite. Of these adsorbents silicic acid–celite was the most satisfactory. It afforded better separations of mixtures of derivatives, faster percolation rate and easier desorption of the derivatives. In the case of alumina various percentages of alcohol in Skellysolve B or benzene were required to develop the chromatograms, and to desorb the derivatives it was necessary to heat the adsorbent with a solution of 80% alcohol-water. Under these conditions of desorption the derivatives underwent hydrolysis and transesterification to give a mixture from which the aluminum salt of *p*-phenylazobenzoic acid and ethyl-*p*-phenylazobenzoate were identified.

Table II shows the results obtained by the adsorption of 15 pairs of aryl *p*-phenylazobenzoates on mixtures of alumina-celite and silicic acid–celite. The first member of each separable pair listed in Table II was the most strongly adsorbed derivative. Six pairs marked +++ were separated sufficiently on at least one of the adsorbents to make two zones visible with a colorless zone between. Two pairs marked ++ formed a continuous band on at least one of the adsorbents. Sectioning of this with subsequent elution yielded an almost homogeneous top and bottom section with intervening sections of varying composition. Three pairs marked + formed a continuous band on at least one of the adsorbents, but sectioning of this gave impure top and bottom materials which were of different melting points, showing that a mixture was initially present. Finally, eight pairs marked "minus" gave no separation on one or both of the adsorbents as there was no significant difference in the melting points of the material from the top and bottom sections of the continuous band. Those pairs which

(1) This paper is based on work presented by J. M. T. in partial fulfillment of requirements for an undergraduate research course offered in the Department of Chemistry of Central State College.

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