that the conformational change observed in α-lactalbumin occurs *just* below the isoelectric region makes it difficult to reject the view that a subtle alteration of the molecular surface of the protein occurs in the isoelectric region. Clearly, further study of the isoelectric protein will be required to answer this question.

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Metabolites of *p*-Aminobenzoic Acid. IV. Structure of "Metabolite I" and Aryl Hydroxylation Prior Hydroxymethylation of the Benzene Ring*

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The previously designated, biologically active *p*-aminobenzoic acid metabolite I ($C_{28}H_{30}N_4O$) is not a metabolite, but rather, is chemically synthesized from four *p*-aminobenzyl alcohol moieties that are formed by the direct enzymatic reduction of *p*-aminobenzoic acid. The structure of the tetramer is proved to be *N*-{*N*-[*N*-(*p*-aminobenzyl)-*p*-aminobenzyl]-*p*-aminobenzyl}-*p*-aminobenzyl alcohol by chemical and proton nuclear magnetic resonance studies. The presence of a hydroxymethyl group in the tetramer and the metabolic substrate role of *p*-aminobenzyl alcohol suggested that aniline hydroxylation may be preceded by prior hydroxymethylation. This intermediate step is verified and the following new metabolic pathway is elucidated: aniline → *p*-aminobenzyl alcohol → *p*-hydroxyaniline.

Investigations of the metabolism of *p*-aminobenzoic acid by acid-fast bacteria resulted in the isolation of *p*-hydroxyaniline, aniline (trace amounts), and two crystalline compounds previously designated metabolites I and II (Sloane *et al.*, 1951, 1954; Sloane, 1961). All the above compounds accumulated in the medium after the metabolism of added *p*-aminobenzoic acid. Metabolite II was identified as *p*-aminobenzyl alcohol (Sloane and Untch, 1962). Further metabolism of this compound, which is formed by enzymatic reduction of *p*-aminobenzoic acid, resulted in the formation of *p*-hydroxyaniline (Sloane *et al.*, 1963). It has now been shown that the biologically active, previously designated metabolite I, hereafter called compound I, is not a metabolite, but rather is chemically synthesized from the metabolic product, *p*-aminobenzyl alcohol. The occurrence of compound I in all fermentation experiments with *p*-aminobenzoic acid was therefore caused by chemical synthesis that occurred during the isolation procedures employed.

In this paper we determine the structure of compound I, suggest a possible role for this compound in aniline hydroxylation, and prove that enzymatic hydroxylation of aniline is preceded by hydroxymethylation of the benzene ring.

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Results and Discussion

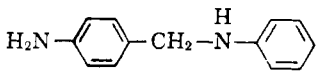
The degradation products of compound I and substrate used for biological studies reported in this paper originated from synthetic compound I. Synthetically prepared compound I was proved to be identical with the material ("metabolite I") previously isolated from fermentation experiments with *p*-aminobenzoic acid by superimposable infrared and ultraviolet spectra, melting behavior, and biological activity (Sloane, 1961). Compound I is spontaneously deposited as crystalline material from a weakly acidic buffer solution of *p*-aminobenzyl alcohol. Initially, compound I was crystallized from methanol to give micro crystals, mp 198–199° (Sloane, 1961). Later, it was found that benzene is a better solvent for recrystallization. The melting point of compound I is not discrete and depends upon the conditions employed when making the determination.[1]

Identification of Isolated Degradation Products of Compound I.—Treatment of compound I with 8 N

[1] The following observations were made using evacuated capillary tubes: preheated bath (140°) mp 238–242°; preheated bath (150°), transition 152°, mp 238–241°; preheated bath (170°), immediate melting, gas evolution with resolidification, mp 240–242.5°.

H_2SO_4 and refluxing the solution for 10 hours produced a mixture of aromatic amines. One of these amines, designated A, was isolated as its oxalate salt. Basification of an aqueous solution of the oxalate salt provided the free base, which was purified by repeated sublimation. The elemental analyses and equivalent-weight determination of compound A, so purified, mp 90.5–92° (corrected), gave it the formula $C_{13}H_{14}N_2$. Its infrared spectrum (see Fig. 1) indicated the presence of a *para*-substituted phenyl group and NH and/or OH. The ultraviolet spectrum of compound A, λ_{243}^{MeOH}, ϵ = (20,000), λ_{288}^{MeOH}, ϵ = (1500), was similar to that of *p*-toluidine.

On the basis of these data, the following structural assignment for compound A was made:



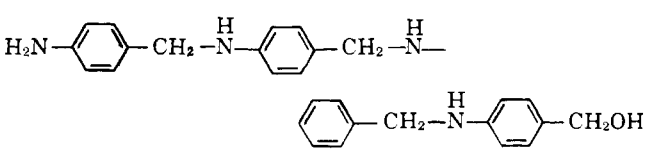
However, the synthesis of this compound, *N*-(*p*-aminobenzyl)aniline, had been described previously (Paal and Springer, 1897) and reported to have a melting point of 49–50°, which differs greatly from the melting point of compound A.

Therefore *N*-(*p*-aminobenzyl)aniline was synthesized according to the method of Paal and Springer (1897). The crude product, after sublimation, melted at 56–57°. An infrared spectrum of this sublimate showed the presence of a significant amount of an *N*-acetate (this impurity undoubtedly was responsible for the low melting point reported by Paal and Springer, 1897). Hydrolysis of the product with aqueous sulfuric acid and isolation of the product as its oxalate salt, followed by three successive sublimations of the free base, gave material melting at 89.5–91° (corrected). A mixture melting point of synthetic *N*-(*p*-aminobenzyl)aniline and compound A, resulting from the degradation of compound I, was undepressed. Infrared spectra of *N*-(*p*-aminobenzyl)aniline, synthetic and compound A, were identical in all respects (Fig. 1).

Another product resulting from the acid hydrolysis of compound I is formaldehyde, which was obtained by steam distilling an 8 N H_2SO_4 solution of compound I and isolating the formaldehyde (31% crude yield) as its dimedon derivative.

Structural Assignment of Compound I.—The elemental analysis of compound I previously reported (Sloane, 1961) was verified with synthetically prepared compound I (see Experimental). The molecular weight of compound I as previously reported (Sloane, 1961) was taken to be 438. However, these prior molecular weight determinations showed marked solvent dependence and variance. Therefore a further molecular weight determination, using a recently available vapor pressure osmometer, was made and gave a value of 410.

Based on these combined data (fragmentation products, analyses, and molecular weight), including that previously reported (Sloane, 1961), compound I was assigned the following structure.



Verification of the Structural Assignment by Nuclear Magnetic Resonance.—This structure for compound I was proved by obtaining its proton NMR spectrum. A sample (*ca.* 20% solution in pyridine-d_5 containing tetramethylsilane as the internal reference) showed the spectra displayed in Figure 2a,b. The absorption pattern centered at *ca.* τ = 2.9 (Fig. 2a) is due to the hydrogens of the aryl rings; the multiplet at τ = 4.25 and that at τ = 4.85 are attributed to the hydrogens bonded to N and O; the peak at τ = 5.17 is due to the hydrogens of the methylene group adjacent to O; the absorption at τ = 5.73 is due to the hydrogens of the methylene groups adjacent to N, with areas of 16.34:5.97:2.02:6.0 (theory, 16.0:6.0:2.0:6.0), respectively. The NMR spectrum of the sample containing deuterium oxide (which eliminates absorption due to exchangeable hydrogens and provides a further proof that the assignments are correct, i.e., NH and OH) is displayed in Figure 2b. The assignments of the absorptions are the same as for those of Figure 2a (note the slight downfield chemical shifts due to added deuterium oxide). The hydrogens bonded to N and O have been exchanged and appear in the absorption due to water. The ratio of the areas under the absorptions in Figure 2b are 16.2:1.9:6.0 (theory, 16.0:2.0:6.0). The ratios of the areas under the absorptions shown in Figure 2a have not been corrected for the small amount of solvent impurity present in the pyridine-d_5, whereas the ratios reported for the absorptions shown in Figure 2b have been corrected for the amount of solvent impurity present. These proton NMR spectra confirm the above structural assignment.

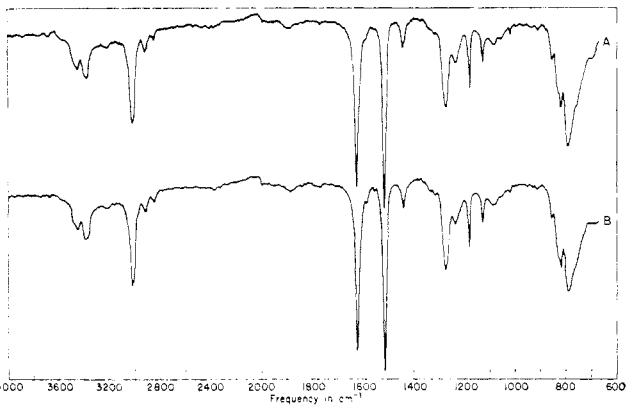


FIG. 1.—Infrared absorption spectra of *N*-(*p*-aminobenzyl)aniline. Curve A, material from acid degradation of compound I; curve B, synthetic material.

After the identification of compound I, it was found that this compound had probably been isolated previously. Thiele and Weil (1895) reported that the reduction of *p*-nitrobenzyl chloride in hydrochloric acid gave an amorphous base which analyzed for $(C_7H_7N)_4$. Treatment of this base with hydrochloric acid, following by basification to give the free base, gave a compound which analyzed for $(C_7H_7N)_4 \cdot H_2O$ and was postulated to be a hydrate. It is likely that this compound was I. The reported data by these investigators (Thiele and Weil, 1825) are consistent with this supposition. Apparently this same compound I was obtained later by Bamberger (1925) by a different synthetic route; however the correct structure was not given.

Biological Properties of Compound I.—The chemically formed compound I that arises from *p*-aminobenzyl alcohol has been shown to be biologically active (Sloane, 1961). Its biological activity appears to be related to the terminal aryl hydroxymethyl group. Compound A, *p*-amino-*N*-benzylaniline (similar structurally to compound I but lacking the aryl hydroxymethyl group), is biologically inactive. The data

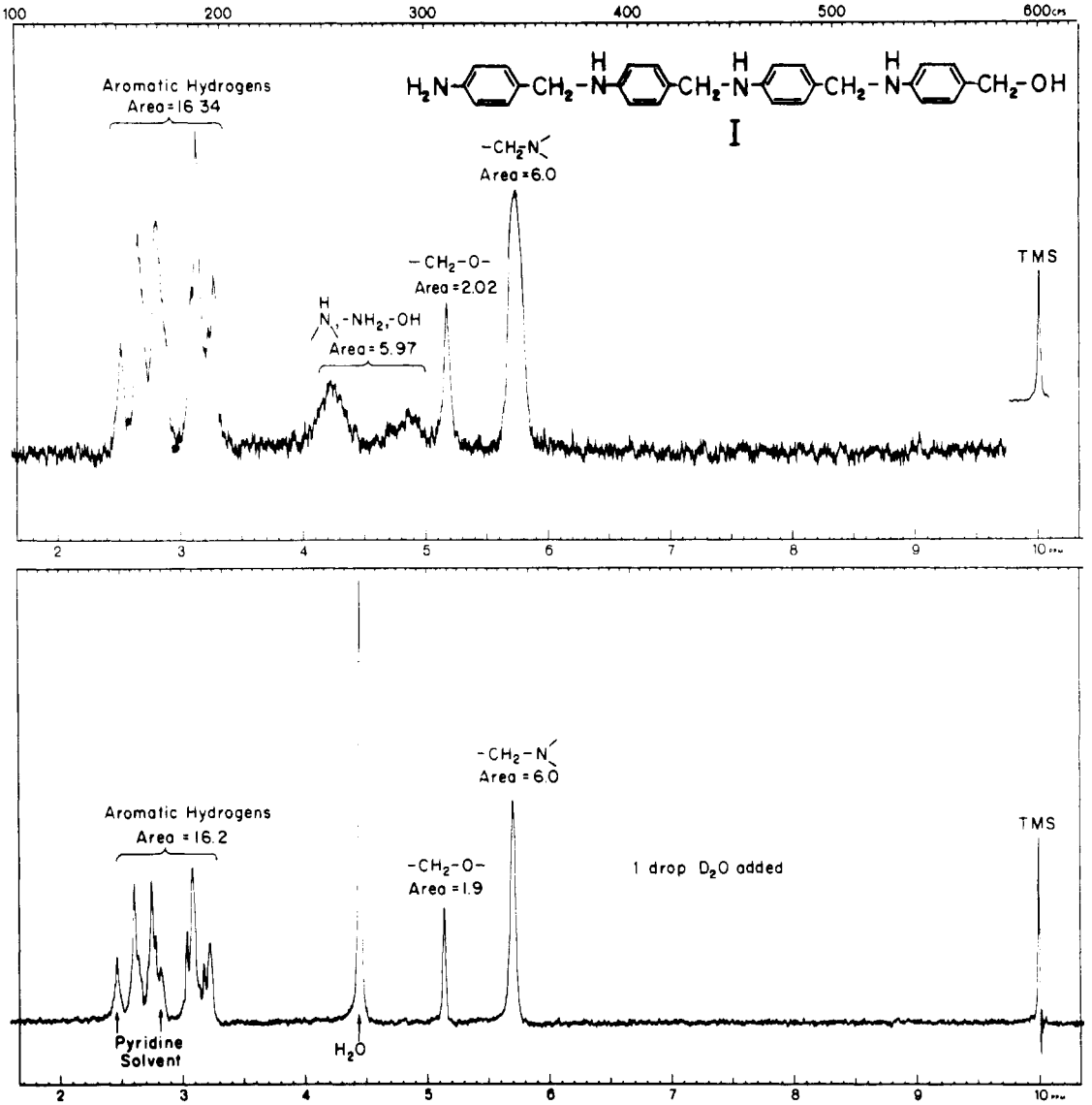


FIG. 2.—Proton NMR spectra of compound I. Upper curve (2a) obtained in pyridine-d_5; lower curve (2b) obtained in pyridine-d_5 after the addition of D_2O.

plotted in Figure 3 compare the activity of compound I and compound A in the aniline hydroxylation system. It can be seen that compound I markedly stimulates aniline hydroxylation and that the amount of *p*-hydroxyaniline formed is a linear function of the concentration of compound I added.

Enzymatic Hydroxymethylation of Aniline.—Two previous observations suggest that aniline may be hydroxymethylated prior to the formation of the hydroxylated end product, *p*-hydroxyaniline: (a) that the addition of compound I reverses the chlortetracycline inhibition of aniline hydroxylation (Sloane, 1961), and (b) that this hydroxylated end product is formed from *p*-aminobenzyl alcohol as substrate in the presence of the antibiotic (Sloane, 1961; Sloane and Untch, 1962). Evidence that this new metabolic pathway does occur was obtained by allowing the microorganism to metabolize [^{14}C]aniline and isolating [^{14}C]-*p*-aminobenzyl alcohol after the addition of cold carrier. The ring-^{14}C-labeled *p*-aminobenzyl alcohol was isolated as its *N*-benzoyl derivative (Sloane, 1964) which was recrystallized (chloroform) to constant specific activity. These results are summarized in Table I.

Since Sloane *et al.* (1963) previously proved that *p*-aminobenzyl alcohol is enzymatically converted to *p*-hydroxyaniline in the bacterial system, the pathways for the formation of the same end product, *p*-hydroxyaniline, from both aniline and *p*-aminobenzoic acid have now been demonstrated. Further, Sloane (1964) reported that aniline is also hydroxymethylated by guinea pig liver microsomes and the resulting intermediate, *p*-aminobenzyl alcohol, is converted to *p*-hydroxyaniline in the presence of added reduced triphosphopyridine nucleotide.

In view of these results, the likelihood that other enzymatic hydroxylations proceed via hydroxymethylation appears enchanced. Other systems are currently being investigated to determine the generality of this pathway.

EXPERIMENTAL

Infrared spectra were obtained from Nujol and halocarbon mulls or chloroform solutions and recorded with Beckman (Model IR-4) or Perkin-Elmer (Model 21) spectrophotometers. Ultraviolet spectra were obtained on a Cary Model 14 spectrophotometer. Proton

TABLE I
HYDROXYMETHYLATION OF [^{14}C]ANILINE TO [^{14}C]-*p*-AMINOBENZYL ALCOHOL BY WASHED CELLS OF *Mycobacterium tuberculosis* (ATCC 607)

Substrate	Radioactivity Added (dpm)	Carrier, *p*-Aminobenzyl Alcohol, Added (mg)	Total Radioactivity in *N*-Benzoyl-*p*-aminobenzyl Alcohol (dpm)
[^{14}C]Aniline			
Experiment 1	1.11×10^8	537.0	7.12×10^4 [a]
Experiment 2	1.11×10^8	513.8	6.20×10^4 [a]
[^{14}C]Aniline			
Autoclaved cells (1 hour at 121°)	1.11×10^8	512.5	1.47×10^3 [b]
[^{14}C]Aniline			
No cells	1.11×10^8	507.2	94 [c]

[a] The counts in the 8- to 10-mg samples were greater than 10 times the background counts. [b] The counts in 8- to 10-mg samples were *ca.* 9 cpm above the background count of 17 cpm. [c] The counts in *ca.* 20-mg samples were only several cpm above background count.

NMR spectra were obtained on a Varian Associates Model A-60 spectrometer. Chemical shifts are given in ppm downfield from tetramethylsilane. Melting and decomposition points are uncorrected except where noted.

Synthesis of Compound I.—One g of *p*-aminobenzyl alcohol (Kaplop Laboratories, Detroit, Mich.) was dissolved in 150 ml H_2O and 30 ml 0.2 M potassium phosphate buffer (*p*H 6.5); the *p*H was adjusted to 6.5, 1 ml of ethyl acetate was added, and the solution was thoroughly shaken and kept at 21° for 10 days. The resulting crystalline material (200 mg) was collected and washed with H_2O and then lyophilized. An analytical sample (recrystallized three times from benzene) melted at *ca.* 240° (decomp).

Anal. Calcd for $C_{28}H_{30}N_4O$: C, 76.69; H, 6.90; N, 12.78; Found:[2] C, 76.68; H, 7.04; N, 12.62. The molecular weight[3] was determined to be 410 (pyridine) with a Mechrolab Model 301A osmometer (theory, 438).

The infrared absorption spectrum of this compound was identical in all respects to that of the material obtained from the fermentation experiments of *p*-aminobenzoic acid and previously published (Sloane, 1961). The ultraviolet absorption spectrum exhibits absorption maxima at 258 (ϵ, 60,000) and 295 (ϵ, 10,000) mμ in ethanol at 1.4×10^{-5} mole/liter.

Isolation of Formaldehyde from Degradation of Compound I.—A sample of compound I, 296.4 mg (0.677 mmole), was dissolved in 80 ml of 8 N H_2SO_4 and steam distilled into a saturated solution of dimedon. The distillation was continued until all the dimedon derivative had been formed. The crude material was collected, washed, and dried (yield, 61 mg). The material, recrystallized from ethanol-water, melted at 190–191°. The melting point of an authentic sample (formed from formaldehyde and dimedon) was 190–191°. An admixture of the derivative obtained from the degradation of compound I and the formaldehyde-dimedon condensation product melted at 190–190.5°.

[2] Analysis by Micro-Tech Laboratories, Skokie, Ill.

[3] Performed by Schwartzkopf Microanalytical Laboratory, Woodside, N. Y.

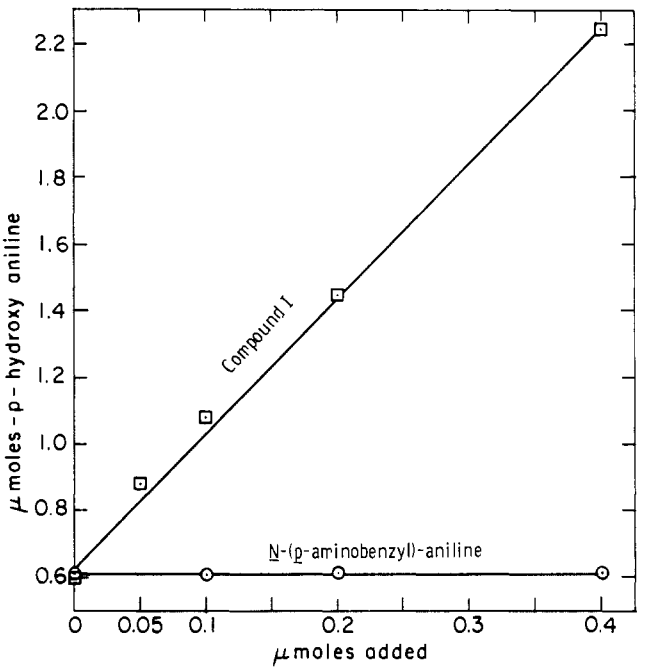


FIG. 3.—Effect of compound I and compound A (*N*-(*p*-aminobenzyl)aniline) on the enzymatic formation of *p*-hydroxyaniline from aniline by resting cells of *Mycobacterium tuberculosis* (ATCC 607) at *p*H 4.0, after shaking for 18 hours at 21°, as described in the text. The amount of *p*-hydroxyaniline formed is the average of two flasks at each level of compounds I and A studied. The concentration of *p*-hydroxyaniline was determined by the method previously described (Sloane *et al.*, 1951).

Isolation of Compound A from the Degradation of Compound I.—Compound I (610 mg) was dissolved in 100 ml 0.2 N H_2SO_4, and then 44 ml H_2O and 46 ml 36 N H_2SO_4 were added and the solution was refluxed for 10 hours. The solution was cooled to room temperature, 600 ml H_2O was added, the solution was cooled to 0°, and the *p*H was adjusted to 8.3 ± 0.2 by adding NaOH. The precipitate was removed by filtration and the filtrate was extracted with ethyl acetate; the ethyl acetate solution was then dried with $MgSO_4$. An oxalate salt was obtained by adding oxalic acid in ethyl acetate to the above extract. The resulting oxalate, after washing with ethyl acetate and drying (154 mg), was dissolved in 20 ml cold 1 N HCl. Basification of this solution with NH_4OH gave an oily amine, which was extracted into ether. The ether solution was dried ($MgSO_4$) and evaporated to dryness *in vacuo*, and the residue was sublimed (mercury diffusion pump) at 78°. A white crystalline material resulted (20 mg). This material, after three successive sublimations (75°) melted at 90.5–92° (corrected). The melting point was unchanged after the second sublimation.

Anal. Calcd for $C_{13}H_{14}N_2$: C, 78.75; H, 7.12; N, 14.13. Found:[4] C, 78.49; H, 7.20; N, 14.37. The equivalent weight (performed by Mr. J. Kerns, Mellon Institute) obtained by titration with perchloric acid was 100 (theory for two basic groups, 99).

N-(p-Aminobenzyl)aniline.—*N*-(*p*-Aminobenzyl)aniline was obtained by reduction of the nitro compound with iron filings and glacial acetic acid as described by Paal and Springer (1897). The nitro compound was prepared essentially by the method of Alway and Walker (1903).

[4] Analyses performed by Alfred Bernhardt, Microanalytical Laboratory, Muhlheim, Germany.

The infrared absorption spectrum ($CHCl_3$) of the crude *N*-(*p*-aminobenzyl)aniline showed the presence of an appreciable amount of *N*-acetylated material. The mixture was dissolved in ether and extracted with *ca.* 25 ml of 1 N HCl. After the addition of 6 ml of 12 N HCl, the aqueous acidic solution was refluxed for 2.5 hours, cooled, neutralized with NaOH, and extracted with ethyl acetate. To this dried ($MgSO_4$) ethyl acetate solution was added a solution of oxalic acid in ethyl acetate. An oxalate salt (620 mg) resulted which was collected, dried, and dissolved in 60 ml of 1 N HCl. The solution was made slightly basic with NH_4OH and extracted with ether. The ethereal solution was dried and evaporated to dryness *in vacuo*. The residue was sublimed at 82° (mercury diffusion pump). The resulting sublimate was resublimed at 78° to give a white solid material (87 mg), mp *ca.* 80–88°. Resublimation (75°) gave initially an oil (*ca.* 10 mg), which was discarded, and continued sublimation gave a white crystalline solid, mp 89.5–91 (corrected). Resublimation of this material gave a crystalline product with the identical final melting point.

An admixture of synthetic *N*-(*p*-aminobenzyl)aniline and compound A melted at 90–91.5° (corrected).

Enzymatic Formation of [^{14}C]*-p-Aminobenzyl Alcohol from* [^{14}C]*Aniline by Resting Cells of Mycobacterium tuberculosis (ATCC 607).*[5]—Washed cells were prepared as previously described (Sloane *et al.*, 1951), stored in the frozen state (−15°) for varying periods, and thawed at room temperature immediately before use. The cells (approximately 250 mg, dry wt) were suspended in 10 ml of buffer solution at *p*H 6.0 and shaken at 21° for 18 hours as previously described (Sloane, 1961). Fifteen flasks were used in each experiment, each flask contained 10.7 μmoles of aniline with 50 μc of [^{14}C]-aniline sulfate (5.10 μc/μmole) supplied by Nuclear Chicago Corp. After centrifugation, the cells were washed with H_2O and the washings were added to the supernatant medium. "Cold" carrier, *p*-aminobenzyl alcohol (503.9 mg), was added to the supernatant, the *p*H was adjusted to 10, and the solution was extracted with ethyl acetate. The ethyl acetate layer was dried ($MgSO_4$) and the oxalate was formed upon the addition of a solution of oxalic acid in ethyl acetate. The oxalate was collected, washed, and dried (510 mg). The oxalate was dissolved in 20 ml of 2 N NaOH and benzoylated by the dropwise addition of 1 ml of benzoyl chloride with constant stirring at room temperature. Stirring was continued for 2 hours. The benzoyl derivative (*N*-benzoyl-*p*-aminobenzyl alcohol) was extracted into ether. The ether solution was dried ($MgSO_4$) and concentrated to dryness *in vacuo* (381 mg). The crude material was crystallized from hot water (98 mg) and finally from hot chloroform as previously described (Sloane, 1964). The samples were recrystallized six times; the counts of recrystallized samples 4, 5, and 6 were identical. The melting points of the samples were 155–156°[6] (reported 150–151°, recrystallized from hot H_2O, Thiele and Dimroth, 1899).

The radioactivity in the samples was determined with a Packard Tri-Carb scintillation counter as previously described (Sloane *et al.*, 1963; Sloane, 1964).

[5] Abbreviation used in this work: ATCC, American Type Culture Collection.

[6] An authentic sample prepared in our laboratory melted at 155–156°, recrystallized from $CHCl_3$. An admixture melting point of an authentic sample and the derivative prepared from addition of cold carrier to medium was not depressed.

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