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# Identification of Cyanoacetic Acid as a Urinary Metabolite of β-Aminopropionitrile[1]

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Urine of rats and rabbits administered β-aminopropionitrile (BAPN) contained a metabolite, not present in control urines, which was detected by formation of a red color with diazotized sulfanilic acid. The metabolite was isolated and identified as cyanoacetic acid. A complex of two moles of cyanoacetic acid with one mole of urea was also isolated from the urine and identified with a synthetic sample. When $C^{14}$-cyano labeled BAPN was administered to rats, about 80–90% of the total administered radioactivity appeared in the urine within 24 hours, and 30–50% of this total was extractable from strongly acidified urine with ethyl acetate. The only radioactive substance detected in such extracts was cyanoacetic acid.

In studies of the metabolism of β-aminopropionitrile (BAPN) by the rat, a urinary metabolite was observed[2] which gave rise to a bright orange-pink zone on paper chromatograms of the urine which were sprayed with diazotized sulfanilic acid.[3] This metabolite was isolated from both rat and rabbit urine and was first obtained[4] as a crystalline solid, $C_7H_{10}N_4O_5$ (I), melting at 94–96°. After further fractionation the metabolite was obtained as a crystalline product, $C_3H_3NO_2$ (II), melting at 67–68°. Compound II was identical with cyanoacetic acid.[5] After II was identified, Compound I was determined to consist of a complex of one mole of urea and two moles of cyanoacetic acid. The addition of petroleum ether[6] to an ethyl acetate solution of 2 moles of cyanoacetic acid and one mole of urea results in formation of nice crystals, m.p. 94–96°, identical with I. Baum[7] previously reported I to result when a mixture of 2 moles of cyanoacetic acid and 1 mole of urea was melted, his product having m.p. 94–95°.

Prior to the actual isolation, the properties of the metabolite of BAPN were studied by administering $C^{14}$-cyano labeled[8] BAPN to white rats. The bulk of the radioactivity was rapidly excreted in the urine in the form of the metabolite, unchanged BAPN and possibly other products. The metabolite was separated from BAPN by extraction from strongly acidified urine by ethyl acetate or ether. Hydrolysis of the urine was unnecessary, as extraction was unaffected by hydrolysis in 1 *N* sulfuric acid at 100° for 70 minutes. Such extracts were chromatographed on papers developed with three different solvent systems[9] and in each case the radioactivity and orange-pink zones coincided. The compound was found to be distillable from the ethyl acetate extract under high vacuum at temperatures below 80° and was thus greatly concentrated.

The final isolation was carried out on non-radioactive materials. Rabbits were especially suitable for this work, not only because of their larger size but also because they converted more of the administered BAPN into the metabolite. The metabolite from rabbits was chromatographically the same as that excreted by rats. The larger-scale isolation from rabbit urine, which later was repeated on rat urine, consisted in extraction of the acidified urine with ethyl acetate, distillation of the metabolite from the extract under high vacuum, and crystallization of I by addition of petroleum ether to an ethyl acetate solution of the distillate.

Compound I displayed a strongly acidic nature,[10] *pK ca.* 2.5, and had a neutral equivalent of about 116. It showed only end absorption in the ultraviolet and lacked absorption characteristic of phenols.[11] Since we were unable to assign a structural formula to the compound at this stage, Compound I was examined on paper chromatograms. An "impurity"[12] identified as urea was detected as a trailing green spot by use of a phenol–hypochlorite spray[13] or as a yellow spot with the *p*-dimethylaminobenzaldehyde[3] spray.

The urea contaminant was eliminated by adsorbing I on a strong base anion-exchange resin and eluting with 0.1 *M* sodium acetate. Following extraction of the acidified eluate with ethyl acetate and removal of the solvent, the solid residue was sublimed *in vacuo*. Compound II was thus obtained as a colorless solid which following recrystallization from ethyl acetate–petroleum ether was identical with cyanoacetic acid in chromatographic migration and color reactions. The infrared spectra corresponded closely and the melting point of a mixture of II and authentic cyanoacetic acid was undepressed. Simultaneously with the isolation of II, it was observed[5] that only cyanoacetic acid, of a large number of nitriles tested, gave a direct orange-pink color with diazotized sulfanilic acid (*i.e.*, without passage through the animal.) The behavior of I and II on paper chromatograms was identical and was duplicated by cyanoacetic acid.

It thus appears that at least in the rat and rabbit BAPN is partly metabolized by conversion of the

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(2) J. J. Lalich, *Federation Proc.*, **17**, 414 (1958).

(3) M. D. Armstrong, K. N. F. Shaw and P. E. Wall, *J. Biol. Chem.*, **218**, 293 (1956).

(4) J. T. Garbutt, J. J. Lalich, S. H. Lipton and F. M. Strong, *Federation Proc.*, **17**, 226 (1958).

(5) S. H. Lipton, J. J. Lalich and F. M. Strong, THIS JOURNAL, **80**, 2022 (1958).

(6) The petroleum ether used was Skellysolve B, b.p. 60-71°.

(7) F. Baum, *Ber.*, **41**, 525 (1908).

(8) E. D. Schilling and F. M. Strong, *J. Org. Chem.*, **22**, 349 (1957).

(9) The three systems butanol:acetic acid:water (8:2:2), isopropyl alcohol:ammonia:water (8:1:1), and benzene:propionic acid:water (2:2:1) as given in reference 3 were employed.

(10) We wish to thank Dr. R. M. Bock for the electrometric titration of this compound.

(11) Because of the strongly positive color test with the diazotized sulfanilic acid reagent,[3] we erroneously believed the metabolite to be an unknown phenol in early stages of the work.

(12) It was not until after free cyanoacetic acid was identified that the $C_7H_{10}N_4O_5$ compound was recognized as a complex of cyanoacetic acid and urea.

(13) R. J. Block, E. L. Durrum and S. Zweig, "A Manual of Paper Chromatography and Paper Electrophoresis," Academic Press, Inc., New York, N. Y., 1955, p. 255.

primary amino group to a carboxyl, possibly by transamination followed by oxidation. The proportion of the administered dose converted into cyanoacetic acid obviously differs with the species of animal studied and quite possibly also with the time and method of administration, dosage level, age of the test animals and other variables. In the present work no attempt to ascertain this proportion has been made other than to estimate roughly the radioactivity appearing in the urine after administration of $C^{14}$-cyano labeled BAPN and its fractionation by solvent extraction of the urine at low and high *p*H values. For rats, the fraction of the total urinary radioactivity extractable at *p*H 10–11 (presumably due to unchanged BAPN) was approximately 20–45%, while that from strongly acidified urine (presumably cyanoacetic acid) was about 30–50%. The previous conclusion[14] that unchanged BAPN is not a major excretory product of rats requires modification in the light of these findings.

Rabbits are much more efficient than rats in converting BAPN into cyanoacetic acid; in fact unchanged BAPN could not be detected at all in rabbit urine. Other species such as the turkey,[15] guinea pig[16] and mouse[16] have also been found to oxidize BAPN to cyanoacetic acid, but no estimate of the extent of the conversion has been made. Likewise a series of other aliphatic nitriles has been investigated with respect to their metabolic degradation by rats and other animals, and preliminary results have been reported.[5] Efforts to extend this study to additional compounds and to put the results on a quantitative basis are currently in progress.

## Experimental

**Excretion of $C^{14}$-cyano Labeled BAPN by Rats.**—Female ether-anesthetized white rats, weighing approximately 150 g., were given by intraperitoneal injection a neutralized 1% aqueous solution containing 20 mg. of $C^{14}$-cyano labeled BAPN hydrochloride, specific activity 14,000 counts per minute per mg.[17] The rats were supplied with water only and kept in wire metabolism cages over glass funnels. The urine accumulated from 6 rats during a 24 hr. period was pooled and filtered through glass wool. Radioactivity recovered in the urine totaled 80–90% of the administered dose in several independent experiments.

**Detection of Unchanged BAPN in Rat Urine.**—An aliquot of the above rat urine was adjusted to *p*H 10–11 with 10 *N* sodium hydroxide and then extracted with ether in a continuous liquid–liquid extractor for 24 hr. Radioactivity determined in such ether extracts equaled 20–45% of the total radioactivity of the urine. Following evaporation of the ether and solution of the extract in a small volume of alcohol, paper chromatograms of the extract were run in the 1-butanol:acetic acid:water (8:2:2) system.[3] When the papers were sprayed with a 0.20% solution of ninhydrin in 1-butanol, a green area was observed at $R_f$ *ca.* 0.25.[18] Authentic BAPN on the same paper gave a spot with the identical $R_f$ and color. When radioactivity was measured along the paper strip, the zone of maximum radioactivity was found to coincide with the BAPN zone, and no other radioactive areas were found on the paper.

Final confirmation of the presence of unchanged BAPN in rat urine was obtained by actual isolation. An ether extract obtained as above from a quantity of rat urine corresponding to a dose of 0.33 g. of BAPN was concentrated to 100 ml. and saturated with dry hydrogen chloride gas. Evaporation of the mixture to dryness and several recrystallizations of the tan colored solid residue from absolute ethanol gave 9 mg. of white crystals, m.p. 161–163°. The product showed no melting point depression when mixed with a known sample of BAPN hydrochloride.

**Detection of a New Metabolite of BAPN in Rat Urine.**—Rat urine which had been extracted at *p*H 10–11, as described above, was acidified to *p*H 1 with sulfuric acid and further extracted with ether for 24 hr. in a continuous liquid–liquid extractor. Radioactivity passing into the ether under these conditions equaled approximately 35% of the radioactivity excreted in the urine.

The reddish-brown residue obtained after evaporation of the ether from the extract was dissolved in a few milliliters of alcohol, and small aliquots were spotted on the base line of a sheet of Whatman No. 1 filter paper. The paper chromatogram developed in the butanol:acetic acid:water system was sprayed with diazotized sulfanilic acid.[3] This reagent was prepared by mixing equal volumes of (a) 5% aqueous sodium nitrite and (b) a 1% solution of sulfanilic acid in 1.2 *N* hydrochloric acid and after cooling 5 min. under the cold water tap, diluting with an equal volume of water.

The paper was sprayed with the diazotized sulfanilic acid, allowed to dry in the draft of an open hood and then sprayed with a 10% aqueous sodium carbonate solution. A bright orange-pink zone, $R_f$ *ca.* 0.55, appeared following the sodium carbonate spray. The radioactivity was determined along the paper strip and the single zone of radioactivity coincided with the orange-pink zone.

Rat urine from control animals which received no BAPN was carried through the extraction described above, and when examined on paper chromatograms the orange-pink zone was absent.

When developed in the benzene:propionic acid:water (2:2:1, organic phase) or the isopropyl alcohol:28% aqueous ammonia:water (8:1:1) systems[3] the zone of maximum radioactivity also coincided with the orange-pink zone detectable with diazotized sulfanilic acid. $R_f$ values observed in both of these solvent systems ranged from about 0.27 to 0.39 in various runs. It thus appeared that the substance giving rise to this zone was a direct metabolite of BAPN and not just a product whose excretion was stimulated by the administration of BAPN.

When paper chromatograms of the metabolite were sprayed with 2% *p*-dimethylaminobenzaldehyde in 1.3 *N* hydrochloric acid[3] and allowed to stand overnight, a yellow color appeared at the same position as the orange-pink zone produced by diazotized sulfanilic acid. The metabolite was also found to be detectable on paper chromatograms as a blue color if diazotized *p*-nitroaniline[19] was used as the spray. The acidic nature of the metabolite caused it to be revealed as a red zone on chromatograms sprayed with methyl red–brom thymol blue.[20]

**Extraction and Distillation from Rat Urine.**—Studies of the conditions suitable for extraction of the metabolite from rat urine were carried out with 0.5-ml. aliquots of urine containing a total of *ca.* 9000 c.p.m. of radioactivity. At neutral *p*H values no radioactivity was extractable. At *p*H 2.0, approximately one-third less metabolite was extractable than at *p*H 1. Since the metabolite thus appeared to be strongly acidic, urine used in isolation studies was routinely made 1 *N* in sulfuric or hydrochloric acid prior to extraction.

A study was made of the effect of heating urine acidified as above to hydrolyze possible conjugates of the metabolite. However, aliquots heated at intervals of 0–70 minutes at 100° were found to yield identical radioactivities in the solvent extracts. This result indicated that hydrolysis was unnecessary and also that the metabolite was stable to hydrolysis in 1 *N* acid. Of various solvents used for this extraction, ethyl acetate proved more effective than ether, and non-polar solvents such as petroleum ether were least

(14) E. D. Schilling, J. T. Garbutt, J. J. Lalich and F. M. Strong, Abstracts, 130th meeting American Chemical Society, Atlantic City, September, 1956, p. 30-C.

(15) D. N. Roy and H. R. Bird, unpublished.

(16) H. W. Sievert and F. M. Strong, unpublished.

(17) We wish to thank Dr. W. C. Liu for the preparation of the $C^{14}$-labeled BAPN used in this study.

(18) This $R_f$ value varied over a range of approximately 0.20–0.34 in different runs.

(19) H. S. Bray, W. V. Thorpe and K. White, *Biochem. J.*, **46**, 271 (1950).

(20) R. E. B. Duncan and J. W. Porteous, *Analyst*, **78**, 641 (1953). The reagent is prepared by mixing a formalin solution containing 0.2% each of methyl red and brom thymol blue with 4 volumes of ethanol and adjusting to *p*H 5.2.

effective. The ethyl acetate extract from one sample of acidified rat urine contained 48% of the total urinary radioactivity.

A small-scale attempt to steam distil the metabolite from acidified urine was unsuccessful, negligible radioactivity being distillable. When subjected to mild heat under high vacuum, 50–90% of the radioactivity of the ethyl acetate extract of acidified urine was found to be distillable below 80°.

**Larger Scale Isolation from Rabbit Urine.**—Non-radioactive BAPN was used. Rabbits weighing 2.5–3.5 kg. were given at 4-hr. intervals two intraperitoneal injections of 0.25 g. of BAPN fumarate[21] in 4 ml. of water. Urine was collected for the next 1–2 days, during which time the animals were given water but no food. About 250 ml. of urine was obtained per rabbit.

The filtered urine, acidified with either sulfuric or hydrochloric acid to 1 *N* concentration, was extracted in a separatory funnel 6 times using for each extraction a volume of fresh ethyl acetate equal to one-half the volume of urine. The combined ethyl acetate extracts were centrifuged to eliminate emulsion, washed 4 times with small volumes of water and concentrated to about 20 g. of a brown sirupy residue by evaporation under reduced pressure at room temperature. When examined on paper chromatograms developed in the butanol:acetic acid:water system and sprayed with diazotized sulfanilic acid, this residue produced a strong orange-pink zone at the usual $R_f$ and also a larger faster-migrating yellow zone of $R_f$ *ca.* 0.90.

The residue was equally divided among 8 to 10 250-ml. conical filter flasks and dried under vacuo at room temperature in these flasks, so as to obtain a thin film on the bottom. A 25 × 200 mm. test-tube was fitted into the neck of each flask by means of a rubber stopper so that the bottom of the test-tube extended to within 1–2 cm. of the oily film. This tube when filled with ice served as a cold finger for condensation of the distillate.

The flasks were evacuated by means of an oil pump and mercury diffusion pump to about 0.1 μ and heated by immersion in an oil-bath held at 40–60°. The distillate obtained after 1 hr., which amounted to only a small fraction of the total residue and consisted of a light yellowish oil, was washed from the cold finger by means of ethyl acetate. A small amount of ethyl acetate-insoluble solid was filtered off and the filtrate decolorized with a little Darco G60 charcoal. Upon addition of sufficient petroleum ether to the ethyl acetate solution to produce a faint turbidity and storage at 4° overnight, a white crystalline solid (I) appeared.

Examined chromatographically, this appeared to be the desired metabolite, since the only zone revealed by the diazotized sulfanilic acid was the orange-pink zone. The product was filtered off and washed first with 1:4 ethyl acetate:petroleum ether and then petroleum ether. The yield obtained from 750 ml. of urine and 1.5 g. of BAPN fumarate was about 60 mg. This compound (I) following recrystallization from ethyl acetate–petroleum ether melted at 94–96°. Qualitative detection of elements indicated that only C, H and N were present. Electrometric titration[10] indicated a single acid dissociation, $pK$ *ca.* 2.5, and equivalent weight 116 ± 10. Absorption in the ultraviolet consisted of a non-characteristic end-absorption below 300 mμ. For analysis[22] a sample was dried at 28° and 0.2 mm. for 48 hr.

*Anal.* Calcd. for $C_7H_{10}N_4O_5$: C, 36.52; H, 4.34; N, 24.36; mol. wt., 230.2. Found: C, 36.52, 36.71; H, 4.21, 4.91; N, 23.46, 23.71; neut. equiv., 122, 116.

The isolated solid, m.p. 94–96°, was very soluble in water and had a strong tendency to separate as an oil during purification attempts. Since it appeared to be hygroscopic and melted over a 2° range, there was doubt as to its purity. Attempts were therefore made to convert it to a salt with hexylamine. However, this salt was too soluble and could not be crystallized. It was also found that the metabolite could not be precipitated from aqueous solution with either neutral or basic lead acetate. The solid was subjected to resublimation at 40–60° and 0.1 μ and found to be unchanged in analysis. Following a work-up of 1900 ml. of rat urine, a crystalline preparation was obtained which was identical to that obtained from rabbit urine.

Evidence for an impurity in the isolated solid described above was obtained by paper chromatography. When sprayed with the *p*-dimethylaminobenzaldehyde reagent, a chromatogram developed in the butanol:acetic acid:water system indicated the presence of a small yellow zone which trailed just behind the zone occupied by the metabolite. This impurity was found to give a green color with the phenol–hypochlorite reagent[13] for urea and moreover was found to occupy the same position as a reference sample of urea.

**Purification by Ion Exchange.**—The metabolite (I) was found to be adsorbed on Dowex 1 resin and the urea impurity to appear in the effluent. For purification of an approximately 0.5-g. quantity of I, a 3 cm. diameter by 2 cm. deep bed of Dowex 1-X10, 200–400 mesh resin in the acetate form was used. The resin was supported in a Corning No. 36060 Pyrex glass funnel with coarse sintered glass plate. The metabolite fraction was dissolved in water, neutralized to litmus by addition of solid sodium bicarbonate and diluted to a concentration of about 1%. Adsorption on the resin was accomplished at a flow-rate of about 0.5 ml./min., following which the resin was washed with about 100 ml. of water. The effluent was checked with diazotized sulfanilic acid and negative results indicated complete adsorption of the metabolite. Elution with 0.1 *M* sodium acetate was carried out at a rate of about 0.5 ml. per minute, the eluate being collected in 10-ml. fractions. Fractions 1–20 contained no metabolite, while the bulk of it appeared in fractions 21–60.

Recovery from the eluate was carried out by acidifying with 3 ml. of concentrated sulfuric acid per 100 ml. of eluate and extracting 10 times in a separatory funnel with small volumes of ethyl acetate. After washing the pooled ethyl acetate extracts several times with small volumes of water, the ethyl acetate was removed by evaporation at room temperature. The residual slightly tan-colored solid was then subjected to vacuum sublimation at 40–50°, and $10^{-4}$ mm. for 1 hr. The pure white solid obtained weighed 84 mg. and melted at 65–68°. When recrystallized from ethyl acetate–petroleum ether it had m.p. 67–68°.

*Anal.* Calcd. for $C_3H_3O_2N$: C, 42.36; H, 3.56; N, 16.47. Found: C, 42.33; H, 3.68; N, 15.42.

The infrared spectrum was compared with that of an authentic sample of cyanoacetic acid and showed no significant differences. There was no melting point depression of a mixture of the isolated compound and authentic cyanoacetic acid. A comparison on paper chromatograms showed the metabolite to behave in all respects the same as known cyanoacetic acid.

**Preparation of Complex of Urea and Cyanoacetic Acid.**—A 760-mg. quantity of cyanoacetic acid (9 mmoles) and 240 mg. urea (4 mmoles) were dissolved in about 10 ml. of warm ethyl acetate. Upon addition of petroleum ether to incipient turbidity and cooling, well-formed cubical crystals separated. After cooling in the ice-box these were collected by filtering, washed with ethyl acetate–petroleum ether (4:1) and dried. A second crop was obtained from the mother liquor. The combined weight of these crystals, m.p. 94–96°, was 740 mg.

*Anal.* Calcd. for $C_7H_{10}N_4O_5$: C, 36.52; H, 4.34; N, 24.26. Found: C, 36.83; H, 4.45; N, 23.97.

Comparison of infrared tracings of this synthetic product and of isolated I revealed only a single point of difference (a moderately strong band at 6 μ in the tracing from I) presumably attributable to the presence of an impurity. Both tracings differed widely from the infrared spectrum of cyanoacetic acid.

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(21) BAPN fumarate ($(H_2NCH_2CH_2CN)_2HOOCH{=}CHCOOH$) was generously supplied by the Abbott Laboratories, North Chicago, Illinois.

(22) Microanalyses were carried out by C. W. Beazley, Micro-Tech Laboratories, Skokie, Illinois.