tized dogs every 5-8 sec. (phased with respiration) results in reproducible dye-dilution curves during cardiovascular equilibrium. The calculated cardiac output does not vary from determination to determination more than ± 5%. The method is useful in the investigation of problems requiring rapidly repeated estimations of cardiac output.

1. Gilford, S. R., Gregg, D. E., Shadle, O. W., Ferguson, T. B., Marzetta, L. A., *Rev. Sci. Instr.*, 1953, v24, 696.

2. Hamilton, W. F., *Am. J. Physiol.*, 1932, v102, 551.

3. Hamilton, W. F., Moore, J. W., Kensman, J. M., Spurling, R. G., *ibid.*, 1932, v99, 534.

4. Fox, I. J., Brooker, L. G. S., Heseltine, D. W., Essex, H. E., Wood, E. H., *Proc. Staff Meet., Mayo Clinic*, 1957, v32, 463, 553.

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Metabolism of Aminonitriles and Related Compounds by the Rat.* (25378)

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During our studies on metabolism of β-amino-propionitrile (BAPN), the rat lathyrism factor, considerable variation in severity of symptoms was observed among individual animals(1). The rat eliminated unchanged BAPN in the urine and also a metabolite identified as cyanoacetic acid (CAA)(2). Since the latter compound produced none of the symptoms of BAPN toxicity when fed to weanling rats(3), it presumably is a detoxification product. Using a method for quantitative determination of CAA(4), a study has been carried out on relationship between efficiency of conversion of BAPN into urinary CAA and severity of skeletal deformities. The metabolism of a number of organic nitriles related to BAPN has also been investigated to determine whether other nitriles are likewise detoxified by conversion into CAA. A preliminary account of part of the latter work has been reported(5).

Methods and materials. BAPN was used as the crystalline fumaric acid salt (2 BAPN · 1 fumaric acid), aminoacetonitrile as the hydrogen sulfate acid salt, and 2,2′-iminodipropionitrile (IDPN) as the free base.† The aminonitrile analogs, 4-aminobutyronitrile hydrochloride (mp 144.5-145.5°) and 5-aminovaleronitrile hydrochloride (mp 121-122.5°) were synthesized from corresponding chloronitriles by procedure of Goldberg and Kelley (6). Analysis of 4-aminobutyronitrile hydrochloride. Calc'd for $C_4H_9N_2Cl$: N, 23.23; Cl, 29.40. Found: N, 23.50; Cl, 29.50. Analysis of 5-aminovaleronitrile hydrochloride. Calc'd. for $C_5H_{11}N_2Cl$: N, 20.81; Cl, 26.34. Found: N, 20.84; Cl, 26.70. The intermediate 4-phthalimidobutyronitrile melted at 79-81° instead of 65-66° as reported(6) and the intermediate 5-phthalimidovaleronitrile had mp 77.5-79°. Analysis of 4-phthalimidobutyronitrile. Calc'd. for $C_{12}H_{10}O_2N_2$: N, 13.09. Found: N, 13.90. Analysis of 5-phthalimidovaleronitrile. Calc'd. for $C_{13}H_{12}O_2N_2$: N, 12.27. Found: N, 12.62. Propionitrile, n-butyronitrile, n-valeronitrile, n-hexanenitrile, succinonitrile, and CAA were Eastman products. Ethylene cyanohydrin was furnished by American Cyanamid Co. *Animal experiments.* Sprague-Dawley female rats were used for studies of relationship between urinary CAA levels and severity of skeletal deformities. Older rats weighing 90-300 g were used for study of metabolism of various organic nitriles related to BAPN. The weanling rats (6/group) were fed one of 3 basal diets which differed only in their protein composition (see Table I). Test diets corresponding to the 3 basals were prepared by addition of 3 g of BAPN fumarate to 1 kg of diet. Feeding was *ad lib.* except during

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† These compounds were kindly supplied by Abbott Labs., North Chicago, Ill.

TABLE I. Basal Diet A.*

Ingredient	Amt
Casein (Borden's), "vitamin-free"	120 g
Cerelose (dextrose)	780 "
Corn oil	60 "
Salts (Wesson's)	40 "
Choline chloride	1 "
DL-Methionine	1 "
L-Cystine	1 "
Thiamine • HCl	25 mg
Riboflavin	3 "
Pyridoxine • HCl	25 "
Calcium pantothenate	20 "
Nicotinic acid	10 "
i-Inositol	10 "
Biotin	.5 "
Folic acid	2 "
Menadione	10 "
Vitamin A acetate	6000 IU
" D	60 "
α-Tocopherol	100 mg

* Basal diets A, B, and C were alike except that diet B had crude instead of "vitamin-free" casein, and diet C had 240 g crude casein and 660 g cerelose. All minor components were mixed into the dry mixture, except for fat-soluble vitamins which were blended into corn oil before mixing with dry ingredients.

collection of urine. Older rats were maintained on commercial Purina pellet diet. Nitriles were injected into these animals intraperitoneally as neutral aqueous solutions or suspensions of 1-2 ml volume, equivalent to dose of 20-50 mg/rat. *For collection of urine* rats were placed for 18 hours in wire metabolism cages over large glass funnels. A glass baffle at funnel outlet and over the bottle receiving urine, deflected the solid feces and thus minimized contamination. Urine was collected from weanling rats after they had been kept on control or test diets for 5 weeks. In rats to which organic nitriles were administered, urine collection was made immediately following injection of the chemical being studied. During collection period the animals were given water *ad lib.* but no food. At end of collection period the funnels were rinsed with a few ml of water, and the combined urine and wash water filtered through glass wool. The slightly diluted urine samples so obtained were then examined for CAA content either by the quantitative method(4), or qualitatively by chromatographing on paper as previously described(2). In the latter case duplicate chromatograms were sometimes prepared and sprayed with 0.1% ferric chloride, 3% hydrogen peroxide reagent to detect thiocyanate(7). *Colorimetric assay for CAA of urine from rat receiving radioactive BAPN.* Urine was collected from a rat injected with C^{14}-cyano labeled BAPN(2). Parallel radioactivity and CAA measurements were carried out on an ethyl acetate extract of acidified urine. By the colorimetric assay a value of 2.50 μM of CAA/ml of urine was found. Calculations based on radioactivity of the extract and the known specific activity of administered BAPN showed that 2.63 μM of CAA could have been present. The close agreement indicated that the ethyl acetate extract of acidified urine contained radioactivity mainly in the form of CAA. *Analysis of urine for CAA.* A 1 ml volume of urine, acidified with 0.1 ml of 1:1 (v/v) sulfuric acid:water, was extracted 6 times with 2 ml portions of ethyl acetate in 6″ test tube or 15 ml centrifuge tube. After separation of layers by settling or centrifuging, the ethyl acetate layer was withdrawn using a glass tube with drawn-out capillary tip. The combined ethyl acetate extracts were washed twice with 0.2 ml of water, then evaporated to dryness in 25 ml beaker in the draft of laboratory hood. The residue was dissolved in a small measured volume of distilled water and used for quantitative colorimetric determination of CAA as previously described(4). *Determination of skeletal deformities.* Animals on BAPN and basal diets were examined for skeletal deformities by manual palpation, primarily noting the everted sterna. Estimations of severity of deformities were expressed numerically as follows: normal, 0; minimal, 1; moderate, 2; severe, 3. Rats with grade 0, 1, or 3 sternal deformities were selected from different groups for urine collection and urinary CAA analysis. At termination of experiment, postmortem examinations were performed on all animals and deformity ratings as estimated on living animals were verified or modified, by visual inspection of skeletal structures.

Results. Urinary CAA and its relationship to severity of skeletal deformities. In Table II results on relationship between quantity of urinary CAA and severity of bony deformities in rat lathyrism are presented. Urinary CAA

TABLE II. Relationship between Urinary CAA in Rat Lathyrism and Severity of Bony Deformities.

Rat No.	Diet	Supplement	Time on diet (days)	Rat wt (g)	Urinary CAA (mg/18 hr)*	Severity of bony deformities
M- 1	Basal A	None	37	135	.17	0
1	"	"	39	139	.17	0
1	"	"	49	164	.24	0
4	"	"	51	157	.09	0
16	"	.3% BAPN	39	120	1.10	1
16	"	"	49	130	.76	3
10	"	"	51	123	.91	1
21	Basal B	None	35	128	.08	0
33	"	.3% BAPN	37	96	.73	3
33	"	"	47	106	.62	3
33	"	"	49	106	.67	3
32	"	"	39	101	.70	3
32	"	"	49	112	.63	3
34	"	"	37	76	.31	3
35	"	"	37	105	1.18	1
D- 1	Basal C	None	49	166	.20	0
13	"	.3% BAPN	49	138	1.95	1
15	"	"	49	129	2.38	1
16	"	"	49	135	1.70	1

* Urine from rats on unsupplemented basal diets contained no CAA; the values shown in this table represent "apparent CAA" due to interfering substances present in normal rat urine (see text).

values are given as total amount excreted in 18 hours. In urines from normal rats on basal diets A, B and C, not receiving BAPN, the observed absorbancy values were calculated as "apparent CAA," and varied from 0.08 to 0.24 mg/18 hour urine sample. Since examination of extracts of such normal rat urines on paper chromatograms failed to indicate presence of CAA, these values represent interfering substances. CAA values of test urine samples were not corrected for this "apparent CAA."

Range of CAA excretion values for rats with minimal (grade 1) bony deformities was 0.91 to 1.18 mg on the 12% protein diets and 1.70-2.38 mg on 24% protein diet. For rats with maximal (grade 3) osseous changes, observed in these experiments on the 12% casein diets, urinary CAA excretions were definitely lower, being 0.62-0.76 in all cases except one which was 0.31 mg. Animals with moderate (grade 2) bony changes were not studied.

The higher urinary CAA excretion by rats with minimal osseous deformities suggests that these animals were better able to detoxify BAPN by converting it into CAA. A quantitative study of urinary excretion of BAPN by rats having symptoms of varying severity would be an interesting supplement to this work. The higher CAA excretion of rats on 24% as compared with 12% casein diet, and the associated reduction of severity of the symptoms, support the previous observations of Dasler(8) that high levels of casein exert a protective effect.

Excretion of CAA following administration of organic nitriles. Tables III and IV present data on urinary CAA excretion following intraperitoneal injection of various organic nitriles related to BAPN. The qualitative results of Table III were obtained by chromatographing ethyl acetate extracts of the various urines on paper(2). Nitriles, besides BAPN, which were thus found to give rise to CAA *in vivo* included IDPN, ethylene cyanohydrin, succinonitrile, 5-aminovaleronitrile, and n-valeronitrile. Traces of CAA were also detected in urines of some animals which received n-hexanenitrile and 4-aminobutyronitrile, although previously CAA was not observed following injection of the latter(5).

Quantitative determinations in Table IV were made subsequently to confirm and extend the qualitative results. As emphasized above, interfering substances in normal rat urine result in a blank, "apparent CAA" value, the approximate magnitude of which may be judged from data on control rats.

TABLE III. Urinary Cyanoacetic Acid following Injection of Various Aliphatic Nitriles in Rats.

Compound	Dosage of nitrile/rat mg	Dosage of nitrile/rat μM	Approx. wt of rats* (g)	Intensity of CAA zone in chromatogram of urine	Remarks
Aminoacetonitrile · HCl	30	320	300	0	
BAPN · HCl	"	280	100	+++	
4-Aminobutyronitrile · HCl	20, 42	170, 350	150, 300	0, 0	
"	30, 50	250, 420	100, 290	tr, tr	
5-Aminovaleronitrile · HCl	", "	220, 370	", 300	tr, +	
N-Propionitrile	50	910	180		Animal died after inj.
N-Butyronitrile	20, 33	290, 480	100, 160	−−−, 0	100 g animal died overnight
N-Valeronitrile	33, 50	400, 600	", 200	+, +	*Idem*
"	20	240	150	+	
N-Hexanenitrile	", 50	210, 520	", 175	0, tr	175 g animal died
IDPN	"	160	140	+++	
Ethylene cyanohydrin	"	280	300	+++	
Succinonitrile	15†	190	"	+	

* All except marked animals (+) are female. † Given in drinking water. Approx. dose.

Another source of error was inefficiency of extraction procedure as applied to urine. Determinations of recovery of 50 μg quantities of CAA added to 1 ml of normal rat urine indicated that only 75% of the CAA was recovered. These 2 errors would tend to counterbalance each other to some extent, but no effort has been made to apply any correction factor to values shown in Table IV.

Expressed as mole % of administered nitrile, urinary CAA values following injection of BAPN and IDPN were 11.1 and 15.2, respectively. The excreted CAA resulting from 4 to 6 carbon alkyl- and amino-nitriles accounted for 2% or less of administered nitriles. Although CAA apparently represents only a minor metabolic product of 4 to 6 carbon nitriles related to BAPN, its appearance in urine demonstrates that both amino- and alkyl-nitriles undergo some degradation from the end of the molecule opposite the cyano group.

Early studies on nitrile metabolism have emphasized direct attack on the nitrile group (9) with conversion either to a carboxyl group, or to thiocyanate. Although we observed traces of thiocyanate (R_f *ca* 0.25 in a butanol:acetic acid:water system, 4:1:1 by volume) in all rat urines, including normals, no marked increase appeared on chromatograms corresponding to any particular nitrile. The major form of excretion of most of the

TABLE IV. Quantitative Determination of Nitrile Excreted in Urine as Cyanoacetic Acid by the Rat.

Compound	Dosage (μM/animal)	Wt of animal (g)	Urinary CAA* (μM after 18 hr)	Fraction recovered as CAA (mole %) avg	Intensity of CAA zone on chromatograms
BAPN	230	93, 105	51.8	11.2	+++
4-Aminobutyronitrile · HCl	250	89, 91	2.9	.58	—
5-Aminovaleronitrile · HCl	220	96, 96	4.4	1.0	+
N-Butyronitrile	300	153, 162	1.1	.18	—
N-Valeronitrile	245	148, 146	7.4, 11.9	2.0	+
N-Hexanenitrile	205	144, "	2.0, 3.5	.67	—
IDPN	160	135, 144	51.1, 47.3	15.4	+++
None (control)	0	128, 164	.9, 3.8		—

* Correction for apparent CAA of normal rat urine has not been made. Where one figure is given it represents avg of 2 values in close agreement. In other cases, the 2 values represent individual determinations. In each case, however, determinations were made on pooled urine from the 2 rats used for each compound, and CAA excretion values are totals for both animals.

nitriles is not disclosed by present studies. Unchanged nitriles and ω-carboxy-nitriles which have undergone no degradation of the carbon chain may represent the major types of urinary excretion products. Identification of α-cyanopropionic acid and unchanged 2-cyanopropylamine as urinary excretion products of 2-cyanopropylamine have been reported(10).

Summary. 1. Among weanling rats receiving 0.3% dietary BAPN fumarate those showing less severe skeletal deformities tended to excrete relatively larger amounts of cyanoacetic acid (CAA) in urine, and vice versa. Increasing dietary casein level from 12 to 24% resulted in less severe skeletal lesions and higher urinary CAA excretion for a given dosage of BAPN. 2. Appreciable urinary excretion of CAA followed injection of the following organic nitriles into rats: 2,2′-iminodipropionitrile (IDPN), ethylene cyanohydrin, succinonitrile, 5-aminovaleronitrile, and n-valeronitrile. Traces of CAA resulted from injection of n-hexanenitrile and 4-aminobutyronitrile.

1. Merkow, L. P., M. S. Thesis, Univ. of Wisconsin, 1959.

2. Lipton, S. H., Lalich, J. J., Garbutt, J. T., Strong, F. M., *J. Am. Chem. Soc.,* 1958, v80, 6594.

3. Lalich, J. J., *Science,* 1958, v128, 206.

4. Sievert, H. W., Lipton, S. H., Strong, F. M., *Arch. Biochem. Biophys.,* in press.

5. Lipton, S. H., Lalich, J. J., Strong, F. M., *J. Am. Chem. Soc.,* 1958, v80, 2022.

6. Goldberg, A. A., Kelley, W., *J. Chem. Soc.,* 1947, 1369.

7. Block, R. J., Durrum, E. L., Zweig, S., *A Manual of Paper Chromatography and Paper Electrophoresis,* Academic Press, N. Y., 1955, p319.

8. Dasler, W., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 485.

9. Williams, R. T., *Detoxication Mechanisms,* John Wiley and Sons, N. Y., 1949, Chapt. 8.

10. Roy, D. N., Lipton, S. H., Bird, H. R., Strong, F. M., PROC. SOC. EXP. BIOL. AND MED., in press.

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Development of Resistance by Cell Cultures to Photodynamic Action. (25379)

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During studies of poliovirus by plaque assay technic(1) it was observed that the uppermost layer of monkey kidney (MK) culture would frequently degenerate within 48 hours after application of overlay medium. The present study was undertaken to determine which factors controlled this cell degeneration.

Materials and methods. Cell cultures. Monolayer cultures of MK(2) in 2 oz. bottles were grown in Medium 199 containing 2% calf serum.* *Media.* Four preparations of Medium 199(3) were employed in these experiments—Medium 199 containing no dye, Medium 199 containing 1:400,000 phenol red, plaque Medium 199 containing no dye, and plaque Medium 199 containing 1:50,000 neutral red. *Light source.* In most experiments a fluorescent desk lamp containing two 15 watt daylight-type tubes was placed 9 inches above the cell cultures. Light intensities were measured by a Weston light meter model 715. *Extraction of neutral red from cells.* MK cultures in 2 oz. bottles were fed 5 ml fluid Medium 199 containing 1:50,000 neutral red and incubated in the dark at 36°C for 48 hours. Each culture was rinsed 3 times with BSS, placed in contact with 1.0 ml of 2-fold concentrated Medium 199, frozen and thawed twice and incubated at 36°C for 4 hours. Cell debris was removed by low speed centrifugation and the clarified plaque medium mixed with agar before application to fresh cultures.

Results. To determine whether degeneration of the uppermost layer of MK cultures resulted from overheating of top bottles by

* The cultures were prepared by the cell culture laboratory of Division of Biologics Standards, N.I.H. under supervision of Dr. Paul Gerber.