Beta-Aminoisobutyric Acid, A Possible Factor in Pyrimidine Metabolism.*† (19000)

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The unknown substance previously reported(1) as a major ninhydrin-reacting component in a number of urine chromatograms, particularly in those of patients with malignancy, has now been isolated from the urine of a normal subject(2) and 2 cancer patients and identified as beta-aminoisobutyric acid (BAIB). Preliminary animal experiments indicate that its precursor is a 5-methylpyrimidine.

Experimental. Isolation and identification of beta-aminoisobutyric acid. Urine samples from cancer patients were collected in bottles containing thymol and stored in the freezer. When several specimens containing high concentrations of the unknown compound had been collected from a single patient they were pooled and fractionated in ion-exchange columns. The large variety and high concentrations of extraneous substances in urine made the fractionation more difficult than in the case of protein hydrolysates(3), but ion exchange appears to be the method of choice for separating the small quantity of amino acids in urine from the much larger quantity of strong electrolytes and non-electrolytes. Generally, our best results were obtained as follows: The urine was diluted 2-fold or more, filtered, and passed through a large column (diam. 2-4 cm; ht. 50-70 cm) filled with coarse particles of a cation exchanger of the sulfonic acid type (Amberlite IR-105, Amberlite IR-120, or Dowex 50). The column was washed with water and the amino acids eluted with dilute NH_4OH (0.1 to 0.3 N), the eluent being collected in tubes containing thymol. The band of amino acids occupied only a small portion of this first column and the fronts were often quite irregular (with precipitates sometimes forming local dams which had to be broken by stirring the column) so usually little or no fractionation of the individual amino acids occurred, and all the alkaline ninhydrin-positive fractions were pooled and concentrated *in vacuo* to remove ammonia, and then all the amino acid fractions were combined and diluted for passage through a second column. The second column (diam. 1-2 cm, ht. 40-60 cm) contained 60-80 mesh particles of an anion exchanger of the strong base type (Amberlite IRA-400). The pooled fractions from the first column were added, the column was washed with water, and the amino acids were displaced with 0.5 N NH_4OH or 0.16 N acetic acid. (Acid elution sometimes caused formation of gas bubbles, presumably CO_2, in such quantity as to interfere with the flow and necessitate their removal by occasional stirring of the column). Usually some separation of the various amino acids occurred at this stage and only the fractions found chromatographically to contain significant concentrations of the unknown compound were pooled, concentrated *in vacuo,* and diluted. Final ion exchange fractionation was carried out in a column 3-10 mm in diameter and 40-60 cm high, filled with sulfonic acid-type resin of 80-100 mesh. The selected eluate of the previous column, a water wash, and

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1. Fink, K., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 692.

2. Crumpler, H. R., Dent, C. E., Harris, H., and Westall, R. G., *Nature,* 1951, v167, 307.

3. Partridge, S. M., and Westall, R., *Biochem. J.,* 1949, v44, 418.

dilute ammonia were passed through at a rate of 1 ml per minute, or less, and the eluate was collected in small fractions.

The best ion exchange fractions contained the unknown compound as the only ninhydrin-reacting component, but the usual procedures failed to produce crystals, and concentration and chromatography revealed the presence of colored and fluorescent substances. Accordingly, some of the best fractions were streaked across large sheets of washed filter paper, chromatographed in butanol, acetic acid, and water (2:1:1), and the areas containing the ninhydrin reacting substance were isolated and eluted as previously described(1) or by Soxhlet extraction with methanol.

A solution of the unknown compound prepared from the urine of one cancer patient by ion exchange and chromatography yielded 67 mg of a crystalline benzenesulfonyl derivative when subjected to the Schotten-Baumann procedure(4). This derivative, when recrystallized from water or toluene (0.2 ml per 10 mg), melted at 83.0 to 83.6°C‡ and yielded equivalent weight values of 254, 247, and 247. (Equivalent weight calculated for the benzenesulfonyl derivative of a 4-carbon, mono-amino, mono-carboxylic acid 243.3).

Analysis§—$C_{10}H_{13}NSO_4$.

Calculated: C 49.36, H 5.39, N 5.76

Found: C 49.49, H 5.63, N 5.68

Hydrolysis of a small portion of the derivative overnight at 100°C in 12 N HCl yielded a ninhydrin-reacting compound with the original chromatographic properties. The isolated substance could be distinguished from the available 4-carbon mono-amino, mono-carboxylic acids, alpha-amino-n-butyric acid, alpha-aminoisobutyric acid, and gamma-amino-n-butyric acid by its chromatographic properties and reaction to cupric ion.¶ Its stability to acid hydrolysis served to distinguish it from beta-amino-n-butyric acid(5), and comparison of the chromatographic behavior of glycine and sarcosine appeared to rule out N-methyl alanine, N-methyl beta-alanine, N-dimethyl glycine, and N-ethyl glycine as possible structures for the unknown. The remaining isomer, beta-aminoisobutyric acid, was synthesized following the procedure of Clemo and Melrose(6) by HCl hydrolysis of the 5-methyl-4,5-dihydrouracil obtained from the condensation of urea and ethyl methacrylate. The synthetic amino acid was chromatographically indistinguishable from the urinary compound and gave the same reaction to copper. A benzenesulfonyl derivative of the synthetic BAIB, prepared as before, melted at 80-83°C, and a mixture of the synthetic and natural derivatives melted at 79-82.5°C.

The "best fractions" from the urine of a second cancer patient yielded about 15 mg of clean crystals of the free amino acid after the fractions were evaporated, dissolved in hot butanol and twice recrystallized. The isolated compound melted at 181-184°C, a synthetic sample of BAIB prepared by Pollack(7)‖ melted at 177-178°C, and a mixture of the natural and synthetic compounds melted at 163-178°C. The infra-red absorption spectra of the natural and synthetic compounds showed only slight differences, similar to those shown by the spectra of *l* and *dl* leucine,†† and as the two preparations also appeared to be identical in their reaction to copper and their chromatographic behavior in various solvents, it was assumed that the melting point variation was due to optical isomerism in the natural BAIB. This assumption appears to

4. Keston, A. S., Udenfriend, S., and Cannan, R. K., *J. Am. Chem. Soc.*, 1949, v71, 254.

‡ All melting points corrected.

§ Elek Micro Analytical Laboratories, Los Angeles.

¶ The previous paper(1) failed to make clear that it was a *variation* of the Crumpler and Dent(5) copper technic, with the amino acids remaining in the copper-brushed area of a one-dimensional chromatogram, in which ninhydrin gave a peculiar color reaction with the unknown compound. The two-dimensional technic described by Crumpler and Dent results in the usual color reaction.

5. Crumpler, H. R., and Dent, C. E., *Nature*, 1949, v164, 441.

6. Clemo, G. R., and Melrose, T. A., *J. Chem. Soc.*, 1942, 424.

7. Pollack, M., *J. Am. Chem. Soc.*, 1943, v65, 1335.

‖ The authors are indebted to Dr. L. J. Reed who sent us a sample of Pollack's synthetic BAIB.

†† We are indebted to Dr. R. E. Nusbaum of the U.C.L.A. Atomic Energy Project for measuring and studying the infra-red absorption spectra.

have been verified by the recent publication of Crumpler, Dent, Harris, and Westall(2), whose isolated BAIB was laevorotatory, melted at 183-184°C, and required racemization before melting point and X-ray diffraction data showed its identity with Pollack's (7) synthetic BAIB.

*Clinical studies.*** First morning urine specimens were collected in bottles containing thymol, an aliquot was stored at —20°C for possible rechecking, and the remainder stored at 5°C until the chromatograms were set up, which was rarely more than 2 hours after the specimen was voided. Duplicate two-dimensional filter paper chromatograms were prepared from 25 μl aliquots of the fresh urine specimens, one with known amino acids added as markers, using water-saturated phenol as the first solvent and the upper phase from a mixture of tertiary butanol, secondary butanol, and water (1:5:5.6) as the second solvent. The density of the ninhydrin-developed spots was determined with a photoelectric cell, and the urinary concentration of the various amino acids then estimated from standard curves prepared from similar chromatograms containing a known number of micromoles of the various amino acids. The standard curves could be reproduced within about ±20%, and addition of known amounts of various amino acids to urine indicated that much of our data on urinary amino acids also falls within this range of accuracy. Overlapping, streaking, or distortion of the spots on urine chromatograms can increase the error markedly, however, and the routine urinary data, particularly as regards amino acids present in very high or very low concentrations, are considered as potentially in error by a factor of two or more.

Data on BAIB levels in urine specimens from a group of 140 apparently healthy subjects (physicians, nurses, laboratory personnel, and members of an Air Force National Guard unit) are shown in Table I. The variability in concentration of the more common urinary amino acids and in the chromatographic development is to some extent compensated for by subdividing the group according to the ratio of BAIB to alanine shown by each chromatogram. In 63% of the group studied BAIB was not detectable, indicating urinary levels less than about 0.15 micromole per liter, the ninhydrin test being only about half as sensitive for BAIB as for most of the simple alpha-amino acids. In 29% of the cases BAIB was detectable but showed concentrations less than twice those of alanine. Eight individuals of the 140 showed BAIB levels 2 to 3 times their alanine levels, and 3 subjects showed still higher BAIB/alanine ratios.

TABLE I. Urinary Excretion of beta-Aminoisobutyric Acid by 140 Apparently Normal Humans.

No. of cases	BAIB/alanine ratio	Avg BAIB conc., millimoles/liter
88	BAIB not detectable	<.15
41	.3–1.9	.3
8	2–3	.4
1	5	2.5
2	12–13	3

Medical histories of individuals included in the control group were not requested, but the individual who showed the highest BAIB concentration was at first dubious about contributing a control specimen because of a family history of malignancy. This individual has remained in good health during the 1½ year period since the first observation. One of the early members of our control group has regularly shown a high BAIB level for the past 2 years (about one millimole per liter, 5 times his alanine level), but was omitted from the control group reported here since he recently noted the appearance on his leg of a small, rapidly growing nodule which was subsequently excised and diagnosed as a primary fibrosarcoma. Several individuals in the normal group were checked at infrequent intervals for periods up to 2½ years and were found to show little variation in their BAIB excretion. In day-to-day studies of patients with neoplastic disease, on the other hand, extreme variations in the urinary BAIB level have occasionally been noted, and the level

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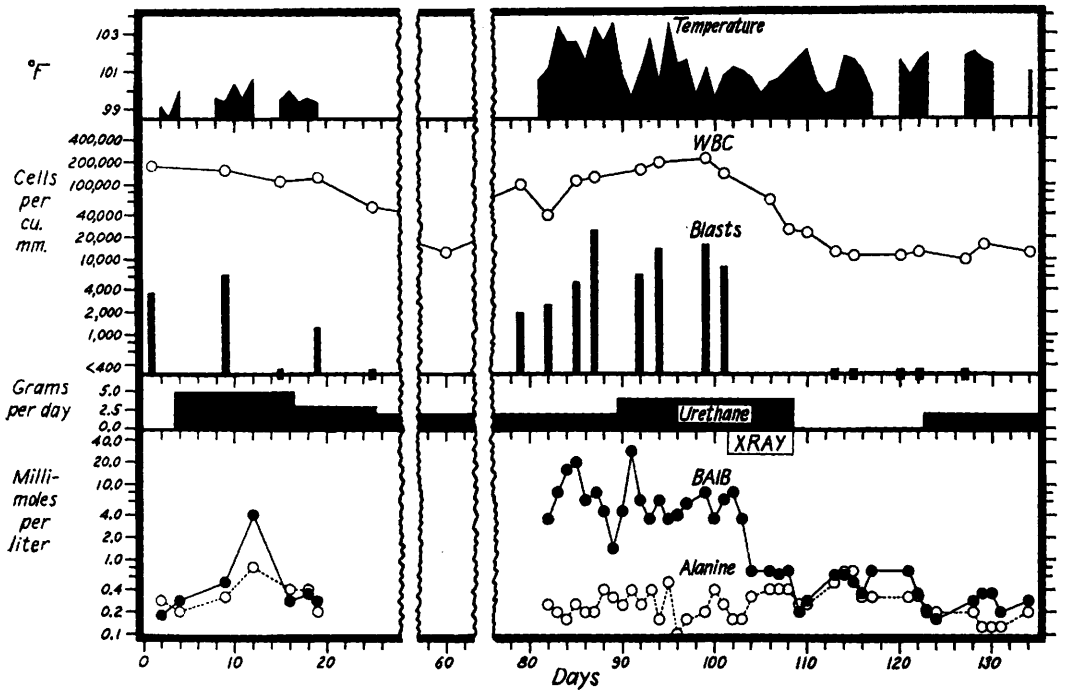

FIG. 1. Data on case of chronic myelogenous leukemia. Note that the blood cell counts and the urinary levels of alanine and BAIB (beta-aminoisobutyric acid) are plotted on logarithmic scales while the body temperature and urethane dosage are on arithmetic scales. The patient was acutely ill and bed-ridden during period from about 80-105 days but felt well and was ambulatory during the remainder of the observation periods.

seems to be affected by therapeutic regimes and the clinical state of the disease. The factors responsible for the variations are not clear, however, and although over a thousand urine specimens from cases of malignancy have been analyzed with duplicate 2-dimensional chromatograms, it appears that an intensive study of the relevant case histories as well as the collection of a larger quantity of data will be required before it can be accurately determined whether BAIB is significantly related to any given type of neoplasm or other disease.

The variability of BAIB excretion seen in some patients is illustrated in Fig. 1, which shows a portion of the clinical data obtained during 2 flareups of a case of chronic myelogenous leukemia. A marked elevation of urinary BAIB was found in only one specimen (day No. 12) during the first of the two episodes shown, and it was accompanied by a moderate elevation of other urinary amino acids. The patient had been hospitalized on the basis of his blood count at the beginning of this period (note that both the blood counts and the urinary amino acid levels are plotted on logarithmic scales) but he continued to feel well, was allowed to leave the hospital on weekends, and after about three weeks was discharged to return to his work and continue urethane therapy at home. His blood picture gradually improved, but on the 81st day an exacerbation of the leukemic process occurred and the patient was acutely ill for about three weeks, with a very high total white blood cell count, a high proportion of immature cells, fever, and a greatly enlarged spleen. Following an increase in urethane dosage and X-ray to the spleen the patient again became ambulatory, showed a drop in white count and blast cells, gained 10 pounds in 2 weeks, was allowed to go home on weekends, and finally was discharged with instructions to continue urethane therapy. During the acute phase of this second episode the urinary BAIB reached levels as high as 30 millimoles per

TABLE II. Urinary Excretion of beta-Aminoisobutyric Acid, Alanine, and Leucines by Rat No. 1 Under Various Dietary Regimes.

Day	Diet	Urinary concentration (μM/20 ml) BAIB*	Alanine	Leucines	Urine vol (ml)	Rat wt (g)
1	chow	0	2	0	16	250 (est.)
2	40% DNA†	20	24	6	19‡	
3 day	"	120	2	2	2	
3 night	chow	2	2	1	17	
4	"	2	3	1	27	
5	"	0	3	2	10	
6	"	0	2	2	22	205
7	8% thymine§	4	2	1	32	
8	"	3	2	1	34	
9	chow	5	4	2	17	
10	"	0	3	2	18	
11	"	0	2	1	13	215
12	"	0	2	2	14	
13	"	0	2	2	14	
14	10% DNA	3	2	1	16	231
15	"	3	2	1	20	237
16	"	6	2	1	26	236
17	"	5	4	1	25	230
18	"	3	2	1	25	232
19	"	3	2	1	20	241
20	chow	6	2	2	14	229
21	"	0	2	1	16	252
22	"	0	1	1	14	251

* The chromatogram spot reported in this column has been identified as BAIB (beta-Aminoisobutyric acid) with reasonable certainty in the case of the 40% DNA diet, and it is probable, but less certain, that the new spot which appeared during the thymine and 10% DNA feedings was also BAIB.

† Desoxyribonucleic acid from sperm (Nutritional Biochemicals Corp., Cleveland, Ohio). The rat consumed 4.2 g at the 40% level and 8.3 g at the 10% level.

‡ Diarrhea, sample contaminated with feces.

§ Rat consumed 1 g of thymine.

liter (rechecked for accuracy with smaller aliquots), about a 200-fold increase over some previously determined levels, and then fell again as the patient's clinical condition improved. The great variation in BAIB excretion was not caused by a generalized aminoaciduria, for the levels of other regularly detectable amino acids were found to show only about 3 to 10-fold variations in the urine specimens of this patient.

Animal experiments. Adult male albino rats were placed in metabolism cages and fed finely ground purina dog chow for a day or two to permit collection of control urine specimens. During an experimental period the dry test substance was thoroughly mixed with powdered chow. The urine was collected under toluene and chromatograms were prepared as in the clinical studies. The excretion levels of BAIB in the urine of two rats subjected to various dietary regimes are shown in Tables II and III, and data on the excretion of alanine and the leucines are included for comparison. Neither rat excreted detectable amounts of BAIB during the preliminary basal diet period. The 40% desoxyribonucleic acid (DNA) diet resulted in a marked loss in weight, a short period of diarrhea, a brief and generally small rise in the concentration of several urinary amino acids, and the appearance of a very high concentration of BAIB. The 40% ribonucleic acid (RNA) diet likewise caused a drop in body weight and production of very soft (but not liquid) feces, but it failed to produced notable changes in amino acid excretion. A 10% DNA diet was well tolerated by the rat for several days and chromatograms prepared during this period showed a rather light spot in the position taken by BAIB. The 8% thymine diet produced a similar spot, but not as dark as would have been expected from feeding DNA containing an equivalent quantity of thymine.

TABLE III. Urinary Excretion of beta-Aminoisobutyric Acid, Alanine, and Leucines by Rat No. 2 Under Various Dietary Regimes.

Day	Diet	Urinary concentration (μM/20 ml) BAIB*	Alanine	Leucines	Urine vol (ml)	Rat wt (g)
1	chow	0	2	2	26	300
2	40% DNA†	4	7	8	22	
3	"	40	2	2	22	
4	"	70	5	2	28‡	
5	chow	10	4	2	31	267
6	"	0	4	2	21	
7	"	0	5	2	29	
8	"	0	3	2	21	305
9	40% RNA§	0	3	1	32	283
10	"	0	3	1	18	269
11	"	0	2	1	10	274
12	chow	0	5	2	22	
13	"	0	3	2	37	304
14	"	0	3	2	30	317
15	"	0	2	2	18	313

* beta-Aminoisobutyric acid.

† Desoxyribonucleic acid from sperm (Nutritional Biochemicals Corp., Cleveland, Ohio). Rat consumed 8.2 g.

‡ Diarrhea, sample contaminated with feces.

§ Ribonucleic acid (Nutritional Biochemicals Corp., Cleveland, O.). Rat consumed 7.7 g.

Rat urines generally yield poor chromatograms, and the area of the chromatograms occupied by BAIB is potentially a crowded one(1), but there is very little doubt that the intense spot produced by the 40% DNA diet actually was BAIB, for its chromatographic behavior in a number of solvents, its stability to acid hydrolysis, and its reaction to copper appeared to be identical with that of the BAIB isolated from urine of humans with neoplastic disease. Although the 8% thymine and 10% DNA diets each produced an acid-stable compound which gave a spot in the chromatographic position of BAIB, and these spots are listed as BAIB in Table II, the concentrations in these cases were so low that a partial purification will be required before extensive chromatographic and chemical tests can establish the identity of the spots beyond reasonable doubt.

Discussion. There appears to be no doubt that many human subjects do excrete BAIB and that some of them excrete a greater amount of it than of any other free amino acid. In addition to the data presented in this paper, Crumpler *et al.*(2) have reported that about 5% of their large group of normal subjects showed consistently high urinary levels of BAIB and that a study on relatives of some of these indicated the involvement of a genetic factor. Some of the available data suggest that BAIB may be related to the cancer process, but a determination of whether the suggested relationship is real and of metabolic significance will require further study.

A "blackboard survey" of conceivable BAIB precursors pointed to the 5-methylpyrimidines as likely possibilities. In preliminary animal experiments a diet high in 5-methylpyrimidine—containing DNA caused BAIB excretion and appeared to support the hypothesis, especially since a similar diet in which the DNA was replaced by RNA failed to show the effect and thus served as a control which tended to rule out some possible complicating factors such as an adenine effect on the kidney(8). A diet high in free thymine caused excretion of a substance tentatively identified as BAIB, but at a lower concentration than might have been expected on the basis of the probable combined thymine content of the DNA diet. It is possible that the free thymine was poorly absorbed or inefficiently catabolized, and isotopic studies or feeding experiments with thymidine, 5-methylcytosine(9,10), and other compounds may

8. Bendich, A., Brown, G. B., Philips, F. S., and Thiersch, J. B., *J. Biol. Chem.*, 1950, v183, 267.

9. Wyatt, G. R., *Nature*, 1950, v166, 237.

clarify the point. No evidence has been obtained to indicate which of several conceivable biochemical pathways might be involved in the formation of BAIB from a 5-methylpyrimidine.

The hypothesis(2) of a genetically determined error in kidney resorption seems logical as an explanation of the high BAIB levels in some normal individuals, but it does not appear to be consistent with clinical data showing marked lowering of the BAIB excretion following therapeutic regimes more likely to injure than to improve kidney function. The studies described suggest, as one alternative hypothesis, that a high BAIB level might indicate an abnormal rate or type of 5-methylpyrimidine catabolism.

Summary. (1) A compound frequently noted in a chromatographic study of urinary amino acids has now been isolated from the urines of two cancer patients and identified as beta-aminoisobutyric acid (BAIB). (2) In a group of 140 apparently healthy subjects, 52 individuals showed detectable urinary levels of BAIB (>0.15 millimole per liter), and three of these showed concentrations over 1 millimole per liter. Individual control subjects studied at intervals for periods up to 2½ years showed little fluctuation in BAIB excretion. (3) In individual patients with neoplastic disease, the urinary BAIB level occasionally varied markedly and appeared to bear some relationship to the neoplastic process. Such variations are illustrated by a report of a case of chronic myelogenous leukemia. (4) Theoretical considerations pointed to a 5-methylpyrimidine as a likely precursor of BAIB, and evidence favoring this view was obtained by rat experiments in which BAIB excretion was produced by a diet containing large amounts of desoxyribonucleic acid but not by one similarly enriched in ribonucleic acid.

10. Johnson, T. B., and Coghill, R. D., *J. Am. Chem. Soc.*, 1925, v47, 2838.

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Effect of Cortisone on the Mast Cells of the Rat. (19001)

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Recent observations have stressed the important role played by cortical hormones, especially cortisone, in the collagen diseases and in the biochemistry of the mesenchymal changes in dermal connective tissue both in man and in laboratory animals under normal and pathological conditions. These changes concern both the fibrillar and cellular constituents of the connective tissue and the homogeneous interfibrillar ground substance. There are two prevalent opinions concerning the physiological significance of the mast cells: some authors believe that these cells produce the hyaluronic acid of the ground substance; according to others the mast cells produce heparin.

In view of the evident changes caused by cortisone on acid muco-polysaccharides (hyaluronic acid) of the tissues, we wished to ascertain whether these histochemical and biochemical changes were related to the morphology of the mast cells.

Material. For this purpose, 36 unselected adult male rats, fed on a fixed and balanced standard diet, were divided in groups of 6 animals. Three groups were injected subcutaneously with cortisone ("Ciba") 10-5-1 mg/day per animal for 3-20-70 days respectively. For the same period 3 groups of control animals were given similar doses of physiological saline alone. At the end of experiment all animals were killed with a sharp blow on the head. Specimens were taken from the skin of the neck and from the muscles of the thigh and myocardium; these were fixed in Carnoy fluid, cut in series, and stained with an aqueous