Quantitative Plasma Amino Acid Values in Leukemic Blood

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QUALITATIVE CHANGES in blood proteins and amino acid nitrogen have long been observed in patients with leukemia. Okada and Hayashi[1] determined the amino acid nitrogen content of deproteinized blood from advanced leukemia cases. They found that the amino acid nitrogen increased or decreased in a fairly consistent manner according to the number of white blood cells present. Values for the non-protein nitrogen levels in the blood plasma of patients with acute, chronic lymphatic and chronic granulocytic leukemia have since been established by other authors.[2, 3]

Baldridge and Barer[4] found an increased oxygen consumption in patients with this disease and suggested that protein catabolism was the major factor contributing to an increased metabolic rate. The relationship of increased basal metabolism to protein catabolism and amino acids levels, however, has not yet been clarified. Until now, the lack of more specific methods for the quantitative determination of the individual amino acids present in blood has been a limiting factor in the further understanding of amino acid metabolism in leukemia.

Quantitative determinations of several free amino acids in blood plasma were made by Waisman et al.[5] Using the microbiologic method of Henderson, Brickson, and Snell,[6] comparisons of the free amino acids were made between normal individuals and leukemic patients before and during remission periods. Their results showed significant increases in phenylalanine, tyrosine and isoleucine in patients with acute leukemia. The values for valine were also sufficiently different to indicate a possible abnormality in the metabolism of this amino acid.

This report presents additional data on the estimation of free amino acids present in the blood plasma of normal and leukemic subjects determined by paper chromatographic micro-analysis and two chemical methods.

METHODS

The paper chromatographic method suggested by Levy,[7] and elucidated by Frankel-Conrat et al.,[8] utilizing derivatives of the 1-fluoro-2, 4 dinitrobenzene* has been modified for the estimation of the free amino acids present in blood plasma. Twenty to 30 ml. of whole blood, drawn in syringes moistened with 1:5000 heparin, were obtained from apparently normal hospital personnel and from patients with laboratory proven cases of leu-

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* FDNB will be used to designate 1-fluoro-2, 4₁ dinitrobenzene, and DNP the 2,4-dinitrophenyl radical.

kemia. The heparinized blood was centrifuged and the plasma, if not used at once for analysis, was frozen and stored. A 4 ml. plasma aliquot was mixed with 4 ml. of a 4 per cent solution of perchloric acid and allowed to stand for 15 minutes and then centrifuged. The precipitate was washed with 0.5 ml. of a 1 per cent perchloric acid solution and the mixture again centrifuged. The combined supernatant liquids were shaken with three 5 ml. volumes of ether* to remove any fat-like material present. The resulting suspension was clarified by further centrifugation. The supernatant liquid was transferred to a 30 ml. beaker, adjusted to pH 9.0 with dilute sodium hydroxide, and 3 ml. of pH 9 buffer† added. This solution was placed in a 40C. oil bath and stirred with a magnetic stirrer. A suspension of 0.13 ml. of FDNB in 0.3 ml. of n-butanol was added to the buffered protein-free supernatant. The reaction was allowed to proceed for 80 minutes at 40 C. A few drops of 0.5 N sodium hydroxide was usually added during this period to maintain pH 9.

The solution of the DNP-amino acids was transferred to a 60 ml. separatory funnel and the unreacted FDNB extracted with ether. The solution was acidified with 0.5 ml. of 5 N HCl and the DNP-amino acids were removed by ether extraction. Five ml. portions of ether were used and the process repeated until the ether extract was colorless. The combined ether extracts were adjusted to 25 ml. and three 3 ml. aliquots removed for spotting.

The ether solution was first evaporated to dryness and the residue dissolved in a milliliter of acetone. The acetone solution was applied in small volumes to the lower left-hand corner of an 11¼" x 18½" piece of Whatman No. 52 filter paper. This paper was preferred to Whatman No. 1 since it was found to produce spots which were smaller in area and more compact.

An ascending technique for the first dimension consisted of a solvent system containing toluene, chloroethanol and pyridine, which had been equilibrated with 0.8 N aqueous ammonia (5:3:1.5:3 by vol.). A 1.5 M aqueous phosphate buffer was utilized as the irrigating fluid for the second dimension. The DNP-amino acid spots were eluted from the paper with 1 per cent sodium bicarbonate solution by heating in a water bath at 55–60 C. for 30 minutes. The optical density of the cooled solution was then measured at 360 mμ (385 mμ for DNP-proline). To eliminate any possible source of error which might occur at the low amino acid concentrations used, a series of standard curves were prepared for each amino acid determined. To estimate the extent of loss occurring during the protein precipitation and fat extraction steps, recovery experiments were also performed. The percentage recovery for these steps averaged 85 per cent. The amino acid levels tabulated in the "results" section have not been corrected for this loss.

The Chemical Determination of Arginine and Tyrosine

The separation of DNP-tyrosine from dinitroaniline can not always be achieved with the Levy procedure. DNP-arginine is also estimated with difficulty since it remains in the aqueous phase. Therefore, most of the samples used for the chromatographic study were also examined for arginine and tyrosine by two chemical methods. The free tyrosine in blood plasma was determined by the procedure of Udenfriend and Cooper.[9] Under appropriate conditions, 1-nitroso 2-napthol reacts with compounds having p-hydroxyphenyl groups to produce a yellow solution whose optical density can be determined at 450 mμ. A modification of the Sakaguchi reaction as suggested by Gilboe and Williams[10] was utilized to measure the levels of arginine in blood plasma. To an alkaline solution of the protein free plasma filtrate, 8-hydroxy quinoline was added. When the solution was treated with NaOBr a red color developed, the optical density of which was read at 500 mμ.

Results

Blood plasmas from normal and leukemic individuals were analyzed by the Levy procedure modified according to the methods described above. The fol-

* All ether used has been first shaken with ferrous chloride to remove peroxides.

† 21.3 ml. of 0.5 NaOH and 50 ml. of 0.5 M H_3BO_3 diluted to 100 ml.

lowing tables summarize the estimation of several of the free amino acids present in blood plasma. To establish a mean value as well as a range for these amino acids in normal subjects, plasma samples from student nurses, medical students and patients with mild emotional problems were analyzed. These control values have been incorporated into the tables to facilitate comparison with leukemic groups. No distinction has been made as to the ages of the subjects analyzed since the plasma amino acid levels of the normal controls were not found to vary with the age of the subjects studied. The blood plasmas of both treated and untreated leukemic subjects were examined. An attempt was made to determine the amino acid levels in the same individual before and after medication was administered. While this was done in some cases many of the untreated leukemic subjects were discharged from the hospital before any medication was given and did not return for further examination. In general therefore, more amino acid values were available for the treated cases. Patients who had received any type of therapy such as 6-mercaptopurine, blood transfusions or x-ray treatment have been designated as treated patients.

Observed Differences in the Plasma Amino Acid Levels Between Normal and Leukemic Subjects as Determined by the Paper Chromatographic Method

The untreated, treated and control plasma amino acid levels have been compared after obtaining the mean value of each amino acid concentration and calculating the standard error of the mean. The amino acid values for the leukemic subjects as a group (i.e., untreated plus treated cases and recorded as "total cases" in the tables) were statistically compared with the control group. The significance of the differences were determined by using the "t" test. Any "p" values less than 0.05 were considered significant.

The DNP derivatives of leucine and isoleucine appeared as one spot on the chromatogram. In order to make some comparison between normal and leukemic subjects regarding these two amino acids, the spot was determined as "leucine." The mean levels of "leucine" were high in all untreated leukemics as compared with the controls, however, only the cases with the acute disease showed significant increases in the "leucine" value.

Plasma Amino Acid Levels in Normal and Acute Leukemia Subjects (Table 1). The acute leukemia patients as a group were found to have increased levels of glutamic acid and phenylalanine whereas the concentrations of asparagine and threonine were lower than normal. Serine values had a tendency to be low but were at the borderline of significance. This was also found to be true for the chronic lymphatic and chronic myelogenous leukemic subjects studied. No differences in the levels of tryptophane and valine were observed among the acute, chronic lymphatic or chronic myelogenous leukemic groups.

Several observations can be made from the mean amino acids values in the leukemic subjects before and following treatment. Following treatment, the levels of glutamic acid and phenylalanine decreased whereas the concentrations of proline and asparagine increased. Only proline was further increased from the established normal level.

Plasma Amino Acid Levels in Normal and Chronic Lymphatic Leukemia Subjects (Table 2). The plasmas of chronic lymphatic leukemia patients were found to

TABLE 1.—*Plasma Amino Acid Levels in Normal and Acute Leukemia Subjects*

Amino Acid	Number of Subjects	Plasma Levels (mg./100 ml. plasma)	
		Mean and S.E.	Range
Alanine			
Untreated Cases	7	2.2 ± 0.2	1.5–3.2
Treated Cases	4	2.0 ± 0.7	1.0–4.0
Total Cases	11	2.1 ± 0.3	1.0–4.0
Normal Controls	16	2.1 ± 0.1	1.5–2.6
Asparagine			
Untreated Cases	9	4.3 ± 0.5	2.3–6.4
Treated Cases	6	5.1 ± 0.9	1.6–8.3
Total Cases*	15	4.6 ± 0.5	1.6–8.3
Normal Controls	17	6.2 ± 0.3	4.5–9.0
Glutamic Acid			
Untreated Cases	9	1.5 ± 0.3	0.4–3.7
Treated Cases	6	1.1 ± 0.2	0.4–1.8
Total Cases*	15	1.3 ± 0.2	0.4–3.7
Normal Controls	17	0.7 ± 0.04	0.4–1.0
Phenylalanine			
Untreated Cases	9	1.4 ± 0.2	0.7–1.8
Treated Cases	6	1.1 ± 0.1	0.7–1.7
Total Cases*	15	1.3 ± 0.4	0.7–1.8
Normal Controls	17	0.8 ± 0.02	0.6–1.0
Proline			
Untreated Cases	9	1.9 ± 0.1	1.1–2.3
Treated Cases	6	2.3 ± 0.3	0.8–6.7
Total Cases	15	2.0 ± 0.1	0.8–6.7
Normal Controls	17	1.7 ± 0.1	1.1–3.0
Serine			
Untreated Cases	9	0.8 ± 0.1	0.4–1.2
Treated Cases	6	0.9 ± 0.1	0.5–1.4
Total Cases	15	0.8 ± 0.1	0.4–1.4
Normal Controls	17	1.0 ± 0.04	0.7–1.3
Threonine			
Untreated Cases	9	0.9 ± 0.1	0.5–1.3
Treated Cases	6	1.0 ± 0.2	0.5–1.3
Total Cases*	15	0.9 ± 0.1	0.5–1.3
Normal Controls	17	1.2 ± 0.1	0.9–1.8
Tryptophane			
Untreated Cases	8	0.6 ± 0.1	0.3–1.0
Treated Cases	3	0.5 ± .0.1	0.4–0.7
Total Cases	11	0.6 ± 0.1	0.3–1.0
Normal Controls	16	0.6 ± 0.1	0.4–1.2
Valine			
Untreated Cases	9	2.1 ± 0.2	1.3–2.8
Treated Cases	6	2.1 ± 0.3	1.3–2.7
Total Cases	15	2.1 ± 0.1	1.3–2.8
Normal Controls	17	1.9 ± 0.1	1.3–3.3

* Difference significant, "P" value less than 0.05.

TABLE 2.—*Plasma Levels of Amino Acids in Normal and Chronic Lymphatic Leukemia Subjects*

Amino Acid	Number of Subjects	Plasma Levels (Mg./100 ml. plasma)	
		Mean and S.E.	Range
Alanine			
Untreated Cases	7	2.9 ± 0.4	1.4–4.7
Treated Cases	8	2.9 ± 0.4	1.3–5.3
Total Cases*	15	2.9 ± 0.3	1.3–5.3
Normal Controls	16	2.1 ± 0.1	1.5–2.6
Asparagine			
Untreated Cases	7	7.0 ± 1.0	2.2–9.8
Treated Cases	9	6.2 ± 0.9	1.5–9.3
Total Cases	16	6.6 ± 0.7	1.5–9.8
Normal Controls	17	6.2 ± 0.3	4.5–9.0
Glutamic Acid			
Untreated Cases	7	1.3 ± 0.4	0.6–3.0
Treated Cases	9	1.9 ± 0.8	0.4–7.1
Total Cases*	16	1.7 ± 0.5	0.4–7.1
Normal Controls	17	0.7 ± 0.04	0.4–1.0
Phenylalanine			
Untreated Cases	7	1.1 ± 0.2	0.8–1.9
Treated Cases	9	1.0 ± 0.1	0.8–1.4
Total Cases*	16	1.1 ± 0.1	0.8–1.9
Normal Controls	17	0.8 ± 0.02	0.6–1.0
Proline			
Untreated Cases	7	2.3 ± 0.1	1.0–3.8
Treated Cases	9	2.9 ± 0.2	2.1–3.6
Total Cases*	16	2.6 ± 0.2	1.0–3.8
Normal Controls	17	1.7 ± 0.1	1.1–3.0
Serine			
Untreated Cases	7	0.9 ± 0.1	0.6–1.6
Treated Cases	9	0.8 ± 0.1	0.4–1.2
Total Cases	16	0.8 ± 0.1	0.4–1.6
Normal Controls	17	1.0 ± 0.04	0.7–1.3
Threonine			
Untreated Cases	7	1.3 ± 0.2	0.6–2.1
Treated Cases	9	1.1 ± 0.1	0.7–1.4
Total Cases	16	1.2 ± 0.3	0.6–2.1
Normal Controls	17	1.2 ± 0.1	0.9–1.8
Tryptophane			
Untreated Cases	5	0.5 ± 0.1	0.3–1.0
Treated Cases	7	0.7 ± 0.1	0.3–1.2
Total Cases	12	0.6 ± 0.1	0.3–1.2
Normal Controls	16	0.6 ± 0.1	0.4–1.2
Valine			
Untreated Cases	7	1.8 ± 0.1	1.4–2.3
Treated Cases	9	1.9 ± 0.2	1.0–3.6
Total Cases	16	1.8 ± 0.1	1.0–3.6
Normal Controls	17	1.9 ± 0.1	1.3–3.3

* Difference significant, "P" value less than 0.05.

TABLE 3.—*Plasma Amino Acid Levels in Normal and Chronic Myelogenous Leukemia Subjects*

Amino Acid	Number of Subjects	Plasma Levels (mg./100 ml. plasma)	
		Mean and S.E.	Range
Alanine			
Untreated Cases	3	3.3 ± 0.7	2.0–4.5
Treated Cases	3	3.0 ± 0.4	2.2–3.6
Total Cases*	6	3.2 ± 0.4	2.0–4.5
Normal Controls	16	2.1 ± 0.1	1.5–2.6
Asparagine			
Untreated Cases	5	5.4 ± 0.9	2.0–7.3
Treated Cases	4	5.7 ± 1.5	1.2–7.6
Total Cases	9	5.5 ± 0.8	1.2–7.6
Normal Controls	17	6.2 ± 0.3	4.5–9.0
Glutamic Acid			
Untreated Cases	5	1.6 ± 0.6	0.5–3.9
Treated Cases	4	2.2 ± 1.5	0.5–6.7
Total Cases*	9	1.8 ± 0.7	0.5–6.7
Normal Controls	17	0.7 ± 0.04	0.4–1.0
Phenylalanine			
Untreated Cases	5	1.2 ± 0.1	1.0–1.3
Treated Cases	4	0.9 ± 0.1	0.7–1.0
Total Cases*	9	1.0 ± 0.1	0.7–1.3
Normal Controls	17	0.8 ± 0.02	0.6–1.0
Proline			
Untreated Cases	4	2.7 ± 0.5	1.7–3.8
Treated Cases	4	2.8 ± 0.5	1.7–3.8
Total Cases*	8	2.7 ± 0.3	1.7–3.8
Normal Controls	17	1.7 ± 0.1	1.1–3.0
Serine			
Untreated Cases	5	0.8 ± 0.1	0.5–1.1
Treated Cases	4	0.8 ± 0.1	0.4–0.9
Total Cases	9	0.8 ± 0.1	0.4–1.1
Normal Controls	17	1.0 ± 0.04	0.7–1.3
Threonine			
Untreated Cases	5	1.5 ± 0.2	0.9–1.9
Treated Cases	4	1.2 ± 0.1	1.0–1.3
Total Cases	9	1.3 ± 0.1	0.9–1.3
Normal Controls	17	1.2 ± 0.1	0.9–1.8
Tryptophane			
Untreated Cases	3	0.6 ± 0.2	0.4–0.9
Treated Cases	4	0.5 ± 0.1	0.3–0.7
Total Cases	7	0.5 ± 0.1	0.3–0.9
Normal Controls	16	0.6 ± 0.1	0.4–1.2
Valine			
Untreated Cases	5	1.9 ± 0.2	1.4–2.6
Treated Cases	4	1.8 ± 0.2	1.4–2.4
Total Cases	9	1.9 ± 0.2	1.4–2.6
Normal Controls	17	1.9 ± 0.1	1.3–3.3

* Difference Significant, "P" value less than 0.05.

have increased levels of glutamic acid, alanine, phenylalanine and proline. Glutamic acid and proline values were higher for the treated chronic lymphatic cases than for the untreated cases. Although the mean value for asparagine in the untreated cases was found to be higher than that established for the normal, the mean value for the treated subjects was equal to the normal level.

Plasma Amino Acid Levels in Normal and Chronic Myelogenous Leukemia Subjects (Table 3). The comparison of the treated and untreated plasmas show that the levels of asparagine and glutamic acid were increased in the treated subjects. Only asparagine rose toward normal. Alanine, phenylalanine and threonine all decreased following treatment.

TABLE 4.—*Tyrosine Levels in Normal and Leukemic Subjects*

	Number of Subjects	Plasma Levels (mg./100 ml. plasma)	
		Mean and S.E.	Range
Normal Controls	17	1.1 ± 0.04	0.8–1.5
Acute Leukemias			
Untreated Cases	8	1.5 ± 0.1	1.0–2.2
Treated Cases	7	1.8 ± 0.1	1.5–2.3
Total Cases*	15	1.7 ± 0.1	1.0–2.3
Chronic Lymphatic Leukemias			
Untreated Cases	6	1.3 ± 0.1	1.0–1.6
Treated Cases	8	1.2 ± 0.1	0.7–1.5
Total Cases	14	1.2 ± 0.3	0.7–1.6
Chronic Myelogenous Leukemias			
Untreated Cases	4	1.3 ± 0.1	1.0–1.6
Treated Cases	1	0.9	
Total Cases	5	1.3 ± 0.1	1.0–1.6

* Difference significant, "P" value less than 0.05.

TABLE 5.—*Arginine Levels in Normal and Leukemic Subjects*

	Number of Subjects	Plasma Levels (mg./100 ml. plasma)	
		Mean and S.E.	Range
Normal Controls	16	1.7 ± 0.1	1.3–2.4
Acute Leukemias			
Untreated Cases	9	1.5 ± 0.1	0.9–1.7
Treated Cases	10	1.8 ± 0.1	1.2–2.2
Total Cases	19	1.6 ± 0.1	0.9–2.2
Chronic Lymphatic Leukemias			
Untreated Cases	7	1.7 ± 0.2	1.0–2.4
Treated Cases	11	1.7 ± 0.1	1.1–2.4
Total Cases	18	1.7 ± 0.4	1.1–2.4
Chronic Myelogenous Leukemias			
Untreated Cases	4	1.3 ± 0.02	0.9–1.6
Treated Cases	5	1.4 ± 0.1	1.2–1.5
Total Cases*	9	1.4 ± 0.1	0.9–1.6

* Difference significant, "P" value less than 0.05.

Tyrosine Levels in Normal and Leukemic Subjects (Table 4)

Both the untreated and treated acute leukemia cases had higher than normal tyrosine concentrations and this increase was statistically significant. No marked differences were noted in the chronic lymphatic or chronic myelogenous groups.

Arginine Levels in Normal and Leukemic Subjects (Table 5)

Only the chronic myelogenous leukemic patients showed changes in the level of this amino acid. Both the untreated and the treated subjects gave low mean values when compared to the normal mean and when examined as a group, this decrease proved to be significant.

Discussion

It is of interest that the patients with acute leukemia had high values for phenylalanine and tyrosine as determined by the chromatographic method. This confirms the earlier work by Waisman et al.[5] when a microbiological method for the determination of amino acids was used. The structural similarity of phenylalanine to tyrosine suggests that an elevated level of one would likely result in an increased concentration of the other. This is apparently true in acute leukemia when both amino acid values were found to be high. At present we have no explanation why only phenylalanine was higher in the chronic granulocytic and chronic lymphatic cases.

Because it has been firmly established that both tyrosine and phanylalanine are important in the formation of melanin, the elevated levels of these two amino acids had increased significance to us when we continued to observe skin hyperpigmentation in some of our patients.[11] This pigmentation has now been observed in acute leukemia patients treated with folic acid antagonists or 6-mercaptopurine.

The higher glutamic acid values in all types of leukemia bears strongly on the other studies done in this laboratory. The enzymes glutamic acid dehydrogenase (GAD) and glutamic oxalacetic acid transaminase (GOAT) were determined in the blood fractions from all types of leukemic patients.[12] The white blood cells (WBC) from these patients contained significantly higher GAD than WBC from normal subjects but no changes in GOAT were observed. It is not yet possible to speculate whether the elevated plasma glutamic acid can be related to the increased WBC levels of GAD.

The high "leucine" values found in the acute leukemia cases can be reasonably attributed to the concentration of isoleucine since Waisman et al.[5] found this amino acid to be significantly higher in acute leukemia patients than in normal individuals. However, no explanation for this change in concentration can be offered at this time. It is also too early to give an explanation for the lower than normal amounts of asparagine and threonine in acute leukemic patients. The elevated proline values in the chronic leukemias is of interest, but also defies explanation at present.

The opportunity to use more quantitative methods for amino acid analysis has thus provided the basis upon which further work can be planned. It seems evident that before any mechanism can be postulated to explain the altered amino acid levels, additional data must be sought in the enzyme systems involved in

amino acid metabolism, both in normal and leukemic patients. These studies are now in progress in this laboratory.

Summary

1. Quantitative methods for analysis of free amino acids in plasma have been performed on blood from normal and leukemic individuals.

2. An increase in the glutamic acid phenylalanine and "leucine" levels was found in the blood plasma of patients with acute leukemia.

3. Both the chronic myelogenous and the chronic lymphatic leukemia patients had high levels of glutamic acid, phenylalanine, alanine and proline.

4. Lower than normal levels of asparagine and threonine were found in the plasma of patients with acute leukemia.

Summario in Interlingua

1. Methodos quantitative pro le analyse del libere aminoacidos in plasma ha essite applicate a sanguine ab individuos normal e ab individuos leucemic.

2. Un augmento in le nivellos de acido glutamic, de phenylalanina, e de "leucina" esseva trovate in le plasma de patientes con leucemia acute.

3. Le patientes con chronic leucemia myelogene e etiam illes con chronic leucemia lymphatic monstrava alte nivellos de acido glutamic, de phenylalanina, de alanina, e de prolina.

4. Nivellos subnormal de asparagina e de threonina esseva trovate in le plasma de patientes con leucemia acute.

REFERENCES

[1] Okada, S., and Hayashi, T.: Studies on the amino-acid nitrogen content of the blood. J. Biol. Chem. *51:* 121, 1922.

[2] Sandiford, K., Boothby, W. M., and Giffin, H. Z.: The amino-acid nitrogen in the blood and its possible relation to the elevation of the metabolism in myelogenous leukemia. J. Biol. Chem. *55:* xxiii, 1923.

[3] LeBianco, S., and Sala, I.: Amino acidemia and amino aciduria in the acute leukemia of childhood. Lattanta *25:* 649, 1954.

[4] Baldridge, C. W., and Barer, A.: Relationship between oxygen consumption and nitrogen metabolism. II. In leukemia. Arch. Int. Med. *51:* 589, 1933.

[5] Waisman, H. A., Pastel, R. A., and Poncher, H. G.: Amino acid metabolism in patients with acute leukemia. Pediatrics *10:* 653, 1952.

[6] Henderson, L. M., and Snell, E. E.: A uniform medium for determination of amino acids with various microorganisms. J. Biol. Chem. *172:* 15, 1948.

[7] Levy, A. L.: A paper chromatographic method for the quantitative estimation of amino acids. Nature *174:* 12,6 1954.

[8] Fraenkel-Conrat, H., Harris, J. E., and Levy, A. L.: Recent Developments in Techniques for Terminal and Sequence Studies in Peptides and Proteins, *in* Methods of Biochemical Analyses. D. Glick, ed. New York, Interscience Publishers, 1955.

[9] Udenfriend, S., and Cooper, J. R.: The chemical estimation of tyrosine and tyramine. J. Biol. Chem. *196:* 227, 1952.

[10] Gilboe, D. D., and Williams, J. N., Jr.: Evaluation of the Sakaguchi reaction for quantitative determination of Arginine. Proc. Soc. Exper. Biol. & Med. *91:* 535, 1956.

[11] Waisman, H. A., Richmond, J. B., and Zimmerman, A. A.: Hyperpigmentation in acute leukemia treated with folic acid antagonists. Proc. Soc. Exper. Biol. Med. & *75:* 332, 1950

[12] ——, Monder, C., and Williams, J. N., Jr.: Glutamic acid dehydrogenase and glutamic-oxalacetic transaminase of blood in leukemia and cancer. Cancer Research *16:* 344, 1956.