

## The Effect of 6-Mercaptopurine on the Incorporation of Labeled Amino Acids into Cellular Protein of Chronic Granulocytic Leukemia Leukocytes

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**F**RANTZ and Zamecnik<sup>1</sup> pointed out that in vitro incorporation of C<sup>14</sup>-leucine and C<sup>14</sup>-alanine into white cell proteins was about six times greater in acute leukemic cells, as compared with normal leukocytes. Similar findings were obtained by Weisberger et al.<sup>2,3</sup> using S<sup>35</sup>-L-cystine and S<sup>35</sup>-L-methionine. Baker, Zamecnik and Stephenson<sup>4</sup> showed that cells from cases of chronic myelogenous leukemia were able to incorporate significantly greater amounts of amino acid and to maintain incorporation capacity longer than control cells. It was suggested that amino acid incorporation into cellular protein could be used to test viability of leukocytes.

Winzler et al.<sup>5</sup> studied the in vitro metabolism of human normal and leukemic leukocytes in the presence of various anti-tumor drugs. Radioactive glycine incorporation into cellular protein was used to measure cellular activity. Their data showed that the rate of glycine incorporation was approximately five times greater in chronic granulocytic leukemic cells than in normal leukocytes. In vitro addition of 6-mercaptopurine to fresh cells did not always inhibit uptake of radioactive amino acid. It was concluded that a four-hour incubation with labeled amino acid could not be used as a screening test for anti-tumor drugs. Observations in our laboratory using 100 µg./ml. of 6-mercaptopurine indicate that this drug gives erratic effects on the rate of labeled leucine incorporation, usually causing some inhibition but occasionally causing stimulation. The question of whether 6-mercaptopurine plays any role in vivo which might alter amino acid incorporation into cellular protein has not been answered. The purpose of this paper is to report our observations on two hospitalized cases of chronic granulocytic leukemia treated with 6-mercaptopurine with repeated determinations of the white cell count and the rate of labeled amino acid incorporation.

### MATERIALS AND METHODS

#### *Separation of White Cells*

Blood was aspirated into vacutainers containing appropriate amounts of ACD solution (25 ml. per 100 ml. blood) and allowed to stand at 5 C. for three hours. In most instances, satisfactory separation of white cells from red cells was obtained. If needed, 0.1 mg. phytohemagglutinin per 100 ml. blood was added and if adequate separation was not

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achieved it was accomplished by slow (700 rpm) centrifugation. The white cells suspended in their own plasma were counted and adjusted to a concentration of 20 million white blood cells in 0.7 ml. of plasma. Red cell contamination was insignificant.

L-valine-1-C<sup>14</sup> and L-leucine-1-C<sup>14</sup> were obtained from the California Corporation for Biochemical Research, Los Angeles, Calif. The valine had a specific activity of 2.9  $\mu\text{C}/\mu\text{M}$  and the leucine had a specific activity of 1.4  $\mu\text{C}/\mu\text{M}$ . Solutions of these amino acids in saline were made up in concentrations of 10  $\mu\text{M}$  per ml. 0.1 ml. (1  $\mu\text{M}$ ) per tube was added to 0.7 ml. cell suspension and the final volume was adjusted to 1 ml. with saline. Control experiments indicated that the plateau for incorporation rate in four hours was attained under these conditions. The amount of valine or leucine in the plasma was determined by microbiological assay and had no significant effect on the amino acid incorporation rates shown in figures 1 and 2. The tubes were incubated for four hours in air at 37 C.

### *Treatment of Cells After Incubation*

The following multi-step procedure was necessary to separate unincorporated labeled amino acid from cellular protein and lipid.

1. The incubated cells were centrifuged and the plasma decanted.
2. The cells were suspended in 5 ml. 5 per cent TCA (W/V) and extracted with shaking in a water bath at 90 C. for 30 minutes.
3. The precipitated protein was centrifuged and step 2 repeated with 10 ml. 5 per cent TCA. The protein was centrifuged and the supernatant discarded.
4. The precipitate was suspended in 10 ml. 95 per cent EtOH, incubated with shaking in a water bath for 30 minutes at 70 C.
5. The centrifuged precipitate was suspended in ethanol, chloroform, ether solution (2:2:1) and allowed to stand for 30 minutes at 30 C.
6. The centrifuged precipitate was treated with 95 per cent EtOH as in step 4.
7. The centrifuged precipitate was treated as in step 3.
8. The centrifuged precipitate was dissolved in the final volume of 2 ml. 0.1 N NaOH.
9. The solution was treated with 2 ml. 10 per cent TCA.
10. The centrifuged precipitate was washed with 10 ml. EtOH to remove TCA, centrifuged, and allowed to drain inverted for 15 minutes.
11. The precipitate was dissolved in a final volume of 2.0 ml. 0.1 N NaOH and used for assay of C<sup>14</sup> and protein by the biuret method.<sup>6</sup> C<sup>14</sup> was assayed by plating on planchets and counting in an open window gas flow well counter. Control data indicated insignificant incorporation of amino acid into plasma protein and it has been reported<sup>4</sup> that there is no significant incorporation into red cell protein.

### RESULTS

Case 1 (R. B.), a 53 year old colored male plumber, was admitted to the Veterans Administration Hospital on January 10, 1961, as a case of suspected leukemia. The patient asserted he was in perfect health. The history and physical examination were non-contributory. The admission blood count showed a WBC count of 92,000 per cu. mm. with 43 per cent polys, 24 per cent bands, 7 per cent metamyelocytes, 4 per cent promyelocytes, 11 per cent myelocytes, 6 per cent lymphocytes, 3 per cent monocytes and 2 per cent basophils. The hemoglobin was 11.8 Gm. A bone marrow study showed a "highly cellular marrow compatible with chronic granulocytic leukemia". The patient remained under hospital observation and treatment for 96 days. The pertinent data are shown in figure 1.

The amino acid incorporation studies on this patient were done with labeled L-valine. The average pre-treatment incorporation rate was 0.345  $\mu\text{M}$  L-valine

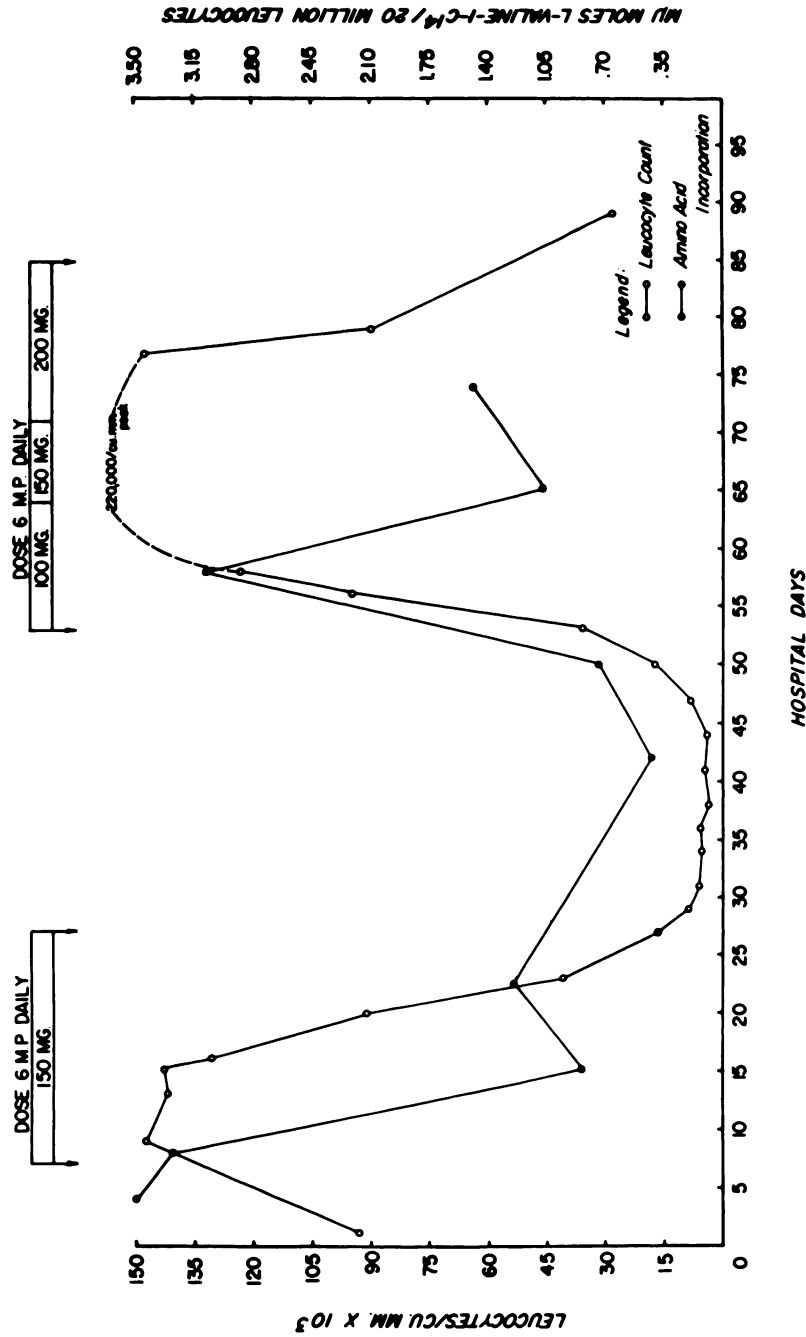


Fig. 1.—Incorporation of L-valine-C<sup>14</sup> by 20 x 10<sup>6</sup> leucocytes in chronic granulocytic leukemia.

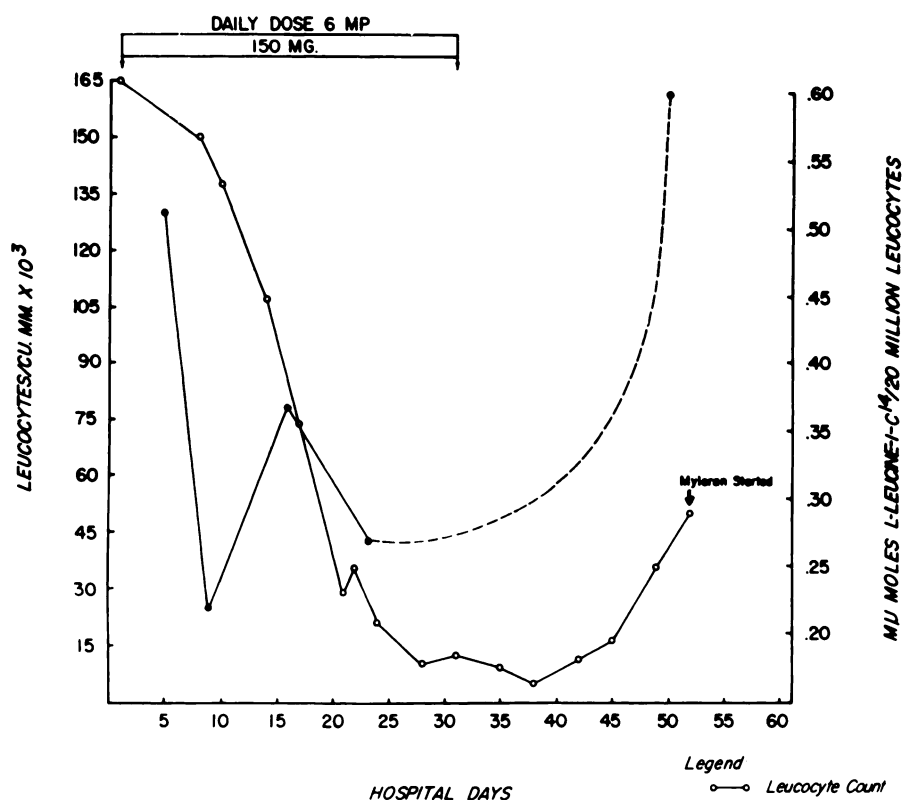


Fig. 2.—Incorporation of L-leucine-1-C<sup>14</sup> by  $20 \times 10^6$  leukocytes in chronic granulocytic leukemia.

per 20 million leukocytes. Treatment with 6-mercaptopurine was started on the seventh hospital day in doses of 50 mg. 6-mercaptopurine orally, three times a day and was continued daily until the 27th hospital day. Seven days after treatment was started, the incorporation fell to  $0.076 \mu\text{M}$  L-valine per 20 million leukocytes, and the average incorporation rate for 40 days after treatment was started was 22 per cent of the initial rate. If one compares these rates with those reported for normal and leukemic cells,<sup>4,5</sup> it can be stated that the rate of incorporation of this amino acid was reduced to "normal" levels. On the 17th hospital day, the leukocyte count began to fall rapidly but did not reach normal levels (9,000 WBC per cu. mm.) until the 29th day, and the differential count continued to show immature cells until the 34th day when the hemogram showed 5800 WBC per cu. mm. with 71 per cent polys, 1 per cent bands, 19 per cent lymphocytes, 4 per cent monocytes, 2 per cent basophils and 3 per cent eosinophils. Thus, decrease in the rate of amino acid incorporation preceded the decrease in leukocyte count by several days. This finding indicates damage to an important cellular function.<sup>4</sup>

The leukocyte count began to rise on the 50th hospital day with the appearance of immature cells in the peripheral blood and reached a peak on the

67th hospital day when the hemogram showed 221,000 total WBC per cu. mm. with many immature cells. Treatment with 6-mercaptopurine was begun again on the 53rd hospital day in doses of 100 mg. per day, increased to 150 mg. per day on the 64th day and to 200 mg. on the 71st day.

A rapid rise of amino acid incorporation rate to the pre-treatment level occurred on the 55th day at the time of the rise in leukocyte count, but dropped to a low level on the 65th day when the leukocyte count was at peak levels. On the 89th day, the leukocyte count was 29,000 per cu. mm. with immature cells still present. On the 97th day, these were 5,000 leukocytes with 54 per cent polys, 19 per cent lymphocytes, 3 per cent monocytes and 24 per cent eosinophils. As in the first response to treatment, the decrease in amino acid incorporation rate preceded the fall in leukocyte count. This exacerbation and response indicate that the decrease in amino acid incorporation rate not only precedes decrease in leukocyte count but may occur while the leukocyte count and immaturity of the cells are increasing.

Case 2 (F. P.), a 68 year old colored male, was admitted to the Veterans Administration Hospital on August 29, 1960, with a chief complaint of abdominal pain for five days. The abdominal pain was aggravated by standing and walking. One month prior to admission, the patient had noted ready fatigability and weakness and some anorexia. There had been a 10 pound weight loss in the previous three months. Physical examination showed a chronically ill patient. Several lymph nodes were palpable on both sides of his neck. Abdominal distention was present and the spleen was felt 4 cm. below the left costal margin. The liver was enlarged 4 cm. below the right costal margin in the mid-clavicular line. Varicosities were present in both lower extremities. The initial hemogram showed RBC 3.39 million per cubic mm. with a hemoglobin of 11.5 Gm. The total WBC count was 165,000 per cu. mm. with 45 per cent polys, 16 per cent bands, 8 per cent metamyelocytes, 5 per cent promyelocytes, 13 per cent myelocytes, 2 per cent lymphocytes, 9 per cent basophils and 2 per cent eosinophils. The patient was treated with 150 mg. 6-mercaptopurine for 29 days and the rate of amino acid incorporation was followed using  $C^{14}$ -L-leucine. The data are diagrammed in figure 2.

The white cell count decreased to normal levels with normal differential by the 34th hospital day, showing 9300 WBC per cu. mm. with 68 per cent polys, 31 per cent lymphocytes, and 1 per cent monocytes. 6-mercaptopurine was stopped on the 32nd day. The first amino acid incorporation was unfortunately not done until the 4th day after 6-mercaptopurine treatment was started and was done three more times with an average of 55 per cent of the initial rate. Leukocytes from two normal adult males assayed for their incorporation of  $C^{14}$ -L-leucine showed incorporation of 0.200 and 0.233  $\mu$ M per 20 million leukocytes. The average incorporation rate after 6-mercaptopurine in this case was 0.279  $\mu$ M with a low of 0.218  $\mu$ M per 20 million leukocytes. The last incorporation rate was done on the 50th hospital day when escape from the effects of 6-mercaptopurine treatment manifested itself and the rate of incorporation was 70 per cent greater than the initial rate. The data on this patient, while not as complete as in Case 1, substantiate the observations made on Case 1.

## DISCUSSION

The exact mechanism of action of 6-mercaptopurine in leukemia is still unknown. It has been demonstrated to interfere with growth of bacterial and mammalian cells in culture.<sup>7,8</sup> Elion et al.<sup>9</sup> have suggested that it is incorporated into nucleic acid. Brockman<sup>10,11</sup> has postulated that the synthesis of 6-mercaptopurine ribonucleotide constitutes the lethal synthesis. Davidson has arrived at a similar conclusion<sup>12</sup>

The data in this paper indicate that there is significant interference in the *in vitro* amino acid incorporation into cellular protein of chronic granulocytic leukemic cells when the patient is given 6-mercaptopurine orally. The rate of incorporation may be a measure either of protein synthesis or of enzymatically catalysed amino acid exchange with preexisting cellular components. The methods of isolation used in these experiments indicate that the incorporated amino acid is an integral component of cellular protein. Indeed, Baker, Zamecnik and Stephenson<sup>4</sup> regard such *in vitro* incorporation as an indicator of "an active metabolic process" dependent on the viability of the cell. The data support the observation that cells of chronic granulocytic leukemia have a significantly higher rate of amino acid incorporation than do normal leukocytes. The concentration of labeled L-amino acid required to reach a plateau beyond which increased concentration of L-amino acid failed to increase incorporation was 1.0  $\mu\text{M}/\text{ml}$ . This agrees favorably with the concentration of 3.0  $\mu\text{M}/\text{ml}$  found by others<sup>4</sup> using labeled DL-amino acid with cells in salt solution rather than plasma.

It is of some interest that a latent period of several days exists between the time oral therapy with 6-mercaptopurine is started and the time of demonstrable interference with amino acid incorporation. The significance of this observation is not clear at this time. Changes in the rate of amino acid incorporation are not synchronous with changes in the cell count since marked decrease in incorporation rate may be noted for several days before there is a decrease in circulating leukocytes. This indicates that the decrease in leukocytes occurs only after their ability to incorporate amino acids is interfered with. In view of these data, it is understandable that Winzler et al.<sup>5</sup> failed to find significant inhibition of  $\text{C}^{14}$ -glycine incorporation in a four hour incubation of leukemic cells with  $\text{C}^{14}$ -glycine in the presence of 6-mercaptopurine. The exact mechanism of interference with amino acid incorporation is unknown but the observations shed additional light on the biological action of 6-mercaptopurine.

## SUMMARY

The leukocytes of chronic granulocytic leukemia incorporate labeled valine and leucine at a higher rate than normal leukocytes. 6-mercaptopurine causes significant decrease in the rate of amino acid incorporation into cellular protein of leukemic cells. The onset of a sharp decrease in the amino acid incorporation rate by granulocytic leukemic cells occurs only after several days of therapeutic oral doses of 6-mercaptopurine. The decrease in incorporation rate precedes the decrease in circulating leukocytes by several days, indicating that damage to a vital function of these cells occurs before their disappearance

from the blood stream. The decrease in the amino acid incorporation rate persists as long as the leukemia is in remission and even after therapy has been stopped; it exists until exacerbation occurs. Increase in incorporation accompanies exacerbation of the leukemic cell count. A possible action of 6-mercaptopurine is its role in interference with amino acid incorporation into cellular protein of chronic granulocytic leukemia cells.

#### SUMMARIO IN INTERLINGUA

Le leucocytos de subjectos con chronic leucemia granulocytic incorpora marcate valina e leucina plus rapidemente que leucocytos ab subjectos normal. Le administration de 6-mercaptopurina causa un significative relentamento del incorporation de amino-acidos ad in le proteina cellular de cellulos leucemic. Le declaration de un acute reduction del rapiditate del incorporation de amino-acidos per cellulas a leucemia granulocytic occorre solmente post plure dies de therapeutic doses oral de 6-mercaptopurina. Le relentamento del incorporation precede le reduction del circulante leucocytos per plure dies, lo que indica que damnification del function vital de iste cellulas occorre ante lor disparition ab le circulation del sanguine. Le relentamento del incorporation de amino-acido persiste durante le curso del remission e mesmo post le suspension del therapia. Illo continua usque al occurrence de un exacerbation. Un acceleration del incorporation accompania le exacerbation del numeration de cellulas leucemic. Un action possibile de 6-mercaptopurina es su rolo in le interferentia in le incorporation de amino-acido in le proteina cellular de cellulas a chronic leucemia granulocytic.

#### REFERENCES

1. Frantz, I. D., and Zamecnik, P. C.: Use of  $C^{14}$ -labeled amino acids in the study of peptide bond synthesis. Symposium on Nutrition 2: Plasma Proteins, Springfield, Charles C Thomas, 1950.
2. Weisberger, A. S., and Levine, B.: Incorporation of radioactive L-cystine by normal and leukemic leukocytes in vivo. *Blood* 9:1082, 1954.
3. —, Suhrland, L. G., and Griggs, R. C.: Incorporation of radioactive L-cystine and L-methionine by leukemic leukocytes in vivo. *Blood* 9:1095, 1954.
4. Baker, W. H., Zamecnik, P. C., and Stephenson, M. L.: In vitro incorporation of  $C^{14}$ -dl-leucine into normal and leukemic white cells. *Blood* 12: 822, 1957.
5. Winzler, R. J., Wells, W., Shapira, J., Williams, A. D., Bornstein, I., Burr, M. J., and Best, W. R.: Metabolism of human leukocytes in vitro. II. The effect of several agents on the incorporation of radioactive formate and glycine. *Cancer Res.* 19:377, 1959.
6. Layne, E.: *Methods in Enzymology*. New York, Academic Press, 1957, vol. 3: p. 450.
7. Elion, G. B., Hitchings, G. H., and Vanderwerff, H.: Antagonists of nucleic acid derivatives. *J. Biol. Chem.* 192:505, 1951.
8. Biesele, J. J.: Effects of 6-mercaptopurine on experimental tumors in tissue culture. *Ann. New York Acad. Sc.* 60:228, 1954.
9. Elion, G. B., Bieber, S., and Hitchings, G. H.: The fate of 6-mercaptopurine in mice. *Ann. New York Acad. Sc.* 60:297, 1954.
10. Brockman, R. W., Sparks, C., Hutchison, J. D., and Skipper, H. E.: A mechanism of resistance to 8-azaguanine I. Microbiological studies on

- the metabolism of purines and 8-azapurines. *Cancer Res.* 19:177, 1959.
11. —: A mechanism of resistance to 6-mercaptopurine: Metabolism of hypoxanthine and 6-mercaptopurine by sensitive and resistant neoplasms. *Cancer Res.* 20:643, 1960.
12. Davidson, J. D.: Studies on the mechanism of action of 6-mercaptopurine in sensitive and resistant L1210 leukemia in vitro. *Cancer Res.* 20:225, 1960.

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