increased in mice of this age group when treated with methylcholanthrene. These experiments demonstrated that mice of the F strain, when painted with methylcholanthrene will develop between 97 and 200 days of age a type of leukemia characteristic for mice of an older age group (300-700 days).

*Summary.* Leukemia appeared at an earlier age than in controls when mice of the F strain were painted twice weekly with methylcholanthrene. Myelogenous leukemia, which does not occur in untreated F mice before 300 days of age, appeared as early as 97 days after birth in treated mice. Only 3 cases of leukemia occurred in 184 mice of non-leukemic strains treated in a similar manner. The effectiveness of methylcholanthrene in influencing the appearance of leukemia in young mice depended on the genetic susceptibility of mice to the disease.

## 11656

## Microestimation of Leucine, Isoleucine, and Valine.

RICHARD J. BLOCK, DIANA BOLLING AND A. A. KONDRITZER.†

*From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, New York City.*

Arginine, histidine, lysine, cystine, methionine, tyrosine, tryptophane, phenylalanine, glycine, alanine, and threonine can all be determined by available micro methods[1, 2] in only 2 to 4 g of protein, but until recently methods were not available for the estimation of 3 of the essential amino acids: leucine, isoleucine, and valine in small quantities of protein. Last year Fromageot and Heitz[3] described a procedure for the determination of leucine and valine, involving deamination to the corresponding hydroxy acids with nitrous acid, oxidation to acetone with chromate, and measurement of the acetone colorimetrically after reaction with salicylaldehyde. Leucine and valine were estimated in the presence of each other by carrying out the oxidation with chromate on 2 aliquots under conditions such that the proportionate yield of acetone from the 2 amino acids differed

† Research Associate in Biochemistry, Neuro-Psychiatric Institute, Hartford Retreat, Hartford, Conn.

1 Block, R. J., *The Determination of the Amino Acids,* Burgess Publishing Company, Minneapolis, Minn., Rev. Ed., 1938.

2 Block, R. J., and Bolling, D., *J. Biol. Chem.*, 1939, **130**, 365.

3 Fromageot, C., and Heitz, P., *Enzymologia,* 1939, **6**, 258.

considerably. We were unsuccessful in obtaining consistent results with this procedure. In the present study we have made extensive changes in and simplified the procedure of Fromageot and Heitz and in addition have found conditions which permit the determination of isoleucine by measuring the ethylmethylketone formed in the oxidation of the corresponding hydroxy acid. All 3 amino acids can be determined in as little as 100 mg of protein for no one of the other amino acids recognized as occurring in proteins yields acetone or ethylmethylketone under these conditions.

*Hydrolysis and Deamination.* The protein is hydrolyzed with 10 volumes of 8 N sulfuric acid and the excess acid is removed with barium hydroxide. The precipitate of barium sulfate is centrifuged and thoroughly washed with hot water. The filtrate and washings are reduced *in vacuo* to 50 cc. Amino acid solutions prepared by other methods of hydrolysis are suitable provided the hydrolyzing agent does not yield compounds which would interfere in later steps. Five or 10 cc aliquots of the solution are removed and the amino acids are deaminated with an excess of sodium nitrite, a few drops of sulfuric acid being added to keep the solution acid to Congo paper. The excess nitrous acid is destroyed by warming the solution on the steam bath from 10 to 15 minutes.

*Oxidation.** Instead of oxidizing with chromate under 2 different conditions (under pressure and with continuous distillation)[3] we employ 2 different oxidizing agents: chromate and permanganate.

A. 2 g of $K_2Cr_2O_7$ are dissolved in 20 cc of 5 N sodium acetate buffer at pH 4.7 in a 3 x 20 cm test tube into which a stream of air is passed in order to aspirate the acetone and ethylmethylketone into 2 water traps connected in series and kept in an ice bath. The oxidation mixture is boiled and the solution of hydroxy acids is introduced from a burette at such a rate that the volume remains constant. Time: 30 minutes. The aeration and boiling are continued until the volume of the oxidizing solution is reduced to 10 cc (30 minutes). The aqueous solutions in the traps are combined and diluted to volume. Under these conditions valine yields 47% and leucine 6% of the theoretical amount of acetone. Isoleucine yields 52% of the theoretical amount of ethylmethylketone.

B. 2 g of $KMnO_4$ are dissolved in 20 cc of M phosphate buffer at

* During the course of this investigation the following oxidizing agents have been tried alone or in combination, $Ce(HSO_4)_4$; $Ce(HSO_4)_4$-$I_2$; $(CH_3COO)_4Pb$; $PbO_2$; $K_2Cr_2O_7$-$CH_3COOH$; $K_2Cr_2O_7$-NaOH; $K_2Cr_2O_7$-$H_2SO_4$; $KMnO_4$-KOH; $KMnO_4$-$H_2SO_4$; $KMnO_4$-$H_3PO_4$; $MnO_2$; $MnO_2$-$H_3PO_4$; $KIO_4$; $KIO_3$; $HIO_4$; $Ag_2O$; Caro's reagent; $K_2S_2O_8$-$AgNO_3$-$H_2SO_4$; $H_2O_2$; $H_2O_2$-$FeSO_4$; and others.

pH 6.8 and the oxidation is carried out in the same way as described above. Under these conditions valine yields 50% and leucine 36% of the theoretical amount of acetone. Isoleucine yields 52% of the theoretical amount of ethylmethylketone.

*Estimation of Leucine and Valine.* The amounts of acetone present in the chromate and permanganate distillates are determined by precipitation with Denigés' reagent or colorimetrically by condensation with salicylaldehyde.[4] Under these conditions ethylmethylketone fails to give a color. Some of the data obtained are given in Table I. The amounts of valine and leucine are calculated from the following equations: Valine = 4.85 $Acetone_{K_2Cr_2O_7}$-0.81 $Acetone_{KMnO_4}$; Leucine = 7.54 ($Acetone_{KMnO_4}$-$Acetone_{K_2Cr_2O_7}$).

TABLE I.
Oxidation of Leucine, Isoleucine, and Valine by $K_2Cr_2O_7$ and $KMnO_4$.

| | $K_2Cr_2O_7$ % | $KMnO_4$ % |
|---|---|---|
| Leucine | 6.7,5.9,5.9,6.3,6.2 | 36,34,32,39,34,41 |
| Isoleucine | 50,50,49,53,51,55,56,53 | 54,52,52,47,53,53,52,53 |
| Valine | 49,48,44,49,49,45 | 53,48,50,53,47 |

*Estimation of Isoleucine.* A. Acetone is precipitated with Denigés' reagent from a portion of the aqueous solution obtained from either the chromate or permanganate oxidation of the hydroxy acids. The filtrate is diluted to a volume such that aliquots of 1 to 2 cc will contain from 0.005 to 0.05 mg of ethylmethylketone. Such aliquots are diluted to 8 cc, 4 cc of absolute alcohol are added, and after cooling to 0°, 4 cc of concentrated sulfuric acid are introduced. The solution is mixed and 2 cc of 10% alcoholic salicylaldehyde are added. The tube is stoppered and, after mixing is placed in a water bath at 50° over night. The color is read using a 520 mu filter. A calibration curve covering the range of 0.005 to 0.05 mg of ethylmethylketone is prepared. Isoleucine=3.5 x ethylmethylketone.

B. Although acetone gives color with salicylaldehyde in the presence of sulfuric acid, the amount is much less than that yielded by ethylmethylketone. Therefore, it is possible to determine ethylmethylketone directly without removal of acetone by means of calibration curves. Both procedures should be employed.

The recovery of leucine, isoleucine, and valine, when a mixture of these 3 amino acids and glycine was oxidized in the proportions 24, 6, 8, and 250 respectively, was leucine+2%, valine+7%, and isoleucine+6% for chromate oxidation and +1% for permangan-

[4] Block, R. J., and Bolling, D., *The Determination of the Amino Acids*, Burgess Publishing Company, Minneapolis, Minn., Second Ed., 1940.

ate oxidation above the amounts expected from the formulae. It should be stressed that standard conditions must be developed to ensure the same percentage oxidation each time.

Up to the present 35 proteins have been analyzed for leucine, isoleucine, and valine (cf. [4], Table II for some of the results).

## 11657

## Rapid Absorption of Substances Injected into the Bone Marrow.

L. M. TOCANTINS

*From the Division of Hematology, Jefferson Medical College and Hospital, Philadelphia.*

Substances injected into the marrow cavity of the tibia of the rabbit and of the sternum of man appear to find their way immediately into the general circulation. That material so administered is taken up and utilized as rapidly as if it had been injected intravenously has been demonstrated in the following ways:

1. Blood replacement by intramedullary injection. A rabbit was bled of 20% of its calculated blood volume, by puncturing the heart and aspirating the blood slowly into a solution of sodium citrate. Twenty-four hours afterwards a needle with bevelled stylet was placed into the marrow cavity of the upper portion of the tibia, and blood, freshly removed from another animal, was introduced in an amount equivalent to that withdrawn the preceding day. The injection was allowed to proceed at the rate of about 5-7 cc per minute. Slight twitching of the leg after the injection started was the only disturbance observed in the animal. Seven animals were treated as described; 2 other animals were bled and allowed to recover spontaneously. Of the 7 treated animals 4 recovered their original (previous to the bleeding) erythrocyte and hemoglobin level within 24 hours after the intramedullary injection, 2 within 48 hours, and one died as a result of a hemopericardium. The last mentioned animal was the only fatality among all the animals that received various substances by the intramedullary route. None of the other animals showed any sign of distress during or after the experiments. In Fig. 1 is illustrated the response of the erythrocytes, hemoglobin and reticulocytes of a treated and an untreated animal.

2. Injections of glucose in experimental hypoglycemia. Four rabbits were rendered hypoglycemic by intravenous injections of a dose