STUDIES ON HUMAN BLOOD PROTEINS*
I. THE Isoleucine DEFICIENCY OF HEMOGLOBIN

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The need and importance of securing adequate substitutes for human blood protein preparations now so extensively and successfully employed in the treatment of traumatic or hemorrhagic shock are obvious. Our approach to this problem is based on the assumption that the preparation of suitable substitutes might be facilitated by an accurate knowledge of the amino acid make-up of the blood proteins. The study of human hemoglobin was undertaken first because it can be isolated in a well defined crystalline form. Accordingly, crystalline hemoglobin was prepared from human red blood cells and analyzed chemically for tyrosine, cystine, alanine, and the ten amino acids currently considered necessary for physiological maintenance of the human adult (1). This study, the details of which will be reported later, revealed that the isoleucine N constitutes approximately 0.5 per cent of the total human hemoglobin N.

This interesting finding prompted us to check the chemical result by bioassay in the rat which has been shown by the use of amino acid mixtures to require dietary isoleucine for both growth (2) and maintenance of adult weight (3). The results of these experiments are reported here and confirm the deduction made from the analytical data, namely, that a diet in which human red blood cell histone comprised the protein moiety would fail to support growth in the immature rat and weight balance of the adult animal unless supplemented with isoleucine. Since supplementation of the deficient diet with d(-)-isoleucine failed to support normal growth in the immature rat, it is apparent that only the L variety of this amino acid is available to the animal.

EXPERIMENTAL

Animals—These observations were made on rats from a hybrid colony of albino and hooded Norwegian rats that have been in use in this labora-

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tory for some years. Individual litters were divided so as to obtain an equitable distribution of weight and sex in the deficient and control groups. During the experiments, they were kept in individual cages which were not designed in such a way as to prevent coprophagy. The weight measurements were made weekly.

Preparation of Diets—The composition of the diets is shown in Table I. The protein moiety of the diet was composed of a tryptophane and cystine reinforced acid hydrolysate of crystalline human hemoglobin prepared as described below. In order to maintain the protein level of the diets con-

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<td>Diets</td>
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<tr>
<td>Human hemoglobin</td>
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<td>Brewers' yeast</td>
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<td>Sucrose</td>
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<td>Starch</td>
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<td>Agar</td>
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<td>Cod liver oil substitute*</td>
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<td>Salt mixture†</td>
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<tr>
<td>l(-)-Tryptophane</td>
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<td>l(-)-Cystine</td>
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<td>dL-Isoleucine</td>
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<td>d(-)-Isoleucine</td>
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* Mead Johnson and Company.
† The salt mixture employed had the following composition (measured in gm.): NaCl 18.9, CaHPO₄, anhydrous, 25.0, MgSO₄, anhydrous, 6.86, KHCO₃ 44.4, KCl 2.88, Fe³⁺ citrate, U. S. P., 2.21, CuSO₄, anhydrous, 0.24, MnSO₄, anhydrous, 0.15, KI 0.015, NaF 0.03.

stant, a reduction was made in the amount of hydrolysate added to the diet proportionate to the amount of amino acid supplementation. Owing to uncertainties regarding the B complex vitamins, brewers' yeast was used instead of a mixture of the synthetically available components of this vitamin group. The amount of isoleucine introduced into the diet in this manner is apparently negligible with respect to the needs of the rat. Except when indicated all of the animals were fed ad libitum and the amount of food consumed daily recorded.

Preparation of Human Hemoglobin Hydrolysates—Human hemoglobin was prepared from human red blood cells by the method of Zinoffsky (4).
10 liters of red blood cells, processed 1 liter at a time, yielded 700 gm. of crystalline hemoglobin which contained 13.96 per cent nitrogen, uncorrected for moisture and ash.

500 gm. of the hemoglobin were hydrolyzed under a reflux for 24 hours with 2 liters of 20 per cent sulfuric acid. After cooling, the hydrolysate was alkalinized by the slow addition of a solution containing 310 gm. of calcium oxide (technical) in 2 liters of water. The mixture was stirred well and resulted in the evolution of ammonia. After standing overnight it was filtered and the calcium sulfate cake washed by resuspension in 2 liters of warm water. The major portion of the pigments remained adsorbed on the calcium sulfate. The combined filtrate and washings were concentrated in vacuo at 50–60° to approximately 1 liter. The ammonia-free concentrate was now made neutral to litmus with 50 per cent sulfuric acid, cooled under the tap, and the resulting calcium sulfate filtered off.

The l(-)-tryptophane, l(-)-cystine, and dl-isoleucine used for supplementation of the diets were obtained from Merck and Company, Inc., and their purity checked in this laboratory. The d(-)-isoleucine used in these experiments was kindly prepared for us by Hoffmann-La Roche, Inc., by enzymic resolution of the racemate. This product was characterized by the nitrogen content and the optical activity of a 3.1 per cent aqueous solution.

Results—In the preliminary experiment, eight immature rats were placed on Diet A. The weight losses caused by Diet A (Fig. 1) revealed its nutritional inadequacy. The animals were then separated into four groups and each group was fed one of four test diets prepared by supplementing Diet A singly with the racemic forms of isoleucine, leucine, valine, and threonine. The results of this experiment made it evident that supplementation with isoleucine alone permitted resumption of growth in the animals. This initial observation was checked by the fact that the animals fed the threonine-, valine-, or leucine-supplemented diets also gained weight when given the isoleucine-supplemented diet, and further confirmed by the data on five additional animals which are not included in Figs. 1 to 4. These experiments demonstrated conclusively that Diet A, and hence human red blood cell globin, is deficient in isoleucine. Feeding of the

C_{6}H_{13}O_{2}N. Calculated, N 10.68; found N 10.65
Locquin (0), [α]_{D}^{20} = -10.55°; found [α]_{D}^{20} = -12.25°
deficient diet to rats of different ages demonstrated that isoleucine is required for growth by the immature rat and for maintenance of weight by the adult rat (Fig. 2). Representative growth curves of another group of animals which were paired fed indicate that a 50 per cent reduction in the isoleucine supplement, Diet C, results in a proportionate loss of nutritional value of the diet (Fig. 3).

In order to test the availability of $d(-)$-isoleucine some of the animals on Diet A or B were changed to Diet D. The growth curves of these animals (Fig. 4) show that a slight growth stimulation is obtained from Diet D. This might be due to an actual slight utilization of the $d$ form by the rat or the presence of traces of the $l$ form in the resolved product. The latter probability is made more plausible by the observation that animals on Diet B lost weight when placed on Diet D.

Comment

Experiments on the nutritional adequacy of human plasma proteins reported in abstract by Stare and associates (7) during the course of this
Fig. 2. The effect of the isoleucine-deficient diet on rats of various ages. All animals were fed Diet A throughout. The figures in parentheses denote animal weight, those in brackets the average daily food intake.

Fig. 3. Growth effect of one-half reduction of the isoleucine supplement. Animals were fed Diet B or C as indicated. The figures in parentheses denote weight of animals, those in brackets the average daily food intake.
study show that the plasma proteins are also poor in isoleucine. The fact that their animals gained an average of 0.9 gm. daily on the plasma protein diets would indicate the isoleucine content of these proteins to be much greater than that of hemoglobin. It would appear therefore that only small amounts of isoleucine are required for the regeneration of human plasma proteins and hemoglobin. The rapid recovery from traumatic or hemorrhagic shock achieved by the administration of human blood protein preparations would seem to corroborate this interpretation of the experimental data.

**FIG. 4.** The availability of d (-)-isoleucine for growth in the rat. Animals were fed Diet A or B as indicated and changed to Diet D at the arrow. The figures in parentheses denote the animal weight in gm., those in brackets the average daily food intake.

Quite apart from the immediate purpose of the study, these findings afforded us an opportunity to test the biological availability of d(-)-isoleucine. Although our data on this point are not so conclusive as might be desired, they would appear to confirm the findings of Rose (8) that d(-)-isoleucine is not utilized for growth by the rat.

**SUMMARY**

Human hemoglobin has been shown by bioassay in the rat to be deficient in isoleucine. Consideration of current knowledge suggests that only small amounts of isoleucine are required for the formation of hemoglobin and the plasma proteins. d(-)-Isoleucine does not appear to be available for growth in the immature rat.
BIBLIOGRAPHY
