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Plasma Free-Amino acids as an index of protein nutrition

An Evaluation of Whitehead's Method [1]

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SUMMARY

The purpose of this work was to evaluate the blood plasma free-amino acid ratio of Whitehead as an indicator of protein nutrition. The method was applied to children and adults suffering from severe protein-calorie malnutrition, at admission to the hospital and during recovery, and to children with chronic malnutrition, from a low socio-economic group in Central America. In addition, a rat experiment was conducted with diets of varying amounts and quality of dietary protein. The results indicate that Whitehead's amino acid ratio is valuable to assess protein nutrition under the conditions tested.

INTRODUCTION

In areas where protein malnutrition is prevalent, methods are needed for detecting inadequacy of protein nutriture before the clinical signs of kwashiorkor become obvious. Investigations of the blood plasma free amino acids of children with

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kwashiorkor in several parts of the world showed alterations which were typical for the disease, regardless of differences in the major source of dietary protein (1-5). Changes in the plasma ratio of non-essential to essential amino acids were observed and were caused generally by a reduction in leucine, isoleucine and valine, with relatively little change in the level of non-essential amino acids (1-5).

Arroyave (6) found changes in the free amino acid pattern of plasma resulting from feeding a nitrogen-free diet. It has been suggested that an altered plasma amino acid pattern might be an indication of potential kwashiorkor. Whitehead (7) proposed that the ratio of certain non-essential to essential amino acids might serve as an early diagnostic test of protein deficiency and that the severity of the alteration in the ratio might be indicative of the degree of protein malnutrition before overt clinical signs appear.

Since complete amino acid analyses of plasma are impractical for field studies, Whitehead (7) proposed a simplified chromatographic procedure. Amino acids were separated on paper, and the group containing primarily leucine, isoleucine and valine was compared with the group containing primarily glycine, serine and taurine.

The purpose of the present studies was to evaluate Whitehead's procedure as a means of distinguishing between population groups known to differ with respect to protein nutriture. The fasting plasma amino acid ratios of Whitehead, as well as several other parameters of nutritional adequacy, were determined under the following conditions: 1) recovery from kwashiorkor; 2) field studies of pre-school children in a low income rural area, characterized by a poor protein intake, and in an orphanage with an adequate diet; 3) recovery from adult malnutrition; 4) a short term rat experiment to test the effect of quality and quantity of protein in the diet under controlled laboratory conditions.

MATERIALS AND METHODS

In each study, fasting blood serum was prepared and analized for amino acids according to the method of Whitehead (7), with the following modifications: 1) Whatman No. 1 or No. 4 filter paper was used; 2) sufficient serum for duplicate

assays was deproteinized, the ethanol was evaporated and the protein-free extract was dissolved in 120 μl of 10% isopropanol; 3) two 25 μl aliquots were applied to *each* of two 2" wide paper strips; 4) a blank strip was chromatographed simultaneously with the papers containing the samples. For elution, two tubes containing blanks corresponding in paper area to that cut for each group of amino acids, were prepared. Optical density (O.D.) of each blank was subtracted from the appropriate sample value. The subtraction of the O. D. of blanks gives a more accurate result. As would be expected the effect is more marked with samples which have a low concentration of the essential amino acid spot. This is illustrated in figure 1. It is obvious that if a large sample of serum is applied to the paper, which is the usual practice in Dr. Roger Whitehead's

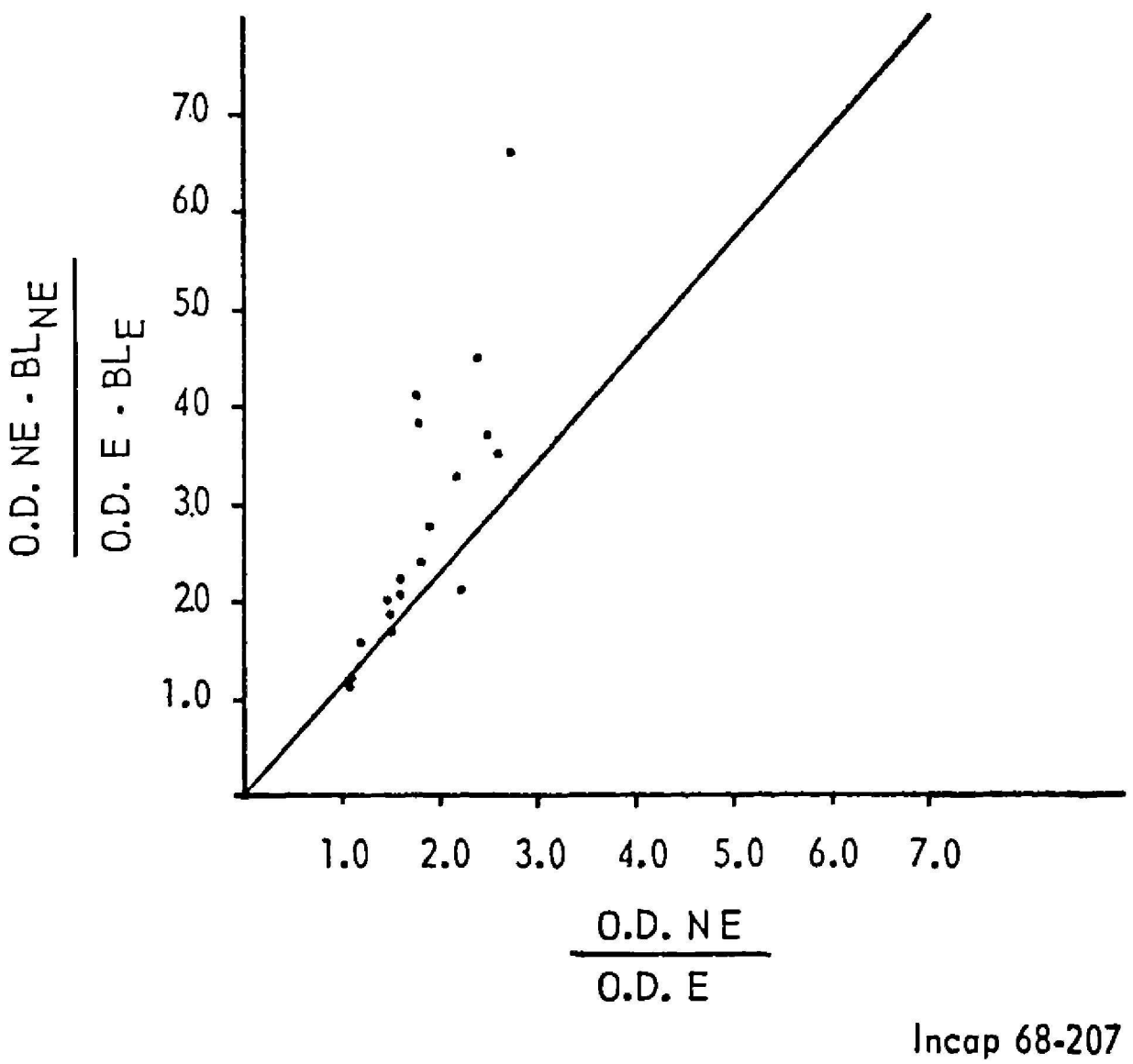

Fig. 1.—Effect of failing to substract "blanks" on the non-essential to essential serum amino acid ratio (NE/E).

laboratory[4], the error introduced by failing to subtract a blank is reduced. We believe, however, that the O.D. of blanks ought to be subtracted in order to avoid this complication entirely, regardless of the size of the serum sample.

The amino acid ratio was computed with the O.D. of the spots containing primarily glycine, serine and taurine in the numerator and the O.D. of the spot containing primarily leucine, isoleucine and valine in the denominator. Throughout this article, we will refer to the ratio so obtained as the non-essential to essential amino acid ratio (NE/E).

Total serum proteins were determined according to the method of Lowry and Hunter (8), and serum albumin by paper electrophoresis in barbital acetate buffer pH 8.6 at an ionic strength of 0.075 using 110 volts for 16 hours. Colorimetric estimation was carried out with amido-black staining and subsequent elution in 0.01 N sodium hydroxide.

Study 1 - Recovery from Kwashiorkor

The technique of Whitehead for determining the serum ratio of certain amino acids was first evaluated under the extreme conditions of acute kwashiorkor and subsequent recovery. Blood samples which had been obtained from five children upon entering the hospital and at intervals throughout recovery were analyzed for the amino acid ratio. The five children ranged in age from 1 years 8 months to 3 years 4 months. All have been diagnosed as moderate kwashiorkor with moderate amounts of edema[5].

RESULTS

Figures 2 through 6 show the relationship between NE/E ratio, serum protein and albumin, protein intake and weight gain during recovery. Initial NE/E ratios ranged from 6.57 - 3.72. (With average of 4.63.) The ratios of all five children showed a generally steady improvement (decrease) in the course of recovery. Final ratios ranged from 2.16 - 1.20 with

4 Personal communication.

5 These patients were under the pediatric care of Dr. Doroty Wilson, Biomedical Division, INCAP. Present address: Ministerio de Trabajo, Previsión Social y Salud Pública, Departamento de Salud Pública, República de Panamá.

an average of 1.86. The ratios followed the same trends taken by total serum protein, serum albumin and weight gain (after decline due to loss of edema). In most cases, the children were fed 0.8 g/kg body weight per day of milk protein during the first few days of recovery, and increased after about one week to 2 g/kg body weight per day. Milk and sometimes a vegetable mixture (Incaparina) with protein of high biological value were fed.

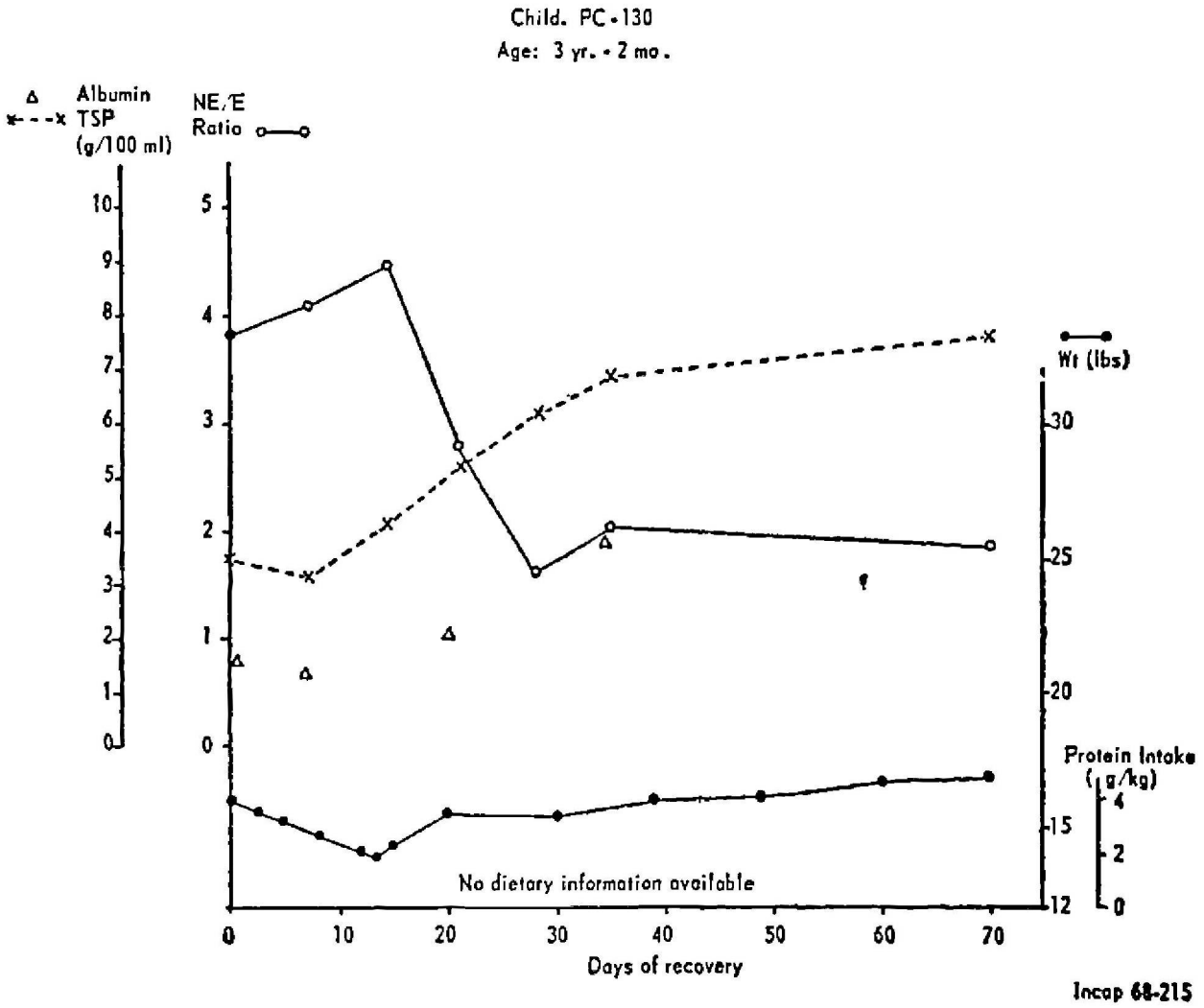

Fig. 2.—Biochemical changes during recovery from kwashiorkor.

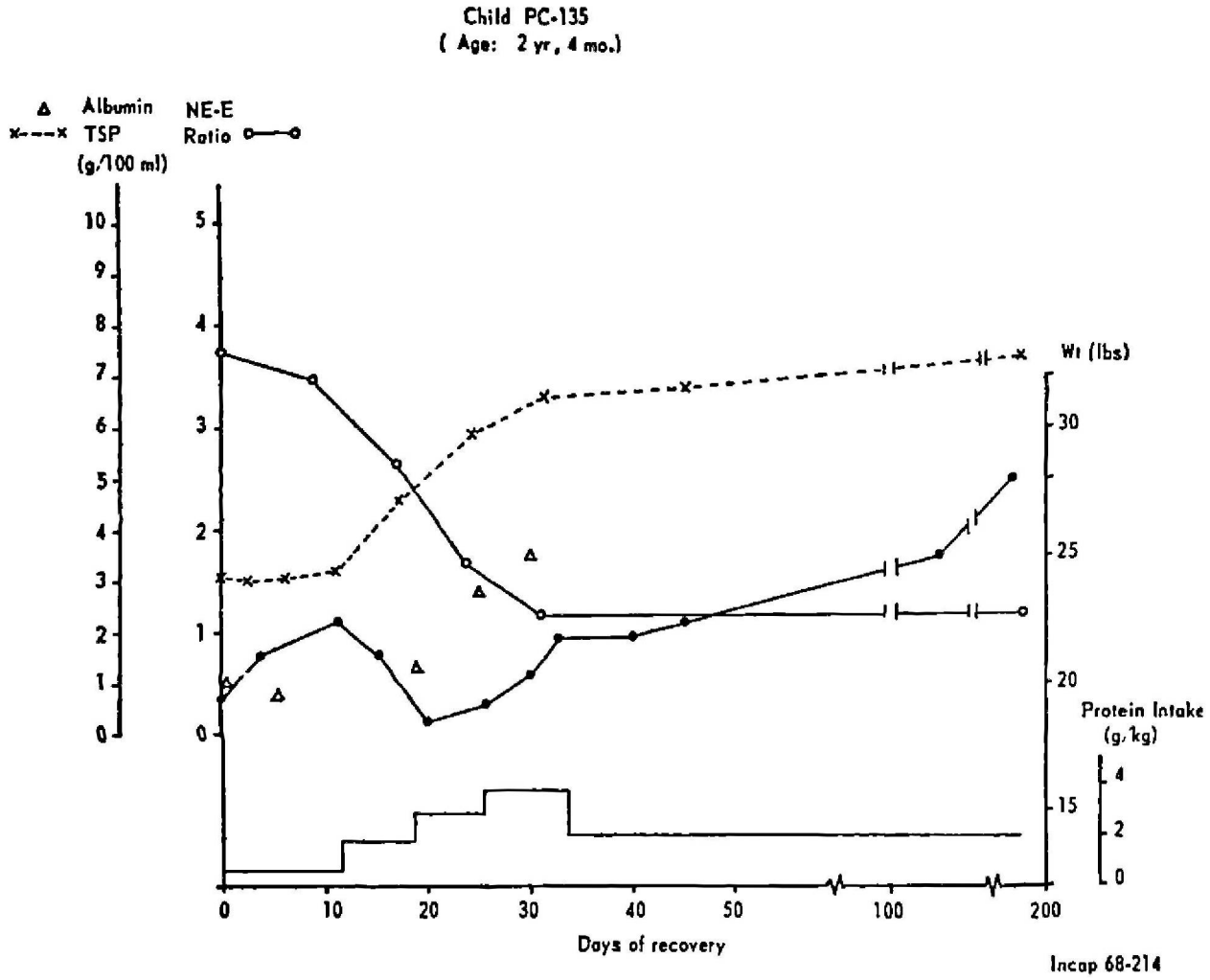

Fig. 3.—Biochemical changes during recovery from kwashiorkor.

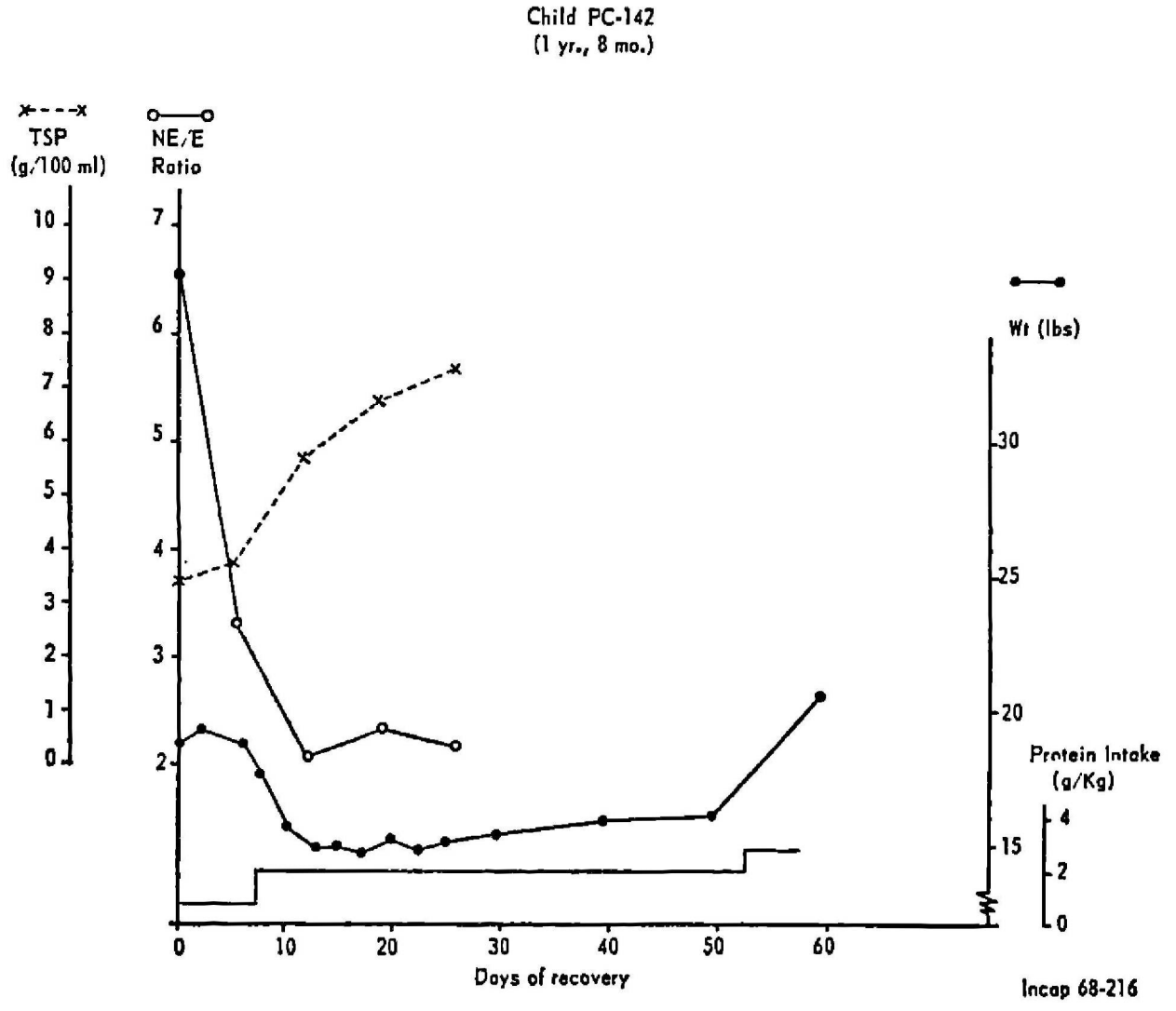

Fig. 4.—Biochemical changes during recovery from kwashiorkor.

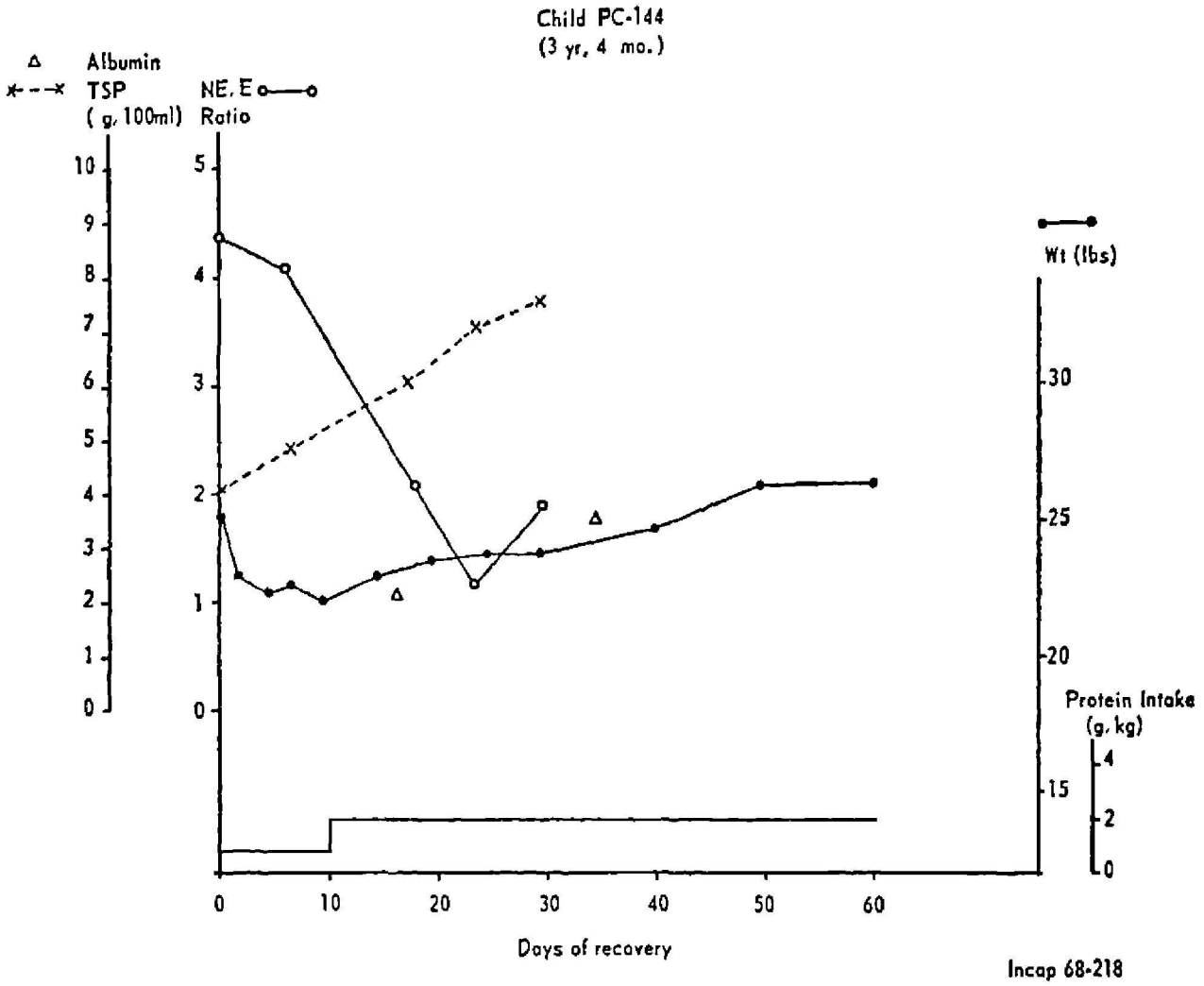

Fig. 5.—Biochemical changes during recovery from kwashiorkor.

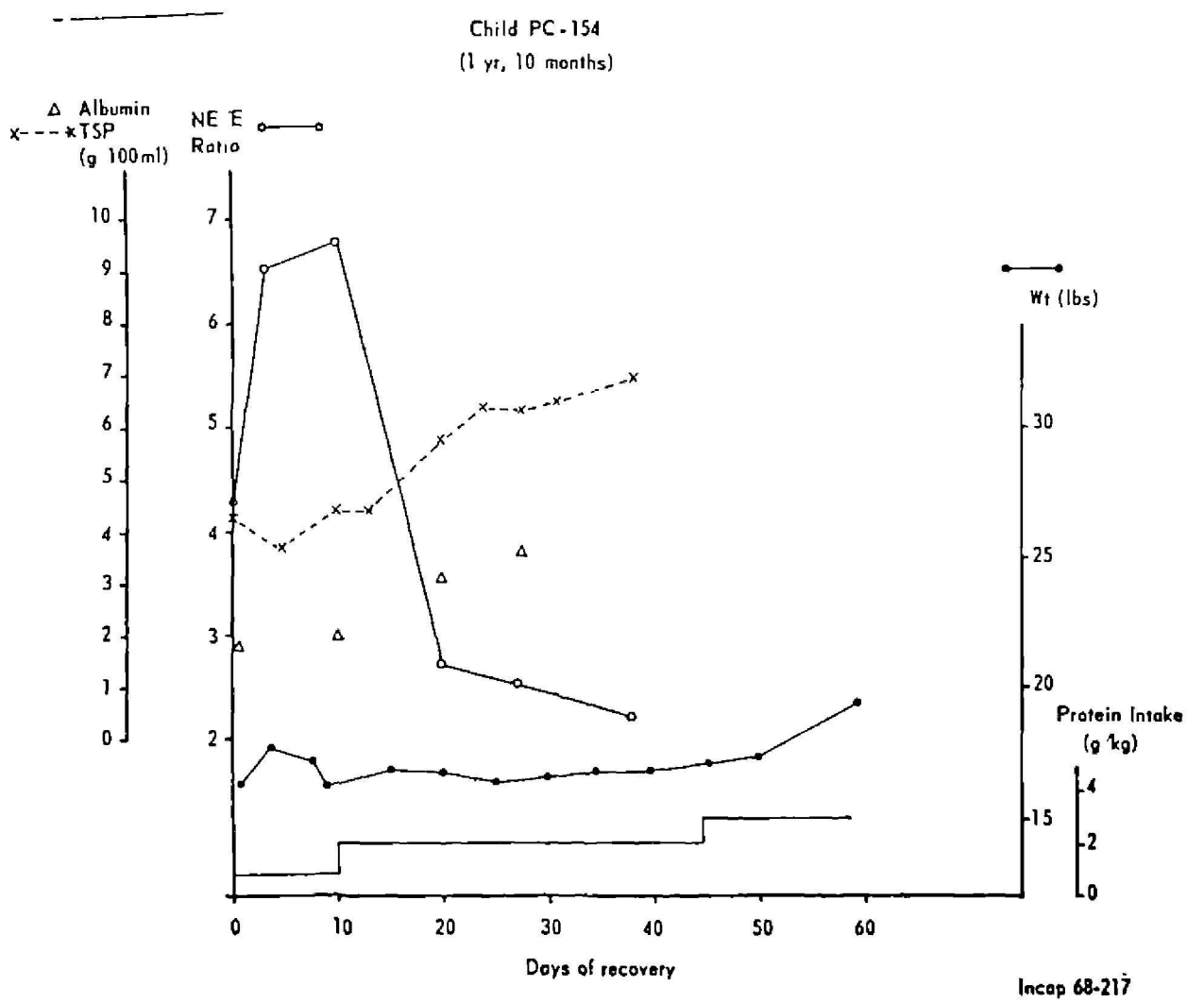

Fig. 6.—Biochemical changes during recovery from kwashiorkor.

DISCUSSION

It was apparent that the NE/E ratio was sensitive to small changes in improvement from kwashiorkor, a condition in which the serum amino acid pattern is known to be severely disturbed. The fact that it is a ratio determination eliminates the effect of plasma volume changes which in many instances alter serum protein and albumin concentration values. These results are in remarkable agreement with those obtained in African kwashiorkor patients reported by Whitehead (9).

The ability of the NE/E ratio to reflect gradual improvement in protein nutriture suggested that it may also be useful in detecting small changes in blood *prior to* the appearance of clinical signs of kwashiorkor.

Study 2 - Pre-school Children

The purpose of this study was to determine the usefulness of the NE/E ratio as a means of detecting chronic protein malnutrition in population groups. The pre-school children in the two groups were of similar racial and socio-economic background. Information about their diets was obtained from a seven day dietary survey which had been conducted several months before.

One group was composed of thirty-four pre-school children from a low-income rural area of Guatemala. Their diet was inadequate in many nutrients, including protein (Table I)[6]. The second group was composed of twenty-two pre-school children from an orphagane in Guatemala City. Their diet was adequate in all nutrients when compared with the NRC recommendations (Table I). All children included in this group had been receiving the orphanage diet for at least two months. Since many of the children had probably entered the orphanage in an undernourished state, they were not typical of a group which had always received an adequate diet. However, with respect to a diet of two months or longer duration, these children provided a definite contrast with the rural children.

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6 Dietary data kindly given to us by Miss Marina Flores, Chief of the Dietary Research Unit, INCAP.

TABLE I

CHARACTERISTIC DAILY AVERAGE NUTRIENT INTAKE* OF THE TWO GROUPS OF PRE-SCHOOL CHILDREN

Nutrient		Cons./person	NRC recommendation	% of NRC
LOW INCOME RURAL (11 children)				
Protein (total)	g	24.8	41.8	59
% animal protein		22	—	—
Calories		785	1209	65
Fat	g	12.0	—	—
Carbohydrate	g	150	—	—
ORPHANAGE (41 children)				
Protein (total)	g	55.4	45.0	123
% animal protein		61	—	—
Calories		1324	1330	100
Fat	g	26.0	—	—
Carbohydrate	g	220	—	—

* Averages are based on a 7-day Dietary Survey.

PROCEDURE

A 0.5 - 1.0 ml blood sample was taken from the finger tip before breakfast. Serum was removed and analyzed for total proteins and the remainder was frozen until analyzed as indicated before. The height and weight of the children were determined.

Resuls

The classification of nutritional status on the basis of body weight is given in Table II. Several children from both groups were classified with second degree malnutrition and only seven had weights in the normal range. Both groups had mean percent deficits in weight greater than the 10% according to

the standards adopted by INCAP (10). The orphanage children were less deficient in both height and weight than the rural children.

Study of the blood serum (Table III) showed a significantly lower NE/E ratio in the orphanage (2.07; S.D.=0.55) than in the group of rural children (2.89; S.D.=0.71). Rutishauser and Whitehead (11) found an average of 1.90 with

TABLE II

CLASSIFICATION OF MALNUTRITION IN PRE-SCHOOL CHILDREN ON THE BASIS OF BODY WEIGHT

Degree of Malnutrition	Relative weight % of normal	Orphanage		Low Income Rural	
		N	%	N	%
Normal	90 — 110	4	18	3	9
First	75 — < 90	15	68	17	48
Second	60 — < 75	3	14	15	43
Third	< 60	—	—	—	—

TABLE III

COMPARISON OF NE/E RATIO WITH OTHER MEASURES OF NUTRITIONAL STATUS IN TWO GROUPS OF PRE-SCHOOL CHILDREN

Parameter Studied	Low Income Rural (Inadequate Intake)	Orphanage (Adequate Intake)
No. of children	35	22
NE/E Amino Acid ratio	2.89 (0.71)[1,2]	2.07 (0.55)[1,2]
Total Serum Protein g/100 ml	6.54	7.04
Age in months	54.6	57.2
Weight deficit (%)	22	18
Height deficit (%)	12	10

[1] Standard deviation.

[2] $t = 4.88$ with 55 degrees of freedom ($t = 2.67$ for $P < 0.01$).

a standard deviation of 0.50 for 195 children of similar ages who had been successfully treated for kwashiorkor or marasmus. We found 1.83 for our recovered kwashiorkor patients. These values are only slightly lower than those found in the orphanage children. On the other hand, the rural children had a much higher ratio. The difference was significant at the 1% level. Ten well-nourished European children studied by Whitehead and Dean (7) gave an average ratio of 1.5 with a range between 1.2-1.8. On the basis of the distribution found in the 195 African children recovered from protein-calorie malnutrition, however, Rutishauser and Whitehead conclude that ratios of three or above are definitely abnormal (11). Thirteen of the 35 children in the rural population had values of 3.0 or over (37% prevalence). In the orphanage, only one of the 22 (4%) had ratios above 3.0.

The difference in the distribution of ratios is shown in figure 7. The difference in the distribution of serum proteins was less striking (figure 7).

DISCUSSION

The children in the orphanage could not be considered adequately nourished on the basis of height and weight. This retardation in physical development indicates that, before entering the institution, they were suffering from a degree of malnutrition similar to the children in the low income rural group. The NE/E amino acid ratio was, however, very sensitive to distinguish between the two groups, reflecting the improvement in protein nutrition produced by the superior diet in the orphanage.

Study 3 - Recovery from Adult Malnutrition

In a manner similar to the study of recovery from kwashiorkor, the NE/E ratios of blood serum were determined under the extreme condition of malnutrition in the adult. It was necessary to determine whether or not older individuals, with demands for repletion and maintenance of body protein which differed from those of children responded in the same way.

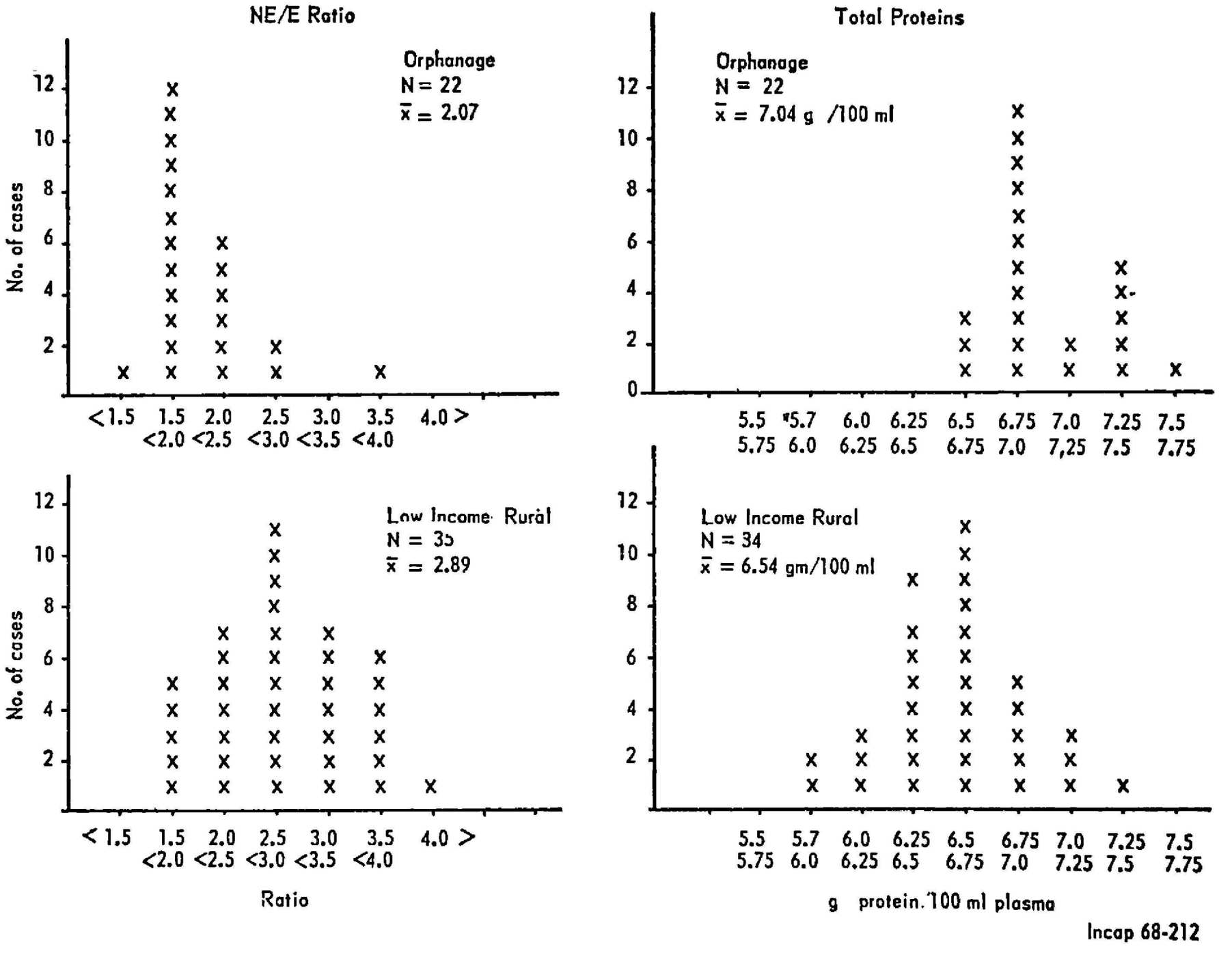

Fig. 7.—Serum free amino acid index (NE/E ratio) and total serum proteins in two groups of pre-school children having different nutritional characteristics.

The blood serum of five patients aged 13-84 years, who had been hospitalized with severe malnutrition was analyzed[7].

RESULTS

Comparisons of NE/E ratios with other parameters of nutritional status are given in figures 8 through 12. Initial ratios ranged from 2.94 - 1.92. The decrease in ratios during recovery followed the pattern of improvement given by total serum proteins and albumin, with one exception. Case AM-9 was an 84-year-old man whose age may have classified him differently with respect to protein metabolism. It is possible that

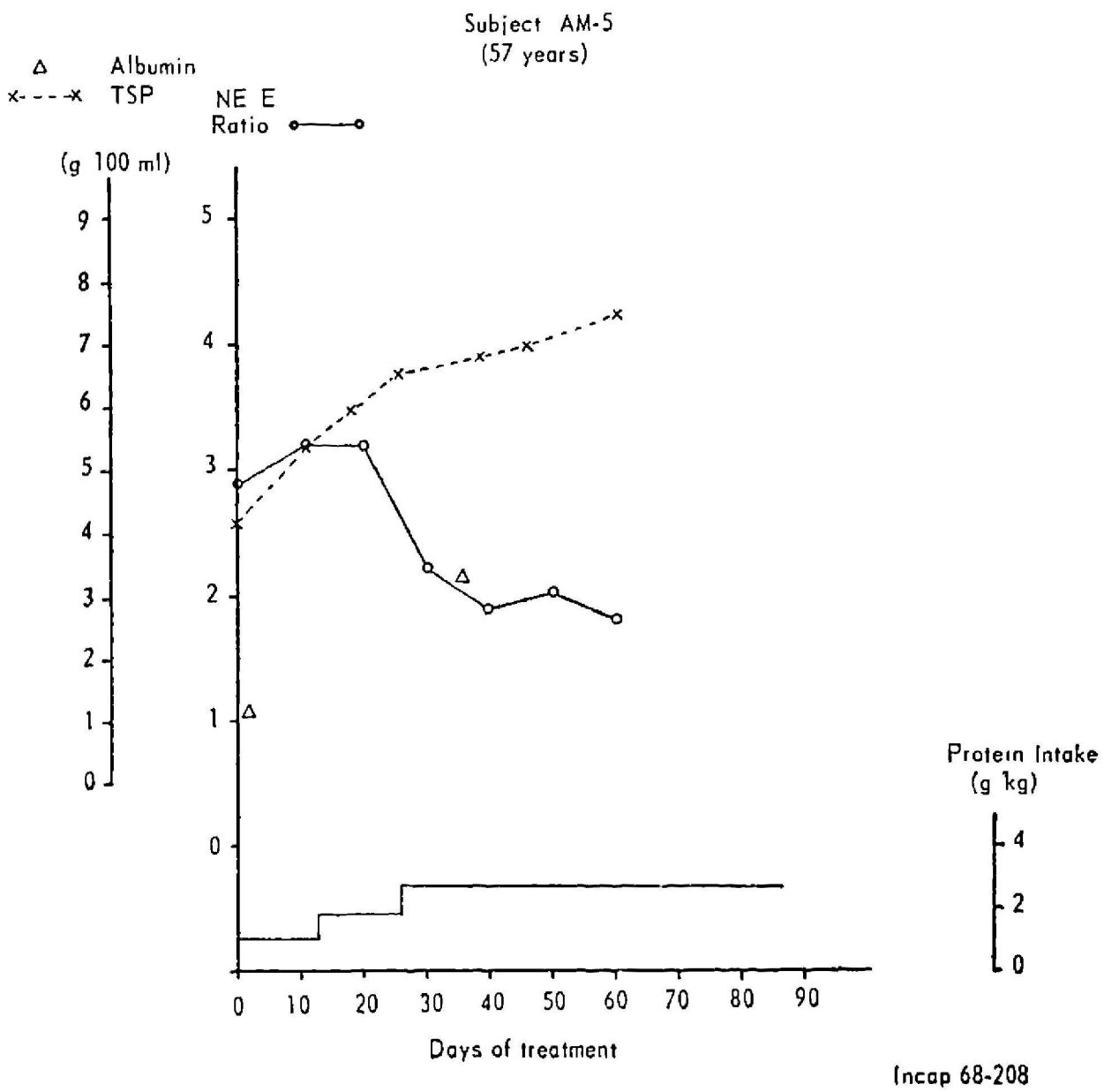

Fig. 8.—Biochemical changes in adults during recovery from severe malnutrition.

7 These patients were under study by Dr. Fernando Viteri, Chief of the Division of Biomedics, INCAP.

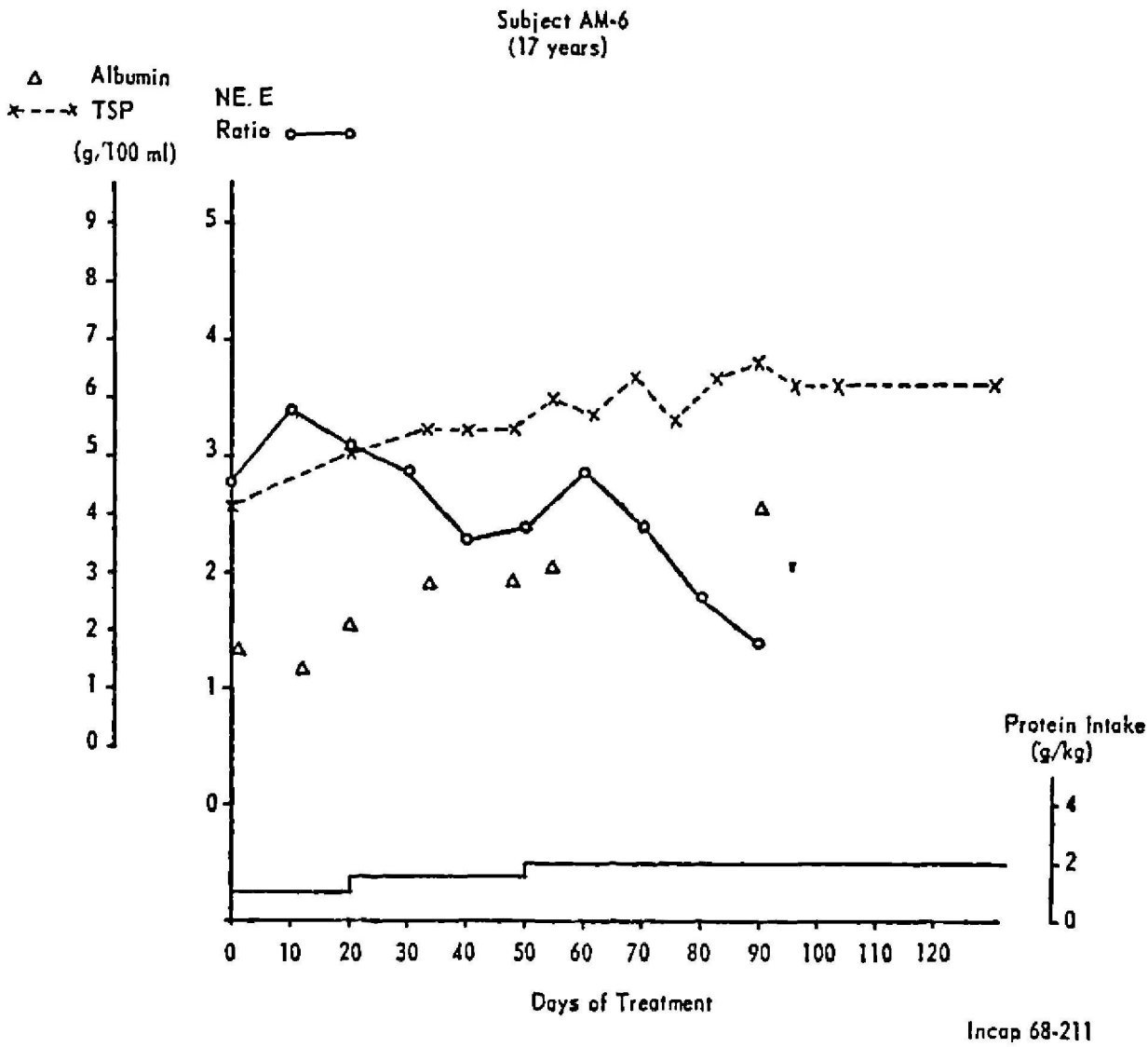

Fig. 9.—Biochemical changes in adults during recovery from severe malnutrition.

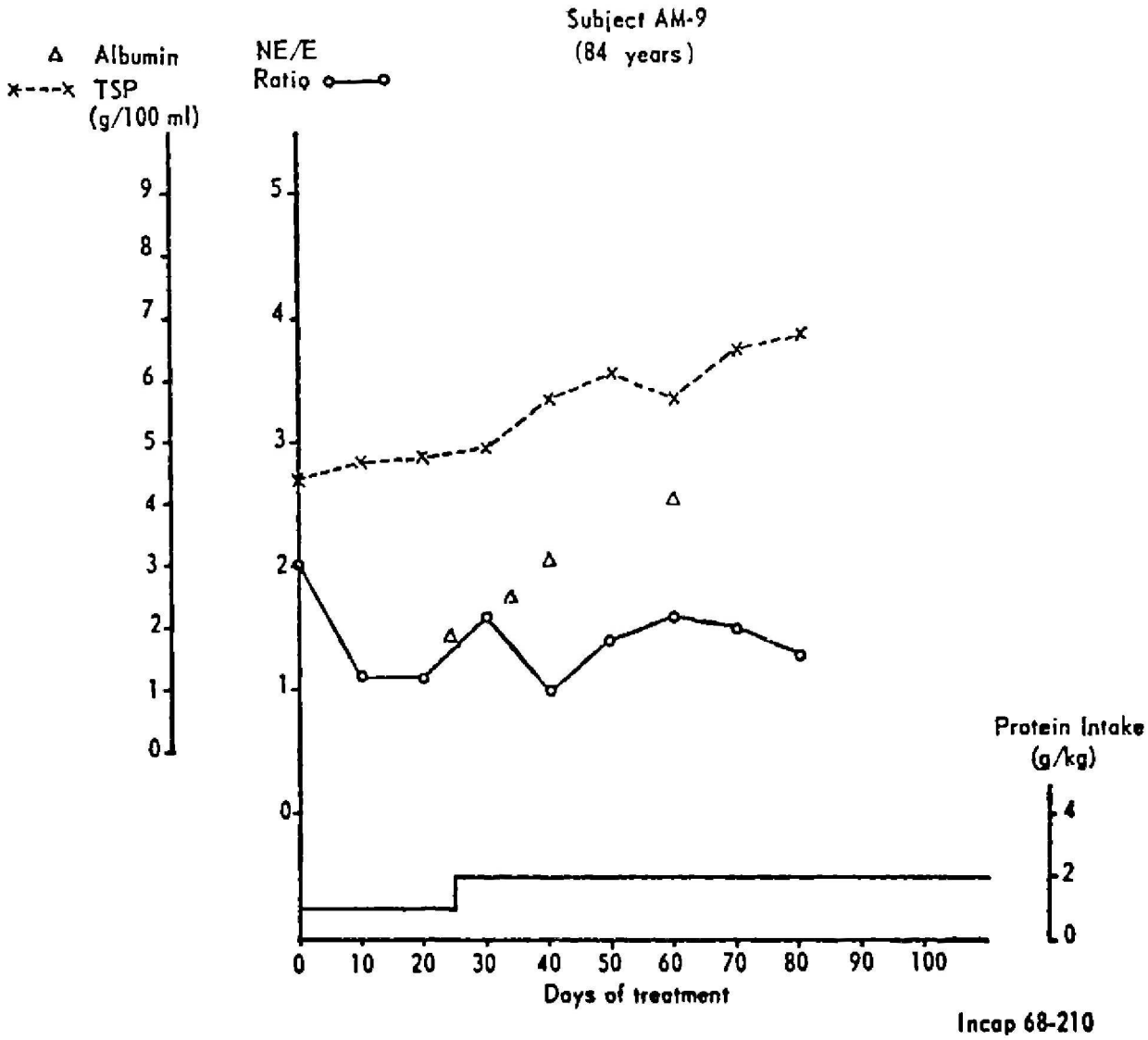

Fig. 10.—Biochemical changes in adults during recovery from severe malnutrition.

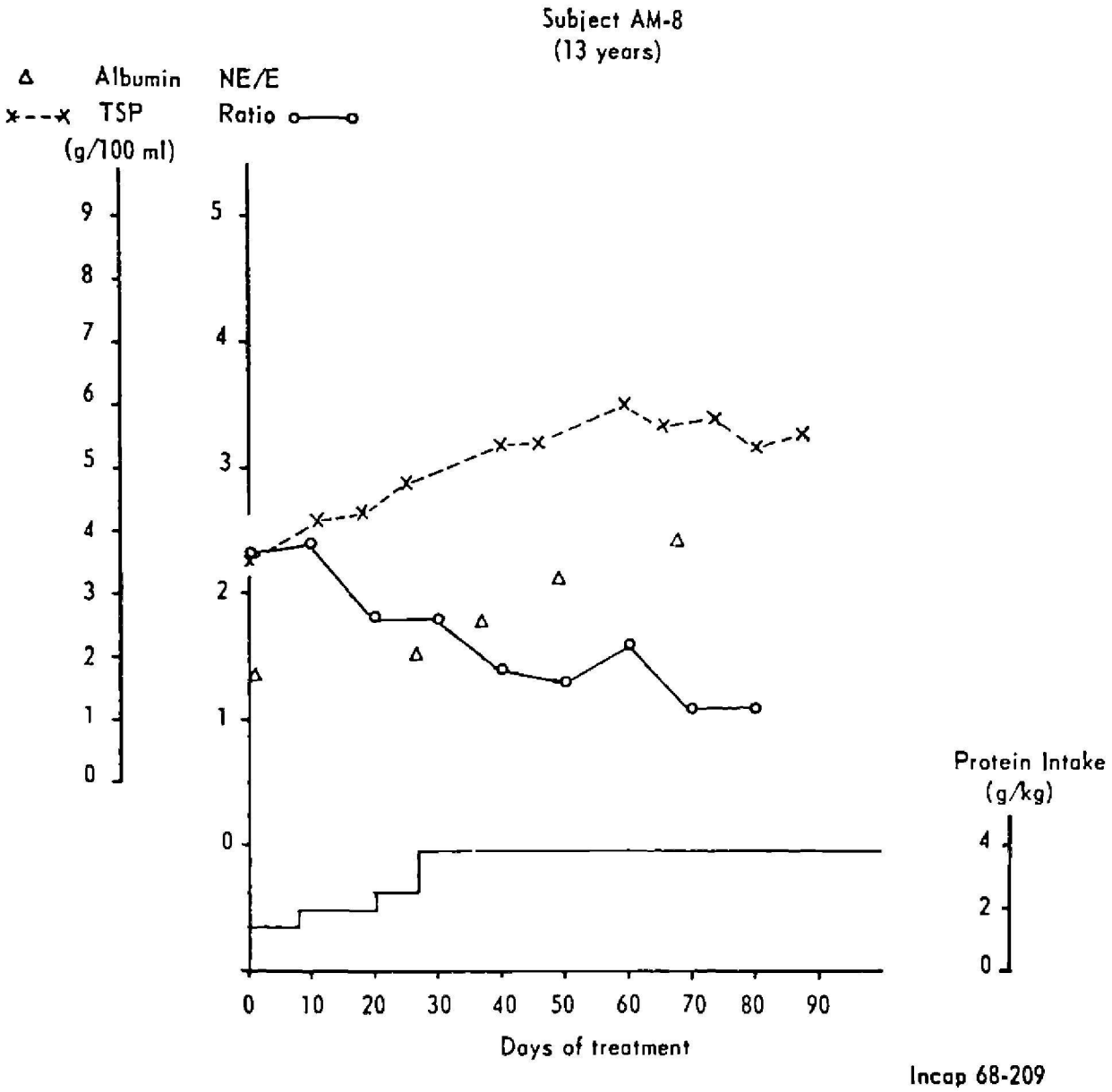

Fig. 11.—Biochemical changes in adults during recovery from severe malnutrition.

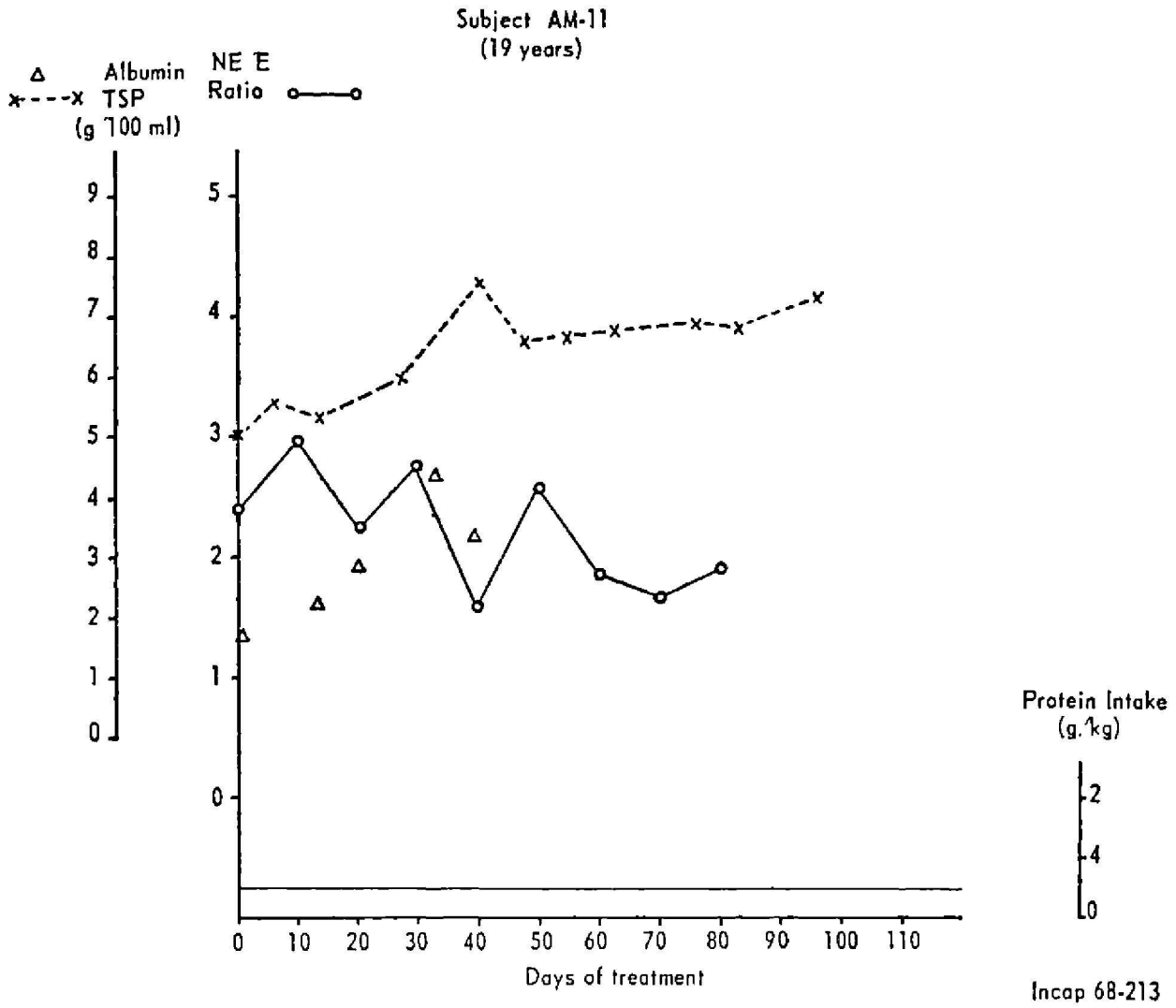

Fig. 12.—Biochemical changes in adults during recovery from severe malnutrition.

he was in a state of protein catabolism during the first few weeks, a fact which might expain the high ratios during the early stages of recovery. It is known, in fact, that in starvation and in the acute phase of measles (catabolic conditions), the changes in plasma amino acids are the reverse of those found in kwashiorkor, i. e. the essential amino acids are elevated while the non-essentials are depressed (12).

DISCUSSION

The NE/E ratio of blood serum permitted detection of gradual improvements in nutritional status of adults as well as children, although, their initial ratios were not as elevated. Similar response by the two groups following the severe metabolic stress of malnutrition suggests that the ratio is a crit-

ical indicator of protein nutriture. It is possible that the NE/E ratio could be used in evaluation of nutritional status of adult populations as effectively as in populations of children.

Study 4 - Experimental Animals

The purpose of this study was to evaluate the use of the fasting NE/E ratio in blood serum under more controlled conditions than those found in human populations by making the quality and quantity of protein the only significant dietary variable. Male rats of the Sprague-Dawley strain, average weight 88.8 grams, received a stock diet[8] prior to the experiment. During the experimental period, diets[9] were offered *ad libitum* and contained either 5% corn protein, 5% casein or 20% casein. Serum NE/E ratios were determined initially, after one week, and after two weeks. On the day of sampling, animals were fasted for eight hours, anesthetized with ether and 1-2 ml of blood was taken by heart puncture. Preparation and analysis of blood was the same as previously described.

RESULTS

Weight gain and NE/E ratios are given in Table IV. The change of diet from stock to purified diet produced a weight loss in animals receiving 5% of either protein source. The greater weight loss with 5% casein was accompanied by a lower food consumption. Adaptation to a new diet was probably partly responsible for the increase in all NE/E ratios during the first week and may have obscured particular effects of the diet on the ratio.

During the second week, NE/E ratios reflected differences in both quality and quantity of dietary protein. The ratios were significantly different in all three groups (Table IV), in spite of the fact that neither protein source supported growth when fed at the 5% level. The ratio declined more rapidly during the second week in the serum of animals receiving a

8 Stock diet (22% protein): Purina, Guatemala, 42%; Corn 55%; minerals (Salmina) 3.0%.

9 Non-protein diet was as follows: 65% or 80% cornstarch; 10% cottonseed oil; 4 ml vitamin mix in alcohol (Manna, L. and S. M. Hauge, J. Biol. Chem., 202:91, 1953): 4%.

TABLE IV

THE EFFECT OF PROTEIN QUALITY AND QUANTITY ON THE NE/E AMINO ACID RATIO IN BLOOD SERUM OF RATS

Diet	ONE WEEK			TWO WEEKS		
	Weight Gain	Ratio $\bar{X}$	S. D.	Weight Gain [2]	Ratio $\bar{X}$	S. D.
5% Corn Protein	(4)[1] — 5.2 ± 1.7	3.31	0.87	(5) — 4.6 ± 2.1	8.96	4.44 [3]
5% Casein	(4) — 9.8 ± 3.5	3.29	0.62	(4) — 12.5 ± 2.8	4.53	1.21 [3]
20% Casein	(6) 41.2 ± 5.9	2.63	0.41	(6) 91.0 ± 5.7	2.17	0.51

Initial average weight: 88.8 g.
Initial ratio: 1.60; S. D. = 0.31 (8 rats).

[1] Number of rats in parenthesis.
[2] Two weeks gain.
[3] "t" significant at the 1% level ($P<0.01$) when compared with the 20% casein group at two weeks.

small amount of poor protein than in the serum of animals receiving a small amount of a high quality protein.

DISCUSSION

The NE/E ratio in fasting serum of rats reflected variations in dietary protein quality and quantity after a period of one week. The changes in ratios during the second week indicated that after adaptation to a diet of low protein quality or quantity, serum ratios continue to reflect dietary differences. Evidence obtained from this very short term study suggested that the NE/E ratio was very sensitive for detecting differences in protein quality and quantity under controlled laboratory conditions.

COMMENTS

Whitehead's technique for estimating the ratio of two groups of amino acids in blood plasma was evaluated under several types of conditions. The method appeared to be sensitive to smal changes in the relative serum concentrations of particular essential and non-essential amino acids which accompanied dietary differences. The procedure is relatively simple and seems to be an effective means of detecting different protein nutrition of population groups. In cases where serum amino acid pattern was severely altered, as the result of disease, the ratio responded to dietary treatment in individual cases.

The technique demands further study, possibly under the following conditions: 1) A field study of groups of children or adults suspected or having, on the average, various grades of chronic malnutrition; 2) longitudinal studies in which various measures for improvng the nutritional status of population groups are being investigated; 3) laboratory investigation of longer duration, in which the balance of nutrients, especially carbohydrate: protein, as well as the source of protein, are studied under controlled conditions.

RESUMEN

Los aminoácidos libres del plasma como índice de la nutrición proteica

El propósito de este trabajo fue evaluar la razón de los aminoácidos libres del plasma, propuesta por Whitehead como indicador de nutrición

proteica. El método se aplicó a niños y adultos que padecían de malnutrición proteico-calórica severa, al ingreso al hospital y durante la recuperación; y a niños con malnutrición crónica pertenecientes a un grupo de nivel socio-económico bajo en el área rural de Centro América. Además, se llevó a cabo un experimento con ratas alimentadas con dietas que diferían en cantidad y calidad de proteínas. Los resultados indican que la razón de aminoácidos de Whitehead es de valor para evaluar el estado nutricional proteico bajo las condiciones de este estudio.

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