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ANNUAL PROGRESS REPORT

—February 1969

EXTENDING PROTEIN CONCENTRATES FOR CHILD FEEDING BY ADDITION OF INEXPENSIVE SIMPLE NITROGEN SOURCES

Contract No. AID/csd-1441

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SUMMARY AND CONCLUSIONS

Comparison of the essential amino acid and total nitrogen requirements of children and adults, as reported in the scientific literature, with the concentration of essential amino acids found in milk and other good protein sources of animal origin leads to the conclusion that for healthy individuals beyond infancy the concentration is higher than the physiological requirement. Under these circumstances, it should be possible to replace part of the nitrogen of these protein sources with nitrogen from any utilizable source, including simple and relatively inexpensive nitrogen containing compounds such as glycine, glutamic acid and ammonium citrate.

Nitrogen balance studies with twenty-one young adults at M.I.T. fed milk as a sole nitrogen source indicated that at least 20%, and in some subjects greater than 25%, of the nitrogen of milk could be so placed without affecting nitrogen retention. In order to allow a margin of safety in the studies with children, a 20% dilution was employed with seven children aged four to nine years in studies at M.I.T. with similar results. While these results indicated that a 20% dilution is effective with children, the number of subjects was too small to give much indication of the extent of individual variation. For this reason a still conservative dilution, 10%, was recommended for nitrogen balance studies during the repletion of malnourished and younger children in the Institute of Nutrition of Central America and Panama. Once again there were no differences in nitrogen retention between

periods with the milk formula diluted or indiluted with non-specific nitrogen.

It is probable that the children would have given the same results with a higher level of dilution, but this was not tested. These results give reasonable assurance that the nutritional contribution of milk as a sole source of protein can be extended to a significant degree by the addition of simple and relatively inexpensive nitrogen sources.

Background Statement:

On the basis of a consideration of the published values for the requirements for individual essential amino acids per g total dietary nitrogen for infants, children and adults (see Holt and Snyderman, 1967; Chan and Waterlow, 1966; FAO, 1965; and shown in Table 1), it appears that the concentrations of the individual essential amino acids in cow's milk protein are in excess of requirements. Therefore, studies were undertaken, with the assistance of Contract No. AID/csd-144-, to determine the extent to which skim milk protein could be isonitrogenously replaced by a simple source of non-specific nitrogen without changing the protein quality of the milk protein.

The studies conducted to date may be catagorized as follows:

1. Studies with mentally retarded children at M. I. T.

2. Studies conducted in collaboration with the Institute of Nutrition of Central America and Panama (INCAP) on children recovering from kwashiorkor.

3. Studies in young adult men at M. I. T.

4. Limited studies in rats conducted at M. I. T. to gain a further understanding of the effects of substituting cow's milk protein with non-specific nitrogen.

For convenience, the results obtained from the above studies are reported below in Sections 1, 2, 3, and 4, respectively.

Table 1

Calculated essential amino acid (g) to total nitrogen (g) ratio (E/T_N) in infants, children, and adults from published values on amino acid and nitrogen requirements

Age Group	Total EAA mg/kg	Total N for Growth or Maintenance mg/kg	E/T_N	% EAAN of Total N
Infants (ca. 1 yr)	760[1]	200[2]	3.8	48
Older Children (10-12 yrs)	260[3]	100[4]	2.3	33
Adults	64[5]	46[6]	1.4	16

1. From Holt and Snyderman (Nutr. Abstr. Rev. 35: 1, 1965).
2. From Chan and Waterlow (Brit. J. Nutr. 20: 755, 1966).
3. From Nakagawa et al (J. Nutr. 86: 833, 1965).
4. From FAO (Nutrition Studies No. 37, FAO, Rome, 1965) not allowing for growth.
5. Mean for men and women, from FAO (Nutrition Studies No. 16, FAO Rome, 1957).
6. From Young and Scrimshaw (Brit. J. Nutr. 22: 9, 1968) includes only urine and fecal N.

SECTION 1

STUDIES ON THE EFFECT OF MILK PROTEIN DILUTION IN CHILDREN AT M. I. T.

Studies on the effect of dilution of skim milk protein were commenced on fourteen mentally retarded children. Of these fourteen children, seven were found to be unsatisfactory for study and returned, within two weeks of admission to the M. I. T. Clinical Center, to their respective State schools.

Details of experimental design, diet, and analytical procedures were previously described in the 1967 Annual Progress Report and are further described in Section 3 of this report.

Details are given in Table 2 for the seven children who were studied during the entire experimental period.

Table 2

Age and weight of children and experimental design used for studies of milk protein dilution[1]

Subject	Age (yrs.)	Initial Wt. (lb.)	Final Wt. (lb.)	Days on Baseline Diet	Days on Dilution
P. K.	9	61.5	58.5	18	20
W. M.	6	33.0	36.0	26	83
C. R.	5	33.0	35.0	25	32
R. O'C.	4	34.0	36.0	33	32
B. L.	5	41.0	43.0	29	40
S. D.	9	35.0	37.0	25	12
J. W.	5	22.0	23.5	40	30

1. Dilution at 20% level.

Results on urinary nitrogen excretion for these subjects during the baseline and milk dilution periods are summarized in Table 3.

Table 3

Effects of dilution of skim milk protein with glycine and diammonium citrate on urinary nitrogen excretion by children

Subject[1]	Dilution of Protein, %	
	0	20
P. K.	2.14 ± 0.24	2.09 ± 0.59
W. M.	0.77 ± 0.14	0.70 ± 0.12
C. R.	0.81	0.81
R. O'C.	1.03 ± 0.1	0.92 ± 0.08
B. L.	1.17 ± 0.04	1.08 ± 0.2
S. D.	0.86	0.89
J. W.	0.66	0.42

1. All subjects fed milk protein as the major protein source (90% total dietary N) at a level equivalent to 0.45g protein/kg body wt. /day. Figures are mean ± std. dev. for daily urinary nitrogen excretion (g/day) for periods of four or more consecutive days.

Using nitrogen excretion as the major parameter for assessing the effect of a 20% dilution of cow's milk protein with non-specific nitrogen (glycine and diammonium citrate, see Section 3), it appears that this level of dilution does not alter the efficiencies of dietary nitrogen utilization. It appears, therefore, that cow's milk protein, when fed at 0.45g protein/kg body wt., contains about 20% essential amino acid N in excess of the essential amino acid requirements of these children.

These results agree well with those described below (Section 3) for young adult men. As revealed by clinical examination and routine clinical chemistries, all children maintained good health throughout the experimental period

SECTION 2

STUDIES ON THE EFFECT OF MILK PROTEIN DILUTION IN CHILDREN AT INCAP

This investigation began at INCAP on 1 January 1968, with the following aims:

1. To study, in protein depleted children, the utilization of milk protein diluted with non-essential nitrogen. The criteria used were nitrogen balance complemented by clinical evaluation of the children. Theoretically, by the use of protein depleted children, differences could be detected between proteins of similar quality which, in fully repleted individuals would be unnoticed. The sensitivity of the depleted child to the availability of essential nitrogen should be greater due to the acute and specific need of essential nitrogen for rapid protein synthesis of young depleted organisms.

2. To test the supplementary value of milk protein versus milk protein whose essential nitrogen has been diluted 10% (milk-diluted 10 with non essential nitrogen, when added to a typical diet consumed by pre-school children in Central America. The protein in this typical diet is known to be of poor quality, mainly because of deficiencies in lysine, tryptophan and methionine.

The present report is concerned with the first aim, since work designe to meet objective number 2 is now in progress.

Four children during the early phase of recovery from severe protein-

calorie malnutrition (PCM) have been studied. Their ages in years were: 5-11/12, 1-5/12, 2-11/12 and 2-0/12. Initial recovery from PCM was satisfactorily obtained in these children by the routine care established at INCAP's Clinical Center and a dietary intake of three grams of protein and 120 calories/Kg/day. Twenty percent of the calorie intake was supplied by vegetable fat. The protein source was 50% from milk and 50% as calcium caseinate. A mineral-vitamin supplement was also administered. Ten days after the child had lost clinical edema the child was placed on 1 g. milk protein and 120 cal/Kg/day. Twenty percent of the calories were supplied by vegetable fat. Vitamins and minerals were supplied in adequate amounts. After a period of thirteen days on this diet, during which a four day adaptation period and two consecutive three days balance periods were carried out, the protein source was changed from milk to milk whose nitrogen was diluted 10% with glycine and diammonioum citrate. The total nitrogen, calories, vitamins and minerals supplied by the diet remained unchanged.*

Three consecutive nitrogen balances were performed on these children on the "milk diluted 10%" without an adaptation period upon the change in diet. At the completion of the last balance, the children were

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* Only child PC193 varied from this scheme in that the caloric intake during the period of 1 g protein intake was reduced to 100 Cal/Kg/day. This was done in order to reduce a tendency of this child to vomit, as it occurs in some children when caloric intake is disproportionately high in relation to protein intake. This caloric intake is still adequate for recovery from kwashiorkor and did not make caloric intake limiting.

placed again on milk protein and three consecutive three days nitrogen balances were again performed, without an adaptation period. This diet was identical to that used before "milk diluted 10%" was introduced. (Fig. 1)

The children were clinically evaluated daily and continued on the study only if they did not present evidence of concurrent diseases or untoward effect of the diet. Total serum proteins, albumin and urinary creatinine excretion were measured periodically.

RESULTS

The results presented here are on four children who fulfilled all the above criteria and on whom the study was carried to completion. The children accepted the diet easily and it caused no clinical alterations.

Nitrogen Balances

Tables 1-4 show the results obtained in the four children studied. Nitrogen absorption and retention are expressed as percent of intake. Figures 2 and 3 show the percent nitrogen absorbed and retained in the four children respectively.

Table 5 shows the average nitrogen absorbed and retained by each child with each diet.

Table 6 presents the average obtained from the four children together with regard to nitrogen absorbed and retained during the three dietary periods.

Figure 4 depicts the daily urinary nitrogen excreted by each child during the 24 days covering the three study periods.

No differences were detected by the nitrogen balance technique between milk and "milk diluted 10%" in protein depleted children. The constancy of urinary nitrogen excretions in these children brings further evidence to the similar behaviour of the children during the three study periods.

Apparent nitrogen absorption varies primarily due to fecal nitrogen excretion and this factor influences the apparent total nitrogen retention more than changes in urinary nitrogen. These variations are due to individual patterns of fecal nitrogen excretion during the early recovery phase of malnutrition in each child and are not due to variations in the diet. The use of children who are still protein depleted for the evaluation of nitrogen sources has this limitation, particularly when nitrogen intake is low. In this case, changes in fecal endogenous nitrogen that are known to occur in children with PCM can account for a large proportion of the fecal nitrogen. The variation in apparent nitrogen absorption and retention in these children, therefore, is greater than that expected in fully recovered ones.

Clinical Evaluation

The four children studied did not show any clinical alteration from either of these diets. However, the rate of weight gain was slightly reduced in the four children while they were receiving 1.0g.Prot/Kg/day

This slower weight gain was not accentuated during the period on "milk diluted 10%". In a similar fashion, creatinine excretion did not rise as rapidly as in children who are maintained on high protein intakes during this phase of recovery. Total serum proteins decreased in two of four children, particularly in child PC196 who remained close to nitrogen equilibrium during the study. These biochemical changes are no more accentuated during the period on "milk diluted 10%".

As stated earlier, we are proceeding with the second aim of INCAP's commitments in order to have this study completed before the middle of this year.

TABLE 1

NITROGEN BALANCES

PC 193
Age 5 y. 11 m.

Weight Kg	Source of Protein	Protein Intake g/Kg/d.	N. Intake mg/Kg/d.	N. Absorption %	N. Retention %
12.73	Milk	0.99	159	91.0	39.1
12.78	Milk	0.99	158	54.0	1.6
12.82	Milk-Dil. 10%	0.95	152	87.2	33.7
12.89	Milk-Dil. 10%	0.98	158	78.0	32.8
12.89	Milk-Dil. 10%	0.98	158	78.0	32.8
13.24	Milk	1.00	159	71.7	37.3
13.21	Milk	0.91*	145*	76.9	30.3
13.23	Milk	0.87*	139*	56.1	5.6

* Intake lower because of emesis

TABLE 2

NITROGEN BALANCES

PC 194
Age 1 y. 5 m.

Weight Kg	Source of Protein	Protein Intake g/Kg/d.	N. Intake mg/Kg/d.	N. Absorption %	N. Retention %
7.43	Milk	1.00	160	75.9	16.8
7.98	Milk	0.99	170	78.9	30.2
8.07	Milk Dil. 10%	1.03	164	81.9	30.6
8.22	Milk Dil. 10%	1.00	159	79.8	22.4
8.29	Milk Dil. 10%	0.99	158	61.7	3.8
8.37	Milk	1.01	162	66.5	18.5
8.52	Milk	1.00	156	76.2	25.8
8.62	Milk	0.98	155	75.5	19.2

TABLE 3

NITROGEN BALANCES

PC 196
Age: 2 y. 11 m.

Weight Kg	Source of Protein	Protein Intake g/Kg/d	N. Intake mg/Kg/d	N. Absorption %	N. Retention %
9.06	Milk	1.01	161	73.9	-1.1
9.16	Milk	1.00	158	72.8	-9.2
9.26	MIlk Dil.10%	0.98	155	68.6	17.2
9.33	Milk Dil.10%	0.98	154	57.9	-0.5
9.44	Milk Dil.10%	0.97	150	75.3	17.2
9.50	Milk	0.99	158	72.0	22.2
9.60	Milk	1.01	151	54.1	-2.1
9.74	Milk	0.99	152	57.8	3.1

TABLE 4

NITROGEN BALANCES

PC 197
Age: 2 y.

Weight Kg.	Source of Protein	Protein Intake g/Kg/d	N. Intake mg/Kg/d	N. Absorption %	N. Retention %
8.12	Milk	0.98	153	87.3	24.7
8.22	Milk	0.96	148	84.1	32.3
8.33	Milk Dil.10%	0.99	156	87.2	29.2
8.48	Milk Dil.10%	0.98	152	85.6	22.4
8.60	Milk Dil.10%	0.96	146	83.1	23.5
8.70	Milk	1.00	157	85.9	34.1
8.84	Milk	0.99	153	83.7	33.0
8.95	Milk	0.97	148	89.7	44.2

TABLE 5

AVERAGE NITROGEN INTAKE, ABSORPTION % AND RETENTION % OF EACH CHILD DURING THE THREE STUDY PERIODS

Child No.	Study Period	Protein Source	Nitrogen Intake mg/Kg/d.	Nitrogen Absorbed %	Nitrogen Retained %
PC 193	1	Milk	158	72.5	20.4
	2	Milk Dil. 10%	154	81.1	33.1
	3	Milk	148	68.2	24.4
PC 194	1	Milk	165	77.4	23.5
	2	Milk Dil. 10%	160	74.5	18.9
	3	Milk	158	72.7	21.2
PC 196	1	Milk	160	73.4	-5.2
	2	Milk Dil. 10%	153	67.3	11.3
	3	Milk	154	59.2	7.7
PC 197	1	Milk	151	85.7	28.5
	2	Milk Dil. 10%	151	86.6	25.0
	3	Milk	153	86.4	37.1

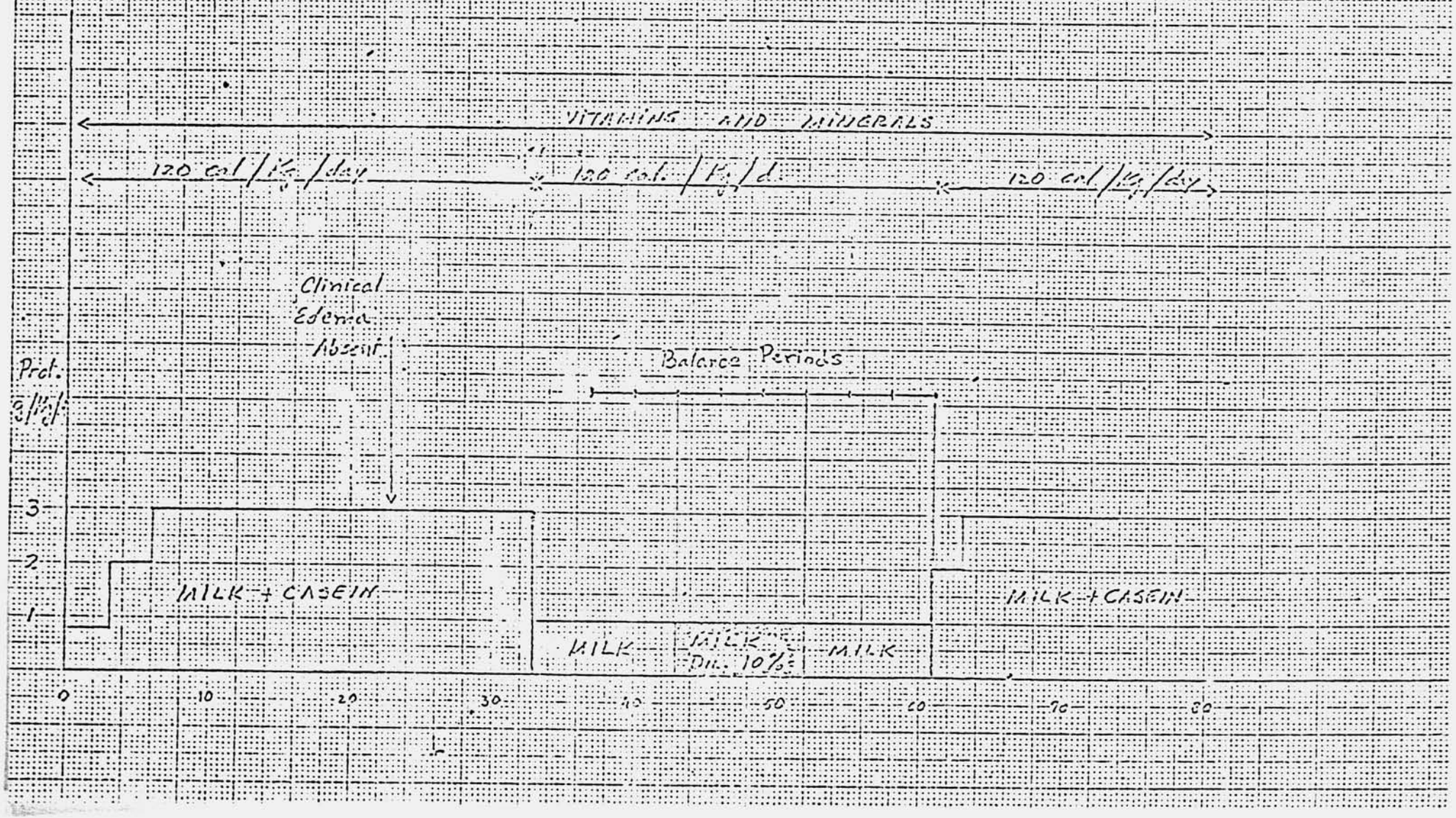
TABLE 6

AVERAGE NITROGEN ABSORPTION AND RETENTION IN PERCENT OF INTAKE

FOR THE FOUR CHILDREN STUDIED, IN EACH DIET

Protein Source	NITROGEN		
	Intake mg/Kg/d.	% Absorption	% Retention
Milk	158.4	77.2	16.8
Milk Dil. 10%	155.2	77.1	22.1
Milk	152.9	72.2	22.6

Plan of Study of Depleted Children



SECTION 3

STUDIES IN YOUNG ADULT MEN AT M. I. T.

The detailed report given below describes the results obtained with young adult men studied for the effects of milk protein dilution with non-specific nitrogen.

The report has been accepted for publication: "Partial Dietary Replacement of Milk Protein by Non-Specific Nitrogen in Young Adult Men," by Scrimshaw, Young, Huang, Thanangkul and Cholakos, in the Journal of Nutrition, May 1969.

INTRODUCTION

Most of the work done on human protein requirements has dealt with the essential or indispensable amino acids. But the other component of the protein requirement, nonspecific nitrogen (1-4), which is furnished from excess essential amino acids and by the nonessential amino acids, merits further investigation. A review of the published values for the essential amino acid requirements of adult man (2, 5, 6) suggests that the concentrations of essential amino acids per gram of total nitrogen in high quality protein foods such as eggs, meat and milk are higher than required when these foods are the sole source of dietary protein. Moreover, a number of investigators have shown in experimental animals that nonessential amino acids are more effective in meeting nonspecific nitrogen requirements than are the essential amino acids (7, 8). In order to investigate further the interrelationship between these 2 components of man's protein needs, we have undertaken a series of studies in which various amounts of the test dietary protein are replaced, isonitrogenously, by a source of nonspecific nitrogen. The present work with milk protein extends our previous observations made with egg (9) and beef protein (10).

In establishing the amino acid requirements of man, investigators have used various sources of supplemental nitrogen including glycine or urea (11), glycine in

combination with diammonium citrate and other nonessential amino acids (12, 13), or a mixture of several nonessential amino acids (14). Although these studies suggest that a number of individual nonessential amino acids can meet the nonspecific nitrogen needs as effectively as a mixture of nonessential amino acids, the issue is far from settled (15). In the present series of experiments, we have isonitrogenously replaced milk protein first by a mixture of glycine and diammonium citrate, and then by a mixture of nonessential amino acids patterned as in cow's milk protein, and compared the effects.

MATERIALS AND METHODS

Subjects. The 21 male college students (table 1) who participated in these studies were in good health as determined by medical history and examination, and continued their normal daily activities throughout the experimental period. Three of the subjects (W.I., K.E., J.R.) participated in more than 1 experiment.

Diet. The composition of the diet, based principally on a liquid formula, is shown in table 2. Dried nonfat kim milk protein supplied 90% of the total dietary protein;

oatmeal supplied the remainder. It was found necessary to include a small amount of oatmeal to maintain a stable formula of agreeable consistency. The diet furnished 0.38 g protein/kg/day. The net protein utilization (NPU) of the skim milk protein by rat assay (16) was determined to be 71 ± 2 compared with 63 ± 2 for unsupplemented casein used as a standard. The daily diet was divided into 4 approximately equal portions.

"Dilution" of the dietary protein. As in our previous studies (9, 10), the dietary nitrogen was replaced ("diluted") by substituting part of the dietary nitrogen with either a mixture of glycine and diammonium citrate (in which each compound supplied equal amounts of nitrogen) or a mixture of nonessential L-amino acids based upon the proportions of amino acids in whole dried nonfat skim milk protein (17). The composition of this mixture is shown in table 3.

Experimental sequence. The subjects were first fed the basal (0% dilution) diet which supplied 0.38 g protein/kg body weight. This rate was calculated to supply nitrogen at the minimum daily N requirement as discussed previously (9, 10). The duration of the first basal period varied from 14 to 18 days, depending upon the time required for the subject to reach an apparently steady state of N metabolism as revealed by urinary N excretion.

Three experiments were conducted. The first 2, with 7 subjects each, evaluated the effects of replacing

20 and 25%, respectively, of dietary protein by a nonspecific nitrogen source, a mixture of glycine and diammonium citrate. The third experiment, with 10 subjects, explored the effects of 25% isonitrogenous replacement of milk protein by a mixture of nonessential amino acids (table 3). The dilution period lasted for 20 or 21 days after which the subjects were returned to the basal diet for a period of 7 to 10 days. After the second basal period the experiment was terminated.

Handling and analyses of samples. Urine was collected for complete 24-hour periods. Total dietary nitrogen, urinary nitrogen, and creatinine were analyzed as described previously (9, 10). Urea nitrogen (10) and total sulfur (18) were measured on pooled urine samples and serum total protein determined (19). On the final day of each dietary period a blood sample was taken at 8 a.m. after an overnight fast (9 to 10 hours), and plasma free amino acids measured by ion exchange chromatography using a Technicon Amino Acid Analyzer. The plasma was prepared for analysis as described by Mechanic et al. (20). Tryptophan was determined according to the procedure of Duggan and Udenfriend (21). Results for urinary nitrogen were evaluated by the "t" test and by the paired "t" test for plasma amino acids.

RESULTS

Total urinary nitrogen excretion during the experimental periods of both the 20 and 25% replacement of milk protein by glycine and diammonium citrate is given in table 4. For the 20% replacement (experiment 1) no significant increase in N excretion was observed in any of the 7 subjects at this dilution when compared with the initial basal period. However, 3 subjects showed a significantly lower urinary N excretion during the final basal period than during the initial basal period.

During the 25% dilution period (experiment 2), 3 of the 7 subjects showed a significantly higher urinary N excretion compared with the first basal period, and most of the subjects showed a decreased rate of urinary nitrogen excretion when they were returned to the basal diet at the end of the experiment (table 4). The increased N retention during this final period, as compared with the dilution period, was statistically significant ($P < 0.01$) in 4 of the 7 subjects (table 4). The results suggest that the N of the diet diluted 20% with glycine and diammonium citrate was not as well utilized as that of the 20% diluted and basal diets. The decreased urinary N excretion during the second basal period of the experiment compared with the first period suggests that dilution of the milk

protein may have resulted in a depletion of the body's pool of an essential amino acid(s) during the period of replacement by nonspecific nitrogen.

As summarized in table 5, urea excretion followed the same trends as total urinary N excretion during the experimental periods of experiments 1 and 2. Subjects fed a 25% dilution diet showed a higher rate of urea N excretion during the dilution period, and most of them showed a lower urea N excretion upon return to the undiluted diet. The results for total urinary S excretion and the total urinary N/S ratio are also given in table 5. Total sulfur excretion was decreased by about 10% during the 25% dilution period compared with the first basal period, and for some subjects sulfur excretion tended to return to initial levels during the final period on the basal diet.

Subject D.K. showed a substantially higher total urinary S excretion during the 20% dilution period (table 5) and a markedly decreased total S excretion when returned to the undiluted diet despite opposite trends in total S-amino acid intake. This subject also showed a significant increase in N retention when returned to the basal diet during the final period of the study. There may have been an interaction between the nonspecific N source and the utilization of S-amino acids in this individual. Moreover, 3 of

the 4 subjects in experiment 2 (P.D., K.E., M.S.) showing lower total urinary N output during the second basal period also showed a decrease in total urinary S output. These results suggest that above a certain level which varies with individuals, the dilution of milk protein with glycine and diammonium citrate as a nonspecific N source influences the utilization and metabolism of the sulfur amino acids which are limiting in this protein (1).

The results given in tables 4 and 5 show that total N and urea N output increased during a 25% dilution with glycine and diammonium citrate and decreased to a variable degree when subjects were returned to the basal diet. The question remained as to whether the glycine and diammonium citrate mixture was the most effective source of nonspecific N. Accordingly, a mixture of nonessential amino acids (table 3) was tested at the 25% level of dietary protein dilution. In this third experiment (table 6) only 2 of the 10 subjects showed a statistically significant decrease in urinary N output following return to the basal diet after the 3-week period of 25% dilution with the NEAA mixture. Three subjects showed a lower urinary N excretion during the second basal period as compared with the initial basal period. Subject K.E. previously showed a similar response following a 25% dilution of the dietary N with

glycine and diammonium citrate (table 4).

Urinary N output during the dilution period did not increase significantly above that of the initial basal period, except in the case of 2 subjects. However, in 1 case (I.S.) a relatively low rate of excretion was observed during the first basal period, and during the second basal period urinary N output was the same as that during the dilution period. The low initial basal level of N excretion was probably atypical but the reason is unknown.

The results for urea N, and total urinary S excretion for the 10 subjects given nonessential amino acids as the source of nonspecific N are summarized in table 5. During the dilution period, total S excretion was lower than during the initial basal period. The mean decrease in total urinary S excretion was slightly greater during this period than in the corresponding period of experiment 2 involving use of glycine and diammonium citrate.

In contrast to results obtained in experiment 2 (table 5), total urinary S excretion did not appear to decrease further during the second basal period for K.E., D.H., and J.R. These 3 subjects showed increased N retention in the final basal period as compared with the initial basal period. In experiment 2 with glycine and

diammonium citrate, a lower S output was observed during the second basal period for the 3 subjects (P.D., K.E., M.S.) who showed increased N retention after the 20-day period of dilution. These results suggest that the mixture of NEAA may be a slightly more effective source of nonspecific N for the isonitrogenous replacement of skim milk protein than glycine and diammonium citrate.

Serum total protein and the concentrations of free amino acids determined for subjects studied at a 25% level of replacement with the 2 sources of nonspecific N are summarized in table 7. No significant differences ($p > 0.05$) in the concentrations of these blood constituents were detected. During dilution with nonessential amino acids (experiment 3) only alanine increased, but because of high variability this change was not statistically significant.

DISCUSSION

The results of urinary nitrogen excretion in the present experiments indicate that for most human subjects, milk protein, when fed at approximately the minimum

requirement level (0.38 g protein/kg/day), can be replaced with 20% of nitrogen from nonspecific nitrogen sources without affecting its nutritive value. The extent of replacement possible varied with the subjects from about 20% to at least 25%; this may reflect variability in the requirement for individual essential amino acids (5, 6). The influence of dilution appeared to be partially affected by the source of nitrogen utilized for the replacement of milk protein nitrogen, because glycine and diammonium citrate resulted in a greater increase in urinary N excretion during the 25% dilution period than the isonitrogenous mixture of nonessential amino acids.

Kofranyi and Jekat (22) in their study with adult subjects concluded that only 10 to 15% of milk protein could be replaced with ammonium citrate without a change in the value of the protein. The difference between their results and ours may be due to their limited number of subjects and use of ammonium citrate alone as the nonspecific N source. Clark et al. (13) observed in young adults given 9.0 g nitrogen/day (of which approximately half was provided by the supplementary nonspecific nitrogen) that N retention was depressed when diammonium citrate was fed, but not when glycine alone was given. Although none of the 7 young adults in the present study appeared to retain less nitrogen when 20% of milk protein was replaced with the glycine and

diammonium citrate mixture, 3 appeared to show additional retention when returned to the basal diet. This latter observation may mean that an amino acid depletion had occurred which was not apparent from measuring urinary N excretion alone during the dilution period. However, this possibility was not reflected by a reduction in the concentration of essential amino acids in fasting plasma samples. The results suggest that milk protein can be replaced to a greater extent in most young adults than indicated by the study of Kofranyi and Jekat (22).

Snyderman et al. (2) studied infants from 3 weeks to 5 months of age and found that weight gain and nitrogen balance could be maintained despite a partial substitution of evaporated cow's milk protein by glycine or urea. Although their experiments were not designed to determine the extent to which milk protein nitrogen could be replaced by nonessential nitrogen, it appears from their data that 20% of cow's milk protein could be isonitrogenously replaced by either glycine or urea without reducing infant growth. However, this is probably an overestimate since the level of protein intake in their study was above minimum requirements for growth (23).

In establishing amino acid requirements of man, investigators have used different sources of supplemental nitrogen (11-14). Swendseid et al. (24) have reported

that glycine alone is not well utilized by man as a nonspecific N source; however, they have observed that a combination of glycine and diammonium citrate was as effective a source of nonspecific N as a mixture of nonessential amino acids. Experiments with weanling rats have shown that different sources of nonspecific nitrogen can affect growth rate in the presence of minimal amounts of essential amino acids (25, 26). Swendseid et al. (27) have shown that the growth rate of rats may be depressed by a low protein diet supplemented with 7.5% glycine. The results of our study with young men suggest that there may be some difference in the utilization of glycine and diammonium citrate compared with a mixture of nonessenti l amino acids when included as nonspecific nitrogen supplements in a diet supplying N at approximately the minimum requirement.

In experimental animals glycine alleviates the growth-depressing effect of a high methionine intake (28, 29) and conversely, methionine protects against excess glycine (30). Sulfur amino acids are limiting in cow's milk protein and therefore, under conditions of a relatively low intake of these amino acids in the present studies, a comparison of the effects of glycine-diammonium citrate and a mixture of nonessential amino acids on urinary nitrogen excretion was considered

appropriate. Although a close relationship between urinary nitrogen and sulfur excretion has been noted by a number of investigators (31, 32), a difference in the pattern of excretion between these elements has also been reported (33). Our results with subjects who reacted to a 20 or 25% dilution with glycine-diammonium citrate by showing changes in urinary sulfur excretion suggest that this nitrogen source may affect S-amino acid metabolism at the level of protein intake used in this study. Further studies are required to evaluate more fully the significance of these observations.

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FOOTNOTES

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[2]This manuscript is contribution no. 1385 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

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[4]Present address: Faculty of Medicine, Chiengmai Hospital, Thailand.

ABSTRACT Skim milk protein was replaced isonitrogenously by glycine and diammonium citrate, or by a mixture of nonessential amino acids in the test diet of 21 male college students; the effect of this replacement on urinary nitrogen and sulfur excretion and on fasting plasma amino acid concentration was studied. The subjects were fed a constant nitrogen intake equivalent to 0.38 g protein/kg body weight. Skim milk protein furnished 90% of the daily protein; oatmeal furnished the remainder. In experiments 1 and 2, glycine and diammonium citrate replaced 20 and 25% of total dietary nitrogen, respectively. In the third experiment, a mixture of nonessential amino acids replaced 25% of dietary nitrogen. The 20% replacement with glycine and diammonium citrate did not increase urinary nitrogen excretion in any of the 7 subjects, but 3 of the 7 showed lower urinary nitrogen excretion after return to the basal diet. A 25% replacement with glycine and diammonium citrate increased urinary nitrogen excretion in 3 of 7 subjects and 4 subjects showed lower nitrogen output during the final basal period. The 25% replacement with nonessential amino acids did not change urinary nitrogen excretion but 3 of 10 subjects showed a variable decrease in nitrogen excretion during the second basal period. As a replacement for 25% of total dietary protein, nonessential amino acids appeared

a more effective nonspecific nitrogen source than glycine and diammonium citrate. Fasting plasma amino acid levels were unchanged during the experimental periods.

TABLE 1

Age, height, weight, and caloric intakes of young men studied for the effects of nonspecific nitrogen replacement of milk protein

Exp.	Subj.	Age	Height	Body weight Beginning	Body weight End	Caloric intake[1]
		yr	cm	kg		kcal/kg body weigh
1	W.D.	21	183	69.4	68.8	47
	W.I.	19	165	66.6	64.4	52
	D.K.	23	183	67.1	67.7	47
	C.K.	23	170	60.0	59.5	44
	R.R.	19	180	74.6	70.5	42
	L.D.	21	173	77.7	77.1	40
	R.B.	20	173	85.4	85.0	34
2	P.D.	19	181	72.1	71.2	51
	K.E.	24	180	76.4	76.9	44
	N.D.	19	162	61.3	62.6	42
	T.J.	27	175	68.0	69.4	49
	J.R.	22	183	75.1	75.7	45
	M.S.	18	177	71.4	72.6	45
	R.W.	23	179	74.1	74.0	49
3	K.E.	24	180	82.4	83.5	42
	D.H.	20	167	84.2	84.1	42
	J.L.	19	181	78.5	78.9	42
	S.M.	22	163	71.0	71.2	45
	J.R.	22	183	77.7	78.4	44
	E.R.	19	177	77.7	77.4	46
	G.S.	23	185	88.1	88.8	50
	I.S.	20	187	65.6	67.5	48
	L.D.	22	173	77.1	75.9	42
	W.I.	19	165	69.1	67.5	50

TABLE 1 (continued)

[1] Calculated from food composition tables (Watt, B.K. and A.L. Merrill. 1963. Composition of Foods. Agriculture Handbook 8, Washington, D.C.) and data supplied by the manufacturers of some of the dietary components.

TABLE 2

Composition of Diet Given to Young Men Studied for the Effects of Replacement of Milk Protein with Nonspecific Nitrogen

Ingredient	Amount/day[1,2]
	g
Nonfat dried skim milk[3]	65.6
Oatmeal[4]	110.8
Dexin[5]	130.0
Oil[6]	140.0
Vanilla	10.0
Lemon juice	5.0
NaCl	1.0
Avicel[7]	5.0
K_2HPO_4	3.5
H_2O	500.0

[1]Amount for a 70 kg subject. Intake for each subject adjusted according to body weight.

[2]In addition, corn starch cookies and Danish dessert (corn starch, sucrose, and artificial flavoring and coloring), and carbonated beverages were given according

to individual caloric needs. Supplemental iron (1 tablet supplying 14 mg Fe, kindly supplied by Smith, Kline and French, Philadelphia) and a Unicap (Upjohn Co., Kalamazoo, Michigan) multivitamin capsule were given daily to all subjects. Each capsule is stated to contain (mg): A, 1.5; D, .012; B_1-HCl, 2.5; B_2, 2.5; C, 50; Nicotinamide, 20; Ca pantothenate, 5; pyridoxine hydrochloride, 0.5; cobalamin, 0.002. Subjects were also allowed Trident Gum (donated by American Chicle Co., New York). Five or six salt tablets, each supplying 1 g NaCl, were given to provide additional sodium required.

[3]Carnation Co., Van Nuys, California.

[4]Dried, cooked Buckeye Homogenized Rolled Oats, kindly supplied by Quaker Oats Co., Barrington, Illinois.

[5]Bouroughs Wellcome and Co., Tuckahoe, New York. Approximate composition: dextrins 75%; maltose 24%.

[6]Corn Oil, Mazola, Best Foods Division, Corn Products Co., New York, New York.

[7]F.M.C. Corporation, Marcus Hook, Pennsylvania. Microcrystalline alpha cellulose.

TABLE 3

Composition of mixture of nonessential amino acids used to dilute milk protein

Amino acid	% by weight
L-arginine HCL	6.15
L-histidine HCL, H_2O	4.44
L-alanine	5.80
L-aspartic acid	12.28
L-glutamic acid	39.26
Glycine	3.33
L-proline	18.72
L-serine	9.93
Total	100.

TABLE 4

Effect of Dilution of Milk Protein with Glycine and Diammonium Citrate on Urinary Nitrogen Excretion (g/day) by Young Men (experiments 1 and 2).

ubj.	N Intake	% Dilution of Dietary Protein			
		0	20	25	0
	g/day				
W.D.	4.22	3.92±0.65(6)[1]	4.09±0.86(13)	----	4.14±1.08(7)
W.I.	4.08	4.61±0.38(5)	4.08±0.52(11)[a*]	----	3.70±0.44(7)[a*]
D.K.	4.09	4.25±0.54(9)	4.14±0.39(11)	----	3.59±0.36(7)[b]
C.K.	3.36	2.69±0.67(6)	2.89±0.32(8)	----	2.98±0.42(6)
R.R.	4.56	3.76±0.44(7)	4.26±0.60(9)	----	3.89±0.52(6)
L.D.	4.80	5.36±0.33(8)	5.50±0.23(9)	----	5.09±0.67(9)
R.B.	4.91	4.43±0.44(7)	4.48±0.48(8)	----	3.74±0.38(9)[b]
I.D.	4.39	4.11±0.16(5)	----	4.67±0.30(9)[a**]	4.02±0.32(10)[1]
K.E.	4.65	4.42±0.33(8)	----	4.51±0.22(10)	3.95±0.47(10)
N.D.	3.73	3.14±0.27(6)	----	3.52±0.52(10)	3.19±0.34(9)
T.J.	4.14	3.49±0.22(5)	----	3.67±0.30(6)	3.37±0.24(10)
J.R.	4.57	4.12±0.17(5)	----	4.66±0.33(9)[a**]	4.16±0.37(9)[b]
M.S.	4.35	3.44±0.30(6)	----	4.27±0.33(11)[a**]	3.52±0.28(9)[b]
R.W.	4.51	5.07±0.50(7)	----	4.88±0.34(11)	4.65±0.60(10)

Mean ±Standard Deviation for number of observations shown in parentheses.

Total length of dietary periods were 14-16, 20, and 7-10 days for the 0%, 20 or 25%, and 0% dilution periods, respectively.

Significantly different from first basal period.

TABLE 4 (continued)

[b] Significantly different from dilution period.

* $P < 0.05$

** $P < 0.01$

TABLE 5

Effect of dilution of milk protein with nonspecific nitrogen on urinary urea, sulfur (g/day) and the total N/S ratio in young men[1]

		% Dilution of protein by glycine and diammonium citrate								
		0			20			0		
Exp.	Subj.	Urea N	S	N/S	Urea N	S	N/S	Urea N	S	N/S
1	W.D.	2.3	.255	15.4	2.9	.292	14.0	2.2	----	----
	W.I.	3.1	.351	13.1	2.4	.285	14.3	2.2	.351	10.5
	D.K.	2.7	.351	12.1	2.3	.516	8.0	1.4	.123	29.2
	Mean	2.7	.319	13.5	2.5	.364	12.1	1.0	.237	19.9
2		0			25			0		
	P.D	2.8	.357	11.5	3.1	.324	14.4	2.2	.264	15.2
	K.E.	2.9	.348	12.7	3.0	.318	14.2	2.3	.281	12.8
	N.D.	2.2	.224	14.0	2.4	.198	17.8	1.9	.216	14.8
	T.J.	2.7	.324	10.8	2.8	.246	14.9	2.0	.240	14.0
	J.R.	2.9	.333	12.4	3.5	.273	17.1	2.5	.297	14.0
	M.S.	1.9	.252	13.6	2.4	.252	16.9	2.2	.221	15.9
	R.W.	3.0	.369	13.7	3.5	.342	14.3	3.5	.363	12.8
	Mean	2.6	.315	12.7	3.0	.279	15.7	2.4	.260	14.2
	Std Dev	0.4	.055	1.2	0.5	.051	1.5	0.5	.050	1.2

[1]Analysis on pooled urine samples for periods shown in tables 4 and 6

TABLE 5 (continued)

Exp. 3		% Dilution of protein by nonessential amino acids								
		0			25			0		
Exp.	Subj.	Urea N	S	N/S[2]	Urea N	S	N/S	Urea N	S	N/S
	K.E.	2.6	.327	13.1	3.0	.285	15.6	2.6	.321	11.9
	D.H.	3.6	.423	12.3	2.9	.342	12.8	3.6	.384	11.5
	J.L.	3.0	.393	11.2	2.9	.282	15.2	2.7	.339	11.8
	S.M.	2.5	.282	13.9	2.6	.216	17.3	2.6	.276	13.9
	J.R.	2.7	.303	14.0	3.0	.306	13.7	2.6	.297	12.8
	E.R.	2.5	.303	12.4	3.0	.261	15.9	3.1	.297	14.1
	G.S.	2.4	.324	14.9	2.9	.273	16.3	2.1	.318	11.6
	I.S.	1.5	.294	9.4	3.2	.336	12.8	3.5	.318	14.3
	L.D.	3.2	.411	11.4	3.3	.363	12.6	3.3	.375	12.3
	W.I.	2.0	.341	11.8	2.3	.285	11.5	2.5	.341	11.3
	Mean	2.6	.339	12.4	2.9	.295	14.4	2.9	.326	12.5
	Std Dev	0.6	.050	1.6	0.3	.042	1.9	0.5	.033	1.1

[2]Ratio of total urinary nitrogen to total urinary sulfur. Values for total nitrogen taken from tables 4 and 6.

TABLE 6

Effects of dilution of milk protein with a mixture of nonessential amino acids on urinary nitrogen excretion by young men (Exp. 3)

Subject	N intake	% of dilution of dietary protein		
		0	25	0
	g/day	g N/day		
K.E.	5.01	4.30±0.20(8)[1]	4.46±0.21(8)	3.84±0.32(9)[a**b]
D.H.	5.12	5.22±0.58(8)	4.38±0.43(5)[a*]	4.40±0.44(7)[a**]
J.L.	4.77	4.42±0.33(7)	4.28±0.33(9)	4.01±0.69(9)
S.M.	4.32	3.93±0.36(10)	3.74±0.38(10)	3.85±0.60(9)
J.R.	4.73	4.25±0.20(6)	4.19±0.33(9)	3.80±0.31(7)[a**]
E.R.	4.73	3.77±0.96(10)	4.14±0.46(9)	4.19±0.57(9)
G.S.	5.38	4.38±0.67(12)	4.45±0.80(9)	3.70±0.73(9)
I.S.	3.99	2.78±0.10(8)	4.29±0.55(9)[a**]	4.55±0.45(9)[a**]
L.D.	4.77	4.70±0.45(6)	4.56±0.66(8)	4.61±0.65(8)
W.I.	4.16	4.04±0.62(5)	3.27±0.47(6)	3.85±0.44(7)

[1]Mean ± standard deviation for number of observations shown in parentheses. Total length of 0, 25, and 0% dietary periods were 16-18, 20-21, and 9 days, respectively.

[a]Significantly different from first basal period.

[b]Significantly different from dilution period.

*$p < 0.05$.

**$p < 0.01$.

TABLE 7

Serum total protein and free amino acids in fasting plasma of young men studied for effects of dilution of milk protein with nonspecific nitrogen

Amino acid	% Dilution of protein					
	0	25	0	0	25	0
	Nonessential amino acids			Glycine and DAC		
Essential						
Threonine	186±24[1]	166±42	174±33	204±31	211±26	181±26
Valine	198±25	219±64	210±26	205±29	174±32	182±40
Isoleucine	76±14	88±27	77±7	86±12	65±6	63±12
Leucine	124±15	146±44	134±18	134±25	115±20	112±23
Tyrosine	62±9	68±24	71±10	77±22	57±11	55±13
Phenylalanine	63±7	67±19	62±5	64±14	55±8	55±11
Lysine	187±36	222±75	201±27	198±28	164±24	179±29
Tryptophan	65±8	72±12	74±9	61±7	57±10	63±12
Nonessential						
Aspartic	67±13	65±15	52±13	70±10	70±8	64±7
Serine and Glutamine	180±52	232±119	189±66	149±68	188±22	169±20
Glutamic	313±38	334±37	312±73	341±50	309±55	289±40
Glycine	307±59	308±123	294±25	335±25	401±51	312±56
Alanine	666±136	809±289	643±188	557±115	490±127	501±118
Histidine	120±10	122±30	117±26	117±21	92±12	100±4
Arginine	99±32	130±24	106±20	121±47	91±19	74±[illegible]
Serum Protein (g/100ml)	7.1±.4	7.2±0.2	7.3±0.4	7.0±0.3	6.9±0.3	7.0±0.[illegible]

TABLE 7 (continued)

1. μmoles per liter. Mean ± standard deviation for samples taken at 8:00 a.m. after a 9-10 hour fast at the end of each period.

APPENDIX I

SUPPLEMENTARY STUDIES IN RATS OF THE EFFECTS OF DILUTION OF MILK PROTEIN WITH NON-SPECIFIC NITROGEN SOURCES

A limited number of experiments, described below, were conducted in rats to extend our observations made in children and young adult males on the effects of dilution of milk protein by non-specific nitrogen sources.

The results suggest that both weanling and young adult rats are more sensitive to the dilution of milk protein than are children or young adult men. The differential response of human subjects and rats to dilution of milk protein N by non-specific nitrogen may be related to differences in the requirements for the sulfur amino acids in these two species. This hypothesis requires further experimentation.

The results of the experiments obtained with rats have been submitted for publication in the Journal of Food Science. Our results confirm and extend those of Daniel, Desai, Venkat Rao, Swaninathan and Parpia (J. Food Science 33: 432, 1968) also obtained with rats given cow's milk and egg proteins.

SUMMARY OF RAT STUDIES

A number of experiments studied growth of weanling rats and urinary nitrogen excretion in young adult rats when cow's milk protein was partially replaced by varying levels of non-specific nitrogen. A mixture of nonessential L-amino acids (NEAA) or a mixture of diammonium citrate and glycine (DAC-Gly) was used as a source of non-specific nitrogen. Substitution of the 15% milk protein diet to the extent of 10% slightly reduced growth; significant growth reduction occurred with substitutions of 20% and greater. Supplementation of the diets containing 10.5% milk protein and non-specific nitrogen with sulfur amino acids did not restore growth to the maximum rate obtained with the 15% milk protein diet. Additional supplementation with tryptophan further improved growth slightly but not to the maximum rate. Several other essential amino acids, alone or in combination, had no apparent effect. Based on urinary nitrogen excretion, comparable results were obtained with young adult rats by substituting non-specific nitrogen for milk protein in the diets. The reduced performance following substitution of milk protein with non-specific nitrog n may be due partly to decreased utilization of sulfur amino acids and possibly to decreased utilization of all essential amino acids.

INTRODUCTION

When essential amino acids form the sole dietary source of protein for rats, a lower growth rate is observed than when the same level of dietary N is supplied by a well-balanced amino acid mixture containing approximately equal amounts of essential and nonessential amino acids (Rose *et al.*, 1948; Stucki *et al.*, 1962; Young *et al.*, 1968). Animal proteins of high biological value may contain some or all of the essential amino acids, expressed per unit of total protein nitrogen, in excess of required amounts for the rat; Bender (1965) suggested that egg protein contains a surplus of 10-20% of all essential amino acids. In this case, essential amino acids are catabolized to meet calorie needs or are used for synthesis of nonessential amino acids and other N-containing compounds. Therefore, isonitrogenous replacement of the excess essential amino acids with non-specific nitrogen (from nonessential amino acids or other utilizable N sources) should not decrease dietary N utilization and animal performance. For man, studies in this laboratory (Scrimshaw *et al.*, 1966; Huang *et al.*, 1966) and in others (Snyderman *et al.*, 1962; Kofranyi *et al.*, 1964) suggest that high quality protein N can be partially replaced with non-specific nitrogen without reducing nitrogen retention.

In the present series of experiments, weanling rats were fed dried skim milk as the source of dietary protein. A mixture of nonessential L-amino acids (NEAA), patterned as in cow's milk, or a mixture of diammonium citrate and glycine (DAC-Gly) was used as non-specific nitrogen to replace part of the protein nitrogen. The influence of the protein nitrogen replacement on growth rate was studied. Some experiments evaluated the effects of using several essential amino acids to supplement the diets containing the non-specific N sources.

We also evaluated the influence of age because available data indicate that for both man (Rose, 1957; Holt *et al.*, 1965) and rats (Rama Rao *et al.*, 1964; Smith *et al.*, 1967) the requirements for essential amino acids per g total nitrogen differ in young and adults.

EXPERIMENTAL AND RESULTS

Methods. A preliminary experiment (Table 1) confirmed general experience that maximum growth rates are achieved with a diet containing 15% protein from unsupplemented skim milk. This concentration of dietary protein was used, therefore, as a control level in the growth studies. The net protein utilization (NPU) of skim milk protein by rat assay (Bender *et al.*, 1957) was 71±2 compared with 63±2 for unsupplemented casein as a standard.

-Table 1-

Male weanling rats (Charles River Laboratories, North Wilmington, Mass., CD strain) were fed the experimental diets *ad libitum* for 14 days. The basal diets contained: dried skim milk[1], 28.1 to 50.5% (10-18% protein); corn oil, 10%; salt mixture, 5% (Rogers *et al.*, 1965); vitamin mixture, 0.5% (Rogers *et al.*, 1965); choline chloride, 0.2%; and dextrin:sucrose (2:1 weight ratio) to make a total of 100%. In all diets lactose was partially substituted for the dextrin:sucrose mixture to achieve the lactose level present in the control diet (15% skim milk protein). Weekly weight gain and daily food intake were recorded.

The essential amino acid content of the basal diet, calculated from the data of Orr *et al.* (1957), and the essential amino acid requirements for rat growth as reported by Rama Rao *et al.* (1964) are given in Table 2.

-Table 2-

The diet appears to meet all requirements although it is borderline for the sulfur amino acids, methionine and cystine. In some experiments methionine and cystine[2] were added in a ratio by weight of 3 to 1 in order to reach a calculated sulfur amino acid content of 0.65% in the diet.

Experiments 1 and 2. Experiment 1 was designed to determine the extent to which a mixture of NEAA could

isonitrogenously replace a 15% milk protein diet without affecting growth in weanling rats. Body weight gains are summarized in Table 3. A highly significant ($P<0.01$) decrease in growth, PER, and food efficiency was observed only for the 30% substitution in this experiment.

-Table 3-

Therefore, a second experiment was conducted with larger groups of rats to evaluate the effect of 20% and 30% replacements of milk protein N by two sources of non-specific nitrogen; the results are summarized in the lower half of Table 3. Significant ($P<0.01$) decreases in growth rate, FE, and PER occurred for both sources of nitrogen at the two substitution levels.

Experiment 3. Because the sulfur amino acids, methionine and cystine, are limiting in cow's milk protein for weanling rats, a third experiment was conducted to determine the influence of DAC-Gly substitution of milk protein N in diets supplemented with sulfur amino acids. Table 4 summarizes these results. Comparison of groups 5 and 9

-Table 4-

reveals that inclusion of DAC-Gly in the diet resulted in a lower weight gain ($P<0.05$) than when the diet contained 10.5% milk protein alone. This effect appeared to be related to the reduced food intake, particularly because efficiency of food conversion was similar for both groups.

Growth rates were similar for diets containing 10.5% milk protein, with or without added DAC-Gly, supplemented with 0.15-0.28% sulfur amino acids (compare group 6 vs 10, 7 vs 11). The highest level of S-amino acid supplementation reduced body weight gain significantly when the diet contained DAC-Gly (group 8) but not when the only other dietary N was milk protein (groups 4 and 12). These findings suggest that the DAC-Gly mixture affected utilization of sulfur amino acids at the high and possibly at the low dietary sulfur amino acid levels. Although addition of 0.15-0.28% S-amino acids to 10.5% milk protein diets containing DAC-Gly allowed weight gains which were similar to the 10.5% milk protein diets without the added non-specific nitrogen, growth rate remained lower than that on the 15% milk protein diet which supplied the same level of total N and was supplemented with sulfur amino acids.

Experiments 4 and 5. A study was conducted with addition of various essential amino acids to diets containing 30% of total dietary N from the NEAA mixture. The added amino acids achieved the concentrations calculated to be present in the 15% milk protein diet. Arginine, lysine, or threonine addition to the diets (Expt. 4, Table 5) failed to improve weight gain significantly.

-Table 5-

The effects of adding tryptophan, tryptophan and threonine, or a combination of several essential amino

acids, were also studied (Expt. 5, Table 5). Tryptophan significantly ($P<0.05$) improved the growth rate, mainly as a consequence of higher food intake (group 13). Addition of tryptophan and threonine together or in combination with arginine and lysine did not result in a higher growth rate than that achieved with tryptophan alone.

Experiment 6. DAC-Gly replaced from 10-50% of skim milk protein N while concentrations of 0.65% sulfur amino acids and 0.21% tryptophan were maintained as in the control diet. The results, summarized in Table 6, show that a 10% replacement of milk protein produced a small but

-Table 6-

nonsignificant ($P>0.05$) decrease in growth rate of weanling rats. A significant reduction ($P<0.01$) in growth rate did occur when 20% of the protein was replaced by DAC-Gly; proportionately greater reductions were observed with still higher rates of replacement by DAC-Gly despite the unchanged amounts of sulfur amino acids and tryptophan in the diet.

Experiment 7. In young adult rats isonitrogenous replacement of milk protein with the DAC-Gly mixture was studied by monitoring changes in urinary nitrogen excretion. The basal diet contained 4% milk protein, while the remaining dietary components were similar to those in

previous experiments. Lactose was maintained at the basal (4% milk protein) level in all diets. Except for sulfur amino acids, the basal diet contained concentrations of essential amino acids in excess of those suggested by Smith et al. (1967) for maintenance in young adult rats. These authors suggested 0.29% for the total sulfur amino acids, whereas the present 4% milk protein diet supplied 0.13%. However, with a 30% DAC-Gly substitution, isoleucine and threonine also were probably deficient.

The rats, housed individually in metabolic cages, were given 10g of the basal diet daily for an initial 10-day adaptation period followed by a 10-day control interval (Period 1). For 10 days (Period 2) after the control phase, group 1 was maintained on the basal diet, while groups 2, 3, and 4 were given diets in which the DAC-Gly mixture replaced 10, 15, and 20% of the milk protein, respectively. During the final 10 days (days 31-40, Period 3) all groups were returned to the basal 4% milk protein diet. On day 11 of the experiment, daily urine collections began. Two 5-day pooled samples of urine were prepared for each period and analyzed for nitrogen by the Kjeldahl method. Rats were weighed at the beginning of the study (mean weight 200g) and at the end of each dietary period. They showed little change in weight during the entire 40-day span.

-Table 7-

Table 7 shows that 15 or 20% substitution of milk protein with DAC-Gly resulted in a significant increase ($P<0.01$) in levels of urinary nitrogen excretion during the first five days of Period 2 (days 21-25). During the last five days of this period, urinary nitrogen excretion decreased for groups 2 and 3 (10 and 15% substitution) but was still high for group 4 (20% substitution). When the rats were returned to the basal diet (Period 3) a sharp reduction in urinary N output occurred in groups 2-4, indicating improved retention of dietary nitrogen on the basal diet as compared with partial replacement of skim milk protein N by non-specific nitrogen. Urinary nitrogen excretion during the last five days of Period 2 was significantly greater ($P<0.01$) for rats fed the diet containing 20% of N from DAC-Gly compared with other groups. However, the level of urinary nitrogen excretion was not significantly higher ($P>0.05$) for rats fed diets with the 10 and 15% substitution than for rats given the basal diet throughout (group 1). In contrast to these results, the reduced urinary N excretion following return to the basal diet was highly significant ($P<0.01$) for all groups given DAC-Gly. The control group (group 1), maintained throughout the experiment on the basal diet, did not show a significant change in urinary N excretion ($P>0.1$) between Periods 2 and 3.

Experiment 8. Because the maintenance requirement of young adult rats for S-amino acids (methionine and cystine) may be higher than the amount supplied by the basal diet (Smith et al., 1967), the effect of partial replacement of milk protein nitrogen by DAC-Gly was studied while a constant dietary level of S-amino acids and tryptophan was maintained. In this study, the basal diet and 20% and 30% levels of substitution were utilized. The experimental design was similar to that of Experiment 7, except that six rats were used per dietary group. Table 8

-Table 8-

shows values of urinary nitrogen excretion for the last five days (15-20) of Period 1 during feeding of the basal 4% protein diet, for days 25-30 of Period 2, and for the first five days (days 31-35) of Period 3. The results show that even with the concentration of S-amino acids and tryptophan maintained at the level present in the basal diet, urinary nitrogen excretion increased for both levels of substitution with DAC-Gly. However, maintenance of the dietary S-amino acid and tryptophan levels appeared to result in a smaller urinary nitrogen increase following 20% replacement than was observed in the earlier experiment without supplemental dietary S-amino acids (Table 7).

DISCUSSION

Growth depression resulted from isonitrogenous substitution of cow's milk protein by non-specific nitrogen, either a mixture of nonessential L-amino acids, patterned as in cow's milk, or DAC-Gly.

Various investigators have examined the interrelationship between glycine and methionine. Glycine is known to alleviate the growth depressing effects of high levels of dietary methionine (Hardin et al., 1951; Roth et al., 1950; Benevenga et al., 1967; Wertz et al., 1968) and methionine protects against excess glycine (Wertz et al., 1968). In the present studies, DAC-Gly was utilized as a source of non-specific nitrogen. The additional dietary glycine may have limited the utilization of the sulfur amino acids and resulted in the lower growth rates achieved when DAC-Gly was added to the 10.5% milk protein diet. Growth rate was reduced to the same extent following the 20% or 30% substitutions of milk protein nitrogen by both DAC-Gly and the NEAA mixture. Also, in rats given the methionine-supplemented diets containing 10.5% milk protein plus non-specific nitrogen, growth rates remained lower than the rate of rats on the 15% milk protein diets with supplementary methionine and cystine. Therefore, in addition to possible alterations in sulfur amino acid metabolism, decreased utilization of all essential amino

acids may have been responsible for the reduced growth rates following replacement of the milk protein by the non-specific nitrogen.

The essential amino acid pattern of the test protein probably is an important factor modifying response to substitution of that protein by non-specific nitrogen. Braham et al. (1968) observed that addition of nonessential amino acids or DAC to egg protein did not influence weight gain of rats. Addition of either or both of these nitrogen sources to casein, soybean, or cottonseed protein decreased weight gain. These authors suggest that the response to nonessential amino acids or DAC depends largely on the essential amino acid pattern of the protein tested. Moran et al. (1967) observed a depression in egg production and weight gain of hens when DAC was added to a 10% protein diet of which half the protein was supplied by corn meal and half by soybean meal. Recently Daniel et al. (1968) have reported that addition of L-glutamic acid to diets containing 5.0 to 8.5% milk protein adversely affected growth rates of rats. On the other hand, Venkat Rao et al. (1964) showed that, although weight gain was proportional to the quantity of egg protein in the diet, addition of L-glutamic acid to the egg protein diets did not adversely affect growth rates in this case.

The present findings showed that tryptophan supplementati of the 10.5% milk protein diet containing added DAC-Gly

and sulfur amino acids resulted in a small improvement of growth rate. Addition of several other essential amino acids suspected as being limiting in the methionine-supplemented 10.5% milk protein diets, with or without added non-specific nitrogen, failed to improve growth rates. However, Swendseid *et al.* (1962), using an 8% casein diet supplemented with methionine, found that the growth depression resulting from addition of several sources of non-specific nitrogen disappeared after addition of tryptophan and threonine, except when glycine was the source of non-specific nitrogen. In the present study the higher growth rates of control rats fed the 15% milk protein diet, compared with the controls used by Swendseid *et al.* (1962), may explain the failure of tryptophan and threonine supplementation of diets containing the non-specific nitrogen to restore growth to the maximum rate achieved with the 15% milk protein diet.

A 10% substitution of milk protein N, with or without supplementary sulfur amino acids, only slightly and nonsignificantly reduced the growth rate in the present study. Proportionately greater, significant reductions were observed at higher levels of substitution. These results differ from those in children (Snyderman *et al.*, 1962) and adult men (Kofranyi *et al.*, 1964; Scrimshaw *et al.*, unpublished results), who apparently are able to

tolerate at least a 10-20% replacement of milk protein with non-specific nitrogen.

When urinary nitrogen excretion was the major parameter used for determining the effects of milk protein substitution by non-specific nitrogen in young adult rats, the results also indicated lessened N utilization (Tables 7 and 8). The increase in urinary nitrogen excretion was greater with higher levels of substitution. Th. s adverse effect on nitrogen balance was not prevented by maintenance of the S-amino acid and tryptophan content of the diets at the level present in the control 4% milk protein diet.

Substitution of the milk protein nitrogen by non-specific nitrogen sources at the 10 or 15% levels, without additional sulfur amino acids, resulted in increased urinary nitrogen excretion. However, a partial adaptation to protein replacement with DAC-Gly appeared to occur, as indicated by the decreased rate of urinary nitrogen excretion during the latter part of the replacement period. These results with young adult rats are, therefore, generally similar to results obtained with weanling rats.

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FOOTNOTES

1. Dried nonfat skim milk, Carnation Company, Van Nuys, California.

2. All L-amino acids used in these studies were purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

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Table 1. Effect of dietary level of skim milk protein on the fourteen-day growth rate of weanling rats.

Group no.	Dietary skim milk protein %	Food intake g/day	Weight gain[1] g/14 days	FE[2]	PER[3]
1	10	8.8	45.5 ± 3.3	36.9	3.69
2	12	8.7	56.4 ± 1.5	46.2	3.86
3	15	9.9	76.4 ± 4.0	54.8	3.67
4	18	9.6	77.9 ± 2.1	57.8	3.22

[1]Beginning weight of rats was 45 ± 2g. Weight gain is mean ± standard error for 8 rats per group.

[2]Food efficiency (FE) is (g gain per g food intake) x 100.

[3]Protein efficiency ratio (PER) is g gain per g protein consumed.

Table 2. Calculated % of essential amino acids in experimental diets compared with requirements for young growing rats.

mino acid	Experimental diets					Amino acids required[2]
		% of milk protein N replaced				
	Basal[1]	10	15	20	30	
	% of diet					
soleucine	0.96	0.86	0.82	0.77	0.67	0.55
eucine	1.47	1.32	1.25	1.18	1.03	0.70
ysine	1.17	1.05	0.99	0.94	0.82	0.90
-amino acids Methionine + cystine)	0.50	0.45	0.43	0.40	0.35	0.50
romatic amino acids Phenylalanine + tyrosine)	1.49	1.34	1.27	1.19	1.04	0.72
hreonine	0.69	0.62	0.59	0.55	0.48	0.50
ryptophan	0.20	0.19	0.18	0.17	0.15	0.11
aline	1.03	0.93	0.88	0.83	0.72	0.55
rginine	0.55	0.50	0.47	0.44	0.39	----
istidine	0.39	0.35	0.33	0.31	0.27	0.25

[1] Calculated values from data of Orr et al. (1957).

[2] From Rama Rao et al. (1964).

Table 3. Fourteen-day growth of weanling rats during isonitrogenous replacement of skim milk by nonessential amino acids or glycine and diammonium citrate.

	Level of milk protein in diet	Nitrogen source	% total dietary N from added N source	Food intake g/day	Weight gain g/14 days	FE %	PER
Expt. 1[1]	15.00	----	0	9.4	73.8 ± 4.0	56.1	3.74
	13.50	NEAA[2]	10	9.3	69.1 ± 2.2	53.1	3.54
	12.75	NEAA	15	9.4	66.8 ± 1.9	50.8	3.38
	12.00	NEAA	20	10.0	67.0 ± 2.3	47.9	3.19
	10.50	NEAA	30	9.3	56.0 ± 2.4	43.0	2.87
Expt. 2[3]	15.0	----	0	9.8	68.0 ± 2.4	49.6	3.30
"	12.0	NEAA	20	9.3	53.4 ± 2.2	41.0	2.73
"	12.0	AC-Gly[4]	20	9.9	56.8 ± 2.1	41.0	2.73
"	10.5	NEAA	30	8.9	48.3 ± 2.0	37.8	2.58
"	10.5	DAC-Gly	30	9.5	49.5 ± 2.5	37.2	2.48

[1]Beginning weight of rats was 36 ± 2 g. Weight gain is mean ± standard error for 8 rats per group.

[2]Amino acid (g) per 100g mixture: L-alanine, 6.5; L-aspartic, 13.72; L-glutamic, 44.03; glycine, 3.72; L-proline, 20.90; L-serine, 11.09.

[3]Beginning weight of rats was 48 ± 3 g. Weight gain is mean ± standard error for 14 rats per group.

[4]Diammonium citrate and glycine mixture. Each compound supplied equal amounts of N per unit weight of mixture.

Table 4. Fourteen-day growth of weanling rats on diets containing skim milk protein supplemented by S-amino acids, with and without added glycine and diammonium citrate (Experiment 3).

roup no.	Level of milk protein in diet %	% total dietary N from added DAC-Glycine	Methionine & cystine added[1]	% dietary methionine & cystine[2]	Food intake g/day	Weight gain[3] g/14 days	FE %	PER
1	15.0	--	----	0.50	10.3	73.3 ± 4.5	50.8	3.49
2	15.0	--	0.15	0.65	10.6	79.8 ± 3.2	53.8	3.55
3	15.0	--	0.28	0.77	10.6	81.1 ± 4.1	54.6	3.58
4	15.0	--	0.43	0.92	10.7	81.3 ± 3.1	54.3	3.52
5	10.5	30	----	0.35	8.9	49.0 ± 4.1	39.3	2.62
6	10.5	30	0.15	0.50	9.8	62.7 ± 5.1	45.7	3.02
7	10.5	30	0.28	0.65	11.1	73.3 ± 3.2	47.2	3.09
8	10.5	30	0.43	0.77	10.7	64.6 ± 2.1	43.1	2.79
9	10.5	--	----	0.35	11.2	61.0 ± 4.2	38.9	3.71
10	10.5	--	0.15	0.50	10.9	67.1 ± 3.5	44.0	4.13
11	10.5	--	0.28	0.65	11.3	73.7 ± 4.8	46.6	4.32
12	10.5	--	0.43	0.77	12.2	78.9 ± 5.1	46.2	4.23

[1] Methionine and cystine were added in a weight ratio of 3:1.

[2] Calculated values.

[3] Beginning weight of rats was 45 ± 3g. Weight gain is mean ± standard error for 7 rats per group.

Table 5. Fourteen-day growth of weanling rats on diets containing skim milk protein supplemented with sulfur amino acids, NEAA, and various essential amino acids.

Group no.	Level of milk protein in diet[1] %	% total dietary N from added NEAA mixture[2]	Essential amino acid added %		Food intake g/day	Weight gain[3] g/14 days	FE	PER
Experiment 4								
1	15.0	--	--------		12.1	85.6 ± 3.0	50.5	3.34
2	10.5	30	--------		12.1	68.4 ± 2.3	40.4	2.64
3	10.5	30	L-Arg.	0.12	12.3	71.0 ± 3.1	41.2	2.67
4	10.5	30	L-Lys.	0.13	11.9	69.0 ± 2.2	41.1	2.68
5	10.5	30	L-Threo.	0.10	12.3	75.4 ± 2.7	43.8	2.84
6	10.5	--	--------		12.2	70.4 ± 3.1	41.2	3.81
7	10.5	--	L-Arg.	0.12	13.4	74.9 ± 3.3	39.9	3.66
8	10.5	--	L-Lys.	0.13	13.6	70.2 ± 2.5	36.9	3.37
9	10.5	--	L-Threo.	0.10	12.2	71.8 ± 2.3	42.0	3.86

(continued)

Table 5, continued.

Group no.	Level of milk protein in diet[1] %	% total dietary N from added NEAA mixture[2]	Essential amino acid added %	Food intake g/day	Weight gain[3] g/14 days	FE %	PER
Experiment 5							
1	15.0	--	--------	11.7	80.7 ± 2.7	49.2	3.25
2	10.5	30	--------	11.3	65.4 ± 2.9	41.3	2.70
3	10.5	30	L-Threo. 0.10	11.1	63.0 ± 3.3	40.5	2.63
4	10.5	30	L-Try. 0.06	12.5	74.0 ± 4.8	42.3	2.75
5	10.5	30	L-Threo. 0.10	11.5	71.0 ± 4.2	44.1	2.85
			+ L-Try. 0.06				
6	10.5	30	L-Threo. 0.10	11.1	68.3 ± 4.2	44.0	2.80
			+ L-Try. 0.06				
			+ L-Arg. 0.12				
			+ L-Lys. 0.13				

[1]15% milk protein diets were supplemented with 0.15% methionine and cystine (3:1) and the 10.5% milk protein diets with 0.3%.

[2]Composition of the NEAA mixture is given in Table 3.

[3]Beginning weight of rats was 45 ± 3g. Weight gain is mean ± standard error for 8 rats per group in Experiment 4 and 7 rats per group in Experiment 5.

Table 6. Fourteen-day growth of weanling rats fed diets with different levels of DAC-Glycine replacement of skim milk protein supplemented with sulfur amino acids and tryptophan (Experiment 6).

Group no.	Level of milk protein in diet %	Amino acid added %	% total dietary N from added DAC-Glycine	Food intake g/day	Weight gain[1] g/14 days	FE %	PER
1	15.0	Met-Cys 0.15	0	11.2	84.8 ± 2.4	54.1	3.57
2	13.5	Met-Cys 0.20	10	11.1	[illegible] ± 2.5	52.3	3.43
		+ Try 0.02					
3	12.0	Met-Cys 0.25	20	10.3	68.4 ± 4.4	47.4	3.10
		+ Try 0.04					
4	10.5	Met-Cys 0.30	30	9.7	61.0 ± 2.3	44.9	2.92
		+ Try 0.06					
5	7.5	Met-Cys 0.40	50	8.3	35.0 ± 2.8	30.1	1.94
		+ Try 0.10					

[1]Beginning weight of rats was 55 ± 3g. Weight gain is mean ± standard error for 7 rat per group.

Table 7. Effect of 40 days' replacement of milk protein by DAC-Glycine on urinary nitrogen excretion in young adult rats (Experiment 7).

Days of expt.[2]	% of milk protein N replaced[1]			
	0 (1)	10 (2)	15 (3)	20 (4)
	Mg urine N per day[3]			
16-20	40.3 ± 1.2	42.8 ± 2.0	42.4 ± 1.6	40.4 ± 1.5
21-25	40.0 ± 1.0	43.7 ± 2.0	46.7 ± 1.6	45.9 ± 1.3
26-30	38.0 ± 1.4	39.7 ± 1.9	41.1 ± 1.2	44.5 ± 1.1
31-35	37.0 ± 0.9	35.6 ± 1.0	36.0 ± 1.1	36.0 ± 1.3
36-40	38.7 ± 0.8	35.6 ± 1.8	36.6 ± 1.3	37.3 ± 1.0

[1]Group number is in parentheses.

[2]During days 16-20 and 31-40 all rats were given the basal 4% milk protein diet. During days 21-30 groups of rats were fed the basal diet or a diet containing 10, 15, or 20% of milk protein N replaced with DAC-Glycine.

[3]Mean ± standard error for 10 rats per group.

Table 8. Urinary nitrogen excretion in young adult rats fed milk protein diets, supplemented with sulfur amino acids and tryptophan, following partial replacement with diammonium citrate and glycine for 40 days (Experiment 8).

Days of expt.[1]	% of milk protein N replaced		
	0	20	30
	Mg urine N per day[2]		
16-20	44.0 ± 1.1	42.3 ± 1.6	42.5 ± 1.7
25-30	40.9 ± 2.1	45.5 ± 2.3	47.8 ± 1.2
31-35	39.5 ± 1.6	38.9 ± 2.7	35.5 ± 1.8

[1]During days 16-20 and 31-35 all rats were given the basal diet and during days 25-30, rats were fed the basal diet or diets containing 20 and 30% of total dietary N from DAC-Glycine. All diets contained 0.13% methionine and cystine and 0.06% tryptophan.

[2]Mean ± standard error for 6 rats per group.

RECOMMENDATIONS

The results justify continuation of this study along two lines:

a. Demonstration that the contribution of milk to the protein value of a mixed diet is not diminished by isonitrogenous substitution by glycine and diammonium citrate at the 10% level. This is now being done at INCAP as part of the terminal stage of the present AID contract.

b. Additional studies in children one to four, to determine whether there are any real differences between children of this age and adults in their ability to use the nitrogen of a milk/glycine/diammonium citrate mixture either alone or as a component of a mixed diet. These again should be done initially at M. I. T. and confirmed in INCAP utilizing malnourished children.

c. Extension of the dilution studies to formula fed infants to be sure that a 10% level of dilution will not impair the protein value of milk protein for infants. There is now reason to believe that such a trial would be wholly successful.

d. With completion of the additional research indicated above, there is every reason to believe that the addition of non-specific nitrogen could be recommended as a safe and nutritionally effective way to increase the protein value of a given amount of dried skim milk for human feeding.

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