EFFECTS OF DIETARY SUPPLEMENTS IN PREVENTING OR AUGMENTING THE PRODUCTION OF CATARACTS IN RATS BY 1,4-DIMETHANE-SULFONOXYBUTANE [1]

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It has been reported by Solomon, Light and de Beer ('55) that busulfan, 1,4-dimethanesulfonoxybutane,[2] will produce cataracts when fed to growing rats. As reviewed by Shlaifer ('54), it is well known that the lens structure is susceptible to nutritional disturbances generated by certain vitamin deficiencies or high dietary levels of a particular monosaccharide, galactose. It was therefore decided to investigate the possibilities of altering the course of the 1,4-dimethanesulfonoxybutane cataract by modifying the diet through the addition of various substances capable of altering metabolic processes.

Busulfan was synthesized by Timmis ('50) during a search for anticarcinogenic chemicals (Haddow and Timmis, '53). It was found to inhibit the growth of Walker rat carcinoma no. 256 and to depress the number of circulating neutrophils. Clinical trials have shown that this compound is useful in the treatment of chronic myelocytic leukemia in humans (Galton, '53; Galton and Till, '53; Wintrobe et al., '54; Petrakis et

[1] Presented in part at the 20th annual meeting of the American Institute of Nutrition at Atlantic City, N. J., April, 1956. See Proceedings of the Federation of American Societies for Experimental Biology, *15:* no. 1, Part II, p. 738, 1956.

[2] Supplied as "Myleran" Brand Busulfan (1,4-Dimethanesulfonoxybutane), Burroughs Wellcome & Co., (U.S.A.), Inc. Tuckahoe 7, N. Y.

al., '54). These and other effects are similar to those following radiation treatment and 1,4-dimethanesulfonoxybutane has therefore been classed as a radiomimetic drug (Elson, '55; Bergel, '55). However, no cataracts attributable to the drug have been discovered in any other species including humans up to the present time.

This report describes the production of cataracts which appeared in rats during prolonged toxicity tests of busulfan using dose levels over 5 times the recommended therapeutic amount. The prevention and augmentation of these opacities with dietary and other supplements are likewise discussed. Since the cause of cataract formation is still an enigma, any pertinent information may prove useful in the study of this condition.

EXPERIMENTAL

a. Toxicity and cataract production. Recently weaned male albino rats [3] were fed diets of ground Fox Chow with meat meal [4] containing amounts of busulfan ranging from 2.5 to 1,000 mg of drug per kilogram of diet. For the lower dosage levels a stock diet containing 100 mg per kilogram was first prepared. This was then diluted further to obtain the desired final concentration of the drug. Ten rats were used on each dose level. Hematological studies and autopsies were conducted, where possible, at the end of the experimental periods of 84 and 182 days. Blood sugar values were determined on the rats receiving the dose of 10 mg per kilogram of diet. Although an ophthalmoscope was used at first to detect opacities it was soon found that the cataracts were readily discernible without aid and so were recorded as they appeared grossly.

b. Non-reversibility of busulfan cataracts. In order to determine whether or not the cataracts were easily reversible, rats receiving doses of 15 and 20 mg of busulfan per kilogram of diet were transferred to the drug-free control diet as soon

[3] Carworth Farms.
[4] Purina.

as lens opacities were observed. Surviving animals were observed on this drug-free regime for an additional 12 weeks.

C. Prevention and augmentation of cataract formation with dietary and other biological supplements. Various substances were tested for their ability to delay death or cataract formation due to busulfan at dose levels of 10, 15 and 20 mg per kilogram of diet. Five or 10 rats were used in each group and the supplements were incorporated in the diet or injected as indicated. Likewise different levels of galactose in conjunction with lower dosages of the drug were tested for abilities to augment the opacity formation.

RESULTS

a. Toxicity and cataract production. The survival time of the rats depended on the dose level of busulfan (table 1). The larger amounts produced death in a relatively short time while smaller doses were less lethal or had no effect upon the animals. Doses between 31 and 1,000 mg per kilogram of diet caused death before cataract formation. Those between 7.5 and 20 mg per kilogram of diet produced cataracts and growth impairment while those of 5 mg and lower had no significant effects even after 26 weeks on test.

At the end of 84 days the rats receiving 10 mg of busulfan per kilogram of diet were autopsied. In general the animals appeared slightly paler in skin color and somewhat smaller than their untreated counterparts. When the cataracts were observed under slight magnification (10 ×) it was seen that both the lens and lens epithelium were opaque with a diffuse milky appearance. There was an increased vascularity of the iris which became quite apparent in front of this opaque background. It was also observed that the femurs were more brittle than those of the controls and the epiphyses were not entirely closed or calcified.

Individual hematological values including blood sugar determinations were obtained from the animals receiving the dose of 10 mg of the drug per kilogram of diet (table 2). It was found that the average blood sugar value for the treated

TABLE 1

Cataract formation in relation to growth and food and drug intake of rats

DOSE OF BUSULFAN	AVERAGE INITIAL WEIGHT	AVERAGE FINAL WEIGHT	AVERAGE SURVIVAL TIME	CATARACT FORMATION	TOTAL FOOD INTAKE	DRUG INTAKE
mg/kg diet	*gm*	*gm*	*days (range)*		*gm/rat*	*mg/kg body wt/day* [1]
1,000.	47.7	37.0	7.8 (7– 9)	none	4.9	16.9
500.	47.8	37.3	8.9 (7–12)	none	14.5	21.8
250.	48.3	42.0	12.5 (12–13)	none	37.6	17.9
125.	48.0	49.0 [2]	15.0 (14–16)	none	77.1	13.1
62.5	51.0	80.0	17.1 (11–20)	none	85.1	3.9
31.3	50.9	100.0	25.3 (20–33)	none	179.	2.2
20.0	45.1	188.8	50.4 (28–70)	3 after 8 wks.[3]	949.	1.6
15.6	50.4	206.6	Killed at 84 days (51–> 84)	4 after 9 wks.[4]	990.	0.890
10.0	45.3	252.8	Killed at 84 days	4 after 10 wks.[5]	1,155.	0.544
Controls	48.0	313.2	Killed at 84 days	none	1,305.	none
7.5	48.9	292.2 [6]	Killed at 182 days	2 after 11 wks.[6]	2,506.	.353
5.0	48.6	320.8	Killed at 182 days	none	2,814.	.241
2.5	48.1	362.2	Killed at 182 days	none	2,924.	.111
Controls	48.3	353.6	Killed at 182 days	none	2,821.	none

[1] Based on the final body weight.

[2] These rats gained up to an average of 55 gm in a week before losing weight and then dying. Animals on lower dosage likewise gained slightly before losing weight and dying.

[3] Cataracts developed in the eyes of three remaining rats after 8 weeks. All rats died before 84 days.

[4] Cataracts developed in the eyes of 4 of 5 rats remaining after 9 weeks. Five other rats died before 84 days.

[5] Cataracts developed in the eyes of 4 of 9 rats remaining after 10 weeks. One rat died before 84 days.

[6] One death at 20 weeks. The final weight value is significantly different from that of the controls, the "t" value being 3.64.

animals was 122.2 mg per 100 ml of blood as compared to that of 106.2 mg for the controls. This was a significant difference, since the "t" value corresponded to $P = 0.05$. Hemoglobin, red and white cell counts were lower in the test animals but cataract formation did not appear to be related directly to these lower values. The differential count revealed a lymphocytosis, and bone marrow studies likewise revealed a picture of anemia with definite lack of completion of the hemopoietic

TABLE 2

Blood studies of rats receiving 10 mg of busulfan/kg diet for 84 days

FINAL BODY WT.	BLOOD SUGAR	HEMOGLOBIN	RED BLOOD CELLS	WHITE BLOOD CELLS
gm	*mg/100 ml*	*gm/100 ml*	*x 10 ^{6}/mm ^{3}*	*x 10 ^{3}/mm ^{3}*
269 [1]	135	9.6	2.67	9.85
246	120	11.4	4.62	6.90
239 [1]	124	12.9	5.28	7.05
227	127	11.8	6.74	9.80
228	111	15.1	6.75	4.50
227	120	3.5	1.09	2.90
287	113	14.0	6.07	7.35
288	120	12.8	4.81	0.00
259 [1]	120	3.0	—	—
293 [1]	132	13.8	6.16	2.10
Controls: Average values with ranges for 9 animals.				
297 (262–331)	106 (83–131)	15.0 (14.4–16.4)	7.86	5.55

[1] Cataracts.

cycle. The presence of basophils in large numbers in 6 of the marrow examinations may be considered a response very similar to that found in x-ray therapy.

Histological sections of the spleen and lymph nodes showed necrosis in focal areas and numerous regions of extramedullary hematopoiesis. The glomeruli of the kidney appeared normal but necrosis was present in the epithelium of the proximal convoluted tubules and in the loops of the tubules. The liver was surprisingly normal.

The eye sections showed the development of cataracts from the very first epithelial proliferation in the lens to mature opaque types. The earliest pathological changes appeared to

be an increase in the mitoses of the lens epithelium especially at the equatorial portion. This was followed by vacuolization in the subcapsular cortex, anteriorly, and perinuclear vacuolization. Finally, diffuse feathery opacities were found subcapsularly, anterior and posterior, as well as perinuclear.

b. Non-reversibility of busulfan cataracts. In the two groups of rats fed diets containing 15 or 20 mg of busulfan per kilogram of diet the cataracts developed within 8 to 9

TABLE 3

Non-reversibility of cataracts

DOSE OF BUSULFAN	AV. INITIAL WT.[1]	8 WK WT.	TOTAL 8 WK. FOOD INTAKE	DRUG INTAKE [2]	AV. SURVIVAL TIME	CATARACT FORMATION	NO. OF CATARACTS REVERSED BY CONTROL DIET
mg/kg diet	*gm*	*gm*	*gm/rat*	*mg/kg body wt/day*	*days*		
15	40.1	205.5	712	0.93	> 147 [3]	10 in 63 days	0
20	39.9	163.7	593	1.29	75	8 in 63 days [4]	0
Controls	48.3	237.8	793	—	> 147	none	–

[1] Ten rats per group.

[2] For a period of 8 weeks.

[3] Two animals died after being placed on the drug free diet following cataract formation. Eight were autopsied after 147 days on experiment. No cataract regression could be seen during the last 84 days on the drug free diet.

[4] Two animals died before cataract formation and the remaining 8 died within 30 days after being placed on the drug free diet.

weeks. During the subsequent realimentation period with the animals receiving no drug, those on the 20 mg dose all died within an additional 4 weeks. Those on the 15 mg level survived and resumed a slow growth but no regression was noted in the cataracts during the additional 12 weeks on the control diet (table 3).

c. Attempts to prevent cataract formation with dietary and other biological supplements. From the results listed in table 4 it could not be concluded that any of the supplements

tested were active in preventing cataract formation or early death when fed at the level of 20 mg per kilogram of diet. Only the antibiotic, oxytetracycline,[3] gave some indication of slightly prolonging life and producing better growth.

With a lower level of busulfan, 10 mg per kilogram of diet, it appeared that of the supplements added only cod liver oil, a vitamin mixture, thiourea, β pyridylcarbinol tartrate, hydrocortisone and a vegetable oil-casein diet prevented cataract formation. However, deaths occurred in the groups receiving the β pyridylcarbinol and hydrocortisone during the initial 16-week period (table 5). When the dose was increased at that time to 20 mg per kilogram of diet only the two fatty type diets continued to afford protection for 8 additional weeks to all of the original animals. Although the fat increased the caloric content per unit weight of diet and thereby reduced the amount of food eaten the actual drug consumption was still within the range that produced cataracts when the fat content was only that of the Fox Chow diet. The 30% galactose supplement displayed its usual cataractogenic activity in this test and the thyroxin at the 100 mg dosage was especially lethal.

The protective action of fat was verified by using an even more critical level of busulfan, 15 mg per kilogram of diet, from the start of the experiment (fig. 1). Cod liver oil prevented the appearance of cataracts for 21 weeks even though the animals consumed 711 μg of busulfan per kilogram of body weight per day. However, two of the 10 animals died before the end of the test. One of the rats receiving the corn oil diet was autopsied after it appeared to have a slight opacity at the end of 8 weeks on test. The remaining 9 exhibited no cataract formation. The busulfan intake for this group was 651 μg per kilogram of body weight per day, well within the range for cataract production. At the end of the 21-week period one animal in each group had become quite pale indicating depressed hematopoietic activity. An equivalent amount of vitamins A and D found in the cod liver oil was given as a

[3] 'Terramycin' Brand Oxytetracycline, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

TABLE 4

Supplements given in attempts to prevent cataract formation or death
(20 mg Busulfan per kg diet, 5 rats per group)

SUPPLEMENT/KG DIET	INITIAL WT.	WT. OF SURVIVORS AT 8 WK.	SURVIVAL TIME	NO. OF RATS WITH CATARACTS [1]	CALCULATED TOTAL 8 WK. FOOD INTAKE	DRUG INTAKE
	gm	*gm*	*days*		*gm/rat*	*mg/kg body wt. (8 wk.)/day*
Armour liver extract No. 2, 10 gm	45.2	161.6	55.8 (40–84)	1	550.	1.21
Salt mix No. 2, 5 gm	45.6	159.3	51.0 (40–61)	2	557.	1.25
Yeast, autolysate Conc. No. 8 (Nat. Yeast Corp.), 5 gm	46.4	183.0 [2]	33.4 (26–54)	– [2]	567.	1.15
Adenine sulfate, 200 mg	46.2	177.0	44.0 (26–59)	1	502.	1.01
Desiccated whole liver, 50 gm	41.0	163.3	60.8 (26–96)	1	565.	1.23
Oxytetracycline, 500 mg	40.4	211.5	79.4 (42–117)	4	690.	1.16
Riboflavin, 250 mg	40.8	148.0	60.8 (43–87)	1	518.	1.25
Vitamin supplement,[3] 50 ml	39.8	164.3	63.8 (40–98)	2	588.	1.28
DL-Methionine, 500 mg	40.2	163.6	58.8 (44–79)	2	545.	1.18
Cod liver oil, 50 ml	40.6	162.4	57.2 (56–70)	4	652.	1.43
Nicotinic acid, tryptophan and ascorbic acid, 500 mg each	46.0 (3 rats)	151.0	61.0 (54–65)	–	527.	1.25
Vitamin B_{12}, 1 mg	49.0	150.0 [2]	30.4 (13–47)	– [2]	395. (43 days)	1.25
Estradiol, 10 mg	48.0	125.0	67.8 (47–83)	4	488.	1.39

[1] All other rats died before cataract formation.

[2] All rats died before 8 weeks.

[3] Vitamin supplement:

2-methylnaphthoquinone	0.01 gm	Choline chloride	50.00 gm
Calcium pantothenate	1.00 gm	Biotin	0.01 gm
Thiamine chloride	0.25 gm	Inositol	2.00 gm
Riboflavin	0.25 gm	Water	100.00 ml
Pyridoxine HCl	0.10 gm	Ethyl alcohol	to 500.00 ml
Nicotinamide	2.00 gm		

TABLE 5

Supplements given in attempts to prevent cataract formation or deaths (10 mg busulfan/kg diet for 16 weeks followed by 4 or more weeks of a 20 mg/kg diet dosage — 5 rats per group)

SUPPLEMENT/KG DIET	INITIAL WT.	10 mg busulfan/kg diet for 16 weeks				Subsequent 20 mg busulfan/kg diet for 4 weeks			
		Wt. and No. of survivors at 16 Wks.	Deaths during period	Cataracts formed [1]	Drug intake	Wt. and No. of survivors after 4 additional wks.	Deaths during period	Cataracts formed [1]	Drug intake
	gm	*gm*			*µg/kg body wt./day*	*gm*			*µg/kg body wt./day*
Liver powder,[2] 50 gm sodium desoxy, nucleic acid, 1 gm	48.6	287 (4)	0	1	466	290 (2)	0	2	879
Amino acid mixture [3]	47.0	280 (4)	0	1	488	265 (2) [4]	0	2	904
Oxytetracycline HCl, 500 mg	47.2	297 (4)	0	1	470	283 (4) [4]	0	0	899
(+) Galactose, 30%	47.0	218 (4) (91 days)	5	5	611	. . . . .	.		
Estradiol, 10 mg	46.8	207 (4)	0	1	578	215 (3)	0	1	1,126
Methyl testosterone 100 mg	47.8	237 (4)	0	1	552	255 (1)	0	3	801
Cod liver oil, 100 ml	49.0	300 (5)	0	0	414	315 (5)	0	0	870
Vitamin mixture [5]	47.0	295 (5)	0	0	490	302 (4)	0	4	976
Thiourea, 50 mg	45.8	307 (5)	0	0	468	288 (5)	2 in 5th wk.	1 in 5th wk.	866
β Pyridylcarbinol tartrate, 50 mg	46.4	304 (3)	2	0	475	273 (2)	1 and 1 in 5th wk.	0	869
Hydrocortisone, 2 mg/kg body wt./day–s.c.	46.0	288 (4)	1	0	490	285 (4)	0	2 in 6th wk.	853
Vegetable oil,[6] Casein diet	46.0	352 (4)	1 in [7] 2 wks.	0	305	334 (4)	0	0	513
Thyroxin, 100 mg	46.2	160 (1) (79 days)	4	1	1,185	. . . . .			. . .
Cholestorol, 1 mg	46.8	316 (2)	0	3	469	315 (1)	0	1	1,199
BAL,[8] 5 mg/kg body wt./day	46.6	282 (2)	0	3	485	285 (2)	0	0	1,004

[1] Rats were autopsied as soon as cataracts became evident.
[2] Desiccated whole liver no. 35 Wilson & Company, Chicago, Illinois.
[3] 2 gm choline chloride, 500 mg DL methionine and 100 mg of glutathione, nicotinic acid amide, L cystine and L cysteine·HCl per kilogram of diet.
[4] All died within 4 more weeks.
[5] 500 mg riboflavin, 500 mg ascorbic acid, 1 mg vitamin B_{12} and 50 ml of vitamin supplement (table 6) per kilogram of diet.
[6] 20% vegetable oil — 20% casein diet, Nutritional Biochemicals Corp., Cleveland, Ohio.
[7] This death at two weeks could not be considered as due to drug action.
[8] British Anti Lewisite or dimercaprol, administered by subcutaneous injection.

concentrate to another group but no inhibition of cataract formation was obtained. Only one rat survived for more than 12 weeks without cataract formation in the group receiving drug alone in the Fox Chow diet. These observations are summarized in table 6.

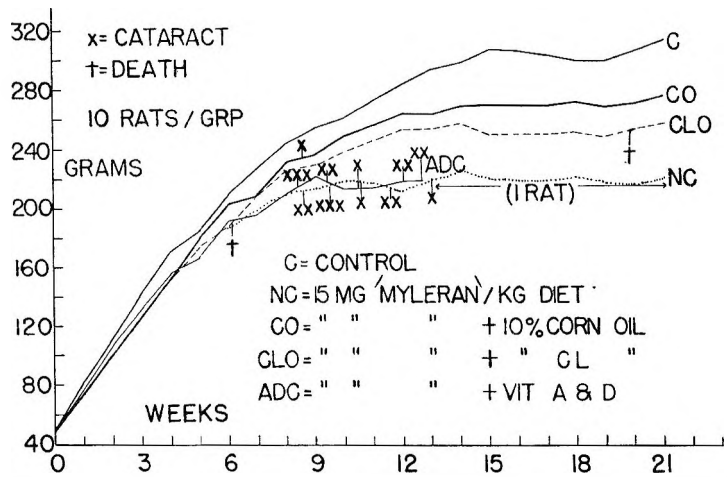

Fig. 1 Effect of 10% corn and cod liver oil on toxicity and cataract formation with busulfan.

TABLE 6

Prevention of cataracts with fat supplements (15 mg busulfan per kg diet)

10 rats/group

SUPPLEMENT	TIME	DEATHS	CATARACTS	DRUG INTAKE
	days			*μg/kg body wt./day*
Corn oil, 10%	147	0	1	651
Cod liver oil, 10%	147	2	0	711
Vitamin A and D concentrate	84	0	10	921
Busulfan, 15 mg	84	0	9	958
Busulfan, 10 mg	70	0	4	540

When galactose was added to the test diet only the 20% level caused cataracts when the dose of busulfan was 5 mg per kilogram of diet (fig. 2). At the higher busulfan dose of 10 mg per kilogram of diet both the 10% and the 20% amounts

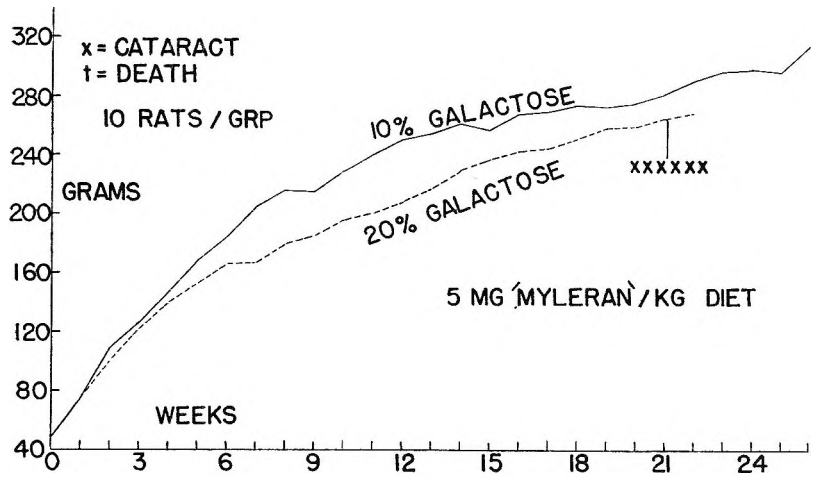

Fig. 2 Augmentation of cataract production with galactose (5 mg busulfan /kg diet).

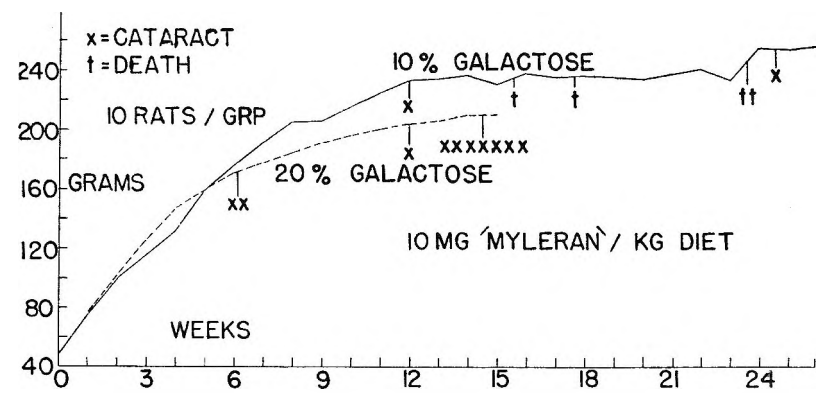

Fig. 3 Augmentation of cataract production with galactose (10 mg busulfan /kg diet).

TABLE 7

Effects of combinations of galactose and busulfan (10 rats/group)

BUSULFAN	GALACTOSE	TIME	DEATHS	CATARACTS	DRUG INTAKE
mg/gk diet	*%*	*days*			*µg/kg body wt./day*
5	10	182	0	0	234
5	20	154	0	6	268
10	10	182	4	2	506
10	20	104	0	10	649

of galactose augmented cataract formation (fig. 3), the latter value greatly reducing the time required for their appearance. These results are summarized in table 7.

DISCUSSION

From the data presented it may be seen that a new chemical tool has been found for use in the study of cataract formation. Doses of busulfan in amounts many times the therapeutic levels used for treating chronic myelocytic leukemia in humans have produced lens opacities in young and growing rats. The actual drug intakes causing cataracts vary between 350 and 1,600 μg per kilogram of body weight per day as compared with the maximum recommended human doses of only some 60 μg per kilogram. Up to the present time no cataracts in humans attributable to the use of this drug have been found. In fact there may be species difference (Bettman, '46; Barnes and Denz, '54) and strain variations (Mitchell et al., '37) with respect to cataract formation.

Although the blood sugar is slightly elevated in the treated animals it does not appear to be great enough to be the cause of cataract formation according to the criteria of Patterson ('53). This form of cataract also does not appear to be the result of any systemic deficiency of amino acids or proteins as discussed by Hall et al. ('48) and Pike ('51) or of vitamins, especially riboflavin, as reviewed by Day et al. ('38). It would seem more logical to class it with substances such as epinephrine (Suden, '40), naphthalene (Fitzhugh and Buschke, '49) and dinitrophenol (Robbins, '44; Bettman, '46) or anoxia (Bellows and Nelson, '44) all of which appear to have a direct action on lens metabolism. The above deficiencies, of course, may also act in this manner by interfering with certain metabolic systems directly in the lens. These possible actions on oxidation processes have been comprehensively discussed by Bourne ('37), Buschke ('43) and Shlaifer ('54). It would be of interest to evaluate the insulin activity in these drug affected animals in order to determine any decrease in the amount of the hormone which might lead to cataract forma-

tion (Patterson, '54, '55a) although the galactose type seems to be independent of insulin level (Mitchell et al., '37). The idea of an allergic type of cataract produced by busulfan should not be overlooked (Bentolila et al., '52). Calcium and thyroxin levels could also be profitably studied (Brand, '50; Giroud and de Rothchild, '51). Lens metabolism studies (Harris et al., '54 and Christiansen and Leinfelder, '52) may likewise be useful in examining opacities produced by busulfan.

The actual cellular development of busulfan cataracts is quite comparable to that caused by diabetes (Patterson, '52), galactose and various toxic agents (Robbins, '44; Buschke, '43) in the peripheral appearance of vacuoles and opacities, whereas the amino acid-deficient types of cataracts usually exhibit initial opacities toward the center of the lens (Day et al., '38).

The prevention of cataracts in itself has been a prolific field for research. The effect of food and drugs has been discussed by Moore ('40). Von Sallmann ('52) has obtained some protection from x-ray damage to rabbit lenses by pretreatment of the animal with cystine, glutathione or thiourea. However, in the present experiment with 1,4-dimethanesulfonoxybutane, which has been continuously administered in the diet to the animals, these adjuncts as well as many others failed to change the course of toxicity or cataract production, even though this chemical has been classed as a radiomimetic drug when administered in single doses (Bergel, '55). Fat in the diet has been found to alleviate cataracts in rats (Charalampous and Hegsted, '50; Rodriguez and Krehl, '51; Nieman, '55) and this protection is evident from the above data in busulfan treated rats at the critical dosage levels used. The toxicity of this drug, therefore, must affect the animal in a different manner than that of sodium fluoride in which case the dietary fat actually increases the toxicity (Miller and Phillips, '55).

According to Patterson ('55b) galactose accelerates cataract formation and this action is confirmed in the present experiment. It would likewise be interesting to see if other

members of the cataractogenic sugar family acted in a similar manner with busulfan.

SUMMARY

Oral administration of 1,4-dimethanesulfonoxybutane in a dose range between 350 and 1,600 μg per kilogram of body weight per day to growing male albino rats depressed hematopoeisis with accompanying cataract formations.

The cataracts were irreversible and resembled those produced by irradiation and certain toxic chemicals such as naphthalene. Although blood sugar levels were slightly elevated it could not be concluded that this was the causative factor for the cataract production.

Among the dietary and other biological substances tested in rats only the fats delayed toxicity symptoms and possibly prevented the appearance of cataracts at critical intake levels of busulfan up to 711 μg per kilogram of body weight per day for 21 weeks. Galactose, on the other hand, augmented cataract production even when the busulfan was given at doses as low as 268 μg per kilogram of body weight per day.

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THE EFFECT OF AGE ON THE PROTEIN AND METHIONINE REQUIREMENTS OF THE RAT [1,2]

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That the protein requirement for maximum growth decreases with increasing age and body weight has been known for some time. Mitchell et al. ('36–'37) found that in order to induce maximum nitrogen retention in 40- to 50-lb. pigs confined in metabolism cages more than 26% of dietary protein was required; for 100-lb. pigs, about 22%; for 150-lb. pigs, about 17%; and for 175- to 200-lb. pigs, about 15%. Reber et al. ('53) found that a ration containing 41% casein produced maximum weight gains and feed efficiency for the very young pig, but as the pigs approached 8 weeks of age 20% casein was used as efficiently as higher levels. Since the protein requirement is a summation of amino acid requirements, the latter must also decrease with increasing age.

It appears highly probable that proportions existing among amino acids in the protein requirement change as the animal reaches mature size, because the requirements for the individual amino acids will depend to a large extent, if not

[1] The data reported in this paper are taken from a thesis of the same title, prepared under the guidance of Dr. H. H. Mitchell, and submitted by E. W. Hartsook in the year of 1954 to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Nutrition.

[2] An unabridged version of this paper received one of the Ciba Foundation s Awards for 1954–55 for research relevant to the problems of ageing

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entirely, on the kinds and amounts of tissues being synthesized and maintained at the time. The work of Osborne and Mendel ('16, '19) presents good evidence for the existence of a large growth requirement and a slight maintenance requirement for lysine. Mitchell ('47) found that diets in which lysine-deficient cereal proteins were the nitrogen source had higher biological values for the mature rat than for the growing rat. Mitchell ('50) showed that beef muscle, casein and peanut flour (all deficient in methionine-cystine) had lower biological values for the mature rat than for the growing rat. He explained the difference as due to the sustained requirement of the constituents for keratin synthesis (the sulfur-containing amino acids, principally cystine) coupled with the decreased total amino acid requirements in the mature rat as compared to the growing rat. Womack et al. ('53) found that cystine could meet approximately three-fourths of the total requirements for the sulfur-containing amino acids in the mature rat, whereas in the young rat Womack and Rose ('41) reported that only one-sixth of the requirement for the sulfur-containing amino acids could be met by cystine. This indicates a decreased requirement for methionine as age progresses coupled with an increased requirement for cystine, evidenced by the added ability to utilize preformed cystine in the diet at the advanced ages.

In order to establish an amino acid requirement for growth of a given species of animal at a given period of its life-span it seems advisable to determine simultaneously the minimum amount of the amino acid in a diet which carries the minimum amount of protein necessary just to promote maximum nitrogen retention (Mitchell, '43). In the case of the adult animal it seems advisable to measure simultaneously the minimum amount of the amino acid contained in a diet with a protein content just necessary to promote nitrogen equilibrium.

The investigations to be reported here were initiated to develop mathematical means of assessing protein and amino acid requirements precisely at different ages in order to

determine whether the requirements of the sulfur-containing amino acids change significantly in relation to the other amino acids with changes in the age of the animal.

GENERAL EXPERIMENTAL PROCEDURES

Three experiments were conducted. The first was a rat growth experiment in which requirements for dietary protein and sulfur-containing amino acids were estimated from daily rates of carcass deposition of the nutrients of interest and estimates of maintenance requirements for animals aged from weaning to 102 days. The second (young rats) and third (mature rats) experiments employed the nitrogen balance technic and provided by statistical means estimates of requirements of the nutrients of interest while their dietary concentrations were being simultaneously varied.

All experiments were conducted in an air-conditioned room maintained at 78 ± 2° F. The animals were housed in individual screen bottom cages except when they were undergoing collection periods, when appropriate metabolism cages were used.

The diets employed were of the semi-synthetic type. The protein source was Labco casein. The nitrogen analysis was done by the Kjeldahl-Wilfarth-Gunning method (A.O.A.C., '50); the ether extract, by a 48-hour extraction of the dry sample with petroleum ether (Skellysolv F) in a Soxhlet apparatus; the moisture determination, by heating for 5 hours at 105° C.; the methionine assay, by the method of Lyman et al. ('46); and the cystine determination, by a modification of the Lyman methionine procedure where 400 mg of DL-methionine were substituted for 200 mg of L-cystine per liter of double-strength medium.

Experiment 1. This experiment was planned to estimate body deposition of nitrogen and the sulfur-containing amino acids in rats growing normally on an adequate diet. Thirty male weanling albino rats were given the experimental diet

ad libitum. The percentage composition of the experimental diet was: protein [4] 20, corn oil [5] 12, cerelose 40, sucrose 19.9, vitamin premix (Hartsook and Johnson, '53) 0.5, mineral mix 446 (Spector, '48) 4, woodflock 2, and choline 25% dry mix 1.6. The vitamin premix was used throughout all experiments and was found to be adequate as judged by the criteria of normal growth and the absence of any deficiency symptoms.

The food consumption was determined weekly, as was the body weight. When the mean weight of the group of animals had increased from 20 to 25 gm above the mean weight last determined, the two rats that were nearest to the mean weight of the group were etherized, their gastrointestinal tracts were completely emptied, and the carcasses were analyzed for total nitrogen and for methionine and cystine. The assay procedures used resulted in excellent recovery of added methionine (96%) and good recovery of added cystine (80%).

An equation of the type $W = a - be^{-ct}$, where W = mean body weight, t = age in days, e is the base of natural logarithms, and a, b, and c are constants, was fitted to the growth data by the method of least squares.

The equation follows:

$$W = 530 - 828e^{-0.0197t}. \qquad (1)$$

The above type of equation is the one used by Brody ('45) to describe the self-inhibiting phase of growth. Differentiation of the fitted equation and evaluation of the resulting differential dW/dt at an age of 40 days indicates a daily rate of gain of 7.4 gm.

[4] Supplied by Labco casein containing 88.46% crude protein (N × 6.25) by analysis, supplemented with DL-methionine of 97.8% purity at the rate of 3.2% of pure methionine. The DL-methionine used throughout this work was supplied by Merck and Company, Inc., Rahway, New Jersey, through the courtesy of Dr. Harold H. Draper. The casein contained 3.2% methionine and 0.25% cystine per 16 gm of nitrogen.

[5] Vitamin A and D concentrate oil (Distillation Products Industries, Rochester New York) and α-tocopheryl acetate were added to the corn oil so that the final diet contained 2,000 I.U. of vitamin A, 200 I.U. of vitamin D, and 10 mg of vitamin E per 100 gm.

The data for mean daily food consumption at increasing body weights is well described by the quadratic equation

$$F = 15.5 + 0.120\ (W - 186) - 0.000201\ (W^2 - 40{,}987) \qquad (2)$$

in which F is the daily intake of food in grams when the rats attained a body weight of W in grams.

Equations of the type Y (carcass component in mg) = $a - be^{-ct}$ (age in days) were fitted by the method of least squares to the data for the nitrogen, methionine and cystine contents of the rats, with the following results:

$$\text{Nitrogen, } Y = 13623 - 27426e^{-0.027t} \qquad (3)$$

$$\text{Methionine, } Y = 1741 - 3583e^{-0.028t} \qquad (4)$$

$$\text{Cystine, } Y = 1535 - 3908e^{-0.034t}. \qquad (5)$$

Figure 1 shows the relationship existing between the net N requirement and age for the experimental animals. Entirely similar graphic representations for net methionine and net cystine requirements are omitted for the sake of economy of space. The values upon which the lower (solid) curve of figure 1 is based were obtained by evaluation of equation (3) at values of t within the range covered by this experiment and at extrapolated values beyond the range studied.

The center curve represents the summation of the lower curve and estimated values for the maintenance requirement. The net maintenance requirements were arrived at as follows: The N requirement was calculated from the data of the metabolism experiment with mature rats to be described below by multiplying the N maintenance requirement of 1.05 mg N/weight$^{0.75}$/day found in that experiment by the calculated weight$^{0.75}$ of the rats at the various ages. The requirements for methionine and cystine were calculated by apportioning the maintenance requirement of methionine of 40.05 mg/weight$^{0.75}$ (kg)/day, found by Nasset and Anderson ('51), between methionine and cystine in proportion to their concentrations in rat muscle tissue on the fresh weight basis and the weight$^{0.75}$ at the particular age. The concentrations of methionine and cystine of rat muscle tissue used for the calculations were 0.475 and 0.221%, respectively, reported by Dunn et al. ('49) and Lee and Lewis ('34).

The upper curve (short dashes) of figure 1 represents the summation of the center curve and estimated values for N lost from the body as shed hair. The amounts of N, methionine, and cystine lost from the carcass as shed hair were estimated by multiplying the body surface area in square centimeters of the rats by a factor of $\frac{(70 \times \text{gm of component per 100 gm of hair})}{307}$. The

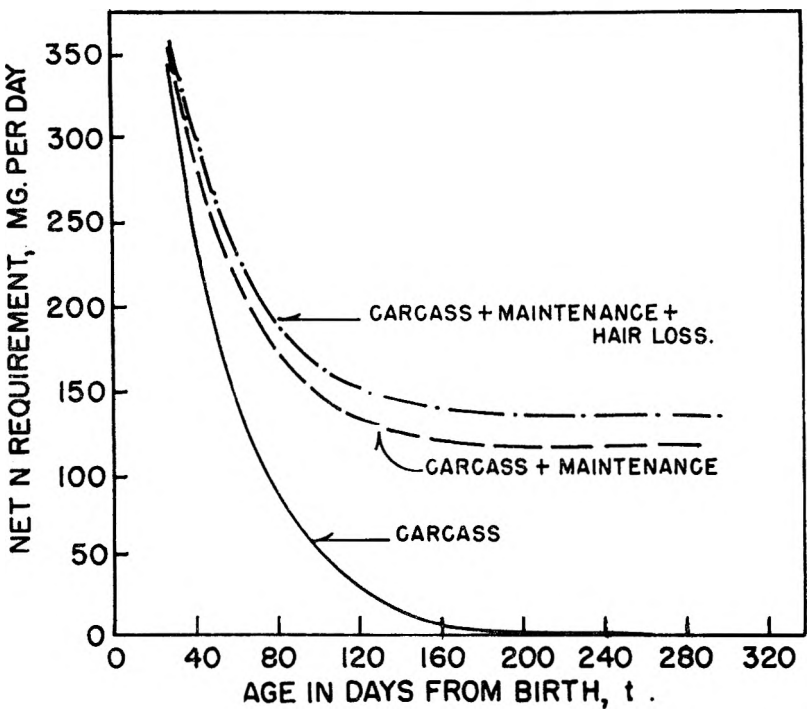

Fig. 1 Relation of net N requirement to age in days from birth of rats fed an adequate diet ad libitum.

figures of 70 and 307 represent the average amount of hair per day recovered by Mitchell ('34) in the feces of rats during periods of ad libitum feeding and the mean surface area in square centimeters of the rats involved, respectively. The figure of 70 mg of hair loss per day, which is the only quantitative figure on hair shedding by rats found in the literature, includes only hair contained in the feces; it is probably

a minimum value. Butcher ('34) found that hair cycles in the albino rat occur approximately every 35 days, with the resting period and the growing stage each being about 17 days in length. According to the data of Smuts et al. ('32) the mean weight of the hair coat of rats fed adequate diets and having a mean body weight of 143 gm and a mean surface area of 246 cm^2 was 2.451 gm. If it is assumed that the entire hair coat is shed during a hair cycle, then the rats of Smuts et al. would have shed $\frac{2.451 \times 1000}{35} = 70.03$ mg

TABLE 1

Average daily net requirements of nitrogen, methionine, and cystine by rats at selected ages

AGE IN DAYS	AVERAGE DAILY NET REQUIREMENTS IN MG/DAY				RATIO OF CYSTINE REQUIRED TO METHIONINE REQUIRED	RATIO OF METHIONINE + CYSTINE REQUIRED TO N REQUIRED
	Nitrogen	Methionine	Cystine	Methionine + cystine		
30	360	49	54	103	1.11	0.286
38.3 [1]	315	41	46	87	1.12	0.276
50	263	34	38	72	1.12	0.274
75	195	25	29	54	1.16	0.277
100	162	21	25	46	1.19	0.284
200	134	18	24	42	1.33	0.313
300	134	18	24	42	1.33	0.313
342 [1]	134	18	24	42	1.33	0.313

[1] These animals are comparable in age to the animals with values obtained in the metabolism studies.

of hair per day. The rats of Mitchell ('34) were larger than those of Smuts (mean body weight of 206 gm and mean surface area of 307 cm^2) and therefore should have shed a proportionately greater amount of hair according to the ratio existing between the surface areas of the animals concerned. These calculations confirm in a striking fashion the value used here for the daily loss of hair in the albino rat.

Average daily net requirements for N, methionine, cystine, and methionine + cystine at selected ages, together with the ratios of cystine requirement to methionine requirement and methionine + cystine requirement to N requirement are given in table 1. Values in this table relating to the growth study,

comparable as to age of animals with values obtained in the metabolism studies, have been marked with an asterisk (*).

A dietary protein requirement may be computed from the net N requirement by multiplying the latter by 6.25 and dividing by mean daily food consumption and a biological value of 90 [6] for casein adequately supplemented with respect to methionine. Dietary requirements for the sulfur-containing amino acids may be calculated in the same manner from net requirements. The selected biological value of 90 was based on the values of 89, 96, and 83 found by Mitchell ('24) and Brown ('49) using adult rats and by Kik ('38) using young rats, respectively. That biological values applicable to proteins of a diet are also applicable to their constituent amino acids is subject to some doubt, but since definite information in regard to the question is lacking, such calculation was made and submitted for what it may be worth. The resulting dietary requirements of protein and of methionine + cystine appear graphically in figure 2, and there the relationships of such dietary requirements to age are shown to be exponential in character.

Metabolism experiments. The experimental diets contained uniformly 5% lard, 1.5% cod-liver oil, 0.5% wheat germ oil, 10% sucrose, 39% corn starch, 4% salts no. 446, 0.5% NaCl, 0.5% vitamin premix, 1.6% choline 25% dry mix, and 2% woodflock. The diets were completed by the addition of varying amounts of protein, cerelose, and corn oil. The protein was included in the experimental diets at levels varying from 1.6 to 4.5% in the case of the mature rats of experiment 3 and at levels varying from 10 to 22% in the case of the young rats of experiment 2. In both experiments each dietary level of protein was supplemented with DL-methionine at the rate of 0, 2, 4, and 6% of the

[6] The use of the same biological value for casein adequately supplemented with methionine for diets through a range of protein levels from 10 to 22% may be justified on the assumption that at the higher levels the absorbed protein used for maintenance and growth is still utilized to the extent of 90%. That portion not so utilized will be deaminated and the nitrogen excreted in the urine.

protein. The additional casein in the higher protein diets was always added at the expense of cerelose. Sufficient corn oil was added so that the total of corn oil and the ether extractives of casein amounted to 5% of the diet, thereby maintaining the fat content of the diets at 12%. Standardization diets contained laboratory-prepared whole egg protein, petroleum-ether-extracted at low temperature, as the sole source of protein.

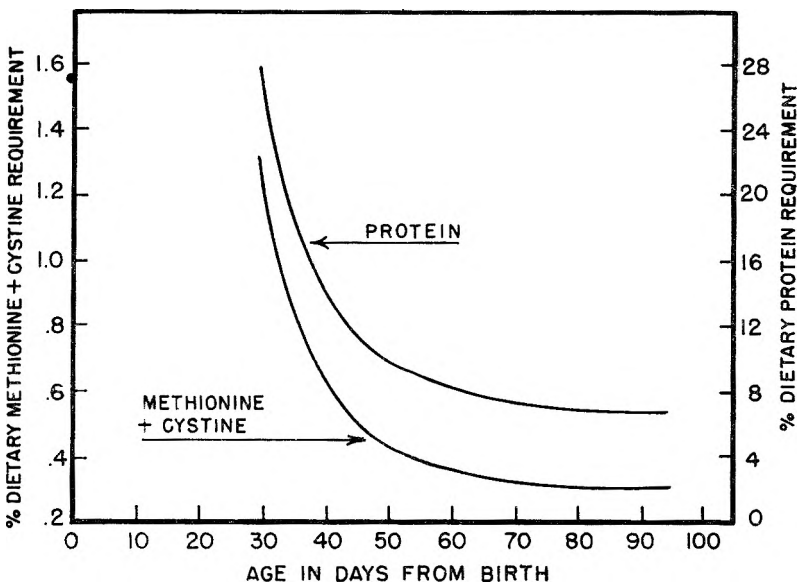

Fig. 2 Relation of the dietary protein and dietary methionine + cystine requirements to age in days from birth of rats (experiment 1) fed an adequate diet ad libitum.

Since in these experiments it was necessary that the responses of rats of differing body weights be pooled, a unit was needed which would allow responses of animals or groups of animals of varying body weights to be equitably compared. The unit selected was N balance (mg)/body weight (gm) raised to the 0.75 power (N bal./wt.$^{0.75}$).

Mathematical functions best describing the data at hand were used in interpreting the experimental results. Regres-

sion equations, linear, exponential, or quadratic, were fitted by the method of least squares, either in the original form or after appropriate transformation, using the method of weights when necessary. Fitting regression equations by the method of weights is a modification of a method given by Goulden ('52). The calculation of variances of dependent and independent variables followed the procedures of Snedecor ('46) or followed derivations given by Hartsook ('54).

Experiment 2. Estimation of requirements for the young rat. Four lots of 16 weanling rats each were received at weekly intervals. The animals were all 22 days of age on the day received and upon receipt were assigned randomly to each of 4 treatments and were fed an 8% protein (dried defatted whole egg) diet for a 7-day period, a 4% egg protein diet for a 14-day period, and either diets containing 10, 14, 18 or 22% casein for a 14-day period. Diets within each series contained either 0, 2, 4, or 6% of DL-methionine supplement, expressed as a percentage of the protein. During the last 7 days of the experimental periods, excreta were collected.[7] During all periods the animals were given daily weighed amounts of diet determined in accordance with equation (2).

In the case of the 10% protein diets, the mean responses to the three highest methionine supplementation levels were shown by an analysis of variance to be significantly greater than the mean response to the lowest level, but were not significantly different from one another (see figure 3). From these findings it was concluded that 10% protein diets could be improved by 2% methionine supplementation (expressed as a percentage of the protein), but not further improved by additional increments of methionine. Therefore, a straight line was fitted by the method of least squares to the first two arrays of data and a horizontal line was fitted to the last two arrays of data. The equations of both of these lines, as

[7] Plans for collection equipment kindly supplied by Dr. Doris H. Calloway, Nutrition Division, Food and Container Institute, Quartermaster Corps, Chicago, Illinois.

well as the points of data, are presented in figure 3. The fit of the regression equations to their respective arrays is highly significant ($P < 0.001$). The Y and Z values of the intercept of the fitted lines are 29.36 ± 0.32 mg and $0.189 \pm 0.015\%$, respectively.

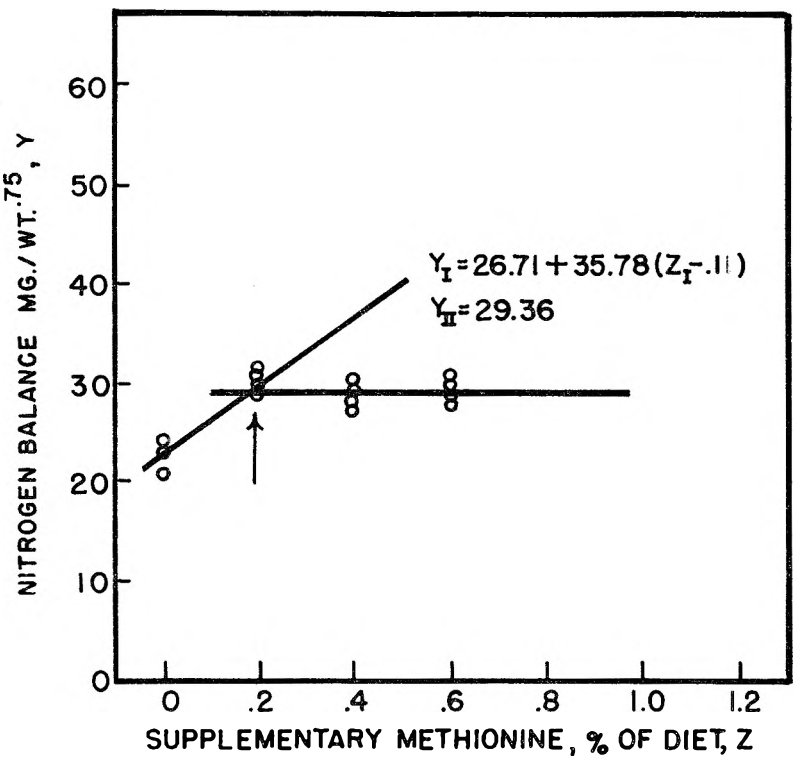

Fig. 3 Relation of N bal./wt.$^{0.75}$ to percent supplementary methionine at a 10% level of dietary protein. In figures 3, 4, 5, 6 and 7 the significance of the point(s) on the curve(s) indicated by the arrow(s) and its (their) coordinates is discussed in the text.

In the case of the 14% protein diets, it was found that the third array of data was not consistent with the other arrays (see figure 4). Moreover, the second and 4th arrays were found to be on the general plateau of N balances found for the entire experiment; this made it appear highly probable that the third array should also be on the plateau. The

first array was significantly lower ($P < 0.01$) than the second, while the second array was not significantly lower ($0.1 < P < 0.2$) than the 4th. An exponential function of the type $Y = a - be^{-cZ}$ fitted to all 4 arrays of data did not yield a satisfactory fit (i.e., the regression did not account for a significant portion of the sum of squares of deviations of Y from its mean), but a similar function fitted to the first, second, and 4th arrays of data did yield a satisfactory fit. The third array was, therefore, eliminated from further con-

TABLE 2

Maximum values of N balance (mg)/weight$^{0.75}$ (Y), minimum methionine supplementation (Z) resulting in maximum N balances, and percentages of dietary protein (X) found with young rats fed methionine-supplemented casein diets

% PROTEIN (X)	MAXIMUM N BALANCE (MG)/WEIGHT$^{0.75}$ (Y)			MINIMUM METHIONINE SUPPLEMENTATION (EXPRESSED AS A PERCENTAGE OF THE DIET) RESULTING IN MAXIMUM N BALANCES (Z)		
	N Balance	Variance	Weight	% Methionine	Variance	Weight
10	29.36 [1]	0.10	9.99	0.189 [1]	0.000229	4,367.61
14	38.74 [1]	0.91	1.10	0.488 [2]	0.116	8.64
18	39.04 [1]	0.52	1.94	0.537 [3]	0.0367	27.23
22	38.68 [1]	0.38	2.65	0	–	–

[1] $P = 0.001$.
[2] $0.1 < P < 0.2$.
[3] $P = 0.05$.

sideration, and the latter fitted equation is given in figure 4. Brody ('45) considers the practical maximum of such a function to be reached at 98% of the value of the asymptote, and since at approximately this value of a, the value of Z loses significance, 98% of the asymptotic value was taken to be the maximum N bal./wt.$^{0.75}$ and the corresponding value for supplementary methionine was calculated. These values of Y and Z with their standard deviations are, respectively, 38.7 ± 0.97 mg and 0.49 ± 0.34% of the diet. The value of Y was highly significant (table 2), but Z was found only to border on significance. Since weighted values were to be

used in the following steps of the interpretation, the value of Z was accepted as the best one available.

The method of interpretation of data for the 18% protein diets was entirely similar to that for the 10% protein diets and for the sake of brevity is not presented graphically. The

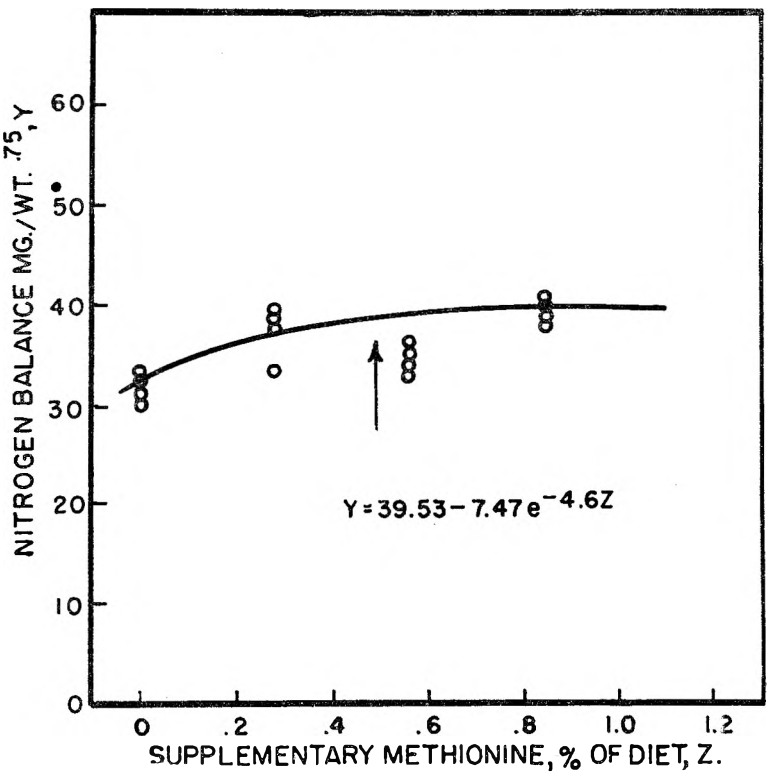

Fig. 4 Relation of N bal./wt.$^{0.75}$ to percent supplementary methionine at a 14% level of dietary protein.

intercept values for Y and Z appear in table 2, where it will be noted that the value of Y is significant at the 0.00[illegible] level and the value of Z is significant at the 0.05 level.

The mean responses (N bal./wt.$^{0.75}$) for the 22% protein diets at the various methionine supplementation levels were not significantly different from one another, and the slope of a line passing through the 4 arrays of data was found to be

not significantly different from zero ($P > 0.8$), showing that methionine supplementation of a diet containing this amount of protein is without significant effect on nitrogen balance. The equation of the regression line became $Y = 38.68$. The value of Y was highly significant. The responses, Y, for the 22% protein diets are not shown graphically.

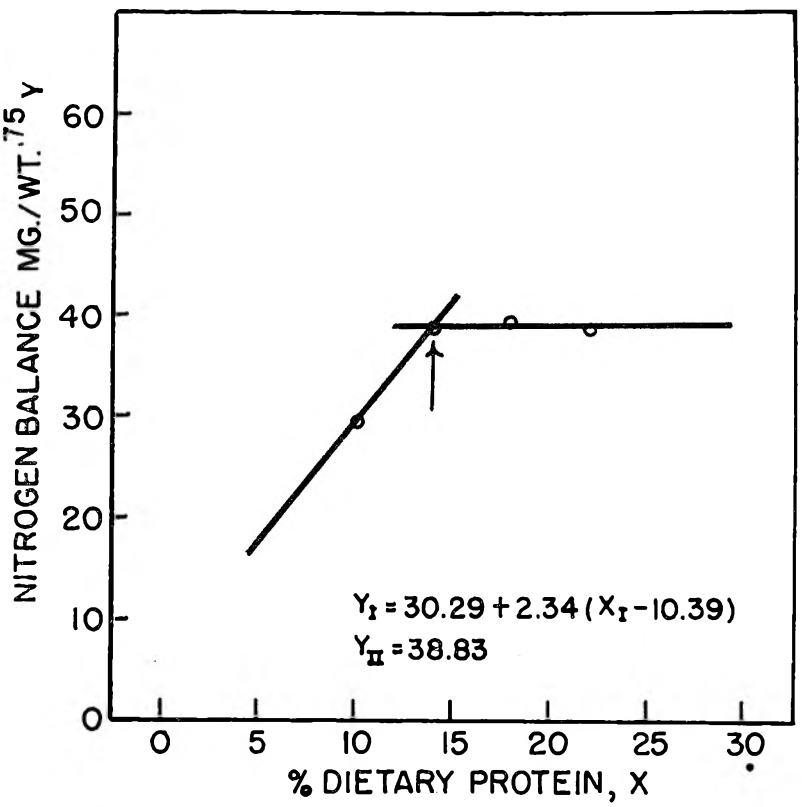

Fig. 5 Relation of maximum N bal./wt.$^{0.75}$ determined at various methionine supplementation levels (Y_1, Y_2, Y_3 and Y_4) to percent of dietary protein.

From table 2 it can be seen that the N bal./wt.$^{0.75}$ was not improved significantly when the dietary level of protein was increased above approximately 14%. Short of having additional information from many rats, the best approximation of the protein requirement is apparently the point of

intersection of lines drawn through the first two points and the second two points, respectively. The variance of each maximum N bal./wt.$^{0.75}$, Y, for the respective protein levels was converted to a weight by taking the reciprocal of the variance; these weights were used in fitting a regression line to the first two points, and in obtaining a mean value for the last two points. Plots of these two lines, together with the optimum values of Y previously calculated, appear in figure 5. The point of intersection has the coordinates: X_R (percentage of dietary protein) = 14.04 and Y_R (N bal./wt.$^{0.75}$ at the value of the protein requirement) = 38.83. The variance and standard deviation of X_R were found to equal 0.21 and 0.46, respectively.

In table 2 are also found the variances of the optimum levels of methionine supplementation at each dietary protein level. These variances were converted to weights in the same manner as previously described for protein. An exponential function, $Z = 0.54 - 52.38e^{-0.5x}$, where Z = percent of methionine supplementation expressed as a percentage of the diet and X = percent of dietary protein, was fitted to the first three points of data by the method of weights (see figure 6). The Z values upon which the fitting of the equation was done were the minimum levels determined in the interpretations of the first three series of experimental diets, and the X values were the protein percentages of the individual series of diets. The minimum level of methionine supplementation resulting in maximum N balances was taken as the Z intercept of the protein requirement value, 14.04, and is designated as Z_R, the requirement for supplementary methionine. Z_R was found to equal 0.49, and its variance and standard deviation to equal 0.023 and 0.15, respectively.

Experiment 3. Estimation of requirements for the mature rat. Twenty-eight adult male rats, weighing from 308 to 401 gm (mean = 361 gm) and approximately 300 days of age were removed from the stock diet, randomly assigned to 4 groups of 7 animals each, placed on a 16% casein diet for a 7-day period, given the experimental diets for a 14-day

period, given a 3.9% protein (dried defatted whole egg) diet for a 14-day period, and finally given the experimental diets for an additional 14-day period. The experimental diets contained three levels of casein, 0 (low egg-protein diet), 4.0 to 4.5% and an intermediate level ranging from 1.6 to 2.0%. At each protein level, except the 0 level, 0, 2, 4, and 6% of DL-methionine supplement (expressed as a percentage of the

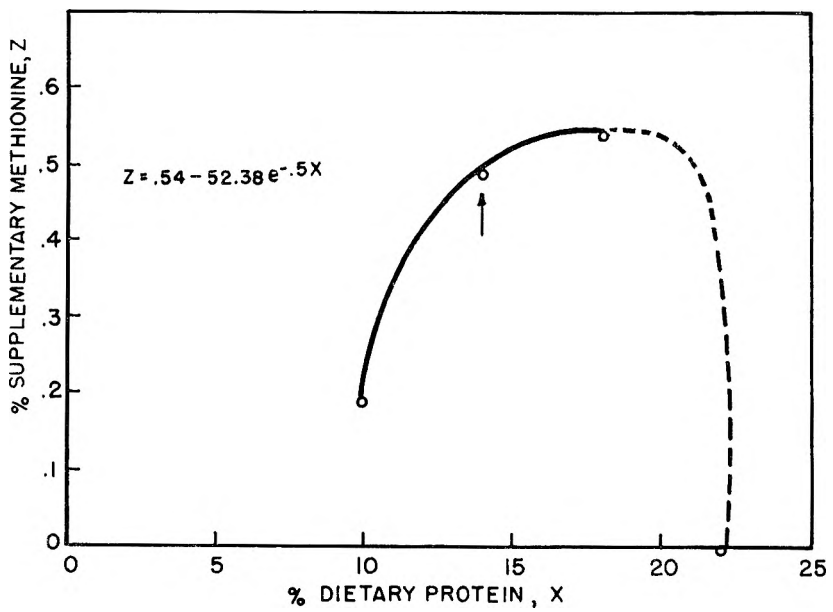

Fig. 6 Relation of minimum methionine supplementation levels (Z_1, Z_2, Z_3 and Z_4) to percent of dietary protein.

casein in the diet) were tested in N balance studies extending over 14 days during the last 7 days of which the excreta were collected.

All rats were given 14 gm of diet per day in all periods, an amount that maintained body weight almost constant. Rats showing symptoms of respiratory disease or refusing in excess of 0.6 gm of food per 7 days were not used in the interpretation of the results of the experiment.

Direct results obtained just prior to the feeding of egg-protein diets, and reversals obtained after such feeding, were analyzed separately.

In interpreting this experiment a modification of a procedure proposed by Melnick and Cowgill ('37) for evaluating the minimum amount of dietary protein necessary for N equilibrium in the adult dog receiving an adequate caloric intake was used. In the work reported here a minimum percentage of dietary protein as well as a minimum amount of methionine supplementation necessary for N equilibrium was desired; therefore, N bal./wt.$^{0.75}$ was plotted against percentage of protein in the diet for each level of methionine supplementation for both the direct and reversal periods. The regressions were linear and for each set of observations it was possible to estimate (1) the level of dietary protein necessary for nitrogen equilibrium and (2) the level of methionine supplementation, expressed as a percentage of the dietary protein, required in each case to attain N equilibrium at the lowest protein intake. Values for protein decrease to a minimum and then increase only slightly as the level of methionine supplementation increases from 0 to 6% of the protein.

The course of these curves for direct and reversal periods, shown in figure 7, is well described by the quadratic equations given in the figure. The decrease in X with changes in Z from 2 to 4% were highly significant, but the decreases in X as Z changed from 0 to 2% were not significant. Hence, the first two values of X for $Z = 0$ and $Z = 2$ were pooled in fitting the quadratic regression lines to the data by the method of weights. The fitted regression lines are shown in figure 7. In order to determine the point at which X reached its minimum value for each curve, the first derivative (dX/dZ) of the equation was equated to zero and solved for Z. These values of Z were substituted in the original regression equations, and corresponding values of X were calculated. The coordinates of the minima are: for the protein requirement, $X_{min} = 3.18 \pm 0.10\%$ of the diet, for the methionine require-

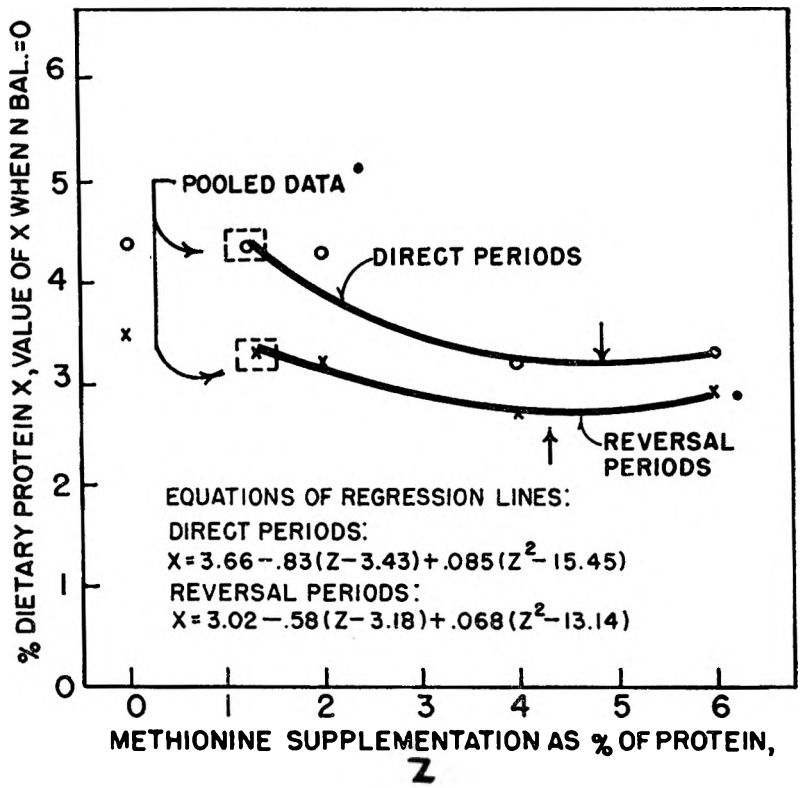

Fig 7 The protein and supplementary methionine requirements of the mature rat. Regression of levels of dietary protein necessary for N equilibrium at the levels of methionine supplementation used on the percent of methionine for mature rats in direct and reversal periods.

TABLE 3

The estimated dietary methionine and dietary methionine + cystine requirements of non-depleted and partially nitrogen-depleted adult rats

COMPONENT	NON-DEPLETED DIRECT PERIODS [1]		PARTIALLY DEPLETED REVERSAL PERIODS [2]	
	% of Protein	% of Diet	% of Protein	% of Diet
Methionine (total)	7.80	0.248	7.80	0.212
Cystine (from casein and total)	0.25	0.008	0.25	0.007
Methionine + cystine (total)	8.05	0.256	8.05	0.219

[1] Indicated protein requirement = 3.18%

[2] Indicated protein requirement = 2.72%.

ment, $Z_{min} = 4.85 \pm 0.52\%$ of the protein in the case of the direct periods; and in the case of the reversal periods, for the protein requirement, $X_{min} = 2.72 \pm 0.07\%$ of the diet, for the methionine requirement, $Z_{min} = 4.31 \pm 0.34\%$ of the protein. The values appended to the above estimates are standard deviations.

The estimates of the methionine requirements in the direct and reversal periods are indistinguishable statistically, but those for the protein requirements are significantly different. This considerable difference in the amount of protein required to achieve N balance during the direct and reversal periods is in agreement with the work of Allison et al. ('49) and of Allison ('51).

In table 3 the requirements found for the sulfur-containing amino acids are expressed both as a percentage of the protein and of the diet for the direct and reversal periods. Since the minimum percentages of methionine supplementation evaluated in the direct and reversal periods were found not to differ significantly, they have been pooled to obtain the entries of columns 2 and 4 of the table.

DISCUSSION

In table 4 are assembled data obtained from the metabolism experiments. Tests of significance were run, comparing the experimental values obtained. The difference between protein requirements found in the two experiments was highly significant ($t = 19.2$, $P < 0.001$). The methionine + cystine requirement of young rats was found to be significantly greater than that of mature rats when both requirements were expressed as a percentage of the diet ($t = 4.8$, $P < 0.001$). The total methionine + cystine requirement of mature rats was found to average greater than that of young rats when both requirements were expressed as a percentage of the protein, but the difference between averages was not statistically significant ($0.3 < P < 0.4$).

The dietary protein and methionine + cystine requirements found in the rat growth experiment (experiment 1) are presented graphically in figure 2.

The requirements in terms of dietary concentrations were converted to requirements in terms of milligrams per day by multiplying the former by the daily food consumption. The ratios between methionine + cystine requirement and N requirement (both expressed as milligrams required per day) for the mature and young rats were found to be 0.503 and 0.437, respectively. The ratio of the value for the greater age to the value for the lesser age was $\frac{0.503}{0.437} = 1.15$. It will be recalled that the ratios of net methionine + cystine requirement to net N requirement recorded in table 1 for rats of the growth experiment at ages of 342 and 38.3 days were 0.313 and 0.276, respectively. The ratio of the value for the greater age to the value for the lesser age was $\frac{0.313}{0.276} = 1.13$. The fact that the two ratios cited stand in excellent agreement is indicative that keratin synthesis assumes an increasingly important role in protein biosynthesis as maturity is approached and latter attained.

TABLE 4

Dietary protein and methionine + cystine requirements determined in metabolism experiments with young and mature rats

AGE OF RATS USED	EXPERIMENT NUMBER	MEAN AGE IN DAYS	MEAN BODY WEIGHT	DAYS (CALCULATED FROM MEAN BODY WEIGHT) [1]	PROTEIN REQUIREMENT (% OF THE DIET)	METHIONINE + CYSTINE REQUIREMENT	
						% of diet	% of protein
			gm				
Young	2	53	141	38	14.04	0.98	7.0
Mature	3	342	362	81	3.18	0.26	8.0

[1] Using equation (1).

CONCLUSIONS

The conclusions based on feeding male albino rats diets containing varying levels of casein, 12% fat and 2% bulk, with varying supplements of methionine follow:

1. The protein requirement (casein plus adequate methionine), expressed as a percentage of the diet, decreases in an exponential fashion as age increases from about 1½% to about 3.2%.

2. The methionine plus cystine requirement, expressed as a percentage of the diet, decreases in an exponential fashion as age increases from about 0.98 to 0.26%. As a percentage of the protein requirement it increases from 7.0 to 8.0%.

3. Evidence presented (although no individual portion reaches statistical significance within itself) is unanimous in indicating that keratin synthesis assumes an increasingly important role in protein biosynthesis as maturity is approached and later attained.

ACKNOWLEDGMENTS

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RESISTANCE OF MOUSE TISSUE SULFHYDRYL TO ALTERATIONS BY CHANGES IN DIETARY INTAKE OF SULFUR AMINO ACIDS [1]

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The likelihood of death occurring in mice or rats subsequent to x-irradiation of the whole body may be appreciably decreased by prior administration of various sulfhydryl compounds [see Patt ('53) for references], in amounts sufficient to bring about temporary marked increases in tissue sulfhydryl concentrations (Cronkite, Chapman and Brecher, '51). The present research was initiated to determine: (a) to what extent significant alterations in mouse tissue sulfhydryl could be secured by dietary procedures, and (b) whether the alterations which were secured would be significantly associated with changes in likelihood of death occurring subsequent to whole body x-irradiation.

It should be noted that Smith, Ackermann and Alderman ('52) have reported that the susceptibility of rats to lethal effects of whole body x-irradiation was relatively little affected by considerable variation in their dietary intake of protein (casein) and the sulfur amino acids, cystine and methionine. On the other hand Jennings ('49) has reported that rats kept for about 10 weeks on a very low protein diet were much more susceptible to lethal effects of whole body

[1] This research was supported in part by AEC Contract AT(30-1)-1758 and NIH Grant-in-Aid RG-3740(C2).

x-irradiation than corresponding control rats. Neither group of workers estimated tissue sulfhydryl concentrations of control and test animals.

METHODS

Chemical

Each tissue sample was frozen on dry ice immediately on excision and held wrapped in aluminum foil in a deep freeze to time of analysis, not more than 24 hours later. Protein-free metaphosphoric acid extracts were secured and analyzed for non-protein sulfhydryl (NPSH) by the nitroprusside method of Grunert and Phillips ('51), standardized against glutathione. Values obtained are shown in the tables as glutathione equivalents in milligrams per cent. Each tissue extract was prepared using pooled tissues of a particular kind, e.g., liver, obtained from all of the mice or the rat weanlings in a given control or test group.

Diets

Prior to their experimental use, mice were kept on a diet of rat pellets,[2] protein content not less than 20%, which were fed ad libitum with water.

Nitrogen-containing substances and sulfur amino acid of diets. The percentages of these substances present in the more important synthetic diets employed are indicated in table 1. Methionine derived from protein was the L form, whereas the free amino acid employed was the DL form. However, Wretland and Rose ('50) have reported that the D and L forms of methionine have equivalent dietary values.

A protein, derived from soy beans,[3] constituted the chief source of nitrogen in diets DA1 to DX3 inclusive (table 1). This protein is markedly deficient in its content of sulfur amino acids; it contains 0.6% of L-cystine and 1.0% of L-methionine according to the manufacturer's analysis. All

[2] Rockland.

[3] Drackett Assay C-1 protein, The Drackett Company, Cincinnati, Ohio.

other essential amino acids except tryptophan are present in amounts which the experiments of Rose ('38) indicate are satisfactory for the growth of rats. Free L-cystine and DL-methionine were added to diets DA1, DA2 and DA3 in amounts judged sufficient to render them adequate in sulfur

TABLE 1

Nitrogen substances in certain of the diets

DIET [1]	PORTION OF THE DIET ESTIMATED PRESENT AS:			
	Soybean protein [2]	Free amino acids		
		L-Cystine [3]	DL-Methionine [3]	Other
	%	%	%	%
	Experiments with mice			
DA1	18.8	0.2 [4]	1.0 [4]	0.0
DA2	13.8	0.2	1.0	0.0
DA3	8.8	0.2	1.0	0.0
DD2	13.8	0.0	0.0	1.2 [5]
DD3	8.8	0.0	0.0	1.2
DD4	6.3	0.0	0.0	1.2
DX1	18.8	0.2 [6]	3.0 [6]	0.0
DX2	18.8	1.2	1.0	0.0
DX3	18.8	1.2	3.0	0.0
AA (Control)	0.0	0.25	1.25	18.5
AD (No sulfur amino acids)	0.0	0.00	0.00	20.0 [5]
	Experiments with rats			
R (Control)	0.0	0.0	1.0	13.9
R minus methionine	0.0	0.0	0.0	12.9 [7]
R minus valine	0.0	0.0	1.0	11.7 [7]
R minus isoleucine	0.0	0.0	1.0	11.9 [7]
R minus threonine	0.0	0.0	1.0	12.9 [7]

[1] For a more detailed description of diets, see Methods, pg. 198.

[2] Drackett Assay C-1 Protein, The Drackett Products Company, Cincinnati, Ohio.

[3] To estimate *total* in the diet add the amount contributed by the soybean protein, which contains 0.6% of L-cystine and 1.0% of L-methionine.

[4] This amount was added to make the diets adequate in sulfur amino acids.

[5] L-Glutamic acid was substituted for free L-cystine plus free DL-methionine, to supply nitrogen equivalent to that in the sulfur amino acids of the other diets.

[6] Added in amounts to make the combined sulfur amino acids in excess of nutritional needs.

[7] An equal weight of sucrose was substituted for the omitted amino acid.

amino acid content, and to diets DX1, DX2 and DX3 in amounts judged to be in excess of nutritional needs. Free glutamic acid, 1.2%, was substituted for the sulfur amino acids in sulfur amino acid-deficient diets DD2 and DD3, in order to make these diets equivalent in nitrogen content to the corresponding sulfur amino acid adequate diets DA2 and DA3.

Pure amino acids constituted the only source of nitrogen in diets AA to R-T inclusive (table 1).

Diets AA and AD, fed to mice, contained: L-arginine, 0.5%; glycine, 0.5%; L-histidine, 1.0%; DL-isoleucine, 2.25%; L-leucine, 2.0%; L-lysine, 2.0%; DL-phenylalanine, 3.0%; L-threonine, 0.25%; DL-tryptophan, 0.5%; and DL-valine, 2.5%. Control diet AA also contained 0.25% L-cystine, 1.25% DL-methionine and 4.0% L-glutamic acid, whereas sulfur amino acid-deficient diet AD contained 5.5% L-glutamic acid, but neither cystine nor methionine.

Diets R to R-T inclusive of table 1 were fed to rat weanlings. Control diet R contained: L-arginine, HCl, 0.6%; DL-histidine HCl, 1.1.%; DL-isoleucine, 2.0%; DL-leucine, 3.2%; L-lysine HCl, 0.85%; DL-methionine, 1.0%; DL-phenylalanine, 1.5%; DL-threonine, 1.0%; DL-tryptophan, 0.45%; and DL-valine, 2.2%.

Sucrose was substituted for DL-methionine in sulfur amino acid-deficient diets R-S, for valine in valine-deficient diet R-V, for isoleucine in isoleucine-deficient diet R-I, and for threonine in threonine-deficient diet R-T.

Non-nitrogenous substances in the diets. Diets DA1 to AD inclusive of table 1, fed to mice, contained: (a) cod liver oil, 2%;, as source of vitamins A and D; corn oil, 1%, as source of vitamin E, and lard oil, 5%; (b) pure vitamins, as follows: choline, 0.2%; riboflavin, 0.001%; thiamine, 0.001%; pyridoxine, 0.001%; pantothenic acid, 0.004%; niacin, 0.008%; folic acid, 0.0003%; biotin, 0.00001%; and menadione, 0.00002 %; (c) the salt mixture of Hubbell, Mendel and Wakeman ('37), 4%; and (d) sucrose, in amount sufficient to bring the sum total of *all* dietary constituents to 100%.

Diets R to R-T inclusive of table 1, which were fed to rat weanlings, contained: (a) corn oil, 5%; (b) 1.05% $NaHCO_3$; (c) pure vitamins, as follows: choline, 0.2%; thiamine, 0.0005%; riboflavin, 0.001%; pyridoxine, 0.0005%; pantothenic acid, 0.004%; and niacin, 0.005%; (d) USP XIV salt mixture, 4.0%; and (e) sucrose, in amount sufficient to bring the sum total of *all* dietary constituents to 100%.

Feeding and care of test animals

Mice were kept in open-mesh wire cages. The daily food consumption by the 4 or 5 mice in each particular cage was estimated as follows: 5 gm per mouse of the appropriate diet was placed in an opal jar of inside diameter 1.75″ and height 1.5″. This jar was placed inside another opal jar of 3.5″ inside diameter and 3.75″ height. The weight of the food left in the two jars was determined 24 hours later. The difference in the two weights was used as the measure of the day's food consumption for the mice in that cage. Our experience, and that of experimenters in the Mellon Institute Toxicology Laboratory, Pittsburgh, Pa., has been that mice do not remove and scatter appreciable amounts of food from this double jar arrangement, even when they are confronted with diets which are not palatable.

The mice used were males, 6 to 10 weeks of age.[4] The animals used in any one experiment were segregated into cage-groups in such a way that all of the different groups had practically identical average body weights at the beginning of the experiment. These average weights ranged from 19 to 24 gm.

The procedures for rat weanlings[5] were similar to those for mice, except that the daily food consumption was not estimated.

[4] Carworth Farms.
[5] Holtzmann.

X-Irradiation of mice

Prior to x-irradiation, one or two control mice, previously kept on a diet judged adequate in sulfur amino acid content, and one or two test mice, previously kept on a diet judged to be grossly inadequate in sulfur amino acid content, were placed in each of 16 plastic cages, each cage being 7″ long, 1″ high and 1.5″ wide. These cages were piled on top of each other in such a way that the center of each cage was, as nearly as possible, 36″ from the point source of the horizontal x-ray beam generated by a 30° angle tube. The Picker x-ray machine employed,[6] was run at 205 KV, 15 ma using 1.0 mm Al plus 0.25 mm filtration. Victoreen meters indicated an average x-ray dose of 14 r per minute. The estimated whole body x-ray doses estimated as having been employed in the different experiments are shown in table 5.

EXPERIMENTAL

Sulfur amino acid-deficient diets. The data obtained in experiments involving the use of diets deficient in sulfur amino acids have been summarized in tables 2 and 3. In those experiments in which the soybean protein formed the chief source of dietary nitrogen, the lowest of the liver NPSH values secured were for mice which had been placed for *one day only* on a diet deficient in sulfur amino acids. The data indicate that a metabolic adjustment occurred for mice kept on this kind of diet for periods longer than one day. This adjustment was of such a nature that the liver NPSH gradually returned toward normal values, in spite of the continuing severe sulfur amino acid deficiency.

From the percent decreases in liver NPSH associated with the use of sulfur amino acid-deficient diets (last two columns of table 2), it may be concluded that more drastic decreases in liver NPSH were induced by placing mice on a pure amino acid diet *devoid* of sulfur amino acids (diet AD) than by placing them on a soybean protein sulfur amino acid-deficient

[6] Courtesy of the Pathology Department, University of Pittsburgh.

diet. The rather high liver NPSH value which was obtained for mice kept on diet AD for 7 days indicates that even for these mice a metabolic adjustment had occurred, leading to a partial return of the liver NPSH toward the original level.

From a comparison of the summarized data (table 3) obtained for: (a) mice fed the soybean protein diets and (b) mice fed the pure amino acid diets, it is apparent that low liver NPSH values were correlated with the use of

TABLE 2

The effect of a dietary deficiency of sulfur amino acids on non-protein sulfhydryl (NPSH) of mouse liver, with special reference to duration of deficiency

DAYS ON DIET BEFORE SACRIFICE	LIVER NPSH, AS MG % GSH EQUIVALENT [1]				% DECREASE IN LIVER NPSH ASSOCIATED WITH USE OF DEFICIENT DIET	
	Protein experiments		Amino acid experiments			
	Adequate diet DA_2 or DA_3	Deficient diet DD_2 or DD_4	Adequate diet AA	Deficient diet AD	Protein exps.	Amino acid exps.
1	263 (4)	124 (4)	283	140	53	51
2	277 (2)	184 (2)	269 (3)	114 (3)	34	58
3	237 (2)	142 (2)	277 (4)	106 (4)	40	62
4	279 (2)	172 (2)	331	154	38	53
7	267 (4)	182 (4)	312	211	32	32
14	278 (2)	203 (2)	322	95 [2]	27	70 [2]
21	292 (2)	199 (2)	—	—	32	—

[1] Figure in brackets after liver NPSH value indicates number of separate analyses contributing to value, each analysis being for a single extract prepared from livers pooled from all mice (4 or 5) in a given cage-group.

[2] Mice very apathetic, bedraggled and underweight.

diets low in sulfur amino acids, rather than with food intakes and weight changes exhibited by mice on these particular diets. It is also apparent that the decreases in mouse liver NPSH concentration induced by sulfur amino acid deficiency were far greater than the decreases (if any) induced by this deficiency in the other tissues studied (kidney, spleen and heart).

Diets unusually rich in sulfur amino acids. Data obtained in experiments of this type, in which the soybean protein constituted the chief source of dietary nitrogen, are shown

TABLE 3

Summary of data obtained in experiments using sulfur amino acid-deficient diets

FACTOR	PROTEIN EXPERIMENTS				PURE AMINO ACID EXPERIMENTS			
	n [1]	Adequate diets DA_2 and DA_3	Deficient diets DDD_2 and DD_3	p [2]	n [1]	Adequate diet AA	Deficient diet AD	p [2]
Liver NPSH	18	271.2 ± 5.9	173.4 ± 8.6	< 0.001	11	288.1 ± 14.4	122.2 ± 11.4	< 0.001
Kidney NPSH	18	169.4 ± 2.4	164.2 ± 3.1	NS [3]	11	160.8 ± 6.4	136.1 ± 6.2	0.02
Spleen NPSH	17	122.9 ± 2.1	114.8 ± 1.9	0.01	6	118.0 ± 4.7	94.5 ± 4.7	0.03
Heart NPSH	18	43.4 ± 1.3	41.0 ± 1.3	NS	7	41.0 ± 1.4	33.8 ± 2.2	0.03
Food intake (grams per mouse per day)	18	2.88 ± 0.06	3.27 ± 0.11	0.01 [4]	11	2.15 ± 0.13	1.89 ± 0.14	NS
Wt. change (grams per mouse per day) [5]	18	+ 1.50 ± 0.049	— 0.073 ± 0.061	0.01 [4]	11	— 1.05 ± 0.16	— 1.49 ± 0.18	NS

[1] Number of comparisons made.

[2] Probability that difference of means was due to chance.

[3] Difference not statistically significant (p greater than 0.05).

[4] In the Drackett protein experiments, sulfur amino acid deficiency resulted in weight loss in spite of an increase in food intake.

[5] Plus values, weight gain; minus values, weight loss.

in table 4. It is apparent from these data that a considerable increase in the content of methionine or L-cystine, or both, over the amounts of these sulfur amino acids provided in control diet DA1, failed to induce a measurable increase in the NPSH concentration of mouse liver, kidney or spleen. In fact, appreciably lower liver NPSH values were found for mice fed the most highly supplemented diet, DX3, than for mice fed the control diet DA1.

Very similar observations were made in another series of experiments lasting one to 4 days, in which tissue NPSH values of mouse liver, kidney, spleen and heart were com-

TABLE 4

Tissue NPSH not significantly increased by use of diets highly supplemented with sulfur amino acids

DIET [1]	AV. FOOD INTAKE	AV. WT. CHANGE	AV. TISSUE NPSH VALUES, AS GSH EQUIV., MG % [2]		
			Liver	Spleen	Kidney
DA1	3.2	+ 0.23	306	105	170
DX1	3.0	— 0.17	265	106	182
DX2	3.4	+ 0.16	268	119	166
DX3	2.4	— 0.33 [3]	224 [3]	104	143

[1] For meaning of symbols, see Methods and table 1.

[2] Averages for groups of mice sacrificed at 1, 3, 7 and 14 days after beginning of dietary regime.

[3] Mice appeared increasingly unkempt and bedraggled as experiment continued.

pared for (I) mice fed a control diet containing 20% casein, or (II) a test diet containing 20% casein plus: (a) 1% L-cystine, (b) 5% L-cystine, (c) 1% L-cystine HCl plus 2.5% DL-methionine, or (d) 2% L-cysteine HCl plus 5% DL-methionine.

Variation in sulfur amino acid content of diet on protein sulfhydryl as percentage of extractable liver protein. Procedures employed in estimating extractable liver protein sulfhydryl, as a percentage of the extractable protein, have been described elsewhere (Beck, Linkenheimer and Marraccini, '54). In the present experiments, protein sulfhydryl was found to constitute about 0.2% of the extractable protein,

regardless of the previous dietary status of the mice contributing livers subjected to analysis. It would appear that the sulfhydryl content of extractable mouse liver protein is not easily altered by dietary procedures.

Combined effects of (A) dietary deficiency in sulfur amino acids and (B) trauma, on liver NPSH values. Work in this laboratory (Beck and co-workers, '52, '54; Linkenheimer, '54) has established that within a few hours after induction of severe trauma, mice and rats exhibit markedly decreased liver NPSH values. The present experiments have afforded a very simple dietary procedure for bringing about a similar decrease in liver NPSH, without injury.

In an experiment designed to test for combined effects of: (a) sulfur amino acid dietary deficiency, and (b) tourniquet trauma, on mouse liver NPSH, the following average liver NPSH values were obtained: (I) 5 controls, 262 mg%; (II) 4 mice placed on diet AD, devoid of sulfur amino acids, for 28 hours, 124 mg%; (III) 5 mice sacrificed one hour after removal of hind leg ligatures, and 4 hours after ligatures had first been applied, 129 mg%; and (IV) 5 mice subjected to both procedure (II) and procedure (III) above, 142 mg%.

These data indicate that part of the liver NPSH is much more labile than the rest, and that once this labile NPSH has disappeared from the liver, as for example by placing mice on diet AD for 24 hours, it cannot be made to disappear again, as by induction of trauma.

X-ray experiments. Pertinent data have been summarized in table 5. It is apparent that the x-radiation-induced mortality rate for mice which had been placed on a sulfur amino acid-deficient diet for a few days, and which control tests indicated had markedly decreased liver NPSH values, was not appreciably or significantly different from the mortality rate induced in mice which had been maintained throughout on diets adequate in their sulfur amino acid content. It should be noted that mice kept on diet AD, completely devoid of sulfur amino acids, possessed spleens less than half

as heavy as those of mice maintained on a diet adequate in its sulfur amino acid content.

Rat weanling experiments. Two experiments were performed. In the first the special diet period was 14 days. The liver NPSH values for weanling rats were as follows: control amino acid diet R, 268 mg%; diet without valine, 283 mg%; diet devoid of isoleucine, 207 mg%; and for the diet devoid of threonine, 310 mg%. The same liver NPSH value of 217 mg% was secured for each of the control groups of experiment 2. One control group was fed diet R for three days, the other

TABLE 5

Effect of short-term sulfur amino acid deficiency on: (a) liver NPSH values, and (b) susceptibility to semi-lethal whole body x-irradiation

EXP.	DIET	DAYS ON DIET PRIOR TO IRRADIATION	ESTIMATED LIVER NSPH AT TIME OF IRRADIATION [1]	ESTIMATED AV. X-RAY DOSE (r)	NO. OF 30-DAY SURVIVORS / No. of irradiated mice
VII	DA1	1	276	560	2/31
	DD4	1	124	560	5/30
X	AA	2	247	504	7/24
	AD	2	106	504	5/24
XII	AA	3	218	504	5/23
	AD	3	85	504	7/23

[1] Data for mice on same diet as x-irradiated mice, but sacrificed shortly before x-irradiation. Each value is for 8 to 10 pooled livers.

for 14 days. The test group fed diet R-S, devoid of sulfur amino acids, for three days gave a liver NPSH value of 78 mg%, that fed diet R-S for 14 days a liver NPSH value of 39 mg%. Only a *sulfur* amino acid dietary deficiency resulted in marked decrease in liver NPSH. Appreciable to marked weight losses occurred in association with each of the specific amino acid deficiencies, whereas weanlings fed the complete diet, R, showed good weight gains over a 14-day test period.

DISCUSSION

Data presented in this paper indicate that the mouse possesses intracellular homeostatic mechanisms which are remarkably effective in maintaining total concentrations of nonprotein sulfhydryl compounds of kidney, spleen and heart within rather narrow limits, characteristic for each tissue, in spite of drastic alterations in dietary intake of the sulfur amino acids, cystine, cysteine or methionine or both. It should be noted that each of these amino acids has been demonstrated to be a good source material for incorporation into the naturally occurring cysteine-glutathione group of non-protein sulfhydryl compounds of animal tissues (Umbreit, '52).

In relation to dietary effects on tissue NPSH, liver constitutes a special case. Leaf and Neuberger ('47) have reported that in the rat, dietary supplementation with large amounts of sulfur amino acids resulted in an appreciable increase in liver glutathione, estimated by both specific and non-specific methods, while a dietary deficiency in these amino acids resulted in marked decrease in liver glutathione. We have failed to find an increase in mouse liver NPSH with moderate to high sulfur amino acid dietary supplementation. Mice placed on very highly supplemented diets actually exhibited significantly lower NPSH values than did the corresponding controls, and appeared to be unhealthy. This is not entirely surprising in view of the finding by Earle and Victor ('42) that liver hemorrhage and necrosis are induced by prolonged excessive dietary intake of L-cystine.

A marked decrease in mouse liver NPSH did occur in association with sulfur amino acid dietary deficiency. However, even in relation to mouse liver NPSH, homeostatic mechanisms appeared to be operating, since mice subjected to sulfur amino acid deprivation over a period of several days actually exhibited a considerable return of liver NPSH toward normal values.

It is well established (Patt, '53) that prior administration of large amounts of various sulfhydryl compounds results

in a significant decrease in mortality induced in mice and other species by whole body x-irradiation. Organ shielding experiments (Jacobson, '52) indicate that the spleen and liver are particularly important in relation to naturally existing resistance to lethal effects of whole body x-irradiation. Since in the present experiments mice fed diets low in sulfur amino acids exhibited very low liver non-protein sulfhydryl concentrations, and spleens less than half as large as those exhibited by control mice, it would not have been a matter of surprise if the mice fed the diets low in sulfur amino acids had exhibited increased susceptibility to whole body x-irradiation. Actually no effect of the sulfur amino acid dietary deficiency on susceptibility to whole body x-irradiation was found. Since liver protein sulfhydryl is appreciably greater than liver non-protein sulfhydryl (Beck, Linkenheimer and Bianconi, '54), it is possible that the diet-induced changes in *total* liver sulfhydryl were unimportant in relation to sulfhydryl action against x-ray-induced deaths. It is also possible that sulfhydryl protective action is exerted predominantly at some site other than the liver or spleen or both, or that it is non-specific in nature, and replaced by an adjustment of unknown nature, occurring simultaneously with a diet-induced marked decrease in concentration of liver non-protein sulfhydryl.

SUMMARY

The only appreciable change in mouse tissue sulfhydryl concentration found to occur in association with alteration in dietary content of sulfur amino acids was a marked decrease in liver non-protein sulfhydryl, occurring in association with use of diets deficient in sulfur amino acids.

In rat weanlings, a marked decrease in liver non-protein sulfhydryl occurred in association with a dietary lack of sulfur amino acids, but not with a lack of valine, of isoleucine, or of threonine.

Mice placed on sulfur amino acid-deficient diets which were effective in markedly decreasing liver non-protein sulfhydryl,

exhibited susceptibilities to semi-lethal x-irradiation indistinguishable from those exhibited by mice maintaned on diets adequate in sulfur amino acid content.

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THE RELATIONSHIP OF VITAMIN B_6 TO SERUM PROTEIN AND NONPROTEIN NITROGEN IN THE RAT DURING PREGNANCY [1]

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Numerous investigators have reported changes occurring in serum protein levels (Plass and Matthew, '26; Robinson et al., '51; Dieckmann, '52; Macy and Mack, no date) and in nonprotein nitrogen and urea levels (Plass, '24; Stander '24; McGanity et al., '49; Dieckmann, '52; de Alvarez and Richards, '54) in complicated and uncomplicated human pregnancies. However, there have been no reports on the serum protein values of pregnant rats and there is a paucity of data on serum nonprotein nitrogen values (Parsons, '30). The present study was undertaken to investigate protein and nonprotein nitrogen levels in the serum of the rat during pregnancy. Specifically, we were interested in studying both the effects of depleting maternal vitamin B_6 stores prior to mating and the effects of a pyridoxine deficiency during pregnancy on the concentration of protein and nonprotein nitrogen in the serum. Data collected on maternal nitrogen retentions, liver weight, moisture and nitrogen content, as well as data on the offspring of rats subjected to a pyridoxine deficiency during pregnancy, were reported recently (Ross and Pike, '56).

EXPERIMENTAL METHOD

Female albino rats of the Sprague-Dawley strain were maintained on laboratory chow [3] until they attained a weight of

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[3] Purina.

approximately 200 gm. When regular estrous cycles were established the animals were randomly divided into two main groups of 50 animals each. The main groups were randomly divided into 5 diet groups containing 10 animals each. Group I, referred to hereinafter as the non-depleted group, was maintained on laboratory chow until the day mating was confirmed; group II, the depleted group, was subjected to a prior depletion period (vitamin B_6-deficient basal ration plus 0.5 mg% of desoxypyridoxine) for at least 7 days before mating. The length of the depletion period varied from 7 to 30 days because of irregularity of estrous cycles in animals subjected to vitamin B_6 deficiency. A group of animals that could not be bred because of cessation of the estrous cycle after 8 days on the depletion diet was sacrificed after 32 to 60 days of depletion and the serum analyzed as it was for the pregnant animals.

On the morning that mating was confirmed by the presence of sperm in the vaginal smear the animals were placed in individual cages and then received the basal ration to which was added one of the 5 supplements shown in table 1. Except for the prior depletion period, there was no difference in the diets offered the two main groups.

On the 22nd day of pregnancy the animal was sacrificed, the heart exposed and blood taken by heart puncture for immediate serum analyses. Serum was analyzed for total protein by a micro-Kjeldahl method employing direct Nesslerization, and for albumin by a method which involved the separation of the albumin and globulin fractions by precipitation with Na_2SO_4 (Hawk, Oser and Summerson, '47). Globulin values were determined by difference. Nonprotein nitrogen concentration of the serum was determined by the method of Folin and Wu ('19).

The data were analyzed statistically by means of analysis of variance. Comparisons were made between the two main groups to test for the effects of vitamin B_6 depletion prior to pregnancy, and within each main group to test for the effect of the level of vitamin B_6 during pregnancy. There are

TABLE 1

Composition of experimental diets

BASAL RATION				SUPPLEMENTS [1]		
	%	*Vitamin mixture* [2]	*mg*			
Casein, vitamin test [3]	26	Thiamine	2.0	*Diet*	*Desoxy-pyridoxine mg %*	*Pyridoxine mg %*
Sucrose and vitamin mixture	9.85	Riboflavin	2.0			
Cornstarch	34	*p*-Aminobenzoic acid	200.0	1	0.5	0
Hydrogenated fat [4]	19	Niacin	10.0	2	0	0
Corn oil [5]	5	Pantothenic acid	8.0	3	0	0.4
Salt mixture [6]	4	Biotin	0.04	4	0	0.8
Agar	2	Inositol	400.0	5	0	1.2
L-cystine	0.15	Vitamin B_{12} triturate [7]	4.0			
Vitamin ADE mixture [8]	+	Folic acid	0.4			
		Choline	400.0			
		Naphthoquinone	1.0			

[1] Incorporated into diet.

[2] Made up to 9.85 gm with sucrose.

[3] Labco.

[4] Crisco.

[5] Mazola.

[6] Hawk and Oser — Science, *74:* 369, 1931.

[7] Trituration of 0.1% crystalline vitamin B_{12} in mannitol (Merck).

[8] ADE mixed in corn oil contained 5,000 I.U of A, 400 I.U. of D_3 and 10 mg of alpha tocopherol in two drops and was administered two drops per rat every three days.

data for less than 10 animals in some of the diet groups due to refusal to mate or pseudo-pregnancies. However, all the analyses were corrected for disproportionality among the groups.

RESULTS AND DISCUSSION

The results of all the serum analyses are shown in table 2.

The average total protein values per 100 ml of serum were lower on all the diets in the depleted than in the non-depleted group, but there was little difference within the two main groups. Analysis of variance showed that only the differences due to depletion were significant ($P = 0.01$). However, the data from the depleted animals that could not be bred indicate that there are no changes in the concentration of total protein of the serum due to vitamin B_6 deficiency per se, and this confirms the report from Beaton's laboratory ('53a). It appears, therefore, that the reduction in the concentration of total protein in the serum observed in the depleted pregnant animals is due to the combined effects of pregnancy and depletion.

The average values for albumin per 100 ml of serum varied little between the depleted and non-depleted groups. Statistically the slight differences in serum albumin content were not significant. The serum albumin values observed for all of the pregnant animals were lower than those for the depleted animals that could not be bred. It appears, therefore, that serum albumin is reduced during reproduction in the rat. Further, it appears from the data obtained that the maintenance of albumin levels during pregnancy is not affected by the vitamin B_6 deficiency imposed under the conditions of this study.

The concentrations of globulin per 100 ml of serum were lower for all the animals in the depleted than for those in the non-depleted group. The lowest values appeared in the depleted animals maintained during gestation on the desoxypyridoxine-supplemented and pyridoxine-free rations. Analysis of variance showed that the differences were significant

TABLE 2

Average protein and nonprotein nitrogen levels of the serum

PYRIDOXINE SUPPLEMENT	NON-DEPLETED					DEPLETED					
	No. of animals	Total protein	Albumin	Globulin	NPN	No. of animals	Total protein	Albumin	Globulin	NPN	No. of depletion days
mg %		*gm %*	*gm %*	*gm %*	*mg %*		*gm %*	*gm %*	*gm %*	*mg %*	
0 [1]	8	6.31	3.09	3.31	34.5	8	5.80	3.29	2.51	24.4	12
		± 0.19 [2]	± 0.15	± 0.21	± 2.5		± 0.32	± 0.17	± 0.30	± 1.3	± 1
0	10	6.45	3.22 [3]	3.07 [3]	35.3	8	5.40	3.22 [4]	2.12 [4]	27.3	14
		± 0.27	± 0.12	± 0.20	± 2.5		± 0.30	± 0.24	± 0.31	± 1.1	± 2
0.4	10	6.15	3.22	2.93	33.8	8	5.71	3.02 [5]	2.72 [5]	31.9	14
		± 0.29	± 0.12	± 0.26	± 1.6		± 0.18	± 0.14	± 0.12	± 1.7	± 2
0.8	10	6.10 [6]	3.11 [3]	2.89 [6]	34.5	7	5.85	3.46 [5]	2.51 [5]	32.4	19
		± 0.23	± 0.14	± 0.25	± 1.4		± 0.24	± 0.12	±0.26	± 1.1	± 3
1.2	10	6.39	3.56	2.82	34.7	8	5.81	3.32	2.50	30.0	16
		± 0.14	± 0.14	± 0.23	± 2.0		± 0.15	± 0.10	± 0.17	± 1.6	± 3
0 [1,7]						5	6.65	3.80	2.85	34.6	52
							± 0.35	± 0.23	± 0.35	± 1.3	± 4

[1] Plus 0.5 mg % desoxypyridoxine.
[2] Standard error of the mean.
[3] Average for 9 animals.
[4] Average for 7 animals.
[5] Average for 6 animals.
[6] Average for 8 animals.
[7] These animals could not be bred because of cessation of estrous cycles after 8 days on the depletion diet.

due to the effect of prior depletion ($P = 0.01$) but were not significant due to diet. The highest concentration of globulin per 100 ml of serum was observed in the non-depleted animals maintained on the pyridoxine-deficient diets during pregnancy. When the animals were depleted prior to mating, thereby producing what would appear to be a more severe tissue deprivation of vitamin B_6, there were less marked elevations of serum globulin. The concentration of globulin in the serum of the animals that could not be bred but were subjected to the stress of pyridoxine deficiency was similar to that observed for animals receiving vitamin B_6 and subjected to the stress of pregnancy.

Marked changes in the nonprotein nitrogen in the blood usually have been shown to be due to altered urea levels, since urea constitutes the largest fraction of the nonprotein nitrogen constituents. Although only total nonprotein nitrogen was determined in this study, for the purposes of this discussion it is assumed that any differences in the levels of nonprotein nitrogen were due to variations in urea.

It may be observed that the average nonprotein nitrogen concentrations in the serum of the depleted animals were lower than those for the non-depleted group. The sera of the depleted animals receiving the diet containing desoxypyridoxine had the lowest average nonprotein nitrogen content; as the pyridoxine intake increased, the percentage concentration of nonprotein nitrogen increased. Analysis of variance showed that the differences in nonprotein nitrogen were significant for the effects of depletion on the response to the desoxypyridoxine-supplemented and pyridoxine-free rations ($P = 0.01$). There was a significant interaction ($P = 0.05$) indicating that the effect of depletion had some influence on the response to these diets. The differences observed between the depleted and non-depleted groups receiving pyridoxine were not significant.

The concentrations of nonprotein nitrogen in the sera in both the non-depleted and depleted groups were lower than those observed by others in non-pregnant animals (Parsons,

'30; Hawkins, MacFarland and McHenry, '46). The depleted animals that could not be bred also had lower nonprotein nitrogen concentrations than those reported for non-pregnant rats, indicating that a vitamin B_6 deficiency also leads to a reduction in nonprotein nitrogen. This is in contrast to reports that an increase in blood urea occurs as a result of a vitamin B_6 deficiency (Beaton et al., '53b; Caldwell and McHenry, '53). The reduction in the nonprotein nitrogen concentrations in the sera observed either as a result of the vitamin B_6 deficiency, or as a result of pregnancy, was intensified by combining the effects of the deficiency and pregnancy. This compound effect was similar to that previously noted for total protein in the serum.

It is of interest that these data on pregnant rats show a trend similar to that reported for humans. The slight decrease in the concentration of total protein which was observed for the pregnant animals is similar to the decrease in total protein reported for uncomplicated human pregnancy (Plass and Matthew, '26; Robinson et al., '51; Dieckman, '52; Macy and Mack, no date). The intensification of the reduction in the concentration of total protein of the serum observed in the depleted animals is similar to what has been reported in complicated human pregnancies (Robinson et al., '51; Macy and Mack, no date). It appears, also, that serum albumin is reduced during pregnancy in the rat as it is in the human (Dodge and Frost, '38; Rinehart, '45). Further, the changes in serum globulin concentration are similar to those observed in human pregnancy; increases in the concentration of serum globulin in uncomplicated pregnancy with peak levels in mild complications (Robinson et al., '51; Macy and Mack, no date) and a tendency to decrease as the severity of the complications progress (Dieckmann, '52). The decrease in the concentration of nonprotein nitrogen in the serum of the pregnant rats is similar to the decrease reported for pregnant women (Plass, '24; Stander, '24). The significant decrease in nonprotein nitrogen levels of the depleted animals is similar to the de-

crease in blood urea values reported by some investigators for toxemic patients (Plass, '24; McGanity et al., '49).

SUMMARY AND CONCLUSIONS

The effects of depleting maternal vitamin B_6 stores prior to mating and of a pyridoxine deficiency during pregnancy on the concentrations of serum protein and nonprotein nitrogen in the serum of the rat were investigated.

Depletion of maternal vitamin B_6 stores prior to mating or pyridoxine deficiency during pregnancy or both, lead to changes in serum protein and nonprotein nitrogen coneentrations in the rat which are similar to those reported for the toxemias of pregnancy.

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INFLUENCE OF DIET COMPOSITION ON CALORIC REQUIREMENTS, WATER INTAKE AND ORGAN WEIGHTS OF RATS DURING RESTRICTED FOOD INTAKE[1]

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The literature contains few reports on food intake, water consumption and organ weights of animals on restricted diets. It is well known, of course, that humans on restricted food intake develop adaptive mechanisms. When these were first examined experimentally about 50 to 80 years ago, it was found that starvation leads to reduced energy requirements. In the rat in particular, this problem was studied more recently by Swift and French ('54) and by Quimby ('48). However, interest has centered mainly around questions of energy requirements and basal metabolism and surprisingly little work has been done about other adaptive changes such as those in the growth patterns of individual organs, or about the influence of the main nutrients on the adaptive mechanisms.

If feeding can be restricted just to the point where the weights of the animals are kept constant, there is the advantage that some data can be more specifically compared inasmuch as there are no differences in growth rates or body weights to be considered.

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Thus it seemed to be desirable to study the influence of diets high in protein, fat, or carbohydrate upon food consumption, water intake, and organ weights in rats whose weights had been kept constant.

EXPERIMENTAL

The experiments were carried out on male albino rats derived from a homogenous colony. They were delivered by the dealer when they weighed about 50 gm (a little over three weeks of age) and were immediately placed on a purified diet containing 30% lactalbumin, 10% commercial lard, 54% cerelose, 4% salts (USP II), 2% roughage,[3] and liberal amounts of all known vitamins.[4] This diet permitted excellent growth. Eight days after delivery, the rats were earmarked and weighed; 4 days later, they were reweighed. Matching groups of 8 rats each were formed whose average weights at 31 days and again at 35 days were identical. They were now housed in single unit cages with wire bottoms, weighed daily except Sunday, and given the appropriate amounts of food to keep their weights constant. It was possible to maintain the average weight of the rats in each group within 2 gm throughout the periods of examinations, which sometimes lasted 4 months.

Water intake was determined by the difference in weight of the full and partially empty bottles, which had been fitted with machine-made stems permitting no dripping but free drinking.

The composition of the three main diets is given in table 1. The caloric values of the diets were calculated by assuming that carbohydrate and protein yielded 4 and fat, 9.2 Cal. per gram. These values seem to be commonly accepted; but even if somewhat different values, such as those found by Thomson

[3] Alphacel, powdered and extracted rice bran hull, supplied by Nutritional Biochemicals Corp., Cleveland, Ohio.

[4] We are indebted to Dr. Leo A. Pirk of Hoffmann-La Roche, Inc., Nutley, N. J. for most of the synthetic vitamins used in these experiments. Barnett Laboratories, Long Beach, California, kindly supplied us with the crystalline beta-carotene and Dr. M. L. Tainter of the Sterling Winthrop Research Institute of Rensselaer, N. Y., gave us the crystalline vitamin D_2.

and Munro ('55) were preferable, our conclusions would not be altered. The cellulose content of the diets has been disregarded in calculating their caloric value because, even in the unlikely case that the rats utilized 10% of the cellulose in the diet containing 11% of this material the caloric value of the diet would have been increased by less than 1%.

In comparing the utilization of the various diets, it was convenient to calculate the calories needed by the rat for the maintenance of 1 gm of body weight during one week. Earlier studies had shown that the rat needs 1 gm of a "normal" diet over and beyond that required for weight maintenance to gain 1 gm of body weight. With this in mind, it was pos-

TABLE 1

Composition of experimental diets [1]

DIET	CASEIN	CERELOSE	LARD	CELLULOSE	SALTS
	%	%	%	%	%
High fat	20	0	65	11	4
High protein	70	0	20	6	4
High carbohydrate	15	75	None (2% Linoleic acid)	4	4

[1] Plus all accessory food factors.

sible to correct the weekly food intake for minor changes in the body weight. Thus, the total weekly individual food intake was increased or decreased by 1 gm for each gram of body weight lost or gained by the rat during the week. This gram for gram correction was used only in the case of the high-protein and high-carbohydrate diets. For the rats on the high-fat diet, the food intake was modified by 0.5 gm for each gram of change in body weight. The corrected weekly individual food intakes of each group were averaged, divided by the average weight of the group, and converted to calories. This value of calories per gram body weight per week was used for comparing the caloric requirements of the various groups. Because the requirements for weight main-

tenance are sensitive to changes in room temperature, comparisons were made only of groups running simultaneously.

RESULTS AND DISCUSSION

When the caloric requirements for weight maintenance were studied on rats given a purified diet including 30% casein, 10% lard, and 54% cerelose for a period of 4 months,, they were found to decline from 1.9 Cal./gm body wt./week to about 1.0 Cal. within the first 5 weeks and to remain fairly constant thereafter. This was in complete agreement with Quimby's findings.

In figure 1 are shown the average caloric requirements and the individual water intakes of groups of rats maintained on high-fat, high-carbohydrate, and high-protein diets. The caloric requirements of the animals on the high-fat and high-carbohydrate diets were initially similar; those for the high-protein diet were lower. The requirements of all groups declined but those for the high-fat diet, more rapidly, so that they eventually were equal to the requirements for high protein. The comparitively lower utilization of carbohydrates by animals on restricted food intake was also suggested in the studies of Rice et al. ('56).

The outcome was not related to the variations in protein intake because, in absolute amounts, the animals on the high-carbohydrate diet consumed only a fraction of that eaten by the rats on high-protein but twice as much as that consumed by the animals on the high-fat regimen.

It may be worth while to emphasize that the depression of the caloric requirements was given only with the fresh fat. We have previously demonstrated that the inclusion of autoxidized fats or their fractions prevents the decline of the caloric requirements during a period of restricted food intake (Kaunitz et al., '56).

The differences in caloric requirements brought on by high-fat, protein, or carbohydrate diets went hand in hand with characteristic differences in water intake shown in figure 1. The weekly intake in milliliters is plotted on a logarithmic

scale, which, for the eye, minimizes differences. However, it is obvious that there existed marked differences among the three groups. The animals on the high-fat diet consumed least, on the average, 45 ml weekly in the earlier weeks and later only 36 ml. Next are the high-carbohydrate animals

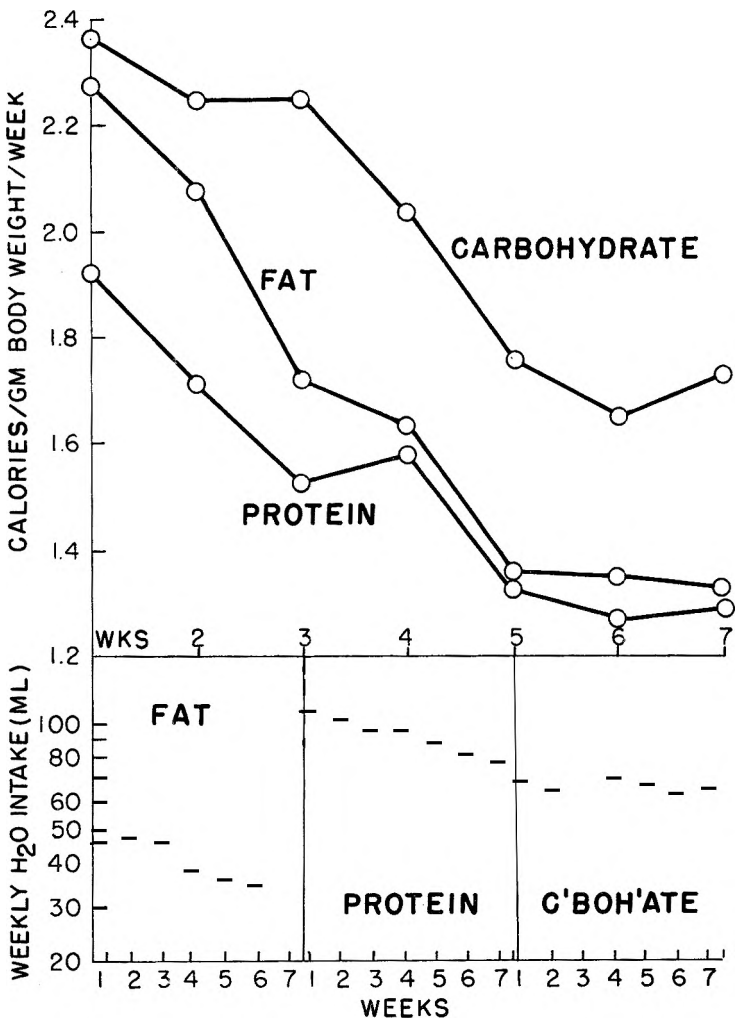

Fig. 1 Average weekly caloric requirements for weight maintenance and weekly individual water intakes of groups of 8 male rats kept at constant weight on diets high in fat, protein, or carbohydrate; — represents average for the group.

with average intakes of 65 ml throughout the whole period. The high-protein group had the highest consumption, about 105 ml in the earlier and 75 to 85 ml in the later weeks.

It has been known that protein intake is one of the determining factors for thirst; and, in these experiments, the animals on high-fat diets, having the lowest protein intake, had the lowest water intake and the animals on the high-protein diet drank most. However, the water intake was not linearly related to the protein intake. The animals on high protein ate at least 5 times the amount of protein consumed by those on the high-fat diet. Yet the water intakes differed by a factor of only two and one-half. Furthermore, the substantial decrease in protein intake of all groups during the time of restricted feeding on account of the decrease in caloric requirements was accompanied by only slight decreases in the water intakes of the high-fat and high-protein groups. The water intake of the high-carbohydrate group remained constant throughout and was only slightly below that of the high-protein group although the protein intakes of the two groups varied by a factor of 3.

For this reason, one must assume that the variations in water intake are not only caused by the differences in protein consumption but are also related to the main constituent of the diet. One wonders whether the effect of fat in reducing thirst has clinically found sufficient attention in conditions where a low water intake is desirable.

When the studies of food consumption and water intake were terminated, the animals were immediately sacrificed and their organs weighed. In table 2 are presented organ weight data calculated per 100 gm of body weight. Such a calculation has some degree of justification in view of the narrow range of the body weights. However, the organ weights were also plotted against the body weights on a log — log scale and the results compared with data for animals which had been allowed to eat freely of a purified control diet. Inferences were drawn only when both methods indicated significant differences.

Liver weights of all groups tended to be low or even subnormal, a consequence of food restriction. As was to be expected, the kidneys of the animals fed the high-protein diet were heaviest and differed significantly from those of the

TABLE 2

Organ weights, per 100 gm of body weight, of male rats kept at constant weights by restricted feeding of diets high in fat, protein or carbohydrate [1]

ORGAN	HIGH FAT 24 animals av. wt. 94 gm range 74–108 gm σ = 8.0			HIGH PROTEIN 15 animals av. wt. 91 gm range 75–108 gm σ = 9.0			HIGH CARBOHYDRATE 16 animals av. wt. 100 gm range 90–110 gm σ = 5.0		
	Av. Wt.	Range	SE	Av. Wt.	Range	SE	Av. Wt.	Range	SE
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Liver (normal range, 3.6–5.4 gm)	3.6	2.9–4.3	0.08	3.8	2.9–5.3	0.15	3.6	2.9–4.2	0.11
Kidneys (normal range, 0.75–1.2 gm)	1.0	0.6–1.3	0.03	1.2	0.9–1.5	0.03	0.9	0.7–1.0	0.03
Adrenals (normal range, 9–27 mg)	27	15–36	1.0	28	17–36	1.4	22	15–28	0.9
Testes (normal range, 0.6–1.4 gm)	1.4	0.5–2.2	0.10	1.1	0.2–2.0	0.16	0.9	0.5–1.5	0.06
Thymus (normal range, 240–550 mg)	80	35–107	5.0	93	60–134	10.0	77	58–105	3.7

[1] "Normal" refers to male rats of 100 gm which had eaten a highly purified control diet ad libitum. The standard deviation, σ, $= \sqrt{\frac{\Sigma d^2}{n-1}}$ where Σd^2 denotes the sum of the squares of the differences of the individual values from the mean and n, the number of values. The standard error, SE, $= \frac{\sigma}{\sqrt{n}}$

animals fed the high-carbohydrate diet. The adrenals of the animals on the high-fat diet were significantly heavier than those of the animals on the high-carbohydrate diet.

The testicular weights of the high-fat animals were, in more than half the cases, above the upper limit of the normal

spread and differed significantly from those of the other two groups. Thus, on the high-fat diet, the testes had increased in size although the body weight had remained constant. The thymus weights of all animals were considerably below normal, again an expected consequence of food restriction.

SUMMARY

1. The influence of high-fat, high-carbohydrate, and high-protein diets on the caloric requirements, water intake, and organ weights of rats kept at constant weight by restricted feeding was studied.

2. The caloric requirements for all diets declined during the first 5 weeks and became constant thereafter. On the high-protein and high-fat diets, the animals were eventually able to maintain their weight with 25% fewer calories than those on the high-carbohydrate diet.

3. The water intake was highest on the high-protein and lowest on the high-fat diet.

4. The adrenal weights of the animals on the high-fat diet were higher, on the average, than those of the animals on the high-carbohydrate diet, with those of the high-protein rats being in between. The renal weights were highest among the high-protein animals. The testicular weight of the high-fat animals was significantly higher than that of the animals on the high-carbohydrate diet.

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ASCORBIC ACID UTILIZATION BY WOMEN

RESPONSE OF BLOOD SERUM AND WHITE CELLS TO INCREASING LEVELS OF INTAKE IN TWO GROUPS OF WOMEN OF DIFFERENT AGE LEVELS [1]

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The response of blood serum and white cells to changes in ascorbic acid intake has been reviewed by Steele et al. ('55) and Morse et al. ('56). Steele et al. ('55) found that the ascorbic acid content of the white cells and serum increased significantly when 40 mg of ascorbic acid per day were given for 7 to 11 days, following an intake of 30 mg for 11 to 14 days. Morse et al. ('56) reported that the average white cell levels paralleled the rise in serum levels in a group of 19 women subjects when the intake of ascorbic acid was increased from 33 mg per day to 58 mg and then to 83 mg per day. Correlation between serum and white cells was statistically significant at these levels of intake. An intake of 133 mg per day caused no further increase in average white cell levels, but did produce a significant rise in average serum levels.

The present study is concerned with the relationship between the ascorbic acid level in blood serum and white cells

[1] Supported in part by funds from Regional Project NE-16, Relationship of Nutrient Intake to Nutritional Status in Human Subjects; a cooperative study involving Agricultural Experiment Stations in the Northeastern Region.

[2] Credit is due Dr. Geoffrey Beall, Professor of Statistics, and Dr. Mary L. Greenwood, Associate Professor of Foods and Nutrition, University of Connecticut, for verification of the statistical treatment of the data.

with subjects receiving smaller graded levels of supplementation than in the previous study. Two groups of women of different age levels are compared.

PROCEDURE

Subjects. Two groups of women, patients at a state training school and hospital for the handicapped,[3] served as subjects during the winter of 1954 to '55. Fifteen had an average age of 31 years (range 28 to 34) and 13 had an average age of 64 years (range 56 to 77). All were in good physical health, and were mentally capable of cooperating in the study. The subjects were examined at the beginning and end of the study for clinical signs of vitamin C deficiency.

Dietary ascorbic acid. Throughout the 4 months of the study, the subjects were on the regular institution diet except that all foods high in ascorbic acid, such as citrus fruits, tomatoes, pineapple, and raw cabbage were omitted. This provided a somewhat restricted and relatively constant level of dietary ascorbic acid. Other fruits and vegetables were always served to the subjects in place of those omitted.

The food intake of each subject was recorded on 16 scattered days near the beginning of the study. Food consumption was recorded in terms of the number of servings, or fraction of serving, of each food. Servings were weighed at intervals to determine size of portions. On 44 days, scattered throughout the period, samples of food were collected in the dining hall for analysis for total ascorbic acid by an adapta-

[3] Mansfield State Training School and Hospital, Mansfield Depot, Connecticut. Thanks are extended to the medical and dietary staffs of the school as follows: To Dr. Gail F. Moxon, M.D., and Dr. Harriet Bixby, M.D., resident doctors, for the physical examinations; to Dr. Joseph E. Nowrey, M.D., resident doctor, and his assistants for taking the venous blood samples; to Dr. Luke Grotano, D.D.S., resident dentist, for the dental examinations; to Mrs. Pauline Duckett, chief dietitian, and to the dietary staff of the women's dining room, for cooperation in the collection of dietary data and of food samples; to the 28 mentally retarded women who served so cheerfully and cooperatively as subjects; and to Dr. Neil A. Dayton, M.D., Superintendent of the Training School, for making the institution available for the study and for his continued interest and encouragement in research work.

tion of the 2,4-dinitrophenylhydrazine method of Roe and Kuether ('43). The collection days were determined by the nature of the institution menu. Dietary ascorbic acid intake of each subject was calculated using the food values obtained by analysis. Calories, protein, fat, carbohydrate, minerals and other vitamins were calculated using the U.S.D.A. Agriculture Handbook no. 8 (Watt and Merrill, '50). The average intakes of these nutrients met the recommendations of the National Research Council by 100% with the exception of iron which was 96% of the recommended allowance.

Ascorbic acid supplementation. After 7 weeks on the diet restricted in ascorbic acid, the subjects were given daily ascorbic acid supplements, beginning with 15 mg and increasing every 14 days to 25, 40, 50 and 75 mg, respectively.

Serum and white cell determinations. Serum and white cell ascorbic acid determinations were made on venous blood samples at the end of the 7-week adjustment period without vitamin C supplementation, and at the end of each two-week test period on the 5 levels of supplementation. The blood samples were always taken at 10:00 A.M., three to 4 hours after an ascorbic acid-free breakfast. Preparation and analysis of the serum samples were carried out according to the procedure outlined in the Northeast Regional Publication on Techniques ('51). The blood samples for white cell determinations were prepared in quadruplicate and the determinations made by the method of Bessey (Gyorgy, '50).

RESULTS

Ascorbic acid intake. The average daily ascorbic acid intake from food was 32 mg for all the women, based on the 16 days on which food intake was recorded. This average daily dietary intake, plus the vitamin supplementation at the 5 levels, brought the average total daily ascorbic acid intakes for the 5 supplemental periods to 47, 57, 72, 82 and 107 mg respectively. This increased intake produced no noticeable changes in the mild symptoms, exhibited by a few subjects, which might have been interpreted as due to vitamin C deficiency.

Ascorbic acid levels in serum and white cells. The young women had an average serum ascorbic acid level of 0.33 ± 0.04 mg per 100 ml of serum at the end of the unsupplemented period of 7 weeks. The older women had a slighty lower level of 0.24 ± 0.03 mg at the end of the unsupplemented period.

The young women had an average white cell ascorbic acid level of 25.6 ± 1.16 mg per 100 gm of white cells after the period on the low vitamin C intake, while the older group had an average white cell level of 22.2 ± 2.31 mg at the same time.

The results of supplementing the dietary intake for 5 two-week periods are given in table 1 and presented graphically in figure 1.

TABLE 1

Average ascorbic acid intakes, serum levels, and white cell levels.

Total intake	SERUM LEVELS		WHITE CELL LEVELS	
	Young women	Older women	Young women	Older women
mg/day	*mg/100 ml*	*mg/100 ml*	*mg/100 gm*	*mg/100 gm*
32	0.33 ± 0.04 [1]	0.24 ± 0.03	25.6 ± 1.16	22.2 ± 2.31
47	0.57 ± 0.04	0.45 ± 0.04	24.4 ± 1.21	23.2 ± 1.38
57	0.60 ± 0.04	0.54 ± 0.05	35.2 ± 1.80	29.5 ± 1.70
72	0.89 ± 0.07	0.84 ± 0.07	35.3 ± 1.46	34.9 ± 2.80
82	1.54 ± 0.08	1.16 ± 0.09	33.3 ± 1.33	34.6 ± 2.01
107	1.76 ± 0.07	1.42 ± 0.10	32.8 ± 0.80	34.7 ± 2.14

[1] Mean ± standard error.

DISCUSSION

The average rise in serum ascorbic acid for the group of young women was steady and was significant with every increasing level of intake except on the second level, namely, the 57-mg intake (see fig. 1). At this level of intake the white cell ascorbic acid rose significantly to its peak for the entire period, namely, about 35 mg per 100 gm of white cells.

Individual responses to increasing levels of intake were reflected in the serum of all of the young women on the first level of intake of 47 mg following a 15-mg supplementation. With the increase of an additional 10 mg, making a total of 57 mg, the white cell levels of all but one of the young women rose, while only 9 showed a rise of serum levels. The white

cell level of one woman had already reached its peak of 34 mg on the 47-mg intake. On the next three levels of supplementation all but one or two subjects showed a rise in serum level at each change in intake.

The average rise in serum ascorbic acid in the older women was steady and significant in each supplemental period. The average white cell ascorbic acid paralleled the serum ascorbic

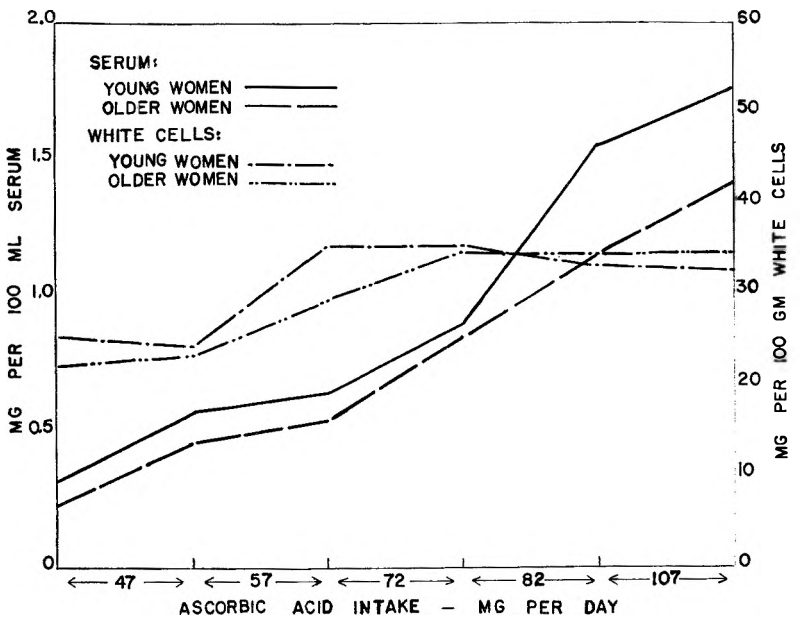

Fig. 1 Average values for ascorbic acid in blood serum and in white blood cells for 15 women near 31 years of age and 13 women near 64 years of age, on a dietary intake of 32 mg, with daily supplements of 15, 25, 40, 50, and 75 mg, each for a two-week period.

acid more closely than in the young group. It reached its peak of about 35 mg for the group on the 72-mg intake, as compared with the 35 mg on the 57-mg intake for the young women.

In the older group, individual responses were similar to those in the young group for serum ascorbic acid. All showed a rise on the 47-mg intake and on the 72-mg intake; all but

two rose on the other levels. Increase in white cell ascorbic acid was found in all but one subject on the 57-mg intake, and in all but three on the 72-mg intake when the average high point was reached.

The above discussion indicates that, at low levels of intake, small increases, such as 10 or 15 mg, resulted in a rise in serum ascorbic acid in the majority of instances. In this study, it seemed to be necessary to go above the level of 47 mg to get a significant average rise in white cell ascorbic acid. In the study by Steele et al. ('55) a significant rise in white cell ascorbic acid was found on increasing the intake from 30 to 40 mg. Their subjects had been maintained at a much lower level of intake previous to supplementation and for a longer period of time than the subjects in this investigation. The smaller increases in levels of intake of ascorbic acid in the present study showed that saturation of white cells may take place on a lower intake level than was exhibited in the earlier study, in which the peak was reached after an intake of 83 mg per day (Morse et al., '56).

Since the average white cell level of the young group reached its peak (35.2 mg) on the 57-mg intake, two weeks earlier than the older group, this might indicate that age had some influence on the rate of uptake of ascorbic acid by white cells. However, no significant difference due to age was noted when a *t* test was applied to the following data:

AVERAGE DAILY ASCORBIC ACID INTAKE	AVERAGE WHITE CELL LEVELS [1]	
	Young group	Older group
57 mg	34.8	29.0
72 mg	35.3	33.6
	$\overline{D}_2 = 0.518$	$\overline{D}_1 = 4.628$

The question is whether $\overline{D}_1$ is really greater than $\overline{D}_2$. It is not significantly so since $0.05 < P < 0.10$, or P lies between 0.05 and 0.10.

[1] These averages differ slightly from the average values given in table 1 because of using only complete pairs in calculating $\overline{D}$.

In contrast to the earlier study of serum and white cell ascorbic acid levels which showed significant correlation at the 33, 58, and 83-mg levels of intake in the group of 19 women (Morse et. al., '56), there was significant correlation in only two instances in the present study. The group of 15 young women showed correlation significant at the 5% level between serum and white cell ascorbic acid on the intake of 32 mg ($r = 0.60$) and also on the intake of 47 mg ($r = 0.60$). The smaller increases in level of supplementation used in this study and the smaller number of subjects may account for the lack of correlation in the other instances.

For the young group, the regression of white cell level, Y, on serum level, X, for the 86 pairs of determinations is:

$$Y = 26.56 + 4.83\ X$$

For the older group, the regression of white cell level, Y, on serum, X, for the 75 pairs of determinations is:

$$Y = 21.88 + 10.26\ X$$

The above equations for the separate groups of women show that the older group experienced a more pronounced rise in white cell ascorbic acid with respect to serum level than the young group, i.e., for every milligram rise in serum the white cells rose 10.26 mg. The regression for the two groups combined as one group is:

$$Y = 24.52 + 6.90\ X$$

A test for possible difference in regression between the two groups was made by the method of residual squares. This showed that age difference was not significant since the F value lies between 0.05 and 0.10.

SUMMARY

Following 7 weeks on a 32-mg intake of ascorbic acid the average serum ascorbic acid level of 15 young women was 0.33 mg per 100 ml. It rose to 1.76 mg during a period of 10 weeks in which the ascorbic acid intake was increased gradually to 107 mg per day. The average white cell ascorbic acid

rose from 25.6 mg per 100 gm of white cells to 35.2 mg during the first 4 weeks when the intake had reached 57 mg per day, and thereafter remained stationary.

The average serum ascorbic acid level for 13 older women, on the same levels of intake, rose from 0.24 to 1.42 mg during the 10-week period. Their average white cell ascorbic acid rose from 22.2 to 34.9 mg during the first 6 weeks when the intake had reached 72 mg per day, and thereafter remained stationary.

Correlation between serum and white cell ascorbic acid levels was significant only in the young group on intakes of 32 mg and 47 mg of ascorbic acid per day. There was no significant difference in uptake of ascorbic acid by the white cells in the young group as compared with that of the older group. Difference in regression of white cell level on serum level between the two groups was not significant.

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NUTRITIONAL PROPERTIES OF THE MOLECULARLY DISTILLED FRACTIONS OF AUTOXIDIZED FATS [1]

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In previous nutritional work on autoxidized fats, special attention was paid either to the polymer fraction left as a residue after molecular distillation (Kaunitz et al., '55) or to the whole autoxidized fat. It was found that the effects of the polymer fraction or of the whole autoxidized fat could be counteracted by the addition of fresh fat to the diet (Kaunitz et al., '55) and that the caloric requirement of the rat for weight maintenance was increased when such fats were consumed.

The distillate fraction of autoxidized fats, obtained by molecular distillation, had previously been studied only briefly and had not seemed to be particularly remarkable. The further studies to be reported below, however, show that this fraction is also of interest nutritionally.

EXPERIMENTAL

The studies were carried out on albino rats of a homogeneous colony. Weanling males, when they weighed 40 to 50 gm,

[1] Aided by a grant from Schenley Laboratories, Inc., and by a fellowship from Swift and Company.

[2] From the Department of Pathology and the Institute of Research in Animal Diseases, Columbia University, New York, N. Y.

[3] A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

were placed on a purified diet containing 30% of lactalbumin and 10% of fresh lard. At the age of 5 weeks, they were distributed into matching groups using procedures reported before (Kaunitz et al., '54).

Commercial lard and refined cottonseed oil were aerated at 95°C. for 200 to 300 hours and then distilled, using alembic distillation for the removal of volatile products, followed by molecular distillation. For the latter, temperatures up to 280°C. were employed. In one instance, a hydrogenated cottonseed oil which had been used for deep fat frying for 80 hours at 190°C. was distilled.

Unless otherwise stated, the experimental diets contained 30% alcohol-extracted casein, 10% fat, 54% dextrose, 4% salts (U.S.P. no. 2), and 2% cellulose, as well as liberal amounts of all known accessory food factors [4] in amounts described before (Kaunitz et al., '54).

RESULTS AND DISCUSSION

In figure 1 are given the average growth curves of groups of 8 male rats which had been maintained on diets containing various fats. The logarithm of the weight in grams is plotted against the reciprocal value of the age; the advantages of this method have been pointed out by Zucker and Zucker ('42).

The animals receiving autoxidized cottonseed oil lost weight rapidly and died after two to 4 weeks. When 10% of fresh fat was added to the diet containing 10% of the oxidized oil, none of the animals died during the period of observation; they were even able to grow. This has previously been described as the protective effect of fresh fat. One group of animals received 10% of the distillate from the molecular distillation of the sample of hydrogenated cottonseed oil which had been used for deep fat frying. These animals grew essentially as well as did those on fresh cottonseed oil. How-

[4] Doctor Leo A. Pirk of Hoffmann-La Roche, Inc., Nutley, New Jersey, very kindly supplied us with most of the synthetic vitamins used. Vitamin D_2 was supplied by the Sterling-Winthrop Research Institute, Rensselaer, N. Y., and the crystalline beta-carotene, by the Barnett Laboratories, Long Beach, California.

ever, when the distillate was combined with oxidized cottonseed oil, growth was significantly below that of the animals receiving both fresh and oxidized cottonseed oils. Also, in contrast to the latter group, some of the animals died toward

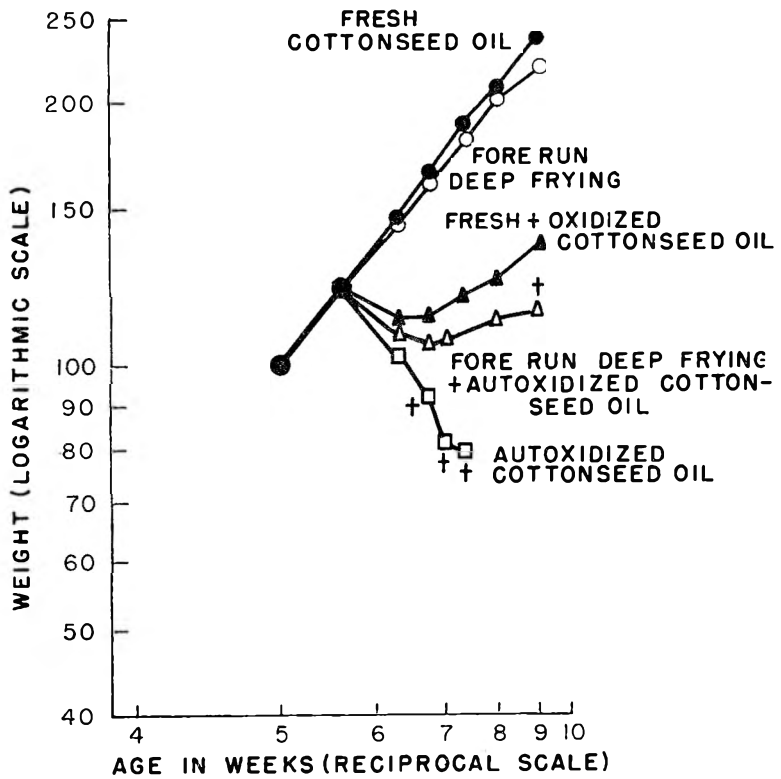

Fig. 1 Influence of the distillate from the molecular distillation of a hydrogenated vegetable oil after its use for deep frying for 80 hours. Each curve is based on the average of 8 well-matched male rats. After the third week of the experiment, the difference in weight of the groups fed oxidized plus fresh fat and oxidized plus distillate is statistically significant.

the end of the period of observation. Therefore, the distillate, while permitting nearly normal growth when included in the diet as the only fat, had lost a high degree of its protective effect.

Six very similar experiments were carried out with the molecular distillation fractions of highly autoxidized cotton-

seed oil or highly autoxidized lard. These distillates usually permitted good, although not quite optimum, growth when used as the sole fat source. Significantly, all of the distillates had lost their protective effect against highly autoxidized cottonseed oil to a degree very similar to that shown in figure 1.

This loss of protective effect could not have been caused by the molecular distillation process. The undistilled autoxidized cottonseed oil containing 40% polymeric "residue" and 60% "distillate" fraction led to rapid deterioration of the animals, whereas a mixture of 40% polymeric residue and 60% fresh oil permitted acceptable growth. Thus, the lack of protective action of the distillate was discernible before the oil had undergone the heating necessary for molecular distillation.

When rats, by daily weighing and restricted feeding, are maintained at a weight constant within 3 gm, it has been observed that the caloric requirements for such weight maintenance decline rapidly within the first few weeks if "good" diets are used (Quimby, '48). It has been shown (Kaunitz et al., '56) that the caloric requirements for weight maintenance do not decrease when the residue fraction of a molecularly distilled autoxidized fat is included in the diet. In figure 2 is shown a similar experiment with fresh fat and the molecular distillate of the hydrogenated vegetable oil which had been used for deep frying. The requirements are expressed as weekly calories per gram of body weight and are the average values for each group of 8 animals. For the calculation of the caloric values of the diets, it was assumed that a factor of 9.2 Cal. per gram could be used for both fats. It seemed reasonable to assume that the caloric value of the distillate did not differ greatly from that of normal fat because, when the distillate was included in a diet as the only fat source and the animals were permitted to eat freely, (1) the resulting growth was only slightly below that of animals fed fresh fat and (2) the food intakes were similar. However, even if

the caloric value of the distillate is slightly below that of the fresh lard, this would not lead to different conclusions.

As can be seen from figure 2, the caloric requirements of both groups declined steeply during the first 4 weeks of observation. The net energy value of the diet containing distillate was lower than that of the diet containing fresh fat, although the difference was not as pronounced as that between the groups fed polymeric residue and fresh fat. However, it

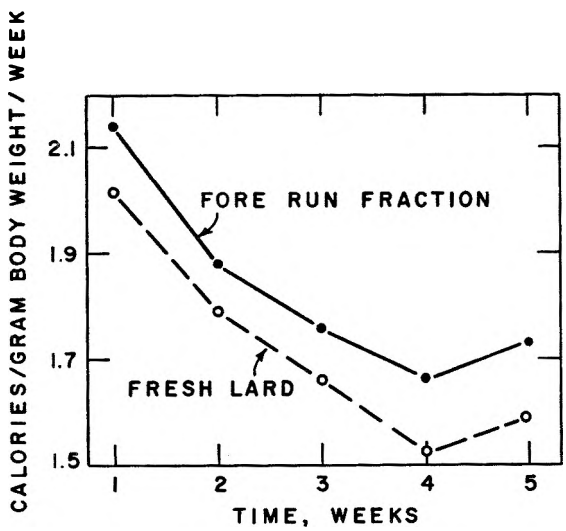

Fig. 2 Influence of fresh lard and the distillate from the molecular distillation of a hydrogenated vegetable oil previously used for deep frying for 80 hours upon the caloric requirements of matching male rats maintained at constant weight. Each curve is based on the average of 8 animals.

may be of some interest that, with a chemically altered but essentially atoxic fat, the animal's caloric requirement for weight maintenance is increased.

When the animals maintained at constant weight were sacrificed at the end of the experiment, their kidneys, livers, and adrenals were weighed. Figure 3 shows log — log plots of organ weights against body weights. The parallel lines give the limits of the spread in organ weight of male rats fed a complete, unrestricted diet. The weights of the livers and

kidneys of the animals on the distillate were within normal limits, although somewhat above those of the animals fed fresh fat. The adrenals of the two groups scarcely differed from one another. In contrast, the livers, kidneys, and adrenals of the animals given the residue fraction substantially

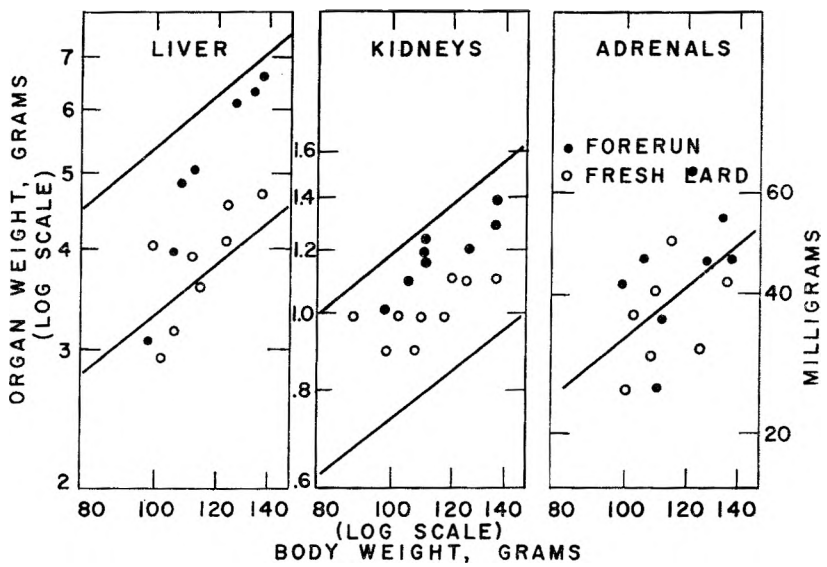

Fig. 3 Influence of fresh lard and distillate from the molecular distillation of a hydrogenated vegetable oil previously used for deep frying for 80 hours upon the organ weight-body weight relationships of male rats kept at constant weight by restricted feeding for 5 weeks. On the log — log plot, the parallel lines indicate the upper and lower limits of the spread in organ weight of rats with unrestricted intakes of a control diet containing fresh lard.

exceeded the upper limit of the normal (Kaunitz et al., '56). These results also show that the distillate itself is hardly toxic.

This low toxicity again became evident in studies with low-protein diets. In earlier work (Kaunitz, '53), it was pointed out that weanling rats placed on diets containing only 5% of casein and fresh fat maintained their weight for several weeks and grew slowly thereafter. Ten per cent of a sample of oxi-

dized lard which was atoxic to rats when included in a diet containing 30% of casein led to rapid weight loss and death when fed in a diet containing only 5% of casein. When 10% of the distillate was included in a diet with 5% of casein, growth of the rats was similar to that of the controls receiving fresh fat.

The chemical changes in the fats responsible for the described effects are not as yet understood. This problem is being actively investigated.

SUMMARY

1. Lard and refined cottonseed oil which had been aerated at 95°C. for 200 to 300 hours and a sample of hydrogenated vegetable oil which had been used commercially for deep fat frying for 80 hours at 190°C. were molecularly distilled at 280°C. The distillates were used in nutritional experiments.

2. When the distillates were included in purified diets containing either 5 or 30% casein, the resulting growth of most of the weanling male rats fed these diets was only slightly below that of matching rats receiving fresh lard.

3. In contrast, distillate added to the nonvolatile polymeric residue from the molecular distillation of autoxidized fats had a protective effect markedly below that of fresh fats.

4. The net energy value of the diet containing distillate was lower than that of the diet containing fresh fat.

5. Liver, kidney and adrenal weights of rats fed distillate were within the normal spread for these organs and were only slightly higher than those of the controls, thereby supplying additional evidence for the low toxicity, if any, of these fractions.

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Doctor Waldo C. Ault of the eastern Regional Laboratory of the U. S. Department of Agriculture has greatly helped this work with his advice, suggestions, and criticisms.

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SOME FACTORS AFFECTING CELLULOSE DIGESTION BY RUMEN MICROORGANISMS *IN VITRO*

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A previous report from this laboratory revealed that cellulose digestion by rumen microorganisms *in vitro* was markedly stimulated by such fishery by-products as whale solubles, herring solubles and herring stickwater as well as by a mixture of 18 amino acids (MacLeod and Brumwell, '54). Although much of the effect of the fishery by-products could be ascribed to their amino acid content, some evidence was obtained that other unknown factors might also be present and capable of stimulating cellulose digestion. The present study was undertaken to investigate this possibility further.

In the previous study a marked stimulation of cellulose digestion was obtained with the various supplements tested only when a more dilute inoculum of rumen liquid than it had previously been the custom to use (cf. Burroughs et al., '51) was employed. It was evident that additional information regarding the nutritional requirements of rumen microorganisms could be obtained only if an active inoculum washed as free as posible of rumen liquid was used.

Various compounds or groups of compounds have been reported to stimulate cellulose digestion by rumen microorganisms *in vitro*. These include glucose (Hoflund et al., '48) various water-soluble vitamins, purines and pyrimidines

(Hall et al., '53; Bentley et al., '54), an amino acid mixture (MacLeod and Brumwell, '54), urea (Belasco, '54), some steroid compounds (Brooks et al., '54) and certain short-chain fatty acids (Bentley et al., '55; Bryant and Doetsch, '55).

Using a well-washed inoculum of rumen microorganisms, a chemically defined medium has been developed incorporating at their optimum levels those factors found to be effective in this study in stimulating cellulose digestion. The medium developed was then used to determine whether various natural materials have a capacity to produce a further stimulation of cellulose digestion. In the course of this study it was established that three amino acids, namely leucine, isoleucine and valine were primarily responsible for the strong stimulation of cellulose digestion previously shown to be produced by a mixture of 18 amino acids (MacLeod and Brumwell, '54).

EXPERIMENTAL METHODS

In vitro rumen fermentations were carried out in a series of 18 × 150 mm test tubes essentially as described previously (MacLeod and Brumwell, '54). Cellulose digestion was measured by determining the difference in the weight of a roll of vegetable parchment (dialyzer paper) before and after fermentation.

Preparation of the inoculum. Inocula washed with varying degrees of thoroughness have been employed in certain of the more recent studies of cellulose digestion by rumen microorganisms *in vitro* (Bentley et al., '55; Cheng et al., '55).

The following procedure, developed in this laboratory, was found to give rise to a cellulolytically active, washed inoculum of rumen microorganisms. Rumen liquid obtained at an abattoir from the paunches of freshly killed cattle was strained through cheese-cloth to remove gross particles. The strained liquid was centrifuged at low speed (125 × G) in a Servall SS-1 centrifuge for 5 minutes to remove as much non-bacterial matter as possible. The supernatant liquid was then cen-

trifuged at high speed (25,000 × G) for 25 minutes to sediment bacterial cells. The cells were resuspended in a solution containing glucose, cysteine and the same salts as were present in the basal medium. The concentrations of the latter in the wash solution were the same as those in the basal medium while glucose was present at a concentration which would provide the optimum level of glucose to the fermentation medium when the inoculum was added to the tubes. The resuspended cells were centrifuged, the supernatant removed and the cells again suspended in the wash solution. This operation was repeated twice. The cells were finally diluted with a volume of wash solution usually equal to one-fifth of the volume of the rumen liquid from which the cells were originally obtained and 2.5 ml of this suspension was added to each assay tube.

To ensure that the inocula used from assay to assay had approximately the same initial activity, a means of comparing the activity of the various inocula was developed. The time required for a given volume of the washed suspension of rumen microorganisms to reduce a solution of triphenyl tetrazolium chloride under standard conditions was determined. In this test 2.5 ml of the washed suspension was added to 2.5 ml of a solution containing 0.25 ml of 0.1% triphenyl tetrazolium chloride, 0.3 ml of 10% glucose and 1.0 ml of M/15 phosphate buffer. The time required to reduce the dye at 40° C. was then determined. Reduction times obtained with the inocula were correlated with the rates of the corresponding fermentations. Inocula reducing the dye in times ranging from 4 to 6 minutes were found to be the most satisfactory. Suitable adjustments in the volume of the suspensions could usually be made to obtain inocula having reducing times falling within this range.

Basal medium. The composition of the chemically defined medium found in this study to be capable of promoting the best cellulose digestion by rumen microorganisms *in vitro* is presented in table 1. Cellulose was added to the medium as a roll of vegetable parchment. Glucose was incorporated

into the medium with the inoculum. The mixture of salts used was the same as that employed previously (MacLeod and Brumwell, '54). The remaining components of the medium were added at levels established by experiment to be optimum under the conditions employed in this study.

TABLE 1

Composition of fermentation medium

COMPONENT	AMOUNT/20 ml
Cellulose	500 mg
Glucose	20 mg
Urea	18.45 mg
Cysteine	15 mg
Amino acid mixture [1]	4.0 ml
Vitamin mixture [2]	1.0 ml
Salts A [3]	1.71 ml
Salts B [4]	0.17 ml
$CaCl_2$	0.646 mg

[1] Amino acid mixture: DL-alanine, 2 gm; L-aspartic acid, 400 mg; L-glutamic acid, 1 gm; DL-threonine, 400 mg; DL-serine, 400 mg; glycine, 200 mg; L-lysine, 400 mg; L-methionine, 200 mg; L-cystine, 200 mg; L-arginine, 400 mg; L-proline, 200 mg; L-histidine, 200 mg; DL-phenylalanine, 400 mg; L-tyrosine, 200 mg; L-tryptophan, 200 mg; L-valine, 200 mg; L-leucine, 200 mg; L-isoleucine, 400 mg, in total volume of 500 ml.

[2] Vitamin mixture: riboflavin, 10 mg; pyridoxal, 2 mg; calcium pantothenate, 10 mg; thiamine, 10 mg; niacin, 10 mg; *p*-aminobenzoic acid, 2 mg; biotin, 100 μg; folic acid, 100 μg; vitamin B_{12}, 15 μg, in a total volume of 100 ml.

[3] Salts A: Na_2HPO_4, 86 gm; $NaHCO_3$, 26.25 gm; KCl, 3.75 gm; NaCl, 3.75 gm; $MgSO_4$, 1.30 gm; in a total volume of 1000 ml.

[4] Salts B: $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 3.89 gm; $MnSO_4 \cdot 4H_2O$, 0.80 gm; $ZnSO_4 \cdot 7H_2O$, 1.43 gm; $CuSO_4 \cdot 5H_2O$, 0.625 gm; $CoCl_2 \cdot 6H_2O$, 0.337 gm, in 1000 ml of solution.

Fermentation technique. The technique employed in this investigation was only a slight modification of that described previously (MacLeod and Brumwell, '54). In the present investigation smaller test tubes (18 $\times$ 150 mm) were used. Each tube in each assay was prepared in duplicate. Before inoculation CO_2 gas was bubbled through the contents of each tube to produce anaerobic conditions and at the same time to lower the pH of the medium to the starting value of 6.9. To maintain anaerobic conditions and still provide a

means of making allowances for the production of fermentation gases, the tubes were capped with small rubber balloons.

It was found in preliminary studies that there was no particular advantage to adjusting the pH of the tubes during the course of the fermentation so long as the fermentation did not proceed too far. In this study the fermentation was terminated when visual inspection indicated that approximately 30 to 50% of the cellulose had digested in those tubes which were fermenting most rapidly.

EXPERIMENTAL

Response to amino acids. The previous study had revealed that cellulose digestion by rumen microorganisms was strongly stimulated by the addition of a mixture of 18 amino acids to the fermentation medium (MacLeod and Brumwell, '54). A similar response to amino acids was obtained using a washed inoculum of rumen microorganisms and the level of the mixture producing maximum stimulation in the medium was approximately the same as found previously to be optimum. To determine which of the amino acids in the mixture were responsible for the effect obtained, the 18 amino acids were first subdivided into three groups of 6 each. These groups were tested alone and in combination for their ability to promote cellulose digestion. It can be seen, table 2, that only one of the three groups, group C, was active and in this experiment it proved to be more active than the complete mixture. When the 6 amino acids in group C were divided into the two sub-groups C_1 and C_2, sub-group C_2 was found to have the same activity as that of C. Sub-group C_2 contained leucine, isoleucine and valine. Omission of each of the amino acids in turn from group C_2 indicated that each was making a contribution to the response produced by the mixture in this experiment.

Because the types of organisms composing the rumen population can be present in the rumen in differing proportions (Gall et al., '53) especially if the feeding regimen of the animals is not well controlled, one might expect some varia-

tion in the response of the population to growth factors from one time to another. For this reason, experiments of the type illustrated were repeated using inocula obtained from rumen liquid collected on numerous different occasions. On each occasion the sub-group containing leucine, isoleucine and valine was found to be responsible for most or all of the activity of the complete mixture. When the three amino acids

TABLE 2

The effect of groups of amino acids on cellulose digestion by rumen microorganisms in vitro

ADDITIONS TO MEDIUM [1]	% CELLULOSE DIGESTED [2]
None	7
Complete mixture	23
Group A	8
Group B	3
Group C	35
Group C_1	6
Group C_2	32
C_2 minus valine	18
C_2 minus leucine	8
C_2 minus isoleucine	12

[1] The medium of table 1 was used with the amino acid mixture omitted and the total nitrogen content of each tube maintained at 17.5 mg by the addition of an appropriate level of urea.

The various groups shown contained the following amino acids: A — alanine, aspartic acid, glutamic acid, threonine, serine, glycine; B — lysine, methionine, cystine, arginine, proline, histidine; C — phenylalanine, tyrosine, tryptophan, valine, leucine, isoleucine; C_1 — phenylalanine, tyrosine, tryptophan; C_2 — valine, leucine, isoleucine.

[2] Incubation time = 72 hours.

were tested alone and in combination on the different occasions, however, somewhat more variable results were obtained. Usually the three amino acids together were better than each tested singly or in pairs. In one case, however, the three amino acids were all equally active when tested individually while in another certain pairs were as good as all three. At no time was it found possible to obtain appreciable cellulose digestion without any of the three amino acids being present.

The results of the experiment recorded in table 2 indicate that in this case the complete amino acid mixture was less effective than the combination of leucine, isoleucine and valine in promoting cellulose digestion. In general, however, the complete mixture had at least the same and sometimes slightly more activity than the more limited number of amino acids. For this reason the complete mixture of amino acids rather than the combination of leucine, isoleucine and valine alone was included routinely in the preparation of the basal medium.

TABLE 3

A comparison of the ability of certain amino acids and fatty acids tested alone and in combination to promote cellulose digestion by rumen microorganisms in vitro

ADDITIONS TO MEDIUM [1]	% CELLULOSE DIGESTED [2]
None	29
Valine + leucine + isoleucine (12 μM of each)	51
Valine + leucine + isoleucine (24 μM of each)	47
Valeric + isovaleric acids (12 μM of each)	38
Valine + leucine + isoleucine + valeric acid + isovaleric acid (12 μM of each)	44

[1] See table 2.

[2] Incubation time = 91 hours.

Bentley et al. ('55) found that cellulose digestion by rumen microorganisms could be stimulated by certain short-chain fatty acids present in rumen liquid as well as by proline and valine. Bryant and Doetsch ('55) observed that *Bacteroides succinogenes,* a cellulolytic bacterium isolated from the bovine rumen, required a combination of a 5- to 8-carbon straight-chain fatty acid and a branched-chain fatty acid which could be either isobutyric, isovaleric or dl-α-methyl-n-butyric acid for growth. It was of interest to know whether the effect of these volatile fatty acids on cellulose digestion by rumen microorganisms was related to or independent of the response of the organisms to leucine, isoleucine and valine.

The results in table 3 show the response to a combination of valine, leucine and isoleucine when these were tested at the level at which they were present in the complete mixture used in the basal medium. Doubling the concentration of the amino acids had a slightly inhibitory effect. Valeric and isovaleric acids tested in combination at the same level as the three amino acids promoted cellulose digestion but not to quite the same extent as the amino acids. The addition of the fatty acids to a medium containing the three amino acids caused no further stimulation of cellulose digestion but actually a decrease similar to that obtained by adding an excess of the three amino acids.

There was thus no evidence of synergism when the amino acids and the short-chain fatty acids were tested together, but rather it would appear from these results that the two types of compounds were being used interchangeably in promoting cellulose digestion by the rumen microorganisms.

Response to vitamins. Hall et al. ('53) found that biotin and vitamin B_{12} were stimulatory to cellulose digestion *in vitro,* while Bentley et al. ('54) reported a response to biotin, vitamin B_{12} and *p*-aminobenzoic acid.

A mixture of 9 vitamins when added to the fermentation medium used in this study consistently stimulated cellulose digestion by the rumen microorganisms. Some difficulty, however, was encountered in establishing which vitamins singly or in combination contributed to the stimulation produced by the mixture. The results of one experiment, shown in table 4, reveal that on this occasion the omission of either pyridoxal, thiamine or niacin lowered the response of the micro-organisms to the vitamin mixture. In other experiments folic acid and *p*-aminobenzoic acid also appeared to play a role. Throughout these experiments, pyridoxal (replaceable by pyridoxamine or pyridoxine) was consistently effective in stimulating cellulose digestion. To ensure maximum stimulation from the water-soluble vitamins, the complete mixture was included in the basal medium at a level slightly in excess of that producing an optimum response.

Response to glucose. Hoflund et al. ('48) found that 0.1 to 0.2% of glucose in the medium stimulated cellulose digestion by rumen microorganisms *in vitro* while higher levels depressed it.

The effect of adding increasing concentrations of glucose to the fermentation medium used here is shown in table 5. In this experiment glucose was omitted from the wash solution used to prepare the inoculum.

TABLE 4

Effect of vitamins on cellulose digestion by rumen micro-organisms

ADDITIONS TO MEDIUM [1]	% CELLULOSE DIGESTED [2]
None	20
Complete vitamin mixture	36
minus riboflavin	33
minus pyridoxal	27
minus calcium pantothenate	35
minus thiamine	27
minus niacin	29
minus *p*-aminobenzoic	34
minus biotin	36
minus vitamin B_{12}	34
minus folic acid	35

[1] The medium of table 1 was used with the vitamin mixture omitted.
[2] Incubation time = 64 hours.

TABLE 5

Effect of increasing levels of glucose on cellulose digestion by rumen microorganisms in vitro

GLUCOSE ADDED [1]	% CELLULOSE DIGESTED [2]
mg/20 ml	
0	19
10	25
20	24
30	29
40	24
80	22
160	19

[1] The medium of table 1 was used with glucose omitted.
[2] Incubation time = 72 hours.

The results show that stimulation of cellulose digestion was obtained in the same range of glucose concentrations as that observed by Hoflund et al. The optimum response was obtained over a range of 10 to 40 mg per tube (0.05 to 0.2%) while decreased effects were observed at higher concentrations. Although the magnitude of the stimulation was not great, it was found that the routine inclusion of glucose at a 0.1% level in the medium greatly improved the reproducibility of all assay results.

TABLE 6

The relation of the cellulolytic factor added to the response of rumen microorganisms to urea

UREA ADDED [1]	CELLULOLYTIC FACTOR ADDED		
	Isovaleric + valeric acids	Amino acids Restricted [2]	Amino acids Complete [3]
mg/20 ml	*% cellulose digested* [4]		
0	11	16	27
18	27	26	36
27	30	35	38
36	32	39	42
45	34	38	25
54	28	23	0

[1] The basal medium used was that of table 1 with urea and the amino acid mixture omitted.

[2] Leucine + isoleucine + valine added at the level present in the complete mixture.

[3] Complete mixture of 18 amino acids (see table 1).

[4] Incubation time = 89 hours.

Response to urea. The presence of urea along with various food proteins in *in vitro* rumen fermentation studies has been found to improve cellulose digestion over that observed with the food proteins alone (Belasco, '54). In the present study, the level of urea needed for maximum stimulation of cellulose digestion varied depending on the "cellulolytic factor" employed. More was required if the combination of valeric and isovaleric acids was added than if amino acids were used, table 6. Even in the presence of the mixture of 18 amino acids the addition of urea was quite strongly stimulatory. Further increases in urea concentration beyond the level re-

quired for maximum activity brought about a decrease in the amount of cellulose utilized, in a manner similar to that observed by Belasco ('54). In the presence of the complete amino acid mixture, not only was less urea required for maximum cellulose digestion but also less was needed to produce inhibition. Thus there is a comparatively narrow range of nitrogen concentration producing optimum cellulose digestion, a factor to be considered when testing the growth-promoting activity of natural materials.

TABLE 7

Effect of various supplements on cellulose digestion by rumen microorganisms in the complete synthetic medium

SUPPLEMENT TO SYNTHETIC MEDIUM [1]	EXP. 1	EXP. 2	EXP. 3
		% cellulose digested	
None	33	41	46
Whale solubles	42	45	52
Herring solubles	35	41	54
Yeast extract	37	40	44
Enzymatic casein	36	41	36
Beef liver extract	41	48	..
Malt extract	28	38	..
Herring stickwater	..	..	51

[1] The complete medium of table 1 was used as the basal medium. Each supplement was tested at a series of levels providing from 1.7 to 13.1 mg of nitrogen per tube.

Effect of miscellaneous supplements. Purines and pyrimidines as well as various steroid compounds have been reported to stimulate digestion by rumen microorganisms *in vitro* (Bentley et al., '54; Brooks et al., '54). Adenine, guanine, uracil and xanthine added alone and in combination to this basal medium at a level of 400 µg per tube had no stimulatory effect on the fermentation. The addition of cholesterol at a level of 400 µg per 20 ml of medium both alone and in combination with Tween 40 likewise did not affect cellulose digestion under the conditions used here.

Addition of natural materials to the fermentation medium. When all of the compounds found in this study to be active in

promoting cellulose digestion were combined at levels producing maximum activity, the medium shown in table 1 was obtained. To determine whether additional factors were active in stimulating cellulose digestion, supplements of various natural materials were added to the synthetic medium. The results obtained in three different experiments are summarized in table 7. Since the margin between the optimum and the inhibitory level of nitrogen in the medium was small and somewhat variable, each supplement was tested at a number of different levels in each experiment. The level of supplement at which a maximum response was obtained varied from one experiment to the next. The results reported in table 7 represent the maximum response obtained for each supplement in each experiment. It can be seen that certain of the supplements, particularly whale solubles and beef liver extract produced an appreciable stimulation of cellulose digestion over that obtained in the synthetic medium alone. The amounts of supplement required to produce a response, however, were quite large, ranging from 30 to 60 mg per tube.

DISCUSSION

In this study leucine, isoleucine and valine have been found to be interchangeable with short-chain fatty acids in promoting cellulose digestion by rumen microorganisms *in vitro*. Bentley et al. ('55) have found valine to be active in stimulating cellulose digestion in the absence of fatty acids. In studying the growth requirements of *Bacteroides succinogenes*, a cellulose digesting microorganism isolated from the bovine rumen, Bryant and Doetsch ('55) obtained a response of the organism to short-chain fatty acids in a medium containing an enzymatic digest of casein. The same brand of enzymatic casein at the level used by the latter authors has been found in this study to produce maximum cellulose digestion by rumen microorganisms and all attempts to increase the extent of digestion by adding either leucine, isoleucine and valine or short chain fatty acids have been unsuccessful. The difference in response obtained can probably be ascribed

to the fact that in the cases where amino acids and fatty acids have been found to be interchangeable a mixed culture of rumen microorganisms was used. Hungate ('50) has described 7 and Huhtanen and Gall ('53) 9 different bacteria or strains of bacteria isolated from the bovine rumen capable of digesting cellulose or fibre. It is of course possible that fatty acids are able to stimulate one and the amino acids another of these strains or group of strains. Knowledge of the growth requirements of other cellulolytic bacteria which have been isolated from the rumen in pure culture should prove of interest in connection with this possibility. The similarity in structure of the active amino acids and fatty acids, however, appears to be more than coincidental and it is likely that, in the mixed culture, organisms associated with the cellulose digesters can convert the appropriate amino acids to the specific fatty acids required by at least one strain of cellulose digesting microorganism. Evidence for the production of volatile fatty acids from amino acids by microorganisms from the rumen of sheep has been demonstrated by El-Shazly ('52). Of particular interest in connection with the work reported here is the fact that the latter author has postulated that the branched-chain C_4 and C_5 volatile fatty acids in the rumen are formed from valine, leucine and isoleucine as a result of Stickland type reactions.

Whether the response to supplements of natural materials, which was obtained in the fermentation, is due to one or more specific factors present in the supplements or to a more generalized response to the addition of large quantities of preformed growth factors to the medium has not been established. The relatively large amounts of the supplements required to obtain the responses observed suggests that the latter explanation could account for the effect. To make it profitable to pursue this phase of the problem further inocula obtained under more reproducible conditions than are presently available to this laboratory would be required.

SUMMARY

Using a washed inoculum of rumen microorganisms a chemically defined medium has been developed incorporating at their optimum levels those factors found to be effective in this study in stimulating cellulose digestion. In this medium responses to amino acids, short-chain fatty acids, various vitamins, glucose and urea have been demonstrated.

A combination of valine, leucine and isoleucine was found to be primarily responsible for the strong stimulation of cellulose digestion previously shown to be produced by a mixture of 18 amino acids. The combination of leucine, isoleucine and valine could be used interchangeably with and was somewhat more effective than the volatile fatty acids tested in promoting cellulose breakdown.

Of the various vitamins tested, vitamin B_6 was the most consistent in its ability to stimulate cellulose digestion by rumen microorganisms in the medium used.

The level of urea required for maximum stimulation of cellulose digestion varied depending on the cellulolytic factor employed. More was required if the combination of valeric and isovaleric acids was added than if amino acids were used. Optimum cellulose digestion occurred over a comparatively narrow range of nitrogen concentration in the medium. Above this range inhibition occurred.

When various natural materials, in particular whale solubles and beef liver extract, were added to the synthetic medium, some further stimulation of cellulose digestion over that obtained in the synthetic medium alone resulted. Relatively large amounts of the supplements were required to produce the additional response.

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THE AMINO ACID REQUIREMENT OF THE LAYING HEN

I. THE DEVELOPMENT OF A FREE AMINO ACID DIET FOR MAINTENANCE OF EGG PRODUCTION [1]

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Very little is known about the amino acid requirements of the laying hen (Bird et al., '54; Almquist, '52). This paucity of information has been primarily the result of an inability to formulate a free amino acid diet on which hens would maintain egg production. Attempts to formulate such a diet have been reported only by Wisconsin workers (Ingram et al., '50a) who found that egg production ceased in two to 4 days when hens were fed amino acid mixtures. Grau and associates ('48, '49) have also reported difficulties with purified diets for laying hens.

It is the purpose of the present report to discuss studies from this laboratory that have led to the successful formulation of a free amino acid diet suitable for studying the qualitative and quantitative amino acid requirements of the chicken for egg production.

EXPERIMENTAL

Single Comb White Leghorn hens from the University flock which were laying in clutches of two eggs or more were used

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in these studies. The birds were maintained in individual cages in a temperature regulated room and allowed water ad libitum. When not on an experimental diet, the hens were fed an all-mash ration.[2]

In view of the findings by Almquist ('47) that chicks fed free amino acid diets exhibited depressed appetites, birds were force-fed in the early experiments in order to maintain a constant nutritive intake for body maintenance and egg formation. Force-feeding was carried out at the rate of

$$Y = 37\ W^{0.7}$$

where Y = grams of diet per day

and W = body weight to the nearest 0.1 lb. at the beginning of an experiment. This formula is based on an arbitary feed intake of 25 gm per pound body weight for a 3.6 lb. bird, laying at a normal rate.

The force-feeding was accomplished with the aid of a metal funnel attached to a $\frac{3}{8}$ in. polyethylene tube, the latter extending into the crop. The diet was mixed in a beaker with equal parts of water by weight and was then poured into the funnel. Diet adhering to beaker and funnel was rinsed out with a wash bottle. When force-feeding was employed, birds were allowed access to the diet and were fed 6 times per day at two-hour intervals.

Five different basal diets were employed in the development of the adequate diet and are given in table 1. The amino acids essential for the chick (Almquist and Grau, '44), arginine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine together with alanine, aspartic acid, cystine, proline, serine and tyrosine were used to develop the diet. The amino acid components were thoroughly mixed with the basal diet preceding each experimental trial and the total made up to one

[2] Ground yellow corn, 35.5; ground wheat, 20; ground oats, 20; soybean oil meal, 5; meat scrap, 7.5; alfalfa meal, 6; steamed bone meal, 2; mineral concentrate (Mico. Limestone Corporation of America), 3; salt, 0.35; vitamin A & D oil, 0.4; vitamin B_{12}-antibiotic feed supplement, 0.25.

hundred parts with the addition of glucose (cerelose) or starch. Sodium bicarbonate was added to neutralize the HCl radicals of the basic amino acids on a molecular basis at the beginning of the study; later bicarbonate addition was standardized at 1% in all diets.

Two birds were used per treatment; these were repeated until satisfactory conclusions could be drawn.

TABLE 1

Composition of basal diets

INGREDIENT	DIET				
	A	B	C	D	E
	%	%	%	%	%
Glucose (cerelose)	56.96	31.96	18.96	....	....
Corn starch	....	20.00	30.00	51.96	51.96
Corn oil	5.00	5.00	8.00	10.00	12.00
Fiber	5.00	5.00	5.00	5.00	3.00
Mineral mix [1]	5.34	5.34	5.34	5.34	5.34
Limestone	2.50	2.50	2.50	2.50	2.50
Vitamin A, D and E concentrate [2]	0.10	0.10	0.10	0.10	0.10
Choline Cl	0.10	0.10	0.10	0.10	0.10
Vitamin mix [3]	0.15	0.15	0.15	0.15	0.15
Variable ingredients (amino acids, antiacid adsorbent,[4], bicarbonate, starch)	24.85	29.85	29.85	24.85	24.85
	100.00	100.00	100.00	100.00	100.00

[1] Percentage of diet: $CaCO_3$, 0.3000; $Ca_3(PO_4)_2$, 2.8000; K_2HPO_4, 0.9000; $MgSO_4 \cdot 7H_2O$, 0.2500; $Fe(C_6H_6O_7)_2 \cdot 6H_2O$, 0.1400; $ZnCl_2$, 0.0020; KI, 0.0040; $CuSO_4 \cdot 5H_2O$, 0.0020; H_3BO_3, 0.0009; $CoSO_4 \cdot 7H_2O$, 0.0001; $MnSO_4$, 0.0650; NaCl, 0.8800.

[2] Supplies per kilogram of diet: 10,000 IU vitamin A, 600 IU vitamin D_3 and 5 IU alpha tocopheryl acetate.

[3] In milligrams per kilogram of diet: thiamine HCl, 25; riboflavin, 16; Ca pantothenate, 20; vitamin B_{12}, 0.02; pyridoxine HCl, 6; biotin, 0.6; folic acid, 4; inositol, 100; *p*-amino benzoic acid, 2; 2-methyl naphthoquinone, 5; ascorbic acid, 250; niacin, 150.

[4] Gelusil, an aluminum hydroxide-magnesium trisilicate preparation, courtesy of Warner-Chilcott Laboratories, New York, N. Y.

RESULTS

The first amino acid mixture to be tried with basal diet A was based on the published requirements for the growing chick. The force-feeding of this diet resulted in immediate stasis of the digestive tract and coma developed after two days. Upon being sacrificed, the birds showed hemorrhagic and necrotic areas in the liver. Crop and proventriculus were also affected, as indicated by many bleeding ulcers. The stasis of the digestive tract was similar to that described by Spector and Adamstone ('50) in rats on an amino-acid-deficient diet.

Noticeable improvement in the general condition of the birds was achieved upon the addition of higher levels of isoleucine and lysine as well as by a change in the source of the amino acids.[3] Supplementation with 1% of an aluminum hydroxide-magnesium trisilicate preparation,[4] originally introduced to control the ulcer condition, further improved the diet. With these changes, coma, liver necrosis and ulcer formation were prevented and food passage was normal; nevertheless, egg production always ceased within 4 days, as had been the experience of Ingram et al. ('50a) under ad libitum feeding conditions.

Caloric intake. The greatest improvements toward a successful ration were made by increasing the energy content. Basal diet B contained 20% starch added at the expense of an equal amount of glucose (cerelose).[5] This change resulted in continued egg production for 6 to 7 days instead of the 4 day limit previously observed. In basal diet C, energy was further increased by raising the starch level at the expense of glucose, as well as by a substantial increase in the corn oil from 5 to 8%. On this diet one bird maintained egg production for the entire two-week experimental period, laying 10

[3] With the exception of glycine, arginine, and methionine, all amino acids were henceforth purchased from Nutritional Biochemicals Incorporated, Cleveland, Ohio.

[4] Gelusil, courtesy of Warner-Chilcott Laboratories, New York, N. Y.

[5] The change provided a small increase in caloric intake since Anderson and Hill ('55) have indicated that cornstarch provides 10% more metabolizable energy than glucose (cerelose).

eggs in 14 days. To the authors' knowledge, this is the first report of a hen maintaining normal production for this length of time on a diet of free amino acids.

As a result of these experiments it was apparent that most of the difficulty in maintaining egg production on amino acid diets was related to the caloric intake of the birds. Rose, Coon and Lambert ('54) have shown that humans require progressively more energy to maintain positive nitrogen balance as the diet is changed from casein to hydrolyzed casein to an amino acid mixture.

Basal diet D was next employed; it contained starch as the only carbohydrate and 10% of corn oil. It soon became evident that this diet was rather well balanced, since most, but not all, hens began eating it by themselves at a normal rate, at the same time maintaining normal egg production over the two-week experimental period.[6]

A typical experiment showing ad libitum feed consumption and egg production for 10 hens on this diet is shown in table 2. It will be noticed that all birds that ate well *throughout* the experimental period continued to lay, while those that did not consume enough nutrients did not maintain production. This was confirmed in several other experiments not listed here. Thus, the adequacy of a diet seems to be reflected in the voluntary feed consumption of hens during the first week. Egg production may not be affected until the second week due to the presence of ova in various stages of development as well as other protein stores. This is best illustrated in table 3 which demonstrates that birds on imbalanced diets laid only a few eggs and nearly all of them stopped production within the first week. In view of these consistent findings, a two-week experimental period was chosen for these studies.

To determine the optimum dietary energy level, 12 and 15% corn oil additions were next studied. The 15% level

[6] Egg quality studies revealed no differences in size or quality before, during, and after the experimental period. Ingram et al. ('50b) have found that dietary changes in amino acids do not influence egg composition.

of corn oil appeared too high, since birds placed on this diet consumed less feed and laid at a much reduced rate. On the other hand, the birds on the 12% corn oil level laid at a good rate (9 eggs/bird/14 days), their production being signifi-

TABLE 2

Egg production and feed consumption on a diet of free amino acids

BIRD NO.	DAILY EGG PRODUCTION DURING 14-DAY EXPERIMENTAL PERIOD														TOTAL EGGS
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
861	x	x	x											x	4
899	x	x	x		x	x	x		x	x		s[1]			9
887	x	x		x	x	x				x					6
883	x		x	x	x		x	x		x	x	x	x		10
897		x	x		x		y	s		x	s		x	x	9
873	x	x	x		x	x			x	x	x	x	x	x	11
865	x		x	x		x	x		x	x		x	x		9
868	x	x	x		x	x									5
880	x		x	x		x	x		x		x	x	x		9
898	x		x	x	x	x		x	x		x	x		x	10
	DAILY FEED CONSUMPTION (gm/day)														AV.
861	6	25	30	77	83	106	93	86	84	90	86	87	83	85	73
899	102	87	72	75	98	98	91	101	99	77	81	78	93	86	88
887	68	59	62	92	84	61	102	76	70	66	75	83	105	81	77
883	54	75	86	86	95	107	113	93	100	100	95	78	100	95	91
897	56	67	34	97	109	98	100	101	97	109	100	97	129	102	93
873	48	78	105	120	101	79	142	109	118	120	113	89	88	103	101
865	65	41	73	68	100	92	77	88	62	59	74	77	63	67	72
868	31	41	55	63	31	52	57	21	27	50	60	82	61	51	49
880	0	48	72	51	117	68	136	100	51	108	96	63	122	95	80
898	55	110	108	108	107	75	108	116	94	98	98	74	88	91	95

[1] Soft shell egg.

TABLE 3

The effect of dietary amino acid and energy imbalances on the number of eggs laid

NUMBER OF EGGS LAID BEFORE PRODUCTION CEASED	NUMBER OF BIRDS	DAYS BEFORE PRODUCTION CEASED
1	4	1–2[1]
2	12	2–4
3	20	4–6
4	8	5–7
5	1	8
6	1	9

[1] Range.

cantly greater ($P < 0.05$) than that of the birds on the 15% corn oil diet. Basal diet E containing 12% corn oil was therefore adopted as the basis of a ration which would contain a balance of amino acids suitable for the maintenance of egg production in a large proportion of the birds.

TABLE 4

Composition of successful free amino acid diet

INGREDIENT		AMINO ACIDS [3]	
	%		%
Corn starch	55.91	DL-alpha Alanine	1.0
Corn oil	12.00	L-Arginine HCl	1.3
Fiber	3.00	L-Aspartic acid	0.5
Mineral mix [1]	5.34	L-Cystine	0.3
Limestone	2.50	L-Glutamic acid	3.5
Vitamin A, D and E		Glycine	1.0
concentrate [1]	0.10	L-Histidine HCl	0.6
Choline Cl	0.10	DL-Isoleucine	2.0
Vitamin mix [1]	0.15	L-Leucine	1.4
Antiacid adsorbent [2]	1.00	L-Lysine HCl (95%)	1.2
Sodium bicarbonate	1.00	DL-Methionine	0.4
	81.10	DL-Phenylalanine	1.0
		L-Proline	0.5
		DL-Serine	1.0
		DL-Threonine	1.0
		DL-Tryptophan	0.4
		L-Tyrosine	0.6
		DL-Valine	1.2
			18.9

[1] For composition see table 1.

[2] Gelusil, aluminum hydroxide-magnesium trisilicate.

[3] The arginine and glycine used were generously supplied by Merck & Co., Rahway, N. J ; DL-methionine was obtained through the courtesy of Dow Chemical Corp., Midland, Mich. All other amino acids were purchased from Nutritional Biochemicals Inc., Cleveland, Ohio.

Such a complete diet was developed and is listed in table 4. Typical results with this diet are shown in table 5. It can be seen that bird 878, which discontinued laying for 8 days, apparently consumed too little feed at the outset of the experiment; upon adjusting to a normal feed intake egg production was resumed. Thus, this diet is considered adequate

for maintaining normal egg production in most hens, enabling one to study the qualitative and quantitative amino acid requirements of the laying hen.

TABLE 5

Egg production and feed consumption on a diet of free amino acids

BIRD NO.	REPLICATE	DAILY EGG PRODUCTION DURING 14-DAY EXPERIMENTAL PERIOD													TOTAL EGGS
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
857	1	x	x	x	x		x	x	x		x	x	x	x		11
874		x			x		x		x	x		x	x		x	8
908	2	x		x	x			x	x		x	x			x	8
878			x	x									x		x	4
		DAILY FEED CONSUMPTION (gm/day)														AV.
857	1	96	108	100	118	114	109	102	86	109	101	106	99	82	85	101
874		49	87	94	79	84	93	89	71	84	80	71	61	61	65	76
908	2	74	76	87	96	117	131	82	113	96	120	99	73	83	110	97
878		23	21	27	78	101	94	118	107	123	122	134	118	126	126	94

DISCUSSION

The levels of amino acids used in this diet are not intended to represent the amino acid requirement of the laying hen; nor has an attempt been made to establish the most efficient levels of amino acids for egg production. Such data will be reported at a later time. Rather, the levels used in this diet represent only an acceptable balance of amino acids which will maintain egg production.

The level of lysine in particular is higher than the requirement of 0.52% of the diet reported by Ingram et al. ('51). Attempts to decrease the level below 0.91% (actual L-lysine) failed to maintain egg production. Calculations from practical diets would suggest that the requirement for L-lysine is not as great as the level used in this diet would indicate. This discrepancy requires further study to clarify the factors involved in the utilization of this amino acid.

Similarly, the isoleucine level of this diet is higher than the requirement for isoleucine as determined by Miller et al. ('54) on a practical diet.

Microbiological assay of the lysine and isoleucine samples yielded correct analytical values which suggests that impurities are not involved in this problem.

With the development of a complete diet that would maintain egg production, an attempt was made to evaluate the results obtained in the early studies. On repetition of these studies, with amino acids from a different source, it was found that the coma, necrotic liver, and also the ulcerative conditions were probably due to contamination in one or more amino acids. The possibility of heavy metal contamination and poisoning is suggested particularly by the liver condition.

Despite the fact that amino acids from a particular source seemed to account also for the ulcerative condition, it was interesting to note that an antiacid adsorbent [7] continued to be a necessary constituent of the present diet. Attempts to eliminate it from the diet resulted in early stoppage of feed consumption and egg production. The beneficial effect of this product lies possibly in its magnesium content since Wisconsin workers (Heinicke et al., '56; Benton et al., '55) have shown the importance of added magnesium in the utilization of amino acids for the growth of the guinea pig and a similar growth-promoting effect in chicks on free amino acid diets upon the addition of the ash from gelatin.

Sodium bicarbonate was also necessary for the success of the diet. The action of sodium bicarbonate may be to neutralize the HCl radical on the basic amino acids, although Rose et al. ('50) have found in their human studies that neutralization of the acid group was not essential. Another action might be to bring the sodium-potassium ratio into more acceptable balance. Heinicke et al. ('56) have also demonstrated a beneficial effect from extra potassium supplementation on amino acid utilization in the guinea pig.

[7] See footnote 4, page 263.

Because of the high cost of this diet, only a relatively small number of animals could be employed in each experiment. This has not been a serious handicap in this and the following studies since agreement among birds on the same treatment has generally been excellent. The two-week experimental period was chosen as sufficient time to demonstrate the adequacy of a diet to maintain egg production. As already demonstrated by Ingram et al. ('50a), hens receiving an incomplete diet will stop production in a very short period. That this finding was not restricted to free amino acid diets is illustrated in table 6 showing the same poor performance of

TABLE 6

Effect of amino acid-deficient protein diet on egg production

BIRD NO.	TYPE OF DIET	EGG PRODUCTION	
		No added leucine	0.5% added leucine
882	15% Drackett protein	2 [1]	5 [2]
885	15% Drackett protein	3 [1]	8 [2]

[1] Stopped laying within 4–5 days.

[2] Number of eggs laid in 10-day period after recovery from leucine-deficient period; when returned to practical diet, production continued.

hens on a leucine-deficient soybean [8] protein diet. Later studies (Johnson and Fisher, '56) showed that birds could be continued on these amino acid diets for 30 days without any change in rate of production.

In accordance with existing custom, the amino acid content of the diet has been given as a percentage of the total diet. Although this expression of the amino acid levels is adequate here, especially since the weight range of the birds was a very narrow one, it is entirely unsatisfactory for quantitative expression. Energy requirement is proportional to a power of body weight; yet for maintenance purposes Rose and associates have never found any relationship between body weight

[8] Drackett Assay Protein. The Drackett Products Company, Cincinnati, Ohio.

or surface area and amino acid requirement. More important with regard to the hen, the amino acid requirements for a single egg are not dependent upon the body weight of the hen laying that egg but total feed consumption is dependent on body weight, which again emphasizes the importance of a better expression for the amino acid requirement than percentage of the diet. Therefore, it is proposed that the amino acid requirement for egg production be expressed in grams per day. The body weight and weight of eggs produced in a given length of time should be reported also to evaluate the effect of these factors on the total requirement. When enough data of this type become available in the future, the proper functions relating maintenance and production requirements can be formulated for each amino acid. This practice will be adopted in the quantitative aspects of this study in this laboratory.

Mention should be made of the "appetite" or "taste" for the amino acid diets exhibited by the hens. It seems abundantly clear that taste plays no important role in the consumption of these diets; nor does appetite appear to be anything but a reflection of the adequacy of the dietary balance. In this respect one is reminded of a similar response by many species of animals to certain B-vitamin deficiencies.

Egg production provides a very sensitive measurement of the amino acid requirements of the laying hen. The deposition of large quantities of protein as well as of other nutrients requires a delicate balance of many dietary factors. By means of the present diet these factors and their interrelationships may be studied critically using egg production as the sensitive criterion.

Besides the obvious application to the study of amino acid requirement, the diet lends itself equally well to a systematic study of the hen's requirement for other nutrients such as minerals, vitamins, and unidentified growth factors — a subject which is receiving much current attention by poultry nutritionists. In respect to unidentified factors required by the hen, several hens have been artifically inseminated after

they had been on an amino acid diet for 4 weeks. Eighty-three per cent fertility was obtained on a total of 12 eggs and of the 10 fertilized eggs, 9 normal chicks were hatched. Since it would seem unlikely that any unknown factors could be present in the free amino acid diets, the hen must either not require such factors for egg production and hatchability or else the bird has a sufficient store for at least a month.

SUMMARY

A free amino acid diet has been developed which was readily consumed in adequate amount by most hens with concomitant maintenance of normal egg production. The factors found to be of greatest importance in the formulation of this diet were the purity of amino acid source and the caloric level of the diet. In the latter respect, starch and 12% corn oil were used to maintain a high energy level with the amino acid mixture. Egg production provided an extremely sensitive criterion of amino acid requirement since on deficient diets production always ceased within one week. The usefulness of the free amino acid diet in studying amino acid requirements as well as other nutrients is emphasized.

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THE AMINO ACID REQUIREMENT OF THE LAYING HEN

II. CLASSIFICATION OF THE ESSENTIAL AMINO ACIDS REQUIRED FOR EGG PRODUCTION [1]

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Although the essential amino acid requirement for growth and maintenance in several animal species including man (Rose, '38; Almquist, '52; Rose et al., '55) has been the subject of considerable study, little information is available on the amino acid requirement for productive purposes as exemplified by milk production in the dairy cow and egg production in the hen. By injecting carbon 14-labeled bicarbonate and acetate intravenously into dairy cows, Black et al. ('52) indirectly classified those amino acids which could not be synthesized by the lactating dairy cow.

Lysine, methionine, tryptophan, leucine and isoleucine (Almquist, '52; Miller et al., '54) have been shown to be essential for egg production with the use of proteins deficient in one or more amino acids. The development of a free amino acid diet (Fisher and Johnson, '56) on which normal egg production could be maintained has made possible direct classification of all essential amino acids required for egg production.

EXPERIMENTAL AND RESULTS

Single Comb White Leghorn hens from the university flock laying in clutches of two eggs or more were used in this study.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Poultry Husbandry, New Brunswick. Supported in part by a grant in aid from the Cooperative Grange League Federation Exchange, Inc., Ithaca, N. Y.

Details of procedure and source of amino acids employed have been described previously (Fisher and Johnson, '56).

In the first experiment alanine, aspartic acid, cystine, glycine, proline and serine were simultaneously omitted from a mixture of 18 amino acids (Fisher and Johnson, '56). The nitrogen content of this diet in the first experiment and of all subsequent diets employed in the present study was standardized at 2% with the addition of ammonium citrate. The complete diet is shown in table 1. In table 2 detailed egg

TABLE 1

Composition of complete diet for maintenance of egg production

AMINO ACIDS [1]		BASAL DIET	
	%		%
L-Arginine HCl	1.3	Starch	58.41
L-Glutamic acid	3.5	Corn oil	12.00
L-Histidine HCl	0.6	Mineral mix [2]	5.34
DL-Isoleucine	2.0	Fiber	3.00
L-Leucine	1.4	Limestone	2.50
L-Lysine HCl (95%)	1.2	Dibasic ammonium citrate	1.50
DL-Methionine	0.8	Antacid adsorbent [2]	1.00
DL-Phenylalanine	1.0	Sodium bicarbonate	1.00
DL-Threonine	1.0	Vitamin A, D, and E concentrate [2]	0.10
DL-Tryptophan	0.4	Vitamin mix [2]	0.15
L-Tyrosine	0.6		
DL-Valine	1.2		

[1] Sources listed by Fisher and Johnson ('56).

[2] Composition given by Fisher and Johnnson ('56).

production beyond the second week together with total production for a 30-day experimental period are given for three hens maintained on the modified diet and for one hen maintained on the 18 amino acid diet. Although a two-week experimental period had been shown previously to be fully satisfactory in determining the adequacy of free amino acid diets, it was felt necessary to demonstrate that birds could maintain egg production well beyond the two-week period.

The results in table 2 clearly demonstrate (a) that alanine, aspartic acid, citrulline, cystine, glycine, hydroxyproline, proline and serine are not essential for egg production when the

nitrogen intake is held at 2%; and (b) that hens will continue in production during a 30-day-experimental period.

Attempts to maintain egg production by replacing tyrosine with higher levels of DL-phenylalanine (2.0, 2.5, and 3.0%) were unsuccessful. At levels of 1.6 and 2.0% of the L-isomer of phenylalanine in place of the racemic form (DL), one out of two birds at each level maintained good egg production (7 and 8 eggs/bird/14 days, respectively) throughout the two-week experimental period. Despite the fact that tyrosine was only replaceable by phenylalanine with difficulty, it must be considered a non-essential amino acid (see discussion).

TABLE 2

Maintenance of egg production on free amino acid diets for a 30-day experimental period

BIRD NO.	DAYS ON EXPERIMENT BEYOND 2 WEEKS																TOTAL EGGS FOR 30 DAYS
	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
12 amino acids in diet																	
900					x	x		x	x		x		x	x			14
966	x		x	x		x	x		x		x	x		x		x	20
905				x			x						x		x	x	10
18 amino acids in diet																	
852	x		x	x		x		x		x		x		x			15

The omission of glutamic acid from the amino acid mixture listed in table 1 did not interfere with ad libitum consumption of an adequate amount of feed (table 3). To date, however, no bird on a glutamic acid-free diet has maintained satisfactory egg production throughout the two-week experimental period; some hens stopped laying at the end of the first week and others maintained only a reduced rate of production (table 3). Glutamic acid must therefore be classified as an *essential amino* acid for egg production (in the presence of the amino acids listed in table 1) according to Rose's ('38) definition of an essential amino acid.

Removal of any one of the remaining 10 amino acids (listed below) from the diet (table 1) resulted in immediate disrup-

tion of feed consumption. This observation demonstrates clearly the essentiality of each one of these amino acids. To obtain additional information regarding the effect on egg production of omitting these amino acids from the diet, the following experiment was designed: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine were individually omitted from the otherwise complete diet (table 1). These diets were then force-fed to pairs of hens in adequate amounts during a 5-day period after which time the birds were returned to the prac-

TABLE 3

Egg production and feed consumption on a glutamic acid-free diet

BIRD NO.	DAILY EGG PRODUCTION DURING 14-DAY EXPERIMENTAL PERIOD														TOTAL EGGS
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
967		x	x		x			x				x		x	6
863	x	x	x				x		x				x		6
910	x		x		x	s[1]									4
874	x		x	x		x									4
	DAILY FEED CONSUMPTION (gm/day)														AV.
967	54	54	68	94	94	115	119	120	116	111	113	71	82	85	93
863	70	60	78	104	95	81	56	60	56	35	24	53	42	57	62
910	88	96	106	122	120	116	77	103	88	117	82	95	98	100	101
847	10	24	28	91	93	135	102	113	97	132	91	78	71	92	83

[1] Soft shell egg.

tical laying ration. The time interval between the last egg on the experimental diet and the first egg on the practical diet offered critical proof of dietary essentiality. The data from this experiment are shown in table 4. The average pause in egg production due to the omission of *any* one of the amino acids listed was 9.8 ± 1.40 days, while control hens continued laying throughout, with an average time interval (as explained above) of only 1.9 ± 1.50 days. The difference between these means is highly significant with $P << 0.001$. Therefore, the essential amino acids for egg production in the hen as determined in these studies are: arginine, glutamic acid, histi-

dine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

TABLE 4

Effect on egg production of force-feeding for 5 days a diet from which an essential amino acid has been omitted

AMINO ACID OMITTED	REPLICATE 1		REPLICATE 2	
	Number of eggs	Pause in production	Number of eggs	Pause in production
		days		*days*
Arginine	2 [1]	9 [2]	2 [1]	12 [2]
Histidine	1	11	2	10
Isoleucine	2	10	3	10
Leucine	2	9	3	10
Lysine	2	8	3	died [3]
Methionine	3	9	2	9
Phenylalanine + tyrosine	4	8	2	12
Threonine	2	died [3]	2	9
Tryptophan	3	10	1	12
Valine	2	11	3	7

SUMMARY		
Average egg production, per hen	Complete diet [4] 2.6 ± 0.49	One amino acid omitted [5] 2.3 ± 0.71
Average pause in production, days	1.9 ± 1.50	9.8 ± 1.40

[1] Eggs laid during 5-day experimental feeding period.

[2] Days from last egg on experimental diet to first egg after return to practical diet.

[3] Died on last day of experimental period; liver and reproductive tract abnormal.

[4] Average of 10 birds.

[5] Average of birds listed above.

DISCUSSION

A comparison of the essential amino acids required for egg production with those required for growth and maintenance makes it evident that they do not differ markedly from those of the growing chick. Only glycine, which is essential for maximum growth, is not essential for egg production. Particularly interesting is the observation that glutamic acid is essential for *maximum* egg production, just as it has been shown necessary for *maximum* growth in chicks, mice and rats

(Almquist and Grau, '44; Maddy and Elvehjem, '49; Rose et al., '48). Since glutamic acid is not required for maximum growth when the other non-essential amino acids are added to the 10 essential ones for the rat, Rose et al. ('48) classified glutamic acid as a dispensable amino acid, although it is considered an *essential* component of diets containing only the 10 essential amino acids. If glutamic acid is classified a dispensable amino acid, it *necessitates* providing other non-essential amino acids in the diet. In the present study using 11 amino acids, glutamic acid must be considered an essential amino acid, although the possibility that it could be replaced with other amino acids considered non-essential is not discounted.

Difficulty was experienced in demonstrating the non-essentiality of tyrosine in the presence of increased levels of phenylalanine. The recent work of Armstrong ('55) which indicated very inefficient conversion of phenylalanine to tyrosine in the rat suggests that even higher levels of phenylalanine than have been tried in the present study would permit normal egg production in all hens. On the other hand, Benton et al. ('56) have shown that phenylalanine at high levels (approached in this study when the DL form was used) acts as an antagonist towards other amino acids. Thus, the combination of an inefficient conversion and amino acid antagonism exhibited by phenylalanine may explain the present observation.

The remarkable uniformity in the egg production pause resulting from the omission of any one essential amino acid (glutamic acid was not included in this experiment; however, see table 3) is similar to the uniform daily loss in body weight exhibited by chicks when fed diets lacking one of the essential amino acids (Almquist, '47). This suggests that as for growth, the amino acid requirement for egg protein synthesis is an aggregate one in that all amino acids must be present simultaneously.

During the 5-day experimental period, hens on the control diet tended to lay slightly more eggs (table 4) than those

on a diet from which one of the essential amino acids were removed; the difference, however, was not statistically significant. The remarkable uniformity in results on either the complete or incomplete diets supports the adequacy of short-period studies with free amino acids diets. The fact that hens are able to lay very few eggs on a deficient diet suggests that they have small protein stores which became rapidly depleted on a deficient diet. Egg formation thus offers a unique opportunity for studying protein synthesis and amino acid turnover *in vivo;* it is hoped that knowledge of the essential amino acids required for this process as well as the availability of a free amino acid diet will enhance such studies in the future.

SUMMARY

The classification of amino acids according to their essentiality for the laying hen has been studied by use of a free amino acid diet. Arginine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine were found to be essential for egg production. With the exception of glutamic acid, omission of any one of the above amino acids resulted in immediate disruption of feed consumption and a 10-day pause in production when such incomplete diets were force-fed for only 5 days. Although feed consumption was not affected by the omission of glutamic acid, normal egg production could not be maintained. Tyrosine could be replaced by phenylalanine only with difficulty. The hen does not require glycine for egg production. This is in contrast to the need of the growing chick for this amino acid.

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FURTHER EVIDENCE ON THE REQUIREMENT OF THE CHICK FOR UNIDENTIFIED MINERALS[1]

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Morrison, Scott and Norris ('55) discovered that, when a mixture of materials containing unidentified chick growth factors is fed to chicks, a portion of the growth response obtained from them is due to a mineral constituent(s) of the mixture not previously reported to be required by animals. At approximately the same time, Dannenburg, Reid, Rozacky and Couch ('55) found that the ash of corn distillers' dried solubles promoted increased growth in chicks. The studies presented in this report confirm and extend the original observations made by these groups of investigators and indicate that the mineral(s) involved is concerned in bone formation.

EXPERIMENTAL

The purified basal diet used in most experiments reported herein contained the following ingredients per 100 gm: glucose,[2] 61.30 gm; purified isolated soybean protein,[3] 25.57 gm; hydrogenated fat,[4] 3 gm; ground cellulose,[5] 3 gm; DL-methio-

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[2] Cerelose.

[3] Drackett Assay Protein C-1.

[4] Hydora, Lever Bros.

[5] Solka-Floc, The Brown Company, Berlin, N. H.

nine, 0.7 gm; glycine, 0.3 gm; $CaHPO_4$, 2.151 gm; $CaCO_3$, 1.492 gm; KH_2PO_4, 0.867 gm; NACl, 0.6 gm; $MgSO_4$, 0.25 gm; $FeSO_4.7H_2O$, 0.0333 gm; $MnSO_4.H_2O$, 0.0333 gm; KI, 0.26 mg; $CuSO_4.5H_2O$, 1.67 mg; $ZnCl_2$, 1.0 mg; $CoCl_2.6H_2O$, 0.17 mg; $Na_2MoO_4.2H_2O$, 0.83 mg; choline Cl, 0.15 gm; inositol, 25.0 mg; niacin, 5.0 mg; calcium pantothenate, 2.0 mg; α-tocopheryl acetate, 2.0 mg; thiamine HCl, 1.0 mg; riboflavin, 1.0 mg; pyridoxine HCl, 0.45 mg; folic acid, 0.40 mg; menadione, 0.05 mg; biotin, 0.02 mg; vitamin B_{12}, 2.0 μg; vitamin A, 500 I.U.; vitamin D_3, 37.5 I.C.U. The mineral compounds in the mineral mixture used in the first few experiments were reagent grade except the dicalcium phosphate ($CaHPO_4.2H_2O$) and the calcium carbonate, which were U.S.P. grade. The quantity of dicalcium phosphate used in the mineral mixture was adjusted for water of hydration. In all subsequent experiments reagent grade minerals were used exclusively. The isolated soybean protein was purified by repeated washings at its isoelectric point of pH 4.6. The supernatant liquid was decanted after each washing. The mineral content of the soybean protein was reduced from 2.8 to 1.3% by the purification process. In a few studies purified casein plus the necessary essential amino acids, or a mixture of purified casein and purified isolated soybean protein, was used as the source of protein in the basal diet. The casein was purified by repeatedly washing in a brine solution at pH 2.5, followed by several washings at the isoelectric point of pH 4.7. The basal diet contained, by calculation, adequate or excess quantities of the nutrients known to be required by the chick.

All additions to the basal diet were made in such a way as to maintain the protein level constant at 20.5%. In experiments in which the minerals of the unidentified growth factor supplements were added to the basal diet, substitution was made at the expense of glucose. The minerals were obtained by burning off much of the organic matter of the crude materials in an evaporating dish, followed by incineration in a

muffle furnace at 525°C. for 4 hours. The inorganic residue then was cooled and brought to pH 7 with glacial acetic acid.

Either New Hampshire × Barred Plymouth Rock or White Plymouth Rock chicks of hens fed diets which produce chicks deficient in unidentified growth factors, according to Waibel, Morrison and Norris ('55), were used in most studies. In a few experiments, however, White Plymouth Rock chicks obtained from a commercial hatchery were used. The chicks were brooded in electrically heated, galvanized metal battery brooders with raised screen floors, and feed was supplied ad libitum. Distilled or demineralized water was supplied in metal watering pans sprayed with plastic paint, except in several of the experiments conducted at the beginning of the investigation. The chicks were identified by numbered wing bands at the start of the experiment and weighed individually at weekly intervals thereafter until they were 4 weeks of age, at which time most of the experiments were terminated. The quantity of feed consumed by the chicks was recorded.

RESULTS AND DISCUSSION

In studies conducted to prove the adequacy of the basal diet in nutrients known to be necessary, it was found that the addition of linoleic acid, increased quantities of all known essential amino acids, or all known essential vitamins, had no influence on growth. The addition to the basal diet of increments of reagent grade magnesium sulfate failed to increase the rate of growth. In several experiments, it was found that increased quantities of sodium or potassium, either alone or in combination, as the chloride or sulfate salts, did not improve growth. Alteration of the calcium and phosphorus content of the basal diet by the addition of 1% of dicalcium phosphate failed to increase chick growth. Additional chlorine had no effect on growth. The addition to the basal diet of 50 or 100% more of the essential trace elements (iron, copper, manganese, cobalt, iodine, zinc), essential major elements (calcium, phosphorus, magnesium, potassium, sodium, chlorine) or the complete mineral mixture did not appreciably

increase growth. Although molybdenum has been shown to be an integral part of certain enzyme systems, the results of two experiments showed that the addition of molybdenum to the basal diet failed to promote a growth increase. Edwards et al. ('55) were also unable to obtain any growth response from the addition of molybdenum to a purified chick diet similar to the diets used in the present studies. Inasmuch as Almquist and Mecchi ('40) had previously reported growth stimulation in chicks from sodium acetate, and because glacial acetic acid was used to neutralize the ignited materials, 0.20% sodium acetate was added to the basal diet, but this failed to improve growth. Since Machlin ('55) found that under certain conditions sulfate *per se* stimulated the growth of chicks, 0.17% sulfate was added to the basal diet, but it failed to influence the rate of gain.

Although it was not possible to increase chick growth by increasing the amounts of the essential nutrients in the basal diet, it was found, in confirmation of previous work from this laboratory, that the addition to the basal diet of 18% of a mixture of 5 unidentified growth factor supplements consisting of 6 parts of corn distillers' dried solubles, 3 parts of fish solubles, 3 parts of dried whey product, 3 parts of forage juice, and 3 parts of penicillin mycelium meal elicited a highly significant ($P < 0.01$ by analysis of variance) growth response at 4 weeks of age. The combined results of a number of experiments are given in table 1. Chicks fed the basal diet supplemented with 5 sources of unidentified growth factors grew for the most part as rapidly, and frequently more rapidly, than those fed a good quality commercial chick ration.

The results further showed that a considerable portion of the growth response produced by the addition to the basal diet of sources of unidentified growth factors is due to an unknown mineral nutrient or nutrients contained in them, since a highly significant ($P < 0.01$) growth response was also obtained from the inorganic portion of the crude materials. In addition to stimulating growth, the mixture of 5 unidenti-

fied growth factor supplements, or its ash, produced a highly significant ($P < 0.01$) increase in efficiency of feed utilization.

In other experiments, the results of which are also presented in table 1, the addition to the basal diet of 6% of a composite sample of corn distillers' dried solubles, or the minerals supplied by 6% distillers' dried solubles, promoted highly significant ($P < 0.01$) growth increases at 4 weeks of age. The efficiency of feed utilization was also increased.

TABLE 1

Evidence of an unidentified mineral(s) required for chick growth

TREATMENT	AV. WT. 4 WKS.	GAIN OVER BASAL	GAIN/ FEED
	gm	*%*	*gm*
A. *Response to 5 UFS*[1] *and the ash of 5 UFS*			
Basal	306 (13)[2]	...	0.498
+ ash 5 UFS[3]	380 (8)	24.2	0.571
+ 5 UFS	416 (8)	35.9	0.595
B. *Response to DDS*[4] *and the ash of DDS*			
Basal	298 (12)	...	0.500
+ ash 6% DDS[5]	355 (9)	19.1	0.543
+ 6% DDS	368 (9)	23.5	0.562

[1] UFS = mixture of unidentified factor supplements.

[2] Number of lots of approximately 20 chicks each.

[3] Ash amounted to approximately 2% of total diet when fed at a level equivalent to 6% distillers' dried solubles, 3% fish solubles, 3% dried whey product, 3% forage juice and 3% penicillin mycelium meal.

[4] DDS = distillers' dried solubles.

[5] Ash amounted to approximately 0.6% of total diet when fed at a level equivalent to 6% distillers' dried solubles.

The experiments on the ash of distillers' dried solubles were not conducted simultaneously in all instances with those on the ash of the mixture of unidentified factor supplements. Although an apparent difference in percentage growth responses was observed, this is not believed to be significant, since in three comparable experiments the average growth (368 gm) obtained at 4 weeks with the ash of the mixture of unidentified factor supplements was no greater than that (375 gm) obtained with the ash of distillers' dried solubles.

A highly significant difference ($P < 0.01$) between the growth response promoted by the 5 unidentified factor sources and that obtained from the ash of these materials was observed in the experimental work summarized in table 1. In later work, the results of which are presented in table 2, larger quantities of ash than those usually fed, prepared either from 5 unidentified growth factor supplements or 3 unidentified growth factor supplements (6 parts of corn distillers' dried

TABLE 2

Differentiation of unidentified organic and inorganic chick growth factors

TREATMENT	AV. WT. 4 WKS.	GAIN OVER BASAL
	gm.	%
Experiments with UFS [1] ash		
Basal	287 (4) [2]	...
+ 1.20 or 2.0% ash [3]	323 (4)	14.5
+ 1.80 or 3.0% ash	323 (4)	14.5
+ 3 UFS	382 (2)	38.2
Experiments with DDS [4] ash		
Basal	305 (4)	...
+ 0.56% ash	352 (3)	17.7
+ 0.75 or 0.85% ash	347 (3)	15.8
+ 5 UFS	408 (3)	38.9

[1] UFS = mixture unidentified factor supplements.

[2] Number of lots of approximately 20 chicks each.

[3] Smaller quantity of ash from mixture of 3 UFS; larger quantity from mixture of 5 UFS.

[4] DDS = distillers' dried solubles.

solubles, 3 parts of fish solubles and 3 parts of dried whey product) did not further stimulate growth. On the other hand, the intact unidentified growth factor supplements significantly ($P < 0.01$) increased the growth of chicks over that obtained with the ash. Larger quantities of the ash of distillers' dried solubles also failed to increase chick growth above the responses obtained from the amount usually supplied. In contrast the growth of the chicks supplied the 5 unidentified growth factors supplements was significantly ($P < 0.01$) greater than that obtained with the ash of the distiller's

dried solubles. Therefore, the results showed that an unidentified organic factor(s), as well as an unidentified inorganic essential(s), is required for maximum chick growth.

Although our data suggest the existence of an unidentified organic growth factor(s) in distillers' dried solubles, there was no statistically significant difference between the growth response obtained from the intact solubles and that obtained from the ash. However, our failure to show a significant response from the organic factor in distillers' dried solubles may have been due to a deficiency of the organic factor(s) present in the other materials in the mixture of 5 unidentified factor supplements. Novak and Hauge ('48a, b) and Dannenburg et al. ('55) have obtained evidence that corn distillers' dried solubles contains an unidentified organic factor(s).

That the distribution of the unidentified inorganic essential(s) may be widespread is suggested by the fact that chick growth responses from diets believed to be adequate in the known nutrients have been obtained from the minerals of dried whey (Couch et al., '55), fish meal (Tamimie, '55), feather meal (Menge et al., '56), and gelatin (Benton et al., '55). The minerals of fish solubles were also found to promote an increase in growth at this laboratory.

Carcass analysis studies showed that the additional gain observed in chicks fed the basal diet supplemented with the ash of 5 unidentified growth factor supplements was not due to increased water retention. The percentages of moisture, protein, ether extract, and ash in the carcasses of chicks fed the basal diet were found to be 71.93, 17.61, 7.32 and 2.80 respectively. The corresponding values for the chicks fed the basal diet plus the ash of 5 unidentified factor sources were 73.18, 17.76, 5.83 and 2.77% respectively.

Highly depleted chicks fed the basal diet exhibited a bone malformation, characterized by enlargement and elongation of the intertarsal (hock) joint. In repeated experiments, it was observed that an average of 26% of the chicks fed the basal diet exhibited the leg bone malformation, while the average incidence of the syndrome in chicks receiving the basal

diet plus 5 unidentified growth factor supplements, or the ash of these 5 supplements, was only 2% and 7% respectively.

In further studies the percentage ash (45.07%) in the dry fat-free tibiotarsae of chicks fed the basal diet was found to be significantly lower ($P < 0.02$) than that (47.30%) of chicks of equal weight fed the basal diet plus the ash of 5 unidentified growth factor supplements. Although the tibiotarsae of chicks fed the basal diet were slightly shorter than those of chicks of equal weight fed the basal diet plus the ash of 5 unidentified growth factor supplements, the difference was not found to be statistically significant. The breaking strength (5.99 kg) of the tibiotarsae of chicks receiving the ash was greater ($P < 0.10$) than that (5.43 kg) of chicks receiving the basal diet. These results are similar to those reported by Caskey, Gallup and Norris ('39) on the effect of manganese deficiency on bone development. However, in the present studies, additional manganese had no effect upon growth or incidence of leg malformation, and slipped tendon, or perosis, has been only rarely observed.

Studies on the blood of chicks receiving the basal diet or the basal diet supplemented with the ash of 5 unidentified growth factor supplements showed that the ash had no effect on the amount of hemoglobin, volume of red cells or red cell count. At 4 weeks of age, the results of these determinations on the blood of chicks fed the basal diet were 9.5 gm%, 26% and 2.06 million/mm^3, respectively. The corresponding values for chicks fed the basal diet plus the ash of 5 unidentified growth factor supplements were 9.8 gm%, 26% and 2.13 million/mm^3, respectively.

Attempts to increase chick growth by feeding a mixture of reagent grade minerals which was prepared in the proportions indicated by the spectrographic analysis of the ash of distillers' dried solubles failed to promote increased growth, in contrast to the finding of Reid, Rozacky and Couch ('55). The spectrographic analysis of the ash of distillers' dried solubles reported by Couch and associates ('55) and that obtained through the courtesy of James McGinnis of the State

College of Washington were used in formulating the mixture of reagent grade minerals. The reconstituted mineral mixture contributed the following elements to the diet in parts per million: Al, 3.0; B, 1.8; Ba, 0.48; Ca, 60.0; Cr, 0.036; Cu, 9.6; Fe, 60.0; Pb, 0.12; Mg, 120.0; Mn, 3.0; Mo, 0.13; Ni, 0.12; P, 180.0; K, 180.0; Si, 60.0; Ag, 0.024; Na, 180.0; Sr, 120; Ti, 0.24; V, 0.12; Zn, 3.0. The results of growth studies conducted on this phase of the problem are given in table 3. The discrepancy in the results obtained by the two groups of workers may pos-

TABLE 3

Effect of synthetic mineral mixtures on chick growth

TREATMENT	AV. WT. 4 WKS.	GAIN OVER BASAL
	gm	%
Response to reconstituted mineral mixture		
Basal	297 (4) [1]	. . .
+ ash 5 UFS [2]	333 (4)	12.1
+ mineral mixture [3]	295 (4)	0
Response to Hoagland and Snyder's mineral solution		
Basal	280 (5)	. . .
+ ash 5 UFS	336 (5)	23.1
+ mineral solution [4]	314 (5)	14.0

[1] Number of lots of approximately 20 chicks each.
[2] UFS = mixture unidentified factor supplements.
[3] Formulated on the basis of spectrographic analyses.
[4] Added to basal diet at a level $\cong$ 150 ml/kg.

sibly be explained by differences in reagent grade mineral salts used in making up the reconstituted mineral mixtures.

Although a mineral mixture formulated on the basis of spectrographic analyses did not increase growth, the data in table 3 show that the complex mineral solution of Hoagland and Snyder ('33) was partially as effective in increasing chick growth as the mineral portion of the mixture of 5 unidentified growth factor supplements.

In further studies designed to determine the biologically active component(s) in the ash of the 5 unidentified growth factor supplements, the addition of salts of aluminum, arsenic,

barium, beryllium, bismuth, boron, bromine, cadmium, cerium, cesium, chromium, cobalt, fluorine, indium, iridium, lead, lithium, mercury, nickel, platinum, rhodium, rubidium, ruthenium, selenium, silicon, silver, strontium, tantalum, tellurium, thorium, tin, titanium, tungsten, vanadium, yttrium or zirconium, did not influence growth at the levels used. These included the mineral elements not known to be required in animal nutrition which are present in the salt solution of

TABLE 4

Results of studies on isolation of unidentified mineral(s) in the ash

TREATMENT	AV. WT. 4 WKS.	GAIN OVER BASAL
	gm	%
Response to water insoluble fraction		
Basal	285 [1]	...
+ ash 5 UFS [2]	324	13.7
+ water soluble portion	304	6.7
+ water insoluble portion	333	16.8
Response to cation exchanger elutrient		
Basal	321	...
+ ash 3 PFS [3]	347	8.1
+ IR-120 [4] effluent of ash	311	— 3.1
+ IR-120 elutrient of ash	351	9.3

[1] Average duplicate lots of 20 chicks per treatment.

[2] UFS = mixture unidentified factor supplements.

[3] Distillers' dried solubles, 6 parts; fish solubles, 3 parts; dried whey product, 3 parts.

[4] Amberlite resin IR-120, Rohm and Haas Company.

Hoagland and Snyder ('33). Many of the above elements have also been tested for biological activity by Dannenburg et al. ('55) and Reid, Rozacky and Couch ('55) with results similar to those obtained by us.

In fractionation studies, the results of which are given in table 4, evidence was obtained that the active component(s) in the ash of the mixture of 5 unidentified growth factor supplements is present in that portion insoluble in boiling water. The ash was treated with 5 volumes of boiling water for 30 minutes, filtered, and the insoluble residue again extracted

with an additional 5 volumes of boiling water for 30 minutes. Under these conditions, it was found that approximately 35% of the ash was water soluble. In a further experiment, the ash was prepared by a wet-ashing procedure, wherein the crude unidentified growth factor supplements were digested with concentrated nitric acid. The results indicated that ash prepared by this procedure was fully as active biologically as ash prepared by the usual incineration procedure.

In an additional study, the ash was prepared by the wet-ashing procedure, dissolved in nitric acid at pH 2.35, and passed through a strong cation exchanger [6] in the hydrogen form. The cations retained on the column were eluted with 10% nitric acid. The elutrient and effluent were concentrated, neutralized, and added to the basal diet. The results of the experiment are given in table 4. They indicated that the growth-promoting effect of the ash is due to a cation(s) since the increased growth obtained with the cationic fraction was highly significant ($P < 0.01$).

Studies have also been conducted to determine if the effect of the unidentified minerals in the ash of unidentified growth factor supplements is a direct one or mediated through the microflora of the intestinal tract. The results of the work revealed no consistent effect of the ash on the numbers of aerobes, anaerobes, coliforms, lactobacilli, streptococci or clostridia in either the entire intestinal tract or sections of it. The ash, furthermore, exerted no effect on the pH of the intestinal tract. The results suggested, therefore, that the primary effect of the ash is exerted directly upon the tissues of the chick rather than indirectly through effects on the intestinal microflora.

SUMMARY

In further work on unidentified chick growth factors results were obtained which indicated that the basal diets supplied the chicks were adequate in amino acids, known vitamins and

[6] Amberlite IR-120, Rohm and Haas Company.

minerals, previously reported to be needed by animals. No evidence of an imbalance was obtained in the studies on required minerals. As a consequence it has been found, in confirmation of previous reports by this laboratory, that when a mixture of unidentified growth factor supplements is fed to chicks the growth response which is observed is due to the presence in the materials of both unidentified organic and inorganic constituents. The results of the investigation showed, therefore, that a mineral or minerals not hitherto considered to be essential in the nutrition of the chick is present in the mineral portion or ash of certain crude feedstuffs. The unidentified mineral(s) was found to be involved in bone formation. Evidence was also obtained which indicated that the unknown mineral nutrient(s) is present in the boiling water-insoluble fraction of the ash of the mixture of unidentified factor supplements, and that it is cationic in acid solution. No consistent effect of the ash on the intestinal microflora of the chicks or the pH of the intestinal contents was observed.

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NUTRITIVE VALUE OF PROTEIN AND TUMOR-HOST RELATIONSHIP IN THE RAT [1]

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Previous studies have emphasized the variable responses of different types of tumors to the diet of the host, one of the most interesting responses being associated with the kind and quantity of dietary protein (Mider, '51; Le Page et al, '52; Tannebaum and Silverstone, '53; Babson, '54; Babson and Winnick, '54; Greenlees and Le Page, '55). These authors and others have presented data that can be interpreted to mean that some tumors deplete the body of labile protein stores more than others, the depleting effect of a growing neoplasm varying with the size and nature of the tumor. Sherman et al. ('50) suggested, for example, that the Walker carcinoma 256 in rats depleted these stores after the tumor had become 10% of the total body weight.

Recent reports from our laboratories have emphasized the effect of dietary protein upon tumor-host interrelationships and upon the response of tumor and normal tissues to chemotherapy with the ethylenephosphoramides (Allison et al., '54, '55, '56; McCoy et al., '56). Studies involving a transplanted sarcoma in the rat, for example, demonstrated that although the growth of this neoplasm was not easily affected by dietary protein, it developed most rapidly in animals fed a semi-synthetic diet containing 12% casein. Supplementing

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with methionine or still better with methionine plus guanidoacetic acid favored the growth of normal tissues and, if the growth of these tissues was maximum, the development of the tumor was depressed. The favorable response to chemotherapy was also maximum under these conditions of optimum development of normal tissues (Allison et al., '55; Allison, '56). The Walker carcinoma 256 was another tumor that grew rapidly at the expense of normal tissues and was resistant to ethylenephosphoramide therapy. The Flexner-Jobling carcinoma, on the other hand, under the conditions of our experiments was most susceptible to this type of therapy, the majority of the tumors completely regressing (Crossley et al, '53) and they seemed to be affected more than the other two tumors by the kind and amount of protein in the diet (Babson, '54).

The following experiments were done, therefore, to further characterize these three tumors according to the effect of dietary protein upon their development in the host, hoping thereby to derive a better basis for evaluating the effects of various drugs and diets upon protein metabolism in the tumor and in the host.

METHODS

The semi-synthetic diet and the methods of transplanting and measuring the development and growth of the tumor were described previously (Allison et al., '54). Three different tumors were used in these studies, the Sarcoma R-1, the Walker carcinoma 256, each transplanted into male Wistar rats, and the Flexner-Jobling carcinoma transplanted into male Sprague-Dawley rats. Ten rats containing one type of tumor were placed on each diet at the time of transplantation. The groups were pair-fed the diet containing different proteins, wheat gluten, casein, beef, and egg albumin. These proteins were of the same type or source reported in previous studies from our laboratory (Allison, '55), the protein forming 12% of the dry diet. The effects on the growth of the tumor and body of supplementing casein with 0.7% methionine

were studied also, using techniques described previously (Allison et al., '54). Similarly groups were fed the 12% casein diet supplemented with mixtures of 0.7% methionine plus 0.7% guanidoacetic acid, 0.7% methionine plus 1.28% glycine, and 0.7% methionine plus 1.95% ammonium citrate (Allison et al., '56). The tumors were allowed to develop to average approximately 17 to 20 gm in rats fed the egg albumin, tumor sizes which approached lethal proportions in animals fed the 12% casein diet. To develop tumors of this size required three weeks in rats bearing the Sarcoma R-1, 11 days in rats with the Walker carcinoma 256 and 28 days in the Sprague-Dawley rats with the Flexner-Jobling carcinoma. Tumors one gram or less in size at the end of the experimental period were considered as not taking.

RESULTS

The protein efficiencies (grams gained per gram of nitrogen intake) for normal and sarcoma-bearing rats are illustrated in figure 1. The white bars record the protein efficiencies for groups of normal rats, pair-fed isocaloric diets containing equivalent amounts of wheat gluten, casein, beef, or egg albumin. These protein efficiencies agree well with those determined previously in rats and they illustrate the relative nutritive values of these proteins for growth of the soft tissues of most animals (Allison, '55). The black bars record the gain in weight of the body per gram of nitrogen intake in sarcoma-bearing rats. This gain, calculated by subtracting the weight of the tumor from the total weight of the tumor-bearing rat, was of the same order as in normal rats but less in magnitude, a reduction that is to be expected because of the utilization of nitrogen by the growing sarcoma. The presence of the sarcoma increased the protein efficiencies of the tumor-bearing animals much above the controls as illustrated by the bars with slanted lines.

The weight of the sarcoma, 21 days after transplantation, was slightly but significantly smaller in animals fed the diet containing egg albumin than in animals fed other proteins

in the diet (table 1). These results are in agreement with other data obtained using this sarcoma, data which demonstrated the sarcoma developed slower in animals where normal tissues developed maximally (Allison et al., '56). The rate of growth of the body with respect to tumor was lowest

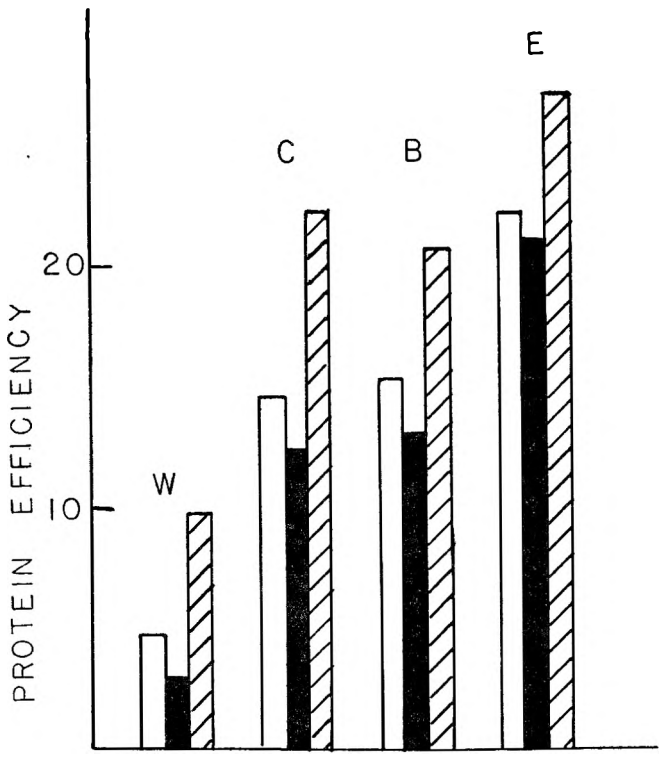

Fig. 1 Protein efficiencies (grams gained in weight per gram nitrogen intake) in normal rats (white bars), body of sarcoma-bearing rats (black bars), and whole sarcoma-bearing rats (bars with slanted lines), the rats being pair fed different proteins — wheat gluten, W; casein, C; beef, B; and egg albumin, E.

(0.5) in animals fed wheat gluten, intermediate (1.6) in those fed casein or beef and highest (3.4) in sarcoma-bearing rats fed egg albumin (table 1). Previous experience has demonstrated that the welfare of the animal, together with resistance to therapeutic stress, increased as the ratio increased (Allison et al., '54, '55, '56).

TABLE 1

Average data obtained on groups of tumor-bearing rats, pair fed so that the nitrogen and caloric intakes were approximately constant for each experiment. Gain in body weight over the experimental period was calculated as equal to total weight minus the weight of the tumor

DIET	SARCOMA (21 DAYS)			WALKER CARCINOMA 256 (11 DAYS)			F-J CARCINOMA (28 DAYS)		
	No. animals	Tumor	$-\frac{\Delta \text{ body}}{\text{tumor}}$	No. animals	Tumor	$\frac{\Delta \text{ body}}{\text{tumor}}$	No. animals	Tumor	$\frac{\Delta \text{ body}}{\text{tumor}}$
		gm			*gm*			*gm*	
Wheat gluten	8	24.7 ± 3.6	0.5 ± 0.1	8	18.3 ± 1.3	0.1 ± 0.2	9	5.3 ± 1.6	1.1 ± 1.4
Casein	8	30.4 ± 4.0	1.6 ± 0.3	8	23.4 ± 3.0	1.7 ± 0.3	6	11.3 ± 2.2	5.0 ± 1.2
Beef	9	28.2 ± 2.1	1.7 ± 0.2	9	21.9 ± 1.0	1.4 ± 0.3	7	18.6 ± 4.0	3.1 ± 0.8
Egg albumin	9	20.0 ± 1.0	3.4 ± 0.3	9	17.7 ± 1.5	2.5 ± 0.2	7	20.0 ± 3.2	4.4 ± 2.1

Feeding these same diets to rats bearing the Walker carcinoma 256 resulted in tumor growth and body weight gains similar to those found for the sarcoma-bearing animals (table 1). The Walker tumor, for example, was slightly but significantly smaller in rats fed albumin than in those fed casein. The rate of gain of the body with respect to the tumor was very low (0.1) in animals fed wheat gluten, was intermediate in animals fed casein or beef (1.4 to 1.7) and was highest (2.5) in tumor-bearing rats fed egg albumin.

The data recorded in table 1 demonstrate, on the other hand, that the Flexner-Jobling carcinoma responded as did the body to the type of protein in the diet, growing poorly in rats fed wheat gluten and most rapidly in animals fed egg albumin. The results suggest that this carcinoma did not grow at the expense of normal tissues as much as the other two neoplasms. a suggestion which is supported by the high body weight gain/tumor ratios in animals fed beef, casein, and egg proteins. The larger carcinoma in the animals fed egg albumin may have had a sufficient depleting effect to keep the ratios from increasing above those found for casein and beef, an increase that would be expected on the basis of relative protein efficiencies. These results using the three types of tumors demonstrate that, under the experimental conditions used here, the Flexner-Jobling carcinoma is affected more by the protein in the diet than are the sarcoma or the Walker neoplasm. Possibly tumors vary as do different normal tissues in their interrelationship with the body metabolic pools. In animals fed a protein-free diet, for example, some tissue proteins are depleted more than others and some are not depleted at all (Allison, '55). It may be significant that the ethylenephosphoramides, which seem to have some inhibiting effect upon protein anabolism (Allison et al., '54; McCoy et al., '56), are much more effective in reducing the growth and causing the regression of the Flexner-Jobling carcinoma than the other two types of tumors.

Increase in liver protein has been used as a measure of nutritive value of the dietary protein (Addis et al., '36; Kos-

terlitz and Campbell, '45–'46; Harrison and Long, '45) . Total liver protein was lowest in normal or sarcoma-bearing animals fed wheat gluten and highest in those fed egg albumin, data illustrated in figure 2. The relatively higher liver protein, however, in the tumor-bearing animals than in the controls has been observed by others (Yeakel and Tobias, '51; Le Page et al., '52; Babson, '54). Such an increase in liver

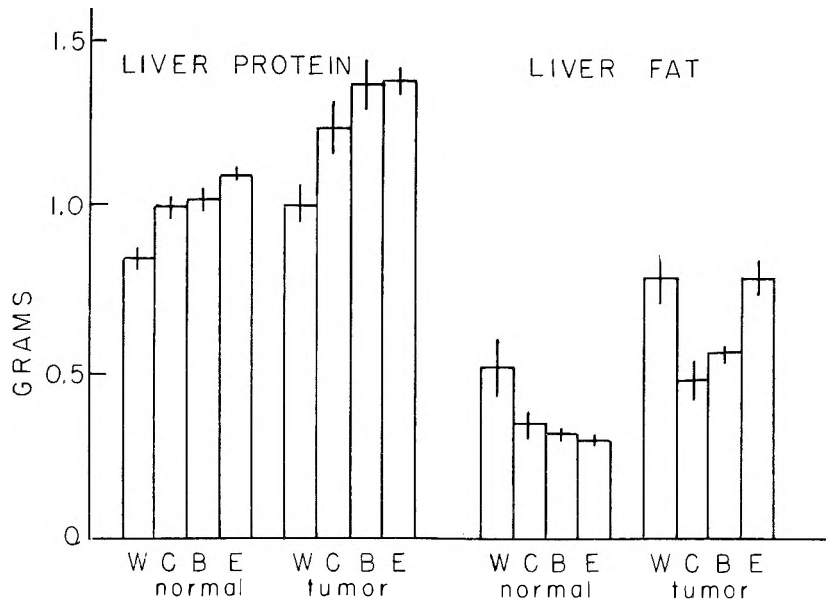

Fig 2 Total liver protein and fat in normal and sarcoma-bearing rats fed wheat gluten, W; casein, C; beef, B; and egg albumin, E. The standard errors of the averages are illustrated by the lines through the bars.

protein is associated with the high catabolic activity of the tumor-bearing animal when the tumor has reached depleting and lethal proportions (Allison et al., '56). The suggestion has been made that liver nitrogen increases at a time when this organ is involved in coping with split products from large necrotic tumors (Sherman et al., '50). Greenlees and Le Page ('55) did not observe enlarged livers in their studies since "the tumors never constituted more than 7 per cent of total body weight, and toxic effects from necrotic tissue

should be negligible.'' The suggestion has been made also that the sarcoma growing at the expense of normal tissues, created an overall amino acid imbalance in the animal (Allison, '55), thereby increasing both total liver protein and liver fat.

The higher liver fat in the normal animals fed wheat gluten is a common response to this deficient diet. The relatively higher liver fat in the tumor-bearing animals possibly is correlated with the formation and transport of lipid and lipoprotein in the presence of the large tumor. A marked lipemia developed in the sarcoma-bearing animals fed 12% casein, a diet which seemed to be optimum for the development of this tumor (see also Mider, '51). The addition of methionine to the diet reduced the lipemia and also slowed the development of the tumor (Allison et al., '54, '56).

Data have been presented to show that supplementation of the casein diet with an optimum amount of DL-methionine, or still better with a mixture of DL-methionine and guanidoacetic acid, improved the growth of normal tissues in the sarcoma-bearing rats (Allison et al., '56). Under these conditions, as with the improved growth of normal tissues in animals fed egg albumin, the sarcoma developed most slowly. Similarly the Walker carcinoma 256 developed more slowly and the normal tissues grew more rapidly in tumor-bearing animals fed casein supplemented with 0.7% DL-methionine than in rats fed unsupplemented casein. Adding glycine or ammonium citrate in equivalent amounts of nitrogen did not change significantly the supplementing effect of methionine. Adding 0.7% guanidoacetic acid with the 0.7% DL-methionine, however, reduced the size of the tumor significantly and increased the body gain/tumor ratio markedly (see figure 3). This effect of a mixture of methionine and guanidoacetic acid was most evident when the tumor became sufficiently large to produce a marked depleting effect, at a time when the catabolic activity as measured by urea nitrogen excretion was relatively high (see Allison et al., '56). The urea nitrogen excretion, for example, in these animals 11

days after transplantation of the tumor, when the excretion of urea would have been relatively low in a depleted rat (Allison et al., '56) was as follows: tumor-bearing rats fed 12% casein, 21 mg.; animals fed casein supplemented with methionine, 19.5 mg; those fed casein supplemented with methionine plus glycine, 30 mg; those fed methionine plus

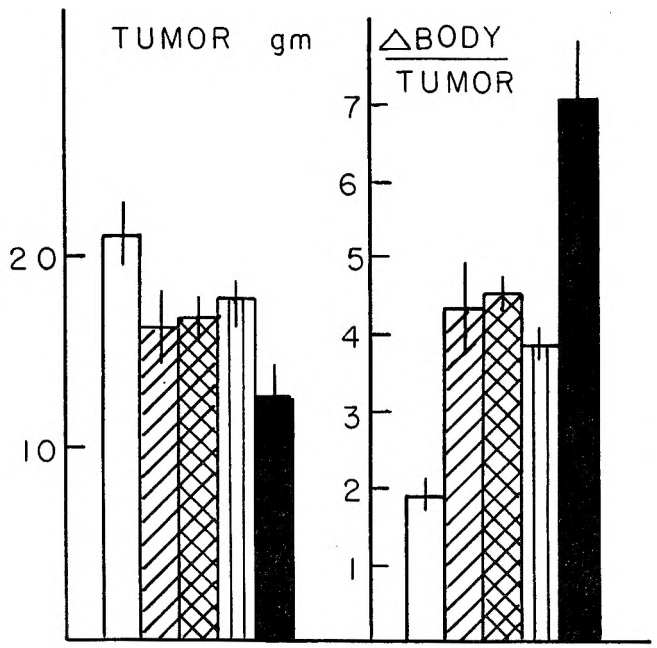

Fig. 3 The effect of supplementation upon the development of the Walker carcinoma 256 and upon the increase in weight of the body with respect to the tumor. White bars record average data for tumor-bearing rats fed diet containing 12% casein. Supplementation with methionine, bars with slanted lines, with methionine plus glycine, bars with crossed lines; methionine plus ammonium citrate, bars with vertical lines; methionine plus guanidoacetic acid, black bars.

ammonium citrate, 31 mg; and sarcoma-bearing rats fed methionine plus guanidoacetic acid, 11 mg urea nitrogen/rat/day. As in other experiments this reduction in catabolic activity (as measured by urea nitrogen) in the presence of a mixture of methionine and guanidoacetic acid was not observed until the tumor was large and, thereby, at a time

when a marked stress was being placed upon the animal. No reduction in the growth of the Flexner-Jobling carcinoma nor increase in the already relatively large body weight gain/tumor ratio was observed when rats were fed casein supplemented with either methionine or a mixture of methionine and guanidoacetic acid. Rather, supplementation of this type tended to improve the growth of the tumor slightly, as was the case in Flexner-Jobling carcinoma-bearing animals fed a protein of high nutritive value such as egg albumin.

SUMMARY

The effects of dietary protein upon the development of three types of transplanted tumors were studied in the rat. A sarcoma developed at essentially the same rate in rats fed diets containing wheat gluten, casein or beef but was slightly depressed in animals fed egg albumin. The bodies of the rats, however, developed according to the nutritive value of the protein, growing poorly in animals fed wheat gluten and at a maximum in those fed egg albumin. During the terminal stages the sarcoma increased catabolism and depleted normal tissues but increased relatively the liver protein and liver fat above pair fed controls. Similar effects on tumor and body growth were observed in rats bearing the Walker carcinoma 256. The development of the Flexner-Jobling carcinoma, on the other hand, was very poor in animals fed wheat gluten and maximum in rats fed egg albumin, a response that was more like the body tissues. Supplementation of the casein diet with methionine or methionine plus guanidoacetic acid had beneficial effects upon the development of normal tissues in the presence of the sarcoma or Walker carcinoma 256, but was much less beneficial in the presence of the Flexner-Jobling carcinoma. The suggestion was made that tumors may vary as do normal tissues in their interrelationships to body metabolic pools, some tissue proteins being depleted more than others, some not at all in the presence of inadequate amino acid intake. A possible correlation between protein anabolic, catabolic influence of these

tumors and the effects of ethylenephosphoramides was also suggested.

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Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award.

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