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THIRTY-SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

A STUDY OF THE NUTRITIVE VALUE OF PROTEINS FROM DIFFERENT SOURCES IN THE FEEDING OF AFRICAN CHILDREN [1]

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(Received for publication March 4, 1958)

The study of the nutritive value of new sources or combinations of proteins attracts more and more interest in the world. This is especially true in underdeveloped countries where the problem of protein malnutrition is very often acute. With this object in mind, we have tried to estimate the supplementation value to a basic diet of different protein-rich foods. The first foods to be tested in this laboratory have been milk, peanut and soybean flour, and a combination of beans and peanuts. The nutritive value of milk is well known and its protein is widely used in the treatment of protein malnutrition, especially kwashiorkor (DeMaeyer, '54). It has been included in this study since there is actually a trend to use it as a reference protein. The other foods were selected because they represent either the main sources of proteins in this part of the country (Kivu Province, Belgian Congo) or, like soya, are frequently used as supplementary foods in schools or hospitals.

Several methods have already been proposed for the estimation of the nutritive value of proteins, and an excellent review has recently been published by Allison ('55) on this

[1] This work was aided in part by a grant from the Food and Nutrition Board, National Academy of Sciences — National Research Council of the United States.

[2] Institut pour la Recherche Scientifique en Afrique Centrale.

subject. In our investigation, we have used the nitrogen balance technique and have estimated the value of the supplementation by an analysis of the nitrogen retention.

SUBJECTS

The study was conducted on African children from the Bashi tribe living around Lwiro. These people are in the habit of coming to the dispensary of the Institute when they are sick. Eighteen children, recovering from kwashiorkor, were selected for the study. They are described in table 1.

TABLE 1

Sex and approximate age of children in balance experiment

CODE NO	SEX	AGE	CODE NO	SEX	AGE
		yrs.			*yrs.*
D 4	boy	3½	D 59	boy	5 [1]
D 8	boy	7 [1]	D 81	girl	3½
D 9	boy	5½	D 83	boy	4½
D 14	girl	4	D 85	boy	4 [1]
D 17	boy	3½	D 93	boy	3
D 20	girl	12	D 96	boy	4
D 22	girl	3½	D 98	girl	4
D 27	girl	6	D 101	boy	5½ [1]
D 49	girl	5½	D 104	girl	3½

[1] Approximately.

All except one (D 20), were children from three to 7 years old. D 20 was first included in the experimental group on the criterion of her weight. Later on, when her age became known, the child was nevertheless kept in the group, since the experimental data were consistent with those of the younger children. The experimental subjects were all former cases of kwashiorkor who had been brought to the clinical investigation center for treatment, and these children were thus at different stages of protein depletion. It is known that the retention of nitrogen is very high at the beginning of the treatment and tends to decrease thereafter (DeMaeyer and Vanderborght, '56). The balances to be described in this paper were, therefore, never performed before the 5th week of treatment. By that time,

the nitrogen retention for a given protein remains fairly constant, although slight variations caused by different levels of protein repletion may occur.

The experimental group was hospitalized at the clinical investigation center of the Institute where it is possible to keep the children under strict supervision. A small garden, level with the wards, allowed some physical activity. All the children were given a physical examination at their entrance, including an x-ray of the chest. Malaria is seldom seen in this part of the country; nevertheless each child was given antimalarial drugs as he or she came in. The microscopic examination of the feces showed the usual pattern of infestation (DeMaeyer and Vanderborght, '56), ascaris and trichiuris being the most frequent, but hookworms were not found. No attempt was made to worm the children. Only once was a treatment applied when taenia eggs were seen, since the presence of proglottids in the stools was a possible source of error in the nitrogen determinations. Ascarides, when present in the stools, were carefully removed, but they were not very often observed.

The children were trained to the discipline of the balance technique during the initial period of their hospitalization. No use was made of any kind of metabolic bed, since we feared this device might put a psychological stress upon the children, inconsistent with physiological processes. It was possible to teach the subjects to urinate and to pass stools in different pans in order to get complete 24-hour collections of urine and stools. The children were thus kept in an environment similar to the one prevailing in their ordinary life and were able to continue a reasonable amount of physical activity.

Each balance period lasted 5 days, beginning on a Monday morning and ending at the same time on the following Saturday. With each change of diet the subjects were first kept for 9 days on the diet, and it was only on the morning of the 10th day that the balance really began. At that time the children emptied the bladder as completely as possible and passed a stool. The latter was quite easy, as they usually

have from three to 4 bowel movements per day. These specimens were discarded and the collection started afterwards. The same procedure was repeated each day at the same hour (usually 8 A.M.), the stools and urine being added this time to the collection of the preceding day in order to complete the 24-hour samples. The procedure was followed with special care on the Saturday morning at the end of the balance period.

The 24-hour collections of stools and urine were kept in the refrigerator, the former mixed with 0.1 N HCl and the latter under a layer of toluene.

ANALYSES

Nitrogen in food. A sample of each food was taken every day for analysis. However, when the food was bought in lots, as in the case of milk, soya or peanut flour, the analysis was repeated only with each change of lot.

Nitrogen in urine and stools. Nitrogen determinations were made on each 24-hour sample. The urine analysis was performed on a 5 or 10 ml aliquot, according to the expected nitrogen content. The stools were first homogenized in a Waring Blendor in presence of 0.1 N HCl to secure a semiliquid paste. A 5-gm sample of this mixture was then used for the determination.

Ashing of foods, urine and stools was performed in the presence of concentrated sulfuric acid and a catalyst mixture composed of potassium sulfate and mercuric sulfate (Hiller et al., '48). When it was completed, the acid residues were brought to a predetermined volume (usually 500 ml) of which an aliquot was taken for the determination of the ammonia (Keys, '40; Ma and Zuazaga, '42).

THE DIET

The diet was mixed. It was composed of a basic portion, which remained almost constant throughout the experiment, and of a variable part which was formed by the supplementary food. The experimental foods were skim milk, a combination

of beans and peanuts, peanut flour and soybean flour. The composition of the basic diet is shown in table 2. In addition, each child received a daily dose of a vitamin and mineral mixture [3] and a weekly dose of 50,000 I.U. of vitamin A. The basic diet provided each child with an average of 8 gm of proteins per day or between 0.44 and 0.57 gm of proteins per kilogram of body weight per day.

The foods to be tested were fed in varying amounts, at least two and very often three different levels of intake being used on each child. The diet (basic portion plus supplement) was designed to provide a minimal Caloric intake of 75 Cal./kg body wt./day. Very often it reached a level of 110 Cal./kg/day.

The foods tested were prepared in the following manner: skim milk (spray dried) was brought into solution by the addition of water and homogenized in a Waring Blendor; beans (bought in a local market) were cooked for one to two hours in an open pan. Small additions of water were made from time to time in order to prevent burning. The final preparation was almost dry and no juice was left in the pan; peanuts (bought in a local market) were first slightly roasted and then ground in a mortar. The resulting paste was cooked for a short time with water; peanut flour [4] was cooked for a few minutes with water; soybean flour [5] was cooked for about 10 minutes with water. The weight of the preparation was usually three to 4 times that of the dry flour.

RESULTS

No allowance has been made in the computation of the results for the loss of nitrogen through sweat, since this is a temperate climate where the average temperature is about 22°C.

[3] The vitamin and mineral mixture was formed of aneurin, 5 mg; lactoflavin, 2 mg; niacin, 20 mg; adermin, 2 mg; Ca pantothenate 3 mg; folic acid, 5 mg; ascorbic acid, 5 mg; vitamin B_{12}, 30 μg; Ca gluconate, 10 gm.

[4] The peanut flour was given by UNICEF. It was produced by Unilever Company in England and bears the UNICEF Code PF-4.

[5] The soybean flour was purchased from British Soya Products, London.

TABLE 2

Approximate composition of the basic portion of the experimental diet

	MEAN CONSUMPTION PER DAY IN GRAMS				WEIGHT IN GRAMS AND RELATIVE PERCENTAGE OF PROTEINS SUPPLIED PER DAY BY THE BASIC PORTION OF THE DIET							
	Experimental groups [1]				Experimental groups							
	I	II	III	IV	I		II		III		IV	
Rice	50	41	47	49	3.80	47%	3.12	42%	3.57	47%	3.72	46%
Banana flour	35	30	31	34	0.91	11%	0.78	11%	0.81	11%	0.89	11%
Bread	28	29	25	28	2.38	29%	2.47	33%	2.13	28%	2.38	30%
Fresh banana [2]	80	80	80	80	1.04	13%	1.04	14%	1.04	14%	1.04	13%
Red palm oil	27	25	30	26	..	..	..	..	..	..	..	..
Butter	19	21	15	19	..	..	..	..	..	..	..	..
Sugar	30	30	30	30	..	..	..	..	..	..	..	..
Orange or lime	1 [3]	1	1	1	..	..	..	..	..	..	..	..
Mean body weight of the children in kilograms	14.2	16.9	13.3	15.2	Total 8.13	100%	7.41	100%	7.55	100%	8.03	100%

[1] The first group received the basic diet plus milk; the second the basic diet plus a combination of beans and peanuts; the third the basic diet plus peanut flour and the 4th the basic diet plus soya flour.

[2] Weight of the edible portion.

[3] Juice of one orange or lime.

All the figures shown in the tables are means calculated from the data collected each day during the 5-day balance periods. The following formulas have been used in the presentation of the results:

(1) Nitrogen absorbed = Nitrogen Intake — Fecal Nitrogen

(2) Balance or retention =
Nitrogen Intake — (Urinary Nitrogen + Fecal Nitrogen)

$$(3)\quad \text{Absorption in } \% = \frac{\text{Nitrogen Intake} - \text{Fecal Nitrogen}}{\text{Nitrogen Intake}} \times 100$$

$$(4)\quad \text{Corrected absorption in } \% = \frac{\text{Nitrogen Intake} - (\text{Fecal Nitrogen} - \text{Endogenous Fecal Nitrogen})}{\text{Nitrogen Intake}} \times 100$$

The endogenous fecal nitrogen has been determined on a group of children of the same age fed a protein-free diet (providing less than 8 mg of nitrogen/kg/24hr.). The average value was found to be 42 mg/kg/24hr. The regression equations have been calculated by the method of least squares.

The experimental scheme included 4 groups that differed according to the supplementary food introduced into the diet. The first group received milk. Twenty-eight balances were performed on 11 children. The nitrogen supplied by the milk ranged from 64.2 to 88.5% of the total intake; the second group received a combination of beans and peanuts. Twenty-five balances were performed on 7 children. The percentage of nitrogen supplied by the combination of beans and peanuts ranged from 60 to 92%. Peanuts and beans were always provided in a 1/1 ratio (dry weight). In one instance however (last balance of D 49), the child could eat only 75 gm of peanuts for 100 gm of beans. Since the experimental data were consistent with those gained previously, the balance was included in the analysis.

The third group received peanut flour. Ten balances were performed on 4 children. The peanut flour supplied from 68.9 to 82.7% of the total nitrogen intake; the 4th group received soybean flour. Thirty-two balances were performed on 14 children. The soybean flour supplied from 66.8 to 86.7% of the total nitrogen intake.

The results of the balances are listed in tables 3, 4, 5 and 6. The regression equations of the nitrogen retention to the absorbed nitrogen and to the nitrogen intake have been calculated for each group. They are as follows:

Nitrogen retention/nitrogen absorbed: for milk: $y = -106 + 0.782x$; for beans + peanuts: $y = -128 + 0.716x$; for peanut flour: $y = -103 + 0.653x$ and for soybean flour: $y = -106 + 0.619x$.

Nitrogen retention/nitrogen intake: for milk: $y = -144 + 0.709x$; for beans + peanuts: $y = -159 + 0.595x$; for peanut flour: $y = -121 + 0.533x$ and for soybean flour: $y = -129 + 0.528x$.

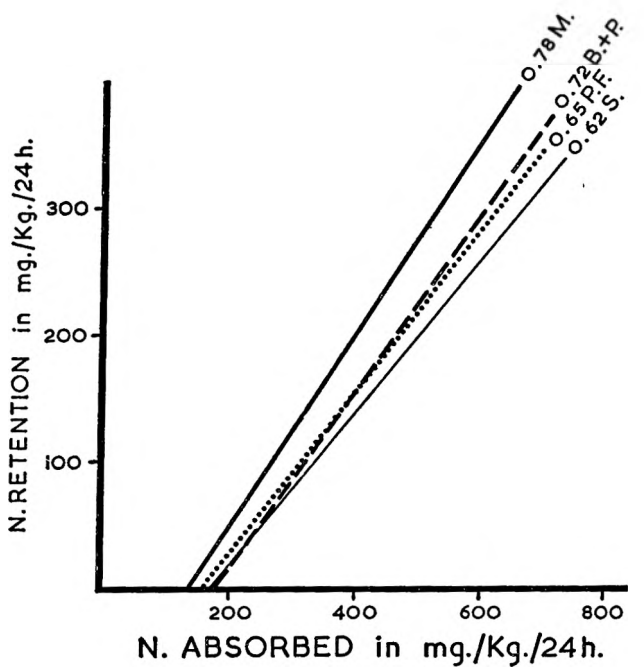

Fig. 1 The regression lines of the nitrogen retention versus the nitrogen absorbed for the different types of supplementation.

M. = Basic diet plus skim milk
B. + P. = Basic diet plus combination of beans and peanuts
P.F. = Basic diet plus peanut flour
S.F. = Basic diet plus soybean flour

These regression equations have been illustrated in figures 1 and 2.

The apparent and corrected absorptions and the percentages of nitrogen retained at different levels of intake are listed in table 7. The percentages of nitrogen retained have been illustrated in figure 3.

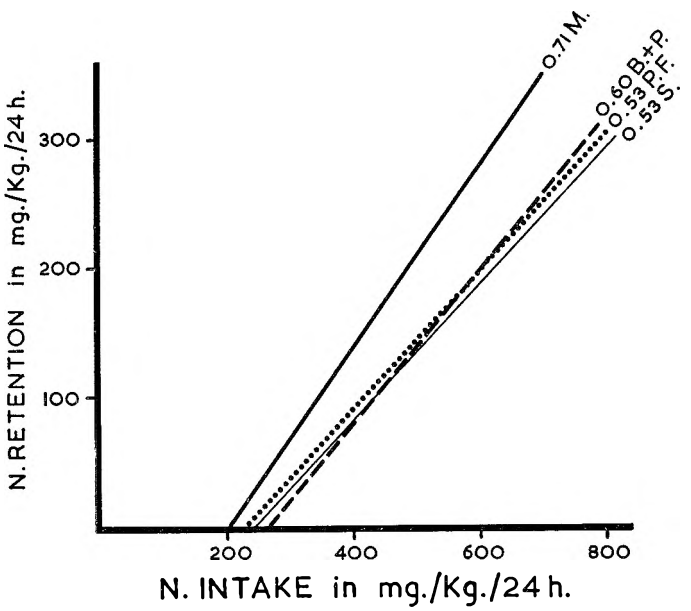

Fig. 2 The regression lines of the nitrogen retention versus the nitrogen intake for the different types of supplementation.

M. = Basic diet plus skim milk
B. + P. = Basic diet plus combination of beans and peanuts
P.F. = Basic diet plus peanut flour
S.F. = Basic diet plus soybean flour

DISCUSSION

Absorption. The absorption figures found in this experiment are lower than those usually found in white children. According to reports from Macy's laboratory, the fecal nitrogen does not normally exceed 10% of the nitrogen intake. High fecal values in African children have, however, been

TABLE 3

Balance studies with milk as the supplementary food

NO. OF CHILD	WEIGHT [1]	NITROGEN PROVIDED BY MILK	NITROGEN INTAKE	URINARY NITROGEN	FECAL NITROGEN	NITROGEN ABSORBED	NITROGEN RETENTION
	kg	*%*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*
D 4	9.67	64.2	388	148	61	327	179
D 4	10.01	75.1	528	188	59	469	281
D 8	17.61	75.2	501	184	79	422	238
D 8	18.12	84.1	824	241	123	701	461
D 8	18.23	79.8	648	225	94	554	329
D 8	18.27	86.5	970	305	104	866	561
D 9	11.15	85.2	907	296	154	753	457
D 9	11.32	85.9	887	215	157	730	515
D 14	11.37	82.8	711	324	96	615	291
D 14	13.37	85.6	753	218	116	637	419
D 14	13.32	85.3	758	269	104	654	385
D 17	12.72	83.4	678	264	174	504	240
D 17	13.05	83.5	660	275	119	541	266
D 17	13.07	83.9	656	247	101	555	307
D 20	14.72	83.3	587	205	157	430	225
D 20	15.52	83.1	558	206	134	424	218
D 20	15.95	83.0	543	199	130	413	214
D 20	16.16	72.3	326	160	119	207	47
D 20	16.65	88.5	758	193	112	646	453
D 22	11.32	83.2	770	202	76	694	492
D 93	12.41	85.5	822	233	160	662	429
D 93	12.75	85.1	805	285	110	695	410
D 93	13.03	74.8	448	225	136	312	87
D 98	16.21	69.2	292	152	92	200	48
D 98	16.46	81.6	488	206	67	421	215
D 101	14.91	81.8	537	138	69	468	330
D 101	15.27	69.1	310	131	72	238	107
D 104	13.86	81.6	579	248	38	541	293

[1] All figures are means calculated from the data recorded everyday during the 5-day balance periods.

TABLE 4

Balance studies with a combination of beans and peanuts as the supplementary food

NO. OF CHILD	WEIGHT [1]	NITROGEN PROVIDED BY COMBINATION OF BEANS AND PEANUTS	NITROGEN INTAKE	URINARY NITROGEN	FECAL NITROGEN	NITROGEN ABSORBED	NITROGEN RETENTION
	kg	*%*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*
D 8	20.00	60	305	211	82	223	12
D 8	22.00	75	447	206	97	350	144
D 8	22.00	75	453	242	101	352	110
D 8	22.00	69	355	232	80	275	43
D 8	23.00	92	536	261	98	438	177
D 9	10.44	85	629	189	177	452	263
D 9	10.47	78	445	195	103	342	147
D 14	12.37	73	402	190	108	294	104
D 14	12.70	82	591	266	146	445	179
D 14	12.84	74	389	231	141	248	17
D 17	18.82	72	280	162	109	171	9
D 17	19.39	84	466	221	144	322	101
D 17	19.64	83	463	203	110	353	150
D 17	20.20	78	347	177	121	226	49
D 17	21.00	92	599	275	154	445	170
D 20	19.37	70	268	158	101	167	9
D 20	19.65	92	64.	254	151	494	240
D 20	19.67	82	429	237	123	306	69
D 20	19.71	83	435	210	111	324	114
D 22	12.72	78	370	181	87	283	102
D 22	12.85	79	357	230	123	234	4
D 22	13.06	87	595	276	141	444	168
D 22	13.10	89	659	281	146	513	232
D 49	12.36	89	529	235	153	376	141
D 49	12.82	89	558	281	145	413	132

[1] All figures are means calculated from the data recorded every day during the 5-day balance periods.

TABLE 5

Balance studies with peanut flour as the supplementary food

NO. OF CHILD	WEIGHT [1]	NITROGEN PROVIDED BY PEANUT FLOUR	NITROGEN INTAKE	URINARY NITROGEN	FECAL NITROGEN	NITROGEN ABSORBED	NITROGEN RETENTION
	kg	*%*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*
D 93	12.41	82.7	689	292	174	515	223
D 93	12.28	82.7	701	284	139	562	278
D 93	12.41	70.2	412	195	130	282	87
D 93	12.46	70.1	407	190	99	308	118
D 96	13.81	82.7	608	279	158	450	171
D 96	13.79	82.2	611	274	152	459	185
D 96	13.04	71.7	397	209	134	263	54
D 96	12.94	70.8	381	218	109	272	54
D 101	15.01	68.9	314	177	65	249	72
D 104	14.44	82.2	570	258	57	513	255

[1] All figures are means calculated from the data recorded every day during the 5-day balance periods.

TABLE 6

Balance studies with soybean flour as the supplementary food

NO. OF CHILD	WEIGHT [1]	NITROGEN PROVIDED BY SOYBEAN FLOUR	NITROGEN INTAKE	URINARY NITROGEN	FECAL NITROGEN	NITROGEN ABSORBED	NITROGEN RETENTION
	kg	*%*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*	*mg/kg/24 hr.*
D 8	19.76	71.2	415	281	103	312	31
D 8	20.00	83.5	723	297	92	631	334
D 8	20.00	82.9	719	327	66	653	326
D 8	20.00	69.9	409	301	88	321	20
D 9	10.45	79.7	693	245	129	564	319
D 14	12.22	81.1	594	218	114	480	262
D 17	18.21	86.7	623	349	127	496	147
D 17	16.74	80.1	447	309	101	346	37
D 17	12.84	84.6	713	389	219	494	105
D 17	12.64	84.3	738	349	154	584	235
D 17	13.26	83.7	681	425	135	546	121
D 17	13.06	74.1	437	295	124	313	18
D 20	17.50	87.7	674	261	122	552	291
D 20	18.23	74.8	325	179	98	227	48
D 20	18.97	88.8	696	307	109	587	280
D 22	11.76	82.8	734	429	130	604	175
D 22	12.58	85.3	558	305	87	471	166
D 27	13.23	83.9	687	289	121	568	277
D 27	14.09	83.8	651	305	143	508	203
D 27	14.23	84.2	646	296	138	508	212
D 59	14.64	84.3	623	211	167	456	245
D 59	14.57	84.0	618	241	177	441	200
D 59	15.07	84.6	617	337	154	463	126
D 81	12.31	84.4	768	265	175	593	328
D 81	12.48	67.6	361	145	128	233	88
D 83	15.89	84.5	597	316	128	469	153
D 83	15.23	66.6	294	147	117	177	30
D 85	15.15	84.8	630	300	150	480	180
D 85	14.53	66.8	310	155	92	218	63
D 98	15.83	69.3	305	141	54	251	110
D 101	15.15	69.3	321	168	81	240	72
D 104	14.57	82.4	574	267	51	523	256

[1] All figures are means calculated from the data recorded every day during the 5-day balance periods.

TABLE 7

Nitrogen absorption and percentage of retention for the different types of experimental foods

	APPARENT ABSORPTION	CORRECTED ABSORPTION	% OF NITROGEN RETAINED IN THE BODY						
			Nitrogen intake in mg/kg/24 hr.						
			300	400	500	600	700	800	900
	% of intake	*% of intake*							
Milk	81.89	89.28	23.0	35.0	42.2	46.8	50.3	52.9	54.9
Peanuts + beans	72.82	82.52	6.7	19.8	27.8	33.0	36.9	39.6	41.9
Peanut flour	75.50	84.38	12.7	22.8	28.8	32.8	35.7	37.9	39.6
Soybean flour	77.74	85.86	9.7	20.5	27.0	31.3	34.4	36.6	38.4

found by Bray ('53) and in African adults by Holmes et al. ('54). The elevation may be caused to a certain extent by a high level of endogenous nitrogen. An investigation that we have made on a group of 9 children of approximately the same age, fed a protein-free diet, gave an average value of 42 mg/kg/24hr. for the endogenous fecal nitrogen.[6] Using this value of 42 mg in order to correct the figures for fecal

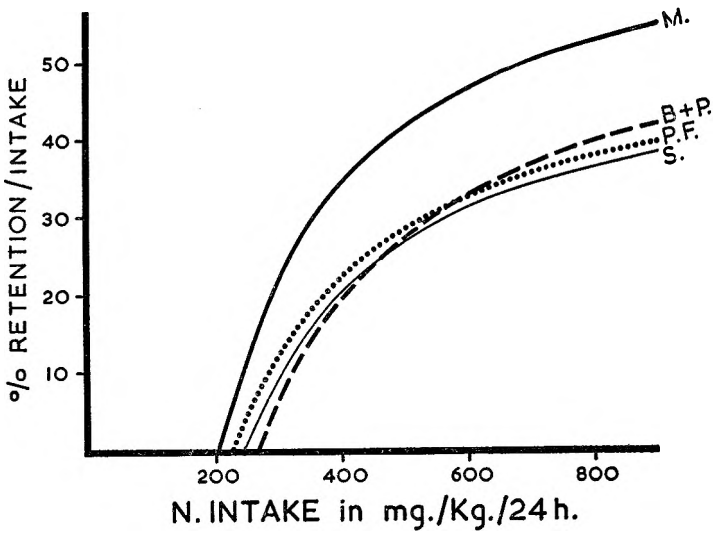

Fig. 3 The percentage of nitrogen retained at different levels of nitrogen intake.

M. = Basic diet plus skim milk
B. + P. = Basic diet plus combination of beans and peanuts
P.F. = Basic diet plus peanut flour
S.F. = Basic diet plus soybean flour

nitrogen, we find that the percentages of absorption nevertheless remain lower than normal. It seems thus that a slight defect of the absorption process does exist, caused either by a digestive failure or by a primary lack of absorption through the intestinal wall, or by both. A failure of the pancreatic secretion in cases of kwashiorkor has already been described by Thompson and Trowell ('52), and it is highly probable

[6] DeMaeyer and Vanderborght, unpublished data.

that a deficiency of that sort is partially responsible for the high fecal values which have been found in this experiment.

The degree of absorption varies with the nature of the proteins fed. A diet supplemented with milk is significantly better absorbed than one containing proteins from purely vegetable sources. The lowest absorption occurs when a combination of beans and peanuts is introduced into the diet. This is not surprising, since beans have a high content of cellulose. The significance of the differences of absorption between the various diets has been calculated with the following results ("t test" of Student):

	DIFFERENCE OF ABSORPTION	t	P	n (number of balances)
Milk vs. soybean flour	4.15	2.286	0.02–0.05	60
Milk vs. peanut flour	6.39	2.465	0.01–0.02	38
Milk vs. beans and peanuts	9.07	5.102	<0.001	53

Retention. The nitrogen retention has been correlated with the nitrogen absorbed and with the nitrogen intake as shown in figures 1 and 2. A first-degree equation or a straight line expresses the experimental data perfectly, and there is no indication that another mathematical expression would be more suitable. This means that between the experimental limits, the ratio of increase of the nitrogen retention to the nitrogen either absorbed or ingested remains precisely the same. The relative depletion of the protein stores possibly explains the fact that the ratio does not tend to decrease.

The slope of the line representing the relationship between the nitrogen retention and the nitrogen absorbed may be called "k." Its value is very similar to that of the classical term "biological value," which is often represented by "K." These values of "k" are respectively 0.78, 0.72, 0.65 and 0.62, when skim milk, beans and peanuts, peanut flour, or soybean flour are introduced into the diet. It is to be remembered, however, that the diet used was a mixed one and that these figures do not express the value of one but of a sum of various proteins. When the retention is correlated with the nitrogen

intake, the same type of equations may be calculated. In this case the slope of the line combines the concept of biological value with that of digestibility, and we have used the term "utilization ratio" to define it. Its significance is close to the "net protein utilization." The ratios are respectively 0.71, 0.60, 0.53 and 0.53 for the proteins tested. It is obvious that owing to a poor digestibility, some of the foods lose part of their value. This actually happens with the combination of beans and peanuts (0.60 versus 0.72).

The relative percentages of nitrogen retained in the body for each type of diet are illustrated in figure 3. These have been calculated by using the regression equations of the nitrogen retention to the nitrogen intake, which give the expected retention for each level of nitrogen intake. From these data, it is easy to calculate the percentages of retention.

It appears thus that milk has obviously the highest supplementary value; the combination of beans and peanuts comes next. Soybean flour and peanut flour have about the same value. It is possible, however, that owing to the short cooking, an anti-enzyme factor is responsible for the relatively low value of soybean flour.

SUMMARY

A study of the supplementation value of different protein-rich foods has been conducted on African children aged from 3 to 7 years and recovering from kwashiorkor. The diet consisted of a basic portion that included rice, bread, banana flour, butter, palm oil, sugar and fruits, supplying an average of 25% of the nitrogen intake. The rest of the nitrogen was supplied by the supplementary food which was either skim milk or a combination of beans and peanuts or peanut flour or soybean flour. The retention of the nitrogen absorbed was highest when milk was introduced into the diet; the combination of beans and peanuts came next, followed by peanut and soybean flour. When the retention was correlated with the nitrogen intake (the digestibility was thus taken into account) the foods might still be classified in the same manner,

although some of them lost part of their value. That actually happened for the combination of beans and peanuts. The experiment demonstrates clearly that nitrogen absorption and retention is highest when milk is introduced into the diet. It shows also that a combination of beans and peanuts has a higher supplementary value than peanut or soybean flour given alone.

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THE NUTRITIONAL VALUE OF A SYNTHETIC DIET STERILIZED BY GAMMA RAYS, AS MEASURED BY REPRODUCTION AND LIFE SPAN OF RATS

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The Office of the Surgeon General of U. S. Army has carried out an investigation to determine the nutritional properties of foods which have been sterilized by gamma radiation from fission products. This laboratory has participated in this overall program on radiation preservation of foods since September 1954. One study in the program was to determine the nutritional value of an irradiated synthetic diet for reproduction and its effect on longevity in rats. The results of this study are described in this report.

EXPERIMENTAL

Four generations of rats, both in the reproduction and longevity studies, received a synthetic diet which had been irradiated with 2.79×10^6 rad of gamma rays. The same number of animals received the non-irradiated diet as a control. The composition of the diet is given in table 1. The diet was mixed in the laboratory, sealed in no. 2 plain tin cans, stored overnight at $-4°$F, packed in canvas packers with dry ice and shipped to the Materials Testing Reactor at Idaho Falls, Idaho. After the diet had come to ambient temperature half of each shipment was irradiated. This portion was designated the irradiated diet. The other half was not irradiated and it was used as the control diet. The irradiated and control diets

were repacked in the canvas packers with dry ice and returned to the laboratory where both diets were stored in a freezer until fed to the rats. New lots of the diet were shipped to the Irradiation Center at intervals of 4 to 8 weeks. Diets and water were supplied ad libitum.

Poling and co-workers ('55) have reported that diets containing irradiated beef lost vitamin E during storage and in view of the possibility that this vitamin and also vitamin A

TABLE 1

Composition of diet

CONSTITUENTS	AMOUNT	VITAMINS PER 100 GM	
	gm		
Soybean protein [1]	30.0	Vitamin A	3000 I.U.
Cerelose	56.1	Vitamin D	400 I.U.
Wood pulp	3.0	Alpha-tocopherol	2.5 mg
Mineral mixture [2]	5.0	Menadione	0.75 mg
DL-Methionine	0.3	Thiamine-hydrochloride	1.0 mg
Corn oil [3]	5.5	Riboflavin	1.0 mg
Choline chloride	0.1	Pyridoxine-hydrochloride	1.0 mg
Inositol	0.01	Calcium pantothenate	4.0 mg
		Niacin	5.0 mg
		Folic acid	0.1 mg
		Biotin	10.0 μg
		Vitamin B_{12}	2.5 μg

[1] Drackett C-1 protein, The Drackett Products Company, Cincinnati, Ohio.
[2] Richardson and Hogan ('46).
[3] Mazola.

might be destroyed during irradiation or while the diet was stored, each rat in the irradiated and control groups received one drop of a mixture of oleum percomorphum, alpha tocopherol acetate and corn oil [1] twice weekly. One drop of this mixture supplied approximately 300 I.U. of vitamin A and 0.25 mg of alpha tocopherol.

Reproduction. In the reproduction study 20 females each received the irradiated and control diets in each of the 4 generations. Five females and one male were kept in a stock cage. All animals were weighed weekly and females which were

[1] Mazola.

TABLE 2

Total litters, and young observed in four generations of rats receiving an irradiated and a non-irradiated synthetic diet

GENERATION	DIET	LITTERS	YOUNG				
			Born	Observed [1]	Weaned		Av. wt.
		no.	*no.*	*no.*	*no.*	*%*	*gm*
Parent	Irradiated	156	1128	956	856	89.5	34.3
	Control	134	911	807	762	94.4	38.7
F_1	Irradiated	134	890	801	733	91.5	36.4
	Control	117	762	683	639	93.6	38.2
F_2	Irradiated	122	864	733	589	80.3	34.6
	Control	144	917	811	750	92.5	38.2
F_3	Irradiated	91	585	503	389	77.3	35.4
	Control	113	700	622	516	83.0	37.3
Total	Irradiated	503	3467	2993	2567	84.7	35.1
	Control	508	3290	2923	2667	90.9	38.2

[1] All litters were reduced to 8 young at birth.

pregnant, as indicated by a marked increase in weight, were put into a maternity cage containing shredded paper so that the mother could make a nest for the young. All litters were reduced to 8 young at birth, and the young were weighed and weaned at 21 days.

The total number of litters and young produced by 20 females per group in each generation and the average weaning weight of the young are given in table 2. The average number of litters and average number of young produced per female are given in table 3. Approximately 500 litters and 3400 young

TABLE 3

Average number of litters, and average number of young born, observed, and weaned per female receiving irradiated and control diets

GENERATION	DIET	AVERAGE PER FEMALE			
		Litters born	Young born	Young observed	Young weaned
		no.	*no.*	*no.*	*no.*
Parent	Irradiated	7.8	56	48	43
	Control	6.7	46	40	38
F_1	Irradiated	6.7	45	40	37
	Control	5.9	38	34	32
F_2	Irradiated	6.1	43	37	29
	Control	7.2	46	41	38
F_3	Irradiated	4.6	29	25	19
	Control	5.7	35	31	26

were born to females receiving the irradiated and control diets. An overall total of 84.7% of the young were weaned from females receiving the irradiated diet and 90.9% were weaned from those receiving the control diet. Fewer young were born to females in the F_3 generation and a smaller percentage of these young were weaned than in the other three generations. The percentage of young weaned was consistently higher in the control group in each of the 4 generations, but the differences between the irradiation and control groups were greater in the F_2 and F_3 generations than in the parent and F_1 generations. Furthermore, the young from mothers receiving the

control diet were an average of two to 4 gm heavier at weaning age than those from mothers receiving the irradiated diet.

The mortality in the females ranged from 10 to 30% and in general was higher in the groups receiving the control diet. Animals which did not die were sacrificed when it was obvious that they were moribund. Most of these animals were over 20 months old when they were sacrificed.

Longevity. Ten female and 10 male rats received the irradiated and control diets in each generation in the longevity study. The reproduction and longevity studies were conducted concurrently and the animals in both series received diets which were mixed, irradiated and stored under identical conditions. Five males or 5 females were kept in a stock cage and received the diet and water ad libitum.

The weight of each animal and amount of diet consumed by the animals in each cage were recorded weekly. The average weights of the animals receiving the irradiated and control diets for an experimental period of 80 weeks are summarized in the curves in figure 1. The males in the control group were heavier than those in the irradiated group for 80 weeks or longer in each generation. Data for a longer period were not plotted because many of the animals either died or were sacrificed soon after this time. The females in the irradiated group in the F_1 generation weighed slightly more than those in the control group; but the females in the parent, F_2 and F_3 generations weighed slightly more in the control group than in the irradiated until 60 to 70 weeks. After this time females in the irradiated group weighed as much or slightly more than those in the control group.

A summary of animals which died or were sacrificed with an average age, in days, at the time of death or at the time of sacrificing, is given in table 4. These data indicate that there was no important difference in the life span of animals receiving the irradiated and control diets. Similar data for the animals in reproduction study agreed with those given in table 4.

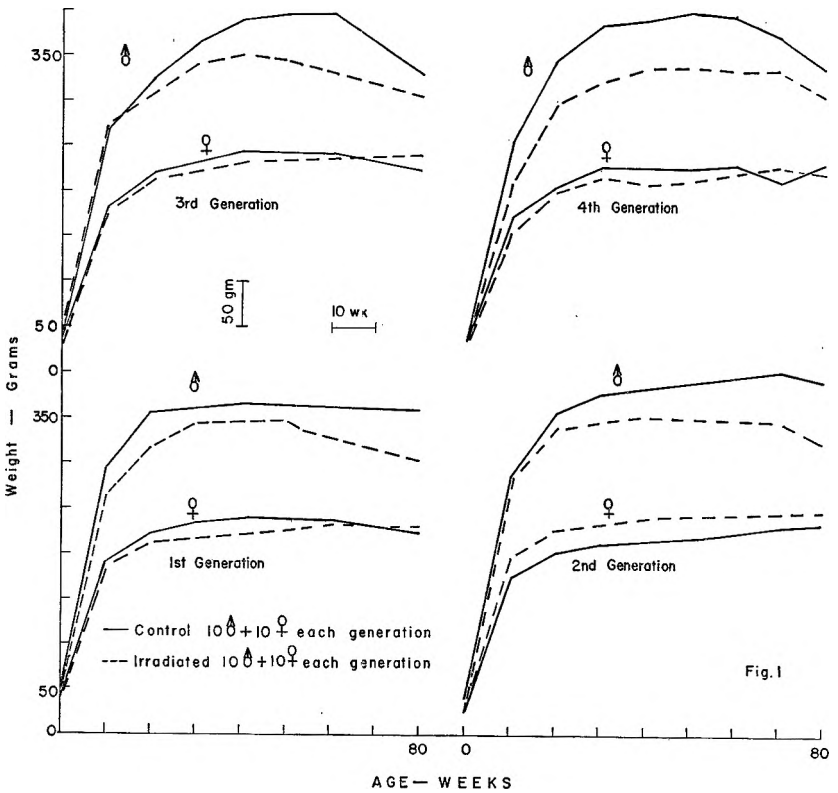

Fig. 1 Average weights of rats receiving irradiated and control diets.

DISCUSSION

Since all animals received a supplement of vitamin A and E the effect of irradiation on these nutrients was not considered in this investigation.

The results of practically every test in both the reproduction and longevity study, with an occasional minor exception, showed that the nutritive value of the irradiated diet was slightly less than that of the non-irradiated or control diet. The difference between the two diets, however as measured by growth of animals, number of young born and weaned and average weaning weight of the young, was very small and it was concluded that the irradiation process did not seriously

damage the nutritive value of the diet. Animals consumed essentially the same amount of irradiated and control diets, therefore the difference between the effects of the two diets could not be attributed to food intake. There was no evidence from mortality or appearance of the animals that the irradiated diet was toxic. Other experiments which were carried out

TABLE 4

Summary of final disposition of rats in the longevity study

GENERATION	DIET	SEX [1]	DIED		SACRIFICED	
				Av. age		Av. age
			%	*days*	%	*days*
Parent	Irradiated	M	80	727	20	809
		F	40	654	60	809
	Control	M	50	608	50	809
		F	70	711	30	812
F_1	Irradiated	M	30	734	70	745
		F	20	680	80	760
	Control	M	30	598	70	753
		F	20	573	80	756
F_2	Irradiated	M	—	—	100	635
		F	—	—	100	673
	Control	M	20	433	80	622
		F	40	557	60	662
F_3	Irradiated	M	—	—	100	608
		F	10	574	90	598
	Control	M	30	487	70	651
		F	20	519	80	560

[1] There were 10 males and 10 females in each group in each of the 4 generations.

in an attempt to identify the specific nutrient involved were unsuccessful. No comparison was made of the nutritive value of a diet sterilized by irradiation and one sterilized by some process commonly used for the preservation of foods. It is believed that the difference between the irradiated and controlled diets was no greater than would be expected if the diet had been preserved by some other process.

SUMMARY

Four generations of rats were used to determine the nutritional properties of a diet treated with sterilizing doses of ionizing radiation from fission products. In the reproduction study a total of 80 females receiving the irradiated diet produced 3467 young and weaned 84.7% of 2993 young which were allowed to remain with their mothers. The same number of females receiving the control diet produced 3290 young and weaned 90.9% of 2923 young.

The average weight of young from mothers receiving the control diet was approximately 3 gm more than that of those from mothers receiving the irradiated diets. The life span of the animals in both groups was essentially the same. In general, the data showed that the nutritional value of the irradiated diet was slightly less than that of the non-irradiated, but the difference was very small, and probably would be of comparatively little practical importance.

ACKNOWLEDGMENTS

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Acknowledgment is made to Merck and Company, Rahway, N.J., for generous supplies of vitamins and to Lederle Laboratories, Division of American Cyanamid Co., Pearl River, New York, for folic acid.

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THE EFFECT OF ZINC AND POTASSIUM IN THE NUTRITION OF *TENEBRIO MOLITOR*, WITH OBSERVATIONS ON THE EXPRESSION OF A CARNITINE DEFICIENCY [1]

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Fraenkel reported in 1948 the discovery of a new growth factor, named at first B_T, which was required by the larvae of the mealworm, *Tenebrio molitor*. B_T was subsequently identified as carnitine (Carter et al., '52). In the absence of carnitine the insects failed to grow and died at an early age. Using these criteria, a method for the assaying of carnitine by the *Tenebrio* test was worked out (Fraenkel, '56) and the carnitine content of many natural materials was determined (Fraenkel, '53, '54). However, starting with the summer of 1953, the *Tenebrio* test for carnitine suddenly failed to work. The insects no longer died in the absence of carnitine and grew badly even when carnitine was present (Fraenkel, '56; Fraenkel and Leclercq, '56; Fraenkel and Friedman, '57). Meanwhile the importance of carnitine in the nutrition of *Tenebrio* and related insects had been confirmed by workers in Belgium (Leclercq, '54) and Germany (Fröbrich, '53). Subsequently difficulties in the testing for carnitine also developed at the University of Liège. A joint investigation with Dr. Leclercq (Fraenkel and Leclercq, '56) showed that the following three conditions influenced the carnitine deficiency of *Tenebrio*:

[1] This investigation was supported in part by grants from the Public Health Service (E533 C 3 through 7), the Office of Naval Research (1834) (07) and the Office of the Surgeon General, Department of the Army (MD-308).

1. Growth and survival of *Tenebrio* depended on the sample of casein used, suggesting the presence of carnitine in "vitamin-free" casein.

2. Different strains of *Tenebrio* reacted differently to a carnitine deficiency.

3. The larvae required another growth factor in addition to carnitine which was supplied by a water-insoluble fraction of yeast. The presence of this unknown factor not only enhanced growth, with carnitine present, but also hastened death of the insects, with carnitine absent.

While these three conditions played important roles in the carnitine syndrome, further inconsistencies arose in a number of cases. An investigation was then instituted into the nature of the insoluble yeast factor. While this was in progress, the growth-promoting activity of insoluble yeast became less marked and proved inferior to certain liver fractions and other natural materials. The solution to a very puzzling situation was reached when it was discovered that the insoluble yeast factor was, in fact, multiple, that the missing factors were inorganic in nature, and that the addition of zinc and potassium was essential for normal growth and the development of a carnitine deficiency. The present paper describes the steps by which the importance of zinc and potassium in the diet of *Tenebrio* was recognized, and also deals with the circumstances which affect the expression of a carnitine deficiency.

MATERIALS AND METHODS

1. Composition and preparation of the diets. The basic diet was the same as stated in earlier publications (Fraenkel, '56) and consisted of: casein,[2] 20; glucose, 80; cholesterol, 1; salt mixture,[3] 2 and the following vitamins in micrograms per gram of diet: thiamine, 25; riboflavin, 12.5; nicotinic acid, 50; pyridoxine, 12.5; pantothenic acid, 25; choline chloride, 500; folic acid, 2.5 and biotin, 0.25.

[2] Labco.

[3] McCollum and Davis no. 185, except where stated otherwise (in McCollum and Simmonds, '18).

In the majority of the experiments to be described the diets contained carnitine, except where stated otherwise. In the earlier tests of table 1, natural L-carnitine (3 μg/gm) was used, while most later tests contained synthetic DL-carnitine (5 μg/gm). A detailed description of the methods involved in the *Tenebrio* assay for carnitine has been given elsewhere (Fraenkel, '56). However, the procedures have been changed in certain details, and since modifications were introduced as the result of the present investigation, and since the strict adherance to certain procedures is essential for obtaining reproducible results, the basic steps in the preparation of tests will be restated here.

The dry ingredients of the diet are weighed out into a mortar and ground together in the following order: casein + cholesterol + salt mixture, and finally glucose. This mixture is submitted to further mixing in a "Minnimill."[4]

Until about two years ago, the salts were suspended in a small quantity of water and mixed thoroughly with the casein. The mixture was dried in an oven and ground to a fine powder in a Wiley mill. This procedure of "salting" the casein was once believed to ensure better mixing and at one time this product appeared to support better growth than the dry mixture of casein and salts. In the course of the present investigation it was recognized that the method of dry mixing not only gave more consistent results, but sometimes gave rise to better growth, as will be shown later (table 10).

The experiments were carried out in one-ounce wide-mouth bottles, each containing 3 gm of food and 10 larvae. The diet was first weighed into the bottles in lots of 6 gm. When an experiment was to be started, the vitamins were dissolved in water in amounts which would add the above named quantities to 1 gm of the diet, employing 0.05 ml/gm. Carnitine was, according to the nature of the experiment, either added to the vitamin mixture or pipetted into the diets separately in solution, and other ingredients were also added in solution or suspension wherever practicable. Liquid additions were used

[4] Fisher Scientific Company, Pittsburgh, Pennsylvania.

in volumes which amounted to a total of 0.1, or at the most 0.15 ml/gm of diet. After addition of the liquids the diets were mixed with a spatula, placed into the constant temperature-humidity chamber (30°C, 65 ± 5% R.H.) where they remained throughout the duration of the tests, and two days later were ground in a mortar. The content of each bottle was then subdivided into two bottles and 10 larvae were placed into each.

2. *Age and sizes of larvae.* In most experiments larvae newly emerged from the eggs were grown in groups of 300 to 400 larvae on 25 gm of the basic diet described above, without carnitine, and then placed on the experimental diets at the age of three to 4 weeks when they had reached a weight of about 2 mg, and had not yet begun to die as a result of the carnitine deficiency. In the course of the present investigation the basic diet successively gave rise to inferior growth and larvae ceased to die prematurely. The period of initial growth in bulk was frequently prolonged up to 8 weeks when the weights were frequently below 1.5 mg. The initial weight of larvae on an experimental diet was important when comparing final weights attained after a definite period. In most experiments, however, what mattered was not so much the absolute age at which a certain weight was reached, but the relative weight in a simultaneous series of tests. In the experiments to be described, the initial weights are not given but the final weights are recorded when the larvae on the best diets weighed 50 to 70 mg. This was usually attained 8 weeks after larvae were started on experimental diets, but on occasion it took longer. Weights beyond 50 to 70 mg are not very significant, because 3 gm of food does not allow optimal growth for 10 larvae beyond this size. The larvae were weighed in fortnightly intervals by groups of living larvae per bottle. (See table 11).

The original reason for the procedure of starting experiments with 4-week-old larvae, and discontinuing them after about 8 weeks on the experimental diets was the great saving in time and material, while, at the same time, obtaining per-

fectly significant and reproducible results. Towards the close of the presently described series of experiments, when the difficulties referred to in the introduction had been largely resolved and the insects grew on a synthetic diet at a greatly enhanced rate, several experiments were performed with larvae freshly hatched from the egg, and were in some cases continued on larger amounts of food until the larvae were fully grown (120 to 140 mg) or had completed their development (tables 11–13).

All the weights stated in this paper are averages of the surviving larvae on a particular diet, out of 20 starting larvae. Normally 16 to 20 larvae survive, in the presence of carnitine. To save space, the number of survivors is not always given, except where this represents an important aspect of a result.

3. Brands of casein, and other proteins used. In most of the tests to be described, Labco casein, vitamin-free was used. As had been pointed out before (Fraenkel and Leclercq, '56), different samples and brands of casein gave different results. The same batch of Labco casein, sometimes referred to as Labco 1955, was used in all the tests described in this publication, except for any of the earlier ones in table 1, or where stated otherwise. For comparison, two older samples of Labco, which in the past had been successfully used in the testing for carnitine, were occasionally used. One of these, referred to as "Rutgers" casein, was a sample of Labco which had been prepared for the Rutgers University evaluation of proteins project, 1946-50. The other, called Labco 1952, was obtained in 1952 and "salted" in February 1953. Other proteins used on occasion included "vitamin-free casein from Hoffmann-La Roche,[5] lactalbumin,[6] and soybean protein.[7] Two other casein samples, called "Wisconsin" casein and "Cornell" casein, were obtained from the Department of Biochemistry of the University of Wisconsin and the School of Nutrition, Cornell University where they had been exhaustively purified to a "vitamin-free" condition.

[5] Bâle.

[6] General Biochemicals Inc., Chagrin Falls, Ohio.

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

4. Salt mixtures used. In almost all the older experiments, and all the tests described in this paper, except where stated otherwise, McCollum-Davis no. 185 salt mixture constituted 2% of the dry diet. This was a tradition followed in over 15 years of nutrition work with insects, and had, up to the present investigation never appeared to give unsatisfactory results. The McCollum-Davis mixture contains calcium and iron in organic salts, is somewhat low in potassium, compared with other salt mixtures, and does not contain trace elements, except as impurities (table 1). As will be shown later, the potassium content, as supplied with 2% salts, would have been somewhat marginal, but some of the difficulties to be reported in due course would probably never have arisen, if the commercially obtained sample which was used during the past two to three years had not by mistake contained only half the stated amount of potassium (see later discussion of potassium as an active principle). In the course of the present investigation the original McCollum-Davis salts formula was also made up in the correct proportion and tested.

The composition of other salt mixtures which were used is given in table 1. Most of them contain various trace elements, but only three contain zinc. Wesson's salt mixture was extensively used in certain experiments, and in order to test the effect of traces, a similar mixture, containing only the major components, was made up and tested without the trace components. Trace components were tested in various combinations and quantities, but most of these tests, which yielded negative results, are not described. However, in certain tests, the 5 trace components of Wesson's mixture (table 1) were used in amounts equivalent to a level of 10% of the Wesson's salt mixture in the diet, and the following additional trace elements were added in amounts of 0.01% of the diet: Co $(No_3)_2$, H_3BO_4, and $(NH_4)_6\,Mo_7O_{24}$. The designation "traces" always refers to the mixture of these 8 compounds (tables 7, 9 and 13).

5. Ash fractions. At a certain stage of the investigation the ashes of liver powder and other materials were tested. Ash-

TABLE 1

Composition of the salt mixtures used in this study

	MC COLLUM-[1] DAVIS	WESSON [2]	HUBBELL-[1] MENDEL-WAKEMAN	JONES-[1] FOSTER	PHILLIPS-[1] HART	GLISTA [3]	FRAENKEL [4] (GSF)
KH_2PO_4		31.0	21.2	38.89			70
K_2HPO_4	25.78				32.2	16.84	
KCl		12	11.2				
NaH_2PO_4	9.38						
NaCl	4.67	10.5	6.9	13.93	16.7	16.47	10
$MgSO_4$	7.19	9.0	1.6	5.72	10.2 [5]	4.68 [5]	10
$MgCO_3$			2.5				
$Ca(H_2PO_4)_2 \cdot 2H_2O$	14.6				7.5		8
$Ca_3(PO_4)_2$		14.9				52.4	
$CaCO_3$		21.0	54.3	38.14	30.0	5.61	
Ca lactate	35.9						
$FePO_4 \cdot 2H_2O$		1.47	2.0				2
$FeSO_4 \cdot 7H_2O$				2.7			
Fe citrate (ic)	3.19				2.75	2.6	
$MnSO_4$		0.2	0.035	0.445 [6]	0.51	1.22 [7]	
$AlK(SO_4)_2 \cdot 12H_2O$		0.09	0.017				
$CuSO_4 \cdot 5H_2O$		0.39	0.009	0.048	0.03	0.037	
NaF		0.57	0.1				
KI		0.005	0.008	0.079	0.08	0.07	
$ZnCl_2$				0.03	0.025	0.037	1 [10]
$CoCl_2$				0.002 [8]	0.005	0.0019 [9]	
H_3BO_7						0.01	
Zinc content as per cent of salt mixture				0.014	0.012	0.018	0.5
Zinc content as per cent of diet, if 2% salt mixture is added				0.00028	0.00024	0.00036	0.01
Potassium content as per cent of salt mixture	11.57	15.19	11.94	11.13	14.45	7.55	20
Potassium content as per cent of diet, if 2% of salt mixture is added	0.23	0.31	0.24	0.22	0.29	0.15	0.4

[1] Nutritional Biochemicals Corporation.
[2] General Biochemicals, Inc.
[3] Department of Animal Nutrition, University of Illinois.
[4] This investigation.
[5] $MgSO_4 \cdot 7H_2O$.
[6] $MnSO_4 \cdot 2H_2O$.
[7] $MnSO_4 \cdot H_2O$.
[8] $CoCl_2 \cdot 6H_2O$.
[9] $CoSO_4 \cdot 7H_2O$.
[10] Added separately in solution.

ing was done by the dry ashing method in a muffle oven. Concentrated hydrochloric acid was then added and mixed with the ash with the aid of a glass rod. The resulting solution-suspension was cautiously heated over a hot plate until all fumes of HCl had gone. The water-soluble fraction, which proved active, was then fractionated into soluble and insoluble constituents by either adding K_2CO_3 until a precipitate no longer formed, or by treating with H_2S in acid or alkaline solution. The ash constituted about 5 to 6% of liver powder.

6. Yeast and liver preparations used. Following the recognition of the growth-promoting property of the insoluble yeast fraction, many yeast preparations, both commercially obtained or prepared by ourselves, were tested. The insoluble yeast preparation used throughout this investigation was the same sample which had been prepared in 1948 from fresh brewers' yeast, by exhaustively boiling in water for one hour, filtering and repeating this procedure 9 times. Insoluble and soluble fractions were also prepared from dried brewers' yeast and active bakers' yeast with varying results. Since these results became dated by later experiments, details about the preparation and effect of these fractions have been omitted.

Subsequently various liver fractions were found to be as active as, or more so, than yeast fractions and more suitable to use in fractionation procedures because of their greater solubility. A liver protein fraction [8] designated as a water-insoluble protein fraction of whole fresh liver proved highly active in amounts of 1%. However, in spite of the label, 90% of this preparation dissolved in water, and the activity rested in the water-soluble fraction. Several potent fractions were prepared from this solution by precipitating the activity in alcohol and removing inactive protein material with trichloracetic acid (TCA). The origin of this "liver protein fraction" could never be elucidated and since it became subsequently no longer available it was abandoned in favor of a liver powder

[8] Nutritional Biochemicals Corporation, Cleveland, Ohio.

from another source,[9] which at first proved to be highly active in amounts of 1 to 2%. The activity was extracted in TCA in the cold, precipitated by alcohol and distributed between different fractions. Since all the work with yeast and liver fractions later became dated, when the potency of certain inorganic salts was discovered, a detailed description of the various preparations from liver fractions has been omitted.

RESULTS

1. Changes in growth responses between 1952 and 1957. Table 2 gives a summary of typical sets of experiments, carried out during the past 5 years with basic diets to which yeast, insoluble yeast fraction, carnitine or liver powder had been added in various proportions and combinations. It starts with a typical test from November 1952. Larvae failed to grow and many died on the basic diet, while the addition of carnitine produced a well-defined effect on survival and growth (662). In April 1953 there was still a striking carnitine effect (685), but soon afterwards (691) the larvae began to survive in the absence of carnitine, and the growth rate was poor with or without carnitine. As late as 1954 (713) the addition of 2% yeast or insoluble yeast fraction frequently improved growth strikingly, but subsequently such addition, with and without carnitine, became less and less effective. The insoluble yeast fraction was on many occasions (702, 737, 783) more active than yeast, and liver powder proved still more active (713, 783). Finally, starting about June 1956 (791) additions of both yeast and liver, even in amounts up to 4% proved very inferior to certain other additions. This is also illustrated in some of the more recent tests (tables 4, 5 and 6).

Strikingly different were the results of tests in which "Rutgers" casein or salted Labco 1952 was used. In some experiments good growth resulted even on basic diets alone (701, 758). The addition of insoluble yeast fraction enhanced the mortality (748b, 758) or growth (748b, 766b) at a time when

[9] Prepared by the Viobin Corporation, Monticello, Illinois.

TABLE 2

Changes in the effect on growth and survival of Tenebrio molitor of a basic diet, and with the additions of carnitine, yeast, the water-insoluble yeast fraction, and liver powder

The tests cover a period of 5 years, 1952 to 1957. The figures represent the numbers of surviving larvae, out of 20, and their average weight, after approximately 12 weeks

EXP.	DATE [1]	BASIC DIET		2% YEAST		4% YEAST		2% INSOLUBLE YEAST		CARNITINE		2% INSOLUBLE YEAST CARNITINE		2% LIVER POWDER		4% LIVER POWDER		REMARKS
		no.	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*	
662	11-17-'52	5	14.8	18	72.3					18	48.1							
685	4-27-'53	1	8	20	54.2					17	25.2							
691	8- 1-'53	11	4.4	19	48.7					19	7.7							
694	8-23-'53	16	6.3	15	49.5					20	7.1							
701	11-30-'53	16	67	20	80					20	34							"Rutgers" casein
702	11-30-'53	16	11	19	27	19	73	12	51	20	13							
713	2-15-'54	19	7.7	20	33.7									19	63.0			
737	4-26-'54	15	10.7	18	15.9			14	65.5									
748a	7- 9-'55	13	6.7					11	14.1	13	6.0	18	14.3					
748b	7- 9-'55	9	14.4					4	39.5	15	11.9	15	87.3					"1953" Labco
758	11- 8-'55	14	41.1					3	57.3	15	36.6	18	131.4					"1953" Labco
766a	12-19-'55	15	2.9					15	6.8	16	3.2	16	4.2					
766b	12-19-'55	14	8.7					17	40.9	20	8.0	15	27.9					"Rutgers" casein
783	4-10-'56	17	5.7	15	12.3			15	35.0					16	83.7			
791	6- 2-'56	19	4.9	17	10.1	20	15.7							19	22.0			0.7% liver ash = 65.5 mg
818	1-21-'57			18	15.8	14	26.2	15	3.0	19	2.1	15	2.1	19	11.6	18	27.8	2% Wesson salt + $ZnCl_2$ = 68.7 mg
823	2-25-'57	13	6.7					11	14.1	13	4.4			20	27.7	20	42.5	

[1] Started on experimental diets after 4 weeks on the basic diet.

it had little or no effect in diets compounded with casein of a later date.

Of many fractions tested in the period up to the spring of 1954 the following gave good growth responses: 2% liver fraction "2,"[3] 2% liver protein fraction,"[8] 2% lactalbumin, 2% fibrin, 2% wheat germ, 2% gluten, 1 to 2% liver powder, 1% water insoluble residue from whey, and the TCA soluble fractions of liver powder or lactalbumin.

2. *Evidence of the multiple nature of the missing growth factor.* In attempts to concentrate and fractionate the active principle of various preparations it was discovered that the activity could be (a) precipitated by alcohol from a solution of "liver protein fraction," (b) extracted from liver powder or lactalbumin by TCA, and (c) was not precipitated by TCA from aqueous solution. Evidence then accumulated to the effect that active preparations made from "liver protein fraction" or liver powder could be separated into two fractions, each of which was relatively inactive, but of great activity when recombined. Several examples of such fractionations are given in table 3. The alcohol precipitate of the water-soluble portion of "liver protein fraction" was highly active. This precipitate did not entirely redissolve in water. The water-soluble and insoluble fractions so obtained were relatively inactive singly, but highly active when combined (772). The same was true with the water-soluble and water-insoluble fraction of liver powder (771). A TCA extract of liver powder which was highly active was fractionated with alcohol. In one test (785) the alcohol-insoluble fraction, which was active, was further divided into the water-soluble and insoluble fractions, which on recombination regained full activity. In another test (789) the alcohol-soluble and insoluble fractions alone were inactive, but fully active when recombined. It was thus proved that at least two active principles were involved. In these fractionations a considerable concentration of activity had been achieved, and in one case (785) a striking growth response was obtained by the addition of 0.04% of a

[8] See footnote p. 368.

water-insoluble fraction from liver powder, a concentration of about 50 times. This particular fraction, and some other insoluble fractions, were highly crystalline and appeared inorganic. This material lost little weight on heating on a hot plate and remained active. This observation proved the turning point in the analysis. The original liver powder was then

TABLE 3

The effect of certain fractions derived from liver, added singly or in combination, on the development of Tenebrio molitor

The larvae were grown for the first 5 to 6 weeks on the basic diet (about 2 mg average weight) and continued on the experimental diets for another 8 weeks. Number surviving out of 20, and average weights

EXP.	NO ADDITION		FRACTION I		FRACTION II		FRACTION I + II	
	no.	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*
771	18	5.3	20	9.9	17	9.8	18	61.6
772	17	4.8	16	7.1	16	20.0	10	54.5
785	17	5.5	19	5.7	20	42.8	19	73.8
789	16	5.1	18	5.1	19	10.1	17	66.8
808	15	4.6	14	9.5	16	9.1	19	58.4

771. Liver (Viobin), I = water-soluble fraction, 0.4%; II = water-insoluble fraction, 1.5%.
772. Liver protein fraction, alcohol precipitate of water-soluble fraction; I = water soluble, 0.38%; II = water insoluble, 0.26%.
785. Liver (Viobin), TCA-soluble fraction, insoluble in alcohol; I = water-soluble, 0.2%; II = water-insoluble, 0.04%.
789. Liver (Viobin) TCA soluble fraction; I = alcohol-soluble, 0.25%; II = alcohol-insoluble, 0.2%.
808. Liver (Viobin) ash, H_2O soluble, treated with H_2S at alkaline reaction; I = H_2S-soluble, 0.2%; II = H_2S-insoluble, 0.03%.

Concentrations of fractions expressed as percentage of dry matter in dry diet.

ashed. This ash in suitable concentrations proved as active as any organic preparation, thus the inorganic nature of the missing factor was revealed.

3. Work with ashes and ash fractions. The preparation of ash samples was described on p. 366, and the activity of liver ash is given in table 4. In test 806, optimal growth ensued upon addition of 1.2% of ash, while the same result in test 815 (not given) was achieved with 0.6%. Of this preparation,

TABLE 4

Growth of the larvae of Tenebrio molitor in the presence of various fractions prepared from dried liver powder. Average weights of larvae after 12 weeks. All additions to diets are expressed as percentage of dry weight of the dry diet

	EXPERIMENT				
	806	811	814		820
	mg	*mg*	*mg*		*mg*
No addition	2.5	4.2	2.5		3.0
2% liver powder	14.8	14.7	13.7		
4% liver powder			23.9		
$ZnCl_2$ [1]	4.1	3.8	2.1		
Liver ash 0.3%	8.1				
Liver ash 0.6%	17.0				
Liver ash 1.2%	62.7	54.7			
Liver ash, soluble in water or 0.01 to 0.1 N HCl				K_2CO_3, 0.02%	4.9
K_2CO_3-soluble fraction [2,3]	6.6	7.5	9.6	K_2CO_3, 0.1%	8.8
K_2CO_3-insoluble fraction [2,4]	8.9	6.4		K_2CO_3, 0.5%	7.1
K_2CO_3-soluble + insoluble	32.3	73.0	62.2	K_2CO_3, 0.2% + $ZnCl_2$	4.8
K_2CO_3-soluble + $ZnCl_2$	36.6	73.5	65.2	K_2CO_3, 0.2% + $ZnCl_2$	44.1
K_2CO_3-insoluble + $ZnCl_2$	4.2			K_2CO_3, 0.5% + $ZnCl_2$	45.6

[1] $ZnCl_2$ = 0.02% of diet.

[2] Saturated solution of K_2CO_3 added drop by drop until no more precipitate formed.

[3] K_2CO_3-soluble fraction. 806: 0.35%; 811: 0.24%; 814: not determined.

[4] K_2CO_3-insoluble fraction. 806: 0.04%; 811: 0.01%; 814: not determined.

the whole activity resided in the water-soluble fraction, while the water-insoluble fraction was entirely inactive. The water-soluble fraction was then fractionated in various ways. In one set of experiments, K_2CO_3 in saturated solution was added drop by drop until the amount of precipitate, thus formed, no longer increased. The K_2CO_3-soluble and insoluble fractions when tested singly, were inactive, but proved highly active when recombined (table 4, 806, 811, 814). The choice of K_2CO_3 as the alkaline precipitating agent at first obscured the fact, only to be discovered later, that the potassium ion was indeed one of the two factors missing in the diet.

Another fractionation of the soluble part of liver ash was carried out with H_2S. In acid solution, the H_2S filtrate was fully active. In slightly alkaline solution, both the H_2S-soluble and insoluble fractions alone proved inactive, but highly active when recombined (table 3, 808). This suggested that one of the factors belonged to the group of metals the sulfide of which is soluble in acid, but not in alkaline solution. The multiple nature of the active principle was thus again proved.

Along with liver ash, the activity of other ashes was also investigated. In one experiment (815) the following substances were ashed: liver powder (6% ash), dried milk (7% ash), dried egg (5% ash) and whole wheat flour (1.4% ash), and tested in the following amounts in the diet: liver ash, 0.6%; milk ash, 0.7%; egg ash 0.5% and flour ash 0.14%. Of them, liver ash alone was highly active at the levels tested.

4. Recognition of zinc as one of the active principles. In earlier tests salts of all the known or suspected trace elements were tested for their activity, and were found entirely inactive, except for $ZnCl_2$ which showed a very slight activity. The effect of zinc was then tested in different combinations. In most instances in which $ZnCl_2$ was added to a basic diet which contained 2% of McCollum-Davis salt mixture, it had no effect (tables 4, 5 and 6). However, it was highly effective in diets made up with Wesson's salt mixture. A diet which contained the major components of Wesson's mixture, plus zinc, was as efficient as the whole Wesson mixture plus zinc. $ZnCl_2$

was then tested in combination with the K_2CO_3 fractions from liver ash. It proved highly active when added to the K_2CO_3-soluble fraction, and entirely inactive in combination with the K_2CO_3-insoluble fraction (table 4, 806, 811, 814). This proved that zinc ion was one of the active principles under investigation, a conclusion entirely consistent with results obtained earlier. Thus it would be precipitated by H_2S in alkaline, but not in acid solution, and would precipitate with K_2CO_3 as the carbonate.

It was also then found that zinc gave an excellent growth effect (46.6 mg) on a diet which contained double (4%) amounts of McCollum-Davis salt mixture, while little (6.4 mg) growth took place on 4% of the salt mixture alone.

5. Recognition of potassium as the other active principle. When the Wesson salt mixture, or a mixture of its major components, was discovered to induce excellent growth in the presence of $ZnCl_2$, while McCollum-Davis mixture + zinc gave a very poor result, the single constituents of the Wesson mixture, excluding the traces, were added to a basic diet which contained McCollum-Davis salt mixture + $ZnCl_2$, in exactly the amounts that would be contained in 2% of Wesson's salt mixture. The two potassium salts in the mixture, KCl and KH_2PO_4, proved highly active, while none of the other salts showed any effect (table 5). It was thus concluded that the McCollum-Davis salt mixture must have been highly deficient in potassium in the quantities used. It was then also shown that K_2CO_3, in combination with $ZnCl_2$ was as active as the K_2CO_3-soluble fraction of liver ash (table 4, 820).

The observation of a glaring potassium deficiency in a diet which contained 2% of McCollum-Davis salt mixture proved at first puzzling. This mixture contains 11.57% potassium, and Wesson's mixture 15.19% potassium. This difference did not seem to account for the striking difference in the results. The sample of McCollum-Davis mixture, which had been used during the past two to three years, was then analyzed for potassium by flame photometry and found to contain about half the amount of potassium it should have contained. This proved

TABLE 5

Growth of Tenebrio molitor on a basic diet which contains 2% of McCollum-Davis salt mixture, and on the same diet with the addition of liver powder, $ZnCl_2$, the major constituents of Wesson's salt mixture or its constituents added singly. Average weight of larvae after 12 weeks.

Experiment 817

ADDITIONS TO BASIC DIET	WEIGHTS OF LARVAE	TOTAL K [1]
	mg	*mg/gm*
No addition	3.7	1.16
2% liver powder	17.0	
0.02% $ZnCl_2$	4.4	1.16
0.02% $ZnCl_2$ + Wesson major components, 2%	90.7	5.32
0.02% $ZnCl_2$ + NaCl 2.0 mg/gm	3.7	1.16
0.02% $ZnCl_2$ + KCl 2.5 mg/gm	93.7	2.4
0.02% $ZnCl_2$ + KH_2PO_4 6.6 mg/gm	72.5	2.92
0.02% $ZnCl_2$ + $Ca_3(PO_4)_2$ 3.0 mg/gm	4.1	1.16
0.02% $ZnCl_2$ + $CaCO_3$ 4.2 mg/gm	4.7	1.16
0.02% $ZnCl_2$ + $MgSO_4$ 1.8 mg/gm	4.7	1.16
0.02% $ZnCl_2$ + $FePO_4$ 0.8 mg/gm	5.1	1.16

[1] See text, p. 375, bottom.

TABLE 6

Effect of graded doses of $ZnCl_2$ on the growth of Tenebrio molitor larvae. Average weights of larvae, in milligrams, after 12 weeks. Additions of $ZnCl_2$ expressed as percent of the dry diet.

ADDITIONS TO DIET	EXPERIMENT 814	EXPERIMENT 822	EXPERIMENT 829
	mg	*mg*	*mg*
No addition	2.6	2.4	2.6
2% (814, 822) or 4% (829) liver powder	13.7	14.2	32.5
$ZnCl_2$, 0.02%, no additional K	2.1	2.5	
K_2CO_3 filtrate of liver ash (814) or KCl 0.5% (822 and 829)	9.6	5.6	5.8
+ 0.000075% $ZnCl_2$			9.2
+ 0.00015% $ZnCl_2$		17.3	10.4
+ 0.0003% $ZnCl_2$		24.2	22.5
+ 0.0006% $ZnCl_2$	39.0	41.3	56.6
+ 0.00125% $ZnCl_2$	60.0	80.2	94.0
+ 0.0025% $ZnCl_2$	52.6	62.3	84.5
+ 0.005% $ZnCl_2$	55.5	77.2	82.1
+ 0.01% $ZnCl_2$	61.5	65.2	
+ 0.02% $ZnCl_2$	65.3		

to be due to an error by the manufacturer who used KH_2PO_4 instead of K_2HPO_4.[10]

Subsequently diets were tested with hand-mixed McCollum-Davis mixture made up in the correct fashion and they proved to be as active as could be expected from the corrected potassium level of the diet.

6. Quantitative zinc requirements. The quantitative requirements for zinc were assessed in three different tests (table 6). The first experiment (814) was performed at a time when the requirement for potassium was as yet unknown, and potassium was added to the diet as a K_2CO_3 filtrate from liver ash. In the two other experiments (822 and 829) the diets contained 0.5% of KCl. The results of the three tests agreed closely. The minimum dosage of $ZnCl_2$ for optimal growth was 0.00125%. However a noticeable effect was achieved with as little as 0.00075% of $ZnCl_2$, i.e. about one third of one part per million of zinc. It seems hardly believable that such a tiny amount should not be present as an impurity in a diet composed of materials which had not been specifically purified in this respect.

7. Quantitative requirements for potassium. After the requirement for additional potassium in the standard basic diet had been recognized, a test was carried out in which graded doses of KCl and KH_2PO_4 were added to that diet (table 7, 821). Subsequently, this test was repeated, but on a basic diet which contained the major constituents of Wesson's salt mixture, exclusive of potassium salts and traces, but with $ZnCl_2$ added (table 7, 829). In experiment 821 the calculations were made on the assumption that the McCollum-Davis salt mixture contained only half the amount of potassium it should have contained. The results of these 4 tests agreed fairly well. In table 7, the lowest amounts of potassium to produce optimal growth are italicized. The figures obtained were 1.67, 2.37, 2.42 and 2.94 mg of potassium/gm of diet. Amounts be-

[10] This is so stated in the Diets Manual of Nutritional Biochemicals Corporation and was confirmed in correspondence with the manufacturers. Thus it was satisfactorily explained why the diet was deficient in potassium.

yond 6.7mg/gm seem to have an inhibitory effect. These figures agree well for the two salts tested, suggesting that the inhibition was produced rather by the potassium ion than the anion moieties of the salts.

On the basis of these quantitative data it is clear that, of the various salt mixtures tested at the 2% level, those of McCollum-Davis (in correct composition), of Wesson, of Hubbell, Mendel and Wakeman, of Jones and Foster, and of Phillips and Hart are marginal in potassium for *Tenebrio* and that of Glista is rather low (see table 1). Four percent of any of these mixtures should fulfill the potassium requirements.

TABLE 7

Growth of Tenebrio molitor larvae on diets which contain either 2% of the major constituents of Wesson's salt mixture, exclusive of KH_2PO_4 or of McCollum-Davis salt mixture, and with the addition of graded doses of KCl or KH_2PO_4. Average weights after 12 (829) or 11 (821) weeks, in milligrams. All diets contain 0.02% of $ZnCl_2$.

KCL	AV. WT. OF LARVAE	TOTAL K/GM DIET	KH_2PO_4	AV. WT. OF LARVAE	TOTAL K/GM DIET
mg/gm diet	*mg*	*mg*	*mg/gm diet*	*mg*	*mg*
		Experiment 829			
		2% Wesson salt mixture, exclusive of traces and of KH_2PO_4			
—	2.3	—	—	2.3	—
1.6	56.4	0.84	2.05	7.3	0.59
3.2	100.5	*1.67*	4.1	67.3	1.18
6.4	102.9	3.35	8.25	95.1	*2.37*
12.8	76.6	6.7	16.5	92.4	4.74
25.6	50.9	13.4	33.0	70.0	9.48
12.8 + tr.[1]	72.5	6.7	16.5 + tr.[1]	92.1	4.74
		Experiment 821			
		2% McCollum-Davis No. 185			
—	2.3	1.16	—	2.3	1.16
0.31	4.6	1.32	0.77	8.7	1.38
0.62	11.7	1.48	1.54	16.8	1.61
1.25	31.6	1.79	3.1	53.5	2.06
2.5	72.9	*2.42*	6.2	82.2	*2.94*
5.0	84.4	3.68	12.4	79.7	4.72

[1] The trace constituents of Wesson's salt mixture were added in an amount corresponding to 5% of the salt mixture.

8. Experiments with salt mixtures. After the qualitative requirements of zinc and potassium for *Tenebrio* had been established, the effects of several salt mixtures, in amounts of 2 and 4% and with the addition of KCl and $ZnCl_2$, were tested (tables 8 and 1). The McCollum-Davis salt mixture showed an absolute deficiency of zinc and potassium on both levels. With only half the stated amount of potassium in the salt mixture, there would not have been enough of potassium at the 2% level, but one would have expected a better growth response at the 4% level. In fact, in several earlier tests, 4% of this mixture plus $ZnCl_2$ had given a good growth response.

The salt mixture by Hubbell, Mendel and Wakeman required additional zinc, but no additional potassium. This was to be expected from its composition, as it contains no zinc and, at 2%, just sufficient potassium (tables 8 and 1).

The Jones-Foster salt mixture is low in potassium and zinc. Even at 4% it did not satisfy the zinc requirements, which is consistent with the data in table 1. At 2% it proved highly deficient in potassium, although its potassium content was marginal, but the potassium deficiency which arose at 4% cannot be explained from the data in table 1.

The diet containing the Phillips-Hart salts is very low in zinc and satisfactory in potassium, at the 2% level (table 1). It was, however, at this level the most efficient of the diets tested. The addition of zinc at both levels proved highly beneficial, as was to be expected (table 8). The potassium content, at the 2% level was adequate. The growth response at the 2 and 4% levels, without additions, was very similar to what had been obtained before (table 6) with similar levels of zinc and adequate amounts of potassium.

The date obtained with the Glista mixture were the least consistent of the various salt mixtures tested. This may be connected with the observation that even on the complete diet growth was not very satisfactory. The tests with the Glista mixture were run on a different occasion (exp. 822) from the other tests in table 8, but larval weights up to 80 mg were also reached in the 822 series (table 6). In interpreting the re-

TABLE 8

The effect of the addition of zinc or potassium on the growth of Tenebrio molitor fed synthetic diets compounded with different salt mixtures. The first 4 columns refer to the same experiment (832) and the last (Glista) to another (822). Amount of salts in the diets: 2 or 4%. Numbers of larvae surviving, out of 20, and average weights after 12 weeks.

TREATMENT	M.D.[1]		H.M.W.[2]		J.F.[3]		P.H.[4]		GLISTA [5]	
	no.	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*
2% salts	19	3.2	17	7.7	20	8.1	20	35.4	19	15.4
4% salts	18	8.4	19	6.0	19	25.0	20	40.9	19	34.5
$ZnCl_2$, (0.02%), 2% salts	19	4.2	20	95.6	19	12.9	20	76.7	18	38.6
$ZnCl_2$, 4% salts	19	5.7	20	93.5	17	49.0	20	71.6	20	32.6
KCl (0.5%), 2% salts	19	6.1	16	4.6	18	14.7	19	19.9	20	18.9
$ZnCl_2$ + KCl, 2% salts	19	75.5	19	101.2	20	82.6	19	73.2	18	46.0

[1] M.D. = McCollum and Davis (J. Biol. Chem., *33:* 55, 1918).
[2] H.M.W. = Hubbell, Mendel and Wakeman (J. Nutrition, *14:* 273, 1937).
[3] J.F. = Jones and Foster (J. Nutrition, *24:* 245, 1942).
[4] P.H. = Phillips and Hart (J. Biol. Chem., *109:* 657, 1935).
[5] In Fisher et al. (J. Nutrition, *52:* 14, 1954).

TABLE 9

Growth of Tenebrio molitor larvae on a newly composed salt mixture (GSF)[1] in the presence or absence of Zn, 8 trace elements,[2] and carnitine. Numbers surviving and average weight after 12 weeks. All diets contain carnitine, except those of column 2.

Experiment 833

TREATMENT	NO ADDITION		WITHOUT CARNITINE + $ZnCl_2$ 0.02%		+ $ZnCl_2$ 0.02%		+ $ZnCl_2$ + traces [2]	
	no.	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*
1% GSF salts	18	9.9	2	7.0	18	36.4	18	40.6
2% GSF salts	18	6.6	4	14.5	19	28.0	19	34.7
4% GSF salts	20	6.9	3	19.6	19	45.7	18	35.6

[1] GSF, this paper, table 1.
[2] Trace elements: salts of Mn, Cu, Al, F, Co, I, B and Mo.

sults we assume that the differences in growth response between 32 and 46 mg may possibly not be significant. It would appear then that this mixture contained sufficient zinc at the 4%, but not the 2% level, which is consistent with its stated zinc content (table 1). It would contain sufficient potassium at both levels, which is not inconsistent with other data, as its potassium content at the 2% level, 0.15%, is close to the lowest figure of potassium requirements obtained (0.167%).

The results obtained with different salt mixtures therefore in general agree with the data on requirements of zinc and potassium, and with the stated amounts of these elements in the mixtures. The few inconsistencies may well be due to weighing inaccuracies or errors in the experimental procedure.

Following these tests a new salt mixture (table 1) was devised which contained the major dietary cations and anions in one combination each, except for PO_4, which was represented in three salts. The general idea behind this mixture was to exclude unnecessary constituents in order to reduce the total amounts of mixture required, to have each of the constituents present in only one combination to facilitate individual omissions, to omit all the trace constituents in order to test their importance, and to ensure adequate supplies of the important elements. This mixture, as one would expect, was highly deficient in zinc at the 1% to 4% levels, but with the addition of zinc produced optimal results even at the 1% level (table 9). The addition of 8 trace elements, Mn, Cu, Al, Co, Mo, F, I and B did not seem to have a significant effect. The final weights reached by the 12th week were not as high as on some of the other diets; this, however is probably of no significance. On two later occasions, when the new salt mixture was used at the 2% level, it produced as good growth as a McCollum-Davis mixture supplemented with potassium and zinc (table 13). In the absence of carnitine, but the presence of zinc, the larvae died rapidly. This will be discussed later.

At this point it was considered advisable to re-examine the various methods by which salt mixtures had been added to diets in the past. This experiment was carried out with the

standard basic diet and 2% of McCollum-Davis salt mixture. In one series the salts were ground dry into the dry diet, in a second series they were pipetted into the diet in a solution-suspension and mixed in, and in the third series they were added to the casein in a solution-suspension, mixed, and the mixture subsequently dried in an oven and ground (as described earlier). There was hardly a difference in the results obtained by the first and third methods, very little growth on the basic diet, and with the addition of $ZnCl_2$ or KCl alone, but good growth with the addition of both. In the second series, growth in the absence of added potassium was very much better than in the other series, suggesting that by supplying potassium in a solution it becomes more available. It is, however, not obvious why the same result should not have occurred in the third series (table 10).

The results certainly show that the method of adding salts may greatly influence results, but do not suggest that the presently practiced method of adding salts dry is inferior and should be abandoned in favor of any other method.

9. Production of large Tenebrio larvae deficient in zinc and potassium. After the requirements of *Tenebrio* for zinc and potassium had been established, it became a matter of interest to raise insects which were deficient in zinc or potassium in order to study their metabolism. For this purpose larvae were grown on a natural diet (wheat flour plus 5% of yeast) to a weight of about 15 mg (about 5 weeks) and then transferred to the basic diet to which zinc and potassium singly or combined had been added. It was expected that the accumulated stores of minerals would allow insects to increase in weight until such stores became exhausted. The insects were weighed in fortnightly intervals (table 11).

Without additions the insects grew very slowly, and with $ZnCl_2$ they grew only slightly faster. But in both instances growth continued steadily throughout the test. These diets, however, contained low levels of potassium (1.1 mg/gm). It can be expected that growth would soon have come to a standstill if the larvae had been transferred to a diet completely de-

TABLE 10

Growth of the larvae of Tenebrio molitor on synthetic diet which had the McCollum-Davis salt mixture incorporated by three different methods. Quantities used in diets: McCollum-Davis mixture, 2%; $ZnCl_2$, 0.02%; KCl, 0.5%. Numbers (out of 20) and average weight of larvae after 12 weeks.

Experiment 831

TREATMENT	SALTS ADDED DRY		SALTS ADDED IN SUSPENSION		"SALTED" CASEIN [1]	
	no.	*mg*	*no.*	*mg*	*no.*	*mg*
No addition	18	2.7	19	9.1	20	4.1
+ $ZnCl_2$	19	3.2	20	25.3	18	4.4
+ KCl	20	9.5	20	6.0	20	5.6
+ $ZnCl_2$ + KCl	20	65.4	20	69.0	20	48.6

[1] Casein with salts mixed in suspension, dried in oven, and then ground.

TABLE 11

Growth of Tenebrio molitor larvae with or without the addition of zinc and potassium. The larvae were grown on a natural diet (wheat flour plus yeast) to an average weight of 15 mg and then transferred to the diets.

Experiment 824

DATE OF WEIGHINGS, 1957	NO ADDITION		+ 0.02% $ZnCl_2$		+ 0.5% KCl		+ $ZnCl_2$ + KCl	
	Av. wt.		Av. wt.		Av. wt.		Av. wt.	
	no.	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*
2–25	40	15.5	40	15.4	40	15.3	40	15.1
3–11	40	24.8	40	26.5	40	44.4	40	42.5
3–28	39	31.3	39	36.4	40	65.3	39	92.2
4– 9	38	34.5	38	42.5	40	66.1	39	113.7
4–25	38	36.5	38	48.4	40	66.2	36	126.2
5–10	38	40.1	38	53.7	40	64.8	36	131.7

void of potassium. With added potassium the larvae grew at a normal rate during the first weighing period, but then slowed down and stopped growing entirely when the zinc deficiency came into play. Growth was continuous and rapid on the diet supplied with zinc and potassium.

It is therefore possible to produce insects which are deficient in zinc or potassium at a weight at which they are far more suitable for analytical and metabolic investigations than those which become deficient early in their life and never grow beyond a few milligrams.

9. *Effect of different samples of proteins.* It was stated in an earlier publication (Fraenkel and Leclercq, '56) that a carnitine deficiency depended on the kind of protein used, suggesting the presence of impurities of carnitine in certain of the proteins employed. In those experiments the diets contained the insoluble yeast fraction. With the recognition of the importance of zinc and adequate doses of potassium in the diet, the question of the adequacy of the protein with regard to growth and the expression of a carnitine deficiency was again open to test. An experiment was then set up with 4 different samples of casein and soybean protein [7] as the protein sources, with 2% of McCollum-Davis salt mixture [11] and the addition of combinations of zinc, potassium and carnitine. (table 12).

On the Labco (1955) casein diet the results were as expected, little growth with the addition of zinc or potassium alone and optimal growth with both present. The diets with "Rutgers" Labco casein and Hoffmann-La Roche casein (see p. 365) gave rise to much better growth in the absence of zinc and potassium, and optimal growth ensued when potassium alone was added. These two samples of casein therefore must have contained adequate amounts of zinc. The casein from the Biochemistry department of Wisconsin gave results similar to those just mentioned, except for the fact that growth was altogether somewhat slower. The casein from Cornell

[7] See footnote p. 365.

[11] Low in potassium, see footnote 10.

TABLE 12

The effect of casein from different sources on the manifestation of deficiencies of zinc, potassium and carnitine in the development of Tenebrio molitor. All diets contain 5 μg/gm of DL*-carnitine, except where stated otherwise. All diets contain 2% McCollum-Davis no. 185 salt mixture.*[1] *Number of larvae, out of 20, and average weight after 12 weeks.*

Experiment 827

ADDITIONS TO BASAL DIET	LABCO (1955) CASEIN		RUTGERS (LABCO)		HOFFMAN-LA ROCHE CASEIN		WISCONSIN CASEIN		CORNELL CASEIN		DRACKETT PROTEIN	
	no.	*mg.*	*no.*	*mg*	*no.*	*mg.*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*
No addition	19	3.6	19	17.2	17	16.7	16	24.3	14	7.7	17	26.1
+ $ZnCl_2$, 0.02%	19	3.4	18	20.1	19	16.2	19	12.4	17	18.9	18	25.6
+ KCl, 0.5%	17	10.3	19	100.9	20	105.3	20	60.1	18	8.6	19	48.9
+ $ZnCl_2$, 0.02% + KCL, 0.5%	18	106.8	18	93.3	20	109.1	20	82.5	15	75.1	20	109.8
+ $ZnCl_2$, 0.02% + KCl, 0.5%, no carnitine	13	88.15	17	63.3	19	68.5	14	56.1	15	52.3	17	79.9

[1] See footnote 10 of text.

University resembled Labco ('55) with good growth only after the addition of both zinc and potassium. For reasons unexplained there was some growth response to zinc even in the absence of potassium. The final weights were very similar to those on Wisconsin casein. Insects grown on Drackett protein did not respond to zinc alone but doubled their weight when potassium alone was added, and quadrupled it with both zinc and potassium. Drackett protein therefore seemed to contain enought zinc to give a growth response in the presence of adequate potassium, but not sufficient for optimal growth.

A somewhat puzzling situation is presented by the differences in the efficiency of the basic diet without added zinc or potassium. With 4 of the proteins, Rutgers, Hoffmann-La Roche, Wisconsin and Drackett, growth greatly exceeded that on Labco (1955) and Cornell. This would suggest that these 4 samples of proteins contained small amounts of potassium and zinc. This is not at all a likely proposition in view of the high solubility of potassium salts and the ease with which they can be removed by washing. Neither can we assume that the Labco (1955) and Cornell samples had suffered as to their nutritional efficiency in the process of their preparation, since the former gave optimal growth upon addition of zinc and potassium. On the other hand, it is not obvious why a deficiency in the protein should be compensated for by additional potassium.

All the tests reported in table 12, so far discussed, contained carnitine. The effect of carnitine was only investigated on the complete diets to which zinc and potassium had been added. In perfect agreement with earlier tests, already discussed, in no instance was the mortality high in the absence of carnitine, though it varied somewhat from protein to protein. In every single case, however, growth was faster in the presence of carnitine.

10. The expression of a carnitine deficiency in relation to the composition of the diet. The starting point of this investigation was the failure to produce a clear-cut carnitine deficiency on diets which up to 5 years ago had invariably pro-

TABLE 13

Growth of Tenebrio molitor larvae on various synthetic diets and in the presence or absence of carnitine, liver powder, the ash from liver powder or a mixture of trace elements. The larvae were started immediately after hatching from the egg. Number of larvae surviving, out of 20, and average weight after 10 weeks. Additions: salt mixtures 2%, $ZnCl_2$ 0.02%, KCl 0.5%, liver powder 4%, liver ash 0.5%, traces, carnitine 5 µg/gm.

TREATMENT	EXPERIMENT 841						EXPERIMENT 842			
	Casein MD [1] salts + $ZnCl_2$ + KCl		Casein GSF [2] salts + $ZnCl_2$		Drackett protein GSF salts + $ZnCl_2$		Casein MD salts + $ZnCl_2$ + KCl		Casein GSF salts + $ZnCl_2$	
	no.	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*	*no.*	*mg*
Without $ZnCl_2$ and KCl, or $ZnCl_2$							19	1.8	5	5.3
same, + carnitine							18	2.2	17	10.1
No addition	13	78.6	4	28.2	2	14.5	14	69.3	3	20.3
+ carnitine	16	102.4	19	96.5	16	59.4	17	104.9	19	110.1
+ traces (841) or liver ash (842)	7	78.2	0	—	2	35.0	13	65.2	1	20.0
same, + carnitine	14	96.1	13	92.3	18	70.5	19	98.6	18	107.2
+ liver powder	17	117.0	10	101.9	15	61.5	20	128.1	18	104.4
same, + carnitine							20	119.3	20	97.7

Control larvae: 841, Whole meal flour + 4% liver powder: 10 larvae = 101.5 mg
842, Whole meal flour: 18 larvae = 46.9 mg
Whole meal flour + 4% liver powder: 19 larvae = 128.5 mg

[1] McCollum-Davis salt mixture.

[2] Fraenkel (table 1) salt mixture.

duced it. This was in part explained as being due to the presence of impurities of carnitine in casein; the results reported earlier (Fraenkel and Leclercq, '56) were, however, not entirely consistent. The present investigation has already quoted several instances where growth clearly suffered through a lack of carnitine (table 12). In tests with the new salt mixture (table 9) a high mortality resulted from the omission of carnitine, in the presence of zinc. This suggested a difference in the expression of a carnitine deficiency according to the kind of salt mixture used. This was submitted to further testing (table 13). The proteins used were Labco casein 1955 and Drackett. All diets contained adequate supplies of potassium and zinc, except where stated otherwise. In every single case where the diet was prepared with the new salt mixture a severe mortality arose in the absence of carnitine, while there was little mortality in the corresponding tests made up with the McCollum-Davis salt mixture. The one case of a higher mortality upon the addition of traces in experiment 841 was not confirmed in the corresponding test where liver ash was added (in experiment 842). From this the conclusion is inevitable that the expression of a carnitine deficiency is also dependent on the nature of the salt mixture used. It should also be emphasized that these carnitine deficiencies arose with the use of a casein which, from earlier experiments (Fraenkel and Leclercq, '56) was presumed to contain some carnitine.

In the tests reported in table 13 positive controls were also run on a natural diet which, from previous experience, was expected to allow optimal growth, namely, whole meal flour plus 4% of yeast. Growth on these natural diets was about the same as that on the synthetic diet to which zinc, carnitine, and where necessary, potassium, had been added. This demonstrates the perfect nutritional adequacy of the synthetic diet.

DISCUSSION

This investigation was prompted by two major problems: (1) Why did *Tenebrio* larvae fail to grow on a synthetic diet

which up to 5 years ago had supported excellent growth? (2) Why did the absence of carnitine from the diet fail to produce a clear-cut deficiency as it invariably did until 5 years ago? The first question has been completely answered. When zinc and potassium salts were added to the diet, the insects grew as well as, and probably better than ever before. The second question was partially answered in a former publication (Fraenkel and Leclercq, '56). The carnitine deficiency developed if the casein employed was free of carnitine, and was enhanced with certain strains of insects. The present investigation has added to this the surprising fact that a carnitine deficiency depended also on the kind of salt mixture used. With a certain brand of casein and a strain of larvae (Urbana) which had been employed throughout the whole investigation, only a mild carnitine deficiency (no increased mortality, but slight decrease in weight) developed when the McCollum-Davis salt mixture was used, but a severe mortality where the salt mixture was either a newly designed one, or that of Wesson. This immediately raises the question of whether the commercial McCollum-Davis mixture contained carnitine. This question cannot be dismissed outright, since this mixture employs two organic salts, calcium lactate and iron citrate, in large quantities, but in view of the very small quantities of carnitine required by *Tenebrio,* less than 1 μg/gm of diet, it would seem a hopeless undertaking to search for traces of carnitine in the salt mixture. The possibility also exists that an expression of the carnitine deficiency depends on the total balance of minerals present, a suggestion which might be open to investigation but is not so far supported by any evidence. For all practical purposes the problem has been solved. A severe carnitine deficiency can be induced in *Tenebrio,* as long as a suitable salt mixture is used.

There could never have been any doubt about the need of insects, like all organisms, for potassium. However, a qualitative requirement has previously been shown only for *Drosophila,* and in experiments which are not entirely conclusive (Loeb, '15; Sang, '56). The quantitative requirements of

Tenebrio for potassium, which in 4 tests fell between 0.17 and 0.29% of the diet, were rather similar to those reported for the chick, 0.4% (Burn et al., '53), the rat, 0.18% (Shaw and Phillips, '55), and the baby pig 0.27% (Meyer et al., '50).

A nutritional requirement for zinc has never been shown before for any insect, though it had been suggested from observations on the growth of certain caterpillars on foliage deficient in zinc (Creighton, '35) and on the concentration of zinc in the ovaries of mosquitoes (Lang, '57). In our experiments the addition of zinc was absolutely limiting for growth and a maximum response resulted from a dose of 6 p.p.m. However, as little as 0.37 p.p.m. had a noticeable growth effect. The quantitative requirement for zinc has never been convincingly stated for any higher animal (Beardsley, '58). Todd et al. ('34) reported that rats developed symptoms of a zinc deficiency on diets containing 0.3 to 1.6 p.p.m. of zinc. The symptoms were relieved by additions of 10 p.p.m. of zinc. According to several authors, hogs developed a zinc deficiency on practical rations which contained 30 to 35 p.p.m. of zinc. Doses of 80 to 100 p.p.m. of zinc relieved these symptoms (Anon., '57). Beardsley ('58) found a semi-synthetic diet with 8.6 p.p.m. of zinc insufficient for pigs, but 103.5 p.p.m. were sufficient. This author also determined the zinc content of Labco casein as 36 p.p.m. and of Drackett protein as 62 p.p.m.

In view of the striking growth responses of *Tenebrio* to zinc in amounts upwards of 0.37 p.p.m. it is difficult to understand how a zinc deficiency could have developed on a diet containing 20% of Labco casein. This is the more surprising since a requirement for any of the other trace elements, Cu, Mn, Co, Al, Mo, F, I, or B could not be demonstrated. There has been no convincing evidence that the addition of salts of these elements, or of liver ash, which in all probability contained these elements in addition to others, further improved the diet. There is no reason to suppose that all of these elements are unnecessary for an insect. Indeed, it cannot be doubted that insects require copper as a constituent of phenol-

oxidase. If some of these elements, as one must presume, were present as impurities, it is surprising that zinc, which is effective in such small amounts, should not have been present simultaneously. The possibility remains that zinc may have been present in a combination in which it was not available. This suggestion is also supported by results of tests with Drackett protein which suggested that this protein might contain sufficient zinc for a growth response, but not sufficient for optimal growth (table 12). Beardsley ('58) reported 62 p.p.m. of zinc in Drackett protein. A parallel case, about the requirement of *Drosophila* for copper, but its non-availability in yeast, has been described by Paulson et al. ('52).

In view of the fact that the zinc requirements of higher animals are rather greater than those of *Tenebrio,* and that the zinc content of three salt mixtures even at the 4% level was insufficient for *Tenebrio,* it follows that the zinc content of these salt mixtures at this level is entirely insufficient for higher animals. This consideration may well apply to other trace elements, the quantity of which in the commonly used salt mixtures is of a similar order.

The importance of zinc in the diet of an insect is not surprising in view of recent observations on the presence of zinc in important enzyme systems, such as carbonic anhydrase, lactic dehydrogenase, glutamic dehydrogenase, alcohol dehydrogenase, and carboxypeptidase (Vallee, '55). A study of these enzymes in zinc-deficient *Tenebrio* larvae will possibly give a clue as to the biochemical basis of this deficiency. Considering the ease with which zinc-deficient *Tenebrio* larvae can be produced (see table 11) *Tenebrio* may well constitute a very favorable subject for studies in this field.

On the basis of these observations we can now proceed to reconstruct the sequence of events leading to the impasse which prompted this investigation. Up to the spring of 1953 the basic diet must have contained sufficient zinc and potassium, and no carnitine. Then, at first, the diet became deficient in zinc and, at the same time, contained more carnitine. The only missing factor then was zinc and this could be successfully

supplied by adding a small amount of yeast or the water-insoluble yeast fraction, liver, or almost any not well-purified natural material. Doubling the salt mixture at this stage could have had no beneficial effect because of the absence of zinc. A former publication (Fraenkel and Leclercq, '56) contains many instances where a carnitine deficiency developed on a casein dating from 1952, and where relatively good growth resulted after the addition of carnitine, indicating that the diets must have contained adequate amounts of zinc. A recent test with Rutgers' Labco casein also clearly shows the presence of adequate supplies of zinc in this sample (table 12).

The addition of insoluble yeast and similar fractions in small amounts (2%) became ineffective when a potassium deficiency was added to the zinc deficiency. This was due to the use of a sample of a commercially-prepared salt mixture which contained only one half the expected amount of potassium. Exactly when this occurred can only be inferred from the results, but inconsistencies in a transition period two to three years ago must have occurred through the simultaneous use of different bottles of McCollum's salt mixture on different occasions. At that stage, then, with insufficient potassium in the diet, the addition of zinc alone, or in organic material, had no effect because these additions carried insufficient potassium, and doubling the salt quota was still ineffective because the diet was lacking in zinc. Only when yeast or liver were added in large amounts was there sufficient potassium to make the diet satisfactory.

This investigation teaches the nutritionist the multiple lesson that the composition of a salt mixture, however time honored, should not be taken for granted, that samples of casein which were no doubt purified with the best possible care and technique may still contain appreciable amounts of materials supposed to be absent, that mixtures prepared commercially have to be checked, that the method of mixing salts into a diet and the combination in which an element occurs, may be deciding factors, and that in the search for unknown nutritional factors ashes of natural materials ought to be tested.

SUMMARY

The investigation was prompted by the failure of the larvae of the mealworm, *Tenebrio molitor* to grow on synthetic diets and to develop deficiency symptoms in the absence of carnitine. The failure to grow was recognized as being caused by deficiencies of zinc and potassium. Zinc is an absolutely limiting factor, with 6 p.p.m. required for optimal growth, but with as little as 0.37 p.p.m. giving a growth response. The potassium requirements were determined as falling between 1.7 and 2.9% of the diet. A method for growing insects deficient in zinc or potassium has been described. The effects of different salt mixtures have been largely accounted for by their content of zinc and potassium. The effects of different proteins in the diet were accounted for by their content of zinc and carnitine. A new salt mixture has been described which at the 1 to 2% level allows optimal growth. Of 9 trace elements tested, zinc was the only one which could be demonstrated as being required as an addition to the diet. A clear-cut carnitine deficiency developed when Wesson's or the newly developed salt mixtures were used, but failed to develop in the presence of the McCollum-Davis salt mixture. *Tenebrio* grew on a semi-synthetic diet consisting of casein, glucose, cholesterol, 8 vitamins of the B complex and carnitine, and Wesson's or the new salt mixture, plus zinc, as well as on the best natural diets.

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EFFECT OF CERTAIN NECROSIS-PREVENTING FACTORS ON HEMOLYSIS IN VITAMIN E-DEFICIENT RATS AND CHICKS[1]

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Rose and György ('52) observed that the erythrocytes of vitamin E-deficient rats hemolyzed when exposed to dialuric acid *in vivo* or *in vitro*. Hemolysis was prevented when α-tocopherol was given to the animal or added to a suspension of vitamin E-deficient erythrocytes *in vitro*. The lysis could also be prevented *in vitro* by β, γ and Δ-tocopherols, by a variety of hydroquinones and by two estrogenic hormones. However, when fed, only the tocopherols gave demonstrable protection, the activity being greatest for α-tocopherol.

Christensen and Dam ('51) showed that the addition of 0.126% of methylene blue to a vitamin E-deficient rat ration resulted in marked but not complete protection against hemolysis induced by dialuric acid. Moore et al. ('53) on the other hand, reported that methylene blue had no protective effect in vitamin E-deficient rats. In the chick, Christensen et al. ('56) observed no lysis when vitamin E-deficient erythrocytes were exposed to dialuric acid, even though the chicks showed symptoms of encephalomalacia. Muytjens ('56), however, observed a marked lysis of the red blood cells of chicks on a diathesis-inducing diet (Scott et al., '55) high in torula yeast.

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Experiments on the induction of liver necrosis by diets low in vitamin E showed that the syndrome could be prevented by additions to the diet of cystine, methionine, some antioxidants, brewers' yeast or selenium (György et al., '50; Schwarz, '51; Schwarz and Foltz, '57; Gitler et al., '57), as well as by tocopherol. The present experiments describe the effects of these substances on hemolysis in rat erythrocytes. Attempts were made to determine whether their action was independent of tocopherol or one of sparing the vitamin, and in addition, the rate of vitamin E depletion in the chick was followed by means of the hemolysis test.

METHODS

The procedure used for the determination of hemolysis *in vitro* was that described by György ('51) with slight modifications. The washed cells from 0.2 ml of blood were suspended in 15 ml of saline, and 2 ml aliquots diluted to 10 ml with water, saline-buffer [3] or saline-buffer plus dialuric acid.[4] After incubation the tubes were centrifuged and the percentage of hemolysis calculated from the amount of liberated hemoglobin read in an Evelyn colorimeter (Gitler, '58).

EXPERIMENTAL

Level of tocopherol and hemolysis. Holtzman strain male weanling rats were housed in wire cages and fed the following diet A ad libitum: casein 200, dextrin 630, salts (Wesson, '32) 40, cellulose 30, lard 90, cod liver oil 10, choline 1.0 and complete vitamins with the exception of vitamin E.[5]

[3] Saline-buffer solution: equal parts of phosphate buffer pH 7.4 (0.2 M KH_2PO_4, 50.00 ml; 0.2 M NaOH, 39.34 ml, water to make 200 ml) and 0.9% sodium chloride.

[4] Dialuric acid solution: 1 mg per ml in saline-buffer solution. Dialuric acid synthesized according to the procedure of Biltz and Damm ('12). In our initial experiments dialuric acid was replaced by a solution containing 1 mg per ml each of alloxan and cysteine. Used 0.2 ml per tube.

[5] The composition of the vitamin mixture in milligrams per kilogram was: inositol 1000, calcium pantothenate 20, niacin 10, menadione 4, riboflavin 3, thiamine 2, pyridoxine 2.5, biotin 0.1, folic acid 0.2 and vitamin B_{12} 0.001.

Hemolysis was determined at the end of one week on this diet, and the rats were then given single doses of α-tocopherol by stomach tube. Hemolysis was determined 24 and 72 hours after the administration of the tocopherol. The results (table 1) show that 0.1 mg of α-tocopherol was not effective in diminishing the rate of hemolysis, but that 0.25 mg and all higher doses caused the hemolysis to fall from original values of 84 to 99% to 1.0 to 1.2% by 24 hours. At 72 hours after the administration of the tocopherol, the hemolysis values for

TABLE 1

Effect of oral administration of α-tocopherol on the hemolysis of rat erythrocytes by dialuric acid in vitro

RAT	PREDOSAGE HEMOLYSIS	DOSE OF VITAMIN E [1]	HEMOLYSIS	
			1 Day	3 Days
	%	*mg*	%	%
1	96	none	96	95
2	95	none	95	94
3	98	0.1	95	96
4	84	0.1	94	93
5	99	0.25	1.1	70
6	95	0.25	1.0	63
7	87	0.5	1.1	44
8	88	0.5	1.0	40
9	89	1.0	1.0	10
10	88	1.0	1.0	10
11	90	5.0	1.0	1.2
12	91	5.0	1.2	1.3

[1] Each dose was dissolved in 0.3 ml of olive oil.

all pairs, except that receiving the 5-mg dose, increased roughly in proportion to the original dose of tocopherol given.

The effect of antioxidants, methionine, cystine and selenium on hemolysis. Gitler et al., ('57) showed that the antioxidants N-N′-diphenyl-*p*-phenylenediamine (DPPD) and methylene blue but not 2,6-ditertiarybutyl-4-methyl phenol (BHT) prevented the development of liver necrosis in rats fed torula yeast diets deficient in α-tocopherol. Schwarz ('51) and Schwarz and Foltz ('57b) have described the prevention of necrosis by the addition to such diets of methionine, cystine or

selenium. Accordingly, these substances were tested for their ability to replace or spare α-tocopherol in the prevention of hemolysis. The procedure was to deplete weanling male rats of their stores of vitamin E by placing them on diet A for varying periods, after which they were fed a torula-SSL yeast [6] basal diet [7] of similar composition to that used in liver necrosis experiments (Gitler et al., '57) plus the substances to be tested.

Table 2 shows that in the absence of vitamin E most of the substances fed — methylene blue, BHT, rutin, methionine, cystine or selenium — failed to prevent the erythrocytes from being lysed by dialuric acid. Selenium was ineffective even though the depletion period was shortened to 7 days. The feeding of DPPD, on the other hand, completely prevented hemolysis. The period to recovery due to DPPD, however, lengthened as the period of vitamin E depletion was increased (table 2, lines 6, 7 and 8 vs. lines 9 and 10).

Yeast and hemolysis. Male weanling rats were placed on diet A to deplete their original stores of vitamin E, hemolysis was determined after one week and the rats were divided into three groups of three rats each. The rats were given 5 mg of α-tocopherol in 0.3 ml of olive oil by stomach tube and placed on the experimental diets. These were either diet A containing casein, or yeast diets containing torula-SSL [6,7] or brewers' yeast.[8] In experiments A and B the diets contained 18% of crude protein (N $\times$ 6.25) while in experiments C and D the casein, torula-SSL and brewers' yeast were increased at the expense of the dextrin to supply 30% of crude protein.

[6] Torula-SSL yeast (*Torulopsis utilis*) was commercial "feed grade" yeast obtained through the courtesy of Dr. P. L. Pavcek, Lake States Yeast Corp., Inc., Rhinelander, Wisconsin. Grown aerobically on spent sulfite liquor it contained an average of 45% of crude protein (N $\times$ 6.25).

[7] Torula-SSL yeast basal diet in grams per kilogram: torula-SSL yeast 400, salts (Wesson, '32) 40, lard 90, cod-liver oil 10, cellulose 30, complete vitamins (see footnote 5) and dextrin to 1000 gm.

[8] Brewers' yeast (*Saccharomyces cerevisiae*) was obtained from the Pabst Laboratories, Milwaukee, Wisconsin. It contained an average of 50% of crude protein (N $\times$ 6.25).

TABLE 2

Hemolysis[1] *of erythrocytes from vitamin E-deficient rats fed various substances*

LINE NO.	EXP. NO.	ADDITIONS TO THE TORULA BASAL	LEVEL PER 100 GM DIET	PRE-EXP. DEPLETION	PERCENT HEMOLYSIS[2]					
						Days on experimental diets				
					Pre-exp.	1	3	7	11	21
				days						
1	1	None	—	7	96	92	—	—	96	—
2	2	None	—	42	93	88	—	—	88	—
3	3	None	—	7	—	—	89	—	82	—
4	4	None	—	14	—	—	90	92	—	—
5	5	None	—	42	86	—	88	—	87	—
6	1	DPPD[3]	0.196 gm	7	91	0.1	—	2.1	2.8	—
7	3	DPPD[3]	0.3 gm	7	—	—	2.6	—	1.2	1.6
8	4	DPPD[3]	0.5 gm	14			2.8	3.1		
9	2	DPPD[3]	0.3 gm	42	93	—	87	—	3.0	2.6
10	5	DPPD[3]	0.3 gm	42	88	85	7.3	3.1	—	—
11	2	BHT[4]	0.3 gm	42	95	—	90	—	89	90
12	4	BHT[4]	0.5 gm	14	—	—	97	97	—	—
13	4	Methylene blue	0.5 gm	14	93	—	94	93	—	—
14	4	Rutin	0.4 gm	14	—	—	87	94	—	—
15	5	DL-Methionine	0.5 gm	42	86	90	91	92	90	—
16	5	L-Cystine	0.5 gm	42	85	86	85	89	87	—
17	3	Se as Na selenite	0.3 mg	7	—	—	89	—	90	91
18	3	Se as Na selenite	0.5 mg	7	—	—	88	—	86	87
19	2	Se as Na selenite	0.3 mg	42	97	—	88	—	84	86
20	2	Se as Na selenite	0.6 mg	42	96	—	86	—	88	88
21	3	α-tocopherol	3 mg	7	—	—	1.0	—	1.6	1.5
22	4	α-tocopherol	3 mg	14	—	—	2.9	2.7	—	—
23	2	α-tocopherol	3 mg	42			0		3.7	2.8
24	5	α-tocopherol	3 mg	42	—	—	2.3	2.6	—	—

[1] Dialuric acid added *in vitro*.

[2] The ranges of values for replicate determinations were usually within 6 percentage points for averages above 85%; for averages under 10% the range was usually within two percentage points (Gitler, '58); three rats per group.

[3] DPPD = N-N'diphenyl-*p*-phenylenediamine.

[4] BHT = 2,6-ditertiarybutyl-4-methylphenol.

Hemolysis was determined at 1, 3, 7 and 11 days after the oral administration of the α-tocopherol. According to this criterion, the rate of depletion of vitamin E was not affected by either of the yeasts (table 3). Hemolysis was essentially the same whether the diet contained casein, a commercial brewers' yeast that is highly active against necrosis, or a sample of torula-SSL devoid of such activity.

TABLE 3

The effect of yeasts and casein on the resistance of rat erythrocytes to hemolysis by dialuric acid in vitro

EXP. NO.	SOURCE OF PROTEIN	DIETARY PROTEIN LEVEL [1]	HEMOLYSIS % [2]				
			Pre-exp.	Days on exp. diets [3]			
				1	3	7	11
		%					
A	Casein	18	82	0.9	4.4	44	83
B	Casein	18	86	—	2.5	40	84
C	Casein	30	75	0.1	2.6	25	65
D	Casein	30	78	—	1.4	24	81
A	Torula yeast	18	82	1.5	4.6	41	85
B	Torula yeast	18	78	—	2.7	40	86
C	Torula yeast	30	78	0.3	0.6	22	67
D	Torula yeast	30	77	—	1.2	14	79
A	Brewers' yeast	18	82	1.3	6.4	45	86
B	Brewers' yeast	18	92	—	2.6	42	84
C	Brewers' yeast	30	78	0.1	0.8	21	73
D	Brewers' yeast	30	77	—	1.2	29	84

[1] N × 6.25.

[2] Three animals were used per group; the ranges between replicates, narrow in most cases, have been recorded elsewhere (Gitler, '58).

[3] Animals received a single dose of 5 mg α-tocopherol in 0.3 ml olive oil at zero days.

Hemolysis in the chick. In conjunction with experiments on exudative diathesis, the erythrocyte-hemolysis test was applied to depleted chicks. Day-old chicks, the progeny of a cross between New Hampshire males and Single Comb White Leghorn hens, were used in the two experiments reported. They were housed in electrically heated batteries and given

feed and water ad libitum. Publications by Bird ('43) and Schwarz et al. ('57a) provided two diets that produced exudative diathesis; the diet of Bird was based on skim milk and casein while that of Schwarz consisted of the high torula yeast diet first employed by Scott et al. ('55). Selenium was added to the diets as sodium selenite at 1 mg Se per kilogram of diet. Vitamin E was added as the succinate [9] at levels equivalent to 40 mg per kilogram of α-tocopherol. Hemolysis was determined *in vitro* by the procedure described for rats.

Although selenium was able to prevent the symptoms of exudative diathesis, the blood from the chicks still hemolyzed to the extent of 41 and 44% on Bird's diet and 51 and 68% on that of Scott et al., this being equivalent to the rate of hemolysis obtained when the basal diets were fed without added selenium (table 4, lines 1–6 and 9–12). Vitamin E on the other hand lowered the rate of hemolysis to a very low level on either basal diet (lines 7, 8 and 13, 14). The rate of hemolysis also did not show any correlation to the symptom of deficiency observed. Thus when Bird's basal diet was fed, three cases of encephalomalacia resulted (line 2), while when NaCl was added, 8 out of 10 birds showed exudative diathesis (line 4). Nevertheless the rate of hemolysis in the two groups was very similar.

The higher rate of hemolysis observed with the diet of Scott et al. as compared to that of Bird may have been due to differences in the rate of growth between the chicks on these diets: at 4 weeks the chicks on the torula diet had an average weight of from 223 to 312 gm while on Bird's diet the averages ranged from 173 to 196 gm.

DISCUSSION

These results add to the growing body of evidence that several symptoms of vitamin E deficiency may develop independently of one another, and that "substitutes for vitamin E" correct only certain of these symptoms, while tocopherol

[9] Obtained through the courtesy of Dr. P. L. Harris, Distillation Products Industries, Division of Eastman Kodak Co., Rochester, New York.

TABLE 4

The relationship between the symptoms of vitamin E deficiency, selenium and rate of red blood cell lysis in chicks

NO.	EXP.	EXPERIMENTAL DIET	SURVIVAL	EM [1]	ED [2]	HEMOLYSIS % Days on diet	
						14	28
1	1	Bird	8/10	—	—	—	35
2	2	Bird	7/10	3	—	12	38
3	1	Bird + NaCl [3]	3/10	—	6	—	37
4	2	Bird + NaCl	2/10 [6]	—	8	11	39
5	1	Bird + NaCl + Se [4]	10/10	—	—	—	41
6	2	Bird + NaCl + Se	10/10	—	—	16	44
7	1	Bird + NaCl + vitamin E [5]	10/10	—	—	—	2.6
8	2	Bird + NaCl + vitamin E	10/10	—	—	2.0	3.1
9	1	Scott	3/10 [6]	—	7	—	53
10	2	Scott	2/10	—	8	30	57
11	1	Scott + Se [4]	10/10	—	—	—	51
12	2	Scott + Se	8/10	2	—	34	68
13	1	Scott + vitamin E [5]	10/10	—	—	—	3.2
14		Scott + vitamin E	10/10	—	—	2.1	3.0

[1] EM = encephalomalacia.

[2] ED = exudative diathesis.

[3] The sodium chloride was given orally at levels of 0.017 ml/gm body weight using a solution containing 20 gm NaCl/100 ml of water. The administration was given during the second week on experiment.

[4] One milligram of Se as sodium selenite per kilogram of diet.

[5] Forty milligrams of α-tocopherol per kilogram of diet as α-tocopherol succinate.

[6] Remaining birds died during 5th week of experiment.

itself corrects all of them. In the present study one of these symptoms, induced hemolysis, developed at about the same rate whether the basal diet contained casein, a brewers' yeast that prevents necrosis at a low level (5%) in the diet or a torula yeast that does not prevent necrosis. Three p.p.m. of selenium, a mineral which prevents hepatic necrosis at only 1 p.p.m. in the diet, failed to diminish the sensitivity of erythrocytes to dialuric acid. Methionine, which minimizes necrosis, likewise failed to influence hemolysis.

Selenium also fails to prevent encephalomalacia in the chick (Gitler, '58), although it is effective against exudative diathesis whether produced on diets high in yeast (Schwarz, et al. '57; Patterson et al., '57) or in the absence of yeast (Bird's diet, table 4). Alterations in the mineral mixture shifted the physical symptoms of vitamin E deficiency in the chick from encephalomalacia to diathesis without any effect on induced hemolysis.

Diphenyl-*p*-phenylenediamine (DPPD) on the other hand shows protective activity not only against hepatic necrosis (Gitler et al., '57) but also against induced hemolysis (table 2) and against stiff lambs' disease (Draper and Johnson, '55). On the other hand it was found to have no protective activity in the prevention of resorption (Ames et al., '56; Johnson, '55) and may even show a deleterious effect in gestation (Oser and Oser, '56). A similar type of principle may be present in low concentrations in bakers' yeast as indicated by the results of Forbes and György ('57). These workers found less hemolysis on 40% of bakers' yeast than when 18% was fed; the active principle was soluble in ether and gave a positive Emerie-Engel reaction. The data in table 2 of our experiments suggest that DPPD may have been sparing traces of α-tocopherol, since the recovery of resistance to hemolysis was very slow in rats fed DPPD after prolonged vitamin E depletion.

Although contrary views have been expressed, the present results suggest that hemolysis may be useful as an index of vitamin E deficiency in the chick. However the degree of

hemolysis (40 to 68%) was not as high as in apparently healthy rats depleted for only one week (85 to 98%), even though chicks in the deficient groups were dying with encephalomalacia or diathesis. Thus hemolysis appears to be a more sensitive end point in the rat than in the chick.

SUMMARY

The rate of depletion of vitamin E in rats and chicks was followed by determining the sensitivity of the erythrocytes to the hemolyzing action of dialuric acid *in vitro*. This rate was not affected by replacing dietary casein with either brewers' or torula yeast up to levels as high as 60% of yeast. Selenium, methionine, cystine, methylene blue and 2,6-ditertiarybutyl-4-methylphenol (BHT) did not alter the rate of hemolysis under the conditions employed. However, N-N'-diphenyl-*p*-phenylenediamine (DPPD) prevented or minimized hemolysis even when the rats had been depleted of vitamin E for long periods of time. These results are contrasted to the activities of these compounds against liver necrosis.

Preliminary experiments showed that hemolysis may also be used as an indication of vitamin E-depletion in chicks.

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THE ACTIVITY OF CERTAIN WATER-SOLUBLE VITAMINS AFTER EXPOSURE TO GAMMA RADIATIONS IN DRY MIXTURES AND IN SOLUTIONS

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In studies to determine the nutritive properties of irradiated foods, it was observed that chicks receiving an irradiated synthetic diet consistently grew at a slower rate than those receiving the untreated diet. Tests were then carried out to determine the specific nutrients affected by the irradiation process. These tests included the effect of irradiation on choline and folic acid in the complete diet and when a solution of these compounds was irradiated, as well as the effect of irradiation on a mixture of thiamine, riboflavin, pyridoxine, calcium pantothenate and folic acid mixed with casein and irradiated dry, and when irradiated in aqueous solution. The results of these studies are summarized in this report.

EXPERIMENTAL

Day-old crossbred Leghorn cockerels were used as the experimental animals. Twenty chicks per group were started and two trials were run in each test. The experimental diets and water were supplied ad libitum. The composition of the basal diet is given in table 1.

Irradiated, untreated and control diets. The diets were mixed in the laboratory, sealed in no. 2 plain tin cans and stored overnight at -4°C. Approximately 70 cans were packed

in insulated canvas packers, each with 70 lbs. of dry ice and shipped to the Materials Testing Reactor, Idaho Falls, Idaho. One portion was irradiated at a dose level of 2.79×10^6 rad of gamma rays, and this portion was designated the irradiated diet. The non-irradiated portion was designated the untreated diet. The control diet was mixed in the laboratory as needed with ingredients that were not shipped to the Irradiation Center. This procedure was used with the complete diets, solutions of choline and folic acid, a dry mixture of B vitamins, and solutions of a mixture of B vitamins.

TABLE 1

Composition of basal diet

CONSTITUENTS	DIET	VITAMINS PER 100 GM	
	%		
Cerelose	53.1	Vitamin A	3000 I.U.
Casein (vitamin-free)	25.0	Vitamin D	425 I.U.
Gelatin	8.0	Alpha-tocopherol	1.0 mg
Fat [1]	5.5	Menadione	0.75 mg
Wood pulp	3.0	Thiamine·HCl	1.0 mg
Mineral mixture [2]	5.0	Pyridoxine·HCl	1.0 mg
Methionine	0.1	Riboflavin	1.0 mg
Choline chloride	0.3	Ca-pantothenate	3.0 mg
Inositol	0.01	Niacin	2.0 mg
		Folic acid	0.2 mg
		Biotin	0.02 mg
		Vitamin B_{12}	1.0 μg

[1] Mazola.

[2] Richardson and Hogan ('46).

Effect of irradiation on choline. A diet containing 300 mg of choline per 100 gm and a solution containing 300 mg of choline per milliliter were used to determine the effect of ionizing radiation on choline. In studies with the complete diet the irradiated and untreated diets were diluted with a choline-deficient diet. The choline equivalents in the final irradiated and untreated diets were zero, 100, 200 and 300 mg per 100 gm of diet. With the choline solution the irradiated and untreated solutions were mixed in a diet in which the ingredients had not been irradiated.

The average weight, mortality and severity of perosis of chicks receiving the various experimental diets are summarized in table 2. Since the average weight and severity of perosis were no greater in the chicks receiving the irradiated diets, or the irradiated choline solution than in those receiving the untreated diets or untreated choline solution, it was concluded that choline was not destroyed by the irradiation process. These observations differed from those of Lemmon, Parsons and Chin ('55) who reported that choline chloride and certain of its analogs were highly sensitive to ionizing radiations. Lemmon et al. ('55) irradiated 200 to 300 mg of the various compounds in a solid state. In the present study a

TABLE 2

Effect of irradiation on choline

TREATMENT	CHOLINE EQUIVALENT	AV. WEIGHT [1]	MORTALITY	PEROSIS	
				Incidence	Severity [2]
	mg/100 gm	*gm*	*%*	*%*	*%*
		A. Complete diet [3] irradiated			
Irradiated	300	204	0	16	3.6
Untreated	300	254	5	8	1.6
Control	300	251	2.5	—	—
Control	0	112	7.5	100	47.2
Irradiated	200	227	2.5	15	4.1
Untreated	200	242	2.5	28	9.4
Irradiated	100	242	0	27	7.1
Untreated	100	231	0	31	9.4
		B. Solution [3] irradiated			
Irradiated	300	230	0	5	0.6
Untreated	300	247	0	0	0
Control	300	268	0	0	0
Irradiated	200	267	0	0	0
Untreated	200	239	0	0	0

[1] Experimental period 4 weeks.

[2] Severity = sum of values (0, 1, 2, 3 and 4 positive) of right and left legs of all chicks in a group × 100 divided by the number of chicks × 8.

[3] Diet contained 300 mg of choline chloride per 100 gm and solution contained 300 mg per ml.

total of 1.2 gm of choline chloride was irradiated at a time in the diet and 120 gm in solution. This difference in the total mass of choline irradiated at a time may account for the apparent differences in the effect of the irradiation.

The data in table 2 show that chicks receiving the irradiated diet before dilution with the untreated deficient diet weighed an average of 204 gm, while those receiving the untreated diet before dilution weighed 254 gm. This difference in the weight of the chicks suggests that irradiation decreased the nutritive value of the diet, but the data for the diluted diets indicate that some constituent other than choline was damaged by the irradiation. For example, chicks receiving the irradiated diet containing 300 and 200 mg choline equivalent per 100 gm of diet weighed less than those receiving the untreated diet. When 100 mg choline equivalent per 100 gm was supplied, the chicks receiving the irradiated diet were heavier than those receiving the untreated diet. If the choline had been completely destroyed by the irradiation process, the average weight of the chicks receiving the irradiated diet would have been essentially the same as those reciving the deficient diet. On the other hand, if only part of choline had been destroyed, and if no other constituent was damaged, the chicks receiving the irradiated diet containing 200 or 300 mg equivalent of choline should have grown much faster than those receiving 100 mg equivalent of choline. These data support the conclusion that some constituent other than choline was damaged by the irradiation process.

Folic acid. The method used to study the effect of radiation on folic acid in the diet and in solution was the same as that used for choline. Preliminary tests showed that 50 μg of folic acid per 100 gm of diet was a borderline amount, and any destruction of the vitamin could be demonstrated more easily than when a large excess was present. As in the case with choline the irradiated and untreated diets were diluted with a diet which had the same composition as the original diet except that no folic acid was added. The folic acid-deficient diet was added to both the irradiated and untreated diets so

that the diluted diets contained 50.0, 37.5, 25.0, 12.5 and zero μg of folic acid per 100 gm of diet. When the folic acid solution was used the level of folic acid in the diet was 50 and 25 μg per 100 gm of diet, respectively. The average weight, mortality and hematocrit values for the chicks receiving the irradiated, untreated, control and deficient diets are summarized in table 3.

TABLE 3

Effect of irradiation on folic acid

TREATMENT	FOLIC ACID EQUIVALENT	AV. WT.[1]	MORTALITY	HEMATOCRIT
	μg/100 gm	*gm*	*%*	*%*
	A. Complete diet irradiated			
Irradiated	50	323	5	21
Untreated	50	396	1.7	23
Control	50	362	3.4	22
Control	0	111	87.5	11
Irradiated	37.5	326	0	20
Untreated	37.5	406	0	23
Irradiated	25.0	301	1.7	18
Untreated	25.0	348	0	21
Irradiated	12.5	186	17	12
Untreated	12.5	217	10	13
	B. Folic acid solution irradiated[2]			
Irradiated	50	336	0	22
Untreated	50	339	5	23
Control	50	361	10	23
Irradiated	25	223	15	12
Untreated	25	336	0	22

[1] Experimental period 5 weeks.

[2] Solution contained 50 μg folic acid per milliliter.

The chicks receiving the untreated diet always grew faster than those receiving the irradiated diet. This difference in weight of the chicks suggested that some folic acid was destroyed, but the differences in hematocrit values were small and probably do not indicate a major loss of the folic acid. When 50 μg of folic acid in solution were added per 100 gm

of diet, the rate of growth, mortality and the hematocrit values were the same for the groups receiving the irradiated, untreated, and control solutions. However, when the amount was reduced to 25 μg per 100 gm of diet, chicks receiving the irradiated solution weighed 223 gm and had an average hematocrit value of 12% while those receiving the untreated solution weighed 336 gm and had a hematocrit value of 22%. This latter value is the same as that obtained when 50 μg of folic acid was added. These data indicate definite destruction of 25 to 50% of the folic acid when it was irradiated in solution.

Irradiation of a mixture of B vitamins. Two series of tests were run to determine the effect of irradiation on a mixture

TABLE 4

Growth and mortality in chicks receiving B vitamins mixed with vitamin-free casein and irradiated dry

TREATMENT	VITAMIN MIXTURE [1] PER 100 GM	AV. WT. AT 6 WKS.	MORTALITY	
			%	Av. age at death
	gm	*gm*		*days*
Irradiated	1.0	503	10.0	24
Untreated		525	0.0	..
Irradiated	0.3	326	10.0	21
Untreated		307	5.0	14

[1] One gram of the mixture contained the same vitamins and amounts as 1.0 ml of solution, footnote 1, table 5.

of thiamine, riboflavin, pyridoxine, pantothenic acid and folic acid. In one test the vitamins were mixed with vitamin-free casein and irradiated dry. In the other the vitamins were irradiated in solution. The amount of each vitamin per 1.0 gm of the dry mixture and per 1.0 ml of solution are given in footnote 1, table 5. The amounts of dry mixture per 100 gm of diet are given in table 4, and the amounts of solution per 100 gm of diet in table 5.

The growth and mortality of chicks receiving 1.0 and 0.3 gm of the irradiated dry mixture are summarized in table 4. The differences in the rate of growth and mortality of the chicks

receiving the irradiated and untreated vitamins at each level were small and did not indicate an important destruction of any vitamin by the irradiation process.

The rate of growth and mortality of chicks receiving diets containing different levels of the irradiated and untreated vitamin solutions are summarized in series A, table 5. When the diet contained 1.0 ml of the solution, the chicks receiving the irradiated solution grew as rapidly as those receiving the untreated solution, but when the solutions were reduced to a level (0.5 or 0.3 ml per 100 gm of diet) which supplied a marginal amount of the vitamins, the mortality rate in the group receiving the irradiated solution was three to 4 times higher than in the group receiving the untreated solution. Many of the chicks died with typical symptoms of polyneuritis which suggested that a relatively large amount of the thiamine had been destroyed by the irradiation process.

In view of these results two series of tests were run to determine whether the activity of other vitamins in the solution was also affected by irradiation. The basal diet used in both tests contained 0.3 ml of the irradiated vitamin solution. The diet in one test, series B, table 5, was supplemented with various combinations of untreated vitamins that were present in the original solution. The amount of a vitamin supplied per 100 gm of diet was the same as that in 1.0 ml of the original solution. The mortality rates in percent after supplementation with non-irradiated vitamins were as follows: no supplement 92; thiamine 85; thiamine and riboflavin 90; thiamine, riboflavin and pyridoxine 50; thiamine, riboflavin, pyridoxine, and pantothenic acid 43; and thiamine, riboflavin, pyridoxine, pantothenic acid and folic acid 2.5. Obviously there was some destruction of each vitamin in the mixture, but it was impossible from these data to estimate the amount destroyed.

A second test was then run by a slightly different procedure in an attempt to obtain information about the relative amount of each vitamin destroyed. In this procedure the basal diet containing 0.3 ml of irradiated solution was supplemented with a high level of all the untreated vitamins in the original

TABLE 5

Growth and mortality in chicks receiving a vitamin mixture irradiated in solution

TREATMENT	ORIGINAL VITAMIN SOLUTION [1]	VITAMIN SUPPLEMENT	AV. WT.[2]	MORTALITY	
				%	Av. age at death
			gm		*days*
		A. Level of vitamin solution			
Irradiated	1.0	—	385	5.0	21
Untreated	1.0	—	391	7.5	29
Irradiated	0.5	—	350	48.0	14
Untreated	0.5	—	353	10.0	29
Irradiated	0.3	—	260	92.0	14
Untreated	0.3	—	298	33.0	14
		B. Irradiated vitamins supplemented with untreated vitamins [3]			
Irradiated	0.3	none	260	92.0	14
Irradiated	0.3	B_1	177	85.0	23
Irradiated	0.3	$B_1 + B_2$	215	90.0	17
Irradiated	0.3	$B_1 + B_2 + B_6$	325	50.0	23
Irradiated	0.3	$B_1 + B_2 + B_6 +$ CAP	260	43.0	32
Irradiated	0.3	$B_1 + B_2 + B_6 +$ CAP, FA	420	2.5	5
		C. Irradiated vitamins supplemented with untreated vitamins, then one vitamin at a time omitted			
Irradiated	0.3	$B_1 + B_2 + B_6 +$ CAP, FA	420	2.5	5
Irradiated	0.3	$- + B_2 + B_6 +$ CAP, FA	439	65.0	16
Irradiated	0.3	$B_1 + - + B_6 +$ CAP, FA	390	12.5	8
Irradiated	0.3	$B_1 + B_2 + - +$ CAP, FA	384	25.0	11
Irradiated	0.3	$B_1 + B_2 + B_6 +$ —, FA	406	10.0	23

[1] One milliliter of solution supplied the following amounts of vitamins per 100 gm of diet: B_1 (thiamine-hydrochloride) 1.0, B_2 (riboflavin) 1.0, B_6 (pyridoxine) 1.0, CAP (calcium pantothenate) 3.0, FA (folic acid) 0.05.

[2] Total 40 chicks, two trials 20 chicks per group each, experimental period 6 weeks.

[3] Individual vitamin supplied per 100 gm of diet was the same as in 1 ml of original vitamin solution.

mixture. One vitamin, with the exception of folic acid, was then omitted from the supplement at a time. These data are summarized in series C, table 5. The mortality rate in percent when the various individual vitamins were omitted was as follows: none 2.5, thiamine 65, riboflavin 12.5, pyridoxine 25.0, and pantothenic acid 10. These data confirm the conclusion that there was some destruction of each vitamin when the solution was irradiated, and show that there was a greater destruction of thiamine and pyridoxine than of riboflavin and pantothenic acid. It was impossible to estimate accurately the amount of each vitamin destroyed by the procedure used in this study.

The observation that thiamine, riboflavin, and pyridoxine were destroyed by irradiation is in agreement with that of Day et al. ('57a, b) who reported that an average of 64.0% of the thiamine, 10% of the riboflavin and 25% of the pyridoxine were destroyed when raw ground beef was irradiated with 3 million rep of gamma rays.

The results in series B and C, table 5, show that there was some destruction of each vitamin when the solution was irradiated. However, the mortality rate when the vitamin was added to the deficient diet, series B, was much greater in every case than when it was omitted from the complete vitamin supplement, series C. A multiple deficiency probably was present in series B, while only a single deficiency existed in each case in series C. The differences in response in the two two tests obviously is due to the difference between a multiple and a single deficiency. These data suggest that it may be necessary to supplement diets composed entirely of irradiated foods with a complete vitamin mixture even though destruction of some of the vitamins may be relatively small.

SUMMARY

The effect of ionizing radiations on choline, folic acid, thiamine, riboflavin, pyridoxine and pantothenic acid was investigated using baby chicks as experimental animals. The vitamins were irradiated under various conditions with a dose of 2.79×10^6 rad of gamma rays.

There was no evidence that any of the vitamins were destroyed when the synthetic diet containing the vitamins or when a mixture of the vitamins with vitamin-free casein was irradiated. Choline was not destroyed when an aqueous solution containing 300 mg of choline chloride per ml was irradiated. Twenty-five to 50% of the folic acid in a solution containing 50.0 μg/ml was lost by the irradiation process. There was no evidence of destruction of thiamine, riboflavin, pyridoxine, pantothenic acid and folic acid when the vitamins were mixed with casein and irradiated dry, but there was some destruction of each vitamin when irradiation was carried out in an aqueous solution. The loss of thiamine in the solution was largest and the loss of pyridoxine was slightly higher than that of riboflavin and pantothenic acid.

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INTERACTIONS OF B VITAMINS ON GROWTH OF RATS

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The effects of a deficiency of one vitamin would not ordinarily be expected to be highly dependent on the presence or absence of another vitamin in the diet, since the symptoms of deficiency of each vitamin are usually quite distinct. Nevertheless, antagonistic or synergistic interactions between vitamins may occur to a greater or less extent. While several mechanisms can be proposed whereby vitamins can be synergistic, it is more difficult to conceive of one which could explain vitamin antagonism. It was therefore unexpected when Beznák and van Alphen ('55) reported that animals deficient in both thiamine and pantothenic acid failed to show weight loss as soon as animals deficient in thiamine alone.

The present paper describes a study of the interactions of thiamine, riboflavin, pyridoxine and pantothenate as shown by growth of the rat.

METHODS

Male weanling rats of the Sprague-Dawley strain, weighing between 35 and 60 gm, were used in these experiments. The basal diet, which has abundant amounts of vitamins, is shown in table 1. Animals were made deficient by omission of one or two of the B vitamins from this diet. When penicillin was added to the diet, the level was 50 mg per kilogram, while when ascorbic acid was used, 5% was added at the expense of

sucrose. Animals were housed in individual screen-bottom cages, and growth and food intakes were measured for a period of 4 weeks.

TABLE 1

Basal diet

CONSTITUENT	AMOUNT
	gm
Casein [1]	240
Hydrogenated vegetable oil [2]	200
Sucrose	520
Salts [3]	40
Vitamins [4]	—

[1] "Vitamin-low," Nutritional Biochemicals.

[2] "MFB," Wesson Oil and Snowdrift Sales Co.

[3] Jones and Foster ('42).

[4] The following were added per kilo of diet: thiamine hydrochloride 30 mg, pyridoxine hydrochloride 30 mg, riboflavin 30 mg, calcium pantothenate 40 mg, niacin 40 mg, inositol 800 mg, folic acid 30 mg, biotin 3 mg, vitamin B_{12} 12 μg, 2-methyl-naphthoquinone 60 mg, vitamin A 52,000 I. U., vitamin D 10,000 I. U., α-tocopherol 22 mg, *p*-aminobenzoic acid 400 mg, and choline chloride 800 mg.

RESULTS

The effects on growth of single deficiencies and combinations of deficiencies are shown in figure 1. The results on larger numbers of animals are summarized in table 2. It will be seen that while a combined deficiency of two vitamins in general produces poorer growth than either single deficiency alone, the difference is marked only in the combination of deficiencies of pyridoxine and pantothenate, and significant in only two other combinations: those of thiamine and riboflavin, and of thiamine and pyridoxine. The growth curves of combinations with thiamine are of particular interest, since they show both the early growth retardation characteristic of the other deficiencies, and the marked later weight loss characteristic of thiamine deficiency.

Of the vitamins in the basal diet, 8 (niacin, biotin, folic acid, inositol, choline, *p*-aminobenzoic acid and vitamins B_{12} and K) can be omitted singly from the diet with little effect on weight gain in 4 weeks, although when all are omitted, the

effect is measurable. The experiment in table 3 was designed to determine whether interactions betweeen these 8 vitamins and the 4 principal B vitamins occur. The results show poorer growth in all cases when the 8 were omitted, but the effect

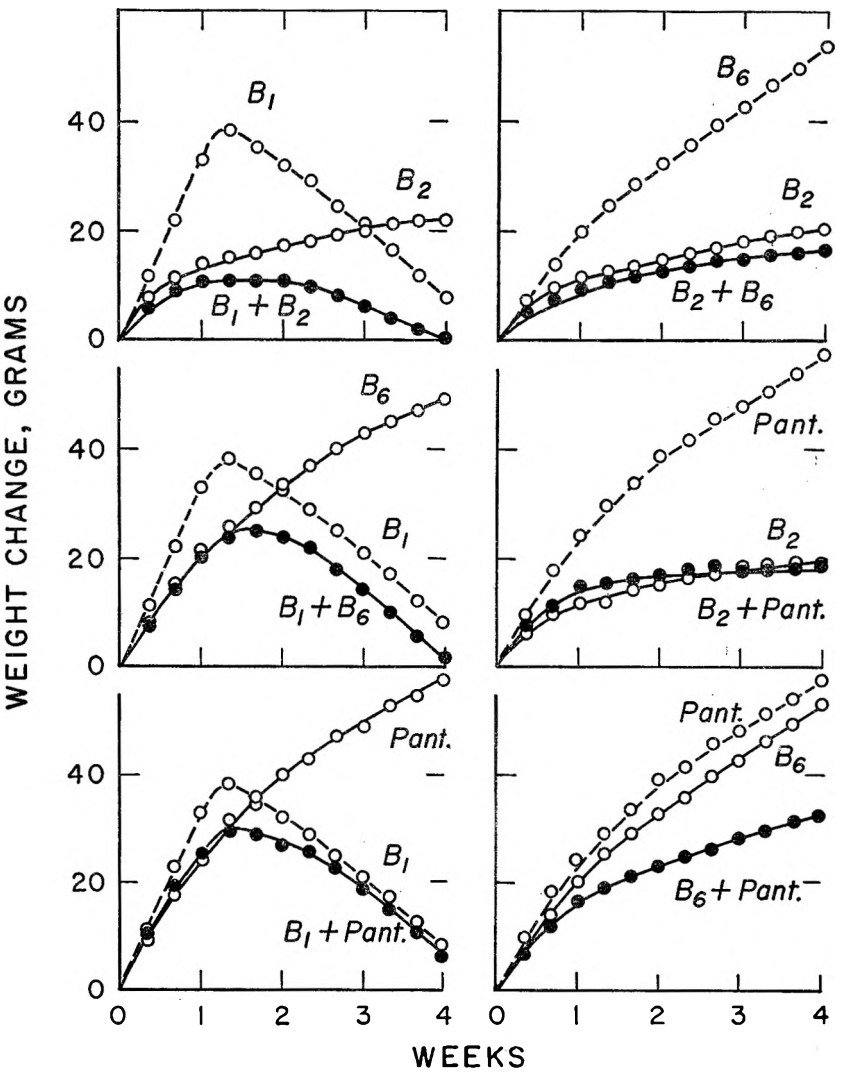

Fig. 1 Weight change of animals in single and combined deficiencies. Each curve is the mean weight change of 30 animals. Open circles = single deficiency; filled circles = combined deficiency.

TABLE 2

Comparison of weight change in single and combined deficiencies

EXP.	DEFICIENCY	NO. OF ANIMALS	WEIGHT CHANGE [1]	DIFFERENCE BETWEEN SINGLE AND COMBINED DEFICIENCY [1]
			gm/4 wks.	*gm/4 wks.*
1	Thiamine	28	8.1 ± 1.2	
	Riboflavin	29	22.6 ± 2.8	
	Thiamine plus riboflavin	30	0.3 ± 1.2	7.8 ± 1.7
2	Thiamine	38	8.8 ± 1.0	
	Pyridoxine	39	50.9 ± 2.6	
	Thiamine plus pyridoxine	40	2.2 ± 1.0	6.6 ± 1.4
3	Thiamine	48	8.4 ± 1.1	
	Pantothenate	50	61.0 ± 2.8	
	Thiamine plus pantothenate	49	6.6 ± 1.4	1.8 ± 1.5
4	Riboflavin	40	18.2 ± 1.6	
	Pyridoxine	39	50.5 ± 3.2	
	Riboflavin plus pyridoxine	39	14.8 ± 1.8	3.4 ± 2.4
5	Riboflavin	40	17.6 ± 1.5	
	Pantothenate	38	56.3 ± 3.6	
	pantothenate	40	17.2 ± 1.5	0.4 ± 2.1
	Riboflavin plus			
6	Pyridoxine	58	54.4 ± 2.6	
	Pantothenate	57	57.1 ± 2.8	
	Pyridoxine plus pantothenate	60	30.6 ± 1.8	23.8 ± 3.2

[1] Mean and standard error of the mean.

TABLE 3

Effect of omission of 8 "other vitamins"[1] on weight change in B vitamin deficiencies

B VITAMIN DEFICIENCY	WEIGHT CHANGE		
	Other vitamins added	Other vitamins omitted	Difference
	gm/4 wks.	*gm/4 wks.*	*gm/4 wks.*
None	170.2 ± 4.8 [2]	143.8 ± 5.7 [2]	26.4 ± 7.4 [2]
Thiamine	4.4 ± 1.7	−2.1 ± 1.6	6.5 ± 2.3
Riboflavin	52.0 ± 7.7	41.3 ± 5.7	10.7 ± 9.6
Pyridoxine	81.0 ± 6.7	76.2 ± 7.1	4.8 ± 9.8
Pantothenate	83.7 ± 12.8	80.8 ± 13.0	2.9 ± 18.2

[1] "Other vitamins" were niacin, *p*-aminobenzoic acid, folic acid, B_{12}, K, choline, inositol and biotin.

[2] Mean and standard error of the mean of 10 animals per group.

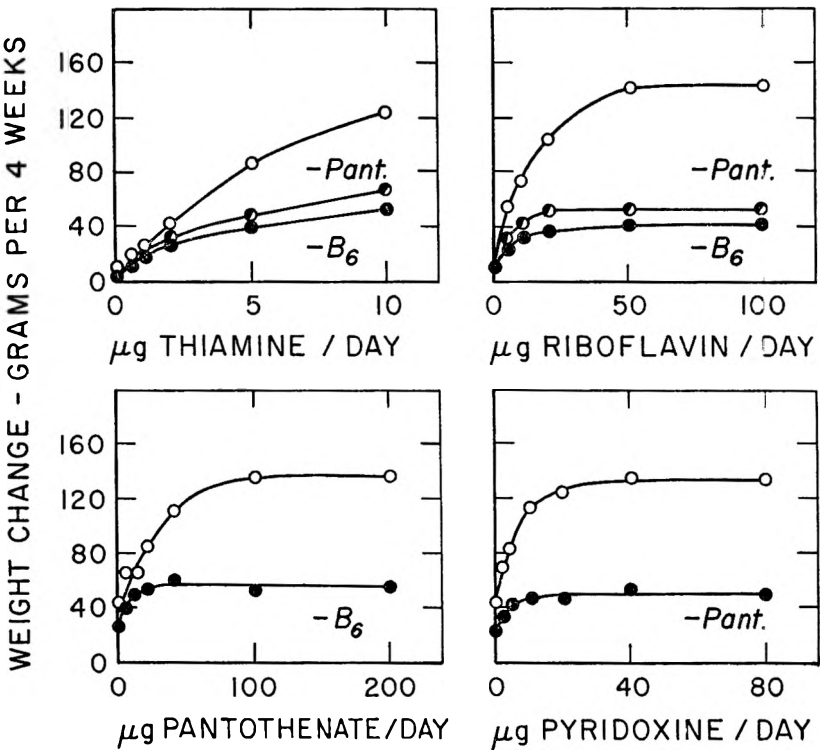

Fig. 2 Effect of graded levels of a vitamin on weight change in a second deficiency. In each case, the upper curve represents animals on a diet lacking only the vitamin being injected at the levels shown; the lower curves represent animals on diets which also lack pyridoxine or pantothenate. Each point is the mean weight change of 10 animals.

was much greater when none of the 4 principal B vitamins was omitted.

The effects of adding penicillin or ascorbic acid, both of which are known to change requirements for B vitamins, (Lih and Baumann, '51; Sauberlich, '52; Daft and Schwarz, '52) are shown in table 4. Penicillin produced increased growth in all single and combined deficiencies, but did not appear to influence interactions of the vitamins.

Ascorbic acid in the diet increased growth in all cases except pyridoxine deficiency and the combined deficiency of pyridox-

TABLE 4

Comparison of single and combined deficiencies with penicillin or ascorbic acid in the diet.

DEFICIENCY	ADDITIONS: None	Penicillin	None	Ascorbic acid
	gm/4 wks.	*gm/4 wks.*	*gm/4 wks.*	*gm/4 wks.*
Thiamine	17.4 ± 4.1 [1]	61.9 ± 7.1 [1]	10.2 ± 1.9 [1]	41.8 ± 3.4 [1]
Riboflavin	27.2 ± 3.2	43.8 ± 4.8	14.5 ± 2.0	53.8 ± 5.5
Pyridoxine	65.5 ± 8.5	99.5 ± 9.3	41.8 ± 3.0	43.5 ± 2.1
Pantothenate	70.3 ± 8.8	105.0 ± 10.2	47.8 ± 2.5	77.7 ± 10.6
Thiamine plus riboflavin	3.9 ± 3.0	27.4 ± 5.4	−0.4 ± 1.7	33.3 ± 5.0
Thiamine plus pyridoxine	6.3 ± 2.6	48.5 ± 3.7	7.0 ± 1.6	25.1 ± 4.4
Thiamine plus pantothenate	18.2 ± 5.1	59.1 ± 7.3	10.0 ± 3.1	36.9 ± 7.4
Riboflavin plus pyridoxine	26.5 ± 4.7	46.4 ± 8.6	11.1 ± 1.2	31.1 ± 2.8
Riboflavin plus pantothenate	25.7 ± 3.2	38.9 ± 6.5	13.9 ± 2.4	42.2 ± 5.0
Pyridoxine plus pantothenate	39.5 ± 4.0	63.7 ± 4.4	32.4 ± 4.4	31.2 ± 2.7

[1] Average weight change of 10 animals and standard error of the mean.

ine and pantothenate. There appeared to be some synergism between riboflavin and pyridoxine in the presence of ascorbic acid.

Since certain of these vitamins appear to be synergistic in their action on growth, it was of interest to determine whether deficiency of one vitamin could "spare" another. In a deficiency of one vitamin, growth and metabolism are retarded, and thus requirement for a second vitamin might be lowered. These points were tested in the experiments shown in figure 2. In general, deficiency of one vitamin lowered the "requirement" for a second, if requirement is defined at that intake per day allowing maximal growth under the circumstances. If requirement is defined as that intake per gram of food allowing maximal growth, lowering of requirement was not evident because of the lower food intake found in deficiency. However, neither pantothenate nor pyridoxine deficiency affected requirement for thiamine. Intakes of thiamine in excess of 10 μg per day were found to have no appreciable effect on growth during a 4-week period.

DISCUSSION

Previous studies of interactions of B vitamins have chiefly stressed the effects of an excess of one vitamin in precipitating deficiency of another. Several clinical reports (Richards, '45) suggest that therapy with one vitamin in human deficiency may lead to precipitation of symptoms of another deficiency. Similarly in animals, it has been found necessary to make the diet adequate in other respects to elicit symptoms of certain deficiencies (Richards, '45). However, Unna and Clark ('43) could find no effects of large excesses of other vitamins on the course of deficiencies of thiamine, riboflavin, pyridoxine and pantothenate. Richards ('45) reported that a large excess of thiamine made the effects of pyridoxine deficiency more severe. Cimino ('49) found that thiamine deficiency was slightly less severe if a large amount (1 mg per day parenterally) of niacin was given. Young et al. ('55) have studied interrelationships of

pyridoxine and vitamin E, but no interaction on growth was measurable.

In our experiments, growth in a combined deficiency was in no case better than in the corresponding single deficiencies, and thus no antagonism was found. Instead, where interaction occurred, the effect of the vitamins was synergistic. Of the mechanisms of synergism which can be postulated, the following appear reasonable:

1. For statistical reasons, an apparent synergism might occur between two vitamins giving approximately the same growth, even though the effects of deficiency were otherwise wholly independent. Thus, part of the animals who show better than average growth in pyridoxine deficiency would be expected to show poorer than average growth in pantothenate deficiency, and part of those showing better than average growth in pantothenate deficiency would show poorer than average growth in pyridoxine deficiency. The net result would be that combined deficiency would give poorer average growth than either single deficiency. This statistical effect is not great, however, and is not sufficient to explain the synergism between pyridoxine and pantothenate.

2. Synergism may have a dietary origin under certain circumstances. Thus in figure 1, it is evident that riboflavin, pyridoxine and pantothenate deficiencies all result in growth retardation earlier than does thiamine deficiency. This retardation and consequent low food consumption might result in a lower thiamine intake which could later cause a more severe thiamine deficiency. This explanation is not applicable to diets such as those used here where the level of vitamins in the diet is very low unless they are added. However, it is still possible that the early growth retardation in deficiencies combined with thiamine deficiency may be related in some unknown way to the lower over-all growth found in combined deficiency.

3. Synergism might have its origin in an effect on bacterial synthesis. Thus intestinal bacteria which can synthesize pantothenic acid might require pyridoxine, or those synthesizing pyridoxine might require pantothenate. If this were true, one

might anticipate that a deficiency of one vitamin would be more severe if the second vitamin were injected rather than supplied orally, assuming no passage of the vitamin into the intestinal tract occurred. This hypothesis was tested in the case of pyridoxine and pantothenate deficiency. The results, shown in table 5, indicate that injected pyridoxine is, at most, only slightly less effective than oral pyridoxine in pantothenate deficiency, and the same is true of injected and oral pantothenate in pyridoxine deficiency.

TABLE 5

Comparison of effect of injection and oral administration of a vitamin in a second deficiency

DIETARY DEFICIENCY	VITAMIN INJECTED	WEIGHT CHANGE
		gm/4 wks.[1]
Pyridoxine	None	64.7 ± 5.4
Pyridoxine plus pantothenate	50 μg pantothenate per day	52.2 ± 3.2
Pantothenate	None	71.4 ± 5.5
Pyridoxine plus pantothenate	20 μg pyridoxine per day	66.4 ± 4.9
Pyridoxine plus pantothenate	None	34.7 ± 3.0

[1] Mean and standard error of the mean.

4. Synergism of vitamins might occur for metabolic reasons. Since growth is a complex process, different vitamin deficiencies may affect growth in different ways. More specifically, one deficiency may limit the utilization of a certain metabolic pathway, which can be compensated for to some extent by an alternate pathway. If a second deficiency limits the alternate pathways, the results of combined deficiency would be more severe than either single deficiency.

While there is no evidence in these experiments for the correct explanation of the cases of synergism here shown, one additional point is of interest. It will be noted in table 4, that penicillin or ascorbic acid promotes growth in all combined deficiencies except one. Since ascorbic acid promotes growth in pantothenate deficiency, but not in pyridoxine deficiency or in combined pyridoxine and pantothenate deficiency, it appears

probable that the limiting factor in the combined deficiency is the amount of pyridoxine.

SUMMARY

Interactions between the vitamins thiamine, riboflavin, pyridoxine and pantothenate were sought by comparing the growth of rats on diets deficient in two vitamins with diets deficient on a single vitamin. No antagonistic interactions were found. Thiamine and riboflavin and thiamine and pyridoxine had some synergistic action on growth, but the only marked interaction found was a synergistic effect of pyridoxine and pantothenate.

No evidence of interaction between the 4 principal B vitamins and niacin, biotin, inositol, *p*-aminobenzoic acid, folic acid, choline and vitamin B_{12} and K was obtained. Penicillin in the diet increased growth in all single and combined deficiences, while 5% of ascorbic acid increased growth in all cases except pyridoxine and combined pyridoxine and pantothenate deficiencies.

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STUDIES ON THE TOXICITY OF *INDIGOFERA ENDECAPHYLLA*

II. TOXICITY FOR MICE

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INTRODUCTION

In a previous paper (Hutton et al., '58) we have confirmed that strains of *Indigofera endecaphylla* cause severe liver damage when fed to rabbits. We have reported that a toxin is present in the seed as well as in the herbage, and that we were unable to detect β-nitropropionic acid in the seed by Cooke's ('55) method. This finding, together with our failure to produce severe liver damage by force feeding synthetic β-nitropropionic to rabbits, led us to the conclusion that β-nitropropionic acid was not the toxin present in *I. endecaphylla,* as claimed by Cooke ('55). Morris et al. ('54) were the first to find that β-nitropropionic acid was a toxin present in *I. endecaphylla,* using chicks in the test for toxicity.

The work described in this paper was designed to test the toxicity of *I. endecaphylla* for mice, with a view to developing a biological method for analyzing various fractions of *I. endecaphylla* so as to isolate and identify the toxic principle.

MATERIALS AND METHODS

The experiments were done with a strain of *I. endecaphylla* introduced from Ceylon and designated C.P.I. 18557. A special

meal [1] was used as a control ration or for compounding rations containing *I. endecaphylla* fractions.

Adult female mice of the Brisbane Hospital strain, 17 to 24 weeks old, and weighing between 20 and 30 gm were used and were housed in wooden cages with wire mesh at top and bottom. Four grams of dry food was offered daily to each mouse, food intake being calculated daily by weighing residues. Water was provided ad libitum, and mice were weighed at least twice weekly.

Livers were removed from the mice as soon as possible after they died or were killed and then fixed in 10% formol saline. Paraffin sections were prepared and stained with hematoxylin and eosin. Frozen sections were stained with Sudan IV to demonstrate fat.

The histological changes seen in the livers were assessed and given arbitrary subjective gradings under the headings of cell degeneration, cell necrosis, fatty change, regeneration and fibrosis. The first three were graded — slight +, moderate ++, moderately severe +++, and severe ++++. Regeneration was assessed by increase in mitotic cells and binucleate cells and graded + to ++++. Fibrosis was graded as follows: some parenchymal collapse, +; parenchymal collapse with appearance of lobulation, ++; lobulation with bands of connective tissue infiltrated by inflammatory cells and fibroblasts and showing bile duct proliferation, if moderately severe, +++; if severe, ++++.

None of the animals to be described developed fibrosis of a degree sufficient to be graded +++ or ++++. The end point of the toxic effect in these experiments can be considered to be where the mouse livers showed in addition to degenerative, necrotic and fatty changes, a proportion of regenerative and necrotic processes with ratings of ++ or +.

[1] Red Comb Special Mix No. 3 (manufactured by Poultry Farmers Coop. Soc. Ltd., Brisbane) containing cereal meal (maize, barley, and wheat) 42%, bran and pollard 35%, meat meal 15%, liver meal 7%, calcium carbonate and salt 1%, vitamin A and riboflavin. The mix contained 19.3% crude protein and 4.5% crude fat.

RESULTS

Seed

(a) *Initial experiments.* *I. endecaphylla* seed fed alone or ground with meal proved very palatable to mice. Several feeding experiments with seed were done, but only representative ones are quoted here.

In the initial experiment (table 1) pairs of mice were fed a series of diets containing respectively 50% seed, 50% heated seed, 15% endosperm, 30% seed coat, and 50% white clover seed (*Trifolium repens* L.) made up with meal. Surviving animals were killed at 14 days and those which became moribund before this time were killed as indicated in the table.

Histological examination showed evidence of gross liver damage. The parenchymal liver cells were swollen and showed increased granularity. Nuclei were grossly swollen in some areas, showing fragmentation and eosinophilic inclusions. Necrosis of liver cells was a common feature and this tended to occur most frequently towards the periphery of the liver lobules, though it did not always show the clear cut periportal distribution noted previously in the rabbit (Hutton et al., '58). Fatty degeneration was a variable feature and was usually mid-zonal in distribution. When necrosis of liver cells had been severe, there was evidence of stromal collapse giving a distorted lobulation. Fibroblastic and inflammatory cell infiltration of these collapsed areas was minimal; cellular infiltration of the portal tracts was not marked.

A common picture was a prominent central vein surrounded by a cuff of swollen but reasonably preserved cells with more pronounced degenerative and fatty change in the mid-zonal areas and some necrotic cells outside this with evidence of stromal collapse. Variation in degree of these changes between various areas resulted in a general distortion of liver architecture with lobules of various sizes orientated about the central veins (fig. 1A). A normal mouse liver is shown in figure 1B.

These experiments demonstrated that the mouse liver was quite sensitive to the toxin of *I. endecaphylla.* Though there was variation between individual animals in the response to

TABLE 1

Result of feeding diets containing ground I. endecaphylla seed to paired mice for 14 days

DIET	MEAN FOOD INTAKE		WT. MOUSE		SURVIVAL	HISTOLOGICAL LIVER CHANGES [1]				
	1st wk.	2nd wk.	Initial	Final		Degeneration	Necrosis	Fatty change	Regeneration	Fibrosis
	gm/day	*gm/day*	*gm*	*gm*	*days*					
1. 50% 18557 seed plus 50% meal	1.6	1.2	21.0	16.5	11	+++	+	+	0	+
			24.5	17.5	13	++	+	0	0	0
2. 50% 18557 seed (heated at 121°C and 15 lb pressure for 30 min.) plus 50% meal	2.5	1.8	26.0	19.0	14	++	++	++	0	0
			26.5	19.0	14	++++	++	++	0	+
3. 4 gm/day fed from mixture of 18 gm endosperm (from 60 gm seed) plus 102 gm meal	3.1	2.4	24.0	18.0	9	++	+	0	0	0
			21.0	17.0	14	+++	++	0	0	++
4. 4 gm/day fed from mixture of 37 gm seed coat (from 60 gm seed) plus 83 gm meal	2.9	2.8	30.0	23.5	14	+++	++	++	0	+
			29.0	21.5	14	++	++	++	+	++
5. 50% white clover seed plus 50% meal	3.0	3.6	24.0	23.5	14	0	0	0	0	0
			26.0	23.5	14	0	0	0	0	0

[1] Degeneration, necrosis and fatty change were graded: slight +; moderate ++; moderately severe +++ and severe ++++. Regeneration was assessed by increase in mitotic cells and binucleate cells and graded + to ++++. Fibrosis was graded as follows: some parenchymal collapse +; parenchymal collapse with appearance of lobulation ++; lobulation with bands of connective tissue infiltrated by inflammatory cells and fibroblasts and showing bile duct proliferation, if moderately severe +++, if severe ++++.

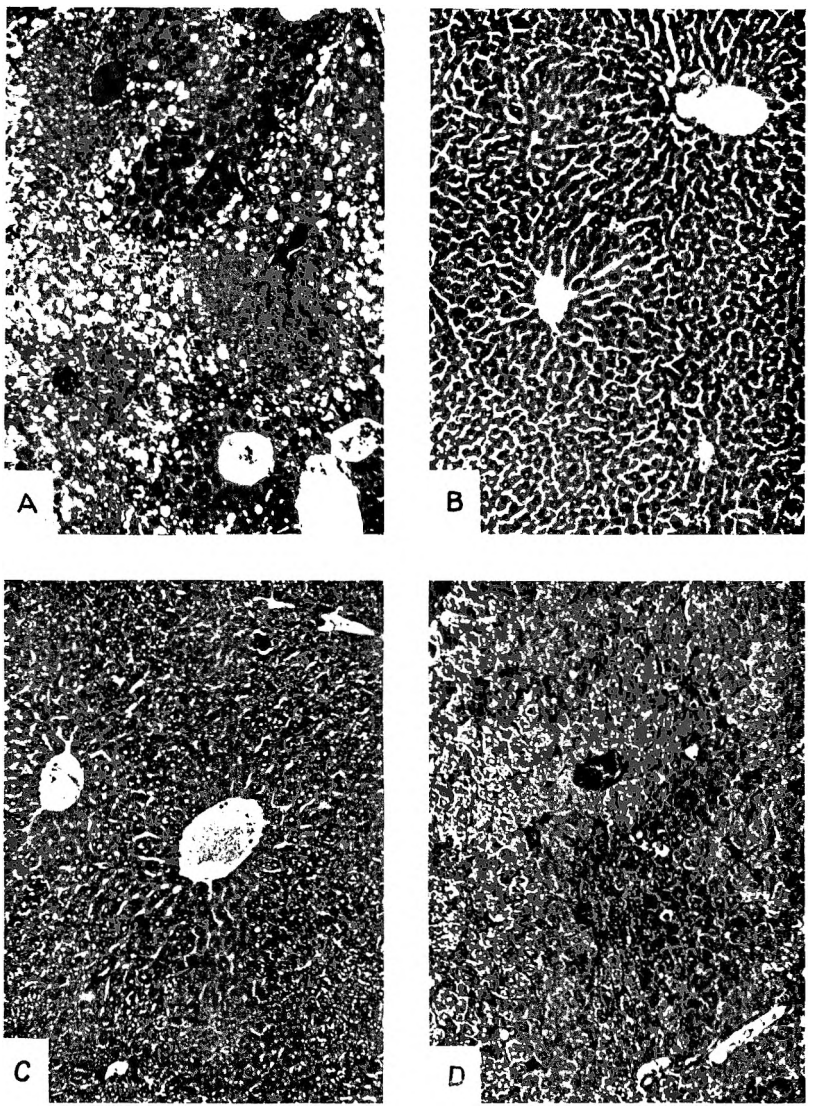

Fig. 1A Liver; H & E; ×100. Mouse fed 50% seed. Gross swelling of liver cells, fatty degeneration and some stromal collapse.

Fig. 1B Liver; H & E; × 100. Normal mouse.

Fig. 1C Liver; H & E; × 100. Mouse 24 hours on 50% seed. Swelling of liver cells with two small foci of liver cell necrosis.

Fig. 1D Liver; H & E; × 100. Mouse 48 hours on 50% seed. Gross swelling and degenerative changes in liver cells.

the diets offered, it was clear that seed, endosperm, and seed coat were all highly toxic, as was the heated seed. The fact that the heated seed was as toxic as the unheated indicates the presence of a heat-stable toxin.

(b) *Development of liver damage on 50% seed.* Mice were offered 4 gm per day of a mixture of 50% seed with meal. It was intended to kill two animals each 24 hours to watch the development of the liver damage. Mean weight at the beginning was 25 gm, with a mean daily weight loss of 0.8 gm per mouse. The mean daily food intake decreased from 2.0 to 0.5 gm. The maximum survival time was 7 days. After 5 days the surviving mice were obviously ill with eyes almost closed and very sensitive to light.

The liver from animals killed at 24 hours showed some generalized swelling of parenchymal cells, most marked at the periphery of the lobules, with an occasional necrotic cell present. The sinusoids appeared compressed by the swollen liver cords (fig. 1C).

By 48 hours, severe liver damage was obvious with swollen degenerate cells and cell necrosis, mainly periportally (fig. 1D). At 72 hours similar changes could be seen, with some inflammatory cell infiltration about groups of necrotic cells.

Changes on the 4th and 5th days were similar except that areas of cellular necrosis were now present in mid-zonal as well as periportal areas, and fatty degeneration appeared more obvious in the surviving cells (fig. 2A). Some areas of stromal collapse with early cell infiltration could be seen. Similar changes were seen in the animals killed on the 6th and 7th day, and none of the livers showed any appreciable evidence of cell regeneration.

(c) *Development of liver damage on 12.5% seed.* Mice were offered 4 gm daily of a mixture of 12.5% seed and meal. It was intended to kill animals at regular intervals but this was not always possible because some animals became moribund as indicated in table 2. The mean daily food intake per mouse on this diet remained relatively stable at 2.5 gm per day till just before death. It did not show the sharp drop seen

TABLE 2

Histological liver changes in mice fed a diet containing 12.5% I. endecaphylla seed for periods of 6 to 83 days

TIME FED DIET	STATE OF ANIMAL AT DEATH	MEAN WT./MOUSE		HISTOLOGICAL LIVER CHANGES				
		1st half feeding period	2nd half feeding period	Degenerative changes	Cell necrosis	Fatty changes	Regeneration	Fibrosis
days		*gm*	*gm*					
6	Moribund	24.0	24.0	++	+	0	+	0
14	Active	29.0	26.0	++	+	+	+	+
14	Active	23.3	20.5	++	+	+	0	+
21	Active	22.0	17.2	++	++	++	+	++
24	Moribund	22.7	20.5	++	++	+	+	+
26	Active	21.9	19.7	+++	++	++	+	++
28	Active	29.2	21.1	++++	++	+	++	+
29	Moribund	22.5	20.8	++	++	++	+	+
43	Moribund	19.1	16.4	++	+	0	+	0
44	Active	21.6	17.2	++	++	+	+	++
83	Moribund	19.4	18.4	++	+	+	+	+

with the 50% meal. Weight loss was not so pronounced as on the 50% seed. Nevertheless liver damage developed rapidly, one animal being moribund after 6 days. Table 2 shows the result of this experiment.

Though this experiment showed some individual variation of the animals to the diet (one animal being moribund at 6 days and another surviving 83 days) the average survival time

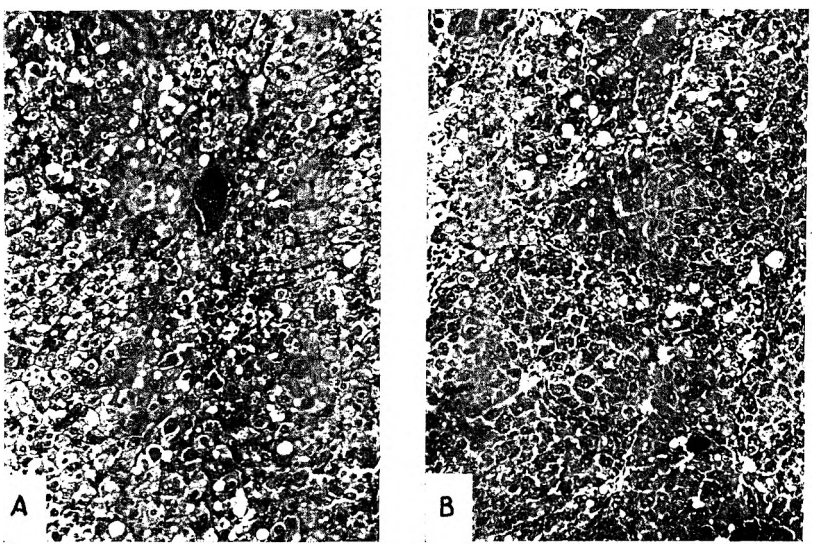

Fig. 2A Liver; H & E; × 100. Mouse 4 days on 50% seed. Marked degenerative changes with fatty infiltration and many necrotic cells.

Fig. 2B Liver; H & E; × 100. Mouse 26 days on 12.5% seed. Swollen liver cells in irregular nodules separated by connective tissue strands.

was of the order of 4 weeks. The development of the liver lesions was similar to that described with the 50% seed, though it progressed at a slower rate. In keeping with the longer survival periods, regenerative and fibrotic processes were more evident in this group than in the group fed 50% seed (fig. 2B).

Herbage

I. Endecaphylla herbage, whether green or dried, was unpalatable to mice and insufficient was eaten from diets con-

taining it to cause liver damage. For example, a diet containing 50% dried *I. endecaphylla* herbage ground up finely with meal was eaten at a rate of less than 1 gm per mouse per day and the animals eventually died of starvation. Histological examination failed to show evidence of liver damage. However a few areas containing fine fat droplets were present as is commonly found in animals dying of starvation.

However it was found that the dried herbage was less unpalatable if it were first extracted with chloroform, which removes the β-nitropropionic acid. A diet containing 36% of the chloroform-extracted residue was offered to 4 mice at the

TABLE 3

Result from feeding a diet containing 36% chloroform-extracted dried residue of I. endecaphylla herbage to 4 mice for 10 days

MEAN WT. MOUSE		HISTOLOGICAL LIVER CHANGES				
Initial	Final	Degeneration	Necrosis	Fatty change	Regeneration	Fibrosis
gm	*gm*					
32.5	21.0	+++	+	+++	0	+
30.0	21.5	++	+	+	0	+
33.0	23.0	+++	++	+	0	+
38.5	26.0	+++	++	++	0	++

rate of 4 gm per mouse per day. The approximate daily intake per mouse was only 1.2 gm and all the animals lost weight rapidly. After 10 days, two were moribund and the other two were listless, so all were killed. All 4 mice showed evidence of severe liver damage comparable to that obtained when diets containing 50% seed were fed. It was evident from the amount of liver necrosis that the changes were not due to the suboptimal food intake. Details of this experiment are set out in table 3.

An attempt was made to feed the chloroform-soluble extract to mice but this proved highly unpalatable even when mixed with meal in the ratio of 1:15. Mice offered this mixture died of starvation without any evidence of liver damage.

It seems, therefore, that the unpalatability of the whole herbage for mice is not soley due to its toxic principle.

DISCUSSION

It is clear that the mouse liver is susceptible to the toxic principle present in the seed of *I. endecaphylla* and that an assessment of the degree and type of liver damage shows a definite relationship with the amount of toxic material fed. The seed and herbage of *I. endecaphylla* will produce similar liver damage in the rabbit (Hutton et al., '58). Experiments with rabbits, mice, and sheep [2] leave little doubt that the liver is the main organ affected by the toxin of *I. endecaphylla,* confirming the reports of earlier investigators (Emmel and Ritchey, '41; Nordfeldt et al., '52). Therefore it appears that a study of the toxin or toxins of *I. endecaphylla* cannot logically be dissociated from a study of the histological liver damage produced in susceptible animals, as has been the case with the chick test described by Rosenberg and Zoebisch ('52) and used by Morris et al. ('54) and Cooke ('55).

The production of liver damage in mice by feeding *I. endecaphylla* has not been described previously. Though the seed is quite palatable to mice, green or dried herbage was quite unpalatable and mice refused to eat it. However, a chloroform-extracted residue of herbage was eaten in sufficient quantity to produce severe liver damage comparable to that produced by feeding seed.

From a large number of experiments we have found that mice survive only 6 to 13 days on a diet of 50% seed, and this gives a good indication of the toxicity of the seed samples, provided intercurrent diseases are excluded as causes of death by pathological examination. After 5 to 6 days on 50% seed, the eyes of the mice gradually close and appear very sensitive to light. In this regard it is interesting to note that bilateral corneal opacities occurred in our sheep experiments, progressing to corneal ulceration in severe cases.

[2] Unpublished data.

The seeds of *I. endecaphylla* fail to give a positive reaction for β-nitropropionic acid by Cooke's ('55) method. The chloroform extracted residue of the herbage is free from β-nitropropionic acid but retains its toxicity. For this and other reasons (Hutton et al., '58) it seems that β-nitropropionic acid is not the toxic principle present in *I. endecaphylla* as has been claimed by Morris et al. ('54) and Cooke ('55).

Further work is in progress to isolate and identify the toxic principle.

SUMMARY

1. Severe liver damage can be produced in mice by feeding the seed of *I. endecaphylla*. The toxicity of seed is not reduced by heating it strongly.

2. Similar liver damage can be produced by feeding the chloroform-extracted residue of the leaf which is free from β-nitropropionic acid.

3. Assessment of the degree and type of histological liver damage produced is a reliable index of the toxicity of the material fed.

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AVAILABILITY TO MAN OF AMINO ACIDS FROM FOODS

I. GENERAL METHODS [1]

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Minimum quantitative amino acid requirements of human subjects have been determined with diets containing mixtures of highly purified amino acids in place of protein (Rose et al., '55; Leverton et al., '56a, b, c, d, e; Jones et al., '55, '56; Swendseid et al., '56; Swendseid and Dunn, '56). It is conceivable that requirements established using diets of natural foods may differ from those determined using synthetic diets.

That amino acids from certain foods may be utilized less efficiently than purified amino acids has been indicated by the results of numerous animal studies (Schweigert and Guthneck, '54; Guthneck et al., '53; Gupta and Elvehjem, '57; Lushbough et al., '57). Since the ultimate goal of amino acid requirement studies is to make possible the translation of these requirements into quantitative terms relative to particular foods, the desirability of determining availability to man of individual amino acids from specific foods is apparent.

GENERAL CONSIDERATIONS

The investigation reported in this paper deals with an attempt to devise a method suitable for determining the

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availability of individual amino acids from specific foods. Since negative nitrogen balance results when the intake of any essential amino acid is insufficient to meet the body's needs, the minimum quantity of an amino acid which will support nitrogen equilibrium has been considered as the requirement. This method should also be applicable to the study of availability of amino acids in food. By determining the quantity of a purified and of a bound amino acid (protein-amino acid) needed for nitrogen equilibrium, the relative availability of the amino may be ascertained.

It is recognized that the validity of the proposed method depends upon whether or not subjects respond in the same manner to diets which provide amino acids only in purified form as they do to diets of approximately equal composition which provide a portion of the amino acids from food. A diet containing some natural food differs from a purified amino acid diet in two important ways; namely, (a) part of the amino acids are provided in bound form as they occur in the protein of the food and (b) certain nonessential amino acids are added by the protein unless all known amino acids are given in the purified amino acid diet.

The purpose of this investigation was to determine whether or not subjects do respond in like manner to the two types of diets described above. The amino acid composition of the basal diet to be used in making this comparison was an important consideration. Accordingly, two separate studies were conducted, using two basal diets which differed in their quantitative content of essential amino acids. Basal diet I contained generous quantities of purified essential amino acids so that the subjects were not dependent upon amino acids from the natural food to supply their minimum daily requirements. Basal diet II contained moderate amounts of the purified essential amino acids so that during the period when a natural food was supplied the minimum daily requirements were met by a combination of purified and bound amino acids.

The response to basal diet I should indicate whether or not nitrogen balance is influenced by the presence of an intact

protein in a diet containing quantities of purified amino acids sufficient to maintain nitrogen equilibrium. The response to basal diet II should indicate whether nitrogen balance can be supported when part of the required amino acids are provided in free form and part in bound form; i.e., whether or not an intact protein is digested and absorbed rapidly enough to be used for protein synthesis along with amino acids ingested in the free form.

STUDY I

Experimental

Experimental plan. Study I was of 30 days duration. A diet of natural food containing 9 gm of nitrogen was fed prior to the start of the experimental period. For the first 15 days of the experimental period, subjects were given the control diet, basal diet I; for the next 10 days, the wheat diet; and for the last 5 days, basal diet I.

Diets. Basal diet I consisted of a few low-nitrogen fruits, cornstarch, sucrose, butter fat, vegetable fat, jelly, a carbonated beverage and mineral and vitamin supplements, as well as purified amino acids and diammonium citrate (table 1). The 8 essential amino acids and cystine, tyrosine, arginine and histidine were given in amounts equivalent to those found in 20 gm of egg protein (table 2). The total nitrogen intake was raised to 9 gm daily by additions of glycine and diammonium citrate, each furnishing equivalent amounts of nitrogen.

The wheat diet was also semi-synthetic in nature and contained the same quantities of the 12 amino acids and of total nitrogen as basal diet I. The main difference in the two diets was that the 12 amino acids in the wheat replaced equal amounts of the 12 purified amino acids in basal diet I (table 2). Each subject was given daily 125 gm of all-purpose white, wheat flour which had been made into a shortbread.[2] The total

[2] Recipe: All-purpose wheat flour, 125 gm; butter fat, 60 gm; sugar, 75 gm; baking powder, 7.6 gm; mineral mix, 1.8 gm; sodium chloride, 2 gm; mucilose flakes, 5 gm. Mixed and baked in a 9-inch pan for 30 minutes at 325°F.

nitrogen intake from the two diets was equalized by adjusting the intake of glycine and diammonium citrate. One-fourth of the daily intake of amino acids and of additional sources of nitrogen was fed at breakfast and three-eighths each at lunch and dinner.

The Caloric intake was such that the subjects neither lost nor gained weight. At the beginning of the experimental

TABLE 1

Composition of basal diets

INGREDIENTS	BASAL DIET I	BASAL DIET II
	gm	*gm*
Orange juice	100	100
Applesauce	100	100
Peaches	100	100
Lemon juice	75	75
Baking powder [1]	7.6	7.6
Mineral mix [2]	3.6	3.6
Glycine	18.8	18.7
Diammonium citrate	28.4	28.3
Glutamic acid	—	19.2
Amino acid mix	14.9	8.4
Wafers [3]	One batch	One batch
Butter fat [4]		
Jelly [4]		
Sugar [4]		
Carbonated beverage [4]		
Decafeinated coffee [5]		
B-complex vitamins [6]	One capsule	One capsule
Vitamins A and D [7]	One capsule	One capsule

[1] Recipe for daily amount: 2.05 gm $NaHCO_3$; 2.86 gm $Ca(H_2PO_4)_2 \cdot H_2O$; 2.69 gm cornstarch.

[2] The mineral supplement of Leverton et al. ('56a) was used. One-half was incorporated into the wafers and one-half into the lemon juice.

[3] Recipe for daily amount: cornstarch, 100 gm; sugar, 40 gm; mucilose flakes, 4 gm; mineral mix, 1.8 gm; baking powder, 7.6 gm; fat, 40 gm; NaCl, 2.0 gm; water, 60 ml.

[4] Amount varied, depending on the Caloric requirement of individual.

[5] As desired.

[6] Each capsule contained the following: thiamine, 3.0 mg; riboflavin, 2.5 mg; nicotinamide, 20.0 mg; pyridoxine, 1.0 mg; pantothenic acid, 4.0 mg; ascorbic acid, 50 mg; vitamin B_{12}, 20 μg; folic acid, 100 μg.

[7] Each capsule contained 5000 I.U. vitamin A and 500 I.U. vitamin D.

TABLE 2

Amino acid composition of diets in study I and study II

AMINO ACIDS	STUDY I			STUDY II		
	Basal diet I	Wheat diet		Basal diet II	Corn diet	
	Purified amino acids	Purified amino acids	Amino acids in wheat	Purified amino acids	Purified amino acids	Amino acids in corn
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
L-Arginine·HCl	1.596	1.031	0.565	0.992	0.398	0.594
L-Histidine·HCl·H_2O	0.593	0.048	0.545	0.492	0.176	0.316
L-Lysine·HCl	1.749	1.348	0.401	1.434	1.138	0.296
L-Tryptophan	0.300	0.216	0.084	0.273	0.218	0.055
L-Phenylalanine	1.260	0.456	0.804	0.434	0.000	0.434
L-Methionine	0.800	0.542	0.258	0.419	0.189	0.230
L-Threonine	0.860	0.465	0.395	0.364	0.021	0.343
L-Leucine	1.840	0.728	1.112	1.462	0.000	1.462
L-Isoleucine	1.540 [1]	0.867	0.673	0.637	0.212	0.425
L-Valine	1.440	0.665	0.775	0.526	0.000	0.526
L-Tyrosine	0.900	0.300	0.600	0.900	0.226	0.674
L-Cystine	0.480	0.180	0.300	0.450	0.279	0.171

[1] Given as 3.080 gm of DL-isoleucine.

period, the subjects were given diets which provided 45 Cal./kg of body weight. When some of the subjects showed a tendency to lose weight, slight adjustments in Caloric intake were made. Except for the first 5 days, the Caloric intake of all subjects was constant throughout the experimental period and ranged from 45 to 47 Cal./kg of body weight. Care was taken to keep the Caloric intake of each subject the same on basal diet I and the wheat diet.

Subjects. Seven college women ranging in age from 17 to 19 years served as subjects. All subjects carried on normal activities and were considered in good health on the basis of a physician's examination. Pertinent information concerning the subjects is given in table 3.

Methods. Nitrogen analyses were made by the boric acid modification of the Kjeldahl method (Scales and Harrison, '20). Urine samples were preserved under toluene and were

TABLE 3

Vital statistics and Caloric intakes of subjects

CODE NO.	AGE	HEIGHT	WEIGHT	CALORIC INTAKE
	yrs.	*cm*	*kg*	*Cal./kg*
		Study I		
1	18	166	49	45
2	18	141	43	47
3	18	170	66	46
4	18	161	57	47
5	18	166	64	46
6	17	166	64	45
7	19	174	59	46
		Study II		
8	18	166	65	36 [1]
9	21	160	48	40 [1]
11	20	164	64	38 [1]
13	20	168	66	30 [1]
14	19	168	73	35 [1]
15	20	168	64	38 [1]

[1] Subjects were started on Caloric intakes comparable to those of study I; reductions were necessary to prevent weight gains. For the last 20 days of the study each subject received the Caloric intake indicated above.

analyzed daily. Fecal samples were collected in plastic containers and immediately frozen, and aliquots of 5-day fecal composites were analyzed. Carmine was used as a marker. The nitrogen content of all foods, amino acid mixes and additional sources of nitrogen was determined daily. Creatinine determinations were made on daily urine samples by the method of Folin ('14). Amino acid composition of the flour and corn was determined microbiologically by the method of Steele et al. ('49).

RESULTS

The mean daily nitrogen balance of each individual subject of study I is shown in table 4. During the first 5 days on basal diet I the mean nitrogen balance of all subjects was -0.35 gm. The mean nitrogen balances of the subjects improved as they continued on basal diet I, mean retentions of +0.12 gm and +0.28 gm being observed for the following two 5-day periods. During the next 10 days when the subjects were fed the wheat diet, the mean nitrogen retention increased to +0.74 gm daily. A return to basal diet I was accompanied by a slight decrease in nitrogen retention, the mean nitrogen balance being +0.44 gm daily.

It has been observed consistently in this laboratory that a marked increase in nitrogen excretion occurs when subjects are transferred from a diet of natural foods to a synthetic diet and that several days are required for complete adjustment to the synthetic regime. Thus, comparisons between basal diet I and the wheat diet were made during the last 15 days of the experimental period. The results of the "t" test (Snedecor, '56) indicate that the difference between the mean nitrogen balances on basal diet I and the wheat diet is not significant ($t = 2.13$).

STUDY II

Experimental

Experimental plan. Study II was of 55 days duration. During the first 5 days the subjects received a diet of natural foods containing 10 gm of nitrogen. For the next 10 days the

subjects were fed the control diet, basal diet II, in order for them to adjust to the synthetic regime (table 1). The remaining 40 days were considered the test period, and during this time 10-day periods on basal diet II were alternated with 10-day periods on the corn diet. One-half of the subjects ingested the corn diet during the first 10 days while the remainder of the subjects ingested basal diet II.

Diets. Basal diet II was the same as basal diet I with the following exceptions; (a) the quantities of the 12 amino acids given were less, and (b) glutamic acid, as well as glycine and diammonium citrate, was given as a source of nitrogen (table 1). Glutamic acid was included because it was believed it would make the amino acid mixture more palatable. The essential amino acids, with the exception of valine, were given in amounts approximately double the reported minimum requirements for young women (table 2). Valine was given in an amount tentatively reported to be the minimum requirement for young women (Leverton, '54). Since studies are being planned to determine the availability of valine from common foods, it seemed desirable to ascertain whether or not this amount of valine was sufficient to maintain nitrogen equilibrium for a relatively long period. The nitrogen content of this diet was raised to 10 gm daily by additions of glycine, diammonium citrate and glutamic acid. Of the nitrogen supplied by these three compounds, glycine and diammonium citrate each furnished 40% and glutamic acid 20%.

The corn diet was also a semi-synthetic one and contained the same amount of the 12 amino acids as basal diet II. The corn diet differed from basal diet II in that the 12 amino acids in the corn replaced equal quantities of the purified amino acids (table 2). The corn diet contained only 5 purified essential amino acids. The quantities of valine, leucine and phenylalanine in the corn equaled the quantities of these amino acids in basal diet II; the amount of threonine in the corn approached that given in the basal diet, and only an insignificant amount of this amino acid was given in purified form (21 mg per day). Each subject received 110 gm ground,

TABLE 4

Nitrogen balance data for subjects in study I

DIET	DAYS	MEAN NITROGEN BALANCE						
		Subjects						
		1	2	3	4	5	6	7
		gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*
Basal diet I	1–5	—0.04	—0.45	—0.67	—0.72	—0.37	0.19	—0.39
Basal diet I	6–10	0.77	0.12	—0.44	0.29	—0.05	—0.02	0.07
Basal diet I	11–15	0.72	0.24	—0.09	0.34	0.29	—0.35	0.84
Wheat diet	16–25	0.81	0.26	0.72	0.96	0.69	0.62	1.12
Basal diet I	26–30	0.82	0.31	0.71	—0.03	0.14	0.43	0.69

TABLE 5

Nitrogen balance data for subjects in study II

DIET	DAYS	MEAN NITROGEN BALANCE					
		Subjects					
		8	9	11	13	14	15
		gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*
Natural food	1–5	1.04	2.14	0.24	1.31	0.42	0.72
Basal diet II	6–15	—0.33	—0.52	—1.21	—0.09	—0.27	—0.59
Corn diet	16–35 [1]	—0.30	0.22	—0.25	0.27	—0.15	—0.27
Basal Diet II	16–35 [1]	0.01	—0.03	—0.26	0.04	—0.46	—0.54
Corn diet	36–55 [1]	—0.05	0.18	0.00	0.21	0.12	—0.15
Basal diet II	36–55 [1]	—0.30	0.05	—0.22	0.08	—0.27	—0.37

[1] One-half the subjects received the corn diet during the first 10 days of each period and basal diet II during the second 10 days; the other half of the subjects received the diets in reverse order.

degerminated corn each day. After adding sufficient water to obtain a desirable consistency, the cornmeal was steamed for 40 minutes and was served as a cereal. The total nitrogen content of the two diets was equalized by appropriate adjustments in glycine, diammonium citrate, and glutamic acid. The amino acids and other sources of nitrogen were distributed among the three meals as stated for study I.

When the subjects of study II were placed on the synthetic diet, they were given 45 Cal./kg body weight. Caloric adjustments were made as necessary to maintain body weight.

Subject. Six healthy women 18 to 21 years of age were subjects in study II (table 3).

Methods. The methods used were the same as those used in study I.

RESULTS

Table 5 shows the mean daily nitrogen balance of each subject in study II. The mean daily nitrogen balance of the subjects on the diet of natural food was +0.98 gm. Nitrogen excretion of all subjects increased markedly during the first 10 days on basal diet II. Apparently all subjects adjusted to the synthetic regime within 10 days since nitrogen excretion decreased and remained constant throughout the following 40-day test period. During the first 20 days of the test period the mean nitrogen balances of all subjects were –0.21 gm daily on basal diet II and —0.08 gm on the corn diet. During the last 20 days of the test period the corresponding mean nitrogen balances were —0.17 and +0.05 gm respectively. The corn diet resulted in a slight but consistent improvement in nitrogen balances. According to the results of the "t" test (Snedecor, '56) the data are inconclusive; the difference between the two diets appears to be statistically significant during the last 20 days of the test period ($t = 5.45$) but not for the first 20 days; i.e., days 16 to 35 ($t = 1.30$).

In contrast to the subjects of study I, all subjects tended to gain weight on the initial Caloric intake of 45 Cal./kg of body weight. It was necessary to make repeated reductions in

Calories for all subjects during the first 30 days on the semi-synthetic diets. No Caloric adjustments were necessary during the last 20 days of the study, at which time the Caloric intakes for individual subjects ranged from 30 to 40 Cal./kg of body weight.

DISCUSSION

The results of the present investigation indicate that availability of an amino acid in food can be determined by comparing nitrogen balances of subjects fed an equivalent amount of a bound and of a free amino acid. The demonstration that each of the basal diets supported nitrogen equilibrium and that the responses to the diets containing natural food were comparable to those of the corresponding basal diets suggests that the presence of a small amount of natural food in a semi-synthetic diet does not profoundly affect nitrogen balance. However, the slight improvements in nitrogen retention of subjects on the wheat and corn diets cannot be ignored. If the food does exert a real improvement, it seems the beneficial effects would be minute when small amounts of food are fed. The relative merits of an amino acid in food and of a purified amino acid can best be determined when a subject is given this amino acid in an amount somewhat below his requirement; negative nitrogen balance results when an essential amino acid is given in insufficient amounts to meet the body's needs, and the severity of the nitrogen loss increases as the amount of the amino acid available for use is decreased.

When the wheat diet was given in study I, it can be assumed that subjects were not dependent upon the wheat for their minimum requirement of any essential amino acid. However, the subjects on the corn diet in study II relied upon the corn for their entire supply of valine, phenylalanine and leucine and for most of the threonine. This situation is comparable to that which will be encountered in an availability study; the individual amino acid being studied should be given at several levels of intake so that each subject can be studied below as well as above his requirement. Increasing the level of the bound amino acid for which availability is being determined

results in an increase of all other amino acids in bound form and a decrease in purified amino acids. Decreasing the quantity of this amino acid has the opposite effect.

As long as amino acid requirements are met, the quantities of essential amino acids provided in the control diet may be varied appreciably. When the basal diet contains liberal quantities of purified amino acids, as in study I, a portion of these can be replaced by an intact protein without affecting nitrogen balance to any significant degree. Furthermore, study II shows that a combination of bound and of free amino acids is used efficiently, even in an extreme case when all of certain amino acids are ingested as bound amino acids and almost all of certain others as purified ones. Apparently the intact protein of corn is digested and absorbed rapidly enough to be metabolized along with free amino acids.

In order to maintain body weight, the subjects in study I required more Calories than those of study II. Expressed as Calories per kilogram of body weight, values for subjects on study I ranged from 45 to 47; whereas, on study II they varied from 30 to 40, with a mean of 36. In other words, subjects of study I required an average of 500 Cal. per day more than subjects of study II. Rose et al. ('54) compared Caloric requirements of subjects receiving 8 essential amino acids plus additional sources of nitrogen with the requirements of subjects fed all the amino acid components of casein. No differences between the Caloric requirements of these subjects could be detected; in both cases, considerably more Calories were required than on diets of natural casein. Nevertheless, it seems that differences in the composition of the basal diets used in the present studies may have influenced Caloric needs. The only two obvious differences between the diets were in the quantities of the essential amino acids and in the compounds providing additional nitrogen. Since the reason for higher Caloric requirement on synthetic diets is unknown, it is interesting to speculate as to whether or not the presence of relatively large amounts of glutamic acid in basal diet II reduced Caloric requirement.

SUMMARY

The replacement of a portion of the 12 amino acids in purified diets by those in wheat or in corn resulted in a slight improvement in nitrogen balance of human subjects.

Since this improvement was slight and of uncertain significance, the results of the present investigation indicate that availability of an amino acid in natural food can be determined for man by feeding in alternate periods an equivalent amount of a purified and of a bound amino acid in food. Either basal diet I, containing liberal quantities of essential amino acids, or basal diet II, containing moderate quantities of essential amino acids, appears suitable for use as a control diet in studying the availability of an amino acid in food.

It seems that man is able to use efficiently a combination of bound (protein-amino acids) and of purified amino acids even when all of certain amino acids, i.e., valine, leucine and phenylalanine, are ingested as bound amino acids from corn and when a major portion of certain others, i.e., lysine and tryptophan, are ingested in purified form.

Differences in the composition of the basal diet in the present investigation may have affected Caloric requirement.

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AVAILABILITY TO MAN OF AMINO ACIDS FROM FOODS

II. VALINE FROM CORN [1]

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Although information concerning the amino acid composition of foods is now readily available and minimum quantitative requirements for the essential amino acids have been reported for men (Rose et al., '55) and for women (Leverton et al., '56 a, b, c, d, e; Swendseid et al., '56; Swendseid and Dunn, '56; Jones et al., '55, '56), the best use of any given protein supply cannot be made until the relative availability of each essential amino acid in the protein is known. Analytical data of amino acid composition do not agree with data obtained from rat growth studies presumably due to variation in the availability of certain of these amino acids (Deshpande et al., '55). The existence of species differences makes it mandatory that the availability of these amino acids to man be investigated. This report deals with an attempt to determine the availability of valine in corn for human subjects using the method previously described (Linkswiler et al., '58).

EXPERIMENTAL

The experimental procedure was designed to measure the availability of valine in corn by feeding in alternate periods

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a basal diet containing purified valine and one containing the same amount of valine from corn. Availability of valine was determined from the nitrogen balance performance of the subjects. The experimental interval was 60 days in length, consisting of a 20-day adjustment period and a 40-day test period. During the adjustment period a natural diet was fed for 5 days and a semi-synthetic diet for 15 days in order to determine the ability of the subjects to maintain nitrogen equilibrium and to allow time for them to adjust to the dietary regime. During the test period the intake of valine was decreased stepwise until the subjects went into negative nitrogen balance, thus establishing the valine requirement of each subject. Since it is believed that a more valid comparison could be made between the diets when the subjects were definitely in negative nitrogen balance, intakes of valine somewhat below the required amounts were subsequently given. The method used in this study is based on the fact that when an amino acid is present in insufficient amounts to meet the body's needs, negative nitrogen balance results and that the severity of the nitrogen loss is increased as the amount of the amino acid available for use is decreased.

The subjects were given a semi-synthetic diet (basal diet) adequate in all known dietary essentials except valine. The initial level of this amino acid was slightly below that suggested as the minimum daily requirement by Leverton et al. ('56b). A 5-day period on the basal diet containing a given amount of purified valine was alternated with a 5-day period during which the same amount of valine was supplied by corn. If an intake of an essential amino acid is sufficiently low for a relatively long period of time, it is possible that the body may become depleted in this amino acid, resulting in an increased nitrogen loss as the experimental period progresses. In order to avoid any possible bias in interpretation, each time the amount of valine was decreased part of the subjects ingested the corn diet during the first 5 days and the basal diet during the last 5 days, while the remainder of the subjects were fed the diets in reverse order.

Subjects. Seven college women ranging in age from 19 to 24 years cooperated in this study. The subjects carried on their normal routine throughout the experiment. On the basis of a physician's examination all were considered to be in good health. Descriptive information concerning the subjects is presented in table 1.

Diets. A natural diet containing 10 gm of nitrogen was fed prior to the start of the experimental period in order to acquaint the subjects with the experimental setup and to allow more time for them to adjust to a definite nitrogen intake.

TABLE 1

Vital statistics and the Caloric intakes of each subject

SUBJECT	AGE	WEIGHT		HEIGHT	CALORIC RANGE
		Initial	Final		
		kg	*kg*	*cm*	
18	19	62.7	61.9	167.6	2483–2510
19	20	59.8	59.5	161.3	2193–2203
20	19	93.3	92.7	175.3	2735–2752
21	20	65.8	65.2	172.7	2462–2483
22	19	68.9	68.5	160.0	2666–2709
23	20	55.7	55.2	167.6	2194–2217
24	24	46.8	47.3	157.5	1872–1910

The basal diet consisted of a few fruits low in nitrogen, cornstarch, sucrose, especially prepared butterfat, vegetable fat, jelly, carbonated beverage, amino acids, additional sources of nitrogen and vitamin and mineral supplements (Linkswiler et al., '58). Coffee was allowed as desired, and the nitrogen content of the quantity used by individual subjects was determined. The amino acid composition of the basal diet is given in table 2. With the exception of valine, all essential amino acids were given in amounts approximately double those reported to be the minimum daily requirements for young women (Leverton, '54; Leverton et al., '55; Jones et al., '55; Swendseid and Dunn, '56). The leucine intake was slightly more than double the recent minimum requirement reported by Leverton et al. ('56e). Because of the high leucine

TABLE 2

The quantities and sources of amino acids given on the basal diet and on the corn diet at two levels of corn intake

AMINO ACIDS	BASAL DIET	CORN DIET			
		109 GM CORN		44 GM CORN	
	Purified amino acids	Amino acids in corn	Purified amino acids	Amino acids in corn	Purified amino acids
	gm/person/day	*gm/person/day*	*gm/person/day*	*gm/person/day*	*gm/person/day*
L-Lysine·HCl	1.434	0.282	1.152	0.114	1.320
L-Methionine	0.419	0.220	0.199	0.089	0.330
L-Tryptophan	0.273	0.052	0.221	0.021	0.252
L-Phenylalanine	0.434	0.413	0.021	0.167	0.267
L-Threonine	0.364	0.363	0.000	0.147	0.217
L-Leucine	1.462	1.390	0.072	0.561	0.901
L-Isoleucine	0.637 [1]	0.404	0.233	0.163	0.474
L-Valine	— [2]	0.500	0.000	0.200	0.000
L-Arginine·HCl	0.992	0.565	0.427	0.228	0.764
L-Histidine·HCl·H_2O	0.492	0.301	0.191	0.121	0.371
L-Cystine	0.450	0.163	0.287	0.066	0.384
L-Tyrosine	0.900	0.641	0.259	0.259	0.641

[1] Given as 1.274 gm of DL-isoleucine.

[2] Variable, 500 to 200 mg.

content of corn, this amount of leucine was given so that the amount of corn in the diet could be raised if necessary without changing the total amino acid intake. Arginine, histidine, cystine and tyrosine were also given. Glycine, diammonium citrate, and glutamic acid were added to bring the total nitrogen intake to 10 gm daily. Of the nitrogen supplied by these three compounds, glycine and diammonium citrate each furnished 40% and glutamic acid 20%.

The corn diet was also semi-synthetic in nature. The main difference between the two diets was that the 12 amino acids in the corn, as determined by microbiological assay in this laboratory, replaced equal amounts of purified amino acids, thus keeping constant the intake of the 12 amino acids (table 2). It is apparent that as the amount of corn was decreased during the experimental period, the ratios between the amino acids supplied by corn and by purified amino acids were altered; e.g., when 109 gm of corn were fed, all of the threonine given was present in the corn itself; whereas, when 44 gm of corn were given, 0.217 gm of threonine was in crystalline form. The total nitrogen intakes from the corn and basal diets were equalized by adjusting the intake of glycine, glutamic acid and diammonium citrate. The amounts of corn given in successive periods were 109, 98, 76, 65, and 44 gm and contained respectively 1.55, 1.39, 1.08, 0.92, and 0.63 gm of nitrogen and 500, 450, 350, 300 and 200 mg of valine. On the corn diet all valine was supplied by the corn except the 30 mg in the low-nitrogen foods which were constant throughout the experiment. The ground corn was served as a cereal and was prepared by steaming for 40 minutes.

One-fourth of the day's allowance of the amino acids and of the additional sources of nitrogen was fed at breakfast and three-eighths each at lunch and dinner. On the basal diet, low nitrogen foods furnished 0.20 gm of nitrogen, the 12 amino acids, 1.16 gm and the additional sources of nitrogen, 8.50 gm. The Caloric intake was sufficient to maintain body weight. A preliminary study indicated that most subjects required approximately 38 Cal. per kilogram of body weight, and at the

beginning of the experiment all subjects were given diets providing this many Calories. The Caloric intake of only two subjects needed to be adjusted and this occurred during the 15-day adjustment period. Care was taken to keep both diets of equal Caloric value throughout the test period.

Methods of assay. Nitrogen determinations were made daily on aliquots of 24-hour urine collections. Urine was collected under toluene and was kept refrigerated until the sample was complete, and analyses were made immediately upon completion of the 24-hour samples. Fecal samples were collected in plastic containers and frozen immediately. Carmine was used as an aid in marking fecal composites, and nitrogen analyses were made on aliquots of the 5-day composites. The nitrogen content of all foods, amino acids, and additional sources of nitrogen was determined daily. The boric acid modification of the Kjeldahl procedure was employed (Scales and Harrison, '20). Creatinine determinations were made on daily urine samples by the method of Folin ('14). Amino acid composition of the corn and the valine content of low-nitrogen foods were determined microbiologically by the method of Steele et al. ('49). Valine was determined using both *Leuconostoc mesenteroides* and *Leuconostoc citrovorum* as test organisms, and the respective values obtained were 4.6 and 4.5 mg of valine per gram of corn. Valine recoveries ranged from 98 to 101%.

RESULTS

The mean daily nitrogen balance of each subject observed on 5 different levels of valine during the 40-day test period is presented in figure 1. During the test period, 5 levels of valine were fed in the following order: 530, 480, 380, 330, and 230 mg. The valine intake included 30 mg daily supplied by the low-nitrogen foods present in the diet. The mean daily nitrogen balances for all subjects receiving purified valine were 0.0, +0.05, —0.20, —0.12, and —0.43 gm/day on respective intakes of 530, 480, 380, 330 and 230 mg. Corresponding mean nitrogen balances for subjects receiving equivalent amounts of

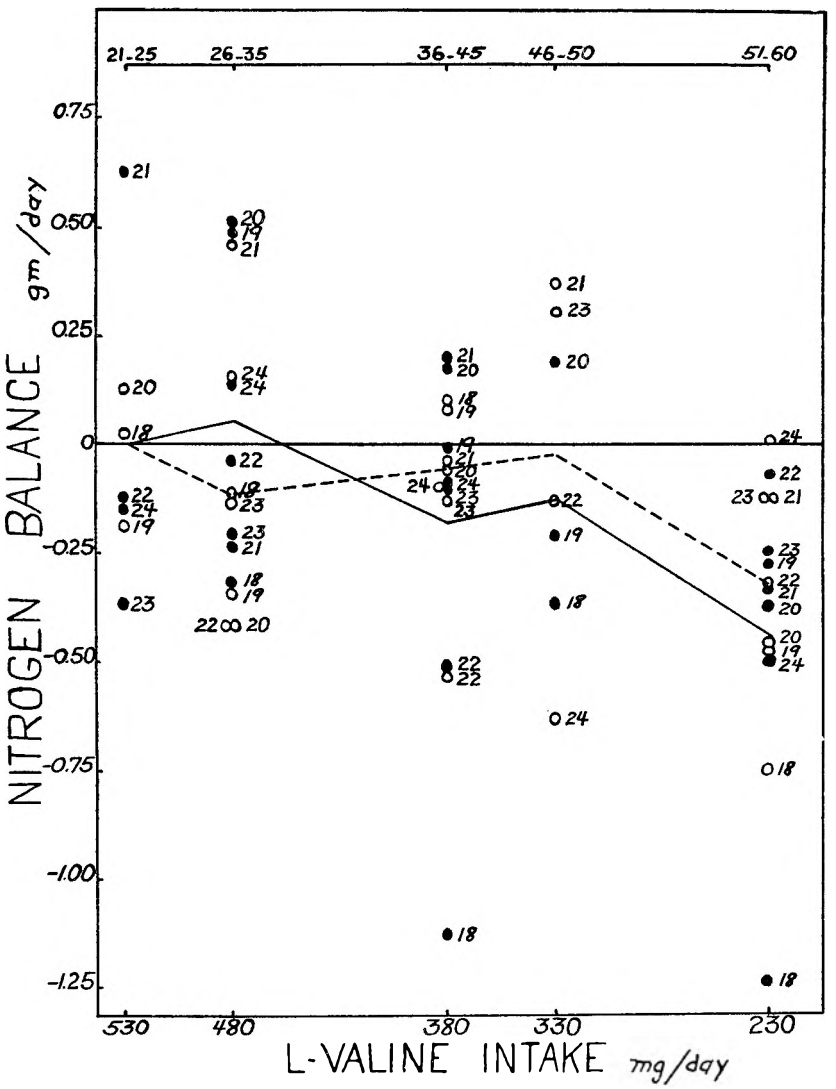

Fig. 1 Individual and average nitrogen balances of 7 subjects on varying intakes of valine. The numbers in the body of the graph refer to subjects. Individual nitrogen balances when valine was supplied by corn ○; or by crystalline valine ●. Curve 1: Average daily nitrogen balance when valine was given by corn — — — —; Curve 2: Average daily nitrogen balance when purified valine was given ————.

valine from corn were 0.0, —0.11, —0.09, —0.02, and —0.31 gm/day.

When 530 mg of valine per day were fed, all subjects, whether receiving valine from corn or purified valine, fell within the "zone of equilibrium," defined by Leverton et al. ('56a) as the state in which nitrogen output is within 95 to 105% of the nitrogen intake. When the valine content of the diet was decreased to 480 mg/day, all subjects remained in nitrogen equilibrium according to this concept. The three lowest levels of valine fed in this study appeared to be less satisfactory than the higher levels for maintenance of nitrogen equilibrium as evidenced by (1) an increased number of negative nitrogen balances as opposed to positive balances and (2) by the downward slope of the line connecting the mean nitrogen balances of all subjects.

At each level of valine intake where nitrogen balances fell outside the "zone of equilibrium," the difference between the performance of subjects receiving the two diets was tested for statistical significance (Snedecor, '56). Although the mean nitrogen balances of subjects receiving the corn diet appeared slightly higher than the mean nitrogen balances of subjects receiving the diet containing purified valine, the difference in performance on the basal diet and the corn diet was not significant at any level of valine intake ($t = 0.953$ at the 230 mg level). Therefore, it appears that the valine in steamed corn is as well utilized as the purified valine.

In certain subjects, variability in nitrogen retention was observed from one period to the next. Subject 22 lost more than 0.5 gm of nitrogen daily while consuming 380 mg of valine from either diet, but showed mean nitrogen balances of —0.14 (corn diet) and —0.31 gm (basal diet) at the 230 mg valine level. Subject 18 consistently lost less nitrogen on the corn diet than on the basal diet. For example, while ingesting 380 mg of valine, this subject showed nitrogen balances of —1.12 gm on the basal diet and +0.08 gm on the corn diet. In most cases, however, no consistent difference in nitrogen

balance performance of individual subjects between the two diets was readily apparent.

Figure 2 indicates that nitrogen from corn was readily absorbed. Mean fecal nitrogen ranged from 0.59 to 0.74 gm daily on the corn diet and from 0.57 to 0.70 gm on the basal diet. When these same subjects consumed a diet of natural foods, the fecal nitrogens averaged 1.2 gm daily, approximately double the values found on either of the two experimental diets.

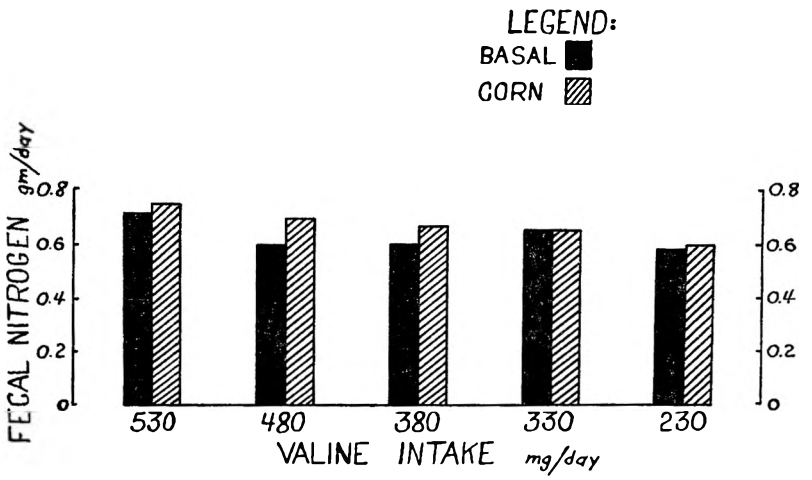

Fig. 2 Mean fecal nitrogen: basal vs. corn diet.

DISCUSSION

Although several investigators have determined utilization of certain amino acids in common foods for the rat, it is difficult to compare the results of these workers because different methods have been used to determine utilization. The utilization values reported for several foods ranged from 49 to 98% for lysine (Guthneck et al., '53), from 48 to 83% for methionine (Schweigert and Guthneck, '54), and from 75 to 132% for tryptophan (Lushbough et al., '57; Gupta and Elvehjem, '57) when growth or protein repletion studies were used to assess availability of an individual amino acid. The availability of all essential amino acids for the rat was esti-

mated simultaneously by determining the fecal excretion of these amino acids on different test foods (Kuiken and Lyman, '48). While the validity of this method has been questioned because of the possible influence of bacterial action in the intestinal tract upon the amino acid composition of fecal material, Gupta and Elvehjem ('57) obtained similar results when growth and fecal excretion of tryptophan were used as indices of availability.

Due to the possible existence of species differences, data obtained in availability studies on laboratory animals cannot be used for assessing amino acid utilization in man. Supplementation of rice with lysine and threonine improved its biological value for rats; however, attempts to improve the nutritional quality of rice for man by supplementation with these two amino acids gave inconclusive results (Hundley et al., '57). That valine from corn may not be equally available to man and the rat is indicated by a comparison of the results of the present study with those of Geiger et al. ('52) who reported that valine from zein was partially unavailable to the rat. In the present study valine in corn appeared to be completely available to the human subjects studied.

The recent demonstration in rat growth studies that the biological value of rice cannot be predicted from data on amino acid composition emphasizes the need for knowledge concerning availability for man of amino acids in cereal proteins. Supplementation of certain proteins with amino acids which appear to be limiting may result in a food of lower biological value than the original protein. Diets that are unbalanced with respect to certain amino acids have produced deleterious effects in animals (Elvehjem, '56). Hundley et al. ('57) inferred that the negative nitrogen balances observed when rice was enriched with certain amino acids may have been due to an amino acid imbalance. Supplementation of certain foods with the amino acid(s) which appears to limit the biological value of the protein has been proposed. The effects of fortifying particular foods with essential amino acids can be determined only by carefully planned investigations with

human subjects. Before supplementation of any foods with the apparently limiting amino acid(s) is begun, it is important to know the relative availability of each amino acid in the protein.

Although the present study was not concerned primarily with the determination of valine requirement, the experimental design used was such that requirement may be estimated. The question of the criterion to be used in assessing requirement is worthy of consideration. Rose has chosen as requirement that amount of amino acid which permits all subjects to attain a slightly positive nitrogen balance, whereas Leverton has suggested as requirement that amount of amino acid which allows no subject to excrete more than 105% of the nitrogen intake. According to the latter concept, all subjects in the present study were in nitrogen equilibrium on 480 mg of valine, an amount somewhat lower than the 650 mg minimum daily requirement suggested (Leverton et al., '56b). If the data are evaluated by Rose's interpretation, the subjects were not in equilibrium and the valine requirement exceeds 530 mg, the highest level given in this study. The minimum daily valine requirement for men as established by Rose et al. ('55) is 800 mg.

Nitrogen losses of the magnitude previously reported (Leverton et al., '56b) were not observed by the present investigators even though valine intakes were considerably less. In the former study, the mean nitrogen loss of subjects given 375 mg of valine was greater than 0.5 gm per day while mean losses on the present study were 0.20 (basal diet) and 0.09 gm (corn diet) for subjects receiving 380 mg of valine and 0.43 (basal diet) and 0.31 gm (corn diet) for subjects receiving 230 mg of valine. However, the amount of valine required to maintain nitrogen equilibrium in young women has been found to vary considerably and the slight differences in performance between the subjects on the two studies may be due entirely to individual differences since the number of subjects studied by each group of investigators was relatively small. It is also possible that differences in the diets may have affected the

results obtained. The composition of the diets used by the two groups of workers differed both in the content of essential amino acids and in the sources of additional nitrogen. Leverton and co-workers provided essential amino acids equivalent to those in 20 gm of egg protein and gave glycine as the source of additional nitrogen; whereas, in the present study essential amino acids were provided in quantities which were approximately double the reported requirements for women, and additional nitrogen was furnished by a mixture of glycine, diammonium citrate and glutamic acid.

Studies on amino acid requirement and utilization have been conducted under specific experimental conditions. Therefore, any requirement or utilization value reported is applicable only under those particular conditions. A better understanding of the complex interrelationships existing between nutrients will result from a thorough investigation of factors affecting amino acid requirement and utilization.

SUMMARY

The availability of valine in corn for human subjects has been determined by feeding in alternate periods a diet containing valine in purified form and one containing the same amount of valine in corn. Nitrogen balance performance of the subjects was compared on these two diets as the valine intake was decreased from 530 to 230 mg daily. From the data obtained in this experiment, it appears that the valine in corn is as well utilized as purified valine.

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THE EFFECT OF DIETARY FAT ON THE FATTY ACID COMPOSITION OF CHOLESTEROL ESTERS IN RAT LIVER[1,2]

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The possible influence of dietary fat on various phases of cholesterol metabolism has recently received much consideration. Reports of a relationship between the polyunsaturated fatty acid components of fat and cholesterol metabolism have been available since 1923 when Bloor ('23) found that the cholesterol in the plasma of various species of animals was associated with the highly unsaturated fatty acids. Later, Kelsey and Longenecker ('41) extended these observations in their report that the cholesterol esters of cow plasma contained approximately 62% of dienoic fatty acids.

The dependence of the type of fatty acids stored in various tissues of rats on the type of fat fed in the diet has been the

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subject of several reports. Nunn and Smedley-MacLean ('38) reported that the livers of fat-deficient rats contained no arachidonic or higher unsaturated fatty acids. Supplementation of the diet with essential fatty acids produced polyunsaturated fatty acids in the liver. Rieckehoff and coworkers ('49), however, found considerable amounts of polyunsaturated fatty acids in tissues of rats fed a fat-free diet. When these rats were supplemented with corn oil or cod-liver oil, increased quantities of tetraenoic, pentaenoic and hexaenoic fatty acids were deposited in tissue lipids, mainly as phospholipid fatty acids. When Sinclair and Chipman ('47) maintained rats on a diet rich in elaidin, the livers incorporated elaidic acid in the tissue lipids, with a larger amount in the cholesterol esters than in the triglycerides.

Recent studies from this laboratory (Alfin-Slater et al., '54) have emphasized the role of the essential fatty acids in the transport of cholesterol and in the regulation of normal cholesterol levels in liver and blood. Diets containing fats rich in essential fatty acids have maintained normal cholesterol levels in rats (Aftergood et al., '57). An investigation was undertaken to correlate cholesterol concentrations in liver with the fatty acid composition of liver cholesterol esters. In this report, the fatty acids present in combination with cholesterol in liver of rats maintained on diets containing fats with varying proportions of essential fatty acids have been characterized; and they have been compared with the fatty acids occuring in the dietary fat and the total lipid content and cholesterol concentration in the liver.

EXPERIMENTAL

Weanling male rats of the University of Southern California strain were divided into 6 groups of 15 animals each. These animals were maintained on the following experimental dietary regimens for 16 weeks: group I, a commercial Laboratory Chow;[5] group II, fat-free diet; group III, fat-free

[5] Purina.

diet; each rat is supplemented daily with 100 mg of linoleate; group IV, 15% cottonseed oil diet; group V, 15% lard diet and group VI, 30% hydrogenated coconut oil diet. The composition of the diets is shown in table 1.

In a separate experiment, rats maintained on a diet containing 15% of cottonseed oil for 14 weeks were fed a fat-free diet for one week and then sacrificed. Similarly, animals main-

TABLE 1

Composition of diets

COMPONENT	FAT-FREE	15% FAT	30% FAT
Lard or cottonseed oil	—	15.0	
Hydrogenated coconut oil [1]	—	—	30.0
Sucrose	71.53	52.53	31.46
Casein, vitamin test	20.0	24.0	28.0
Celluflour	4.0	4.0	5.0
Salt mixture [2]	4.0	4.0	5.0
Choline	0.24	0.24	0.28
Vitamin mixture [3]	0.19	0.19	0.22
Vitamins A, D, E [4]	0.04	0.04	0.04

[1] Kindly supplied by Vegetable Oil Products Co., Los Angeles, California.

[2] Wesson, '32, obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

[3] The vitamin mixture consisted of 38.57% *p*-amino-benzoic acid, 31.88% inositol, 12.75% ascorbic acid, 4.59% thiamine hydrochloride, 3.82% niacin, 3.82% Ca pantothenate, 1.72% riboflavin, 1.72% pyridoxine, 0.64% folic acid, 0.32% menadione, 0.16% biotin, and 0.00004% vitamin B_{12}; Merck and Co. and Nutritional Biochemicals Corp.

[4] The mixture contains 50 gm Nopsol, Nopco Chemical Co., Harrison, N. J. (100,000 U.S.P. units vitamin A and 20,000 U.S.P. units vitamin D per gram) and 225 gm mixed tocopherols (34%)

tained on a fat-free diet for 14 weeks were then given a diet containing 15% of cottonseed oil for one week, after which they were sacrificed.

At the end of the experimental period, the animals were sacrificed under anesthesia.[6] The livers of the rats were removed, and the lipids were extracted with Skellysolve B in a Waring Blendor according to the method of Thompson et al. ('49). All extracts were analyzed for total lipids gravi-

[6] Nembutal, Abbott.

metrically, and for cholesterol by a modified Sperry-Schoenheimer method reported by Nieft and Deuel ('49). Pooled lipid samples from livers of 4 rats were employed for the determination (in duplicate or, in some cases, in triplicate) of the fatty acid composition of the isolated cholesterol esters.

The cholesterol ester fraction of the liver lipids was separated on a silicic acid column by a modification, developed in this laboratory, of the procedure of Fillerup and Mead ('53). The lipids were applied in normal pentane to the column, pre-washed successively with 4 column volumes of methanol, acetone, ether and *n*-pentane; the cholesterol esters were recovered by removal of the solvent under reduced pressure on a water bath at 60°C and were hydrolyzed by refluxing with 0.1 N sodium ethoxide for 6 hours. The cholesterol which precipitated was separated from the sodium salts of the fatty acids by filtration. The solution was further extracted with a solvent mixture composed of chloroform, ether and Skellysolve B (1:3:6). The mixed fatty acids were subsequently extracted with Skellysolve A from the soap solution after acidification with 10% sulfuric acid. The extracts were washed free from acid, dried over anhydrous sodium sulfate, and the mixed fatty acids were recovered by removal of the solvent under reduced pressure at 60°C. The fatty acids were dissolved in chloroform, and suitable aliquots were used for determination of iodine number by the Wijs procedure (Wijs, 1898). The individual polyunsaturated fatty acids were determined spectrophotometrically as described in an earlier communication (Mukherjee et al., '57).

RESULTS AND DISCUSSION

Table 2 records the fat content of the diet, the amount of fatty acids ingested per rat per day (calculated on the basis of food consumption and the percentage of these acids in the dietary fat), the liver cholesterol concentration, and liver total lipid content of the various groups of rats at the end of the 16-week experimental period. It can be observed that an elevated total cholesterol level obtains in the liver of animals in the absence of fat from the diet (group II), and also

when fat with relatively small amounts of essential fatty acids and large amounts of saturated fatty acids are included in the diet (group V). The addtion of linoleate to rats fed a fat-free diet maintains a normal liver cholesterol concentration. Normal values are also obtained when the animals are fed the cottonseed oil diets. These data confirm previous observations reported by this laboratory concerning the dependence of normal liver cholesterol concentration on the presence of fat in the diet (Alfin-Slater et al., '54). The fact that hydrogenated coconut oil, containing only small amounts of unsaturated fatty acids, yields atypical results has been explained in an earlier paper (Deuel et al., '55). The possibility exists that short-chain fatty acids may esterify with cholesterol to maintain the normal cholesterol transport, although slight elevations in cholesterol concentration are present in this group (group VI) as well.

It is evident from table 3 that the fatty acid composition of the cholesterol esters in rat liver is very susceptible to variability depending on the presence or absence of fat in the diet, and on the nature of the fatty acids in the dietary fat. In the case of animals in group I, maintained on a commercial chow diet (receiving approximately 6% of dietary fat which contains 12.5% essential fatty acids, as determined spectrophotometrically), about 20% of the cholesterol is esterified with di- and polyunsaturated fatty acid. There is also a preponderant amount of monounsaturated fatty acid, 67%, and a rather low concentration, approximately 13%, of saturated fatty acid. When the animals are maintained on a fat-free diet (group II), there are very small amounts of di- and polyethenoid fatty acids present in combination with cholesterol, the principal unsaturated component being the monoethenoid acid. There is a marked decrease in the total unsaturation as is evidenced by the lower iodine number of the mixed fatty acids of the cholesterol esters; this is reflected by an increase in the saturated fatty acid component of the ester. This elevated saturated fatty acid content is accompanied by an increase in esterified cholesterol concentration and lipid content in the liver of these animals (table 2).

TABLE 2

The effect of the amount and kind of dietary fat on cholesterol and lipid content of the liver

	DIET [1]	FAT IN DIET	FATTY ACID INGESTED [2]			CHOLESTEROL IN LIVER [3]			TOTAL LIPID IN LIVER [3]
			S	M	P	Total	Free	Free	
		%	*mg/rat/day*			*mg/gm*	*mg/gm*	%	*mg/gm*
I	Commercial chow [4]	6	140	385	75	2.46 ± 0.03	2.04 ± 0.05	82.9	45.0 ± 4.6
II	Fat-free	0	0	0	0	4.39 ± 0.04	2.14 ± 0.04	48.7	57.9 ± 3.3
III	Fat-free + linoleate		0	0	100	2.69 ± 0.05	1.82 ± 0.09	67.6	54.6 ± 3.4
IV	Cottonseed oil	15	465	300	735	2.51 ± 0.10	1.83 ± 0.06	72.9	53.2 ± 4.2
V	Lard	15	768	837	255	3.25 ± 0.02	2.42 ± 0.09	74.5	58.4 ± 3.6
VI	Hydrogenated coconut oil	30	2910	90	—	2.87 ± 0.11	2.18 ± 0.10	76.0	55.0 ± 2.6

[1] Animals were fed the respective diets for 16 weeks except the group fed the lard diet where animals were sacrificed after 12 weeks.

[2] S = Saturated, M = Monoethenoid, and P = Polyethenoid.

[3] Including standard error of the mean.

[4] Purina.

TABLE 3

A comparison between the fatty acid composition of the dietary fat and the fatty composition of liver cholesterol esters

DIET	CATEGORY	IODINE VALUE	FATTY ACID COMPOSITION [1]				RATIO S : D
			S	M	D	P	
			%	%	%	%	
Commercial chow [2]	Dietary lipid	80	23.5	64	12.5	—	2 : 1
	Liver cholesterol esters	103	12.6	67	17.1	3.3	0.7 : 1
Fat-free	Dietary lipid	—	—	—	—	—	—
	Liver cholesterol esters	67	26.6	72.8	0.4	0.2	—
Fat-free + linoleate	Dietary lipid	181	—	—	100	—	—
	Liver cholesterol esters	100	19.3	58.4	18.1	4.2	1 : 1
Cottonseed oil	Dietary lipid	108	31.0	20.0	49.0	—	0.6 : 1
	Liver cholesterol esters	104	16.9	60.6	18.2	4.9	1 : 1
Lard	Dietary lipid	64	39.0	48.0	12.0	1.0	3 : 1
	Liver cholesterol esters	54	55.0	35.0	10.0		5.5 : 1
Hydrogenated coconut oil	Dietary lipid	2.5	97	3	—	—	32 : 1 [3]
	Liver cholesterol esters	26	71	29	—	—	3 : 1

[1] S = Saturated, M = Monoethenoid, D = Diethenoid, and P = Polyethenoid excluding diethenoid.

[2] Purina.

[3] Ratio S : M

When 100 mg of linoleate is administered daily to rats maintained on a fat-free diet (group III), the lipid picture undergoes several changes. In addition to the prevention of the fat-deficiency symptoms present in the animals of groups II, and the maintenance of the cholesterol and total lipid levels to within values exhibited by the control group (group I), the iodine number, as well as the composition of the fatty acids in combination with cholesterol, compare favorably with the control values. This has been observed previously in determinations on blood of rats fed similar diets (Mukherjee et al., '57).

Animals in group IV receiving a 15% cottonseed oil diet, containing approximately 10 times as much polyunsaturated fatty acids as the diet of group I, and 7 times as much polyunsaturated fatty acid as group III, also show no abnormalities so far as the cholesterol level and fatty acid composition of esterified cholesterol is concerned; approximately 20% of cholesterol is combined with essential fatty acids. The iodine number of the mixed fatty acids of the esters, 104, compares with the value of 103 obtained from the mixed fatty acids in group I.

When 15% of lard (containing more than three times as much linoleic acid as the commercial chow diet) is used in the diet (group V), however, in addition to the increased liver cholesterol content, a survey of the fatty acid composition of the cholesterol ester in the livers of animals of this group indicates that only 10% of the cholesterol is combined with diethenoid fatty acids (half that obtained in groups I, III and IV). It would seem, therefore, that the essential fatty acid content of the fat is not the sole regulatory factor, since, despite the ingestion of presumably adequate amounts of essential fatty acid, there is a large increase in the saturated fatty acid component of the ester commensurate with a large decrease in iodine number of the mixed fatty acids. However, this group of animals is receiving much higher amounts of saturated fatty acids than the rats in group IV receiving the cottonseed oil diet; the ratio of saturated fatty acid to es-

sential fatty acid in these two groups being respectively 3 : 1 for lard and 0.6 : 1 (or 3 : 5) for cottonseed oil. Thus, the ingestion of larger amounts of saturated acid present in lard results in the incorporation of higher amounts of saturated fatty acids in the cholesterol esters of the liver lipid of rats receiving the 15% lard diet.

This fact is further emphasized by the results obtained in group VI where the rats are maintained on a 30% hydrogenated coconut oil diet. These animals are receiving nearly 3,000 mg of saturated fatty acids and no polyunsaturated fatty acid; here, the cholesterol esterifies mainly with sat-

TABLE 4

The effect of change in diet on fatty acid composition of liver cholesterol esters after one week

DIET	IODINE VALUES	LIVER CHOLESTEROL ESTERS % FATTY ACIDS [1]			
		S	M	D	P
15% Cottonseed oil	104	16.9	60.6	18.2	4.9
15% Cottonseed oil followed by fat-free for one week	89	21.2	63.4	12.4	3.0
Fat-free	67	26.6	72.8	0.4	0.2
Fat-free followed by 15% cottonseed oil for one week	86	28.9	55.4	10.7	4.8

[1] S = Saturated, M = Monoethenoid, D = Diethenoid, and P = Polyethenoid.

urated fatty acids (70%). Ingestion of excessive amounts of saturated fatty acids has again resulted in an incorporation of increasingly higher amounts of saturated acids in the liver cholesterol esters. The iodine value of the liver fatty acids is 26; furthermore, monounsaturated fatty acids constitute 29% of all acids in the liver cholesterol esters but only 3% in the dietary fat.

In an attempt to determine the rate at which changes in the fatty acid content of the liver cholesterol esters occur as a result of changes in dietary regimes, groups of animals were maintained on either a fat-free diet or a diet containing 15% of cottonseed oil for a 14-week period, after which time the diets were reversed for one week. The results are

shown in table 4. When rats which have been maintained on a fat-free diet for a period of 14 weeks are given a 15% cottonseed oil diet for *only one week,* the iodine number and the diethenoid acid content of the mixed fatty acid of the liver cholesterol esters increase. The diethenoid acid concentration observed here is approximately half of that which occurs in the liver cholesterol esters of animals maintained regularly on the 15% cottonseed oil diet. The marked effect of essential fatty acids on the regulation of the fatty acid composition of cholesterol esters is once again apparent; the essential fatty acids in the cottonseed oil tend to restore the fatty acids of the cholesterol ester of rats on a fat-free diet towards a normal pattern. When the 15% cottonseed oil diet is replaced by a fat-free diet, the composition of the fatty acid of the cholesterol esters is considerably altered, there being the expected decrease in the diethenoid acid content and a decrease in total unsaturation of the mixed fatty acids. Since the depletion of the essential fatty acids from rat tissues ordinarily takes from 12 to 16 weeks, it is not surprising that livers of these animals still retain appreciable amounts of essential fatty acid even when they are maintained on a fat-free diet for a week.

SUMMARY

1. The differences in fatty acid composition of cholesterol esters in the liver of rats have been compared in adult rats fed from weaning on diets containing different dietary fats.

2. In diets which adequately maintain an animal, and which yield "normal" values for cholesterol concentrations in various tissues (e.g., a commercial chow,[5] a fat-free diet supplemented with linoleate, and a synthetic diet containing 15% of cottonseed oil) approximately 20% of the cholesterol in the liver occurs esterified with polyunsaturated fatty acids.

3. Animals fed a diet containing 15% of lard, which besides adequate amounts of essential fatty acids, contains larger amounts of saturated fatty acid, tend to accumulate

[5] See footnote 3, page 470.

cholesterol in the liver. Increased proportions of cholesterol are esterified with the more saturated fatty acids.

4. Diets containing highly saturated fats, such as hydrogenated coconut oil, result in the formation of large amounts of saturated fatty acid esters of cholesterol deposited in the liver. Approximately 70% of the cholesterol is combined with saturated fatty acid.

5. In the absence of fat from the diet, there is very little polyunsaturated or essential fatty acid found in the cholesterol esters in the rat liver. The essential fatty acid content of the liver cholesterol esters can be increased by supplementing the diet of animals with linoleate or cottonseed oil.

6. When a fat-free diet and a diet containing fat are interchanged for even short periods, considerable readjustment in the fatty acid composition of the liver cholesterol esters of the rat is observed.

7. The composition of the dietary fat exerts a marked influence on the fatty acid pattern exhibited by the cholesterol esters in the liver.

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RELATIONSHIP OF PROTEIN LEVEL TO THE MINIMUM LYSINE REQUIREMENT OF THE RAT [1]

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INTRODUCTION

Examination of the literature reveals that most investigators have found an increased requirement for various amino acids as the protein level of the diet is raised. Thus, Grau ('48) found an almost linear increase in lysine requirement of chicks as the protein content of the diet was raised from 5 to 30%. A similar observation was made by Almquist ('49) in tests with methionine. Twining et al. ('55) and Grau and Kamei ('50) also observed increased methionine requirements with increased protein levels, but in these instances a straight line relationship was not found. In the latter paper, Grau and Kamei ('50) also found non-linear increases in lysine requirement as the protein level was increased. Almquist and Merritt ('50), in chick studies, found a linear relationship between arginine requirement and protein levels in the range of 15 to 30% of protein.

In the pig, Brinegar et al. ('50) observed a lysine requirement of 0.6%, using a ration containing 10.6% of protein and

[1] Journal Paper no. 1185, Purdue Agricultural Experiment Station. The experimental data in this paper are taken from a thesis submitted by R. Bressani in partial fulfillment of the requirements for the Ph.D. degree in Biochemistry. A preliminary report appeared in Federation Proc., *15:* 544, 1956.

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a requirement of 1.2%, using a ration containing 22% of protein. More recently, Becker et al. ('57) obtained an isoleucine requirement of 0.46% at a 13.35% protein level and a requirement of 0.65 at a 26.70% protein level in the weanling pig. They assumed a linear relationship between dietary protein and isoleucine, and concluded that the isoleucine need (expressed as percentage of the protein) within the protein range studied could be expressed by the equation $Y = 4.5 - 0.076\ X$, where Y is the isoleucine need, and X is the percentage of dietary protein.

In the weanling rat, Forbes et al. ('55) concluded that the minimum amount of L-isoleucine required to promote maximum efficiency of protein utilization in diets containing 8.49 to 15.30% of crude protein as amino acids was 2.6% of the crude protein, irrespective of the total nitrogen content of the diet.

Almquist ('52) reviewed evidence to show that as the percentage of total protein in the diet of the chick increased from 10 to 40, the percentage of lysine required (expressed as percentage of the total protein) decreased from approximately 5.8 to 4.8, the total sulfur amino acids from approximately 4.5 to 3.3%.

In the experiments reported below, the weanling rat was used as the test animal, and lysine was the limiting amino acid.

EXPERIMENTAL

Preliminary tests showed that commercial oven-dried corn gluten, when adjusted to the essential amino acid composition of whole egg protein with purified amino acids, supported good growth in young white rats. This mixture was therefore used in these studies.

The corn gluten [3] was analyzed for nitrogen by the A.O.A.C. micro-Kjeldahl method ('50), and assayed for its content of 18 amino acids by the micro-biological method of Steele et al. ('49). The analytical data are shown in column 2, table 1.

[3] Kindly supplied by Dr. E. L. Powell, American Maize Co., Roby, Ind.

Column 3, table 1, shows the amounts of the essential amino acids in whole egg protein.

The rations tested contained 4, 8, 12, 16, 20, 24, 32 and 40% of crude protein and 3.75, 7.50, 11.25, 15.00, 18.75, 22.50, 30.00 and 37.50% of corn gluten, respectively (see columns 1 and 3, table 2). The levels of a mixture of purified essential amino acids which raised the "active" essential amino acid content

TABLE 1

Amino acid content of corn gluten

AMINO ACID	AMOUNT IN CORN GLUTEN [1] (DRY WT. BASIS)	AMOUNT IN WHOLE EGG PROTEIN [2]
	%	%
Alanine	5.23	..
Arginine	1.61	6.4
Aspartic acid	4.00	..
Cystine	0.60	2.4
Glutamic acid	14.20	..
Glycine	1.50	..
Histidine	0.87	2.1
Isoleucine	2.15	8.0
Leucine	10.09	9.2
Lysine	1.01	7.2
Methionine	1.08	4.1
Phenylalanine	4.01	6.3
Proline	5.82	..
Serine	3.30	..
Threonine	2.03	4.9
Tryptophan	0.21	1.5
Tyrosine	3.01	4.5
Valine	2.50	7.3

[1] Total nitrogen, 9.16%.
[2] Mitchell and Block ('46).

of the corn gluten (table 1) to that of an equal weight of whole egg protein (table 1) are shown in column 4, table 2. This mixture contained appropriate amounts of all of the "essential" amino acids except leucine and lysine. Leucine was supplied in adequate amounts by the corn gluten, and L-lysine monohydrochloride [4] was added separately as shown in column

[4] du Pont.

TABLE 2

Partial composition of rations [1]

PROTEIN	TOTAL LYSINE	CORN GLUTEN	AMINO ACID MIXTURE [2]	L-LYSINE·HCL ADDED	DIAMMONIUM CITRATE	CERELOSE
%	% × 10	%	%	%	%	%
4	1,2,3,4,5,6	3.75	1.57	0.09–0.74	0.81–0.00	47.78–47.94
8	2,3,4,5,6,8	7.50	3.13	0.17–0.96	1.43–0.43	41.77–41.98
12	4,6,7,8,10,12	11.25	4.63	0.38–1.43	1.45–0.16	36.29–36.53
16	5,7,8,9,11,13	15.00	6.21	0.46–1.52	2.26–0.97	30.07–30.30
20	7,8,9,10,11,12	18.75	7.72	0.67–1.33	2.66–1.86	24.20–24.34
24	5,7,8,9,10	22.50	9.24	0.69–2.01	3.15–1.53	18.42–18.72
32	6,8,10,12,14	30.00	12.33	0.40–1.45	5.33–4.04	5.96– 6.18
40	6,8,10,12,14,16	37.50	15.41	0.29–1.61	6.94–5.33	3.86– 4.15

[1] See text for other ingredients.

[2] L-Arginine·HCl, L-histidine·HCl·H_2O, DL-isoleucine, DL-methionine, L-phenylalanine, DL-threonine, DL-tryptophan, DL-valine.

5, table 2, to provide the "active" lysine levels listed in column 2, table 2.

L-Phenylalanine (see footnote 2, table 2) was added to compensate only for differences in levels of phenylalanine, and no L-tyrosine was added to raise the level of this amino acid to that in whole egg protein (table 1). DL-Methionine was considered completely "active," compensated only for differences in levels of methionine and no L-cystine was added. DL-Tryptophan was considered to be completely "active," and the D-components of DL-isoleucine, DL-threonine and DL-valine were considered completely inactive. Diammonium citrate (column 6, table 2) was added where required to raise the total nitrogen to the appropriate level of crude protein (column 1, table 2).

Inasmuch as Rosenberg and Culik ('55) showed changes in lysine requirement with changes in the energy content of the ration, the diets were formulated to be nearly isocaloric, containing approximately 410 kilocalories of gross energy per 100 gm.

The diets were supplemented with the vitamin mixture of Manna and Hauge ('53) plus 22 μg of crystalline vitamin B_{12} per 100 gm of food, and the mineral mixture of Heinemann et al. ('46).

All diets contained the following ingredients in constant percentages: cod liver oil 2.00, corn oil 7.00, celluflour 2.00, and mineral mixture 5.00. In all but the 40% protein diet, 30% of cerelose was also present. This cerelose contained the vitamin mixture which had been dried on it as an 80% alcohol solution. In the 40% protein ration, 20% cerelose, enriched with the vitamin mixture, was present. Other ingredients were added in the amounts shown in table 2. The total amount of cerelose in each ration was the sum of the amount indicated above and that shown in table 2.

Weanling albino rats from the department colony (Wistar strain) were used in the feeding tests. Six rats (three males and three females) weighing between 45 and 55 gm were selected for each lysine level. The rats were placed in individual cages in an air-conditioned room maintained at 25°C

and fed *ad libitum* for 28 days. Weight gains and food consumption were determined every 7 days. The feeding tests with the 24, 32, and 40% protein diets had to be repeated because all of the lysine levels selected were too high for estimation of minimum lysine requirements. The data from the first feeding tests at these three protein levels are therefore omitted.

RESULTS

At the 4% protein level, average individual total gains of the 6 rats at each lysine level were in the range of 0 to 4 gm for 28 days. Growth response was therefore inadequate at this protein level. Weight gains at higher protein levels are shown in table 3. Maximum weight gains were observed at the 16, 20, and 24% protein levels, suggesting that the minimum protein requirement is at 16% or between 12 and 16% on this type of diet. In table 3, the highest lysine increments

TABLE 3

Weight gains at different lysine and protein levels

LYSINE LEVELS, %	PROTEIN LEVELS, %						
	8	12	16	20	24	32	40
0.2	9[1]	...	...	...	...	...	...
0.3	26	...	...	...	...	...	...
0.4	*41*	49	...	...	...	...	...
0.5	*56*	...	61	...	52	...	...
0.6	63	*103*	...	...	...	*73*	*65*
0.7	..	*119*	111	*112*	*98*	...	...
0.8	59	119	*126*	*125*	*122*	*115*	*106*
0.9	..	...	*139*	133	130	...	...
1.0	..	122	...	132	134	115	120
1.1	..	...	130	136	...	...	...
1.2	..	121	...	138	...	120	129
1.3	..	...	137	...	...	...	...
1.4	..	...	...	...	...	117	121
1.6	..	...	...	...	...	...	127
(LSD—1%)	12.1	9.5	17.8	12.7	13.5	13.7	21.3
(LSD—5%)	8.9	7.0	13.1	9.4	9.9	10.1	15.7

[1] Average individual total gain, 6 rats, 28 days, in grams.

showing growth responses significant at either the 1% or 5% level are indicated for each protein level by the weight gains shown in italics.

With 8% of dietary protein, the highest lysine increment showing significant growth response was the increment between 0.4 and 0.5% of lysine. In order to predict the minimum lysine requirement, lysine levels (table 3) were plotted on the abscissa and average individual total gains on the ordinate of a graph (fig. 1). The point of intersection of

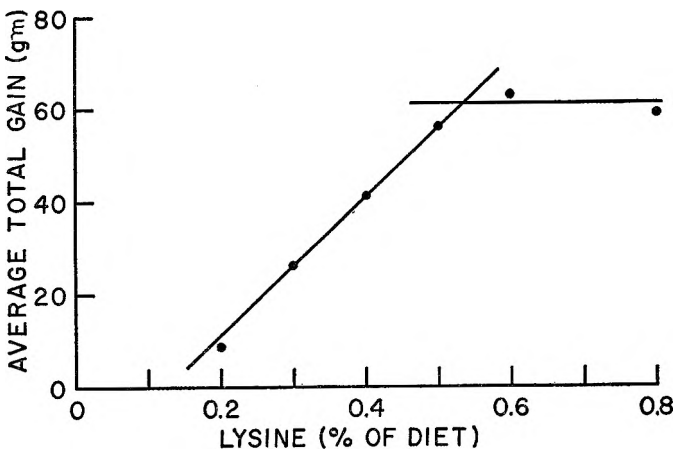

Fig. 1 Lysine requirement of rats receiving 8% protein diets.

the ascending and horizontal lines was assumed to be the minimum lysine requirement. The requirement value from this graph plot is 0.54% of lysine. This represents 6.75% of the crude protein content of the diet.

Similar graph plots based on the data in table 3 were constructed to determine the minimum lysine requirements at higher protein levels. The results are summarized in figure 2, where the percentage of protein in the diet is plotted against the minimum lysine requirement in percentage of the diet (as determined by graph plot) at each protein level. This curve shows that the lysine requirement increased rapidly in the

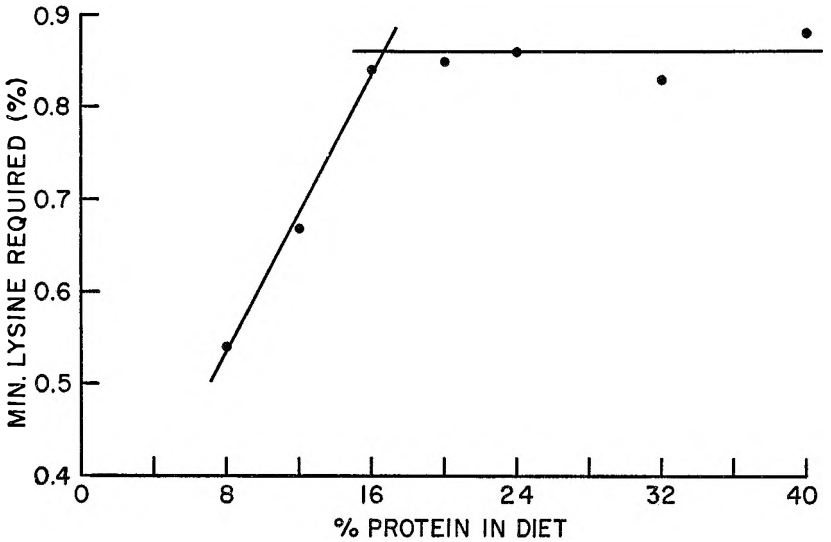

Fig. 2 Relationship of minimum lysine requirement expressed as a percentage of the diet to protein content of the diet.

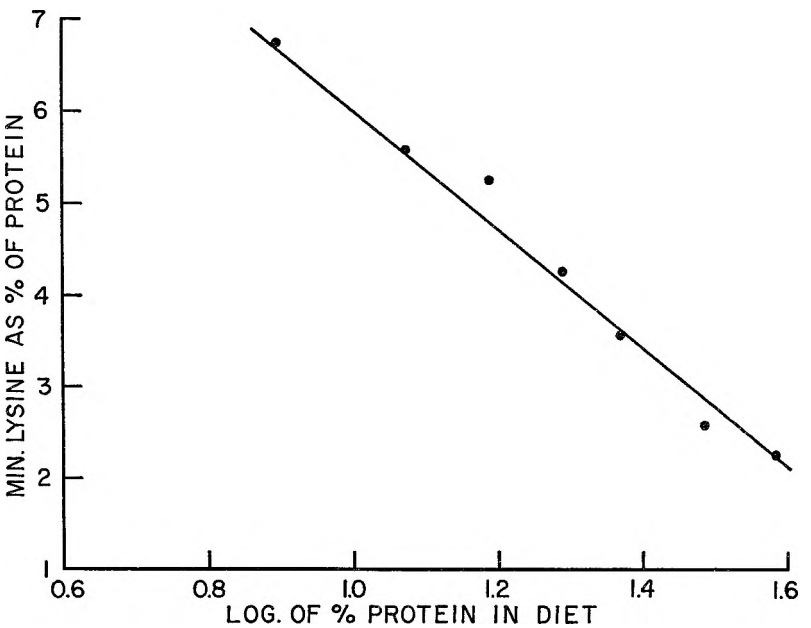

Figure 3

8 to 16% protein range, but remained essentially constant in the 16 to 40% protein range.

When the lysine requirement values (fig. 2) were expressed as percentage of the protein and plotted against the logarithm of the percentage of protein in the diet, a straight line with a negative slope was obtained (fig. 3). The relationship within the protein range studied can be expressed best by the equation $Y = 13 - 6.8 \log X$, where Y is the lysine need (as percentage of the protein) and X is the percentage of protein in the diet.

At all protein levels except the 4% level, food and protein efficiency values reached a maximum at or near the minimum lysine requirement value. Food efficiency values were at a maximum, and essentially constant (0.40–0.43 gm gained per gram of food consumed) at and above the minimum lysine requirement value in the range of 16 to 40% of protein.

DISCUSSION

As indicated in the literature review, most investigators have found a constant increase in requirement for various amino acids as the protein level of the diet is raised. In the present feeding tests, lysine requirement increased steadily with increases in protein level until the protein level in the diet was adequate for maximum weight gains. At adequate protein levels, the lysine requirement remained essentially constant.

In contrast to Becker et al. ('57) who found a linear relationship between isoleucine requirement and protein levels, our data suggest a linear relationship between lysine requirement and the logarithm of the protein level. In addition, our regression curve has a slope which is more steep.

It is possible that our experimental diet is an "ideal" diet for testing the relationship of lysine requirement to total protein level. The different protein levels were essentially isocaloric, thus avoiding the changes in lysine requirement due to changes in energy content of the diet as shown recently by Rosenberg ('57).

A second property of our experimental diet is its favorable balance of essential amino acids. The ratio of corn gluten to purified amino acids was adjusted so that the total mixture had the balance of essential amino acids found in whole egg protein. The deleterious effect of amino acid imbalances on gain and body composition of rats has been emphasized recently by Elvehjem ('56).

It is possible also that the rate of release of a limiting amino acid in the digestive tract may vary from one protein to another. In our studies, the bulk of the lysine was readily available.

The perfection of amino acid balance in a particular combination of amino acids, proteins, or proteins and amino acids, may perhaps be determined by plotting the lysine (or other amino acid) requirement against a wide range of protein levels, and checking the curve against that shown in figure 2.

The increased amino acid requirements noted with all increments of protein and amino acid combinations by other workers are undoubtedly valid; it is possible, however, that their diets represent deviations from the "ideal" combination of protein and amino acids.

SUMMARY

Weanling rats were fed isocaloric diets in which corn gluten supplied 54% of the nitrogen, purified amino acids approximately 32%, and diammonium citrate approximately 14%. Except for lysine, all diets contained the proportions of essential amino acids found in whole egg protein. Minimum lysine requirements were determined at 4% increments from 8 to 24%, and at 32 and 40% crude protein. The minimum lysine requirements expressed as a percentage of the diet remained essentially constant in the protein range of 16 to 40%. Minimum lysine requirements were (in per cent) 0.54, 0.67, 0.84, 0.85, 0.86, 0.83, and 0.90 with 8, 12, 16, 20, 24, 32, and 40% of total protein (N $\times$ 6.25), respectively. Expressed as a percentage of the total protein, the lysine requirements were 6.7, 5.6, 5.2, 4.2, 3.6, 2.6, and 2.2%, respectively.

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