AUGUST 10, 1959

THE JOURNAL OF NUTRITION

VOLUME 68 NUMBER 4

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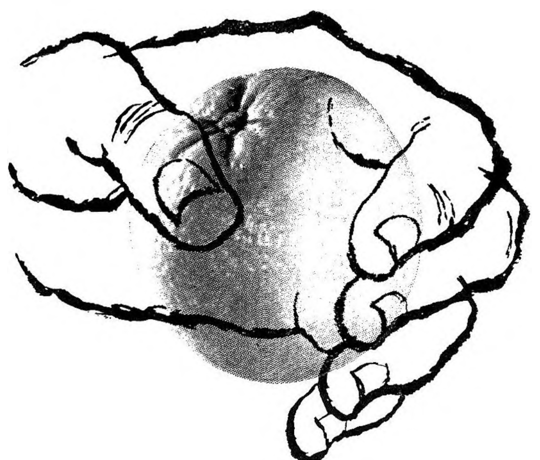

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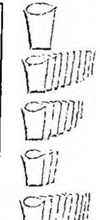

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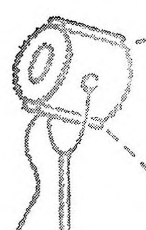

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EFFECTS OF FAT DEFICIENCY ON PITUITARY-GONAD RELATIONSHIPS [1]

T. C. PANOS,[2] G. F. KLEIN [3] AND J. C. FINERTY [4]
The University of Texas Medical Branch, Galveston

(Received for publication November 3, 1958)

INTRODUCTION

Fat deficiency was first shown to have an adverse effect on the reproductive ability of rats by Burr and Burr ('30), when they described irregular ovulation, decreased fertility and decreased sexual activity in these animals. Since that time, information has been accumulated to describe further the structural and functional changes induced by fat deficiency in the reproductive organs.

Evans, Lepkovsky and Murphy ('34a) reported abnormal gestation periods and increased abortion and neonatal death rate in fat-deficient female rats. In males (Evans et al., '34b), they found degenerating testes which histologically showed absence of spermatozoa and the presence of multinucleated giant cells. They and other authors (Maeder, '37) concluded that the changes induced were due primarily to a deficiency of the sex hormones. On the other hand, Greenberg and Ershoff ('51) concluded that the primary failure was in the production of pituitary gonadotrophins: following the use of chorionic

[1] This investigation was supported in part by a research grant (A-380) from the Institute for Arthritis and Metabolic Diseases, National Institutes of Health.

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gonadotrophin, they reported reversal of secondary sexual atrophy observed after 20 weeks of a fat-free diet.

In investigating the endocrine changes of fat deficiency, Panos and Finerty ('53, '54) found that "wheel nuclei," a sign of gonadotrophin insufficiency (Selye, Collip and Thomson, '33), appeared in the interstitial cells derived from the spindle cells of persistent thecae internae of atretic ovarian follicles of fat-deficient female rats. Different cell counts of the anterior pituitary glands showed an increase of basophiles and a decrease of acidophiles in the deficient animals. In male rats, changes described included atrophy and degeneration of the seminiferous tubules from which spermatozoa were absent, and the presence of multinucleated giant cells and vacuoles in the tubules. However, no significant decrease in the size of the sex accessory organs was evident.

Changes in the reproductive system of animals fed diets deficient in essential substances other than fat are of interest, and in many instances resemble the changes of fat-deficiency. These include testicular degeneration in vitamin A and E deficiencies and inanition (Mason, '33; Siperstein, '20) in pyridoxine deficiency (Emerson and Evans, '40) and in biotin deficiency (Katsch et al., '55). Also, decreased activity of sex accessory organs has been reported in deficiencies of vitamin A (Mayer and Goddard, '51), vitamins B (Moore and Samuels, '31), biotin (Katsch et al., '55) and in starvation (Pazos and Huggins, '45). In addition, an increased gonadotrophic activity of the hypophysis has been found by assay in several deficiency states. These include starvation (Rinaldini, '49), deficiencies of vitamin E (Nelson, '33; P' An et al., '49) and vitamin B_6 (Wooten et al., '55) and also in fat deficiency (Klein, '56). Cellular changes in the anterior hypophysis resembling those appearing after castration have been reported, especially in vitamin E deficiency (Nelson, '32; Koneff, '39), while increased percentage of basophiles has been reported also in inanition (Pearse and Rinaldini, '50) as well as in fat deficiency.

The findings mentioned above illustrate the multiplicity of factors which may be responsible for reproductive deficiency. Inanition could result in general anabolic inadequacy as well as in specific metabolic defects. Specific nutritional deficiencies, on the other hand, may lead to primary gonadal dysfunction due to lack of essential materials or to failure of the hypophysis to secrete or release gonadotrophins, causing degeneration secondarily of gonads and accessory organs. Primary gonadal failure may reside either in the failure of the Leydig cells to secrete androgenic hormone (with subsequent tubular atrophy) or in defective spermatogenesis, leading to tubular degeneration without affecting the interstitial cells.

Mason ('33) concluded that testicular atrophies of vitamin A and E deficiencies were not secondary to a deficiency of pituitary hormones because of histological differences between the testicular degeneration after hypophysectomy and that induced by A or E deficiency, as well as on the basis of a failure of injections of pregnancy urine and daily pituitary implants to effect testis repair.

The present experiment was designed to provide information concerning these problems in relation to fat deficiency, as well as to describe the reproductive system changes in more detail, with special reference to time of development.

METHODS AND MATERIALS

Maintenance of animals. Two-hundred fifty male albino rats of the Holtzman strain were obtained at weaning for these experiments. They were grouped according to diet as follows: (1) "Fat-free" group — maintained on a diet completely free of fat, allowed to eat ad libitum. (2) "Fat-free + fat, isocaloric" group — maintained on a diet identical in composition to the fat-free diet, but with fat substituted for sucrose to provide 30% of the calories and limited to a daily caloric intake equal to that of the "fat-free" group. (3) "Fat-free + fat, ad lib." group — maintained on the "fat-free + fat" diet but allowed to eat ad libitum. (4) "Fat-

free + fat, equal weight'' group — maintained on the ''fat'' diet, but with intake limited to maintain body weight equal to that of the ''fat-free'' group. (5) ''Chow'' group — maintained ad libitum on laboratory chow,[5] approximately 6% fat by weight. Compositions of the ''fat-free'' and the ''+ fat'' diets are shown in table 1. Each of the dietary groups was established with a definite control purpose in mind. The ''isocaloric'' group was maintained to eliminate differences due strictly to variations of caloric intake, the ''equal weight'' group to detect the effects due to starvation, and the ad libitum group as an optimal nutrition group. The ''chows'' were maintained as an accepted ''normal'' diet control.

TABLE 1

Composition of diets

COMPONENT	% OF WEIGHT		% OF CALORIES		CALORIES/KG	
	Fat-free	+ fat	Fat-free	+ fat	Fat-free	+ fat
Sucrose	75.2	55.7	80	50	3008	2230
Casein	18.0	21.4	20 (casein + cystine)	20 (casein + cystine)	720	855
Cystine	0.3	0.4			12	14
Choline	0.4	0.5	—	—	—	—
Fat	0.0	14.8	0	30	0	1333
a-Cellulose	2.0	2.4	—	—	3740	4432
Inositol	0.1	0.1	—	—	Caloric equivalent: 1 gm of fat-free food = app. 0.84 gm of ''+ fat'' food.	
Salt mixture	4.0	4.7	—	—		

Vitamins were added in the following amounts per kilogram of food: thiamine chloride 5, riboflavin 10, pyridoxine 5, calcium pantothenate 50, nicotinic acid 25, para-aminobenzoic acid 10, biotin 2, inositol 1,000, folic acid 2, vitamin B_{12} with mannitol 1.8, menadione 50, crystalline vitamin A acetate 2, vitamin D_2[6] 20 and alpha-tocopherol[7] 100 mg. The last three vitamins were added after being dissolved in absolute alcohol; this mixture was made up in small amounts

[5] Purina Laboratory Chow.

[6] Drisdol ®.

[7] Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

at frequent intervals and was stored in the freezer. With an average daily food consumption of 60 Cal. (about 15 gm), the daily intake of vitamins A, D and E was 32 μg (100 units), 0.3 mg (3 units) and 1.6 mg respectively.

Injection of hormones and autopsy. Preliminary observations of the condition of the testes were made after 9, 12 and 15 weeks on the diets. This was done by sampling the "fat-free" and "+fat, ad lib." groups by autopsy at these times. Eight animals from each group were killed and examined, with removal and weighing of pituitary glands, testes, prostates and seminal vesicles, all of which were prepared for microscopic examination.

After 15 weeks on the diets, some evidence of testicular degeneration began to appear in the "fat-free" group. To determine the effects of hormones on this degeneration, three groups of 8 "fat-free" animals were injected, beginning at 18 weeks, with one of the following preparations: (a) Testosterone,[8] 100 μg, (b) Chorionic gonadotrophin (CG),[9] two units or (c) 0.6 mg of pituitary tissue in saline (PE), prepared by mashing and drying fresh glands as described by Kupperman et al. ('41). Chorionic gonadotrophin was used for its luteinizing properties and pituitary extract was used because most of its activity is expressed as FSH. Injections were given subcutaneously daily for two weeks, at the end of which time (20 weeks on the diets) the injected "fat-free" animals and an equal number of uninjected animals from all control groups were killed and examined.

This same series of injections was then begun in remaining animals of all except the "chow" group, and was carried on for three weeks. At the end of this time (23 weeks on the diets) all animals, injected and uninjected, were autopsied. Testes, prostates and seminal vesicles were fixed in Bouin's solution and stained with hematoxylin and eosin. Pituitary glands were fixed in formol-saline and stained with PAS-Methyl blue technique (Rennels, '57).

[8] Testosterone, U.S.P.

[9] Antuitrin "S"; Parke, Davis.

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RESULTS AND DISCUSSION

Analysis of results obtained involves consideration of both gross and microscopic observations and correlation of these with time and dietary factors.

Autopsy data

Weight data are expressed in the tables both as absolute values and as relative values (mg/100 gm body weight). This is done in an effort to detect those differences which are due simply to variation in body size.

Analysis of data obtained from serial autopsies (table 2) reveals the following effects of a fat-free diet: (1) Body weights are significantly lower in the "fat-free" group, an effect which has been demonstrated as early as two weeks on the diet (Panos and Finerty, '54) and which can be related to the increased metabolic rate induced by fat deficiency (Panos, Finerty and Wall, '56). (2) In the control group, whereas the body weight continues to increase after the 9th week, the absolute weight of the testes remains the same; in the fat-deficient animals, the body weight increases slightly after the 9th week, but testicular weight progressively decreases, especially after the 20th week. On a relative basis, the progressive decrease in testicular size in fat-deficient animals is masked by the sharply decelerated body growth, so that the testes are relatively heavier than in controls until 23 weeks on the diet. Thus, testicular weight is maintained through the 9th dietary week in spite of failure in normal rate of body weight gain. After 9 weeks, progressive testicular failure and degeneration occurs without relation to body weight, suggesting a progressive deficiency of some requirement essential for testicular growth or maintenance. (3) The prostate glands of the "fat-free" group appear to be small throughout the test period and, in contrast to the testes, apparently are correlated with body weight, since on a relative basis there are no significant differences from those of the control groups. In other words, the weight of

TABLE 2

Serial autopsies

WEEKS ON DIET	9th		12th		15th		20th		23rd	
GROUPS (8 ANIMALS EACH)	FF	FF + FAT	FF	FF + FAT	FF	FF + FAT	FF	FF + FAT	FF	FF + FAT
Absolute wts.										
Body, gm	268	348	293	376	285	383	292	426	300	430
S.D.[1]	± 24	± 20	± 21	± 18	± 4	± 36	± 17	± 26	± 22	± 27
P	< 0.05		< 0.05		< 0.05		< 0.05		< 0.05	
Testes, mg	2906	3039	2785	3099	2693	3027	2610	3155	2380	3069
S.D.	± 177	± 105	± 147	± 177	± 141	± 252	± 433	± 201	± 476	± 104
P	> 0.05		< 0.05		< 0.05		< 0.05		< 0.05	
Prostate, mg	321	409	413	477	389	621	431	631	310	649
S.D.	± 61	± 65	± 72	± 86	± 68	± 136	± 97	± 111	± 84	± 233
P	< 0.05		> 0.05		< 0.05		< 0.05		< 0.05	
Sem. ves., mg	334	346	318	303	270	352	357	354	287	325
S.D.	± 40	± 35	± 68	± 44	± 39	± 66	± 82	± 41	± 49	± 25
P	> 0.05		> 0.05		< 0.05		> 0.05		> 0.05	
Relative wts.										
	mg/100 gm		*mg/100 gm*		*mg/100 gm*		*mg/100 gm*		*mg/100 gm*	
Testes	1085	873	952	825	945	788	894	742	792	715
S.D.	± 68	± 67	± 86	± 63	± 106	± 53	± 132	± 83	± 153	± 67
P	< 0.05		< 0.05		< 0.05		0.02		> 0.05	
Prostate	119	105	141	126	137	161	146	149	104	151
S.D.	± 18	± 57	± 18	± 21	± 20	± 30	± 25	± 30	± 84	± 35
P	> 0.05		> 0.05		> 0.05		> 0.05		> 0.05	
Sem. ves.	124	99	109	81	95	92	123	83	95	76
S.D.	± 15	± 13	± 27	± 26	± 15	± 16	± 32	± 11	± 17	± 10
P	< 0.05		0.05		> 0.05		< 0.05		> 0.05	

[1] Standard deviation.

the prostate is related more to growth than to age or testicular size, and suggests a decreased androgen production coincident with impaired body growth. (4) The seminal vesicles of the two groups appear to be about equal in absolute size, but relatively larger in the "fat-free" than in the control groups. This is in contrast to the prostates which

TABLE 3

Weights in uninjected control groups at 20-week autopsy [1]

GROUPS	BODY	TESTES	PROSTATE	SEMINAL VESICLES
Absolute wts.	*gm*	*mg*	*mg*	*mg*
Fat-free	292 ± 17 [2]	2610 ± 433	431 ± 97	357 ± 82
Chow	441 ± 29	3697 ± 116	855 ± 140	735 ± 53
P	< 0.05 [3]	< 0.05 [3]	< 0.05 [3]	< 0.05 [3]
Ad libitum	426 ± 26	3155 ± 201	631 ± 111	354 ± 41
P	< 0.05 [3]	< 0.05 [3]	< 0.05 [3]	> 0.05
Isocaloric	402 ± 22	3247 ± 32	528 ± 97	342 ± 16
P	< 0.05 [3]	< 0.05 [3]	< 0.05 [3]	> 0.05
Eq. wt.	322 ± 22	3358 ± 118	518 ± 101	299 ± 32
P	< 0.02 [3]	< 0.05 [3]	> 0.05	> 0.05
Relative wts.		*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
Fat-free	—	894 ± 132	146 ± 25	123 ± 32
Chow	—	841 ± 85	198 ± 32	172 ± 9
P		> 0.05	0.05 [3]	0.05 [3]
Ad libitum	—	742 ± 83	149 ± 30	83 ± 11
P		0.05 [3]	> 0.05	0.05 [3]
Isocaloric	—	814 ± 68	131 ± 21	85 ± 9
P		> 0.05	> 0.05	0.05 [3]
Equal wt.	—	1047 ± 160	160 ± 26	93 ± 2
P		> 0.05	> 0.05	> 0.05

[1] Eight animals per group.
[2] Standard deviation.
[3] Significant differences.

are considered to be more accurately measurable (Loraine, '50).

In table 3 are presented data of all groups after 20 weeks. As expected, body weight and size of testes and accessories are greater in control groups than in the "fat-free" group. Two other points to be noted are: the "chow" group shows apparently greater body weight and larger testes, prostates and seminal vesicles than do the other control groups; and

there is no significant difference between accessory organs of the "fat-free" and "equal weight" groups. Also, the body weight average of the animals from the "equal weight" group is seen to be slightly greater than that of the "fat-free" group, a technical fault which is felt not to affect the value of the group as starvation controls.

TABLE 4

Weights in uninjected control groups at 23-week autopsy[1]

GROUPS	BODY	TESTES	PROSTATE	SEMINAL VESICLES
Absolute wts.	*gm*	*mg*	*mg*	*mg*
Fat free	300 ± 22[2]	2380 ± 476	310 ± 84	287 ± 49
Chow	450 ± 17	3417 ± 285	593 ± 62	374 ± 29
P	< 0.05[3]	< 0.05[3]	< 0.05[3]	< 0.05[3]
Ad libitum	430 ± 27	3069 ± 104	649 ± 233	325 ± 25
P	< 0.05[3]	< 0.05[3]	< 0.05[3]	> 0.05
Isocaloric	409 ± 13	3366 ± 169	531 ± 92	345 ± 54
P	< 0.05[3]	< 0.05[3]	< 0.05[3]	> 0.05
Equal wt.	334 ± 7	2861 ± 196	366 ± 91	257 ± 48
P	< 0.05[3]	< 0.05[3]	> 0.05[3]	> 0.05
Relative wts.		*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
Fat-free	—	792 ± 153	104 ± 84	95 ± 17
Chow	—	760 ± 64	132 ± 17	83 ± 9
P		> 0.05	> 0.05	> 0.05
Ad libitum	—	715 ± 67	151 ± 35	76 ± 10
P		> 0.05	> 0.05	> 0.05
Isocaloric	—	825 ± 40	129 ± 20	84 ± 14
P		> 0.05	> 0.05	> 0.05
Equal wt.	—	865 ± 75	110 ± 32	79 ± 16
P		> 0.05	> 0.05	> 0.05

[1] Eight animals per group.
[2] Standard deviation.
[3] Significant differences.

Autopsy of uninjected control groups at 23 weeks (table 4) showed the same findings as at 20 weeks, except that the difference between the "chow" group and the other controls was much less. Accessory organs of "equal weight" group again were not different from those of the "fat-free" group, which seems to substantiate the observation made above, correlating body growth and size of accessory organs.

Results of hormone injections into fat-deficient animals from the 18th to 20th weeks are presented in table 5. This shows stimulation of the accessories by chorionic gonadotrophin and pituitary extract, but not by testosterone. Significant reduction in testicular weight was observed following injection of testosterone, at this time, a finding characteris-

TABLE 5

Weights in fat-free injection groups at 20-week autopsy [1]

GROUPS	BODY	TESTES	PROSTATE	SEMINAL VESICLES
Absolute wts.	*gm*	*mg*	*mg*	*mg*
Uninjected	292 ± 17 [2]	2610 ± 433	431 ± 97	357 ± 82
CG [3]	287 ± 28	2596 ± 266	558 ± 144	428 ± 84
P	> 0.05	> 0.05	< 0.05 [4]	0.05 [4]
Pituitary ext.	285 ± 47	2413 ± 186	529 ± 27	377 ± 34
P	> 0.05	> 0.05	< 0.05 [4]	> 0.05
Testosterone	303 ± 23	2278 ± 163	411 ± 79	306 ± 34
P	> 0.05	< 0.05 [4]	> 0.05	> 0.05
Relative wts.		*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
Uninjected	—	894 ± 132	146 ± 25	123 ± 32
CG	—	919 ± 183	196 ± 55	149 ± 29
P		> 0.05	< 0.05 [4]	< 0.05 [4]
Pituitary ext.	—	840 ± 139	188 ± 20	133 ± 8
P		> 0.05	> 0.05	< 0.05
Testosterone	—	747 ± 90	136 ± 18	101 ± 9
P		< 0.05 [4]	> 0.05	< 0.05

[1] Eight animals per group.
[2] Standard deviation.
[3] Chorionic gonadotrophin.
[4] Significant differences.

tically produced in normal animals and observed in similarly injected control animals at 23 weeks (see below). This latter finding would suggest that gonadotrophin, at least of the FSH type, is being secreted in the fat-free animal after 18 weeks. Enlargement of sex accessory organs induced by CG and PE indicates, on the other hand, that the Leydig cells are capable of responding to stimulation with produc-

tion of endogenous androgenic hormone. There was no change in body weight.

Injection of the same hormones from the 20th to the 23rd weeks produced more striking effects in the "fat-free" group (table 6). First, there was a pronounced stimulation of body growth in the testosterone-injected group. The same hor-

TABLE 6

Weights in fat-free injection groups at 23-week autopsy[1]

GROUPS	BODY	TESTES	PROSTATE	SEMINAL VESICLES
Absolute wts.	*gm*	*mg*	*mg*	*mg*
Uninjected	300 ± 22[2]	2380 ± 476	310 ± 84	287 ± 49
CG[3]	301 ± 27	2611 ± 331	705 ± 106	499 ± 56
P	> 0.05	> 0.05	< 0.05[4]	< 0.05[4]
Pituitary ext.	317 ± 21	2398 ± 331	483 ± 76	360 ± 31
P	> 0.05	> 0.05	< 0.05[4]	< 0.05[4]
Testosterone	350 ± 26	3176 ± 123	416 ± 57	374 ± 24
P	< 0.05[4]	< 0.05[4]	< 0.05[4]	< 0.05[4]
Relative wts.		*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
Uninjected	—	792 ± 153	104 ± 84	95 ± 17
CG	—	866 ± 84	236 ± 40	167 ± 33
P		> 0.05	< 0.05[4]	< 0.05[4]
Pituitary ext.	—	756 ± 103	153 ± 24	114 ± 12
P		> 0.05	> 0.05	< 0.05[4]
Testosterone	—	908 ± 47	117 ± 14	107 ± 8
P		< 0.05[4]	> 0.05	> 0.05

[1] Six animals per group.
[2] Standard deviation.
[3] Chorionic gonadotrophin.
[4] Significant differences.

mone produced an increase in the weights of the testes and accessory organs. The increase in testicular size (fig. 1) was significant even on a relative weight basis, indicating a trophic effect independent of its anabolic effects on body growth. The marked trophic responses produced by testosterone injections in the "fat-free" group strongly substantiate the indication that androgen levels of these animals are

abnormally low, since it is a well known fact that hormones are most effective when their level in body fluids and tissues is low (Selye, '49). It is also notable that CG and PE produced significant stimulation of the accessory organs, indicating that the Leydig cells are capable of function, in spite of evidence for decreased activity in the uninjected animals. Maintenance of testicular size (i.e., prevention of progressive degeneration) by CG is apparent from reference to figure 1.

The contrasting effects produced by testosterone injection in the 20- and 23-week groups is paradoxical. The latter

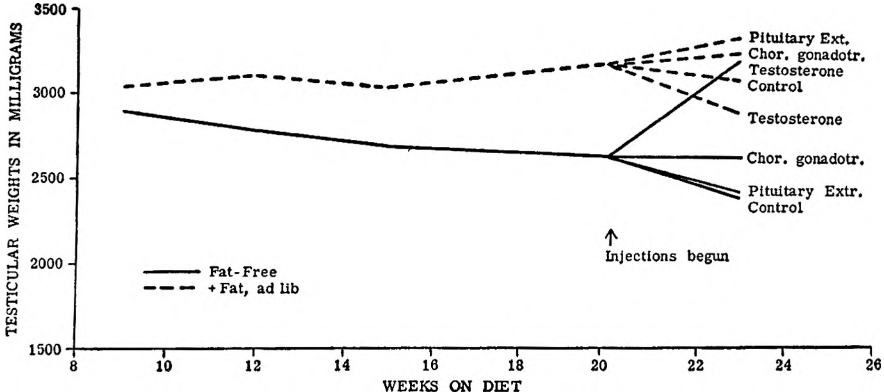

Fig. 1 Hormone injections — influence on testicular weights.

received injections over a longer period of time, and had been fat-deficient two weeks longer before injections began. It is probable that the testicular effects of this deficiency worsen sharply after the 20th week (cf. weight and microscopic changes). It is also probable that androgen deficiency becomes more pronounced after 20 weeks, resulting in the marked body weight response to testosterone at 23 weeks.

The effects of injecting the hormones in the control group are shown in tables 7, 8, and 9. It can be seen that PE was most effective in stimulating accessory organ size, as well as the size of the testes in the "equal weight" group. In con-

trast to its effects on fat-deficient animals, testosterone consistently induced a suppression of testicular weight in the control groups, probably representing suppression of endogenous gonadotrophin (Ludwig, '50). This was in face of the fact that the dosage used was not high enough to stimulate the accessory organs, a finding which suggests that the

TABLE 7

Weights in injection groups fed added fat ad libitum at 23-weeks autopsy [1]

GROUPS	BODY	TESTES	PROSTATE	SEMINAL VESICLES
Absolute wts.	*gm*	*mg*	*mg*	*mg*
Uninjected	430 ± 27 [2]	3069 ± 104	649 ± 233	325 ± 25
CG [3]	470 ± 42	3229 ± 261	831 ± 187	487 ± 107
P	> 0.05	> 0.05	> 0.05	< 0.05 [4]
Pituitary ext.	463 ± 44	3305 ± 48	820 ± 64	475 ± 52
P	> 0.05	< 0.05 [4]	> 0.05	< 0.05 [4]
Testosterone	456 ± 19	2879 ± 207	617 ± 80	357 ± 15
P	> 0.05	> 0.05	> 0.05	< 0.05 [4]
Relative wts.		*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
Uninjected	—	715 ± 67	151 ± 35	76 ± 10
CG	—	694 ± 72	179 ± 44	105 ± 29
P		> 0.05	> 0.05	< 0.05 [4]
Pituitary ext.	—	716 ± 53	179 ± 8	103 ± 6
P		> 0.05	< 0.05 [4]	< 0.05 [4]
Testosterone	—	631 ± 51	135 ± 24	78 ± 9
P		< 0.05 [4]	> 0.05	> 0.05

[1] Six animals per group.
[2] Standard deviation.
[3] Chorionic gonadotrophin.
[4] Significant differences.

pituitary is more sensitive to lower hormone levels than are the accessory organs, as has been shown to be true of estrogen injections (Byrnes and Meyer, '51). These findings should be contrasted with the increase in both testes and accessory organs produced by injection of testosterone in fat-deficient animals at 23 weeks (table 6).

Chorionic gonadotrophin also was effective in increasing accessory organ size. In addition, this hormone induced a

TABLE 8

Weights in injection groups fed added fat, isocaloric, at 23-week autopsy [1]

GROUPS	BODY	TESTES	PROSTATE	SEMINAL VESICLES
Absolute wts.	*gm*	*mg*	*mg*	*mg*
Uninjected	409 ± 13 [2]	3366 ± 169	531 ± 92	345 ± 54
CG [3]	388 ± 27	3342 ± 195	528 ± 116	367 ± 51
P	> 0.05	> 0.05	> 0.05	> 0.05
Pituitary ext.	380 ± 23	3219 ± 134	705 ± 44	447 ± 15
P	< 0.05 [4]	> 0.05	< 0.05 [4]	< 0.05 [4]
Testosterone	407 ± 20	2766 ± 304	599 ± 114	351 ± 37
P	> 0.05	< 0.05 [4]	> 0.05	> 0.05
Relative wts.		*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
Uninjected	—	825 ± 40	129 ± 20	84 ± 14
CG	—	864 ± 80	135 ± 31	94 ± 14
P		> 0.05	> 0.05	> 0.05
Pituitary ext.	—	851 ± 67	186 ± 12	118 ± 11
P		> 0.05	< 0.05 [4]	< 0.05 [4]
Testosterone	—	682 ± 151	148 ± 31	87 ± 6
P		< 0.05 [4]	> 0.05	> 0.05

[1] Six animals per group.
[2] Standard deviation.
[3] Chorionic gonadotrophin.
[4] Significant differences.

marked deterioration in the size of the testes of the "equal weight" group, in contrast to its effects in the "fat-free" and "ad lib." groups. This is difficult to explain, but the time of administration may have been long enough to induce suppression, known to occur with chronic CG administration, possibly due to antihormone formation (Selye, '49).

Histology of testes and accessory organs

Histological examination of the testes from the 9th to 23rd weeks revealed progressive degenerative changes occurring in the seminiferous tubules of the fat-deficient animals. These changes apparently are very similar to those produced by vitamin E deficiency as described by Mason ('39). Earliest changes to appear were intercellular vacuola-

tion and some decrease of tubular size. This was followed by a loss of sperm from the tubular lumina and formation of bead-like spermatids which later fused to form a syncytium, or multinucleated giant cell. Changes were present

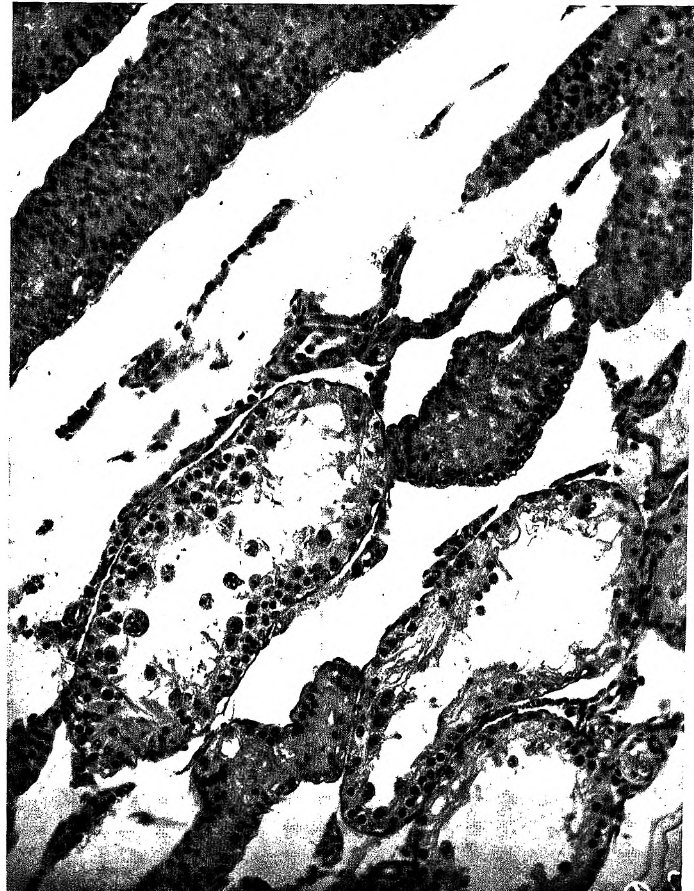

Fig. 2 Testis of fat-deficient animal after 23 weeks, showing tubular atrophy, sloughing of germinal epithelium and giant cell formation. × 150.

occasionally as early as the 12th week, but only at 15 weeks were they common to most specimens examined. By this time, a decreased number of spermatozoa in some tubules was observed, and in some sections, fragmentation of ger-

minal cells had occurred, with sloughing of debris into the tubular lumina. By the 20th week, all testes of the fat-deficient animals showed some degeneration, ranging from vacuolation and shrinkage to giant cell formation and com-

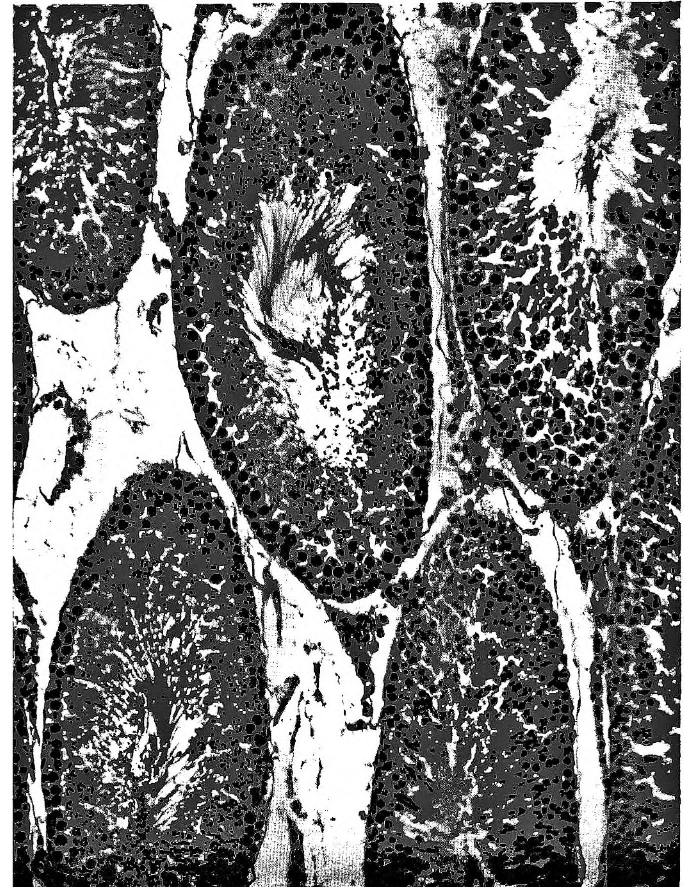

Fig. 3 Testis of "+ fat, ad lib." animal showing normal tubular structure after 23 weeks. × 150.

plete tubular atrophy. At this time, approximately one-fourth of the specimens examined showed the extreme degree of degeneration characterized by the formation of multinucleated giant cells and tubular atrophy. None of the hormone

injections completed at this time (20 weeks) appeared to affect the histological picture in regard to tubular structure.

After 23 weeks, all specimens from the fat-deficient group again revealed degenerative changes. At this time, the ad-

Fig. 4 Testis from "fat-free" group, injected with testosterone for 3 weeks, showing sperm in tubules. × 150.

vanced stages were more common, and approximately one-half of the testes examined showed giant cell formation and marked tubular atrophy as shown in figure 2 in contrast to the control testes in figure 3. Hormone injections completed at this

time were effective in changing the histological picture. The most important effect noted was the alteration of testicular structure in the testosterone-injected group, in accordance with the weight stimulation noted above (figs. 4, 4a. These

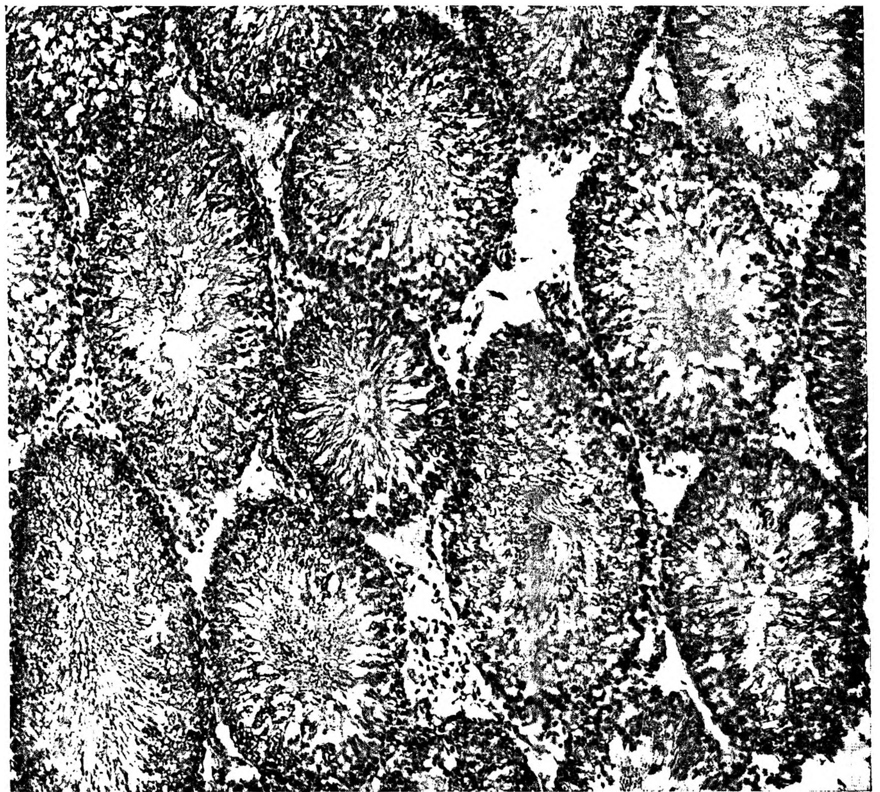

Fig. 4a Testis from "fat-free" rat, injected with testosterone from 20 to 23 weeks. Note sperm in tubules and in small tubule just left of center the "shrinkage" of Sertoli cells. × 150.

glands were characterized by great variation in tubular epithelium. Certain of them appeared distended with fluid and consisted only of Sertoli cells and spermatozoa. Others consisted mainly of densely-packed secondary spermatocytes. Almost all tubules contained spermatozoa and were rounded and fully-packed in contrast to those of "fat-free" animals

which were irregular, empty of spermatozoa and shrunken in appearance.

Chorionic gonadotrophin seemed also to exert some stimulatory effects in that only one-fourth of the testes examined

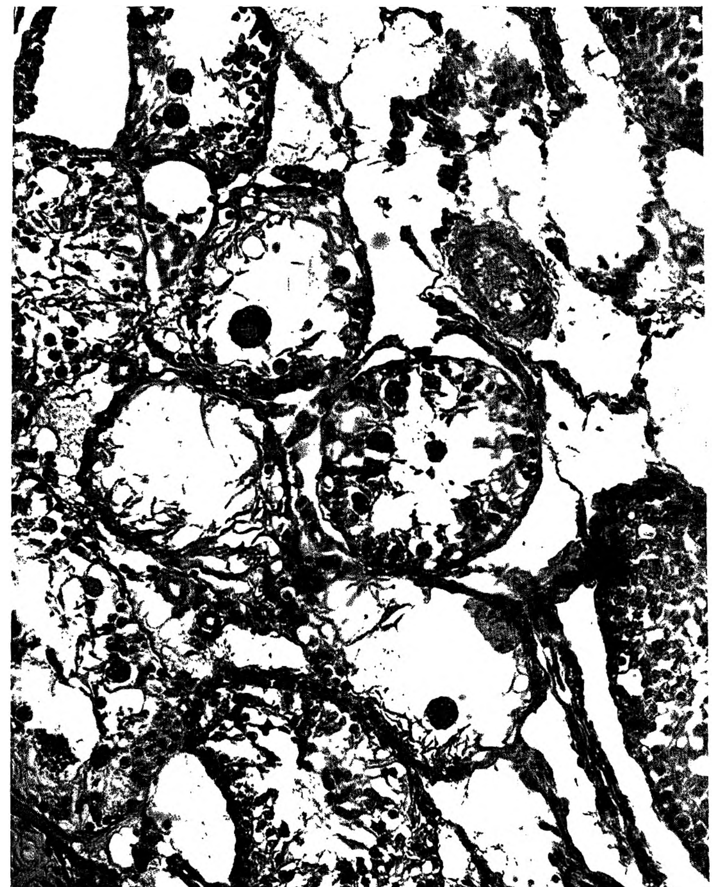

Fig. 5 Testis from "fat-free" group injected with pituitary extract for 3 weeks, showing an extreme degeneration of germinal epithelium. × 150.

showed extreme degenerative changes. Examination of testes after pituitary extract (fig. 5) revealed an incidence of degeneration about the same as for uninjected "fat-free" animals. Examination of the control groups showed that the "chow"

and "isocaloric" groups had completely normal appearing testes, including all of those injected with the various hormone preparations.

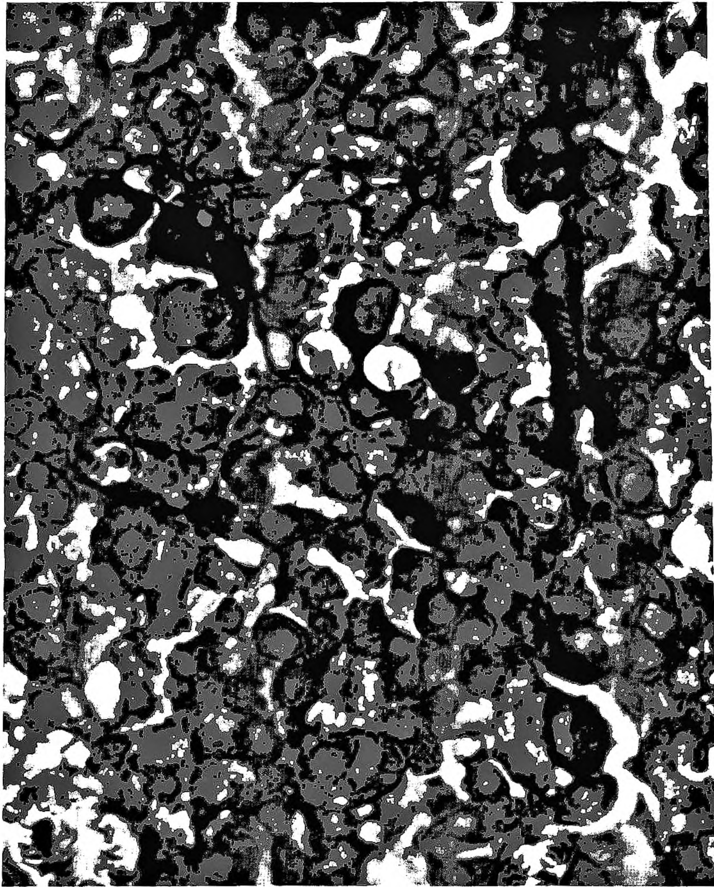

Fig. 6 Field from anterior hypophysis of "+ fat, ad lib." animal after 9 weeks, showing "normal" cytoarchitecture. × 675.

The "equal weight" (starvation control) group, however, showed some degenerative changes. At 20 weeks, there was no more than minimal early tubular shrinkage in a few of the specimens examined. At 23 weeks, the uninjected "equal weight" group showed early degenerative changes in some of

the specimens, but none showed extreme atrophy or giant cells. In the CG-injected group, however, several specimens showed severe tubular degeneration. These findings correlate with the weight changes reported above. Testosterone failed to exert any gross or microscopic reparative influence, in striking contrast to its effects on testes of fat-deficient animals. Pituitary extract, while it caused only slight improvement in the histologic picture, did produce significant increase in testicular size (table 9).

TABLE 9

Weights in "equal weight" injection groups at 23-weeks autopsy [1]

GROUPS	BODY	TESTES	PROSTATE	SEMINAL VESICLES
Absolute wts.	*gm*	*mg*	*mg*	*mg*
Uninjected	334 ± 7 [2]	2861 ± 196	366 ± 91	257 ± 48
CG [3]	303 ± 33	1587 ± 280	630 ± 133	465 ± 100
P	> 0.05	< 0.05 [4]	< 0.05 [4]	< 0.05 [4]
Pituitary ext.	334 ± 10	3280 ± 143	533 ± 68	369 ± 53
P	> 0.05	< 0.05 [4]	< 0.05 [4]	< 0.05 [4]
Testosterone	337 ± 16	2511 ± 189	373 ± 55	294 ± 18
P	> 0.05	< 0.05 [4]	> 0.05	> 0.05
Relative wts.		*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
Uninjected	—	865 ± 75	110 ± 32	79 ± 16
CG	—	619 ± 88	209 ± 34	152 ± 18
P		< 0.05 [4]	< 0.05 [4]	< 0.05 [4]
Pituitary ext.	—	985 ± 84	159 ± 54	111 ± 13
P		< 0.05 [4]	> 0.05	< 0.05 [4]
Testosterone	—	696 ± 106	111 ± 8	88 ± 6
P		< 0.05 [4]	> 0.05	> 0.05

[1] Six animals per group.
[2] Standard deviation.
[3] Chorionic gonadotrophin.
[4] Significant differences.

The testicular damage in this starvation control group is different from that in the fat-deficient condition, as indicated by lack of pituitary changes, the failure to respond to testosterone injection, and the harmful effects of CG administration. In spite of the absence of pituitary changes histolog-

ically (see below), this condition strongly suggests an FSH deficiency because of the stimulatory effects produced by PE injections.

Examination of the interstitial cells for evidence of change in their functional activity was done, although no attempt was made to count or measure these cells. There were no apparent differences in quantity of interstitial tissue between experimental and control groups. However, characteristic cells (Hooker, '48) with large vesicular nuclei and moderately abundant, active cytoplasm were somewhat less apparent in "fat-free" groups after 23 weeks, there being a higher incidence of deficiency or "wheel" cells present. It also appeared that some stimulation in the number and activity of interstitial cells in the "fat-free" group resulted from administration of CG, which also caused an increase in the size of testes and accessories (table 6).

Histological examination of the prostates of the fat-deficient animals revealed flattened epithelium, reduced papillary folding and lighter nuclear staining in comparison with controls. These changes were conspicuous in the 20- and 23-week groups but were present only occasionally at 12 and 15 weeks. Such findings are interpreted to indicate a deficient androgenic stimulation (Turner, '55).

Pituitary cytology (figures 6, 7, 8, 9 and 10)

Examination of pituitary glands stained by the PAS technique with methyl blue counterstain allows the distinction of two gonadotrophic basophiles, termed PAS-red and PAS-purple gonadotrophs (Rennels, '57), which have been associated with production of LH and FSH, respectively (Hildebrand et al., '57).

In the fat-deficient group at 9 weeks (fig. 7) there was a marked increase in the number of basophilic cells, with an apparently normal predominance of PAS-purple cells. "Castration" or "signet-ring" cells similar to typical ones following germinal epithelium degeneration (Van Wagenen, '25)

also were observed at this time, but only to a limited extent. Subsequent examinations done on glands from the 12- and 15-week autopsy groups (figs. 8 and 9) showed progression of the changes with an increased number and size of "signet-ring"

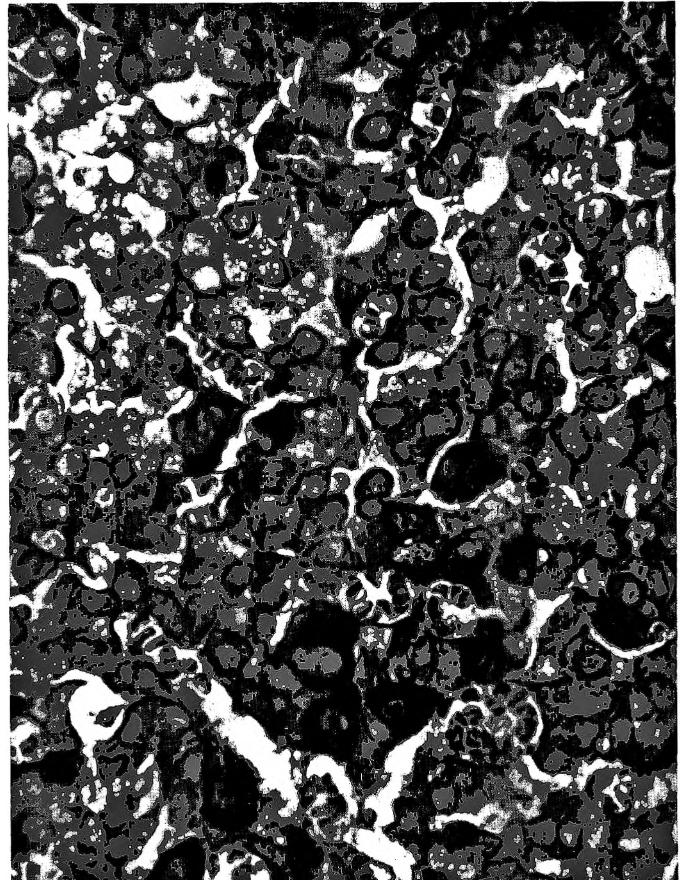

Fig. 7 Field from anterior hypophysis of "fat-free" animal after 9 weeks, showing increased number and size of basophiles. × 675.

cells. Previous work from this laboratory has demonstrated increased FSH potency of pituitaries after 9 weeks of fat deficiency, without obvious evidence of increased LH potency at this time (Klein, '56). After 9 weeks, there was a progres-

sive increase in the ratio of PAS-red to PAS-purple cells to the extent that, at 23 weeks, only occasional purple cells could be found, while the PAS-red cells were very numerous.

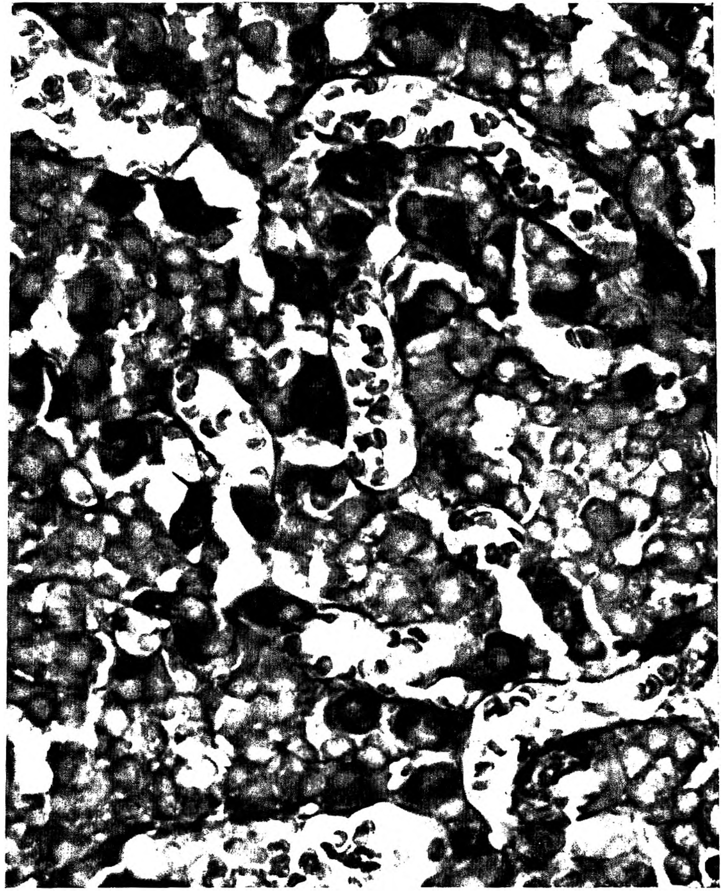

Fig. 8 Field from anterior hypophysis of "fat-free" animal after 12 weeks, showing increased number and size of basophiles and early vacuolization. × 675.

Under these circumstances, associating red and purple cells with LH and FSH, respectively, as mentioned above, it may be inferred that increased LH potency would be expected, as indicated by reports in long-term castrate rats (Hildebrand et al., '57). The decrease of purple cells would indicate a pro-

gressive failure of FSH production and correlates in time with the testicular degeneration which occurs, as described above. As has been suggested by Rinaldini ('49), the pituitary cellular changes possibly represent a storage of the

Fig. 9 Field from anterior hyophysis of "fat-free" animal after 15 weeks, showing more pronounced castration-like changes. × 675.

gonadotrophic hormones, with subsequent atrophy of target organs, due to inadequate release. This is in contrast to the changes following castration, where increased circulating levels of the hormones have been described (Lower and

Hicken, '34). Examination of hypophyses from control groups revealed no changes from the expected normal picture. This is true for equal weight controls in spite of some evidence of testicular degeneration.

Fig. 10 "Castration" cells, showing large cytoplasmic inclusion vacuoles. × 1425.

The injection of testosterone into the "fat-free" group autopsied at 23 weeks induced a reversal of the "castration" picture, so that cytoarchitecture appeared normal. This striking change, when coupled with effect of testosterone in

restoring body and testicular weight in fat-deficient animals (table 6) strongly suggests that a primary defect in chronic fat deficiency is impairment of hormone synthesis at the testicular level. The ability of testosterone to prevent or reverse effects of castration on the pituitary is well known. Chorionic gonadotrophin also reduced the size and apparent activity of the cells, though no gross suppression in number could be detected. Injections of PE did not alter the picture noticeably from that of fat deficiency.

CONCLUSIONS

Changes occurring in fat-deficient rats revealed in the results above may be summarized:

1. Castration-like changes appeared in the anterior pituitary glands as early as 9 weeks on the diet.
2. After 9 weeks, the sizes of accessory sex organs were not different on a relative weight basis from the controls, though they were absolutely smaller.
3. Testicular size was maintained in spite of body growth suppression until 10 weeks on the diet, with progressive atrophy thereafter. The atrophy was most pronounced after 20 weeks.
4. Injections of testosterone after the 20th week resulted in reversal of the basophilic changes in the pituitary. Striking increase in testicular weight (to control level) occurred with restoration toward normal histology. There was also a significant gain in body weight.
5. Stimulation of accessory sex glands by chorionic gonadotrophin indicates functional ability of Leydig cells, although there was only moderate improvement in histology of testes and pituitary. Also, there was no accompanying gain in body weight.

While the above-described effects were produced by feeding a fat-free diet, it is important to consider the role of other deficiencies as primary or contributory etiologic factors. This is particularly true for vitamin E, in view of the similarites

of testicular histopathology produced by deficiency of this vitamin. Reference to descriptions of the diet elsewhere in this article indicates that the amounts of all the fat-soluble vitamins fed were more than adequate. The efficiency of absorption was not tested, but there is no reason to question the efficacy of the bile salt mechanism (Greaves and Schmidt, '37). Absolute proof would depend on determination of vitamin E content of blood and tissues, which was not done. However, the following considerations indicate that the findings described in this experiment are due to fat deficiency and not to vitamin E deficiency:

(a) Experience with the use of the same diet as described in this experiment in a large number of rats (about 2000) has yielded at no time evidence of deficiency of any of the fat-soluble vitamins and specifically none of the following classical signs of vitamin E deficiency: paresis or paralysis, muscle dystrophy or liver necrosis, brown pigmentation. Testicular changes are similar to those described for vitamin E deficiency but, as indicated by the results presented in this report, appear to be more or less completely reversible, in striking contrast to vitamin E deficiency.

(b) The "isocalorically-fed" group received the same amount of vitamins as the "fat-free" group, with 30% of the calories as cottonseed oil. There were no signs of any abnormality in these animals, a finding of particular significance with respect to vitamin E deficiency which has been demonstrated to be accelerated in production and exaggerated in severity by concomitant feeding of large amounts of unsaturated fatty acids (Harris and Mason, '55).

(c) In the "starvation control" group of animals which received less vitamins than the "fat-free" group but 30% of the calories as fat, the testicular histology was normal after 20 weeks on the diet.

The fundamental cause of these changes is presumably a deficiency of one or more of the three hormones known to be involved in the pituitary-testicular axis, namely FSH, LH and testosterone. There is no doubt that testosterone, given

in replacement amounts, achieved the most substantial restorative changes, namely, a marked stimulation of body growth, a reversal of the pituitary castration-like changes and an impressive increase in testicular size with pronounced improvement in testicular histology. This is strong evidence for the primary deficiency of testosterone in these animals. It cannot be stated, however, that this is the only hormone deficiency present. The extent of tubular damage indicates a deficiency also of FSH, which is known to maintain spermatogenesis to the secondary spermatocyte stage (Engle and Levin, '42). It has been noted, however, that injections of PE, which is predominantly FSH in action, did not result in improvement of the histologic picture of testicular degeneration in fat-deficient animals. Testosterone perhaps corrected not only the androgen deficiency, but also the FSH deficiency by reversing the castration-like pituitary changes.

On the other hand, chorionic gonadotrophin was able to achieve, to a lesser extent, most of the same effects as did testosterone. These include moderate improvement of testicular and pituitary histology, marked stimulation of the accessories, but not an increase of body size. In addition, this hormone appeared to stimulate the interstitial cells of the testes histologically. This suggests that the interstitial cells are capable of functioning. The reason the effects were not so marked as in testosterone administration may have been because of a time lag during which the interstitial cells were being restored to function. This could explain the increased size of the more responsive accessory organs without an increase of body weight.

Thus two possible mechanisms emerge, acting independently or in combination. One represents a primary hypogonadism, with changes in pituitary and seminiferous tubules occurring secondarily; the other represents a primary pituitary failure to secrete adequate gonadotrophic hormones, with decrease of androgens and degeneration of seminiferous tubules occurring secondarily. Evidence adduced in the experiments

herein reported does not permit categorical identification of the primary mechanism.

SUMMARY

Male albino rats were kept on a fat-free diet for 23 weeks. During this time, progressive reproductive system changes were observed by serial autopsies at 9, 12, 15, 20 and 23 weeks. The results show that testicular weight was maintained in spite of decelerated body weight gain until the 10th week on the diet, after which progressive degeneration occurred, resulting in failure of spermatogenesis. The size of the sex accessory organs correlated with body weight. A pituitary stain demonstrating FSH-producing and LH-producing gonadotrophic cells revealed an increased number and activity of both types after 9 weeks. Thereafter, progressive basophilic changes occurred with an increased number and size of "signet-ring" cells. The number of FSH-producing cells gradually decreased and that of LH-producing cells increased until, by the 23rd week, the latter comprised the large majority of gonadotrophs.

The effects of administering chorionic gonadotrophin pituitary extract and testosterone were observed. Injections of testosterone after the 20th week resulted in reversal of the basophilic changes in the pituitary. Striking increase in testicular weight (to control level) occurred with restoration toward normal histology. There was also a marked increase in body weight. Injections of gonadotrophin resulted in similar but much less striking changes, indicating that the Leydig cells were capable of functional response. Injection of pituitary extract caused stimulation of the accessory reproductive organs but had no apparent gross or histologic effects on the testes or anterior hypophysis.

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THE INCREASED SEVERITY OF ATHEROSCLEROSIS IN RABBITS ON A LACTOSE-CONTAINING DIET [1]

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(Received for publication January 24, 1959)

It has been reported that the absorption of cholesterol is increased by the addition of 40% of lactose to the diet of the male albino rat (Wells, '57; Wells and Cooper, '58). It is known that high levels of dietary lactose are toxic to the rat (DeGroot and Engel, '57); also, that the animals are resistant to experimental atherogenesis by conventional methods (Horlick and Havel, '48). Therefore, studies were conducted to measure the efficacy of a lactose-containing diet in the development of atherosclerosis in another species, namely, the rabbit. The results of these studies reported here demonstrate a harmful relationship between dietary lactose and the degree of atherosclerosis induced in the rabbit.

METHODS AND EXPERIMENTAL

Two separate series of identical experiments were conducted with male rabbits of the New Zealand White strain.[2] Series I consisted of 16 animals and series II consisted of 20 animals weighing approximately 1350 and 1400 gm each respectively. Each series was divided into two groups, and the animals were housed in individual cages over wire screens. The food cups were attached to the outside of the cage per-

[1] This investigation was supported by a research grant (H-2458, C2) from the National Institutes of Health, U. S. Public Health Services.

[2] Obtained from a local supplier.

mitting only the head of the rabbit to pass. Since only negligible spilling occurred, an accurate record of diet intake was possible. The composition of the diet was essentially the same as that reported by Hove and Herndon ('57) consisting of 45% soybean meal[3] (analysis: protein, 44%, minimum, fat, 0.5% minimum), 29.35% sucrose, 5% cellulose,[4] 5% salts,[5] 13% cottonseed oil, 2% cod liver oil, 0.35% cholesterol, 0.2% choline Cl, 0.1% vitamin mix, and ample oral supplements of α-tocopheryl acetate. The diets for each group were identical with the exception that groups 2 and 4 received 29.35% of lactose[6] at the expense of sucrose. The individual food consumption was recorded throughout each experiment which lasted 7 and 8 weeks for series I and II respectively. At two-week intervals and at the termination of the experiment, the free and total serum cholesterol of each rabbit was determined by the Sperry-Webb procedure ('50). The rabbits were sacrificed by electrocution and the blood removed from the heart and liver. After weighing the trimmed liver, a sample was taken for free and total cholesterol analysis by a method previously described (Wells and Baumann, '54). The heart and aorta with connecting branches were removed, the aorta incised longitudinally and the upper thoracic arch was evaluated for degree of atherosclerosis. The procedure employed in this study is based on the actual measurement of the plaques of each rabbit by three independent workers, and the average of their total area of plaque formation is related to the total thoracic aorta area as percentage of involvement score (0 to 100%). Although somewhat crude, we feel that this method of scoring atherosclerosis provides a measurement which can be estimated with greater sensitivity and accuracy than can be estimated by certain inspection methods of grading, e.g., zero to 4.

[3] Glidden.

[4] Alphacel, Nutritional Biochemicals Company.

[5] Wesson.

[6] Mallinckrodt.

RESULTS

Preliminary attempts to use a casein diet in rabbit studies were unsuccessful because of the extreme variation in anorexia and from deleterious effects of occasional diarrhea. The diet of Hove and Herndon, which contains a high level of soybean meal proved to be especially useful. The rabbits on the lactose diets showed no signs of diarrhea distress. All the animals gained weight favorably, but in both series, the lactose group consumed somewhat less food (series I, lactose 50.9 gm/day vs. sucrose, 52.5 gm/day and series II, lactose, 55.9 gm/day vs. sucrose, 60.4 gm/day), and finished the experiment on the average of 100 to 140 gm lighter than their respective sucrose controls (series I, group 1, 2250 gm vs. group 2, 2108 gm and series II, group 3, 2560 gm vs. group 4, 2468 gm).

Serum cholesterol

The effect of a lactose-containing diet can be seen as early as two weeks after initiation of the experiments. Total serum cholesterol values of the lactose groups averaged 100 to 180 mg % higher than their sucrose controls (table 1, series I, groups 1 and 2, 336 mg % vs. 437 mg %, P was not significant and series II, groups 3 and 4, 173 mg % vs. 374 mg %, $P = 0.001$). This early hypercholesterolemia characteristic of the lactose-fed group was continuous throughout the length of the experiments (final week, groups 1 and 2, 467 mg % vs. 751 mg %, $P = 0.005$ and groups 3 and 4, 488 mg % vs. 685 mg %, $P = 0.02$). The serum cholesterol values of the 4th and 6th weeks are omitted from table 1 but are intermediate between the second and final weeks of the experimental period.

Liver cholesterol

We have found that the calculation of liver cholesterol concentration is most accurate and reliable when the sterol is determined on the basis of dry, fat-free residue (Wells and Baumann, '54). This method eliminates the biological variation in water and or fat content which can easily mask the

TABLE 1

The effect of dietary lactose on serum and liver cholesterol in the rabbit

GROUP NO.[1]	DIET[2]	SERUM CHOLESTEROL 2nd week		SERUM CHOLESTEROL Final week		LIVER CHOLESTEROL	
		Free	Total	Free	Total	Free	Total
		mg%	*mg%*	*mg%*	*mg%*	*mg/gm*[3]	*mg/gm*[3]
Series I							
1	Sucrose	137 ± 64[4]	336 ± 139[4]	232 ± 109[4]	467 ± 190[4]	10.6 ± 2.5[4]	35.0 ± 25.6[4]
2	Lactose	177 ± 64	437 ± 142	401 ± 73	751 ± 127	16.0 ± 4.2	80.0 ± 35.5
Series II							
3	Sucrose	50 ± 29	173 ± 71	263 ± 135	488 ± 225	10.2 ± 3.2	34.2 ± 22.9
4	Lactose	147 ± 49	374 ± 103	386 ± 105	684 ± 179	16.6 ± 4.3	81.4 ± 46.9

[1] Group 1 is directly comparable with group 2 and group 3 with group 4, each consisting of male rabbits of the New Zealand strain, 8 for groups 1 and 2 and 10 for groups 3 and 4.

[2] See text for composition of diet.

[3] Milligrams per gram of dry, fat-free tissue.

[4] Standard deviation, $\sigma = \sqrt{\Sigma X^2 - \frac{(\Sigma X)^2}{N} \Big/ N - 1}$.

true cellular concentration. The total liver cholesterol concentrations were similar in both series (table 1, groups 1 and 2, 35.0 mg/gm vs. 80.0 mg/gm, P = 0.001 and groups 3 and 4 34.2 mg/gm vs. 81.4 mg gm, P = 0.02). The concentration of free liver cholesterol was higher in each lactose-fed group (groups 1 and 2, 10.6 mg/gm vs. 16.0 mg/gm, P = 0.01 and groups 3 and 4, 10.2 mg/gm vs. 16.6 mg/gm, P = 0.005).

TABLE 2

The effect of dietary lactose on the degree of atherosclerosis in the rabbit

GROUP NO.	DIET [1]	ATHEROSCLEROSIS SCORE [2]	NO. OF ANIMALS WITH > 10% SCORE	NO. OF ANIMALS WITH < 10% SCORE
		%		
Series I				
1	Sucrose	11.8 ± 25.7 [3]	1	7
2	Lactose	40.6 ± 29.0	6	2
Series II				
3	Sucrose	11.5 ± 17.0	3	7
4	Lactose	56.5 ± 42.0	7	3

[1] Diets described in text.

[2] Thoracic arch score, 0 to 100% involvement.

[3] Standard deviation, $\sigma = \sqrt{\Sigma X^2 - \frac{(\Sigma X)^2}{N} / N - 1}$.

Atherosclerosis

The atherosclerosis score was observed in both series to be significantly higher in the lactose-fed groups (table 2, groups 1 and 2, 11.8% vs. 40.6%, P = 0.001 and groups 3 and 4, 11.5% vs. 56.5%, P = 0.01). There was observed variation in each group as indicated by the columns listing the number of animals in each series with a score greater or less than 10% (table 2). Individual data showed that there was a very good correlation with the cholesterol level at two weeks and the eventual atherosclerosis score. Thus it appears that the success of a lactose-containing diet in promoting greater atherosclerosis in the rabbit is related to the promptness of the hypercholesterolemia during the experimental period.

DISCUSSION

One can not ascribe the increased atherogenesis to a higher absolute intake of cholesterol by the lactose groups. If instead, one takes the position that in the rabbit as well as in the rat (Wells and Cooper, '58) the effect of lactose is related to the absorption of cholesterol, several possibilities to explain this mechanism may be offered. One of the effects of a lactose diet in the rat coincidental with severe diarrhea is the lowering of pH in the caecum (Wells and Cooper, '58). The pH value of the caecal and small intestine content of the rabbits in series II, however, were not found to be significantly different. We attribute this to an adaptation of the rabbit to the environment of the digestive tract as evidenced by the lack of diarrhea and by good growth of the animals throughout the experiments. Since the active site of cholesterol absorption reported by Swell et al. ('58) and confirmed by us by an alternate method [7] is located in the second quarter of the small intestine of the rat, any pH effect in the rabbit can not be given serious consideration at this time. A second effect which could be of possible importance is the intestinal tract motility. If the motility of the digestive tract were slowed down by the addition of lactose in the diet, one could suggest that the increased cholesterol absorption was only a result of increased absorption time. On the contrary, Fischer and Sutton ('49) reported that food residues passed through the digestive tract more rapidly when lactose was included in the diet than when starch was similarly given. We have studied the passage of a meal of Norit-A stabilized by gum ghatti through the digestive tract of rats (previously fed either lactose or sucrose diets for 5 days) and confirm the observation that motility is greater in the lactose-fed group than in the sucrose controls.[8] A third effect which we have observed in our studies of cholesterol absorption in the rat is the significantly greater flow of lymph in animals on a

[7] Unpublished results.
[8] Unpublished results.

lactose diet compared to sucrose controls. Whether the lymph flow is related to cholesterol absorption, as cause or effect, is currently unknown although the studies of Vahouny and Treadwell ('57) do not associate elevated cholesterol absorption with a parallel increase in lymph flow. Recently we have become aware of the presence of an Ehrlich-positive contaminant of the commercial milk sugar [9] used in all our diet studies. This observation is suggestive of the presence of neuramin-lactose isolated from milk (Zilliken et al., '56) and mammary glands (Trucco and Caputto, '54), and makes it necessary to repeat the present study using purified lactose. Studies are presently under way to determine the effect of lactaminic acid itself on cholesterol absorption and conversely of a possible lactose interaction with lactaminic acid or similar substances of the intestinal cell wall membrane.

The present finding in the rabbit may present an explanation for the report by Bragdon ('52) that suckling rabbits have elevated serum cholesterol levels. The application of high dairy product-cholesterol-containing diet in human studies is characterized by a scarcity of experimental data. There is one old report that the tribesmen of the Khirghiz Steppes who subsist on a diet exceedingly rich in meat and mare's milk, show a high rate of atherosclerosis (Kuczynski, '25). It would be interesting to subject the findings of recent world health surveys (Keys et al., '55) to an analysis for the existence of a possible correlation between the consumption of dairy products with and without excessive cholesterol-containing foods (meats, eggs, etc.) and the incidence of atherosclerosis.

SUMMARY

1. Two separate series of rabbits of the New Zealand White strain were fed a 0.35% cholesterol-containing diet with either 29.35% sucrose or lactose as the carbohydrate for periods of 7 or 8 weeks.

[9] Mallinckrodt.

2. At all time intervals studied, the serum cholesterol levels of the lactose-fed groups were consistently higher than the corresponding values of the sucrose-fed controls. The total liver cholesterol concentration of the lactose-fed groups was more than double that of the sucrose-fed animals.

3. The atherosclerosis score for lactose-fed rabits was 40.6% and 56.5% involvement in contrast to 11.8% and 11.5% for the sucrose-fed controls.

ACKNOWLEDGMENT

We express our thanks to Mr. George Meindl for technical assistance in this study.

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CHANGES IN HEMOGLOBIN, HEMATOCRIT AND PLASMA PROTEIN IN VITAMIN B_6-DEFICIENT RATS DURING PREGNANCY [1,2]

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(Received for publication January 26, 1959)

Our laboratory has previously reported on plasma protein values at term in both control and vitamin B_6-deficient pregnant rats (Ross and Pike, '56). In this study, weekly changes in the concentrations of total protein of the plasma, in hemoglobin and hematocrit were observed in control and vitamin B_6-deficient pregnant and non-pregnant rats. The study was planned and the data analyzed to ascertain the effects due to pregnancy, to the presence of vitamin B_6 in the diet and to the prior depletion of vitamin B_6 stores.

EXPERIMENTAL PROCEDURE

Female albino rats of the Sprague-Dawley strain were maintained on laboratory chow until they attained a weight of approximately 200 gm. When regular estrous cycles were established, the animals were randomly divided into 8 groups. The groups serving as non-pregnant controls each contained 6 animals and there were 10 animals each in the groups that were mated.

For a three-week period, one group of non-pregnant rats received the basal diet (table 1) with a supplement of 0.8 mg %

[1] Home Economics Research Publication no. 164.
[2] Supported in part by a grant from The Nutrition Foundation, Inc.
[3] Mary Swartz Rose Fellow, 1955–56.
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of pyridoxine; and another group received the same basal diet with a supplement of 4 mg% of deoxypyridoxine. Two groups of pregnant rats were treated similarly, one on each of the supplements, from the day of mating throughout the three week gestation period.

In order to observe the effect of depletion of vitamin B_6 stores prior to pregnancy, thereby intensifying the deficiency,

TABLE 1

Basal ration

CONSTITUENT	AMOUNT	CONSTITUENT	AMOUNT
	%	Vitamin mixture [1]	*mg*
Casein, vitamin test [2]	26	Thiamine	2.0
Sucrose and vitamin mixture	9.85	Riboflavin	2.0
Cornstarch	34	*p*-Aminobenzoic acid	200.0
Hydrogenated fat [3]	19	Niacin	10.0
Corn oil [4]	5	Pantothenic acid	8.0
Salt mixture [5]	4	Biotin	0.04
Agar	2	Inositol	400.0
L-Cystine	0.15	Vitamin B_{12} triturate [6]	4.0
Vitamin ADE mixture [7]	+	Folic acid	0.4
		Choline	400.0
		Naphthoquinone	1.0

[1] Made up to 9.85 gm with sucrose.

[2] Labco.

[3] Crisco.

[4] Mazola.

[5] Hawk-Oser; Science, *74:* 369, 1931.

[6] 0.1% trituration of crystalline B_{12} in mannitol (Merck).

[7] Mixture in corn oil contained 5,000 I.U. of vitamin A, 400 I.U. of vitamin D_3 and 10 mg of α-tocopherol in two drops and was administered two drops per rat every three days.

two groups of animals received the deoxypyridoxine-containing diet for 6 to 26 days prior to mating. The depletion period varied because of irregularity of estrous cycles after approximately one week on the deoxypyridoxine supplement. Following mating, one group was continued on the deoxypyridoxine supplement and the other received the basal diet to which pyridoxine was added. Non-pregnant controls were treated similarly except for a uniform depletion period of 21 days.

Blood was collected in capillary tubes from the tip of the tail on the first, 8th, 15th and 21st days. Specific gravities of whole blood and of plasma were determined in gradient tubes according to the method of Lowry and Hunter ('45). Hemoglobin and hematocrit values were calculated according to the method of Van Slyke et al. ('50). The data were analyzed by an analysis of variance with corrections for disproportionality amongst the groups.

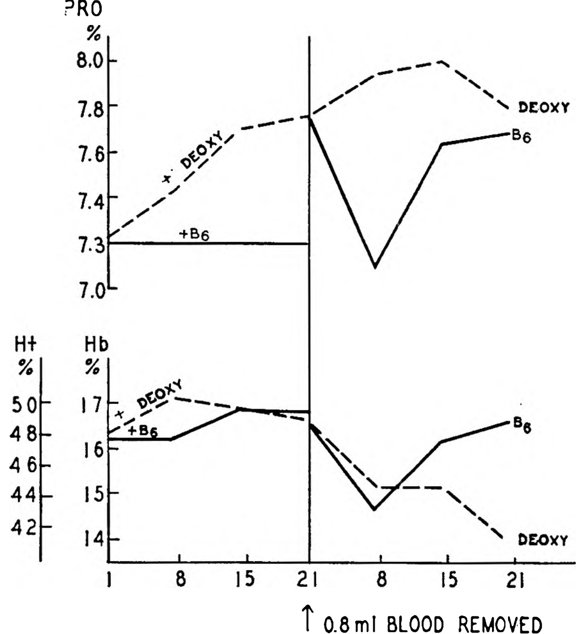


Fig. 1 Total protein, hemoglobin and hematocrit in non-pregnant rats.

RESULTS AND DISCUSSION

Figure 1 shows the changes in the concentrations of total protein in the plasma, and in hemoglobin and hematocrit observed in non-pregnant rats. The animals receiving the vitamin B_6 supplement served as the non-pregnant controls. The concentration of total protein in the plasma in this group remained constant during the period of observation. However, the group receiving the deoxypyridoxine supplement showed a

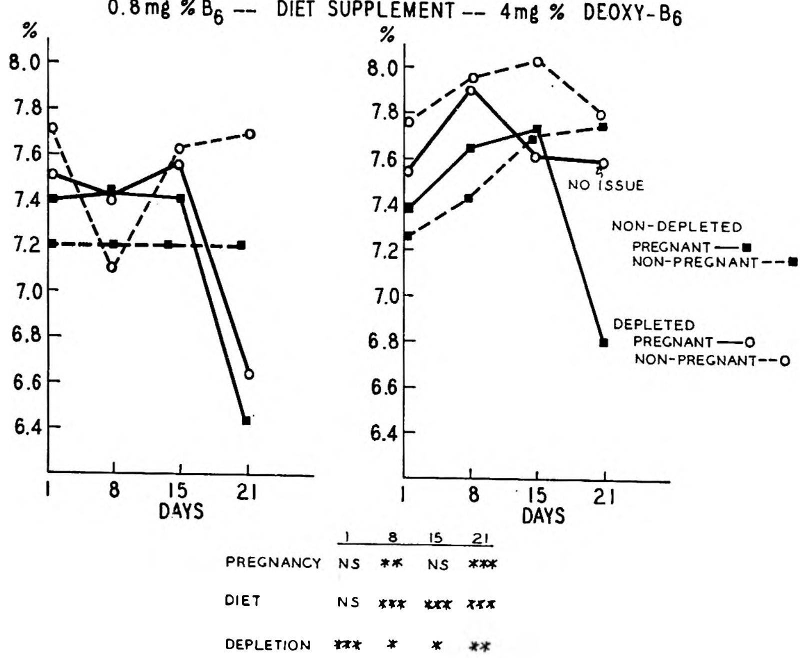
marked increase in the concentration of total protein in the plasma. This group also showed an early increase in hematocrit which suggests that the initial effect was one of hemoconcentration. In addition, it is possible that these animals may have been unable to draw upon plasma proteins for tissue utilization; consequently, the concentration of total protein increased steadily. After the initial rise in hemoglobin and hematocrit, these values leveled off, suggesting a decreased ability on the part of the animals receiving deoxypyridoxine to synthesize both hemoglobin and blood cells.

Since another aspect of this study, which will be reported at a later date, involved the withdrawal of blood samples at intervals on the 21st and 22nd days, we were able to observe the response of the depleted animals to the loss of approximately 0.8 ml of blood. On the 22nd day, half of the animals on the deoxypyridoxine diet were continued on this diet, and the other half received the basal diet with the pyridoxine supplement. The group remaining on deoxypyridoxine continued to show an increase in the concentration of total protein. This may have been due, in part, to the reduction in blood volume and, in part, to the continued accumulation in the plasma of proteins which could not be utilized by the tissues. Hemoglobin and hematocrit levels fell sharply after the blood loss and continued to fall, indicating an inability on the part of the deoxypyridoxine-fed animals to regenerate both hemoglobin and blood cells.

The addition of pyridoxine to the diet after depletion led to a marked fall in the concentration of total protein and in the levels of hemoglobin and hematocrit. It is suggested that this was due to the restoration of blood volume following pyridoxine feeding. During the following two weeks, total protein, hemoglobin and hematocrit rose indicating a restored ability to synthesize plasma protein, tissue protein, hemoglobin and blood cells. At the end of the 6-week period, hemoglobin and hematocrit were restored to the levels observed at the beginning of the experiment. However, the concentration of total protein was greater at the end of the experimental period.

This could have been due to the increased tissue synthesis accompanying the growth of these animals during the rehabilitation period.

Figure 2 shows the changes in total protein of the plasma in the pregnant rats compared to their non-pregnant controls. The shape of the curves for the pregnant groups is essentially the same as those for the comparable non-pregnant animals



	1	8	15	21
PREGNANCY	NS	**	NS	***
DIET	NS	***	***	***
DEPLETION	***	*	*	**

Fig. 2 Total protein of the plasma.

from day one to 15: level for the non-depleted animals receiving the pyridoxine supplement, and a rising line for the animals receiving deoxypyridoxine. Since the depletion period for the pregnant animals was of shorter duration, the readjustment in blood volume following the feeding of pyridoxine, as indicated by the fall in the concentration of total protein of the plasma on the 8th day, was less dramatic. The highly significant ($P = 0.001$) decrease in the concentration of total

protein during the last week of pregnancy was probably due to the hemodilution which normally occurs, as well as to the utilization of plasma proteins as a source of protein for tissue synthesis and fetal growth. The drop did not occur in the depleted group maintained on deoxypyridoxine during pregnancy. Half of this group did not implant and the other half showed implantation sites at term but complete resorption of the young. In effect, pregnancy was terminated before the end of the second week.

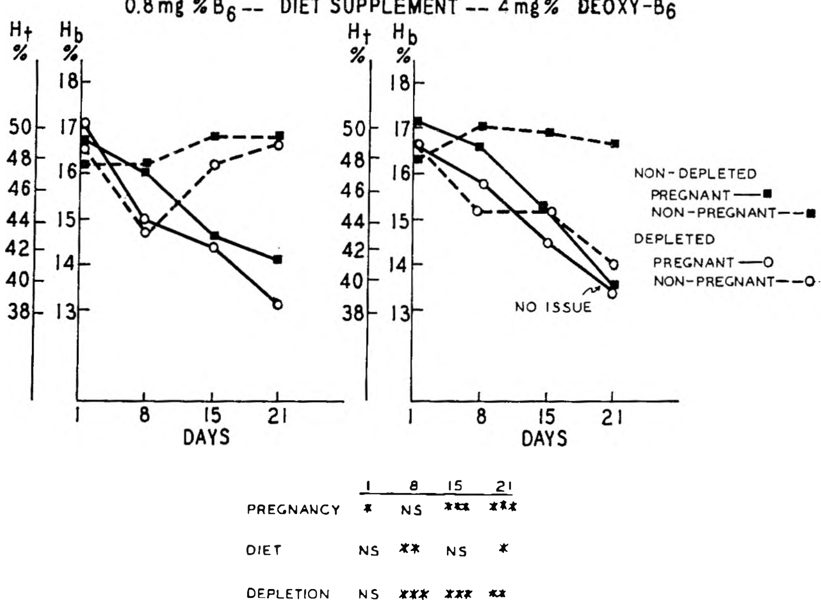
Whereas the highly significant effects due to pregnancy which were observed on the 21st day produced a lowering in the concentration of total protein in the plasma, the effects due to diet ($P = 0.001$) and to depletion ($P = 0.01$) produced an increase in total protein concentration. Both non-pregnant and pregnant animals deprived of vitamin B_6 showed a higher concentration of total protein of the plasma than their pyridoxine-fed controls.

The levels of hemoglobin and hematocrit responded quite differently to the effects of pregnancy, diet and depletion (fig. 3). Although hemoglobin and hematocrit levels declined by the 8th day, the highly significant decrease in the concentration of hemoglobin and in hematocrit due to pregnancy ($P = 0.001$) did not become apparent until the 15th and 21st days. This was probably due both to fetal demands and to the hemodilution of late pregnancy. The significant effect due to diet on the 8th day ($P = 0.01$) appears to be a combination of the restoration of blood volume in the depleted animals receiving pyridoxine and the early effect of hemoconcentration in the non-depleted animals receiving deoxypyridoxine.

The effects of depletion were highly significant on the 8th and 15th days ($P = 0.001$) and retained significance on the 21st day ($P = 0.01$). This effect of depletion upon the concentration of hemoglobin and blood cells was independent of fetal development, since these low levels were observed even in the group which, because of the severity of the vitamin B_6 deprivation, was unable to produce young. These data indicate that the response to a severe vitamin B_6 deficiency in the non-

pregnant animal (the depleted group maintained on the deoxypyridoxine supplement) is similar to the response evoked by pregnancy in animals maintained on an adequate diet both before and during the gestation period.

The interpretation of the findings in this study is supported by data on direct quantitative measurements of changes in blood volume presented in a preliminary report from this lab-



	1	8	15	21
PREGNANCY	*	NS	***	***
DIET	NS	**	NS	*
DEPLETION	NS	***	***	**

Fig. 3 Hemoglobin and hematocrit.

oratory (Brown and Pike, '58). There is hemodilution in late pregnancy in animals receiving pyridoxine, but less change in blood volume in deoxypyridoxine-fed pregnant animals. This difference appears to be due to the counterbalancing influences of pregnancy and pyridoxine deficiency upon blood volume. The tendency toward a reduction in blood volume in deoxypyridoxine-fed animals is indicated by the increase in the concentration of total protein in the plasma and by increased hematocrit. However, the hematocrit elevation is not

sustained due to an apparent loss of ability to synthesize hemoglobin and blood cells.

SUMMARY AND CONCLUSIONS

Weekly changes in the concentration of total protein of the plasma, in hemoglobin and in hematocrit were observed in control and vitamin B_6-deficient pregnant and non-pregnant rats.

Non-pregnant rats receiving a deoxypyridoxine supplement exhibited a tendency toward early hemoconcentration evidenced by a marked increase in the concentration of total protein in the plasma and an increase in hematocrit. Continued administration of the deoxypyridoxine led to a decreased ability to synthesize both hemoglobin and blood cells.

Administration of pyridoxine to animals previously depleted of their vitamin B_6 stores led to a restoration of blood volume as indicated by a marked fall in the concentration of total protein and in the levels of hemoglobin and hematocrit. This was followed by a return of both hemoglobin and hematocrit to the levels observed at the beginning of the experiment. The concentration of total protein at the end of the experimental period was higher than at the beginning and was probably due to the increase in tissue synthesis during the rehabilitation period.

The response of the pregnant and non-pregnant controls was similar during the first two weeks. During the third week, the pregnant animals exhibited a marked fall in the concentration of total protein, hemoglobin and hematocrit indicating hemodilution of late pregnancy.

The effect of depletion upon the concentration of hemoglobin and blood cells appears to be independent of fetal development.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Merck, Sharpe and Dohme Research Laboratories for their kindness in supplying vitamins for this study.

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SOME EFFECTS OF ISONICOTINIC ACID HYDRAZIDE-INDUCED VITAMIN B_6 DEFICIENCY IN PREGNANT RATS [1,2]

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(Received for publication January 27, 1959)

Our laboratory has reported several studies of vitamin B_6 deficiency during pregnancy in which deoxypyridoxine was the antimetabolite (Ross and Pike, '56a, '56b; Pike and Brown, '59). In the present study isonicotinic acid hydrazide (INH) was employed as the vitamin B_6 antagonist. In addition to reporting the effects of INH on reproductive performance, these effects will be compared with those produced by deoxypyridoxine.

EXPERIMENTAL PROCEDURE

Sprague-Dawley rats were maintained on laboratory chow until they attained a weight of approximately 200 gm. Estrous cycles were followed by means of daily vaginal smears. When regular cycles were established, the animals were randomly divided into groups of 10 animals each. The day that mating was confirmed by the presence of sperm in the vaginal smear was considered the first day of pregnancy and the animals were placed in individual cages. One group of animals was offered the basal diet (table 1) which was supplemented with 0.8 mg% of pyridoxine. These animals served as controls. Another group received the basal diet supplemented

[1] Home Economics Research Publication no. 165.
[2] Supported in part by a grant from The Nutrition Foundation, Inc.

with 50 mg/kg of INH administered by daily intraperitoneal injection. The deoxypyridoxine group received 4 mg% of deoxypyridoxine incorporated into the basal diet. The diets were offered ad libitum and weekly food intake records were kept.

Two additional groups of animals, one pregnant and one non-pregnant, were started on the basal diet supplemented

TABLE 1

Basal ration

CONSTITUENT	AMOUNT	CONSTITUENT	AMOUNT
	%		*mg*
Casein, vitamin test [2]	26	*Vitamin mixture* [1]	
Sucrose and vitamin mixture	9.85	Thiamine	2.0
Cornstarch	34	Riboflavin	2.0
Hydrogenated fat [3]	19	*p*-Aminobenzoic acid	200.0
Corn oil [4]	5	Niacin	10.0
Salt mixture [5]	4	Pantothenic acid	8.0
Agar	2	Biotin	0.04
L-Cystine	0.15	Inositol	400.0
Vitamin ADE mixture [7]	+	Vitamin B_{12} triturate [6]	4.0
		Folic acid	0.4
		Choline	400.0
		Naphthoquinone	1.0

[1] Made up to 9.85 gm with sucrose.

[2] Labco.

[3] Crisco.

[4] Mazola.

[5] Hawk-Oser; Science, *74:* 369, 1931.

[6] 0.1% trituration of crystalline B_{12} in mannitol (Merck).

[7] Mixture in corn oil contained 5,000 I.U. of vitamin A, 400 I.U. of vitamin D_3 and 10 mg of α-tocopherol in two drops and was administered two drops per rat every three days.

with 100 mg/kg/day of INH. These groups were not continued because of the severity of the convulsive seizures produced. However, observations on a few of these animals will be presented.

Blood from the tip of the tail was collected in heparinized capillary tubes on the first, 8th, 15th and 21st days of pregnancy. Specific gravities of whole blood and plasma were obtained in gradient tubes according to the method of Lowry

and Hunter ('45), for the determination of hemoglobin and total protein.

During the second and third weeks of pregnancy, urine was collected in pans containing boric acid infiltrated filter paper. Feces were removed daily and held in 50% sulfuric acid until the week's collection for each animal was completed. No collections were made during the first week since this was considered an adjustment period. Nitrogen was determined by the Kjeldahl method.

Young were removed on the 22nd day of gestation by abdominal section. Individual weights were obtained for the live young in each litter and they were fixed for further study. Undeveloped implantation sites and resorptions were recorded.

The data were analyzed to show the significance of the differences between the control and INH groups, the control and deoxypyridoxine groups and between the INH and deoxypyridoxine groups.

RESULTS AND DISCUSSION

No dermatitis was observed in any of the animals receiving 50 mg/kg/day of INH. However, two of the three animals given 100 mg/kg/day of INH that survived for the 22-day experimental period showed slight alopecia. This had not been previously reported as a result of INH administration and would, of necessity, require confirmation by a larger experimental group. Dermatitis and alopecia were severe in the groups receiving deoxypyridoxine.

The absence of alopecia has been reported as one of the differences between INH and deoxypyridoxine administration (Boone et al., '55); however, these workers used a level of 50 mg/kg/day and the data in this study confirm the absence of alopecia at that level.

Another point of distinction between INH- and deoxypyridoxine-induced vitamin B_6 deficiency is the occurrence of convulsions following INH administration. Here again, there is variation between the data in this study and that of Boone

et al. ('55). Whereas they reported convulsions in animals receiving 50 mg/kg/day of INH, this did not occur in the pregnant animals in this study. Perhaps the metabolic adjustments occurring during pregnancy may have prevented their occurrence or, the possibility exists that the slight differences in the protein and fat levels in the two studies might account for the differences in response. When 100 mg/kg/day of INH was administered to pregnant animals, severe convulsions were observed in one animal surviving the experimental period but there was no issue or evidence of implantation sites. In two animals that survived 9 and 10 days, no convulsions were actually observed, but there was evidence of convulsions followed by death in one. In the non-pregnant animals that received 100 mg/kg/day, severe convulsions were observed. Two of 4 animals survived the 22-day experimental period despite convulsions; one died following convulsions on the 15th day and one was found dead after 11 days.

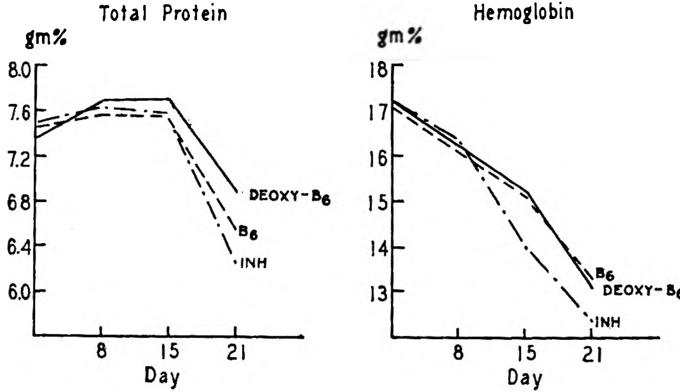

Fig. 1 Mean concentrations of total protein and hemoglobin.

The changes observed in total protein and hemoglobin during the pregnancy period are shown in figure 1. The fall in both total protein and hemoglobin observed between the 15th and 21st days is a typical response to the hemodilution of pregnancy which is known to occur and which has been measured quantitatively in our laboratory (Brown and Pike, '58).

Although the INH group showed a greater decrease in percentage of hemoglobin, this was of no statistical significance. However, the percentage of total protein on the 21st day was significantly lower ($P = 0.02$) in the INH group when compared to the group receiving deoxypyridoxine. This finding will be discussed in relation to fetal growth.

Food intakes and changes in body weight during the gestation period in both the control and vitamin B_6-deficient groups are shown in table 2. Both food intake and weight gain in the control group during each of the three weeks of pregnancy were significantly greater ($P = 0.001$) than in either of the vitamin B_6-deficient groups. However, there were no significant differences between the INH and deoxypyridoxine groups.

Table 3 shows the nitrogen balances during each of the last two weeks of pregnancy. Whereas the control group averaged a gain of over one gram of nitrogen during the two-week period, both vitamin B_6-deficient groups were in negative nitrogen balance. These differences were highly significant ($P = 0.001$). The differences between the INH group and the deoxypyridoxine group were not statistically significant. Each of the vitamin B_6 antagonists administered during pregnancy not only prevented the normal retention of nitrogen, but led to loss of maternal nitrogen despite fetal growth. It appears that the breakdown of maternal protein tissue made available a supply of amino acids for the rapidly metabolizing fetal tissue.

The extent of fetal growth at the expense of maternal tissue is shown in table 4. The weight of the litter accounts for approximately half of the total maternal weight gain of control animals during pregnancy; the remainder can be accounted for by the weight of the placentae, amniotic fluid, and the growth of maternal tissues. The expected relationship between maternal weight gain and litter weight was apparent in the group receiving pyridoxine. In the groups receiving the antagonists, however, maternal weight gain was exceeded by the weight of the litter. Maternal weight gains in the INH

TABLE 2

Weekly food intakes and weight gains

DIET SUPPLEMENT	WEEK 1		WEEK 2		WEEK 3	
	Food intake	Weight gain	Food intake	Weight gain	Food intake	Weight gain
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
Vitamin B_6	114 ± 22 [1]	30 ± 2	96 ± 13	21 ± 9	86 ± 14	56 ± 16
Deoxypyridoxine	72 ± 9	2 ± 8	61 ± 9	7 ± 5	48 ± 11	7 ± 7
INH [2]	66 ± 6	− 2 ± 7	56 ± 8	4 ± 8	42 ± 8	8 ± 15

[1] Standard error of the mean.
[2] Isonicotinic acid hydrazide.

TABLE 3

Nitrogen balance during pregnancy

DIET SUPPLEMENT	SECOND WEEK	THIRD WEEK	TOTAL SECOND AND THIRD WEEKS
	mg	*mg*	*mg*
Vitamin B_6	+ 348 ± 175 [1]	+ 757 ± 262	+ 1106 ± 305
Deoxypyridoxine	+ 112 ± 69	− 273 ± 156	− 161 ± 189
INH [2]	+ 57 ± 134	− 254 ± 262	− 198 ± 301

[1] Standard error of the mean.
[2] Isonicotinic acid hydrazide.

TABLE 4

Maternal weight gain and data on litters

	DIET SUPPLEMENT		
	Vitamin B_6	Deoxypyridoxine	INH [1]
Maternal weight gain, *gm*	107 ± 17 [2]	17 ± 9	10 ± 23
Litter weight, *gm*	56 ± 8	23 ± 7	39 ± 15
Litter size, *no.*	12 ± 2	9 ± 3	10 ± 5
Fetal weight, *gm*	4.6 ± 0.5	2.7 ± 0.5	3.5 ± 0.9
Resorptions, *no.*	0.9 ± 1.2	2.9 ± 1.3	1.2 ± 1.5
Maternal weight gain minus litter weight, *gm*	+ 51 ± 14	− 6 ± 13	− 23 ± 10

[1] Isonicotinic acid hydrazide.
[2] Standard error of the mean.

and deoxypyridoxine groups were not significantly different from each other, but each of these groups made significantly smaller weight gains than the control group ($P = 0.001$). In fact, both the INH and deoxypyridoxine groups had a net loss of maternal tissue at term. This was particularly marked in the group of animals receiving INH. In this group, the total weight of the litter was approximately 4 times the gain in maternal weight. These findings are in accord with the suggestions of Hammond ('44) that tissues of high metabolic activity such as the placenta, fetus and brain, can draw on maternal tissues of low metabolic activity, so that in nutrient shortage there is actual loss of weight in maternal tissues other than brain, placenta and fetus.

A comparison of the average number of live young in the litters, the number dead or resorbed and the average weight of the individual fetus is also shown in table 4. There was no significant difference in the size of the litters produced by the INH animals compared to either the controls or the deoxypyridoxine groups. However, the group receiving deoxypyridoxine had significantly fewer young than the controls ($P = 0.01$). The deoxypyridoxine group also had a significantly greater number of dead or resorbed fetuses than either the control ($P = 0.01$) or INH groups ($P = 0.02$). The limitations exerted upon fetal growth were most severe in the case

of deoxypyridoxine, and less significant for INH. Whereas the antagonism produced by the administration of INH appeared to exert its effect primarily upon the maternal organism, permitting fetal growth to proceed at the expense of maternal tissue, the effects of deoxypyridoxine were apparent both in curtailing the number of live young and in reducing the average weight of the individual fetus. These effects are strongly apparent when the differences between maternal weight gain and litter weight are observed. If the increase in maternal tissue, exclusive of the weight of the litter shown for the pyridoxine-containing diet is accepted as the normal, the net losses observed in the groups receiving each of the vitamin B_6 antagonists are highly significant. In addition, the differences in the response to the two antagonists are readily apparent. The net loss of maternal tissue in the group receiving INH far exceeded the net loss observed for the deoxypyridoxine group. This increased breakdown of maternal tissue apparently made available the necessary amino acid constituents for the increased fetal growth observed in the INH group. This increased fetal growth may also have been responsible for the greater fall observed in plasma proteins in this group. It appears that the fetal tissue was able to exert a priority in obtaining the necessary nutrients for its metabolic needs.

The explanation for this difference in response to the two antimetabolites is not readily apparent. It has been postulated by Biehl and Vilter ('54) and by Umbreit ('55) that INH combines with pyridoxal phosphate to form a hydrazone. Boone et al. ('55) reported no evidence for the formation of pyridoxal isonicotinoyl hydrazone in a chromatographic study of plasma and urinary metabolites in rats receiving INH. However, they suggested that the compound may have been hydrolyzed prior to or during the process of chromatography. Gradual hydrolysis of this compound has been reported *in vitro* (Prescott et al., '57; Harrison and Feiwel, '56). If INH combines with pyridoxal phosphate to form a hydrazone, it is the coenzyme that is not available; whereas deoxypyridox-

ine competes with pyridoxal phosphate for position on the apoenzyme (Umbreit and Waddell, '49).

Since maternal weight gains and nitrogen balances were similar in the INH and deoxypyridoxine groups, the explanation for the significantly greater litter weights and fetal weights in the INH group and the significantly greater sacrifice of maternal tissue for fetal development probably lies in the mechanism of action of the two antimetabolites. It is probable that a differential exists in the sensitivity of metabolic pathways to the antagonistic influences of INH and deoxypyridoxine. It would be expected, therefore, that the amino acids made available to the fetal tissue at the expense of maternal tissue could vary quantitatively and qualitatively. Since it is recognized that the amino acids essential for tissue synthesis and for tissue maintenance are different, the increased growth of the fetus in the INH-induced vitamin B_6 deficiency, in comparison to deoxypyridoxine, might be related to the availability of a better distribution of amino acids essential for the rapidly developing fetal tissue.

The possibility also exists that the placenta might act as a barrier for INH and not for deoxypyridoxine or, that the fetus might possess some means whereby it could rid itself of INH. This could explain the presence of INH in amniotic fluid in the rabbit (Welles et al., '53). If the INH entered the fetal circulation in combination with pyridoxal as the hydrazone and underwent hydrolysis in the fetus, the pyridoxal would then be available for fetal growth and the INH could be excreted into the amniotic fluid.

In distribution and excretion studies in mice, Roth and Manthei ('52) found that labeled INH was rapidly absorbed following subcutaneous injection, transported via the blood and excreted chiefly through the kidneys. Highest activity was found in the kidneys, liver, skin and lungs. The localization of INH in the skin suggested to these authors the possibility of storage there. Although concurring on tissue concentrations and excretion rates, Boone et al. ('56) did not find high concentrations in the lungs and skin and expressed doubt

that the skin served as a storage depot in the rat. The dilution volume of INH, from isotope dilution calculations, approximated that of body water and these authors suggest that in all probability INH and its metabolites pass from the blood into extracellular fluid and also into intracellular water. If this is so, then the possibility exists that INH entering the fetus as the hydrazide could find its way into the amniotic fluid via the kidneys and perhaps the cord and skin. This is in accord with the suggestion that amniotic fluid is of fetal origin secreted by the amnion and cord with some additions from voided fetal urine (Evans and Marriott, '49; Palliez et al., '56; Harrison and Malpas, '53). The latter authors also suggest the possibility that amniotic fluid is produced by the fetal skin.

Since it has been observed that transaminase activity of placental tissue is higher than cord blood and that, in turn, is higher than maternal blood (Glendening et al., '55), it appears that fetal tissue has the ability to concentrate these enzymes. If, in addition, fetal tissue were endowed with the ability to excrete INH into the amniotic fluid, fetal growth could then proceed at the expense of the maternal tissue during INH administration. There would be no reason to suppose that deoxypyridoxine would be metabolized in a manner different from pyridoxine. Therefore, if the antagonist competes with the vitamin for position on the protein moiety of the enzyme, both maternal and fetal tissue would be affected equally.

SUMMARY AND CONCLUSIONS

A comparison was made during pregnancy of vitamin B_6 deficiency induced by isonicotinic acid hydrazide (INH) and by deoxypyridoxine.

Compared to control animals, both INH and deoxypyridoxine groups had significantly lower nitrogen retention, maternal weight gain, total litter weight and fetal weight.

There were no significant differences in nitrogen balance and maternal weight gain in the two deficient groups but total

litter weight and the net loss of maternal tissue was significantly greater in the INH group than in the deoxypyridoxine group.

There were no differences in the concentration of hemoglobin and hematocrit among the groups but the concentration of total protein in the plasma was significantly lower in the INH group at term.

The suggestion was made that the difference in response to the two antimetabolites might be due to a difference in their mechanisms of action or to the ability of the fetus to rid itself of INH by excreting it into the amniotic fluid.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Merck, Sharp Dohme for their kindness in supplying vitamins for this study.

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EFFECT OF ANTIBIOTICS ON THE GASTRO-INTESTINAL ABSORPTION OF CALCIUM AND MAGNESIUM IN THE RAT [1]

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(Received for publication February 11, 1959)

INTRODUCTION

Calcium absorption is enhanced by the presence in the intestinal tract of several chemically dissimilar substances. In rats, bile (Lengemann and Dobbins, '58), lysine (Wasserman et al., '56), and lactose (Outhouse et al., '38) have been shown to increase the uptake of this cation from the intestine. Murrary and Campbell ('55) found that the addition of aureomycin to diets of rachitic rats increased the response to vitamin D. Migicovsky et al. ('51) reported that dietary penicillin increased the calcium absorption index of chicks fed a low calcium diet. In the present study using paired-feeding techniques, the effects of chloramphenicol and neomycin on calcium absorption were studied in rats on adequate vitamin D intakes. Outhouse et al. ('38) noted an increased renal excretion of magnesium in rats fed a lactose diet, suggesting that magnesium absorption may be modified by agents which influence the absorption of calcium. In this study magnesium balance studies were similarly carried out.

METHOD

Rochester colony albino rats with initial weights between 125 and 150 gm were housed in individual metabolism cages.

[1] Supported by a research grant (RG-4996) from the National Institutes of Health.

Water was supplied ad libitum and food provided daily in double feeding cups. The diet consisted of 60% carbohydrate (galactose or glucose), 21% casein, 14% fat [2] and 4% salt (Wesson modification of Osborne and Mendel salt mixture) and a complete vitamin supplement.[3] Experimental and control animals were pair-fed diets which were identical except that an antibiotic (1 mg per gm of diet) was added to the experimental diet. Paired-feeding balance studies were carried out over 7-day periods. Hot distilled water was used to flush cages between periods; acid-washed charcoal served as fecal marker.

Urine and stools were collected separately. Urine was diluted to volume and sodium, potassium and calcium determinations were carried out by flame photometry. Magnesium analyses were done by modification of the Titan yellow method (Orange and Rhein, '51). Stools were homogenized, ashed with nitric acid and the cations determined in the same manner as for urine. Diets were ashed in nitric acid and analyzed for calcium and magnesium. Absorption was calculated as the difference between ingested and fecal calcium and magnesium during each balance period.

For fecal fat analysis homogenized, acidified stool samples were extracted with ether and alcohol. The ether-alcohol extract was evaporated to dryness and the petroleum ether-soluble lipids determined gravimetrically. Free fatty acids were measured by titration.

Marrow-free bone samples for analysis were prepared from the shaft of the femur. Bone was scraped to remove non-osseous tissue and marrow removed from femur by compressed air and blotting. Bone samples were dried at 105°C and ashed at 550°C for 48 hours.

[2] Crisco.

[3] Each kilogram of diet contained: Menadione, 50 mg; thiamine, 10 mg; pyridoxine, 40 mg; calcium pantothenate, 25 mg; nicotin amide hydrochloride, 50 mg; para-amino benzoic acid, 50 mg; folic acid, 0.6 mg; biotin, 0.6 mg; riboflavin, 20 mg; inositol, 100 mg; vitamin B_{12}, 0.010 mg; choline chloride, 1 gm; and 19 gm of U.S.P. cod liver oil.

RESULTS

1. Condition of animals. Rats maintained on the glucose diets for periods up to 4 weeks were active and consistently gained weight. Those fed galactose had galactosuria and polyuria with polydipsia and gained weight at approximately half the rate of the glucose-fed animals. Lenticular cataracts developed after three weeks of galactose feeding. No animals showed diarrhea; some receiving antibiotic had soft stools but water loss did not appear excessive and fecal sodium and potassium values were not elevated.

2. Calcium absorption. In preliminary paired-feeding balance studies, galactose-fed animals absorbed the same percentage of dietary calcium (30%) as glucose-fed control animals but the addition of 3% of sulfasuxidine to the diet reduced fecal calcium excretion.

Further studies of the effect of antibacterial agents on calcium balance were carried out using chloramphenicol. The balance studies are summarized in table 1. Ingestion of chloramphenicol (1 mg/gm diet) resulted in a greater calcium absorption than that of pair-fed animals not receiving the antibiotic. A similar improvement in calcium absorption was found when this antibiotic was added to the glucose diet. No other dietary carbohydrates have been tried.

The antibiotic effect is presumably localized in the gastrointestinal tract because a negligibly absorbed antibiotic, Neomycin, is as effective in increasing calcium absorption as chloramphenicol (table 1).

Calcium intake and antibiotic dosage. The daily intake of antibiotic in experiments in which calcium balance was improved averaged 10 mg per animal or 70 mg per killigram per day. Rats fed chloramphenicol at one-fifth this dosage absorbed the same percentage of calcium as the control animals (table 1). No intermediate dosages have been tried.

On a 60% glucose low-calcium diet (0.2 mg/week) the fecal calcium of antibiotic-fed animals was not significantly different from that of control animals. Calcium absorption when the calcium intake was doubled by increasing the salt content

TABLE 1

Calcium balances with and without antibiotic

ANTIBIOTIC	DIETARY CARBOHYDRATE	NO. OF PAIRS OF ANIMALS	CALCIUM BALANCE MEQ/7 DAYS			S.E.[1]
			Average intake	Absorbed		
				Antibiotic-fed	Control	
Chloramphenicol, 1 mg/gm diet	galactose	7	3.14	2.13	1.02	± 0.20
Chloramphenicol, 1 mg/gm diet	glucose	7	5.90	4.90	3.70	± 0.28
Chloramphenicol, 1 mg/gm diet	glucose	7	13.71	3.49	0.70	± 1.20
Chloramphenicol, 1 mg/gm diet	glucose	7	0.2	—.45	—.90	± 0.20
Chloramphenicol, 0.2 mg/gm diet	glucose	6	5.4	1.60	1.70	± 0.6
Neomycin 1 mg/gm diet	glucose	7	6.8	2.90	1.40	± 0.34

[1] Standard error of mean difference between two groups.

of the diet is shown on table 1. The difference is significant at the 10% level. The effect of modifying calcium intake is being investigated further.

Magnesium absorption. Magnesium absorption is also significantly enhanced by the addition of antibiotic (table 2). All studies were carried out on animals fed a 60% glucose diet. In general magnesium absorption was found to be improved under the same conditions that resulted in enhanced calcium absorption. Neomycin was effective in increasing magnesium absorption in the second but not the first period. Certain findings suggest that the action of the antibiotic on magnesium absorption is not the same as on calcium absorption. In any one animal the absorption of one but not the other may be enhanced. In any one balance period the antibiotic-fed animal may have a significantly greater absorption of one but not the other. Renal excretion of the two ions is also different.

Renal excretion. The renal excretion of calcium and magnesium in these animals is summarized in table 3. The excre-

tion rates reported are taken from periods in which the absorption of these two ions was enhanced in antibiotic-fed animals. Animals fed galactose diets without enhanced calcium absorption show elevated urinary excretion of this ion

TABLE 2

Magnesium balance studies

ANTIBIOTIC	PERIOD	NO. OF PAIRS OF ANIMALS	MAGNESIUM BALANCE MEQ/7 DAYS			S.E.[1]
			Average intake	Absorbed		
				Antibiotic-fed	Control	
Chloramphenicol 1 mg/gm diet	1	7	4.12	3.68	2.81	± 0.12
Chloramphenicol 1 mg/gm diet	2	7	4.29	3.78	3.15	± 0.17
Neomycin 1 mg/gm diet	1	8	2.95	2.61	2.57	± 0.13
Neomycin 1 mg/gm diet	2	9	2.84	2.46	1.97	± 0.10

[1] Standard error of mean difference between two groups.

TABLE 3

Urinary calcium and magnesium excretion

DIET	NUMBER OF ANIMALS	MEQ/24 HRS.	
		Antibiotic-fed	Control
	Calcium		
60% glucose	20	0.019 ± 0.002 [1]	0.021 ± 0.007
60% galactose	8	0.046 ± 0.005	0.072 ± 0.005
	Magnesium		
60% glucose	21	0.194 ± 0.018	0.104 ± 0.008
60% galactose	8	0.335 ± 0.009	0.198 ± 0.011

[1] Mean ± standard error.

(Handler, '47) and in studies in this laboratory to be reported the same was shown to be true for magnesium. Urinary calcium excretion in the glucose-fed animals is not changed by feeding of chloramphenicol. In the animals fed galactose renal losses are lower in the experimental animals than in control

animals not fed the antibiotic. Urinary magnesium losses are increased in animals receiving antibiotic in diets containing either carbohydrate during periods of improved magnesium absorption. The renal excretion is elevated to the point that over-all retention of this ion is not improved in animals receiving antibiotic.

Duration of effect. It is probable that the increased calcium absorption produced by antibiotics does not persist for extended periods of time. In successive balance studies animals ingesting antibiotic showed a significantly greater absorption of this cation during the first but not the second period

TABLE 4

Calcium and magnesium absorption in successive balance periods [1]

PERIOD	DAY	INTAKE	ABSORBED		S.E.[2]
			Antibiotic	Control	
		Ca balance			
1	1–7	5.93	4.89	3.86	0.28
2	15–21	5.26	3.18	2.98	0.63
		Mg balance			
1	1–7	4.16	3.68	2.81	0.12
2	15–21	3.66	3.16	2.54	0.12

[1] Seven pairs of rats in each group.
[2] Standard error of mean of differences.

(table 4). Magnesium absorption was significantly higher in experimental animals in both periods.

No significant increase was found in the bone ash of animals maintained on diets containing this concentration of antibiotic. Two groups of 21-day old rats were fed the diet ad libitum. On the 28th day all animals were sacrificed and femurs removed for analysis. The ash content of the femurs of both groups was 58.9%. Hantsook ('56) also found no increase in the carcass gain of calcium in weanling rats fed aureomycin for a 5-week period. Outhouse et al. ('37) did find a higher ash content in rachitic animals fed lactose as compared to sucrose. Perhaps differences in calcium absorption induced

by lactose persist for longer periods of time or are magnified by a concomitant vitamin D deficiency.

Intestinal pH. The intestinal and cecal contents two hours following feeding were collected under oil and the pH determined. The average of the pH of the contents of small intestine of 6 rats from the control and experimental groups was 6.68 ± 0.10; the average pH of the colon contents in both groups was 7.11 ± 0.16. This is consistent with the finding that intestinal pH is not modified by agents as bile salts (Lengemann and Dobbins, '58) that also enhance calcium absorption.

Fecal fat. Animals receiving antibiotic excreted significantly less ether-soluble total fat and free fatty acid in the stool than pair-fed control rats. Animals ingesting chloramphenicol had a total fecal fat excretion of 312 mg; pair-fed control animals, 568 mg per 14 days. The standard error of the differences is $\pm$ 53 mg. In the experimental group free fatty acids (as stearic) made up 55% of the total as compared to 49% for the control group. The decreased excretion of fat is believed to be related to the change in intestinal flora brought about by the antibiotics. During the period in which fat excretions were measured the antibiotic-fed animals absorbed 1 meq more of Mg^{++} and Ca^{++} than controls while the difference between the two groups in stool fatty acid (calculated as stearic acid) was 0.5 meq. The reduction in fecal fat is probably not a significant factor in the mechanism by which calcium absorption is enhanced.

Cecal contents. The cecal and to a lesser extent the colonic contents of the antibiotic-fed animals were found to be greater in size and weight than those of pair-fed controls. In table 5 are summarized the solid and water contents of the ceca and colons of rats maintained on the diets for 8 days and sacrificed 24 hours following the last feeding. The small bowel contents of the two groups were, by inspection, not different. As antibiotic-fed animals did not have excessive stool water losses, water exchange in the cecum and colon must progress at a different rate in antibiotic-fed as compared to the control animals.

TABLE 5

Water and solid content of cecum and colon

DIET	NO. ANIMALS	H_2O CONTENT		SOLIDS CONTENT	
		Cecum	Large bowel	Cecum	Large bowel
		gm	*gm*	*gm*	*gm*
Glucose + antibiotic	7	2.33 ± 0.18 [1]	1.21 ± 0.15	0.71 ± 0.08	0.43 ± 0.03
Glucose	7	0.78 ± 0.11	0.66 ± 0.06	0.39 ± 0.04	0.38 ± 0.06
Galactose + antibiotic	4	3.21 ± 0.60		0.94 ± 0.12	
Galactose	4	1.27 ± 0.23		0.30 ± 0.08	

[1] Mean ± standard error of the mean.

DISCUSSION

The mechanism by which antibiotics enhance calcium absorption is unknown. Intestinal pH is not a factor. Though fecal fatty acid excretion was lower in antibiotic-fed animals this is not believed to be related to the increased calcium and magnesium absorption. The differences in absorption might be accounted for by a decreased gastrointestinal secretion with less endogenous calcium entering the intestine. Fecal calcium excretion in antibiotic-fed rats is not significantly different from that of control animals (0.65 mg per 7 days as compared to 1.10 mg per 7 days). A decreased fecal excretion would be predicted whether decreased secretion or greater absorption or both played a role in the improved absorption found. Further experiments on the effect of antibiotic on secretion are being carried out. The striking increase in the water content or the colon of antibiotic-fed animals was the only difference observed between experimental and control animals. That lactose feeding produces this change (Morgan et al., '38) and that both agents enhance calcium absorption raise the possibility that the mechanism involved may be the same.

As magnesium absorption is sometimes enhanced in the absence of improved calcium absorption the mechanisms involved may be different. Further work including the effect of antibiotics with other dietary carbohydrates is being carried out.

SUMMARY

1. Rats fed complete diets containing 60% galactose or glucose show greater absorption of calcium when a broad spectrum antibiotic such as neomycin is included in the diet.

2. Absorption of magnesium is also increased. The additionally absorbed magnesium is excreted in the urine resulting in no overall improvement in utilization.

3. The duration of the antibiotic effect appears to be less than one month.

4. The mechanism of the effect is unknown. The only difference noted was that animals ingesting antibiotics had greatly distended ceca, predominantly due to increased water content.

ACKNOWLEDGMENT

The author is grateful for the technical assistance of Katherine Srokose.

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URINARY EXCRETION OF AMINO ACIDS BY THE SAME WOMEN DURING AND AFTER PREGNANCY [1]

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(Received for publication November 20, 1958)

Reports concerning the comparisons of amino acid excretions in pregnant and nonpregnant women are not in agreement in respect to the particular amino acids that are excreted in larger amounts during pregnancy. Wallraff et al. ('50) found that "free" arginine, histidine, phenylalanine, serine, threonine, tryptophan and tryosine and "bound" glutamic acid and tryptophan were excreted in the urine in significantly higher amounts by pregnant than by nonpregnant women. Sheft and Oldham ('52) reported that the amounts of total histidine, methionine and threonine excreted by pregnant women were markedly higher than the amounts reported in the literature for nonpregnant individuals. Miller et al. ('54) found that the median excretion values for "free" arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, trypophan and valine were all higher for pregnant than for nonpregnant women and lower for lactating than for nonpregnant women.

With the exception of 4 women in the latter study, who supplied at least one sample while nonpregnant, pregnant and

[1] Contribution 1152, University of Massachusetts Agricultural Experiment Station. This study was part of a Northeast Regional Project (NE-16, Relationship of Nutrient Intake to Nutritional Status in Human Subjects); a cooperative study involving agricultural experiment stations in the Northeastern Region and supported in part by regional funds.

lactating, comparisons of the amino acid excretions of pregnant and nonpregnant women have not been made with the same individuals in the different stages of the reproductive cycle but have been made between different groups of individuals. Because there are wide variations among individuals in the excretion of amino acids, comparisons between small groups of different subjects may not reveal effects of pregnancy on amino acid excretion. In the present study, in order to eliminate the effect of variations among individuals, comparisons were made between the amounts of the amino acids excreted by the same women during pregnancy and in the post partum period.

EXPERIMENTAL

Thirteen normal healthy women between the ages of 21 and 36 years and between the weights of 108 and 155 pounds were subjects for this study. Eight of the women (group 1) furnished urine samples in early pregnancy (second or third month), in late pregnancy (8th month) and in the 4th or 5th month post partum. The other 5 women (group 2) provided urine samples in late pregnancy and in the post partum period only. None of the women was lactating.

The results of a preliminary study on three subjects indicated that there were daily fluctuations in excretion of amino acids as well as changes in excretion throughout pregnancy. It appeared that the analysis of several 24-hour urine samples within a given period, rather than the analysis of one sample, would give more reliable data on which to base comparisons of amino acid excretions between periods. At least 5 complete 24-hour urine samples were analyzed for each subject within a 14-day span in each period.[2] Acid and alkaline hydrolysates of each urine sample were prepared by autoclaving suitable aliquots in 2N HCl or 2N NaOH for 5 hours

[2] The authors are indebted to Dr. Walter D. Foster (formerly Regional Biometrician, West Virginia Agricultural Experiment Station, Morgantown, West Virginia) for the statistical interpretation of the data obtained on the preliminary study.

at 15 pounds pressure. The acid hydrolysates were used for the assay of all amino acids except tryptophan for which the alkaline hydrolysate was used. *Leuconostoc mesenteroides* P-60 was the organism employed in the assay of leucine, lysine, methionine, phenylalanine and tryptophan, according to the method of Steele et al. ('49); *Streptococcus faecalis* R for threonine with the medium of Henderson and Snell ('48); and *Lactobacillus plantarum* 17–5 for isoleucine and valine with medium 1 of Sauberlich and Baumann ('46).

Dietary records were kept by the women for 7 nonconsecutive days in each period. Protein intakes for each woman were calculated from tables of food composition (Watt and Merrill, '50; Bowes and Church, '51).

RESULTS AND DISCUSSION

Data obtained from the individual and group average daily protein intakes and amino acid excretions for the experimental subjects are presented in table 1. The differences between the post partum and early pregnancy values, between the early and late pregnancy values, and between the post partum and late pregnancy values were tested for statistical significance (paired "t" test); the average differences and their statistical significance are shown in table 2. The differences in amino acid excretions between the two periods of pregnancy and the post partum values were calculated as percentage "increases" above the post partum values rather than as percentage decreases from the pregnant to post partum values. This procedure seemed logical as, with the exception of lysine, the excretions of the amino acids increased to some extent from early to late pregnancy and the higher excretions in pregnancy undoubtedly represent a true increase above normal nonpregnant values. Because of the inability to obtain preconception values for the subjects, the excretion values for the nonlactating mothers in the 4th or 5th month post partum have been substituted as the only available values which approximate normal nonpregnant amino acid excretions for these subjects.

TABLE 1

Amino acids excreted by women in early and late pregnancy and after the third month post partum

(Each value is the average of five nonconsecutive days for each subject in each period)

SUBJECT NO.	PROTEIN INTAKE	ISO-LEUCINE	LEUCINE	LYSINE	METHIO-NINE	PHENYL-ALANINE	THREO-NINE	TRYPTO-PHAN	VALINE
	gm/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*
				Early pregnancy					
Group 1									
1	58.6	12.6	17.9	70.1	8.9	18.0	108.9	40.5	18.1
2	46.6	13.5	23.2	220.5	9.7	26.2	125.2	48.5	25.9
3	64.9	11.3	18.1	133.0	6.7	23.9	83.0	43.7	20.9
4	69.9	8.6	17.5	28.3	5.3	20.7	38.6	27.0	20.2
5	59.8	14.8	21.8	50.0	9.2	19.1	51.5	30.6	23.4
6	73.9	22.3	36.0	241.1	7.8	38.1	85.6	41.7	22.9
7	101.1	28.5	33.9	100.2	12.3	25.6	124.9	37.8	36.3
8	62.8	21.1	22.5	123.3	10.8	21.1	129.3	42.2	27.8
Average	67.2	16.6	23.9	120.8	8.8	24.1	93.4	39.0	24.4
				Late pregnancy					
Group 1									
1	73.5	18.2	23.0	107.7	11.8	27.9	213.7	44.2	26.8
2	61.3	11.4	18.4	148.5	8.1	19.6	142.0	45.8	20.5
3	51.1	21.2	24.7	114.8	10.3	28.3	122.8	49.0	24.6
4	76.3	18.0	28.4	62.3	6.9	21.5	98.5	27.4	28.0
5	47.6	22.7	27.7	47.5	12.0	25.8	87.5	25.8	34.9
6	77.5	20.9	42.6	162.1	6.8	39.1	140.2	54.5	24.9
7	100.4	27.6	31.5	98.7	14.2	27.1	149.8	49.4	36.3
8	87.4	23.7	29.1	118.2	10.2	25.4	163.4	48.2	31.5
Average	71.9	20.5	28.2	107.5	9.9	26.8	139.7	43.0	28.6

Group 2									
9	71.9	16.6	19.8	69.0	9.2	27.0	187.4	61.8	28.9
10	50.9	18.3	33.0	326.3	10.7	41.9	210.9	102.6	31.7
11	61.2	15.1	21.5	52.9	8.5	27.9	252.1	69.8	29.2
12	76.2	14.0	21.2	46.6	9.9	19.8	120.3	36.9	20.1
13	67.5	17.1	19.2	47.1	7.0	22.9	137.5	44.7	22.3
Average	65.5	16.2	22.9	108.4	9.1	27.9	181.6	63.2	26.4
Post partum									
Group 1									
1	63.4	11.1	22.9	43.3	4.8	19.2	36.3	13.8	17.6
2	64.3	12.5	19.1	185.2	7.8	15.9	54.6	22.3	19.4
3	55.2	12.4	17.1	43.4	8.5	14.1	35.3	12.7	16.7
4	56.5	12.9	21.9	43.5	6.8	17.2	39.9	12.4	19.6
5	65.8	18.4	29.0	51.6	10.8	22.5	44.7	13.8	19.3
6	87.3	17.5	31.4	149.4	5.6	29.9	51.3	17.6	20.5
7	108.7	13.1	28.4	89.6	8.0	13.8	52.3	17.5	28.0
8	75.5	8.7	16.9	54.8	5.6	16.1	42.2	8.0	18.0
Average	72.1	13.3	23.3	82.6	7.2	18.6	44.6	14.8	19.9
Group 2									
9	61.0	14.5	17.8	47.8	6.3	16.9	38.0	17.5	19.2
10	56.9	15.1	27.5	80.5	10.7	22.1	53.8	19.8	27.5
11	56.1	10.6	18.7	36.2	6.9	16.6	42.2	13.8	14.6
12	56.0	14.5	19.7	42.4	6.6	20.0	31.5	12.1	16.3
13	68.8	10.0	18.9	41.3	6.9	13.5	33.1	9.1	19.0
Average	59.7	12.9	20.5	49.6	7.5	17.8	39.7	14.5	19.3

Quantity and quality of dietary protein. The average daily protein intakes of the subjects varied from 47 gm for subject 2 in early pregnancy to 109 gm for subject 7 in the post partum period. The average daily protein intakes of the groups did not differ significantly between the periods. However, the average daily protein intakes of some of the individual subjects showed considerable change from one period to another; changes of 15 gm or more occurred for 6 women. The amount of protein consumed per kilogram of body weight per day varied from 0.8 to 1.6 gm. Ten subjects had protein intakes equal to or greater than 1.0 gm/kg body weight in all periods; only one subject fell below this level in more than one period.

The subjects had no unusual food habits; all of them ate foods of animal origin in varying quantities. The average daily intakes of animal protein for the subjects ranged from 27 gm for subject 2 to 76 gm for subject 7. This intake represented 58 to 70% of the total protein eaten and varied from 0.47 to 1.1 gm/kg body weight per day.

Daily variation in amino acid excretion of individual subjects. Because at least 5 24-hour urine samples were obtained for each woman in each period it was possible to observe the daily variation in amino acid excretion for individual subjects. Within a given period, the daily amounts of a particular amino acid excreted by an individual were fairly uniform; the majority of the values fell within $\pm$ 15% of the average excretion of that amino acid by the individual for that period. In cases in which the average excretion of an amino acid was very nearly the same in different periods there was considerable overlapping of values from one period to another.

Variations in amino acid excretions among individuals. There was a wide range in the amounts of a specific amino acid that were excreted in one period, and the extent of the range in excretion varied for the different amino acids. The amino acid showing the greatest range in excretion was lysine; in early pregnancy subject 6 excreted 241 mg daily, which was more than 8 times the 28 mg excreted by subject 4. For all other amino acids, the maximum excretion varied from 1.5

TABLE 2

Mean difference in daily amino acid excretions between the early pregnant (EP) and post partum (PP) periods, the early pregnant and late pregnant (LP) periods, and the late pregnant and post partum periods

	ISO-LEUCINE	LEUCINE	LYSINE	METHIONINE	PHENYLALANINE	THREONINE	TRYPTOPHAN	VALINE
Group 1								
Mean difference, EP minus PP, mg	3.3 ± 2.6 [1]	0.6 ± 0.5	38.2 ± 14.3	1.6 ± 1.0	5.5 ± 2.0	48.8 ± 11.0	24.2 ± 2.4	4.5 ± 1.2
Increase above PP, %	24.8	2.6	46.2	22.2	29.6	109.4	163.5	22.6
Significance levels [2]	n.s.	n.s.	0.05	n.s.	0.05	0.005	0.001	0.01
Mean difference, LP minus EP, mg	3.9 ± 2.3	4.2 ± 1.8	-13.3 ± 15.2	1.1 ± 0.7	2.7 ± 1.7	46.4 ± 9.7	4.0 ± 2.2	4.2 ± 1.8
Increase above EP, %	23.5	18.0	−11.0	12.5	11.2	49.7	10.2	17.2
Significance levels	n.s.	0.10	n.s.	n.s.	n.s.	0.005	n.s.	0.05
Mean difference, LP minus PP, mg	7.2 ± 2.0	4.9 ± 1.9	24.9 ± 13.5	2.7 ± 0.9	8.2 ± 1.5	95.1 ± 14.4	28.2 ± 3.7	8.7 ± 1.6
Increase above PP, %	54.1	21.0	30.0	37.5	44.1	213.2	190.0	43.7
Significance levels	0.01	0.05	n.s.	0.025	0.001	0.001	0.001	0.001
Group 2								
Mean difference, LP minus PP, mg	3.3 ± 1.3	2.4 ± 0.9	58.7 ± 46.9	1.6 ± 0.7	10.1 ± 3.2	141.9 ± 21.4	48.7 ± 9.9	7.1 ± 2.2
Increase above PP, %	25.6	11.7	118.3	24.1	56.7	357.4	335.9	36.8
Significance levels	0.10	0.05	n.s.	0.10	0.05	0.005	0.01	0.05

[1] Standard error.

[2] Paired "t" test.

to 3.5 times the minimum excretion in any one period. Differences in amino acid excretion among individuals have been generally observed, but the factors which control the levels of excretion have not been definitely established. Berry ('53) presented evidence which suggested that some of the differences between individuals in excretion of urinary constituents may be genotypically conditioned. There is conflicting evidence concerning the influence of age, body weight or size, and diet on the excretion of amino acids (Thompson and Kirby, '49; Steele et al., '50; Wallraff et al., '50; Sheft and Oldham, '52; Fowler et al., '57; Waddill et al., '57).

From the data obtained in the nonpregnant period in the present study an evaluation of the relationships between the amount excreted of each of the amino acids and the ages, weights, and protein intakes of the 13 subjects was made by computing the partial correlation coefficients. No significant relationship was found between the level of excretion of any of the 8 amino acids and the protein intakes and weights of the subjects. A significant correlation appeared to exist between level of threonine excretion and age ($P < 0.05$). From these findings it is evident that the levels of amino acids that were excreted by these women were not generally related to age, weight, or protein intake.

Influence of pregnancy on amino acid excretion. Comparisons of the average excretions of amino acids in the pregnant and nonpregnant periods (group 1) indicated that pregnancy stimulated an increased output of amino acids. The excretion of all amino acids did not increase to the same extent and did not show the effects of the stimulation in the same periods. The average excretions for lysine, phenylalanine, threonine, tryptophan and valine were significantly higher in early pregnancy than in the post partum period. A further significant increase occurred for threonine and valine between early and late pregnancy. With the exception of lysine, the maximum excretion of amino acids occurred in late pregnancy. The significance levels (table 2) indicate that it is improbable that the differences in excretion between the late-pregnant and

post partum periods occurred by chance for this group of subjects. Although the difference was not statistically significant, the average excretion of lysine was less in late pregnancy than in early pregnancy; this finding is in agreement with that of Miller et al. ('54), but contrary to that of Haga and Ishida ('56) who reported that the urinary excretion of lysine increased throughout pregnancy.

The results obtained for group 2 generally corroborated the findings for group 1. The average amounts of the individual amino acids excreted in late pregnancy and in the post partum period for groups 1 and 2 agreed fairly well; threonine and tryptophan were higher in late pregnancy, and lysine was lower in the post partum period for group 2. Comparison of the values obtained for these women with the values reported in the studies cited above show a higher value for lysine and lower values for leucine and methionine in the present study.

The largest percentage differences between the post partum and pregnancy values were obtained for threonine and tryptophan. For group 1 the mean excretions in late pregnancy represented increases above the nonpregnant values of 213 and 190% for threonine and tryptophan, respectively. The percentage increases of the other 6 amino acids were of much smaller magnitude; in late pregnancy the range was from 21% for leucine to 54% for isoleucine. For group 2, the percentage differences between the post partum and late pregnancy values for lysine, threonine, and tryptophan were much greater than for group 1, being 118, 357, and 336%, respectively.

The differences in the magnitude of the increases in excretion of amino acids found in the present study are in accord with those of Miller et al. ('54) who reported that "pregnancy stimulated an increased output of amino acids but the degree of stimulation varied for each particular amino acid."

SUMMARY

Eight indispensable amino acids were determined in the urine of 13 women; 8 women (group 1) furnished samples

in early and late pregnancy and in the 4th or 5th month post partum, and the other 5 women (group 2) furnished samples in late pregnancy and in the post partum period only.

All the amino acids were excreted in higher amounts during pregnancy than in the post partum period. The greatest average increases above the post partum values were found for threonine and tryptophan, which were 213 and 190%, respectively, for group 1 and 357 and 336%, respectively, for group 2 in the late pregnancy period. Average increases for the other amino acids in early pregnancy ranged from 3% for leucine to 46% for lysine; in late pregnancy the range was from 21% for leucine to 54% for isoleucine for group 1, and from 12% of leucine to 118% for lysine for group 2.

The amount of amino acid that was excreted by an individual was not generally related to protein intake, weight, or age.

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VITAMIN THERAPY IN MICE WITH AN HEREDITARY MYOPATHY (DYSTROPHIA MUSCULARIS) [1]

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(Received for publication January 22, 1959)

The hereditary dystrophic syndrome in mice includes lesions of the skin (periocular inflammation), abnormal nervous reflexes, fasciculation, diminished growth rate of bone and other non-muscle tissue, and early death, in addition to the pronounced myopathy (Michelson et al., '55; Baker et al., '58). In an effort to formulate a unifying hypothesis as to the primary lesion in this disease, attention has been drawn to the similarities between the dystrophic syndrome and the disease pattern which develops as a result of certain vitamin deficiencies, particularly of vitamins A and E (Baker et al., '58).

An hereditary disease could bring about a vitamin deficiency if any of the following conditions existed: (a) appetite centers were maloperative; (b) gastrointestinal absorptive processes were deranged; (c) vitamins were inactivated or excreted at an excessive rate; (d) vitamins failed to be incorporated into an active functional group within cells. In the present experiments an attempt was made to determine which of the dystrophic symptoms, if any, might result from an hereditarily-induced impairment of the digestion, absorption, excretion or destruction of vitamins A and E. In order to prevent or minimize tissue damage resulting from

[1] Aided by a grant from Muscular Dystrophy Associations of America, Inc.

such impairment vitamin supplementation was initiated at an early age and in amounts 10-fold the usually accepted requirements.

METHODS

Heterozygous, normal, male and female mice (strain 129) were obtained from Jackson Memorial Laboratory, Bar Harbor, Me. Nine litters of mice, produced by 7 pairs of breeders, were used in the present experiment. Seven of these litters received the vitamins listed below, while the other two served as untreated controls. All suckling mice were allowed to remain with their mothers until the approximate age of 30 days, when they were removed and segregated with respect to sex.

Water and food were available to mother and litter *ad libitum*. The food consisted of a pelletized laboratory chow [2] of the following composition: 25% protein, 6% fat, and 47.5% "nitrogen-free extract" (presumed to be largely carbohydrate in nature).

Vitamin administration was begun, on the average, 7 days after birth (range 5 to 12 days) and was continued on a daily or alternate-day schedule. Control litters were injected in a like manner with normal saline and sterile sesame oil. The mice were weighed and then injected intraperitoneally with aqueous B-complex vitamin solution (0.19 ml/20 gm/day); subcutaneously injected with the oil-soluble vitamins (0.10 ml/20 gm/day); tube-fed orally with an emulsion of vitamins A, D and E (Lewis et al., '50) (in some cases containing choline chloride) (0.063 ml/20 gm/day); and tube-fed unsaturated fatty acids (0.011 ml/20 gm/day). The small volumes for oral tube feeding were delivered by means of a microsyringe and polyethylene tubing. The quantities of vitamins and supplements administered with the estimated multiples of the daily requirement are summarized in table 1.

The sterile, aqueous vitamin solution for intraperitoneal injection was made from a commercially available vitamin B complex solution containing ascorbic acid [3] to which was

[2] Purina Laboratory Chow, Ralston Purina Co., St. Louis, Missouri.

[3] Not required by the mouse.

TABLE 1

Vitamins and supplements administered

VITAMIN OR SUPPLEMENT	QUANTITY GIVEN ON BASIS OF 20-GM MOUSE	MULTIPLE OF DAILY REQUIREMENT [1]	VEHICLE	MODE OF ADMINISTRATION [2]
Vitamin A	200 I.U.	10	oil	SC
Vitamin A	200 I.U.	10	aqueous	PO
Vitamin D	20 U.	10	oil	SC
Vitamin D	40 U.	20	aqueous	PO
Vitamin E (*dl*-α tocopherol)	1 mg	10	oil	SC
Vitamin E (*dl*-α tocopheryl acetate)	1 mg	10	aqueous	PO
Thiamine	0.5 mg	25	aqueous	IP
Riboflavin	0.4 mg	7.5	aqueous	IP
Pyridoxine	0.3 mg	15	aqueous	IP
Cyanocobalamine	0.8 μg	10	aqueous	IP
Calcium pantothenate plus pantothenyl alcohol	2.3 mg	11.5	aqueous	IP
Niacinamide	4.0 mg	40	aqueous	IP
Folic acid	100 μg	10	aqueous	IP
Biotin	6 μg	10	aqueous	IP
Synthetic vitamin K	200 μg	10	aqueous	IP
Ascorbic acid [3]	5 mg		aqueous	IP
Unsat. fatty acids	10 mg	1	oil	PO
Choline	43.5 mg	10.9	aqueous	PO

[1] Based on Spector ('56) except in the case of unsaturated fatty acids which are based on the quantities given by Turpeinen ('38) for rats.

[2] SC, subcutaneously; PO, orally; IP intraperitoneally.

[3] Not required by normal mouse.

added additional calcium pantothenate, vitamin B_{12}, folic acid, synthetic vitamin K[4] and biotin.

When folic acid in the form of the sodium salt was added to vitamin B complex (which was acidic) folic acid precipitated out as an extremely fine material. This was readily and homogeneously dispersed by shaking immediately before it was used.

Commercially available[5] aqueous "solutions" of vitamins A, D and E were mixed in the requisite proportions. When

[4] Menadiol tetrasodium phosphate.

[5] Aquasol A and D and Aquasol E, U. S. Vitamin Corp., New York, N. Y.

choline chloride was administered it was combined with these vitamin solutions. The addition of the large concentrations of choline chloride to the above solutions caused the separation of an oily layer. With thorough mixing immediately before administration, however, the mixture remained homogeneous during the feeding.

The unsaturated fatty acid mixture consisted of mixed fatty acids from linseed oil (containing approximately 88% of unsaturated acids, largely linolenic acid, and small amounts of arachidonic acid). No antioxidant was added.

Observations of the mice were made either daily or on alternate days. One or more of the following criteria were used to recognize the onset of the disease: muscular weakness, abnormal reflexes, skin lesions (mainly around the eyes), decreased growth rate. The diagnosis was considered to be established, however, by the appearance of paralysis of the hind limbs.

RESULTS

Despite the fact that massive vitamin therapy was initiated prior to recognition of any dystrophic symptoms, 12 out of 38 treated mice became definitely dystrophic. Only one of the 10 mice comprising the two control litters developed dystrophy. The over-all incidence of the disease, 27% (13 of the 48 mice) may be compared with the value of 21% found by Russell and Silvers in extensive studies of matings between known heterozygous animals of this strain.[6] The theoretical incidence of the disease which should result from such matings is 25%.

Evidence of muscular paralysis was taken as the final criterion for establishing the diagnosis of the disease, and paralysis was almost invariably associated with abnormal reflex movements of the hind limbs (flexion rather than extension when the animal was suspended by the tail). Therefore, early therapy with vitamins of the type and dosage used

[6] Personal communication from Dr. E. S. Russell, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. This finding was reported by Russell and Silvers at the 1957 AMA convention.

in this study failed to prevent the appearance of paralysis and the abnormal reflexes which are typical symptoms of this disease.

Another abnormality commonly observed in untreated dystrophic mice (Michelson et al., '55; Baker et al., '58) is convulsive backward extension of the neck and head. Only one of the 12 treated mice developed this defect, and this occurred as a relatively late symptom. One control dystrophic animal also showed this behavior as a late manifestation of the disease. The low incidence of this finding in the treated dystrophics is of questionable significance, since 9 of the 12 dystrophic animals died (6 as a result of a laboratory accident) at a relatively early stage of the disease.

Careful examinations were made for two other characteristics of the disease, growth changes and skin lesions, both of which might be expected to be corrected by vitamin therapy if the dystrophic syndrome reflected an avitaminosis. There was no evidence that vitamin therapy overcame the impaired growth rate. However, because of the small number of animals in the study and because of large variations in body weight from litter to litter, as well as within individual litters, a definite conclusion regarding this aspect of the disease could not be reached. Skin lesions, on the other hand, were observed in 5 of the 12 treated dystrophics, and in 4 of these the changes in the fur were the first recognizable symptoms. In several cases, evidence of slight muscular weakness was noted on the same day that an abnormal coat of fur was recorded. Periocular inflammation and denudation of the eyelids were not recorded. If the latter symptoms are late sequelae of the disease they would have been undetected because of the short duration of this experiment. Abnormalities of the eye such as the presence of conjunctivitis and ptosis of one eye were observed in two treated dystrophics. Apparently, early vitamin treatment with large multiples of the daily requirement failed to prevent the appearance of early skin lesions in the dystrophic mice.

A number of treated mice were classified as "normal" though they were observed to have occasional and variable muscular weakness. None of these, however, developed a persistent paralysis, nor did they have abnormal leg reflexes or skin lesions. Three of 7 mice in one treated litter were considered as borderline cases during the experiment but were finally included with the normal group. Soon after the termination of the experimental period the observation was made that if a hind leg of any of the 7 mice in this litter was extended backward, the limb was not returned to its usual position immediately, in contrast to normal mice. The heterozygous, normal mother was observed to behave in the same abnormal manner.

DISCUSSION

The disease syndrome in hereditarily dystrophic mice is similar to that reported for young mice raised on vitamin-deficient diets (Baker et al., '58). In order to explain how an hereditary disease might result in an apparent vitamin deficiency, 4 hypotheses were suggested as stated in the introduction. Of these 4 possibilities, three may now be eliminated. Thus, appetite centers are operative, since dystrophic mice eat *ad libitum,* at least as much food per unit of body weight as normal litter-mate controls (Baker et al., '58). Neither deranged gastrointestinal absorption nor abnormal destruction of vitamins seem likely possibilities since it has been shown here that parenteral administration of vitamins in amounts 10 times the normal requirement failed to prevent the onset of the dystrophic syndrome. Thus, the 4th hypothesis, namely that the vitamins fail to be incorporated into some necessary functional structure at the cellular level remains compatible with the supposition that these mice suffer from an hereditarily-induced vitamin deficiency.

SUMMARY

Seven litters of mice were bred from animals known to be heterozygous with respect to the dystrophic gene. These lit-

ters were treated parenterally with massive doses of vitamins. Treatment was initiated when the average mouse age was 7 days; at this time no dystrophic symptoms were manifest. Of the 38 mice treated, 12 developed evidence of muscular degeneration and abnormal reflexes which are associated with the dystrophic syndrome in untreated animals. This frequency (12 dystrophics per 38 mice) was greater than that observed in two untreated control litters (one dystrophic per 10 mice); however, the former incidence approximates that usually seen when large populations of untreated mice are studied. There was no indication that early vitamin therapy could prevent the appearance of such other dystrophic symptoms as skin lesions, convulsive seizures, and early death. The significance of these data is discussed as related to the hypothesis that the dystrophic syndrome may result from an hereditarily-induced vitamin deficiency.

ACKNOWLEDGMENTS

We wish to express our appreciation to the U. S. Vitamin Corporation for generous supplies of Aquasol A & D, and Aquasol E. Also, we wish to thank Mary A. Pogue and Laurence V. Johnson for expert assistance.

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EFFECTS OF THE PREVENTION OF COPROPHAGY IN THE RAT

VI. VITAMIN K[1]

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(Received for publication January 23, 1959)

The rat is extremely resistant to the development of vitamin K deficiency although hypoprothrombinemia has been noticed occasionally, when large groups are fed vitamin K-deficient diets (Greaves, '39). The consistent development of vitamin K deficiency in this species has been accomplished only by the creation of abnormal situations whereby absorption from the intestine is blocked, synthesis in the lower intestine is inhibited, or systemic functioning is altered (reviewed in Sebrell and Harris, '54). It has long been recognized that coprophagy could be the means whereby the rat obtains sufficient vitamin K to prevent deficiency symptoms from developing and the studies reported here were designed to investigate this hypothesis.

PROCEDURES AND RESULTS

The rats were obtained as male weanlings from either Holtzman or Charles River Breeding Colony.[2] In a few experiments where females were used this is noted in the description of the study. Coprophagy was prevented by the use of a small plastic cup that covered the anus so as to

[1] This research has been supported in part by funds provided by the State University of New York, The Nutrition Foundation and the National Science Foundation.

[2] C. D. Strain.

prevent the rat from obtaining the freshly extruded fecal pellets (Barnes et al., '57). Individual raised-wire screen-bottom cages were used (except where noted otherwise) and food and water were supplied *ad libitum*. A highly purified diet as described below was fed in all studies except where otherwise noted.[3]

Prothrombin times were determined in most experiments by a slight modification of the one stage micro method described by Hoffman and Custer ('42), using whole blood from the tail. Prothrombin times were checked several times using oxalated plasma from heart blood by the one stage method of Quick ('38). Good agreement between the two procedures has always been obtained. Prothrombin times were never run for more than 180 seconds and therefore the average values in extremely depleted animals are frequently lower than true values since they would include some animals with actual prothrombin times in excess of 180 seconds.

The first attempts to produce vitamin K deficiency by the prevention of coprophagy were unsuccessful. At this time the purified diet that was being used contained 15% hydrogenated vegetable oil.[4] It was recognized that most fats contain some vitamin K, so this fat was removed from the diet and 1% stripped corn oil[5] was added as the sole source of fat. With this low-fat diet, vitamin K deficiencies in the young animals were obtained routinely as shown in figure 1. In the two experiments shown in this chart, male weanling rats that were obtained from Holtzman were given the vitamin K-free diet immediately upon being received in the

[3] The vitamin K-deficient diet contained per 100 gm: casein (vitamin test, G.B.I.), 25; cerelose, 68; salts (Hubbell, Mendel and Wakeman), 4; choline dihydrogen citrate, 0.3; B vitamins in finely ground sucrose, 2; fat soluble vitamins in stripped corn oil (Distillation Products, Inc.), 1. The B vitamin mixture contained in 2 gm sucrose: thiamine·HCl, 0.4 mg; riboflavin, 0.8 mg; pyridoxine·HCl, 0.4 mg; Ca pantothenate, 4.0 mg; niacin, 4.0 mg; inositol, 20.0 mg; biotin, 0.02 mg; and vitamin B_{12} (crystalline), 0.03 mg. The fat-soluble vitamins in stripped corn oil contained per 1.0 gm: vitamin A acetate, 0.31 mg; vitamin D (calciferol), 0.0045 mg and α-tocopherol, 5.0 mg.

[4] Primex.

[5] Distillation Products, Inc.

laboratory. Two days later they were placed in individual cages with wide-mesh screen bottoms and tail cups were attached. There were 30 rats in experiment A and 6 in experiment B. Prothrombin times and body weight were measured at weekly intervals. The non-symmetrical rise and fall of prothrombin that is illustrated appears to be typical of the pattern of uncomplicated vitamin K deficiency in the rat. In experiment A, 9 of the original 30 animals died by the

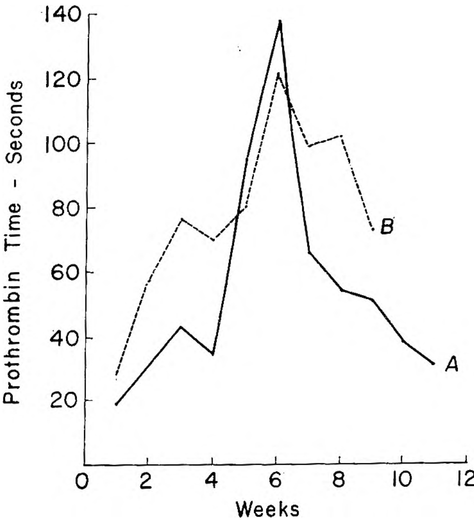

Fig. 1 Prothrombin time changes in rats receiving a vitamin K-free diet with prevention of coprophagy. Two experiments — Group A, 3 rats; Group B, 6 rats.

11th week, and in experiment B, two of the 6 had died by the 9th week. In order to rule out the selection of rats with low prothrombin times as a result of the high mortality rates, the averages have been recalculated by including only those animals that survived the entire experimental period. These average prothrombin times are shown in figure 2 and it will be noted that the same general pattern of rise and fall is still evident. In other words, the prothrombin times actually increased and then decreased during approximately three months after weaning.

On two occasions rats from the C.D. strain of the Charles River Breeding Colony were subjected to the regimen that has just been described. Peak prothrombin times at the third and 6th week were found, but the highest prothrombin times were about 35 seconds. One could interpret this as the development of a mild deficiency, but obviously this is of an entirely different order of magnitude from the results obtained with Holtzman rats. Since the two colonies originated

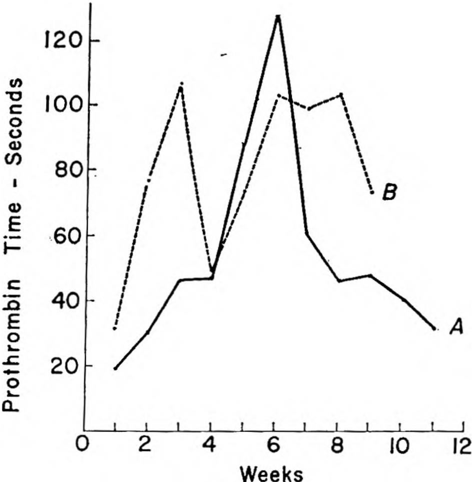

Fig. 2 Prothrombin time changes in rats receiving a vitamin K-free diet with prevention of coprophagy. Same experiments as in figure 1, but averages calculated from rats surviving the experimental periods. Group A, 21 rats; group B, 4 rats.

from the same parent strain, it seems logical to "guess" that the difference was due to the relative amounts of vitamin K storage from the two colony diets.

The influence of vitamin K storage upon the subsequent development of a deficiency of the vitamin is not clear. Possibly some of the confusion comes from the use of prothrombin times as the sole criterion of deficiency, since it is now established that variables other than prothrombin concentration are involved. Six groups of 10 rats each were caged individually and fed the vitamin K-free diet from weaning

age. At intervals up to 12 weeks individual groups were fixed with fecal collection cups so as to prevent coprophagy, and prothrombin times were measured after one week, or until a rise was obtained. The results are shown in figure 3. No change was seen until the rats had been on the vitamin K-free diet for between two and three weeks. Prevention of coprophagy after three weeks caused an immediate increase

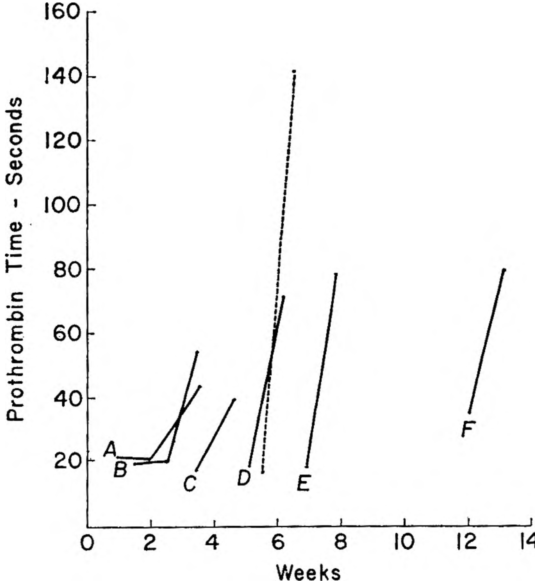

Fig. 3 Effect of preventing coprophagy after varying periods of time on a vitamin K-free diet.

in prothrombin times. The high initial value in group F was due to one rat that had developed a spontaneous hypoprothrombinemia. The broken line in figure 3 represents a group of 15 rats that were treated in the manner just described, but were from an experiment run at a different time. The dramatic rise in prothrombin time that was found when coprophagy was prevented after approximately 6 weeks on the vitamin K-free diet was accompanied by the death of 9 out of the 15 rats with extensive bleeding. Here again is

an illustration of the great variation that may occur in different experiments.

In another study two rats were caged together; one with and one without the fecal collection cups. The vitamin K-free diet was fed *ad libitum* to 6 pairs in each of two experiments. In the first experiment fine-mesh window screening was placed on the floor of the cages so as to prevent feces from falling through, and in the second experiment the standard one-half-inch mesh wire-bottom cages were used. The primary purpose of the study was to investigate the cause of the slight growth depression that uniformly has accompanied the prevention of coprophagy. The rats with fecal collection cups would have access to the "left-over" feces of the control rat and the presence or absence of hypoprothrombinemia would provide an index of the consumption of feces under these conditions. The prothrombin time responses are shown in figure 4. In the upper portion (graph 1) the paired rats were kept on fine window screen and it is evident that sufficient feces were consumed by the rats with the fecal collection cups to prevent the development of vitamin K deficiency. In the lower portion (graph 2) rats in groups A and B were caged together, while group C rats were individually caged and coprophagy-prevented for comparison. All rats were in cages with one-half-inch wire-screen bottoms. The conventional rats (group A) maintained normal prothrombin times as expected. With fecal collection cups the rats apparently were able to get some fecal material from their conventional cage partners, as evidenced by the lower prothrombin-time curve than that of the individually caged rats (group C). However, quite evidently they were not protected from vitamin K deficiency as completely as in the first study. The most interesting outcome of this study was that in both of the experiments the typical depression in growth rate was found in the groups with fecal collection cups.

It has been suggested that a side chain must be added to menadione so as to convert it to vitamin K_1 or K_2 before it

can exert its vitamin K activity (Martius and Nitz-Litzow, '55). Furthermore, Gustafsson ('59) has found that under certain conditions menadione is slow in its action or not effective in overcoming the vitamin K deficiency that is developed in the germ-free rat. This observation implies that

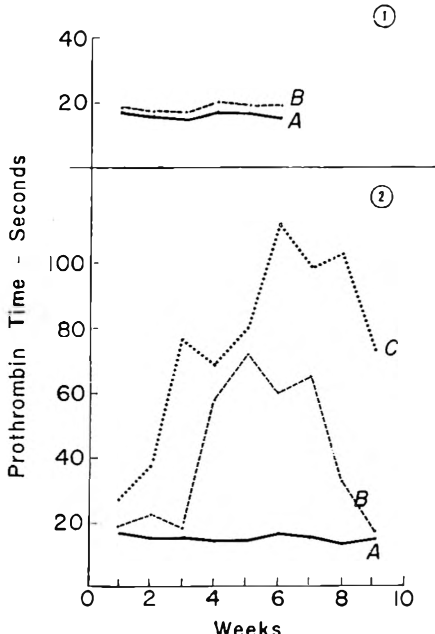

Fig. 4 Development of vitamin K deficiency in rats when caged together; one with fecal collection cups and the other without. Experiment 1 — Fine mesh window screen on the cage floor. Group A, conventional rats; group B coprophagy-prevented. Experiment 2 — Standard one-half inch mesh cage floor. Group A, conventional rats; group B, coprophagy-prevented. Group C for comparison were caged individually and coprophagy-prevented.

the conversion of menadione to vitamin K may take place by intestinal microbial synthesis. Since the coprophagy-prevented rat receiving a diet devoid of vitamin K develops a K deficiency, while on the same diet its conventional control does not, vitamin K that is synthesized within the large intestine cannot be absorbed to any appreciable extent. There-

fore, this type of rat should provide an excellent means for examining the phenomenon in more detail.

Three groups of 12 rats each were caged individually and fed the vitamin K-free basal diet with menadione added at three levels: 0.1, 1.0 and 10.0 mg per 100 gm of diet. Coprophagy was prevented in one-half of the rats in each group. The high levels of menadione were used so as to cover the possibility that at such concentrations the menadione might inhibit bacterial growth and biosynthesis of vitamin K. Prothrombin times were determined at weekly intervals for 4 weeks and it was found that they remained normal in all groups. The second part of this study was to measure the immediate response of vitamin K-deficient rats to small single doses of menadione or vitamin K. No attempt was made to grade the dosages down to a point where specific quantitative requirements could be calculated. Both menadione and vitamin K_1 were dissolved in ethanol and diluted so that the dose to be administered by stomach tube was contained in 0.5 ml of 5% ethanol per 100 gm body weight. It was determined that ethanol at 20% concentration had some effect in causing an immediate lowering of the prothrombin time, but 5% ethanol by itself was essentially inactive. Under these conditions a single dose of 1.0 μg of either menadione or vitamin K_1 per 100 gm body weight returned the prothrombin time to normal within 18 hours. Although the minimal effective dose was not determined quantitatively, it was found in two separate experiments that with single doses of vitamin K_1 in 5% ethanol by stomach tube, 0.5 μg per 100 gm body weight reduced prothrombin time to normal within 18 hours, but 0.1 μg was without effect. It can be concluded that under conditions of complete prevention of coprophagy and within the limits of the experimental procedures employed, menadione was active in overcoming vitamin K deficiency in an amount that approached the minimal effective dose of vitamin K_1.

There has been an occasional reference in the literature to the incidence of spontaneous vitamin K deficiency that de-

velops in the rat being maintained on a vitamin K-free diet (Greaves, '39). Apparently this fact is not too well recognized by the experimental nutritionist, for at least one review of vitamin requirements for the rat states that vitamin K is not essential in the diet (Brown and Sturtevant, '49). As a by-product of the studies that are reported here, it was possible to examine the incidence of spontaneous hypoprothrombinemia that developed among the conventional animals used as controls in the various experimental groups that received vitamin K-free diets for periods of 5 to 12 weeks after weaning. The most striking observation was the extreme variability among separate studies. For example, in 7 different studies there were 4 representing a total of 39 rats in which no instance of hypoprothrombinemia was observed. In three studies representing 45 rats, a total of 19 developed signs of vitamin K deficiency. However, 16 of the 19 came from one study employing 20 rats. It would seem to be foolish to calculate the percentage incidence of spontaneous hypoprothrombinemia with such extreme variability, but it is obvious that it can occur in rats maintained in cages with raised wire bottoms.

DISCUSSION

The most confusing observation associated with the development of vitamin K deficiency in the coprophagy-prevented rat is the fall in prothrombin time that was noted consistently. Since it has been shown that a deficiency of vitamin K can affect proconvertin and plasma thromboplastin component (PTC) (Naeye, '56) as well as prothrombin, it is obvious that the one-stage prothrombin time determination does not adequately measure the entire metabolic defect. If changing concentrations of three blood clotting components are proceeding as vitamin K deficiency develops, this may in some manner explain the rise and fall of prothrombin time. However, it must be kept in mind that the greatest mortality rate was associated with the highest prothrombin times, even though it was occasionally noted that rats would die

with massive hemorrhage when prothrombin time was only slightly elevated above normal.

Another explanation might be that after a prolonged period of preventing coprophagy sufficient microbial growth may take place in the small intestine so as to provide vitamin K at a location within the intestinal tract where it can be absorbed. In the development of other deficiencies such as biotin, the longer coprophagy is prevented, the more severe the deficiency becomes. Furthermore, it has been noted consistently that prothrombin time passed through a peak at approximately three weeks post-weaning and then decreased before rising to the second and highest peak at about 6 weeks. If a change in intestinal flora accounted for the first dip, there should not have been a second rise in prothrombin time unless the microbial population was undergoing a concomitant change.

The most convincing evidence that nutrients synthesized by intestinal microflora are directly utilized by man has been furnished by the pattern of prothrombin time changes in the human infant (Dam et al., '41). The prothrombin time rises following delivery and this is followed by a drop after three to 5 days. Since the fall in prothrombin time is believed to coincide with the development of microflora in the intestinal tract it has been presumed that newly synthesized vitamin K was responsible. Although this has been considered a very logical explanation, a rise and subsequent fall in prothrombin time has been observed in germ-free chicks (Luckey, Pleasants and Reyniers, '55) and the same pattern has now been shown for the coprophagy-prevented rat.

Perhaps the second major point that deserves some discussion has to do with the relative activity of menadione and vitamin K_1 in the rat in which coprophagy is prevented. If the supposition is correct that vitamin K arising from microbial synthesis in the intestinal tract is not available to the rat, any vitamin K that may result from bacterial conversion of menadione should not be available either. On this basis these studies make it unlikely that menadione is effective as

vitamin K only after conversion to one of the side chain forms of vitamin K by the intestinal flora. Whether or not such a conversion is effected at some other site is not pertinent to the studies reported here.

The extreme variability in the development of vitamin K deficiency that has been observed may be due in part to some shortcoming in the technique of preventing coprophagy. However, it would seem that any such studies in the rat should involve large groups and repetitive experiments. This may be a difficulty in the use of germ-free rats and may offer an explanation for the reported conclusion that such animals do not develop vitamin K deficiency (Luckey et al., '55) although the diet that was employed was claimed to have contained 2 to 10 μg of vitamin K per 100 gm. This amount of vitamin K might have been sufficient to prevent deficiency from developing in the rat according to the present studies, but chicks that were fed a similar diet did show signs of deficiency.

SUMMARY

1. When rats are prevented from eating their feces and are fed a vitamin K-free diet, vitamin K deficiency uniformly develops.

2. The extent of the vitamin deficiency as measured by prolongation of the prothrombin time is variable, but it does appear to pass through one and possibly two peaks during the first 12 weeks after weaning.

3. Menadione added to an otherwise vitamin K-free diet prevents the development of vitamin K deficiency in the rat that cannot practice coprophagy.

4. In a single-dose 18-hour test, menadione and vitamin K_1 are both active in returning the prothrombin time to normal at a level of 1.0 μg per 100 gm body weight.

5. It has been confirmed that a small but extremely variable number of conventional rats develop vitamin K deficiency when fed a vitamin K-free diet.

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STUDIES ON THE EFFECT OF QUANTITY AND TYPE OF FAT ON CHICK GROWTH [1]

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(Received for publication January 26, 1959)

Russell, Taylor and Polskin ('40) found that the reduction of substances in a practical chick diet, soluble in diethyl ether to 0.1% or less, did not retard the growth of chicks significantly up to 14 weeks of age when provision was made for the vitamins removed by the extraction process. Davis and Upp ('41) observed, on the other hand, that chicks fed a diet which had been extracted with isopropyl ether for 30 hours grew somewhat more slowly, in spite of replacement of extracted fat-soluble vitamins, than chicks fed the same diet supplemented with fat.

Reiser ('50a) reported that chicks fed a purified, fat-free diet required polyunsaturated fatty acids for growth. The average mortality at three weeks of age of chicks given unsaturated fats was 36.5%, whereas that of the chicks supplied methyl palmitate, bayberry tallow or the basal diet varied from 78 to 100%. The results of Reiser are open to question, however, as much of the observed mortality may have been caused by thiamine deficiency. The "alpha-protein" [2] used in the basal diet has been found by O'Dell et al. ('52) and

[1] The experimental work reported in this paper was supported in part by grants to Cornell University by Cooperative G.L.F. Exchange, Ithaca, N. Y.; Distillers Feed Research Council, Cincinnati, Ohio; Foremost Dairies, Inc., San Francisco, Cal.; and The Squibb Institute for Medical Research, New Brunswick, N. J.

[2] The Glidden Company, Chicago, Illinois.

others to contain sufficient sulfite to destroy thiamine. Thus the reported differences in results may have been caused by the sparing effect of unsaturated fats upon the requirement for this vitamin.

Subsequently Reiser ('50b) obtained evidence which suggested that the chick is unable to synthesize linoleic and linolenic acids. The quantity of these fatty acids in the bodies of 4-week-old chicks given a fat-free diet appeared to be no greater than the amount present in the eggs of hens fed either a stock diet or a purified fat-free diet.

Carver and Johnson ('53) reported that chicks fed a purified casein diet or a purified soybean diet containing 0.05 and 0.04% of fat, respectively, as determined by hexane extraction, require factors present in unsaturated fats for maximum growth. Crude corn oil, refined corn oil, soybean oil, wheat germ oil, an oleic acid concentrate and a linolenic acid concentrate were found to contain these factors in varying concentrations.

Bieri and associates ('56) presented evidence which indicated that the chick requires highly unsaturated fat in the diet in order to grow normally. Chicks fed a diet containing no corn oil grew poorly after 6 to 8 weeks of age. Depigmentation of feathers occurred from about the 8th week and scaliness of skin was observed in several chicks after 16 to 20 weeks of age.

Yacowitz ('53) found that chicks fed a diet consisting largely of corn meal and solvent-process soybean meal and containing 2.49% of ether-extractable substances grew at a somewhat faster rate and had better feed conversion when the diet was supplemented with 2.5 and 5% of cottonseed oil, soybean oil or lard. Denton, Lillie and Sizemore ('54) reported that chicks fed a diet composed for the most part of corn, soybean meal and alfalfa meal, grew at a faster rate when lard was added to the diet.

Rosenberg and Baldini, Sunde and Bird, and Runnels ('55) in a collaborative study conducted in three laboratories observed improved growth in chicks as well as improved feed

conversion by adding 3 to 5% fat to diets of a practical character containing approximately 2.5 to 3.0% of fat. Rosenberg and Baldini at Stine Laboratory, E. I. du Pont de Nemours and Company used corn oil and prime tallow in their work, Sunde and Bird at Wisconsin white grease, and Runnels at Delaware yellow grease. Donaldson and associates ('57) showed that the addition of 10% of stabilized yellow grease to practical chick diets containing 3.8 to 4.6% of fat promoted increased growth. Corn oil was also found to stimulate growth. Vondell and Ringrose ('58) obtained evidence of a significant increase in growth in chicks fed a practical diet containing 15.7% of added tallow over that of chicks fed a similar diet with the same energy-protein ratio which contained 2% of added tallow.

Baldini and Rosenberg ('57) reported that prime tallow added to a practical diet did not stimulate growth in chicks or reduce the amount of feed consumed per unit of gain when the protein and energy contents of the diets were maintained constant by substituting fat and cellulose for cerelose. They attributed the effect of adding fat to the chick diet entirely to the caloric value of the fat. Increasing the fat content of the diet without increasing caloric content, however, increased the fat content of the chick bodies by 12.6%.

Arscott, Weswig and Schubert ('57) showed that the fat fraction of dried egg yolk significantly stimulated chick growth. The diet, the chief ingredients of which were soybean meal and sucrose, probably contained approximately 0.5% of fat. Menge, Lillie and Denton ('57) demonstrated the existence of a chick growth factor in the substances removed from dried egg yolk by common fat solvents. The factor was shown not to be identical with oleic acid, linoleic acid or lecithin. It was present in the non-phospholipid and non-saponifiable portions of the yolk extract. The factor was destroyed by ultraviolet irradiation and by hot but not by cold saponification.

The results of the research work in which either fat-extracted or fat-free diets were fed indicate that, although the

essential fatty acids are probably required by the chick, the requirement is quite small. They also indicate the likelihood that the chick at time of hatching is supplied with a sufficient quantity of the essential fatty acids through its body reserves and the unabsorbed egg yolk to meet its needs during the first few weeks of life. Therefore, the effect of the growth-stimulating properties of fat on chicks fed either purified, fat-free diets or practical diets of low-fat content appears to be caused by some other characteristic of fat.

The growth-stimulating effect of fat has also been observed frequently in experimental work with the rat, using diets that were adequate in essential fatty acids. The effect has been obtained both in experiments in which ad libitum feeding was employed and in experiments in which variations in energy intake were avoided by the paired-feeding of isocaloric diets. The work has been reviewed by Deuel ('57).

In an attempt to develop a purified, high energy diet which would be more suitable for use in studies of chick unidentified growth factors, experiments were conducted with several vegetable oils, hydrogenated vegetable oil and lard. The results, which are presented in this report, provide further evidence on the growth-stimulating property of fat for the chick.

EXPERIMENTAL

In this investigation male White Plymouth Rock chicks, Cobb strain, were used in experiments 1, 2, 3, 5, 6 and 7. In experiments 4 and 8, chicks of the Cobb strain White Plymouth Rock hens, mated to Red Vantress males, were used. With the exception of experiments 5 and 7, these chicks were obtained from hens, maintained at the Cornell University poultry plant, which were fed a simplified diet composed essentially of yellow corn meal, soybean meal (50% protein), corn gluten meal and necessary vitamin and mineral additions. All chicks were identified with numbered wingbands and placed on experiment at approximately one day of age. The chicks were weighed by lot at the start and individually each week thereafter until the experiments were terminated.

The duration of the experiments was 4 weeks. The chicks were housed in electrically heated zinc-coated battery brooders equipped with wire-mesh floors to prevent coprophagy. Feed and water were supplied ad libitum. The feed consumed by each lot of chicks was recorded at time of weighing. Duplicate lots of chicks were subjected to each treatment in experiments 1, 3, 4, 5, 6, 7 and 8, and triplicate lots in experiment 2. In experiments 1 and 4, each lot contained 20 chicks, in experiment 2, 10 chicks, and in the remaining experiments, 14 or 15 chicks.

TABLE 1

Composition of basal diets

INGREDIENT	5% FAT	10% FAT	20% FAT
	%	%	%
Glucose (Cerelose)	53.34	45.21	28.93
Purified soybean protein [1]	31.05	33.38	38.06
Corn oil [2]	5.00	10.00	20.00
Cellulose [3]	3.00	3.23	3.67
DL-Methionine	0.70	0.75 [4]	0.88
Glycine	0.30	0.32 [4]	0.37
Mineral mixture [5]	5.43	5.84	6.65
Vitamin mixture [6]	1.16	1.25	1.42
2,6-Ditertiary butyl-4-methylphenol (BHT)	0.02	0.022	0.025
Metabolizable energy, Cal./100 gm	324	349	398
Protein, gm	27.9	30.0	34.2
Calories/gm protein	11.6	11.6	11.6

[1] Drackett Assay C-1.

[2] Mazola.

[3] Solka Floc.

[4] In experiments 6, 7 and 8 the methionine and glycine contents of the 10% fat diets were 0.84 gm per 100 gm and 0.36 gm per 100 gm respectively.

[5] The grams per 1000 gm of Analytical Reagent Grade chemicals in the mineral mixture were as follows: 396.13 $CaHPO_4$, 274.77 $CaCO_3$, 159.67 KH_2PO_4, 110.50 NaCl, 46.04 $MgSO_4$, 6.13 $FeSO_4 \cdot 7H_2O$, 6.13 $MnSO_4 \cdot H_2O$, 0.05 KI, 0.31 $CuSO_4 \cdot 5H_2O$, 0.18 $ZnCl_2$, 0.03 $CoCl_2 \cdot 6H_2O$, 0.15 $Na_2MoO_4 \cdot 2H_2O$.

[6] The grams or units per 1000 gm of vitamins, diluents and glucose in the vitamin mixture were as follows: 129.79 choline Cl, 21.63 inositol, 4.33 niacin, 3.46 *d*-Ca pantothenate, 5.71 α-tocopheryl acetate, 0.87 thiamine·HCl, 0.87 riboflavin, 0.39 pyridoxine·HCl, 0.35 folic acid, 0.083 menadione sodium bisulfite, 0.017 biotin, 0.0043 vitamin B_{12}, 431,000 I.U. vitamin A, 86,200 I.C.U. vitamin D, 1.90 diphenyl-*p*-phenylenediamine (DPPD), 121.14 vitamin A, D and E diluents, 709.53 glucose (Cerelose).

The composition of the basal diets fed the chicks in experiments 1, 2 and 3 is given in table 1. In composing the diets the protein was adjusted with each change in fat content so as to maintain a constant energy-protein ratio. A constant relationship between the energy content of the basal diets and the minerals and vitamins included in them was also maintained. The diets fed in experiment 4 contained the same ingredients as the basal diets of experiments 1, 2 and 3 and possessed the same energy-protein ratio and other energy relationships. The control diets supplied in experiments 6, 7 and 8 were identical with the basal diet containing 10% of corn oil used in experiments 1, 2 and 3. The metabolizable energy values of Anderson, Hill and Renner[3] were used in calculating the energy content of the purified diets. The protein content of all diets was somewhat in execess of known requirements. The soybean protein used in the diets was purified by repeated washings with tap water at the isoelectric point (pH 4.6) with a final washing with demineralized water, after which it was pressed and dried in a forced draft electrically heated oven at a temperature of 65°C.

In order to study the effect of quantity of fat on the response of chicks to unidentified growth factors (UGF), each of the diets used in experiments 1, 2, 3, 6, 7 and 8 was fed with and without 10% of a supplement (UGF) of crude sources of these factors. The supplement was composed of 50% of corn distillers' dried solubles, 25% of fish solubles and 25% of dried whey product. After mixing, these materials were dried and ground to 20 mesh. The dried mixture was added to the supplemented diets in such a manner as to maintain protein content and energy-protein ratios constant.

The oils and fat used in the investigation were corn oil,[4] soybean oil,[5] cottonseed oil,[6] peanut oil,[7] hydrogenated vege-

[3] Unpublished results, Cornell University Agricultural Experiment Station.

[4] Mazola.

[5] Capitano.

[6] Wesson.

[7] Planters.

table oil,[8] and lard.[9] The stripped corn oil and the distillates of corn oil nsed in experiment 8 were supplied by Distillation Products Industries. These fractions were prepared by molecular distillation. The stripped corn oil was composed largely of glycerides and represented about 80% of the original oil. The first molecular distillate consisted of the most volatile 10% of the oil and the second distillate the second most volatile 10%. The distillates contained most of the non-saponifiable material in the corn oil.

The treatments used in the experiments are given in the tables of results. Comparable treatments in experiments 6 and 7 were combined, since the differences between them were not significant. In tables 2, 5 and 6 are presented results showing the effect of supplying sources of unidentified growth factors in the basal diets. The experimental diets fed in experiments 4 and 8 contained 50 mg/kg of zinc rather than approximately 5 mg/kg. This increase was made because of the discovery during the course of the investigation that under conditions which eliminated the possibility of contact with zinc-coated utensils, 5 mg/kg of zinc was inadequate, and therefore might not always be adequate for chicks in zinc-coated battery brooders.

The results of each experiment were subjected to analysis of variance according to Snedecor ('56). Afterwards, Duncan's multiple range test (Federer, '55) was applied to the results where necessary in order to locate the sites of significant differences in growth.

RESULTS AND DISCUSSION

Experiments 1, 2 and 3 were conducted to determine if greater growth differences could be attained in chicks fed a basal diet only and those fed the diet supplemented with sources of unidentified factors, by increasing the energy content by means of corn oil. The results of the experiments are presented in table 2. The results showed that the growth of

[8] Wesson Oil and Snowdrift Sales Company, New Orleans, Louisiana.

[9] Snow Cap.

the chicks fed the diet containing 10% of corn oil was greater than that of the chicks supplied the diet containing 5% of corn oil but that no further increase was obtained by supplying the diet containing 20% of corn oil except in experiment 1, when the unsupplemented basal diet was fed. The gain per unit of feed consumed, however, improved with each increase in fat content. The growth increases obtained in experiments 1 and 2 were found to be highly significant statistically ($P < 0.01$ and $P < 0.025$, respectively). The average difference in growth of the chicks supplied the 5% corn oil basal diet and

TABLE 2

Effect of fat level on growth of chicks fed basal and supplemented diets

TREATMENT	AV. 4 WK. WT.			GAIN/FEED, AV. 3 EXP.
	Exp. 1	Exp. 2	Exp. 3	
	gm	*gm*	*gm*	
	Basal diet			
5% corn oil	391 (37)[1]	418 (29)	—	0.63
10% corn oil	414 (37)	451 (30)	450 (28)	0.69
20% corn oil	468 (38)	—	448 (29)	0.77
	Basal diet plus UGF[2]			
5% corn oil	479 (39)	485 (28)	—	0.66
10% corn oil	535 (40)	533 (28)	524 (30)	0.72
20% corn oil	535 (39)	—	510 (30)	0.81

[1] Number of survivors.

[2] Unidentified growth factor supplement.

that of the chicks fed the diet supplemented with sources of unidentified growth factors was 19.3% as compared to 21.2 and 14.2% for the other comparable treatments. No evidence was obtained, therefore, that the higher energy basal diet was more suitable for use in studies of unidentified growth factors.

Improved growth was obtained at all levels of corn oil and of energy content by supplementation with sources of unidentified growth factors. These growth increases in each of the three experiments were found to be highly significant ($P < 0.01$). The possibility that the better growth was due to the small amount of added zinc provided by the sources of

unidentified growth factors was ruled out by the results of experiment 7, in which the basal diet contained 50 mg/kg of added zinc. In this experiment an increase in growth amounting to 21% was obtained by supplementing the basal diet with the unidentified factor supplements. Although studies on the potassium requirement of chicks fed high energy diets indicate that all of the basal diets fed in these experiments were slightly deficient in potassium, subsequent work in which the potassium content of the diet was increased indicates that the response from the sources of unidentified factors was due only in part to a deficiency of this mineral.

TABLE 3

Effect of fat level on growth of chicks fed basal diets

(Experiment 4)

K:ME [1]	AV. WT., 4 WKS.		
	3% Fat	10% Fat	20% Fat
	gm	*gm*	*gm*
47.4	260 (25) [2]	282 (20)	309 (20)
59.5	436 (26)	458 (25)	410 (25)
70.8	501 (28)	530 (25)	518 (28)
112.6	526 (27)	590 (27)	597 (28)
141.4	572 (28)	610 (28)	600 (28)

[1] Ratio of milligrams of potassium per 100 Cal. of metabolizable energy.

[2] Number of survivors.

The discovery that increasing the corn oil content of the basal diet from 5 to 10% increased the growth rate of the chicks has been confirmed in experiment 4 on the effect of fat on the potassium requirement of the chick. In this work purified diets containing 3, 10 and 20% corn oil were fed. The results of the experiment are presented in table 3. The potassium-energy ratio of each potassium variable was maintained constant at all fat levels in order to assure uniform potassium intake. Regardless of the amount of potassium supplied, improved growth was obtained by increasing the fat content from 3 to 10%. The growth increase was highly significant ($P < 0.01$). No further increase in growth, how-

ever, was obtained by increasing the fat content from 10 to 20%.

A similar finding was made in experiment 5, designed to develop an improved practical chick diet. The results of the work are presented in table 4. The practical type diet fed the chicks was composed largely of corn and dehulled soybean meal and contained 3.4% of fat. When this diet was supplemented with 7.5% of soybean oil in such a way as to maintain the energy-protein ratio constant, the growth of the chicks at 6 weeks was increased from 953 to 1050 gm, and better feed efficiency was observed. The growth increase was found to be highly significant ($P < 0.01$).

TABLE 4

Effect of fat level on growth of chicks fed practical diets

(Experiment 5)

TREATMENT	AV. WT.		GAIN/FEED	
	4 wks.	6 wks.	4 wks.	6 wks.
	gm	*gm*		
Basal diet [1]	505	953 (38) [2]	0.53	0.48
+ 7.5% soybean oil	536	1050 (40)	0.63	0.56

[1] Contained 3.4% fat, mostly from corn.

[2] Number of survivors.

Experiments 6 and 7 were undertaken to determine if soybean oil, cottonseed oil, peanut oil, lard and hydrogenated cottonseed oil were approximately of equal value to corn oil in promoting chick growth. Corn oil, soybean oil and cottonseed oil were compared in experiment 6 and corn oil, soybean oil, lard and hydrogenated vegetable oil in experiment 7. All the fats were supplied in the diets at a level of 10%, and energy-protein ratios were maintained constant. The energy contents of the fats were calculated from the metabolizable energy values of Renner and Hill ('58). Soybean oil, cottonseed oil and peanut oil, due to similarity in chemical and physical characteristics, were assumed to have approximately the same metabolizable energy content as corn oil, since actual values were lacking at the time the experimental work

was conducted. Since lard contains about the same amount of metabolizable energy as the vegetable oils used in the study, except hydrogenated vegetable oil, the energy content and the protein content of the lard diet were almost the same as those of the diets containing the non-hydrogenated vegetable oils.

TABLE 5

Effect of various fats on growth of chicks fed basal and supplemented diets
(Experiments 6 and 7)

TREATMENT	AV. 4 WK. WT.		GAIN/FEED	
	Basal	+ UGF	Basal	+ UGF
	gm	*gm*		
10% corn oil (6,7)[1]	434 (56)[2]	586 (58)	0.66	0.70
10% soybean oil (6,7)	450 (56)	575 (57)	0.66	0.68
10% cottonseed oil (7)	438 (29)	605 (29)	0.65	0.69
10% peanut oil (7)	457 (28)	582 (30)	0.66	0.68
	445	587	0.66	0.69
10% lard (6)	438 (26)	534 (28)	0.63	0.65
10% hydrogenated vegetable oil (6)	407 (25)	503 (27)	0.61	0.61

[1] Experiment.
[2] Number of survivors.

The results of the experimental work, presented in table 5, showed that, either with or without sources of unidentified factors in the diets, all of the non-hydrogenated vegetable oils were of approximately equal value in promoting chick growth and improving feed efficiency, and that hydrogenated vegetable oil was much less effective. Lard, on the other hand, promoted growth equal to the non-hydrogenated vegetable oils in the absence of unidentified factors but was intermediate between the hydrogenated oil and the non-hydrogenated oils when the factors were supplied. The differences in growth obtained in experiment 7 between the chicks fed the supplemented diet with corn oil and soybean oil and those fed the supplemented diet with hydrogenated vegetable oil were found to be highly significant ($P < 0.01$). The growth differences between the non-hydrogenated vegetable oils and lard were also found to be significant ($P < 0.05$). In a later experi-

ment, not reported herein, in which hydrogenated vegetable oil was compared with soybean oil, a growth depression was obtained in chicks fed the unsupplemented basal diet. The growth depression in this instance was found to be significant ($P < 0.05$). The results showed, therefore, that the growth-promoting effect of vegetable oils is markedly reduced during or by hydrogenation.

The improved growth obtained in this investigation by increasing the quantity of non-hydrogenated vegetable oil in the chick diet from 5 to 10% was not due to lack of capacity of the chicks to eat more feed. Chicks fed the practical type diet (table 4) containing 7.5% of soybean oil attained approximately the same weight at 4 weeks of age as the chicks fed the purified diet (table 2) containing 10% of corn oil and sources of unidentified factors (536 vs. 531 gm), yet they consumed approximately 100 gm more feed per chick (789 vs. 682 gm). The greater quantity of feed consumed by chicks fed practical type diets in attaining growth weights comparable to those of chicks fed more concentrated purified diets has been frequently observed at this laboratory.

The results suggest that the improved growth of the chicks supplied 10% of corn oil as compared with 5% of the fat (table 2) was due to a more optimum concentration of energy unrelated to the capacity of the chick to consume feed. Absolute energy level, however, was not the determining factor since the quantity of energy per gram of feed in the practical diet (table 4) containing 7.5% of soybean oil was less than the concentration of energy per gram of feed in the supplemented purified diet (table 2) containing 10% of corn oil (3.36 vs. 3.49 Cal./gm) although growth of the chicks as just pointed out was almost identical. This possibility also appears to be ruled out by the results obtained with the supplemented diet by supplying 10% of lard (table 5) in comparison with those obtained with 10% of non-hydrogenated vegetable oils, as the energy content per gram of diet was approximately the same (3.45 vs. 3.49 Cal./gm) in both instances. In spite of this the lard diet failed to promote growth, when included

in the supplemented diet, equal to that obtained with the non-hydrogenated vegetable oils.

In view of the favorable results obtained by supplying 10% of corn oil, an attempt was made to determine if the growth-promoting effect was due to the glyceride fraction or to the most volatile fractions. The results of this work, presented in table 6, showed that either with or without a source of unidentified factors in the diets the glyceride fraction of corn oil promoted growth equal to that obtained with the intact

TABLE 6

Effect of corn oil fractions on growth of chicks fed basal and supplemented diets
(Experiment 8)

TREATMENT	AV. 4 WK. WT.		GAIN/FEED	
	Basal	+ UGF	Basal	+ UGF
	gm	*gm*		
10% corn oil	484 (29)[1]	588 (30)	0.75	0.71
10% stripped corn oil[2]	489 (30)	564 (29)	0.70	0.74
9% stripped corn oil + 1% of 1st molecular distillate[3]	476 (30)	562 (30)	0.73	0.71
9% stripped corn oil + 1% of 2nd molecular distillate[4]	484 (28)	573 (30)	0.72	0.69
8% stripped corn oil + 1% each of 1st and 2nd molecular distillate	486 (28)	564 (27)	0.73	0.71

[1] Number of survivors.
[2] Residue of first and second molecular distillations, chiefly corn oil glycerides, approximately 80% of original oil.
[3] First most volatile 10% of corn oil.
[4] Second most volatile 10% of corn oil.

corn oil. No further improvement in growth was obtained by adding the most volatile fractions of the corn oil to the glyceride fraction. These results showed that the improved growth obtained by supplying 10% of non-hydrogenated vegetable oils in the diet of chicks was due to the glycerides present in them, or to an unknown substance not readily removed by molecular distillation. Whether or not any of the growth-promoting effect of corn oil remained in the most

volatile fractions containing most of the non-saponifiable material, was not revealed by the results.

Since Scott, Amato and Bray ('58) have obtained evidence that both egg yolk fat and corn oil promote chick growth to approximately the same extent when fed on an isocaloric basis in isonitrogenous diets, the results obtained with the corn oil fractions appear to be contrary to the finding of Menge, Lillie and Denton ('57) who showed that the non-saponifiable fraction of egg yolk fat produced by cold saponification contains an unidentified chick growth factor. The discrepancy in results may be caused, however, by differences in the effect of saponification and molecular distillation upon separation of the growth factor from the glyceride fraction of fat, or by differences in the physical and chemical characteristics of egg yolk fat and vegetable oils.

The increased growth obtained by raising the quantity of corn oil in the diet from 5 to 10% also suggests the possibility that the lower specific dynamic action of fat in comparison with carbohydrate produced a saving in energy which was used in bringing about greater gains in weight. This has been called "associated dynamic action" by Forbes and Swift ('44) when determined at planes of nutrition above maintenance. The efficiency of energy utilization, however, was not improved in this work by increasing dietary fat as measured by gain in weight, but appeared to be increased by supplementation with unidentified factors. This is made evident by calculating the grams gain per 100 Cal. of metabolizable energy from the data presented in table 2. These values for the lots of chicks fed the basal diets containing 5, 10 and 20% of fat were 19.4, 19.7 and 19.3 gm, respectively, and for the supplemented diets 20.5, 20.8 and 20.5 gm, respectively. Therefore, the improvement in growth does not appear to have been due to the sparing effect of increased quantities of fat on energy output.

On the other hand, the increased caloric efficiency of the supplemented diets, because they promoted more rapid growth, may have been caused by a greater utilization of

metabolizable energy for gain in weight relative to maintenance than occurred when the basal diets were fed. The greater caloric efficiency of the supplemented diets may also have been caused by the fact that these diets were more nutritionally complete than the basal diets, as a consequence of which a better utilization of metabolizable energy was obtained through reduction of heat losses.

The possibility exists, of course, that some increase in the efficiency of utilization of metabolizable energy which is not measurable by growth occurred in this work. Evidence of this possibility has been obtained by Carew and Hill ('58) in experiments with chicks which showed that corn oil substituted on an isocaloric metabolizable energy basis for glucose[10] increases the efficiency of energy utilization. Using a procedure in which energy intake was equalized, no improvement in growth was observed on substituting 10 and 20% of corn oil for isocalorically equivalent amounts of glucose. The results of tissue analysis showed, however, that significantly greater energy gains consisting of greater fat gains and lower protein gains were produced by the diets containing corn oil.

Another possible explanation of the improved growth obtained by increasing the fat content of the diet from 5 to 10% is that it was caused by previously unrecognized growth-stimulating characteristics of some of the known components of the glyceride fraction of the vegetable oils studied in this work. Since the growth promoting properties of vegetable oil were decreased by the process of hydrogenation, it would seem that the unsaturated fatty acids of the glycerides were implicated, rather than the saturated ones. However, in preliminary work with coconut oil using the supplemented diet, as good growth was obtained with this highly saturated fat as was obtained with unsaturated vegetable oils, when both types were supplied at a level of 10%. Therefore, if the work with coconut oil is confirmed, it seems unlikely that the growth-promoting effect is due to one or more fatty acids, in view

[10] Cerelose.

of the widely differing composition of the unsaturated vegetable oils and coconut oil.

Since the completion of the experiments discussed in this report, Rand, Scott and Kummerow ('58) have presented further evidence of the growth-promoting properties of corn oil. Purified diets containing either 0.5 or 1.0% of corn oil were used in the investigation. On pair-feeding isocaloric diets, the inclusion of 7.5 to 8% or more of corn oil in them promoted growth increases which were reported to be statistically significant. In experiments in which non-isocaloric diets were fed, significant growth increases were obtained with 8.3% of corn oil both with ad libitum feeding and with equalized caloric intake, but no further increase was observed with either 18.1 or 30.7% fat. Rand, Scott and Kummerow ('58) assumed that 0.5% of corn oil is sufficient to meet the chick's need for essential fatty acids and concluded, therefore, that the effects observed in their work were not caused by a deficiency of these substances. Since previous experimental work supports this assumption, the results reported by these investigators and those presented in this report appear to be in close agreement.

SUMMARY

In experiments conducted with chicks highly significant growth increases were obtained by increasing the vegetable oil content of purified diets from 3–5 to 10% and practical diets from 3.4 to 10.1%. The responses were obtained with purified diets supplemented with sources of unidentified growth factors and with basal purified diets. The growth of chicks fed hydrogenated vegetable oil was significantly less than that of chicks fed an equal quantity of corn oil or soybean oil, indicating that the growth-promoting effect of the oil was markedly reduced during hydrogenation. Lard also appeared to have less of this effect when added to purified diets supplemented with unidentified factors. The fraction of corn oil composed chiefly of glycerides not readily subject to molec-

ular distillation was found to promote growth to approximately the same extent as the intact oil.

The evidence indicated that the improved growth was not due to a lack of capacity of the chicks to eat more feed, or to a more optimal concentration of energy unrelated to food capacity. The evidence also indicated that the response was not due to the lower specific dynamic action of fat making more energy available for growth. The effect, therefore, appeared to be caused either by an unknown substance not removed by molecular distillation or by heretofore unrecognized growth-promoting characteristics of some of the known components of fat.

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CHANGES IN TOTAL NITROGEN CONTENT OF SOME ABDOMINAL VISCERA IN FASTING AND REALIMENTATION[1]

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(Received for publication January 30, 1959)

The free amino acid mixture in the small intestine is not greatly affected by the test meal ingested (Nasset et al., '55; Nasset, '56, '57). A sufficient amount of endogenous protein appears to move into the lumen, from digestive glands and mucosa, to conceal peculiarities of amino acid composition of the test meal. If this assumption is valid the digestive tract should be capable of rapid protein synthesis and should contain a relatively large and mobile protein reserve. Protein loss as a result of fasting or ingesting a non-protein diet was extensively investigated by Addis et al. ('36a, b, '39a, b). They found that liver lost 20 and 40% of its protein in two and 7 days of fasting. Their gravimetric determination of "protein" included variable amounts of ash and possibly other non-protein material and was probably no more precise than the simple determination of total N as used in our investigation. Addis et al. included stomach, intestines, pancreas, spleen, bladder and abdominal fat under the heading of alimentary tract. The protein loss in this category, therefore, was not typical of a specific organ and it was reported to be only about three-fourths as great as that lost by liver.

[1] Aided by a grant from the National Institutes of Health.

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The experiments described below provide information on the loss and gain of N in specific digestive organs during fasting and realimentation.

METHODS

Male albino rats of the Wistar strain (255 to 295 gm) were caged individually in a room at 26 to 28°C. They were fed a complete commercial ration [3] for a week before being used for experiment. Control animals were taken directly from full feeding and anesthetized lightly with ether. Blood was taken by cardiac puncture after which the thorax was opened and the aorta and vena cava divided to allow most of the remaining blood to pool in the thorax. The abdominal cavity was then opened and pancreas, stomach, small intestine and liver, removed quickly in that order. Fat, mesentery and omentum were excluded from tissue samples. Stomach and gut were opened, their contents gently washed out in two changes of cold Ringer's solution, and blotted gently for one minute on a paper towel. These viscera were placed in bottles and weighed. Samples were taken later for moisture (100 to 105°C for 24 hrs.) and N determination (duplicate, micro Kjeldahl). Moisture and N of the whole animal, less samples mentioned above, were determined according to Miller and Bender ('55). Obviously total N yields only an approximation of the protein present in a tissue but, for comparative purposes and when the changes are as great as those listed below, the error introduced by this method is not great.

Animals were analyzed in the same way after various periods of fasting, consuming a non-protein diet, and realimentation subsequent to these procedures. Fasting rats consumed only water and were sacrificed at 24, 48, 96 and 192 hours. Others were fed a non-protein diet for 24, 48, 96, 192 and 384 hours. The course of early recovery from the effects of fasting or non-protein diet was followed by analyzing groups of animals after 24, 48 and 96 hours of realimentation. The recovery diet contained the following: egg albumin

[3] Purina Fox Chow.

TABLE 1

Nitrogen content of whole rats, blood and abdominal viscera after fasting

DETERMINATION	CONTROL [1]	FASTING			
		24 hrs.	48 hrs.	96 hrs.	192 hrs.
Number of rats	21	8	7	9	10
Body wt., initial, gm	293 ± 12 [2]	286 ± 3.6	284 ± 6.9	277 ± 3.6	284 ± 0.6
end of fast, gm		266 ± 7.2	244 ± 5.5	225 ± 0.4	193 ± 4.0
Whole rat, dried, gm	88.7 ± 1.39	82.9 ± 13.6	73.8 ± 1.61	68.1 ± 1.89	54.8 ± 1.43
Total N, gm	9.16 ± 0.14	8.76 ± 0.28	8.13 ± 0.15	7.93 ± 0.10	7.14 ± 0.17
Whole blood, mg N/100 ml	3.29 ± 0.02	3.34 ± 0.10	3.41 ± 0.07	3.44 ± 0.06	3.69 ± 0.11
Stomach wt., gm [3]	1.23	1.25	1.22	1.15	1.27
Total N, mg	34.8 ± 1.07	33.2 ± 0.15	31.6 ± 1.22	31.0 ± 1.31	31.9 ± 1.01
Small gut wt., gm	6.12	5.15	4.74	4.07	2.78
Total N, mg	155 ± 3.7	132 ± 6.2	124 ± 6.4	106 ± 5.1	72 ± 1.7
Pancreas wt., gm	0.87	0.83	0.68	0.61	0.53
Total N, mg	31.0 ± 1.2	28.7 ± 2.7	22.4 ± 2.1	20.8 ± 1.1	17.2 ± 2.3
Liver wt., gm	11.5	8.60	7.44	7.24	4.89
Total N, mg	375 ± 7.0	317 ± 5.2	278 ± 7.6	263 ± 7.2	186 ± 1.3

[1] Taken directly from food and sacrificed.

[2] Mean ± standard error of the mean.

[3] Weights of viscera and their total N content were computed on the basis of a standard metabolic body size of 0.380 $kg^{3/4}$ which corresponds to a body weight of 275 gm.

12.8%, sucrose 56.9%, cottonseed oil 17.4%, salts (U.S.P. XIII) 4.5%, vitamin mixture 2.6% and cellulose flour 5.8%. The non-protein diet was made by omitting egg albumin and adding an equal weight of sucrose. The animals were allowed 121 Cal./day/$kg^{3/4}$, based on their initial non-fasting body weights, which is enough for maintenance of body weight. Food was usually more than 95% consumed.

RESULTS

Table 1 summarizes the results obtained from control and fasted animals. In this and subsequent tables the body weights and total N in the whole rat and blood are the averages as determined directly. The changes in stomach, small gut, pancreas and liver were of primary interest and therefore their weights and N contents were computed on a comparable basis. The mean body weight of all rats used was 275 gm and hence the "standard rat" was taken as one of equivalent metabolic body size, i.e., 0.380 $kg^{3/4}$, and the observed values were corrected accordingly. The stomach gave up very little of its N in 192 hours of fasting but the other three organs lost about half of their N under the same conditions and organ weights followed an approximately parallel course. Nitrogen in blood increased steadily as fasting progressed.

The effects of realimentation after fasting are presented in table 2. Whether the fast was 96 or 192 hours in length the responses to refeeding were qualitatively alike. The stomach and pancreas either failed to gain or lost weight and total N in the first day of refeeding; the small gut and liver began to recover at once. Four days of realimentation under these conditions was insufficient to permit recovery of original body weight or total N content. The concentration of N in blood decreased steadily during refeeding.

Feeding a non-protein diet was well tolerated for 96 hours. As indicated in table 3, body weights and total body N changed very little in that time. At the end of 384 hours, however, body weight was reduced 21% and body N 16%. The stomach, which resisted change during a fast (table 1), decreased stead-

TABLE 2

Nitrogen content of whole rats, blood and abdominal viscera after realimentation

REALIMENTATION [1]	AFTER 96 HRS. FASTING			AFTER 192 HRS. FASTING		
	24 hrs.	48 hrs.	96 hrs.	24 hrs.	48 hrs.	96 hrs.
Number of rats	8	9	8	6	7	9
Body weight, initial, gm	275 ± 7.5 [2]	278 ± 7.1	297 ± 8.9	273 ± 6.5	281 ± 4.8	282 ± 4.2
end of fast, gm	215 ± 5.2	221 ± 4.7	229 ± 5.3	180 + 4.1	176 + 7.0	186 + 4.2
end of realimentation, gm	232 ± 4.2	238 ± 5.1	255 ± 5.7	192 ± 5.8	217 ± 9.0	232 ± 4.0
Whole rat (dried), gm	71.0 ± 1.55	74.6 ± 2.85	80.3 ± 1.08	52.5 ± 0.97	61.2 ± 3.32	67.7 ± 2.71
Total N, gm	7.69 ± 0.10	7.66 ± 0.14	7.55 ± 0.34	6.31 ± 0.03	6.95 ± 0.17	7.04 ± 0.27
Whole blood, mg N/100 ml	3.38 ± 0.04	3.34 ± 0.21	3.30 ± 0.02	3.41 ± 0.07	3.32 ± 0.07	2.98 ± 0.22
Stomach wt., gm [3]	1.11	1.10	1.15	1.20	1.23	1.22
Total N, mg	29.9 ± 1.46	30.1 ± 0.49	32.0 ± 0.53	30.9 ± 0.45	33.0 ± 1.08	33.3 ± 0.88
Small gut wt., gm	4.70	5.04	5.68	3.95	4.93	6.07
Total N, mg	114 ± 3.3	136 ± 0.6	138 ± 3.9	94.7 ± 4.2	118 ± 3.2	154 ± 8.8
Pancreas wt., gm	0.69	0.77	0.81	0.60	0.61	0.74
Total N, mg	20.4 ± 0.53	24.6 ± 0.61	27.2 ± 0.64	17.2 ± 0.98	18.0 ± 0.82	22.9 ± 0.59
Liver wt., gm	10.2	9.85	9.80	9.00	12.2	13.4
Total N, mg	255 ± 0.6	272 ± 6.0	285 ± 3.7	212 ± 7.4	267 ± 12.4	318 ± 6.5

[1] Complete diet containing 12.8% egg albumin.

[2] Mean ± standard error of the mean.

[3] Weights of viscera and their total N content were computed on the basis of a standard metabolic body size of 0.380 $kg^{3/4}$ which corresponds to a body weight of 275 gm.

TABLE 3

Nitrogen content of whole rats, blood and abdominal viscera after feeding non-protein diet[1]

DETERMINATION	TIME ON NON-PROTEIN DIET				
	24 hrs.	48 hrs.	96 hrs.	192 hrs.	384 hrs.
Number of rats	13	13	14	6	6
Body weight, initial, gm	272 ± 4.4[2]	272 ± 3.3	266 ± 3.2	282 ± 2.2	281 ± 5.5
final, gm	259 ± 4.0	260 ± 4.0	253 ± 4.1	241 ± 5.4	221 ± 6.0
Whole rat (dried), gm	80.7	83.7	84.1	76.8	71.7
Total N, gm	8.46 ± 0.11	8.59 ± 0.14	8.42 ± 0.11	7.89 ± 0.11	7.82 ± 0.14
Whole blood, mg N/100 ml	3.35 ± 0.03	3.38 ± 0.06	3.44 ± 0.04	3.45 ± 0.04	3.24 ± 0.14
Stomach wt., gm[3]	1.16	1.13	1.06	0.99	0.90
Total N, mg	32.5 ± 1.65	31.3 ± 0.40	28.1 ± 0.47	26.5 ± 1.07	24.3 ± 0.45
Small gut wt., gm	5.96	6.01	5.48	4.73	4.22
Total N, mg	147 ± 4.7	148 ± 1.5	136 ± 1.2	117 ± 4.8	105 ± 6.0
Pancreas wt., gm	0.73	0.71	0.67	0.65	0.51
Total N, mg	23.0 ± 0.49	21.1 ± 0.81	20.5 ± 0.28	20.4 ± 0.76	15.8 ± 0.89
Liver wt., gm	10.6	11.0	9.85	8.87	7.73
Total N, gm	301 ± 2.9	287 ± 6.0	255 ± 11.4	242 ± 11.7	206 ± 11.8

[1] Complete diet except for absence of protein or other source of N.

[2] Mean ± standard error of the mean.

[3] Weights of viscera and their total N content were computed on the basis of a standard metabolic body size of 0.380 $kg^{3/4}$ which corresponds to a body weight of 275 gm.

TABLE 4

Nitrogen content of whole rats, blood and abdominal viscera after realimentation subsequent to non-protein diet

REALIMENTATION [1]	AFTER 192 HRS. ON NON-PROTEIN DIET			AFTER 384 HRS. ON NON-PROTEIN DIET		
	24 hrs.	48 hrs.	96 hrs.	24 hrs.	48 hrs.	96 hrs.
Number of rats	7	7	6	9	9	10
Body weight, initial, gm	246 ± 8.0 [2]	288 ± 2.6	277 ± 6.5	247 ± 6.1	254 ± 4.0	280 ± 2.7
end of non-prot. diet, gm	219 ± 5.9	258 ± 4.8	246 ± 11.6	205 ± 3.7	202 ± 4.1	238 ± 4.7
end of realimentation, gm	224 ± 5.8	264 ± 2.1	261 ± 9.5	209 ± 4.0	213 ± 4.5	252 ± 4.8
Whole rat (dried), gm	76.3 ± 3.35	84.2 ± 1.22	81.3 ± 2.45	74.3 ± 2.13	75.5 ± 3.91	79.2 ± 2.70
Total N, gm	7.42 ± 0.24	8.10 ± 0.19	8.49 ± 0.20	7.00 ± 0.14	6.92 ± 0.13	7.93 ± 0.23
Whole blood, mg N/100 ml	3.03 ± 0.08	3.14 ± 0.07	2.88 ± 0.04	3.11 ± 0.05	3.07 ± 0.05	2.92 ± 0.06
Stomach wt., gm [3]	1.03	1.05	1.27	0.99	1.08	1.15
Total N, gm	29.2 ± 0.69	28.8 ± 0.57	33.3 ± 0.75	26.8 ± 1.45	28.1 ± 0.71	29.8 ± 0.90
Small gut wt., gm	6.02	6.17	6.87	5.28	5.43	6.11
Total N, mg	147 ± 3.8	147 ± 5.7	167 ± 7.2	133 ± 2.5	138 ± 5.4	150 ± 5.3
Pancreas wt., gm	0.63	0.68	0.71	0.55	0.62	0.64
Total N, mg	18.4 ± 0.41	21.3 ± 0.98	22.1 ± 0.89	15.7 ± 0.78	18.2 ± 1.59	19.9 ± 0.73
Liver wt., gm	10.1	10.2	10.5	10.5	9.91	9.93
Total N, mg	261 ± 8.9	268 ± 8.4	285 ± 8.0	253 ± 6.0	264 ± 11.2	266 ± 0.6

[1] Complete diet containing 12.8% egg albumin.

[2] Mean ± standard error of the mean.

[3] Weights of viscera and their total N content were computed on the basis of a standard metabolic body size of 0.380 $kg^{3/4}$ which corresponds to a body weight of 275 gm.

ily in weight and N content. The same was true of the pancreas. The small gut and liver were not much affected early but at the end of the non-protein diet they were, like the stomach and pancreas, reduced to roughly two-thirds or three-fourths of their original weight and N content.

Table 4 shows that realimentation during 4 days with the egg albumin diet, after the non-protein diet, leads to prompt and complete recovery of N by the small intestine. The liver and stomach recoveries are slower and less complete. Pancreatic recovery is delayed beyond the first 24 hours and the first response appears to be a diminution of total N content.

DISCUSSION

In 192 hours of fasting the rats lost an average of 2.019 gm of their total N. The 4 abdominal viscera studied lost 0.289 gm N in this time and together weighed 19.74 gm at the beginning. These organs, representing only 7.2% of the body weight, contributed 14.3% of the N that was lost. The small gut, liver, pancreas and stomach lost respectively 54, 51, 45 and 8% of their total N. It seems logical to associate these losses with atrophy of disuse but the behavior of the stomach failed to fit this concept. Perhaps it may continue in fasting to synthesize nitrogenous compounds, such as intrinsic factor, which are not directly related to processes of digestion. What is the basic stimulus for degradation of tissue protein in starvation? Is it related to lowered amino acid concentration in the blood and, if it is, why is it not effective in the stomach? Histologic sections of the small gut were prepared in an attempt to localize the loss of N. Both mucosal and muscular layers seemed to get thinner in starvation but sectioning, fixing and staining artifacts made quantitative measurements unfeasible.

Realimentation after fasting or ingestion of a non-protein diet affected the viscera in different ways. It is obvious that the classical protein-sparing effects of carbohydrate and fat do not occur in the stomach and pancreas. These and other effects are best illustrated in figure 1 in which percentile

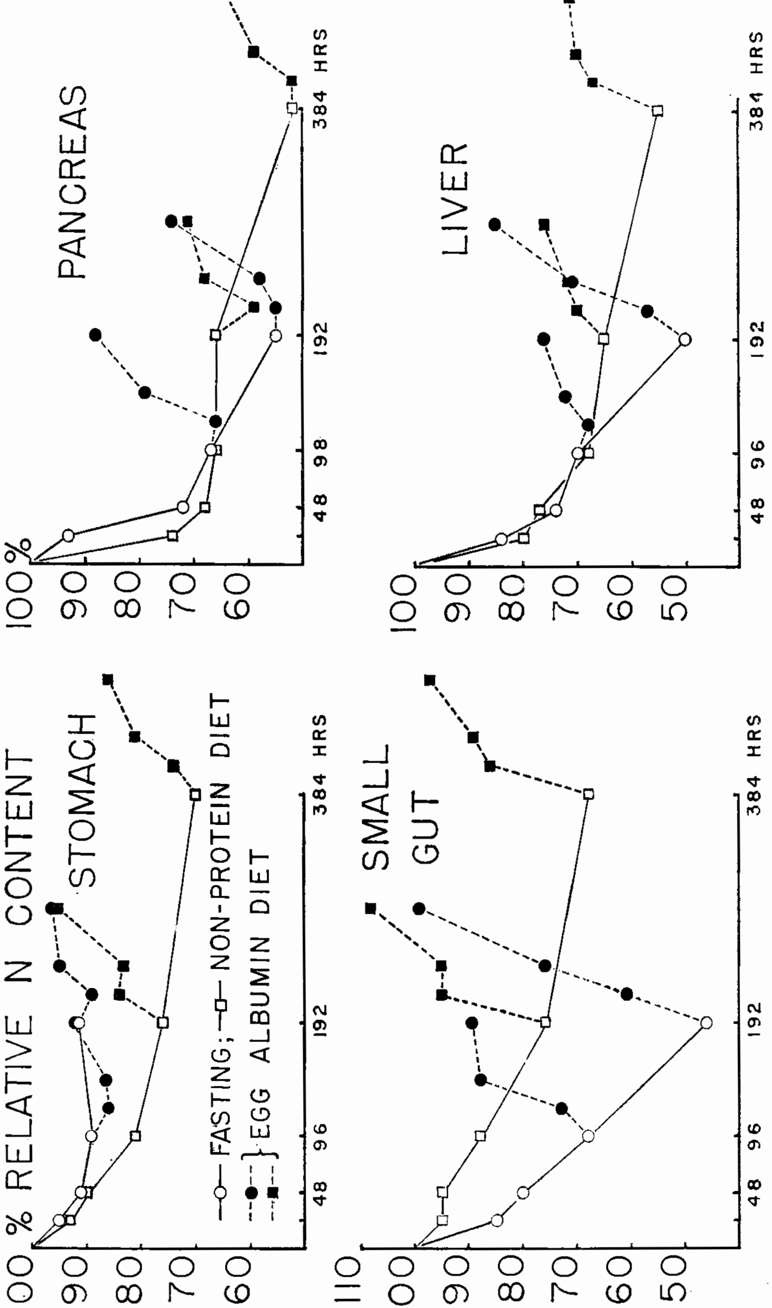

Fig. 1 Relative changes in N content of stomach, pancreas, small gut, and liver in fasting and after feeding non-protein or complete diet, containing egg albumin as source of protein.

change from control in total N content is plotted against time. The stomach consistently lost more N during non-protein diet feeding than in fasting. The immediate effect on the pancreas was a very severe loss of N which slowed considerably after 96 hours of non-protetin diet. The stimulus of food ingestion doubtless caused both stomach and pancreas to secrete more enzymes and mucoproteins than in the fasting state.

The small intestine exhibited some interesting variations in response. Ingestion of the non-protein diet was accompanied by a striking sparing of N in this organ. The food stimulus to secretion must have been adequate here as well as in the stomach and pancreas but the essential difference lies in the dual function of the gut, namely, simultaneous secretion and absorption. The data suggest that the intestinal mucosa retains amino acids which are en route from lumen to portal blood. When the animal eats a non-protein or any other type of diet the salivary glands, stomach, pancreas and small intestine contribute to the gut contents a variety of endogenous proteins which are probably hydrolyzed and largely recovered. Certainly very little protein is lost in the stool. The intestinal mucosa, by virtue of its absorptive function, has first choice of the amino acids as they pass through. During realimentation recovery of N was most rapid in the small gut and it was more than 90% complete in 96 hours.

If the small gut removes amino acids from the assortment being absorbed, the amino acid pattern of portal blood could differ from that of the intestinal contents. Scanty data now available lend some support to this idea. Denton and Elvehjem ('54) analyzed portal blood for amino acids two and one-half hours after feeding zein to a dog. Their data were used to compute the relative molar concentrations of the amino acids in portal blood. These values were compared with those reported by Nasset el al. ('55) for composition of jejunal contents three hours after feeding zein to a dog and were found to be different. Among the essential amino acids the greatest discrepancies were in the relative concentrations of the leucines and valine. This problem is more difficult than

is suggested by this simple comparison. The amino acids in the portal blood represent an unknown mixture of those supplied by arterial blood and those contributed by the process of absorption. The curves in figure 1 are based on fasting animals or those ingesting a non-protein diet. In the normal animal the stimulus to intestinal secretion exists whenever feeding occurs and the mucosa may always withdraw from the absorption stream some of the amino acids required to resynthesize the enzymes and other proteins that were secreted by the mucosa in response to feeding.

Figure 1 indicates that 96 hours of realimentation induced recovery of N most rapidly and most completely in the small gut. The liver was next and the stomach and pancreas seemed slowest to start. This order can almost be inferred from the anatomy of the circulatory system. The gut has first choice of amino acids and transports the excess through the mucosal cells to the portal vein from which the liver withdraws some of the remainder. The amino acids which escape the liver into the hepatic vein are then distributed in the pulmonary and systemic circulations to other organs and tissues. This means that stomach and pancreas must compete with the rest of the body for their share of the amino acids required to fill their depleted stores of nitrogenous compounds. It is regrettable that it is necessary at present to deal with such a non-specific item as total N. The results suggest, however, that large and rapid movements of protein and amino acids must occur in these abdominal viscera. Observations of changes in specific enzyme activity, such as amylase or proteinase, would help in more detailed interpretation of the data in regard to secretion. Secretions contribute an important share of the endogenous protein found in the gut lumen during digestion. Another important source of endogenous protein is sloughed mucosa. Judging from the relative frequency of mitoses Leblond and Walker ('56) estimated that all of the protein in the mucosa is turned over in less than three days. They stated that the mass of mucosal cells released into the human gastrointestinal tract may be as much as one-half pound per day.

SUMMARY

1. Total N was determined in stomach, pancreas, small gut and liver of rats after 24, 48, 96 and 192 hours of fasting. After 192 hours of fasting the greatest loss of N (54%) occurred in the small gut and the smallest loss (9%) occurred in the stomach.

2. Identical determinations were made after feeding a non-protein diet for the same intervals plus another of 384 hours. The small intestine in 192 hours lost less N (24%) than in fasting and the stomach lost more (24%). In pancreas and liver the differences between fasting and feeding non-protein diet were not so pronounced.

3. Realimentation always brought prompt and complete recovery of N content of the small gut. Stomach and pancreas either lost more N or failed to gain in 24 hours of refeeding. Liver began to recover promptly but not as rapidly as the small gut.

4. The data suggest that the small intestine during absorption withdraws amino acids directly for its own replenishment and transports the excess into portal blood from which the liver has the next opportunity to withdraw amino acids. The stomach and pancreas, being dependent on the systemic circulation for their supply of amino acids, are the slowest to recover their stores of N.

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EFFECT OF POTASSIUM IODIDE AND VITAMIN A ON THE THYROIDAL UPTAKE AND URINARY EXCRETION OF I^{131} IN GOITROUS SUBJECTS ON THE ISLAND OF KRK

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(Received for publication January 27, 1959)

The incidence of goitre on the island of Krk, the only island on the Adriatic where endemic goitre is found, varies quite sharply in its intensity between the various parts of the island. Whereas the northern regions show an incidence of goitre among the adult population as high as 46%, there is much less goitre toward the southern parts of the island with an almost goitre-free area in the southwestern region. In a recent paper about the examination of goitre on this island (Buzina et al., '59) we reported that the radio-iodine uptake curves showed an increased avidity of the thyroid for iodine. The maximum I^{131} uptake for 24 hours varied between 36 and 57% of the administered dose, being the lowest in the non-goitrous southwestern areas and the highest in goitrous northern areas. All those values of I^{131} uptake were higher than the values for normal subjects from a nonendemic area in Zagreb, indicating that the whole area of the island of Krk may be considered as an area of disturbed iodine metabolism. The difference in the I^{131} uptake seemed not to be because of the difference in the exogenous intake of iodine since a calculation of the daily iodine supply from foodstuffs consumed in those areas showed no substantial variation.

In a previous paper, Horvat and Maver ('58) reported that the vitamin A supply varied greatly between the goitrous and non-goitrous areas of the island, suggesting that a deficiency of vitamin A in the northern parts could be a factor in the development of goitre. In attempting to examine the role of vitamin A in the development of goitre, they administered 3,000 I.U. of vitamin A to the school children of the town of Krk and after three months of treatment they obtained a reduction of 45% in the incidence of goitre, as compared with a control group.

The possibility that vitamin A might play a role in the etiology of goitre on the island was studied by following the I^{131} uptake in goitrous subjects on the island before and after the treatment with vitamin A and iodine.

The last substance was introduced to test the influence of KI on the thyroid function of goitrous subjects from this part of the island where disturbances of iodine metabolism had already been established (Buzina et al., '59). This was of interest since iodination of table salt has recently become obligatory in this country.

MATERIALS AND METHODS

Twenty-one goitrous subjects without clinical symptoms of hyper- or hypothyroidism were hospitalized. Eighteen of them had an enlarged thyroid of grade I, one subject had goitre of grade II, and two subjects were with nodular goitre, according to WHO's Goitre Commission classification ('53).

After administration of I^{131} the 48-hour uptake curve was followed and 48-hour urine was collected. Then, the subjects were divided into two groups, one of 11 subjects treated with 4,000 I.U. of vitamin A daily and the second one of 10 subjects receiving 150 μg of KI daily. After three months of administration of vitamin A and KI respectively, another dose of I^{131} was given and the 48-hour thyroid uptake and urinary excretion were determined.

The I^{131} uptake was determined by means of a Geiger-Mueller Bismuth cathode. The determination of radio-iodine in urine was done in a Marinelli beaker.

RESULTS

Among 11 subjects in the group having received vitamin A, three subjects showed a lower I^{131} uptake than before the treatment, whereas all the 10 subjects of the second group treated with iodide showed a lower uptake of radio-iodine. Two subjects in the vitamin A-treated group showed a decrease in the size of their goitre.

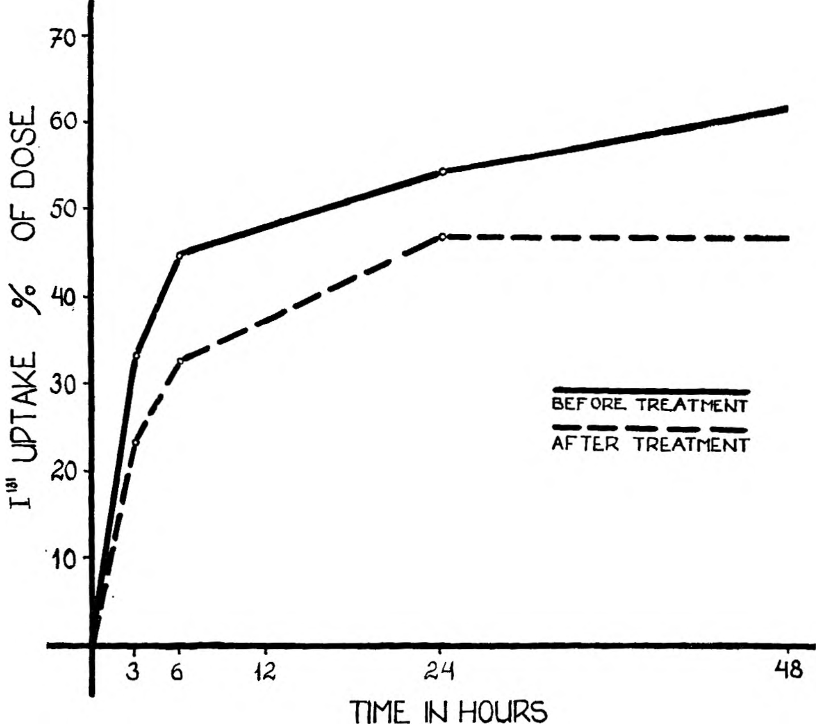

Fig. 1 The thyroidal I^{131} uptake before and after three months of treatment with 4,000 I.U. of vitamin A.

The I^{131} uptake curves for the two groups of subjects before and after treatment with vitamin A and iodine respectively are shown in figures 1 and 2. The first group (fig. 1) represents the 11 subjects whose initial I^{131} uptake reached its maximum after 24 hours with a mean value of 59.8% and a standard deviation of $\pm$ 10.0. After three months of treat-

ment with 4,000 I.U. of vitamin A the radio-iodine uptake curve kept almost at the same level, being a little steeper at the first three- and 6-hour intervals. The mean 24-hour intake was 59.03% with a standard deviation of 9.26. The second group (fig. 2) represents 10 subjects who were treated with

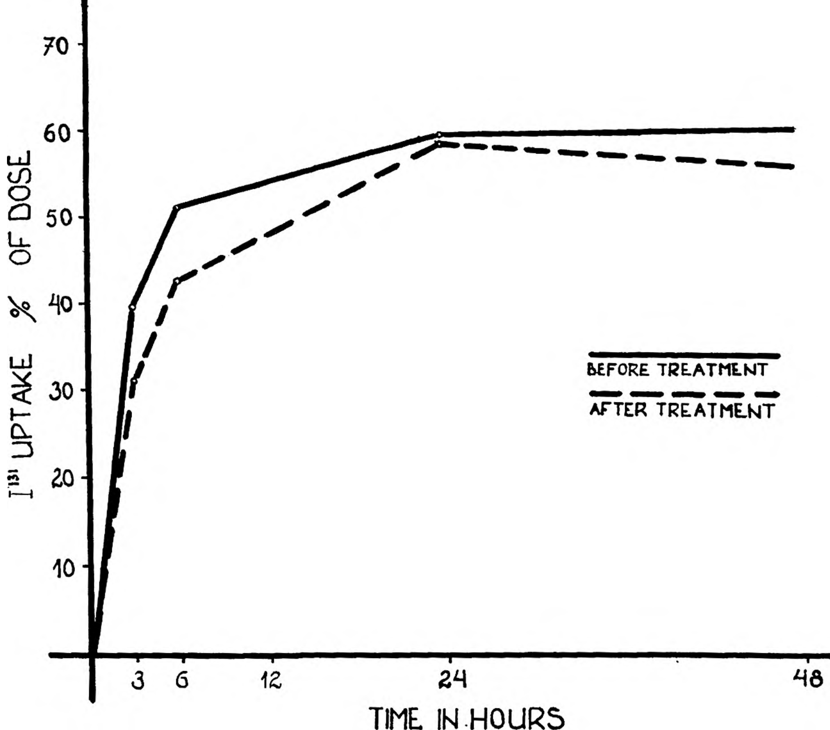

Fig. 2 The thyroidal I^{131} uptake before and after three months of treatment with 150 μg of KI daily.

150 μg of KI during three months. Before the administration of iodide the thyroid I^{131} uptake reached its maximum after 48 hours showing that 61.9% ± 9.5 of the administered dose had been trapped by the thyroid. After three months of iodide intake the iodine uptake was less rapid and there was a tendency towards a new plateau at the level of 46.7% ± 5.8 of the administered dose.

The 48-hour urinary radio-iodine excretion is shown in table 1. There was a slight increase after three months of treatment with vitamin A (about 4%) while an increase of about 9% occurred in the group having received 150 µg of KI.

TABLE 1

The 48-hour urinary I^{131} excretion in subjects after three months of treatment with vitamin A and iodine respectively

	URINARY I^{131}	
	Before treatment	After treatment
	%	%
Vitamin A-treated group (4,000 I.U. daily)	35.6	39.5
Iodine-treated group (150 µg KI daily)	39.6	48.1

DISCUSSION

The relation of vitamin A to the development of endemic goitre is still obscure. That a deficiency of vitamin A and carotene might be considered as an etiological factor has been reported by many authors (Richard, '51; Haubold, '50; Eggenberger, '54) but very few controlled experiments in which the beneficial influence of vitamin A on the reduction of goitre in humans are described (St. Loup, '52; Horvat and Maver, '58). The failure of vitamin A administration to reduce goitre in school children was reported by Scrimshaw ('57).

If the lack of an essential metabolite or the presence of a goitrogenic agent is the cause of hyperplasia of the thyroid gland, and consequently followed by increased avidity of the gland for iodine, a correction of these factors should reduce the avidity of the gland for iodine showing a tendency of the I^{131} uptake curve to reach a lower plateau.

In a study on endemic goitre in Mendoza, Stanbury and his colleagues ('54) were able to show a close relationship between iodine deficiency and the incidence of goitre in that area. With an administration of KI in amounts of 150, 500, and 1,500 µg daily to goitrous subjects, the I^{131} uptake curve

showed a return to a new equilibrium. The net fall after a daily dose of 150 μg was 18% in 50 days on an average.

In a recent study on goitre on the island on Krk (Buzina et al., '59) we showed that goitre had appeared in areas where the I^{131} uptake exceeded 41% in 24 hours. Thus the new desirable plateau for the goitrous areas of the island should at this point reach an equilibrium if at least hyperplasia of the gland, as a visual sign of disturbed iodine metabolism, is to be prevented. From figures 1 and 2 it is evident that the iodine supply was able to reduce the 24-hour I^{131} uptake bringing it to a new plateau of 46%. The vitamin A intake, however, showed no effect on the thyroidal 24-hour I^{131} uptake.

According to these results the role of vitamin A deficiency, as a possible goitrogenic factor in the development of goitre in these parts of the island remains undetermined. But, as already mentioned, the iodine suppply does not differ in the various parts of the island and the existence of some factors disturbing the iodine utilization is still suspected. The tendency of iodine to lower the I^{131} uptake curve toward a new plateau indicates, however, that the action of those factors can be counteracted by additional supply of iodine.

SUMMARY

The effect of iodine and vitamin A on the I^{131} thyroidal uptake and urinary excretion among goitrous subjects from the island of Krk was studied during a period of three months. The administration of 150 μg of KI daily caused a 15% reduction in the I^{131} uptake of 10 subjects, whereas a daily supply of 4,000 I.U. of vitamin A reduced the I^{131} thyroidal uptake in only three out of 11 subjects without decreasing the average values of the group as a whole.

At the end of the study the urinary excretion of I^{131} was 8.5% higher in the group having received 150 μg of KI versus an elevation of 3.9% in the group receiving 4,000 I.U. of vitamin A.

The results of this study, using radioactive iodine, do not support the suggestion that a low intake of vitamin A is a factor in the occurrence of goitre on the island of Krk.

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INFLUENCE OF DIETARY POTASSIUM AND SODIUM ON CESIUM-134 AND POTASSIUM-42 EXCRETION IN SHEEP [1,2,3]

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(Received for publication February 9, 1959)

Since cesium is one of the more important long-lived fission products, there has been interest in obtaining data on the metabolism of this element in the animal body.

Cesium, as an alkali metal, might be expected to behave similarly to sodium, potassium and rubidium, chemically. Cesium and potassium are known to enter the solute complex, participating in ion antagonism, osmosis, permeability regulation, maintenance of the colloidal state and similar physiological phenomena (MacLeod and Snell, '50). An increase in dietary potassium has been reported to result in an increased excretion of cesium-134 in the rat (Mraz and Patrick, '57), rabbit (Williams and Patrick, '57) and pig (Mraz et al., '58). Mraz et al. ('57) reported that dietary potassium increased the rate of excretion of body cesium in the rat even when the diet was deficient in sodium, while dietary sodium increased its excretion only when the diet was adequate in potassium.

[1] This manuscript is published with the permission of the director of the University of Tennessee, Agricultural Experiment Station, Knoxville.

[2] This work was completed under contract no. AT-40-1-GEN-242 between the University of Tennessee, College of Agriculture and the Atomic Energy Commission.

[3] The radioactive materials used in this work were obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Comission.

This study was initiated to ascertain if dietary sodium and potassium would similarly influence cesium-134 and potassium-42 excretion in sheep.

EXPERIMENTAL

The two basal rations, one a purified and the other a natural feedstuffs ration, shown in table 1, were used in three metabolism experiments involving 48 yearling western crossbred wethers.

In the first experiment, 16 wethers were divided into 4 groups of 4 wethers each. One group was fed purified basal

TABLE 1

Sheep rations used

INGREDIENTS	BASAL 1	BASAL 2
Casein	4.0	—
Urea	3.5	—
Soybean oil meal	—	5.0
Cornstarch	25.0	—
Cerelose	30.0	—
Yellow corn	—	59.0
Alfalfa meal	—	10.0
Cottonseed hulls	—	25.0
Wood pulp	30.0	—
Corn oil	2.9	—
Mineral supplement	3.4 [1]	1.0 [2]
Vitamin supplement	1.2 [3]	+ [4]

[1] Composition of mineral mixture in grams: $CaHPO_4 \cdot 2H_2O$, 1350; $K_3C_6H_5O_7 \cdot H_2O$, 829; NaCl, 378; $CaSO_4 \cdot 2H_2O$, 184; $MgCO_3$, 143; $Fe(C_6H_5O_7) \cdot 6H_2O$, 58; $MnSO_4 \cdot 6H_2O$, 3; KI, 1.7; $CuSO_4 \cdot 5H_2O$, 0.7; $CoCI_2 \cdot 6H_2O$, 0.6; $ZnCl_2$, 0.5; CaF_2, 0.5; MoO_3, 0.085.

[2] Mineral mixture supplied per 100 lb. of ration: NaCl, 0.5 lb.; Ca, 0.166 lb.; P, 0.083 lb.; Mn, 0.0033 lb.; I, 0.00012 lb.; Fe, 0.00035 lb.; Cu, 0.00025 lb.; Co, 0.00010 lb.

[3] Vitamin supplement supplied per pound of ration: vitamin A, 9080 I.U.; vitamin D_2, 1135 U.S.P. units; α-tocopherol acetate, 5 mg; choline chloride, 454 mg; ascorbic acid, 0.13 mg; biotin, 0.32 mg; calcium pantothenate, 0.25 mg; folic acid, 1.67 mg; inositol, 0.84 mg; nicotinic acid, 0.17 mg; *p*-aminobenzoic acid, 83.5 mg; pyridoxine hydrochloride, 21.0 mg; riboflavin, 41.8 mg; thiamine hydrochloride, 21.0 mg; vitamin B_{12} triturate, 0.13 mg and 2-methyl-1, 4 naphthoquinone 8.35 mg.

[4] Irradiated yeast added at a level of 4 gm/100 lb. of diet supplying 720 USP units vitamin D_2 per lb. of ration.

ration 1, (table 1), containing 0.0086% of sodium and 0.0037% of potassium, and the remaining groups were fed this basal ration supplemented with 0.25% of sodium or 0.32% of potassium, or a combination of the two, all in the chloride form. These ingredients were substituted for cornstarch in the basal ration. The sheep were maintained on their respective rations for two weeks before receiving two millicuries (mc) of potassium-42 and 100 microcuries (μc) of cesium-134 orally. The potassium-42 dosage contained 18.8 mg of potassium while the cesium-134 dosage was carrier-free.

Sixteen wethers divided into groups of 4 wethers were also used in the second experiment. One group was fed purified basal ration 1 supplemented with 0.25% of sodium and the other groups were fed the sodium-supplemented ration with either 0.15, 0.30 or 0.60% of potassium in the chloride form. The sheep remained on their respective rations for a period of 12 days before oral administration of 2 mc of potassium-42 containing 10.6 mg of potassium and 100 μc of carrier-free cesium-134.

In the third experiment, the natural feedstuffs basal ration 2, shown in table 1, that contained 0.68% of potassium and 0.27% of sodium was fed to 16 wethers for a period of two weeks before dosing orally with 100 μc of carrier-free cesium-134. Immediately after dosing, 4 wethers each received 0, 0.68, 1.36 and 2.04% additional potassium in the citrate form in the basal ration daily for the duration of the trial.

In all three experiments, the wethers were fed and offered water twice daily. Excreta were collected daily for a period of 5 days after administration of the radionuclides, and the cesium-134 and potassium-42 content was determined as described by Mraz et al. ('58). For ease of recording, data have been reported utilizing a maximum of three significant figures and are not indicative of the sensitivity of the experiments. Standard deviations were determined by the method described by Dean and Dixon ('51), for small numbers.

RESULTS AND DISCUSSION

In the first experiment, the deficiency of potassium in the ration reduced the appetite of the sheep. They consumed an average of 1.7 kg of feed in 5 days as compared to 3.1 kg for those receiving potassium. Consequently fecal output was only 0.4 kg for the sheep on the potassium-deficient ration compared to 1.3 kg for those receiving potassium. A diuresis was evident in those sheep fed the potassium deficient ration. It may be observed in table 2 that, when potassium was added to the ration, both urinary and fecal potassium-42 and cesium-134 were significantly increased over the rations with no potassium supplementation. The addition of sodium to the ration supplemented with potassium significantly increased urinary and decreased fecal excretion of the radionuclides. Total excretion of potassium-42 also increased, while total excretion of cesium-134 was not appreciably affected.

As the potassium content of the ration increased in the second experiment, feed consumption also increased from a low of 1.5 kg of basal ration to a high of 3.5 kg of the 0.60% potassium-supplemented ration over the 5-day trial period. Fecal output increased from a low of 0.3 kg to a high of 2.0 kg on these rations. Data presented in figures 1 and 2 showed that there was greater excretion of both radionuclides as the potassium content of the ration increased. However, potassium-42 excretion did not appreciably increase until 0.30% of potassium had been added to the ration, while 0.15% of potassium sufficed to produce an appreciable change in cesium-134 excretion. The greatest increase in both potasium-42 and cesium-134 excretion was observed in the 0.60% level of supplementation, primarily due to an increase in urinary excretion of these radionuclides.

The natural feedstuffs ration proved to be more palatable to the sheep than was the purified ration, for they consumed an average of 6.2 kg of ration over the 5-day trial period and had an average fecal output of 4.2 kg. The level of dietary potassium had little influence on feed consumption or fecal

TABLE 2

Potassium-42 and cesium-134 content of excreta from sheep fed varying levels of sodium and potassium

SUPPLEMENT TO NA AND K DEFICIENT BASAL	48-HOUR K^{42} EXCRETION [1]			120-HOUR CS^{134} EXCRETION [1]		
	Urinary	Fecal	Total	Urinary	Fecal	Total
None	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	6.7 ± 2.8	5.0 ± 4.0	11.7 ± 4.1
0.25% Na	0.3 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	8.8 ± 1.0	6.1 ± 2.9	14.9 ± 3.7
0.32% K	0.9 ± 0.6	2.4 + 0.6	3.3 ± 0.2	14.3 ± 0.9	25.7 ± 2.6	40.0 ± 3.5
0.25% Na						
0.32% K	4.5 ± 0.7	1.1 ± 0.3	5.6 ± 0.8	20.5 ± 3.8	15.6 ± 4.8	36.1 ± 5.1

[1] Expressed as mean per cent ± standard deviation of orally administered radionuclide dose.

output. As the dietary potassium level increased from 0.68% of potassium in the basal ration to 2.72% of potassium on the highest level of supplementation, urine production increased from a low of 2.3 kg to a high of 6.9 kg. The addition of 1.36

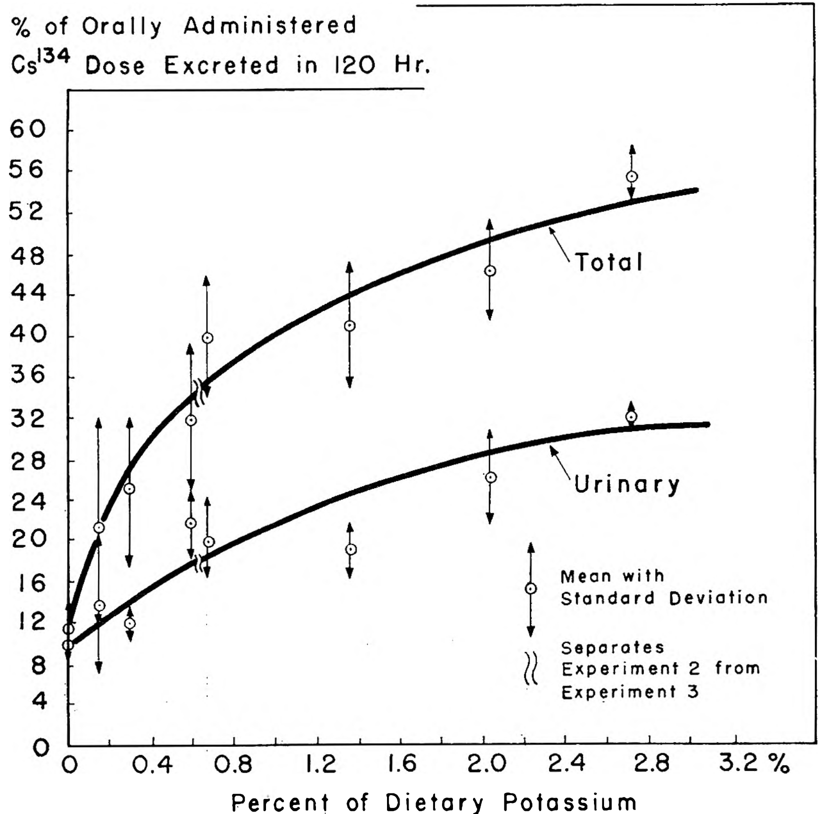

Fig. 1 Cesium-134 content of excreta from sheep fed varying levels of potassium.

and 2.04% of potassium to the basal ration increased total excretion of cesium-134, primarily from urinary excretion (fig. 1).

Therefore, it would appear that sheep react to potassium supplementation similarly to rats (Mraz et al., '57; Mraz and Patrick, '57), swine (Mraz et al., '58) and rabbits (Williams and Patrick, '57) by increasing excretion of cesium-134 and

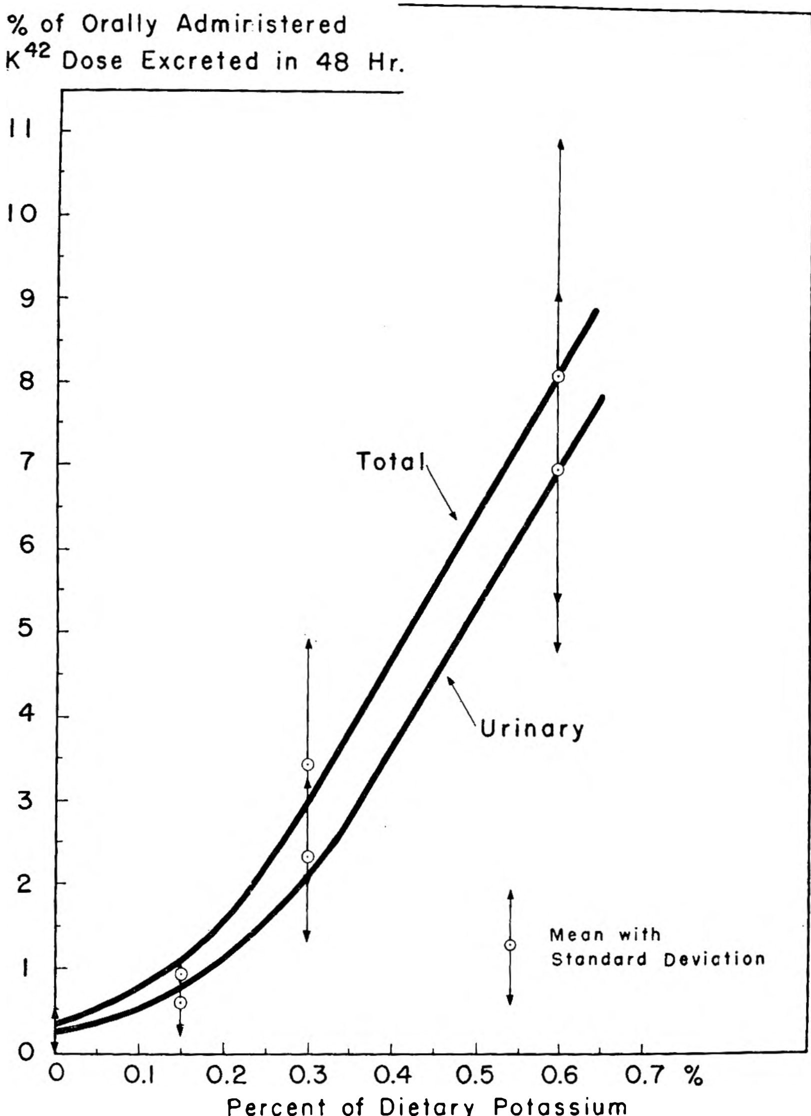

Fig. 2 Potassium-42 content of excreta from sheep fed varying levels of potassium.

potassium-42 when potassium is added to a potassium deficient ration. A deficiency of dietary sodium tends to prevent the absorption of potassium from the gut. The fecal cesium-134 and potassium-42 decrease when sodium is added to a

sodium deficient ration adequate in potassium whereas urinary excretion of cesium-134 and potassium-42 increases. Sodium supplementation had a greater influence on potassium-42 excretion than it did on cesium-134 under the above conditions. The addition of sodium to a potassium-deficient ration had little effect on excretion of either potassium-42 or cesium-134. These results clearly demonstrate that cesium excretion can be significantly increased by altering ration constituents. These findings are important in view of the biological interest in increasing the excretion of fission products which might be consumed accidentally.

SUMMARY

Forty-eight yearling wethers were used in the three investigations reported on to test the influence of dietary potassium and sodium upon the metabolism of orally administered potassium-42 and cesium-134 in sheep.

Dietary potassium significantly increased excretion of both potassium-42 and cesium-134 in both the presence and absence of dietary sodium. Dietary sodium did not appreciably affect excretion of potassium-42 and cesium-134 in the absence of dietary potassium but did change the excretory pattern of these radionuclides in the presence of dietary potassium.

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NUTRITION OF SALMONOID FISHES

VII. NITROGEN SUPPLEMENTS FOR CHINOOK SALMON DIETS [1]

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(Received for publication February 13, 1959)

INTRODUCTION

In the development of purified diets for amino acid studies with various animals it was found to be of advantage to supply non-essential amino acid nitrogen as a single compound. Numerous investigators have studied the conversion of nitrogen compounds to non-essential amino acids. Frost ('50) reported that the ability of the rat to convert essential amino acid nitrogen to non-essential amino acids was not as high as the ability to convert other sources of nitrogen to these compounds. Rose et al. ('49) indicated that ammonium compounds were more efficient than urea or glycine. Diammonium citrate has been used advantageously as a nitrogen supplement in studies with swine (Mertz, Beeson and Jackson, '52). More recently Birnbaum, Winitz and Greenstein ('57), working with rats, have made a very extensive study of individual amino acids, urea and ammonium acetate as sources of nitrogen. In these studies the nitrogen supplement compound supplied 62.3% of the nitrogen and the remaining 37.7% was supplied by the 10

[1] Journal Paper no. 1379 of the Purdue Agricultural Experiment Station. The experimental data in this paper are taken from a thesis submitted by Donald C. DeLong in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry, Purdue University.

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essential amino acids. These workers found that L-alanine, L-arginine·HCl, ammonium L-glutamate, L-glutamine, ammonium L-asparate and L-proline were the most efficient. Ammonium acetate was more effective for promoting growth than urea or glycine but was only 80% as effective as the most efficient compounds. Additional studies by these workers (Winitz, Greenstein and Birnbaum, '57) confirmed the high efficiency of L-arginine as a nitrogen source even in diets containing combinations of all of the non-essential amino acids in addition to the essential amino acids.

Recent work (DeLong, Halver and Mertz, '58) has indicated that the protein requirement of chinook salmon *(Oncorhynchus tshawytscha)* is very high in comparison with that of other animals. This high protein requirement greatly increases the expense of purified diets in which all components are defined. Such diets are, however, desirable for fundamental nutrition studies. For this reason, this work has been directed toward finding a relatively inexpensive source of nitrogen that is available in pure form. In addition to economical advantages, this study provides another area of nitrogen metabolism which can be compared with that of warm-blooded animals.

EXPERIMENTAL

The methods of diet preparation and general feeding techniques were the same as those previously reported by Halver ('57). The composition of the diets is shown in table 1. The basal diet contains 20% of crude protein made up of casein and gelatin supplemented with an amino acid mixture to give the balance of indispensable amino acids found in whole egg protein. Diet 1 contains 40% of this balanced protein. Diets 2 to 5 contain 20% of the balanced protein and 20% of "crude protein"[3] (N × 6.25) supplied only as the single compounds studied: L-arginine·HCl, glycine, urea or diammonium citrate. Diets 1 to 5 were isonitrogenous.

[3] The term "crude protein" is used here to indicate other sources of nitrogen.

For the feeding trial, 6 groups of two hundred chinook salmon each, averaging 3.5 to 3.7 gm were hand-counted into experimental hatchery troughs. Water from a spring at Hagerman, Idaho, which had a constant temperature of 58°F, was used in these experiments. The fish were fed a slowly-sinking diet expelled through a garlic press into the upper portion of the water. Each feeding was continued as long as

TABLE 1

Composition of nitrogen supplement diets

DIETS	BASAL	1	2	3	4	5
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
Casein	28.5	60.0	28.5	28.5	28.5	28.5
Gelatin	7.15	15.0	7.15	7.15	7.15	7.15
Amino acid mixture [1]	15.68	32.9	15.68	15.68	15.68	15.68
Dextrin	138.67	82.1	107.95	94.92	120.61	72.77
Corn oil	12.5	12.5	12.5	12.5	12.5	12.5
Cod liver oil	5.0	5.0	5.0	5.0	5.0	5.0
Minerals [2]	10.0	10.0	10.0	10.0	10.0	10.0
CMC [3]	25.0	25.0	25.0	25.0	25.0	25.0
Vitamins [2]	7.5	7.5	7.5	7.5	7.5	7.5
Water	250.0	250.0	250.0	250.0	250.0	250.0
Arginine·HCl			30.72			
Glycine				43.75		
Urea					18.08	
Diammonium citrate						65.9

[1] The same as in diet used for protein requirement studies (DeLong, Halver and Mertz, '58). This mixture balances the "essential" amino acid content of casein and gelatin to the balance found in whole egg protein.

[2] Mineral and vitamin mixtures the same as reported previously (Halver, '57).

[3] Carboxymethylcellulose.

fish accepted food. Fish were fed three times daily on a rigid schedule. The entire population of each trough was weighed bi-weekly. Troughs were cleaned daily without removing the fish, and were drained, cleaned and disinfected during the bi-weekly weighing period.

At the end of the experimental period (6 weeks), samples were taken for gross examination of various organs and tissues, for histological examination and for proximate analysis (method of Wood et al., '57).

RESULTS

The average individual weight gains obtained in 6 weeks with the use of the diets containing nitrogen supplements are summarized in table 2. The bi-weekly weight gains are plotted in figure 1. The 20% protein diet was the basal diet of this experiment and it can be seen that a supplement of an additional 20% of the balanced protein greatly increased growth. Weight gains on the 40% balanced protein diet are 2.7 times the weight gains on the 20% balanced protein diet (basal diet). When the 20% balanced protein diet was sup-

TABLE 2

Growth and mortality of experimental fish

DIET	AV. INITIAL WT.	AV. FINAL WT.	AV. GAIN [1]
	gm	*gm*	*gm*
Basal	3.63	4.41	0.78 (1)
1	3.71	5.81	2.13 (1)
2	3.52	5.10	1.58 (1)
3	3.57	4.69	1.12 (0)
4	3.64	4.35	0.71 (3)
5	3.67	3.47	− 0.20 (2)

[1] Fish mortality within parentheses.

plemented with 20% of crude protein (crude protein = nitrogen content × 6.25) as L-arginine·HCl, the average gain was 2.0 times that obtained on the basal diet. A 20% crude protein supplement, as glycine, also showed some stimulation and gave an average gain that was 1.4 times that with the basal diet. An urea supplement of the same magnitude had no significant effect on weight gain. A supplement of diammonium citrate caused a weight loss as shown in figure 1.

Mortality on all lots of fish was very low as shown in table 2. Representative gross post-mortem examination, histological examination and proximate analysis (see Wood et al., '57) failed to show any change among the lots of fish.

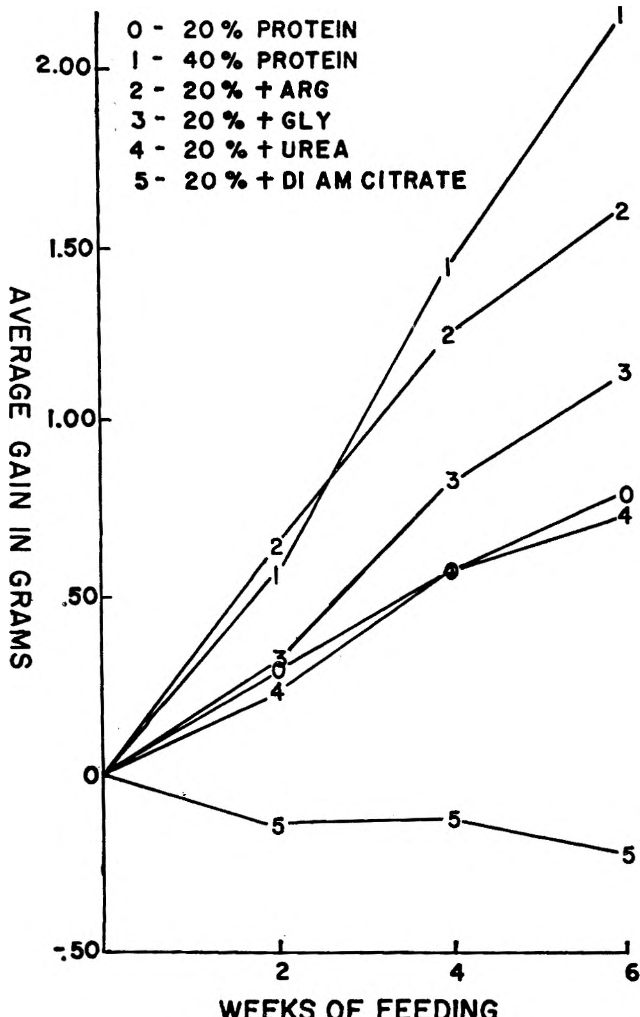

Fig. 1 Growth of experimental fish.

DISCUSSION

The diets used in this study with salmon differed from those used in cited work with rats since the present diets had some whole protein which supplied a portion of the non-essential amino acids. It is still of interest to compare the utilization of the compounds used as nitrogen supplements

in fish with their utilization in rats (data of Birnbaum, Winitz and Greenstein, '57). Arginine produced maximum gains as a supplementary compound in both rats and fish. Growth on the arginine diet containing 40% of crude protein (diet 2) was not as good as that on the 40% balanced protein (diet 1), which might indicate that one of the essential amino acids in the 20% balanced protein diet was limiting. However, it could also indicate that, if the amino acids are in perfect balance, a diet containing more than 20% of the balanced protein is required for maximum growth. This needs further study.

The ability of the fish to use glycine was about equal to that observed for rats. Urea in the diet of the rat permitted 40% of the weight gain obtained with arginine-supplemented rations (Birnbaum, Winitz and Greenstein, '57) whereas urea had no growth-promoting action in these studies on fish. Ammonium salts, when used as a supplement to the basal diet in rats, gave weight gains equal to 80% of that obtained with arginine-supplemented rations. In contrast, ammonium salts depressed growth of the fish. These data indicate that the chinook salmon is not able to utilize urea or diammonium citrate as a source of nitrogen for growth.

SUMMARY

The efficiency of L-arginine·HCl, glycine, urea, and diammonium citrate as nitrogen supplement compounds in chinook salmon diets was studied. Six lots of 200 fish each, average weight 3.8 gm, were used. Diets containing 20% balanced protein and 40% balanced protein were compared with diets containing 20% balanced protein and 20% "crude" protein as L-arginine·HCl, glycine, urea or diammonium citrate. Chinook salmon appeared similar to other animals in the ability to convert arginine and glycine to non-essential amino acids, but in contrast to other animals, seemed unable to convert urea or diammonium citrate to these compounds for growth.

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FURTHER OBSERVATIONS ON FACTORS AFFECTING L-CYSTEINE DESULFHYDRASE ACTIVITY IN CHICK LIVER

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(Received for publication February 18, 1959)

The presence of cysteine desulfhydrase in microorganisms (Kallio, '51; Delwiche, '51) and mammalian liver (Braunshteĭn and Azarkh, '51) has been known for a number of years. Recently the presence of the enzyme has been demonstrated in chick liver, with evidence to indicate that the nature of the ration fed may affect the level of activity (Goswami et al., '57).

Since it has been suggested that changes in the quantities of enzymes may be a general means by which metabolism is regulated (Knox, '51) the present study was initiated to investigate the effect of dietary, hormonal and stress factors on the activity of cysteine desulfhydrase. The data presented here show that its activity was markedly influenced by alteration of the nutritional status of the chick, by the administration of a number of hormones and by subjecting chicks to stress.

MATERIALS AND METHODS

The chicks used in these experiments were White Plymouth Rocks which, depending on the type of treatment used, varied from day-old to 6 weeks of age. Feeding and management were the same as described previously (Goswami et al., '57)

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TABLE 1

Composition of basal rations

	1	2	3	4	5	6	7	8
	%	%	%	%	%	%	%	%
Sucrose	60.7	84.4	88.7	60.7	38.7	40.7	54.6	38.8
Vitamin-test casein [1]	18	3	—	5	5	38	18	18
Gelatin	10	1.3	—	23	45	10	10	10
Corn oil	4.75	4.75	4.75	4.75	4.75	4.75	4.75	4.75
Fish oil (2250A, 300D)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Salts V [2]	6	6	6	6	6	6	6	6
DL-Methionine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Vitamins [3]	+	+	+	+	+	+	+	+
Urea	—	—	—	—	—	—	6.1	—
Ammonium citrate	—	—	—	—	—	—	—	21.9

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

[2] Briggs et al. ('43).

[3] Vitamins were added at the following levels per 100 gm of ration: thiamine hydrochloride 0.4, riboflavin 0.6, calcium pantothenate 2.0, pyridoxine hydrochloride 0.3, niacin 5.0, biotin 0.03, folic acid 0.5, 2-methyl-1,4-naphthoquinone 0.05, vitamin B_{12} 0.003, vitamin E acetate 1.0, choline chloride 150.0 mg.

except in the experiments in which the effects of feed or water restriction were studied. The composition of the purified basal rations used is shown in table 1. In several of the trials the chicks were fed a stock ration containing 20% protein.

Cysteine desulfhydrase activity was measured by the method described by Thompson and Guerrant ('53) except that indirect titration was used to estimate the H_2S liberated. The cadmium sulfide formed was acidified with 6N HCl, a known excess of N/300 I_2 was added and the excess iodine was titrated with N/300 sodium thiosulfate.

RESULTS AND DISCUSSION

Treatments used and results obtained in a series of 9 experiments are summarized in tables 2 and 3.

Effect of alterations in nitrogen portion of the ration on enzyme activity

The effects of alterations in the ration involving level of protein, quality of protein and supplementation with non-protein nitrogenous compounds were studied in the first three experiments.

In the first experiment cysteine desulfhydrase activities of livers of chicks fed a low protein (lot 2) and a protein-free ration (lot 3) were compared to those of chicks fed the control ration (lot 1). After 7 days, enzyme activities on the low-protein and protein-free rations were 75 and 58% respectively of those of chicks fed the control ration. This reduction in activity as a result of feeding low-protein or protein-free rations is similar to that observed with other enzymes (Knox et al., '56).

The second experiment was designed to study the effect of protein quality on level of enzyme activity. Since it was previously observed (Goswami et al., '57) that increased cysteine desulfhydrase activity was associated with the feeding of high levels of protein of good quality, it seemed pos-

TABLE 2

The effect of various treatments on cysteine desulfhydrase activity

EXP.	LOT	RATION FED	TREATMENT	AGE OF CHICKS AT START OF TEST	NO. LIVERS ASSAYED	DAYS ON TREATMENT	CYSTEINE DESULFHYDRASE ACTIVITY (μG H_2S LIBERATED IN 2 HRS.) per gm fresh liver ± S.E.[1]
1	1	1	Control	3 wk.	3	7	43 ± 1.6
	2	2	Low protein	3 wk.	3	7	32 ± 2.0
	3	3	Protein-free	3 wk.	3	7	25 ± 1.6
2	4	1	Control	day-old	2	7	51 ± 0.70
	5	4	23% gelatin ration	day-old	2	7	57 ± 0.70
	6	5	45% gelatin ration	day-old	2	7	81 ± 2.0
3	7	1	Control	day-old	7	21	40 ± 3.5
	8	6	High protein	day-old	6	21	78 ± 5.9
	9	7	Urea	day-old	4	21	41 ± 5.9
	10	8	Ammonium citrate	day-old	4	21	50 ± 1.5
4	11	Stock	Control	6 wk.	8	4	45 ± 2.2
	12	Stock	Inject cysteine (2 mM daily) intraperitoneally	6 wk.	7	4	70 ± 3.0
	13	Stock	Inject methionine (2 mM daily) intraperitoneally	6 wk.	4	4	44 ± 2.4
	14	Stock	Inject histidine (2 mM daily) intraperitoneally	6 wk.	3	4	60 ± 1.6
	15	Stock	Inject glutamic acid (2 mM daily) intraperitoneally	6 wk.	3	4	45 ± 3.1
	16	Stock	Inject arginine (2 mM daily) intraperitoneally	6 wk.	3	4	45 ± 3.1

5	17	Stock	Control	3 wk.	3	5	43 ± 1.6
	18	Stock	Inject cortisone acetate (3 mg daily) subcutaneously	3 wk.	7	5	69 ± 4.9
	19	Stock	Inject cortisone acetate (3 mg daily) + vitamin B_{12} (10 μg daily) subcutaneously	3 wk.	7	5	47 ± 1.6
	20	Stock	Inject histamine diphos- (1 mM daily) intraperitoneally	3 wk.	5	5	66 ± 2.4
	21	Stock	Inject epinephrine (1 mM daily) intraperitoneally	3 wk.	5	5	43 ± 3.8
6	22	Stock	Control	3 wk.	2	0	37 ± 3.0
	23	Stock	Water withdrawal 24 hrs.	3 wk.	2	1	62 ± 1.4
	24	Stock	Water withdrawal 48 hrs.	3 wk.	2	2	54 ± 3.0
	25	Stock	Water withdrawal 96 hrs.	3 wk.	2	4	88 ± 3.0
7	26	Stock	Control	6 wk.	9	0	44 ± 3.4
	27	Stock	Starved 3 days	6 wk.	6	3	61 ± 3.1
	28	Stock	Starved 6 days	6 wk.	10	6	162 ± 10.3
	29	Stock	Starved 9 days	6 wk.	2	9	139 ± 3.0
	30	Stock	Starved 12 days	6 wk.	2	12	77 ± 2.5
8	31	Stock	Control	4 wk.	4	—	41 ± 2.6
	32		Starved 5 days	4 wk.	4	—	109 ± 4.3
	33		As 32 + inject dextrose (6 gm daily) intraperitoneally	4 wk.	2	—	41 ± 3.5
	34		As 32 + inject dextrose (6 gm on 4th and 5th day) intraperitoneally	4 wk.	3	—	76 ± 1.6

[1] Standard error of mean.

sible that lowering the quality of protein might tend to lower the enzyme activity. The data for experiment 2 show that lowering the quality of the protein by substituting gelatin for a part of the casein in the ration without increasing the level of protein had no effect on enzyme activity (lot 5) as compared to the control ration (lot 4); however, increasing the level of protein by the addition of gelatin caused a marked increase in enzyme activity (lot 6).

In order to determine whether the increased activity was dependent on an increased level of protein in the ration or whether other sources of nitrogen might have a similar effect, a third experiment was conducted to ascertain the effect of addition of non-protein nitrogenous compounds. Increasing the level of casein in the ration resulted in a high level of activity (lot 8); however, addition of urea (lot 9) or ammonium citrate (lot 10) to provide a level of nitrogen equivalent to that in the high-protein ration failed to produce an increase in activity as compared to the control (lot 7). It, therefore, seems likely that the increased activity was caused by a higher level of protein in the ration.

Effect of injection of amino acids and hormones

Increases in enzyme levels in the organism have been reported (Knox, '51) to occur not only from administering substrate, but also in response to other amino acids and hormones. In the 4th experiment the effects of intraperitoneal injection of neutralized solutions of a number of amino acids including the enzyme substrate, L-cysteine, were tested. It was noted that administration of L-cysteine (lot 12) or L-histidine (lot 14) resulted in an increase in enzyme activity over that in the control group (lot 11), while DL-methionine (water suspension) (lot 13), L-glutamic acid (lot 15) and L-arginine (lot 16) had no effect. The increased activity associated with the injection of L-cysteine suggests that this enzyme may be classified as an adaptable enzyme. The mode

of action of histidine is not clear, but information in the literature on adaptive synthesis of enzymes in animals (Knox, '51) plus evidence in a recent report (Schayer et al., '56) of the presence of histidine decarboxylase in the mast cells of the peritoneal fluid of rats capable of converting histidine to histamine, and the fact that histamine injections raised the liver cysteine desulfhydrase level of chicks in the next experiment suggest the possibility that similar decarboxylation of histidine to histamine may have occurred.

Since cortisone, histamine and epinephrine have been reported (Dietrich, '54; Knox and Auerbach, '55) to influence the activity of enzymes in the animal body, effects of their injection in three-week-old chicks were assessed in experiment 5. Determination of the effect of simultaneous injection of vitamin B_{12} with cortisone was of interest because this vitamin is reported (Feng and Meites, '55) to counteract the protein catabolic action of cortisone.

The results obtained indicated that administration of cortisone (lot 18) resulted in an increase in cysteine desulfhydrase activity, a change similar to that observed with other enzyme systems (Knox et al., '56). Histamine (lot 20) stimulated the activity of the enzyme, but epinephrine (lot 21) had no effect on cysteine desulfhydrase levels. No explanation is apparent for the failure of epinephrine to stimulate desulfhydrase activity, particularly in view of the fact that it has been shown that both epinephrine and histamine cause the release of adrenocorticotrophic hormone. It is interesting to note that simultaneous injection of cortisone and vitamin B_{12} (lot 19) resulted in levels of enzyme activity comparable to those of the control group (lot 17). It seems possible that this effect may be related in some way to the observation of Feng and Meites ('55) that this vitamin counteracts protein catabolism associated with the administration of cortisone thus serving to prevent an increase in free amino acids, including cysteine, in the tissues.

Effect of water restriction and fasting on enzyme activity

Since injection of cortisone and histamine indicated that changes in enzyme activity might be controlled by a hormonal mechanism involving the adrenal gland, experiments were conducted to study the effect of stress imposed by restriction of drinking water and by fasting.

Withdrawal of water (experiment 6) for one, two or 4 days (lots 23 to 25) resulted in a stimulation in enzyme activity as compared to the initial level (lot 22). Similarly, starvation for periods of three, 6, 9 and 12 days (experiment 7) resulted in a marked increase in desulfhydrase activity during the first 6 days to levels equal to or exceeding those recorded for newly hatched chicks (Goswami et al., '57). As starvation was extended beyond 6 days, the level of enzyme activity declined. Baxter et al. ('57) did not observe any material change in cysteine desulfhydrase levels in rats starved for 24 to 48 hours.

Since starvation eventually results in utilization of tissue protein as a source of energy, it seemed possible that administration of a source of energy, by virtue of its protein-sparing effect might counteract the increased enzyme activity resulting from starvation. This, coupled with the reported inhibitory effect of sugars on the activity of certain adaptive enzymes in microorganisms (Epps and Gale, '42; Magasanik, '55) prompted us to test (experiment 8) the effect of daily administration of dextrose on cysteine desulfhydrase activity. The results obtained indicated that daily injection of dextrose solution in water throughout a 5-day fasting period (lot 33) resulted in maintenance of liver cysteine desulfhydrase activity equivalent to that of birds which were not starved (lot 31); while administration of dextrose during the 4th and 5th day of starvation (lot 34) gave enzyme activities midway between those for the control group (lot 31) and those for chicks starved for 5 days (lot 32).

Effect of feeding thyroprotein and thiouracil on enzyme activity

The observation that cysteine desulfhydrase activity was affected by caloric intake suggested that administration of thyroactive or antithyroid compounds, because of their influence on energy metabolism, might also affect the activity of the enzyme. Experiment 9 was conducted to ascertain the effect of supplementing the ration with thyroprotein or thiouracil on cysteine desulfhydrase activity. The treatments used and the results obtained are summarized in table 3.

The chicks fed the ration supplemented with iodinated casein [2] showed a marked depression in cysteine desulfhydrase activity (lot 36) as compared to the control group (lot 35) while those fed the ration containing thiouracil (lot 37) showed an increased level of enzyme activity. After three weeks the total liver cysteine desulfhydrase activity in the thiouracil group was nearly 5 times as great as in the control group. This may be attributed partly to the increased activity per gram of liver and partly to the great increase in liver size as a result of thiouracil feeding. The effect of feeding thiouracil on liver size has been reported previously (Moreng and Shaffner, '49).

SUMMARY

L-cysteine desulfhydrase activity in chick liver was reduced by feeding either a low-protein or a protein-free ration and was increased by feeding a high-protein ration rich in gelatin. Increasing the nitrogen levels of the ration by the addition of urea or ammonium citrate had little or no effect on the enzyme activity of chicks fed the rations.

Intraperitoneal administration of L-cysteine or of L-histidine resulted in increased activity; DL-methionine, L-glutamic acid and L-arginine were not effective.

The injection of cortisone or histamine resulted in an increase in cysteine desulfhydrase activity. The increased

[2] Protamone.

TABLE 3

Comparison of the effect of iodinated casein[1] and thiouracil feeding on cysteine desulfhydrase activity of chick liver

EXPERIMENT	LOT	DAYS ON TREATMENT	RATION FED	TREATMENT	CYSTEINE DESULFHYDRASE ACTIVITY (μH_2S LIBERATED IN 2 HRS.)	
					Per gm fresh liver[2]	Per organ
9[3]	35	7	1	Basal	43 ± 1.4[4]	379 ± 12.9[4]
	36	7	1	Basal + 0.05% iodinated casein	16 ± 1.5	111 ± 11.0
	37	7	1	Basal + 1% thiouracil	44 ± 1.2	530 ± 15.1
	35	14	1	Basal	31 ± 1.7	317 ± 17.6
	36	14	1	Basal + 0.05% iodinated casein	19 ± 2.0	139 ± 14.6
	37	14	1	Basal + 1% thiouracil	47 ± 3.6	819 ± 63.3
	35	21	1	Basal	36 ± 2.8	522 ± 42.1
	36	21	1	Basal + 0.05% iodinated casein	22 ± 1.5	265 ± 18.5
	37	21	1	Basal + 1% thiouracil	89 ± 5.9	2448 ± 181.8

[1] Protamone.

[2] Individual determinations on 4 chicks in each lot.

[3] Chicks were three weeks old at start of experiment.

[4] Standard error of mean.

activity due to cortisone administration was prevented by simultaneous injection of vitamin B_{12}.

Stress resulting from restriction of water intake or fasting caused an increase in enzyme concentration. Intraperitoneal administration of dextrose during fasting arrested the increase.

Feeding of thyroprotein resulted in a marked depression in cysteine desulfhydrase concentration, while inclusion of thiouracil in the ration increased activity of the enzyme.

ACKNOWLEDGMENT

Grateful acknowledgment is made to the National Research Council, Canada, for a postdoctorate fellowship to one of us (M.N.D.G.) during the course of this work.

The vitamins used were kindly supplied by Merck & Co. Ltd., Montreal, Quebec; Lederle Laboratories, Pearl River, New York; and Hoffmann-La Roche, Inc., Nutley, New Jersey.

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INVITATIONS FOR NOMINATIONS FOR 1960 AMERICAN INSTITUTE OF NUTRITION AWARDS

Nominations are requested for the 1960 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed and (2) a statement as convincing as possible as to the basis for the nomination, stating the eligibility of the candidate (this may include the pertinent bibliography of the most appropriate and significant recent papers on which the nomination is based, but such bibliography is not necessary unless later requested by the Nominating Committee). Reprints are not required, nor are seconding statements. *Five copies of all documents* must be sent to the chairman of the appropriate Nominating Committee *before December 1, 1959,* to be considered for the 1960 awards.

General Regulations for A. I. N. Awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award.[1] A Jury of Award composed of A. I. N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant

[1] Including recipients of the former Mead-Johnson award. These are listed at the end of this notice.

the award. An award is usually given to one person but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made at the annual dinner at the annual meeting.

1960 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1,000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are: 1944 — E. V. McCollum; 1945 — H. H. Mitchell; 1946 — P. C. Jeans and Genevieve Stearns; 1947 — L. A. Maynard; 1948 — C. A. Cary; 1949 — H. J. Deuel, Jr.; 1950 — H. C. Sherman; 1951 — P. György; 1952 — M. Kleiber; 1953 — H. H. Williams; 1954 — Agnes Fay Morgan and A. H. Smith; 1955 — A. G. Hogan; 1956 — F. M. Strong; 1957 — no award; 1958 — L. D. Wright; 1959 — H. Steenbock.

Nominating Committee:

C. A. Baumann, *Chairman*
G. V. Mann
E. E. Snell

Send nominations to:

Dr. C. A. Baumann
Dept. of Biochemistry
University of Wisconsin
Madison, Wisconsin

1960 Osborne and Mendel Award

The Osborne and Mendel Award of $1,000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are: 1949 — W. C. Rose; 1950 — C. A. Elvehjem; 1951 — E. E. Snell; 1952 — Icie Macy Hoobler; 1953 — V. du Vigneaud; 1954 — L. A. Maynard; 1955 — E. V. McCollum; 1956 — A. G. Hogan; 1957 — G. R. Cowgill; 1958 — P. György; 1959 — Grace A. Goldsmith.

Nominating Committee:

D. M. HEGSTED, *Chairman*
J. S. DINNING
GRACE A. GOLDSMITH

Send nominations to:

DR. D. M. HEGSTED
Harvard School of Public Health
1 Shattuck St.
Boston, Mass.

Former recipients of the Mead-Johnson Award presented by A.I.N. are: 1939 — C. A. Elvehjem; 1940 — W. H. Sebrell, Jr., J. C. Keresztesy, J. R. Stevens, S. A. Harris, E. T. Stiller, and K. Folkers; 1941 — R. J. Williams; 1942 — G. R. Cowgill; 1943 — V. du Vigneaud; 1944 — A. G. Hogan; 1945 — D. W. Woolley; 1946 — E. E. Snell; 1947 — W. J. Darby, P. L. Day, and E. L. R. Stokstad; 1948 — F. Lipmann; 1949 — Mary S. Shorb and K. A. Folkers; 1950 — W. B. Castle; 1951 — no award; 1952 — H. E. Sauberlich.

INVITATION FOR NOMINATIONS FOR 1960 AMERICAN INSTITUTE OF NUTRITION FELLOWS

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows will be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by January 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April. The following persons have been elected previously as Fellows of the Society:

Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Eugene F. DuBois (1958)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Albert G. Hogan (1959)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
William C. Rose (1959)
Harry Steenbock (1958)
Robert R. Williams (1958)

Fellows Committee:

COSMO G. MACKENZIE, *Chairman*
PAUL GYÖRGY
W. D. SALMON
KARL FOLKERS
E. W. MCHENRY

Send nominations to:

DR. COSMO G. MACKENZIE
Dept. of Biochemistry
Univ. of Colorado School of Medicine
Denver 20, Colorado

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THIS NUMBER COMPLETES VOLUME 68

THE JOURNAL® OF NUTRITION

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PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America