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EXPERIMENTS UPON VITAMIN A

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The experiments here described relate chiefly to questions concerning the storage of vitamin A in the body and the bearing of such storage upon the problem of the quantitative determination of vitamin A in foods. For the sake of brevity we omit the review of previous work on the storage of this vitamin which has been done from a more qualitative or a more physiological point of view.

Vitamin A cannot be determined satisfactorily in a manner parallel to that employed in the determination of vitamin B by means of the rat-growth method,[1] for even at weaning time young animals may already have a considerable store of vitamin A in the body and thus be able to continue to grow for some time upon a diet freed from vitamin A.[2] In order to give the maximum quantitative value to experiments of this character it was important that a diet be devised which should be as free as possible from vitamin A but in all other respects well adapted to the support of good growth in the young rat.

Experimental Diet Otherwise Adequate but Free from Vitamin A

Previous experiments in this Laboratory[3] had shown that patent flour contains no detectable amount of vitamin A. Such flour was adopted as the main source of energy of our experimental diet since it is better relished by the experimental animal than pure starch and carries sufficient protein to reduce appreciably the amount of specially purified protein which must be prepared.

Following Osborne and Mendel we have used extracted meat residue as the protein concentrate for these experiments, and dried brewery yeast as the source of vitamin B. We have also used the salt mixture described by Osborne and Mendel.[4]

The ingredients were mixed in the following proportions (Diet 97–34): patent flour, 82%; extracted meat residue, 10%; dried brewery yeast, 4%; salt mixture,[4] 4%; total, 100%.

Since Osborne and Mendel have shown[5] that the yeast does not furnish any detectable amount of vitamin A even when fed in over ten times the

[1] Sherman and Spohn, THIS JOURNAL, **45**, 2719 (1923). Sherman and Grose, *ibid.*, **45**, 2728 (1923).

[2] Sherman and Smith, "The Vitamins," Chemical Catalogue Company, New York, 1922. Steenbock and others, *J. Biol. Chem.*, **56**, 327 (1923).

[3] Experiments by Miss Hazel E. Munsell, not yet published.

[4] Osborne and Mendel, *J. Biol. Chem.*, **37**, 572 (1919).

[5] Osborne and Mendel, *ibid.*, **45**, 145 (1920).

proportion here used, the only ingredient of this experimental diet whose purification required study was the meat residue. In our hands extraction with hot alcohol as recommended by Osborne and Mendel gave better satisfaction than the method of destroying the vitamin by heating the protein in contact with air as used in the English laboratories.

Under the conditions of our experiments, more than 12 hours' heating at 105° appeared to be necessary for the destruction of the small amount of vitamin A contained in the meat residue and the animals did not seem to thrive so well upon the material which had been thus heated as upon that which had been freed from vitamin A by alcoholic extraction.

For the removal of vitamin from the dry, finely ground meat residue, three different methods of extraction were tested.

In the first method the dry meat residue was boiled under a reflux condenser for one hour with twice its weight of 95% alcohol, then poured while boiling hot upon a large filter and filtered rapidly by means of suction. This process was repeated a second and a third time upon each portion of the meat residue, which was then dried in the air and used air-dry in the food mixture above described.

In the second method the extraction with hot, 95% alcohol was made continuous by the use of a Koch extractor which is essentially a modified coffee percolator with a hollow lid arranged as a condenser and the long central tube of the percolator removed. Thus the meat residue is continuously extracted with hot condensed alcohol at the temperature of and surrounded by alcohol vapor. Heat was applied by means of a steam-bath so regulated that alcohol dripped steadily and rapidly from the condenser onto the meat residue. The last hour of extraction was always with a fresh portion of alcohol, to ensure the washing down of any trace of extracted material which might have been splashed upon the lower surface of the strainer. A total of 12 hours' extraction in this apparatus was employed. This was probably more than sufficient; but in order to be sure of completeness of extraction no attempt was made to reduce the time to a minimum. A test of the completeness of the extraction was made by combining the second and third methods and greatly extending the time of extraction as described below.

In the third method of extraction the dry meat residue was first washed on the filter thrice with acetone at room temperature, using in all 2 cc. of acetone for each gram of meat residue and allowing the acetone to run through slowly, employing suction only to ensure thorough removal of the solvent at the end of the washing, after which the material was extracted twice with boiling alcohol as in the first method described above.

In order to ascertain whether meat residue would yield any further vitamin A than is removed by these methods, a portion was extracted in the percolator first for 12 hours with acetone, then for 12 hours with

alcohol, then for 60 hours longer with alcohol, making a total of 12 hours' hot extraction for each of seven successive days. The meat residue thus extracted gave no different results in quantitative feeding trials than that which had been extracted for 12 hours according to the second method of extraction here described, showing that under the conditions obtained here extraction for 12 hours was ample.

Using meat residue thus extracted in the basal diet above described, the diet was shown to be fully adequate for rats in all other respects, since on supplying vitamin A in the form of butterfat excellent growth and successful reproduction and rearing of young were obtained.

From the experimental evidence here but briefly cited, it may be said that a basal diet free from vitamin A and adequate in all other respects can be prepared for quantitative experimental work (upon rats) by the methods just described.

Relative Quantities of Vitamin A Stored in Young Rats when Weaned at Four Weeks of Age

Since in this Laboratory rats to be used for experimental purposes are regularly separated from their mothers at the standard "weaning" age

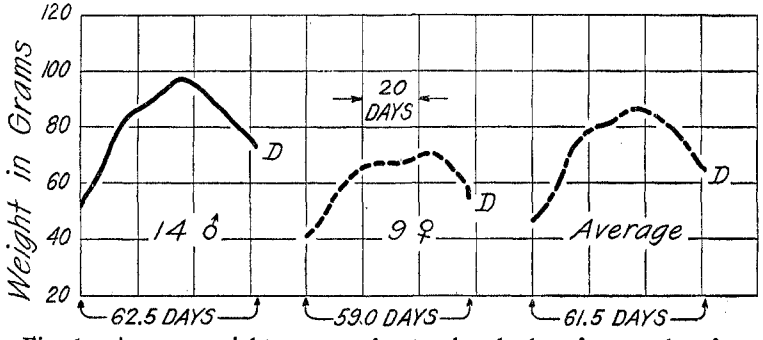

Fig. 1.—Average weight curves of rats placed when four weeks of age upon diet free from vitamin A. The initial point of the curve is the weight at four weeks, when transferred from the stock diet to the experimental diet. The figures at the bottom of the curve show the average survival periods in days.

of four weeks, it becomes important to determine the influence of size, sex and family diet upon the relative stores of vitamin A in the body at this age as measured by the number of days that such rats can survive upon the basal diet above described which is adequate in other respects but carefully freed from vitamin A. Table I and Fig. 1 show the survival periods of rats of different sizes and both sexes but all from the same diet.

It will be seen from Table I that individual differences among rats at this age are considerable, even when comparison is confined to animals of approximately the same size. Hence, the importance of frequent

TABLE I

SURVIVAL PERIODS OF RATS FROM DIET 13M PLACED UPON VITAMIN-A-FREE DIET WHEN FOUR WEEKS OLD

Body wt. at 4 weeks, g		70	61	60	56	55	50	48	47	46	45
Survival period, days.	Males	75	59	60	69	57	77	55	64	59	77
									73	61	
	Females	..	..	..	..	..	..	..	54	..	60
Body wt. at 4 weeks		44	43	42	40	39	37	34	32	30	..
Survival period, days.	Males	49	64	..	..	44	..	..	..	..	..
		60									
	Females	67	80	66	70	..	73	54	47	41	..
				65					38		
				54							

repetitions of tests if quantitative results are sought, since natural variability is a large factor in individual tests even if accompanied by controls. In making repeated tests each should if possible have a control animal from the same litter so that a more or less than average stamina due to family trait shall not become a source of error. (It may be noted, for example, that the extreme cases in Table I, a male of 45 g. which lived 77 days, and a female of 43 g. which lived 80 days, were members of the same litter.)

Compared with the individual differences Table I shows no constant or significant difference between males and females except that the larger males tend to show a slightly longer survival period. In Fig. 1 the average initial weight of the males is considerably above that of the females, and it is apparently due to this difference in size rather than to sex that the males in this particular series show a slightly longer average survival period than the females.

The influence of the family diet upon the average survival period of the four-weeks-old rat when placed upon a diet adequate in all other respects but free from vitamin A is shown in Table II.

TABLE II

INFLUENCE OF PREVIOUS FOOD ON AVERAGE SURVIVAL PERIOD OF FOUR-WEEKS-OLD RATS PLACED ON DIET FREE FROM VITAMIN A

	Previous diet	Number of cases	Survival days
A.	($^1/_6$ dry milk, $^5/_6$ wheat)	11	34
B.	($^1/_3$ dry milk, $^2/_3$ wheat)	14	51
D.	($^2/_3$ dry milk, $^1/_3$ wheat)	7	64

The diets A, B and D are mixtures of dry whole milk and ground whole wheat with increasing proportions of milk and therefore of vitamin A. The corresponding increase in body store of vitamin A, even at four weeks of age, is plainly apparent in the increasing average survival periods, namely, 34, 51 and 64 days, respectively. Along with the difference in survival period the differing store of vitamin A in the body results also

in markedly different growth curves on the same vitamin-A-free diet, as shown by the cases of individual animals from Diets A and D represented in Fig. 2.

It will be seen that the rat from Diet A made very little growth upon the experimental diet free from vitamin A, while the rat from Diet D placed on the same diet at the same age grew rapidly and practically tripled his weight by virtue of the vitamin A previously stored in his body.

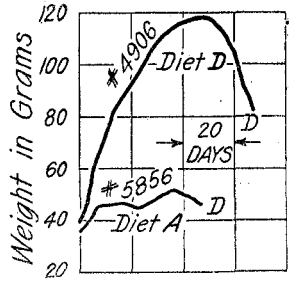

Fig. 2.—Weight curves of rats from different stock diets (Diet A, relatively poor in fat-soluble vitamin, and Diet D, relatively rich in fat-soluble vitamin) placed at the same age (four weeks) upon the same diet free from vitamin A (Diet 47–34).

It is plain that unless the factor of varying storage in the body of the young rat is very carefully safeguarded it is likely to be an extremely important source of error in studies of vitamin A and especially in attempts to make such studies quantitative.

Because of the large differences shown by rats of the same age when placed on the same diet, partly individual and partly due to previous diet or family history, we have felt forced to abandon attempts to make quantitative experiments upon vitamin A with rats placed directly at weaning time upon the foods to be tested, as in similar studies of vitamin B, and have recently followed the practice of Drummond and Zilva in beginning each experiment by keeping the rat upon a vitamin-A-free diet until his stored surplus of vitamin A is exhausted as indicated by cessation of growth before beginning the feeding of the food to be tested.

Storage of Vitamin in the Body of the Rat after Weaning at Four Weeks of Age

That diet also influences the storage of vitamin A in the body after the age at which the above comparisons were made is clearly indicated in Fig. 3 which summarizes the histories of twin brother rats which at weaning time were placed on Diets A and B, respectively, and later at the same adult age were transferred to the same diet deficient in vitamin A. Their opportunities for storage up to four weeks of age had been identical; but the difference in their diet at later ages resulted in such different storage of vitamin A in their bodies that one was able to survive nearly twice as long as the other when both were later subjected to the same vitamin-A-deficient diet. Such experiments as this make it plain that vitamin A is needed at all ages and that the apparently lesser need of the adult than of the growing animal may be largely due to the fact that he has had more opportunity to lay up a store of vitamin A in his body.

As shown in Table II the average survival period of rats from Diet A

when placed on vitamin-A-free diet at 28 days of age was 34 days; but for rats taken from the same diet at 61 days the average survival period was 76 days; and for those taken at 100 days the average period of survival was 94 days.

Similarly, rats taken from Diet B at 28 days survived an average of 51 days; taken at 78 days, survived an average of 124 days; and taken at 132 days they survived an average of 157 days.

Thus the store of vitamin A in the body as indicated by these survival periods continues on either Diet A or B to increase with the age of the rat for at least a very considerable time beyond the weaning age and beyond the ages at which feeding experiments have ordinarily been begun; and at any given age within the range covered by our experiments the rats

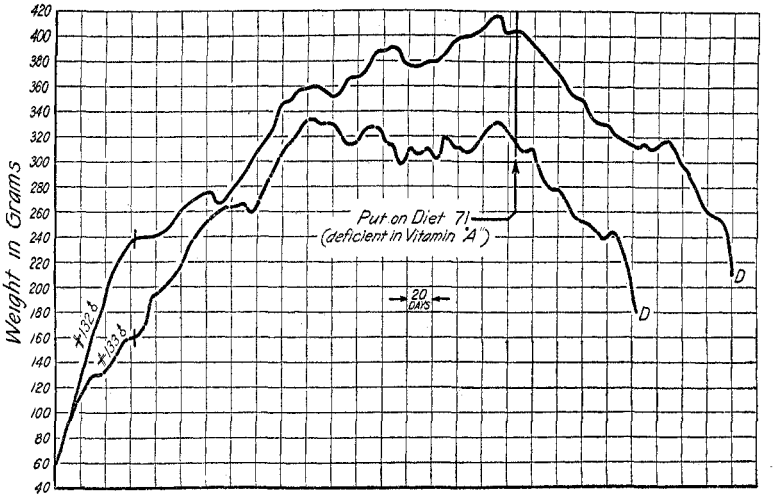

Fig. 3.—Weight curves and survival periods of twin rats kept from weaning time until full grown upon two different stock diets and then placed upon the same experimental diet deficient in vitamin A. Diet of No. 132 contained 1/3 milk solids. Diet of No. 133 contained 1/6 milk solids.

which have had more vitamin A in their food continue to show a larger store of this vitamin in their bodies.

Some of the experiments with older rats were made before the standard basal ration above described had been perfected and were carried out with different vitamin-A-deficient diets, not all of which were equally adequate in their mineral content. In this connection an experiment was made which, while of preliminary nature from the standpoint of the development of strictly quantitative technique proved, nevertheless, to be extremely instructive.

Four healthy male rats of the same litter were fed from weaning time until they were 130 days old and nearly full grown upon Diet B described

above plus fresh green string beans *ad libitum*, and were then placed upon four different deficient diets, all lacking vitamin A but differing in their mineral content. These four diets were as follows: Diet 23—patent flour, 100 parts; sodium chloride, 2 parts; Diet 83—patent flour, 95; sodium chloride, 2; calcium lactate, 3 parts; Diet 84—patent flour, 95; sodium chloride, 2; calcium lactate, 2.9; ferric citrate, 0.1 part; Diet 85—patent flour, 95; sodium chloride, 2; calcium lactate, 2.5; potassium phosphate, 0.4; ferric citrate, 0.1 parts.

The four rats had grown well and so uniformly that they were excellently matched at the beginning of the survival experiment. Their weight curves

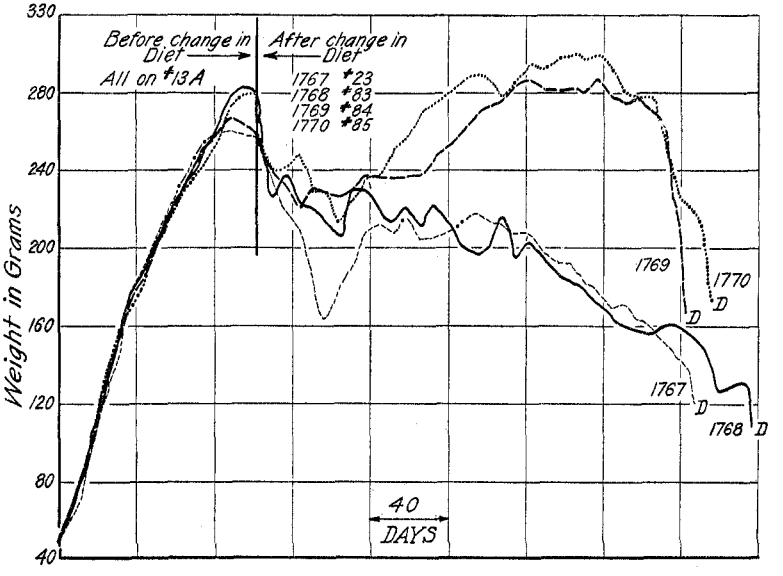

Fig. 4.—Weight curves of four rats of the same litter raised to practically adult size upon the same stock diet (Diet 13 A) and then placed upon four different experimental diets all equally devoid of vitamin A but with widely varying mineral content; showing uniformity of survival period as measure of previously stored vitamin A, notwithstanding differences in mineral content of diet.

are shown in Fig. 4 in which the time of death of each is indicated by the termination of the curve and the letter D. It will be seen from this figure that when first placed on the deficient diets all four rats lost weight rapidly. Subsequently their weight curves fluctuated in various ways; but notwithstanding this the survival periods in all four cases were strikingly similar. After death the calcium content of each rat was determined with the results given in Table III.

Thanks are due Miss F. L. MacLeod and Miss E. L. McCollum for these quantitative determinations of calcium.

TABLE III

CALCIUM CONTENT OF RATS ON FOUR DEFICIENT DIETS

Rat no.	1767	1768	1769	1770
On diet	23	83	84	85
Ca content, g.	1.82	2.78	2.78	3.72

From the results of numerous analyses of normal rats made in this Laboratory it may be presumed that each of these rats contained when placed on deficient diet about 3 g. of calcium. No. 1767 must, therefore, have lost nearly half of his body calcium before his death. In contrast with this Nos. 1768 and 1769 evidently lost only small amounts and No. 1770 apparently continued to gain in calcium content while on the diet free from vitamin A, evidently because he received, as the others did not, a well-balanced intake of calcium and phosphorus in his food. In spite of these large differences in calcium metabolism of the four rats, due to differing mineral contents of their vitamin-A-deficient diets, the survival periods were nearly the same and such differences as appear are not in the order of adequacy of the mineral contents. That, notwithstanding the differences in mineral intake and mineral metabolism, the rats starting with presumably the same body stores of vitamin A should all die from lack of this vitamin after such strikingly uniform survival periods is significant evidence of the importance of the survival period as an indication of the relative amount of vitamin A which the body had been able to store from its previous diet.

Summary

The experiments described deal chiefly with the storage of vitamin A in the body and the bearing of this upon methods for the quantitative determination of vitamin A in foods.

An experimental diet devoid of vitamin A but adequate in all other respects to support excellent nutrition in the experimental animals (rats) is described.

Young rats placed upon this experimental diet at a uniform weaning age of four weeks show very different growth curves and survival periods according to the vitamin A content of the diet of the family from which they come.

The differing stores of vitamin A in the bodies of experimental animals, even at early ages, have undoubtedly been a very serious disturbing factor in much of the earlier work, as has also been shown by Steenbock and his co-workers in publications appearing while this paper was in preparation.

The body can also store vitamin A at later ages; and in the older as well as the younger animals the lengths of time that individuals can survive upon a diet otherwise adequate but devoid of vitamin A serves as a good indication of the relative amounts of this vitamin which the body had been

able to store, and presumably, therefore, of the relative amounts contained in the previous diet.

Even when the diets devoid of vitamin A were strikingly different in their mineral content and their ability to support the mineral metabolism of the body, the vitamin deficiency of the experimental diet and the vitamin content of the preceding diet together determined the survival period.

The investigation is being continued with reference both to the storage and distribution of vitamin A in the body and the quantitative determination of this vitamin in foods.

NEW YORK, N. Y.

NOTE

Certain Reactions of Tetryl.[1]—Next to trinitrotoluene, tetryl or 2,4,6-trinitro-phenylmethyl-nitramine is the most important of the military explosives. At the present time it is probably used by all the great nations as the booster explosive for high explosive shells. Its reactions, then, are not without interest; and mention of its comportment with acids is conspicuously absent from the chemical literature. The vacuum heat test, in which the sample is heated and the evolved gases are collected and measured, is becoming a standard test for tetryl; it is well known that many commercial samples of tetryl contain occluded acid; and a knowledge of the comportment of tetryl with strong sulfuric acid may have considerable bearing upon the interpretation of the heat test.

Mertens[2] observed that when tetryl is heated in various solvents, nitric oxide is evolved and methylpicramide (2,4,6-trinitro-monomethylaniline) is formed. He obtained his best yields by heating tetryl in phenol at 160°, but the product was relatively impure. With aniline, dimethylaniline, glycerol, paraffin and nitrobenzene, he obtained very impure tar-like mixtures. We have verified his results and have found that methylpicramide is produced when tetryl is refluxed in capryl alcohol solution (methylhexylcarbinol) or in *n*-butyl alcohol. With the latter solvent, 46 hours of boiling was required to obtain enough methylpicramide to identify by a mixed melting point. It seems unlikely that tetryl enters into any reaction with those solvents, especially paraffin and nitrobenzene, and the formation of methylpicramide may possibly be due to the action of water fortuitously present or more probably to the action of water and acid produced by the decomposition of another portion of the tetryl.

[1] The experimentation was carried out in connection with a contract between the Ordnance Department and the Massachusetts Institute of Technology, and the present note is published by permission of the Chief of Ordnance.

[2] Mertens, *Ber.*, **19**, 2123 (1886).