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# Improvement of Vitamin A Deficient Diets for Use in Bioassays.

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The use of vitamin A-free diets of the U.S.P. (United States Pharmacopoeia) and B.P. (British Pharmacopoeia) types for biological assay of foods and feeds possessing vitamin A activity has been questioned recently by various investigators. They have pointed out that these diets do not permit normal growth in rats even when a generous amount of vitamin A is supplied as a supplement. Several workers have, in turn, proposed modifications in, or entirely new formulas for, such vitamin A-free diets. Gridgeman[1] has cited some of this work.

From the standpoint of the improvement of purified diets fortified with all of the known vitamin B factors, enhanced growth in rats has been obtained both by the use of liver extract and by increased level of casein in the diet. In the first instance, Hartman and co-workers[2,3] have conducted numerous experiments dealing with a growth factor lacking in diets such as the U.S.P. vitamin A assay diet but present in a wide variety of natural products. They have prepared highly active concentrates of the factor from liver extract which promote enhanced growth in rats maintained on the usual synthetic diet used in vitamin A assay. That casein may carry varying amounts of the factor is shown by differences in growth obtained with different casein preparations as purified and used in various laboratories, commercial and otherwise. McIntire *et al.*[4] also suggested that liver extract supplied a missing factor in, or otherwise corrected, a deficiency condition. Bosshardt *et al.*[5] have concluded that extracts of liver and pancreas contain a growth-promoting substance for mice which is distinct from the recognized B factors and not present in yeast in appreciable amounts. Zucker and Zucker,[6] on the other hand, obtained improved growth by increasing the casein content from 18 to 28% or thereabouts and suggest that the missing factor "is associated whether as an impurity or an essential amino acid, with protein of good quality." Deuel *et al.*[7] have obtained better growth by substitution of mackerel protein for casein in the U.S.P. vitamin A assay diet.

The foregoing evidence plus other accessory facts seems to show rather definitely that the U.S.P. XII diet lacks some growth factor or factors other than vitamin A. The work reported here is the result of experiments designed to include this new factor in the vitamin A-free diet. Although the substance has not been isolated as a pure compound, it is possible to include it in the form of natural products, while still maintaining a vitamin A-free diet. This may not be fully satisfactory, but should contribute to accuracy in the assay of many materials.

*Experimental.* The feeding tests were conducted with male and female rats, 21 to 26 days of age. Equal numbers of the 2 sexes were used in each group and the rats were further distributed in the groups as evenly as possible with regard to age, weight, and genetic history.

Modifications were made in the basal vitamin A-deficient diet, regularly used in the

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2 Hartman, A. M., Dryden, L. P., and Cary, C. A., *J. Biol Chem.*, 1941, liv.

3 Hartman, A. M., and Cary, C. A., *J. Dairy Sci.*, 1942, **25**, 679.

4 McIntire, J. M., Henderson, L. M., Schweigert, B. S., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 98.

5 Bosshardt, D. K., Ciereszko, L. S., Buffington, A. C., and Arnow, E. L., *Arch. Biochem.*, 1945, **7**, 1.

6 Zucker, T. F., and Zucker, L., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 136.

7 Deuel, H. J., Jr., Hrubetz, M. C., Johnston, C. H., Rollman, H. S., and Geiger, E., *J. Nutrition*, 1946, **31**, 187.

TABLE I.
Composition of Rat Diets.

| Ingredients* | % of ingredient in diet No. | | | |
|---|---|---|---|---|
| | 568 | 687 | 747 | 748 |
| Casein | 18 | 9 | 9 | 9 |
| Pork muscle | | 9 | | |
| Sardine meal | | | 12 | |
| Blood fibrin | | | | 9 |
| Dextrin | 66 | 66 | 63 | 66 |
| Yeast, non-irr. | 7 | 7 | 7 | 7 |
| " irr. | 1 | 1 | 1 | 1 |
| Salt mixture | 3 | 3 | 3 | 3 |
| Peanut oil | 5 | 5 | 5 | 5 |

* Alpha tocopherol was added to all diets at the rate of 3 mg per 100 g.

laboratory and essentially the U.S.P. XII diet, to determine the effects produced by the use of various materials known to contain the growth factor already discussed. One of these materials was lean pork obtained from fresh hams. The pork was first cooked, then dehydrated, extracted with ethyl ether in a percolator, and finally ground. Blood fibrin was also prepared for use by grinding and extracting with ethyl ether. The third material used as a substitute for casein was ether-extracted fish (sardine) meal. The casein as used in vitamin A feeding tests in this laboratory is first extracted 3 times with acidulated water and then with 95% alcohol (hot) for 24 hours in a large metal Soxhlet extractor.

The composition of the basic experimental diets is given in Table I. In the feeding tests, groups of rats were fed the diets as given in Table I. Rats on diets 747 and 748 also received orally 0.05 ml of liver extract 3 times per week. Four parallel groups received the same 4 diets plus a supplement of cod liver oil (series A) to supply vitamin A at a level of 50 I.U. per rat per day. Another (series B) received the cod liver oil as in (series A) plus liver extract fed by dropper to each rat at the rate of 0.05 ml 3 times per week. As a control, one group of rats received the complex stock colony diet made up mainly of natural and other concentrate feeds.

*Results.* Preliminary experiments with a diet of the U.S.P. type such as diet 568 in Table I supplemented with vitamin A, showed that increasing the extracted casein from 18% to 23% and adding 0.2% of *l*-cystine failed to produce increased growth. The animals averaged little more than 15 g per week over an 8-week growth period, while animals fed a stock colony diet averaged nearly 25 g per week. This seemingly indicated that the casein and yeast furnished adequate protein, but failed to provide some other necessary factor or factors.

Feeding results of the comparisons of the diets given in Table I are presented in Table II. That the basal casein diet, plus vitamin A (diet 568A), is deficient for optimum growth is indicated plainly by the relatively poor mean gains over a period of 8 weeks. This deficiency in growth appears to have been corrected by addition of 0.05 ml of the liver extract, since the difference in growth on diet 568B and on the stock colony diet is probably not significant. Increased growth was also obtained in diet Series A and B with extracted pork muscle, sardine meal, and blood fibrin. The best gains were made on the supplemented sardine meal diets. While a complete statistical analysis of the data has not been made, it appears evident that the sardine meal was superior to the pork. Both supplied the growth factor contained in the liver extract since the addition of the latter material in diet treatments 747B and 748B but omission in 747A and 748A had little if any effect on growth. That sardine meal, pork muscle, and liver extract were free of vitamin A activity was shown by the animals fed diets 687 and 747. All of the animals on these diets showed severe symptoms of vitamin A deficiency, being just as

TABLE II.
Results of Rat Feeding Tests Lasting 8 Weeks.

| Diet treatment | Diet No. | No. animals | | Avg wt gain (g) |
|---|---|---|---|---|
| | | Start | Close | |
| Basal casein diets | | | | |
| No supplement | 568 | 8 | 5 | 46.2 |
| + 50 I.U. of A* | 568A | 8 | 8 | 135.3 |
| + .05 ml L.E.† and 50 I.U. of A. | 568B | 14 | 14 | 172.4 |
| Pork diets | | | | |
| No supplement | 687 | 8 | 3 | 60.0 |
| + 50 I.U. of A. | 687A | 8 | 8 | 162.0 |
| + .05 ml L.E. and 50 I.U. of A. | 687B | 8 | 8 | 163.4 |
| Sardine meal diets | | | | |
| + .05 ml L.E. | 747 | 5 | 1 | 28.0 |
| + 50 I.U. of A. | 747A | 6 | 6 | 191.8 |
| + .05 ml L.E. and 50 I.U. of A. | 747B | 6 | 6 | 193.1 |
| Blood fibrin diets | | | | |
| + .05 ml L.E. | 748 | 6 | 6 | 181.0 |
| + 50 I.U. of A. | 748A | 6 | 6 | 192.8 |
| + .05 ml L.E. and 50 I.U. of A. | 748B | 6 | 6 | 190.2 |
| Stock colony diet | | | | |
| No supplement | — | 14 | 14 | 183.7 |

* A—vitamin A.
† L.E.—liver extract.

pronounced as on diet 568. This was not true of the animals fed diet 748, indicating that extraction failed to remove A-activity from the blood fibrin.

It should be noted that the known B complex factors are furnished in the diets given in Table I by the 8% of high potency yeast. The work reported by Hartman and Cary[3] along with unpublished data obtained in this laboratory show that the addition of all of the known B complex vitamins, including pteroyl glutamic acid, to this type of diet will not produce normal growth. Accordingly it seems evident that the results reported here show that the U.S.P. type of diet is deficient in at least one unknown factor. The data further indicate means by which the diet may be made complete so that it can be used in obtaining more satisfactory vitamin A assays.

*Summary.* Results of experiments are given which show that for optimum growth in rats, it is necessary to add at least one factor besides vitamin A to the U.S.P. vitamin A-free diet. This factor, which has been described by other investigators, is present in liver extract. Replacement of half of the casein in the basal diet either with pork or sardine meal was likewise effective and the addition of liver extract to these ingredients did not give any added benefit. Unfortunately, extraction with ether failed to remove the vitamin A from blood fibrin to make possible its use. Excepting the groups fed blood fibrin the sardine meal diet with supplements promoted the best gains of all the remaining groups.