XL. THE RELATIONS OF VITAMINE TO LIPOIDS.

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Although vitamine, i.e. the substance which prevents beri-beri and avian polyneuritis, has been shown to be present in higher concentration in various tissues rich in lipoid, e.g. liver, cardiac muscle, brain (ox) and egg-yolk, than in voluntary muscle (ox, fish) which is poor in lipoid [Cooper, 1913, 1] it is now generally believed that the active substance as isolated from foodstuffs is not a lipoid. It contains no phosphorus or carbohydrate [Cooper and Funk, 1911; Funk, 1911, 1914; Edie, Evans, Moore, Simpson, Webster, 1912] and is therefore neither a phosphatide nor cerebroside. MacLean [1912] furthermore showed that lecithin, which had previously been found by Schaumann [1910] to be slightly curative, had still less effect after purification, and he concluded that the anti-neuritic substance is not a lipoid but is present in lipoids as an impurity extractable by simple methods. Cooper [1913, 2] subsequently found that when the fats and lipoids (alcoholic extract) of horse voluntary muscle were treated with ether the bulk of the anti-neuritic substance contained therein was precipitated and by a complex fractionation of the active precipitate a substance was isolated, small doses of which readily cured polyneuritis in pigeons. 4 g. of the original alcoholic extract exerted a rapid curative action in neuritic pigeons, but after one extraction with ether as much as 12 g. of the fats and lipoids (ether-soluble fraction) had only a slight ameliorative effect. More than 66 per cent. of the total amount of active material present in the original extract could thus be removed by simple treatment with ether and was thus apparently not in chemical union with the lipoids. Similar results were obtained when the flesh was not air-dried at 30° before extraction with alcohol [Cooper, 1913, 1]. This showed that the anti-neuritic substance was not split off from a lipoid complex during the process of air drying.

The above facts however do not exclude the possibility that, although

the anti-neuritic substance is preformed in the diet, in the nervous system it in part becomes chemically combined with other substances to form a lipoid.

Experiments on similar lines have therefore been carried out with cardiac muscle and brain, and the results obtained are set forth in the present communication. The work with heart muscle was undertaken primarily with the object of isolating a sufficient amount of the anti-neuritic substance for a study of its chemical nature, the cardiac muscle being substituted for voluntary muscle on account of its higher content of active material [Cooper, 1913, 1].

It must first be pointed out however that according to Vedder and Williams [1913] dry and wet beri-beri are caused by a deficiency in the dietary of distinct substances. In birds as a rule only dry beri-beri occurs, and, although infantile beri-beri is almost exclusively of the wet type, no signs of this condition were observed by the author even in very young chickens fed on polished rice. In this paper we are therefore only concerned with the substance which prevents and cures dry beri-beri and avian polyneuritis.

I. PREPARATION OF AN ANTI-NEURITIC SUBSTANCE FROM CARDIAC MUSCLE.

150 lbs. of ox-heart were minced, dried at 30° by means of an electric fan, ground, and thoroughly extracted with 95 % alcohol. The filtered extract, from which the alcohol was removed by evaporation *in vacuo*, was treated with an excess of ether, and the material precipitated possessed marked anti-neuritic properties, 0.2 g. given orally rapidly curing pigeons affected with polyneuritis. The procedure subsequently followed was a modification of that employed in the fractionation of horse-flesh and is briefly described below.

The ether-insoluble precipitate was taken up with 95 % alcohol which dissolved a considerable amount of material. The insoluble residue contained both inorganic and organic matter, but was inactive. The soluble fraction however was strongly curative and was next treated with excess of ether, which completely precipitated the anti-neuritic substance. The precipitate was dissolved in water and allowed to stand in a vacuum desiccator for several days. A white crystalline substance slowly separated, but this had no curative action. The supernatant liquor was then treated with basic lead acetate until no more precipitate was formed. As in the case of horse-flesh, the active substance was not precipitated by this reagent, and the filtrate

possessed marked curative properties. So far the method was identical with that employed in the case of horse-flesh, but at this stage a departure from the original process was made. It was found in the previous work that the addition of silver nitrate after the lead acetate precipitation only partially precipitated the curative substance. This manipulation was therefore postponed in the subsequent technique with the object of ascertaining what influence intermediate fractionations might have upon the precipitability of the substance by the silver salt. The lead acetate filtrate after the removal of excess of lead by dilute sulphuric acid was therefore evaporated *in vacuo* and the concentrated solution treated with excess of 95 per cent. alcohol. By this procedure some material was separated, but all the anti-neuritic substance remained in solution. The filtrate was evaporated to a syrup and treated with acetone, which precipitated practically all the active substance. The filtrate possessed only slight curative properties, whilst 0.15 g. of the precipitated substance given orally rapidly cured pigeons affected with polyneuritis. The substance was insoluble in benzene, ether, and chloroform, but dissolved readily in water. The aqueous solution was next treated with silver nitrate solution, which however still only precipitated a portion of the active substance, and the latter could furthermore only be partially precipitated from the filtrate by the addition of baryta. The first precipitate (with silver nitrate only) was decomposed with dilute hydrochloric acid and silver nitrate again added to the filtered solution. On this occasion the substance was not precipitated to any appreciable degree and the filtrate was strongly curative. The filtrate after removal of excess of silver as the chloride was concentrated *in vacuo* and was then treated with acetone. A substance was thereby precipitated, 0.04 g. of which given orally quickly cured polyneuritis in pigeons whilst 0.008 g. cured by subcutaneous injection. The filtrate from the third precipitate (obtained with silver nitrate and baryta) was concentrated and treated with 95 per cent. alcohol, and a substance was precipitated, 0.04 g. of which given orally also cured polyneuritis. The manipulations are set forth schematically below.

(The thick lines represent the main course of the active substance during the fractionations.)

The substances separated in the above way from filtrates II and III and the solution obtained by decomposition of precipitate III were now combined and treated with 95 per cent. alcohol, which precipitated a small amount of material possessing no curative properties. The filtrate was evaporated and acetone added. This caused the precipitation of a substance (5 g.) 0.04 g. of which administered orally readily cured neuritic pigeons. The filtrate was only slightly curative.

On standing a few days in a sulphuric acid desiccator the substance however ceased to have any curative action, and even large doses only prolonged the lives of neuritic birds. It was thus of little interest to carry out a chemical analysis of the substance.

It is evident therefore that the anti-neuritic substance is extremely labile and with the chemical methods at present at our disposal its isolation will prove a very difficult matter. Further work must be carried out to ascertain the conditions favourable to the existence of the active substance. For this reason and also on account of cost it is doubtful if highly purified preparations will be of much therapeutical value.

An inexpensive method however of preparing a non-toxic highly curative solution suitable for use in the treatment of human beri-beri was described in a previous communication [Cooper, 1914, 2].

II. THE RELATIONS OF VITAMINE TO LIPOIDS.

The curative power of the original alcoholic extract of cardiac muscle was determined before and after treatment with ether. It was found that about 3 g. of the extract readily cured polyneuritis in pigeons, but after the extract was treated with ether 4 to 8 g. of the fats and lipoids (ether-soluble fraction) had little or no curative action. The small amount of active substance still retained by them could not be removed by a second extraction with ether, but by treating the ether-soluble fraction with acetone the phosphatides were precipitated and as much as 10 g. had not the slightest effect upon neuritic pigeons. These observations support the conclusion of MacLean (p. 347).

Different observations however were made in the case of brain. 13 kilos. of ox-brain were minced, dried at 30° for 16 hours, and extracted with alcohol on the shaking machine. The filtered extract was concentrated

in vacuo to remove the alcohol, and when dry weighed 730 g. The extract was next treated with excess of ether, which precipitated 100 g. of a white substance. 0.6 g. of this rapidly cured polyneuritis in pigeons, but 0.4 g. had only a slight action.

This fraction from brain was less curative than the corresponding fractions from voluntary muscle and cardiac muscle, 0.2 g. of which was quite sufficient to bring about recovery. It also differed from the voluntary muscle preparations in not being completely soluble in water. The ether-soluble fraction from brain, on the other hand, unlike the analogous fractions from muscle, possessed marked curative properties, 2-3 g. being sufficient to cure pigeons affected with polyneuritis. The fats and lipoids still retained their activity even when extracted three times with ether.

On treating the ether-soluble fraction with acetone however the active substances were present in the acetone-soluble fraction, 1-3 g. of which readily cured, and, as was observed in the case of cardiac muscle, the precipitated phosphatides in doses of 5 to 10 g. possessed no detectable curative power. The phosphatides had no curative effect even after hydrolysis with 5 per cent. sulphuric acid, a process which does not affect the active substance. This shows that the negative results were not due to the indigestibility of the lipoids.

Similar results were obtained when alcoholic extracts of sheep-brain and egg-yolk, were treated with ether. There would thus appear to be present in brain and egg-yolk an ether- and acetone-soluble substance which possesses a marked affinity, chemical or adsorptive, for vitamines, and which may function as a carrier in the living organism.

The residues left after extracting the ox- and sheep-brain with alcohol were retained, and experiments carried out to ascertain to what extent the anti-neuritic substance contained in brain was extracted by alcohol.

13 kilos. of the ox-brain yielded 1680 g. of residue after exhaustion with alcohol. It was previously found [Cooper, 1914, 1] that the addition of 6 g. of ox-brain to the polished rice diet (1/20th body-weight ration daily) was sufficient to prevent polyneuritis in pigeons for at least 50 days.

Two pigeons were therefore fed daily on the above ration of polished rice and an amount of the brain residue equivalent to 12 g. of brain (1.5 g.). Both birds developed polyneuritis in 30 days. It is evident from this result that the greater part of the anti-neuritic substance contained in ox-brain is extracted by 95 per cent. alcohol.

1900 g. of the sheep-brain yielded 238 g. of alcohol-insoluble material.

It was previously observed [Cooper, 1913, 1] that the addition of about 8 g. of sheep-brain to a 1/20th body-weight ration of polished rice daily prevented polyneuritis in pigeons for 50 days. Three pigeons were therefore fed daily on the above ration of rice and an amount of the brain residue equivalent to 15 g. of the original brain (2 g.). All the birds developed polyneuritis within 30 days. As in the case of ox-brain, the bulk of the anti-neuritic substance is evidently extracted along with the fats and lipoids by 95 per cent. alcohol.

It having been demonstrated that brain phosphatides when purified possessed no curative power, experiments were next carried out to ascertain if the anti-neuritic substance were more firmly combined with certain other constituents of brain. Cholesterol prepared by crystallisation from an acetone extract of brain was first tested on neuritic pigeons, but 1 g. was found to exert no curative action.

1 kilo. of ox-brain was next minced and without drying repeatedly extracted with absolute alcohol at 40°. The extract was filtered and kept at 0° for 12 hours. By that time a yellowish precipitate (protagon) separated out. This was filtered off and washed with cold alcohol, the residue weighing 15 g. 2 to 5 g. of this deposit however had no effect at all upon neuritic pigeons.

5 g. were then treated with ether. The insoluble residue (3 g.) containing cerebroside (cerebrone) had no curative action, and kephalin (1 g.) precipitated from the ethereal extract by the addition of absolute alcohol also proved to be inactive. The original alcoholic extract after removal of the "protagon" was concentrated *in vacuo*, and the residue treated with ether. As before, the precipitate was curative. It was extracted with absolute alcohol at 0°, and the undissolved substance (1.5 g.) containing cerebrosides was again inactive. The alcohol-soluble fraction was concentrated *in vacuo* and when dry weighed 5 g. This was highly curative, 0.25 to 0.5 g. being adequate to cure polyneuritis.

The original ether-soluble fraction containing the phosphatides and fats of the brain was freed from ether and treated with acetone. The precipitated phosphatides were dissolved in a little chloroform and again reprecipitated with acetone. The phosphatides in doses of from 4 to 8 g. were inactive but 1 to 2.5 g. of the acetone-soluble fraction readily cured pigeons, thus confirming the observations detailed earlier in the paper.

This work was then repeated on a larger scale, 3 kilos. of ox-brain being used. Similar results were obtained, protagon (even when hydrolysed)

kephalin, cerebrone, and the phosphatides again being found to be ineffective when given to neuritic pigeons in approximately the above mentioned doses.

The failure of protagon, kephalin, cerebrone, cholesterol and phosphatides to cure polyneuritis is of interest in relation to the researches of Stepp [1909, 1911, 1913] who has found that food extracted with alcohol-ether is deleterious to the health of mice, and that the harmful effect of the diet is not checked by the addition of lecithin, cholesterol, kephalin, cerebrone, and phytin. When however egg-yolk or alcoholic extracts of egg-yolk or brain are added the animals remain healthy. Of these substances cholesterol, kephalin, and cerebrone as stated above do not contain vitamine, and lecithin [MacLean, 1912; Cooper, 1913, 2] and phytin [Schaumann, 1910; Cooper and Funk, 1911] when purified are also practically free from the active substance.

Egg-yolk and alcoholic extracts thereof on the other hand contain the essential substance [Cooper, 1913, 1], as also do alcoholic extracts of brain [Funk, 1912; Cooper, 1913, 2]. It is therefore probable that the harmful effects of diets consisting of food extracted with alcohol-ether is due not to the deficiency in the diets of lipoids, but to a deficiency of vitamine which is extracted by alcohol along with the lipoids.

The main conclusion that emerges from these observations is that vitamine does not apparently enter into the constitution of the lipoids of the brain, but may possibly be stored therein until required in the nutrition of the nervous system.

To what extent lipoids can adsorb the active substance from an *aqueous* medium is a point to be settled by further experiment. Meanwhile it may be noted that asbestos (Ural) does not appreciably adsorb vitamine from solutions of autolysed yeast (acid reaction).

SUMMARY.

1. Although infantile beri-beri is almost exclusively of the wet type, young chickens fed on polished rice develop symptoms of the dry form only, like adult birds.

2. A method is described for the preparation from cardiac muscle of a substance small amounts of which cure polyneuritis in pigeons. The substance however was extremely unstable and lost its activity in a few days after isolation.

3. As in the case of voluntary muscle, the bulk of the anti-neuritic

substance contained in the fats and lipoids (alcoholic extract) of cardiac muscle can be separated therefrom by means of ether.

4. In the case of brain, however, the anti-neuritic substance can only be partially precipitated from the alcoholic extract by treatment with ether, but can be entirely removed from the phosphatides by the subsequent addition of acetone. An explanation of this is offered.

5. Purified brain phosphatides possess no curative power even after hydrolysis with acid. This shows that their inability to cure polyneuritis is not due to incomplete digestion.

6. Protagon (even when hydrolysed), kephalin, cholesterol, and cerebrone similarly have no curative action.

7. The bulk of the anti-neuritic substance contained in sheep- and ox-brain can be extracted by 95 per cent. alcohol.

CONCLUSION.

The results show that vitamine does not enter into the constitution of the lipoids of brain and muscle, but may possibly be adsorbed in the lipoids and so stored, until required by the organism.

It would thus appear that the deleterious effect of lipid-free diets observed by Stepp is due not to the deficiency of lipoids, but to the mechanical removal of vitamine during the alcohol-ether extractions.

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