XLVII. A STUDY OF THE CHEMICAL NATURE OF VITAMIN B₆ AND METHODS FOR ITS PREPARATION IN A CONCENTRATED STATE.

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THE term vitamin B₆ has been given by György [1934] to that part of the vitamin B₂ complex which is responsible for the cure of the specific dermatitis developed by young rats fed on a vitamin B-free diet supplemented with purified vitamin B₁ and lactoflavin. The object of this paper is to give an account of the study which has been made of this dietary factor.

PART I. EXTRACTION OF VITAMIN B₆.

The method of assaying vitamin B₆ was described by György [1935, 1]. It consists of finding the minimum daily dose of the test material needed to cure the rat of the specific "acrodynia-like" dermatitis. The amount necessary is defined as one unit. This method has been used in all our experiments and proved to be satisfactory. The biological tests are shown in Table I, the experimental details of the extraction of the vitamin being set out in the experimental section.

Previously György [1935, 1] had shown that fresh fish muscle was a good source of vitamin B₆. The results obtained with salmon, haddock and herrings are shown in Table I. As aqueous extracts of fish muscle contain relatively small amounts of solid material in comparison with aqueous extracts of yeast or liver (from which the water-soluble vitamins are often prepared), it was thought that fish would prove an excellent source for the preparation of concentrates. With this object in view both salmon and haddock muscle were extracted by boiling water; but with both materials less than 25% of the vitamin was found to be extracted (Table I, Exps. 2, 3 and 4).

Attempts were next made to extract the vitamin by means of alcohol, but again no more than 25% of the vitamin could be obtained in solution. In the first place, extraction with cold alcohol was tried. This proved unsuitable, and in a later experiment minced herring muscle was boiled under a reflux condenser with 50% alcohol containing 1% HCl. Unfortunately a similar result was obtained (Exps. 5 and 6).

As it appeared to be very difficult to get the vitamin into solution in anything approaching quantitative amounts by simple extraction, autolysis of the tissue was next tried. This method gave variable results. If only toluene were used as an antiseptic, 50-70% of the vitamin was obtained in solution, but the autolysis mixture developed a putrid smell, indicating that some bacterial decomposition had occurred. If chloroform in addition to toluene were used bacterial action was prevented, but only 25% of the activity was recovered in solution (Exps. 7, 8 and 9).

In order to ascertain if it were possible to obtain all of the vitamin in solution after the tissue had been completely broken down, herring muscle was hydrolysed by the action of papain. In this manner 75 % of the vitamin was recovered in solution (Exp. 10).

Table I.

Exp.	Material tested	Amount fed	Healing of acrodynia	Weekly increase in g.	% of activity extracted
1	Fresh salmon	0.5 g.	Yes	8	
	Haddock	0.5 g.	Yes	3	
	Fresh herring	0.5 g.	Yes	6	
2	Boiled water extract of salmon I (2 ml. = 1.5 g.)	2.0 ml. 3.0 ml. 4.0 ml.	No Yes Yes	- 1 5 6	<25
3	Boiled water extract of salmon II (1.5 ml. = 2 g.)	1.5 ml. 4.0 ml.	No Yes	0 10	<25
4	Boiled water extract of haddock	2.0 g.* 3.0 g.*	No No	- 3 - 1	<17
5	Alcoholic extract of salmon (1 ml. = 1 g.)	1.0 ml. 2.0 ml. 3.0 ml.	No Improved Yes	- 2 6 9	<25
6	Alcoholic HCl extract of herring (1 ml. = 0.6 g.)	2.0 ml. 3.0 ml. 4.0 ml.	No No Yes	3 3 4	<25
7	Herring autolysed with toluene (putrid) (1 ml. = 2.5 g.)	0.2 ml. 0.3 ml. 0.4 ml.	No Yes Yes	0 3 5	65-70
8	Herring autolysed with CHCl ₃ and toluene (1 ml. = 1 g.)	0.75 ml. 1.0 ml. 1.5 ml. 2.0 ml.	No No No Yes	1 2 - 1 7	25
9	Herring autolysed with toluene (1 ml. = 1 g.)	0.75 ml. 1.0 ml. 1.5 ml. 2.0 ml.	No Yes Yes Yes	2 7 7 6	50
10	Herring digested with papain (1 ml. = 0.53 g.)	1.5 ml. 2.0 ml. 3.0 ml. 4.0 ml.	Improved Yes Yes Yes	3 4 4 6	75
11	Wheat germ	0.2 g.	Yes	8	
12	Alcoholic HCl extract of wheat germ (1 ml. = 0.5 g.)	0.75 ml. 1.0 ml. 1.0 ml. 1.5 ml. 1.5 ml. 2.0 ml. 2.5 ml.	No Yes No Yes Yes Yes Yes	- 7 2 5 2 3 3	40
13	Chloroform and toluene wheat germ autolysate (1 ml. = 0.25 g.)	0.75 ml. 0.75 ml. 1.0 ml. 1.5 ml. 1.5 ml. 1.5 ml. 2.0 ml.	Yes Yes Yes Yes Yes Yes Yes	2 5 5 6 6 5 10	80-100

* Equivalent in fresh haddock.

Although hydrolysis with papain brought nearly all of the vitamin into solution, the method was of little use for application on a large scale for the preparation of concentrates. Our attention was therefore turned to other materials from which the vitamin might be extracted more easily. It was found

that wheat germ was exceedingly rich in vitamin B₆, containing approximately 5 units per g. (Exp. 11). We first attempted to extract the vitamin by employing the method used by Guha and Drummond [1929] for vitamin B₁, which consists of boiling the wheat germ with 50 % alcohol containing 1 % HCl, under a reflux condenser for 30 min. This method resulted in the extraction of only 40 % of the vitamin (Exp. 12). This was a better yield than was obtained from fish, but it was still thought desirable to find a method of extraction which would give almost quantitative yields. Autolysis was next tried and was found to give yields of 80–100 %. Equally good results were obtained when either a mixture of chloroform and toluene or toluene alone was employed as antiseptic. This method was finally adopted as the standard procedure for the preparation of active extracts of the vitamin.

DISCUSSION.

The results of the experiments described above may be interpreted as meaning that vitamin B₆ is largely combined in some way with the tissue in which it occurs, since the greater part is not easily extracted by ordinary solvents. In fish muscle less than 25 % of the vitamin exists in the soluble state, and more is liberated by autolysis; but for complete extraction it is necessary to digest the tissue with papain. With wheat germ, again, only 40 % is easily extracted, but all is liberated by autolysis. This behaviour is in contrast with that of the other water-soluble vitamins, such as vitamin B₁, lactoflavin and ascorbic acid, which are easily extracted, but is similar to that of factor X of Boas [1927] and vitamin H of György [1931] (the factor necessary to neutralise the toxic action of dried egg white), which is only rendered soluble by digestion with papain [György, 1935, 2]. Vitamin B₆ therefore is not properly speaking a water-soluble vitamin but occupies a position intermediate between the water-soluble group and vitamin H, which is insoluble in its natural state. No information has been obtained concerning the nature of the union between vitamin B₆ and the tissue, but possibly the vitamin is attached to the protein as a prosthetic group which is not easily split off.

EXPERIMENTAL. (For results see Table I.)

Exp. 2. 200 g. of minced salmon were boiled with water for a few minutes, then filtered, and the filtrate adjusted so that 1 ml. = 0.75 g.

Exp. 3. 200 g. of minced salmon were boiled with water for approximately 30 min., then filtered and adjusted so that 1 ml. = 1.3 g.

Exp. 4. 200 g. of minced haddock muscle were boiled with water for 30 min., filtered and adjusted so that 1 ml. = 1 g.

Exp. 5. 200 g. of minced salmon were shaken with cold 97 % alcohol, allowed to stand for a short time and then filtered. The residue was again extracted with alcohol and filtered. The combined filtrates were evaporated to remove the alcohol and made up so that 1 ml. = 1 g.

Exp. 6. 300 g. of minced fresh herring were extracted with three times their volume of 50 % alcohol containing 1 % HCl by boiling under a reflux condenser for 30 min. The alcohol was evaporated from the filtrate and the volume adjusted so that 1 ml. = 0.6 g. of fresh herring.

Exp. 7. About 7 kg. of minced herring were placed in large stone jars with twice their volume of water. Toluene was added and the jars were placed in a warm room at 20–25° for 14 days. The autolysed tissue was then filtered off and the filtrate evaporated down by boiling. A precipitate which appeared was filtered off. The volume was adjusted so that 1 ml. was equivalent to 2.5 g. of fresh herring. The autolysed tissue had a strongly putrid smell.

Exp. 8. The details of this experiment were similar to the previous one except that chloroform was added in addition to toluene. The final volume of the filtrate was adjusted so that 1 ml. = 1 g.

Exp. 9. Repetition of Exp. 7. Volume of filtrate adjusted so that 1 ml. = 1 g.

Exp. 10. 200 g. of minced fresh herring were mixed with twice their volume of water. 1 g. of a papain preparation and also a small amount of toluene were added. The mixture was incubated at 37° for 24 hours. The small amount of insoluble residue was filtered off and the filtrate adjusted so that 1 ml. = 0.53 g. of fresh herring.

Exp. 12. 200 g. of wheat germ were extracted with 1400 ml. of 50% alcohol containing 1% HCl by boiling under reflux for 30 min. The extract was filtered and the alcohol evaporated off *in vacuo*. A slight precipitate which appeared after evaporation of the alcohol was filtered off and the filtrate made up so that 1 ml. was equivalent to 0.5 g. of wheat germ.

Exp. 13. 200 g. of wheat germ were thoroughly mixed with 1400 ml. of water. Chloroform and toluene were then added, the mixture shaken for a few moments to ensure that the anti-septics were properly distributed throughout the viscous mixture. It was then incubated at 37° for 4–5 days. At the end of this period the autolysed material was filtered and the filtrate evaporated and adjusted so that 1 ml. = 0.25 g. of wheat germ.

PART II. THE CHEMICAL NATURE OF VITAMIN B₆.

The behaviour of the vitamin towards chemical reagents.

Previous to the commencement of this work little was known about the chemical behaviour of vitamin B₆, for in the earlier work on "vitamin B₂" investigators were chiefly dealing with lactoflavin. György *et al.* [1933; 1934] had noticed that their "complementary factor" which supplemented lactoflavin and vitamin B₁, and which was thought at that time to be vitamin B₄, could be adsorbed only partly on fuller's earth from impure solutions such as milk or yeast extract. These authors also found that adsorption was made more quantitative by using purified concentrates, high acidity and large amounts of fuller's earth. Later György [1934; 1935, 1] named the factor vitamin B₆ as it had markedly different properties from those ascribed by Reader to vitamin B₄, *e.g.* stability towards alkali and heat and cure of specific "acro-dynia-like" dermatitis in the rat. The anti-pellagra factor for chickens of Elvehjem and Koehn [1935] is distinct from lactoflavin and has also been shown in a previous paper [Birch *et al.* 1935] to be different from vitamin B₆ and so has no bearing on the present problem. The only other work dealing with the chemical behaviour of this vitamin is that of Chick *et al.* [1935], who state that "the activity is found present in the dialysate after dialysis through a cellophane membrane and is not removed by precipitation with lead acetate either at p_H 4.0 or 8.0." We have been able to confirm these findings, as is shown in Table II, which summarises our observations. The experimental details are given in the experimental section, and the biological tests are shown in Table III.

We have not recorded the exact potencies of our most active preparations. The most active were of the order of 1 mg. and were obviously still very impure. We have been concerned in this paper with studying the chemical nature of the vitamin rather than with endeavouring to obtain preparations of a high activity. In order to do this it is of course necessary to obtain the vitamin in a moderately concentrated form. We therefore first studied the method for adsorbing the vitamin from crude extracts.

It was found that the vitamin could be adsorbed by fuller's earth from acid solution and eluted again by treatment with alkali. A fairly large amount of fuller's earth was necessary: approximately 1 g. to 10 units must be used in order to get anything approaching quantitative yields. This behaviour of the vitamin with fuller's earth is different from its behaviour with acid clay, on which it is apparently not adsorbed. Also it was noticed on one occasion that with a sample of fuller's earth obtained from Germany an active concentrate

Table II.

Reagent	Conditions	Result
Norite charcoal	p_H 6	Not adsorbed
English fuller's earth	p_H 2.5 or 5	Adsorbed, activity recovered on elution with $Ba(OH)_2$
"	p_H 9	Not adsorbed
Lead acetate	p_H 4.5 and 8	Not precipitated, activity recovered in filtrate
Silver nitrate	p_H 4 and 7	Not precipitated, activity recovered in filtrate
Mercuric nitrate	p_H 3	Not precipitated, activity recovered in filtrate
Electrodialysis in multi-compartment cell	—	Activity migrated to the cathode
Phosphotungstic acid	p_H 1	Precipitated
Picric acid	—	Not precipitated
Alcohol	Extraction of an alkaline gummy residue	Partly extracted
Acetone	Extraction of an alkaline gummy residue	Not extracted
Amyl alcohol	Extraction of an alkaline solution	Activity remained in water phase
Ether	Extraction of an alkaline solution	Activity remained in water phase
Benzoyl chloride	In alkaline solution according to the Schotten-Baumann procedure	No activity remained in filtrate
Nitrous acid	In dilute HCl	Not inactivated

Table III.

Exp.	Material tested	Amount fed ml.	Healing of "acrodynia-like" dermatitis	Weekly increase in g.	Remarks
1	Charcoal eluate (volume = 140 ml.)	0.8	No	— 3	No activity recovered
1a	Filtrate from charcoal adsorption (volume = 200 ml. approx.)	1.0 2.0	Yes Yes	7 10	About 50% of activity recovered
2	Pb acetate precipitate decomposed with H_2SO_4 (volume = 200 ml.)	0.3 0.5 0.8	No No No	— 0.5 — 0.5 2.5	No activity recovered
2a	Pb acetate filtrate decomposed with H_2S (volume = 1000 ml.)	0.5 0.75 1.0	No Yes Yes	1 5 4	Over 70% activity recovered
3	Fuller's earth eluate (volume = 200 ml.)	0.20 0.25	Yes Yes	2 2	About 25% activity recovered
3a	Fuller's earth filtrate	2.0	No	0	Over 50% activity lost
4	Fuller's earth eluate from adsorbate at p_H 2.5 (volume = 250 ml.)	0.5 2.0	Yes Yes	5 6	50% activity recovered
4a	Fuller's earth filtrate from adsorbate at p_H 2-3	1.0 2.0	No No	— 10 — 5	Over 50% activity removed
4b	Fuller's earth eluate from adsorbate at p_H 5 (volume = 250 ml.)	0.5 1.0 2.0	Yes Yes Yes	4 2 2	50% activity recovered

Table III (*cont.*).

Exp.	Material tested	Amount fed ml.	Healing of "acrodynia-like" dermatitis	Weekly increase in g.	Remarks
4c	Fuller's earth filtrate from adsorbate at p_H 5	2.0	No	0	Over 50% activity removed
4d	Fuller's earth eluate from adsorbate at p_H 9	1.0	No	Died	Not active
4e	Fuller's earth filtrate from adsorbate at p_H 9	2.0	No	Died	
5	AgNO ₃ filtrate (volume = 500 ml.)	1.0	Improved	0	50% activity remained
		2.0	Yes	2	
5	AgNO ₃ filtrate (volume = 500 ml.)	0.5	Yes	2	Over 50% activity recovered
		1.0	Yes	4	
5a	AgNO ₃ precipitate at p_H 4 (volume = 250 ml.)	0.5	No	Died	Not active
		0.5	No	Died	
5b	AgNO ₃ precipitate at p_H 7 (volume = 250 ml.)	0.5	No	Died	Not active
		0.5	No	Died	
6	Hg(NO ₃) ₂ filtrate (volume = 500 ml.)	0.5	Yes	4	Over 75% activity recovered
		0.75	Yes	2	
		1.0	Yes	6	
6a	Hg(NO ₃) ₂ precipitate (volume = 250 ml.)	0.75	No	Died	Not active
		1.0	No	Died	
7	Phosphotungstic precipitate (volume = 100 ml.)	0.2	Yes	0	Over 50% activity recovered from precipitate
		0.2	Improved	Died	
		0.4	Yes	5	
7a	Phosphotungstic filtrate (volume = 120 ml.)	0.4	No	- 2	Less than 30% activity in filtrate
8	Picric acid precipitate (volume = 100 ml.)	0.5	No	Died	Not active
9	Alcoholic extract of treacle (volume = 1000 ml.)	0.5	Yes	1	Over 50% activity removed
		1.0	Yes	4	
		2.0	Yes	4	
10	Acetone extract of solution from Exp. 9 (volume = 100 ml.)	0.1	No	0	
10a	Acetone residue (volume = 500 ml.)	0.5	Yes	2	
11	Amyl alcoholic extract of alkaline treacle (volume = 100 ml.)	0.5	No	- 9 Died	No activity removed
		1.0	No	- 8 Died	
12	Ether extract of alkaline treacle	1.0	No	- 1	
13	Filtrate after benzoylation	0.75	No	1.0	75% activity destroyed
		1.5	No	0.5	
		2.0	No	1.0	
14	Solution after treatment with nitrous acid	0.5	Yes	3	No loss of activity
		1.0	Yes	5	

was not obtained. However with material obtained from The British Drug Houses, Ltd., several satisfactory preparations have been made. It is possible therefore that a special type of fuller's earth is necessary in order to get proper adsorption of the vitamin. Attempts to adsorb the vitamin on norite charcoal at p_H 6 have failed, the activity always remaining in the filtrate. This result was rather surprising as it had been previously noted that the vitamin was present in the vitamin B₁ concentrates prepared according to the method of Kinnersley and Peters [1928]. However, although this method proved fruitless, we have not entirely abandoned the use of charcoal as an adsorbent; for it is well known that adsorption of a substance by this material is variable, and a slight modification of technique might result in its successful application.

The vitamin is apparently not precipitated by salts of the heavy metals such as lead, mercury or silver, but is precipitated from acid solution by phosphotungstic acid. This latter reaction suggests that it is a base, which supposition is borne out by the results of the electrodialysis experiment. It is probable however that its basic properties are not dependent on the presence of a primary amino-group for the vitamin is not inactivated by nitrous acid. On the other hand, treatment with benzoyl chloride according to the Schotten-Baumann method for the benzoylation of amino- and hydroxyl groups inactivates the vitamin. As the vitamin is not affected by nitrous acid it is possible that the destruction of the activity on benzoylation is through the involvement of a hydroxyl group.

Although the vitamin appears to be basic in nature we have not been able to precipitate it by picric acid or extract it from strongly alkaline solution by organic solvents such as ether or amyl alcohol. Its solubility in acetone has not been accurately determined, but the extraction with this solvent of a gummy residue obtained by the evaporation of an alkaline solution of the vitamin failed to bring much of the vitamin into solution, nearly all the activity remaining in the residue.

Electrodialysis experiments as a means of determining the chemical nature of the vitamin.

The object of an electrodialysis experiment is to determine whether the substance under consideration is an acid, base, ampholyte or neutral substance. If it happens to be an ampholyte an approximate measure of its isoelectric point is also obtained. This method was used by Williams and Waterman [1929] and by Birch and Guha [1931] for investigating the chemical nature of vitamin B₁ and McKinnis and King [1930] also used it for vitamin C.

Altogether three electro-dialysis experiments were carried out on vitamin B₆ (see Experimental section, p. 313). The first had to be stopped after a few hours as the concentrate used passed too high a current, but the activity was found to have migrated towards the compartment at the cathode end of the cell. In the second experiment which was carried out on a purer concentrate, electrodialysis was continued for 4 days. At the end of this period all the activity was found to have migrated to the cathode, the recovery of activity being approximately 35 %. A third experiment gave essentially similar results. It is thus evident that vitamin B₆ is either a simple base or an ampholyte having a highly alkaline isoelectric point.

Pure substances tested for vitamin B₆.

The following substances have been tested for vitamin B₆ activity but all have been found inactive in the day-dose indicated. Adenine 1 mg., adenylic acid 1 mg., yeast nucleic acid 0.2 g., choline 10 mg., betaine 10 and 50 mg. Some similarity between the chemical properties, and also the distribution in foodstuffs, of vitamin B₆ and choline made it seem possible that the two might be identical. For instance failure to precipitate the vitamin by silver or mercury excluded the purine or pyrimidine type of compound. The alkaloidal and the amino-types are excluded by failure to extract the vitamin from alkaline solution by organic solvents and by the stability of the vitamin towards nitrous acid. The curative action of fats, mention of which is made below, also indicated that choline might be concerned in some way with vitamin B₆ activity. However, pure choline chloride when fed to the deficient animals did not exert any curative action.

The sparing action of fat.

Hogan and Richardson [1934] have reported that rats fed with vitamin B which had been subjected to intense ultraviolet irradiation developed a severe dermatitis. From the description given by these authors the dermatitis appears to be similar in all respects to that produced by vitamin B₆ deficiency. In a later communication Hogan and Richardson [1935] stated that the dermatitis could be cured by wheat germ oil. This conclusion differed from our observation, for we had found that the curative factor was water-soluble rather than fat-soluble. This observation of Hogan and Richardson pointed to the supposition that two factors might be necessary for the cure of the dermatitis. However, the discrepancy between these observations has now been explained; for it has been found that fat exerts a remarkable sparing action on vitamin B₆. Thus, if a rat which has developed the dermatitis on a diet containing little fat (10% butter fat) is given small amounts of vitamin B₆ (under 1 unit) no cure is produced; if extra fat is now supplied (10 drops of linseed oil) a cure will be brought about. When a diet from which fat has been rigorously excluded is fed to rats the typical "acrodynia-like" dermatitis can be produced even while the animals are receiving relatively large amounts of vitamin B₆. This is shown in Fig. 1. By

Fig. 1. Production of dermatitis in rats fed on fat-free diets containing vitamin B₆. When lard is added as indicated by arrows the dermatitis is cured and growth is resumed. Diet 1 = 20% caseinogen, 20% lard, 55% sucrose, 5% salts + 3 units vitamin B₁ and 10 γ lactoflavin daily + 1 drop halibut-liver oil per week. Diet 2 = 10% caseinogen, 9% butter fat, 66% sucrose, 4% salts, 2% agar agar, 1% C.L.O. + 3 units vitamin B₁ and 10 γ lactoflavin daily. Diet 3 = 25% caseinogen, 70% sucrose, 5% salts + 0.5 ml. alcoholic yeast extract daily + 1 drop halibut-liver oil per week. Diet 4 = 25% caseinogen, 70% sucrose, 5% salts + 1.0 ml. alcoholic yeast extract daily + 1 drop halibut-liver oil per week.

The yeast extract was made by extracting 500 g. dried yeast with 1600 ml. of 60% alcohol for 24 hours. The residue was filtered off and washed. The filtrate and washings were then evaporated to 500 ml. and extracted with ether to remove traces of fat.

supplying as little as 20 drops of lard per day to these animals growth may be restored and the symptoms cured. The possibility still remains that fats may contain appreciable amounts of vitamin B₆. However, animals develop the specific dermatitis even when receiving 30% of linseed oil or lard in the diet although the onset of the symptoms is slightly delayed and the dermatitis is not so severe.

The nature of the substance present in fats which exerts the curative action on vitamin B₆-deficient animals has not yet been determined, but results indicate that it may possibly be linoleic acid. The work of Burr and Burr [1930] has shown that linoleic acid is necessary for the normal growth of the young rat and symptoms similar to those described by these authors, *e.g.* scaly tail and scurfy appearance of the skin have often been noticed in vitamin B₆-deficient animals. In addition butter fat which has been chiefly used in diets for the production of vitamin B₆ deficiency contains only small amounts of linoleic acid, whilst linseed oil and lard which contain about 30% and 14% respectively of this substance are particularly potent in exerting a curative action on vitamin B₆-deficient animals. It is hoped to publish a further account of these observations in a later paper.

EXPERIMENTAL. (For results see Table II.)

Exp. 1. 2000 ml. of an autolysed extract of herring containing about 3 units per ml. were brought to p_H 6 and then treated with two successive lots of 25 g. of norite charcoal. Each lot was filtered off and both together were eluted with 50% alcohol adjusted to p_H 1 with HCl. The eluate was evaporated to dryness and then made up for testing.

Exp. 2. 700 ml. of an autolysed extract of herrings containing 3 units per ml. were treated with lead acetate at p_H 4.5. The precipitate was filtered off and decomposed with H₂SO₄ and H₂S. It was then made up to 200 ml. for testing. The filtrate was adjusted to p_H 8 and more lead acetate added to ensure complete precipitation; only a slight precipitate was obtained. The filtrate was then treated with H₂S to remove the lead and made up to 1 litre for testing.

Exp. 3. The filtrates from the norite charcoal adsorption and the lead acetate precipitation were combined so as to give about 4000 units in 3 litres of solution; they were then treated with two successive lots of 100 g. of English fuller's earth. The fuller's earth was filtered off, washed with water and then eluted with a mixture of pyridine 30, methyl alcohol 60, acetone 30 and water 60. The eluate was evaporated to dryness and made up to 200 ml. for testing.

Exp. 4. 3 litres of autolysed wheat germ extract, after precipitation with lead acetate, containing approximately 1 unit per ml., were divided into three equal parts. The first part was adjusted to p_H 2.5, the second to p_H 5 and the third to p_H 9. Each lot was then treated with 100 g. of English fuller's earth. The fuller's earth was filtered off and each lot eluted with the pyridine mixture given above. The eluates were evaporated to dryness and made up to 250 ml. for testing. The activity was found in the eluates from the p_H 2.5 and 5 adsorptions, but in the filtrate from the p_H 9 adsorption. Later experiments were carried out using barium hydroxide solution for eluting the fuller's earth and it was found to give as good results as did the pyridine mixture. A sample of fuller's earth of German origin was used in one experiment and no active eluate was obtained.

Exp. 5. 400 ml. of a solution obtained from a fuller's earth eluate containing 1000–2000 units were adjusted to p_H 4. Silver nitrate was added until maximum precipitation was obtained. The precipitate was filtered off and the filtrate then adjusted to p_H 7, more silver nitrate being added to ensure complete precipitation. This precipitate was also filtered off and both precipitate and filtrate were decomposed with H₂S. The precipitates were each made up to a volume of 250 ml. and the filtrate to 500 ml.

Exp. 6. Part of the filtrate from the silver precipitation was adjusted to approximately p_H 3 by means of nitric acid; mercuric nitrate dissolved in nitric acid was then added until no more precipitate appeared; the solution was then brought back to p_H 3 with NaOH and the precipitate filtered off. Mercury was removed from the precipitate by treating with H₂S, the precipitate being made up to 250 ml. and the filtrate to 500 ml.

Exp. 7. 100 ml. of a solution after precipitation with silver and mercury, containing about 700 units, were made acid to p_H 0–1 with HCl. Phosphotungstic acid dissolved in water was added until almost complete precipitation was obtained. The precipitate and filtrate were each decomposed with barium hydroxide and made up for dosing. Volume of filtrate = 120 ml., volume of precipitate = 100 ml.

Exp. 8. A solution containing 2000 units of vitamin B₆ was obtained by extracting alkaline treacle with alcohol. After evaporation of the alcohol the extract was made up to 800 ml., and 10 g. of picric acid dissolved in hot water were added. On standing in the cold, a crystalline precipitate appeared, which was filtered off and then decomposed by dissolving in warm HCl and extracting the picric acid with benzene. The HCl solution was neutralised and made up to 100 ml. for testing. The filtrate was made acid with HCl and extracted with benzene. It was found impossible to remove all the picric acid from the filtrate, and when made up for dosing the animals refused to take it properly. It was therefore impossible to say how much activity remained. A further attempt to obtain a precipitate of the vitamin with picric acid was made, using a more purified solution containing 20 units per ml. However, after addition of the picric acid no precipitate was obtained, and the solution still retained at least 50 % of its activity, after the picric acid had been removed by benzene.

Exp. 9. 900 g. of black treacle, which contained about 3600 units of vitamin B₆, were made alkaline by heating on a water-bath with solid barium hydroxide. It was then transferred while hot to a Winchester bottle and 2 litres of 97 % alcohol were added. The mixture was shaken by a mechanical shaker for 20 min. The alcohol extract was then poured off from the semi-solid syrup and a second extract was made. The extracts were combined and evaporated *in vacuo* to remove the alcohol and were then made up to 1 litre. Any barium present was removed with sulphuric acid.

Exp. 10. An alcoholic extract of treacle made according to the procedure given in Exp. 9 was evaporated to a sticky mass and then extracted 5 times by stirring with cold acetone. Although a considerable amount of colour was extracted all the activity was found to remain in the residue.

Exp. 11. 200 g. of treacle were diluted with an equal amount of water and made alkaline with NaOH. This solution was then extracted 5 times with amyl alcohol in a separating funnel. The amyl alcohol was evaporated off and the extracted material dissolved in 100 ml. of water for testing.

Exp. 12. The procedure given in Exp. 11 was repeated, with the use of ether instead of amyl alcohol.

Exp. 13. 500 ml. of an autolysed extract of wheat germ after precipitation with lead acetate, containing approximately 1 unit per ml., were made alkaline with NaOH and then treated with benzoyl chloride according to the Schotten-Baumann method for the benzoylation of NH₂ and OH groups. While the solution was still alkaline a large sticky precipitate appeared. On making acid, a further crystalline precipitate came down, which was also filtered off. The filtrate was then extracted with ether to remove the benzoic acid formed and was adjusted to 200 ml. for testing.

Exp. 14. A solution containing 3 units per ml., obtained from the cathode compartment of an electrodialysis of a vitamin B₆ fraction, was made acid with HCl and then treated with a 10 % solution of sodium nitrite. The mixture was heated on a water-bath for 1 hour, further amounts of sodium nitrite being added until no more gas was evolved. The solution was allowed to stand overnight and the excess of nitrite was removed by means of urea. The volume was then adjusted so as to equal the original volume used.

Electrodialysis. The apparatus used by us for electrodialysis was similar to that described by Williams and Waterman [1929]. It consisted of an ebonite cell divided into 10 compartments by 9 parchment paper membranes. The 8 centre compartments held volumes of about 90 ml. each, whilst the anode and cathode held 200 ml. Platinum electrodes were used and a potential of 50 volts was generally employed.

It is necessary in performing an electrodialysis experiment of this kind to use a concentrate of the vitamin which is free from large amounts of ionisable impurities. If such impurities are present a large current is passed when the potential is applied and high concentrations of acid and alkali are formed at the anode and cathode. Under these conditions, instead of getting a gradual rise in p_H between the anode and cathode an abrupt change is obtained, which renders it impossible to determine the isoelectric point of an ampholyte with any degree of accuracy. Furthermore a considerable amount of heat is generated by a large current which, coupled with the high degree of alkalinity and acidity, is liable to inactivate the vitamin.

In the first place a preliminary experiment was carried out with a solution obtained by eluting an activated fuller's earth preparation. This concentrate passed too high a current owing to the presence of inorganic salts, and the experiment had to be stopped after a few hours. However, it was found that the activity had collected in the compartments towards the cathode end of the cell. In a later experiment an alcoholic extract of a fuller's earth eluate, taken down to dryness, was used. This preparation contained sufficient activity for the purpose and only contained small amounts of inorganic salts. The details of this experiment are as follows.

An alcoholic extract of material obtained from the elution of an activated fuller's earth preparation was evaporated to remove the alcohol and then made up to 1020 ml. This solution contained approximately 1500 units; 50 ml. were placed in both the anode and cathode compartments and 90 ml. in each of the centre compartments.

Electrodialysis was then carried out for 4 days. The current at the commencement of the experiment was 0.1 amp., but it fell considerably during the experiment until only a few milliamps were passed.

The volumes of the solutions in the various compartments and their p_H values at the end of the experiment were as follows:

Cathode 1,	202 ml.	p_H 10-12
Comp. 2,	45 ml.	p_H 7.4
„ 3,	85 ml.	p_H 5.0
„ 4,	73 ml.	p_H 4.5
„ 5,	74 ml.	p_H 3.9
„ 6, 7,	152 ml.	p_H 3.4
„ 8,	77 ml.	p_H 2.4
„ 9,	78 ml.	p_H 2.2
Anode 10,	134 ml.	p_H 2.0
Total	920 ml.	

On feeding the various fractions to rats it was found that the cathode solution contained approximately 3 units per ml. whilst the solutions from all other compartments were inactive in 1 ml. (See Table IV.)

Table IV.

Compartment	Amount fed	Healing of acrodynia	Weekly increase in g.
Cathode 1	0.25	Improved	1
	0.33	Yes	3
	0.50	Yes	3
„ 2	0.75	No	-1
	1.0	No	0
„ 3	0.5	No	-2
	1.0	No	Died
„ 4	0.25	No	-4
	0.5	No	-7
„ 5	2.0	No	-1
„ 6, 7	1.0	No	0
„ 8	1.0	No	-2
„ 9	1.0	No	-2
Anode 10	1.0	No	-3
	2.0	No	-2

A second experiment was conducted in a similar manner to the one described above and again the activity was found only in the cathode.

SUMMARY.

Part I.

The quantitative extraction of vitamin B_6 from fish muscle and wheat germ has been studied. With the use of boiling water or alcohol only 25 % is extracted from fish, about 40 % from wheat germ.

On autolysis, quantitative extraction is obtained from wheat germ, but the yields from fish muscle are variable. For complete extraction from fish muscle, hydrolysis of the tissue by papain is necessary.

Part II.

An account of the behaviour of vitamin B₆ towards various chemical reagents is given.

1. The vitamin is not precipitated by salts of lead, mercury or silver, or by picric acid.

2. It is adsorbed on fuller's earth from acid solution, is precipitated by phosphotungstic acid and migrates towards the cathode on electro dialysis.

3. It is inactivated by benzylation but not by the action of nitrous acid.

4. It is soluble in ethyl alcohol but is not extracted from a concentrated watery solution by acetone, amyl alcohol or ether.

5. From a consideration of these properties it is suggested that the vitamin does not contain a primary amino-group but is of a basic nature and possibly contains a hydroxyl group.

6. The observation by other workers that the vitamin is present in fats may be explained by our observation that fat has a sparing action on the vitamin.

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