STUDIES ON THE VITAMIN B_2 COMPLEX

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by

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Historical Introduction.

Beriberi, the Oriental disease, now known to be caused by deficiency of vitamin B_1 was recognised by the Chinese as early as 2000 B.C. [1]; it was, however, little more than half a century ago that it was first suggested that the disease had a dietary origin. In 1884, Takaki[2] almost completely eradicated beriberi from the Japanese Navy by introducing meat and legumes into the ration issued to the men; previously this ration had consisted mainly of polished rice. Takaki considered that the improvement was due to the introduction of more and better protein into the diet.

Eijkman in 1897 [3] observed that chickens receiving a diet of polished rice developed a paralytic disease which resembled beriberi in man; this disease of

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* Nomenclature. The vitamin B complex consists of the essential dietary factors contained in aqueous extracts of yeast, and the vitamin B_2 complex includes these dietary essentials of the vitamin B complex which are not inactivated by autoclaving for 5 hours at 120° at pH5. Since vitamin B_1 (aneurin) is probably the only essential nutrient present in yeast extracts destroyed by such autoclaving, the vitamin B complex may be regarded as being comprised of aneurin and the vitamin B_2 complex.

chickens was prevented when rice polishings were fed with the polished rice. Some years later Eijkman shared the view of Grigns[4], that the disease of chickens and also beriberi were due to deficiency of some substance present in whole rice but absent from polished rice. These discoveries of Eijkman are of very great importance; in these experiments we find not only the first real step towards the final elucidation of the etiology of beriberi, but also the first production of a deficiency disease in experimental animals.

Proof of the correctness of the theory of Eijkman, that beriberi is a deficiency disease, was afforded by experiments by Fletcher[5], Fraser and Stanton[6] and others, who showed that beriberi developed when the food consumed consisted mainly of polished rice, but not when the staple article of diet was rice from which the bran had not been removed.

The well known experiments of Hopkins[7] were published in 1912; he showed that the rat could not survive when given a diet of purified proteins, carbohydrates, fats and salts; but that the addition of a small percentage of dried milk, to supply "accessory

food factors" rendered that diet wholesome for rats. In that year Funk[8] introduced the name "vitamine".

An important advance in the field of vitamin research was the realisation of the multiplicity of vitamins. McCollum and his associates[9] in 1915 proved that the rat required at least two vitamins, one fat-soluble and the other water-soluble. The fat soluble "vitamin" of butter fat has since been found to be complex and to consist of vitamins A and D; the water-soluble "vitamin" has proved to be made up of several essential factors, which are included in the vitamin B complex.

The earliest indications of the complex nature of water-soluble vitamin B are found in the experiments of Mitchell[10], Emmett and Luros[11] and Kinnersley and Peters.[12] Generally, these investigators noted that various vitamin preparations had not always the same potency, when tested by methods involving (a) the curing of polyneuritis in birds and (b) the growth stimulation of young rats. Final proof that vitamin B is not a single substance was supplied by Smith and Hendrick[13] in 1926. They found that yeast after autoclaving was no longer potent in the prevention of polyneuritis in rats, but that the growth stimulating properties of the autoclaved yeast for young rats, maintained on certain diets, was unimpaired.

The etiology of pellagra, a disease associated with the eating of maize, was meantime being investigated by Goldberger et al[14]. A disease of dogs, named black tongue, was produced by feeding a diet similar to that commonly consumed in districts where pellagra was common. Since pellagra and blacktongue were cured by the same foodstuffs (milk, green vegetables, yeast, etc.), it was concluded that these diseases had similar etiologies, being caused by deficiency of a material termed "pellagra preventive" factor; adsorbates obtained by treatment of extracts of autoclaved yeast with fuller's earth were rich in this factor. Goldberger and Lillie[15] noted a dermatitis in rats, maintained on a diet of purified materials, containing an aqueous alcoholic extract of maize, as source of the antineuritic vitamin. This disease, which had some resemblance to human pellagra, was cured by fuller's earth adsorbates from autoclaved yeast extracts, and it was therefore concluded that the pellagra preventive factor was probably identical with the rat growth factor of Smith and Hendrick[13].

Experiments by Chick and Roscoe[16] in 1927 completed the proof that vitamin B was composed of at least two factors. Rats which received a Peters' antineuritic

vitamin concentrate as source of vitamin B ceased to increase in bodyweight after some time and developed dermatitis; the addition of autoclaved yeast to the diet cured the dermatitis and caused a resumption in growth. The antineuritic more heat-labile factor of the vitamin B complex was named vitamin B_1 and the more heat-stable growth-stimulating and dermatitis-preventing factor, vitamin B_2.

Before passing to consideration of vitamin B_2, which has since been found to be a complex containing several factors, brief mention must be made of the achievements, of both scientific and medical importance, which have attended the efforts of those who have chosen to investigate vitamin B_1. This was the first vitamin to be isolated in a pure state. In 1926 Jansen and Donath[17] prepared the crystalline vitamin from rice polishings, and later other investigators isolated this substance from other materials. The chemical nature of vitamin B_1 was elucidated mainly by the work of Williams in America and Windaus in Germany; the vitamin was proved to be the product of the condensation of 2-methyl-4-amino-5-chloromethylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole. Synthesis of vitamin B_1 was

achieved, almost simultaneously, in 1936 by Williams et al[18], Andersag and Westphal[19] and, in this country, by Todd and Bergel.[20] The work of Peters and his collaborators has gone far to prove that in the animal body the vitamin is connected with the metabolism of pyruvic acid. Lohmann and Schuster[21] proved that cocarboxylase is the pyrophosphoric ester of vitamin B_1.

It is incorrect to term a known chemical compound, a vitamin and, therefore, the use of the name vitamin B_1 should be discontinued; the name, aneurin, suggested by Jansen and Donath, who first isolated this compound, is now most commonly employed in Europe but some investigators do not favour this name.

The discovery that vitamin B_2 contains more than one factor was made in 1929 by Chick and her collaborators,[22,23] It was first observed that a concentrate made from egg-white, although it cured rat dermatitis, was less effective in promoting growth of young rats than was an extract of yeast autoclaved at pH5; this indicated that an essential factor, additional to that contained in egg-white concentrates, was present in the above yeast extracts. Later experiments showed that yeast extracts, autoclaved at pH10, were alone inactive as source of vitamin B_2 in rat growth tests, but greatly

enhanced the growth promoting action of egg-white concentrates. The factor contained in alkaline autoclaved yeast extracts was named factor Y and the term vitamin B_2 complex was introduced.

Reader [24,25] suggested in 1929 that rats required in addition to vitamins B_1 and B_2 a factor, named vitamin B_4, the heat stability of which was between those of vitamins B_1 and B_2. Subsequent work has not completely confirmed these earlier findings, but some workers still use the term; it is possible that this factor in reality was riboflavin, the least stable factor of the vitamin B_2 complex.[26]

In 1933 the yellow fluorescent pigment, riboflavin (lactoflavin) was isolated from egg-white and other materials by Kuhn et al,[27] and was shown to possess vitamin activity, when administered to rats receiving, as source of vitamin B_1, an alcoholic extract of cereals. It was first suggested by the discoverers that this compound had full vitamin B_2 activity, but soon they found that this was not the case. Ellinger and Koschara[28] had simultaneously isolated this substance from whey, and a year earlier Warburg and Christian[29] had investigated this same substance which is contained in their yellow

oxidation enzyme. Within two years from the time of the isolation of riboflavin, its chemical constitution had been elucidated and synthesis achieved, principally by Kuhn and his collaborators and, to a less extent, by Karrer and his collaborators.[30] This substance (6:7-dimethyl-9-(d-1'-ribitylisoalloxazine) is the product of condensation of a substituted alloxazine and reduced d-ribose. Riboflavin-5-phosphoric acid is the prosthetic group of the old yellow oxidation enzyme of Warburg and Christian; riboflavin, therefore, was the first substance found to be a dietary essential and also be concerned with enzymic activity.

The proof that riboflavin requires supplementation to attain full vitamin B_2 activity for the rat is found in the experiments of György *et al*[31], who supplied this supplement as the filtrate obtained by treating liver extracts with fuller's earth; they named this supplementary factor, vitamin B_4. Later György[32,33] used Peters' vitamin B_1 and B_4 concentrate as source of supplement, and he found that rats deprived of this supplement developed a dermatitis somewhat similar to that described by Goldberger and Lillie[15]; the addition of Peters' concentrate cured this dermatitis and the factor responsible for this effect György named vitamin B_6.

He identified this factor with what had previously been named vitamin B_4 by György *et al*.[31]

Chick *et al*[34] also investigated the supplementation of riboflavin. They recognised the egg-white concentrate previously employed to be essentially an impure solution of riboflavin. The sources of supplement used were (a) yeast extracts, autoclaved at pH 5 at 120° for 5 hours and subsequently treated with fuller's earth at pH 1; (b) yeast extracts autoclaved at pH 10. Both of these fractions promoted the growth of rats receiving riboflavin and also cured what was named "florid" dermatitis, developed on diets deficient in supplement; it was thought that the factor present in these materials was identical with György's vitamin B_6.

The study of the vitamin B_2 complex had reached this stage when the investigations described in the following pages were begun. The vitamin B_2 complex was then recognised to contain riboflavin and one other essential dietary factor, which was a growth factor for rats and cured the florid type of rat dermatitis; this factor was named vitamin B_6 by György and supplementary factor by Chick *et al*.

Further fractionation of the vitamin B_2 complex - the dietary essentials of the vitamin B_2 complex required by the rat.

It had been established[34] that young rats could be reared satisfactorily, for several weeks from weaning, on a synthetic diet in which the B-vitamins were provided by the following three materials.

(1) Small daily doses (= 0.3-0.6 g. yeast, dry wt.) of Peters' vitamin B_1 concentrate from yeast, or 10-15 μg. daily of aneurin.

(2) Small daily doses, 10-20 μg. of crystalline riboflavin.

(3) A heat-stable supplement contained in the filtrate from yeast extracts after treatment with fuller's earth, given in daily amount equivalent to 0.5 g. yeast, dry weight. This material was called the yeast fuller's earth filtrate fraction.

Animals receiving the above supplements remained healthy and no dermatitis developed.

The experiments, designed to effect purification and elucidate the chemical nature of the factor present in the above yeast fuller's earth filtrate fraction (p. 114), indicated that this factor was not identical with György's vitamin B_6. It had been found by Birch and György[35]

that vitamin B_6 had basic properties; this factor migrated to the cathode on electrodialysis and was readily precipitated by phosphotungstic acid. The factor present in the yeast filtrate fraction we found had no basic properties and, on the other hand, its precipitation with barium hydroxide in alcohol suggested an acidic nature. Vitamin B_6 was destroyed by exposure to light and was readily adsorbed by fuller's earth; the yeast fuller's earth filtrate factor was not destroyed by light and, of course, was not adsorbed by fuller's earth.

The experiments carried out in the Lister Institute by Copping[36] proved that the dermatitis which developed in rats receiving riboflavin only of the vitamin B_2 complex, was more effectively cured by alcoholic extracts of cereals than by the fuller's earth filtrate fraction. This indicated that at least one additional factor, not contained in yeast fuller's earth filtrate, was present in alcoholic extracts of cereals.

Final proof that yeast fuller's earth filtrate fraction did not supply all the nutrients of the vitamin B_2 complex, excluding riboflavin, was obtained by comparing the growth-rates of young rats receiving

as sources of the vitamin B complex (a) untreated yeast extract; (b) autoclaved yeast extract and aneurin; (c) yeast fuller's earth filtrate, riboflavin and aneurin. The growth-rates of the rats in this last group were very considerably lower than those of the rats in the other two groups, and, therefore, an essential nutrient must have been absent from the diet of the animals in the last group.

From the above experiments it was apparent that the fuller's earth had removed from the autoclaved yeast extract, a further essential nutrient required by the rat. A fraction was prepared from the fuller's earth adsorbate, which had growth promoting and dermatitis-curing properties for rats; this fraction was free from riboflavin. We named this fraction and the essential factor it contained, "yeast eluate fraction" and "yeast eluate factor" respectively. Later experiments (p.111) proved that eluate factor is identical with vitamin B_6.

The essential factors of the vitamin B_2 complex contained in extracts of liver were examined. Preparations named liver filtrate fraction and liver eluate fraction having biological properties identical

with the corresponding fractions from yeast, were prepared.

It appeared, for some time, that the vitamin B_2 requirements of the rats were satisfied when the animals received riboflavin, yeast or liver filtrate factor and yeast or liver eluate factor. Rats receiving these factors, given as synthetic riboflavin, the yeast fuller's earth filtrate fraction and the yeast eluate fraction, increased in bodyweight as rapidly as rats which received autoclaved yeast extracts as source of the vitamin B_2 complex; further, rats receiving these separated fractions were successfully mated and the mothers reared their young satisfactorily.

Quite recently, however, we have shown that there exists at least one further factor of the vitamin B_2 complex, required for growth of the rat. During the last two years we have been engaged in the purification, not only of filtrate factor and eluate factor, but also of our basal diet. We now find that rats receiving the purified filtrate factor, purified eluate factor and riboflavin, as sources of the vitamin B_2 complex, do not increase in bodyweight as rapidly as do rats receiving autoclaved extracts of yeast. There must, therefore,

exist a further essential nutrient for the rat of the vitamin B_2 group. As yet no name has been given to this factor, and it will be referred to as "additional factor". A concentrate of "additional factor" was prepared by extracting a liver extract with fuller's earth and then with amyl alcohol; the fuller's earth removed the eluate factor and the amyl alcohol the filtrate factor, while "additional factor" remained in the residue.

Recently nicotinic acid has been found to be an essential nutrient of the vitamin B_2 complex required by the dog, the pig, the monkey and man; we have been unable to demonstrate that this substance is a dietary factor for the rat. A combination of nicotinamide and adenylic acid was also inactive in rat experiments.

At present, therefore, four factors of the vitamin B_2 complex, essential for the rat are recognised: (1) riboflavin; (2) eluate factor; (3) filtrate factor; (4) "additional factor".

Methods and Results.

A. Separation of yeast filtrate factor and yeast eluate factor from autoclaved extracts of yeast.

Rat growth tests were carried out by the methods generally employed in the Lister Institute.[34] Young rats, weaned at 21 days, weighing between 40 and 50 g. received a basal diet consisting of commercial light white casein 100, rice starch 300, cotton-seed oil 60, lard 15, salt mixture (McCollum's No. 185) 25 and water 500; the diet was steamed for 3 hr. The lard was added to the diet to ensure an adequate supply of the essential unsaturated fatty acid in the hope of eliminating scaly tails and associated conditions[37,38]. The diet was supplemented by 0.08-0.1 ml. of cod liver oil daily, to supply vitamins A and D, and by 10-15 μg. of aneurin. It was found that aqueous solutions of aneurin could be kept free from moulds and without loss of activity if solutions containing 1 mg. per ml. in N/1000 HCl, were stored in the cold. More dilute solutions, also containing N/1000 HCl, suitable for dosing were made from the stock solution at least once weekly.

In preparing litters for this work we did not find it necessary, in order to render the young rats sensitive

to deficiency of the B_2-vitamins, to remove the yeast from the stock diet of the mothers during the whole of the lactation period, as was previously the custom in this laboratory. The mothers received the full stock breeding diet, except during the last week of lactation, when yeast was not included.

The rats were weighed 3 times weekly. Their growth had usually ceased by the end of the second week after weaning, and when the weight had remained stationary for several days, the animals received the vitamin B_2 supplements; the effects on the growth rates were observed.

Comparison of the growth-promoting action of the B-vitamins provided as (a) untreated aqueous yeast extract (b) yeast extract autoclaved at pH5, supplemented by aneurin and (c) the fuller's earth filtrate from autoclaved yeast extract supplemented by aneurin and riboflavin.

Preparation of the yeast fuller's earth filtrate fraction. A dilute acetic acid yeast extract, prepared as described by Chick and Roscoe[39], was autoclaved at pH 5 at 120° for 5 hr. to destroy vitamin B_1. 1 l. of this extract (1 ml. = 0.5 g. yeast, dry wt.) was adjusted to pH 1.4 by addition of approximately 20 g. of H_2SO_4. 50 g. of fuller's earth

(B.D.H. for adsorption purposes) were added and after stirring at intervals for 30 min. the fuller's earth was filtered off. The adsorption was repeated and the final filtrate was treated with Ba $(OH)_2$ to remove sulphate and adjusted to pH 3 for storage (1 ml. = 0.5 g. yeast, dry wt.).

A litter of rats was divided into 3 groups as follows: 2 males received doses of an untreated aqueous yeast extract equivalent to 1 g. yeast, dry wt. daily; 2 males received similar doses of the yeast extract after autoclaving at pH 5 at 120° for 5 hr. supplemented by 10-20 µg. daily of aneurin; 2 males and 2 females received doses of the yeast fuller's earth filtrate ~~(see Edgar et al. 1937)~~, equivalent to 1-1.5 g.yeast, dry wt. daily, supplemented by 10-20 µg. daily of aneurin and 50-75 µg. daily of riboflavin.

The growth rates of the rats receiving the untreated yeast extract (curve A, Fig. 1) were almost identical with those of the rats having autoclaved yeast extract supplemented by aneurin (curve B); both pairs showed rapid and steady growth during the experimental period of 8 weeks. The rats receiving the fuller's earth filtrate supplemented by riboflavin and aneurin showed lower growth rates (curves C and C^1); , when these doses

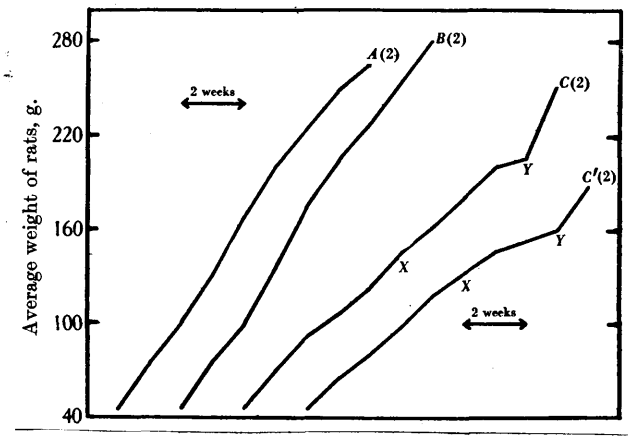

Fig. 1. Growth of young rats on a basal diet free from B-vitamins and receiving daily as sources of B-vitamins: curve A (♂ rats): untreated yeast extract = 1g. yeast dry wt.; curve B (♂ rats) autoclaved yeast extract = 1 g. yeast, dry wt. + 10-20 µg. aneurin; curve C (♂ rats) and C′ (♀ rats) yeast fuller's earth filtrate = 1 g. yeast, dry wt. + 50 µg. riboflavin + 10-20 µg. aneurin. At X the doses were increased to fuller's earth filtrate = 1.5 g. yeast, dry wt. and 75 µg. riboflavin. At Y the doses were changed to autoclaved yeast extract = 1 g. yeast, dry wt. + 20 µg. aneurin. The figures in brackets indicate the number of rats from which the [redacted] were derived.

TABLE I.

Growth of rats receiving the constituents of the vitamin B_2 complex as different fractions from yeast extract.

Unless otherwise stated, 10-20μg. aneurin were given daily.

	No. of rats.		Ribo-flavin daily	Yeast preparation given	Daily dose as equivalent of yeast, dry wt.	Average weekly gain in weight over period of 4 weeks. g.	
	♂	♀	μg.		g.	♂	♀
Exp. 1	2	0	0	Untreated yeast extract; no additional vitamin B_1	1.0	31.1	-
	2	2	0	Autoclaved yeast extract	1.0	31.3	22.75
	2	2	50	Fuller's earth filtrate	1.0	19.3	18.3
	1	1	75	Fuller's earth filtrate	1.5	19.25	17.0
Exp. 2	1	3	50	Purified fuller's earth filtrate	1*	22.5	18.5
	2	3	50	Fuller's earth eluate	2*	12.1	11.9
	3	5	50	Fuller's earth filtrate + fuller's earth eluate	1 2	32.6	24.0

						Average weekly gain in weight over period of 2 weeks. g.	
							♀
	-	1	0	Fuller's earth eluate;	2	-	8.5
				after 2 weeks, given fuller's earth filtrate in addition	1	-	8.5
	-	1	0	Fuller's earth eluate + fuller's earth filtrate	2 1	-	3.5

* Dose doubled after 2 weeks.

were replaced by untreated yeast extract a sharp increase in growth rate resulted immediately. Further experiments, summarized in Table I, Exp. 1, confirmed these results.

These experiments demonstrate that autoclaving yeast extracts at pH 5 does not destroy any of the B-vitamins concerned with growth other than aneurin, and that treatment with fuller's earth removes from an

autoclaved yeast extract at least one growth factor in addition to riboflavin.

Investigation of the yeast fuller's earth adsorbate.

Preliminary experiments in which fuller's earth adsorbate was itself fed directly to young rats in addition to the fuller's earth filtrate and riboflavin suggested that the adsorbate had some additional growth-promoting action. The growth responses were, however, irregular, and seemed to indicate that elution of the adsorbed material from fuller's earth was not accomplished satisfactorily in the alimentary canal of the rats. Eluates were therefore prepared, the following method being finally adopted.

The fuller's earth adsorbate was made by adding 50 g. of fuller's earth (B.D.H. "for adsorption purposes") to 1l. of aqueous yeast extract (1 ml. = 0.5 g. yeast, dry wt.), which had been autoclaved at pH 5 at 120° for 5 hr. and then adjusted to pH 1.4. This adsorbate was washed twice by grinding in a mortar with 250 ml. of 0.1 N HCl and then, after filtering, was thoroughly mixed with 600 ml. of 0.1 N $Ba(OH)_2$ in a mortar. After standing for 16 hr. in the cold, the fuller's earth was filtered off and the elution repeated. The eluates were

neutralized with H_2SO_4 immediately after being filtered from the fuller's earth. In order to remove the riboflavin present, the combined eluates were treated at pH 8 with a slight excess of a solution of basic lead acetate (containing approximately 7 g. basic lead acetate). After standing 16 hr. in the cold, the precipitate was filtered off, the filtrate treated with H_2S, the lead sulphide filtered off and the final filtrate reduced in vacuo to a volume of 250 ml. (1 ml. = 2 g. yeast, dry wt.). This material will be referred to as the yeast fuller's earth eluate.

For the following experiments the yeast fuller's earth filtrate was further purified by three additional adsorptions with fuller's earth at pH 3, 50 g. fuller's earth per litre of filtrate being used for each adsorption.

The additional growth-promoting action of the eluate fraction on rats receiving aneurin, riboflavin and the purified fuller's earth filtrate was striking (Table I, Exp. 2 and Fig. 2). Rats receiving daily the eluate solution (= 2 g. yeast, dry wt.) supplemented by the filtrate fraction (= 1 g. yeast, dry wt.) and 50 µg of riboflavin showed weight increases of about 24-32 g. weekly, according to their sex, over a 4-week period (curves A' and A). This is a growth rate of the same

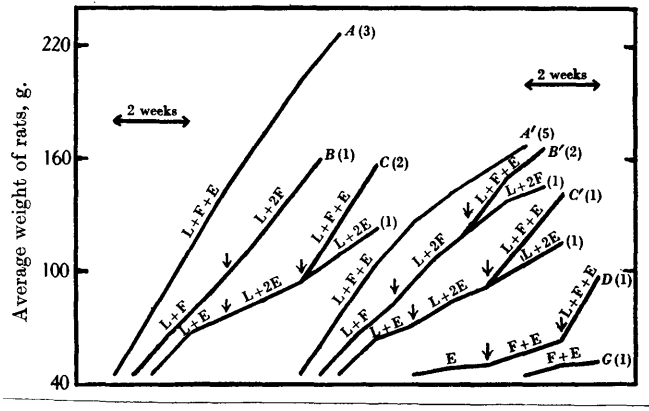

Fig. 2. Growth of young rats on a basal diet free from B-vitamins and receiving daily 10-20 μg. aneurin and one or more of the following components of the vitamin B_2 complex: L= 50 μg. riboflavin. F = purified fuller's earth filtrate = 1 g. yeast, dry wt. E = fuller's earth eluate = 2 g. yeast, dry wt. The arrows indicate the points at which the doses were changed. Curves A, B, C, ♂ rats. Curves A', B', C', D, G, ♀ rats. The figures in brackets indicate the number of rats from which the growth curves were derived.

order as that attained by rats maintained on experimental diets with untreated yeast extract as source of the B-vitamins, or on a good mixed diet of natural foodstuffs. The growth rate obtained with litter-mate rats receiving riboflavin supplemented only by the fuller's earth filtrate (1 g. yeast, dry wt. daily) was approximately 15-20 g. weekly for 4 weeks (curves B' and B), and was

little affected by doubling the dose of the filtrate fraction, but it was greatly enhanced by the addition of the eluate fraction. Feeding of the eluate fraction in doses equivalent to 2 g. dried yeast daily supplemented by riboflavin caused an initial increase in growth (curves C and C') similar to that obtained when the filtrate fraction was fed with riboflavin but, after about 10 days when the animals had gained approximately 30 g. the growth slackened sharply; doubling the eluate dose at the end of 2 weeks had little or no effect on the growth rate, while the addition of the filtrate fraction to the diet caused a marked increase.

Since only a slight or negligible effect on the growth rate was observed by doubling the doses of either the filtrate fraction or the eluate fraction it may be assumed that a fairly complete separation of these two growth-promoting vitamins had been attained. The eluate fraction, having been treated with basic lead acetate, contained no riboflavin, and rats receiving this fraction and aneurin only did not show any increase in weight, nor did they grow when these were supplemented with the filtrate fraction (curves D and G). This illustrates the fact that riboflavin is the most active of the growth-promoting vitamins of the B_2 group, since in its

absence all growth was checked, while the absence of one or both of the other factors resulted only in limitations of the growth if the riboflavin were present in the diet.

The regularity of the growth response of the animals to the various factors was striking, the effects being evident in 1 week or even less. When all three vitamin B_2 components were present, the average increase in weight during the first week was 28-35 g.; in the absence of either the eluate fraction or the filtrate fraction it was 18-23 g. By the end of the second week a difference was apparent, absence of the filtrate fraction resulting in a marked slackening in the growth rate during the second week, while in the absence of the eluate fraction the initial suboptimum growth rate was maintained for 3-4 weeks.

More satisfactory results have more recently been obtained using a preparation of yeast filtrate factor which was purified by amyl alcohol extraction, a procedure which has also proved effective in the purification of the factor required for the growth and prevention of dermatitis in chicks receiving a heated grain diet[40,41]. The following method has been employed by us.

The fuller's earth filtrate (4 l. of concentration 1 ml. equivalent to 0.5 g. dry yeast) was concentrated in open trays at 37° to one-fourth of its original volume. H_2SO_4 was added to pH 1 and the extract was shaken with 2 l. amyl alcohol. After separation, the amyl alcohol extract was shaken with 500 ml. water to which was added just enough NaOH to make the aqueous layer alkaline to thymol blue. The aqueous extract was separated, neutralized with HCl and the amyl alcohol again extracted with a second portion of alkali. The amyl alcohol was now transferred back to the yeast extract and the extraction repeated until the yeast filtrate had been extracted 6 times, the amyl alcohol being extracted with the alkali after each treatment of the yeast extract. The combined aqueous extracts of the amyl alcohol, containing the filtrate factor, were evaporated to small volume and treated with 4 volumes of 96% alcohol. The precipitated salts were removed by filtration and the alcohol was distilled off <u>in vacuo</u>. A small amount of a gummy material which separated was discarded; in the final product 1 ml. was equivalent to 2 g. dry yeast.

Rats receiving this preparation of yeast filtrate factor increased in bodyweight less rapidly than did animals receiving the crude fuller's earth filtrate, and

a more striking effect on the growth-rate was observed when the yeast eluate fraction was added (see TableIII, p. 35).

B. Preparation from liver extracts of fractions containing the same dietary essentials for the rat as yeast eluate fraction and yeast filtrate fraction.

Fractionation of liver extracts, by the methods successfully employed for the separation of the factors present in yeast extracts, did not yield liver fractions with biological properties similar to those of the yeast fractions. By other methods, however, separation of the essential factors of the vitamin B_2 complex present in liver was accomplished; preparations were obtained which were biologically undistinguishable from yeast filtrate fraction and yeast eluate fraction.

The rat-growth tests were carried out by the method previously described (p. 15). During the first week after weaning the young animals received the basal diet supplemented by 0.08 ml. cod liver oil and 10 μg. aneurin each daily. Thereafter, the experimental procedure was varied to some extent.

Yeast filtrate fraction and yeast eluate fraction were prepared by the methods already described on (pp. 25 and 20). All the liver fractions were prepared from liver residues obtained in the manufacture of the pernicious anaemia factor[42]; these were kindly

supplied to us by Messrs. Glaxo Laboratories. Three residues have been investigated.

Liver residue I. This residue was the filtrate resulting when a liver extract, obtained by extraction of liver with aqueous acetone and subsequent removal of the acetone, was treated with charcoal. Experiments with rats showed that this residue probably contained all of the factors of the vitamin B_2 complex with the exception of riboflavin, since rats receiving the basal diet supplemented by cod liver oil, aneurin, riboflavin and this liver residue grew as well as did rats receiving that diet with whole yeast or liver extract as the source of the B_2-vitamins.

Liver residue II. The liver extract made by aqueous extraction of liver, freed from acetone and reduced to small volume was extracted with phenol. The fraction insoluble in phenol was liver residue II. This liver fraction was also rich in members of the vitamin B_2 complex, although it contained less of the liver factor corresponding to yeast filtrate factor than did liver residue I.

Liver residue III. The aqueous layer, obtained when the above phenol extract was shaken with water and ether, was treated with charcoal. Liver residue III

was the filtrate from the charcoal. This residue contained considerable amounts of the rat dietary factor corresponding to the yeast filtrate factor and only traces of other factors.

Liver residue I has been mainly used in this investigation.

Fractionation of liver residue I with fuller's earth.

The liver fraction (1 l.; 1 ml. = 5 g. fresh liver) was adjusted to pH 1.2 by the addition of H_2SO_4, and 50 g. fuller's earth ("specially selected, activated", Fuller's Earth Union) was added. After stirring at intervals for 30 min. the adsorbate was removed by filtration. The adsorption was repeated, and the filtrate, after treatment with $Ba(OH)_2$ to remove H_2SO_4 was ready for administration to rats.

The first adsorbate was washed twice with 500 ml. of N/10 HCl and suspended in 700 ml. of 2% $Ba(OH)_2$. After several hours at 0^o, the eluate was removed by filtration, and the adsorbate was again eluted with 500 ml. of 2% $Ba(OH)_2$. The combined eluates, freed from Ba with H_2SO_4, were adjusted to pH 8 and treated with an excess of basic lead acetate (8 g. in 40 ml. of H_2O). The resulting precipitate was filtered off and the excess lead removed from the filtrate with H_2S.

The PbS was filtered off and the filtrate reduced *in vacuo* to 250 ml. (1 ml. = 20 g. fresh liver).

Young rats, prepared as described, having received the basal diet and the cod liver oil and aneurin supplements for 1 week, were each given additional daily supplements of 50 μg. of riboflavin and either 1 ml. of the liver fuller's earth filtrate (= 5 g. fresh liver) or 1 ml. of liver fuller's earth eluate (= 20 g. fresh liver).

The animals receiving the liver fuller's earth filtrate gained in weight at an average rate of 30 g. weekly for 3 weeks (Table II). This growth rate is nearly double that obtained when rats receive the diet supplemented by yeast fuller's earth filtrate (see Table III), indicating that the liver filtrate contains other dietary essentials for the rat in addition to those present in yeast filtrate fraction. The addition of liver fuller's earth eluate fraction to the diet after 3 weeks did, however, cause an increase in the growth rate, showing that the liver fuller's earth filtrate was deficient in a growth factor for rats which was contained in the liver fuller's earth eluate fraction.

The rats which received the liver fuller's earth eluate fraction from the end of the first week after

TABLE II. Effect of fractionation of liver residue I with fuller's earth.

Each rat (male) received daily 10-15μg. aneurin and 50μg riboflavin.

No. of rats.	Daily supplement given during period of 3 wks.	Av. weekly wt. increase of group during 3-week period (g).	Additional daily supplement given during 4th. week	Av. weekly wt. increase of group during 4th. week (g).
3	Fuller's earth filtrate from liver residue I (= 5g. fresh liver)	35, 29, 27	Fuller's earth eluate from liver residue I (= 20g. fresh liver)	37
3	Fuller's earth filtrate from liver residue I (= 5g. fresh liver)	33, 28, 29	None	26
4	Fuller's earth eluate from liver residue I (= 20g. fresh liver)	22, 15, 10	Yeast fuller's earth filtrate not purified by amyl alcohol extraction (= 1g. dry yeast)	25

weaning, gained less in body weight than those receiving filtrate. Here again, however, the growth rate was somewhat in excess of that occurring after administration of yeast eluate (see Table III), suggesting the presence of other dietary essentials in this liver fraction. The addition of yeast filtrate fraction to the diet caused a striking increase in the growth rate, which proved that the dietary essential contained in the yeast filtrate fraction was a limiting factor in this liver fuller's

earth eluate fraction.

The above procedure therefore, which is essentially the same as that which was employed in the separation from yeast of the yeast eluate and yeast filtrate factors, did not yield fractions from liver with the same biological properties as those from yeast.

Preparation from liver residue I of fractions biologically similar to the yeast filtrate and yeast eluate fractions.

Since fuller's earth fractionation of liver extract did not yield fractions corresponding to the yeast fractions, other methods of separation had to be employed. The liver preparations described below with activities similar to those of the yeast filtrate and eluate fractions we have named liver filtrate fraction and liver eluate fraction, respectively.

Liver filtrate fraction. Liver residue I (500 ml.; 1 ml. = 10 g. fresh liver), adjusted to pH 1 with H_2SO_4, was extracted six times with 800 ml. portions of amyl alcohol. The combined extracts were then shaken three times with 1 l. portions of water containing enough NaOH to make the aqueous layer alkaline to thymol blue. The combined aqueous extracts were neutralized with HCl, evaporated in vacuo to about 100 ml. and treated with

.

4 vol. of 96% alcohol. The precipitated salts were filtered, and the filtrate, after removal of alcohol in vacuo was adjusted to 800 ml. (1 ml. = 6 g. of fresh liver).

Liver eluate fraction. To the residue from the amyl alcohol extraction of liver residue I diluted with 3 vol. of water (now 1 ml. = 2.5 g. fresh liver) and readjusted to pH 1.2, 150 g. of fuller's earth were added. After stirring for 30 min., the adsorbate was collected on a Büchner funnel and washed thoroughly with N/10 HCl; it was then twice eluted with 1 l. and 500 ml. portions of 2% $Ba(OH)_2$. To the combined eluate, freed from Ba with H_2SO_4 and adjusted to pH 8 with NaOH, an excess of basic lead acetate (25 g.) was added. The resulting precipitate was filtered, and the lead removed from the filtrate with H_2S. The filtrate was then reduced in volume in vacuo to 200 ml. (1 ml. = 25 g. fresh liver).

Comparison by the rat growth method, of the eluate and filtrate fractions from liver with those from yeast.

The rats used in these growth tests were prepared in the usual manner. After the usual depletion period of 1 week during which they received supplements of cod liver oil, and aneurin, each male rat was given the

additional daily supplements of 50 μg. of riboflavin, and either yeast filtrate fraction purified by amyl alcohol extraction equivalent to 2 g. dry yeast or the above liver filtrate fraction equivalent to 6 g. fresh liver; each female rat received riboflavin and either yeast eluate fraction equivalent to 2 g. dry yeast or the above liver eluate fraction equivalent to 12 g. fresh liver.

The two groups of male rats receiving the filtrate fractions increased in weight at approximately the same rate during the 2-week period during which they received this diet (see Table III). The average weight increases of the animals in the two groups receiving the eluates from yeast and liver for the 2-week period were also the same. Certain of the rats of the two groups which had received the different filtrate fractions were now each given an additional daily supplement of yeast eluate fraction (= 2 g. dry yeast) and the others were given the liver eluate fraction (= 12 g. fresh liver). In all cases a marked increase in the growth rate resulted, and all animals continued to increase in weight at approximately the same rate during the 2-week test period, irrespective of the sources of filtrate and eluate fractions in their diets.

TABLE III. Comparison of eluate and filtrate fractions from liver with those from yeast.

Each rat received daily 10-15μg. aneurin and 50 μg. riboflavin. The supplements indicated in the table were given daily in the following equivalents: liver filtrate fraction = 6 g. fresh liver; liver eluate fraction = 12 g. fresh liver; yeast filtrate fraction, purified by amyl alcohol extraction = 2 g. dry yeast; yeast eluate fraction = 8 g. dry yeast.

No. of rats.	Sex.	Daily supplement given during preliminary period	Av. weekly wt. increase of group for preliminary period of 2 weeks g.	Additional daily supplement given during subsequent period of 2 weeks	Av. weekly increase during subsequent period of 2 weeks g.
2	♂	Liver filtrate fraction	19, 13	Liver eluate fraction	32, 23
2	♂	Liver filtrate fraction	22, 20	Yeast eluate fraction	27, 26
5	♂	Yeast filtrate fraction	17, 14	Liver eluate fraction	25, 24
6	♂	Yeast filtrate fraction	18, 12	Yeast eluate fraction	24, 25
8	♀	Liver eluate fraction	16, 10	Liver filtrate fraction	25, 27
2	♀	Liver eluate fraction	22, 12	Yeast filtrate fraction	22, 21
10	♀	Yeast eluate fraction	20, 10	Liver filtrate fraction	24, 18
6	♀	Yeast eluate fraction	21, 13	Yeast filtrate fraction	22, 21

The rats which had received the eluate supplements during the first 2 weeks were given supplements of the filtrate fractions; certain animals of the two groups were each given doses of yeast filtrate fraction (= 2 g. dry yeast) and the others were given the liver filtrate

fraction (= 6 g. fresh liver). Again, rises in the growth rates of all animals occurred, the weight increases being the same whether the animals received their fractions from yeast or liver.

The above experiments prove beyond reasonable doubt that liver filtrate and liver eluate fractions, prepared as described, contain the same dietary essentials for the rat as the fractions prepared from yeast.

Preparation of liver eluate and filtrate fractions from other liver residues.

Liver eluate fraction from liver residue II. The liver residue II was treated with fuller's earth without previous extraction with amyl alcohol; the adsorbate was eluted with $Ba(OH)_2$ and the eluate purified by treatment with basic lead acetate as described above. When tested on rats this fraction proved to be contaminated with filtrate factor, which, however, was easily removed by extraction of the preparation with amyl alcohol. The product had then the same growth-promoting properties for rats as the yeast eluate fraction.

Liver filtrate fraction from liver residue III. Amyl alcohol extraction of liver residue III by the method

described for the preparation of liver filtrate fraction from liver filtrate I, yielded a preparation with the same growth-promoting properties as the yeast filtrate fraction.

C. The existence of a further rat dietary essential of the vitamin B_2 complex, additional to riboflavin, eluate factor and filtrate factor.

The principal difficulty encountered in the preparation of "vitamin B-free" diets is the obtaining of a vitamin-free protein of high nutritive value. Light white casein or "Glaxo ashless extracted" casein were found satisfactory for certain investigations although it was recognized that these caseins were probably not free from B-vitamins.

Supplee et al[43] demonstrated that the washing of casein with a solution of NaCl removed riboflavin, and it is our experience that this process also removes other factors of the vitamin B complex from the casein.

"Glaxo ashless extracted" casein (2.5 kg.) was stirred for 30 min. with a solution containing 600 g. NaCl and 30 ml. glacial acetic acid in 30.1 tap water. The casein was allowed to settle for 1 hr. and the supernatant liquor was poured off. The washing with the salt solution was repeated 6 times. The casein was then pressed dry on Büchner funnels and stirred into 96% alcohol (5 l.). The alcohol was removed by filtration and the casein spread in thin layers on the open bench and dried in a current of air.

In the following experiments casein, purified by the above method, was used; the cooked starch diet fed to the rats was otherwise the same as previously employed.

Comparison of the growth-promoting action of the B_2-vitamins provided as (a) riboflavin, eluate factor and filtrate factor and (b) crude extracts of yeast or liver.

It had been realised for some time that the growth-rate of rats, which had received, as sources of the vitamin B_2 complex, adequate amounts of riboflavin purified eluate factor and purified filtrate factor for periods of about 4 weeks, increased very markedly when crude extracts of yeast or liver were added to the diet of the animals. This indicated that crude yeast and liver extracts contained an unknown factor not present in these purified fractions.

More striking results were obtained by comparing the growth-rates of young rats receiving the above purified fractions as sources of the vitamin B_2 complex with those of animals receiving either autoclaved extracts of yeast or crude extracts of liver.

The rats at weaning received the basal diet and supplements of 10 µg. aneurin and 0.08 ml. cod liver oil.

Certain animals then received additional daily supplements of 50 µg. riboflavin, purified yeast eluate factor, equivalent to 4 g. dry yeast, and yeast filtrate factor, purified by amyl alcohol extraction, equivalent to 4 g. dry yeast; other animals were given autoclaved yeast extract equivalent to 1-2 g. dry yeast and still others were given crude extracts of liver (liver residue I p.28) equivalent to 6 g. fresh liver.

The animals receiving the crude yeast or liver extracts increased in bodyweight at a much greater rate than those animals given the separated fractions (Table IV). The difference was apparent even at the end of the first week, and it became more definite during the subsequent weeks. The male rats given the crude yeast and liver fractions maintained their original high growth rate for the 4 week test period, while those receiving the purified fractions increased in weight at a diminished rate during the second week; during the subsequent weeks the growth rate of the second week was maintained. The high growth-rate of the female rats receiving the crude liver and yeast fractions was maintained only for 2 weeks; at the end of that period the animals weighed about 120 g. and therefore a slackening of the growth-rate had to be expected, since the adult female rat weighs

TABLE IV.

Growth of rats receiving the essential factors of the vitamin B_2 complex as (a) riboflavin, and purified yeast eluate and filtrate fractions, (b) autoclaved extracts of yeast and (c) crude extracts of liver.

All rats received 15 µg. aneurin.

	No. and sex of rats.	daily supplements of the vitamin B_2 complex	Av. weekly increase in wt. of group for 4 weeks subsequent to dosing g.
A.	4♂	50 µg. riboflavin + purified yeast eluate fraction = 4g dry yeast + purified yeast filtrate fraction = 4g. dry yeast	25, 17.8, 17.8, 18
	3♀	"	26, 17.7, 13.3, 13.
B.	4♂	25 µg. riboflavin + autoclaved yeast extract = 1-2 g. dry yeast	31.8, 28, 28, 31.6.
	2♀	"	39, 31.5, 20, 14.5.
C.	1♂	25 µg. riboflavin + liver residue I = 6 g. fresh liver.	33, 35, 36, 43.
	1♀	"	31, 29, 21, 14.

only 150-200g.

Further proof of the existence of "additional factor" was obtained by observing the increases in the growth-rates, which followed administration of various filtrate factor preparations from liver to rats receiving riboflavin and eluate factor of the vitamin B_2 complex; the purer were the preparations of liver filtrate factor the smaller were the increases in the growth-rates. The following is a typical experiment.

Young rats were given for a period of 2 weeks daily supplements of 50 µg. riboflavin and yeast eluate fraction equivalent to 2 g. dry yeast. Certain animals then received a crude liver extract while others were given the additional supplement of a purified filtrate factor preparation made from the crude material.

Addition of the crude material caused the growth-rate to rise from about 8 g. to about 30 g. weekly while addition of purified filtrate was followed by an increase in the rate of gain in bodyweight from 8 g. to about 20 g. weekly (Table V). There was no question of filtrate factor being a limiting factor in these experiments, since no increased growth rate was observed when the filtrate factor supplements were doubled.

Comparison of the effect on growth of rats, receiving riboflavin and eluate factor, of the addition to the diet of crude and purer preparations of filtrate factor.

Each rat received 15 μg aneurin, 50 μg riboflavin and yeast eluate fraction = 2 g. dry yeast.

No. of rats.	Av. weekly wt. increase for group during the 2 weeks previous to giving filtrate factor preparation. g.	Filtrate factor given.	Av. weekly wt. increase for group during the 2 weeks subsequent to giving filtrate factor. g.
2	18, 7	crude = 6g. fresh liver	32, 26
2	14, 8.5	crude = 12g. fresh liver	29, 28
2	15.5, 9	purified = 12g. fresh liver	19, 16.5
2	16.5, 7.5	purified = 24g. fresh liver	21.5, 14.5

The following method for the biological determination of "additional factor" has been provisionally adopted.

Young rats for 1 week from weaning received the basal diet aneurin and cod liver oil. The animals were then

given for a period of 2 weeks the further supplements of 50 µg. riboflavin, yeast eluate fraction equivalent to 2 g. dry yeast and purified yeast filtrate fraction equivalent to 2 g. dry yeast. Some animals then received the material to be tested for "additional factor" others which served as positive controls were given autoclaved yeast extract and yet others received no further supplement and served as negative controls; an increase in the growth-rate of the animals receiving the test material indicated "additional factor" activity (see Table VI). Purified preparations of eluate and filtrate factors from liver may be used instead of the yeast preparations.

Few experiments have been carried out on this "additional factor". A concentrate of the factor, however, has been obtained by treating liver residue III (p. 28), acidulated to pH 1, first with fuller's earth to remove eluate factor and then with amyl alcohol to remove filtrate factor; the "additional factor" remained in the residue being neither adsorbed by fuller's earth nor extracted by amyl alcohol. The increased growth-rate which followed administration of this residue to rats receiving liver eluate factor and filtrate factor was definite and was comparable with that observed following administration of autoclaved extract of

TABLE VI.

Tests for additional factor

Each rat received 15 µg aneurin, 50 µg. riboflavin, liver eluate factor = 12 g. fresh liver and purified liver filtrate factor equivalent to 24 g. fresh liver.

No. of male rats.	Average weekly wt. increase of group for preliminary period of 2 weeks. g.	Additional supplement given	Av. weekly wt increase of group during 2 week test period g.
3	22, 20.3	Fuller's earth and amyl alcohol residue= 32g. fresh liver	32.7, 31.7
3	22, 21	None	23.7, 17.7
2	22.5, 24.5	Autoclaved extract of yeast = 2g. dry yeast	36, 29

yeast (Table VI)

From these experiments and others not described we now know that the earlier described fuller's earth filtrates from yeast extracts contained at least 2 factors essential for the rat; one, now named yeast filtrate factor, is extracted by amyl alcohol and the other, now named "additional factor" which is not extracted. Crude eluate fractions are probably also contaminated with a little "additional factor".

D. Nicotinic acid, the derivatives of nicotinic acid and adenylic acid in the nutrition of the rat.

(a) Earlier work on nicotinic acid and other pyridine derivatives.

Warburg and Christian[44] found that nicotinamide was contained in the molecule of codehydrogenase II, prepared from red blood cells. Codehydrogenase II is the coenzyme required in the dehydrogenations in which the lumboflavin-bearing yellow oxidation enzyme also plays a part. Nicotinamide has also been found present in the cozymase molecule[45]. Knight[46] has more recently proved that nicotinic acid is one of the essential growth factors contained in the high-vacuum distillate from yeast extracts, required for the growth of Staphylococcus aureus. Since the two identified members of the vitamin B complex required by rats, aneurin and riboflavin, have been proved to be essential growth factors for certain micro-organisms and since the close relationship between certain vitamins and enzymes is well established, it seemed possible that either yeast eluate factor or yeast filtrate factor might be identical with nicotinamide or some related compound.

We have investigated nicotinic acid, nicotinamide and codehydrogenase II, the last kindly supplied by

Prof. Warburg, to find if these compounds could replace either the yeast filtrate factor or the yeast eluate factor in rat-growth experiments.

Since these experiments were completed Elvehjem et al[47] made the important discovery that canine black-tongue is cured by nicotinic acid and that curative concentrates from liver have yielded biologically active crystals identified as nicotinamide.

METHODS AND RESULTS

Growth of young rats was again the criterion employed. The basal diet used was the usual one having light white casein as source of protein. Vitamin B_1 was supplied as aneurin, except in a few of the earlier experiments, in which Peters's concentrate was used. The rats also received riboflavin, either the synthetic product or the natural, prepared from liver extract.

The nicotinic acid used in the following experiments was prepared by oxidation of nicotine[48], M.P. 232°. Nicotinamide was obtained by the method of Pollak[49] and was crystallized from benzene containing a trace of absolute ethyl alcohol, M.P. 127°. The sample of codehydrogenase II used in these experiments had 60% of the enzymic activity of pure codehydrogenase II. (Private communication from Prof. Warburg).

The nicotinic acid and other pyridine derivatives were investigated for vitamin activity by the following three types of experiment. The compounds were fed to rats receiving the basal, vitamin B-deficient diet and

(a) only aneurin and riboflavin of the vitamin B complex
(b) aneurin, riboflavin and our yeast filtrate factor,
(c) aneurin, riboflavin and our yeast eluate factor.

(a) Effects of nicotinic acid, nicotinamide and codehydrogenase II on the growth of rats receiving only aneurin and riboflavin of the vitamin B complex.

The animals at weaning received the basal diet supplemented by aneurin. When growth had ceased, after approximately 10 days, the animals were given 12 µg. daily of riboflavin supplemented by varying doses of the pyridine derivatives; control animals received 12 µg daily of riboflavin only. The weights of the animals were recorded over a 4-week period. Codehydrogenase II was further tested by feeding it to two animals which had received the basal diet supplemented by aneurin and riboflavin for 3 weeks.

Comparison of the growth rates (Table VII) reveals that no significant increase in the growth rate was effected by addition of any of the above pyridine

TABLE VII. Effects of nicotinic acid, nicotinamide and codehydrogenase II on the growth of rats receiving aneurin and riboflavin (12 μg. daily) of vitamin B complex.

No. of rats.	Aneurin given as (daily)	Pyridine derivatives given (daily dose)	Av. weekly gain in wt. over a 4 week period g.	No. of rats in control group receiving no pyridine derivatives	Av. weekly gain in wt. over a 4-week period g.
2	Peters's conc.=0.3-0.6g. dry yeast	1mg. nic. acid	8.1	2	7.1
9	" "	5mg. nic. acid	6.7	10	5.5
5	Crystalline vitamin (5 μg)	5mg. nic. acid	4.0	3	3.2
2	Peters's conc.=0.3-0.6g. dry yeast	1mg. nic. amide	7.4	2	7.1
4	" "	5mg. nic. amide	5.8	6	5.3
2	Crystalline vitamin (5 μg)	40 μg codehy.	2.25	4	4.75
2	" "	100 μg codehy.	2.5	4	4.75
2	" "	500 μg "	1.0*	-	-

* These 2 rats, prior to dosing with the codehydrogenase II, had received aneurin and riboflavin for 3 weeks, the average weekly gain being 2.7 g. The figure 1.0 g. weekly entered in the table was the average weekly weight increase during 2 subsequent weeks while receiving the codehydrogenase in addition to aneurin and riboflavin.

derivatives. In the case of nicotinic acid a slightly increased growth rate was noted in rats receiving 1 or 5 mg. daily, but the difference between these animals and the controls was so slight that the increase can hardly be regarded as significant. Feeding of 1 and

5 mg. daily of nicotinamide also caused no significant increase in the growth rate, while the rats which received 40µg. or 100µg. daily of the codehydrogenase II preparation actually grew at a slower rate than the control animals. In the case of two rats, which had received riboflavin and aneurin only of the B-vitamins for 3 weeks previously, the addition of 500µg. daily of the codehydrogenase II preparation was followed by a decrease in the growth rate. All the rates of weight increase shown in Table VII are markedly subnormal.

The addition of the yeast filtrate factor to the diet of rats receiving only aneurin and riboflavin of the vitamin B complex causes a marked increase in growth rate (p. 22) while the addition of the yeast eluate factor under similar circumstances causes a smaller, but still significant increase (p. 23). It is, therefore, clear that neither the yeast filtrate factor nor the eluate factor can be replaced by any of the above pyridine derivatives.

(b) <u>Effects of nicotinic acid, nicotinamide and codehydrogenase II on the growth of rats receiving aneurin, riboflavin and the yeast filtrate factor of the vitamin B complex.</u>

Two types of experiment were employed.

In the first series, A, the rats at weaning received the basal diet supplemented by aneurin until all weight increase had ceased; they then received in addition daily 50 µg. of riboflavin, yeast filtrate factor corresponding to 1 g. dry yeast and 1 mg. of nicotinic acid or nicotinamide while the control animals received no added pyridine derivative. The growth rates of the animals in the three groups over a 4-week period were nearly identical (Table VIII A, Fig. 3). The addition of the yeast eluate factor to the diet of the rats receiving nicotinic acid, however, resulted in an immediate increase in the growth rate.

TABLE VIII

Effects of nicotinic acid, nicotinamide and codehydrogenase II on the growth of rats receiving daily aneurin (10-15μg), riboflavin (50μg) and yeast filtrate fraction (= 1g. dry yeast) as sources of the B-vitamins

Series	No. of rats	Pyridine derivatives (daily dose)	Av. weekly wt. increase of the group during 3 wks. subsequent to dosing g.	Additional supplement (S) given later, daily	Av. wt. increase of group during the week after giving S g.
A	3	1mg. nic. acid	27, 21, 20.6	Yeast eluate factor (= 1g. dry yeast)	36.5 (2 rats)
	3	1mg. nic. amide	24.7, 21, 19.3	-	-
	3	-	25, 20, 18.7	Yeast eluate factor (= 1g. dry yeast)	30 (2 rats)

	No. of rats	Av. weekly wt. increase in group during 2 preliminary weeks (g.)	Additional supplement (S') (daily dose)	Av. weekly wt. increase of group after receiving S'
B	3	18.7, 15.3	2mg. nic. acid	16, 15.3
	3	20, 18	2mg. nic. amide	15, 11.7
	2	17.5, 15	500μg. codehyd.	20, 14.5
	3	19.3, 16	None	15.3, 13.7
	3	19, 19.7	Yeast eluate factor (= 1g. dry yeast)	28, 23.7

In the second series of experiments, B, the rats received the basal diet supplemented by aneurin, riboflavin and yeast filtrate factor for 2 weeks; they then received daily in addition 2 mg. of nicotinic acid or 2 mg. of nicotinamide or 0.5 mg. of the codehydrogenase II preparation for a further 2-week period. As controls, 3 animals were maintained on the basal diet supplemented by the above three vitamins of the B complex for the whole of the 4-week period, while others received, in addition to the three vitamins, the yeast eluate factor

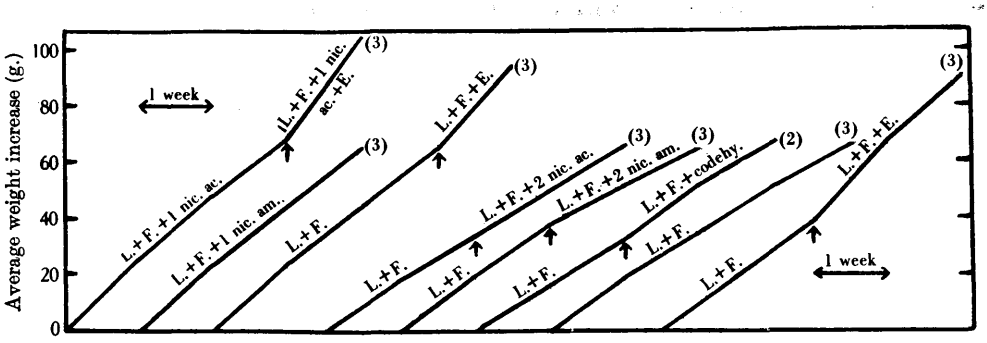

Fig. 3. Effects of nicotinic acid (1mg. daily = 1 nic.ac. 2 mg. daily = 2nic.ac.), nicotinamide (1mg. daily = 1 nic. am.; 2mg. daily = 2nic.am.) or codehydrogenase II (0.5mg. daily=codehy.)compared with that of yeast eluate factor (corresponding to 1g. dry yeast daily=E.)on the growth rate of young rats receiving aneurin (10-15γ daily), riboflavin(50γ daily=L)and yeast filtrate factor(corresponding to 1g.dry yeast daily=F). The arrows indicate the points at which the doses were changed. The figures in brackets indicate the number of rats from which the growth curves were derived.

at the period when the experimental animals received the pyridine bases. The addition of nicotinic acid, nicotinamide or codehydrogenase II to the diets of the above animals did not cause any significant increase in the growth rate, while the addition of yeast eluate factor caused the usual increase previously recorded by us (Table VIII,B, Fig. 3).

The yeast eluate factor therefore cannot be replaced by nicotinic acid, nicotinamide or codehydrogenase II.

(c) Effect of nicotinic acid and nicotinamide on the growth of rats receiving aneurin, riboflavin and yeast eluate factor of the vitamin B complex.

Rats received the basal diet supplemented by aneurin from time of weaning until they ceased to grow, when the diet was supplemented by the daily addition of 50µg. riboflavin and of yeast eluate factor corresponding to 1 g. dry yeast. After 2 weeks certain animals were given a supplement of 2 mg. nicotinic acid and others a supplement of 2mg. of nicotinamide while the remainder served as controls and received no further addition to the diet. All the animals were observed for a further period of 2 weeks, and at the end of this period, in addition to all previous supplements they received yeast filtrate factor corresponding to 1 g. dry yeast. No

significant increase in the growth rate resulted on the addition of the pyridine derivatives, while the subsequent addition of the yeast filtrate factor caused the usual growth response (see Table IX, Fig. 4; cf. p. 22.).

Therefore neither nicotinic acid nor nicotinamide can replace the yeast filtrate factor.

Although it is shown above that neither nicotinic acid, nicotinamide nor codehydrogenase II can replace either our yeast eluate factor or our yeast filtrate factor it cannot be assumed that these pyridine derivatives may not, nevertheless, be dietary factors for rats, for it is possible that either or both the yeast eluate factor and the yeast filtrate factor are complex in nature and may contain one of these pyridine derivatives as an essential constituent. Since yeast contains cozymase and other pyridine derivatives it seems likely that one or both of the yeast fractions containing these dietary factors will contain nicotinamide or other pyridine derivatives, and, indeed, since this work was completed we have isolated nicotinamide from the yeast eluate fraction. (Found: C,59.4; H,5.0; N,22.1%. $C_6H_6ON_2$ requires: C,59.0; H,4.9; N,22.9%) M.P. 127°; mixed with authentic specimen of nicotinamide, 127°. M.P. of chloroaurate, 234-236°

(decomp.); M.P. of chloroaurate of authentic specimen of nicotinamide, 235-237° (decomp.).

TABLE IX. Effects of nicotinic acid and nicotinamide on the growth of rats receiving daily, aneurin (10-15 µg.), riboflavin (50 µg.) and yeast eluate fraction (= 1g. dry yeast) as sources of the vitamin B complex.

No. of rats	Av.wkly.wt. increase of group for 2 preliminary weeks (g.)	Pyridine derivatives (daily dose)	Av.wkly.wt. increase of group for 2 weeks after giving the pyridine derivatives (g.)	Av.wt.increase of the group for the week after giving yeast filtrate factor (=1g. dry yeast) (g.)
3	14, 8·7	2 mg.nic.acid	4·7, 5·3	16
3	14, 6·7	2 mg.nic.amide	4·3, 3·3	17·3
3	16·5, 8	none	5, 6	15

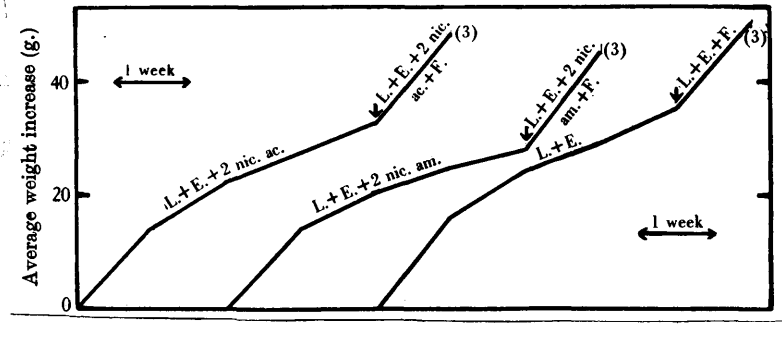

Fig. 4. Effect of nicotinic acid (2mg. daily=2nic.ac.) or nicotinamide (2 mg. daily = 2 nic.am.) compared with that of yeast filtrate factor (corresponding to 1 g. dry yeast daily = F.) on the growth rate of young rats receiving aneurin (10-15 µg daily), riboflavin (50 µg daily = L.) and yeast eluate factor (corresponding to 1 g. dry yeast daily = E.) Other details as in Fig. 3.

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(b) Later work on nicotinamide and adenylic acid in the nutrition of the rat.

Since the above experiments were completed the importance of nicotinic acid and its derivatives in nutrition has been realised. The work of Elvehjem et al[47] showed that nicotinic acid cured blacktongue in dogs, and nicotinic acid has also been proved to be an essential nutrient for the pig,[50] the monkey[51] and man[52,53,54]; pellagra is at least partially due to nicotinic acid deficiency.

Many papers on the question of whether nicotinic acid either alone or in combination with adenylic acid has vitamin potency for the rat have appeared. Frost and Elvehjem[55] reported that nicotinamide and adenylic acid from yeast or heart had growth promoting properties when administered, either separately or simultaneously, to rats receiving a basal diet which contained 12 per cent. of white corn. Later Oleson et al[56], however, found no growth-stimulating action of nicotinic acid when given to rats on an entirely "synthetic" diet containing riboflavin. Euler and Malmberg[57] stated that adenylic acid had no effect on the growth rate of rats receiving a diet containing riboflavin; but that nicotinamide prolonged the life of rats receiving

riboflavin and a yeast fuller's earth filtrate. More recently cozymase was found by Euler et al[58] to have a growth-stimulating effect when administered to rats receiving riboflavin and a fuller's earth filtrate of an autoclaved acidulated yeast extract. György[59] has attributed an anaemia of rats, termed panmyelophthisis, to deficiency of nicotinic acid in the diet.

In agreement with our experiments described above, Cook et al[60] also failed to find that nicotinic acid alone and in combination with adenylic acid had vitamin activity for rats. Chick et al[61] noted no increased growth when rats, fed on a maize diet, received nicotinamide, and Chick[62] was also unable to demonstrate that nicotinamide had a growth stimulating effect, when fed to rats receiving a purified synthetic basal diet containing sucrose as source of carbohydrate. Helmer and Fouts,[63] on the other hand, reported that nicotinic acid did increase the growth rate of rats receiving a maize diet containing riboflavin and liver filtrate fraction. Dann and Subbarow[64] stated that nicotinic acid given to rats receiving a diet containing riboflavin had no growth promoting action and did not prevent rat dermatitis, i.e. could not replace vitamin B_6.

We, therefore, have extended our former experiments

by investigating a combination of yeast adenylic acid and nicotinamide for vitamin activity.

METHODS AND RESULTS

The rat growth technique was again employed. Light white casein or Glaxo ashless extracted casein were used in the basal diet. All animals received 10 µg. aneurin and 0.08 ml. cod liver oil daily from the time of weaning until a bodyweight of 100 g. was attained; the daily supplements were then increased to 15 µg. and 0.1 ml. respectively.

The yeast adenylic acid was obtained from Messrs. British Drug Houses and the nicotinamide from Messrs. Hoffmann La Roche. These compounds were tested for vitamin activity by administration to rats receiving supplements of (a) riboflavin, (b) riboflavin and yeast eluate fraction and (c) riboflavin and yeast filtrate fraction.

(a) Addition of yeast adenylic acid and nicotinamide to the diet containing riboflavin.

Six litter male rats received for 1 week from weaning the basal diet supplemented by aneurin and cod liver oil. Three animals which served as negative controls then each received a daily supplement of 50 µg.

TABLE X

Effect of combination of yeast adenylic acid and nicotinamide on the growth-rate of rats receiving daily 10-15 μg aneurin, 50 μg riboflavin, and various other factors of the vitamin B_2 complex.

	No. of rats.	Daily supplements	Av. weekly wt. increase of group during 6 weeks subsequent to dosing g.
A	3	2mg. adenylic acid + 2mg. nicotinamide*	10.3, 4.7, 3.7, 2.3, 2.3, 1.7
	3	none	15, 6.7, 5, 4, 1, 1.3

	No. of rats.	Daily supplements given during preliminary period of 2 or 3 weeks	Av. wkly wt. increase during preliminary pe period g.	Av.	Additional daily supplement added at end of preliminary period (S_1)	Av. wkly. increase during 3 wks. after giving S_1	Av. g.	Further supplement added (S_2)	Av. wt. increase during wk. after giving S_2 g.
B	3	yeast eluate factor = 2g. dry yeast	14.0 9.3	-	2mg. adenylic acid + 2mg. nicotinamide	3, 6.3, 4	4.4	yeast filtrate factor = 1g. dry yeast	16.7
	1	"	16.0 7.0	-	yeast filtrate fraction = 1g. dry yeast	13, 18, 16	15.7	-	-
C Exp. 1.	3	yeast filtrate factor = 1g. dry	20.3, 21 13.7	18.3	2mg. adenylic acid + 2mg. nicotinamide	15, 17, 13.7	15.2	yeast eluate factor = 2g. dry yeast	24.3
Exp. 2.	4	"	19.2, 15.2	17.2	"	18.2, 15.2 11.8	15.7	-	-
	2	"	18.5, 16.0	17.2	none	16, 16.5, 17	16.5	-	-
	2	"	21.5, 15.0	18.2	yeast eluate factor = 2g. dry yeast	31.5, 28.5	30	-	-

* After 1 week supplements of adenylic acid and nicotinamide reduced to 1 mg. of each daily.

riboflavin, 2 mg. of yeast adenylic acid and 2mg. of nitotinamide, the doses of the last 2 of these compounds after 1 week were changed to 1 mg. of each daily. The body weights of the animals were observed for 6 weeks (Table X A). The average total weight increase during the 6 weeks subsequent to the animals having adenylic acid and nicotinamide was 25 g. while the corresponding average for the control rats was 33 g. This difference, however, cannot be regarded as significant and there was no apparent differences in the general condition of the rats of the 2 groups; all the animals were much undersized but were active. Two rats of the control group receiving [riboflavin only] developed the characteristic florid rat dermatitis[36] after 6 and 15 weeks respectively from the beginning of the experiment, while 2 of the animals receiving riboflavin, adenylic acid and nicotinamide developed the dermatitis after 8 and 15 weeks respectively. Addition of adenylic acid and nicotinamide therefore did not influence either the growth of the rats or the development of dermatitis.

(b) Addition of yeast adenylic acid and nicotinamide to the diet containing riboflavin and yeast eluate factor.

Four litter-mate female rats received at weaning the basal diet supplemented by aneurin and cod liver oil

and for a preliminary period of 2 weeks additional supplements of 50 μg. riboflavin and yeast eluate fraction equivalent to 2 g. dry yeast were given. Three of the rats then each received for a further period of 3 weeks 2 mg. yeast adenylic acid and 2 mg. nicotinamide daily, while one animal received yeast filtrate fraction not purified by amyl alcohol extraction, equivalent to 1 g. dry yeast. This last animal showed the usual growth response but no increased growth rate followed the administration to the test animals of adenylic acid and nicotinamide (Table X B). At the end of the 3 week period addition of yeast filtrate fraction to the diet of these animals caused a striking increase in growth rate, indicating that these amounts of adenylic acid and nicotinamide, although unable to stimulate growth, did not inhibit the growth of the animals when the missing nutrient was supplied. The increase in growth rate caused by addition of filtrate factor, however, was not as great as that which follows administration of unfractionated yeast extracts to such animals; therefore, these compounds cannot replace that factor of the vitamin B_2 complex required in addition to riboflavin, eluate factor and filtrate factor.

(c) Addition of yeast adenylic acid and nicotinamide to the diet containing riboflavin and yeast filtrate fraction.

Three rats, which had received the basal diet with aneurin and cod liver oil for 1 week from weaning, were each given daily supplements of 50 μg. riboflavin and yeast filtrate fraction not purified by amyl alcohol extraction, equivalent to 1 g. dry yeast, for a period of 3 weeks; 2 mg. of yeast adenylic acid and 2 mg. of nicotinamide were then given daily for a further period of 3 weeks, but there was no increase in the growth rate, which, however, did occur when a daily ration of yeast eluate fraction equivalent to 2 g. of dry yeast was added (Table X C. Exp. 1).

In a second experiment 8 litter-mate rats, treated as usual for the first week after weaning, each received daily 50 μ g. riboflavin and the yeast filtrate fraction equivalent to 1 g. dry yeast for a period of 2 weeks. Four of the animals then each received the additional daily supplements of 2 mg. adenylic acid and 2 mg. nicotinamide for 3 weeks, 2 negative control animals received no additional supplement during that period and 2 positive control animals received yeast eluate fraction equivalent to 2 g. dry yeast daily. The animals receiving the adenylic acid and nicotinamide increased

in bodyweight at approximately the same rate as the negative controls while the growth rates of the 2 positive control rats increased from an average of 18.2 g. to 30 g. weekly, on addition of the eluate factor (Table X C. Exp. 2).

The results of the above experiments afford no evidence that yeast adenylic acid and nicotinamide administered simultaneously have any growth promoting activity for rats deprived of the yeast eluate factor or yeast filtrate factor of the Vitamin B_2 complex; neither do these compounds prevent rat dermatitis. In later experiments (p. 90) we found no evidence that alloxazine-adenine-dinucleotide has any nutritional activity other than that due to the riboflavin it contains. Since this coensyme contains also adenylic acid in its molecule, these experiments also suggest that adenylic acid is not an essential nutrient for the rat; additional support to this view was provided by the earlier experiments (p. 48) which showed that codehydrogenase II (adenine-nicotinamide-dinucleotide) had no demonstrable nutritive value for rats. It is of course possible that if nicotinamide and yeast adenylic acid were required by the rat in small amounts these might be contained in our purified yeast fractions or in our basal diet in such

amounts that the rats were independent of a further supply, but it is at least certain that these compounds do not replace our yeast filtrate or yeast eluate factors in the diet of the rat. Recent experiments, not reported, and those described above also indicate that nicotinamide and adenylic acid cannot replace "additional factor" which, with riboflavin, eluate factor and filtrate factor, satisfy the Vitamin B_2 requirements of the rat. The question whether nicotinamide and adenylic acid are essential nutrients for the rat will be decided only when all other factors are available in a pure state.

We have made no attempt to repeat the experiments of Frost and Elvehjem[55], who found that a combination of yeast or heart adenylic acid and nicotinamide had very marked growth promoting properties for rats receiving a diet which differed from that used by us principally in that it contained 12 per cent. of white corn. The possibility cannot be excluded that white corn may have some virtues for the rat which render that animal capable of benefiting from the administration of adenylic acid and nicotinamide; it appears to us, however, that the introduction of such complicated materials as cereals in basal diets used in the study of the different members of the vitamin B complex renders interpretation

of the results difficult.

DISCUSSION

During the past few years investigation of the vitamin B_2 complex has been pursued in many laboratories all over the world. Yeast, liver, rice polishings and other materials have been used by the different workers as sources of the B-vitamins, different methods of fractionation have been employed and the resulting fractions have been tested for vitamin activity with different animals; new terms have been introduced, often without justification. For those and other reasons the literature of the vitamin B_2 complex has become probably more confused than that of any other biochemical subject. It is quite impossible to mention all the work which has been published and, therefore, only the papers which most concern the present studies will be considered.

The work on the separation of yeast filtrate fraction and yeast eluate fraction from autoclaved extracts of yeast was almost completed when communications bearing on this subject appeared from other laboratories. Euler and Malmberg[57] suggested that two supplements for riboflavin besides aneurin are present in yeast extracts,

though adequate experimental evidence was not provided for this hypothesis. Lepkovsky et al[65] showed that the vitamin B_2 requirements of rats were satisfied only when (1) riboflavin, (2) an eluate prepared from the fuller's earth (adsorbates of rice bran extracts and (3) a fuller's earth) filtrate from aqueous liver extracts were supplied. These results were supported by Halliday and Evans[66] whose work further indicated the presence of the above supplementary factors, (2) and (3), in alcoholic extracts of wheat.

The work of Lepkovsky et al requires special consideration. These investigators employed methods of fractionation which were essentially similar to those we adopted, but different materials were used as sources of the dietary essentials.

It was, of course, immediately recognised that the essential factor, named factor 1, present in eluates from rice bran fuller's earth adsorbates was probably identical with the essential factor present in our yeast fuller's earth ~~filtrate~~ eluate fraction and also with György's vitamin B_6. Proof that such was the case has since been forthcoming; factor 1 was isolated in a crystalline state by Lepkovsky[67], vitamin B_6 was isolated by Keresztesy and Stevens[68], György[69] and Kuhn and Wendt[70] and we have obtained crystals from our yeast eluate fraction

identical with those of the hydrochloride of a specimen of factor 1. Further, crystalline factor 1 completely replaced yeast eluate fraction in the diet of the rat.

The exact relationship of factor 2 of Lepkovsky et al[65], that factor present in liver extracts after exhaustive extraction with fuller's earth, to our crude yeast fuller's earth filtrate fraction has not been finally established. We have found that liver fuller's earth filtrates have growth promoting properties for the rat not possessed by fuller's earth filtrate from autoclaved extracts of yeast (p. 30). The methods of preparation of liver filtrates used by Lepkovsky et al, however, were somewhat different from those we employed and, therefore, we cannot be certain that our liver filtrates contained the same dietary essentials as the liver filtrates of the Californian workers. We have more recently obtained from yeast and liver fuller's earth filtrates, by extraction of these with amyl alcohol, fractions which for the rat were nutritionally similar; the essential nutrients for the rat in these fractions we named yeast and liver filtrate factors respectively. Factor 2 certainly must have contained liver filtrate factor, but from our subsequent work it appears that factor 2 must also have contained what we now refer to as "additional factor". Crude

yeast fuller's earth filtrates have much less "additional factor" than the corresponding preparations from liver and therefore our yeast fuller's earth filtrate fraction probably differed from factor 2 principally in that the latter may have contained more "additional factor".

The relationship of our yeast or liver filtrate factor to the factor required for growth and prevention of dermatitis of chicks, maintained on a heated grain diet, first found in liver fuller's earth filtrate by Elvehjem and Kuhn[40, 71] and later investigated and named "filtrate factor" by Lepkovsky and Jukes[41, 72] is uncertain. The factors have many common properties but since certain differences between the factors have been discovered, it is probable that they will eventually prove distinct. Our rat factor is adsorbed by norite charcoal while the chicken factor is not adsorbed; the barium salt of the rat factor is insoluble in 96% alcohol, while that of the chick factor is soluble. A recent publication by Woolley et al[73] also indicates that the factors will eventually prove distinct.

Elvehjem et al[74] have claimed that the rat requires, in addition to other recognized factors of the vitamin B_2 complex, a further factor which they named "factor W". This factor was present in alcohol-ether precipitates of

liver extracts, and also in fuller's earth filtrates from liver[55]. The diet employed in these investigations contained 12 per cent. of white corn which was the only source of what we name eluate factor and also filtrate factor; it is unlikely that this amount of maize would supply the rat's optimal need of eluate factor and certainly filtrate factor must have been almost entirely absent from the diet since we found (unpublished experiments) that maize is almost devoid of the rat dietary essential we name filtrate factor. Frost and Elvehjem[55] found that "factor W" as contained in liver fuller's earth filtrates, contained two factors which were separated by treatment with mercury salts; they indicated that these rat dietary essentials, separated by mercury precipitation, might be adenylic acid and nicotinamide respectively. Because of the inclusion of white corn in the diet used by these workers, their results are extremely difficult to interpret. It seems, however, that the concentrates employed as source of "factor W" must have contained at least two of the essential nutrients we recognized namely, filtrate factor and "additional factor".

Even more difficult to interpret are the experiments described in a very recent publication from the same laboratory[56]. Liver fuller's earth filtrate together

with liver fuller's earth eluate when administered to rats receiving a basal diet similar to the one we employed, had not the growth promoting properties possessed by the unfractionated liver extract. These workers suggest that the factor missing in the separated fractions is "factor W", in spite of the fact that in previous experiments,[55] liver fuller's earth filtrate, made in a very similar manner, was the source of "factor W". We find that liver fuller's earth filtrate and liver fuller's earth eluate supply all the nutrients of the vitamin B_2 complex required by the rat (p. 30).

The relationship of the rat dietary essentials of the vitamin B_2 complex to the pigeon factors, vitamin B_3 and vitamin B_5, first described by Williams and Waterman[75] and Carter et al[76] respectively, is an interesting problem which will probably be solved in the near future. It appears that vitamin B_5 has properties similar to eluate factor and it seems likely that these factors will prove to be identical.

The interesting question of whether nicotinic acid or other related pyridine derivatives is an essential nutrient of the rat is still unanswered. We do know that nicotinamide does not replace filtrate factor, eluate factor or "additional factor" in the diet

of the rat, but only when all essential nutrients have been obtained in a pure state and basal diets free from nicotinic acid are available, will the essential or non-essential nature of nicotinic acid be finally decided. However, the rat thrives and even reproduces when given diets on which pigs, dogs and man sicken and die through deficiency of nicotinic acid and therefore, there exists at least a quantitative difference between the nicotinic acid requirements of the rat and those of the pig, the dog and man. Any one of the following three possibilities might explain why the rat is different from those other animals; (1) the rat may be specially economical with nicotinic acid and thereby be able to thrive on diets containing only small amounts of that compound; (2) the rat may obtain a supply of nicotinic acid from that which may be synthesised by microorganisms in the animal's alimentary tract; (3) nicotinic acid may be synthesised in the tissues of the rat.

EXPERIMENTS ON RIBOFLAVIN

A. The estimation of riboflavin by rat growth methods.

Riboflavin is perhaps the most important factor of the vitamin B_2 complex. Young rats given a diet devoid of that factor but otherwise complete do not thrive. After about 2 weeks the animals refuse food and, unless riboflavin is administered, death follows in a few days. Riboflavin deficiency in dogs leads to a condition which has been named "yellow liver".[77] The recent experiments of Sebrell and Butler[78] have proved that riboflavin is a dietary essential for man. It is, therefore, a matter of very considerable importance, that a reliable method for the estimation of riboflavin in foodstuffs should be available. Physical and physico-chemical methods for the determination of riboflavin have been extensively employed[30]. Some investigators have simply estimated the riboflavin in extracts of foods either fluorimetrically or colorimetrically, but more often the riboflavin has been first converted to lumiflavin which was estimated fluorimetrically, after extraction with chloroform. There are many objections to the use of physical and chemical methods of determination of essential nutrients in foodstuffs. The test employed may not be specific for the factor and inert substances may influence the results;

imperfect extraction of the essential nutrient from the foodstuff or partial destruction of the factor during that process is liable to occur; the vitamin may exist in different chemical combinations in the foodstuffs and certain of these might not be estimated by the method of determination employed. The physical and physico-chemical methods of determination of riboflavin are certainly unsatisfactory for very different values for the same materials have been reported by different workers. It is probable that most of the values found by the non-biological methods are too low.

It has been claimed that the rat growth method of determination of "vitamin B_2" described by Bourquin and Sherman,[79] estimates riboflavin and the values of "vitamin B_2" activity found by that method are readily converted to riboflavin values.[80] All the recognised factors of the vitamin B_2 complex, influence the growth-rate of rats and, therefore, reliable biological determination of riboflavin can only be achieved when a basal diet is employed which contains an adequate supply of all the essential nutrients of the vitamin B_2 complex, excluding riboflavin. In the Bourquin-Sherman diet an alcoholic extract of wheat is the sole source of those supplementary factors and that, according to our experience, must be

almost devoid of filtrate factor.

Several papers had appeared on the growth promoting action of riboflavin for rats before these experiments were completed. Kuhn et al obtained weight increases in young rats of about 10 g. weekly over a 4-week period by supplementing the Bourquin-Sherman diet with 10 µg daily of riboflavin and with an aqueous yeast extract which had been treated with fuller's earth. Since the Bourquin-Sherman diet contains an alcoholic extract of wheat as a source of aneurin, this must be regarded as an additional source of supplementary material. Euler et al,[82,83] reported a similar weight increase when employing a synthetic diet supplemented only by the fuller's earth filtrate from yeast extracts. An average weight increase of approximately 11 g. weekly for 4 weeks was obtained by György[33] when 10µg daily of riboflavin were fed with "vitamin B_6" as contained in large doses of Peters's vitamin B_1 concentrate from yeast, but the growth rate was not enhanced by doubling the riboflavin dose. Ansbacher et al[84] however, found that the growth rate of rats was increased with increasing doses of riboflavin, when supplemented by an extract from rice polishings.

The earlier experiments on the growth promoting action of riboflavin described below were completed

before it was realised that so many essential nutrients of the vitamin B_2 complex existed. Various materials as sources of supplementary factors of the vitamin B_2 complex were used, but from the growth-rates obtained, it is now apparent that not one of these sources supplied all the supplementary factors. These experiments, therefore, did not provide a satisfactory method for the estimation of riboflavin foodstuffs, but they very clearly indicated how regularly the growth-rates of rats vary with the amounts of riboflavin the animals receive.

With the discovery of the bipartite and later the tripartite nature of the supplement, which with riboflavin, constitute the vitamin B_2 complex, other materials were introduced into the diet with a view to producing a diet suitable for the estimation of riboflavin. We first added crude yeast fuller's earth filtrate and yeast eluate fractions to the diet; these materials supplied all the essential nutrients of the vitamin B_2 complex required by rat excluding riboflavin. As in the earlier experiments graded growth-responses to graded doses of riboflavin were obtained, but the growth-rates observed were much higher than those in the earlier experiments.

The preparation of the above materials is a tedious and costly procedure and we have, therefore, searched

for other more easily prepared sources of the supplementary materials. Treatment of extracts of liver or yeast with norite charcoal at pH 5 yielded materials which were devoid of riboflavin but contained adequate amounts of the other factors of the vitamin B_2 complex. Rats receiving our basal diet and norite charcoal filtrates from aqueous extracts of liver or yeast did not increase in bodyweight; when, however, riboflavin was added to the diet the growth-rate was similar to that observed when the animals received untreated extracts of yeast or liver as source of the B_2 vitamins.

Growth promoting properties of riboflavin for rats receiving as sources of the supplementary factors of the vitamin B_2 complex one of the following materials: (1) fuller's earth filtrate from yeast extract; (2) that material purified by adsorption on norite charcoal; (3) alcoholic extract of wheat germ.

The basal diet used in these rat growth experiments contained light white casein as source of protein. In certain experiments vitamin B_1 was supplied as aneurin and in other experiments as a Peters' concentrate. The young rats at weaning received only vitamin B_1 of the

vitamin B complex and when growth had stopped the animals were given riboflavin and the supplementary materials. The growth-rates of the rats were observed for 3-4 weeks.

Preparation of supplementary materials.

Fuller's earth filtrate from yeast extract. This was prepared as previously described (p. 16).

Eluate from the norite adsorbate of the fuller's earth filtrate. 1 l. of the above fuller's earth filtrate was extracted 4 times with 12 g. portions of norite charcoal at pH 2.5. The norite adsorbate was washed with N/1000 HCl, dried in a vacuum desiccator and extracted with glacial acetic acid on a water-bath until the extract was nearly colourless (3 extractions). The acetic acid was removed *in vacuo* and the light brown residue dissolved in 167 ml. of water (1 ml. = 3g. yeast, dry wt.).

Alcoholic extract of wheat germ. Wheat germ was air-dried to constant weight at 37°, defatted with ether and exhaustively extracted in a Soxhlet extractor with hot absolute alcohol. The alcohol was removed *in vacuo* and the remaining gummy material dissolved in water (1 ml. = 1 g. wheat germ, dry wt.).

RESULTS

Growth-promoting action of riboflavin without supplement.

The administration of 12µg daily of riboflavin

to rats fed on the basal diet unsupplemented by other members of the vitamin B_2 group caused a small but definite increase in weight during the first 2 weeks, after which the growth of the animals practically stopped. The animals receiving vitamin B_1 as the Peters's concentrate showed a slightly greater weight increase, average of 6.7 g. weekly for 3 weeks, than

TABLE XI.

Growth of rats receiving 12 µg. daily of riboflavin with no supplement.

Vitamin B_1 given as	No. of rats	Average weekly increase in wt over 3 weeks. g.	Standard deviation
Peters's concentrate from yeast (=0.3-0.6g. yeast, dry wt. daily)	50	6.7	1.7
Aneurin (10 µg daily)	26	5.3	2.3

those having aneurin 5.3 g. weekly (Table XI). This indicates the presence of a small amount, doubtfully significant, of some growth factor, in addition to aneurin, in the Peters's concentrate.

Growth-promoting action of riboflavin when supplemented.

Table XII shows the weight increase of rats given varying amounts of riboflavin supplemented by the different extracts. With the supplementary doses fed without riboflavin the rats showed only a slow weight increase approximately 3.5 g. weekly; this indicated either an absence of riboflavin from these solutions or a very low content.

When the diet containing riboflavin was supplemented by the above heat-stable fractions the growth of the rats seemed to depend on the amount of riboflavin given and, for a given dose, was the same, within experimental error, for the supplementary materials tested. Rats receiving 6 µg. riboflavin daily, supplemented by the preparation from yeast extract or wheat germ showed average weekly increases in weight of 9.6 and 10.0 g. respectively, and those having 12 µg. riboflavin daily, of 12.4 and 12.2 g. respectively. Increasing the doses of the supplements beyond a certain amount did not cause increased growth, as was demonstrated by experiments in which daily doses of 12 µg. riboflavin were supplemented by fuller's earth filtrate in doses ranging from the equivalent of 0.25 to 1.0 g. of yeast, dry wt., daily, or by wheat germ extract equivalent to 1.0-2.0 g. wheat germ dry wt.

Since the degree of growth appeared to be independent of the source or quantity of the supplements when these were given beyond a certain minimum amount, it seems probable that the different extracts contained the same growth factors although the possibility of this being a coincidence is not excluded.

Care was taken in all cases to give sufficient amounts of the supplementary materials, and the doses were increased with increasing amounts of riboflavin, in case the resulting augmented growth stimulated by the increased ration of lactoflavin might raise the requirements of the supplement. It can be seen from Table XII that the optimum dose of wheat germ extract for the 12μg riboflavin level lay between 0.5 and 1.0 ml. so that the 1.5 ml. dose given with larger doses of riboflavin was considered to be adequate.

TABLE XII

Growth of rats receiving aneurin, various doses of riboflavin and other heat-stable dupplements from yeast or wheat germ.

Daily dose of ribo-flavin µg.	Supplement given	Equivalent of yeast or wheat germ, dry wt. g.	No. of rats	Average weekly increase in wt. over 4 weeks. g.	Standard deviation	Average for group g.
0	Yeast fuller's earth filtrate	0.5	6	3.9	-	3.5
	Wheat germ extract	1.5-2.0	5	3.2	-	
6	Yeast fuller's earth filtrate	0.75	3	9.6	-	9.8
	Wheat germ extract	1.5	4	10.0	-	
9	Wheat germ extract	1.5	4	10.7	-	10.7
12	Eluate from norite adsorbate of yeast fuller's earth filtrate	1.0	6	12.5		
"	Yeast fuller's earth filtrate	0.25	2	11.7		
		0.5	26	12.3	1.7	12.3
		0.75	2	12.6		
"	Wheat germ extract	0.5	2	7.6		
		1.0	4	12.6		
		1.5	3	11.7	1.5	
		2.0	2	12.4		
25	Eluate from norite adsorbate of yeast fuller's earth filtrate	3.0	15	15.6	2.5	16.6
"	Yeast fuller's earth filtrate	0.75-1.0	6	17.4	2.4	
"	Wheat germ extract	1.5	2	16.7	-	
37	Yeast fuller's earth filtrate	1.0	3	18.9	-	18.9
50	Yeast fuller's earth filtrate	1.0	8	18.2	3.4	18.2
75	Yeast fuller's earth filtrate	1.5	2	18.1	-	18.1

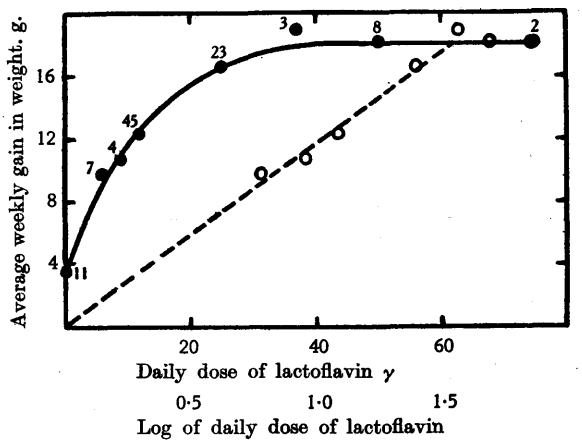

Fig. 5. Growth of young rats on a basal diet free from B-vitamins, receiving vitamin B_1 as either 0.05-0.1 ml. daily of Peters's B_1 concentrate from yeast or 10-20μg. daily aneurin and graded doses of riboflavin supplemented by yeast fuller's earth filtrate or alcoholic extract of wheat germ

The figures by each point on the curve indicate the number of rats (♂ and ♀) on each dose of riboflavin. The continuous line represents the growth plotted against the daily dose of riboflavin and the dotted line the growth plotted against the log. of the daily dose.

Increasing the daily dose of riboflavin from 6 μg to 37μg caused the average gain in body weight to increase from 9.8 to 18.9 g. weekly, over a 4-week

period. The increases in weight showed a fair proportionality to the riboflavin dose, and the experimental points derived from all observations with the different supplementary materials lay on a smooth logarithmic curve (Fig. 5). The optimum daily dose of riboflavin under these conditions was approximately 40 µg. Growth was not increased beyond an average of 18 g. weekly, over a 4-week period, when much larger doses were given.

Growth promoting action of riboflavin for rats receiving as sources of the vitamin B_2 complex, crude yeast fuller's earth filtrate fraction and yeast eluate fraction.

In these experiments the basal diet used contained glaxo ashless extracted casein. The method of preparation of crude yeast fuller's earth filtrate fraction and yeast eluate fraction is described earlier (pp. 16 and 20). The young rats at weaning received the basal diet and the usual aneurin and cod liver oil supplements. After about 10 days growth had ceased and the animals then were each given daily in addition the allotted amount of riboflavin, crude yeast fuller's earth filtrate fraction, equivalent to 1g. dry yeast and

and yeast eluate fraction, equivalent to 2 g. dry yeast. The growth-rate of the animals were observed for a period of 4 weeks.

Results.

As in the previous experiments the weight increase which followed administration of the vitamin B_2 supplements was dependent on the dosage of riboflavin. The rats given no riboflavin increased in bodyweight at the rate of about 5 g. weekly while those given 50 μg. riboflavin increased at the rates of about 23 and 30 g. weekly, according to whether the rats were females or males respectively. The growth rates of rats receiving intermediate doses of riboflavin showed a fair proportionality to the riboflavin dose (Table XIII). Again the growth response curve was a logarithmic one and the optimum response was again obtained when about 40 μg. daily was given to the rats. However, all the growth rates observed were considerably higher than those obtained in the previous experiments in which the supplements supplied were deficient in certain factors of the vitamin B_2 complex.

TABLE XIII

Growth of rats receiving daily 10-15 µg. aneurin, various doses of riboflavin, crude yeast fuller's earth filtrate fraction, equivalent to 1 g. dry yeast and yeast eluate fraction, equivalent to 8 g. dry yeast.

Daily dose of riboflavin µg.	No. of male rats.	Av. wkly. wt. increase for group over 4 week period g.	No. of female rats.	Av. wkly wt. increase for group over 4 week period g.
0	2	4.5	4	5.9
3	3	9.0	3	6.9
6	3	13.8	3	13.1
12	5	19.8	6	16.6
25	2	25.0	2	23.0
37	2	27.8	2	21.0
50	9	30.3	8	22.7

The basal diet and supplements used in these growth tests supplied adequate amounts of all B vitamins with the exception of riboflavin. The growth rates of the rats receiving the large doses of riboflavin were comparable with those of rats receiving unfractionated extracts of yeast or liver. Confirmation of the adequacy of this diet was obtained from breeding experiments.

Male and female rats received our basal diet and optimal amounts of aneurin, riboflavin, crude yeast fuller's earth filtrate fraction and yeast eluate fraction; after about 6 weeks the animals were mated. Healthy litters were born and those were successfully weaned without changing the diet of the mothers. The young were raised to maturity and were again mated, both the bucks and the does being taken from the litters of the mothers which had received the "synthetic" diet. Again litters were born and successfully weaned, but the animals of this second generation were not completely normal; their condition was reminiscent of that observed in animals deprived of the essential unsaturated fatty acids[38]. However, since we did succeed in obtaining 2 generations there could have been no serious deficiency in the diet these animals received.

Growth promoting properties of riboflavin for rats receiving as source of supplementary factors of the vitamin B$_6$ complex, either yeast or liver extracts which had been treated with norite charcoal.

Preparation of the norite charcoal filtrate of extract of liver. Minced liver was extracted with a 50% acetone-water mixture, the acetone was removed in vacuo and the volume of the extract adjusted so that 1 ml. was

equivalent to 6 g. fresh liver. To 500 ml. of this extract at pH 5, 10 g. norite charcoal was removed by filtration; the filtrate was treated with a second portion of 10 g. charcoal. The filtrate from the second charcoal treatment was yellow in colour.

Preparation of the norite charcoal filtrate of extract of yeast. 500 ml. of autoclaved yeast extract, 1 ml. equivalent to 2 g. dry yeast was adjusted to pH 5 and extracted twice with 10 g. portions of charcoal. The final filtrate was light brown in colour.

The Basal diet employed in these experiments contained casein which had been purified by repeated treatment with salt solution as previously described (p. 38). The rats at weaning received the basal diet and the usual supplements of aneurin and cod liver oil until bodyweight increase ceased. The animals were then each given daily the allotted amount of riboflavin and either the above charcoal treated liver extract, equivalent to 6 g. fresh liver or the charcoal treated yeast extract, equivalent to 2 g. dry yeast. The growth-rate of the rats was observed for 4 weeks.

Results.

The rats which received the liver or yeast extracts

but no riboflavin increased very little in bodyweight and at the end of the 4-week period the average total gain was about 10g. On the other hand, rats which received daily 50μg. of riboflavin increased in bodyweight at rates comparable with those of rats receiving untreated extracts of liver or yeast; the average total bodyweight increase during the 4 week period was about 130 g. and 100 g. for male and female rats respectively. Rats given smaller amounts of riboflavin increased in bodyweight at lower rates. Again the growth response curve to added amounts of riboflavin was a logarithmic one. No appreciable difference in growth-rate was observed between the animals receiving the liver and yeast extracts.

The above experiments provide a simple method for the biological determination of riboflavin. Since the basal diet and the yeast and liver extracts and other supplements supply all the essential nutrients, excluding riboflavin, required by the rat for optimal growth, the estimation of riboflavin in foodstuffs would not be interfered with by the supplementary factors present in the foodstuffs.

B. Alloxazine-adenine-dinucleotide in the nutrition of the rat.

Warburg and Christian[85] have recently isolated from liver and yeast a riboflavin containing dinucleotide (alloxazine-adenine-dinucleotide) which is the coenzyme of d-amino acid dehydrogenase, of xanthine dehydrogenase and of other enzymes[86,87,88]. It was to be expected that this important coenzyme would replace riboflavin in the diet of the rat, and it seemed possible that it might also possess the vitamin activity of some other member of the B complex. We were able to investigate this problem through the kindness of Professor Warburg, who put an adequate amount of the coenzyme at our disposal.

Methods and Results.

Growth of young rats was the criterion emplyed. The basal diet used was that containing casein purified by washing with salt solution. All animals received daily 10-15 μ g. aneurin and 0.08-0.1 ml. cod liver oil.

The yeast and liver eluate fractions and the yeast filtrate fractions were prepared by the methods previously described (pp. 16, 25, and 30). The sample of alloxazine-adenine-dinucleotide received from Professor Warburg was a preparation of the monobarium salt obtained from

yeast; it contained 5 per cent. of impurities. The material was fed to rats as the sodium salt, prepared by the addition of Na_2SO_4 to a solution of the barium salt.

Four separate experiments were carried out. Groups of rats received one of the following combinations of vitamin B_2 fractions; (a) yeast eluate fractions and yeast filtrate fraction, (b) riboflavin and yeast eluate fraction, (c) riboflavin and yeast filtrate fraction, (d) riboflavin, yeast eluate and yeast filtrate fractions. The effect of the addition to the diets of alloxazine-adenine-dinucleotide on the growth rate of the rats in these groups was observed.

(a) Addition of a alloxazine-adenine-dinucleotide to the diet containing yeast eluate fraction and yeast filtrate fraction.

These experiments were carried out to determine how efficiently the coenzyme replaced riboflavin in the diet of the rat.

Previous experiments had shown that rats receiving diets containing yeast filtrate and yeast eluate fractions, but no riboflavin, failed to grow, but gained rapidly in weight when riboflavin was subsequently added to the diet. (Compare fig. 2/p.22). The following experiment carried out in a similar manner with alloxazine-adenine-

dinucleotide instead of riboflavin, indicated that this coenzyme could replace riboflavin in the diet of the rat. Two rats which received only yeast eluate and filtrate fractions as sources of the vitamin B_2 complex increased in weight at an average rate of 2.1 g. weekly for 3 weeks; after receiving an additional daily supplement of an amount of alloxazine-adenine-dinucleotide equivalent to 20 μg. riboflavin, the average weekly gain in weight during two subsequent weeks was 15.0 g.

A quantitative comparison of the vitamin activities of riboflavin and the coenzyme was, however, thought desirable.

The curve of response of bodyweight-increase to riboflavin-dosage of animals receiving yeast eluate and yeast filtrate fractions as sources of the supplementary factors of the vitamin B_2 complex is a logarithmic one, similar to that obtained when yeast filtrate fraction was the only supplementary factor given (see p.83). We found optimum growth only when 40 μg. riboflavin daily was administered to each rat; the growth-rate, however, was most sensitive to small changes in the riboflavin content of the diet when the animals received only about one quarter of the optimal amount.

Accordingly comparison of the relative vitamin

potencies of riboflavin and alloxazine-adenine-dinucleotide was carried out by comparising the increase in bodyweight of rats receiving only 6 or 12 μg. of riboflavin, with that of animals receiving amounts of coenzyme equivalent to 6 or 12 μg. of riboflavin.

Animals from two litters of 8 rats each received at weaning the basal diet supplemented by aneurin and cod liver oil. For the first few days the animals increased slightly in bodyweight but this increase ceased after 7-10 days. Four of the animals from one of the litters now each received daily doses of yeast filtrate fraction purified by amyl alcohol extraction (= 2 g. dry yeast), of yeast eluate fraction (= 2 g. dry yeast) and an amount of alloxazine-adenine-dinucleotide equivalent to 6 μg. riboflavin. Their 4 litter mates received the same doses of yeast filtrate and eluate fractions, but instead of the coenzyme, 6 μg. of riboflavin daily. The rats of the second litter were similarly treated, but received double the amounts of coenzyme and riboflavin. The growth rate of all rats was observed for 4 weeks after the supplements were given.

All rats showed immediate growth responses when the supplements were added (Table X IV A). The 4 animals receiving 6 μg. daily of riboflavin maintained an average

growth rate of 8.3 g. weekly for the 4 week period, and those receiving an equivalent amount of coenzyme one of 7.4 g. weekly. Similarly, the two groups of animals receiving daily 12 μg. of riboflavin or alloxazine-adenine-dinucleotide equivalent to that amount of riboflavin increased in bodyweight at approximately the same rate, the average weekly weight increases being 15 g. and 13.9 g. respectively. It is therefore concluded that, when administered orally, alloxazine-adenine-dinucleotide replaces riboflavin in the diet of the rat, and that equimolecular amounts of the coenzyme and riboflavin have the same vitamin potency.

(b) Addition of alloxazine-adenine-dinucleotide to the diet containing riboflavin and yeast eluate factor.

Eleven rats received for one week from weaning the basal diet supplemented by aneurin and cod liver oil, and during a further preliminary period of 2 weeks each received daily in addition 50μg. riboflavin and yeast eluate fraction equivalent to 2 g. dry yeast. During the subsequent test period of 2 weeks, 4 of the animals received the additional supplement of an amount of alloxazine-adenine-dinucleotide equivalent to 20μg. of riboflavin daily, 2 received an amount equivalent to 40μg. riboflavin, 1 serving as a positive control

received yeast filtrate factor not purified by amyl alcohol extraction equivalent to 1 g. dry yeast, while 4 rats served as negative controls and received no further supplement. In this experiment the addition of alloxazine-adenine-nucleotide to the diet has no effect in maintaining growth in the 6 rats receiving it (Table XIV, B), the weekly weight increases being no greater than those of the negative controls. In the positive control rat, however, the growth rate was restored.

TABLE XIV.

Effect of alloxazine-adenine-dinucleotide on the growth rate of rats receiving aneurin and various other factors of the vitamin B complex.

	No. of rats	Daily supplements of the Vitamin B complex			Av. weekly wt. increase of group during 4 weeks subsequent to giving supplements. g.	Mean wkly wt. increase g.
A.	4	yeast eluate factor (=2g. dry yeast)	+ yeast filtrate factor (=2g. dry yeast)	+ 6 µg. riboflavin	11.2., 6., 8.2., 8	8.3
	4	"	"	+ alloxazine-adenine-dinucleotide = 6 µg riboflavin.	9.5., 4., 8.5., 7.8	7.4
	4	"	"	+ 12 µg riboflavin	19., 14., 14.5., 12.5	15.0
	4	"	"	+ alloxazine-adenine-dinucleotide = 12 µg. riboflavin	18.5., 15.2., 12.2., 9.8	13.9

	No. of rats	Daily supplements given during preliminary period of 2 weeks	Av. weekly wt. increase during preliminary period g.	Av.	Additional supplement added daily (= S)	Av. weekly wt. increase during the 2 weeks after giving S. g.	Av.
B.	4	50 µg riboflavin + yeast eluate factor (=2g. dry yeast)	18.5., 9.8	-	alloxazine-adenine-dinucleotide = 20 µg riboflavin	9.0., 5.5.	7.2
	2	"	21.5., 8.5	-	alloxazine-adenine-dinucleotide = 40 µg riboflavin	11.5., 2.5	7.0
	4	"	20.2., 10	-	None	9.5., 5	7.2
	1	"	20., 12	-	yeast filtrate factor =1g. dry yeast	17., 18	17.5
C.	3	50 µg riboflavin + yeast filtrate fraction =1g. dry yeast	16., 13.3	14.6	alloxazine-adenine-dinucleotide=24 µg riboflavin	15., 13.7	13.3
	2	"	14.5., 12.5	13.5	liver eluate factor = 12g. fresh liver	23.5., 22.5	23

	No. of rats	Daily supplements the vitamin B_2 complex	Alloxazine-adenine dinucleotide given daily	Av. wt. increase of group during 4 weeks subsequent to dosing	Av.
D.	4	50 µg riboflavin + eluate factor = 2g. dry yeast + yeast filtrate factor = 2g. dry yeast	equivalent of 24 µg riboflavin	19.8., 20.5., 20.5., 21.2	20.5
	3	"	None	22., 19.3., 21., 22.7	21.2

(c) Addition of alloxazine-adenine-dinucleotide to the diet containing riboflavin and yeast filtrate factor.

Five rats were maintained for 1 week from weaning on the basal diet supplemented by aneurin and cod liver oil, then for the further preliminary period of 2 weeks each received additional daily supplements of 50 μg. riboflavin and of yeast filtrate factor not purified by amyl alcohol extraction, equivalent to 1 g. dry yeast. During the subsequent test period of 2 weeks daily supplements of the coenzyme equivalent to 24 μg. of riboflavin were given to 3 of the rats, and eluate fraction equivalent to 12 g. fresh liver, to the other 2 animals which served as positive controls. The addition of the coenzyme caused no increase in the growth rate, while the addition of the eluate fraction caused the usual marked increase (Table XIV, C).

(d) Addition of alloxazine-adenine-dinucleotide to the diet containing riboflavin, yeast eluate factor and yeast filtrate factor.

Seven male litter-mate rats at weaning received the basal diet supplemented by aneurin and cod liver oil for 1 week. Four of the animals then each received daily 50 μg. riboflavin, yeast eluate fraction equivalent to 2 g. dry yeast, yeast filtrate fraction purified by amyl

alcohol extraction, equivalent to 2 g. dry yeast and alloxazine-adenine-dinucleotide equivalent to 24 μg. riboflavin. Three animals which served as negative controls received all these fractions except the coenzyme. There was no significant difference in the rate of growth of the 2 groups (Table XIV, D), the average weekly gains in weight during a 4 week period being 20.5 and 21.2 g. respectively.

The usual weekly weight increase of similar male rats receiving unfractionated extracts of yeast or liver as sources of the whole vitamin B_2 complex is more than 30 g. The amounts of riboflavin and yeast filtrate and eluate factors administered were more than enough to supply the animal's needs of those factors; this experiment proves, therefore, (a) that at least one factor of the vitamin B_2 complex, in addition to those factors, is required by the rat and (b) that alloxazine-adenine-dinucleotide cannot replace this additional factor.

The above experiments indicate that the only vitamin activity possessed by alloxazine-adenine-dinucleotide is that which it has by virtue of the riboflavin it contains bound in its molecule, and that when it is administered orally its vitamin potency is identical

with that of an equimolecular amount of riboflavin.

Warburg and Christian[85] suggest that alloxazine-adenine-dinucleotide is probably the form in which riboflavin functions in the animal body, but it is also possible that riboflavin phosphoric acid or free riboflavin or other unknown compounds of riboflavin may be concerned with processes in the living cell. These experiments show, however, that riboflavin and alloxazine-adenine-dinucleotide, when administered orally to the rat, are converted with equal ~~deficiency~~ of into the compound or compounds which function in the animal's tissues.

Experiments on the eluate factor (vitamin B_6, factor 1) of the vitamin B_2 complex.

Determination of eluate factor by a rat growth method.

Male rats were found more satisfactory for this test. The basal diet used was that previously described containing either Glaxo ashless extracted casein or that washed with salt solution. The method of preparation of the young rats was similar to that used in other experiments. During the first week after weaning the animals each received 10-15μ g. aneurin and 0.08-0.1 ml. cod liver oil. For a period of 2 weeks the animals now received daily additional supplements of 50μ g. riboflavin and an amount of the yeast filtrate fraction equivalent to 2 g. dry yeast; these amounts were sufficient to supply the animals' requirements. An immediate growth response resulted and during the first week the animals usually increased in body weight by about 20 g. A slight fall in the rate of growth occurred in the second week during which the weight increase was usually about 15 g. Certain animals then received additional supplements of the materials to be tested for eluate factor activity, others (negative controls) were given no added supplement, while yet others (positive controls) were given an amount of a tested yeast eluate fraction equivalent to 2 g. dry

yeast, this amount being known to supply the animals' requirements of eluate factor. The test was continued for a further 2-week period. Even after 2 days a sharp increase in the growth rate of the animals now receiving eluate fraction was noted, and the increased growth rate was maintained during the 2-week period, the average weekly gain usually being 25-30 g. (Table XV). The negative control animals generally continued to increase in weight at the rate observed towards the end of the preliminary period of 2 weeks, this rate being about half of that of the positive control animals. Comparison of the growth rates of the animals receiving the test material with those of the negative and positive control animals indicated the vitamin potency of the tested material. As far as was practicable, animals used in a single test were taken from the same litter, although usually no great difference was observed from litter to litter. Reliable results were obtained when 3 animals, together with one positive and one negative control animal, were used for each test.

In the earlier experiment in which the basal diet contained unwashed "Glaxo ashless extracted" casein as source of protein and the unpurified yeast fuller's earth filtrate as source of filtrate factor, the

slackening in the growth rate during the second week of the preliminary period was less marked than that observed when the more highly purified casein and purer filtrate factor preparations were introduced into the basal diet. The body-weight increase observed during the 2-week period following administration of eluate factor was also slightly less in the case of the animals receiving the purer diet; however, the growth response obtained when eluate factor was added to the diet was more striking and tests using the purified diet were generally more satisfactory.

It was observed that the increased growth rate resulting from the addition of eluate fraction varied with the dose given. In one experiment the average total weight increase of 4 rats receiving a suboptimal dosage of eluate fraction was 40 g. for the 2-week test period, that of 5 rats receiving twice the above dosage was 49 g., while 4 rats given 4 times the dosage increased in body weight by 55 g. during the 2-week period. From this and other experiments not recorded it appeared that the growth response curve was the usual logarithmic one.

TABLE XV. Tests for eluate factor.

Each rat (male) received daily 10-15γ. aneurin and 50γ. riboflavin

No. of rats	Filtrate fraction, preparation and amount given daily	Av. wkly wt. increase of grp. for preliminary period of 2 wks. g.	Additional supplement	Av. wkly wt. increase of grp. during test period of 2 wks g.
Unwashed casein diet:				
52	Yeast filtrate fraction (= 1g. dry yeast)	21,19	Eluate fractions from yeast or liver	29,25*
36	" "	21,18	None	16,16†
4	Yeast filtrate fraction purified by extraction with amyl alcohol (= 2g. dry yeast)	21,14	Eluate fractions from yeast or liver	27, 27
Washed casein diet:				
9	Yeast filtrate fraction (= 1g. dry yeast)	19,16	"	27,[illegible]
6	" "	20,19	None	15,15
8	Yeast filtrate fraction purified by extraction with amyl alcohol (2 g. dry yeast)	16,12	Eluate fractions from yeast or liver	22,22
6	" "	19,13	None	12,10

* The standard error of the average total weight increase for the 2-week test period ($\sigma/\sqrt{n}$) = 1.08.

† The standard error of the average total weight increase for the 2-week test period ($\sigma/\sqrt{n}$) = 1.89.

The unit of eluate activity we have adopted is based on the potency of an amount of our standard eluate fraction equivalent to 2 g. dry yeast. This amount when given daily to a rat prepared as described above, produces a growth response of approximately 90% of the maximum obtainable.

The rat growth method for the determination of factors of the vitamin B complex has been so extensively used in the past that the use of this criterion for the study of new factors of the vitamin B complex requires no further justification. In the case of aneurin, the rat growth method, although perhaps more tedious than some others, is certainly one of the more reliable biological methods for the determination of that vitamin. The biological determination of riboflavin has been almost exclusively carried out by rat growth methods.

Although curative methods for vitamin determination have the advantage of specificity in cases where a specific group of symptoms is involved, growth methods which employ uniform healthy young animals appear to be the more reliable. Curative methods necessitate the use of sick animals and the response effected by administration of the missing vitamin is largely dependent on the degree to which the pre-existing deficiency had affected the general health of the animal.

For the estimation of vitamin B_6, which we now realize is identical with our yeast eluate factor and factor 1, the cure of the dermatitis developed by rats deprived of the vitamin has been most extensively used. In our laboratory, when using this method, we have encountered the disadvantages of curative methods referred

to above. Further, we have also noted occasional spontaneous cures in our animals and have also found that the specificity of yeast eluate factor for the cure of this dermatitis is not complete. Cures of dermatitis developed on diets deficient in yeast eluate factor and riboflavin, or deficient in yeast eluate factor and yeast filtrate factor have frequently been obtained by the addition of riboflavin or of yeast filtrate factor, respectively. In carrying out the rat curative test, therefore, the supply to the animal of all the other vitamin B factors must be adequate. Since the animals used in curative tests are usually on experiment for many weeks, during which they must be carefully observed, the time required for these tests is much greater than the simple routine growth test described.

The purification of yeast eluate factor.

It was soon realised that yeast eluate factor was almost certainly identical with Vitamin B_6[32] and with factor 1[65]. The chemical properties of the yeast eluate factor were similar to those of vitamin B_6[35], highly purified concentrates of our factor also cured the florid type of rat dermatitis (Chick, unpublished

experiments) and its growth promoting action for rats was very similar to that of factor 1.

About two years ago experiments were begun which, it was hoped, would terminate in the isolation of the yeast eluate factor in a chemically pure state. Good progress was made and about one year ago we had progressed so far that we were hopeful that isolation of the pure vitamin would be achieved in a short time. At that time, however, the isolation of factor 1 was reported by Lepkovsky[67]; and immediately there followed publications from three different laboratories announcing the isolation of Vitamin B_6[68,69,70]. The identity of vitamin B_6 with factor 1 was proved.

Dr. Lepkovsky very kindly presented us with a generous sample of his crystalline factor 1 and we found that this material completely replaced our yeast eluate factor in the diet of the rat. Since the biological identity of yeast eluate factor and factor 1 was thus established, we did not pursue further our independent experiments on the isolation of yeast eluate factor. However, by submitting our concentrates to the methods of fractionation found successful for factor 1, we obtained a small amount of a crystalline material which appeared to be identical with the hydrochloride prepared

from factor 1. The identity of vitamin B_6, factor 1 and yeast eluate factor is therefore established.

Very recently Kuhn et al[89,90,91] have proved that vitamin B_6 (eluate factor) is 2-methyl-3-hydroxy-4:5-dihydroxymethylpyridine. It is interesting that vitamin B_6 should be chemically related to nicotinic acid (3-carboxypyridine).

Experimental.

The rat growth method for estimation of eluate factor described above was employed throughout.

The starting material used in these experiments was the yeast eluate fraction, prepared by elution of a yeast fuller's earth adsorbate with barium hydroxide and purified by treatment with basic lead acetate (see p.20). It was further purified as follows.

Precipitation with phosphotungstic acid, further treatment with fuller's earth and reprecipitation with phosphotungstic acid. To 2 l. of yeast eluate fraction (1 ml. = 2 g. dry yeast), adjusted to pH 1.2 with H_2SO_4, a solution of 57 g. of phosphotungstic acid in warm water was added and, after standing overnight in the cold, the precipitate was collected and washed with 1 per cent. H_2SO_4. This phosphotungstate, dissolved in 300 ml. acetone, was decomposed by adding gradually cold saturated aqueous

$Ba(OH)_2$ until the solution remained alkaline after standing for some time. Barium phosphotungstate was removed by filtration and the filtrate, freed from acetone by distillation *in vacuo* was adjusted to pH 1.2 by the addition of H_2SO_4; the precipitated $BaSO_4$ was filtered off and the filtrate treated twice with 50 g. portions of fuller's earth and each adsorbate after washing with N/10 H_2SO_4 was twice eluted with 0.1 N $Ba(OH)_2$. H_2SO_4 was added to the combined eluates to pH 1.2 and, after removal of the $BaSO_4$, a solution of 50 g. phosphotungstic acid in warm water was added. The phosphotungstate separated by filtration, was decomposed as previously and, finally, the excess barium was removed with H_2SO_4, care being taken to avoid an excess of H_2SO_4. This solution contained the greater part of the eluate factor present in the original eluate fraction, and was found to be a convenient concentrate for certain biological experiments.

Extraction with acetone. The water was removed from the above solution by distillation *in vacuo* and the residue was thoroughly dried by repeated addition of absolute alcohol, followed by removal of the alcohol *in vacuo*. The residue was then extracted with 200 ml. acetone by heating under a reflux condenser for 2 hours;

the extraction was repeated with three further portions of acetone. The combined acetone extracts were reduced in volume to 50 ml. On standing, 160 mg. of adenine separated, M.P. 340° (decomp.). M.P. of flavianate 278° (decomp.);M.P. of flavianate of authentic specimen of adenine,278° (decomp.). This adenine, administered to rats receiving riboflavin and filtrate factor in daily amounts of 1 mg. had no growth stimulating effect.

The mother liquor from the adenine contained the eluate factor; the material which was insoluble in acetone was inactive.

Extraction with chloroform. The gummy residue obtained by removal of acetone from the above mother liquor, from which the adenine had separated, was extracted four times with 50 ml. portions of chloroform. The chloroform extract was reduced to 10 ml. when 66 mg. of a substance separated which after purification was found to be nicotinamide (see p. 55). The mother liquor from the nicotinamide crystallised was biologically active. The gummy material insoluble in chloroform was inactive.

Treatment with palladium chloride. The chloroform soluble portion, from which the nicotinamide had crystallised, was freed from chloroform and dissolved in 50 ml. water. To this solution, heated on the water

bath, an excess of a 2% solution of palladium chloride in dilute HCl (55 ml.) was added. After standing overnight in the cold the precipitate was collected. The filtrate, freed from palladium chloride by shaking with freshly precipitated silver, was completely colourless and possessed the eluate factor activity. The material obtained by decomposition of the palladium precipitate was biologically inactive.

Precipitation with gold chloride. The above filtrate from the treatment with palladium chloride was reduced in volume to 20 ml. and to this solution heated on the water bath, 10 ml. of a 3% solution of $HAuCl_4$ was added. On cooling, a precipitate appeared which, after standing overnight in the cold, was filtered off, washed with water and decomposed with H_2S; the filtrate from the gold precipitate was also decomposed with H_2S. The eluate factor was contained in the decomposed gold precipitate; the filtrate was almost completely inactive.

This decomposed gold precipitate, which was active in rat tests, had a very small dry weight and the percentage of vitamin in this material must have been great.

The experiments had reached this stage when the

papers announcing the isolation of factor 1 and vitamin B_6 appeared. The various yeast eluate factor concentrates available in this laboratory, which consisted of materials from different stages in the above purification were therefore mixed and submitted to a purification process essentially similar to that employed by Lepkovsky[92] in the isolation of factor 1. After some difficulty a small amount of a crystalline hydrochloride was obtained, M.P. 201-203°; the M.P. when mixed with a specimen of the hydrochloride of factor 1, prepared from a sample of factor 1 supplied by Dr. Lepkovsky, being 202-203°. Our crystals had the same crystalline form as those of the hydrochloride of factor 1, and also gave similar red colorations with $FeCl_3$ (compare Kuhn and Wendt[93]; Keresztesy and Stevens[94]). Unfortunately there was not enough of our material for chemical analysis, and to obtain more would have entailed work on quantities of yeast that could not be easily dealt with in our laboratory.

<u>The activity of Factor 1 in our rat growth tests for eluate factor on our "B-free" basal diet with cod liver oil and aneurin.</u>

Rats were prepared as for eluate factor tests and received yeast filtrate factor and riboflavin for a

TABLE XVI.

Growth promoting action of factor 1 for rats receiving a "B-free" basal diet with supplements of 10-15 μg. aneurin, 50 μg. riboflavin and yeast filtrate fraction equivalent to 1-2 g. dry yeast.

No. of rats.	Av. weekly wt. increase of the group during 2 preliminary weeks	Av.	Factor 1 (μg. daily)	Av. weekly wt. increase of the group during 2 wks. subsequent to dosing factor 1. g.	Av.
4	17, 12.5	14.7	5	20, 19.8	19.9
5	19.6, 13.6	16.6	10	26.2, 22.8	24.5
4	16.5, 14.5	15.5	20	28, 24.8	26.4
*6	18, 12	15	Yeast eluate fraction = 2g. dry yeast	24, 25	24.5

* Taken from Table III (p. 35)

preliminary period of two weeks. Some of the animals then received the additional daily supplement of 5 μg. factor 1, others 10 μg. and other 20 μg. In all cases increases in the growth rate of the animals occurred (Table XVI). The extent by which the growth rate of

the animals was increased by administration of factor 1 was of the same order as that which has been repeatedly observed following administrations of our yeast eluate factor to rats receiving the same diet with the same supplements. Crystalline factor 1 also cured the florid type of rat dermatitis (Chick, unpublished experiments).

The growth-rate increase following administration of 20 μg. daily of factor 1 (free base) was somewhat greater than that observed with 10 μg. daily which in turn was considerably greater than that observed with 5 μg. daily. The optimal daily requirements of the rat for factor 1 in growth experiments appears therefore to lie between 10 μg. and 20 μg. daily and is probably nearer 10 μg. Dimick and Schreffler[95] found 10 μg. of factor 1 (free base) produced nearly optimal growth; Kuhn and Wendt[96] stated that the rate of weight increase observed following administration of 2.5-10 μg. daily of vitamin B_6 hydrochloride increased with the dose of the vitamin given.

Experiments on the Purification and Nature of Filtrate Factor from Yeast and Liver.

The earlier experiments on filtrate factor were carried out before it was realised there was more than one factor of the vitamin B_2 complex, additional to riboflavin. The source of filtrate factor then employed was the filtrate obtained by treatment of autoclaved extracts of yeast with fuller's earth. It is now realised that this material contains, in addition to yeast filtrate factor, appreciable amounts of that factor we have provisionally named "additional factor". However, it now appears that the biological test we then employed estimated the filtrate factor and the presence of "additional factor" did not interfere with the biological tests.

After it had been proved that yeast eluate factor was a dietary essential for the rat, that factor was introduced into the diet used in the animal tests for filtrate factor, and a very much improved test resulted.

In later experiments filtrate factor, as present in extracts of yeast and liver, has been intensively studied, and highly purified concentrates of the factor from yeast and liver have been prepared. The factors as present in these two materials, although biologically identical

(see p. 33) have proved to be chemically distinct.

Early experiments on yeast filtrate factor as contained in fuller's earth filtrates from autoclaved extracts of yeast.

Young rats were prepared for the routine testing of this factor by the method previously described (p. 15). The basal diet contained light white casein as source of protein. Each rat received daily 0.1 ml. of cod liver oil and either 10 μg. of aneurin or an adequate amount of Peter's vitamin B_1 concentrate. After being constant in weight for several days, they received the test doses supplemented by 12 μg. daily of riboflavin; 3 rats were used for each test and the growths were observed for a period of 4 weeks. The results were generally taken as positive when the average weekly weight increase was 10 g. or more. That amount of riboflavin alone produces weight increases of approximately 6 g. weekly for a period of 3 weeks. Some variation has been noted occasionally in the growth responses of rats from different litters, but within a litter, growth responses were fairly regular, and therefore the different preparations obtained from one fractionation were tested on litter-mates, one rat of the litter being given riboflavin alone as a control.

More stress was laid on the relative growths shown by litter-mates dosed with the various fractions and the riboflavin control rat than on the actual increases in weight obtained.

The fuller's earth filtrate was prepared as described (p. 16) by extraction of 1 l. of aqueous yeast extract (1 ml. = 0.5 g. yeast, dry wt.), autoclaved for 5 hr. at 120° at pH 5, adjusted to pH 1.4, with 2 portions of 50 g. each of fuller's earth.

The experimental details of all the processes investigated are given below, and the results of the animal tests are given in Table XVII.

Exp.1. Stability. (a) To acid. To 200 ml. of the fuller's earth filtrate 10 g. of concentrated H_2SO_4 were added and the mixture was immersed for 2 hr. in a vigorously boiling water-bath. The sulphuric acid was removed with $Ba(OH)_2$. Some darkening occurred during the heating, but there was no loss of activity.

(b) To alkali. 20 g. of very finely ground $Ba(OH)_2$, $8H_2O$ were added to 200 ml. of fuller's earth filtrate previously adjusted to pH 8 by the addition of $Ba(OH)_2$. The mixture was heated as above for 2 hr., after which the $Ba(OH)_2$ was removed with H_2SO_4. No darkening

occurred, and the vitamin was unaffected.

Exp. 2. Stability to light. (a) Sunlight. Clear glass bottles containing fuller's earth filtrate adjusted to pH 3 and pH 10 were exposed daily during 3 summer months to direct sunlight, thereby receiving about 200 hr. of sunshine. Controls in dark glass bottles wrapped in brown paper were also kept under the same conditions. All solutions at the end of this period were fully active.

(b) Ordinary electric light. Fuller's earth filtrate was exposed at pH 3 or 10 for ~4 hr. in very thin layers at a distance of 21 cm. from an Osram 500 W. gas-filled clear lamp, the solutions being kept cool by a fan. A control solution at pH 10 was not irradiated. There was no destruction of vitamin by this irradiation either at pH 3 or 10.

(c) Ultraviolet light. Very thin layers of fuller's earth filtrate at pH 3 and 10 were placed 14 cm. distant from a quartz mercury lamp and irradiated for 10 hr., cooling being effected by standing the dishes in running water. Control solutions at pH 3 and 10 were also exposed with thick glass screens between the lamp and the solutions. There was no destruction of vitamin.

Exp. 3. Adsorption experiments. (a) Fuller's earth at pH 10 and 8. 100 ml. portions of fuller's earth filtrate were adjusted to pH 8 and approx. 10. Each portion

was treated twice with 10 g. portions of fuller's earth (B.D.H. "for adsorption purposes"). Both filtrates were fully active, showing that little or no vitamin had been adsorbed.

(b) $Al(OH)_3C\gamma$. The adsorbent was prepared according to the method of Willstätter et al[97]. 200 ml. of fuller's earth filtrate, adjusted to pH 4, were cooled in ice and treated with 27 ml. of a suspension of $Al(OH)_3$ $C\gamma$ (1 ml. = 18.5 mg. Al_2O_3). After shaking for 10 min. the $Al(OH)_3$ was centrifuged out. The adsorption was repeated 4 times. The total adsorbate was washed with 50 ml. M/10 acetate buffer at pH 4 and suspended in 100 ml. M/4 Na_2HPO_4. After 14 hr. the $Al(OH)_3$ was centrifuged down and again eluted with an equal volume of Na_2HPO_4. The eluates were mixed. No activity was detectable in the eluate whilst the filtrate appeared to have its full activity.

(c) Norite charcoal. 400 ml. of fuller's earth filtrate were adjusted to pH 2.5 with HCl and treated 4 times with 5 g. portions of norite. The norite was washed once with N/10 HCl and then eluted with 250 ml. of 0.25 N NaOH, being allowed to stand overnight. The elution was repeated with an equal volume of 0.25 N NaOH. In another experiment elution was accomplished by heating

the norite on a water-bath with glacial acetic acid.

Further adsorptions with norite were carried out at pH 8.2, 7 and 1.2, also using 0.25 N NaOH as eluant. Adsorption was best at pH 2.5 and 1.2. At pH 8.2 and 7 little adsorption occurred. Good elution was obtained with NaOH or glacial acetic acid, but in one experiment using 0.1 N $Ba(OH)_2$ as eluant, the eluate was inactive.

Exp. 4. Electrodialysis. The alcohol-soluble portion from 400 ml. of fuller's earth filtrate, dissolved in 800 ml. H_2O (pH 2.6), was put in the centre chamber of a 3-chambered cell divided by cellophane membranes which were permeable to the factor. The anode and cathode chambers were filled with distilled water and all chambers were cooled by coils through which cold water flowed. The current was approximately 1-1.5 amp. and the voltage was 100-200 V. After 1½ hr. fresh water was put in the anode and cathode chambers and the dialysis continued for a further 5½ hr. No attempt was made to regulate the pH in the chambers. The solution in the centre chamber remained at approximately pH 2.6. The pH of the anode solution fell to 2.4 while that of the cathode solution rose to approximately 11 before the fluid in the chambers was changed and to pH 9.2 after the fresh water had been added. At the end of the dialysis approximately 65% of

of the nitrogen in the original material had migrated to the cathode, while 5% was in the anode solution. The remainder was in the centre chamber solution. The animal tests were not very satisfactory, but it appeared that most of the activity remained in the centre chamber.

Exp. 5. Sublimation in high vacuum. 400 ml. of fuller's earth filtrate were adsorbed with norite charcoal at pH 2.5 and the activity eluted with 0.25 N NaOH as described in Exp.3 (c). The eluate, adjusted to pH 7, was evaporated to dryness *in vacuo* and the residue transferred to a vacuum tube, which was then kept in a vacuum desiccator over P_2O_5 for 2 days. A tube cooled by a rapid flow of cold water was inserted inside the vacuum tube, which was evacuated to 0.05 mm. The apparatus was heated in a metal-bath to 150° for 4 hr. The vacuum remained good during the heating. A partially crystalline acid sublimate was obtained on the surface of the cooled tube. This was biologically inactive, even when large doses were fed. There was some charring of the residue, but it retained some vitamin activity.

Exp. 6. Solubilities. (a) 96% Ethyl alcohol. 140 ml. of fuller's earth filtrate were evaporated to dryness on the water-bath and finally in a desiccator. The hard resin-like material was divided as finely as

possible and extracted for 20 hr. with 96% ethyl alcohol in a Soxhlet extractor. The alcohol was removed from the extract *in vacuo* and the remaining gum was taken up in water. The greater part of the activity was found in the alcohol-soluble portion.

In a second experiment, the gum which separated from the alcohol extract as the extraction proceeded, was removed and tested separately; it contained no activity.

(b) Acetone. Dried fuller's earth filtrate was extracted with purified acetone in a Soxhlet extractor for 30 hr. The extract was quite inactive even when fed in large amounts. The portion insoluble in acetone was active.

(c) Precipitation with acetone. 100 ml. of a solution of the vitamin were prepared by elution with glacial acetic acid of a norite absorbate obtained from 600 ml. of fuller's earth filtrate and were mixed with 1 l. of acetone. After standing overnight in the cold the acetone solution was decanted from the gummy precipitate. Acetone was removed from both fractions *in vacuo*. Some activity appeared to be present in the precipitate, but the greater part was found in the soluble portion.

Exp. 7. Precipitation with precipitants for bases.

TABLE XVII. Results of tests of fractions prepared from fuller's earth filtrate from yeast

Each rat received 10 µg. aneurin or 0.05-0.1 ml. of Peter's vitamin B_1 concentrate and 12 µg. riboflavin.

Exp. No.	Treatment	Fraction	Equivalent of dry yeast given daily	Av. wkly increase in wt. over 4 weeks	No. of rats	Av. wkly increase in wt. over 3 wks. of litter control rat receiving riboflavin only.	Result of test
			g.	g.		g.	
1(a)	Heating with 5% H_2SO_4 for 2 hr.	-	0.5	11.5	3	7.6	+
(b)	Heating with 10% $Ba(OH)_2,8H_2O$ for 2 hr.	-	0.5	12.2	3		+
2(a)	Visible light (3 months in sun, pH 3)	-	0.5	13.3	2		+
	Control at pH 3	-	0.5	12.75	1	5.3,8.0	+
	3 months in sun, pH 10	-	0.5	12.5	2		+
	Control at pH 10	-	0.5	12.75	2		+
(b)	24 hr. under 500 W. lamp, pH 3	-	0.5	10.75	3	5.6,3.0	+
	24 hr. under 500 W. lamp, pH 10	-	0.5	12.3	3		+
(c)	Ultraviolet light, 8 hr. at pH 3	-	0.5	11.4	3		+
	Control at pH 3	-	0.5	11.4	3	8.6	+
	8 hr. at pH 10	-	0.5	12.5	3		+
	Control at pH 10	-	0.5	12.2	3		+
3(a)	Adsorption with fuller's earth at pH 8	Filtrate	0.5	12.5	4	7.7	+
	Adsorption with fuller's earth at pH 10	Filtrate	0.5	10.2	4		+
(b)	Adsorption with $Al(OH)_3Cl$ at pH 4	Filtrate	0.5	11.7	3	7.4	+
		Na_2HPO_4 eluate	0.5	8.5	2		-
(c)	Adsorption with norite charcoal, pH 2.5	Filtrate	1.0	12.4	3	6.3	+
		NaOH eluate	1.0	11.5	3	5.6	+
		Acetic acid eluate	1.0	11.9	3	6.3	+
		$Ba(OH)_2$ eluate	1.0	7.6	3	-	-
	pH 1.2	Filtrate	1.0	11.4	3	5.0	+
		NaOH eluate	1.0	13.2	3		+
	pH 7.0	Filtrate	0.5	16.0	2	7.4	+
	pH 8.2	Filtrate	1.0	10.8	3	7.3	+
		NaOH eluate	1.0	8.4	3		-
4	Electrodialysis pH 2.6	Centre	1.0	8.6	3	6.3	±
		Anode	1.0	5.4	3		-
		Cathode	1.0	6.3	3		-
5	Sublimation <u>in vacuo</u> at 150°	Sublimate	10.0	2.0	1	*	-
		Residue	2.0	8.6	2	5.0	±
6(a)	Extraction of dried fraction with 96% ethyl alcohol	Extract	0.5	13.3	4		+
		Residue	0.5	9.6	3	8.5	±
		Sparingly soluble fraction	1.0	5.9	2	5.7	-
(b)	Extraction of dried fraction with acetone	Extract	2.0	6.5	2		-
		Residue	0.5	9.1	3	4.0	±
(c)	Precipitation with 10 vol. of acetone	Filtrate	2.0	10.9	2		+
		Precipitate	2.0	9.7	3	7.0	±
7(a)	Precipitation with picric acid	Filtrate	1.0	10.3	3		+
		Precipitate	1.0	7.5	5	7.3	-
(b)	Precipitation with pierolonic acid	Filtrate	1.0	12.3	3		+
		Precipitate	1.0	4.4	3	7.0	-
(c)	Precipitation with Reinecke acid	Precipitate	1.0	4.3	3	-	-
(d)	Precipitation with flavianic acid	Filtrate	1.0	13.8	3		+
		Precipitate	1.0	6.4	3	7.7	-
(e)	Precipitation with phosphotungstic acid	Filtrate	0.5	13.1	3	8.7	+
8 (a)	Precipitation with copper acetate	Filtrate	1.0	8.0	3		+
		Precipitate	1.0	1.8	3	2.7	-
(b)	Precipitation with lead acetate (pH 4)	Filtrate	0.5	11.2	3		+
		Precipitate	0.5	9.1	2	4.7	±
	Precipitation with lead acetate (pH 8)	Filtrate	0.5	11.75	2		+
		Precipitate	0.5	7.8	2		-
(c)	Precipitation with mercuric sulphate	Filtrate	0.5	10.1	2		+
		Precipitate	0.5	7.2	3	8.7	-
(d)	Precipitation with silver sulphate	Filtrate	1.0	12.1	3		+
		Precipitate	1.0	8.7	3	9.7	-
(e)	Precipitation with $Ba(OH)_2$ in 90% alcohol	Filtrate	2.0	7.7	3		-
		Precipitate	2.0	10.0	3	7.3	+
	Precipitation with $Ba(OH)_2$ in 80% alcohol	Filtrate	1.0	9.3	3		+
		Precipitate	1.0	9.0	3	3.7	+
9	Treatment with acetic anhydride	Water-insoluble fraction	1.0	6.4	3		-
		Water-soluble fraction	1.0	8.4	3	5.7	±

* This rat received 18 µg. riboflavin daily for 3 weeks and grew an average of 3 g. weekly; after receiving the sublimate it grew only 4 g. in 19 days.

(a) Picric acid. 400 ml. of fuller's earth filtrate were adjusted to pH 7.2 and a hot solution of 7 g. of picric acid in 100 ml. H_2O added. After standing overnight in the cold the crystalline picrate precipitate was filtered off and the excess picric acid was extracted from the acidified filtrate with ether. The picrate precipitate was decomposed by suspending in dilute HCl and extracting with ether. There was no activity in the decomposed precipitate while the filtrate was active.

(b) Picrolonic acid. 400 ml. of fuller's earth filtrate were mixed with a solution of 5 g. of picrolonic acid in 100 ml. of 96% alcohol (pH of the mixture was 4.5). The heavy precipitate was filtered after standing overnight in the cold, the filtrate freed from picrolonic acid with ether by the usual method and the picrolonate precipitate decomposed with acid and extracted with ether. All the vitamin activity was in the filtrate.

(c) Reinecke acid. 15 g. of Reinecke acid in 300 ml. H_2O at 60^0 were added to 400 ml. of fuller's earth filtrate (pH 4.5). On standing a heavy precipitate was deposited, which was filtered off, after being kept overnight in the cold, and dissolved in 100 ml. of 50% acetone; 130 ml. of 1% Ag_2SO_4 were added to the solution. The Ag reineckate was filtered off, the acetone removed

on a water-bath and the excess Ag precipitated with HCl. This decomposed precipitate was inactive when fed to animals.

(d) Flavianic acid. 25 g. of flavianic acid, dissolved in 125 ml. H_2O were added to 400 ml. of fuller's earth filtrate, NaOH being added to pH 4.5. A heavy precipitate was obtained, which was filtered off after standing overnight in the cold. The filtrate was treated with 100 g. of basic lead acetate and the pH adjusted to 8, the lead salts were filtered off and excess lead removed from the filtrate with H_2S. The flavianic acid precipitate was decomposed by dissolving in H_2O and adding basic lead acetate at pH 8. The filtrate had full biological activity, whilst the decomposed precipitate was inactive. Filtrates from flavianic acid precipitations at more acid reactions were also active.

(e) Phosphotungstic acid. 200 ml. of fuller's earth filtrate were mixed with a solution of 10 g. of purified phosphotungstic acid in 200 ml. H_2O and con. H_2SO_4 was added to pH 1. After standing 24 hr. with occasional stirring, the precipitate was filtered off. The filtrate, after removal of the excess phosphotungstic acid by the usual means, was found to have vitamin activity approximately equal to that of the original fuller's earth filtrate.

(d) Silver sulphate at pH 7. 400 ml. of fuller's earth filtrate at pH 7 were shaken with 20 g. of finely ground Ag_2SO_4 for 6 hr. at room temperature. The pH was maintained at 7 by the addition of small quantities of NaOH. After standing overnight in the cold a dark brown precipitate was filtered off; silver ions were present in the filtrate. The precipitate was decomposed with H_2S and the filtrate was also treated with H_2S. The activity was present in the filtrate.

(e) Barium hydroxide in 90% ethyl alcohol. The glacial acetic acid eluate, obtained by adsorbing 600 ml. of fuller's earth filtrate with norite and eluting as described under Exp. 3 (c) (p. 118) was freed from acetic acid and taken up in 100 ml. of H_2O. 900 ml. of absolute ethyl alcohol were added and then 0.3 N $Ba(OH)_2$ very gradually, the mixture being stirred vigorously and alcohol added to maintain the alcohol concentration at 90%. After the addition of approximately 140 ml. of the $Ba(OH)_2$ solution no further precipitate appeared on addition of more $Ba(OH)_2$; the pH was 8.5. After keeping overnight in the cold the precipitate was filtered off, washed with alcohol, dissolved in water and the barium precipitated with H_2SO_4. The alcohol was removed from the filtrate in vacuo and the excess

barium precipitated. The greater part of the activity was present in the decomposed precipitate, whilst the filtrate was inactive. In another experiment, the precipitation was carried out in 80% alcohol and the activity was shared about equally between the filtrate and precipitate.

Exp. 9. Effect of heating with acetic anhydride. The alcohol-soluble portion from 400 ml. of fuller's earth filtrate was dried to a gummy residue; 5 g. of anhydrous sodium acetate and 100 g. of freshly distilled acetic anhydride were added, and the mixture was heated under anhydrous conditions for 3 hr. on a water-bath; the gum gradually dissolved. The acetic anhydride was then removed *in vacuo* on a water-bath. 150 ml. H_2O were added and the gum partially dissolved. The insoluble portion was thoroughly extracted with water and hydrolysed in the cold with 0.5 N NaOH. The water-insoluble acetyl fraction was not active, whilst the fraction which was water-soluble after treatment with acetic anhydride showed some activity, although it was difficult to test since it contained a great deal of acetate.

Later experiments on yeast filtrate factor.

Estimation of filtrate factor. After it was realised that yeast eluate factor is a dietary essential for the rat, this factor was introduced into the diet and a very much improved test for filtrate factor resulted. The method of estimation of filtrate factor finally adopted resembled that used for the estimation of eluate factor (p. 100). The diet used contained, as source of protein, either Glaxo ashless extracted casein or that casein purified by washing with salt solution (p. 38). Rats of both sexes having received the basal diet supplemented only by aneurin and cod liver oil for one week from weaning, received daily for 2 weeks 50 μg riboflavin and a dose of yeast eluate fraction equivalent to 2 g. dry yeast. By the end of the first week the animals had generally increased in weight by approximately 20 g. ; during the second week of this period, however, a very marked slackening in the growth rate occurred, and by the end of the week the growth rate was usually about 7 g. weekly, although over the whole of the second week weight increases of about 10 g. were generally obtained. Certain animals then received the added supplement of the material to be tested for filtrate factor activity. Negative control animals were given no added supplement, while

positive control animals received either the yeast fuller's earth filtrate fraction from 1 g. dry yeast or the preparation of filtrate fraction purified by extraction with amyl alcohol, equivalent to 2 g. dry yeast. The test proper lasted for 2 weeks, and the presence of filtrate factor in the test material was indicated by an immediate increase in the growth rate, unmistakable even within 2-3days. The growth rate increased to about 22 g. weekly, and this was maintained for the 2-week period (Table XVIII). The negative control animals usually continued to increase in body weight at the rate of about 7 g. weekly. Reliable results were obtained when 3 animals, exclusive of controls, were used for each test. As far as possible animals were taken from the same litter but we found little variation between the litters. A certain difference existed between the males and females and this had to be considered in planning the tests. The growth response varied with the amount of filtrate fraction given (see Table XIX). The tests in which the purer casein was used were generally more satisfactory, as the growth rate diminished more regularly during the second week of preparation when the animals were receiving the riboflavin and eluate factor supplements.

TABLE XVIII. Tests for filtrate factor.

Each rat received daily 10-15 μg. aneurin, 50 μg. riboflavin and yeast eluate fraction (= 2 g. dry yeast).

No. of rats	Sex	Av.wkly wt. increase of group for preliminary period of 2 weeks	Additional supplement given during test period	Av.wkly wt. increase of group during test period of 2 wks
		g.		g.
Unwashed casein diet:				
6	♂	22, 12	Filtrate fractions from yeast or liver	25, 26
3	♂	19, 10	None	7, 5
39	♀	20, 10	Filtrate fractions from yeast or liver	22, 19*
20	♀	18, 10	None	6, 5†
Washed casein diet:				
10	♂	18, 11	Filtrate fractions from yeast or liver	21, 19
14	♂	19, 9	None	9, 6
7	♀	19, 8	Filtrate fractions from yeast or liver	19, 16
20	♀	18, 8	None	5, 4

* The standard error of the average total weight increase for the 2-week test period ($\sigma/\sqrt{n}$) = 0.88

† The standard error of the average total weight increase for the 2-week test period ($\sigma/\sqrt{n}$) = 1.11.

TABLE XIX. Growth response of rats to graded doses of filtrate factor as contained in amyl alcohol extracts from (a) yeast filtrate fractions, and (b) liver filtrate fractions.

Each rat (female) received daily 10-15 μg. aneurin, 50 μg. riboflavin and yeast eluate fraction (= 2 g. dry yeast)

	No. of rats	Av. wkly wt. increase of group for preliminary period of 2 weeks	Daily filtrate supplement given during test period	Av. wkly wt increase of group during test period of 2 weeks
		g.		g.
(a)	4	23, 12	Equiv. of 1g. dry yeast	14, 20
	3	22, 11	Equiv. of 2g. dry yeast	19, 22
	2	23, 13	Equiv. of 6g. dry yeast	22, 18
(b)	2	17, 5	Equiv. of 3 g. fresh liver	11, 13
	2	22, 9	Equiv. of 6 g. fresh liver	23, 18

The unit of filtrate factor activity we have adopted is based on the potency of an amount of our standard filtrate fraction, purified by amyl alcohol extraction, equivalent to 2 g. dry yeast. This amount, when given daily to a rat prepared as above, produces a growth response of approximately 90% of the maximum.

Extraction of yeast filtrate factor with amyl alcohol at various hydrogen ion concentrations. The extraction of yeast filtrate factor by amyl alcohol at pH 1 was described above (p. 25). Further experiments were carried

out at pH 3 and pH 7, similar procedures being adopted. At pH 7 the amyl alcohol extracted no filtrate factor, at pH 3 only a small amount was extracted and at pH 1 nearly all the activity was extracted. (Table XX, a).

Table XX. The effect of hydrogen ion concentration on the extraction of yeast filtrate factor with amyl alcohol.

Each rat received daily 10-15 µg. aneurin, 50 µg. riboflavin and yeast eluate fraction = 2 g. dry yeast.

	No. of rats.	Av. wkly. wt. increase of group for 2 preliminary weeks g.	Amyl alcohol extract given = 2 g. dry yeast daily (S)	Av. wkly. wt. increase for 2 weeks subsequent to giving S. g.
a	2	11.5, 6	pH 7	8.5, 8.5
	2	19, 6.5	pH 3	10.5, 16
	2	22, 9	pH 1	18, 22
b	3	20, 8.7	pH 3	8.3, 12.3
	4	18.2, 11	pH 1*	21, 20

* Extraction carried out on the residue from the extraction at pH 3.

In a second experiment extraction with amyl alcohol at pH 3 was followed by extraction at pH 1; the extract at pH 3 was only slightly active and that at pH 1 was highly active (Table XX, b).

Treatment of the yeast filtrate factor, purified by amyl alcohol extraction, with lead acetate.

To 40 ml. yeast filtrate fraction purified by amyl alcohol extraction at pH 4 (1 ml. = 25 g. dry yeast) an excess of neutral lead acetate solution was added (40 g. lead acetate). The precipitated lead salt was filtered off and the filtrate adjusted to pH 8 by addition of NaOH; the resulting precipitate was again removed by filtration. The lead salt fractions were decomposed

TABLE XXI. Treatment of yeast filtrate factor with lead acetate.

Each rat received daily 10-15 μg. aneurin, 50 μg. riboflavin and yeast eluate fraction = 2 g. dry yeast.

No. of rats.	Av. wkly wt. increase of group for 2 preliminary weeks. g.	Lead fraction given = 4 g. dry yeast daily. (S)	Av. wkly wt. increase for 2 weeks subsequent to giving S. g.
2	17, 9	precipitate at pH 4	18, 21
2	17, 9.5	precipitate at pH 8	5.5, 5
2	18.5, 9	filtrate	19, 15

with H_2S and lead was removed from the filtrate by the same reagent. The filtrate factor activity was almost exactly equally divided between the decomposed first

lead precipitate and the filtrate; the second lead precipitate was almost completely inactive (Table XXI).

In the earlier experiments using the original test method it was found that most of the activity was not precipitated by lead acetate (p. 125). A further experiment was therefore carried out in which the total lead salts were precipitated at pH 8; the filtrate factor was almost equally divided between the decomposed precipitate and the filtrate. Since the test method employed in these latter experiments was superior to that previously used, it is concluded that yeast filtrate factor is partially precipitated by lead acetate.

Other experiments have been carried out. The results obtained using the original test method have been confirmed. The stability of yeast filtrate factor to treatment with 5% H_2SO_4 at 100° has been verified.

Experiments on liver filtrate factor.

The test method employed was identical with that used in the later experiments on yeast filtrate factor. The sources of liver filtrate factor were liver residue I and liver residue III(p. 28).

Extraction of filtrate factor from liver residue I with amyl alcohol at various hydrogen ion concentrations.

As in the experiments with the yeast factor, extractions

TABLE XXII. The effect of hydrogen ion concentration on the extraction of liver filtrate factor by amyl alcohol.

Eact rat received daily 10-15μg. aneurin, 50 μg. riboflavin and yeast eluate factor = 2 g. dry yeast.

	No. of rats.	Av. wkly wt. increase of group for 2 preliminary weeks. g.	Amyl alcohol extract given = 3 g. fresh liver daily. (S)	Av. wkly wt. increase for 2 weeks subsequent to giving S. g.
a	3	16.3, 9	pH 7[1]	15, 16
	3	15, 3	pH 3	16, 15
	3	16, 9.3	pH 1	20, 16.7
b	3	18, 11	pH 3[2]	20. 16.3
	3	19.3, 9.3	pH 1[2]	9.7, 10

1 Equivalent to 6 g. fresh liver daily.
2 Extraction carried out on the residue from the extraction at pH 3.

were carried out at pH 7, pH 3 and pH 1; in a second experiment extraction at pH 3 was followed by extraction at pH 1. The liver filtrate factor was

partially extracted at pH 7 and at pH 3 and pH 1 it was readily extracted (Table XXII, a); in the experiment where extraction at pH 3 was followed by extraction at pH 1 most of the filtrate factor had already been removed by the extraction at pH 3 (Table XXII, b).

Treatment of liver filtrate factor purified by amyl alcohol extraction with lead acetate. 200 ml. of the amyl alcohol extract of liver residue III (1 ml. = 250 g.

TABLE XXIII. Treatment of liver filtrate factor with basic lead acetate.

Each rat received 10-15 µg. aneurin, 50 µg. riboflavin and yeast eluate factor = 2 g. dry yeast.

No. of rats.	Av.wkly wt. increase for 2 preliminary weeks. g.	Fraction given daily = 24 g. fresh liver (S)	Av.wkly wt. increase for 2 weeks following administration of S. g.
2	16.5, 9	basic Pb.ac.ppt.	4, 7
2	14.5, 10.5	alcohol ppt.	2.5, 2
10	16.5, 9.5	filtrate	20, 18.5

fresh liver) adjusted to pH 8 was treated with an excess of hot saturated basic lead acetate solution (160 g. basic lead acetate). The resulting precipitate was filtered off and 4 volumes of 96% ethyl alcohol added; a further precipitate was thrown down and was removed by filtration. The 2 precipitates were decomposed with H_2S. Alcohol

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was removed from the filtrate in vacuo and the excess lead was precipitated with H_2S. The vitamin activity was contained in the filtrate; both decomposed lead precipitates being found to be inactive (Table XXIII).

Many other experimentshave been carried out on liver filtrate factor. In general that factor was found to possess properties similar to those of yeast filtrate factor. However, certain differences have been observed. In addition to the differences in extractability of the factors by amyl alcohol and the differences in solubility in water of the lead salts, described in the preceding pages, it has been observed that there are marked differences in solubility of the barium salts of the yeast and liver factors in aqueous alcohol, the yeast salt being insoluble in 90% alcohol and the liver salt being soluble in 90% alcohol but only sparingly soluble in 98% alcohol. The yeast factor is not destroyed by heating in presence of 5 or 10% H_2SO_4 for 2 hours at 100°, while the liver factor is completely destroyed by such treatment.

Highly active concentrates of liver filtrate factor have been prepared; those contain the rat day dose of the vitamin associated with only a fraction of a milligram

of solid matter. Isolation of the vitamin in a pure state should be achieved at an early date.

The following procedure has yielded our most active concentrates of liver filtrate factor.

1. Liver residue III (p.28) was treated with fuller's earth at pH 1.

2. The fuller's earth filtrate was extracted with amyl alcohol at pH 1, and the filtrate factor recovered from the amyl alcohol by treatment with NaOH.

3. Treatment with basic lead acetate at pH 8 precipitated inert lead salts and further inactive lead salts were thrown down by addition of 4 volumes of ethyl alcohol.

4. Mercuric acetate was then added, and an inactive precipitate was removed by filtration.

5. The filtrate, freed from mercury, lead and acetate, was treated at pH 2 with absolute alcohol and the filtrate factor activity passed into the absolute alcohol.

6. Hot saturated $Ba(OH)_2$ was added to the alcoholic solution of filtrate factor and the vitamin was precipitated as the barium salt. The final concentration of alcohol in this precipitation was 98%.

7. The barium precipitate was suspended in 80% alcohol; the active salt dissolved and a large amount of inert

material was filtered off.

8. The active barium salt fraction was freed from barium. Quinine was added and inactive crystalline quinine salts separated in large amounts. These were removed and the filtrate, freed from quinine, was found to be very potent in our rat growth tests. The rat day dose of filtrate factor in this concentrate is associated with about 0.5mg. of solids.

Discussion

The exact relationship between the filtrate factors as contained in autoclaved extracts of yeast and untreated extracts of liver is undecided. The factors have the same growth promoting properties for rats (p. 33) and recent experiments have shown that they have no supplementary action for each other in rat growth experiments; therefore, for the rat they are biologically identical. However, the factors are certainly chemically distinct, since they differ in (1) their extractability with amyl alcohol, (2) the solubility of their lead salts in water, (3) the solubility of their barium salts in aqueous alcohol and (4) their stability to H_2SO_4. The properties of the factors, nevertheless, suggest that they are chemically closely related. They behave similarly

when treated with fuller's earth, norite charcoal and other adsorbents, both are acidic in nature and neither have basic properties, and the solubilities of the acids and of their salts in various solvents although not always identical are generally very similar.

The extraction experiments with amyl alcohol suggest that the yeast factor is a stronger acid than the liver filtrate factor. Since the yeast factor was not extracted by amyl alcohol at pH 3, it probably exists as a salt at that hydrogen ion concentration, while at pH 1 it probably is present in the free state. The liver factor, on the other hand, appears to exist as the free acid at pH 3. It was thought that the yeast factor might have two acidic groups and that the liver factor might differ from the yeast in that one of these acidic groups might be esterified. However, treatment of the liver factor with 5% alcoholic KOH did not change its extractability with amyl alcohol and, therefore, this hypothesis is probably incorrect. A further possibility is that the liver factor may be a hydroxy acid and the yeast factor may be the phosphoric ester of that acid. If this is the case then (1) the phosphoric ester must be very stable to treatment with acid, and (2) the phosphoric acid group must stabilise the yeast factor to treatment with $HgSO_4$; these conditions render it

improbable that this theory will finally prove correct.

There are other theories which might be advanced but the exact relationship between the yeast and liver filtrate factors will be proved only when these have been obtained in a pure state, and their chemical constitutions have been established.

The properties of the liver filtrate factor suggest that this compound may be a hydroxy acid and may possibly be a derivative of a sugar; such compounds occur in considerable amounts in liver. The purest concentrates contain very small amounts of nitrogen and that which is present does not appear to be significant. Filtrate factor therefore may be found to differ from the other vitamin B factors in that it may contain no nitrogen in its molecule. It may be more closely related to ascorbic acid than the other dietary essentials of the vitamin B complex.

Although the rat has been most extensively used in the study of the vitamin B_2 requirements of mammals, in recent years a considerable amount of knowledge has accumulated concerning the needs of man, the dog, the monkey and the pig of the essential nutrients of the vitamin B_2 complex; we have studied the pig in this respect.

The earlier studies of the vitamin B_2 complex were generally carried out with the view to elucidate the etiology of pellagra. It was soon recognised that the rat was not a suitable animal for this study since that animal thrived and even reproduced when fed pellagra-producing diets[61,98,99,100]. It was realised that canine blacktongue was probably the dog equivalent of human pellagra[14]. Investigations from this laboratory showed that a disease with an etiology similar to that of pellagra was produced in pigs by feeding these animals on a diet composed mainly of maize[61,100]. The animals developed severe dermatitis and diarrhoea was usually observed; untreated animals died of the disease and at autopsy lesions in the large intestine were regularly found. The disease was prevented and cured by autoclaved extracts of yeast; the factor present in these extracts was adsorbed by fuller's earth and was present in our

fuller's earth eluate fraction[61]. After Elvehjem et al[47] had shown that nicotinic acid cured blacktongue in dogs we found that that compound cured our diseased pigs most dramatically (see Chick et al[50], plate I). Since we have isolated nicotinamide from our yeast eluate fraction (p. 55) there remains no doubt that nicotinic acid or a related compound is the essential nutrient deficient in the diet we fed our pigs.

Very recently we have investigated the requirements of the pig for other essential nutrients of the vitamin B_2 complex[101]. The animals received a "synthetic" diet similar to that we fed our rats (p. 15) supplemented by cod liver oil, aneurin, riboflavin and nicotinic acid. Animals receiving this diet did not thrive and became ill, but when the diet was further supplemented by our liver eluate and liver filtrate fractions (pp. 32 and 33) the pigs remained healthy and increased in bodyweight at an almost normal rate; addition of either the eluate fraction or the filtrate fractions improved the diet, but after some time the animals receiving the diet supplemented by either the eluate or filtrate fraction ceased to gain in weight and characteristic symptoms developed.

The pigs which were deprived of the eluate fraction developed a severe microcytic anaemia and became epileptic;

subsequent addition of the eluate fraction cured the anaemia and the epilepsy and restored the growth-rate. Deprivation of filtrate factor caused severe nervous symptoms. The hind quarters became paralysed and death followed. One animal recovered when given filtrate fraction and eventually became a healthy pig although certain signs of the nerve disorder remained; the remaining animals were so ill that they were unable to benefit from the filtrate fraction when it was administered. Filtrate factor deficiency also caused anaemia in pigs, the number of red blood cells and amount of haemoglobin both being reduced.

From the work of other investigators it seems probable that the pig also requires aneurin[102] and riboflavin[103]. The vitamin B needs of the pig are therefore similar to those of the rat except that the rat probably does not require nicotinic acid.

An interesting deficiency disease in monkeys which is cured by extracts of yeast and liver has been studied by Wills et al[104,105]. These animals when fed a diet of polished rice, white bread and margarine develop a macrocytic anaemia which is probably the monkey equivalent of tropical macrocytic anaemia, a disease prevalent in India and in other countries. Our yeast filtrate

fraction cured the monkey disease[105] and recently (unpublished experiments) it has been found that highly purified concentrates of our liver filtrate factor cure the disease as effectively as crude extracts of yeast or liver; the liver eluate fraction had no curative action. Filtrate factor deficiency in rats also causes an anaemia which is similar to the monkey anaemia (unpublished experiments) and deficiency of this factor also caused an anaemia in pigs[101] and therefore it appears probable, that filtrate factor is an important factor in the maintenance of normal blood. It seemed possible that filtrate factor might prove to be the extrinsic factor of the pernicious anaemia factor but this does not seem to be the case, since purified preparations of the pernicious anaemia factor do not replace filtrate factor in the diet of the rat (unpublished experiments).

Eluate factor deficiency also caused blood changes in the pig but in this case the anaemia was microcytic in character[101]; we have not, however, observed an anaemia in rats deprived of that factor. Deficiency of eluate factor (factor 1) in puppies, never-the-less, produced an anaemia similar to that we observed in pigs; crystalline factor 1 cured this anaemia of puppies.[106,107].

Our knowledge of the needs of man for factors of

the vitamin B_2 complex has been advanced considerably in the last 2 years. It is now recognised that nicotinic acid has an important therapeutic value in pellagra[52,53,54] although it seems that nicotinic acid is not the only deficiency in pellagra[108]. The important investigations of Sebrell and Butler[78] have proved that riboflavin is also a human dietary essential. It seems probable that filtrate factor will prove to be the dietary essential deficiency of which causes tropical macrocytic anaemia, since that factor cures the similar anaemia of monkeys.[105]

Among the most outstanding advances made in recent years in the field of biochemistry have been those made in vitamin research. Little more than a decade ago little was known about the vitamins and they were regarded as rather mysterious substances which somehow were essential for health. Now much of mystery has gone; several of the vitamins have been isolated in a pure state and have been synthesised in the laboratory. Vitamin D has been shown to be closely related to the sterols, the sex hormones and the carcinogenic hydrocarbons while vitamin A is related to the carotinoid pigments. Even more striking have been the discoveries concerning the B vitamins; at least 3 of these compounds probably owe their biological importance to the fact that they are

required by the organism for the synthesis of coenzymes. The pyrophosphoric ester of aneurin is cocarboxylase; riboflavin is contained in the yellow oxidation enzyme and in the molecule of alloxazine-adenine-dinucleotide, an important coenzyme which functions in several oxidising systems; cozymase and codehydrogenase II, both of which take part in certain enzymic oxidations, contain nicotinamide bound in their molecules.

The B vitamins are essential nutrients of micro-organisms. Aneurin, riboflavin, nicotinamide and vitamin B_6 have been proved to be required by various micro-organisms. Many laboratories are now engaged in the study of the nutritional needs of micro-organisms and it is possible that the results obtained may be of great importance in the final elucidation of the vitamin B_2 complex, which may be achieved in the not too distant future.

SUMMARY

1. When these experiments were commenced the vitamin B_2 complex was recognised to consist of riboflavin and a supplementary material. Investigation of extracts of yeast and liver has proved that this supplement is tripartite in nature and consists of factors named eluate factor, filtrate factor and "additional factor"; these have been separated from each other.

2. Although nicotinic acid is an essential nutrient of man, the dog, the pig and the monkey, neither nicotinic acid nor any related compound has been found to have vitamin activity for the rat.

3. Biological methods for the determination of riboflavin, eluate factor and filtrate factor are described.

4. Eluate factor was isolated from extracts of yeast and proved identical with factor 1 (Lepkovsky) and vitamin B_6 (György).

5. The chemical properties of filtrate factor as contained in extracts of yeast and liver has been investigated; purified concentrates have been prepared. The factors as present in extracts of yeast and liver are biologically identical in rat growth experiments but are chemically distinct.

6. Alloxazine-adenine-dinucleotide replaced riboflavin in the diet of the rat but had no other vitamin B_2 activity.

7. The need of the pig for factors of the vitamin B_2 complex has been studied.

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