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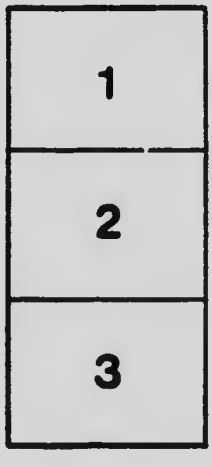

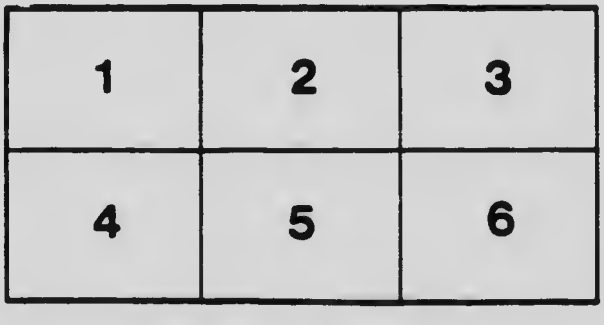

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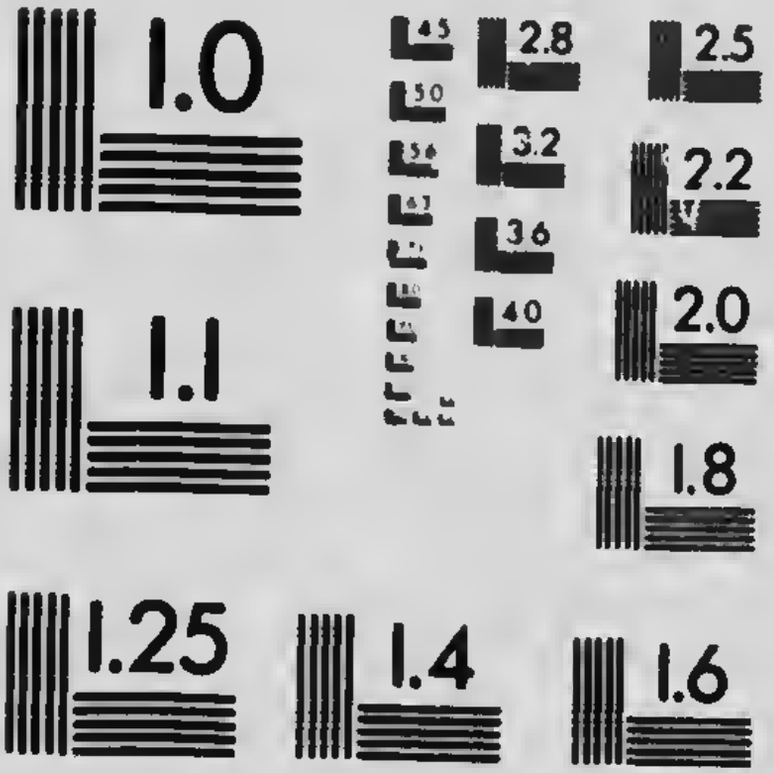

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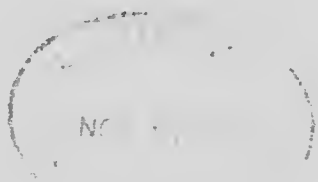
The Relation of Vitamines to the Growth of Young Animals.

BY

A. BRUCE MACALLUM.

From the Department of Pathological Chemistry
University of Toronto



From the Transactions of the Royal Canadian Institute, Toronto

THE UNIVERSITY OF TORONTO PRESS, TORONTO

1919

The Relation of Vitamines to the Growth of Young Animals.

A. BRI[illegible] LUM
From the Departme[illegible] ological Chemistry,
Univers[illegible] Toronto

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy in the University of Toronto.

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1919

THE RELATION OF VITAMINES TO THE GROWTH OF YOUNG ANIMALS

By A. BRUCE MACALLUM

From the Department of Pathological Chemistry
University of Toronto

The last decade of research in the field of animal nutrition has led to the discovery of a new class of substances in the food stuffs constituting the animal dietary. These compounds have been designated "Vitamines, Accessory Factors of the Diet, Exogenous Hormones of the Diet". They are present in infinitesimal quantities in certain articles of the diet, but their *rôle* in the metabolic cycle is one of the greatest importance. Subsequent investigation has shown that they are essential for the well-being and even the life of the organism itself. Without these indispensable elements the animal cell is unable to maintain its activities unimpaired, or the adolescent subject to attain normal growth. Continued deprivation leads to disease and ultimately to cessation of life.

The discovery of these compounds was the result of a generation's work on the etiology of two diseases—Beri-beri and Scurvy. These are now known as "Deficiency Diseases". Each of these pathological conditions is due to the dietary deficiency of a specific substance, which in the case of beri-beri is known as the "Anti-neuritic Vitamin" (Funk); "Water Soluble B substance" (McCollum). In the case of scurvy this element is called the "Antiscorbutic Substance". A third factor associated with fats of animal origin has been subsequently discovered, but its deficiency results in a general malnutrition of a chronic type complicated with Xerophthalmia.

I. Beri-beri is a disease which was formerly widespread throughout Asiatic countries where polished rice formed the staple and very often the only article of diet for long periods. It has appeared in various parts of North and South America, of late years, in isolated places where the population is forced to live on white flour and preserved meats during the winter months. The earliest observations regarding the etiology of this disease were made in the Orient where rice is put through a milling process, technically spoken of as "polishing", which removes the embryo, pericarp and outer layer of the grain which contains the aleurone granules. The finished article consists almost wholly of the

starchy part of the endosperm. The object of the polishing process is to obtain a product which can be readily cooked.

The relation of the diet to the incidence of the disease was first pointed out by Wernich (1878), Van Leent (1880), and Vordermann (1898). Their conclusions indicated that the disease is always associated with a diet of highly polished rice.

Eykmann (1897, 1) was able to induce this disease in fowls which were fed entirely on polished rice. He designated this condition "Polyneuritis Gallinarum". It could be relieved when whole rice grain was substituted for the polished article. Furthermore addition of the pericarp or polishings had a similar effect (1987, 2). For this reason, he considered it analogous to human beri-beri. The curative substance was extracted from the polishings by means of distilled water. Phytin was isolated from this extract in large quantities, but this proved ineffective when administered to fowls, in which the disease had been artificially induced. When the aqueous extract was dialysed the active substance passed into the dialysate. It remained in solution when the extract was diluted with alcohol. If the solutions were heated to 120° C. for a short time they failed to exert any beneficial influence on the course of the disease (1906).

Fraser and Stanton (1909, 1911) were able to cure the poly-neuritis of fowls by treatment with rice polishings. They were able to prevent the incidence of the disease by addition of the polishings to a diet of polished rice. The antineuritic compound could be dissolved in alcohol, was dialysable and stable to the action of acids.

Grijns (1901, 1909) repeated and confirmed Eykmann's work. He was able to demonstrate the presence of an antineuritic compound in the Katjang Idoe bean and meat. When these were heated above 120° C. their curative properties disappeared.

Breaudet (1901) used rice polishings in the treatment of human beri-beri with great success.

Hulschoff Pol (1910) found that the aqueous extract of Katjang Idoe bean possessed curative properties. He attempted to obtain a concentrated fraction of this substance. The extract was treated with lead acetate, but the filtrate, after filtering off the lead precipitate, still retained its antineuritic properties. This solution yielded a crystalline compound designated "X" acid, but no reference is given regarding its therapeutic effect.

Eykmann (1913) used yeast as an antineuritic and was able to extract the active substance with 88% alcohol. Funk (1912) was able to separate only a portion of the beri-beri vitamine from yeast by this method.

Subsequent investigations have indicated the presence of the antineuritic vitamine in a number of food stuffs such as yeast, lentils, barley, egg yolk, beef muscle, ox heart, brain and muscle, fish muscle, cheese and milk (Cooper 1913, 1914). Wheat, maize and rice germ, turbot roe, egg yolk, desiccated egg (whole), malt extract, raw beef, dried peas, pea flour, lentils, potatoes and yeast (Chick and Hume 1917, 2). Honey, nectar and corn pollen (Dutcher and Adams 1918, 2).

Attempts have been made to isolate the vitamine in a pure form by chemical means. The various fractions are tested for their activity by administering to pigeons in whom the disease has been artificially induced. The rice polishings and yeast have been first extracted with ether to remove the fats. This does not remove any of the vitamine. The material is extracted with alcohol and the alcohol removed by evaporation, yielding a syrupy residue. The antineuritic compound is probably an organic base since it withstands hydrolysis by strong mineral acid (Funk and Cooper 1911). The substance also remains active when yeast is autolysed at 35° C. for twenty-four hours (Cooper 1914, 2).

Funk (1911) hydrolysed the residue from the alcoholic extract of fat free rice polishings with 20% sulphuric acid for twenty-four hours. The diamino acid fraction was precipitated with phosphotungstic acid and the antineuritic compound was completely carried down with the precipitate. The phosphotungstates were decomposed with baryta and redissolved. The solution was concentrated to dryness and the residue dissolved in alcohol. This alcoholic solution was treated with alcoholic mercuric chloride. This yielded a voluminous precipitate which carried down part of the vitamine, while the remainder stayed behind in solution. The active substance was isolated from both these fractions, after separation from the mercuric chloride, by means of silver nitrate and baryta. This precipitate was taken, the silver removed with hydrogen sulphide, and the resulting solution concentrated in vacuo over sulphuric acid. A crystalline substance was finally obtained which was extremely powerful. Four milligrammes were sufficient to effect a cure in a case of avian polyneuritis. 1.1 grammes of this substance were obtained from 54 kilos of rice polishings. This compound gave none of the reactions characteristic of the amino acids. It was soluble in alcohol, slightly soluble in hot water and insoluble in cold water. It was not precipitated from solution by picric acid, nor did it melt below 200° C.

By the use of the above process, omitting the precipitation with mercuric chloride, a crystalline compound was obtained from the alcohol soluble fraction from dried yeast (Funk 1912, 2). This substance melted at 233° C. Similarly a crystalline compound, free from ash, was isolated from rice polishings (M.P. 231), milk (M.P. 230) and ox brain

(M.P. 203). The silver-baryta fraction from lime juice was also curative, but no crystalline compound could be prepared from it.

Since the phosphotungstic acid precipitates a large quantity of inactive material, advantage has been taken of the solubility of the phosphotungstates of some of the bases in acetone. Extraction of the phosphotungstate precipitate with this liquid dissolves a large quantity of this precipitated mass, leaving behind an insoluble residue which contains all the antineuritic compound, together with a minimal quantity of adventitious material (Funk 1914, 4).

Vedder and Williams (1913) also used a similar method to isolate the vitamine from rice polishings. They showed, however, that a silver-baryta method does not completely precipitate all the antineuritic compound. The solution, after removal of this fraction, still contains sufficient vitamine to protect a pigeon from the onset of beri-beri for a considerable time. A case of dry human beri-beri was cured by treatment with the crystalline compound isolated from the silver-bary fraction.

Funk analysed these crystalline compounds and assigned the tentative formula $C_{17}H_{20}N_2O$ to them. In view of the fact that these compounds gave insoluble phosphotungstates and salts with the silver-baryta mixture, he is inclined to believe that these compounds belong to the pyrimidine group (1912, 2).

Further investigation of these crystalline antineuritic bases obtained from yeast and rice polishings showed that they could be decomposed into several compounds. These latter had lost their antineuritic properties. Three substances were obtained from the vitamine from rice polishings and two from that prepared from yeast. In each case one of these compounds was nicotinic acid (Funk 1913, 3).

Suzuki, Shimamura and Odake (1912) extracted the rice polishings with ether, then with alcohol. The residue of the alcoholic extract was dissolved in water and treated with phosphotungstic acid. They found the vitamine in the phosphotungstic precipitate. The filtrate was negative. Decomposition of the precipitate and concentration of the precipitated bases yielded a syrupy residue which they designated "Rohoryzanin I". This was several times more active than the residue from the original alcoholic extract of the yeast. The Rohoryzanin I was dissolved in water and treated with tannic acid. This yielded a dense precipitate which, after removal of the tannic acid and concentration of the solution, gave a sticky residue which was again still more active than the Rohoryzanin I. The filtrate from the tannic acid precipitate had no curative action. The fraction precipitated by tannic acid was termed "Rohoryzanin II". This latter compound, when dissolved in

aqueous solution, gave an insoluble picrate from which a crystalline antineuritic substance was obtained. This, however, is contrary to the results published by Funk (1911).

Eadie, Moore, Simpson, Evans and Webster (1912) attempted to precipitate the active element from the alcohol soluble fraction of rice meal, katjang bean and yeast with basic and normal lead acetate without success. After removal of the lead, the filtrate was found to contain all the vitamine. It could not be isolated as a benzoyl compound, nor as an insoluble platinum salt. Hot alcoholic extracts of yeast gave inactive residues. They obtained a curative substance from yeast by treating solutions of the alcohol soluble fractions with lead acetate, filtering, removing the lead from the filtrate and treating it with silver nitrate and baryta. Decomposition of the silver salt, concentration of the solution, yielded a hygroscopic residue, insoluble in acetone and ether. This compound was extremely active, 6 mgr. being sufficient to cure a beri-beri pigeon. The crude substance was recrystallized with alcohol. The purified substance consisted of feathery crystals which, however, contained barium and phosphorus, possibly as an impurity. Analysis indicated a formula $C_7H_{17}N_2O_8$, which these authors term "Torulin".

Funk (1913, 1) using, as a working basis, his theory that the vitamine was a pyrimidine compound, investigated the antineuritic properties of a number of purines and pyrimidines. Most of these displayed mild curative powers, but were in no way comparable to that exhibited by the vitamine. Chamberlin and Vedder (1913) also showed that some purines were slightly antineuritic.

The methods used by the above authors are expensive, tedious and unwieldy. Another method has been elaborated which takes advantage of the capacity of the vitamine to adhere to adsorption media. Chamberlin and Vedder (1914) observed that filtering through animal charcoal removed part of the vitamine from the solutions which they treated in this manner. It can also be removed from autolysed yeast liquor by treatment with fuller's earth and dialysed iron (Harden and De Zilva 1918, 2). Seidell has utilized this property to prepare a stable form of the vitamine for therapeutic use. Lloyd's reagent (a hydrated aluminium silicate used by Lloyd to absorb alkaloids from solution) is the medium employed. When a mixture of autolysed yeast liquor is agitated with this compound the vitamine can be removed almost completely. Fifty grammes of the reagent mixed with the liquor, which has been rendered acid ($\frac{1}{2}$% HCl), will remove practically all the vitamine from solution. The reagent is filtered off and biological tests show that it has retained all the antineuritic properties of the original liquor.

Emmet and McKim (1917) separated the vitamine by adsorption with Lloyd's reagent and fuller's earth. Kieselguhrs do not exhibit any adsorptive capacity towards the antineuritic compound.

Sugiura (1918) dialyzed the aqueous extract of yeast against air. The gradual evaporation of the dialysate on the outer surface of the membrane deposited a colourless, powdery residue which was sometimes crystalline. Biological tests showed that this powder displayed highly active antineuritic properties.

A number of experimenters have called attention to the association of active vitamine solutions with a specific colour reaction. Suzuki (1912) mentions the indigo blue colour given by ammoniacal solutions of rohoryzanin with phosphomolybdic acid. Funk (1913, 3) discovered that vitamine solutions gave a blue colour reaction with the Folin phenol and uric acid reagents.

The depth of the colour was also a measure of the activity of the antineuritic substance. Funk and Macallum (1913) have used these reactions in the qualitative analysis of a number of purine and pyrimidine bases to obtain some clue regarding the relationship of these colour reactions to the constitution of the vitamine. This work has been carried on still further by Nicolet and Lewis (1913). Eddy (1917) has developed a method for the quantitative estimation of the vitamine in activated fuller's earth based on the colour depth given by the phenol reagent. He confirms Funk's results regarding the relation between the antineuritic activity and the intensity of colour given by the reaction.

The compounds adsorbed from autolysed yeast liquor by Lloyd's reagent can be separated and redissolved when the activated reagent is suspended in alcoholic solutions of sodium hydroxide (5%). The inert reagent is filtered off and the alcoholic solution contains the vitamines. Fractionation of these solutions showed that adenin was the principal constituent. This compound possessed no antineuritic properties. These reappeared, however, when the adenin was heated with sodium ethylate in a sealed tube. The reappearance of the activity was accompanied by the development of a positive reaction with Folin's uric acid reagent (Williams and Seidell 1916). These authors conclude that the active substance prepared by heating the inactive adenin is an isomeric form of the latter.

Voegtlin and White (1916) heated pure adenin with sodium ethylate in a sealed tube but were unable to find any antineuritic power in the final product.

Sugiura (1918) calls attention to the relation of the degree of curative power of the depth of colour given by phosphotungstic and phosphomolybdic acids with vitamine solutions.

Attempts have been made to prepare synthetical antineuritic compounds. Several of the hydroxypyridines have shown a slight curative action when freshly prepared. Their therapeutic power gradually diminishes and disappears after these compounds have been stored for several days. a-hydroxypyridine was synthesized in the form of needle-shaped crystals. These displayed curative action on polyneuritic pigeons. After standing for a few days the crystals changed to a granular form. This latter type had no antineuritic properties (Williams 1917). This author holds that these two types of crystals are isomers. He concludes that the antineuritic form of the hydroxypyridine is a pseudo betaine and considers this configuration essential for the exhibition of antineuritic activity.

Harden and De Zilva (1917) have also prepared these two crystal forms of hydroxypyridine by Williams' method. They were unable to obtain any curative action from either types of crystals.

McCollom, Simmonds and Pittz (1918) made histological examinations of the cords of pigeons in the acute stage of beri-beri. While the major portion of the cells in the cord had degenerated, they were interspersed by an occasional healthy cell. They believe that the hydroxypyridines stimulate the activity of these healthy cells and would thus account for the antineuritic power displayed by Williams' hydroxypyridine preparation.

Voegtlin, Sullivan and Meyers (1916) found that corn bread which had been baked with sodium bicarbonate as a leavening agent produced beri-beri in pigeons, when it formed their sole diet. They ascribe the destruction of the vitamine to the combined action of the heat and the carbonate produced from the bicarbonate during the baking process.

A special feature of this antineuritic vitamine is its reaction to heat and the action of alkalis. It is extremely stable to the action of acids. It will remain unaltered in mineral acid solutions (20%) even when the temperature is raised to the boiling point and maintained for twenty-four hours (Funk 1911, 1912, 2). Its activity remains unimpaired by the action concentrated hydrochloric acid at 98° C. (Steenbock 1917). Strong alkalis do not affect its curative powers at ordinary temperatures (Steenbock 1917). It can withstand the action of dilute alkalis at room temperature without loss of therapeutic power towards beri-beri, but its weight maintaining power is destroyed by this treatment (Williams and Seidell 1916). Vitamine solutions become inactive after the use of baryta and ammonia when these are used in the isolation process (Vedder and Williams 1913).

It is rendered inactive by temperatures at or above 120° C. for one to two hours' duration (Eykmann 1906, Grijns 1901, Schaumann 1911).

Partial destruction occurs at temperature ranges between one hundred and one hundred and fifteen degrees Centigrade (Shiga and Kusama 1911).

Commercial feeding stuffs (rice grain and meals from staple cereal grains) are usually deficient in vitamine content. When these are milled to such an extent that the phosphorus content is reduced below 0.5% (Fraser and Stanton 1909, 1910; Voegtlin, Sullivan and Meyers 1916; Voegtlin, Meyers and Lake 1918, 1, 3). The fat content of cereal foods is a reliable index of its vitamine content, and these two generally run parallel (Voegtlin, Meyers and Lake 1918, 3).

It has not yet been definitely determined whether the antineuritic substance is a mixture or an individual element. Emmet and McKim (1917) believed that there were two compounds, an antineuritic substance and a second which is responsible for the maintenance of weight in healthy pigeons. They find that this latter compound is not adsorbed to any appreciable extent by Lloyd's reagent. Williams and Seidell (1916) also hold that there are two elements, the antineuritic and the weight-sustaining factor. This latter (property) undergoes destruction when the vitamine has been subjected to the action of dilute alkalis at room temperature for a short interval of time.

Very little is known concerning the actual *rôle* of these anti-polyneuritic substances in the animal metabolism. Gryns (1901, 1909) believed that it was essential for the metabolism of the tissues of the nervous system. Funk (1912, 1) made nitrogen and phosphorus estimations of the brains of normal and beri-beri pigeons. In the latter case, the phosphorus was lower compared to the nitrogen, than in the case of normal birds. This indicated a loss of lipoids from the nervous system as a result of the disease. Schaumann (1911) suggests that it may act on the metabolism of the nervous system after the manner of a hormone.

There is a direct relation between the incidence of the disease and the amount of carbohydrate in the diet. Abderhalden and Lampe (1913) found that it took longer to develop the neuritis in pigeons whose diet was boiled polished rice than in the case of birds in whom the disease was induced by a diet of uncooked rice. Funk (1914, 1, 2) ascribes this result to the fact that the pigeons fed on the cooked rice, making due allowance for the water content, actually consumed a smaller amount of carbohydrate than the controls on the plain rice. He found that the disease appeared in a much shorter period when the diets contained large quantities of carbohydrates. Pigeons on a low carbohydrate diet were the last to develop the disease.

Pigeons fed on a vitamine-free diet develop a hyperglycemia while the glycogen content of the liver diminishes. Treatment of these cases

with yeast vitamine causes the hyperglycemia to diasappear and raises the glycogen content of the liver to normal levels.

The catalase content of the tissues is diminished by 50% in polyneuritic pigeons. Treatment with the antineuritic substance tended to bring the tissues to the normal condition. The vitamine does not act as a catalase activator but stimulates the production of this substance by the tissues (Dutcher and Adams 1918, 1, and Collatz 1918).

Ullman (1918, 1, 2) tested the pharmacological action of vitamine preparations. These stimulated the digestive glands, increasing the amounts of their respective secretions. It increased the tone of both smooth and striated muscle. Its action is upon the nerve centres since its effects are inhibited by atropine.

The tissues of pigeons suffering from beri-beri are never entirely deprived of their vitamine reserve. An alcoholic extract of a pigeon which died during an attack of beri-beri yielded a residue which, injected intramuscularly, effected a cure in a beri-beri pigeon (Funk 1914, 2).

II. Scurvy is now definitely accepted as a deficiency disease although its relation to the diet has been known for over half a century. The first experimental work in this subject is that of Holst and Frolich (1912). They produced a disease in guinea pigs, by confining them to a diet of grain and water, which was identical with the human type of the disease. They studied the distribution of the antiscorbutic element in the various food stuffs and their results show that it is limited to fresh fruits and vegetables. Chick and Hume (1917, 2) and Rhodes (1918) have repeated and confirmed the work of Holst and his co-workers. In addition to the food stuffs mentioned by Holst, they find that the antiscorbutic factor is also present in fresh meat and eggs. Small quantities are present in milk. Fresh yeast is deficient in antiscorbutic powers. Harden and De Zilva (1918, 5) could not obtain any antiscorbutic action with beer.

Lime juice and lemon juice have been the classical remedies used in the treatment of this disease for generations. Lemon juice is the more active of these two, while grape juice is also slightly antiscorbutic. Lime juice which had been stored several months with preservatives had become inactive (Chick and Rhodes 1918).

The antiscorbutic power of lemon juice is not associated with the organic acids which it contains. Lemon juice, freed from the organic acids, rendered slightly acid, can be evaporated to dryness. The residue contains the antiscorbutic factor in a very potent condition. The lemon juice can be stored for two weeks without injuring its activity to any important extent (Harden and De Zilva 1918, 1).

Holst and Frolich (1912) hold that the acid reaction of the lime juice is the factor which determines its stability when subjected to the action of heat and prolonged storage.

The peculiar characteristics of the antiscorbutic substance are its thermolability and its reaction to desiccation and prolonged storage. Milk (slightly antiscorbutic) displays a partial loss in its protective and curative power after being heated at 100 to 112° C. for various periods of time (10 minutes to 2 hours). The rate of depreciation varies directly as the time and the temperature range. Autoclaving milk at 120° C. for a period of one hour causes total destruction of its antiscorbutic element (Frolich 1912).

Pasteurized milk can produce scurvy in infants when it forms the sole article of diet for a lengthy period, especially when it has been stored for more than twenty-four hours after pasteurizing (Hess 1917, 1918, 3; and Fish 1914).

Cabbage and cabbage juice showed a deterioration in its antiscorbutic qualities when heated at 110 to 120° C. The cabbage was more resistant to the action of the heat than the juice. Desiccated cabbage was quite inactive (Chick and Hume 1917, 2).

Holst and his co-workers hold that desiccation is the most important factor in the destruction of the antiscorbutic properties of food stuffs. This is the case even when they are dried at quite low temperatures (37° C.) (Chick and Hume 1917, 2). Cabbage, dried at low temperatures, and subsequently boiled at ranges below 100° C. was found to be inactive (Delf and Skelton 1918).

The American experience does not bear out these claims *in toto*. Dried cabbage is partially curative, especially when dehydrated at low temperatures (37° to 45° C.) (Cohn and Mendel 1918). Desiccated vegetables display a slightly diminished activity (Hess and Unger 1918, 2). Tomatoes dried at 35° to 60° C. retain practically all their antiscorbutic properties (Givens and McCluggage 1917). Cooking, prior to desiccation, usually brings about a complete loss of curative powers (Givens and Cohn 1918).

Prolonged [illegible]ge and the action of preserving media both exert an unfavourable ac[illegible]on the antiscorbutic power of food stuffs. Cabbage desiccated and stored for several months was inactive (Holst and Frolich 1912). Pasteurized milk stored for more than twenty-four hours is no longer antiscorbutic and storage is the principal factor influencing the depreciation of this property (Hess 1913, 3). Preserved lime juice and cabbage dried at low temperatures and stored for five weeks have very weak antiscorbutic powers (Chick, Hume and Skelton 1918). Lemon

juice after two weeks storage gave evidence of slight depreciation in its activity (Harden and De Zilva 1918, 1).

Furst (1912) was the first to ascertain the fact that dried legumes, which were inactive in this state, acquired antiscorbutic powers when soaked in water and allowed to germinate. This has been confirmed by Chick and Hume (1917, 2). They hold that in the dry condition the seeds have lost the antiscorbutic power originally present, but this is recreated by the renewal of the cellular activity initiated by the germination process. In their opinion the scurvy vitamine is only associated with cells undergoing active metabolism which are rendered turgid by absorption of water. They state that all vegetables are antiscorbutic, which are stored with the cells in this turgid condition.

Various attempts have been made to isolate and identify the antiscorbutic compound, but without success. Its general properties have been ascertained from solutions and vegetable extracts, which biological tests have shown to be anti scorbutic. It is soluble in alcohol and $\frac{1}{2}\%$ acetic acid. It cannot be separated by dialysing its solutions, and is insoluble in petroleum ether (Holst and Frolich 1912). It can be extracted from food stuffs with 95% alcohol (Hess and Unger 1918, 2). It is soluble in 66% alcohol solutions (Harden and De Zilva 1918, 1).

The scurvy vitamine has an identity peculiar to itself and can be differentiated from the antineuritic and fat soluble accessories. Scurvy has been produced even when these two latter compounds have been present in the diet (Cohn and Mendel 1918). The fat soluble and the antineuritic vitamine are incapable of exerting any antiscorbutic power (Hess and Unger 1918, 2; Givens and Cohn 1918). Mixtures of autolysed yeast and lemon juice, after treatment with fuller's earth, still show undiminished antiscorbutic power, but the silicate has removed all traces of the antineuritic substance. The antiscorbutic power of lemon juice and yeast mixtures is not affected after treatment with dialysed iron and fuller's earth, but these substances quantitatively remove the antineuritic compound. The antiscorbutic substance cannot be separated from lemon juice when this is passed through a Berkfeldt filter (Harden and De Zilva 1918, 2).

The scurvy vitamine, unlike the antineuritic substance, does not exert any effect when injected into the peritoneum (Holst and Frolich 1912), or intramuscularly (Harden and De Zilva 1918, 1).

McCollum and Pitz (1917) state that scurvy is not a deficiency disease, but due to the texture of the diet which causes an impaction of faeces in the caecum of the guinea pig. They are of the opinion that the scurvy symptoms are secondary to the autointoxication following such a condition. They attained a considerable degree of success by administer-

ing laxatives, intestinal antiseptics, and compounds which rendered the faeces more pliable, to guinea pigs with experimental scurvy. Pitz (1918) obtained a number of cures after adding certain carbohydrates to the diet of the scorbutic guinea pig. This investigator contends that the carbohydrates, which relieved the scurvy, effected this change by altering the types of intestinal flora and consequently preventing the formation of autointoxication products by the putrefactive organisms.

The work of these authors has been repeated and the results do not bear out the statements of McCollum and Pitz. Roughage and texture of the diet are not connected in any way with the production of experimental scurvy (Hess 1918, 1; Cohn and Mendel 1918). Givens and McCluggage (1919) arrive at similar conclusions and believe that it is a specific deficiency disease. Harden and De Zilva (1918, 1, 3) demonstrate the falsity of McCollum's conclusions that the laxative action of citrates on the impacted intestine can account for its curative action on experimental scurvy. They obtained relief of this condition by the use of lemon juice from which they had removed all the citric acid. Their results with carbohydrates do not confirm the work of Pitz. They ascribe the curative action of the compounds used by McCollum and Pitz as antiscorbutics, to an entirely different factor. These latter investigators fed their animals on a basal diet which included as much milk as the guinea pigs were able to consume. Chick, Hume and Skelton (1918, 2) showed that at least 100 grm. of milk per day must be consumed to prevent the onset of scurvy when animals are fed on a scorbutic diet. Harden and De Zilva are of the opinion that the milk was the factor which relieved the disease in the case of the animals used by McCollom. They state that if the daily intake of milk had been measured in the case of the animals in which cures were obtained by McCollum and Pitz, that the consumption of the milk might well have exceeded the 100 grm. daily quantity, and been responsible for the successful results which the American authors reported.

The principal factor in the production of the scorbutic symptoms is referred to the degenerated condition of the capillary walls as a result of a scorbutic diet for long periods. The blood, in infantile scurvy, shows very little variation from the normal. The clotting power is very slightly diminished. The scorbutic haemorrhages are the result of diminished resistance of the capillary walls to slight increases in the blood pressure. The scorbutic vessel walls respond to such increases in pressure by permitting the blood to escape into the surrounding tissues (Hess and Fish 1914).

The major portion of the work in experimental scurvy was carried out prior to the discovery of the beri-beri vitamine and the field this

discovery opened up. The results and conclusions of Holst and his co-workers must now be regarded with some reserve. Their basal diets, which induced scurvy, were probably deficient to a certain extent in the antineuritic and fat soluble vitamine. These combined deficiencies would ultimately produce a symptom complex in which the scurvy would be complicated by other deficiency diseases. The workers at the Lister Institute have surmounted this objection by the use of a basal diet which includes the consumption of 100 grm. of milk per day by their guinea pigs. This gives a true scorbutic condition uncomplicated by degenerative changes resulting from antineuritic and fat soluble deficiencies.

III. The earliest reference to the effect of accessory factors in the growth of young animals is found in the work of Lunin (1881). He was investigating the *rôle* of inorganic salts in the metabolism. In the course of his experiments, he had occasion to use diets of desiccated milk and artificial diets similar in composition to the milk powder, as it was then known. He noted that the artificial mixtures lacked some essential compound whose presence in the milk powder enabled the animals to grow. He made no investigation regarding this compound.

Hopkins (1912) was the first to show conclusively the existence of an element indispensable for the growth of young animals, which had previously, with the above-noted exception, escaped notice. He fed rats on a basal diet of pure casein, lard, starch and a salt mixture. This in itself, while sufficient in quality and the energy requirements of these known compounds, was unable to allow young rats to grow for more than a week, or to sustain life for more than a month or five weeks. Additions of small quantities of milk (1 to 3 c.c.) to the daily ration of the basal diet resulted in a normal increment in growth comparable in all respects to that of rats fed on natural food mixtures. The solids in these quantities of milk would not account for the change which demonstrated the presence of some element in minute quantities in the milk, which had hitherto remained undiscovered.

Stepp (1909, 1912, 1912-13, 1914) published a series of papers containing results obtained by feeding mice with a mixture of natural food stuffs, which had been extracted with alcohol and ether to remove the liquids and fats. This extracted diet would not support life for more than a few weeks. When the extracted material was added to the diet, it enabled the animal to maintain life in normal growth. Stepp considered that the lipoids, removed by the extraction process, were the essential factors in maintaining the animals in normal condition.

Addition of pure lethicins and cholesterin to the extracted diet did not make good the deficiency. Neither did butter or neutral fat. He concluded that the essential factor, if a liquid, was one hitherto unknown. Milk could replace the extracted material with equally good results, but the essential compound was associated with the plasma instead of the ether soluble portion. The growth stimulating factor, present in the ether-alcohol extract, was not in evidence when the extracted material was boiled in 95% alcohol or water for two days. The essential substance cannot be a liquid since Landers (1915) has demonstrated that pure lipoids possess no growth-stimulating powers. Stepp concluded that this indispensable compound was a thermolabile lipoid, hitherto unknown, which could not be synthesized by the animal tissues. Lately (1914) he subscribes to the opinion that the growth factors are the combined action of lipoids and vitamines.

McCollum and Davis (1913) fed rats on mixtures of casein lard, carbohydrates and salt mixtures, which they assumed, were chemically pure. Rats fed on this diet grew for sixty days, and then ceased. Resumption of growth followed when 5 to 10% of the ether soluble portions of egg yolk and butter fat were added to the basal diets That the diets contained some trace of the growth accessories is indicated by the long preliminary period of growth. Hopkins (1912) used a basal diet of purified substances which enabled the rats to grow for eight days only, after the initiation of the experiment. McCollum admits that, prior to 1915, all his diets contained traces of the growth accessories which accounted for this preliminary period of growth. The commercial lactose which was used to form part of the carbohydrate in the diets contained sufficient of the growth factors to enable the animals to carry on for the first two months on these diets (McCollum and Davis 1915, 2, 3).

McCarthur and Luckett (1915) used a food mixture of purified substances, free from lipoids, but failed to obtain any growth when lard, olive oil or butter was added to these artificial diets. The ether-alcohol extracts of egg yolk when added to the basal diets enabled the animals to resume growth. The ether soluble portion of the residue of the extract (the lipoids and fats) had no effect when added to the artificial food mixture. The growth factor could be extracted with cold alcohol, but was not so efficient when the extracts were made with hot alcohol. They conclude that the antineuritic vitamine is the factor responsible for the growth of their animals.

Osborne and Mendel (1913, 1914, 3; 1915, 3) purified butter fat by melting at 45° C. and centrifuging off the watery portion and the solids, at a high rate of speed. This gave a clear yellow oil which solidified

when cool. They fed animals on an artificial diet which enabled them to grow for three months followed by a cessation and associated with xerophthalmia. In some cases they declined and died. Substitution of a portion of the lard in the food mixture with this purified butter fat corrected all these pathological features, stimulated the resumption of normal growth, cleared up the xerophthalmia and enabled the animals to attain their normal growth and expectation of life. Cod liver oil and beef fat possessed these same properties. On the other hand, almond oil and lard failed to exert any beneficial influence when added to the diets of these animals.

This growth-promoting factor in the butter fat was uninjured after bubbling live steam through the fat for two and one-half hours. They separated out the higher melting point fat from the mixture by recrystallising with alcohol. The evaporation of the alcoholic solution left behind a clear yellow oil, which contained all the growth-promoting properties of the original butter fat. This yellow oil became inactive after being stored for several months.

Steenbock (1918) found that this butter fat accessory was absent from lard. Butter which was not salted, but stored for several months, had lost its growth-promoting properties.

The fat soluble accessory can be removed from the butter fat when it has been shaken with twenty consecutive portions of warm distilled water (McCollum, Simmonds and Steenbock 1917). The washings do not exhibit any growth-inducing properties and apparently this procedure renders it inactive. Molten butter fat, agitated for two hours with distilled water, or heated at 100° C. for the same length of time, fails to stimulate growth (Steenbock, Kent and Boutwell 1918).

This accessory is also found in slight traces in large seeds such as the wheat, corn and oat grain. Small seeds, the millet, flax and hemp are fairly well supplied with this element (McCollum, Simmonds and Pitz 1917). Vegetable oils, derived from the sunflower and cotton seed, wheat grain and olive oil are deficient in growth-inducing properties (McCollum and Kennedy 1916). The leaves of alfalfa and cabbage are extremely active and this accessory is evidently prepared and stored where the metabolic activity of the plant is at its maximum. Extraction of the oils from plant tissues by expression and fat solvents leaves the fat soluble accessory behind in the plant tissues. It is present in the tissues of the plant in a combined form which is liberated by the action of the digestive juices of the animal and stored with its reserve of fat, from which it can be separated by the usual fat solvents (McCollum Simmonds and Pitz 1917).

The Soy bean contains a considerable amount of this fat soluble accessory (Osborne and Mendel 1917, 1; Daniels and Nichols 1917).

The growth-promoting property associated with fats can be isolated from animal tissues by the usual fat solvents. It has been found in the ether extracts of dried pig heart and kidney (McCollum and Davis 1915, 2) and testicle (1916, 1).

Butter substitutes which are made from beef fats or oleo oils are physiologically equivalent to butter in their growth-stimulating properties. Those prepared from vegetable oils are inadequate in this respect. Even margarines made from oleo oils vary in their fat accessory content (Osborne and Mendel 1915, 3; Drummond and Halliburton 1917; Steenbock, Kent and Boutwell 1918).

The activity of butter, stored for long periods, ultimately deteriorates (Steenbock, Kent and Boutwell 1918).

McCollum and Davis (1915, 3) and Kennedy (1916) and Simmonds and Pitz (1918) find, that in addition to this fat soluble accessory, a second factor is essential for the normal growth of animals. McCollum designates the fat accessory as the fat soluble "A", and the second factor the water soluble "B" compound. Both of these must be present in the diet to enable the animal to run a normal course of existence. This water soluble factor is soluble in alcohol, but not in ether. Food stuffs containing the "B" compound can be extracted with ether to remove the fats, and then extracted with alcohol to obtain this substance. It is practically insoluble in acetone, benzene and ethylacetate. It has been isolated by the above investigators from coagulated fat-free egg yolk, wheat embryo and milk whey. Its presence has also been demonstrated in rolled oats, potato juice, dried kidney and cabbage. It is present in small quantities in the navy bean. This factor is identical with the antineuritic vitamine, since it effects cures in cases of avian polyneuritis (McCollum, Simmonds and Pitz 1918, 1), a view confirmed by the work of Drummond (1917, 1).

This water soluble growth accessory is present in milk (Hopkins 1912; McCollum and Davis 1915, 5; Osborne and Mendel 1918, 1) and protein-free milk powder (Osborne and Mendel 1917, 1). Osborne and Mendel found that the milk was not as active as yeast. They could not duplicate the results of Hopkins (1912) with milk, while the protein-free milk powder ranked equal to yeast in its content of the water soluble vitamine (Osborne and Mendel 1918, 1).

The presence of the water soluble accessory has also been demonstrated in dried pig heart, liver, kidney and brain. Muscle tissue contains only small traces of this active compound (Osborne and Mendel 1917, 2; 1918, 1, 2). This is in accordance with the results of Cooper (1913, 1914).

who found that it was necessary to use large quantities of muscle tissue to effect cures in experimental beri-beri. Eddy has shown that alcohol extracts of pancreas are able to stimulate growth in young rats, which have been fed on a vitamine-free diet (1916).

The water soluble accessory is contained in fresh vegetable food stuffs. Osborne and Mendel have shown that cabbages, alfalfa and timothy possess small quantities of this element, while spinach is fairly active in this respect (1919).

Various attempts have been made to isolate the water soluble factor in a concentrated form. Eddy (1916) used the method by which Funk isolated the antineuritic vitamine. He used alcoholic extracts of pancreas. The precipitation with phosphotungstic acid gave a precipitate whose organic residue had a slight stimulating action on the growth of young rats fed on an accessory-free diet. Further subdivision of this residue by silver nitrate resulted in complete destruction of the growth-promoting property. The filtrate from the phosphotungstic precipitate proved negative. Lloyd's reagent adsorbed practically all the growth of sustaining element from these extracts of the pancreas.

Drummond (1917, 1) found that phosphotungstic acid carried down the water soluble accessory from yeast extracts, but its activity had undergone partial diminution. The accessory was also associated with the acetone insoluble fraction of the phosphotungstic acid precipitate, a property shared in common with the antineuritic vitamine. The dialysate from yeast was treated with silver nitrate and the resulting precipitate gave a residue which was feebly active. The filtrate was negative. Lead acetate failed to precipitate the water soluble accessory.

The isolation of the vitamines by precipitation with phosphotungstic acid and metal salts has always resulted in a gradual depreciation of the activity of the accessory factor with each successive step in the process. Vedder and Williams (1913) state that the baryta used to decompose the phosphotungstates is the principal factor in the deterioration. To avoid this, lead acetate has been substituted for baryta (Funk and Drummond 1914).

Drummond (1917, 1) states that the voluminous precipitates resulting from the separation of the precipitating reagents adsorb the major portion of the vitamine, so that the loss can be accounted for in this way.

Paccini and Russell (1918) obtained an alcoholic solution of the water soluble accessory from cultures of typhoid bacilli. These extracts give the blue reaction with Folin's uric acid reagent, while the culture media were negative to this reaction. The presumption is that the organisms can synthesize this accessory from a medium deficient in this respect.

Rosenheim (1917) extracted peat, which had been rotted by the action of aerobic soil organisms, and found that this extract increased the rate of growth of Primula seedlings as compared with control plants. 60 c.c. of the extract was sufficient to bring this about. The extract from rotted peat gave a voluminous precipitate with phosphotungstic acid. A large portion of the precipitate was insoluble in acetone. This latter fraction gave a positive reaction with the Folin phenol reagent. Extract of the unrotted peat gave only a trace of precipitate with phosphotungstic acid. The soil organisms are probably the agency responsible for the synthesis of a large portion of the material thrown down by the phosphotungstic acid. Possibly they are also responsible for the synthesis of the accessory compounds in the rotted peat, but the unrotted peat extract was not investigated as to its growth-promoting properties.

The water soluble accessory in stimulating the growth of young rats, appears to act primarily through stimulation of the appetite. Its addition or removal from the food mixture has always been followed by an increase or decrease in the food consumption. Hopkins (1912) stated that cessation of growth took place prior to the decline in the food intake and concluded that its effect on the appetite was secondary, a view supported by Osborne and Mendel (1917, 1). Drummond (1918) found that he could obtain increased food consumption of diets, deficient in this accessory, by stimulating the appetite by means of flavouring agents. The increased consumption of these deficient diets was not accompanied by any corresponding increase in growth. This demonstrated clearly that the accessory did not act through stimulation of the appetite, but created the demand for increased food consumption by its action at some point in the metabolic cycle.

In addition to the fat and water soluble accessories, Harden has demonstrated that rats cannot attain their maximum growth, unless the antiscorbutic factor is also included in the diet. His animals grew at a greater rate when fed on a diet containing the three accessories than animals whose diets contained only the fat and water soluble accessories (1918, 4).

Drummond (1918) studied the nitrogenous metabolism of rats fed on diets deficient in the water soluble factor. The only variation from the normal was a creatinuria due to the wasting of the muscle tissue when the animals were losing weight. There was also a slight fall in the body temperature. Extracts of rapidly-growing tissues such as the rat embryo and Rous sarcoma were unable to furnish sufficient supply of growth vitamine, and extracts of some of the endocrine glands were likewise inadequate.

The animal tissues are unable to synthesize these accessory factors. Young breast fed rats are unable to grow when these compounds are absent from the diet of the mother (McCollum, Simmonds and Pitz 1916, 2).

In the case of malignant tumors, which are able to grow very rapidly and attain relatively large sizes, it might be presumed that these tissues could synthesize their own accessories; especially as they are often supported by very cachectic and emaciated hosts. Fink (1914) investigated the reaction of the Rous sarcoma of chickens to the presence or absence of the growth accessories in the diet. These experiments were unsatisfactory, but the tendency was for the tumor inoculations to give a larger number of takes and grow more rapidly when the plane of vitamine intake of the hosts was at its highest level. Benedict and Rahe (1917) used rats which were on artificial diets whose vitamine content and food intake could be accurately regulated. The tumor inoculations grew more rapidly and attained much larger sizes on rats fed a diet containing an abundant supply of accessories, than in cases where the accessory content of the diet was regulated so as to provide only for the maintenance of weight of the host. In these cases the inoculation showed a lower percentage of takes and the tumors grew slowly but at the expense of the tissues of the host, upon which it depended for its vitamine supply. Drummond (1917, 2) found that tumors grew upon rats, whose accessory supply was restricted or cut off, until the reserve of vitamines in the tissues of the host were exhausted. The tumor growth reacted most readily when the water soluble factor was removed from the diet of the host. Exclusion of the fat soluble accessory from the diet of the animal permitted the tumor to grow for quite a lengthy period before its decline set in. Thus, tumor cells are similar to those of ordinary tissue both in nutritional requirements and synthetical power.

EXPERIMENTAL.

Technique of Keeping the Animals Undergoing Experiment.

The animals used in the following series of experiments were albino rats. In a few cases, grey and white and black and white animals were employed. Male rats were used in preference to females, where possible, since the former grow more rapidly and attain greater weights.

The rats take readily to the synthetic diets and can be fed such mixtures immediately after weaning. Some animals have been put on these diets when a fortnight old and 14-18 grammes in weight.

The cages used are a modification of the type used by Hopkins at Cambridge. This cage is shown in Fig. 1. It is constructed with a detachable top; and a bottom of galvanized iron wire, soldered to the

frame. The wire forming the bottom is spiralled in concentric rings from the centre to the rim, and is soldered to two cross supports at right angles to one another. The feeding devices are a pair of arms projecting laterally from the sides of the cage. This arm is made up a horizontal part with a vertical member at its extremity. There is a hinged door at the intersection of the horizontal and vertical portion for the introduction and removal of the food receptacles which are placed in the vertical portion of the arm.

As rats are inclined to scatter their food, experience has shown that this can be avoided by devices which prevent the rat turning around while feeding. These are conical-shaped tunnels which are inserted in the horizontal part of the arm from within the cage. Three sizes are provided to accommodate the smallest and largest sizes of rats capable of use. These tunnels are shown in the above-mentioned illustration.

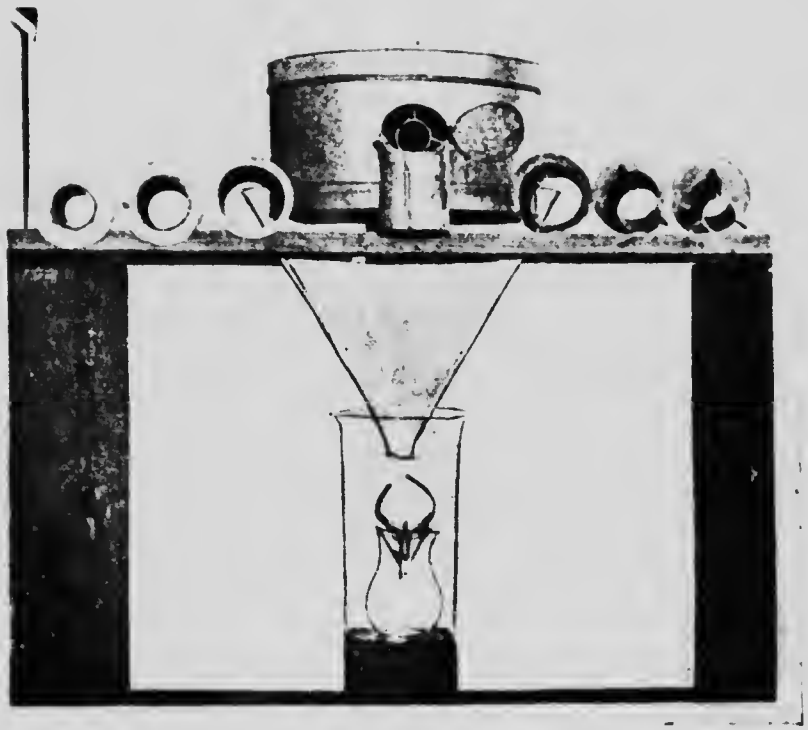
The glass apparatus below the glass funnel is used to separate the liquid excreta from the faeces and particles of food mixtures. The liquids adhere to the bulb and trickle down and drop into the small glass vessel, while the solids rebound from the glass bulb into the large beaker. This device was invented by Paine at the Cancer Hospital Research Institute, London.

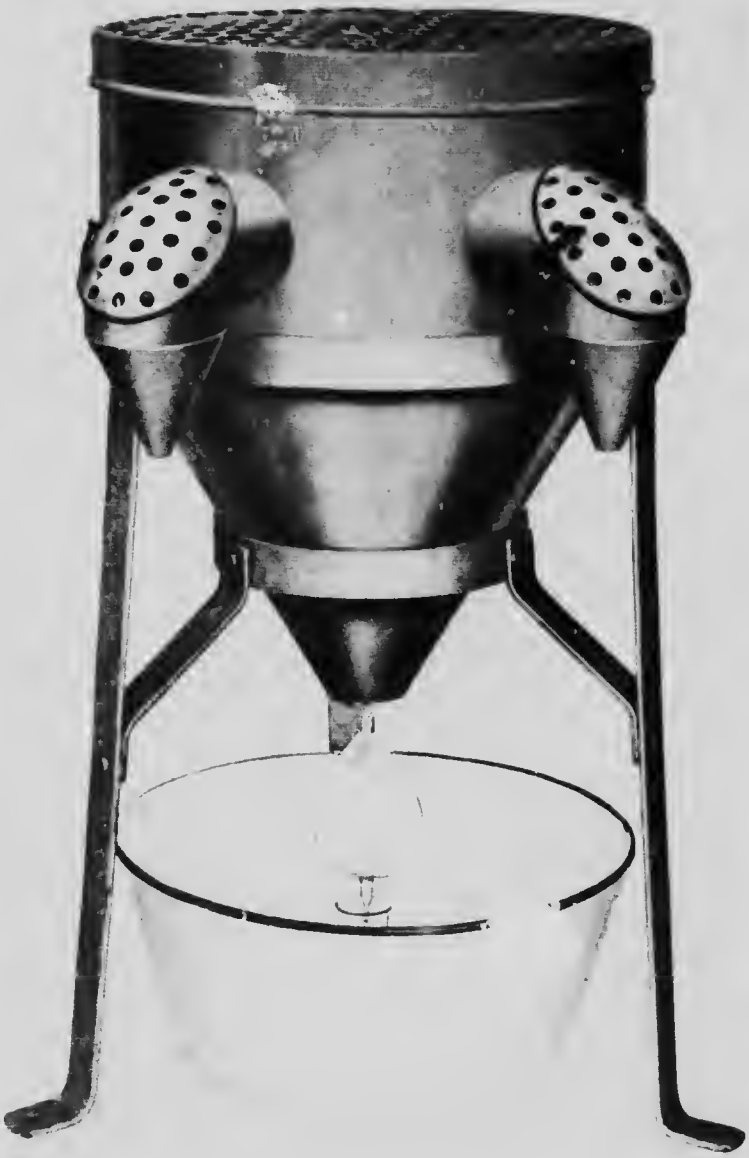
The cage, as modified by the writer, is shown in Fig. 2. The various parts of the cage, "knocked down", are illustrated in Fig. 3.

The lateral feeding arms have been placed on the same side of the cage at an angle of 35°. Spaced in this way, they can be refilled and cleaned more conveniently than in the original type. The vertical part of the arm is funnel shaped and has an orifice at the bottom. The food receptacle can be removed and held beneath the aperture of the funnel-shaped part of the arm, and the particles of the food mixture brushed from the horizontal portion of the compartment into the vertical part, caught in the food pan, and weighed back with practically no loss. This enables one attendant to look after a large number of animals with the minimum of labour and time.

The cage proper is 11"x6" (Fig. 3, 3) and is constructed of sheet zinc. A flange ½" wide runs around the bottom, projecting inwards towards the centre of the cage. This supports the wire grid which forms the bottom of the cage.

The bottom of the cage (Fig. 3, 5) is detachable and constructed of brass wire spiralled from the centre to the rim with ½" interspaces. The spiral is soldered to heavy cross supports. The whole is heavily nickle plated and highly polished, with the result that food particles and excreta do not adhere to it but drop through to the funnel.





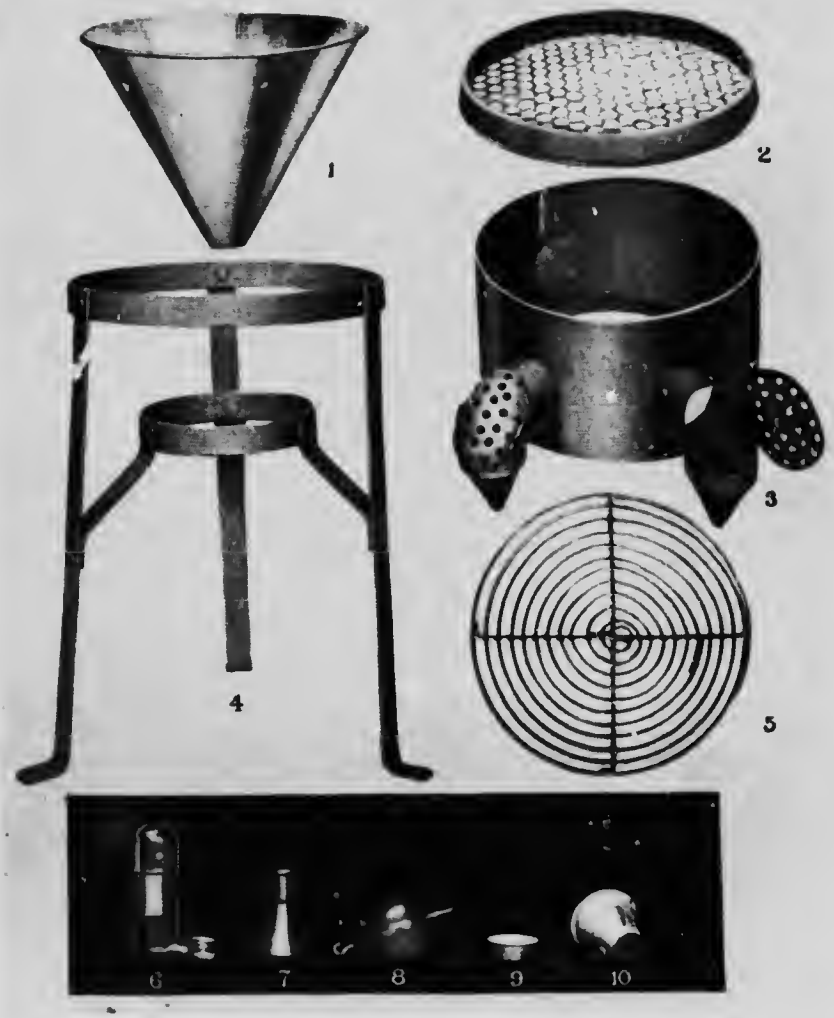


Fig. 3

The cage rests on an 11″ tin funnel (Fig. 3, 1) which is a stock article with the lower tubular portion removed. Tin was utilized in place of glass because war conditions rendered this type unobtainable.

The top of the cage (Fig. 3, 2) is a perforated zinc sheet fastened to a zinc rim ¾″ deep, which fits snugly over the top of the cage.

The funnel is supported upon a ¾″ strap iron stand (Fig. 3, 4) 15″ high, which is coated with aluminum paint.

The bulb for separating the fluid and solid excreta is shown in Fig. 3, 8. It is 2½″ x 2″ and is suspended by means of the hook to a brass wire soldered across the opening in the bottom of the funnel.

The object of the tunnels in the feeding arm is to prevent the animals scattering the diet mixtures, and contaminating them with their excreta. Experience has proven that only two sizes are necessary. One (shown in the illustration, Fig. 3, 10) used for small sizes of rats, has the aperture at the narrow end ¾″ in diameter. The size used for rats up to 250 grm. in weight has an aperture 1¼″ across.

Hopkins placed the water receptacle in one of the lateral arms. The author found that in this case (two rats were kept in one cage) a good deal of struggling resulted, since both animals strove to feed at the same time. Consequently considerable amounts of food were lost through being carried into the cage. This was avoided by utilizing both arms for food containers and providing a glass drinking fountain, held against the side of the cage by a nickle plated spring brass clip (Fig. 3, 6).

The food container (Fig. 3, 9) is made of spun aluminum. It is 1⅛″ diameter at the top and tapers down to 1″ at the bottom. It will hold up the 20 grm. of the food mixtures. Two were used for each cage. They are very light and are all approximately the same weight, so that the daily weighing out and back of the food mixtures is rapidly carried out.

In actual practice one attendant, with a supply of duplicate parts, can change the food containers and contaminated parts of the cages in a very short time. The knocked down feature of the cage renders all the parts accessible for cleaning and sterilization. This latter condition is essential for the success of this type of feeding experiment.

Rats do not as a rule scatter the diets, but are very clean feeders. In the few cases where the diets were clawed about and scattered, the animals had been on a restricted diet for some time and were not in a healthy condition. No contamination of the food or water occurred.

THE DIET MIXTURES.

The diets are made up of mixtures of a protein, fat, carbohydrate and a salt mixture. These diets furnish the energy requirements of the

organism and are sufficient, with the exception of the vitamine content, for all the needs of the animal nutrition.

These diet mixtures have been employed for some years by various investigators. Hopkins (1912) was the first to recognize the possibility of contamination by substances whose presence could not be demonstrated by the usual methods of chemical analysis. He purified his proteins and carbohydrates by extraction with alcohol and used purified fats. His salt mixtures were prepared by ashing the natural food stuffs which previous experience had shown to be fully adequate in all respects. Diet mixtures made of these purified substances did not support life for more than a few weeks when administered to rats. On the other hand, diets made from commercial preparations of these substances enabled the rats to carry on for a much longer period.

McCollum (1913) stated that his rats grew on these artificial diet for about sixty days, but later (McCollum and Davis 1915, 3) he found that his lactose preparations, presumed pure, contained enough of the growth accessories to enable the rats to grow during the earlier period of the experiment. This was the case even when the diet contained only 10% of lactose. When pure dextrin was substituted for the lactose, these diets did not bring about any increase in growth.

Drummond (1916) detected traces of nitrogenous compounds in commercial preparations of lactose. He was able to demonstrate that lactose containing these impurities had feeble growth-inducing properties.

Hopkins and Neville (1913) found that diets made from commercial preparations of these food stuffs could be rendered free of accessory substances by extraction with alcohol.

Osborne and Mendel (1912, 1, pp. 356, 361) claim to have obtained successful growth in the case of three rats fed on a diet made up of apparently pure materials. They further state that diets free from "hypothetical hormones" enable animals to attain their maximum growth increment. This is not in accordance with Hopkins' experience (1912) for he found that rats could not grow for more than a week or live longer than forty days on diets made up of materials previously extracted with alcohol.

In the major portion of their work on the growth and metabolism of rats on these artificial diet mixtures, Osborne and Mendel used a protein-free milk powder to furnish a supply of salts and lactose. This was present to the extent of 28% of their food mixtures. Subsequently (1917, 1), they have ascertained that this dried milk residue contained the water soluble growth accessory in amounts equal to those present

in yeast. This latter substance gave excellent results in stimulating growth in rats when mixed with the basal diet.

Commercial casein was used by the writer as the protein constituent in the diet mixture. It was rendered accessory-free by refluxing for six hours with boiling 95% alcohol. The hot mixture was filtered, washed with 95% alcohol and ether. It was then dried by spreading out on a table and the ether allowed to evaporate spontaneously.

Commercial lard was used as a source of fat. It was autoclaved f three hours at 120° C. to inactivate any possible trace of accessories. This procedure was omitted later on (rats 200-29) without untoward results, since lard was subsequently shown to be free of any trace of accessories (Osborne and Mendel 1915, 1; Drummond and Haliburton 1917, 1).

Sugar and commercial cornstarch formed the carbohydrate component of the food mixtures. These compounds were used as purchased, without undergoing further purification.

The salt mixture is one used by Osborne and Mendel (1913, 1, p. 317), which experience has proven suitable for the saline requirements of growing rats. Mixture IV, minus the lactose, is the preparation used by the writer.

Casein was selected as the protein, since it contains both tryptophane and lysin. Both these amino acids are essential for the maintenance of normal metabolism in animals. Henriques (1908) used a diet mixture in which the protein was represented by a mixture of amino acids obtained by hydrolizing certain proteins. When the hydrolysis had proceeded to such an extent that the amino acid mixture gave a negative tryptophane reaction, the animals failed to maintain the nitrogen balance. Hopkins and Willocks (1906) were unable to get rats to live longer than a few weeks when the protein in the diet was one which did not contain the tryptophane radicle. Addition of this particular amino acid to the food mixtures, enabled the animals to remain alive, converting the loss in weight into a balance or slight gain.

Osborne and Mendel (1912, 1; 1914, 1, 2; 1915, 2) have carried out a lengthy series of experiments on the importance of isolated amino acids in the animal metabolism. They find that tryptophane is necessary for the maintenance of weight and that the animals decline when this compound is not included in the constitution of the protein of the diet. Both tryptophane and lysin must be present in the amino acid complex of the protein of these artificial diets in order that the animal may be enabled to grow.

Hopkins and Ackroyd (1910) have shown that histidin and arginin must also form part of the protein complex of the diet, to enable the animal to grow at a normal rate.

Casein must be present in these diet mixtures to the extent of 18% in order to furnish the protein requirements of growing animals (Osborne and Mendel 1913, 1).

THE FAT SOLUBLE GROWTH FACTOR.

The investigations of Hopkins (1912), had previously shown that the usual constituents of the diet, hitherto considered sufficient for all requirements, were not alone responsible for the growth of adolescent animals. The recent work of Funk on the etiology of beri-beri had opened up a new experimental field in animal nutrition which enabled many problems to be investigated from a fresh point of view. The hypothesis assumed by the writer was that the phenomena of growth in adolescent animals were brought about by some dietary factor similar to, and possibly belonging to the same group as the antineuritic vitamine discovered by Funk. Accordingly a series of investigations was initiated on this basis, when a paper by McCollum (1913) appeared, followed shortly after by that of Osborne and Mendel (1913), in which they ascribe peculiar growth-promoting properties to butter fat. While these properties might be peculiar to this group of fats, it was also possible that the butter, being prepared from milk, might have retained by occlusion, some of the vitamines from the milk. It was considered advisable to consider this phase of the growth question before proceeding further. Accordingly the first step was to ascertain the presence of possible traces of accessories in the butter fat.

Twelve kilos of butter were melted at 45° C. and centrifuged at 6,500 revolutions per minute for forty-five minutes. The molten fat had been clarified, forming a clear yellow oil underlain by a layer of water and solids deposited by the centrifugation. The oil was pipetted off.

The aqueous layer was collected and filtered through a moist paper. Its volume was 1,300 c.c. It had an odour resembling that of cheese. Two volumes of 95% alcohol were added and a white gelatinous precipitate came down which consisted for the most part of casein. The precipitate was centrifuged off and the liquid concentrated by vacuum distillation to 200 c.c. and filtered through a moist paper. The total nitrogen of this fraction was 2.23 grm.

The butter fat was [illegible]ed in the following manner to remove possible traces of vitamines. [illegible] was melted at 45° C., 500 c.c. portions were mixed with 500 c.c. of ac[illegible]one and poured into two litres of water which contained ½% of hydrochloric acid (Kahlbaum's C.P.). This yielded a fine emulsion which was agitated for thirty minutes by a mechanical shaker. The mixture was then put in a separatory funnel and the layers allowed to separate at 37° C. The aqueous layer was drawn off

and filtered through a moist paper. This process was repeated until the whole of the fat from 25 kilos had been treated in this way.

The aqueous extracts were united and concentrated by vacuum distillation to about 150 c.c. at 37°-40° C. and filtered through a moist paper. The solution was clear orange yellow and gave off fumes of hydrochloric acid. It was made up to 200 c.c.

10 c.c. of this solution gave 0.8 c.c. N 10 sulphuric acid by the Keldjahl, while the control on the reagents required 0.2 c.c. The whole solution contained 0.0168 grms. total nitrogen.

20 c.c. of the solution were evaporated to dryness in vacuo over sulphuric acid. The residue was coloured deep brown and was hygroscopic. It was mixed with copper oxide and the nitrogen determined by the Dumas method. It yielded 1.6 c.c. of nitrogen at 19° C. and 756 mm. The whole solution contained 0.0234 grms. nitrogen.

The solution gave no colour with the Folin phenol reagent and was negative to the uric acid reagent. It gave a precipitate with the phosphotungstic acid. The quantity of the precipitate was insufficient to provide for further subdivision.

The butter fat, after the above treatment was extracted a second time. One kilo of the butter fat was refluxed with 1,500 c.c. of ½% hydrochloric acid for six hours. This process was repeated on the whole mass of fat. The aqueous solutions were separated from the fat, filtered through a moist paper and concentrated by vacuum distillation to 200 c.c. 10 c.c. of this solution gave 0.7 c.c. N 10 sulphuric acid by the Keldjahl. The blank required 0.2 c.c. The total solution contained 0.014 grms. of nitrogen.

20 c.c. were evaporated to dryness in vacuo over sulphuric acid and the nitrogen of the residue, which was brown and hygroscopic, was determined by the Dumas method. This gave 0.222 grms. nitrogen for the whole solution.

The solution gave a positive phenol and uric acid reaction. A precipitate came down when treated with phosphotungstic acid.

It is clear that the method used by Osborne and Mendel does not completely purify the butter fat of nitrogenous compounds which might possibly be traces of vitamines. Subsequently (Osborne and Wakeman 1915) have found small traces of nitrogen and phosphorus in the butter fat after centrifugation. The quantities of impurities represented by these amounts of phosphorus and nitrogen are not considered by them as being able to influence the course of growth in animals whose diets contain their purified butter fat.

The fat purified by the double extraction with ½% hydrochloric acid was used as the fat fraction in a number of feeding mixtures of casein,

sugar, starch, fat and a salt mixture. A large number of preliminary experiments showed that this mixture would not enable the animals to grow longer than a week, after which they declined in weight and died after 30-45 days subsistence on these diets. These food mixtures behaved as if they were completely free of accessories. Butter itself gave the same result, with the exception that the rats lived a few days longer on the average.

It is well to consider at this stage that both McCollum and Osborne and Mendel obtained their results from butter fat on rats who consumed a diet that enabled them to grow for two or three months before cessation set in. The addition of the butter fat enabled the animals to resume the delayed growth. Obviously the initial diets contained traces of some growth-stimulating compound. This was shown subsequently when McCollum and Davis (1915, 3) discovered that their lactose contained appreciable quantities of the water soluble accessory. Osborne and Mendel included in their dietaries a dried residue from milk (freed from proteins) to the extent of 27%. This has subsequently shown excellent growth-inducing properties (1917, 1). In both these cases the result obtained from the action of the butter fat was a summation of two factors, one present in the basal diet, and another supplied by the butter fat.

To ascertain the validity of this hypothesis the preliminary series of experiments was repeated on a number of selected rats. Yeast vitamine was supplied to a number of controls by adding 2-6% of dried brewer's yeast to the diets. A series was run, using as the fat fraction of the diets, butter fat, rectified first by the centrifugation method of Osborne and Mendel and then by the double extraction process previously described. A second series was started in which ordinary butter was used in place of the rectified butter fat, while a third series was carried out, using butter, with the addition of the dried yeast.

The diets had the following composition:

Diets	No. 1	No. 2	No. 3	No. 4
Casein	22%	22%	22%	22%
Sugar	10%	10%	10%	10%
Starch	33%	33%	31%	27%
Butter	30%		30%	30%
Butter fat (purified)		30%		
Agar	2%	2%	2%	2%
Salt Mixture	3%	3%	3%	3%
Yeast (dry)			2%	6%

The calorific value of the dietary intake and the fuel value of the faeces was ascertained by combustion determinations in an adiabatic calorimeter. The calorific value of the food indicated in the tables is the quantity actually consumed by two animals for the corresponding period.

EXPERIMENT I.—Fig. 4, Table 1. The curves represent the average weight of two rats each of which was fed on Diet 1 (butter). As represented by the upper curve, the rats showed a slight initial gain in weight and maintained for about 20 days; then a rapid decline set in with fatal termination at the 36th day. The lower curve represents the average

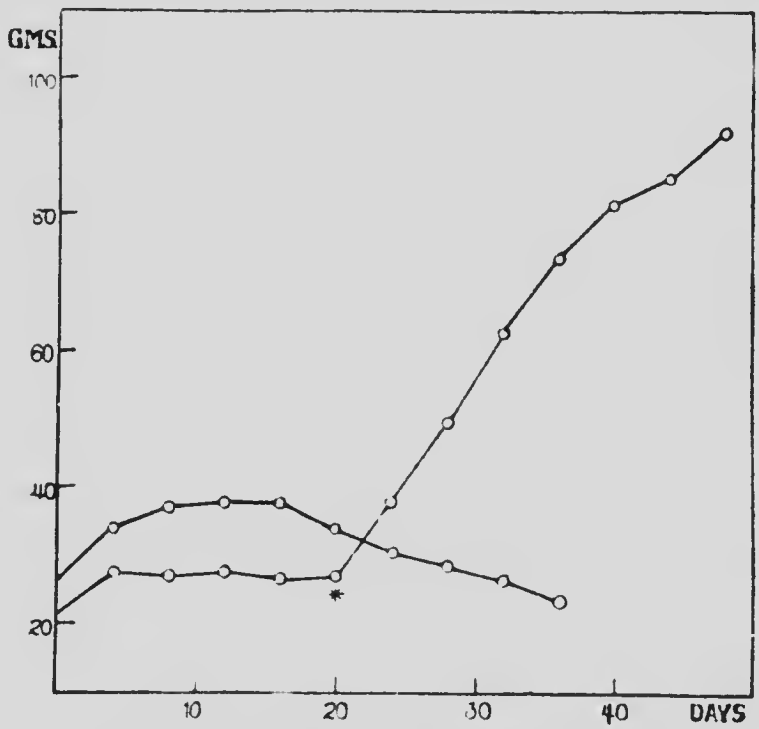


FIG. 4. Upper Curve, Rats 25 and 26. Lower Curve, Rats 27 and 28. At the point * Diet I was changed to Diet IV. Rats 25 and 26 died [illegible] days on Diet I.

weight of two rats which were changed from Diet 1 to Diet 2 on the 20th day. The effect of the addition of the dry brewer's yeast was striking. The rats suddenly recovered and grew normally up to the end of the experiment. The food intake and intestinal absorption are recorded in Table 1

TABLE I.

SEE GROWTH CURVES, FIG. 4.

Day.	Upper Curve, Rats 25 and 26. Weight. (Aver.)	Food.* (Cals.)	Faeces. (Cals.)	Lower Curve, Rats 27 and 28. Weight. (Aver.)	Food.* (Cals.)	Faeces. (Cals.)
0	26 5 grm.			21 .0 grm.		
4	32 .2	120 5	3 .67	27 .5	89 .2	3 .09
8	36 .5	95 4		26 .5	74 .7	
12	37 .5	103 .7	3 .37	27 .5	91 .7	2 .26
16	37 .0	103 .6		26 5	85 .5	
20	33 .0	125 .1	2 66	27 .0	134 .2	5 .42
24	30 .5	111 .2		38 .0	229 .5	
28	28 .5	74 .5	4 .36	49 .5	223 .4	9 .75
32	26 5	53 .2		62 5	285 .8	
36	23 .5	52 2	2 23	73 .5	308 .8	16 .69
40	Died			81 .5	286 .5	
44				85 .0	288 .5	
48				92 .0	309 .5	

*The food intake is the amount consumed by the two animals.

EXPERIMENT II.—Table 2, Fig. 5. In this experiment, purified butter fat has been used. Each curve represents the average weight of two male rats. The results are similar to those of the first experiment, only

TABLE 2.

SEE GROWTH CURVES, FIG. 5.

Day.	Upper Curve Rats 31 and 32. Weight. (Aver.)	Food.* (Cals.)	Faeces. (Cals.)	Lower Curve, Rats 29 and 30. Weight. (Aver.)	Food.* (Cals.)	Faeces. (Cals.)
0	43 .5 grm.			29 .5 grm.		
4	49 0	176 .8	8 .34	36 .5	141 .2	4 .25
8	52 .0	143 .9		39 .5	99 .9	
12	48 5	127 .8	7 .35	42 0	97 .2	3 .58
16	46 .0	129 .8		39 .0	101 .8	
20	44 .5	129 6	8 09	36 .0	96 .9	3 .43
24	42 0	131 5		31 5	96 .6	
28	42 5	109 0	3 95	48 0	229 5	8 .04
32	39 5	77 2		58 .5	290 .2	
36	36 .5	68 2		74 .5	324 .3	
40	32 .5	36 .7		86 0	285 .0	
44	Died			94 .5	304 .2	
48				103 .0	328 .8	

*The figures indicate the amount consumed by the two animals.

the maintenance period was slightly shortened. The same marked recovery was observed when the animals were changed from Diet 2 to Diet 4.

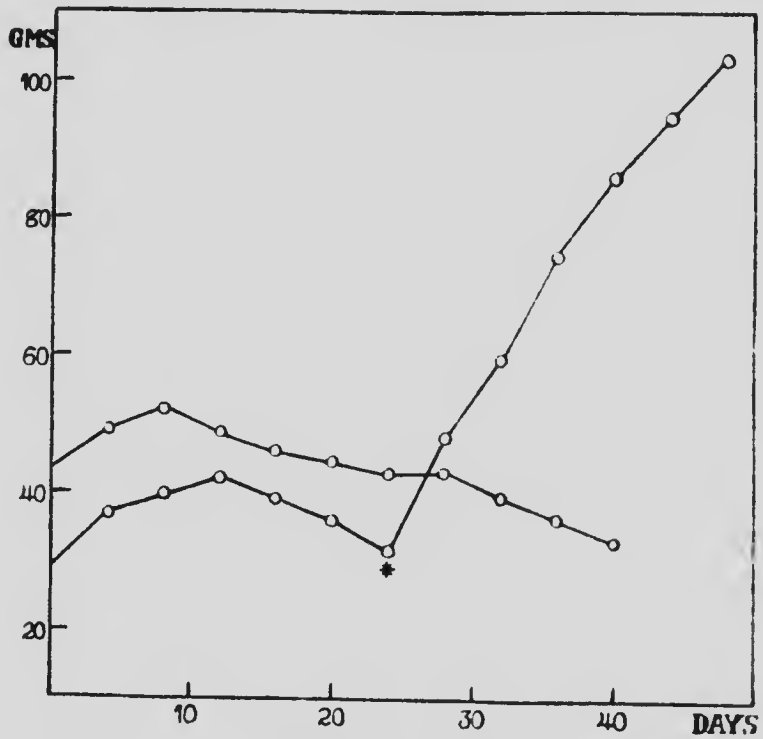


FIG. 5. Upper Curve, Rats 31 and 32. Lower Curve, Rats 29 and 30. At the point * on the lower curve, Diet II was changed to Diet IV. Rats 31 and 32 fed after 40 days on Diet II

EXPERIMENT III.—Table 3, Fig. 6. In this experiment the upper curve represents the weight of two male rats, and the lower curve that of two female rats. The first 20 days Diet 3 was used, being then replaced by Diet 4. This was continued in one set to the end of the experiment (lower curve). In the other experiment Diet 4 was replaced by Diet 2 (without yeast) after 32 days; the growth ceased abruptly and the animals began to decline. No marked difference was noticed between diets containing 2 and 6 per cent. of yeast.

It is obvious, from a consideration of the curves and tables, that the purified butter fat and even the butter itself had no influence on the growth of the animals. While the ordinary butter showed a slight superiority over the purified butter fat, in that it enabled the animals

TABLE 3.—SEE GROWTH CURVES, FIG. 6.

Day.	Upper Curve, Rats 33 and 34. Weight. (Aver.)	Food.* (Cals.)	Faeces. (Cals.)	Lower Curve, Rats 35 and 36. Weight. (Aver.)	Food.* (Cals.)	Faeces. (Cals.)
0	20.0 grm.			18.5 grm.		
4	29.0	151.2	5.31	27.5	136.2	3.41
8	35.0	117.3		32.0	97.9	
12	37.0	132.4	7.30	37.0	137.1	4.39
16	41.5	137.2		39.5	148.1	
20	43.5	161.5	6.45	42.5	178.7	7.44
24	47.0	217.5		49.5	236.8	
28	59.0	233.5	8.06	58.0	230.9	11.13
32	69.0	262.5		69.5	259.5	
36	72.5	225.5	9.53	80.0	323.5	13.68
40	74.5	187.7		87.5	302.2	
44	71.0	169.6		90.0	276.5	
48	68.5	180.9		94.0	310.4	

*The figures indicate the amount consumed by the two animals.

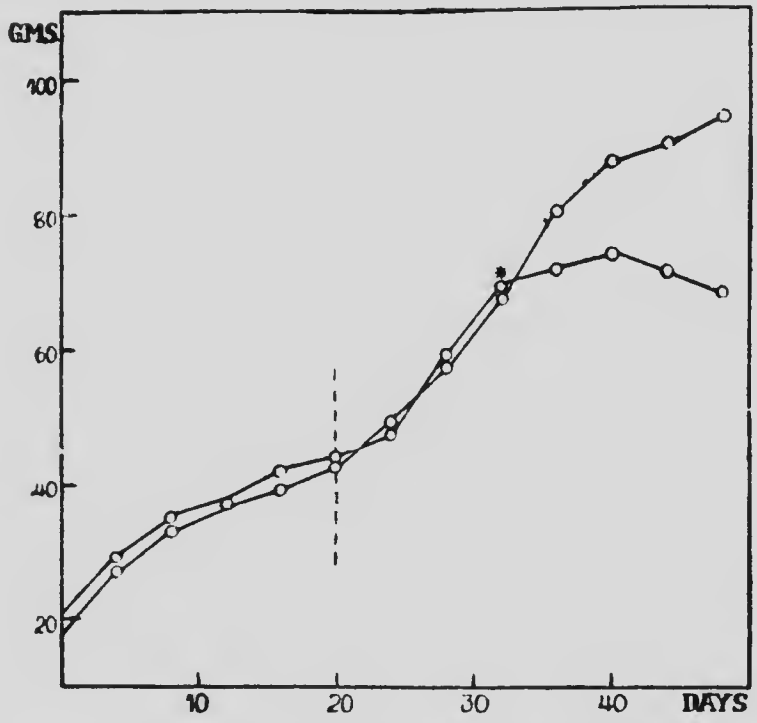


FIG. 6. Upper Curve, Rats 33 and 34. Lower Curve, Rats 35 and 36. To the left of the dotted line Diet III; to the right, Diet IV. At the point * Rats 33 and 34 were changed from Diet IV to Diet II.

to maintain the initial gain in weight for a slightly longer period, yet the death of the animals ensued at practically the same time in both cases.

Consequently, butter fat and butter itself cannot contain an accessory factor, for which, by itself, can enable the animal to attain its normal growth. On the other hand, the animals reacted most readily to the addition or removal of the yeast from the food mixtures. Since yeast is an excellent antineuritic, it may be possible that the antipolyneuritic vitamine is responsible for these remarkable effects on the growth curves. This assumption has subsequently been confirmed by McCollum, Simmonds and Pitz (1918, .) and Drummond (1917, 1).

THE RELATIVE VALUE OF LARD AND BUTTER FAT IN THE NUTRITION OF GROWING ANIMALS.

There was a possibility that in the preceding experiments regarding the growth-promoting power of butter fat, the duration of the experiment (48 days) might not be of sufficient length to permit the special features attributed to the purified butter fat to exert any marked difference. Accordingly a series of experiments was initiated in which diets, similar to those in the preceding set, were used with the exception that the fat was supplied in the form of pure lard. This had been autoclaved at 120° C. for three hours in order to eliminate the activity of the vitamines that might possibly be present in lard. Dried yeast was supplied to furnish its quota of the growth accessories, whose presence had been demonstrated in this compound by the previous experiments.

These animals grew rapidly for about 48 to 52 days, but then ceased to make any further gain in weight. They were able to maintain this gain for a period of two to three weeks, then declined, and ultimately died. Increasing the initial amount of yeast in the diet did not enable the rats to resume their growth. Xerophthalmia made its appearance coincident with the cessation of growth. Treatment with zinc sulphate solutions held this eye condition in abeyance for a time, but this ultimately brought about the fatal termination of all the animals in this experiment.

The food intake maintained a fair average and, contrary to the case of rats declining as a result of a deficient diet, did not immediately show any diminution in food consumption sufficient to account for the cessation of growth and ultimate death of the animals. Marked diminution in the food intake occurred shortly before the death of the animals.

EXPERIMENT IV.—Table 4. Eight male rats were used, all in the neighbourhood of 20 grms. in weight. Rats 42 and 48 died in the early stages of the experiment as a result of asphyxiation by food particles.

Rats 41 grew from 20 to 89 grms. in 64 days and died on the 70th day, after a slight decline in weight. Rats 43 and 44 grew rapidly for 48 days, then declined slowly for 20 days and died between the 64th and 68th day. Rats 45 and 46 ran approximately the same course. Rat 47 was the hardiest specimen in the lot, since he grew for 60 days, attaining a weight of 110 grms. This animal was put on Diet 2 on the 80th day

TABLE 4.

Diet.	No. 1.	No. 2
Casein	22%	22%
Starch	30%	27%
Sugar	10%	10%
Lard	30%	30%
Salt	3%	3%
Agar	2%	2%
Yeast (dried)	3%	6%

All male rats. Rats 41-46 and 48, Diet No. 1 throughout. Rat No. 47, 0-79th day, Diet No. 1; 80-112th day, Diet No. 2.

Day.	Weight.		Average Daily* Food Intake. (Calories.)	Weight.		Average Daily* Food Intake. (Calories.)
	41	42		43	44	
0	20.0 grm.	20.0 grm.		22.0 grm.	23.0 grm.	
4	27.5	26.0	39.3	28.0	27.5	41.8
8	33.5	30.5	38.5	31.0	33.5	36.8
12	36.5	31.5	34.5	35.0	37.0	40.4
16	37.5	Dead	22.0	40	37.0	39.3
20	41.5		24.8	45.0	43.0	44.1
24	47.0		27.0	47.0	48.0	44.5
28	47.0		22.0	52.0	51.0	46.3
32	56.0		25.2	58.0	60.0	52.4
36	62.0		29.3	64.0	62.0	50.2
40	72.0		28.1	70.0	70.0	52.1
44	76.0		26.8	74.0	75.0	49.7
48	79.0		29.2	78.0	78.0	51.7
52	79.0		28.3	75.0	79.0	46.8
56	85.0		31.6	77.0	78.0	39.2
60	88.0		28.3	76.0	70.0	38.9
64	89.0		22.5	67.0	65.0	15.5
68	84.0		24.8	Dead	Dead	
	Dead 70th Day					

*The figures indicate the amount consumed by the two animals.

Day.	Weight.		Average Daily Food Intake (Calories)	Weight.		Average Daily Food Intake (Calories)
	45	46		47	48	
0	20.0 grm.	18.0 grm.		28.0 grm.	22.0 grm.	
4	26.5	25.5	38.3	36.0	26.0	36.0
8	31.0	27.0	34.1	48.0	28.0	45.5
12	34.0	33.0	37.6	54.0	32.0	45.2
16	38.0	36.0	38.4	57.0	Dead	39.4
20	40.0	37.0	43.1	63.0	18th	28.6
24	46.5	43.0	41.8	65.0	Day	25.3
28	50.0	48.0	42.7	73.0		30.1
32	51.0	52.0	43.5	78.0		28.6
36	55.0	58.0	45.4	83.0		40.6
40	60.0	66.0	45.3	90.0		32.7
44	61.0	65.0	42.4	95.0		31.5
48	59.0	69.0	40.6	102.0		34.1
52	64.0	72.0	40.3	104.0		35.7
56	65.0	70.0	39.8	106.0		35.2
60	65.0	73.0	36.6	110.0		31.0
64	64.0	70.0	40.7	110.0		32.5
68	Dead	Dead		110.0		37.6
72	60th Day			107.0		37.7
76				105.0		
80				103.0		
84				101.0		
88				102.0		
92				103.0		
96				100.0		
100				90.0		
104				80.0		
108				80		
112				72.0		

in the hope of stimulating further growth, since his diet contained double the original quantity of dried yeast. This conferred no benefit since the animal gradually declined in weight until the 112th day when the experiment was stopped. In all cases death was brought about by an extensive purulent panophthalmitis and undoubtedly the fatal termination was a result of the toxaemia consequent from the eye trouble.

It was evident that in the case of these animals, there was a deficiency in some factor of the diet mixtures which might account for these untoward results.

It is possible that:

(1) The yeast, whose age is uncertain, may not be sufficient in respect to its accessory content to support the animals beyond the initial ninety days of their life.

(2) The quantity of salts may not be sufficient to provide for the skeletal growth after the animal has reached a certain weight.

(3) Some special feature of the butter fat might be necessary to rectify a deficiency in the lard.

Accordingly, experiments were initiated, in which these factors were varied and their effect on the growth curve noted. After a short period the salt content of the diet was doubled without any corresponding benefit. The eye symptoms appeared in the animals of this series after sixty days and would have undoubtedly resulted in the death of the animals. At this point fresh, moist brewer's yeast was substituted for the dried article. This immediately eliminated the eye symptoms and enabled the animals to grow at a normal rate for the balance of the experiment. In one set purified butter fat was used in place of lard, but these animals also required the fresh yeast preparation to keep them in a normal condition. They were somewhat less susceptible to attacks of the eye trouble and were in a slightly better condition than the animals whose diet contained lard.

The moist yeast was prepared by taking the brewer's yeast fresh from the vats and centrifuging off as much as possible of the fluid portion. The yeast cake was then pressed to a firm cake in a screw press. The total nitrogen of the pressed cake was determined and an amount added to the diet mixtures equivalent to the quantity of dried yeast previously used, based on its total nitrogen content.

EXPERIMENT V.—Tables 5 and 6, Fig. 7. Rats 49-54 were placed on the diets whose composition is given at the head of the appended tables.

Rats 49 and 50 (Table 5) were placed on diets containing 6% of dried yeast for the first 68 days. At this point the eye symptoms made their appearance. They were then placed on a diet containing moist brewer's yeast in amounts equal to 1% of the dried article as determined by the nitrogen content. The rats at once reacted to the change in diet and maintained their usual rate of growth. About the 98th day the growth curve began to flatten and the yeast content of the diet was increased to the equivalent of 3% of dry yeast. This did not increase the rate of growth, since the animals showed no marked additional gain. At the 68th day the salt content was increased from 3 to 6% but no extra advantage was obtained.

Rats 51 and 52 (Table 5) were put on a diet containing 3% of dried yeast for the first 68 days. They grew at the same rate as 49 and 50

and the eye symptoms appeared at about the same time in both cases. On the 69th day, the fresh yeast was included in the diets with very successful results, the animals running the same course as 49 to 50. Increasing the quantity of moist yeast on the 99th day did not confer any additional benefit, as shown by the curves. On the 53rd day the salt mixture in the diets was raised to 6%, but no special effect could be noted as a consequence.

Rats 53 and 54 (Table 6). In the case of these animals the diets contained purified butter fat (Osborne and Mendel). The first 52 days the diet mixtures contained 3% of dried yeast, from the 53rd to the 68th day the yeast formed 6% of the total intake. At this point there was a slight tendency for the animals to follow the same course and display the same symptoms observed in the four preceding animals. From the 69th day to the 100th day the diets contained moist brewer's yeast, equal to 1% of the dried article. At the 101st day the fresh yeast content was raised to the equivalent of 3% dry yeast and this diet was continued to the end of the experiment. After the 69th day the salt ration was raised from 3 to 6% of the total food intake.

In all cases the use of dried yeast brought the animals to the point where further administration would have brought about a fatal termination. The use of the moist brewer's yeast, in place of the dried article, immediately relieved the eye troubles and enabled the animals to proceed at a normal rate for the duration of the experiment. Increasing the amount of moist yeast for the final 50 days did not confer any additional benefit as far as could be seen in the appearance or growth of the animals. The increase of the salt content probably did not have any effect in increasing the growth rate, since in the later periods of this research grown animals were able to maintain themselves on diets which contained 3% of the salt mixture.

In the case of the two animals whose diet contained butter fat there was a slight improvement in the appearance and behaviour of the animals in their capacity to resist the eye infection. Slight attacks of the latter trouble made their appearance but were easily controlled by the application of dilute zinc sulphate solutions. These animals, being females, naturally did not grow at the same rate or attain the same size as the other animals of its series. In any case no marked advantage conferred by the action of the butter fat was noticeable from a comparison of the growth curves during the experimental period of 150 days.

The most important feature of this series is the marked improvement resulting from the use of moist yeast. It is apparent that the prime factor, responsible for the failure of the animals in experiment IV. was the dried yeast. The drying process has evidently resulted in a partial

TABLE 5.

Diet	No. 1	No. 2	No. 3	No. 4	No. 5
Casein	22%	22%	22%	22%	22%
Sugar	10%	16%	10%	10%	10%
Starch	30%	27%	27%	29%	27%
Lard	30%	30%	30%	30%	30%
Salt	3%	3%	6%	6%	6%
Agar	2%	2%	2%	2%	2%
Yeast (dry)	3%	6%	3%		
Yeast (moist) equivalent to				1% of Dry Yeast	3%

Rats 49 and 50, males, 0-68th day, Diet No. 2; 69-98th day, Diet No. 4; 99-150th day, Diet No. 5. Rats 51 and 52, males, 0-52nd day, Diet No. 1; 52-68th day, Diet No. 3; 69-98th day, Diet No. 4; 99-150th day, Diet No. 5.

Day.	Weight.		Average Daily Food Intake. (Calories	Weight.		Average Daily Food Intake. (Calories.)
	49	50		51	52	
0	44 grm.	46 grm.		41 grm.	46 grm.	
4	55	58	77.4	62	55	78.2
8	81	85	92.4	72	65	81.3
12	96	100	103.2	91	82	67.2
16	104	110	105.7	102	91	97.1
20	119	124	110.3	110	100	102.2
24	132	137	110.5	119	110	106.8
28	146	150	110.0	132	120	100.3
32	150	158	110.0	138	128	100.8
36	162	163	109.3	148	138	112.2
40	170	174	114.6	159	147	116.2
44	173	176	114.3	160	150	115.2
48	177	180	111.1	163	150	117.5
52	178	184	95.4	170	158	108.6
56	182	188	110.8	176	161	120.3
60	185	191	103.8	178	163	106.6
64	186	176	78.7	175	164	96.0
68	184	183	90.0	192	167	120.7
78	199	200	115.9			116.3
84	201	201	98.5	206	190	108.5
100	207	205	85.0	209	192	91.0
120	200	205	105.7	219	199	103.7
140	193	201	76.3	216	186	85.4
150	192	203	75.8	221	199	67.4

* The figures indicate the amount consumed by the two animals

TABLE 6.

Diet.	No. 1	No. 2	No. 3	No. 4
Casein	22%	22%	22%	22%
Sugar	10%	10%	10%	10%
Starch	30%	27%	20%	27%
Lard	12%	12%	12%	12%
Butter Fat (Osborne and Mendel)	18%	18%	18%	18%
Salt	3%	3%	6%	6%
Agar	2%	2%	2%	2%
Yeast (dry)	3%	6%		
Yeast (moist) equivalent to			1%	3%
			of Dry Yeast	

Rats 53 and 54, females, 0-52nd day, Diet No. 1; 53-68th day, Diet No. 2; 69-100th day, Diet No. 3; 101-150th day, Diet No. 4.

Day.	Weight.		Average Daily Food* Intake. (Calories.)
	53	54	
0	43 grm.	41 grm.	
4	54	55	74.3
8	64	63	66.2
12	78	75	72.0
16	80	80	77.0
20	85	87	73.5
24	90	92	76.8
28	96	95	81.1
32	100	101	79.2
36	106	106	88.9
40	112	112	87.3
48	112	112	87.2
56	119	116	83.7
64	123	126	95.2
84	138	135	79.8
100	144	146	76.5
120	149	147	79.2
150	47	153	92.8

*The figures indicate the amount consumed by the two animals

deterioration of its growth-promoting properties, so that it cannot support the growing animals for a longer period than 50-60 days. It was noted that in all cases where moist yeast was employed there was after a time a diminution in the food intake and a corrresponding slacken-

ing in the growth rate. When a fresh diet was made up from a freshly-prepared yeast preparation the consumption at once increased, followed by a slight increment in the rate of growth

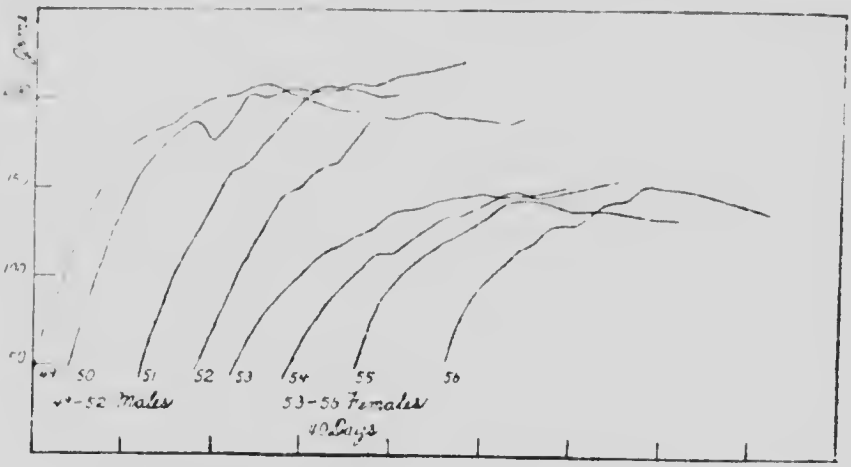


FIG. 7. Rats 50-52 Diet mixtures contain lard
Rats 53-54 Diet mixtures contain butter fat
Rats 55-56. Diet mixtures contain lard. Casein has been purified by McCollum's method.

SEPARATION OF THE GROWTH-PROMOTING ELEMENT FROM THE YEAST.

The close relationship between avian polyneuritis and the phenomena of growth in adolescent animals is indicated by the results obtained by the curative action of yeast in cases of beri-beri, in the first place, and the action of this article in stimulating the growth of young animals as instanced by the results of the foregoing experiments. This suggests the possibility of separating out the growth-promoting element from the yeast by methods which have been successful in the preparation of the antineuritic vitamine.

Phosphotungstic acid was used as the precipitating medium for the first attempt to isolate the accessory growth factors. This compound or group of compounds was carried down with the diamino acid fraction which was precipitated by the use of this reagent. The filtrate after removal of the excess phosphotungstic acid gave evidence of possessing infinitesimal traces of the growth element, since the animals maintained the initial gain in growth which results from the reserve of the accessories stored in the tissues prior to the experiment.

Attempts to subdivide the residue precipitated by the phosphotungstic acid by means of silver nitrate and baryta solutions did not

give successful results. The treatment with phosphotungstic acid and the manipulations incidental to the removal of this reagent and solution of the residue had effected a partial destruction of the activity of the growth element. This partial loss was rendered almost complete by further treatment with the silver salts.

The yeast was prepared by hydrolysing moist brewer's yeast with 10% sulphuric acid for 6 hours at 90-95° C. The mixture was filtered and an equal volume of water added to the filtrate. A saturated solution of phosphotungstic acid was added until no further precipitate resulted. After 24 hours the precipitate was filtered on the pump and well washed with 5% sulphuric acid.

The precipitate was suspended in water and decomposed in the ordinary way with baryta and the barium phosphotungstate filtered off. The solution, after removal of the slight excess of baryta with dilute sulphuric acid, was neutralized with a dilute solution of sodium bicarbonate. It was then concentrated by vacuum distillation and a sticky hygroscopic mass was obtained. This was standardized by determining the total nitrogen and adding such quantities to the diet mixtures as were equivalent to the stated quantities of dried yeast which was also standardized by this method.

The filtrate from the phosphotungstic precipitate was freed from the phosphotungstic acid by precipitating as the barium salt, and the slight excess of the latter eliminated with dilute sulphuric acid and the solution likewise neutralized with bicarbonate solution. The final solution was concentrated by vacuum distillation and standardized in the same manner as the diamino acid fraction. This residue from the filtrate was brown and hygroscopic and was added to the basal diet mixtures to test its physiological activity.

EXPERIMENT I.—Table 7, Fig. 8. Rats 80 to 83 were placed on diets containing the residue precipitated by phosphotungstic acid. These animals grew slowly and doubled their original weight in 36 days. The method used in the preparation of this residue has caused a loss of approximately one-third of the activity of the growth substances as compared with those of the original yeast. It has been the writer's experience that rats of this age double their weight in twenty-one to twenty-six days when fed on diets containing fresh yeast preparations.

Rats 85 and 84 were supplied with diets containing the residue from the phosphotungstic acid filtrate. They made a slight initial gain of about 40% of their original weight in twenty-eight days and at this point were placed on a diet containing the precipitated residue, and a striking increase in growth resulted. In the following 14 days they attained an increment just double their original weight.

This series shows that the phosphotungstic acid precipitates the major portion of the growth-promoting elements along with the diamino acids. The filtrate still contains a sufficient amount of the accessory factors to enable the rat to maintain its initial gain in weight. The treatment incidental to the isolation of this element lowers its activity by about a third.

The appearance of the animals was even more striking than the difference exhibited by the growth curves. The animals which were fed on the diets containing the residue from the phosphotungstic precipitate were sleek, lively and in every way like healthy individuals. On the contrary, those on the diet containing the filtrate residue had all the appearances of animals suffering from a dietary deficiency. Their fur was coarse and matted and the animals took little interest in their surroundings. The change from the deficient to the adequate diet was marked by the disappearance of these features and the rats eventually assumed a normal appearance.

In the same issue of the journal in which these results were published, Eddy (1916) communicated a paper which contains an account of his attempts to isolate the growth-promoting factor from the alcohol soluble residue of the pancreas. He finds that it is thrown down by phosphotungstic acid and similarly that further subdivision of the precipitated residue results in the loss of the physiological activity of the accessory growth factors.

Owing to the difficulty of obtaining a concentrated form of the growth factor by the use of reagents and metal salts by which the natural organic bases are usually prepared, an alternative method was utilized. Seidell (1916, 1917) has utilized the adsorptive capacity of silicates towards the antineuritic vitamine as a means of separating an active fraction from autolysed yeast liquors. The silicate with the adsorbed material was fed to pigeons suffering from experimental beriberi and was followed by prompt relief.

Funk (1916) also effected a separation of the growth-promoting element in autolysed yeast through adsorption by means of Lloyd's reagent. The rats fed on the diets containing the unadsorbed residue were able to make slight gains and maintain them for a considerable period. He noted that the activated silicate was not so efficient as the original yeast liquor.

Eddy (1916) used this method to obtain the growth factor from the alcohol soluble residue of the pancreas. He used the method outlined by Seidell for the preparation of the yeast vitamine. The addition or removal of the activated silicate from the pure basal diets of the experimental animals was accompanied by corresponding increases or

TABLE 7.

Diet.	No. 1	No. 2
Casein	22%	22%
Sugar	10%	10%
Starch	24%	24%
Fat (Lard)	30%	30%
Salt	6%	6%
Agar	2%	2%
Residue Phosphotungstic Acid Precipitate equivalent to		6% of Dry Yeast
Residue Phosphotungstic Acid Filtrate equivalent to	6% of Dry Yeast	

Rats 80, 81, 82, males. Rat 83, female, 0-36 days, Diet No. 1.

Day.	Weight.		Average Daily* Food Intake. (Calories.)	Weight.		Average Daily* Food Intake. (Calories.)
	80	81		82	83	
0	24 grm.	25 grm.		20 grm.	30 grm.	
4	31	33	29.8	23.5	37	27.8
8	36	40.5	33.5	28	43	32.8
12	36	43	35.7	29	45	33.3
16	38	44.5	40.5	29	48	40.5
20	40	48	28.6	31	51	38.3
24	42	49	30.1	34	54	30.2
28	44.5	52	27.8	38	57	35.0
32	46	54	28.7	40	59	27.4
36	49	56	25.0	42.5	61	20.8

Rat 84, female. Rat 85, male, 0-28 days, Diet No. 2; 28-39 days, Diet No. 1.

Day.	Weight.		Average Daily* Food Intake. (Calories.)
	84	85	
0	45	45	
4	48	49	28.6
8	53	54.5	46.3
12	55	55	42.9
16	55	55	35.9
20	57	57	50.9
24	58	58	48.6
28	59	61	47.9
32	69	68	54.5
36	84	82	70.3
39	89	90	66.3

*The figures indicate the amount consumed by the two animals.

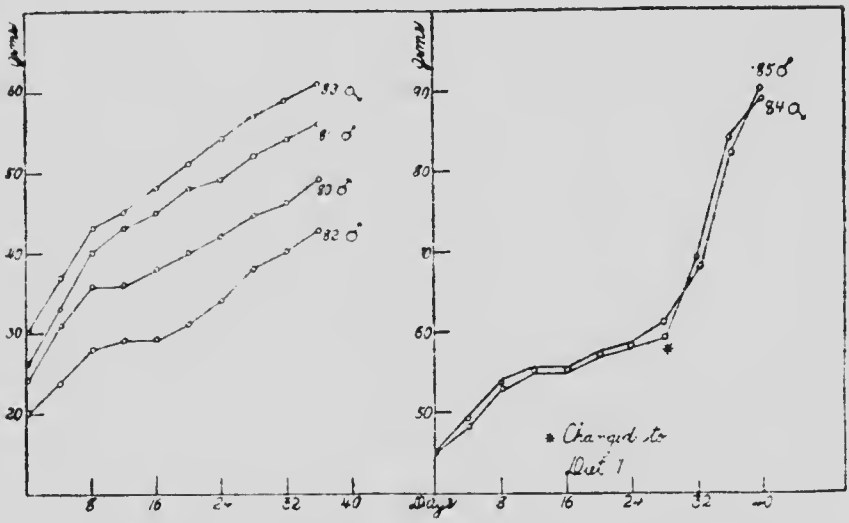


FIG. 8. Rats 80-83. Diet I containing bases from yeast precipitated by phosphotungstic acid.
Rats 81-85. Diet II containing bases from yeast not precipitated by phosphotungstic acid.

losses in weight. The time during which these additions or deficiencies were supplied was short—a period of five to eight days—and the results while indicating the fraction containing the accessory, were unsatisfactory, since the quantity of this element adsorbed by the silicate might not be sufficient to carry the animal through a longer experimental period.

Brewer's yeast, fresh from the vat, was put in an incubator at 37° C. for 48 hours. It was then filtered and the filtrate placed in cold storage. 10 c.c. of this filtrate sufficed to enable young animals of about 20 grms. in weight to double their original weight in three weeks, when added to basal diet mixtures.

The autolysed yeast liquors was shaken with Lloyd's reagent for one hour in a bottle agitated by mechanical means. The mixture was let stand and the supernatent liquid siphoned off after the silicate had settled. This was taken up with N 100 hydrochloric acid which was equal in volume to that of the original yeast liquor. It was twice washed by decantation by these weak acid solutions. The residue was collected on a buchner and washed three times with small portions of distilled water and with two portions of 95% alcohol. The residue was then air dried and finally dehydrated over sulphuric acid in vacuo.

In all previous communications, 50 grms. of the reagent were used to separate out the accessories from one litre of the yeast liquor. In the following experiments two concentrations were used—75 and 12

grms. of the reagent per litre of autolysed yeast solution. In each case the yeast liquor after this treatment was examined to ascertain the presence or absence of any growth accessories.

The nitrogen content of the autolysed yeast liquor, the activated Lloyd's reagent and the yeast liquor after treatment with the reagent, was determined by the Keldjakl method. Then both these latter fractions were added to the basal diets, in such quantities that the nitrogen content of the additions were equal to 10 c.c. of the autolysed yeast liquor which was the unit required to render 100 grms. of the basal diet adequate for a normal growth in rats.

EXPERIMENT II.—Table 8, Fig. 9. Rats 202-3. In this series the autolysed yeast was treated with 75 grms. of Lloyd's reagent per litre. When the diets containing the activated reagent were fed to these animals the result was extremely satisfactory for the early period of the experiment. These animals grew at a normal rate for the first twenty days, and grew slowly for about forty days longer. With the hope of stimulating a more rapid rate of growth, the diet containing butter fat was substituted for that con[illegible] the lard on the 50th day, but without any effect as the rats be[illegible]cline shortly after. Point (2) on the curve indicates the change [illegible]he lard to the butter diet. At point (1) on the chart drinking wate[illegible]used, which contained 10% filtered orange juice, which was followed by a slight increment in growth.

TABLE 8.

LLOYD'S REAGENT 75 GRM. PER LITRE OF AUTOLYSED YEAST LIQUOR.

Diet.	No. 1	No. 2	No. 3	No. 4
Casein	22%	22%	22%	22%
Starch	33%	33%	33%	33%
Sugar	10%	10%	10%	10%
Lard	30%		30%	
Butter Fat		30%		30%
Salt	3%	3%	3%	3%
Agar	2%	2%	2%	2%
Lloyd's Reagent Ppt. equivalent to			10 c.c. Autolysed Yeast Liquor	10 c.c.
Lloyd's Reagent Fft. equivalent to	10 c.c.	10 c.c. Autolysed Yeast Liquor		

Rats 200, 201, males, 0-59 days, Diet No. 1; 60-72 days, Diet No. 2. Rats 202, 203, males, 0-59 days, Diet No. 3; 60-72 days, Diet No. 4. 45th day drinking wa[illegible]er contains 10% orange juice which was increased to 20% on the 61st day.

Day.	Weight.		Food Intake* for Period. (Grammes.)	Weight.		Food Intake* for Period. (Grammes.)
	200	201		202	203	
0	41 grm.	45 grm.		38 grm.	38 grm.	
4	45	49	41.5	49	40	45.2
8	50	49	44.1	61	51	51.8
12	52	51	42.4	69	56	51.9
16	52	52	35.1	74	62	50.5
20	53	53	40.4	78	60	54.3
24	53	54	38.1	82	76	57.1
28	49	49	42.1	83	78	54.5
32	50	50	42.7	82	75	51.4
36	52	52	39.0	83	77	48.2
40	53	52	36.5	83	79	48.7
44	53	53	42.9	88	83	45.9
48			52.2	88	84	43.4
52	62	65	41.3	92	86	50.2
56	62	64	47.9	94	91	45.2
60	65	60	50.4	88	86	42.1
64	65	59	39.6	84	88	40.3
68	65	55	39.5	90	85	35.1
72	65	48	38.2	91	86	36.6
		Died 73rd Day				

*The figures indicate the amount consumed by the two animals.

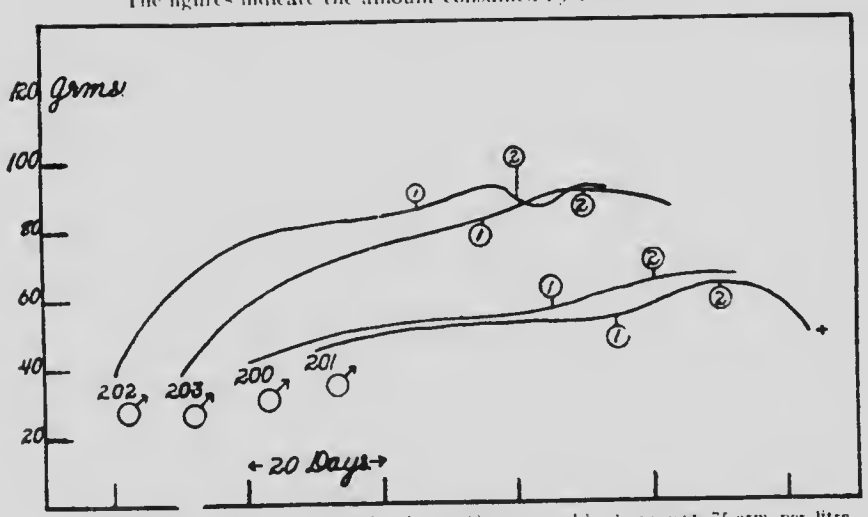


FIG. 9. Rats 200-201. Diets contain autolysed yeast filtrate from Lloyd's reagent, 75 grm. per litre.
Rats 202-203. Diets contain activated Lloyd's reagent 75 grm. per litre of autolysed yeast liquor.
(1). Drinking water contains 10% filtered orange juice.
(2). Drinking water contains 20% filtered orange juice.
Animals changed from lard to butter fat diet.

On the 60th day the amount of orange juice in the drinking water was doubled, without any further improvement being noticed.

Rats 200-1. These animals were fed on diet which contained the yeast liquor which had previously been treated with the above-stated quantity of Lloyd's reagent. These rats grew very slowly and the initial gain was not very great. There was, however, a sufficient quantity of the growth element to keep the animal alive for 72 days, since the basal diet would not maintain life more than 35 days on the average. Xerophthalmia developed about the 40th day. On the 45th day orange juice was added to the drinking water with the result that the eye condition cleared up. It reappeared about the 60th day, and the amount of orange juice in the drinking water was increased, but without effect, since the xerophthalmia persisted and developed into a panopthalmitis, which was followed by the death of rat 201 on the 93rd day. At this point the experiment was terminated.

EXPERIMENT III.—Table 9, Fig. 10. Rats 204-7. In this experiment the accessory growth factors were prepared by treating autolysed yeast liquor with 125 grms. of Lloyd's reagent per litre of yeast solution.

Rats 206-7 were fed on the diets containing the activated Lloyd's reagent. They grew rapidly for the first forty days and doubled their initial weight during the first twenty-five days, and were like normal rats in every respect. At the point (1) on the chart, drinking water containing 10% filtered orange juice was administered to the animals, which was increased to 20% on the 61st day, but this addition was without any noticeable effect on the course of the experiment. Diets containing purified butter fat were used after the 60th day, but with no consequent improvement.

Rats 204-5 were given diet containing the yeast liquor from which the accessories were separated by means of 125 grms. of Lloyd's reagent per litre. These mixtures did not enable the rats to make more than a 15 grm. gain on their initial weight. The usual signs of a deficiency appeared in a short time. Contemporaneously with rats 200-1 the usual eye symptoms appeared, which were temporarily relieved by the substitution of the drinking water by 10% orange juice, which was increased to 20% on the 61st day. Diets containing purified butter fat were used after the 60th day. In spite of these changes, the eye symptoms reappeared about the 60th day and persisted with increasing severity until the experiment was terminated on the 70th day. Had the animals been kept much longer on these diets, death would have ensued.

The result of these experiments indicate that Lloyd's reagent is an admirable medium for obtaining a concentrated preparation of [illegible]

TABLE 9.

LLOYD'S REAGENT 125 GRM. PER LITRE OF AUTOLYSED YEAST LIQUOR.

Diet.	No. 1	No. 2	No. 3	No. 4
Casein	22%	22%	22%	22%
Starch	33%	33%	33%	33%
Sugar	10%	10%	10%	10%
Lard	30%		30%	
Butter Fat		30%		30%
Salt	3%	3%	3%	3%
Agar	2%	2%	2%	2%
Lloyd's Reagent Ppt. equivalent to			10 c.c.	10 c.c.
Lloyd's Reagent Fft. equivalent to 10 c.c.		10 c.c.		
				Autolysed Yeast Liquor

Rats 204-6, females. Rats 207, male. Rats 204-5, 0-59th day, Diet No. 1; 60-70th day, Diet No. 2. Rats 206-7, 0-59th day, Diet No. 3; 60-70th day, Diet No. 4. 48-60th day, drinking water contains 10% orange juice; 61-70th day, drinking water contains 20% orange juice.

Day.	Weight.		Food Intake* for Period. (Grammes.)	Weight.		Food Intake* for Period. (Grammes.)
	204	205		206	207	
0	41 grm.	50 grm.		37 grm.	18 grm.	
4	47	57	52.9	50	23	49.2
8	53	61	51.5	59	26	33.1
12	58	63	48.0	69	30	40.6
16	60	64	44.5	77	33	41.4
20	57	60	40.0	80	34	46.1
24	51	50	38.6	81	35	46.1
28	48	50	37.5	83	35	46.2
32	50	53	32.9	90	40	47.7
36	54	55	47.7	94	47	45.4
40	62	62	43.7	100	52	50.1
44	65	65	43.9	100	55	47.2
48	67	65	46.8	100	57	48.8
52	64	65	45.0	105	59	53.2
56	64	60	40.2	105	57	45.3
60	60	58	36.0	105	55	48.1
64		Dead	28.0	108	55	45.3
70	55	64th Day	27.3	100	50	71.4

*The figures indicate the amount consumed by the two animals.

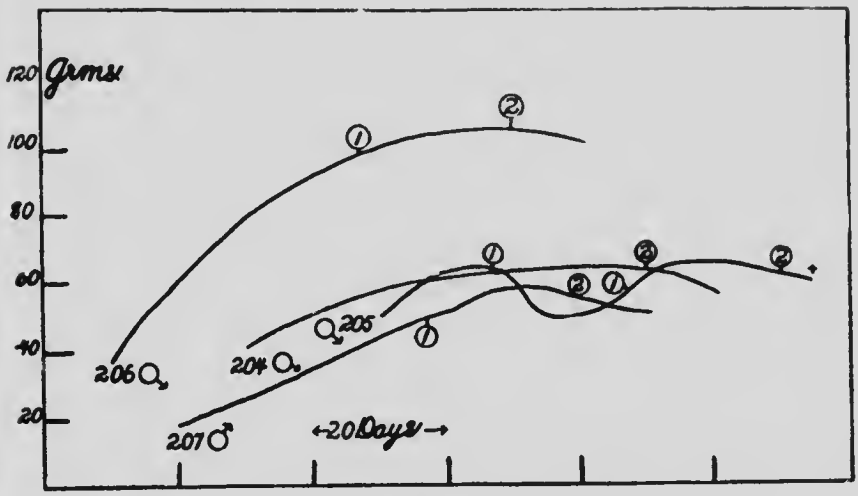


FIG. 10. Rats 204-205. Diets contain autolysed yeast filtrate from Lloyd's reagent, 125 grm. per litre.
Rats 206-207. Diets contain activated Lloyd's reagent, 125 grm. per litre.
(1). Drinking water contains 10% orange juice.
(2). Drinking water contains 20% orange juice.
Animals changed from lard to butter fat diet.

growth accessories from yeast. Considered on the basis of the time required for the animals to double their initial weight, the results obtained by the use of this activated reagent may be considered as equal to those observed in rats fed on normal diets. The use of greater quantities of Lloyd's reagent apparently resulted in an increased adsorption of the growth substances, for in the case where 125 grms. of the silicate per litre of the yeast liquor was used, the activated reagent gave better results.

The Lloyd's reagent brought down from one-quarter to one-third of the total nitrogen of the yeast liquor, depending on the quantity of the reagent used. It is possible that 75 grms. per litre concentration was not quite sufficient to adsorb all the growth fraction. When 125 grms. of the reagent per litre of yeast were used the amount of nitrogen brought down were correspondingly greater and the behaviour of the animals indicated an increased adsorption of the growth accessories. But even in this case, the adsorption was not absolutely perfect, since the animals maintained their life for the experimental period, when subsisting on diets containing the filtrate from the yeast liquor after treatment with the above concentration of the reagent. Had the yeast liquor been completely freed of vitamines by the above method, the animals would not have lived more than five weeks.

It is evident that none of these methods of separation which were used in attempts to isolate the growth substances will quantitatively remove these elements from their solutions. In the case of the antineuritic vitamine, the biological test would not demonstrate the presence of infinitesimal traces of this compound. In the case of the growth factors, the deficiency of the method is evident. Since these experiments are carried on for a period of time sufficient to demonstrate an appreciable effect, through the summation of a series of subminimal stimuli due to the extremely minute traces of the growth accessories, which the separating agency cannot remove.

The writer is not of the opinion that yeast contains several growth-promoting agencies. Emmet and McKim (1917) and Williams and Seidell (1916) believed that there are two factors, one antineuritic and the second whose function is to maintain weight. The former authors hold that this latter factor is not adsorbed by Lloyd's reagent. A cursory inspection of the writer's results would tend to support the theory that there are two elements, a growth-promoting factor and a weight-maintaining factor, which remains in the yeast liquor after agitation with the reagent. Diets which contain the inactivated yeast liquor gave evidence of slight weight-maintaining factors, but from the writer's previous experience with rats subsisting on diets of varying degrees of deficiency, he is of the opinion that there is only a single factor which is incompletely removed by the methods used in the above series of experiments. The maintenance factor observed in the inactivated yeast liquor is the effect of a continuous administration of subminimal quantities of the growth factor left behind by the precipitating media.

The growth accessory is one that is readily adsorbed by the voluminous precipitates resulting from the action of phosphotungstic acid on simple organic bases and various silicates and colloids. Its removal by these agencies is due to the physical process of adsorption, which is the principal factor in facilitating the isolation of this element. On the other hand, its precipitation by silver salts when the solution is rendered alkaline seems to point towards a chemical combination with the silver and formation of an insoluble salt with this precipitating agency.

THE TOXIC EFFECT OF PROLONGED ADMINISTRATION OF LLOYD'S REAGENT.

In the previous series of experiments the animals declined during the final period of the experiment, even when the diet contained the growth accessory in a concentrated form. In some cases the animals received, in addition to this, the antiscorbutic factor (orange juice) and the fat soluble compound (purified butter fat). In spite of these additions the

decline in the final stage of the experiment persisted and could not have been due to an accessory deficiency. The only other possible factor responsible for the decline was the presence of the inassimilable Lloyd's reagent in the diets. Accordingly a further series of experiments was carried out in which the basal diets were reinforced with 10-20 c.c. of autolysed yeast per 100 grms. of the diet mixture. This series was the control. Another series was initiated in which the animals were fed on basal diets with the autolysed yeast liquor, as described above, but with the addition of five grms. of Lloyd's reagent to every 100 grms. of the basal diet. In all previous cases where the silicate was used, it was present to the extent of 4-6 grms. to every 100 grms. of the diet.

EXPERIMENT I.—Table 10, Fig. 11. Rats 208-11. These animals were fed the diet indicated in the table. The first 60 days of the experiment the diets contained 10 c.c. of autolysed yeast liquor per 100 grms. of diet. On the 61st day (point 2 on curve) the autolysed yeast was increased to 20 c.c. for the remainder of the experiment and the fat soluble accessory was supplied by putting the animals on Diet 2 on the 64th day at the point (3) on the curve. The increase in the supply of yeast liquor and the addition of the fat soluble accessory stimulated an increase in the consumption of the diets and resulted in a slight gain in weight which had, however, been preceded by a slight decline in the growth curve. In no case did the animals attain a weight a little more than double their original weight in a period of 84 days. On the whole, taking into consideration the presence of the accessories, this experiment was extremely unsatisfactory as regards the growth increment.

TABLE 10.

EXPERIMENT TO SHOW THE TOXIC EFFECT OF LLOYD'S REAGENT.

Diet.	No. 1	No. 2
Casein	22 grm.	22 grm.
Sugar	33	33
Starch	10	10
Lard	30	
Butter Fat		30
Salt	3	3
Agar	2	2
Lloyd's Reagent	5	5

All female rats. Rats 208-11, 0-60th day, 10 c.c. autolysed yeast liquor to 100 grm. diet; 61-91st day, 20 c.c. autolysed yeast liquor to 100 grm. diet; 0-64th day, Diet No. 1; 65-91st day, Diet No. 2.

Day.	Weight.		Food Intake* For Period. (Grammes.)	Weight.		Food Intake* For Period. (Grammes.)
	208	209		210	211	
0	60 grm.	72 grm.		48 grm.	37 grm.	
7	78	72	95	75	61	105.5
14	74	72	107.7	69	58	100.2
21	87	80	98.3	80	70	108.2
28	95	87	99.8	87	74	105.5
35	106	89	100.3	95	83	106.9
42	100	83	96.4	97	83	102.6
49	100	87	88.1	100	85	100.3
56	100	86	97.0	100	75	98.2
63	110	90	125.0	100	65	102.9
70	105	85	111.7	100	65	107.6
77	120	95	125.2	105	80	122.2
84	125	100	140.0	110	80	139.5

*The figures indicate the amount consumed by the two animals.

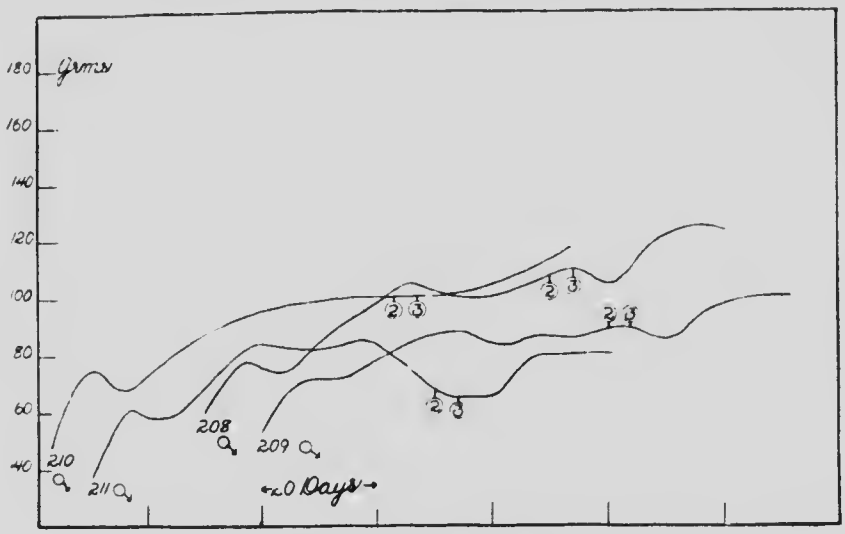


FIG. 11. Stock diet containing autolysed yeast and Lloyd's reagent.
(2). Autolysed yeast increased from 10-20 c.c. per 100 grm. diet.
(3). Animals changed from lard to butter fat diet.

EXPERIMENT II.—Table 11, Fig. 12. Rats 212-15. These were controls to the preceding experiment and the animals were fed basal diets reinforced with autolysed yeast liquor, but without addition of the Lloyd's reagent. The amount of autolysed yeast was increased

from 10 to 20 c.c. per grms. diet, on the 66th day in the case of rats 212-13 and on the 47th day for rats 214-15 (point 2 on curve). At the point (3) on the chart (the 65th day for rats 212-13, the 51st day for rats 214-15) the butter fat accessory was supplied by the use of Diet 2. These variations were introduced to make this experiment strictly comparable to Experiment I. In all cases the changes were made simultaneously.

TABLE II.

CONTROL FOR THE TOXICITY OF LLOYD'S REAGENT (TABLE 10).

Diet.	No. 1	No. 2
Casein	22%	22%
Sugar	10%	10%
Starch	33%	33%
Lard	30%	
Butter Fat		30%
Salt	3%	3%
Agar	2%	2%

Rats 212, 213, females, 0-59th day, 10 c.c. autolysed yeast liquor mixed with 100 grm. of the diet; 60-91 day, 20 c.c. autolysed yeast liquor mixed with 100 grm. of the diet; 0-64th day, Diet No. 1; 65-91st day, Diet No 2; Rats 214, 215, females, 0-46th day, 10 c.c. autolysed yeast liquor mixed with 100 grm. of the diet; 47-77th day, 20 c.c. autolysed yeast liquor mixed with 100 grm. of the diet; 0-50th day, Diet No. 1; 51-77th day, Diet No. 2.

Day.	Weight.		Food Intake* For Period. (Grammes.)	Weight.		Food Intake* For Period. (Grammes.)
	212	213		214	215	
0	58 grm.	58 grm.		50 grm.	45 grm.	
7	77	75	86.9	84	70	100.9
14	81	77	107.5	100	85	104.5
21	98	95	100.8	115	100	126.6
28	110	104	107.6	120	105	124.9
35	116	110	120.4	125	110	126.7
42	120	110	113.6	133	108	135.2
49	130	120	117.9	142	110	140.6
56	128	112	122.9	152	117	140.6
63	130	120	132.9	165	125	152.3
70	132	125	125.1	180	140	181.0
77	140	140	151.4	185	145	200.8
84	145	150	147.7			
91	145	150	147.7			

*The figures indicate the amount consumed by the two animals.

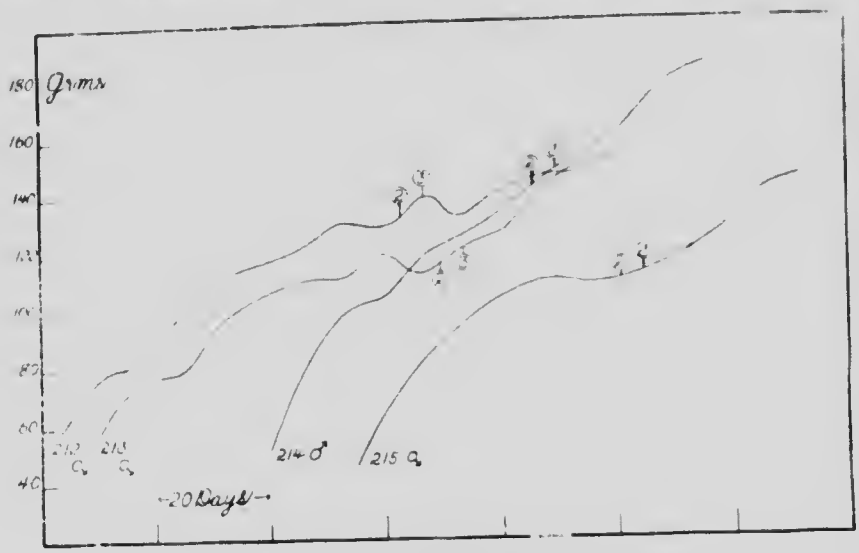


FIG. 12 Stock diet containing 0% autolysed yeast
(2) Autolysed yeast increased from 10-20 cc. per 100 grm diet.
(3) Animals changed from lard to butter fat diet.

This control series demonstrated that this diet could support the rats in normal condition during the whole of the experimental period, since these animals almost trebled their original weight during the experiment. They were normal in appearance and behaviour; and the growth rate was that usually observed in animals fed with natural food mixtures.

This series of experiments fairly demonstrates the toxic effect produced by prolonged administration of Lloyd's reagent. A glance at the charts shows this result in the most striking fashion. Since the animals consumed from 10-15 grms. of food per day, the amount of reagent in the diet of rats 208-11 varied from 0.4-0.6 grms. daily. The records of food intake of both sets of animals shows that the consumption was approximately the same, both in the controls, and the animals whose diets included the Lloyd's reagent; consequently the consumption was sufficient to permit the latter set to grow at the same rate as the controls. The toxic effect of the silicate did not consist in producing a series of metabolic changes which would lead to diminished intake, followed by a corresponding retardation in the growth rate; but acted in some fashion as yet unknown.

The growth obtained in rats 212-15 by the use of autolysed yeast as a source of accessories, demonstrates the superiority of this substance for such a purpose. In comparison with the previous experiments, in which whole yeast was employed to enable the animals to attain their

usual growth, the writer is of the opinion that the autolysis improves the growth-promoting properties of yeast. Cooper (1914, 2) and Seidell (1917) conclude that autolysed yeast is more active than the original dry or moist mass of yeast cells as an antipolyneuritic [illegible] accordance with the writer's experiences regarding [illegible] common properties of the brewer's yeast, as illustrated [illegible] result.

The fact that the growth element from the [illegible] by methods similar to those by which the antineuritic [illegible] been prepared, points to the conclusion that both [illegible] belong to the same group, or are in all likelihood identical with one another. This view coincides with that expressed by McCollum, Simmonds and Pitz (1918, 1) who found that their water soluble B accessory which is the factor prepared from yeast by [illegible] so the element which effects relief when administered to [illegible] polyneuritis. This view is shared by Drummond (1917, 1).

With regard to the action of the fat soluble [illegible] the experience of the author does not coincide with that of McCollum, Osborne and Mendel and Drummond. The animals which received this compound did not show any marked advantage over the animals whose diet was purified, with the exception of an increased resistance to infection and a slightly superior external appearance. The effect comparable to that described by these authors was noted in case of the animals were ingesting the fat soluble accessory. It is perhaps possible that the fresh yeast and the autolysed yeast liquor may contain small quantities of the fat soluble accessory and thus account for the similarity of the results observed from the use of diets containing the lard and butter fat. Again, the comparatively short experimental period (5 months) may be insufficient to establish the difference between the lard and the butter fat to assert it. Osborne and Kennedy (1916) state that the fat soluble factor [illegible] for long periods, when it is only a question of maintaining the animals. The results so far obtained by the writer indicate that the fat soluble accessory has [illegible] antineuritic action.

ARE [illegible] SEINS VITAMINE-FREE?

[illegible] which must be considered in the preparations of accessory-free proteins, is the possible traces of the accessory compounds present as impurities in the commercial preparations of [illegible] which constitute these basal diets. It has been customary for investigators to treat the carbohydrates and casein preparations by extraction with cold or hot alcohol to render

them vitamine-free. McCollum and Davis (1915, 4) have called attention to the possibility of destroying the nutrititive efficiency of the casein itself, when the extracts are made with hot alcohol, or when the casein is autoclaved at temperatures above 100° C. They purified casein by extraction with distilled water for a period of a week or so and states that this process renders the protein accessory-free, without injuring the casein itself.

The writer has always extracted the commercial casein by refluxing with boiling alcohol for six hours. The alcohol extract was always tinged yellow and when cool, deposited a considerable quantity of white or yellow flocculent feathery flakes. The casein, after extraction, had usually changed from a light gray to a brown colour. It was, therefore, advisable to determine whether the casein had been adversely affected by the action of the hot alcohol.

Some casein was taken from a lot, which was subsequently purified by alcoholic extraction, and purified by the method of McCollum and Davis. It was put in a cotton bag and immersed in distilled water. The water was charged daily and the bag was well kneaded several times daily to insure intimate contact with the water. After a week, the contents of the bag were put on a filter and the water drawn off at the pump. The mass was washed once with cold alcohol and allowed to dry at room temperature.

Diet mixtures were made up containing this washed casein and fed to rats 55-6, Table 12, Fig. 7. These animals were put on the experimental diet at the same time as those whose growth curves are recorded in Fig. 7, and the experiments were carried on simultaneously. Lard was used to supply the fat in the diet. These animals did not show any superiority when compared with those others whose diets contained casein, refluxed with boiling alcohol. This latter procedure, therefore, had not affected the nutritive properties of the casein proper.

TABLE 12.

Diet.	No. 1	No. 2	No. 3
Casein (McCollum)	22%	22%	22%
Sugar	10%	10%	10%
Starch	27%	29%	27%
Lard	30%	30%	30%
Salt	3%	6%	6%
Agar	2%	2%	2%
Yeast (dry)	6%		
Yeast (moist), equivalent to		1%	3% of Dry Yeast

Rats 55 and 56, females, 0-59th day, Diet No. 1; 60-87th day, Diet No. 2; 88-140th day, Diet No. 3.

Day.	Weight.		Average Daily Food* Intake. Calories
	55	56	
0	48 grm.	52 grm.	
4	67	71	75 1
8	80	82	92 2
12	89	91	88 4
16	96	96	77 2
20	104	102	86 0
24	107	104	74 9
28	109	109	94 3
32	115	116	79 8
40	120	120	88 4
60	140	133	86 3
80	144	143	78 4
100	136	150	79 4
140	133	136	61 9

*The figures indicate the amount consumed by the two animals

Commercial casein preparations were also used just as they were purchased, without purification. Diets which contained these casein preparations were fed to various animals. The diets were otherwise accessory-free. Two samples of casein were used—Eimer and Amend's and the Digestive Ferments Co.—in both cases the animals made the usual initial gain, then declined and were taken off these diets at the 39th day, when they had receded to their original weight. In no case could any sign of the accessory factor be detected and consequently these casein preparations could be considered vitamine-free (Table 13, Rats 222-5).

Diets were employed which contained dried egg albumen (Merck) as the protein fraction. These likewise failed to show any trace of the accessories and this preparation was therefore vitamine-free. The animals were declining in weight at the 20th day (Table 13, Rats 228-9).

Fibrin was prepared by washing the blood clot, till free from haemoglobin, soaked in 95% alcohol, filtered and dried in an oven at 90° C. This was used to furnish the supply of protein. The animals on diets containing this protein showed all the evidences of a vitamine deficiency. There was also a deficiency in some other respects, for the animals made no initial gain but immediately receded to about 80% of their original weight. The urine of these animals had a greenish tinge (Table 13, Rats 220-1).

TABLE 13.

Diet.		
Protein	22%	Rats 224-5, males. Protein is casein (Eimer and Amend)
Starch	33%	Rats 222, male; 223, female. Protein is casein (Difco)
Sugar	10%	Rats 220, 221, males. Protein is equal parts of fibrin and
Lard	30%	egg albumen (Merck)
Salt	3%	kats 226 and 227, females. Protein is fibrin
Agar	2%	Rats 228 and 229, males. Protein is egg albumen (Merck)

Day	220	221	222	223	224	225
0	45	40	100	100	90	100
7	48	52	120	107	105	107
14	45	50	140	120	112	115
21	45	43	140	125	118	120
28			132	100	110	110
35			115	85	100	100
39			108	78	80	70

Day.	226	227	228	229
0	100	96	40	40
7	98	86	55	57
14	95	85	54	52
21	92	87	45	50
28	95	78		
35	80	68		
39	80	70		

A diet similar to the above was fed to 4 animals (Table 14). The accessories were supplied in the form of autolysed yeast liquor. This mixture enabled animals to grow at the normal rate for 21 days and demonstrated that the diet was fully adequate for this period.

TABLE 14.

Diet	
Fibrin	11%
Egg Albumen	11%
Starch	33%
Sugar	10%
Lard	30%
Salt	3%
Agar	2%

To each 100 grm. of the above mixture 10 c.c. of autolysed yeast was added and worked into a stiff paste.

Rats 216 and 218, males; 217 and 219, females.

Day.	216	217	218	219
0	18 grm.	35 grm.	37 grm.	38 grm.
7	36	45	52	55
14	46	55	58	60
21	50	60	55	68

It is evident that these commercial protein preparations were free from all trace of accessory growth substances. The alcoholic extraction used by the writer to eliminate these compounds had no appreciable effect on the nutritive value of the protein in spite of the vigorous treatment it had undergone. The casein used by the writer throughout all these experiments was a commercial preparation manufactured by Eimer and Amend.

THE ENERGY REQUIREMENT OF GROWING ANIMALS.

The writer has kept a record of the dietary intake, measured in calories, of a number of animals. These records were prepared over a period of two years and a selection of the results obtained is tabulated in Tables 15 and 16.

In Table 15 the figures have been compiled to illustrate the gain in growth per 1,000 calories of food intake and the cost in calories for each gramme of new tissue added to the weight of the animal. The animals whose dietary records are included in these tables are recorded in Tables 1-7 and Table .

The animals fall into two main groups according to their requirements for the formation of new tissue and their economy in utilizing the dietary intake.

The first group includes all the rats who are under 50 grms. in weight and when the experiments were conducted during the spring and fall, during the warm weather. The experimental period in this class was under fifty days.

In all these cases, the cost per gramme of new tissue was practically the same. Rats 27-36 gained from 50 to 61 grammes per thousand calories, and the cost of one gramme of new tissue ranged from 16.4 to 20 calories. Rats 27 to 30 were on a deficient diet the first 20 days and did not grow, beyond a slight initial gain, followed by a slight decline. During the last 28 days these rats were put on an adequate diet and grew rapidly, ultimately attaining the same approximate weight, as the animals who grew continuously throughout the experimental period. Rats 35

and 36 grew normally and are the controls for this series. Rats 33 and 34 utilized their energy intake less efficiently than the others in this group, probably owing to the fact that they had been placed on an inadequate diet the final twelve days of the experiment and the growth curve flattened and declined as a result. Rats 80 86 did not display the average economy in dealing with their calorific intake. They were consuming diets containing the isolated fractions which had been prepared from yeast, and the accessories had undergone a partial depreciation, as a consequence. Animals 80-83 consumed diets containing the bases carried down by phosphotungstic acid. This fraction included the growth accessories but the rats required a longer time than usual to

TABLE 15.

Rat No	Original Weight	Gain in Grammes	Total Calories Ingested	Gain in Grammes per 1,000 Cals	Cost per 1 grm. New Tissue in Calories
27 and 28	21 grm.	71	1203	55	17
29 and 30	29.5	73 5	1197	61	16 4
33 and 34	20	54 5	1088	50	20
35 and 36	18.5	75	1318	57	17.5
Rats 27-30 were stunted the first 20 days of the experiment and grew normally the last twenty-eight days. 33 and 34 were put on a deficient diet the last twelve days and lost weight. 35 and 36 grew normally throughout and are controls.					
80 and 81	24 5 grm.	28	560	50	20
82 and 83	25	26	590	44	22 6
85 and 86	45	44 5	951	47	21 3
Rats 80-83 grew throughout the experiment, while 85-6 were stunted the first twenty-eight days and grew rapidly the remaining eleven.					
45 and 46	19 grm.	44	822	55	18.5
47	28	72	1435	49 5	20
41	20	69	1214	56 7	17 7
43 and 44	22 5	55 5	1094	50 6	21.6
49 and 50	45	161	4163	39	25.7
51 and 52	43.5	154 5	4165	37 2	27 0
53 and 54	42	94 5	3252	29	34
55 and 56	50	93 5	3334	28	36

45-6 grew 40 days while 47 reached its maximum on the 40th day. 41 grew for 64 days and 43-4 for 48 days. 49-56 reached their maximum weight on the 80th day.

double their initial weight. Rats 85 and 86 were stunted the first 28 days and grew the last 11 days, during which time they attained a figure double their initial weight. The principal feature of this series is the fact that the ultimate cost of growth was confined to narrow limits even when the animals had been stunted during the earlier period of the experiment.

The second class includes rats which were undergoing experiments of longer duration than the above series, or where the diet ultimately displays an inadequacy. In the case of all the animals of this second class, the results are calculated from the data recorded during the period in which the subject reached its maximum growth and do not include the final period when the curve was almost flat and the gain for the period was only a few grammes. Rats 41-7 were fed on diets which finally proved inadequate so that the animals ultimately died. These animals resemble those of the first class in their metabolic economy. The remaining members (rats 49-56) grew for a longer period and terminated their maximum rate of growth about the 80th day. These animals utilized the energy less efficiently, the cost of new tissue being 30-100% more than that observed in all previous cases. This may be accounted for in part by the fact that this experiment was carried out during the winter months and the animals were kept in metal cages without any bedding. The room temperature often fell to a low level and some of the energy undoubtedly went to balance the heat lost through radiation.

The energy intake per 100 grms. live weight of rat (Table 16, cf. Fig. 4-6) yields interesting information. In these short term experiments the energy intake is approximately the same, with slight variations, whether the animals are growing or declining in weight. In the case of rats 25-34 and 80-86 the intake for the 8-12 day period ranged from 30-46 calories per 100 grms. live weight. Rats 25, 26, 31 and 32 were kept on accessory-free diets throughout and eventually died. During the 24-28th day interval they were declining but nevertheless the energy consumption remained at the same level. Rats 27-28 and 29-30 were consuming inadequate diets and declining during the early period of the experiment. On the 20th and 24th day, respectively, they were given food mixtures sufficient for all requirements and the decline was immediately converted into a rapid growth increment. This was accompanied by a corresponding energy intake, an increase of 75% for rats 27-8 and 100% in the case of 29 and 30, over that of the early period of the experiment. In these cases there was a compensatory increment to the growth rate, since these animals reached approximately the same weight in the last 20-24 days as that attained by animals who were growing throughout the whole experimental period. In the final

part of the experiment, when the animals had accommodated themselves to the adequate diet and had regained their normal condition, the intake had fallen to normal levels. The series 80-86 ingested from 39 to 46 calories per 100 grms. live weight during the 8-12th day period. The intake in rats 80-83 fell slightly during 24-28th day.

TABLE 16.

CALORIFIC INTAKE PER 100 GRM. LIVE WEIGHT OF RAT.

Rats.	Period 8-12th Day.	Period 24-28th Day.	Period 44-48th Day.
25 and 26	39.5 cals.	35 cals.	Dead
31 and 32	32.0	32 4	Dead
27 and 28	41	65	46.5
29 and 30	39 6	81.5	41 5
33 and 34	46.0	51 1	32 2
80 and 81	46 0	30 0	
82 and 83	46 2	38 3	
84 and 85	39.4	40 5	38.2 (36-9th day)

Rats.	12th Day	48th Day	64th Day.	100th Day.	120th Day	140th Day.	150th Day.
49 and 50	57	31 3	21.7	21	26	19.5	19.3
51 and 52	43 2	56	28 3	22.3	25 8	21.3	16.4
53 and 54	51 5	38.8	38.5	31.9	26 7		31.0
55 and 56	51 5	37.5	31 0	27.7	31.0	22.8	

The energy consumption of animals in experiments of long duration follows a different course. The calorific requirements display a tendency to shrink with the increasing duration of the experiment. The requirements in the early period were normal, as compared with the other animals included in the table, but steadily diminished so that at the end of the experimental period they were ingesting from a half to a third of the original calorific intake per 100 grms. live weight. In spite of the diminishing intake the animals grew rapidly until the 80th day when the rate slowed down and resulted in only a small gain in weight for the balance of the experiment. During the latter part of the experiment rats 51 and 52 consumed only 10.4 calories per 100 grm. live weight yet maintained weight and health (150th day). The energy requirements during the period prior to the 40th day were about 10% higher than usual, partly on account of the fact that these animals were put on the diets during the month of February, and had to balance a loss of body heat through radiation as the temperature fell several occasions low enough to cause discomfort.

These figures are in accordance with the findings of Hopkins (1912). The energy requirements of his animals ranged between 40 and 60 calories per 100 grms. live weight. These experiments were carried out on young animals and were of short duration - 20 to 50 days. In one series, Protocol VII., the duration was 60 days, but in this case the energy intake gradually declined as time went on.

These calculations illustrate a much-discussed feature of all these growth experiments. Investigators have always noticed that the appetite corresponded very closely to the increment or decline in growth. It becomes a question whether the appetite is directly affected as the result of the presence or absence of the growth accessories, or by metabolic conditions consequent therefrom. Examination of the Tables 1-3 shows that about 95% of the energy intake is assimilated from the intestine in every case, whether the animal is growing or declining. Therefore it cannot be a question of the action of the growth-promoting factor in this respect. Hopkins also finds that 90% of the energy intake is assimilated in all cases. The writer concludes that the function of the accessory growth factor is connected with the tissue metabolism, stimulating the formation of new tissue and creating a demand for increasing intake which reflects itself on the appetite. In the case of rats who were declining and ultimately died (25, 26, 31 and 32), the intake was 27-35 calories per 100 grms. live weight. This was amply sufficient for growth since rats 49-50 were still growing when the energy consumption had fallen as low as 21 and 22.3 calories per 100 grms. live weight. This demonstrates conclusively that the decline in growth sets in before the diminished consumption per unit weight becomes noticeable. When rats are removed from a deficient to an adequate diet, an immediate response is seen in the form of an increased appetite. Hopkins points out that these growth factors may stimulate consumption as an indirect result of a direct effect on growth, while diminished consumption may be seen to follow rather than to precede the decline in weight, and must be the effect rather than the cause of the cessation of growth.

Drummond (1918) finds that the rate of growth varies with the energy intake, verifying the law that the calorific requirements are directly related to the amount of live tissue weight. His figures show that rats may grow when the intake falls below 29 calories per 100 grms. live weight. Rats grew at the usual rate when the consumption fell as low as 25 calories and slow growth was noted when the intake had fallen to 19 calories per 100 grms. live weight.

The tendency for the energy intake per unit weight to decline in prolonged experiments, in addition to the reason previously mentioned, may be in part accounted for by the findings obtained from the investiga-

tions regarding the energy requirements of human beings. It is known that boys, prior to the age of puberty have a basal metabolism 20% higher than that of adults. This period corresponds to the first 60 days in the life of the rat, and examination of the calorific intake shows a decline after this period which goes on steadily as the animal approaches maturity. This seems to indicate that the basal metabolism of rats may follow a course similar to that of human beings, but no proof of this assumption has been submitted as yet.

These observations illustrate the intimate relationship between the requirements of growing tissue and the instinctive appetite of the animal. While it is not yet certain whether the appetite regulates itself by variations in the growth impulse of the tissues, apparently the tissue metabolism creates the demand for energy which is followed by a response in the appetite, when the tissues are making new additions to their mass.

SUMMARY.

(1) The butter fat, purified by the method of Osborne and Mendel, still contains traces of nitrogenous compounds.

(2) The growth factor in the butter fat is unable, alone, to bring about the normal growth increment in young animals.

(3) Butter fat shows no nutritive superiority, compared to lard, in feeding experiments of short duration. In prolonged experiments there is a slight improvement as compared with the results obtained with lard.

(4) Dried yeast preparations contain sufficient quantities of the accessory growth factor to sustain growing animals for a short time. For prolonged feeding experiments, fresh whole yeast or autolysed yeast liquor is essential for the maintenance of the normal growth rate.

(5) The growth-promoting factor can be isolated from hydrolysed yeast by means of phosphotungstic acid. This method entails a partial diminution in the activity of the growth accessory. It can also be removed from autolysed yeast with Lloyd's reagent, but larger quantities are necessary than those previously stated necessary for the removal of the antineuritic vitamine. The accessory growth factor is similar to, and most probably identical to the beri-beri vitamine.

(6) Prolonged ingestion of the activated Lloyd reagent causes an inhibitory effect of the growth of the animal, due to toxic properties of the inassimilable silicate.

(7) The extraction of the commercial casein preparations with boiling alcohol to render them accessory-free does not affect the nutritive properties of the protein itself. Commercial preparations of caseins, egg albumen and fibrin are vitamine-free.

(8) The growth cost in terms of energy requirements per unit weight of new tissue, is the same for experiments of short duration, regardless of the rate of growth. The energy requirements per unit weight of live tissue is the same, within narrow limits, in animals kept in the same experimental conditions whether the animals are growing, maintaining or declining. The energy requirement of young animals is slightly higher during the period prior to the development of the secondary sexual characteristics.

(9) The function of the growth accessory is not connected with intestinal assimilation. It has an action at some point in the tissue metabolism, creating a train of events which are reflected upon the appetite, which responds, as a consequence, regulating the energy requirements to the demand.

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