CCLXXXV. THE WATER-SOLUBLE B-VITAMINS OTHER THAN ANEURIN (VITAMIN B₁), RIBOFLAVIN AND NICOTINIC ACID REQUIRED BY THE PIG

BY HARRIETTE CHICK, THOMAS FOTHERINGHAM MACRAE, ARCHER JOHN PORTER MARTIN AND CHARLES JAMES MARTIN

From the Institute of Animal Pathology, Cambridge, and the Division of Nutrition, Lister Institute, London

FROM the results of previous work [Birch *et al.* 1937; Chick *et al.* 1938, 1, 2] it was concluded that young pigs could be reared successfully on a diet of maize and purified casein with the addition of a suitable salt mixture, provided that nicotinic acid were also given. Similar experiments made on rats showed that the addition of nicotinic acid was not required for this species [Chick *et al.* 1938, 2].

The fact that an extraneous source of nicotinic acid or its amide does not appear to be required by the rat is responsible for the misleading results of researches into the aetiology of pellagra in which this animal was used. The dog, the pig and the monkey appear to resemble more closely the human being in their requirement of nicotinic acid in their food and consequently in their failure to maintain health on diets consisting too largely of maize.

The vitamin requirements of different species of animals being so various, it seemed worth while to ascertain what other heat-stable, water-soluble, accessory factors in addition to nicotinic acid and riboflavin, are required by pigs. Whatever they may be, it is clear from our previous observations [Chick *et al.* 1938, 1, 2] that they are contained in whole maize in sufficient amount when it comprises 80 % of the diet.

Edgar & Macrae [1937] have concluded that at least two other "B₂-vitamins" are necessary for rats. One of these they call "filtrate", the other "eluate" factor. Fractions containing these two factors were originally derived from yeast but Edgar *et al.* [1938, 2] have also separated similar fractions, using somewhat modified methods, from a water-acetone extract of liver.

In the present enquiry we are dealing with "filtrate fraction" and "eluate fraction" as described by Edgar and Macrae and prepared by their methods. Fractions derived by similar methods from yeast, liver and rice polishings have been prepared by Lepkovsky *et al.* [1936] and by other workers. The relation of these to those employed by us will be discussed later.

EXPERIMENTAL METHODS

It was first necessary to find a simple basal diet suitable for young pigs which would contain an adequate amount of vitamins A, D and E and of linoleic acid but which was as free as possible from water-soluble vitamins. To the basal diet a mixture containing vitamin B₁ (aneurin), riboflavin and nicotinic acid, all

given as pure chemicals, could be added as required. The following was found satisfactory:

Purified maize starch	...	...	71
Purified casein	...	...	21
Cod liver oil	...	...	2·5
Cotton seed oil	...	...	2·5
Salt mixture	...	...	3

It contained protein 18·1%, fat 6·0%, carbohydrate 64% and had a nutrient ratio of 1 to 4·2. As the pigs increased in weight the proportion of casein was gradually reduced to 15%. At first wood-charcoal was supplied as an unobjectionable form of roughage. This was found unnecessary and later discarded.

The maize starch was the purest sample obtainable and had a nitrogen content of 0·03%. It was prepared from dent white maize by the following process. The grain was steeped in a warm dilute solution of SO_2, then broken; the embryos were separated by flotation, the fibre by sieving and the protein by a process of differential sedimentation in an alkaline medium. Finally the starch deposit was washed by decantation until the nitrogen (protein) content was reduced to the required figure, after which it was filtered and dried rapidly in a current of dust-free air.

The cod liver oil was certified to contain per g. 1000 I.U. of vitamin A and 100 I.U. of vitamin D. The casein was "Glaxo ashless extracted" casein, described by the manufacturers as a casein of the "self soured" type, thoroughly washed with dilute acetic acid and afterwards extracted with hot alcohol; it contained 94% protein.

The salt mixture used was based on that recommended by Hubbell et al. [1937] who found it adequate for rats on a similar diet. It contained $CaCO_3$ 38%, bone ash 23·2%, NaCl 10·5%, KCl 27·2%, KI 0·008% and Fe_2O_3 0·84%. At first it formed 3% of the diet. Later it was realized that for rapidly growing pigs this mixture was low in phosphorus, the proportion of which in the diet, allowing for that in the casein, was less than 0·3%. The deficiency was rectified by mixing one-fifth of its weight of sodium dihydrogen phosphate (NaH_2PO_4, $2H_2O$) with that of the original salt mixture and adding 4% of the mixture to the diet. The phosphorus in the salt mixture was thus increased from 3·9 to 6·6% and that in the diet to 0·4%.

The riboflavin used was a sample of pure synthetic material kindly given by Messrs Hoffmann-La Roche, Basle, to whom we are also indebted for the specimen of nicotinic acid used.

The aneurin was pure synthetic hydrochloride provided by Prof. A. R. Todd.

The yeast was a commercial preparation of dried brewer's yeast.

Filtrate and eluate fractions prepared from liver

The laboratory preparation from yeast of the large amounts of filtrate and eluate fractions required in these experiments was not feasible and we had therefore to seek other sources. It was found by Edgar et al. [1938, 2] that fractions with similar biological properties, as tested on rats, were present in liver and were contained in the residues discarded during the commercial manufacture of some of the preparations used in the treatment of pernicious anaemia. These liver residues were kindly put at our disposal by Messrs Glaxo Laboratories, Ltd., who also prepared some of the fractions.

The liver residue available to us at the beginning of our experiment was the filtrate from the charcoal treatment of an aqueous acetone extract of liver.

Filtrate fraction was prepared from this by simple extraction of the acidulated liver residue with amyl alcohol, the filtrate fraction passing into the amyl alcohol. Eluate fraction was prepared from the material which had been extracted with amyl alcohol, by adsorption on fuller's earth and subsequent elution with barium hydroxide.

While the experiments were in progress the above liver residue became unobtainable, but two new residues were available. The first residue, that portion of an aqueous acetone extract of liver which was unextractable by phenol, was found to be a good source of eluate fraction and towards the end of our experiments we prepared an eluate fraction from this residue by adsorption on fuller's earth, elution with barium hydroxide and subsequent extraction of the eluate with amyl alcohol to remove any filtrate factor. The second residue, the filtrate from charcoal treatment of the phenol-extractable portion of the aqueous acetone extract, was rich in filtrate fraction and extraction of this residue with amyl alcohol gave the purified preparation of filtrate fraction which was used in the latter part of these experiments.

The amounts of eluate and filtrate fractions administered to the pigs were based on the requirements of rats found by Edgar & Macrae [1937] and Edgar et al. [1938, 1]. The animals in Exp. I (heavier pigs) received 100 "rat doses"[1] of filtrate fraction and 100 "rat doses" of eluate fraction daily. For the pigs in Exp. II we could not afford such a liberal allowance of eluate; they received 100 rat doses of filtrate and 50 rat doses of eluate daily. These allowances were chosen as being roughly proportional to the food intake of the pigs compared with that of the rats. At the beginning of the experiment each pig consumed about 50–100 times as much dry food as a young rat. The above allowances, however, were not increased or decreased as the pigs consumed more or less food during the course of the experiment.

The amounts given would appear to have been adequate, as 5 pigs (nos. 60, 62, 63, 65, 66) grew well when receiving both fractions. The dose of filtrate, at least, was probably not excessive, as pigs (nos. 60, 63) in which the dose of filtrate was reduced for 2 weeks showed an immediate diminution in growth, which was restored as quickly when the dosage was increased.

All the pigs received the basal diet and, except for those having yeast (see below), daily doses of aneurin 1 mg., riboflavin 2 mg. and nicotinic acid 25 mg. The doses of aneurin and riboflavin, like those of eluate and filtrate fractions, were calculated on the requirements of rats. The daily dose of 25 mg. nicotinic acid was adopted on the basis of previous experiments with pigs [Chick et al. 1938, 1] in which 60 mg. daily produced an immediate and striking effect in animals which had been depleted for 2–3 months; we decided that this dose would be excessive as a prophylactic. Sebrell et al. [1938] found 10 mg. nicotinic acid twice weekly an effective prophylactic dose to prevent black-tongue in dogs of about 6 kg. weight.

The pigs were divided into groups of 2–4 animals; one group received in addition to the above a daily dose of Edgar and Macrae's filtrate fraction, a

---

[1] The "rat doses" of filtrate factor and eluate factor, referred to in this paper, are equivalent to the units of these factors defined by Edgar et al. [1938, 1]. The unit of eluate factor is the potency of an amount of standard eluate fraction equivalent to 2 g. dry yeast and the unit of filtrate factor is the potency of an amount of standard filtrate fraction purified by amyl alcohol extraction, also equivalent to 2 g. dry yeast. When rats, whose growth rates are limited by deprivation of either filtrate factor or eluate factor, receive daily 1 unit of the appropriate missing factor, immediate growth responses occur of approximately 90 % of the maximum.

second group received doses of their eluate fraction, a third group received both fractions and a fourth group yeast. The growth and health of these various groups were then studied.

General management of the animals

The general management of the animals has been previously described in detail [Birch et al. 1937]. The pigs receiving the same diet lived together in the same stall; these stalls consisted of commodious built-in rooms with concrete flooring and attached to each was a separate open run. The animals had no bedding, but a wooden platform was provided for them to sleep on. The diet was made up in batches of 50 lb. at a time; it was merely stirred with water to a thin cream and fed cold. The appropriate amounts of aneurin, lactoflavin, nicotinic acid and the various fractions for the whole group were stirred in immediately before feeding. The animals were fed twice daily up to the limit of their appetite. If any food was left at breakfast a corresponding diminution was made in their supper, so that all was consumed.

EXPERIMENTAL RESULTS

Experiment I

The initial object of this experiment was to ascertain what would happen to young pigs fed upon a diet of starch, casein, cod liver oil and cotton seed oil supplemented with (1) dried yeast and (2) aneurin, riboflavin and nicotinic acid. The experiment was begun on 2 May 1938. A litter of 8 pigs, 12 weeks old and of weights varying between 51 and 70 lb., was divided into 2 groups of 4 each.

The first group contained the lightest pigs (nos. 53, 54, 56 and 57). They had 4 % of dried yeast added to their diet and the casein and starch were correspondingly reduced. With the exception of no. 53, which died after 5 weeks and was found at autopsy to have a number of abscesses in the mesentery, these pigs made steady growth from the 2nd week onwards and gained an average of 10·9 lb. weekly. After 9 weeks the experiment was discontinued, as it was evident that 4 % yeast supplied all the B-vitamin requirements over this period (see Fig. 1).

The second group (nos. 50, 51, 52 and 55) received the same diet supplemented by aneurin, riboflavin and nicotinic acid. At first the animals grew well, but in all cases growth ceased abruptly after 3–4 weeks. These pigs did not manifest any symptoms of illness but it was clear that aneurin, riboflavin and nicotinic acid did not supply all that was required. From the 7th week the eluate fraction (100 rat day doses daily) was added to the diet of nos. 50 and 55 and the filtrate fraction (100 rat day doses daily) to that of nos. 51 and 52.

Pigs receiving eluate fraction

Unfortunately no. 55 developed an acute pneumonia shortly afterwards and died. At autopsy, extensive solidification of both lungs, purulent pleurisy and pericarditis were found. This pig cannot therefore be further considered.

Pig no. 50, after a resumption of growth which lasted 5 weeks, lost its appetite, suffered intermittently from diarrhoea with the passage of blood[1] and began to lose weight rapidly. The loss of weight continued although the diarrhoea abated. It was noticed to be weak in its hindquarters and moved with

[1] At autopsy this pig was found to have an infection of the mucous membrane of the large gut with the nematode Oesophagostomum dentatum.

a peculiar swaying gait. The weakness in the hindlegs increased until the animal had difficulty in maintaining equilibrium whilst eating or drinking and could not raise the hindlimbs sufficiently to scratch its body. At the end of the 14th week, that is 8 weeks after receiving the eluate fraction, the filtrate fraction was also added to its diet. A slight temporary improvement in appetite and general condition followed but the feebleness soon returned and the paresis of the hind quarters increased. Finally food was refused and the animal was killed.

Pigs receiving filtrate fraction

Pigs nos. 51 and 52 responded at once to the addition of filtrate fraction and for 1 month increased in weight at nearly the normal rate. At the end of this time growth ceased abruptly and during the next month No. 51 grew not at all and no. 52 but 7 lb. Both pigs then showed another spurt in growth which lasted for 3 weeks, during which each gained 20–25 lb. At the 10th week from the time they first received filtrate fraction growth again ceased in both of them (see Fig. 1). The first spurt of growth was understandable but the second was not. The latter coincided with the administration of a new preparation of filtrate fraction and ceased abruptly when that supply was exhausted. The most obvious interpretation would be that this particular preparation of filtrate fraction had been imperfectly fractionated, but we could not discover, by experiments upon rats, that it contained any eluate fraction. Pigs nos. 51 and 52 were occasionally observed to have epileptic fits.

Experiment II

This experiment, which was a continuation of the preceding one and over-lapped it, was begun on 30 May 1938 with 11 9-week old pigs from a litter which had just been weaned; their weights ranged from 25 to 39 lb.

Two of the lightest pigs were used to find out how much dried yeast was needed to supplement the basal casein-starch diet. From the rate at which nos. 54, 56 and 57 of Exp. I were growing we concluded that 4 % in the diet was perhaps excessive. After 3 weeks on the basal diet, the diet of nos. 59 and 67 therefore had 2 % yeast added to it. The subsequent growth was, however, very poor, and after a further 3 weeks the yeast supplement was increased to 6 %. Immediate increase in growth rate followed and the average gain in weight soon exceeded 10 lb. per week. When this occurred the amount of yeast was reduced to 4 %, and the rate of growth was maintained. We conclude, therefore, that the amount of yeast required to supply all the B-vitamins for pigs of this age, on so simple a diet as that here used, is between 2 % and 4 % and is nearer 4 %.

The remaining 9 pigs of this litter were used to ascertain the effect of giving the filtrate fraction and the eluate fraction alone and in combination. As the pigs were a month younger than those of Exp. I it was thought likely that the effects might be more striking.

For the first 3 weeks all 9 pigs received the basal diet with the additions only of aneurin, riboflavin and nicotinic acid, in the same dosage as was employed with the first litter. This procedure was adopted as a measure of economy, for we had difficulty in making enough of the eluate and filtrate fractions to keep pace with the requirements of the animals and it was thought that depletion of their reserves during this preliminary period would abbreviate the experiments. The pigs showed a slow, subnormal rate of growth.

At the end of the preliminary period 3 pigs (nos. 58, 61 and 64) received the eluate fraction in daily amounts equivalent to 50 rat day doses, 3 pigs (nos. 60,
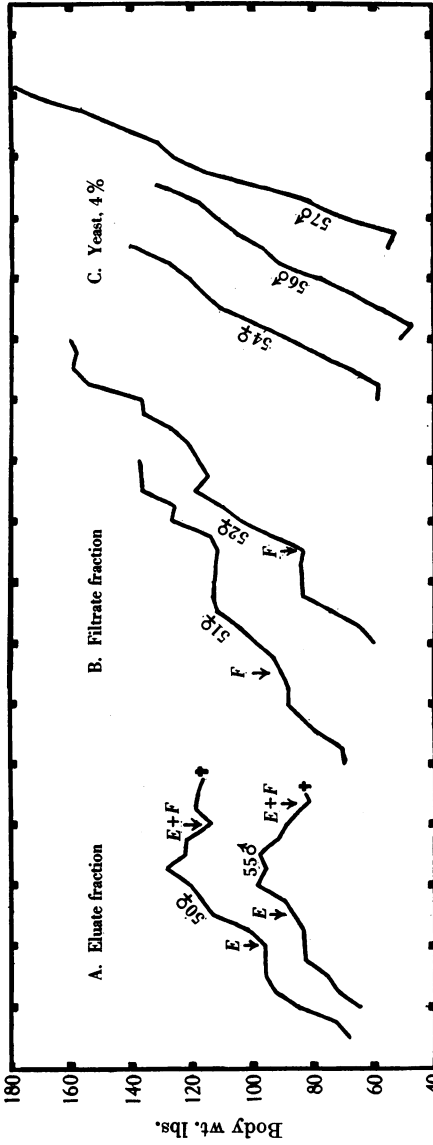
Fig. 1. Exp. I. Growth curves of young litter-mate pigs receiving a basal diet of purified casein, purified maize starch, cottonseed oil, cod liver oil and salt mixture, with daily rations of aneurin 1 mg., riboflavin 2 mg. and nicotinic acid 25 mg. Curves A, supplemented with 100 rat day doses of eluate fraction daily. Curves B, supplemented with 100 rat day doses of filtrate fraction daily. Curves C, basal diet supplemented with 4 % dried yeast. E signifies addition of eluate-fraction; F signifies addition of filtrate fraction; + signifies death. Divisions on the abscissa indicate periods of 4 weeks.
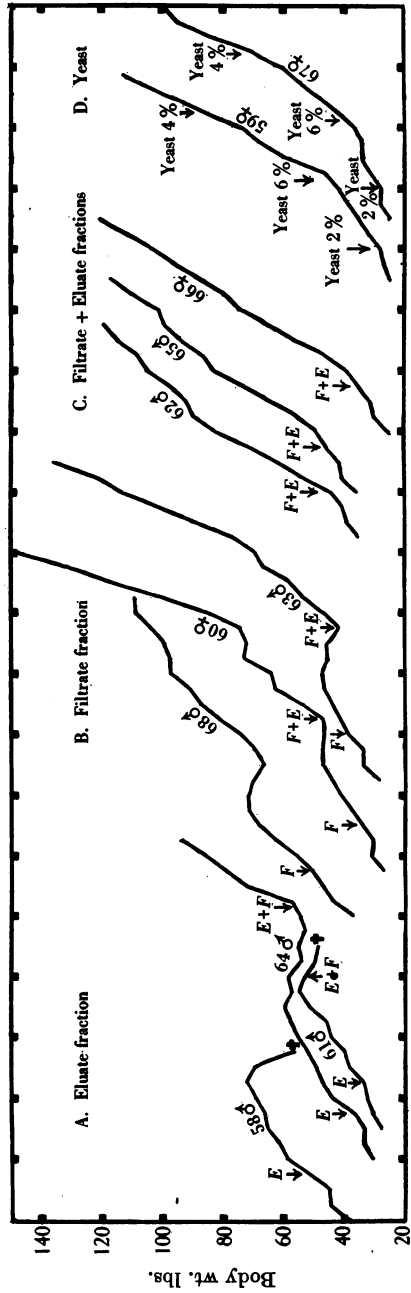
Fig. 2. Exp. II. Growth curves of young litter-mate pigs receiving a basal diet of purified casein, purified maize starch, cottonseed oil, cod liver oil and salt mixture, with daily rations of aneurin 1 mg., riboflavin 2 mg. and nicotinic acid 25 mg. Curves A, supplemented with 50 rat day doses of eluate fraction daily. Curves B, supplemented with 100 rat day doses of filtrate fraction daily. Curves C, supplemented with both fractions. Curves D, basal diet supplemented with dried yeast. E signifies addition of eluate fraction; F signifies addition of filtrate fraction; + signifies death. Divisions on the abscissa indicate periods of 4 weeks.

63 and 68) received the filtrate fraction in similar dosage and 3 pigs (nos. 62, 65 and 66) received both fractions. The growth curves of all these pigs are shown in Fig. 2.

Pigs receiving eluate fraction only

The pigs receiving eluate fraction only (nos. 58, 61 and 64) grew subnormally for 6–7 weeks, the average weight increase being about 3 lb. weekly. Afterwards appetite failed and there was an abrupt cessation of growth and a decline in weight; nos. 58 and 61 died 2–3 weeks later. Shortly before growth ceased, it was noticed that all 3 pigs had weakness of the hind quarters and sagging of their backs. These symptoms increased day by day and the gait became unusual, the hind quarters swinging as they walked. It appeared as if the back end of the pig were loosely attached to the rest of the body. The hind legs were slightly flexed at all 3 joints so that the hind portion of the body was lowered. The clumsiness of the movements of the hindquarters was most noticeable when turning and increased day by day, the condition becoming progressively worse until, after about 10 weeks from the commencement of the experiment, nos. 58 and 61 were almost completely paralysed in their hindquarters. They were unable to raise them from the ground and if lifted could not remain standing. It was a flaccid palsy and at no time spastic.

For some weeks the animals pulled themselves about with their front legs dragging the collapsed hinder end of their bodies, which were rotated laterally. At this stage the deep reflexes were absent and the pigs appeared to be insensitive over the distal half of their hindlimbs, since pricking, either superficial or deep, elicited no response. The fore quarters and head and neck were unaffected. The pigs could still eat but had to be fed by hand. No. 61 was given filtrate fraction when it was severely ill. It became brighter and for a day or two its appetite improved, but it died a few days later.

The third pig in this group, no. 64, maintained its weight rather longer. The early symptoms of damage to the nervous system occurred at about the same time as in the others but did not develop to the same extent. This animal had the same swinging gait and clumsiness of the hindquarters and often fell when turning quickly, but it could always stand or get up on its hindlegs, perhaps after one or two unsuccessful efforts. The paresis of the left hindleg was greater than that of the right and the animal would stand on 3 legs, swinging it loosely back and forward. It remained in much the same condition for a month. 13 weeks after receiving the eluate fraction it was given the filtrate fraction also, in amounts equivalent to 200 rat doses daily. Improvement in the general condition of the animal occurred immediately. Its appetite returned and during the next week it consumed 3 times as much food as before and put on 15 lb. in weight (see Fig. 2). At the same time it became much stronger and learnt to make the best of its disablement. The paresis of the left leg and clumsiness in movement, however, remained.

Pigs receiving filtrate fraction only

The 3 pigs (nos. 60, 63 and 68) which were given filtrate fraction only, remained in better condition than those having the eluate fraction. The improved rate of growth which occurred when filtrate factor was first added, after the pigs had been 3 weeks on the basal diet alone, lasted 3–4 weeks. For the next month, however, weights remained stationary. Nos. 60 and 63 were then given the eluate fraction in daily amounts equal to 50 rat doses. Their appetite immediately increased and they started growing well, their weekly increase in weight steadily increased and in 6 weeks reached 15 lb.

No. 68 continued to have filtrate factor only and from 12 August to 5 September 1938 the same unaccountable spurt in growth occurred as was shown at the same time by nos. 51 and 52 of the previous litter (see above, p. 2211). We at first suspected the particular sample of filtrate fraction, but after that sample had been replaced by another, no. 68, after a stationary period, again resumed a moderate rate of growth (Fig. 2), suggesting that our suspicions were unfounded and that intermittent growth may be characteristic of deficiency of the eluate fraction.

None of the pigs deprived of the eluate factor seemed particularly ill but they all suffered from epileptic fits. These were first observed in no. 63, 7 weeks after the commencement of the experiment, when growth had just ceased. During the next week no. 60 was seen in a fit and 3 weeks later no. 68. It is impossible to say how frequently the fits occurred as the animals were not under observation during the greater part of the 24 hr.

Only one fit was observed in nos. 60 and 63 after they were receiving the eluate fraction as well as the filtrate, and that occurred on the day following administration of the former. In the case of no. 68, which was kept without eluate for 20 weeks, the epileptic attacks became more frequent, as many as 5 being observed in 1 week and 2 on 1 day.

The pigs receiving the filtrate fraction only were also noticed to have become pale. Estimations of haemoglobin in their bloods 2–4 weeks after they had ceased to grow (8 weeks after the beginning of the experiment) showed it to be less than 60 % of the amount usually present in the blood of normally fed pigs of this age or in the blood of the pigs which were receiving yeast.

Pigs nos. 60 and 63 received the eluate fraction as well as the filtrate fraction from the 10th week onwards; the anaemia was steadily repaired and 8 weeks later their blood was nearly normal.

Details concerning the blood of all these pigs will be found on p. 2217, Table III below.

Pigs receiving both filtrate and eluate fractions

The 3 pigs (nos. 62, 65 and 66) which received both eluate and filtrate fraction at the same time as the preceding groups received one only of these fractions, remained healthy and grew steadily during the 11 weeks they were observed after these additions were made to their diets. They had no nervous symptoms and their blood remained normal. They did not, however, grow so well, although they ate as much, as those receiving yeast. Their average increase in weight per week was 7 lb. whereas that of the latter was 8·5 lb. It was also noted that their appetite diminished towards the end of the period whereas it should have become greater, having regard to their increased body weight.

We are able to compare the gain of weight per unit of food consumed by the pigs in these 2 groups over periods of 4 weeks, during which all the animals were of approximately the same weight. The pigs to which yeast was given gained 15·5 kg. and consumed 22 kg. of dry food. Those having both eluate and filtrate gained 16 kg. and consumed 29 kg. food. If 0·5 kg. of dry food per day be subtracted as the maintenance allowance for pigs of this weight—an amount representing the average amount of this diet required to maintain weight—the productivity, $\dfrac{\text{Wt. gained}}{\text{Food consumed} - \text{maintenance allowance}}$, was 1·4 for the diet with yeast and 1·0 for the diet with eluate and filtrate.

These facts suggest either that the supplements of aneurin, riboflavin, nicotinic acid, eluate fraction and filtrate fraction were not given in adequate amount,

or that, when combined, they do not possess quite all the nutritive virtues of whole yeast, a conclusion similar to that reached by Edgar *et al.* [1938, 3] from their experiments with rats.

HAEMATOLOGICAL OBSERVATIONS

Anaemia from lack of mixtures of B-vitamins has been recorded in rats by Damianovich *et al.* [1923], Whitehead & Barlow [1929], Sure *et al.* [1931] and by György *et al.* [1937]; in pigeons by Barlow [1927] and by Hogan *et al.* [1937]; in dogs by Rhoads & Miller [1933], Spies & Dowling [1935] and by Fouts *et al.* [1938]; in monkeys by Day *et al.* [1935], Wills & Stewart [1935] and by Wills & Evans [1938]; in pigs by Miller & Rhoads [1935], Birch *et al.* [1937] and by Wintrobe *et al.* [1938].

The observations of Miller & Rhoads [1935] and of Wintrobe *et al.* [1938] are particularly pertinent to the present investigations because they concerned pigs, and those of Fouts *et al.* [1938] because they cured the anaemia with a fraction from rice polishings containing the "rat anti-dermatitis factor" (vitamin B_6). As will be seen below (p. 2221), Edgar and Macrae's eluate fraction which prevented or cured the microcytic anaemia we encountered in our pigs also contains vitamin B_6.

Systematic observations on the blood of the pigs were not carried out from the beginning of our experiments but as after 8–10 weeks on the deficient diets all the pigs were observed to be paler, determinations of haemoglobin and examinations of blood films were undertaken. These showed that a considerable degree of anaemia existed both in the animals deprived of eluate fraction and in those deprived of filtrate fraction and that the anaemia of the former was associated with the presence of large numbers of small corpuscles, $3–4\mu$ in diameter (microcytic). Henceforth, a more complete examination of their bloods was made from time to time. The observations are set out below in tabular form (see Tables I–III). Incomplete as they are, they permit of some definite conclusions concerning the effect on the blood picture of deprivation of Edgar and Macrae's filtrate and eluate fractions.

The blood was drawn from a vein either of the ear or of the tip of the tail. The haemoglobin determinations were made with a Sahli haemometer and checked with a Haldane CO haemoglobinometer. Fresh standards were prepared of such strength that the 100 mark on the tubes corresponded to 18·5 vol. O_2 or 13·8 g. haemoglobin per 100 ml. blood. The error of the pipette was ascertained by drawing human blood to the 20 c.mm. mark, allowing it to clot, weighing the pipette filled with blood and subtracting the weight of the pipette. The specific gravity of the blood was assumed to be 1·060. The graduated tubes were calibrated by adding approximately 1 and 2 g. water, weighing them and noting the levels in the tubes.

The corpuscular volume was ascertained by centrifuging bloods containing 0·2% potassium oxalate in tubes of 1 mm. bore and 10 cm. length for half an hour at 4000 r.p.m. The proportion of the length of the column of corpuscles to that of the blood was multiplied by 1·1 to compensate for the shrinkage of the corpuscles due to this strength of oxalate [Wintrobe, 1931].

The mean corpuscular vol. in μ^3

$$= \frac{\text{Vol. corps.}}{\text{No. of corps.}} \text{ per } \mu l. \times 10^9.$$

Dr Lucy Wills very kindly measured the red corpuscles and plotted Price Jones curves from films taken from pigs nos. 61 and 64 which had been deprived

of filtrate fraction and from pigs nos. 63 and 68 which had been deprived of eluate fraction, and compared them with films taken from normal pigs of about the same age. The mean diameter, standard deviation and coefficient of variability arrived at from this laborious procedure, for which we cannot be too grateful, were:

No. pig	Mean diameter μ	Standard deviation	Coefficient of variability
Normal	5·87	0·48	8·2
61	5·83	0·69	11·8
64	6·25	0·49	7·9
63	5·64	0·69	11·8
68 (19. viii. 38)	5·16	0·98	18·9
68 (13. ix. 38)	5·54	0·84	15·9

The films supplied were poor ones, which perhaps accounts in some measure for the high standard deviation from the mean diameter. The changes in mean diameter are consistent with the results obtained by the haematocrit method, which by itself gives no indication of the variability in size of the corpuscles.

The data obtained from the examination of the blood of pigs nos. 59 and 67 will serve as a standard of reference (see Table I). These pigs taken from the same litter had been fed for 2 months on the basal diet supplemented by yeast; they were growing rapidly and were in excellent condition. The figures are close to some recently obtained by Mr Parry (personal communication) at the Institute of Animal Pathology, Cambridge, for healthy pigs somewhat younger than the present ones fed on an ordinary good diet. Mr Parry's data were obtained by methods precisely similar to ours. They also correspond closely with the averages of those found by Miller & Rhoads [1935] for their 15 young pigs before they received a deficient diet. We therefore assume that they may be taken as fair average values for normal pigs.

Table I. *Blood examination of normal pigs*

No. pig	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb, g. per 100 ml. corpuscles	Remarks
I. Pigs nos. 59 and 67 after 10 weeks on basal diet supplemented by yeast; age 5 months; wts. 108 and 95 lb. respectively						
59	7·8	12·9	43	55	30	Films show little scatter in the size of cells; most appear to be about 6μ in diameter
67	8·1	12·4	44	54	28	
II. Pigs nos. 62, 65 and 66, after 3 weeks on basal diet and 10 weeks on basal diet supplemented by both eluate and filtrate fractions; age 5 months; wts. 115, 109 and 112 lb. respectively						
62	7·5	11·8	40	53	29	Most cells appear to be around 6μ in diameter
65	8·1	12·4	42	52	29	
66	8·0	12·9	45	56	28	

The effect of deprivation of filtrate fraction was to produce a moderate degree of anaemia in which both the number of blood corpuscles and the concentration of haemoglobin were reduced to about 2/3 of their normal amounts. The size of the corpuscles underwent little change. This type of anaemia is similar to that observed by Wintrobe et al. [1938] in pigs receiving minimal amounts of yeast. On receiving filtrate fraction, the composition of the blood of pig no. 64 (the only surviving pig in this group) showed little improvement after

Table II. *Effect on blood of deprivation of filtrate fraction*

Pigs nos. 58, 61 and 64. At the date (15. viii. 38) of the first observation they were 3 months old and had received the basal diet for 3 weeks, followed by 8 weeks on the same diet supplemented by eluate fraction; wts. 60, 50 and 60 lb., respectively. From 23. ix. 38 onwards no. 64 received filtrate fraction also.

No.	Date	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb, g. per 100 ml. corpuscles	Remarks
58	15. viii. 38	—	7·9	—	—	—	Paralysed, losing wt.; died 17. viii. 38
61	15. viii. 38	—	7·0	—	—	—	Paralysed, losing wt.; died 25. viii. 38
64	15. viii. 38	—	9·6	—	—	—	Partial paralysis; wt. stationary
64	13. ix. 38	6·3	8·5	30	48	28	Films show abnormal scatter, mean diameter about 6μ
64	21. ix. 38	5·6	7·7	29	52	26	—
64	1. x. 38	5·4	6·9	26	48	26·5	Filtrate fraction also given from 23. ix. 38
64	11. x. 38	5·9	7·6	30	51	25	—

Table III. *Effect on blood of deprivation of eluate fraction*

Pigs nos. 60, 63, 68. At the date of the first observation (25. vii. 38) they were 4 months old and had received the basal diet for 3 weeks, followed by 5 weeks on the same diet supplemented by filtrate fraction only; wts. 47, 46 and 72 lb., respectively, and weight stationary. From 8. viii. 38 onwards nos. 60 and 63 were given eluate fraction also.

No.	Date	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb, g. per 100 ml. corpuscles	Remarks
68	25. vii. 38	—	7·9	—	—	—	—
68	19. viii. 38	6·5	6·1	24	37	25	Numbers of small corpuscles of about 4μ diam.
68	13. ix. 38	7·0	5·8	24	34	24	—
68	21. ix. 38	7·1	4·2	23	32	17·5	—
68	11. x. 38	11·3	6·3	27	24	23·5	Numbers of small cells much increased
60	25. vii. 38	—	6·8	—	—	—	Amisocytosis: large numbers of small corpuscles of about 4μ diam.
63	25. vii. 38	—	7·1	—	—	—	

From 8. viii. 38 nos. 60 and 63 received eluate also

No.	Date	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb, g. per 100 ml. corpuscles	Remarks
60	19. viii. 38	7·8	6·7	28	36	24	—
63	19. viii. 38	7·1	7·2	26	36	27	Still numbers of small corpuscles of about 4μ diam.
60	13. ix. 38	—	8·7	—	—	—	—
63	13. ix. 38	—	8·9	—	—	—	—
60	21. ix. 38	—	9·8	—	—	—	—
63	21. ix. 38	—	9·6	—	—	—	—
60	5. x. 38	8·8	11·7	37	42	31	Numbers of small corpuscles diminished
63	5. x. 38	8·3	12·0	33	40	36	—
60	15. x. 38	8·4	12·1	40	47	30	Few small corpuscles seen
63	15. x. 38	7·9	11·8	37	42	32	—

3 weeks, but this does not necessarily mean that its blood-forming tissue has not responded to the addition. Immediately after it received the filtrate fraction this pig started to grow at a great rate and added 25 lb. (45 %) to its body weight during the first 18 days and presumably the blood volume was also considerably increased (see Table II).

The effect of deprivation of eluate fraction was at first to cause a small decrease in the number of blood corpuscles and a considerable decrease in the size. In the case of pig no. 68, in which the deprivation was continued longest, the number of corpuscles increased later (11. x. 38) to about 50 % in excess of the usual number and their mean volume was reduced to about one half. The large proportion of small corpuscles ranging about 4μ in diameter was at once obvious on inspecting a dried film or when counting them in the haemocytometer. The amount of haemoglobin per ml. blood was half, and per unit of corpuscular volume three-quarters, of the normal, the result being a microcytic hypochromic anaemia. In those pigs, nos. 60 and 63, in which after 8 weeks the deprivation of eluate fraction was made good, the small corpuscles disappeared from the blood, slowly at first and then steadily, until after 7 weeks their size was nearly normal. At the same time the amount of haemoglobin increased and approached normality (see Table III).

Here again the initial slowness of recovery in the blood picture must again be considered in the light of the concomitant sudden increase in growth rate, and presumably in blood volume, brought about by restoration of the missing factor in the diet.

Comparison of our results with those of others leads us to conclude that there is more than one substance in the vitamin B_2 complex, the lack of which embarrasses the blood-forming tissues.

The anaemia observed by Wintrobe et al. [1938] in pigs fed on a synthetic diet not unlike that used by us, to which diminishing quantities of yeast were added as the experiment proceeded, was of the simple type which occurred in pigs nos. 58, 61 and 64 (see p. 2217, Table II above). These were the animals from which filtrate fraction was withheld. The number of red blood corpuscles and the amount of haemoglobin in their blood fell together without any obvious change in the dimensions of the red cells.

The anaemia in dogs suffering from black-tongue, produced by feeding them on Goldberger's pellagra-producing diet, was found by Rhoads & Miller [1933] to be of the macrocytic type. Later, when Miller & Rhoads [1935] turned their attention to pigs on a similar diet, they found the same evidence of increased activity of the bone marrow but the anaemia was microcytic in some animals and macrocytic in others.

Wills & Stewart [1935] produced a profound macrocytic anaemia by long-continued feeding of monkeys on polished rice, margarine, white bread and cod liver oil. Some success was obtained by treating the monkeys with Edgar and Macrae's filtrate fraction [Wills & Evans, 1938]. As it is the eluate fraction which prevents or cures the microcytic anaemia we have encountered, it is difficult to see the connexion between their experiments and ours. Nor is the precise relationship obvious between our observations and those of György et al. [1937] on aplastic anaemia in rats receiving a synthetic diet, beyond the fact that the anaemia was cured in both instances by an "impure preparation of vitamin B_6". Purified preparations of vitamin B_6, whilst curing the dermatitis characteristic of deficiency of this vitamin in rats, had no effect on the anaemia. In a later paper György [1938] concluded that the anti-anaemic substance was nicotinic acid. The anaemia in our pigs nos. 60, 63 and 68 deprived of eluate fraction,

was not aplastic in type and as it developed in spite of a daily ration of nicotinic acid, there does not appear to be much correspondence between these observations on rats and our own.

The recent observation of Fouts *et al.* [1938] that hypochromic microcytic anaemia, accompanied by convulsive fits, occurred in puppies maintained on a synthetic diet, do, however, afford an almost complete parallel with some of our experiments on pigs. Their basal diet included vitamins A and D, vitamin B_1, either as crystalline material or as purified concentrate, and riboflavin. The remaining constituents of the vitamin B_2 complex were provided as (1) a liver extract which contained the chick anti-dermatitis filtrate factor [Elvehjem & Koehn, 1935; Lepkovsky & Jukes, 1935; 1936] and nicotinic acid, and (2) an extract of rice polishings containing the "rat anti-dermatitis factor" (vitamin B_6). The activities of their preparations were controlled by experiments on rats and chickens. Four puppies which received the basal diet and liver extract only (Lepkovsky's filtrate factor + nicotinic acid) developed a severe anaemia and exhibited convulsive fits. The anaemia was cured when extract of rice polishings was added to the diet. Preparations (1) and (2) above correspond broadly to our "filtrate" and "eluate" fractions, respectively, except that in our preparations from yeast or liver the nicotinic acid or amide originally present in these sources was contained in the eluate fraction, being adsorbed together with it on fuller's earth at pH 1·3 and subsequently eluted, whereas in the preparation used by Fouts and his colleagues nicotinic acid was present in the filtrate after adsorption of their liver extract with Lloyd's reagent (see also below, p. 2222).

The results of the experiments of Hogan *et al.* [1937] are also difficult to bring into line, partly because no details are given. Pigeons were fed on a diet of casein, sucrose, cellulose and cod liver oil and, when polyneuritis had developed a concentrated preparation of vitamin B_1 was given. This cured the polyneuritis but about 2 months later the birds became profoundly anaemic. Neither riboflavin nor "anti-dermatitis concentrate" (?chick anti-dermatitis factor) was curative.

The anaemia occurring in pigs after gastrectomy, which has been studied by Bence [1933; 1934; 1936] and by Petri *et al.* [1937], should perhaps be mentioned, as this may be partly due to "conditioned deficiency", although the former author adduces evidence to show that it is caused by deprivation of Castle's intrinsic factor, which is manufactured in the mucous membrane of the normal stomach. According to Petri *et al.* [1937] the pigs manifested symptoms suggestive of pellagra and Bence's interpretation is not to be regarded as sufficient. An interesting pathological point is that, for the first year after the operation, the anaemia was of the hypochromic microcytic type associated with hyperplasia of the bone marrow. Bence reports that it subsequently became macrocytic and hyperchromic. The pigs studied by Petri and his colleagues were not under observation long enough for this result to be confirmed.

The confusion indicated in the précis of literature given above will not be cleared up until it is possible to employ pure substances in nutritive experiments in which one deficiency only is present. Even then it will probably be found that results obtained with one species of animal cannot necessarily be applied to another.

Nervous symptoms in pigs deprived of eluate factor and of filtrate factor

All the pigs in these groups had nervous manifestations; those deprived of eluate fraction had fits and those deprived of filtrate fraction became paralysed.

Altogether, some 30 fits were recorded and it is probable that more occurred when nobody was there to observe them. Their severity and frequency increased with the length of time the animals had been deprived of eluate fraction. They were typical epileptic fits as seen in the human being. The pig ran round in apparent terror, screamed (epileptic cry) and dropped as if shot, making no effort to guard the fall; tonic spasm followed during which the legs were extended. The pig became deathly white. This first stage was succeeded by a clonic stage, during which the limbs were jerked about violently; there was grinding of teeth and sometimes discharge of urine. The comatose stage followed; respiration was at first deep and stertorous, gradually becoming shallow. The pig looked as if dead but in a few minutes the colour improved, consciousness was recovered and the animal got up languidly and resumed his previous occupation, e.g. feeding. The duration of the fits was from a few minutes to a quarter of an hour. In the intervening periods the pigs appeared to be quite well. Minor epileptic manifestations were also observed in which the animals appeared dazed, butted into obstacles, tottered about without always actually falling and then rapidly regained consciousness. After the eluate fraction was added to pigs nos. 60 and 63 the epileptic seizures ceased.

In the experiments on puppies reported by Fouts et al. [1938] convulsions were associated with deprivation of a filtrate fraction somewhat similar to our own, prepared from liver; some of the puppies died during the fits. In experiments of earlier workers on dogs deprived of B-vitamins, convulsions were observed towards the termination of their lives, but as far as we know the only record of epilepsy being caused by a nutritive deficiency is one by Sheehy & Senior [1930], in a group of pigs deprived of vitamin D and shielded from ultraviolet rays by glass. The fits occurred from the seventh week of the experiment onwards and three of the animals died from them.

The paralytic symptoms which developed in the pigs deprived of filtrate fraction are described above (p. 2213) in the history of the animals in this group. It was a flaccid paraplegia confined to the hindquarters and accompanied by some disturbance of sensation. Once developed it was not cured by giving filtrate fraction (see pig no. 64).

Posterior paralysis of swine has been not infrequently encountered in young pigs in the United States. A good description of the disease was given by Wehrbein [1916] who found demyelination in the spinal cord and peripheral nerves. The symptoms described correspond closely with those observed in pigs nos. 58, 61, 64. ·Sporadic cases of posterior paralysis in pigs are also recorded by Doyle [1937]. Some of these occurred under conditions which rendered it improbable that the cause was nutritional in origin and Doyle therefore suggested that there was an infective form of the disease. The discovery of some scattered areas of small cell infiltration in the nerves and spinal cord supported this view.

Paraplegias have been reported in swine as the result of an inadequate supply of vitamin A by Hughes et al. [1928], Dunlop [1934], Eveleth & Biester [1937] and Foot et al. [1938]. The nervous symptoms observed by Dunlop were uncommonly like those seen by us, but they took a much longer time to develop. Deficiency of vitamin A can hardly have been responsible for the paralysis of our pigs which were deprived of filtrate fraction. Each pig received daily 25 g. or more of a reliable cod liver oil certified to contain more than 1000 I.U. vitamin A per g. The requirements of the pig for this vitamin were carefully determined by Dunlop [1935] who concluded that 6000 I.U. daily affords a 50 % margin for safety.

The recently published observations on pigs of Wintrobe et al. [1938] have a close bearing upon our own. The basal diet, consisting of purified casein, butter,

sucrose, cod liver oil and salts, was similar to that used by us. At first, 3 g. yeast per kg. body-weight were given daily, this dose being subsequently gradually reduced to 0·1 g. per kg. As the yeast was reduced, crystalline aneurin and riboflavin were given. After about 200 days the animals manifested nervous symptoms. These consisted of progressive ataxia, with some loss of deep and superficial sensation but without much motor weakness. Histologically there was severe degeneration of the posterior columns of the spinal cord, the dorsal ganglion cells and the peripheral nerves.

From a scrutiny of the diet it would appear that the pigs in these experiments of Wintrobe and his colleagues were gradually deprived of nicotinic acid and of the vitamins contained in our eluate and filtrate fractions. The symptoms were more of the sensory type than in our pigs, but further comment is deferred until the nervous systems of our animals have been examined by appropriate histological methods. Until that has been done we also postpone consideration of many interesting papers on the nervous lesions produced in other animals by feeding on diets deficient in some or all of what is conveniently called the vitamin B_2 complex.

Comparison of our eluate and filtrate fractions with those of other investigators

The fractions containing water-soluble B-vitamins other than aneurin and riboflavin which have been described by various investigators have mostly been prepared from aqueous extracts of liver, yeast and rice polishings by treatment with fuller's earth, but the conditions under which the adsorptions have been carried out have varied.

It is unlikely that the extracts of liver, yeast and rice polishings from which these fractions have been separated were nutritionally equivalent and it is quite possible, even should they be so, that the individual factors were present in the various extracts in different chemical combinations. The amount and composition of other materials present differ and affect the results of the adsorption processes. Further, fuller's earth is by no means a standard product, the adsorptive properties of different samples varying considerably. It is therefore not surprising to find that after fractionation of extracts of liver, yeast and rice polishings in various laboratories with the use of fuller's earth, the different essential nutrients are not always found in the corresponding fractions. For example, in the case of yeast, the fuller's earth adsorbate contains the greater part of the nicotinamide or other active pyridine base, as has been demonstrated in experiments on dogs and pigs, while in the case of some extracts of liver and of rice polishings these compounds have been found present in the fuller's earth filtrates. Similarly, filtrates obtained after treatment of liver extracts with fuller's earth are by no means nutritionally equivalent to those obtained by exactly similar methods from yeast when tested on the rat [Edgar et al. 1938, 2].

Comparison of the liver fractions used in this work with fractions prepared by others is therefore very difficult. When tested on rats, they had the same action as the filtrate and eluate fractions prepared from yeast by means of fuller's earth [Edgar et al. 1938, 2].

The eluate fraction certainly contained vitamin B_6, the rat anti-dermatitis factor of György [1935]. We found that the crystalline factor 1 of Lepkovsky [1938], which is identical with vitamin B_6, completely replaced our eluate fraction in the diet of the rat [unpublished results]. The rice polishings eluate used in the experiments on dogs by Fouts et al. [1938], in which a nutritional anaemia similar to that now observed in pigs was noted, would be similar to our liver eluate factor, in so far as they both contained vitamin B_6. It is

probable that our eluate fraction also contained nicotinamide [cf. Dann & Subbarow, 1938]. Since, however, our pigs received nicotinic acid in their basal diet, the possibility that nicotinamide was the essential nutrient supplied by the eluate fraction is excluded. Pigs deprived of eluate fraction were certainly deprived of vitamin B_6 but, until the experiments can be repeated with the use of crystalline vitamin B_6, the question whether all the symptoms observed are due to a deficiency of this vitamin must remain unanswered.

Our filtrate fraction apparently contained the nutrient required for growth of rats named factor 2 by Lepkovsky et al. [1936]. The growth-promoting action of their factor 2 for rats was very similar to that possessed by our liver filtrate fraction.

Lepkovsky & Jukes [1936] first gave the name "filtrate factor" to the substance obtained after treatment with fuller's earth of an extract of beef liver. This substance had a specific effect in the prevention and cure of the dermatitis described by Elvehjem & Koehn [1935] which occurs in chicks receiving a diet of yellow maize, wheat middlings and casein heated for 144 hr. at 100°. Lepkovsky & Jukes showed that their filtrate factor withstood autoclaving at pH 5·0, was not precipitated by lead acetate at pH 6·6 or by barium hydroxide, was not adsorbed on norite charcoal or lead sulphide and was extracted by amyl alcohol. Although in many respects our filtrate factor possessed similar properties to these, it is uncertain whether the chick "anti-dermatitis factor" which was also present in Lepkovsky's factor 2 and is possibly identical with the rat growth filtrate factor of Edgar and Macrae, would be contained in all the preparations of "filtrate fraction" used by us. From the properties described for this chick factor, the earlier batches used by us ought to have contained it, but the later batches made from phenol extracts would only have contained it if it is extractable by phenol. It is most improbable that our filtrate fraction would contain nicotinamide or other pyridine bases, since one step in its preparation was extraction from strong acid solution by amyl alcohol. The liver filtrate fractions used by Fouts et al. [1938] would probably contain all factors present in our filtrate fraction, and nicotinamide in addition. The relation of our fractions to the Elvehjem factor W [Elvehjem et al. 1936; Frost & Elvehjem, 1937] is obscure.

As was the case with our eluate fraction it is impossible to tell at present whether the vitamins in our filtrate fraction which are essential for the rat and the pig, respectively, are identical.

SUMMARY

1. Pigs can be reared on a synthetic diet containing purified casein, purified starch, cottonseed oil, cod liver oil and a suitable salt mixture, and optimum growth can be obtained, when 4 %, but not 2 %, dried yeast is added.

2. When the above diet was supplemented by aneurin, riboflavin and nicotinic acid, growth ceased after 3–5 weeks according to the age and reserves of the animals at the beginning of the experiment.

3. If to the diet supplemented as described under 2 was added either the eluate fraction of Edgar and Macrae or their filtrate fraction, prepared from liver, growth proceeded at about one-third the normal rate, but was checked or arrested after 4–6 weeks.

4. When the eluate fraction was added to the diet of pigs which had previously received the filtrate fraction only and whose growth was arrested, there was an immediate and continuous gain in weight.

5. Addition of filtrate fraction to the diet of those pigs which had previously received only eluate fraction was followed by immediate improvement in appetite and rapid increase in weight in the one case in which paralysis was slight and the animal was not too ill to respond. The other 3 animals died in a few days after the filtrate was administered.

6. When, after about 3 weeks on the basal diet supplemented as described under 2, both eluate and filtrate fractions were given, there was an immediate response in food intake and good growth was observed for about 5 weeks, after which there was no further increase in appetite and the rate of gain in weight slackened accordingly. It is therefore probable that yeast contains some unidentified essential nutrient for the pig in addition to aneurin, nicotinic acid, riboflavin and what may be contained in the eluate and filtrate fractions.

7. Pigs maintained on the basal diet supplemented by eluate only, developed a flaccid palsy of the hindquarters.

8. Pigs receiving supplements of filtrate only, developed a microcytic anaemia and had typical epileptic fits. On receiving supplements of the eluate the blood returned to normal and the fits ceased.

9. In addition to aneurin, nicotinic acid and probably riboflavin, at least two further water-soluble vitamins are necessary for the nutrition of the pig. These vitamins are contained in the filtrate and eluate fractions of Edgar and Macrae; whether the whole activity of the eluate fraction is to be accounted for by the vitamin B_6 which it contains, cannot be decided until this vitamin is available in the pure state in sufficient quantity to be tested on pigs.

REFERENCES

Barlow (1927). *Amer. J. Physiol.* **83**, 237.
Bence (1933). *Wien. med. Wschr.* **83**, 1055.
—— (1934). *Z. klin. Med.* **126**, 127.
—— (1936). *Z. klin. Med.* **130**, 275.
Birch, Chick & Martin (1937). *Biochem. J.* **31**, 2065.
Chick, Macrae, Martin & Martin (1938, 1). *Biochem. J.* **32**, 10.
—— —— —— —— (1938, 2). *Biochem. J.* **32**, 844.
Damianovich, Bianchi & Savazzin (1923). *C.R. Soc. Biol., Paris,* **88**, 377.
Dann & Subbarow (1938). *J. Nutrit.* **16**, 183.

Day, Langston & Shukers (1935). *J. Nutrit.* **9**, 637.
Doyle (1937). *J. Amer. vet. med. Ass.* **90**, 656.
Dunlop (1934). *J. agric. Sci.* **24**, 435.
—— (1935). *J. agric. Sci.* **25**, 217.
Edgar & Macrae (1937). *Biochem. J.* **31**, 886.
—— El Sadr & Macrae (1938, 1). *Biochem. J.* **32**, 2200.
—— —— —— (1938, 2). *Biochem. J.* **32**, 2225.
—— —— —— (1938, 3). *Chem. Ind.* **57**, 1111.
Elvehjem & Koehn (1935). *J. biol. Chem.* **108**, 709.
—— Koehn & Oleson (1936). *J. biol. Chem.* **115**, 707.
Eveleth & Biester (1937). *Amer. J. Path.* **13**, 257.
Foot, Golding & Kon (1938). *Nat. Inst. Res. Dairying*, Publ. No. 462.
Fouts, Helmer, Lepkovsky & Jukes (1938). *J. Nutrit.* **16**, 197.
Frost & Elvehjem (1937). *J. biol. Chem.* **121**, 255.
György (1935). *Biochem. J.* **29**, 741.
—— (1938). *Proc. Soc. exp. Biol. N.Y.*, **37**, 732.
—— Goldblatt, Miller & Fulton (1937). *J. exp. Med.* **66**, 579.
Hogan, Richardson & Johnson (1937). *J. biol. Chem.* **119**, Scientific Proc. l.
Hubbell, Mendel & Wakeman (1937). *J. Nutrit.* **14**, 273.
Hughes, Aubel & Leindhart (1928). *Tech. Bull. Kansas State Coll.* no. 23.
Lepkovsky (1938). *J. biol. Chem.* **124**, 125.
—— & Jukes (1935). *J. biol. Chem.* **111**, 119.
—— —— (1936). *J. biol. Chem.* **114**, 109.
—— —— & Krause (1936). *J. biol. Chem.* **115**, 557.
Miller & Rhoads (1935). *J. clin. Invest.* **14**, 153.
Petri, Boggild, Soeborg-Ohlsen & Wanscher (1937). *Acta Path. microbiol. scand.* **14**, 111.
Rhoads & Miller (1933). *J. exp. Med.* **58**, 585.
Sebrell, Onstatt, Fraser & Daft (1938). *J. Nutrit.* **16**, 355.
Sheehy & Senior (1930). *J. Dept. Agric. Ireland*, **30**, 1.
Spies & Dowling (1935). *Amer. J. Physiol.* **114**, 25.
Sure, Kik & Smith (1931). *Proc. Soc. exp. Biol., N.Y.*, **28**, 498.
Wehrbein (1916). *J. Amer. vet. med. Ass.* **49**, 2, 238.
Whitehead & Barlow (1929). *Amer. J. Physiol.*, **89**, 542.
Wills & Evans (1938). *Lancet*, II, 416.
—— & Stewart (1935). *Brit. J. exp. Path.* **16**, 444.
Wintrobe (1931). *J. Lab. clin. Med.* **17**, 899.
—— Mitchell & Kolb (1938). *J. exp. Med.* **68**, 207.

Note added 7 December 1938. Since the above paper was sent to press we have received the report of work by Hughes [1938, *Hilgardia*, **11**, 595], on the requirements of the pig for different members of the vitamin B complex. Although his basal diet and supplements were not as highly purified as those used by us, his observations were made on more animals, 83 young pigs in all; the results are in general agreement with our own. Vitamin B_1, riboflavin given as whey adsorbate, nicotinic acid and "rice bran filtrate" (Lepkovsky's factor 2) were all needed to supplement a basal diet of polished rice, purified casein, salt mixture and cod liver oil. The whey adsorbate, given as source of riboflavin, doubtless contained our "eluate factor". The growth observed with the above supplements was superior to that occurring when 3 % yeast was added to the basal diet. This is in agreement with our observations indicating 4 % yeast as the minimum required to ensure an adequate supply of all the B-vitamins.