CCLXXXVI. THE WATER-SOLUBLE B-VITAMINS

XII. THE PREPARATION FROM LIVER OF FRACTIONS CONTAINING THE SAME DIETARY ESSENTIAL FACTORS FOR THE RAT AS YEAST ELUATE FRACTION AND YEAST FILTRATE FRACTION

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(Received 1 November 1938)

IN this and certain other laboratories yeast has been employed as a source of the B-vitamins, whilst some workers have favoured the use of liver. Similar experimental methods have been used for the fractionation of liver and yeast extracts, and of these adsorption with fuller's earth has been most generally employed. We have obtained by this means two fractions from autoclaved yeast extracts containing distinct rat dietary essentials. These we have called yeast eluate factor and yeast filtrate factor [Edgar & Macrae, 1937].

The identity of yeast eluate factor, vitamin B₆ [György, 1935] and factor 1 [Lepkovsky *et al.* 1936] is established [Edgar, El Sadr & Macrae, unpublished]; the relationship of the yeast filtrate factor to similar factors described by other workers is discussed in the preceding paper [Edgar *et al.* 1938].

Fractionation of liver by the methods we used to separate the factors present in yeast extracts did not yield fractions with biological properties corresponding to those of the yeast fractions. However, by other methods we have obtained fractions from liver extracts biologically similar to the yeast eluate and filtrate fractions. The present paper describes these experiments.

EXPERIMENTAL

In general the rat growth method used in the testing for yeast eluate and yeast filtrate factors has been employed [Edgar *et al.* 1938]. The preliminary preparation of the rats was carried out as described, the young animals receiving for the first week after weaning the same basal diet and daily supplements of 0.08 ml. of cod liver oil and 10 μ g. of aneurin. Thereafter, the experimental methods were varied to some extent, as is described in the separate experiments.

Yeast eluate fraction and yeast filtrate fraction were prepared by the methods described in the previous paper [Edgar *et al.* 1938]. All the liver fractions were prepared from liver residues obtained in the manufacture of the pernicious anaemia factor [Laland & Klem, 1936]; these were kindly supplied to us by Messrs Glaxo Laboratories. Three residues have been investigated.

Liver residue I. This residue was the filtrate resulting when a liver extract, obtained by extraction of liver with aqueous acetone and subsequent removal of the acetone, was treated with charcoal. Experiments with rats showed that this residue probably contained all of the factors of the vitamin B₂ complex with the exception of riboflavin, since rats receiving the basal diet supplemented by cod

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liver oil, aneurin, riboflavin and this liver residue grew as well as did rats receiving that diet with whole yeast or liver extract as the source of the B₂-vitamins.

Liver residue II. The liver extract made by aqueous acetone extraction of liver, freed from acetone and reduced to small volume was extracted with phenol. The fraction insoluble in phenol was liver residue II. This liver fraction was also rich in members of the vitamin B₂ complex, although it contained less of the liver factor corresponding to yeast filtrate factor than did liver residue I.

Liver residue III. The aqueous layer, obtained when the above phenol extract was shaken with water and ether, was treated with charcoal. Liver residue III was the filtrate from the charcoal. This residue contained considerable amounts of the rat dietary factor corresponding to the yeast filtrate factor and only traces of other factors.

Liver residue I has been mainly used in this investigation.

Fractionation of liver residue I with fuller's earth

The liver fraction (1 l.; 1 ml. = 5 g. fresh liver) was adjusted to pH 1.2 by the addition of H₂SO₄, and 50 g. fuller's earth ("specially selected, activated", Fuller's Earth Union) was added. After stirring at intervals for 30 min. the adsorbate was removed by filtration. The adsorption was repeated, and the filtrate, after treatment with Ba(OH)₂ to remove H₂SO₄, was ready for administration to rats.

The first adsorbate was washed twice with 500 ml. of *N*/10 HCl and suspended in 700 ml. of 2% Ba(OH)₂. After several hours at 0°, the eluate was removed by filtration, and the adsorbate was again eluted with 500 ml. of 2% Ba(OH)₂. The combined eluates, freed from Ba with H₂SO₄, were adjusted to pH 8 and treated with an excess of basic lead acetate (8 g. in 40 ml. of H₂O). The resulting precipitate was filtered off and the excess lead removed from the filtrate with H₂S. The PbS was filtered off and the filtrate reduced *in vacuo* to 250 ml. (1 ml. = 20 g. fresh liver).

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

The animals receiving the liver fuller's earth filtrate gained in weight at an average rate of 30 g. weekly for 3 weeks (Table I). This growth rate is nearly double that obtained when rats receive the diet supplemented by yeast fuller's

Table I. *Effect of fractionation of liver residue I with fuller's earth*

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

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

earth filtrate (see Table II), indicating that the liver filtrate contains other dietary essentials for the rat in addition to those present in yeast filtrate fraction. The addition of liver fuller's earth eluate fraction to the diet after 3 weeks did, however, cause an increase in the growth rate, showing that the liver fuller's earth filtrate was deficient in a growth factor for rats which was contained in the liver fuller's earth eluate fraction.

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

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

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

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

Liver filtrate fraction. Liver residue I (500 ml.; 1 ml. = 10 g. fresh liver), adjusted to pH 1 with H_2SO_4 , was extracted six times with 800 ml. portions of amyl alcohol. The combined extracts were then shaken three times with 1 l. portions of water containing enough NaOH to make the aqueous layer alkaline to thymol blue. The combined aqueous extracts were neutralized with HCl, evaporated *in vacuo* to about 100 ml. and treated with 4 vol. of 96% alcohol. The precipitated salts were filtered, and the filtrate, after removal of alcohol *in vacuo*, was adjusted to 800 ml. (1 ml. = 6 g. of fresh liver).

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

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

The rats used in these growth tests were prepared in the usual manner. After the usual depletion period of 1 week during which they received supplements of cod liver oil and aneurin, each male rat was given the additional daily supplements of 50 μg . of riboflavin and either yeast filtrate fraction purified by amyl alcohol extraction equivalent to 2 g. dry yeast or the above liver filtrate fraction equivalent to 6 g. fresh liver; each female rat received riboflavin and either yeast

eluate fraction equivalent to 2 g. dry yeast or the above liver eluate fraction equivalent to 12 g. fresh liver.

The two groups of male rats receiving the filtrate fractions increased in weight at approximately the same rate during the 2-week period during which they received this diet (see Table II). The average weight increases of the animals in

Table II. *Comparison of eluate and filtrate fractions from liver with those from yeast*

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

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

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

The rats which had received the eluate supplements during the first 2 weeks were given supplements of the filtrate fractions; certain animals of the two groups were each given doses of yeast filtrate fraction (= 2 g. dry yeast) and the others were given the liver filtrate fraction (= 6 g. fresh liver). Again, rises in the growth rates of all animals occurred, the weight increases being the same whether the animals received their fractions from yeast or liver.

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

Preparation of liver eluate and filtrate fractions from other liver residues

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

Liver filtrate fraction from liver residue III. Amyl alcohol extraction of liver residue III by the method described for the preparation of liver filtrate fraction from liver filtrate I, yielded a preparation with the same growth-promoting properties as the yeast filtrate fraction.

DISCUSSION

Considerable confusion in the literature concerning the vitamin B complex has resulted from the indiscriminate use of yeast, liver, rice bran, wheat germ and other materials as sources of factors in the complex. Fractions prepared in the same way from different materials have sometimes been finally proved to have quite different biological properties. Confusion has also been caused by certain authors assuming that effects noted in different experimental animals after deprivation of the same fraction are due to lack of the same factor. As each additional factor in the vitamin B complex is discovered, the need for caution in generalizing conclusions drawn from such results becomes more apparent.

In this laboratory we have realized for some time that the dietary essentials of the vitamin B complex contained in yeast fuller's earth filtrates are by no means always the same as those contained in liver fuller's earth filtrates. Experiments on pigs [Chick *et al.* 1938] and dogs [Koehn & Elvehjem, 1936] have proved that, while yeast fuller's earth filtrates contain little or no nicotinic acid or other pyridine derivative with similar biological properties, liver fuller's earth filtrates are rich in these substances. The marked difference, reported in this paper, between the fuller's earth filtrates as sources of rat dietary factors, cannot be due to the difference in the amounts of nicotinic acid derivatives they contain, since we have not been able to show that nicotinic acid or its derivatives have growth-promoting properties for rats [Macrae & Edgar, 1937]. Lepkovsky *et al.* [1936] reported that the preparation of liver fuller's earth filtrates free from factor I (yeast eluate factor) was comparatively easy, but it is possible that in our experiments all of that factor was not removed from the liver filtrate. Further biological investigation of liver fuller's earth filtrate fraction must be carried out before the relationship of the dietary factors contained in it to those of other vitamin fractions is clarified; the proof of the biological identity of one fraction from liver with our yeast filtrate fraction is one step in that direction. Since extractability from acid aqueous solution is a common property of the rat filtrate factor and the chick antidermatitis factor [Elvehjem & Koehn, 1935], the probability that these factors will eventually prove identical is increased. The relationship between our rat dietary factors and those required for growth and maintenance of pigeons [Carter & O'Brien, 1937] is an interesting problem which will probably be solved in the near future.

Only when all factors of the vitamin B complex are obtained in a pure state will the vitamin B problem be finally solved. The isolation of yeast eluate factor (vitamin B₆, factor I) in a pure state [Lepkovsky, 1938; Keresztesy & Stevens, 1938; György, 1938; Kuhn & Wendt, 1938; Edgar, El Sadr & Macrae, unpublished] has proved an important step. Since the crystalline vitamin completely replaces our eluate fraction in the diet of the rat, it seems probable that vitamin B₆ is the only essential nutrient for the rat present in our yeast and liver eluates. The possibility, however, still exists that other essential nutrients may be common contaminants of our eluate fraction, our filtrate fraction and even our basal diet.

SUMMARY

1. Fuller's earth treatment of liver extracts did not yield fractions with the same dietary essentials for the rat as did similar treatment of autoclaved yeast extracts.

2. Fractions with the same growth-promoting properties for rats as the yeast eluate fraction and yeast filtrate fraction were obtained from liver extracts by other methods.

We are very grateful to Dr H. Chick for her valuable advice and criticism. We wish to thank Messrs Glaxo Laboratories, Ltd. for gifts of large amounts of liver extracts and Messrs Hoffmann-La Roche for gifts of riboflavin.

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