CCIX. OBSERVATIONS ON THE LIVER FILTRATE FACTOR OF THE VITAMIN B_2 COMPLEX

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(*Received 13 August 1939*)

We have been engaged for some time on the purification of the liver filtrate factor of the vitamin B_2 complex [Edgar *et al.* 1938, 2] and in view of recent interesting publications [Hoffer & Reichstein, 1939; Subbarow & Hitchings, 1939; Woolley *et al.* 1939] dealing with obviously closely related factors we desire in the interest of clarity to place on record this interim report of our own experiments.

Our recent studies show that at least three unknown members of the vitamin B complex are required for normal growth by rats receiving the three already identified members, aneurin, riboflavin and vitamin B_6 [Edgar *et al.* 1938, 3; El Sadr *et al.* 1939]. One of these is our liver filtrate factor which can be extracted from acidified aqueous liver extracts by amyl alcohol, but is not adsorbed by fuller's earth; a second factor is neither adsorbable by fuller's earth nor extractable by amyl alcohol and is therefore contained in the residue obtained by successive treatment of liver extract with fuller's earth and amyl alcohol; the existence of at least one further factor is proved by the inferiority of the growth response of rats to optimal amounts of both the above fractions compared with that which results from administration of unfractionated liver extract.

The liver filtrate factor is unstable both to acids and alkalis, especially at high temperatures. It is neither adsorbed nor destroyed by treatment with fuller's earth at *p*H 1–3; it is, however, adsorbed by relatively large amounts of norite charcoal, from which it can then be eluted by cold aqueous NaOH. Treatment of preparations of the factor from liver with salts of lead, silver and mercury yields bulky precipitates, but the activity remains in the filtrates.

The factor is lacking in any definite basic properties, as shown by its failure to be precipitated from aqueous solution by phosphotungstic acid. It shows a tendency to be adsorbed from alcoholic solutions by inert materials, which probably accounts for the appearance of the major portion of the activity in the precipitate when concentrates of the factor previously neutralized with NaOH are treated in alcoholic solution with phosphotungstic acid. The same explanation may account for the precipitation of the factor from alcoholic solution by barium hydroxide.

Transference of the factor from aqueous solution to amyl alcohol takes place at acid reactions and is reversed by the presence of alkali, indicating that we are dealing with an acid. The neutral methyl ester given by the action of diazomethane is only slightly less active biologically than the free acid, which can be recovered completely by hydrolysis with dilute aqueous Na_2CO_3. The factor is also extracted from acidified solutions by ether, but even after continuous extraction for 43 hr. about half of the activity remains in the aqueous layer. Much inactive material can be precipitated by addition of ether to certain alcoholic concentrates of the

factor; when ether is replaced by ethyl acetate most of the activity remains in solution, but some is carried down with the precipitate. The factor is sparingly soluble in chloroform, for if such ether-precipitated concentrates are treated with that solvent almost all the material and most of the activity pass into solution, but the small residue remaining shows a very high degree of potency; in this way we have obtained material active in daily doses of 180 μg. per rat. The hydrophilic nature of the factor is shown by its failure to separate from aqueous solution in the form of a salt with either quinine or brucine.

The liver filtrate factor readily yields a chloroform-soluble acetyl derivative which shows only slight biological activity, but which is hydrolysed smoothly by alcoholic ammonia with but little loss of activity. The presence of hydroxy groups is also suggested by the action of acetone. According to the conditions (dryness, *p*H) this solvent causes more or less complete inactivation of the factor, strongly suggesting the formation of the acetone compound of a glycol.

These properties fit very closely with those described for pantothenic acid by Williams and his collaborators [Williams *et al.* 1938; 1939] and for the chick anti-dermatitis factor by Woolley *et al.* [1938]. Jukes [1939] has already shown that these two substances are probably identical, and Subbarow & Hitchings [1939] have suggested that pantothenic acid is one of the substances present in liver concentrates which influences the growth of rats. We consider that this is very likely, though it should be remembered that the pantothenic acid tested by the above workers may not have approached homogeneity, since it was apparently required in a very much higher dose (8 mg. per rat per week) than that we have found necessary with our best preparations (1·2 mg. per rat per week).

The relationships with "factor W", further properties of which were recently reported on by Frost & Elvehjem [1939], are not clear, and it seems likely that these workers were dealing with a mixture of nutritive factors. Some of the properties they report tally well with those of our liver filtrate factor, whilst others, such as the failure to be extracted with ether from acid solution and the failure to yield a hydrolysable acetyl derivative, virtually exclude the identity. The above authors also claim that concentrates of the chick antidermatitis factor have been prepared which show no growth-stimulating action on the rat; this appears to be in direct contradiction to the conclusions of Subbarow & Hitchings [1939]. We are at present engaged in testing the identity or otherwise of our liver filtrate factor with pantothenic acid.

Experimental

The rat growth method for the determination of filtrate factor previously described [Edgar *et al.* 1938, 1] was employed. In our previous work we added the test dose of filtrate factor to the diet of the rats after they had received aneurin, riboflavin and the vitamin B_6 concentrate for 2 weeks. We now find, however, that better results are obtained when the running out period is continued until the rate of weight increase is 7 g. weekly or less; this generally occurs during the third week. Animals given an active dose of the filtrate factor then increase in weight at the rate of approximately 20 g. weekly, which rate is maintained during the 2-week test period. Negative control animals continue to increase in weight at the rate of about 6 g. weekly. Animals which have been deprived of filtrate factor for many weeks respond satisfactorily to added doses of that factor and therefore we have been able to use for further tests animals which had previously acted as negative controls or had received inert fractions; thereby we have effected a saving in animals. Very regular growth responses have resulted on administration of our filtrate factor concentrates and reliable

results have been obtained when only 2 animals were used in each test; nevertheless, we have generally used 3, 4 or more animals to test each fraction. Since we employed such a small number of animals for each test, strictly quantitative results were impossible; we consider, however, that our results were roughly quantitative and that our experimental error was not more than $\pm 25\%$.

Where practicable we have stated the dry weights of our concentrates, together with the number of rat day doses they contained; however, it is not easy to free the concentrates from water and therefore the dry weights are only approximate. To indicate the losses involved in the various fractionations we have also stated the amount of original fresh liver tissue which has yielded that amount of concentrate containing 1 rat day dose of vitamin.

Preparation of liver filtrate concentrates from liver residue III

A convenient source of liver filtrate factor was found in the liver residue previously named residue III [Edgar *et al.* 1938, 2]. This material, which is that portion of an aqueous extract of liver extractable by phenol and not adsorbed by small amounts of charcoal, contains about one-third of the amount of the filtrate factor present in whole liver extract. All evaporations were carried out *in vacuo* below 40°.

3 l. of liver residue III from 500 kg. of fresh liver, adjusted to *p*H 2 by the addition of H_2SO_4, were stirred for 30 min. with 500 g. of fuller's earth ("specially selected, activated", Fuller's Earth Union) and filtered; the fuller's earth treatment was repeated. The filtrate (*p*H 2) was extracted with 8 l. of amyl alcohol; the extract was in turn extracted first with 1 l. H_2O to which was added just enough NaOH to make the aqueous layer alkaline to thymol blue, and then with 500 ml. H_2O. The whole process was repeated ten times using the same amyl alcohol. The combined aqueous extracts from the amyl alcohol, which had been adjusted to *p*H 8 with H_2SO_4 immediately on separation, were evaporated to 2 l. This fraction contained about 25,000 rat day doses of liver filtrate factor.

A slight excess of a hot solution of basic lead acetate (1200 g. in 3 l. H_2O) was now added to the above material and after stirring and standing overnight in the cold, the copious precipitate, which contained less than one-sixteenth part of the activity, was removed by filtration. The bulk of the lead was removed from the filtrate by addition of H_2SO_4, the filtrate from the $PbSO_4$ was adjusted to *p*H 7 with NaOH and then treated with an excess of a solution of mercuric acetate (40 g. in 250 ml. H_2O) and the inert precipitate filtered off. Excess Hg was removed from the filtrate by H_2S and the filtrate from the HgS evaporated to 1 l. To this solution, adjusted accurately to *p*H 3 by the addition of H_2SO_4, 4 l. of 96% alcohol were added and, after standing overnight in the cold, the precipitated salts (mainly Na_2SO_4) were filtered off. The filtrate was evaporated and, when nothing further distilled, about 200 ml. of absolute alcohol were added and the solution again evaporated; this treatment with absolute alcohol followed by evaporation was repeated three times to remove acetic acid and water.

The final gummy concentrate (concentrate A) weighed approximately 70 g. and contained about 20,000 rat day doses of liver filtrate factor; the rat day dose was therefore associated with about 3·5 mg. of dry matter and was contained in the material from approximately 25 g. fresh liver. This method of concentration has been repeated five times and the final material has been reasonably constant in weight and activity.

Much inactive material could be removed from concentrate A by precipitation with phosphotungstic acid in 5% H_2SO_4, or better by filtration at *p*H 1 through a column of fuller's earth (1250 g.), the column being washed with water. The

filtrate, which was promptly neutralized with barium hydroxide, contained practically all the original activity and on evaporation yielded a product of which 1·7 mg. = 1 rat day dose (concentrate B). When filtration through fuller's earth is employed, the initial treatment of liver residue III with fuller's earth may be dispensed with.

Stability to acid. To 1·6 g. of concentrate A, 5 ml. H_2O and 5 ml. 20 % H_2SO_4 (by weight) were added and the mixture was heated at 100° for 2 hr. The neutralized material when fed to rats in daily amounts equivalent to 96 g. fresh liver evoked no growth response.

Stability to alkali. Concentrate A was heated in the same manner as above in the presence of 5 % NaOH instead of 10 % H_2SO_4. There was no demonstrable activity in the material thus treated when fed at the level of 48 g. fresh liver daily.

Adsorption with norite charcoal. To 20 ml. of a solution of the amyl alcohol extract of liver residue III (1 ml. = 240 g. fresh liver containing 12 rat day doses) at *p*H 2·5, 1 g. of norite charcoal was added. After 30 min. the norite was filtered off and washed with *N*/100 HCl; the adsorption was repeated a further three times with 1 g. portions of norite. The combined adsorbates were eluted first overnight with 100 ml. 1 % NaOH, then with 50 ml. 1 % NaOH. The eluates were neutralized with HCl. The filtrate from the norite contained the rat day dose in the material from 60 g. of fresh liver and therefore about two-thirds of the activity had been adsorbed. The eluates were active at a level equivalent to 48 g. fresh liver and therefore contained about 42 % of the original activity.

Precipitation with barium hydroxide in alcohol. To a solution of 70 g. concentrate A in 4 l. absolute alcohol, 120 g. of $Ba(OH)_2, 8H_2O$ dissolved in the minimum amount of hot water were added gradually with very rapid stirring. The final concentration of alcohol in the solution, allowing for the water of crystallization contained in the barium hydroxide, was 96·5 %. The precipitate was filtered off after 16 hr. at 0° and suspended in 500 ml. water in which it partially dissolved. 2 l. 96 % alcohol were then added and after standing in the cold for a few hours the insoluble barium salts were removed by filtration. The barium was removed from all fractions by balancing out exactly with H_2SO_4. The fraction, which was insoluble in 96·5 % alcohol but soluble in 77 % alcohol, weighed 18 g. and contained about 10,000 rat day doses of liver filtrate factor (1 rat day dose equivalent to 50 g. liver), i.e. it contained about half the activity of the concentrate from which it was prepared and about one-quarter of the original dry matter. Solutions of this material gave an inactive precipitate with Ag_2SO_4 at *p*H 7, the filtrate being active. The fraction soluble in 96·5 % alcohol had 2500 rat day doses associated with 38 g. dry matter while that insoluble in 77 % alcohol was completely inactive.

Treatment with alkaloids. Attempts made to obtain the active principle from concentrates of varying potency as crystalline quinine or brucine salts failed completely whether the acid concentrate was treated with the free alkaloid or the concentrate neutralized by NaOH was treated with the alkaloid salt. Although several alkaloid salts were separated, the activity remained in the solutions.

Precipitation with phosphotungstic acid in alcohol. To a solution in 100 ml. alcohol of 1·63 g. of a preparation of concentrate B (neutralized with NaOH), which was less potent than other preparations of this material and contained the rat day dose associated with 3·94 mg. dry matter, an alcoholic solution of phosphotungstic acid was added till no further precipitation occurred. The bulky precipitate was filtered off, dissolved in water and decomposed with $Ba(OH)_2$. After removing excess Ba with H_2SO_4 the solution was evaporated. The residue

(0·77 g.) contained two-thirds of the activity of the starting material, while the gum (0·89 g.) obtained on working up in similar fashion the filtrate from the phosphotungstic acid precipitate contained only about one-eighth of the original activity. This treatment therefore effected some purification. Concentrate B, if not previously neutralized with NaOH, gave no precipitate with alcoholic phosphotungstic acid and we therefore consider that the active material in the above experiment was probably carried down by adsorption.

Precipitation with acetone. To 1·78 g. of concentrate A, dissolved in 10 ml. absolute alcohol, 110 ml. acetone were added. The precipitate (1·2 g.) contained slightly less than one-quarter of the original activity, while the filtrate on evaporation yielded a residue (0·57 g.) containing about one-quarter of the original activity. This inactivation was not observed when concentrate A was used in the form of its neutral sodium salt.

Precipitation with ethyl acetate. 1 g. of concentrate B containing about 550 rat doses was treated with 100 ml. hot absolute alcohol and, after keeping for some time at 0°, a small amount of undissolved material was removed by filtration. The alcohol was removed by evaporation, the residue taken up in 10 ml. absolute alcohol, cooled to 0° and the resulting precipitate was filtered off and washed with 3 ml. absolute alcohol. The combined precipitates obtained by these treatments weighed 154 mg. and was biologically inactive.

The filtrate from the above was evaporated, the residue taken up in 6 ml. absolute alcohol, 100 ml. ethyl acetate were added and after 3 hr. at 0° the precipitate was filtered off. The precipitate (242 mg.) contained 80 rat day doses while the filtrate (614 mg.) contained 300 rat day doses. Ethyl acetate therefore gave no satisfactory fractionation.

Precipitation with ether. 500 mg. of concentrate B containing about 275 rat day doses were dissolved in 10 ml. absolute alcohol and after cooling 150 ml. dry ether were added. After 30 min. the precipitated material (240 mg.) was filtered off and was found to contain no activity when 3·2 mg. equivalent to 96 g. fresh liver were fed daily to rats.

Fractionation with chloroform. The residue from the filtrate from the preceding experiment was extracted by shaking for 30 min. with 100 ml. chloroform (undried) and the chloroform solution decanted from insoluble material adhering to the sides of the vessel. The chloroform-soluble portion (240 mg.) contained 150 rat day doses, each associated with 1·6 mg. of dry matter, while that insoluble in chloroform (10 mg.) contained 56 rat day doses of filtrate factor. The rat day dose in the latter fraction was therefore associated with only about 180 μg. of solids; however, the loss of activity in the preparation of this fraction was high. In a further experiment in which the chloroform extraction was less thorough about half the activity remained in the chloroform-insoluble fraction but in this case the rat day dose was associated with 400–500 μg. of dry matter.

Effect of hydrogen ion concentration on extractability of liver filtrate factor by amyl alcohol. In the preparation of concentrate A we obtained satisfactory extraction of liver filtrate factor with amyl alcohol at *p*H 2. In other experiments we extracted crude liver preparations (liver residue I [see Edgar *et al.* 1938, 2]) at various hydrogen ion concentrations with amyl alcohol using a procedure similar to that already described (p. 1683). At *p*H 1 and *p*H 3 the activity was extracted satisfactorily but the extract obtained at *p*H 7 contained only about one-half of the activity present in those obtained by extraction of the acidified solutions.

Extraction with ether from aqueous solution. 4·4 g. of concentrate A dissolved in 20 ml. H_2O adjusted to *p*H 2·5 with H_2SO_4 were continuously extracted with ether during 15 hr. The ether was changed and the extraction continued for a

further 28 hr. The first ether extract (1·2 g.) contained about one-third of the activity present in the starting materials, the second (250 mg.) about one-eighth and the residue between one-quarter and one-half.

Acetylation experiments. 3·36 g. of concentrate A were taken up in a mixture of 2 ml. pyridine and 50 ml. acetic anhydride, the solution was heated on the water bath for 2 hr. and then evaporated. The residue was completely soluble in chloroform and was partitioned between this solvent (100 ml.) and water (100 ml.). Chloroform phase: this was evaporated to dryness and a portion (one-third) heated on the water bath for 30 min. with 100 ml. water to which had been added 5 ml. of 3*N* HCl. The resulting solution on evaporation yielded a gum which had little or no biological activity (inactive in amount = 96 g. fresh liver). The remaining two-thirds of the material from the chloroform phase was hydrolysed by heating at 100° in a sealed tube with alcoholic ammonia (saturated at 0°). The solvent and excess ammonia were removed and the residue freed from acetamide by extracting its aqueous solution with chloroform. Evaporation of the aqueous layer gave a brownish syrup (0·8 g.) which was active when fed to rats at a level equivalent to 48 g. original liver. Aqueous phase: this was hydrolysed with alcoholic ammonia at room temperature for 48 hr. and gave a residue fully active at a dosage equivalent to 96 g. fresh liver.

In another acetylation experiment with concentrate A the acetylated product was hydrolysed using sodium methoxide according to the method of Zemplén & Pacsu [1929]. The regenerated material was fully active when fed at a level equivalent to 48 g. fresh liver (i.e. 75 % activity recovered in first experiment and at least 50 % in second).

The unhydrolysed acetyl fraction when fed in an amount equivalent to 48 g. of the original liver evoked only a very slight response and twice this amount also produced a suboptimal response (average weekly weight increase of 8·8 g. and 15 g. respectively, compared with 6 g. weekly for negative control rats and 23 g. weekly for rats receiving the hydrolysed material equivalent to 48 g. original liver daily). Whether this activity is due to unacetylated material or whether the acetyl derivative actually possesses slight biological activity is not known.

Treatment with diazomethane. 1 g. of concentrate B in 50 ml. absolute alcohol was treated with an excess of diazomethane in ethyl alcohol and allowed to stand for 30 min.; the solution was then neutral to litmus. The solvent was removed and the residue taken up in 20 ml. absolute alcohol. One-half of this material was heated at 30° for 2 hr. with 100 ml. *N*/20 Na_2CO_3, the solution cooled, neutralized with H_2SO_4 and evaporated. Both the hydrolysed and unhydrolysed portions when fed to rats at levels equivalent to 48 g. original liver evoked growth responses. The rate of weight increase produced by the hydrolysed material was about 18 g. weekly; the unhydrolysed material produced growth rates averaging 16·5 g. weekly. This difference may not be significant.

Summary

1. Liver filtrate factor is not precipitated by salts of lead, mercury, silver, quinine or brucine, but is precipitated from alcoholic solution by barium hydroxide.

2. The factor is not adsorbed even on exhaustive treatment with fuller's earth; it is, however, adsorbed by large amounts of norite charcoal. Amyl alcohol and ether extract the factor from acidified solutions.

3. By combination of several methods of concentration a material has been obtained containing the rat day dose of the vitamin associated with about 180 μg. of dry matter.

4. On acetylation liver filtrate factor yields a product easily extractable from aqueous solution by chloroform and possessing only feeble activity; mild hydrolysis of this material gives a product having biological activity comparable with that of the starting material. Treatment of a liver filtrate factor concentrate with diazomethane does not appreciably affect the biological activity.

We wish to thank the Medical Research Council for a personal grant to H. G. H. and gratefully acknowledge gifts of large amounts of liver extracts from Messrs Glaxo Laboratories, Ltd., and of riboflavin from Roche Products, Ltd.

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