RESEARCH PAPERS

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PART II

THE "NINHYDRIN-REACTING" HYDROLYTIC FRAGMENT OF VITAMIN B_{12}

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WE have previously reported[1] that hydrolysis of vitamin B_{12} with 20 per cent. hydrochloric acid at 100°C., followed by examination of the hydrolysates by unidimensional paper-strip chromatography using the technique described by Consden, Gordon and Martin[2], revealed the presence of *one* "ninhydrin-reacting" substance which could not be identified with any of the known amino-acids. Collateral studies by Brink *et al.*[3] in America, although confirming the absence of amino-acids in the hydrolysates, did not substantiate the existence of the "ninhydrin-reacting" substance. We were therefore led to re-examine and extend our observations on this fragment of the B_{12} complex and now report further data which fully supports our earlier conclusions.

Rigorous purification of crystalline samples of vitamin B_{12} failed to alter the pattern of our results. Paper chromatograms of the hydrolysates, as before, invariably revealed one spot on treatment with ninhydrin, the intensity of which, however, depended markedly on the nature of the irrigation solvent employed. Thus, pronounced purple spots were obtained with solvents consisting of, or containing, the aliphatic acids. Faint or hardly visible spots, in contrast, resulted when *n*-butyl alcohol or phenol were employed. Brink *et al.*[3], it should be added, used phenol as their irrigation solvent, a fact no doubt explaining the difference between the two sets of results.

Again, in another series of experiments the coloured moiety[1] produced by hydrolysis of vitamin B_{12} was quantitatively extracted from the diluted hydrolysate with *n*-butyl alcohol, and the colourless cobalt-free aqueous phase examined spectroscopically. Selective absorption in the ultra-violet was observed with bands and inflections at 2850, 2768, 2690, 2585 and 2500 Å (for convenience this colourless cobalt-free hydrolytic material will subsequently be referred to as "the 285 component"), similar to certain fine structure bands present in the ultra-violet absorption spectrum of vitamin B_{12} itself. A paper chromatogram prepared from the aqueous phase, and irrigated with *iso*-butyric acid, when developed with ninhydrin, gave the typical purple spot obtained using the total hydrolysate. A second chromatogram, run parallel with the first, when examined under a low pressure mercury resonance lamp fitted with a Corning 9863 glass filter[4], showed a clearly visible localised pale-blue fluorescent area occupying a position corresponding to the "ninhydrin spot." The ultra-violet absorption of the eluate from this fluorescent area, moreover, showed selective absorption substantially the same as that of the aqueous solution from which the chromatogram was prepared.

We were thus led to believe that the "ninhydrin-reacting" substance was responsible for the fluorescence and identical with "the '285' component." In later experiments, however, when *n*-butyl alcohol-acetic acid was employed as the irrigation solvent for the chromatograms, an entirely different result was obtained. The control strip showed the "ninhydrin-reacting" area as before, but this area was no longer fluorescent when examined using the Corning filter. Furthermore, an eluate prepared from it was now found to be transparent to ultra-violet light, an observation which excludes an aromatic structure for the "ninhydrin-reacting" substance and leads to its formulation as an aliphatic base. The chromatogram, however, showed at least two blue fluorescent zones separated from each other and from the "ninhydrin-reacting" area. Eluates from these zones showed absorption spectra similar to each other and to "the 285 component," which had been resolved under these experimental conditions into at least two structurally related substances[5].

We have already reported our failure to identify the "ninhydrin-reacting" substance with any of the naturally occurring amino-acids[1] from which it differs in giving very pale spots with ninhydrin on chromatograms irrigated with phenol (*vide supra*). In addition, studies on its chromatographic behaviour led to the tentative conclusion that the compound might be slightly volatile. We therefore turned our attention to the related amino-alcohols which are known to exist in combination in many naturally occurring products (e.g., ethanolamine in gramicidin[6] and cephalin[7]; 2-aminopropanol in ergometrine[8]). The ninhydrin-reacting" substance proved to be different from ethanolamine, as separation of the two occurred on paper chromatograms irrigated with phenol and with collidine respectively. When the behaviour of 2-aminopropanol was investigated, R_F values *identical* with those of the "ninhydrin-reacting" substance were obtained on chromatograms irrigated respectively with *iso*-butyric acid, *n*-butanol-acetic acid and phenol (see experimental part). Slightly different results were given using collidine. Separation of a mixture of the two substances did not occur, a single, nearly circular spot being obtained with ninhydrin. 2-Aminopropanol itself, on the other hand, gave rise to an elongated zone.

Whilst these experiments were in progress we had noted, *inter alia*, that substantial quantities of ammonia were formed when vitamin B_{12} was heated with acid or alkali. Ammonium chloride was therefore present in acid hydrolysates of vitamin B_{12}. We therefore examined the effect of adding ammonium chloride to the 2-aminopropanol prior to paper chromatography in order to simulate the ionic environment of the "ninhydrin-reacting" substance. Irrigation with collidine, followed by development with ninhydrin, now gave a nearly circular spot identical in R_F value with the vitamin B_{12} "ninhydrin-reacting" hydrolytic fragment. As 2-aminopropanol and the "ninhydrin-reacting" substance appear to have identical partition coefficients in four different solvent systems, it seems difficult to avoid drawing the conclusion that the two substances are identical. A final decision on this point must, of course, rest on a rigid chemical comparison.

Finally, parallel hydrolyses of a sample of vitamin B_{12} (Merck) (prepared by Merck & Co., Inc., and kindly sent to us through the courtesy of Dr. Randolph T. Major) and of our product, followed by detection of the " ninhydrin-reacting " fragment on paper chromatograms irrigated with 65 per cent. *iso*-butyric acid and with *n*-butyl alcohol-acetic acid respectively, have given identical results as shown in the accompanying photograph (Fig. 1). Our previous conclusions[1] are thus reaffirmed and the presence of a " ninhydrin-reacting " fragment in hydrolysates of vitamin B_{12} firmly established.

EXPERIMENTAL

Whatman No. 1 filter paper was used for all chromatograms. Solvents for irrigation were saturated with water, with the exception of *iso*-butyric acid which was used as a 65 per cent. aqueous solution " Analar " Grade phenol was employed. Collidine (2:4:6-trimethyl pyridine) was purified by the method of Consden *et al.*[9]. Other solvents were purified by distillation. Chromatograms, after irrigation, were dried in air, sprayed with

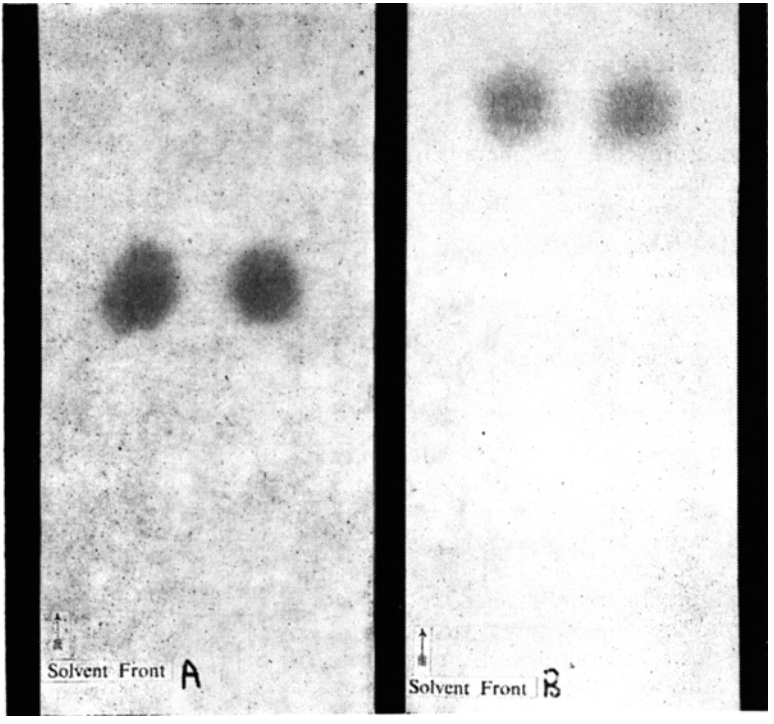

FIG. 1.—Photograph showing identity of behaviour on paper chromatograms on irrigation with (A) *iso*butyric acid and (B) *n*-butyl alcohol-acetic acid of 'ninhydrin-reacting' substances present in hydrolysates of (1) Vitamin B_{12} (Merck) (left hand spot on each paper) and (2) Vitamin B_{12} isolated by authors (right hand spot on each paper). Only the lower parts of the chromatograms are reproduced.

a freshly prepared 0·1 per cent. solution of ninhydrin in aqueous *n*-butyl alcohol and then heated for 15 minutes at 90°C. in order to develop the colours. R_F values obtained with *iso*-butyric acid, *n*-butanol, and collidine were reproducible to within 5 per cent. Greater variations were observed with phenol, and in places of R_F values the positions of spots relative to the position occupied by valine, used as a marker, are given.

Purification of crystalline vitamin B_{12}.—A sample (30 mg.) of twice recrystallised vitamin B_{12} was chromatographed on a column (1·4 cm. diam.) consisting of a mixture of aluminium silicate and kieselguhr. The sharply defined red band was developed to a distance of 12 cm. down the column, when it was dissected out, eluted, and the vitamin B_{12} recovered and recrystallised twice from aqueous acetone yielding 21 mg. of repurified crystalline product.

Paper chromatography of acid hydrolysates of vitamin B_{12}.—(1) 3 mg. of the sample of crystals prepared as described above was hydrolysed for 6 hours with 0·5 ml. of 20 per cent. hydrochloric acid in a sealed tube at 100°C. The product was evaporated to dryness *in vacuo* and the coloured residue treated with 100 microlitres of distilled water. Five microlitre quantities of this solution were spotted on to paper strips in the usual way. The strips were irrigated overnight and then developed with ninhydrin with the following results.

iso-*Butyric acid.*—A pronounced purple spot having R_F 0·76, was obtained superimposed upon the tail of the pigment present, which formed a pale orange-red streak extending almost to the solvent front. A sample of vitamin B_{12} (Merck), hydrolysed and chromatographed in the same way, gave an identically placed spot (paper strip A in Fig. 1).

Phenol.—The spot obtained was very weak indeed and appeared near the head of the pigment streak. It occupied a position in front of that of valine, run alongside and used as a marker. A significant change in the position of the spot did not occur when the vessel at the bottom of the chamber contained 50 per cent. acetic acid, but the intensity of the colour produced with ninhydrin was greatly increased. A weak spot was also obtained by substituting the acetic acid in the vessel by 5 N hydrochloric acid. In this case, however the " ninhydrin-reacting " substance travelled more slowly and was located a short distance behind valine, again used as a marker.

n-*Butyl alcohol.*—A pale spot appeared with R_F 0·20. The intensity and position was not altered by the presence of ammonia or potassium cyanide in the chamber. The pigment did not migrate.

Collidine.—A blue spot was obtained having R_F 0·34.

n-*Butyl alcohol-acetic acid.*—*n*-Butyl alcohol (4 vols.), acetic acid (1 vol.), and water (5 vols.) were mixed and the upper layer used as the solvent. Intense purple spots were obtained both with our sample of vitamin B_{12} and with vitamin B_{12} (Merck) as shown in Fig. 1 (paper strip B).

Caproic Acid.—In the one experiment carried out, a pronounced bluish-purple spot was obtained having R 0·53.

(2) Specimens of vitamin B_{12} were hydrolysed with 20 per cent. hydrochloric acid for (a) 5 days at room temperature and (b) 6 hours at 150°C. The " ninhydrin-reacting " substance was detected on chromatograms prepared from both these hydrolysates.

(3) 3·2 mg. of vitamin B_{12} were hydrolysed with 1 ml. of 20 per cent. hydrochloric acid by heating in a sealed tube at 100°C. for $6\frac{3}{4}$ hours. The hydrolysate was diluted to 10 ml. with distilled water and a 7 ml. portion extracted 4 times with aqueous *n*-butyl alcohol (3 ml. each extraction). This treatment removed the coloured moiety completely, leaving a colourless aqueous phase the ultra-violet absorption spectrum of which showed bands and inflections at 2850, 2768, 2690, 2585 and 2500 Å. Part of the solution was examined for the presence of cobalt with negative results. A portion (0·75 ml.) was taken to dryness *in vacuo*, the residue dissolved in 20 microlitres of water, and equal volumes (10 microlitres each) of this solution dispensed as two spots, 5 cm. apart, on a paper strip. After irrigation overnight with *iso*-butyric acid, the chromatogram was cut longitudinally down the centre and one half developed with ninhydrin. A purple spot appeared having R_F 0·76. Examination of the other strip under the ultra-violet light transmitted by a low-pressure mercury resonance lamp fitted with a Corning 9863 filter revealed a pale blue fluorescent spot with the same R_F value of 0·76. Black patches were not observed, thus indicating the absence of certain purines and pyrimidines.[4] The fluorescent area of the paper was cut out, eluted with 0·1 N hydrochloric acid and the ultra-violet absorption spectrum of the eluate examined. This was substantially the same as that of the aqueous phase from which the chromatogram was prepared.

Different results were obtained by the use of *n*-butyl alcohol-acetic acid as the irrigation solvent. In this case, development of one paper strip with ninhydrin gave rise to an intense purple spot, as already described, whilst two and possibly three fluorescent spots were revealed on the companion strip when viewed under the Corning filter. These fluorescent spots were well separated from each other and also from that area of the paper containing the substance capable of giving a colour with ninhydrin. An eluate from the latter area did not show any selective ultra-violet absorption. That a " ninhydrin-reacting " substance was, in fact, present was shown by the appearance of a typical ninhydrin spot on a chromatogram prepared from this eluate.

(4) When total hydrolysates of vitamin B_{12} were chromatographed using phenol as the irrigation solvent, the very weak spot produced with ninhydrin occupied a position in which it could easily be confused with, and even mistakenly regarded as part of, the pigment streak also present. The spot was more readily seen if the coloured moiety in a vitamin B_{12} hydrolysate was first removed by extraction with *n*-butyl alcohol.

Comparative behaviour of the " ninhydrin-reacting " fragment and 2-aminopropanol on paper chromatograms.—5·6 mg. of vitamin B_{12} were

hydrolysed with 20 per cent. hydrochloric acid for 6 hours in the usual way, the hydrolysate diluted, and the pigment quantitatively extracted with *n*-butyl alcohol. The product obtained on evaporation of the aqueous layer was dissolved in 250 microlitres of distilled water and this solution [solution (a)] used in the preparation of chromatograms. A 0·2 per cent. aqueous solution of 2-aminopropanol [solution (b)] was also employed.

Three spots consisting of (i) 3 microlitres of solution (a), (ii) 5 microlitres of solution (b), and (iii) a mixture of 3 microlitres of solution (a) and 5 microlitres of solution (b) were placed 2·5 cm. apart along the starting line of each paper strip (10 cm. wide and 50 cm. long). Irrigation of the strips was allowed to proceed until the solvent front had travelled in every case a distance of not less than 40 cm. The following results were obtained:

iso-*Butyric acid.* —The R_F values of the purple stops corresponding to (i) and (ii) were identical, namely, 0·76. A single spot corresponding to (iii) was obtained with the same R_F value of 0·76.

n-*Butyl alcohol -acetic acid.*—Three purple spots corresponding to (i), (ii) and (iii) were observed with identical R_F values of 0·33.

Phenol.—Acetic acid (50 per cent. v/v) was included in the chamber in order that easily visible colours should be given with ninhydrin. Valine spotted near one edge of the paper served as a marker. The apparently identical purple spots corresponding to (i), (ii) and (iii) occupied a position just in front of that of valine.

Collidine.—Both (i) and (iii) gave rise to blue spots with almost identical R_F values of *ca.* 0·32. 2-aminoproponal (ii) alone gave a blue elongated zone the limits of which extended from R_F 0·26 to R_F 0·44. The addition of several micrograms of ammonium chloride to spot (ii) prior to irrigation of the paper resulted in the appearance of a single nearly circular spot having an R_F value of 0·32.

Detection of ammonia formed on hydrolysis of vitamin B_{12}.—(a) On gently warming a mixture of 1 mg. of vitamin B_{12} and 100 microlitres of N sodium hydroxide, ammonia was evolved, detected by the appearance of a brown coloration on a piece of filter paper moistened with Nessler's reagent and held over the mixture.

(b) 10 mg. of vitamin B_{12} were hydrolysed with 0·5 ml. of 20 per cent. hydrochloric acid at 100°C. for 6 hours, the solution diluted to 10 ml., and the pigment extracted with *n*-butyl alcohol. The aqueous layer gave a colourless crystalline residue on evaporation to dryness *in vacuo*. Part of this residue was gently heated in an ignition tube with a free flame, when a white sublimate formed on the cooler upper sections of the tube. Another part of the residue, dissolved in a few microlitres of water, was mixed with one drop of cold N sodium hydroxide, and the ammonia evolved detected as described above.

Summary and Conclusions

1. Our previous conclusion that acid hydrolysis of vitamin B_{12} leads to the formation of a " ninhydrin-reacting " substance is confirmed.

2. The behaviour of this " ninhydrin-reacting " substance and of 2-aminopropanol on paper chromatograms has been examined.

3. The results appear to show that these two substances are identical.

4. Evidence has been obtained for the presence in acid hydrolysates of vitamin B_{12} of material showing selective absorption with bands and inflections at 2850, 2768, 2690, 2585 and 2500 Å (referred to as " the 285 component ").

5. This " 285 component " is resolved into at least two structurally related substances by chromatography employing *n*-butyl alcohol-acetic acid as the irrigation solvent.

6. Formation of ammonia occurs during acid or alkaline hydrolysis of vitamin B_{12}.

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