RESEARCH PAPERS

THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS

PART 1

THE LIBERATION OF PHOSPHORUS AS PHOSPHATE FROM VITAMIN B_{12} BY ACID HYDROLYSIS

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WORK in these laboratories on the anti-pernicious anæmia factor present in liver has led to the isolation from anahæmin[1] of a clinically active red crystalline compound which we have characterised by:—

(i) lack of a definite melting-point, the crystals darkening at 190° to 250°C.

(ii) an R_F value of 0·1 when run on unidimensional paper-strip partition chromatograms using *n*-butyl alcohol as a solvent.

(iii) characteristic light absorption, an aqueous solution showing maxima at 550 mμ (shoulder at *ca.* 520 mμ), 361 mμ, and 278 mμ, with inflections at 322 mμ and 304 mμ.

(iv) the presence of nitrogen, phosphorus and cobalt in the molecule (see also Rickes *et al.*[2]; Smith[3]).

(v) a cobalt content of 4·0 per cent. for material dried *in vacuo* at 76°C.

(vi) the presence of one ninhydrin-reacting substance detected in hydrochloric acid hydrolysates run on paper-strip partition chromatograms.

The identity of our compound with the substance to which the name vitamin B_{12} was first applied[4] has now been established by a direct comparison with an authentic specimen, kindly carried out for us by Merck Laboratories, Inc., through the courtesy of their Vice-President and Research Director, Dr. Randolph T. Major.

More detailed examination of the paper-strip chromatograms obtained in (vi) above revealed the presence of phosphate on them, an observation which led us to undertake a quantitative study of the liberation of this ion from vitamin B_{12} during its hydrolysis with hydrochloric acid. The solutions obtained on hydrolysis, however, are deeply coloured owing to the survival of a pigmented cobalt-containing fragment of the B_{12} molecule to which we have already referred in an earlier publication[1]. The problem of estimating phosphate liberated on hydrolysis thus resolved itself into a search for a micro-method which would permit quantitative separation of the inorganic ion from the pigmented material both of which are present in the hydrolysate. We ultimately achieved this by a new application of the unidimensional paper-strip chromatography technique. In addition, we have carried out some exploratory experiments on the behaviour of

phosphates on chromatograms irrigated with a number of solvents, the results being reported below (Experimental (i)).

The method finally adopted for the estimation of phosphate consisted in spotting the vitamin B_{12} hydrolysate on to strips of filter-paper, irrigating with *iso*butyric acid, locating phosphate on the guide strips by means of the ammonium molybdate and benzidine spot test reagents[5], and estimating the phosphate colorimetrically after its elution from the paper.

We were unable to obtain evidence for the liberation of phosphate from vitamin B_{12} when the compound was hydrolysed with 20 per cent. hydrochloric acid at room temperature for periods as long as 17 days. Phosphorus must therefore be present in a fairly stable form. At 100° C., however, liberation of phosphorus as phosphate took place and appeared to be essentially complete after about 6 hours, values corresponding to a phosphorus content in vitamin B_{12} of *ca.* 2·0 per cent being obtained. This was equivalent to all the phosphorus present in vitamin B_{12}, as total phosphorus determinations gave results ranging between 1·9 and 2·3 per cent. of phosphorus.

We have previously reported that vitamin B_{12} contains 4·0 per cent. of cobalt[1]. This figure, taken in conjunction with a phosphorus content of 2·1 per cent., shows that the atomic ratio cobalt : phosphorus in the vitamin B_{12} molecule is 1 : 1. The anti-pernicious anæmia factor isolated by Smith and Parker[6], it may be added, is reported by Smith[3,7] to have a cobalt : phosphorus ratio of 1 : 3. It follows from this that the compound described by Smith and Parker[6] must differ from vitamin B_{12}, a conclusion supporting the view that at least two hæmatopoietic factors containing cobalt may be obtained from liver. Further work is required to establish whether in fact both these compounds co-exist in liver or whether, as is possible, one of them is a clinically-active artefact produced from the other during the process of isolation.

Experimental.

Whatman No. 1 filter paper was used for all chromatograms. Solvents used for irrigation were saturated with water, with the exception of *iso*butyric acid which was employed as a 65 per cent. aqueous solution. Phosphorus was estimated colorimetrically by the method of Fiske and Subbarow[8]. A "Spekker" photoelectric absorptiometer with an Ilford Spectrum filter No. 608 was used throughout for colour intensity comparisons, in conjunction with at least two phosphate standards. These were freshly prepared on each occasion and gave absorptiometer readings satisfactorily coinciding with points on a previously constructed calibration curve.

(i) *Detection, behaviour, and quantitative estimation of the phosphate ion on paper-strip chromatograms.* Disodium hydrogen phosphate, potassium dihydrogen phosphate, diammonium hydrogen phosphate, and phosphoric acid were employed as sources of phosphate ions. Phosphate was readily detected on the chromatograms using the ammonium molybdate and benzidine reagents employed in spot-test techniques[5]. For this purpose, the chromatogram, after irrigation with the solvent, was dried

and lightly sprayed with a reagent consisting of a solution of 5 g. of ammonium molybdate in a mixture of 100 ml. of water and 35 ml. of concentrated nitric acid. The presence of phosphate on the paper, in quantities exceeding 2μg. of phosphorus, was indicated by the appearance of a yellow spot or zone, which changed to blue after spraying with a solution prepared from 50 mg. of benzidine (or benzidine hydrochloride) in 100 ml. of 10 per cent. acetic acid, followed by exposure of the area to ammonia vapour. Diffusion or running of the coloured spot or zone was minimised by spraying very lightly with the two reagents, whilst excessive exposure to ammonia was avoided as this tended to reduce the intensity of the blue colour.

n-Butyl alcohol, *iso*butyric acid, collidine, and phenol were employed as solvents in the present investigation.

n-*Butyl alcohol.* Migration of phosphate did not occur.

iso*Butyric Acid.* Slightly elongated spots falling within the region R_F 0·20 to R_F 0·25 were obtained with phosphoric acid and with all the phosphates. The diammonium hydrogen salt gave, in addition, a second smaller spot at R_F 0·3.

Collidine. Elongated tapering zones, extending from the source to points determined by the nature of the cations present were obtained. The phosphoric acid and diammonium hydrogen phosphate zones were longest, terminating at R_F 0·20, whilst those formed by the potassium and disodium salts terminated at R_F 0·17 and R_F 0·13, respectively.

Phenol. The major portion of each phosphate migrated as a spot to *ca.* R_F 0·1, but a small fraction resisted migration and remained as an annular ring at the source. It was important that all traces of phenol be removed from these chromatograms before application of the ammonium molybdate reagent, otherwise the chromatograms turned black or brown.

Experiments directed to the quantitative estimation of phosphate were limited to chromatograms irrigated with *iso*butyric acid as, when used for the chromatography of acid hydrolysates of vitamin B_{12}, complete separation of the phosphate and pigmented material occurred.

The technique used for locating phosphate and preparing "cuts" for elution followed that described by Consden, Gordon and Martin[9]. Twenty microlitres of an aqueous solution containing a known amount of phosphate (equivalent to 10 to 20μg. of phosphorus) were distributed on 8 spots, 1 cm. apart, along a starting line drawn 6 cm. from one end of a paper strip 12 cm. wide. After irrigation of the paper with *iso*butyric acid a "cut" containing the phosphate was trimmed to a point at one end and eluted by the method of Dent[10]. Phosphate in the eluate was estimated colorimetrically by the method of Fiske and Subbarow[8]. Substantially quantitative recoveries (96 to 102 per cent.) were obtained in all of 14 estimations using both phosphoric acid and its salts.

In current paper-strip chromatography technique, acid hydrolysates are evaporated to dryness to remove hydrochloric acid and the residues dissolved in distilled water before being spotted on the paper strips. In quantitative work such a procedure involves the risk of errors arising during evaporation and re-solution. In order to establish whether this

procedure could be eliminated during estimation of phosphate in acid hydrolysates of vitamin B_{12}, control experiments were carried out in which phosphoric acid dissolved in 20 per cent. hydrochloric acid was spotted directly on the paper strips. The spots so obtained were allowed to dry at room temperature for at least 1 hour before irrigation with *iso*butyric acid. Chromatograms prepared in this way showed fairly uniform areas of waterlogging within which, however, the phosphate could be located in approximately its normal position. Elution, followed by estimation, resulted in substantially quantitative recoveries as before. Some degree of waterlogging could therefore be ignored in these estimations. It followed from this that acid hydrolysates of vitamin B_{12} could be employed *directly* for phosphate estimations and that the usual procedure involving removal of hydrochloric acid by evaporation and re-solution of the residues was unnecessary.

(ii) *The liberation of inorganic phosphorus from Vitamin B_{12} during hydrolysis with hydrochloric acid.* Sealed tubes containing 3 to 3·5 mg. of vitamin B_{12} dissolved in 200 microlitres of 20 per cent. hydrochloric acid were, with one exception, heated at 100°C. for increasing periods of time, cooled to room temperature, and opened. Twenty microlitres of each hydrolysate were withdrawn with a micropipette and dispensed on to a paper strip (12 cm. wide) in a series of 8 spots. An additional 2 to 3 microlitres of solution were placed near one edge of the paper to serve as a " guide " for the location of phosphate. The papers were irrigated overnight with 65 per cent. *iso*butyric acid and air-dried. The phosphate was located by means of the guide strip and fell within the region R_F 0·2 to R_F 0·3, well removed from the pigmented zones which appeared further down the chromatograms. The area containing the phosphate was cut out, eluted, and phosphorus determined colorimetrically[8]. The main results of duplicate phosphorus estimations obtained from pairs of chromatograms arising from each hydrolysate are given in the table below.

Experiment	(a) Phosphorus content of vitamin B_{12} per cent.	Time of hydrolysis	Temperature	(b) Phosphorus liberated as percentage of vitamin B_{12} used	Ratio (b)/(a)
1	1·9	17 days	18-23°	Nil	0
2	2·1	1 hour	100°	0·6	0·29
3	2·1	2 hours	100°	1·0	0·48
4	2·3	3 ,,	100°	1·7	0·74
5	—	6 ,,	100°	1·9	—
6	1·9	12½ ,,	100°	2·0	1·05
7	2·0	25 ,,	100°	2·1	1·05

(Specimens of vitamin B_{12} used in experiments 1, 5, 6 and 7 were dried *in vacuo* at room temperature. Crystals dried *in vacuo* at 76°C. were employed in experiments 2, 3 and 4.)

Total phosphorus was estimated by dispensing aliquot portions of each hydrolysate on to small pieces of filter paper and determining phosphorus on them by the perchloric acid digestion method of King[11]. Colour intensities, which reached a maximum 20 minutes after the addition of the aminonaphtholsulphonic acid reducing agent, were measured with a

"Spekker" photoelectric absorptiometer as before. These estimations were carried out in triplicate, the mean results being given in the above table.

Summary and Conclusions

1. Phosphate is found in acid hydrolysates of vitamin B_{12}.

2. Such hydrolysates are deeply coloured and, in order to determine phosphate in them colorimetrically, it has been necessary to develop a new application of the unidimensional paper-strip partition chromatogram technique.

3. The atomic ratio of cobalt : phosphorus in vitamin B_{12} is found to be 1 : 1.

4. From a comparison of this result (3 above) with the values recorded by Smith [3] for the anti-pernicious anæmia factor of Smith and Parker[6], it is concluded that the latter compound is not identical with vitamin B_{12}.

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