THE PREPARATION, DETECTION AND SEPARATION OF SOME VALENCY STATES OF VANADIUM AND COBALT BY PAPER CHROMATOGRAPHY

by

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INTRODUCTION

Recent work with *Neurospora crassa*[1], *Escherichia coli*[2] and higher plants[3] has shown that the enzyme nitrate reductase contains Mo, and that the element undergoes a valence change ($Mo^{VI} \rightleftharpoons Mo^{V}$) when nitrates are reduced by these organisms.

Mo is also essential for nitrogen fixation by *Azotobacter*[4], but the Mo requirement of this organism can be partly replaced by V. It was, therefore, of interest to prepare and separate the valency states of V for use in biochemical studies which are being undertaken in this laboratory.

It is well known that Co is a constituent of Vitamin B_{12}, which is produced by microorganisms, and recently it has been established by X-ray absorption analysis that the metal is present in the trivalent form[5]. Thus the separation of the valence states of Co was a useful preliminary to a study of possible valence changes of the element during microbial metabolism[6].

Independent investigations upon the separation of the valency states of several elements, including V and Co, by paper chromatography have also been carried out by POLLARD and co-workers[7,8] in the Department of Chemistry of the University of Bristol. The author is grateful to Mr. A. J. BANISTER for telling him of the work before publication.

EXPERIMENTAL

Vanadium

Preparation of vanadium solutions

V^V chloride solution was prepared by acidifying a solution of ammonium vanadate with 2*N* HCl, and a V^V sulphate solution by dissolving 4-5 g of vanadium pentoxide in a mixture of sulphuric acid (20 ml) and water (10 ml) by heating. The sulphate solution was cooled and diluted to 400-500 ml with distilled water, and filtered (Whatman No. 41 paper).

V^{IV} chloride ($VOCl_2$) solution was prepared by heating vanadium pentoxide with hydrochloric acid (d = 1.18) until the concentrated solution ceased to give a V^V spot when analysed by paper-strip chromatography (see below). A V^{IV} sulphate solution was prepared by dissolving 4 g of vanadyl sulphate ($VOSO_4.2H_2O$) in 500 ml of water and adding 2 ml sulphuric acid (d = 1.84), or by electrolytic reduction of V^V sulphate solution using Pt electrodes and a current of 6-7 A in a cell with a porous anode chamber as described by WARDLAW AND SYLVESTER[9].

V^{III} solutions were made by the following methods:

(a) V metal powder was heated with hydrochloric acid ($d = 1.18$), and the green solution diluted with ethanol, filtered and evaporated under reduced pressure from a water bath or at ordinary pressure in a slow stream of CO_2. The prepared solution contained Fe (as $FeCl_2$) as a contaminant from the vanadium metal employed.

(b) Reduction of an acid V^{IV} solution by zinc dust, followed by the removal of Zn^{+2} ions by chromatography, using an eluant consisting of: ethanol 100 ml, ether 100 ml, hydrochloric acid ($d = 1.18$) 20 ml. Then 2 g of $VOSO_4.2H_2O$, were dissolved in 20 ml of water, and the solution mixed with 40 ml of eluant. The mixture was left in contact with zinc dust with occasional agitation until the solution was blue-green or blue in colour and reduced 0.5% copper sulphate to copper (due to V^{II}). Excess of zinc dust was removed by filtration A cellulose column 45-50 cm in length and 3.5 cm in diameter was prepared by packing a suspension of Whatman cellulose in ethanol under compressed air in the usual way. A quantity of pure eluant, followed by a solution of fuchsin in the eluant were allowed to soak into the cellulose in turn. The zinc-reduced vanadium solution was then placed on the column, and when this had sunk into the cellulose a 500 ml reservoir of eluant was inverted into the top of the column, and elution allowed to proceed. The fuchsin band together with Zn^{+2} ions moved ahead of the vanadium, and was rejected after breakthrough. The vanadium fraction was collected when the eluate commenced to reduce sodium molybdate solution to "molybdenum blue". During the run V^{II} was continually oxidized to V^{III}, which moved ahead of the former, and by the time the fraction had been collected all the vanadium was in the trivalent state. The eluate was concentrated by distillation from a water-bath under reduced pressure or at ordinary pressure in a stream of CO_2. Paper-strip analyses, using the same eluant, were performed upon the concentrated eluate fractions. The strips were developed by dipping them into a solution of oxine in ether and allowing them to dry in air. *Rf* values were 0.24 for V^{III} (rusty brown spot) and 0.94 for Zn^{II} (yellow fluorescence over ammonia in u.v. light[10]). Zinc was found to be concentrated in the initial fuchsin-coloured fraction and absent from the V^{III} fraction.

(c) An acid V^{V} or V^{IV} sulphate solution was prepared and electrolysed as described above until a *slight* precipitate of copper was obtained (due to the presence of V^{II}) when a few drops of the catholyte was added to 1-2 ml of 0 5% w/v solution of copper sulphate. On standing, the trace of divalent vanadium soon oxidized.

(d) V metal powder was heated in a stream of Br_2 vapour carried by dry CO_2, and the black sublimate of VBr_3 was dissolved in a warm mixture of acetone and water (80 vols : 20 vols).

V^{II} solutions were prepared by reducing solutions of the other valencies, containing a small excess of hydrochloric acid, with Zn dust. When excess of Zn was removed by filtration, the lavender solution soon oxidized, and was deemed too unstable for experiments of any duration.

Reducing power of the valencies

A few drops of the chloride solution of each valency were added in turn to 2-3 ml of the following reagent solutions in separate test tubes (a) 2% sodium molybdate to which one tenth of the volume of $2N$ HCl had been added to give a pale yellow colour, (b) 0.5% w/v copper sulphate, (c) mercuric chloride (saturated solution), (d) potassium nitrate ($0.1M$) with sulphanilamide (1% in N HCl) and N-(1 naphthyl) ethylenediamine dihydrochloride (0.001%)[11]. Results are set out in Table I.

TABLE I

Reagent solution	V^{V}	V^{IV}	V^{III}	V^{II}
(a)	no action	no action	blue (Mo blue)	blue (Mo blue)
(b)	no action	no action	*white precipitate of Cu_2Cl_2	brown precipitate of Cu
(c)	no action	no action	*white precipitate of Hg_2Cl_2	black or grey precipitate of Hg
(d)	no reduction	no reduction	pink, due to reduction of NO_3^-	pink, due to reduction of NO_3^-

* In strongly acid solution precipitation is inhibited; buffering with sodium acetate is then advisable.

V^{III} appeared to reduce Cu^{+2} and Hg^{+2} ions to the univalent state only.

Estimation of the strength of vanadium solutions

The method of TALVITIE[12] using 8-hydroxyquinoline was modified, and the estimations were carried out in the absence of iron, which caused serious interference and could not be satisfactorily removed by chemical extraction methods.

A standard solution of V^{V} containing 1000 μg V per ml was prepared by warming 0.2294 g of "AnalaR" ammonium vanadate with water, and adding 2*N* sodium hydroxide until a clear solution was obtained. The solution was then acidified with 10 ml of 2*N* HCl* and diluted to 100 ml with distilled water. Volumes of this standard ranging from 0.1 ml to 0.7 ml were then mixed with 1 ml of 2*N* HCl and shaken with 50 ml aliquots of 1% w/v 8-hydroxyquinoline in pure chloroform at two minute intervals for 15 minutes. The chloroform extracts were then filtered, and a calibration curve obtained on a Hilger "Spekker" absorptiometer using Ilford No. 604 green filters and a drum setting of 1.30.

The use of a phthalate buffer as recommended by TALVITIE[12], was found to suppress the colour of the vanadium oxine complex under the conditions employed here, and so was omitted in the estimations.

The strengths of V^{IV} and V^{III} solutions were determined by digesting measured aliquots (0.1-0.5 ml) with a mixture of nitric acid (20 ml), sulphuric acid (2 ml) and perchloric acid (5 ml) until most of the latter had been fumed off. The resulting orange-yellow digest containing the vanadium in the pentavalent state was cooled, mixed with 50 ml of water and treated with sodium hydroxide solution until just alkaline to phenolphthalein. The mixture was then neutralized with 2*N* HCl and 1 ml of the acid added in excess. The solution was allowed to stand for 10-15 minutes until it was colourless and the vanadium was estimated by extraction with a 1% w/v solution of oxine in chloroform as described above. The error obtained in the determination of duplicate aliquots (0.05 ml) of a V^{III} solution containing 9700 μg V per ml was 0.6%.

Separation of the valencies by paper-chromatography

Valencies differing in charge by more than 1 were found to interact with one another, and so separations were achieved between V^{V} and V^{IV} and between V^{IV} and V^{III}. Downward chromatography upon acid-washed Whatman No. 1 paper-strips was employed for the separations.

Eluants containing ether have been successfully used by POLLARD *et al.*[8], and in this work those containing acetone and ether were employed. The very volatile nature of these solvents rendered the reproduction of results difficult at times, and it was sometimes observed that the best results and the most compact spots were obtained when a small chromatography jar (30 cm × 12 cm diameter) was used without the preliminary equilibration of the air in the jar with the eluant vapour. Previous equilibration had the effect of reducing movement and causing tailing. This effect could also be produced by increasing the acetone or ether content of the eluant and it is suggested that the escape of vapour from the trough, followed by its adhesion to the wet strip increased the concentration of the volatile constituent on the strip during the run, and caused the detrimental effect upon the separations. Chromatograms were developed by dipping them into a solution of 8-hydroxyquinoline in ether and allowing them to dry in air. A black spot appeared almost at once for V^{V},

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* A yellow colour at this stage was allowed to fade before proceeding, in order to avoid low results.

a dark yellowish-brown one for V^{IV} gradually turning black, and a rust-coloured spot for V^{III} which turned black over a period of several hours.

V^{III} spots became blue or yellow upon chromatograms sprayed with acidified sodium molybdate or potassium thiocyanate solutions respectively. The corresponding colours for V^{IV} spots were pale greenish brown and pale blue.

Solvent mixtures recommended for the separations of the valencies with approximate R_F values are given in Table II.

TABLE II

Valencies separated		*Solvent mixture*		*R_F values (approx.)*	*Notes*
V^V and V^{IV}	(a)	Ethanol acetic acid 20% sodium acetate solution	: 50 ml : 20 ml : 30 ml	V^V 0.50 V^{IV} 0.80 (20 cm run)	Solvent (a) preferred because of the less volatile nature of the organic constituent.
	(b)	acetone ether acetic acid 20% sodium acetate solution	: 84 ml : 42 ml : 25 ml : 50 ml	V^V 0.40 V^{IV} 0.70 (20 cm run)	Replacement of the ether by isopropyl ether gave inferior results (c.f. cobalt separations below).
V^{IV} and V^{III}	(c)	ethanol acetic acid 40% sodium acetate solution	: 50 ml : 5 ml : 10 ml	V^{IV} 0.68 V^{III} 0.97 (15 cm run)	(c) preferred to (d). It was essential to buffer the vanadium solutions to pH 6-7 with 40% sodium acetate to get a satisfactory separation.
	(d)	acetone acetic acid 40% sodium acetate solution	: 50 ml : 10 ml : 5 ml	V^{IV} 0.64 V^{III} 0.97 (17.5 cm run)	

Quantitative investigation of V^V-V^{IV} separations

Chromatograms loaded with a mixture of 0.1 ml of V^V solution (3480 µg V per ml) and 0.1 ml of V^{IV} solution (3530 µg V per ml) were run in solvent mixture (b) (see Table II). An attached "pilot" strip was run alongside each of the main chromatograms in the usual way[13]. After the run the chromatograms were dried and the pilot strips cut off and developed by dipping them into an ethereal solution of 8-hydroxyquinoline. The vanadium-bearing bands were then cut out from the main chromatograms and digested in a hot mixture of nitric acid (50 ml) and sulphuric acid (5 ml) until all solid matter had disappeared. Perchloric acid (5 ml) was then added and the mixture heated until nearly all of the latter had been fumed off. After cooling, the vanadium estimation was carried out as described previously. Two typical results are given below in Table III. A blank chromatogram was treated as one bearing vanadium. Bands of the same area as the valency zones were excised and digested, and allowance made for the readings in the vanadium analyses.

TABLE III

	V^V put on	*V recovered*	*V^{IV} put on*	*V recovered*
Experiment 1	348 µg	372 µg	353 µg	326 µg
Experiment 2	348 ,,	360 ,,	353 ,,	343 ,,

It was noted that a faint yellow tail was usually present behind the V^V spots. This was presumed to be colloidal vanadium pentoxide which had been precipitated from the acidified vanadate solution by the buffering action of the sodium acetate in the solvent mixture and so the part of the main chromatogram bearing the tail was always included in the V^V digest. No backward tail was produced with V^{IV} (or V^{III}) spots. Exclusion of sodium acetate from the solvent mixture gave inferior separations of V^V and V^{IV}.

The results, which were not regarded as being very satisfactory, suggested that the high values usually obtained for V^V might be due to some adsorption of V^{IV} (vanadyl) ions by the precipitated vanadium pentoxide tail which also caused low values for V^{IV}. It is impossible to get a truly representative "blank" determination as another sheet of filter paper has to be used, and this is suggested as being another source of error.

V^{III} solutions underwent oxidation too readily on the chromatogram to permit a separation from V^{IV} to be investigated quantitatively.

Interaction of vanadium and molybdenum valencies

It was necessary, prior to biological application, to determine interactions between the valency-states of V and Mo. These tests were carried out using chloride solutions, and were based upon the reducing properties of the V valencies given in Table I, the reactions of Mo valencies with potassium thiocyanate, and the fact that copper sulphate and mercuric chloride were reduced to cuprous or mercurous chloride by Mo^{III} but were unaffected by Mo^V. The conclusions drawn from these tests are summarized in Table IV.

TABLE IV

(— no action, + interaction)

	V^V	V^{IV}	V^{III}	V^{II}
Mo^{VI}	—	—	+	+
Mo^V	+	—	—	+
Mo^{III}	+	—	—	—

Cobalt

For biological purposes it was desired that the Co in the valency solutions should not be in the form of co-ordinate complexes. As the Co^{+3} ion itself is unstable, a solution of the weakly-complexed acetate was used for the work.

Preparation of cobalt solutions

Co^{II}. Cobaltous acetate tetrahydrate (1.0 g) was dissolved in water, 1 ml of glacial acetic acid was added and the volume made up to 100 ml with distilled water. This solution contains 2370 µg of Co per ml.

Co^{III}. 50 ml of ice-cold Co^{II} solution (prepared as above) was added gradually with shaking to an ice-cold mixture of saturated potassium bicarbonate solution (9 ml) and 100 volume hydrogen peroxide (1.5 ml). Acetic acid (2 ml) was added, and when effervescence had ceased the solution was diluted to 100 ml with distilled water. This solution contained 1185 µg Co per ml.

Estimation of the strength of Co solutions. The strength of both valency solutions was estimated colorimetrically as the blue thiocyanate compound (Co^{II}) in acetone.

Reagents required

1. *80% acetone.* A mixture of acetone 80 vols. and water 20 vols.

2. *Thiocyanic acid.* 40 ml of hydrochloric acid (d = 1.18) was mixed with an ice-cold solution of potassium thiocyanate (40 g) in water (40 ml). Acetone (400 ml) was added to the mixture (which was kept ice-cold during the addition) and after being allowed to stand in the refrigerator for an hour, the precipitated potassium chloride was removed by filtration through a Whatman No. 41 filter paper.

3. *Standard Co solution.* 0.4034 g of AnalaR cobaltous chloride hexahydrate was dissolved in distilled water, and the solution diluted to 100 ml in a graduated flask. This solution contains 1000 μg of Co per ml

A Hilger "Spekker" absorptiometer was calibrated by mixing volumes of this standard solution ranging from 0.2 ml to 2.0 ml with 10 ml of thiocyanic acid reagent and diluting to 50 ml with 80% acetone. Readings were obtained on the drum using Ilford No. 607 orange filters and a drum-setting of 1.30. The Co concentration range in the acetone solutions was 4-40 μg per ml.

The strength of the Co^{II} acetate solution was checked by treating an 0.5 ml aliquot in the manner described. A value of 2375 μg Co per ml was obtained. A similar check was performed upon 0.5 ml of the Co^{III} acetate solution giving a value of 1187 μg Co per ml.

The presence of hydrochloric acid was found to suppress the colour of the cobalt thiocyanate (presumably because of dissociation) and iron, which caused serious interference, could not be effectively suppressed by stannous chloride in the acetone medium employed. A possible explanation for this is that stannous chloride is present in acetone as a covalent compound, thereby rendering the reducing power of the Sn^{II} ion ineffective.

Chromatographic separation of the valency states

For qualitative experiments more concentrated Co^{II} and Co^{III} solutions were prepared. For a Co^{II} solution 10 g of cobaltous acetate tetrahydrate and 5 ml of acetic acid were dissolved in distilled water, finally diluting to 50 ml and filtering. To prepare the Co^{III} solution, 10 ml of this Co^{II} solution (ice-cold) was slowly added to an ice-cold mixture of saturated potassium bicarbonate solution (35 ml) and 100 vol. hydrogen peroxide (5 ml), followed by 6 ml of glacial acetic acid. Freshly prepared Co^{III} acetate solution, when analysed by paper-strip chromatography (see below) gave a very faint Co^{II} spot.

When it was desired to remove residual hydrogen peroxide from the Co^{III} solution immediately, a small quantity of a suspension of metallic silver (prepared by mixing solutions of silver nitrate, sodium hydroxide and hydrogen peroxide, and centrifuging and washing the precipitate) was added; the mixture was shaken for a short time and then filtered from the silver. By allowing hydrogen peroxide solutions of different strength to stand over precipitated silver, an approximate minimum concentration of 1 part by weight of H_2O_2 in 10,000 volumes of solution could be detected by the escape of oxygen bubbles from the surface of the metal.

Spots of the mixed valency solution were run on acid washed Whatman No. 1 paper strips by downward chromatography.

The eluant used was prepared by warming powdered magnesium acetate (1.5 g) with ethanol (60 ml) and acetic acid (3.5 ml) until the solid had dissolved The solution was cooled, 30 ml of diethyl ether was added, and the mixture filtered and stored in the refrigerator. The diethyl ether could be replaced by 30 ml of iso-propyl ether with virtually the same results, but with *n*-butyl- or *n*-amyl ether, the movement of the spots was reduced and resolutions were inferior. When the *iso*-propyl ether : ethanol ratio in the eluant was increased to 40 : 50 or 50 : 40, good results were obtained, provided that the separations were carried out on unwashed Whatman No. 1 strips. An increase in the ethereal constituent imparts a swifter flow-rate to the eluant, and this seems to be enhanced by the acid-washing of the paper. Consequently, the flow is too great, and poor resolutions are obtained. The Co separations, like the vanadium ones (*q.v.*), were sometimes impaired by the previous saturation of the air in the chromatography jar with the eluant vapour.

The inclusion of magnesium acetate in the eluants improved the separations and was prompted by the discovery of POLLARD and co-workers[14] that the addition of ammonium salts of fatty acids to solvents used for the separation of alkaline earth elements resulted in better resolutions. Ammonium or potassium acetate was not sufficiently soluble in the eluants employed for the cobalt separations to permit the use of either of these salts, and so magnesium acetate was employed instead.

The dried strips were developed by dipping them into either a solution of oxine in ether and drying in air (yellow spots) or into the thiocyanic acid reagent (blue spots). A spray of potassium iodide in dilute sulphuric acid detects Co^{III} as a brown spot, due to the liberation of iodine.

When the strips were sprayed with a 0.2% solution of 2-nitroso-1-naphthol-4-sulphonic acid in 50% ethanol, which has been found to be a very sensitive detecting reagent for cobalt by POLLARD *et al.*[15], it was observed that the Co^{III} zone tailed back to the starting point. The approximate R_F values of the main spots were 0.9-0.95 for Co^{III} and 0.55-0.60 for Co^{II} (13-14 cm run).

A quantitative investigation was made by the usual method[13]. The Co valency bands were ashed with nitric and perchloric acid, and then fumed to dryness with hydrochloric acid. The residues were taken up in a few drops of water, 10 ml of thiocyanic acid reagent was added, and the solution diluted to 50 ml with 80% acetone. The Co concentrations were then measured colorimetrically on the "Spekker". A blank chromatogram was treated in a parallel manner. The values for the Co^{II} fraction were always high, and those for the Co^{III} fraction low, due presumably to the gradual reduction of the latter on the paper during the run

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SUMMARY

Solutions containing penta-, tetra- or trivalent vanadium were prepared and their vanadium strength estimated The reducing power of all the vanadium valencies upon Cu^{+2} and Hg^{+2} salts, acidified molybdate, and nitrate was investigated Separations between the valency states, which included a quantitative investigation of a V^{V}-V^{IV} separation, and between V^{III} and Zn by paper chromatography are described. The interaction of vanadium valencies with some Mo valency-states are summarized.

A chromatographic separation of Co^{II} and Co^{III} as acetates, including a brief quantitative investigation, is also described.

RÉSUMÉ

Des solutions, renfermant le vanadium penta-, tétra-, ou trivalent, ont été préparées; la teneur en vanadium de ces solutions a ensuite été déterminée. Le pouvoir réducteur du vanadium, à ces différentes valences, sur les sels de Cu^{+2} et de Hg^{+2}, le molybdate en solution acide et le nitrate, a été examiné. Des séparations entre les différents stades de valence (comprenant une recherche de séparation quantitative de V^{V}-V^{IV}) et entre V^{III}-Zn par chromatographie sur papier sont décrites. Les interactions du vanadium et du molybdène, à différentes valences, sont signalées

Une séparation chromatographique de Co^{II}-Co^{III} comme acétates, avec une brève étude quantitative, est également décrite

ZUSAMMENFASSUNG

Fünf-, vier- oder dreiwertiges Vanadin enthaltende Lösungen wurden hergestellt und ihr Vanadingehalt bestimmt. Das Reduktionsvermögen der verschiedenen Wertigkeiten des Vanadins gegen Salze von Cu^{+2} und Hg^{+2} sowie gegen Molybdat in saurer Lösung und gegen Nitrat wurde untersucht. Papierchromatographische Trennungen der verschiedenen Wertigkeitsstufen (mit Inbegriff einer quantitativen Untersuchung der Trennung V^{V}-V^{IV}), sowie von V^{III}-Zn wurden beschrieben. Die Wechselwirkungen zwischen den verschiedenen Wertigkeiten des Vanadins und einigen Wertigkeiten des Molybdäns wurden zusammengefasst.

Eine chromatographische Trennung der Acetate von Co^{II} und Co^{III}, sowie eine kurze quantitative Untersuchung dieser Trennung wurden ebenfalls beschrieben.

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