Bio-analytical studies of the bleomycins

BIO-ANALYTICAL STUDIES OF THE BLEOMYCINS

by

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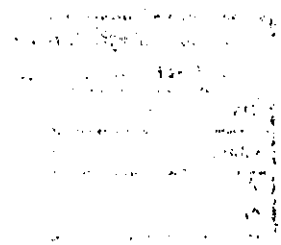
This is to certify that except where otherwise stated the work described in this thesis is believed to be original. It has not been submitted, in full or in part, to this, or any other institution for a degree.



TO MY MOTHER AND

THE MEMORY OF MY FATHER

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1. INTRODUCTION

1.1 General Aspects

Since the development of penicillin by Florey and Chain (1) for therapeutic use, there has been an almost continuous search for new antibiotics. In such a search various strains of Streptomyces verticillus native to the soil of a coal mining district of Japan were investigated. From one such sample an antibiotic complex was isolated as a copper containing blue powder which inhibited Gram-positive and Gram-negative bacteria and Erhlich carcinoma. This group of closely related antibiotics were termed the bleomycins (2); they were soluble in water and methanol but insoluble in other organic solvents. The bleomycins were identified as sulphur-containing, basic glycopeptides (2) and could be resolved by ascending paper chromatography into two groups, the A and B type bleomycins. The bleomycins have the general structure shown in Fig. 1 and differ in structure from one another only in their terminal amine components (Table 1) (3) (4). The bleomycins have a close structural relationship with another group of antibiotics, the phleomycins (Fig. 2). The phleomycins are also produced by certain strains of Streptomyces verticillus and like the bleomycins are isolated as copper containing blue powders (5). Both groups of antibiotics have similar biological and chemical properties and inhibit the growth of certain experimental tumours (6) (7).

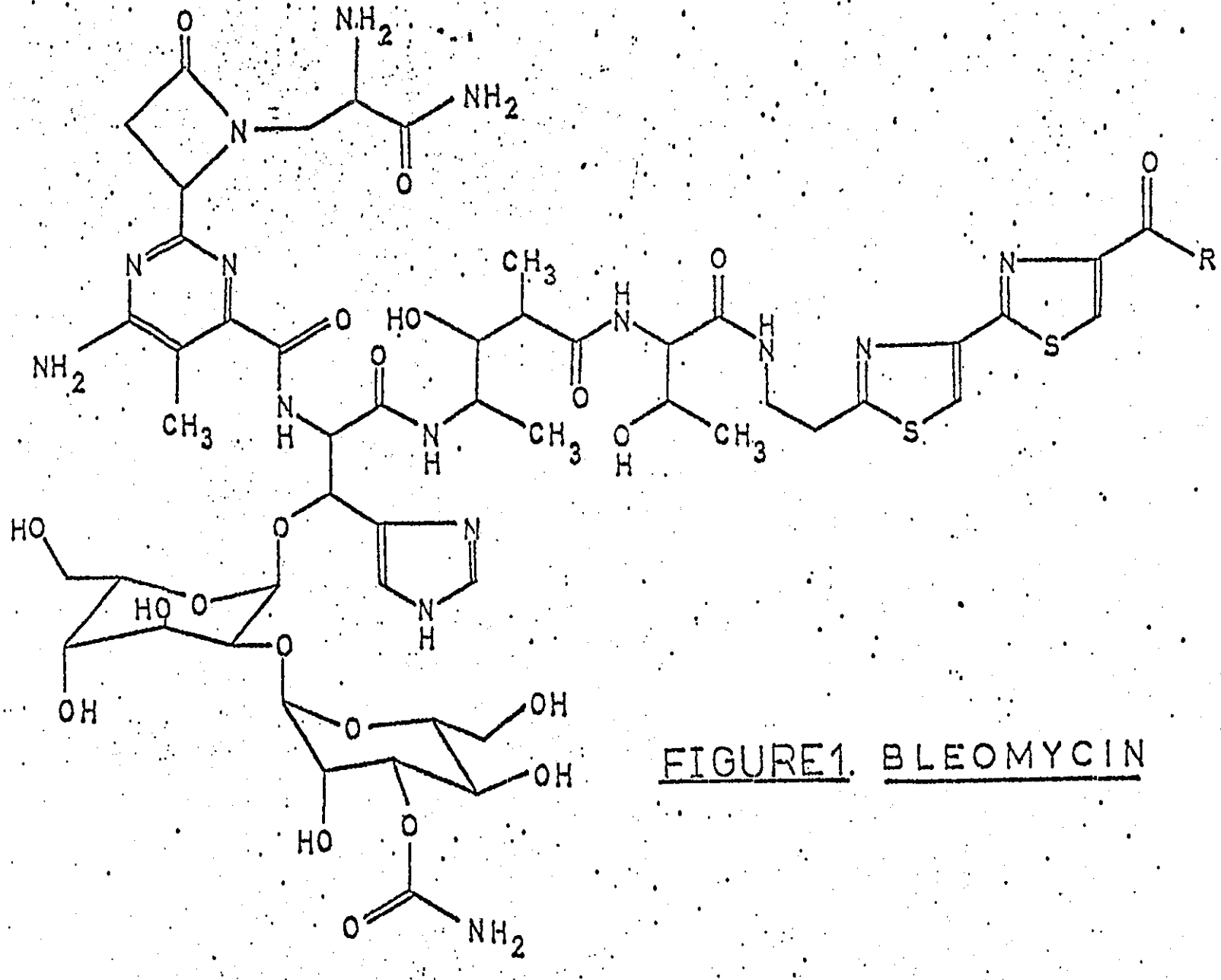


FIGURE 1. BLEOMYCIN

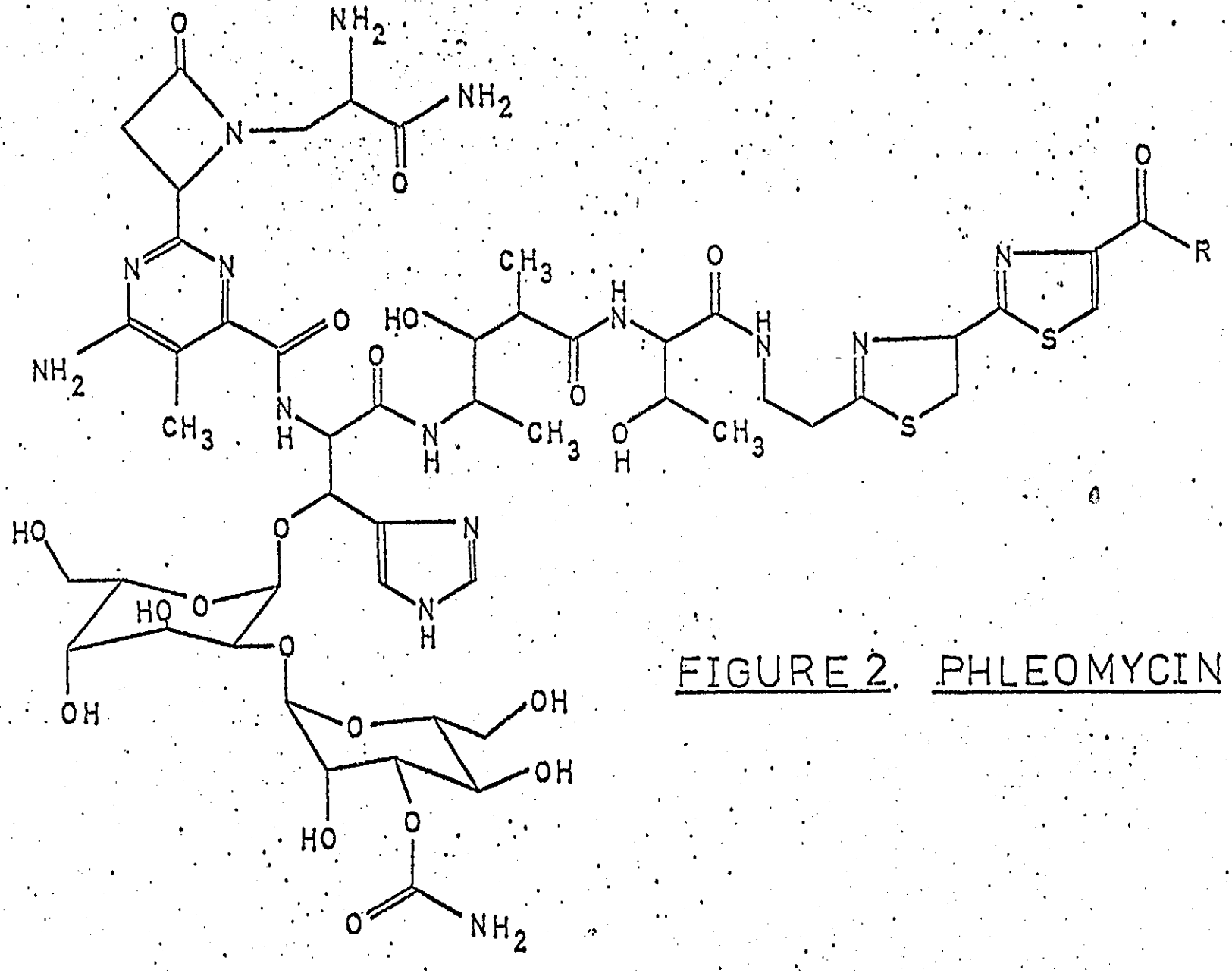
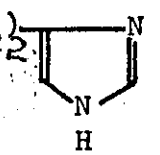


FIGURE 2. PHLEOMYCIN.

Table 1 Terminal Amine Parts of the Bleomycins

Bleomycin	Terminal Amine	Name of Parent Amine
A_1	$-NH-(CH_2)_3-SO-CH_3$	(3-aminopropyl)-methyl sulphonium
Desmethyl A_2	$-NH-(CH_2)_3-S-CH_3$	(3-aminopropyl)-methyl sulphide
A_2	$-NH-(CH_2)_3-\overset{+}{S}(CH_3)-CH_3$	(3-aminopropyl)-dimethyl sulphonium salt.
A'_2-a	$-NH-(CH_2)_4-NH_2$	Putrescine
A'_2-b	$-NH-(CH_2)_3-NH_2$	Diaminopropane
A'_2-c	$-NH-(CH_2)_2-$ 	Histamine
A_5	$-NH-(CH_2)_3-NH-(CH_2)_4-NH_2$	Spermidine
A_6	$-NH-(CH_2)_3-NH-(CH_2)_4-NH(CH_2)_3-NH_2$	Spermine
B'_1	$-NH_2$	Ammonia
B_2	$-NH-(CH_2)_4-NH-\overset{NH}{\overset{\parallel}{C}}-NH_2$	Agmatine
B_4	$-NH-(CH_2)_4-NH-\overset{NH}{\overset{\parallel}{C}}-NH_2-(CH_2)_4-NH-\overset{NH}{\overset{\parallel}{C}}-NH_2$	N-(4-amino-butyl)-N'-(4-guanido-butyl)-guanidine

The phleomycins were considered to exert their anti-tumour effect by inhibiting DNA synthesis (8) and were thought to have considerable potential as therapeutic anti-tumour agents but unfortunately they produced irreversible renal toxicity in dogs (7). The bleomycins also showed renal toxicity but unlike the phleomycins, this toxicity was reversible (7). Later Ichikawa and co-workers found that bleomycin had a therapeutic effect on squamous cell carcinoma (9).

The major therapeutic uses of bleomycin are in the treatment of squamous cell carcinoma and lymphomas e.g. Hodgkin's disease. It is apparently not successful against lung cancer by itself but bleomycin-containing combinations appear to be active in the treatment of lung squamous cell carcinoma (26) (27). It is active against many experimental tumours e.g. Ehrlich carcinoma, solid and ascites sarcoma 180, spontaneous lymphosarcoma in dogs. This antineoplastic activity probably results from interference with DNA activity and DNA synthesis (22).

Bleomycin is supplied commercially as a freeze-dried, copper-free mixture of several bleomycins, it consists of at least 65% bleomycin A_2 and approximately 25-32% bleomycin B_2. Upper limits are set for bleomycins A'_2 and B_4 (less than 1%) since these bleomycins are exceptionally toxic. The need to purify the bleomycin complex from the culture filtrate of Streptomyces verticillus and to separate the various bleomycins from each other gave rise to considerable investigations.

1.2 Purification of bleomycins

There are several purification steps in the commercial extraction of bleomycins from the culture filtrates of Streptomyces verticillus (10). The filtrate is first passed down an Amberlite IRC-50 cation-exchange resin in the H^+ form and the bleomycins adsorbed onto the resin. They are then eluted with dilute hydrochloric acid. The complex is then dissolved in methanol, converted to the copper (II) chelates and then subjected to alumina column chromatography using methanol as the mobile phase. The next step in purification involves Sephadex G-25 column chromatography which splits the antibiotic complex into two fractions A and B. Carboxymethyl-Sephadex C-25 column chromatography using an aqueous ammonium formate [0.05 - 1.0 M] gradient elution procedure resolves each fraction into several components: fraction A is resolved into six active components A_1 - A_6 and fraction B split into five components B_1 - B_5. Each separated fraction has been investigated by paper chromatography, thin-layer chromatography and high-voltage electrophoresis (10) Table 2). The t.l.c. procedure has been extensively used to separate copper-free bleomycins and copper-chelated bleomycins. More recent separation procedures involve high pressure liquid chromatography (11) (12). H.p.l.c. has been used to resolve several complex mixtures of antibiotics (13) and has the merit of being a relatively mild yet simple procedure. The h.p.l.c. methods used to

Table 2

Rf and Rm values obtained for each copper (II) bleomycin component (10). The Rf values were obtained using Silica Gel G and 10% Ammonium acetate-methanol (1:1). The Rm values refer to high voltage electrophoresis (2000 V and 25 mA), buffer system formic acid-acetic acid-water (25:75:900 by volume, pH 1.8). The Rm values were calculated taking L-alanine as the reference compound (Rm = 1)

Bleomycin Component	Rf	Rm
A_1	0.74	0.66
A_2	0.40	0.79
A_3	0.13	0.91
A_4	0.49	0.92
A_5	0.51	0.84
A_6	0.30	0.84
B_1	0.75	0.58
B_2	0.68	0.74
B_3	0.68	0.80
B_4	0.60	0.78
B_5	0.52	0.86

resolve the bleomycin complex have so far been relatively lengthy procedures and take several hours to achieve separation.

1.3 Structural features

The structures of the bleomycins have been elucidated (14) (15) (16) (17) (18) (60). The peptide moiety of the bleomycins (Table 3) consists of (i) L-β-amino-alanine amide linked via a β-amino group to β-methine of (4-amino-6-carboxy-5-methyl-pyrimidin-2-yl) propionic acid which is considered to form a β-lactam ring with the β-amino group.

(ii) L-erythro-β-hydroxyhistidine

(iii) (2S,3S,4R)-4-amino-3 hydroxy-2-methyl-n-valeric acid.

(iv) L-threonine.

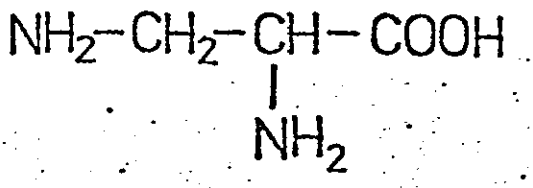
(v) 2'-(2-aminoethyl)2,4'-bithiazole-4 carboxylic acid and terminal amine.

The sugar component of the bleomycins (Table 4) consists of 2-O-(3-O-carbamoyl-α-D-mannopyranosyl)-L-gulose and is linked via an α-glycoside bond to the hydroxyl group of β-hydroxy histidine.

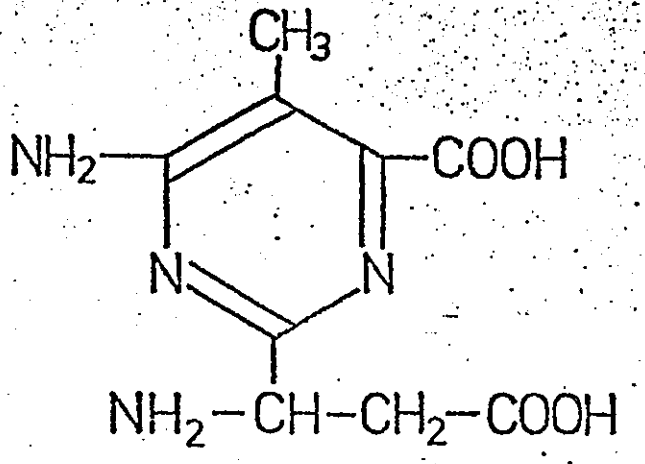
The major structural difference between the bleomycins and the phleomycins is the sulphur containing heterocyclic ring system, bleomycins have two thiazole rings (bithiazole acid) while phleomycins have a dihydrobithiazole component. It is possible to convert certain phleomycins into the corresponding bleomycin (15) by treating an aqueous solution of the phleomycin with manganese dioxide e.g. phleomycin D_1 can be converted into dehydrophleomycin D_1 (Bleomycin B_2).

Table 3. Components of the peptide moiety of the bleomycins :

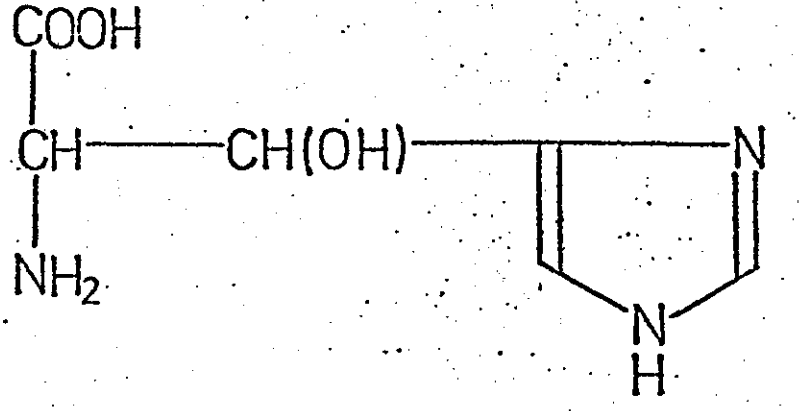
L-β-aminoalanine



β-amino-β-(4-amino-6-carboxy-5-methyl-pyrimidin-2-yl) propionic acid



L-erythro-β-hydroxyhistidine



4-amino-3-hydroxy-2-methyl-n-valeric acid

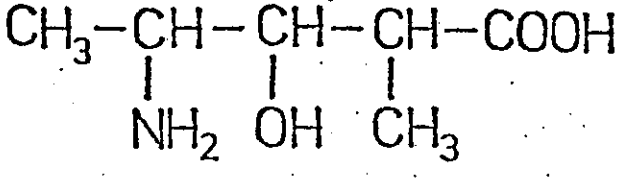
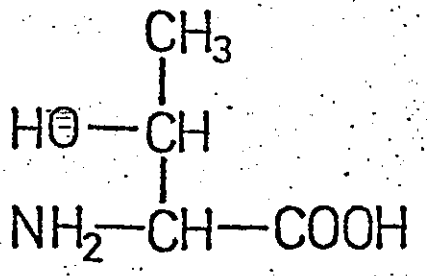


Table 3 (cont.)

L-threonine



2'-(2-amino ethyl)2,4' bithiazole-4-carboxylic acid

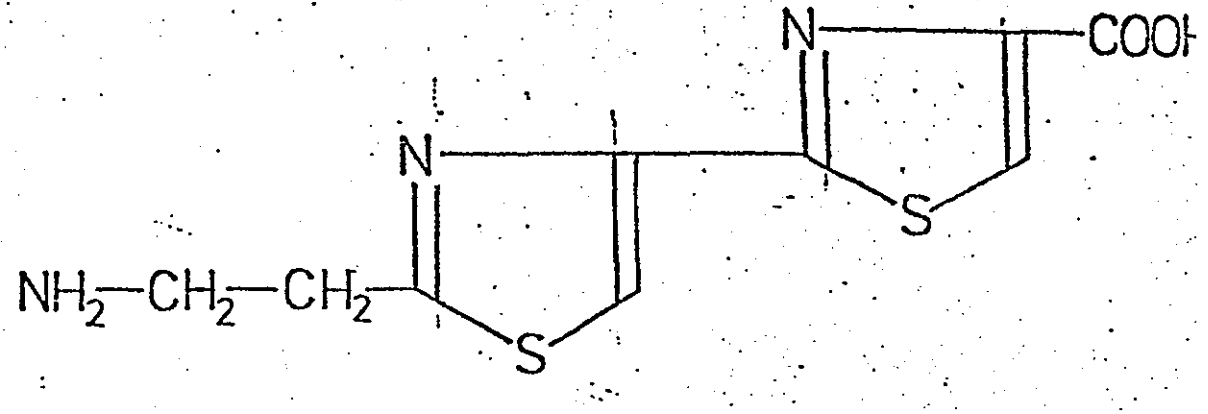
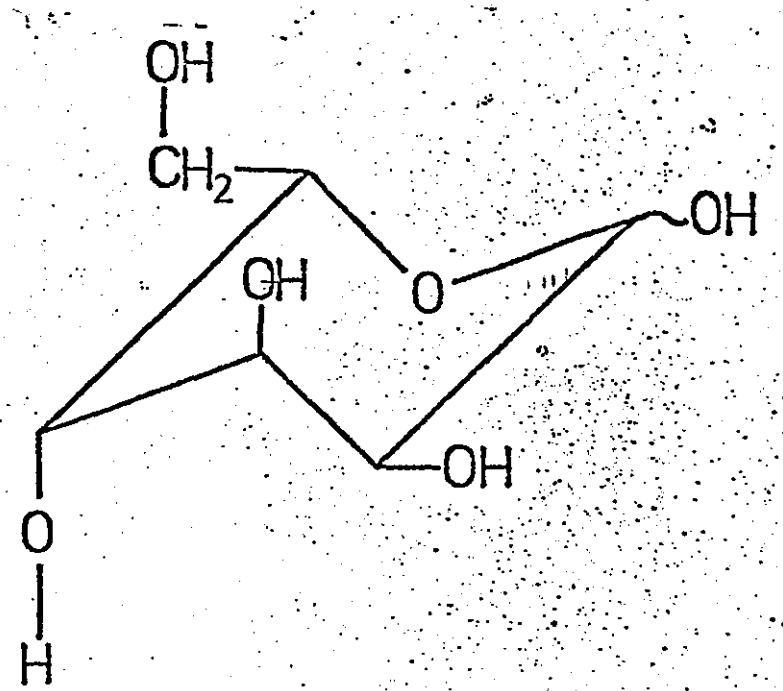
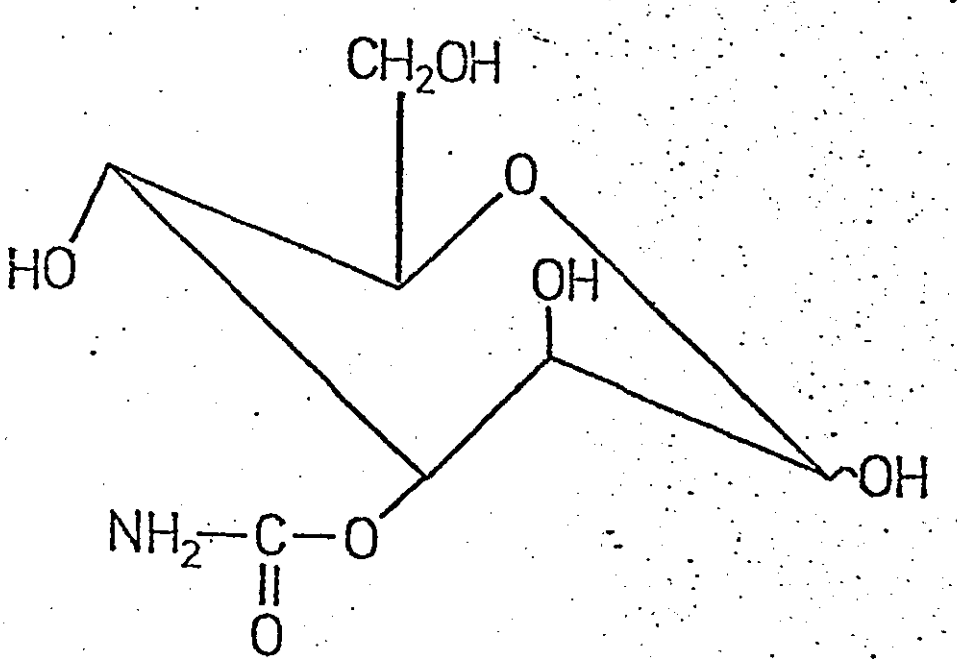


Table 4. Sugar components of bleomycins

L-Gulose



3-O-Carbamoyl-D-Mannose



The amine composition of the bleomycin complex may be altered by adding a particular amine e.g. spermine to the culture media (3). This not only gave rise to the preferential production of the bleomycin containing the added amine e.g. bleomycin A_6 but also suppressed the production of other bleomycins. Bleomycins B_4 and B_6 contain more than one guanido group in their terminal amine component (Table 1) and like the phleomycins cause irreversible kidney toxicity in dogs (19), hence it becomes necessary to remove these compounds from the bleomycins produced for therapeutic uses. It is possible that by adding a particular amine to the culture filtrate that the production of bleomycins B_4 and B_6 may be prevented and thus produce a less toxic but still potent anti-tumour bleomycin complex.

Other studies have investigated the possibility of producing novel bleomycins by enzymic methods (20). The enzyme agmatine aminohydrolase is found in a certain strain of Fusarium anguioides SHERBAKOFF and hydrolyses bleomycin B_2 to bleomycinic acid (R = H, fig. 1) and agmatine. This enzyme is specific for bleomycin B_2, all other bleomycins are not hydrolysed by this enzyme. The bleomycinic acid can be chemically converted into artificial bleomycins. Bleomycinic acid like the bleomycins forms a strong chelate with copper (II) ions but unlike the bleomycins, bleomycinic acid has only a weak anti-microbial activity against Mycobacterium (20). This suggests that the terminal amine part of the bleomycin molecule is somehow involved in the anti-microbial

activity of the antibiotic and also is not involved in the chelation of metal ions.

The copper-binding sites of the bleomycin molecule are as yet unclear. Studies on the pK'_a values of bleomycin by potentiometric titration have yielded three basic functions 2.9, 4.7 and 7.3. These have been assigned to the 4-amino pyrimidine, imidazole and β-aminoalanine groups respectively (21). The pK'_a 4.7 was assigned to the imidazole group because the chemical shift of the imidazone-2-proton was sensitive to the pH change from 4 to 6. Umezawa (21) has suggested that the α-amino group of the β-amino alanine component is a site of copper (II) binding since the pK_a of this group is lost when copper (II) is bound to bleomycin. The carboxamide group of β-amino alanine seems to play a role in the attack of DNA by bleomycin since an enzyme which inactivates bleomycin in certain tissues (23) removes this group and the resulting product does not attack DNA (24). Copper (II)-bleomycin does not bind to DNA and it is reasonable to assume that the manner in which the copper (II) is bound alters the conformation of the bleomycin molecule and prevents it binding to DNA.

The 3-O-carbamoyl group on the sugar component of bleomycin has been implicated in copper (II) binding, this group undergoes a transfer under alkaline conditions and migrates to the 2-position (25). This rearrangement takes place in copper-free bleomycin A_2 but no interconversion takes place in copper-chelated bleomycin A_2 indicating that the

carbamoyl group is involved in the chelation of copper (II) ions.

Copper-chelated bleomycins can be converted into the copper-free form by treatment with hydrogen sulphide (2). Both the copper-chelated and copper-free bleomycins show similar activity in inhibiting the growth of animal and bacterial cells but the latter form appears to be less toxic when used for human therapeutic investigations.

1.4 Biochemical Toxicity of bleomycins

The toxicity (LD_{50}) of the copper (II)-chelated bleomycins in rats and mice was found to about one-third that of the copper (II)-free bleomycins (24). Major changes observed in experimental animals were inflammation of the skin, severe weight loss, loss of hair and slight liver toxicity (9) (22) (27) (29). When the administration of bleomycin to these animals was stopped, all these effects disappeared. Long term investigations on young pure bred beagles showed that the major chronic long term effects of bleomycin toxicity were interstitial pneumonia and pulmonary fibrosis of the lungs (19). The toxicity of bleomycin was found to be related to the dose schedule, Jorgensen (29) studied mice which had been subjected to a transplanted dermoid carcinoma and obtained almost identical results with respect to tumour growth and animal toxicity when the bleomycin was administered by daily injection for three days or by twice weekly injections for three weeks. However, cyclic treatment i.e. six injection within 48 hours once a week for three weeks,

was found to give the best therapeutic index of the three dose schedules, giving a slightly reduced therapeutic effect but markedly decreased toxic effects.

Bleomycins also exhibit histamine-like effects in experimental animals when injected at relatively high doses, a transient decrease in blood pressure occurs which is probably due to peripheral vasodilation.

In marked difference to most anti-neoplastic drugs, bleomycin only causes minimal bone marrow damage (30). The major toxic effect in bleomycin treated patients is pulmonary toxicity (31) and is the cause of death in about 10-25% of the patients treated (32) (33). A majority of treated patients suffer from hyperpyrexia, this tends to occur several hours after the dose of bleomycin has been given (28).

1.5 Mode of Action

Bleomycin has created considerable interest in that it has a therapeutic effect on certain tumours but its mode of action has also probably stimulated interest in several fields of research. It was observed that bleomycins inhibited DNA synthesis in *E. coli*, Ehrlich ascites tumour and HeLa cells (34). Even more interest was taken when *in vitro* studies (35) showed that copper-free bleomycins and phleomycins were physically bound to DNA. Copper-free bleomycins after binding to DNA gave rise to strand-scission of the DNA with a resultant decrease in its melting temperature T_M, this was obtained with single and double-stranded DNA from several sources (36).

This strand scission was inhibited by the presence of EDTA, copper (II), cobalt (II) and zinc (II) ions (37) but enhanced by the presence of sulphydryl compounds e.g. 2-mercaptoethanol and hydrogen peroxide. The chelating of bleomycin to the divalent ions prevented the binding of bleomycin to DNA (37).

Umezawa (65) has recently proposed a possible mechanism which may account for such inhibition and activation of bleomycin on DNA. It is considered that when inside cells the copper (II) ions chelated to bleomycin molecules are removed by interaction with a low molecular weight, sulphydryl containing compound (possibly cysteine) releasing free bleomycin and reducing the copper (II) ions to copper (I) ions.

The copper (I) ions are then thought to be either bound to high molecular weight cellular material or reoxidised back to the copper (II) oxidation state and then bound to high M.Wt. cellular material.

When poly d (TA) poly d (TA) DNA or poly (dA) poly (dT) DNA was subjected to copper-free bleomycin attack in the presence of dithiothreitol, no release of phosphate groups was detected but thymine residues were released (38).
When poly d(GC) poly d(GC) DNA, poly (dG). poly (dC) DNA or naturally occuring RNA and synthetic polyribonucleotides were investigated, no degradation of the nucleic acids was observed.
It has been proposed (38) that the degree of degradation of polynucleotides by bleomycin was directly proportional to the concentration of dA and dT in the polynucleotide and that

thymine was released by bleomycin from the thymine residues of thymidine containing polynucleotides. The product of this degradation would be an athyminic nucleic acid and it is considered that when the release of thymine residues from the nucleic acid reached a certain level, strand scission of the DNA occurs. It is known that athyminic nucleic acids contain non-glycoside deoxyribose residues which are rich in aldehyde groups. These aldehyde groups are the main enzyme inhibitory sites of apurinic nucleic acids (38), so it is reasonable to regard that the inhibition of DNA synthesis by bleomycins (34) could be due to the aldehyde-rich athyminic DNA inhibiting the activity of enzymes which use DNA as a substrate or as a template e.g. DNA polymerase (39) (40).

Bleomycin markedly inhibits an ATP-dependent DNA ligase prepared from rat ascites hepatoma (41). This enzyme has been implicated in DNA replication, recombination and repair, it is possible that if the primary action of bleomycin is the scission of DNA in vivo, the repair of the damaged DNA will be impaired if the DNA ligase is inhibited and thus increase the lethal effect on rapidly-dividing cells.

1.6 Tissue Distribution

The tissue distribution of the bleomycins has been investigated using both a microbiological assay (42) (43) for bleomycin or radioisotope techniques e.g. tritiated bleomycin (11) and ^{57}Cobalt-bleomycin. All studies have shown relatively low concentrations in liver and spleen and relatively high concentrations in skin and lung tissues. There appeared to be a variation in tissue distribution with different bleomycins, bleomycin A_2 was at its highest tissue level in skin while bleomycins B_1 and B_4 had their highest levels in lung tissue (42). A study (11) on tumour-bearing female rats indicated that bleomycins A_2 and B_2 had high tumour to non-tumour ratios and high tumour concentrations while bleomycins A_1 and demethyl A_2 were not markedly concentrated by the tumour. It was considered that the difference in tumour concentration of these bleomycins was due to some extent to the charged terminal amine group of bleomycins A_2 and B_2 and the neutral terminal amine groups of bleomycins A_1 and demethyl A_2. The same study showed that the blood concentration of bleomycin showed an exponential decrease with a half-life of less than 100 minutes; there appeared to be a direct relationship between tumour bleomycin concentration and the blood bleomycin level and hence the tumour levels decreased with time.

Several tissues have shown an ability to inactivate bleomycins. The tissues with the highest rate of inactivation appear to be mouse liver and gastrointestinal tissue (46). Mouse muscle, skin and lung appear to be less active and it seems that human tissues have a similar order of activity. The rate of degradation appears to vary with different bleomycins with bleomycin A_5 being more rapidly inactivated in mice than bleomycin A_2, Bleomycin A_5 was found to be metabolised in rabbits and oxidised at its terminal amine component (spermine) to a carboxylic acid (47).

Bleomycins and their metabolites were found to be excreted in the urine but the rate of excretion has been found to vary. Nevertheless excretion takes place fairly rapidly after administration of the dose and about 24 - 47% of the dose is excreted in the urine (48) within the first 24 hours.

The inactivation of the bleomycin appears to be due, at least partially, to the presence of an enzyme (21). This enzyme was extracted from mouse liver and partially purified by ammonium sulphate precipitation and affinity chromatography. It appears to hydrolyse the carboxamide group of the β-amino alanine component of bleomycin (21), it was a labile enzyme and also hydrolysed lysinamide yet it differed from leucine aminopeptidase. It seems to preferentially inactivate bleomycin B_2, it is of note to find that this enzyme has been found to exhibit high activity in liver tissue yet has low activity in lung and skin tissue.

I.7 Bleomycin Tumour Scanning

The detection of a malignant disease in its early stages has been one of the aims of nuclear medicine. If the metabolism of malignant cells differ from normal cells it is possible that certain compounds will be localised in such cells. If such a compound were to be labelled with an isotope of the right physical properties it might be possible to locate the tumour. Bleomycin has certain properties that make it possible to be used as a diagnostic radio pharmaceutical for the localisation of tumours (49) it is concentrated in certain tumours and readily chelates with certain divalent ions. The first radionuclide bleomycin complex used for tumour localisation was ^{57}Cobalt-labelled bleomycin (50) and it was reported that this complex could be used to locate several types of tumours. It was also reported that the ability to locate a certain type of tumour was not related to the tumour response to bleomycin therapy. Unfortunately ^{57}Cobalt has certain disadvantages as a radio-label e.g. its long half-life of 270 days and this prompted the use of other radioisotopes. Several such isotopes have been investigated e.g. ^{62}Zinc, ^{111}Indium and ^{67}Copper. It has been reported that ^{59}Fe did not form a stable complex with bleomycin (51). The ^{62}Zinc-bleomycin and the ^{67}Copper-bleomycin complexes were found to be of little use for tumour localisation because of their low tumour: blood ratios (52).

^{111}Indium-bleomycin complex (53) was found to be strongly concentrated within certain tumours e.g. lymphomas (54), while this complex showed a lower tumour specificity and stability than the ^{57}Cobalt complex it had certain advantages as a tumour-locating compound:

(i) ^{111}Indium is a cyclotron produced radioisotope.

(ii) It decays by electron capture with a 67½ hour half-life, giving an optimum scanning time of 48-72 hours.

(iii) It emits two forms of gamma rays, both of which are within the required energy use of rectilinear scanners.

As stated previously, ^{111}Indium forms a weaker chelate with bleomycin than ^{57}Cobalt with the result that a considerable proportion of the radiolabel becomes bound to transferrin in the blood. This transferrin slowly releases ^{111}Indium to the bone marrow and hence gives rise to a high background of ^{111}Indium. In addition the radiation absorbed dose to certain tissues e.g. liver is substantial (1.61 rads. mCi^{-1}) and certain workers (55) have suggested that this complex should only be used with patients with known tumours in order to obtain a measure of the primary tumour and metastases or for patients that other clinical tests suggest that they have a considerable possibility of having a malignancy.

1.8 Assay Techniques

The first methods devised to assay bleomycins in biological tissue and fluids were the classical microbiological assay procedures. The discovery of biological inactivation of the bleomycins in experimental animals, and the resultant disadvantages of the microbiological assays led to the development and use of radio-immunoassay procedures. These assays will now be considered and in addition an assay to estimate the enzymic inactivation of bleomycins.

The ability of the bleomycins to inhibit a wide range of bacteria has been utilised to develop microbiological bioassays to estimate levels of bleomycins in blood, urine and tissues. The first bioassay (2) employed Mycobacterium phlei NIHJ as the test organism and used the cylinder plate technique, but apparently no detailed description of the procedure was published. Fujita and Kimura (56) used the same technique but used spores of Bacillus subtilis ATCC 6633 as the test organism. They claimed that the assay had a limit of detection of 0.1 ug. cm^{-3}, they investigated the tissue distribution, excretion and inactivation of bleomycin in man. An improved microbiological assay for the estimation was claimed by Pittilo and his co-workers (43) who used a strain of E. coli ATCC 9637 which was resistant to ethionine. This organism was used in the assay due to its high sensitivity to bleomycin, it seemed that the addition of guanine to glucose-salts agar medium increased the sensitivity of the

organism to inhibition by bleomycin. Other advantages were the linear dosage response and the rapid growth rate, nevertheless this assay had a similar limit of detection to the assay procedure of Fujita and Kimura. In these assay procedures, the diameter of the inhibitory zone was affected by several factors which included lot number of antibiotic, composition of the media and diluent. Other disadvantages were the lengthy incubation periods and interference from other antibiotics present in the biological specimens.

The search for more sensitive and more rapid methods of analysis for bleomycins in biological fluids led to the development of radio-immunoassays (57) (58). The first radio-immunoassay developed for bleomycin used ^{125}Iodine labelled bleomycin, the basis of the assay was a competitive binding assay between the ^{125}I-bleomycin and unlabelled bleomycin for antibody binding sites. The sensitivity and precision of this assay were superior to those of the microbiological assays and there was no significant interference from the several other anti-tumour compounds used in conjunction with bleomycin in cancer therapy. The more recent radio-immunoassay (58) uses ^{57}Cobalt-bleomycin, this has certain advantages over the ^{125}Iodine-bleomycin procedure in that the 270 day half-life of ^{57}Cobalt rules out the necessity of monthly labelling which has to take place with the ^{125}Iodine radiolabel. Also ^{57}Cobalt is very tightly bound to bleomycin and very little exchange between ^{57}Cobalt and other metal ions present in biological samples takes place.

It is possible that the part of the bleomycin molecule active in the microbiological assays may not be identical to those groups in the molecule which are recognised in the immunoassays since the latter assays do not pick up the inactivation of bleomycin which has been reported by microbiological assays (48) (56).

An assay (59) has been devised to estimate the enzymic inactivation of bleomycin which is based on the in vitro inhibition by active bleomycin on the DNA-dependent DNA polymerase. The greater the concentration of active bleomycin in the assay, the lower the activity of the enzyme and thus a lower rate of DNA synthesis. The procedure used involved the determination of the active bleomycin concentration in a given aliquot of bleomycin

(i) after incubation in the absence of DNA polymerase and (ii) after incubation with the enzyme.

(iii) calculation of the reduction in potency of bleomycin using a plot of incorporation of ^{3}H-TMP to DNA against bleomycin concentration. The DNA of the tissue samples containing the inactivating enzymes interferes with the determination and thus has to be destroyed prior to the determination by treatment with micrococcal nuclease. Studies using this assay system indicated that the activity of the bleomycin inactivating enzyme in mouse tissue was: liver 53%, spleen 36%, lung 28% and skin 1%.

1.9 Aims of Present Work

The plasma concentrations and urine levels of bleomycin in patients undergoing bleomycin therapy are of considerable interest since they may give an indication of the amount of bleomycin left in the body and hence that amount which is potentially available to the tumour. These levels are also important since bleomycins have toxic effects and the onset of toxic effects in patients is quite variable. The dose level of bleomycin can be 30 mg injected intra-muscularly (61) and since approx. 25 - 50% of the dose is excreted in the first 24 hours after injection the urinary concentration of bleomycin may be in the range of 7.5 - 15.0 mg. dl^{-1} of urine (assuming volume of urine excreted in 24 hours to be 1.0 l.). In view of the variable excretion and the differing tumour: blood ratios of the various bleomycins, there is a need not only to determine total bleomycin levels but also the individual bleomycins.

As mentioned earlier, several techniques have been employed to monitor bleomycin therapy. The microbiological assays suffer from lengthy incubation periods, lack of sensitivity and variations in media and diluent preparations. The ^{125}Iodine-bleomycin radioimmunoassay is said to be superior to the microbiological assays but apparently it only estimates metal-free bleomycin molecules, in addition it does not detect enzymic inactivation of bleomycins. The other radioimmunoassay which utilises ^{57}Cobalt-bleomycin appears to be the best available analytical technique for monitoring bleomycin levels in the biological fluids.

It is possible to use other techniques which are less sensitive provided a pre-concentration step is employed prior to analysis. The investigation of possible concentration techniques e.g. ultrafiltration, freeze-drying are thus worthy of investigation. Purification procedures which may remove interfering substances are also to be considered e.g. ion-exchange using cellulose-phosphate or gel filtration.

Bleomycins have been separated by t.l.c. and high voltage electrophoresis, the separated bleomycins have been detected by ultra-violet absorption at 260 nm. It may be possible to improve the sensitivity of such procedures by employing more sensitive spray reagents. This should be of benefit to routine quality control studies of bleomycin production. Gas-liquid chromatography seems to be unsuitable for the separation of bleomycins in view of their polarity and molecular weights ($\simeq$ 1,500 daltons) and to obtain a separation it is probable that conversion of the bleomycins into volatile derivatives would be required. High pressure liquid chromatography appears to be worthy of investigation since it has been able to yield rapid separations of complex mixtures of polar compounds. Again, such a method should be of use in quality control studies and if coupled with an appropriate pre-concentration or purification step may be of use in investigating bleomycin levels in biological fluids.

An interesting feature of the chemistry of the bleomycins is their ability to bind several transition metal ions. Metal-binding of bleomycins is essential for their use in the radio diagnosis of cancers since the bleomycins are used to transport the radioactive metal to the tumour tissue. The copper (II) chelates of bleomycins were used for chemotherapy but since it was discovered that this form is more toxic than the copper-free bleomycins, the latter preparation is now used. Copper (II) ions form strong complexes with the bleomycins and even if copper (II)-free bleomycins are injected intravenously into a human being there is evidence that the bleomycins interact with serum copper. Serum copper levels in man are approximately 1 ug. cm^{-3} but over 95% of this is tightly bound to the protein ceruloplasmin and the remainder is loosely associated with albumin and amino-acids. Nunn (62) has observed that the non-ceruloplasmin bound copper rapidly chelates with the bleomycins. In order to obtain a more complete understanding of the chemistry of bleomycin there is a need to investigate the stoichiometric relationship of copper (II) ions with bleomycin molecules and also to identify which particular groups on the bleomycin molecules which bind to copper (II) ions. The binding of copper (II) and cobalt (II) ions to bleomycin may be investigated by spectrophotometric methods since both chelates absorb in the visible range. Such procedures would involve the method of continuous variation and the mole-ratio method. In addition electron

spin resonance studies on the copper (II) bleomycin may suggest the nature of the groups which are involved in binding copper (II) ions.

The affinity of bleomycin and some of the terminal amine compounds for copper (II) ions can be investigated by potentiometric titration studies in order to provide an estimation of their stability constants. These may indicate possible role(s) of the terminal amines of bleomycins in metal-binding. The bleomycins may be regarded as polyamines by virtue of them having several amine and amide groups. Studies (63) (64) suggested that high molecular weight, water-soluble polyamines were effective in inhibiting the growth of experimental tumours by virtue of being incorporated into the fibrin network present in the tissue by the enzyme transglutaminase. This incorporation inhibited clot stabilisation and hence the growth of the tumour. Bleomycin and copper (II) bleomycin contain amine groups, have a relatively high molecular weight and are water soluble compounds hence an investigation of their possible influences on the transglutaminase-fibrin enzyme system was considered worthy of study. The transglutaminases used in this study are transglutaminase /Factor XIII/ and a tissue transglutaminase isolated from rat lung. Lung tissue was chosen because of its low content of bleomycin inactivating enzyme and for its response to bleomycin therapy when invaded with squamous cell carcinoma.

CHAPTER 2

LUMINESCENCE STUDIES OF BLEOMYCINS

INTRODUCTION

Luminescence may be defined as a spontaneous emission of radiation by a substance during the transition from an electronically excited state to the ground state. Luminescence phenomena includes fluorescence and phosphorescence, the major difference between these phenomena is the duration of the emission of radiation. In fluorescence, the emission of radiation lasts for a few nanoseconds to a microsecond while in phosphorescence it can persist from a tenth of a millisecond to one second and in somes cases it may be even longer.

Luminescence analysis has found increasing application in analytical investigations since the sensitivity and selectivity of the technique has been increasingly appreciated. Also a considerable number of commercial instruments ranging from simple filter instruments to recording prism or grating spectrofluorimeters have become commercially available over the last twenty years. Such machines may be modified so that they can also be used for phosphorescence analysis (66).

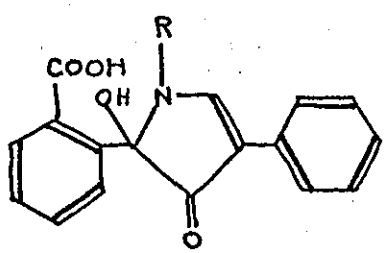
The development of luminescence spectroscopy as an analytical technique has to some extent paralleled the considerable growth of biological and clinical analysis over the last two decades. Indeed, the first modern spectrofluorimeter was developed in order to analyse biological materials (67). One use of luminescence analysis is the estimation

and detection of pharmaceuticals in biological fluids, some of these compounds have been administered to patients in small doses in the therapeutic treatment of certain disease states. Sometimes it is possible to carry out a luminescence assay after performing relatively simple extraction and fractionation procedures and obtain sensitive estimations of such substances as catecholamines in urine (68), morphine and quinine in urine (69).

Reagents have been employed which react with non-fluorescent compounds to produce a fluorescent derivative of the compound of interest. Only three of the essential amino acids have natural luminescent properties but a number of reagents such as o-phthaldehyde (70) and 1-dimethyl-aminonaphthalene-5 sulphonyl chloride (71) react with most amino acids to form fluorescent derivatives. In recent years there has been considerable interest in the development of new fluorimetric reagents for the estimation of amino-acids and related compounds. One such reagent is 'fluorescamine' (4-phenylspirofuran-2-(3H), 1-phthalan-3,3'-dione) which is sold under the trade name Fluram^{R}. Fluorescamine reacts with amino-acids and other primary amines to yield fluorescent derivatives (72), it was developed after intensive studies on the medically important estimation of serum phenylalanine. This assay depends on the reaction of phenylalanine with ninhydrin in the presence of copper (II) ions at pH 5.8 (73). The intensity of the fluorophore (λ_{ex} 365 nm, λ_{em} 515 nm) was enhanced when small peptides such as L-leucyl-L-alanine

were added to the sample. It was found (74) that the reaction between phenylalanine and ninhydrin gave rise to phenylacetaldehyde which then reacted with excess ninhydrin and any primary amine present to yield highly fluorescent products which were considered to be pyrrolinones (Fig. 3).

Figure 3. Product of fluorogenic ninhydrin reaction with a primary amine



Fluorescamine was later synthesized and replaced the fluorogenic ninhydrin reaction, it reacts with primary amines to form similar fluorophors (λ_{ex} 390 nm, λ_{em} 475) as were produced in the ninhydrin-phenylacetaldehyde reaction.

The following mechanism has been proposed for the reaction of fluorescamine and primary amines (75).

$$\text{Fluorescamine} + \text{Primary Amine} \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} [\text{F-A}] \xrightarrow{K_2} \text{P}$$

[F-A]: intermediate complex

P: fluorescent product

(K_1, K_{-1}, K_2 - rate constants)

If fluorescamine is in great excess, the intermediate complex is non-fluorescent and there is a rapid equilibrium between the reactants and the intermediate complex compared with the rate of product formation it could be shown that the rate of product formation dP/dt

$$\frac{dP}{dt} = \frac{K_1 K_2 (F) (A_T)}{K_I + K_1 (F)} = - \frac{d A_T}{dt}$$

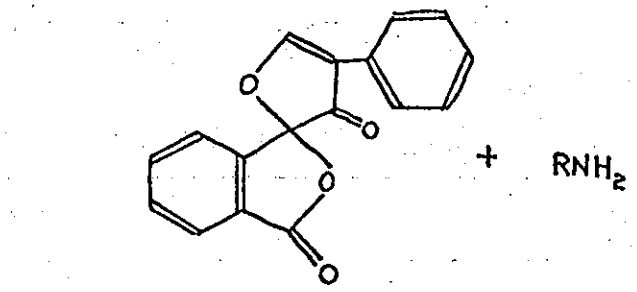
A_T = Sum of primary amine concentration present in free form and in the intermediate complex.

The rapid and reversible addition of the primary amine across the double bond of fluorescamine yields a non-fluorescent intermediate which then undergoes several rearrangements to form the final fluorophor (Fig. 4).

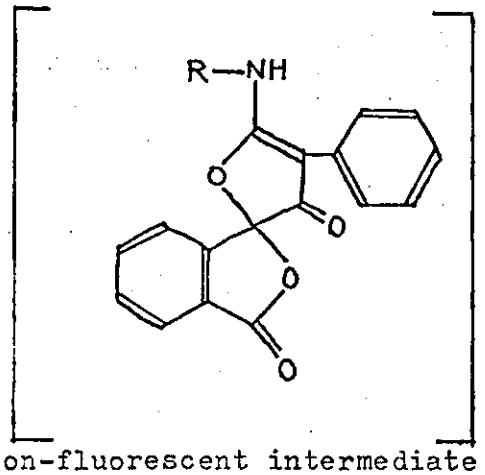
The reaction has a half-life of 200-500 m.sec. at room temperature while the excess fluorescamine is destroyed in aqueous solutions within a half-life of several seconds to yield non-fluorescent derivatives.

Fluorescamine has been used to assay various primary amine containing compounds such as amino acids, peptides and proteins (72). Fluorescamine can also be used to estimate secondary amines provided they are converted into primary amines by reaction with N-chlorosuccinimide, such a procedure has enabled the assay of the imino acid, proline (76) by reaction with fluorescamine.

Figure 4. Scheme (75) for the reaction of fluorescamine with a primary amine

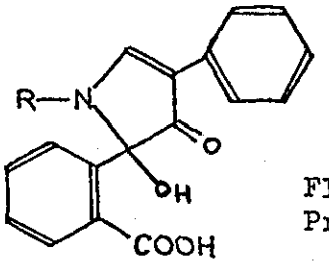


fluorescamine



non-fluorescent intermediate

several steps



Fluorescent Product

Fluorescamine can replace ninhydrin as the reagent in the automated ion-exchange analysis of amino acids (74) and the h.p.l.c. of polyamines (77). Stein et al. (78) developed an h.p.l.c. system to assay for amino-acids using fluorescamine which was more sensitive than previous systems due to the use of a luminescence detector system. The high cost of fluorescamine compared with that of ninhydrin and o-phthaldehyde has to some extent limited the use of fluorescamine. One way of minimising the amount of fluorescamine used and hence the cost in a chromatographic separation is to label the primary amines of interest with fluorescamine prior to chromatographic separation (79). The t.l.c. of amino-acids and peptides pre-labelled with fluorescamine have been investigated (80) and also the h.p.l.c. of pre-labelled polyamines (77) (81).

Fluorescamine has found considerable use as a spray reagent for the location of separated amino-acids (82) and peptides (83) on t.l.c. plates. It has also been used as a reagent to detect amphetamines in urine by t.l.c. (160). The sensitivity is greater than that obtained with ninhydrin but the fluorescence rapidly decreased and within several hours the positions of the primary amino compounds could not be detected on the t.l.c. plate. Felix and Jimenez (82) observed that when peptide-resin (from solid phase peptide synthesis) was allowed to react with fluorescamine, pre-treatment of the peptide-resin with triethylamine enhanced

and stabilised the fluorescence of the resin by preventing conversion of the fluorophor to the non-fluorescent γ - lactone. This observation was applied to the location of amino acids on t.l.c. plates and it was found that an improvement in the sensitivity and stability of the spots took place.

Several pharmaceuticals containing aromatic or aliphatic primary amine groups have been investigated for their solution fluorescence when reacted with fluorescamine and for their detection on t.l.c. (84). Kusnir and Barna (85) found that fluorescamine was able to detect at picomole levels in aqueous solution certain basic antibiotics such as streptomycin and ampicillin.

The bleomycins contain primary amino groups and as such may be possible compounds for labelling with fluorescamine. The solution fluorescence, the use of fluorescamine as a reagent to locate separated bleomycins on t.l.c. and the t.l.c. of pre-labelled bleomycins are aspects which may yield useful information.

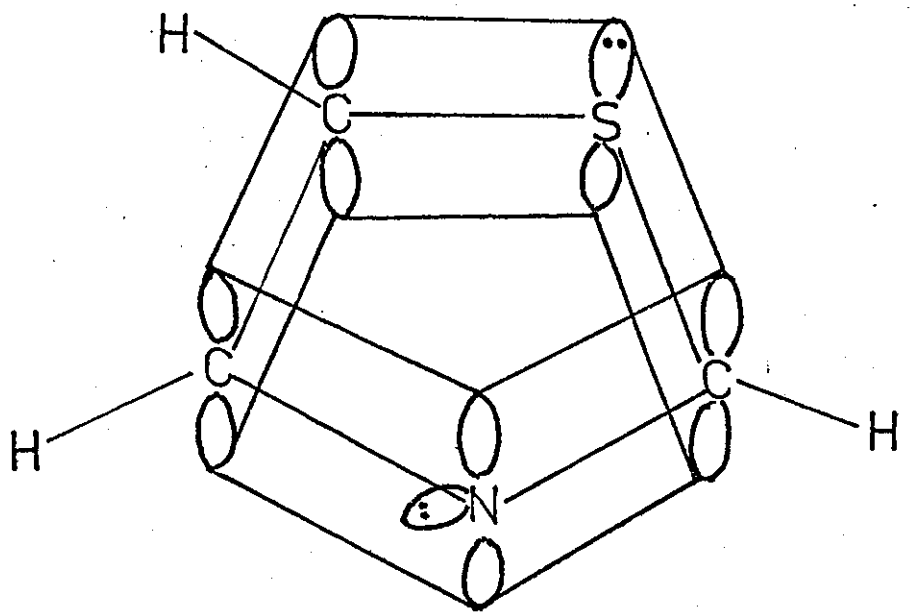
There is no mention in the literature of phosphorescence or low temperature luminescence studies being applied to the bleomycins. Several antimetabolites have been investigated (86) for phosphorescence and some were found to have analytically useful phosphorescence, one of these compounds was 2-amino-4 methyl pyrimidine (λ_{ex} 302 nm, λ_{em} 438 nm) and it is of interest that one of the components of bleomycin is 4-amino-5-methyl pyrimidine. Also the 4-amino pyrimidine group does

fluoresce weakly after n - π^* excitation (87). The bleomycins also contain a bithiazole heterocyclic ring system which is considered to exist as a planar structure in the molecule (17). The thiazole molecule has the following molecular orbital arrangement (88), Fig. 5.

Each of the three carbon atoms present in the thiazole molecule contributes a p_z-electron to the molecular orbital while the azole nitrogen donates one electron and the sulphur atoms two electrons to complete the aromatic sextet and forming a stable closed shell of six delocalised π electrons.

Thiazole is structurally related to thiophene and pyridine, but it resembles the latter compound in both chemical and physical properties (89). Since pyridine in ethanol exhibits phosphorescence (λ_{ex} 310 nm, λ_{em} 440 nm) (90) it could be expected that thiazole and bithiazole would exhibit similar phosphorescence. In most unsaturated N-heterocyclic compounds investigated, the lowest energy electronic transition is n,- π^* and such compounds are phosphorescent but not fluorescent (89). An examination of the possible low temperature luminescence of the bleomycins may provide useful data since phosphorescent location of separated components by t.l.c. may have analytical advantages.

Figure 5- Electronic structure of thiazole



MATERIAL AND METHODS

Reagents

Fluorescamine (4-phenyl spiro [furan-2(3H), 1'-phthalan]-3,3' dione) was obtained from Roche Products Ltd., London, U.K. as Fluram^{R}. The fluorescamine was used as a solution in dioxane. The solvents used were reagent grade, redistilled in glass, triple distilled water was used and kept in glass containers. All the chemicals (reagent grade) involved in the preparation of buffers were obtained from B.D.H. Ltd., Poole, U.K. The silica gel 60 t.l.c. plates (0.25 mm thickness) were supplied by E. Merck, Darmstadt, West Germany

Equipment

The fluorescence studies were carried on a Baird Atomic Fluorispec and the low temperature luminescence studies were performed on a modified Baird-Atomic Fluorispec SF 100E. The reference solution used in both situations was quinine sulphate 1.0 ug. cm^{-3}.

Methods

Solution fluorescence

The conditions for the reaction using fluorescamine in solution were similar to those described for the fluorimetric estimation of proteins (92). A solution of bleomycin (Lot 004) in phosphate or borate buffer was placed in 50 mm x 10 mm glass tube. While the tube was agitated on a vortex mixer, 0.5 cm^{3} of fluorescamine in dioxane was quickly added

to the bleomycin solution from a 1 cm^3 capacity glass syringe. A reagent blank was also performed and the reactions carried out at room temperature. After mixing, the solutions were left for ten minutes in the dark and then their fluorescences were measured.

Thin layer chromatography

The bleomycin-fluorescamine derivatives were produced by placing 20 ul of bleomycin in a phosphate buffer of pH 8.0 from a Hamilton microsyringe and then adding 50 ul of fluorescamine (20 mg. 100 cm^3 dioxane) while the test tube and contents were being agitated on a vortex mixer. An aliquot of the resulting solution (5 ul) was applied to the aluminium-backed silica gel t.l.c. plate. The solvent system used was A.R. methanol : 0.02M phosphate buffer pH 8.0 (7:3 v/v). The chromatogram was developed in the dark. After development, the plate was air-dried in a stream of cold air for 30 minutes and then sprayed with 10% triethanolamine in chloroform. The plate was again dried in a stream of cold air for 5 minutes and then resprayed again with 10% triethanolamine in chloroform. The plate was again air-dried and then examined under a long wave (366 nm) ultraviolet light and the green-yellow fluorescent spots outlined with a pencil. The R_f values were then determined.

Fluorescamine used as a spray reagent

The bleomycin were subjected to silica gel t.l.c. using either A.R. methanol : 10% aqueous ammonium acetate (1:1 v/v) or A.R. methanol : 10% aqueous sodium acetate

trihydrate (1:1 v/v) as mobile phases. The bleomycins were streaked across the origin and dried using a cold stream of air. The chromatograms were developed to a height of 15 cms, the plates were then removed, the solvent fronts were marked and the plates dried in a stream of cold air for 30 minutes. The plates were examined under short wave (260 nm) ultraviolet light and the positions of the bleomycins obtained. The Rf values of these components were determined. One half of the plate was covered with a glass plate and the other half sprayed with a solution of 10% triethanolamine in chloroform, the plate was then air-dried in a stream of cold air for 5 minutes. The exposed half of the plate was then sprayed with 0.05% fluorescamine in dioxane, then it was air-dried and then re-sprayed with 10% triethanolamine in chloroform. The plate was then dried at room temperature and then examined under long wave (366 nm) ultraviolet lamp. The fluorescent zones were located, outlined and the Rf values determined and compared with those obtained for the short-wave U.V. investigation.

Low Temperature Phosphorescence

The bleomycin sample dissolved in 0.1M phosphate buffer pH was mixed with an equivalent volume of A.R. ethanediol. The silica sample tube (internal thickness 2 nm) was washed in A.R. conc. nitric acid, rinsed with tapwater, distilled water twice with triple distilled water, 1:1 v/v ethanediol : triple distilled water and finally with the test solution. The open silica tube was then filled with

an aliquot of the test solution to a height of at least 4 cm. and then carefully lowered into liquid nitrogen and the contents of the tube frozen to yield a glassy solid. The tube and contents were then placed into the phosphorimeter sample compartment.

RESULTS

Solution fluorescence

The fluorescence produced from the reaction between the bleomycins and fluorescamine had an optimum wavelength of excitation of 390 nm and an optimum emission wavelength of 481 nm (Fig. 6). The influence of the pH on the reaction with fluorescamine was investigated (Fig. 7). The bleomcyin fluorophors were found to be reasonably stable for the first 30 minutes after reaction but a slow progressive decrease in fluorescence intensity then occurred (Fig. 8). The sensitivity of the procedure was decreased if the fluorescamine concentration used was greater than 300 mg. dl^{-1}. The method gave a linear response up to 200 $\mu g. cm^{-3}$ bleomycin and the limit of detection (twice the background standard deviation)was determined to be 0.4 $\mu g. cm^{-3}$ bleomycin. Fluorescamine did not react with copper (II) bleomycin samples.

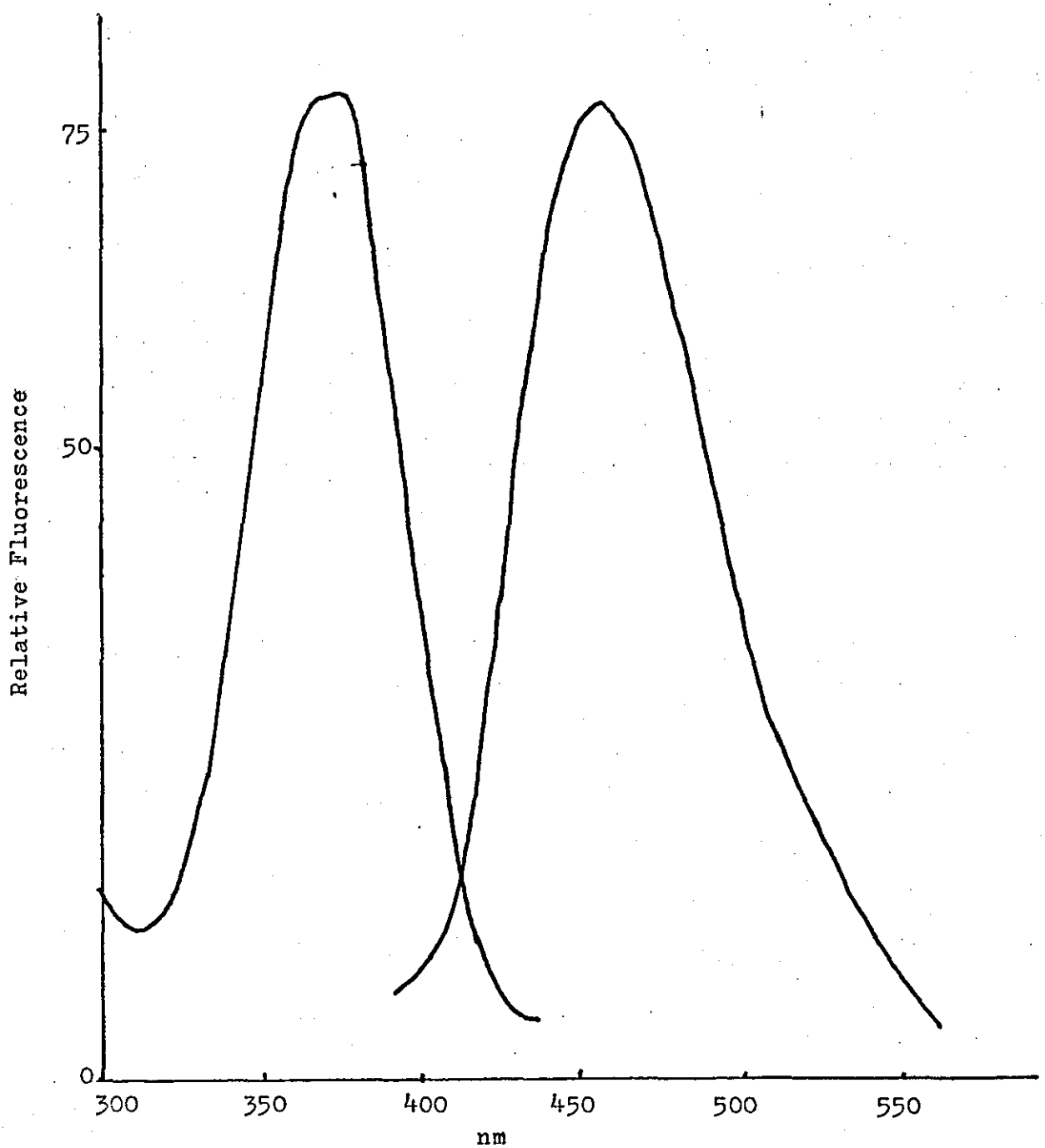
Thin-layer Chromatography

The t.l.c. of the fluorescamine derivatives of bleomycin gave the three Rf values (Table 5) the blank solution (no bleomycin present) also yielded a fluorescent spot. The t.l.c. of fluorescamine labelled bleomycin B_2 derivatives yielded two fluorescent spots, one of which had the same Rf as the spot obtained with the blank solution.

Figure 6. Excitation and emission spectrum of fluorescamine labelled bleomycin (uncorrected)

Spectra obtained in 0.05M phosphate buffer pH 8.0

Bleomycin conc. 0.5 mg. cm^{-3}.



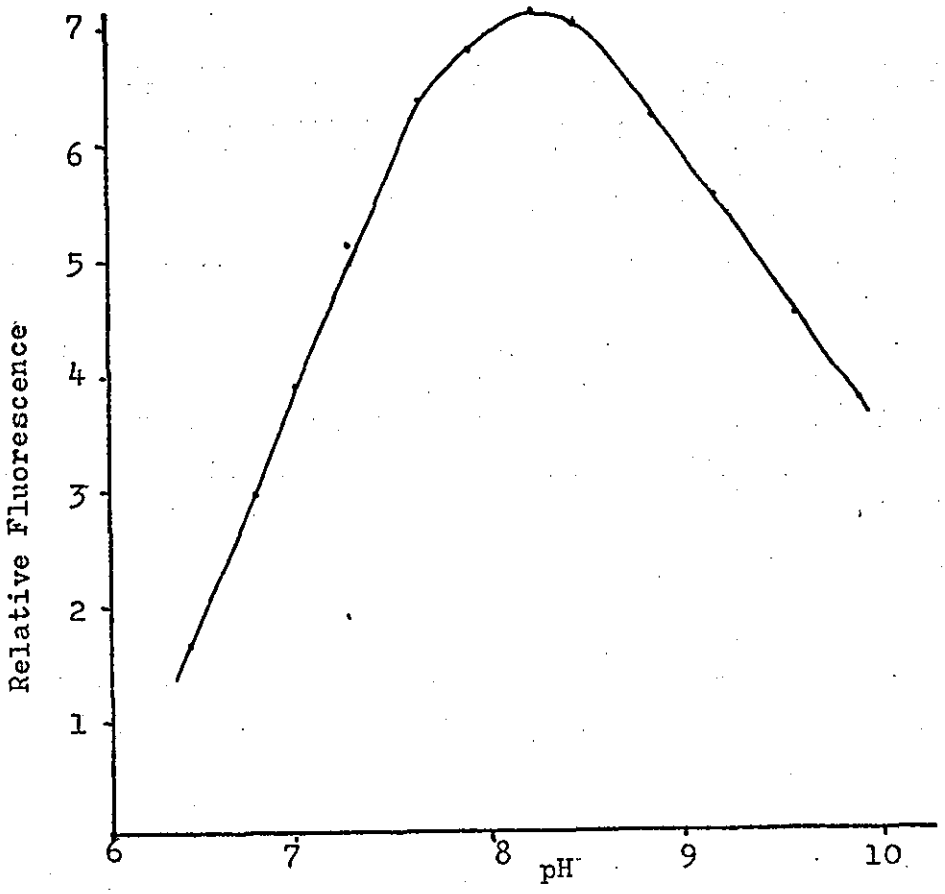


Figure 7. Fluorescence versus pH of bleomycin-fluorescamine reaction

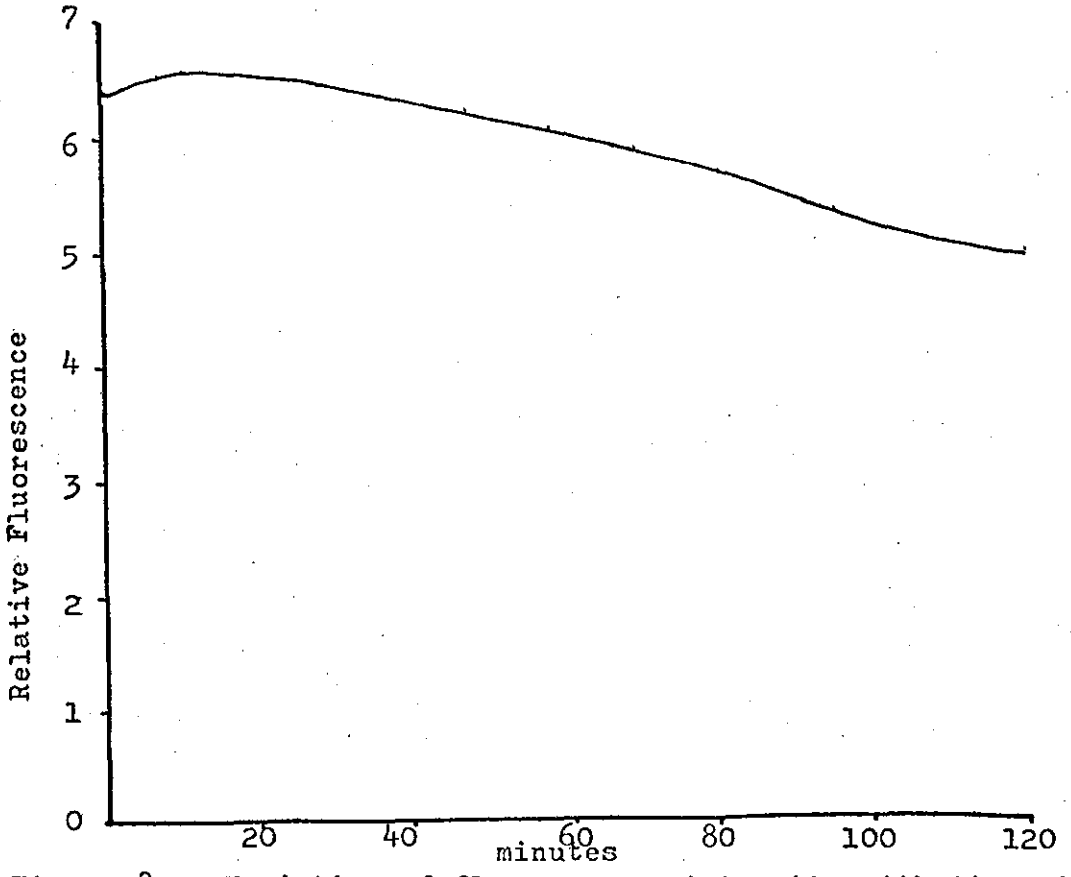


Figure 8. Variation of fluorescence intensity with time at room temperature

Table 5. Rf values of fluorescamine-labelled bleomcyins

Solution	Rf values		
Bleomycins	0.33	0.38	0.83
Bleomycin B_2	-	0.38	0.83
Buffer only	-	-	0.83

Mobile phase – A.R. Methanol : 0.02M Phosphate buffer pH 8.0 (7.3 v/v).

Stationary Phase – Silica gel G t.l.c. plates (thickness 0.25 cm).

Detection – Fluorescence on irradiation with U.V. light 360 nm.

Use of Fluorescamine as a Spray Reagent

The fluorescamine spray reagent located the major bleomycins (A_2, A_1, B_2 and DMA_2) when the fluorescamine sprayed t.l.c. plates were illuminated under U.V. light of 360 nm wavelength, the fluorescamine could not locate copper (II) bleomycins. When the bleomycin samples were applied as compact spots rather than streaks, the bleomycin A_2 spot was located by fluorescence when 10 µl bleomycin sample of concentration 100 µg cm^{-3} was applied. When the absorbance of 260 nm U.V. irradiation location procedure was used to locate separated bleomycins, bleomycin A_2 could be located when a 10 µl solution of bleomycin (600 µg. cm^{-3}) was applied as a compact spot to the t.l.c. plate.

Low temperature luminescence

The spectra obtained are shown in Fig. 9. The maximum excitation wavelength of the bleomycin mixture is 304 nm while the maximum emission wavelength is 390 nm. The spectra were investigated at several pH values but no significant change in spectral characteristics were observed. The limit of detection was determined to be 4×10^{-6} g. of bleomycin and the response was linear up to 160 ug bleomycin. No low temperature luminescence was obtained with copper (II) bleomycin samples.

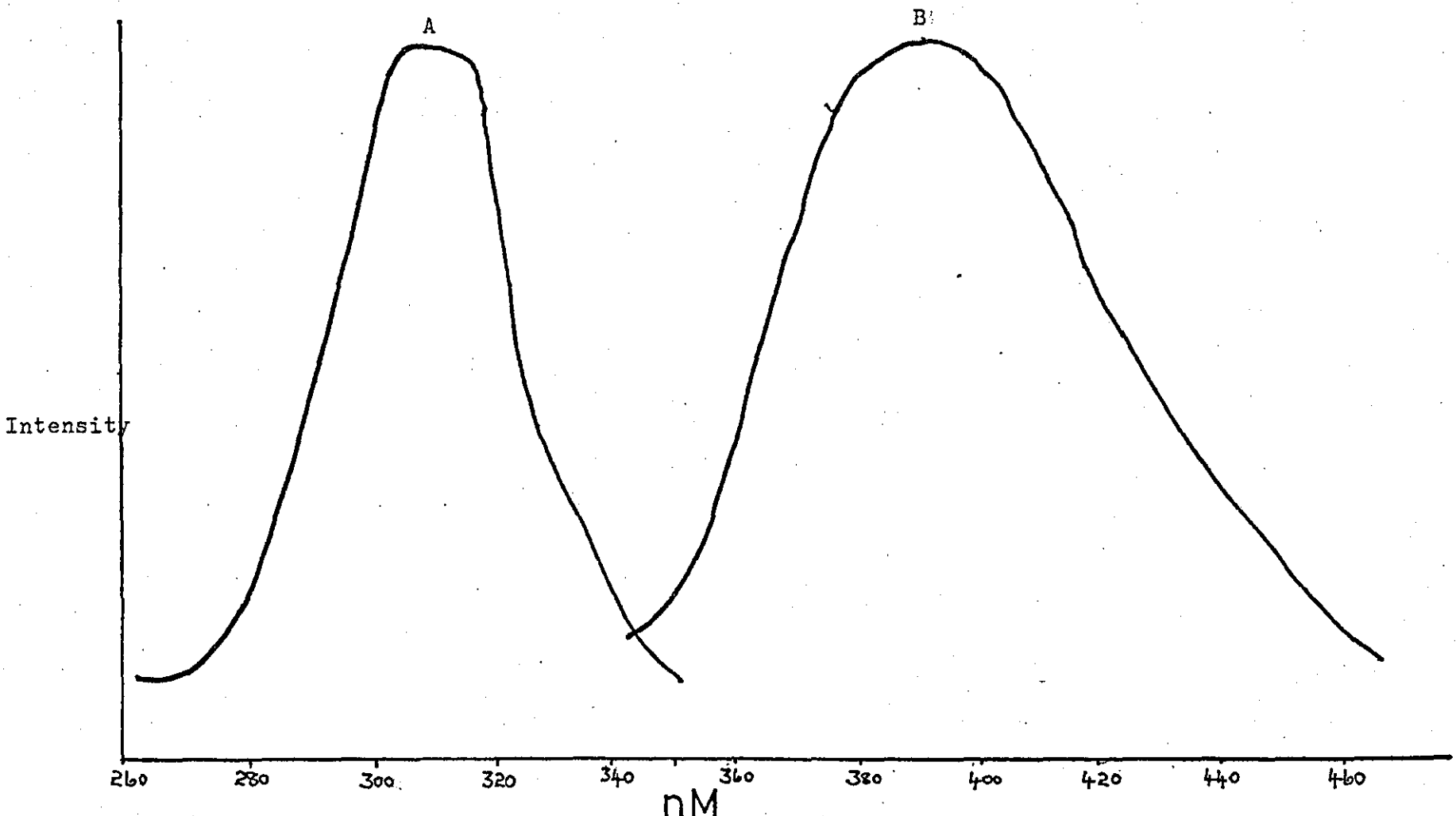


Figure 9. Total luminescence spectrum of bleomycin at 77°K

A, excitation: E, emission

DISCUSSION

Solution fluorescence

The bleomycins reacted with fluorescamine to produce fluorescent products which had an optimum excitation wavelength of 391 nm and an optimum wavelength of emission of 481 nm. These values are typical of the fluorescence characteristics of fluorescamine derivatives of primary amines; in addition the optimal pH for maximum fluorescence was pH 8.3 - 8.4 and this was within the range of pH 8.0 - 8.5 which peptides yield maximum fluorescence with this reagent.

The amine groups present in the bleomycin molecule e.g. bleomycin A_2 are the 4-amino group of pyrimidine moiety and and α-amino group of β-amino alanine component, while some of the terminal amines also have primary amine groups e.g. bleomycins A_5, A_6, B'_1. Samejima *et al.* (91) studied the fluorescence of various amines and obtained no fluorescence with adenine, creatine and guanidine but obtained considerable fluorescence with polyamines such as spermine and putrescine. The fluorescence of the bleomycins with fluorescamine is probably due to the α-amino group of β-alanine. This grouping is considered to be involved in the chelation of copper (II) ions and it is of note that no fluorescence was obtained with solutions of copper (II) bleomycin.

Fluorescamine is a sensitive reagent and can certainly detect low concentrations of metal free bleomycins in aqueous

solution but unfortunately it lacks selectivity since it reacts with most compounds which have primary amine groups.

T.l.c. of bleomycin-fluorescamine derivatives

This procedure was found to be simple and sensitive. The fluorescent spots obtained were made more stable by spraying the chromatograms with 10% triethanolamine in chloroform. The two major bleomycins (A_2 and B_2) were separated by this procedure and B_2 was identified by using standard bleomycin B_2 sample. The fluorescent spot which was obtained with all solutions even the blank solution may be due to an interaction with ammonia or a primary amine impurity in the buffer.

It was found that if the development of the chromatograms was carried out in daylight the fluorescence of the primary amine components was considerably reduced when compared with identical chromatograms developed in the dark.

This procedure may have some potential if an h.p.l.c. method is used for the separation of the fluorescamine derivatives using fluorimetric detection system. Such a system has already been developed for the h.p.l.c. of fluorescamine derivatives of polyamines (77).

Fluorescamine as a spray reagent

The location of bleomycins after separation on t.l.c. by fluorescamine is a simple, rapid and sensitive method. The stability and sensitivity of the fluorescence was found to be improved by pre-spraying with 10% triethanolamine in chloroform.

Using this procedure separated bleomycins could be detected by illumination with U.V. light of 366 nm and is superior in terms of sensitivity to that of examination of separated bleomycins by U.V. light of 260 nm. This procedure could be used to identify bleomycins in quality control studies and if each bleomycin-fluorescamine derivative has the same quantum efficiency it is possible that such a procedure could be made semi-quantitative by using a t.l.c. fluorimetric scanning system.

Low temperature luminescence

The excitation maximum of 304 nm of bleomycin is quite similar to those obtained by amino-pyrimidine derivatives (302 nm) and that of pyridine (310 nm) but the emission maxima (390 nm) is of lower wavelength than of those exhibited by amino-pyrimidine derivative (438 nm) and pyridine (440 nm). Although the thiazole molecule has similar chemical and physical properties to that of pyridine, it is possible that the bithiazole structure may have slightly dissimilar spectral characteristics and in addition a different solvent system was employed.

The low temperature luminescence is probably a n, π^* transition in view of its short half life and the properties of the 4 amino-pyrimidine and thiazole molecules.

CHAPTER 3

ELECTROPHORETIC STUDIES OF THE BLEOMYCINS

INTRODUCTION

Bleomycins have several ionizable groups and thus migrate under the influence of an applied voltage at appropriate pH values. High voltage electrophoresis was used by Umezawa *et al.* (10) in their purification studies to separate copper (II) bleomycins (Table 2) at a pH of 1.8 using L-alanine as the reference compound. Later van der Poll *et al.* (93) employed cellulose acetate electrophoresis to separate ^{57}Cobalt-bleomycins at pH 3.0 into two components but when an acetate buffer system of pH 5.6 was used, the two components were poorly resolved. The components were located by virtue of their radioactivity. There are few electrophoretic studies on bleomycin to be found in the literature and none using polyacrylamide gel electrophoresis (PAGE). PAGE has provided a relatively simple system of high resolution for the separation and characterisation of proteins and larger peptides on the basis of their size and charge (94) (95). Rapid diffussion and loss of small molecules from the gel matrix during conventional staining and destaining procedures have prevented PAGE being used to study oligopeptides and glycopeptides such as bleomycin.

A method circumventing the problems of staining and destaining is to label the components of a mixture of low molecular peptides in such a manner that they may be visualised

by their own fluorescent properties. Such a fluorescent label was used by Talbot and Yphantis (96) to investigate peptide mixtures, they used dansyl chloride (dimethyl naphthalene-5-sulphonyl chloride) to fluorescently label peptides. This procedure was found to be sensitive but time-consuming and unfortunately the results obtained were difficult to interpret due to contamination of the peptide samples with fluorescent breakdown products.

Vandekerckhove and Van Montagu (97) used fluorescamine (4-phenylspiro [furan-2(3H), 1'-phthalan] -3,3' dione) (Fig. 10) a non-fluorescent compound which reacts with peptides and proteins to form highly fluorescent products (92). They separated these fluorescamine-labelled peptides by means of sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and visualised the separated compounds by illumination of the gels at 365 nm. This procedure was sensitive and also because the hydrolysis products of fluorescamine were non-fluorescent the fluorescamine-labelled peptides were produced free of fluorescent contaminants.

Fluorescamine modified oligo-peptides were also studied by Rosemblatt et al. (98), who found that polyacrylamide gel electrophoresis of these derivatives was a rapid, sensitive and reproducible method which overcame many of the problems arising with conventional staining techniques. They investigated the effect of varying pH and gel concentrations (% acrylamide) and found that at low pH values (below pH 7.5) the fluorescamine-labelled peptides took a considerable time to

achieve separation while at pH values (pH 9.8) the negatively charged peptides migrated very rapidly and gave rise to a fluorescent tail which markedly affected resolution. The optimum pH values for separation of the fluorescamine labelled peptides was found to be in the range pH 8.9 - 9.4. Molecular sieving occurred during the electrophoretic separation of the oligopeptides in acrylamide gels and because of this it was found that the best acrylamide gel concentrations for resolution of oligopeptides were 12 - 16%.

Electrophoresis on polyacrylamide gels in the presence of sodium dodecyl-sulphate (SDS) was shown by Weber and Osborn (94) to be a reliable method of estimating protein molecular weight. PAGE of peptides was shown by Ornstein (99) and Davis (100) to be dependent not only on the charge but also on the size of the molecules. When dodecyl sulphate ions bind to proteins (103), the individual charge pattern of each protein or peptide was changed by the SDS anions which made all the molecules negatively charged. The number of SDS anions bound by a protein or peptide, and hence the overall negative charge was related to the molecular size of the molecule.

Nunn (101) observed the gel filtration properties of the bleomycins and found that although the difference between the molecular weights of bleomycins A_2 and B_2 was less than 1%, there was a considerable difference in their elution profiles. He suggested that this difference was due to differences in the sizes (Stokes radius) of the two molecules.

It was considered possible that if this observation was correct it might be possible to resolve these two bleomycins by subjecting them or their fluorescamine labelled derivatives to SDS-polyacrylamide gel electrophoresis as well as investigating the performance of fluorescamine labelled bleomycins on convential polyacrylamide electrophoresis.

Conventional SDS-PAGE of unlabelled bleomycins was also investigated, the separated bleomycins were located by means of conventional gel staining procedures using Coomassie Brilliant Blue G250.

MATERIAL AND METHODS

Materials

Acrylamide, N,N'-methylene-bis-acrylamide, ammonium persulphate, N,N,N',N'-tetra methyl ethylene diamine (TEMED), sodium dodecyl sulphate, alanine, bromophenol blue and glycerol were obtained from BDH Ltd., Poole, U.K. Coomassie brilliant blue G.250 (C.I. 142655) was obtained from Raymond Lamb, North Acton, London N.W.10 6JL.

The Shandon Analytical polyacrylamide electrophoretic apparatus and Vokam power pack were obtained from Shandon Scientific Products Ltd., Runcorn, U.K.

Methods

Polyacrylamide Gel Electrophoresis of Bleomycin-Fluorescamine Derivatives.

10 µl. of an aqueous bleomycin sample was mixed with 30 µl. of 0.05 M sodium phosphate buffer pH 8.0 containing glycerol to give a final concentration of 50% (v/v) in a 2.0 cm x 0.3 cm glass tube. While the solution was being rapidly mixed on a Vortex mixer, 8 µl of a freshly prepared solution of fluorescamine in acetone (3 mg. cm^{-3}) was rapidly added from a 10 µl. Hamilton micro-syringe. The solution was mixed for a further 30 seconds then allowed to stand at room temperature in the dark for 10 minutes. A blank solution and a reference solution were also prepared by the same method except that 10 µl. of double distilled water was used instead of the bleomycin sample in the blank solution

and 10 µl. of 10^{-3} M alanine was used in the reference solution.

The running gel acrylamide was prepared by dissolving 20.0 g. acrylamide and 0.625 g. of methylene-bis-acrylamide in 100 cm^3 of water. After the components had dissolved the solution was filtered and collected in a brown bottle and kept, except when in use, at 4°C. The ammonium persulphate was freshly prepared by dissolving 40 mg. in 10 cm^3 distilled water. The TEMED solution was prepared by dissolving 0.1 cm^3 in 9.9 cm^3 distilled water. Solution A was 0.40M Tris-HCl pH 8.9 buffer. The running gel compositions were prepared as follows:

% Running Gel	Water (cm^3)	Acrylamide (cm^3)	Solution A	TEMED	Per-sulphate
7.5	9.0	9.0	3.0	1.5	1.5
10.0	6.0	12.0	3.0	1.5	1.5
12.5	3.0	15.0	3.0	1.5	1.5

The acrylamide, water, and solution A were mixed in the proportion indicated and then deaerated. TEMED was then added to the mixture and the solution again deaerated. The ammonium persulphate was then added, the solution mixed and the degassed solution placed in each of eight glass gel tubes (7.5 cm x 0.6 cm) held vertically and sealed at the lower end with a rubber gasket. The tubes were filled to within 1 cm. of the top, taking care not to produce any bubbles. About 0.5 cm^3 of a solution A/8 (1 cm^3 solution A and 7 cm^3 distilled water) was carefully layered on top of each gel in order to exclude

oxygen and to give the gel on setting a flat surface. The gel polymerised in about 20 minutes.

After the gel had formed the A/8 solution above the gel was drained off and the upper gel surface rinsed several times with A/8 solution. The top of each gel was then dried with a tissue and each tube filled with solution A/8. The rubber gasket covering the bottom of the gel tube was carefully removed and the upper end of each tube was then inserted in the upper buffer reservoir. Approximately 300 cm^3 of solution A/8 was placed in the lower buffer reservoir, this was then covered by the lower electrode assembly. The upper reservoir was then placed, so that the lower ends of the running tubes passed through the holes in the lower electrode assembly. Twenty microlitres of the sample was layered on top of the gel. This was repeated for another 2 tubes, then 20 µl. of blank solution was applied to two of the gel tubes, 20 µl. of alanine reference solution was applied to two of the gel tubes and finally 10 µl. of bromophenol blue (0.2% in 10% sucrose) applied to the last tube. The upper reservoir was then filled with about 250 cm^3 of buffer solution. The lid-electrode assembly was placed on the upper reservoir. The positive lead of the Vokam power pack was connected to the lower electrode and the negative lead connected to the upper lid electrode. The electrophoretic conditions used were a potential of 200 v which gave a current of 2.5 mA per tube. The progress of electrophoresis could be monitored by following the migration of the bromophenol blue marker

visually and the migration of the leading fluorescent band by illumination with long wave ultra-violet radiation. After the bromophenol blue marker had migrated about 5 cms, the current was switched off and the tubes quickly removed from the electrophoresis apparatus, dried with tissue and examined under long wave U.V. radiation (365 nm), the positions of the green-yellow fluorescent bands were then noted.

Gel electrophoresis was also carried out at an acidic pH. The following solutions were prepared according to Devenyi and Gergely (102).

Solution A

48 cm^3 of 1M potassium hydroxide, and 17.2 cm^3 of glacial acetic acid and 4.0 cm^3 of N,N ,N ' N '-tetra methyl-ethylene diamine, made up to 100 cm^3 with distilled water.

Solution B

0.13 g. of methylene-bis-acrylamide was dissolved in distilled water, followed by 20.0 g. of acrylamide. The solution was made up to 100 cm^3 with distilled water. The solution was then filtered and stored in a brown bottle.

Solution C

This was prepared just before use. 0.28 g. of ammonium persulphate was made up to 25 cm^3 with distilled water.

Electrode buffer (pH 4.5)

16.1 g. of β-alanine was dissolved in distilled water, 4.0 cm^3 of glacial acetic was added and the solution made up to a volume of 500 cm^3 by the addition of distilled water.

The gels were prepared by mixing 2.0 cm^3 of solution A and 12.0 cm^3 of solution B. This mixture was then degassed and then 2.0 cm^3 of solution C added. The tubes were filled in a manner previously described. The fluorescamine derivatives of bleomycin were prepared by the method already described except that potassium phosphate (0.05M) was used instead of sodium phosphate. Before the samples were placed on top of gels they were diluted with an equal volume of electrode buffer.

Bromophenol blue was not used as a marker because the solution was electrophoresed towards the negative electrode. Electrophoresis was carried out for 80 minutes at a current of 4 mA per tube. At the end of the run, the current was switched off and the positions of the fluorescent bands observed by examination under long wave ultra-violet radiation.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis of Bleomycin-Fluorescamine Derivatives

Electrophoresis was carried out on 12.5% gels, sodium dodecyl sulphate (SDS) was distributed throughout the gel and tank buffer at a concentration of 0.1%. The system used was that of Weber and Osborn (93).

Solution A (Gel buffer)

A solution of 7.8 g of sodium dihydrogen phosphate monohydrate and 38.6 g. of disodium hydrogen phosphate hexahydrate pH 7.5 with 2M sodium hydroxide was made up to 1 litre with double distilled water. Sodium dodecyl sulphate was added to this solution to a final concentration of 0.2% (w/v).

Solution B

A 33.3% (w/v) solution of acrylamide in distilled water.

Solution C

A 2.25% (w/v) solution of N,N'-methylene-bis-acryl-amide in distilled water.

Solution D

An aqueous solution of ammonium persulphate (10 mg. cm^{-3}) prepared just before use.

Solution E

The tank buffer was the gel buffer diluted 1:1 with distilled water.

The gels were polymerised by mixing 15 cm^3 of gel buffer, 11.0 cm^3 acrylamide, 2.5 cm^3 of bisacrylamide. This solution was deaerated, then 1.5 cm^3 of solution D and 0.045 cm^3 of TEMED added. The solution was again deaerated and then transferred to eight glass tubes (7.5 cm long x 0.6 cm bore) held vertical and sealed at the bottom with

parafilm. Each gel was filled to within 1 cm. of the top and the gel overlayed with distilled water. Polymerisation took place within 20 minutes but the gels were left for one hour before use after which time the overlayering water was removed and each gel washed with distilled water.

Bleomycin (1 mg.) was dissolved in 100 µl of 0.1M phosphate buffer pH 8.5 containing 0.1% SDS. The solution was heated to 100°C for 3 minutes to ensure complete complexing of SDS and bleomycin. After cooling in ice, 5 µl. of 1 mg. cm^{-3} of fluorescamine in dimethyl sulphoxide was added rapidly from a 10 µl Hamilton syringe, the mixture being stirred in a Vortex mixer while the fluorescamine was added. A further 5 µl. of the fluorescamine was added to the solution. The labelling was checked using long wave ultra violet light. 100 µl of 0.2 g. cm^{-3} sucrose solution was added to the tube again with mixing followed by 5 µl. of tracking dye (bromophenol blue 5 mg. cm^{-3}). Fifty microlitres of the fluorescent sample was carefully layered on top of each gel followed by 5% sucrose on top of the gel. Electrophoresis was started at 1 mA/gel for the first 30 minutes then it was increased to 8 mA/gel for 3 hours. The migration distances of the bleomycin fluorescamine SDS complexes were then measured by observation under long-wave U.V. light.

SDS-PAGE of bleomycin samples followed by staining of gel with Coomassie Brilliant Blue G250

Solutions used were the same as those in the previous experiment except that there was no labelling of bleomycin with fluorescamine. At the end of the electrophoresis, the gels were removed from the tubes and the position of the tracking dye was marked by placing a thin piece of wire through the gel at the appropriate position.

The bleomycins were visualised by staining for 1 hour in 0.2% (w/v) Coomassie Brilliant Blue G250 (C.I. 42655) in a glacial acetic acid-ethanol-mixture (10:4 by vol).

The excess Coomassie Brilliant Blue was removed by repeated washings in a solution of glacial acetic acid-ethanol-water mixture (8:25:65 by volume).

Results

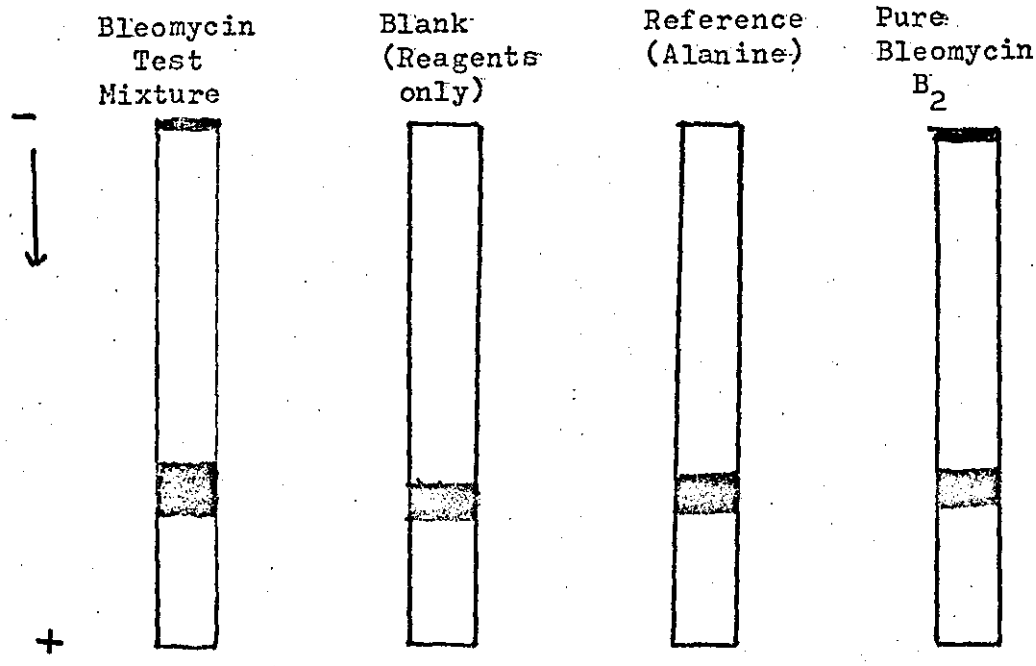
Gel electrophoresis of fluorescamine-labelled bleomycins gave electrophoretic patterns that varied with the acrylamide concentration of the gel used (Fig. 10). With the 7.5% acrylamide the commercial bleomycin sample components either remained at the origin or migrated with the buffer front. When a 12.5% or 16% gel was used, the fluorescamine-labelled commercial bleomycin sample gave two fluorescent bands one of which remained at the origin and the other (weakly fluorescent) migrated towards the anode but migrated some distance behind the buffer front. The bleomycin B_2 sample gave a fluorescent band which remained at the origin in the 12.5% and 16% gels. The fluorescent band at the origin was assigned to the fluorescamine derivatives of bleomycins A_2 and B_2. The anodic fluorescent band of the bleomycin mixture was assigned to bleomycin A_1.

A fluorescent band which migrated with the buffer front at all the various gel concentrations was obtained when the reagent blank solution was labelled with fluorescamine. No primary amines were present when the blank solution was labelled with fluorescamine and this suggests that an impurity might be present. A similar result was obtained when the t.l.c. of bleomycin-fluorescamine derivatives was investigated.

Figure 10. Gel electrophoresis of fluorescamine-labelled bleomycins at pH 8.9 and at varying acrylamide conc.

The following patterns were obtained by visualisation of gels under U.V. light (365 nm).

7.5% Gel



12.5 and 16% Gels

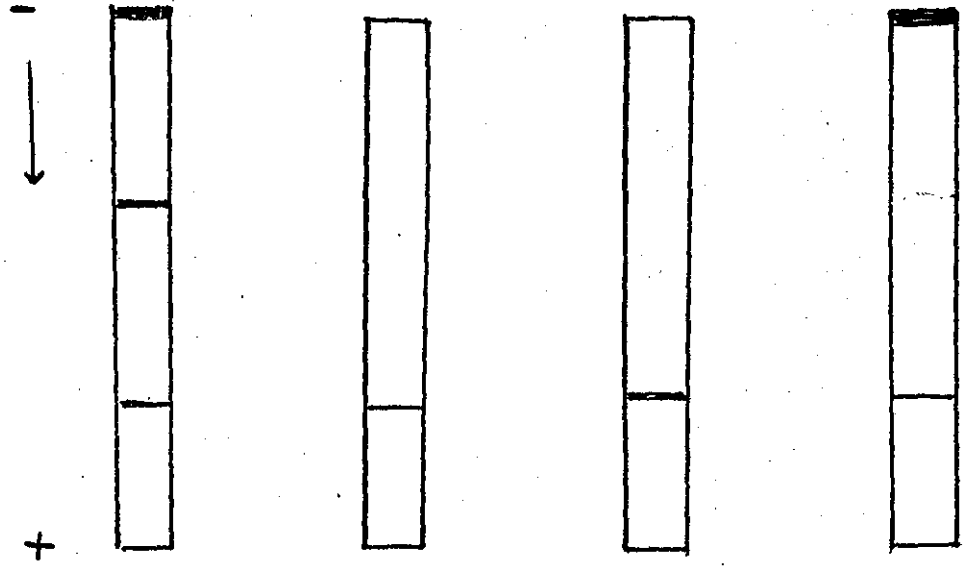
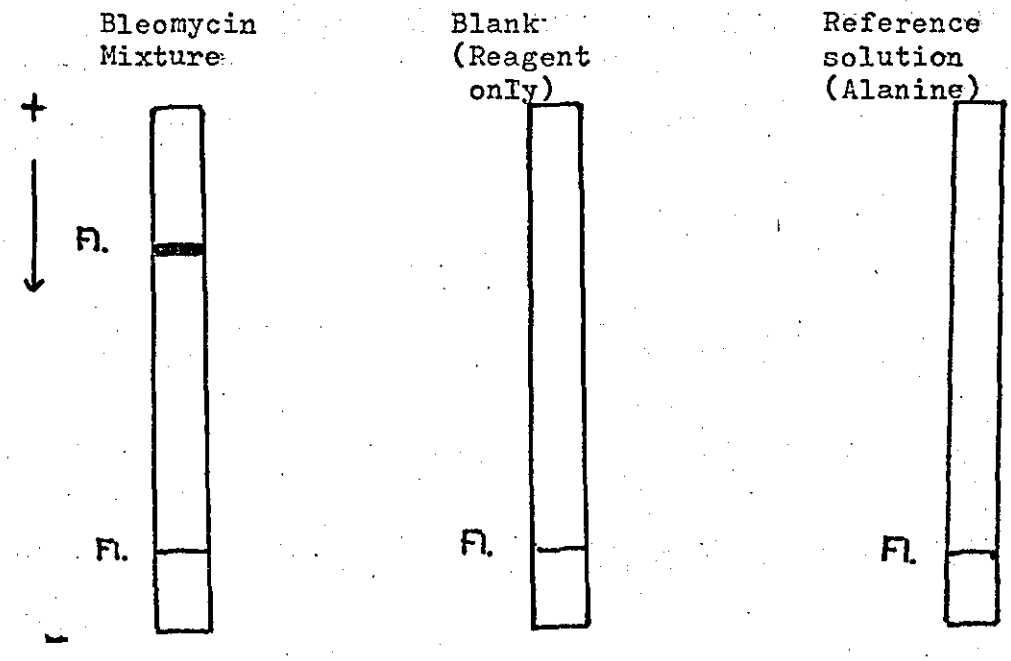


Figure 11- Polyacrylamide gel electrophoresis of fluorescamine-labelled bleomycins at pH 4.5 and at 15% acrylamide concentration



Fl - Fluorescence observed

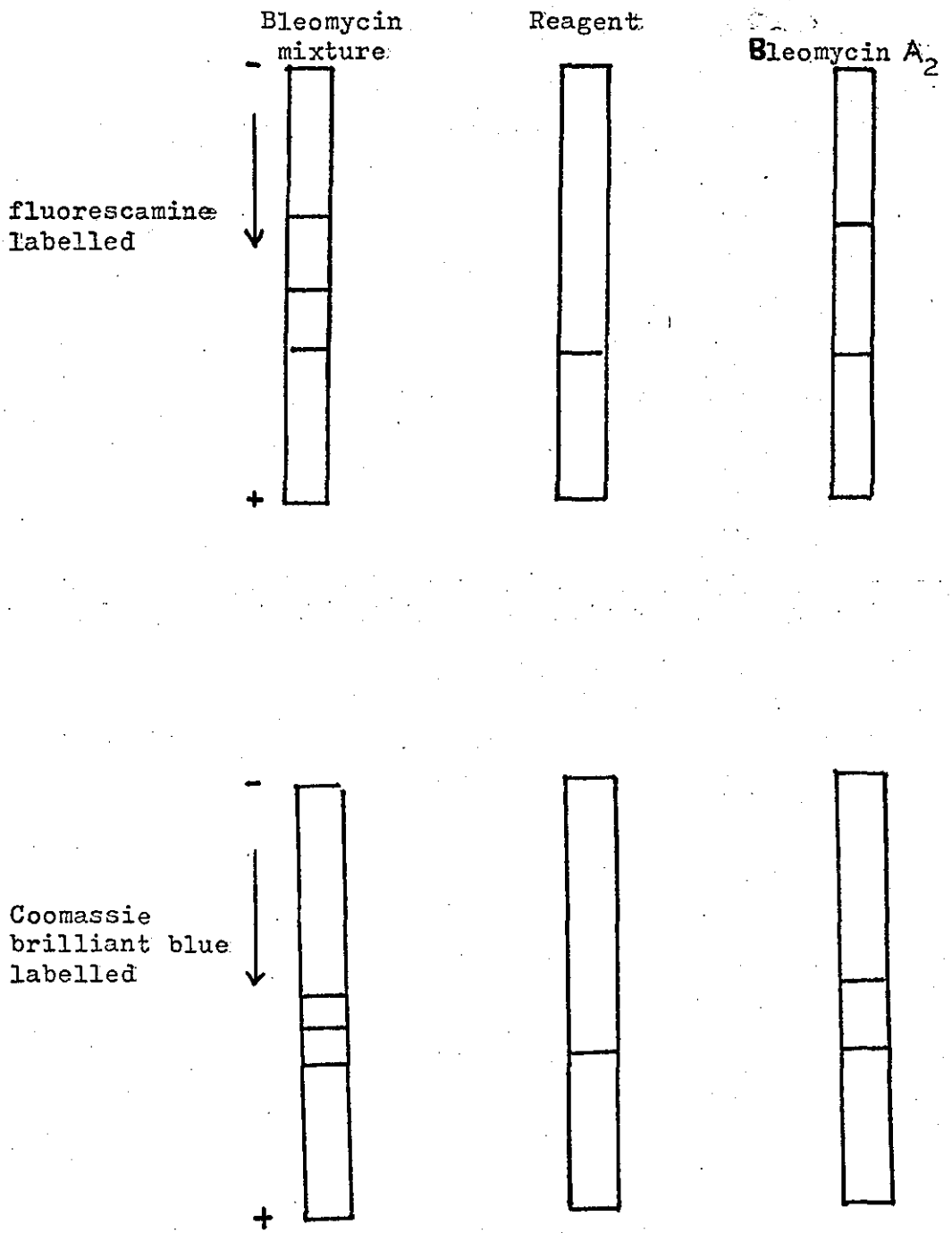
and

Gel electrophoresis of bleomycin of the bleomycin-fluorescamine derivatives in 15% acrylamide gels and at a pH of 4.5 gave rise to two weakly fluorescent bands (Fig. 11). Both migrated towards the cathode, the fast moving band was assigned to the impurity reaction with fluorescamine since the reagent blank also gave this band. The other slower moving band coincided with that obtained with the bleomycin B_2 sample. Since no other fluorescence bands were observed it was considered that in the bleomycin mixture, both the major bleomycins (A_2 and B_2) migrated together. The limit of detection was determined to be 3 mg. cm^{-3} for the bleomycin mixture. The fluorescence obtained at this pH was weaker than that obtained at the optimum pH 8.9.

SDS-PAGE of the bleomycin mixture at pH 7.6 resolved the mixture into two bands. Bleomycin A_2 sample produced a single band which had a similar mobility to the slower band of the bleomycin mixture. The faster band was assigned to bleomycin B_2. (Fig. 12)

When fluorescamine dissolved in acetone was used, the acetone sometimes reacted with the polyacrylamide gel when the sample was layered on the gel. This was overcome by using dioxane or dimethylsulphoxide solutions.

Figure 12. SDS-Polyacrylamide gel electrophoresis of bleomycins and bleomycin-fluorescamine derivatives at 12.5% acrylamide



Discussion

Fluorescamine modification of peptides gave these compounds extra negative charges and it was found that the best conditions to separate fluorescamine labelled peptides was at high acrylamide concentrations (12 - 16%) and in the pH range 8.9 - 9.4 (98). When fluorescamine labelled bleomycins were investigated under similar conditions, the major bleomycin components (A_2 and B_2) remained at the origin while the bleomycin A_1 derivative migrated through the 12.5% gel towards the anode. It appears that the bleomycin A_1-fluorescamine derivative has an overall negative charge at pH 8.9 while the A_2 and B_2 derivatives have a net charge of zero. The bleomycin A_2 and B_2 have charged terminal amines while the A_1 does not and this may explain the difference in electrophoretic mobility of these fluorescamine derivatives at this pH.

When the separation was carried out at pH 4.5, both bleomycin A_2 and B_2 derivatives were positively charged and migrated through the gel towards the cathode. A single, weak fluorescent band was obtained, no zone due to bleomycin A_1 was observed.

The SDS-PAGE of the bleomycin and bleomycin-fluorescamine derivatives separated the commercial bleomycin mixture into two bands which were assigned to bleomycins A_2 and B_2

respectively. The greater mobility of bleomycin B_2 compared with that of bleomycin A_2 suggested that the latter bleomycin had a greater molecular size than that of bleomycin B_2. Nunn (101) obtained a similar observation when studying the gel filtration of individual bleomycins and proposed that although bleomycin A_2 and B_2 have almost similar molecular weights, bleomycin A_2 has a far greater Stokes radius than bleomycin B_2. The sensitivity of both SDS gel electrophoresis were not very satisfactory but the fluorescamine derivative technique proved to have greater sensitivity than the traditional staining procedure.

The electrophoresis of fluorescamine derivatives of oligopeptides may find general application since oligopeptides have tended not be studied by gel electrophoresis due to the lack of suitable staining procedures. Fluorescamine should be a useful reagent for labelling oligopeptides since it can label primary amino groups with high sensitivity. The development of fluorimetric methods of scanning gels (103) should enable such separations to be made semi-quantitative.

CHAPTER 4

HIGH PRESSURE LIQUID CHROMATOGRAPHY OF THE BLEOMYCINS

INTRODUCTION

High pressure liquid chromatography (h.p.l.c.) has undergone considerable developments (104) over the last few years to become an analytical technique capable of separating complex mixtures and having sensitive, low dead volume detectors. H.p.l.c. offers shorter separation times and greater resolution than open column chromatography or thin layer chromatography. Involatile substances can be analysed directly unlike gas-liquid chromatography which requires the preparation of volatile derivatives. Thus h.p.l.c. is particularly suitable for the analysis of complex mixtures of polar, relatively high molecular weight compounds such as the bleomycins.

There have been several h.p.l.c. studies of antibiotics including griseofulvin (105) bacitracin (106) and the tetracyclines (107). The bleomycins have also been investigated, Eckelmann *et al.* (11) studied the h.p.l.c. of metal-free bleomycins and ^{57}Cobalt-bleomycins using several stationary phases. Most of the stationary phases including the reverse-phase, cation-exchange and ligand-exchange were found to be unsatisfactory. No separation was achieved with the ligand-exchange stationary phase (Chelex-100 in the copper (II) form) due to strong absorption.

Separation was achieved on cation exchange resins at relatively high temperatures (60°C) and high ionic strengths (0.1 - 1.0M ammonium formate) but even under these conditions the analysis times were excessive. The identification of the separated fractions was made more difficult by the need to freeze-dry large volumes of solution in the recovery process and by the presence of relatively large amounts of ammonium formate. Eckelmann and his co-workers found that the best system to separate the bleomycins was the use of a silica gel (Porasil A) stationary phase and a mobile phase of 0.3% aqueous ammonium formate:methanol (1:1). The only technical details provided were that the separation was carried out at room temperature and at a flow gradient of 1-5 cm^3 $min.^{-1}$ mobile phase. In a later paper (12) further details were supplied, the bleomycins as their ^{57}Cobalt chelates were separated into two major components (bleomycins A_2 and B_2) and two minor components, bleomycins A_1 and dimethyl A_2 (DMA_2), the entire separation took six hours. Rzeszotarski and his co-workers (12) also investigated the reverse-phase separation of the bleomycins on a μ-Bondapak C_{18} stationary phase and eluted with 5mM ammonium formate in aqueous methanol, the methanol concentration was increased from 15% to 95% during the analysis. The separation took place at room temperature and took 4 hours. The bleomycin mixture was again separated into two major components (BLM-A_2 and BLM-B_2) and two minor components (BLM-A_1 and BLM-DMA_2).

The presence of ammonium formate was considered to be of critical importance for the elution and resolution of the bleomycins, it was thought it suppressed the ionisation of the bleomycins and increased the polarity of the mobile phase. The relative volatility of ammonium formate compared with salts like sodium chloride was beneficial when the major fractions were collected after h.p.l.c. separation and freeze-dried prior to identification by comparison of the R_f values of the residues with those of the bleomycin mixture on silica gel t.l.c. (10).

The analysis of bleomycins so far developed for h.p.l.c. take a considerable time to attain separation. There is a need therefore for a rapid analytical separation of bleomycins by h.p.l.c. in quality-control studies of the commercial bleomycin mixture.

MATERIALS AND METHODS

Materials

Ammonium formate and A.R. Methanol were obtained from B.D.H. Ltd., Poole, U.K. Silica gel t.l.c. plates (0.25 mm thickness) were obtained from E. Merck, West Germany. The chromatography columns used were μ Porasil, a fully porous, small particle (diameter 10 μm) silica packing, and μ Bondapak C_{18} a 10 micron particle packing with octadecylsilane chemically bonded to a solid silica support. Both columns (dimension 300 mm x 4 mm i.d.) were purchased pre-packed from Waters Associates (Inst.) Ltd., Stockport, U.K. The h.p.l.c. apparatus consisted of a Waters Associate Model 660 solvent programmer, a M6000 chromatography reciprocating pump and model U6K septumless injector, model 440 absorbance detector fitted with a 254 nm interference filter and an LKB flat bed recorder operating on 10 mV range.

All water used was double-distilled and all h.p.l.c. solvents were filtered through Whatman glass fibre filter GF/C and also degassed prior to being used in h.p.l.c. system. A stainless steel sintered filter (20-30 μm) was used to remove coarse, particulate matter from the delivery system taking the solvents to the solvent programmer. At the end of each chromatographic run, the system was washed with a methanol:water (1:1 v/v) mixture for at least twenty minutes. The samples were injected onto the system by means of a Pressure-Lok 10 μl liquid micro syringe.

The solvents were prepared daily as it was found that the separations attained were adversely affected when aged mobile phases were used.

The bleomycin standards were generously provided by Dr. N. Nunn. Bleomycin B_2 standard was pure but the bleomycin A_2 standard contained bleomycin A_1 and B_2 as impurities. Bleomycin B_2 constituted approximately 10% of the bleomycin A_2 standard sample.

Methods

The solvent delivery system was filled with the appropriate solvent by pulling the solvent through by means of a glass syringe, this also enabled any trapped air-bubbles to be removed. The column was filled with solvent by forcing solvent through it by means of a syringe. When it was considered that there were no air-bubbles trapped in the system, the reciprocating pump was started up and solvent forced through the system at the appropriate flow-rate and the pressure of the system noted. After twenty minutes, the system was ready for the injection of samples and the detector switched to the required sensitivity. If the sample was to be investigated under isocratic conditions the programmer was set at the appropriate flow rate; if a gradient elution separation was required the appropriate gradient system was chosen and the time taken to achieve the gradient also selected. The microsyringe was filled and emptied several times with appropriate solution and then filled to the required

sample volume, the outside of the needle was wiped with a paper tissue to remove excess solution, the injector system switched from "COLUMN" to "FILL" and the syringe needle inserted into the injector septum. The required sample volume was injected into the loop system, the syringe was then withdrawn and the injector switched to "COLUMN". The sample was swept into the column and at the same instant an electronic signal sent to the recorder to mark the time of injection on the recorder chart.

The course of the chromatographic separation was traced out on the recorder chart. If a separated component was required, for further study e.g. t.l.c., the recorder chart was monitored to observe when the peak was being traced out and a clean test-tube was placed below the outlet tube and the eluate collected until it could be seen that the recorder pen was returning to the base line. The eluate was then freeze-dried prior to t.l.c. investigation. The t.l.c. method used to investigate the components was that of Umezawa et al. (10) and the retention values (Rf) were compared with those of the bleomycin mixture which was separated on the same t.l.c. plate.

RESULTS

A typical h.p.l.c. chromatogram of the bleomycin mixture using μ Porasil stationary phase is shown in Fig. 13. An identical result was obtained with a mixture of bleomycins in the copper (II) form. If a gradient elution system was used the retention time for the most strongly retained component could be reduced Fig. 14. The various components were collected after elution from the detector, subjected to freeze-drying and then t.l.c. The Rf values of these components were compared with the Rf values of certain bleomycins Table 6 Component 2 gave an Rf value which identified it as bleomycin B_2. Component 3, the most strongly held component, gave two spots on the t.l.c., the major spot corresponded to bleomycin A_1 and the other to bleomycin A_2. Other workers (11) had previously observed that freeze-drying partially demethylated bleomycin A_2 and the resulting demethyl A_2 was oxidised to yield bleomycin A_1 Fig. 15. This would explain the presence of bleomycin A_1 in the fraction containing component 3. The identity of the two major components were also investigated by using bleomycin standards Fig. 17.

Figure 13. Isocratic h.p.l.c. of bleomycin using μ Porasil

Column - μ Porasil (300 mm x 0.4 mm); flow rate - 2 cm^3 min^{-1}; mobile phase - 0.3% ammonium formate-methanol (1:1); sample - 10 ul. of 0.4 mg. cm^{-3} aqueous bleomycin: absorbance range 0 - 0.10.

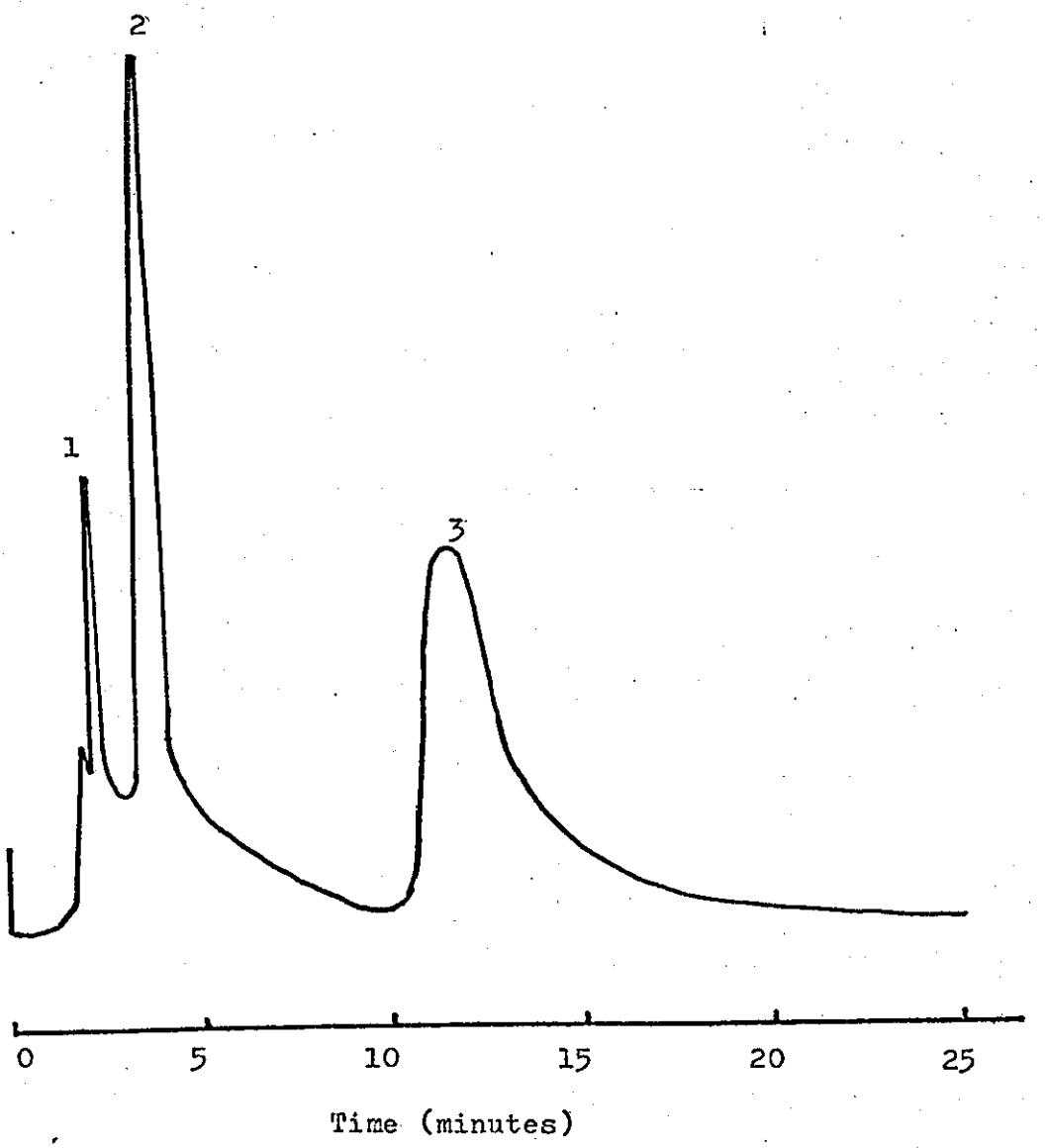
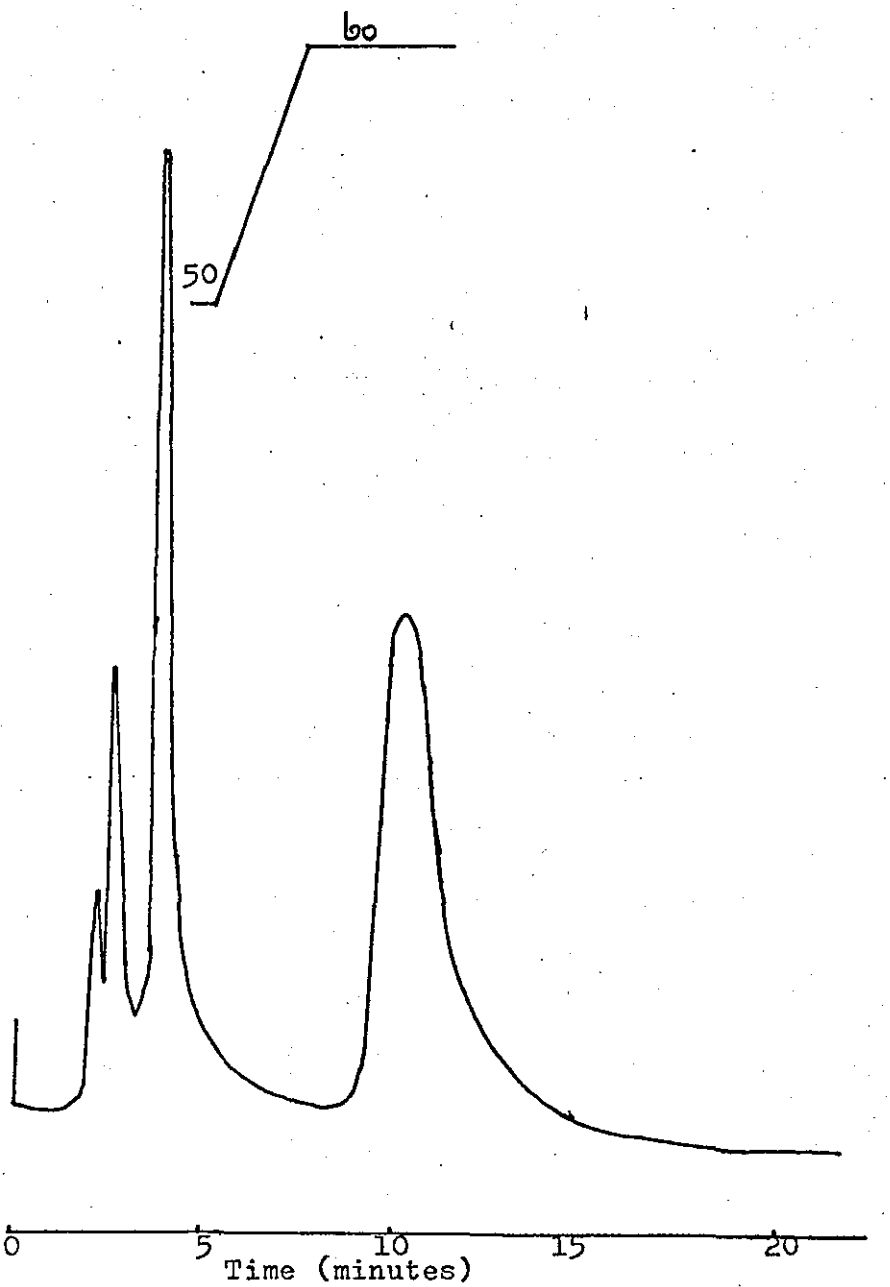


Figure 14. Gradient elution of aqueous bleomycin using μ Porasil

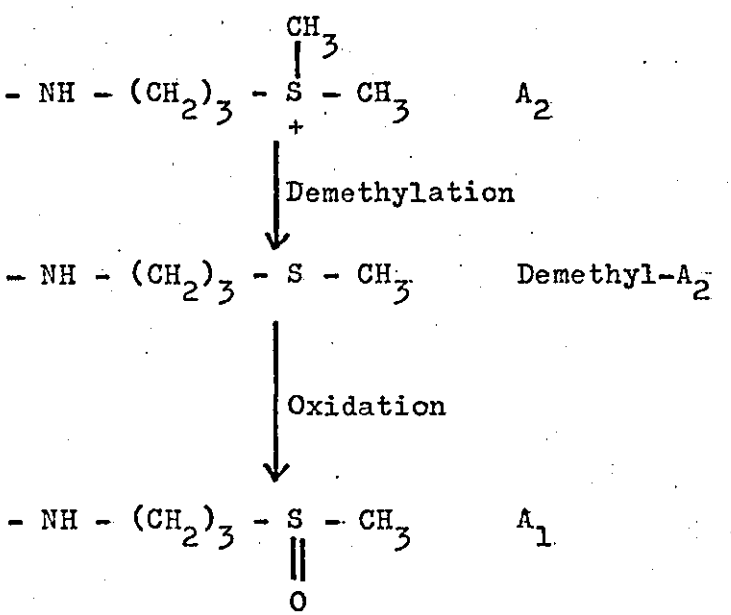


Column - μ Porasil (300 mm x 0.4 mm); flow rate - 2 cm^3 min^{-1}; mobile phase - first five minutes, 0.3% ammonium formate-methanol (1:1) then linear gradient change in 3 minutes to (60) 0.3% ammonium formate-methanol (3:2); sample - 10 ul 0.4 mg cm^3 aqueous bleomycin; absorbance range 0 - 0.20.

Table 6. Retention times of Rf values of the freeze-dried components after μ Porasil chromatography.

H.p.l.c. Component	Retention time (mins)	Bleomycin	Rf obtained	Rf value (10)
2	4	B_2	0.68	0.68
3	11½	A_2	0.40 0·72	0.40
1	3	A_1	0.72	0.74

Figure 15. Conversion of Bleomycin A_2 to Bleomycin A_1



Further confirmation was obtained by subjecting the bleomycin mixture to silica gel t.l.c. The aqueous solution was streaked across the origin and evaporated by a cold stream of air. After chromatographic separation, the plate was dried for one hour in a stream of cold air. It was then examined under ultra-violet light (265 nm) and the zone corresponding

Figure 16. Plot of peak area against total bleomycin concentration

Column - μ Porasil 300) mm x 0.4 mm: flow rate - 2.0 cm^3 min^{-1}; mobile phase - 0.3% ammonium formate-methanol (1:1); Sample volume - 10 ul; absorbance range 0 - 0.05.

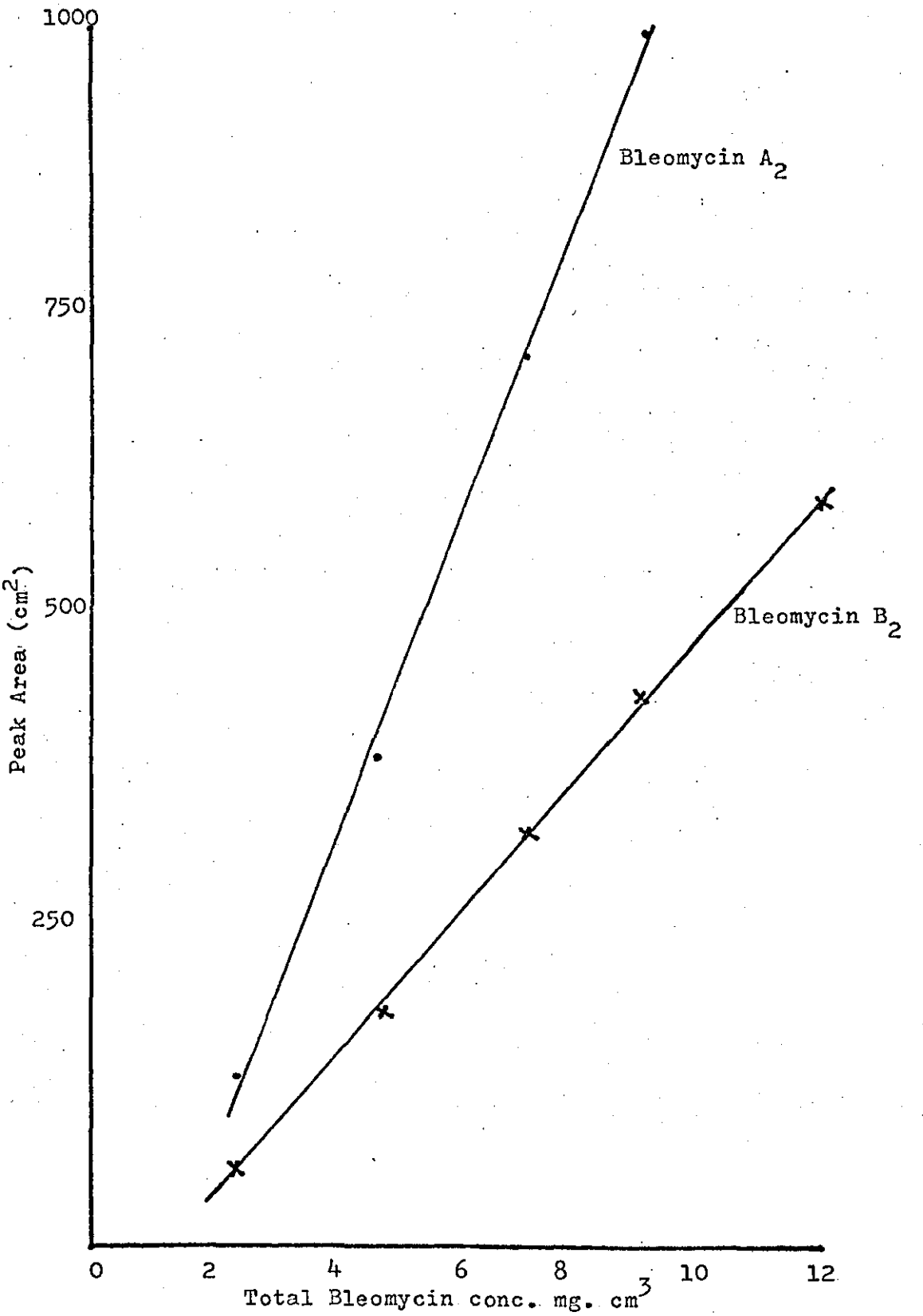
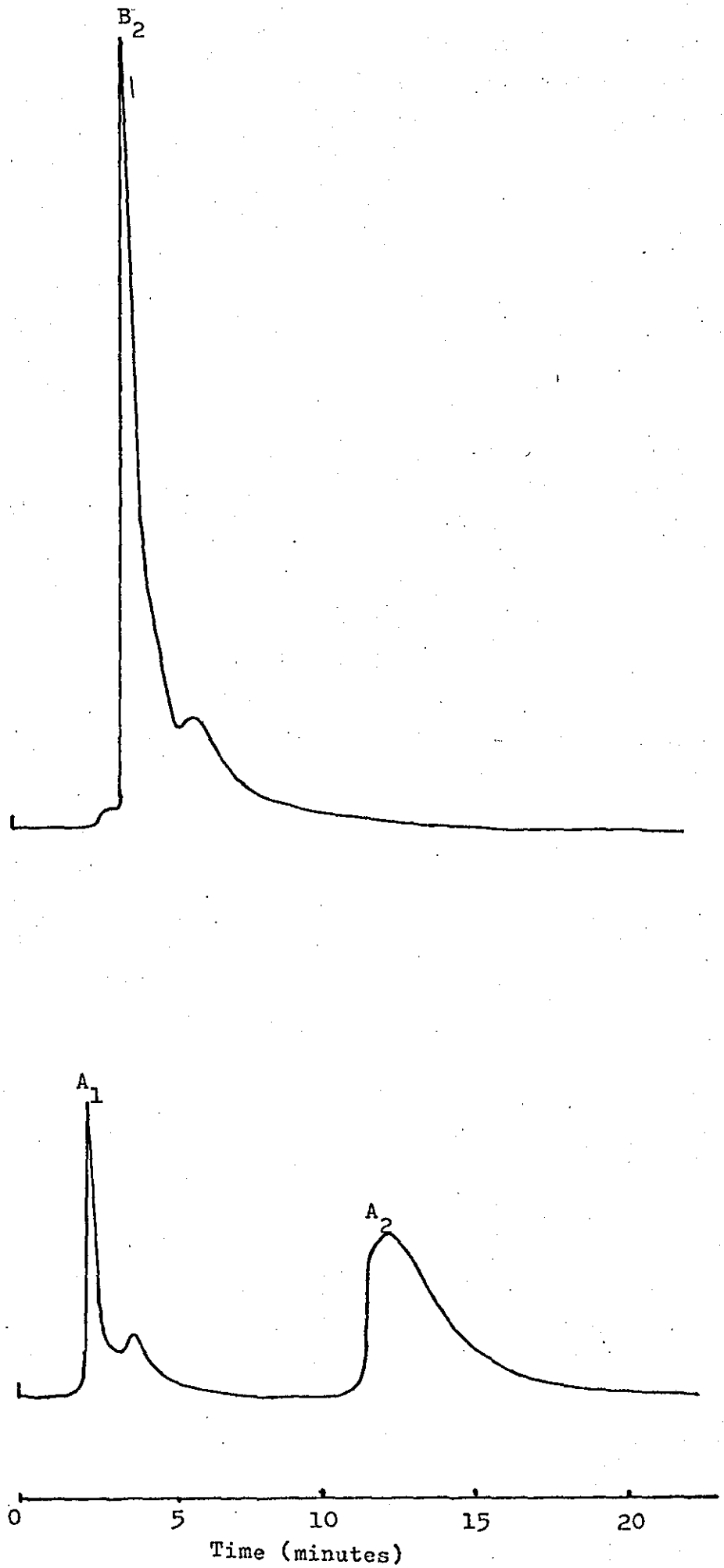


Figure 17. μ Porasil h.p.l.c. of bleomycin standards



to bleomycin B_2 component was carefully scraped off, placed in a clean glass centrifuge tube, mixed with the minimum amount of 50% aqueous methanol and centrifuged at 1000 g. for 5 minutes. The supernatant was removed and filtered through Whatman glass fibre filter GF/C; an aliquot was then injected onto the h.p.l.c. and gave a peak of retention time identical to bleomycin B_2.

The limit of detection of bleomycin B_2 was estimated to be 0.04 $\mu g. cm^{-3}$ (5 μl sample) and the retention times of the separated components were determined Table 6. The plot of peak area ($\frac{1}{2}$ base x height) against increasing bleomycin load Fig. 16 indicated that the bleomycin mixture could be quantitated by this technique in spite of the badly tailed peak exhibited by bleomycin A_2. A comparison was made of the percentage composition of a bleomycin mixture determined from the h.p.l.c. results with the values obtained by the manufacturer's who used microbiological assay and t.l.c. techniques Table 7.

Table 7. Percentage composition of bleomycin mixture

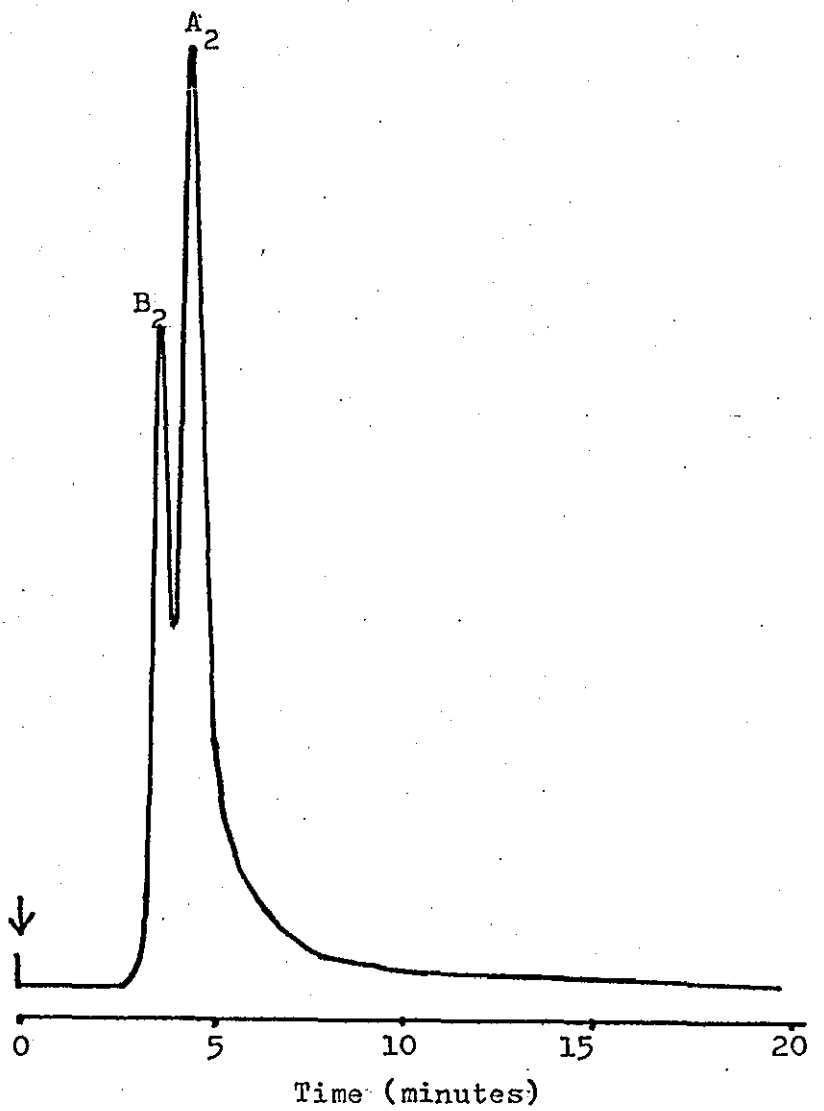
Bleomycin component	H.p.l.c. (%)	Manufacturer's analysis (%)
A_2	61	63.9
B_2	33	30.9
A_1 and DMA_2	6	3.6

Note. The percentage composition of the mixture was determined from the h.p.l.c. results by estimation of the areas under the respective peaks. Bleomycin sample Lot Number U11A54.

The second stationary phase investigated was the reverse-phase column μ Bondapak C_{18} which consisted of a monomolecular layer of octadecylsilane chemically bonded to a porous silica support. In order to separate the polar bleomycins on this non-polar stationary phase, a counter-ion had to be added to the mobile phase in order to suppress the ionization of the bleomycins and hence make them less polar. Three such counter-ions were employed in aqueous methanol solution-1-heptane sulphonate, perchlorate and formate. The methanol-formate separation obtained is shown in Fig. 18. The 1-heptane sulphonate affected the column efficiency and a deterioration in column performance was observed, it was possible that this was due to the acidity of this counter-ion solution even though the column was always washed at the end of a chromatographic run for at least twenty minutes with 50% aqueous methanol. When the formate-aqueous methanol mobile phase was subjected to a gradient elution procedure, an increase in U.V. absorbance was obtained when the 95% aqueous methanol-5 mM formate was the mobile phase. This was considered to be due to the less polar bleomycins present in the mixture namely bleomycins A_1 and demethyl A_2 but were not identified by t.l.c. probably because of the small amounts of these components present.

Figure 18. Reverse-phase h.p.l.c. of bleomycins using C_{18} μ Bondapak

Column, C_{18} μ Bondapak (300 mm x 4 mm); flow rate 1.0 cm^3 min^{-1}; mobile phase - 80% aqueous methanol containing 5 mM ammonium formate; sample 10 ul aqueous bleomycin 10 mg cm^3; absorbance range 0-0.10.



The two major components were identified by comparing their retention times (Table 8) with the standard bleomycin solutions and by mixing the two standard solution Fig. 19.

Table 8. Retention times of bleomycin A_2 and B_2 obtained with C_{18} μ Bondapak reverse-phase column

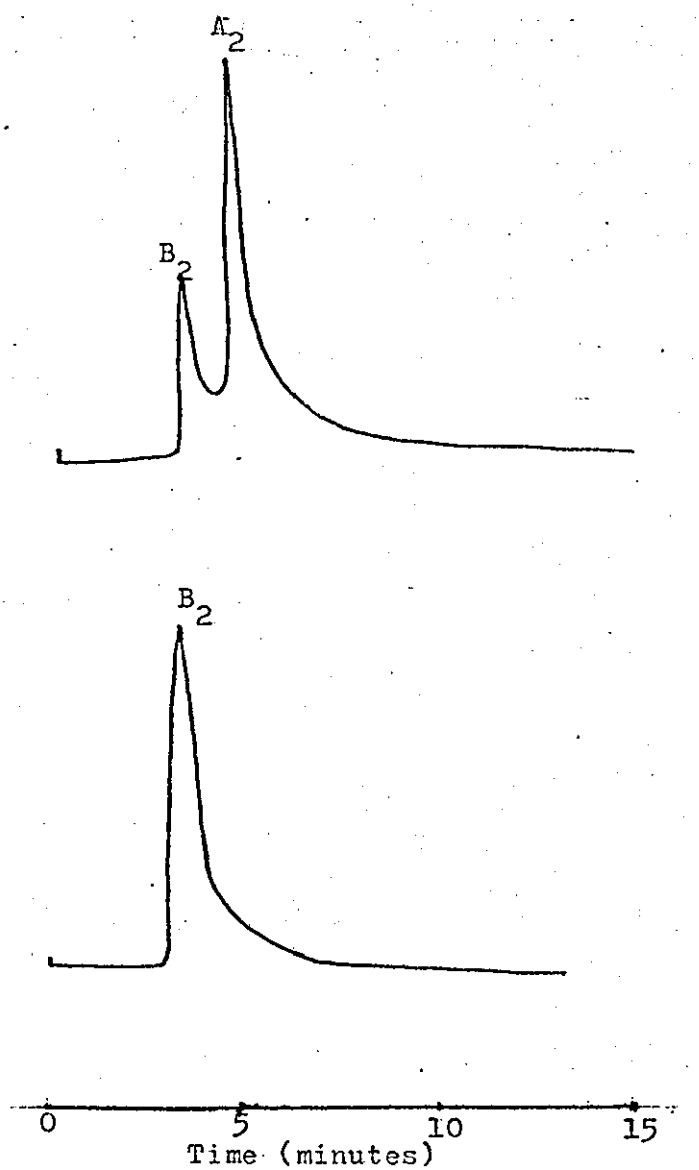
Bleomycin	Retention time (mins)
A_2	$4\frac{3}{4}$
B_2	$3\frac{1}{2}$

Conditions - flow rate - 1 cm^3 min^{-1}, pressure - 2000 p.s.i., mobile phase - 80% aq. methanol containing 5 mM ammonium formate.

The peak eluted first was also identified as being due to bleomycin B_2 by subjecting the eluted fraction to t.l.c. and Rf of the component was found to be 0.68.

When perchlorate was used as the mobile phase counter ion a single fast moving peak was obtained and when this eluted fraction was subjected to t.l.c., bleomycins A_2 and B_2 were found to be present. It is of note that in the latter case the separated components although detected on the t.l.c. by ultra-violet absorption (260 nm), did not fluoresce when sprayed with fluorescamine.

Figure 19. Reverse-phase h.p.l.c. of bleomycin standards using C_{18} μ Bondapak



DISCUSSION

The h.p.l.c. separation of commercial bleomycin using μ Porasil as a stationary phase resolved the complex within twenty minutes into two major components A_2, B_2 and minor component A_1. The copper (II) bleomycins gave a similar separation pattern to that of the copper-free bleomycins. This study emphasised the need to carry out the chromatographic separation and isolation of the bleomycin components under mild conditions in order to prevent chemical modification of the bleomycin A_2 molecule. Reasonable symmetry was obtained with the earlier peaks due to bleomycins A_1 and B_2 but the later peak due to bleomycin A_2 was badly tailed. This tailing probably arose due to specific adsorption of this component onto certain favourable sites on the silica surface. The presence of the polar function group $-\overset{+}{S}-(CH_3)_2$ in the bleomycin A_2 probably influenced the tailing. The separation times obtained for the bleomycin components were far quicker than those obtained by previous workers (11), the entire separation was attained in less than twenty minutes while that of Rzeszotarski et al. (11) took six hours. The column dimensions used were far greater (1220 mm x 9.5 mm) than those used in this study (300 mm x 4.6 mm). The stationary phase used by Rzeszotarski et al. was Porasil A, a totally porous silica particle packing of diameter 37-75 um, suitable for large scale preparative work but giving slow analysis times.

The stationary phase used in this study was μ Porasil, a porous small particle packing (10 um) which is designed for fast analyses.

There was also reasonable agreement between the percentage composition of bleomycin mixture determined from h.p.l.c. studies compared with the values obtained by microbiological studies.

The ion-pair studies using the counter-ions 1-heptane sulphonate and perchlorate were of limited application since the former affected column performance and its use had to be discontinued while latter counter-ion did not separate the bleomycins and results suggested that the bleomycins were modified by the perchlorate. The formate ion-pair system used gave a rapid separation of the major bleomycin components (A_2 and B_2) but the less polar bleomycins (A_1 and DMA_2) were only eluted from the column with a mobile phase of 95% aqueous methanol (5 mM ammonium formate). The elution of the bleomycin B_2 before bleomycin A_2 was unexpected, it was thought that the more polar bleomycin A_2 would be the less strongly held of the two bleomycins. Although this procedure would not detect the prescence of the more toxic bleomycins (e.g. bleomycin B_4) which can be found in commercial bleomycin preparations it should be of use in examination of the two major components, bleomycins A_2 and B_2 which constitute about 90% of the preparation.

CHAPTER 5

PURIFICATION PROCEDURES

INTRODUCTION

The plasma and urine levels of bleomycins are of considerable importance since they can indicate how much of the therapeutic dose remains in the patient's body. The plasma level is also believed to be an indication of the bleomycin available to the tumour tissue (11). The ability of h.p.l.c. to bring about a rapid separation of microgram quantities of bleomycins in aqueous solutions led to the consideration of analysing bleomycin levels in physiological fluids using this technique. The detection system available (U.V. absorbance at 254 nm) required sample "clean-up" in order to minimise interference in the assay from other biological compounds present in physiological fluids. The bleomycins are compounds which are soluble in water and methanol but are insoluble in less polar organic solvents e.g. chloroform and carbon tetrachloride, hence they cannot be purified by organic solvent extraction procedures as can for instance the barbiturates (108). Distillation procedures do not seem to be applicable since bleomycins are relatively involatile (decomposition temperature approx. 196°C) (2).

A possible method of removing some of the possible interfering compounds is ion-exchange chromatography. CM-Sephadex C-25 and Amberlite IRC-50 (H^+ form) have been used to purify bleomycins obtained from the culture filtrate of

S. verticillus (10). Since there is a possibility of the bleomycins being denatured by interaction with strongly hydrophobic matrices in polystyrene based exchange resins, a cellulose based resin - cellulose phosphate might provide a better alternative.

Cellulose phosphate was used by Kremzner (109) to purify the aliphatic polyamine spermidine N-(3-amino-propyl)-1,4-butanediamine from biological tissues prior to fluorimetric analysis. This method was later modified by Abe and Samejima (110) to determine spermidine and spermine in animal tissues. The tissue of interest was homogenised with cold 2% perchloric acid and then centrifuged. The resulting supernatant was then subjected to cellulose phosphate chromatography followed by a step-wise elution procedure. The fraction containing the polyamines was then evaporated to dryness, the residue dissolved in 100 µl of a water-methanol (1:1 v/v) mixture then subjected to t.l.c. The separated compounds and an internal standard were located by spraying with fluorescamine and scanned by a fluorimeter equipped with a t.l.c. scanner and a recorder.

One of the purification steps used in the original study (2) of the bleomycins was gel chromatography using Sephadex G-25 which split the bleomycin mixture into two major groups A and B. The exclusion limit for peptides and dextrans with Sephadex G-15, the gel immediately below G-25 in the pore size, is a molecular weight of approximately 1,500. Bleomycins have an approximate molecular weight of 1,500 and may be regarded as glycopeptides so it is possible

that they may be eluted in the void-volume of a G-15 column. If this were the case, it would be possible to separate bleomycins from possible low molecular weight interfering compounds.

Ultrafiltration could be employed to remove low molecular interfering compounds from the bleomycins. This procedure is rapid and reasonably efficient and it has the additional advantage of being a mild procedure. The retention of a molecule by a filter can be expressed by the rejection coefficient 6

$$6 = 1 - \frac{C_f}{C_b} \quad \frac{\text{(Solute conc. in filtrate)}}{\text{(Solute conc. in bulk solution)}}$$

The rejection coefficient is a function of the molecular weight of the molecule, total membrane exclusion is 1 while 6 = 0 represents complete permeation of solute. Nevertheless the grading of membranes on the basis of molecular weight cut-off is quite arbitary and it would be more sensible to express the retentivity or rejection coeffient in terms of molecular dimensions.

There is no mention in the literature (to date) of the use of iso-electric focusing (IEF) of copper (II) bleomycins or metal-free bleomycins. Although it may be of no apparent use in the estimation of bleomycin levels in biological fluids it could find application in the purification of copper (II) bleomycins on a relatively large scale. The IEF method employed in this study is analytical polyacrylamide gel IEF.

MATERIALS AND METHODS

Materials

Cellulose phosphate was obtained from Sigma Chemicals.

Sephadex G-15 was supplied from Pharmacia (G.B.) Ltd., London.

Ampholine pH 3.5 - 10.0. Supplied as a 40% solution from L.K.B. Ltd.

The Amicon Model 52 ultrafiltration cell was purchased from Amicon Ltd., High Wycombe, Bucks.

Glass barrel econo-columns 4 x 0.7 cm. and 30 x 1.0 cm. were obtained from Bio-Rad Laboratories Ltd., Bromley, Kent.

Methods

Preparation of cellulose phosphate

10 g. of cellulose phosphate was poured onto the surface of 0.5 M NaOH (300 cm^3) and left for ten minutes, it was then diluted to 600 cm^3 by the addition of 1M NaCl. It was then filtered on a Buchner funnel using a Whatman No. 54 filter paper and the residue washed with distilled water and then resuspended in distilled water. Sufficient 1M HCl was added to make solution strongly acid (universal indicator paper) and the cellulose phosphate immediately filtered and washed free of acid by filtering with water (universal indicator paper). The resin was converted to the Na^+ form by resuspending in 0.5M NaOH and then filtered and washed free of alkali with water. The ion-exchanger was washed with 300 cm^3 of 0.5M acetic acid. The excess

acid was washed free by rinsing with distilled water. The H^+ form was then suspended in distilled water and packed under pressure in a 4 x 0.7 cm glass tube to a volume of approximately 0.5 cm^3. The test sample was prepared by placing 3.0 cm^3 aliquot in a "Centriflo" conical ultrafiltration membrane (CF-25, nominal cut-off M.Wt. 25,000) placed inside a plastic collection vessel and centrifuged at 3,000 r.p.m. for 15 minutes in a Griffin-Christ centrifuge. The ultrafiltrate was collected inside the plastic vessel and a 1.0 cm^3 aliquot was diluted with 1 cm^3 of 0.01 M sodium phosphate buffer pH 6.0 containing 0.5% sodium chloride. This solution was applied to the column. A stepwise elution procedure was carried out:

(i) 10.0 cm^3 of 0.01M sodium phosphate buffer containing 0.5% sodium chloride.

(ii) 6.0 cm^3 of 0.1M HCl.

(iii) 2.0 cm^3 of 1M HCl.

Certain compounds e.g. several amino acids, polyamines, bleomycins and creatinine were investigated to see in what fractions they were eluted from the column. The compounds were subjected to Silica gel t.l.c. using 1:1 10% ammonium acetate: A.R. methanol as the mobile phase. The compounds were located on the dried separated t.l.c. plate by spraying with fluorescamine and alkaline picrate reagent. In the urine samples "spiked" with bleomycin, the 1M HCl eluate was neutralised with solid sodium hydrogen carbonate and freeze-dried. The residue was dissolved in 200 µl of a mixture of 1:1 water: A.R. methanol. Aliquots of the solution was subjected to silica gel G t.l.c. and µ Porasil h.p.l.c.

Preparation of Sephadex G-15 column

10 g. of Sephadex G-15 was placed in a clean 100 cm^3 glass beaker. 40 cm^3 of 0.9% NaCl in water was added and the beaker and contents placed on a boiling water bath in order for the gel to swell. The 1.0 x 30 cm column was mounted vertically and the dead space under the polyethylene disc support and the tubing filled with 0.9% NaCl by pumping the liquid up the outlet tubing and through the bed support. The outlet tubing was then closed.

The swollen gel was poured carefully down the wall of the column until the column was completely full. The eluant reservoir was connected to the column and the last few traces of air removed via the air-vent in the column top piece. The top piece was then connected to the eluant reservoir (Mariotte flask) via plastic tubing and the eluant drawn through the column until the gel had stabilised. The column top piece was then removed and a filter paper disc placed on the gel surface. The column packing was examined and its void volume determined by injecting 0.5 cm^3 of 2 mg. cm^3 of Blue Dextran 2000 in a 0.9% NaCl solution containing 10% sucrose onto the surface of the gel. The top of the column was then carefully filled with 0.9% NaCl and re-connected to the Mariotte flask. The progress of the eluant was monitored visually. A 0.5 cm^3 aliquot of the bleomycin under test (containing 10% sucrose) was then injected onto the column and the progress of the eluate monitored using an LKB Uvicord II at 254 nm

Isoelectric Focusing

The following solutions were required:

LKB Ampholine pH 3 - 10; 40% solution.

Acrylamide solution: 30 g. acrylamide and 1 g. of NN'-methylene bis acrylamide in 100 cm^3 distilled water. Solution was filtered prior to it being used.

Ammonium persulphate, freshly prepared 1% ammonium persulphate solution.

Electrolytes, 0.2% sulphuric acid, 0.4% ethanolamine.

Eight gel tubes (8.0 x 0.8 cm) were prepared by sealing one end with parafilm and held in a vertical position. The following solutions were mixed and re-aerated.

16.8 cm^2 water

6.0 cm^3 acrylamide

0.6 cm^3 ampholine

1.2 cm^3 persulphate

The mixed solutions were placed in the gel tubes. Water was layered over the top of each gel without causing mixing at the interface. The gels set in about thirty minutes, the water was then poured off and the gels placed in the electrophoresis apparatus.

The electrolysis was first carried out under constant current conditions using not more than 2 ma per tube; the potential was monitored so that it did not exceed 300 volts. The pH gradient was established in about 20 minutes, the current was switched off and 0.1 cm^3 of 6 mg cm^3 bleomycin, 6 mg cm^3 copper (II) bleomycin and 3 mg cm^3 copper (II) chloride were applied separately to each two gel tubes.

The current was then switched back on and 60 minutes were allowed to focus the compounds. When focusing had been attained, the current was switched off and the tubes removed from the apparatus. The copper (II) bleomycin, copper (II) chloride and bleomycin bands were located by observation. One of the gels which did not contain any test sample was cut with a razor blade into 2-3 mm slices and each slice placed in distilled water (2 cm^3). Also the blue zone obtained with copper (II) bleomycin was cut out and placed in 2 cm^3 distilled water. The solutions were left for one hour and then the pH of the solutions measured on a standardised Pye 290 pH meter.

Ultrafiltration

Two membranes were investigated, Diaflo UM05 with a cut-off of M.Wt 500 and UM2 which has a cut-off of MWt 1000. The membranes were carefully removed from their protective envelopes and soaked for 5 minutes in 25% glycerol/water, they were then rinsed in separate labelled beakers of distilled water for 100 minutes. The water was changed every 20 minutes in order to remove the glycerol.

The membrane under test was placed in Amicon Model 52 ultrafiltration cell with the glossy side towards the solution. The test solution (25 cm^2) was placed in the cell, the top of the cell was placed in position and cell clamped.

Stirring was started and the cell was connected by tubing to the Nitrogen cylinder and the gas pressure switched on. The gas pressure was set at 25 lbs. sq. in. The ultrafiltrate was collected in a 50 cm^3 graduated flask and 15 cm^3 collected. The gas was then switched off. The absorbance of the original solution and the solution left in the cell were measured at 292 nm in a Unicam SP 500 using 1 cm path length silica cells. The retention R was determined using the formula

$$R = \frac{\log_e \frac{Cf}{Co}}{\log_e \frac{Vo}{Vf}}$$

Cf = Conc. of compound in filtrate

Co = Original conc. of compound in solution

Vo = Volume of original solution

Vf = Volume of filtrate

RESULTS

Cellulose phosphate

The acidic and neutral amino acids aspartic acid and glycine were eluted with the 0.01 M sodium phosphate buffer (pH 6.0) containing 0.5% sodium chloride while the basic amino acid histidine was located with 0.1M HCl and 1.0M HCl eluates. Lysine, arginine, creatinine, the polyamines spermine and putrescine were located in the 1.0M eluates. The bleomycins were eluted with the 1.0M HCl eluate and when the neutralised fraction was subjected to silica gel G t.l.c. using (1:1 v/v) 10% ammonium acetate: A.R. methanol the bleomycins could be detected by spraying the dried plate with 0.05% fluorescamine in dioxane. The Rf values obtained were 0.40 and 0.70 respectively.

Normal urine samples "spiked" with bleomycin (4 mg. 100 cm^{-3}) was subjected to cellulose phosphate chromatography and the neutralised 1.0M HCl was freeze dried, the residue dissolved in 100 ul 1:1 methanol;water. Aliquots were subjected to t.l.c. and u Porasil h.p.l.c. The h.p.l.c. procedure utilised a U.V. absorption detector (254 nm) and the expected bleomycin pattern was completely obliterated by contaminants. The t.l.c. method yielded a faint spot at Rf 0.70 which indicated the presence of bleomycin B_2 but no bleomycin A_2 could be located. When an equivalent t.l.c. plate was sprayed with the alkaline picrate reagent (111) a large orange spot was detected with an Rf value of approximately 0.68 - 0.78.

Sephadex G-15 gel filtration

Figure 21 represents the pattern of the separations obtained. The Kav values for bleomycin and bleomycin B_2 were calculated to be 0.03 and 0.07 respectively.

Iso-electric focusing

The patterns obtained with the various samples are shown in Fig. 22.

A single blue band was obtained with the copper (II) bleomycin in the pH range 4.0 - 4.2. Several blue zones were obtained with the copper (II) chloride sample in the alkaline pH part of the gel. No evidence of focusing was observed in gels containing the metal-free bleomycins. Treatment of these gels with 10% TCA produced no precipitation bands.

Ultrafiltration

R values of 0.16 and 0.24 were obtained for copper (II) bleomycin and bleomycin with UM05 membrane filter. Both samples gave R values of zero with membrane filter UM2.

Figure 20. pH gradient along IEF polyacrylamide gel

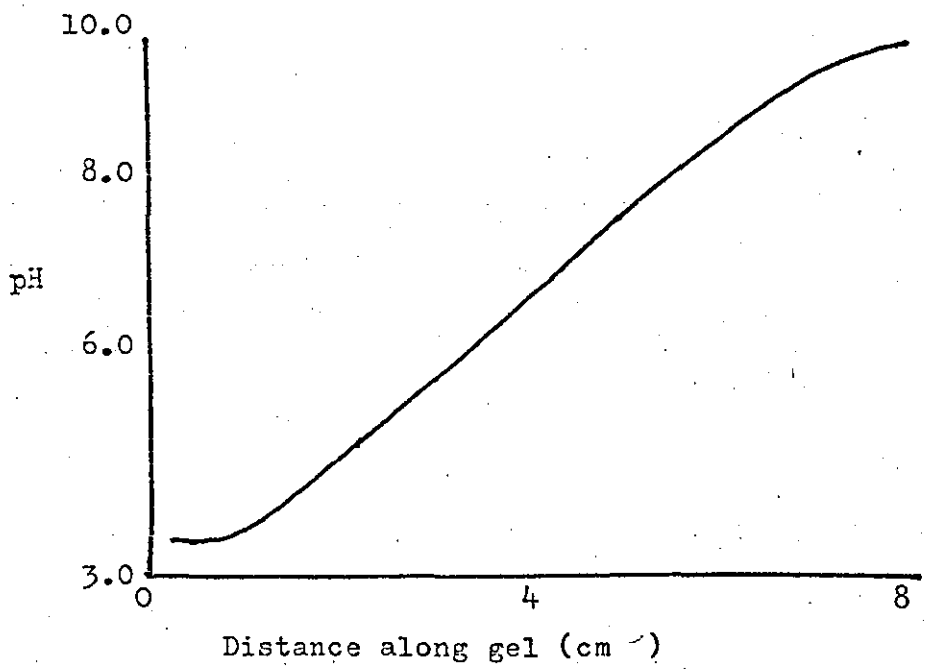


Figure 21. Gel filtration on G-15 of metal-free bleomycins

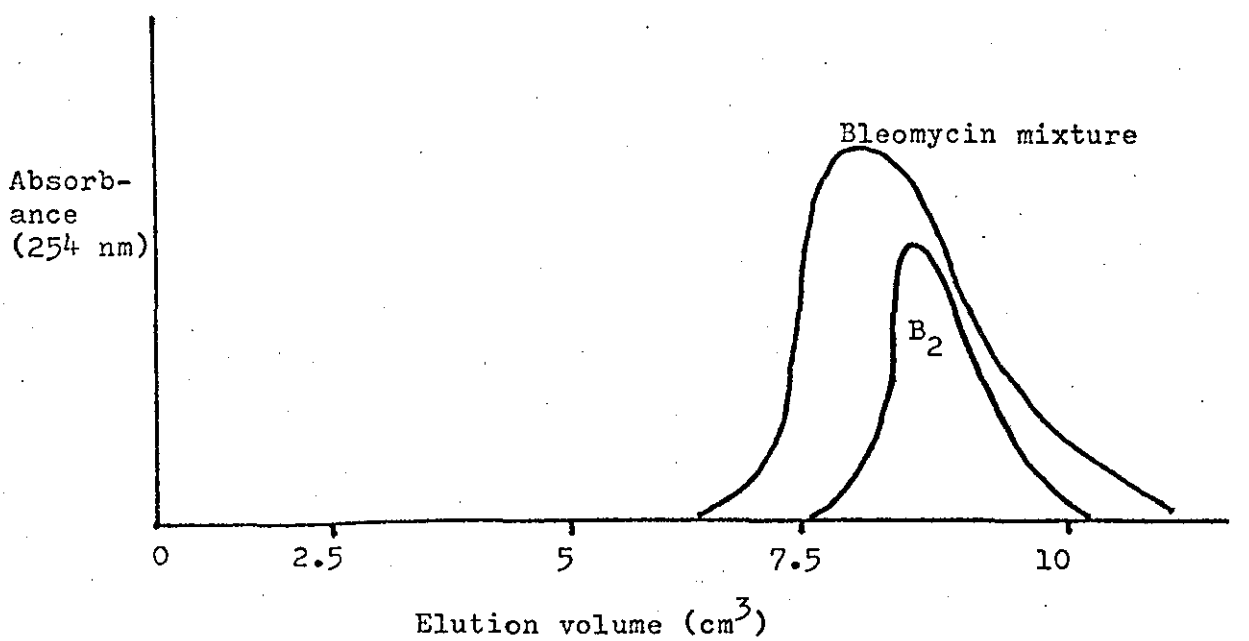
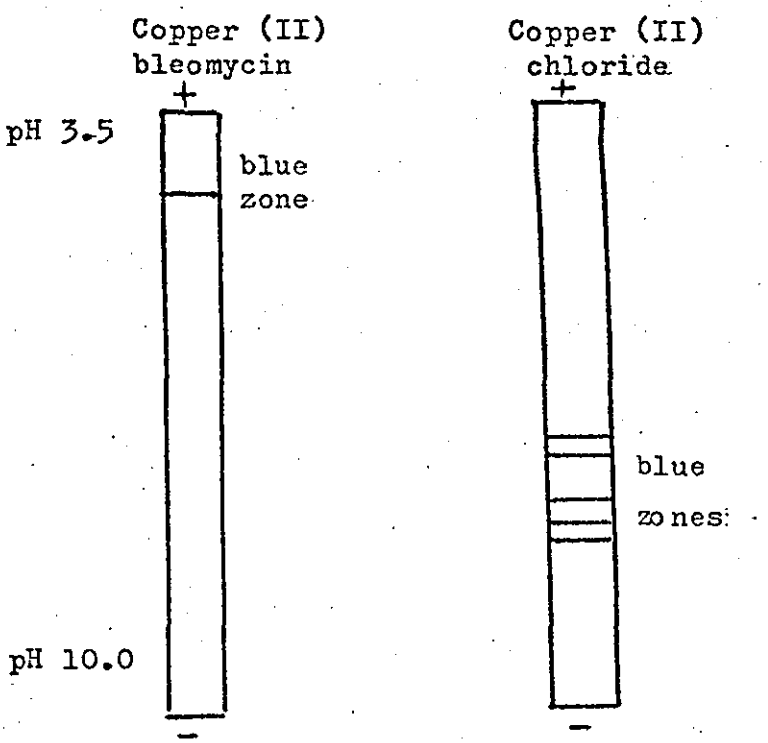


Figure 22. Isoelectric Focusing Pattern of Copper (II) bleomycin.



DISCUSSION

Cellulose phosphate chromatography

The bleomycins were concentrated in the 1.0M HCl fraction and the eluates from aqueous solutions after neutralisation gave a typical bleomycin pattern when aliquots were subjected to h.p.l.c. using u Porasil stationary phase. After freeze-drying the expected conversion of bleomycin A_2 to bleomycins DMA_2 and A_1 took place and this was observed on the h.p.l.c. system. The h.p.l.c. of the freeze-dried neutralised eluate from bleomycin "spiked" normal urine did not yield the bleomycin B_2 component since the absorbance of this compound was overwhelmed by interfering compounds. T.l.c. studies indicated that a major interfering compound was creatinine, one of the major nitrogen containing excretory products which occurs in considerable amounts in blood and urine (normal values 0.1 - 1.4 mg. 100 cm^{-3} and 130 mg. 100 cm^{-3} respectively) (112). It is obvious that h.p.l.c. using a Ultra-violet absorption detection system is of little use for investigating bleomycin levels in urine and blood at the sensitivity required unless compounds such as creatinine are removed by a pretreatment process. Creatinine does not fluoresce with fluorescamine so it may be feasible to employ a fluorescence detector and after the eluate leaves the h.p.l.c. column react it with fluorescamine; bleomycin reacts with fluorescamine giving fluorescent products which could then be detected using an excitation wavelength of 390 nm and an emission wavelength of 481 nm. It must be realised

that there still could be interference if other primary amine compounds were eluted with the same retention times. Another objection is the partial conversion of bleomycin A_2 into other bleomycin types when the eluate is freeze-dried.

Sephadex G-15 gel filtration

The elution volume of the bleomycin B_2 sample was slightly greater than that of the mixed bleomycin sample. The 0.9% saline solution was used to minimise any possible interactions with the gel due to the positive charge on the bleomycin (113). Nunn (101) has investigated bleomycin A_2 and found it to have a smaller elution volume than the bleomycin mixture. Nunn explained the difference in elution volumes of the two major bleomycins as being due to the greater Stokes radius of the bleomycin A_2 resulting from the terminal amine group of bleomycin A_2 projecting out into the solvent away from the bulk of the molecule.

Iso-electric focusing

The pI (isoelectric point) of copper (II) bleomycin was determined to be pH 4.0 - 4.2. This is difficult to accept since a far higher value could be expected from the pKa' values of bleomycin A_2 are 2.9, 4.7, and 7.3 (21). The blue bands obtained with copper (II) chloride occur in the alkaline region and suggested that the blue band obtained in the acidic region of the gel when copper (II) bleomycin is the sample is not due to free copper (II) ions binding with the ampholine.

In addition the sample was added after the gel had been formed in order to minimise free radical modification of the sample (114). It is known that excess persulphate migrates to the anode and can interfere with the sample. Again the gels were pre-run in order to prevent such interactions. Nunn (personal communication) obtained a similar result when he carried out the iso-electric focusing of ^{61}Copper (II) bleomycins.

The absence of a single focusing zone for the copper-free bleomycins can be explained by the consideration of the structures of bleomycins A_2 and B_2. Both have positively charged terminal groups (Table 1) which render these molecules positively charged over the pH range 3.5 - 10.0.

Ultrafiltration

Both the membrane filters used were permeable to the copper (II) bleomycins and the metal-free bleomycins. It is understood that the cut-off points of these filters are not sharp with respect to differences in molecular shape. It is possible that bleomycins are quite flexible molecules and could be forced through a membrane filter when a globular peptide of similar molecular weight would be retained. The R values for copper (II) bleomycin and bleomycin with the UM05 are quite similar and the difference obtained is within the limits of the experimental error. Nunn (101) compared

the Sephadex G-15 elution volumes of ^{61}Copper-bleomycin A_2 and bleomycin B_2 with those of metal-free bleomycins and observed that there was no detectable change in elution volume. The ultrafiltration results and the gel filtration studies suggest that there is no significant difference in the size and shape of the bleomycin molecule when it is chelated to copper (II) ions or is in the metal-free form.

CONCLUSIONS.

It was hoped that a suitable purification step in conjunction with h.p.l.c. could be used to estimate bleomycins in biological fluids. Unfortunately this objective could not be satisfactorily realised due to the inability to remove interfering compounds. Another disadvantage was that a freeze-drying process was employed to concentrate the so-called pure fraction and this converted bleomycin A_2 to bleomycin A_1 and DMA_2. It is clear that a successful non-radioisotopic method will not be easy to devise due to (i) the presence of several bleomycins in the commercial preparation. (ii) the alteration of bleomycin A_2 during freeze-drying (iii) difficulties in devising a relatively rapid method of purifying bleomycins. (iv) chemical instability of bleomycins at pH extremes.

CHAPTER 6

ENZYME INVESTIGATIONS

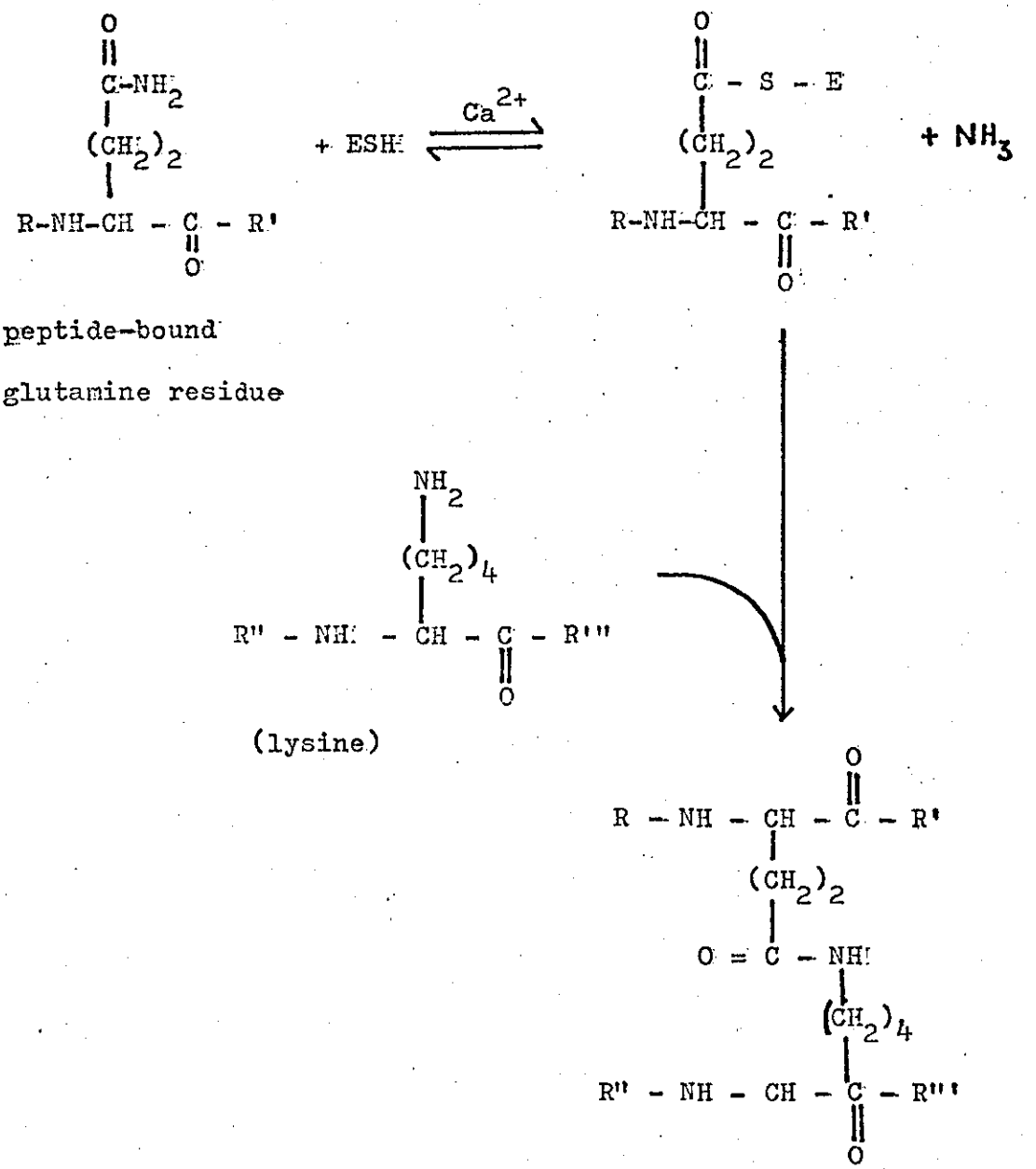
INTRODUCTION

The transglutaminases (amine:R-glutamine transferases (hydrolysing)) are a group of enzymes which catalyze an acyl transfer reaction between the γ-carboxamide groups of peptide-bound glutamine residues (acyl donors) and various primary amine groups (acyl acceptors) to produce mono-substituted γ-amide bonds of peptide-bound glutamic acid and releasing ammonia (Fig. 23).

Transglutaminase activity was originally discovered in the livers of various animals (115) but was later found to be widely distributed in other animal tissues (116). The specific functions of transglutaminases found in various tissues are not clear and only in plasma (117,119) and hair follicles (118) has a definite biological function been assigned.

Fibrin Stabilising Factor (blood coagulation factor XIII) exists as a zymogen form of transglutaminase in blood plasma and platelets. It is converted into the active enzyme by proteolytic cleavage by the enzyme thrombin. The resultant transglutaminase catalyses the formation of ϵ-(γ-glutamyl)-lysine cross-links between fibrin monomers giving rise to an insoluble polymer matrix. The hair follicle enzyme is thought to function in the formation of ϵ-(γ-glutamyl)-lysine cross-links between

Figure 23. General mechanism of action of the transglutaminase enzymes



Key:

ESH refers to the thiol transglutaminase enzyme.

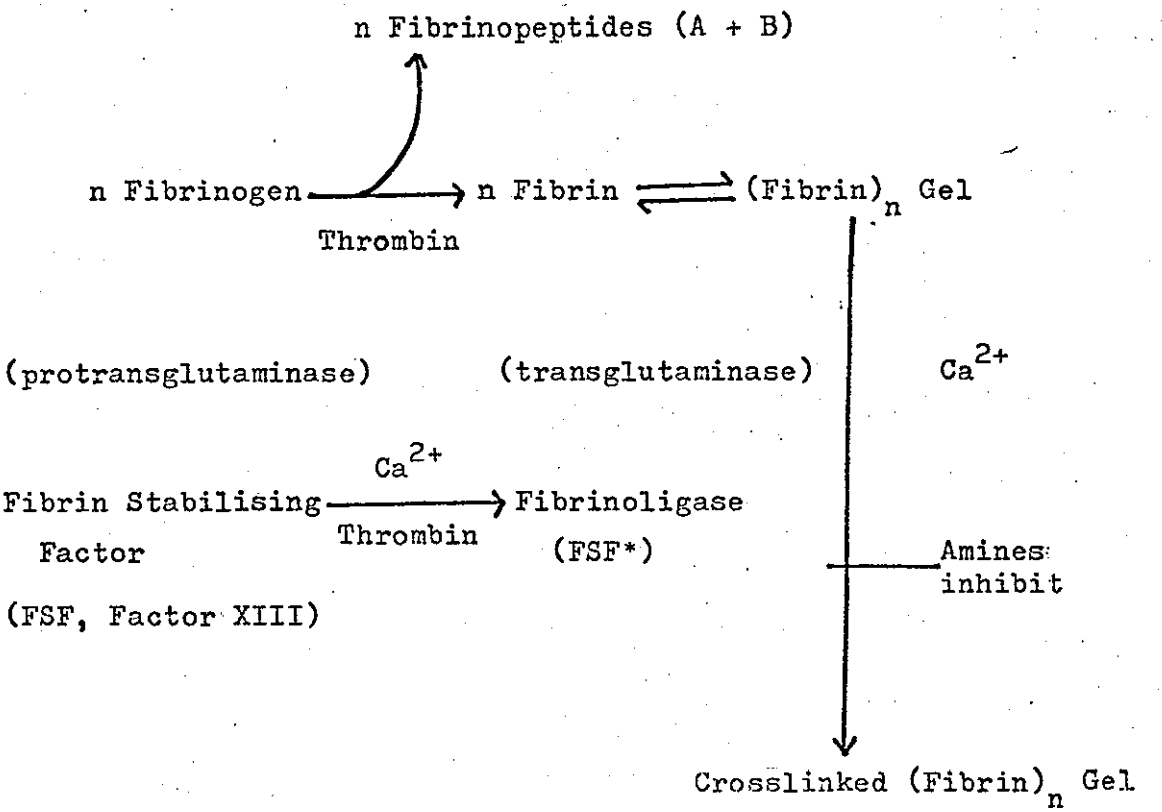
R, R', R'', R''' are all peptides in proteins.

keratin chains of the hair follicle.

The physiological function of the plasma transglutaminase is to cross-link fibrin in order to produce a fibrin aggregate. The initial fibrin aggregate is converted into an insoluble polymer (clot) which is physically stronger and is less susceptible to proteolysis than the initial aggregate (121, 122) Fig. 24. The insoluble aggregate acts as a stable haemostatic plug which prevents blood loss from intact blood vessels and prevents excessive bleeding from severed blood vessels.

The clotting process may be summarised as follows:- (i) The enzyme or enzymes responsible for the formation of insoluble fibrin exist in plasma in an inactive precursor form. (ii) Plasma transglutaminase requires calcium ions for activity, it is inactivated by sulphydryl agents e.g. iodoacetate and its ability to make fibrin insoluble is inhibited by synthetic primary amines. (iii) Products of the enzyme catalysed primary amine incorporation into fibrin are glutamic acid monosubstituted amides. (iv) The enzyme forms γ-glutamyl-ϵ-lysine peptide bridges in fibrin concurrent with insolubilisation. The solubility of the different forms of fibrin are defined by the solubility in dilute acid (e.g. 1% monochloroacetic acid) and in solutions of denaturing compounds e.g. urea.

Figure 24. Clotting of Fibrinogen in Vertebrates



The above actions closely follow those determined for liver transglutaminase except that this enzyme does not occur in a zymogen form but in an active form.

The transglutaminases studied in this work were plasma transglutaminase [Factor XIII] and a tissue transglutaminase isolated from rat lung. Lung tissue was used due to its ability to accumulate bleomycins and because of its response to bleomycin therapy when invaded with squamous

cell carcinoma (9, 26). Another factor in favour of lung tissue was its low activity with respect to bleomycin inactivating enzyme (23).

Evidence that transglutaminases are involved in the proliferation of tumour tissue has been reported in several papers (63, 64, 123, 124). Yancey and Laki (63, 64, 123) established that transglutaminases were required for the stabilisation of the fibrin network required by solid tumours during proliferative growth and demonstrated that polyamines and monodansyl cadaverine possess inhibitory activity against this type of neoplasm. They established that high molecular weight, water soluble polyamines were the most efficient of the compounds tested.

It is considered that the catalytic mechanism (Fig. 23) of transglutaminase enzymes proceeds via an acyl enzyme thiolester intermediate which is deacylated after nucleophilic attack by a donor amine or by water if no amine is present. Donor amines can either be a lysine of a nearby fibrin molecule or a synthetic amine (H_2N R). Synthetic amines inhibit the cross-linking of fibrin since they act as pseudo-donors competing with the natural donor for the acceptor sites. To act as an inhibitor a synthetic amine must satisfy the specificity requirement of the acyl-enzyme intermediate in order to combine with it and also be sufficiently nucleophilic to be effective in the aminolysis of the intermediate. Several amines have been found to be competitive inhibitors of the cross-linking reaction and by using labelled

amine donors e.g. ^{14}C-monodansyl cadaverine (N-(5-amino-pentyl)-5-dimethylamino-1-naphthalene sulphonamide) it has been shown that they are incorporated into the fibrin network (117). Since these amines do not prevent fibrin aggregation only the final cross-linking step, the extent of inhibition can be regarded as a function of clot solubility. Such amines can also be incorporated into water-soluble proteins which contain glutamine residues e.g. casein. Such reactions can be made quantitative by using labelled amines and this forms the basis of several analytical assays (125, 126, 127, 128, 129). In addition to native proteins, chemically modified forms e.g. N,N'-dimethyl (130), in which lysine side chains are excluded from participating in cross-link formation are used.

The substrate for plasma transglutaminase is fibrin which is formed from plasma fibrinogen by the action of the enzyme thrombin. Mammalian fibrinogen (M.Wt 330,000) consists of three pairs of polypeptide chains held together by disulphide bonds (131). These chains are termed the α, β and γ chains (M.Wts. 67,000, 56,000 and 45,000 respectively). Fibrin aggregates are found after limited proteolytic cleavage by thrombin causing fibrinopeptides A and B to be released from the α and β chains respectively. The cross-linking of fibrin chain is considered to take place by reduction of disulphide bridges (132) to allow γ chains to rapidly form γ-γ dimers followed by the α chains forming α-polymers of high molecular weight at a slower rate. The β chains

do not participate in the cross-linking of fibrin by plasma transglutaminase during the time in which the α- and γ-chains are cross linked. The cross-linking of fibrin in purified systems can be investigated by sodium dodecyl sulphate (SDS) disc gel electrophoresis of the reduced chains (140). This enabled the inhibition of the cross-linking of fibrin by synthetic amines to be investigated. This method may be used to investigate the effect of bleomycin and copper (II)-bleomycins on plasma transglutaminase activity.

MATERIAL AND METHODS

Reagents

All reagents used with protein and enzyme preparations were stored below $4^{o}C$ unless otherwise stated. Thrombin, Topical Bovine was obtained from Parke-Davis and Co. Fibrinogen was kindly supplied by Dr.M.Griffin, (Trent Polytechnic). [1,4-^{14}C] - putrescine dihydrochloride ([1,4-^{14}C] tetra methylenediamine dihydrochloride) was supplied at a specific activity of 62 mCi m mol^{-1} from the Radiochemical Centre, Amersham, U.K.

Monodansylcadaverine (N-[5-Aminopentyl] - 5-dimethylamino-1-naphthalene sulphonamide used as a fluorescent pseudo-donor substrate in the enzyme assays was supplied by I.C.I. Ltd., Alderley Park, Macclesfield, U.K. Dithiothreitol used to protect the enzymes by keeping their sulphidryl groups in an active, reduced state, was obtained from Sigma Chemicals.

Buffers

In all studies with fibrinogen or fibrin, the term "Tris buffer" refers to 50 mM Tris/HCl pH 7.4 containing 100 mM NaCl so that the ionic strength is equivalent to the physiological level. The term "Tris" refers to Trizma base supplied by Sigma Chemicals.

Methods

Preparation of Fibrinogen

Fibrinogen was dissolved in 50 mM Tris 100 mM NaCl, pH 7.4 as a solution of about 15 mg. cm^{-3} and then dialysed against 20 volumes of the same buffer at $4^{o}C$ with stirring. 2 cm^{3} aliquots of the dialysed fibrinogen was placed in plastic tubes which were capped and frozen until required. This contained enough factor XIII as an impurity as not to merit further addition of the enzyme.

Thrombin assay

Thrombin (10,000 NIH units) was dissolved in 20 cm^{3} 50 mM Tris/100 mM NaCl, pH 7.4 and dialysed with stirring against twenty volumes of the same buffer at $4^{o}C$. The thrombin was assayed for its ability to clot a fibrinogen solution and its activity expressed in NIH units. The assay system utilised a fibrinogen solution which consisted of 4 cm^{3} distilled water, 2 cm^{3} 1.5% fibrinogen and 2 cm^{3} 0.3M KCl. An aliquot of this fibrinogen solution was mixed with 0.2 cm^{2} of the thrombin solution, both solutions having been prewarmed to $37^{o}C$. Calcium ions were added to a final concentration of 5 mM. The time was recorded from the time of mixing to the production of a small, white, diffuse, fibrous aggregate. One NIH unit of thrombin being defined as that concentration which produces a clot in 15 seconds. By means of a suitable dilution factor of the thrombin solution with respect to its clotting time, the activity of the thrombin

solution could be estimated. The thrombin solution was stored in 2 cm^3 aliquots containing 200 NIH units of thrombin at -25°C until required.

Preparation of Rat Lung Transglutaminase

Rats of mixed sex (Wistar derived) weighing between 200-250 g. were used. They were killed by a sharp blow on the back of the head followed by section of the carotid artery. The lungs were removed, perfused with cold 0.15M NaCl and then placed in cold 0.15 M NaCl. The bronchi were removed from the lungs and the remaining tissue weighed, from this stage the lungs were kept at 4°C. With a pair of sharp scissors the lungs were cut into small pieces (1-2 mm squares) and added to a cold glass homogeniser tube together with three volumes of sucrose buffer (0.25 M Sucrose/1 mM EGTA/1 mM Tris) pH 7.4. The suspension was then homogenised with a Potter-Evehjem homogeniser fitted with a loose-fitting teflon pestle. When the homogenate attained a soup-like consistency it was transferred to a tight-fitting glass homogeniser tube and the homogenisation continued for only two up and down strokes of the pestle.

The homogenate was centrifuged at 1000 g. ($\bar{r}$ ave 28 cm) for 10 minutes. The supernatant was decanted and the pellet resuspended in a further 10 cm^3 of sucrose buffer and re-homogenised with one up and down stroke of the loose fitting pestle. The suspension was again centrifuged at 1000 g ($\bar{r}$ ave 28 cm) for a further 10 minutes. The supernatant

was decanted and added to the initial supernatant and retained.

The pellet was washed a further three times with 5 cm^3 washes of sucrose buffer and centrifuged each time at 1000 g, each supernatant was retained for assay. The final nuclear pellet was resuspended in 5 - 10 cm^3 of the sucrose buffer. The collected supernatants were centrifuged at 75,000 g. for 60 minutes, the supernatant was decanted and used for enzyme assays. The protein content of the supernatant and nuclear pellet were determined using the method of Lowry et al. (133). Enzyme assays were performed on the same day as the enzyme samples were prepared since the enzyme was found to be quite labile.

The "soluble" transglutaminase enzyme consisted of the high speed supernatant combined with the washings from the nuclear pellet, the "bound" transglutaminase consisted of enzyme activity remaining in the 'nuclear pellet'.

Quantitation of amine incorporation using a modification of the filter paper assay of Lorand et al. (129).

This technique was used to investigate the effect of copper (II) bleomycin and metal-free bleomycins on lung transglutaminase activity. Transglutaminase activity was investigated by using a filter paper assay procedure for the radioactive amine incorporation into protein. The method involved the acid precipitation of the acceptor protein onto a small piece of filter paper, followed by removal of the free, non-protein bound radioactive amine substrate by washing

and then after drying the filter square, determination of the protein-bound radioactivity by scintillation counting.

The radio-active labelled pseudo-substrate used was [1,4-^{14}C] putrescine dihydrochloride and the pseudo-acceptor was N,N'-dimethyl casein. The alkylation of the free amino groups of lysine prevents casein from acting as an amine donor and cross-linking itself. The following solutions were made up in 50 mM Tris-HCl, (pH 7.4), buffer and stored frozen:

Dithiothreitol (DTT) 38.5 mM, $CaCl_2$ (100 mM)

EDTA (50 mM), N,N'-dimethyl casein (5.4 mg cm^{-3})

The dry contents of a single ampoule of [1,4-^{14}C) Putrescine dihydrochloride were dissolved in 1 cm^3 of 0.8 mM unlabelled putrescine in 50 mM Tris-HCl, pH 7.5 and also stored frozen.

The assay of each sample was carried out in duplicate, in the first reaction mixture $CaCl_2$ which was necessary for transglutaminase activation was present while in the second reaction mixture EDTA replaced $CaCl_2$ and this reaction mixture acted as a control. The reaction mixtures were made up in Autoanalyzer plastic sample cups fitted with polythene caps.

10 ul of DTT, 5 ul of $CaCl_2$, 20 ul of N,N-dimethyl casein, 20 ul of ^{14}C-labelled putrescine and 10 ul of test substance or double distilled water were pipetted into the appropriate plastic vial with gentle mixing between each addition. The test substance was one of the following

solutions made up in tris-HCl pH 7.4 buffer -3.3 mM bleomycin, 3.3 mM copper (II)-bleomycin. If the assay was carried out in the absence of an inhibitor, 10 ul of double distilled water was used. After allowing the reaction mixture to equilibrate at 37°C on a metal block unit, 35 ul of the enzyme preparation was added, the vial capped and spun on a Vortex mixer. Samples of the reaction mixture were removed from the plastic vials at appropriate time intervals and placed on a pre-cut pencil-labelled Whatman 3 MM filter paper square (1 cm^2). These samples were immediately dropped onto a copper-wired basket submerged in a beaker of ice-cold 10% Trichloroacetic acid (TCA). The contents of the beaker were continuously stirred and the filter papers washed for 20 minutes after the addition of the last paper. The basket and filter paper squares were transferred to a 5% solution of ice-cold TCA. The washing procedure was continued according to the following treatments:-

5 minutes each with one change of cold 5% TCA,
5 minutes each with ethanol-acetone (1:1 v/v) and
5 minutes with acetone. At least 5 cm^3 of wash solution was used for each filter-paper added. The above procedure removed any radioactivity from the filter papers which were not protein bound. The filter papers were then placed on a foil tray and dried at 80°C for 30 minutes. In order to obtain the total radioactive label applied to each sample "totals" were taken of each pair of samples by removing 10 microlitres from each reaction vial at the end of the

incubation process and was placed on a pre-cut 1 cm^3 filter paper as above but taking it immediately through the drying stage, thus ignoring the washing procedure. The squares were placed in separate plastic inserts to which was added 4 cm^3 of a scintillation fluid (3 g. of 2,5-diphenyl oxazole and 0.3 g. of 1,4-di- [2-(5-phenyloxazoyl)-] benzene per litre of toluene). Each of the plastic inserts were placed in a glass vial and placed in a Packard (Model 3330) scintillation spectrometer set to count ^{14}C (gain 6.8, window 50-1000) for ten minutes per sample. All pipetting of microlitre quantities was carried out using Finpipettes with Eppendorf disposable plastic tips except for the sampling which was carried out using Corning disposable 10 microlitre glass pipettes.

Fibrin Cross-linking Assay

The effects of copper (II) bleomycin and bleomycin on the activity of factor XIII (plasma transglutaminase) on fibrin polymerisation and the pattern of fibrin cross-linking was investigated using sodium dodecyl sulphate (SDS) - disc gel electrophoresis. The fibrinogen samples were exposed to fibrinoligase activity and the cross-linking allowed to proceed at 37°C until the enzyme activity was terminated at the required time by freezing the sample in ethanol/dry ice mixture. The state of the reaction at that point in time can be examined by gel electrophoresis.

Reactions were performed in plastic tubes containing in a final volume of 0.310 cm^3 the following components:

	Test (ul)	Control (ul)
Calcium chloride 100 mM	5	0
EDTA 100 mM	0	5
Dithiothreitol 180 mM	5	5
Potential Inhibitor 10 mM	100	100
Fibrinogen 1.33%	100	100

The potential inhibitor was one of the following: 10 mM bleomycin, 10 mM Copper (II)-bleomycin, 10 mM Dansyl cadaverine. All these substances were made up in 50 mM Tris-HCl pH 7.4.

Reactions were started by the addition of 100 ul thrombin solution. The reactions were incubated at 37°C throughout the experiment and the reactions were terminated by removing a reaction tube from the water bath at the required time and immediately placed in an ethanol/dry ice mixture to freeze the contents of the tube. The freeze-dried contents were then dissolved in 0.4 cm^3 of 3% w/v SDS/9M Urea/3% 2-mercapto-ethanol solution prior to SDS polyacrylamide gel electrophoresis.

S.D.S. polyacrylamide gel electrophoresis:

The polyacrylamide gels were prepared according to the scheme on page 61 except that the tank buffer used was 0.1 M sodium phosphate pH 7.1.

A 10 µl aliquot of a sample obtained from the cross-linking experiment, was carefully laid on top of the flat horizontal gel surface. 5 µl of bromophenol blue (0.05% in 10% sucrose) was added as a marker and 5% sucrose overlayed to the top of the tube. The gels were run at a current of 10 mA per tube and by following the progress of the marker stain to 0.5 cm from the base of the gel, the maximum separation could be attained. The gels were removed from the tubes and fixed in 50:50 methanol:acetone for 12 hours. They were then removed from this solution and placed in 0.4% Coomassie brilliant blue G in 38% methanol; 7% acetic acid and 54.6% distilled water for 4 hours. They were then destained in 38% methanol:7% acetic acid in water overnight and then transferred in 25% methanol:7% acetic acid destaining solution after this period. Later they were stored in 5% methanol:7% acetic acid. The stained gels were scanned in a Unicam S.P. 1800 spectrophotometer with a scanning attachment, the wavelength of light used was 600 nm and the slit width 1.5 mm.

RESULTS

(i) Effects of bleomycin and copper (II) bleomycin upon fibrin polymerisation by Factor XIII.

The gel electrophoretic patterns of fibrin polymerisation as a function of time in the presence and absence of inhibitors are shown in Fig. 25. The control system shows that the cross-linking of fibrin by Factor XIII involves the conversion of γ chains to $\gamma-\gamma$ dimers and α-chains to α-polymers, while for the duration of the experiment the β chains remain relatively unaltered.

Examination of the semi-quantitative results obtained by scanning the stained gels at 600 nm showed that the incubation mixtures containing bleomycin and copper bleomycin gave rise to marked inhibition of α-polymerisation by Factor XIII but had no detectable effect upon the formation of $\gamma-\gamma$ dimers. The decrease in peak area of the α-monomer with respect to time of incubation is shown in Fig. 26. Variations in the amount of staining of gels and in loading volume were minimised by employing the β-monomer peak as an internal standard and for comparative purposes all peak areas were adjusted with respect to a constant value for the unchanged β-monomer. The decrease in area of the α-monomer peak over 60 minutes incubation period showed approximately 59%, 15% and 18% inhibition of α-polymer formation for copper (II) bleomycin, bleomycin and monodansyl cadaverine respectively when compared with the control Fig. 26.

(ii) Effects of bleomycin and copper (II) bleomycin on the [1,4-^{14}C]-putrescine incorporation into N',N'-dimethyl casein by rat lung transglutaminase.

Both bleomycin and copper (II) bleomycin were shown to inhibit "soluble" transglutaminase; a Lineweaver-Burk plot of the reciprocal of the initial velocity against the reciprocal of the [1-4 ^{14}C]-putrescine concentration is shown in Fig. 27. Bleomycin and copper (II) bleomycin gave plots which were characteristic of competitive inhibition with enzyme inhibitor constants (K_i) of 3.8×10^{-4} M and 1.4×10^{-4} M respectively. The Michaelis constant (K_m) of the "soluble" transglutaminase enzyme was computed to be 2×10^{-4}M which was in good agreement with the literature value for guinea pig liver transglutaminase (129).

The equivalent studies on the "bound" transglutaminase enzyme found in the nuclear pellet are shown in Fig. 28. Again copper-bleomycin was found to have an K_i of 8.9×10^{-4}M. The Michaelis constant (K_m) of the bound enzyme for [1,4-^{14}C]-putrescine was calculated as 4.1×10^{-4}M. Bleomycin had negligible inhibitory activity upon the "bound" enzyme and probably indicates the binding of bleomycin to the large amount of DNA present in this enzyme fraction (37). It is to be noted that copper-bleomycin does not appear to bind to DNA (36).

The possible effect of free copper (II) ions on lung transglutaminase activity was investigated since it was reported that guinea pig liver transglutaminase was rapidly inactivated by copper (II) ions (134) in the presence of calcium ions. Reaction systems using the "soluble" transglutaminase were set up containing varying amounts of (1,4-^{14}C)-putrescine (as described in Fig. 27) with Cu $(No_3)_2.H_2O$ present at a fixed concentration of 0.33 mM. Inactivation of the enzyme occurred at all concentrations of (1,4-^{14}C)-putrescine except 0.8 mM where only 16% of the original enzyme activity remained. The result indicated the susceptible nature of lung transglutaminase to free copper (II) ions and although it confirms that the majority of copper is probably bound during copper (II) bleomycin inhibition it does not eliminate the possibility that small amounts of free copper (II) ions may be present; which may account for the greater inhibitory power of copper (II) bleomycin over free bleomycin.

DISCUSSION

The effect of copper bleomycin upon rat lung transglutaminase showed that this compound was an effective competitive inhibitor of (1,4-^{14}C) putrescine incorporation into N,N' dimethyl casein. Studies on the "soluble" enzyme gave an enzyme inhibitor constant for copper (II) bleomycin which was similar to the Michaelis constant for (1,4-^{14}C) putrescine which indicated a similarity in the affinity of these two compounds for the "soluble" lung transglutaminase binding site. The lack of inhibition exhibited by bleomycin when the "bound" enzyme was used probably reflects the binding of bleomycin to DNA present in the enzyme fraction (39) (45). This effect was not shown by copper (II) bleomycin and supports the observation (36) (24) that copper (II) bleomycin does not bind to DNA.

The observation that copper (II) bleomycin was a more potent inhibitor than free bleomycin may be due to a greater rigidity imposed on the bleomycin backbone when the antibiotic was bound to copper (II) and may minimise the steric effects of glycopeptide chain when it approached the enzyme binding site. It must be realised that the presence of small amounts of free copper (II) ions may be present and could account for the increased potency of copper (II) bleomycin over free bleomycin.

The fibrin cross-linking experiments showed that bleomycin and copper (II) bleomycin were effective inhibitors of α-polymerisation when compared with the standard inhibitor monodansylcadaverine (135), no inhibition of γ-dimerisation was shown by either compound. The results seem to be of some significance when one considers the work of McKee et al. (1932) who reported that the stability of the fibrin gel matrix was only complete when α-polymer formation started.

The catalytic mechanism of the transglutaminases has been established (136) (137) to proceed via a thio ester intermediate which arose between the thiol group of the enzyme and the γ-glutamyl component of the acceptor protein while amide formation resulted in aminolytic deacylation of the acyl enzyme by the primary amine. Most of the inhibitors of the transglutaminases (136) (138) were primary amines which served as pseudosubstrates for the enzyme and became incorporated into the γ-glutamyl acceptor sites of the receptor protein e.g. monodansylcadaverine. Observations obtained with monotosylated and dansylated alkyl diamine compounds (138) (139) indicated that an alkyl side chain of 0.5-0.6 nm was the optimum for the primary amine donor. For enhanced inhibition, an attachment of a bulky apolar substituent to the primary amine was thought to be necessary for the binding of the inhibitor to a hydrophobic centre near to the catalytic site of the enzyme.

The bleomycins in the bleomycin mixture have a variety of terminal amines (Table 1) and one can only speculate which component or components exhibited inhibition characteristic of competitive inhibition or pseudo-substrate. The only bleomycins which seem to be capable of acting as a pseudo-substrate are bleomycin A'_{2-a} and A'_{2-b} but these occurred at a concentration of less than 1%. Bleomycin B_2 which accounts for approx. 30% of the complex may meet the requirements of the enzyme by having a terminal amine side chain in the order of 0.5-0.6 nm which is linked to a large apolar group (thiazole ring) but it is unlikely that bleomycin B_2 would be effective in aminolysis of the acylenzyme intermediate by virtue of the overall positive charge of the guanidine group (pK≈13) at neutral pH. It thus seems unlikely that bleomycin B_2 would act as a pseudo-substrate for trans-glutaminases, however its ability to bind to the acyl intermediate would indicate its capacity to function as a competitive inhibitor of (1,4-^{14}C)-putrescine.

Figure 25. The effects of bleomycin, copper bleomycin and dansylcadaverine upon the cross-linking of fibrin by Factor XIII.

Electrophoresis was performed in 1.75 nm pore size gels, CuB, B and DC indicates fibrin polymerisation in the presence of 2.7 mM copper bleomycin, bleomycin and mono-dansylcadaverine respectively. N, indicates the control reaction system without inhibitor. EDTA shown above the gel column indicates that EDTA was included in the reaction system instead of calcium.

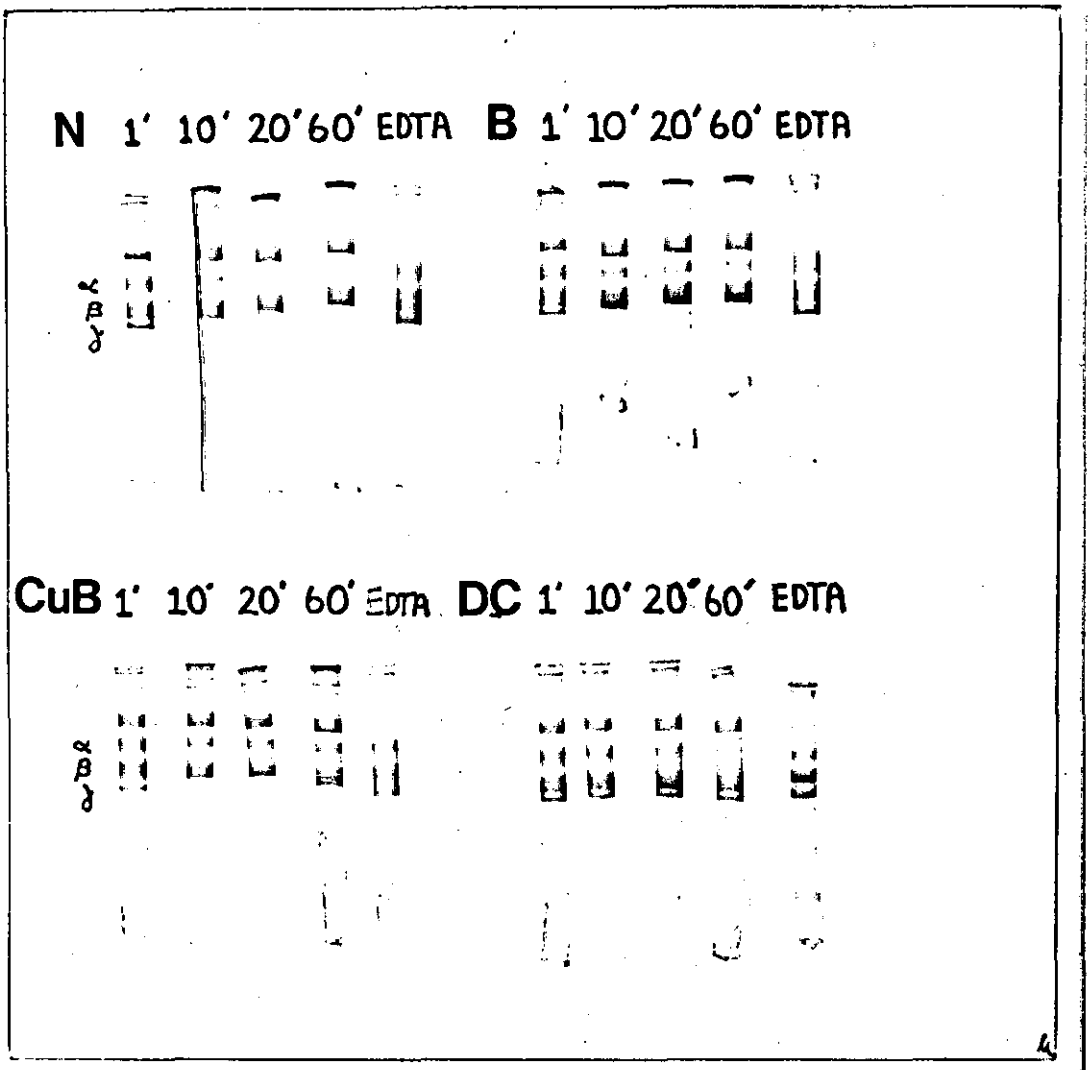

N 1' 10' 20' 60' EDTA
B 1' 10' 20' 60' EDTA
α
β
γ
CuB 1' 10' 20' 60' EDTA
DC 1' 10' 20' 60' EDTA
α
β
γ

Figure 26. Comparative analysis of the polymerisation of the α-monomer during fibrin cross-linking.

The symbols ▼, ▽ and ○ indicates fibrin polymerisation in the presence of 2.7 mM copper bleomycin, bleomycin and monodansyl cadaverine respectively; ● indicates the control system without inhibitor.

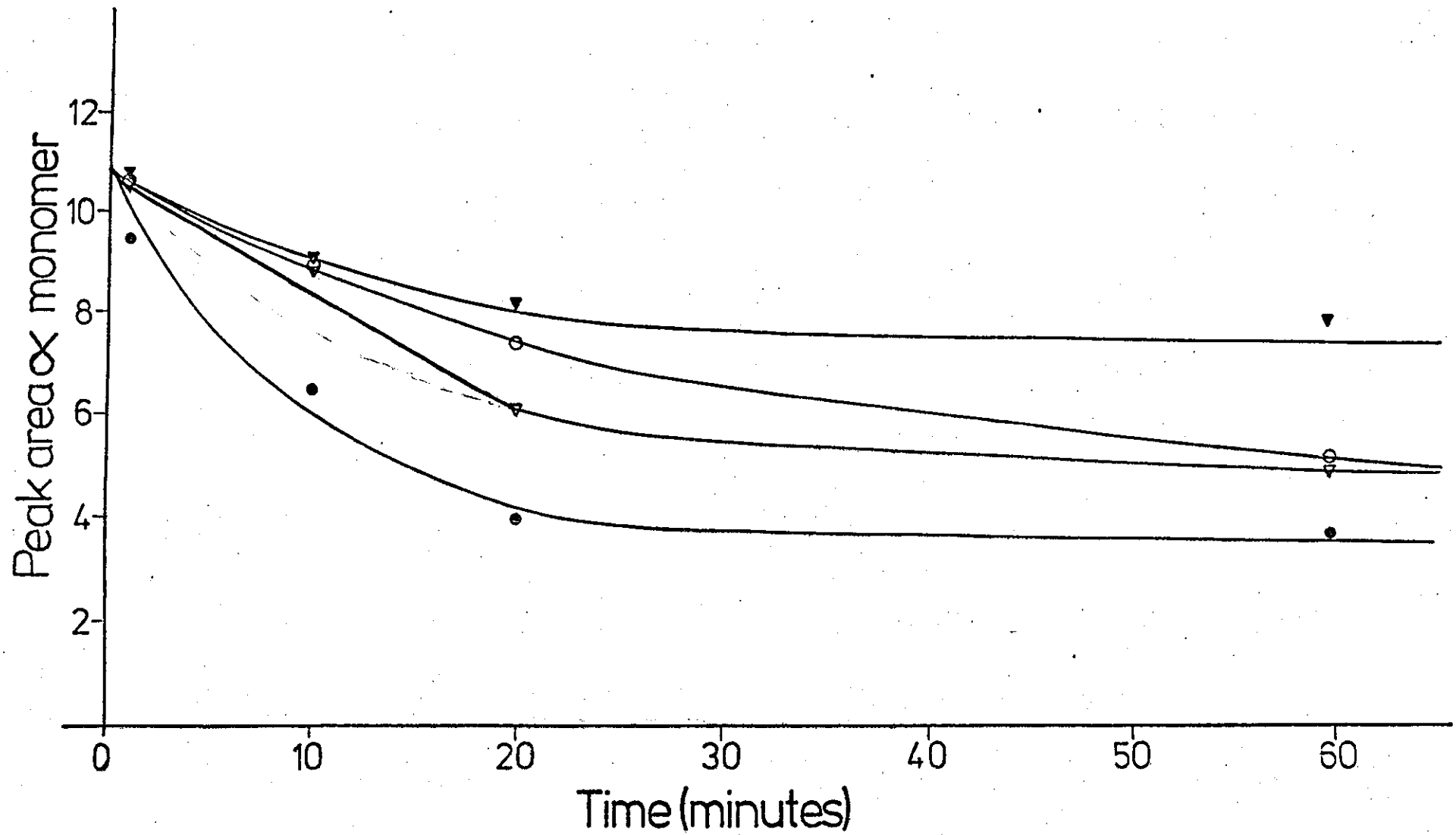

Peak area α monomer
12
10
8
6
4
2
0
10
20
30
40
50
60
Time (minutes)

Figure 27. Lineweaver-Burk plot showing the effects of bleomycin and copper bleomycin upon "soluble" rat lung transglutaminase.

The conditions of assay were as those described in the Methods. (1,4-^{14}C)-putrescine was supplied to the reaction mixture at the concentrations indicated (0.5 - 8 x 10^{-4}M). Reactions carried out at 37° were started by the addition of 100 ug of "soluble" enzyme fraction. In the series of experiments shown: □ , represents the control system without inhibitor; • in the presence of 0.165 mM bleomycin; ○ , in the presence of 0.33 mM bleomycin; ▽ in the presence of 0.165 mM copper bleomycin and ▽ , in the presence of 0.33 mM copper bleomycin. Inset, represents calculation of Ki by the method of Dixon.

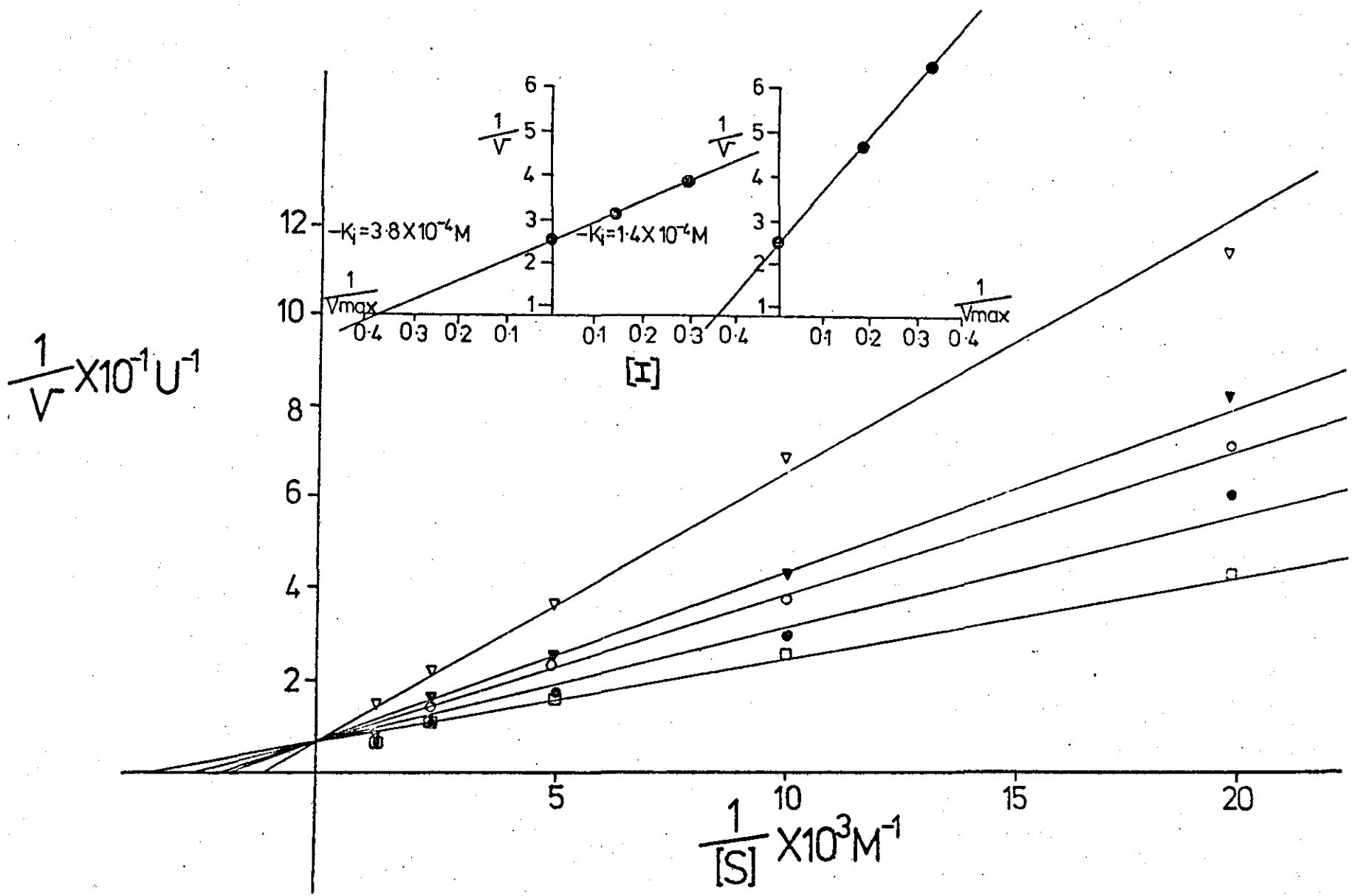
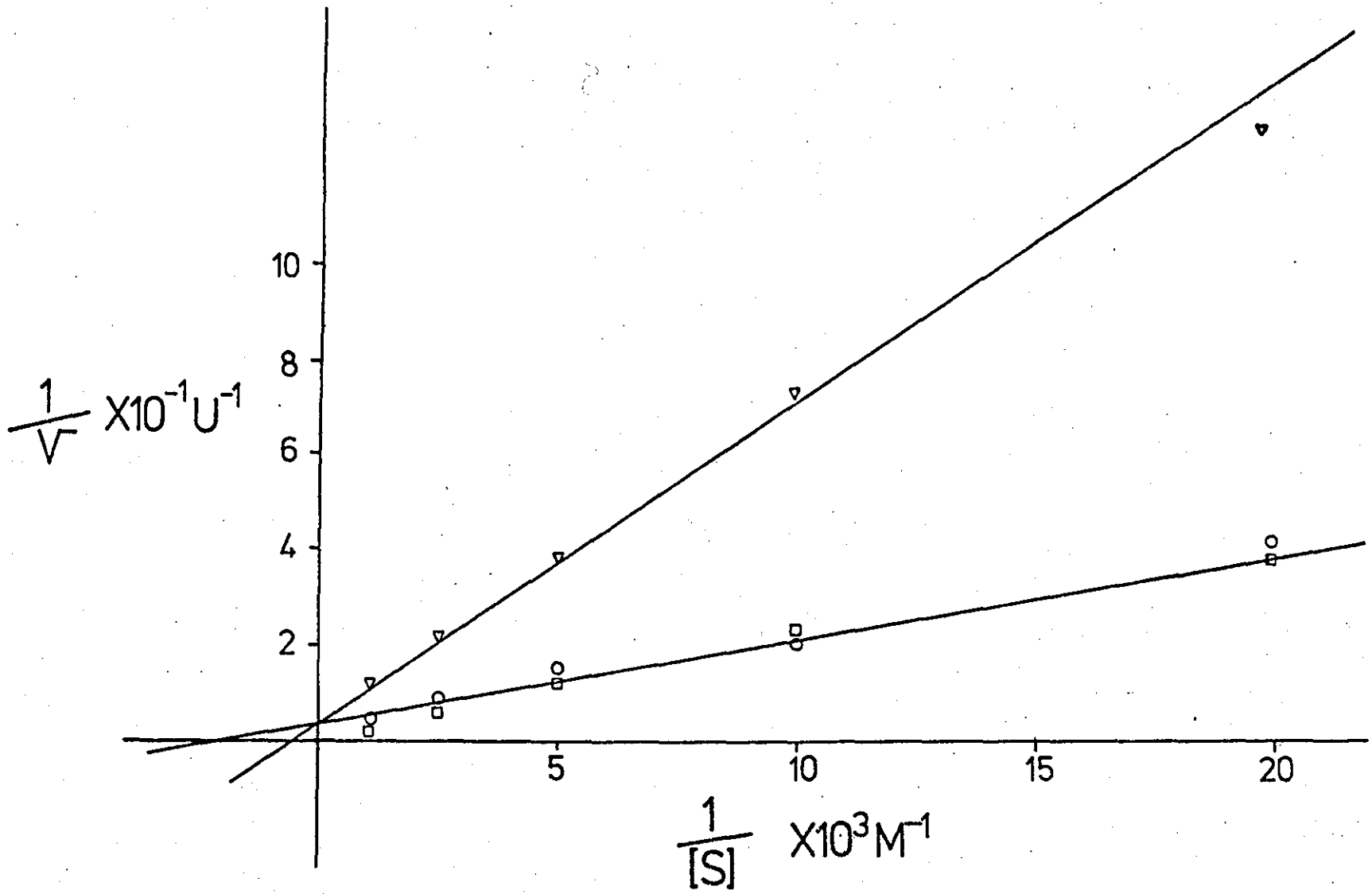

$\frac{1}{V} X10^{-1} U^{-1}$
12
10
8
6
4
2
5
10
15
20
$\frac{1}{[S]} X10^{3} M^{-1}$
$-K_i = 3{\cdot}8 X10^{-4} M$
$-K_i = 1{\cdot}4 X10^{-4} M$
$\frac{1}{V}$
$\frac{1}{Vmax}$
0·4 0·3 0·2 0·1
0·1 0·2 0·3 0·4
[I]

Figure 28. Lineweaver-Burk plot showing the effects of bleomycin and copper bleomycin upon "bound" rat lung transglutaminase.

The conditions of assay were as those described in Fig. 27. In the Series of experiments shown: □ represents the control system without inhibitor; ○ in the presence of 0.33 mM bleomycin; ▽ in the presence of 0.33 mM copper bleomycin.


1/V X10⁻¹U⁻¹
10
8
6
4
2
5
10
15
20
1/[S] X10³M⁻¹

CHAPTER 7

METAL-BLEOMYCIN CHELATE STUDIES

INTRODUCTION

An important aspect of bleomycin chemistry is the ability to chelate several metals, indeed bleomycins are isolated from the culture filtrate of *Streptomyces verticillus* as copper (II) chelates. This metal-chelating ability has been put to use in the radio-location of tumours, the bleomycins are used as carrier molecules to take a metal radio-isotope to the tumour. Several radioactive metals have been used to label the bleomycins for tumour imaging, ^{57}Cobalt and ^{111}Indium have found the most extensive use but ^{111}Indium appears to form a far weaker chelate with bleomycins than does ^{57}Cobalt. Indium-bleomycin is broken down in vivo and the released ^{111}Indium binds to transferrin in the blood (141). This is thought to explain the localisation of ^{111}Indium obtained from ^{111}Indium-bleomycin in the bone marrow of patients and for the slower clearance from blood than ^{57}Cobalt-bleomycin. Although the ^{111}Indium radioisotope has far superior physical characteristics to ^{57}Cobalt, ^{57}Cobalt-bleomycins seem to yield better tumour-imaging results (55).

Copper (II) like cobalt ions forms a strongly bound chelate with the bleomycins and although the interaction of the bleomycins with metal ions is not clearly understood, Umezawa (21) suggested that a possible site of copper (II)

binding is the α-amino group of the β amino alanine moiety since the pKa' of this group disappears when copper (II) is bound to bleomycin. The 3-O-carbamyl group can by rearrangement be transferred to position 2, but this re-arrangement is prevented if the bleomycin is bound to copper (II). This suggests that this group may be involved in the chelation of copper (II) ions. There is also the possibility that some of the amine groups of the terminal amine group may be involved in the binding of metal ions and it must be realised that although it appears that all bleomycins bind copper (II) ions strongly there could be different binding sites in different bleomycins due to the presence of different terminal amines.

There is clearly a need for further investigation of the nature of the stoichiometry of the copper (II) bleomycin and cobalt-bleomycin complexes since there appears to be little evidence of a quantitative study. The stoichiometry of these complexes can be investigated by visible spectroscopy using the method of continuous variation (142) and the mole-ratio method (143). The stoichiometry may also be investigated by potentiometric titration, in addition the stability constants of certain polyamine terminal amine groups namely putrescine and spermine could be examined to consider their metal-chelating potential. The implication of particular groups on bleomycin being involved in binding metal ions can be studied by electron-paramagnetic resonance studies of copper (II) bleomycin powder and by consideration of the visible spectra.

The principle of the method of continuous variation is that the ratio of the metal-ion and ligand is varied between 0 and 1 at constant total concentration C where $C = C_L + C_M$; and the absorbances of the solutions of different composition are measured at a particular wavelength. These absorbances are then plotted against the mole-fraction of the ligand L. If a single complex ML_n is formed and provided the Beer-Lambert law is obeyed and the absorbances are measured at a wavelength where neither the metal ion nor the ligand absorb but the complex does; then n can be determined from the abscissa of the maximum of the curve (X_{max}).

In the mole-ratio method, a series of solutions are prepared in which the concentration of the metal ion is kept constant while that of the ligand is varied. The absorbances of the solutions are measured at a suitable wavelength and a graph of absorbance against the ratio of variable and constant concentrations prepared. If only a single stable complex is formed then the absorption increases in a linear manner with the increasing mole-ratio and then becomes constant, the abscissa of the point of intersection of the two tangents indicates the number of ligand molecules in the complex.

The protonation constants of bleomycin (commercial preparation), terminal polyamines putrescine and spermine were investigated by potentiometric titration procedures. The principle used is that a solution of known concentration of the conjugate acid of the base to be investigated is titrated with a strong base (NaOH) and the reaction followed

by monitoring the pH. A pH-titration curve is drawn up and the protonation constants calculated.

The stability constants of the copper (II) chelates of the above compounds were investigated by potentiometric titrations. The first part of the study is identical to the determination of the protonation constants of these compounds but in addition a second titration is performed on a solution of similar constitution but also containing copper (II) ions in the presence of a copper ion selective electrode (144, 145) and an additional calomel electrode. If the complex-formation occurs mainly in the pH region 3-10 (i.e. the complex is stable) and the concentration of ligand L is not much larger than the concentration of copper (II) ions then a considerable difference between the two pH-titration curves is obtained. If the protonation constants of the ligand are known then it is possible using these curves to calculate the stability constants.

The electron paramagnetic resonance studies were carried out on dry copper (II) bleomycin powder. It is known that copper (II) ions form the strongest chelates with most ligands among all divalent ions, as exhibited by the Irving-Williams series (146). Copper (II) ions successfully compete with other metal ions and protons in the activation of enzymes or bind to essential groups at the active site and cause inhibition. Due to these effects copper is quite poisonous because it affects key metabolic processes.

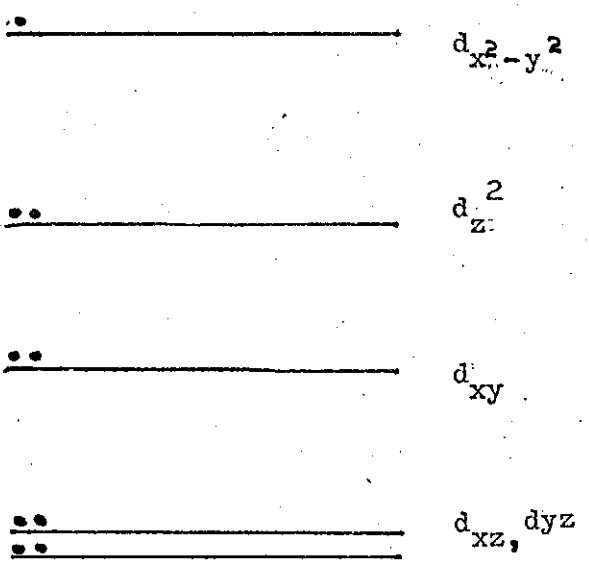
In aqueous solutions under aerobic conditions copper (II) is the most stable ion of copper in the presence of biological ligands. The copper (II) ion often has a co-ordination number of 4, with the ligands arranged at the corners of a square but it is also possible to have two extra ligands which are bound less strongly and having their valence bonds directed perpendicular to the plane of the square. This latter arrangement gives the copper (II) complex a tetragonal complex.

Copper (II) ions have one less electron than can be included by the five d-orbitals and thus it has one unpaired electron in its mononuclear complexes. Such complexes are paramagnetic and are characterised by a spin of $\frac{1}{2}$.

Because this d^9 configuration involves a single electron hole, the electron cloud surrounding the copper (II) ion is not spherically symmetrical and ligands of copper (II) tend to produce irregular, elongated octahedral co-ordination due to ligands near to the half-filled orbital being subject to greater attraction by the positive nucleus than those ligands close to filled orbitals.

Electron paramagnetic resonance studies (147) suggest that the electron hole in copper (II) complexes is found in the $d_{x^2-y^2}$ orbital. The energy-level diagram for the d-orbitals in an irregular octahedral complex of copper (II) is shown in Fig. 29.

Figure 29. Energy level diagram for d-orbitals in a distorted octahedral ligand field. The dots represent electrons and show the ground-state configuration for a d^9 ion e.g. Copper (II)

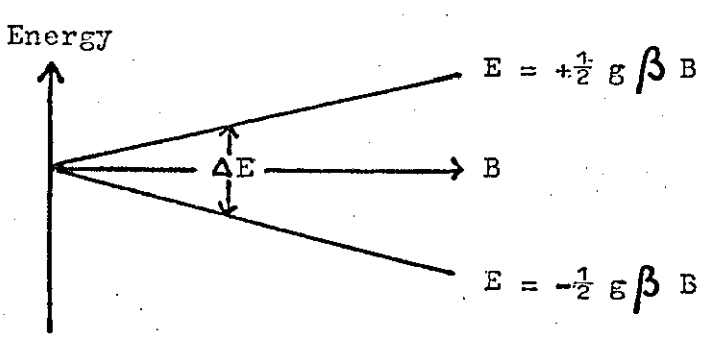


In the spectra of copper (II) complexes, three absorption bands would be expected. These correspond to transitions from the three filled levels to the $d_{x^2-y^2}$ orbital but usually only two bands are seen in the visible spectra because the absorption bands are broad and tend to overlap or because one band is formed in the near infra-red. In complexes of lower symmetry as many as four d - d bands may be present.

Electron paramagnetic resonance (e.p.r.) is used to study compounds containing unpaired electrons, for copper complexes it is restricted to complexes of copper (II) and high spin compounds of copper (III). Electron paramagnetic resonance spectroscopy is based on the spin properties of the electron and for a single electron this is designated by the spin quantum number, S, which has the value of $\frac{1}{2}$. The spin gives the electron a magnetic moment which in an external field can have two different directions; the components along the vector representing the applied field are $+\frac{1}{2}\ g\beta$ and $-\frac{1}{2}\ g\beta$ where g is a dimensionless quantity and β is the Bohr magnetron, a quantity containing the charge and mass of the electron and Planck's constant.

The energy of the magnetic dipole produced by the spinning electron equals the negative of the product of the strength of the applied magnetic field, B, and the component of the electron magnetic moment along the field. For a single electron in an external field one obtains the following energy scheme.

Figure 30. Energy diagram for an electron in an external magnetic field B



The energy difference ΔE between the two levels is $g \beta B$. When electromagnetic radiation of energy $h\nu$, equals ΔH, absorption of radiation takes place.

$$h\nu = g \beta B$$

Consideration must also be made of the nuclear spin; this is characterised by the quantum number I and the corresponding magnetic moment has (2I+1) values of its components along the field axis in an applied magnetic field. A single absorption line can be split into (2I + 1) lines of equal intensity (hyperfine structure) and the distance between the lines designated by a hyperfine splitting constant A (cm^{-1}). The two natural isotopes of copper (^{63}Copper and ^{65}Copper) have $I = 3/2$ and four hyperfine lines would be expected in the spectra of copper (II) complexes.

Copper (II) complexes are not spherically symmetrical and tend to have the ligands arranged in the form of an irregular octahedron. Absorption properties depend on the molecular orientation relative to the applied magnetic field and because the symmetry is tetragonal, two g values designated g_{11} and $g_{\perp}$ are required to characterise the spectra.

The hyperfine splitting $A_{\perp}$ which arises from the nuclear magnetic moment of copper (II) at $g_{\perp}$ is usually very small and often shows no splitting. A_{11} the nuclear hyperfine splitting at g_{11} is usually quite large and has typical values of 10-25 mK. The magnitude of A_{11} and g_{11} depend on several parameters, one of which is the nature of the ligands of copper (II) and these values can be used to assign structure (148).

METHODS AND MATERIALS

Apparatus

The potentiometeric titration apparatus used for the determination of protonation constants and metal-chelate formation constants involved a pH meter, a titration cell, a ten-cm^3 microburette and a constant temperature bath (25°C). The pH meter employed was a Corning-Eel digital 110 expanded scale model (Evans Electroselenium, Halstead, Essex) and the electrodes used were an E.I.L. all purpose pH and an E.I.L. saturated calomel reference electrode. The metal-chelate formation constant studies utilised a Radiometer PHM 64 Research pH meter operating on the 1500 mV scale as the ion-selective meter. The copper (II) sensitive electrode was a Radiometer Selectrode F 300 type and the reference electrode was an E.I.L. saturated calomel type.

The ultra-violet-visible spectra of the copper (II) and cobalt(II) chelates of the bleomycins were obtained on a Perkin Elmer 402 ultra-violet spectrophotometer which had been calibrated for wavelength by means of a Holmium filter. The fixed wavelength studies were carried out on a Unicam SP 600 which had been calibrated for wavelength by means of Didymium filter using wavelengths 573 mm and 588 mm. 1 cm^3 path length matched glass cells were used for the visible absorption spectroscopy.

Reagents

The chemicals used in potentiometric titration studies were as given below.

A solution of perchloric acid (approx. 0.005 M) containing sodium perchlorate (0.21M). The hydrogen-ion concentration was determined by titration with standard sodium hydroxide (0.1M) using phenolphthalein as indicator.

Carbonate-free sodium hydroxide was prepared according to the method of Kolthoff and Sandell (149). This solution (approx. 0.1M) was standardised by titration against potassium hydrogen phthalate using phenolphthalein as indicator.

A standard copper (II) perchlorate solution (approx. 0.01M) was prepared by dissolving the required weight of copper (II) perchlorate hexahydrate (B.D.H. Chemicals Ltd., Poole, Dorset) in water. The solution was standardised by titration against (i) EDTA using pyrocatechol violet as indicator (150) and (ii) standard sodium thiosulphate.

Potentiometric Determination of Protonation Constants

The potentiometric titrations were carried out in a 400 cm^3 pyrex glass jacketed titration cell with water at 25.0 $\pm$ 0.1°C circulating through the outer jacket. A perspex cover was placed on the cell to minimise evaporation. Holes in the cover allowed the introduction of electrodes and burettes into the solution.

The general procedure was as follows: the pH meter was standardised immediately before use with B.D.H. standard buffer solutions of pH 4.005 $\pm$ 0.005 and pH 6.986 $\pm$ 0.01

at 25°C. A weighed amount of the compound under study was placed in the clean, dry titration cell. A plastic covered bar magnet was placed in the cell. 100 cm^3 of 0.005M perchloric acid (0.21M in sodium perchlorate) was placed in the cell followed by 5.00 cm^3 of water. The cover was then placed in position. The two electrodes were then positioned in the solution and the solution gently stirred. The tip of the burette containing the sodium hydroxide solution was positioned in the cell so that it was below the surface of the solution. The burette was gravity filled from a 4 dm^3 polyethylene bottle. The contents of the burette and the bottle were protected from atmospheric carbon dioxide by guard tubes filled with soda-lime asbestos. The contents of the cell were allowed to reach 25°C and the titration carried out by small increments of sodium hydroxide. The pH meter reading of the solution was noted after the solution had reached equilibrium.

The protonation constant log K_H^* is defined by

$$H^+ + L \rightleftharpoons HL^+$$

$$K_1 = \frac{[HL]}{[H^*][L]}$$

$$H^+ + H_{n-1}L \rightleftharpoons H_nL$$

$$K_n = \frac{[H_nL]}{[H^+][H_{n-1}L]}$$

\+ For convenience charges are omitted from species other than the hydrogen ion.

* All constants determined in this work are equilibrium concentration constants.

In the titration of a ligand containing nH^+ dissociable hydrogen ions, the number of protons bound to the ligand $\bar{n}$ is

$$\bar{n} = \frac{(n - a)\ C_{HnL} + [OH^-] + [H^+]}{C_{HnL}}$$

where a = degree of neutralisation.

Potentiometric Determination of Copper (II) Chelate Formation Constant

The chelate formation constants of copper (II) ions with the polyamines, spermine putrescine and bleomycin were investigated. The procedure was similar to that used in the potentiometric determination of these compounds, except that the 5.00 cm^3 of water was replaced by 5.00 cm^3 of a 0.01M copper (II) ion solution. The ion-selective copper (II) electrode was calibrated by immersing in standard copper (II) solutions of concentrations of 10^{-2} - 10^{-5}M. The titrations were carried out at a molar ratio of ligand to metal of approximately 7:1.

The overall metal-chelate formation constant β_{qpn} for species is defined by

$$\beta_{qpn} = \frac{[M_q H_p L_n]}{[M]^q [H]^p [L]^n}$$

The stepwise formation constants (151) are defined by :

$$K_n = \frac{[ML_n]}{[ML_{n-1}][L]}$$

The average ligand number $\bar{n}$ is determined by $\bar{n} = \frac{[L \text{ bound}]}{C_M} = \frac{(a - a_o)}{C_M} C_{HL}$

where a_o is the degree of neutralisation of an acid to a given pH and

a is the degree of neutralisation of an acid in the presence of the metal ion.

Method of Continuous Variation

The visible spectra of copper (II) bleomycin is shown in Fig. 31. From an examination of this spectra it was decided to investigate the copper (II) bleomycin chelate in the region 560 - 610 nm since neither bleomycin or copper (II) nitrate absorbed in this range at the concentrations used. A series of solutions containing various volumes of 2×10^{-3}M bleomycin and 2×10^{-3}M copper (II) nitrate were prepared in 0.01M acetate buffer pH 4.5, the final volume of each solution being 3.0 cm^3. The absorbances of the solutions at 560 nm 590 nm and 610 nm were recorded using a solution of 0.01M acetate buffer as the blank solution. A graph of absorbance at 590 nm against the mole-fraction of bleomycin was plotted Fig. 32.

Method of Mole-Ratio

A series of solutions of varying bleomycin : copper (II) ratios from 0:1 to 2.5 : 1.0 were prepared in acetate buffer pH 4.5. All the solutions were made to 10.0 cm^3.

The absorbances were recorded at 590 nm using 0.01mM acetate buffer pH 4.5 as the blank solution. A graph was plotted of absorbance at 590 nm against the mole ratio of bleomycin : copper (II) Fig. 33.

The cobalt chelate of bleomycin was also studied. The visible spectra of cobalt bleomycin was recorded Fig. 34 and a series of solutions of bleomycin : cobalt (II) chloride of varying ratios from 0:1 to 2.5 : 1.0 were prepared. The absorbances were recorded at 580 nm using 0.01mM acetate buffer pH 4.5 as the blank solution. A graph was plotted of absorbance at 580 nm against the ratio of bleomycin : cobalt (II) Fig. 35.

Electron Paramagnetic Resonance studies

Copper (II) bleomycin was prepared in aqueous solution by mixing copper (II) nitrate to bleomycin at molar ratio of 1 : 1.2 (the latter quality was used due to the possible error in weighing out bleomycin) in the minimum volume of distilled water. The solution was placed in a dessicator containing silica gel. The dessicator was evacuated and left until the blue copper (II) bleomycin remained as a dry solid. This solid was ground with an agate pestle and mortar and the resulting powder sent to the Chemistry Dept., University of Leicester where its e.p.r. spectra was determined Fig. 36 and assignments made.

Figure 31. Visible Spectra of Copper (II) Bleomycin

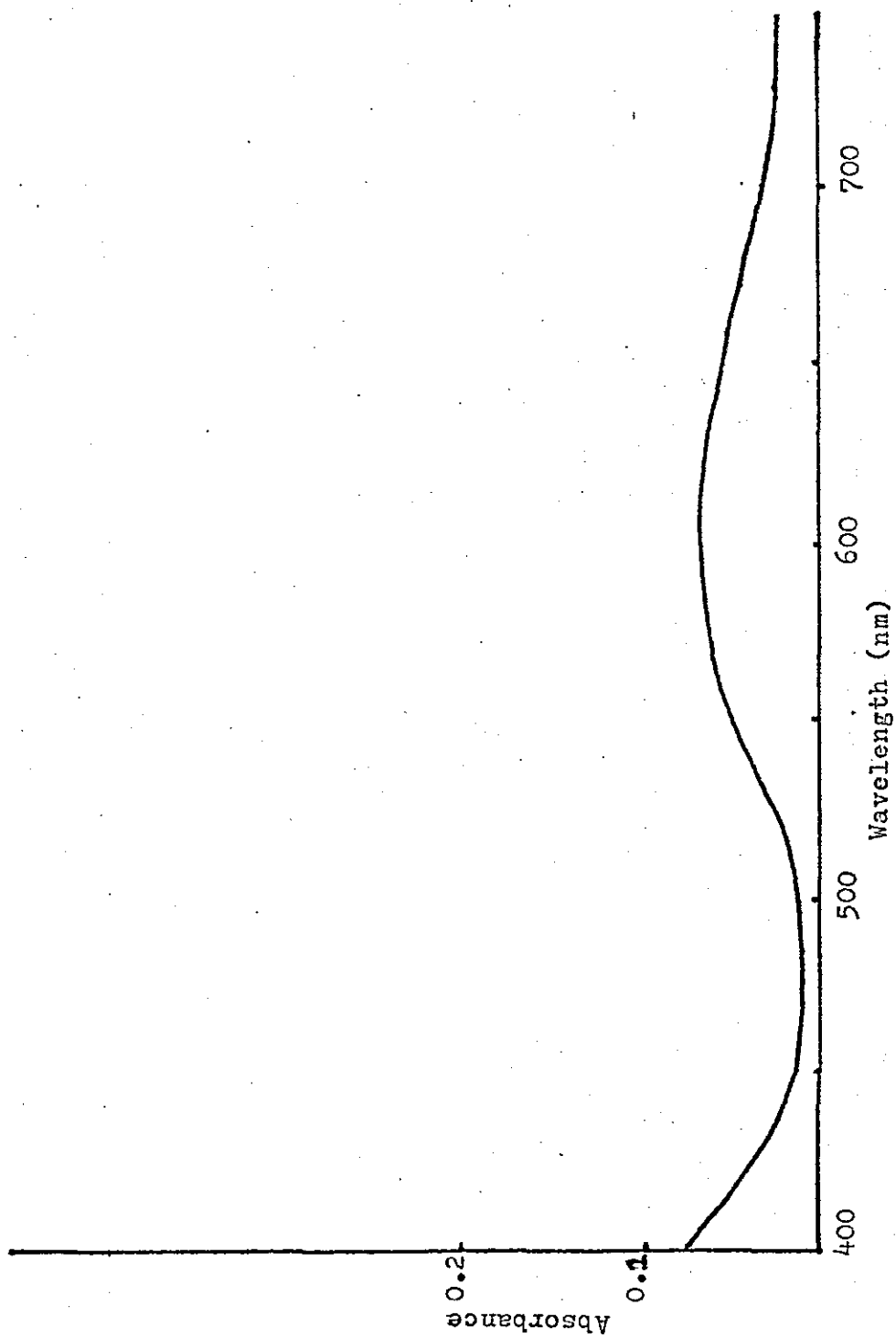


Figure 32. Continuous Variation Plot for Copper (II) - Bleomycin Complex ($C = C_{Cu} + C_{BLM} = 0.002M$ at pH 4.5)

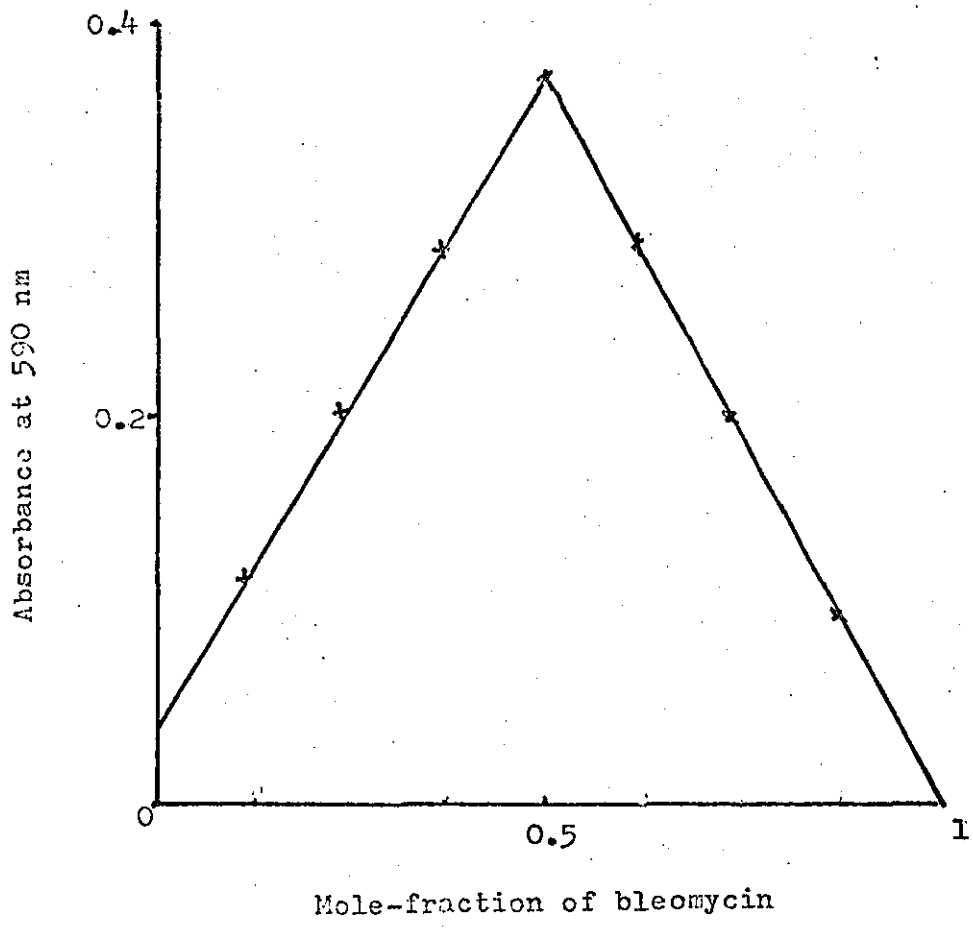


Figure 33. Mole-Ratio Plot for the Copper (II)-Bleomycin Complex

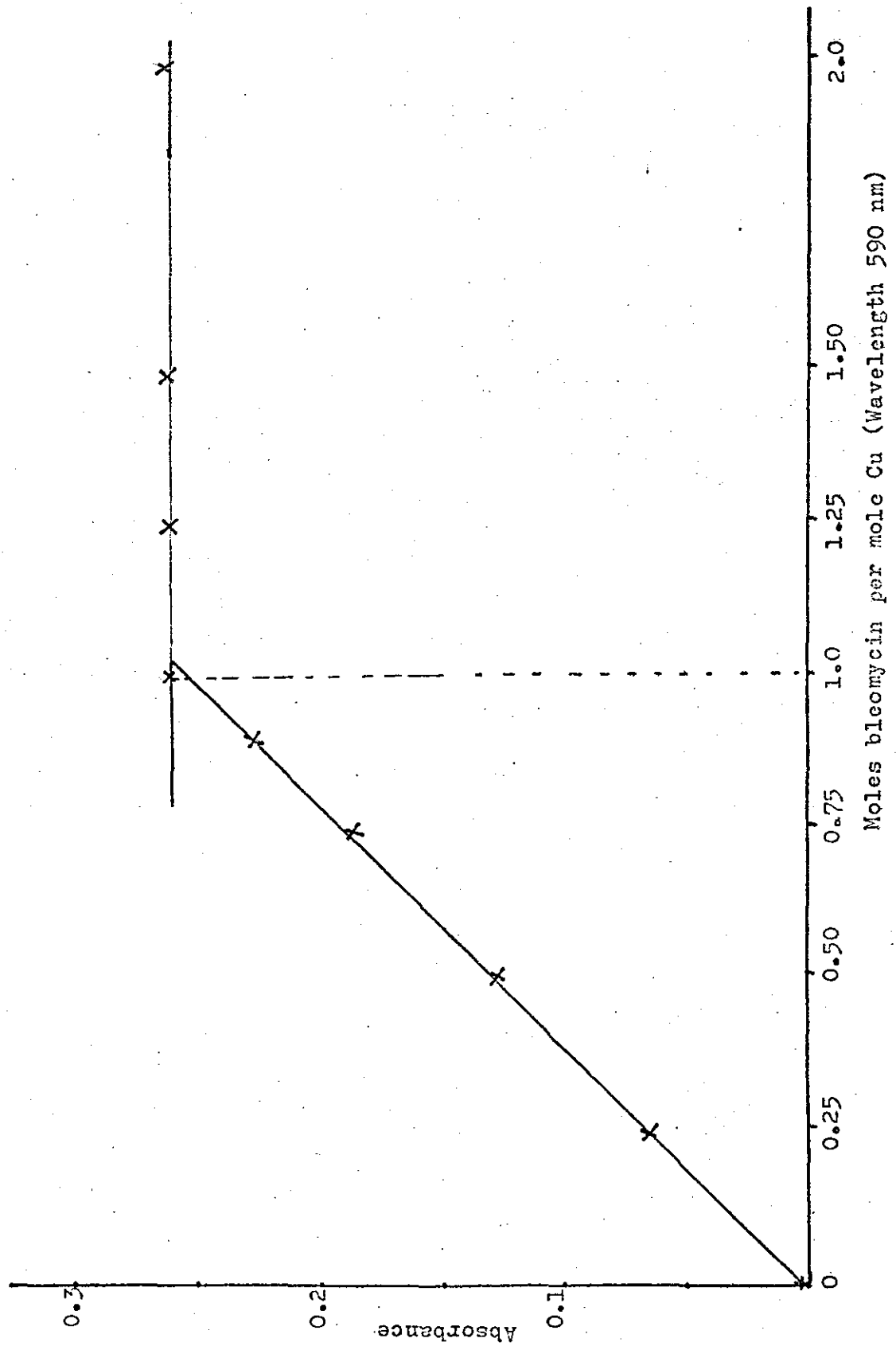


Figure 34. Visible Spectra of Cobalt Bleomycin

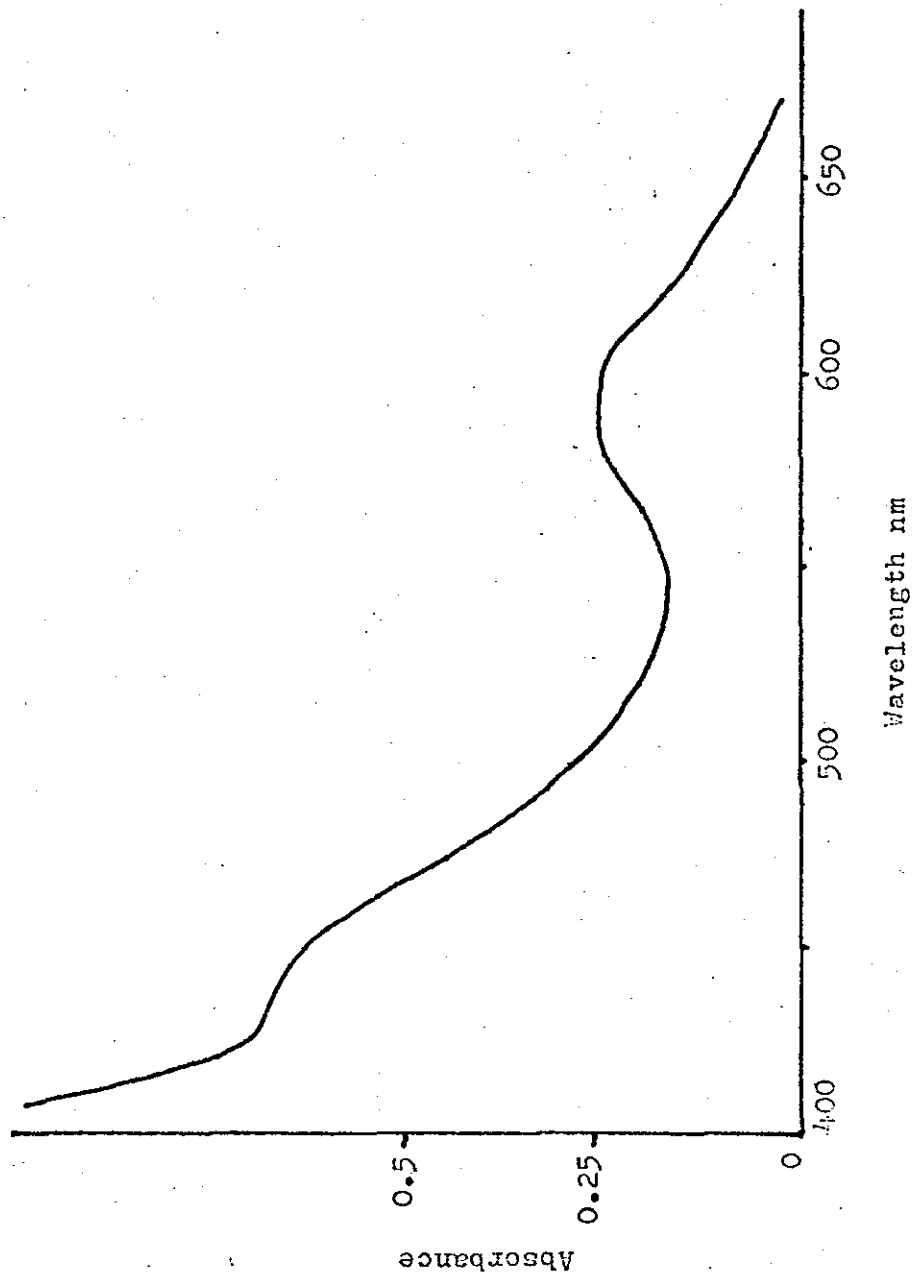


Figure 35. Mole-Ratio Plot for Cobalt-Bleomycin Complex

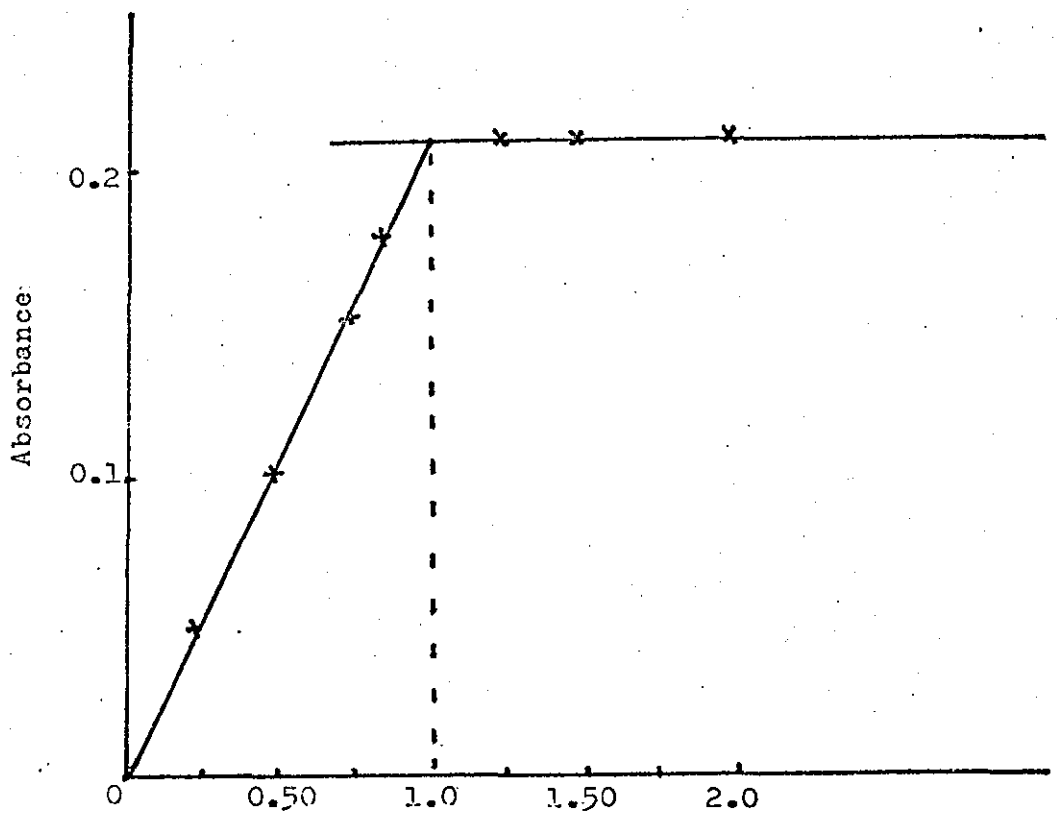
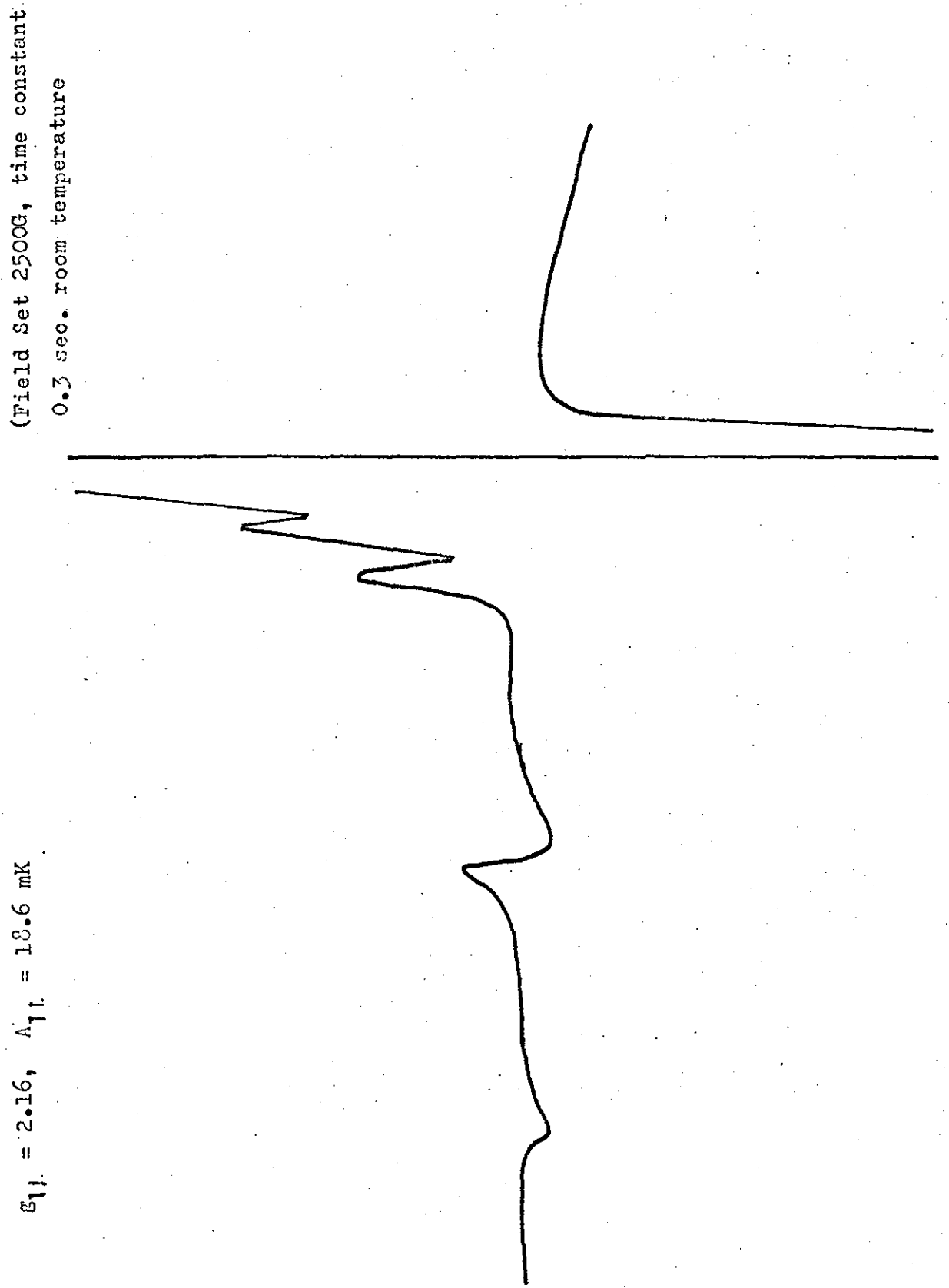


Figure 36. E.p.r. Spectrum of Copper (II) Bleomycin



RESULTS

The method of continuous variation suggested that the copper (II) bleomycin chelate exists as a 1:1 complex. The intersection of the two straight lines is approximately 0.5; the reason why it is not exactly 0.5 is probably due to errors in weighing out the bleomycin by assuming the molecular weight to be 1,484 (pure bleomycin A_2). The sharpness of the two straight lines indicate that the complex is quite stable and when the ratio of copper (II) to bleomycin is 1:1 the plot indicates that all the copper ions present are in the form of the complex.

The mole-ratio studies indicated that both the copper (II) and cobalt bleomycins exist as 1:1 complexes. In addition, the straight-line nature of the two graphs indcate that both complexes are quite stable.

The protonation constants determined for putrescine (1,4-diaminobutane) and spermine (4,9-diazo dodecane-1, 12-diamine) are shown in Table 9. Because the original values determined for the logarithm of the consecutive protonation constants were less than 3.0, a correction was necessary to determine accurately the values of the constants, the relaxation method of Watkins and Jones (152) was used. The $\bar{n}$ - pH plots are shown in Figs. 40 and 41. The titration curve for the bleomycin sample is shown in Fig. 39, no marked inflections are apparent and this coupled with the presence of several different ionisable groups made it not possible to determine the protonation constants.

Figure 37. Titration curves of Spermine (I=0.1, 25 C)

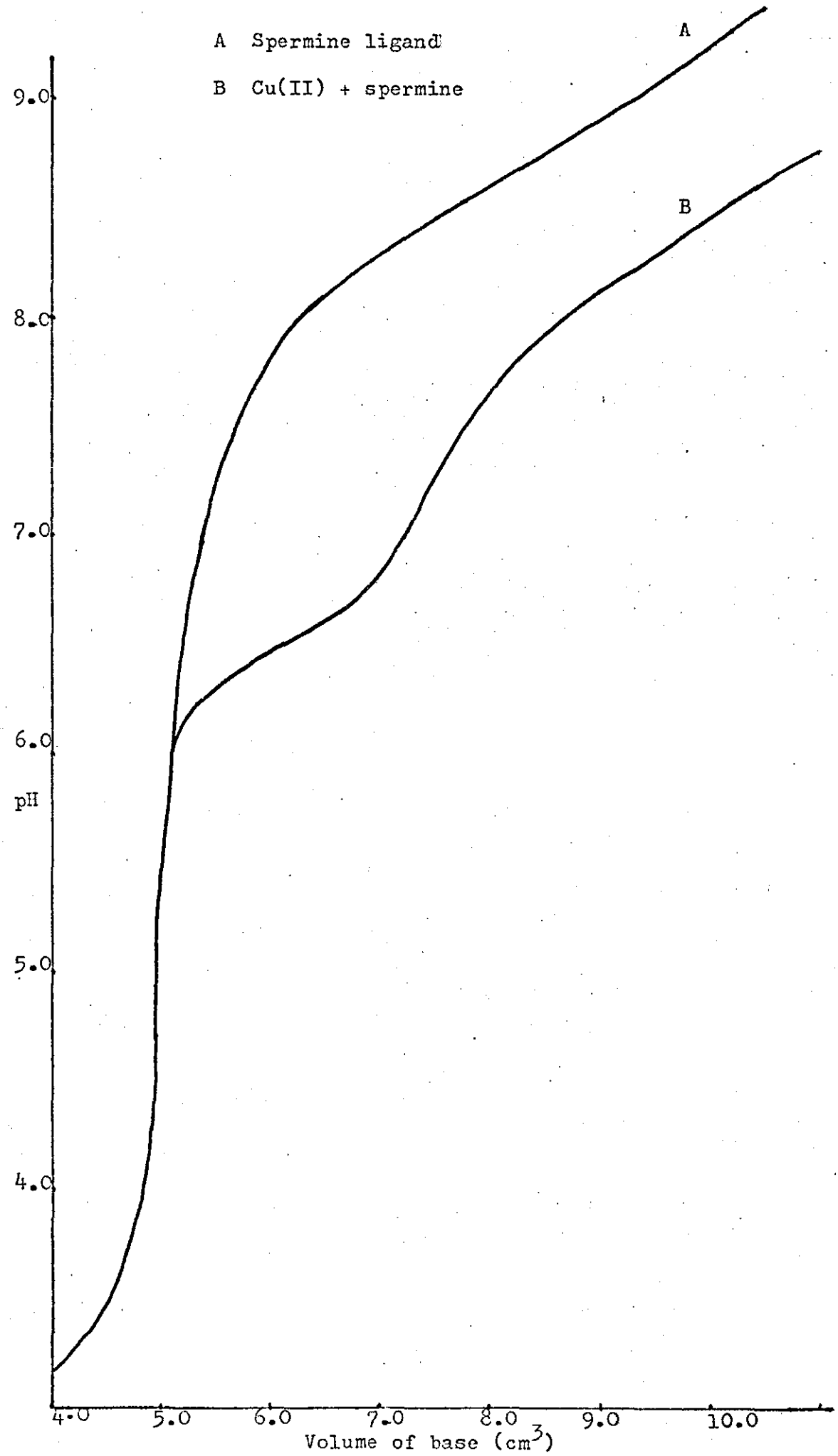


Figure 38. Titration Curves of Putrescine (I=0.1, 25°C

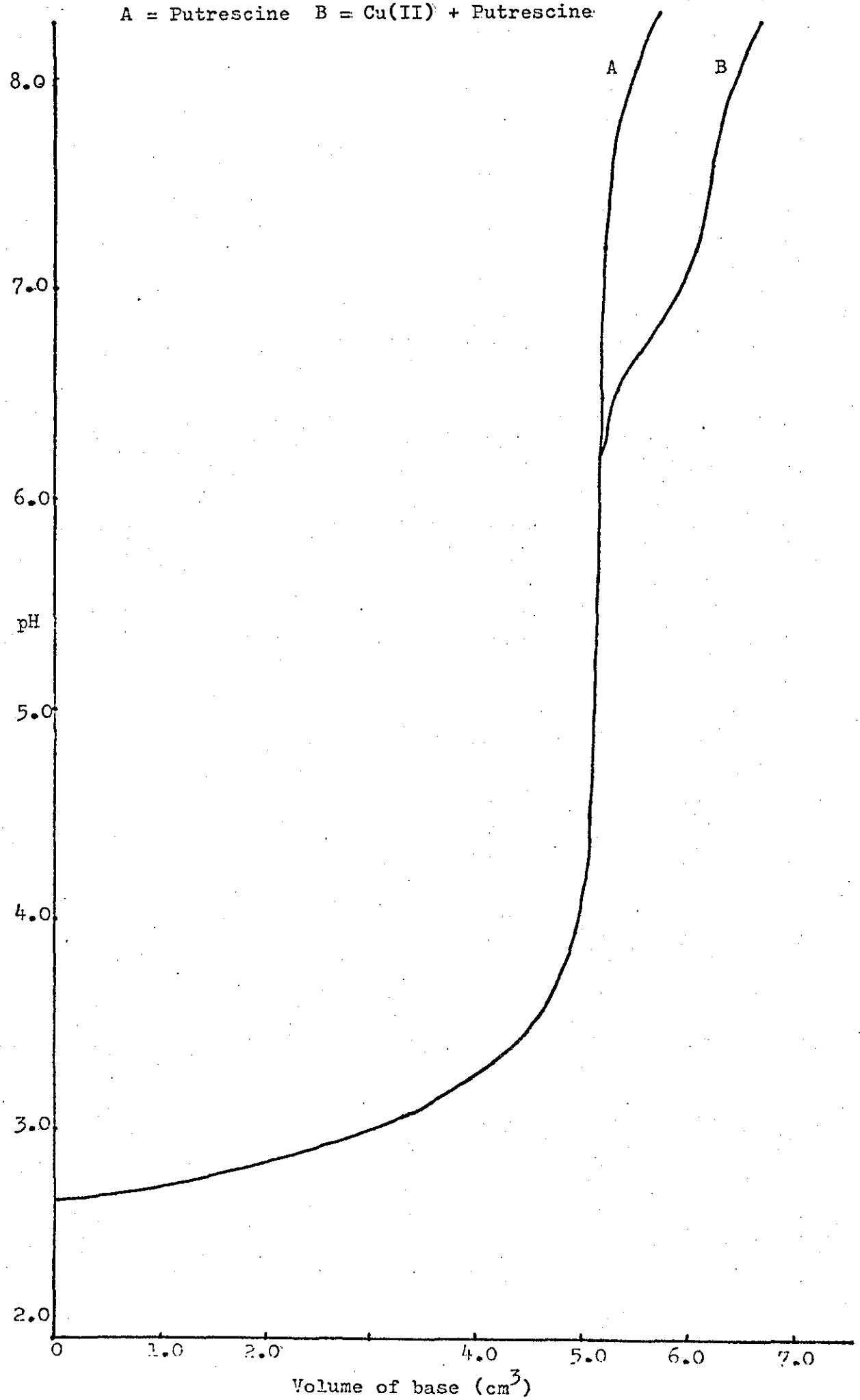


Figure 39. Titration curve of bleomycin in water, 25°C, I = 0.1

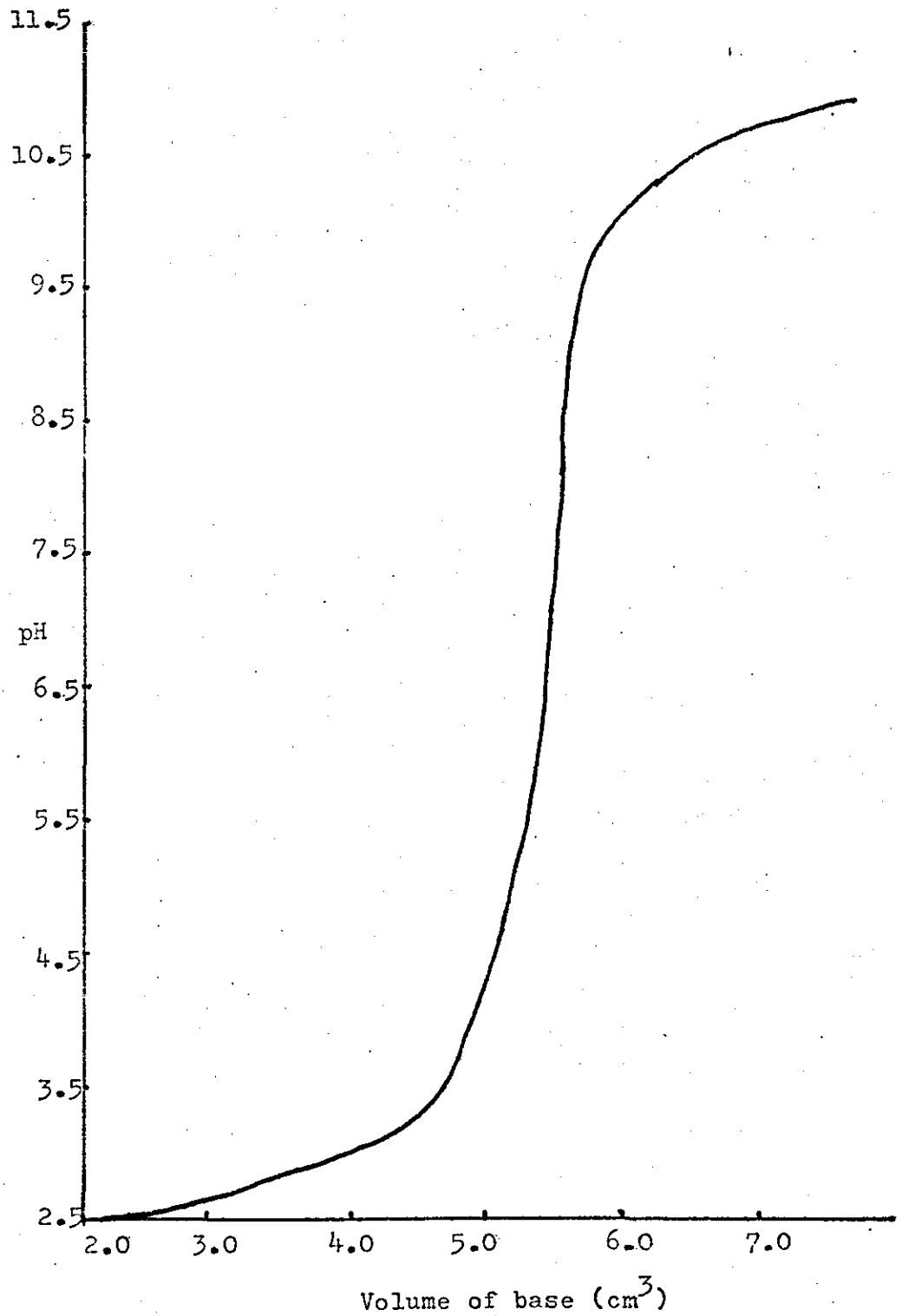


Figure 40. Formation curve of the Protonation of Putrescine

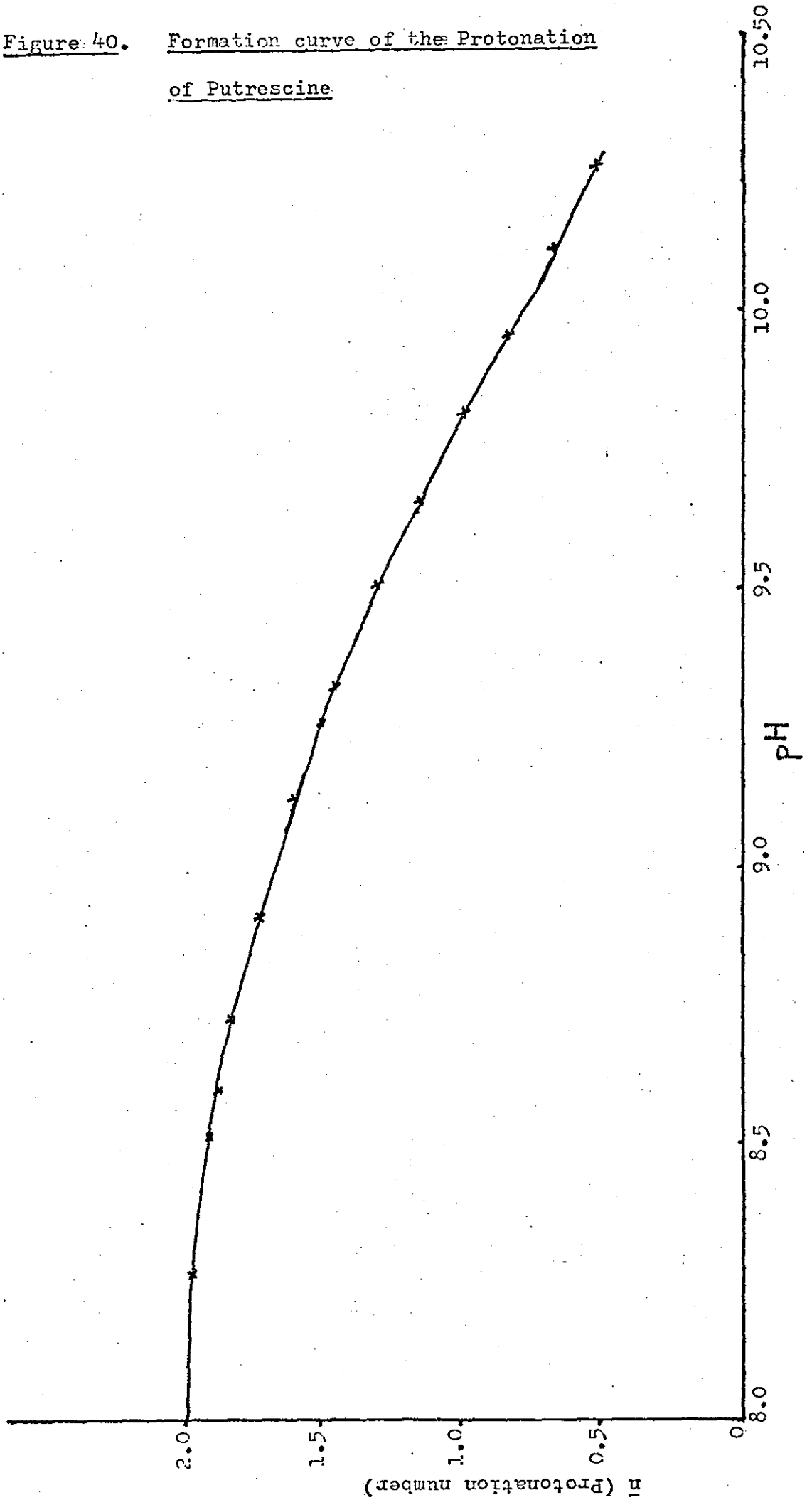


Figure 41. Formation Curve for the Protonation of Spermine

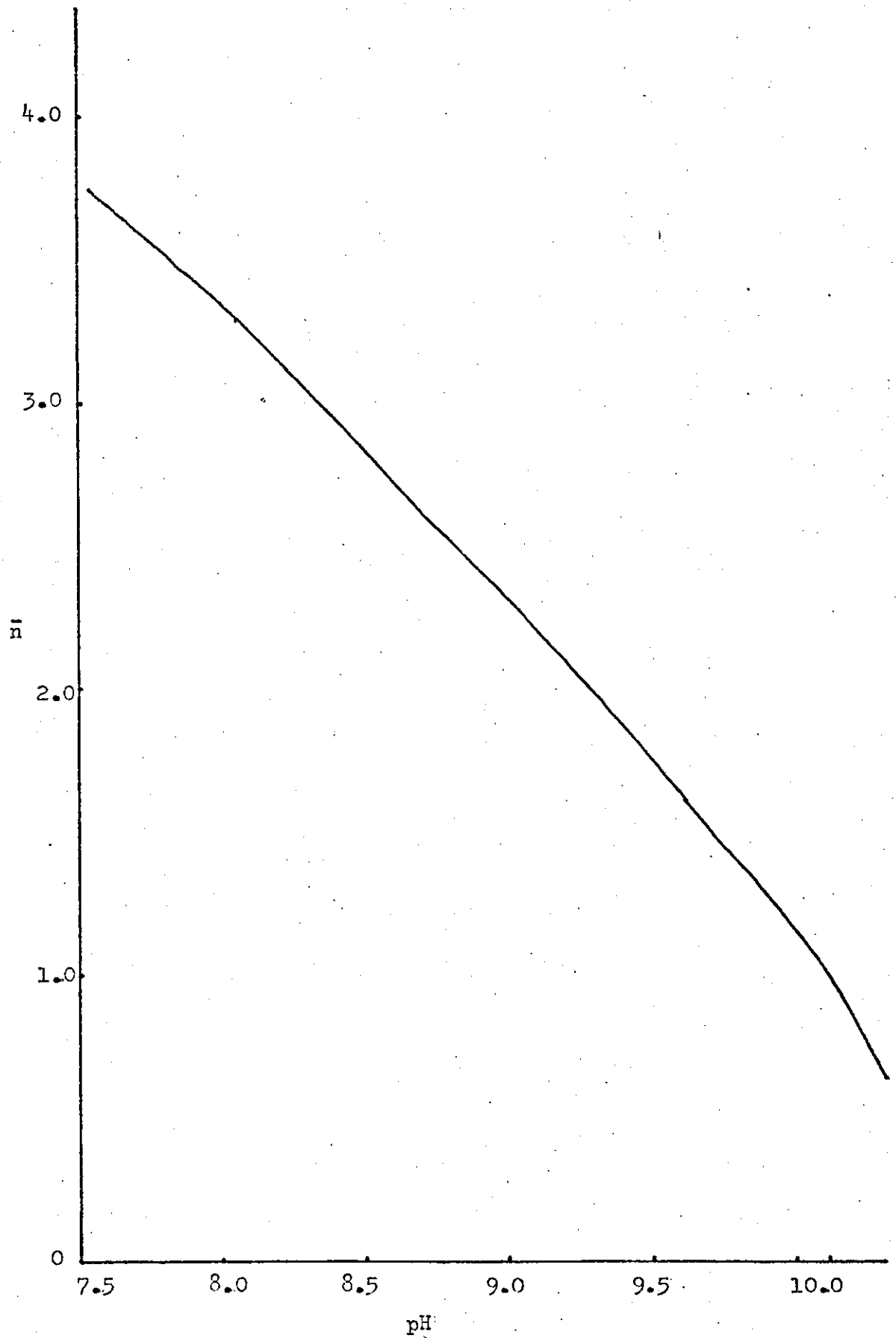


Table 9. Protonation Constants of Putrescine and Spermine

Temp. 25°C, ionic strength 0.1

Polyamine	log K	(± 0.1) Refined	Literature Values (158, 159)
Putrescine	10.3	10.5	10.72
(1,4-diaminobutane)	9.25	9.25	9.24
Spermine	10.3	10.5	10.80
(4,9,diazododecane-1,12-diamine)	9.7	9.9	10.02
	8.8	8.8	8.85
	7.8	7.8	7.96

Table 10. Chelate Formation Constants of Copper (II) complexes of Putrescine and Spermine

Polyamine	Stability Constant		Literature Values (159)
Putrescine (1,4-diaminobutane)	7.4	1,0,1	-
Spermine (4,9-diazo-dodecane-1,12-diamine)	14.5	1,0,1	14.7
	9.80		9.99

The chelate formation constants for the copper (II) complexes of the polyamines putrescine and spermine were determined and are shown in Table 10. In the potentiometric titration of bleomycin in the presence of copper (II) ions, the addition of copper (II) ions resulted in the immediate formation of a bright blue solution, no precipitate

was formed. The copper (II) ion-selective electrode indicated that at a pH less than 3, the copper ions were removed from solution and presumably were chelated by the bleomycin present. This ability of bleomycin to chelate copper (II) ions at such an acid pH indicates that the bleomycin molecule has considerable affinity for copper (II) ions and that the complex formed is quite stable.

The e.p.r. spectra of copper (II) bleomycin powder were run at room temperature. A_{11} was determined to be 18.6 mK, while g_{11} was calculated to be 2.16. No superfine structure was observed from ^{14}N but the marked asymmetry and broadening of $\|$ features could be due to unresolved ^{14}N hyperfine. Only two $\|$ features could be detected in the spectra and these were considered to be the $+ \frac{3}{2}$ and $+ \frac{1}{2}$ features. No features to the low field of these features were observed in the spectra and it was considered that the $- \frac{1}{2}$ and $- \frac{3}{2}$ features were hidden among the perpendicular features.

The data presented was accurate to the first order i.e. limits of the high field approximation, all data used in references (148, 153) were also of first order and examined the copper (II) e.p.r. of proteins and artificial polypeptides.

DISCUSSION

Both the continuous variation and mole-ratio methods indicated that copper (II) bleomycin exists as a 1:1 complexes, in addition the mole-ratio method suggested that cobalt bleomycin is also a 1:1 complex. These experiments also showed that both metals are strongly chelated by bleomycin. These results agree with the observations of Nunn (154), Nunn has also considered that the exceptional stability of cobalt bleomycin arises from the oxidation of cobalt (II) to cobalt (III) while the ion is chelated to bleomycin. Copper (II) bleomycin shows an absorption band in the visible spectrum in the region 585 - 610 nm. The molar extinction coefficient at 590 nm was calculated to be 370 M^{-1} cm^{-1}. Bleomycins are unique glycopeptide molecules but it is possible to make an approximate comparison with polypeptides and proteins e.g. ribonuclease. The visible absorption spectra of copper (II) bleomycin is similar to that obtained when copper (II) is chelated to ribonuclease. The absorption spectra is not intense in the region 600 - 625 nm as that exhibited by the "blue" ("type I") copper electron transfer proteins e.g. ceruloplasmin, ascorbate oxidase. These proteins have an molar extinction coefficient of 1000 - 5000 M^{-1} cm^{-1} at 600 - 626 (155) and it is considered that these proteins have an unusual co-ordination system with copper (II). It is considered that the binding sites consist of 3N and 1S of which two of the nitrogen binding

sites are imidazoles. Breslow and Girotti (156) investigated the binding of copper (II) ions to ribonuclease by comparing the visible spectra obtained with model systems of copper (II) low molecular weight chelates with that of ribonuclease. Copper (II) chelates with a maximum wavelength of absorption in the visible region of 590 - 600 nm with molar extinction coefficient of approximately 300 M^{-1} cm^{-1} were considered to be either bound to a ligand system consisting of one imidazole and two peptide nitrogen bonds or to a system of four NH_3 groups.

The e.p.r. spectra of copper (II) bleomycin was compared with values obtained for first order spectra of natural polypeptides and artificial polypeptides (148). The A_{11}, g_{11} values suggest that the ligands binding the copper (II) ion to bleomycin are either two nitrogen and two sulphur containing groups or four nitrogen containing groups.

Possible sulphur ligands in bleomycin could be the two sulphur atoms in the bithiazole ring system. It seems that when bleomycin is chelated to copper (II) ions it does not attack DNA. It has been suggested that the bithiazole ring system is implicated in the binding of bleomycin to DNA(157) and hence if this part of bleomycin is chelated to copper (II) it may not be able to bind to DNA.

The α-amino group of the β-amino-alanine moiety and the 3-O-carbamoyl group have been implicated in copper (II) chelation but there appears to be no evidence for the

involvement of the histidine (imidazole) component of bleomycin. The pKa' values of bleomycins are 4-aminopyrimidine (pKa' 2.9), imidazole (pKa' 4.7) and β aminoalanine (pKa' 7.3). The potentiometric titration study of copper (II) bleomycin indicated that considerable chelation occured even at pH 2.7. This suggests that the above 3 nitrogen containing groups and the 3-O-carbamoyl group could be involved in the chelation of copper (II) ions. It must be realised that several of the terminal amine groups e.g. guanidine could also be involved in chelation but as yet there is no evidence of such an involvement.

The protonation constants of putrescine (1,4-diamino butane) and spermine (4,9-diazadodecane-1,12-diamine) agree with literature values (158, 159); in addition the copper (II) stability constants calculated for spermine agree with the literature values (159). There appears to be no previous calculation of copper (II) stability constants of putrescine in the literature, this may be due to problems of precipitation of hydrolysis products during the titration. This was overcome by working at a putrescine:copper (II) ratio of 9:1.

SUMMARY

The reaction of fluorescamine with the bleomycins gave rise to fluorescent products which exhibited typical characteristics of fluorescamine derivatives of primary amines. Fluorescamine was also used as a spray reagent to locate separated bleomycins on t.l.c. plates and bleomycin-fluorescamine derivatives were separated on t.l.c. plates. The gel electrophoresis of bleomycin and bleomycin-fluorescamine was studied and it was shown that S.D.S. gel electrophoresis could resolve commercial bleomycin mixtures and fluorescamine labelled bleomycins into two fractions.

The silica gel h.p.l.c. procedure separated commercial bleomycin preparation into its major components, this method being rapid and sensitive. The reverse phase h.p.l.c. procedure also proved a rapid procedure for separating the major bleomycins of the commercial bleomycin product. Attempts were made to separate and estimate the bleomycins in biological fluids using cellulose phosphate as a purification step prior to silica gel h.p.l.c. This procedure proved to be more difficult than expected and interference from several biological compounds was obtained, of which a major component was found to be creatinine. The silica gel h.p.l.c. procedure should prove suitable for quality control studies.

The effects of bleomycin and copper (II) bleomycin on rat lung transglutaminase and on fibrin cross linking by factor XIII were investigated. Both bleomycin and copper (II) bleomycin proved to be competitive inhibitors of [1,4-^{14}C] - putrescine incorporation into N,N-dimethyl-casein when catalysed by the "soluble" rat lung trans-glutaminase. Only copper (II) bleomycin was found to be an effective competitive inhibitor of [1,4-^{14}C]-putrescine when studying particle "bound" rat lung transglutaminase. Both bleomycin and copper (II) bleomycin were shown to be inhibitors of α-monomer polymerisation of fibrin cross-linking by Factor XIII.

The metal-bleomycin studies showed that both copper (II)-bleomycin and cobalt-bleomycin to exist as 1:1 chelates. Spectrospcopic studies suggest that copper (II) may be bound to four nitrogen containing ligands or two nitrogen and two sulphur containing ligands when complexed with bleomycin.

FUTURE WORK

At present studies are being carried out on the mass-spectroscopy of bleomycin B_2 and its tetra-methyl silyl derivative(s). It is hoped to follow this study with a mass-spectroscopy investigation of copper (II) bleomycin, it may be possible with these investigations to obtain additional information on the groups which chelate to copper.

Although bleomycin is considered to have its major influence on cell metabolism by binding to DNA and causing chain breakage of DNA, it may have other effects. Polyamines are known to play important roles in protein synthesis e.g. activation of amino-acyl t-RNA formation (161) and induction of the association of ribosomal sub-units (162). The conversion of polyamines into their respective aldehydes is catalysed by copper (II) - containing enzymes - diamine oxidase (histaminase) and serum amine oxidase. The former enzyme occurs in high concentrations in kidney tissue (163) small cell lung carcinoma (164) and medullary thyroid carcinoma (165). Both enzymes are readily inhibited by copper (II) chelating agents and it is intended to investigate the effect of bleomycin on the activities of these enzymes.

The development of an h.p.l.c. system to assay bleomycin in biological fluids seem to require the use of a fluorimetric detector. A fluorimetric h.p.l.c. system has

recently been devised to assay gentamicin levels in serum (166) using o-phthaldehyde as the fluorescent label; a pre-h.p.l.c. purification step involving silicic acid was used. It is intended to investigate such a system for bleomycin using fluorescamine as the fluorescent label if a flow-through micro-fluorimetric cell can be obtained.

The discovery (167) that DNA chain breakage and release of free bases by bleomycin were enhanced by iron (II) and iron (III) ions raised the possibility that free radicals are involved in bleomycin action. Sausville et al. (168) observed that molecular oxygen was required while Ishida and Takahashi (167) proposed that O_2^- was one of the chemical mediators responsible for the enhancement of the DNA chain breakage by bleomycin.

Udenfriend et al. (169) have proposed a system for hydroxylation involving iron (II) ions, EDTA, ascorbic acid and molecular oxygen. The molecular oxygen can be replaced by hydrogen peroxide and ascorbic acid can be replaced by other electron donors (169); the system retains good hydroxylating activity even when iron-EDTA is replaced by copper (I) ions. There is still argument as to the nature of the hydroxylating agent of the Udenfriend system; Staudinger et al. (170) have suggested that $HO_2^{\bullet}$ radicals are involved while other workers (171) have proposed that $^-O\text{-}O^{\bullet}$ is the hydroxylating agent. It is possible that a similar system to that of Udenfriend may operate when bleomycin attacks DNA, bleomycin replacing EDTA in the system.

Lown and Sim have recently demonstrated (172) that free radicals are involved when bleomycin attacks DNA and it is proposed to investigate further such an effect on model systems, in particular to observe its effects on tritiated DNA and on lipid peroxidation.

In addition it is intended to investigate the stoichiometry of the copper (II), bleomycin, ascorbic acid system using an oxygen electrode.

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