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THE EFFECTS OF BLEOMYCIN AND COPPER BLEOMYCIN UPON TRANSGLUTAMINASE ENZYMES

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Abstract—The effects of bleomycin and copper (Cu) bleomycin upon rat lung transglutaminase together with their effects upon fibrin crosslinking by factor 13 have been investigated. Bleomycin and Cu bleomycin proved to be effective competitive inhibitors of [1,4-^{14}C]-putrescine incorporation into *N,N*-dimethylcasein when catalysed by 'soluble' rat lung transglutaminase. The enzyme inhibitor constants (K_i) for bleomycin and Cu bleomycin were calculated as 3.8×10^{-4} M and 1.4×10^{-4} M respectively; these values were found to be in close agreement with the Michaelis constant (K_m) of the 'soluble' enzyme for [1,4-^{14}C]-putrescine, calculated as 2.0×10^{-4} M.

Studies using particle 'bound' rat lung transglutaminase revealed only Cu bleomycin to be an effective competitive inhibitor of [1,4-^{14}C]-putrescine. An approximate K_i of 8.9×10^{-4} M was calculated for Cu bleomycin and a K_m of 4.1×10^{-4} M for [1,4-^{14}C]-putrescine. Subcellular localisation of rat lung transglutaminase revealed the particle 'bound' enzyme to be predominantly associated with a fraction containing nuclei, membranes and cell debris.

The effects of bleomycin and Cu bleomycin upon fibrin crosslinking by factor 13 illustrated both compounds to potent inhibitors of α-monomer polymerisation when compared with the standard inhibitor monodansylcadaverine.

The enzyme transglutaminase [EC 2.3.2.–] catalyses an acyl transfer reaction between the γ-carboxamide groups of protein bound glutamine and various primary amine groups yielding new γ-amide bonds of glutamic acid and ammonia [1].

Covalent crosslinks involving the ϵ-amino group of lysine and glutamine ϵ-(γ-glutamyl)lysine stablize structural proteins of many tissues including fibrin stabilisation during wound healing, wool follicles and epidermal proteins during the final stages of keratinization [2–5]. Although transglutaminases specific for these tissues have been identified the function of a group of transglutaminases widely distributed in mammalian tissue is still poorly understood, although certain physiological functions have been postulated [1, 6–8].

Evidence to suggest that transglutaminase enzymes are involved in the proliferation of tumour tissue has been reported in a number of papers [9–12]. Yancey and Laki [9–11] established that transglutaminases were necessary for stabilisation of the fibrin network required by solid tumours during proliferative growth and demonstrated that polyamines and the substituted primary amine *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalene sulphonamide (monodansylcadaverine) possessed inhibitory activity against this type of growth. Other reports have connected transglutaminase activity with abnormalities arising in membrane proteins of liver hepatomas following transformation from the normal cell [12].

The bleomycins are a closely related group of water soluble glycopeptides each differing in a terminal amine side chain, and are obtained from the culture of a strain of *Streptomyces verticillus*. Their use in the therapy of certain types of tumour has been well documented together with their use as tumour imaging compounds [13–16]. The mode of action of bleomycin is thought to be associated with the causing of strand scissions in DNA; the splitting off of thymine bases from the DNA molecule and inhibition of DNA-dependent DNA polymerases [17–20].

The fact that these processes are inhibited by the binding of metal ions to bleomycin such as Cu and cobalt may be important with respect to recent evidence [21–23]. This suggested that a large proportion of the bleomycin present in the body during bleomycin therapy is probably Cu bleomycin owing to the high affinity of bleomycin for the Cu ion.

In the work reported here the effects of bleomycin and Cu bleomycin upon transglutaminase enzymes has been examined in order to determine another possible role of bleomycin in antitumour activity.

The transglutaminases chosen for this work were the plasma transglutaminase (Factor 13) and a tissue transglutaminase isolated from rat lung. Lung tissue was chosen because of its capabilities to accumulate bleomycin and because of its responsiveness to bleomycin therapy when invaded with squamous cell carcinoma [24]. Another factor in this tissue's favour was its low content of a particular aminopeptidase enzyme reported as a bleomycin inactivating enzyme [25].

MATERIALS AND METHODS

Materials. Freeze dried bleomycin sulphate in 5 mg ampoules was kindly supplied by Lundbeck Ltd, Luton, U.K. (Batch No. 004). Solutions of bleomycin were made up to the required concentration in 50 mM Tris–chloride buffer (pH 7.4) or 50 mM Tris–100 mM NaCl buffer (pH 7.4). [1,4-^{14}C]-putrescine was purchased from the Radiochemical Centre, Amersham, *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalene sulphonamide (monodansyl-

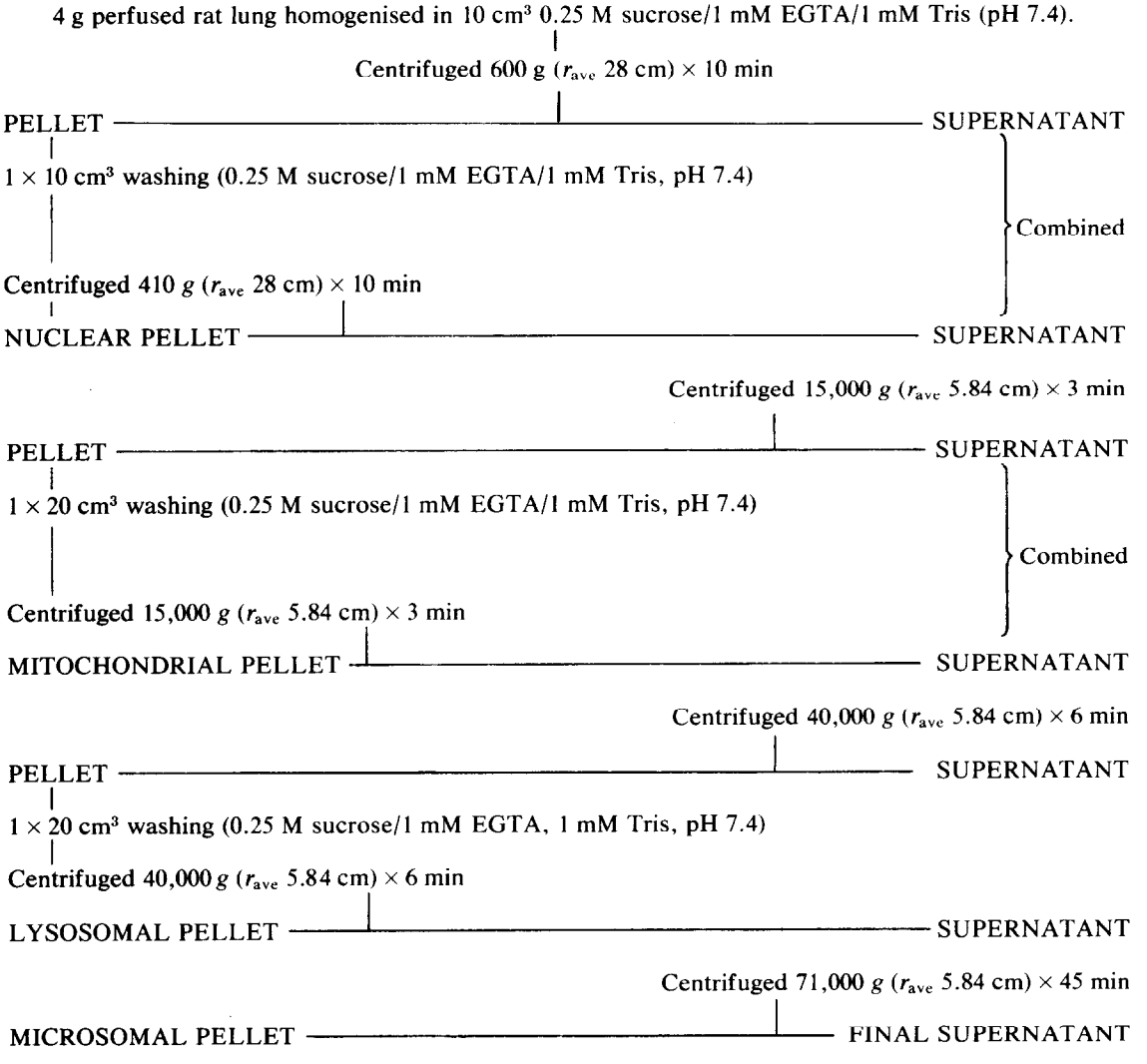
cadaverine) was kindly supplied by I.C.I., Pharmaceuticals, Macclesfield. Copper bleomycin was prepared by dissolving the required amount of bleomycin in an appropriate volume of $Cu(NO_3)_2.H_2O$ solution so that a molar ratio of 4:3 was given for bleomycin to Cu.

Animals. Rats of mixed sex (Wistar derived) weighing between 200–250 g were used in all experiments.

Preparation of rat lung transglutaminase. Lungs were removed, perfused with cold 0.15 M NaCl, cut into small pieces and homogenised in 3 volumes of 0.25 M sucrose/1 mM EGTA/1 mM Tris–chloride (pH 7.4) with a mechanically driven Potter–Elvejham homogeniser. The homogenate was centrifuged at 71,000 *g* (r_{ave} 5.84 cm) for 45 min at 4° on a M.S.E., superspeed 50 centrifuge and the resultant supernatant decanted and assayed for transglutaminase activity by the putrescine incorporation into casein method.

Supernatants prepared in this manner revealed only small amounts of transglutaminase activity, the majority of the enzyme activity remaining in the high speed spin pellet. This result was contrary to that obtained for guinea pig liver transglutaminase an enzyme commonly regarded as 'soluble'[26]. Subcellular fractionation studies were therefore carried out upon rat lung to determine other subcellular localisations of the transglutaminase enzyme which might be used for bleomycin inhibition studies.

Fractionation procedure for rat lung. Rat lungs were prepared as described earlier and fractionated according to the following procedure:



Enzyme assays. Glucose-6-phosphatase [E.C. 3.1.5.9] was assayed according to the method of de Duve *et al.* [27]. Cytochrome oxidase [E.C. 1.9.3.2.] was assayed according to the spectrophotometric method of Appelmans *et al.* [28]. Lactate dehydrogenase [E.C. 1.1.1.27] was assayed according to the method of Henry *et al.* [29]. Acid phosphatase [E.C. 3.1.3.2.] was assayed according to the procedure of Appelmans *et al.* [28].

Transglutaminase activity was measured by a modification of the "filter paper assay" of Lorand *et al.* [30]. The reaction mixture incubated at 37° contained in a final volume of 0.1 cm³, 50–400 μg of enzyme fraction, 5 mM $CaCl_2$, 3.85 mM dithio-

threitol, 500 μg of *N',N*-dimethylcasein, 1.2 mM putrescine containing 2.5 μCi of [1,4-^{14}C]-putrescine (specific activity 62 mCi/m-mole), 30 mM Tris–chloride buffer (pH 7.4). Controls were used substituting 2.5 mM EDTA for $CaCl_2$. Samples of 10 μl were taken from the reaction at appropriate time intervals enabling a linear rate of putrescine incorporation to be measured. Radioactive samples were counted in a Packard (Model 3330) Scintillation Spectrometer in a scintillation cocktail containing 3 g of 2,5-diphenyloxazole (PPO) and 0.3 g of 1,4-di-[2-(5-phenyloxazoyl)-]-benzene (POPOP) per litre of toluene.

Fibrin polymerisation by Factor 13. Reaction mixtures contained in a volume of 0.3 cm^3, 0.45% (w/v) fibrinogen, 15 mM $CaCl_2$ (or 5 mM EDTA), 25 NIH units of thrombin, 3 mM dithiothreitol, and 100 mM NaCl/50 mM Tris buffer (pH 7.4). Reactions carried out at 37° were started by the addition of thrombin. Reactions were stopped at various time intervals by freezing in dry ice-ethanol and the frozen contents of the tubes lyophilised. Each lyophilised reaction mixture was dissolved in 0.4 cm^3 of 1% (w/v) sodium dodecyl sulphate–10 M Urea—5% (w/v) 2-mercaptoethanol and 10–20 μl used for electrophoresis.

Preparation of fibrinogen. Bovine fibrinogen was prepared by the method of Blombäck and Blombäck [31]. The preparation contained sufficient amounts of contaminating plasma transglutaminase (Factor 13) to render further additions unnecessary for performing crosslinking experiments. Bovine topical thrombin (Parke Davis) was used for Factor 13 activation and the conversion of fibrinogen to fibrin.

Chemical determinations. Protein was measured by the method of Lowry *et al.* [32]. DNA was determined by the diphenylamine method of Giles and Myers [33].

Polyacrylamide gel electrophoresis. Sodium dodecyl sulphate gel electrophoresis was carried out according to the procedures of Weber and Osborn [34]. Prior to staining, gels were fixed in a mixture of ethanol–10% (w/v) trichloroacetic acid (1/1 by volume) and then stained with Coomassie brilliant blue (G-250) and cleared by washing in glacial acetic acid–ethanol–water (7:28:55 by volume). Stained gels were scanned at 600 nm using a Unicam S.P. 1800 spectrophotometer with scanning attachment, slit width 1.5 mm.

Electron microscopy. Samples for electron microscopy were fixed in 2% (v/v) glutaraldehyde, washed in 0.1 M sodium phosphate buffer (pH 7.2) and post fixed in Millonigs buffered osmium tetroxide [35]. Specimens were further dehydrated in graded alcohols and propylene oxide and embedded in Araldite CY212 resin (Polaron Equipment Ltd, Watford, U.K.) in cupped embedding moulds. Sections were made through the entire pellet on a Reichert OMU2 ultramicrotome.

After staining in lead citrate followed by uranyl acetate [36] the sections were examined in a A.E.I. model 802 transmission electron microscope.

Analysis of bleomycin complex by HPLC. Quantitative analysis of bleomycin used in these studies was carried out according to the procedure of Williams *et al.* [37].

RESULTS AND DISCUSSION

Fractionation of rat lung. The subcellular distribution of marker enzymes together with DNA and transglutaminase are shown in Fig. 1. Acid phosphatase, cytochrome oxidase and glucose-6-phosphatase were found to be predominantly associated with the "lysosomal", "mitochondrial" and "microsomal" fractions respectively showing a reasonable degree of separation between fractions. Enzyme recoveries compared to the original homogenate were always in the region of 85–95 per cent.

Transglutaminase activity was distributed in all fractions although predominantly associated with the "nuclear fraction", a fraction found to contain membrane components, nuclei, collagen and cell debris when examined by electron microscopy (Fig. 2). This localisation of transglutaminase activity is in direct contrast to the subcellular localisation claimed for guinea pig liver transglutaminase an enzyme reported to be associated with the cytosol of the liver cell [38].

Substitution of EDTA for EGTA into the sucrose medium revealed that a large proportion of the lung transglutaminase activity could be solubilised when the nuclear pellet was repeatedly washed with 0.25 M sucrose/1 mM EDTA/1 mM Tris, pH 7.4 (Table 1). This may indicate the binding of the enzyme through the mediation of divalent cations from which Ca can probably be excluded. Whether the enzyme is bound to its normal cellular locality or alternatively non-specifically bound to insoluble debris by virtue of the homogenisation procedure is a question still to be answered.

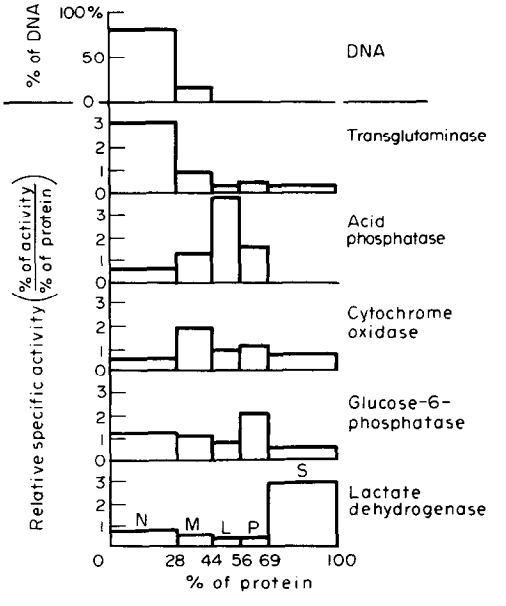


Fig. 1. Subcellular distribution of transglutaminase, acid phosphatase, cytochrome oxidase, glucose-6-phosphatase, lactate dehydrogenase and DNA in rat lung. Enzyme activities were measured by the procedures described in Methods. The distribution of DNA is represented by per cent DNA in fraction against per cent protein in fraction. *N*, represents the 'nuclear fraction'; M, the 'mitochondrial fraction'; L, the 'lysosomal fraction'; P, the 'microsomal fraction' and S, the particle free supernatant.

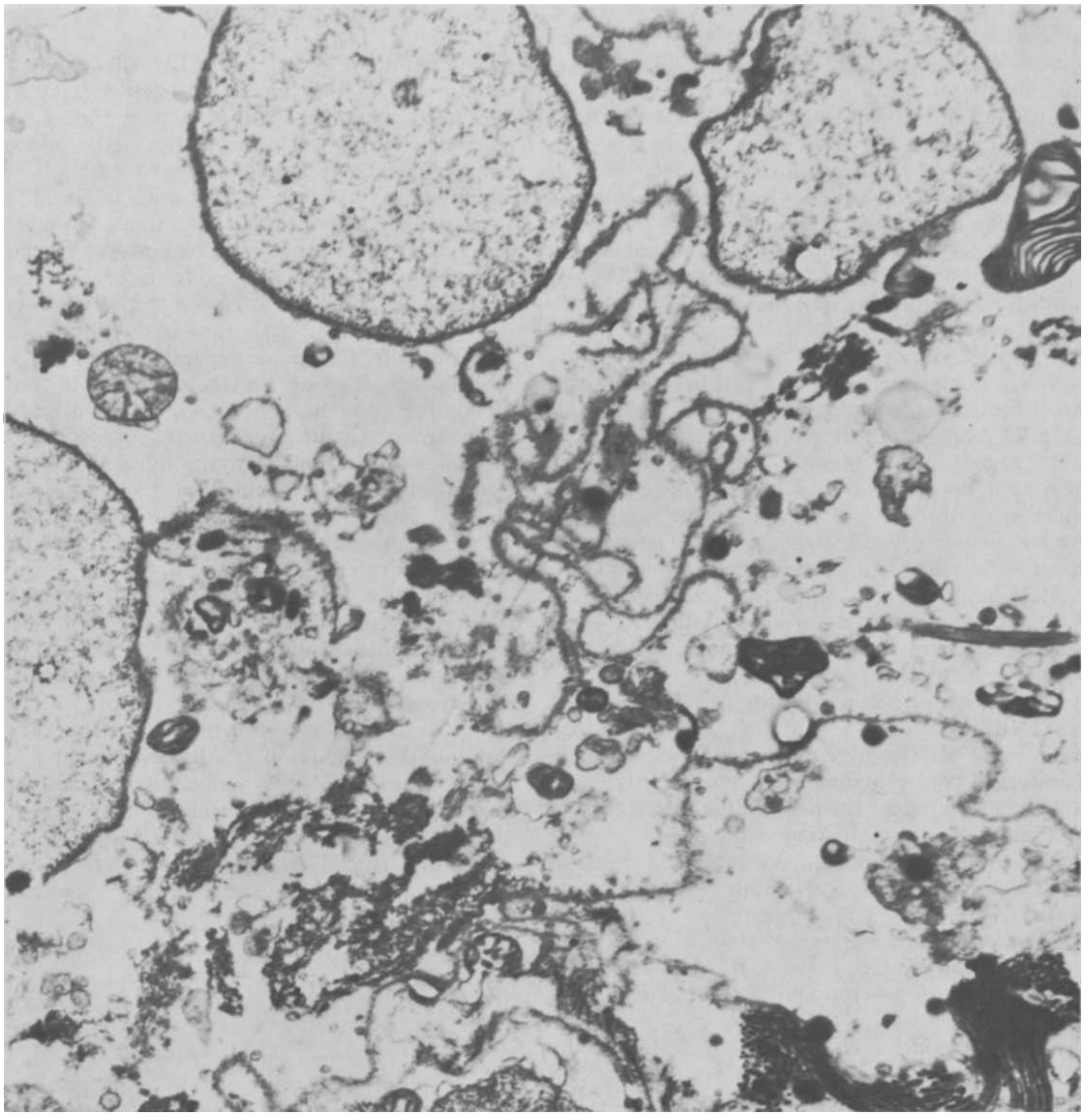


Fig. 2. Electron micrograph of 'nuclear pellet' from rat lung (15000 ×).

Table 1. Solubilisation of particle bound rat lung transglutaminase by washing with sucrose medium containing EDTA

	Volume cm^3	Total protein, mg	Total activity	Sp. Act. *U/mg
Original homogenate	13	473	23819	50.4
First supernatant	9.2	184	699	3.8
Washed 'nuclear pellet'	8.5	180	21600	120
Wash 1	3.5	31.5	945	30
Wash 2	4	28	1988	71
Wash 3	4	20.4	4528	222

* Unit of enzyme activity equals nmol (1,4-^{14}C)-putrescine incorporated per hr under conditions of assay.

Perfused rat lung (3.2 g) was homogenised by the procedures previously described using 5 cm^3 of 0.25 M sucrose/1 mM EDTA/1 mM Tris pH 7.4. The homogenate was centrifuged for 10 min at 600 *g* (r_{ave} 28 cm) and supernatant removed. The remaining pellet was resuspended in 4 cm^3 of the original sucrose medium and centrifuged as before. The supernatant was removed and combined with the original supernatant (First supernatant). This procedure was repeated three times using 4 cm^3 of sucrose medium (wash 1, 2 and 3). The remaining pellet was resuspended in 5 cm^3 of sucrose medium (washed nuclear pellet) and each fraction assayed for transglutaminase activity by the procedures described in Methods.

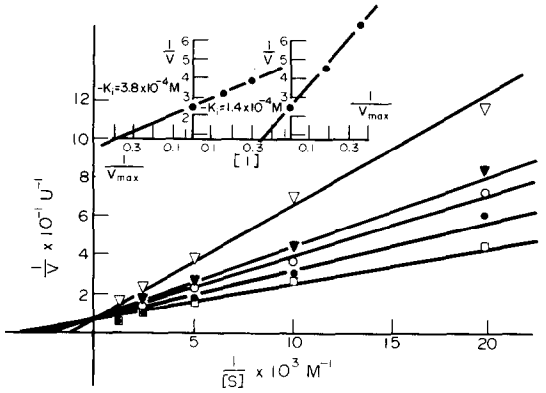


Fig. 3. Lineweaver–Burk plot showing the effects of bleomycin and Cu bleomycin upon 'soluble' rat lung transglutaminase. The conditions of assay were as those described in Methods. [1,4-^{14}C]-Putrescine was supplied to the reaction mixture at the concentrations indicated ($0.5–8 \times 10^{-4}$ M). Reactions carried out at 37° were started by the addition of 100 μg 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 Cu bleomycin and ▽, in the presence of 0.33 mM Cu bleomycin. Inset, represents calculation of K_i by the method of Dixon.

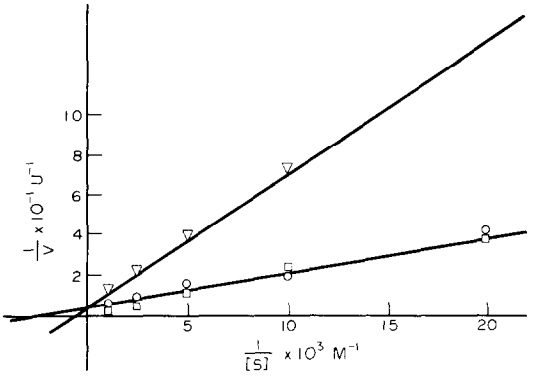


Fig. 4. Lineweaver–Burk plot showing the effects of bleomycin and Cu bleomycin upon 'bound' rat lung transglutaminase. The conditions of assay were as those described in Fig. 3. In the series of experiments shown: □, represents the control system without inhibitor; ○, in the presence of 0.33 mM bleomycin; ▽, in the presence of 0.33 mM Cu bleomycin.

This bimodial distribution of the enzyme prompted investigations into the effects of bleomycin and Cu bleomycin being carried out upon lung transglutaminase using both 'soluble' and 'bound' enzyme. The 'soluble' enzyme consisted of the high speed spin supernatant combined with the washings from the 'nuclear pellet', the 'bound' enzyme consisted of the enzyme activity remaining in the 'nuclear pellet'.

Inhibition of lung transglutaminase with Cu bleomycin and bleomycin. The effects of bleomycin and Cu bleomycin upon lung transglutaminase activity were observed by looking at the inhibition of [1,4-^{14}C]-putrescine incorporation into *N,N'*-dimethylcasein in the presence of fixed concentrations of bleomycin and Cu bleomycin. A Lineweaver–Burk plot of the results obtained for the soluble transglutaminase are shown in Fig. 3. Bleomycin and Cu bleomycin gave plots 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' enzyme for [1,4-^{14}C]-putrescine was calculated as 2×10^{-4} M a value in good agreement with that claimed for the guinea pig liver transglutaminase[30].

Similar studies using the 'bound' enzyme found in the nuclear pellet are presented in Fig. 4. The effects of Cu bleomycin upon transglutaminase activity gave results in agreement with those obtained for the 'soluble' enzyme, competitive inhibition was shown with an approximate K_i for Cu bleomycin calculated as 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 appeared to have negligible inhibitory activity upon the 'bound' enzyme at a concentration of 0.33 mM and probably reflects the binding of bleomycin to the large amount of DNA present in the enzyme fraction [17, 18].

The observation that Cu bleomycin proved to be a more potent inhibitor than bleomycin alone may be the result of the stability conferred to the glycopeptide chain upon the binding of Cu thus eliminating any steric effects the glycopeptide chain may exert when approaching the enzyme binding site [22].

An alternative explanation may be the release of small amounts of free Cu during substrate binding. Reports that guinea pig liver transglutaminase was rapidly inactivated by Cu salts in the presence of Ca caused us to investigate the susceptibility of lung transglutaminase to free Cu [39].

The effects of Cu upon lung transglutaminase activity. Reaction systems using the 'soluble' transglutaminase were set up containing varying amounts of (1,4-^{14}C)-putrescine (Fig. 3) with $(Cu(NO_3)_2 \cdot H_2O$ present at a fixed concentration of 0.33 mM.

Inactivation of the enzyme was found to occur at all concentrations of (1,4-^{14}C)-putrescine except that of 0.8 mM where only 16 per cent of the original enzyme activity remained. This result indicates the susceptible nature of lung transglutaminase to free copper and although confirming that the majority of Cu is probably bound during copper bleomycin inhibition it does not fully eliminate the possibility that small amounts of free Cu (II) ions are present, which may account for the increased potency of the Cu bleomycin over free bleomycin.

The effects of bleomycin and Cu bleomycin upon fibrin polymerisation by Factor 13. Inhibition of Factor 13 was determined by observing the effects of bleomycin and Cu bleomycin upon fibrin polymerisation. The gel-electrophoretic patterns of fibrin polymerisation as a function of time in the presence and absence of inhibitors are shown in Fig. 5. A standard inhibitor of Factor 13 monodansylcadaverine was also included in the reaction systems [40]. In the crosslinking reaction by Factor 13 the control system showed that γ-chains were transformed to γ-dimers and α-chains to α-polymers, but β-chains remained unchanged [41].

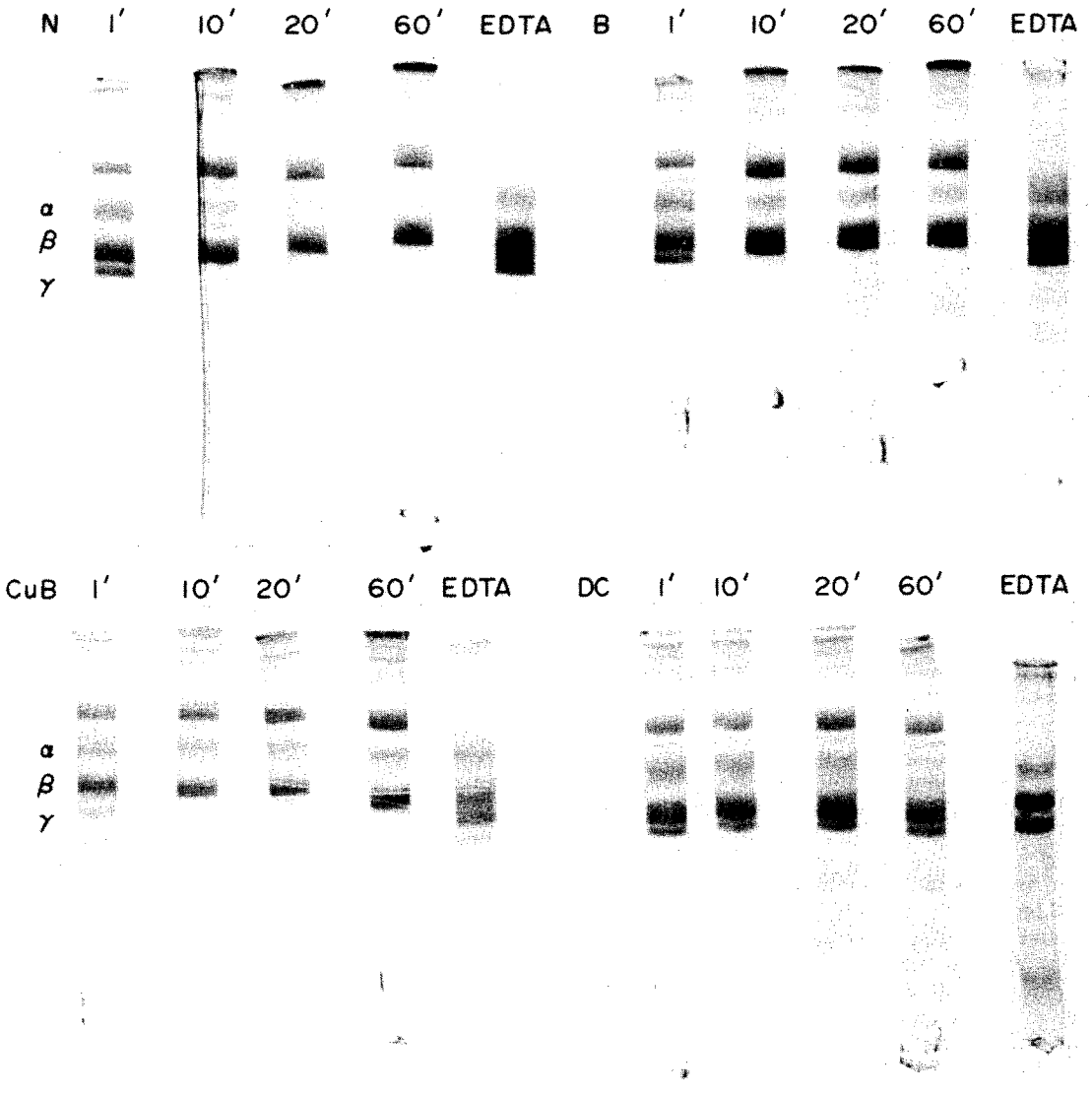


Fig. 5. The effects of bleomycin, copper bleomycin and dansylcadaverine upon the crosslinking of fibrin by Factor 13. Electrophoresis was performed in 1.75 nm pore size gels, Cu B, B and DC indicates fibrin polymerisation in the presence of 2.7 mM Cu bleomycin, bleomycin and monodansylcadaverine respectively. 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 Ca.

Incubation mixtures containing bleomycin and Cu bleomycin revealed marked inhibition of α-polymerisation by Factor 13 but no detectable change in the formation of γ-dimers could be shown when semi-quantitative results were obtained by scanning the stained gels at 600 nm. The decrease in peak area of the α-monomer with respect to time of incubation of reaction system is shown in Fig. 6. Anomalies occurring during the loading and staining of gels were eliminated to a large extent by using the β-monomer as an internal standard. For comparative analysis, 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 min incubation time revealed 59, 15 and 18 per cent inhibition of α-polymer formation for Cu bleomycin, bleomycin and monodansylcadaverine respectively when compared with the control (Fig. 6).

This result would confirm the results shown with the lung enzyme with regard to the potency of the two inhibitors in that Cu bleomycin proved to be a more potent inhibitor than bleomycin alone. Although no inhibition of γ-dimerisation could be shown with either compound the result that α-polymerisation was markedly inhibited is significant when considering the results of McKee *et al.* [41] who reported that the stability of the fibrin gel matrix was only complete when the onset of high mol. wt α-polymer formation occurred.

Folk *et al.* [26, 42] established that the catalytic mechanism of transglutaminases proceeds via a thiol ester intermediate between the thiol group of the enzyme and the γ-glutamyl moiety of the acceptor protein, amide bond formation results by aminolytic deacylation of the acyl enzyme by virtue of a primary amine. The majority of inhibitors of transglutaminases [40, 43] with emphasis upon fibrin

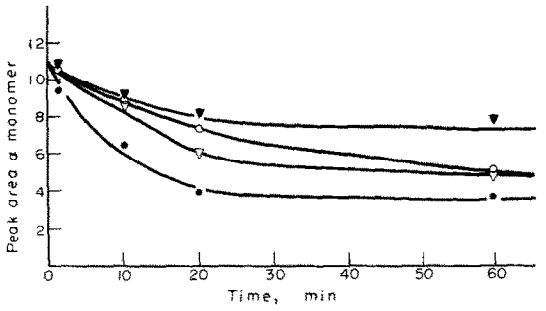
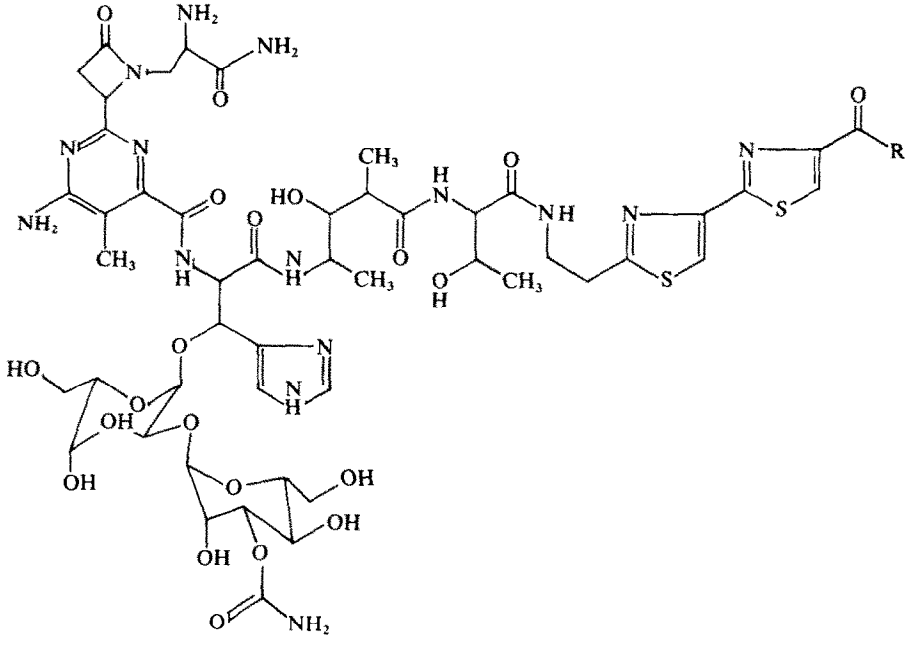


Fig. 6. Comparative analysis of the polymerisation of the α-monomer during fibrin crosslinking. The symbols ▼, ▽, and ○, indicate fibrin polymerisation in the presence of 2.7 mM Cu bleomycin, bleomycin and monodansylcadaverine respectively; ● indicates the control system without inhibitor.

crosslinking are primary amines which serve as pseudosubstrates for the enzyme and actually become incorporated into the γ-glutamyl acceptor sites of the recipient protein e.g. monodansylcadaverine. Observations obtained with compounds in the monotosylated or dansylated alkyl diamine series indicated an optimal alkylside chain length of 0.5–00.6 nm for the primary amine donor [43, 44]. A further requirement for enhanced inhibition was the attachment of a large apolar substituent to the primary amine thought to be necessary for binding to a hydrophobic centre near the catalytic site of the enzyme.

Analysis of bleomycin used for inhibitor studies. Separation of the bleomycin complex into its individual components each differing in its amine side chain is shown in Fig. 7. Assuming a comparative molar extinction coefficient at 254 nm for the individual components separated, the sample contained approximately 57% bleomycin A_2, 35% bleomycin B_2 and 7% bleomycins A_1 and DMA_2. The unknown peak associated with bleomycin B_2 has not been identified but is thought to be a mixture of bleomycin A_{2-a} and bleomycin A_{2-b} (Table 2).

Owing to this variety of amine side chains present in the bleomycin complex one can only speculate at this stage which components are exhibiting inhibition characteristics of a typical competitive inhibitor



Bleomycin	R (Terminal amine)
A_1	$-HN-CH_2-CH_2CH_2-\overset{\overset{O}{\|}}{S}-CH_3$
demethyl-A_2	$-HN-CH_2-CH_2-CH_2-S-CH_3$
A_2	$-HN-CH_2-CH_2-CH_2-\overset{\overset{CH_3}{\|}}{\overset{+}{S}}-CH_3$
B_1	$-H_2N$
A_{2-a}	$-HN-CH_2-CH_2-CH_2-CH_2-NH_2$
A_{2-b}	$-HN-CH_2-CH_2-CH_2-NH_2$
B_2	$-HN-(CH_2)4-NH-\overset{\overset{NH}{\|}}{C}-NH_2$

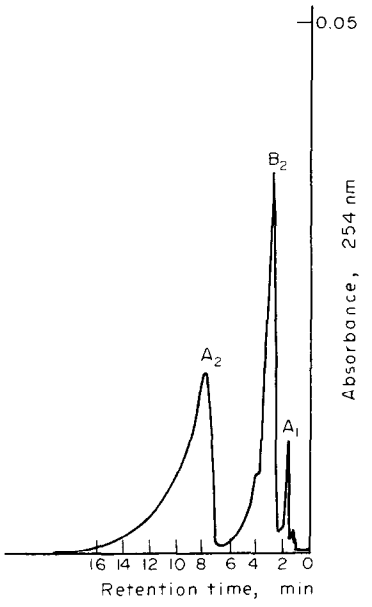


Fig. 7. Analysis of bleomycin complex by HPLC. Aqueous bleomycin (5 μl of a 4 mg/cm^3 solution) was loaded onto a column of μ Porasil (30 cm × 4 mm), flow rate 2 cm^3/min^{-1}, pressure 2,500 psi; absorbance range 0–0.05 (254 nm), mobile phase 0.3% (w/v) ammonium formate–methanol (1:1 by volume).

or pseudosubstrate. Compounds capable of fulfilling the role of pseudosubstrate namely bleomycins A_{2-a} and A_{2-b} have only been tentatively identified. Bleomycin B_2 a component present as approximately 35 per cent of the complex may satisfy the specificity of the enzyme by virtue of a terminal amine side chain in the order of 0.5–0.6 nm which is linked to a large apolar group represented by the thiazole ring. The ability of bleomycin B_2 to be effective in aminolysis of the acylenzyme intermediate is unlikely considering the overall positive charge of the guanidine group at neutral pH (approximate *p*Ka [13–14]). It therefore seems unlikely this compound would act as a pseudosubstrate; however its ability to bind to the acyl enzyme intermediate would reflect its ability to act as a competitive inhibitor of [1,4-^{14}C]-putrescine.

These findings may have illustrated another possible role of bleomycin and Cu bleomycin in their antitumour activity. An interesting conclusion which can be drawn from these results is the therapeutic potential of bleomycin not only in cancer therapy but in other disorders where transglutaminase enzymes have been implicated i.e. thrombosis and atherosclerosis [45, 46]. Substitution of the terminal amine side chain of the bleomycin molecule with various amines of different side chain length and differing basicity would certainly merit further study with regard to the new compounds inhibitory activity against transglutaminases.

Other studies which have prompted further interest are investigations into the inhibitory potential of the bleomycins against proteolytic enzymes reported to be sensitive to guanidyl amines [47, 48]. Invasive proteases have been reported to be required for the proliferation of certain tumours into surrounding tissues [49]. The question arises therefore whether bleomycin or Cu bleomycin have any inhibitory effect upon this type of proteolytic enzyme.

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