Biochem. J. (1971) **123**, 773–787
Printed in Great Britain

Physicochemical Properties of Vancomycin and Iodovancomycin and their Complexes with Diacetyl-L-lysyl-D-alanyl-D-alanine

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(*Received* 8 *March* 1971)

Electrometric and spectrophotometric titrations showed vancomycin to contain groups having pK values of about 2.9, 7.2, 8.6, 9.6, 10.5 and 11.7. Of these the four last-named were phenolic. Titration above pH11 and below pH1 was irreversible and antibiotic potency was destroyed. Combination with the specific peptide diacetyl-L-lysyl-D-alanyl-D-alanine hindered the titration of the first three phenolic groups. Spectrophotometric titration of iodovancomycin showed that the phenolic group with pK 9.6 was the one iodinated. The stability of the vancomycin–peptide complex in the range pH1–13 showed that complex-formation occurred only when carboxyl groups were ionized and the phenolic groups were non-ionized. The complex was formed in concentrations of urea up to 8M, of potassium chloride up to 4M, of sodium dodecyl sulphate up to 1%, and at temperatures up to 60°C. From titration curves, organic chlorine and iodine analysis, and combination with peptide, a minimum molecular weight for vancomycin of 1700–1800 was estimated. Optical-rotatory-dispersion and circular-dichroism experiments suggested that vancomycin has only limited conformational flexibility. Both vancomycin and its complexes with peptide exhibited properties suggesting aggregation. Vancomycin and iodovancomycin can be fractionated into a main fraction and at least three minor components. The isolation of these fractions salt-free is described and their antibiotic properties are shown to correlate with their ability to form complexes with peptide.

Vancomycin, an antibiotic obtained from cultures of *Streptomyces orientalis*, prevents the growth of Gram-positive bacteria by inhibiting the biosynthesis of the cell-wall mucopeptide (Reynolds, 1961, 1966; Anderson, Matsuhashi, Haskin & Strominger, 1965, 1967), although its precise mechanism of action is uncertain. Jordan (1965) found vancomycin to be strongly bound by the walls of *Staphylococcus aureus* within seconds and put forward the hypothesis that vancomycin, by binding to the walls *in vivo*, prevented the introduction of new structural molecules into it during growth. Chatterjee & Perkins (1966) found vancomycin to form complexes with nucleotide-*N*-acetylmuramyl-pentapeptide precursors and Perkins (1969) further studied the complex-formation reaction and found it to be specific for peptides with D-alanyl-D-alanine at the carboxyl terminus. He found the dissociation constant of the complex to be about 10^{-6}–10^{-7} and suggested that attachment of vancomycin by complex-formation with D-alanyl-D-alanine termini either with lipid intermediate or on un-cross-linked chains in the wall could account for the inhibition of mucopeptide biosynthesis and hence of growth.

The object of the present work is to describe the behaviour of vancomycin–peptide complexes under a variety of circumstances. Since very little is known about the chemistry of vancomycin itself parallel studies of the properties of vancomycin and the vancomycin–peptide complex were carried out.

MATERIALS AND METHODS

Chemicals. The peptide used throughout this investigation was *NN'*-diacetyl-L-lysyl-D-alanyl-D-alanine, which was synthesized as described by Nieto & Perkins (1971) and recrystallized from ethanol–hexane, m.p. 202–204°C (Kofler apparatus, corr.).

Sodium dodecyl sulphate was purchased from Matheson, Coleman and Bell (Cincinnati, Ohio, U.S.A.); all other chemicals were AnalaR quality from British Drug Houses Ltd., Poole, Dorset, U.K.

Antibiotics. Vancomycin hydrochloride (Vancocin HCl, lot XK3076) was kindly given by Eli Lilly and Co. Ltd., Basingstoke, Hants., U.K. Iodovancomycin was prepared as follows: 16mg of vancomycin hydrochloride was dissolved in 2.0ml of water and vigorously shaken with 0.25ml of a 0.04M solution of iodine in carbon tetrachloride for 30min. The pH was checked at 10min intervals and adjusted to pH6 with dilute aq. NH_3 soln. The aqueous

layer containing iodovancomycin was fractionated in a CM-Sephadex C-50 column as described below. Radioactive iodovancomycin was prepared by the same procedure.

Fractionation of vancomycin and iodovancomycin. The procedure of Best, Best & Durham (1968) was modified as follows. A column of CM-Sephadex C-50 was equilibrated with 200ml of 0.5M-ammonium acetate buffer, pH7.8, and then washed with 500ml of glass-distilled water. (The column was 2.5cm in diameter and its length varied from 40cm in water to about 12cm in 0.5M-ammonium acetate.) The sample (16–500mg) was dissolved in water (3–10ml), the solution was adjusted to pH5–6 and applied to the column. Elution was started with a linear gradient from water to 0.15M-ammonium acetate buffer, pH7.8 (250ml in each chamber). This gradient eluted the first two fractions; after elution of the second peak the eluent was changed to 0.5M-ammonium acetate, pH7.8, and the third and fourth peaks emerged. The flow rate of this type of column varied with the salt concentration from about 40 to 25ml/h. Fractions of 5ml were collected and E_{280} was continuously measured by means of a Uvicord absorptiometer (LKB, Stockholm, Sweden). Homologous fractions were pooled and desalted as follows: the fractions were concentrated under vacuum at 35–40°C until a thick syrup was obtained. This was dissolved in 96% (v/v) ethanol (1 vol. for each 4 vol. of original unconcentrated fractions), and acetone (2 vol.) was added slowly with swirling. Addition of acetone after the first volume had been added had to be quite slow and mixing good, otherwise crystallization of ammonium acetate ensued.

The precipitate that appeared after addition of 2 vol. of acetone was left to stand for 1h, collected by centrifugation and washed twice with ethanol–acetone (1:4, v/v). Similar desalting of radioactive iodovancomycin showed 61% of the fraction to appear in the precipitate. Fraction I was not soluble in ethanol. It was desalted by continuous-flow electrophoresis as described by Perkins & Nieto (1970).

Antibiotic potencies. Antibiotic potencies were tested against *Bacillus subtilis* W23 grown in glucose–salts medium as described by Perkins & Nieto (1970).

Electrometric titration. Electrometric titration was carried out with a Radiometer Titrator TTT1C as pH meter at 20±0.1°C by the method of separate solutions (Kenchington, 1960). No scale expansion was used and the accuracy and reproducibility of the readings was ±0.02pH unit. A Radiometer combination electrode (GK 2021 C) was used throughout and Na^+ was carefully excluded. The electrode was calibrated before each measurement with standard buffers of pH4.00, 6.50 and 9.24 at 20°C (Bates, 1964) and checked for drift after each measurement with a buffer in the range concerned. Temperature control was maintained to ±0.1°C by circulating water through the jacket compartment of the titration vessel.

Solutions of samples and titrating agents were made up in glass-distilled water, boiled and cooled under N_2. Potassium hydroxide (0.1–1.0M) was standardized against potassium hydrogen phthalate, and HCl (0.5–1.0M) against standardized KOH. The solutions of sample and titrating agents were made 0.02M in KCl. Additions of acid or alkali were performed with a 5µl microsyringe (Terumo; Shandon). The concentration of vancomycin was 0.80mg/ml in the range pH4–10 and 2.67mg/ml above and below these values: 2.0 and 3.0ml samples were used respectively. Titrations in the alkaline range were carried out under continuous bubbling with N_2 previously equilibrated with 0.02M-KCl. Blank titrations were carried out under identical conditions and the net number of protons released was calculated (Kenchington, 1960). Results obtained by continuous titration fitted perfectly on the curve obtained by the separate solutions method.

Spectrophotometric titration. Extinction measurements were made in a Unicam SP.700 double-beam spectrophotometer. All the results were obtained by difference spectrophotometry with a reference sample at pH5.10 adequately diluted with solvent to compensate for additions of titrant to the sample. The extinction values were determined with an accuracy estimated as ±0.003 unit.

Titration of vancomycin was carried out by the separate solution method with the same solutions as for electrometric titration and the difference spectra (2mm cells) were scanned immediately after the pH was read. A concentration of 0.80mg/ml was always used. Reversibility and time-dependency were studied by doing reverse titration at chosen pH values at different times.

Iodovancomycin and vancomycin–peptide complex were titrated in a continuous manner, other conditions being the same as above. For the formation of the complex a molar ratio of peptide to vancomycin of 1.5/1 was used.

Optical rotatory dispersion and circular dichroism. The spectropolarimeter was a Polarmatic 62 apparatus (Bellingham and Stanley, London N.15, U.K.) fitted with a Hewlett–Packard 7005B X–Y recorder. The settings were as follows: slits, 1mm; scale, 50m° or 100m°; scan time, 25 or 37min with time-constants of 3 or 2 respectively. The cell compartment was maintained at 25°C by circulating water from a constant-temperature bath. The solutions were filtered through fine-pore sintered glass. Replicate spectra in every set were obtained without moving the cell from the holder or altering its position in any way.

Circular dichroism on analogous solutions was performed in a Jouan dichrograph (mark II; Roussel-Jouan Co., Paris, France).

U.v. difference spectroscopy. Combination between peptide and vancomycin was observed as described by Perkins (1969) except that since the peptide used in this work did not absorb in the range 250–330nm the tandem arrangement of cells was not employed.

The concentration of vancomycin varied from about 0.04 to 2.00mg/ml. The concentration in each experiment was calculated from E_{280}. The light-pathway was chosen to give an E_{280} value in the range 1.0–1.8. A Unicam SP.700 double-beam spectrophotometer was used; slit adjustment was automatic; damping selector and resolution were set at position 2. Unless otherwise stated the experiments were carried out at room temperature (25–27°C) in 0.02M-sodium citrate buffer, pH5.1. When dilution was higher than 0.5% an appropriate correction was made in the value of the difference. Peptides or other reagents (1–10µl) were added with Carlsberg micropipettes, with parallel dilutions in the reference beam.

Stability of the complex. The stability of the vancomycin–peptide complex with pH was studied in two ways: (*a*) formation of complex at a given pH value; (*b*) behaviour of the complex formed in water on varying the pH. In both

types of experiment complex-formation was followed by u.v. difference spectroscopy and O.R.D.* In Expt. (*a*) vancomycin was dissolved in 0.02M buffers with a range of pH values from 1.0 to 12.0. The buffers utilized were as follows, all at a concentration of 0.02M: pH2.39, glycine–HCl; pH3.13–6.17, sodium citrate; pH7.09, pH8.10, pH10.21, pH10.62 and pH11.72, potassium phosphate; pH9.10, glycine–KOH; pH1.3 was in 0.07M-HCl and pH12.94 in KCl–KOH. Concentrations of vancomycin around 0.26mg/ml were used. In Expt. (*b*) vancomycin–peptide complex was formed in water in the sample cell and the behaviour of the difference spectrum observed upon addition of acid and alkali to both sample and reference cells. Binding in the acid and neutral range was determined by using the difference in E_{282} or in $[\alpha]_{2930}$ induced upon addition of peptide. In the basic region, where phenolic groups are ionized, $E_{301-310}$ was used.

To study the stability of the complex in salts, urea and sodium dodecyl sulphate, vancomycin was dissolved in a buffered solution (0.02M-citrate buffer, pH5.1) of the compound and complex-formation studied by u.v. difference spectroscopy or O.R.D. or both.

Stability of the complex at different temperatures was examined by u.v. difference spectroscopy in 0.02M-citrate buffer, pH5.1. The sample and reference cell holders were heated by circulating water from a constant-temperature bath, giving equal and constant temperatures in both cells within ±0.1°C in the range 20–60°C. A 10.0ml sample of a vancomycin solution (0.047mg/ml) was introduced into each cell (4cm pathway) and allowed to equilibrate at the temperature of choice; then titration with peptide was carried out as usual. Thermal perturbation was carried out according to Bello (1969) in 0.02M-citrate buffer, pH5.1.

Association constants. These were calculated for the reaction vancomycin+peptide = vancomycin–peptide complex. They were calculated on the assumption that the vancomycin and complex were present as monomers. The association constants were calculated from experiments with dilute solutions of antibiotics (0.04–0.06mg/ml) and 4cm pathway cells. A total volume of 10ml of solution in 0.02M-citrate buffer, pH5.1, was used. The value of the difference in E_{283} (ΔE_{283}) was used as a measure of the amount of complex formed. The maximum value of ΔE_{283} corresponding to 100% complex present was determined by addition of a large excess of peptide (5 molar excess; mol.wt. of vancomycin 1800). Slits were set at 0.32–0.36mm. Damping selector and resolution were set at 2.

Chlorine and iodine analysis of vancomycin and iodovancomycin. Elementary analyses of Cl and I were carried out on samples of fraction IV (see the Results section) of both vancomycin and iodovancomycin (free base) equilibrated with the laboratory atmosphere. Percentages of chlorine and iodine were corrected for volatile matter and ash. Vancomycin contained 4.0% organic chlorine. Iodovancomycin had 6.7% iodine, 3.7% chlorine. Ash: vancomycin 1%, iodovancomycin 0.5%. Loss of weight on drying at 65°C under vacuum over P_2O_5 until constant weight: vancomycin 17%, iodovancomycin 21%.

Determination of extinction coefficients. $E^{1\%}_{1cm}$ values at 280nm were estimated from the slope of plots of extinction/concentration. Four dilutions in 0.02M-citrate buffer, pH5.1, were made and the extinction was measured in a Unicam SP. 500 spectrophotometer with the light-paths chosen to obtain a reading in the region of 0.3–0.5 extinction unit. Concentration values were corrected for loss of weight on drying and ash (see above). Loss of weight for the batch of vancomycin hydrochloride used in this work was 5%; ash was 0.5%. Extinction coefficients were: vancomycin 45.0±1; iodovancomycin 44±1; vancomycin hydrochloride 46.0±1.

RESULTS

Fractionation of vancomycin and iodovancomycin. Vancomycin hydrochloride as supplied by the manufacturers contains more than one substance. Best *et al.* (1968) introduced a fractionation based on absorption on CM-Sephadex and elution with ammonium carbonate solution. This procedure was modified to allow better recovery of salt-free

* Abbreviations: O.R.D., optical rotatory dispersion; C.D., circular dichroism.

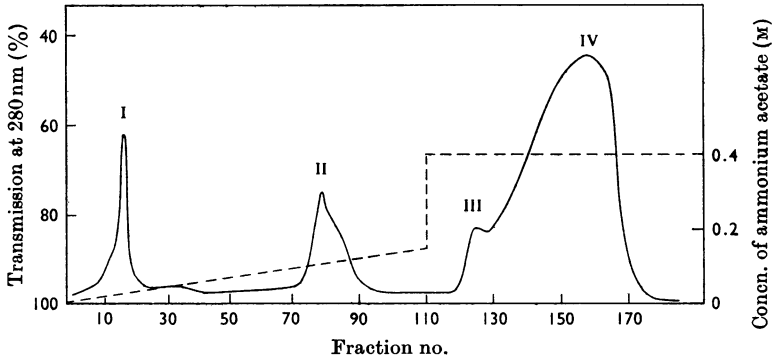

Fig. 1. Fractionation pattern of commercial vancomycin hydrochloride on a CM-Sephadex C-50 column. Average flow rate, 66ml/h; volume of the fractions, 5.5ml. ——, % of transmission at 280nm; – – –, concn. of ammonium acetate (M).

Table 1. *Fractionation of vancomycin hydrochloride on a CM-Sephadex C-50 column and properties of the fractions*

Yield after precipitation for fractions II, III and IV and continuous flow electrophoresis for fraction I (see the Materials and Methods section) is expressed as mg of fraction recovered from 100 mg of vancomycin hydrochloride. Minimal inhibitory concentration for *B. subtilis* W23 was determined as indicated in the Materials and Methods section. The culture flask contained 20 ml of a bacterial suspension (150 μg dry wt./ml when the antibiotic was added). K_A is the association constant of the complexes with peptide. Combining mol. wt. for all the fractions was estimated as 1800. Temperature 26°C.

Fraction	Yield (mg)	Isoionic pH	$E^{1\%}_{1cm}$ at 280 nm	$10^6 K_A$	Minimal inhibitory concn. (μg/ml)
I	6–8	4.7 (0.32 mg/ml)	41	0.30 ± 0.05	2–3
II	3–4	6.8 (0.27 mg/ml)	47	0.8 ± 0.05	2–4
III	2–3	6.5 (0.78 mg/ml)	48	0.7 ± 0.06	
IV	50–55	7.2 (0.27 mg/ml)	45	1.5 ± 0.2	0.25–0.50
Unfractionated vancomycin hydrochloride	100	4.5 (0.27 mg/ml)	46		0.25–0.50

components; Fig. 1 shows the course of fractionation and Table 1 lists the properties of the separated components. The fractionation of iodovancomycin affords a similar picture except that fraction I is increased and split into three similar peaks and fraction II is decreased.

When the yield of the precipitation reaction is taken into account (see the Materials and Methods section) fraction IV is seen to represent 85–87% of the total vancomycin hydrochloride on a weight basis. Best *et al.* (1968) reported that, on running this fraction again, all the other fractions in the same proportions as in the original vancomycin were found. In the present work fraction IV kept at −20°C as a solid did not show any detectable amount of the other fractions 2 months after being isolated. On the other hand another portion kept as a frozen solution in 0.02 M-citrate buffer, pH 5.1, for the same period of time, with occasional thawing for a few hours, showed on refractionation traces of fractions II and III. It thus seems that at least fractions II and III are somehow derived from fraction IV.

The shapes of the u.v. and O.R.D. spectra of the fractions were very similar, differing only in intensity. Some differences observed in fractions I and II resembled those produced by high pH values (see below), but exposing vancomycin hydrochloride for 1 h to pH 10.6, for 24 h to pH 9.0 or for 2 h to concentrated ammonia did not produce higher proportions of fractions I and II.

Fraction IV was used in most of this work and will be referred to as vancomycin. The same applies to iodovancomycin. The commercial product is referred to as vancomycin hydrochloride.

Electrometric and spectrophotometric titrations. Higgins, Harrison, Wild, Bungay & McCormick (1958) examined the acid–base titration of vancomycin and concluded that carboxyl, amino and

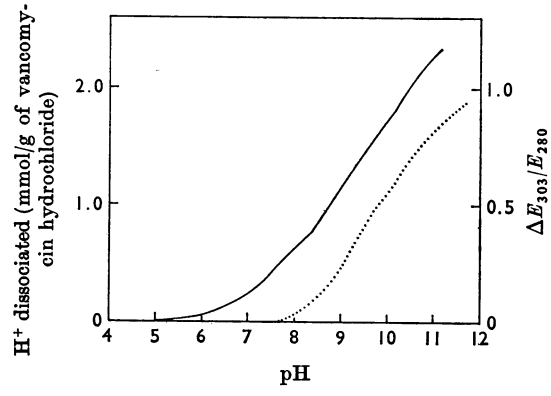

Fig. 2. Electrometric (——) and spectrophotometric (···) titration of vancomycin. Experimental points were obtained by the method of separate solutions as indicated in the Materials and Methods section at an average distance of 0.1–0.2 pH unit. The deviations from the curve drawn on the figure were ± 0.003 and ± 0.02 ordinate unit for the spectrophotometric and electrometric titrations respectively. The ordinate for the spectrophotometric titration represents the increment in E_{303} divided by the E_{280} for the same solution at pH 5.1.

phenolic groups were present. Since we wished to ascertain how complex-formation with specific peptide might affect these functional groups, we first titrated vancomycin alone and attempted to determine which of the groups were phenolic by parallel spectrophotometric titration. Fig. 2 shows the electrometric and spectrophotometric titrations of vancomycin in the basic region. One group with pK 7.1–7.2 titrates without the development of any phenoxide absorption and is therefore probably an amino group. Thus up to pH 7.8, before any phenolic groups have titrated, 1 mol of H^+ has dissociated

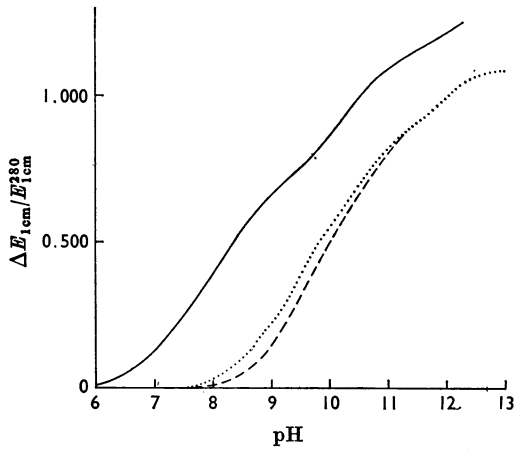

Fig. 3. Spectrophotometric titrations of iodovancomycin (——), vancomycin (···) and vancomycin–peptide complex (– – –). Titration was carried out as indicated in the Materials and Methods section. The difference in extinction ΔE was measured at 303nm for vancomycin and its complex with diacetyl-L-lysyl-D-alanyl-D-alanine and at 307nm for iodovancomycin. The ordinate scale represents the ratio described in Fig. 2.

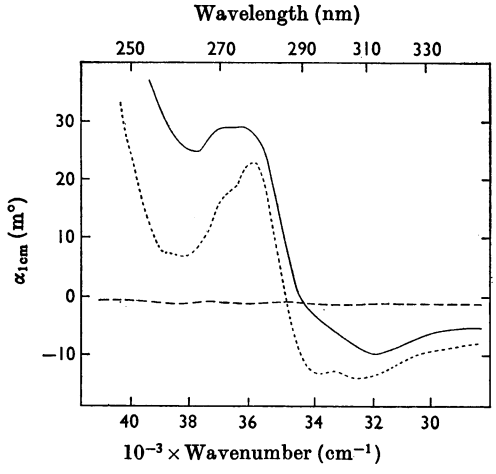

Fig. 4. O.R.D. curves for vancomycin (2.5ml of a 0.27mg/ml solution in a 1cm pathway cell) (——) and its complex with diacetyl-L-lysyl-D-alanyl-D-alanine (as above plus 30μl of 0.020M-peptide) (···) at pH9.10. The solvent (– – –) was 0.02M-glycine–KOH buffer.

per 1800g of vancomycin. Then between pH7.8 and pH9.9 a further 2mol of H^+ dissociate and phenoxide absorption develops, suggesting that two phenolic groups are ionizing for each amino group previously ionized. Between pH9.9 and pH10.9 another 1mol of H^+ dissociates, accompanied by further development of phenoxide absorption, and spectrophotometric titration up to pH13 (Fig. 3) suggested that at least one more phenolic group ionized in this region. The estimated approximate pK values for the phenolic groups are 8.7, 9.6, 10.5 and 11.7.

As expected, iodination of vancomycin affected the titration curves, and the spectrophotometric titration of iodovancomycin is shown in Fig. 3. Elementary iodine analysis indicated that only one iodine atom had been introduced per 1900g, or in other words only one phenol per amino group has been substituted. The curve is most easily interpreted as iodination of the phenolic group with pK9.6; iodination changes the pK of this group to approx. 7.4 and produces an increment in extinction of the phenoxide ion. E_{280} was virtually unchanged (see the Materials and Methods section) and the E_{307}/E_{280} ratio for this group was about 0.37 compared with 0.27 for untreated vancomycin.

The effect of peptide-complex formation on the acid–base titration of vancomycin is shown in Fig. 3. The titration of the first three phenolic groups was apparently hindered by complex-formation with peptide. This observation was reflected in the O.R.D. spectra of vancomycin and its complex with peptide shown in Fig. 4.

In addition to these changes, observed at alkaline pH values, the u.v. spectrum of vancomycin was also perturbed on addition of acid (Fig. 5*a*). Vancomycin was titrated in the acidic region and Fig. 5(*b*) shows the electrometric titration of a carboxyl group accompanied by a progressive change in $\Delta E_{max.}$ (294nm). Titration of the carboxyl group of vancomycin could induce a perturbation of the aromatic chromophores either if the carboxyl group was attached to or located near to the aromatic groups (Wetlaufer, 1962), or if variation of charge in the carboxyl group induced a change in the conformation of vancomycin that modified the interactions involving the aromatic chromophores. However, the C.D. spectrum of vancomycin in acid is very similar to the one in neutral solution (see below), hence alterations in conformation are probably rather limited.

Electrometric titration of vancomycin was also reported by Lomakina, Muravyeva & Yurina (1970*b*). The main features of the titration curve, the total number of titratable groups and the approx. molecular weight were as reported here but the group with pH 8.5 was assigned to an amino function, whereas the present spectrophotometric results indicate that it is a phenolic group.

Stability of vancomycin at various pH *values.* Vancomycin is known to be unstable at 37°C at alkaline pH values (Higgins *et al.* 1958). To see how well the decreased antibiotic potency of alkali-treated samples correlated with the ability to form

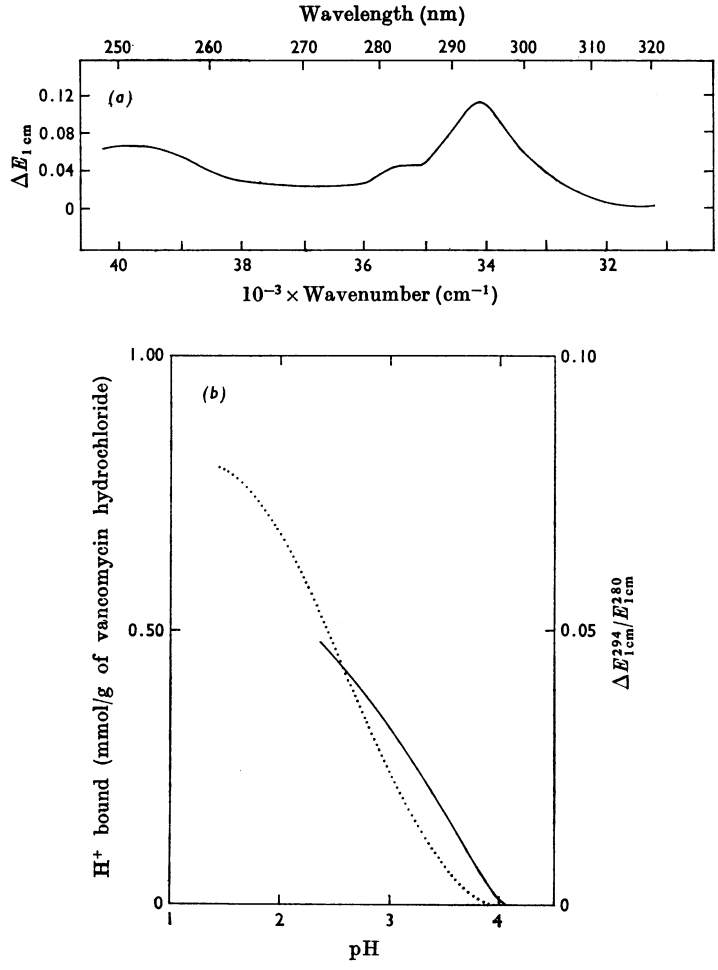

Fig. 5. (*a*) Acid difference spectrum of vancomycin. The concentration of vancomycin in both sample and reference cells was 0.286 mg/ml. The pH values were 1.51 in the sample cell, 5.10 in the reference cell. (*b*) Electrometric titration of vancomycin in the acid region (——) and difference in E_{294} (···) induced by addition of acid to vancomycin. Experimental points were obtained every 0.1–0.2 pH unit. Deviations from the curve were as in Fig. 2.

a complex with specific peptide, samples of vancomycin were held at various alkaline pH values before being returned to pH 5.1. The effect on the u.v. absorption is shown in Fig. 6. All samples retained 100% of their antibiotic potency and ability to form a complex with $N^{\alpha}N^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-alanine, with the exception of the one exposed to pH 12.8 for 36 h, which lost both.

Optical rotatory dispersion and circular dichroism of vancomycin. Spectrophotometric titration indicated the presence of several phenolic groups in vancomycin and measurement of O.R.D. in the alkaline range of pH values showed that these chromophores were also asymmetric (Fig. 7). The lack of an isosbestic system and the fact that changes in O.R.D. occurred over the whole range in which the phenolic groups titrated indicated that most or all of these groups were associated with asymmetric centres. The simplest explanation would be that the phenols were chemically distinct, but complex interaction of rotational groups is not excluded. Measurement of C.D. spectra in the near u.v. (Fig. 8) showed no evidence for direct phenol–phenol interaction, there being a single relatively symmetrical dichroic band with a minimum at 285 nm.

C.D. spectra in the far u.v. (Fig. 9) showed a very intense system of bands of opposite sign and

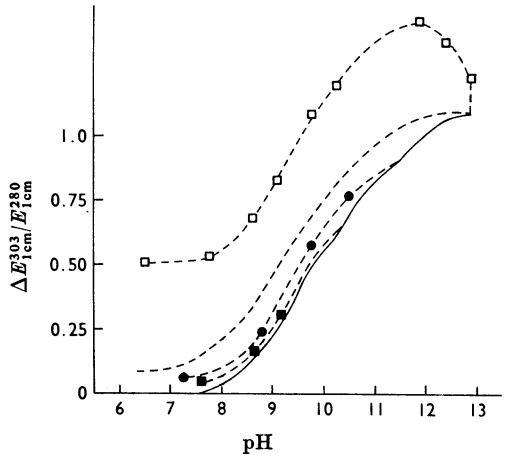

Fig. 6. Reverse spectrophotometric titration of vancomycin after: – – –, 5min at pH12.85; □ – – □, 36h at pH12.85; ● – – ●, 4 days at pH11.45; ■ – – ■, 4 days at pH10.30 (temperature in all cases was 20–22°C); —— represents the forward instantaneous spectrophotometric titrations also shown in Figs. 2 and 5(*a*).

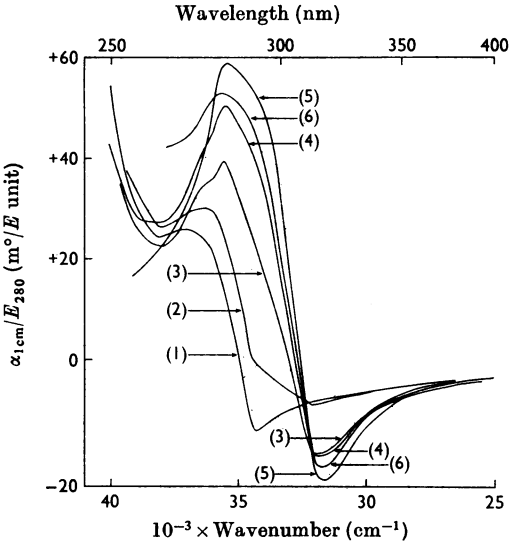

Fig. 7. O.R.D. curves for vancomycin hydrochloride in 0.02M buffer solutions at various pH values: (1) pH8.1; (2) pH9.1; (3) pH10.2; (4) pH 10.6; (5) pH11.8; (6) pH12.9. The ordinate is rotation per unit of extinction of the sample at 280nm and pH5.0.

approximately equal overall intensity. Such a system is characteristic of strongly interacting transitions that would require the physical proximity of the chromophores, e.g. stacking or perhaps a

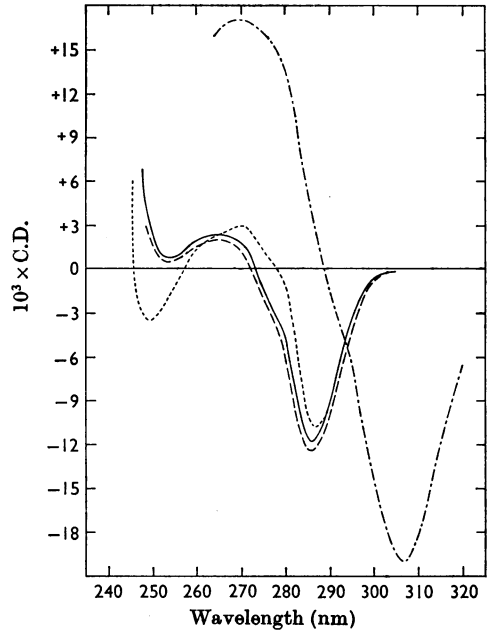

Fig. 8. Near-u.v. circular-dichroism spectra of vancomycin hydrochloride at pH5.2 (——), pH1.4 (– – –), pH11.7 (– · –) and of vancomycin–peptide complex at pH5.2 (···). The concentration of vancomycin hydrochloride was 0.27mg/ml and the light-path 1cm. Complex was formed by adding 1.5 molar excess of peptide. Circular dichroism is expressed as absolute values of the difference in extinction of right- and left-hand circularly polarized light.

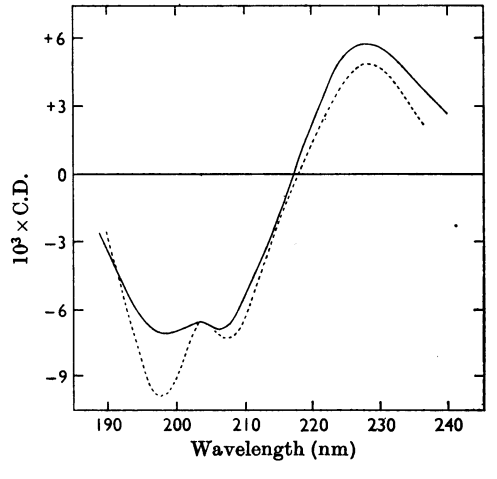

Fig. 9. Far-u.v. circular dichroism of vancomycin hydrochloride (——) and vancomycin–peptide complex (···); concentration of vancomycin, 0.090mg/ml; light-path, 1mm. Other conditions were as described in Fig. 8.

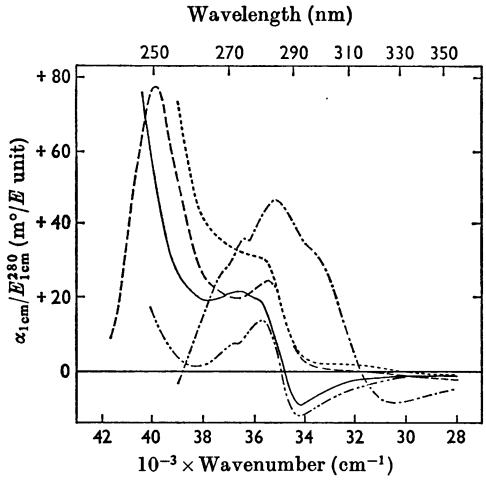

Fig. 10. O.R.D. spectra of: ——, vancomycin at pH 5.1; – · · –, vancomycin–peptide complex at pH 5.1; · · ·, iodovancomycin at pH 5.2; – – –, iodovancomycin–peptide complex at pH 5.2; – · – ·, iodovancomycin at pH 11.9.

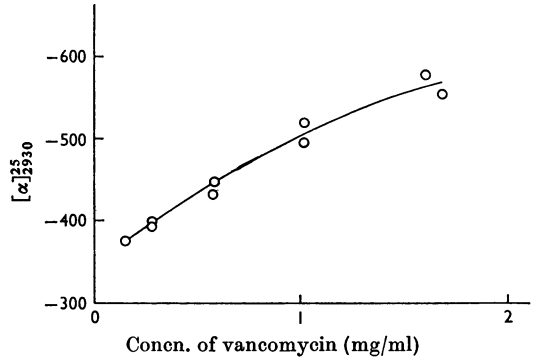

Fig. 11. Concentration-dependence of the specific rotation of vancomycin in 0.02M-citrate buffer, pH 5.1. Spectropolarimeter settings: 100m° scale; 37min scan; time-constant, 2; slits, 1mm. The light-pathway was varied to give an extinction in the range 1–1.8.

biphenyl type of arrangement. This mechanism gives strong dichroism in asymmetrically stacked chromophores (Bayley, Neilsen & Schellman, 1969). However, the insensitivity of the spectrum to certain organic solvents argues against simple physical stacking but would be expected with a cyclic, biphenyl or head-to-tail arrangement.

Vancomycin did not undergo any drastic change in symmetry properties on addition of the specific peptide (Figs. 8 and 9). However, there were some characteristic perturbations in the O.R.D. and C.D. both in the near and far u.v. regions. There is thus the possibility of direct interaction, shielding of ionizable groups changing the intrinsic properties of the chromophores or of minor changes in shape of the vancomycin molecule. The substitution of one of the phenolic groups by iodine also affected the rotation but once again the addition of specific peptide made the rotation considerably more negative below 270nm (Fig. 10).

Aggregation of vancomycin. The specific rotation of vancomycin in the negative branch of the aromatic Cotton effect was concentration-dependent, although no concentration dependence was apparent from 250 to 280nm, and the change in specific rotation at 293nm with concentration is shown in Fig. 11. This concentration-dependence, which was also supported by evidence from C.D. spectra, presumably indicates aggregation. Extrapolation to zero and infinite concentration gave $[\alpha]^{25}_{2930}$ as about −350° for the totally non-aggregated species and about −700° for totally aggregated vancomycin. A concentration of about 10mg/ml produces

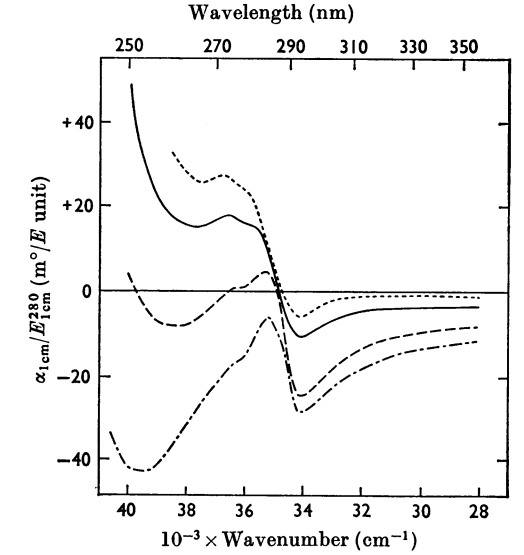

Fig. 12. O.R.D. spectra of vancomycin in: 0.02M-citrate buffer, pH 5.1 (——); in buffered 4M-KCl, pH 5.2 (– – –); in buffered 0.24% sodium dodecyl sulphate, pH 5.1 (· · ·); in buffered 4M-KCl plus 1.5 molar excess of *NN'*-diacetyl-L-lysyl-D-alanyl-D-alanine (– · – ·). The O.R.D. spectrum in 8M-urea was virtually identical with that in buffer. The E^{280}_{1cm} for the vancomycin solutions was about 1.2. The spectropolarimeter settings were the same as in Fig. 11.

total aggregation as judged by this method. The molecular weight of vancomycin as determined by sedimentation (McCormick, Stark, Pittenger, Pittenger & McGuire, 1956) has been reported as about 3300. Although no details of the method were given, presumably a concentration higher than 5mg/ml was used in the centrifuge run and hence vanco-

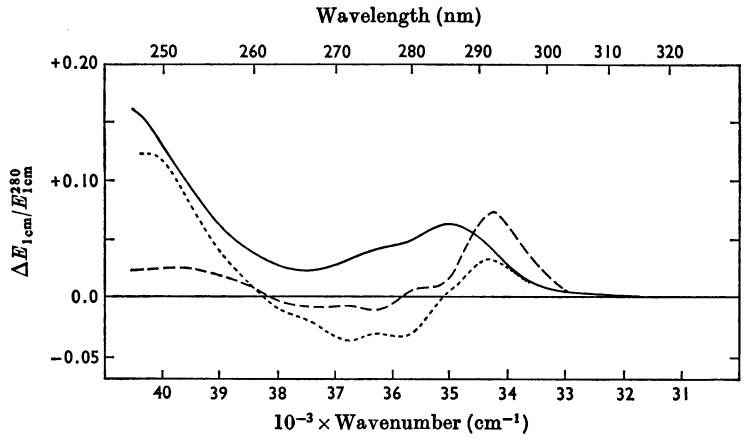

Fig. 13. Difference spectra induced on vancomycin by: ——, increasing concentration (sample: 2.260 mg/ml solution in 0.02 M-citrate buffer, pH 5.1, 1 mm pathway; reference: 0.226 mg/ml in same buffer, 10 mm pathway); – – –, 8 M-urea (0.235 mg/ml vancomycin solution in buffered 8 M-urea, pH 5.1, against same concentration in buffer alone); ···, 4 M-KCl (0.266 mg/ml vancomycin solution in buffered KCl, pH 5.1, against same concentration in buffer alone). The spectrophotometer settings were: scan speed, 3; chart speed, 80 in/h; resolution, 2; damping, 2.

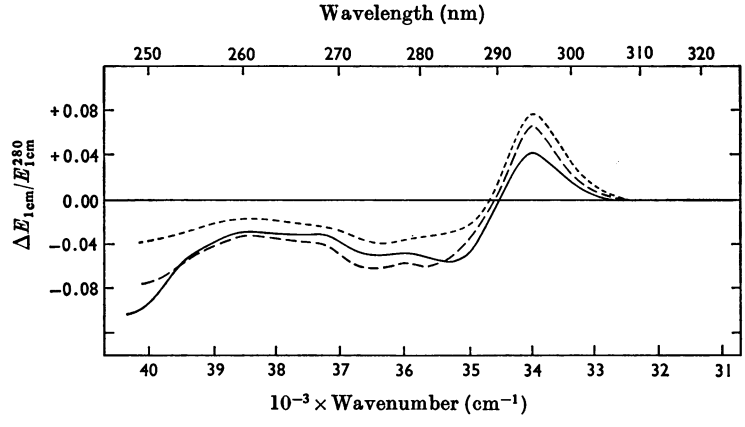

Fig. 14. Difference spectra induced by the addition of 1.3 mol of *NN'*-diacetyl-L-lysyl-D-alanyl-D-alanine/mol of antibiotic to the sample cell. Cells contained vancomycin solutions in 0.02 M-citrate buffer, pH 5.1. Vancomycin concentration: 0.270 mg/ml (——), 0.390 mg/ml (– – –), 0.270 mg/ml in buffered 2 M-KCl (– · – ·). Spectrophotometer settings were as in Fig. 13.

mycin was highly or totally associated. If the associated species is a dimer, an approximate value for the association constant can be calculated from the values of rotation at intermediate concentrations. The value obtained was $8.0(\pm 1) \times 10^2$ for solutions at 26°C and pH 5.1 in 0.02 M-citrate buffer.

Conformational flexibility of vancomycin. The alteration produced by peptide in the O.R.D. and C.D. spectra of vancomycin raises the problem of the conformational flexibility of the vancomycin molecule. The O.R.D. of vancomycin was obtained in the following solvents, all in 0.02 M-citrate buffer, pH 5.1: 0.2, 2.0 and 4.0 M-potassium chloride; 0.12, 0.24 and 0.89% sodium dodecyl sulphate; 4 M- and 8 M-urea. 8 M-Urea influences the O.R.D. of vancomycin very little. Only the negative branch of the Cotton effect was affected in a manner similar to the change produced by increased concentration. However, the corresponding u.v. difference spectrum (Fig. 13) compared closely with one produced

by 20% glycol. Hence, the small effect of urea may well be an effect of increased refractive index on the aromatic side chains exposed to the solvent.

Potassium chloride, on the other hand, produced a strong perturbation in both the O.R.D. and absorption spectra (Figs. 12 and 13). Since peptide combines with vancomycin in potassium chloride (Fig. 12) no extensive conformational change in the binding site can have occurred and perhaps the perturbations induced by potassium chloride are due to either (*a*) the effect of ionic charges on the vancomycin chromophores or (*b*) a small conformational change, the vancomycin molecule contracting to minimize the unfavourable contact between solvent and its hydrophobic regions. High concentrations of salt were needed to produce an effect; the perturbation caused by 0.2M-potassium chloride was almost unnoticeable.

Sodium dodecyl sulphate affected the O.R.D. spectrum in an opposite manner to potassium chloride (Fig. 12). The changes brought about by the detergent could be attributable to a number of mechanisms, such as deaggregation of the vancomycin molecules or shielding of the chromophores from the solvent by hydrophobic bond formation between the antibiotic and the dodecyl chains. At the same time a small expansion of the molecule could occur. The peptide-binding site of vancomycin cannot have been greatly affected by the presence of sodium dodecyl sulphate, since complex-formation with peptide took place at all concentrations of the detergent.

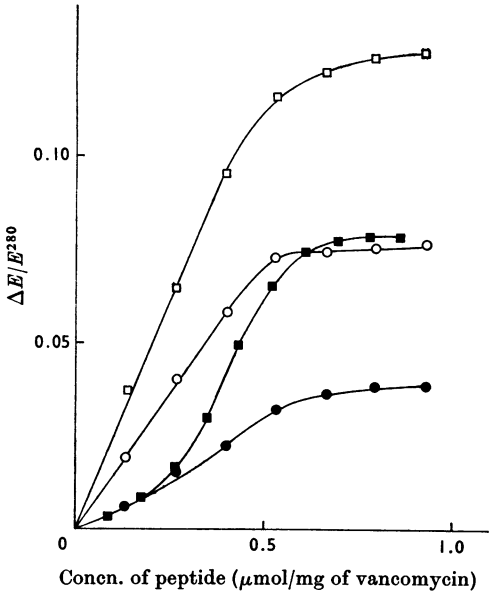

Fig. 15. Spectrophotometric titration of vancomycin with 0.02M-*NN'*-diacetyl-L-lysyl-D-alanyl-D-alanine. Cells contained 2.5ml of a solution (0.225mg/ml) of the antibiotic in 0.02M-citrate buffer, pH5.1. The difference in extinction induced on addition of peptide at 293nm (●), 283nm (○) and 250nm (□) has been plotted against the peptide/vancomycin ratio. The difference in E_{293} in an analogous experiment with a stronger solution of vancomycin (0.440mg/ml) has also been plotted (■) to emphasize the sigmoid character of the spectral change at this wavelength. Spectrophotometer settings were as in Fig. 13.

Combination of peptide with vancomycin and iodovancomycin as shown by difference spectroscopy. In earlier work (Perkins, 1969) the combination between vancomycin and mucopeptide precursors, or partial analogues consisting of acylated alanine peptides, was studied by difference spectroscopy. In the present investigation the analogue chosen as a standard was *NN'*-diacetyl-L-lysyl-D-alanyl-D-alanine. The u.v. difference spectrum produced upon addition of this peptide to vancomycin is shown in Fig. 14. It is seen to be composed of a positive difference at 293–294nm, two minima at 283 and 273nm and a bigger negative difference in the far u.v., of which the difference seen around 250nm is only a shoulder. In principle, the amount of complex present could be determined from a plot of the difference ΔE at any of these extremes against the amount of peptide added. Such plots are shown in Fig. 15. The developments of the differences at the minima correlate very well with each other, but the maximum at 293 increases non-linearly. A possible explanation is that the vancomycin–peptide complex aggregates. Fig. 16 shows a concentration difference spectrum of the complex. The positive differences with maximum values at 281 and 293nm and in the far u.v. are seen to account for the decrease in the minima and the increase of the maximum in the complex difference spectrum when concentration is increased.

The value of $E^{1\%}_{1cm}$ at 293nm at the plateaux of titration curves similar to the ones in Fig. 15 was obtained by carrying out titrations of vancomycin with peptide at various concentrations. These values were plotted against the concentration of complex as for the specific rotation with vancomycin alone (see Fig. 11). Extrapolation to zero and infinite concentrations gave values of $E^{1\%}_{1cm}$ at 293nm of 0.84 and 7.0 respectively. From these values the association constant for dimerization of the complex was calculated to be $K_A = 7.4 \pm 0.5 \times 10^2$, a value similar to the one quoted above for the dimerization of vancomycin alone.

The non-linear development of the maximum at 293nm and its influence on the minimum at 282nm cause the increments of ΔE_{282} on addition of peptide to be 'non-equivalent'. They are bigger for small peptide/vancomycin ratios, becoming smaller as the ratio increases. This effect of aggregation, very

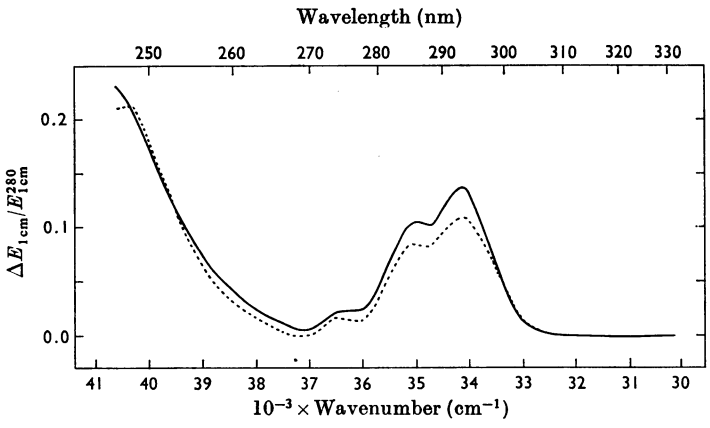

Fig. 16. Difference spectrum induced on the complex of vancomycin and *NN'*-diacetyl-L-lysyl-D-alanyl-D-alanine by (*a*) ionic strength (···) and (*b*) increasing concentration (——). (*a*) The reference cell contained a solution (2.6ml) of vancomycin (0.68mg) and peptide (1.25μmol) in 0.02M-citrate buffer, pH5.1. The sample cell contained the same solution made 4M with respect to KCl. (*b*) The reference cell contained a solution (3.2ml) of vancomycin (0.68mg) and peptide (0.36μmol) in 0.02M-citrate buffer, pH5.1; light-path, 10mm. The sample cell contained a solution (0.32ml) of vancomycin (0.68mg) and peptide (0.36μmol) in 0.02M-citrate buffer, pH5.1; light-path, 1mm.

small up to a concentration of vancomycin of about 0.2mg/ml, becomes stronger as the concentration is increased and has to be taken into account when attempting to determine the stoicheiometry of the binding from the difference spectral changes.

Fig. 17(*a*) is the result of titrations of vancomycin and iodovancomycin with peptide in very dilute solution, and Fig. 17(*b*) shows a Scatchard plot (Edsall & Wyman, 1958) of the binding data. The molecular weight was assumed to be 1800 for vancomycin and 1900 for iodovancomycin. The slope is very steep in both cases, and consequently the value for the association constant is not very accurate. Values of $K_A = 1.5 \pm 0.2 \times 10^6$ for vancomycin and $0.74 \pm 0.1 \times 10^6$ for iodovancomycin are the best present estimates for the peptide used throughout this work at pH5.1. The intercept with the x axis gives a value of between 1.00 and 1.02 sites per assumed molecular weight in both cases.

Complex-formation at various pH *values.* Changes of pH affect the u.v. absorption, rotational properties and stability of vancomycin. Nevertheless, complex-formation with peptide will occur over a considerable range of pH values (Fig. 18). As soon as phenol ionization occurs (cf. Fig. 3) the complex becomes less stable, and the same is true at low pH values where ionization of carboxyl groups might be expected to be prevented. Other results (Nieto & Perkins, 1971) suggest that at least the carboxyl group in the peptide substrate must be ionized for complex-formation to take place. Although the pK value of the carboxyl group in the substrate lies in the region of 3–4, it could be lowered considerably by complex-formation with vancomycin. C.D. spectra in acid (Fig. 8) suggest that conformational changes cannot affect the stability profile in Fig. 18.

Stability and ionic strength. Perkins (1969) showed that 10mM-Ca^{2+} or Mg^{2+} ions and 85mM-Mg^{2+} did not affect the formation of complex as shown by paper chromatography or electrophoresis. We have extended those results by using u.v. difference spectroscopy and O.R.D. Neither Ca^{2+} nor Mg^{2+} nor Cu^{2+} had any effect. These facts rule out any specific effect of bivalent cations on complex-formation.

The effect of ionic strength has been studied in the presence of potassium chloride at pH5.1. From experiments in dilute vancomycin solution in 4M-potassium chloride and a Scatchard plot an association constant of $K_A = 7.0 \times 10^4$ was calculated. This is more than an order of magnitude lower than the value in 0.02M buffer (see above) and may well be related to the effect of ionic strength on vancomycin alone. Table 2 shows the affinity of the peptide for vancomycin at intermediate salt concentrations, expressed as a percentage of the affinity in 0.02M-citrate buffer, pH5.1.

Stability in the presence of sodium dodecyl sulphate. Table 3 shows the variation of the stability of the complex at various detergent concentrations. The stability is seen to decrease with increasing detergent concentration, reaching a minimum value at a concentration of about 1%, then increasing again as the concentration of sodium dodecyl sulphate is

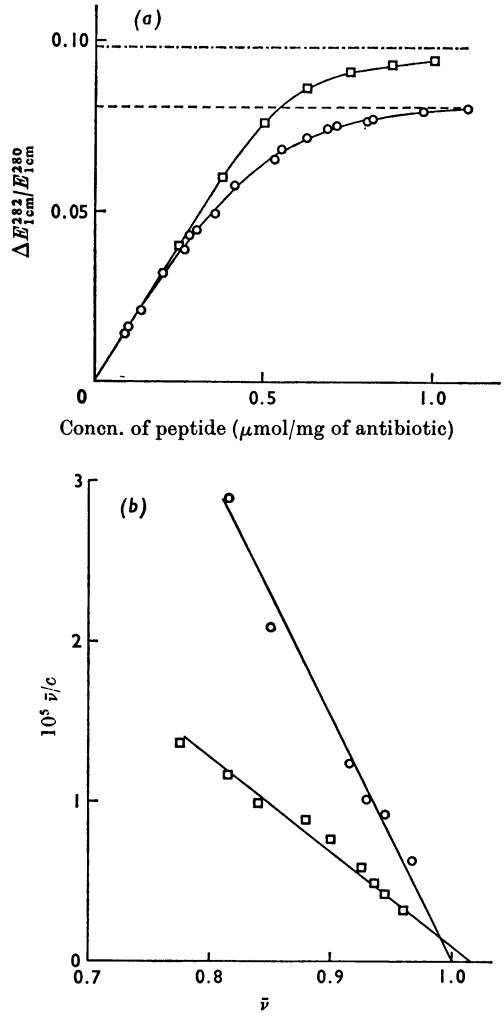

Fig. 17. (*a*) Titration in dilute solution of vancomycin (0.0545 mg/ml) (○) and iodovancomycin (0.0600 mg/ml) (□) with 0.025 M-diacetyl-L-lysyl-D-alanyl-D-alanine. – – – and –·– represent the respective values of ΔE obtained with excess of peptide. (*b*) Scatchard plot of these results: $\bar{v}$ is the spectral difference at any point divided by the value of the difference at saturation. The assumption is made that the difference is proportional to the number of available binding sites occupied. *c* is the molar concentration of free peptide. The mol.wt. was assumed to be 1800 for vancomycin and 1900 for iodovancomycin.

raised. A possible explanation for these results is that sodium dodecyl sulphate binds to the hydrophobic regions of vancomycin causing vancomycin molecules to disaggregate, at the same time blocking mechanically the access of peptide to its binding site on vancomycin. Below its critical micelle concentration the sodium dodecyl sulphate would bind to vancomycin in proportion to the detergent concentration. Once micelle formation had occurred, the amount of free detergent available to 'solvate' the vancomycin molecule would be relatively low and the peptide complex could form more readily again.

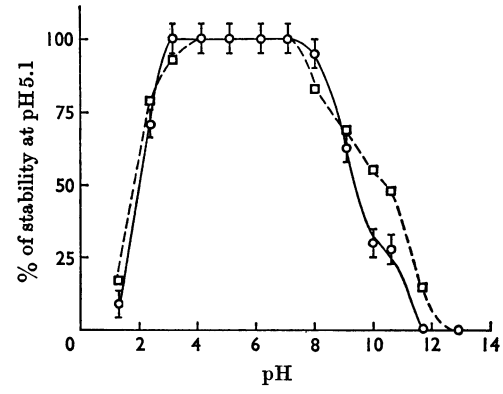

Fig. 18. Stability of the vancomycin–diacetyl-L-lysyl-D-alanyl-D-alanine complex with pH as determined by O.R.D. (○) and difference spectrophotometry (□). Stability is reported as the percentage of the association constant observed at pH 5.1. Vertical lines represent the range of individual results.

Complex-formation in the presence of urea. The stability of the complex formed in the presence of different concentrations of urea at pH 5 is shown in Table 4. Urea does not alter the conformation of vancomycin, but because hydrogen bonding may be important in the complex-formation (Nieto & Perkins, 1971), one might expect a much stronger effect on combination than the one found. However, it is already recognized that urea may have little significant influence on hydrogen bonds (Klotz & Shikama, 1968).

Stability at various temperatures. Table 5 shows the dissociation constants and relative stability of the complex at temperatures in the range 25–50°C at pH 5.1 as estimated by u.v. difference spectroscopy. As a control of the chemical stability of vancomycin itself thermal perturbation as described by Bello (1969) was carried out. The perturbation induced in the u.v. absorption spectrum by increasing the temperature to 60°C varied linearly with the temperature, and was readily reversible after heating at each temperature for 1h (the duration of any of the experiments carried out for determining the stability of the complex). Prolonged heating (1–3 days at 70°C), however, introduced non-linear changes, increasing the extinction very much in a manner similar to the changes produced by exposure to high pH values, again suggesting chemical degradation.

Table 2. *Variation with ionic strength of the stability and aggregation of the vancomycin–peptide complex*

The experiments were performed in 0.02M-citrate buffer, pH5.0, at 26°C. Percentage aggregation was:

$$100(\Delta E^{1\%}_{1cm} \text{ at } 293\,nm)/(\Delta E^{1\%}_{1cm(A)} - \Delta E^{1\%}_{1cm(F)})$$

where $\Delta E^{1\%}_{1cm}$ at 293nm was the specific extinction difference observed on addition of peptide to vancomycin in KCl soln., $\Delta E^{1\%}_{1cm(A)}$ is the same parameter for fully aggregated complex determined by extrapolation (value 7.0±0.3) and $\Delta E^{1\%}_{1cm(F)}$ is the value for unaggregated complex also determined by extrapolation (value 0.84±0.1). K_A values for 0, 2.0 and 4.0M-KCl were calculated as indicated in the Materials and Methods section. K_A in 0.2M-KCl was estimated as described by Perkins (1969) and was therefore subject to a larger error. Stability was calculated from the K_A values, that in the absence of added KCl being taken as 100%.

Concn. of KCl (M)	$E^{1\%}_{1cm}$ at 293nm	K_A (l.mol^{-1})	Aggregation (%)	Stability (%)
0.0	1.95	1.5×10^6	32	100
0.2	2.08	1.1×10^6	34	73
2.0	3.80	1.8×10^5	62	12
4.0	5.92	7.0×10^4	96	5

Table 3. *Stability of vancomycin–peptide complexes in the presence of sodium dodecyl sulphate*

Experiments were performed at 26°C in 0.02M-citrate buffer, pH5.1. Approximate association constants for complex-formation with added peptide were calculated as described by Perkins (1969). Stability was calculated as described in Table 2.

Concn. of detergent (%, w/v)	K_A (l·mol^{-1})	Stability (%)
0.00	1.5×10^6	100
0.10	4.0×10^5	27
0.38	1.3×10^5	9
0.95	2.5×10^4	2
9.50	1.4×10^5	9–10

Table 4. *Stability of the vancomycin–peptide complex in urea solution*

Experiments were performed at 26°C in 0.02M-citrate buffer, pH5.0. K_A was calculated as indicated in the Materials and Methods section. Stability was calculated as described in Table 2.

Concn. of urea (M)	K_A (l·mol^{-1})	Stability (%)
0.0	1.5×10^6	100
4.0	3.7×10^5	25
8.0	5.0×10^4	3

From a plot of log K_A against $1/T$(°K) the enthalpy change for the association reaction can be estimated as $\Delta H = -1700\pm200$ cal/mol and from the K_A value at 25°C the change in free enthalpy (Gibb's free energy) ΔG can be determined as -8500 ± 100 cal/mol. The entropy change for complex-formation at 25°C deduced from the above values is $\Delta S = 23$ cal/°K. Complex-formation is thus an exothermic process but it is also entropy-driven. The association itself must restrict the freedom of both vancomycin and peptide and the entropy change associated with it should be negative. The most likely explanation left is that combination in some way produces a disordering effect on the solvent.

Table 5. *Stability of the vancomycin–peptide complex at different temperatures*

Experiments were performed in 0.02M-citrate buffer, pH5.1. K_A was determined from Scatchard plots as indicated in the Materials and Methods section. The error is approx. $\pm0.2\times10^6$ for 26°C and 30°C and $\pm0.2\times10^5$ for 40°C and 50°C. Stability was calculated as described in Table 2.

t (°C)	K_A (l·mol^{-1})	Stability (%)
26	1.5×10^6	100
30	9.0×10^5	60
40	5.0×10^5	33
50	3.3×10^5	20

DISCUSSION

The interaction of vancomycin with specific peptide is complex. Some factors that may be involved are ionic interaction, formation and possibly breakage of hydrogen bonds, steric repulsion (Nieto & Perkins, 1971), hydrophobic bond formation and aggregation of vancomycin and the vancomycin–peptide complex. The interaction of these factors in the absence of detailed knowledge of the chemical structure of vancomycin is difficult to interpret. However, the progress made in the

chemistry of the related antibiotic ristomycin (Lomakina, Bognar, Brazhnikova, Sztaricskai & Muravyeva, 1970*a*) encourages the hope that before long the precise way in which these antibiotics form complexes with peptide related to the structure and biosynthesis of bacterial cell walls will be understood.

A possible model for the binding would involve hydrogen-bonding between the phenols of vancomycin and the carbonyl groups in the peptide (Nieto & Perkins, 1971), thus explaining the decreased ionization of phenols after complex-formation. It is equally possible that the phenolic hydroxyl groups lie inside a cleft at the binding site and that the peptide merely shuts off the cleft and hinders the access by solvent. In the latter model, ionization of the phenols would decrease the affinity by electrostatic repulsion.

As shown by acid–base titration the phenols in vancomycin behave anomalously. It is not possible to identify unambiguously which of the phenolic groups belong to 'simple' phenols and which to chlorophenols; the position of the maximum of absorption of the phenoxide ion changes from 301 to 306nm as titration proceeds, which suggests that the higher pK value phenols are chlorophenols. If this were true their behaviour would be even more anomalous.

The spectrophotometric titration curve for iodovancomycin indicates that the phenol with pK 9.6 is the one iodinated. This iodination, as described in the Materials and Methods section, takes place very easily; also a diazonium coupling reaction described by Perkins (1969) takes place under adverse conditions (acid pH) very easily, and presumably the same phenol is responsible for it. The last two results suggest that this phenol is highly susceptible to nucleophilic attack and this is difficult to reconcile with a pK value of 9.6.

A comparison of the u.v. difference spectra induced by peptide on vancomycin and iodovancomycin suggests that the phenol iodinated is responsible for the positive difference at 293nm that correlates with aggregation. Since the interaction associated with this perturbation is not necessary for complex-formation to take place and seems rather to be associated with some kind of steric hindrance (Nieto & Perkins, 1971), this phenol may be in the 'surface' of the molecule. The fact that iodination prevents neither combination with peptide nor antibiotic activity is thus reasonable.

The observation of difference spectra during complex-formation between specific peptide and vancomycin or ristocetin (Perkins, 1969) has been confirmed by Best, Grastie & McConnell (1970). These authors found that 1 mol of peptide combines with 3200g of vancomycin, in contrast with the value of about 1800g observed in the present work. It is not possible to account for this discrepancy, however, since Best *et al.* (1970) do not give details of their titration experiments. The value of 1800g has been amply confirmed by the work with a range of analogous peptides (Nieto & Perkins, 1971).

Studies on ristomycin and actinoidin (Lomakina, Spiridonova, Bognar, Puskas & Sztaricskai, 1968*c*; Lomakina, Muravyeva & Puskas, 1968*b*; Lomakina *et al.* 1968*a*; Lomakina, Zenkova & Yurina, 1969; Lomakina *et al.* 1970*b*) have brought progress in the chemistry of the vancomycin group of antibiotics. Indeed, Lomakina *et al.* (1970*a*) have proposed a tentative structure for the aglycone of ristomycin A, an antibiotic closely related to the ristocetins. The central feature is a cyclic dipeptide composed of two diaminodicarboxylic acids, each consisting of two residues of hydroxylated phenylglycine joined by an ether link. Additional hydroxyl groups are also present, so that all four aromatic rings are phenolic. A structure of this general type would be consistent with the results reported here for vancomycin.

A sample of ristomycin A kindly supplied by Professor G. F. Gause was tested for complex-formation with diacetyl-L-lysyl-D-alanyl-D-alanine by the difference spectrum method. The resulting curve was closely similar to that observed with ristocetin B (Perkins, 1969). Hence it may reasonably be argued that results obtained for the combining site of one of these antibiotics may serve as a guide in the elucidation of the others.

We are deeply indebted to Dr P. M. Bayley for expert advice and invaluable help in obtaining the C.D. data. Excellent technical assistance was provided by Mr I. D. Bird. Thanks are given to Mrs G. Ostler and Miss S. Lathwell for microanalytical data.

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