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COUMARIN ANTIBIOTIC BINDING TO HUMAN ALBUMIN

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Abstract—Equilibrium dialysis has been used to measure the binding of clorobiocin (18631 R.P.) to human albumin. A program has been written in BASIC for a Wang system 2200 that will find the binding constants and the number of binding sites by an iterative procedure. A second program calculates the percentage free drug in equilibrium with whole serum at any concentration of drug that is required. At 4% albumin concentration and 10^{-5}M drug, clorobiocin is found to be 99.99 per cent bound, novobiocin 99.59% and coumermycin A_1 99.63% bound. These calculated figures are found to compare reasonably well with literature values.

Clorobiocin [1] is an extremely potent antibiotic *in vitro* [2]. In the presence of serum its activity is greatly reduced*. In fact clorobiocin is bound more tightly to serum than novobiocin and coumermycin A_1, two other members of the 4-hydroxycoumarin family of antibiotics, which are themselves bound between 95 and 99 per cent at drug concentrations between 5 and 20 μg/ml [3–5]. At clorobiocin concentrations between 10^{-4}M and 10^{-5}M in equilibrium with plasma, the free drug concentrations is in the range 10^{-8} to 10^{-9}M. In the context of a synthetic modification program for the antibiotic [6, 7] it became important to monitor the binding. To measure this low level of drug accurately by physiochemical means, radiolabelling of the drug would have been required. As an alternative we decided to develop another method of measuring drug binding to protein based on equilibrium dialysis.

Equilibrium dialysis has been proposed as the most effective method of measuring protein binding [8, 9]. The techniques available are either tedious and inaccurate, involving the tying of a large number of dialysis bags or expensive, if an instrument has to be purchased. We developed some equilibrium dialysis units which are reliable, easy to use, and inexpensive. They can be used to measure the binding of any ligand to macromolecule, and we have studied the binding of salicylic acid [10] and acebutolol [11] to plasma proteins. Clorobiocin was too tightly bound to use whole plasma, so we used low concentrations of human albumin (H.A.) to obtain sufficient data to allow the calculation of binding constants and number of binding sites by an iterative computer program written in BASIC for a Wang system 2200. These values were then put into a program, given to us by Dr. R. F. Mais, to give the percentage free drug in equilibrium with whole plasma [12].

* J. A. Sharp, personal communication.

MATERIALS AND METHODS

Equilibrium dialysis. The equipment consisted of two specially constructed Perspex blocks, between which was sandwiched a Spectrapor semi-permeable membrane, forming the complete equilibrium dialysis unit with six compartments (figure 1 in ref. 10). To carry out an experiment 5 units were used to accommodate 28 concentrations of drugs and two blanks (to allow for non-specific ultraviolet absorbance). The units were then placed in a water-bath at a suitable temperature.

For binding measurements clorobiocin and coumermycin A_1 were initially dissolved in methanol at 10^{-2}M concentration and diluted 1 in 100 into 0.05M phosphate buffer. The presence of 1% methanol was found not to alter the drug binding. The sodium salt of novobiocin was dissolved directly in buffer. 2 ml of drug solution were placed on one side of the membrane and 2 ml of human albumin (H.A.) solution on the other. Concentrations of drug between 10 and 100 μM were used. Clorobiocin binding was assayed with a concentration of 0.3 mg/ml, novobiocin at 0.5 mg/ml, and coumermycin A_1 at 1 mg/ml albumin. A round plastic cap was placed on each compartment to prevent evaporation, and the units were shaken in a water-bath at 37° for 8 hr and then allowed to stand at 25° for 8 hr.

Preliminary experiments to test the rate of equilibration indicated that molecules of weight lower than 200 daltons equilibrated in about 2–3 hr, while clorobiocin, molecular weight 695, takes at least 8 hr at 37°. The molecular weight of novobiocin is 612 while that of coumermycin A1 is 1152. In order to ensure equilibration in each case therefore, the units were left a further eight hours at 25°. The lower temperature was used to minimise the risk of deterioration of the protein, although Cannon, Olitzky and Inkpen [13] could find no change in the electrophoretic pattern of serum stored at 30° for 4 days, compared with −20° for 6 months. Since all three compounds are broad spectrum antibiotics [5] it was not thought necessary to test for bacterial contamination.

The drug concentrations were determined by measuring the ultraviolet absorbance at 315 nm of aliquots taken from both sides of the membrane. This wavelength is not the absorbance maximum but is the wavelength at which there is no change in absorbance when drugs bind to H.A., as measured by different spectrum. The free concentration was subtracted from the total drug concentration on the albumin side to give the amount of bound drug. These data points were then fed into the computer program to give binding constants and the number of binding sites.

Calculations. Binding constants and the number of binding sites were obtained from the binding data with an iterative computer program devised in our laboratories. The raw data were fitted by non-linear regression analysis to the equation:

$$r = \frac{n_1 k_1 T}{1 + k_1 T} + \frac{n_2 k_2 T}{1 + k_2 T},$$

where r is bound, T is free drug, n_1, n_2 are the number of primary and secondary binding sites respectively, and k_1, k_2 the respective binding constants. The inclusion of a third set of binding sites was found not to improve the fit.

Arbitrary values for n_1, n_2, k_1, k_2 were entered, and a standard deviation (S.D.) of the curve from the points calculated. The value n_1 is increased by 5 per cent, k_1 and k_2 are kept constant and the best value for n_2 is selected on the basis of minimal S.D. If this S.D. is lower than the previous one, then n_1, is further increased by 5 per cent and the best value for n_2 obtained. If the second S.D. is higher, then a 5 per cent reduction in n_1 is made and calculation carried out as before. The values selected for n_1 then continue to fall by 5 per cent until the S.D. starts to rise, at which point 1 per cent increments are made to n_1 until the lowest S.D. and corresponding n_2 value are obtained. This value for n_2 is retained while first k_1 and then k_2 are treated in the same way to achieve one complete iteration. The whole process is repeated. At the next stage n_2 takes the place of n_1 and the process is repeated. A second iteration follows. The process is considered complete when an entire cycle of four iterations (2 on n_1 and 2 on n_2) does not improve the S.D. by more than 0.1 per cent. The minimum S.D. obtained (M.S.D.) is reported in Table 1 as an average of the three or more runs done on each compound. The percentage free compound at 10^{-5}M in equilibrium with 4 per cent albumin was then computed [12]. This indicated how much free compound would be present in plasma.

Difference spectrum. 1 ml each (Emil green line pipettes) of drug solution at 36 μM and albumin at 0.3 mg/ml (final concentration in both cases) were placed either side of the junction in a split compartment cell (Light-path) with 1 cm path-length. The cells were placed in a Cary 17 recording spectrophotometer and a base-line obtained. The sample cell was inverted and the two compartments thoroughly mixed. The difference spectrum was thus obtained. The reference cell was inverted, thoroughly mixed, and scanned to show that the final base-line did not differ significantly from the original. Any scans that did differ by more than 0.005 units at any wavelength from 230–350 nm were discarded.

Preparation of monomeric H.A. Samples of H.A. as supplied were found to contain dimerized material, and the dimer bound clorobiocin less tightly (see Table 2). It was necessary therefore to isolate the albumin monomer before use. 400 mg of H.A. were loaded on to a Sephadex G.100 column (53 × 2.5 cm), and eluted with 0.05 M phosphate buffer pH 7.4 at a flow-rate of 10 ml/hr at 4°. 15 ml fractions were collected and the ultraviolet absorbance read at 280 nm. Two peaks were obtained—one eluting after 135 ml had passed through the column, and the second after 180 ml. The fractions from the second peak were combined and concentrated in an Amicon 402 ultrafiltration cell over a PM-10 membrane. When the volume had fallen to about 30 ml, 150 ml of distilled water were passed through the cell to remove the buffer. The solution was freeze-dried and the protein stored at −20° as advised by Pickart and Thaler [14]. A figure of 0.531 for the absorbance at 280 nm of a 1 mg/ml solution H.A. was used throughout [15]. In order to keep the H.A. in a state resembling the physiological it was not delipidated.

Materials. Clorobiocin was obtained from Rhone-Poulenc by kind permission of Dr. Jolles. Novobiocin sodium salt was from B.D.H., coumermycin A_1 was obtained from Bristol/Myers, while Miles supplied the H.A. as fraction V crystallized from outdated human plasma. All other reagents were analytical reagent grade.

The original equilibrium dialysis units were made in our workshops. They are now available from Universal Scientific, 231, Plashet Road, London, E13 0QU, UK.

RESULTS

A representative run is shown in Table 1 for novobiocin binding to albumin. The original drug concentration refers to the concentration of drug placed on one side of the membrane. When the drug has equilibrated the concentration either side of the membrane would be half the original. Taking the average of both sides after incubation (bound + free and free) we can see that there are effectively no losses due to binding to the Spectrapor membrane and cell materials.

Figure 1 shows a Scatchard plot for clorobiocin binding to human albumin. The iterative procedure indicates two sets of binding sites, one tightly bound with few sites, the other less tightly bound with a larger number of sites. The data for all three compounds together with published data for novobiocin [15] are shown in Table 2, where number of experiments indicates the number of runs of 28 concentrations of drug carried out as described in the Methods section. From this data, at a drug concentration of 10^{-5}M novobiocin was calculated to be 0.41 per cent free, clorobiocin was 0.01 per cent, and coumermycin A_1 was 0.37 per cent free. All these figures have been calculated with respect to equilibrium with 4% H.A., and have been revised with respect to those published earlier [7].

The difference spectrum for clorobiocin binding

Table 1. Clorobiocin binding data

Initial drug concn (μm)	Free drug concn (μm)	Bound and free drug concn (μm)
8.0	0.81	6.86
	0.81	6.86
10.0	1.48	8.09
	1.71	8.67
15.0	3.24	10.71
	3.24	10.81
20	5.99	13.76
	5.33	13.85
25	8.09	17.57
	8.09	17.95
30	10.38	20.00
	10.62	20.00
35	13.14	23.23
	13.19	23.33
40	15.47	25.85
	15.33	25.61
45	17.90	29.99
	18.19	29.23
50	20.14	34.47
	19.99	34.02
55	23.14	39.66
	23.33	35.85
60	25.14	39.66
	25.23	39.84
80	36.37	51.42
	35.71	51.42
100	46.99	62.85
	47.04	60.94

to H.A. is shown in Fig. 2. The main features are troughs at 250 and 300 nm and a peak at 325 nm. The molar absorptivity of clorobiocin at 315 nm, where there is no absorbance change on complex formation with the range of drug concentrations under study, was 15,600. The ultraviolet spectrum of clorobiocin in phosphate buffer and in dioxan is given in Fig. 3. The maximum absorbance is observed at 278 nm in buffer and 272 nm in dioxan, and is characteristic of the pyrrole and coumarin nuclei [16, 17]. The shoulder at 305 nm in buffer appears to have moved to 335 nm in dioxan.

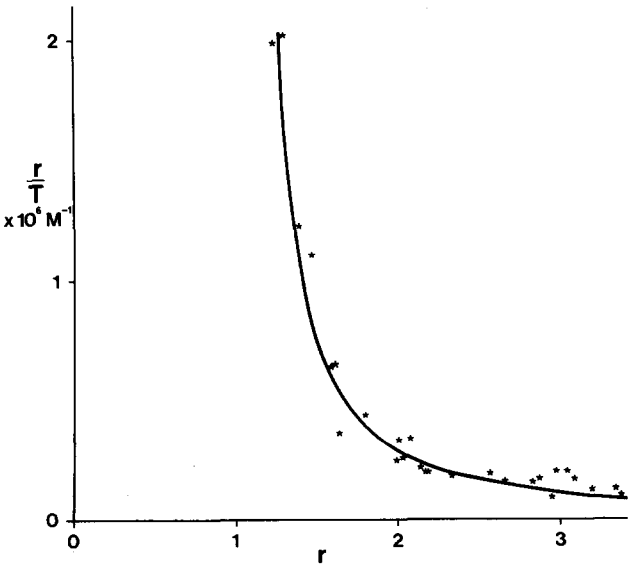

Fig. 1. Scatchard plot for the binding of clorobiocin to human albumin (0.3 mg/ml) in 0.05M phosphate buffer, pH 7.1, 25°. *Represent the experimental points and the solid line is the calculated line of least squares best fit.

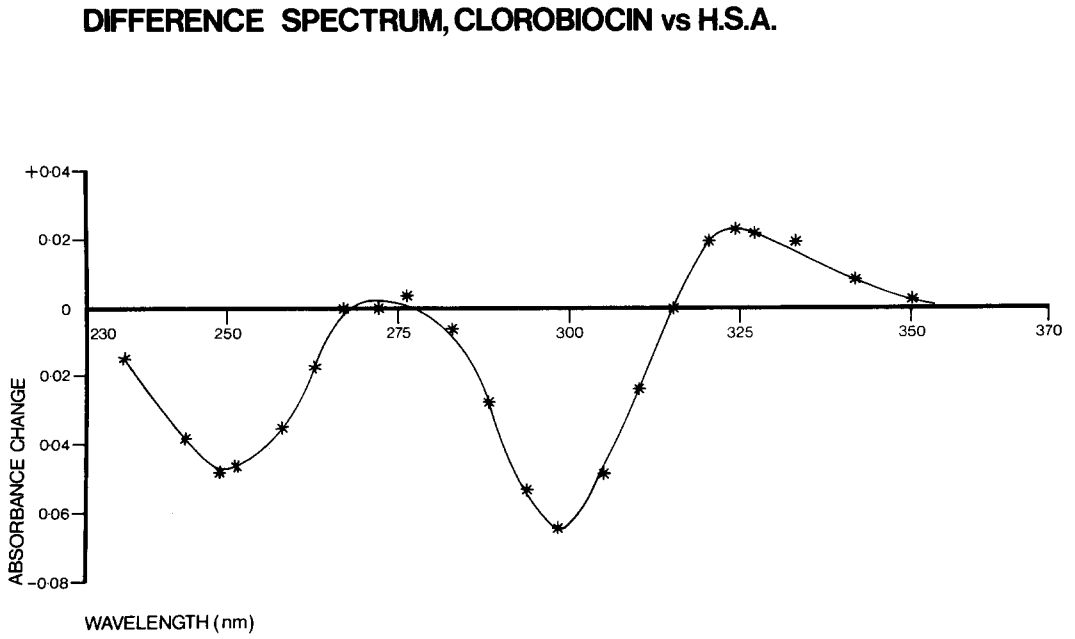

Fig. 2. Clorobiocin (36 μM) was mixed with H.A. (0.3 mg/ml) (final concentration in both cases) in 0.05M phosphate buffer pH 7.4, 25°. *Represent the points at which the absorbance change was measured from the spectrophotometer trace. The line was drawn to connect these points.

Table 2. Data for antibiotics binding to human albumin

Compound	No. of experiments	n_1	n_2	k_1 litre/mole $\times 10^{-6}$	k_2 litre/mole $\times 10^{-4}$	M.S.D.
Novobiocin*	3	1.18 ± 0.12	3.15 ± 0.38	0.25 ± 0.11	3.28 ± 0.62	1.403
Novobiocin†	—	1.0	1.0	37.1	3.90	—
Clorobiocin I	8	1.97 ± 0.24	6.33 ± 1.32	41.9 ± 26.5	2.32 ± 0.64	1.334
Clorobiocin II	4	2.77 ± 0.38	4.48 ± 0.03	3.72 ± 1.91	1.78 ± 0.64	1.395
Coumermycin A_1	3	1.21 ± 0.28	2.15 ± 0.38	0.25 ± 0.03	7.37 ± 2.64	1.611

I Binding to albumin monomer and II dimer.
* This work.
† Brand and Toribara [15].

DISCUSSION

The figures for the number of binding sites and binding constants for novobiocin agree reasonably well with those obtained by Brand and Toribara [15], who used equilibrium dialysis at 30° albeit with twice the concentration of H.A.

The number of binding sites (n) and binding constants (k) on human plasma albumin for the 4-hydroxycoumarin antibiotics is similar to the figures for n of 1.6 to 2.6 and for k of 5.9 to 42×10^4 litres/mole obtained for the warfarin group of anticoagulants by O'Reilly [18]. The latter group also contains the 4-hydroxycoumarin nucleus. It is probable that both series bind to the same site on human albumin, and it would be interesting to know whether there is a competition between the two series of compounds. Dicoumarol is partially displaced by a wide series of acidic drugs [19].

Godfrey and Price [5] reported that coumermycin A_1, temperature and drug concentration unstated, was 98–99 per cent bound to fresh human serum as measured by both equilibrium dialysis and a microbiological plate assay. Scholtan and Schmidt [3] found that 3 per cent of novobiocin at 200 μg/ml and 37° was free in equilibrium with serum using equilibrium dialysis, while Rolinson and Sutherland [4] found 0.8 per cent free at 100 μg/ml drug concentration using ultrafiltration with human serum at room temperature. Clorobiocin at 100 μg/ml is 0.08 per cent free by ultrafiltration with human serum and bacteriological assay at 25°*. The calculation of percentage free drug from the initial binding data thus gives results of the correct order of magnitude, and confirms the usefulness of the equilibrium dialysis units. Although the binding constants in some cases lie outside the experimental concentration limits and thus are extrapolations, the accuracy

* J. A. Sharp, personal communication.

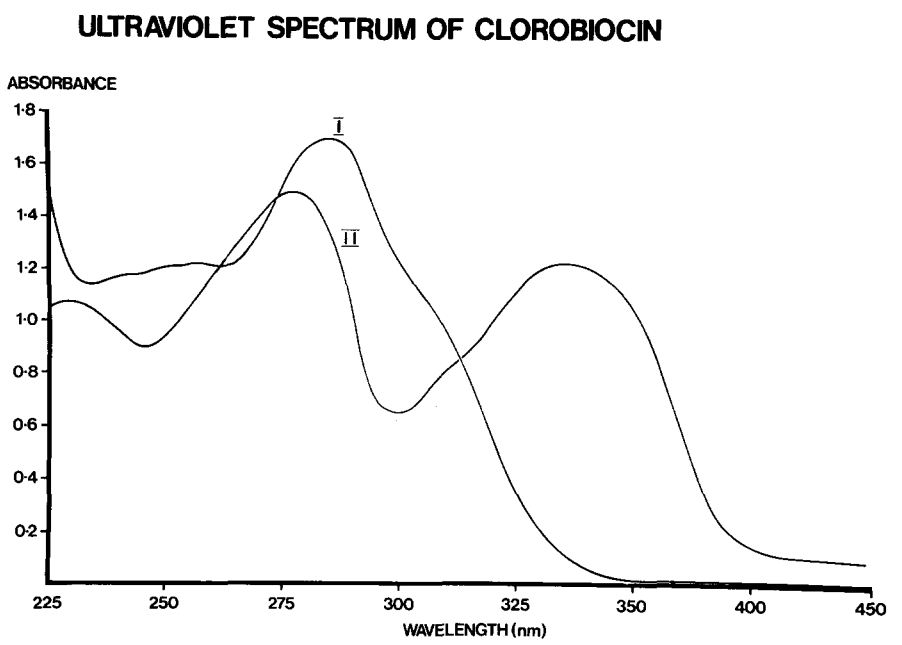

Fig. 3. The spectrum of clorobiocin (50 μM) is shown in 1 cm path length cells: I: 0.05M phosphate buffer, pH 7.4. II: dioxan.

of the extrapolations is borne out by the agreement with *in vitro* results with whole serum.

We should however be aware that there are situations where this extrapolating technique will not work i.e. when the major binding protein for the drug is not albumin. Propranolol binds primarily to a α-acid glycoprotein [20]. The discrepancy between the binding of propranolol to human plasma of 90–96 per cent [21] and 42 per cent to solutions of human albumin at physiological concentrations [22] is thereby explained. In addition there may be inhibitors of albumin binding in blood, and thus the extrapolation would over-estimate the percentage bound drug in plasma.

Fletcher and Spector [23] criticise the use of non-integral coefficients for n_1 and n_2. We obtain non-integral coefficients possibly because we use H.A. with lipid attached. To use delipidated H.A. would be to reduce, we believe, the physiological validity of our data.

The mode of binding of clorobiocin to H.A. may be indicated by the difference spectrum. The trough at 250 nm coincides with the absorbance maximum of the *p*-hydroxybenzamide portion of the molecule. The trough at 300 nm is close to the main peak of the 4-hydroxycoumarin group [16, 17]. The increased absorbance at 325 nm is of interest as it resembles the increased absorbance in that region when clorobiocin is dissolved in dioxan, and when novobiocin is acidified [24]. It is likely that in both of these situations the spectrum is that of the unionized species, since, at physiological pH, novobiocin is present as a singly-charged anion with a pK_a of 4.3 [25]. This suggests that the binding site on the albumin molecule is hydrophobic, a conclusion also reached for the warfarin anti-coagulants by O'Reilly [18]. In addition the binding site may also contain a cationic group which is able to protonate the 4-hydroxy group on the coumarin nucleus—the source of the negative charge. This is the group that appears to make a critical contribution to the binding of the warfarin type of structure [18]. The lipophilicity of the substituents on the antibiotic molecule then modulates the binding [7].

The probable involvement of different parts of the molecule in the binding spectra suggests that the entire molecule is concerned in the binding process. This conclusion is consistent with our finding that the more lipophilic analogues of clorobiocin bind to H.A. more tightly, but the position at which the lipophilicity is changed is not critical [7].

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REFERENCES

1. L. Ninet, F. Benazet, Y. Charpentie, M. Dubost, J. Florent, D. Mancy, T. Preud'homme, T. L. Threlfall, B. Vuillemin, D. E. Wright, A. Abraham, M. Cartier, M. de Chezelles, D. Godard and J. Theilleux, *C. R. Acad. Sci. Paris* (C), **275**, 455 (1972).
2. D. Mancy, L. Ninet and J. Preud'homme, British Patent 1,208,242 (1970).
3. W. Scholtan and J. Schmidt, *Arzneim-Forsch.* **13**, 288 (1963).
4. G. N. Rolinson and R. Sutherland, *Br. J. Pharmac.* **25**, 635 (1965).
5. J. C. Godfrey and K. E. Price, *Adv. appl. Microbiol.* **15**, 231 (1972).

6. C. J. Coulson and V. J. Smith, *Enzyme Microb. Technol.* **1**, 193 (1979).
7. C. J. Coulson and V. J. Smith, *J. Pharm. Sci.* **69**, 799 (1980).
8. W. E. Lindup, *Biochem Soc. Trans.* **3**, 635 (1975).
9. H. Kurz, H. Trunk and B. Weitz, *Arzneim.-Forsch.* **27**, 1373 (1977).
10. T. J. Coombs and C. J. Coulson, *Biochem. Soc. Trans.* **6**, 1048 (1978).
11. T. J. Coombs, C. J. Coulson and V. J. Smith, *Br. J. Clin. Pharmac.* **9**, 395 (1980).
12. R. F. Mais, S. Keresztes-Nagy, J. F. Zaroslinski and Y. T. Oester, *J. Pharmac. Sci.* **63**, 1423 (1974).
13. D. C. Cannon, I. Olitzky and J. A. Inkpen, in *Clinical Chemistry, Principles and Technics* (Eds. R. J. Henry, D. C. Cannon and J. W. Winkelman) 2nd edn, p. 442. Harper and Row, Hagerstown, Maryland (1974).
14. L. Pickart and M. M. Thaler, *Biochem. biophys. Res. Commun.* **74**, 961 (1977).
15. J. G. Brand and T. Y. Toribara, *Archs. Biochem. Biophys.* **167**, 91 (1975).
16. M. J. Mehta, R. S. Hegde, M. A. Bhatt, D. J. Patel and S. L. Bafna, *J. appl.* Chem. **19**, 29 (1969).
17. J. Mendez and M. I. Lojo, *Microchem. J.* **13**, 506 (1968).
18. R. A. O'Reilly, *Molec. Pharmac.* **7**, 209 (1971).
19. J. H. Perrin, J. L. Vallner and D. A. Nelson, *Biochem. Pharmac.* **24**, 769 (1975).
20. K. M. Piafsky, O. Borga, I. Odar-Cederlof, C. Johansson and F. Sjoquist *New Engl. J. Med.* **299**, 1435 (1978).
21. J. L. Anderson, D. C. Harrison, P. J. Meffin and R. A. Winkle, *Drugs* **15**, 271 (1978).
22. C. A. Applegren, K. O. Borg, R. Elofsson and K. A. Johansson, *Acta Pharmac. Suec.* **11**, 325 (1974).
23. J. E. Fletcher and A. A. Spector, *Molec. Pharmac.* **13**, 387 (1977).
24. J. G. Brand and T. Y. Toribara, *Molec. Pharmac.* **8**, 751 (1972).
25. R. M. Smith, J. J. Perry, G. C. Prescott, J. L. Johnson and J. H. Ford, *Antibiotics Annual* 43, (1957–1958).