Table IV—Mean Total Plasma Drug Levels (Expressed as Nordiazepam) after Oral Administration of 6.5 mg of Clorazepate Monopotassium (I) and 7.5 mg of Clorazepate Dipotassium (II) to Dogs (12 Dogs/Compound)

Compound	Plasma Nordiazepam[a], μg/ml											Area under Curve[a]	
	0 hr	0.5 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	12 hr	24 hr	0–4 hr	0–12 hr
I													
Mean	0	0.23	0.51	0.47	0.34	0.22	0.10	0.05	0.02	0.01	0	1.12	1.41
SD		±0.21	±0.24	±0.19	±0.13	±0.09	±0.05	±0.03	±0.02	±0.02		±0.43	±0.56
Range		(0.00 −0.69)	(0.24 −1.16)	(0.28 −0.94)	(0.17 −0.58)	(0.07 −0.36)	(0.02 −0.20)	(0.01 −0.10)	(0.01 −0.07)	(0.00 −0.05	0.00 −0.01	0.60 −2.10	0.71 −2.80
II													
Mean	0	0.36	0.61	0.50	0.35	0.21	0.11	0.05	0.03	0.01	0	1.26	1.56
SD		±0.15	±0.14	±0.17	±0.13	±0.09	±0.06	±0.03	±0.02	±0.01		±0.34	±0.53
Range		(0.10 −0.62)	(0.42 −0.85)	(0.25 −0.84)	(0.16 −0.60)	(0.08 −0.36)	(0.02 −0.21)	(0.01 −0.11)	(0.00 −0.08)	(0.00 −0.04)	0.00 −0.00	0.64 −1.82	0.68 −2.34

[a] No significant differences were found at the 0.05 level.

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Hansch Analysis of Interaction of Haptens with Benzylpenicilloyl Antibodies

A. E. BIRD

Abstract □ Hansch analysis is applied to inhibition data obtained from the interaction of penicilloic and penilloic acid haptens with benzylpenicilloyl specific antibodies. Significant regressions with partition coefficient and molar volume parameters indicate the importance of hydrophobic and steric effects in these interactions.

Keyphrases □ Benzylpenicilloyl antibodies—interaction with penicilloic and penilloic acid haptens, Hansch analysis of inhibition data, hydrophobic and steric effects □ Penicilloic and penilloic acid haptens—interaction with benzylpenicilloyl specific antibodies, Hansch analysis of inhibition data □ Hansch analysis—interaction of haptens with benzylpenicilloyl antibodies □ Structure–activity relationships—interaction of penicilloic and penilloic acid haptens with benzylpenicilloyl specific antibodies

Munro *et al.*[1] studied the cross-reaction with benzylpenicilloyl specific antibodies of the penicilloic (I) and penilloic (II) acids obtained from several penicillins (III). They determined the extent of this interaction by measuring the inhibition by the haptens of agglutination of sensitized erythrocytes caused by the antibodies.

[1] A. C. Munro, M. G. Chainey, and S. R. Woroniecki, to be published.

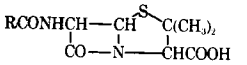
In this paper the data so obtained are subjected to Hansch quantitative structure–activity analysis to evaluate the role of various physicochemical properties of the haptens in their interaction with the antibodies. Published applications of Hansch analysis to hapten antibody interactions appear to be confined to a single paper in which Kutter and Hansch (1) showed the importance of steric factors in such interactions.

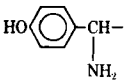
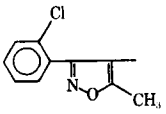
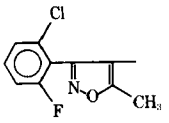
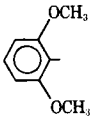
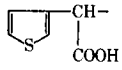
EXPERIMENTAL

The term C in log $1/C$ in Table I is the molar concentration of hapten required to give 50% inhibition of hemagglutination by benzylpenicilloyl specific antibodies.

Hydrophobic effects are modeled by log P, where P is the octanol–water partition coefficient of the parent penicillins in their unionized form. Values of P are not available for the penilloic or penicilloic acids, but the variation of P with changes in the side chain is expected to be the same for these compounds as for the parent penicillins. Log P for penicillin G (benzylpenicillin), cloxacillin, methicillin, phenethicillin, penicillin V (phenoxymethyl penicillin), and propicillin are measured values from Bird and Marshall (2). The log P values for ampicillin and carbenicillin are calculated from those for penicillin G with the appropriate hydrophobic frag-

Table I—Inhibition Data and Physicochemical Parameters for Inhibition of Erythrocyte Agglutination by Penilloic and Penicilloic Acids



Parent Penicillin	R	log P[a]	MV[a]	pKa[a]	Penicilloic Acids log 1/C Obs.[b]	Penicilloic Acids log 1/C Calc.[c]	Penicilloic Acids Δ log 1/C	Penilloic Acids log 1/C Obs.[b]	Penilloic Acids log 1/C Calc.[d]	Penilloic Acids Δ log 1/C
Amoxicillin		0.04	134.3	1.9	3.11	3.15	−0.04	—	—	—
Ampicillin		0.90	128.7	1.8	3.71	3.77	−0.06	—	—	—
Carbenicillin		1.26	145.0	5.0	4.43	3.74	0.69	3.41	2.98	0.43
Cloxacillin		2.44	193.4	3.8	3.84	3.73	0.11	2.63	2.77	−0.14
Floxacillin		2.30	204.8	3.8	3.41	3.33	0.08	2.81	2.51	0.30
Methicillin		1.13	152.9	3.7	3.41	3.44	−0.03	2.63	2.80	−0.17
Penicillin G	$C_6H_5CH_2$–	1.76	114.5	4.3	5.22	4.95	0.27	4.14	3.74	0.40
Penicillin V	$C_6H_5OCH_2$–	2.01	125.0	3.2	4.92	4.94	−0.02	3.53	3.69	−0.16
Phenethicillin		2.19	147.2	3.2	4.43	4.59	−0.16	3.23	3.41	−0.18
Propicillin		2.58	169.4	3.2	4.14	4.44	−0.30	3.11	3.24	−0.13
Ticarcillin		0.94	136.5	5.0	3.11	3.63	−0.52	2.63	2.96	−0.33

[a] See text for sources of these constants. [b] From A. C. Munro, M. G. Chainey, and S. R. Woroniecki, to be published. [c] Calculated from Eq. 2. [d] Calculated from Eq. 4.

mental constants and proximity effect (3). For example:

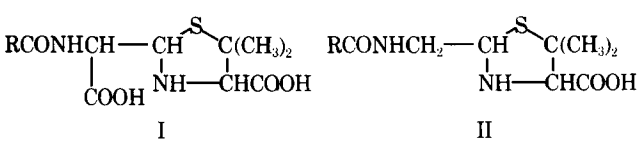
$$\log P(\text{ampicillin}) = \log P(\text{penicillin G}) - f(CH_2) + f(NH_2) + f(CH) + \text{p.e.} \quad \text{(Eq. 1}a\text{)}$$

$$\log P(\text{ampicillin}) = 1.76 - 0.52 + (-1.38) + 0.24 + 0.8 = 0.9 \quad \text{(Eq. 1}b\text{)}$$

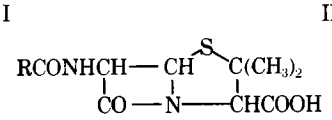
The proximity effect (p.e.) is used because the amino or carboxy group is separated by one carbon from the side-chain amide function. Log P for amoxicillin was calculated as for ampicillin plus a Hansch π term for the hydroxy group [−0.50, from substituted phenylacetic acids (4)]. Log P for floxacillin was calculated from that for cloxacillin plus a π term for the fluoro group (0.14, from substituted nitrobenzenes[2]). Log P for ticarcillin was calculated as for carbenicillin minus 0.32, which is the difference in log P between benzene and thiophene (5).

The molar volume of the side chain was used as a parameter, giving a measure of variations in size of the haptens. These values were calculated from the atomic volumes of Le Bas given by Partington (6). Where a range of values was given (*e.g.*, ring sulfur 21.6–22.5), the average was used. An average nitrogen value of 12.9 was used for the nitrogen of the isoxazole ring. The fluorine value used for floxacillin was from Exner (7) because there is no value in Le Bas' list. Exner's values for chlorine and bromine are very similar to those of Le Bas, so the fluorine value should be consistent.

The pKa values of the side-chain acids (R—COOH) were used as a measure of the variation in electronic effects with a change of side chain. The values for phenylacetic, phenoxyacetic, and phenylmalonic acids are from the literature (8). Phenylmalonic acid has pKa values of 2.6 and 5.0. The relevant value for carbenicillin was taken as that at which one carboxy group is ionized, *i.e.*, 5.0, because the side-chain carboxy of carbenicillin is ionized at the pH of the inhibition experiments. The pKa values for the side chains of amoxicillin, ampicillin, methicillin, phenethicillin, and cloxacillin are approximate values measured in these laboratories. The values for the side-chain acids of floxacillin, propicillin, and ticarcillin



I II



III

[2] A. E. Bird and A. C. Marshall, unpublished data.

were assumed to be the same as those for cloxacillin, phenethicillin, and carbenicillin, respectively.

Regression equations were obtained in the usual way by computer calculation using the method of least squares. Initially the penicilloic and penilloic acids were treated separately, but a final equation was calculated for all data.

RESULTS AND DISCUSSION

Penicilloic Acids—Linear regressions with log P, pKa, and molar volume (MV) had correlation coefficients (r) of 0.458, 0.189, and 0.371, respectively, and the t test showed that they were not statistically significant at the 90% level. Two-parameter equations in log P and pKa and in pKa and MV were also not significant, with correlation coefficients of 0.469 and 0.441, respectively; log P and MV gave:

$$\log(1/C) = \underset{(\pm 0.425)}{0.977 \log P} - \underset{(\pm 0.011)}{0.024\ \mathrm{MV}} + 5.961 \quad \begin{matrix} n & r & s \\ 11 & 0.899 & 0.350 \end{matrix} \quad \text{(Eq. 2)}$$

where n is the number of data points, s is the standard deviation from the regression, and the figures in parentheses are the 95% confidence limits on the regression coefficients. The t test shows that both terms in Eq. 2 are significant at the 99.5% level.

Addition of a pKa term to Eq. 2 gave:

$$\log(1/C) = \underset{(\pm 0.456)}{0.956 \log P} + \underset{(\pm 0.263)}{0.079\ \mathrm{pKa}} - \underset{(\pm 0.012)}{0.024\ \mathrm{MV}} + 5.725 \quad \begin{matrix} n & r & s \\ 11 & 0.906 & 0.362 \end{matrix} \quad \text{(Eq. 3)}$$

This equation has a higher standard deviation than Eq. 2, and the pKa term is not statistically significant.

Penilloic Acids—Linear regressions were not statistically significant, with correlation coefficients of 0.080, 0.001, and 0.658 for log P, pKa, and MV, respectively. Two-parameter equations in log P and pKa and in pKa and MV were also not significant, with correlation coefficients of 0.115 and 0.675, respectively; log P and MV gave:

$$\log(1/C) = \underset{(\pm 0.562)}{0.506 \log P} - \underset{(\pm 0.011)}{0.017\ \mathrm{MV}} + 4.757 \quad \begin{matrix} n & r & s \\ 9 & 0.828 & 0.333 \end{matrix} \quad \text{(Eq. 4)}$$

The t test shows that log P is significant at 90% and MV at 98%. Addition of a pKa term to Eq. 4 gave:

$$\log(1/C) = \underset{(\pm 0.785)}{0.837 \log P} + \underset{(\pm 0.575)}{0.334\ \mathrm{pKa}} - \underset{(\pm 0.011)}{0.018\ \mathrm{MV}} + 3.101 \quad \begin{matrix} n & r & s \\ 9 & 0.884 & 0.304 \end{matrix} \quad \text{(Eq. 5)}$$

Although this equation has a slightly lower s value than Eq. 4, the pKa term is not statistically justified (t found 1.488; t 90%, 5 df 2.015).

Penicilloic and Penilloic Acids—A regression for the combined data was calculated with log P, MV, and a dummy parameter assigned a value of zero for the penicilloic and of 1 for the penilloic acids; this parameter was introduced to allow for the inherent difference in activity of the two sets of acids:

$$\log(1/C) = \underset{(\pm 0.316)}{0.801 \log P} - \underset{(\pm 0.341)}{0.930\ (0/1)} - \underset{(\pm 0.007)}{0.021\ \mathrm{MV}} + 5.780 \quad \begin{matrix} n & r & s \\ 20 & 0.903 & 0.353 \end{matrix} \quad \text{(Eq. 6)}$$

The t test shows that all three terms are significant at the 99.9% level.

Intuitively, one would not expect $(\log P)^2$ to be a significant factor in describing this type of biological activity, because the inhibition apparently depends on a single-step binding of hapten to antibody. Therefore, multiple interactions in a "random walk" to the active site, which can produce (9) parabolic dependence of activity on the partition coefficient, do not occur. However, $(\log P)^2$ terms were tried in some regressions. For penilloic acids, the two-parameter equation in log P and $(\log P)^2$ had both terms significant at the 95% level, but the r and s values of 0.751 and 0.393, respectively, were poor. In other cases, particularly when $(\log P)^2$ was added to the log P and MV regressions, $(\log P)^2$ terms were not significant.

Hansch (10) pointed out that partition coefficient and molar volume are not independent variables for simple substituents. The collinearity of these variables for the 11 compounds studied here was 33% ($r^2 = 0.33$). This value shows that although the variables are not completely independent, they are not very highly correlated and their use in the same equation to model different aspects of the binding process seems to be justifiable (11).

Thus, Eqs. 2, 4, and 6 are the best regressions obtained. Although they are statistically significant, the standard deviations of these regressions are quite high, due in part perhaps to experimental error in the inhibition data. A twofold dilution method was used to obtain these data, so the experimental error may be quite large and the regressions may be as good as can be expected with data of this accuracy. It is also possible that the effect of the large variations in the side chain of the compounds studied cannot be fully explained by the simple models used. For example, Kutter and Hansch (1) showed that separate parameters are required in regression equations for *ortho*-, *meta*-, and *para*-substituents in benzoic and arsonic acid haptens. This indicated a fine degree of dependence of the interaction with antibodies on the steric properties of the haptens. It is not possible to analyze the present data in such detail. However, some useful conclusions can be drawn from the regression analysis.

The nonsignificance of pKa indicates that electronic effects induced by variation of the side chain can play only a minor part in the binding of hapten to antibody.

The positive and negative coefficients for log P and MV, respectively, show that inhibitory activity increases with an increase in log P and a decrease in MV. This finding is consistent with an increase in hydrophobic binding of hapten to antibody as log P increases and with a decrease in binding due to steric or bulk effects as MV increases. Corresponding regression coefficients in Eqs. 2 and 4 are not significantly different (the 95% confidence limits overlap), indicating that the dependence of activity on log P and MV is similar for penilloic and penicilloic acids, although the data are not sufficiently precise to be certain of this dependence. However, the good fit of all data obtained with Eq. 6 encourages the view that the interaction of both sets of haptens with antibody depends in the same way on log P and MV.

The cause of the near 10-fold difference in the inhibitory activity of corresponding penilloic and penicilloic acids is of interest. The log P and MV values for the penicilloic acid whole molecule (not just the side chain) are less and greater, respectively, than the values for the corresponding penilloic acid. Since inhibitory activity is promoted by an increase in log P and a decrease in MV, penilloic acid would be expected to be more active than the corresponding penicilloic acid if these were the only factors involved in the interaction of the haptens and antibody. Thus, it is clear that some overriding factor (presumably binding *via* the C_6 carboxy group) promotes the activity of penicilloic acids relative to that of penilloic acids.

Examination of the differences between observed and calculated values of log $(1/C)$ in Table I shows particularly high differences for carbenicillin and ticarcillin in the penicilloic acid series and for carbenicillin in the penilloic acid series. The estimated log P values for these compounds may be open to question. However, a systematic error in log P or MV cannot explain the high differences because it would affect both compounds in the same way and the sign of the difference is positive for carbenicillin and negative for ticarcillin. The high differences for these compounds and for benzylpenilloic acid may be due to random errors in the data.

The fragmental constant of the carboxy group used to calculate log P for carbenicillin and ticarcillin relates to an unionized carboxy group (3), so that log P relates to the unionized penicillin. At the pH of the inhibition experiments (7.2), the side-chain carboxy group will be ionized, so log P for the molecule with this group ionized might be more appropriate. Hansch (12) gave a difference in π value between neutral and ionized carboxy groups of 4.1, which gives log P values of −2.84 and −3.16 for carbenicillin and ticarcillin, respectively, with the carboxy group ionized.

Use of these values in log P and MV regressions gave nonsignificant log P terms. Omission of carbenicillin and ticarcillin from the regressions had only slight effects on the regression coefficients and the r and s values given in Eqs. 2 and 4. Thus, it is clear that the activity of the carboxy compounds fits with that of the other

penicillin derivatives when log P for the unionized form is used rather than log P of the apparently more appropriate ionized form. Similar behavior was found in relating log P to the extent of binding of carbenicillin and ticarcillin to serum proteins[2]. Possibly this effect is coincidental due to ionic binding by the side-chain carboxy group to an extent approximately sufficient to offset the low hydrophobic binding due to the ionic nature of this group.

Nishida *et al.* (13) reported binding constants (k_{rel}) relative to benzylpenicilloic acid for binding to benzylpenicilloyl specific antibodies of some penicilloic acids not studied in this work. Included were the penicilloic acids of *o*-, *m*-, and *p*-chloro- and *o*-, *m*-, and *p*-nitrobenzylpenicillins and of methyl- and ethylpenicillins. Equation 2 was used to calculate values of log (1/C) (and of k_{rel} using the experimental C value for benzylpenicilloic acid) for these compounds. This equation cannot distinguish the effect of varying the substituent position in the benzene ring, but values were calculated for chloro- and nitrobenzylpenicilloic acids using the appropriate MV values (131.1 and 143.7, respectively) and log P values (2.47 and 1.48, respectively) calculated from those of penicillin G with Hansch π values of 0.71 and −0.28 for chloro and nitro, respectively (14). The k_{rel} values obtained were 1.02 and 0.06 for the chloro and nitro compounds, respectively. These values are reasonably close to the experimental values of 1.36–5.17 for the three chloro compounds and 0.115–0.33 for the three nitro compounds, especially when it is recalled that Nishida *et al.* (13) used a fivefold dilution technique for their determinations so their results may not be very precise.

The log P value for methylpenicillin was calculated in two ways: (*a*) from log P penicillin G minus π phenyl, 1.76 − 1.89 = −0.13; and (*b*) from log P heptylpenicillin (15) minus 6 π methylene, 3.32 − 3.0 = 0.32. The mean value of 0.22 is used, and log P of ethylpenicillin then becomes 0.72 by addition of π methylene. Substitution of these values with MV values of 25.9 and 48.1 in Eq. 2 leads to k_{rel} values of 2.16 and 1.96 for methyl- and ethylpenicilloic acids, respectively.

These values are very different from Nishida *et al.*'s experimental values of 0.009 and 0.016. Thus, penicilloic acids with such small side chains are outside the range of applicability of Eq. 2. Intuitively, it seems probable that Eq. 2 will be applicable only to a limited range of penicilloic acids, presumably with log P and MV values centered about those of benzylpenicilloic acid because the antibodies were raised to a benzylpenicilloyl antigen. Experiments with antibodies raised to other penicilloyl antigens would be interesting in this context.

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Cytotoxic Principles of *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae)

W. H. MARKS, H. H. S. FONG ˣ, M. TIN-WA, and N. R. FARNSWORTH

Abstract □ Systematic fractionation of the cytotoxic extracts of the roots of *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae) on silica gel column chromatography led to the isolation of three cardenolides: cymarin, strophanthidin, and a strophanthidin glycoside (XS-89). Also isolated during the investigation were β-sitosterol-β-D-glucoside, a mixture of α- and β-amyrins, a mixture of alkanols, and a mixture of plant sterols.

Keyphrases □ *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae)—isolation and identification of cytotoxic principles □ Cytotoxicity—isolation and identification of cymarin, strophanthidin, and strophanthidin glycoside from *Parquetina nigrescens* □ Cardenolides—isolation and identification from *Parquetina nigrescens*

Parquetina nigrescens (Asclepiadaceae), a woody shrub native to Africa, is sometimes referred to as *Periploca nigrescens* (1). Previous studies (2–5) showed it to contain cardiac glycosides of the strophanthidin type. Strophanthidiol-β-glucoside acetates, strophanthidin, strophanthidiol, 16-hydrostrophanthidin, convallatoxin, 17α-strophanthidin, 16-acetoxystrophanthidin, 16-acetoxystrophanthidin rhamnoside, 16-dehydrostrophanthidin, 16-dehydrostrophanthidol, nigrescigenin, tetra-*O*-acetylstrophanthidin-β-D-glucoside, and several incompletely identified cardenolides have been isolated from this plant.

Other species of the genus have also been investi-