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The influence of phospholipids on the transport of antibacterial agents across non-aqueous barriers, and the methods used to quantitate the rate processes†

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Penicillin G and ampicillin are known to bind with phospholipids, which may influence antibiotic transport across the bacterial cell wall. This influence has been investigated *in vitro* in a three-phase cell containing 1-octanol as the non-aqueous barrier. The data have been analysed by an iterative technique using digital computation, in order to quantitate the rate processes. The results indicate that binding of the antibiotics with phospholipids occurs at the oil-water interface which influences the further transport of the antibiotics through the non-aqueous phase. The possible biological significance is discussed.

There are many examples in the literature where knowledge of the factors affecting solute transport rates is essential to an understanding of transport phenomena. Recently (Padfield & Kellaway, 1973a,b,c; Kellaway, Padfield & Marriott, 1973) we have reported differences between phospholipid interaction with penicillin G and ampicillin which could profoundly influence the transport of these antibiotics across the bacterial cell wall to their site of action.

Multi-component and multi-compartment models have been designed to depict transport across lipid layers. Three-phase cells of the type described by Agostini & Schulman (1965) have been widely used (Perrin, 1967; Augustine & Swarbrick, 1970) and have been claimed to possess advantages over the rocking, inverted Y-tube of Khalil & Martin (1967) and Dolusio, Crouthamel & others (1970), since longer-chain alcohols may be used with less likelihood of emulsification. Further, and of particular importance to studies involving amphipathic molecules, as the solvent interfaces neither contract nor expand in area to any significant extent, phospholipids may be added to the system to form an orientated layer at the oil-water interface. This monolayer may represent one half of the bilayer lipid present in biological membranes. Augustine & Swarbrick (1972) have recently reported a correlation between transport in such *in vitro* systems and *in vivo* absorption for a series of sulphonamides.

We have investigated the relation between antibiotic transport and its non-aqueous partitioning, and the influence of phospholipids on this transport particularly to observe if the previously found antibiotic-phospholipid binding occurs at the interface. To adequately quantitate the rate processes it was necessary to critically examine the methods available, and the combined use of graphical analysis and an optimization procedure was developed.

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THEORY

The three-phase cell used is shown in Fig. 1. It may be represented by the following model:—

$$A \underset{k_3}{\overset{k_1}{\rightleftharpoons}} B \underset{k_4}{\overset{k_2}{\rightleftharpoons}} C \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

The kinetics of this system are expressed by the following set of linear differential equations:

$$\frac{dA}{dt} = -k_1A + k_3B \quad .. \quad .. \quad .. \quad .. \quad (2)$$

$$\frac{dB}{dt} = k_1A - (k_2 + k_3)B + k_4C \quad .. \quad .. \quad .. \quad (3)$$

$$\frac{dC}{dt} = k_2B - k_4C \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

where A, B, C are the solute concentrations at time, t, in the respective compartments. If the volumes are maintained in a known ratio and radioisotopes are used, then the volume terms (see Perrin, 1967) are embraced in the total activity (in counts min^{-1}) in each compartment.

The solution of equations (2–4) is complex and requires calculation of the four rate constants (k). The equations have been derived from those described by Frost & Pearson (1961), Nagashima, Levy & O'Reilly (1968), Atkins (1969) and Shipley & Clark (1972) allowing for the system being "closed", hence the elimination rate constant described by those workers does not exist. We have used graphical analysis to provide initial estimates of the rate constants, k, required by a digital computer program designed to minimize the least squares function (f_x) between the experimental and theoretical data by means of iteration.

Swann (1969) has critically reviewed the methods available for minimizing such functions as least squares, iterative methods being those most widely used; direct search methods rely solely on the values of the objective function, whereas gradient methods also use partial derivatives of the function. The solution of equations (2–4) leads to sums of exponentials which are notorious for the long ridges they produce in the sums-of-squares surface (J. A. Nelder, personal communication), and hence a direct search method was chosen. The method of Hooke & Jeeves (1961) consists of a combination of exploratory moves (seeking to locate the direction of any valleys in the surface) and pattern moves (attempting to progress down any such valleys).

MATERIALS AND METHODS

Materials

The antibiotics and phospholipids were used as previously described (Padfield & Kellaway, 1973a). Analar phenol, spectroscopic grade cyclohexane, laboratory

grade 1-octanol, glycerol trioleate, oleyl alcohol (BDH) and cod-liver oil BP (Boots) were used as supplied. Sørensen's phosphate buffer pH 7·4 was used throughout.

Apparatus

The apparatus is diagrammatically represented in Fig. 1; interfacial areas of 19·68 and 20·51 cm^2 were calculated for A–B and B–C respectively. Compartments A and C (containing 30 ml of aqueous phase) and compartment B (containing 50 ml of non-aqueous phase) were stirred at 250 and 60 rev min^{-1} respectively unless

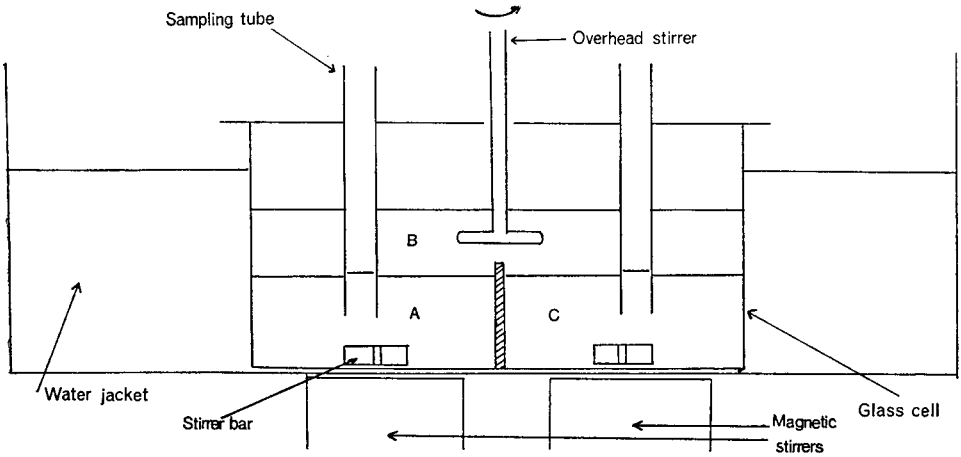

FIG. 1. Schematic representation of three-phase cell.

otherwise stated. The cell was thermostated at 37 ± 0·1°. Samples (0·1 ml) were removed from each compartment at various times and the activity in each determined. Each activity was corrected for its respective compartment volume and, in addition, the values for the activity in compartment B were corrected by a factor of 1·67 to bring them into the same volume terms as A and C. Thus the results express the total activity in each compartment as counts ml^{-1} per 30 ml.

Experimental design

Preliminary studies were undertaken with phenol to investigate the effects of stirring rate and composition of compartments A, B and C. All materials used as non-aqueous barriers were pre-saturated with buffer, and vice versa, before use to reduce water transport due to osmosis (Rosano, Schulman & Weisbuch, 1961).

Antibiotic-phospholipid studies

Rosano & others (1961) reported a technique for investigating carrier transport in these model systems, using a material (e.g. chloroform) which removed water from the non-aqueous phase, thus blocking diffusion transport. In our study 20% v/v chloroform was added to the non-aqueous phase and the effect of addition of phosphatidylcholine (0·8% w/v) on the transport of penicillin G (2% w/v) determined.

Further studies were undertaken to determine the effect of phospholipids (0·05 mM) dissolved in 1-octanol on the transport of the penicillins (0·5 mM) dissolved in buffer from A to C.

Partition coefficients

These were determined for the systems studied by adding 20 ml of the antibiotic-buffer solution to an equal volume of the non-aqueous phase (with or without phos-

pholipid) in sealed flasks placed in a shaking water bath at 37 ± 0·1° for 48 h, a time sufficient for equilibrium to be established.

RESULTS AND DISCUSSION

Effect of experimental design

Rosano, Duby & Schulman (1961) have studied the effect of varying the cell size and its operating conditions and concluded that the interfacial area and general cell dimensions had no effect, in contrast to the observations of Augustine & Swarbrick (1970). Since transport across the interface is the rate-limiting step in this model, transfer into B, and hence into C, will be controlled by the partition coefficient. We have observed in these studies that while stirring is essential, the actual rate is not critical, provided that it is sufficient to significantly reduce the thickness of the stationary diffusion layer and eliminate concentration gradients (Padfield, 1972).

A correlation was observed between the rate of loss of phenol from A into compartments B of different composition, and the partition coefficient (Fig. 2a). Phenol has a very low cyclohexane-water partition coefficient (0·09) which accounts for its slow loss from A and rapid uptake into C from B (Fig. 2b) as a negligible amount is

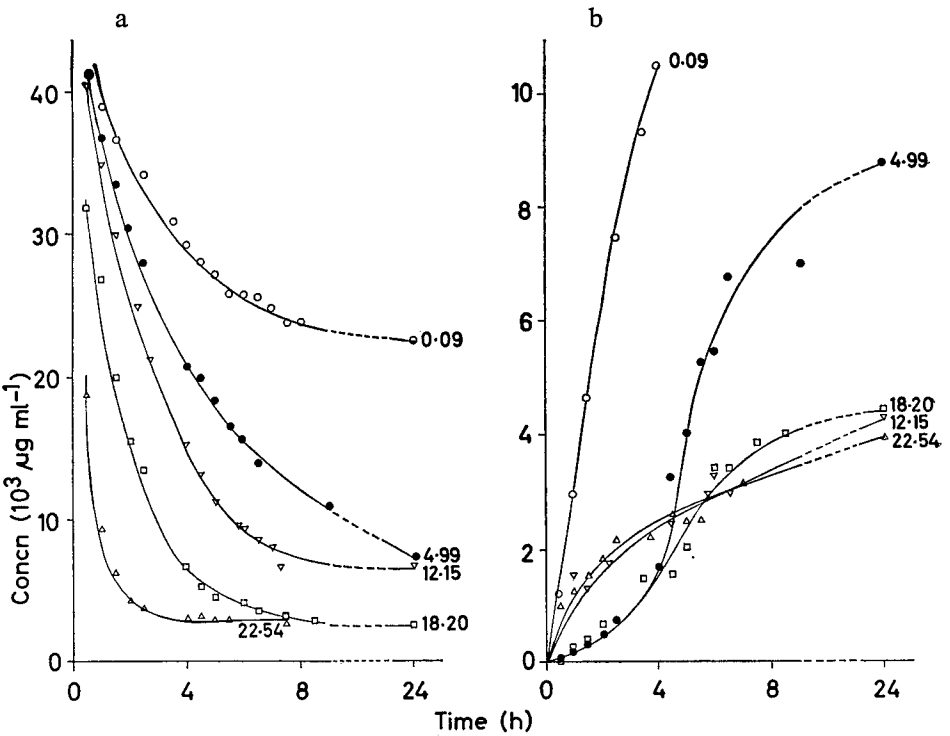

FIG. 2. Effect of composition of B on (a) the loss of 0·05M phenol from A, and (b) the gain of phenol in C. Values given are oil-water coefficients. ○ Cyclohexane; ● cod-liver oil; ▽ glycerol trioleate; □ oleyl alcohol; △ 1-octanol.

retained in B. Cyclohexane has been used by several workers as the non-aqueous phase in this model, as its low retention of drug closely resembles the biological membrane. However at 37°, it evaporates too rapidly in an incompletely closed system for determinations over periods of 24 h. Fatty alcohols may be present in biological membranes and their use is to be preferred since they provide a hydrophilic

component to the system in their ability to form hydrogen bonds with drug molecules; 1-octanol was used in studies of antibiotic transport.

Antibiotic-phospholipid studies

Rosano & others (1961) have distinguished between diffusion and carrier transport in this model, whereby the drug is transported through B by water molecules or a "carrier" (e.g. phospholipid) respectively. The influence of a carrier, phosphatidylcholine, on penicillin G transport in a diffusion-blocked system is shown in Table 1. Penicillin G loss from A and its appearance in B was greatest in the system

Table 1. *Effect of a "carrier" on penicillin G transport in diffusion-free and diffusion-blocked systems.*

System	$k_1(h^{-1})$	$k_2(h^{-1})$	$k_3(h^{-1})$	$k_4(h^{-1})$	K_A[a]	K_B[b]
1-Octanol	0·0093	0·0082	0·1136	0·0009	0·0819	9·1110
1-Octanol–chloroform	0·0052	0·0024	0·0355	0·0347	0·1465	0·0692
1-Octanol–chloroform/PC	0·0149	0·0026	0·0249	0·0066	0·5984	0·3939
1-Octanol–PC	0·0074	0·0001	0·0542	0·0371	0·1365	0·0027

[a] $K_A = k_1/k_3$.
[b] $K_B = k_2/k_4$.
PC = phosphatidylcholine.

1-octanol–chloroform–phosphatidylcholine. The reduction in viscosity due to the presence of 20% chloroform in 1-octanol will be greater than addition of chloroform to 1-butanol used by Rosano & others (1961) and may be contributory to the differences in results obtained. Further, the presence of large amounts of chloroform may change the physical state of the phospholipid in 1-octanol, a condition which may not be satisfactory for a carrier mechanism to be adopted.

Fig. 3 illustrates the transport of the antibiotics through 1-octanol in the presence of phospholipids. Ampicillin loss from A and its appearance in B and C is more rapid than penicillin G, which may be due to its more hydrophilic nature. The partition coefficient $K_{o/w}$ of ampicillin is slightly lower than that of penicillin G (Table 2) and is of the same order as that recently reported by Purich, Colaizzi & Poust (1973); the increased transport rate of ampicillin in this diffusion-controlled

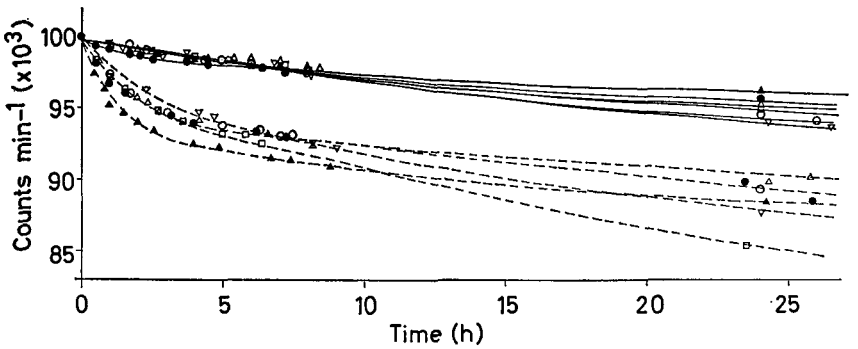

FIG. 3. Effect of phospholipids on the loss of 0·5mM penicillin G (——) and ampicillin (– – – –) into 1-octanol. ○ penicillin G; ▲ penicillin G–PC; △ penicillin G–LPC. □ penicillin G–PE; ▽ penicillin G–PI. ● penicillin G–PS; ○ ampicillin; ▲ ampicillin–PC; △ ampicillin–LPC; □ ampicillin–PE; ▽ ampicillin–PI; ○ ampicillin–PS. See Table 2 for abbreviations.

system may thus be due to its affinity for the polar portion (i.e. the associated water) of the non-aqueous phase. It has been observed previously (Padfield & Kellaway, 1974) that ampicillin has a greater diffusion coefficient than penicillin G and traverses a cellulose acetate membrane more rapidly. Purich & others (1973) have reported that, at the pH of this study (7.4), the anionic form of ampicillin accounts for 75% of the ionic species, which would inhibit its transport through a non-polar layer if not for the presence of the associated water.

Table 2 gives the values of the rate constants (k), calculated by iteration and the equilibrium constant (K). The rate constants were calculated by iteration using initial estimates provided by graphical analysis (Fig. 4). Addition of phospholipid

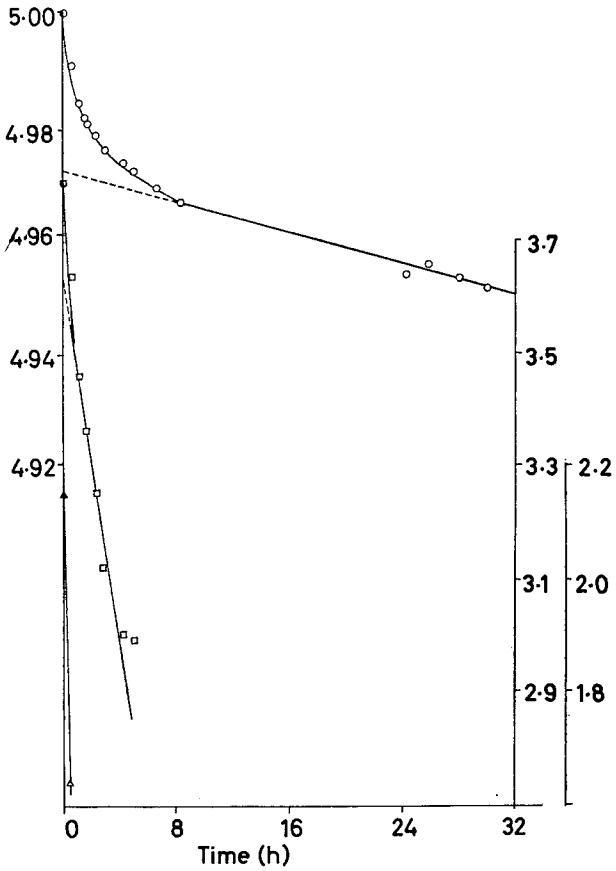

FIG. 4. Graphical analysis for ampicillin transport across 1-octanol–lysophosphatidylcholine barrier. $P = 1640e^{-2.655t} + 4710e^{-0.3788t} + 93650e^{-0.0016t}$ *Ordinates* From the left: ○ Log P; □ Log $(P - Ce^{-\gamma t})$; △ Log $(P - Ce^{-\gamma t} - Be^{-\beta t})$.

results in a decreased loss of penicillin G from A and an increased loss of ampicillin (except where lysophosphatidylcholine is present); these changes are reflected in B and C. The $K_{o/w}$ values (Table 2) do not exactly correspond with these changes, the relation depending on which rate constant is examined. Good correlation is obtained for the forward transfer constant (k_1) (Fig. 5A) whereas a significant correlation between the backward rate constant (k_3) and the partition coefficient is only obtained for ampicillin systems (Fig. 5B); a similar observation is made by plotting K_A against

Table 2. *Effect of phospholipids on the transport of penicillin G and ampicillin across a 1-octanol phase.*

System	$k_1(h^{-1})$	$k_2(h^{-1})$	$k_3(h^{-1})$	$k_4(h^{-1})$	K_A[a]	K_B[b]	$K_{o/w}$
Penicillin G	0·0032	0·0114	0·0371	0·0574	0·0863	0·1986	0·181
Penicillin G–PC	0·0033	0·0564	0·1147	0·0218	0·0288	2·5872	0·257
Penicillin G–LPC	0·0025	0·0286	0·0416	0·0403	0·0601	0·7097	0·195
Penicillin G–PE	0·0029	0·0086	0·0409	0·0216	0·0709	0·3981	0·163
Penicillin G–PI	0·0041	0·0048	0·0491	0·0241	0·0835	0·1992	0·357
Penicillin G–PS	0·0033	0·0121	0·0754	0·0112	0·0438	1·0804	0·331
Ampicillin	0·0088	0·0026	0·0880	0·0757	0·1000	0·0343	0·168
Ampicillin–LPC	0·0592	0·0795	1·2478	0·1045	0·0474	0·7608	0·222
Ampicillin–PE	0·0052	0·0045	0·0063	0·0002	0·8254	22·50	0·149
Ampicillin–PI	0·0123	0·0038	0·0936	0·0987	0·1314	0·0385	0·163
Ampicillin–PS	0·0184	0·0016	0·1766	0·0007	0·1042	2·2857	0·171

[a] $K_A = k_1/k_3$
[b] $K_B = k_2/k_4$

PC = Phosphatidylcholine. PE = Phosphatidylethanolamine PS = Phosphatidylserine. LPC = Lysophosphatidylcholine. PI = Phosphatidylinositol.

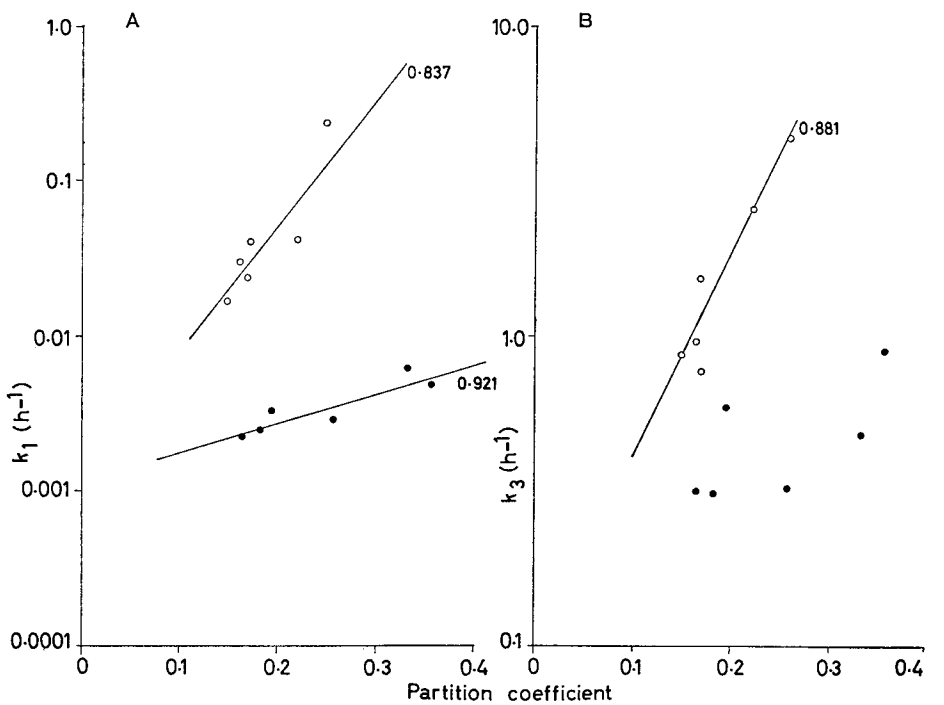

FIG. 5. Semilogarithmic relation between (A) k_1 and (B) k_3 and 1-octanol–water partition coefficient for (●) penicillin G and (○) ampicillin systems. Values given are correlation coefficients.

$K_{o/w}$. In the cases of penicillin G-phosphatidylinositol and penicillin G-phosphatidylserine a significant rise in $K_{o/w}$ is observed, whereas no corresponding changes for ampicillin systems are observed, except for a small change in the presence of lysophosphatidylcholine. Phosphatidylinositol and phosphatidylserine have been observed previously to bind penicillins strongly (Padfield & Kellaway, 1973a). Several authors have reported correlations between rate constants (or their logarithms)

and parameters relating to membrane affinity such as partition coefficient (Augustine & Swarbrick, 1972) and lipid polarity (Khalil & Martin, 1967; Augustine & Swarbrick, 1970). However, although these correlations exist, it may be more valid to compare *in vitro* rate constants with *in vivo* absorption data since these are the dynamic processes, whereas partition is an equilibrium process.

The changes in $K_{o/w}$ and non-linearity of some of the k *vs* $K_{o/w}$ plots indicate the possibility of penicillin G interaction with the phospholipids at the oil-water interface; the complex formed may be held there and hence would lead to interference with the interfacial rate process k_3. This rate constant would be affected to a greater degree than k_1 since the rate process described by equations (2–4) may not apply to the interfacial system if complex formation has occurred. Previous studies have shown binding of penicillin G to phospholipids with reduced binding of ampicillin, confirming hypotheses regarding transfer across the bacterial cell structure (Padfield & Kellaway, 1973a, c). There is apparently greater interaction between ampicillin and lysophosphatidylcholine than between penicillin G and this phospholipid (Table 2), an observation that has been noted previously (Padfield & Kellaway, 1973a; Kellaway & others, 1973).

A possible explanation for the lack of "carrier" transport of the penicillins may lie in the physical nature of the phospholipid in the non-aqueous phase. Since the phospholipid is dissolved in the solvent it does not possess the bilayer form present in sonicated dispersions in water (Finer, Flook & Hauser, 1972) which may be necessary for interaction to occur. A similar observation has been made by Hauser, Finer & Chapman (1970) in an nmr study of alamethicin—phospholipid interaction. This would lend further support to the involvement of hydrophobic interactions (Padfield & Kellaway, 1973a, c) which would not occur in the non-aqueous solvent.

Kinetic analysis

Various authors have used different procedures to determine the values of the rate constants. Graphical analysis (Fig. 4) possesses some inherent disadvantages (see Atkins, 1969) primarily concerning choice of the line best fitted to the data. The advantages of digital computation over graphical analysis are that the parameters are determined simultaneously and are, therefore, all approximately equally well determined, and that subjective errors or bias on the part of the experimenter are removed.

The method of Hooke & Jeeves (1961) has been found to be a very powerful means of calculating the rate constants for this model, especially when good estimates are provided by graphical analysis.

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