A STUDY ON THE RELATION BETWEEN PHYSICOCHEMICAL PROPERTIES AND PHYSIOLOGICAL DISPOSITION OF SOME SEMI-SYNTHETIC PENICILLINS

A Thesis

Presented to the

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Ian M. Rollo

December 1967

ACKNOWLEDGEMENTS

I would like to express my deep appreciation to Dr. Mark Nickerson firstly for the opportunity to carry out this work and, secondly, for providing stimulation both in atmosphere and personal example.

I wish to thank Mrs. Sylvia White for typing this manuscript, and Mrs. Stasha Krzywdzinski and Mr. Roy Simpson for their help in preparing the figures.

The following Companies provided samples of the penicillins used in this study, and to these organizations I am most grateful: Ayerst Laboratories, Beecham Research Laboratories, Bristol Laboratories Inc., Distillers Company (Biochemicals) Ltd., and Wyeth Ltd.

I also wish to express my gratitude to the Canadian Medical Research Council without whose financial support this work would not have been possible.

ABSTRACT

Variation in the structure of the side-chain of penicillins has been shown to be associated with changes in the physicochemical properties of the substances.

Values obtained for ionization constants, oleyl alcohol/water partition coefficients and binding to serum albumin in a series of ten semi-synthetic penicillins varied from 2.78 to 5.41, from 0.87 to > 800, and from 29 per cent to 100 per cent respectively.

The partition coefficient appears to be the most important of these parameters. Decrease in acid strength is due probably to the formation of dimers by highly non-polar penicillins. Increase in extent of binding to albumin is due probably to increase in hydrophobic bonding between highly non-polar penicillins and the protein surface. In certain cases, the formation of re-inforced ionic bonds may supplement non-ionic bonding.

Two phenoxy penicillins and one phenylthio penicillin were selected for a study of physiological disposition. Phenylthiodiphenyl penicillin was, however, rejected because it underwent complete bio-transformation. The resultant substance retained antibacterial activity but lost its resistance to the enzyme β-lactamase.

The rate of absorption of phenoxymethyl and phenoxybenzyl penicillins from rat small intestine was at variance with the properties of the substances and the concept of passive, non-ionic diffusion. A more detailed study excluded possibility that some form

of specialized transport was involved. The data are consistent with a delay in absorption caused by an interaction between penicillin and some component of intestinal epithelium. Absorption may be further hindered by the formation of penicillin micelles. The relatively polar nature of such micelles would tend to reduce the ability of the parent substance to penetrate lipid-like membranes.

Maintenance of high concentrations in the blood is favoured by a numerically large partition coefficient, and the associated high degree of binding to albumin and decrease in acidity. It appears that differences in both biliary and renal tubular secretion also play an important part.

TABLE OF CONTENTS

INDEX TO FIGURES

INDEX TO TABLES

The microbe is so very small
You cannot make him out at all,
But many sanguine people hope
To see him through the microscope
His jointed tongue that lies beneath
A hundred curious rows of teeth;
His seven tufted tails with lots
Of lovely pink and purple spots
On each of which a pattern stands,
Composed of forty separate bands;
His eyebrows of a tender green;
All these have never yet been seen -
But Scientists who ought to know,
Assure us that they might be so -----
Oh! let us never, never doubt
What nobody is sure about.

H. Belloc

1. INTRODUCTION

This study was started because we did doubt what everybody was sure about, or, at least, appeared to be sure about. For example, Weinstein (1965) stated, "it (the phenoxymethyl analogue of benzyl penicillin) is more stable in an acidic medium and, therefore, is better absorbed from the gastrointestinal tract", and further, "Ampicillin is acid stable and therefore, as expected, it is well absorbed after oral administration;".

It appeared to us that very marked differences in apparent degree of absorption occurred with penicillins whose acid stability was of the same order (Rollo, Somers and Burley, 1962), and that even benzyl penicillin, which has some degree of acid lability, should be little affected by its brief stay in the fasting stomach. Also, it appeared to us that the application of some well-established principles of physiological disposition, and the parameters on which they depend, had been lacking in the study of semi-synthetic penicillins.

The concept that there is some relation between structural variation in a series of related chemical substances and the pharmacological actions of those substances, arose soon after it was determined that substances of known chemical structure could be used profitably in the treatment of disease. This realization is of comparatively recent date. Ehrlich's work on the chemotherapy of protozoal infections, his realization that "the ways and means by which drugs are distributed over the body must be of the greatest importance to the rational development of therapeutics" (Ehrlich & Hata, 1911), and the work in Abel's laboratory leading to the isolation of adrenaline and acetylcholine (see Krantz & Carr, 1951), must be regarded as the birth of the scientific pharmacology we know today. Before this, effective chemical treatment of disease was

to a very large extent limited to the use of a few traditional herbal remedies such as extract of ipecachuana for dysentery, cinchona bark for 'the ague' and foxglove leaf for 'dropsy'.

Ehrlich's early ideas of 'receptors' on the surface of pathogenic protozoa to which were attached the 'haptophore' groups of effective antiprotozoal agents, are ideas which persist in modified form today to explain the specificity of diverse types of pharmacologically active agents. Indeed, contemporary thought envisages the action of a drug as being dependent on both an affinity of the drug for a specific receptor and an intrinsic activity once attachment to the receptor has taken place.

So far we have only vague concepts of what factors determine these parameters. Close examination of earlier studies on relation between structure and activity shows how little has the complex interrelation between the drug and all the factors interposed between the drug in the surrounding medium and its site in the tissue been understood. Even less reliance can be placed on deductions from data obtained from responses in intact animals to whom drugs had been administered. In these cases, to the uncertainties inherent in <u>in vitro</u> experiments were compounded unknown differences in physiological disposition.

Of more practical importance, perhaps, would be the ability to determine from previous knowledge what particular molecular structure should possess certain pharmacological properties. Ideally, it should be possible to predict what structural parameters were important for any desired pharmacological effect. Subsequent development should then permit of additions to, or subtractions from, the parent substance to

regulate its physiological disposition.

Little can be said of the requirement for specific pharmacological activity because we know very little of specific drug-receptor relations, however, the same cannot be said of the requirements for variations in physiological disposition. Only too often, however, are new drugs designed on the flimsiest of premises and on the basis of empirical trial.

The importance of physiological disposition in structure/activity studies and on the proper design and use of drugs is obvious when given some consideration. However, scant attention is often paid to this aspect of pharmacology. It is important to know whether the establishment of a rapidly attained and prolonged 'therapeutic' concentration in the blood is a result of ready absorption from the gastrointestinal tract with subsequent binding to plasma proteins; or whether it is a result of recycling via renal tubular reabsorption or biliary secretion. It is important to know to what extent the drug can penetrate into various tissues, and in the case of antibacterial agents and centrally acting drugs, to know how readily it can traverse the blood-brain barrier. It is important to know to what extent biotransformation can account not only for the 'detoxification' of the drug by converting it into a more polar, more readily excreted, substance but, in some cases, for its conversion to the active form of the drug (see Harper, 1964).

It is now quite certain that the various structures inside a cell are separated from the outside environment by an integument, the plasma membrane, which has lipid-like characteristics. The early work of Meyer (1899) and Overton (1901, 1902) established that the rate of diffusion of organic non-electrolytes into the intact cell depended upon

the lipid-water distribution coefficient of the substances. However, the existence of lipid-insoluble substances which penetrate into cells readily, and the marked differences in permeability shown by cells of different species, has tended to obscure the straightforward picture presented by Overton and Meyer. These facts have necessitated the introduction of a pore theory as a supplement to the original lipid theory (Davson and Danielli, 1952). In addition, the absorption from the gastrointestinal tract of glucose (Cori, 1925 and Verzar, 1935) and amino acids (Höber and Höber, 1937), indicated that at least some substances are absorbed by specific metabolic mechanisms.

Up until 1938, data on the rate of absorption of drugs was virtually non-existent. In that year Ellisor and Richardson showed that the penetration of nicotine into goldfish was dependent on the pH of the surrounding medium. Travell (1940), using the isolated loop technique, studied the effect of pH on the rate of absorption of alkaloids in the cat, and came to the conclusion that when the reaction of the gastric juice was strongly acid there was no gastric absorption. The rate of absorption was closely related to the concentration of free undissociated base, so that, as the pH was raised, the rate of absorption was increased. These data were made good use of by Poth, et al. (1942) and Poth and Knotts (1942). They realized that succinylsulphathiazole, although readily soluble in water, was a fairly strong acid and therefore diffused very slowly across cell membranes. Its usefulness as a non-absorbable sulphonamide depends on these properties. Shortly afterwards, Fisher, et al. (1943) carried out a most important study on the effect of change in structure among sulphonamides on the rate of renal excretion. At the

same time, they studied overall distribution, binding to plasma protein, and conjugation and metabolism of the drugs within the animal. Their studies were fundamental to subsequent studies on the physiological disposition of drugs, and confirmed the importance of the dissociation (ionization) constant. However, it was difficult to formulate a coherent theory based on their data to explain the relation between structure, ionization constant, and renal excretion. Höber (1940, 1945) drew attention to the importance of a hydrophobic-hydrophilic structure and lipid solubility in the disposition of a drug in the kidney. Despite this, the exact relation of chemical structure and physicochemical characteristics with the various mechanisms within the kidney was far from clear.

The rapid rate of excretion of benzyl penicillin, at that time a scarce and expensive material, led to the realization that it participated in a proximal tubular secretory mechanism common to a number of organic acids. Beyer and his co-workers (1944) then demonstrated that its rate of excretion could be decreased by the simultaneous administration of sodium p-aminohippurate, a substance which competed for the same secretory pathway. Subsequently, caronamide (Strauss, et al., 1947, Crosson, et al., 1947) and later, probenecid (Beyer, et al., 1951) were introduced into clinical practice to conserve this expensive antibiotic. Alteration of the pH of urine by the administration of acid or alkali was also shown to alter the extent of tubular reabsorption and, hence, the overall rate of excretion of drugs. (Andrews and Cornatzer, 1944; Beyer, et al., 1944).

Up until this time there had been little systematic study of the absorption of drugs from the gastrointestinal tract, at least not enough to lay down guidelines that might predict the behaviour of a series of drugs. More often than not the studies concerned themselves with the absorption of single substances (see Schanker, et al., 1958). In 1957 studies of the distribution of drugs between plasma and gastric lumen confirmed the hypothesis that simple diffusion of the lipid soluble unionized molecule occurred across the equivalent of a lipid membrane (Shore, et al., Hogben, et al., Schanker, et al.). The charged, lipid-insoluble ion could not pass from one side to the other. Of considerable importance was the finding that quite strong organic acids were absorbed to an appreciable extent from the stomach even if the contents were made basic. From such data it was concluded that the continuous secretion of hydrogen ions would maintain the absorbing surface in an acidic state despite the basic mileau.

The passage of most drugs from the intestinal lumen to the blood is also governed by the lipid-solubility of the unionized molecule. The non-polar solvent/water partition coefficients of the unionized form have been shown to be good guides to rates of intestinal absorption (Hogben, et al., 1959; Schanker, 1960; Schedl and Clifton, 1961). However, as for the stomach, evidence has been presented that the distribution of organic electrolytes between plasma and intestinal lumen is a function, not of plasma pH and intestinal lumen pH, but rather of plasma pH and an 'effective' pH probably zoned at the surface of the intestinal epithelial boundary. From such studies it appears that this effective pH is 5.3, a figure appreciably lower than the nearly neutral value of normal luminal contents (Hogben, et al., 1959; Hogben, 1960, Schanker,

1960). This value is consistent with a previous finding that the intestinal mucosa secretes hydrogen ions (Bucher, Flynn and Robinson, 1944).

In addition to passage by simple diffusion, specialized transport systems with some specificity have been shown to exist for several different types of substances. This will be discussed later.

In any study on physiological disposition of drugs, therefore, account must be taken, not only of those factors which regulate passive diffusion but also of the presence in mammals of mechanisms of specialized transport. In this study we have examined some physicochemical properties of a series of penicillins and attempted to relate these to some of the parameters which are important in their physiological disposition.

The substances we used were representative of the many penicillins that are available now for the treatment of bacterial infections. Their history is long and fascinating. Even before Fleming's original observation in 1929 that a contaminating mould lysed colonies of Staphlococcus aureus, many workers had shown that substances produced by one micro-organism could inhibit the growth of another. The first such observation was probably that of Pasteur and Joubert in 1877. They observed the antagonistic effect of aerobic bacteria on the growth of Bacillus antracis, and even found that death of animals from antrax could sometimes be prevented by including some of these aerobic bacteria in the infective dose of the bacillus. Pasteur ascribed the effect to the consumption of oxygen by the aerobic organisms, but Babès (1885) interpreted experiments on growth inhibition of one organism by another as due to a chemical substance produced by the antagonistic organism. Many subsequent workers isolated substances from bacteria which inhibited

the growth of other bacteria, among them Emmerich and Low (1899) - pyocyanase from Pseudomonas pyocyanea, and Nicolle (1907) - a thermostable substance with lytic action against many bacterial pathogens. However, all the substances isolated were useless clinically because of their toxicity towards the mammalian hosts.

Perhaps the first successful therapeutic use of an antibiotic was reported by Vandremer in 1913. He found that filtrates from cultures of Aspergillus fumigatus could attenuate Mycobacterium tuberculosis, and that their use in the treatment of human tuberculosis was attended by some success. Much later work by Jennings (1945) showed that this mould produces four antibiotic substances, one of which, helvolic acid, has some action against the tubercle bacillus in vitro. Gosio in 1896 produced from a Penicillium, a crystalline substance which inhibited the growth of antrax bacilli. He was unable to carry out tests on infection of experimental animals owing to lack of material. However, the substance has since been reisolated under the name mycophenolic acid and found to be useless as a chemotherapeutic agent although quite active in vitro (Florey, et al., 1946).

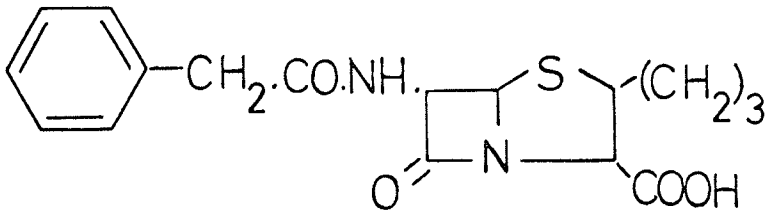
Subsequent to Fleming's discovery of the antibacterial substance penicillin, so named because the contaminating mould belonged to the genus Penicillium, attempts were made to concentrate the active substance by Clutterbuck, Lovell and Raistrick (1932). These attempts were made because Fleming had shown the substance to be almost completely non-toxic to animals and man, and to leucocytes, and had suggested that it would be a useful antiseptic for infected wounds. However, its instability and low concentration resulted in both poor clinical response and lack

of success in purifying the active substance. The advent of World War II spurred the search for improved methods of growing the mould and of harvesting the penicillin in high yield. These efforts in large scale surface culture and improvement in techniques of chemical separation proved successful, and enough material, crude by present-day standards, was prepared to enable investigators to treat infections in experimental animals and man (Chain, et al., 1940 and Abraham, et al., 1941). Despite this success, the material was still crude, containing about 10 per cent active material, and present only in small concentration in the culture medium.

While Fleming's original strain of P. notatum was used in the early production of penicillin it was soon found that other penicillia produced the substance, some in much higher yields. By selection of mutants from one such strain of P. chrysogenum was derived the strain used today. Of considerably greater significance was the development of the submerged culture, or deep fermentation, procedure whereby the mould could be grown in vast tanks of liquid culture medium with proper attention to control of temperature, pH and day-to-day assay of penicillin-content. By the use of these techniques the overall production of the antibiotic was increased by several orders of magnitude (Hirsch and Putnam, 1958). Another considerable step forward was made when it was found that the yield of the antibiotic could be increased further by growth of the mould in a medium containing optimal concentration of phenyl-acetic acid.

In the meantime considerable efforts were being made to eluci-date the structure of penicillin, a task in which conventional methods

of organic chemistry proved inadequate because of two equally possible structures for the hitherto unknown type of compound. Eventually the structure shown below was established by x-ray crytallographic analysis (Crowfoot, et al., 1949).



Benzyl Penicillin, 6-(phenylacetamido) penicillanic acid.

The penicillin nucleus, the 6-aminopenicillanic acid moiety, is the chief structural requirement for antibacterial activity. Modifications in the sidechain are consistent with maintenance of activity but any alteration to the nucleus results in a very marked decrease in activity.

After the structure had been elucidated it became clear that the increased yield of benzyl penicillin following the addition of phenylacetic acid to the culture medium, was due to the incorporation of the exogenous substance by the mould into the final product. Following this, workers in many laboratories tried to produce novel penicillins by incorporating various carboxylic acids into the medium of experimental fermentations. It was hoped that the mould would utilize these to

synthesize usable amounts of substances with properties different from those of the natural penicillins. These attempts were prompted by the following shortcomings of benzyl penicillin. Benzyl penicillin is unstable at pH 2, and has a half-life of ca. 6 minutes at 37°C. This observation led to the conclusion that since benzyl penicillin is poorly absorbed from the gastrointestinal tract much of the administered dose is degraded in the stomach. Benzyl penicillin is excreted very rapidly by the kidney. This is due largely to its secretion by the acid secretory mechanism of the proximal tubules and, probably, to a very limited degree of distal tubular reabsorption. Benzyl penicillin is susceptible to the action of the enzyme β-lactamase produced by certain strains of S. aureus, and, hence, is ineffective in the treatment of staphylococcal disease caused by such strains. Benzyl penicillin is effective against only a limited number of different organisms. Many gram-negative bacilli are insusceptible to concentrations readily attainable in treated patients. It is regarded, therefore, as being a 'narrow spectrum' antibiotic. Benzyl penicillin doesn't penetrate readily into the cerebrospinal fluid. However, in this respect, measurements in normal experimental animals and man have been misleading. The clinical usefulness of benzyl penicillin in, for example, pneumococcal and streptococcal meningitis demonstrates that the inflamed meninges offer much less resistance to the passage of benzyl penicillin than do the normal tissues. This has been confirmed for several penicillins in experimental infections in animals (Ruedy, 1965). Benzyl penicillin is a potent sensitizing agent. Because of indiscriminate, and often unnecessary, use of benzyl penicillin, an appreciably large proportion of the population have been sensitized to the drug. The usefulness of the antibiotic has, therefore, been seriously

compromised (see Rollo, 1966).

Attempts at producing useful novel penicillins by 'semi-artificial biosynthesis' have been remarkably unsuccessful. Although many hundreds, possibly thousands, of new substances have been produced in this way (e.g., Rollo and Somers, 1961), few have possessed beneficial properties. Only three have been introduced into clinical practice.

The first of these was phenoxymethyl penicillin, produced in 1953 by Brandl, Giovannini and Margreiter. It is much more stable in acidic media than benzyl penicillin, half-life 210 minutes at pH 2, 37°C, and is much more readily absorbed from the gastrointestinal tract. It is commonly supposed that the absorption of a larger proportion of the administered dose is a result of a smaller proportion being destroyed in the stomach (Weinstein, 1965), but this is unlikely to be the case. This penicillin has little advantage to offer over benzyl penicillin. In fact, the minimal inhibitory concentration of this penicillin is appreciably greater than that of benzyl penicillin against many pathogenic bacteria (see Barber and Garrod, 1963). The other two which are used clinically are 6-(α-phenoxyphenylacetamido) penicillanic acid (Rollo, Somers and Burley, 1962) and 6-(α-phenoxybutyramido) penicillanic acid (Williamson, Morrison and Stevens, 1961). These two penicillins have similar properties to the one previously mentioned.

The difficulties involved in this method of preparation were twofold. First, the successful isolation of a novel penicillin was dependent on the incorporations of the added side-chain by the mould. Second, to be commercially feasible, the extraction and purification had to be simple and cheap. The isolation of the penicillin nucleus, 6-amino-penicillanic acid opened up a new approach to the problem.

This compound had previously been synthesized and described by Sheehan in 1958. Although it represented a considerable achievement in terms of chemical synthesis, the overall yields were very small. A more practical approach had been described by Sakaguchi and Murao in 1950. They described the hydrolysis of benzyl penicillin by amidases, elaborated by various micro-organisms, with the resultant splitting off of the side-chain from the 6-amino group. This method of producing 6-aminopenicillanic acid was subsequently used by Rolinson and co-workers, and Gourevitch, Hunt and Lein in 1960 for the production of semi-synthetic penicillins. Previous to this, Batchelor, et al. (1959) described the isolation of 6-aminopenicillanic acid in moderate yield from fermentations in which side-chain precursor were deliberately withheld.

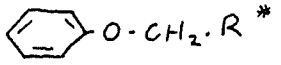
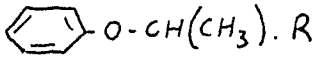
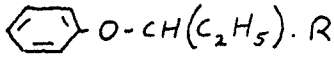
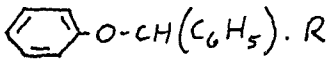
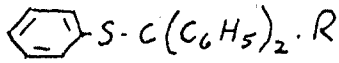
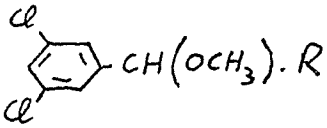
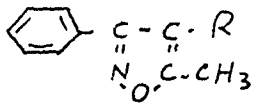
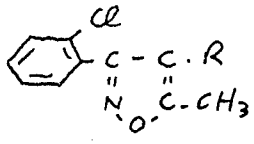
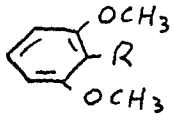
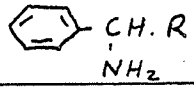
The value of having the free nucleus as a starting material is obvious. The primary amino group in the 6-position can be reacted with virtually any organic acid chloride which the investigator cares to use. The use of this method has resulted in the introduction into clinical practice of many semi-synthetic penicillins which overcome several of the disadvantages of benzyl penicillin (see Weinstein, 1965 and Rollo, 1966).

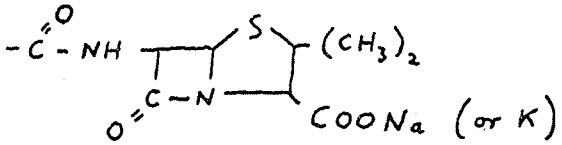
Of the penicillins used in this study, four, 6-(phenoxyacetamido) penicillanic acid, phenoxymethyl penicillin; 6-(α-phenoxybutyramido) penicillanic acid, phenoxypropyl penicillin; 6-(α-phenoxyphenylacetamido) penicillanic acid, phenoxybenzyl penicillin; and 6-(α-phenylthiodiphenylacetamido) penicillanic acid, phenylthiodiphenyl penicillin were prepared by the addition of suitable precursors into the fermentation medium. The remainder were prepared as semi-synthetic penicillins by the reaction of suitable acid chlorides with 6-aminopenicllanic acid.

2. PENICILLINS USED IN THIS STUDY

Table 1 identifies the penicillins used in this study. The first four represent successively increasing substitution on the α-carbon atom of phenoxy-terminated side-chains. The fifth is assumed to belong to the same series since the phenylthio- termination probably confers no different physicochemical properties on the molecule as a whole than does the phenoxy- termination. Attempts made to induce the biosynthesis of phenoxydiphenyl penicillin by introducing the appropriate precursor into the fermentation medium resulted in yields so small as to be practically worthless. The remaining five are representative of the various types of penicillins which have been introduced as semi-synthetic penicillins with novel properties. All the penicillins were used as either their sodium or potassium salts.

Table 1. The series of penicillins used in this study.

Chemical Name	Structural Formula
Phenoxymethyl penicillin	
Phenoxyethyl penicillin	
Phenoxypropyl penicillin	
Phenoxybenzyl penicillin	
Phenylthiodiphenyl penicillin	
3,4-Dichloro-α-methoxybenzyl penicillin	
5-Methyl-3-phenyl-4-isoxazolyl penicillin	
5-Methyl-3-(2-chlorophenyl)-4-isoxazolyl penicillin	
Dimethoxyphenyl penicillin	
α-Aminophenyl penicillin	

* R = 

3. DISC-PLATE ASSAY FOR PENICILLINS

The method depends on diffusion of penicillin from standard filter paper discs (Schleicher & Schuell Co., No. 740-E) placed on the surface of a thin-layer of nutrient agar seeded with the assay organism (Vincent and Vincent, 1944). In this case the organism used was Sarcina lutea, ATCC 9341. The material diffusing into the agar inhibits the growth of the organism so that, after incubation, clear circular zones surround the disc (Figure 1).

The assay plates were constructed for the application of thirty-six sample discs in a six by six array. Each consisted of a base of 1/8 inch-thick glass cemented into walls of 1 inch X 1/4 inch aluminum strip, grooved 1/8 inch from the bottom, and made into a square with internal dimensions of 8 1/4 inches X 8 1/4 inches. Covers were made from 20 gauge aluminum sheet.

Before placing on the agar, the discs were arranged on the surface of a plate-glass sheet measuring 8 inches X 8 inches. To the underside of this glass sheet was pasted a card on which were printed thirty-six numbered spots as a guide for the placing of the discs and samples. The numbers were distributed randomly, as dictated by a table of random numbers, and indicated the position for each sample in triplicate, and for each of four penicillin standards in triplicate. The glass plate was raised off the bench by three rubber bungs (Figure 2).

After 'filling' each disc with 0.05 ml of the appropriate sample or standard, the assay plate was inverted and lowered over the discs until each adhered to the surface of the agar. The plates were then covered and incubated upside-down for 18 hours at 37°C. At the end of this period, the diameter of each zone was measured and the data tabulated. The arithmetical mean values for each standard concentration were plotted

against the logarithm of the concentration to give a straight regression line (Figure 3). The concentrations present in the experimental samples could then be determined by applying the observed data to the standard graph. The accuracy of this method is such that check samples of known concentration can be assayed to within $\pm$5 per cent of the actual figure.

Figure 1. Appearance of assay plate after application of samples and subsequent incubation.

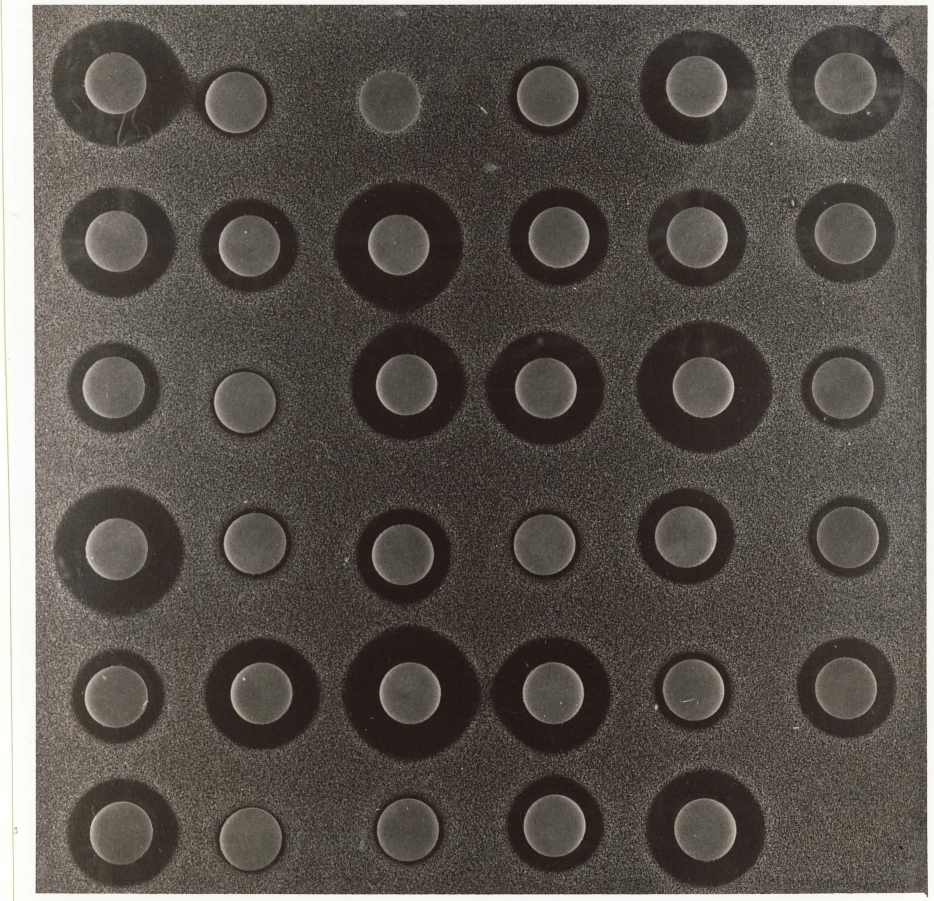


Figure 2. Arrangement of assay-discs for sample placement.

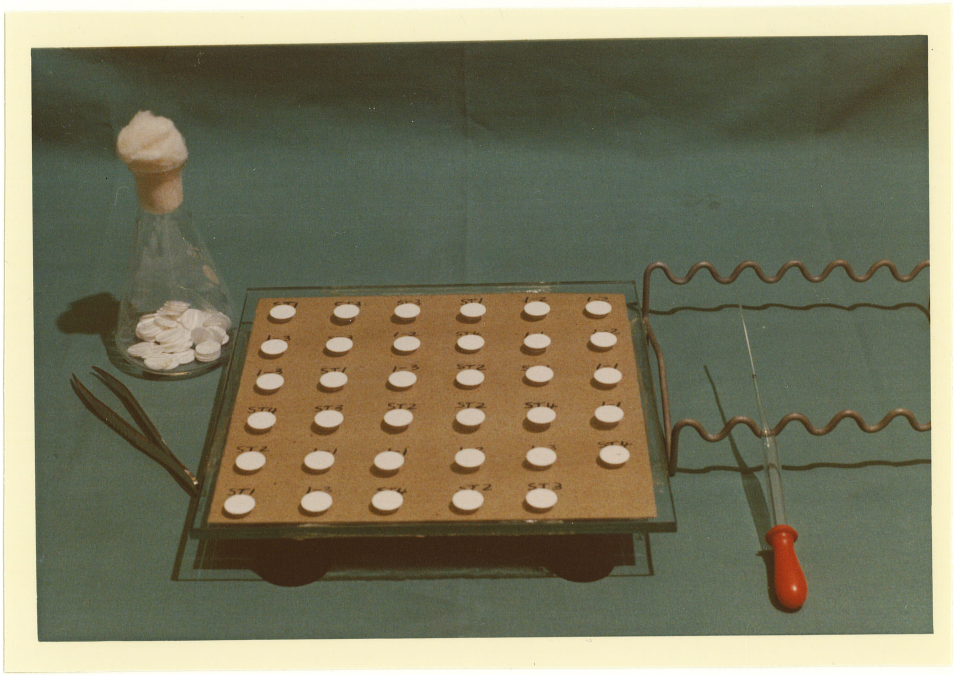
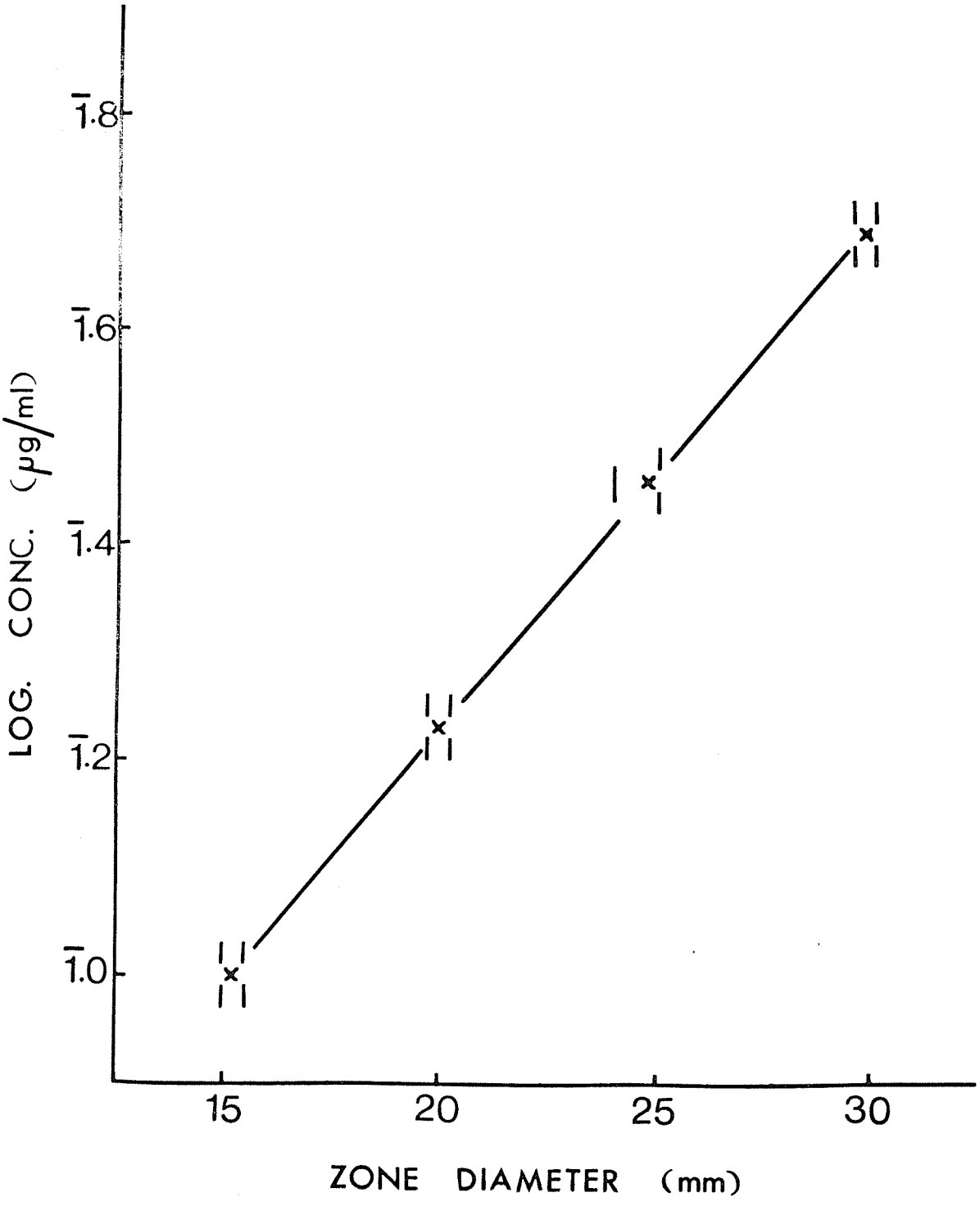


Figure 3. Regression line from disc-plate assay. Each point represents the arithmetical mean of 3 zone diameters. Ordinate - logarithm of concentrations applied to discs. Abscissa - diameter of zone of inhibition about each disc. Vertical bars represent largest and smallest zone diameters in each group.


LOG. CONC. (µg/ml)
1.8
1.6
1.4
1.2
1.0
15
20
25
30
ZONE DIAMETER (mm)

4. PHYSICOCHEMICAL PROPERTIES OF THE PENICILLINS

(A) IONIZATION CONSTANTS

The term 'ionization constant' is used here rather than the more commonly used 'dissociation constant'. By ionization constant is meant that constant which gives numerical expression to the strength of acids and bases. Hence 'ionization' is to be preferred, since 'dissociation' covers such widely differing phenomena as molecules dissociating into atoms, I_2 to 2I at > 700°C, micelles dissociating into monomers, drugs dissociating from cellular receptors, and so on.

The term 'K_a' will be used throughout although, in the past, K_b has been used to designate the strength of bases. It might be objected that the use of K_a for ammonia, for example, represents an equilibrium in which one ion gives rise to another,

$$K_a = \frac{[H^+][NH_3]}{[NH_4^+]}$$

but this objection can be overcome if we define the constants as referring to hydrogen ions only. In fact the more general equation,

$$K_a = \frac{[H^+][B]}{[BH^+]}$$

introduced by Brönsted in 1923 allowed the use of acidic constants for the ionization of bases as well as of acids. The formal similarity

between this last equation and that for acids,

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

shows that the species on the bottom line, in each case, loses a hydrogen ion to furnish a new species which is the original substance minus the hydrogen ion. Consequently, the use of the acidic constant, K_a, relates solely to the affinity of the substance for the hydrogen ion.

It is much more customary now to refer to the negative logarithm of the ionization constant because the constant is numerically very small and hence inconvenient to use. This is termed 'pK_a' and its use provides a very convenient way of assessing the comparative strengths of acids and bases. The stronger an acid is, the lower its pK_a; the stronger a base is, the higher its pK_a. It also provides for the simple estimation of what proportion of an acid or base is ionized in solution of known pH. Thus, from the equation,

$$K_a = \frac{[H^+][A^-]}{[HA]} \qquad \text{(where } A^- \text{ is any anion)}$$

can be obtained the Henderson-Hasselbalch equation,

$$pK_a = pH + \log [HA] - \log [A]$$

(where p means the negative logarithm).

Hence, when pH equals pK_a, the substance is 50 per cent ionized, and, further, when an acid is 10 per cent ionized the pH is one unit less than the pK_a, when 1 per cent ionized the pH is two units less than the pK_a, and so on. The estimation of pK_a values for drugs, which are in the main organic acids or bases, thereby enables a fairly accurate assessment to be made of their state of ionization in any body compartment. Consequently, from the Henderson-Hasselbalch equation, if the pH on both sides of a membrane are known then the ratio of concentrations on both sides at equilibrium is known. Conversely, if the pH on one side of the membrane and the concentration ratio are known, the pH on the other side can be calculated readily.

(i) Methods.

The method used was essentially that described by Albert and Serjeant (1962) as a semi-micro potentiometric titration. Briefly, solutions of the sodium or potassium salts of the penicillins in distilled, de-ionized water were titrated with 0.10 N hydrochloric acid. In order to prevent errors due to the presence of carbonic acid, the water was boiled vigorously for 15 minutes, then cooled to laboratory temperature in a 'carbosorb'-guarded erlenmeyer flask. The titrant was added in 0.05 ml aliquots from a micro-syringe of 0.5 ml capacity, to 10 ml of an 0.005 M solution of the penicillin.

Prior to weighing, the penicillins were dried overnight in a vacuum desiccator over concentrated sulfuric acid. This concentration was deemed adequate since accurate results can be expected if the logarithm of the dilution, in this case 2.3, is not equal to or greater than the expected pK_a. Since the pK_a for benzyl penicillin is known to be

about 2.8, the concentration was thus chosen to conserve material and to avoid problems of precipitation as the ion was converted to the free base.

The mixture was stirred during the addition of titrant and for 1 minute longer, by a slow stream of nitrogen bubbles. The gas was supplied from a glass reservoir of about 500 ml capacity and passed through polyethylene tubing to a glass stirring capillary. There was no need to control the temperature of the solution since the constancy of ambient temperature was quite adequate to maintain the contents of the titration vessel within 0.5C°. The pH values were measured within 0.01 pH unit at the beginning and after each addition of titrant. The electrodes were silver/silver chloride (reference electrode) and glass (measuring electrode) included in a single unit (Beckman combination electrode). The indicating device was a Beckman Model 76 Expanded Scale pH meter.

The meter and electrodes were standarized immediately before and immediately after each titration with Beckman standard buffer solutions. In no case had data to be rejected because of unsatisfactory standardization. The constants were calculated according to the method of Albert and Serjeant for monobasic acids, In those cases where the pH range of the titration fell under pH 4.0, due allowance was made in the calculations for the concentration of hydrogen ions. No 'activity' corrections were necessary since the concentration of penicillin was less than 0.01 M. A value could not be obtained for dimethoxyphenyl penicillin because of its acid lability.

(ii) Results.

These are shown in Table 2. Each result in the table is the arithmetical mean of a set of pK_a values calculated at each pH value measured during the titration. All sets contained 9 values. The figures quoted are the mean values together with the maximum deviations of each set.

The spread about the mean for each determination would not commend our work to the physical chemist. Finer values quoted as absolute measurements for a large number of substances have been obtained using as pure material as could in practice be prepared. In our work no effort was made to purify those substances known to be impure. It was also to be expected that those penicillins supplied as commercial samples would not be completely pure. However, no claim for precision is made for these data nor was this aimed for. The object was to detect gross differences if these were present. Nevertheless, our data are quite consistent with the results published by Rapson and Bird (1963) for the five penicillins common to both studies.

One may conclude that the structure of the side-chain can be altered quite appreciably without having very much effect on the ionization of the carboxylic acid group of the penicillin. Successive substitution on the α-carbon of the phenoxy penicillins from H to CH_3 to C_2H_5 results in substances with virtually the same pK_a. Even quite drastic changes from the usual straight side-chains to cyclic side-chains, as in the isoxazolyl penicillins, result in penicillins with pK_a values essentially the same as those for more conventional penicillins. Such results are to be expected because of the electronic and spatial isolation

Table 2. Ionization constants of nine penicillins.

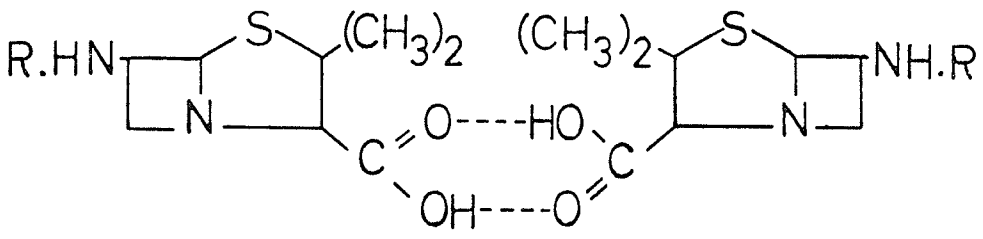
Penicillin	Purity[x]	-COOH, pK_a
Phenoxymethyl	Comm.[+]	2.88 ± 0.26
Phenoxyethyl	Comm.	2.78 ± 0.08
Phenoxypropyl	88.4%	2.88 ± 0.07
Phenoxybenzyl	94.7%	5.41 ± 0.35
Phenylthiodiphenyl	79.5%	5.15 ± 0.47
3,4-Dichloro-α-methoxybenzyl	Comm.	3.30 ± 0.53
5-Methyl-3-phenyl-4-isoxazolyl	85.5%	2.84 ± 0.09
5-Methyl-3-(2-chlorophenyl)-4-isoxazolyl	Comm.	2.95 ± 0.25
α-Aminophenyl	Comm.	2.68 ± 0.12

[+] Comm. (supplied as commercial sample, purity not specified)

[x] The initial concentration of 0.005 M was corrected for purity where this was known. Commercial samples were assumed to be 100 per cent pure.

of the carboxylic acid group from the side-chain.

When the side-chain is markedly increased in bulk as in phenoxybenzyl and phenylthiodiphenyl penicillins, however, there is an appreciable decrease in the acid strength of the carboxyl group. This is surprising in the light of the carboxylic acid group's relative isolation from the side-chain. Most probably the increase in pK_a values is due to the formation of dimers, as -



Attempts to demonstrate dimerization by infra-red spectrophotometry have been frustrated by the insolubility of the penicillins in all solvents lacking absorption in the dimer-indicating region of the spectrum.

(B) PARTITION COEFFICIENTS.

If to a system of two liquid layers made up of two immiscible components is added a third substance which is soluble in both layers, then the substance will distribute or partition itself between the two layers in a definite manner. This property of a substance is its distribution or partition coefficient which is constant for any two solvent system as long as the temperature at which the determination is being made is constant. The coefficient is given numerical value by dividing the concentration present in one layer at equilibrium by the concentration in the other layer. The distribution of a substance between a non-polar solvent and water is usually of greatest interest in biological work, and in this situation the following formula applies:

$$\text{Partition coefficient} = \frac{\text{Concentration in non-polar solvent}}{\text{Concentration in water.}}$$

It is important in the case of organic electrolytes to adjust the pH of water so that all, or a very large proportion, of the substance is present as the unionized molecule. Ionized, and hence charged, molecules will not leave readily the water, or polar, phase. It is important also, in biological work, to determine partition coefficients using several non-polar solvents. The prime interest is how a substance will distribute itself between extracellular water and intracellular water, the two being separated by the lipid-like cell membrane. The use of only one non-polar solvent of low molecular weight might give a misleading picture of the substance's solubility in the lipids of cell

membrane. These consist of fatty acids, triglycerides, lecithin, cholesterol, and related compounds (Davson and Danielli, 1952).

(i) Methods.

Solutions of either the sodium or potassium salt of the penicillins at a concentration of 0.2 mM in distilled water were brought to pH 2.0 by the addition of 1.0 N HCl. At this pH all, or a very large proportion, of each penicillin was present in the unionized form. Ideally the pH should have been reduced even further to reduce the proportion of ionized molecules present in solutions of the penicillins with low pK_a values, but, when this was tried, some of the penicillins decomposed to an appreciable extent. However, the movement of some proportion of unionized substance into the non-polar phase would result in an overall shift in equilibrium towards unionized molecules so that the small proportion of the total present as ionized molecules would be reduced. The error would, therefore, be quite small.

One ounce capped bottles containing the solutions were shaken vigorously in a shaking machine for 15 minutes at laboratory temperature, 22°C. Each bottle contained 10 ml of the penicillin solution and an equal volume of n-heptane, petroleum ether (fraction 60-80°C), or oleyl alcohol. At the end of this time, which had been determined previously as adequate for equilibration, the bottles were centrifuged and the water layer pipetted off. The aqueous solution was then brought to pH 7.0 by the addition of 1.0 N NaOH solution. After appropriate dilution with distilled water, the concentration of penicillin remaining in the water phase was determined by the disc-plate assay described above. In each experiment, identical solutions of the penicillins were

subjected to the same procedure, with the omission of the non-polar phase, so that due allowance could be made for errors caused by the addition of the small volumes of acid and alkali. The assayed concentrations of these penicillins were regarded as the 'control' concentrations.

The partition-coefficient is expressed as:

$$\frac{(\text{'Control' concentration}) - (\text{Conc. remaining in water phase})}{(\text{Conc. remaining in water phase})}$$

In a further series of experiments using oleyl alcohol as the non-polar phase, the pH was adjusted to 5.3 to mimic conditions pertaining at the absorbing surface of the small intestine. It was thought that data from these experiments should give a better indication than an absolute, or near-absolute, partition coefficient of the ability of the penicillins to pass from intestinal lumen to the general circulation.

The non-polar solvents were chosen for the following reasons. N-heptane is a solvent commonly used in studies on partition-coefficients of drugs. Petroleum ether was selected arbitrarily as a mixture of hydrocarbons of different chain lengths. Oleyl alcohol is thought to be representative of the fatty alcohols found in cytoplasmic membranes (Albert et al., 1954).

(ii) Results.

These are shown in Table 3.

All the penicillins were virtually insoluble in n-heptane.

Table 3. Partition coefficients of ten penicillins between water and three non-polar solvents.

	Partition Coefficient			
Penicillin	N-heptane	Pet.Ether	Oleyl alc. pH 2.0	Oleyl alc. pH 5.3
Phenoxymethyl	0 *	0.035	48	2.8
Phenoxyethyl	"	0.11	81	4.6
Phenoxypropyl	"	not done	> 800	5.9
Phenoxybenzyl	"	0.074	> 800	15
Phenylthiodiphenyl	"	0.36	> 800	800
3,4-Dichloro-α-methoxybenzyl	"	0.35	> 800	13.3
5-Methyl-3-phenyl-4-isoxazolyl	"	0.12	63	1.5
5-Methyl-3-(2-chlorophenyl)-4-isoxazolyl	"	0.15	> 800	1.8
Dimethoxyphenyl	"	0.043	11.6	0.19
α-Aminophenyl	"	0.048	0.87	0.08

* All the penicillins were virtually insoluble in this solvent

With the procedure used, there was no difference detectable in the concentrations in the water phase whether or not n-heptane was present. The partition-coefficients for this series of substances between n-heptane and water must therefore be vanishingly small.

The petroleum-ether/water partition coefficients of the penicillins are all numerically low. These data are inconsistent with the readiness with which most of these penicillins are absorbed from the gastrointestinal tract (e.g. see Bond et al., 1963).

The values obtained at pH 2.0 using oleyl alcohol as the non-polar phase, were all numerically much greater than 1, with the exception of that for α-aminophenyl penicillin which was 0.87. These data indicate that all these penicillins in the unionized state, possess an appreciable degree of lipid solubility. The data in the final column of Table 3 show that, at pH 5.3, marked changes have occurred in the distribution of the penicillins. The partition coefficients are now a function not only of the solubility in oleyl alcohol but also of the pK_a values of the different substances. All values have decreased. This was to be expected since very much greater proportions of the molecules of each penicillin were present in ionized form.

(C) BINDING TO SERUM ALBUMIN

A very large number of substances foreign to the body, whether endowed with pharmacological activity or not, interact with plasma proteins. Almost all of those which have been studied adequately have been found to combine with plasma albumin. In most of the cases studied closely it has been found that the drug-protein interaction can be shown to follow an adsorption isotherm. However, in the case of a reversible reaction, which is the case of most drug-protein combinations in which an equilibrium is set up between free and bound drug, the finding of close adherance to the adsorption isotherm does not mean that the forces are physical rather than chemical in nature. In fact Langmuir's adsorption isotherm is simply an extension of the law of mass action, as shown below.

In the equilibrium, $X + P_f \rightleftharpoons XP_f$ where X is the concentration of unbound drug, P_f is the concentration of free binding sites (? receptors), XP_f is the concentration of combined sites, and K is the dissociation constant, then

$$K = \frac{(X)(P_f)}{(XP_f)}$$

Now let n be the total number of sites carried by each protein molecule and P be the molar concentration of total protein. Then nP represents the total concentration of sites, i.e. $(P_f) + (XP_f)$. Substituting $(P_f) = nP - (XP_f)$,

$$nP(X)-(XP_f)(X) = K(XP_f)$$

or $$nP(X) = \left[K + (X)\right](XP_f)$$

Now let r be the moles of drug bound per mole of total protein, or $(XP_f)/P$, then,

$$r = \frac{(XP_f)}{P} = \frac{n(X)}{K + (X)}$$

which is identical in form with the Langmuir adsorption isotherm -

$$y = \frac{x}{m} = \frac{bc}{1/a + c}$$

Hence any reversible reaction, whether physical or chemical, will follow a curve represented by the isotherm as long as the following criteria are observed:

(a) the interaction must be reversible and the data must be obtained at complete equilibrium.

(b) all groups, or sites, must have the same affinity for the drug,

(c) there must be no reduction in binding by electrostatic repulsion due to the approach of subsequent ions of the same charge as that of initially bound drug, and

(d) quantitation is only applicable to interactions of pure drug with pure and homogeneous protein of known molecular weight.

In the light of these requirements it would seem, therefore, to be to little effect to study quantitatively the number of binding sites and the tightness of binding of albumin to the penicillins used in this study. There may be several interactions involving different equilibrium constants, and there may be appreciable effects due to repulsion of 'free' molecules by those already bound. Moreover, the penicillins contained impurities.

It would be useful at this point to examine the various types of bonds possible between albumin and drugs. Briefly, they are as follows; (a) van der Waals' attractive forces with a bond strength of approximately 0.5 kcal/mole but strongly dependent on close approximation of the two molecules, (b) hydrogen bonds with bond strength of from 2 to 5 kcal/mole, (c) ionic bonds with bond strength of 5 kcal/mole, (d) reinforced ionic bonds with bond strength of 10 kcal/mole, (e) covalent bonds with bond strength of from 40 to 140 kcal/mole, and (f) hydrophobic bonds with bond strengths as yet inadequately defined, but implicated as the major force in maintaining the conformation of proteins, for example, (see, West, et al., 1966).

Little is known about the rate constants for association and dissociation between drugs and albumin. However, an indication of their magnitude was given by Froese, Sehon and Eigen (1962). They shifted the equilibrium between an acidic azo dye and bovine albumin by suddenly decreasing the temperature 10C° within 1 μsec. Their calculation of the rate constant showed that dissociation occurs with a 'half-life' of 20 msec. Since the values for K and n for this acid were similar to those of other organic anions binding to bovine albumin, it seems likely that the rate constants for other organic acids will be of a similar order of magnitude.

Because of this short 'half-life', and because of the known ready reversibility of the penicillin-albumin complex, covalent bonding can be excluded from consideration. It is well known that, at body temperature, the greatest strength of bond that can be broken readily is about 10 kcal.

The isoelectric point of albumin is at pH 4.9 so that at pH 7.4 the molecule carries a net negative charge. It was early supposed that only cations would interact under these circumstances but this was soon shown to be incorrect. Ionic combination between groups of opposite charge occurs irrespective of the net charge on the protein, and a great many more anions than cations have actually been shown to interact at physiological pH. For example, Klotz and Walker (1947) have shown that the binding of methyl orange, a sulfonated azo dye, is constant as the pH is varied between 5 and 9, but that complete dissociation occurs between pH 9 and 11.5. This corresponds very closely with the dissociation range of the ϵ-amino group of lysine. Since there is an abundance of the groups in serum albumin, it was concluded that the dye probably combines with them.

However, the properties of individual amino acids may play an unimportant part. The general surface pattern of the protein may be of more account. Binding sites probably depend not only upon individual ionic groups but also upon the properties of nearby non-ionic residues, upon steric hindrance to approaching molecules, and upon surface configurations affecting the strength of van der Waals' and lipophobic forces.

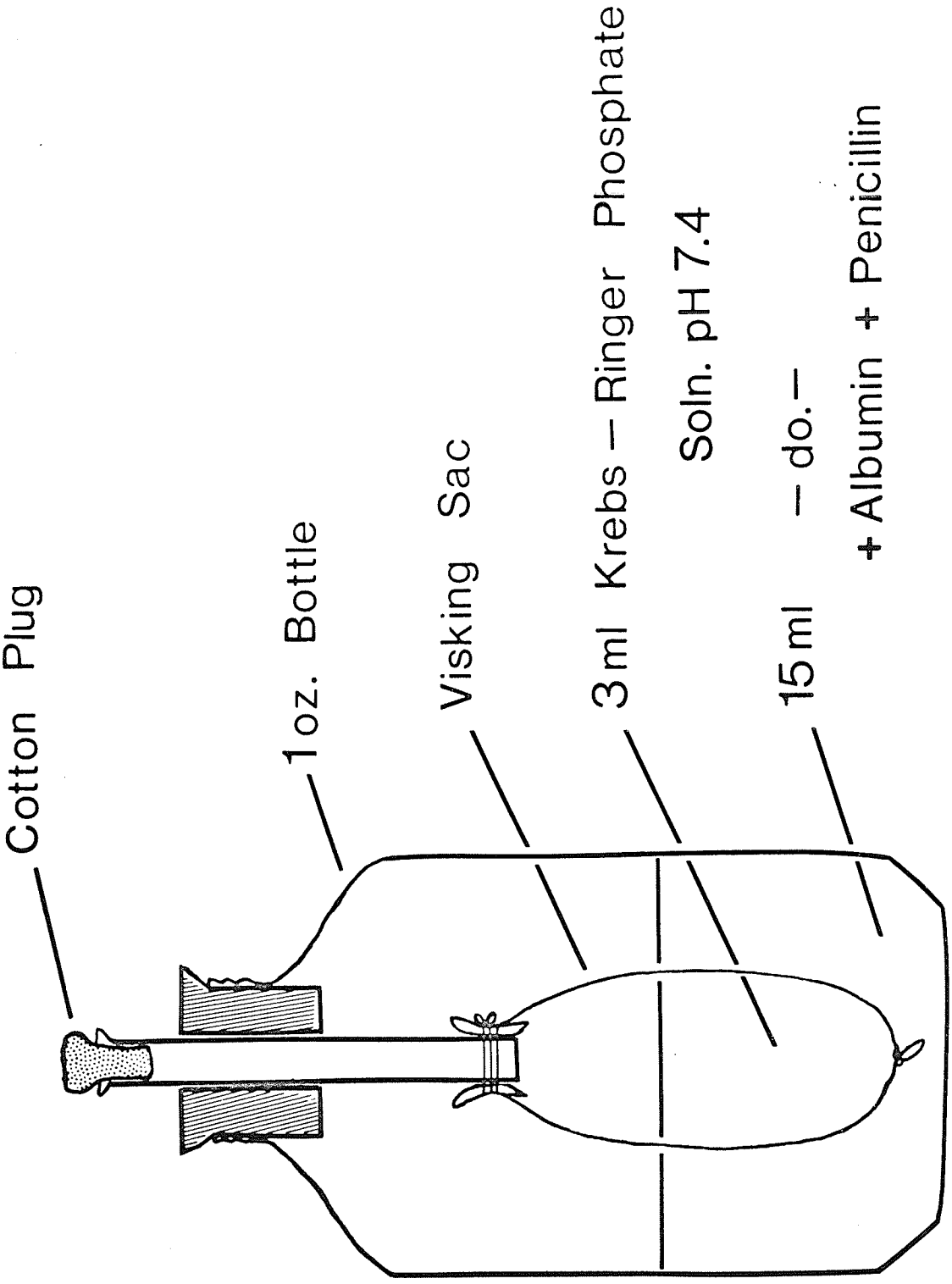
Hence, for binding of penicillins to serum albumin, all the remaining bond types will have to be considered.

(i) Methods.

The apparatus depicted in Figure 4 was used in this series of experiments. Before assembly, the sac prepared from Visking tubing and tied to a short length of glass tubing, was tested for leaks by applying compressed air to the open end and immersing the sac in water. Any allowing bubbles to form were rejected. After assembly in the 1 oz bottle, the whole was autoclaved at 15 lb/sq in for 20 minutes. Batches of these assemblies were set aside to dry at laboratory temperature until a sample, subsequently discarded, felt dry to touch. Hence, errors introduced by water contained within the membrane were minimal. All manipulations from this point on were carried out using aseptic technique and sterile solutions to obviate any possibility of penicillin hydrolysis by penicillinase-producing contaminants. Three millilitres of Krebs-Ringer-Phosphate solution (pH 7.4) were pipetted into the distended sac and allowed to permeate the dry membrane for 15 minutes. After loosening the rubber stopper, 15 ml of the same solution containing the albumin/penicillin mixture were pipetted into the free space of the bottle. The albumin was diluted from a concentrated solution (25 gm/100 ml) of human serum albumin prepared by Connaught Laboratories. Bottles containing no albumin were included in each batch. The stopper was replaced so that the levels of solution within and without the sac were the same.

Groups of bottles were then immersed in a water bath at 37°C and shaken gently. Control bottles containing only penicillin-Krebs-Ringer-Phosphate solution were also included in each group. These solutions were used at the end of the experiment in the preparation of standards for the pad-plate assay. After 1/2, 1, 2, 4 and 6 hours of

Figure 4. Apparatus used in dialysing penicillin-albumin solutions.


Cotton Plug
1 oz. Bottle
Visking Sac
3 ml Krebs – Ringer Phosphate
Soln. pH 7.4
15 ml – do. –
+ Albumin + Penicillin

incubation, 0.05 ml aliquots were removed from the solution inside the sac by means of a calibrated Pasteur pipette and deposited on standard filter paper assay discs. These were stored in the refrigerator until required for assay. A further two 0.05 ml aliquots were taken from the solution at 6 hours so that the estimated figure for the final concentration was based on three samples from each of three bottles. Standards for assay were prepared by dilution from the control solution.

The time of attaining equilibrium could be assessed from the slope of the concentration versus time curve pertaining to each bottle. It always occurred between 4 and 6 hours after the start of the experiment (see Figure 5). All dialysates were tested for the presence of protein by adding ninhydrin reagent at the end of each experiment. In no case did penetration of the membrane by albumin occur.

(ii) Results.

The results obtained are shown in Table 4.

Each figure, representing the percentage of free, or unbound penicillin, was calculated from the formula,

$$\frac{\text{Concentration in dialysate from protein solution}}{\text{Concentration in dialysate from protein-free solution}} \times 100\%$$

The initial concentrations of the penicillins were not equivalent; they were chosen for convenience of assay. Other workers have shown that the proportion of several penicillins bound to plasma proteins varies little, if at all, over a wide range of penicillin concentrations (see Keen, 1965). Indeed, if only one binding site were

Figure 5. Rate of appearance of penicillin in dialysate from a solution containing both penicillin and serum albumin. Ordinate - concentration of penicillin in dialysate. Abscissa - time in hours.

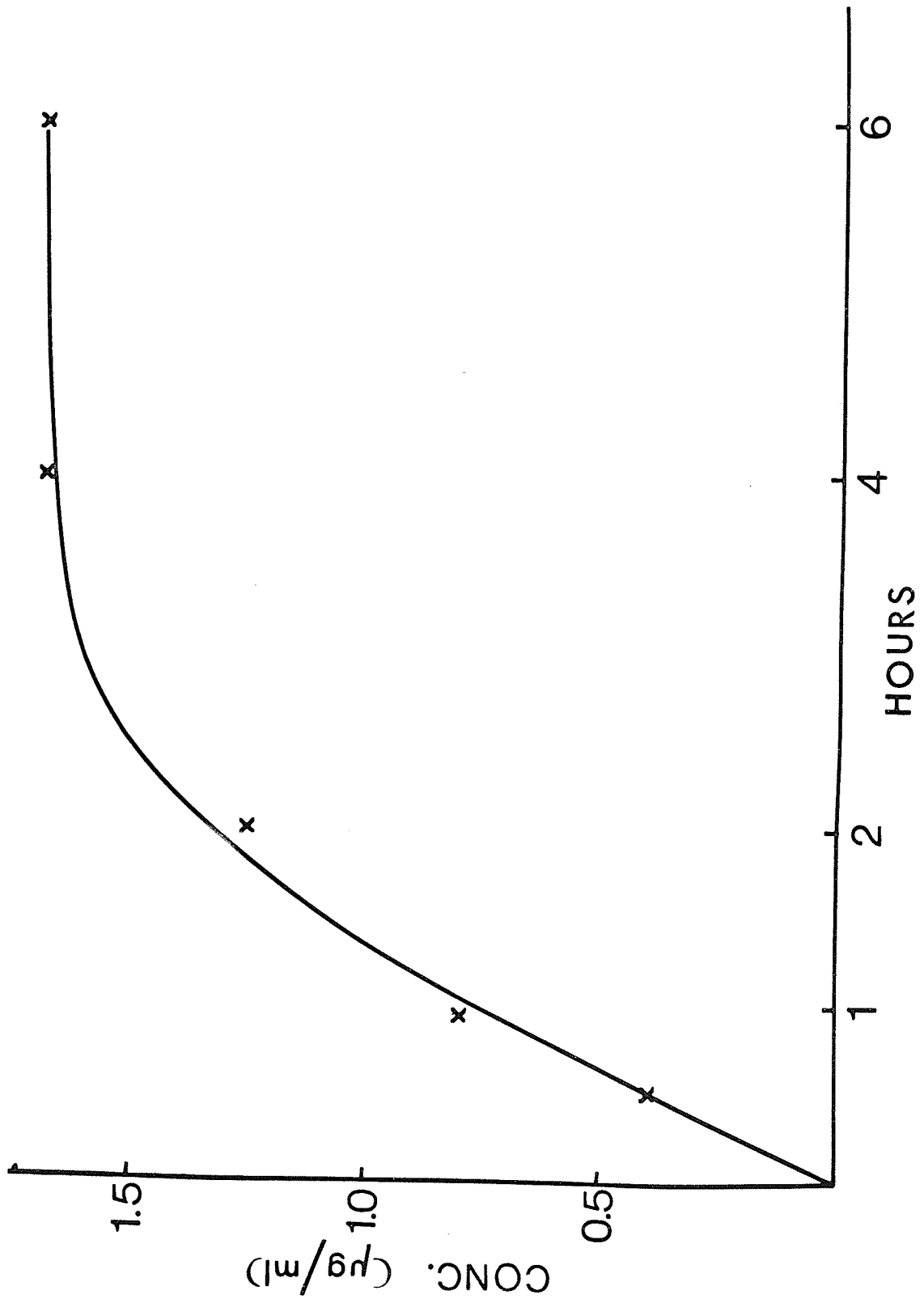

CONC. (µg/ml)
1.5
1.0
0.5
1
2
4
6
HOURS

Table 4. The binding of ten penicillins to human serum albumin. Each figure represents the percentage of unbound substance (arithmetical mean of three experiments).

Penicillin	% Albumin		
	0.25	1.0	4.0
Phenoxymethyl (2) *		50	31
Phenoxyethyl (8)		89	54
Phenoxypropyl (8)		62	28
Phenoxybenzyl (8)		39	18
Phenylthiodiphenyl (80)	87	12	0
3,4-Dichloro-α-methoxybenzyl (2)		44	21
5-Methyl-3-phenyl-4-isoxazolyl (20)		31	20
5-Methyl-3-(2-chlorophenyl)-4-isoxazolyl (12)		71	30
Dimethoxyphenyl (100)		87	71
α-Aminophenyl (10)		100	54

* Figures in parentheses are initial concentrations in μg/ml (corrected for purity)

present on the albumin molecule, so that its carrying capacity were one molar equivalent, the limiting concentration above which the fraction of unbound drug would begin to increase greatly would be about 170 μg/ml. Even the highest concentration in these experiments, 100 μg/ml of dimethoxyphenyl penicillin, is appreciably lower than this.

Human serum albumin only has been used in these experiments. The reasons are two-fold. First, the penicillins are bound almost exclusively to albumin. Thompsett, Schultz and McDermott (1947) have shown this for the early natural penicillins. We have extended this work for several of the semi-synthetic penicillins (Rollo, unpublished). Second, because virtually pure human serum albumin was available from pooled stocks, and because great variation in albumin content appears in serum from even healthy donors.

It is obvious from examination of the data that there are marked differences in the degree of binding of the various penicillins at any one albumin concentration. Further experiments, not reported here, in which smaller concentrations of some of the penicillins were used, show no appreciable differences in the proportions bound. The data also show that, as expected, the degree of binding decreases as the concentration of albumin is decreased. Phenylthiodiphenyl penicillin is, of all the penicillins, the most highly bound. Even at a low concentration of albumin of 1 per cent, only 12 per cent remains unbound. The degree of binding becomes small only when the concentration of albumin is further reduced.

(D) DISCUSSION

It is difficult to delineate what physical properties of the 'bulky' penicillins (see above) cause a decrease in their acid strength. We have assumed, for want of other explanation, that the observed increase in pK_a values is due to a greater propensity for the formation of dimers. The question then arises, why do these penicillins form dimers more readily than their congeners, when it is unlikely that intramolecular changes due to changes in the side-chain could affect a distant carboxylic acid group? One physical difference that has been observed is the very high value obtained for the oleyl alcohol/water partition coefficients of both penicillins.

High values were also obtained for phenoxypropyl, 3,4-dichloro-α-methoxybenzyl, and 5-methyl-3-(2-chlorophenyl)-4-isoxazolyl penicillins. This is associated with a decrease in acid strength for 3,4-dichloro-α-methoxybenzyl penicillin, although this may not be significant because of the large possible error in the estimation. It seems likely that, next to the two phenoxy penicillins already mentioned, this dichloro-substituted penicillin possesses the highest partition coefficient by virtue of the strongly lipophilic-promoting properties of the halogen atoms.

If indeed the tendency to dimer formation is increased by increasing lipid solubility of the side-chain, it could be imagined that an increase in the non-polar characteristics would tend strongly to the squeezing out of those molecules from solution due to the high internal pressure of the water molecules. This squeezing out might result in greater opportunity for association between molecules, rather as fat

droplets in water tend to coalesce to form globules, and hence for the formation of hydrogen-bonded dimers.

In addition to the strongly lipophilic penicillins mentioned above, phenoxymethyl, phenoxyethyl, and 5-methyl-3-phenyl-4-isoxazolyl penicillins also pass quite readily into the non-polar phase. This correlates well with the lipophilic nature of their side-chains. Indeed, the lengthening of the substitution on the α-carbon atom from $H \rightarrow CH_3 \rightarrow C_2H_5$, which might be expected to increase the lipophilic nature of the substances, results in increases in the oleyl alcohol/water partition coefficients from $48 \rightarrow 81 \rightarrow > 800$.

The relatively low value obtained for α-aminophenyl penicillin probably reflects the amphoteric nature of the compound. The pK_a value for the primary amino group has been found to be 7.24 by Rapson and Bird (1963). At pH 2, therefore, virtually all the molecules would be ionized and would tend to stay in the polar phase. Dimethoxyphenyl penicillin also has a relatively low oleyl alcohol/water partition coefficent. This is due probably to a shortening of the side-chain and to the presence of two methoxy groups which have slight hydrophilic-promoting properties.

If the value ten is selected arbitrarily as indicating a high value for the partition coefficient, then only three of the penicillins appear to be highly lipid soluble at pH 5.3. For two of these, phenoxybenzyl and phenylthiodiphenyl penicillins, the high values result from the combination of two factors. These are, (a) the high degree of lipid solubility of the unionized molecule, and (b) the pK_a values, 5.41 and 5.15 respectively, both of which are close to the pH of the water phase. For the third, although only a small proportion of the substance was present in the unionized form, the high degree of lipid solubility

probably results from the strongly lipophilic halogen substitution in positions 3 and 4 of the terminal phenyl ring.

Since the values for partition coefficients obtained at pH 5.3 are all greater than unity, with the exception of the final two in Table 3, it might be expected that all penicillins showing this distribution would be well absorbed from the small intestine. Such is indeed the case. Various published data include all of these substances in the group of 'oral' penicillins (see Weinstein, 1965). The low value obtained for dimethoxyphenyl penicillin is consistent with the low degree of absorption from the small intestine (Pindell, Tisch and Reiffenstein, 1961).

The low value obtained for α-aminophenyl penicillin is inconsistent, however, with its apparently ready absorption after oral administration (Acred, et al., 1962). It might be suspected, therefore, that concentrations appearing in the blood do not reflect only the extent of absorption from the gastrointestinal tract. Since little or no tissue storage or formation of metabolites occurs, most of the penicillin's elimination is dependent on biliary or urinary excretion (Acred, et al., 1962).

High concentrations have been found in the bile of treated animals (Stewart and Harrison, 1961), hence the cycle of absorption-excretion-reabsorption and so on, would tend to augment whatever concentration appeared in the blood. Furthermore, this penicillin is relatively slowly excreted in the kidney (Barber and Garrod, 1963; Kirby and Kind, 1966), and only a small fraction is recovered after oral administration compared to the fraction recovered after parenteral administration (Acred, et al., 1962).

The extent of intestinal absorption, therefore, is probably not great. This conclusion is supported by the fact that peak blood concentrations occur much later than do those of the other 'oral' penicillins (Lynn, 1965). Nevertheless, well controlled comparative studies are required, and these have not been reported.

Binding of benzyl penicillin to serum albumin has been extensively studied since its initial demonstration by Chow and McKee in 1945. The availability of many other penicillins with differing side-chains, and comparison of the various proportions bound, has led to the conclusion that the side-chain is the major determinant of binding (Kunin, 1965). This is supported by the finding that the carboxylic acids of the side-chains showed greater activity as displacing agents than did the parent compound or the 6-aminopenicillanic acid nucleus. In addition, Steinman and Denny (1963) showed that esterfication and amidation of the terminal carboxyl group, produced few changes in binding, an indication that this group is of little importance as a binding site.

Differences in degree of binding have been explained on the basis of steric hindrance (Brandl, Brenner and Knauseder, 1964), and on the basis of reinforcement of van der Waals' forces by increase in molecular weight (Keen, 1965). However, recent data obtained by Fischer and Jarditzky (1965) from a nuclear magnetic relaxation study of inter-molecular complexes have shown that, for benzyl and phenoxymethyl penicillins, the penicillin-albumin interaction is hydrophobic in nature. Their findings ruled against ionic bonds being of any great significance.

Our data confirm that there are very marked differences in degree of binding from penicillin to penicillin. Meaningful comparison of these with previously reported results is difficult. Various

investigators have reported quite widely divergent data on binding to serum proteins. This is scarcely surprising since several different methods have been used for evaluating the degree of binding, e.g., gel filtration, static and continuous dialysis, ultrafiltration and cup-plate assay. Test conditions and interpretation of results have also varied.

Nevertheless, if listed according to degree of binding, our data shows quite good correlation with a summary of data acquired from ten different reports (see Warren, (1965); (Table 5). The only real difference is in the relative positions of the two penicillins first on each list. The figures for percentage binding quoted by Warren for α-aminophenyl penicillin range from 10.2 to 21 per cent while our figure is 46 per cent. The respective figures for dimethoxyphenyl are from 28 to 49.3 per cent and 29 per cent. Obviously our data are consistent for dimethoxyphenyl penicillin but not for the α-aminophenyl compound. We are unable to explain this difference which is outside the range of probable error.

Differences also appear in the ranking of the penicillins fifth, sixth and seventh on the list. These differences are more apparent than real, since the appropriate percentages (with ranges) are,

phenoxypropyl penicillin	88.6	(88-89.3)
5-CH_3-3-phenyl-4-isoxazolyl penicillin	89.1	(86-93.1)
5-CH_3-3-(2-chlorophenyl)-4-isoxazolyl penicillin	91.4	(88-94.6)

hence any one ranking is probably as good as another.

Table 5. Penicillins listed according to increase in binding to plasma proteins.

Listed according to data summarized by Warren (1965)*	Listed according to data presented here
α-Aminophenyl penicillin+	Dimethoxyphenyl penicillin
Dimethoxyphenyl penicillin+	α-Aminophenyl penicillin
Phenoxyethyl penicillin	Phenoxyethyl penicillin
Phenoxymethyl penicillin	Phenoxymethyl penicillin
Phenoxypropyl penicillin+	5-CH_3-3-(2-chlorophenyl)-4-isoxazolyl penicillin
5-CH_3-3-phenyl-4-isoxazolyl penicillin+	Phenoxypropyl penicillin
5-CH_3-3-(2-chlorophenyl)-4-isoxazolyl penicillin+	5-CH_3-3-phenyl-4-isoxazolyl penicillin
Phenoxybenzyl penicillin	Phenoxybenzyl penicillin

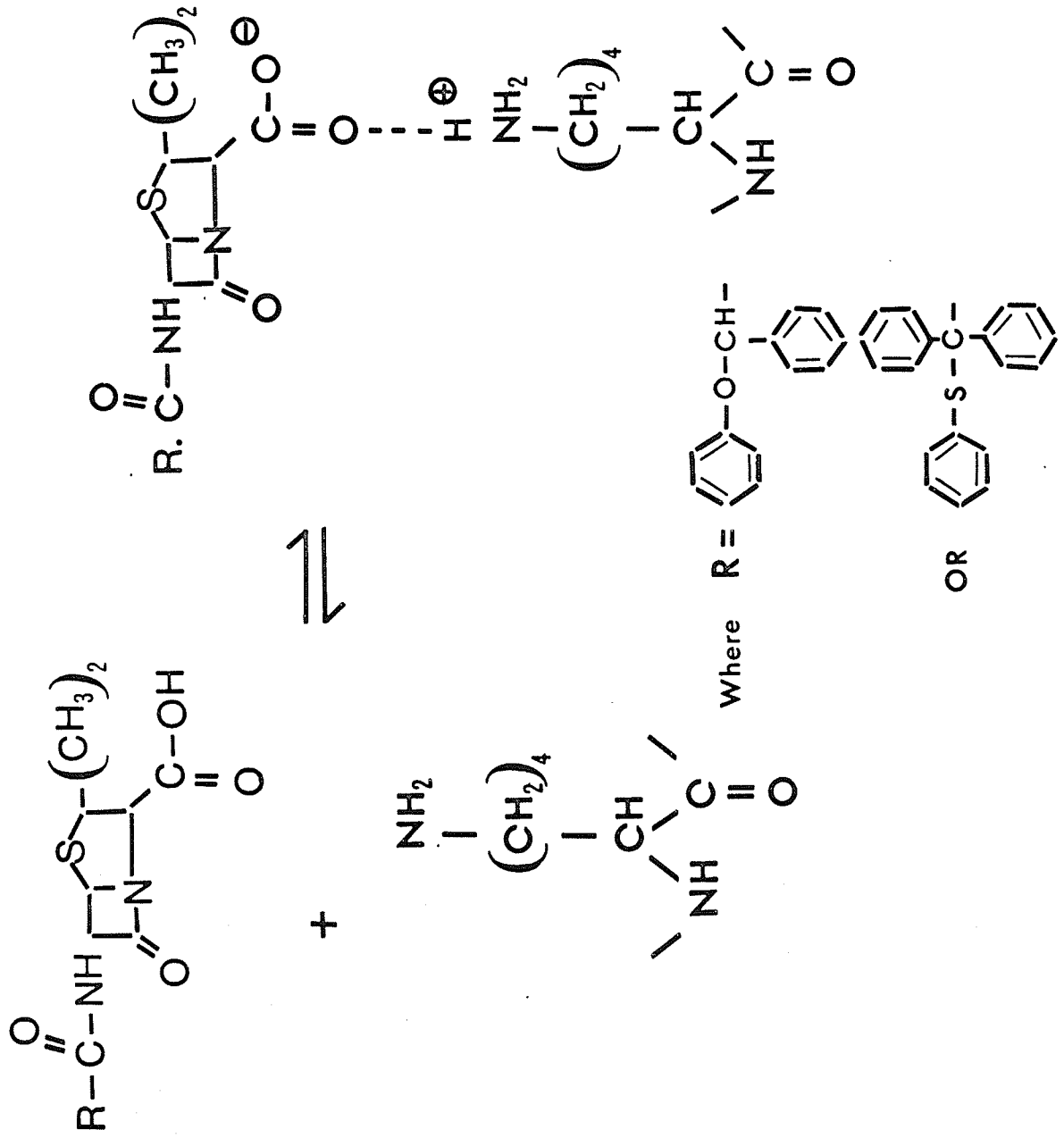
* Ranked according to arithmetic mean of data grouped for each substance.

+ Order differs from ours.

The data pertaining to the phenoxy-penicillins and the closely related phenylthio-penicillin are are especially interesting because the five substances are members of an essentially homologous series. Since the experiments were carried out at pH 7.4, all the penicillins were wholly, or almost wholly, present in the ionized form. Hence, any effect on binding due to ionic bonding between the carboxyl anion of the penicillins and oppositely charged groups on the surface of the protein molecule would have been constant. It seems unlikely, however, in light of Fischer and Jarditsky's data, that any penicillin with an ionization constant similar to that of phenoxymethyl penicillin would be bound to any appreciable degree by such bonds. The same is not necessarily true for the 'bulky' penicillins. We have determined that the acid strengths of these two substances are considerably less than the acid strengths of other members of the series, and have postulated that hydrogen bonding with dimer formation may account for this. If such is the case, we may postulate further that any short-acting ionic bond formed between the ϵ-amino groups in the abundant lysine residues of albumin, and carboxyl groups of the penicillins, would become much stronger and more long-lasting due to the simultaneous formation of hydrogen bonds, the so-called 're-inforced ionic bond' (Figure 6). Such binding could, of course, be supplemented by short range van der Waals attraction and hydrophobic bonding. This is discussed below.

In this series which represents successive substitution on the α-carbon atom of the side-chain by - H, CH_3, C_2H_5, C_6H_5, di-C_6H_5 (assuming that in the last case phenylthio- is equivalent to phenoxy-), it might be assumed that increase in molecular size would result in an

Figure 6. Formation of re-inforced ionic bonds between certain penicillins and ϵ-amino groups of albumin lysine.

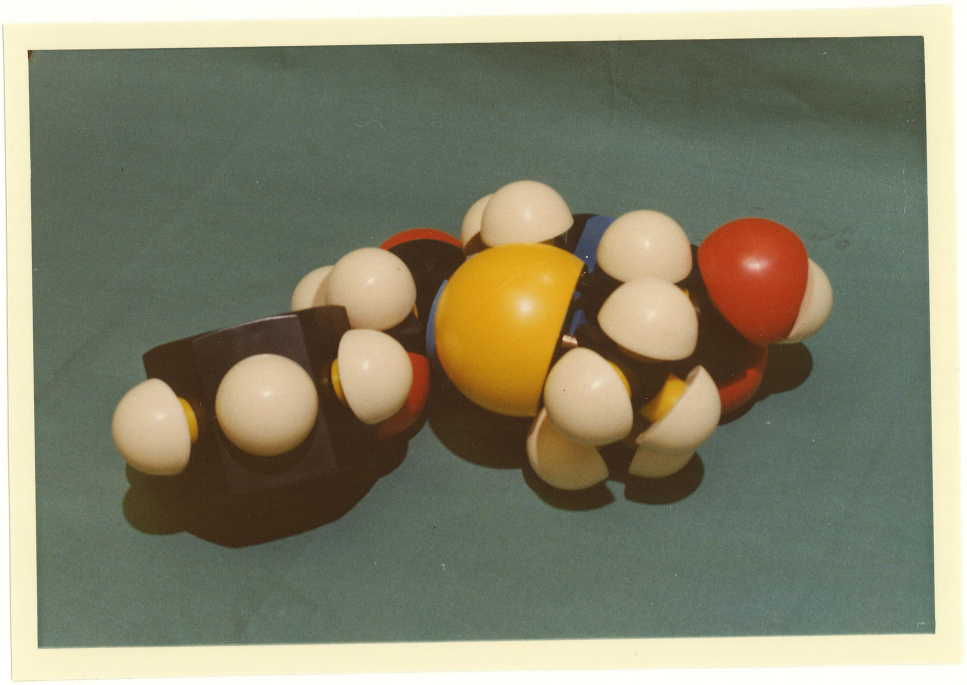

Where R=
OR

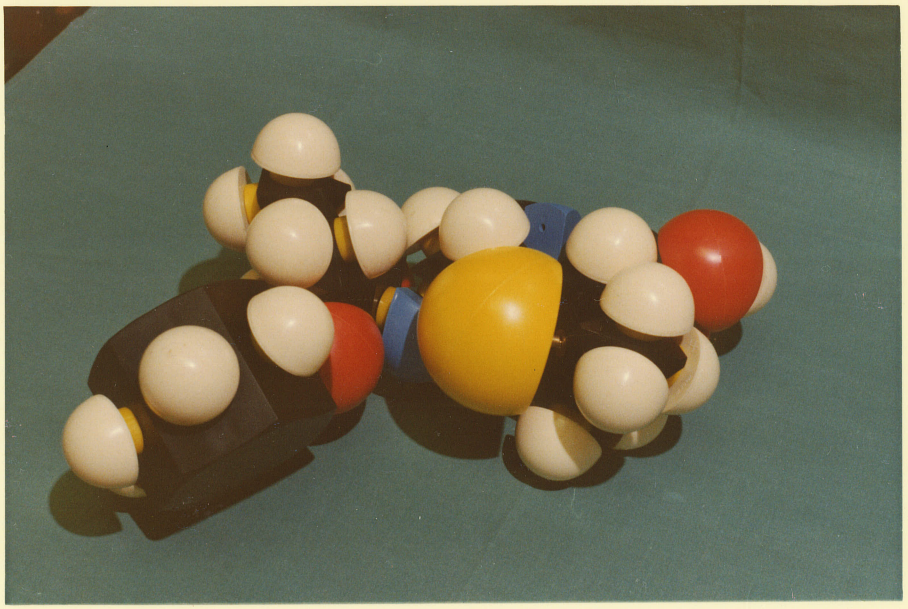
increase in affinity to the protein surface due to an increase in non-specific van der Waals forces. This explanation has been invoked by Keen (1965) for a limited series of three penicillins differing little in molecular weight on the basis of data for barbiturates (Goldbaum and Smith, 1954), fatty acids (Teresi and Luck, 1952), and the early penicillins (Thompsett, Schulz and McDermott, 1947).

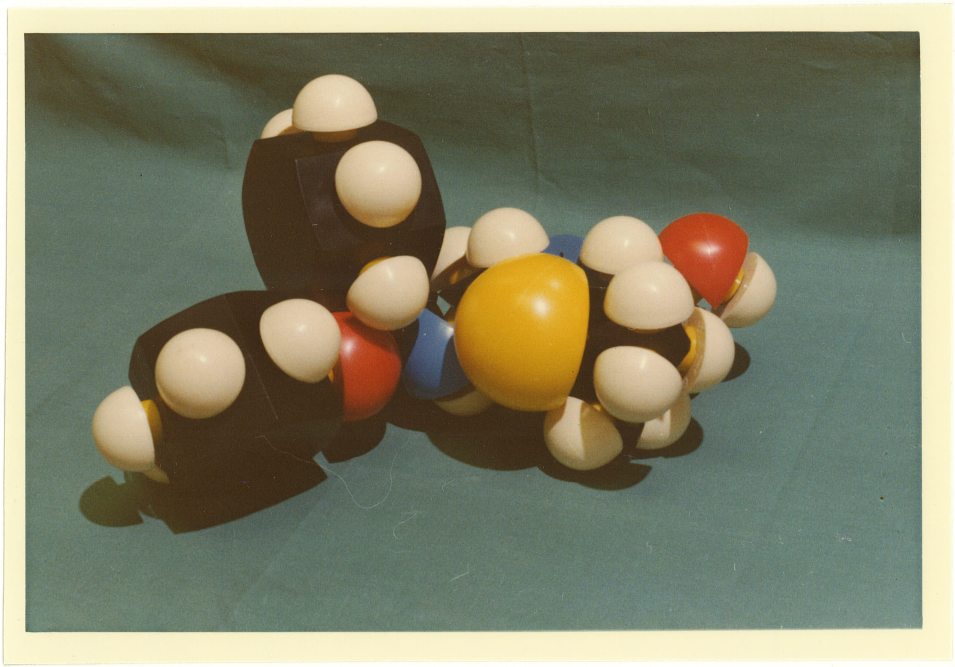
However, such forces vary inversely as the seventh power of the distance between attracting groups and are, therefore, significant only over a very short range. Furthermore, since the terminal aromatic ring would probably make the most sizable contribution to the bonding energy, only those substances in which the ring could be oriented in a position planar to the protein surface would be bound to equivalent degrees. Examination of models constructed from Courtauld space-filling units (Plates 1 to 3) reveals that in no case is an orientation of the terminal phenyl group flush with a flat surface possible. With phenoxymethyl penicillin, the angle of incidence between the plane of the phenyl group and a nearest possible flat plane is quite small. However, successive substitution, as in phenoxyethyl and phenoxypropyl penicillins, results in a nearly regular increase in the angle of incidence between the two planes. With the mono- and di-phenyl substituted substances, the angle of incidence becomes even greater.

If van der Waals forces make an appreciable contribution to the degree of binding, it might be expected, therefore, that increasing substitution on the α-carbon atom would result in a decrease in the proportion of penicillin bound. Such is indeed the case when phenoxymethyl and phenoxyethyl penicillins are compared. It appears, therefore, that

Plates 1, 2 & 3. Courtauld models of phenoxymethyl, phenoxypropyl and phenoxybenzyl penicillins (in that order).







van der Waals bonding plays an appreciable part in the overall forces holding these penicillins to the albumin molecule.

There is, however, a marked increase in the degree of binding of phenoxypropyl penicillin when compared with its nearest neighbour, phenoxyethyl penicillin. This marked increase, instead of a decrease expected on the above basis, is most probably associated with the high value observed for the oleyl alcohol/water partition coefficient. Any decrease in binding due to weakening of van der Waals bonding forces might be more than compensated for by an orientation of the molecule close to the protein surface. This might be expected to occur because of the strongly non-polar nature of the molecule and the all-surrounding presence of the strongly polar water molecules.

Previously to the recent, more definitive, work by Fischer and Jarditsky (1965), hydrophobic bonding had been proposed as being responsible for the binding of several types of substances to serum albumin (see Goldstein, 1949). The interaction of the lower fatty acids (C_2-C_{10}), for example, is clearly favored by increasing length of the hydrophobic chain. It has been proposed that penicillin interactions allow an extension of the same principle. These substances can be regarded as modified fatty acids containing a peculiar cyclic dipeptide as part of the long chain. In the early penicillins, penicillin K with the longest aliphatic chain (n-heptyl) shows the greatest affinity, followed, in turn, by dihydro-F (n-amyl), G (Benzyl), and X (p-hydroxy-benzyl). The difference between X and G has been attributed to the polar hydroxy group of the former which is presumably attracted away from the protein towards the polar phase. Recent thermodynamic data on the binding of a sulfonamide to albumin showed that changes in standard free energy

and entropy occurred which were consistent with the active involvement of van der Waals or hydrophobic binding forces (Clausen, 1966).

The strongly non-polar nature of the side chains of phenoxybenzyl, phenylthiodiphenyl, 3,4-dichloro-α-methoxybenzyl, and 5-methyl-3-(2-chlorophenyl)-4-isoxazolyl penicillins probably accounts for their high affinity to albumin. This, together with the postulated occurrence of re-inforced ionic bonds between the first two named and the ε-amino groups of albumin lysine, would be consistent with the fact that they are, of all, the most highly bound penicillins.

Dimethoxyphenyl penicillin is little bound. This is probably a function of steric hindrance to close approximation of molecule and surface, and of its relatively low lipid solubility in comparison to other, more highly bound, penicillins. α-Aminophenyl penicillin is aberrant in that, while it is appreciably bound, it is only very slightly lipid soluble. The insertion of the amino group would be expected to have an additional, sterically hindering, effect. Since the substance is amphoteric, a possible explanation might involve ionic bonding between the two ionized sites and complementary sites on the surface of the protein. We have no data to support such an explanation.

5-Methyl-3-phenyl-4-isoxazolyl penicillin is completely aberrant. It is highly bound to albumin, yet it is not highly lipid soluble, its spatial structure does not suggest any undue peculiarity that might suit it for a close fit to the protein surface, and it is neither more nor less strongly acidic than the majority of its congeners. Obviously, a more extended study is required to elucidate the nature of the binding of these last two substances to albumin.

Our data also show that, as might be expected, the degree of protein binding decreases as the concentration of albumin is decreased. Phenylthiodiphenyl penicillin is, of all the penicillins, the most highly bound. Even a fourfold decrease in albumin concentration results in only 12 per cent remaining unbound. Only when the concentration is reduced a further fourfold does the degree of binding become small.

Our data were not corrected for Donnan distribution nor for the space occupied by albumin since, at any one albumin concentration, these factors would have been identical. Correction of our data would involve multiplying the concentrations in Table 4 by a factor of 1.036, and multiplying the concentration on the numerator of the equation by a factor of 0.9 (McLean and Hastings, 1935; Keen, 1965). Since we were interested in comparative values for binding rather than absolute figure we have quoted, and used, the uncorrected data.

5. CHOICE OF PENICILLINS FOR FURTHER STUDY

(A) METABOLIC CHANGES

Before going on to study the physiological disposition of at least some of the penicillins and attempt a correlation with their physicochemical properties, it was obvious that a selection had to be made from those in which the properties differed appreciably. The choice was restricted to the phenoxy-penicillins as members of a homologous series. The physicochemical properties of these five penicillins are summarized in Table 6.

Because of differences in ionization constants it is obvious that one or other of the first three should be matched against one or other of the last two. It is also obvious that of the first three, phenoxymethyl penicillin should be chosen because it has the lowest oleyl alcohol/water partition coefficient. It is also appreciably less bound to albumin than are the last two. Phenoxymethyl, phenoxybenzyl and phenylthiodiphenyl penicillins were selected, therefore, for further study. Preliminary experiments, however, showed that phenylthiodiphenyl penicillin was extensively metabolized in the body. It was, therefore, rejected from the group.

Historically, knowledge of biotransformation of drugs dates to the early days of chemotherapy. Ehrlich (1909) had postulated that the trypanocidal activity of atoxyl in vivo was due to reduction of arsenic to the trivalent form, despite this, he did not realize that arsenobenzene derivatives as such have little antimicrobial activity (Ehrlich and Bertheim, 1912). It was not until Voegtlin, Dyer and Leonard showed in 1923 that there was a latent period of several hours after the administration of salvarsan before the parasites disappeared,

Table 6. The physicochemical properties of five closely related penicillins.

Penicillin	-COOH pK_a	Oleyl alcohol/water partition coefficient	% bound to serum albumin
Phenoxymethyl	2.88	48	69
Phenoxyethyl	2.78	81	46
Phenoxypropyl	2.88	> 800	72
Phenoxybenzyl	5.41	> 800	82
Phenylthiodiphenyl	5.15	> 800	100

while the trivalent form was immediately trypanocidal, that it became clear that the parent compound had little activity. Voegtlin and his co-workers concluded that organic arsenicals are immediately toxic only in the arsenoxide form. During the considerable latent period before development of trypanocidal activity, atoxyl and arsphenamine are slowly converted in the tissues of the host to the trivalent form,

$$R\text{-}As(OH)_2 = O \rightarrow R\text{-}As = O \leftarrow R\text{-}As = As\text{-}R$$

More than twenty years elapsed, however, before careful analytical work showed that a trivalent arsenical was present in the urine of animals treated with a pentavalent compound (Crawford and Levvy, 1947). Subsequently, several examples have come to light of pharmacologically effective substances being produced from apparently ineffective parents by biotransformation (see Harper, 1959 & 1962). Perhaps the best known is the hydrolysis of prontosil to the bacteriostatic agent sulfanilamide.

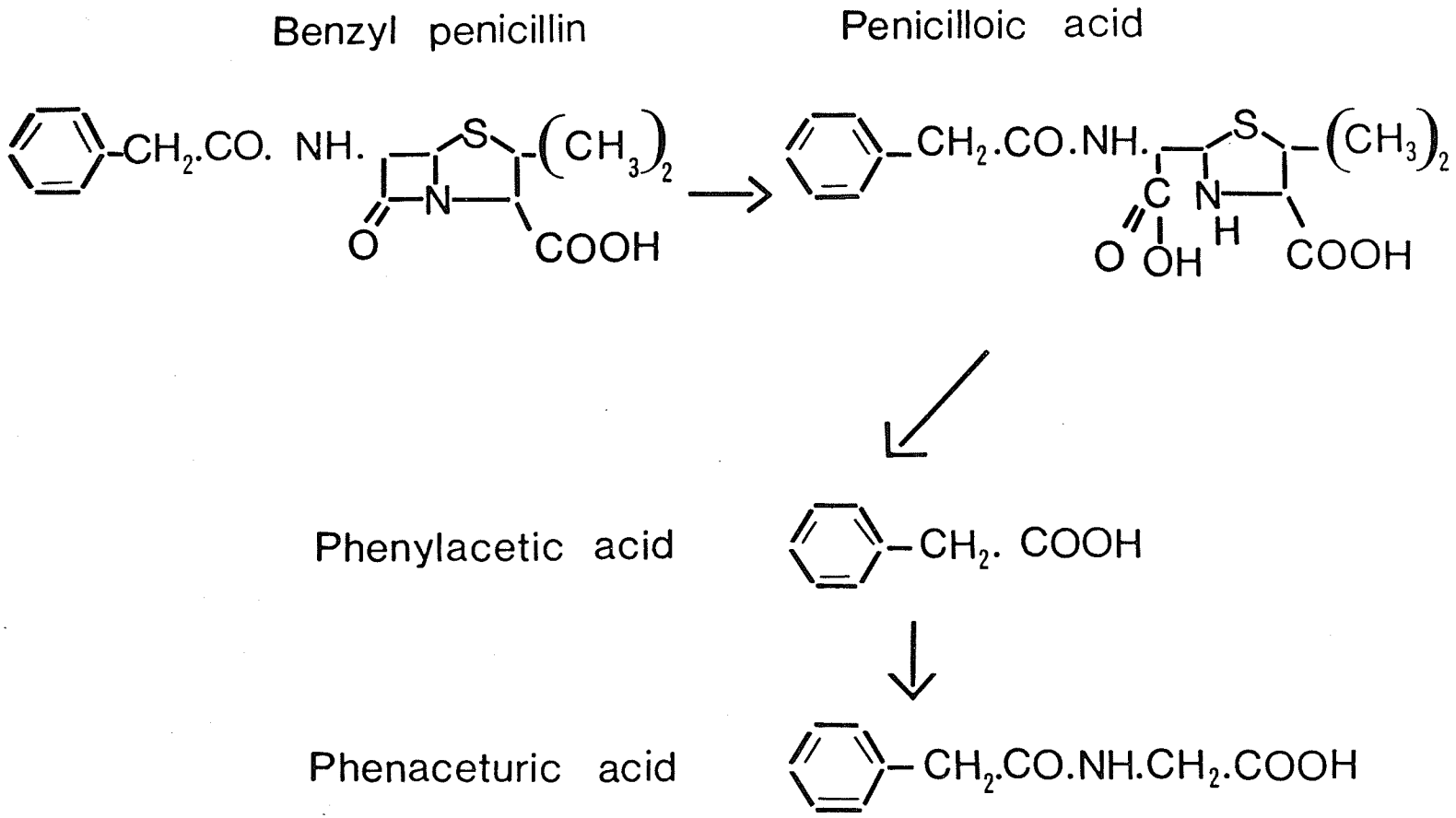
Shortly after it had been shown that prontosil could be used effectively in the treatment of certain bacterial infections (Foerster, 1933; Domagk, 1935), Tréfouël and co-workers (1935) suggested that it was split in the host tissues at the azolinkage to yield p-aminobenzenesulfonamide, the active principle. The basis for this suggestion was that diazotized benzenesulfonamide derivates, quite different from prontosil, still possessed antibacterial properties, but derivatives in which p-aminobenzenesulfonamide was replaced by other groups were inactive. Two years later, Fuller (1937) confirmed the earlier suggestion that the drug was split in the host to the active compound.

Although there are numerous examples of biotransformation converting an inactive or only slightly active substance into a potent derivative, a greater proportion of drugs are transformed into inactive or less active compounds. The reason is not hard to find. The mechanisms of biotransformation (detoxification) most probably were evolutionary developments to assist the organism to rid itself of harmful substances injested with food. Such substances would of necessity be lipid-soluble, otherwise little or none would have passed across the gastrointestinal epithelium. In order to get rid of these rapidly through the excretory system and prevent their penetration into cells, conversion to more polar substances would be required.

The first observation that penicillins are subject to biotransformation was made by Rowlands, Rowley and Stewart in 1948. In treated cats, ^{35}S-labelled benzyl penicillin was wholly excreted in the urine but only about half as the parent material. It appeared that the thiazolidine ring was quite stable since most of the metabolized material appearing as penicilloic acid. A small amount of phenaceturic acid was also formed indicating that penicilloic acid is further split at its peptide linkage (Figure 7) (Walkenstein, Chumakow and Seifter, 1954). Neither penicilloic acid nor phenaceturic acid have antibacterial properties at low concentrations.

Penicillins may also undergo change into substances which retain antibacterial activity, and, in fact, with the exception of dimethoxyphenyl and α-aminophenyl penicillins, this appears to be a fairly general property of penicillins when passed through man. Little is known, however, of the structural changes involved, nor of the differences occurring between species. Vanderbraeghe and co-workers

Figure 7. Biotransformation of benzyl penicillin.


Benzyl penicillin
Penicilloic acid
Phenylacetic acid
Phenaceturic acid

reported in 1962 that 3,4-dichloro-α-methoxybenzyl penicillin was excreted in the urine partly in the form of an active metabolite. In the case of phenoxymethyl penicillin some 10 to 15 per cent of the penicillin in the urine was found to be in the form of the p-hydroxy derivative. Phenoxybenzyl penicillin has been reported to give rise to active metabolites (Rollo, Somers and Burley, 1962), and this has also been shown for some isoxazolyl penicillins (Nayler, et al., 1962). Rolinson and Batchelor (1963) confirmed and extended this work when they showed that phenoxymethyl, phenoxyethyl, phenoxypropyl, phenoxybenzyl, 3,4-dichloro-α-methoxybenzyl, 5-methyl-3-phenyl-4-isoxazolyl, and 5-methyl-3-(2-chlorophenyl)-4-isoxazolyl penicillins all appeared as both parent substance and antibacterial metabolites in the urine of treated human volunteers. Dimethoxyphenyl and α-aminophenyl penicillins did not seem to be converted to active metabolites.

Before going on to examine the physiological disposition of the three penicillins selected, it was necessary, therefore, to examine their fate and reject for in vivo study any that showed more than a minor degree of transformation into active metabolites.

(i) Methods.

White rats, the animals chosen for the remainder of these studies, were treated by stomach tube with aqueous solutions of each of the three penicillins, after overnight fasting. Each animal was given a dose equivalent to 100 mg/kg. One to one and a half hours later, the animals were killed by a blow on the head, and samples of blood were obtained by heart puncture. At the same time, the bladder was washed out with 0.5 ml of 0.85 per cent sodium chloride solution.

Each sample was brought to pH 2.0 with phosphoric acid and shaken well with a small volume of butyl acetate.

Aliquots of the butyl acetate extracts were spotted on to strips of Whatman No. 1 chromatography paper previously impregnated with phosphate buffer at pH 6.2 and dried. After humidification for 15 minutes, the strips were equilibrated in a chromatography tank containing a layer of water-saturated ether for 1 hour, and then developed at laboratory temperature for 2 1/2 to 3 hours.

The chromatographic method is essentially that described by Karnovsky and Johnson (1949). Solutions of the penicillins prepared in water, blood and urine at a concentration of 4 μg/ml were treated in an identical manner and served as standards for comparison.

After drying, the strips were laid carefully on the surface of a nutrient agar suspension of S. lutea, prepared in the same way as described previously. The plates used were similar to the assay plates described, but constructed with 18" x 18" sides. Each plate contained a uniform layer of inoculated agar about 1 mm thick. The strips were left in contact with the agar for 20 minutes to allow diffusion of the penicillins into the agar, then carefully stripped off and discarded. After overnight incubation at 37°C, the clear zones in the agar indicated positions of the penicillins on the original chromatographic strip.

(ii) Results.

The results obtained from such bioautographs are shown on Table 7.

These show that, in the rat, phenoxymethyl and phenoxybenzyl

Table 7. Rf values of three penicillins and a metabolite.

Penicillin	Sample	Rf Values
Phenoxymethyl	Standard	0.56
	Blood) from	0.56
	Urine) treated rats	0.56
Phenoxybenzyl	Standard	0.79
	Blood) from	0.79
	Urine) treated rats	0.79
Phenylthiodiphenyl	Standard	0.87 (+ impurity at 0.72)
	Blood) from	0.76
	Urine) treated rats	0.76

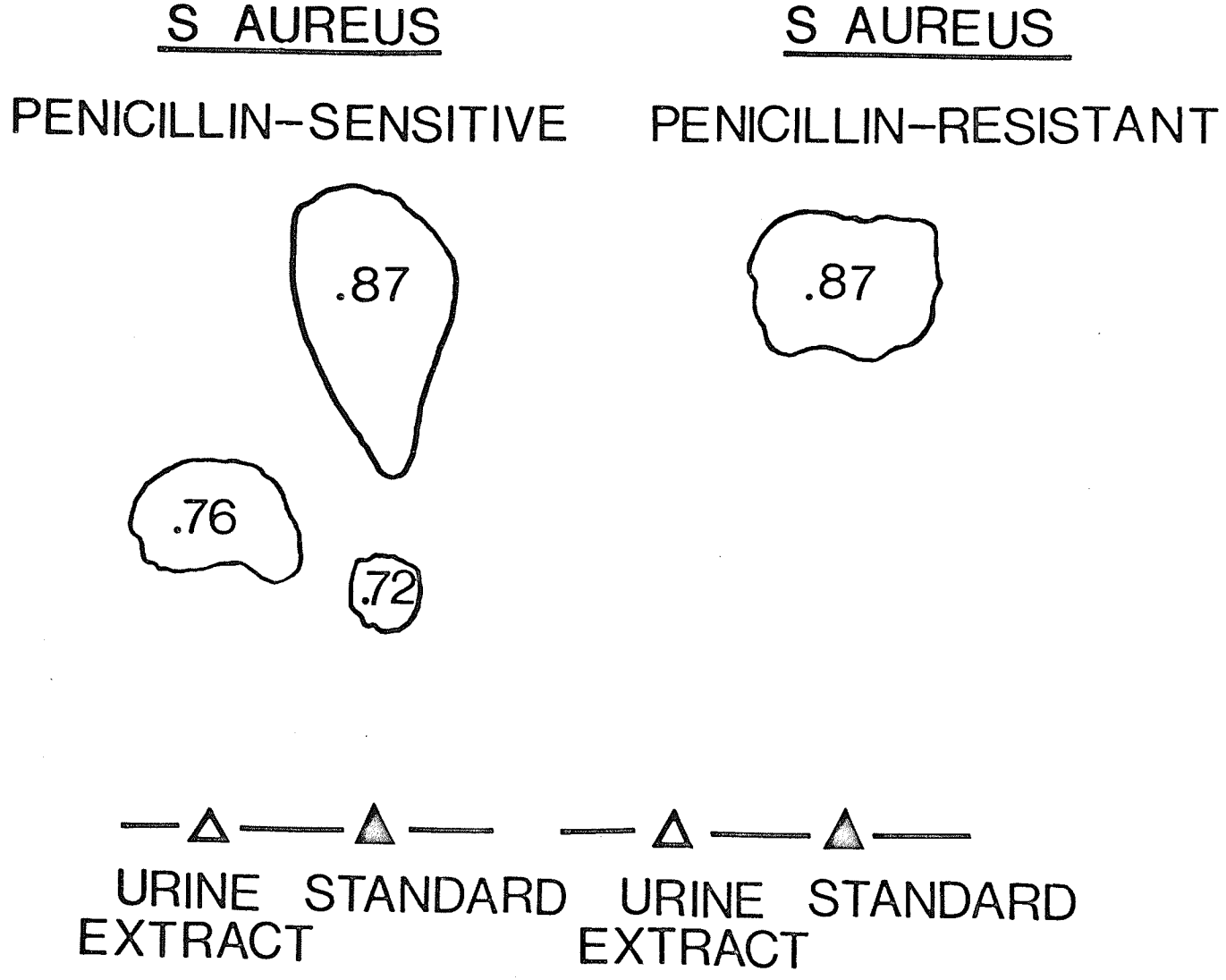
penicillins undergo no, or an undetectable, change into other antibacterial substances. Undoubtedly the corresponding penicilloic acids are formed but the rate of hydrolysis is probably similar for each penicillin.

Phenylthiodiphenyl penicillin, however, underwent extensive biotransformation. Chromatographic separation showed that none of the original material remained. A large proportion appeared in both blood and urine as another antibacterial substance. These data were of particular interest since this penicillin was known to be insensitive to β-lactamase and yet provided no protection when administered to groups of mice infected with a β-lactamase-producing strain of staphylocci (Rollo and Somers, unpublished).

Bioautographs were prepared as before but with two different strains of Staphylococcus aureus as the indicator organism. One strain was a penicillin-sensitive strain, the other a strain producing the enzyme β-lactamase and hence penicillin-resistant. A tracing of these bioautographs is shown in Figure 8.

These tracings show that phenylthiodiphenyl penicillin was transformed into another antibacterial substance which, while retaining activity against normally sensitive strains of organisms (S. lutea and S. aureus), had lost completely its resistance to staphylococcal β-lactamase.

Figure 8. Tracings from bioautographs of phenylthiodiphenyl penicillin (standard) and of a butyl acetate extract of urine from rats treated with the same penicillin. Left: indicator organism, penicillin-sensitive S.aureus. Right: indicator organism, β-lactamase-producing S.aureus.


S AUREUS
PENICILLIN-SENSITIVE
S AUREUS
PENICILLIN-RESISTANT
.87
.87
.76
.72
URINE EXTRACT
STANDARD
URINE EXTRACT
STANDARD

(B) DISCUSSION

Other workers have noted that commercially produced phenoxymethyl penicillin often contains traces of a chromatographically slower moving penicillin which is probably the p-hydroxy derivative (Stephens and Grainger, 1955). It is unlikely that our sample contained this impurity, since, being less lipid soluble, it would have been concentrated to a relatively greater extent in the urine of the treated rats. As it did not appear on the bioautograph it must have been present in the original material in extremely small proportions or not at all.

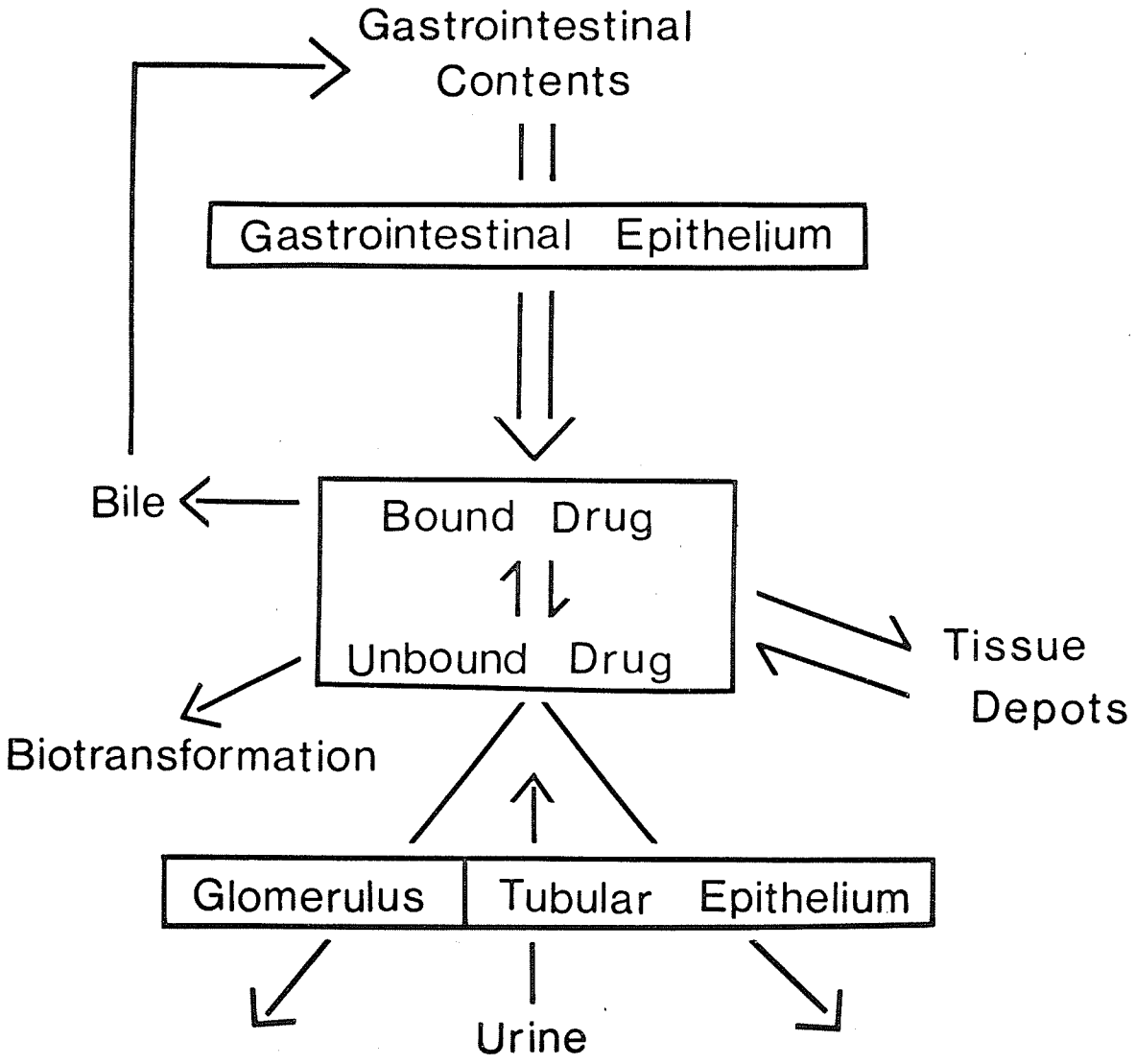
While both phenoxymethyl and phenoxybenzyl penicillins have been reported to undergo biotransformation into other antibacterial substances in the human, our data indicate that, in rats, no such metabolic change takes place. If it does take place, the process is so slow as to be undetectable within the one to one and a half hours of our experiments. Such species specificity both as to type and rate of biotransformation is well recognized (Williams, 1959). Phenylthiodiphenyl penicillin, however, was completely transformed and could not be considered for experiments in vivo. Phenoxymethyl and phenoxybenzyl penicillins were chosen, therefore, for the next part of the study.

6. PHYSIOLOGICAL DISPOSITION OF PHENOXYMETHYL AND PHENOXYBENZYL PENICILLINS AFTER ABSORPTION FROM THE GASTROINTESTINAL TRACT

It is well known that the resultant concentration of a drug in the blood after oral administration depends not only on its rate and degree of absorption from the gastrointestinal tract, but on its participation in several regulatory functions. The effective concentration of any drug present in the plasma may be decreased by binding to plasma proteins, binding to tissue components, secretion in the bile, biotransformation into more polar substances, and filtration, and possibly active secretion, in the kidney. On the other hand effective concentrations may be augmented by release of material bound reversibly to plasma protein and tissues, reabsorption of material secreted in the bile, and reabsorption from the tubular epithelium of the kidney. These factors and their inter-relationship are summarized in Figure 9.

No serious consideration has been given in the past to the part played by these factors in the physiological disposition of penicillins. Previously, we have described how some physicochemical properties relate to gastrointestinal absorption and binding to serum albumin. Now let us examine in some detail the behaviour of the two selected penicillins in the body. Previous data on their behaviour in human volunteers have been reported by Rollo, Somers, and Burley (1962) and Bond, et al., (1963).

Figure 9. Factors influencing the physiological disposition of drugs.


Gastrointestinal
Contents
Gastrointestinal Epithelium
Bile
Bound Drug
Unbound Drug
Tissue
Depots
Biotransformation
Glomerulus
Tubular Epithelium
Urine

(A) ORAL ADMINISTRATION OF PENICILLINS TO INTACT ANIMALS

(i) Methods.

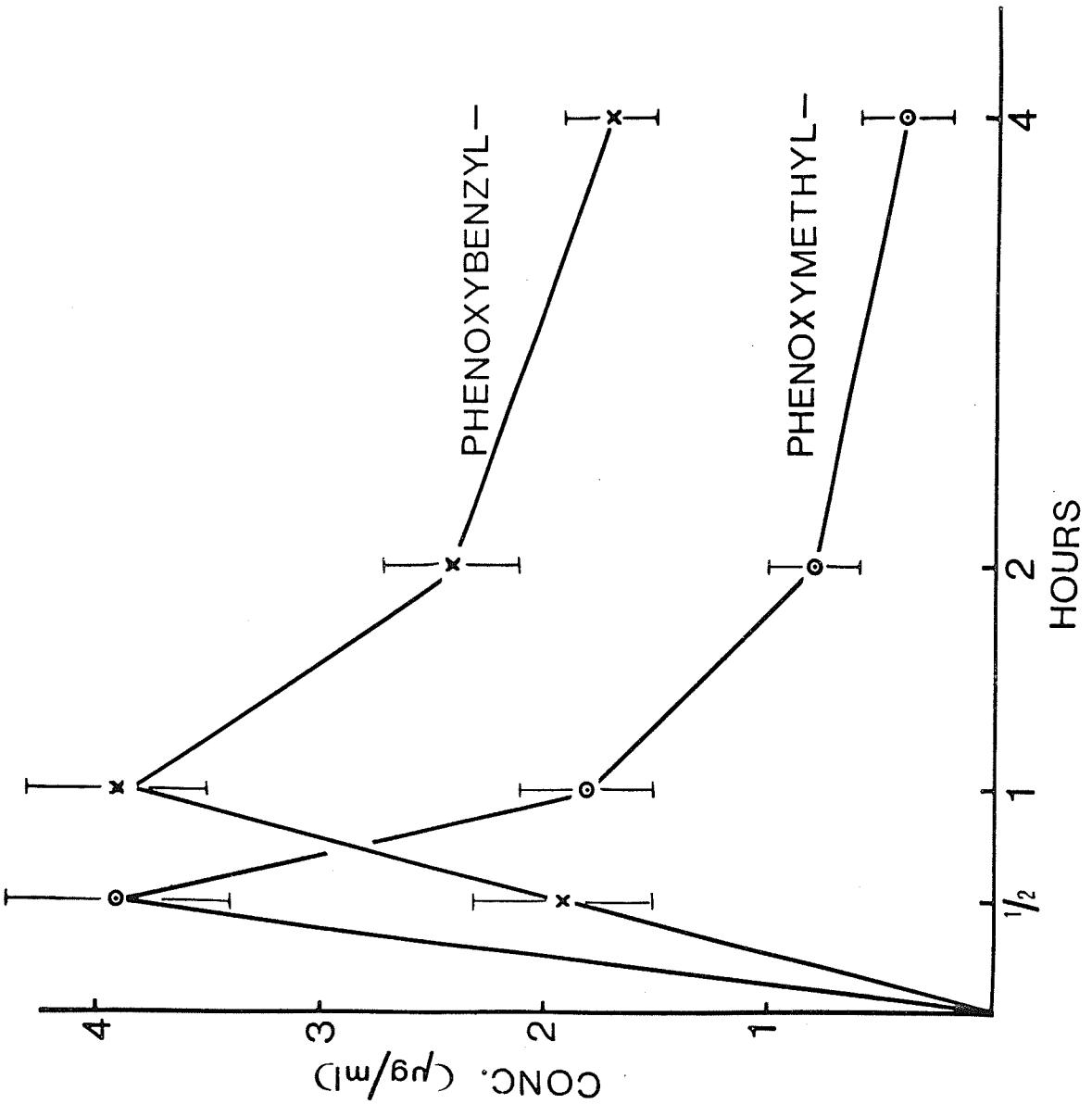
Young adult rats of either sex, weighing from 250 to 300 gm were segregated in wire bottom cages to prevent coprophagy, and fasted for 24 hours. Water was available ad libitum. Aqueous solutions of the potassium salts of either phenoxymethyl or phenoxybenzyl penicillins, prepared freshly for each experiment, were administered to each animal by stomach tube. The concentration was adjusted so that each rat received a dose equivalent to 50 mg/kg (0.25 ml/250 gm body weight). Tail blood samples were taken at 1/2, 1, 2 and 4 hours after administering the dose and heparinized. The total penicillin content of each sample was determined by bio-assay as described above. Standards were made by serially diluting solutions of the appropriate penicillin in heparinized rabbit blood.

(ii) Results.

The results obtained are shown in Figure 10.

These data show that both penicillins attain approximately the same concentration in the blood although earlier sampling might have shown a higher peak concentration for phenoxymethyl penicillin. Two points are notable however. The peak concentration for phenoxybenzyl penicillin was reached appreciably later than that for phenoxymethyl penicillin, and a much higher concentration of phenoxybenzyl penicillin was maintained for the last three hours of the experimental period. At each of the four sampling times the differences between mean values were significant at the < 0.01 level as determined by Student's t test.

Figure 10. Concentrations of phenoxymethyl and phenoxybenzyl penicillins in the blood of intact rats after single oral doses of 50 mg/kg. Each indicated point is the arithmetical mean value from a group of ten animals. The vertical lines signify ± standard error.
Ordinate - concentration of penicillin (μg/ml).
Abscissa - time in hours.


CONC. (µg/ml)
4
3
2
1
½
1
2
4
HOURS
PHENOXYMETHYL-
PHENOXYBENZYL-

There seems little doubt, therefore, that differences in the properties of the two substances have resulted in changes in the interplay of those factors upon which physiological disposition depends. In the next series of experiments some of those factors were eliminated.

(B) INTRADUODENAL ADMINISTRATION OF PENICILLINS TO 'ANURIC' ANIMALS

(i) Methods.

Young adult rats were prepared preoperatively as described in the previous section. The animals were lightly anaesthetized with ether. The abdomen was opened through a mid-line incision. The small intestine was ligated so as to form a sac which extended from the pylorus to 20 cm along its length. Both renal pedicles and the bile duct were ligated. Aqueous solutions of the potassium salts of phenoxymethyl and phenoxybenzyl penicillins were introduced into the sac near the pylorus by means of a fine gauge needle. The dose used was 50 mg/kg, and the concentration was such that 0.1 ml was given for each 100 gm body weight. Tail-blood samples were taken at 1/2, 1, 2 and 4 hours after the start of the experiment and heparinized. The assay procedure was identical with that described in the previous section.

(ii) Results.

The results obtained are shown in Figure 11.

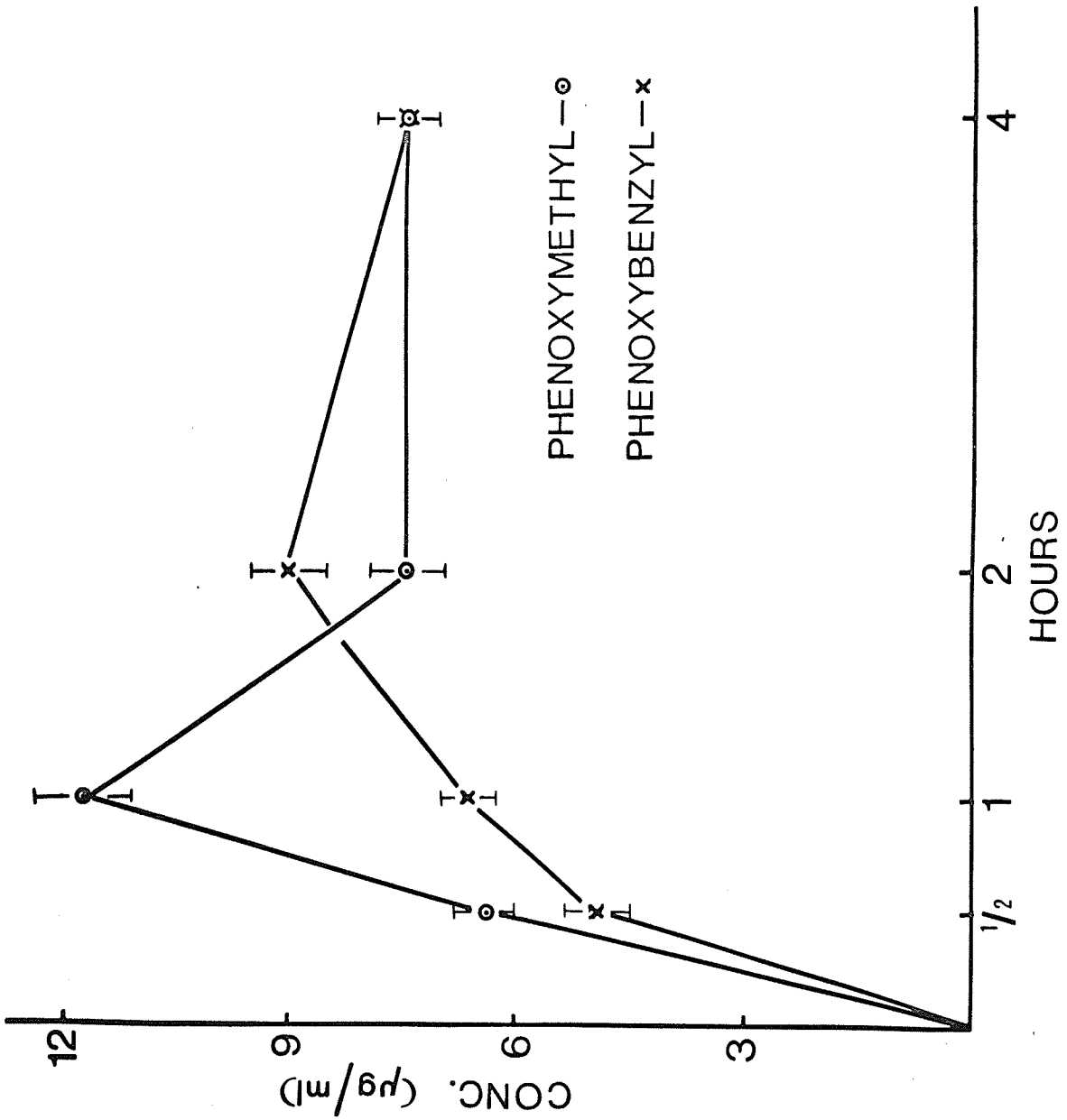
At each of the first three sampling times the differences between mean values were significant at the < 0.01 level as determined by Student's t test.

In this series the marked differences between the blood levels of the penicillins that were observed after the peak concentrations had been achieved in the previous experiments, did not occur. The decrease that did occur may be due to redistribution to tissues and/or to biotransformation to inactive metabolites. The levelling out of the curve

Figure 11. Concentrations of phenoxymethyl and phenoxybenzyl penicillins in the blood of 'anuric' rats after intraduodenal administration of 50 mg/kg. Each indicated point is the arithmetical mean value from a group of ten animals. The vertical lines signify $\pm$ standard error.

Ordinate - concentration of penicillin (μg/ml).

Abscissa - time in hours.


CONC. (µg/ml)
12
9
6
3
1/2
1
2
4
HOURS
PHENOXYMETHYL—⊙
PHENOXYBENZYL—×

for phenoxymethyl penicillin would, however, tend to counter this latter explanation.

Of particular interest are the first parts of both curves. Until peak concentrations are attained, the rate of rise of the curve for phenoxymethyl penicillin is appreciably greater than that for phenoxybenzyl penicillin. The peak concentration for phenoxymethyl penicillin is also much greater than that for phenoxybenzyl penicillin. These facts would suggest that phenoxymethyl penicillin is absorbed more rapidly and more completely than is phenoxybenzyl penicillin.

(C) DISCUSSION

Comparison of Figures 10 and 11 shows that after 2 hours, when presumably virtually all intestinal absorption had ceased, differences in blood concentrations of the two penicillins were much less in the 'anuric' animals than in the intact animals. The data also show that differences in the time required to attain peak concentrations were appreciably greater in the second series of experiments.

In interpreting these results we must make the following assumptions; despite changes in concentration, the equilibrium between tissue depots and unbound penicillin in the blood did not change, and, the rate of biotransformation into inactive metabolites remained the same. These appear to be reasonable assumptions.

If this is the case, then the only difference from one set of experiments to the other is the part played by biliary secretion and renal excretion. Biliary secretion with subsequent reabsorption would tend to maintain the blood concentration. Decrease in proximal tubular transport in the kidney and/or increase in tubular reabsorption would also tend to maintain the blood concentration.

Secretion of substances from blood to bile has many similarities to the secretion of substances by the proximal tubule of the kidney. There appear to be two separate processes; the one concerned with the transfer of organic acids such as phenol red, bromsulphalein and penicillins (see Sperber, 1959), the other concerned with organic bases (Schanker, 1962; Solomon and Schanker, 1962). Both processes have the characteristics of active transport in that transfer occurs agains a concentration gradient, the mechanism is saturable, and competition

between substances transferred by the common system occurs.

Some comparative studies on the biliary secretion of several penicillins have been carried out by Harrison and Stewart (1961). Of those common to this study, there were marked differences in the concentrations appearing in the bile. α-Aminobenzyl penicillin appeared in high concentration (1,385 μg/ml); the concentration of dimethoxyphenyl penicillin was much lower (377 μg/ml). This ratio of approximately 7.2:1 is inversely related to the oleyl alcohol/water partition coefficients of the two substances. These data might suggest that appreciable amounts of more lipid-soluble penicillin, dimethoxyphenyl, were being reabsorbed in passage down the bile duct. However, this seems unlikely since at the pH of bile, 5.7 to 8.6 (Popper and Schaffner, 1957), very little of the penicillin would be unionized. The inverse correspondence of the two ratios is, therefore, probably fortuitous. A more ready acceptance of α-aminophenyl penicillin into a transport system would be a more likely explanation. In fact, Harrison and Stewart have shown that a marked specificity does exist. The L (+) isomer of α-aminobenzyl penicillin appears in the bile at only one quarter the concentration of its stereoisomer.

It seems quite likely, therefore, that differences in molecular structure and properties of the two penicillins would result in differences in rate of biliary secretion. Stewart and Harrison (1961) have shown that one fifteenth of an oral dose of α-aminobenzyl penicillin is secreted by this route within a few hours. This represented a large proportion of the amount actually absorbed from the gut. Hence, biliary secretion with subsequent reabsorption could make an appreciable contribution to the maintenance of penicillin concentration in the blood.

The magnitude of its contribution would depend on the extent to which the different substances participated in the active process.

There is no question that variations in the handling of drugs by the kidney have a very marked effect on the stay of drugs in the body. Indeed Brodie and Hogben (1957) have estimated that excluding extrarenal factors, the half-life of a drug in the extracellular fluid would be 70 minutes if excreted solely by glomerular filtration, 7 minutes if secreted by the tubules and limited by only renal blood flow, and 7 days if the drug were reabsorbed by the tubules to the extent that its concentration in the urine is no greater than in the plasma. Penicillins are both filtered at the glomerulus and secreted by the proximal tubule. Presumably, some degree of tubular reabsorption also occurs (see Weinstein, 1965).

While glomerular filtration and tubular reabsorption of organic electrolytes are passive, non-specific processes dependent on degree of plasma protein-binding for the first process (see Beyer, 1950), and lipid solubility of unionized substance for the second (see Brodie and Hogben, 1957), tubular secretion is an active and much more specific process. Different processes account for acids and bases but we shall confine our discussion to the secretion of organic acids.

The structural requirements of organic acids undergoing active secretion have been examined extensively, and it is now apparent that a wide variety of compounds may be transported by this common mechanism. Indeed, work on aliphatic acylglycines (Schachter, Manis and Taggart, 1955) and oxalic acid (Cattell, et al., 1962) which suggested that these substances also undergo transport, would tend to show that neither

aromatic nor heterocyclic ring structures are absolute prerequisites. Other postulates that, for example, transported molecules must possess both polar and non-polar ends, or that the presence of both an anionic site and a carbonyl (or equivalent) group is a requirement, have not been substantiated. To quote Weiner and Mudge (1964), "at present, for the acid secretory system it is not possible to define a minimal structural requirement other than the compound be an acid". Even this criterion may be inadequate since the secretion of creatinine in, for example, the dog is inhibited by secreted organic acids (O'Connell, Romeo and Mudge, 1962). However, this particular picture is complicated since the secretion of creatinine in the dog is influenced not only by organic acids but also by organic bases and mercurials.

Nevertheless, a degree of structural specificity does exist. In some series of substances the extent of participation can be correlated with increase in molecular size, or size of alkyl substituent. The introduction of hydrophilic groups can interfere with participation (Essig and Taggart, 1960; Volle, et al., 1959; Green, et al., 1959; and Despopoulos, Pendergrass and Stoechkinger, 1963). Moreover, what little data are available suggest that a considerable degree of structural specificity exists in the transport of penicillins. Valentine, Simon and Rantz (1964) have shown that renal clearance of 5-methyl-3-phenyl-4-isoxazolyl penicillin is only 45 per cent that of benzyl penicillin despite a low degree of binding to plasma protein and probably little tubular reabsorption. The secretion of dimethoxyphenyl penicillin is inhibited completely by concentrations of probenecid which do not completely block the secretion of α-aminophenyl penicillin (Acred, et al., 1961; Acred, et al., 1962). However, no really comparative studies have been carried out.

Despopoulos (1965) has suggested that transported substances have a three-point contact with the 'receptor' which involve the two oxygens of the carboxyl group in a reinforced ionic bond and one oxygen of the carbonyl group in a supporting hydrogen bond. He described two classes of 'substrates' corresponding spatially to either the folded or the fully extended state of the 4-aminohippurate side-chain. However, in this thesis which was based on data from a large number of compounds, he did not consider how differences in physicochemical properties could account for substances either fitting or not fitting the general theory. Huang and Lin (1965) have shown that a strong correlation exists between the partition coefficients of substances and the ability of those substances to inhibit competitively the tubular transport of PAH in an isolated kidney tubule preparation. While by no means conclusive these data suggest a connection between this, and possibly other physicochemical parameters, and the affinity of organic acids for the carrier.

The regulation of non-ionic diffusion by changes in pH along the lumen of the tubule is also important. Appreciable reabsorption of the more acidic penicillins could occur only at quite low pH values. Extremes of acidification occur only in the most distal regions of the tubular system (Goltschalk, Lassiter, and Mylle, 1960) which, in the mammalian kidney receive a meagre blood supply (Ullrich, Kramer and Boylan, 1961). Therefore, low-pH-dependent diffusion of a substance out of the nephron in this region may be limited.

The handling of phenoxymethyl penicillin by the kidney may, therefore, be very different from that of phenoxybenzyl penicillin. Differences in protein-binding would alter the proportion of unbound

substance presented to the glomerular membrane, favouring the filtration of phenoxymethyl penicillin. The specificity of the proximal tubular secretory mechanism might favour the secretion of one or other of the substances. We have no evidence on this point. Tubular reabsorption would favour the passage back into the blood stream of the more lipid soluble less ionized substance, hence promoting the reabsorption of phenoxybenzyl rather than phenoxymethyl penicillin.

The net effect of differences in renal, and possibly biliary, handling on the disposition of phenoxymethyl and phenoxybenzyl penicillins becomes very apparent if the differences between the appropriate curves in Figures 10 and 11 are expressed numerically. In Figure 12 we have plotted the ratio of 'anuric' to 'intact' penicillin concentrations against time of sampling. A comparison of the slopes of the two lines reveals that phenoxymethyl penicillin is eliminated from the body more than three and a half times faster than phenoxybenzyl penicillin.

Our data are consistent with data obtained after the administration of the two penicillins to human volunteers (Rollo, Somers and Burley, 1962). These authors commented that the urinary excretion of phenoxybenzyl penicillin followed a similar pattern to that of phenoxymethyl penicillin but was rather slower. No attempt was made, however, to compare the rate of urinary excretion with the concentrations attained in the blood. These data are summarized in Figures 13 and 14. It is obvious from comparison of these figures that, indeed, the rate of urinary excretion is very much less for phenoxybenzyl penicillin than for phenoxymethyl penicillin. Our expression of 'excretion rate', Table 8, shows that during the first six hours after administering the dose, phenoxymethyl penicillin is excreted from two and a half to six times faster than phenoxybenzyl penicillin.

Figure 12. A graphical representation of the differences in rates of elimination of phenoxymethyl and phenoxybenzyl penicillins from the body.

Ordinate - ratio of 'anuric' to 'intact' mean blood concentrations of the penicillins (data from Figures 10 & 11).

Abscissa - time in hours.

(The lines were fitted by eye).

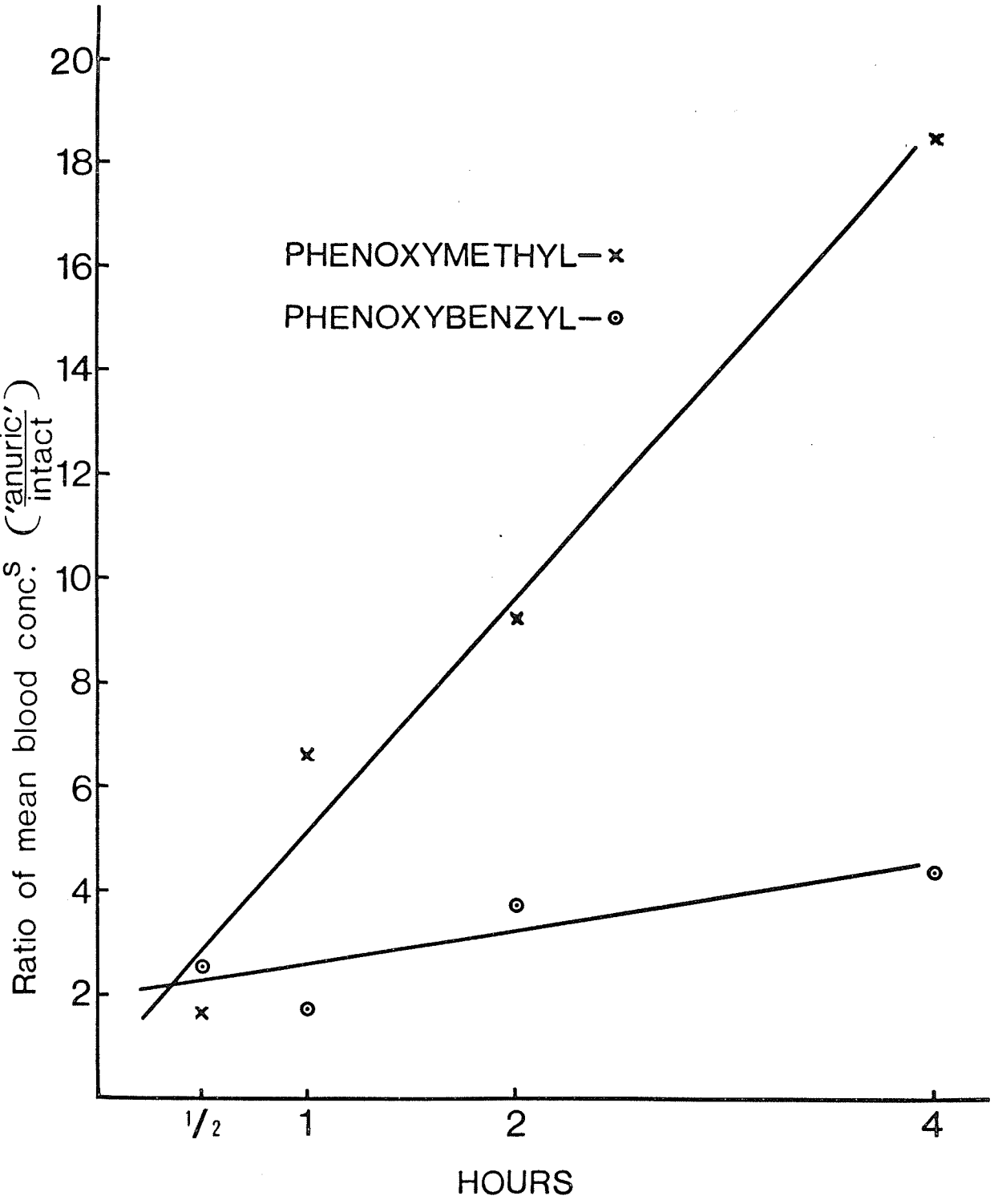

20
18
16
14
12
10
8
6
4
2
PHENOXYMETHYL—×
PHENOXYBENZYL—⊙
Ratio of mean blood conc.s ('anuric'/intact)
1/2
1
2
4
HOURS

Figure 13. Mean blood concentrations after the oral administration of 150 mg phenoxymethyl penicillin and phenoxybenzyl penicillin to human volunteers (after Rollo, Somers and Burley, 1962).

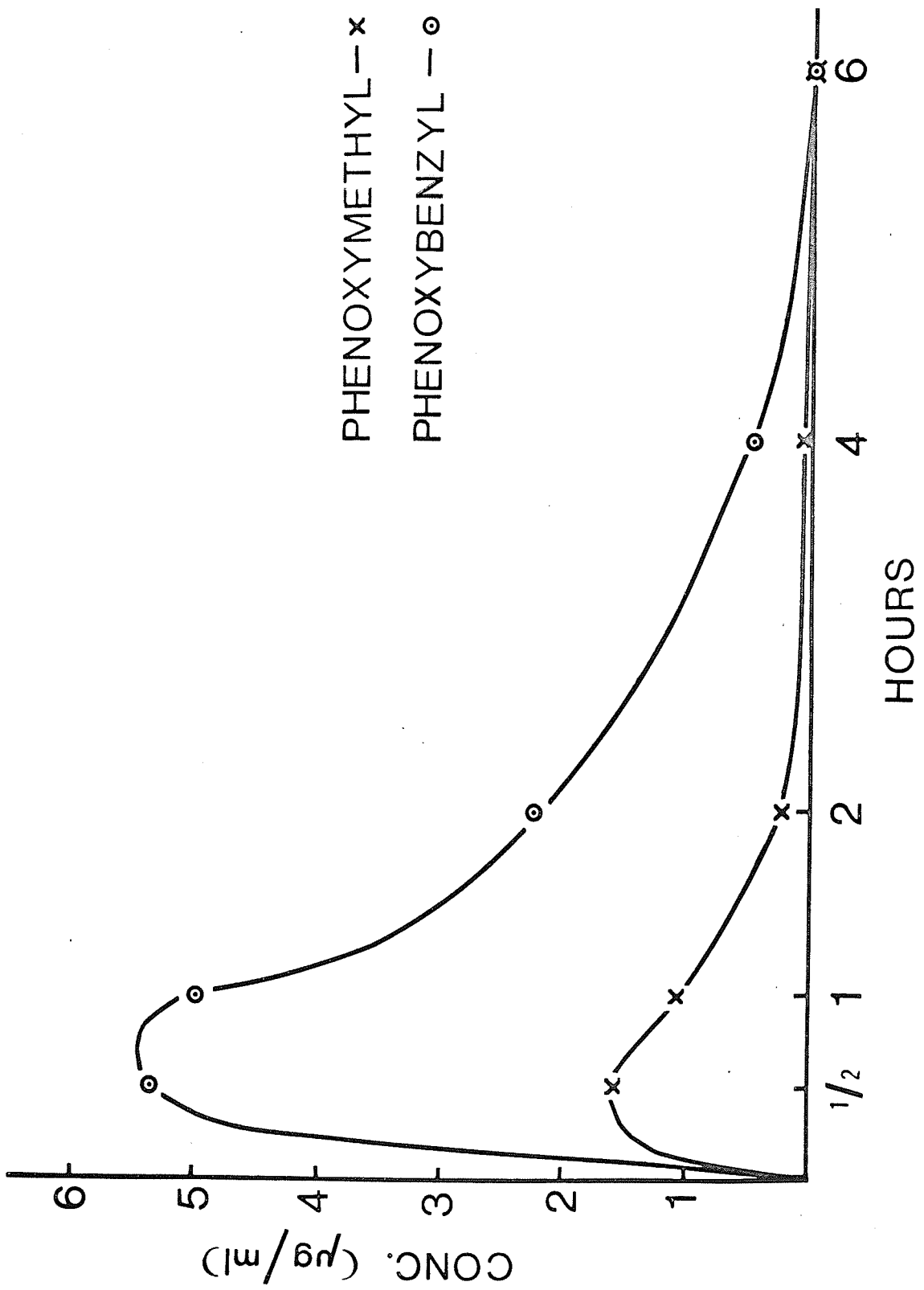

PHENOXYMETHYL — x
PHENOXYBENZYL — o
CONC. (µg/ml)
1
2
3
4
5
6
1/2
1
2
4
6
HOURS

Figure 14. Urinary excretion of phenoxymethyl and phenoxybenzyl penicillins after oral administration of 150 mg to human volunteers (after Rollo, Somers and Burley, 1962).

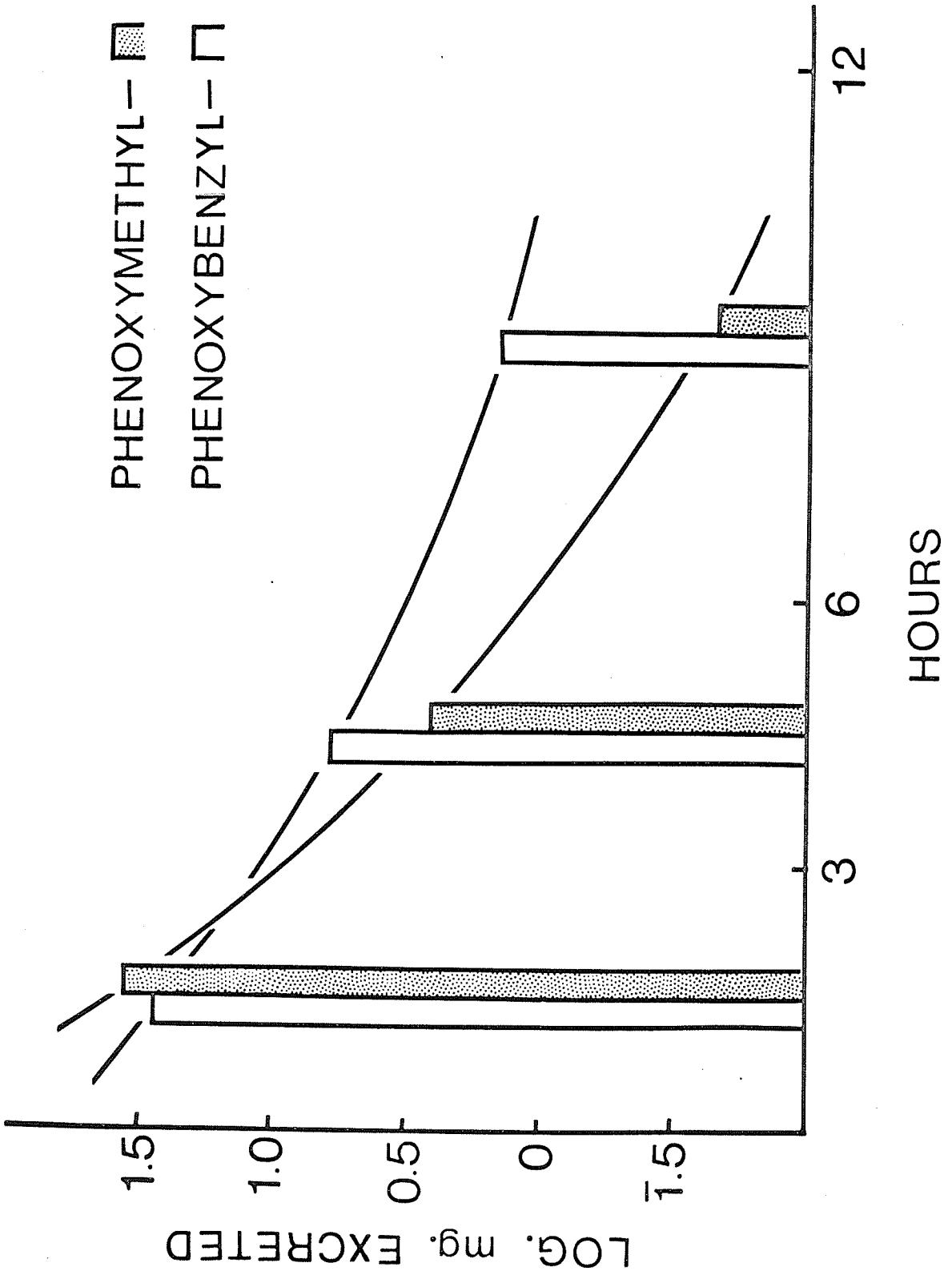

PHENOXYMETHYL-
PHENOXYBENZYL-
LOG. mg. EXCRETED
1.5
1.0
0.5
0
1.5
3
6
12
HOURS

Table 8. Excretion of phenoxymethyl and phenoxybenzyl penicillins as function of blood concentrations. (Data from Figures 13 & 14).

Penicillin	'Excretion Rate' = (mg in urine over period / area* under conc/time curve over same period)	
	0 to 3 hours	3 to 6 hours
Phenoxymethyl penicillin	$\frac{36.18}{1.94} = 19$	$\frac{2.55}{0.18} = 14$
Phenoxybenzyl penicillin	$\frac{28.24}{9.16} = 3.1$	$\frac{6.07}{1.10} = 5.5$

(* expressed in arbitrary units as measured by planimetry from Figure 13).

Interpretation of these figures is complicated by the fact that active metabolites of phenoxybenzyl penicillin appeared in the urine. The parent substance, however, accounted for over 80 per cent of the activity.

So far we have compared and discussed differences in the disposition of the penicillins during the latter part of the experimental periods. Comparison of the rate of rise of the curves during the early part of the experiments depicted in Figure 11 would suggest that phenoxymethyl penicillin is more rapidly and more completely absorbed than phenoxybenzyl penicillin. If the pH at the absorbing surface of the intestinal mucosa is 5.3, it might be expected that the reverse would occur. Indeed when measured at pH 5.3, the oleyl alcohol/water partition coefficients would give to phenoxybenzyl penicillin a five-fold advantage.

These observations suggested to us that the passage of penicillins, and probably other organic acids, may be dependent on some mechanism other than simple diffusion of the unionized, lipid-soluble molecule. This hypothesis is tempting because organic acid transport systems occur in at least three sites in mammals, one in the kidney tubule, a second involving biliary secretion, the third shifting organic acids such as benzyl penicillin from the cerebrospinal fluid back into the bloodstream (Dixon and Rall, 1964). In teleological terms, however, it would seem undesirable to oppose mechanisms serving the removal of particular substances, with a further mechanism promoting their entry. Nevertheless, our data seemed interesting enough to explore this possibility further.

7. MECHANISMS OF INTESTINAL ABSORPTION OF PENICILLINS

Several separate mechanisms account for the active transport of substances from the intestine into the blood. One transports monosaccharides (Höber, 1945), another L-amino acids (Wiseman, 1953), and a third, pyrimidines (Schanker and Tocco, 1960). In addition, the mechanism for the transport of L-amino acids has been subdivided into mechanisms specific for neutral amino acids, basic amino acids, and a group consisting of L-proline, sarcosine, N,N-dimethylglycine, and betaine (Lin, Hagihira and Wilson, 1962). Moreover Newey and Smyth (1963) have suggested that there may be more than one carrier site for neutral amino acids with different specificities. Other processes may facilitate the diffusion of substances through the intestinal epithelium; for example 'solvent drag' may accelerate the movement of urea and other solutes (Fisher, 1955). Despite the presence of these several mechanisms nearly all drugs appear to cross the boundary by simple diffusion of the lipid-soluble, unionized molecule. In only one case, that of the foreign pyrimidines 5-fluorouracil and 5-bromouracil, have drugs been shown to share a system with naturally occuring substances (Schanker and Jeffrey, 1961).

In general, several criteria must be satisfied before active transfer of a substance can be implicated. The transport mechanism becomes saturated when the concentration of the substance transported is increased sufficiently. The substance moves across the membrane against a concentration gradient. The transport mechanism is inhibited non-competitively by interference with cell metabolism, either by the use of metabolic inhibitors or, by decreasing the temperature at which the experiment is carried out. The process shows some degree of chemical specificity. If two substances are transported by the same mechanism, one will inhibit competitively the transport of the other.

(A) RATE OF PASSAGE OF PHENOXYMETHYL AND PHENOXYBENZYL PENICILLINS ACROSS RAT INTESTINE *IN VITRO*

The following experiments were carried out to determine whether or not there was a saturable component in the mechanism of transfer of the two penicillins from mucosal to serosal side of the rat small intestine.

(i) Methods.

Adult rats, fasted for 24 hours, were killed by a blow on the head, and a 20 cm length of upper small intestine removed with a minimum of handling. Each end was tied to form a sac which was filled with 2 ml of solutions of the penicillins in Krebs bicarbonate saline. No glucose was added to the medium since Smyth and Taylor (1958) had shown that its presence promoted the transfer of fluids across the intestinal wall, and, associated with this, the transfer of short-chain fatty acids. The sacs were placed in 50 ml erlenmeyer flasks containing 10 ml of the same saline, and were shaken in a Dubnoff metabolic shaker, 90 oscillations per minute, at 37°C in an atmosphere of 95 per cent oxygen - 5 per cent carbon dioxide. Previously a bubble of the same gas mixture was added to each sac.

Aliquots of 0.1 ml were removed from the suspending medium at 20, 40, 80 and 160 minutes after starting incubation, and their content of penicillin assayed as previously described. The rate of appearance of penicillin on the serosal side was estimated by measuring the slope of the line formed from a semi-log plot of concentration *versus* time.

(ii) Results.

The results obtained are shown in Table 9. A graph of these data is shown in Figure 15.

The shape of these curves suggests that two processes are taking place. At the lower concentrations of both penicillins there is a sharp increase in rate as the concentration is increased. As the concentration is increased further, the increase in rate falls off quite markedly.

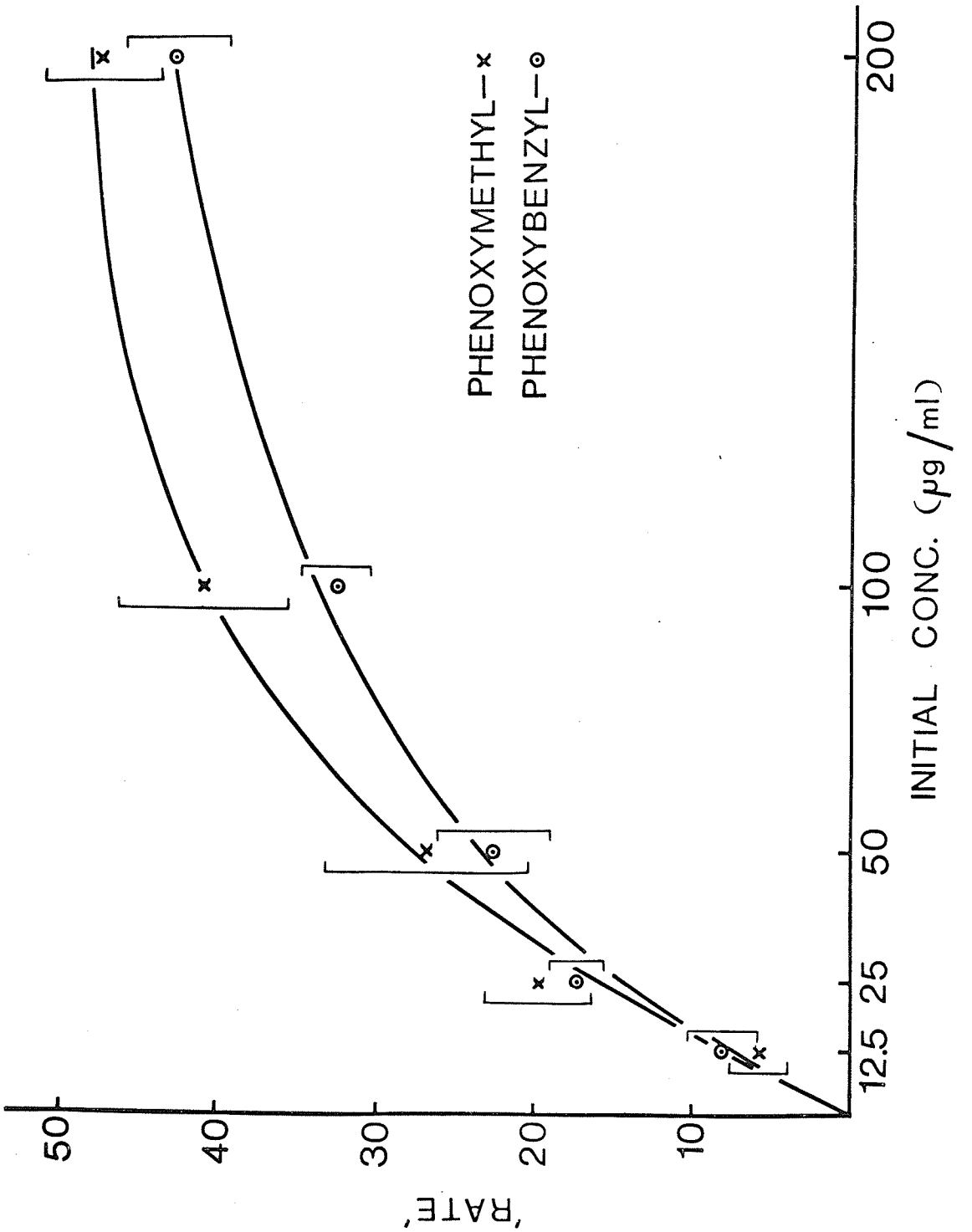
These results suggest that the penicillins may cross the intestinal epithelium by a combination of two processes: (1) A specialized transport system, evident at the lower concentrations, which becomes saturated when the concentration is increased sufficiently (2) A passive process, probably simple diffusion, which is evident at the higher concentrations.

This evidence was sufficiently encouraging to warrant looking for further evidence of a specialized transport mechanism. The next series of experiments was carried out, therefore, to determine whether or not transport of the two penicillins was effected against a concentration gradient.

Table 9. Rate of passage of penicillins through the wall of isolated, uneverted, sacs of rat small intestine.

Penicillin	Conc. in lumen (μg/ml)	No. of Preparations	Mean Rate (degrees of arc) ± S.D.
Phenoxymethyl	12.5	5	5.8 ± 1.7
	25	10	19.8 ± 3.5
	50	8	26.8 ± 6.2
	100	9	40.8 ± 5.2
	200	5	47.4 ± 3.8
Phenoxybenzyl	12.5	11	8.36 ± 1.8
	25	12	17.2 ± 2.4
	50	10	22.6 ± 3.7
	100	11	32.4 ± 2.8
	200	13	42.6 ± 2.7

Figure 15. Rate of passage of penicillins through the wall of isolated, uneverted, sacs of rat small intestine. (Graphical representation of data presented in Table 9.)


PHENOXYMETHYL—×
PHENOXYBENZYL—⊙
'RATE'
50
40
30
20
10
12.5
25
50
100
200
INITIAL CONC. (μg/ml)

(B) DISTRIBUTION OF PHENOXYMETHYL PENICILLIN BETWEEN MUCOSAL AND SEROSAL SIDES OF SACS OF EVERTED RAT SMALL INTESTINE

(i) Methods.

For this series of experiments the technique of Wilson and Wiseman (1954) was used. 20 cm Lengths of rat upper small intestine were carefully everted before the ends were tied so that mucosa formed the outside of the sac, serosa the inside. The sac was filled with penicillin solution and immersed in a solution of the same penicillin at the same concentration. The object of using such everted sacs is twofold. Eversion, with slight distension of the sac, stretches the mucosa and improves its oxygenation. The small volume within the sac, 2 ml as against 10 ml on the mucosal side, causes transport of any solute from the mucosal to the serosal side to produce a relatively large increase in its concentration in the serosal fluid. This, of course, facilitates the detection of transport against a concentration gradient.

Otherwise the preparation, shaking, temperature of incubation and so on were identical with the previous series of experiments. After 30 minutes incubation, the sacs were removed from the flasks, rinsed with saline and aliquots of the contents taken. At the same time aliquots were taken from the suspending solution and the penicillin content of all samples determined by bioassay as before.

(ii) Results.

The results are shown in Table 10.

These results show that at all concentrations tested the ratio of serosal to mucosal final concentrations was less than unity. The

Table 10. Distribution of phenoxymethyl penicillin between mucosal and serosal sides of everted sacs of rat small intestine incubated at 37°C for 30 minutes. Each value is the arithmetical mean ± standard error of a group of 6 experiments.

Concentration of phenoxymethyl penicillin (μg/ml)

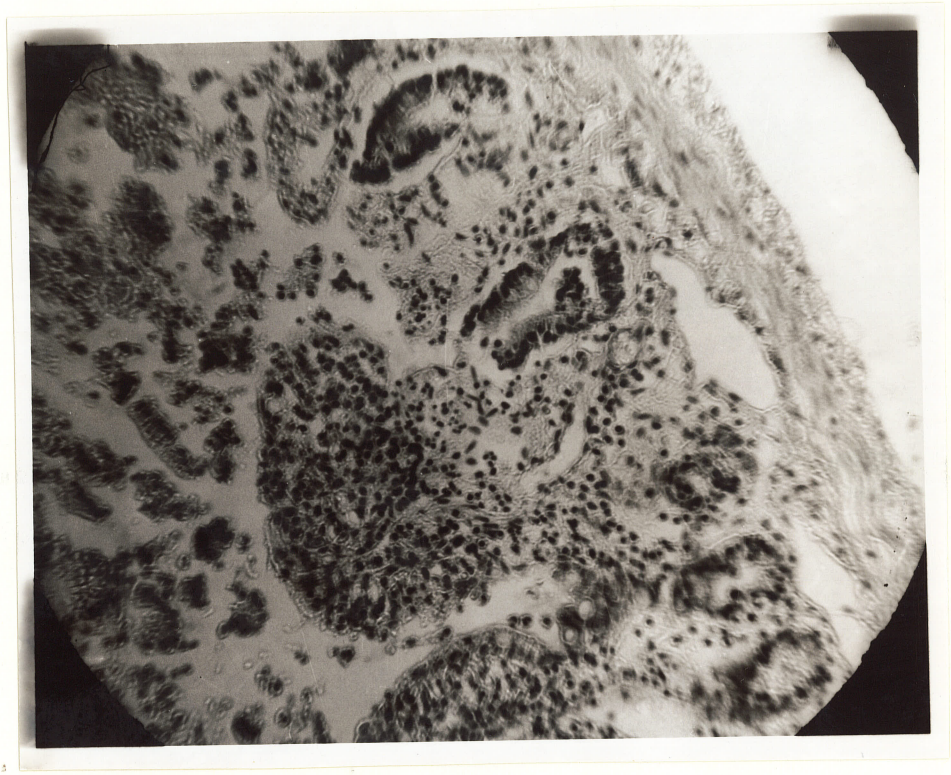
Initial mucosal and serosal solutions	Final mucosal solution	Final serosal solutions	Ratio of serosal to mucosal final concentrations
0.2	0.19 ± 0.03	0.18 ± 0.02	0.95
0.8	0.78 ± 0.09	0.62 ± 0.12	0.80
3.2	3.07 ± 0.19	2.65 ± 0.15	0.86

decrease in numerical value of the ratio from an initial value of unity to less than unity, was due to a slight decrease in concentration on the serosal side rather than an increase on the mucosal side. The differences are, however, small and in the case of the two lower concentrations, statistically insignificant.

These data fitted neither our previous results nor our working hypothesis, therefore an explanation had to be found. It was suspected that, despite the care taken in everting the intestine, some damage to the mucosa might have occurred. Therefore, in a dummy experiment, sections were taken for histological examination from a piece of everted intestine after 30 minutes incubation. Figure 16 is a photomicrograph of a transverse section of one such preparation. Obviously the epithelium had stripped off and dissolution of the mucosa had occurred until little of the normal morphology remained. Only the musculature and a small proportion of the mucosa remained intact. The rest of the tissue had broken down.

This rapid disintegration of the metabolically active layers of the intestine offered good reason why the experiments involving evertion of the sac were unsuccessful. For this reason, subsequent experiments were carried out using normal, non-everted sacs. Sections taken from such preparations up to one hour after starting incubation showed that the integrity of the various layers was well maintained.

Figure 16. Photomicrograph of a transverse section of rat small intestine after eversion and incubation *in vitro* at 37°C for 30 minutes.



(C) DISTRIBUTION OF PHENOXYMETHYL AND PHENOXYBENZYL PENICILLINS BETWEEN MUCOSAL AND SEROSAL SIDES OF SACS OF NONEVERTED RAT SMALL INTESTINE

(i) Methods.

The preparation used was identical with that described in the previous section except that the isolated lengths of intestine were not everted before being tied off into a sac. Immediately after removal from the animal, the intestinal segment was rinsed through with the saline solution while immersed in the same saline solution at 37°C. This was continued until the rinsings were clear. At the end of 30 minutes incubation, aliquots were taken from both mucosal and serosal solutions, and assayed for penicillin content as before.

(ii) Results.

The results obtained are shown in Table 11.

In every case a serosal:mucosal ratio of greater than unity was obtained after 30 minutes incubation. However, without exception, the final concentration on the serosal side remained essentially unchanged from the initial concentration. The increase in serosal:mucosal ratios over unity was caused by a decrease in the mucosal concentration.

In order to exclude the possibility that some energy-dependent mechanism might have been responsible for the observed data, a series of experiments were carried out at different temperatures. If active transport was taking place, changes in the metabolic rate of the tissue corresponding to changes in temperature should be reflected in changes in the final serosal:mucosal ratios.

Table 11. Distribution of phenoxymethyl and phenoxybenzyl penicillins between mucosal and serosal sides of noneverted sacs of rat small intestine incubated at 37°C for 30 minutes. Each value is the arithmetical mean ± standard error of a group of six experiments.

Penicillin	Concentration of penicillin (μg/ml)			
	Initial mucosal & serosal solutions	Final mucosal solution	Final serosal solution	Ratio of serosal to mucosal final concentration
Phenoxymethyl	0.05	0.03 ± 0.01	0.06 ± 0.01	2.0
	0.2	0.13 ± 0.03	0.23 ± 0.05	1.77
	0.8	0.59 ± 0.12	0.86 ± 0.10	1.46
	3.2	2.31 ± 0.16	3.72 ± 0.47	1.61
	12.8	8.82 ± 0.36	14.9 ± 1.5	1.69
Phenoxybenzyl	0.2	0.09 ± 0.02	0.23 ± 0.06	2.56
	0.8	0.52 ± 0.10	0.88 ± 0.08	1.69
	3.2	2.42 ± 0.17	4.06 ± 0.75	1.66
	12.8	7.96 ± 0.28	13.8 ± 0.90	1.73

(D) DISTRIBUTION OF PHENOXYMETHYL PENICILLIN BETWEEN MUCOSAL AND SEROSAL SIDES OF SACS OF NONEVERTED RAT SMALL INTESTINE AT DIFFERENT TEMPERATURES

Since mechanisms of active transport have many of the characteristics of enzyme reactions (see above - criteria for active transport) it is obvious that a change in temperature of the system would have quite a marked effect on the rate at which a substance is transported. Hence, the temperature coefficient, Q_{10}, would probably have a similar numerical value to that of many enzyme reactions (West et al., 1966). Reduction in temperature of our experimental system by 10, 20 or more centigrade degrees should result in significant decreases in the serosal:mucosal ratios if active transport were responsible for at least part of the differences observed so far.

(i) Methods.

The preparation used was identical with that described in the previous section except that different groups of experiments were carried out at temperatures of 37, 23 and 10°C.

(ii) Results.

The results obtained are shown in Table 12.

These data show that over a range of temperature of 27 centigrade degrees there was no significant differences in serosal:mucosal ratios. It is, therefore, unlikely that the passage of, at least, this penicillin is dependent on any metabolic process occurring in the intestinal epithelium.

Table 12. Serosal:mucosal concentration ratios of phenoxymethyl penicillin (3.2 μg/ml) after 30 minutes incubation *in vitro* at various temperatures in noneverted sacs of rat small intestine.

Temperature of incubation	$\frac{\text{Serosal concentration}}{\text{Mucosal concentration}}$ ratios
37°C	1.33*
23°C	1.32
10°C	1.31

* Each value is the arithmetical mean of 8 determinations.

Our data so far have shown that the decrease in final mucosal concentration caused the imbalance between final serosal and mucosal concentrations. It seemed worthwhile, therefore, to determine whether or not there was any relation between the tendency of a penicillin to bind to serum albumin and this imbalance. Two other penicillins were chosen, therefore, so that the total of four represented a wide range of binding to serum albumin. The two chosen were α-aminobenzyl penicillin (46 per cent bound) and dimethoxyphenyl penicillin (29 per cent bound).

(E) DETERMINATION OF FINAL SEROSAL:MUCOSAL CONCENTRATION RATIOS OF FOUR PENICILLINS REPRESENTING DIFFERENT DEGREES OF BINDING TO SERUM ALBUMIN

(i) Methods.

The preparation used was identical to that described in the previous section. The sacs were incubated for 30 minutes at 37°C. Data for the degree of protein binding to albumin have been presented previously (Table 4).

(ii) Results.

The results obtained are shown in Table 13.

The increase over unity in all ratios was due to a decrease in concentration on the mucosal side. The concentration on the serosal side remained essentially constant.

These data show clearly that there is some relation between the decrease in concentration on the mucosal side and the tendency of penicillins to bind to serum albumin. Hence, the magnitude of the serosal:mucosal concentration ratios appears to be dependent on an interaction between the penicillin the lumen of the intestine and some component of the mucosal epithelium.

Indeed preliminary investigation showed this to be the case and led to the following series of experiments.

Table 13. Comparison of percentage binding to serum albumin of four penicillins with the final serosal:mucosal concentration ratios attained after incubation in uneverted sacs of rat small intestine. Each penicillin was used at a concentration of 3.2 μg/ml in the intestinal sac experiments.

Penicillin	% binding to plasma albumin	$\frac{\text{Serosal concentration}}{\text{Mucosal concentration}}$ ratio
Phenoxybenzyl	82	1.72 *
Phenoxymethyl	69	1.33
α-Aminobenzyl	46	1.21
Dimethoxyphenyl	29	1.04

* Each value is the arithmetical mean of 8 determinations.

(F) DETERMINATION OF BINDING OF PENICILLINS TO RAT INTESTINAL MUCOSA

(i) Methods.

The penicillins used were those listed in Table 13. 20 cm Lengths of upper small intestine from fasting rats were opened lengthwise and carefully rinsed with sterile saline. The mucosa from each was scraped off with a microscope slide and transferred to flasks containing the penicillins dissolved in sterile Krebs bicarbonate saline. The contents were briefly homogenized to disperse the mucosa and then incubated at 37°C in a shaking incubator. The concentration of each penicillin was chosen for convenience of assay. As a result of preliminary experiments it was found that consistent data could be obtained when in each flask the mucosa from eight intestinal segments was suspended in a total volume of 15 ml. After six hours incubation the contents of each flask were centrifuged at 1,500 G for 30 minutes. Aliquots were removed from each supernatant solution and assayed for penicillin content as before.

(ii) Results.

In the case of α-aminobenzyl penicillin no precise data could be obtained because of the instability of the penicillin in this preparation.

The results obtained for the other three penicillins are shown in Table 14.

While these data have no absolute value due to the empirical nature of the experiments, there is a direct relation between the degree of binding to intestinal mucosa, the degree of binding to plasma albumin and the decrease in concentration on the mucosal side of isolated intestinal sacs. There is also a direct relation between the degree of mucosal binding

and the oleyl alcohol/water partition coefficients of the penicillins. The mucosal suspension may be regarded to some extent, therefore, as a non-miscible lipid-containing phase analogous to the non-polar solvent, oleyl alcohol, used in our earlier experiments. In one aspect, therefore, our work has come full circle.

Because of the unphysiological nature of the in vitro, intestinal sac preparations described above, the presence of a system other than simple diffusion which might account for the rapid passage of penicillins across the intestinal epithelium could have been missed. For this reason a series of experiments were carried out in vivo in which rat small intestine was perfused with solutions of phenoxymethyl penicillin.

Table 14. The binding of three penicillins to rat intestinal mucosa. (The data in parentheses to the right of the table are from Table 13 and are included for comparison).

Penicillin (conc^{n} in μg/ml)	% binding to mucosa	
Phenoxybenzyl (0.5)	30.9 ± 2.9*	(% protein binding, 82; S/M ratio 1.72)
Phenoxymethyl (0.15)	16.9 ± 1.2	(% protein binding, 69; S/M ratio 1.33)
Dimethoxyphenyl (3.0)	1.18 ± 1.03	(% protein binding, 29; S/M ratio 1.04)

* Each value is the arithmetical mean ± standard error of 5 determinations.

(G) THE ABSORPTION OF PHENOXYMETHYL PENICILLIN FROM THE PERFUSED SMALL INTESTINE OF THE RAT *IN VIVO*

Investigations on the kinetics of absorption of substances from the intestine have been carried out previously by perfusing a length of gut *in situ* and measuring the proportion absorbed from various concentrations. Thus Jervis and Smyth (1955) demonstrated active transport of amino acids from the lumen of the perfused intestine to the bloodstream. Care must be exercised, however, in the interpretation of such data because substances may be metabolized, or taken up and retained, before passing through to the circulating blood. For example, glutamic acid is transaminated during absorption, and appears in the blood largely as alanine (Matthews and Wiseman, 1953; Neame and Wiseman, 1957); small peptides enter the mucosal cells as such but are hydrolyzed and enter the blood as amino acids (Newey and Smith, 1960); cyanocobalamin, complexed to intrinsic factor, is transported to the bloodstream only after a delay of several hours at, and within, the mucosa where it may have arrived by pinocytosis (*see* Herbert, 1965). Direct measurements of the rate of entry of substances into the venous drainage from a loop of intestine may also be made. Hence, Matthews and Smyth (1954) have compared this rate for amino acids with their rate of disappearance from the intestine.

Even without the elaboration of this last technique, the demonstration of the presence or absence of a rate-limiting mechanism is strongly suggestive of the presence or absence of some system other than simple diffusion.

(i) Methods.

Adult rats of either sex, weighing from 250 to 300 gm were fasted overnight and anaesthetized with chloralose/urethane. The abdomen was opened through a mid-line incision and the bile duct cannulated to prevent recycling of absorbed penicillin. The intestine was tied just distal to the pylorus and a glass cannula inserted to permit introduction of the perfusing solution. A second cannula was tied into the intestine at the ileo-caecal junction. The outflow from this was collected for analysis.

A Harvard infusion pump was used to pump the solution through the intestine at a rate of 1.23 ml per minute. Just before entering the intestine the solution was warmed to 37°C by circulation through a thermostatically controlled heat exchanger. The potassium salt of phenoxymethyl penicillin was dissolved in 0.85 per cent NaCl solution containing 0.014 mMol/litre phenol red. This dye is not absorbed by the intestine, so that estimation of its concentration in the outflow permitted a correction to be made for any gain or loss of fluid.

The cannulated intestine was first flushed through with 0.85 per cent NaCl solution until the outflow appeared clear. The saline solution was then replaced by penicillin-phenol red-saline solution, and perfusion continued for a further 30 minutes. At the end of this period, the outflow was collected for 4 ten-minute periods. These four samples, together with an aliquot of the original penicillin-solution, were adjusted to pH 6.0 by the careful addition of 0.1 N HCl. The absorption due to the phenol red in each sample was measured at 435 mμ by means of a Unicam SP600 spectrophotometer. A correction factor could then be applied to

the concentration of penicillin in each sample.

The penicillin content of original solution and samples were determined by the pad-plate method described previously. The proportion absorbed was then calculated by dividing the difference between the concentration of penicillin in the original perfusing solution and sample (corrected), by the concentration in the original solution, and expressed as a percentage. The perfusing solutions contained phenoxymethyl penicillin in concentrations of from 0.6 to 160 μg/ml.

(ii) Results.

The results obtained are shown in Table 15.

These results show two things. First, precision is not a characteristic of this technique. Second, over the very wide range of concentrations used, there was no significant decrease in the proportion absorbed as the concentration was increased. It may be assumed, therefore, that for phenoxymethyl penicillin at least, and probably also for its congeners, absorption from the small intestine is dependent solely on passive diffusion.

Table 15. The proportions of phenoxymethyl penicillin absorbed from solutions perfusing rat small intestine *in vivo*. Each value is the arithmetical mean of a number of observations ± standard deviation.

Concentration of penicillin in perfusing solution (μg/ml)	Percentage absorbed
160	17.4 ± 3.7 (4)*
40	19.0 ± 5.0 (9)
10	19.4 ± 4.6 (9)
2.5	18.1 ± 1.3 (5)
0.6	16.6 ± 2.8 (5)

* Numbers in parentheses represent the number of individual perfusions carried out in each concentration.

(H) DISCUSSION

Briefly our experiments have shown the following. Phenoxybenzyl penicillin is absorbed from the rat intestine *in vivo* at a slower rate than phenoxymethyl penicillin. In isolated intestinal sacs, the rate at which both penicillins traverse the intestinal wall decreases with increase in concentration of penicillin in the lumen. Both these observations point to the participation of some mechanism of transfer other than simple diffusion of the unionized, lipid-soluble molecule. The reasons are as follows. (a) Phenoxybenzyl penicillin has an oleyl alcohol/water partition coefficient and an ionization constant which are more advantageous for passage across the intestinal epithelium than the equivalent properties of phenoxymethyl penicillin, and (b) if simple diffusion only were involved, the rate of passage would have shown a uniform increase as the concentration in the lumen was increased in the *in vitro* experiments.

Despite these indications, subsequent experiments both *in vitro* and *in vivo* showed that transfer against a concentration gradient could not be achieved, nor was there any evidence that the process could be saturated. Indeed, in experiments where initial concentrations on both mucosal and serosal sides of intestinal sacs were the same, the concentration on the mucosal side always decreased without any corresponding increase on the serosal side. Furthermore, the process was not temperature-dependent.

Further investigation showed that decreases in mucosal concentrations were related to the ability of particular penicillins to bind to serum albumin. From this it is concluded that the passage of penicillins

through the epithelium of the intestine may be impeded by interaction with some component of the epithelium in a manner analagous to the interaction between penicillin and albumin. Hence, since lipophilic properties are important both in the readiness with which substances may traverse the intestinal epithelium, and are important in the readiness with which substances may bind to serum albumin, it appears that the rate of passage is the resultant of the two opposing functions. From this it would appear that optimal rate of passage would depend on a nice balance between too little lipid-solubility and too much.

This still leaves unexplained, however, the observation that the rate of passage of penicillins in the in vitro preparation decreased as the luminal concentration increased. At first sight such a change might be ascribed to the participation of some specialized transport mechanism at low concentrations, with subsequent saturation, and decrease in rate, at high concentrations. No evidence was found, however, that such a mechanism existed in our preparations.

The curves depicted in Figure 15 may be regarded in another light. The lower part may represent normal passive diffusion. The decrease in rate at higher concentrations must then represent participation of some inhibitory process. Such a concentration - dependent change can be explained by an alteration in the physical state of the penicillin in solution. If the alteration resulted in a decrease in lipid solubility, increase in concentration would cause a decrease in the ability of the penicillin to cross from one side of the intestine to the other. The result would be an apparent decrease in the permeability of the tissue.

The ability of certain substances to form micelles in aqueous solution is such a concentration-dependent change. In this respect we may regard some of the penicillins as modified fatty acids with a strongly polar group at one end of the molecule attached to the physicochemical equivalent of a lipophilic fatty acid chain.

All fatty acids with chain lengths greater than seven carbon atoms show evidence of colloidal aggregation (micelle formation) above certain concentrations. As the chain length is increased above seven, the tendency to micelle formation increases and becomes evident at progressively lower concentrations. Various forms of micelles have been postulated, for example the 'laminar' micelle of McBain and the 'spherical' micelle of Hartley, (see, Pankhurst, 1953). All, however, envisage a central core of lipophilic groups surrounded on the outside by a layer of hydrophilic groups.

The concentration at which micelles start to form, the critical micelle concentration, differs for each substance, and, in general, decreases as the hydrocarbon chain increases. The addition of neutral non-colloidal electrolytes further reduces the critical micelle concentration of individual substances. For example, the addition of 0.032 M sodium chloride to cetyl pyridinium bromide solution has been shown to reduce its critical micelle concentration from about 0.0009 M to about 0.0001 M. The latter concentration is close to the concentrations shown on Figure 15 where a change in slope of the curve occurred (0.0001 M phenoxymethyl penicillin $\equiv$ 35 µg/ml, and 0.0001 M phenoxybenzyl penicillin $\equiv$ 43 µg/ml.)

We may envisage, therefore, an increasing proportion of micelles being formed once the concentration passed the critical micelle concentration. Such micelles would not pass readily into, and through, the intestinal epithelium because of their polar nature. The net result would be an increasing degree of inhibition to passage and a slowing in the rate of appearance of penicillin on the serosal side of the intestinal sac.

In our in vivo experiments the same conditions did not pertain. The rapid removal and distribution of absorbed penicillin by the blood resulted in the maintenance of a large concentration gradient between mucosal and serosal sides. Any differences in degree of absorption would therefore be small and obscured in the large spread about each mean inherent in the technique.

8. SUMMARY AND CONCLUSIONS

Our study has shown that variation in the structure of the side-chain of penicillins is associated with changes in the physico-chemical properties of the substances. Most of the penicillins studied possess ionization constants that would class them as fairly strong organic acids, however, two members of the group are much less strongly acidic. The decrease in acid strength is associated with an increase in 'bulkiness' of the side-chain. Assuming that electronic and structural configurations are unlikely to influence directly the ionization of the relatively remote carboxyl group, it is proposed that dimerization accounts for the change in this property.

Gross differences were observed in oleyl alcohol/water partition coefficients for the series of substances. Some of the penicillins passed readily into the non-polar phase. Others possess much stronger polar characteristics. These differences are associated with the presence or absence of lipophilic substituents on the α-carbon atom of the side-chain. The use of oleyl aclohol in obtaining these data is noteworthy; the penicillins were all relatively insoluble in non-polar solvents of lower molecular weight. Partition coefficients determined in the oleyl alcohol/water system at a pH of 5.3, the pH at the absorbing surface of intestinal epithelium, were nearly all greater than unity. This property is consistent with the ready absorption of such penicillins from the gastrointestinal tract. Those shown to be much more polar, are very much less readily absorbed from the gastrointestinal tract.

Great variation was shown to occur in the extent to which the various penicillins were bound to serum albumin. The 'bulky' penicillins

were bound to the greatest extent. Although ionic binding has been thought to play little or no part in the binding of penicillins to albumin, we suggest that in these latter cases, other binding components may be supplemented by re-inforced ionic bonds formed between the carboxyl groups of the penicillins and the ϵ-amino groups of lysine. There is some evidence for the participation of van der Waals forces in the binding of lower molecular weight penicillins, but, in the main, binding to albumin is directly associated with partition coefficients. Therefore, our data confirm previous observations that hydrophobic bonding is a major component in the binding of most penicillins. In the case of one amphoteric penicillin this relationship does not hold. The presence of complementary ionized sites on the protein surface may explain its aberrant behaviour.

In selecting penicillins for further study, one of the group was found to undergo extensive biotransformation in the body. The resultant substance was quite different from its parent in that resistance to β-lactamase was lost. Activity against normally penicillin-sensitive strains of organisms was retained.

Experiments *in vivo* and *in vitro* with two selected penicillins showed that persistence in the body could, to some extent, be related to the physicochemical properties of the substances. It seems probable that, in terms of active transport, differences in both biliary and renal tubular secretion play an important part in the maintenance of concentrations of the penicillins in the blood. In terms of passive behaviour in the kidney, differences in physicochemical properties are such as to markedly affect glomerular filtration and tubular reabsorption.

The more highly protein bound of the two penicillins studied is also the more lipid soluble and the less acidic. The association between these parameters has been alluded to above.

The rate and degree of absorption of the two penicillins from the intestine were not in accord with what might have been predicted from their physical chemistry. Our data show that absorption may be delayed by an interaction between the penicillin and some component of the intestinal epithelium. There is some relation between this interaction and the degree of binding to albumin. Because of the association of lipid solubility with protein binding on the one hand, and readiness to penetrate lipid-like membranes on the other, we propose that optimal absorption from the gastrointestinal tract depends on a nice balance between too much lipid solubility and too little.

Some data obtained from in vitro experiments on absorption from intestinal sacs could be interpreted as showing the presence of some system other than passive diffusion. This interpretation could not be supported by further work. Our observations are consistent with the formation of micelles at low concentration. The relatively polar nature of these micelles would tend to reduce the ability of the parent substance to penetrate lipid-like membranes.

BIBLIOGRAPHY

Abraham, E.P., Chain, E., Fletcher, C.M., Florey, H.W., Gardner, A.D., Heatley, N.G. and Jennings, M.A. (1941) Further observations on penicillin. Lancet 2, 177.

Acred, P., Brown, D.M., Turner, D.H. and Wilson, M.J. (1962) Pharmacology and chemotherapy of ampicillin - a new broad-spectrum penicillin. Brit. J. Pharmacol. 18, 356.

Acred, P., Brown, D.M., Turner, D.H. and Wright, D. (1961) Pharmacology of methicillin. Brit. J. Pharmacol. 17, 70.

Albert, A., Hampton, A., Selbie, F. and Simon, R. (1954) The influence of chemical constitution on antibacterial activity. Part VII: The site of action of 8-hydroxy-quinoline (oxine). Brit. J. exp. Path. 35, 75.

Albert, A. and Serjeant, E.P. (1962) Ionization Constants of Acids and Bases. Methuen.

Andrews, J.C. and Cornatzer, W.E. (1944) The effect of acid and alkali on the absorption and metabolism of quinine. J. Pharmacol. 82, 261.

Babès, V. (1885) Concurrence vitale des bacteries. Attenuation de leurs proprietes dans des milieux nutritifs modifies par d'autres bacteries. Tentatives de therapeutique bacteriologique. J. Connaiss. med. Prat. 7, 321.

Barber, M. and Garrod, L.P. (1963) In 'Antibiotic and Chemotherapy', Livingstone.

Batchelor, F.R., Doyle, F.P., Naylor, J.H.C. and Rolinson, G.N. (1959) Synthesis of penicillin:6-Aminopenicillanic acid in penicillin fermentation. Nature, 183, 257.

Beyer, K.H. (1950) Functional characteristics of renal transport mechanisms. Pharmacol. Rev. 2, 227.

Beyer, K.H., Peters, L., Patch, E.A. and Russo, H.F. (1944) Renal elimination of sulfamerazine, sulfamethazine, sulfadiazine and sulfathiazole by the dog. J. Pharmacol. 82, 239.

Beyer, K.H., Peters, L., Woodward, R. and Verwey, W.F. (1944) Enhancement of the physiological economy of penicillin in dogs by simultaneous administration of para-aminohippuric acid. J. Pharmacol. 82, 310.

Beyer, K.H., Russo, H.F., Tillson, E.K., Miller, A.K., Verwey, W.F. and Gass, S.R. (1951) 'Benemid', p-(di-n-propylsulfamyl)-benzoic acid; its renal affinity and its elimination. Am. J. Physiol. 166, 625.

Bond, J.M., Lightbown, J.W., Barber, M. and Waterworth, P.M. (1963) A comparison of four phenoxypenicillins. Brit. med. J. 2, 956.

Brandl, V.E., Brunner, R. and Knauseder, F. (1964) Die eiweissbindung von penicillinen. Arzneimittel-Forsch. 14, 883.

Brandl, E., Giovannini, M. and Margreiter, H. (1953) Untersuchungen über das säurestabile, oral wirksame Phenoxymethyl-penicillin (Penicillin V). Wein. med. Wschr. 103, 602.

Brodie, B.B. and Hogben, C.A.M (1957) Some physicochemical factors in drug action. J. Pharm. Pharmacol. 9, 345.

Bucher, G.R., Flynn, J.C. and Robinson, C.S. (1944) The action of the human small intestine in altering the composition of physiological saline. J. biol. Chem. 155, 305.

Cattell, W.R., Spencer, A.G., Taylor, G.W. and Watts, R.W.E. (1962) The mechanism of the renal excretion of oxalate in the dog. Clin. Sc. 22, 43.

Chain, E., Florey, H.W., Gardner, A.D., Heatley, N.G., Jennings, M.A., Orr-Ewing, J. and Sanders, A.G. (1940) Penicillin as a chemotherapeutic agent. Lancet 2, 226.

Chow, B.F. and McKee, C.M. (1945) Interaction between crystalline penicillin and human plasma proteins. Science 101, 67.

Clausen, J. (1966) Binding of sulfonamides to serum proteins: Physicochemical and immunochemical studies. J. Pharmacol. exp. Therap. 153, 167.

Clutterbuck, P.W., Lovell, R. and Raistrick, H. (1932) Studies in the biochemistry of micro-organisms. Biochem. J. 26, 1907.

Cori, C.F. (1925) The rate of absorption of hexoses and pentoses from the intestinal tract. J. biol. Chem. 66, 691.

Crampton, R.F. and Matthews, D.M. (1964) In 'Absorption and Distribution of Drugs', Livingstone.

Crawford, T.B.B. and Levvy, G.A. (1947) Changes undergone by phenyl arsenious acid and phenyl arsonic acid in the animal body. Biochem. J. 41, 333.

Crosson, J.W., Boger, W.P., Shaw, C.C. and Miller, A.K. (1947) Caronamide for increasing penicillin plasma concentrations in man. J. Amer. med. Assoc. 134, 1528.

Crowfoot, D., Bunn, C.W., Rogers-Low, B.W. and Turner-Jones, A. (1949) In 'The Chemistry of Penicillin', Princeton University Press.

Danielli, J. (1952) The Permeability of Natural Membranes. dition, Cambridge University Press.

(1965) A definition of substrate specificity in renal port of organic anions. J. theoret. Biol. 8, 163.

Pendergrass, L.H. and Stoechkinger, J.M. (1963) nce of analogues of phenylbutazone on renal transport of ohippurate. Am. J. Physiol. 205, 489.

Rall,D.P. (1964) Evidence of active transport of benzyl- llin-C-14 from cerebrospinal fluid to blood. Fed. Proc. 70.

5) Ein Beitrag zur Chemotherapie die bakteriellen ionen. Dtsch. med. Wschr. 61, 250.

09) Über den jitzigen stand der Chemotherapie. Ber. dtsch. Ges. 42, 17.

Bertheim, A. (1912) Über das salzsäure 3.3'-diamino-4.4'- arsenobenzol und seine nächsten Verwandten. Ber.dtsch. Ges. 45, 756.

Hata, S. (1911) The Experimental Chemotherapy of losis , Ribman, London.

d Richardson, C.H. (1938) Penetration of nicotine into sh from solutions of various hydrogen ion concentrations. l. comp. Physiol. 11, 377.

Low, O. (1899) Bakteriolytische Enzyme als Ursache der enen Immunität und die Heilung von Infektionskrankheiten ieselben. Z. Hyg. Infektkr. 31, 1.

ggart, J.V. (1960) Competitive inhibition of renal ort of p-aminohippurate by other mono-substituted tes. Am. J. Physiol. 199, 509.

Jardetsky, O. (1965) Nuclear magnetic relaxation study rmolecular complexes. The mechanism of penicillin to serum albumin. J. Amer. chem. Soc. 87, 3237.

5) The absorption of water and of some solute molecules e isolated small intestine of the rat. J. Physiol. 5.

ast, L., Waterhouse, A. and Shannon, J.A. (1943) The n between chemical structure and physiological disposi- a series of substances allied to sulfanilamide. macol. 79, 373.

Fleming, A. (1929) On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of B. influenzae. Brit. J. exp. Path. 10, 226.

Florey, H.W., Gilliver, K., Jennings, M.A. and Sanders, A.G. (1946) Mycophenolic acid; an antibiotic from Penicillium brevi compactum dierckx. Lancet 1, 46.

Foerster (1933) Sepsis in Anschluss an ausgedehnte Periporites. Heilung durch Streptozon. Zbl. Haut-u. Geschl. Kr. 45, 549. (not checked from original)

Froese, A., Sehon, A.H. and Eigen, M. (1962) Kinetic studies of protein-dye and antibody-hapten interactions with the temp-jump method. Can. J. Chem. 40, 1786.

Fuller, A.T. (1937) Is α-aminobenzenesulfonomide the active agent in prontosil therapy? Lancet 1, 194.

Goldbaum, L.R. and Smith, P.K. (1954) The interaction of barbiturates with serum albumin and its possible relation to their disposition and pharmacological action. J. Pharmacol. exp. Therap. 111, 197.

Goldstein, A. (1949) The interactions of drugs and plasma proteins. Pharmacol. Rev. 1, 102.

Gosio, B. (1896) Quoted from Florey, H.W. (1946) Rev. Igiene Sanit. publ. 7, 825. Brit. med. Bull., 4, 248.

Gottschalk, C.W., Lassiter, W.E. and Mylle, M. (1960) Localization of urine acidification in the mammalian kidney. Am. J. Physiol. 198, 581.

Gourevitch, A., Hunt, G.A. and Lein, J. (1960) Structure-activity relationships in a series of synthetic penicillins. Antibiotics and Chemotherapy, 10, 121.

Green, R.E., Richer, W.E., Attwood, W.C., Kok, Y.S. and Peters, L. (1959) Studies of the renal tubular transport characteristics of N-methylnicotinamide and tetroalkylammonium compounds in the avian kidney. J. Pharm. exp. Therap. 126, 195.

Harper, N.J. (1959) Drug latentiation. J. med. pharm. Chem. 1, 467.

Harper, N.J. (1962) In 'Progress in Drug Research', Vol. 4. Birkhäuser Verlag Basel und Stuttgart.

Harper, N.J. (1964) In 'Absorption and Distribution of Drugs', Livingstone.

Harrison, P.M. and Stewart, G.T. (1961) Excretion of antibiotics in bile. Brit. J. Pharmacol. 17, 420.

Herbert, V. (1965) In 'The Pharmacological Basis of Therapeutics' 3rd Edit. Macmillan.

Hirsch, H.L. and Putnam, L.E. (1958) Penicillin, Medical Encyclopedia, Inc.

Höber, R. (1940) Correlation between the molecular configuration of organic compounds and their active transfer in living cells. Cold Spring Harbor Symp., 8, 40.

Höber, R. (1945) Physical Chemistry of Cells and Tissues, Churchill.

Höber, R. and Höber, J. (1937) Experiments on the absorption of organic solutes in the small intestine of rats. J. cell. comp. Physiol. 10, 401.

Hogben, C.A.M. (1960) The first common pathway. Fed. Proc. 19, 864.

Hogben, C.A.M., Schanker, L.S., Tocco, D.J. and Brodie, B.B. (1957) Absorption of drugs from the stomach. II. The Human. J. Pharmacol. exp. Therap. 120, 540.

Hogben, C.A.M., Tocco, D.J., Brodie, B.B. and Schanker, L.S. (1959) On the mechanism of intestinal absorption of drugs. J. Pharmacol. exp. Therap. 125, 275.

Huang, K.C. and Lin, D.S.T. (1965) Kinetic studies on transport of PAH and other organic acids in isolated renal tubules. Am. J. Physiol. 208, 391.

Jennings, M.A. (1945) Activity of helvolic acid against Mycobacterium tuberculosis. Nature 156, 633.

Jervis, E.L. and Smyth, D.H. (1959) The effect of concentrations of amino acids on their rate of absorption from the intestine. J. Physiol. 149, 433.

Karnovsky, M.L. and Johnson, M.J. (1949) Filter paper chromatography of penicillin broths. Anal. Chem. 21, 1125.

Keen, P.M. (1965) The binding of three penicillins in the plasma of several mammalian species as studied by ultrafiltration at body temperature. Brit. J. Pharmacol. 25, 507.

Kirby, W.M.M. and Kind, A. (1966) Clinical pharmacology of ampicillin and hetacillin. Abstract from - N.Y. Acad. Sci. Conference on 'Comparative Assessment of the Broad Spectrum Penicillins and Other Antibiotics'.

Klotz, I.M. and Walker, F.M. (1947) The binding of organic ions by proteins. Charge and pH effects. J. Amer. chem. Soc. 69, 1609.

Krantz, J.C. and Carr, C.J. (1951) The Pharmacologic Principles of Medical Practice. Williams and Wilkins.

Kunin, C.M. (1965) Inhibitors of penicillin binding to serum proteins. J. lab. clin. Med. 65, 416.

Lin, E.C.C., Hagihira, H. and Wilson, T.H. (1962) Specificity of the transport system for neutral amino acids in the hamster intestine. Am. J. Physiol. 202, 919.

Lynn, B. (1965) The semi-synthetic penicillins. Antibiotica and Chemotherapia 13, 125.

Matthews, D.M. and Smyth, D.H. (1954) The intestinal absorption of amino-acid enantiomorphs. J. Physiol. 126, 96.

Matthews, D.M. and Wiseman, G. (1953) Transmission by the small intestine of the rat. J. Physiol. 120, 55P.

McLean, F.C. and Hastings, A.B. (1935) The state of calcium in the fluids of the body. I. The conditions affecting the ionization of calcium. J. biol. Chem. 108, 285.

Meyer, H. (1899) Zur Theorie der Alkoholnarkose. Arch. exp. Path. Pharmak. 42, 109 and 119.

Nayler, J.H.C., Long, A.A.W., Brown, D.M., Acred, P., Rolinson, G.N., Batchelor, F.R., Stevens, S. and Sutherland, R. (1962) Chemistry, toxicology, pharmacology and microbiology of a new acid-stable penicillin, resistant to penicillinase (BRL 1621). Nature 195, 1264.

Neame, K.D. and Wiseman, G. (1957) The transportation of glutamic and aspartic acids during absorption by the small intestine of the dog in vivo. J. Physiol. 135, 442.

Newey, H. and Smyth, D.H. (1960) Intracellular hydrolysis of dipeptides during intestinal absorption. J. Physiol. 152, 367.

Newey, H. and Smyth, D.H. (1963) Specificity of carriers in intestinal transfer of glycine. J. Physiol. 165, 74.

Nicolle, M. (1907) Action due Bacillus subtilis sur diverses bactéries. Ann. Inst. Pasteur 21, 613.

O'Connell, J.M.B., Romeo, J.A. and Mudge, G.H. (1962) Renal tubular secretion of creatinine in the dog. Am. J. Physiol. 203, 985.

Overton, E. (1901) Studien über die Narkose, zugleich ein Beitrag zur allgemeinen Pharmakologie. Jena, Fischer.

Overton, E. (1902) Beiträge sur allgemeinen Muskel und Nervenphysiologie. Pflüg. Arch. ges. Physiol. 92, 115.

Pankhurst, K.G.A. (1953) Detergent solutions. Monograph No. 5, The Royal Institute of Chemistry.

Pasteur, L. and Joubert, J.F. (1877) Charbon et septicémie. C.R. Acad. Sci. 85, 101.

Pindell, M.H., Tisch, D.E. and Reiffenstein, J.C. (1961) Pharmacological properties of dimethoxyphenyl penicillin. Symposium on Dimethoxyphenyl Penicillin, State University of New York, Syracuse.

Popper, H.P. and Schaffner, F. (1957) Liver: Structure and Function. McGraw-Hill.

Poth, E.J. and Knotts, F.L. (1942) Clinical use of succinylsulfathiazole. Arch. Surg. Chicago 44, 208.

Poth, E.J., Knotts, F.L., Lee, J.T. and Inui, F. (1942) Succinylsulfathiazole, a new chemotherapeutic agent locally active in the gastro-intestinal tract. Arch. Surg. Chicago 44, 187.

Rapson, H.D.C. and Bird, E.A. (1963) Ionisation constants of some penicillins and of their alkaline and penicillinase hydrolysis products. J. Pharm. Pharmacol. 15, 222 T.

Rolinson, G.N. and Batchelor, F.R. (1963) Penicillin metabolites. Antimicrobial Agents and Chemotherapy - 1962, 654.

Rolinson, G.N., Batchelor, F.R., Butterworth, D., Cameron-Wood, J., Cole, M., Eustace, G.C., Hart, M.V., Richard, M. and Chain, E.B. (1960) Formation of 6-aminopenicillanic acid from penicillin by enzymatic hydrolysis. Nature 187, 236.

Rollo, I.M. (1966) Antibacterial chemotherapy. Ann. Rev. Pharmacol. 6, 209.

Rollo, I.M. and Somers, G.F. (1961) Structure-activity relationships in new penicillins. Biochem. Pharmacol. 8, 43.

Rollo, I.M., Somers, G.F. and Burley, D.M. (1962) Bacteriological and pharmacological properties of phenoxybenzylpenicillin. Brit. med. J. 1, 76.

Rowlands, S., Rowley, D. and Stewart, H.C. (1948) Absorption and excretion studies with radioactive penicillin. Lancet 2, 493.

Ruedy, J. (1965) The concentrations of penicillins in the cerebro-spinal fluid and brain of rabbits with experimental meningitis. Can. J. Physiol. Pharmacol. 43, 763.

Sakaguchi, K. and Murao, S. (1950) Penicillin amidase. J. agric. Chem. Soc. Japan 23, 411.

Schachter, D., Manis, J.G. and Taggart, J.V. (1955) Renal synthesis, degradation and active transport of aliphatic acyl amino acids. Relationship to p-aminohippurate transport. Am. J. Physiol. 182, 537.

Schanker, L.S. (1960) On the mechanism of absorption of drugs from the gastrointestinal tract. J. med. pharm. Chem. 2, 343.

Schanker, L.S. (1962a) Concentrative transfer of an organic cation from blood into bile. Biochem. Pharmacol. 11, 253.

Schanker, L.S. (1962b) Passage of drugs across body membranes. Pharmacol. Rev. 14, 501.

Schanker, L.S. and Jeffrey, J.J. (1961) Active transport of foreign pyrimidines across the intestinal epithelium. Nature 190, 727.

Schanker, L.S. and Tocco, D.J. (1960) Active transport of some pyrimidines across the rat intestinal epithelium. J. Pharmacol. exp. Therap. 128, 115.

Schanker, L.S., Shore, P.A., Brodie, B.B. and Hogben, C.A.M. (1957) Absorption of drugs from the stomach. I. The Rat. J. Pharmacol. exp. Therap. 120, 528.

Schanker, L.S., Tocco, D.J., Brodie, B.B. and Hogben, C.A.M. (1958) Absorption of drugs from the rat small intestine. J. Pharmacol. exp. Therap. 123, 81.

Schedl, H.P. and Clifton, J.A. (1961) Small intestinal absorption of steroids. Gastroenterology 41, 491.

Scholtan, W. and Schmid, J. (1962) Die bindung der Penicilline an die eiweißkörper des serums und des gewebes. Azneimittel-Forsch. 12, 741.

Sheehan, N.C. (1958) In 'Amino-acids and Peptides with Antimetabolic Activity', Ciba Foundation Symposium, Churchill.

Shore, P.A., Brodie, B.B. and Hogben, C.A.M. (1957) The gastric secretion of drugs: A pH partition hypothesis. J. Pharmacol. exp. Therap. 119, 361.

Smyth, D.H. and Taylor, C.B. (1958) Intestinal transfer of short-chain fatty acids in vitro. J. Physiol. 141, 73.

Solomon, H.M. and Schanker, L.S. (1962) Hepatic excretion of organic bases. Fed. Proc. 21, 150.

Sperber, I. (1959) Secretion of organic anions in the formation of urine and bile. Pharmacol. Rev. 11, 109.

Steinman, H.G. and Denny, E.L. (1963) Reversible binding of penicillin to serum albumin. Third Intersc. Conf. Antimicrob. Agents and Chemotherapy.

Stephens, J. and Grainger, A. (1955) Paper chromatography of penicillin V. J. Pharm. Pharmacol. 7, 702.

Stewart, G.T. and Harrison, P.M. (1961) Excretion and re-excretion of a broad spectrum penicillin in bile. Brit. J. Pharmacol. 17, 414.

Strauss, E., Richburg, P.L., Saba, P.Z. and Alexander, J.E. (1947) Enhancement of plasma penicillin concentrations by caronamide and sodium benzoate. J. lab. clin. Med., 32, 818.

Teresi, J.D. and Luck, J.M. (1952) The combination of organic ions with serum albumin. VIII. Fatty acid salts. J. biol. Chem. 194, 823.

Thompsett, R., Schultz, S. and McDermott, W. (1947) The relation of protein-binding to the pharmacology and antibacterial activity of penicillins X, G, dihydro F & K. J. Bact. 53, 581.

Travell, J. (1940) The influence of the hydrogen ion concentration on the absorption of alkaloids from the stomach. J. Pharmacol. 69, 21.

Tréfouël, J., Tréfouël, Mme. J., Nitti, F. and Bovet, D. (1935) Activité du α-aminophénylsulfamide sur les infections streptococciques expérimentales de la souris et du lapin. C.R. Soc. Biol. Paris 120, 756.

Ullrich, K.J., Kramer, K. and Boylan, J.W. (1961) Present knowledge of the counter-current system in the mammalian kidney. Prog. cardiovasc. Dis. 3, 395.

Valentine, R.J., Simon, H.F. and Rantz, L.A. (1964) The renal clearance of oxacillin. Proc. Soc. exp. Biol. Med. 115, 1151.

Vanderhaeghe, H., Van Dijck, P., Claesen, M. and De Somer, P. (1962) Preparation and properties of 3,4-dichloro-α-methoxybenzyl penicillin. Antimicrobial Agents and Chemotherapy - 1961, 581.

Vaudremer, A. (1913) Action de l'extrait filtré d'Aspergillus fumigatus sur les bacilles tuberculeux. C.R. Soc. Biol. 74, 278 & 752.

Verzar, F. (1935) Die Rolle von Diffusion und Schleimhautaktivität bei der Resorption von verschiedenen Zuckern ous dem Darm. Biochem. Z. 276, 17.

Vincent, J.G. and Vincent, H.W. (1944) Filter paper disc modification of the Oxford cup penicillin determination. Proc. Soc. exp. Biol. Med. 55, 162.

Voegtlin, C., Dyer, H.A. and Leonard, C.S. (1923) On the mechanism of the action of arsenic upon protoplasm. Publ. Hlth. Rep., Wash. 38, 1882.

Volle, R.L., Huggins, C.G., Rodriguez, G.A. and Peters, L. (1959) Inhibition of the renal tubular transport of N-methylnicotin-