MECHANISTIC STUDIES

OF

AZO-COUPLING OF PYRROLES

AND RELATED COMPOUNDS

A Thesis

presented for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of Science of the

University of St. Andrews

by

PETER THOMAS SHEPHERD, B. Sc.

July 1978

to my parents

ABSTRACT

Chapter One describes the azo-coupling reaction of pyrrole and several methylpyrroles. Part (a) deals with the reaction in aqueous acid solution. The evidence obtained favours attack of the unprotonated pyrrole in an S_E2 mechanism, with a steady state intermediate. A consideration of two linear free-energy relationships shows that little can be deduced about the nature of any transition state in these reactions. However, protonated pyrrole is a good model for the steady-state intermediate. The activating effects of a methyl group towards electrophilic attack at various positions on the ring are calculated. The special reactions of 3,4-dimethyl- and 2,3,4,5-tetramethylpyrrole are discussed. In part (b) the azo-coupling reaction of pyrroles in non-hydroxylitic solvent in the presence of a crown ether is considered. A preparative procedure for the mono-azopyrroles using dicyclohexyl-18-crown-6 as a phase transfer catalyst has been devised and the kinetics of the azo-coupling reaction have been studied in this medium.

The mechanism of the azo-coupling reaction of dipyrrylmethanes in acid solution and in buffer solution (pH $\simeq$ 7) is described in Chapter Two. Where acid is present the methylene bridge of the dipyrrylmethane is cleaved by the proton and the resultant pyrrole monomers go on to react with the arenediazonium ions, so that the observed azo-coupling reaction is a one step process. In the absence of acid it is the arenediazonium ion which brings about cleavage of the methylene bridge. The reaction is studied both in water and in 50% aqueous ethanol; it is found that the reaction rate is virtually unaffected by the change

in solvent.

The subject of Chapter Three is the binding of bromocresol green and bromocresol purple to albumin. The rates of these binding processes have been studied and it has been found that while the binding of bromocresol purple to albumin takes place in one step,the binding of bromocresol green to albumin is a two step reaction, the second being much slower than the first. These results have been rationalised in terms of the different types of binding sites in the albumin molecule and in terms of the different pH's of the bromocresol green and bromocresol purple reagents.

In Chapter Four the azo-coupling reaction of bilirubin is discussed. The effect on the reaction of the binding of bilirubin to albumin is assessed. A mechanism for the azo-coupling reaction is described and is found to be that anticipated from the azo-coupling reactions of the dipyrrylmethanes i.e. rapid cleavage of the central methylene bridge by the proton followed by attack of the oxodipyrromethene fragments thus formed,by the arenediazonium ions. The effect of three commonly used 'accelerators' of the azo-coupling reaction of bilirubin on the rate of reaction is described and it is found that they all cause an increase in the rate but by very different amounts.

Declaration

I declare that this thesis is my own composition, that it is based on the results of experiments carried out by me, and that it has not previously been presented for a Higher Degree.

This thesis describes the results of research carried out in the Department of Chemistry of the United College of St. Salvator and St. Leonard, University of St. Andrews, under the supervision of Dr. A.R. Butler, between October 1975 and June 1978.

July 1978 P.T. Shepherd

Certificate

I hereby certify that Peter T. Shepherd has spent twelve terms of research work under my supervision, has fulfilled the conditions of Ordinance General No. 12 and Resolution of the University Court, 1967, No. 1, and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

A.R. Butler
Director of Research

Acknowledgements

I wish to express my gratitude to Dr. A.R. Butler for his constant help and encouragement over the past three years during the course of this work.

I would also like to thank the other members of the Chemistry Department who have been of assistance and in particular Mr. P. Pogorzelec for the synthesis of several pyrroles. My thanks also go to Mrs. W. Pogorzelec for typing this thesis.

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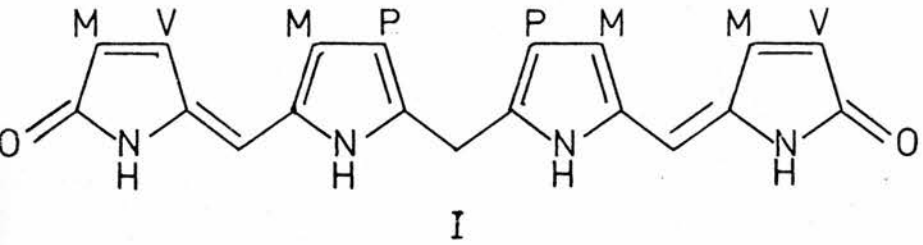
INTRODUCTION

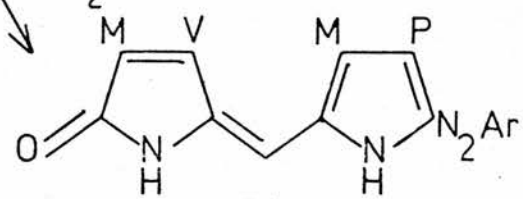


I

Enzyme catalysed esterification

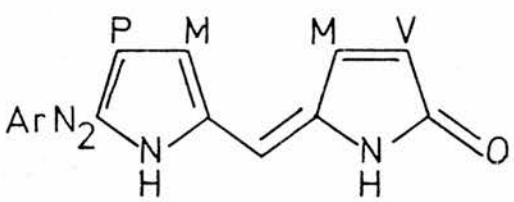
Several steps

H^+ ArN_2^+ III



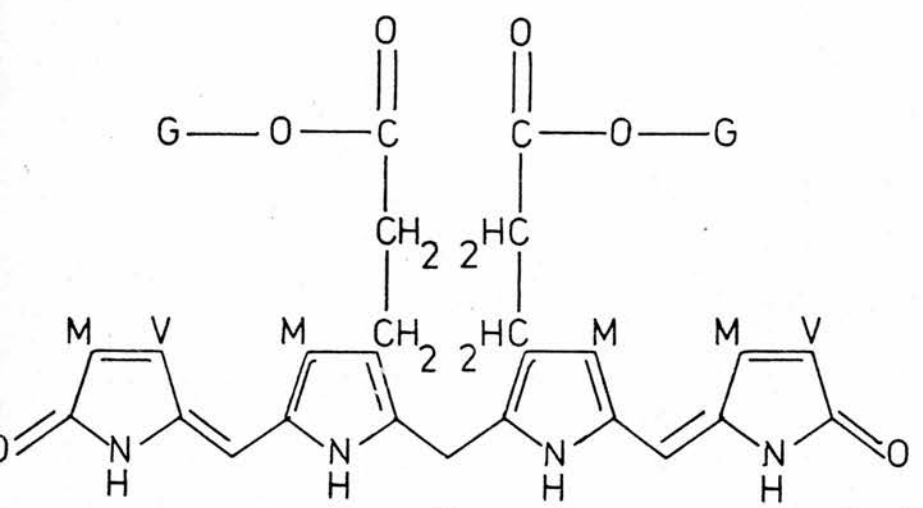
IVa

+



IVb

+ CH_2O



II

H^+ ArN_2^+

Va and Vb
Same as IVa and IVb, except with esterified propionic aci side chains

$M = CH_3$ $V = CH{=}CH_2$

$P = CH_2CH_2COOH$

G = Glucuronic acid

$ArN_2^+ = HO_3SH_4C_6N_2^+$

SCHEME 1

+ CH_2O

The Problem of Bilirubin Determination in Human Serum:

Bilirubin (I), a pyrrole tetramer, is found in low concentrations in normal human serum, and originates primarily from the breakdown of the haeme moiety of haemoglobin[1], although a small amount does come from other sources[2]. The nature of the enzymatic reaction involved in opening the tetrapyrrole ring in the conversion of haeme to bilirubin has not yet been clarified[3] and several conflicting mechanisms have been proposed[4-6].

The bilirubin formed from the breakdown of haemoglobin is transported in plasma bound to protein, albumin being by far the major carrier[7,8], but small amounts of bilirubin have been identified with other serum proteins such as the β-globulins[7,9]. The binding takes place between the primary amino groups in the protein and the carboxylic acid groups of the bilirubin[10], and this is why anionic drugs such as the salicylates reduce the bilirubin transport capacity of plasma since they are competing for the protein binding sites[3,11].

As well as being present in the plasma as the simple molecule (I), bilirubin is also present as the ester of glucuronic acid, the esterification taking place mainly in the liver[12]. The bilirubin ester of glucuronic acid exists in serum exclusively as the diglucuronide (II)[3], the monoglucuronide not having been found in serum, although it must be an intermediate in the formation of (II).

Elevated bilirubin concentrations in serum (hyperbilirubinaemia) are found in those suffering from conditions such as pernicious anaemia, liver disorders and indeed any disease which causes

haemoglobin breakdown or disrupts one or more of the steps between production and excretion of bilirubin[2,3,13,14]. Some diseases, such as haemolytic disorders, result in elevated serum concentrations of unesterified bilirubin (I), whereas certain liver disorders result in elevated concentrations of the bilirubin diglucuronide (II)[3]. The various causes of hyperbilirubinaemia have been dealt with in some detail by Schmid[15].

It is interesting that, although in recent years much new knowledge has been amassed on bilirubin, its clinical determination still relies on a method, admittedly now used with some modifications, devised at the end of the nineteenth century by Ehrlich and others[16,17], long before the chemistry of bilirubin was as well understood as it is today. This method is based on the coupling of bilirubin with diazotised sulphanilic acid (III). However, as more and more accurate methods of bilirubin determination have been demanded, a variety of modifications have been made to the original method, apparently unsystematically, with the result that the value one obtains for bilirubin concentration varies widely, according to which of the many available methods of determination is used[18]. These will now be discussed briefly.

It has long been known that bilirubin reacts with arene-diazonium ions to form an azo-pigment which is blue at strongly acidic and alkaline pH, and red near neutrality[16], ie. it behaves as an indicator. The reaction pathway (Scheme 1) has been established[19,20] and the azo-pigments isolated and identified as (IV) and (V)[21]. The reaction proceeds in two steps, each giving rise to equal amounts of azo-pigment, the central methylene

bridge being released as formaldehyde[22] in the presence of excess arenediazonium ion. It is this diazo-reaction which was first introduced by Ehrlich for the detection of bilirubin in urine in 1883[16] and was then applied to serum by Van den Bergh and Snapper[23] after removal of the serum proteins - which were thought to inhibit the diazo-reaction - by the addition of ethanol, which caused them to precipitate. The test is carried out under the mildly acidic conditions necessary for diazotisation of sulphanilic acid, giving rise to the red azo-pigment. It is the serum test which is clinically the more useful and it is here most of the effort has been spent on modifications and improvement. Having developed this method for serum bilirubin, Van den Bergh[24] later discovered that two types of diazo-reaction could be distinguished, which he described as follows: the 'direct' reaction, in which colour develops within 30 s in the absence of alcohol, and the 'indirect' reaction, requiring the addition of alcohol for colour development. The total colour then existing after the addition of alcohol is the total bilirubin ie. the sum of the direct and indirect reactions. Further breakdown of the serum diazo-reaction - known as the Van den Bergh test - has taken place since[25] into 'prompt direct', 'delayed direct', 'biphasic' etc., but these are minor sub-divisions which I shall not discuss in detail, but instead I will concentrate on the important difference ie. that between the direct and the indirect diazo-reactions.

Many years of research have gone into explaining the difference between the direct and indirect forms of bilirubin in the Van den Bergh test. Several reasons have been put forward[3],

the most plausible being:

(a) the presence in serum of 'inhibitors' and 'accelerators' of the diazo-reaction,

(b) there are two different types of bilirubin present,

(c) bilirubin is present in only one form, but is bound to different substances.

It is now generally accepted that (b) is the correct explanation of the situation and it is the bilirubin diglucuronide (II) which is reacting in the direct reaction along with some of the unesterified bilirubin (I), whereas the indirect bilirubin consists solely of the unesterified bilirubin (I). See Scheme 1.

Although the mechanism of the azo-coupling reaction of bilirubin has long been established in vitro[20] using organic solvents, the reaction involving serum bilirubin is much more complicated because of the different forms of bilirubin present and the fact that bilirubin is bound to molecules of protein. Brodersen[26] studied the reaction in serum and found that it could be described as a series of three first order processes, the first two being related to the bilirubin ester, ie. the direct reaction, and the third, slowest process involving the unesterified bilirubin - the indirect reaction.

There are several variations on the basic diazo-reaction[3], some of which involve precipitation of the serum proteins by the addition of alcohol prior to the reaction with the arenediazonium salt. These methods have the disadvantage that some bilirubin co-precipitates with the protein, leading to underestimation of the total bilirubin. Some methods available avoid protein precipitation.

One such method is the widely employed technique of Malloy and Evelyn[27] in which an alcohol concentration below that which causes protein precipitation was used. The major drawback of this method is the fact that the high dilutions necessary reduce its accuracy at low bilirubin concentrations, and even at these high dilutions there is some problem with turbidity[28]. Other modifications of the Van den Bergh test avoid the need for using alcohol at all, and there are several substances which promote the azo-coupling of unesterified bilirubin in aqueous solution. These include caffeine, sodium benzoate, sodium acetate, urea, and mixtures of these[29]. Such substances are known as 'accelerators' or 'promoters' and, it is claimed, increase both the rate of reaction and colour intensity[29] in the indirect bilirubin case. The results presented in this thesis confirm at least the first of these claims. How these accelerators work remains a mystery since they appear to have little in common chemically, but it is suggested that they alter the bilirubin-protein complex in such a way as to make the bilirubin more open to attack by the arenediazonium ion.

Originally the Van den Bergh test for bilirubin was purely qualitative in nature and it was the need to put it on a quantitative basis which led to the many modifications, the most important of which have been described above, with the result that dozens of procedures employing various combinations of solvents, promoters, arenediazonium salts, pH and reading times are currently in use[3,29-34]. All of them claim to be quantitative but no two yield the same results, and there is a

particularly large discrepancy in the direct bilirubin readings.

Almost all of the diazo-methods used in bilirubin determinations employ diazotised sulphanilic acid (III) as the diazonium salt[32, 35], any differences between the various methods being in the solvents used, accelerators, pH, the order in which the reagents are added and the time allowed for colour development. The last of these is crucial since the same diazo-method can yield very different results if the readings are not taken consistently at the same time[36]. There are many conflicting views, supported by much experimental evidence, on which diazo-method is the most accurate[29, 30] and the choice of method remains largely a matter of personal preference.

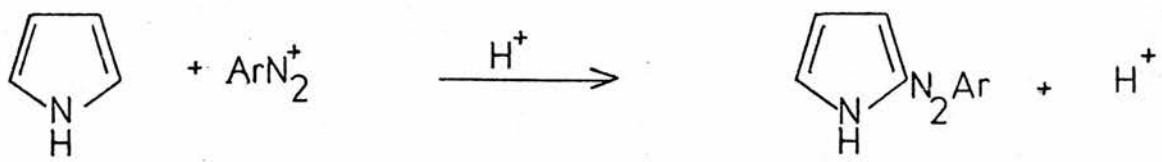
Apart from the Van den Bergh test the other most commonly used method of bilirubin determination is the direct spectrophotometric method[36, 37] since bilirubin in serum has an intense absorption maximum at about 460 nm. This method has the advantage that it is simpler to perform and requires smaller volumes of serum than the diazo-method, but unlike the diazo-method it is unspecific for bilirubin. This is because any haemoglobin and bilirubin decomposition derivatives present also absorb about 460 nm and are therefore included in direct spectrophotometry, resulting in higher values than by the diazo-method, which is specific for bilirubin. Consequently direct spectrophotometry is much less widely used than the diazo-method which still gives the least inaccurate results. But there is much room for improvement in the diazo-method, which has undergone no major alterations since Mather[29]

stated in 1960 that "Bilirubin determinations are perhaps the most notoriously unreliable of any in chemistry". It was one of the principal aims of the work described in this thesis to cast some more light on the nature of the bilirubin diazo-reaction in serum, with a view to suggesting some changes which might improve the Van den Bergh test.

Introduction to the work presented in this thesis:

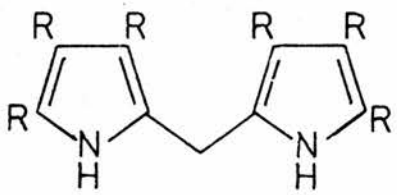
Before going on to investigate the reaction between bilirubin and arenediazonium ions, the ultimate aim of this work, kinetic studies were carried out on the reactions of pyrrole (VI) and several methylpyrroles with arenediazonium ions, and on the azo-coupling reactions of some dipyrrylmethanes (VII). Bilirubin (I) is a pyrrole tetramer and its reaction with arenediazonium ions in acid solution will be typical of the pyrrole nucleus, therefore it is useful to begin a study of this nature with a systematic mechanistic investigation into the corresponding reaction of the simple pyrroles. The dipyrrylmethanes contain that part of the bilirubin molecule which is the site of reaction in the Van den Bergh test - the two methylene bridged pyrrole rings - in a much simpler molecule, and serve as good model compounds for bilirubin. It was hoped, then, that by building up an overall picture of the azo-coupling reactions of pyrroles and dipyrrylmethanes more light would be shed on the Van den Bergh test. This aim was only partly fulfilled by the work presented here.

Pyrroles readily undergo electrophilic substitution, one example of which is their reaction with arenediazonium ions

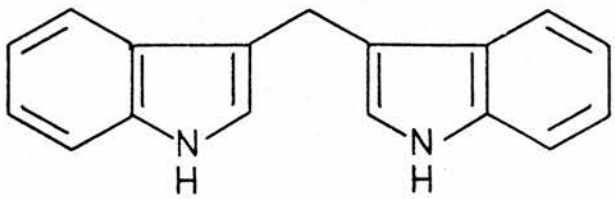


VI VIII

SCHEME 2



VII



IX

(Scheme 2), the product under acid conditions being the intensely yellow coloured mono-azo pyrrole (VIII). This reaction has been known for a long time[38], but until the work published by Treibs and Fritz[39] in 1958 and by Mitsumura et al[40] in 1972 no detailed information on the mechanism and reactivity of the reaction had been reported. A fuller mechanistic study of the reaction is described in the present work[41]. This investigation has yielded interesting information on structure-reactivity relationships in pyrroles, including the activating effect of methyl groups at different positions in the ring and on the nature of the reaction intermediate. Preparation of the mono-azopyrroles (VIII) in an analytically pure form has, until now, proved difficult[42] but a novel method for their preparation involving the use of a crown ether as a phase transfer catalyst is reported in this thesis.

Some new dipyrrylmethanes were synthesised in the course of this work in an effort to obtain the best possible model for bilirubin and a structure which would yield the most interesting mechanistic information. The dipyrrylmethanes have been known for a long time and those with simple alkyl substituents have been synthesised since the 1930's[43]. But, as yet, no investigation has been carried out into their reactions with arenediazonium ions, although a corresponding study of the azo-coupling reaction of di-indolylmethane (IX) was reported recently by Jackson et al[44]. A detailed mechanistic study of the reaction between dipyrrylmethanes and arenediazonium ions is reported in this work.

Bilirubin present in serum is bound to albumin, and since I have suggested that the 'accelerators' in the Van den Bergh test alter the bilirubin-albumin complex in some way, it is of interest to investigate the nature of the binding sites in the albumin molecule. A convenient way of doing this is by studying the binding capacity of albumin for dyes such as bromocresol green. This has been done by Rodkey et al[45,46], who have found that there are two classes of binding sites for bromocresol green, one giving much stronger binding than the other. The binding of bromocresol green by albumin is also used as a method of determining albumin concentration in serum[47-49]. The test is quick and easy to perform but is reported to have the disadvantage that it is non-specific for albumin since bromocresol green is bound by other serum proteins[47,48]. Bromocresol purple is claimed to be more specific for albumin[50], and in a recent paper Pinnell and Northam[51] recommend its use instead of bromocresol green. The binding of both of these dyes to albumin has been studied by stopped-flow spectrophotometry in this work, and the results are reported in Chapter 3.

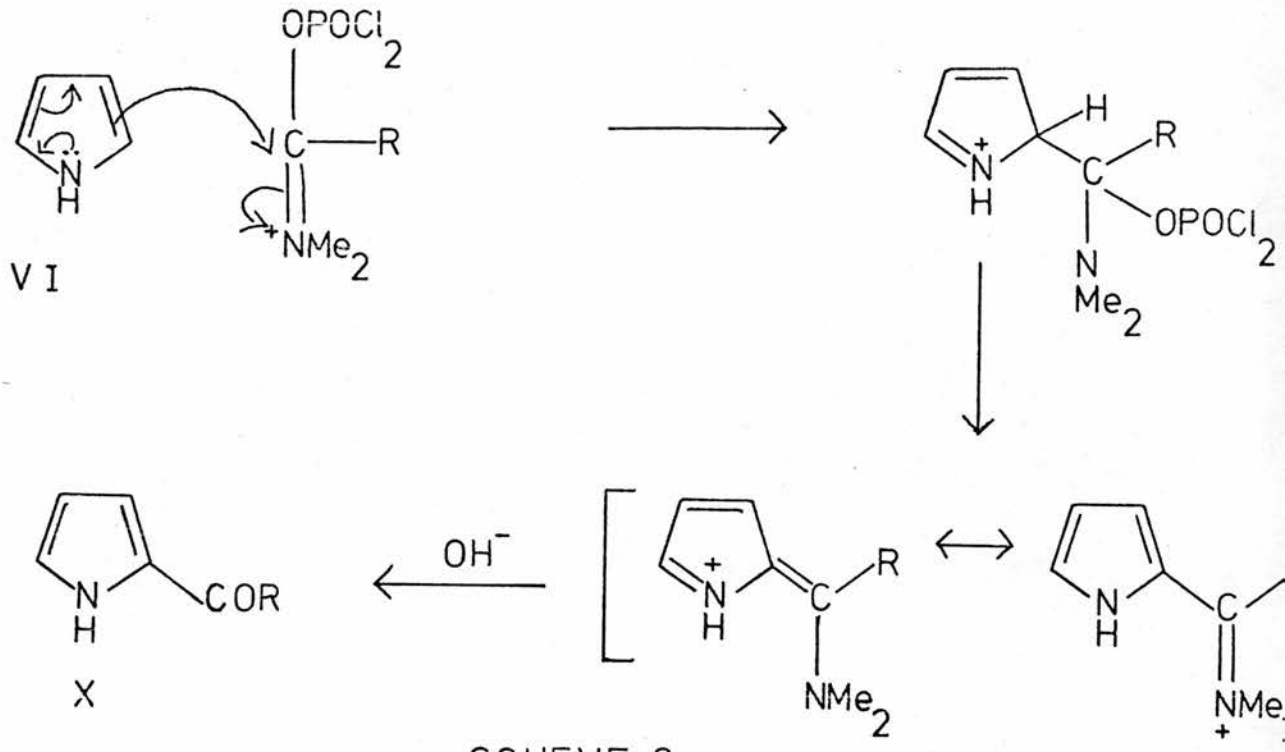
Finally, the azo-coupling reaction of bilirubin itself has been investigated. The effect of binding to albumin on the rate of the reaction is assessed and the influence of some of the more commonly used 'accelerators' described.

CHAPTER 1

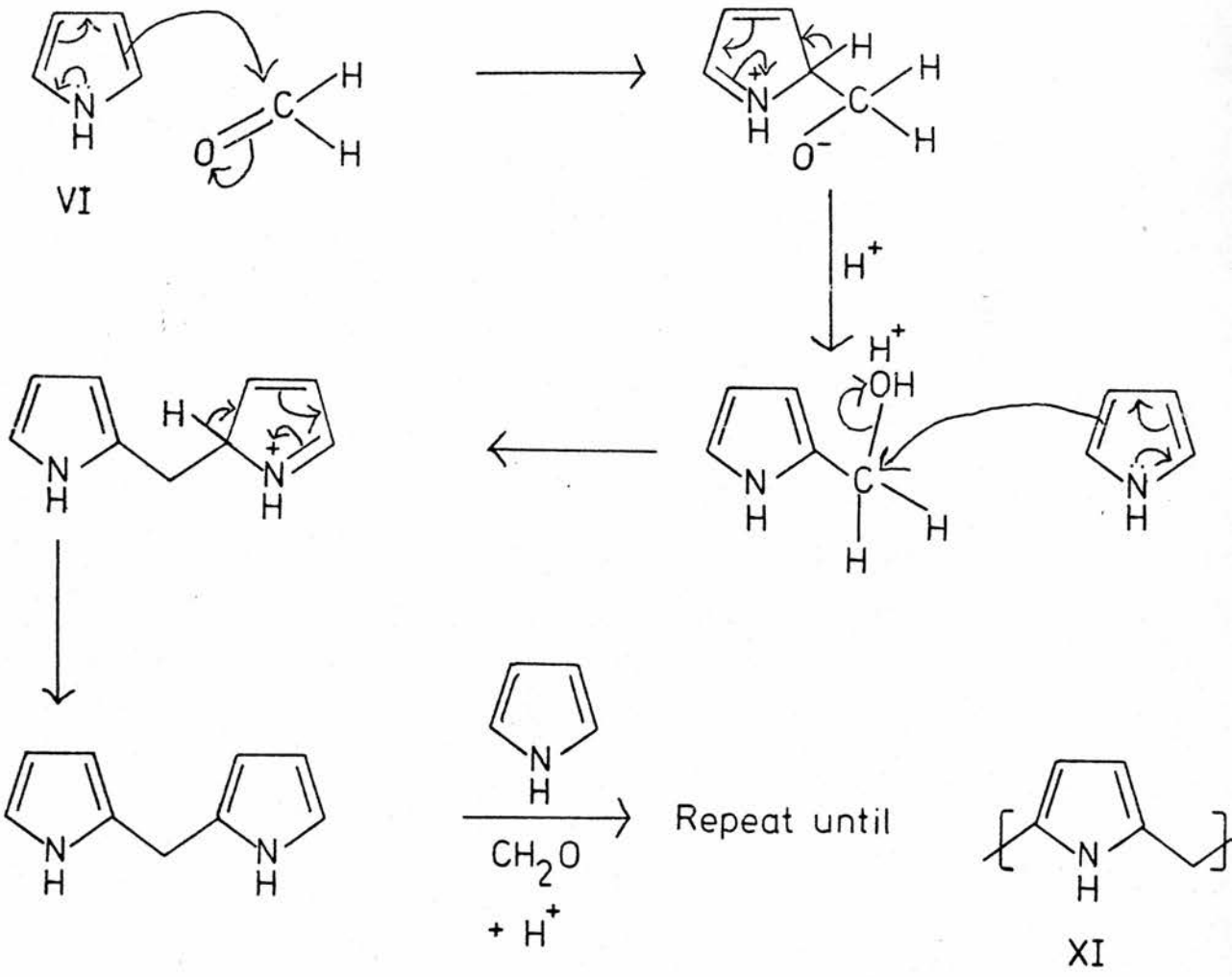
THE REACTION BETWEEN PYRROLES AND ARENEDIAZONIUM IONS

(a) IN AQUEOUS ACID SOLUTION

(b) IN NON-HYDROXYLITIC SOLVENT IN THE PRESENCE OF A CROWN ETHER



SCHEME 3



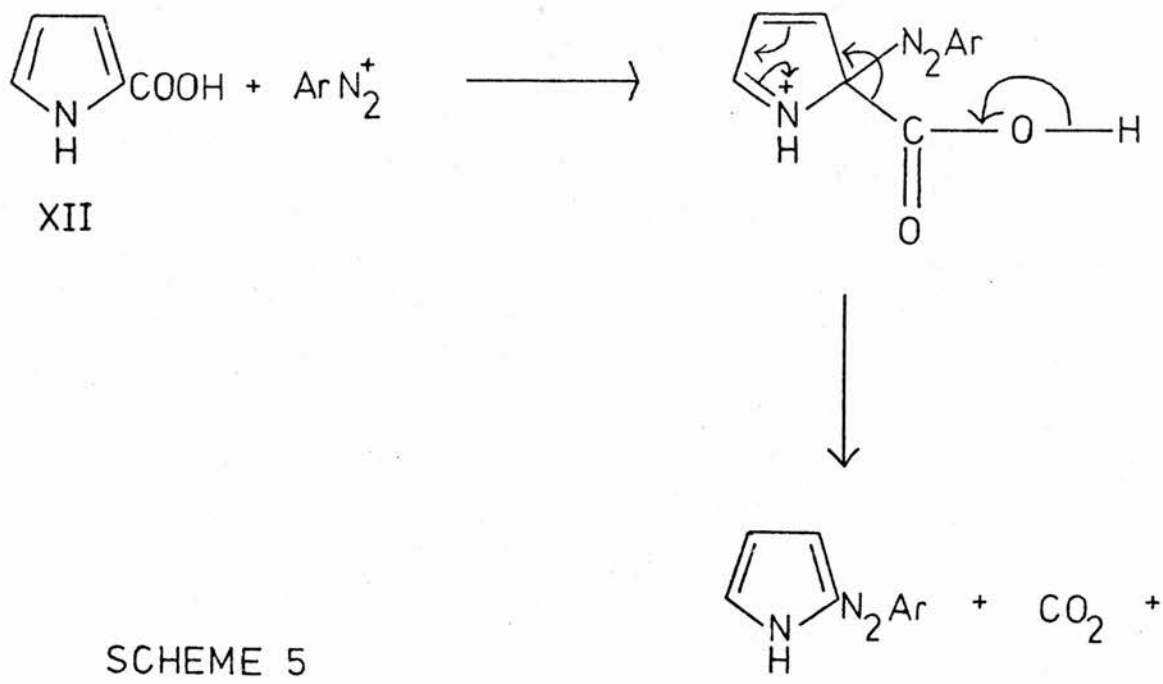
SCHEME 4

INTRODUCTION:

Pyrroles are highly reactive towards electrophiles, in which respect they have been compared to phenols[52]. This reactivity is modified by substituents on the pyrrole ring. In general, the α-positions are more reactive than the β-positions, but the difference is not so great that it cannot be outweighed by electron attracting substituents.

There are many examples which illustrate the reactivity of pyrroles towards electrophiles. Halogenation of the pyrrole ring occurs very readily and invariably leads to polysubstitution, with tetra-substitution being the only easily observed process[53-57]. Acylation[58] and alkylation[59] of pyrroles by electrophilic reagents also proceed readily (Scheme 3). Aromatic compounds which are sufficiently electrophilic can effect substitution of pyrroles[60]. Pyrrole reacts vigorously with formalin[61], giving condensed products of the type (XI) in a reaction which is analogous to the bakelite condensation (Scheme 4). Nitration of pyrroles takes place but since concentrated mineral acids cause polymerisation of pyrroles, ordinary nitration methods cannot be used unless deactivating substituents are present. The pyrrole carboxylic acids and their esters[56,62] and nitropyrroles[63] can be nitrated using concentrated nitric acid.

The ability of pyrroles to couple with arenediazonium cations has long been known and further illustrates the similar reactivities of pyrroles and phenols towards electrophiles[64]. In acid or neutral solution pyrrole forms the 2-mono-azo compound, but in alkali the 2, 5-bisazo derivative is the product of reaction.

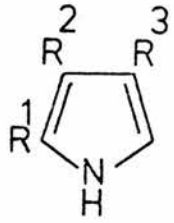


XII

SCHEME 5

VIII

XIII



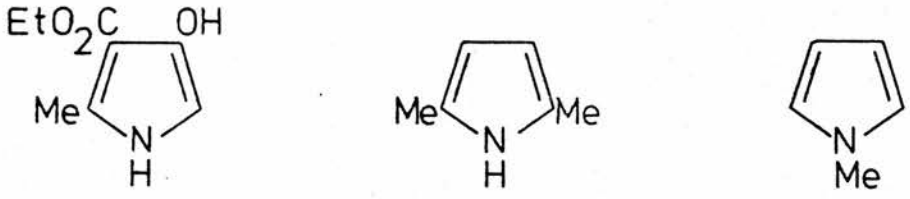
a $R^1 = R^3 = Me$, $R^2 = Et$

b $R^1 = H$, $R^2 = Et$, $R^3 = Me$

c $R^1 = R^2 = R^3 = Me$

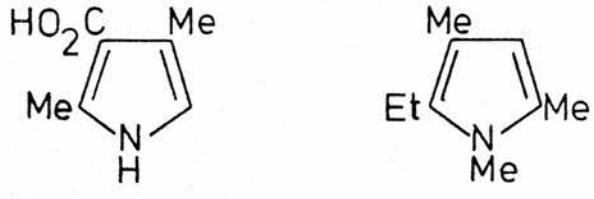
d $R^1 = Me$, $R^2 = R^3 = H$

XIV



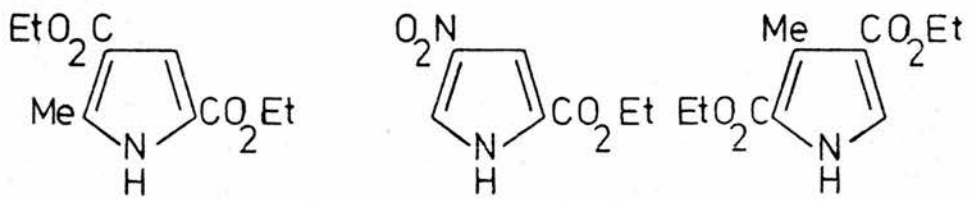
a b c

XV



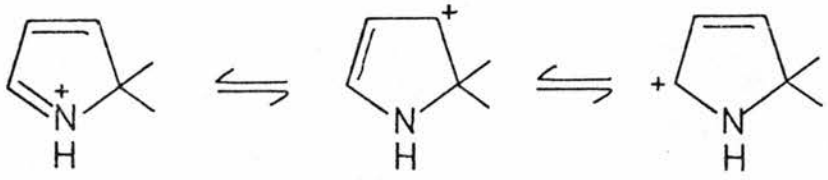
a b

XVI

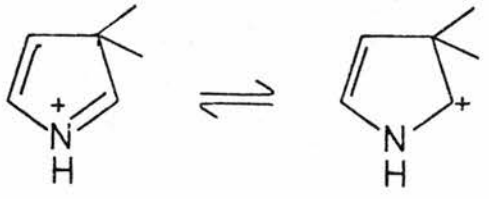


a b c

Only the reaction in acid solution will be considered in the present study. Azo-coupling occurs at the β-position when both α-positions are blocked, as is the case in 2,5-dimethylpyrrole. Pyrrole-2-carboxylic acid reacts with arenediazonium salts to form the same mono-azo derivative as pyrrole, carbon dioxide being eliminated (Scheme 5)[65,66]. 2,3,4,5-Tetramethylpyrrole is reported to react with arenediazonium salts very slowly, resulting in an olive green product which has not yet been identified, although Treibs and Derra-Scherer[67] claim that a 2-methyl group is eliminated in the process. This reaction was investigated further in the work described in this chapter. Treibs and Fritz[39] have carried out an extensive study of the coupling abilities of pyrroles, using three arenediazonium salts of widely differint reactivities. Their study was not quantitative, but they were able to divide the pyrroles studied into four classes (XIII, XIV, XV, XVI) in decreasing order of reactivity towards the arenediazonium ions. The first group (XIII) was several orders of magnitude more reactive than the slowest group (XVI) and this clearly shows how substituents modify the character of the pyrrole nucleus in a way which is expected in terms of their electronic properties; ie. the electron repelling methyl groups activate the pyrrole ring towards electrophilic attack while the electron attracting nitro- and ester substituents bring about deactivation. Treibs and Fritz also examined the replacement of groups other than hydrogen. Readily replaced groups include the carboxyl group[68,69], the sulphonic acid group[70] and the halogen atom[56,68,70]. These are all electron



XVII XVIII XIX



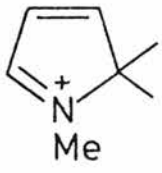
XX XXI

attracting groups and are, in consequence, able to produce a high electron density on the carbon atom to which they are attached. They can then be eliminated as a stable entity. The work reported in this chapter is a more quantitative study than that of Treibs and Fritz and only the highly activated methylpyrroles have been examined. The kinetics of their reactions with up to five different arenediazonium ions are reported.

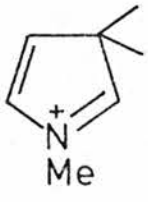
Because the azo-coupling reactions discussed in this chapter are carried out in acid solution it is of some importance to examine the behaviour of pyrroles in acid.

Protonation of pyrrole, like that of indole, occurs at the carbon atom rather than the nitrogen atom and protonation at the α-position is favoured over that at the β-position. Why this is so can be understood in terms of the canonical forms which can be drawn for α-protonated pyrrole and β-protonated pyrrole. In the case of α-protonation, three canonical forms can be drawn (XVII, XVIII, XIX), while for β-protonation only two forms (XX, XXI) may be drawn. Consequently, the α-protonated pyrrole has greater resonance stabilisation and will therefore be favoured. The same argument may be used to illustrate why α-substitution is preferred to β-substitution in electrophilic attack. The α-substituent Wheland intermediate will have more resonance stabilisation.

Pyrrole itself is a very weak base, having a pK_a value of -3.8[71], but methyl substituents markedly increase its basicity, while electron withdrawing substituents make it less



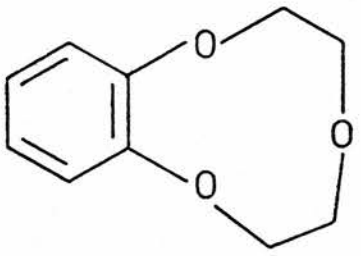
XXII



XXIII

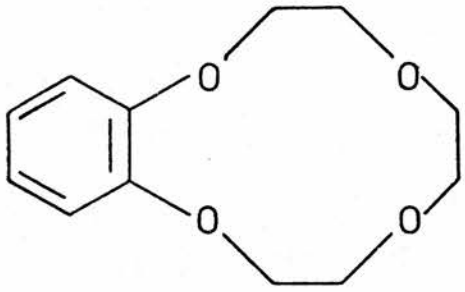
basic. The basicity constants of the methylpyrroles have been calculated by Chiang and Whipple[71] and have been found to vary with the position of methyl substitution in a semi-empirically predictable manner (Appendix 1). A 1-methyl group increases the basicity of both the α- and β-positions, with that of the β-position being more strongly enhanced due to the greater stabilisation of the double bonds by the methyl group in the β-protonated pyrrole (XXII, XXIII). A methyl substituent at the α-position increases the basicity of the adjacent β-position and of the opposite α-position, while a β-methyl substituent increases the basicity of the adjacent α-position. Because of the electron repelling capacity of the methyl group, the basicity of the carbon atom to which it is attached is always reduced. This explains why there is a greater amount of β-protonation (approximately 30%) in 2,5-dimethylpyrrole than in pyrrole itself[72]. Methyl substituents have similar activating and directive effects towards electrophilic substitution and the relevance of this with regard to the reaction of pyrroles with 4-dimethylaminobenzaldehyde (Ehrlich's aldehyde) has been discussed by Alexander and Butler[73]. The data obtained in the work reported in the present thesis allowed the calculation of the activating effect of a methyl group at various positions on the ring towards electrophilic attack by arenediazonium ions.

It has been widely reported that pyrroles polymerise in acid solution[74-78]. The polymerisation process typically involves electrophilic attack by a β-protonated pyrrole cation upon the conjugate base, and therefore requires the presence of protonated and unprotonated pyrrole in solution. The amount



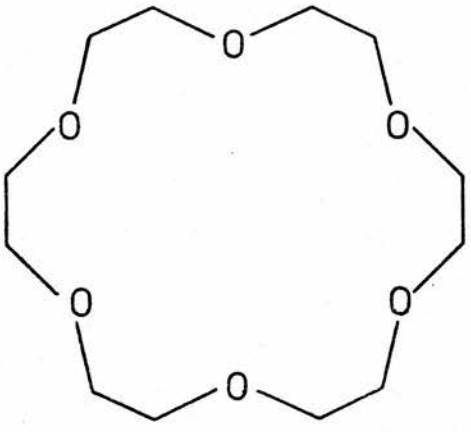
Monobenzo-9-crown-3

XXIV



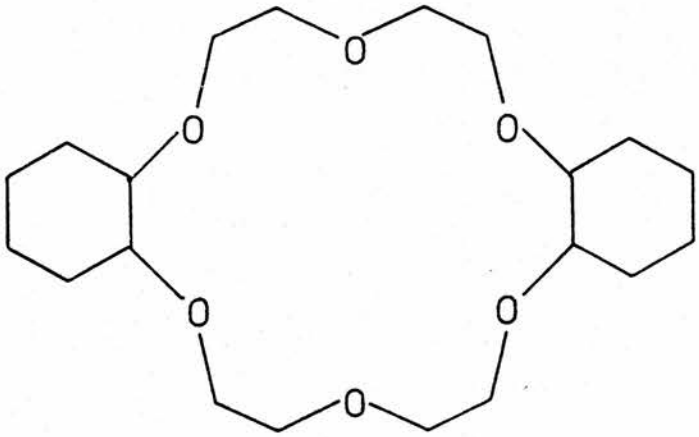
Monobenzo-12-crown-4

XXV



18-Crown-6

XXVI



Dicyclohexyl-18-crown-6

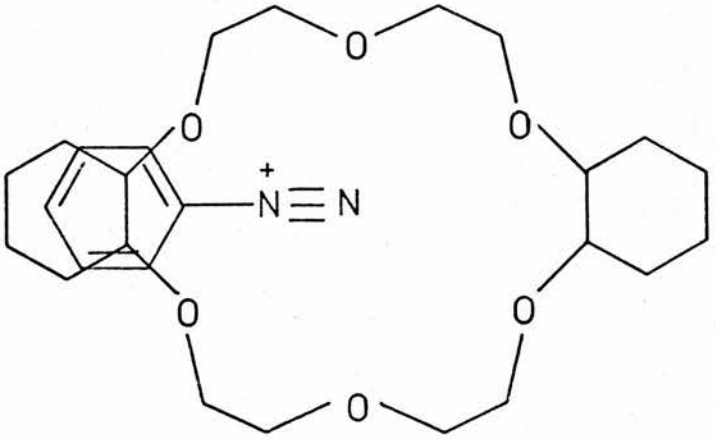
XXVII

of polymerisation taking place will also depend on the concentration of the pyrrole solution, which is very low in this study (about 10^{-4}M). Consequently, polymerisation during the kinetic run is not, in most cases, important here.

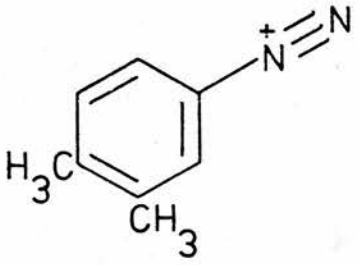
The use of crown ethers as solubilising agents has by now become well established. It was in 1967 that Pedersen[79] recognised the ability of these compounds to complex a variety of cationic substrates. Although no longer the only class of 'crowns', since, for example, Lehn and his co-workers have developed the cryptates[80], the first and still the most important class of crowns are the macrocyclic polyethers. These are molecules of the type $(-CH_2-CH_2-O-)_n$, where n is greater than or equal to 2 (XXIV-XXVII). They are relatively straightforward to prepare, and a comprehensive review of the synthetic routes to them has been made by Gokel and Durst[81].

The complexing ability of the crown ethers appears to be a Lewis acid-base phenomenon in which the basic hetero-atoms surround the complexed cation, the electrostatic interaction of each hetero-atom with the appropriately sized cation being approximately equal[82]. The crystal structures of several crown ether complexes have been determined[81].

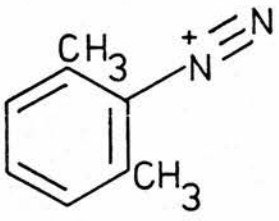
More recently, nmr has been used to examine the complexation of various substrates[83,84], including a detailed study of the complexation of arenediazonium and benzoyl cations with dicyclohexyl-18-crown-6 (XXVII) in non-polar media by Gokel and Cram et al[85,86]. The complex between the arenediazonium ion and the crown ether, although it has not been



XXVIII



XXIX



XXX

isolated, has been shown to be an insertion complex of the type (XXVIII)[86]. The evidence quoted to support this was the fact that p-tolyldiazonium tetrafluoroborate (-N≡N- diameter = 2.4 Å) was complexed by crown ethers with rings of diameter 2.6, 2.8 and 3.75 Å, but not by a crown ether of diameter 2.2 Å. Secondly, 3,4-dimethylbenzenediazonium ion (XXIX) was complexed by a crown ether, but not the sterically hindered 2,6-dimethylbenzenediazonium ion (XXX). Many other types of crown ether complex have been developed and studied. These have been described by Gokel and Durst[81], but it is the arenediazonium ion complex which is of most relevance to the work discussed in this thesis. Gokel and Cram et al[86] have also examined the kinetics of crown ether complex formation with various 'guest' compounds and have calculated guest/host ratios for various arenediazonium ions complexed by dicyclohexyl-18-crown-6. Further studies of this nature are presented in this chapter.

Sam and Simmons[87] first demonstrated the use of dicyclohexyl-18-crown-6 as a phase transfer catalyst by its ability to solubilise potassium permanganate in benzene and then carry out oxidation reactions in this solvent (eg. oxidation of stilbene). Since then many further uses have been found for crown ethers as phase transfer catalysts, the most important of which have been described by Gokel and Durst[81].

In some cases stoichiometric quantities of the crown ether must be used, but in others only catalytic amounts are necessary. As well as promoting liquid-liquid phase transfer catalysis, the crown ethers are also very effective solid-liquid phase transfer

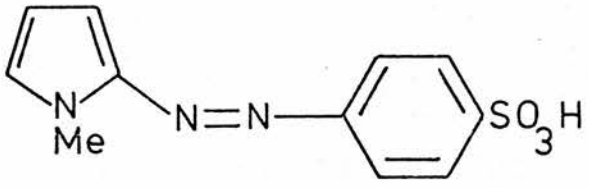
catalysts, and it is as such they are used in the present study. The success of crown ethers as solid-liquid phase transfer catalysts has been attributed to two factors. Firstly, because the crown ether is a two-dimensional structure with many polar sites, it may, when it approaches the crystal lattice, assume the geometry of the complex. Therefore the movement of the 'guest' cation from the lattice to the ligand is small. Secondly, the complexed product of the reaction may be deposited once formed, and the crown ether freed to complex more of the solid reactant.

In the course of this work a large number of methylpyrroles were prepared. All were prepared by literature syntheses noted in the experimental section of this chapter. One method which was of general use was that of Hinman and Theodropulos[88], in which pyrroles containing carboxyl, carbethoxy or carboxaldehyde groups are reduced in good yield to the corresponding methylpyrroles using a large excess of lithium aluminium hydride. The pyrrole starting material was prepared by a Knorr- or Hantzch-type synthesis.

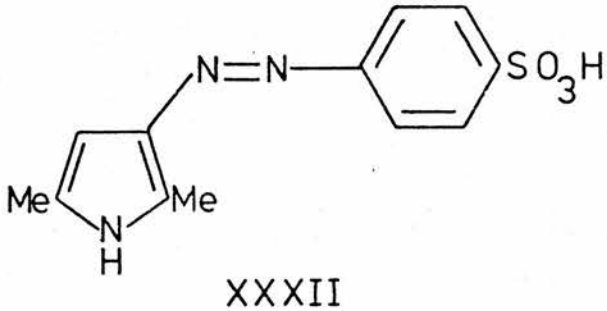
1(a):- REACTION BETWEEN PYRROLES AND ARENEDIAZONIUM IONS IN AQUEOUS ACID SOLUTION

Results and Discussion:

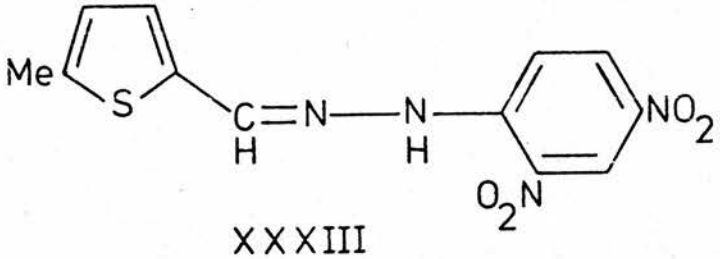
The reaction between pyrroles and arenediazonium ions gives highly coloured products, which under neutral or acid conditions are the mono-azo derivatives[89]. This has been confirmed in the reaction between diazotised sulphanilic acid



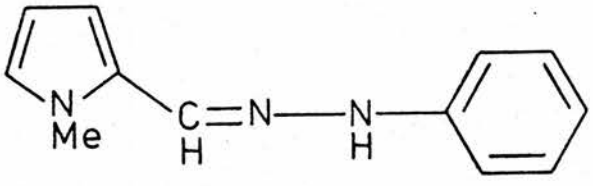
XXXI



XXXII



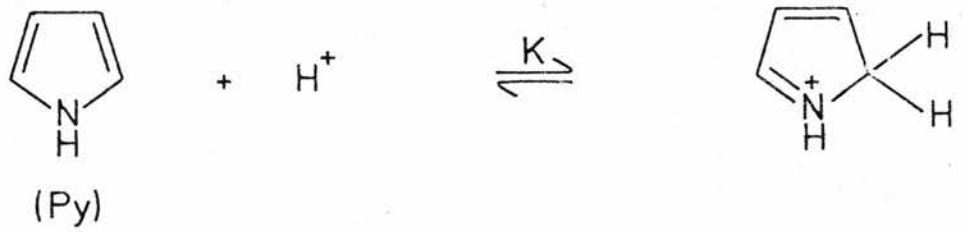
XXXIII

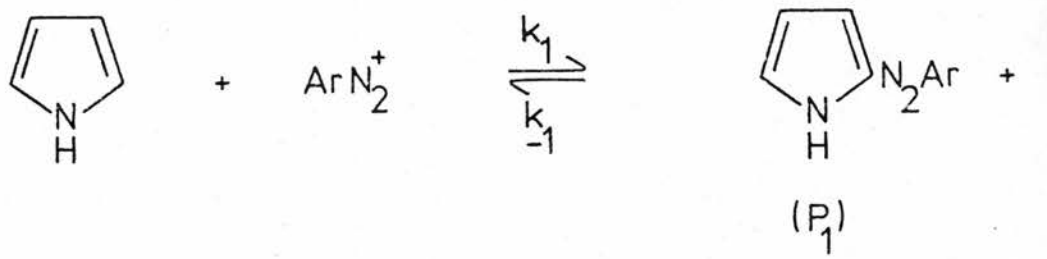


XXXIV

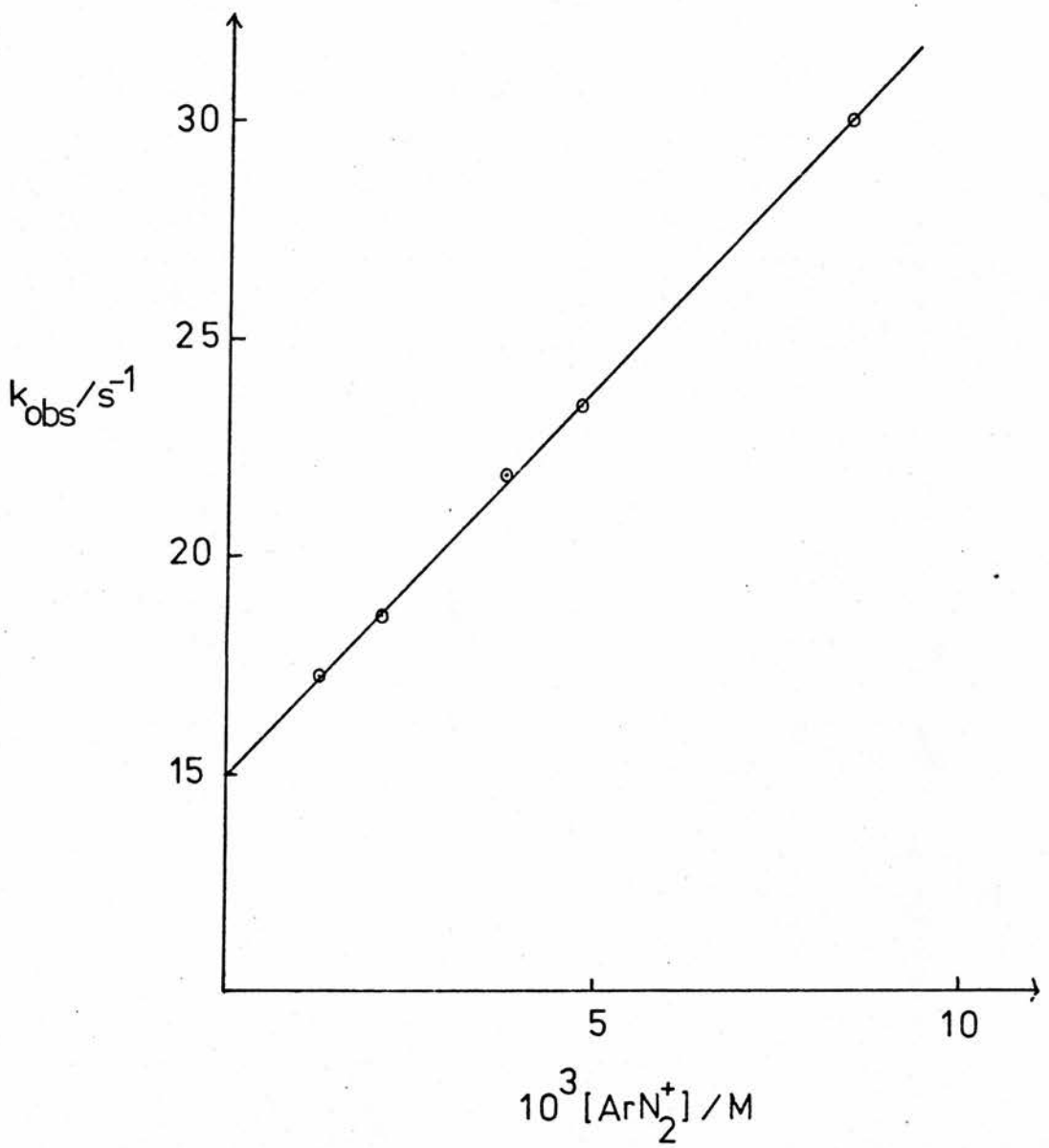
and 1-methyl- and 2,5-dimethylpyrrole, where the products were identified as (XXXI) and (XXXII). It was found impossible to obtain analytically pure samples of these compounds by the method of Kreutzberger and Kalter[42], who reported a similar situation in the preparation of 4-(2,5-diphenylpyrrol-3-ylazo)-benzenesulphonic acid. However, the spectral evidence obtained was good enough to characterise (XXXI) and (XXXII), which are the expected products of reaction. It will be described later in this chapter how analytically pure samples of the mono-azo-pyrroles can be readily obtained by the use of a crown ether as a phase transfer catalyst.

Tedder et al[90] have reported that the reaction between 2,5-dimethylthiophen and 2,4-dinitrobenzenediazonium tetrafluoroborate results in some coupling through the methyl group to give (XXXIII). This compound was readily identified by the N-H stretching band in the ir spectrum. This band was missing in the spectrum of (XXXII) and it can be concluded that, in the case of 2,5-dimethylpyrrole, there is no reaction through the methyl group. Such a reaction may be peculiar to the very strong electrophile used by Tedder et al. An analogous reaction product (XXXIV) was prepared from 1-methylpyrrole-2-carboxaldehyde and was found to have the N-H stretching band (at 3300 cm^{-1}) absent in (XXXI) and (XXXII). As already noted in the introduction to this chapter, the α-positions of the pyrrole ring are more susceptible to electrophilic attack than the β-positions and it is assumed here that reaction leads to the 2(5)-mono-azo compound when possible. When both α-positions are blocked by





SCHEME 6



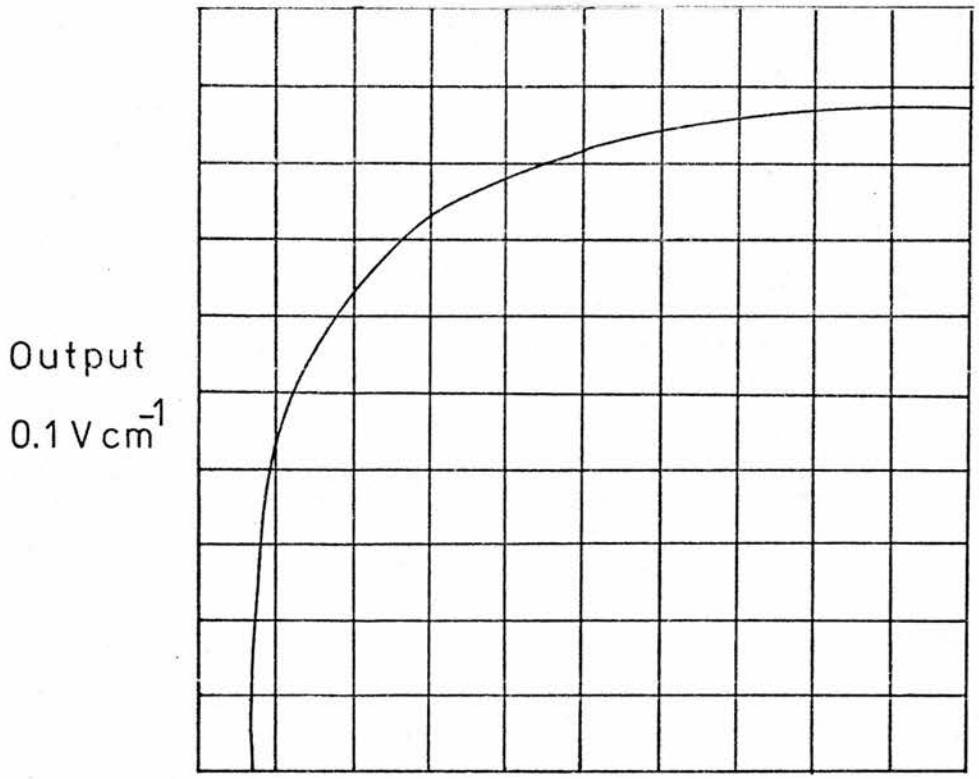
Variation of k_{obs} with concentration of diazotised sulphanilic acid in diazo-coupling with 1,2-dimethylpyrrole at 25°C.

FIGURE 1

electron repelling substituents, such as the methyl group, then attack occurs at one of the β -positions.

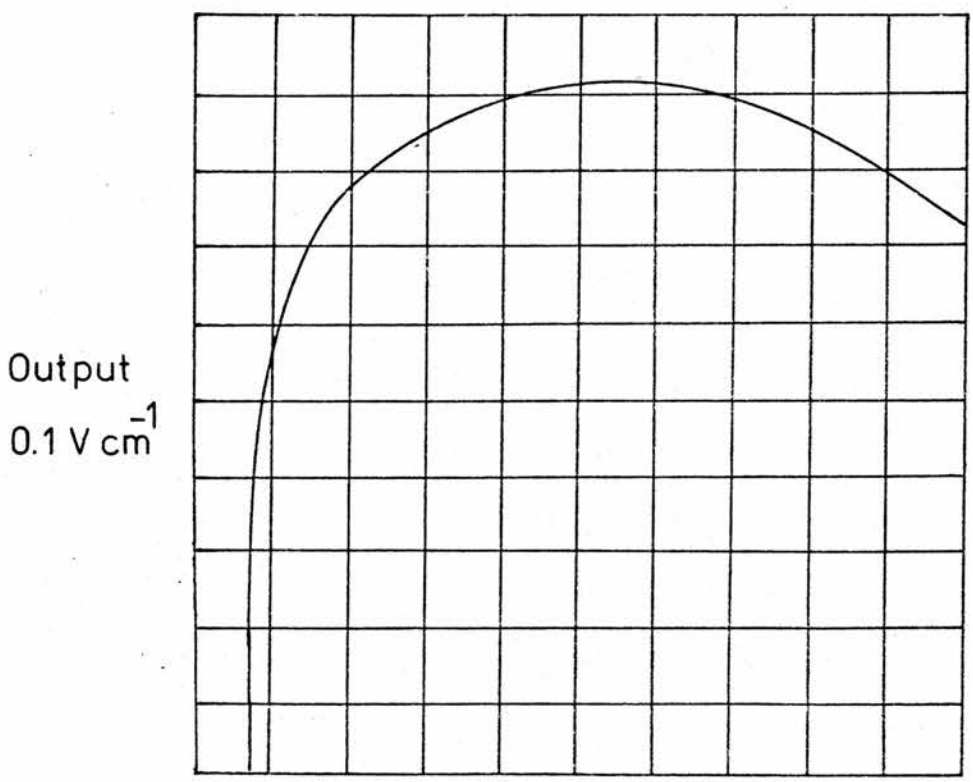
Pyrroles are basic and, under the acid conditions used for diazo-coupling, are partially protonated, but it is reasonable to assume that reaction occurs via the neutral species. This is what was found by Challis and Rzepa[91] in their work on diazo-coupling to indoles. Kinetic evidence, to be described later, shows that diazo-coupling is an equilibrium process and the mechanism shown in Scheme 6 is proposed. Since the products of diazo-coupling reactions are highly coloured, the kinetics of reaction were readily studied by stopped-flow spectrophotometry. In every case the reaction was found to be first order in appearance of product and, with an excess of arenediazonium ion, the rate constant was independent of the initial pyrrole concentration.

The reaction scheme is somewhat unusual in that one of the products of reaction, a hydrogen ion, is already present in a large excess since acid conditions were used for the preparation of the diazonium compounds. The mathematics of the kinetics of the reaction have been analysed in detail in Appendix 2 and from equation (11) it can be seen that the experimentally determined rate constant (k_{obs}) is $k_1[ArN_2^+]/(K[H^+]+1) + k_{-1}[H^+]$. A typical plot of k_{obs} against $[ArN_2^+]$ is shown in Figure 1. That there is an intercept (equal to $k_{-1}[H^+]$) proves that diazo-coupling is an equilibrium reaction. The values of K, the basicity constants for the pyrroles, were obtained from the work of Chiang and Whipple[71]. As the acid concentration was, in most cases, low, the term $[H^+]$ was taken as the molarity of the acid.



Oscilloscope trace of reaction between
1-methylpyrrole and diazotised sulphanilic acid at 25°C

FIGURE 2



Oscilloscope trace of reaction between
3,4-dimethylpyrrole and diazotised sulphanilic acid at 25°C

FIGURE 3

In other cases, where acid concentration was outside the ideal range, the h_o value was used[92]. It is found that the protonation of pyrrole and the methylpyrroles follows H_0''' and H_I acidity functions, but H_0, H_0''' and H_I are similar in the range considered and the semi-logarithmic plot of the indicator ratio $I=[BH^+]/[B]$ against the Hammett acidity function H_0 is also linear[71]. Therefore the h_o value is a reasonable and simple choice of acidity scale.

The kinetics of the reaction of a number of pyrroles with five arenediazonium ions were examined, and the results are given in Table 1. The significance of the values of k_1 and k_{-1} listed will be discussed later. It should be noted at this point that while many of the pyrroles studied gave stable azo-compounds and hence oscilloscope traces of the type shown in Figure 2, a few of the more highly activated pyrroles formed unstable mono-azo-compounds, the yellow colour fading quite rapidly to green or blue, giving oscilloscope traces of the type shown in Figure 3, where absorbance at 450 nm increases in the normal way as the mono-azo-pyrrole is formed and then begins to decrease as it underwent further reaction. This was particularly noticeable in the case of 3,4-dimethylpyrrole, where the fading reaction was so rapid that it could be followed on the stopped-flow instrument. This particular case will be discussed later.

Confirmation of the proposed reaction scheme comes from a study of the acid dependence of k_{obs}. The results for three pyrroles are shown in Table 2. For the reactions of (b), (d) and (i) k_{-1} is zero so that equation (11) simplifies to

$$k_1 [ArN_2^+]/(K[H^+]+1),$$

Table 1

Data for the reactions of various pyrroles with $XC_6H_4N_2^+$ ions at

	X	p-MeO	
Pyrrole	K/l mol^{-1}	k_1	k_{-1}
		l mol^{-1}s^{-1}	
(a) Parent	0.00016	2.8	0.33
(b) 1-Me	0.0013	10.9	2.2
(c) 2,5-Me_2	0.195	9.8	0
(d) 1,2,5-Me_3	0.794	11.8	2.1
(e) α-Me	0.617	129	16
(f) 1,2-Me_2	3.16	374	9.2
(g) 3,4-Me_2	5.00	456	25
(h) 2,3-Me_2	31.5	142	11
(i) 2,3,5-Me_3	100	1.20	0.0038
(j) 2,4-Me_2	398	6800	62
(k) 3-Et-2,4-Me_2	3160	30500	7.0

$[Py]_o = 5 \times 10^{-5}$ M

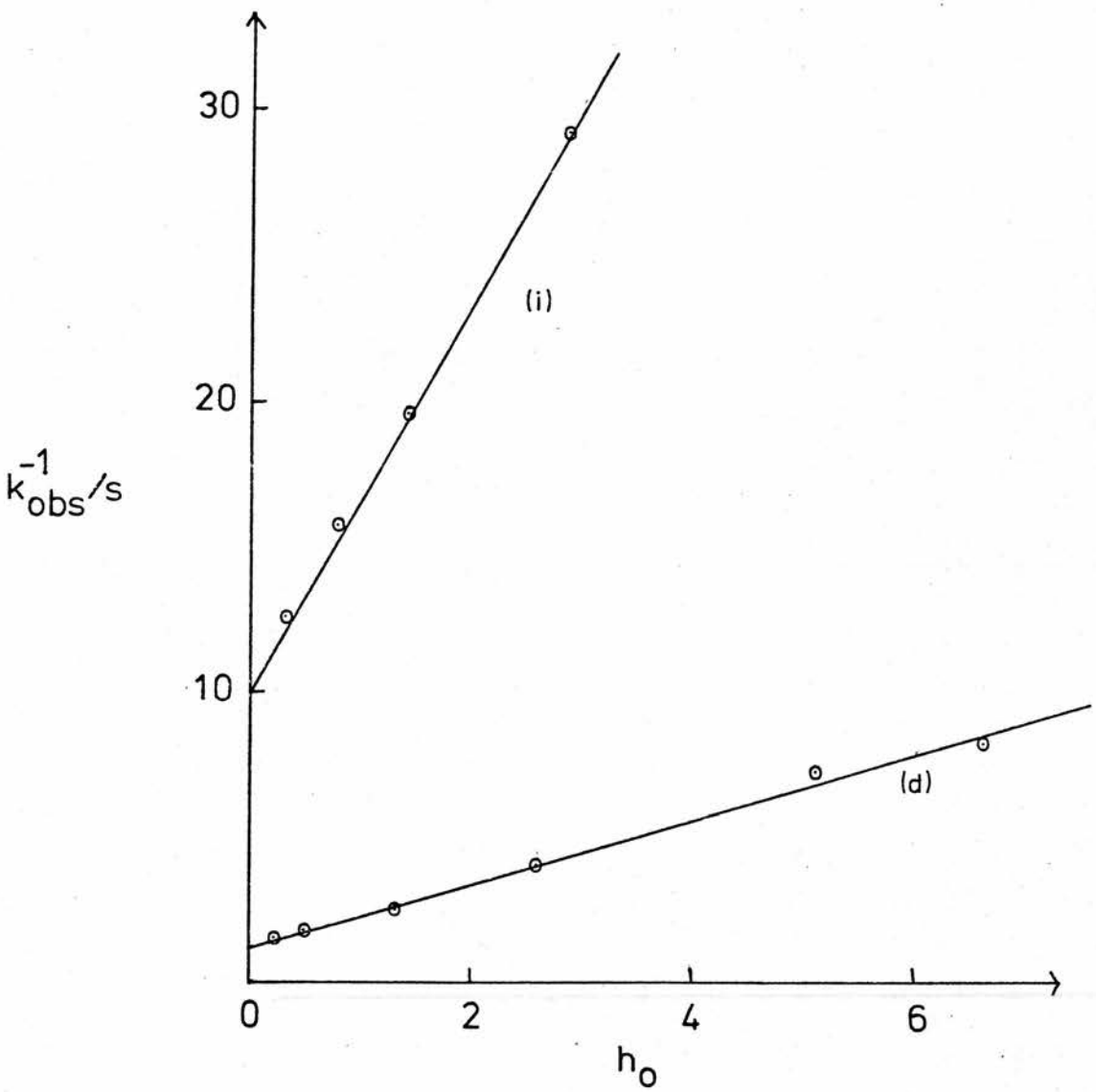
$[XC_6H_4N_2^+] = 0.020$ M

[HCl] = 0.05 M, except for the 4-nitro-compound, where

[HCl] = 0.50 M.

25°C in acid solution

H		p-SO_2OH		p-CN		p-NO_2	
k_1	k_{-1}	k_1	k_{-1}	k_1	k_{-1}	k_1	k_{-1}
$l\ mol^{-1}\ s^{-1}$		$l\ mol^{-1}\ s^{-1}$		$l\ mol^{-1}\ s^{-1}$		$l\ mol^{-1}\ s^{-1}$	
6.8	8.4	23.8	0.60	100	23	220	14
25.2	0	68.0	0	287	19	1000	23
32.5	0	758	0	-	-	-	-
24.4	3.2	744	0	-	-	1600	8.7
280	154	587	245	3640	268	-	-
905	162	1490	310	-	-	-	-
869	400	5270	284	-	-	-	-
532	398	942	120	7140	520	-	-
1.80	1.1	54	1.54	-	-	1300	0.11
13400	48	26500	50	63200	69	-	-
177000	13	-	-	-	-	-	-



Acidity dependence for the diazo-coupling of (d) and (i) with diazotised sulphanilic acid at 25°C.

FIGURE 4

Table 2

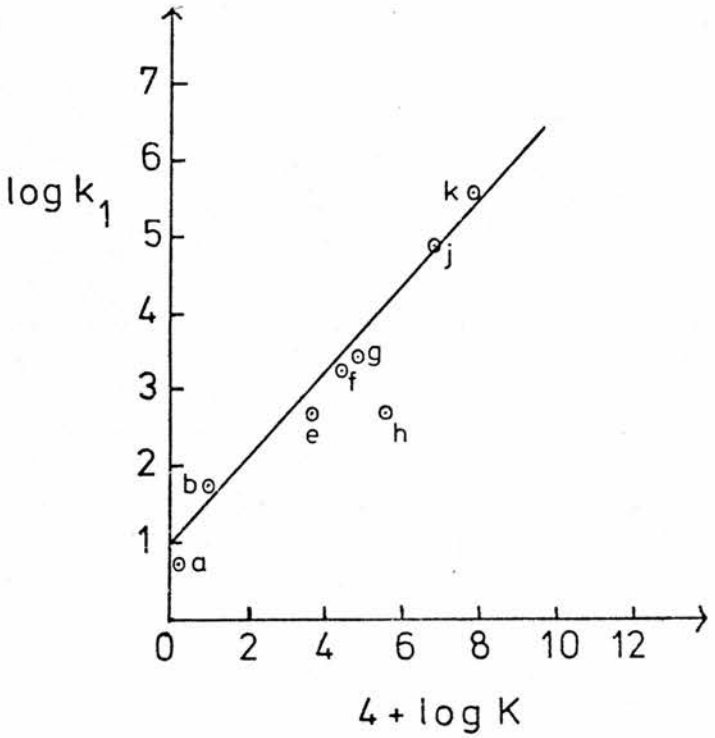
Variation of k_{obs} with acid concentration for the reaction of benzendiazonium-4-sulphonic acid ions with various pyrroles at 25°C

	k_{obs} (s^{-1})		
[HCl]/M	(b) 1-Me	(d) 1,2,5-Me_3	(i) 2,3,5-Me_3
0.025	-	5.7	-
0.050	0.49	5.8	-
0.10	0.68	-	-
0.25	0.79	5.3	0.086
0.50	0.87	4.4	0.067
1.00	0.94	3.1	0.051
1.50	0.79	1.6	0.035
2.00	0.53	1.4	0.033
2.50	0.39	1.0	0.020

$[HO_3SC_6H_4N_2^+] = 0.020$ M

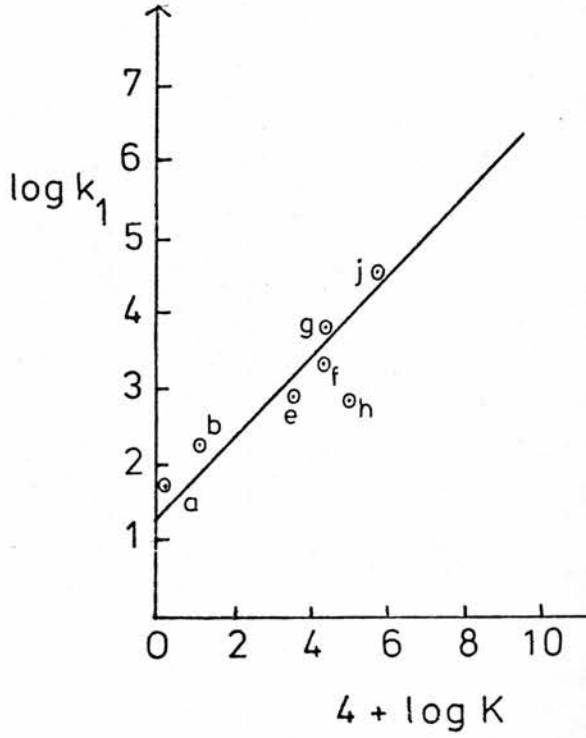
$[Py]_o = 5 \times 10^{-5}$ M

If $K[H^+] \ll 1$, as is the case for (b), then k_{obs} should be independent of acid concentration. The figures in Table 2, column 2, show that this is approximately correct. The small variation of k_{obs} is due to the non-validity of this approximation at high acid concentrations. Complex formation[93] may also complicate the situation. With (d) and (i) the approximation is not valid, but, if the scheme is correct, plots of $1/k_{obs}$ against h_o should be linear. This is seen to be the case from Figure 4 and the proposed reaction scheme can therefore be accepted with confidence.



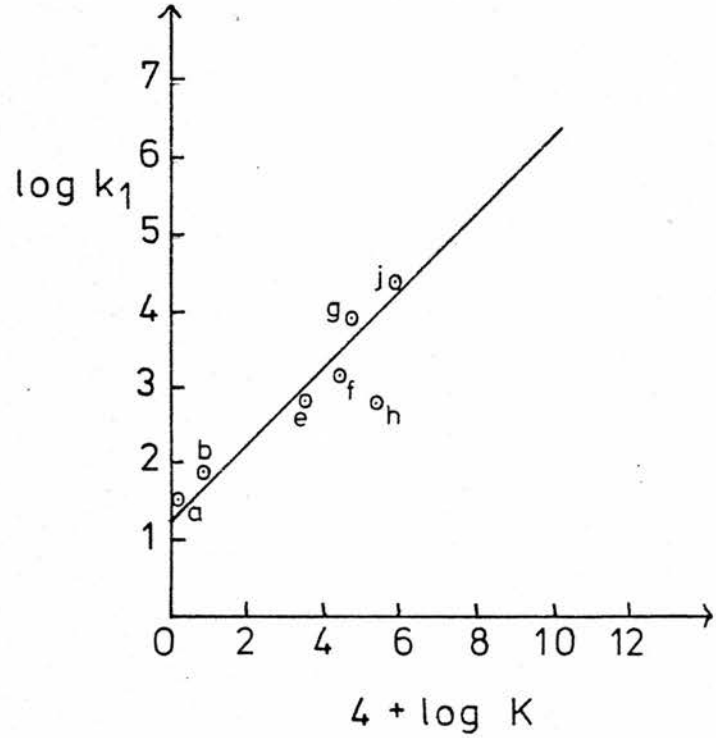
Plot of log k_1 against pyrrole basicity for reaction of various pyrroles with p-methoxybenzenediazonium chloride.

FIGURE 5



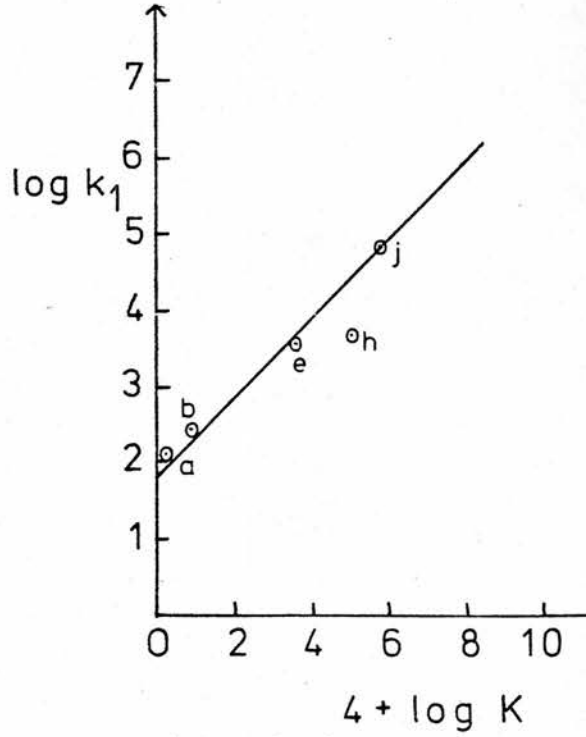
Plot of log k_1 against pyrr basicity for reaction of v pyrroles with benzenedia chloride.

FIGURE 6



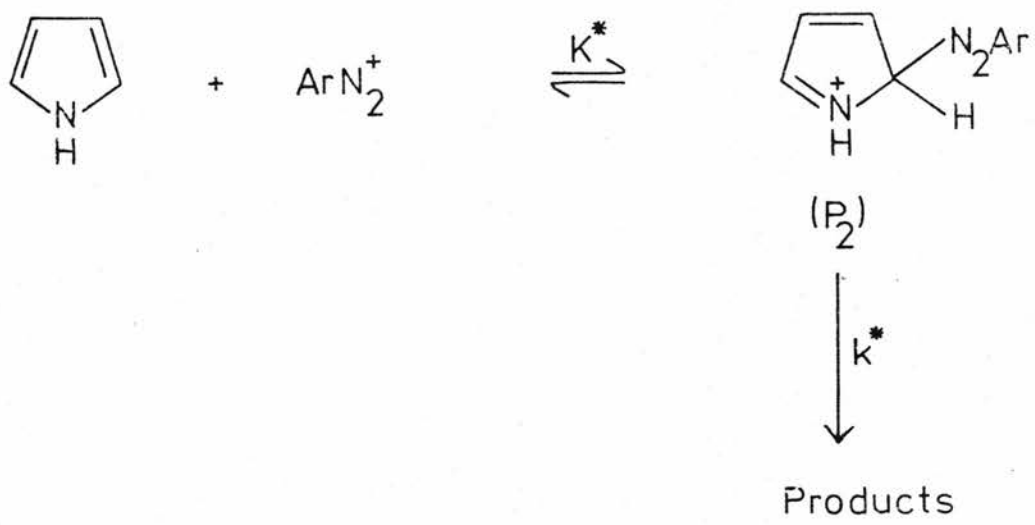
Plot of log k_1 against pyrrole basicity for reaction of diazotised sulphanilic acid with various pyrroles.

FIGURE 7



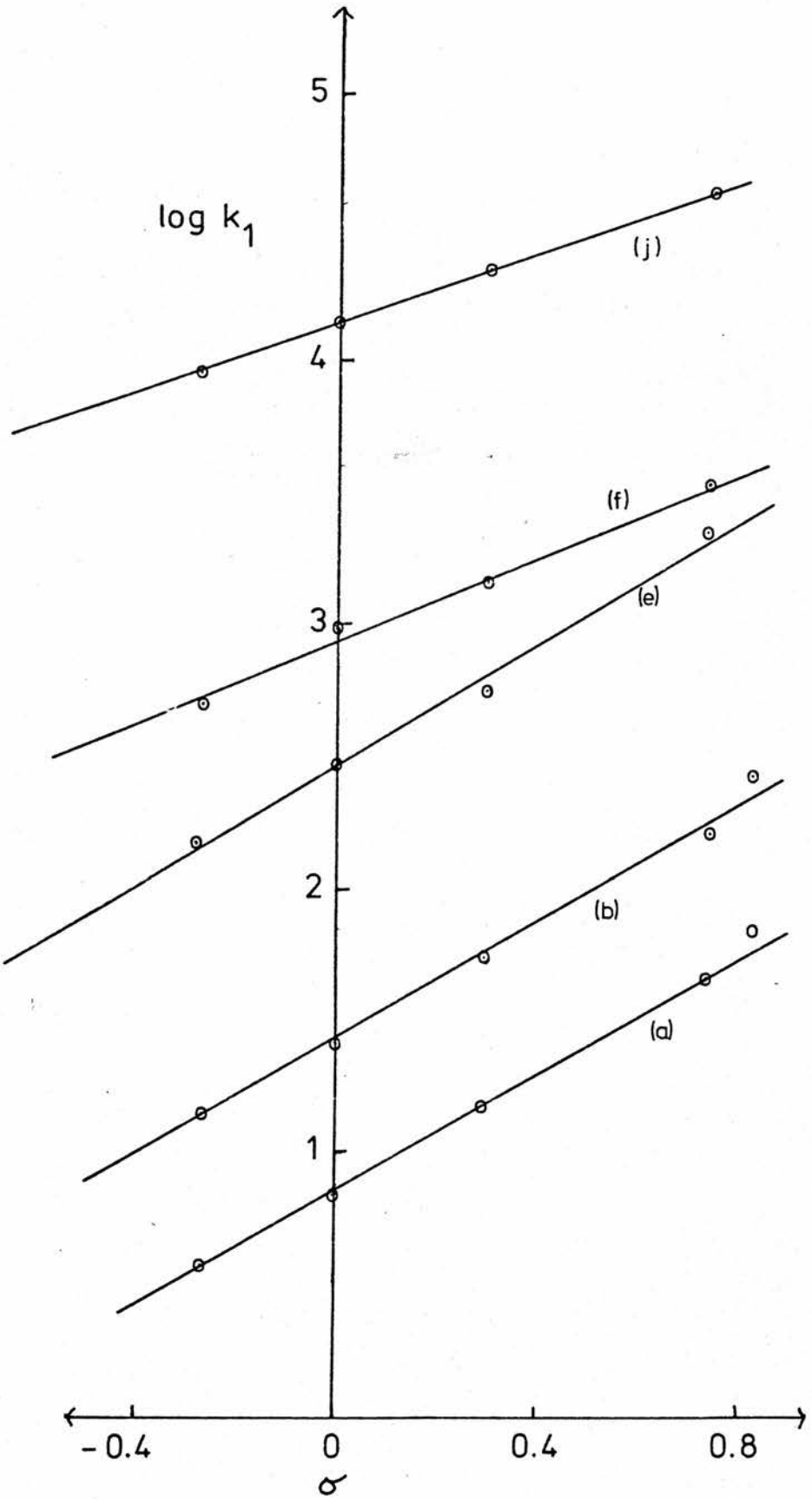
Plot of log k_1 against pyrro basicity for reaction of v pyrroles with p-cyanoben diazonium chloride.

FIGURE 8



SCHEME 7

To return to the figures in Table 1, there is no easily perceived correlation between k_{-1} and K; also the value of the ratio k_1/k_{-1} appears to vary in a random manner. Equally there is no obvious correlation between k_1/k_{-1} and the electrophilicity of the arenediazonium ion. However, when substitution occurs at an α-position, there is an excellent linear correlation between k_1 and the basicity of the pyrrole (K) covering, in some cases, a range of values of K of 10^7. The only exception to this is 2,3-dimethylpyrrole. Plots of $\log_{10} k_1$ vs. $\log_{10} K$ are shown for the reactions of four different arenediazonium ions with various pyrroles in Figures 5, 6, 7 and 8. Values of k_1 for attack at the β-position fall below this line in each case and may lie on a different curve, but there are too few to be certain of this. The slopes of the curves for attack at the 2(5)-position are all ca. 0.5 . A simple deduction can be made from this Brønsted plot: protonated pyrrole is a good model for the Wheland intermediate formed in the diazo-coupling reaction and this intermediate must be a relatively stable species on the reaction pathway. This matter has been discussed in detail by Zollinger[94], who supports the S_E2 mechanism with a steady state intermediate for reactions of this type. Details of this part of the reaction are given in Scheme 7. The rate-determining step is proton loss[95] and so the rate of the overall reaction is $k^*[P_2]$. If, as seems most probable, the equilibrium concentration of P_2 parallels the basicity of the pyrrole and as the overall reaction does this also (the Brønsted plot), k^* must be constant and essentially independent of the pyrrole. This matter will be discussed again later.



Hammett plots for the diazo-coupling of various pyrroles with $XC_6H_4N_2^+$ ions where X is p-MeO, H, p-SO_3H, p-CN, p-NO_2

FIGURE 9

From the accumulated data it is possible to make a series of Hammett plots for pyrroles of different reactivities (Figure 9). Only those reactions involving attack at an α-position have been considered. The ρ -values vary over the range 1.2-1.6. Mitsumura et al[40] report larger ρ -values (ca. 4.3) for the same reaction, but they used only deactivating substituents on the arenediazonium ion. Different ρ -values for electron attracting and donating substituents have been reported previously[96]. For a number of diazo-coupling reactions Sterba and Valter[97] report ρ -values ranging from 2.62 to 4.27, so the value reported in the present study is not an unreasonable one.

A value of σ for the 4-sulphonic acid group is not available. A value was therefore measured from the lowest curve (that for pyrrole) as 0.28 and this was used to obtain points for the remaining four curves. Because of the manner in which these points were obtained, they should be treated with some caution, but they do appear to fit the other curves quite well. Hopkinson and Wyatt[98] obtained a value of 0.56 for the 3-sulphonic acid group.

The significant observation from Figure 9 is that, although the substrates vary in reactivity by a factor of 10^4, the value of ρ remains essentially constant. This appears to contradict the selectivity principle, which asserts that a reactive substrate should be less discriminating (exhibit a smaller ρ -value) than a less reactive substrate. Olah et al[99] have produced extensive experimental evidence to support the selectivity principle, although this evidence has been criticised by Johnson and Schofield[100].

Arnett and Reich[101] have plotted linear free energy relationships for the nucleophilic attack of a series of 3- and 4-substituted pyridines on four alkylating agents of widely varied reactivity and found no variation in selectivity among the nucleophiles on changing the reactivity of the alkylating agent. The evidence produced in the present study suggests that if a stable reaction intermediate is formed the selectivity principle does not apply, because the rate determining step is breakdown of that intermediate.

It has been shown already that k^* is a constant and so the rate of the overall reaction (ie. formation of P_1) depends upon the equilibrium concentration of P_2. Although 2,4-dimethylpyrrole may be less discriminating in its reactions with substituted arenediazonium ions than pyrrole, this will not be reflected directly on the equilibrium concentration of P_2. The Brønsted plots show that the concentration of P_2 for each arenediazonium ion depends on only the basicity of the pyrrole. Clearly the rate of the back reaction (ie. dissociation of P_2) is such that the discriminatory nature of the forward reaction is balanced out. Although this does not necessarily lead to invariant ρ-values, it is clear why the selectivity principle does not apply to reactions with a stable reaction intermediate.

The data in Table 1 allow the calculation of the activating effect of a methyl group on different positions in the pyrrole ring. This was done with the data reported by Alexander and Butler[73] but more results were obtained during the present study and a more complete picture of the situation can now be obtained. The calculated effects are given in Table 3. Where comparison is possible, agreement with the results of Alexander and Butler

is good. Their results are shown in parentheses in Table 3.

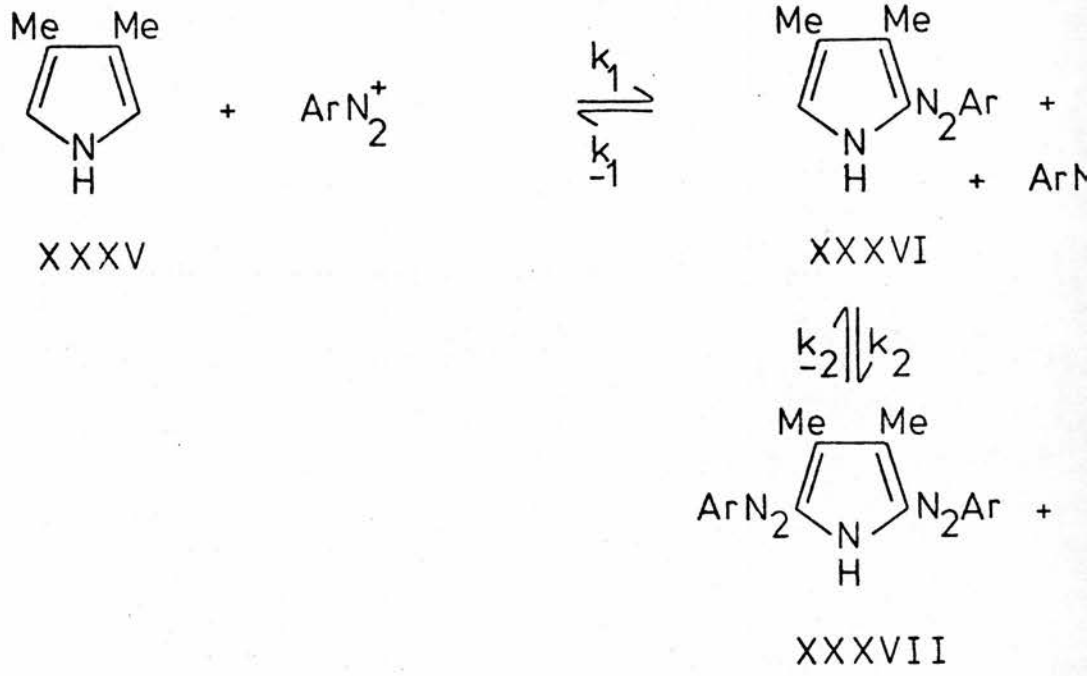
Table 3

Activating effect of a methyl group towards electrophilic attack on the pyrrole ring

Compared compounds	Position of methyl group	Position of attack	Activating Effect X= 4-SO_3H	H	4-OMe	
(b) and (a)	1	2	2.9	3.7	3.9	(4.3)
(f) and (e)	1	2	2.5	3.2	2.9	(3.3)
(e) and (a)	5	2	25	41	47	(43)
(f) and (j)	5	2	18	15	19	(15)
(j) and (e)	3	2	45	48	53	(50)
(h) and (e)	4	2	1.6	2.0	1.1	-
(i) and (c)	4	3	0.03	0.02	0.02	-

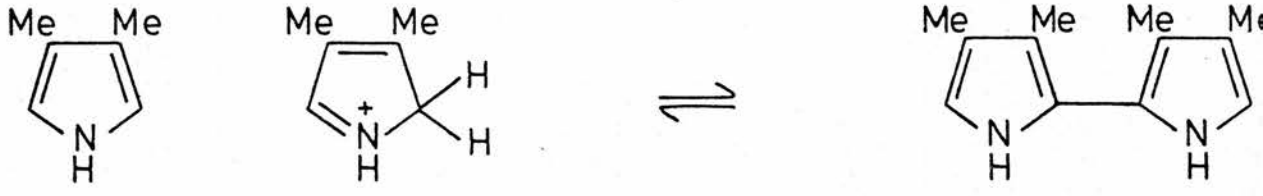
There are too few values to know if the variation of activating effect with electrophilicity is a genuine effect. The only surprising result is that a 4-methyl group deactivates the 3-position. This is probably a steric effect. The 3-methyl group should also deactivate the 2-position for the same reason, but the effect is masked by the hyperconjugative activating effect.

It has been assumed that in all cases the reaction observed was formation of the mono-azo compound and, on the stopped-flow time scale, no subsequent reaction was detected. The only exception to this was 3,4-dimethylpyrrole and, in this case, the initially formed intense yellow colour changed to pale blue, a change which was complete within 3 seconds for reaction with diazotised sulphanilic acid. The rate of disappearance of the



XXXV XXXVI XXXVII

SCHEME 8



XXXVIII XXXIX

SCHEME 9

yellow species has been determined and the results are shown in Table 4. The blue colour is typical of the bis-azopyrroles,

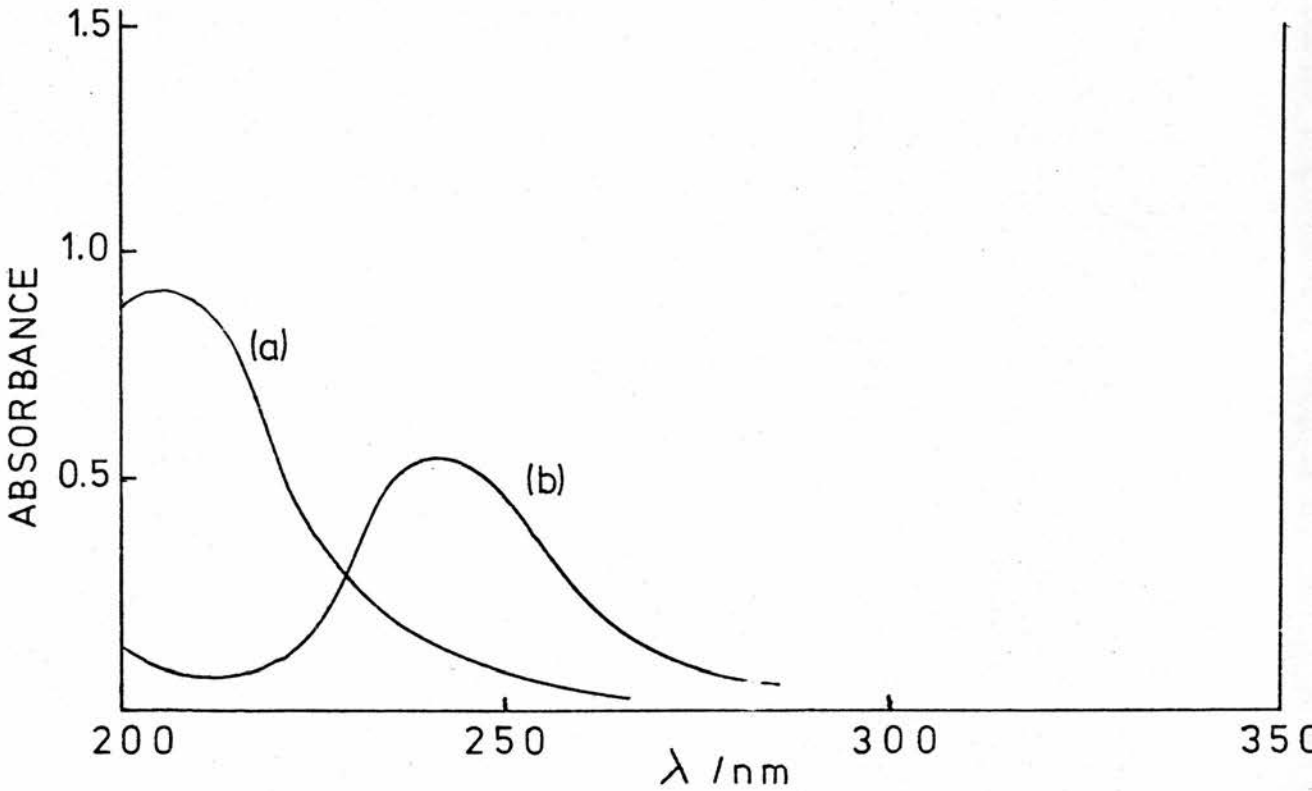
Table 4

Variation of k_{obs} with arenediazonium ion concentration for the disappearance of 4(3,4-dimethylpyrrol-2-ylazo)benzene-sulphonic acid (XXXVI)

$10^3[PhN_2^+]/M$	2.0	4.0	5.0	6.1	10
k_{obs}/s^{-1}	0.19	0.17	0.17	0.16	0.16

which are the normal products of reaction under alkaline conditions. Indeed the spectrum of the blue colour has been recorded and does correspond to that of the bis-azopyrrole, with a λ_{max} = 626 nm, formed under alkaline conditions. The probable reaction is that shown in Scheme 8. The small change in k_{obs} as the arene-diazonium ion concentration is varied would appear to indicate, however, that formation of a bis-azo compound did not occur. But the kinetics of the reaction may be complicated by the fact that 3,4-dimethylpyrrole dimerises very readily in acid solution (Scheme 9)[102]. Attempts to isolate pure products failed, therefore, as this reaction was not the principle concern of the present study it was not investigated further.

There is a reaction between arenediazonium ions and 2,3,4,5-tetramethylpyrrole and Treibs and Derra-Scherer[67] claim that this is accompanied by the elimination of methanol. Over the time scale used in the kinetic experiments in the present work, no reaction was detected, except with 4-nitrobenzenediazonium



U.v. spectrum of (a) unprotonated pyrrole in water and (b) protonat
pyrrole in 5M HCl

FIGURE 10

ions. Even in this case, the reaction was at least 1,000 times slower than with pyrroles with a free ring position. Tlc of the product of reaction showed that it was a complex mixture, including some tarry material. Tedder et al.[90] report that 2,4-dinitrobenzenediazonium ions react with tetramethylthiophen. through a methyl group. This diazonium ion also reacts with 2,3,4,5-tetramethylpyrrole in glacial acetic acid to give two coloured products (tlc), but they were not present in sufficient quantities to isolate. It could well be that there is attack of a methyl group if the ring positions are blocked and a strongly electrophilic arenediazonium ion is used. This matter was investigated further, using a crown ether as a phase transfer catalyst and the results of that investigation are reported in part (b) of this chapter.

I have described above how the protonation of pyrroles occurs in acid solution. Consequently, it is of interest to examine the reaction rate of such a process. The uv spectrum of a protonated pyrrole has a sufficiently different absorbance maximum from the unprotonated pyrrole[71] to make a spectrophotometric method of rate determination quite feasible (Figure 10). This was attempted in the course of the present study by the use of stopped-flow spectrophotometry in the uv region. The rates of protonation of 1-methyl-, 2-methyl-, 2,5-dimethyl- and 2,3,4-trimethyl-pyrroles in HCl solution were studied, but in each case the spectral change due to protonation occurred too rapidly to be followed on the instrument. The rate determining factor was the rate of mixing of the reactants. An investigation of the system

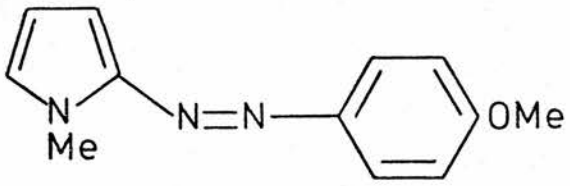
by temperature-jump was then carried out by Hibbert et al[103], but this did not yield any positive results. They found that the ΔH^{o} values for the equilibria studied were small and there was therefore no measurable shift in the position of equilibrium with a change in temperature.

1(b): REACTION BETWEEN PYRROLES AND ARENEDIAZONIUM IONS IN NON-HYDROXYLITIC SOLVENT IN THE PRESENCE OF A CROWN ETHER

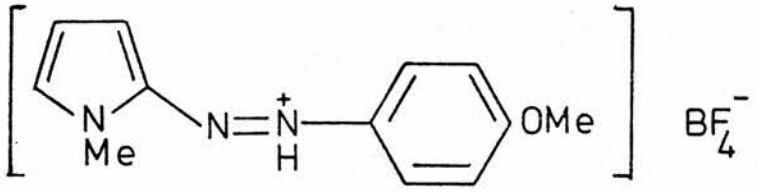
Results and Discussion

The failure to prepare characterisable samples of azopyrroles by the method of Kreutzberger and Kalter[42] is reported in part (a) of this chapter. There is little doubt about the identities of the products, but the analyses for carbon, hydrogen and nitrogen differed significantly from the theoretical values. The possibility of preparing these compounds in a purer state in a non-hydroxylitic solvent was then investigated. From the work of Cram et al[85,86] it is known that arenediazonium ions can be solubilised in dichloroethane or chloroform by the addition of a crown ether. As already noted in the introduction to this chapter, the ether fits like a collar around the $-\overset{+}{N}\equiv N$ group of the guest arenediazonium ion (XXVIII).

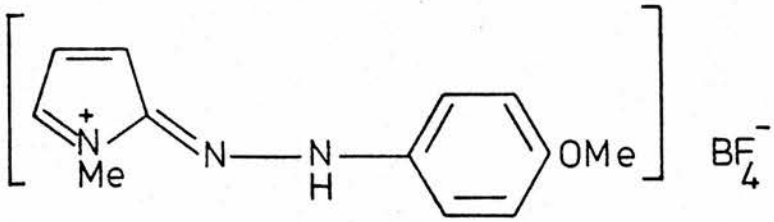
On a preparative scale a chloroform solution of 1-methylpyrrole did not react with 4-methoxybenzendiazonium tetrafluoroborate at an appreciable rate because the latter is essentially insoluble. However, on addition of a little dicyclohexyl-18-crown-6



XL



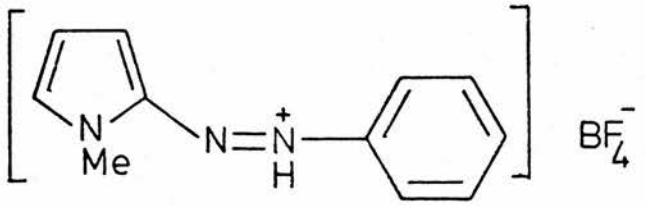
XLI



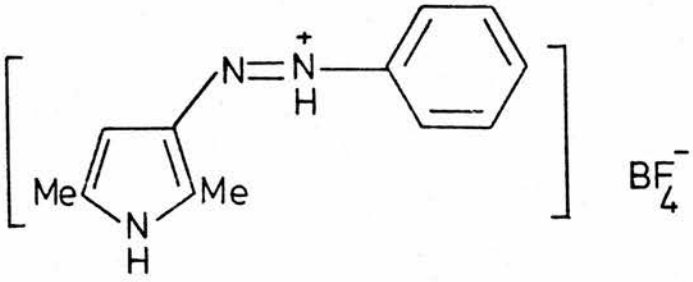
XLII

(XXVII), and after stirring for 15 minutes, reaction was complete. All the diazonium salt had been taken into solution and the product of reaction precipitated. The crown ether acted, therefore, as a phase transfer catalyst. It was quickly established from the elemental analysis of the product that the simple azopyrrole (XL) had not been prepared. A flame test[104] showed the presence of boron and in the ir spectrum there were stretching frequencies characteristic of boron-fluorine bonds. It was concluded, therefore, that what had been obtained was the tetrafluoroborate salt (XLI). This agreed well with the elemental analysis and analytically pure material was obtained without recrystallisation. The ^{13}C nmr spectrum indicated that there was no protonation of the pyrrole ring, although pyrroles are fairly strong bases. Protonation must, then, occur on the azo-group as shown in (XLI), although normally protonation of an azo-group occurs only in concentrated strong acids[94]. With an azopyrrole the positive charge can be delocalised, as in (XLII), and this explains the greater basicity of the azo-group in such compounds. The free azopyrrole (XL) was obtained by treatment of the salt with aqueous ammonia. The analysis of the crystals obtained corresponded to (XL) and the uv/visible spectrum was identical with that of the product of reaction of 1-methylpyrrole and 4-methoxybenzenediazonium ions in aqueous solution.

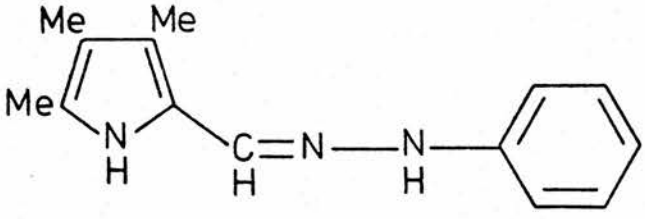
The preparative procedure used previously[42] and described in part (a) of this chapter did not lead to salt formation and it was thought that with (XLI) the 4-methoxy group, as an electron donating group, might have an important effect in this regard.



XLIII



XLIV

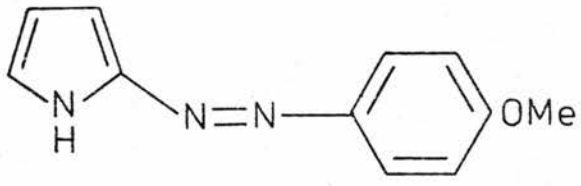


XLV

However, in chloroform and under phase transfer conditions, 1-methylpyrrole and benzenediazonium tetrafluoroborate also form an analogous salt (XLIII). If the 2-, and 5-positions are blocked, attack occurs at the 3(4)-position and the salt (XLIV) was obtained from 2,5-dimethylpyrrole. In every case the salt precipitated from solution could be filtered, washed and analysed without further purification.

In the proton nmr spectra of all the salts obtained there was a broad peak at δ ca. 1.9, which is not present in the spectra of free azopyrroles. The broadness, and the region[105], are typical of a hydrogen bound to nitrogen and are consistent with the postulated position of protonation.

The reaction of 2,3,4,5-tetramethylpyrrole under phase transfer conditions is of special interest. Some reaction occurs, as is evidenced by formation of coloured products[67], but the reaction is very much slower than diazo-coupling with pyrroles possessing a free position, as was the case in aqueous solution. In aqueous solution, to obtain material for product analysis, a very reactive diazonium salt, 2,4-dinitrobenzenediazonium tetrafluoroborate, had to be used. The result was an intractable green tar. Under phase transfer conditions reaction occurred with the unsubstituted benzenediazonium tetrafluoroborate but, unlike the cases described above, no precipitated product was obtained. Removal of the solvent gave another green tar and, by tlc, four components were recognised. By comparison with an authentic sample, one of the spots was identified as that of the phenylhydrazone of 3,4,5-trimethylpyrrole-2-aldehyde (XLV).



XLVI

One reaction of 2, 3, 4,5, -tetramethylpyrrole is, therefore, attack on the 2-methyl group. It is possible that one of the other products is that from attack at the 3(4)-methyl group. Attack of a methyl group was also observed by Tedder et al[90] in the reaction of 2,5-dimethylthiophen with 2,4-dinitrobenzenediazonium tetrafluoroborate. This experiment has been repeated here, using benzenediazonium tetrafluoroborate in chloroform and dicyclohexyl-18-crown-6 as a phase transfer catalyst, but the starting materials were recovered unchanged.

It is of interest to study the kinetics of reaction between pyrroles and arenediazonium ions in dichloroethane solution in the presence of a crown. These conditions are different from those of the synthetic procedure in that all the reactants were in solution. The reaction between pyrrole and 4-methoxybenzenediazonium tetrafluoroborate in dichloroethane with added dicyclohexyl-18-crown-6 occurs at a rate convenient for measurement. The diazonium salt was present at a concentration in a large excess over that of the pyrrole and so the reactions were first order in appearance of product, which was identified as (XLVI) by its spectrum. 4-Methoxybenzenediazonium tetrafluoroborate is sufficiently soluble in dichloroethane for reaction to occur even in the absence of a crown. The value of k_{obs} for reaction with a saturated solution of the diazonium salt is shown in the first column of Table 5 and the effect on k_{obs} of addition of crown to this solution is shown in the remainder of the Table. The concentration of diazonium ion in a saturated solution was determined spectroscopically (see Experimental). The decrease

Table 5

Effect of dicyclohexyl-18-crown-6 concentration on k_{obs} for reaction between 4-methoxybenzenediazonium tetrafluoroborate and pyrrole in dichloroethane at 25°C.

$10^3[CE]_t/M$	0	3.30	6.50	10.00	14.0	17.0
$10^2 k_{obs}/s^{-1}$	3.61	3.67	3.12	1.95	1.29	1.02

$[pyrrole]_o$ = ca. 5×10^{-5} M $[ArN_2BF_4]_t = 2.76 \times 10^{-3}$ M

in the value of k_{obs} as the concentration of crown was increased suggests that, in solution, the bound and unbound form of the diazonium ion exist in equilibrium but it is only the unbound form of the diazonium ion which reacts. The pyrrole is present at such a low concentration that the position of equilibrium will remain essentially unchanged during the course of the reaction. Thus, for reaction in solution, a crown has the effect of lowering the rate of reaction and yet, under heterogeneous conditions, reaction does not occur at an observable rate without addition of a crown.

Further analysis of the results in Table 5 necessitates knowing the equilibrium constant (K) for the binding of the diazonium ion within the crown, ie. equation (12). In this equation $ArN_2^+.CE$ is the crown/diazonium ion complex, while ArN_2^+ is the

$$K = [ArN_2^+.CE]/[ArN_2^+][CE] \qquad (12)$$

unbound diazonium ion. The guest:host ratio[86] for 4-methoxybenzenediazonium tetrafluoroborate and dicyclohexyl-18-crown-6 in dichloroethane-d_4 was measured. The value obtained was

0.8, with a total crown ether concentration of 0.27 M. This gives the ratio of the concentrations of the two species, whether bound or free, and cannot be used directly to calculate the value of K. However, we have good evidence that if a solution of crown in dichloroethane is saturated with the diazonium salt the concentration of unbound diazonium salt remains constant whatever the concentration of crown, and equal to its concentration in a saturated solution in the absence of crown. This evidence comes from the results in Table 6 where k_{obs} is seen to be independent of

Table 6

Effect of dicyclohexyl-18-crown-6 concentration on k_{obs} in dichloroethane solution saturated with 4-methoxybenzenediazonium tetrafluoroborate at 25°C

$10^3[CE]_t/M$	0	3.30	6.50	10.0	14.0
$10^2 k_{obs}/s^{-1}$	3.61	3.50	3.83	3.81	3.77

$[pyrrole]_o$ = ca. 5×10^{-5} M $[ArN_2BF_4]_t$ in absence of crown $= 2.76 \times 10^{-3}$ M

the crown ether concentration for solutions saturated with diazonium salt. This is understandable if the reasonable assumption is made that only the unbound form of the diazonium ion is responsible for reaction. The same assumption is consistent with the results in Table 5. Taking the value of $[ArN_2^+]$ in equation (12) as 2.76×10^{-3} M and the guest:host ratio as 0.8, a value of 1436 l mol^{-1} is obtained for the apparent equilibrium constant K. The significance of the word 'apparent' will be explained later.

With a knowledge of K it is possible to calculate the apparent concentrations of unbound diazonium ion in each of the kinetic determinations in Table 5. The values quoted in Table 5 are total concentrations of bound and unbound diazonium ion and crown. Equation (12) may be rewritten as (13), where $[ArN_2^+.CE]$ is the equilibrium concentration of bound diazonium

$$K = [ArN_2^+.CE]/([ArN_2^+]_t - [ArN_2^+ CE\ ])([CE]_t - [ArN_2^+.CE]) \quad (13)$$

ion. The terms $[ArN_2^+]_t$ and $[CE]_t$ are the total concentration of diazonium ion and crown present, while $([ArN_2^+]_t - [ArN_2^+.CE])$ is the equilibrium concentration of free diazonium ion $[ArN_2^+]$, which is the significant quantity as far as reaction rate is concerned. Equation (13) is a quadratic in $[ArN_2^+.CE]$ and was solved for each value of $[CE]_t$ in Table 5, and then used to calculate $[ArN_2^+]$ in each experiment. If unbound diazonium ion is the reactive species, then the ratio $k_{obs}/[ArN_2^+]$ is the molar rate constant for reaction. The calculations are displayed in Table 7.

Table 7

Calculation of molar rate constants for diazo coupling reaction of pyrrole with p-methoxybenzenediazonium tetrafluoroborate in dichloroethane at 25°C

$10^3[CE]_t$/M	0	3.30	6.50	10.0	14.0	17.0
$10^3[ArN_2^+]$/M	2.76	1.03	0.44	0.27	0.17	0.14
$\frac{k_{obs}}{[ArN_2^+]}$ / l mol^{-1} s^{-1}	13	36	71	72	76	73

The fact that the term $k_{obs}/[ArN_2^+]$ is not constant indicates either that an incorrect assumption has been made, or that the situation is more complex than supposed. The latter is more likely as ion aggregation is certain to occur in a nonpolar solvent like dichloroethane, in which the ions are not heavily solvated[106] and so far, this effect has not been considered. The values of $k_{obs}/[ArN_2^+]$ in Table 7 are consistent with the occurrence of ion aggregation as it will be more pronounced at high concentrations of diazonium ion and become less as crown is added and the free diazonium ion concentration reduced. Ion aggregates have a much lower electrophilicity than monomeric diazonium ions, hence their formation will lead to a drop in the rate of reaction[106].

The value of K was determined at concentrations where ion aggregation is significant and so the value obtained is not the equilibrium constant for binding of the monomeric form. This is why it was called an 'apparent' equilibrium constant earlier. The constancy of the term $k_{obs}/[ArN_2^+]$ at higher crown ether concentration (Table 7) may occur because, at low concentrations of unbound diazonium ion, aggregation is not important. However, the numerical value of the term is not meaningful because the relevant value of K has not been obtained.

Further evidence for the effect of ion aggregation upon the kinetics of reaction comes from another series of experiments. In these the concentration of diazonium ion was varied while that of the crown was kept constant. The results are displayed in Table 8.

Table 8

Effect of 4-methoxybenzenediazonium ion concentration on k_{obs} for reaction with pyrrole in the presence of a crown in dichloroethane at 25°C

$10^3 [ArN_2BF_4]_t/M$	2.50	5.00	7.50	10.0	12.5
$10^2 k_{obs}/s^{-1}$	1.05	1.68	3.31	4.42	5.70

$[crown] = 17.0 \times 10^{-3}$ M $[pyrrole]_o$ = ca. 5×10^{-5} M

As the diazonium ion concentration is lowered by a factor of five the value of k_{obs} falls by the same factor. All other things being equal, the reduction in k_{obs} should be greater as, at constant crown concentration, the proportion of diazonium ion present in the bound form should increase as the concentration of diazonium salt is lowered. However, there is a concomitant decrease in ion aggregation and this leads to an increase in the value of k_{obs}.

In view of the anti-catalytic effect of a crown ether upon the diazo-coupling reaction in solution, it must now be considered why it acts as a catalyst in the preparative procedure. Under heterogeneous conditions the slow step in the reaction may be the rate at which the diazonium salt goes into solution. I suggest that the crown catalyses this step in the reaction by acting as a solid-liquid phase transfer catalyst. The mechanism of such reactions has been considered by Gokel and Durst[81]. They suggest that the crown, since it is a two-dimensional structure with many polar sites, as it approaches the crystal lattice, may assume the approximate geometry of the complex and so the

movement of the cation from the lattice to the ligand is small. In this way the rate of solution is accelerated.

One step in a diazo-coupling reaction is removal of a proton from the Wheland intermediate. In aqueous solution the proton is removed by the base water[95] but it is not obvious what the mechanism in dichloroethane or chloroform as solvent is. As the azopyrrole salt is the product of reaction the proton could be lost by an intramolecular proton transfer. Also, dicyclohexyl-18-crown-6 is a strong base[107] and this may effect proton removal and only reprotonate the azopyrrole as it is precipitated as the salt. In view of the complications of ion aggregation and proton loss it is not possible to compare the rates of diazo-coupling of pyrroles in water and nonpolar solvents. However, the qualitative evidence is that the rates are similar, and this feature of diazo-coupling reactions has been noted previously[108].

EXPERIMENTAL

Materials: Pyrrole, 1-methyl pyrrole, 2,5-dimethyl pyrrole and cryptopyrrole were obtained commercially. 1,2,5-Trimethyl pyrrole[88], 2,3,5-trimethylpyrrole[88], 2,3,4-trimethyl pyrrole[109], 1,2-dimethyl pyrrole[88], 2,3-dimethyl pyrrole[110], 2,4-dimethyl pyrrole[111], 3,4-dimethyl pyrrole[88] and 2-methyl pyrrole[112] were prepared by published methods. Amines were recrystallised

or distilled before use and converted to benzenediazonium salts by reaction with sodium nitrite and HCl at 0°C.

Dicyclohexyl-18-crown-6 was obtained commercially, and was purified before use by column chromatography using an alumina column and hexane as the elutant[107].

The benzenediazonium tetrafluoroborate salts of aniline, p-methoxyaniline, p-nitroaniline and sulphanilic acid were prepared by the method of Vogel[113] as follows. Aniline (9 ml) was dissolved in a mixture of conc. hydrochloric acid (25 ml) and water (25 ml) in a beaker (250 ml). The solution was cooled to 0-5°C in an ice/salt bath and a solution of sodium nitrite (7.3 g) in water (15 ml) was added in small portions; the mixture was stirred vigorously and the temperature kept below 5°C. After 5 min diazotisation was complete. A solution of sodium tetrafluoroborate (15 g) in water (30 ml) was prepared, cooled and slowly added to the stirred diazonium salt solution at such a rate as the temperature was maintained below 10°C. After standing for 10 min with frequent stirring the precipitate of benzendiazonium tetrafluoroborate was filtered off by suction, drained, washed with water, methanol and ether, and dried.

The other three benzenediazonium tetrafluoroborate salts were prepared in the same way using the following amines: p-nitroaniline, sulphanilic acid, and p-methoxyaniline.

Kinetics: A "Canterbury" stopped-flow spectrophotometer was used for the kinetic studies in aqueous solution. A very dilute solution of the pyrrole ($\simeq 10^{-4}$ M) was placed in one arm and

the benzenediazonium salt in standard HCl in the other. The wavelengths used for the various pyrroles were in the range 420-470 nm. The kinetic studies in non-aqueous solvent (AR dichloroethane), using the crown ether as the solubilising agent were carried out in the Unicam SP700 spectrophotometer. The observation wavelength was 390 nm. Spectra of the coloured products were recorded on a Unicam SP800 spectrophotometer.

Rate constants were calculated by the method of Kezdy[114] and Swinbourne[115] (Appendix 3). The reactions were all first order in appearance of product.

The guest:host ratio for 4-methoxybenzenediazonium tetrafluoroborate and dicyclohexyl-18-crown-6 in dichloroethane-d_4 were determined by the ^{1}H-nmr method of Cram et al[86]. The solubility of 4-methoxybenzenediazonium tetrafluoroborate in dichloroethane was determined spectrophotometrically at 320 nm by comparison with a series of solutions of known concentrations.

Kinetic Studies on the Protonation of Pyrroles: The stopped-flow spectrophotometer was used with a uv light source and suitable photomultiplier. The observation frequencies used were as follows: 1-methyl pyrrole λ_{obs}=250 nm, 2-methyl pyrrole λ_{obs}=235 nm, 2,5-dimethyl pyrrole λ_{obs}=245 nm, 2,3,4-trimethyl pyrrole λ_{obs}=264 nm, since these wavelengths correspond to the λ_{max} of the protonated forms of the pyrroles. Analar grade HCl was used. The rates of protonation proved to be too rapid to be followed on the stopped-flow spectrophotometer.

Products:

Preparation of the azo-pyrroles of 1-methyl and 2,5-dimethyl pyrrole by the method of Whetsel et al[116] employed by Kreutzberger and Kalter[42] gave products which were difficult to purify and no satisfactory analyses were obtained. However, spectroscopic evidence indicated that (XXXI) and (XXXII) were the correct structures for these products. The mass spectra M^+ were 265 and 279 respectively, which are correct for the proposed structures. For (XXXI) the nmr spectrum showed (DMSO-d_6 at 35°C) 3.94(3H), 6.34 (1H), 6.76 (2H), and 7.88 (4H). For (XXXII) the nmr spectrum showed (DMSO-d_6 at 35°C) 2.53 (6H), 6.54 (1H), and 7.56 (4H). Neither (XXXI) nor (XXXII) had an absorption in the region 3300-3500 cm^{-1} which is the region of N-H stretch frequency.

The phenylhydrazone of 1-methylpyrrole-2-carboxaldehyde (XXXIV) was prepared by the method of Mann and Saunders[117] and formed white crystals, mp. 124°C (lit.[118] 123°C). This compound had the expected strong absorption at 3300 cm^{-1}.

Analytically pure samples of mono-azo-pyrroles of 1-methyl and 2,5-dimethyl pyrrole were prepared by the use of dicyclohexyl-18-crown-6 as a phase transfer catalyst.

Synthetic Method: The pyrrole (2 g) was dissolved in chloroform (100 ml) and dicyclohexyl-18-crown-6 (0.1 g) was added. To this was added sufficient benzenediazonium tetrafluoroborate to give a 50% excess of the pyrrole. The mixture was then stirred at room temperature for 15 min after which time the product had

precipitated out. The product was filtered off and washed with ether. In every case it was found to be analytically pure without recrystallisation.

Tetrafluoroborate of 4-(1-methylpyrrol-2-ylazo)methoxybenzene (XLI) was obtained as crimson crystals, yield 2.8 g, 73%. Decompose on heating - m/e 215 (M^+ - HBF_4) Found: C, 47.26;, H, 4.70; N, 14.01%. $C_{12}H_{14}N_3OBF_4$ requires: C, 47.56; H, 4.66; N, 13.87%. δ(CD_3CN) 1.90 (1H, s), 3.75 (3H, s), 3.85 (3H, s), 6.31 (1H, s), 6.65 (1H, s), 6.95 (1H s), 7.44 (4H, m); ^{13}C nmr chemical shifts (CD_3CN) at 34.7, 56.3, 109.0, 116.6, 118.0, 121.5, 147.2, 152.0 and 155.8 ppm; ir stretching frequencies at 984 and 769 cm^{-1} due to B-F bonds.

Compound (XLI) (2.0 g) was dissolved in dichloromethane (100 ml) and shaken with ammonium hydroxide (5M, 50 ml). After separation, the organic layer was washed with water, dried ($MgSO_4$), and the solvent removed by evaporation to give yellow crystals of 4-(1-methylpyrrol-2-ylazo)methoxybenzene (XL). Yield 0.5 g, 75% mp. 44-46°C, m/e 215 (M^+). Found: C, 67.14; H, 6.11; N, 19.77% $C_{12}H_{13}N_3O$ requires: C, 66.96; H, 6.09; N, 19.52%. δ($CDCl_3$) 3.75 (3H, s), 3.85 (3H, s), 6.20 (1H, s), 6.60 (1H, s), 6.75 (1H, s), 7.32 (4H, m); ^{13}C nmr chemical shifts at 32.2, 55.5, 99.2, 110.0, 114.6, 124.0, 126.0, 145.1, 147.8 and 160.8 ppm.

Tetrafluoroborate of 1-methylpyrrol-2-ylazobenzene (XLIII) was obtained as dark red crystals, yield 2.1 g, 61%. Decomposes on heating. m/e 185 (M^+ - HBF_4). Found: C, 48.71; H, 4.64; N, 15.56% $C_{11}H_{12}N_3BF_4$ requires: C, 48.39; H, 4.43; N, 15.39%.

$\delta(CD_3CN)$ 1.90 (1H, s), 3.94 (3H, s), 6.95 (1H, s), 7.45 (1H, s), 7.55 (1H, s), 7.93 (5H, m).

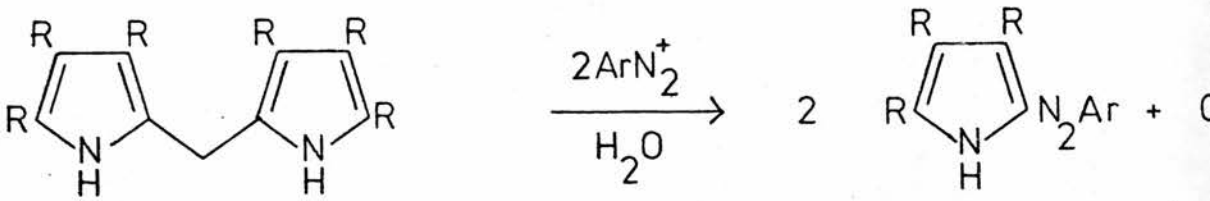
Tetrafluoroborate of 2, 5-dimethylpyrrol-3-ylazobenzene (XLIV) was obtained as dark red crystals, yield 2.4 g, 75%. Decomposes on heating. m/e 199 ($M^+ - HBF_4$). Found: C, 50.44; H, 5.11; N, 15.21% $C_{12}H_{14}N_3BF_4$ requires: C: 50.16; H, 4.91; N, 14.73% $\delta(CD_3CN)$ 1.92 (1H, s), 2.30 (3H, s), 2.68 (3H, s), 6.50 (1H, s), 7.60 (5H, m).

2, 3, 4-Trimethylpyrrole-2-aldehyde was prepared by the reaction of 2, 3, 4-trimethyl pyrrole with dimethylformamide and phosphoryl chloride[58]. Reaction with phenylhydrazine[117] gave the expected phenylhydrazine (XLV), m/e 227 (M^+); δ ($CDCl_3$) 1.90 (3H, s), 2.02 (3H, s), 2.17 (3H, s), 3.53 (1H, s), 5.53 (1H, s), 7.79 (5H, m). Tlc used methanol as solvent.

The reaction between 2, 3, 4, 5-tetramethyl pyrrole and benzenediazonium tetrafluoroborate in the presence of dicyclohexyl-18-crown-6 was carried out as described above, but in this case no product precipitated out. On evaporation of the solvent a dark green tar was left, which was shown by tlc to be a mixture of products. One of the spots in the tlc (using methanol as a solvent) corresponded to 2, 3, 4-trimethylpyrrole-5-phenylhydrazone (XLV). Another three spots were present which could not be identified.

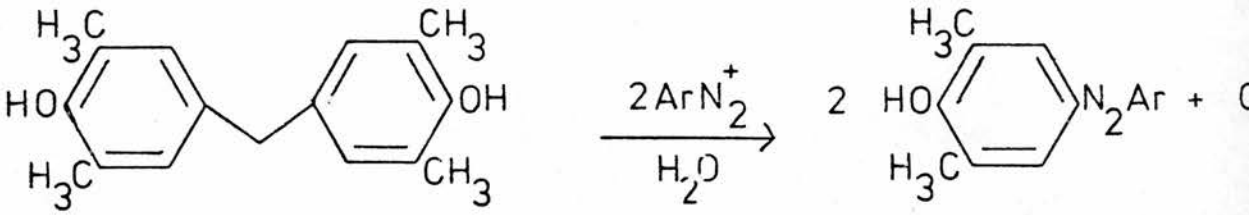
CHAPTER 2

THE REACTION OF DIPYRRYLMETHANES WITH ARENEDIAZONIUM IONS IN ACID SOLUTION



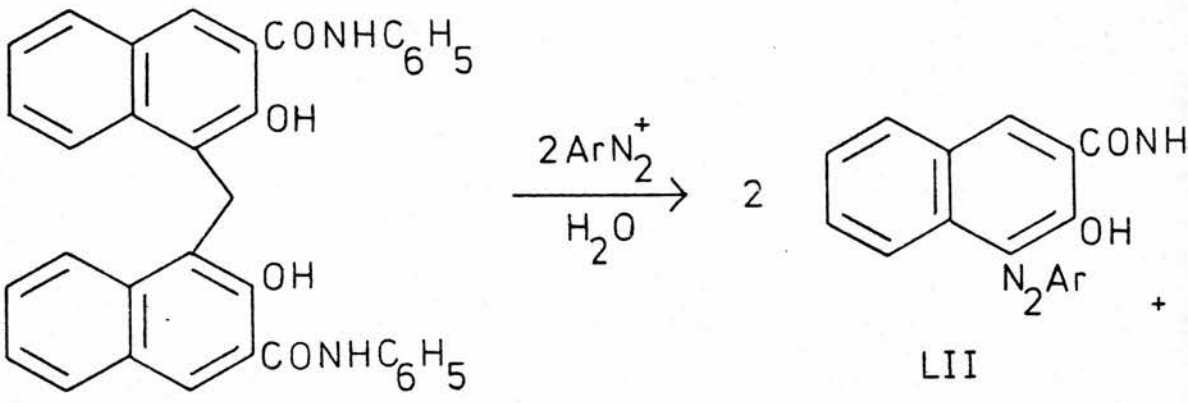
XLVII XLVIII

SCHEME 10



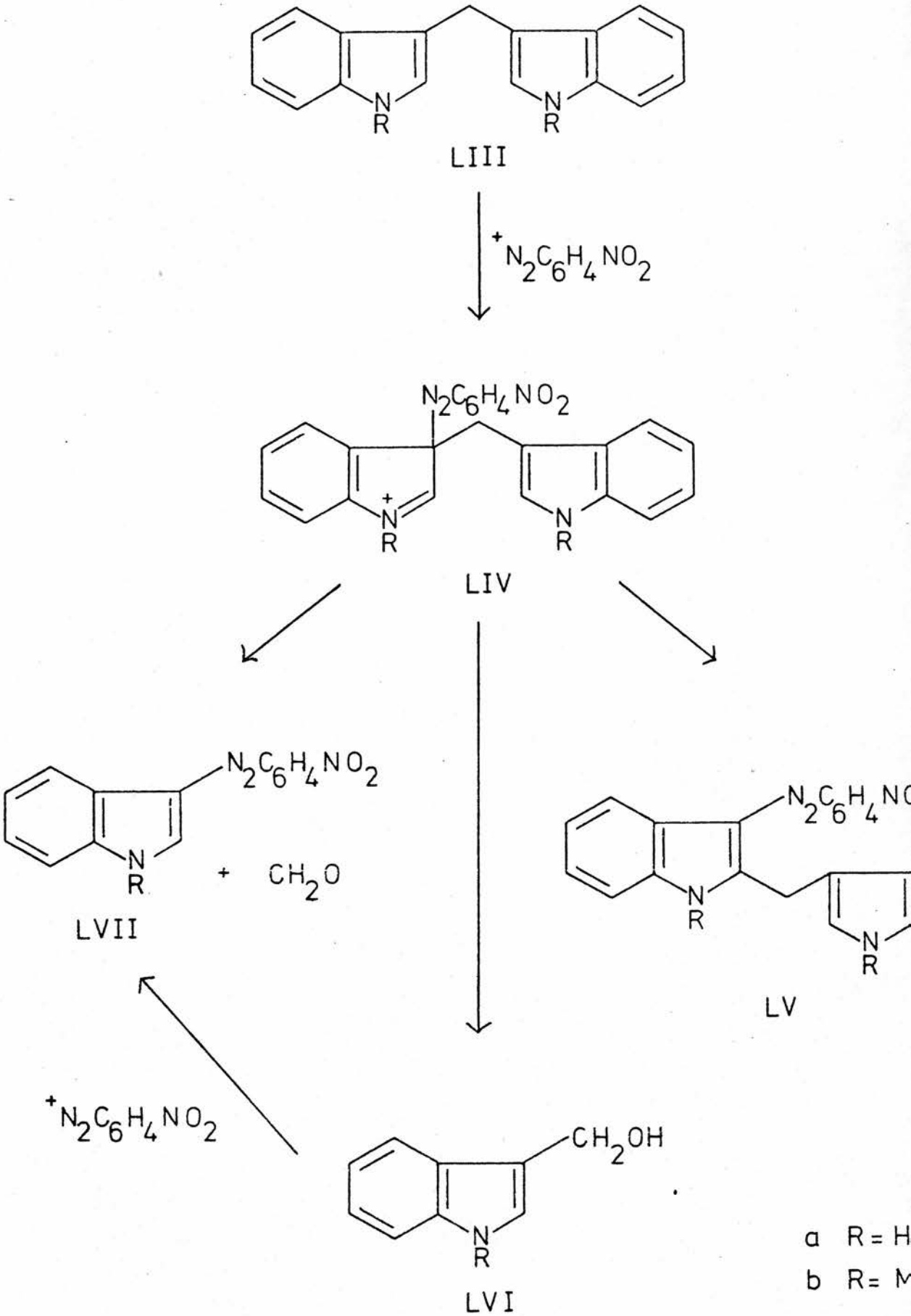
XLIX L

SCHEME 11



LI LII

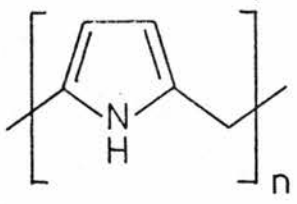
SCHEME 12


LIII
$^{+}N_2C_6H_4NO_2$
$N_2C_6H_4NO_2$
LIV
$N_2C_6H_4NO_2$
LVII
+ CH_2O
$N_2C_6H_4NO$
LV
$^{+}N_2C_6H_4NO_2$
CH_2OH
LVI
a R = H
b R = M

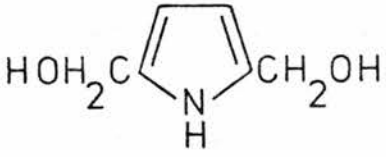
SCHEME 13

INTRODUCTION:

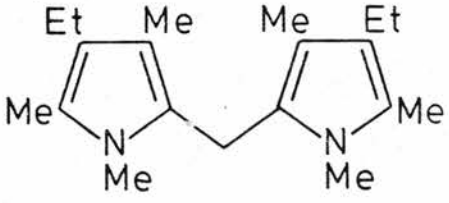
The work discussed in this chapter is the first kinetic study of the reaction between dipyrrylmethanes (XLVII) and arenediazonium ions. A qualitative investigation of the reaction has, however, been carried out by Treibs and Kolm[56] who found that azo-coupling occurs with cleavage of the central methylene bridge of the dipyrrylmethane (Scheme 10). In this respect the dipyrrylmethanes resemble the diphenylmethanes (XLIX) which also react with arenediazonium ions, resulting in the cleavage of the central methylene bridge, which is released as formaldehyde (Scheme 11)[119]. This further demonstrates the similarity in reactivities of phenols and pyrroles towards electrophilic attack. Likewise, dinaphthylmethanes (LI) undergo the same type of azo-coupling reaction (Scheme 12)[120]. The reaction between arenediazonium ions and di-indolylmethanes (LIII) has recently been investigated by Jackson et al[44]. In this case the incoming arenediazonium ion attacks the already substituted 3-position, either displacing the substituent completely, or, alternatively, causing it to migrate to the neighbouring 2-position (Scheme 13). In aqueous solution the 3-benzylazo-indole (LVII) is obtained in greater than 90% yield and the methylene bridging group is released as formaldehyde, which has been isolated as its dimedone derivative. When the reaction was carried out in acetonitrile a mixture of products (LV and LVII) was obtained. The fact that indoles undergo primary electrophilic attack at the 3-position, which, if that position is already substituted, may be followed by a migration of either the incoming electrophile



LVIII



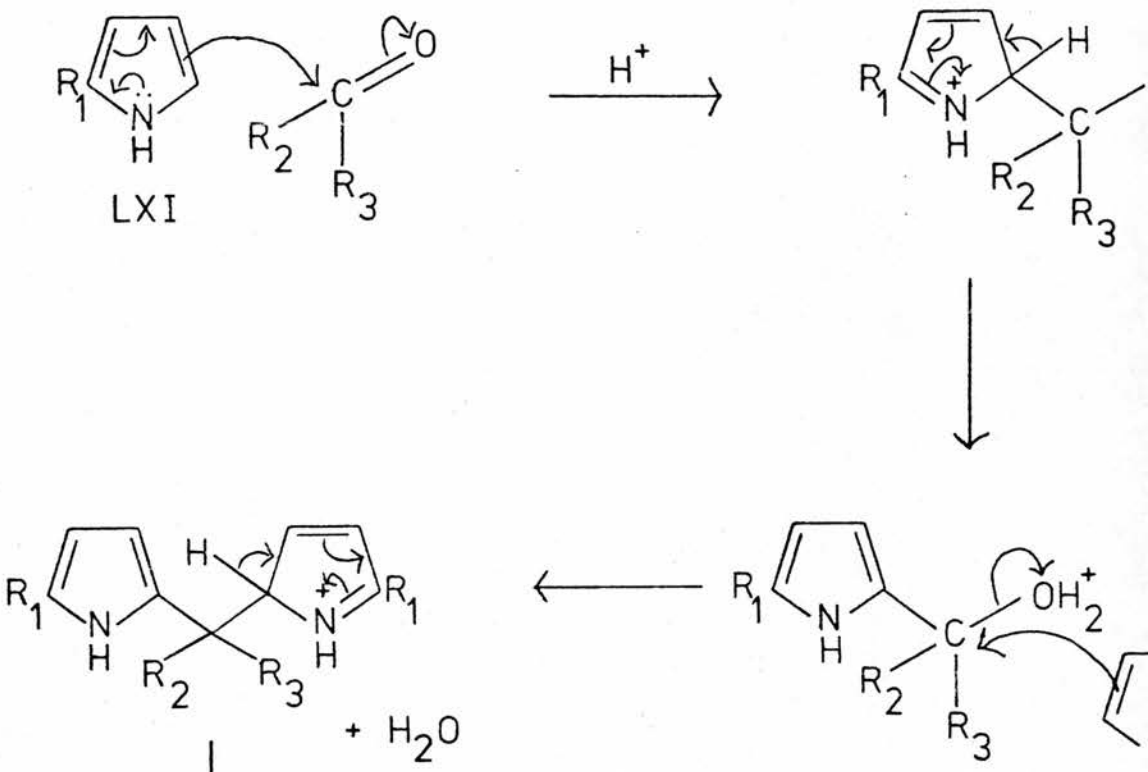
LIX



LX

or of the 3-substituent to the 2-position, is well recorded[44,121]. This is not the case in pyrrole chemistry, where the most reactive site for electrophilic attack is the 2(5)-position and where there is no evidence for intramolecular rearrangement after the initial electrophilic substitution. It is reported in this chapter that the dipyrrylmethanes invariably react with arenediazonium ions to give the simple azopyrroles with the methylene bridge group being released as formaldehyde. Jackson et al point out the similarity between the reactions of di-indolylmethanes and bilirubin with arenediazonium ions. In this respect, the dipyrrylmethanes resemble bilirubin much more closely. Firstly, the methylene bridge in bilirubin is at the 2-position and not at the 3-position; secondly, electrophilic attack of bilirubin occurs at the 2-position; thirdly, there is no evidence for migration of substituents after electrophilic attack of bilirubin. Consequently, the dipyrrylmethanes are good models for investigation of the Van den Bergh test.

A number of synthetic routes to the dipyrrylmethanes are available. The normal products of the acid-catalysed reaction of polysubstituted pyrroles with aldehydes or ketones are the 2,2'-dipyrrylmethanes, but when both the 2- and 5-positions of the pyrrole ring are unsubstituted, further reaction of the dipyrrylmethane can lead to polymeric products. In a reaction analogous to that of phenol with formaldehyde, pyrrole forms 'bakelite-type' polymers (LVIII), via carbinols of the type (LIX)[61,122]. In general, it is difficult to synthesise the dipyrrylmethanes by the condensation of formaldehyde with pyrroles because of the tendency

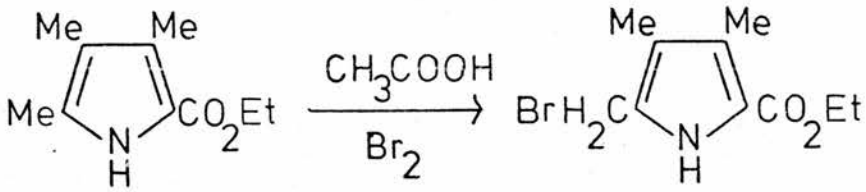


a $R_1=R_2=R_3=Me$

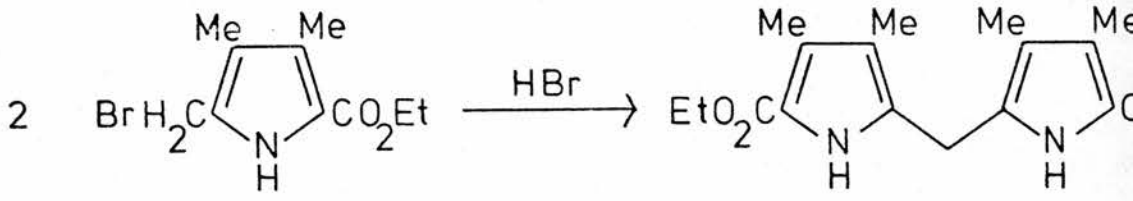
b $R_1=R_2=Me, R_3=H$

SCHEME 14

of both to polymerise in acid solution. The method is only successful where there is a deactivating substituent (eg. CO_2Et) on the pyrrole ring, which inhibits the polymerisation process. The success of Corwin and Quattlebaum[43], who reported the synthesis of 1,1',3,3',5,5'-hexamethyl-4,4'-diethyl-2,2'-dipyrrylmethane (LX) by the reaction between 'old' formalin (containing enough formic acid to turn blue litmus red) and the highly activated 1,2,4-trimethyl-3-ethylpyrrole was not repeated in the present work, polymeric products being obtained instead. When carbonyl compounds other than formaldehyde, such as acetone and acetaldehyde condense with pyrroles in acid solution a much higher yield of the dipyrrylmethane is obtained and these may be easily isolated pure. It has been reported by Treibs et al[123] that pyrroles with unsubstituted 2- and 3-positions condense with aldehydes other than formaldehyde, in the presence of acid, to give bridge-substituted dipyrrylmethanes (LXII(a) and (b)), although bis-dipyrrylmethanes (LXIII) and tris-tripyrrylmethanes (LXIV) as well as polycondensation products are also formed (Scheme 14). In the present study, then, some bridge-substituted dipyrrylmethanes (LXII(a) and (b)) were prepared by this method. These are of interest in order to investigate the effect of bridge-substituents in the azo-coupling reaction and bridge cleavage. However, other methods had to be employed for the synthesis of the bridge-unsubstituted dipyrrylmethanes. King and Brown[124] reported the synthesis of a dipyrrylmethane during the attempted nitration of a pyrrole. The authors claim general applicability for this coupling procedure but it has only been applied to those pyrroles

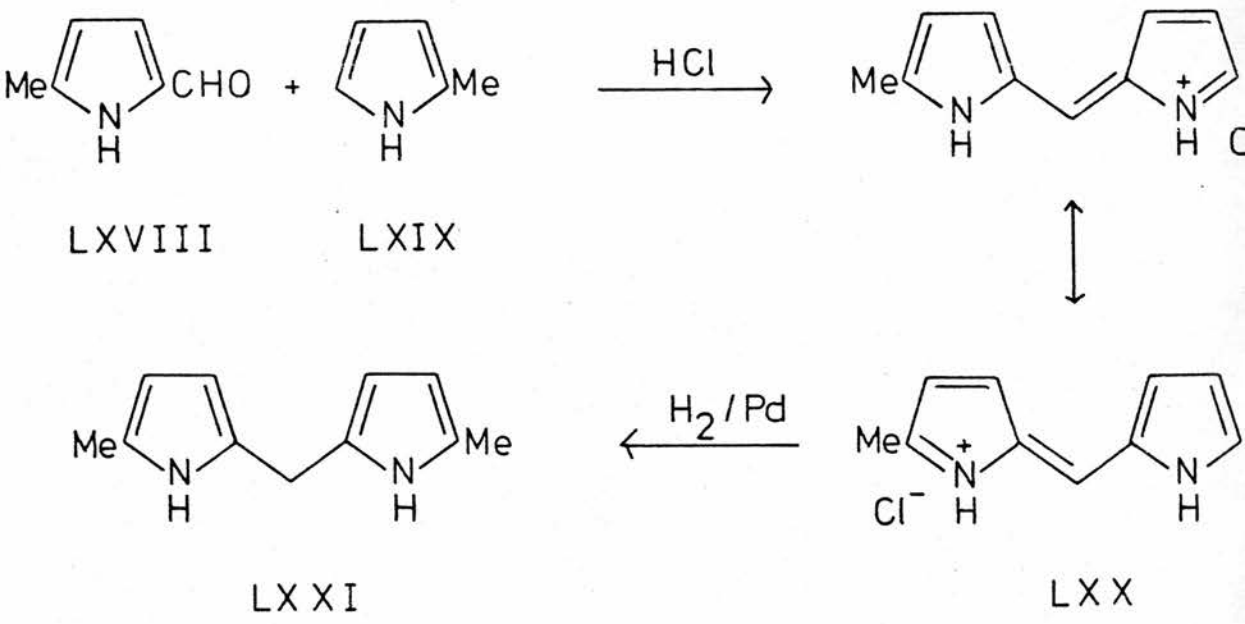


LXV LXVI



LXVII

SCHEME 15



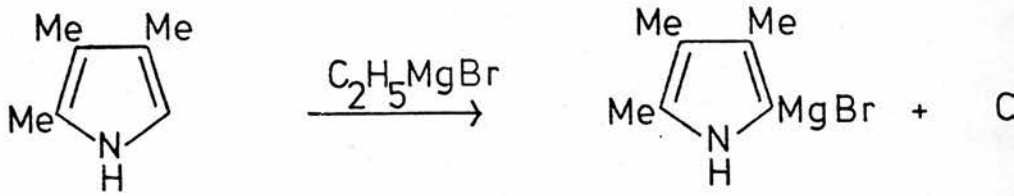
LXVIII LXIX LXXI LXX

SCHEME 16

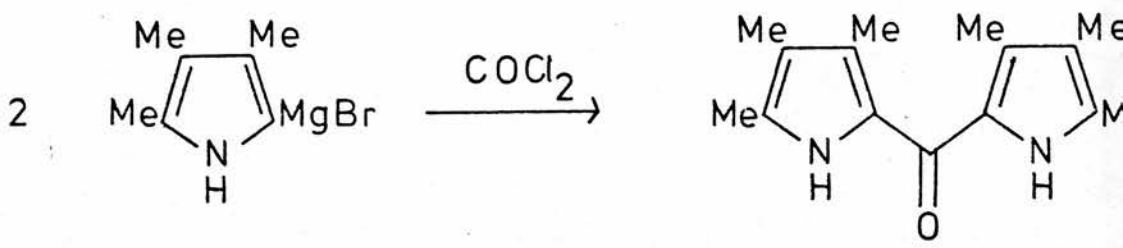
containing deactivating substituents and its utility as a method for synthesis of methyl-substituted dipyrrylmethanes is dubious because of the tendency of the methylpyrroles to polymerise in acid solution. A further synthetic route which falls into this category is that via the α-bromomethylpyrrole (or α-acetoxymethylpyrrole), in which this compound is refluxed in the presence of acid, the dipyrrylmethane (LXVII) precipitating out (Scheme 15)[125,126]. Again a deactivating substituent must be present in the pyrrole ring for this method to succeed.

There are, however, two routes to the dipyrrylmethanes which are of more general use. The first of these proceeds via the dipyrrylmethene, which is then reduced by catalytic hydrogenation to the corresponding dipyrrylmethane (Scheme 16). To prepare the dipyrrylmethene salt (LXX), equimolar quantities of the α-formylpyrrole (LXVIII) and α-unsubstituted pyrrole (LXIX) are stirred in dry ether through which a stream of HCl gas is bubbled[127,128]. The highly coloured dipyrrylmethene salt (LXX) precipitates out. The catalytic hydrogenation procedure, carried out at atmospheric pressure, has been described by Chong et al[129], and pure crystals of the dipyrrylmethane (LXXI) were obtained, but not in good yield (15%).

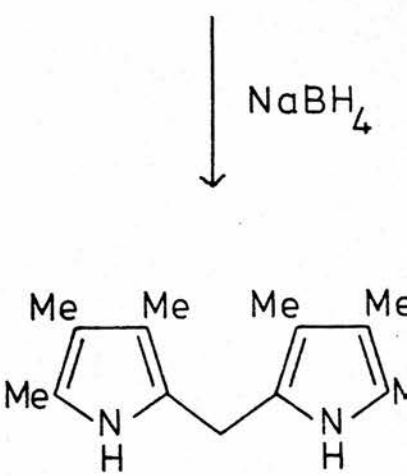
The most satisfactory route to the bridge-unsubstituted dipyrrylmethanes is to proceed via the dipyrrylketone, which may be reduced to the corresponding dipyrrylmethane in good yield by sodium borohydride. The syntheses of several dipyrrylketones have been reported[130,131], but the method applied in the present work is that of Clezy et al[132] in which



LXXII LXXIII



LXXIV



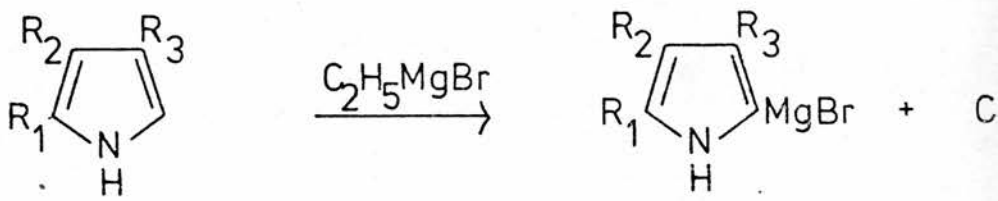
LXXV

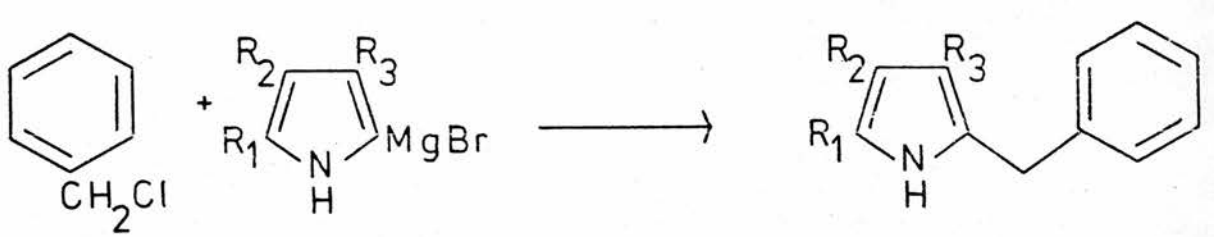
SCHEME 17

3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylketone (LXXIV) is prepared by the reaction of 2,3,4-trimethylpyrryl Grignard reagent (LXXIII) with phosgene solution. This was then reduced to the 3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylmethane (LXXV) with sodium borohydride (Scheme 17)[129].

The reactions between the dipyrrylmethanes thus synthesised and the arenediazonium ions in acid solution were then investigated and the mechanism of the process determined. It was found that cleavage of the methylene bridge did take place, and that this was released as formaldehyde. The electrophilic species causing cleavage of the bridge is the proton, and this cleavage occurs at a much faster rate than azo-coupling. Consequently, the arenediazonium ions are reacting with the pyrrole monomers, the bridging methylene group having already been released as formaldehyde, which was isolated as its dimedone derivative. The kinetic and synthetic evidence leading to this conclusion will be discussed later in this chapter. The assumed reaction intermediate, the 2-hydroxymethylpyrrole could not, however, be isolated because of its high reactivity, but its role could be inferred from the investigation of an analogous reaction - that of a 2-benzylpyrrole with arenediazonium ions, from which benzyl alcohol was isolated.

The 2-benzylpyrroles are similar to the dipyrrylmethanes in that both consist of two aromatic rings joined by a methylene bridging group. The major difference in their reactions with arenediazonium ions lies in the fact that once the bond between the pyrrole ring and the methylene carbon is broken, the benzyl





LXXVI, R_1 = Me, R

LXXVII, $R_1 = R_2 = R$

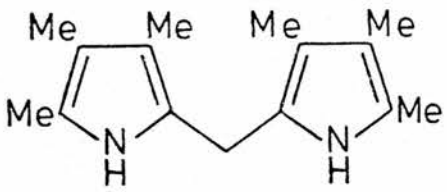
SCHEME 18

alcohol thus formed will not react with arenediazonium ions and can, therefore, be isolated from the reaction. By analogy, if benzyl alcohol is formed in this reaction then the reaction intermediate in the dipyrrylmethane case will be the 2-hydroxymethylpyrrole. That benzyl alcohol is formed was proved in the present study by a radio-isotopic labelling experiment, the results of which will be discussed later in this chapter.

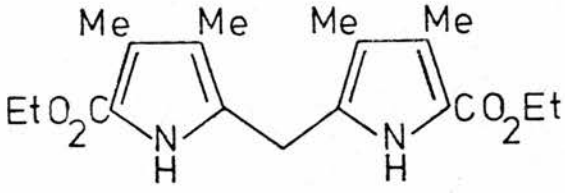
The 2-benzylpyrroles(LXXVI and LXXVII) were prepared from the pyrryl Grignard reagent and benzyl chloride by the method of Hobbs et al (Scheme 18)[133].

RESULTS AND DISCUSSION:

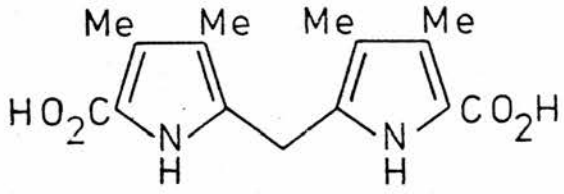
The present study of the azo-coupling of dipyrrylmethanes is the first investigation into the kinetics of this reaction. A mechanism for the process consistent with the results obtained is outlined here. The study was carried out under conditions similar to those of the Van den Bergh test. In the present work the 2,2'-dipyrrylmethanes are used as model compounds for bilirubin since they contain that part of the bilirubin molecule which is the site of reaction in the Van den Bergh test[20,22]. It is known that in the Van den Bergh test, cleavage of the dipyrrylmethane unit takes place, and the exact nature of that cleavage process has been clarified by the studies of the azo-coupling reactions of the dipyrrylmethanes and related compounds reported in this chapter.



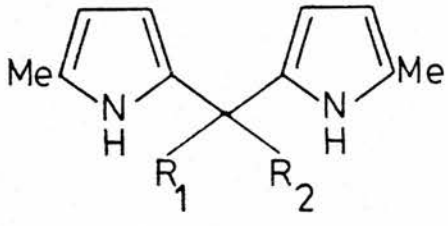
LXXVIII



LXXIX



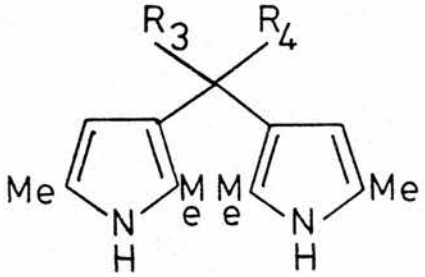
LXXX



LXXXI $R_1=R_2=H$

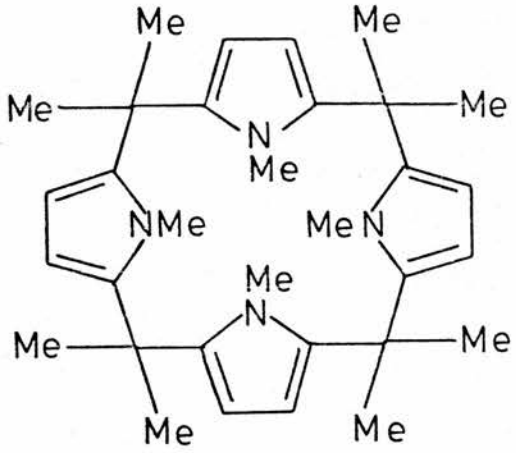
LXXXII $R_1=R_2=CH_3$

LXXXIII $R_1=H, R_2=CH_3$

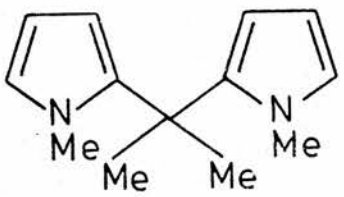


LXXXIV $R_3=R_4=CH_3$

LXXXV $R_3=H, R_4=CH_3$



LXXXVI



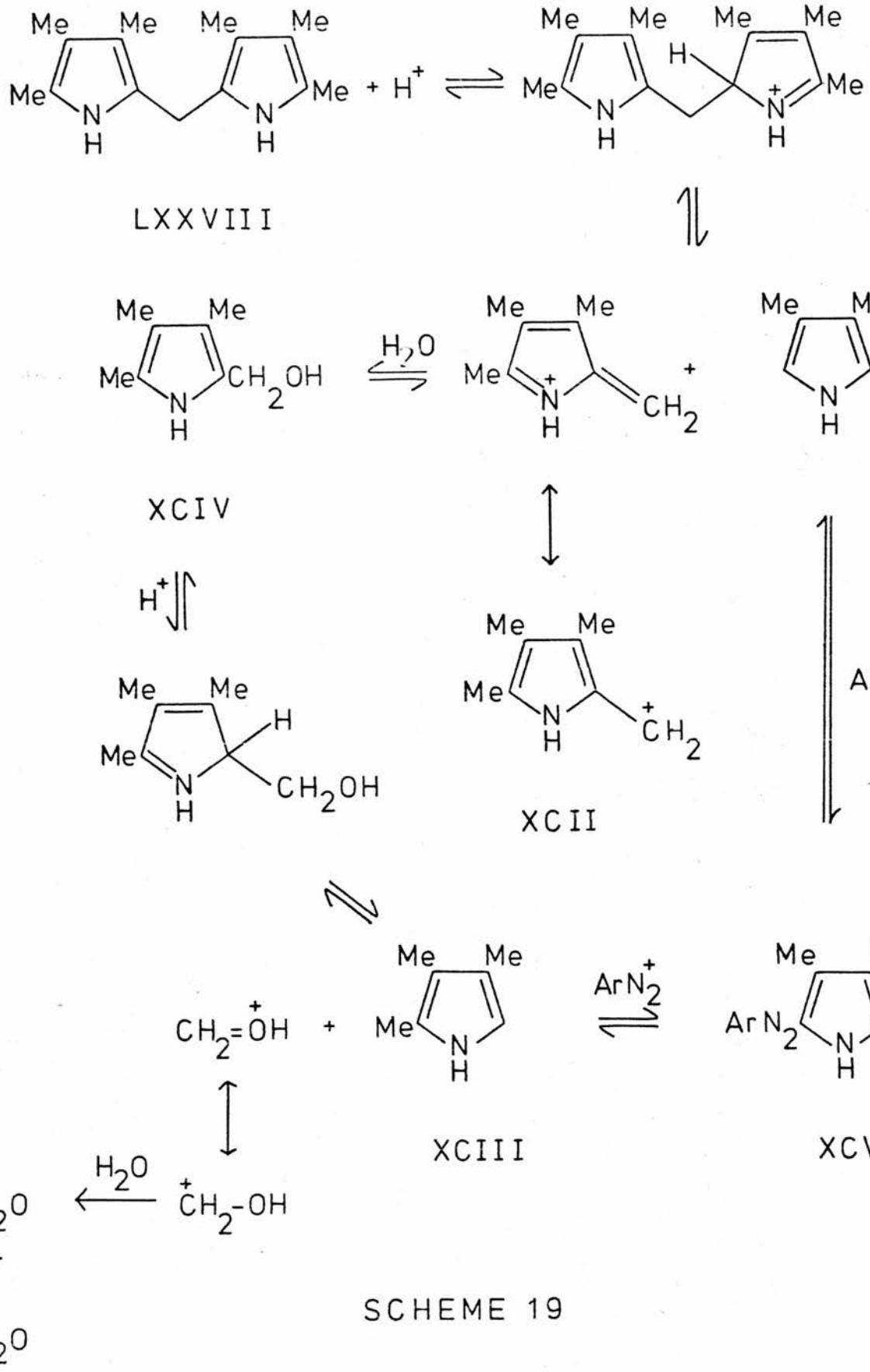
LXXXVII

A series of dipyrrylmethanes (LXXVIII-LXXXV) were synthesised by a variety of procedures. These were described, and their applicability assessed, in the introduction to this chapter. As they are all literature preparations or adaptations thereof, further discussion is not necessary and the methods used in each case are described in the experimental section of this chapter.

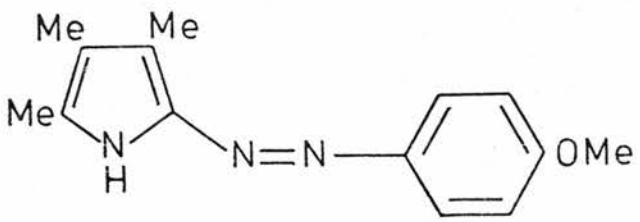
The acid-catalysed condensation product of 1-methylpyrrole and acetone was the previously unreported porphyrin ring-type compound (LXXXVI), which was prepared in the course of the present work during an attempt to synthesise the dipyrrylmethane (LXXXVII). (LXXXVI) is not, however, biologically useful because the pyrrole nitrogens in the ring are all methylated and will not, therefore, complex metal ions.

The dipyrrylmethanes and 2-benzylpyrroles prepared were easily identified from the molecular ion peaks in their mass spectra, and from their ^{1}H-nmr spectra. Many of them proved to be unstable at room temperature and some did not give entirely satisfactory microanalyses because of this.

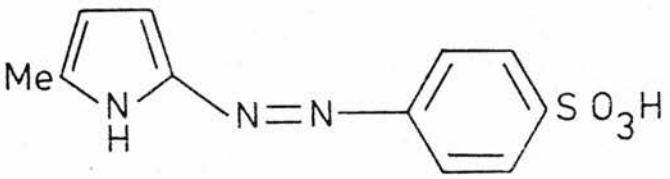
The reactions of several dipyrrylmethanes (LXXVIII-LXXXIII) with arenediazonium ions in aqueous acid solution have been studied. In all of the cases examined the reaction takes place with cleavage of the central methylene bridge. This is released as formaldehyde, which has been isolated as its dimedone derivative in the reaction between (LXXVIII) and excess p-methoxybenzenediazonium tetrafluoroborate, and in the reaction



SCHEME 19



LXXXVIII



LXXXIX

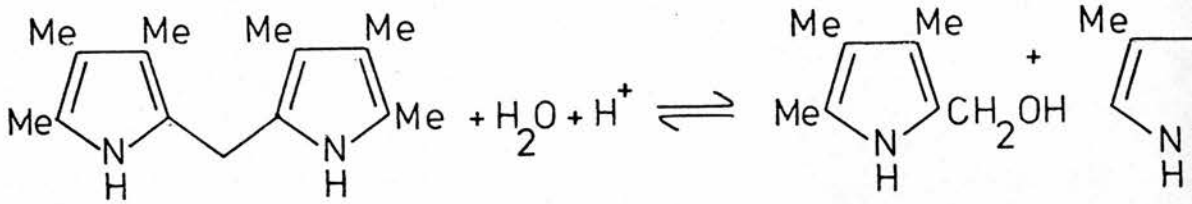
between (LXXXI) and excess diazotised sulphanilic acid, the methyl substituted methylene bridge in (LXXXIII) is released as acetaldehyde in the azo-coupling process and this has also been isolated as its dimedone derivative. The coloured products of the reactions have not been isolated, but spectroscopic evidence indicates that they are the mono-azopyrroles, which are the normal products of reaction under acid conditions[89]. The yellow product of the reaction between (LXXVIII) and p-methoxybenzenediazonium ions has a spectrum identical to that of 4-(2,3,4-trimethylpyrrol-5-ylazo)methoxybenzene (LXXXVIII) (λ_{max} = 468 nm); the reactions of (LXXXI), (LXXXII) and (LXXXIII) with diazotised sulphanilic acid give a coloured product whose spectrum is identical with that of 4-(2-methylpyrrol-5-ylazo)benzenesulphonic acid (LXXXIX) (λ_{max} = 444 nm).

As was the case in the majority of the reactions of the methylpyrroles with arenediazonium ions, no azo-coupling through the methyl groups occurred in the dipyrrylmethanes. The only example of azo-coupling through a methyl substituent occurred in 2,3,4,5-tetramethylpyrrole, where there was no possibility of azo-coupling to the pyrrole ring itself. Methyl substituents make very poor leaving groups and, in general, alkyl groups cannot be displaced by diazo-groups[134]. But where methyl groups are joined to hydroxy or tertiary amino groups, or to a system of conjugated double bonds, then they can be eliminated[135,136].

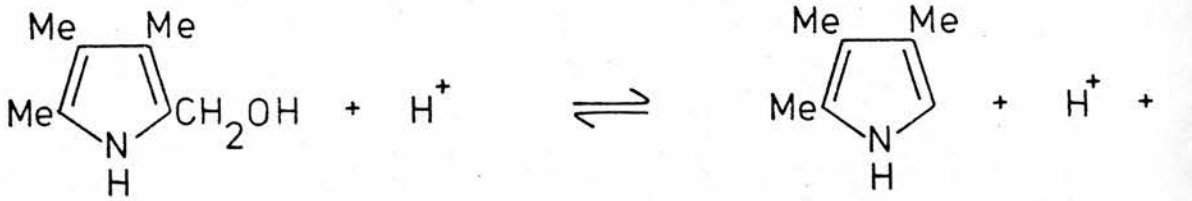
If one postulates Scheme 19 as the mechanism of the reaction of a dipyrrylmethane with arenediazonium ions in acid solution, then it is apparent why the methylene bridge is so readily broken. The leaving group on breaking one half of the methylene bridge is the cationic species

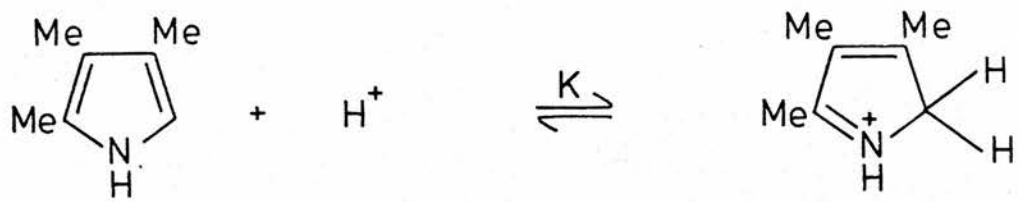
(XCII), which is stabilised by the delocalisation of electrons through the pyrrole ring. This then goes on to react with water to give the 2-hydroxymethylpyrrole (XCIV). When the second part of the methylene bridge is broken, the positively charged $CH_2=\overset{+}{O}-H$ species is released, but this time the charge is mesomerically stabilised by the adjacent –OH group. This then goes on to react with a molecule of water to give formaldehyde. The same reasoning accounts for the azo-coupling reactions of diphenylmethanes, dinaphthylmethanes and di-indolylmethanes described in the introduction to this chapter. The electrophilic attack resulting in cleavage of the methylene bridge in all of these compounds gives rise to resonance stabilised cations as the first and second parts of the methylene bridge are broken. Bilirubin itself reacts in the same way[20,22] (Scheme 1) with arenediazonium ions. It has been established by McDonagh and Assisi[136] that bilirubin also undergoes a reversible acid catalysed cleavage about the central methylene bridge, leading to formation of a mixture of bilirubin isomers. This is further evidence of the susceptibility of the 2-, and 2'-pyrrole ring positions (adjacent to the methylene bridge) towards electrophilic attack. The fact that azo-coupling occurs at the 2-position in dipyrrylmethanes (LXXXI-LXXXIII) rather than the unsubstituted 3- or 4-positions substantiates this.

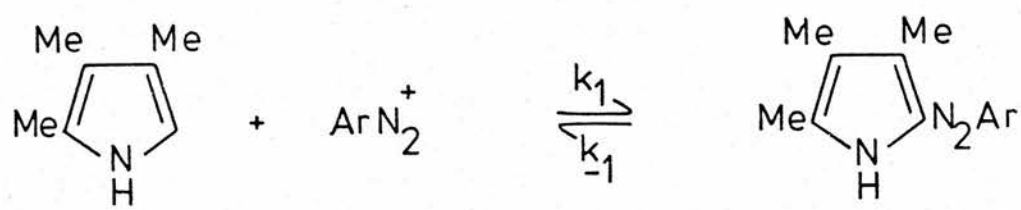
Under the conditions used in the experiments described in this chapter there are two electrophiles present; the proton and the arenediazonium ion. The kinetic evidence now to be described indicates that the proton brings about cleavage of the central methylene bridge of the dipyrrylmethane and that the pyrrole



LXXVIII XCIV XC







XCV

SCHEME 20

monomers thus formed go on to react with arenediazonium ions in the normal way. The mechanism for this process is shown in Scheme 20.

The kinetics of these reactions were followed on the stopped-flow spectrophotometer by observing the appearance of the highly coloured mono-azopyrroles. It was found in all cases that the reaction was first order in appearance of product and, with an excess of arenediazonium ion, the rate constant was independent of the initial dipyrrylmethane concentration. If one assumes that the rate of cleavage of the methylene bridge is much faster than the rate at which the pyrrole monomers subsequently couple with the arenediazonium ions, then the reaction being observed in the stopped-flow spectrophotometer is that between the pyrrole monomers and the arenediazonium ions. If this is the case, the mathematics of the reaction kinetics for the observed reaction are those shown in Appendix 2 for the reaction between pyrroles and arenediazonium ions in acid solution. This assumption leads to the expression for the observed rate constant:

$$k_{obs} = \frac{k_1 [ArN_2^+]}{(K[H^+]+1)} + k_{-1}[H^+] \qquad (11)$$

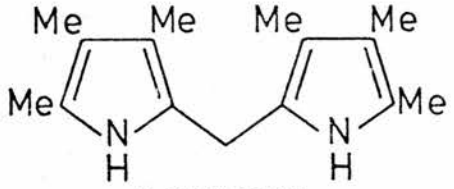
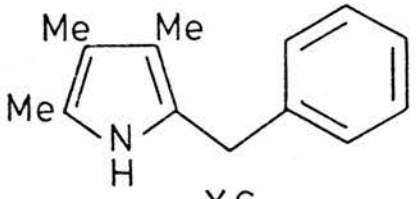
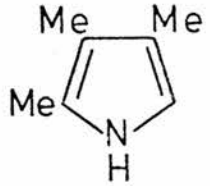
from which values for the second order rate constants k_1 and k_{-1} may be obtained. If the proposed mechanism is correct the rate of reaction of a dipyrrylmethane with arenediazonium ions in acid solution will be the same as that of the corresponding pyrrole monomer and the reaction will be observed as a one step process. Had the arenediazonium ion been the electrophile which brought about cleavage of the methylene bridge, then the reaction would have been observed as a two step process, as the first and second

parts of the methylene bridge were broken, and the resultant molecules of the mono-azopyrroles appeared. The situation where the arenediazonium ion is the only electrophilic species present will be considered later in this chapter.

Table 9 shows the values obtained for the rate constants calculated from equation (11) above for the diazo-coupling reaction of 3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylmethane and some related compounds. The observed reaction is a one step,

Table 9

Data for the reactions of 3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylmethane and related compounds with p-methoxybenzenediazonium ions in acid solution at 25°C

		k_1 (l mol^{-1} s^{-1})	k_{-1} (l mol^{-1} s^{-1})
 LXXVIII	a	47182	6.75
 XC	b	45907	5.62
 XCIII	b	46824	6.31

2,3,4-trimethylpyrrole K = 7943; k_1 and k_{-1}, see equation (11)

a [dipyrrylmethane]$_o$ = 2.5×10^{-5} M, b [pyrrole compound]$_o$ = 5×10^{-5} M

[$MeOC_6H_4N_2^+$] = 0.02 M, [HCl] = 0.05 M

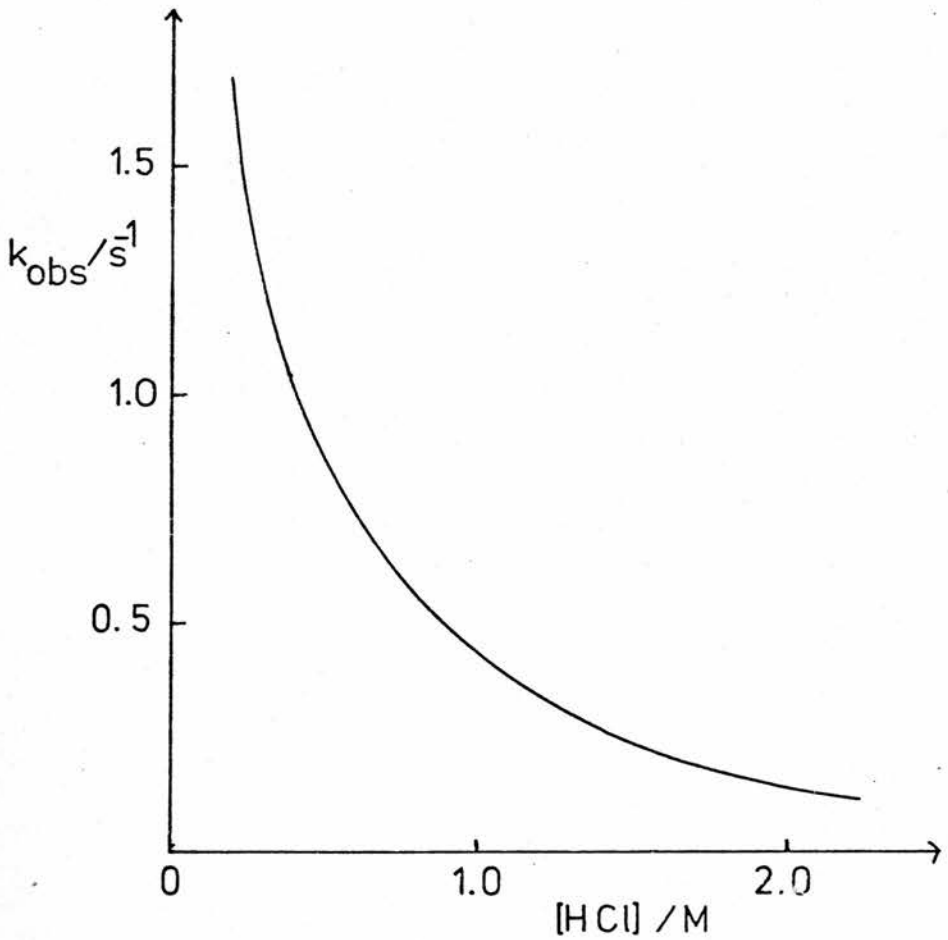


FIGURE 11

Plot of k_{obs} against ac
concentration for t
diazo-coupling react
of LXXVIII, XC an
XCIII with p-meth
benzenediazonium
at 25°C

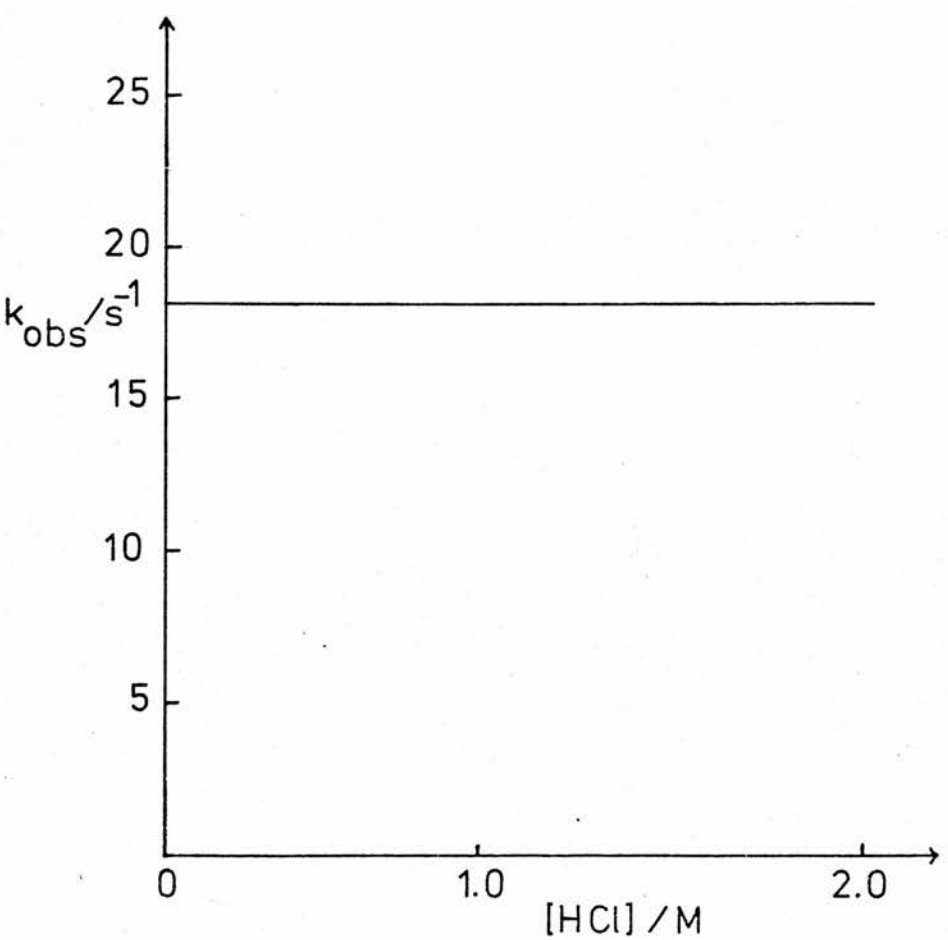
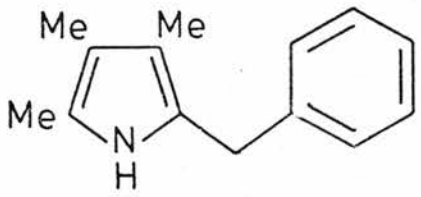


FIGURE 1

Plot of k_{obs} against
concentration for th
diazo-coupling reac
of LXXXI, LXXXII,
and XCVII with dic
sulphanilic acid at

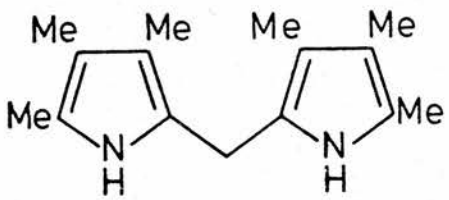


X C

equilibrium process, which implies that a mechanism whereby there is direct attack of the dipyrrylmethane by the arenediazonium ion is unlikely. It can be seen from Table 9 that the values of k_1 for (LXXVIII) and (XC) are essentially the same as that for (XCIII), the 2,3,4-trimethylpyrrole monomer, and the values for k_{-1} are virtually constant also, although they do have a wider spread. This indicates that the same species is reacting with arenediazonium ion in each case and that species is the 2,3,4-trimethylpyrrole monomer. These results, then, are consisted with the proposed reaction scheme.

Further evidence for this mechanism comes from the acidity dependence plots for these reactions. When acid concentration is varied, all others being kept constant (LXXVIII) and (XC) behave in exactly the same way as (XCIII), the same curve being obtained in all three cases (Figure 11). It is therefore reasonable to accept Scheme 20 as representing the reaction mechanism, since the way in which k_{obs} decreases as acid concentration increases can be predicted from equation (11). As $[H^+]$ increases the term containing k_1 decreases and, although the term containing k_{-1} increases, this is relatively small and the net effect of this is to cause the value of k_{obs} to drop.

In the Van den Bergh test the arenediazonium ions are generated <u>in situ</u> by the diazotisation, in the presence of acid, of the corresponding aniline; therefore two electrophiles, the proton and the arenediazonium ion, are always present. This means that the above reaction mechanism (Scheme 20) for the reaction between a dipyrrylmethane and arenediazonium ions



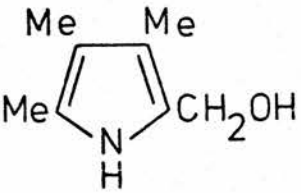
LXXXVIII

+

$MeOC_6H_4N_2^+$

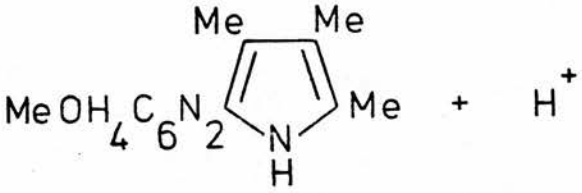
+

H_2O

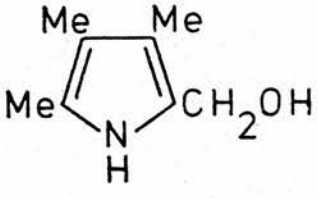


XCIV

+



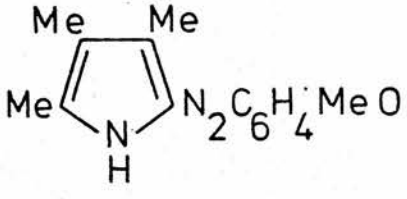
XCVI



XCIV

+

$MeOC_6H_4N_2^+$



XCVI + CH_2O

SCHEME 21

in the presence of acid will also describe the situation in the Van den Bergh test (Scheme 1). However, it is of interest to examine the azo-coupling reaction of dipyrrylmethanes in the absence of acid, with the arenediazonium ion as the only electrophilic species. Bridge cleavage of the dipyrrylmethane must, in this case, be brought about by the arenediazonium ion and the reaction observed will be a two-step process (Scheme 21). The azo-coupling of (LXXVIII) with p-methoxybenzenediazonium tetrafluoroborate was studied in the stopped-flow spectrophotometer. The rate of appearance of the mono-azopyrrole was observed and the reaction did, indeed, take place in two steps. The first step showed good first order kinetics and the results are given in Table 10. The second step showed imprecise kinetics and no rate

Table 10

Data for the reaction between 3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylmethane and p-methoxybenzenediazonium tetrafluoroborate at 25°C in potassium dihydrogen phosphate buffer (pH=7)

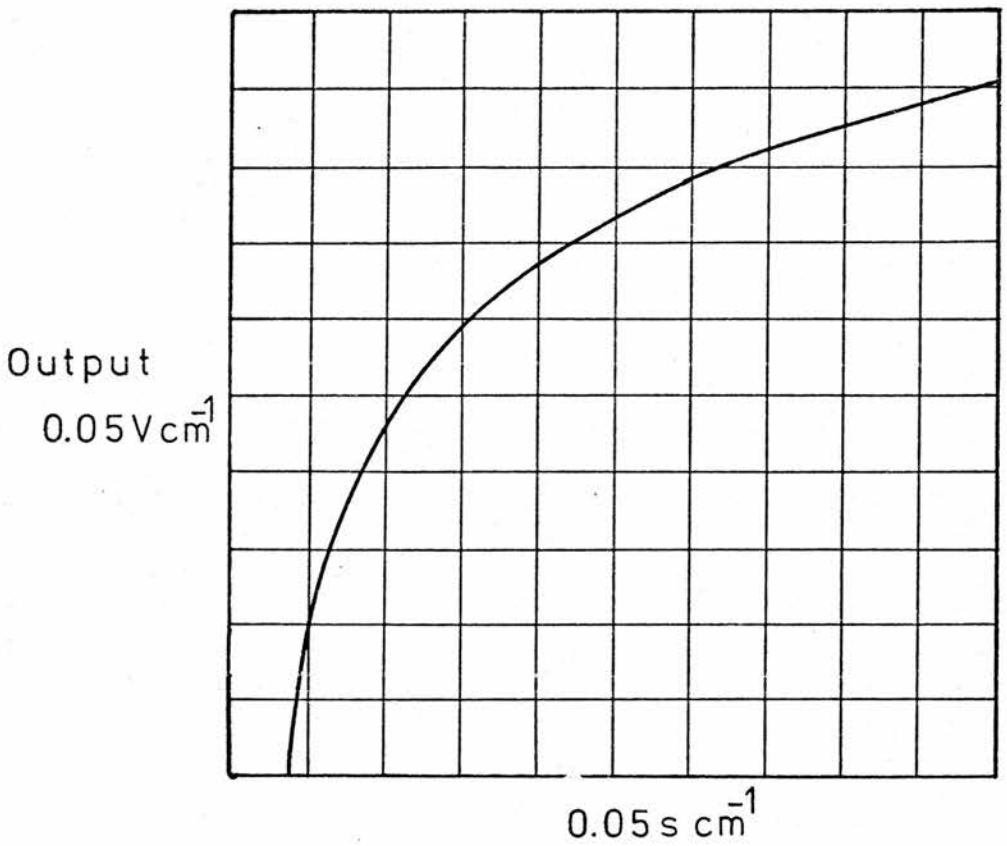
FIRST STEP		SECOND STEP	
k_1 ($l\,mol^{-1}\,s^{-1}$)	k_{-1} ($l\,mol^{-1}\,s^{-1}$)	k_1 ($l\,mol^{-1}\,s^{-1}$)	k_{-1} ($l\,mol^{-1}\,s^{-1}$)
2530	1.60	difficult to determine	

$[MeOC_6H_4N_2^+] = 0.02$ M

$[\text{dipyrrylmethane}]_o = 2.5 \times 10^{-5}$ M

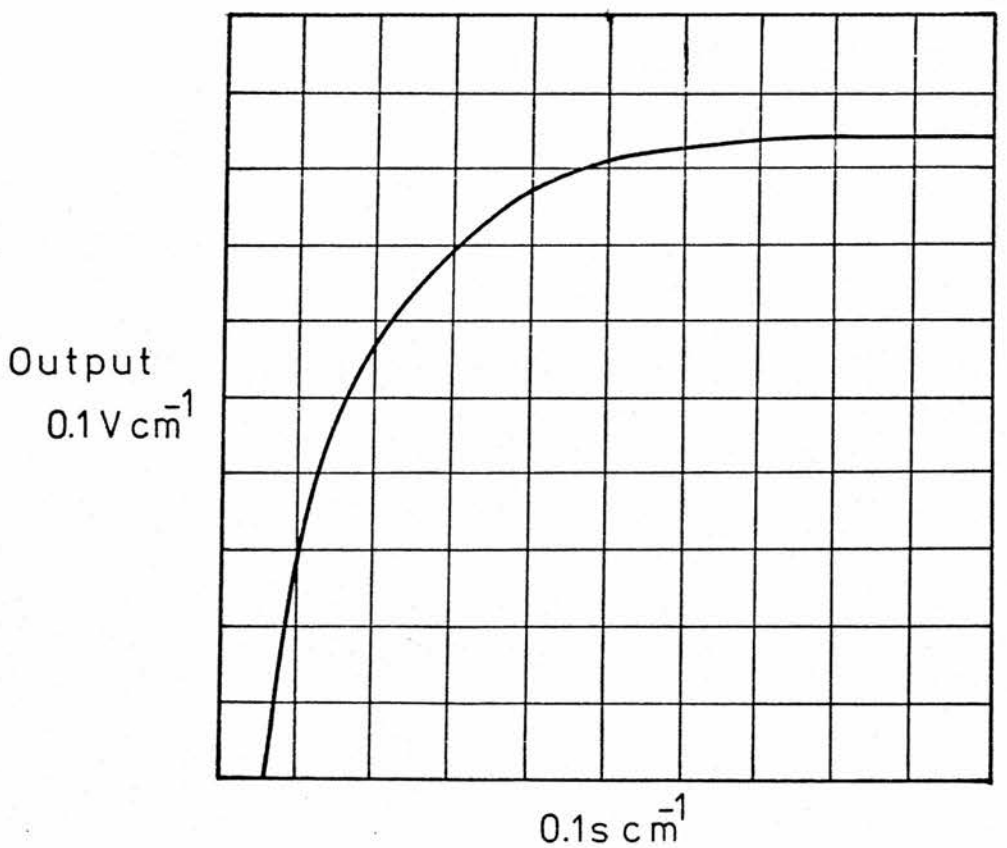
$k_1 = \frac{k_{obs}}{[ArN_2^+]}$

constant could be determined, but this reaction appeared to proceed at a rate which was about one quarter that of the first step. These results, then, are consistent with Scheme 21, and prove that the arenediazonium ion, as well as the proton, can bring about cleavage of the methylene bridge in dipyrrylmethanes.



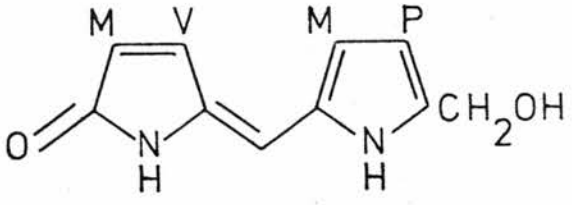
Oscilloscope trace of the reaction between LXXVIII and p-methoxybenzenediazonium tetrafluoroborate in phosphate buffer, pH=7, at 25°C

FIGURE 14



Oscilloscope trace of the reaction between LXXVIII and p-methoxybenzenediazonium ions in 0.05M HCl at 25°C

FIGURE 15



XCVII

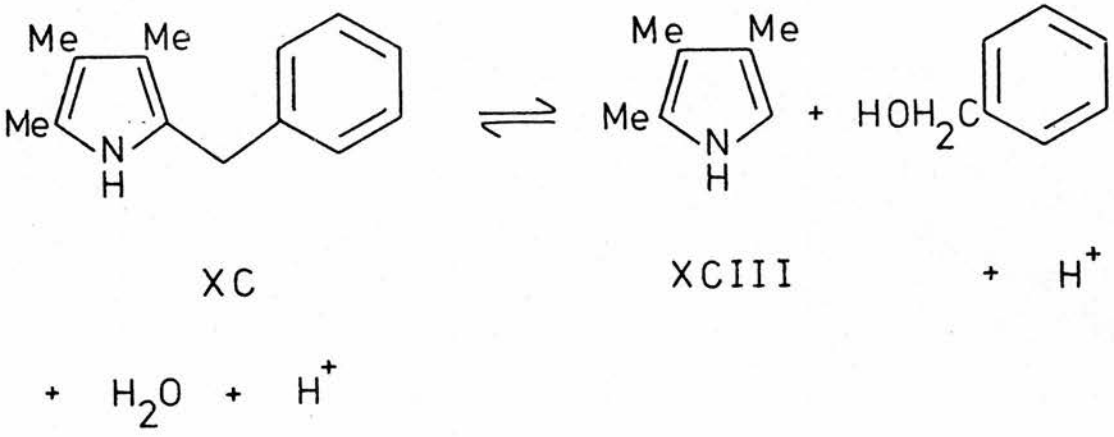
M = CH_3 V = $CH=CH_2$

P = $CH_2CH_2CO_2H$

The type of oscilloscope trace obtained in the stopped-flow study of this reaction is shown in Figure 14. After a first order increase in absorbance from which k_1 and k_{-1} may be calculated, the colour intensity continued to increase due to the second step in the reaction, and this levels off within 1 minute, after which absorbance is constant. This situation may be compared with the azo-coupling reaction of dipyrrylmethanes in the presence of acid, where first order decay curves (Figure 15) were obtained, the reaction occurring in one step.

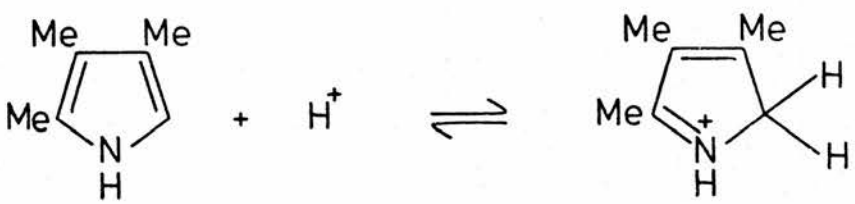
In the reactions described above the role of 2-hydroxymethyl-3,4,5-trimethylpyrrole (XCIV) as an intermediate has been assumed, although there is no direct evidence for its existence. The corresponding intermediate, the 3-hydroxymethylindole (LVI) has been isolated from the reaction of 3,3'-di-indolylmethane with arendiazonium ions by Jackson et al[44] (Scheme 13). Likewise, the intermediate in the bilirubin azo-coupling reaction, the 2-hydroxymethyldipyrrylmethene (XCVII) has been isolated and identified[20,21]. I attempted to isolate (XCIV) from the reaction mixture in this case but was unsuccessful. A sample of (XCIV) was therefore prepared by another route, by the sodium borohydride reduction of the corresponding aldehyde[137]. It proved an extemely unstable species and appeared to decompose rapidly at room temperature; its reaction with arenediazonium ions did not give conclusive, or reproducible, results.

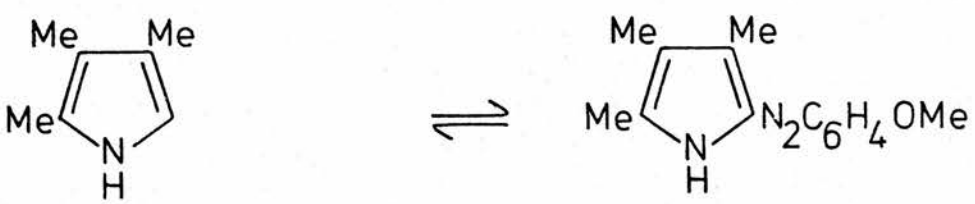
2,3,4-Trimethyl-5-benzylpyrrole (XC) reacts with p-methoxybenzenediazonium ions in the presence of acid in exactly the same way as (LXXVIII) and (XCIII) (Table 9 and Figure 11). It is therefore reasonable to postulate the mechanism shown in



XC ⇌ XCIII + H^+

+ H_2O + H^+





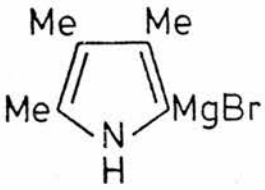
+ $MeOC_6H_4N_2^+$ XCVI + H^+

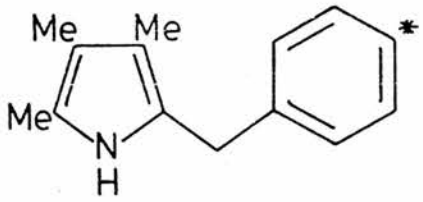
SCHEME 22

9g

$ClCH_2OCH_3$
+ 60% H_2SO_4

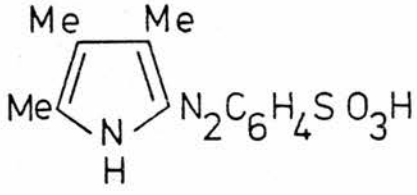
7g, 49%



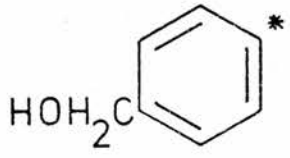


3.2g, 29%

$HO_3SH_4C_6N_2^+$
100% excess



+



0.1ml ≡ 216000 cpm

i.e. 0.154g ≡ 216000 cp

∴ 1mol ≡ 1.774×10^8 c

1.774×10^8 cpm m

1.774×10^8 cpm m

↓ dilute wit
↓ 11.6g ber
alc

Theoretical
molar count = $2.477 \times$

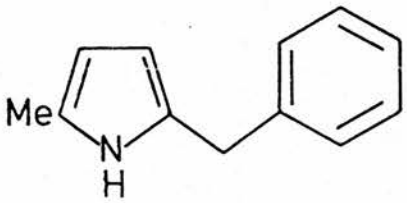
Actual molar count = $1.283 \times$

∴ Molar yield = 52%

SCHEME 23

Scheme 22 for this reaction. Bridge cleavage is much more rapid than attack of the pyrrole monomer by the p-methoxybenzenediazonium ion. This time, however, the second half of the methylene bridge is not broken because the species formed when the first part of the bridge is broken are the pyrrole monomer and benzyl alcohol, which is analogous to the 2-hydroxymethyl-3,4,5-trimethylpyrrole (XCIV) formed in Scheme 20. The difference is that while (XCIV) goes on to react further with acid and arenediazonium ions, the benzyl alcohol is stable and undergoes no further reaction. Consequently it should be isolable from the reaction mixture. To prove that benzyl alcohol was, indeed, formed in this reaction, a sample of 2,3,4-trimethyl-5-benzylpyrrole was prepared with a ^{14}C label in the benzene ring. The preparation of this compound is described in the experimental section of this chapter. Its activity was counted on the liquid scintillation counter.

To isolate the ^{14}C labelled benzyl alcohol the 2,3,4-trimethyl-5-benzylpyrrole prepared above was stirred with an excess of p-methoxybenzenediazonium tetrafluoroborate and the yellow colour due to the mono-azopyrrole developed rapidly. When the reaction appeared to be complete (3 hours of stirring was allowed), the ^{14}C labelled benzyl alcohol was isolated from the reaction mixture by a dilution technique described in detail in the experimental section of this chapter. The activity of this benzyl alcohol was recorded on the liquid scintillation counter (Scheme 23). The expected molar count of the benzyl alcohol was 2.447×10^7 cpm, but the actual molar count was 1.283×10^7 cpm. Therefore the molar



XCI

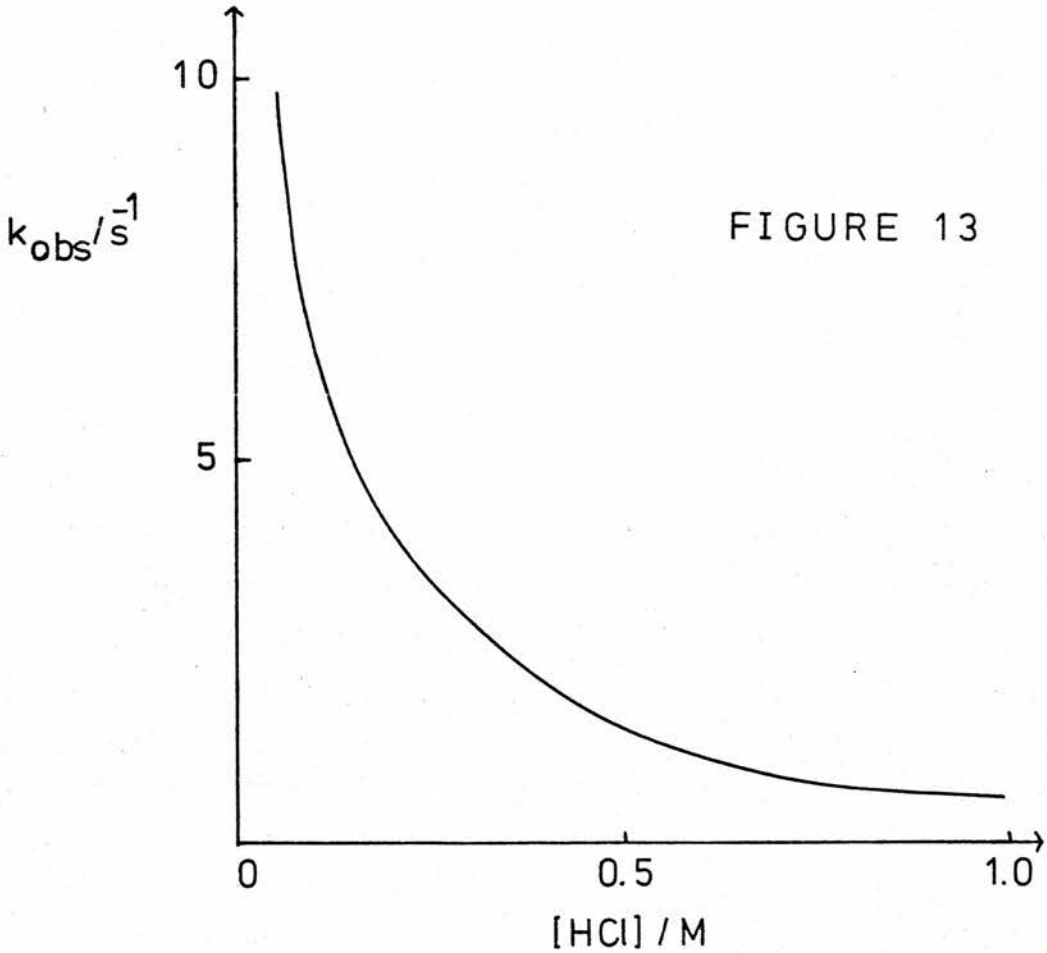
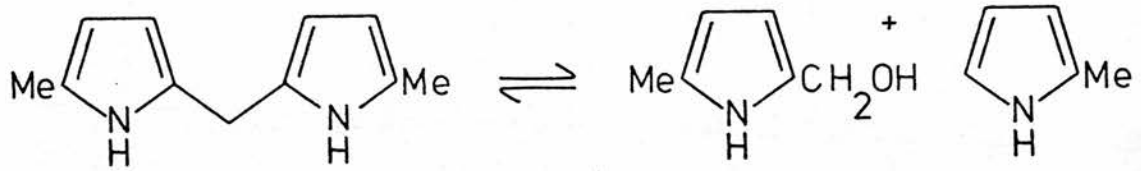


FIGURE 13

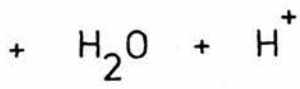
Plot of k_{obs} against acid concentration for the diazo-coupling reaction of LXXVIII and XCIII with p-methoxybenzenediazonium chloride in EtOH/H_2O, 50/50 (V/V) at 25°C

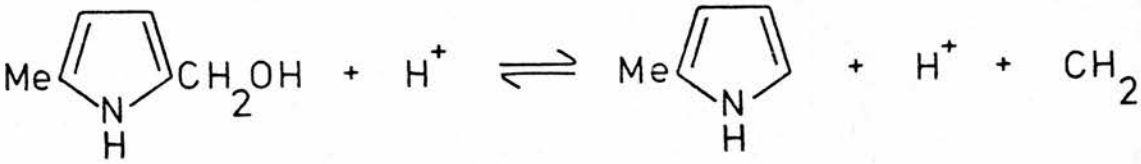
yield of benzyl alcohol in the experiment was 52%. This yield is lower than hoped for, and I do not understand why, but it is sufficiently high to show that the benzyl substituent of the 2,3,4-trimethyl-5-benzylpyrrole is released as benzyl alcohol in the azo-coupling reaction. By analogy, therefore, the intermediate in the azo-coupling reaction of dipyrrylmethanes on cleavage of one half of the methylene bridge is the 2-hydroxymethylpyrrole.

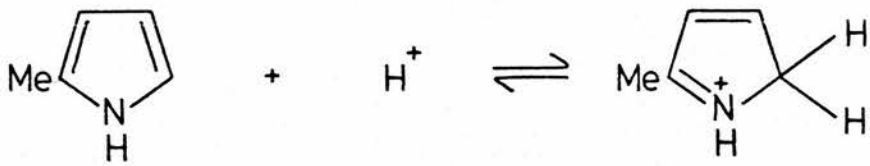
The next set of compounds to be considered are the 3,4-unsubstituted dipyrrylmethanes (LXXXI-LXXXIII) and related compounds (XCI) and 2-methylpyrrole (XCVIII). In spite of the presence of free 3- and 4-positions on the pyrrole rings, cleavage of the methylene bridge still takes place in the azo-coupling reaction and the coloured product of the reaction with diazotised sulphanilic acid is the 4-(2-methylpyrrol-5-ylazo)benzenesulphonic acid (XCIX) i.e. the arenediazonium ion attacks the 2-position of the pyrrole ring. I described earlier in this chapter how the methylene bridge groups of (LXXXI) and (LXXXIII) were isolated as the dimedones of formaldehyde and acetaldehyde respectively. The azo-coupling reactions of the 3,4-unsubstituted dipyrryl and pyrrole compounds were much slower than those of the more fully methylated pyrrole compounds already described (because of the activating effects of the methyl groups) and a more reactive arenediazonium salt, diazotised sulphanilic acid, had to be used in order to obtain a convenient rate of reaction for observation on the stopped-fow spectrophotometer. I have assumed again that bridge cleavage is rapidly brought about by the proton, and that it is the 2-methylpyrrole monomer (XCVIII) which is reacting

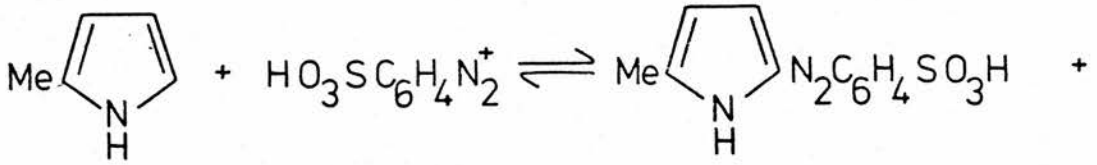


LXXXI XCVIII









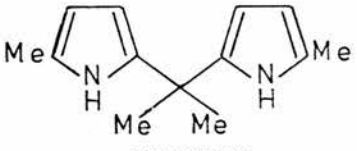
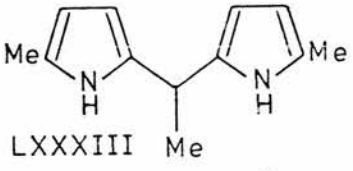
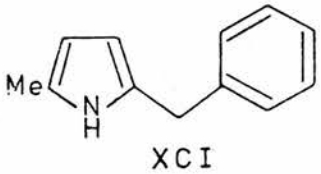
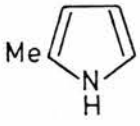
XCIX

SCHEME 24

with the arenediazonium ions (Scheme 24). This being so, the mathematics of the kinetics will be the same as Appendix 2, and the expression for the observed rate constant, k_{obs}, will be equation (11). The calculated second-order rate constants obtained from equation (11) are shown in Table 11. Again the

Table 11

Data for the reactions between various 3,4-unsubstituted dipyrrylmethanes and related compounds with diazotised sulphanilic acid in acid solution at 25°C

		k_1 ($l\ mol^{-1}\ s^{-1}$)	k_{-1} ($l\ mol^{-1}\ s^{-1}$)
 LXXXI	a	565	287
 LXXXII	a	558	213
 LXXXIII	a	570	206
 XCI	b	555	226
 XCVII	b	587	245

a $[\text{dipyrrylmethane}]_o = 2.5 \times 10^{-5}$M, b $[\text{pyrrole}]_o = 5 \times 10^{-5}$M

$[SO_3HC_6H_4N_2^+] = 0.02$ M, $[HCl] = 0.05$ M

observed reaction was a one-step process. The values of k_1 are all about equal, and are similar to the value obtained for k_1 for the reaction between 2-methylpyrrole itself and diazotised sulphanilic acid. The same holds for k_{-1}. Thus the kinetic evidence is consistent with rapid cleavage of the methylene bridge by the proton, followed by a much slower reaction of the 2-methylpyrrole with diazotised sulphanilic acid.

Confirmation of the proposed reaction mechanism (Scheme 24) comes from the acidity dependence plots of the compounds listed in Table 11 (LXXXI-LXXXIII) and (XCI). In all cases k_{obs} behaves in exactly the same way and is independent of acid concentration (Figure 12). This implies that the observed azo-coupling reaction is that of 2-methylpyrrole. Comparison of Figures 11 and 12 indicates that 2,3,4-trimethylpyrrole and 2-methylpyrrole show very different behaviour as acid concentration is varied, but this can be explained in terms of equation (11). 2,3,4-Trimethylpyrrole is a relatively strong base (pK_a=3.9),

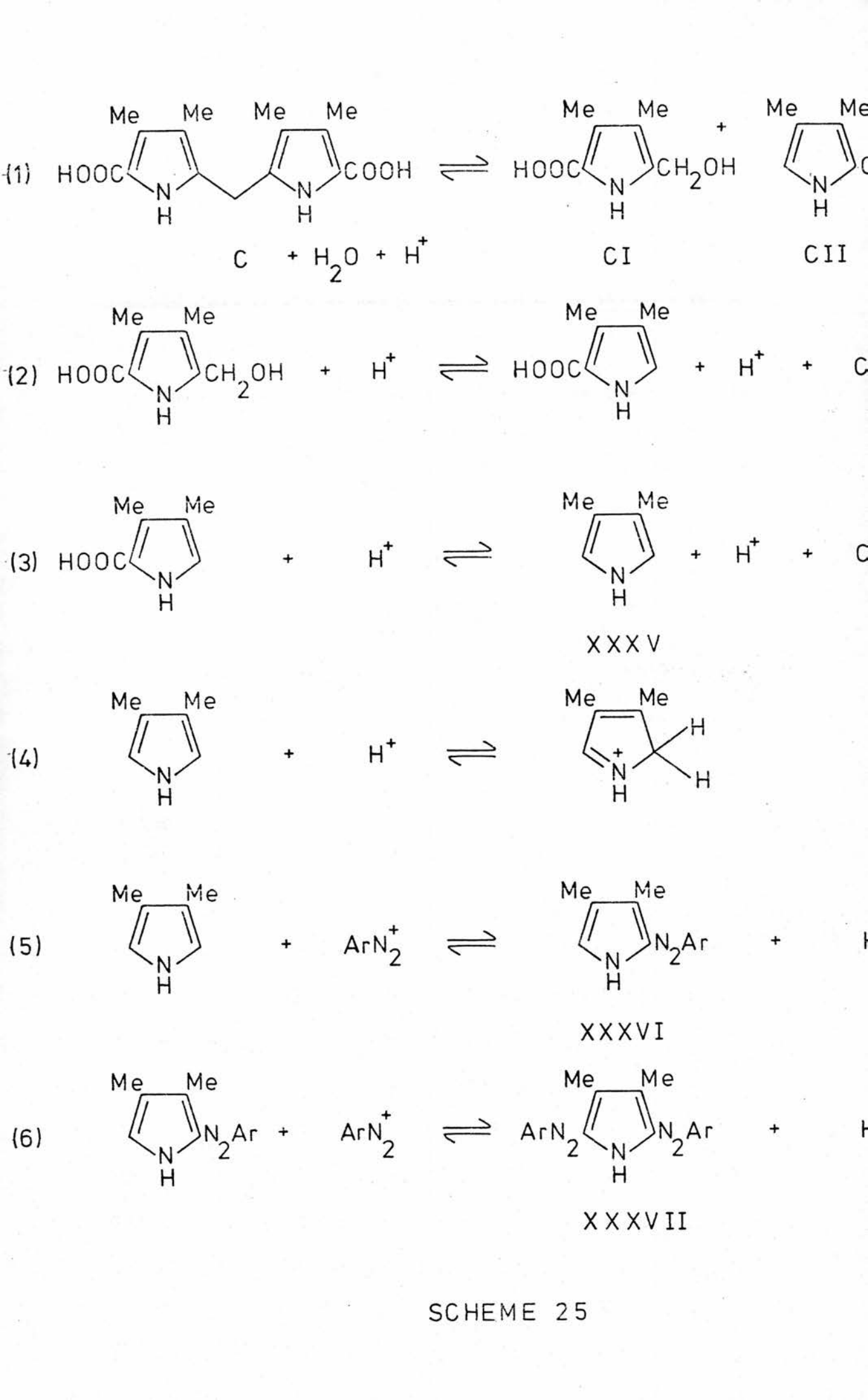
$$k_{obs} = \frac{k_1 [ArN_2^+]}{(K[H^+]+1)} + k_{-1} [H^+] \qquad (11)$$

hence the value for K in equation (11) will be of the order of 10000. Therefore, keeping $[ArN_2^+]$ constant, as $[H^+]$ increases the magnitude of the first term in equation (11) will decrease markedly. An increase in $[H^+]$ will cause an increase in the value in the second term of equation (11), but because k_{-1} is small for the azo-coupling reaction of 2,3,4-trimethylpyrrole (Table 9), this is not large enough to counter the large decrease in the value of the first term as $[H^+]$ becomes greater. The net result, then, is a decrease in

k_{obs} as $[H^+]$ increases. However, 2-methylpyrrole is a weak base (pK_a=-0.21), therefore K is less than 1, and as $[H^+]$ increases, the value of the first term in equation (11) will decrease, but not by as large an amount as with 2,3,4-trimethylpyrrole. Furthermore, k_{-1} is large in the 2-methylpyrrole case, therefore the increase in the value of the second term of equation (11) will have a much greater effect on the value of k_{obs}. The net result in this case is that the decrease in the value of the first term and the increase in the value of the second term as $[H^+]$ becomes larger, balance each other out and k_{obs} does not vary with acid concentration.

Having examined the azo-coupling reactions of dipyrrylmethanes containing only methyl substituents on the pyrrole rings, a much better picture of the reaction taking place in the Van den Bergh test has been obtained. This study has proved that, where acid is present, it is the proton and not the arenediazonium ion which brings about cleavage of the methylene bridge. Unfortunately, it was not possible to observe the rate of bridge cleavage by the proton as it occurred at a rate much faster than the stopped-flow instrument could follow and mixing of the reactants became the rate determining step.

Bilirubin in plasma is present bound to albumin and it is therefore of interest to examine the effect of albumin binding on a dipyrrylmethane. It was necessary to prepare a dipyrrylmethane with a substituent which would bind to albumin, since the dipyrrylmethanes with only methyl substituents contain no such group.


(1)
Me Me Me Me
HOOC COOH
N H N H
C + H2O + H+
Me Me
HOOC CH2OH
+
N H
CI
Me Me
N H
CII
(2)
Me Me
HOOC CH2OH
N H
+ H+
Me Me
HOOC
N H
+ H+ + C
(3)
Me Me
HOOC
N H
+ H+
Me Me
N H
+ H+ + C
XXXV
(4)
Me Me
N H
+ H+
Me Me
H
N H
H
(5)
Me Me
N H
+ ArN2+
Me Me
N2Ar
N H
+
XXXVI
(6)
Me Me
N2Ar
N H
+ ArN2+
Me Me
ArN2 N2Ar
N H
+
XXXVII

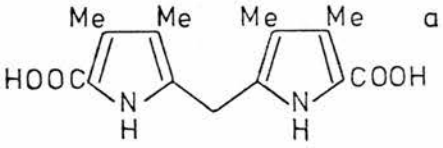
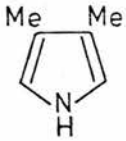
SCHEME 25

The carboxylic acid group is known to bind to albumin and an easily prepared dipyrrylmethane containing this was (LXXX), 3,3',4,4'-tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane.

The kinetics of the azo-coupling reaction of (LXXX) in its unbound form were studied before albumin was incorporated into the system. This time a reaction was observed in two steps. Firstly, the appearance of the yellow colour typical of the mono-azopyrrole, but at a rate much slower than in the 3,3',4,4'-5,5'-hexamethyl-2,2'-dipyrrylmethane case, and a subsequent reaction in which the yellow colour disappears and is replaced by a blue colour which is then stable. The work already described in this chapter has demonstrated that the methylene bridge in dipyrrylmethanes is readily cleaved by acid, and it is also well recorded that the pyrrole carboxylic acids are readily decarboxylated in acid solution[68,69]. If one assumes that, as with methylene bridge cleavage, decarboxylation of the pyrrole by the proton is a much more rapid reaction than azo-coupling, the species which is reacting with arenediazonium ion is the 3,4-dimethylpyrrole (Scheme 25). If this mechanism is correct the second order rate constants for the first azo-coupling step (Scheme 25, step 5) can be calculated using equation (11) and the values for k_1 and k_{-1} thus obtained are shown in Table 12. These agree quite well with the rate constants obtained for the reaction of 3,4-dimethylpyrrole itself, and therefore indicate that the proposed mechanism is correct. The rate of disappearance of the yellow colour of the mono-azopyrrole was followed and was found to be almost independent of the

Table 12

Data for the reaction between 3,3',4,4'-tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane and diazotised sulphanilic acid in acid solution at 25°C

	k_1 ($l\ mol^{-1}\ s^{-1}$)	k_{-1} ($l\ mol^{-1}\ s^{-1}$)
 a	4830	190
 b	5270	284

a $[\text{dipyrrylmethane}]_o = 2.5 \times 10^{-5}$ M $\quad [HCl] = 0.02$ M

b $[\text{pyrrole}] = 5 \times 10^{-5}$M $\quad [SO_3HC_6H_4N_2^+] = 0.02$ M

arenediazonium ion concentration (Table 13). Again this was the situation found with 3,4-dimethylpyrrole itself and has already been discussed in Chapter 1(a) (Table 4). The fading of the yellow colour and the appearance of the blue colour is due to the formation of the 2,4-bis-azopyrrole (Scheme 25, step 6), which is the normal product of reaction under alkaline conditions. It is not surprising that the remaining unsubstituted α-position is attacked by an arenediazonium ion as it is highly activated towards electrophilic attack by the adjacent methyl group, but it is unusual that the rate of formation of the bis-azopyrrole should be independent of arenediazonium ion concentration. I have already described in Chapter 1(a) how 3,4-dimethylpyrrole

Table 13

Variation of k_{obs} with diazotised sulphanilic acid concentration for the disappearance of 4-(3,4-dimethylpyrro-2-ylazo)benzenesulphonic acid at 25°C

$10^3[ArN_2^+]$ /M	2.0	4.0	5.0	8.0	10.0
k_{obs}/s^{-1}	0.21	0.20	0.20	0.18	0.21

$[dipyrrylmethane]_o = 2.5 \times 10^{-5}$M, [HCl] = 0.05 M

readily dimerises in acid solution, and the same may hold for (CIV). If it were the rate of breakdown of this dimer which was the rate determining step in formation of the bis-azopyrrole, then k_{obs} would be independent of arenediazonium ion concentration. The relative rates of steps (1), (2) and (3) in Scheme 25 could not be determined because they all occurred at a rate which was too rapid for observation on the stopped-flow spectrophotometer and it is therefore questionable whether bridge cleavage is more rapid than decarboxylation. But the fact that both of these processes occur much more rapidly than azo-coupling is enough to substantiate the mechanism shown in Scheme 25.

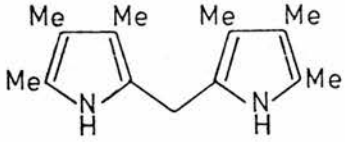
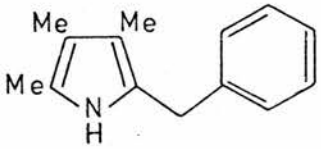
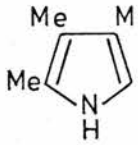
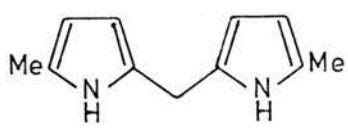
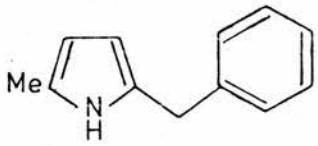
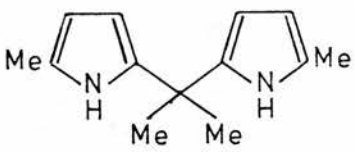
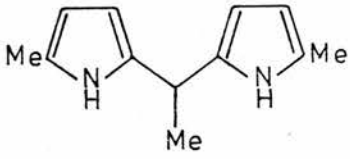
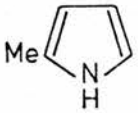
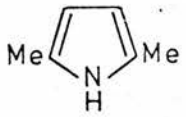
The 3,3',4,4'-tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane was dissolved in a buffer (pH=7) containing an excess of albumin, so that the maximum amount of dipyrrylmethane was albumin-bound. When the azo-coupling reaction of the albumin-bound dipyrrylmethane was studied, it was found that the reaction again took place in two steps. The first step was formation of the yellow colour typical of the mono-azopyrrole, and this again faded to a blue colour in the second step. Decarboxylation

therefore still takes place, even when the carboxylic acid group is bound to albumin. This indicates that the interaction between the carboxylic acid group and the albumin binding site is not very strong since decarboxylation still appears to occur very readily. The reaction was not investigated further because this dipyrrylmethane is not a wholely satisfactory model for bilirubin as it undergoes bis-azo-coupling very readily, whereas the product of reaction in the Van den Bergh test is the mono-azo compound.

The two 3,3'-dipyrrylmethanes (LXXXIV) and (LXXXV) were completely insoluble in water, therefore the kinetics of their azo-coupling reactions were examined in 50% aqueous ethanol (v/v), in which all components of the reaction were soluble. For comparison, therefore, the reaction of the other dipyrrylmethanes and related compounds with arenediazonium ions in acid solution were also studied in this medium; the results are given in Table 14. The 3,3'-dipyrrylmethanes did not exhibit simple first order kinetics and a complicated decay curve was obtained from which no rate constant could be determined. However, the compounds listed in Table 14 did give simple first order kinetics and the reaction observed in the stopped-flow by the appearance of the mono-azopyrrole was again a one step process. Assuming the reaction mechanism to be the same as in aqueous solution (Schemes 20 and 24), values for the second order rate constants k_1 and k_{-1} can be obtained from equation (11). The pK_a values for 2,3,4-trimethylpyrrole and 2-methylpyrrole in 50% aqueous ethanol(v/v) are not known,but they can be estimated approximately[138].

Table 14

Data for the reactions of dipyrrylmethanes and related compounds with arendiazonium ions in acid solution, using 50% aqueous ethanol (v/v) as the solvent at 25°C. Corresponding values for aqueous solution are shown in parentheses.

		k_1 ($l\ mol^{-1} s^{-1}$)	Arenediazonium Ion ($X\text{-}C_6H_4N_2^+$) X
	a	114000 (47200)	MeO
	b	119000 (45900)	MeO
	b	121000 (45800)	MeO
	a	631 (565)	SO_3H
	b	537 (555)	SO_3H
	a	655 (558)	SO_3H
	a	673 (570)	SO_3H
	b	649 (587)	SO_3H
	b	1073 (758)	SO_3H

$^+]=0.02M$ a $[\text{dipyrrylmethane}]_o = 2.5 \times 10^{-5} M$ b $[\text{pyrrole}]_o = 5 \times 10^{-5} M$ $[HCl]=0.05M$

Alcohols weaken both acids and bases; Hall and Sprinkle[139] have plotted the curves of pK_a against decreasing alcohol concentration from 97% to 10% ethanol in water for eighteen aliphatic and aromatic amines. They found that the average depression of pK_a by 50% aqueous ethanol was 0.54 (max. 0.88, min. 0.26). This average was used in the present study to estimate the pK_a of 2,3,4-trimethylpyrrole, 2-methylpyrrole and 2,5-dimethylpyrrole in 50% aqueous ethanol, by subtraction from their pK_a values in water. The values obtained were 3.36, -0.75 and -1.25 respectively. The rate constants calculated using these values are shown in Table 14. Again the observed azo-coupling reaction is a one step process, which implies that it is the pyrrole monomer which is reacting with the arenediazonium ion in each case, hence the reaction mechanism is the same as in aqueous solution. This was confirmed by the acidity dependence plots which were made for (LXXVIII) and (XCIII). These were identical and are shown in Figure 13.

Penton and Zollinger[108] have found that the rates of the azo-coupling reaction of 4-toluenediazonium salts with N,N-dimethylaniline in tetramethylene sulphone, acetonitrile, water and nitromethane at 30°C are the same within a factor of 5. This is a very small variation in rate from solvent to solvent and is attributed to the fact that the reaction does not involve significant solvent reorganisation of the reagents in the formation of the transition state. This is because neither the arenediazonium ions, which are very weak electrophiles, nor the N,N-dimethylaniline, are strongly solvated. Therefore changing solvent has

very little effect on the rate of reaction. It was also found by Penton and Zollinger that the anion of the arenediazonium salt (HSO_4^- or BF_4^-) had no significant effect.

The results obtained for the azo-coupling reactions of pyrroles and dipyrrylmethanes in water and in 50% ethanol may be compared. The second order rate constant (k_1) for the reaction in aqueous solution are shown in parentheses in Table 14. These results confirm the observations of Penton and Zollinger that the rate of reaction is almost independent of solvent because of the weak solvation of the weakly electrophilic arenediazonium ions. The values of k_1 for the reaction of 2-methylpyrrole and its derivatives are particularly good examples with which to illustrate this. Here, k_1 is essentially the same for both solvent systems, with the largest difference being less than 20% in the case of (LXXXII). The value for k_1 for the azo-coupling reaction of 2,3,4-trimethylpyrrole and its related compounds increases by a factor of 3 in 50% aqueous ethanol (v/v); this is still quite a small increase and is not significant because of the uncertainty in the value of the pK_a in this solvent. As 2,3,4-trimethylpyrrole is a strong base, any inaccuracy in determining the pK_a value will have a marked effect on the value obtained for k_1 (equation (11)); the value of 0.54 for the depression of pK_a in 50% ethanol was only an average value, and had this been instead about 0.30, which is well within the range of values obtained by Hall and Sprinkle, then k_1 values of the same order would have been obtained for the azo-coupling reactions of the 2,3,4-trimethylpyrrole compounds in water and in 50% ethanol. 2-Methylpyrrole and

2,5-dimethylpyrrole are weak bases, with K_a less than unity, so that any inaccuracy in estimating their pK_a values in 50% ethanol will not greatly affect k_1, which is virtually independent of K . This is why there is a much better correlation between the values of k_1 for the 2-methylpyrrole compounds in water and in 50% ethanol and for 2,5-dimethylpyrrole in the two solvent systems than for the 2,3,4-trimethylpyrrole compounds.

EXPERIMENTAL:

Materials: Amines were recrystallised or distilled before use and converted to the arenediazonium salts by reaction with sodium nitrite and HCl. The dipyrryl and benzyl pyrrole compounds were synthesised as follows:

3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylmethane (LXXVIII):

(i) 3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylketone: The Grignard reagent of 2,3,4-trimethylpyrrole was prepared by the reaction between the pyrrole (15 g) and ethyl magnesium bromide which had been prepared from magnesium (4.4 g) and bromoethane in dry ether (30 ml). The method used was that of McCay and Schmidt[140]. To the pyrryl Grignard reagent a solution of phosgene in toluene (12.5% w/w, 70 ml) was added slowly and stirring continued for 30 minutes, after which the reaction mixture was poured slowly onto ice (300 g). The dipyrrylketone precipitated out and was filtered off and washed well with alcohol and ether. Recrystallisation

from ethanol gave 3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylketone (7 g, 40%), m.p. 250-252°C (lit. 253-255°C)[141].

(ii) 3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylmethane: Under nitrogen, a solution of the dipyrrylketone (7 g), in refluxing ethanol (95%, 100 ml), containing morpholine (4 ml), was treated with sodium borohydride (6 x 2 g), added portionwise over 3 hours. Water (6 ml) was added 15 minutes after the addition of each portion of the borohydride. The mixture was refluxed for a further 2 hours, poured into water (200 ml) and extracted with ether (4 x 30 ml). The combined ether extracts were washed with water, the solvent removed, and the residue extracted with petroleum ether 40/60. The petroleum ether extracts were then concentrated to give white crystals of (LXXVIII) on cooling. (Yield 1.5 g, 23%) m.p. 85-87°C $C_{15}H_{22}N_2$ requires: C, 78.21; H, 9.63; N, 12.16 Found: C, 77.20; H, 10.99; N, 11.27% m/e 230 (M^+); δ ($CDCl_3$) 1.98 (6H, s), 2.02 (6H, s), 2.17 (6H, s), 3.93 (2H, s).

5,5'-dimethyl-2,2'-dipyrrylmethene hydrochloride (LXX):

(i) 2-methyl-5-formylpyrrole: 2-methylpyrrole (3 g) was formylated at the 5-position using the method of Silverstein et al[112]. (Yield, 1.58 g, 46%) m.p. 67°C (lit. 68°C[142]); δ ($CDCl_3$) 2.40 (3H, s), 6.07 (1H, s), 6.9 (1H, s), 9.3 (1H, s).

(ii) 5,5'-dimethyl-2,2'-dipyrrylmethene hydrochloride[128]: 2-methyl-5-formylpyrrole (4 g) was dissolved in dry ether (400 ml). To this was added 2-methylpyrrole (3 g). The mixture was then stirred at room temperature and HCl was bubbled through the solution at a

moderate rate for 5 minutes. The red crystals of the dipyrrylmethene hydrochloride (LXX) precipitated out, were allowed to settle, were filtered off and washed with ether. (Yield 5.7 g, 62%), decomp. at 130°C. $C_{11}H_{13}N_2^+Cl^-$ requires: C, 63.31; H, 6.28; N, 13.42 Found: C, 62.57; H, 6.94; N, 13.13%. m/e 172 (M^+-HCl)- δ(DMSO-d_6) 2.13 (3H, s), 2.22 (3H, s), 2.57 (1H, s), 5.78 (4H, s).

5,5'-dimethyl-2,2'-dipyrrylmethane (LXXXI)[129]:

5,5'-dimethyl-2,2'-dipyrrylmethene hydrochloride (3 g), was dissolved in absolute methanol (100 ml). To this was added Pd/charcoal (0.6 g), which was washed into the flask with absolute methanol (50 ml). This was then attached to the hydrogenation apparatus and the mixture stirred for 48 hours under hydrogen at atmospheric pressure. When the required amount of hydrogen had been taken up, the hydrogenation was stopped and the Pd/charcoal catalyst filtered off. The bulk of the methanol was then evaporated off and the remaining solution added to water (200 ml). The aqueous solution was then extracted with solvent ether, basified and extracted with ether again. The ether extracts were then dried and concentrated and the product separated from unreacted dipyrrylmethene hydrochloride on an alumina column with solvent ether/methanol 50/50 (v/v) as the elutant. Concentration of the elutant yielded the 5,5'-dimethyl-2,2'-dipyrrylmethane (LXXXI) which was recrystallised from ether/pet. ether. (Yield 0.4 g, 15%) m.p. 84-86°C $C_{11}H_{14}N_2$ requires: C, 75.80; H, 8.10; N, 16.08 Found: C, 75.46; H, 8.39; N, 15.66%. m/e 174 (M^+), δ ($CDCl_3$) 2.15 (6H, s), 3.88 (2H, s), 5.87 (2H,s), 6.19 (2H, s).

2-methyl-5-benzylpyrrole (XCI)[133]:

Ethyl magnesium bromide was prepared from magnesium (2.0 g) and bromoethane (10.8 g) in dry ether (30 ml) under nitrogen. The reaction mixture was cooled to 0°C and a solution of 2-methylpyrrole (5.8 g) in ether (10 ml) was added dropwise with stirring. Stirring was continued at 0°C for another 5 hours after which ammonium chloride solution (0.1 M, 25 ml) was added over 1 hour. The mixture was then separated, the aqueous layer extracted with solvent ether and the combined extracts dried over $MgSO_4$. The solvent was removed and the residue re-distilled to give the 2-methyl-5-benzylpyrrole. (Yield 3.5 g, 29%) b.p. 90-95°C 0.1 mm. $C_{12}H_{13}N$ requires: C, 84.17; H, 7.65; N, 8.18. Found: C, 83.43; H, 6.83; N, 7.21%. m/e 171 (M^+). δ ($CDCl_3$) 2.10 (3H, s), 3.07 (2H, s), 5.85 (1H, s), 6.10 (1H, s), 7.00 (5H, m).

2,3,4-trimethyl-5-benzylpyrrole (XC)[133]:

Prepared by the same method as (XCI) above using 2,3,4-trimethylpyrrole (8 g). (Yield 4.1 g, 35%) b.p. 101-105°C, 0.1 mm) $C_{14}H_{17}N$ requires: C, 84.3; H, 8.60; N, 7.03; Found: C, 83.41; H, 8.23; N, 6.82%. m/e 199 (M^+), δ ($CDCl_3$) 1.98 (3H, s), 2.04 (3H, s), 2.19 (3H, s), 3.00 (2H, s), 7.00 (5H, m)

5,5'-dimethyl-2,2'-dipyrrylethane (LXXXIII)[143]:

2-Methylpyrrole (1 g), acetaldehyde (3 ml) and conc. HCl (1 small drop) were refluxed together over a steam bath for 30 minutes. The excess acetone was then evaporated off over the steam bath and the residue dissolved in solvent ether and filtered. The 5,5'-dimethyl-2,2'-dipyrrylethane was then precipitated from

the solution by the addition of petroleum ether 40/60 and was recrystallised from ether/pet. ether 40/60 (Yield 0.5 g, 22%) m.p. 140-142°C. $C_{12}H_{16}N_2$ requires: C, 76.56; H, 8.56; N, 14.87; Found: C, 75.73; H, 7.90; N, 14.51%. m/e 188 (M^+) δ ($CDCl_3$) 1.16 (3H, s), 2.10 (6H, s), 3.48 (1H, s), 5.85 (2H, s), 6.10 (2H, s).

5,5'-dimethyl-2,2'-dipyrrylpropane (LXXXII):

Same method as for (LXXXII) above. Acetone (3 ml) was used instead of acetaldehyde. (Yield 0.6 g, 24%) m.p. 128-130°C $C_{13}H_{18}N_2$ requires: C, 77.19; H, 8.97; N, 13.85. Found: C, 76.53; H, 8.77; N, 13.00%. m/e 202 (M^+) δ ($CDCl_3$) 1.20 (6H, s), 2.15 (6H, s), 5.87 (2H, s), 6.12 (2H, s).

2,2',5,5'-tetramethyl-3,3'-dipyrrylpropane (LXXXIV):

Same method as (LXXXIII) above. 2,5-Dimethylpyrrole (1 g) and acetone (3 ml) were used (Yield 0.3 g, 24%) m.p. 175-176°C (lit. 174-175°C)[143].

2,2',5,5'-tetramethyl-3,3'-dipyrrylethane (LXXXV):

Same method as (LXXXIII) above. 2,5-Dimethylpyrrole (1 g) and acetaldehyde (3 ml) were used. (Yield 0.5 g, 24%), m.p. 172-174°C. $C_{14}H_{20}N_2$ requires: C, 77.73; H, 9.32; N, 12.95. Found: C, 76.66; H, 9.52; N, 12.61%. m/e 230 (M^+) δ($CDCl_3$) 1.18 (3H, s), 2.12 (6H, s), 2.28 (6H, s), 3.54 (1H, s), 5.93 (2H, s).

Attempted synthesis of 1,1'-dimethyl-2,2'-dipyrrylpropane (LXXXVII):

Same method as (LXXXIII) above. 1-Methylpyrrole (1 g) and acetone (3 ml) were used. The product of the reaction was not

(LXXXVII) but the tetrapyrrole ring compound (LXXXVI) which precipitated out as white crystals which were recrystallised from solvent ether/ethanol. (Yield 1.5 g, 25%) m.p. 187-188°C. $C_{32}H_{44}N_4$ requires: C, 79.16; H, 9.28; N, 11.56; Found: C, 79.19; H, 9.40; N, 11.36%. m/e 484 (M^+) δ ($CDCl_3$) 1.57 (24H, s), 2.84 (12H, s), 5.98 (8H, s).

3,3',4,4'-tetramethyl-2,2'-dipyrrylmethane-5,5'-dicarboxylic acid (LXXX):

This was prepared by the method of Fischer and Walach[125].

3,3',4,4'-tetramethyl-5,5'-dicarbethoxy-2,2'-dipyrrylmethane (LXXIX):

This was prepared by the method of Fischer and Walach[125].

2-hydroxymethyl-5-methylpyrrole (XCVII):

This was prepared from 2-formyl-5-methylpyrrole by the method of Silverstein et al[137].

Preparation of ^{14}C-labelled 2,3,4-trimethyl-5-benzylpyrrole and isolation of the ^{14}C-labelled benzyl alcohol:

(a) ^{14}C-labelled benzyl chloride: ^{14}C-Labelled benzene (9 g) was dissolved in chloromethyl methyl ether (60 g) and to this was added conc. sulphuric acid (25 g), dropwise with stirring. The mixture then stood for a few minutes and was diluted with water (200 ml). The ^{14}C-labelled benzyl chloride separated out and was extracted into methylene chloride/pet. ether 50/50, dried over $MgSO_4$ and the solvent removed. The residue was then redistilled and the pure ^{14}C-labelled benzyl chloride was obtained (b.p. 180-182°C, 760 mm). Yield 7g, 49%.

(b) ^{14}C-labelled 2,3,4-trimethyl-5-benzylpyrrole: Prepared by the same method as (XC) above[133], using the ^{14}C-labelled benzyl chloride (Yield 3.2 g, 49%)

(c) Isolation of the ^{14}C-labelled benzyl alcohol: An aqueous solution of benzenediazonium chloride p-sulphonic acid (12 g, 100% excess) was added to a solution of ^{14}C-labelled 2,3,4-trimethyl-5-benzyl-pyrrole (6 g) in ethanol/solvent ether (100 ml; 70:3) v/v) with stirring. The yellow colour of the mono-azopyrrole developed rapidly and the solution was stirred for 3 hours to ensure complete reaction. Unlabelled benzyl alcohol (10 ml) was added to the reaction mixture, followed by water (100 ml) which caused phase separation to occur. The benzyl alcohol went into the predominantly organic solvent layer. The aqueous layer was extracted with solvent ether and the ether extracts combined, dried over $MgSO_4$ and concentrated to a small volume. Elution on an alumina column with chloroform removed the azopyrrole which remained and the benzyl alcohol obtained on evaporation of the solvent was purified by redistillation (b.p. 90-93°C, 10 mm). The benzyl alcohol thus obtained was tlc pure and its activity was counted on the liquid scintillation counter (See Scheme 23).

Isolation of the dimedone derivative of formaldehyde from the azo-coupling reaction of 3,3',4,4',5,5'-hexamethyl-2,2'-dipyrryl-methane[22]

3,3',4,4',5,5'-hexamethyl-2,2'-dipyrrylmethane (0.2 g) was dissolved in dichloroethane/methanol 50/50 (100 ml) to which a solution of benzenediazonium chloride (0.5 g in 20 ml H_2O) was

added. The mixture was stirred at room temperature for 2 hours, after which it was evaporated to dryness, the solvents being distilled into a solution of dimedone (0.2 g) in methanol (50 ml). The dimedone solution was then refluxed for 30 minutes, cooled, the solvents removed, and the residue was eluted down an alumina column using petroleum ether(60/80)/solvent ether (3:1, v/v) as the elutant. The dimedone derivative of formaldehyde was isolated and was recrystallised from ethanol/water. White crystals (yield 0.16 g, 32%), m.p. 182-184°C. δ ($CDCl_3$) 1.10 (12H, s), 2.31 (8H, s), 3.15 (2H, s).

Isolation of the dimedone derivative of acetaldehyde from the azo-coupling reaction of 5,5'-dimethyl-2,2'-dipyrrylethane:

Same procedure as above. (Yield 0.1 g, 28%), m.p. 138-140°C (lit. 141-142°C)[144]. δ ($CDCl_3$) 1.12 (12H, s), 1.53 (3H, s), 2.26 (1H, s), 2.33 (8H, s).

Kinetics: A "Canterbury" stopped-flow spectrophotometer and a Unicam SP500 were used for the kinetic studies. Observation wavelengths were in the range 420-450 nm. Spectra of the coloured products were recorded on the Unicam SP800 spectrophotometer.

Rate constants were calculated by the method of Kezdy and Swinbourne (Appendix 3).

CHAPTER 3

THE BINDING ABILITY OF ALBUMIN -

A Comparison of the binding of bromocresol green and bromocresol purple to serum albumin

INTRODUCTION:

Bilirubin is practically insoluble in water except at alkaline pH, when it is present as a salt[7]. In plasma bilirubin is solubilised by being bound to a protein and in normal plasma it is bound to more than one protein[7]. By far the greatest part of the bilirubin is bound to albumin, but it has been reported that a little is bound to the α-globulins and the β-globulins[7]. Proof of the interaction between bilirubin and albumin is given by the modification of the visible absorption spectrum of bilirubin in the presence of albumin, characterised by the shift of the peak from 433 to 460 nm[7]. This does not occur in the presence of any other of the plasma proteins, which indicates that these proteins do not bind significant quantities of bilirubin. A photometric titration of bilirubin with bovine serum albumin on one hand, and human serum albumin on the other hand shows that the first binds 20 mg g^{-1}, the second 21 mg g^{-1} [8]. The binding of bilirubin to albumin has been demonstrated by several methods: fluorescence quenching[145], ultrafiltration[146], electrophoresis and ultracentrifugation[147], and precipitation with proteins[148]. There are 40 g l^{-1} of albumin in plasma, which can, theoretically, bind 840 mg of bilirubin[7]. It has been shown that, even in the most severe cases of jaundice, no bilirubin concentrations of more than 500 mg l^{-1} of plasma have been recorded[7], hence, whatever the severity of hyperbilirubinaemia in jaundice, the concentration of albumin in plasma is sufficient to bind all the circulating bilirubin, although, as I have already stated, very small amounts are linked with the α- and β-globulins.

Fluorescence quenching studies of the binding of bilurubin

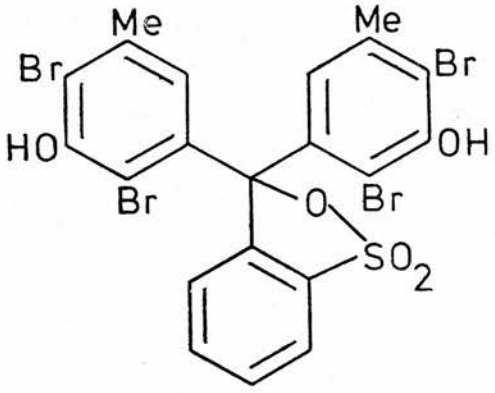
to albumin have been carried out by Levine[145], who found that there are two types of binding site on the albumin molecule for bilirubin. The first site binds one molecule of bilirubin and has a binding affinity (K_a) of 1×10^8 l mol^{-1}, and the second site also binds one molecule of bilirubin, but with a weaker binding affinity, 3.0×10^6 l mol^{-1}. These values did not change within the pH range 7.3-7.5 (blood pH). The albumin binding sites for bilirubin are often assumed to be non-specific sites and will bind any organic anion. Exact information on the structure of these sites is lacking, but the very high affinity for bilirubin, particularly of the first site, suggests a great degree of stereospecificity. While typical man-made anionic drugs have binding affinities of approximately 10^4 l mol^{-1} [149], that for the first bilirubin binding site is 10^8 l mol^{-1} and even the weaker binding site has a K_a of 10^6-10^7 l mol^{-1}. Such K_a values rival the affinity of antibodies for their antigens, where Ka's may be 10^7-10^9 l mol^{-1} i.e. a highly stereospecific interaction. It therefore seems likely that the bilirubin binding sites on albumin possess a high degree of stereospecificity.

Organic anions such as salicylate or 2-(4'-hydroxybenzene-azo)benzoate bind to albumin and have been used as indirect measures of the binding capacity of albumin for bilirubin[150]. Bromocresol green binds to numerous sites on albumin, and has a high affinity for the site of bilirubin binding[151]. However, such dyes are poor molecular analogues of bilirubin and it would appear that such anions estimate the nonspecific capacity to bind organic anions rather than the very specific bilirubin binding capacity of albumin.

They can, therefore, provide no estimate of the bilirubin binding affinity of albumin.

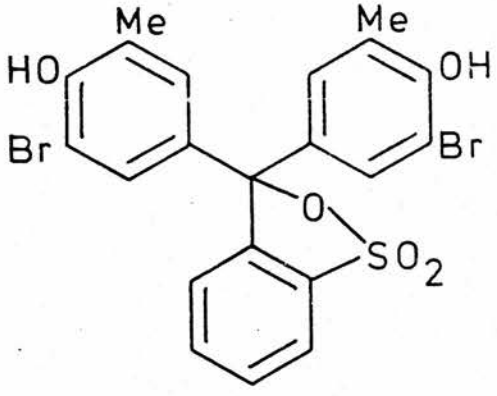
Dye binding methods do, however, yield information on the different types of binding sites present in albumin. The two most suitable dyes for this purpose are bromocresol green and bromocresol purple, which are also used clinically to determine albumin concentration in human serum. The nature and extent of the binding between albumin and bromocresol green has been investigated by Rodkey[45], who studied this process at pH=6.95, and found that there are two classes of binding sites. One binds three molecules of indicator with a $K_a = 7.0 \times 10^5$ $1\ mol^{-1}$, and the other binds five molecules of indicator with a $K_a = 2.0 \times 10^4$ $1\ mol^{-1}$. These binding constants have 'normal' values for the binding of organic anions to albumin, but are much lower than those for the binding of bilirubin, which is much more stereospecific. The amount of dye which is bound by albumin is very pH dependent i.e. at low pH essentially all of the bromocresol green is bound (pH less than 3.5) whereas at high pH (greater than 11) only a small amount is bound.

Measurement by dye binding is an important clinical method for determination of serum albumin concentration. Since Rodkey[152] first described it, the method employing bromocresol green has been the most widely used. When albumin binds bromocresol green the visible spectrum of the dye changes and there is a large increase in absorbance at 632 nm[47]. It is this increase in absorbance, when compared with that brought about by albumin solutions of known concentration, which is a measure of the albumin content of a sample of serum. The principal drawback of this method is that it is not



Bromocresol green

CIII



Bromocresol purple

CIV

specific for albumin since other proteins in serum also react with the bromocresol green reagent under the conditions of most assays[153] and after the initial increase in absorbance at 632 nm due to binding to albumin it continues to increase slowly. Therefore it usually leads to an overestimation of the albumin content of serum when compared with more specific methods such as electrophoresis and immunoprecipitation[154]. Webster[47] has found that the bromocresol green method routinely overestimates albumin by 3 g l^{-1} and suggests that if this quantity is subtracted from the result obtained, a reliable measure of albumin content is given. The overestimation has been variously attributed to reaction with 'acute phase reactants' (other plasma proteins)[155], time allowed for colour development[153], reagent composition and source of specimens[156]. The investigation into the binding of bromocresol green to albumin carried out in the present work was done with a view to clarifying this situation.

Pinnell and Northam[51] have suggested the use of bromocresol purple instead of bromocresol green, since this method did not overestimate albumin. They found that bromocresol purple was much more specific for albumin, and that once the initial increase in absorbance at 600 nm due to the binding of the dye was over, no further increase in absorbance took place and the results obtained gave accurate determinations of albumin concentration. This marked difference in behaviour cannot be due to a difference in structure of the dye as bromocresol green (CIII) and bromocresol purple (CIV) have very similar structures.

It is claimed[51] that the difference in behaviour is due to the different pH's at which of the two reagents are used. Pinnell and Northam[51] suggest that the slow reaction of bromocresol green is due to electrostatic binding to cationic sites on other proteins than albumin. The pH of the bromocresol green reagent is 4.2[153], that of bromocresol purple is 5.2[51]. By increasing the pH, the number of cationic sites available for binding is reduced and the slow reaction will also be reduced. This matter is dealt with in this chapter.

The rates of these two dye binding processes have been investigated on the stopped-flow spectrophotometer in the present work and the results are reported in this chapter. A comparison of the two methods has been made on the basis of the rate data obtained and reasons why there is a slow secondary reaction of albumin with bromocresol green, but not with bromocresol purple, are suggested. Because of the virtually identical behaviour of bovine serum albumin and human serum albumin in these reactions, the former was used in the present study. All investigations were carried out on serum albumin solutions rather than on samples of human serum, so that only the reaction between the dye and the albumin molecule itself was being observed.

RESULTS AND DISCUSSION:

The bromocresol green method for albumin determination, consistently overestimates the albumin concentration[47]. Gustafsson[155] has suggested that bromocresol green reacts with

serum proteins in two steps, the first being an immediate reaction due to albumin, and a second, slower reaction, which he claims is a measure of other species present.

Using the stopped-flow spectrophotometer it was possible to observe the reaction betweeen dye and albumin from the moment of mixing, and for a period of ten minutes afterwards; the time taken for reaction could therefore be determined. The bromocresol green reagent used had a pH=4.2, and was made up according to the method of Webster[47]. Its spectrum before and after mixing with albumin was recorded and these are shown in Figure 16. There is a large increase in absorbance at 632 nm on binding to albumin, with a corresponding decrease in absorbance of the peak at 430 nm. Because the photomultiplier of the stopped-flow instrument appeared to be insensitive at wavelengths greater than 600 nm, the reaction was followed satisfactorily at 590 nm. Reaction conditions were used which reflected almost exactly those of the clinical test[47], except that solutions of bovine serum albumin were used instead of samples of plasma. This eliminated the possibility of the dye binding to any other of the plasma proteins, which would complicate the situation and make any attempt at a kinetic study difficult.

A typical decay curve obtained on mixing the bromocresol green reagent with albumin solution is shown in Figure 17. This shows that the reaction between bromocresol green and albumin is a two step process i.e. a rapid initial reaction followed by a slower increase in absorbance which continues for several minutes. The bromocresol green is in excess and the first step is first order in appearance of product for which k_{obs}, the experimentally

determined rate constant, can be calculated by the method of Kezdy and Swinbourne (Appendix 3). This proved to be 7.19 s^{-1} at 25°C (Table 15) and gave a half-life for the reaction of 0.096 seconds. The initial reaction between bromocresol green and

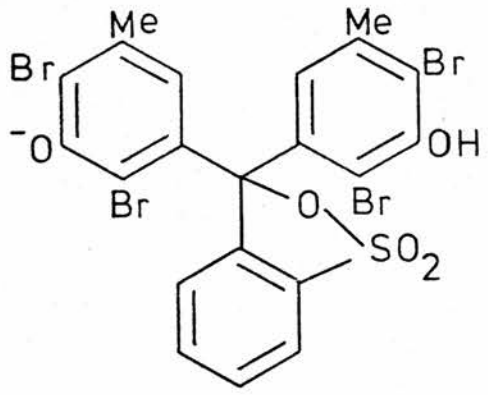
TABLE 15

Data for the immediate reaction between albumin and bromocresol green reagent at five different temperatures

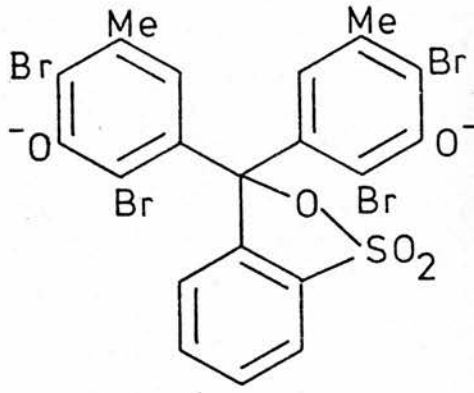
T (°C)	k_{obs} (s^{-1})
15	3.95
20	5.75
25	7.19
30	5.47
35	3.50

[albumin] = 1.51×10^{-6} M [bromocresol green] = 6.22×10^{-5}M pH = 4.2, 'Brij-35' surfactant solution,

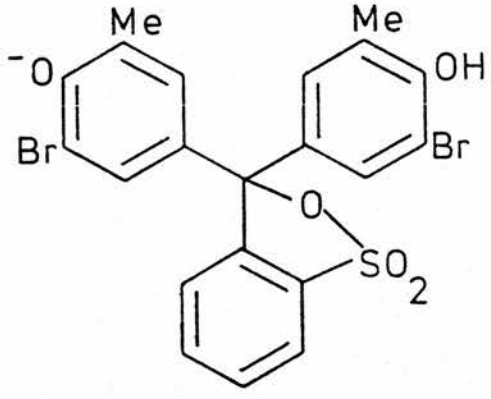
albumin is therefore effectively over within 1 second. The slow reaction which follows this must be due to the binding of more molecules of bromocresol green to albumin by a different mechanism to that involved in the first step. It is known that there are two classes of binding sites for bromocresol green on albumin[45]; one class have a binding affinity (K_a) of 7.0×10^5 l mol^{-1} and bind a total of three molecules of bromocresol green. The other binding sites have a binding affinity of 2.0×10^4 l mol^{-1} and bind five molecules of bromocresol green. The nature of these binding



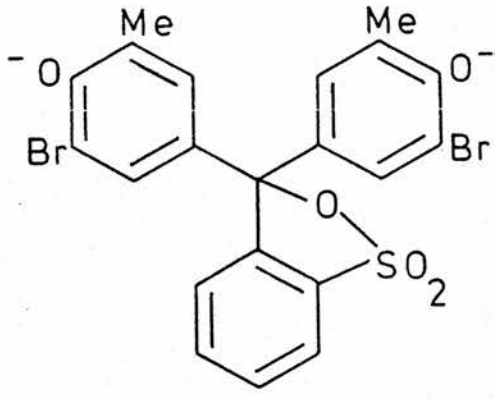
CV



CVI



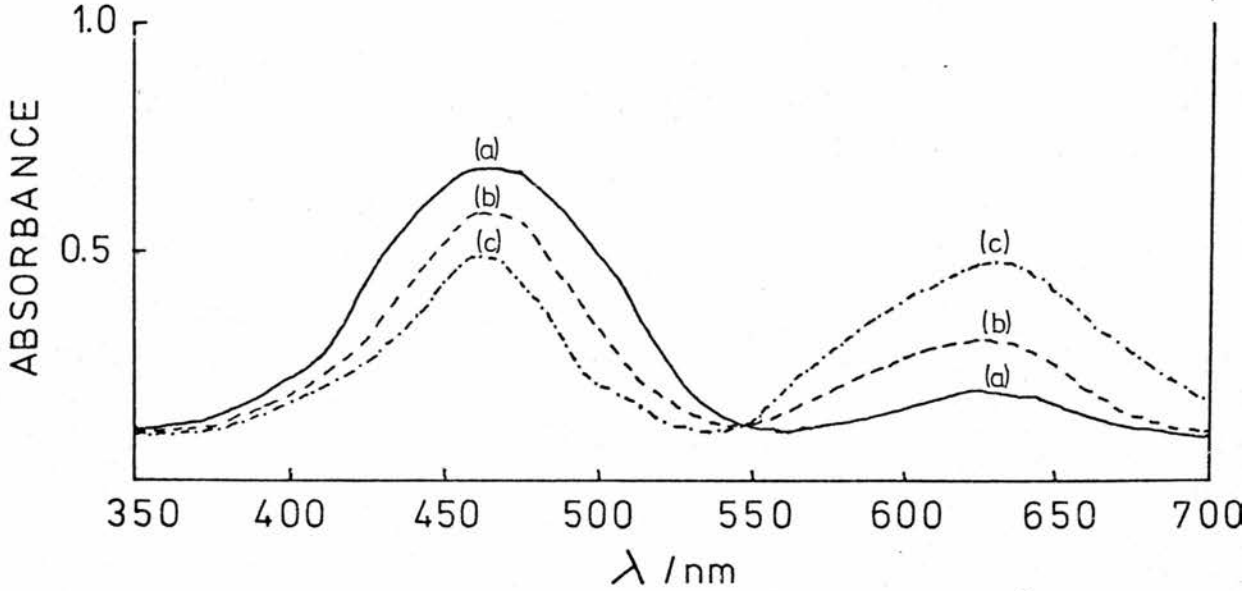
CVII



CVIII

sites on albumin will be dealt with in more detail later in this chapter. Bromocresol green exists both as a univalent (CV) and as a bivalent (CVI) anion, both of which are bound to albumin at the pH at which the test is carried out (4.2)[45]. I would suggest then, that the first step in the above reaction is due to the binding of the bromocresol green to the high affinity binding sites and that the slower increase in absorbance which follows this represents the binding of bromocresol green to the weaker binding sites on the albumin molecule. Hence the slow reaction, described by Webster[47] and attributed by him to a 'non-specific' reaction with other serum proteins can, in fact, be accounted for by a reaction between bromocresol green and albumin itself. Webster describes the 'immediate (30 s) reaction' and claims that this is the true measure of albumin content, but I have used the bromocresol green reagent prepared by Webster and have found that the immediate reaction is effectively over within 1 second at 25°C. Therefore the 'immediate (< 30 s) reaction' should read the 'immediate (< 1 s) reaction'. Since this is very rapid it is impossible to measure absorbance before the secondary process is under way, but the results from the bromocresol green method are obtained by comparison with standard albumin solutions, and presumably the same, two step, reaction is occurring in each case, therefore if readings are taken consistently at the same time after mixing, even although the secondary process is under way, fairly accurate albumin determinations should be given.

In the serum environment, many species other than albumin are present, and it is known that other plasma proteins, such as transferrin



Spectrum of: (a) bromocresol green reagent, 6.22×10^{-5} M

(b) (a) + albumin solution, 1.51×10^{-6} M, with no Brij-35

(c) (a) + albumin solution, 1.51×10^{-6} M, with Brij-35

Succinate buffer, pH = 4.2

FIGURE 16

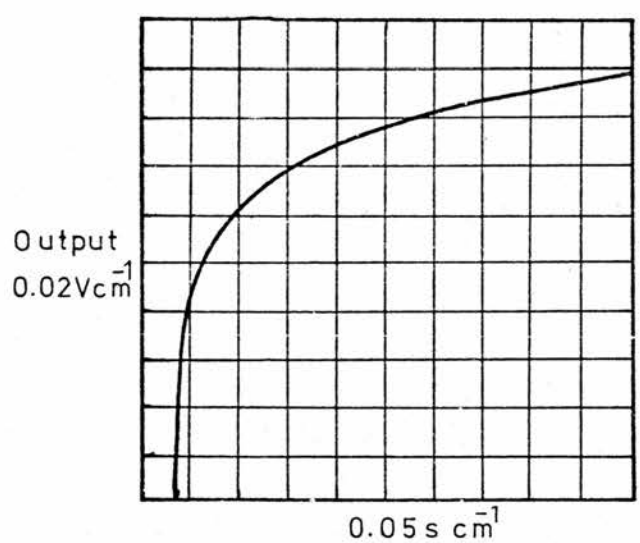


FIGURE 17

Oscilloscope trace of the re
between bromocresol green re
albumin solution at 25°C in
succinate buffer, pH = 4.2
[albumin] = 1.51×10^{-6}M
[bromocresol green] = 6.22×
λ_{obs} = 590 nm

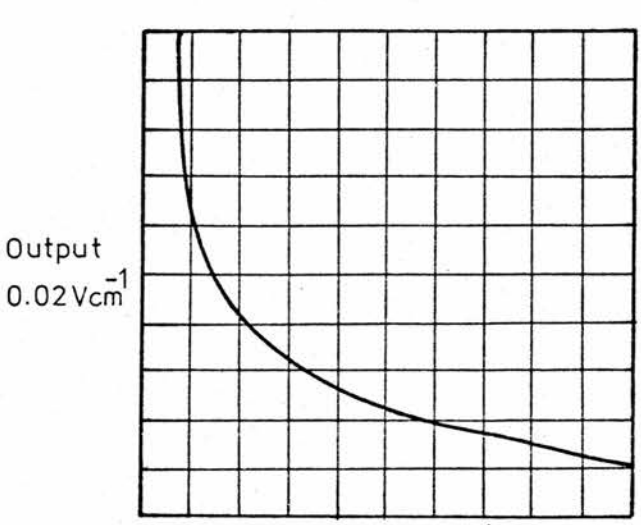


FIGURE 18

Oscilloscope trace of the r
between bromocresol green
and albumin solution at 25
succinate buffer, pH = 4.2
[albumin] = 1.51×10^{-6} M
[bromocresol green] = 6.22
λ_{obs} = 430 nm

and the globulins, bind bromocresol green[51]; this will contribute to overestimation of albumin, even when absorbance is read at the same time after mixing. Webster found a fairly constant overestimation of albumin of 3 g l^{-1}, which was independent of the albumin concentration of the sample. Since he took his readings at a constant time interval after mixing, this cannot be attributed to the secondary reaction between albumin and bromocresol green, but must be due to the other plasma proteins. However, by subtracting 3 g l^{-1} from each result he obtained accurate answers for albumin concentrations which compared well with those determined by the more specific method of immunoprecipitation. But because of the secondary albumin/bromocresol green reaction it is very important that absorbance is read consistently at the same time after mixing so that the correction to be made is the same in each case. Webster suggests allowing 10-20 seconds for colour development, and this would appear a reasonable time interval since the secondary bromocresol green/albumin reaction and the non-specific reaction with other proteins will not be far advanced at this stage.

The decrease in absorbance at 430 nm (Figure 16) as albumin binds bromocresol green was also followed in the present study and an oscilloscope trace of the type shown in Figure 18 was obtained. The pattern is the same as that for the increase in absorbance at 590 nm (Figure 17) i.e. a fast initial reaction which was over in 1 second, followed by a much slower decrease in absorbance which took several minutes for completion. Therefore the decrease in absorbance at 430 nm is probably due to the same

process as the increase in absorbance at 590 nm.

The bromocresol green reagent as prepared by Webster, and used in the above experiments contain 'Brij-35' surfactant. I also prepared a bromocresol reagent without the 'Brij-35', and found that, on mixing with albumin solution, the same reaction took place as above (Table 16); except that the increase in absorbance at 590 nm and the decrease in absorbance at 430 nm were halved, therefore the reaction is not so sensitive in the

TABLE 16

Data for the immediate reaction between albumin and bromocresol green reagent at 25°C in the absence of (a) 'Brij-35' surfactant and (b), sodium chloride

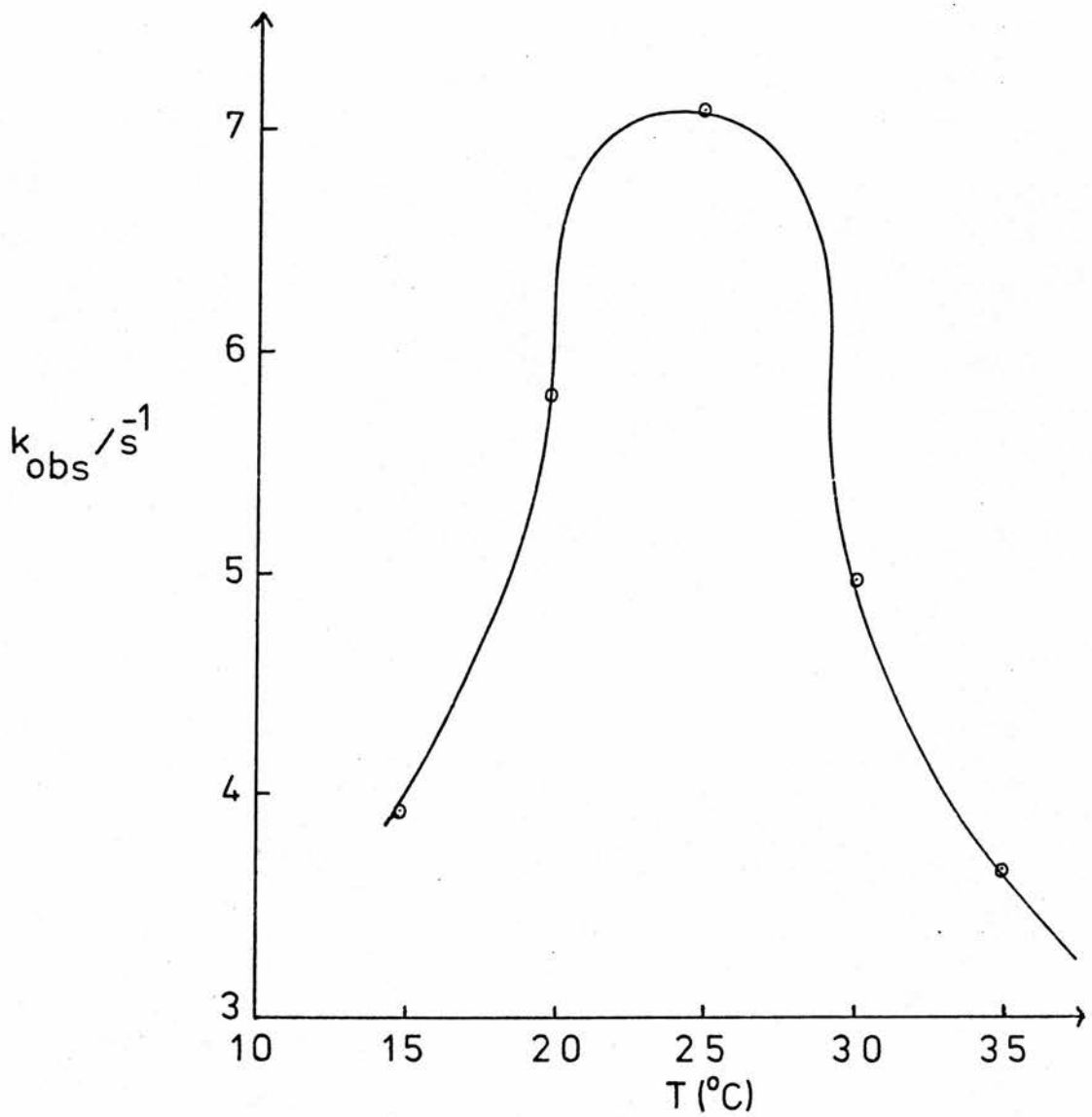
	k_{obs} (s^{-1})
(a) No 'Brij-35'	7.25
(b) No Sodium Chloride	7.36

[albumin] = 1.51×10^{-6} M [bromocresol green] = 6.22×10^{-5} M

succinate buffer, pH = 4.2

absence of the surfactant. Bromocresol green tends to form a green fibrillar precpitate with albumin, an effect which is greatest at the isoelectric point (pH=4.3) of the complex[157]. This precipitation is inhibited, but not completely eliminated, by the presence of 'Brij-35' surfactant. This explains the difference in absorbance in the presence and absence of the surfactant.

Gustafsson[155] reported that the use of sodium chloride solution in the dilution of the serum sample prior to mixing with the bromocresol green reagent causes erroneously high albumin



Plot of k_{obs} against T for the immediate reaction between bromocresol green and albumin.

succinate buffer, pH = 4.2

[albumin] = 1.51×10^{-6} M

[bromocresol green] = 6.22×10^{-5} M

FIGURE 19

values to be given. This was attributed by him to a conformational rearrangement of one or more of the non-albumin proteins so that the rearranged protein will then react more quickly with the bromocresol green reagent as if it were albumin. He therefore suggested that distilled water be used instead of sodium chloride solution in the dilution step. In the present study I have found that the presence or absence of sodium chloride made no difference to the immediate reaction between bromocresol green and albumin itself (Table 16).

The effect of temperature on the rate of reaction was also studied here. Five temperatures were chosen; 15, 20, 25, 30 and 35°C. The value of k_{obs} was calculated for the initial reaction in each case and these values are shown in Table 15. The reaction rate appeared to be a maximum at 25°C and dropped at temperatures above and below this; this implies that the binding process is most favourable at this temperature. A plot of k_{obs} vs T for this reaction is given in Figure 19. The three-dimensional structure of a protein is known to be temperature dependent. The catalytic and binding sites of proteins depend on their structure and hence on temperature. Therefore the binding capacity of a protein is a function of temperature. In the case of albumin, it appears that it assumes the structure most suitable for the binding of bromocresol green at about 25°C. The temperature dependence of the binding of butyl orange to serum albumin has been studied by Takagishi et al[158] who found that the binding constant is a maximum at 18°C, and that above and below this temperature the value of the binding constant drops sharply. Although this result is not directly comparable

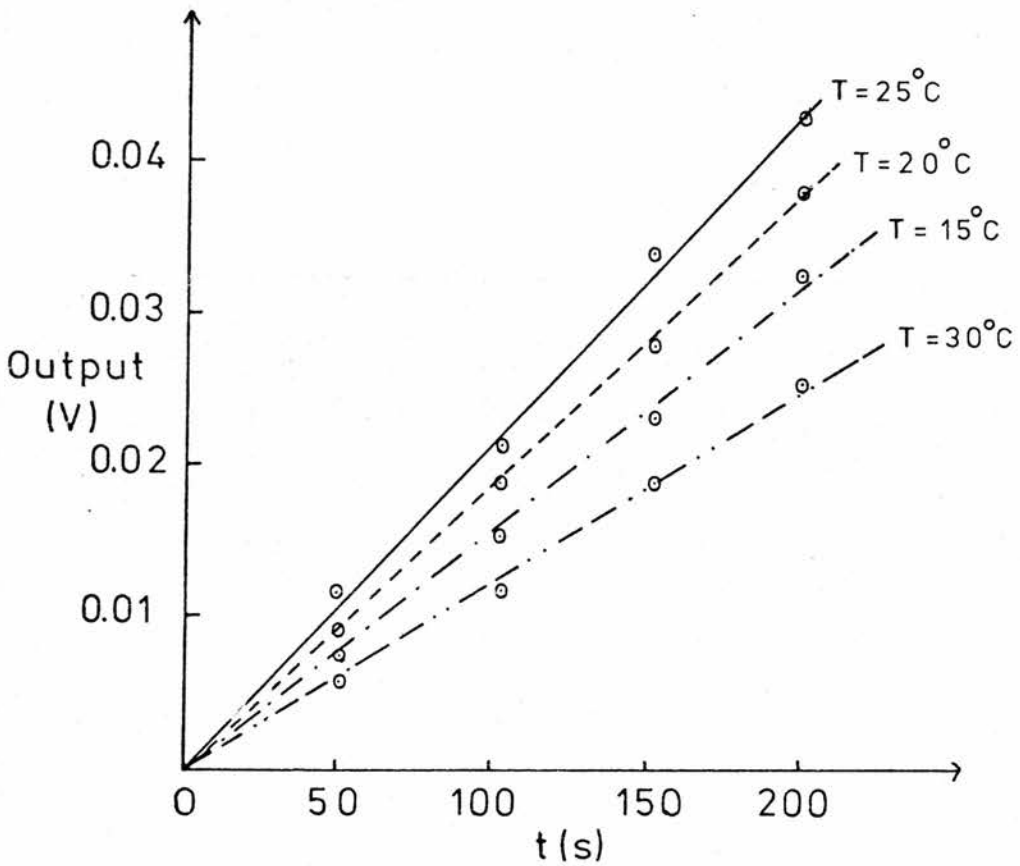
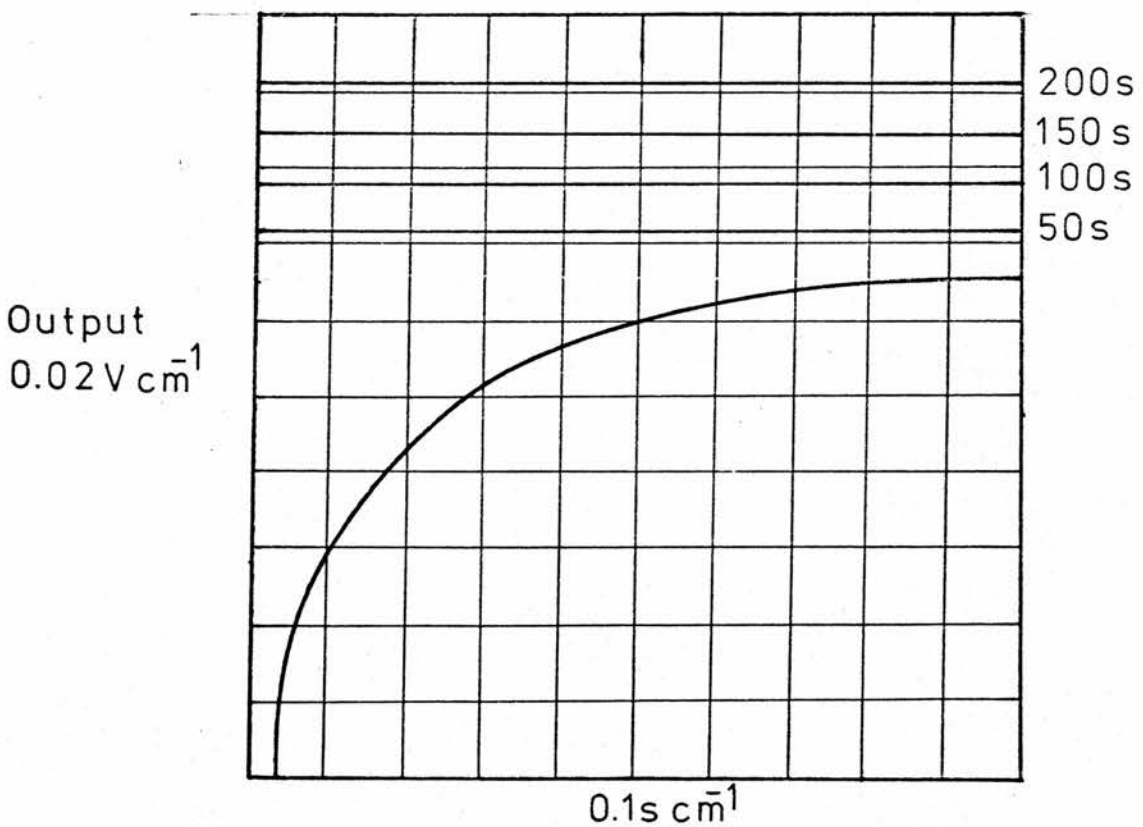


FIGURE 2

Plot of absorban
increase agains
for the slow re
between albumin
bromocresol gre
reagent at 15, 2
and 30°C
[albumin] = 1.51
[bromocresol gre
6.22
succinate buffe
λ = 590 nm



Oscilloscope trace of the reaction between bromocresol green reagent and albumin at 25°C in succinate buffer, pH = 4·2
Oscilloscope retriggered at 50,100,150, and 200s after mixing
[albumin] = $1 \cdot 51 \times 10^{-6}$ M [bromocresol green] = $6 \cdot 22 \times 10^{-5}$ M
λ = 590 nm

FIGURE 21

with mine, since I have determined a kinetic quantity and the binding constant is a thermodynamic property, it does confirm that binding of dye anions to serum albumin is a temperature dependent process and reaches a maximum where the protein structure is most favourable for the binding of that particular dye.

I have already described how the initial, fast reaction, is followed by a much slower increase in absorbance due to more bromocresol being bound by albumin. This process was much too slow to follow satisfactorily on the stopped-flow spectrophotometer, but in order to obtain some idea of the rate of reaction, and the degree of overestimation which could be caused by reading absorbance at different time intervals, the stopped-flow recording system was re-triggered at 50, 100, 150 and 200 seconds after mixing. This was done at 15, 20, 25, 30 and 35°C to observe how temperature affected the second step. Again, the rate of the second step was temperature dependent, the rate of increase in absorbance being a maximum at 25°C, and falling off at temperatures above and below this. But the temperature dependence is not as marked as in the first step, indicating that the binding process taking place here is not so dependent on the protein structure. Reasons for this will be put forward when the nature of the albumin binding sites is discussed later in the chapter. Apart from the readings at 35°C, which were anomalous, the absorbance at 590 nm increase linearly over the 200 second period after mixing of the bromocresol green reagent and the albumin solution (Figure 20). A typical photograph of the type of oscilloscope trace obtained in this experiment is shown in Figure 21. The % increases in

absorbance over that at the end of the initial reaction at 50, 100, 150 and 200 s at 15, 20, 25 and 30°C are shown in Table 17.

TABLE 17

% Increase in absorbance at 590 nm due to the secondary reaction of albumin and bromocresol green

	% Increase over Initial Reaction			
T (°C)	50 s	100 s	150 s	200 s
15	4.2	8.3	15	21
20	8.3	13.0	20	25
25	7.1	14.0	22	29
30	4.6	8.8	13	17

pH=4.2, succinate buffer,

[albumin] = 1.51×10^{-6} M,

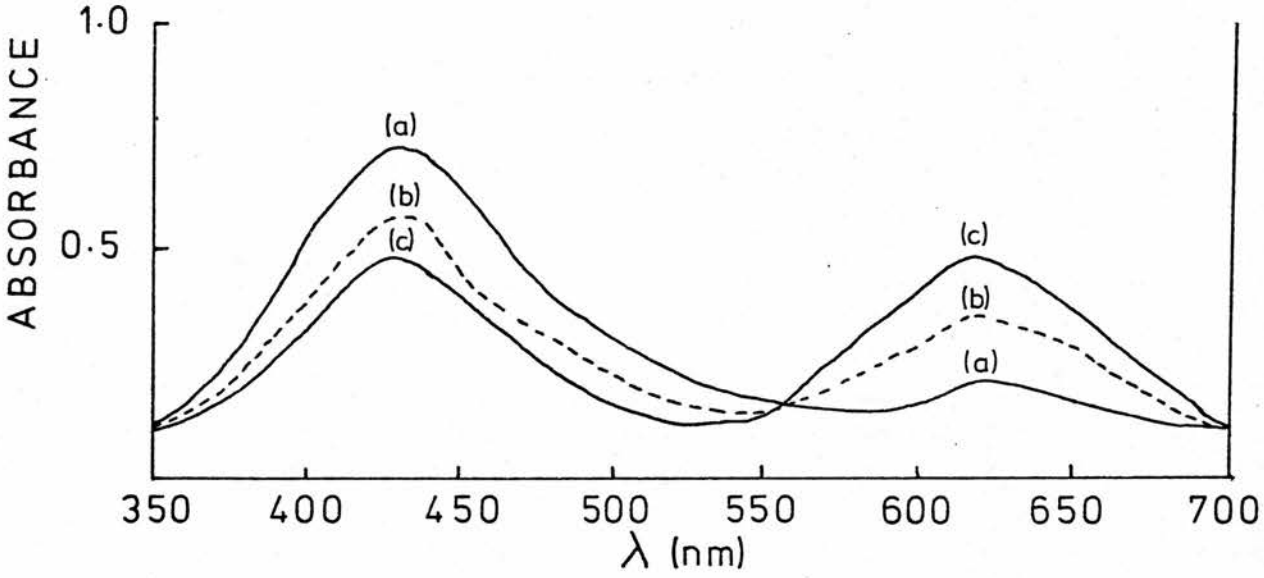
[bromocresol green] = 6.22×10^{-5} M

After 200 s the absorbance had increased by 20-30% over that of the initial reaction, and this went on increasing but was not followed any further because any albumin assays are almost certainly going to be recorded within 200 s of mixing with the bromocresol green reagent. This particular set of data serves to underline the importance of constancy in time allowed for colour development in order to eliminate errors due to the slow reaction between albumin and bromocresol green, when the serum sample results are compared with albumin standards.

It is of interest to examine in more detail the types of binding sites in albumin. Albumin is known to have two general

classes of binding site for anionic molecules other than the fatty acids[151]. Binding at the high affinity sites is thought to be due to a combination of electrostatic and hydrophobic forces, while binding at the low affinity sites, which are more numerous but vary in number, is principally electrostatic. In the binding process the anionic dye molecule is attracted to the cationic centres in the albumin molecule. These consist of the basic amino acid residues such as histidine, lysine and argnine[158]. Pinnell and Northam[51] attribute the slow reaction of bromocresol green with serum to the purely electrostatic (low affinity) binding to cationic sites in the non-albumin proteins. But the same argument can be applied to albumin itself, and the slow reaction between it and bromocresol green can be accounted for by purely electrostatic interaction between the anionic dye and the cationic centres in the albumin molecule.

In recent years it has been emphasised that hydrophobic interactions play an important role in the binding of small molecules by water soluble macromolecules such as albumin. In albumin approximately 40% of the amino acid residues are apolar and the protein has an intrinsic viscosity of near 4 ml g^{-1}, which indicates that it is relatively compact in aqueous solution[158]. Klotz and Shikama[159] found that methyl orange bound to albumin shows an absorption maximum near 435 nm. The free dye in aqueous solution has an absorption maximum at 465 nm. They found that the spectrum of methyl orange in organic solvents is the same as that when bound to albumin and therefore concluded that the dye bound to albumin is in an apolar environment. When I performed the



(a) bromocresol green in succinate buffer, pH = 4·2

(b) „ „ „ chloroform

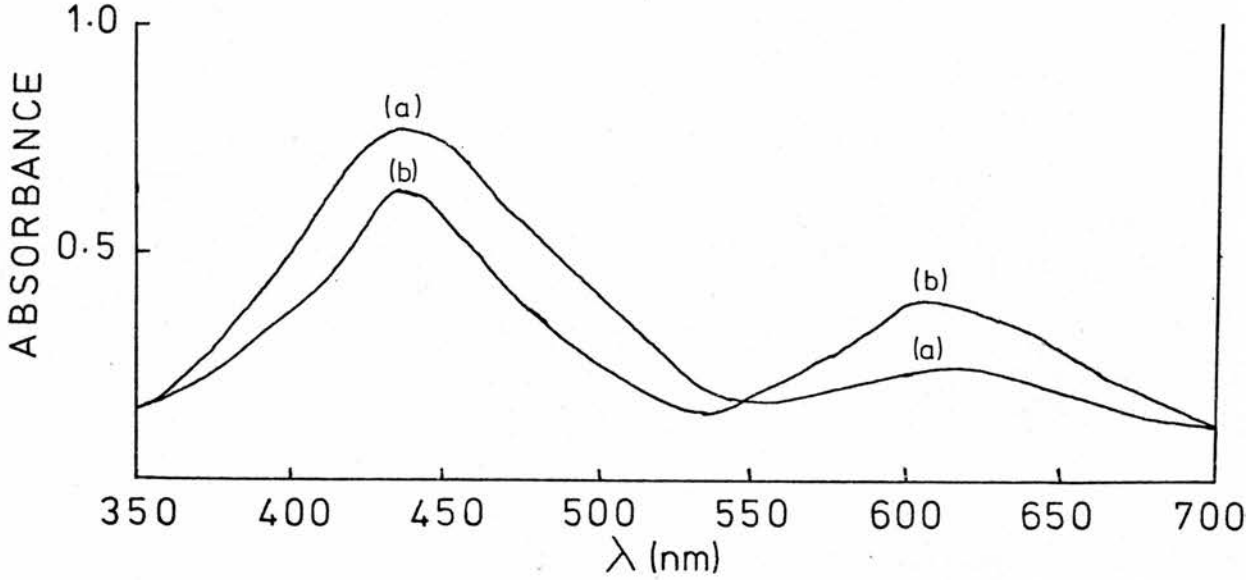
(c) „ „ „ albumin solution

[albumin] = $1 \cdot 51 \times 10^{-6}$M [bromocresol green] = $6 \cdot 22 \times 10^{-5}$M

FIGURE 22

same experiment on bromocresol green the results were not so convincing, and the spectrum of bromocresol green in chloroform (Figure 22), although similar to that of the albumin bound dye was certainly not identical. Therefore, although I would not go so far as to claim that the dye is in a hydrophobic environment, when albumin bound, it is safe to say that hydrophobic forces appear to play a part in the binding of bromocresol green. Swaney and Klotz[160] have shown that albumin provides clusters of apolar side chains which could play a part in the binding of small molecules. Hence it can be seen that the contribution of the apolar parts of the protein to the dye-protein complex will be significant. The high affinity binding sites of the albumin therefore involve not only electrostatic interactions between the cationic side chains of the protein and the dye anions, but are a combination of these and hydrophobic interactions. When Takagishi et al[158] studied the temperature dependence of these interactions for the binding of butyl orange to albumin, the pH used was such that the number of cationic sites was minimised (pH=6.9) and purely electrostatic interactions with the dye anion were not important. Therefore they were observing the high affinity binding process - that involving hydrophobic interactions. This is the type of process which I suggest is taking place in the first step of the reaction between bromocresol green and albumin. I have already described the temperature dependence of the binding of bromocresol green to albumin and how the situation for the first step was similar to that found by Takagishi et al, for the binding of butyl orange. The marked temperature dependence of the high-affinity binding process is due to the need

FIGURE 23

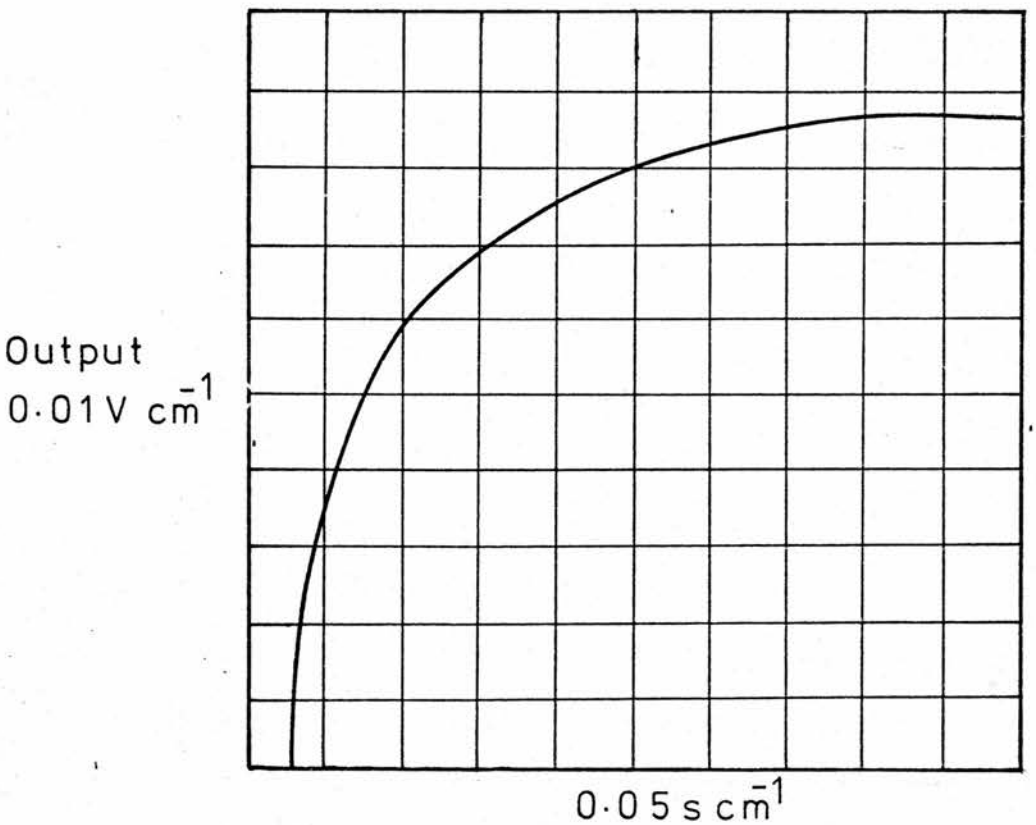


(a) bromocresol purple reagent.

(b) " " " in albumin solution

[bromocresol purple] = 4.0×10^{-5}M [albumin] = 1.51×10^{-6}M

acetate buffer, pH = 5·2



Oscilloscope trace of the reaction between bromocresol purple reagent and albumin at 25°C in acetate buffer, pH = 5·2.

FIGURE 24

for the protein structure to be in exactly the right conformation for maximum interaction of both the electrostatic and the hydrophobic binding processes. Where only electrostatic interaction is taking place the protein structure is not so critical and hence this form of binding is not so dependent on temperature, but much more on pH, since it is determined by the number of available cationic sites in the albumin molecule.

The reaction between bromocresol purple and albumin was also studied on the stopped-flow spectrophotometer. The bromocresol purple reagent was prepared according to the method of Pinnell and Northam[51]. The spectrum of this reagent before and after mixing with albumin solution is shown in Figure 23. The absorbance maximum at 590 nm increases, and that at 440 nm decreases on addition of albumin. The reaction was followed at 590 nm. This time a one step process was observed and there is no slow increase in absorbance after the initial reaction (Figure 24). As with bromocresol green, the reaction is fast, with a half-life of about 0.1 s. The observed rate constant for the reaction at 25°C is shown in Table 18 and with a value of 7.99 s^{-1} is very similar to

TABLE 18

Data for the reaction between bromocresol purple reagent and albumin at 25°C

	k_{obs} (s^{-1})
pH= 5.2, acetate buffer, [ALBUMIN] = 1.5×10^{-6} M	7.99
[Bromocresol purple] = 4.0×10^{-5} M	

that for the bromocresol green-albumin immediate reaction. This

is consistent with the same type of binding process occurring in both reactions i.e. hydrophobic and electrostatic interactions with the high-affinity binding sites on the albumin molecule. Like bromocresol green, bromocresol purple exists as a univalent (CVII) and a bivalent (CVIII) anion. That no secondary reaction occurs between bromocresol purple and albumin is due to the higher pH used for the bromocresol purple reagent (pH=5.3) which reduces the number of cationic sites on the albumin molecule and hence the number of weak dye-protein electrostatic interactions. This confirms the suggestion that the slow step in the bromocresol green reaction is due to low affinity electrostatic binding, both with albumin itself and the other proteins present in serum.

Bovine serum albumin has been used in all of the experiments carried out in the present work, and it is known that its reactivity towards bromocresol purple is less than that of human serum albumin (although its reactivity towards bromocresol green is the same)[51]. Therefore, the increase in absorbance at 590 nm is less and the bromocresol purple method is therefore less sensitive to bovine serum albumin. The probable reason for this is that since, as well as being temperature dependent, protein structures can be pH dependent, in going from the pH of the bromocresol green reagent (4.2) to the pH of the bromocresol purple reagent (5.2) the structure of bovine serum albumin changes in such a way that its ability to bind dye anions is decreased. However this problem does not arise with human serum albumin.

Pinnell and Northam[51] are therefore correct in suggesting the use of bromocresol purple instead of bromocresol green in

albumin determinations, since it is readily bound by the protein but does not suffer the disadvantage of the slow reaction with albumin and other proteins, and the immediate reaction with albumin can be used with confidence to give much more accurate results with no danger of overestimation. The method is, therefore, much more specific for albumin. This, I would attribute entirely to the higher pH of the reagent which minimises electrostatic interactions between the dye and the proteins.

EXPERIMENTAL:

Materials: Bromocresol Green was obtained from Koch-Light Laboratories, Colnbrook, Bucks, and Bromocresol Purple from BDH Chemicals. Both were pH indicator grade. Bovine serum albumin was purchased from BDH Chemicals and was used without further recrystallisation. 'Brij-35" surfactant was obtained from the Sigma Chemical Co.

Preparation of Reagents:

Bromocresol Green Reagent[47]: Succinic acid (4.4444 g) was dissolved in distilled water (330 ml). To this was added bromocresol green (0.043 g) dissolved in sodium hydroxide solution (0.1 M, 1.1 ml), followed by Brij-35 (polyoxyethylene lauryl ether) surfactant (2 ml). After mixing, the pH was adjusted to 4.2 with 10 M sodium hydroxide solution (about 2 ml was required). The solution was then diluted to 500 ml with distilled water and stored at 4°C.

Bromocresol Purple Reagent[51]: Stock solution: Bromocresol purple (0.54 g) was dissolved in absolute ethanol (25 ml) and stored at 4°C.

Brij-35 solution: Brij-35 (5 g) was dissolved in distilled water with warming and the solution diluted to 20 ml.

Stock acetic acid solution: Glacial acetic acid (AR grade, 150 ml) was diluted to 1 litre with distilled water.

Bromocresol purple working reagent: Sodium acetate trihydrate (AR grade, 10 g) was dissolved in distilled water (800 ml). The stock acetic acid solution (10 ml), Brij-35 solution (1 ml), and stock bromocresol purple solution (1 ml) were added and the solution made up to 1 litre with distilled water.

Albumin standard: Bovine serum albumin (0.2 g) was dissolved in sodium chloride solution (9 g l^{-1}, 1 litre).

Kinetics: A 'Canterbury' stopped-flow spectrophotometer was used for the kinetic studies. The reaction between albumin and bromocresol green was followed at λ = 590 nm, as was the reaction between albumin and bromocresol purple. Spectra of the coloured reagents and products were recorded on the Unicam SP800 spectrophotometer. Rate data were calculated by the method of Kezdy and Swinbourne (Appendix 3).

CHAPTER 4

THE REACTION BETWEEN BILIRUBIN AND ARENEDIAZONIUM IONS IN ACID SOLUTION

INTRODUCTION:

The problem of bilirubin determination in human serum has already been discussed in some detail in the introduction to this thesis, where it was concluded that the diazo-method, although far from perfect, remains the most satisfactory procedure since it best combines simplicity and reasonable accuracy. Nowadays, many variations of the original Van den Bergh method are in use in clinical laboratories[29-33], all of which are claimed to yield quantitative results, but no two of them, in fact, give the same results. This is one of the major problems encountered in the application of the diazo-method for bilirubin determination and is due to the different conditions under which each of the procedures is carried out, with a variety of solvents, accelerators, arenediazonium ions and time allowed for colour development.

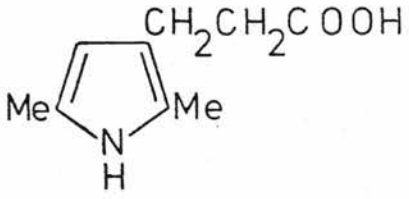
The fact that bilirubin is present in human serum in two forms, the simple bilirubin molecule (I) and the diglucuronide ester of bilirubin (II), results in two distinct diazo-coupling reactions taking place in the Van den Bergh test (Scheme 1). These are the 'direct' reaction which is attributed mainly to (II) and the 'indirect' reaction, which is attributed to (I)[24]. The total bilirubin present in serum is therefore the sum of the 'direct' and 'indirect' determined bilirubin. This has been dealt with in the introduction to the thesis.

In order to be able to study the basic reaction taking place in the Van den Bergh test I have simplified the reaction conditions as much as possible and have examined only the reaction between

the unesterified bilirubin (I) and diazotised sulphanilic acid. The effect of albumin and of several 'accelerators' on this reaction have been studied and the results are reported in this chapter.

It was described in the introduction to the previous chapter, that bilirubin is insoluble in water except at alkaline pH and how it is solubilised in human serum by being bound to albumin. Levine[145] has studied the binding of bilirubin to albumin by a fluorescence-quenching technique and found that there are two classes of binding site on albumin for bilirubin of quite different binding affinities. The high affinity site binds one molecule of bilirubin with a binding affinity (K_a) of 1.0×10^8 l mol^{-1} and the low affinity site also binds one molecule of bilirubin, but with a binding affinity of 3.0×10^6 l mol^{-1}. Brodersen[161] has also found that albumin has two classes of binding sites for bilirubin with similar binding affinities to those determined by Levine. In the past it has often been assumed that the bilirubin binding sites are non-specific sites for organic anion binding but the high K_a (binding affinity) values obtained by Levine are of the same order as the affinities of antigens for their antibodies ($K_a = 10^7$-10^9 l mol^{-1}) and indicate that the sites are highly stereospecific for bilirubin. In view of the fact that bilirubin in serum is bound to albumin, it is important to study the diazo-coupling reaction of bilirubin in the presence of albumin. This was done and the results are reported in this chapter.

Before looking at the diazo-coupling reaction of albumin-bound bilirubin, it was of interest to observe the effect of being

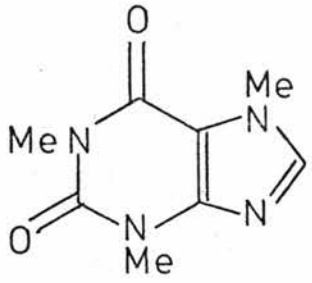


CIX

bound to albumin on a simple pyrrole and its reaction with an arenediazonium ion. A suitable pyrrole (2,5-dimethylpyrrole-3-propionic acid, (CIX)) was synthesised[162] and had an anionic site, the carboxylate group, which bound to albumin. The binding was characterised by a shift in the pyrrole's maximum of absorption from 215 nm to 235 nm, which is the same type of shift - to a longer wavelength - brought about by the binding of bilirubin to albumin. The kinetics of the reaction of 2,5-dimethyl-pyrrole-3- propionic acid (CIX) with diazotised sulphanilic acid both in the presence and absence of albumin were studied; the results are reported in this chapter. Although (CIX) was used in the present study as a model compound for bilirubin it should be noted that, as far as binding to albumin is concerned, no compound can be an entirely satisfactory model for bilirubin. The interaction between bilirubin and albumin is very stereospecific, consequently any organic anion cannot mimic precisely the binding characteristics of bilirubin and whatever model compound for bilirubin is chosen it will not be entirely satisfactory in this respect[145]. The binding affinities of organic anions for albumin are of the order of 10^4-10^5 l mol^{-1} and these are therefore much less strongly bound than bilirubin. However, the fact that (CIX) is bound to albumin will allow some conclusions to be drawn on the effect of albumin binding on the diazo-coupling reaction of a simple monomeric pyrrole, but care must be taken in applying these results to the bilirubin situation because of the very much stronger interaction between bilirubin and albumin.

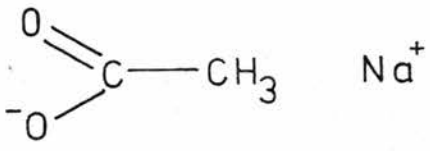
The diazo-coupling reaction of bilirubin was studied in the stopped-flow spectrophotometer under the same conditions as were used in the reactions of pyrroles (Chapter 1) and dipyrrylmethanes (Chapter 2) with arenediazonium ions in order to see whether the data obtained were consistent with the reaction mechanism anticipated by the previous work. The rate of reaction was observed both in the presence, and in the absence of albumin, although in the latter case bilirubin tended to precipitate out of solution as time progressed[7].

I described in the introduction to this thesis how the indirect Van den Bergh reaction has been ascribed to the azo-coupling reaction of unesterified bilirubin (I) which is present in serum bound to albumin. It is this reaction which will be examined in the present work. The reaction is called indirect because it requires the addition of alcohol or an accelerator to serum before it can proceed. In some of the procedures used, enough alcohol is added to bring about denaturation and precipitation of the albumin[3], leaving the free bilirubin molecule in solution and this can then go on to react with the arenediazonium ions. Other methods, such as that of Malloy and Evelyn[27] employ the addition of alcohol to the serum sample, but at a concentration short of that which causes precipitation of the protein. What happens in this case is that the bilirubin/albumin adduct has been broken up due to the change in the conformation of the albumin molecule brought about by the change in solvent, but the medium is still sufficiently aqueous to allow the albumin to remain in solution.

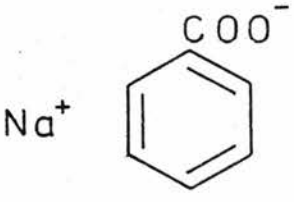


Caffeine

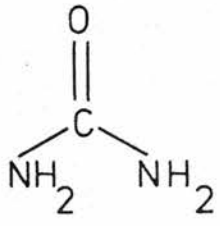
CX



CXI



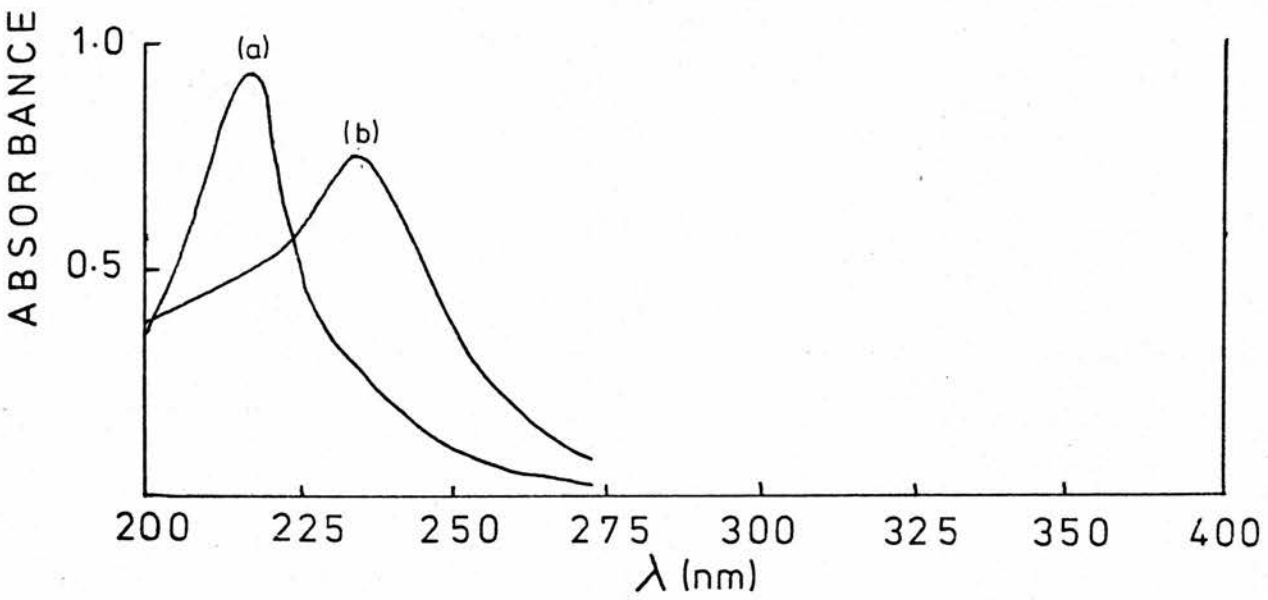
CXII



CXIII

It is much more difficult to determine how the many different accelerators of the indirect Van den Bergh reaction work. No investigation has yet been carried out into the way in which they affect the bilirubin/albumin adduct such that the bilirubin azo-coupling reaction is accelerated. Presumably they must alter the bilirubin/albumin interaction in such a way as to make the bilirubin more open to electrophilic attack, but it is not immediately obvious how, since the many accelerators used appear to have little in common chemically.

Lolekha and Limpavithayakul[29] have investigated the ability of several different accelerating agents to promote the indirect diazo reaction of different types of bilirubin samples. The accelerators investigated were caffeine (CX), sodium acetate (CXI), diphylline,HCl, sodium benzoate (CXII), urea (CXIII) and mixtures of these. Their effect on the diazo-coupling reaction of bilirubin was studied in solutions of bovine serum albumin, human serum albumin and human serum. The accelerators brought about an increase in absorbance due to the formation of the azo-bilirubin (IV) at 600 nm, but by different amounts. Lolekha and Limpavithayakul concluded that sodium acetate was the most efficient (it brought about the biggest increase in absorbance at 600 nm) and was suitable for all types of bilirubin, but the accelerators were more efficient if used in combination rather than individually. Three of these accelerators were investigated in the present study; sodium acetate, sodium benzoate and the caffeine/urea/sodium acetate mixture; their effect on the rate of the diazo-coupling reaction is reported here.



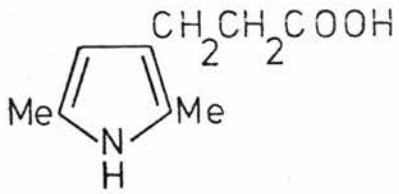
Spectrum of 2,5 - dimethylpyrrole-3-propionic acid:

(a) in phosphate buffer, pH = 7·32

(b) „ „ „ „ „ containing albumin [$2{\cdot}65 \times 10^{-5}$ M

[pyrrole] = 5×10^{-5} M

FIGURE 25



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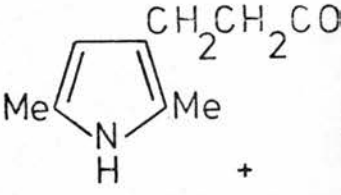
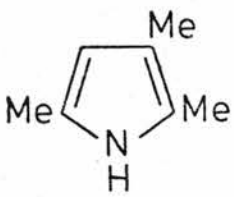


CXIV

SCHEME 26



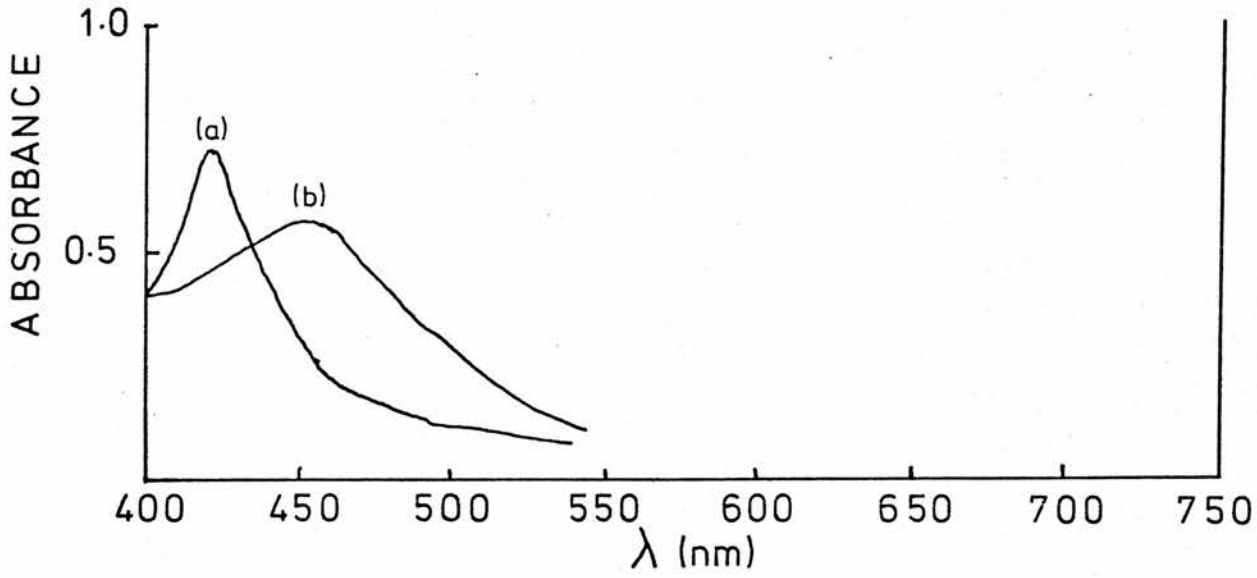
CXV

RESULTS AND DISCUSSION:

In Chapter 1 the kinetics of the diazo-coupling reactions of pyrrole and several methylpyrroles were examined and discussed and a mechanism for the process consistent with the results obtained was postulated (Scheme 6). Likewise in Chapter 2 for the diazo-coupling of dipyrrylmethanes, for which a reaction mechanism was also postulated (Scheme 20). Having studied these two systems I was then in a position to look at the reaction between bilirubin and arenediazonium ions, which is the basis of the Van den Bergh test.

The role of albumin in solubilising bilirubin in human serum has already been discussed, along with the nature of the albumin/bilirubin binding interactions, in the introduction to Chapter 3. The effect of albumin binding on the diazo-coupling reaction of bilirubin will be described in this chapter.

Since bilirubin in human serum is present bound to albumin, it is of interest to examine the effect of albumin binding on the diazo-coupling reaction of a simple pyrrole monomer. A suitable pyrrole was prepared (2,5-dimethylpyrrole-3-propionic acid (CIX)) by the method of Volz and Messner[162]. This pyrrole contained the anionic group - the carboxylate group - necessary for binding to albumin. The pyrrole was dissolved in phosphate buffer (pH=7.32) and its uv spectrum recorded (Figure 25). λ_{max} occurred at 215 nm. Sufficient albumin was then added to bind all of the 2,5-dimethyl-pyrrole-3-propionic acid. At the pH of phosphate buffer (7.32) it may be assumed that this pyrrole will exist as (CXIV) with the albumin-binding characteristics typical of an organic anion.



Spectrum of 4-(2,5-dimethylpyrrole-3-propionic acid-4-ylazo) benzenesulphonic acid:

(a) in phosphate buffer solution, pH = 7·32

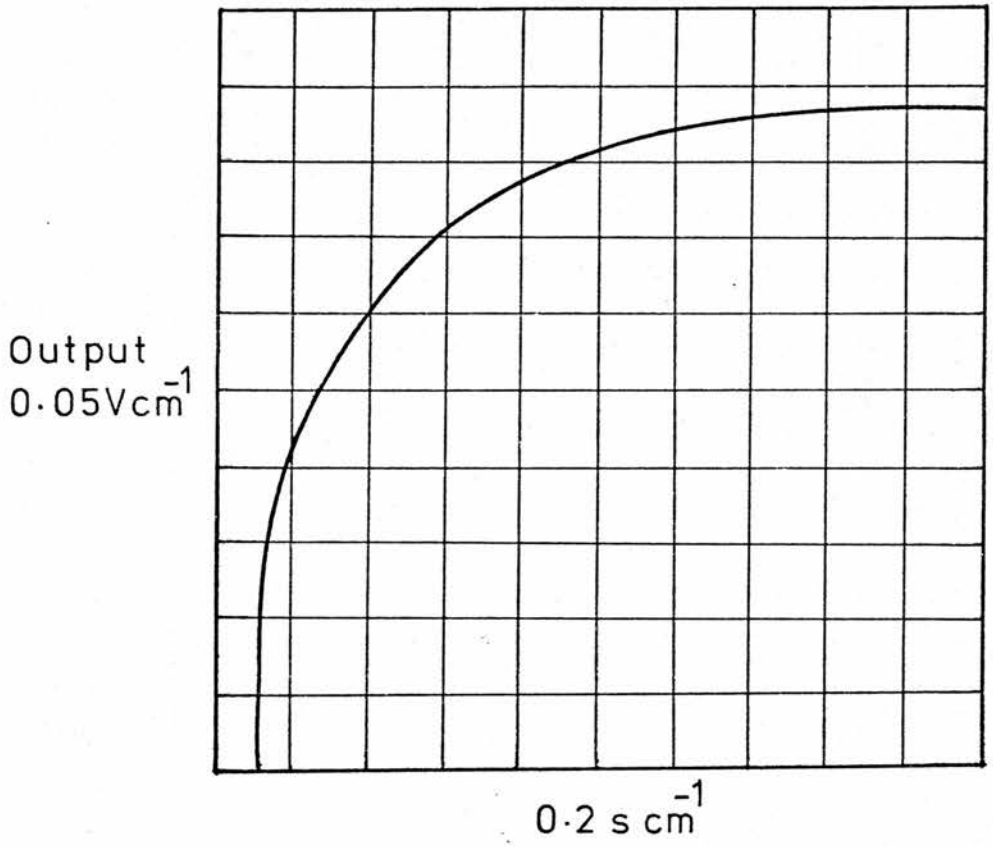
(b) " " " " containing albumin

[pyrrole] = 5×10^{-5}M [albumin] = $2 \cdot 65 \times 10^{-5}$M

FIGURE 26

Rodkey[45] reported that albumin has two types of binding sites for bromocresol green at pH=6.95, one with a higher binding affinity than the other. Altogether these sites bind eight molecules of the dye. These are described as the 'nonspecific sites for organic anion binding'[145] and should, therefore, bind the same number of molecules of (CXIV). This implies that eight molecules of 2,5-dimethylpyrrole-3-propionic acid can be bound by one molecule of albumin. The albumin concentrations used in the present study are 3.8×10^{-5} M and the concentration of 2,5-dimethylpyrrole-3-proprionic acid is 5×10^{-5} M, therefore there is an excess of albumin, which means that all of the pyrrole could be albumin bound. The uv spectrum of 2,5-dimethylpyrrole-3-propionic acid in albumin solution in phosphate buffer (pH=7.32) was recorded. The uv absorption maximum of the pyrrole moved from 215 nm to 235 nm in the presence of albumin (Figure 25). This indicates that there is interaction between albumin and the pyrrole. The addition of more albumin to the solution did not affect the uv spectrum, and λ_{max} remained at 235 nm, therefore there was already sufficient albumin present at 3.8×10^{-5} M to bind all of the 2,5-dimethylpyrrole-3-propionic acid.

On mixing the solution of the albumin-bound 2,5-dimethylpyrrole-3-propionic acid with diazotised sulphanilic acid, the yellow colour typical of the mono-azopyrrole appeared. Therefore diazo-coupling takes place in the normal way when the pyrrole is bound to albumin. The spectrum of the mono-azopyrrole was then recorded and compared with that of the mono-azopyrrole in the absence of albumin (Figure 26). The value of λ_{max} had moved



Oscilloscope trace of the reaction between 2,5-dimethylpyrrole-3-propionic acid and diazotised sulphanilic acid at 25°C

FIGURE 27

from 425nm to 450nm. This indicates that the mono-azopyrrole must also be bound to albumin. Therefore, either the albumin/pyrrole complex is broken down during the diazo-coupling reaction and is re-formed with the mono-azopyrrole, or the diazo-coupling reaction takes place while the pyrrole is bound to albumin. I would suggest that the latter is the more probable situation, since the part of the pyrrole molecule which binds to albumin, the carboxylate group, is quite remote from the site of electrophilic attack of the pyrrole ring (the 4-position), and the fact that it is bound to albumin should not affect the diazo-coupling reaction of the pyrrole.

The kinetics of the reaction between 2,5-dimethylpyrrole-3-propionic acid (both unbound and albumin-bound) and diazotised sulphanilic acid in the presence of acid were followed on the stopped-flow spectrophotometer. In both cases the observation wavelength was 440 nm. The reaction was first order in appearance of product and, with an excess of arenediazonium ion, was independent of the initial pyrrole concentration. The type of oscilloscope trace obtained, in the presence and in the absence of albumin, is shown in Figure 27. The reaction scheme is the same as that for the pyrroles discussed in Chapter 1 (Scheme 6) and the experimentally determined rate constant can be expressed so:

$$k_{obs} = \frac{k_1 [ArN_2^+]}{K([H^+]+1)} + k_{-1} [H^+] \qquad (11)$$

(Appendix 2)

from which the second order rate constants k_1 and k_{-1} for the forward and backward reactions of this equilibrium process can be obtained. These values have been calculated for the

diazo-coupling reaction of the free 2,5-dimethylpyrrole-3-propionic acid and of the same pyrrole when bound to albumin. The results are reported in Table 19. The rate of reaction was

TABLE 19

Data for the reaction between 2,5-dimethylpyrrole-3-propionic acid and diazotised sulphanilic acid at 25°C

2,5-dimethylpyrrole-3-propionic acid	k ($l\ mol^{-1}s^{-1}$)	k_{-1} ($l\ mol^{-1}s^{-1}$)
not bound to albumin	44.0	2.0
bound to albumin	39.1	2.7

$[pyrrole]_o = 5 \times 10^{-5}$ M, $[HO_3SC_6H_4N_2^+] = 0.02$ M, $[HCl] = 0.05$ M, $[Albumin] = 3.8 \times 10^{-5}$ M

virtually unaffected by the binding of the pyrrole to albumin. as can be seen from the second order rate constants in Table 19. This supports the suggestion that the albumin binding site of the 2,5-dimethylpyrrole-3-propionic acid is remote enough from the position of electrophilic attack such that binding to albumin does not affect the diazo-coupling reaction of the pyrrole.

The actual figures in Table 19 should be treated with some caution since I was unable to find the pK_a value for 2,5-dimethylpyrrole-3-propionic acid and have therefore used the pK_a value for 2,3,5-trimethylpyrrole (CXV) (pK_a=2.00) instead, in obtaining the K value for equation (11). This is a reasonable value to use, since the carboxylic acid group is remote from the pyrrole ring and should not, therefore, greatly affect the amount of protonation which takes place in acid solution. I suggest that the activating

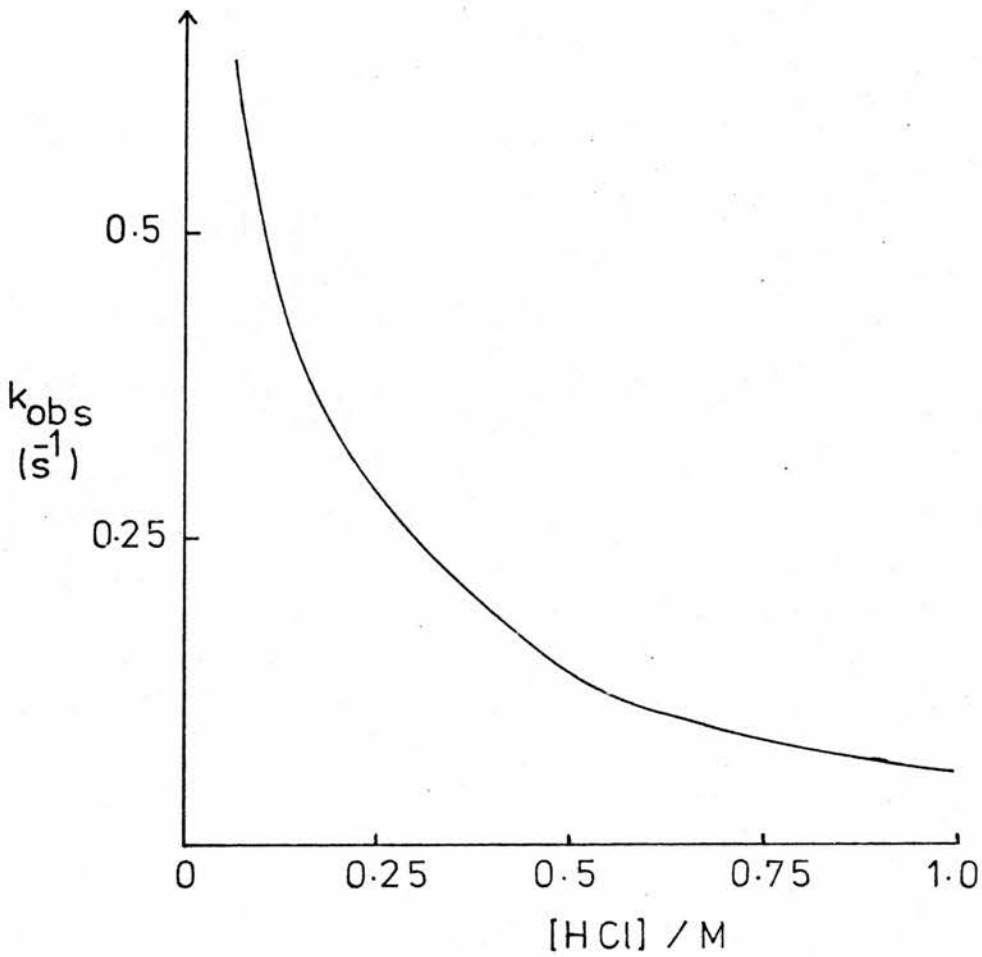


FIGURE 28

Plot of k_{obs} against [
for the reaction of
dimethylpyrrole-3-p
ionic acid with diaz
sulphanilic acid at
$[ArN_2^+] = 0.02M$
$[pyrrole]_0 = 5 \times 10^{-5}M$

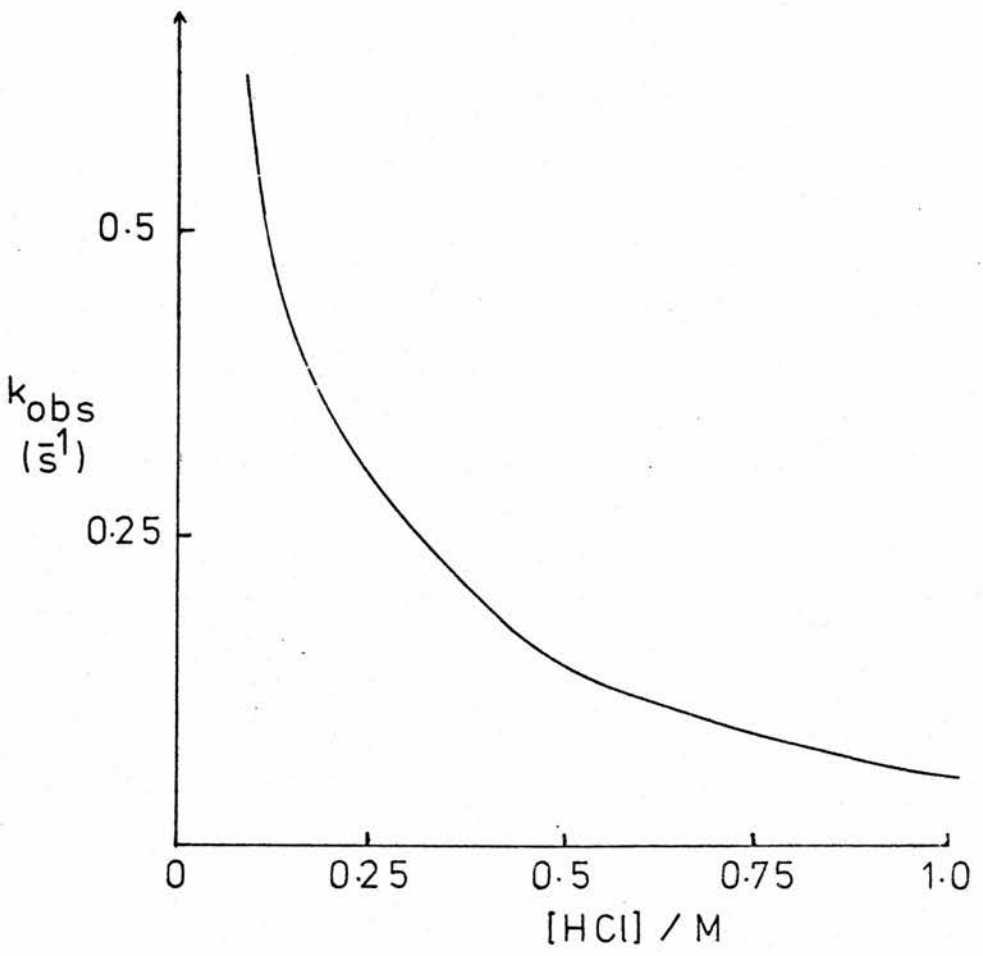


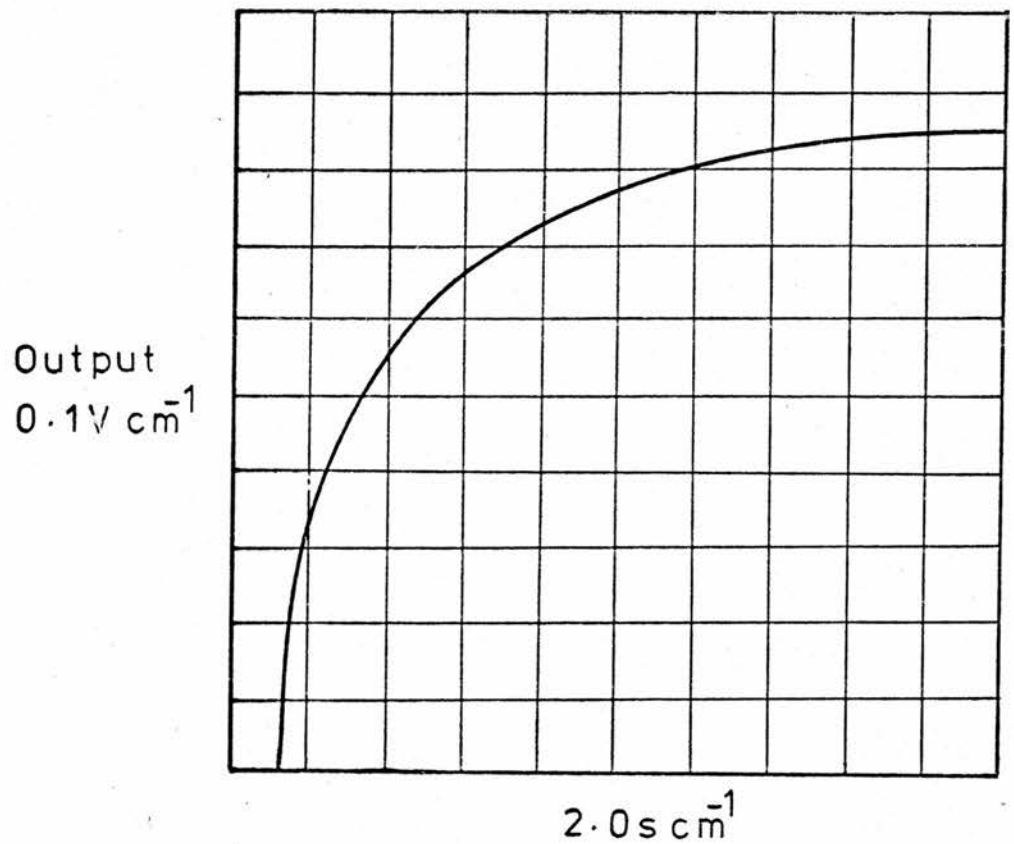
FIGURE 29

Plot of k_{obs} against
for the reaction of
dimethylpyrrole-3-p
ionic acid in albumi
solution with diaz
tised sulphanilic a
at 25°C
$[ArN_2] = 0.02M$
$[pyrrole]_0 = 5 \times 10^{-5}M$
[albumin] = 2·65 × 1

effect of a $-CH_2-CH_2-COOH$ group will not be very different from that of the $-CH_3$ group and the pK_a value of 2,3,5-trimethylpyrrole is close to that of 2,5-dimethylpyrrole-3-propionic acid. But, whether the k_1 and k_{-1} values given in Table 19 are exactly correct or not does not affect the fact that bidning to albumin has virtually no affect on the rate of the diazo-coupling of 2,5-dimethylpyrrole-3-propionic acid. The acidity dependence plots (Figures 28 and 29 confirm this).

The reaction between bilirubin and diazotised sulphanilic acid in acid solution was then examined. The visible spectrum of bilirubin itself has an absorption maximum at 440 nm, which shifts to 460 nm in the presence of albumin[7]. This has been ascribed by Martin[8] to the interaction of bilirubin with albumin. Similarly, the absorption maximum of azobilirubin (IV(a) and (b)) shifts from 540 nm, its absorption maximum in aqueous solution[27], to 580 nm in the presence of albumin[7]. This indicates that not only is bilirubin bound to albumin but that the azobilirubin isomers are also albumin bound. This is the same situation as was observed for 2,5-dimethylpyrrole-3-propionic acid, where both the pyrrole itself, and the azopyrrole were albumin bound.

The kinetics of the reaction between bilirubin dissolved in phosphate buffer (pH=7.32) and diazotised sulphanilic acid were followed in the stopped-flow spectrophotometer at 550 nm. But the situation was complicated by the fact that bilirubin is not soluble in water at this pH (7.32) and precipitated out of solution. Consequently, it was not possible to obtain good reproducible oscilloscope traces and no kinetic data could therefore be



Oscilloscope trace of the reaction between bilirubin in albumin solution in phosphate buffer (pH = 7·32) and diazotised sulphanilic acid at 25°C

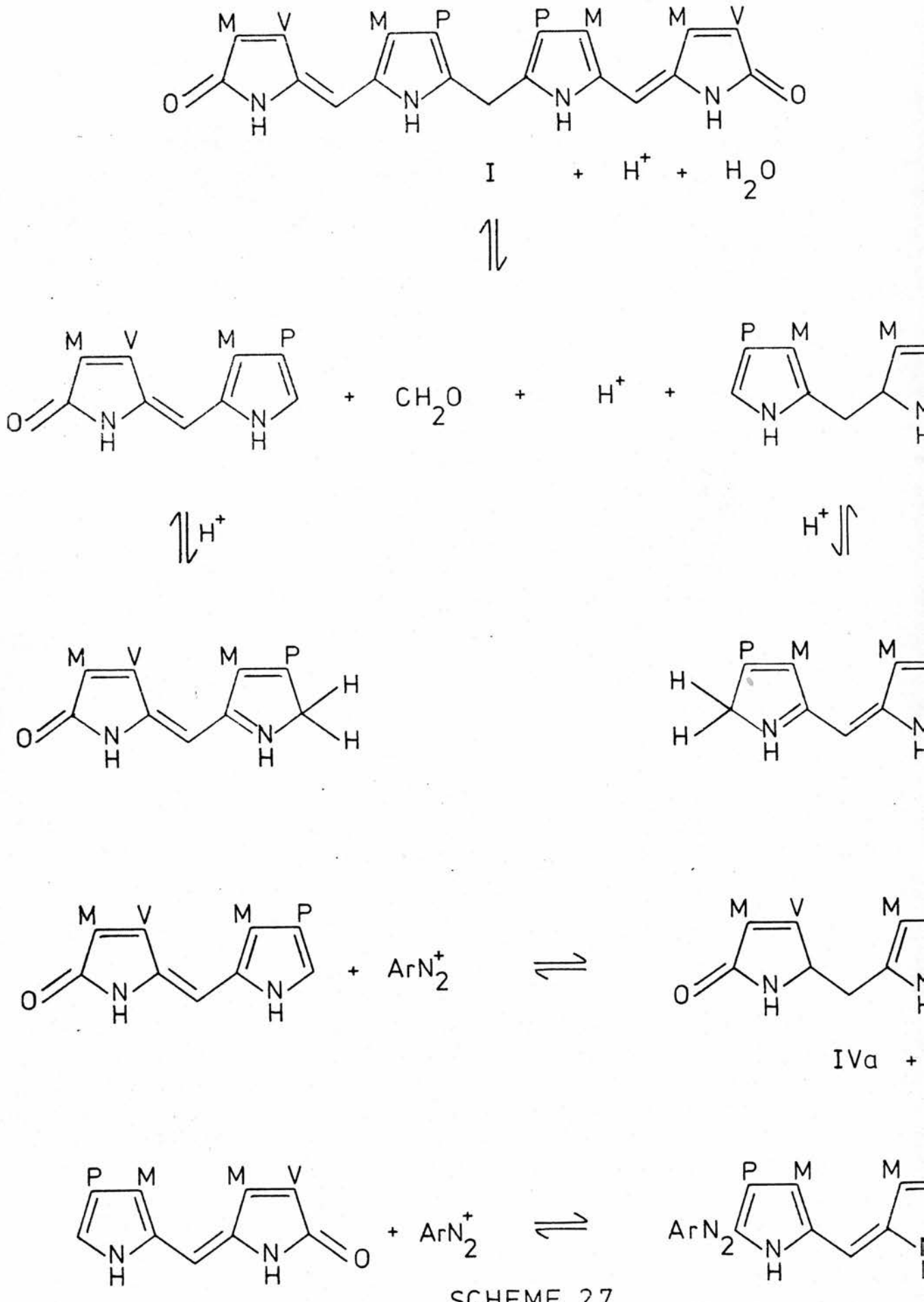
[bilirubin] = $1{\cdot}70 \times 10^{-5}$ M

[albumin] = $2{\cdot}65 \times 10^{-5}$ M

[ArN_2^+] = 0·02 M

[HCl] = 0·05 M

FIGURE 30


M V M P P M M V
O N H N H N H N H O
I + H+ + H2O
M V M P
O N H N H
+ CH2O + H+ +
P M M
N H
H+
H+
M V M P H
O N H N H H
P M M
H H N H
M V M P
O N H N H
+ ArN2+
M V M
O N H
IVa +
P M M V
N H N H O
+ ArN2+
P M M
ArN2 N H

SCHEME 27

obtained for the reaction.

Albumin was, therefore, necessary in order to make bilirubin soluble at the pH of the phosphate buffer (7.32, which is the pH of blood serum) and the kinetics of the diazo-coupling reaction of albumin bound bilirubin were therefore studied. The reaction was again followed on the stopped-flow spectrophotometer at 550 nm. This time satisfactory, reproducible oscilloscope traces of the type shown in Figure 30 were obtained. The observed reaction was a one-step process which was first order in appearance of product and, with an excess of diazonium ion, was found to be independent of the initial bilirubin concentration. The fact that the observed diazo-coupling reaction is a one step process indicates that the mechanism proposed for the reaction of 2,2'-dipyrrylmethanes with arenediazonium ions in acid solution (Scheme 20) also holds for the diazo-coupling of bilirubin in acid solution i.e. the central methylene bridge is cleaved very rapidly and is released as formaldehyde[22] and the two pyrrole monomers (in the case of the dipyrrylmethanes) or the two oxydipyrromethene fragments (in the case of bilirubin) then go on to react more slowly with the arenediazonium ions. The reaction mechanism for the diazo-coupling of bilirubin in acid solution is shown in Scheme 27. From this reaction scheme the experimentally determined rate constant, k_{obs}, may be expressed as $k_1[ArN_2^+]/(K[H^+]+1) + k_{-1}[H^+]$ (11) (Appendix 2), since the reaction mechanism is the same as for the diazo-coupling of dipyrrylmethanes (Scheme 20). However, the oxodipyrromethene fragments of the bilirubin molecule will almost certainly be protonated in

acid solution at the free α-position created on bridge cleavage, and this is taken into account in the above expression for k_{obs}, since, as with the pyrroles and dipyrrylmethanes, it is assumed that the oxodipyrromethene fragments of the bilirubin molecule will react with arenediazonium ions in their unprotonated form. However, the pK_a values for these oxodipyrromethenes is not known, hence K cannot be calculated and it is not, therefore, possible to obtain the true second order rate constant for the process. The reaction was first order in diazotised sulphanilic acid, and a plot of k_{obs} vs $[HO_3SC_6H_4N_2^+]$ was linear from which a value of $k'_1 = \frac{k_{obs}}{[HO_3SC_6H_4N_2^+]}$ could be obtained as well as a value for k'_{-1}. These values are given in Table 20.

What this study has proved then, is that in the Van den Bergh test, the methylene bridge is cleaved by the proton very rapidly and the oxodipyrromethene fragments then go on to react with the arenediazonium ions much more slowly. Further kinetic evidence for the rapid cleavage of the methylene bridge was obtained

TABLE 20

Data for the reaction of albumin-bound bilirubin with diazotised sulphanilic acid at 25°C

k'_1 ($l\ mol^{-1} s^{-1}$)	k'_{-1} ($l\ mol^{-1} s^{-1}$)
6.56	0.059

$[HO_3SC_6H_4N_2^+] = 0.02$ M, $[bilirubin]_o = 1.7 \times 10^{-5}$ M

$[albumin] = 3.8 \times 10^{-5}$ M, $[HCl] = 0.05$ M

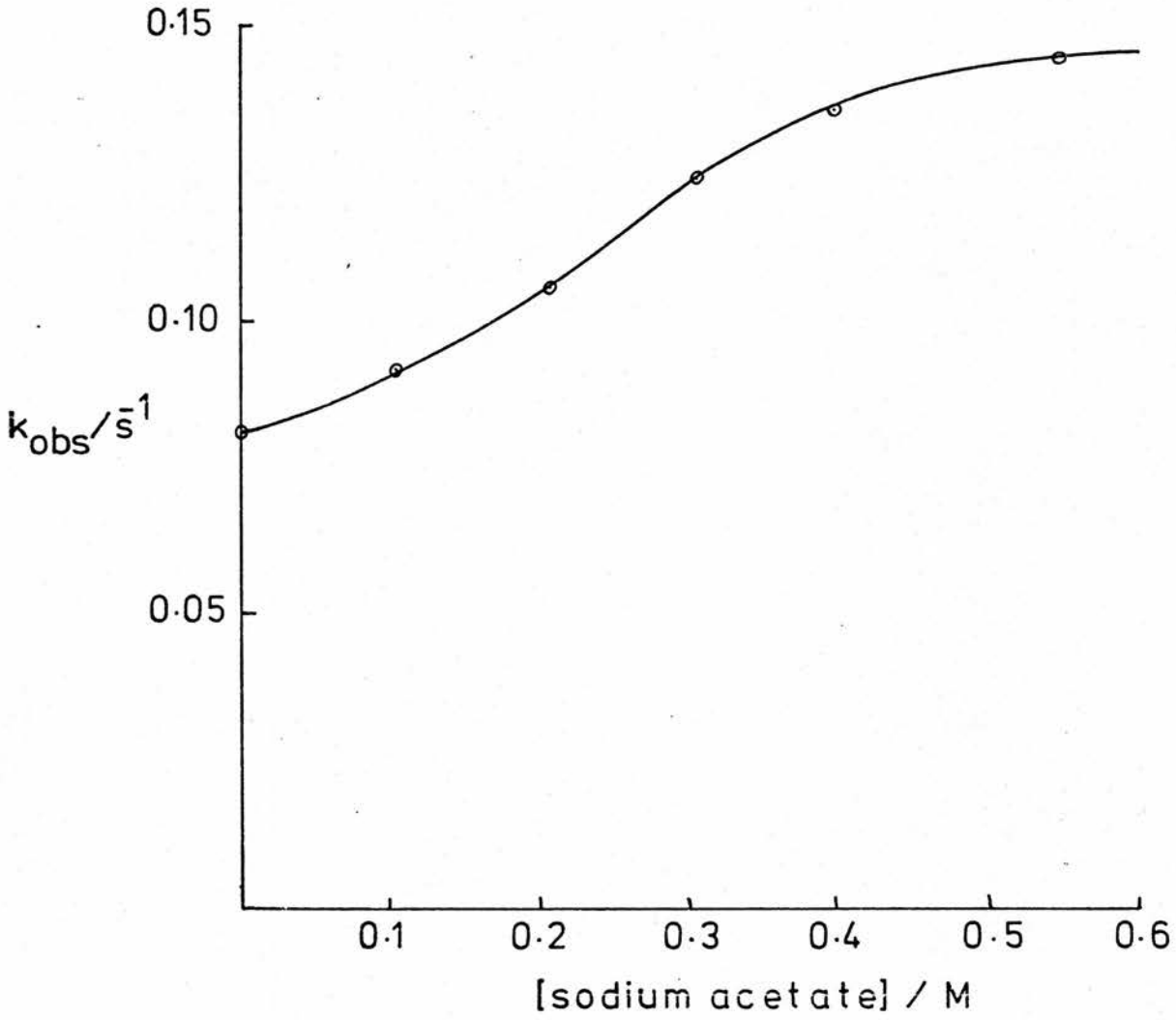
by following the change in the absorption at 460 nm due to the disappearance of bilirubin during the azo-coupling. The change

in absorbance at 460 nm due to the breakdown of bilirubin, occurred at a rate too rapid to be followed on the stopped-flow instrument and was therefore a much faster reaction than the azo-coupling process.

The fact that albumin-bound bilirubin undergoes diazo-coupling is itself surprising, since this is supposed to be the 'indirect' reaction in the Van den Bergh test, which requires the addition of alcohol or an accelerator before it can proceed. It might have been that the very strong binding of bilirubin to albumin[145] would have meant that no diazo-coupling reaction could take place, but this is apparently not so, presumably for the same reason as it did not affect the azo-coupling of 2,5-dimethyl-pyrrole-3-propionic acid, i.e. the part of the molecule which binds to albumin is remote from the site of electrophilic attack. Therefore I suggest that clinical chemists look for some other explanation of the 'indirect' diazo-coupling reaction of serum bilirubin since it is obvious from the present study that albumin-bound bilirubin does react with diazotised sulphanilic acid in the absence of alcohol or an accelerator. There must be some other factor in human serum which is responsible for the difference between the 'direct' and 'indirect' Van den Bergh reactions, which has not yet been taken into account. I am not in a position to suggest what that might be, but it is a matter which certainly requires further investigation.

The bilirubin diazo-coupling reaction occurring under the conditions used in the present study is very different to that of Overbeek and Deenstra[20], who found that the diazo-coupling

reaction of bilirubin with diazotised sulphanilic acid proceeded in two consecutive steps. Their reaction conditions were, however, very different and the experiments were conducted at 20°C in a medium of 60% ethanol, 30% chloroform and 10% water. Overbeek and Deenstra themselves admitted that their conditions did not reflect those of the serum diazo-test. That test is much more clearly emulated by the present study and the results reported here are therefore much more relevant to it. Brodersen[26] has studied the diazo-coupling reaction of bilirubin in serum, and has found that it can be described as a series of three first-order processes, the first two relating to the reaction of the bilirubin diglucuronide (II) and the third, he ascribed to the reaction of the unesterified bilirubin. Only the third reaction is relevant to the present study. Landis and Purdue[163] have investigated the reaction of albumin-bound bilirubin with diazotised sulphanilic acid using caffeine as an accelerator and found that the reaction occurred in two steps, the first of which had an observed rate constant ($k_{obs}=10s^{-1}$) and that the second step was much slower than the first, although no kinetic data is given for this step. During the present work the effect of various accelerators, including caffeine, on the diazo-coupling reaction was investigated and the results are reported later in this chapter. These results do not agree with those of Landis and Purdue although, like them, I have found that the reaction was first order in bilirubin, and with an excess of arenediazonium ion the observed rate constant was independent of initial bilirubin concentration.



Effect of sodium acetate on the rate of reaction between albumin-bound bilirubin in phosphate buffer, pH = 7·32 at 25°C

[bilirubin] = $1·7 \times 10^{-5}$ M [albumin] = $2·65 \times 10^{-5}$ M

[ArN_2^+] = 0·02 M [HCl] = 0·05 M

FIGURE 31

Lolekha and Limpavithayakul[29] have investigated the effect of several accelerators of the 'indirect' bilirubin diazo-coupling reaction on the sensitivity of the reaction i.e. the increase in absorbance at 600 nm due to formation of azobilirubin. This varied from accelerator to accelerator, and they found that sodium acetate was the most efficient accelerator in that it promoted the largest increase in absorbance at 600 nm, although the 'mixed' accelerators such as urea/caffeine/sodium acetate mixtures were still more efficient. Thus far no attempt has been made to understand why these accelerators work and what their mode of action is.

The effect on the rate of the diazo-coupling reaction of albumin-bound bilirubin of the addition of different accelerators was investigated during the present work. The three accelerators chosen were sodium acetate, sodium benzoate and caffeine/urea/sodium acetate mixture.

Firstly, the effect of sodium acetate on the rate of the diazo-coupling reaction will be dealt with. As the sodium acetate concentration was increased, so the observed rate constant, k_{obs}, also increased from 0.082 s^{-1} at [sodium acetate]=0, to 0.135 s^{-1} at 0.55 M sodium acetate (the concentration of sodium acetate in the accelerated used by Lolekha and Limpavithayakul) (Figure 31). Hence, sodium acetate is a true accelerator in that it does speed up the rate of the diazo-coupling reaction of the albumin-bound bilirubin.

The addition of the caffeine/urea/sodium acetate mixture to the reaction mixture also increased the rate of the diazo-coupling

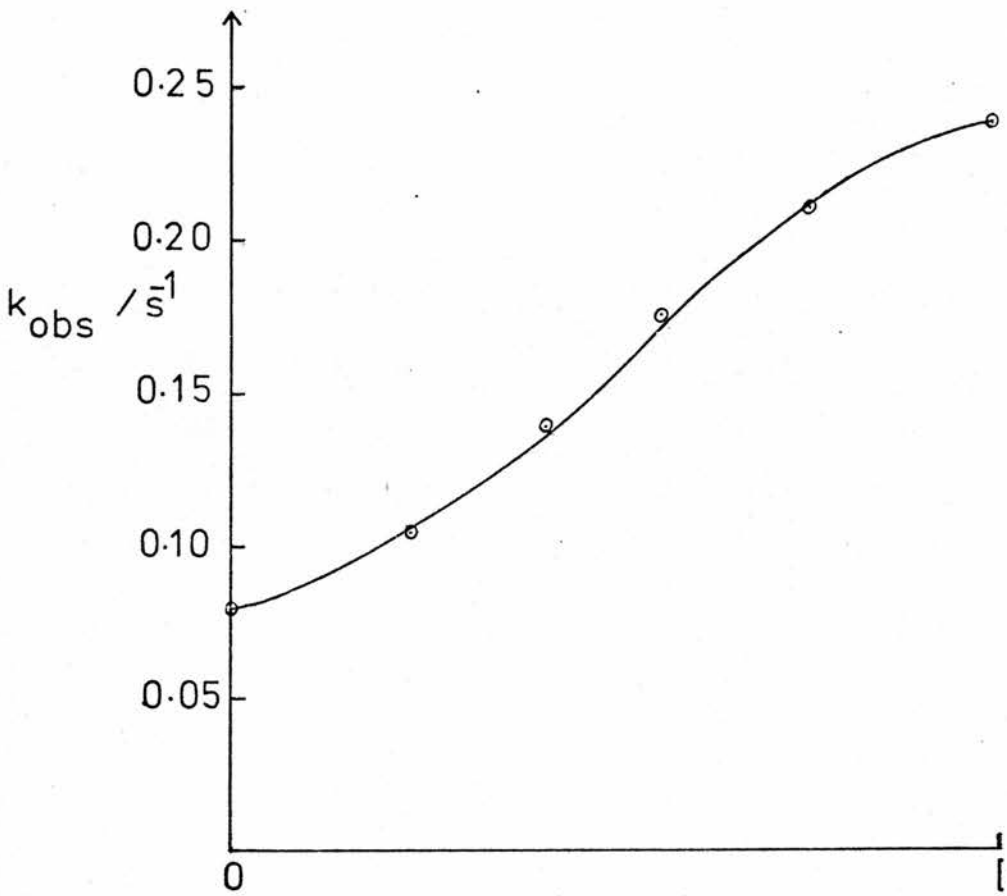


FIGURE 32

Effect of sodium
urea/ caffeine mix
on the rate of r
between albumin-
bilirubin in phosp
buffer, pH = 7·32 a
diazotised sulpha
acid at 25°C

[bilirubin] = $1·7 \times 10^{-}$

[albumin] = $2·65 \times$

[ArN_2^+] = 0·02 M

[HCl] = 0·05 M

[urea] = $8·25 \times 10^{-}$

[caffeine] = $2·32 \times 10^{-2}$

[sodium acetate] = 0·55 M

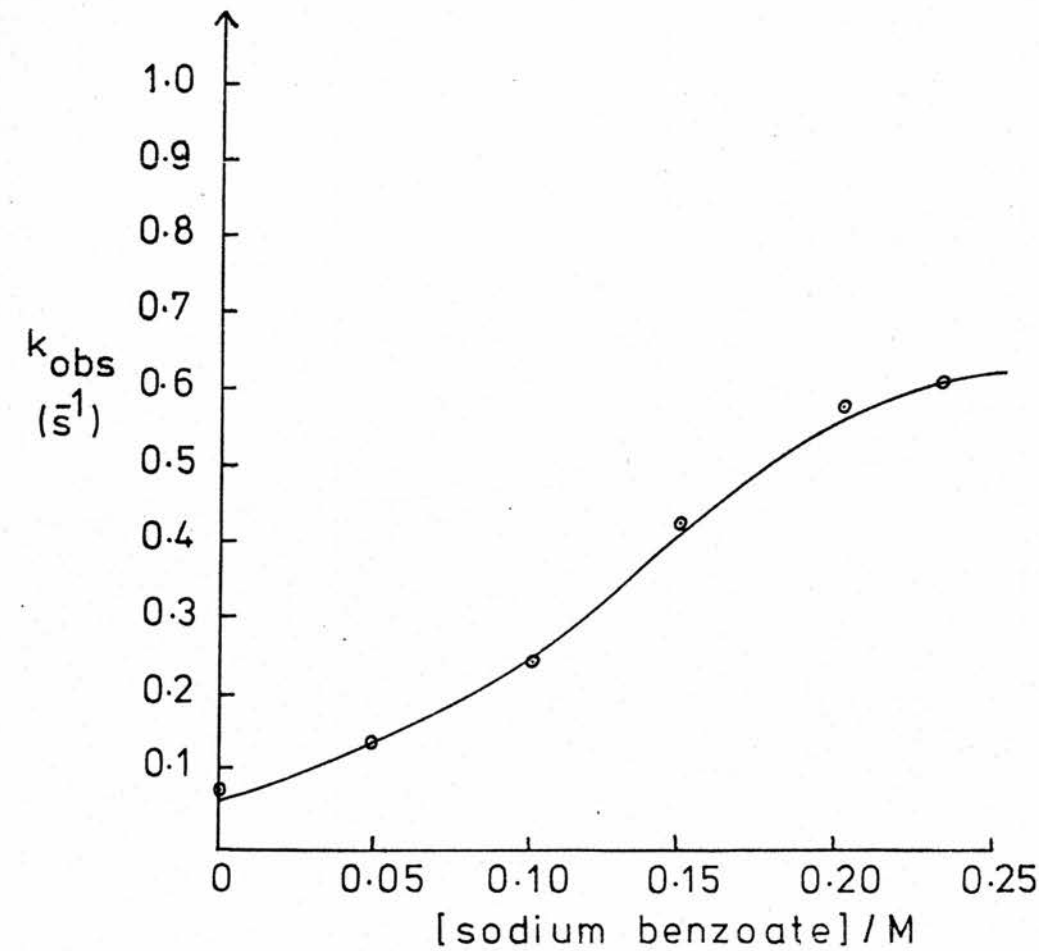


FIGURE 33

Effect of sodium
benzoate on the
of reaction betwe
albumin-bound bi
in phosphate buff
pH = 7·32 and diaz
tised sulphanilic
at 25°C

[bilirubin] = $1·7 \times 10^{-}$

[albumin] = $2·65 \times$

[ArN_2^+] = 0·02 M

[HCl] = 0·05 M

reaction. Again as the concentration of the accelerator was increased, the rate of reaction increased (Figure 32). This time the increase in k_{obs}, as the concentration of the accelerator was increased from zero to a sodium acetate concentration of 0.55 M, a urea concentration of 0.085 M, and a caffeine concentration of 0.023 M (the concentration used in this accelerator by the authors) was from 0.082 s^{-1} to 0.206 s^{-1} i.e. more than double. Therefore again the rate of reaction is accelerated.

Thirdly, as the concentration of sodium benzoate was increased, the rate of the diazo-coupling reaction also increased. This time the value of k_{obs} increased from 0.082 s^{-1} at [sodium benzoate]=0 to 0.57 s^{-1} at 0.23 M sodium benzoate (the concentration of the accelerator as used by Lolekha and Limpavithayakul) i.e. a seven-fold increase in rate (Figure 33).

Although each of the accelerators described above increased the rate of the reaction between diazotised sulphanilic acid and albumin-bound bilirubin they did not alter the pattern of the observed reaction, which remained a one-step, process following first order kinetics. Therefore, as well as causing an increase in absorbance at 600 nm, all three accelerators studied increase the rate of the diazo-coupling reaction of albumin-bound bilirubin.

The question remains to be answered, why do those compounds, and mixtures of them, have such a marked effect on this reaction? In the serum environment, albumin-bound bilirubin reacts only very slowly with diazotised sulphanilic acid[3], while in the present study, albumin bound bilirubin reacts quite readily with diazonium ions. The effect of an accelerator is

apparent in both cases, but is much more marked in the serum environment. I would therefore suggest that, although the accelerator alters the bilirubin-albumin binding interaction in such a way as to make the bilirubin more accessible to electrophilic attack, it must also have an effect in some other way in serum, since here the presence or absence of an accelerator means the difference between reaction or virtually no reaction whatsoever. In the present *in vitro* study, the only effect of the accelerator was to speed up a reaction which already took place quite readily.

It is difficult to understand exactly how these accelerators affect the bilirubin/albumin interaction and why sodium benzoate, which is not as efficient as the other two accelerators in increasing the absorbance of the azobilirubin at 600 nm, causes a much greater increase in the rate of the diazo-coupling reaction.

The binding ability of albumin is very dependent on its conformational structure, which in turn is pH and temperature dependent. It could be that the only effect of some accelerators is to change the pH of the reaction mixture and thus alter the bilirubin/albumin interaction. It is also known that anionic drugs, such as salicylates, compete with bilirubin for the cationic binding sites on albumin and can reduce the binding of bilirubin to albumin[11]. Each of the accelerators examined in the present study has an organic anionic component which could compete with bilirubin for the cationic binding sites in albumin and thus weaken the albumin/bilirubin interaction. However, this matter requires further investigation before it can be fully understood, and if, as I think, other components in serum play a part, it will prove

complicated to do so.

In conclusion, then, the present study into the diazo-coupling reaction of bilirubin has proved useful in a number of ways. Firstly, it has been the first investigation into the reaction under conditions in which bilirubin is found in human serum but without the many other components in serum which can complicate the study so much as to make the results difficult to interpret. Secondly, it has shown the exact mechanism of cleavage of the methylene bridge of bilirubin during the diazo-coupling reaction, which has been verified by comparison with model compounds - the dipyrrylmethanes. Thirdly, the studies of biologically important molecules such as bilirubin in an environment very different to that in which they are found in nature have only slight bearing on the situation in vivo. For example, the investigation into the azo-coupling reaction of bilirubin by Overbeek and Deenstra[20] was carried out in a mixed solvent of ethanol/chloroform/water in order to get all of the components of the reaction into solution, and the reaction mechanism was, therefore, very different to that taking place in the present study, where the bilirubin was bound to albumin, and the reaction was carried out in aqueous solution. As a deeper knowledge of the types of reaction taking place in clinical tests is required, it is becoming increasingly important for the chemist, with his understanding of molecular interactions and reaction mechanisms, to investigate these systems. To take the example of clinical tests on serum, it is impossible to investigate properly the reactions of an individual species present in serum in the serum itself. By isolating the molecule of

interest, and studying it under the sort of conditions in which it will be found in serum, but without the many other components of serum present, which is what has been done in the present study with bilirubin, the exact nature of its reactions in clinical tests can be understood. Although the present study was not an unqualified success, in that I have not been able to suggest any improvements to the Van den Bergh test, it is the type of study which will have to be carried out on biological molecules. Until now clinical chemistry has been a purely empirical science, which has led to the reagents and methods used being needlessly complicated and numerous. A deeper understanding of the chemistry of the various tests used would lead to a much more selective use of reagents.

EXPERIMENTAL:

Materials: Bilirubin (crystalline, from bovine gallstones) was obtained from the Sigma Chemical Co., St. Louis, Mo., USA; albumin (bovine) was obtained from BDH Chemical Ltd., Poole, England, and was used without recrystallisation; caffeine was obtained from BDH Chemicals Ltd, and was used without recrystallisation; Analar urea was obtained from BDH Chemicals Ltd., and was used without recrystallisation. Sodium benzoate and sodium acetate were recrystallised before use. Diazotised sulphanilic acid was prepared as described in Chapter 1: experimental section. 2, 5-Dimethylpyrrole-3-propionic acid was prepared by the method of Volz and Messner[162].

Phosphate buffer solution: 500 ml of 0.1 M potassium dihydrogen-phosphate were made up and to this was added 370 ml 0.1 M sodium hydroxide solution (pH=7.32).

Spectra were recorded on a Pye-Unicam SP800 Spectrophotometer.

Kinetics: A "Canterbury" stopped-flow spectrophotometer was used. The observation wavelength used for the azo-coupling reaction of 2,5-dimethylpyrrole-3-propionic acid was 440 nm. That for the azo-coupling reaction of bilirubin was 550 nm. Rate constants were calculated by the method of Kezdy[114] and Swinbourne[115] (Appendix 3).

Products: It was attempted to prepare the azobilirubin isomers (IVa and b) in chloroform solution from bilirubin and diazotised sulphanilic acid by using a crown ether as a phase transfer catalyst. The method used was that described in Chapter 1: experimental section, but neither the azobilirubins, nor their tetrafluoroborate salts were obtained. A solid product, which could not be identified, precipitated out of the reaction mixture.

APPENDIX 1[71]

Calculation of pK_a values for methyl substituted pyrroles

pK_α = -3.8 and is the pK_a value for protonation at the α-position

pK_β = -5.9 and is the pK_a value for protonation at the β-position

m is the position of the methyl substituents

j is the position of protonation

$$pK_\alpha = -3.8$$

$$pK_\beta = -5.9$$

j

m	2	3	4	5
1	0.7	0.8	0.8	0.7
2	-0.6	(3.6)	(1.4)	3.6
3	2.8	(-0.7)	(1.2)	1.7
4	1.7	(1.2)	(-0.7)	2.8
5	3.6	(1.4)	(3.6)	-0.6

By addition of rows in the above table appropriate to the substitution, and taking as reference points the indicated pK_a's for α- and β-protonation in pyrrole, a set of predicted basicity constants is obtained for any particular case. The values for β-protonation are mostly intuitive estimates and these are shown in parentheses.

APPENDIX 2

The concentration of free pyrrole is given by (1) where

$$[Py] = \frac{([Py]^{o}_{St} - [P_1])}{(K[H^+] + 1)} \qquad (1)$$

where $[Py]^{o}_{St}$ is the initial stoichiometric concentration of the pyrrole. The rate of production of P_1 as the reaction approaches equilibrium is given by (2).

$$\frac{d[P_1]}{dt} = k_1[Py][ArN_2^+] - k_{-1}[P_1][H^+] \qquad (2)$$

As ArN_2^+ and H^+ are present in large excesses they are unchanged during the course of the reaction and (2) simplifies to (3).

$$\frac{d[P_1]}{dt} = k_1' [Py] - k_{-1}'[P_1] \qquad (3)$$

Substitution from (1) gives equation (4) which on rearrangement to (5), can be integrated to give (7). At t=0, $[P_1]=0$ and so the constant

$$\frac{d[P_1]}{dt} = \frac{k_1'([Py]^{o}_{St} - [P_1])}{(K[H^+] + 1)} - k_{-1}'[P_1] \qquad (4)$$

$$= \frac{k_1'[Py]^{o}_{St}}{(K[H^+]+1)} - [P_1] \frac{k_1'}{(K[H^+]+1)} + k_{-1}' \qquad (5)$$

$$= A - B[P_1] \qquad (6)$$

$$-\frac{1}{B[\ln(A - B[P_1])]} = t + \text{constant} \qquad (7)$$

of integration is $-1/B[\ln A]$ and the full integrated equation is (8). This may be simplified by setting the equilibrium condition that

$$\frac{-1}{B[\ln(A - B[P_1])]} = t - \frac{1}{B[\ln A]} \qquad (8)$$

at $[P_1]_e$, $d[P_1]/dt=0$, where $[P_1]_e$ is the final or equilibrium

concentration of product. Applying this to (6), equation (9) is obtained. Substitution in (8) gives (10), which may be

$$A = B[P_1]_e \tag{9}$$

rearranged to (11). Therefore the slope of a plot of

$$\frac{-1}{B[\ln(B[P_1]_e - B[P_1])]} = t - \frac{1}{B[\ln A]}$$

$$-\ln([P_1]_e - [P_1]) = Bt + \ln\frac{B}{A} \tag{10}$$

$-\ln([P_1]_e - [P_1])$ against t (i.e. k_{obs}) is $k_1[ArN_2^+]/(K[H^+]+1) + k_{-1}[H^+]$ (11

APPENDIX 3[114,115]

Kezdy-Swinbourne method for the determination of a first order rate constant (k)

It is often impracticable to measure the initial concentration or concentration after 'infinite' time of a reactant during a kinetic study and the method outlined below overcomes these problems.

Consider a first-order reaction of which observations $(x_0, x_1, x_2, \ldots\ldots x_n \ldots\ldots x_\infty)$ are taken at times $(t_0, t_1, t_2, \ldots\ldots t_n \ldots\ldots t_\infty)$. For a reading (x_n) taken at (t_n)

$$(x_\infty - x_n) = (x_\infty - x_0)\exp.(-kt_n) \qquad (14)$$

Now consider a second series of observations $(x_0, x'_1, x'_2, \ldots\ldots x'_n \ldots\ldots x_\infty)$ taken at times $(t_0, t_1 + \Delta t, t_2 + \Delta t_1 \ldots\ldots t_n + \Delta t \ldots\ldots t_\infty)$ where Δt is a small, constant time interval.

For a reading (x'_n) taken at time $(t_n + \Delta t)$

$$(x_\infty - x'_n) = (x_\infty - x_0)\exp.[-k(t_n + \Delta t)] \qquad (15)$$

Dividing (14) by (15) and rearranging gives

$$(x_\infty - x_n)\exp.(kt_n) = (x_\infty - x'_n)\exp.[k(t_n + \Delta t)] \qquad (16)$$

Therefore,

$$x_n = x_\infty[1 - \exp.(k\Delta t)] + x'_n \exp.(k\Delta t)$$

A straight line is obtained when plotting the observed readings in the first series (x_n) against the corresponding readings in the second series (x'_n), and the rate constant (k) of the reaction can be evaluated from the log of the slope of the line.

For $t=\infty$, $x_n = x'_n = x$, and therefore x_∞ is the point on the line at which x_n and x'_n are equal. Also, if the time of commencement of the reaction is known it is possible to extrapolate back along the line and find the corresponding value of x_0.

The following features should, however, be noted when employing this method:

(1) readings taken towards the end of the reaction are 'telescoped' on the graph and, therefore, are weighted less than earlier readings

(2) the data should be recorded over a period of time greater than $t_{\frac{1}{2}}$ (the half-life of the reaction) and preferably greater than twice this period. The time interval Δt should be in the range $0.5 t_{\frac{1}{2}} \longrightarrow t_{\frac{1}{2}}$.

(3) the method is relatively insensitive to deviations from strict first-order law, so an independent check of this is advisable.

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