DIFFERENTIAL PULSE POLAROGRAPHIC STUDY OF DRUG COMPOUNDS

by

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CONTENTS

SYNOPSIS

The work discussed in this thesis is in three parts. The initial work carried out was to develop a differential pulse polarographic (d.p.p.) method for the determination of disodium cromoglycate (INTAL) in human urine samples. The method eventually recommended involves an ion-pair extraction of the cromoglycate with tri-n-butylbenzylphosphonium chloride into chloroform. After evaporating the chloroform, and dissolving the residue in a buffer solution, down to 0.5 µg INTAL per ml urine can be determined. The method has the distinct advantages of simplicity and speed over the colorimetric method currently in use.

Colorimetric and d.p.p. methods were developed for the determination of sulphaguanidine after derivatization with hypochlorite and phenol. The methods are reasonably selective for sulphaguanidine, and could be applied advantageously for identification as well as determination. Good precision was obtained when the reaction conditions were properly controlled, so that, the procedure may be of value even though the clinical use of sulphaguanidine is declining.

The main work has been concerned with a d.p.p. polarographic study of several cephalosporins and their degradation products. D.p. polarographic methods were developed for the determination of eight cephalosporins in aqueous buffer solutions. The potentialities of d.p.p. for studying the degradation of cephalosporins has been

illustrated. In particular the evolution of hydrogen sulphide has been noted during the degradation of cephalexin and cephradine, and this hydrogen sulphide has been determined by an indirect d.p.p. method. Polarographic reduction peaks due to other degradation products have been identified. The reports of previous workers on non-polarographic methods have been confirmed, and it is now possible to follow the formation and degradation of several degradation products polarographically. The concentration of several products can now be determined for the first time.

On the basis of our degradation studies, an indirect d.p. polarographic procedure has been developed for the determination of cephalexin after hydrolysis in pH 7.4 phosphate buffer solution at 100°C for 1 hour. The recommended procedure has been shown to be superior to two other d.p.p. procedures developed and assessed in the present study. Also the sensitivity of this method is much better than d.c. and cathode-ray polarographic procedures developed by other workers.

Final studies have been made on the degradation of ampicillin (a penicillin of similar structure to cephalexin). Hydrogen sulphide was found not to be formed during the degradation, and the two major degradation products at pH 2.5 observed polarographically were penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine. D.p.p. procedures for the determination of these products have been developed, and the amounts of penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine have been determined in degradation solutions of ampicillin.

GENERAL INTRODUCTION

Direct current polarographic analysis was introduced in 1922 by Professor J. Heyrovsky[1]. Polarography is defined as a method making use of current-potential curves obtained in the electrolysis at a dropping mercury electrode (d.m.e.) of the solution to be analysed. Later, the application of solid electrodes[2], which may be stationary, rotated or vibrated, was introduced. Polarography with solid electrodes is often called voltammetry.

Polarography derives its analytical importance from two characteristics of current-potential curves. First, the half-wave potential (the potential at which the current is one-half the diffusion value) may indicate the identity of the substance which undergoes electron transfer. Second, the magnitude of the diffusion current is governed by the concentration of the electroactive substance.

In d.c. polarography, a very slow moving d.c. potential ramp is applied to the dropping mercury electrode. As the potential of the electrode moves into a region in which an electrode reaction takes place, a current is obtained, and the characteristic S-shaped polarographic wave is developed. However, d.c. polarography is not satisfactory at concentrations below about 10^{-5}M. This is because at such low concentrations, the faradaic current, caused by the reduction of the electroactive species, is reduced to the same order of magnitude as the charging current (current required to charge the electrode double layer). Further, the resolution

becomes very poor in the presence of relatively large concentrations of more easily reduced substance. In order to improve the sensitivity and the resolution, a number of variations on d.c. polarography have been introduced[3]. Pulse polarography which was first introduced by Barker in 1960[4] can be considered as one of the best of these variations. Recently, a number of publications which discuss the theory and application of pulse polarography have appeared[5-10].

Two pulse polarographic techniques are in general use; normal (or integral) pulse polarography (n.p.p.) and differential pulse polarography (d.p.p). In normal pulse polarography, potential pulses of successively increasing amplitude from a fixed initial potential are applied to the working electrode at a fixed time during the drop life or during the timing period (for a stationary electrode). The pulses are usually 50-60 msec in duration and the current is measured (sampled) at some fixed time (usually 40 msec) after pulse application. The time between pulses is usually 0.5 to 5 sec, and sample and hold circuits are utilized to display the sampled current on a conventional recorder[11]. The potential waveform and the resulting current-time behaviour for normal pulse polarography are shown in Fig. 1. The timing of the pulse application is such that it is applied to the electrode for 50-60msec before the drop is dislodged by a mechanical drop timer in the case of d.m.e. or 50-60 msec before the timing sequence ends when a stationary electrode is used. Therefore, when a d.m.e. is used the pulse is applied towards the end of the drop life when the rate of change of the drop area dA/dt is small and the electrode may be considered as a stationary electrode.

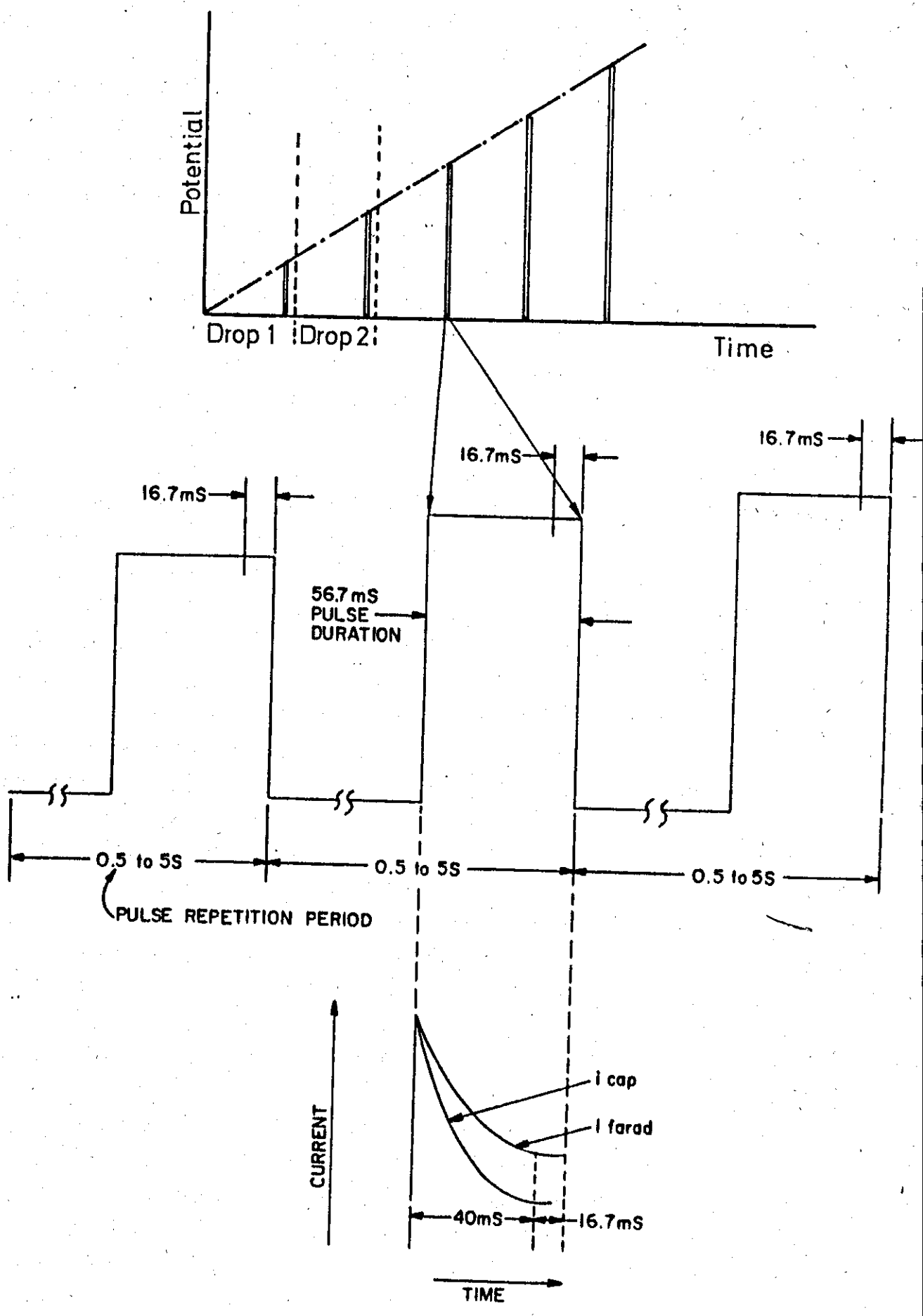

Fig. 1. POTENTIAL WAVEFORM & RESULTING CURRENT FOR PULSE POLAROGRAPHY

There are two sources of current resulting from pulse application: the faradaic current I_f and the capacitance current I_c. The capacitance current decays exponentially, while the faradaic current decays at a much slower rate. In potential regions where a faradaic current is present, the measured current is the sum of $I_c + I_f$. Examination of Fig. 1 shows that after 40 msec of pulse application, the capacitance current I_c is decayed to a negligible value compared with the faradaic current. Thus the measured current, is more nearly a pure faradaic current than is the case with d.c. polarography.

The normal pulse polarographic current on the diffusion plateau is given by Cottrell equation[12].

$$i_{d(n.p.p.)} = n\ F\ A\ D^{\frac{1}{2}} C\ /\ (\pi t)^{\frac{1}{2}} \qquad \ldots\ldots(1)$$

where $i_{d(n.p.p.)}$ is the normal pulse current in microamperes,

- n the number of electron involved in the reduction or oxidation of the electroactive species,
- F the Faraday (96500 coulomb/equivalent),
- A the electrode area in cm^2,
- D the diffusion coefficient of the reacting species in cm^2/sec,
- C the bulk concentration in mM,
- t the time in seconds measured from pulse application, at which the current is measured.

Substituting the area term A for the growing drop and including the factor $(\frac{7}{3})^{\frac{1}{2}}$ of the Ilkovic equation, equation 1 becomes

$$i_{(n.p.p.)} = \frac{462\ nD^{\frac{1}{2}} C m^{\frac{2}{3}} t_d^{\frac{2}{3}}}{t^{\frac{1}{2}}} \qquad \ldots\ldots(2)$$

$$A = (4\pi)^{\frac{1}{3}} (3 \times 10^{-3}/d_{Hg})^{\frac{2}{3}} m^{\frac{2}{3}} t_d^{\frac{2}{3}}$$

where d_{Hg} is 13.534 g/cm^3 at 25°C, m is the mercury flow rate mg/sec and t_d is the drop time in sec.

The sensitivity of normal pulse polarography with respect to d.c. polarography can be obtained by calculating the ratio of the normal pulse current $i_{(n.p.p)}$ from equation (2) and the d.c. current obtained from Ilkovic equation (3)

$$i_{(d.c.)} = \left(\frac{7}{3}\right)^{\frac{1}{2}} \quad 462\ nD^{\frac{1}{2}}C\ m^{\frac{2}{3}}\ t_d^{\frac{1}{6}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

$$\frac{i_{(n.p.p.)}}{i_{(d.c.)}} = \left(\frac{3\ t_d}{7t}\right)^{\frac{1}{2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(4)$$

For reasonable values of t_d and t the ratio $i_{(n.p.p)}/i_{(d.c.)}$ lies in the range 3-6 (Ref. 10). It follows that the analytical signal in pulse polarography is larger than that in classical d.c. polarography by a factor of 3-6. In practice, however, pulse polarography is more sensitive than it is predicted from equation 4. This additional increase in sensitivity arises from the ability of the technique to discriminate against the capacitance current and yield a signal composed mainly of the faradaic component which can be amplified[11].

In differential pulse polarography, a linearly increasing d.c. potential ramp is applied to the working electrode and a fixed-height potential pulse is superimposed near the end of the life of each drop or at the end of the timing period (for a stationary electrode). The current is sampled twice during the life of each mercury drop (or twice during the timing period in the case of stationary electrode). In the Model PAR 174 which has been used throughout this study, the current is measured (averaged) for 16.7 msec just prior to pulse application and again for the same period

starting 40 msec after the pulse has been applied. Zero difference is obtained when the currents are equal. Therefore, only at or near a half-wave potential is there a difference in the current flowing before and after the pulse application. This will give rise to a peaked representation of the data (see Fig. 2 and 3).

The relation between the peak current in differential pulse polarography $i_{(d.p.p)}$ and the normal pulse current $i_{(n.p.p.)}$ is given by equation 5

$$i_{(d.p.p)} = i_{(n.p.p.)} \ (\sigma - 1)/(\sigma + 1) \ldots\ldots\ldots (5)$$

where $\sigma = \exp(-\Delta E/2)nF/RT$, ΔE is the pulse amplitude in mV and other terms are as previously defined.

The ratio $(\sigma - 1)/(\sigma + 1)$ depends only on the pulse amplitude ΔE and the number of electrons n transferred during the electrochemical reaction. This ratio is always less than one.

The pulse amplitude has a remarkable effect on the d.p.p. current. For small pulse amplitude the d.p.p. current increases roughly linearly with ΔE, and therefore an increase in ΔE gives better sensitivity, however, at very large values of ΔE the problem of capacitance current becomes worse.

In n.p.p. the current increases linearly with n while in d.p.p. the current increases linearly with n^2 for a small pulse amplitude. Therefore, for compounds in which the number of electrons involved in the electrochemical reaction is large, higher sensitivity is

obtained than is the case with n.p.p.

The relation between peak potential, Ep, and half-wave potential is given by the relation

$$E_p = E_{\frac{1}{2}} - \frac{\Delta E}{2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(6)$$

For a very small pulse amplitude for a reversible system $E_p = E_{\frac{1}{2}}$.

The peak half-width which is a measure of the resolution is dependent on the pulse amplitude and the number of electron involved. For a large ΔE, (Ca. $\Delta E > 75$ mV) as ΔE increased, the peak width increases, but at small ΔE, the peak half-width is independent of pulse amplitude and inversely proportional to the number of electrons transferred[10].

The above relationships have been derived for a reversible electrochemical reactions and will not necessarily hold for irreversible cases. It is important therefore, to select the experimental conditions carefully to maximize the reversibility of the system by judicious selection of the supporting electrolyte.

The faradaic current in differential pulse polarography is less than that of normal pulse polarography. Nevertheless, the detection limit in d.p.p. is lower than that of n.p.p.. This can be attributed to a number of factors. In normal pulse polarography each pulse has a greater amplitude than the previous one and during the course of the scan the pulses become quite large, which results in a large capacitance current. The capacitance current in differential pulse polarography, however, is considerably less than

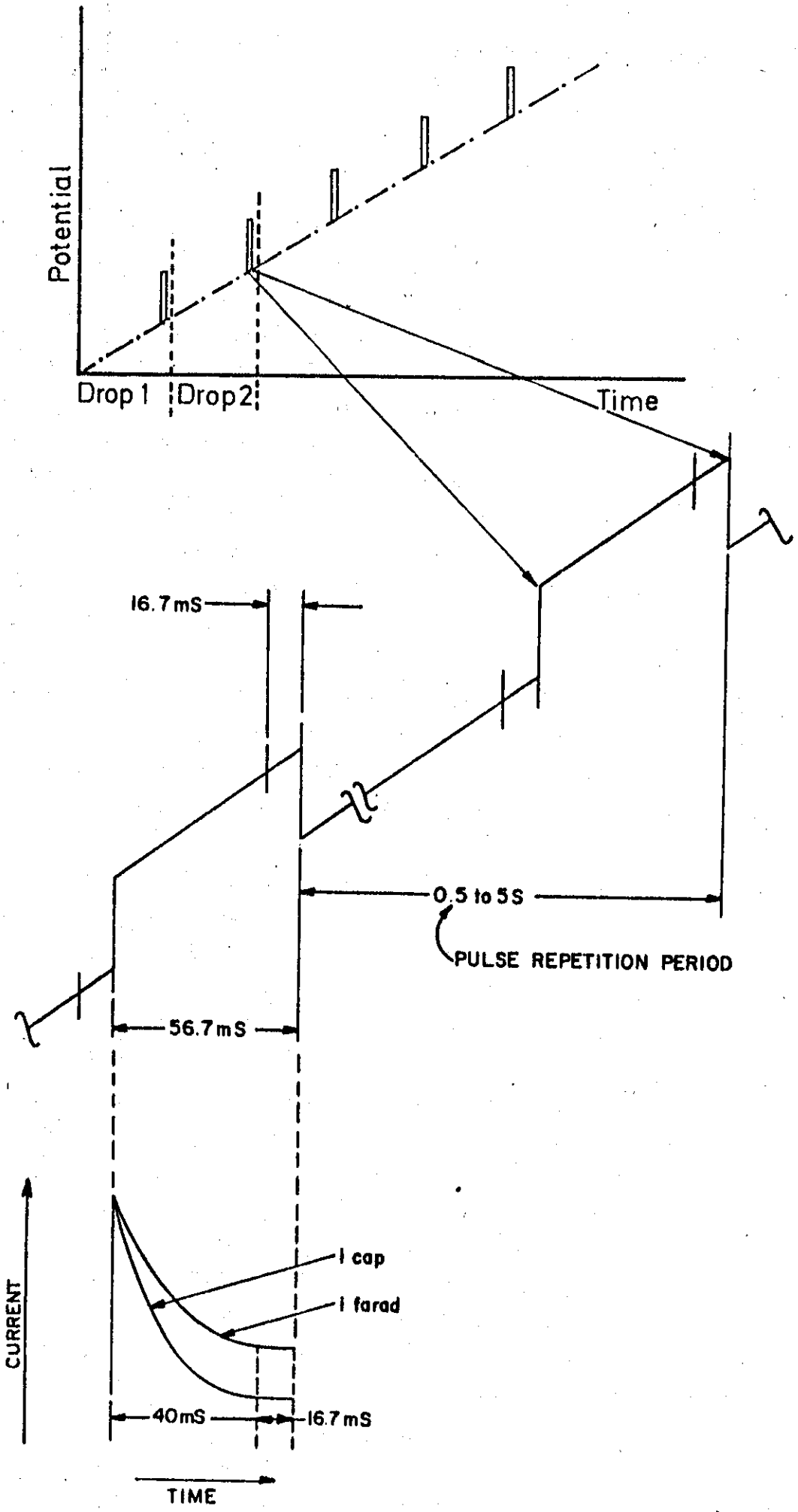

Fig. 2 . DIFFERENTIAL PULSE EXCITATION WAVEFORM & RESULTING CURRENT-TIME BEHAVIOR

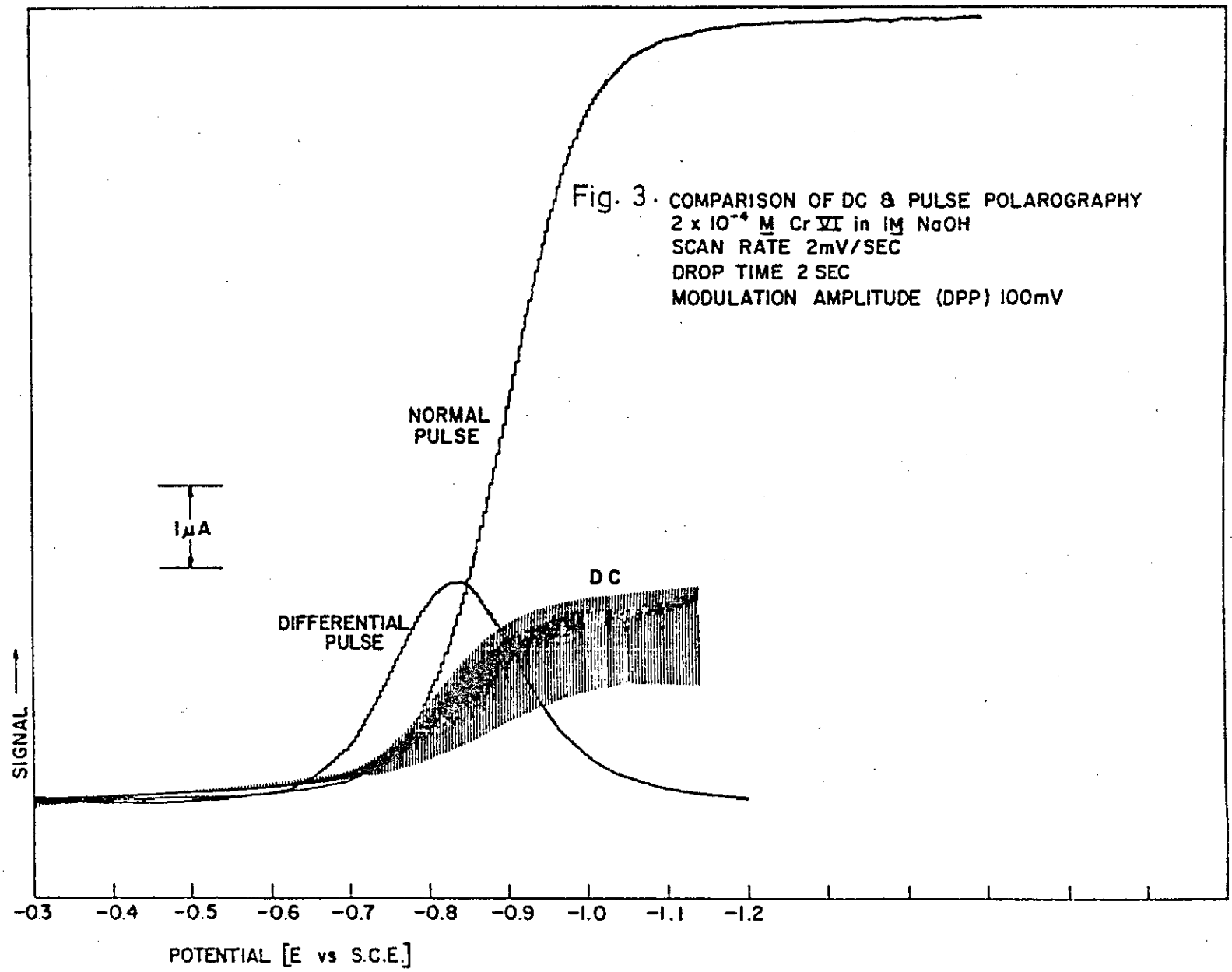

Fig. 3 · COMPARISON OF DC & PULSE POLAROGRAPHY
2×10^{-4} M Cr VI in 1M NaOH
SCAN RATE 2mV/SEC
DROP TIME 2 SEC
MODULATION AMPLITUDE (DPP) 100mV

that for normal pulse polarography owing to the pulse amplitude being small and uniform, and the resulting current which is relatively noise-free is more amenable to signal processing i.e. amplification. Further, differential pulse polarography provides peaks, which are more easily measurable specially at low concentrations, instead of steps as in the case of normal pulse polarography. Thus at high concentrations the limiting current obtained by normal pulse polarography will be larger than the peak current obtained by differential pulse polarography, but the limit of detection by differential pulse polarography will be considerably less than by normal pulse polarography (see Fig. 3).

Polarographic analysis has been used frequently in studying compounds of pharmaceutical importance in the 1950's. Brezina and Zuman[13] Volke[14] and Zuman[15] surveyed the polarographic behaviour and assays of the active constituents of many pharmaceutical preparations in addition to reviewing the application of the technique to biochemistry and medicine. The decreased interest in the application of d.c. polarography during the 1960's and the beginning of this decade, particularly for drug analysis, can be attributed to a number of factors:

1. The low sensitivity and poor resolution of d.c. polarography
2. Increased analytical requirements for trace analysis to provide evidence of purity (in environmental, food and drug analysis).
3. Development of more potent drugs administered in smaller amounts and the tendency for smaller sample size to be available (particularly when blood samples are involved).

4. Emergence of other sensitive instrumental techniques with the desired sensitivity and resolution such as gas-liquid chromatography.

5. Increase in the number of samples and the need for manpower economy.

During the present decade, increasing interest has been shown in the application of polarography and other voltammetric techniques of analysis of organic compounds especially drug compounds. This interest has been brought about mainly by the development of pulse polarography[4] together with the commercial availability of reliable but relatively simple and inexpensive instruments. Moreover, in more recent developments, commercial automatic polarographic analysers such as PAR Model 374 and PAR Model 384-1 were introduced. These instruments incorporate a pressurised mercury electrode in an automatic system, and also contain a microprocessor unit which is capable of subtracting a pre-recorded blank. These developments have extended into the region of trace analysis the lower limit of the range of concentrations over which polarography can be applied. Also, it allows the analysis of large numbers of samples both in pharmaceutical control and in production.

Advantages and limitations of pulse polarography

Polarography and particularly its more sensitive extension, differential pulse polarography of the analysis of pharmaceutical have several distinct advantages which can be summarised as follows:

1. Although large numbers of organic compounds of pharmaceutical importance are polarographically active, and can be determined directly by polarography, other electroinactive compounds can be made amenable to polarography after simple derivatization.

2. The sensitivity and resolution of the technique is sufficiently high to enable the determination of small amounts of polarographically active drug compound or traces of impurities or degradation products.

3. Polarography is a "cold" method of analysis which permits the drug compound of interest to be recovered and subjected to further analytical procedures.

4. Pharmaceutical preparations can in many cases be analysed without separation of the excipients. Also the technique is applicable to the analysis of turbid and coloured solutions.

5. One of the attractive features of polarography is that as we usually record the whole polarogram, any carelessness in handling the electrode, the hydrolysis cell or the presence of any electroactive interfering species will be noticed by the formation of new polarographic waves or the deformation in the wave shape. In contrast, in other techniques such as colorimetry,

UV spectrophotometry and spectrofluorimetry the common practice is not to record the whole spectra, but to carry out the measurements over ranges of few nanometres. The numerical values in this case neither indicate the presence of any interfering absorbing substance, nor it shows how carefully the analysis was carried out.

Differential pulse polarography also has some limitations. In many organic reductions the size of the peak currents (i_p) in d.p.p. is governed by the rate at which the current increases when the potential pulse is applied. For this reason the peak current is highly dependent on the exact composition of the matrix in which the polarography is carried out. A slight change in matrix composition can alter the peak current considerably.

Another problem is the nature of the capacitance background in d.p.p., which has the form of a differential capacitance curve. Adsorption of compounds from the solution alters the double layer capacitance and will increase the capacitance background in particular potential regions. The presence of traces of surface active materials can produce spurious capacitance peaks and/or enhance faradaic peak currents. This will normally only be apparent at high sensitivities.

Differential pulse polarography has recently been successfully applied to the analysis of drug compounds in biological fluids, formulations and dosage forms. The technique was applied either directly for drug compounds which have an intrinsic polarographic activity or after derivatization for those which do not show any

polarographic activity. The number of publications dealing with these applications is steadily increasing. The aim of the present discussion is not to survey these publications, but to point out the potentialities of d.p.p. in studying drug compounds guided by a selection of some important examples. The application of polarography and other voltammetric techniques to the analysis of organic compounds of pharmaceutical importance has been reviewed elsewhere[16-20].

The work of Brooks et al[18] adequately illustrates the usefulness of differential pulse polarography in the analysis of drug compounds and their metabolites in biological fluids, and will be discussed in some detail.

Brooks et al developed a direct d.p.p. procedure for the determination of two N-1-substituted nitroimidazoles, several benzodiazepines "diazepam(Valium), chlorodiazepoxide hydrochloride (Librium) and nitrazepam (Mogadon)" and trimethoprim and its metabolites in blood. Also they employed an indirect d.p.p. procedure to assay phenobarbital, diphenylhydantoin and glibornuride.

As with other methods of analysis, it was necessary to separate the drug species of interest prior to the determination step. The direct differential pulse polarographic procedures for the determination of the first group of drug compounds requires obtaining a protein free filtrate of blood with 10% sodium tungstate in 10% sulphuric acid, followed by extracting the drug into an organic solvent and dissolving the residue after evaporating the solvent into a suitable supporting electrolyte for polarography. The sensitivity of

the assay for both N-1-substituted nitroimidazole is about 0.2 - 0.3μg per ml blood, with a recovery of approximately 50%.

In the case of diazepam, the extraction procedure was not specific enough as the desmethyl metabolite is also co-extracted and interferes in the polarography. Therefore, the authors found it necessary to separate the parent compound from its metabolite by thin layer chromatography. The spots were eluted with ethanol, and the residues of which were analysed by d.p.p. after dissolving in a suitable supporting electrolyte. Similar procedures were used for the determination of Librium, Mogadon and their metabolites in blood samples. The procedure which was developed for the determination of trimethoprim employs selective extraction of the drug into chloroform from blood or urine. For the assay of blood, the drug was back-extracted into sulphuric acid and analysed directly by d.p.p. Urinary trimethoprim was first separated by t.l.c. from its metabolites, eluted with ethanol, and the residue of which was dissolved in sulphuric acid for polarography.

Brooks et al successfully determined phenobarbital, diphenylhydantoin and glibornuride by indirect differential pulse polarographic procedures. The assay involves selective extraction of the drug compound into an organic solvent from whole blood, following suitable "clean-up" of the sample. Each compound was nitrated in 10% KNO_3/H_2SO_4 at 25°C for 1 hour. The nitroderivatives were extracted into a suitable organic solvent and the residues were dissolved in the supporting electrolyte for polarography. The d.p.p. assay of the nitroderivatives of phenobarbital and

diphenylhydantoin enabled these compounds to be determined with a sensitivity of 1-2 µg per ml blood, while a sensitivity of 0.1 - 0.2 µg per ml of blood was obtained for the assay of glibornuride.

D.p.p. have also found application in the determination of drug compounds in formulations. The degree of prior separation required, depends upon the other ingredients present. In some cases simple dissolution is sufficient. In others some form of clean-up is required prior to the determination steps. This is illustrated in the following examples. Oelschläger et al[21] developed d.c. and d.p.p. procedures for the determination of chlordiazepoxide (Librium) in ten different commercial formulations, without the need for initial separation from other drugs or excipients. Teare et al in 1978[22] developed a d.p.p. procedure for the determination of flucytosine in tablets. The drug compound was first extracted from tablets with aqueous hydrochloric acid solution before it was polarographed in phosphate buffer.

The introduction of solid electrodes such as glassy carbon, carbon paste, rotating platinum and silicon rubber based graphite electrodes to the analysis of drug compounds has made voltammetry applicable to a very wide range of drug compounds. Thus many drug compounds which have no reducible functional groups, may be determined by an oxidation process at an indicator electrode, either in aqueous or non-aqueous solutions.

The glassy carbon electrode is now being extensively used in flowing systems for flow-injection analysis and as a detector in HPLC systems[23].

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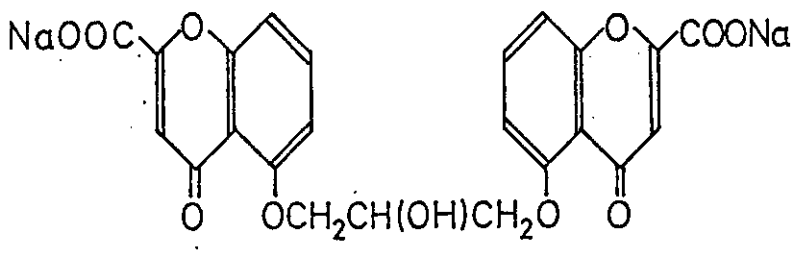
DIFFERENTIAL PULSE POLAROGRAPHIC DETERMINATION OF DISODIUM CROMOGLYCATE (DSCG) IN URINE

Introduction

Intal is the trade name of disodium cromoglycate (DSCG) (the disodium salt of 1,3-di(2-carboxy-4-oxychromen-5-yloxy) propan-2-ol)[1]. DSCG is freely soluble in water up to 5% and has a molecular weight of 512.3



DISODIUM CROMOGLYCATE

DSCG represents a new pharmacologic approach to the treatment of bronchial asthma. The compound is poorly absorbed from the gastro-intestinal tract after oral administration, but after inhalation as a fine powder into the lungs - using a special inhalation device - the compound is well absorbed and rapidly eliminated unchanged in the urine and bile[2]. The absorption, distribution and excretion characteristics in a number of laboratory animals have been reported[3-4].

Analytical methods developed for the determination of DSCG in absorption and metabolic studies include a fluorimetric method for its determination in plasma[2], and a colorimetric method for its determination in urine[2,5]. The intrinsic fluorescence properties of DSCG in aqueous solutions of pH greater than 2 has been made the

basis of the fluorimetric method. The method was applied after a preliminary purification of the plasma to remove naturally occurring fluorescent substances which interfere with the measurements. The clean-up process included precipitation of the plasma protein followed by extraction of DSCG into ethyl acetate. The DSCG was then back-extracted into dilute ammonia solution, and concentrated to a small volume under vacuum. The sample was then applied to a silica gel TLC plate. The DSCG was eluted from the plate with potassium dihydrogen phosphate solution and the fluorescence of the centrifuged supernatent layer was measured at an excitation wavelength of 350nm and an emission wavelength of 450nm.

In the case of urine samples, interfering substances present in urine caused a lack of specificity and precluded the use of the above method for measurement of DSCG. However, a colorimetric method for DSCG determination in urine based on the instability of the chromone ring to alkali has been developed[2]. The method involves the alkaline hydrolysis of DSCG to bis-o-hydroxyacetophenone, which is then reacted with diazotised p-nitroaniline to form an azo compound, having an absorption maximum of 490nm. The clean-up step preceding the colorimetric determination included passing the urine sample after acidification with formic acid (98-100%) through a column of Amberlite ion-exchange resin. Most urinary constituents, but not DSCG, come through the column on washing with aqueous formic acid, DSCG is later eluted with 98% formic acid and the residue after evaporating the eluate is subjected to the hydrolysis process. Curry and Mills[5] reported that, in order to obtain the recovery, precision and accuracy reported by Moss, G.F., et al[2], they found it necessary

to use a radioactive internal standard.

The aim of the present study was to develop a sensitive, precise and rapid polarographic method to determine DSCG in urine samples to replace the time consuming colorimetric method currently in use.

In the present work, the polarography of DSCG has been studied, and a differential pulse polarographic method for the determination of DSCG in urine has been developed. Separation of DSCG from urine can be effected either by extraction of cromoglycic acid from acidic solution into ethyl acetate, or by extraction of di-(tri-n-butyl-benzylphosphonium) cromoglycate from aqueous solution, over a wide pH range into chloroform. In both cases the organic solvent is evaporated and the DSCG is dissolved in a suitable supporting electrolyte for polarography.

Experimental

Equipment. Polarographic measurements were made with a PAR 174 polarographic analyser (Princeton Applied Research Corporation), equipped with a drop timer. Polarograms were recorded with an Advance HR 2000 x-y recorder. Three electrode operation was used with a dropping mercury electrode as working electrode, a platinum counter electrode and a saturated calomel reference electrode. For differential pulse operation, the forced drop time was 0.5 second, the pulse height 50mV and the scan rate 5 mV s^{-1}, except where otherwise indicated. The dropping mercury electrode used had (at an open circuit) a flow rate of 1.76 mg s^{-1} and a drop time of 3.5

sec. in B.R. buffer solution pH 6 at a mercury reservoir height of 68cm. Traces of oxygen in the nitrogen gas used to deoxygenate the solutions were removed by means of vanadium(II) scrubber[6]. Spectrophotometric measurements were made with Pye Unicam SP 8000 and SP 600 spectrophotometers.

Reagents. All chemicals were of analytical reagent grade except where otherwise stated.

Disodium cromoglycate. A pure sample of DSCG, obtained from Fisons Pharmaceutical Ltd., was dried at 100°C in a vacuum oven to a constant weight before use.

Tri-n-butylbenzylphosphonium chloride. Prepare 0.2M stock solution of tri-n-butylbenzylphosphonium chloride (Maybridge Chemical Co. Ltd) by dissolving 15.712g in 250ml distilled water.

Britton-Robinson buffer solution. A stock B.R. buffer solution (pH 1.9) which is 0.04M in boric acid, orthophosphoric acid and glacial acetic acid was prepared. From this stock solution, buffer solutions of varying pH were prepared by the addition of 0.2M sodium hydroxide solution and the pH was measured using a glass electrode connected to an expanded scale pH meter. Distilled methyl alcohol, butan-1-ol (S.L.R), chloroform, ethyl acetate, sodium perchlorate solution (0.4M) and hydrochloric acid solution (2M).

Experimental Technique

Polarography of DSCG in aqueous solutions.

(1) Study of the effect of pH on peak current and peak potentials

Polarographic behaviour of DSCG in the pH range 2-12 was studied in B.R. buffer solutions. At pH values lower than 2, hydrochloric acid solutions were used. For each polarographic run, 1 ml of DSCG stock solution (1×10^{-3}M) was pipetted into a 10-ml calibrated flask, and then diluted to the mark with B.R. buffer of the required pH. After thorough mixing, the solution was placed in the polarographic cell, and deoxygenated with oxygen-free nitrogen for 10 minutes. The solution was blanketed by an atmosphere of nitrogen whilst the polarograms were recorded. Polarograms in d.p. and d.c. modes were obtained for each solution between 0.0 - -1.6V.

(2) Effect of mercury reservoir height on the limiting current.

DSCG stock solution (1ml, 10^{-3}M) was diluted to volume in a 10ml calibrated flask with B.R. buffer pH 6.0, mixed thoroughly, and transferred to the polarographic cell. After deoxygenation for 10 min, d.c. polarograms were obtained at various mercury reservoir heights.

(3) Concentration Effect.

The effect of concentration was studied in B.R. buffer solutions pH 5.80 containing 10% methanol in the range 0.04 - 2.14 µg/ml DSCG.

Determination of DSCG in human urine

The presence of naturally occurring electroactive constituents in the urine necessitated the separation of DSCG from urine before the polarographic determination step. Two approaches were found satisfactory for the separation of DSCG from aqueous standards and urine samples:

(A) Ion-pair extraction of DSCG into chloroform

Procedure. Into a clean, dry 35ml glass-stoppered centrifuge tube, place 2ml of urine sample spiked with DSCG and 5ml of n-butanol. Shake the tube gently for 30 seconds and then centrifuge for 3 min at 2000 r.p.m. Carefully discard the (upper) organic layer with a syringe equipped with a 6in. cannula. To the aqueous layer in the centrifuge tube, add 1ml of B.R. buffer solution (pH 1.9), 2ml of aqueous 0.2M of tri-n-butylbenzylphosphonium chloride and 5ml of chloroform. Shake the tube gently for 30 seconds and centrifuge for 4 minutes at 4000 r.p.m. Carefully transfer the (lower) chloroform layer by means of a syringe to a 35ml glass centrifuge tube. (In order to ensure that only chloroform is drawn into the syringe, draw a small amount of air into the syringe before passing the cannula through the aqueous layer. This air is then expelled when the tip of the cannula is in the chloroform, thus discharging any of the aqueous phase that has entered it). Re-extract the aqueous layer with a second 5ml portion of chloroform in the same way. Evaporate the combined chloroform extracts to dryness in a water-bath under a stream of nitrogen. Dissolve the residue in 0.5ml of methanol and then add 4.5ml of B.R. buffer (pH 5.8). Transfer the solution to the polarographic cell, deoxygenate it with oxygen free nitrogen for

10 minutes, and obtain a differential pulse polarogram between -0.6 and -1.0V. Another approach to recover DSCG from the chloroform layer was found to be satisfactory. This involves the back-extraction of DSCG from the chloroform layer into 2ml of aqueous sodium perchlorate solution (0.4M). Add 3ml of B.R. buffer solution (pH 5.8) to the aqueous extract. Deoxygenate the solution and obtain a d.p. polarogram.

Effect of tri-n-butylbenzylphosphonium chloride concentration on the differential pulse peak heights.

B.R. buffer (4.5ml, pH 5.8), 0.5ml of methanol, and 0.1 ml of DSCG standard solution (100 μg/ml) were pipetted into the polarographic cell. After deoxygenating the solution for 5 minutes, a differential pulse polarogram was obtained. The above experiment was repeated using the same concentrations of DSCG and methanol in the presence of different concentrations of the phosphonium salt.

Spectrophotometric determination of tri-n-butylbenzylphosphonium chloride co-extracted with DSCG.

Aqueous solutions of the phosphonium salt show three absorption bands with λ_{max} at 253nm, 258nm and 265nm. However, the strongest absorption band at 258nm was used for the determination of the phosphonium salt in aqueous solutions.

Standard solutions of the phosphonium salt in distilled water were prepared in the concentration range 1×10^{-3}M - 2×10^{-2}M. The absorbances of the standard solutions were measured against water as a reference at λ_{max} 258nm using a Unicam SP 600 spectrophotometer and 1 cm silica cell.

In each of four 35ml glass-stoppered centrifuge tubes, 2ml distilled water, 1ml of B.R. buffer solution pH 1.9, 0.1ml of DSCG standard solution (100 µg/ml), 2ml of the phosphonium salt (0.2M) and 5ml of chloroform were added. The contents were shaken for 30 seconds, centrifuged for 4 minutes at 4000 r.p.m. and the organic layers were discarded. The aqueous layers were re-extracted with another 5ml portion of chloroform as above, and again the organic layers were discarded. The absorbance of the aqueous layers was then measured at 258nm using a 0.5cm silica cell against water as reference. The amount of phosphonium salt left in the aqueous layer was determined by comparing the absorbance with those of standards.

(B) Procedure with ethyl acetate

Place 2ml of urine sample and 5ml of ethyl acetate in a clean, dry 35-ml centrifuge tube. Shake gently for 30 seconds, carefully remove (by syringe) the (upper) ethyl acetate layer and discard it. Repeat the above extraction using another 5ml portion of ethyl acetate. To the aqueous layer add 2ml hydrochloric acid solution (2M), and extract with two 5-ml portions of ethyl acetate. Evaporate the combined ethyl acetate extract to dryness. Dissolve the residue in 0.5ml methanol and 4.5ml B.R. buffer solution pH 3.4 and obtain a d.p. polarogram as indicated above. (Alternatively, in this case, the DSCG may be back-extracted into 2ml of sodium hydroxide (2M), add 3ml B.R. buffer pH 1.9, deoxygenate for 5 minutes, and record the polarogram).

Results and Discussion

Pure DSCG was examined in B.R. buffer solution as the supporting electrolyte. The peak potentials and peak currents of the differential pulse polarographic peaks obtained for a 10^{-4}M solution of DSCG in B.R. buffer solutions at various pH values are shown in Table 1 and Figure 1.

Table 1. Effect of pH on the peak potentials and peak currents of d.p.p. peaks obtained for 10^{-4}M solution of DSCG in B.R. buffer.

pH	Peak 1		Peak 2		Peak 3		Peak 4	
	$-E_p$(V)	$i_p/\mu A$	$-E_p$(V)	$i_p/\mu A$	$-E_p$(V)	$i_p/\mu A$	$-E_p$(V)	$i_p/\mu A$
0.4	0.53	3.1	0.83	1.30				
0.7	0.58	2.4	0.87	1.50				
1.1	0.61	2.2	0.90	1.50				
1.7	0.63	2.3	0.92	1.50				
3.0	0.70	1.9	1.0	0.80				
4.0	0.78	1.9	1.1	0.10				
5.0	0.88	2.1	1.18	0.10				
6.0	0.96	3.5						
7.0	1.02	4.0						
8.0	1.10	3.1			1.40	0.20	1.60	0.35
9.0	1.17	2.3			1.37	0.70	1.60	0.70
10.0	1.20	0.5			1.35	1.20	1.60	0.95
11.0					1.35	1.40	1.60	0.80
12.0					1.35	1.50	1.62	0.60

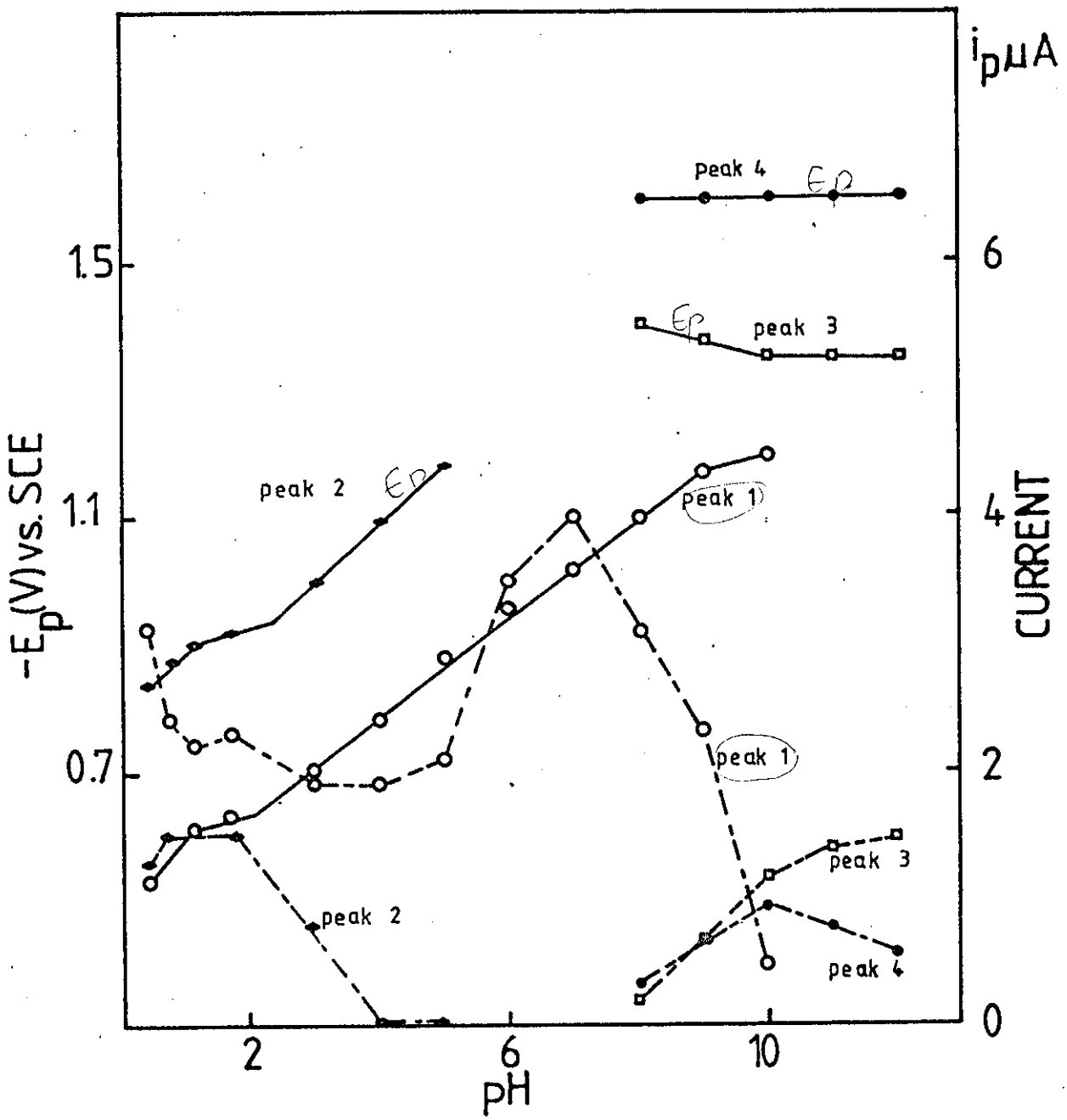


Fig. 1. Effect of pH on the peak potentials and peak currents of the d.p.p. peaks obtained for a 10^{-4} M solution of DSCG in B.R. buffer. Full lines, peak potentials vs pH; broken lines, peak currents vs. pH.

The peak potential of the main (first) peak is (-0.47 - 0.078 pH)V in the pH range 2-9; the change of the peak potential with pH is consistent with an irreversible electrode reaction. The size of the main (first) peak is clearly dependent on pH and is at a maximum at pH 7.

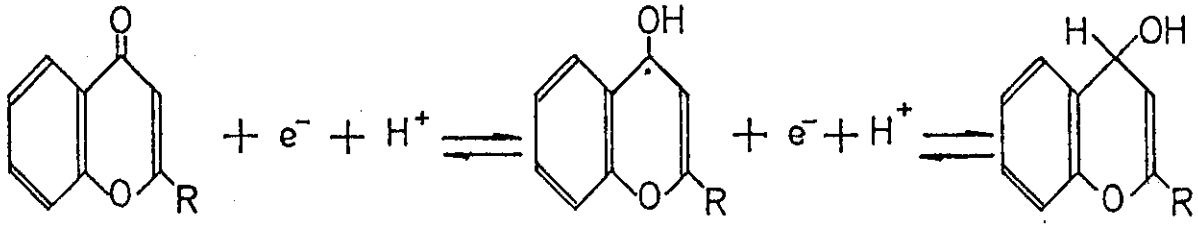
The effect of mercury reservoir height on the limiting current of the main wave is shown in Figure 2. The plot of wave height versus the square root of the height h of the mercury column (measured between the level of mercury in the reservoir and the tip of capillary) which is corrected for the interfacial tension at the surface of the growing drops (h_{back}) gave a straight line passing through the origin indicating diffusion control.

$$h_{corr} = h - h_{back},$$

where $h_{back} = 3.1/m^{\frac{1}{3}}t^{\frac{1}{3}}$ cm.

where m is mercury flow rate (mg/sec), and t is the drop time.

Knoboch,E.[7] has studied the electroreduction of chromone compounds in citrate-hydrochloric acid buffer solutions, and showed that these compounds are reduced irreversibly at the dropping mercury electrode. The reduction process which occurs at the C = O group in position 4 involves a two electron reduction step according to the following schemes:



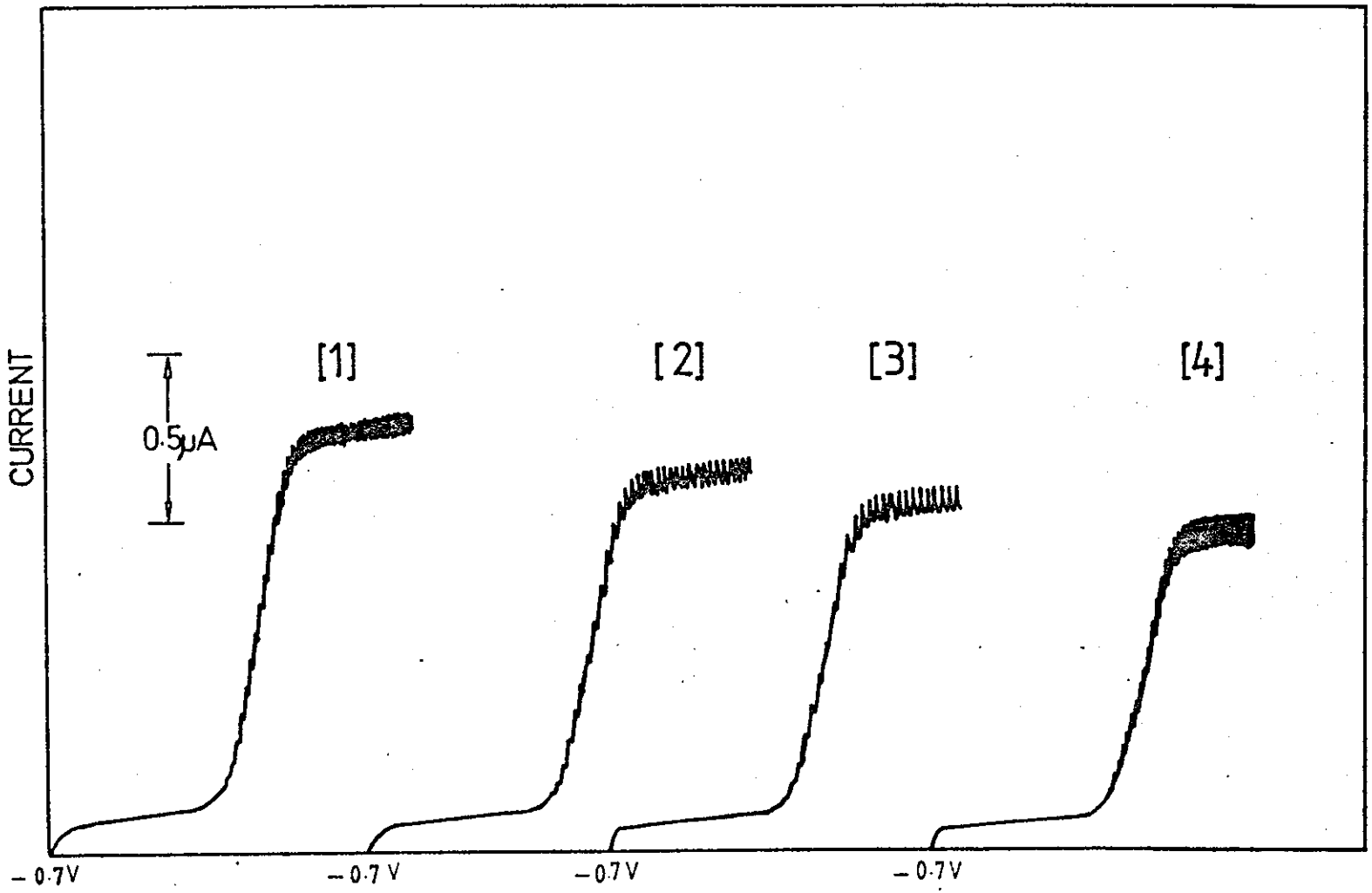


Fig. 2. Effect of mercury reservoir height (h) on the limiting current of the main d.c. polarographic wave. Mercury reservoir hiehgt (h): (1) 76, (2) 60, (3) 52, (4) 44cm.

In addition to the reduction wave, a catalytic wave accompanied by an adsorption pre-wave observed in buffer solution at pH values lower than 4. The catalytic wave was pH dependent and increased in size with increasing pH. However, both waves disappeared in the presence of surfactants.

DSCG, which contains two substituted chromone rings, undergoes electroreduction at the dropping mercury electrode. Two polarographic reduction waves were obtained in B.R. buffer solution at pH values lower than 5. The peak current of the second wave is strongly dependent on pH, and decreases in size considerably as the pH is increased; it reached about 6% of its maximum size at pH 4. However, buffers which also contained glycine and citric acid gave an adsorption wave between the two waves obtained for DSCG in B.R. buffer in the pH range 2 - 5. This wave disappeared when the DSCG concentration was reduced to $< 10^{-4}$M. This effect is shown in Fig. 3.

From the above information, it seems to be that DSCG follows the same polarographic behaviour of chromone compounds studied by Knoboch, E[7].

Typical d.p. polarograms obtained for DSCG in B.R. buffer solution (pH 5.8) containing 10% methanol are shown in Fig. 4. A rectilinear calibration curve was obtained in the concentration range 0.04 - 2.14 µg/ml and is shown in Fig. 5.

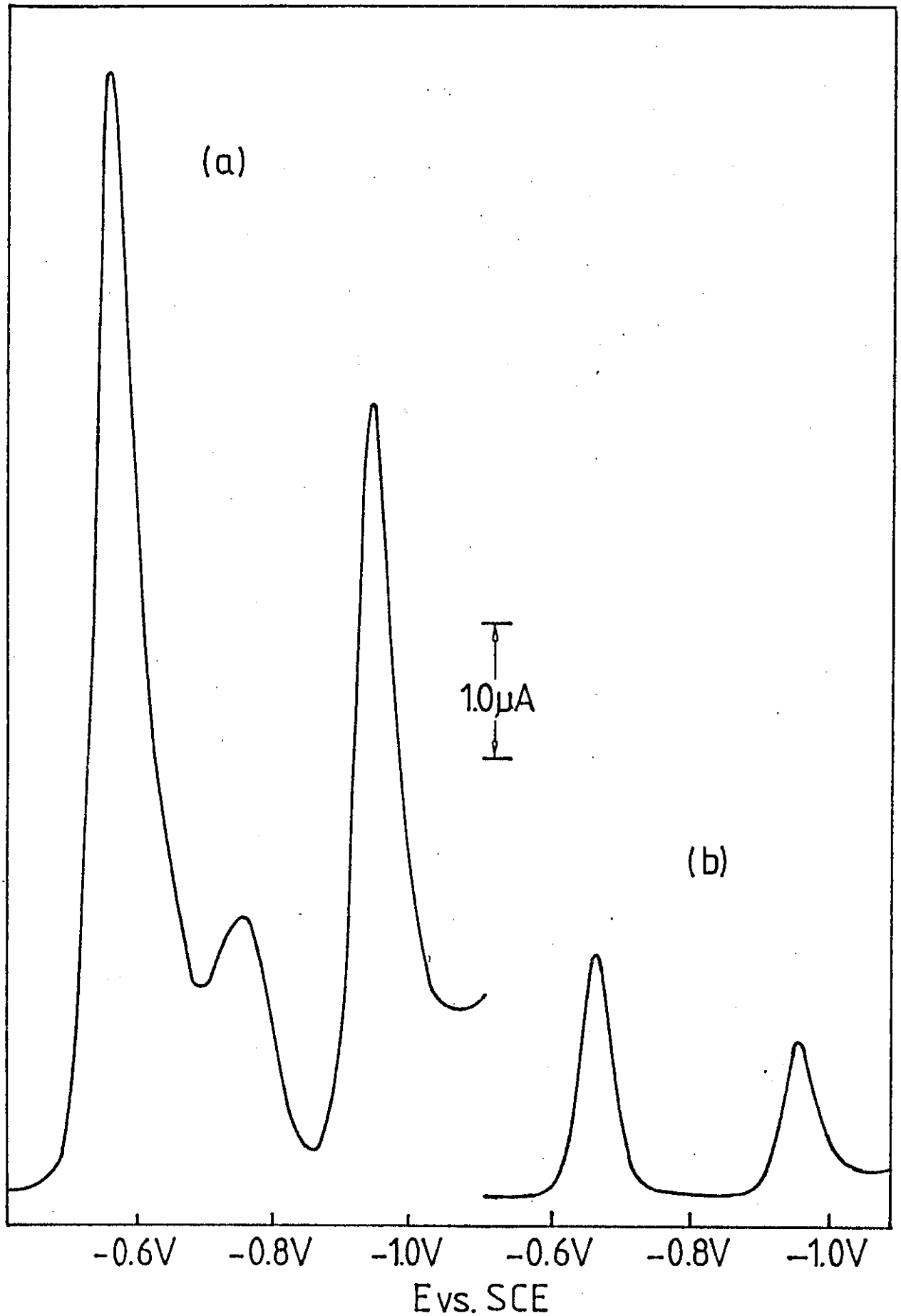


Fig. 3. Effect of DSCG concentration on the second d.p.p. peak of DSCG obtained in a buffer solution containing citric acid, orthophosphoric acid, hydrochloric acid, and glycine, (pH 2.3). DSCG concentration: (a) 1×10^{-4}M (b) 1×10^{-5}M

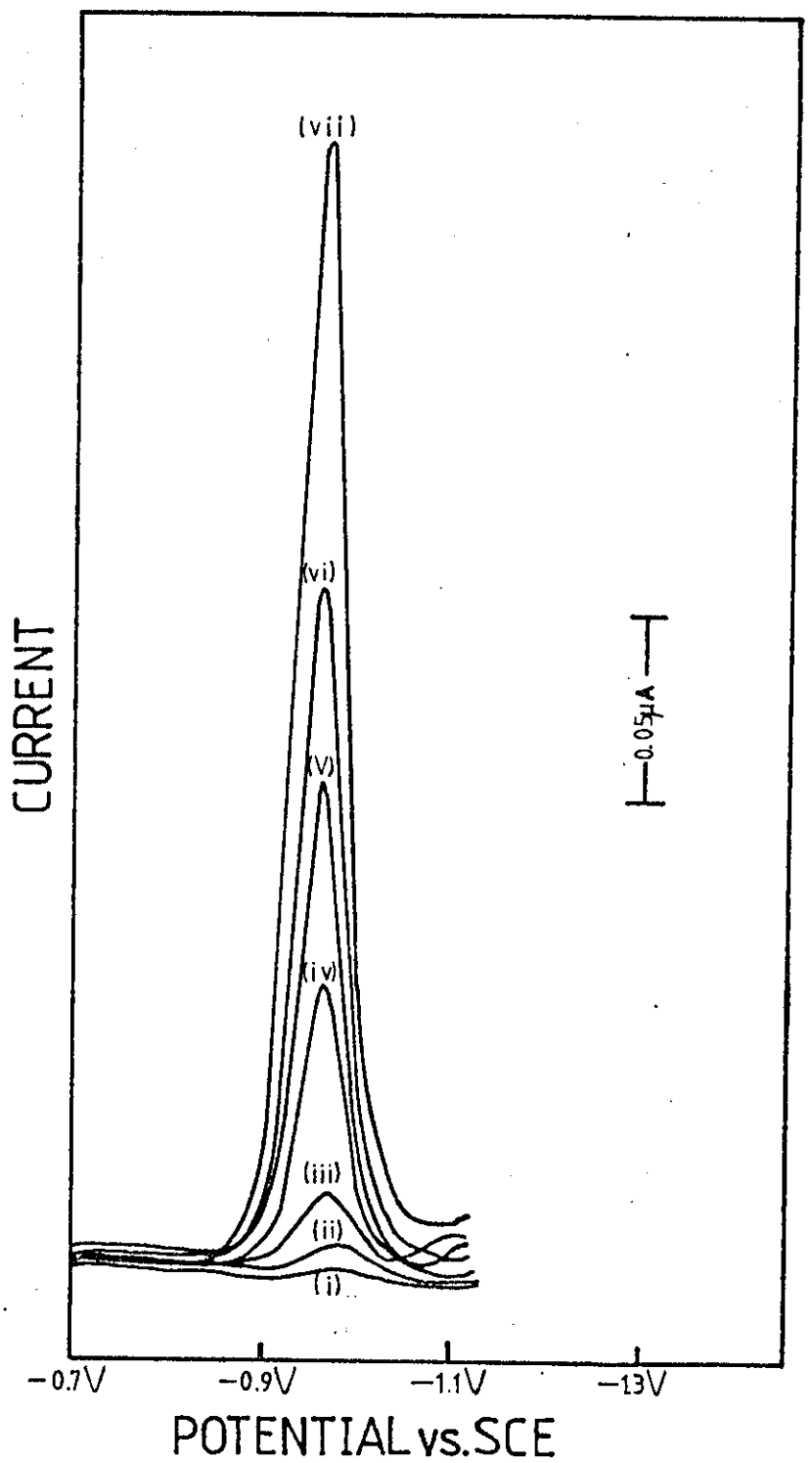
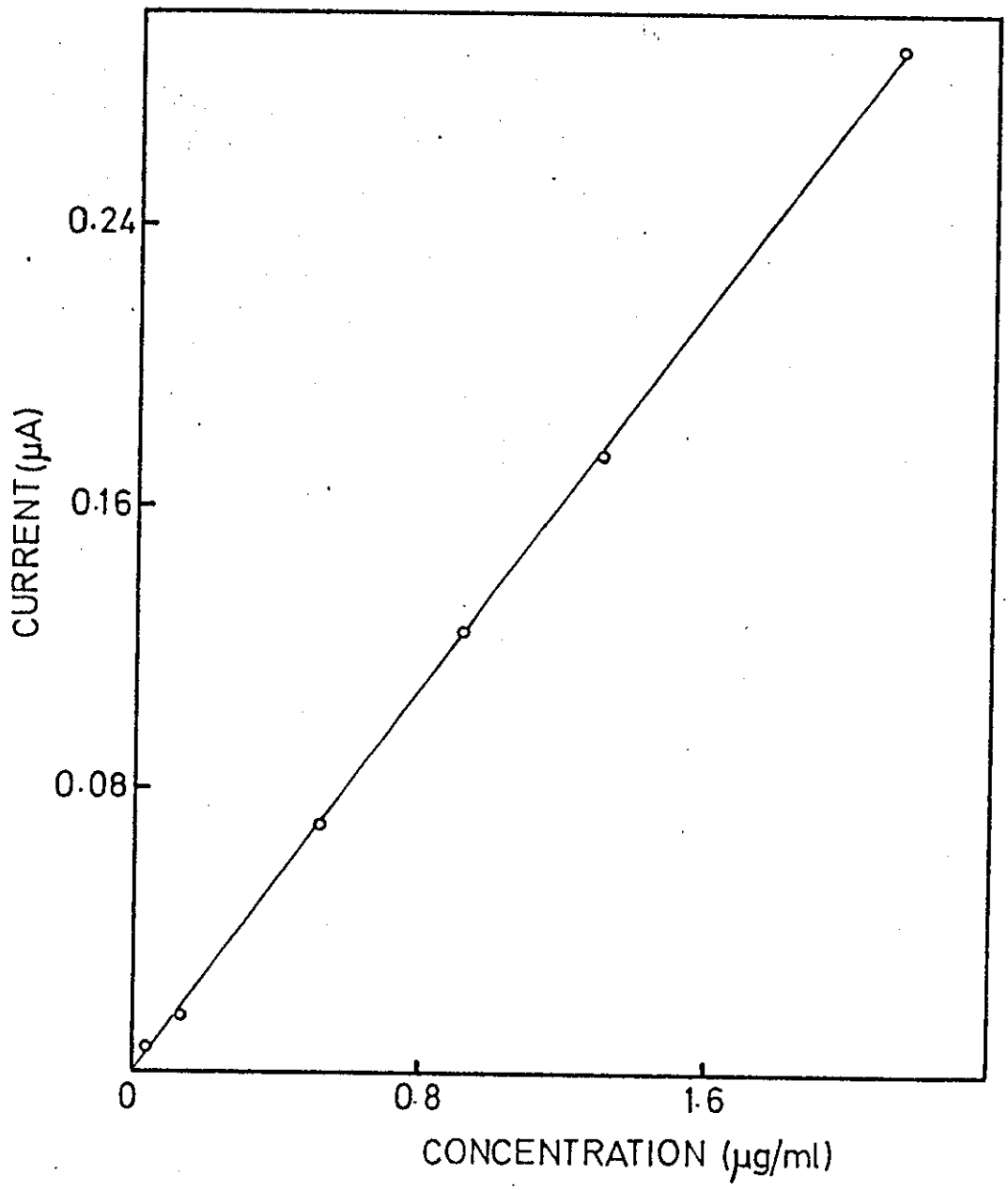


Fig. 4. Typical d.p. polarograms obtained in B.R. buffer pH 5.8 with 10% methanol. DSCG concentration: (i) 0, (ii) 0.04, (iii) 0.14, (iv) 0.53, (v) 0.92, (vi) 1.31, (vii) 2.14 μg/ml.



Fig, 5. Calibration curve of DSCG in B.R. buffer solution with 10% methanol.

Polarography of DSCG in urine

As we expected, it was necessary to separate DSCG from urine before the polarographic determination step. This is due mainly to the presence of naturally occurring electroactive constituents which interfere with the measurements and consequently limit the applicability of the technique for the determination of low drug levels in urine. Ion-pair extraction of DSCG into an organic solvent was tried first. Benzalkonium chloride (Benzyldimethyloctadecylammonium chloride) and tetrabutylammonium chloride were tried first unsuccessfully. Several other phosphonium salts were also tried, but tri-n-butylbenzyl-phosphonium chloride was readily soluble in water and gave low polarographic blanks. This compound was found to extract DSCG effectively from urine samples spiked with DSCG, and from aqueous standards (pH 2-9) into chloroform. However, extraction at an acidic pH was used throughout this study because it has the attendant advantage of lessening the risk of emulsion formation[8].

Attempts were made to extract interferents from the urine into chloroform at the physiological pH of the urine before DSCG was extracted with the phosphonium salt. Good recovery of DSCG was obtained but the base line was no better: a considerable improvement was observed, however, when pre-extraction of the impurities was done with n-butanol. Other extractive clean-up procedures with iso-amyl alcohol and hexane were tried unsuccessfully. No loss of DSCG was observed due to the pre-extraction of the urine samples spiked with DSCG with n-butanol.

Typical differential pulse polarograms obtained from urine samples spiked with DSCG by the above method are shown in Fig. 6a. Polarograms obtained after extraction of aqueous standards are shown in Fig. 6b. Comparison of the heights of these peaks with those obtained with aqueous standards without extraction indicated that the recoveries of DSCG from aqueous standards and from urine samples were 95% and 73% respectively. The relative standard deviation obtained for the determination of DSCG at the 1 µg/ml level in urine (10 determinations) was 4%.

The concentration of the phosphonium salt in the polarographed solution affected the height of the d.p.p. peak of DSCG. Jacobsen, E., et al[9] have shown that surface-active materials have this effect. The effect of different concentrations of phosphonium salt on the peak height of 3.9×10^{-6}M DSCG standard solution is shown in Table 2.

Table 2. Effect of various concentration of the phosphonium salt on the peak height of DSCG standard solution (3.9×10^{-6}M)

Phosphonium salt concentration	Peak height uA
3.9×10^{-6}M	0.34
1.96×10^{-2}M	0.30
3.90×10^{-2}M	0.28
7.80×10^{-2}M	0.24

The peak height of a standard solution of DSCG (3.9×10^{-6}M) in B.R. buffer pH 5.8 was 0.26 µA. From the above information, it can

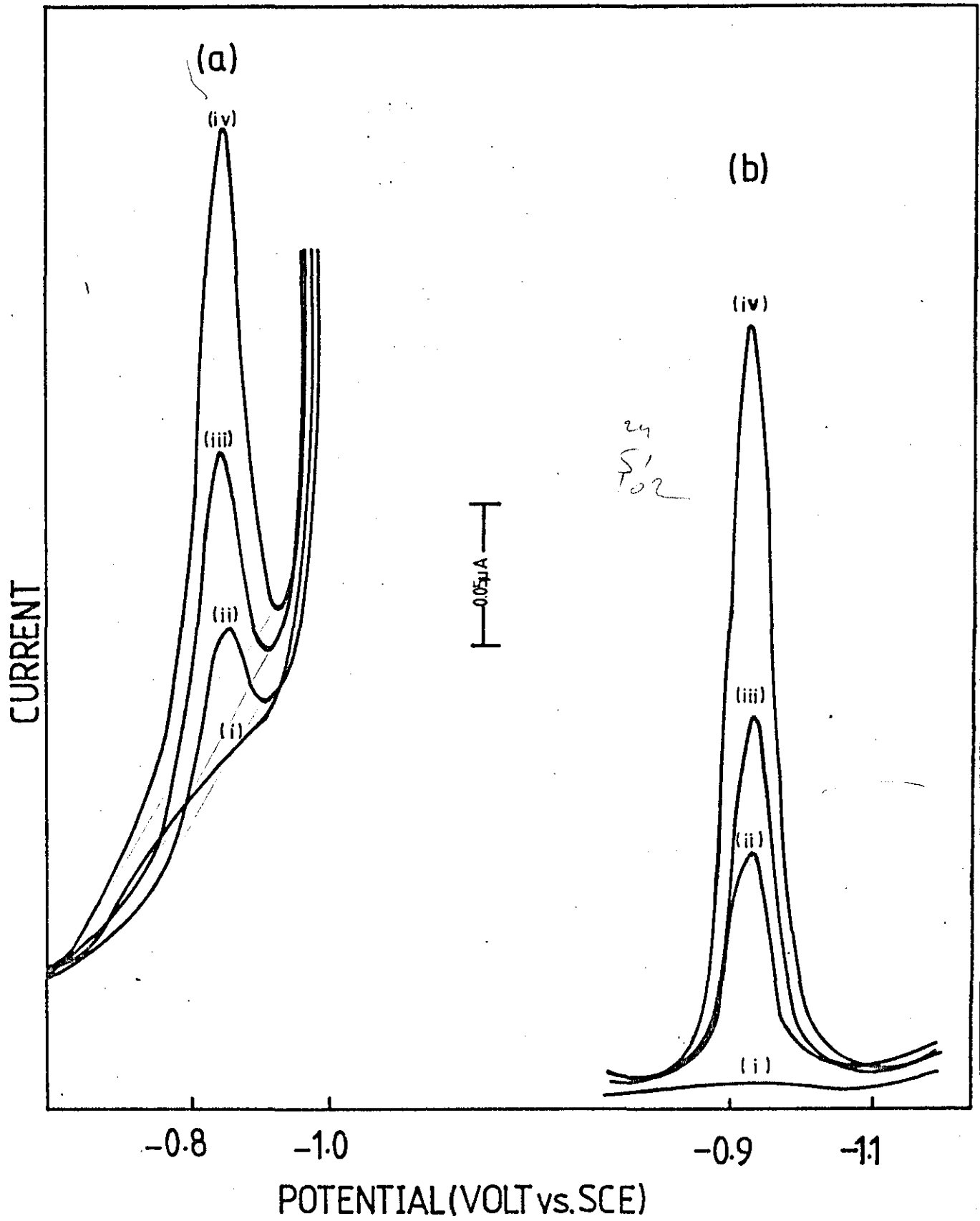


Fig. 6. Typical d.p. polarograms obtained for DSCG: after extraction with tri-n-butylbenzylphosphonium chloride from (a) urine and (b) aqueous standard for DSCG concentration of (i) 0, (ii) 1.75, (iii) 2.5 (iv) 5 μg/ml.

be seen that, the addition of an equimolar amount of tri-n-butylbenzylphosphonium chloride to DSCG at the 10^{-6}M level (pH 5.8) increased the height of DSCG d.p. peak by 30%, much larger concentrations of the phosphonium salt, however, depressed the height of the peak. So, the amount of the phosphonium salt used in the extraction of standards and samples should be controlled fairly closely.

U.V. spectrophotometry, was used to determine the amount of tri-n-butylbenzylphosphonium chloride co-extracted with the DSCG in the chloroform. First, a U.V. spectrophotometric method was developed for phosphonium salt determination in aqueous solutions. A rectilinear calibration curve was obtained over the concentration range $1 \times 10^{-3} - 2 \times 10^{-2}$M. The results are shown in Table 3.

Table 3. Results obtained for the spectrophotometric determination of phosphonium salt in aqueous solutions.

Phosphonium salt concentration.	Absorbance at 258nm
1×10^{-3}M	0.082
2×10^{-3}M	0.157
3×10^{-3}M	0.236
4×10^{-3}M	0.315
1×10^{-2}M	0.780
2×10^{-2}M	1.500

The amount of phosphonium salt co-extracted with DSCG was obtained by subtracting the amount left in the aqueous layer from

the total amount of the phosphonium salt added. In order to determine the amount of phosphonium salt left in the aqueous layer, four identical aqueous standard sample solutions were extracted as recommended above and the absorbance of the aqueous layers were measured. The results are shown in Table 4.

Table 4. Results obtained for the determination of the phosphonium salt left in the aqueous layer.

Sample No.	Absorbance of aqueous layer at 258nm (measured in 0.5 cm cells)
1	0.655
2	0.653
3	0.655
4	0.655

The amount of phosphonium salt left in the aqueous layer was found - by comparison of the absorbance with those of standards - to be 27.5mg; it follows that 98.2mg of phosphonium salt was co-extracted with DSCG. The addition of this amount to an aqueous standard solution of DSCG at 10^{-6}M level left the d.p. peak of DSCG unchanged.

DSCG was found to be effectively extracted as cromoglycic acid ($pK_{a_1} = pK_{a_2} = 2.0$) into ethyl acetate from aqueous standards and urine samples spiked with DSCG. The interferents in the urine were first extracted from urine at the physiological pH of the urine with ethyl acetate before extracting the DSCG as the free acid at lower pH. DSCG was recovered from the ethyl acetate by evaporating the ethyl acetate to dryness then dissolving the residue in 0.5ml of

methanol and 4.5ml B.R. buffer solution pH 3.4. Alternatively the DSCG may be back-extracted into 2ml of 2M sodium hydroxide solution. In this case, add 3ml B.R. buffer (pH 1.9). The latter procedure does not work well with urine samples.

The recovery of DSCG from urine sample by the ethyl acetate method exceeded 95% and the relative standard deviation (10 determinations) at the 1 µg/ml level was 5%. Typical differential pulse polarograms obtained by this method are shown in Fig. 7. The polarograms obtained when the phosphonium salt extraction was used seemed to be better, as indicated by a less steep base line. This may be due to the fact that ion-pair extraction is more selective and the amount of interferents co-extracted are less than in the case of ethyl acetate extraction. Even so, interferents in the urine produced a fairly steep base line and restricted the determination to 0.5 µg/ml DSCG in urine, compared with 0.04 µg/ml DSCG in aqueous standards.

The differential pulse polarographic procedure for the determination of DSCG in urine samples covers approximately the same concentration range as the colorimetric procedure[2,5]. The clean-up procedure recommended for use with the polarographic method, however, is simpler and quicker than that used with the colorimetric method and avoids the use of ion-exchange resins and concentrated formic acid solutions. In addition to the long analysis time of the colorimetric method ($>$ 2 hours), Moss, G. et al[2] reported that the rate of the application of the urine sample to the ion-exchange column was critical: faster rate than about 0.5 ml/min gave lower

recoveries, whereas, slower rates gave higher blank readings. Further, Curry and Mills[5], to obtain sufficient precision when formate-treated resins were used, found it necessary to use a radioactive internal standard to determine the DSCG recovery. In contrast, the precision of the polarographic method is good. The concentration of electroactive constituents in urine samples varies with the time of sampling and by the physiological state of the patient at the time of sampling. In the present work, a higher concentration of the electroactive interferences - indicated by steeper base lines - was observed in early morning samples, so that samples taken later in the day are to be preferred.

Previous authors[10,11] have commented on the fact that half-wave and peak potential of organic compounds in, or after extraction from, biological fluids, can differ from the potentials of the same compounds measured directly in aqueous solutions. This was true in the present work, the peak potential of DSCG extracted from urine being some 60mV more positive than that obtained directly in aqueous buffer at the same pH.

The polarographic method should be readily adaptable to the determination of DSCG in formulations, and should be particularly valuable in stability testing as the drug can be determined without derivatization.

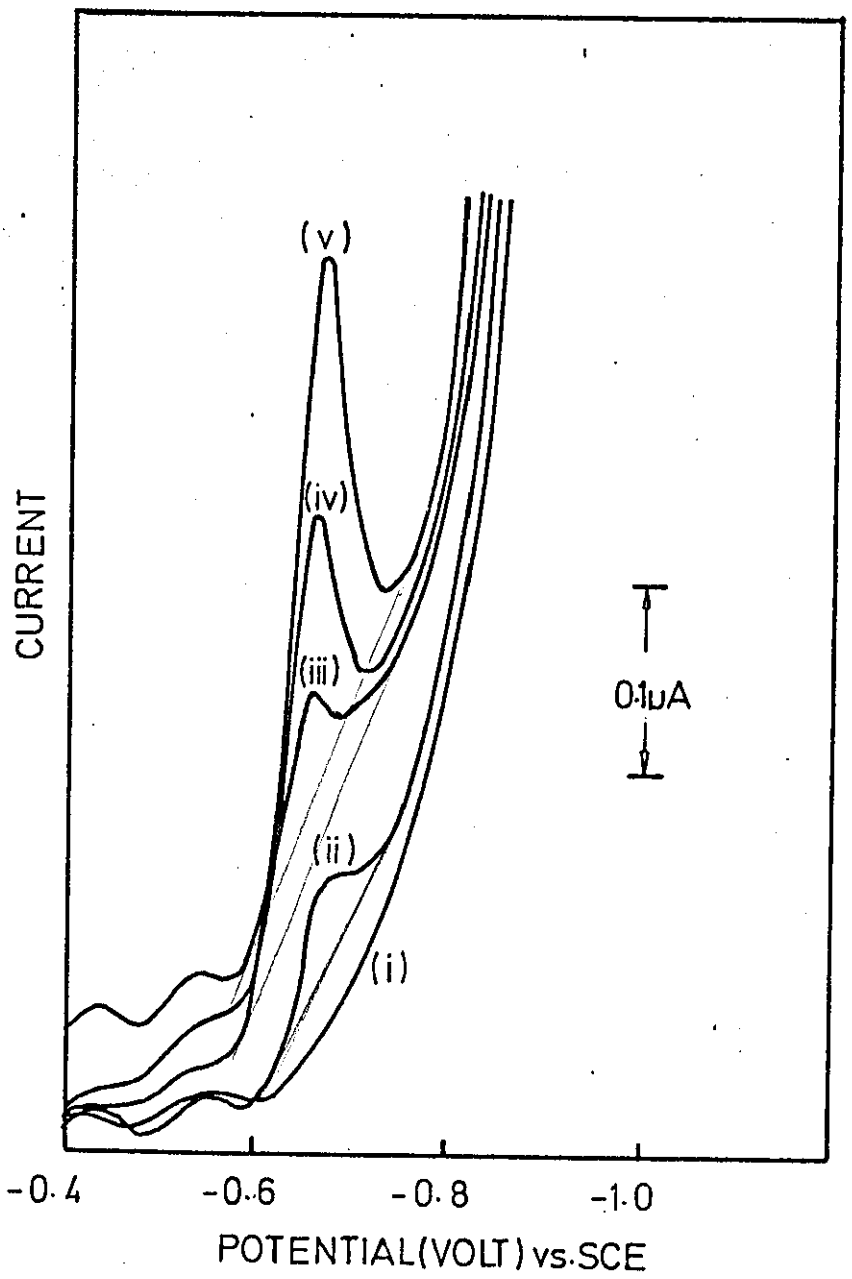


Fig. 7. Typical d.p. polarograms obtained after extraction of cromoglycic acid into ethyl acetate from urine for DSCG concentrations (i) 0, (ii) 0.5, (iii) 1.0, (iv) 2.5, (v) 5 μg/ml.

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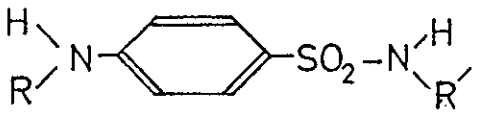
SPECTROPHOTOMETRIC AND DIFFERENTIAL PULSE POLAROGRAPHIC DETERMINATION OF SULPHAGUANIDINE BY REACTION WITH HYPOCHLORITE AND PHENOL

Introduction

The sulphonamides constitute a very important class of antimicrobial agents, and still sustain an important place in the chemotherapy of infectious diseases. Sulphapyridine, the preparation of which was reported in 1938, was one of the earliest sulphonamides to be used with great success in clinical practice for treatment of pneumonia. Over 3300 sulphonamides, however, have since been prepared, but only a few have been accepted for medicinal use[1]. The antimicrobial spectrum of all sulphonamides is essentially the same, but they may vary widely in their absorption, excretion and distribution characteristics.

Sulphaguanidine, which is absorbed to a varying degree from the gastrointestinal tract after oral administration is used for the treatment of local intestinal infections, particularly bacillary dysentery. The use of sulphaguanidine is now largely suppressed by the use of the less toxic sulphonamides, phthalylsulphathiazole and succinylsulphathiazole[2].

Generally, most of the therapeutically useful, antimicrobial sulphonamides are characterised by the following structure:



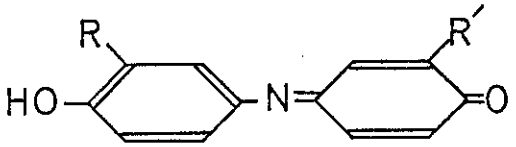
Structure I

The different sulphonamides are formed by varying the substituents R and R′.

A wide range of analytical methods is available for the determination of sulphonamides. Traditionally, these drugs were analysed by paper chromatography and colorimetry[3]. Recently, high performance liquid chromatography was extensively applied to the analysis of sulphonamides in pharmaceutical dosage forms, body fluids and tissues[4].

In this work, the formation of a coloured product possibly an indophenol compound was made the basis of colorimetric and differential pulse polarographic (d.p.p.) methods for the determination of sulphaguanidine.

Indophenols have the general structure II[5]



R=R′=H

Structure II

These compounds are usually intensely coloured, and their physical and chemical properties such as colour, acid-base and redox behaviour are affected by the introduction of various functional groups into the indophenol molecule.

Indophenol derivatives have been used frequently in analytical chemistry. They have been extensively employed for many years as redox indicators[6], as a titrant for the determination of ascorbic acid[7,8] and as acid-base indicator[9]. Some indophenols are used as spray reagents in paper chromatography[10], and as standards in

chromatographic separations[11]. Esters of indophenols have been used as chromogenic substrates for the estimation of acetylcholinestrase activity[12]. 2,6-Dichlorophenolindophenol was used for the detection of bacteriological contamination in foodstuffs[13].

The formation of an indophenol dye when ammonia solution was heated with an alkaline solution of phenol and hypochlorite was used by Thomas in 1912 to determine very low levels of ammonia[14]. Orr in 1924[15] used the reaction for the direct determination of ammonia in urine, and Murray (1925)[16] for determination of blood urea. The reaction has been used by several investigators after slight modifications[17-23]. Fairly recently, it was reported that the addition of sodium nitroprusside as a catalyst does not only accelerate the speed of the reaction but also increased to a maximum the conversion of ammonia to indophenol dye[24-29].

The condensation of quinonechlorimide and phenol to produce an indophenol dye was first described by Hirsh (1880)[30]. This reaction has been made the basis for an extremely sensitive method for the quantitative determination of phenolic compounds[31,32]. Vignoli et al (1965)[33] developed a colorimetric method for the determination of sulphonamides. In that method, a coloured product was formed (suggested to be quinoneimine)when sulphonamides were made to react with chloramine T in presence of phenol. Bratton et al(1939)[34] described a colorimetric method for the determination of sulphanilamide . The method was applicable to all sulphonamides containing a free primary amine group which in the reaction, is diazotised and coupled with N-(1-naphthyl)ethylenediamine dihydrochloride. Welch et al(1965)[35] described a colorimetric method for the determination

of N-acetyl-p-aminophenol in urine. The method involves the hydrolysis of N-acetyl-p-aminophenol with acid to p-aminophenol which is then coupled directly with phenol in the presence of hypobromite to form an indophenol dye.

Automatic[36] and manual[37] colorimetric methods for the determination of paracetamol and phenacetin based on the indophenol reaction have been described. Paracetamol and phenacetin were made to react with acidified hypochlorite solution to form a quinonechlorimide which in turn reacts with phenol to form an indophenol dye. Davis et al (1974)[38] developed an alternative manual procedure for the determination of phenacetin and paracetamol. In this latter procedure, paracetamol and phenacetin were first hydrolysed to p-aminophenol and p-phenetidine respectively, before reaction with hypochlorite to form p-quinonechlorimide, which then undergoes a reaction with phenol to form an indophenol dye. Ellcock and Fogg in 1975[39] described a simple method for the determination of paracetamol. The method involves the spontaneous oxidation of alkaline mixture of p-aminophenol (obtained as a result of paracetamol hydrolysis in hydrochloric acid solution) and phenol with molecular oxygen to form an indophenol dye.

Ellcock and Fogg (1974)[40] found it necessary to introduce sodium nitroprusside as a catalyst when they applied a method similar to that of Davis et al[38] for the colorimetric determination of sulphanilamide. The catalyst was found to be particularly effective in the reaction of sulphanilamide with alkaline phenol and hypochlorite. Finally, Fogg and Ahmed (1978)[41] applied differential pulse polarography (d.p.p.) for the determination of ammonia, p-aminophenol and sulphanilamide at the 10^{-7}M level after indophenol

derivatization. They found that d.p.p. can be conveniently applied as an alternative near the limit of the colorimetric procedure. Ellcock and Fogg[40] noticed that sulphaguanidine gave a green-yellow colour with λ_{max} at 450nm when it reacted with alkaline phenol and hypochlorite solutions in the presence of sodium nitroprusside as a catalyst. This was in contrast with the blue indophenol colour given by sulphanilamide under the same experimental conditions. The authors suggested that, the reaction could be made the basis of a colorimetric method for the determination of sulphaguanidine.

In the present work, the reaction between sulphaguanidine and alkaline phenol and hypochlorite was studied in more detail, particularly, the various factors which affect the coloured product formed. The initial reaction conditions used were those of sulphanilamide assay[40].

On the basis of these studies, a colorimetric method for the determination of sulphaguanidine was developed. Further, the coloured product formed gives a well-defined polarographic wave. This wave was used as the basis for a selective, precise and sensitive procedure for the determination of sulphaguanidine. The polarographic procedure was finally applied to the determination of the sulphaguanidine contents of a commercial sample of Guanimycin Suspension Forte.

Experimental

Equipment. Colorimetric measurements were made with a Pye Unicam SP 600 spectrophotometer using 1 cm cells. Polarographic measurements were made with a PAR 174 polarographic analyser as described for the previous work.

Reagents

All reagents were of analytical reagent grade except where otherwise indicated.

Sodium hypochlorite solution. Dilute 20ml of commercial sodium hypochlorite solution (10-14% available chlorine) to 500ml with distilled water.

Sodium hydroxide solution (10M). Dissolve 40gram of sodium hydroxide in distilled water and dilute the solution to 100ml.

Phenol solution, 3% w/v. Dissolve 3 gram of phenol in 100ml distilled water. (Care should be taken in handling concentrated phenol solutions to avoid contact with skin).

Sodium nitroprusside solution. Dissolve 0.1 gram of sodium nitroprusside in 250ml distilled water.

Standard sulphaguanidine solution, 2.8×10^{-4}M. Dry sulphaguanidine to constant weight at 105°C, dissolve 0.600 gram in a little dilute hydrochloric acid and dilute the solution to 100 ml with distilled water in a volumetric flask. Two further dilution (10ml→100ml) gave a 2.8×10^{-4}M sulphaguanidine standard solution.

Guanimycin Suspension Forte. Supplied by Allen and Hanburys Ltd., Ware, Herts.

Results and Discussion

The following set of experiments were carried out in order to optimise the experimental conditions which produce the maximum colour intensity.

Optimisation of sodium hydroxide concentration

Into six 50ml volumetric flasks, from 1-6 ml of sodium hydroxide solution (10M) were pipetted. The required volume of distilled water was added to each flask to bring the final volume to 6ml. To each of these flasks, 5ml of sodium nitroprusside, 5ml of sulphaguanidine solution (2.8×10^{-4}M), 5ml of 3% w/v phenol solution, and 5ml of sodium hypochlorite solution were added. The flasks were heated in a water bath at 45°C for 15 minutes. The solutions were cooled and diluted to 50ml with distilled water. The absorbance was measured at 450nm against water as a reference in 1 cm cells. The results are shown in Table 1.

Table 1. Effect of sodium hydroxide concentration on the apparent molar absorptivity of the coloured product formed.

Sodium hydroxide concentration(M)	0.38	0.76	1.14	1.52	1.9	2.28
Apparent molar absorptivity/ 10^4 l mol^{-1} cm^{-1}	1.64	1.21	0.68	0.67	0.54	0.57

The optimum concentration of sodium hydroxide is 0.38M.

Optimisation of sodium hypochlorite concentration

The experiment was repeated as before, except that the sodium hydroxide concentration was fixed to 0.38M and different volumes of sodium hypochlorite stock solution were used. The results are shown in Table 2.

Table 2. Effect of sodium hypochlorite concentration on the apparent molar absorptivity of the coloured product formed .

Volume of sodium hypochlorite added (ml)	1	2	5	10	15	20
Apparent molar absorptivity/10^4 l $mol^{-1}cm^{-1}$	0.82	1.41	1.69	1.85	1.64	1.46

The optimum volume of sodium hypochlorite is 10ml.

Optimisation of sodium nitroprusside concentration

Into eight 50ml volumetric flasks, from 0-7 ml of sodium nitroprusside solution (0.04% w/v) were added. The final volume was brought to 7ml with the required volume of distilled water. To each flask, 5ml of phenol solution (3% w/v), 1 ml of sodium hydroxide (10M) 5ml of sulphaguanidine standard solution and 10ml of sodium hypochlorite solution were added, and the experiment carried out as before. The results are shown in Table 3.

Table 3. Effect of sodium nitroprusside concentration on the apparent molar absorptivity of the coloured product formed.

Concentration of sodium nitroprusside solution/per cent w/v	0.0	0.0014	0.0028	0.0042	0.0057	0.0071	0.0086	0.01
Apparent molar absorptivity/10^4 l $mol^{-1}cm^{-1}$	1.75	1.67	1.60	1.67	1.71	1.71	1.73	1.73

From the above information, it is apparent that sodium nitroprusside has no significant catalytic effect on the formation of the coloured product, so, this reagent was omitted subsequently.

Optimisation of phenol concentration

In this experiment, the same concentrations of sulphaguanidine, sodium hypochlorite and sodium hydroxide were used as above in the presence of varying phenol concentrations, but in the absence of sodium nitroprusside. The results are shown in Table 4.

Table 4. Effect of phenol concentration on the apparent molar absorptivity of the coloured product formed.

Phenol concentration/per cent w/v	0.23	0.46	0.58	0.69	0.92	1.15
Apparent molar absorptivity/10^4 l $mol^{-1}cm^{-1}$	0.89	1.14	1.42	1.39	1.23	1.12

From the above results, the optimum phenol concentration is seen to be 0.58% w/v.

Effect of heating time

The effect of heating time on the colour intensity was studied at 45°C at the optimum reagents concentration, namely, sodium hydroxide concentration of 0.38M, sodium hypochlorite solution (10ml of about 0.4% available chlorine), phenol solution concentration of 0.58% w/v, and sulphaguanidine standard solution 2.8×10^{-4}M. Results are shown in Table 5.

Table 5. Effect of heating time on the apparent molar absorptivity of the colour obtained at 45°C.

Heating time/min	5	10	15	20	25	30	35
Apparent molar absorptivity/10^4 l $mol^{-1}cm^{-1}$	1.64	1.64	1.48	1.0	0.75	0.60	0.43

From the above results, the optimum heating time is seen to lie between 5 and 15 minutes.

Stability of the colour obtained with time.

The previous experiment was repeated with 10 minutes heating time, and the absorbance of the coloured product was measured with time at room temperature. The results are shown in Table 6.

Table 6 Effect of standing time at room temperature on the colour intensity obtained after heating for 10 minutes at 45°C

Standing time/min	4	9	13	18	23	29	35
Apparent molar absorptivity/10^4 l $mol^{-1}cm^{-1}$	1.73	1.66	1.61	1.55	1.42	1.41	1.35

Recommended procedure for the colorimetric determation of sulphaguanidine

To a 50ml volumetric flask add, in the following order, 1ml of 10M sodium hydroxide solution, 5ml of aqueous 3% (w/v) phenol solution, 5ml of sulphaguanidine standard solution or sample (contains less than 0.4mg of sulphaguanidine), and 10 ml of sodium hypochlorite solution. Heat the flasks in a water bath at 45°C for 10 min, cool, dilute to 50ml with distilled water, and immediately measure the

absorbance at 450nm in 1-cm cells. Subtract the absorbance of a blank determination containing no sulphaguanidine.

A rectilinear calibration curve obtained with the above procedure in the concentration range 0.5 - 4.5 x 10^{-5}M is shown in Fig. 1. The slope of the calibration curve corresponded to a molar absorptivity of 1.65 x 10^{4} l $mol^{-1}cm^{-1}$ for the coloured product, assuming a 1:1 molar conversion from the sulphaguanidine. The coefficient of variation (eight determinations) at the 2.8 x 10^{-5}M level in the measured solution was 1%.

Polarographic procedure

The procedure adopted was the same as the colorimetric procedure except that 5ml of ethanol was added after the heating step and before dilution to 50ml with distilled water; in the absence of ethanol the polarographic wave is distorted by an adsorption wave. Deoxygenation is affected by passing oxygen free nitrogen gas through the solution for 7 minutes. The d.p.p. curve is then immediately recorded between -0.4 and -0.8V.

Typical differential pulse polarograms are shown in Fig. 2. A rectilinear calibration curve obtained in the concentration range 0.5 - 4 µg/ml as shown in Fig. 3. The coefficient of variation at the 2 µg/ml level was 2.4% (10 determinations).

Application of the polarographic method to determine the sulphaguanidine content of a commercial sample of Guanimycin Suspension Forte.

The sample (1g) was weighed into a 50ml beaker and mixed well with 20ml of distilled water and 2ml of concentrated hydrochloric acid. The mixture was transferred to a 100ml volumetric flask,

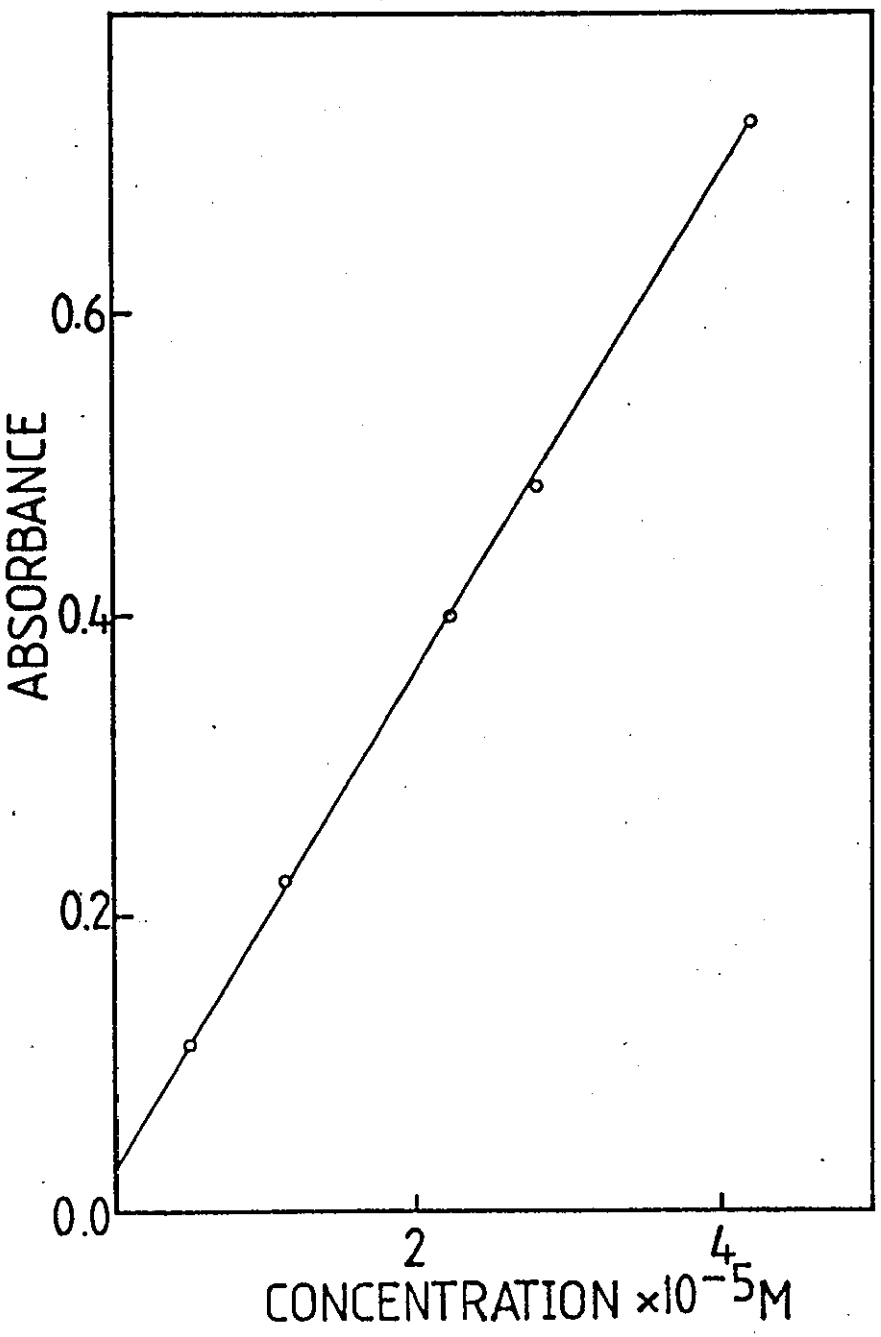


Fig. 1. Calibration graph obtained for sulphaguanidine by the spectrophotometric method.

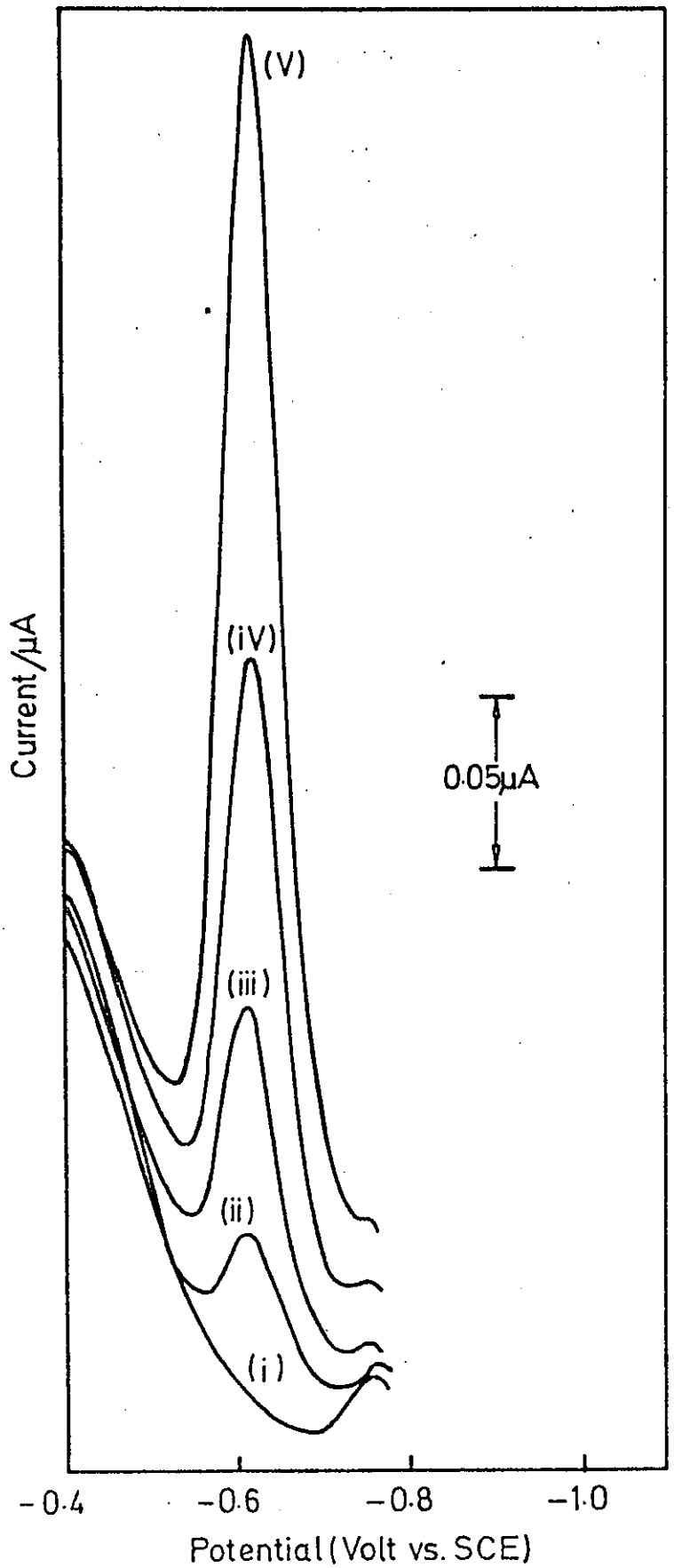


Fig. 2. Typical d.p. polarograms obtained for sulphaguanidine under the reaction conditions of the recommended procedure. (i) 0.0, (ii), 0.5, (iii) 1, (iv) 2, (v) 4 µg/ml

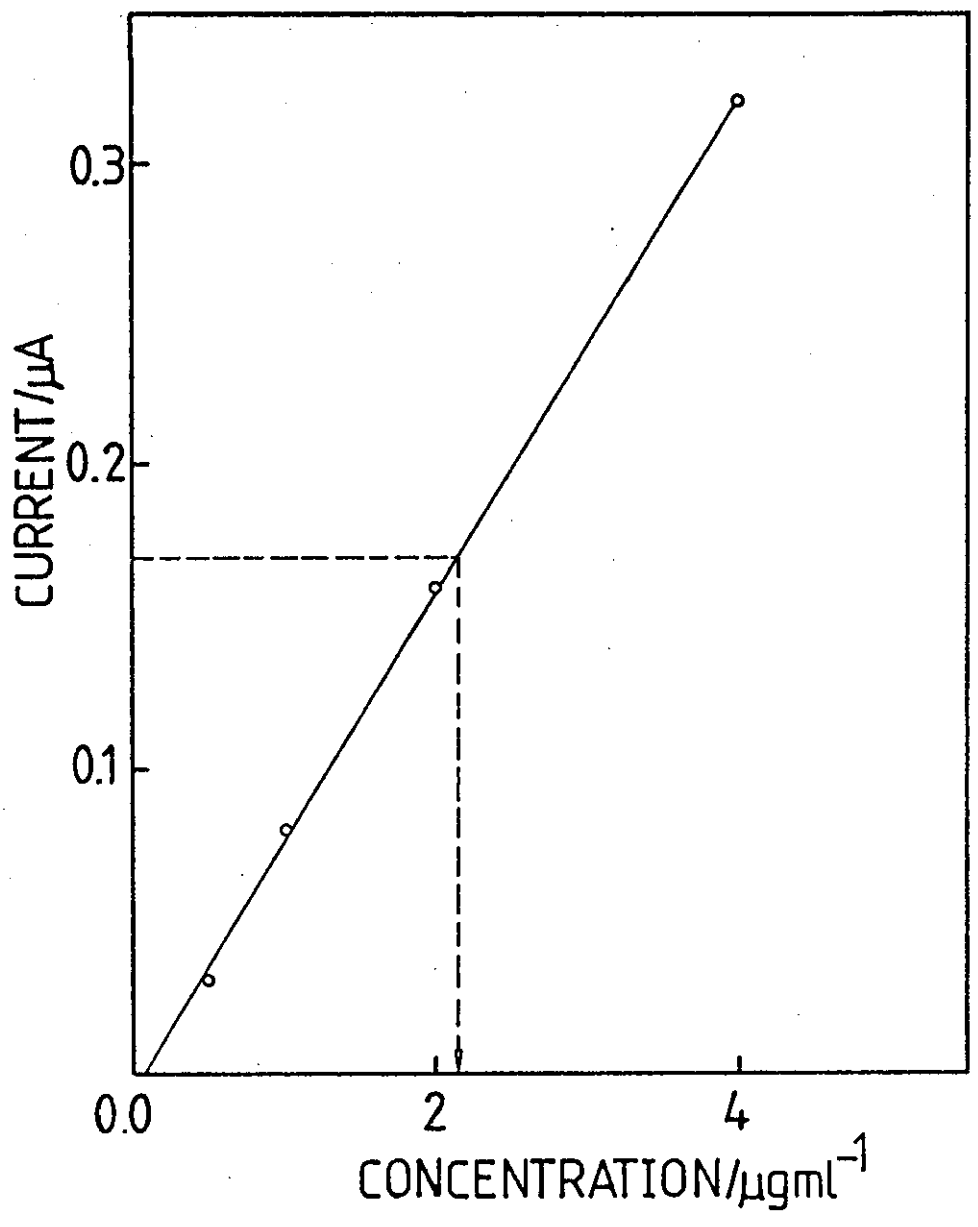


Fig. 3. Calibration graph obtained for sulphaguanidine by d.p.p.

diluted to volume with distilled water, and filtered through a sintered glass crucible; 10ml of the filtrate was diluted to 100ml in a volumetric flask. Further dilutions (10ml—⊶100ml followed by 20ml—⊶100ml) were made, and aliquots (5ml) of the final solution were used for the determination. The sulphaguanidine equivalent in the final solution was found by comparison of the d.p.p. peak height with those of standard to be 2.15 µg/ml. This corresponds to 2.1g of sulphaguanidine in 15ml of Guanimycin Suspension Forte compared with the 1.98g nominal content.

Behaviour of other sulphonamides.

The behaviour of several other sulphonamides under the reaction conditions of the recommended procedure was studied. None of these was observed to react at the same low levels as sulphaguanidine. For sulphanilamide at the 10^{-3}M level the blue indophenol colour and the polarographic peak were observed but the extent of the reaction was much less than would be the case with nitroprusside present[41]. Further, a shift in the position of the polarographic peak of 0.17V to more negative potential was observed under the present experimental conditions. Sulphasolucin gave the same blue indophenol colour and a polarographic reduction wave at -0.5V, but the extent of the reaction was also less than sulphanilamide. Sulphadiazine at the 10^{-3}M level was observed to give the yellow colour and a polarographic wave: the absorbance and peak current obtained were consistent with about 10% of the sulphadiazine reacting. Two other pyrimidine sulphonamides, sulphamerazine and sulphadimidine, and also sulphapyridine gave a slight reaction at the 10^{-2}M level indicating about 1% reaction. No colour reactions or polarographic waves were obtained for

guanidinium chloride and benzene sulphonamide under these reaction conditions. The result for the above sulphonamides and other sulphonamides are summarized in Table 7.

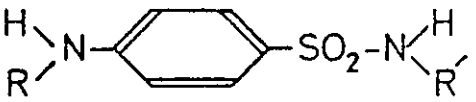
Good precision was obtained using the colorimetric and the polarographic methods, but, the alkalinity of the solution is critical. The addition of 2ml of 10M sodium hydroxide gives an apparent molar absorptivity of only 1.21×10^4 l mol^{-1}cm^{-1} compared with apparent ε of 1.64×10^4 l mol^{-1}cm^{-1} obtained when 1ml of 10M sodium hydroxide was added. So, the concentration of sodium hydroxide solution should be controlled fairly closely for standard and sample. The concentration of phenol and hypochlorite are less critical, but nevertheless, the volumes added should also be controlled quite closely. Calibration graphs should be prepared for each new batch of hypochlorite reagent solution.

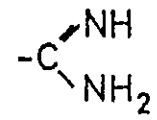
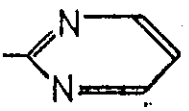
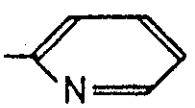
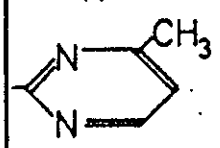
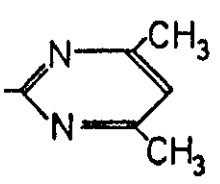
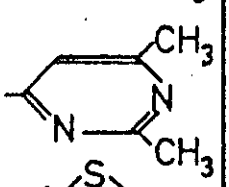
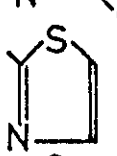
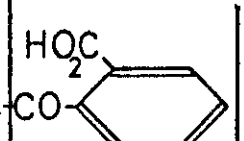
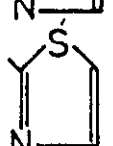
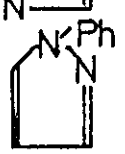
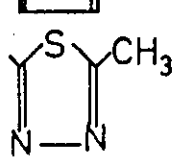
The colour intensity decreases rapidly for heating times greater than 10 minutes (e.g. apparent $\varepsilon = 1.48$ and 0.75×10^4 l mol^{-1}cm^{-1} after 15 and 25 minutes respectively). Loss of colour intensity after cooling to room temperature is much slower (e.g. apparent $\varepsilon = 1.35 \times 10^4$ l mol^{-1}cm^{-1} after heating for 10 minutes followed by a standing period of 35 minutes).

The presence or absence of sodium nitroprusside was found to have no significant affect on the apparent molar absorptivity of the coloured product formed. This was advantageous as nitroprusside gives an appreciable blank owing to the formation of nitropentacyanoferrate in alkaline solution[42].

$$Fe(CN)_5NO^{2-} + 2OH^- \rightleftharpoons Fe(CN)_5NO_2^{4-} + H_2O$$

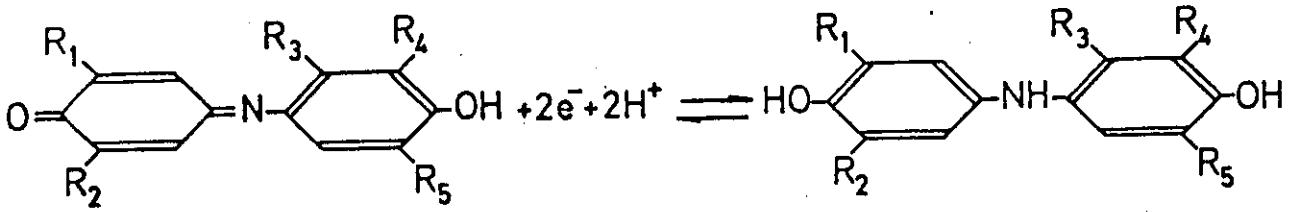
Table 7. Results obtained for number of sulphonamides under the reaction conditions of the recommended procedure



Name	Substituents		Colour Reaction	D.p.p. peak position	Remarks
	R	R′			
Sulphaguanidine	-H		Green-yellow colour	-0.63V	All concentrations.
Sulphanilamide	-H	-H	Blue colour	-0.50V	"
Sulphadiazine	-H		Yellow colour	-0.58V	At high concentration only
Sulphapyridine	-H		"	-0.58V	"
Sulphamerazine	-H		"	-0.60V	"
Sulphadimidine	-H		"	-0.60V	"
Sulphasomidine	-H		No colour	No peak	-
Sulphathiazole	-H		"	"	-
Succinylsulphathiazole	$-CO(CH_2)_2CO_2H$		"	"	-
Phthalylsulphathiazole			"	-0.56V	At high concentration only
Sulphaphenazole	-H		"	-0.56V	"
Sulphamethizole	-H		"	-0.56V	"

Further, Fogg and Ahmed[41] found it necessary to remove excess nitroprusside ion from the reaction mixture by the addition of sodium sulphite solution as the presence of excess sodium nitroprusside gives a d.p. polarographic peak at -0.27V which interferes with the measurements.

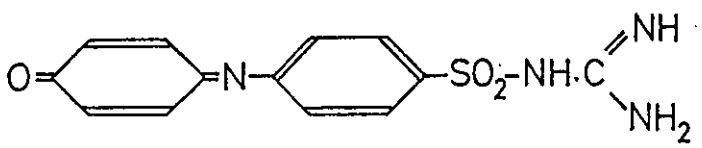
The polarographic behaviour of six indophenols has been studied by Patrick R.A. et al [43]. They suggested that all indophenols were reduced reversibly at the dropping mercury electrode, showing a well-defined polarographic wave. The wave was found to be diffusion controlled and corresponds to the addition of two electrons and two protons according to the following scheme:



The sensitivity of the polarographic method for the determination of sulphaguanidine is about 10 times greater than the colorimetric method, and could be applied advantageously to determine sulphaguanidine in turbid and coloured solutions. Satisfactory results were obtained when the polarographic method was applied to determine sulphaguanidine in Guanimycin Suspension Forte.

The colorimetric and the polarographic methods are more selective than the colorimetric method developed by Bratton et al[34] that is applicable to all sulphanomides that have a free primary aromatic amino group. Both methods described in this work could be applied with advantage in application which require identification as well as determination. The nature of the green-yellow colour is

not known: the colour remains unchanged on acidification. When sulphaguanidine is oxidised with hypochlorite under acid conditions, followed by reaction with phenol in alkali, it gives a blue colour (apparent $\varepsilon = 1.07 \times 10^4$ l mol^{-1}cm^{-1} at 625nm)[38]. The blue product obtained with sulphaguanidine possibly has structure III although this has not been confirmed.



Structure III

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DIFFERENTIAL PULSE POLAROGRAPHIC STUDY OF CEPHALOSPORINS AND THEIR DEGRADATION PRODUCTS

Introduction

Penicillins and cephalosporins, which are commonly referred to as the β-lactam antibiotics, are among the oldest and most important classes of naturally occurring antibiotics used in antimicrobial chemotherapy. The importance of these antibiotics is due to their inhibition of the terminal step in bacterial cell wall synthesis[1]. Cephalosporin C[2] is the parent substance from which the first cephalosporins to find clinical use were derived. Cephalosporin C was discovered at Oxford during a chemical study of the structure of penicillin N[3]. This substance which was found to have antibacterial activity, resembles penicillin N in some of its chemical and biological properties, but differed from it strikingly in others. The antibacterial activity of cephalosporin C *in vitro* was low, but the apparent relationship of the substance to the penicillin family, coupled with its resistance to penicillinase from *staphylococcus aureus*[4], gave it at once a potential clinical interest.

All penicillins which can be made by fermentation have a 6-acylamido group of the general structure $R\text{-}CH_2\text{-}CONH$, while, in the case of cephalosporin C, there are two side chains attached to the cephalosporin nucleus. The isolation of the penicillin nucleus, 6-aminopenicillanic acid (6-APA), in 1959[5], and the cephalosporin nucleus, 7-aminocephalosporanic acid (7-ACA), in 1961[6] has permitted the preparation of numerous semisynthetic penicillins and cephalosporins (by varying the side chain groups) which could not be prepared by biosynthesis.

These chemical modifications have been designed especially to produce penicillins and cephalosporins with more desirable properties, such as improved acid stability, broadened microbiological spectrum, decreased allergenicity and improved metabolic or pharmacological efficiency such as slow excretion, better tissue diffusion and better oral absorption.

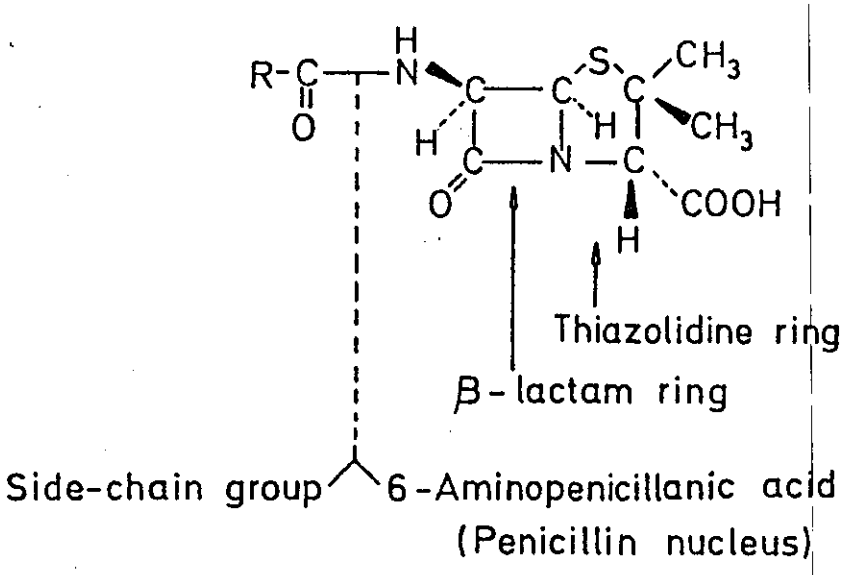
In contrast to the large number of side chains which give penicillins and cephalosporins with high activity, very little change can be tolerated in the nucleus before antimicrobial activity is markedly reduced or lost altogether.

Within the last few years several excellent reviews covering the chemistry [7-12], structure activity relationships[13-15], pharmacology[16-18] and clinical aspects[19] of semisynthetic β-lactam antibiotics have been published.

Structure of β-lactam antibiotics

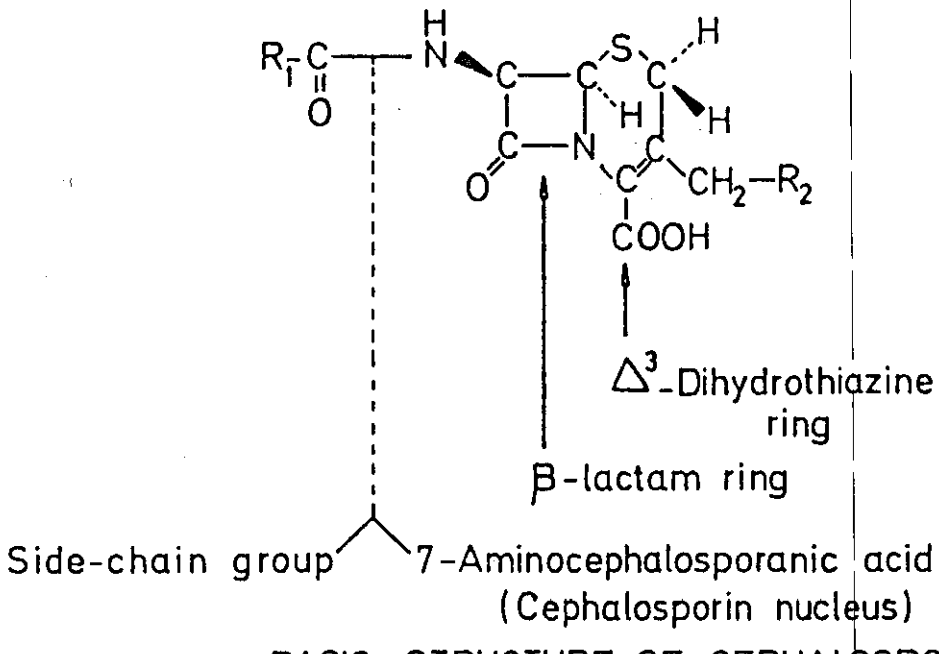
All β-lactam antibiotics are derivatives of a bicyclic ring system. All, with the exception of 6-amino-penicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA), have an acyl group attached as a side chain to the amino group of the β-lactam ring.

The penicillin molecule consists of a nucleus and a condensed side-chain group. The nucleus contains a four-membered β-lactam ring fused with a five-membered thiazolidine ring. The basic structure of penicillin is shown below.



BASIC STRUCTURE OF PENICILLINS

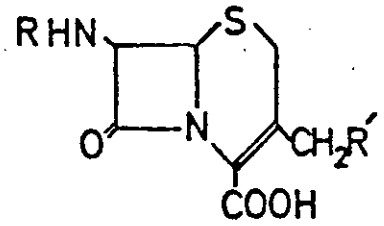
The cephalosporin ring system differs insofar as the four-membered β -lactam ring is fused with an unsaturated six-membered dihydrothiazine ring. The basic structure of cephalosporins is shown below.

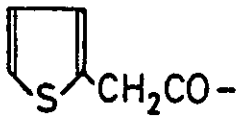
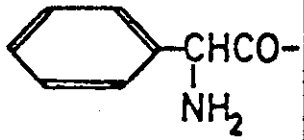
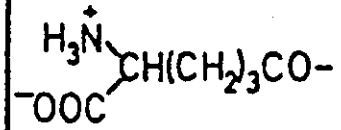
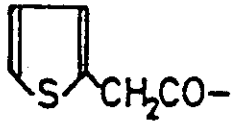
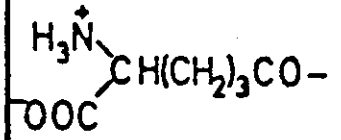
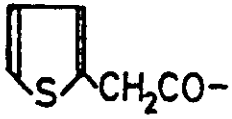
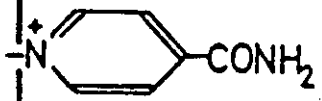
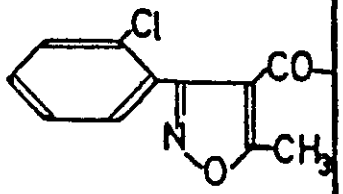
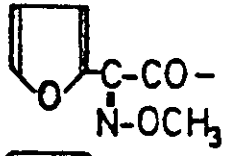
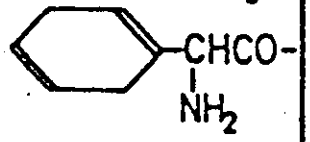


BASIC STRUCTURE OF CEPHALOSPORINS

The different penicillins are formed by altering the 6-amino side-chain and the different cephalosporins by altering the 7-amino side-chain and, in some cases, the 3-methyl substituent. The structure of some cephalosporins of clinical importance have been listed in Table 1.

Table 1.



NAME	SUBSTITUENT		NAME	SUBSTITUENT	
	R	R´		R	R´
CEPHALOTHIN		$-OCOCH_3$	CEPHALEXIN		$-H$
CEPHALOSPORIN C		$-OCOCH_3$	CEPHALORIDINE		
DESACETYL-CEPHALOSPORIN C		$-OH$	CEPHALONIUM		
CEPHOXAZOLE		$-OCOCH_3$	CEFUROXIME		$-OOCNH_2$
7-AMINOCEPH-ALOSPORANIC ACID	H-	$-OCOCH_3$	CEPHRADINE		$-H$
7-AMINODESACET-OXY CEPHALOSP-ORANIC ACID	H-	$-H$			

Analytical methods of determining cephalosporins and penicillins

Many techniques have been described for the analysis of cephalosporins. These techniques in many instances were applied previously to the determination of penicillins. This is due mainly to the structural similarity of cephalosporins and penicillins. The methods of determination can be classified as follows:-

1. Microbiological determinations:
   a. Plate assays.
   b. Photometric assays.
2. Chemical determinations:
   a. Iodometric method.
   b. Hydroxylamine method.
   c. Ninhydrin colorimetric method.
   d. Nicotinamide method.
3. Chromatographic methods:
   a. Paper and thin layer chromatography.
   b. High performance liquid chromatography (HPLC).
   c. Column chromatography.
4. Physicochemical method.

   This category includes U.V., I.R., N.M.R., optical rotatory, x-ray diffraction, spectrofluorimetric and polarographic methods.

Methods in the first two sections provide quantitative results, while those in the other sections serve in addition to identify the β-lactam antibiotic (or its metabolites or impurities). Recently two reviews covering most of the analytical techniques used to assay penicillins and cephalosporins have appeared[20,21].

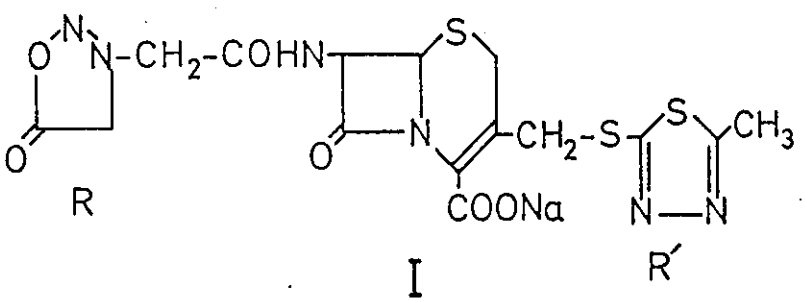
Polarography of cephalosporins and penicillins

Microbiological assay of antibiotics is the accepted technique for measuring antibacterial activity. This technique is time consuming and lacks the capability to function as a rapid screening technique. Often, more information than just antibiotic activity is required. In the development of a new antibiotic species, the analytical chemist is required to develop analytical methods for formulation stability, quality-control and drug metabolism studies. For formulation stability studies a precise and specific method is needed to discriminate between the parent antibiotic and its degradation products, while in quality-control it is necessary to ensure that the antibiotic is not contaminated with unacceptable amounts of starting materials or reaction by-products. In metabolic studies, specific and sensitive methods are required to assay antibiotics in the presence of their metabolites. Polarography and especially differential pulse polarography appears to be a satisfactory technique capable of solving most of the above problems providing that the compounds sought are polarographically active.

The first report of the d.c. polarographic determinations of cephalosporins seems to be that of Jones et al in 1968[22]. The authors showed that cephalosporin C, cephalothin and cephaloridine each exhibits a reduction wave at the dropping mercury electrode (d.m.e).

They suggested that only the cephaloridine d.c. wave could be used for quantitative determinations, while use of the ill-defined waves of cephalothin and cephalosporin C should be restricted to qualitative purposes.

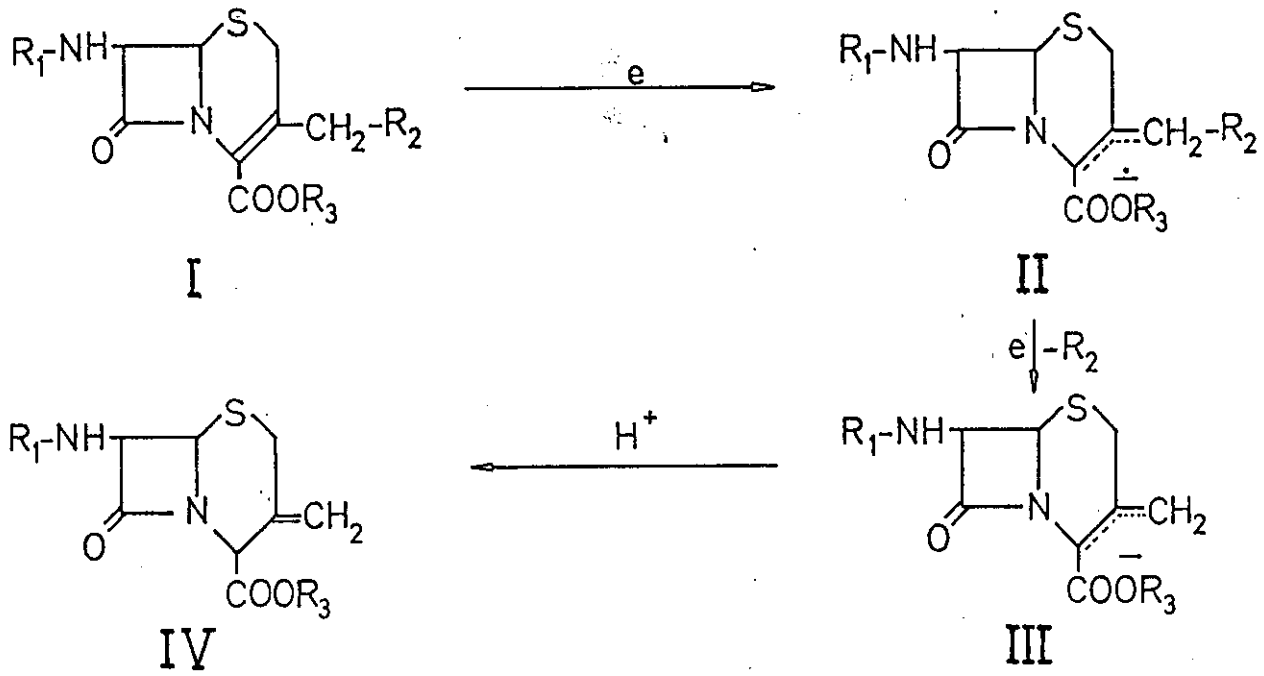
Benner[23] determined several penicillins in addition to cephalothin, cephaloridine and cephalexin in serum by cathode-ray polarography, the latter compound being determined after hydrolysis in sodium hydroxide solution at 50°C for 30 min. He claimed that the peak obtained for cephalexin was very stable and allowedthe determination of cephalexin with an accuracy of ± 1 µg/ml in the range 1-10 µg/ml. Hall[24] has used d.c. polarography and coulometry in a study of the degradation of 3-(5-methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-(2-(3-sydnone)acetamido)-3-cephem-4-carboxylic acid, sodium salt I.



I

He suggested that acid hydrolysis results in the loss of the R'-group at the 3-position and the formation of an α-β -unsaturated lactone, while base and enzymatic hydrolysis opens the β -lactam ring and simultaneously eliminates the R'-group at the 3-position. Ochiai et al [25] have utilized preparative electrolysis and polarography in order to study the mechanism of the cathodic reduction of cephalosporanic acid derivatives containing various substituents at the 3-position. They concluded that the

electrochemical reduction of these derivatives gave the corresponding 3-methylene-cepham derivative IV according to Scheme 1.



Scheme 1

They also suggested that the variation of the R_1-group does not affect the reaction, and the presence of a substituted methyl group at the 3-position is the essential requirement for the electrochemical reduction of these compounds. Hall et al[26] have applied a.c. and d.c. polarography, cyclic voltammetry and coulometry to study the electrochemical behaviour of cephalothin in both aqueous and non-aqueous solutions. Recently, Rickard et al[27] have used d.c. polarography in analytical control and stability testing of the cephalosporins, cefamandole and cefamandole nafate, while controlled potential coulometry was applied as an absolute measure for purity evaluation of these compounds.

A d.c. polarographic procedure for the determination of cephalexin at the 10^{-3}M level after hydrolysis in 5M hydrochloric acid at 80°C has been described by Squella et al in 1978.[28]

Siegerman[29] reported a differential pulse polarographic method for the determination of cephaloglycin in 1M H_2SO_4 at the 1ppm level.

Although penicillins are not polarographically active, they can easily be converted to species that are amenable to polarographic analysis either by derivatization procedures, such as nitrosation[30], or after hydrolysing the penicillins to their polarographically active degradation products[29,31]. Krejci[32] used polarography in a study of the kinetics of penicillin degradation in acidic aqueous solution. The use of oscillographic polarography to follow enzymatic inactivation of penicillins has also been reported[33-34]. Recently, Jemal et al[35,36] studied the polarographic behaviour of benzylpenicillenic acid, and used differential pulse polarography to follow the degradation of this compound in neutral media.

The application of d.c. polarography and its more sensitive successor, differential pulse polaorgraphy to the determination of penicillins and other antibiotics has been reviewed recently[29,37-39].

Degradation of penicillins and cephalosporins

Penicillins and cephalosporins have been known to undergo facile cleavage of their β-lactam bonds in aqueous solution. This has evoked a considerable interest in the chemical degradation of these antibiotics, as the chemical reactivity of β -lactam moiety is linked

with antimicrobial reactivity[40]. Further, the instability of these antibiotics may lead to the formation of some degradation products involved in penicillin and cephalosporin allergy[41-42]. It is known that allergy is mediated by antigen-antibody reaction, and drug molecules of low molecular weight such as penicillins or cephalosporins are nonimmugenic themselves. The capacity of these compounds to induce an immune response depends largely on the ability of these compounds or their degradation product or metabolites formed in vivo to react covalently with proteins or other tissue macromolecules[42]. Once the antibody is formed as a response to exposure of the individual to the immugenic substance, it can react with the antigen and initiate the allergic reaction. It is apparent from the above discussion that a knowledge of the chemical and biochemical behaviour of these antibiotics is an essential requirement for successful study of their allergenicity.

Penicillin degradation

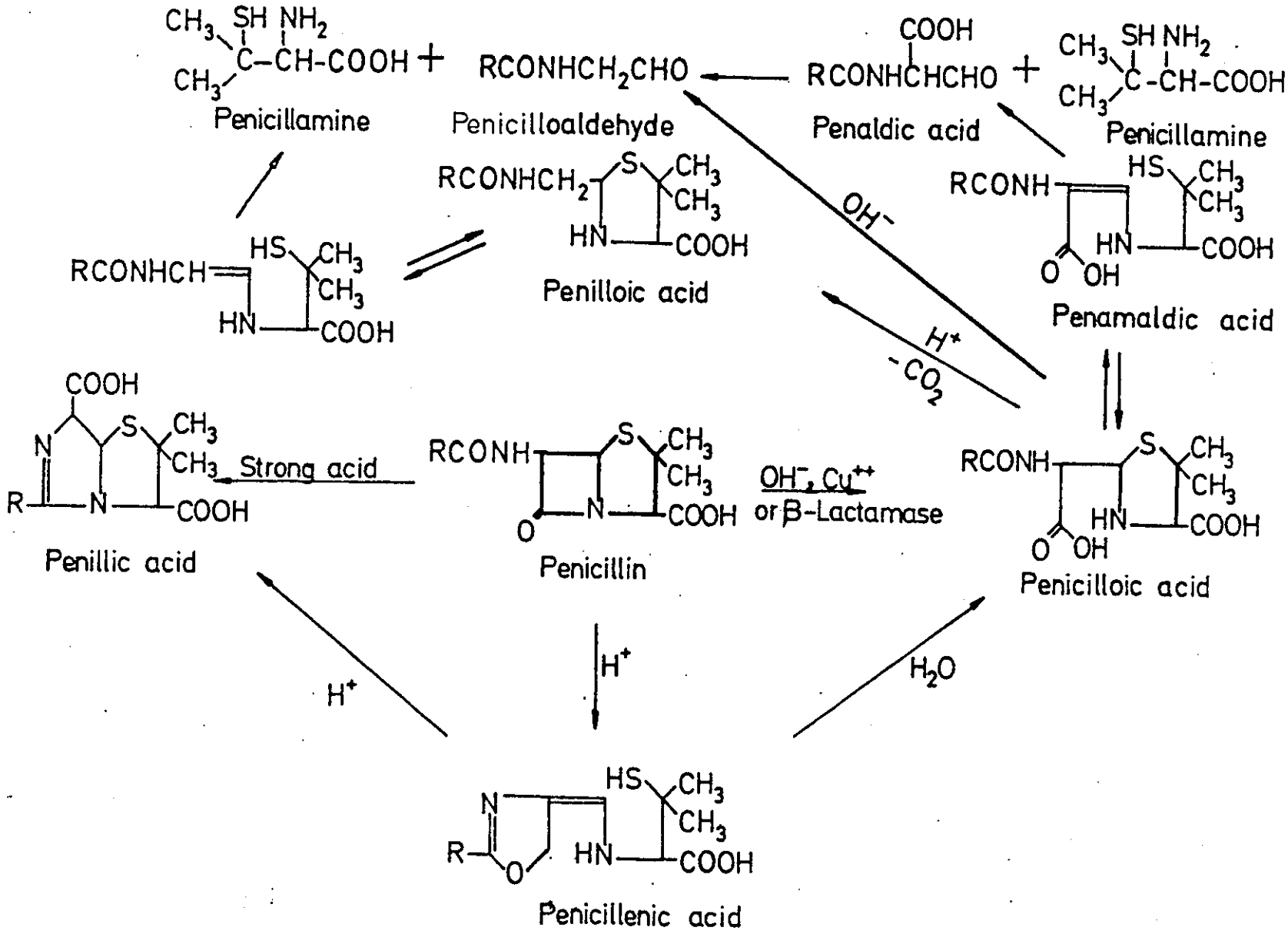
The lability of the penicillin structure has been known from the time of the earlier investigations. This lability was attributed to the high reactivity of the β- lactam moiety of the penicillin due to the nonplanarity of the β-lactam-thiazolidine structure which may cause a great deal of suppression of the usual amide resonance as compared with that caused by the dipolar stabilized forms in the normal β-lactam structure. This reactivity is reflected by the high sensitivity of the penicillins to nucleophilic, electrophilic, and oxidizing reagents and even to water[43].

In alkaline solution, the initial hydrolysis product of penicillin is the biologically inactive penicilloic acid[43]. This compound is stable in alkaline and neutral solution in the form of salts or esters, but on acidification it readily loses one molecule of carbon dioxide, forming the corresponding penilloic acid[43]. Penicilloic acid has been shown to be formed by enzymic hydrolysis of penicillins with β-lactamase[44] and in the presence of cupric ions[45].

In concentrated acid solution, penicillins are hydrolysed to penillic acid, while in dilute acid or neutral solution, penicillenic acid is produced. Penicillenic acid is unstable and under a variety of reaction conditions the compound has been shown to rapidly hydrolyse to penillic acid and 4-hydroxymethyleneoxazol-5(4H)-one[46], penicilloic acid[47] and penamaladic acid[48]. The end hydrolytic degradation products of penicillins are penicilloaldehyde, carbon dioxide and penicillamine. The more common penicillin degradation pathways are shown in Scheme 2.

Ampicillin degradation

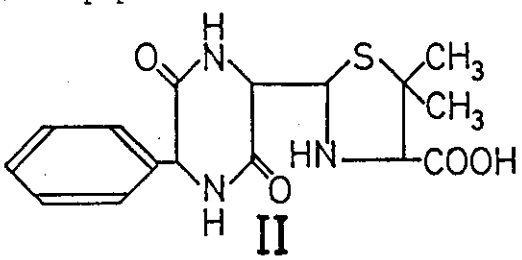
Ampicillin (α-aminobenzylpenicillin) is a semisynthetic penicillin which contains an α-amino group at the 6-side chain and which has been shown to be the most acid stable penicillin so far introduced[49]. Ampicillin was found to be effective against gram-positive and gram-negative organisms[50,51] and has become one of the most widely used antibiotics particularly for the treatment of urinary tract infections.


Penicillamine
Penicilloaldehyde
Penaldic acid
Penicillamine
Penilloic acid
Penamaldic acid
Strong acid
Penillic acid
Penicillin
Penicilloic acid
Penicillenic acid
OH⁻
H⁺
-CO₂
OH⁻, Cu⁺⁺
or β-Lactamase
H⁺
H⁺
H₂O

Scheme 2

Surprisingly, until recently, very limited work has been done on the mechanism of ampicillin degradation. The first study of the kinetics and mechanism of ampicillin degradation seems to be that of Hou and Poole in 1969[52], who used the iodometric assay to monitor the intact penicillin. Depending on classical knowledge of penicillin G hydrolysis and UV spectrophotometric measurements they postulated a degradation pathway for ampicillin similar to that of penicillin G. The authors stated that the amino group side-chain of ampicillin plays a significant role in the rate of degradation but not on the mechanism of degradation.

Jusko (1971)[53] has shown that a fluorescent degradation product is obtained following ampicillin hydrolysis in acidic medium. This fluorescent product was also obtained when the method was applied to an ampicillin sample hydrolysed first with β-lactamase. The addition of formaldehyde was found to increase the yield of the fluorescent product. Jusko tentatively proposed that the fluorescent product was the diketopiperazine derivative II.



II

Compound II, however, was obtained in a high yield after hydrolysis of 6-epi-ampicillin in neutral aqueous solution in the presence of pyridine and acetic acid[54]. Failure of ampicillin to produce the same product under the same conditions was attributed to steric hindrance[54]. Barbhaiya et al[55] reported a fluorimetric assay for amoxicillin in which no formaldehyde was used. This method was found

tp be applicable to ampicillin. Numerous publications have appeared reporting fluorimetric assay procedures based on the formation under different experimental conditions of fluorescent degradation products for the analysis of β-lactam antibiotics having a side-chain containing an α-amino group[55-63].

Several investigators[21,64,65] reported that the fluorescent degradation product is 2-hydroxy-3-phenylpyrazine. Recently, Barbhaiya et al (1978)[66] isolated the fluorescent degradation product obtained from a number of cephalosporins and penicillins containing the α-amino group at the side chain. In all cases the product was shown by unambiguous synthesis to be 2-hydroxy-3-phenyl-6-methylpyrazine.

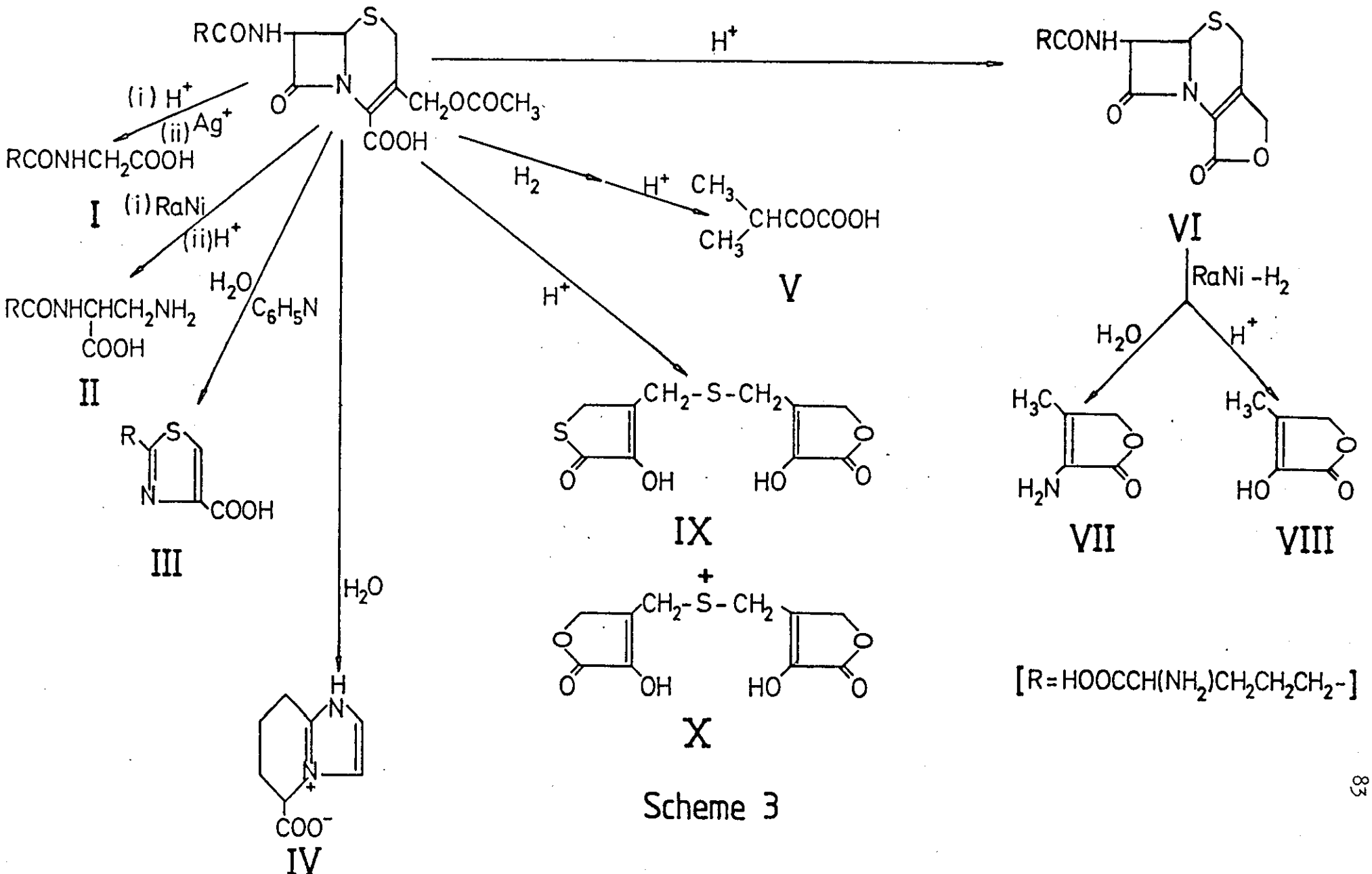
Perrett[67] studied the degradation of ampicillin in acidic medium using an amino acid analyser, and showed that several chromatographic peaks were obtained. A free sulphhydryl group containing compound was also formed. The formation of this compound parallels the increase in the area of a peak exhibiting maximum absorption at 440 nm.

Blazsek-Bodo et al in 1978[68] reported that penicillamine was formed after alkaline hydrolysis of ampicillin and other penicillins. This has been made the basis of a potentiometric method for the determination of several penicillins including ampicillin.

Cephalosporin C degradation

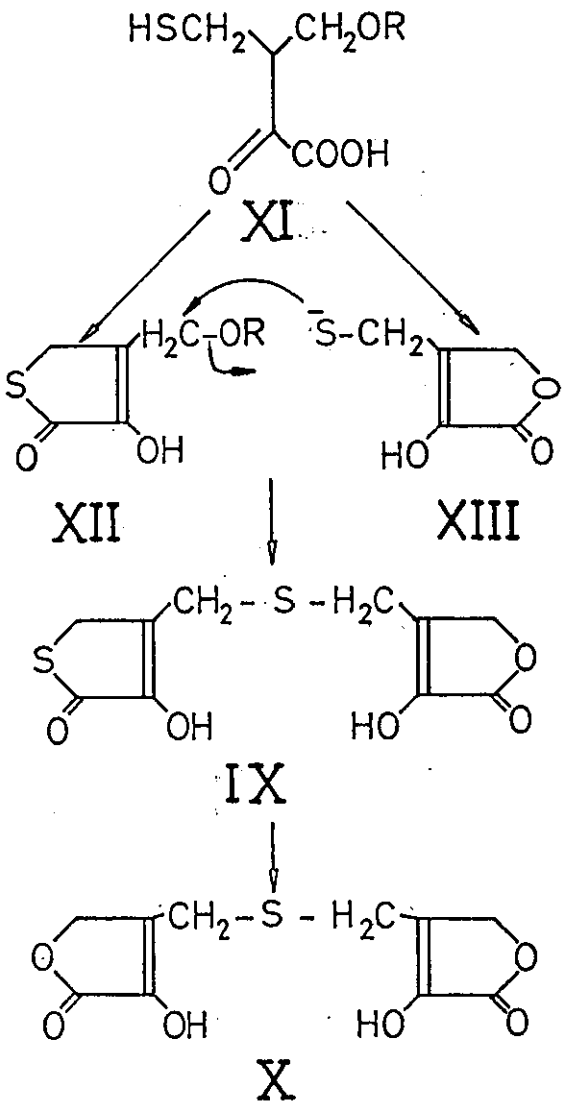
The degradation studies of cephalosporin C played an important role in assigning the structure of this compound. These degradation studies were, in general, combinations of hydrogenation, desulphurization and hydrolysis. Scheme 3 shows most of the degradation pathways of cephalosporin C.

Hydrolysis of cephalosporin C in hot acid followed by oxidation of the neutral fraction with silver oxide yielded δ-amino- δ -carboxy-valerylglycine I. Raney-nickel desulphurization at room temperature, followed by partial hydrolysis with acid degrades cephalosporin C to α- β-diaminopropionic acid II and D- α-amino-adipic acid[69]. Jeffery et al[70] reported that a thiazole carboxylic acid III was obtained when cephalosporin C was kept in neutral pyridine-water solution at 37°C, while imidazole -tetrahydropyridine IV was found to be produced when cephalosporin C was kept in water alone[71]. Hydrolysis of cephalosporin C with Raney-nickel followed by hydrolysis with hot acid gave α-oxovaleric acid V[72]. Cephalosporin C lactone (Cephalosporin C_c) VI was obtained after mild hydrolysis of cephalosporin C with the liberation of acetic acid which was isolated from strong acid solution. If the desulphurization product of cephalosporin C lactone (obtained after treatment of cephalosporin C lactone with Raney-nickel) is treated with water, it gives an aminobutenolide VII, but if treated with acid it produces a hydroxybutenolide VIII[69]. Two sulphur containing lactone compounds IX and X were obtained by hydrolysis of cephalosporin C in 1.25M hydrochloric acid at 100°C for one hour. Abraham and Newton[69]


RCONH
S
N
O
CH2OCOCH3
COOH
(i) H+
(ii) Ag+
RCONHCH2COOH
I
(i) RaNi
(ii) H+
RCONHCHCH2NH2
COOH
II
H2O
C6H5N
R
S
N
COOH
III
H2O
H
N
N+
COO-
IV
H+
H2
H+ CH3
CH3
CHCOCOOH
V
H+
CH2-S-CH2
S
O
OH
HO
O
IX
+
CH2-S-CH2
O
OH
HO
O
X
H+
RCONH
S
N
O
O
O
VI
RaNi -H2
H2O
H+
H3C
O
H2N
O
VII
H3C
O
HO
O
VIII
[R=HOOCCH(NH2)CH2CH2CH2-]

Scheme 3

have suggested a mechanism by which these two compounds might be produced (see Scheme 4). They proposed that the carboxyl group of the hypothetical intermediate XI could lactonize with either the thiol group or the hydroxy group to give compound XII and XIII. Condensation of XII and XIII, as shown, would give compound IX, which undergoes β, γ-elimination of the sulphur from the ketone form of the thiolactone ring of compound IX, followed by elimination of H_2S and relactonization to give compound X.

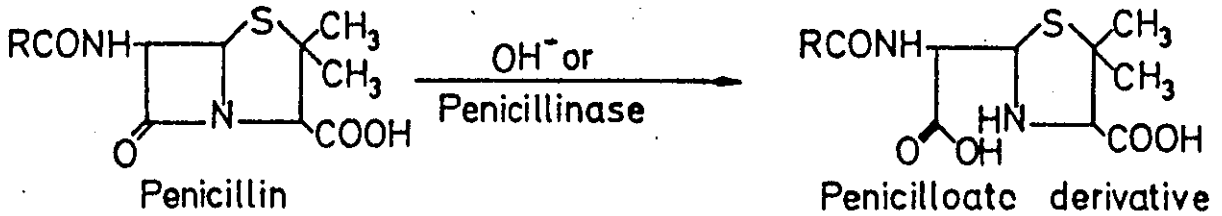


Scheme 4

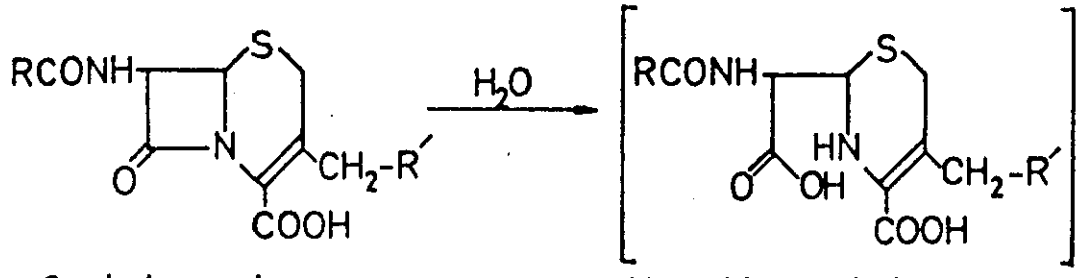
β-lactam cleavage of cephalosporins

Compared with the extensive studies on the degradation reactions of penicillins, relatively little work has been done on the degradation reactions of cephalosporins. Degradation studies are important as they may be associated with allergic reactions to these antibiotics.

In spite of the structural similarity between the penicillins and cephalosporins, there are major differences in the way these compounds undergo β -lactam cleavage. When the β-lactam ring of penicillin is opened by mild alkaline hydrolysis, or with penicillinase, the corresponding penicilloate is mainly produced. This is a well-characterized and relatively stable compound[69]. Cephalosporins are readily attacked similarly by nucleophilic reagents and by cephalosporin β-lactamases, but the initial corresponding degradation product, "cephalosporoate", is unstable and rapidly fragments in aqueous solutions[73], Scheme 5. Recently, cephalosporoate



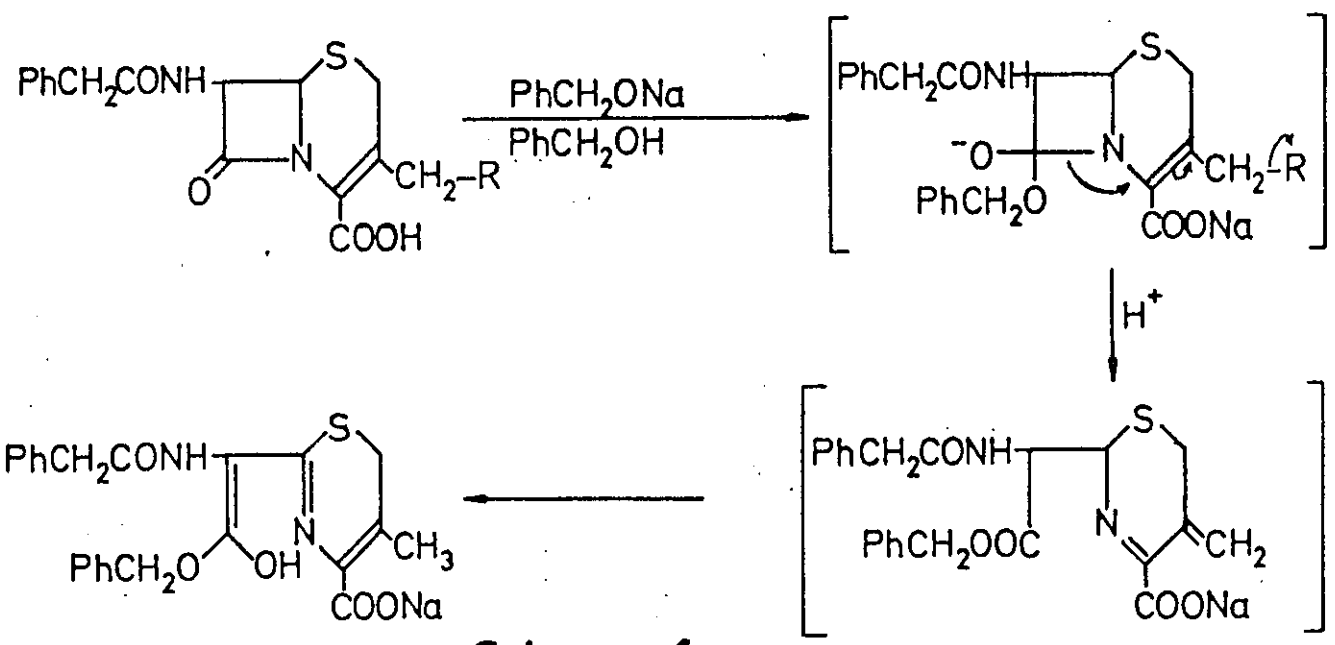
Penicillin — Penicilloate derivative



Cephalosporin — Unstable cephalosporoate

Scheme 5

like compounds were obtained under a variety of experimental conditions. Eggers et al[74] studied the reaction of 7-phenylacetamidocephalosporanic acid (cephaloram) with sodium benzyloxide in benzyl alcohol. The final degradation product (the corresponding cephalosporoate) has lost the acetoxy group with opening of its β -lactam ring according to Scheme 6.



Scheme 6

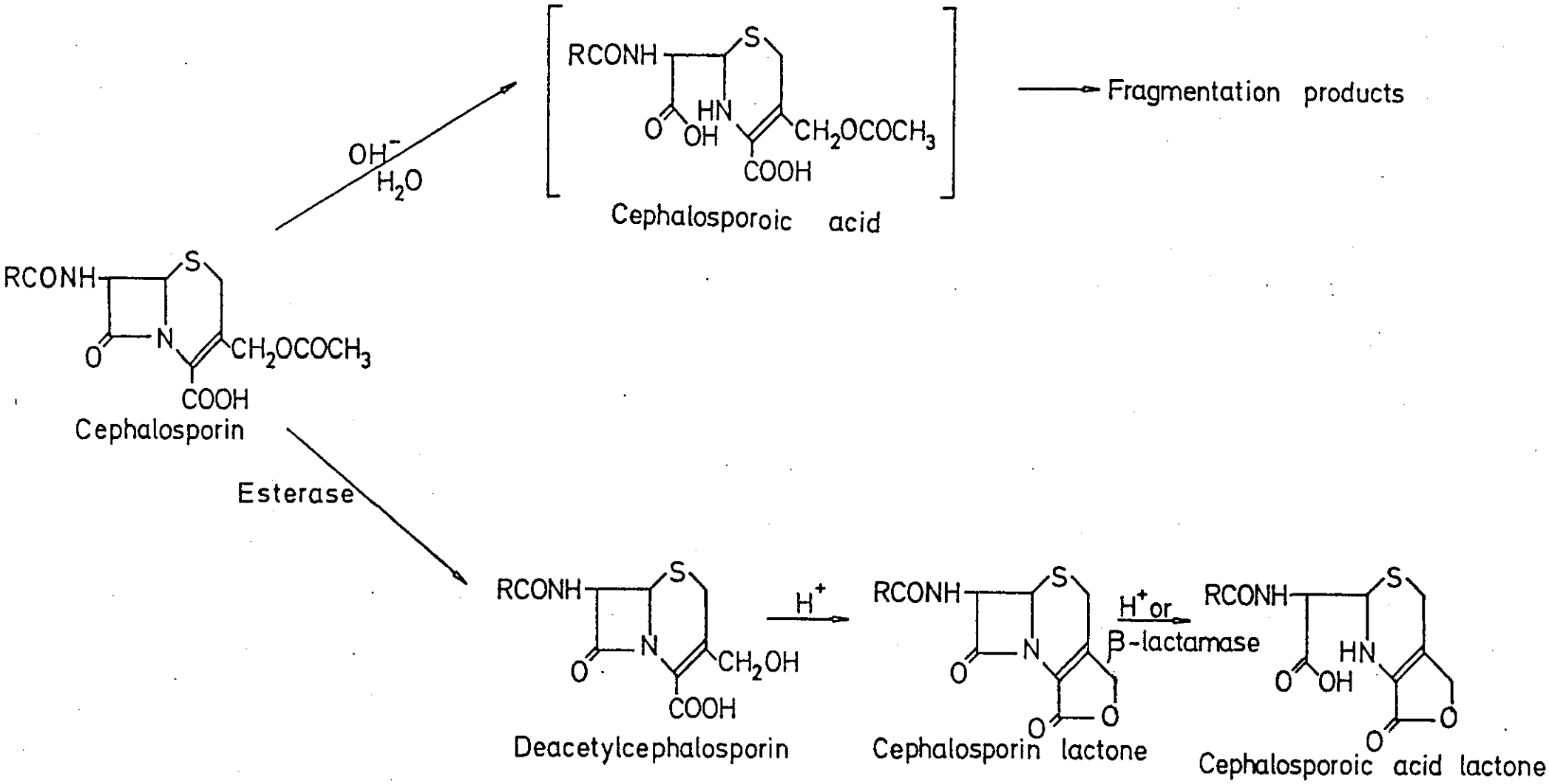
Alkaline hydrolysis of cephalosporin C at room temperature has been shown to produce deacetyl-cephalosporin C in low yield. Higher yields, however, were obtained when cephalosporin C was treated with an acetyl esterase in neutral aqueous solution[75]. Acid hydrolysis of deacetylcephalosporin C at room temperature produced deacetylcephalosporin C lactone. The latter had been shown to produce from cephalosporin C itself[69]. Mild acid hydrolysis of deacetylcephalosporin C lactone opened the β-lactam ring and yielded a relatively stable product which could be regarded as the corresponding cephalosporoate[69]. Newton et al[73] reported that

the corresponding cephalosporoate was obtained following β-lactamase hydrolysis of cephalothin. These reactions are shown in Scheme 7.

Hamilton-Miller et al[76] reported that a labile compound with λ_{max} 230nm was obtained following aminolysis in weakly alkaline aqueous solutions of cephalosporins but not from deacetyl or deacetoxy-cephalosporins. Hydrolysis with β -lactamase results in the formation of similar products from deacetyl cephalosporins and cephalosporins, but not from deacetoxycephalosporins. These degradation products were found to degrade further to compounds tentatively identified as penaldates and penamaldates.

In order to shed more light on the identity of the above degradation products, Hamilton-Miller et al[77] have followed the aminolysis and the enzymic hydrolysis of a number of cephalosporins having different substituents at the 3-position in ND_3 and D_2O by NMR and UV spectrophotometry. They concluded that the aminolysis of 7-n-butyramido-cephalosporanic acid is accompanied by expulsion of the acetoxy group as acetate and the formation of an exo-methylene compound (λ_{max} 230nm) as a semistable intermediate. This compound was found to degrade further to give a penaldate (λ_{max} 270nm). Similar results were obtained with cephalosporin C.

Aqueous aminolysis of 7-n-butyramidodeacetoxycephalosporanic acid and 7-n-butyramidodeacetylcephalosporanic acid, however, gave intermediate deacetoxy and deacetyl cephalosporoates.

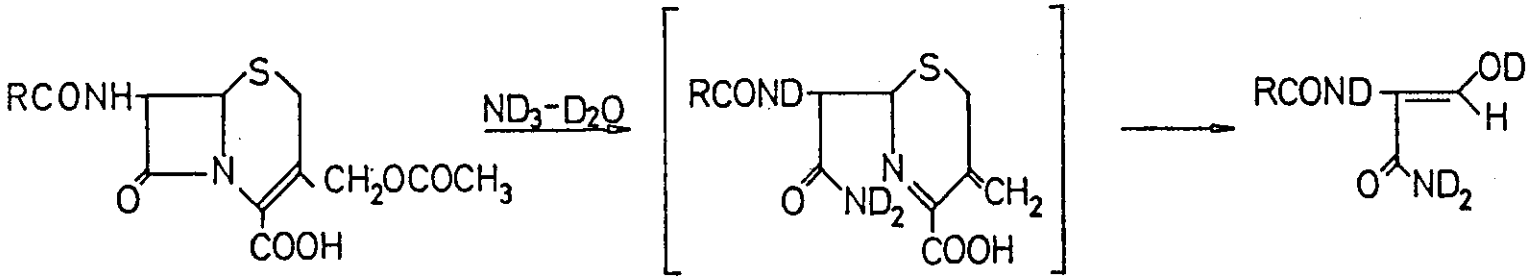

RCONH
S
N
O
CH2OCOCH3
COOH
Cephalosporin
OH⁻
H2O
HN
OH
Cephalosporoic acid
Fragmentation products
Esterase
CH2OH
Deacetylcephalosporin
H+
H+or
β-lactamase
Cephalosporin lactone
Cephalosporoic acid lactone

Scheme 7

β -lactamase hydrolysis of cephalosporin C and deacetylcephalosporin C also gave intermediate with λ_{max} 230nm - a different result from the aminolysis. The investigators speculated that enzymic hydrolysis could have converted the hydroxyl groups of deacetylcephalosporins into better leaving groups resulting in behaviour similar to that of cephalosporin C. Degradation pathways of the above study are depicted in Scheme 8.

The enzymic hydrolysis of cephalosporin C, cephalothin, and cephaloridine was studied by Sabath and coworkers[78]. They showed that opening of the β -lactam ring of these compounds by β -lactamase at pH 7 is accompanied by the spontaneous expulsion of acetate in case of cephalothin and cephalosporin C and the expulsion of pyridine from cephaloridine. Two equivalents of acid per mole of antibiotic were produced. One equivalent is attributable to the β -lactam ring hydrolysis while the other equivalent resulted from the liberation of acetic acid or pyridinium ion. By comparison, Deacetoxycephalosporin C yielded only one equivalent of acid. Recently, Bundgaard[42] showed that during aminolysis and alkaline hydrolysis of cephaloridine, pyridine ion was found to be released quantitatively at exactly the same rate as the cleavage of the β -lactam ring.

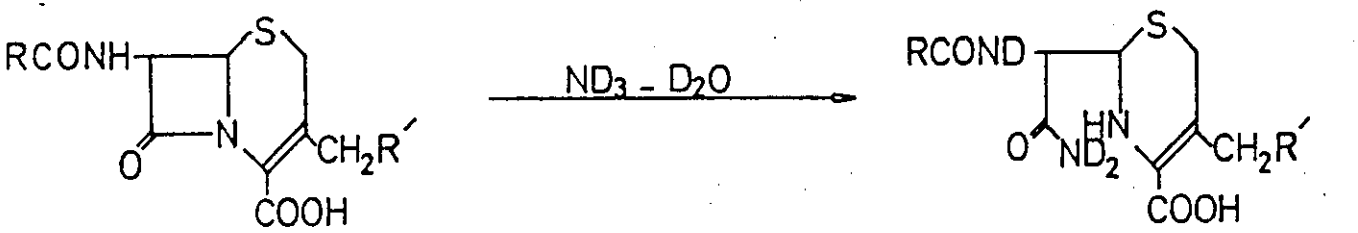
Several reports have appeared recently which detail the degradation of cephalosporins. The degradation of cephalothin was reviewed by Simmons in 1972[79]. Konecny et al[80] reported the kinetics of the degradation of cephalosporin C in a wide pH range at 25°C. Indelicato et al[81] studied the substituent effects of various acylamido side chain moieties upon the alkaline hydrolysis of



7-n-butyramidocephalosporanic acid

exo-methylene intermediate

penaldate



cephalosporoate derivative

R = $CH_3(CH_2)_3$

R´= OH or H

Scheme 8

cephalosporins and penicillins. The kinetics of aminolysis and alkaline hydrolysis of a series of cephalosporins in aqueous solution at 35°C were investigated by Bundgaard[42]. Yamana et al[82,83] reported the kinetics and mechanisms of degradation of six therapeutically useful cephalosporins and other semi-synthetic cephalosporins in acidic, neutral, and alkaline aqueous solution.

Recently, the hydrolysis of several cephalosporins in strongly acidic medium at 100°C was investigated by Bontchev et al[84]. They found that the hydrolysis proceeds with a high rate and results in the formation of definite products with low molecular weights.

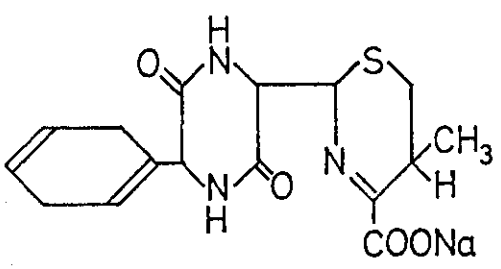
The lability of cephalosporins to ultra-violet light was first reported by Demain in 1966[85], who showed that cephalosporin C loses 90% of its original biological activity in aqueous solution after being irradiated with UV light for 30 min. Maki et al 1977 [86] studied the mode of photodegradation of cephalosporins. They found that UV irradiation through a Pyrex filter of 3-cephem derivatives in alcohols causes a novel photorearrangement, leading to thiazole derivatives, which involves incorporation of alcohols into an intermediate photoproduct.

Cephalexin and cephradine degradation

Cephalexin, 7-(D- α-amino- α-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid, is a new semi-synthetic cephalosporin. It is relatively acid stable and is completely absorbed following oral administration[21,87]. The antimicrobial spectrum of cephalexin is similar to that of cephalothin and cephaloridine but the latter antibiotics are poorly absorbed when administered orally and must therefore be given parenterally. Cephalexin which has the same side chain as ampicillin and has an unsubstituted methyl group at the 3-position in the dihydrothiazine ring was found to be excreted unchanged in urine or bile[88].

Cephradine is similar to cephalexin having a 1,4-cyclohexadienyl group in place of the benzene ring. Its behaviour is similar to cephalexin.

Elegant studies have recently been made of the degradation of cephalexin and cephradine by several investigators. Cohen et al in 1973[89] reported that degradation of cephradine in sodium carbonate solution at 5°C for one week affords the diketopiperazine (2- 6-(1,4-cyclohexadine-1-yl)-2,5-dioxo-3-piperazinyl) -5,6-dihydro-5-methyl-2H-1,3-thiazine-4-carboxylic acid, sodium salt).

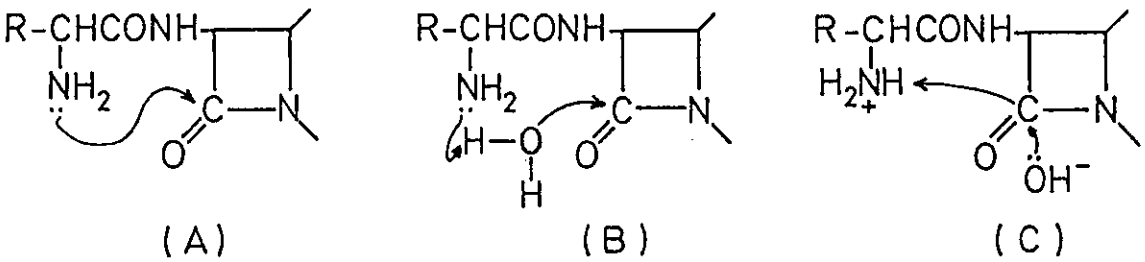


Diketopiperazine derivative

Indelicato et al[81,90] studied the degradation of several cephalosporins in alkaline aqueous solutions at pH 10. They suggested that the degradation of cephalosporins having a side chain containing an α-amino group could take place by the intramolecular nucleophilic attack of the side chain α-amino group on the carbonyl carbon atom of the β-lactam ring. This would result in the formation of diketopiperazine type products. However, they were not able to isolate such degradation products from degraded aqueous solution of cephalexin or cephaloglycin, probably because degradation was performed at pH 10 where the major degradation pathway is a β-lactam hydrolysis. They succeeded, however, in isolating such products from a heated solution of cephalexin trichlorethyl and p-nitrobenzyl esters and cephaloglycin lactone in benzene under reflux overnight. Under the same conditions, ampicillin does not yield a similar cyclic product, but cyclic degradation products from ampicillin[64-66] and 6-epi-ampicillin have been reported[54].

Extensive kinetic and mechanistic studies of the degradation of cephalosporins in aqueous solutions have been reported by Yamana et al[82,83]. They were able to isolate a strongly fluorescent degradation product from the reaction mixture of cephalexin and cephaloglycin at pH 8. This product was tentatively identified as the diketopiperazine derivative. They reported that at neutral pH, cephalexin, cephradine and cephaloglycin hydrolysis is mainly due to intramolecular nucleophilic attack of the side chain amino group on the β-lactam moiety which is superseded in importance at high pH by hydroxide-ion attack on the anionic antibiotics and at low pH by direct water catalyzed hydrolysis of the β-lactam ring.

Yamana et al[83] suggested three possible kinetically indistinguishable mechanisms for α-amino groups participation in the β -lactam ring cleavage of these α-aminocephalosporins. These mechanisms are illustrated in Scheme 9.



Scheme 9

Mechanism(A) Intramolecular nucleophilic attack of the unprotonated side chain amino group on the β-lactam carbonyl moiety.

Mechanism (B). Intramolecular general base catalysis by the amino group of the attack of water molecule on the β-lactam bond.

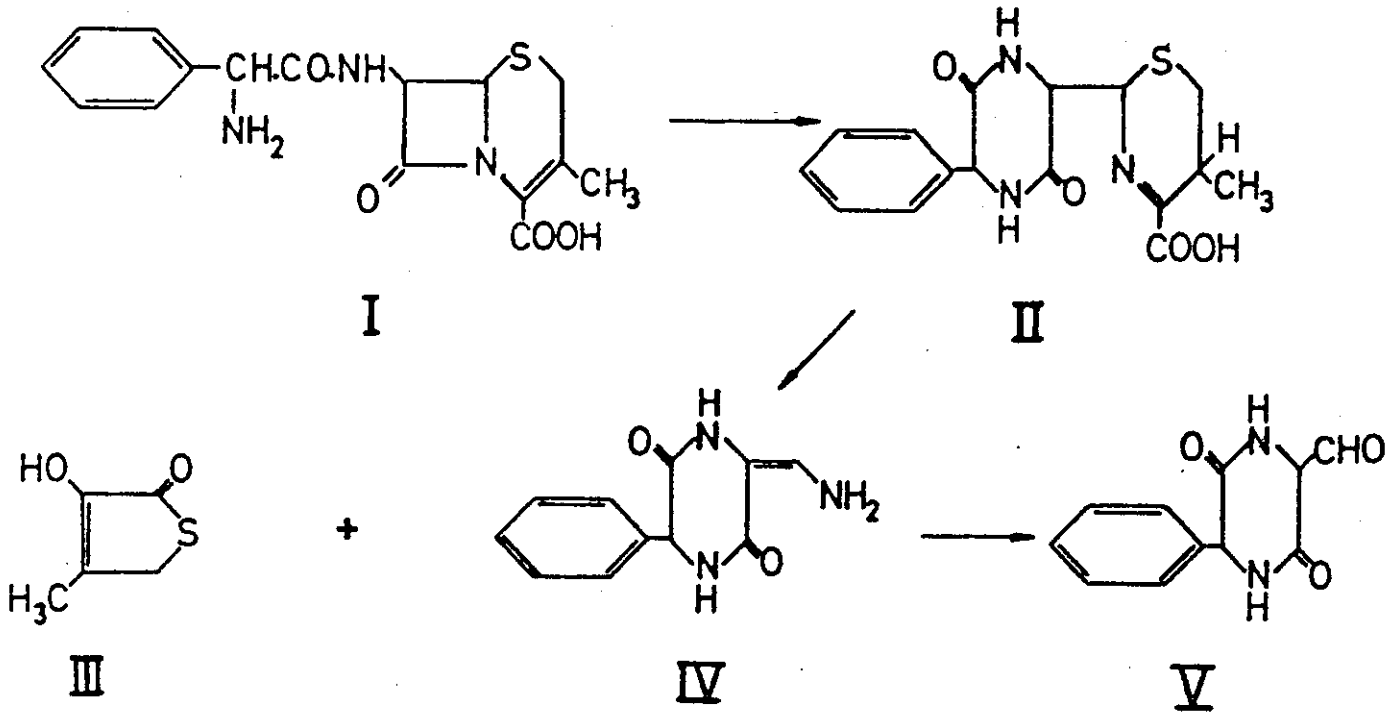
Mechanism (C). Intramolecular general acid catalysis by the protonated amino group of the attack of hydroxide ion on the β-lactam bond.

Bundgaard[91] obtained evidence of the involvement of mechanism (A) in the reaction of cephalexin from isolation of the diketopiperazine derivative, by observing that the side chain amino group was consumed during the degradation and from kinetic analysis of this amino group consumption. Bundgaard showed by primary amino group analysis that at pH 7 and 35°C, a 98% loss of the primary amino group occurs

indicating essentially complete degradation of cephalexin via the diketopiperazine derivative II, whereas at pH above 10 only 32% of the cephalexin degrades by intramolecular aminolysis, the remainder degrading by hydrolysis of the β-lactam ring[91]. After degrading cephalexin in distilled water at pH 3, Dinner[92] isolated 3-hydroxy-4-methyl-2(5H)-thiophenone III and 3-formyl-3,6-dihydro-6-phenyl-2,5(1H,4H)-pyrazinedione V. Bundgaard[93] isolated and characterised a compound which is precipitated during the degradation of cephalexin in neutral aqueous solution as 3-aminomethylene-6-phenyl-piperazine-2,5 dione IV. Bundgaard showed that this compound is a precursor of compound V. Barbhaiya et al[66] reported that the fluorescent degradation product formed by treatment of cephalexin first with sodium hydroxide solution at room temperature and then in pH 5 citrate buffer at 100°C is 2-hydroxy-3-phenyl-6-methyl-pyrazine VI. Bontchev and Papazova[84] found that a thiazine compound VII is formed following cephalexin hydrolysis in 12.5% sulphuric acid solution at 100°C.

Cephalexin degradation products are shown in Scheme 10. The diketopiperazine derivative of cephalexin compound II will be referred to as DKP II throughout the text.

The present investigation was carried out in order to evaluate the usefulness of d.p. polarography for the determination of cephalosporins and for studying their degradation in solution.



Scheme 10

EXPERIMENTAL

Equipment. Polarographic measurements were made with a PAR 174 polarographic analyser (Princeton Applied Research Corporation). For differential pulse operation the forced drop time was 1 sec , the scan rate 2 mV sec^{-1}, and the pulse height 50 mV, except where otherwise indicated. Three-electrode operation was employed using a platinum counter electrode, a dropping mercury working electrode (d.m.e) and a saturated calomel reference electrode. The water jacketed polarographic cell was kept at 25°C by passing water through the jacketed cell from a thermostatically controlled water bath. Solutions for polarography were deoxygenated with nitrogen gas which has previously been passed through a vanadium(II) scrubber.

UV absorption spectrophotometric measurements were made with a Pye Unicam SP8000 and IR measurements were made with a Pye Unicam SP 200. A Baird-Atomic "Fluoricord" fluorimeter was used for the fluorimetric measurements. Potentiometric measurements were carried out with an Orion solid-state sulphide ion-selective electrode (Model 94-16) and an expanded scale Radiometer PHM 64 research pH meter. Thin layer chromatography was done on precoated 0.25mm silica gel F_{254} aluminium sheets (Merck, Germany).

Chemicals

All chemicals were of analytical reagent grade except where otherwise indicated

Samples

Samples of cephalothin, cephaloridine, cephalosporin C, deacetylcephalosporin C, 7-aminocephalosporanic acid (7-ACA), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephoxazole, cephalonium, cefuroxime, cephalexin and penicillamine were obtained from Glaxo Operations (U.K.) Ltd. Samples of cephradine, ampicillin trihydrate and 2-hydroxy-3-phenyl-6-methylpyrazine were kindly provided by E.R. Squibb and Sons Ltd., Beecham Pharmaceuticals Ltd. and Fisons Pharmaceuticals Ltd, respectively.

Preparation of buffer solutions

Britton-Robinson buffer solution. A stock solution of Britton-Robinson universal buffer solution (B.R. buffer) pH 1.9 composed of a mixture of boric acid, orthophosphoric acid and glacial acetic acid, all 0.04M, was prepared; pH adjustment were made with 0.2M sodium hydroxide solution as required.

Phosphate buffer solution (pH 7.4)

The phosphate buffer was prepared by adjusting a 0.5M disodium hydrogen phosphate solution to pH 7.4 by addition of 0.5M potassium dihydrogen phosphate solution.

McIlvain's citrate-phosphate buffer solution

A stock solution of citrate-phosphate buffer was prepared by adjusting 0.1M citric acid solution to the required pH with 0.2M disodium hydrogen phosphate solution.

Sorensen's citrate buffer solution (pH 5)

A stock solution of Sorensen's citrate buffer solution was prepared by adjusting the pH of 0.1M disodium citrate (21.0g citric acid monohydrate) dissolved in 200ml 1M sodium hydroxide and made up to 1 litre with distilled water) to pH 5 with 0.1M sodium hydroxide.

EXPERIMENTAL TECHNIQUES

A. D.P. POLAROGRAPHIC DETERMINATION OF CEPHALOSPORINS

1. Preparation of stock solutions

Except for 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA), stock solutions of cephalothin, cephaloridine, cephalosporin C, deacetylcephalosporin C, cephoxazole, cefuroxime, cephalonium, cephalexin and cephradine were prepared in distilled water. Fresh solutions were usually used before measurements. 7-ACA and 7-ADCA were dissolved in 2ml of 0.2M sodium hydroxide solution and the resulting solutions were immediately made up to volume with pH 2 Britton-Robinson buffer solution. Prolonged contact with sodium hydroxide solution caused extensive degradation, as indicated by the appearance of polarographic peaks of degradation products, and decrease in the height of the 7-ACA peak.

2. Effect of pH

The effect of pH on the peak potentials and peak currents of the above cephalosporins was studied in B.R. buffer solution in the pH range 2-12. For each polarographic measurement, 1 ml of the freshly prepared stock solution containing 100 μg/ml of the drug compound and 10 ml of the buffer solution at the required pH were placed in the polarographic cell. Oxygen-free nitrogen was passed through the solution for 5 min, the solution was then blanketed by an atmosphere of nitrogen whilst recording the d.p. polarograms.

3. Effect of mercury reservoir height

The effect of the mercury reservoir height on the limiting current was studied in the d.c. mode at the optimum pH which gives the highest signal response (μA/μg). D.c. polarograms for each solution of the antibiotics (100μg/ml) were obtained at various mercury reservoir heights.

4. Construction of calibration graphs

Calibration curves were prepared by successive syringe injections of concentrated solution of the antibiotic into a deoxygenated buffer solution at the optimum pH at 25°C.

B. D.P. POLAROGRAPHIC STUDY OF THE DEGRADATION OF CEPHALOSPORINS

I. Degradation of cephalothin and cephaloridine

The degradation of cephalothin (140μg/ml) and cephaloridine (100μg/ml) was studied in B.R. buffer solution at pH 2 and 50°C. In the case of cephalothin the degradation was carried out directly in the polarographic cell, whereas the degradation of cephaloridine was carried out in a separate volumetric flask which was placed in a water bath at 50°C. D.p. polarograms were obtained, for 10ml of the degraded solution at suitable intervals.

II. Degradation of cephalexin and cephradine

1. Effect of temperature on the degradation of cephalexin and cephradine

Initial degradation studies were carried out in phosphate buffer

solution at pH 7.4 and 37°C. Cephalexin and cephradine solutions (100µg/ml) were prepared by dissolving 25mg of the drug compound in 250ml of the above buffer. The flasks were then placed in a water bath at 37°C. At suitable intervals, 10ml of the degraded solution was pipetted into the polarographic cell; solutions were deoxygenated with oxygen-free nitrogen for 10 minutes before recording the polarograms in the differential pulse mode at 25°C between 0.0 and -1.8V.

When the effect of temperature was studied the flasks were placed in a water bath at the required temperature (60°C and 80°C) and d.p. polarograms were recorded as above.

Throughout the degradation studies, a cephalexin concentration of 100µg/ml was used and d.p. polarograms were obtained at pH 7.4 and 25°C irrespective of the temperature and the pH at which degradation was done except where otherwise indicated.

2. Isolation of a sample of diketopiperazine

A sample of diketopiperazine derivative was isolated from a degraded solution of cephalexin as follows:

A solution of 1g cephalexin in 50ml of 0.3M aqueous phosphate buffer solution (pH 7.6) was kept at 35°C for 24 hours. A grey-white precipitate was formed, which was then filtered off through a Whatman No. 1 filter paper. The pH of the filtrate was brought to pH 2.5 with 5M hydrochloric acid and extracted with two 50ml portions of ethyl acetate. After being washed twice with water and subsequently

dried over anhydrous sodium sulphate the combined ethyl acetate extracts were evaporated in a vacuum oven at about 25°C. The residue of evaporation was suspended in 25ml of boiling chloroform and filtered off. The residue was dried in a vacuum oven over phosphorus pentoxide for 24 hours. The identity of the product was confirmed by IR spectrophotometry, fluorimetry, thin layer chromatography and melting point measurement.

3. Polarographic determination of diketopiperazine derivative (DKP II)

a. Effect of mercury reservoir heights on the limiting current

Diketopiperazine stock solution (1ml, 100 µg/ml) was diluted to volume with phosphate buffer solution (pH 7.4) in a 10ml volumetric flask, mixed thoroughly, and transferred to the polarographic cell. The solution was deoxygenated for 10 minutes, and d.c. polarograms were obtained at various mercury reservoir heights at 25°C.

b. Effect of temperature on the wave height of DKP II

The effect of temperature was studied using a diketopiperazine solution of 10 µg/ml in phosphate buffer (pH 7.4). The measurements were carried out at 25, 30, 35, 40 and 45°C. Because of the lability of DKP II, a fresh solution was used each time.

c. Cdnstruction of a calibration graph for the determination of DKP II

Into the clean dry polarographic cell, 10ml of phosphate buffer solution (pH 7.4) was placed. The solution was deoxygenated for 10 minutes, and a blank was recorded. A calibration graph was then obtained by successive syringe injection of 0.25ml portions of

concentrated solution of diketopiperazine (0.5mg/ml). After each injection, the solution was deoxygenated for 2 minutes (to ensure complete mixing) before recording the d.p. polarogram between -0.7V and -1.1V at 25°C.

d. Degradation of diketopiperazine in phosphate buffer solution

A solution of 10mg diketopiperazine in 100ml phosphate buffer (pH 7.4) was prepared in a 100ml volumetric flask. The flask was placed in a water bath at 37°C. Aliquots (10ml) of the degraded solution were drawn at suitable intervals, placed in the polarographic cell and deoxygenated for 10 minutes before recording the d.p. polarogram at 25°C between 0.0 and -1.6V.

4. Isolation of a sample of 2-hydroxy-3-phenyl-6-methylpyrazine

Cephalexin (0.5g) in distilled water (50ml) was treated with sodium hydroxide solution (25ml, 1M) for 10 minutes at room temperature. Hydrochloric acid (25ml, 1M) was then added followed by Sørensen's citrate buffer solution (150ml, pH 5) containing formaldehyde (1% v/v) The mixture was then heated at 100°C for 30 minutes, cooled and extracted repeatedly with ethyl acetate. The combined ethyl acetate extracts were then evaporated, and the residue was crystallized from ethyl acetate. For comparison purposes, d.p. polarograms and UV spectra, were obtained for the prepared sample and a sample of the pure compound, also thin layer chromatography on silica gel F_{254} plate for the two samples were compared, the plate was eluted with acetone-chloroforme (1:1 v/v).

5. Effect of initial cephalexin concentration

The effect of initial cephalexin concentration on the degradation process was studied using cephalexin solutions of 5, 100μg/ml and 5mg/ml all in phosphate buffer at pH 7.4 at 37°C and 80°C. Polarographic measurements were carried out at 25°C as described above.

6. Effect of the presence of hydrogen sulphide and molecular oxygen in the degraded solution of cephalexin.

In this set of experiments, the degradation of cephalexin was carried out in a gas wash bottle whilst passing a slow stream of nitrogen (or air when the effect of dissolved oxygen was being studied) through the solution. The nitrogen or air was presaturated with water in a second wash bottle, both bottles being contained in a water bath at the required degradation temperature. The hydrogen sulphide evolved was stripped from the nitrogen or air by passing it through a stripping solution (10^{-3}M cadmium nitrate solution or 1M sodium hydroxide solution). D.p. polarographic measurements were carried out using 10ml of the degraded solution at suitable intervals. When the amount of hydrogen sulphide was being determined, polarographic measurements were made using a separate solution of cephalexin which was prepared and kept under the same experimental conditions.

7. Determination of hydrogen sulphide evolved during cephalexin degradation.

Two approaches for the determination of hydrogen sulphide were examined:

a. Potentiometric determination of hydrogen sulphide

Preparation of sodium sulphide standard solution:

Due to the hygroscopic nature of sodium sulphide, it is not possible to prepare solutions of exact molarity, and it was necessary to standardise these solutions. All solutions used in this study were prepared in tri-distilled water which was boiled for about one hour and cooled under a stream of oxygen-free nitrogen. An approximately 0.1M solution of sodium sulphide prepared in 0.2M sodium hydroxide solution was standardised against 0.1M silver nitrate standard solution using a sulphide ion-selective electrode. The molarity of silver nitrate solution was confirmed by a routine standardisation procedure using standard sodium chloride solution and potassium chromate as an indicator (Mohr titration)[94].

Recovery of hydrogen sulphide from a standard sodium sulphide solution

An aliquot (0.2ml) of standard sodium sulphide solution (0.0955M) was diluted to 50ml with phosphate buffer solution (pH 7.4), the solution was transferred to a gas wash bottle being placed in a water bath at 37°C. A slow stream of nitrogen which had previously been saturated with water was passed through the solution for 1 hour. The hydrogen sulphide was absorbed from the ensuing nitrogen stream in 50ml of 1M sodium hydroxide solution. The sodium sulphide formed was then directly titrated against a 10^{-2}M silver nitrate standard solution using a sulphide ion-selective electrode.

b. Polarographic determination of hydrogen sulphide

Citrate buffer solution (pH 4.6).

Citrate buffer solution was prepared by dissolving 52.1g citric acid and 28.1g potassium hydroxide in 500ml of distilled water.

Sodium sulphide solution

From a freshly prepared and standardised sodium sulphide solution (0.0952M), a 2ml aliquot was diluted to 100ml with 0.2M sodium hydroxide solution.

Cadmium nitrate solution

A standard solution of cadmium nitrate (1×10^{-3}M) was prepared by dissolving 0.0746g in 250ml of distilled water.

Construction of a calibration graph

Into a clean dry polarographic cell, 10.0ml of cadmium nitrate standard solution (1×10^{-3}M) and 10.0ml of citrate buffer solution were placed. The solution was deoxygenated for 10 minutes and a d.p. polarogram was obtained between -0.4V and -0.8V at 25°C. A calibration graph was obtained by successive syringe injections of sodium sulphide standard solution (1.9×10^{-3}M) into the deoxygenated solution (0.5ml was injected each time). The solution was deoxygenated for a further 30sec after each injection before recording the d.p. polarograms at 25°C.

Hydrogen sulphide evolved during the degradation of cephalexin was absorbed in 10.0ml cadmium nitrate standard solution (1×10^{-3}M). After suitable intervals, the absorbing solution was transferred to the dry polarographic cell, 10.0 ml of previously deoxygenated citrate buffer was added and the polarogram was recorded as above. The concentration of sulphide was obtained by comparing the cadmium peak height with those obtained for standards.

When the potentiometric method was used, the hydrogen sulphide was absorbed in 1M sodium hydroxide solution, and the sodium sulphide formed was immediately titrated against standard solution of silver nitrate. This method, however, was used infrequently and at high sulphide concentrations only.

8. Effect of pH on the degradation of cephalexin

The effect of pH has been studied in citrate-phosphate buffer solution at pH 3 (the buffer was prepared by adjusting 0.1M citric acid solution with 0.5M disodium hydrogen phosphate to pH 3.0) and in disodium hydrogen phosphate-potassium dihydrogen phosphate buffer solution at pH 8.5 and 10. In the latter case, the pH of the phosphate buffer (pH 7.4) was adjusted to pH 10 by the addition of 0.2M of sodium hydroxide solution. The degradation was studied also in distilled water without buffering and in 0.1M sodium hydroxide solution.

III Comparative degradation studies of 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA)

The degradation of 7-ACA and 7-ADCA was studied in 0.1M sodium hydroxide solution at 25°C by d.p. polarography and UV spectrophotometry.

Procedure. Solutions of 7-ACA and 7-ADCA (100 and 86 µg/ml, respectively) were prepared in 0.1M sodium hydroxide solutions in 50ml volumetric flasks. The flasks were placed in a water bath at 25°C. After suitable intervals, an aliquot(2ml) of the degraded solution was transferred to the polarographic cell which contains 10ml of previously deoxygenated B.R. buffer solution pH 1.9 (final pH 2.4). The solution

was then deoxygenated for a further 2 minutes before recording the polarogram in the d.p. mode between 0.0 and -1.2V.

UV absorption measurements were carried out by diluting 1ml of the degraded solution to 6ml with 0.1M sodium hydroxide. The spectrum was recorded using 1cm silica cells against sodium hydroxide solution (0.1M) as reference.

Determination of the amount of hydrogen sulphide evolved during the degradation of 7-ACA and 7-ADCA

Procedure. Solutions of 7-ACA and 7-ADCA were prepared by dissolving 10mg of each compound in 100ml of 0.1M sodium hydroxide solution. The solutions were placed in gas wash bottles being placed in a water bath at 25°C. The solutions were continuously flushed with a slow stream of nitrogen gas which had previously been saturated with water. The hydrogen sulphide was stripped from the nitrogen gas by absorbing it in 10.0 ml of cadmium nitrate standard solution (1×10^{-3}M). After 4.5 hours, each solution was acidified with 2ml of concentrated hydrochloric acid, and the hydrogen sulphide was driven out with nitrogen for a further 30 minutes. The cadmium nitrate solution was transferred to the polarographic cell, and 10ml of citrate buffer (pH 4.6) was added. The solution was deoxygenated for 5 minutes and a d.p. polarogram was obtained at 25°C between -0.4V and -0.8V. The concentration of sulphide was obtained by comparing the cadmium peak height with those of standards.

C. D.P. POLAROGRAPHIC DETERMINATION OF CEPHALEXIN

1. Recommended procedure.

Into 10ml calibrated flasks add aliquots (<5ml) of neutral

cephalexin solution (standard, or sample,) containing less than 30 µg of cephalexin and dilute the solution to 10ml with pH 7.4 phosphate buffer. Place the loosely-stoppered flasks in a boiling water bath for 1 hour, cool and transfer the solution to the polarographic cell. Deoxygenate the solution for 5 minutes and obtain d.p. polarograms between -1.1 and -1.4V at 25°C.

The reproducibility of the method was checked by repeating the experiment ten times at the 2 µg/ml level. On one occasion, the dissolved oxygen was removed from the solution by passing a stream of oxygen-free nitrogen through the solution for 15 minutes before the hydrolysis step.

2. Effect of hydrolysis time on the peak height at -1.26V

In each of four 10ml calibrated flasks, cephalexin solution (0.5ml, 100µg/ml)was diluted to volume with phosphate buffer solution (pH 7.4). The loosely-stoppered flasks were placed in a boiling water bath for various periods of time (45, 60, 75 and 90 minutes). The contents of each flask was cooled, transferred to the polarographic cell and deoxygenated for 5 minutes before recording the d.p. polarogram between -1.1 and -1.4V at 25°C.

3. Effect of standing time at 25°C on the peak height at -1.26V

Cephalexin solution hydrolysed for 1hour as above was left in the polarographic cell at 25°C for 2.5 hours and a d.p. polarogram was recorded again as described above.

4. Effect of pH on the peak potential

Cephalexin in pH 7.4 phosphate buffer solution (50ml, 10 µg/ml) was hydrolysed for 1 hour at 100°C, and d.p. polarograms at various pH values were obtained after adjusting the solution pH to the required value with 1M sodium hydroxide and 1M phosphoric acid solutions.

5. Effect of mercury reservoir height on the d.c. limiting current at -1.26V.

D.c. polarograms were obtained for the hydrolysed cephalexin solution (10µg/ml) at various mercury reservoir height, at 25°C. The effect of raising the temperature on the d.c. wave height was also studied using the same solution.

6. Hydrolysis of cephalexin to form 2-hydroxy-3-phenyl-6-methylpyrazine.

Procedure. To a 10ml volumetric flask an aliquot (typically 1ml) of cephalexin stock solution (10 µg/ml) and 0.5ml of 2M sodium hydroxide solution were added and left for 10 minutes at room temperature. Then 0.5ml of 2M hydrochloric acid solution and 3ml of citrate buffer pH 5 (0.2M citric acid adjusted to pH 5 with 1M sodium hydroxide solution) were added. The flask was loosely stoppered and heated in a boiling water bath for 30 minutes, cooled and diluted to 10ml with phosphate buffer (pH 8.5). The solution was transferred to the polarographic cell and deoxygenated for 10 minutes. A d.p. polarogram was obtained between -0.85 and -1.1V.

The reproducibility of the method was checked at 1 and 5 µg/ml of cephalexin in the measured solution by repeating the experiment several times. Attempts to improve the reliability of the method were made by including formaldehyde (1% v/v) as a catalyst[60],

increasing the heating time to 60 minutes and using different concentrations of sodium hydroxide.

Isolation of a sample of 2,4-dinitrophenylhydrazone of the carbonyl compound responsible for d.p. peak at -1.26V.

Procedure. Cephalexin (0.5g) was dissolved in 100ml of pH 8.5 phosphate buffer and the solution was heated in a water bath at 80°C for 12 hours whilst continuously flushing the hydrogen sulphide formed from the solution by means of a stream of nitrogen gas. After cooling the solution, the grey-white precipitate of 3-aminomethylene-6-phenyl-piperazine-2,5-dione[93] was filtered off, and a saturated solution of 2,4-dinitrophenylhydrazine in methanol/concentrated hydrochloric acid (10:1 v/v) was added to the filtrate. The solution was heated to 60°C for 1 minute and then was allowed to cool to room temperature. The 2,4-dinitrophenylhydrazone was filtered off and was purified on a preparative silica gel TLC plate eluting with 3:1 chloroform-methanol. The main compound had an R_f value of 0.35. The compound was obtained as a solid after extraction into ethanol.

D. D.P. POLAROGRAPHIC STUDY OF THE DEGRADATION OF AMPICILLIN

Methods

Degradation studies were carried out as described previously for cephalexin degradation. Degradation solutions were contained in a suitable wash gas bottles in a water bath at the required temperature. Nitrogen gas, previously saturated with water, was passed through the solution throughout the degradation and the evolution

of hydrogen sulphide was tested for by passing the ensuing nitrogen gas through a standard solution of cadmium nitrate (10^{-3}M). When it was required to study the effect of molecular oxygen on the degradation the nitrogen was replaced by air.

Degradation studies were carried out in pH 7.4 phosphate buffer solution at 37°C and in pH 2.5, 8.5 McIlvain's citrate-phosphate buffer solutions. The effect of temperature on the degradation process was studied at 37°C, 60°C and 80°C in McIlvain's buffer at pH 2.5.

In all cases, 100µg/ml ampicillin trihydrate was used (85.5 µg/ml ampicillin as free acid). Spectrophotometric measurements were carried out using 1 cm silica cells, and the degraded solutions were cooled before recording spectra.

Polarographic determination of penicillamine

Penicillamine gives a well defined anodic wave at -0.14V in citrate-phosphate buffer pH 2.5. The half wave potential was found to be concentration dependent.

a. - Effect of mercury reservoir height on the d.c. anodic wave of penicillamine

Into the polarographic cell, 10ml of citrate-phosphate buffer solution pH 2.5 and 2ml of penicillamine solution (100µg/ml) were placed and deoxygenated for 10 minutes. Polarograms were recorded in the d.c. mode at 25°C and various mercury reservoir heights.

b. - Effect of temperature

The effect of temperature on the d.c. anodic wave was studied using the above solution. The temperature of the polarographed solution was adjusted to the required temperature (25, 30, 35°C) by passing water from the thermostatically controlled water bath through the jacketed polarographic cell.

c. Effect of pH on the half-wave potential, d.c. limiting current, d.p. peak potential and peak height of penicillamine.

The polarographic behaviour of penicillamine in the pH range 2-12 was studied in B.R. buffer solutions; the pH was adjusted to the required value by adding 0.2M sodium hydroxide solution. For each polarographic run, 10ml of the buffer solution (at the required pH value) and 2ml of penicillamine stock solution (100 µg/ml) were placed in the polarographic cell, and deoxygenated for 10 minutes. The solution was blanketed by an atmosphere of nitrogen whilst the polarograms were recorded. Polarograms in the d.c. and d.p. modes were obtained for each solution at 25°C.

d. Concentration effect

The concentration effect was studied in citrate-phosphate buffer at pH 2.5 and 25°C. Two calibration graphs were obtained in the concentration ranges 1.96 - 21.8 µg/ml and 9 - 44.4 µg/ml.

Polarographic determination of 2-hydroxy-3-phenyl-6-methyl-pyrazine

The polarographic behaviour of 2-hydroxy-3-phenyl-6-methyl-pyrazine was studied as above, and calibration graphs were obtained

in citrate-phosphate buffer solutions at pH 2.5 and 25°C in the concentration ranges 1.7 - 8.38 µg/ml and 3.5 - 18.6 µg/ml.

The calibration graphs for this compound and also for penicillamine were used for the determination of the amounts of penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine formed during the degradation of ampicillin.

RESULTS

A. D.P. POLAROGRAPHIC DETERMINATION OF CEPHALOSPORINS

Cephalothin, cephalosporin C, 7-aminocephalosporanic acid, cephoxazole and deacetylcephalosporin C which have a substituted 3-methyl group behave quite similarly in B.R. buffer at pH 2. All of them exhibit a well defined polarographic peak at about -1V at pH 2-4. This peak was found to disappear at pH values higher than 4. The extent of the change of the half wave potential with pH is consistent with the electrode reaction being irreversible. Also the size of the peak is dependent on pH and is at a maximum at pH 2. Analysis of d.c. polarograms with respect to changes in the limiting current with mercury reservoir height indicated diffusion control. The half-wave potentials were found to be concentration dependent, which gives further evidence on the irreversibility of the electrode reaction.

Cephaloridine exhibits two polarographic reduction peaks in the pH range 2-12, but only the first reduction peak has been used for analytical purposes here. The peak potential of the (main) first peak is (-0.79 - 0.068 pH) V in the pH range 2-9. The size of the first peak is pH dependent, and is at maximum at pH 2. Nevertheless, the calibration curve was obtained at pH 4 to reduce the interference effect of the buffer. At pH 8 a polarographic maximum of the first kind was observed on the d.c. polarogram which was not affected by the addition of the maximum suppressor Triton X-100.

Cephalonium shows three polarographic peaks in B.R. buffer. The second and third peaks disappear at pH values higher than 8. The peak potential of the first peak is ((-0.51 - 0.069 pH)V in the pH range 2-9. This peak, which is twice the height of the other two, was used for analytical purposes.

Cefuroxime gives two polarographic peaks: the first peak is about twice the height of the second peak. The second peak at -1.02V (pH 2.5) disappears at pH values higher than 5. This behaviour is similar to that observed with other cephalosporins with a substituted 3-methyl group. This is believed to be due to the reductive elimination of the $^{-}OOCNH_2$ group at the 3-position. The first peak, however, was used for the determination of cefuroxime in B.R. buffer pH 2.5.

7-Aminodeacetoxycephalosporanic acid (7-ADCA) does not give a polarographic peak, but several of its degradation products do. This had been observed when the compound was first dissolved in sodium hydroxide and then B.R. buffer pH 2 added. Cephalexin and cephradine which have unsubstituted methyl groups on the 3-position were found not to give polarographic wave at the dropping mercury electrode.

Results obtained for the cephalosporins studied are summarised in Table 1, and typical differential pulse polarograms obtained in producing calibration curve for each compound at the optimum pH are shown in the Appendix together with graphs showing the effect of pH on the peak potentials (full lines) and peak heights (broken lines) for most of the studied compounds.

Calibration curves (typically 0-3μg/ml) were rectilinear in all cases.

Table 1. Data obtained for the cephalosporins studied

Name	Optimum pH	Position of d.p. peaks (Volt)	Position of peak used for analysis (Volt)	Linear range (μg/ml)
Cephalothin	2	-1.0	-1.0	0.42 - 2.36
Cephaloridine	2	-1.0	-1.0	0.2 - 1.82
Deacetyl-cephalosporin C	2	-0.99	-0.99	0.39 - 1.48
Cephoxazole	2	-0.95	-0.95	0.1 - 1.33
7-ACA	2	-0.99	-0.99	0.1 - 0.86
Cephaloridine	4	-1.06,-1.28	-1.06	0.15 - 3.32
Cephalonium	3	-0.72,-1.0, -1.12	-0.72	0.05 - 0.38
Cefuroxime	2.5	-0.39,-1.02	-0.39	0.1 - 1.08
Cephalexin	2 - 12			
Cephradine	2 - 12			

B. D.P. POLAROGRAPHIC STUDY OF THE DEGRADATION OF CEPHALOSPORINS

I. Degradation of cephalothin and cephaloridine

The applicability of d.p.p. to degradation and stability testing studies has been examined. Preliminary hydrolysis studies of cephalothin and cephaloridine showed that heating of these antibiotics in aqueous B.R. buffer solution at pH 2 and 50°C and 37°C respectively, results in the decrease of the characteristic peak height at about -1V and the appearance of small peaks at less negative potential. D.p. polarograms showing the degradation of cephalothin and cephaloridine are depicted in Fig. 1 and 2.

Previous investigators[42,73,79] reported that the opening of the β-lactam ring of cephalothin and cephaloridine following acid, alkaline or enzymatic hydrolysis is accompanied by the expulsion of the acetoxy and the pyridine side chain on the 3-position to form the corresponding polarographically inactive cephalosporoate lactone[26]. This will explain the decrease in the d.p. peak height with heating time, as the presence of the substituted methyl group on the 3-position is the essential requirement for polarographic activity of these compounds in the absence of other reducible group in the molecule.

II. Degradation of cephalexin and cephradine

The initial degradation studies of cephalexin and cephradine (100 μg/ml) were made in phosphate buffer solutions (pH 7.4) and 37°C without flushing the solution with nitrogen. The degradation of these compounds was followed over a period of 340 hours. D.p.p.

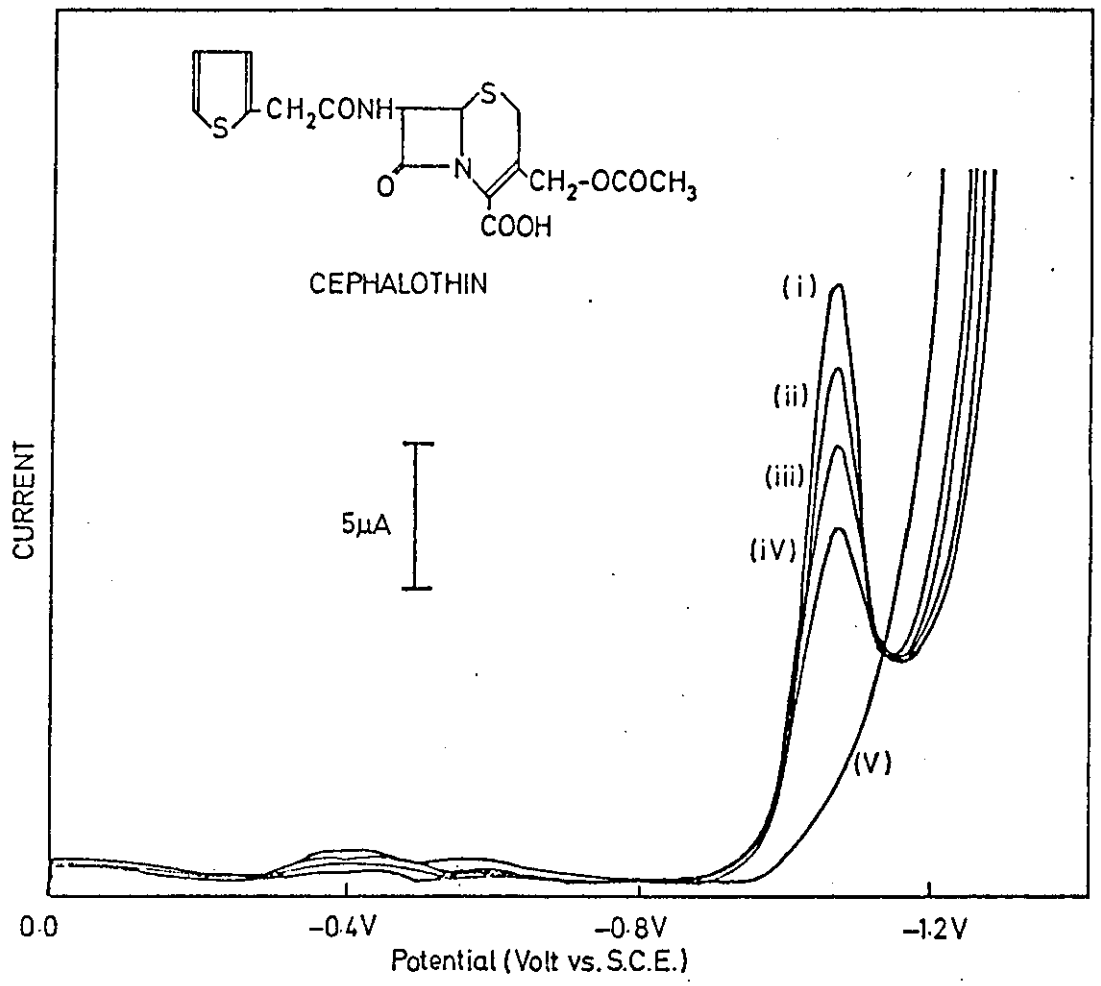


Fig. 1. D.p. polarograms showing degradation of 140μg ml^{-1} solution of cephalothin in B-R buffer at pH 2 at 50°C. Measuremant time (i) 0.5 (ii) 2.0, (iii) 2.5 (iv) 4.5, (v) 18.5 hours.

Degradation was carried out in the polarographic cell and the polarograms were obtained at 50°C

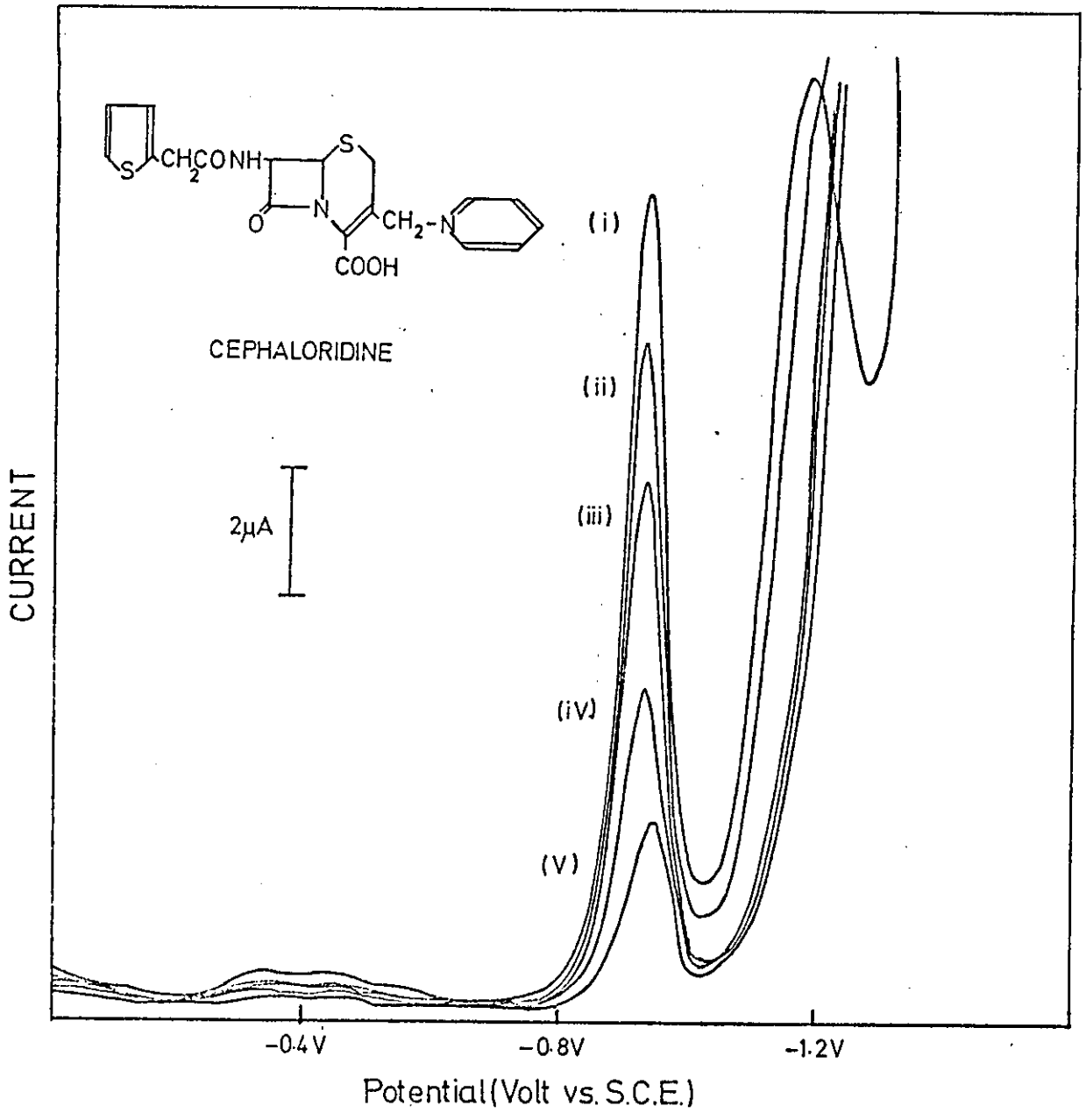


Fig. 2. D.p. polarograms showing degradation of 100μg ml^{-1} of solution of cephaloridine in B-R buffer atpH 2 at 37°C Measurement time: (i) 0, (ii) 17.5, (iii) 27, (iv) 48, (v) 96 hours

Polarograms were obtained at25°C.

curves showing part of the degradation of cephalexin are shown in Fig. 3. The first peak appeared at -0.56V is due to hydrogen sulphide. The large peak at -0.9V is due to diketopiperazine derivative (DKP II). The appearance of the other two peaks at -0.78V and -1.26V will be discussed later. Cephradine as expected, was found to behave in the same manner.

The changes in the peak heights of cephalexin and cephradine degradation products with time are illustrated in Tables 2,3 and Figs. 4, 5.

A sample of the diketopiperazine derivative was isolated from a degraded solution of cephalexin as described by Bundgaard[91]. The product had the following characteristics:

IR (kBr) ν_{max} : 3150, 1770, 1720, 1675, 1450, 1320, 1070, 725 and 695 cm^{-1}, spectrofluorimetric excitation maximum at 360nm and emission maximum at 433 nm (both uncorrected, in 0.1M sodium bicarbonate), thin layer chromatographic analysis on Meric silica gel F_{254} plates with solvent system water-glacial acetic acid-acetone-ethyl acetate (1:2:2:5 v/v) gives R_f value of 0.64. These results are in accord with the findings of Bundgaard[91]. The melting point (145 - 148°C) was less than that quoted 160-162°C. However, the compound gives only one d.p. polarographic peak at -0.9V in phosphate buffer pH 7.4. Evidence that this polarographic wave is diffusion controlled was obtained from studying the effect of mercury pressure and temperature on the d.c. wave height. A straight line which passes through the origin was obtained when the wave height (μ A) was plotted against the square root of the mercury reservoir height (corrected for back-pressure). Also a temperature coefficient typically of 2%

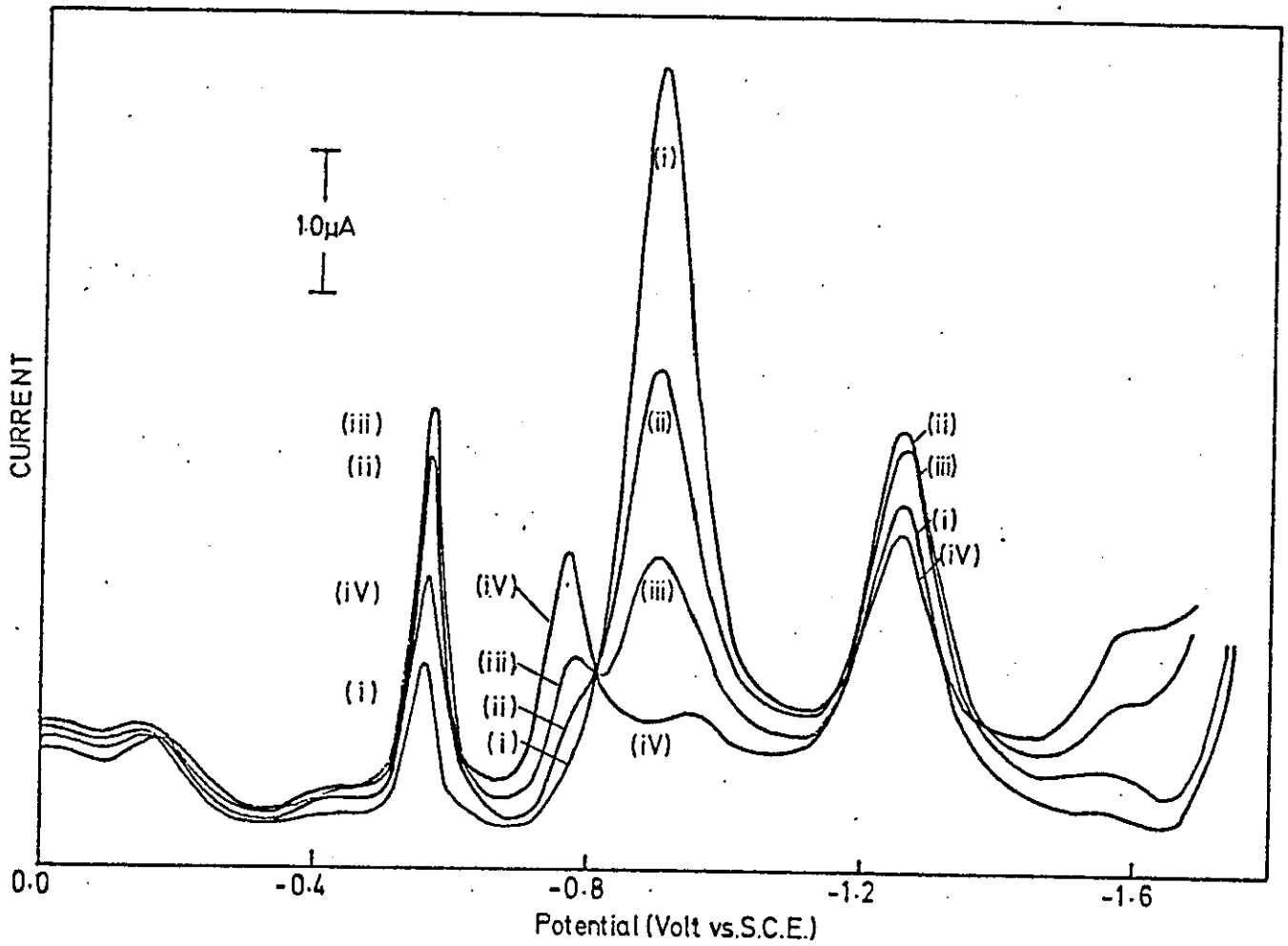


Fig. 3. D.p. polarograms showing part of the degradation of 100μg ml^{-1} of cephalexin in phosphate buffer at pH 7.4 at 37°C.

Measurement time: (i) 24, (ii) 46.5, (iii) 70, (iv) 190 hours

Table 2. Changes of d.p. peak heights of cephalexin degradation products with heating time at 37°C in phosphate buffer solution pH 7.4

Time/hrs	Peak height /μA			
	Peak at -0.56V	Peak at -0.78V	Peak at -0.9V	Peak at -1.26V
0.0	0.0	0.0	0.0	0.0
2.0	0.0	0.0	2.0	0.0
5.0	0.1	0.0	4.3	0.0
7.0	0.15	0.0	5.4	0.1
14.75	0.18	0.0	5.3	0.4
22.00	0.80	0.0	5.4	1.3
24.25	1.00	0.0	4.9	1.6
26.25	1.40	0.0	4.7	1.7
28.00	1.55	0.0	4.5	1.9
31.00	1.70	0.05	4.2	1.95
46.50	2.50	0.10	2.8	2.10
48.50	2.60	0.12	2.75	2.20
50.50	2.50	0.15	2.60	2.20
54.00	1.90	0.20	2.25	2.10
70.00	2.90	0.45	1.50	1.96
75.50	2.60	0.70	0.85	1.96
94.00	2.35	0.75	0.45	1.65
102.00	2.25	0.90	0.40	1.62
121.00	2.25	1.10	0.25	1.50
144.00	2.00	1.20	0.12	1.45
168.00	1.90	1.30	0.01	1.35
190.50	1.60	1.35	0.00	1.35
214.50	1.30	1.35	0.00	1.25
334.00	0.70	1.50	0.00	1.15

Table 3. Changes of d.p. peak heights of cephradine degradation products with heating time at 37°C in phosphate buffer solution pH 7.4

Time/hrs	Peak height /μA			
	Peak at -0.56V	Peak at -0.78V	Peak at -0.9V	Peak at -1.26V
0.5	0.0	0.0	0.0	0.0
2.5	0.0	0.0	0.6	0.0
4.7	0.1	0.0	1.8	0.0
7.3	0.2	0.0	4.8	0.0
22.0	1.6	0.0	4.8	1.2
24.7	1.85	0.0	4.9	1.4
26.1	2.0	0.0	4.9	1.7
28.5	2.2	0.0	4.5	1.7
30.8	2.4	0.0	4.0	1.8
46.5	3.1	0.0	2.6	2.0
48.5	3.0	0.1	2.3	1.9
51.4	3.1	0.1	2.15	2.15
53.8	3.1	0.1	2.0	1.9
70.4	3.5	0.2	1.2	1.7
75.4	3.2	0.3	0.8	1.5
94.4	3.1	0.35	0.4	1.3
102.3	3.0	0.45	0.35	1.25
119.7	3.0	0.50	0.20	1.0
143.4	2.7	0.65	0.1	1.0
164.7	2.4	0.70	0.0	0.9
189.0	2.3	0.75	0.0	0.75
214.0	2.0	0.70	0.0	0.90
334.0	1.4	0.70	0.0	0.80

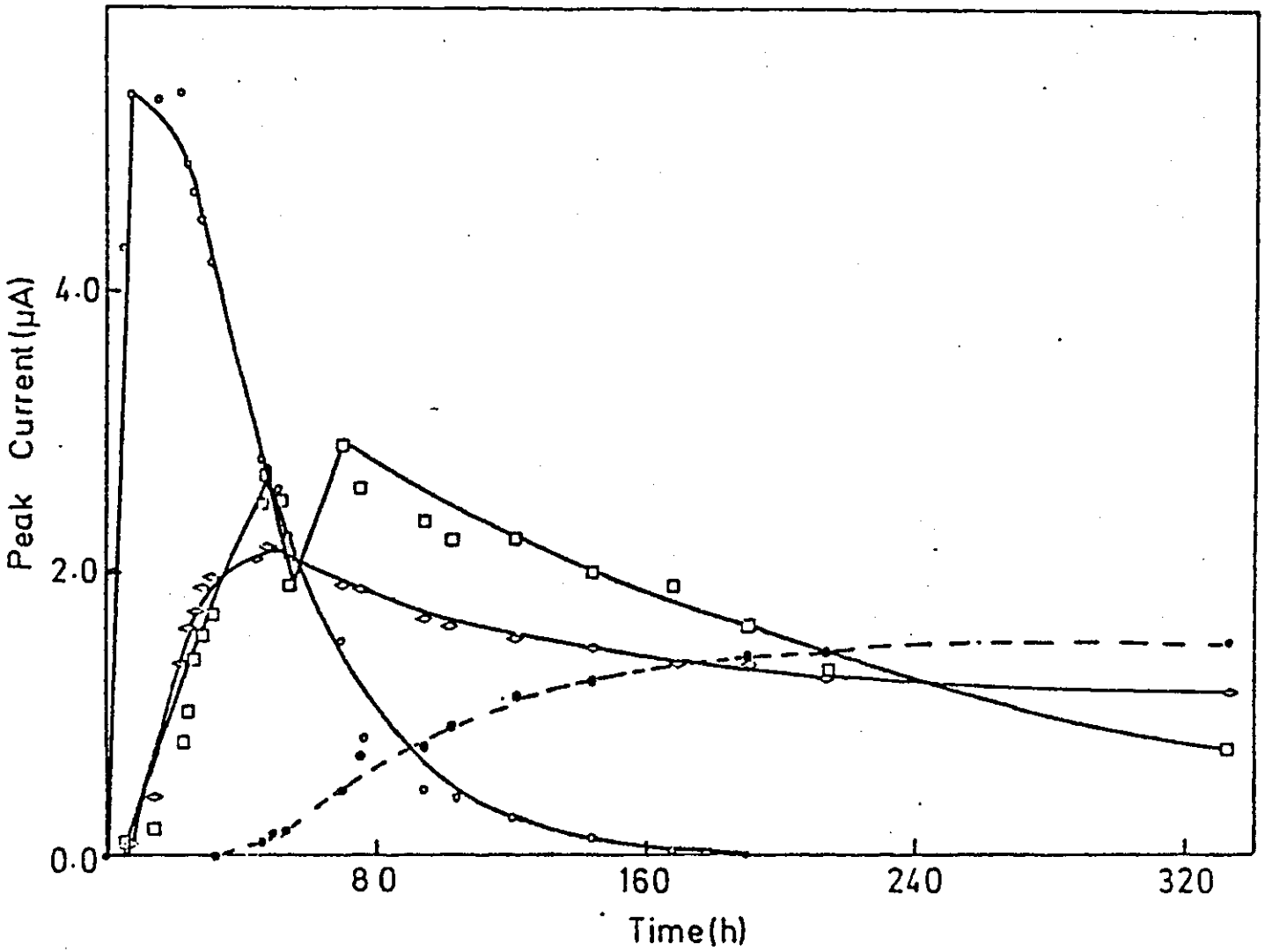


Fig. 4. Plot of peak heights of cephalexin degradation products versus measurement time.

o——o - 0.9V (DKP);□——□ -0.56V(hydrogen sulphide);

◇——◇ -1.26V; ●-----● -0.78V.

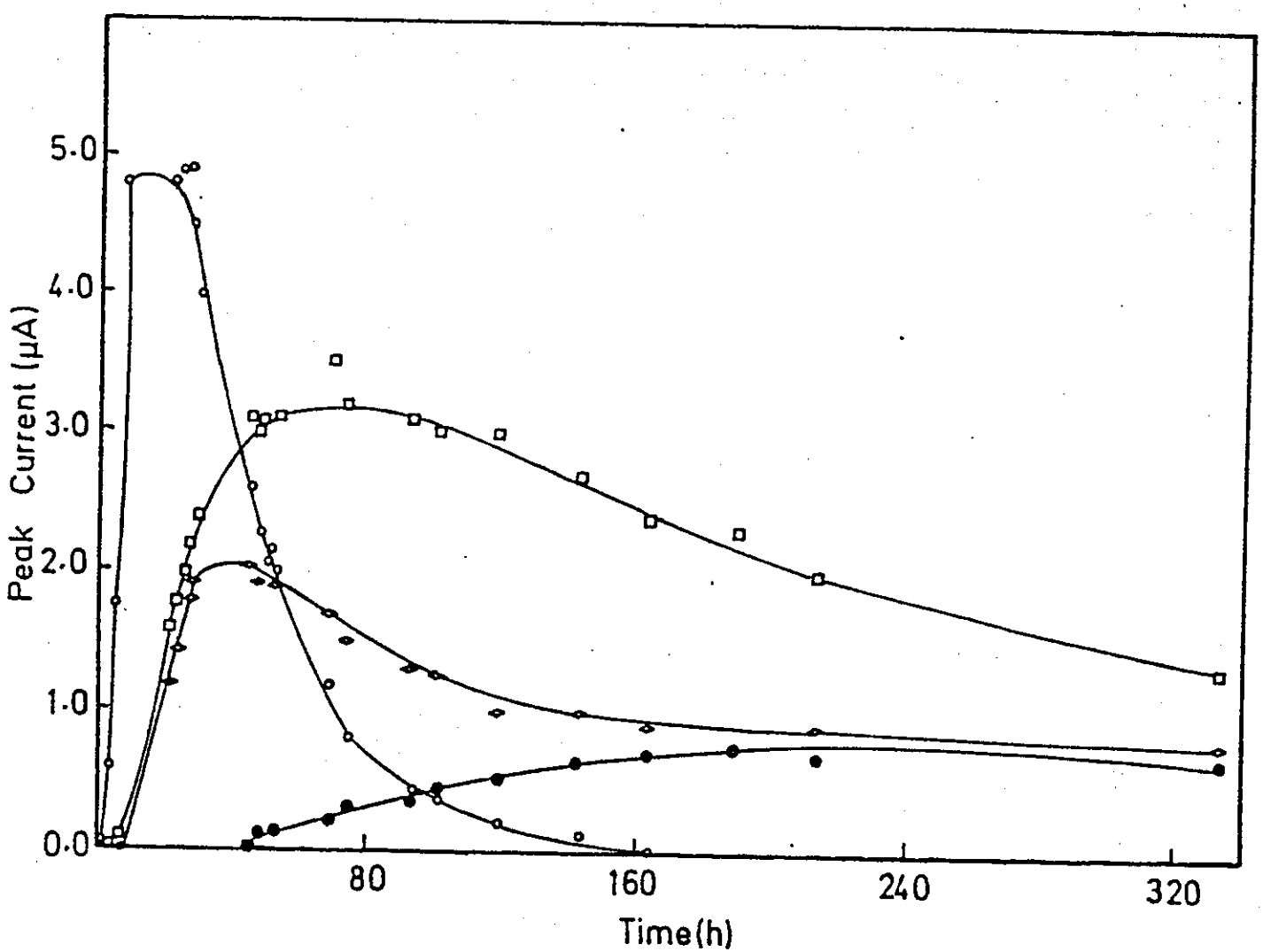


Fig. 5. Plot of peak heights of cephradine degradation products versus measutements time.

Key - the same as Fig. 4.

was obtained. The half-wave potential was found to be dependent on concentration and slightly shifted to more negative potentials as the concentration of DKP II increased. D.p. polarograms obtained for DKP II in phosphate buffer solution pH 7.4 at 25°C are shown in Fig. 6. A rectilinear calibration graph obtained over the concentration range 12 - 84μg ml^{-1} is shown in Fig. 7. This calibration curve was then used to determine the amounts of DKP II formed in degraded solutions of cephalexin.

The degradation of DKP II has also been followed in phosphate buffer pH 7.4 and 37°C. Similar polarographic patterns as those observed in the degradation of the parent compound (cephalexin) were obtained. A plot of peak heights of DKP II and its degradation products versus time is shown in Fig. 8.

The degradations of cephalexin and cephradine under the above experimental conditions were found to follow an apparent first-order reaction with a rate constant of 0.2 and 0.43 hr^{-1} respectively. The rate constants were obtained by plotting the logarithm of peak heights (μ A) of DKP II (formed in the early stages of degradation) versus measurement time (hours).

The degradation of DKP II was also found to follow a first-order reaction with a rate constant of 0.025 hr^{-1}, regardless of whether the degradation was followed for a pure sample of DKP II or for DKP II formed during the degradation of cephalexin. An abrupt increase in the rate constant has been observed between 70-75 hours; then the degradation of DKP II started to follow exactly

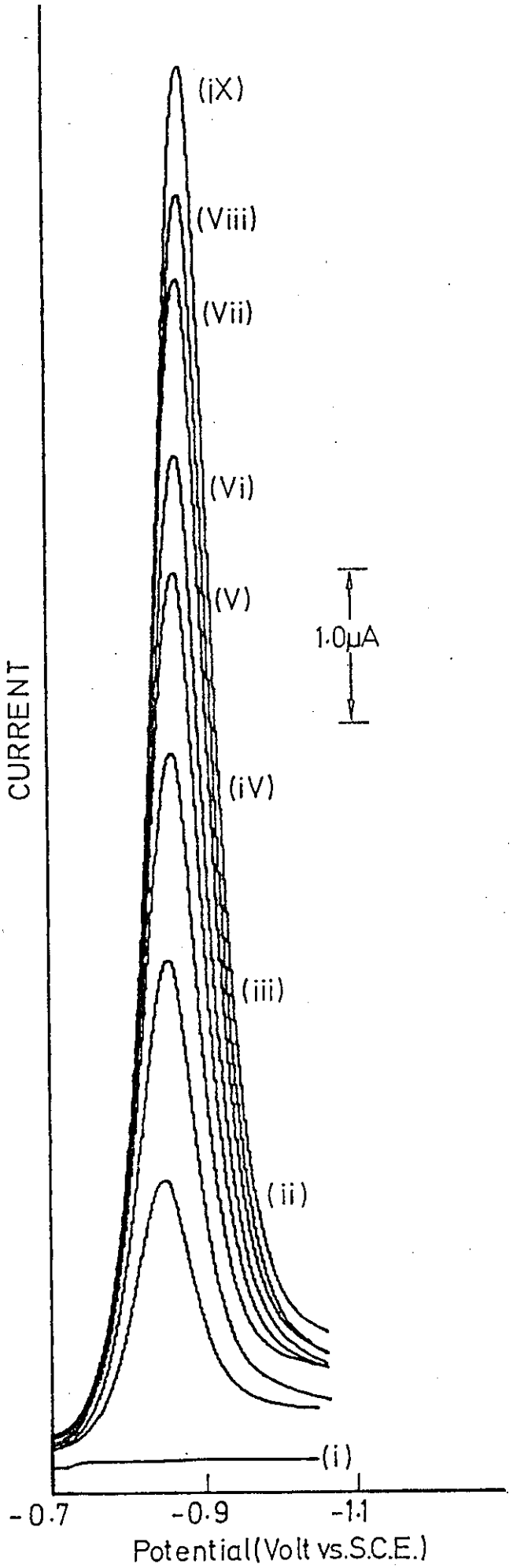


Fig. 6. D.p. polarograms obtained for DKP II in phosphate buffer solution at pH 7.4 and 25°C.
(i) 0; (ii) 12.2;(iii)23.8; (iv) 34.9; (v) 45.5; (vi) 55.6; (vii) 65.2; (viii) 74.5; (ix) 83.3 μg ml^{-1}

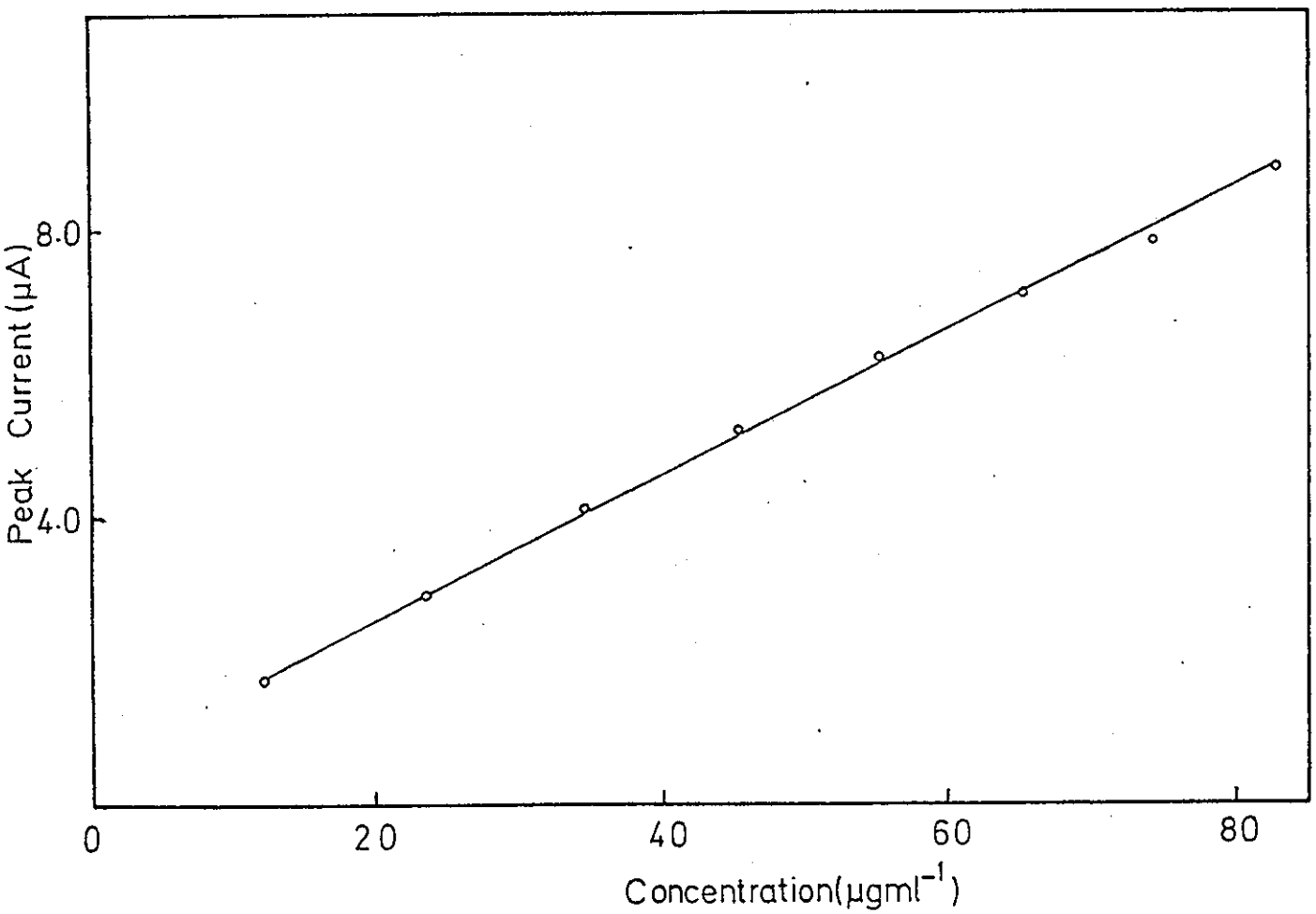


Fig. 7. Calibration graph obtained for DKP II in phosphate buffer solution pH 7.4 at 25°C

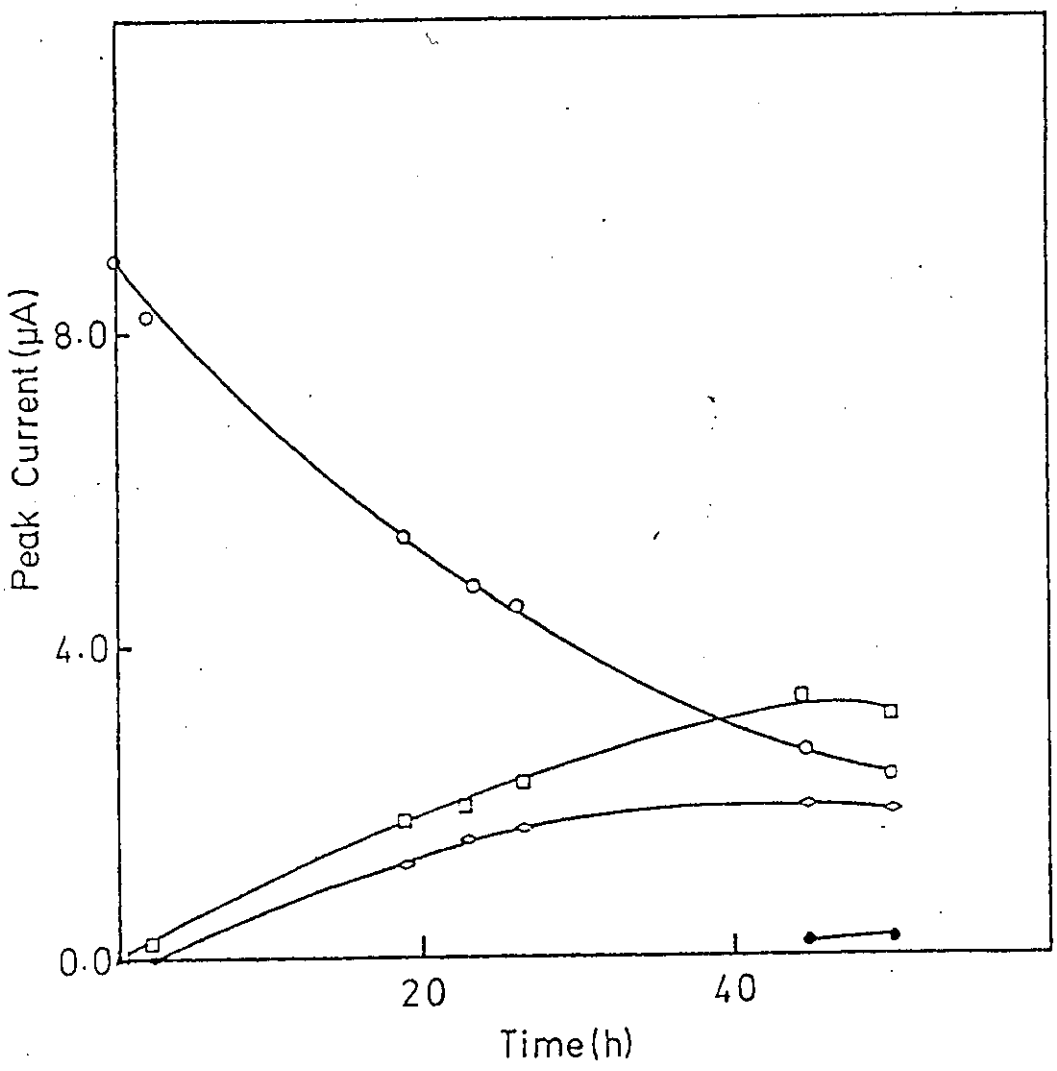


Fig. 8. Plot of peak heights of DKP II and its degradation products versus measurement time.

Key the same as Fig. 4.

the same rate constant as previously. A similar result was obtained for the degradation of DKP derived from cephradine but the rate constant in this case was 0.03 hr^{-1}. The results are illustrated in Fig. 9.

The effect of various factors on the degradation of cephalexin was then studied. First, the effect of increasing the temperature was monitored.

At 60°C the same pattern was obtained, but the degradation is faster. The DKP II peak attains its maximum value after two hours and disappears completely in 22 hours. Also, a high yield of the compound responsible for the peak at -1.26V was obtained. The maximum yield was about double that obtained at 37°C (compare Fig. 4 and Fig. 10).

At 80°C DKP II was formed and degraded within 2.5 hours and the peak at -1.26V was about five times larger than any other peak present, Fig. 11. The peak at -0.78V disappeared after 54 hours degradation. In addition to the peak at -0.16V another peak appeared at -0.96V later in the degradation after DKP II had degraded. The compound responsible for this latter peak was identified by t.l.c., UV spectrophotometry and by comparison with polarograms obtained with a sample of the pure compound as being due to 2-hydroxy-3-phenyl-6-methylpyrazine(VI); this compound had been isolated from degraded cephalexin solutions by Barbhaiya et al[66] and identified by unambiguous synthesis. A d.p. polarogram obtained for a sample of 2-hydroxy-3-phenyl-6-methylpyrazine isolated from degraded solution of cephalexin as described by Barbhaiya et al[66]

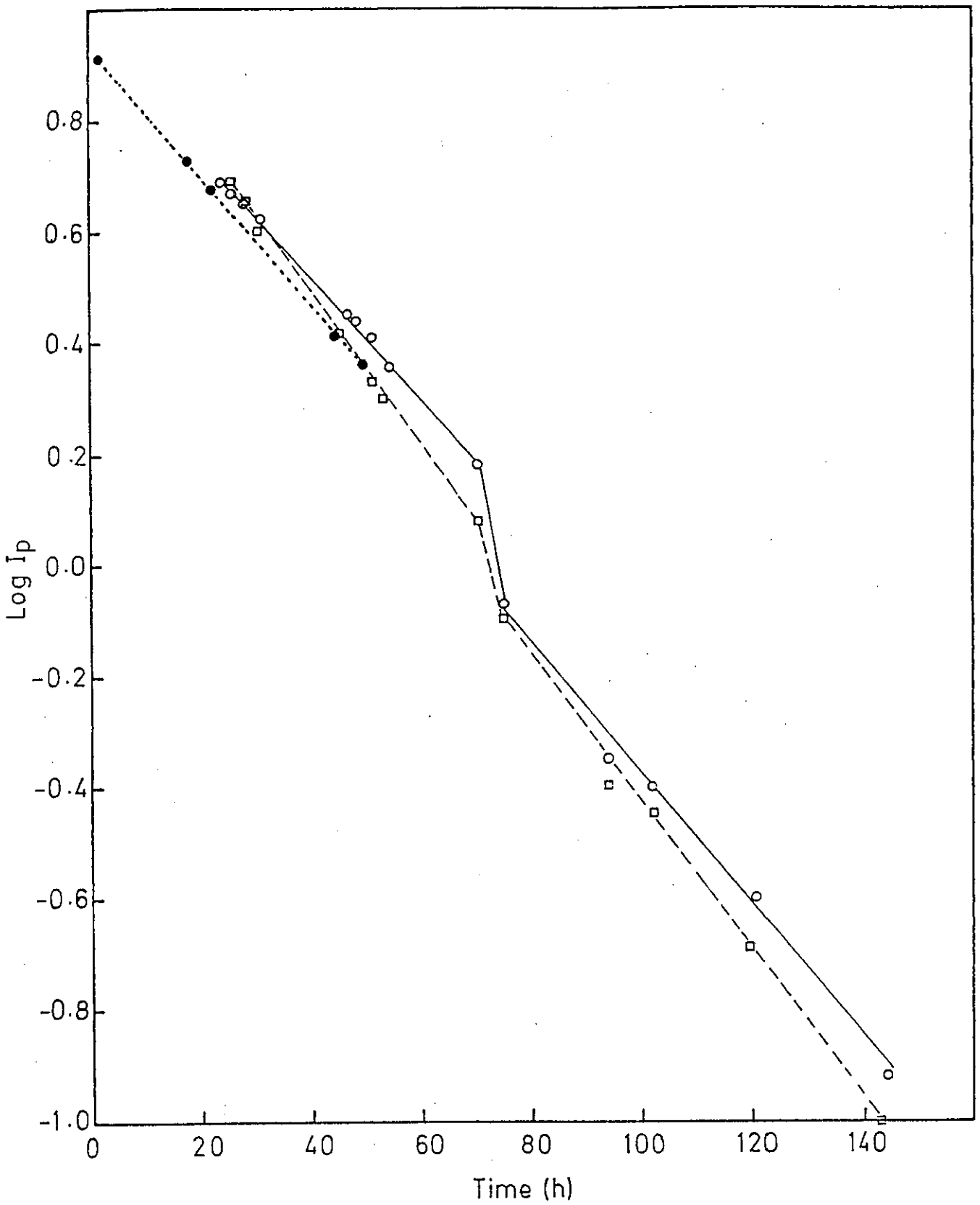


Fig. 9. Plot of logarithm of DKP II peak height. (μA) versus measurements time (hours) obtained for the degradation of pure sample of DKP II ●·····● , degraded cephalexin solution o——o and □---□ degraded cephradine solution all in phosphate buffer pH 7.4 at 37°C

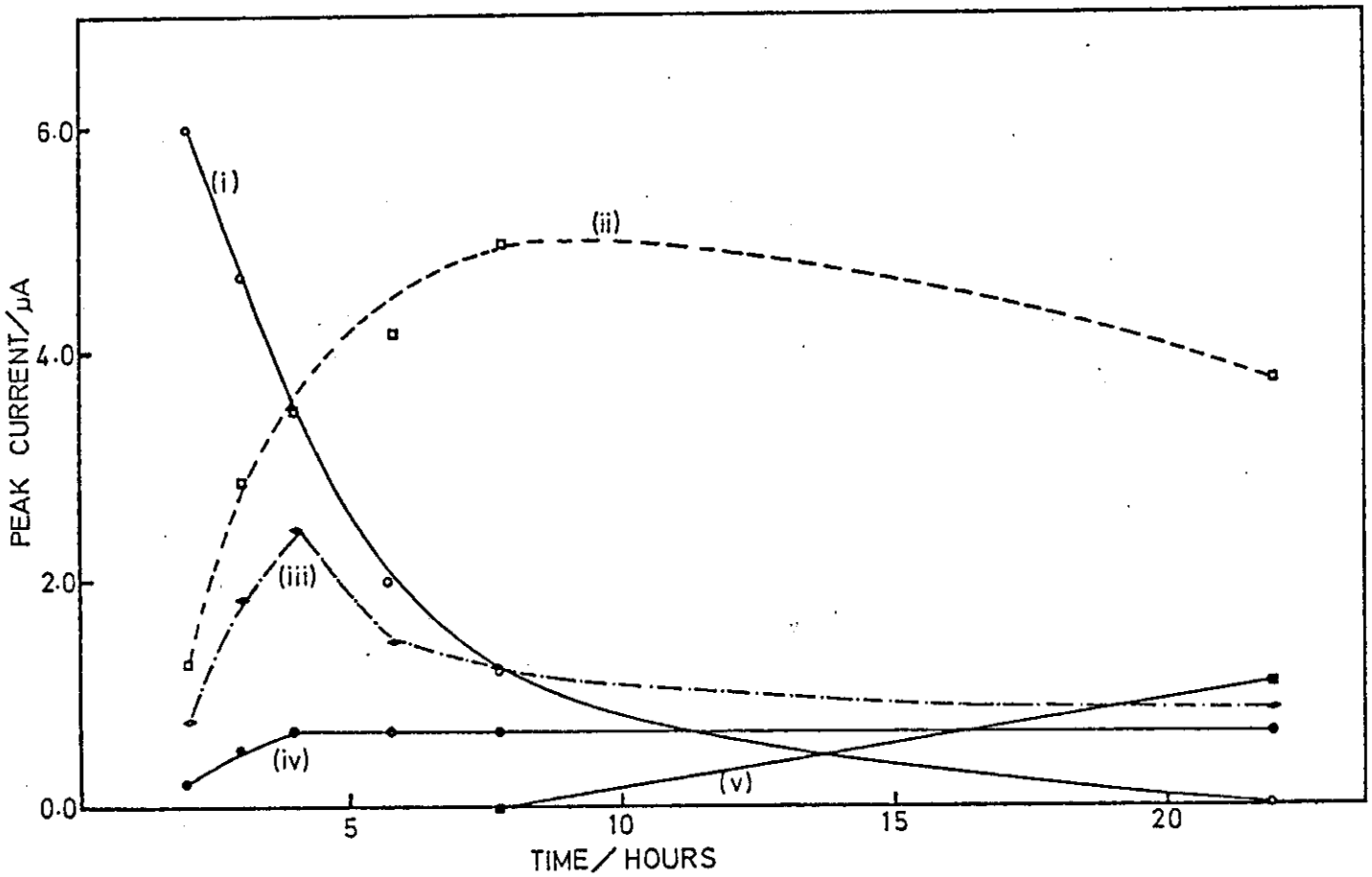


Fig. 10. Degradation of cephalexin (100 g/ml) in 0.5M phosphate buffer at pH 7.4 and 60°C. Peak potentials:(i) -0.9V (DKP II), (ii) -1.26V, (iii) -0.56V (hydrogen sulphide), (iv) -0.16V, and (v) -0.78V.

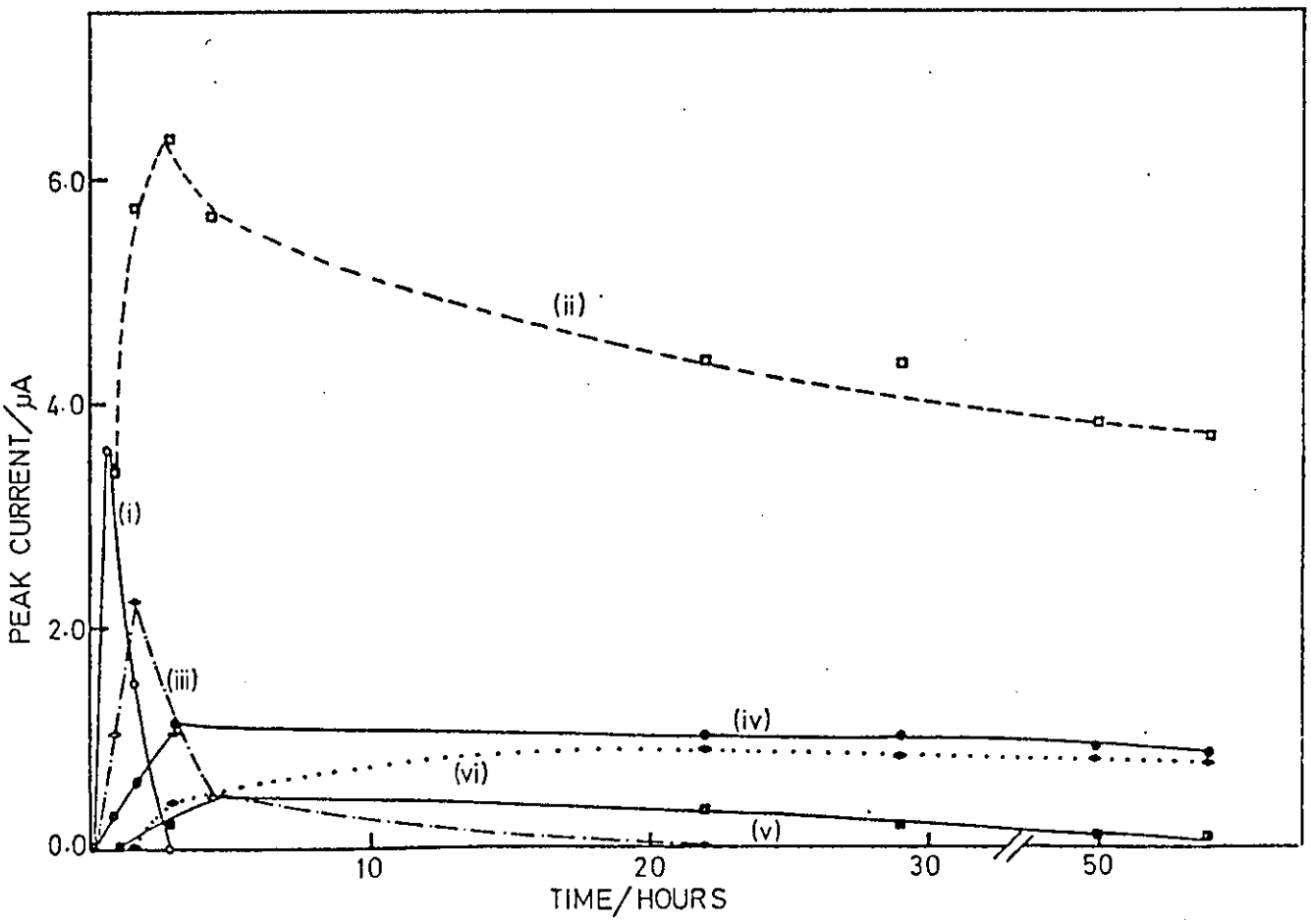


Fig. 11. Degradation of cephalexin (100μg/ml) in 0.5M phosphate buffer at pH 7.4 and 80°C. Peak potentials: (i) -0.9V (DKP II), (ii) -1.26V, (iii) -0.56V (hydrogen sulphide), (iv) -0.16V, (v) -0.78V and (vi) -0.96V (2-hydroxy-3-phenyl-6-methylpyrazine)

is shown in Fig. 12. In Fig. 13, UV spectra of the prepared sample and a pure sample of this compound are compared.

The effect of the initial cephalexin concentration was studied next. Polarograms obtained for 5 μg/ml solutions degraded at 37°C were of similar pattern to those for 100μg/ml solutions degraded at 37°C or 80°C. When 5 mg/ml solutions were degraded at 80°C however, the peak at -1.26V disappeared and a peak appeared at -1.44V (irrespective of the concentration at which the polarography was done). Another difference was the presence of a peak at -1.06V which decayed after about 2hr; the peak at -0.96V then appeared and in turn decayed with time. A peak also appeared at -0.36V early in the degradation of the 5 mg/ml solutions: the compound responsible for this peak degraded more slowly than the DKP II. It disappeared completely after 22 hrs; and two small new peaks appeared at -0.22V and -0.84V after 54 hours.

The presence of molecular oxygen and hydrogen sulphide in the degraded solutions was found to have an effect on the degradation of cephalexin. This is apparent from the modification observed in polarograms obtained in absence of hydrogen sulphide and dissolved molecular oxygen. When nitrogen gas was passed through the degrading solutions to blow off the hydrogen sulphide, in addition to the hydrogen sulphide peak at -0.56V, the peaks at -0.78V (37°C and 80°C) and -0.16V (80°C) are lost also. When, however, the hydrogen sulphide was removed with air instead of nitrogen, the peak at -0.78V and -0.16V were present, indicating that these peaks are probably due to oxidation products produced by reaction

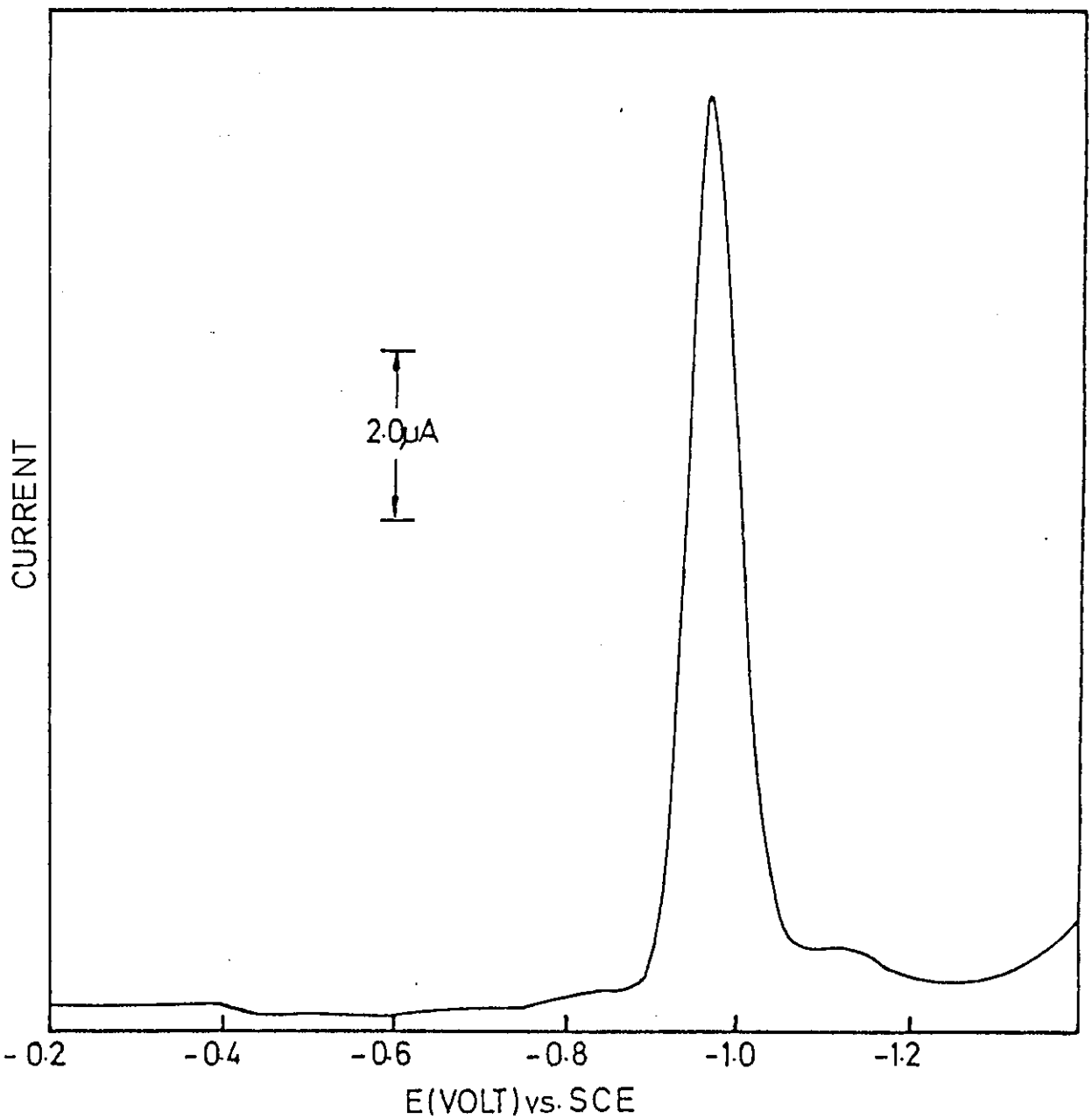


Fig. 12. D.p. polarograms of 2-hydroxy-3-phenyl-6-methylpyrazine in phosphate buffer solution pH 7.4.

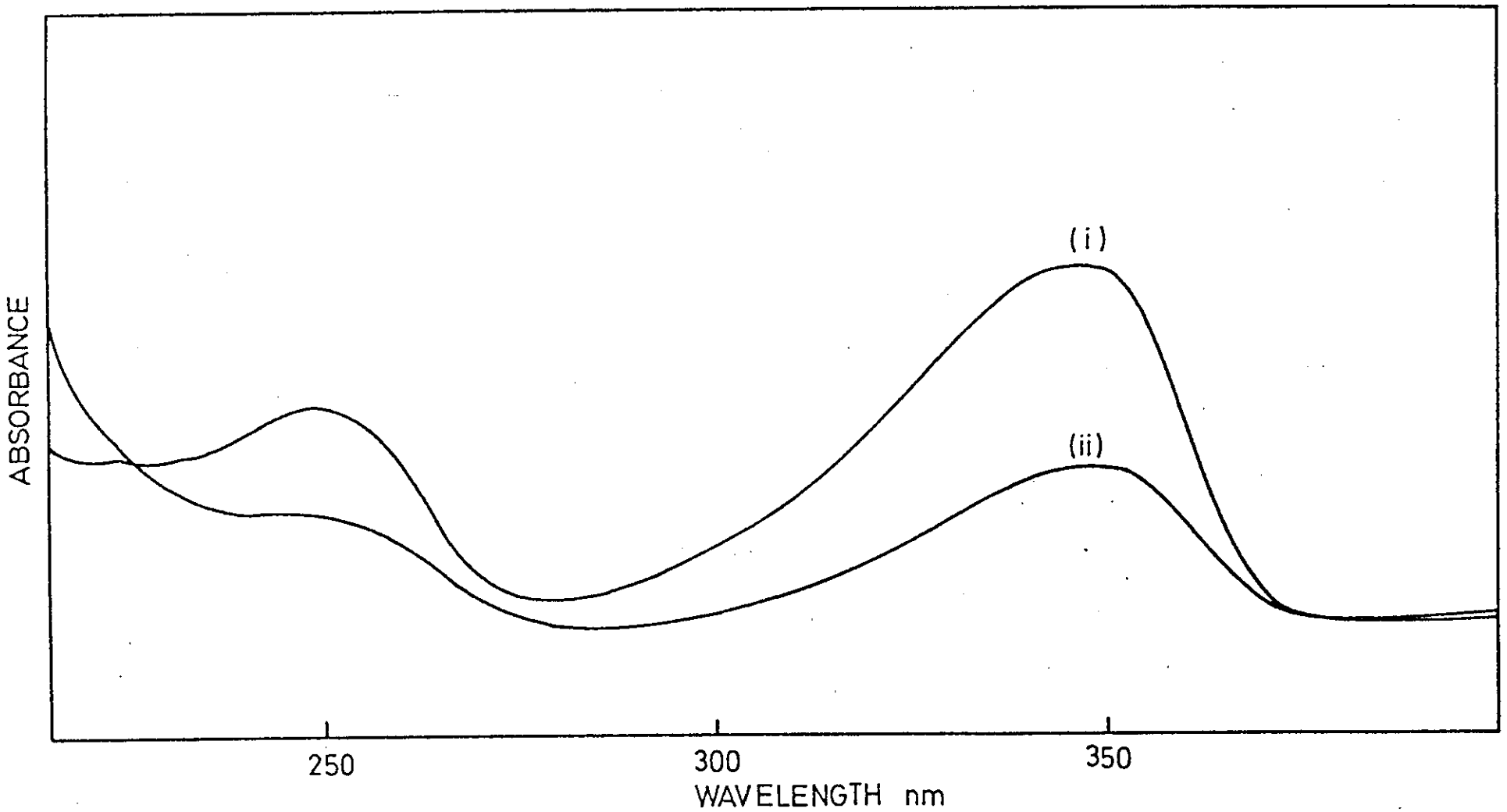


Fig. 13. UV spectrum obtained for 2-hydroxy-3-phenyl-6-methylpyrazine in distilled water.
(i) pure sample, (ii) sample isolated after cephalexin hydrolysis

of other degradation products with molecular oxygen in the solution. In Fig. 14 two polarograms obtained for the degradation of cephalexin in phosphate buffer (pH 7.4) at 37°C are compared: the first was obtained for a solution from which the molecular oxygen and hydrogen sulphide had not been purged, and the second was obtained for a solution through which nitrogen gas had been passed during the degradation.

A further effect of passing nitrogen through the degrading solution of cephalexin at 37°C was that the maximum transient yield of DKP II and the compound responsible for the peak at -1.26V increased by about 35% and 25% respectively. At 80°C, however, the maximum yield of these compounds was increased by 25% and 40% This is illustrated in Fig. 15A and 15B.

In order to determine the amount of hydrogen sulphide evolved during the degradation of cephalexin, two techniques have been considered. At first, potentiometric titration of sodium sulphide formed after absorbing the hydrogen sulphide in 1M sodium hydroxide solution against a standard solution of silver nitrate using a sulphide ion-selective electrode was tried. The method worked well for the determination of high concentrations of sulphide ($>$Ca. 10^{-4}M), but at lower concentrations; the uncertainty in determining the end point has so far precluded the use of this method. Nevertheless, the method was used infrequently to determine large amounts of hydrogen sulphide evolved during the degradation, and also for the determination of the exact molarity of sodium sulphide solutions used later as a standard for the polarographic method.

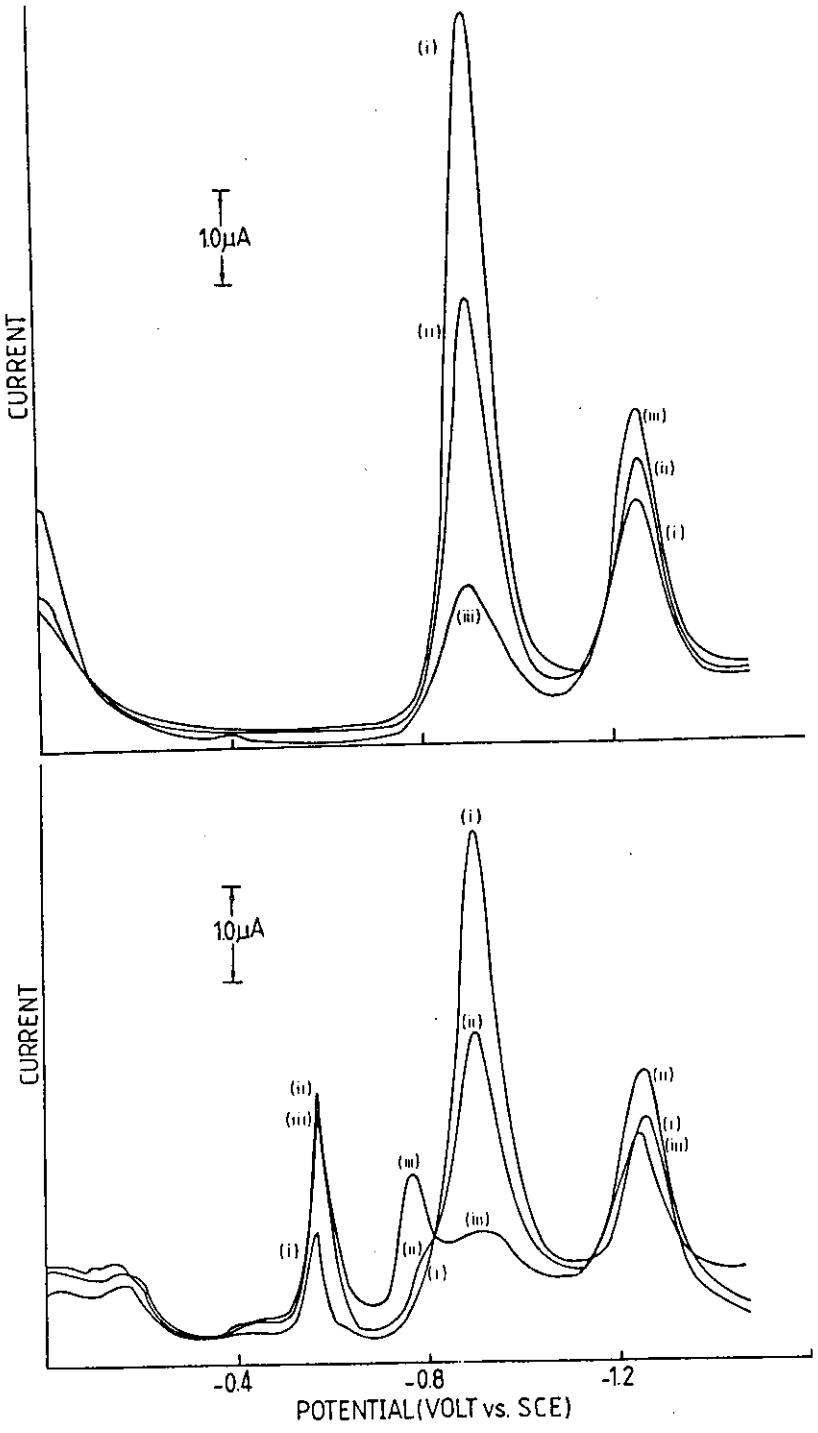


Fig.14. Degradation of cephalexin at 37°C and pH 7.4.
Upper polarograms: Molecular oxygen and hydrogen sulphide removed with nitrogen. Time: (i) 22, (ii) 46, (iii) 119 hours.
Lower polarograms: Molecular oxygen and hydrogen sulphide not removed. Time: (i) 24.5, (ii) 46.5, (iii) 121 hours.

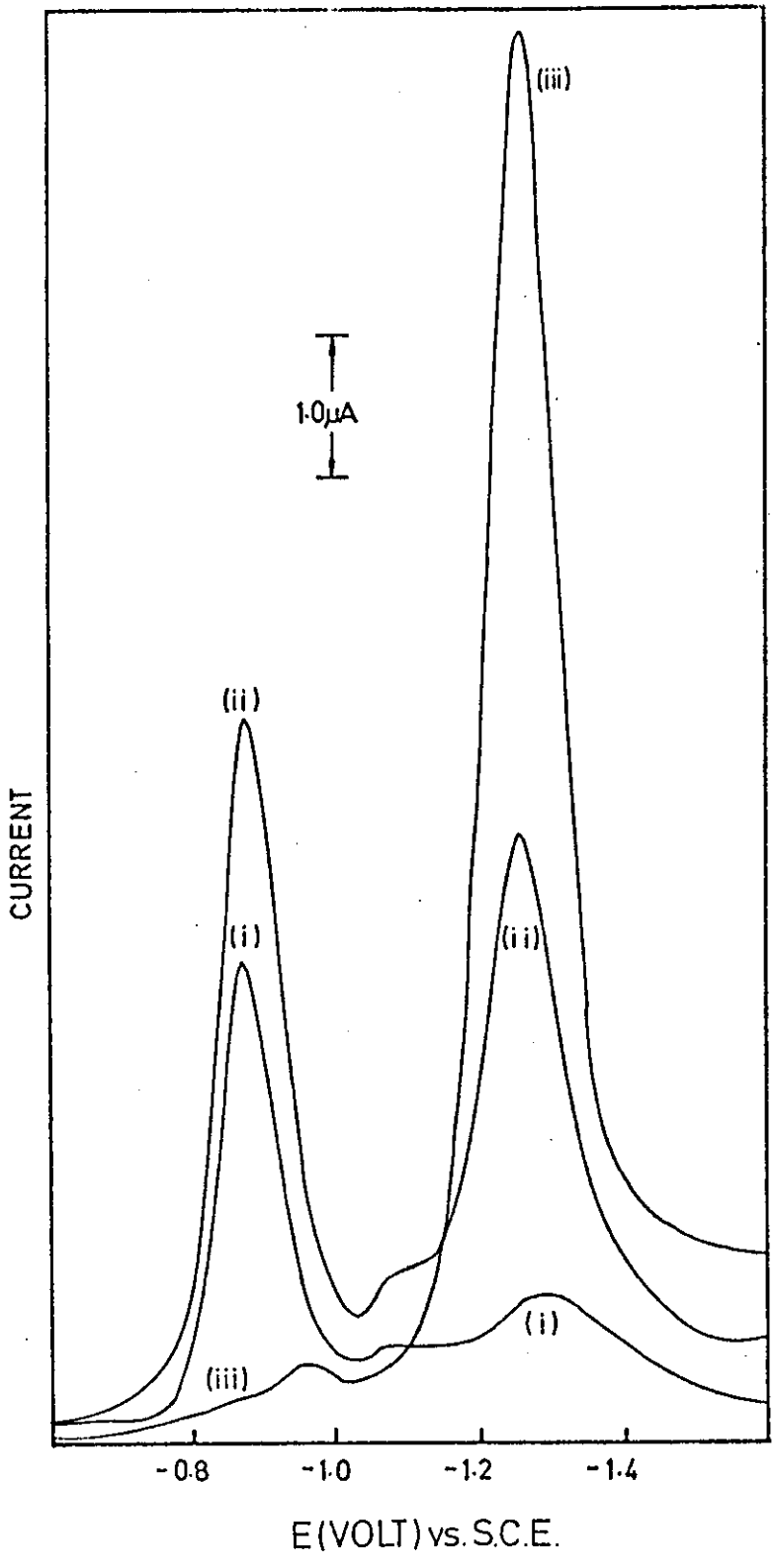


Fig. 15A. Degradation of 100 μg/ml cephalexin in phosphate buffer at pH 7.4 at 80°C in the absence of molecular oxygen and hydrogen sulphide. Measurement time: (i) 18; (ii) 32 and (iii) 148 min.

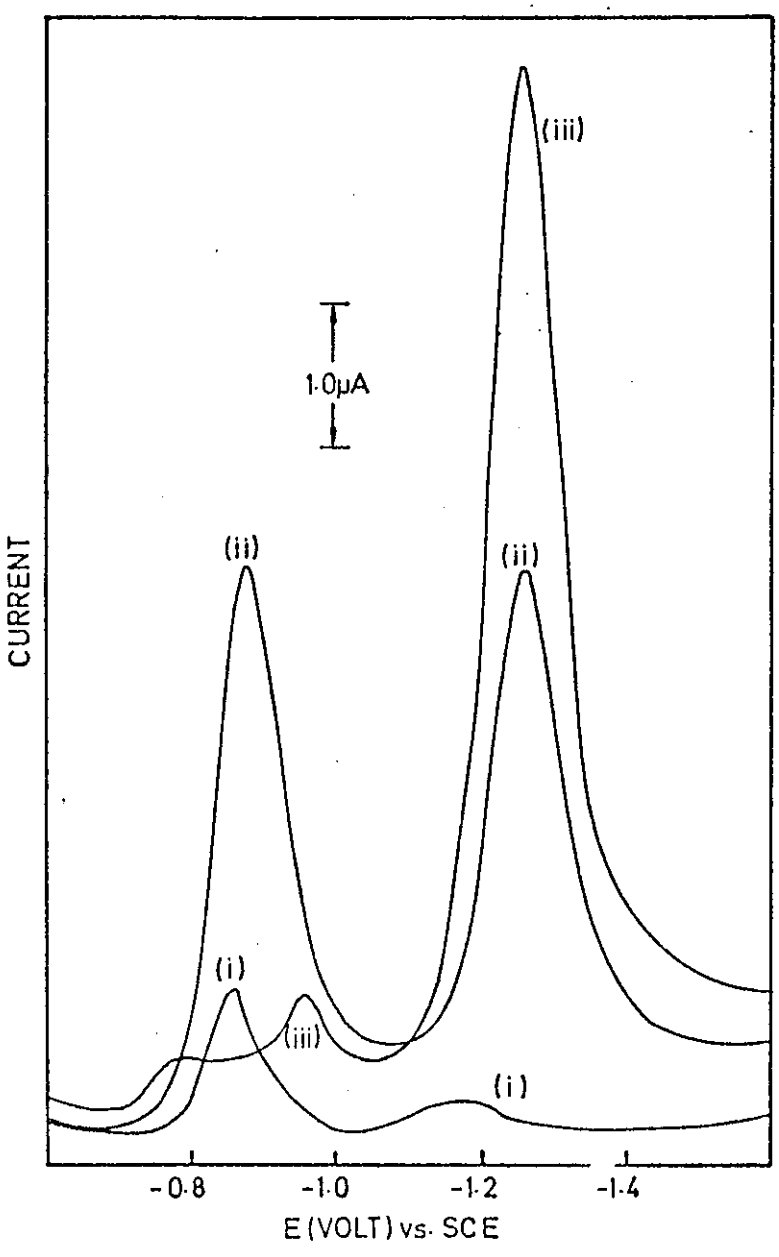


Fig. 15B. Degradation of 100μg/ml cephalexin in phosphate buffer at pH 7.4 at 80°C in the presence of molecular oxygen and hydrogen sulphide. Measurement time: (i) 5, (ii) 35 and (iii) 160min.

Results obtained for standardization of about 0.1M sodium sulphide solution against 0.1M silver nitrate standard solution are shown in Table 4.

Table 4. Standardisation of about 0.1M sodium sulphide (10.0ml) with 0.1M silver nitrate solution using a sulphide ion-selective electrode.

Volume of 0.1M silver nitrate added (ml)	Potential (mV)	Volume of 0.1M silver nitrate added (ml)	Potential (mV)
0.0	-867	17.7	-823
2.0	-867	18.0	-820
4.0	-865	18.2	-817
6.0	-863	18.4	-812
8.0	-856	18.5	-788
10.0	-852	18.7	-761
12.0	-848	18.9	-248
14.0	-843	19.0	+15
16.0	-835	19.2	+102
17.0	-830	19.5	+119
17.5	-826	20.0	+125

From the plot of the potential (mV) versus the volume of silver nitrate, the end point was obtained at 19.1ml of 0.1M silver nitrate. The molarity of sodium sulphide solution is 0.0955M.

The recovery of hydrogen sulphide, when it was purged with nitrogen for one hour from a standard solution of sodium sulphide (3.8×10^{-4}M) at pH 7.4 and 37°C and then absorbed in 50ml of 1M sodium hydroxide, was determined by the potentiometric method. The results are shown in Table 5.

Table 5. Potentiometric titration of sodium sulphide formed against 0.01M silver nitrate standard solution.

Volume of 0.01M silver nitrate added (ml)	Potential (mV)	Volume of 0.01M silver nitrate added (ml)	Potential (mV)
0.0	-823	3.2	-752
0.4	-821	3.4	-394
0.8	-819	3.6	-143
1.2	-816	3.8	-95
1.6	-813	3.9	-77
2.0	-809	4.0	-60
2.4	-803	4.2	+72
2.8	-793		

The end point was obtained at 3.6ml of 0.01M silver nitrate standard solution. The molarity of sodium sulphide solution is 3.6×10^{-4}M; it follows that the recovery of hydrogen sulphide is about 95%.

Due to the unreliability of the potentiometric method to determine low concentrations of hydrogen sulphide, an attempt was made to use the d.p.p. peak of hydrogen sulphide at -0.56V, but the

reproducibility of the calibration curve obtained in this case was unsatisfactory. An indirect d.p.p. method was developed, however. In this method, the decrease in the cadmium peak height at -0.61V when it reacts with sulphide in citrate buffer solution (pH 4.6) was made the basis of a sensitive method for the determination of hydrogen sulphide. Typical d.p. polarograms obtained for cadmium after successive addition of sodium sulphide standard solution are shown in Fig. 16. A rectilinear calibration graph obtained over the concentration range 4.6 - 28 x 10^{-5}M is shown in Fig. 17. This calibration graph has been used throughout this study to determine the amount of hydrogen sulphide evolved during the degradation of cephalexin and other cephalosporins studied.

The results of degradation studies of cephalexin (100µg/ml) in phosphate buffer at pH 7.4 at 37°C in which hydrogen sulphide was removed with nitrogen and determined are depicted in Table 6A, 6B and Fig. 18A, 18B. In Fig. 18A the fate of the sulphur during the degradation is shown.

Table 6A. Amount of hydrogen sulphide evolved during cephalexin degradation at pH 7.4 and 37°C

Time (hours)	Amount of H_2S (Moles x 10^5)	Time (hours)	Amount of H_2S (Moles x 10^5)
2.6	0.05	68.5	1.0
5.5	0.10	76.5	1.02
9.5	0.15	93.5	1.17
22.5	0.40	102.0	1.20
31.5	0.55	119.0	1.28
46.0	0.75	142.0	1.36
51.5	0.85	238.0	1.4

Table 6B. Results obtained for cephalexin degradation in phosphate buffer at pH 7.4 at 37°C in the absence of molecular oxygen and hydrogen sulphide

Time (hours)	Amount of DKP II (Moles x 10^{5})	Peak heights (μ A) at -1.26V
1.5	0.4	0.0
3.5	1.03	0.25
5.25	1.46	0.35
6.25	1.83	0.40
7.08	1.94	0.50
7.75	2.07	0.70
8.16	2.11	1.0
8.66	2.10	1.0
22.16	1.91	1.6
30.16	1.59	2.2
46.0	1.08	2.2
51.5	0.93	2.3
68.5	0.65	2.6
76.5	0.53	2.8
93.5	0.43	2.7
102.0	0.38	2.7
119.0	0.28	2.7
142.0	0.14	2.6
238.0	0.01	1.7

After 240 hours DKP II had degraded completely and about 50% of the total sulphur had been evolved as hydrogen sulphide. Apparently the remaining sulphur remains in the solution in an electroinactive form, possibly as the thiolactone identified by

Dinner[92];supporting evidence for this thiolactone was provided by t.l.c. results which showed the same R_f values as those obtained by Bundgaard[93] and Dinner[92]. In Fig. 18B, the height of the peak due to DKP II and the peak at -1.26V are compared throughout the degradation. A small peak appeared at -0.96V after about 140 hours indicating the formation of some 2-hydroxy-3-phenyl-6-methyl-pyrazine. D.p. polarograms showing the formation of DKP II and its subsequent degradation at 37°C and pH 7.4 in the absence of hydrogen sulphide and molecular oxygen are shown in Fig. 19A and 19B.

The rate constant for the degradation of DKP II (37°C, pH 7.4) based on the height of its polarographic peak changes after 80 hours from 0.021 h^{-1} to 0.012 h^{-1}. This is illustrated in Fig. 20.

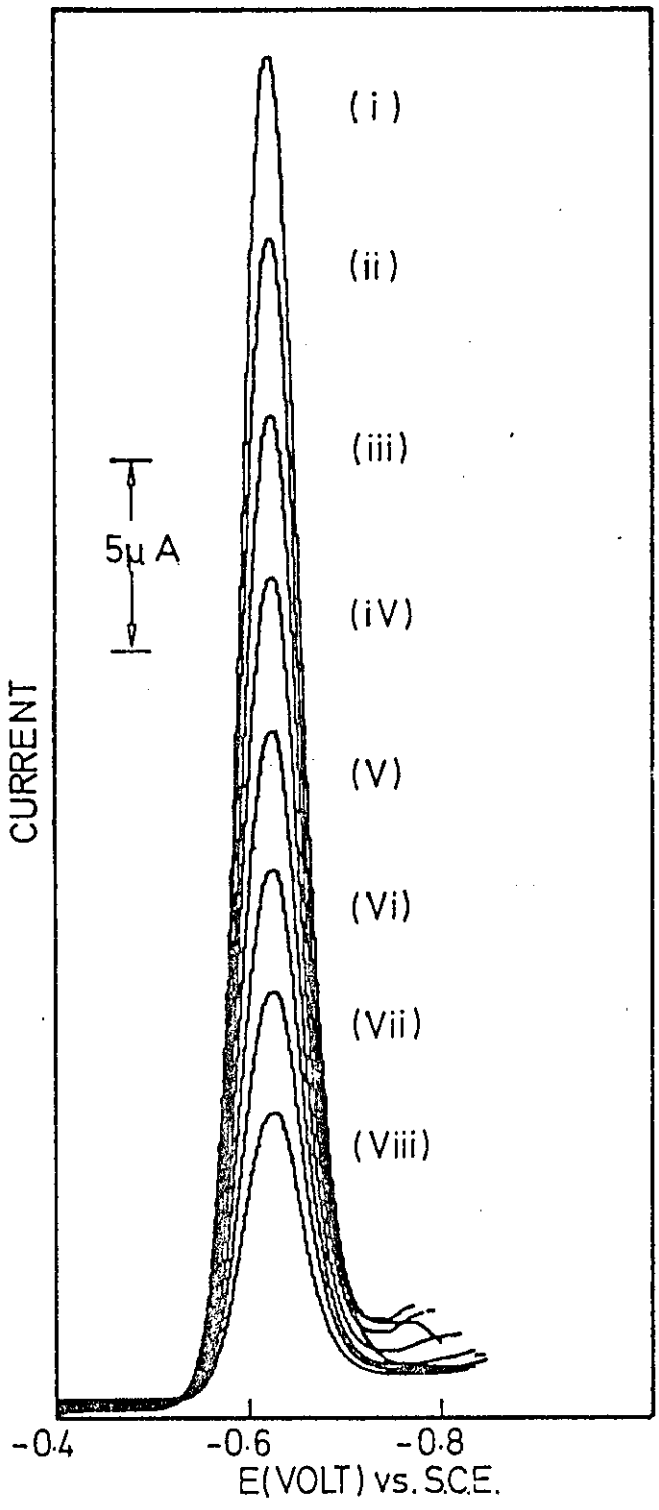


Fig. 16. D.p. polarograms obtained for cadmium in citrate buffer (pH 4.6) after successive syringe injections of sodium sulphide standard solution
Sodium sulphide concentration (i) 0, (ii) 4.6, (iii) 9, (iv) 13, (v) 17, (vi) 21, (vii) 24.7 and (viii) 28 x 10^{-5}M

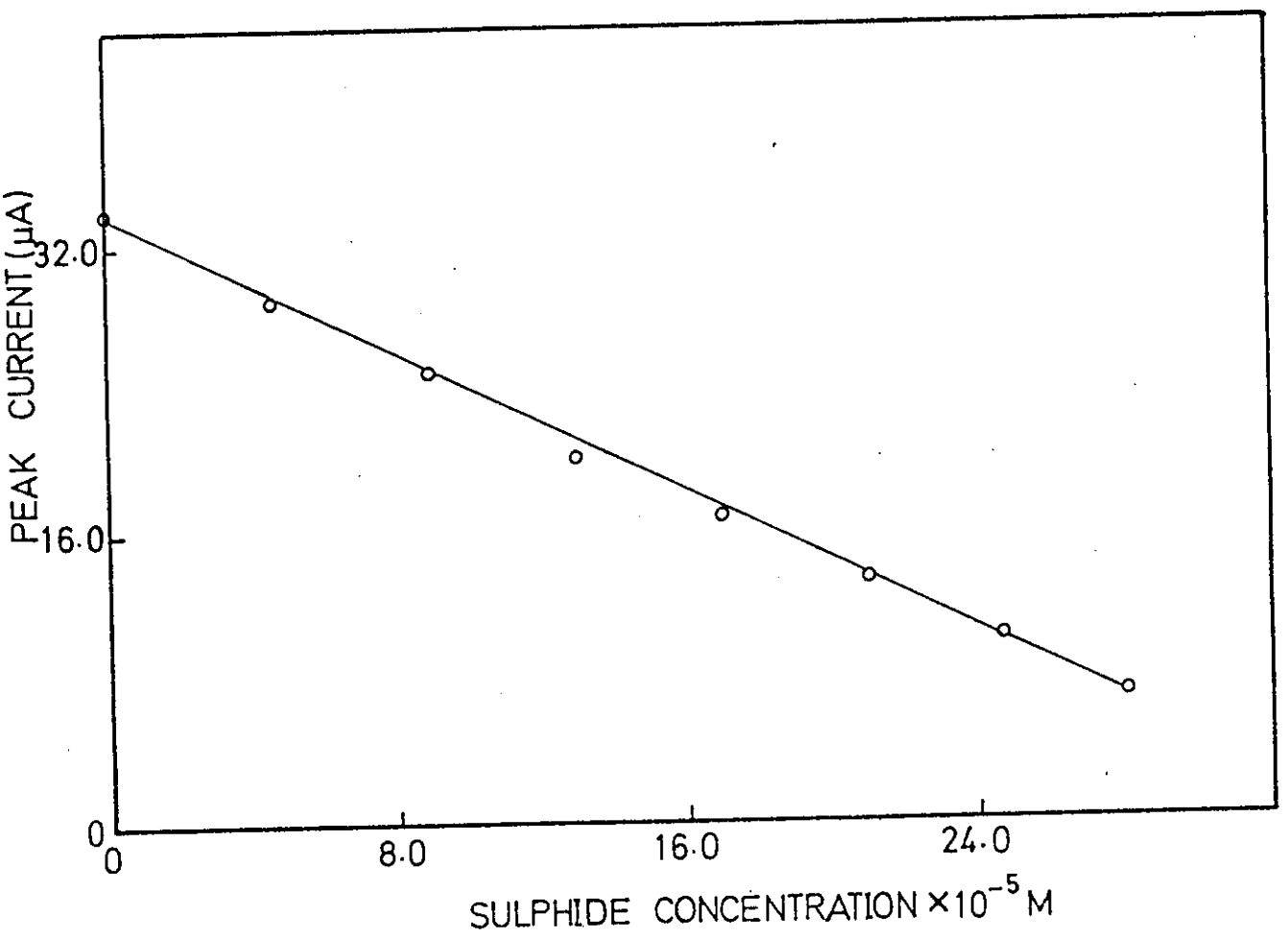


Fig. 17. Calibration graph obtained by the indirect d.p.p. method for the determination of sulphide.

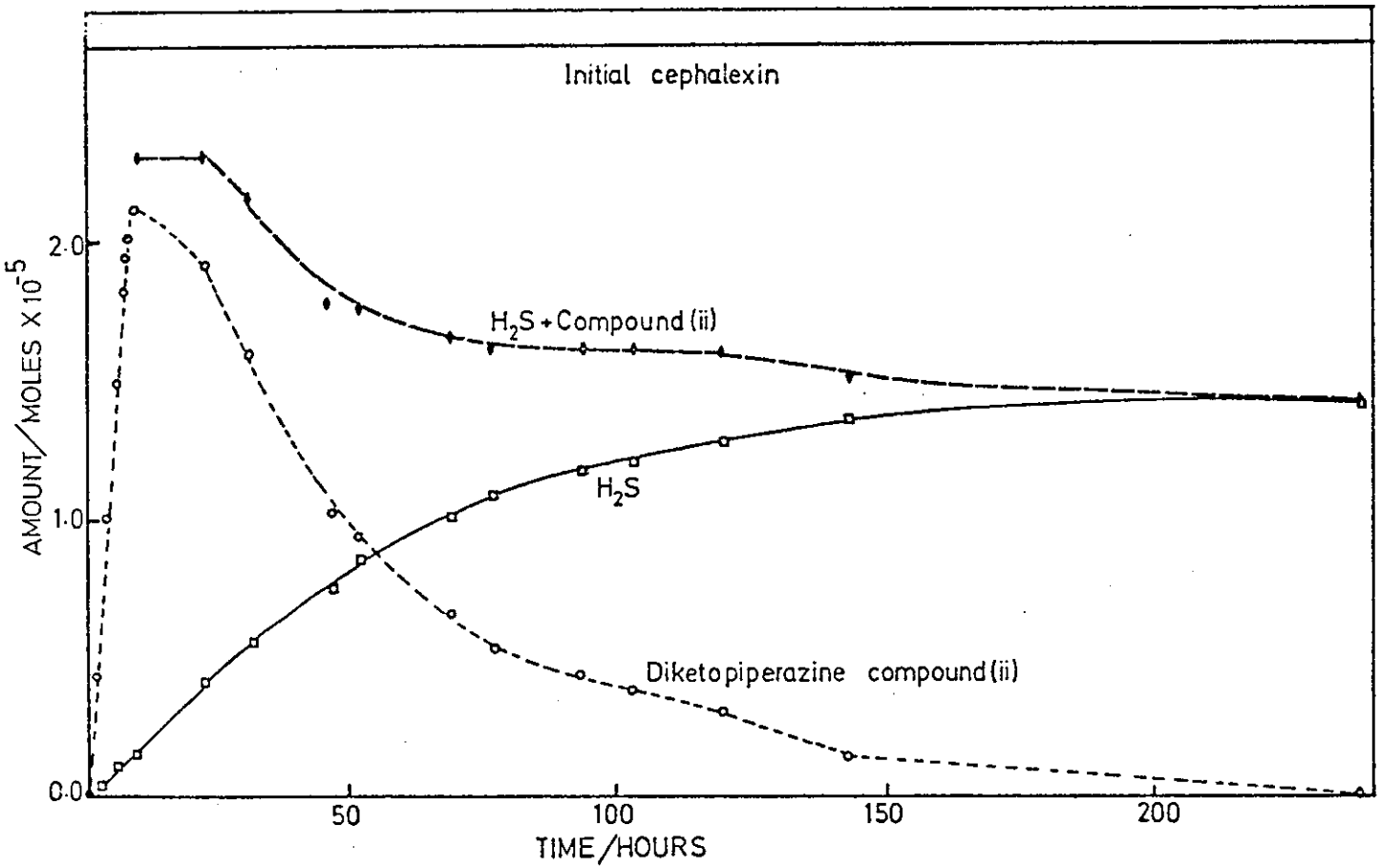


Fig. 18A. Amounts of electroactive sulphur-containing compounds formed during the degradation of cephalexin in phosphate buffer at pH 7.4 and 37°C. Initial cephalexin concentration 100µg/ml

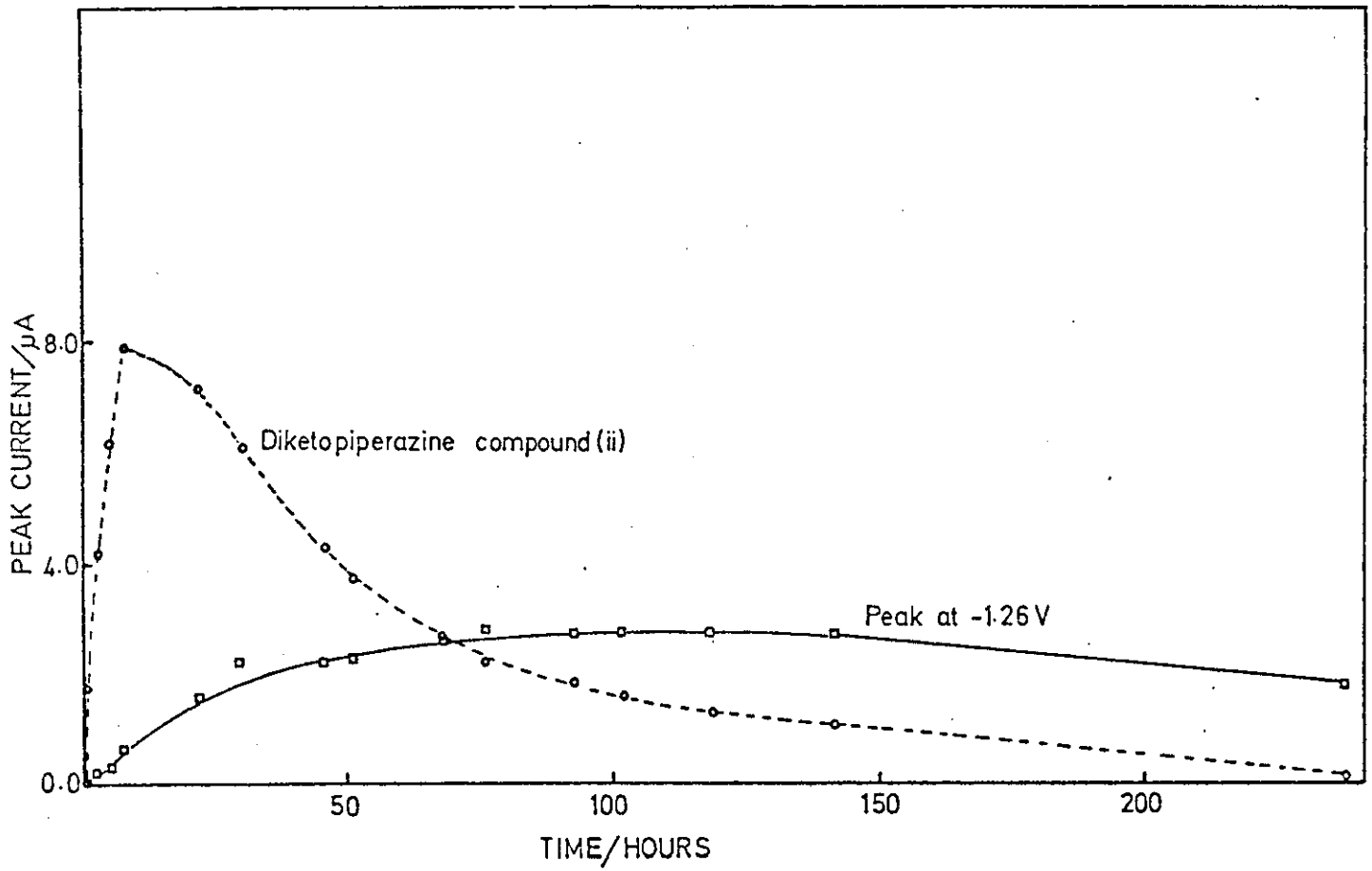


Fig. 18B. Comparison of peak currents of DKP II and the compound responsible for the peak at -1.26V obtained during the degradation of 100μg/ml cephalexin in phosphate buffer at pH 7.4 at 37°C

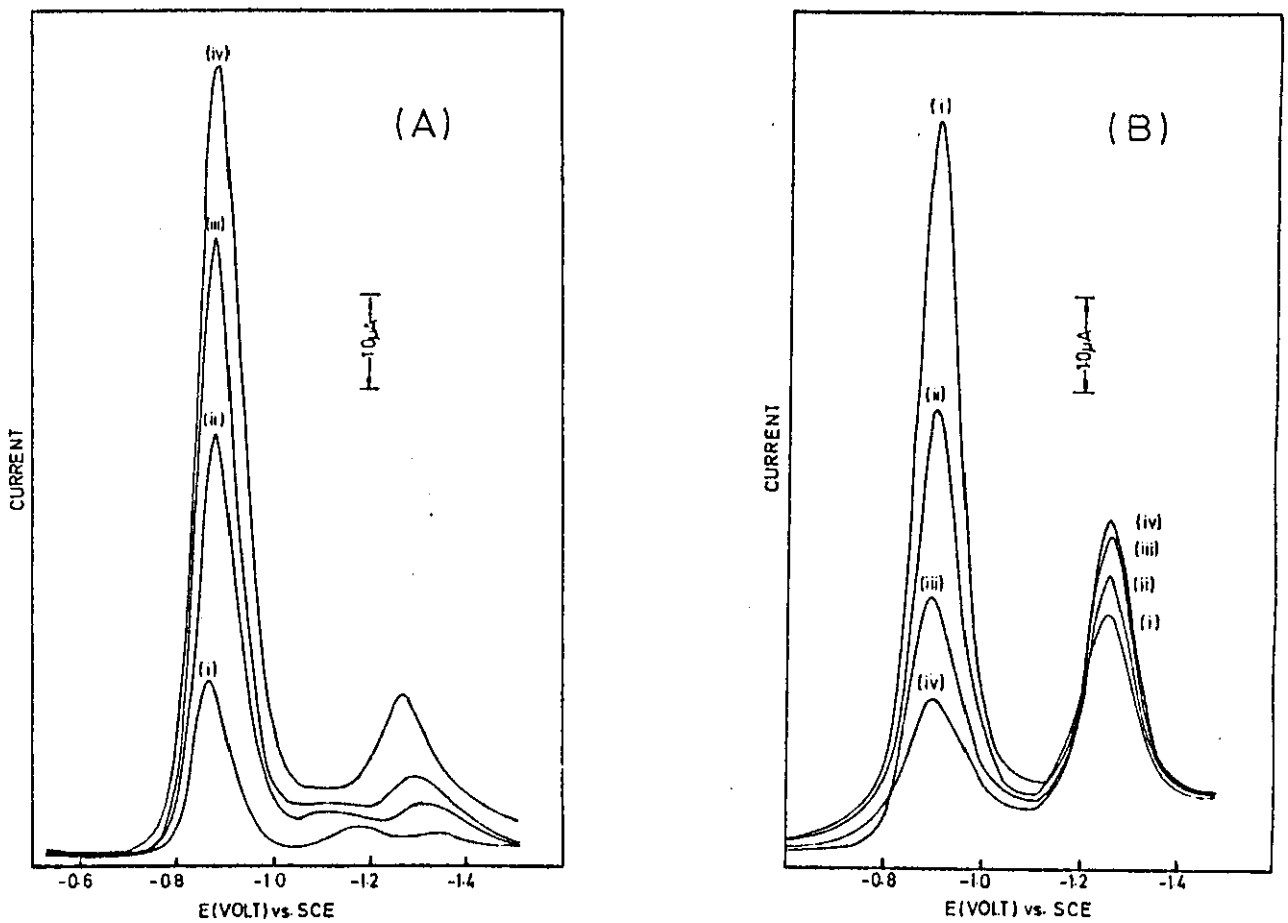


Fig. 19. D.p. polarograms of the degradation of cephalexin showing the formation and degradation of the diketopiperazine derivative (II) at 37°C and pH 7.4

Time: (A); (i) 1.5, (ii) 3.5, (iii) 5.25 (iv) 8.2 hours

(B); (i) 22.2 , (ii) 46.6, (iii) 76.5, (iv) 119 hours

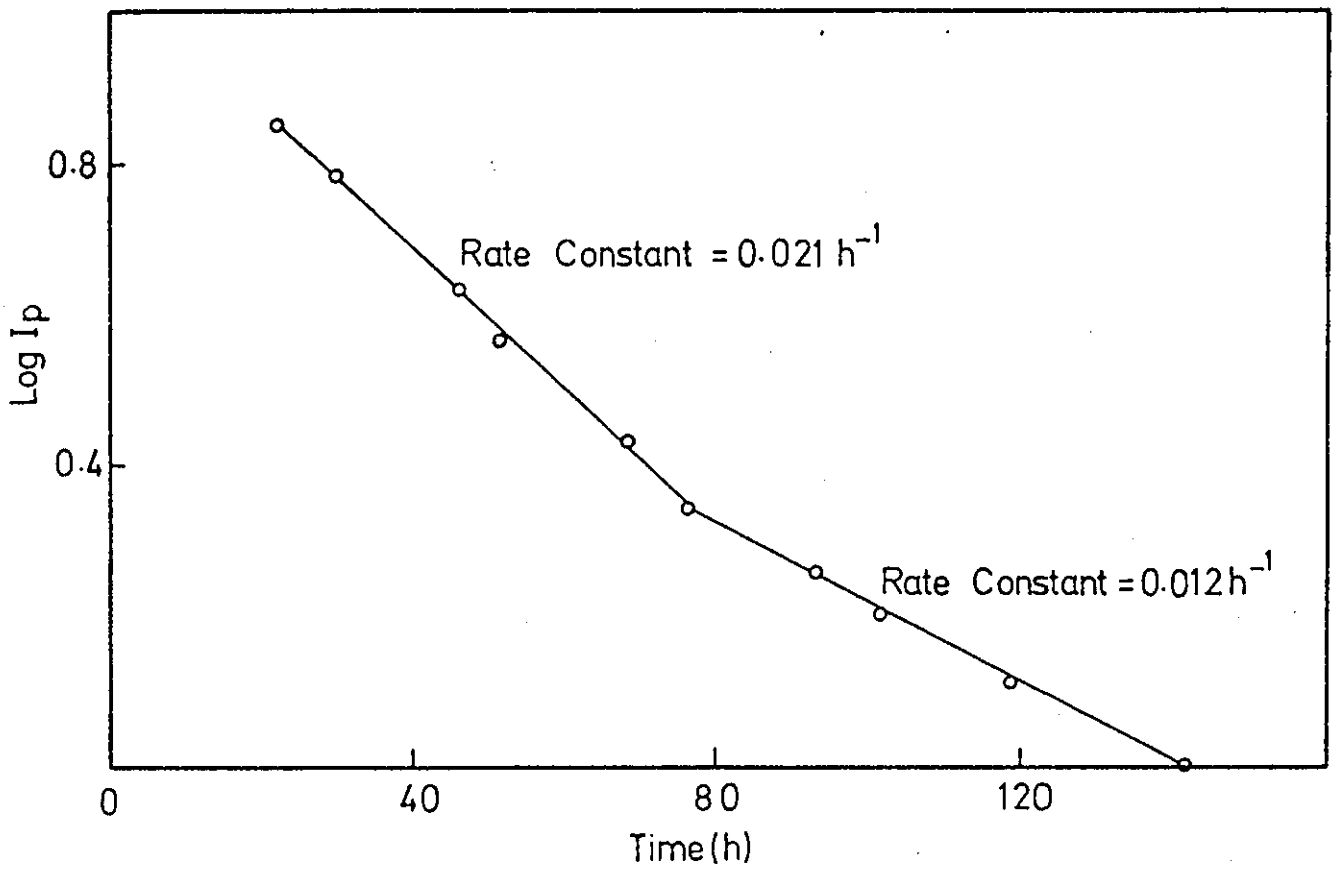


Fig. 20. Plot of logarithm of peak heights (μA) of DKP II versus time (hours)

The effect of flushing with nitrogen on the degradation of cephalexin at 80°C and pH 7.4 was similar. Only three main peaks were observed (-0.9V, -0.96V and -1.26V). Production and degradation of DKP II was rapid. Comparison of peak currents is shown in Fig. 21A. Hydrogen sulphide production was rapid initially (25% recovery of total sulphur after 3 hours), but then slowed down considerably (see Fig. 21B).

The effect of raising the pH of the degradation solution slightly was marked. Results obtained for 100µg/ml cephalexin solutions at pH 8.5 (phosphate buffer) and 80°C are shown in Fig. 22A and 22B. A high proportion of the total sulphur (56%, 78%) was eliminated as hydrogen sulphide within 6 hours and 29 hours respectively. Another significant change in the degradation pattern was the large increase in the yield of 2-hydroxy-3-phenyl-6-methylpyrazine. A small new peak at -1.08V appeared early in the degradation but the compound responsible had degraded completely after 21 hours. D.p. polarograms showing the formation of 2-hydroxy-3-phenyl-6-methylpyrazine in pH 8.5 phosphate buffer and 80°C are shown in Fig. 23.

The effect of pH on the degradation of cephalexin was studied next. When the degradation was carried out in phosphate buffer at pH 10 and 80°C, large peaks appeared at -1.08V and -1.26V after 45 minutes, in addition to small peaks at -0.56V (hydrogen sulphide), -0.96V (2-hydroxy-3-phenyl-6-methylpyrazine) and -0.62V (unidentified peak). With increased heating time the peak at -1.08 became smaller, whereas those at -0.62V, -0.96V and -1.26V increased. The hydrogen

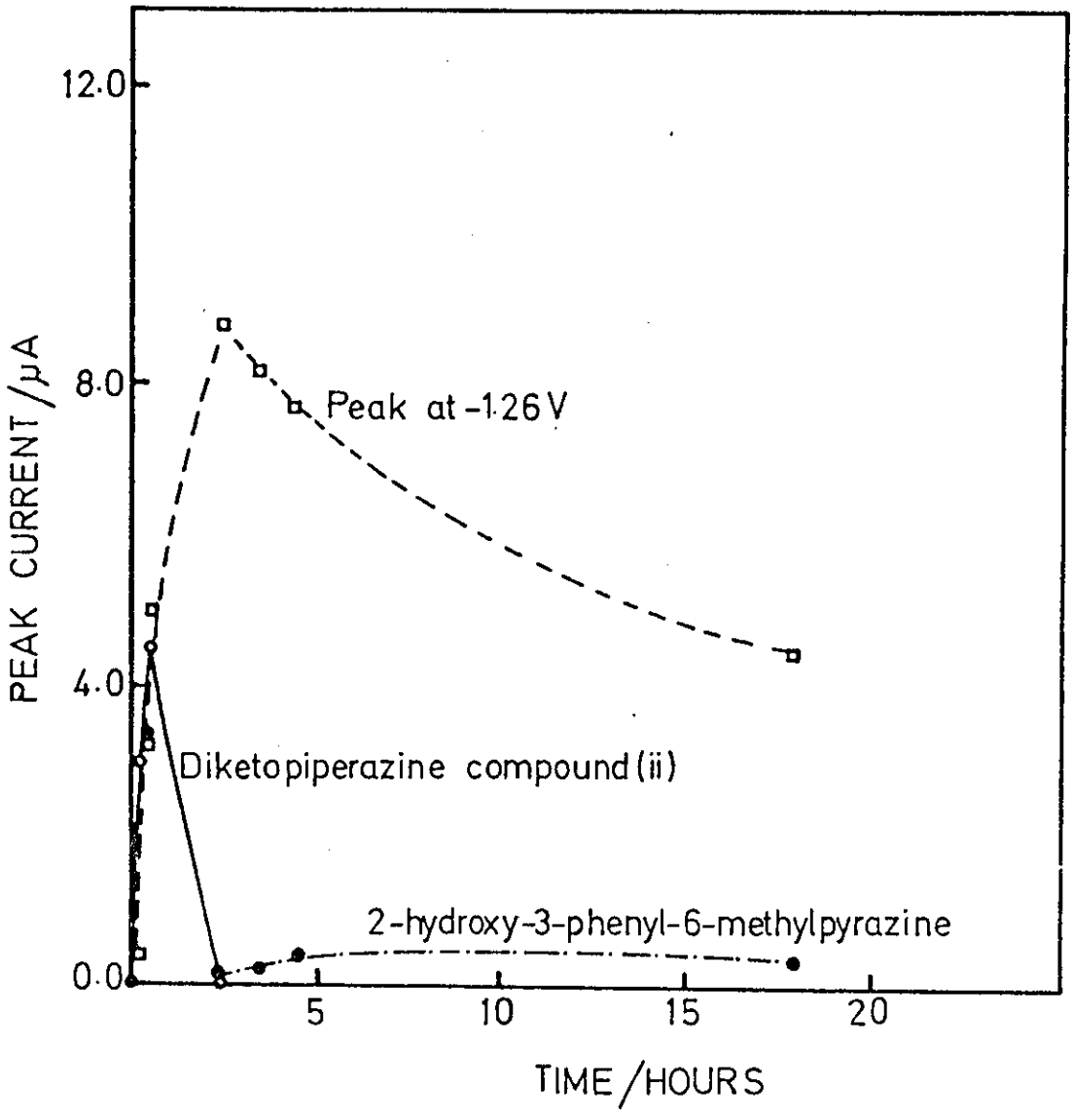


Fig. 21A. Comparison of peak currents of cephalexin degradation products formed in phosphate buffer at pH 7.4 and 80°C

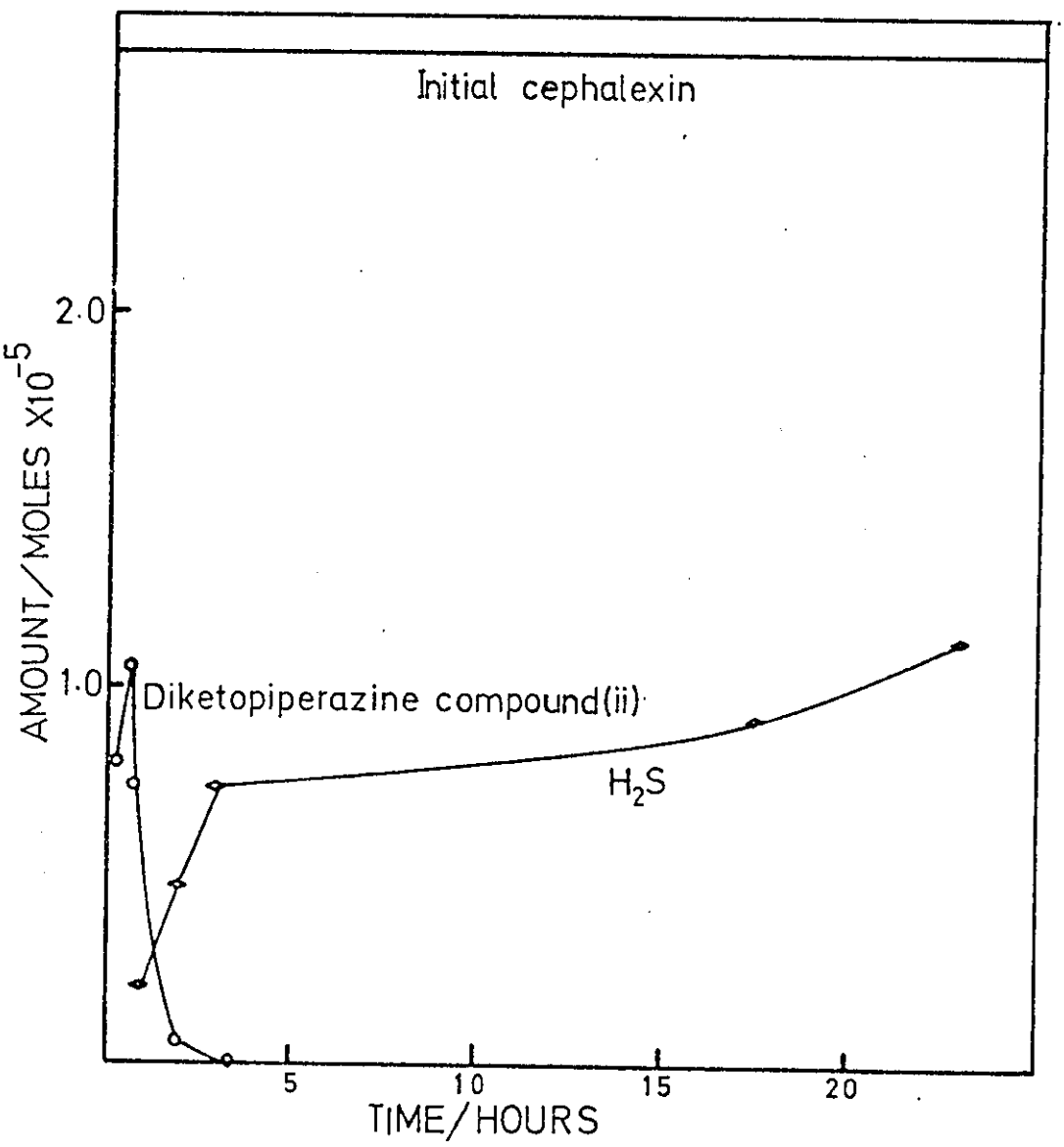


Fig. 21B. Amounts of sulphur containing compounds formed during cephalexin degradation at pH 7.4 at 80°C

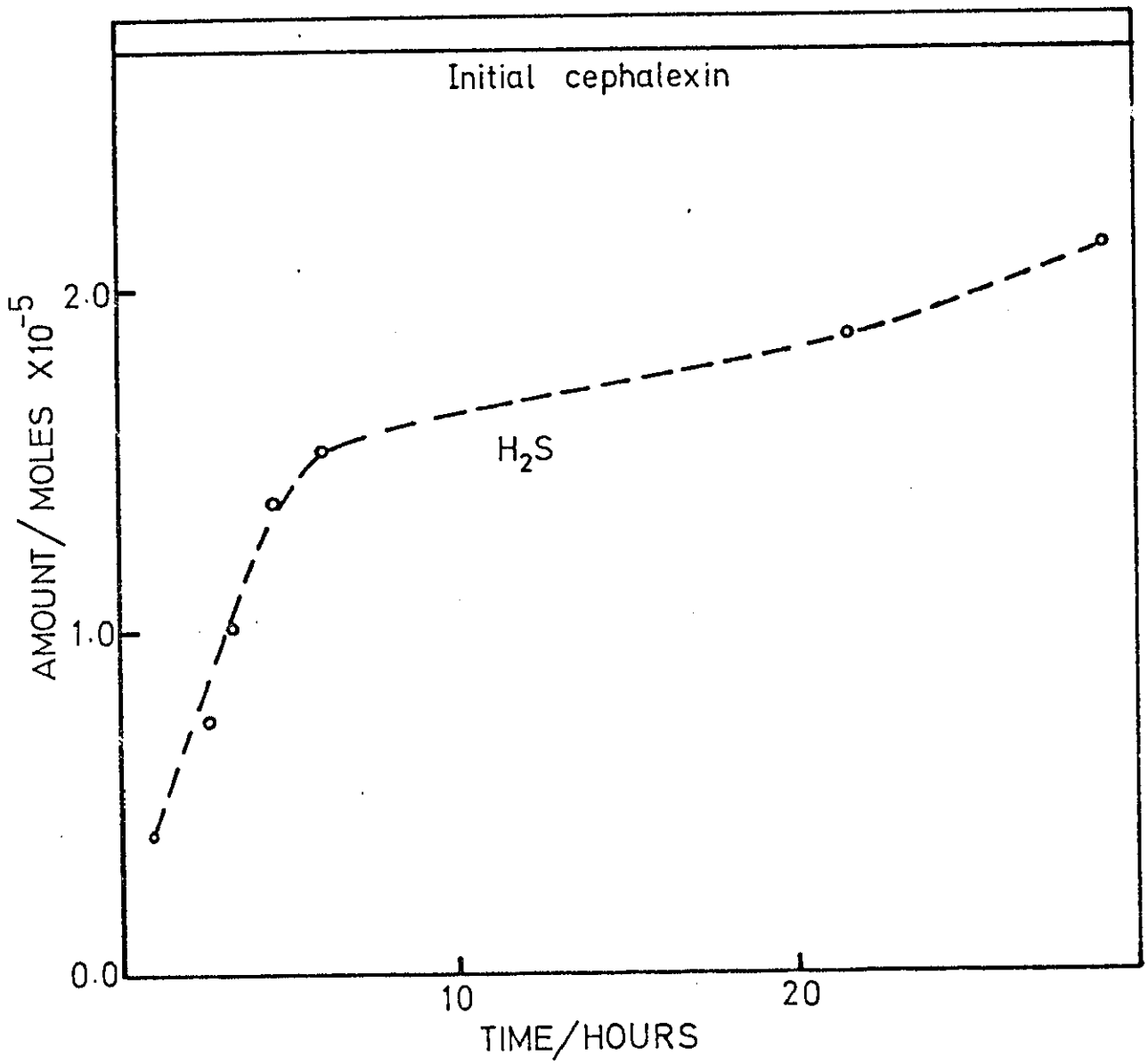


Fig. 22A. Amount of hydrogen sulphide evolved during the degradation of 100µg/ml cephalexin in phosphate buffer at pH 8.5 and 80°C

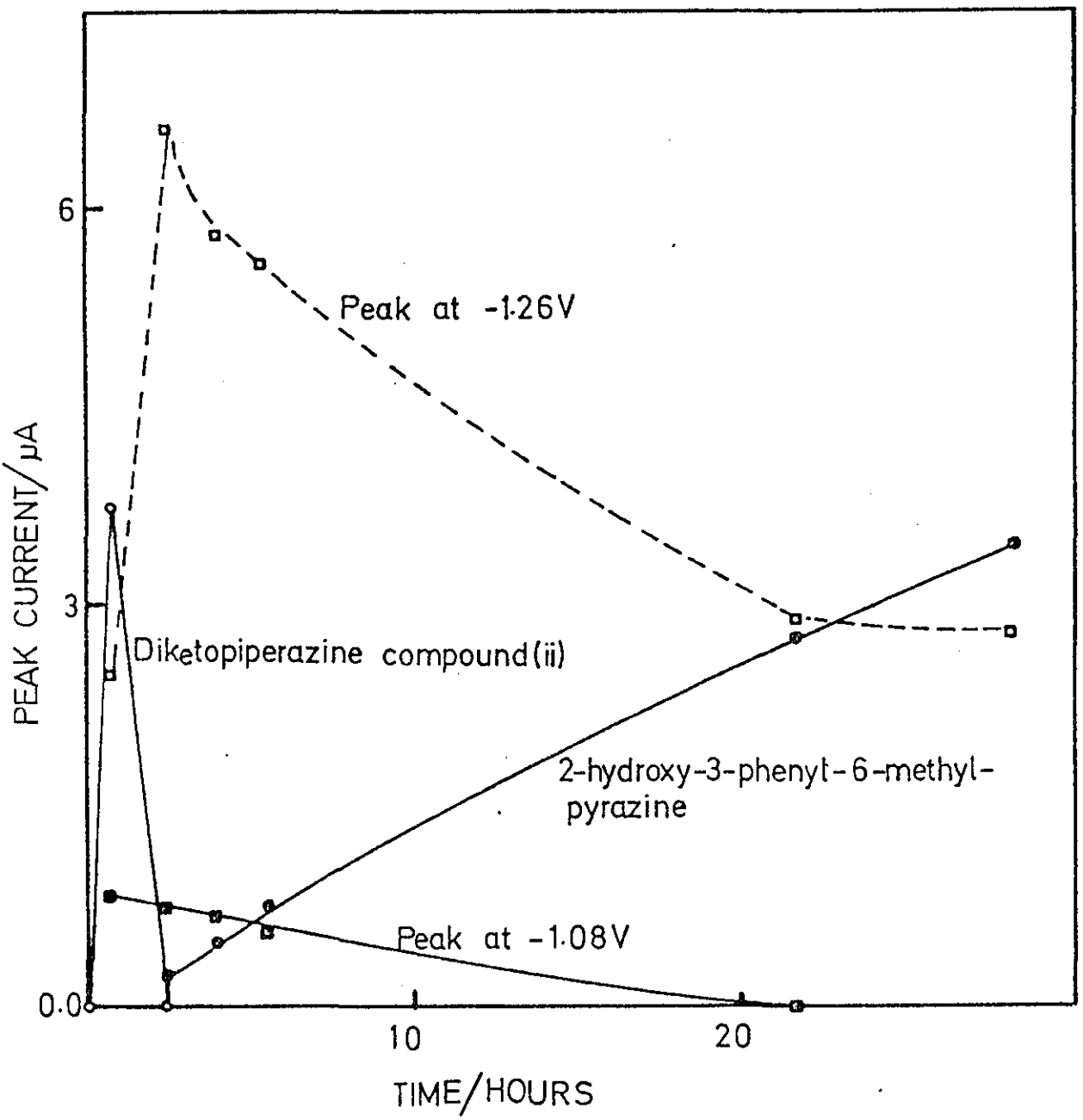


Fig. 22B. Comparison of peak currents of cephalexin degradation products formed in phosphate buffer at pH 8.5 at 80°C

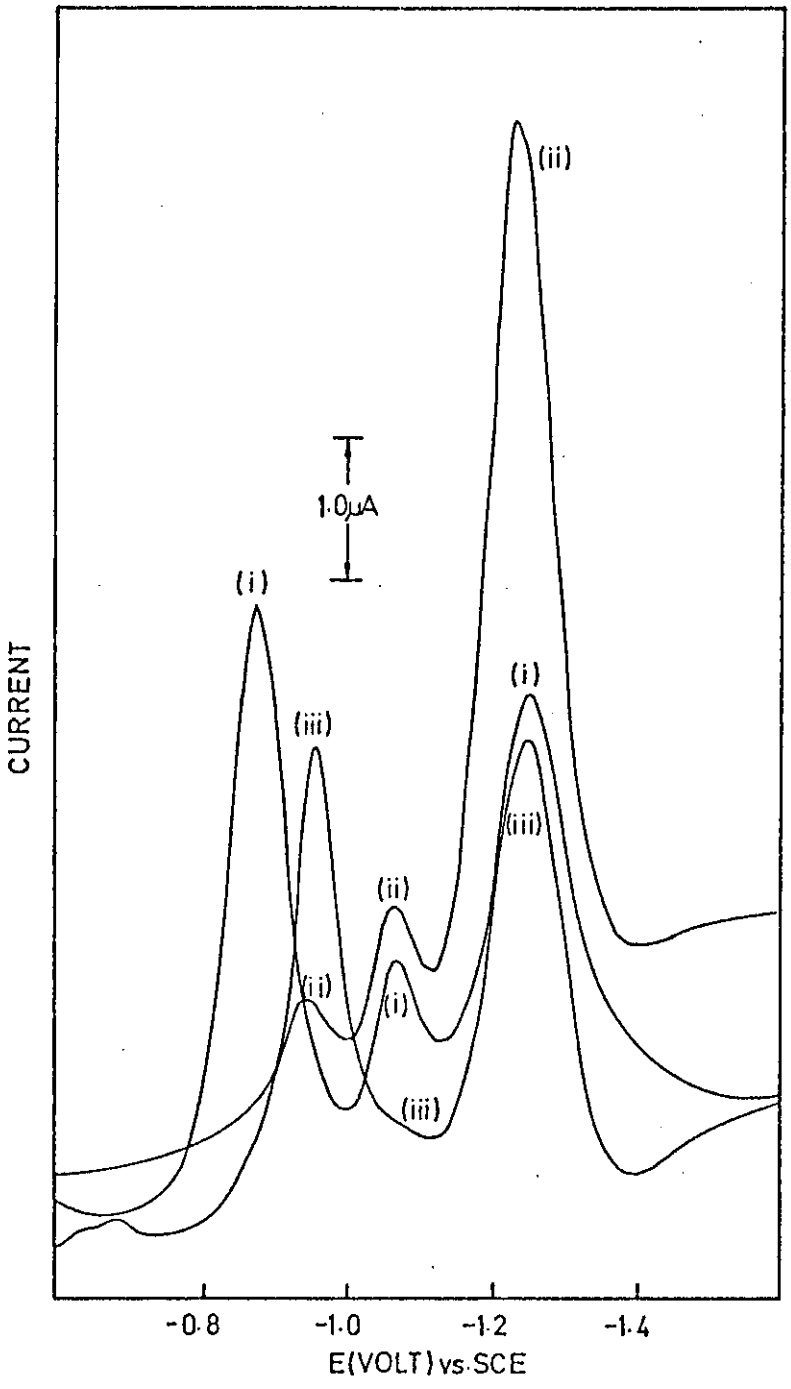


Fig. 23. Degradation of cephalexin at 80°C and pH 8.5 showing the formation of 2-hydroxy-3-phenyl-6-methylpyrazine.
Time: (i) 0.66, (ii) 4.0, (iii) 21.8 hours

sulphide was more difficult to purge in this case and incomplete removal of the gas was evident from the persistence of the peak at -0.56V in polarograms of the degraded solutions. However,the amount of hydrogen sulphide (driven out with nitrogen gas and measured) within the first 5 hours was 10%. When the degradation was carried out under the same experimental conditions but in borate buffer solution at pH 10, the same polarographic pattern was obtained except that the peak at -1.26V disappeared and was replaced by a peak at -1.33V Fig. 24.

Degradation was also studied in distilled water without buffering. The pH of the 100 µg/ml cephalexin solution was 5.7. Hydrogen sulphide was obtained in relatively low yield at 80°C (about 24% of the total sulphur after 30 hours). In addition to the DKP II peak at -0.9V, and 2-hydroxy-3-phenyl-6-methylpyrazine peak at -0.96V, peaks at -0.36V, -1.08V and -1.44V observed under other conditions were again observed. The DKP II peak at -0.9V was found to disappear after 4 hours, while the compound responsible for the peak at -1.08V degraded at a slower rate. The compounds responsible for peaks at -0.36V and -1.44V were major products in degradation at high concentration (10mg/ml).

Degradation in citrate-phosphate buffer solution at pH 3 and 80°C showed a similar polarographic pattern to that in unbuffered solution at pH 5.7, except that the peak at -1.08V was absent. The yield of DKP II and hydrogen sulphide after 4 hours were only 8% and 5% respectively.

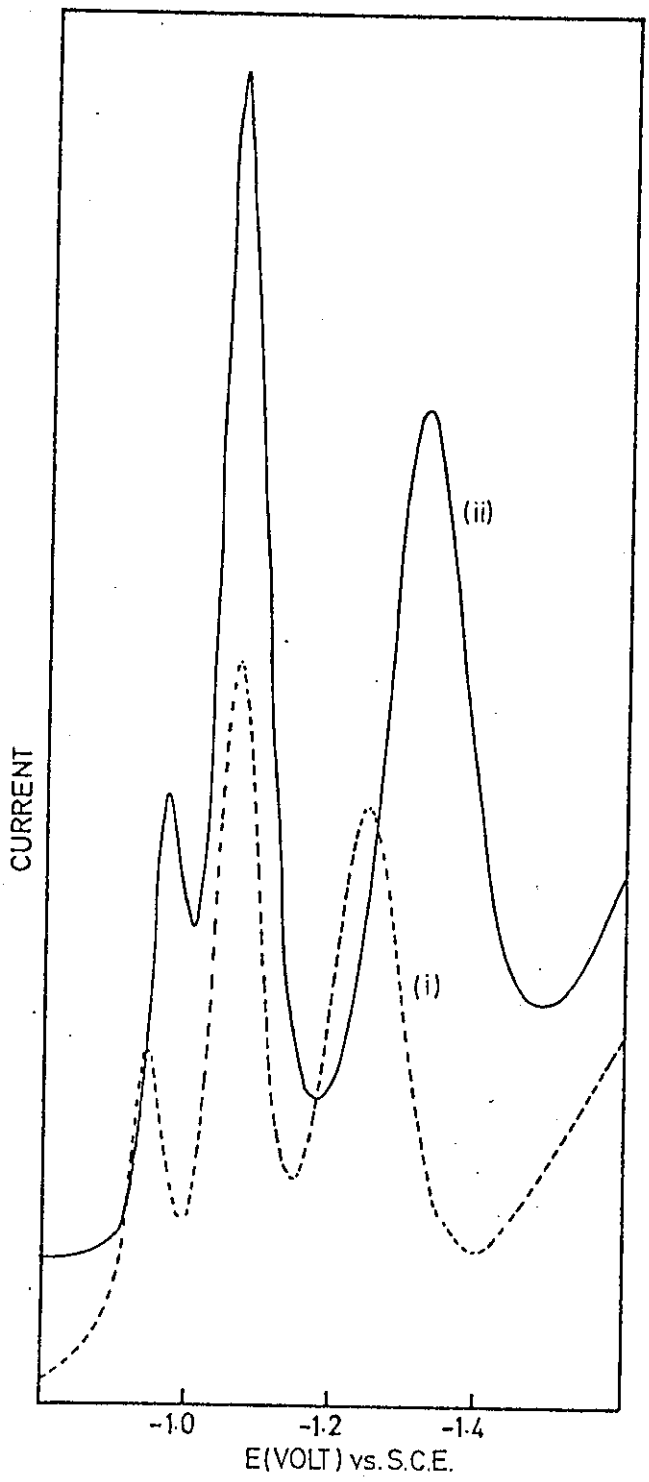


Fig. 24. Comparison of cephalexin degradation pattern at pH 10 and 80°C (i) phosphate buffer, (ii) borate buffer

When the degradation of cephalexin was carried out in 0.01M sodium hydroxide solution at 25°C, in addition to small peaks at -0.13V, -0.36V, -0.56V and -0.62V, the peak of DKP II at -0.9V was observed which overlaps a peak at -0.8V later in the degradation. After 117 hours, when the degraded solution adjusted to pH 5 with citrate buffer and heated in a water bath for 20 minutes at 100°C, a well defined peak at -0.96V due to 2-hydroxy-3-phenyl-6-methylpyrazine was observed in addition to a very small peak at -0.56V.

D.p. polarographic peaks appearing during the degradation of cephalexin are summarised in Table 7.

An attempt was made to prepare a sample of the compound responsible for the peak at -1.26V by heating a concentrated cephalexin solution (1g in 50ml phosphate buffer pH 7.4) at 80°C for 6 hours whilst flushing the solution with nitrogen. Hydrogen sulphide was taken out in 20ml of 1M sodium hydroxide and its amount was determined by the potentiometric method previously discussed. The grey-white precipitate that formed was filtered off, washed with distilled water, dried in an oven at 50°C for 3 hours and then weighed. The identity of this compound, which was identified by Bundgaard[93] as 3-aminomethylene-6-phenyl-piperazine-2,5-dione IV, was confirmed by UV, IR and mass spectrometry. The product had the following characteristics: UV(water-ethanol 99:1), λ_{max} 288nm, IR(kBr) ν_{max}: 3400-2900, 1700, 1640, 1595, 1510, 1450, 1350 and 1300 cm^{-1}, m/e 219, important fragment ions, 217, 189, 188, 171, 132, 118, 112, 106, 91, 85, 79, 77, 69, 63, 57, 55, 51, 41, 39, 30, 29, 16, 14. The products had a melting point higher than 300°C. The yield of this compound and hydrogen sulphide was 57% and 58% respectively. No

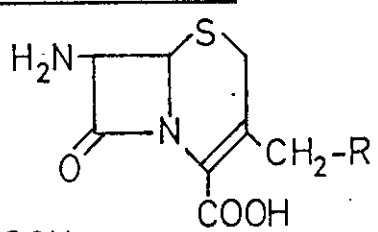
Table 7. Differential pulse polarographic peaks appearing during the degradation of Cephalexin

Peak potential (V)	Remarks about compound responsible
-0.16	Formed in low yield at 37°C and in relatively higher yield at 80°C in pH 7.4 buffer; oxidation product, not formed in absence of oxygen.
-0.22	Relatively minor product formed late in the degradation at high cephalexin level
-0.36	Major product formed early in distilled water at 80°C (pH 5.7). Formed also in high yield at high cephalexin concentration in distilled water at 75°C.
-0.56	Hydrogen sulphide
-0.78	Oxidation product, not formed in absence of oxygen
-0.84	As for peak at -0.22V
-0.90	Diketopiperazine derivative (II): first major degradation product formed.
-0.96	2-hydroxy-3-phenyl-6-methylpyrazine(VI). Forms late in degradation usually but good yields obtained in pH 8.5
-1.08	High yield at pH 10 (80°C) degrades later
-1.26	High yield at 80°C(pH 7.4 and 8.5).
-1.33	Associated with appearance of peak at -1.08V in borate buffer only at pH 10: seems to appear when peak at -1.26V is absent
-1.44	High yield at 80°C (pH 7.4), also at high cephalexin concentration (37°C) as the -1.26V peak is absent, and in unbuffered solution (pH 5.7).

polarographic peaks were observed for this compound in phosphate buffer at pH 7.4. (Due to the extremely low solubility of this compound, it was first dissolved in dimethyl sulphoxide, then diluted with the buffer). Also, peaks were not observed for 3-formyl-3,6-dihydro-6-phenyl-2,5(1H,4H)-pyrazinedione V which is supposed to be formed after acid hydrolysis of compound IV (see Scheme 10).

The filtrate obtained after removing compound IV showed polarographic peaks at -0.36V (0.25μA), -0.56V (0.2μA), -0.96V (0.7 μA) and -1.26V (2.6 μA). Attempts to extract the compound responsible for the peak at -1.26V using chloroform, isoamyl alcohol,ethyl acetate and methylene chloride at various pH values were unsuccessful.

III Preliminary comparative degradation studies of 7-amino-cephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalos-poranic acid (7-ADCA).



7-ACA R=$OCOCH_3$

7-ADCA R= H

The degradation of these compounds has been studied in 0.1M sodium hydroxide solution at 25°C. The course of degradation was followed by d.p.p. and UV spectrophotometry.

7-ADCA which contains an unsubstituted methyl group at the 3-position is not reduced at the dropping mercury electrode, but

some of its degradation products give polarographic peaks. Two polarographic peaks were observed in B.R. buffer at pH 2.4. The first peak at -0.22V formed later in the degradation, whereas the second major peak at -0.42V formed rapidly after 7-ADCA degradation and attained its maximum value within two hours. The compound responsible for this peak degraded slowly with time. In Fig. 25 the changes of peak currents with time are shown. D.p. polarograms showing part of the degradation of 7-ADCA are shown in Fig. 26.

When the degradation was followed by UV spectrophotometry, it was found that the UV absorption band at 265nm due to the intact 7-ADCA disappeared after 110 minutes and a new absorption band at 223nm appeared. The intensity of this band increased with time up to 95 hours. Less than 1% of the total sulphur of 7-ADCA was evolved as hydrogen sulphide after 4.5 hours.

The degradation of 7-ACA appears to follow a different pathway. Marked differences in the polarographic and UV degradation patterns were observed in this case. The degradation of 7-ACA is more rapid than that of 7-ADCA. The degradation of 7-ACA indicated by the loss of the characteristic d.p. peak at -1.05V and the UV absorption band at 265nm was accompanied by the appearance of a new d.p. peak at -0.37V. This peak attains its maximum height within 90 min and then degrades at a faster rate than the 7-ADCA degradation product responsible for the peak at -0.42V. Further, a small peak at -0.22V was observed after the compound responsible for the peak at -0.37V had degraded. D.p. polarograms showing part of the

degradation of 7-ACA in sodium hydroxide solution are shown in Fig. 27. Peak currents of 7-ACA and its degradation products are shown in Fig. 28.

UV absorption measurements showed that the absorption band of 7-ACA had completely disappeared after 70 min degradation. This coincides with the disappearance of the 7-ACA d.p. peak at -1.05V. Also, an absorption band at 225nm which was observed in the early stages of degradation, was replaced by a more stable band at 240nm.

The amount of sulphur evolved as hydrogen sulphide after 4.5 hours was about 6% of the total sulphur present.

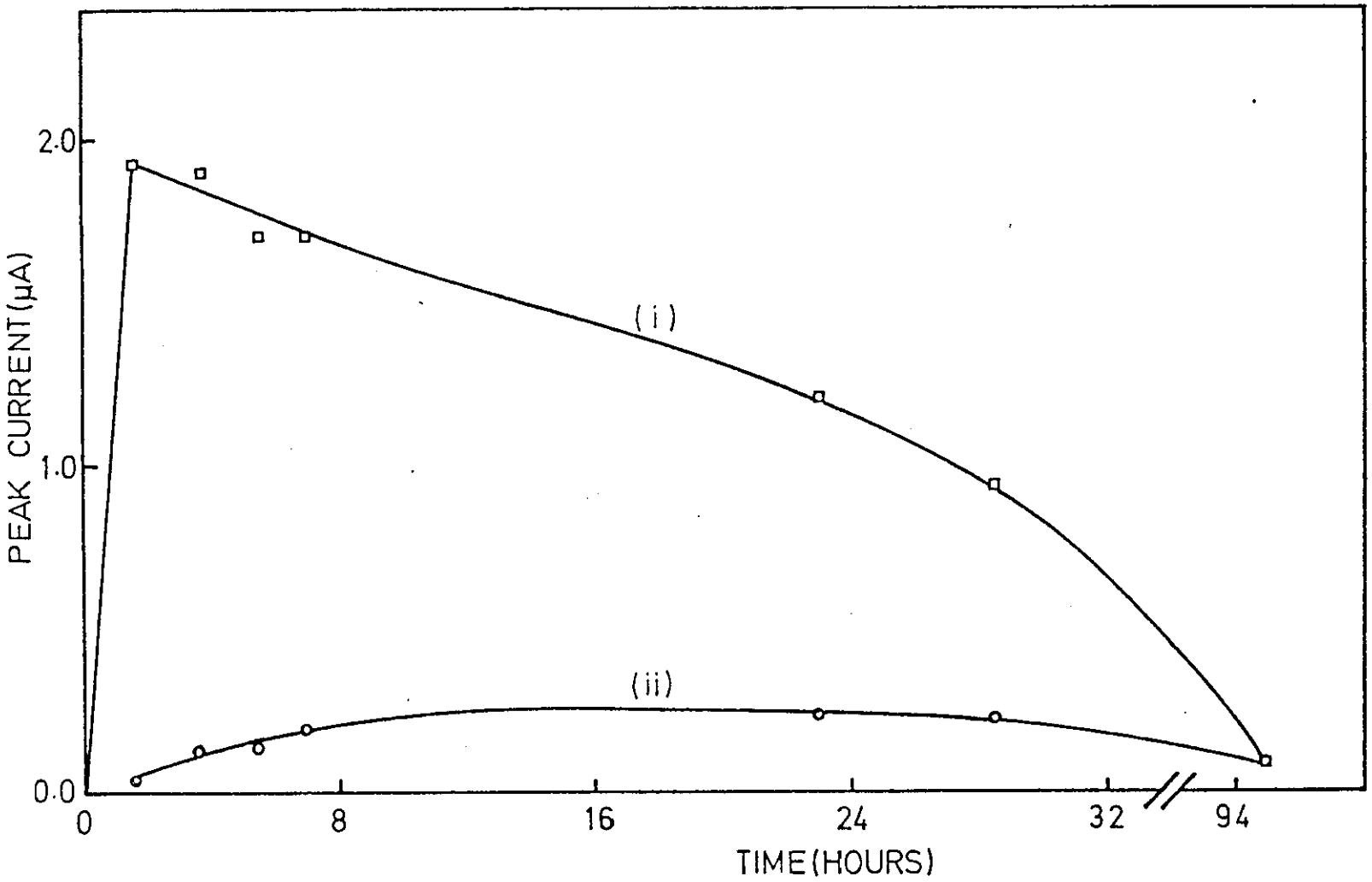


Fig. 25. Comparison pf peak currents of 7-ADCA degradation products.
(i) peak at -0.42V; (ii) peak at -0·22V

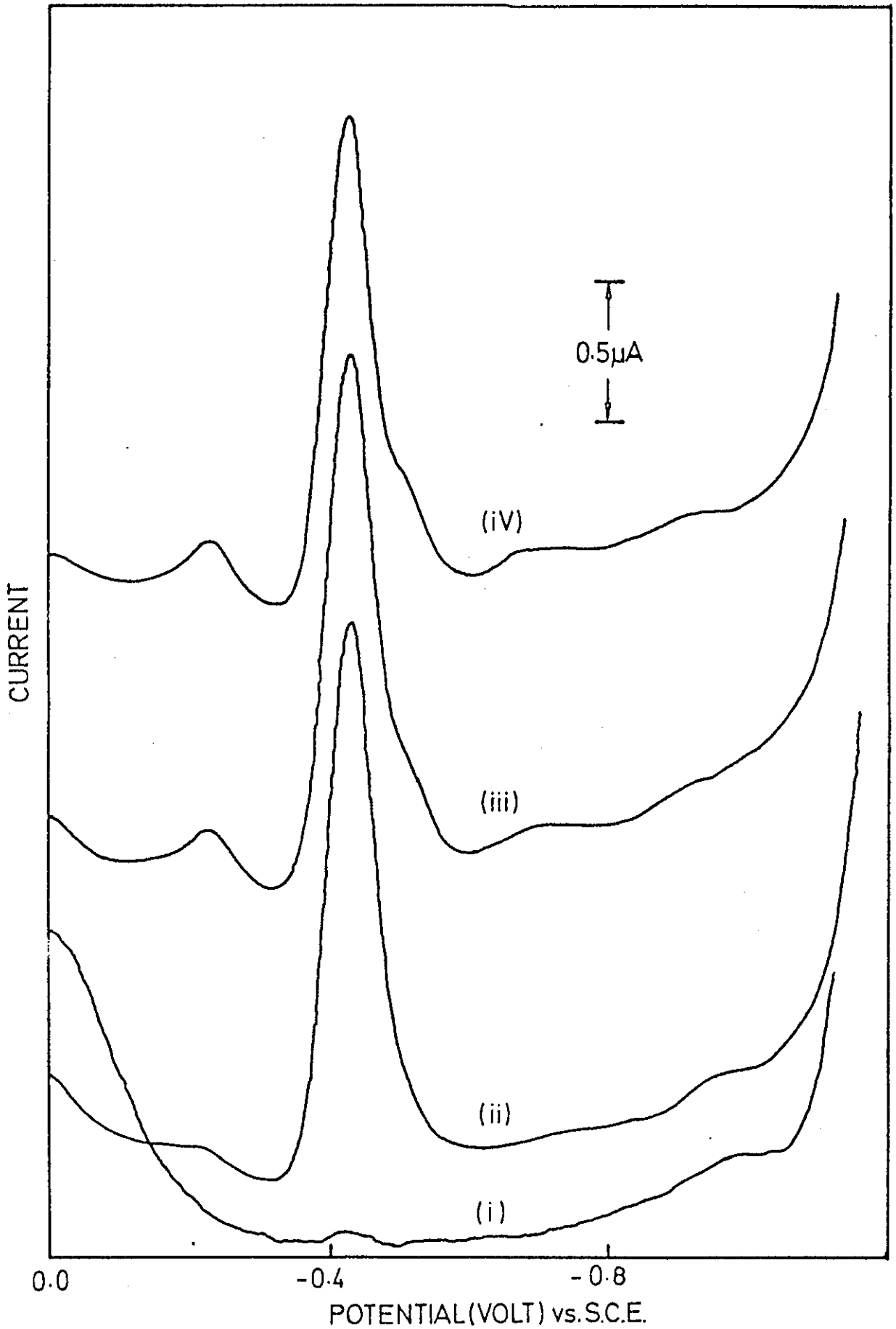


Fig. 26. D.p. polarograms showing part of the degradation of 7-ADCA in 0.1M sodium hydroxide at 25°C; polarograms obtained in B.R. buffer pH 2.4
Measurements time: (i) 0, (ii) 1.7; (iii) 3.7 (iv) 5.5 hours

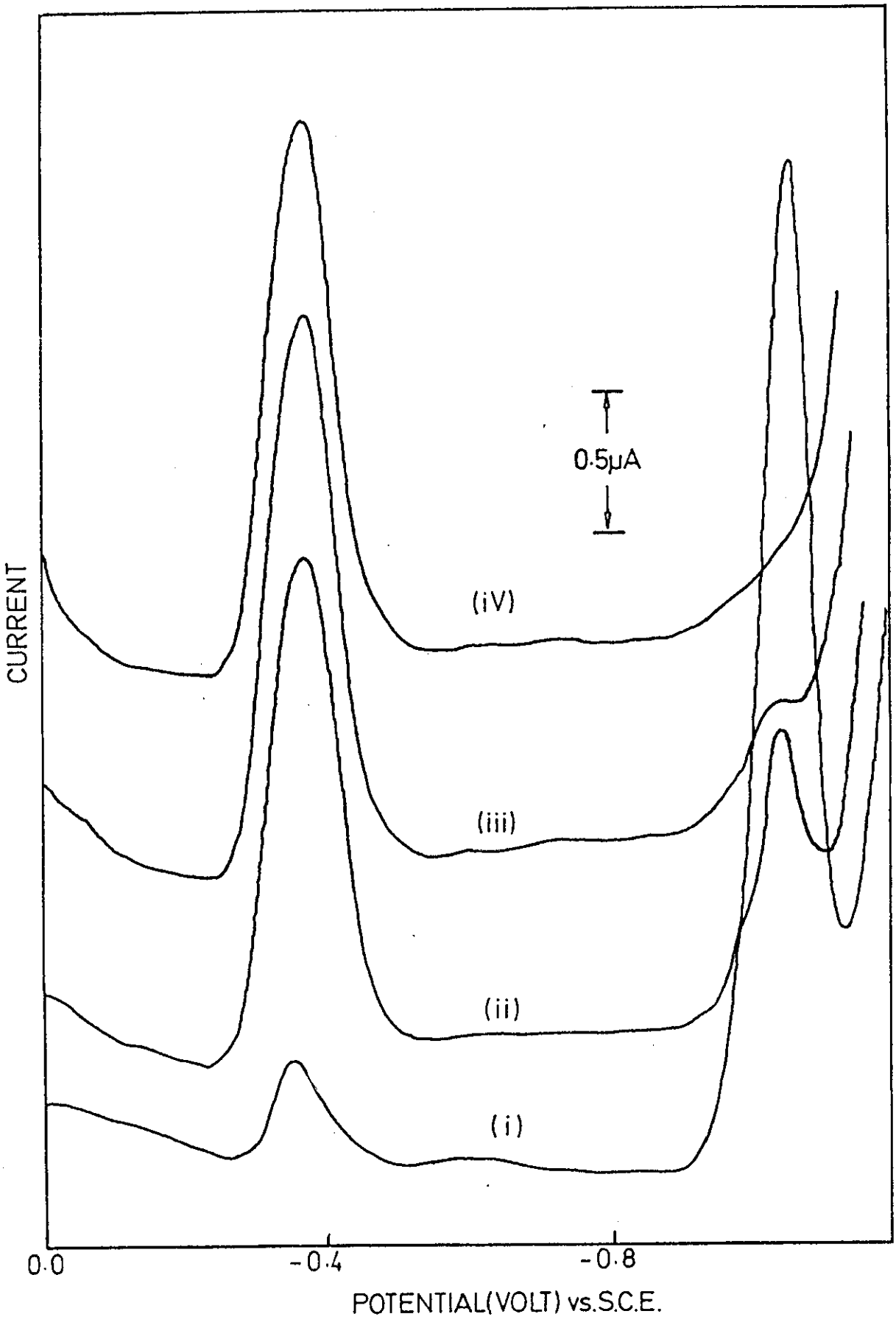


Fig. 27. D.p. polarograms showing part of the degradation of 7-ACA in 0.1M sodium hydroxide at 25°C; polarograms obtained in B.R. buffer pH 2.4.
Measurement time: (i) 0.08, (ii) 0.75, (iii) 1.2, (iv) 1.5hours

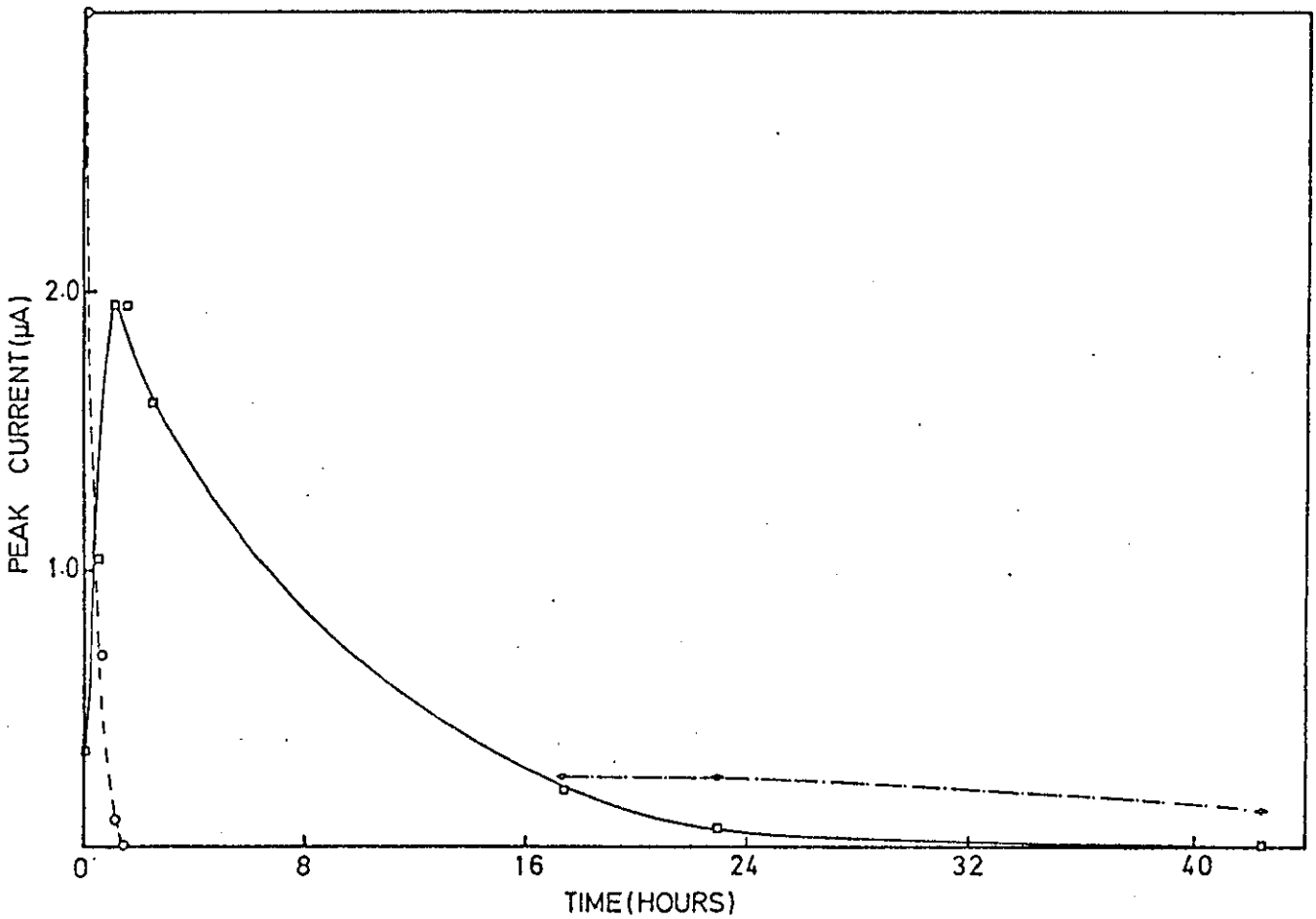


Fig. 28. Comparison of peak currents of 7-ACA and its degradation products

Key: □—□ peak at -0.37V; o----o peak at -1.05V; (7-ACA)

◇-·-◇ peak at -0.22V

C. D.P. POLAROGRAPHIC DETERMINATION OF CEPHALEXIN AFTER HYDROLYSIS IN NEUTRAL PHOSPHATE BUFFER SOLUTION

As was shown earlier, a high yield in solution of the compound responsible for the d.p. peak at -1.26V was obtained after cephalexin hydrolysis in phosphate buffer solution at pH 7.4 and 80°C. This peak has been made the basis of a sensitive and precise d.p. polarographic method for the determination of cephalexin.

The method involves heating cephalexin in pH 7.4 phosphate buffer solution at 100°C for 1 hour before recording the d.p. polarograms between -1.1 and -1.4V at 25°C. Typical d.p. polarograms obtained for cephalexin standards are shown in Fig. 29. The calibration graph obtained over the range 0.4 - 3 µg/ml of cephalexin in the measured solution was rectilinear and is shown in Fig. 30. The coefficient of variation (10 determinations) at the 2µg/ml level was 3.6%. Removal of dissolved oxygen from the solution before hydrolysing the cephalexin had no effect on the height of the peak obtained.

The heating time was found to affect the yield of the compound responsible for this peak, slightly, but negligible error occurs if the heating time is kept within 55 - 75 minutes. Peak heights obtained after 45 and 90 minutes heating were 10% and 5% low respectively. No change in the peak height, however, was observed when the degraded solution was kept at 25°C for 2.5 hours.

The degraded solution of cephalexin gave a positive test with 2,4-dinitrophenylhydrazine indicating the presence of a carbonyl compound. Further, the peak at -1.26V is decreased in steps on

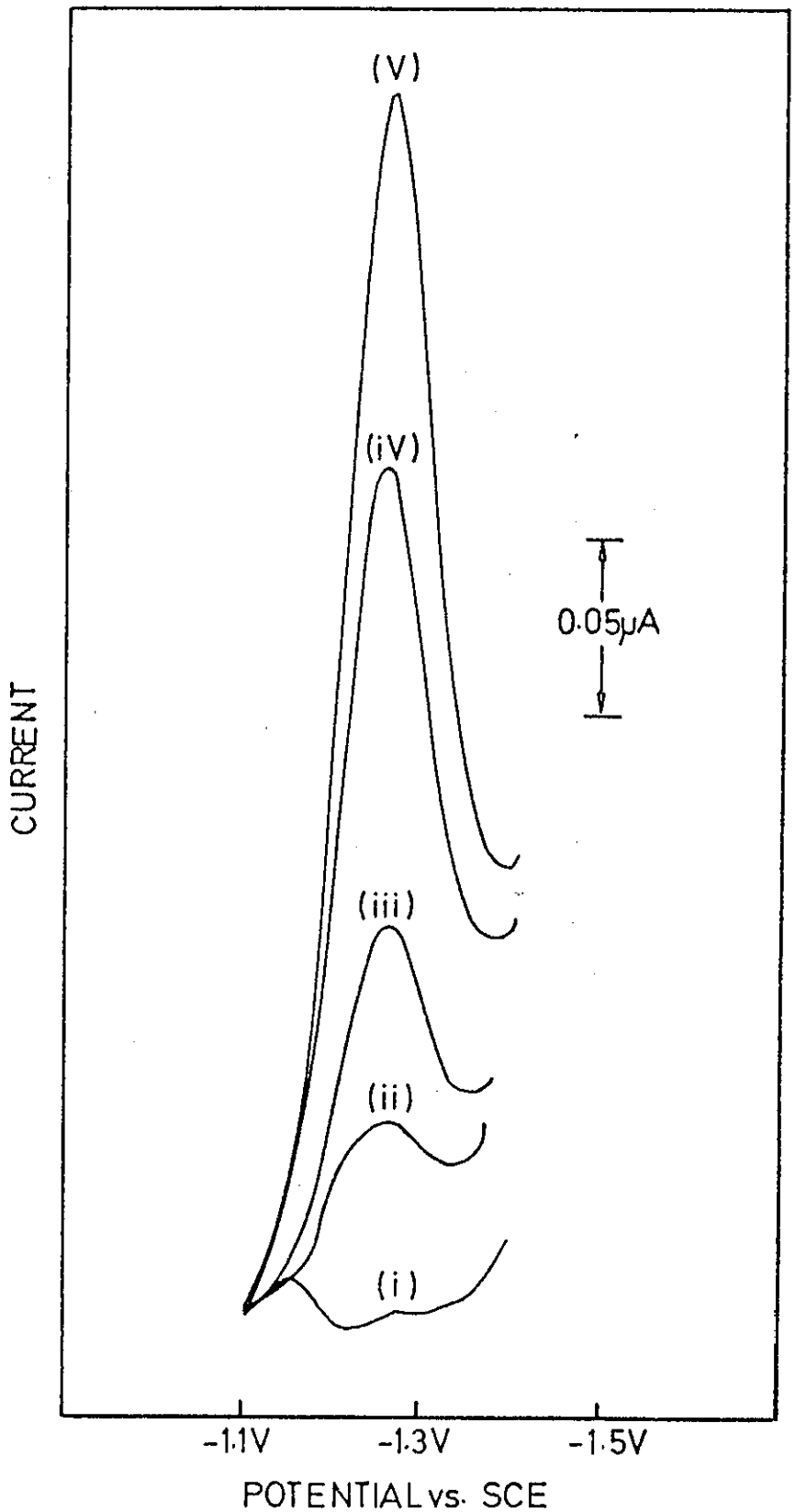


Fig. 29. Typical d.p. polarograms obtained for cephalexin determination by the recommended procedure.
Cephalexin concentration: (i) 0, (ii) 0.4, (iii) 0.8, (iv) 2, (v) 3μg/ml

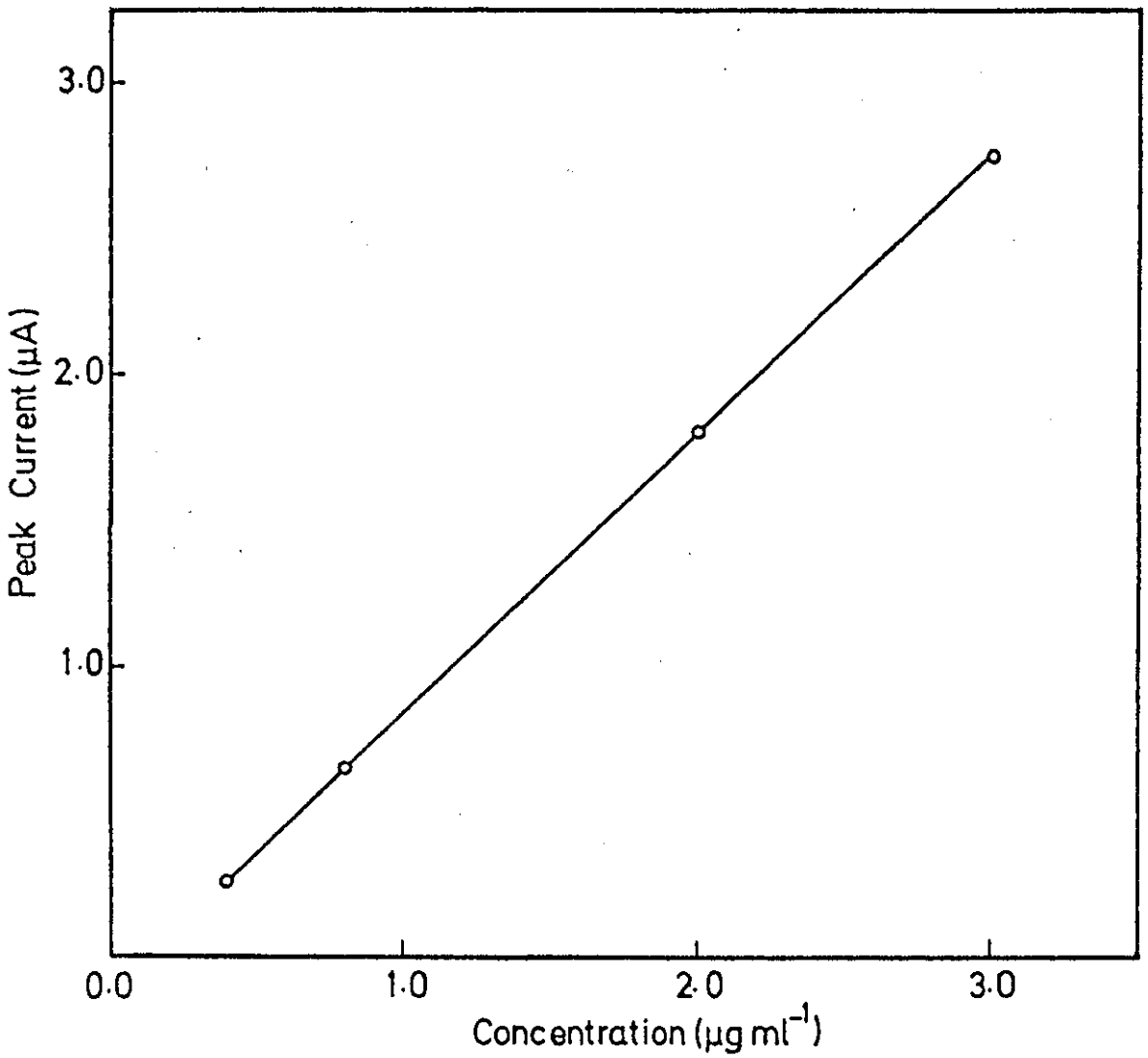


Fig. 30. Calibration graph obtained for the determination of cephalexin after hydrolysis in phosphate buffer pH 7.4

the drop-wise addition of Schiff's reagent, and disappears completely on the addition of an excess amount of this reagent or of a few drops of 45% sodium bisulphite solution. The d.p. peaks of the other degradation products that are present, including 2-hydroxy-3-phenyl-6-methylpyrazine, are unaffected by the addition of these reagents for carbonyl compounds.

The nature of the polarographic wave at -1.26V was studied by d.c. polarography. Analysis of d.c. polarograms with respect to changes in the limiting current with height of the mercury reservoir indicated that the wave is not fully diffusion controlled and probably has some kinetic character. This might be expected if the carbonyl compound is an aldehyde.

In the previous d.p.p. study of the degradation of cephalexin in pH 8.5 phosphate buffer at 80°C, 2-hydroxy-3-phenyl-6-methylpyrazine was shown to be formed at a constant rate during at least the first 30 hours (Fig. 21B). These conditions are clearly unsatisfactory as the basis for an analytical method. It was therefore assumed that the hydrolysis conditions optimised for the fluorimetric procedure[62] would be optimum for the d.p.p. method too. Very unreliable results were obtained when that method was adopted for d.p.p. determination of cephalexin. At relatively high concentrations the precision on some occasions was good (i.e. coefficient of variation was about 5% at the 5µg/ml level) but on other occasions completely different peak heights were obtained. Attempts to improve the reliability and

precision by altering reagent concentrations and heating times and by adding formaldehyde as a catalyst were unsuccessful.

A d.c. polarographic method for the determination of cephalexin based on hydrolysis in 5M hydrochloric acid solution at 80°C for 15 minutes has been suggested by Squella et al[28]. As the hydrolysis time is considerably shorter than that recommended above for hydrolysis in pH 7.4 buffer for 1 hour, this method was considered for adaptation at lower levels using differential pulse mode.

The cephalexin levels used by Squella et al were very high (6×10^{-3}M) and the d.c. currents obtained were disproportionately low. A non-rectilinear calibration graph over the concentration range 0 - 50 µg/ml was obtained in the present work using the conditions of Squella et al, and the coefficient of variation was found to be very high (>8%). The d.p.p. peak was absent at cephalexin concentrations of less than 10 µg/ml. Subsequently it was discovered that the height of the d.p.p. peak continued to increase rapidly for hydrolysis times up to at least 90 minutes. Even using these increased hydrolysis times the method was found to be highly irreproducible.

A sample of 2,4-dinitrophenylhydrazone was obtained for the carbonyl compound responsible for the peak at -1.26V. The hydrazone derivative which purified on a preparative silica gel t.l.c. plate and obtained as a solid after extraction into ethanol had a melting point of >318°C, and shows three UV absorption bands in

ethanol with λ_{max} at 215, 255 and 385 nm. No further work has been done to identify this compound.

D. D.P. POLAROGRAPHIC STUDY OF THE DEGRADATION OF AMPICILLIN

The initial degradation study was carried out at 37°C in pH 7.4 phosphate buffer. Ampicillin itself gave no polarographic wave in the available potential range (0 - -1.6V) but after 1 hour an anodic wave at -0.1V appeared and continued to increase in height after this. Hydrogen sulphide was found not to be formed during the degradation.

The degradation pattern at pH 2.5 citrate-phosphate buffer was different. Two major peaks were obtained: an anodic peak at -0.14V and a cathodic peak at -0.64V. The first anodic peak was shown by comparison with polarograms of a known sample to be due to penicillamine. The peak disappears on addition of a few drops of 0.1M silver nitrate, also the addition of a few drops of 0.1M mercuric chloride results in the formation of a black precipitate possibly due to the formation of mercuric mercaptide of penicillamine. The second peak is due to 2-hydroxy-3-phenyl-6-methylpyrazine. UV absorption spectrophotometric and t.l.c. studies confirm the formation of this latter compound. Degradation was studied at 37°C, 60°C and 80°C, the rate of degradation increases with temperature. The effect of dissolved oxygen was also studied and it was found that the yield of penicillamine was considerably less in its presence. In the absence of oxygen the yields of penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine

(pH 2.5, 80°C, 30 hours) were 20% and 5% respectively. In addition to the peaks due to penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine, an unidentified peak at -0.8V was observed in all degradation solutions at a later stage. In the presence of molecular oxygen, a further transient peak appeared at -0.45V (pH 2.5), the compound responsible for this peak apparently degrading rapidly itself. The peak at -0.8V was much larger in the presence of dissolved molecular oxygen. D.p. and d.c. polarograms of an ampicillin solution (100µg/ml) after degradation at pH 2.5 and 80°C for 3.5 hours are shown in Fig. 31 and 32 respectively.

Results obtained for the degradation of ampicillin in citrate-phosphate buffer at pH 2.5 at 37°C, 60°C and 80°C are shown in Tables 8 - 10. In Fig. 33 and Fig. 34 the amounts of penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine formed during the degradation of 10mg of ampicillin trihydrate in 100ml of citrate-phosphate buffer at pH 2.5 and 60°C in the presence and absence of molecular oxygen are shown. D.p. peak heights of ampicillin degradation products obtained under the same experimental conditions are also compared in Fig. 35 and 36.

Degradation of an ampicillin solution (100µg/ml) at pH 8.5 again followed a different pattern. An anodic peak formed at -0.16V and this decreased in height again as a broad cathodic peak appeared at -1.16V. The anodic peak is thought to be due to a thiol compound formed from the opening of the thiazolidine ring, and the peak at -1.16V to be due to a carbonyl compound formed as this thiol is hydrolysed. Typical d.p. polarograms are shown in Fig. 37.

The polarographic behaviour of penicillamine was investigated using a pure sample. The anodic polarographic wave was shown to be diffusion controlled by mercury height and temperature studies (i_{lim} vs $h^{\frac{1}{2}}$ gives a straight line passing through the origin and a temperature coefficient of 0.8% deg^{-1} was obtained).

The effect of pH on penicillamine d.p. peak potential, peak current, and d.c. wave height is shown in Fig. 38. The peak potential varied rectilinearly over the pH range 2-8 (E_p =-0.03 - 0.059 pH V). The d.p. peak current is more dependent upon the pH than the d.c. wave height.

Typical d.p. polarograms obtained for the determination of penicillamine in citrate-phosphate buffer solutions at pH 2.5 and 25°C are shown in Fig. 39. Rectilinear calibration graphs were obtained over the concentration ranges of 1.96 - 21.8µg/ml and 9 - 44.4 µg/ml. The degradation of penicillamine in the presence of molecular oxygen in citrate-phosphate buffer solution at pH 2.5 and 60°C is shown in Fig. 40: the broad peak which appears at -1.0V is thought to be due to a carbonyl compound.

The polarographic behaviour of 2-hydroxy-3-phenyl-6-methylpyrazine was also studied using a pure sample. The cathodic polarographic wave was shown to be diffusion controlled by mercury height and temperature studies (temperature coefficient in this case is 0.4% deg^{-1}). The effect of pH on peak potential and peak current is shown in Fig. 41, from which it can be seen that the peak potential varies rectilinearly over the pH range 2 - 9 (E_p = -0.49 - 0.067 pH V).

The peak current is highly dependent on pH having a maximum value at pH 3: the peak height decreased to almost zero at pH 11. D.p. polarograms of 2-hydroxy-3-phenyl-6-methylpyrazine in citrate-phosphate buffer solution (pH 2.5) are shown in Fig. 42. Rectilinear calibration graphs were obtained over the ranges 1.74 - 8.4 µg/ml and 1.9 - 11.4 µg/ml.

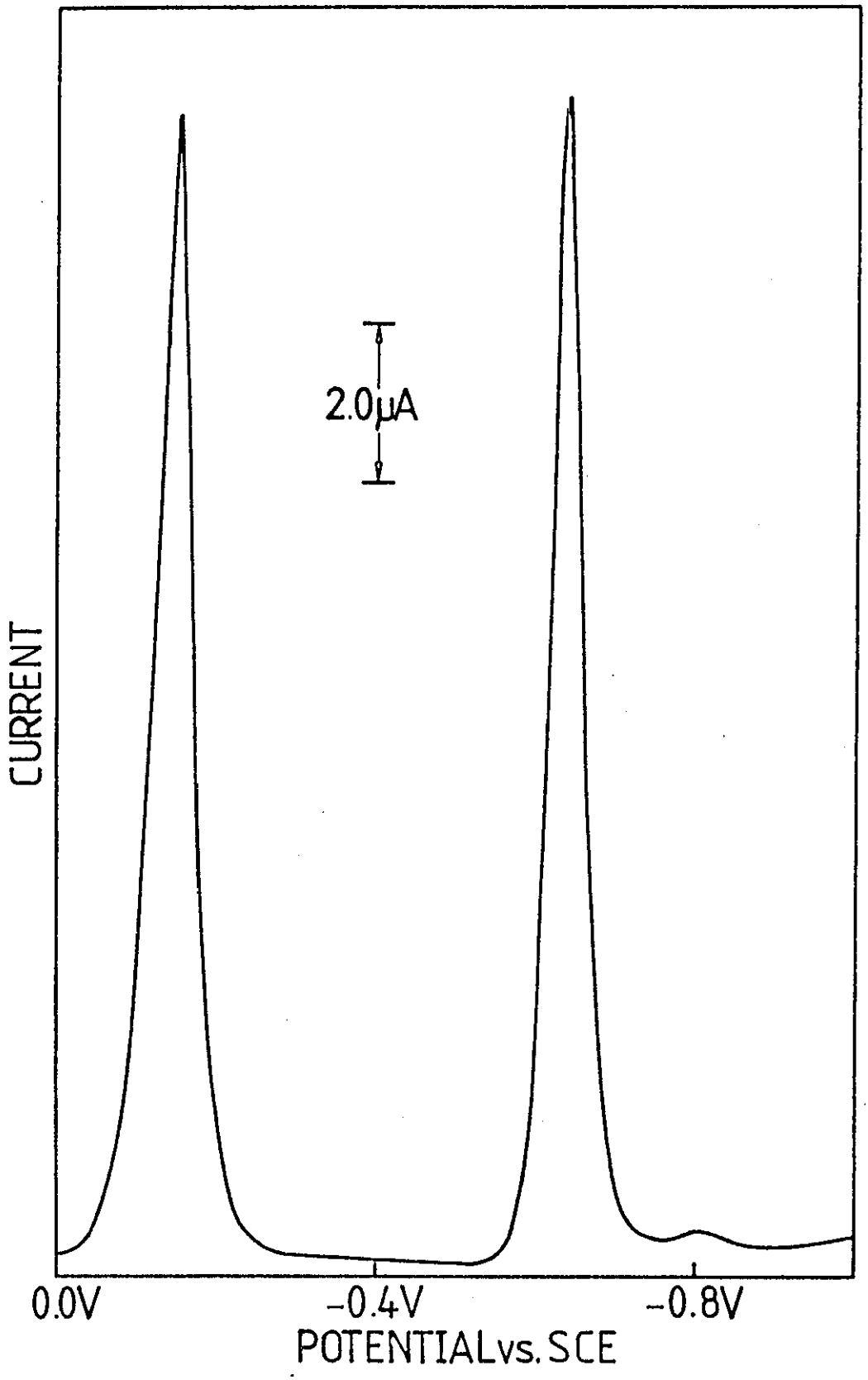


Fig. 31. D.p. polarogram obtained for ampicillin solution (100μg/ml) after degradation at pH 2.5 for 3.5 hours at 80°C

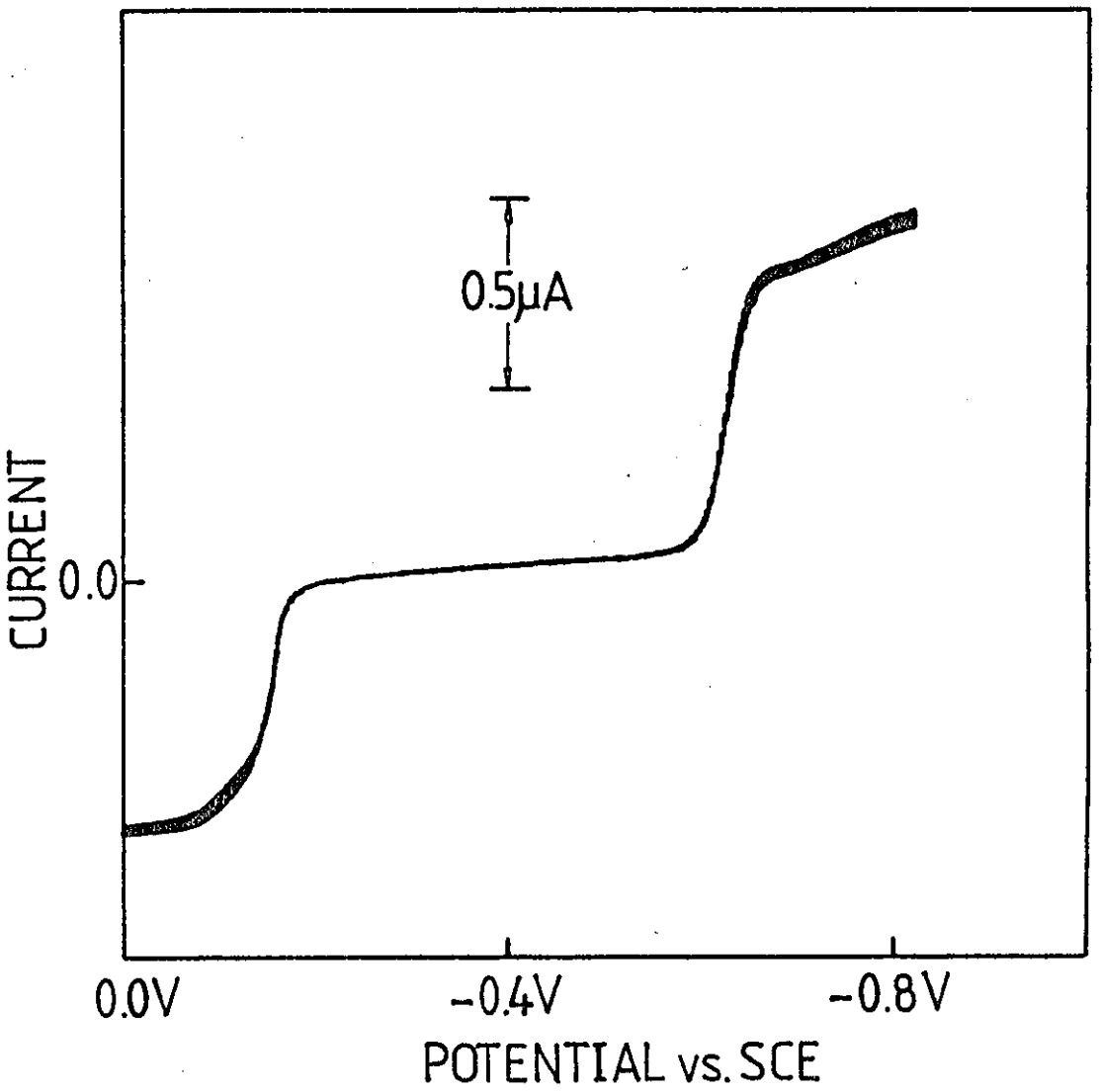


Fig. 32. D.c. polarogram obtained for an ampicillin solution (100µg/ml) after degradation at pH 2.5 for 3.5 hours at 80°C.

(Natural drop time, scan rate 1 mVs^{-1}, low pass filter 3)

Table 8. Data obtained for the degradation of ampicillin (2.45×10^{-5} Moles) in citrate-phosphate buffer solution at pH 2.5 and 37°C in presence of molecular oxygen

Time/hr	Penicillamine		2-hydroxy-7-phenyl-6-methylpyrazine		Peak at -0.45V	Peak at -0.8V
	I_p(μA)	Amount(Mol$\times 10^7$)	I_p(μA)	Amount (Mol $\times 10^7$)	I_p (μA)	I_p (μA)
0.5	0.0	0.0	0.0	0.0	0.0	0.0
1.5	0.0	0.0	0.0	0.0	0.0	0.0
4.25	0.0	0.0	0.0	0.0	0.0	0.0
19.50	0.55	1.5	0.55	0.46	0.3	0.02
22.8	0.80	2.7	1.10	0.86	1.3	0.05
26.2	0.95	3.4	1.30	1.0	0.0	0.05
45.7	1.90	7.8	3.80	2.8	0.0	0.25
89.8	2.50	10.5	8.60	6.98	0.0	0.60
97.2	2.70	11.5	9.55	7.8	0.0	0.75

Table 9. Data obtained for the degradation of ampicillin (2.45×10^{-5} Moles) in citrate-phosphate buffer at pH 2.5 and 80°C in absence of molecular oxygen

Time/min	Penicillamine		2-hydroxy-3-phenyl-6-methylpyrazine		Peak at -0.8V	Absorbance at 350nm
	I_p(μA)	Amount(Molex10^7)	I_p(μA)	Amount(Molex10^7)	I_p (μA)	
12.0	0.0	0.0	0.0	0.0	0.0	0.0
30.0	0.45	1.1	0.2	0.2	0.0	0.0
44.0	1.70	6.8	2.0	1.51	0.15	0.24
66.0	3.0	12.8	4.4	3.25	0.20	0.43
85.0	3.95	17.2	6.1	4.49	0.20	0.62
139.0	10.1	35.3	11.4	9.37	0.30	1.04
191.0	12.8	44.8	13.7	11.30	0.30	1.30
376.0	14.9	52.1	16.3	13.48	0.30	1.64

Table 10. Data obtained for the degradation of ampicillin (2.45×10^{-5} Moles) in citrate-phosphate buffer solution at pH 2.5 and 60°C

(A) In presence of molecular oxygen
(B) In absence of molecular oxygen

(A)

Time/hr	Penicillamine		2-hydroxy-3-phenyl-6-methylpyrazine		Peak at -0.8V
	I_p (μA)	Amount (Mole x 10^7)	I_p (μA)	Amount (Mole x 10^7)	I_p (μA)
0.42	0.0	0.0	0.0	0.0	0.0
1.22	0.1	0.6	0.05	0.1	0.0
2.0	1.0	3.6	1.05	0.82	0.15
6.66	2.9	12.4	5.30	3.9	0.25
9.16	3.5	15.2	8.00	6.5	0.40
23.8	2.6	11.0	15.30	12.7	1.0
30.4	2.0	8.2	16.30	13.5	1.2

Table 10(B).

Time/hr	Penicillamine		2-hydroxy-3-phenyl-6-methylpyrazine		Peak at -0.8V
	I_p (μA)	Amount (Mole x 10^7)	I_p (μA)	Amount(Mole x 10^7)	I_p (μA)
0.42	0.0	0.0	0.0	0.0	0.0
1.22	0.1	0.6	0.05	0.1	0.0
2.0	1.1	4.1	1.30	1.0	0.05
6.66	4.4	19.3	5.7	4.2	0.10
9.16	6.8	23.7	8.6	7.0	0.15
23.8	13.7	47.9	15.2	12.56	0.15
30.4	13.8	48.3	16.0	13.24	0.20

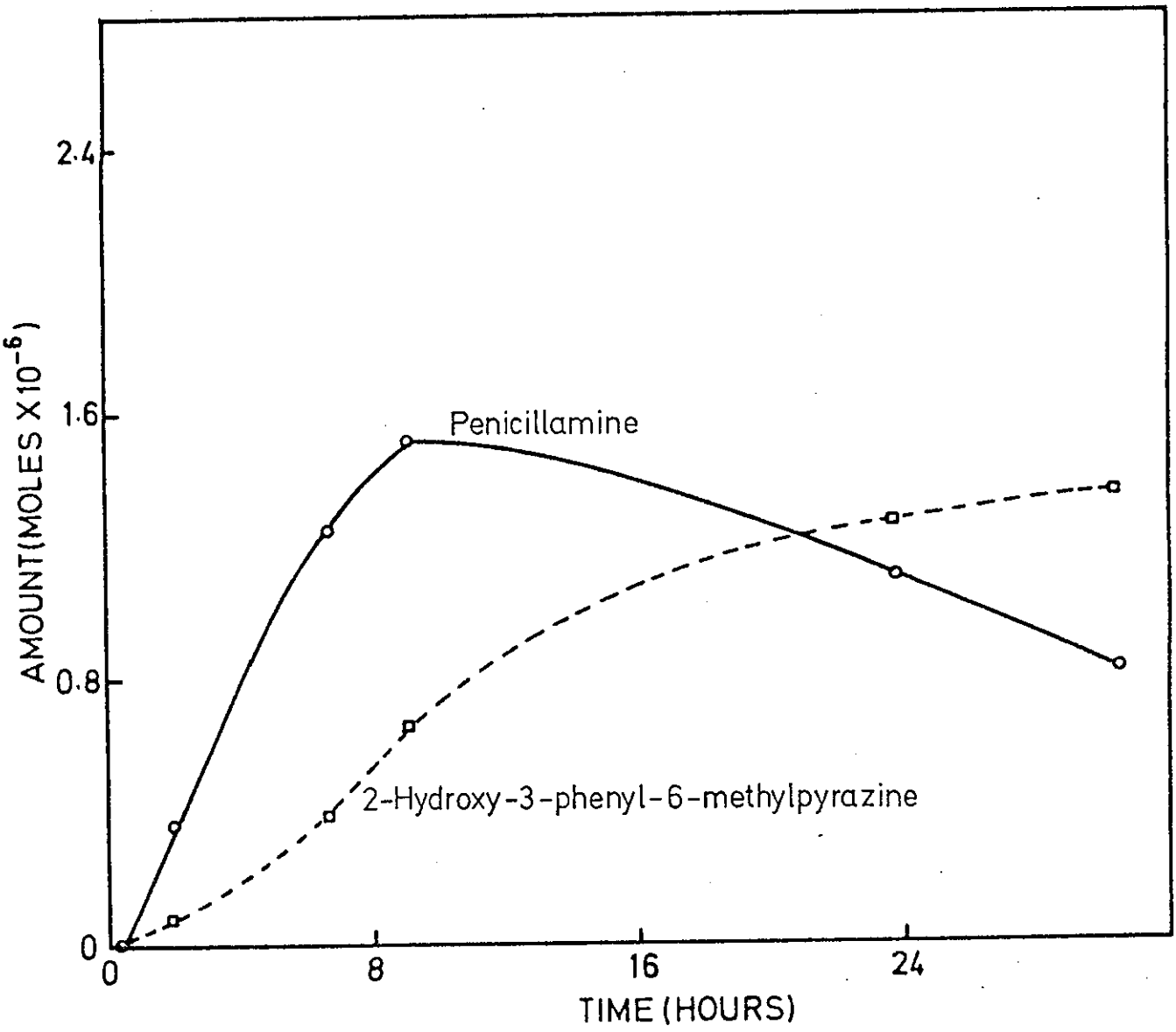


Fig. 33. Comparison of the amounts of penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine formed during the degradation of ampicillin in pH 2.5 buffer at 60°C in presence of molecular oxygen

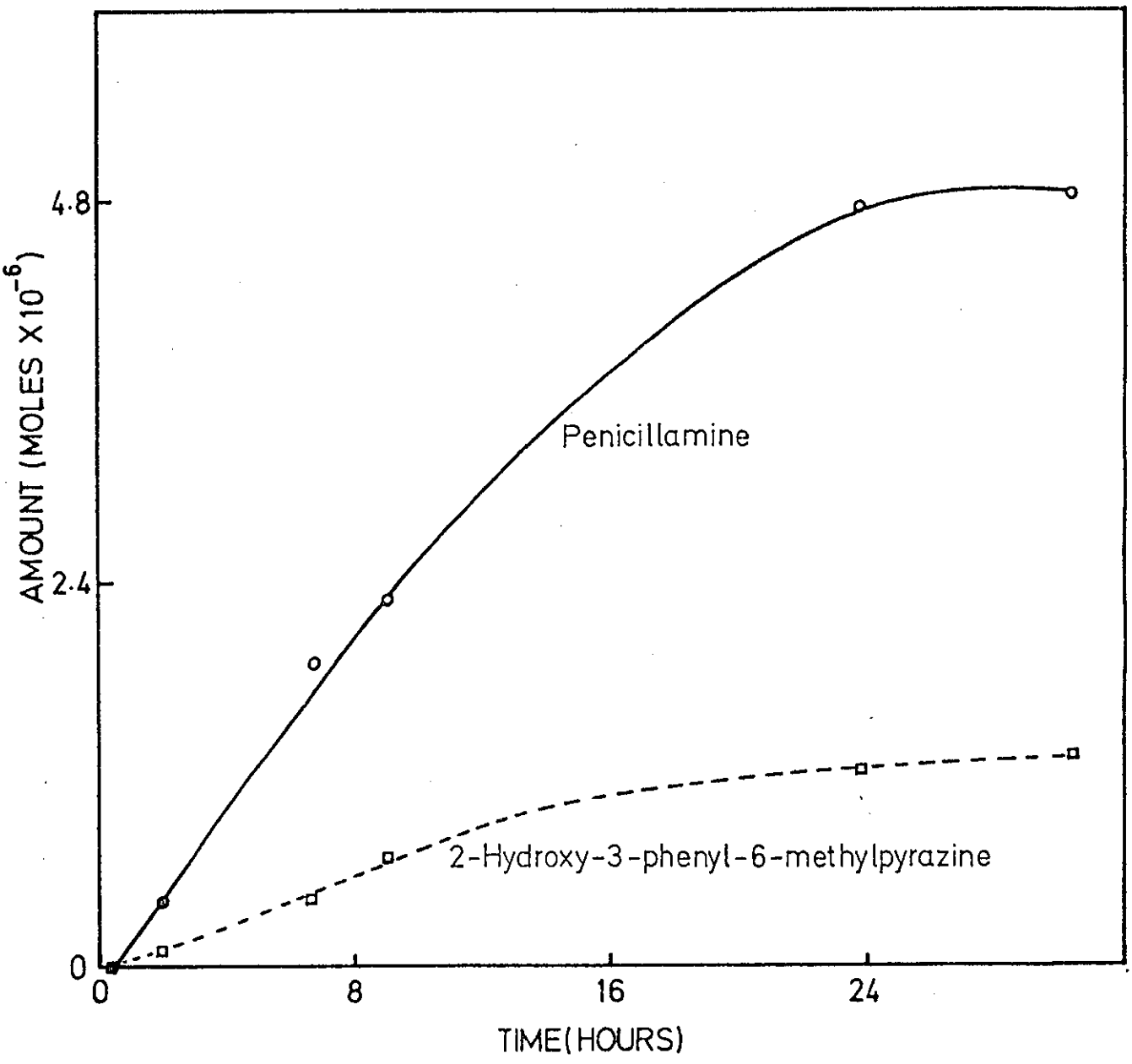


Fig. 34. Comparison of the amounts of penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine formed during the degradation of ampicillin in pH 2.5 buffer at 60°C in absence of molecular oxygen

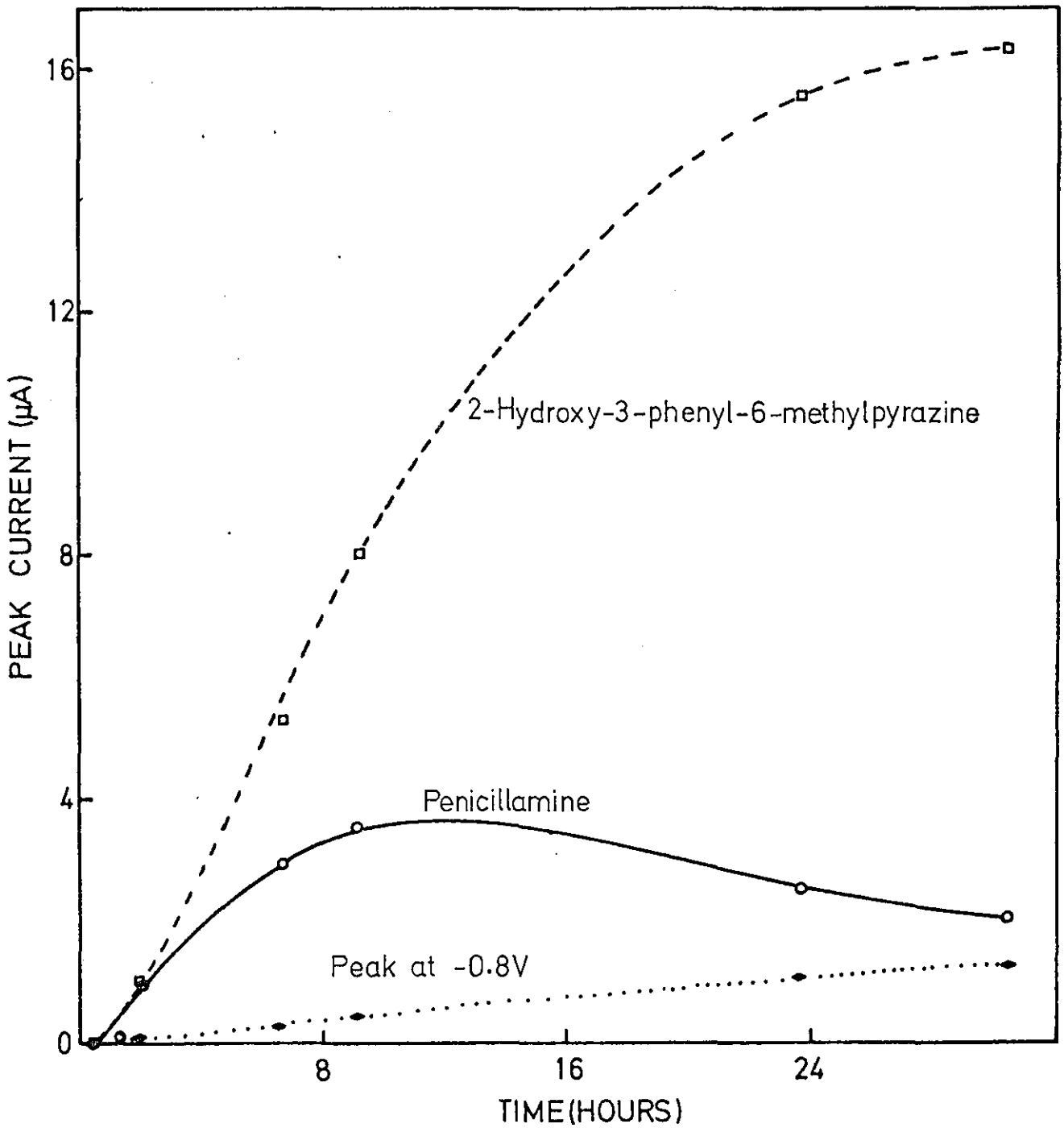


Fig. 35. Comparison of peak height of ampicillin degradation products obtained in pH 2.5 buffer at 60°C in presence of molecular oxygen

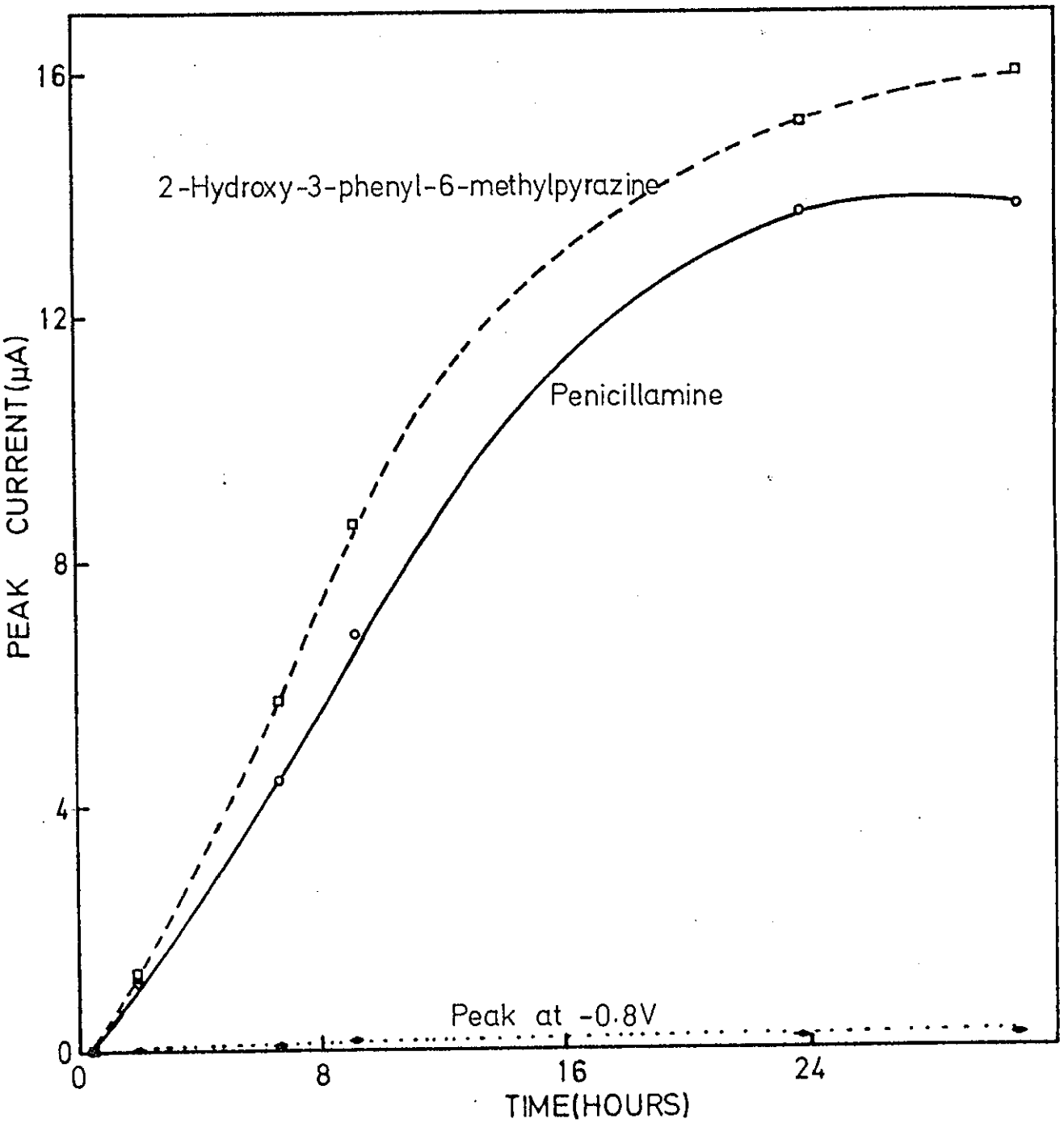


Fig. 36. Comparison of peak heights of ampicillin degradation products obtained in pH 2.5 buffer at 60°C in absence of molecular oxygen.

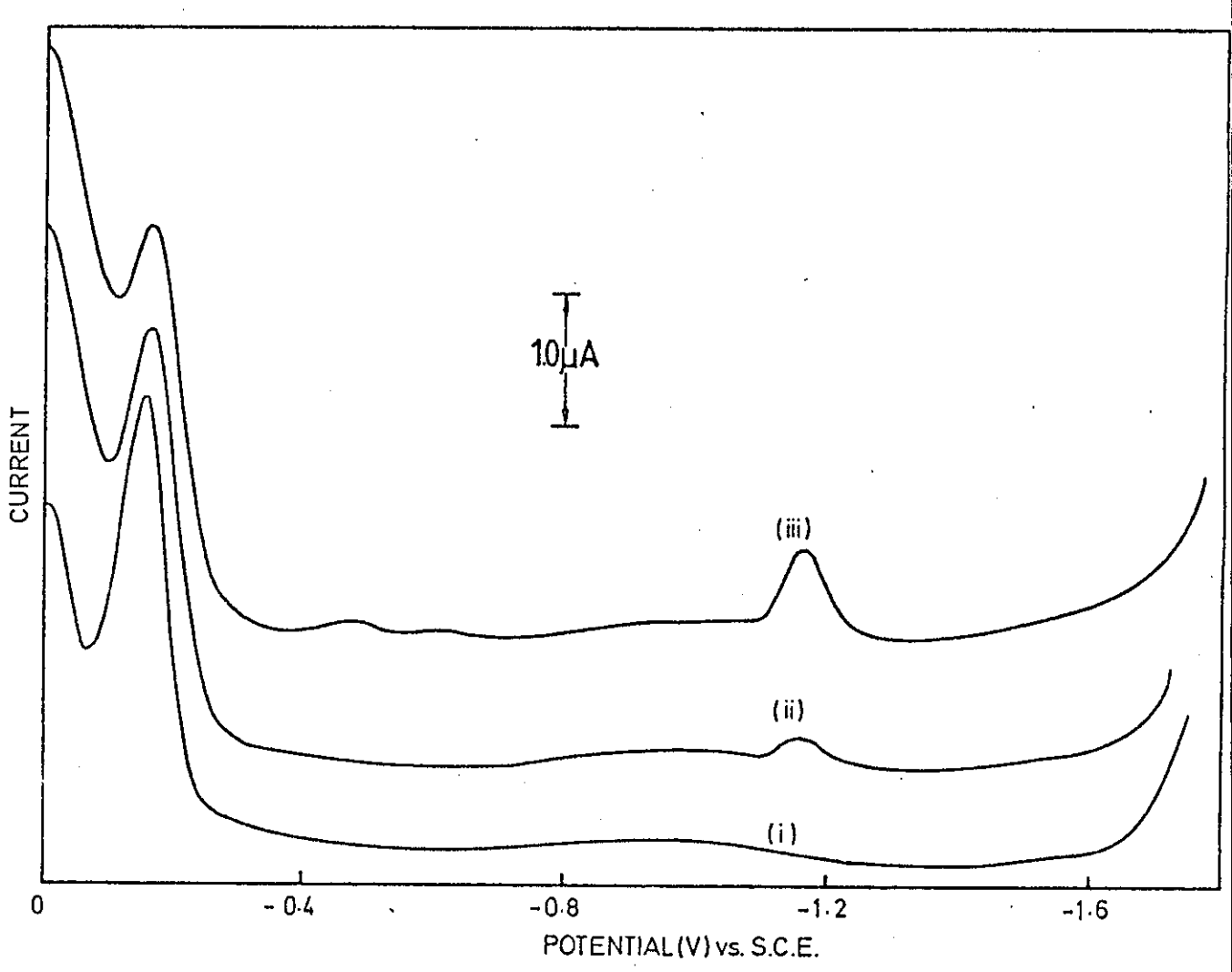


Fig. 37. D.p. polarograms obtained for ampicillin in pH 8.5 citrate-phosphate buffer at 80°C.

Time: (i) 22, (ii) 52, (iii) 163 min

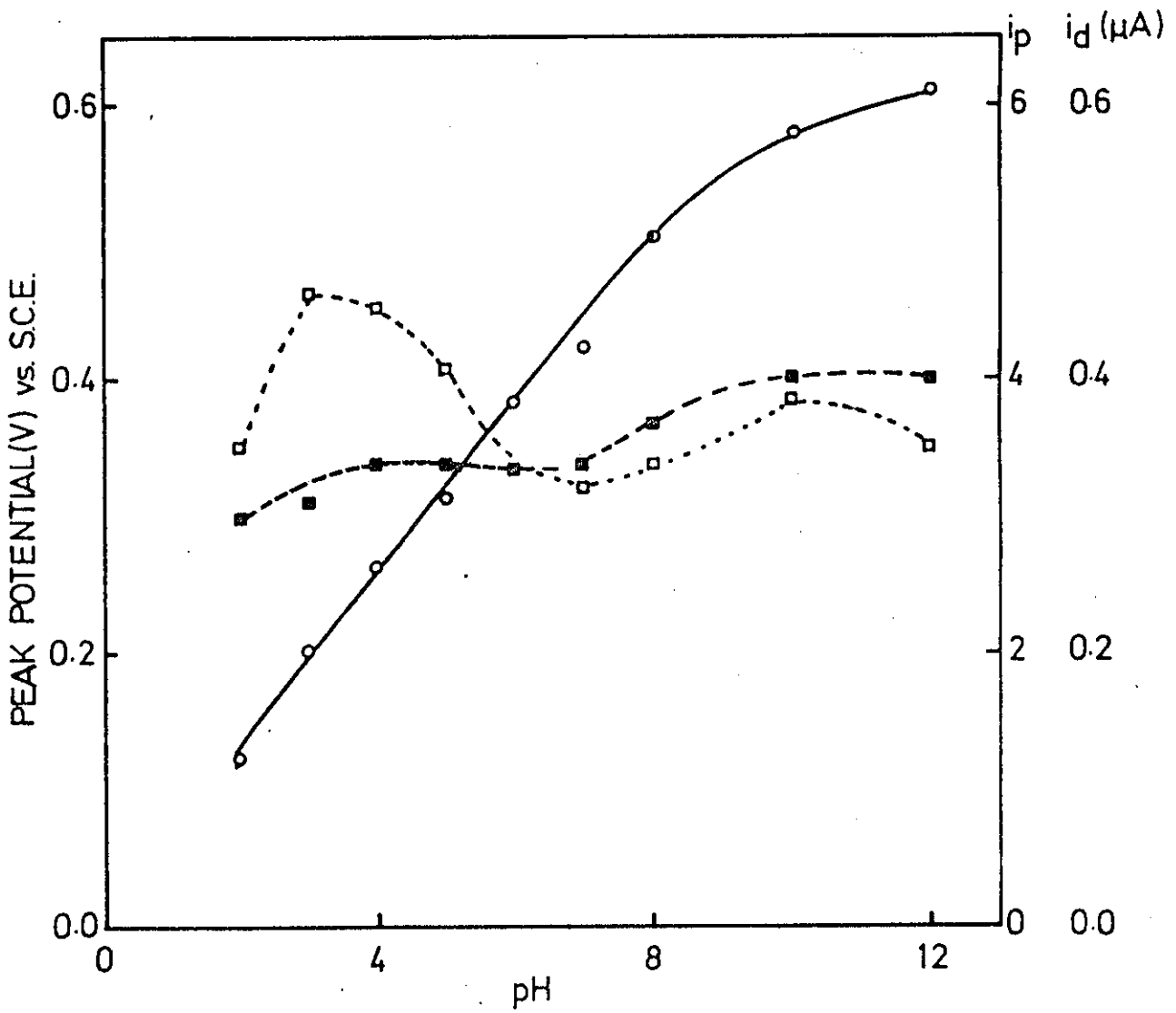


Fig. 38. Effect of pH on the peak potential, peak height and d.c. wave height of penicillamine.

Key: o—o Peak potential vs. pH; □·····□ peak current vs. pH and ■----■ d.c. wave height (μA) vs. pH

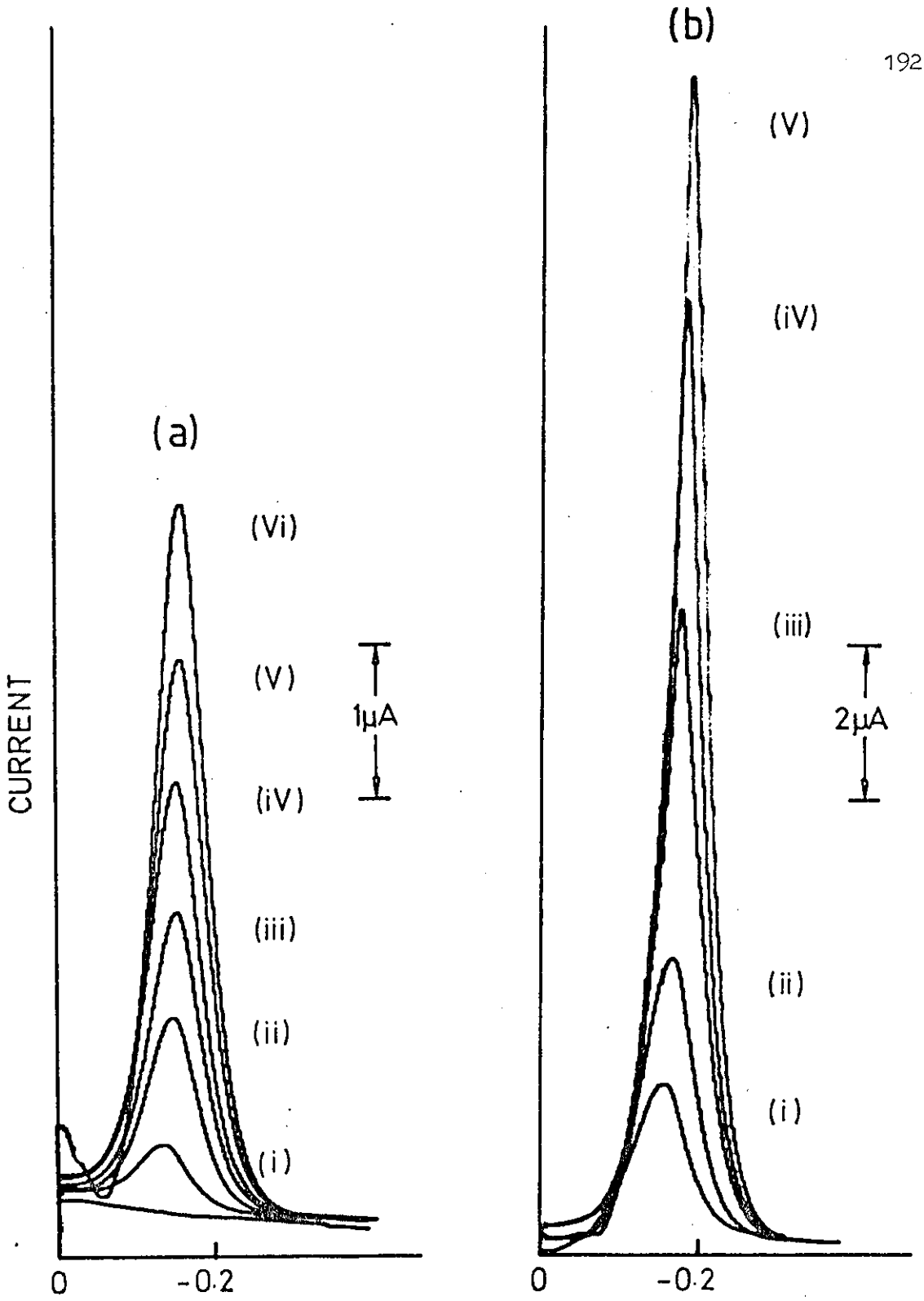


Fig. 39. D.p. polarograms obtained for penicillamine in citrate-phosphate buffer solution at pH 2.5 and 25°C

Concentrations: (a) (i) 1.96, (ii) 5.66, (iii) 9.09, (iv) 13.8, (v) 18, (vi) 21.9μg/ml

(b) (i) 9.09, (ii) 16.7, (iii) 28.6, (iv) 37.5, (v) 44.4 μg/ml

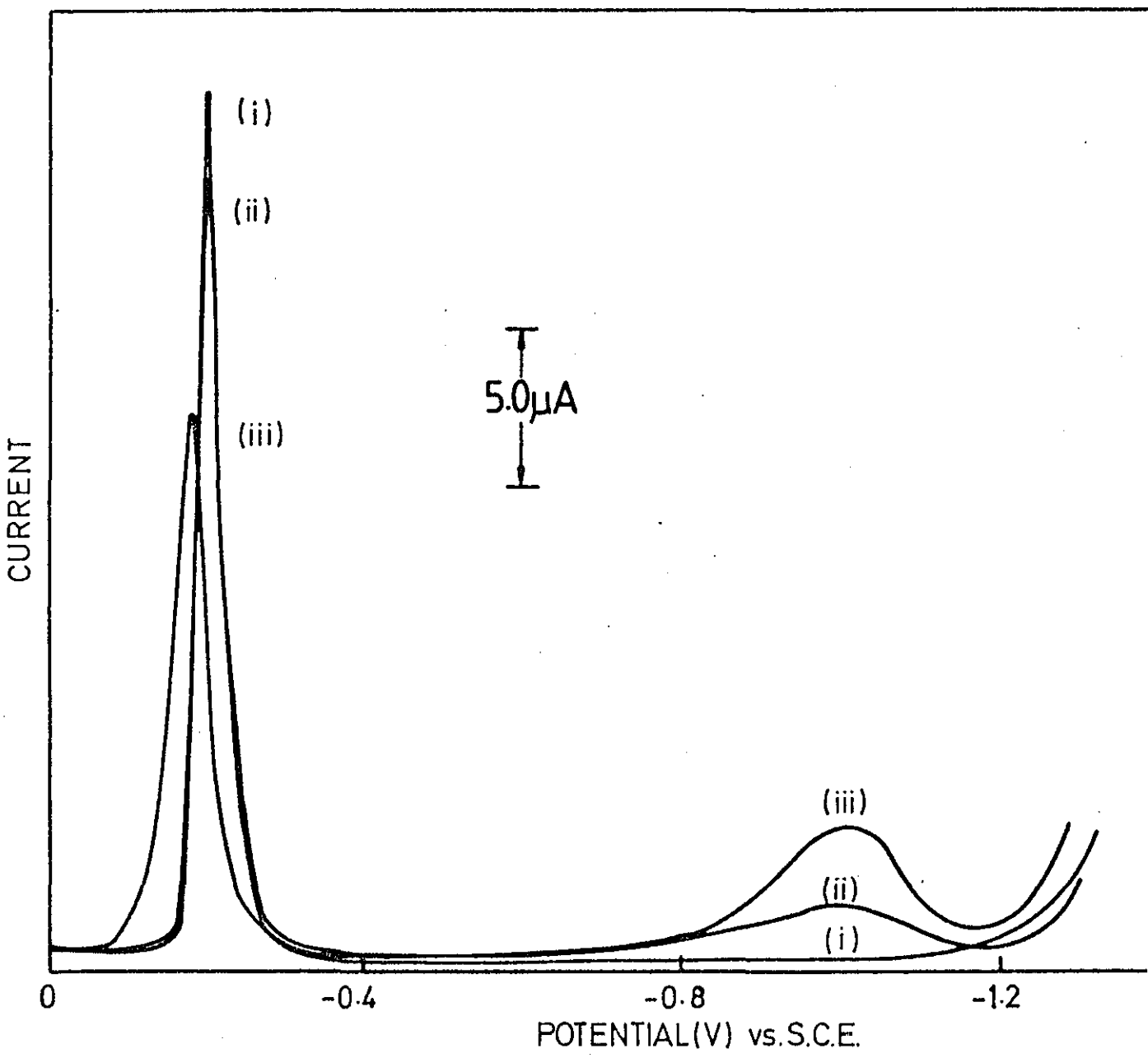


Fig. 40. D.p. polarograms showing the degradation of penicillamine (100μg/ml) in citrate-phosphate buffer pH 2.5 and 60°C in presence of oxygen

Time: (i) 0.0, (ii) 3, (iii) 13.5 hrs

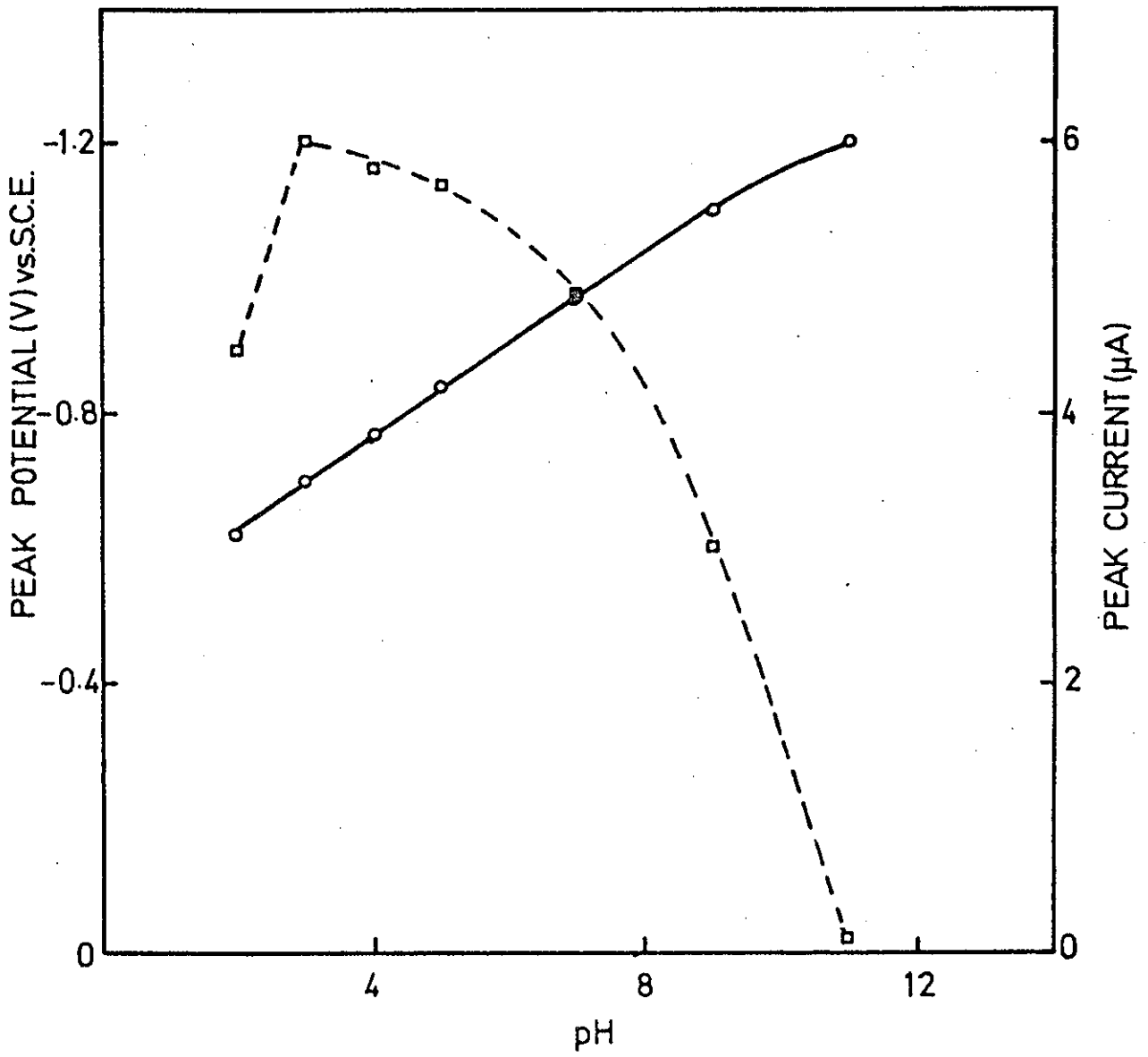


Fig. 41. Effect of pH on peak potential and peak current of 2-hydroxy-3-phenyl-6-methylpyrazine

Key: peak potential vs. pH;

peak current vs. pH

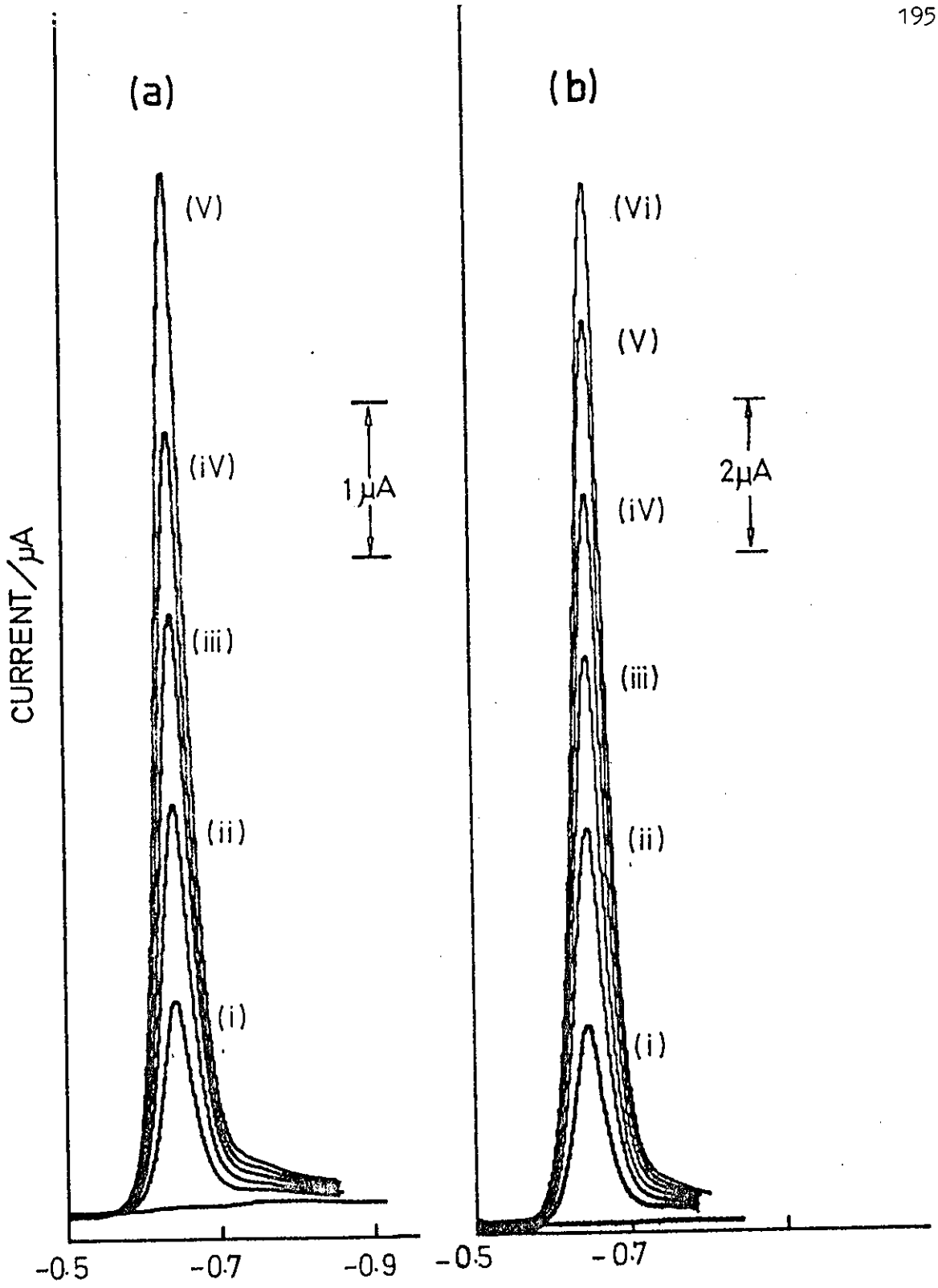


Fig. 42. D.p. polarograms obtained for 2-hydroxy-3-phenyl-6-methyl-pyrazine in citrate-phosphate buffer at pH 2.5

Concentrations: (a) (i) 1.74, (ii) 3.45, (iii) 5.13, (iv) 6.77, (v) 8.38µg/ml

(b) (i) 3.45, (ii) 6.77, (iii) 9.96, (iv) 13, (v) 16, (vi) 18.6µg/ml

GENERAL DISCUSSION

Previous workers[22,23,28] have studied the determination of some cephalosporins by d.c. polarography and cathode-ray polarography. Ochiai et al[25] indicated that cephalosporins with a substituted methyl group at the 3-position of the dihydrothiazine ring are reduced at the dropping mercury electrode at about -1.2V. Thus cephalosporins with an unsubstituted 3-methyl group are not reduced at the d.m.e. in the available potential range. Some cephalosporins have reducible groups elsewhere in the molecule and give polarographic waves at less negative potentials. The present work was carried out to investigate any advantages that d.p.p. might have for the determination of cephalosporins and their degradation products.

Cephalosporins having a substituted methyl group at the 3-position have been determined down to 0.1 µg/ml level using the d.p.p. peaks at -1V despite the peaks being near the cathodic limits under the conditions used. This is illustrated by the results obtained for cephalothin, cephaloridine, 7-aminocephalorporanic acid, deacetylcephalosporin C and cephoxazole contained in Table 1 and by the d.p. polarograms shown in the Appendix. Other cephalosporins which contain other reducible groups elsewhere in the molecule in addition to the substituted methyl group at the 3-position, however, give other d.p. peaks at less negative potentials. For example, cephalonium and cefuroxime showed three and two polarographic peaks respectively. The sensitivity of the d.p.p. method is superior to that of d.c. and cathode-ray polarography reported previously by other workers[22,23] and could replace the

chemical and spectrophotometric methods currently in use. Also, the method can function as a possible check on a microbiological assay particularly when a mixture of two or more dissimilar antibiotics has to be analysed. Without prior separation of the antibiotics, such an analysis would be impossible by the microbiological technique. Further, it was shown using cephalothin and cephaloridine that d.p.p. is a useful technique for following the degradation of such cephalosporins. Degradation can be followed directly in a polarographic cell with no interference from degradation products. It should be noted that the degradation reaction was followed using the decrease in the d.p. peak at -1V; the decrease in the height of this peak arises specifically owing to the hydrolytic elimination of the 3-methyl substituent.

Although cephalosporins which do not have a substituted 3-methyl group, and have no other reducible groups, do not give d.p. reduction peaks themselves, many of their degradation products do. The cephalosporins studied in this category were cephalexin, cephradine and 7-aminodeacetoxycephalosporanic acid (7-ADCA). However, the present studies have been concentrated on cephalexin.

The kinetics and mechanisms of the degradation of cephalexin have been previously studied extensively by other workers[81-84,91-93]. As the cephalexin molecule contains an α-amino group in the 7-position, it undergoes intramolecular aminolysis on degradation, producing a diketopiperazine derivative which is unstable itself and undergoes further degradation. The degradation pathway of cephalexin has been discussed earlier in this thesis.

The main finding of this work is that several cephalexin degradation products are polarographically active. Hydrogen sulphide has been shown to be eliminated during the degradation. Also the kinetics and mechanisms of the degradation reactions are critically dependent upon the experimental conditions under which the degradation is carried out.

The initial degradation product of cephalexin, the diketopiperazine derivative, was isolated from a degraded solution of cephalexin, and a d.p.p. procedure for its determination has been developed. The procedure has been applied to the determination of the diketopiperazine during the degradation. Also, 2-hydroxy-3-phenyl-6-methylpyrazine, which was isolated from degraded solutions of α-aminobenzylcephalosporins and α-aminobenzylpenicillins under special experimental conditions[66] was found to be produced in a good yield under the conditions used in this study.

The present work appears to be the first to report the formation of hydrogen sulphide in the degradation of cephalexin and cephradine, although its odour is apparent in deteriorating sample solutions. Hydrogen sulphide has been found to give a reduction peak at -0.56V. The peak arises owing to formation of mercury(II) sulphide at the surface of the mercury drop which is subsequently reduced to mercury and sulphide.

$$HgS + H^+ + 2e \longrightarrow Hg + HS^-$$

The peak has also been observed in the cathodic stripping analysis of hydrogen sulphide[95]. Youssefi et al[96] used this d.p. peak for

the direct determination of sulphide. When this method was applied here for the determination of hydrogen sulphide, the linearity of the analytical plot and the precision were found to be unsatisfactory. An indirect d.p.p. method, however, has been developed for the determination of sulphide on the basis of the decrease in the cadmium d.p. peak when it reacts with sulphide.

In an attempt to identify positively the hydrogen sulphide evolved during cephalexin degradation, a mass spectrum was taken on a sample of the gas evolved. The m/e peak at 34 (H_2S) was present but the highest peak was at 74; the compound responsible was not identified. That no mercaptans were evolved and absorbed with the hydrogen sulphide in the trapping solution, however, was shown by the analytical results. Mercaptans do not react with cadmium nitrate solution[97], and the polarographic method we applied determines the hydrogen sulphide only. Furthermore, the initial potential of the 1M sodium hydroxide solution after trapping the gas is consistent with the gas being hydrogen sulphide. Any mercaptan present would have given a second end point at about-500mV and this was not observed[97].

A pronounced effect on the kinetics and mechanisms of the degradation of cephalexin was noticed when the experimental conditions of the degradation were varied. Some degradation products were found to be produced only in the presence of molecular oxygen. Other degradation products were formed only at high initial concentrations of cephalexin. Also the pH of the degradation solution was found to affect the yield and the identity of the degradation products.

Cephradine has been found to behave similarly to cephalexin except that the rate of degradation is much faster than that of cephalexin. This increase in the reactivity of cephradine must be attributed to the presence of the 1,4-cyclohexadienyl group in the 7-position which may facilitate the intramolecular nucleophilic attack of the side chain α-amino group on the carbonyl carbon atom of the β-lactam ring. The degradation of the diketopiperazine derivatives of cephalexin and cephradine were found to follow a first order reaction with rate constants of 0.025 and 0.03 h^{-1} respectively. Between 70 and 80 hours the values of the rate constants were found to increase rapidly then attain their original values. However, in the absence of hydrogen sulphide and molecular oxygen, the degradation of DKP II was slightly slower. A first order rate constant of 0.021 h^{-1} was obtained: again an abrupt change in the rate constant to 0.012 was observed after 80 hours degradation, but in this case the new value of the rate constant did not change (see Fig. 9 and 20). This phenomenon has not been satisfactorily explained so far.

At pH 7.4 and 37°C the amount of hydrogen sulphide evolved during cephalexin degradation was about 50% of the total sulphur present. About 40% of the total sulphur was produced in the first 80 hours, and then the production of hydrogen sulphide was reduced considerably (see Fig. 18A). These results coincide with the abrupt change in the rate constant of the degradation of DKP II after 80 hours, and may indicate that the degradation of DKP II follows a different mechanism, i.e. after 80 hours DKP II does not degrade via the thiolactone III. It is suspected that the degradation of this

lactone gives hydrogen sulphide and the carbonyl compound responsible for the d.p. peak at -1.26V. However, this suggestion needs confirmation by studying the degradation of a pure sample of the thiolactone III under the same experimental conditions.

Previous methods for the quantitative determination of cephalexin, cephradine and cephaloglycine include UV spectrophotometry, iodometric method, and hydroxylamine determinations[20,21]. These methods, however, lack sensitivity and selectivity. A sensitive fluorimetric procedure[60] for the determination of these compounds was recently developed but again the selectivity is poor as the method is applicable to all α-aminobenzylcephalosporins and α-aminobenzylpenicillins.

Benner[23] developed a cathode-ray polarographic method for the determination of cephalexin following alkaline hydrolysis. Recently, Squella et al[28] described a d.c. polarographic method for the determination of cephalexin at high concentrations.

In the present work, a d.p. polarographic method of the determination of cephalexin has been investigated, and a procedure based on the formation of a d.p.p. peak at -1.26V following cephalexin hydrolysis was recommended for general use. This procedure is superior to two other d.p.p. procedures, based on hydrolysis to 2-hydroxy-3-phenyl-6-methylpyrazine and on hydrolysis in 5M hydrochloric acid. The sensitivity and precision of the recommended procedure is good. The initial diketopiperazine derivative formed, however, if present in the cephalexin sample, would produce the peak at -1.26V and would interfere. The presence of this compound, however, can be tested for

by polarographing the solution before the hydrolysis step, as it gives a d.p.p. peak at -0.9V (pH 7.4) and its concentration could be determined and allowed for. It is envisaged, therefore, that as well as for determination of low concentrations of cephalexin in a degradation-free system, the d.p. polarographic method could be used with advantage in degradation and metabolic studies.

When the degradations of 7-ACA and 7-ADCA were studied in 0.1M sodium hydroxide, and the course of degradation was followed by d.p.p. and UV spectrophotometry, it was noticed that the rate of degradation of 7-ACA (as indicated by the decrease in the d.p. peak at -1.05V and by UV spectra) was much faster than that of 7-ADCA (as indicated by the increase of the peak of the degradation product at -0.42V and by UV spectra). This increase in the degradation rate of 7-ACA must be attributed to the presence of the acetoxy group in the 3-position of 7-ACA. Previous workers[42,98] indicated that the reactivity of the β -lactam ring is enhanced as the electronegativity of the 3-methyl substituent increased.

In addition to the difference in the degradation rates of 7-ACA and 7-ADCA, marked differences in the polarographic patterns and UV absorption spectra of degraded solutions of 7-ACA and 7-ADCA were observed. Degraded solutions of 7-ACA show an absorption band at 225nm and the compound responsible for this band degraded later and a new stable absorption band at 240nm was observed. The first product may be an unstable intermediate and the second product may correspond to a more stable penaldate degradation product similar to that obtained following 7-n-butyramidocephalosporanic acid degradation

with ND_3 - D_2O (see Scheme 8)[76,77].

Degraded solutions of 7-ADCA, on the other hand, do not show an absorption band at 240nm, but at 223nm. The compound responsible seems to be stable since an increase in the intensity of this band was observed with time. Again, this stable compound may correspond to the cephalosporoate derivative suggested by Hamilton-Miller et al [76,77] (see Scheme 8).

The amount of sulphur evolved after 4.5 hours degradation as hydrogen sulphide from 7-ACA and 7-ADCA represented about 6% and <1% of the total sulphur present respectively. This is consistent with the fragmentation of the molecule in the first case only to form the proposed penaldate. More work is certainly required in order to positively identify these degradation products.

In contrast to the corresponding α-aminobenzylcephalosporin, cephalexin, the α-aminobenzylpenicillin, ampicillin, does not give hydrogen sulphide on degradation at pH 2.5 and 7.4. Analogous compound to diketopiperazine II derivative which might be expected to be formed from ampicillin has not been observed. This compound would be expected to give a d.p. peak near -0.9V (pH 7.4) as does DKP II. Instead in the degradation of ampicillin, penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine are formed at low pH (pH 2.5). The polarographic behaviour of these products has been investigated, and d.p.p. procedures for their determination have been developed. The amounts of penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine formed indicated that the yield obtained in pH 2.5 buffer at 80°C after 30 hours in the absence of oxygen is 20% and 5% respectively.

Therefore, other polarographically inactive species must also be formed including the various polymeric species investigated by Kuchinskas and Levy[99] and Stanfield et al[100].

CONCLUSION

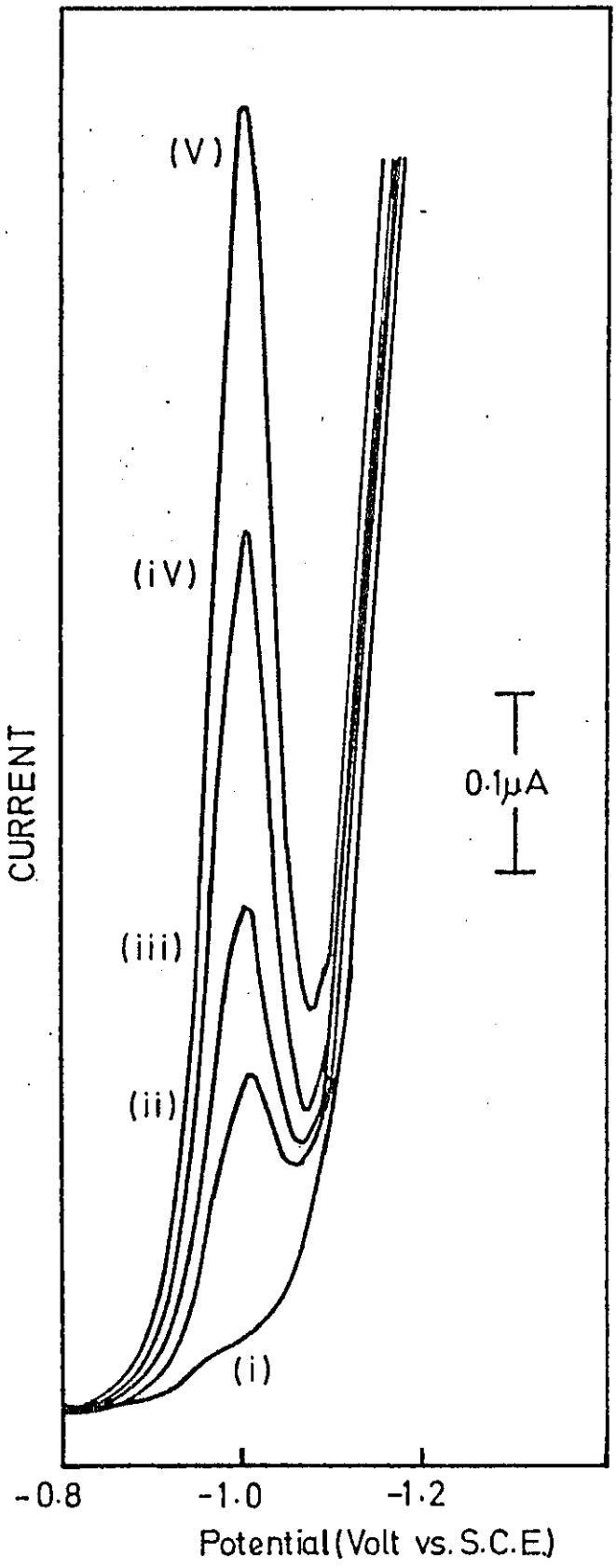
The present work on the degradation of cephalosporins and ampicillin illustrates well the potentialities of d.p.p. in degradation and stability testing studies of pharmaceuticals. Considerable information has been obtained using d.p.p. which would have been difficult to obtain by other methods, in addition to information that has confirmed previous work by other investigators using other methods. D.p. polarography seems to be ideally suited for kinetic studies of cephalosporins and penicillins. The sensitivity of the technique has allowed kinetic measurements to be made at low concentration levels and to measure simultaneously the concentration change for more than one electroactive species of the degradation solution. D.p.p. measurements have enabled us in some cases to detect degradation intermediates and also provided valuable information about the optimum conditions for their preparation.

D.p.p. seems to be competitive with other methods which are usually used to study the degradation kinetics of β-lactam antibiotics. These latter methods which depend upon measurements of the change in the amount of the intact drug compound or, alternatively, on determining the amount of degradation product, either biologically or chemically, lack selectivity and sensitivity and are time consuming. Recently, HPLC with UV detection has been used increasingly for following degradation reaction in aqueous solutions. A possible problem which may arise with the use of HPLC is that compounds that degrade rapidly in aqueous solutions may possibly degrade further, more rapidly, and possibly by a different mechanism, during their passage through an HPLC column.

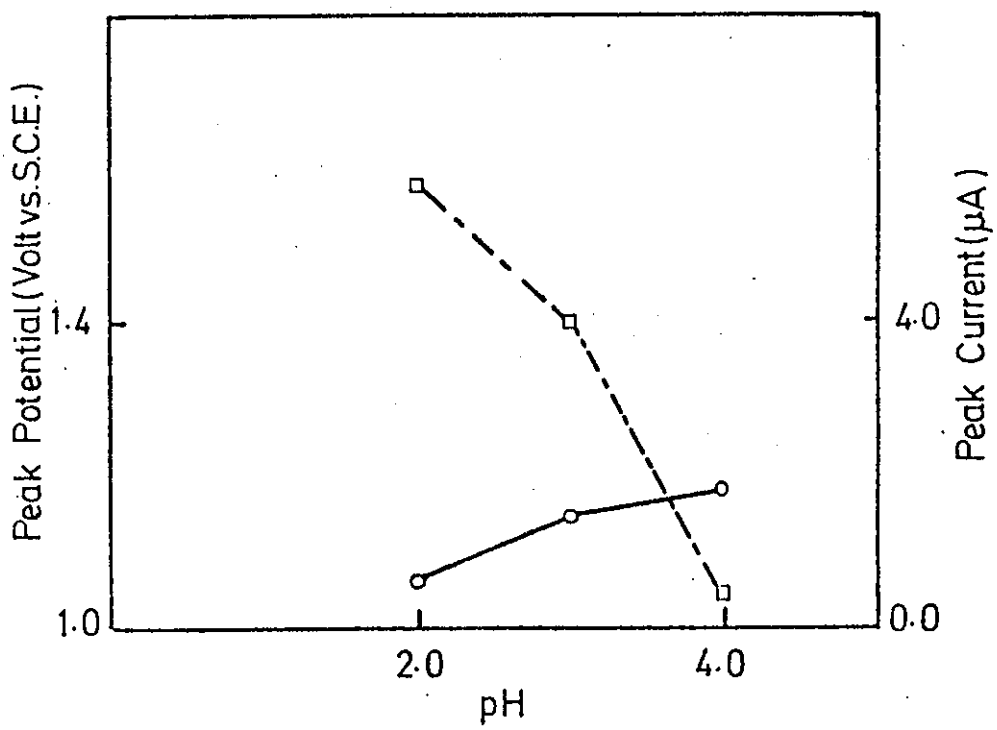
APPENDICES

APPENDIX 1

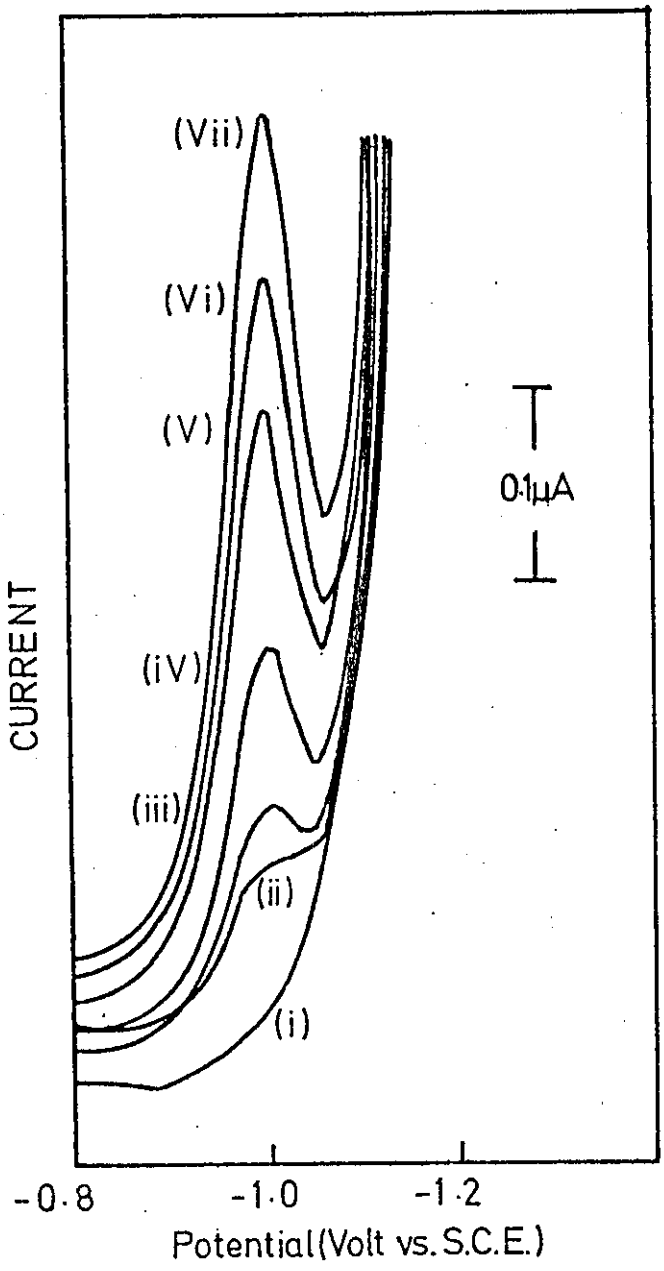
Typical d.p. polarograms obtained for the cephalosporins studied at the optimum pH are shown, together with graphs showing the effect of pH on the peak potentials (full lines) and peak heights (broken lines).



D.p.p. of cephalothin in B-R buffer at pH 2. (i) 0, (ii) 0.42, (iii) 0.82, (iv) 1.61, (v) 2.37 μg ml^{-1}.

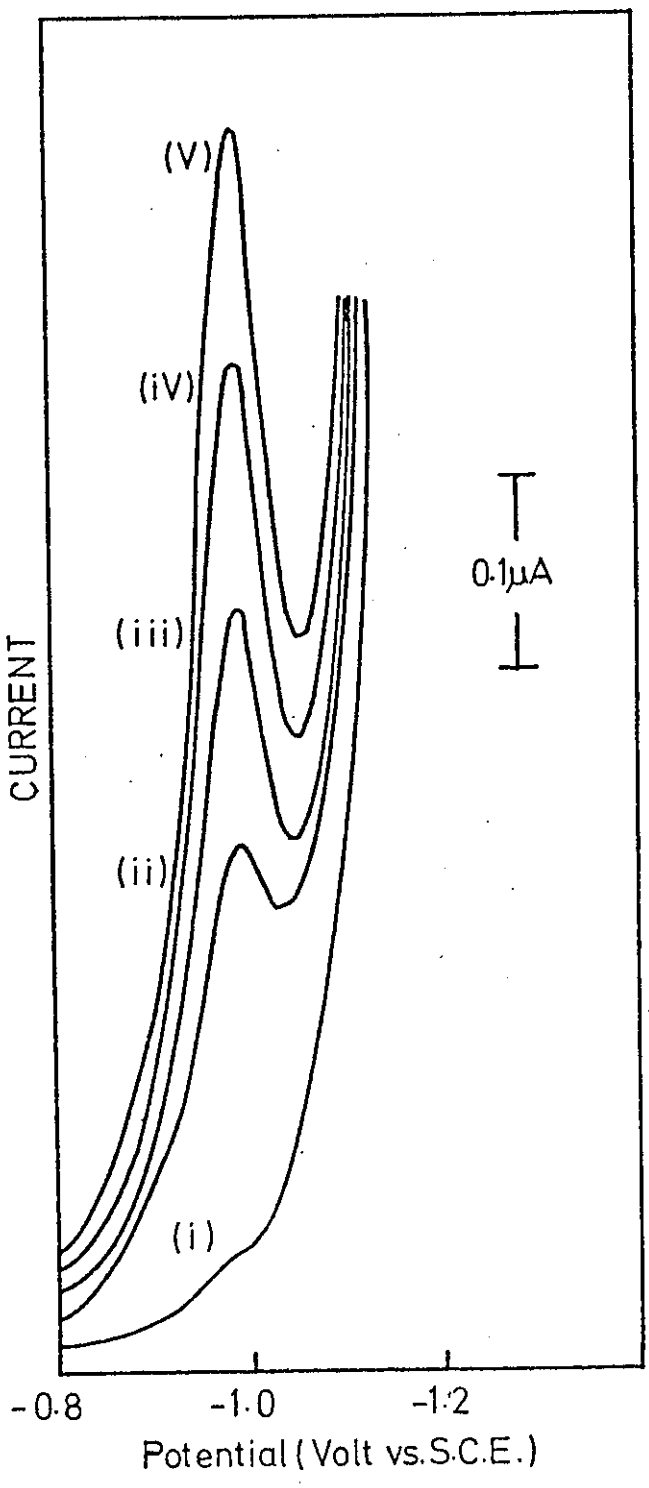


Peak potential and peak current versus pH (B-R buffer)
Cephalothin concentration = 70μg ml^{-1}.



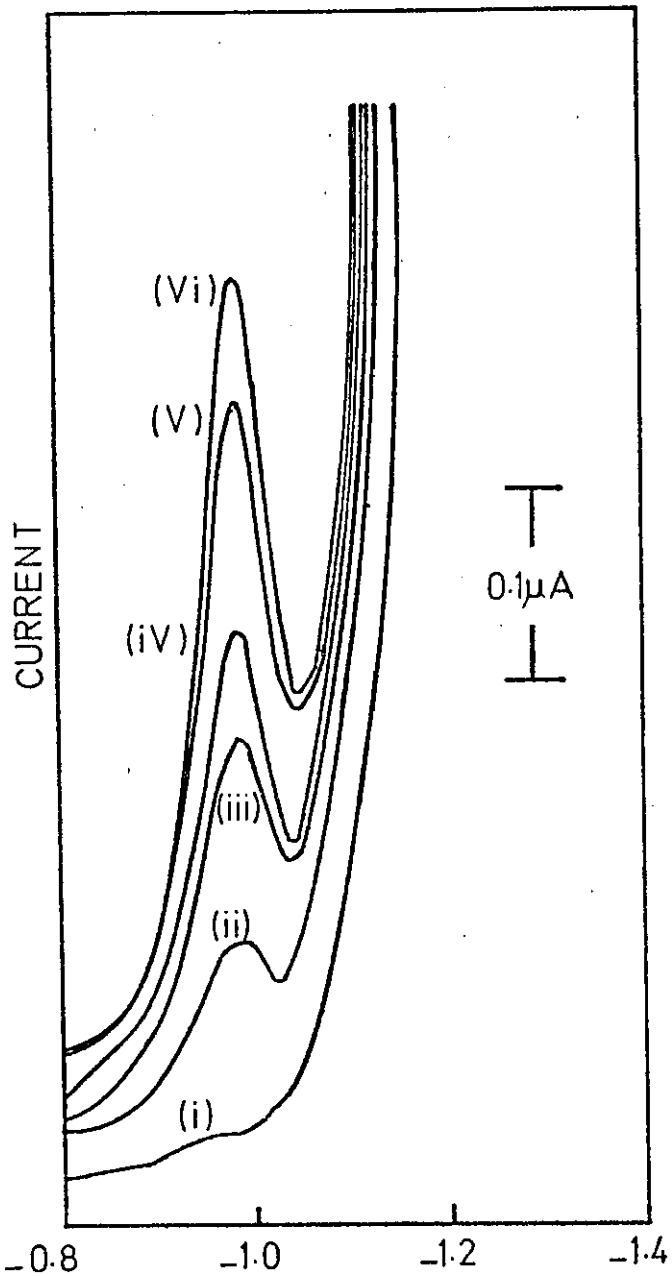
D.p.p. of cephalosporin C in B-R buffer at pH 2. (i) 0, (ii) 0.2, (iii) 0.38, (iv) 0.77, (v) 1.13, (vi) 1.48, (v) 1.82 $\mu g\ ml^{-1}$.

For a 9 $\mu g\ ml^{-1}$ cephalosporin solution $i_p \simeq 0.8\ \mu A$ at pH 2, and $0.5\ \mu A$ at pH 3: the peak disappeared at pH>3.



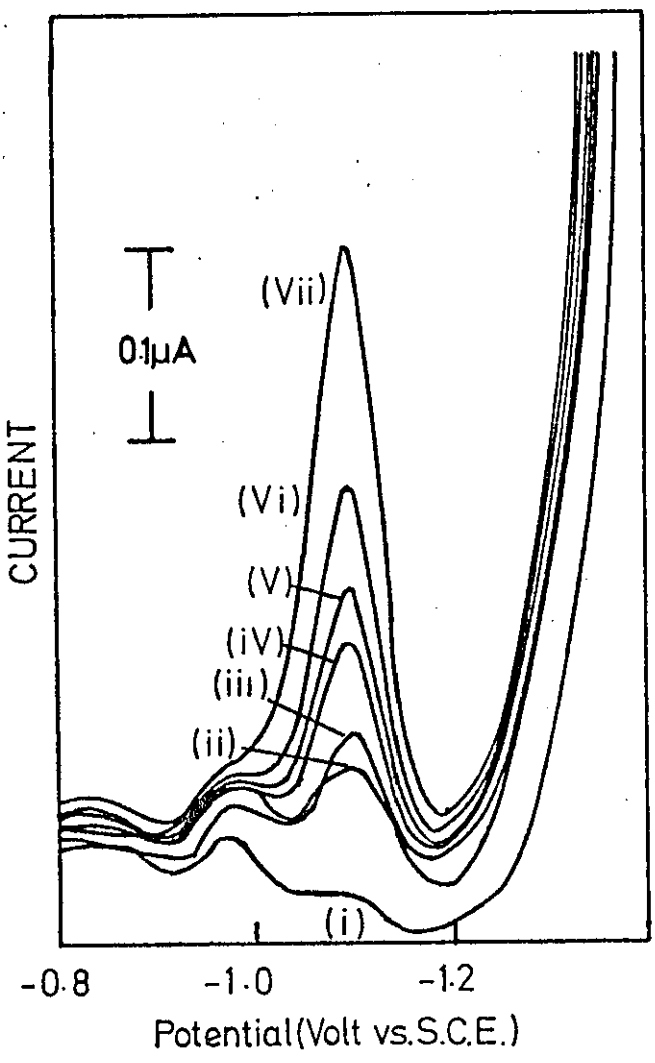
D.p.p. of desacetylcephalosporin C in B-R buffer at pH 2.

(i) 0, (ii) 0.39, (iii) 0.76, (iv) 1.13, (v) 1.48 μg $ml.^{-1}$

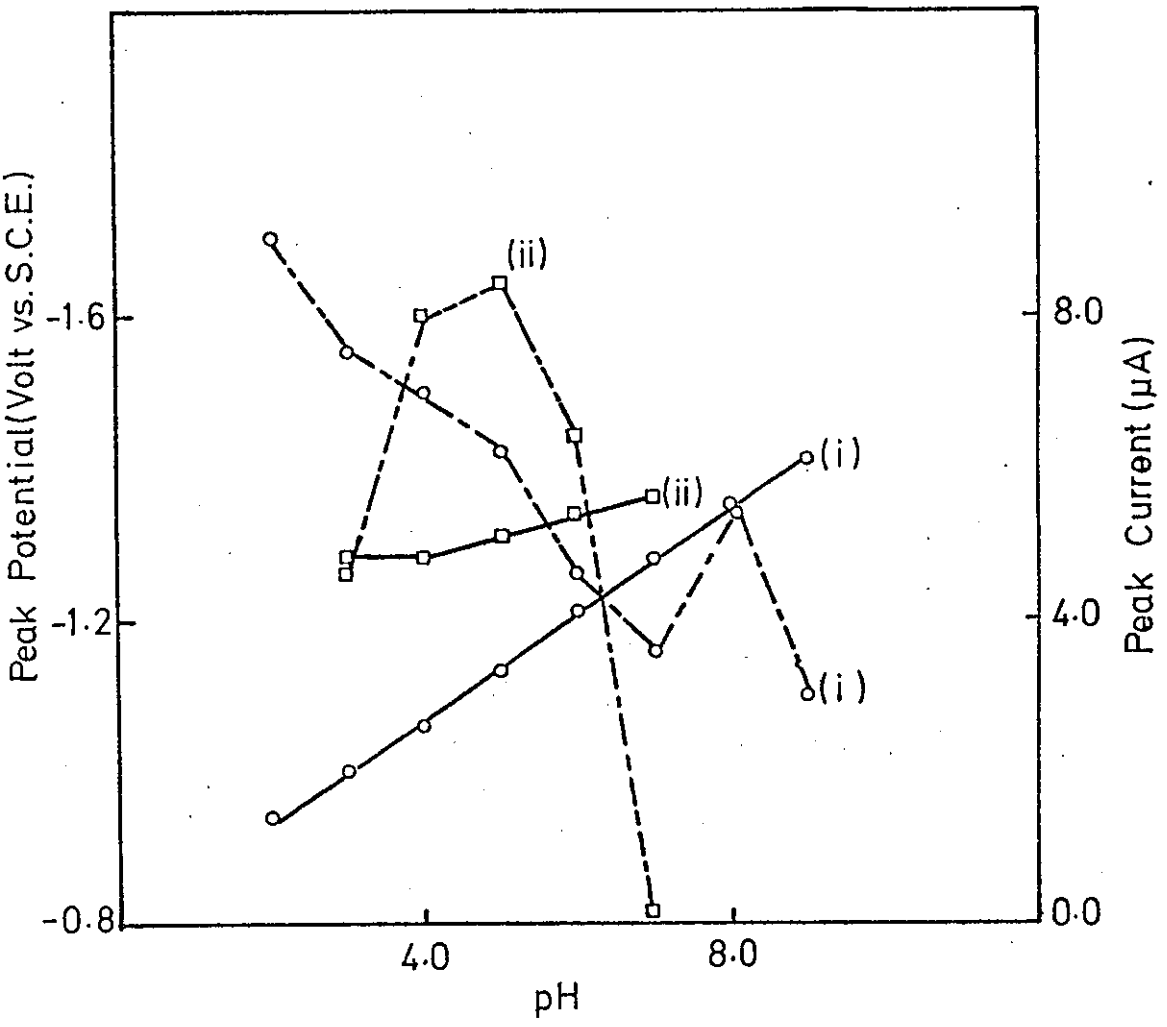


D.p.p. of 7-ACA in B-R buffer at pH 2. (i) 0, (ii) 0.10, (iii) 0.30, (iv) 0.49, (v) 0.65, (vi) 0.86 $\mu g\ ml^{-1}$

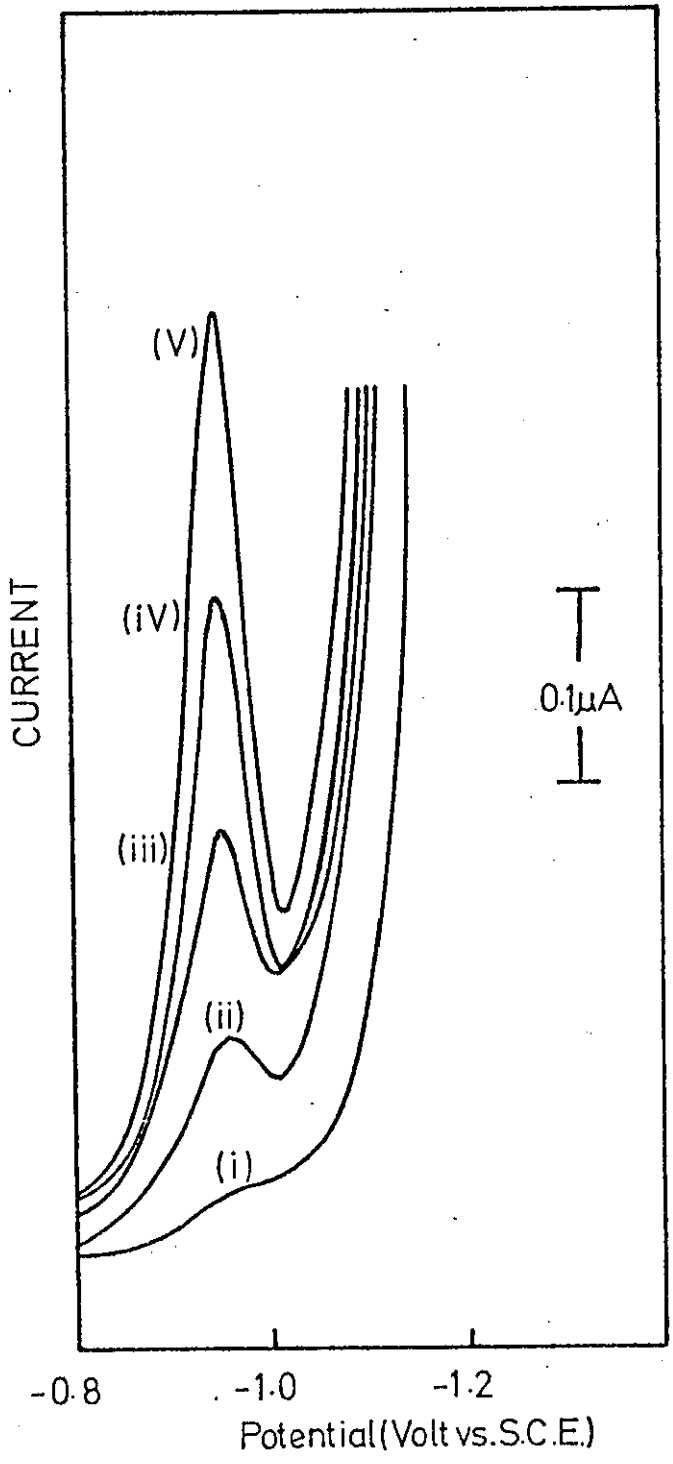
For a 9 $\mu g\ ml^{-1}$ 7-ACA solution i_p = 0.8μA at pH 2 and 0.34 μA at pH 3: the peak disappears at pH≥ 3.



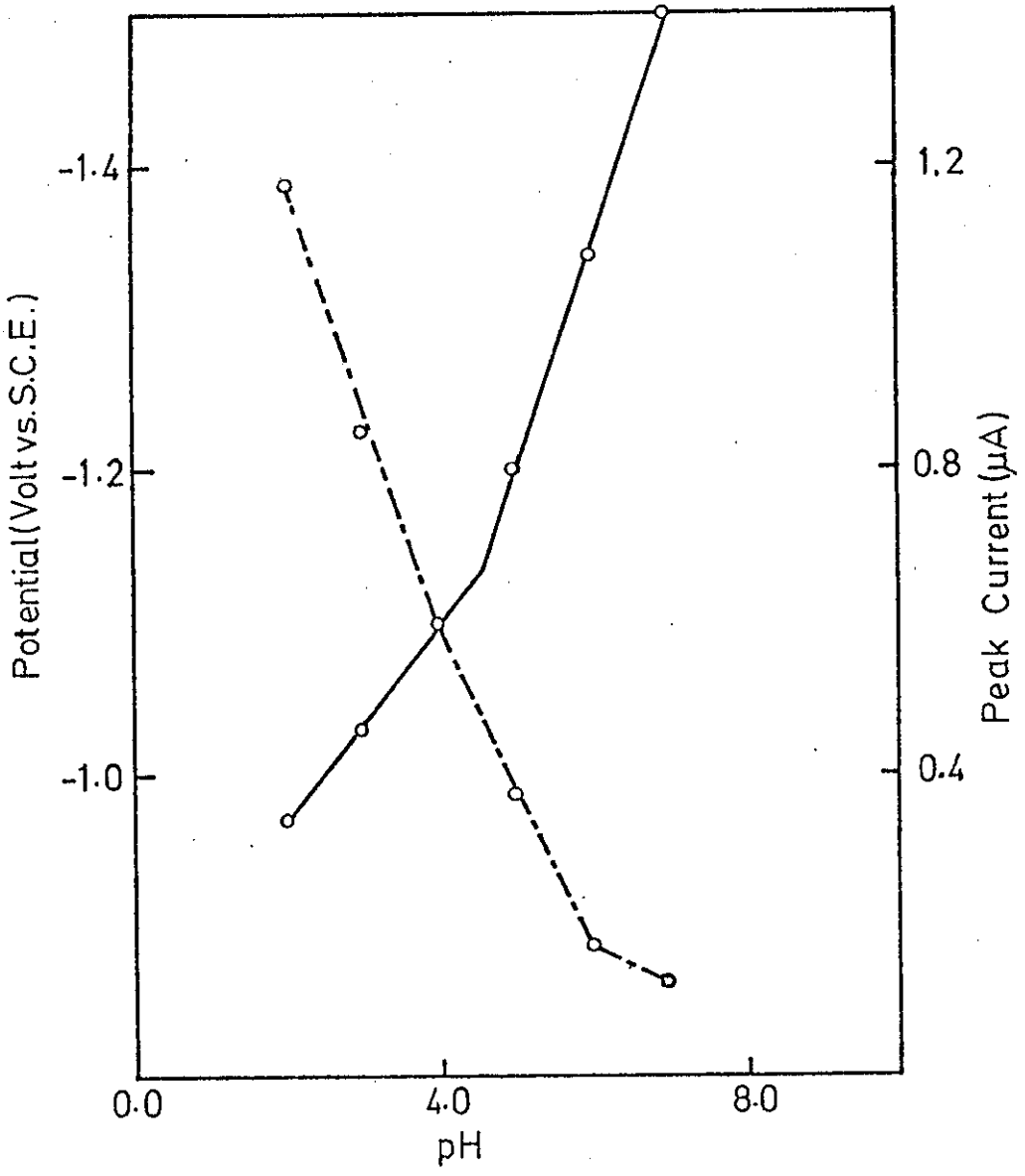
D.p.p. of cephaloridine in B-R buffer at pH 4. (i) 0, (ii) 0.16, (iii) 0.30, (iv) 0.41, (v) 0.57, (vi) 0.79, (vii) 1.32 μg ml^{-1}.



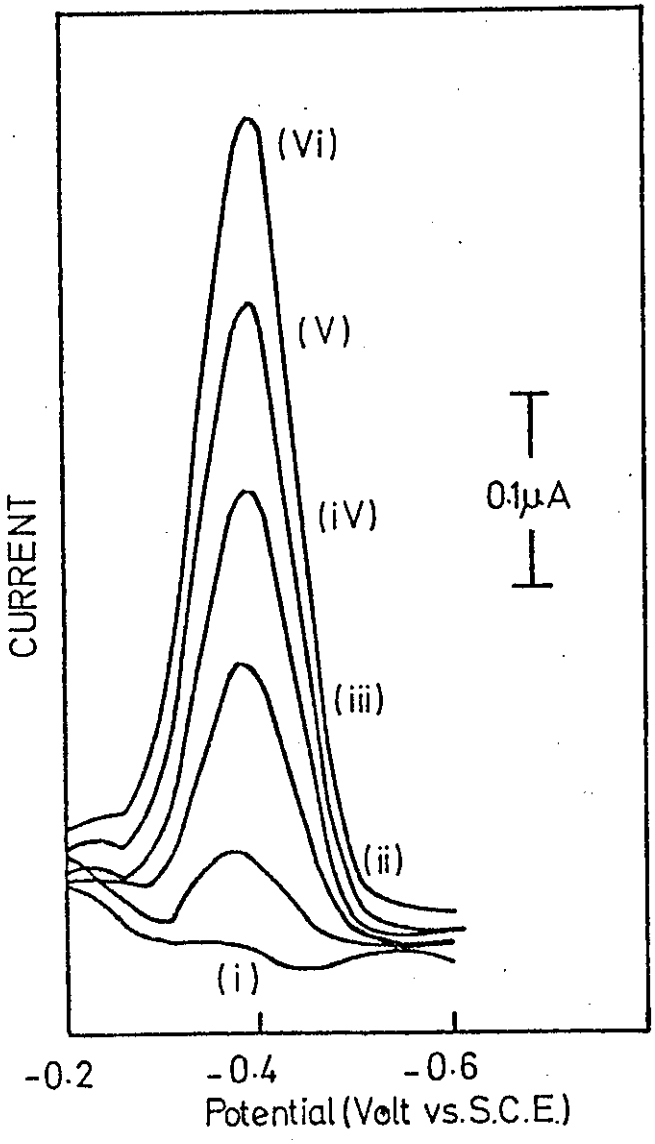
Peak potential and peak current versus pH (B-R buffer)
Cephaloridine concentration = 69 μg ml^{-1}.



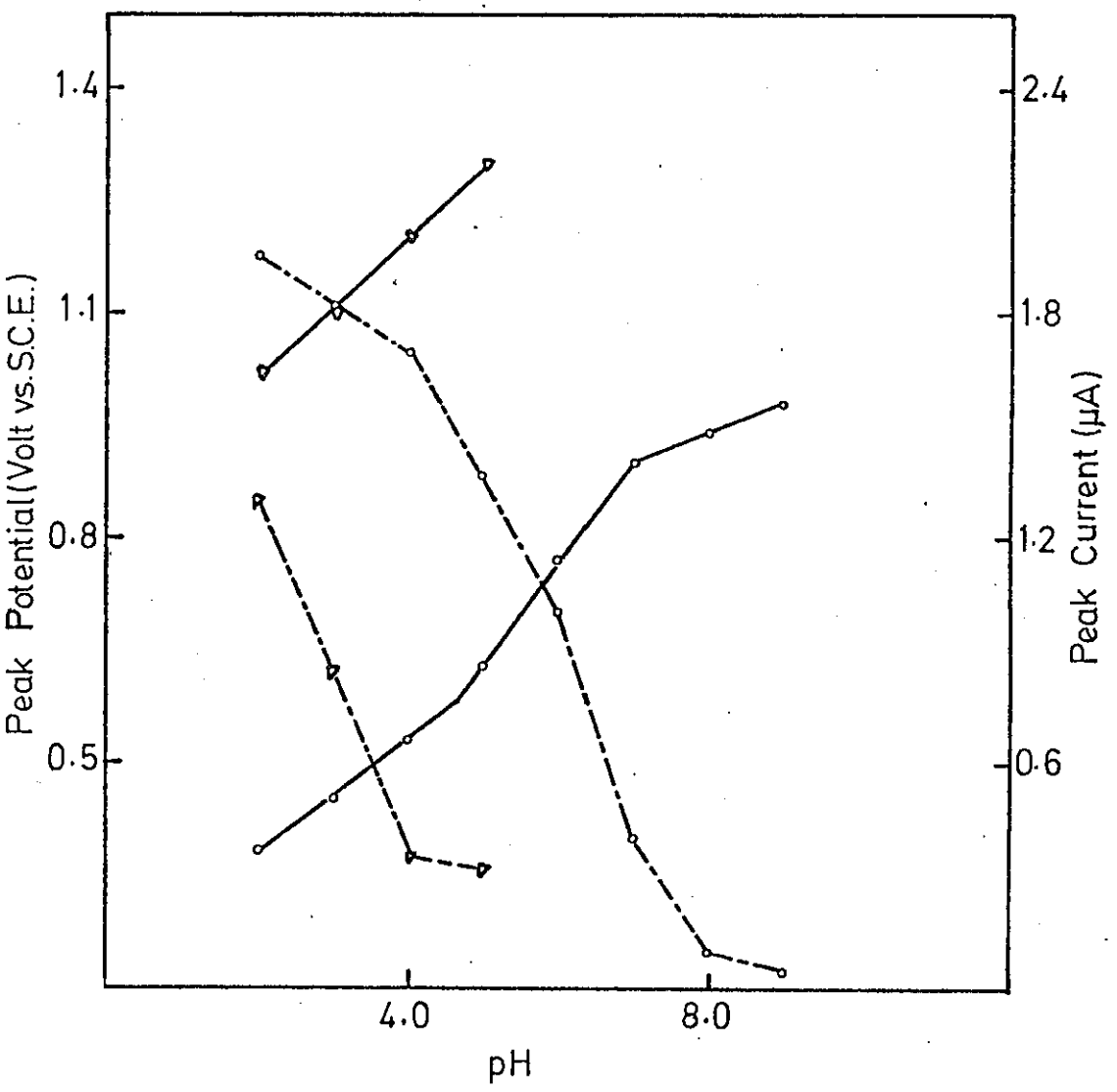
D.p.p. of cephoxazole in B-R buffer at pH 2. (i) 0, (ii) 0.10, (iii) 0.35, (iv) 0.84, (v) 1.33 µg ml^{-1}



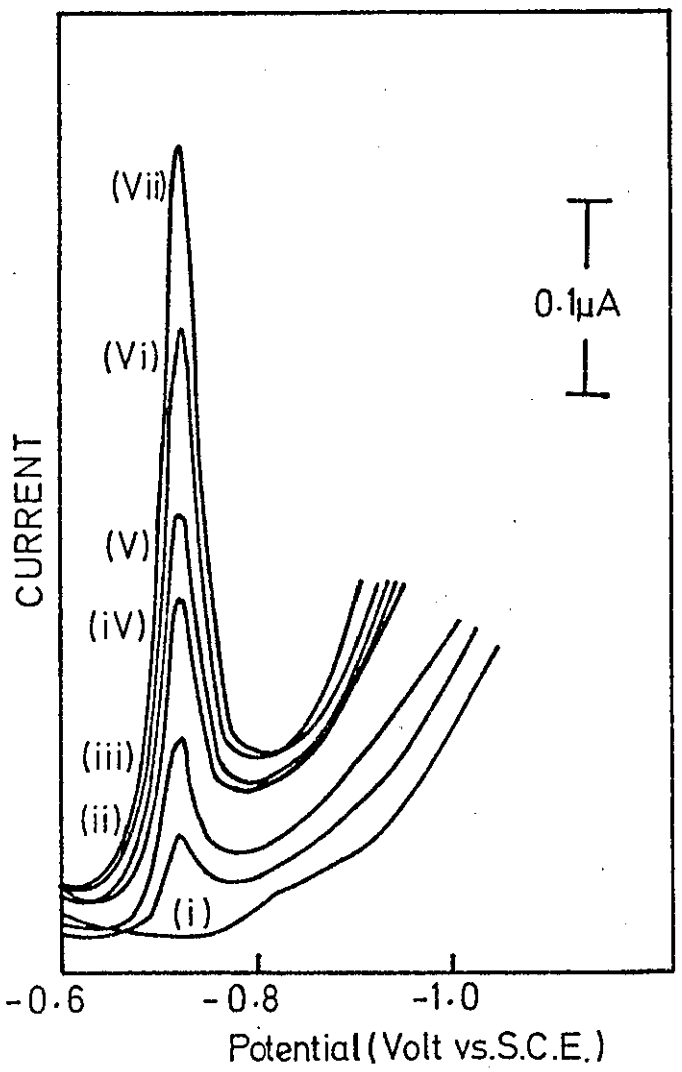
Peak potential and peak current versus pH (B-R buffer)
Cephoxazole concentration = 9 μg ml^{-1}.



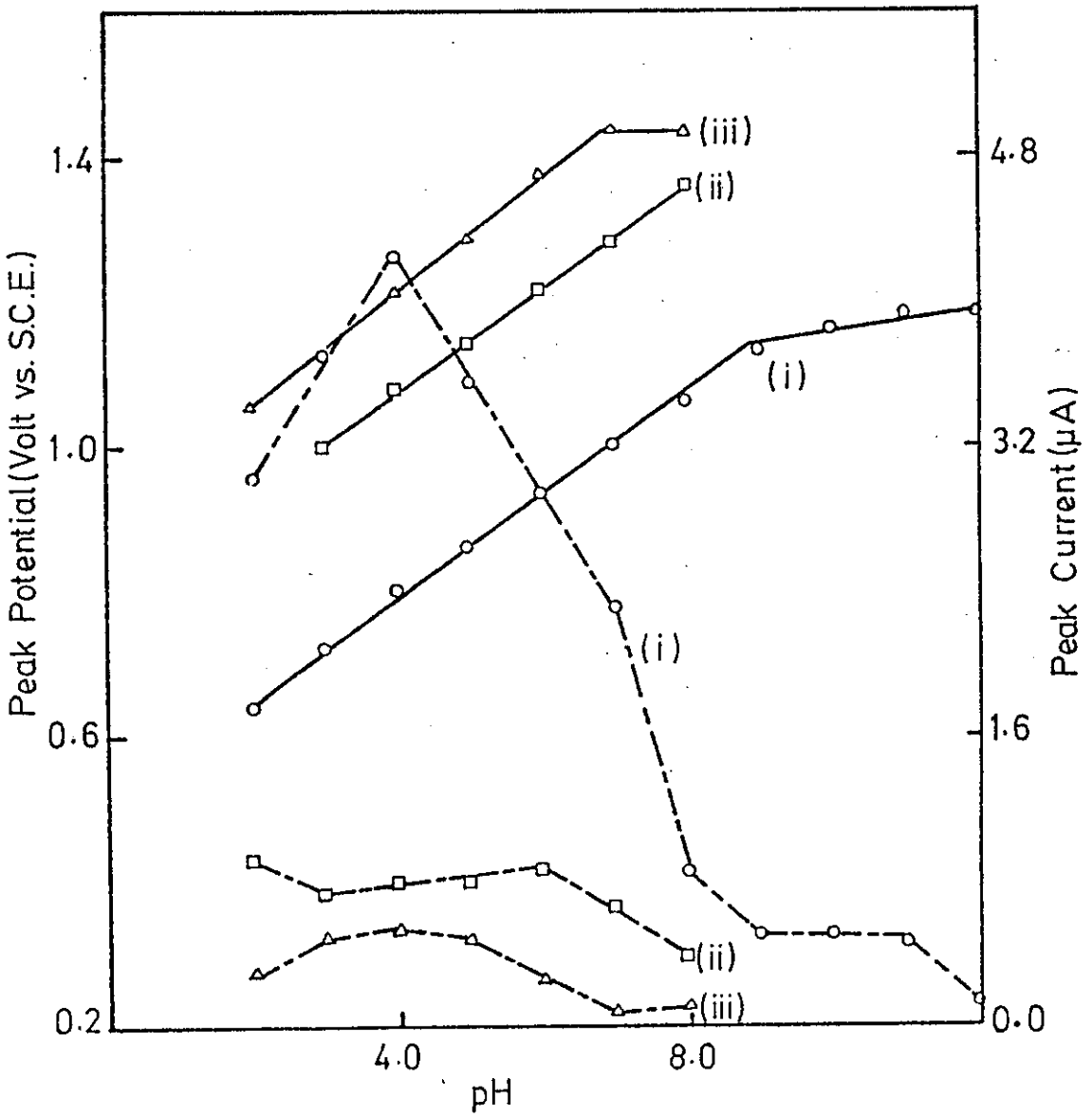
D.p.p. of cefuroxime in B-R buffer at pH 2.5. (i) 0, (ii) 0.10, (iii) 0.35, (iv) 0.59, (v) 0.84, (vi) 1.08 μg $ml.^{-1}$



Peak potential and peak current versus pH (B-R buffer)
Cefurozime concentration = 10μg ml^{-1}.



D.p.p. of cephalonium in B-R buffer at pH 3. (i) 0, (ii) 0.05, (iii) 0.10, (iv) 0.15, (v) 0.20, (vi) 0.29, (vii) 0.38 μg $ml.^{-1}$



Peak potential and peak current versus pH (B-R buffer).
Cephalonium concentration = 9μg ml^{-1}.

APPENDIX 2

Further Comments on Ampicillin Degradation

After this work had been completed, LeBelle *et al*[65] reported the isolation of a fluorescent product following ampicillin degradation. The isolated product was found by unambiguous synthesis and by other analytical techniques to be 2-hydroxy-3-phenylpyrazine. The experimental conditions under which this compound was isolated was slightly different from those of Barbhaiya *et al*[66] in which 2-hydroxy-3-phenyl-6-methylpyrazine was obtained. The present additional study was made in order to see whether this new fluorescent product has the same polarographic and UV absorption characteristics as those of 2-hydroxy-3-phenyl-6-methylpyrazine or not.

In this work the degradation of ampicillin was followed by d.p.p. and UV spectrophotometry under exactly the same experimental conditions as LeBelle *et al*, i.e. hydrolysis of ampicillin (50mg/ml) at pH 11-12 for 2 hours at room temperature, followed by hydrolysis at pH 2 and 50°C for 5 hours. The characteristic d.p.p. peaks of penicillamine and 2-hydroxy-3-phenyl-6-methylpyrazine were again observed but the UV spectrum of the degraded solution was found to be modified. In addition to the broad absorption band at about 260nm, an absorption band with λ_{max} 330nm which overlaps another weaker band at 350nm was observed. A spectrum of a pure sample of 2-hydroxy-3-phenyl-6-methylpyrazine or of a degraded solution of ampicillin in citrate-phosphate buffer at pH 2.5 shows two well defined

absorption bands at 250nm and 350nm (Fig. 1). LeBelle *et al* speculated that the different product obtained by Barbhaiya *et al*[66] is due to the use of formaldehyde as a catalyst (in the latter case) which is incorporated into the final reaction product, but we have shown earlier that 2-hydroxy-3-phenyl-6-methylpyrazine can be formed in the absence of formaldehyde and under different conditions of those of Barbhaiya *et al*. It seems to be that under the experimental conditions of Le Belle *et al* both 2-hydroxy-3-phenylpyrazine and 2-hydroxy-3-phenyl-6-methylpyrazine is formed and these two compounds are indistinguishable by d.p.p. Jusko[53] reported that at low ampicillin concentrations only one fluorescent degradation product was detected, while at higher concentrations several fluorescent products were obtained. No more work has been made in this direction and further investigations need to be carried out to shed more light on the mechanisms by which these fluorescent degradation products are formed.

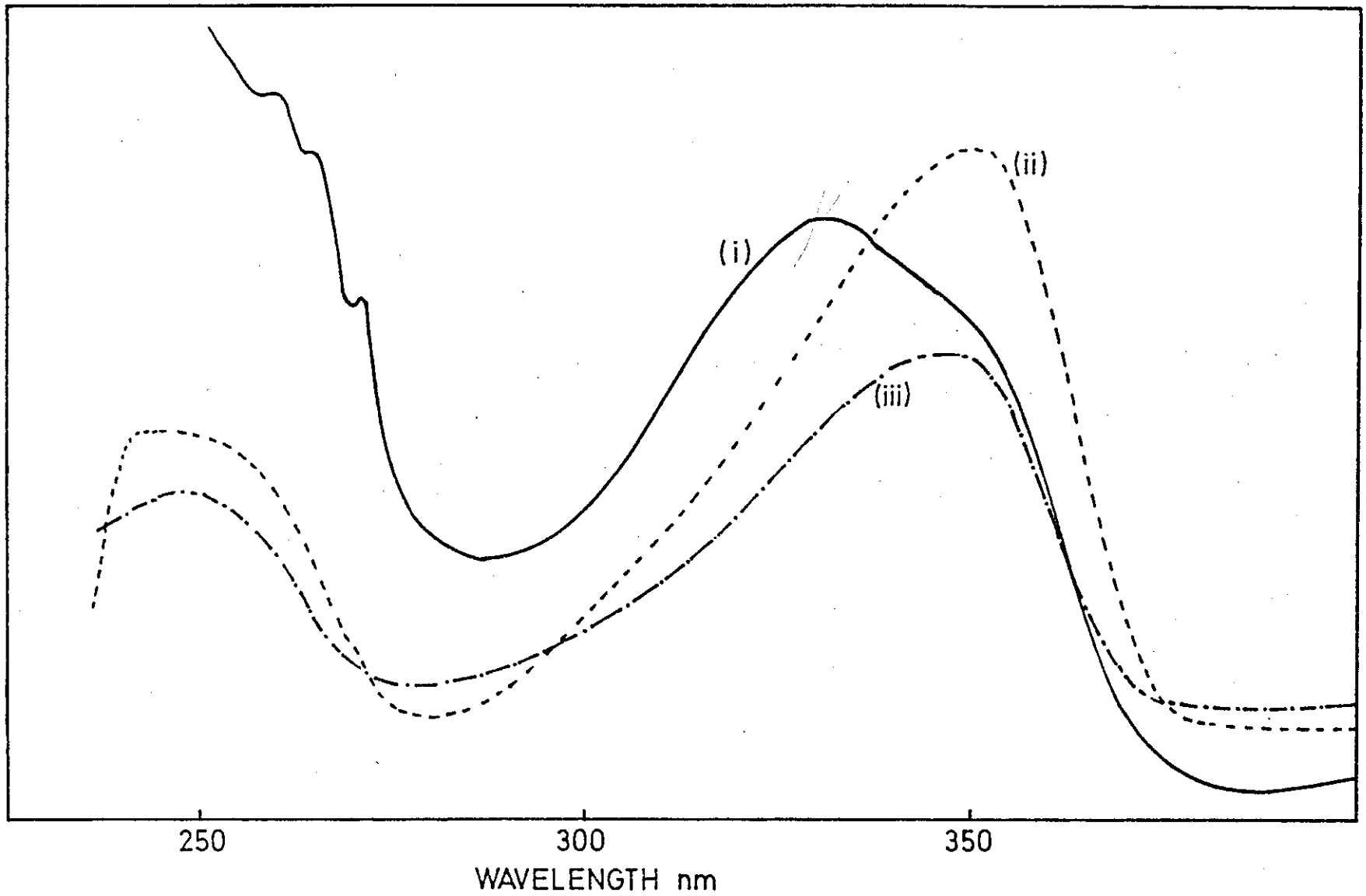


Fig. 1. UV absorption spectra obtained for the fluorescent degradation product of ampicillin: (i) By the method of LeBelle et al; (ii) After hydrolysis in citrate-phosphate buffer pH 2.5; (iii) Pure sample of 2-hydroxy-3-phenyl-6-methylpyrazine

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