THE DETECTION AND DETERMINATION OF DRUGS AND THEIR METABOLITES IN BIOLOGICAL FLUIDS.

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SUMMARY

This thesis is concerned with the detection, identification and estimation of drugs and their metabolites in body fluids. The principal drugs studied were the halogenated drugs chlorbutanol and chloral hydrate.

One widely used technique for the detection of chloral hydrate and its metabolite, trichloroacetic acid, is the Fujiwara test. This test was also known to be capable of detecting chlorbutanol. The reaction conditions were studied to determine the optimum conditions for the detection of chlorbutanol. As part of the investigation into the optimum conditions an investigation was carried out into the mechanism of the Fujiwara reaction. Previously this test was thought to detect only a series of halogenated compounds. This investigation showed, however, that this is not correct. A positive reaction can be obtained if the reaction conditions lead to the attachment of an electrophilic group to the nitrogen atom of a pyridine ring if this group, in the presence of base, gives rise to a conjugated system which can give rise to an ionised species. A modification of this test was also used for quantitative estimation of the metabolites of chloral hydrate.

For the identification of the drugs in body fluids

gas chromatography was found to be the most suitable technique. A number of possible systems were investigated and the most suitable chosen for the routine identification and estimation of these drugs. This technique was preferred to the Fujiwara test for estimations as more than one drug can be readily estimated in the same sample.

The Fujiwara test was used to study the excretion of chlorbutanol by racing greyhounds, in conjunction with a comprehensive study designed to assess all aspects of the drug's effects on these animals. A similar study was carried out using phenobarbitone.

The gas chromatographic technique was used to investigate the effectiveness of the Fujiwara technique developed, and also a number of aspects relating to the handling of samples thought to contain chlorbutanol.

Both the Fujiwara and gas chromatographic techniques were employed in the routine analysis of samples.

A study was also carried out into the rapid detection of phenylbutazone and contraceptive steroids. Existing techniques were found to be satisfactory for the detection of phenylbutazone and its metabolites, and these substances were characterised using these techniques. However a simple, speedy technique is not practical for the detection of contraceptive steroids

in urine samples.

The technique of gas chromatography was also used to identify and estimate a number of volatile materials. This was originally carried out to detect contaminants in the reagents used for the Fujiwara test, but also found an application in a post-mortem analysis. One of the column packings found to be unsuitable for the identification of chlorbutanol was found to be capable of separating a wide range of volatile materials. This range included ethanol and trials were carried out in conjunction with a test of the effect of orally administered fructose upon ethanol metabolism. This column packing was found to be a useful supplement to the standard packing used for the determination of blood and urine ethanol concentrations.

INTRODUCTION

This thesis is concerned with the detection and identification of drugs and their metabolites in body fluids. Although a vast literature exists relating to this type of work, the field is large and has not been investigated evenly. This means that, although basic data is often available for many drugs, further work is required to provide satisfactory techniques for specific purposes.

The techniques employed for this work were those commonly used in analytical toxicology. Since toxicology involves the detection of small quantities of material in a complex biological matrix, chromatographic techniques are very common and both thin layer and gas chromatography were employed. Spectrophotometric techniques generally require a purified sample in much greater quantities than do the chromatographic techniques and were less widely used. Ultraviolet spectrophotometry does not require an absolutely pure sample and is fairly sensitive. Therefore it was of some assistance in this work. Infra-red spectrophotometry is much less sensitive and requires a pure sample and is consequently less widely used. This technique was, however, useful during investigations into an analytical technique.

The work was sponsored by the National Greyhound

Racing Society of Great Britain, so the problems studied are related mainly to the detection of drugged greyhounds. The Society has used analytical toxicology for this purpose for some years, and there are laboratories at a number of race tracks which carry out pre-race testing.

One of the tests employed in the pre-race laboratories is the Fujiwara test which detects a number of halogenated materials. On a number of occasions positive results have been obtained, and it was the purpose of this work to investigate these results further.

The paramount objective of this study was to establish a technique to enable the halogenated material in the samples to be identified. This was thought to be chlorbutanol or one of the metabolites of chloral hydrate, trichloroacetic acid, but this was not known with certainty.

Once the identification technique was established a number of related points were considered. The Fujiwara test was investigated and modified to provide optimum conditions when used to detect chlorbutanol, which to date has been the only drug of this type employed. In the course of the modification of the Fujiwara test a limited investigation into the reaction

mechanism was carried out and a reaction pathway postulated.

Other aspects of the use of the Fujiwara test were more involved with the metabolism of chlorbutanol by the greyhound. Such aspects considered included how quickly the drug actually appeared in the urine after administration and for how long afterwards it was detectable. These were investigated in conjunction with comprehensive trials designed to assess all aspects of the affect of certain drugs upon the racing greyhound. In addition, measurement of the chlorbutanol in the routine samples was carried out with the intent, that in time, some indication of time of administration and dosage may be obtainable from analysis. This needs a large number of samples to be investigated, and since the supply is somewhat unreliable this work has not been completed.

The work carried out in connection with racing greyhounds was not concerned entirely with halogenated drugs. An investigation was made into the detection of contraceptive steroids, with the objective of including a test for these drugs in the pre-race test procedure. From time to time attention was drawn to other drugs, such as phenylbutazone, and their behaviour under the routine test procedures investigated.

Some of the work is not connected directly with greyhound racing. One use made of gas chromatography was the identification of impurities in the reagents used for the Fujiwara test, and this work found use in the detection of trichloroethylene in post-mortem cases. In addition, a gas chromatographic column found unsuitable for the main work was found to be useful for the analysis of samples for ethanol and other volatile materials.

This work therefore is concerned with the application of chromatographic and spectrophotometric techniques to the detection of drugs, and the development of test procedures to detect drugs.

FUJIWARA REACTION - REVIEW OF METHODS

The original report made by Fujiwara (55) appears to have attracted little attention, for in 1923 a paper was published by J. H. Ross (112). In this paper, Ross describes how, by heating chloroform or chloralhydrate with pyridine and aqueous sodium hydroxide solution, a pink colour was produced in the pyridine layer. This is the Fujiwara test but Ross does not refer to Fujiwara.

In the two papers Fujiwara and Ross reported that a positive reaction could be obtained with chloral, bromoform, iodoform, ethylene chloride, Benzoyl Trichloride and trichloroacetic acid as well as with chloroform. However, they realised that this was not the whole range of materials which will cause a positive reaction. Ross postulated that the reaction could be brought about by the group R-CH-X (where X is a halogen atom). Later this was found to be incorrect (130). Trichloroethanol, for example, does not react to give a pink colouration to the pyridine layer (54).

Any analytical technique in which a colour is developed may be tried as a method for the quantitative measurement of one of the reactants. For the quantitative measurement to be a success, the intensity of colour developed must be proportional to the quantity of test material present, and a given quantity

of test material must always produce the same colour intensity. Preferably, also, the reaction should be specific for the material under examination, for instance chloral hydrate reacting with quinaldine ethiodide (7).

The technique given by Ross (112) called for 3 to 5 cc. of 17-25% aqueous sodium hydroxide to be placed in a test-tube and a 2 mm. deep layer of pyridine added. The test substance was then added and the test-tube placed in a boiling water bath, and vigorously shaken. A positive reaction was indicated by a pink colouration of the pyridine layer appearing within a few seconds. When Cole (33) used the Fujiwara reaction quantitatively, he had to standardise reagent strengths and heating times. He had also to increase the volume of pyridine used so that sufficient pyridine layer could be removed to permit measurements to be made by comparison with fuchsin standards.

The method adopted by Cole was similar to that of Ross (112). He used 2.0 ml. of 20% aqueous sodium hydroxide, 1.0 ml. of pyridine and 1.0 ml. of test solution. The test-tube containing this mixture was placed in a boiling water bath so that the water level was above the level of the tube contents, and kept there for one minute. After this time, the tube was

removed and cooled to 20°C for the measurements to be made.

Although Cole managed to obtain measurements with his technique, when Daroga and Pollard (38) investigated means of measuring chloroform and carbon tetrachloride levels in air and soil they found that the pink colour was unstable. The only alternative technique for measuring carbon tetrachloride was the reaction with α-naphthol, which is far less sensitive than the Fujiwara reaction. Daroga and Pollard carried out an investigation into the factors affecting the Fujiwara reaction. These were found to include the sodium hydroxide concentration, the amount of pyridine added, the diluting solvent, and the heating time. As mentioned earlier, they found that the conditions had to be adjusted to suit requirements. They used the Fujiwara reaction to measure chloroform and carbon tetrachloride; Hildebrecht (68) used the same reaction with modified conditions to measure chloroform in carbon tetrachloride.

For their quantitative work, Daroga and Pollard used a Lovibond tintometer rather than the fuchsin standards used by Cole.

One disadvantage of using the Fujiwara test for quantitative work is that the pink colour is unstable.

In the presence of acid the colour is discharged completely, and Daroga and Pollard (38) also found that the colour is unstable in the presence of alkali. In one of their tests the colour decayed from 7.9 units to 2.1 units in 24 minutes. To avoid this, Daroga and Pollard tried diluting the reaction mixture, after heating, with an organic solvent. This improved the stability of the red colour, but a partition of the pink colour between the organic and aqueous phases occurred. The technique they finally adopted was to separate the organic phase, and then dilute it with acetone or water. Under these conditions the pink colour was much more stable, the use of water giving a more intense colour than the use of acetone.

The colour development was also found to be dependent upon the concentration of sodium hydroxide in the aqueous phase. With dilute sodium hydroxide the reaction lacks sensitivity, with concentrations in excess of 20% the red colour decays more rapidly than when using a 20% solution.

A further variable was the quantity of pyridine used. Colour stability was enhanced by using high concentrations of pyridine in the final sample used for measurements. Unfortunately, this made the colours dull and difficult to match.

Later authors found other factors to be of importance when using the Fujiwara reaction quantitatively. However the technique developed by Daroga and Pollard has been widely used. Nearly all other users varied the exact reaction conditions, but all used the idea of separating the pyridine layer from the aqueous layer and diluting with another solvent before making measurements (25, 26). Other users of the technique described by Daroga and Pollard were Habgood and Powell (65). These authors were interested in the determination of carbon tetrachloride, chloroform and trichloroethylene in blood. These compounds were isolated by steam distillation, the chlorinated hydrocarbons distilling over in the first fraction. Whilst the use of 1 ml. of the first fraction of the distillate was capable of detecting the chlorocarbons, extracting the distillate with toluene produced up to a fifty times increase in sensitivity. Further, when determining the carbon tetrachloride concentration, the addition of acetone to the reaction mixture intensified the colour. The addition of acetone had no effect on the colour produced by trichloroethylene and an adverse effect on that produced by chloroform. Using this technique Habgood and Powell obtained recoveries in the range

95 - 106% for each of the three chlorinated hydrocarbons.

In the same year that Habgood and Powell reported their studies using the method of Daroga and Pollard, Webb, Kay and Nichol (130) published a more intensive investigation of the Fujiwara reaction. Like many others, they were interested in the determination of carbon tetrachloride. Their work is distinctive from that of Daroga and Pollard only in scope. They successfully reduced volumes of reagents required, examined a range of possible solvents for dissolving the test material, and examined a range of halogenated materials.

Where the method of Daroga and Pollard called for 1 ml. of test solution to be treated with 10 ml. of pyridine, the method of Webb, Kay and Nichol required only 2 ml. of pyridine to be added to the 1 ml. of test solution. This gives an increase in sensitivity due to less dilution of the pink colour with pyridine.

The authors' findings relating to the optimum concentration of hydroxide parallel those of Daroga and Pollard. These are that whilst sodium hydroxide concentrations in the range 5 - 20% give a good red colour, 25% gives a pink colour and 30% a pale yellow colouration. The total volume of hydroxide used is

also critical, too little causes the colour to be feebly developed.

The authors' investigations regarding the effects of solvents are of more interest. For testing solvents, the authors used a mixture of 5 ml. of 20% aqueous sodium hydroxide solution, 2 ml. of pyridine and 1 ml. of test solution. The conclusion they reached was that whilst 95% ethanol, petroleum ether and dioxane were acceptable, acetone gave a far more intense colour. This is exactly the conclusion reached by Habgood and Powell (65). Webb, Kay and Nichol were able to show that the optimum sodium hydroxide concentration varied from solvent to solvent. For solvents such as acetone, dioxane, ethanol and ether, 10% aqueous sodium hydroxide was optimum, for petroleum ether and toluene 20% sodium hydroxide was preferable. In general, the less miscible the solvent is with water the greater the concentration of sodium hydroxide required.

The wide range of materials tested by these authors indicated that the sodium hydroxide concentration also needed adjusting for each test substrate if optimum results were required. For most materials the optimum sodium hydroxide concentration was in the range 10 - 20%.

Whilst earlier authors, for example Ross (112), Friedmann and Calderone (53) considered that the pink colouration was given by compounds containing the R-CH-X group, the work of Webb, Kay and Nichol (130) showed that this is not so. In general, trichloro groups are more reactive than dichloro groups, whilst, with the exception of 2 4 dinitrochlorobenzene, monochloro groups are not reactive. The test is less sensitive to bromine groups than to chlorine groups.

Shortly after this publication, details of a single phase system appeared. This was by Rogers and Kay (111). All techniques to date had found it necessary to have a separate organic phase where the reaction occurred and an aqueous phase containing the sodium hydroxide. Webb, Kay and Nichol had commented on the fact that the less miscible solvents required stronger sodium hydroxide solutions. Rogers and Kay reported that by shaking one volume of pyridine with two volumes of 15% aqueous sodium hydroxide solution, standing overnight, and separating sufficient sodium hydroxide could be dissolved in the pyridine to carry out the Fujiwara reaction. To use this reagent however, Rogers and Kay dissolved their test substrates in acetone, a water miscible solvent. Perhaps an

immiscible solvent would cause phase separation or precipitation of the dissolved sodium hydroxide. This form of single phase technique permits investigation into the effect of the water and sodium hydroxide concentrations in the organic phase upon the course of the reaction. Rogers and Kay did a certain amount of work in this direction, their findings being that 0.5 ml. of 15% aqueous sodium hydroxide solution in 100 ml. of pyridine was sufficient to produce maximum colour development. In addition, water plays an important part in the colour producing reaction.

In the time between 1914 and 1948, the users of the Fujiwara reaction had been investigating the metabolism and detection of the halogenated hydrocarbons chloroform, carbon tetrachloride and trichloroethylene. Although some work had been done to investigate the ability of the test to detect other materials, this work was mainly directed to improving the test itself, or the users' knowledge of the test.

The Fujiwara test is not at all specific for one material, but in 1948 Butler (24) was able to use the test to investigate the metabolism of chloral hydrate. The chloral hydrate, it was suspected, was metabolised

to trichlorethanol, trichloroacetic acid, and urochloralic acid. These materials could be expected to occur simultaneously, and to complicate the detection even more, trichlorethanol does not give a pink colour under the Fujiwara test conditions. The technique was not simple, but it was effective, using solvent extraction to separate the trichlorethanol, chloral hydrate, and trichloroacetic acid. The trichloracetic acid and chloral hydrate levels were readily determined. To detect the trichloroethanol using this test the trichloroethanol is oxidised to trichloroacetic acid using potassium dichromate-sulphuric acid as oxidising agent.

This technique does not detect urochloralic acid. This was shown to be a trichlorethanol complex by extracting to remove all trichloroethanol from a sample, and hydrolysing with acid. More free trichloroethanol was detected after hydrolysis.

Using this technique, Butler was able to show that in the dog a high proportion of the chloral hydrate "perhaps all not oxidised to the acid (trichloroacetic acid) is reduced to the alcohol (trichloroethanol)".

The colour development technique employed by Butler was a modification of the two phase system of

Daroga and Pollard (38).

Such work as was done by Butler possesses a certain elegance in spite of being somewhat devious and tedious to carry out. A similar piece of work was done to study the excretion of trichloroethylene, trichloracetic acid and trichloroethanol in urine (119). The trichloroethylene was extracted from the urine into toluene, and the Fujiwara reaction carried out employing 1 ml. of toluene with 5 ml. of pyridine and 0.35 ml. of 1% ethanolic potassium hydroxide. The mixture was allowed to stand for 5 minutes at room temperature, 1 ml. of water added and allowed to stand for a further 10 minutes at room temperature. At the end of this time, 3 ml. of 95% Ethanol is added to clarify the turbid solution and the optical density determined within 5 minutes.

This is a very interesting technique as the colour is developed at room temperature, and this colour is attributed to trichloroethylene. To use this technique successfully, one must be careful with the timing of the various steps, and the strength of the potassium hydroxide is critical. Butler tried the use of xylene instead of toluene and found that for a given trichloroethylene concentration the colour intensity when using xylene was 30% less than when

using toluene.

To determine trichloroacetic acid in the urine Seto and Schultze employed a very different technique, that of Powell (102). The most interesting point is the use of a temperature of 65°C for 40 minutes to develop the colour rather than the more often used 100°C for 1 - 5 minutes. It was claimed that this gave a more sensitive and more reproducible test.

Having determined two components of a three component system, Seto and Schultze could determine the trichloroethanol by the difference between the total "trichloro" content of the urine and the sum of trichloroacetic acid and trichloroethylene. It is, of course, necessary that the trichloroethanol should be rendered detectable. To do this, the trichloroethanol was oxidised. However, they considered that the technique of Butler (25) failed to give quantitative results, as did many other oxidations so they developed one using chromic oxide/nitric acid mixture at 37°C for twenty hours.

A very elegant concept, but this approach was soon overtaken by modern instrumental techniques, which reduce the amount of chemical treatment and handling of samples required.

It is generally known that trichloroethanol does

not give a pink colour under the Fujiwara reaction conditions. However, a reaction does occur which creates two absorption bands when measured on an ultraviolet/visible light spectrophotometer. One of these bands occurs in the ultraviolet region of the spectrum at a wavelength of 370 mμ, and also occurs when chloral hydrate and trichloroacetic acid are subjected to the Fujiwara reaction. The other absorption band occurs in the visible part of the spectrum at a wavelength of 440 mμ, corresponding to yellow colour. Trichloroacetic acid and chloral hydrate have a second band in the red part of the visible light spectrum, this being at a wavelength of 540 mμ.

Friedmann and Cooper (154) used these absorption bands when studying the metabolism of chloral hydrate and wished to measure levels of chloral hydrate, trichloroacetic acid and trichloroethanol.

To measure the total "trichloro" level of a sample merely involved heating 5 ml. of pyridine, 1.0 ml. of sample and 2.0 ml. of 10M aqueous potassium hydroxide solution in a boiling water bath for exactly four minutes. After cooling in an ice bath, 3.0 ml. of the pyridine layer and 0.5 ml. of distilled water were used for spectrophotometric measurements at both

370 mμ and 440 mμ wavelengths. The absorption at 370 mμ was due to the combined effect of chloral hydrate, trichloroacetic acid and trichloroethanol. The contribution due to trichloroethanol could be calculated from the absorption at 440 mμ, hence the sum for chloral hydrate and trichloroacetic acid at 370 mμ can also be calculated.

To measure the contribution of trichloroacetic acid, 1.0 ml. of sample was heated with 2.0 ml. of 10M aqueous potassium hydroxide for two minutes on a boiling water bath. This decomposes chloral hydrate but not trichloroacetic acid. After heating, the sample is cooled in an ice bath for two to three minutes, 5.0 ml. of pyridine added and then the procedure is as described earlier, except that it is only necessary to take measurements at a wavelength of 370 mμ.

Friedmann and Cooper (54) investigated the reaction conditions carefully. They were not able to achieve a completely stable chromophore, though using water as a diluent to clarify the pyridine was far superior to the use of acid, base or ethanol. This necessitated the running of standards with samples when carrying out determinations using this technique. However the technique was an improvement over earlier methods, partly because of the simpler sample preparation required

and partly because of the increased sensitivity gained by using the absorption band at 370 mμ which is three to four times as intense as that at 540 mμ. Additionally, this band is more stable than the 540 mμ absorption band.

One disadvantage of this technique, however, is that the absorption of trichloroethanol and chloral hydrate at 370 mμ is not strictly addative, which leads to some error in the results. These errors are accentuated by the absorption bands due to chloral hydrate and trichloroacetic acid at 540 mμ and 370 mμ tailing over the 440 mμ band of trichloroethanol. The procedure was adapted by Cabana and Gessner (26) to overcome these difficulties and to enable urochloralic acid to be determined as well as the other three materials.

They achieved this by ignoring the absorption band at 370 mμ, and only using the bands at 440 mμ and 540 mμ, the advantages being that there is no absorption at 540 mμ due to trichloroethanol, and the total absorption at 440 mμ is the sum of the individual absorptions of such of the compounds as were present in the original sample. The estimation still depended upon the destruction of chloral hydrate by heating with base, 10M aqueous potassium hydroxide being used. To

determine urochloralic acid, the sample was first hydrolysed with 12N hydrochloric acid, the increase in total "trichloro" content over an unhydrolysed sample being attributed to urochloralic acid.

The technique suffered from two defects which were mentioned by the authors. Firstly, the apparent level of trichloracetic acid declined by 10.4% after heating with potassium hydroxide. This presumably was due to the destruction of trichloroacetic acid as well as the chloral hydrate. Secondly, when using tissue homogenates containing known quantities of trichloroacetic acid, the absorption was 40% less than expected. This they attributed to quenching of the chromophore by the tissue supernatant.

Whilst the method of Cabana and Gessner does avoid errors due to spectral difficulties, which are probably smaller than errors due to chemistry anyway, the method of Friedmann and Cooper (54) used the additional sensitivity made available by using absorption bands which occur in the ultraviolet region of the spectrum. Both methods fail to avoid the old problem of instability of the chromophore measured. The only apparent way of avoiding this is to use a derivative of the coloured product, and forming the derivative as rapidly as possible.

Feigl in his book "Spot Tests in Organic Analysis" (47) describes how the colour formed in the Fujiwara reaction may be discharged by the addition of acetic acid to the pyridine layer. Subsequent addition of benzidine then causes a violet colouration.

This reaction was used by Leibman and Hindman (86). They hoped to attain the greater sensitivities achieved by Friedman and Cooper using the absorption band in the near ultraviolet, and to be able to estimate trichloroethanol readily and avoid interference due to tissue extracts. They were, on the whole, successful.

Two variations were developed by Liebman and Hindman. The first was to measure the total "trichloro" content of a sample, as was done by Friedman and Cooper (54). To do this, a mixture of 2 ml. of the test sample, dissolved in water, 2.0 ml. of pyridine and 4.0 ml. of 10M aqueous potassium hydroxide solution were heated in a boiling water bath for exactly three minutes, and then chilled in an ice bath. After chilling, 1.0 ml. of the pyridine layer was removed and added to 0.2 ml. of a 3% solution of benzidine in 88% formic acid. After 30 minutes at room temperature the absorbance could be measured at 530 mμ on a spectrophotometer. The second variation corresponded to the second variation of Friedman and Cooper, 2.0 ml. of

test solution being hydrolysed by 4.0 ml. of 10M potassium hydroxide at 100°C for 2 minutes and then colour development as before.

Liebman and Hindman do not claim that conditions employed by them are optimum, for example, the use of 10M potassium hydroxide instead of 20M. With trichloroacetic acid the colour developed was 92% of that developed using 20M hydroxide, and with trichloroethanol only about half. However, this was to enable them to analyse tissue extracts prepared by tungstic acid precipitation of proteins.

The loss of sensitivity here was more than offset by the gain achieved by using this technique. For a given strength of trichloroacetic acid the absor at 530 mμ using this technique was five to eight times that of the Fujiwara reaction product at the same wavelength and about five times that of the product of the Friedman and Cooper method at 370 mμ.

One major advantage of the technique is the stability of the coloured reaction product. This colour is formed rapidly, 80% of the maximum value being attained in 30 seconds, though thirty minutes are required to achieve maximum colour. After this the colour is stable for four hours and in eighteen hours only decays to 85% of its maximum value.

Not only does this technique give exactly the same colour when reacting with trichloroethanol as when reacting with trichloroacetic acid but a number of other compounds also form exactly the same colour. In all cases, however, there is a variation in the absorbance produced for one micromole of reactant. In general, compounds with only one halogen atom per carbon atom in a molecule showed little, or no reactivity. Trihalo compounds give a greater colour intensity than dihalogen compounds, and chlorine derivatives give a greater intensity than bromine or iodine derivatives.

In determining the optimum reaction conditions, these authors found that the sensitivity of the method increased markedly with the decreasing of the ratio of the pyridine volume to the volume of sample used. For practical purposes, they recommended that the ratio of sample:pyridine:alkali should lie in the range 1:1:1 to 1:1:3.5. Since the sample is in aqueous solution this effectively means a pyridine:aqueous layer ratio in the range 1:2 to 1:4.5. The full significance of the changes of such ratios was highlighted by Lugg (90).

The work of Lugg was in connection with the determination of carbon tetrachloride, chloroform, tetrachloroethane and trichloroethylene. To do this,

he investigated both two phase and single phase methods of carrying out the Fujiwara reaction.

In the two phase system, he found that the intensity of colour produced varied with the strength, and quantity of aqueous sodium hydroxide solution used. This he attributed to variations in the water and sodium hydroxide content of the pyridine layer caused by the changes in the aqueous phase. The optimum concentration of sodium hydroxide in the aqueous phase he found to be 10.75M, and when using this he found that the absorbance reached a maximum and then remained constant as the water content of the pyridine layer did not change. It is worth contrasting these findings with those of, say, Daroga and Pollard who found that sodium hydroxide concentrations in excess of 20% (5M) had an adverse effect on colour development. The use of sodium hydroxide concentrations of greater than 10.75M limited the solubility of sodium hydroxide in the pyridine layer, this limiting the colour development. This limitation of hydroxide solubility in the pyridine layer, and also a reduction in the amount of water dissolved, can be brought about by use of a solvent in the pyridine layer. When a solvent was used the optimum concentration of sodium hydroxide in the aqueous layer was found by Lugg to be 6.25M. This

variation parallels the earlier findings of Webb, Kay and Nichol (130) that each solvent has its own optimum hydroxide concentration.

In the one phase system, the optimum conditions were found to be 20% of water and 0.023% of sodium hydroxide in the pyridine. That the water content was critical may be inferred from the observation of Rogers and Kay (111) that water seemed to play an important part in the colour producing reaction. However, as the water content increases beyond 20% the colour developed decreases.

In the determination of carbon tetrachloride most authors have had acetone present. This is frequently used as an absorbing agent for carbon tetrachloride. Lugg found that for a colour to be developed in the Fujiwara reaction a ketone had to be present. This need not be acetone, in fact Lugg used methyl ethyl ketone. In contrast to this, aldehydes inhibit colour formation.

It is interesting to note there has been some discussion that the colour reaction by carbon tetrachloride was in fact due solely to the presence of chloroform as an impurity in the carbon tetrachloride. This was refuted by Burke and Southern (21) when they found that to produce the same absorbance as was found

when testing carbon tetrachloride, the chloroform impurity would have to amount to 25% of the sample used. When examined by means of an infra-red spectrophotometer no trace of chloroform was detectable. The technique used by these authors was a single phase system not including a ketone.

Conclusions

If the Fujiwara reaction is to be employed in a quantitative manner, the method to be preferred is that of Liebman and Hindman (86). This method gives an increased sensitivity compared with the use of the pink Fujiwara reaction product and also detects trichloroethanol. In addition, the final coloured product is more stable than that of the Fujiwara reaction. This is an advantage in that the critical step is the addition of the benzidine-formic acid reagent which can be performed rapidly, hence with little change of colour. The slower step involving measurements on a spectrophotometer are performed with a stable coloured product. This particularly applies when a number of samples are being analysed at the same time.

It may be that the qualitative screening could employ this technique. In the case of chloral hydrate in dogs, the principal metabolite is trichloroethanol (24, 94) although some trichloroacetic acid is also formed. A considerable improvement in the sensitivity of the screening test can be expected to result from the use of this technique. It does, however, employ benzidine, which is carcinogenic, and the solution in 88% formic acid is unstable, both these factors being

undesirable in a routine procedure.

FUJIWARA - COMPARISON WITH OTHER TECHNIQUES

The Fujiwara reaction is not the only reaction for detecting halogenated hydrocarbons and their derivatives. Archer and Haugas (7) developed a test for chloral hydrate based upon its reaction with quinaldine ethiodide in alkaline solution. This test is suitable for quantitative estimation and is apparently specific for chloral hydrate.

There have also been developed a number of methods based on earlier techniques for the estimation of halogen content. Goodall (61) estimated fluothane by hydrolysis with sodium amylate. The liberated halogens were precipitated with silver nitrate and turbidimetric measurements used.

This technique only liberates 60-80% of the bromine present when determining halothane, so the technique was adapted by Duncan and Raventos (44). They extracted the halothane from blood using petroleum ether and liberated the halide by reacting with Lithium aluminium hydride in diethyl ether. The liberated halide was precipitated with silver nitrate, and the optical density measured. Using this technique, Duncan and Raventos claimed overall recoveries of better than 97%.

A further chemical test for chlorbutanol was developed by Rehm and Mader (107). The chlorbutanol

was steam distilled out of the biological sample and reacted with hydroxyalamine hydrochloride under basic conditions. A colour was then developed using ferric chloride solution. The test was sensitive to 10 mg./ 100 ml. of chlorbutanol and gave recoveries in the range 98-102%. Unfortunately the test is liable to interference from a large number of materials including esters, imides and iron precipitants.

The reaction conditions resemble those for the Fujiwara reaction. The sample is reacted with an amine in the presence of a strong base. The reaction according to Rehm and Mader is of the form shown in Figure 1.

$$CCl_3 - C(CH_3)_2 - OH + NH_2OH + 4NaOH \longrightarrow Na^+ \ ^-O\text{-}NH\text{-}C\text{-}C(CH_3)_2 - OH + 3NaCl + 3H_2O$$

There are, of course, a number of instrumental, as opposed to chemical, techniques for detecting halogenated materials. Inevitably gas chromatography has been used (56, 73, 94, 118) and also infra-red spectrometry (29).

Gas chromatography will be discussed later. Infra-red spectrometry has the great advantage of being able to identify the material under examination unequivocally. In addition, it is capable of making quantitative measurements, simultaneous with identification, with an

accuracy of ± 10% (29). However, the technique is less sensitive than gas chromatography or the Fujiwara reaction.

Discussion

The identity of the material present in urine samples which give a positive Fujiwara reaction requires the use of instrumental techniques. Curry (34) describes how the Fujiwara test may be used to obtain a tentative identification of the material present. However he advises caution, and with so many materials which may give a positive reaction an alternative technique for confirmation of identity is necessary.

When time is the limiting factor, as for example in pre-race testing, it is necessary to use speedy, simple techniques which do not require expensive and sophisticated equipment. The test must also be capable of detecting chlorbutanol. These conditions restrict the choice of techniques to one of the chemical techniques.

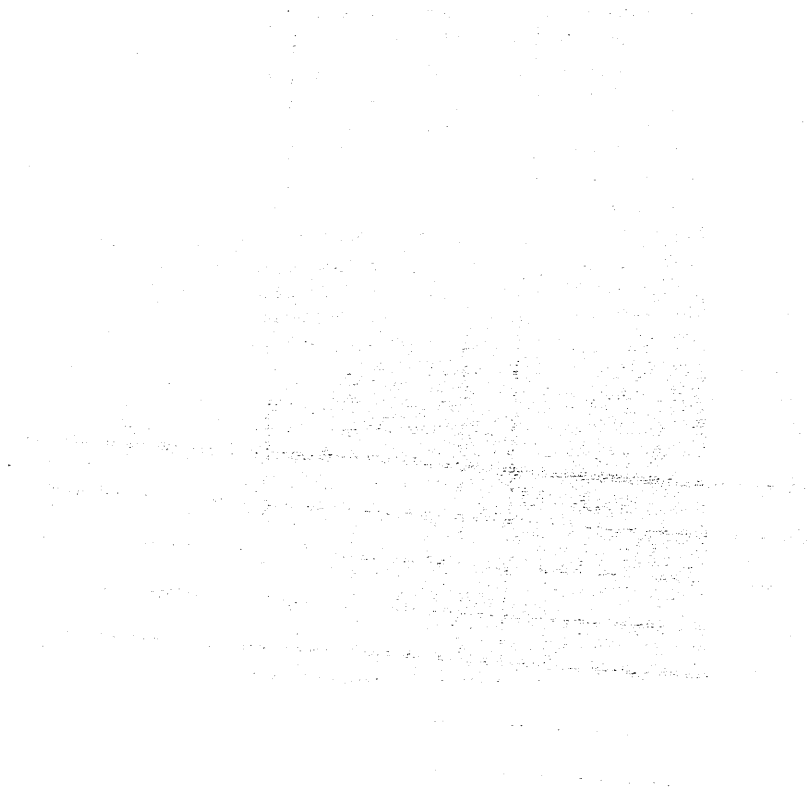
The use of sodium amylate followed by chloride precipitation with silver nitrate is rather a slow process, and prone to interference by chloride ion which is always present in urine samples. Using Lithium aluminium hydride instead of sodium amylate does

not avoid the second difficulty. Additionally, when using lithium aluminium hydride the reaction must be carried out in organic solvents which are completely dry. This is difficult to guarantee. Further, these techniques are relatively slow and cumbersome.

The test proposed by Rehm and Mader (107) was designed to detect and measure chlorbutanol. No doubt other halogenated materials may be detected, such as trichloroacetic acid and trichloroethanol. However, the test described is somewhat sensitive to conditions and also prone to interference by materials which could well be present in urine samples. Steam distillation to separate interfering materials is also time consuming.

The remaining choice is the continued use of the Fujiwara reaction. It is known that this test is capable of detecting chlorbutanol from the work of Webb, Kay and Nichol (130) and Moss and Kenyon (94). However, whilst there are reasons to insist that the pre-race test must be capable of detecting chlorbutanol there is no reason to assume that this drug is exclusively used. That the Fujiwara reaction is capable of detecting other drugs of this type is a further reason for employing this test.

A further advantage of the Fujiwara test is that it is capable of being modified to suit many requirements, or that a given set of requirements can be satisfied in a number of ways. In addition to the more important developments of the original Fujiwara reaction outlined, there have been many other variations used successfully (2, 4, 46, 48, 57, 75, 79, 105, 121).



FUJIWARA REACTION - EXPERIMENTAL

Introduction

One version of this test was already in use as a qualitative screening procedure. Work was carried out to determine whether the test could be used to identify the material present and to establish a technique for measuring the quantity of drug present. While this work was in progress a gas chromatographic technique was being developed, which indicated that the only drug present in the samples under investigation was chlorbutanol. An investigation into the reaction conditions was then carried out to provide optimum conditions for the detection of chlorbutanol.

Identification by means of the Fujiwara Reaction

Introduction

A pink colour is produced in the Fujiwara reaction by chloral hydrate, chlorbutanol and trichloroacetic acid. Trichloroethanol does not give this colour. Identification may be possible by means of small ultraviolet or visible spectrum differences of the reaction product.

Reagents

The reagents used were solutions containing approximately 25 µgms./ml. of chloral hydrate, chlorbutanol, trichloroacetic acid and trichloroethanol in pyridine. The sodium hydroxide used was a 10% aqueous solution.

Procedure

4.0 ml. aliquots of each solution were pipetted into test tubes and 2.0 ml. of the sodium hydroxide solution added to each tube. The tubes were heated in a boiling water bath until a pink colour developed, and then cooled in an ice bath. An aliquot of the pyridine layer was examined against a reagent blank using a Unicam SP800 ultraviolet/visible spectrophotometer.

Results

The peak maximum found for these compounds is given in Table 1.

TABLE 1

Spectra of Some Trichloromaterials in the Fujiwara Reaction

Compound	Observed Peaks (λmax.) mμ.
Chloral hydrate	366, 528
Chlorbutanol	366, 528
Trichloroacetic acid	366, 528
Trichloroethanol	366, 438
Chloroform	366, 528

Discussion

These figures are in close agreement with those reported by other authors (95). Obviously, this simple technique cannot be used for identification. Complex procedures which differentiate between chloral hydrate, trichloroacetic acid and trichloroethanol are available. However, these depend upon treatment with oxidising agents, strong bases, and solvent extraction from aqueous solutions at various pH's. Chlorbutanol is extracted under much the same conditions as trichloroethanol, but is unstable in the presence of strong alkali. Therefore chlorbutanol is not

identifiable by such techniques.

Some use can be made of the rate at which the colour develops under varying reaction conditions. This is affected by the concentration of material present, and is not reliable. Curry (34) recommends caution.

Conclusions

Some entirely different technique is necessary to identify effectively the trichloro material present, but the Fujiwara reaction may be useful as a quantitative process.

Estimation using the Fujiwara Reaction

Introduction

This can be done using either the pink Fujiwara compound or by means of a derivative. Using derivative formation obviously takes longer than working on the pink reaction product. However, it is necessary if the pink reaction product is unstable under the reaction conditions employed. Therefore investigations were carried out to determine the stability of the pink reaction product.

Stability of the Fujiwara Reaction Product

Reagents

Two reagents were used. A 6.25 µg./ml. solution of trichloroacetic acid in pyridine, and 10% aqueous sodium hydroxide solution.

Equipment

Colourimetric measurements were made on a Hilger and Watts H 760 spectrophotometer, using the No. 4 filter.

Procedure

2 ml. of each of these solutions were mixed and heated on a boiling water bath for 5 minutes. The

solutions were cooled, and an aliquot of the organic phase removed for colourimetric measurements.

Results

These are given in Table 2.

TABLE 2

Stability of the Pink Fujiwara Reaction Product

Time from start (hrs/mins)	Optical Density
0.00	0.995
2.15	1.056
3.39	0.663
4.18	0.580
4.40	0.510

Discussion

These results show deterioration of the pink colour. This is of little use in quantitative work. The rise in optical density during the first $2\frac{1}{4}$ hours suggests that a heating time of five minutes is insufficient for maximum colour development.

Conclusion

The Fujiwara reaction product is not stable enough for accurate quantitative work, and so no further work was done upon this. The formation of a benzidine

derivative of the reaction product is reported to give a stable colour (86) so it was decided to investigate this further.

Use of Benzidine Derivative

Introduction

The work involving the Fujiwara reaction product emphasised the critical nature of the heating time and this is the first topic investigated with respect to the derivative formation. Thereafter the reproducibility of the technique was tested and following this calibration graphs were prepared for trichloroacetic acid and trichloroethanol; the two metabolites of most interest in this group.

Effect of Heating Time

Reagents

These were a 2.4 µgrm./ml. solution of trichloroacetic acid in water, pyridine, 10% aqueous sodium hydroxide solution, and freshly prepared 3% benzidine in 88% formic acid.

Procedure

A series of tubes were prepared, each containing 2.0 ml. of the trichloroacetic acid solution, 2.0 ml. of pyridine, and 2.0 ml. of the sodium hydroxide solution. The tubes were placed in a boiling water bath, and a tube removed at intervals as shown in the results. The tubes were cooled in an ice bath, and 1.0 ml. of the organic phase added to 0.2 ml. of the

benzidine solution. After 30 minutes the colour developed was measured on the Hilger and Watts colourimeter, using a reagent blank as reference solution.

TABLE 3

Effect of Heating Time upon Colour Development

Boiling Time (minutes)	Optical Density
2	0.065
2.5	0.202
3.0	0.213
3.5	0.190
4.0	0.156
4.5	0.238

Discussion

The results listed in Table 3 give anomalous figures for 4.5 minutes boiling but indicate that the optimum boiling time is 3.0 minutes. This is in agreement with Liebman and Hindman (86).

Conclusion

The optimum boiling time is 3 minutes, and must be measured accurately if large errors are to be avoided.

Reproducibility of the Colour Development

Reagents

The same reagents were used as were used for investigating the heating time. The benzidine solution was freshly prepared.

Procedure

Eight tubes were prepared each containing 2.0 ml. of the trichloroacetic acid solution, 2.0 ml. of pyridine and 2.0 ml. of 10% sodium hydroxide solution. The tubes were heated on a boiling water bath for three minutes and then cooled in an ice bath. 1.0 ml. of the organic phase was added to 0.2 ml. of benzidine, and the colour allowed to develop for 30 minutes. The colour intensity was then measured on the colourimeter.

Results

The values found were:- 0.037, 0.277, 0.282, 0.303, 0.254, 0.272, 0.304, 0.260.

The value 0.037 appears to be in error. Ignoring this, the average value for the optical density is 0.278, and the range 0.254 to 0.304. This gives an error within ± 10%, which is precise enough for most work.

Conclusion

This technique can be used for the estimation of

trichloro-materials in urine samples, provided the colour developed is proportional to concentration. The results should be accurate to within ± 10%.

Preparation of Calibration Graphs

Introduction

The preparation of calibration graphs serves two functions. The graphs indicate the relationship that exists between the colour developed and the drug concentration. In addition, they provide a ready means of estimating drug concentrations in biological samples providing that the same analytical conditions are used. The two drugs selected are dealt with individually below.

(i) Trichloroethanol

Reagents

The trichloroethanol solution was 22.1 µg./ml. in water. In addition, 10% aqueous sodium hydroxide solution, "Analar" pyridine and freshly prepared 3% benzidine in 88% formic acid.

Procedure

Into each of seven test tubes was pipetted 2.0 ml. of pyridine and 2.0 ml. of sodium hydroxide solution, and also an aliquot of the trichloroethanol solution and sufficient water to form 4.0 ml. of aqueous phase.

The tubes were heated on a boiling water bath for 3 minutes, cooled in ice, and 1.0 ml. of the organic phase added to 0.2 ml. of benzidine solution. The colour was allowed to develop for 30 minutes, and the optical densities measured using the colourimeter.

Results

These are shown in Table 4, and shown plotted on Graph 1.

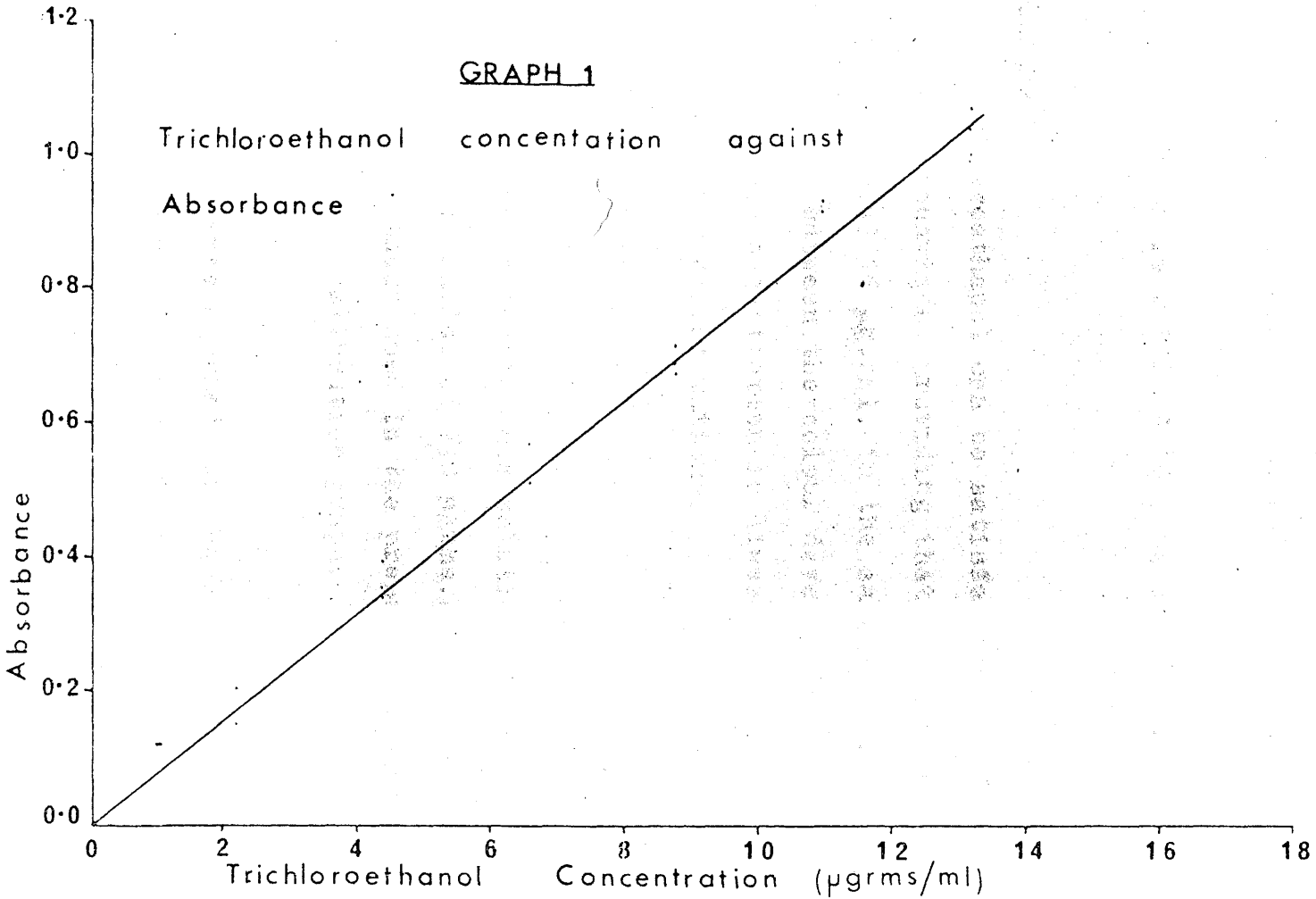
TABLE 4.

Calibration for Trichloroethanol(TCE)

	Optical Density		
µgm/TCE	Run I	Run II	Run III
4.4	0.154	-	0.202
8.8	0.340	0.382	0.352
13.4	0.510	-	0.572
17.6	0.673	0.720	0.690
22.0	0.902	-	0.918
26.6	1.002	1.038	1.074
31.0	1.031	-	1.194

Discussion

The relationship between the colour developed and the trichloroethanol concentration in the sample is linear. This means that trichloroethanol can be readily estimated using this technique if the quantity does not exceed 30 µg. Above this level the sample


GRAPH 1
Trichloroethanol concentation against
Absorbance
1·2
1·0
0·8
0·6
0·4
0·2
0·0
Absorbance
0
2
4
6
8
10
12
14
16
18
Trichloroethanol Concentration (µgrms/ml)

requires dilution as it is possible that the relationship may become non-linear due to consumption of one of the reagents.

The error is within ± 10%, as was found in earlier work.

Conclusions

Trichloroethanol can be satisfactorily estimated using this technique, providing that its concentration does not exceed 15 µg./ml. in the sample. The relationship between the colour developed and trichloroethanol concentration is linear, which facilitates quantitative estimations.

(ii) Trichloroacetic Acid

Reagents

The trichloroacetic acid solution was prepared having a concentration of 6.0 µgrms./ml. The other reagents were those used in the preparation of the trichloroethanol calibration graph.

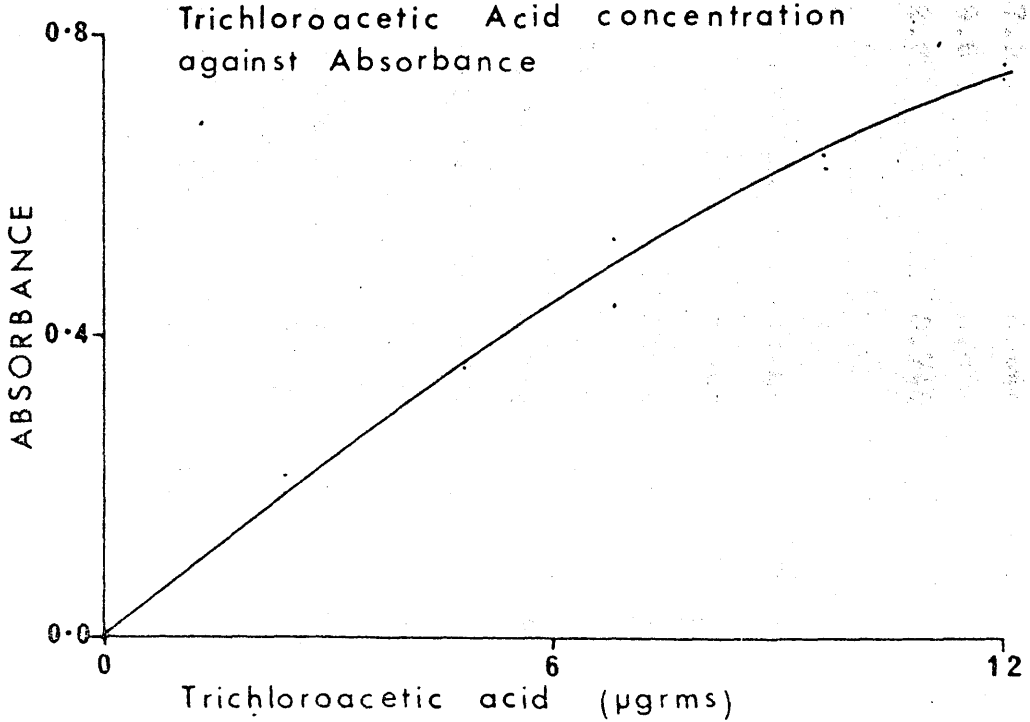
Procedure

Two runs were carried out, each involving five samples. The procedure was that used for the preparation of the trichloroethanol calibration graph.

Results

These are given in Table 5 and shown plotted on

GRAPH 2


Trichloroacetic Acid concentration against Absorbance
ABSORBANCE
0·8
0·4
0·0
0
6
12
Trichloroacetic acid (µgrms)

Graph 2.

TABLE 5

Calibration for Trichloroacetic Acid

Trichloroacetic Acid (µgrms)	Optical Density Run 1	Run 2
2.4	0.192	0.212
4.8	0.357	0.370
7.2	0.444	0.535
9.6	0.651	0.627
12.0	0.746	0.767

Discussion

This graph is not linear, though using up to about 7 µgrms. of trichloroacetic acid it is approximately so. This non-linearity may be due to decarboxylation of the trichloroacetic acid by the sodium hydroxide. This would lead to dichlorocarbene which would react vigorously with the water present.

Conclusion

This procedure can only be used at very low concentrations of trichloroacetic acid, in fact at concentrations of less than 3.5 µgrms./ml., if an approximately linear relationship is to be used.

Discussion

The Fujiwara pink colour is too unstable to be used for accurate quantitative measurements. This

was found in the past by other authors and is noted in the literature survey.

The use of the benzidine derivative, however, provides a much more stable coloured complex, which means that the most important part of the analysis is the accurate control of the heating time. This time is critical, but not sufficiently so as to render the technique impracticable.

When the test substrate is trichloroethanol, a linear relationship between colour developed and trichloroethanol concentration exists up to 15 µgrms./ml. with the procedure used. This was the highest concentration tested.

When the test substrate is trichloroacetic acid however, the relationship between trichloroacetic acid concentration and colour development is non-linear. At the highest concentration tested, 6.0 µgrm./ml., the deviation from linearity is pronounced, but up to 3.5 µgrms./ml. an approximately linear relationship exists. This non-linearity may be due to side reactions resulting from the decarboxylation of the trichloroacetic acid by the sodium hydroxide.

Conclusions

The following procedure was adopted.

To a test tube containing 2.0 ml. of pyridine

and 2.0 ml. of 10% aqueous sodium hydroxide solution is added 2.0 ml. of urine. The tube is placed in a boiling water bath for exactly 3 minutes, cooled in ice, and 1.0 ml. of the organic phase added to 0.2 ml. of a 3% solution of benzidine in 88% formic acid. After allowing the colour to develop for 30 minutes, the optical density is measured against a reagent blank.

The optical density is linearly related to concentration up to 15 µgrms./ml. for trichloroethanol but only up to 3.5 µgrms./ml. for trichloroacetic acid.

Applications of the Measurement Technique

Introduction

The measurement technique was used to investigate two aspects of the pre-race testing procedure. The first was the use of β-glucuronidase in the hydrolysis of the conjugates of chlorbutanol and trichloroethanol. The second application was to improve the test procedure when used to detect chlorbutanol.

Use of β-Glucuronidase

Introduction

Trichloroethanol and chlorbutanol are reported to be excreted, in part at least, in the form of the glucuronide. This is formed to increase the water solubility, and hence the rate of excretion, of the drug. Since the glucuronide may not be extracted by toluene, it was customary to add a little β-glucuronidase solution to the urine sample shortly before carrying out the extraction.

The following test was carried out to assess the effectiveness of this procedure.

Sample

This was a 24 hour urine specimen from a racing greyhound which had been dosed with chloral hydrate. The dose cannot be published for security reasons.

Reagents

The β-Glucuronidase was prepared by dissolving 0.1 grms of the solid in 100 ml. of water. This gave a solution with an activity of 900 Fisherman units/ml. One Fisherman unit is equivalent to the liberation of 1 µgrm. of phenolphthalein from phenolphthalein glucuronide in pH 4.0 acetate buffer at 37°C in one hour.

The analysis involved the use of pyridine, sodium hydroxide solution and benzidine solution as described in the preceding section.

Procedure

Into each of 15 flasks was pipetted 20 ml. of the urine sample. To five was added 0.2 ml. of the β-Glucuronidase solution and to a further five flasks was added 0.2 ml. of concentrated hydrochloric acid. The remaining five flasks were used as controls. All fifteen flasks were placed in a shaking incubator which was maintained at 37°C. One flask from each group was withdrawn after one hour, after two hours, after four hours, after eight hours and after twenty-four hours.

The analysis was carried out using the procedure described in the preceding section, and used 0.5 ml. of urine and 1.5 ml. of water together with 2.0 ml. of sodium hydroxide to form the 4.0 ml. of aqueous phase.

Results

The results are given in Table 6.

TABLE 6

Hydrolysis of Glucuronides in Urine Sample

(a) Group 1. Hydrolysed with β-Glucuronidase

Incubation Time (hours)	µgrms Trichloroethanol		
	1	2	Average
1	28.2	28.6	28.4
2	28.4	-	28.4
4	31.4	29.2	30.3
8	28.2	29.6	28.9
24	398	362	380

(b) Group 2. Hydrolysed with Hydrochloric Acid

Incubation Time (hours)	µgrms Trichloroethanol		
	1	2	Average
1	28.2	28.6	28.4
2	29.4	26.0	27.7
4	27.2	27.6	27.4
8	27.6	27.6	27.6
24	404	406	405

(c) Group 3. Control

Incubation Time (hours)	µgrms Trichloroethanol		
	1	2	Average
2	27.4	27.6	27.5
4	28.2	28.8	28.5
8	27.2	28.0	27.6
24	28.0	28.4	28.2

Discussion

The results show, beyond doubt that hydrolysis does improve the yield of trichloroethanol. However, using β-Glucuronidase at least 24 hours incubation at 37°C is necessary, so the use of β-glucuronidase in the pre-race laboratories serves no useful purpose.

The results also show that hydrolysis with hydrochloric acid is as effective as hydrolysis with β-glucuronidase. This is not unexpected, although the hydrolysis is less specific than when the enzyme is employed. With acid hydrolysis the hydrolysis rate increases with temperature whereas most enzymatic reactions progress most rapidly at 37°C. This gives the possibility of rapid hydrolysis at 100°C; however, the pre-race testing has been satisfactory with no effective hydrolysis, so this would be an unnecessary complication.

The results also give some indication of the extent to which the glucuronide of trichloroethanol occurs.

The technique employed measures the total trichloro-material present in the urine. Due to the non-linearity of the trichloroacetic acid, it is not strictly accurate to estimate the concentrations present from these results, merely to compare the change in

optical density with hydrolysis. However, if the trichloroethanol calibration graph is used, the initial concentration is 6.2 mg./100 ml., and after hydrolysis the concentration is 80 mg./100 ml. This does indicate that the trichloroethanol is the principal metabolite of chloral hydrate, and is largely excreted as the glucuronide conjugate.

Conclusions

The use of β-glucuronidase by the pre-race laboratories serves no useful purpose. If hydrolysis must be carried out it can probably be done much better by using concentrated hydrochloric acid. As the test seems to perform satisfactorily, hydrolysis by any method would appear to be unnecessary.

Test Procedure Development

Introduction

A number of anomalous results have been reported by the pre-race laboratories.

The method used entailed the extraction of about 15 ml. of urine with 2 ml. of toluene. The toluene was added to test-tubes containing 2.0 ml. of pyridine and 2.0 ml. of 10% w/v sodium hydroxide which had been heated in boiling water for 5 minutes to check the reagents. After the addition of the toluene, the tubes were again heated for 10 minutes. If a pink colour did not appear within this time the sample was assumed to be free of trichloro material.

However, on a number of occasions, samples which gave a positive result when repeated did not give a pink colour within 10 minutes, or sometimes not at all. Indeed, on one occasion, the pink colour did not appear until after 45 minutes immersion in boiling water.

Work on a gas chromatographic technique has shown that the only trichloro material occurring was chlorbutanol. An investigation was carried out into the parameters affecting the detection of chlorbutanol by the Fujiwara reaction.

Test of Technique

Introduction

If a series of identical samples are treated identically their observed reaction would be the same. If the observed reactions differ there has been some difference of treatment.

Procedure

A solution of 1 mg./100 ml. of chlorbutanol in water was prepared. Thirteen aliquots of this solution were each extracted with 2.0 ml. of toluene. The toluene from each extraction was then added to a test tube containing 2.0 ml. of pyridine and 2.0 ml. of 10% aqueous sodium hydroxide which had been treated in a boiling water bath for five minutes. The tubes were replaced in a boiling water bath and the time taken for the pink colour to appear in each tube noted. The results are given in Table 7.

Results

TABLE 7

Boiling Times

Time to Positive Reaction (mins.)	No. of Samples Appearing
0.75	1
3.25	1
4.00	2
5.5	1
7.0	1
8.0	1
9.0	1
11.0	3
negative to 45.00	2

Discussion

Some slight variation of the rate of appearance of the pink colour is to be expected. However, the range of times is so great that there must have been some variation in handling technique. The variation is not due to the reagents as the same batches were used throughout.

Conclusion

Some aspect of the procedure is not sufficiently reproducible to ensure a reproducible rate of colour development and is making the test somewhat unreliable.

Sensitivity of the Reaction

Introduction

This parameter affects the strength of solutions used for future investigations, and also defines the volume of sample required for a given chlorbutanol concentration.

Procedure

A number of solutions of chlorbutanol in pyridine were prepared, each containing a different concentration. Two ml. of solution were placed in a test tube together with 2.0 ml. of 10% w/v aqueous sodium hydroxide. The tubes were placed in a boiling water bath and the time for a pink colouration to appear noted. The results are given in Table 8.

Results

TABLE 8

Sensitivity of the Fujiwara Reaction

Chlorbutanol Concentration (mg./100 ml.)	Time to Positive (mins)
9.42	1.5 at room temp.
0.75	2.0
0.38	2.0
0.15	negative to 30 minutes

Discussion

The test is sensitive to about 2.5 µg./ml. of chlorbutanol in the pyridine phase in the absence of other organic solvents. These solvents may, of course, affect the sensitivity of the test. The sensitivity in terms of the minimum detectable concentration of chlorbutanol in the urine depends upon the volume of sample, the volume of extracting solvent, the extraction efficiency, and the volume of pyridine. The nature of the extracting solvent may also affect the sensitivity.

Conclusion

The sensitivity of the test is probably about 2.5 µg./ml. of chlorbutanol in the organic phase. This may, however, be affected by the presence of another organic solvent.

Choice of Extracting Solvent

Introduction

The solvent must fulfil a strict specification. The solvent must not interfere with the Fujiwara reaction, which precludes aldehydes which are reported to inhibit colour formation.

The solvent must have a solubility of less than 5 ml. in 1 litre of water. It is intended to use 2 ml.

of solvent to extract up to 50 ml. of sample and more than about 0.25 ml. loss would be excessive. At the same time, the solvent should separate readily from the urine after shaking and not form an emulsion.

While being insoluble in water, the solvent must also be soluble in pyridine in the presence of sodium hydroxide to form solutions of up to 1:1 strengths. Obviously also the solvent must not form a third layer which would result in the reactants being separated. Most organic solvents will mix with pyridine, but one with a specific gravity of less than 1.0 is required.

The test is a colour reaction, and so the solvent while extracting the trichloro materials must extract very little urinary pigments. These would obscure a weak positive reaction.

The solvent used is toluene as used by Seto and Schulze (119), which is a non-polar solvent. However, chlorbutanol contains polar groups, and so a more polar solvent may solvate the chlorbutanol molecules better, giving a better recovery.

These requirements restrict the choice of solvent to alcohols, esters, ethers and ketones having four to eight carbon atoms in the molecule.

Reagents

The solvents were all of "analar" grade;

a comprehensive list of those tested is given in the results.

Procedure

2.0 ml. of each of these solvents were vigorously shaken with 50 ml. of urine in a 100 ml. separating funnel. The funnels were inspected approximately one minute and fifteen minutes after shaking, and note taken of how well the solvent had separated and if urinary pigments had been extracted.

Results

These are given in Table 9.

TABLE 9

Extraction Solvent for Fujiwara Test

(a) Alcohols

Compound	Solubility	Emulsification	Pigments Extracted
Butanol	Excessive	-	-
Amyl alcohol	Insoluble	Slight	Strong Colouration
Hexanol	Insoluble	Emulsified	Yes
Heptan-1-ol	Insoluble	Emulsified	Yes
Allyl alcohol	Excessive	-	-

(b) Esters

Compound	Solubility	Emulsification	Pigments
Ethyl acetate	Insoluble	Slight	Coloured
Amyl acetate	Insoluble	Slight	Slight

(c) Ketones

i-amyl methyl ketone	Insoluble	Moderate	Slight

(d) Ethers

Methyl phenyl ether (anisole)	Insoluble	Slight	Very little

Discussion

The solvents which may be expected to give the best recoveries cannot be used. These are the homologous alcohols, butanol and amyl alcohol. Butanol is too soluble in urine and amyl alcohol is sufficiently polar to extract a great deal of urinary pigment.

Consideration of solubilities and the amount of urinary pigment extracted restricts the choice to three solvents from these results, amyl acetate, isoamyl methyl ketone and anisole.

Conclusion

The only possible alternative solvents to toluene from these results are amyl acetate, anisole and isoamyl methyl ketone.

Suitability of Solvents in Fujiwara Test

Introduction

On the basis of their lack of solubility and polarity, three solvents have been selected which may be used to extract chlorbutanol from urine. One further important consideration is the behaviour of the solvent in the Fujiwara test. The solvent must be miscible with pyridine, in the presence of aqueous sodium hydroxide, in proportions of up to 50%. In addition, the solvent must not give a positive reaction

in the test, nor prevent a positive reaction occurring if there are materials such as chlorbutanol present.

Reagents

The reagents were "analar" grade samples of the solvents under consideration, and also anisaldehyde which was tried for comparative purposes. The other reagents were pyridine, 10% aqueous sodium hydroxide solution and a 1.0 mg./100 ml. solution of chlorbutanol in pyridine.

Procedure

Eight test tubes were prepared containing 2.0 ml. of the sodium hydroxide solution. 2.0 ml. of pyridine were added to four of the tubes and 2.0 ml. of the chlorbutanol solution to the remainder. 2.0 ml. of each solvent were added to each of one pair of test tubes, and the tubes immersed in a boiling water bath for 15 minutes. They were then removed and inspected.

Results

These are given in Table 10.

TABLE 10

Solvents in the Fujiwara Reaction

Solvent	With Pyridine	With Chlorbutanol solution
Anisaldehyde	Negative	Negative
Amylacetate	Negative	Negative
i-amyl methyl ketone	Pale yellow	Positive
Anisole	Negative	Positive

Discussion

Anisaldehyde was included to verify that aldehydes do interfere in the Fujiwara reaction. This interference would appear to extend to certain other compounds containing a carbonyl group, certainly to amyl acetate.

i-Amyl methyl ketone would appear to react in some manner as the tube containing pyridine became yellow. It did not, however, prevent the normal Fujiwara reaction when chlorbutanol was present.

Anisole did not appear to affect the reaction in any way, neither preventing the normal Fujiwara reaction with chlorbutanol, nor giving any side reactions with coloured products.

Conclusions

Anisole is the only one of the solvents considered

that may be a suitable alternative to toluene. The others either prevent the Fujiwara reaction occurring, or give rise to side reactions.

Rate of Colour Development

Introduction

The work carried out in establishing the quantitative colourimetric technique emphasised how critical the duration of heating is upon the development of the Fujiwara pink colour. This earlier work was carried out using an aqueous phase and a pyridine phase only. In this section the effects of different concentrations of organic solvents, of different organic solvents, and different base strength will be investigated. These are of crucial importance if the technique used in the pre-race laboratories is to give optimum results.

The technique used in the pre-race laboratories involves extracting a volume of urine with 2.0 ml. of toluene, and adding this to a test tube containing 2.0 ml. of pyridine and 2.0 ml. of 10% aqueous sodium hydroxide. The tube is then heated in a boiling water bath for 10 minutes.

In the pre-race laboratories, it is the intensity of the Fujiwara pink colour which is of crucial importance. Any investigation into its rate of development involves quantitative colourimetry; the pink colour itself is unsuitable for this due to its instability. The benzidine derivative was used,

which at any given concentration gives a more intense colouration. This colouration, however, appears to be related to the intensity of the pink colour formed.

Effect of Toluene on Derivative Formation

Introduction

The benzidine derivative forms satisfactorily when the organic phase consists solely of pyridine. The presence of an organic solvent may, however, affect this.

Reagents

A solution of 1.9 mg./100 ml. of chlorbutanol in pyridine was prepared. In addition, 10% aqueous sodium hydroxide, 3% benzidine in 88% formic acid and "analar" toluene were used.

Procedure

Two tubes were prepared containing 2.0 ml. of the chlorbutanol solution, 2.0 ml. of toluene and 2.0 ml. of sodium hydroxide solution. The tubes were heated in a boiling water bath until a pink colour was observed. The tubes were removed and one tube cooled in ice. It was intended, after cooling, to pipette 1.0 ml. of the organic phase into 0.2 ml. of the benzidine solution, but this was not possible. 1.0 ml. of the organic phase was taken from the second tube immediately after

removal from the water bath, and added to 0.2 ml. of benzidine solution. The colour was allowed to develop for 30 minutes.

Results

In the first tube, three phases were formed on cooling; for this reason, 1.0 ml. of the organic phase was not added to the benzidine solution.

In the second tube, two phases were formed during the colour development with benzidine. These were a violet coloured lower phase, and a clear upper (toluene) phase. The addition of 1.0 ml. of pyridine gave a homogenous solution suitable for colourimetry.

Discussion

This separation of the organic phase into two layers means that 1.0 ml. must be removed immediately after heating.

To ensure a homogeneous solution, 1.0 ml. of pyridine must be added after colour development with benzidine. The separation into two phases during the colour development does not appear to impair the reaction in any way.

Conclusion

To use the benzidine derivative when organic solvents are present in the pyridine phase, two modi-

fications of the technique are necessary. First, 1.0 ml. of the organic phase must be removed immediately after heating. Secondly, 1.0 ml. of pyridine must be added after colour development with benzidine solution.

Neither of these modifications affects the colour forming reaction, though of course there is dilution of the chromophore.

Rate of Colour Development without Organic Solvent

Introduction

Previous work has indicated that when using an aqueous solution of trichloroethanol the maximum colour is developed with 3 minutes heating. In this work, a solution of chlorbutanol in pyridine is used for convenience, and this first test is used to establish the course of the reaction in the absence of an organic solvent. These results can then be used as a norm for comparison of the results obtained when an organic solvent is present.

Reagents

A solution of 1.9 mg./100 ml. of chlorbutanol in pyridine, 10% aqueous sodium hydroxide and freshly prepared 3% benzidine in 88% formic acid were used.

Procedure

A series of tubes was prepared containing 2.0 ml. of the chlorbutanol solution and 2.0 ml. of sodium hydroxide solution. The tubes were heated on a boiling water bath for various lengths of time, and on removal 1.0 ml. of the organic phase was pipetted into 0.2 ml. of the benzidine solution. The colour was allowed to develop for 30 minutes, and then the optical density measured on the colourimeter against a reagent blank. This was carried out three times.

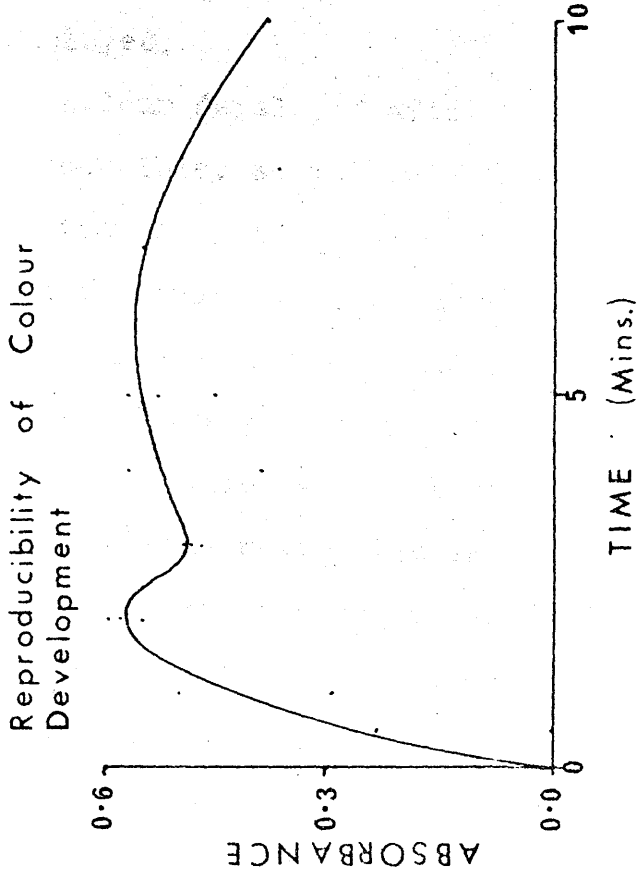
Results

TABLE 11

Progress of Reaction for Chlorbutanol

Time of Heating (minutes)	Optical Densities		
	Run I	Run II	Run III
½	0.005	0.236	-
1	0.294	0.502	-
2	0.591	0.576	0.549
3	0.486	0.490	0.470
4	-	0.393	0.573
5	0.571	0.532	0.455
6	-	-	0.513
7	-	-	0.504
8	-	-	0.373
9	-	-	0.378

GRAPH 3


Reproducibility of Colour Development
ABSORBANCE
0·6
0·3
0·0
0
5
10
TIME (Mins.)

Visual observations made at the time were that in Run II the pink colour was most intense after 2 minutes heating, and in Run III after 4 minutes heating. Further no pink colour was evident in Run III after heating for 6 minutes.

Discussion

The graph indicates that the maximum colour developed using benzidine occurs after the same length of heating as for the maximum pink colour if the first maximum is employed.

The peak colour developed after 2 minutes boiling is fairly reproducible, as for trichloroacetic acid and trichloroethanol. After this time a number of factors appear to complicate matters, and the colour intensity is not reproducible. This can be explained by the breakdown of the pink complex on prolonged heating, and by loss of pyridine due to evaporation.

It is particularly noticeable in Run III that after 6 minutes heating no pink colour is visible, but treatment with benzidine reagent produces 65% of the maximum colour. In fact the optical density corresponds closely to that in Run II after 4 minutes boiling when the pink colour was still clearly visible.

This suggests that the following steps are involved:-

(a) The formation of the pink product (A)

(b) The breakdown of A to B on boiling.

(c) The breakdown of B on boiling.

(d) The breakdown of A on treatment with acid to give C.

(e) The formation of coloured complex D with benzidine

(f) The breakdown of B to give E.

Products E and C could be the same. This would explain the results if the reaction to produce A is rapid a vivid pink colour is produced, and the benzidine complex is almost entirely by way of product C. After further boiling, appreciable quantities of B are formed with loss of A. Thus the pink colour fades and the benzidine colour also as B does not react to produce D.

On further boiling, appreciable quantities of E are formed from B and the colour intensity with benzidine increases as E reacts to give D. At the same time more of A is destroyed and the pink colour fades even more.

On further boiling slow loss in E occurs.

Conclusion

This technique can be used to study the rate of colour formation. However, in the event of more than one maximum being produced the first ought to be used, as this seems to be reproducible.

Care is needed to note those tubes in which

excessive evaporation of the organic phase occurs. In most of the tubes very little if any loss occurs, but in some tubes almost total loss of the organic phase can occur.

Rate of Colour Development with Toluene Present

Introduction

This work was carried out to investigate the effect of toluene upon the rate of colour development. It may be that, in addition to diluting the pink colouration, the toluene affects the rate of reaction.

Reagents

The chlorbutanol solution was 9.4 mg./100 ml. in pyridine. This stronger solution was used to ensure that reasonably large optical densities were obtained in spite of dilutions in the procedure.

In addition, "Analar" pyridine and toluene were used, 10% aqueous sodium hydroxide, and 3% benzidine in 88% formic acid.

Procedure

A series of four tests was carried out involving 20%, 40%, 50% and 60% of toluene in the organic phase.

In each test, a series of tubes was prepared containing 2.0 ml. of sodium hydroxide solution, 0.5 ml. of chlorbutanol solution, the appropriate volume of

toluene and sufficient pyridine was added to form 2.0 ml. of organic phase.

The tubes were immersed in a boiling water bath, and tubes removed at intervals as specified in the results. After removal from the water bath, 1.0 ml. of organic phase was added to 0.2 ml. of benzidine solution. After the colour had been allowed to develop for 30 minutes a further 1.0 ml. of pyridine was added, and the optical densities measured on the colourimeter against a reagent blank.

Results.

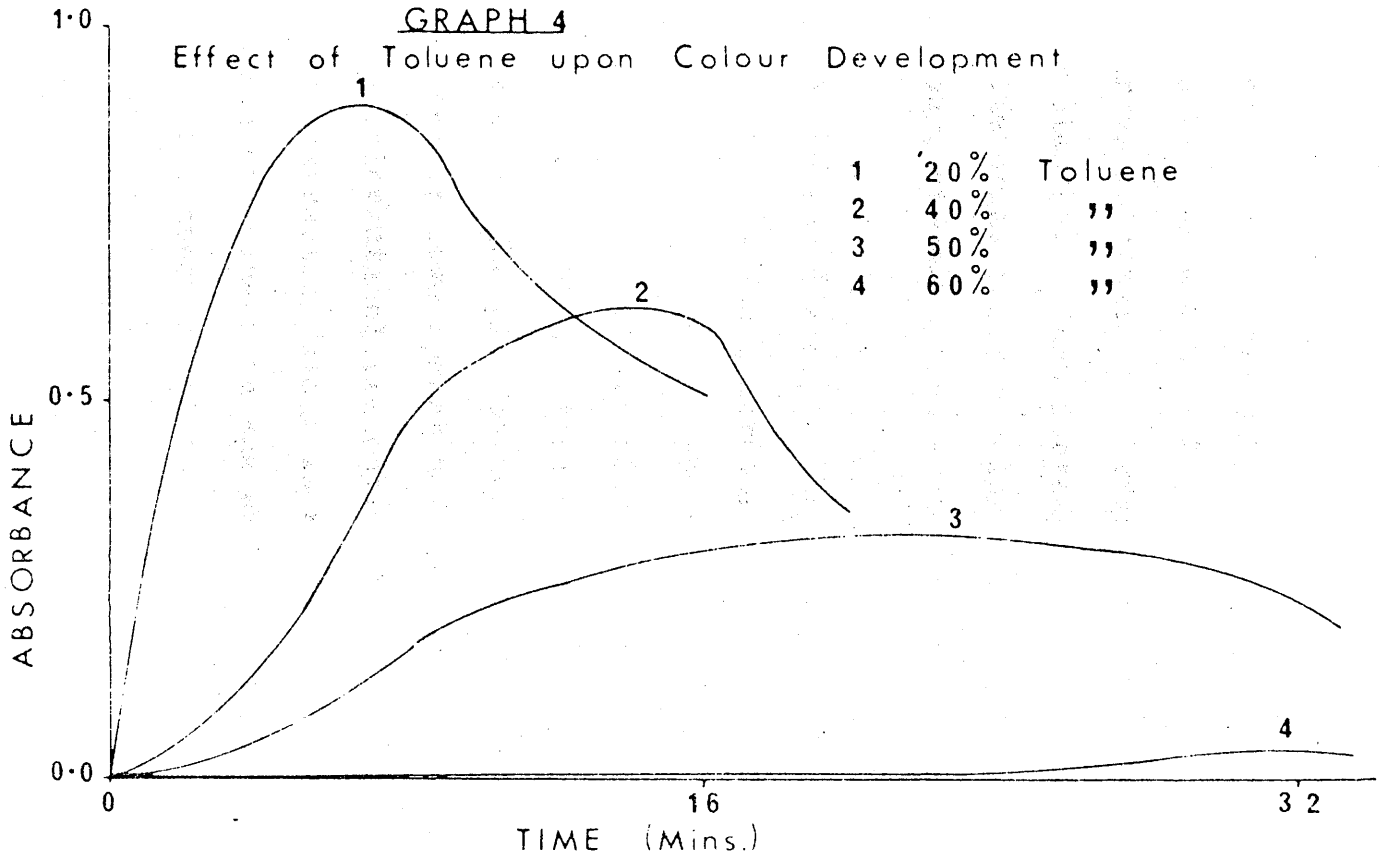
These are shown in Table 12 and Graph 4.

TABLE 12

Effect of Toluene on Colour Development

Toluene Concentration	20%	40%	50%	60%
Boiling Time (mins)	O.D.	O.D.	O.D.	O.D.
2	0.507	0.037	-	-
3	-	-	0.041	-
4	0.786	0.136	-	-
6	0.881	0.281	0.086	-
8	0.873	0.474	-	-
9	-	-	0.193	-
10	0.738	0.428	-	-
12	0.633	0.563	0.255	-
14	0.602	0.622	-	-
15	-	-	0.252	0.003
16	0.510	0.608	-	-
18	-	0.451	0.228	0.003
20	-	0.348	-	-
21	-	-	0.223	0.197*
22	-	0.325	-	-
24	-	-	0.251	0.088*
27	-	-	0.283	0.019
30	-	-	0.252	0.032
33	-	-	0.208	0.025

In the above Table, O.D. stands for optical density. In the tubes leading to results marked *, appreciable evaporation of the organic phase occurred. This means that the results are erroneously high.


GRAPH 4
Effect of Toluene upon Colour Development
1·0
0·5
0·0
ABSORBANCE
1
2
3
4
1 20% Toluene
2 40% ,,
3 50% ,,
4 60% ,,
0
16
32
TIME (Mins.)

Discussion

Visual observations indicated that the maximum pink colour occurred after the same heating time as gave the maximum benzidine colour.

If the two results marked * are excluded, a family of curves is obtained. From these emerges two facts very clearly. That increasing concentration of toluene in the organic layer delays the attainment of maximum colour, and also reduces the maximum colour attained.

These findings would explain the difficulties experienced by the pre-race laboratories. The method gives a toluene concentration of 50% which requires more than the 15 minutes heating stipulated to reach maximum colour. In practice, this would be critical only in rare cases. The pink colour would be visible before maximum colour development had occurred unless there is little chlorbutanol present. The recovery of toluene from the extraction stage is not 100%, lowering the concentration of toluene in the organic phase.

To offset this, however, is the loss of pyridine when heating with sodium hydroxide solution to check the reagents before adding the toluene extract. This would tend to increase the toluene concentration in the organic phase, impairing colour production.

Conclusion

The presence of toluene in the organic phase reduces the rate of colour formation, and also the maximum colour attained. The extent of these effects depends upon the concentration of toluene in the organic phase.

Effect of Anisole upon Colour Development

Introduction

Earlier work was carried out to find an alternative extraction solvent to toluene. Of the solvents studied anisole was considered to be the most suitable. Since the concentration of toluene in the organic phase markedly affects the colour forming reaction, it is possible that anisole will have a similar effect. The following work was carried out to investigate this possibility.

Reagents

These were the 9.4 mg./100 ml. solution of chlorbutanol in pyridine, "Analar" pyridine and anisole, and freshly prepared 3% benzidine in 88% formic acid.

Procedure

The same procedure as was used when investigating the effect of toluene was followed. The concentration of anisole in the organic phase investigated were 0%,

20%, 40%, 50% and 60%.

Results

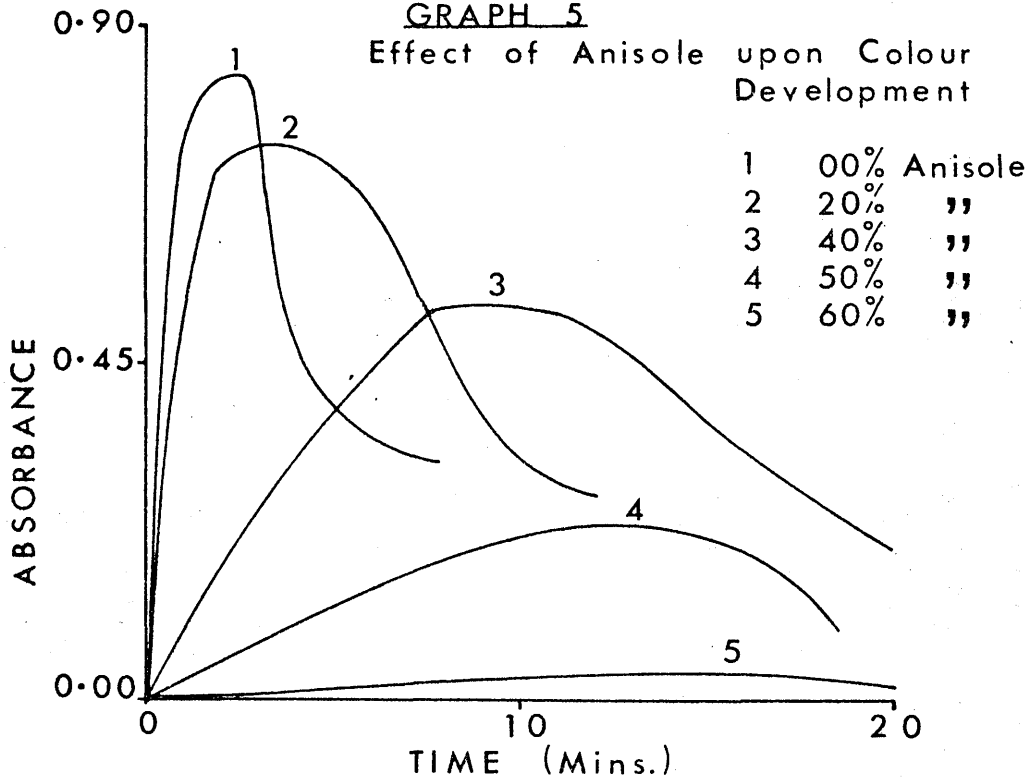
These are given in Table 13 and shown plotted as a graph against time in Graph 5.

TABLE 13

Effect of Anisole on Colour Development

Anisole Concentration	0%	20%	40%	50%	60%
Boiling Time (mins)	O.D.	O.D.	O.D.	O.D.	O.D.
½	0.166	-	-	-	-
1	0.713	-	-	-	-
1½	0.552	-	-	-	-
2	0.302	0.726	0.219	0.048	-
2½	0.846	-	-	-	-
3	0.543	-	-	-	-
3½	0.617	-	-	-	-
4	0.226	0.741	0.304	0.093	-
5	0.734	-	-	-	-
6	0.352	0.673	0.442	0.112	-
8	-	0.317	0.553	0.203	0.021
10	-	0.322	0.429	0.212	0.018
12	-	0.267	0.265	0.237	0.031
14	-	-	0.424	0.232	0.030
16	-	-	0.349	0.105	0.027
18	-	-	0.174	0.126	0.101*
20	-	-	0.240	0.202	0.025

The maximum observed pink occurred after 2½ minutes in Run I, 2 and 4 minutes in Run II, 8 minutes in Run III, 12 minutes in Run IV and 18 minutes in Run V.


GRAPH 5
Effect of Anisole upon Colour Development
1 00% Anisole
2 20% ,,
3 40% ,,
4 50% ,,
5 60% ,,
0·90
0·45
0·00
ABSORBANCE
0
10
20
TIME (Mins.)
1
2
3
4
5

This latter seems anomalous.

Discussion

The results follow the same general pattern as for toluene. There are two points worth noting. For any given solvent concentration, the boiling time to maximum colour is less when anisole is used than when toluene is used. Secondly, that when toluene is used the maximum colour developed is greater than when anisole is used.

This solvent effect has been attributed to the change of solubility of water and of sodium hydroxide in the organic phase. Anisole, being more polar than toluene, will suppress the solubilities less than toluene. The higher the sodium hydroxide concentration the more rapidly the colour develops, but the higher the water concentration the faster it fades.

Conclusion

When using anisole, the colour development is impaired, as it is when toluene is used. When anisole is used the maximum colour occurs sooner but is less intense than when toluene is used.

Effect of Sodium hydroxide concentration

Introduction

The effect of organic solvents in the pyridine has

been attributed to the solvents affecting the solubility of the sodium hydroxide and of water in the organic phase. One other factor which may affect this is the initial concentration of sodium hydroxide in the aqueous phase. This was investigated in the following test.

Reagents

A series of sodium hydroxide solutions were prepared having concentrations of 10%, 20%, 30% and 40% and 50% w/v. In addition a 9.9 mg./100 ml. solution of chlorbutanol in pyridine, pyridine, anisole and freshly prepared 3% benzidine in 88% formic acid was used.

Procedure

One run was carried out using each sodium hydroxide solution.

For each run a series of tubes was prepared containing 2.0 ml. of the selected sodium hydroxide solution, 0.5 ml. of the chlorbutanol solution, 0.4 ml. of anisole and 1.1 ml. of pyridine. The tubes were heated in a boiling water bath for the times shown in the results, and after removal from the water bath the colour was developed as described earlier.

Results

These are shown in Table 14 and plotted in Graph 6.

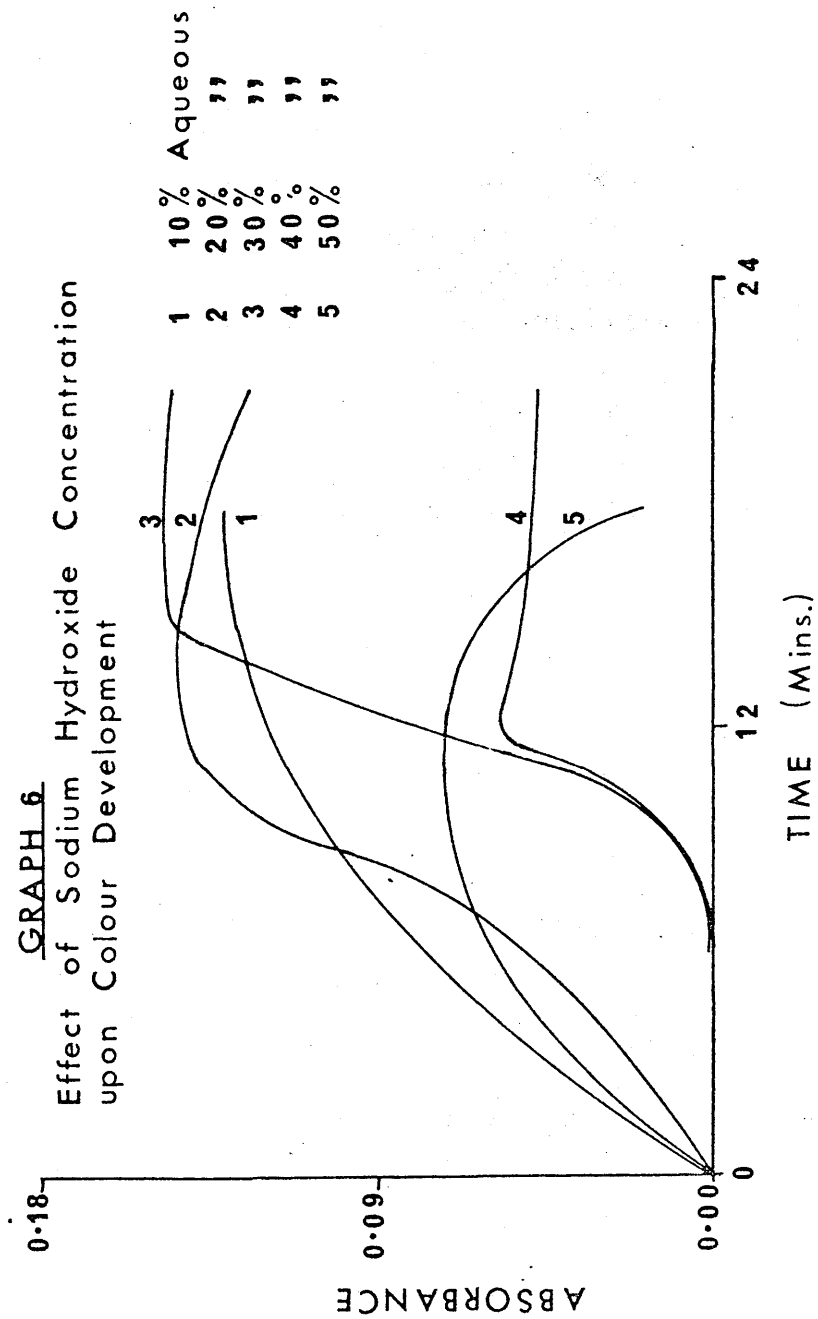

GRAPH 6
Effect of Sodium Hydroxide Concentration upon Colour Development
1 10% Aqueous
2 20% "
3 30% "
4 40% "
5 50% "
0·18
0·09
0·00
ABSORBANCE
0
12
24
TIME (Mins.)

TABLE 14

Effect of Sodium Hydroxide Concentration

Sodium Hydroxide Conc^{n}.	10%	20%	30%	40%	50%
Boiling Time (mins)	O.D.	O.D.	O.D.	O.D.	O.D.
3	0.038	0.024	-	-	-
6	0.083	0.049	-	-	-
9	-	0.113	0.008	0.007	-
12	0.115	0.142	0.067	0.059	0.061
15	0.129	0.129	0.147	0.054	0.073
18	-	0.111	0.132	0.042	0.062
21	-	0.126	0.144	0.047	0.018

Discussion

For sodium hydroxide concentrations in the range 10% to 30% the same maximum colour is reached. Using higher concentrations the maximum colour is less intense and fades more rapidly.

Conclusion

The optimum sodium hydroxide concentration is in the range 10% to 30% w/v.

Effect of Volume of Aqueous Phase

Introduction

The remaining factor requiring investigation is the effect of changing the volume of aqueous phase. It is possible that the sodium hydroxide partitions between the two phases. If the volume of aqueous phase

is too small, this partition may cause an appreciable fall in sodium hydroxide concentration in the aqueous phase, which will in turn limit the sodium hydroxide concentration in the organic phase.

Reagents

These were a 9.9 mg./100 ml. solution of chlorbutanol in pyridine, 10% aqueous sodium hydroxide solution, anisole, and pyridine.

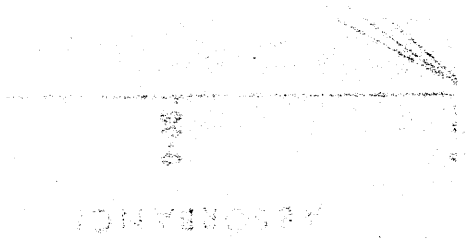
Procedure

Three runs were carried out using 1 ml., 2 ml. and 3 ml. of sodium hydroxide solution.

The procedure for each run was as described for the investigation of the effects of sodium hydroxide concentration.

Results

These are shown in Table 15 and plotted in Graph 7.



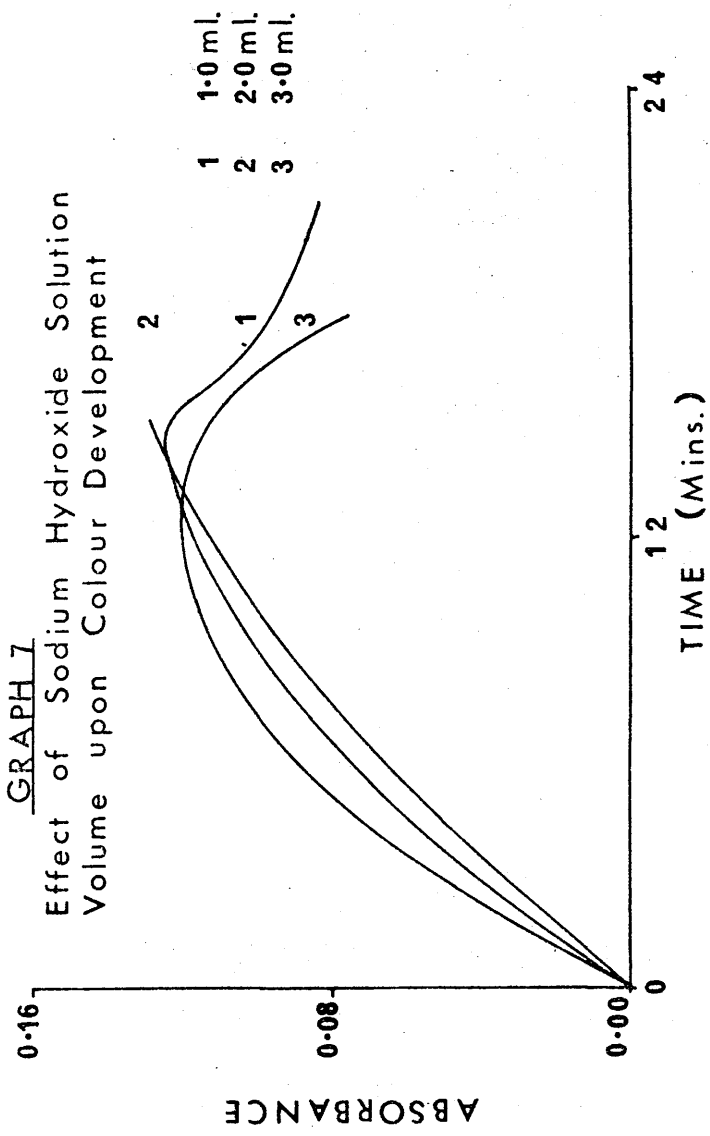

GRAPH 7
Effect of Sodium Hydroxide Solution Volume upon Colour Development
1 1·0 ml.
2 2·0 ml.
3 3·0 ml.
ABSORBANCE
0·16
0·08
0·00
0
12
24
TIME (Mins.)

TABLE 15

Effect of varying the Aqueous Phase Volume

Volume of Aqueous Phase	1 ml.	2 ml.	3 ml.
Boiling Time (mins)	O.D.	O.D.	O.D.
3	0.042	0.038	0.047
6	0.072	0.083	0.090
9	0.101	-	-
12	0.116	0.115	0.120
15	0.124	0.129	0.112
18	0.097	-	0.076
21	0.083	-	-

Discussion

The volume of aqueous phase does not appear to have any effect upon the colour formation although presumably sufficient is required to form a separate phase, effectively saturating the organic layer.

Conclusion

Providing that a separate aqueous phase exists the volume of sodium hydroxide solution does not affect the colour developing reaction.

Calibration Graph for Chlorbutanol

Introduction

The preceding work has been carried out assuming that there is a linear relationship between colour developed and the quantity of reactant. This may not

be so; for example there may be insufficient benzidine present, or the organic solvent may affect the reactions. Unfortunately, this cannot be investigated until the effect of such factors have been determined. Now that this has been done, a check can be made for factors invalidating the above assumption. This is most conveniently done by preparing a calibration graph.

Reagents

The chlorbutanol solution was 9.9 mg./100 ml. in pyridine, and 10% aqueous sodium hydroxide, 3% benzidine in 88% formic acid, anisole and pyridine were used.

Procedure

A series of tubes were prepared containing 2.0 ml. of sodium hydroxide solution, 0.4 ml. of anisole, aliquots of the chlorbutanol solution and sufficient pyridine to form 2.0 ml. of organic phase.

The tubes were placed in a boiling water bath for 4 minutes, removed and 1.0 ml. of the organic phase removed to form the benzidine derivative as described earlier.

Results

These are given in Table 16 and shown plotted on Graph 8.

GRAPH 8

Calibration Graph for Chlorbutanol

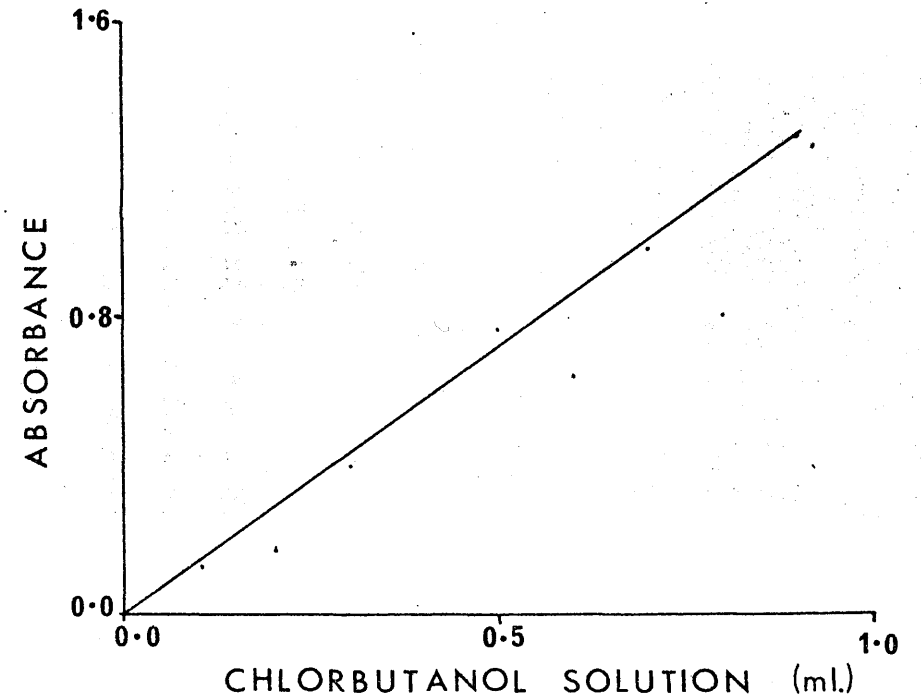


TABLE 16

Calibration Graph for Chlorbutanol

ml. solution used	µgms. chlorbutanol	Optical Density
0.1	9.9	0.121
0.2	19.8	0.166
0.3	29.7	0.400
0.4	39.6	-
0.5	49.5	0.767
0.6	59.4	0.640
0.7	69.3	0.983
0.8	79.2	0.803
0.9	89.1	1.238

Discussion

The results approximate to a straight line. This indicates that the colour reaction fits the Beer-Lambert Law in the presence of the organic solvent. If this were not so the earlier work may not have been valid. In addition, higher chlorbutanol concentrations were used in the preparation of this graph than were used in the earlier work. The linear form of the graph indicates that there was not constraint, such as lack of benzidine.

Conclusion

There is no constraint due to lack of linearity of the colour development in the earlier work.

General Conclusion

The preceding work indicates that even when the Fujiwara test is only used qualitatively, strict attention must be paid to the strengths and volumes of the reagents and to the heating times.

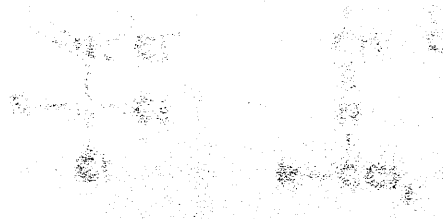
When using toluene, the following procedure is recommended for the pre-race laboratories.

The urine is extracted with 2.0 ml. of toluene, which is then added to a test tube containing 3.0 ml. of pyridine and 2.0 ml. of 10% aqueous sodium hydroxide. The tube is placed in a boiling water bath for 15 minutes and inspected frequently for the appearance of a pink colour in the organic phase.

A separate reagent blank should be used to check the reagents and not pre-heating of the tube to be used for the sample extract. Such pre-heating may lead to loss of pyridine, increasing the subsequent toluene concentration in the organic phase to an unacceptable level.

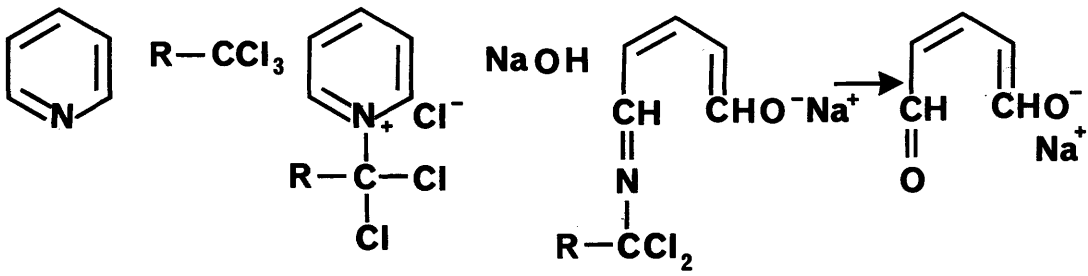
The use of anisole can only be justified if it recovers appreciably more chlorbutanol from the urine sample. If anisole is used, the same procedure should be followed, but 10 minutes heating would be adequate.

FUJIWARA REACTION MECHANISM - REVIEW



Although the Fujiwara reaction was first reported in 1914, the reaction mechanism is still far from being understood. Early workers believed that the appearance of a colour depended upon the presence of the group R.CH.Halogen (53, 112). This was soon shown not to be the case (19, 130).

The earliest postulated mechanism for this reaction was that proposed in 1956 by Feigl (47). In this, Feigl suggests that the reaction product is a Schiffs base of glutaconic aldehyde formed by opening of the pyridine ring in an adduct formed between pyridine and the polyhalogenated compound:-



Subsequent publications concerning the reaction mechanism have either used the above mechanism as a basis for further work, or made no detailed suggestions as to the mechanism, merely reporting related facts.

In the course of developing a novel method for the

determination of methyl chloride Redford-Ellis and Kench (106) carried out a series of experiments using pyridine derivatives. Although the method was based on the Fujiwara reaction, no colour was produced and all their measurements were made using an ultra-violet spectrophotometer at 365 mμ.

First the methyl chloride was trapped in 10 ml. of 98% pyridine-ethanol mixture. When heated at 60°C for 18 hours, the methyl chloride reacted to form N-methyl pyridinium chloride. To this was added 1 ml. of 0.25N alcoholic potassium hydroxide solution and the mixture heated, under a blanket of nitrogen, at 60°C for one hour. After cooling in a water bath, the measurements at 365 mμ were made.

The authors tried using 2, 4 and 2, 6 lutidene and 2, 4, 6 collidine as an alternative to pyridine when forming the N-methyl salt. When using the lutidenes an absorbance, much less than that found using pyridine, was found at 365 mμ. No such absorbance occurred when collidine was used. They also tried using α, β and γ picolines, 2, 4, 2, 6 lutidenes, and 2, 4, 6 collidine as a solvent for the N-methyl pyridinium chloride in the second part of the reaction. Again there was no absorbance when 2, 4, 6 collidine was used, and when using the other substituted pyridines the absorbance at

365 mμ was much less than when pyridine was used.

From these observations the authors decided that one molecule of the N-methyl pyridinium salt reacts with a further molecule of pyridine, and that the positions of both molecules are involved.

Further studies were carried out by Moss and Rylance (95) using chloroform, chloral hydrate, trichloroacetic acid, chlorbutanol, trichloroethylene and trichloroethanol as test substrates. They found that the red product formed by trichloroethylene had a different spectrum in the ultraviolet/visible region to that given by four of the other test substrates. Trichloroethanol, of course, did not produce an absorbance in the visible region. From the spectra produced, and chromatographic examination of the pyridine layer containing the red product, the authors decided that the red products formed from chloroform, chloral hydrate, trichloroacetic acid and chlorbutanol were identical.

This led the authors to conclude that chloroform or dichlorocarbene was an intermediate involved in the formation of the red colour. Trichloroacetic acid and chloral hydrate are known to yield chloroform when treated with hot caustic, and they found that chlorbutanol yielded acetone. Trichloroethanol, however,

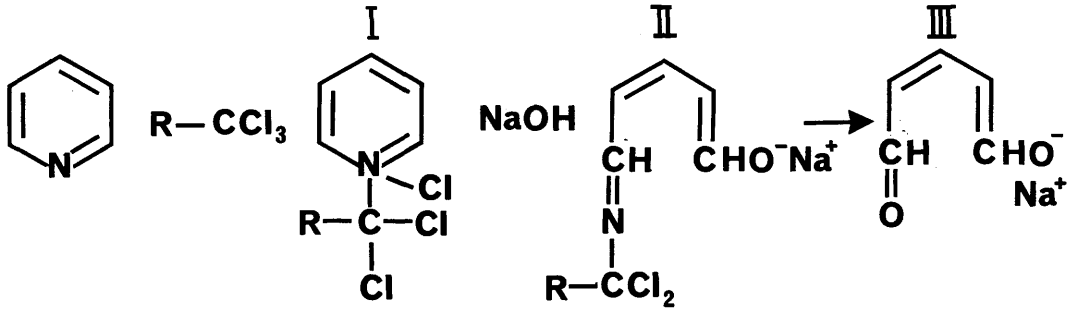
produces mainly glycollic acid under these conditions.

The authors studied the progress of the reaction spectrophotometrically with the six substrates mentioned. The reaction was carried out by heating the reaction mixture

The hot reaction produced two absorbance peaks at 365mμ and about 530 mμ. (540 mμ for trichlorethylene, 430 mμ for trichloroethanol). In the cold, however, three absorbance bands were observed at about 365, 390 - 400 and 530 mμ. The peak at 365 mμ was caused by the decomposition of the red (540 mμ absorbance) material.

The authors outlined the reaction:-

Figure 2



The compound I, formed by the reaction between the trichloro compound and pyridine, absorbing at 390-440 mμ. This is an unstable material, the pyridine ring cleaving to give the compound II absorbing at 540 mμ. This decomposes to give glutaconic aldehyde, compound

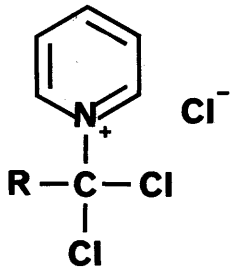
III, absorbing at 365 mμ. Basically, this mechanism is the same as the one proposed by Feigl (47). The authors differ from Feigl, however, in that Feigl implies that the red complex, II above, differs for each trichloro compound investigated. Moss and Rylance (95) however, think it is the same for all. They support this view with the spectrophotometric observations and the chromatographic behaviour of the red compound. However, an ultraviolet spectrophotometer only reacts to certain chromophores, changes in other parts of the molecule being nearly, or completely undetectable by the technique. Small changes in the molecule may also be non-detectable under the chromatographic conditions employed.

These authors refer to the compound I as a closed ring compound, and state that these have not previously been reported. It is interesting to contrast this with the reaction between 2, 4 dinitrochlorobenzene and pyridine in the presence of sodium hydroxide solution. These are the reaction conditions for the Fujiwara reaction and it is reported that a positive reaction occurs (19). A mechanism outline for this reaction is given by R. M. Acheson (3). When pyridine and 2, 4 Dinitrochlorobenzene are heated together at 100°C, 2, 4 Dinitropyridinium chloride is formed. In

the presence of cold aqueous alkali a deep red compound is formed, to which the author attributes a structure corresponding to II of Moss and Rylance. Although Acheson does not refer to it as such, this is the Fujiwara reaction, and the comparatively easy opening of the pyridine ring is facilitated by a strong electron attracting group attached to the nitrogen.

The formation of a pyridinium salt IV rather than

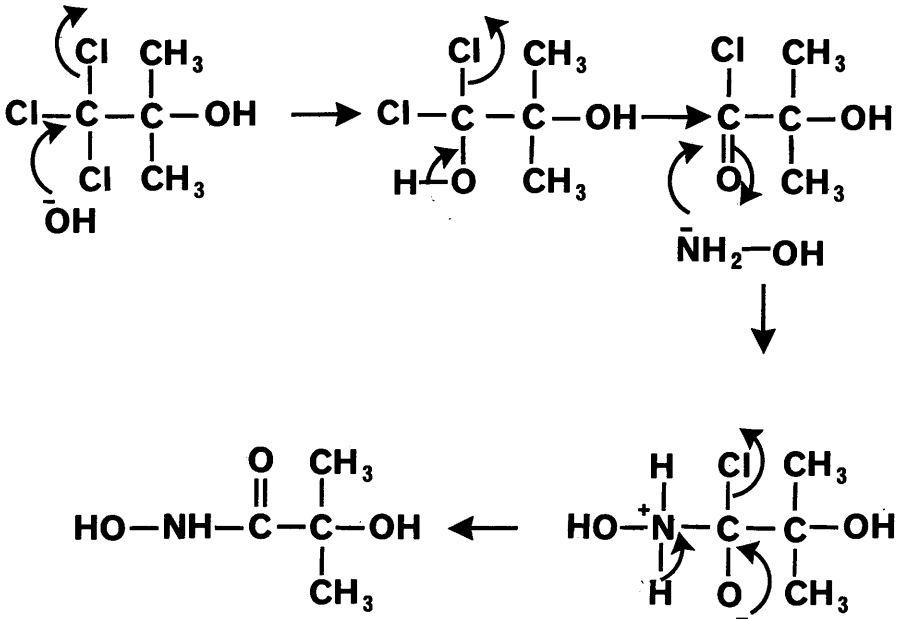
IV



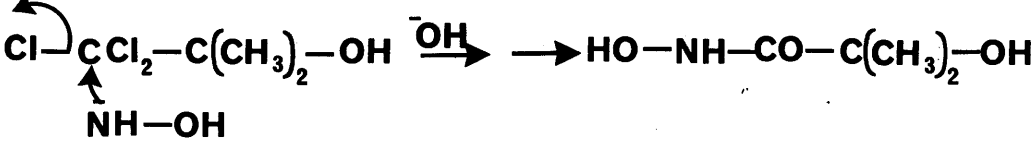
a five covalent nitrogen is probably correct in view of the fact that nitrogen cannot form penta-covalent bands due to the lack of a d orbital. This is also indicated by the work of Redford-Ellis and Kench (106), in the determination of methyl chloride, which has already been referred to.

However, pyridine itself is probably not a strong enough nucleophile to displace chloride ion from the $-CCl_3$ group of chlorbutanol or from most of the other materials which give a positive Fujiwara reaction.

Rehm and Mader (107) in their paper describing a test for chlorbutanol using hydroxylamine hydrochloride, identified the reaction product as Na O-NH-C-C$(CH_3)_2$ -OH. This can arise by two different mechanisms:- Figure 3



The reactive species may be $^{\ominus}$NH -OH (50), in which case the additional electrons may enhance the base strength of the hydroxylamine:-



The first mechanism involves an acid chloride as an intermediate. These are extremely reactive compounds, in aqueous solution being readily hydrolysed to the corresponding acid. However, as acid chlorides, acid anhydrides, imides and esters interfer in this test, it is not unreasonable that an intermediate containing a carbonyl group should be formed.

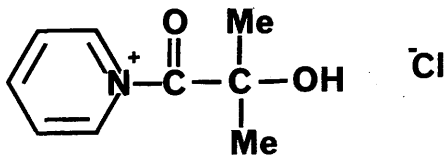
In the second mechanism, the hydroxylamine has to be a sufficiently strong base to displace chloride ion from the molecule. Having displaced one, others may also be displaced by hydroxylamine rather than by base, to give

Since this, apparently, does not occur, the reaction probably proceeds by mechanism 1.

Applying this to the Fujiwara reaction, there are again three possible first stages for the reaction with chlorbutanol.

The chlorbutanol may be decomposed by base to yield acetone and chloroform, the chloroform then reacting with pyridine. This is suggested by Moss and Rylance (95) who found that chloroform was produced by chlorbutanol, chloral hydrate and trichloroacetic acid. Trichloroethanol, which does not produce a red colour in the Fujiwara reaction does not decompose to yield chloroform.

Secondly, the initial reaction may be by the base upon the trichloro group to produce the acid chloride. This then reacts with the pyridine to produce a pyridinium salt having the structure:-

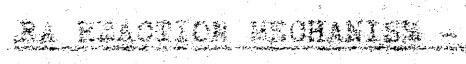


The third possibility that the presence of base and water enhances the nucleophilic strength of the pyridine leading to direct displacement of a chlorine atom attached to the trichloro group.

The reaction in the case of chlorbutanol may involve decomposition to chloroform and acetone. However, if this was a necessary first step then when equimolar amounts of say trichloracetic acid and chloroform are reacted the colour intensity produced by the chloroform would equal, or exceed that produced by trichloroacetic acid. In fact both Webb and others (130) and Liebman and Hindman (86) report that trichloroacetic acid gives the most intense colour.

The subsequent steps depend upon the exact nature of the initial reaction product but could well follow the ring opening steps outlined earlier.

Whatever the exact reaction mechanism, however, there is no doubt that the reaction is affected by conditions including solvents, heating times and base strengths.



FUJIWARA REACTION MECHANISM - EXPERIMENTAL

Introduction

Earlier, three possible reactions of chlorbutanol were considered. Firstly, the decomposition of chlorbutanol to acetone and chloroform, secondly the formation of an acid chloride and, thirdly, the direct replacement of chlorine by pyridine. Of these three possibilities, the third is extremely unlikely as pyridine is not strongly nucleophilic. However, tests were carried out to investigate all three possibilities.

Whatever the early stages of the reaction are, they probably result in the coupling of a strongly electronegative group to the nitrogen atom of the pyridine ring. This renders the ring liable to cleavage and is probably the reaction, but the work of Redford-Elliss and Kench (106) raised doubts.

They found that when using 2, 4, 6 collidine no reaction occurred, and that using other substituted pyridines the absorbance at 365 mμ is much less than when using pyridine. If the Fujiwara chromophore is produced from a pyridine ring residue the substituent groups can be expected to affect the wavelength for maximum absorption. This effect is exemplified by "Woodwards Rules". These may not apply exactly to the

coloured Fujiwara reaction product, but the principle will be the same, so if the absorption maximum is unchanged then the ring residue is probably not involved, certainly not as part of the chromophore.

It was felt that possible reaction mechanisms are as shown below:-

Mechanism I requires that acetone is liberated before the colour forms, and that either two or three moles of chloride ion are liberated from one mole of chlorbutanol.

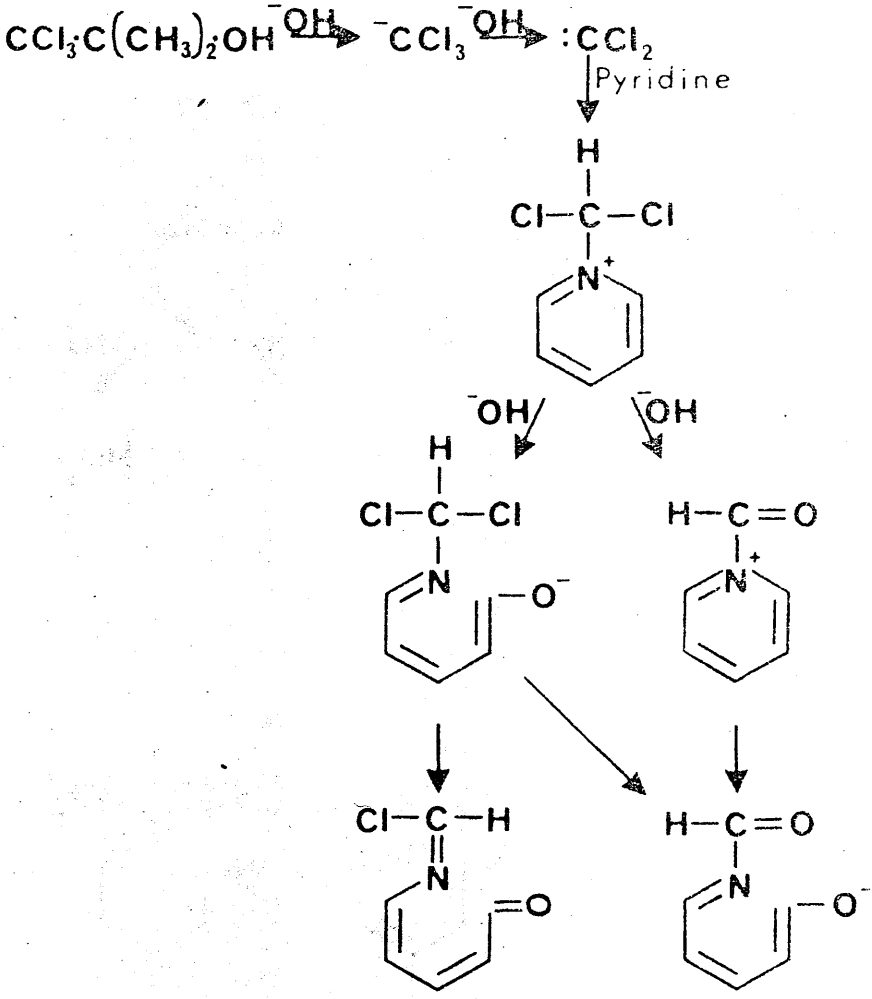
Mechanism II requires pyridine to be a sufficiently powerful nucleophile to displace chloride ion from chlorbutanol. Acetone may, or may not, be liberated, and either two or three moles of chloride ion formed for every mole of chlorbutanol consumed.

Mechanism III requires the liberation of three moles of chloride ion for every mole of chlorbutanol reacting, and acetone may, or may not be liberated.

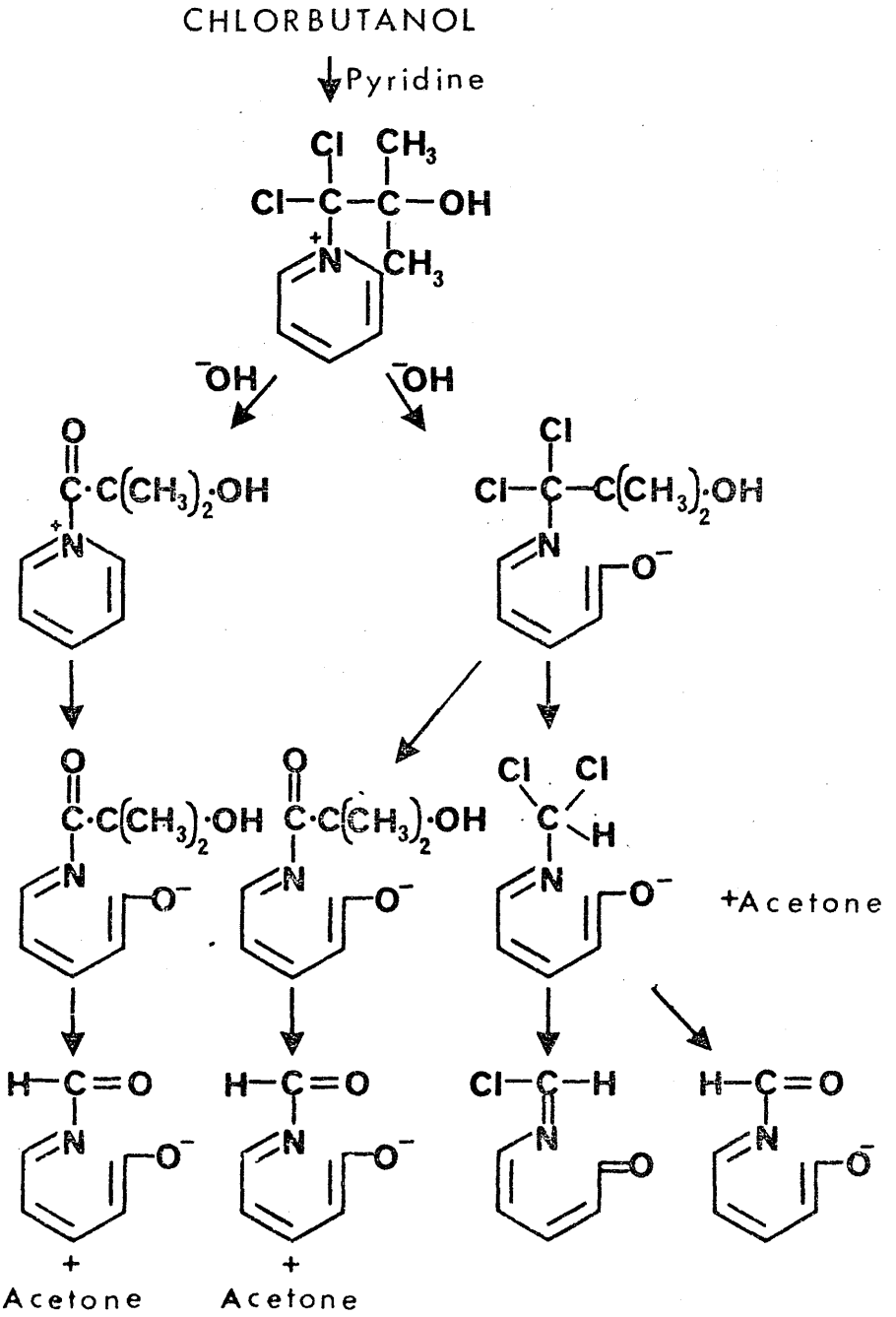
Experiments were designed to test these proposals and are described in detail below.

Possible reaction pathways for chlorbutanol

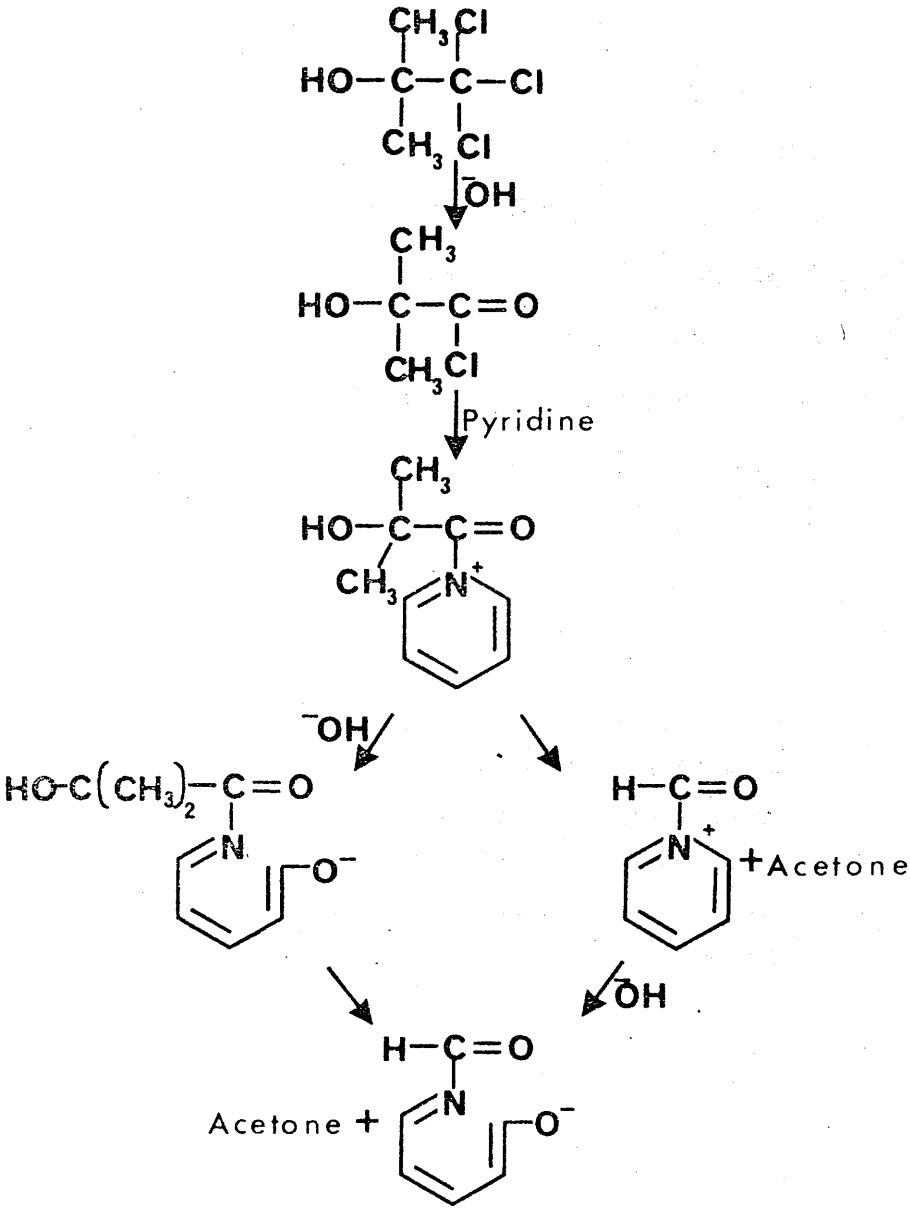
1. Involving Dichlorocarbene



2. Involving direct replacement of Chlorine by Pyridine



3. Involving an Acid Chloride intermediate



Production of Chloroform and Acetone in the Fujiwara Reaction

Procedure

This was carried out on a reaction mixture of pyridine, chlorbutanol, and 10% aqueous sodium hydroxide. The mixture was heated on a water bath under reflux for 15 minutes and then examined by gas chromatography using a Pye 104 machine fitted with a flame ionisation detector. The column was 5' x ¼" packed with 10% PEG 400 on Chromosorb W, and maintained at 90°C. The retention times of the components, relative to n-propanol are given in the results.

Results

The results obtained were as follows.

Compound	Retention Time	Organic Phase	Aqueous Phase
Acetone	0.34	none detected	none detected
Chloroform	0.639	none detected	none detected
Chlorbutanol	not eluted	-	-
Pyridine	2.30	Present	Trace
n-propanol	1.00	-	-

Conclusion

It would appear that the reaction does not proceed by the initial decomposition of chlorbutanol to chloroform and acetone.

Liberation of Chloride Ions

Introduction

The common feature of the materials giving a positive Fujiwara reaction is that they contain halogens. The halogen may be liberated in the form of halide ion or as organic halogen in an eliminated group. In addition, in the polyhalogenated materials, the halogen atoms may be eliminated by different means. The elimination of the chlorine atoms in chlorbutanol was investigated.

Qualitative Tests

These were sought using 0.1N silver nitrate solution to precipitate the chloride ion as silver chloride.

(i) Pyridine and 10% aqueous sodium hydroxide solution

2 ml. of each of these reagents was placed in a test-tube and heated on a boiling water bath for 10 minutes. After cooling the mixture was acidified with nitric acid and a few drops of silver nitrate solution added. No precipitate was formed.

(ii) Pyridine and Chlorbutanol

2 ml. of pyridine containing chlorbutanol were heated at 100°C for 25 minutes. The solution was acidified and silver nitrate solution added. No

precipitate was formed.

(iii) Pyridine, Water and Chlorbutanol

2 ml. of pyridine containing chlorbutanol and 1 ml. of water were heated at 100°C for 25 minutes. The solution was then acidified and silver nitrate solution added. A slight white precipitate formed and at no time was the pink Fujiwara colour observed.

(iv) Sodium hydroxide and chlorbutanol

2 ml. of sodium hydroxide solution containing chlorbutanol were heated at 100°C for 10 minutes. After acidification and addition of silver nitrate solution a heavy white precipitate was formed.

(v) Pyridine, Sodium hydroxide and Chlorbutanol

2 ml. of pyridine containing chlorbutanol and 1 ml. of sodium hydroxide solution were heated at 100°C for 10 minutes. After acidification and addition of silver nitrate solution a heavy white precipitate was formed. During this test the pink Fujiwara colour was observed.

Discussion

The nitric acid/silver nitrate solution itself does not liberate chloride ion from chlorbutanol, and the test reagents do not contain noticeable amounts of chloride ion. Pyridine and water do not liberate much chloride ion from chlorbutanol even with prolonged

heating, and no pink colour was observed. Such chloride ion as was liberated could be due to the slow hydrolysis of the chlorbutanol by the water.

When sodium hydroxide is used, chloride ion is rapidly formed, whether pyridine is present or not. If pyridine is present the pink Fujiwara colour is formed.

Conclusions

The formation of the pink colour involves the liberation of chloride ion. This may be the first step of the reaction.

Origin of the Chloride Ion

Introduction

Although the preceding work has shown that chloride ion is formed in the course of the Fujiwara reaction, it has not shown at which stage it is liberated. It may be liberated as the first stage of the reaction. However, the acidification of the reaction mixture discharges the pink colour, and the chloride ion may be liberated at this stage.

Procedure

5 ml. of pyridine containing chlorbutanol were heated with 5 ml. of 10% aqueous sodium hydroxide solution at 100°C for 15 minutes. A strong Fujiwara

pink colour was formed in the organic phase. The organic phase was washed with 10% sodium hydroxide solution until the washes were chloride free. The organic phase was acidified and tested with 0.1N silver nitrate solution. No precipitate was formed.

Discussion

The chloride ion is more soluble in the aqueous phase than in the organic phase. Washing with aqueous sodium hydroxide solution removes the chloride ion, but does not discharge the pink colouration. If any chloride ion were liberated upon acidification then it would have been detected upon acidification of the chloride free organic phase.

The test also shows that the stability of the pink colouration is not dependent upon there being chloride ion in the organic phase.

Conclusion

The chloride ion is liberated prior to, or during the formation of the pink colouration, and the chloride ion is not essential for the stability of the pink coloured product.

Quantity of Chloride Ion Liberated

Introduction

One molecule of chlorbutanol contains three atoms

of chlorine. During the Fujiwara reaction at least one of these atoms is liberated. Subsequent acidification and treatment with silver nitrate does not liberate more chloride ion. Thus a gravimetric procedure can be used to determine how many of the chlorine atoms are liberated in the course of the reaction.

Reagents

A standard solution of 472.2 mg./100 ml. of chlorbutanol in pyridine, 10% aqueous sodium hydroxide, concentrated nitric acid and 1N silver nitrate solution were used.

Procedure

20 ml. of the chlorbutanol solution were refluxed on a boiling water bath with 10 ml. of sodium hydroxide solution for 12 hours. The reaction mixture was then acidified, excess silver nitrate solution added, and filtered. The precipitate was dried and weighed. This analysis was carried out in duplicate.

Results

Weight of chlorbutanol ($C_4H_7OCl_3\frac{1}{2}H_2O$) in each analysis was 94.4 mg. $\equiv 5.25 \times 10^{-4}$ moles.

Weight of silver chloride (AgCl) precipitate = 221.8 mg. and 221.5 mg. This is equivalent to

1.55×10^{-3} and 1.55×10^{-3} moles of silver chloride. If all the chlorine is liberated as chloride ion 94.4 mg. of chlorbutanol would be expected to produce 1.575×10^{-3} moles of silver chloride.

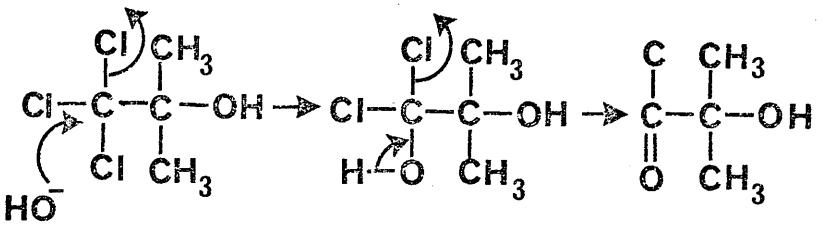
Discussion

Prolonged heating was necessary to ensure complete reaction with the high concentrations and volumes employed. This does not invalidate the results as previous work showed that the chloride is liberated during the colour forming reaction. The very intense colour formed had not noticeably faded at the end of heating.

The results obtained are very near to the theoretical yield expected if all the chlorine is liberated as chloride during the colour forming reaction.

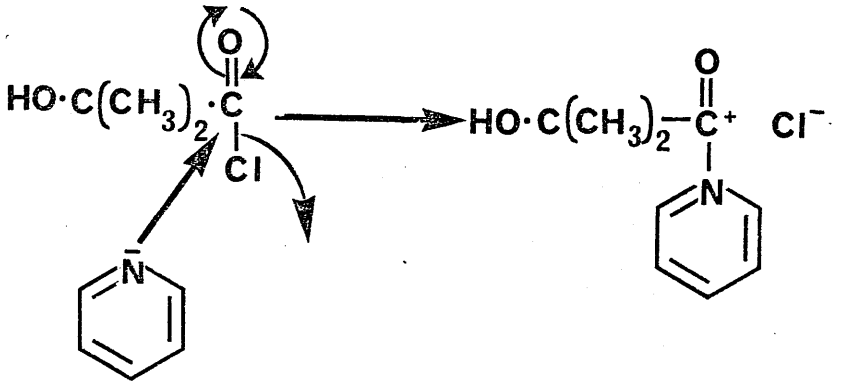
Taken together, the results so far indicate that the first step in the reaction is between chlorbutanol and hydroxyl ion liberating chloride. Pyridine and chlorbutanol do not react, even in the presence of water.

One possible course is an Sn2 displacement of one of the chlorine atoms:-



which yields an acid chloride.

These compounds are very reactive, and when produced in solution react immediately with the solvent, in this case pyridine:-



This is supported by a number of other pieces of evidence:-

(i) The composition of the organic phase, and to a certain extent the strength of the sodium hydroxide solution affect the reaction. Other authors (110) have attributed this to the effect on the solubility of water in the pyridine, which will in turn affect the solubility of the sodium hydroxide in the pyridine. If the sodium hydroxide concentration in the pyridine

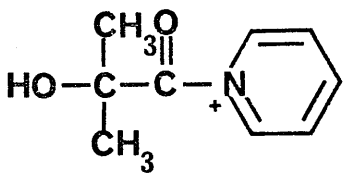
is low, the acid chloride will not, or only slowly, be formed. If the water concentration is high the acid chloride will react to give acetone and sodium formate.

(ii) The reaction parallels that outlined by Rehms and Madder (107) when using hydroxylamine to detect chlorbutanol.

(iii)The reaction outlined is consistent with the reactive properties of the groups involved. It does not involve otherwise unknown entities.

(iv) If the later stages of the reaction do involve opening of the pyridine ring, an electrophilic group is attached to the nitrogen. Such groups in that position facilitate such a reaction.

It must be emphasised that the pyridinium salt:-

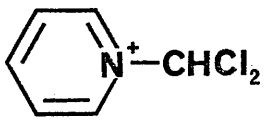


is not the coloured Fujiwara product, but a suggested intermediate.

A second possibility is the decomposition of the chlorbutanol to chloroform and acetone. In fact the products would be acetone and dichlorocarbene:-

$$CCl_3{\cdot}C(CH_3)_2{\cdot}OH \longrightarrow CH_3{\cdot}CO{\cdot}CH_3 + \bar{C}Cl_3 \longrightarrow :CCl_2$$

The dichlorocarbene is an extremely electrophilic entity and will react with pyridine to produce the salt:-



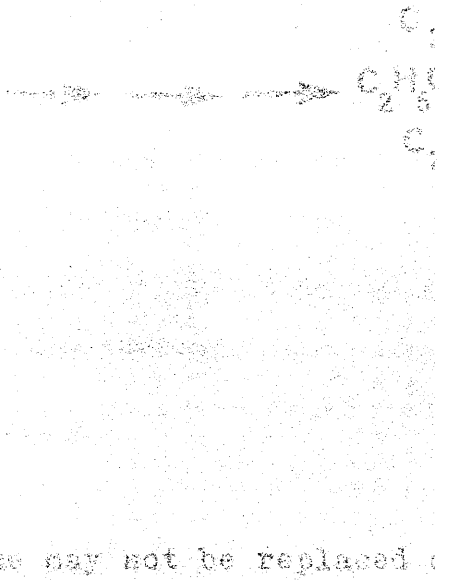
This also attaches an electronegative group to the nitrogen atom of the pyridine ring.

However, no acetone was detected in the reaction mixture.

Conclusion

All three chlorine atoms in the chlorbutanol molecule are liberated as chloride ion during the colour forming steps of the Fujiwara reaction. Since pyridine and chlorbutanol do not react the first step in the reaction must be between chlorbutanol and sodium hydroxide. This reaction does not produce chloroform

or acetone, and so must involve the Sn2 displacement of one of the chlorine atoms. The elimination of a further chlorine atom will yield an acid chloride which would react with pyridine to give a pyridinium salt.

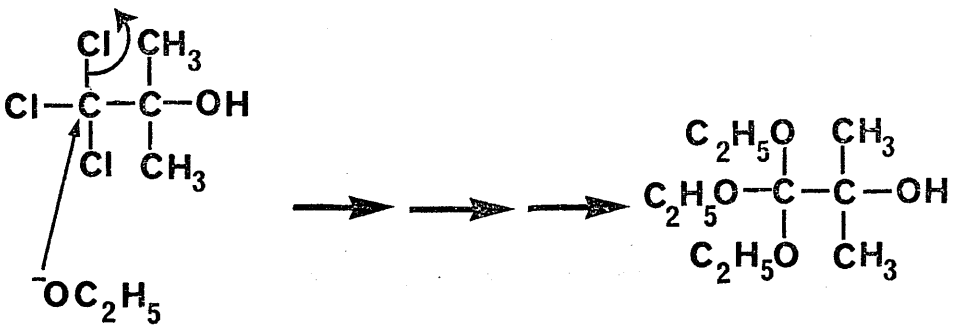


Confirmatory Reactions

(i) Sodium Ethoxide

Introduction

If the first step of the reaction is the replacement of chlorine by pyridine directly then the reaction will proceed as well, if not better, using sodium ethoxide instead of sodium hydroxide. If the reaction proceeds as suggested, the Fujiwara colour will not be formed as the acid chloride cannot be formed.



All the chlorine may not be replaced due to steric hindrance inhibiting the reaction, but the acid chloride cannot be formed.

Procedure and Result

2 ml. of pyridine containing 9 mg. of chlorbutanol were added to 1 ml. of sodium ethoxide and heated in a boiling water bath for 30 minutes. No visible reaction occurred, even after the addition of water.

Discussion

If the reaction were by means of dichlorocarbene this would only be impeded, not prevented by the use of sodium ethoxide. The dichlorocarbene would be formed, some of which would react with the ethanol, but some also would react with the pyridine, giving rise to the pink colour.

Conclusion

This strengthens the belief that the first step in the reaction is the Sn2 replacement of one of the chlorine atoms by a hydroxyl group, and not the decomposition of the chlorbutanol to chloroform, dichlorocarbene and acetone.

(ii) Acetyl Chloride

Introduction

If the Fujiwara reaction proceeds by way of an acid chloride intermediate then an acid chloride should produce a similar reaction product.

Procedure and Results

A little acetyl chloride was added to a few drops

of pyridine. A vigorous reaction resulted forming a cream coloured solid.

The solid was divided into three parts which were tested as follows:-

(i) By adding a little 10% aqueous sodium hydroxide solution and heating on a boiling water bath for 30 minutes. No visible reaction occurred.

(ii) By adding a little pyridine and 10% aqueous sodium hydroxide solution and heating on a boiling water bath for 30 minutes. No visible reaction occurred.

(iii)By adding pyridine and heating on a boiling water bath. On warming a rapid reaction occurred to yield a red, extremely viscous mass. This gave the infra-red spectrum shown in figure 4(a).

An attempt was made to purify part of this viscous mass. A little was dissolved in 10% aqueous sulphuric acid forming a pale yellow solution. A little of this acid solution was made basic with sodium hydroxide solution and gave a base insoluble red oil. This reversible reaction is reminiscent of the behaviour on thin layer chromatography of the reaction product reported by Moss and Rylance (95).

The remainder of the acidic solution was extracted with diethyl ether, which was dried and evaporated off. The product was an orange-red sticky substance which

FIGURE 4a

Intra-Red Spectrum of Acetyl Chloride Reaction Product

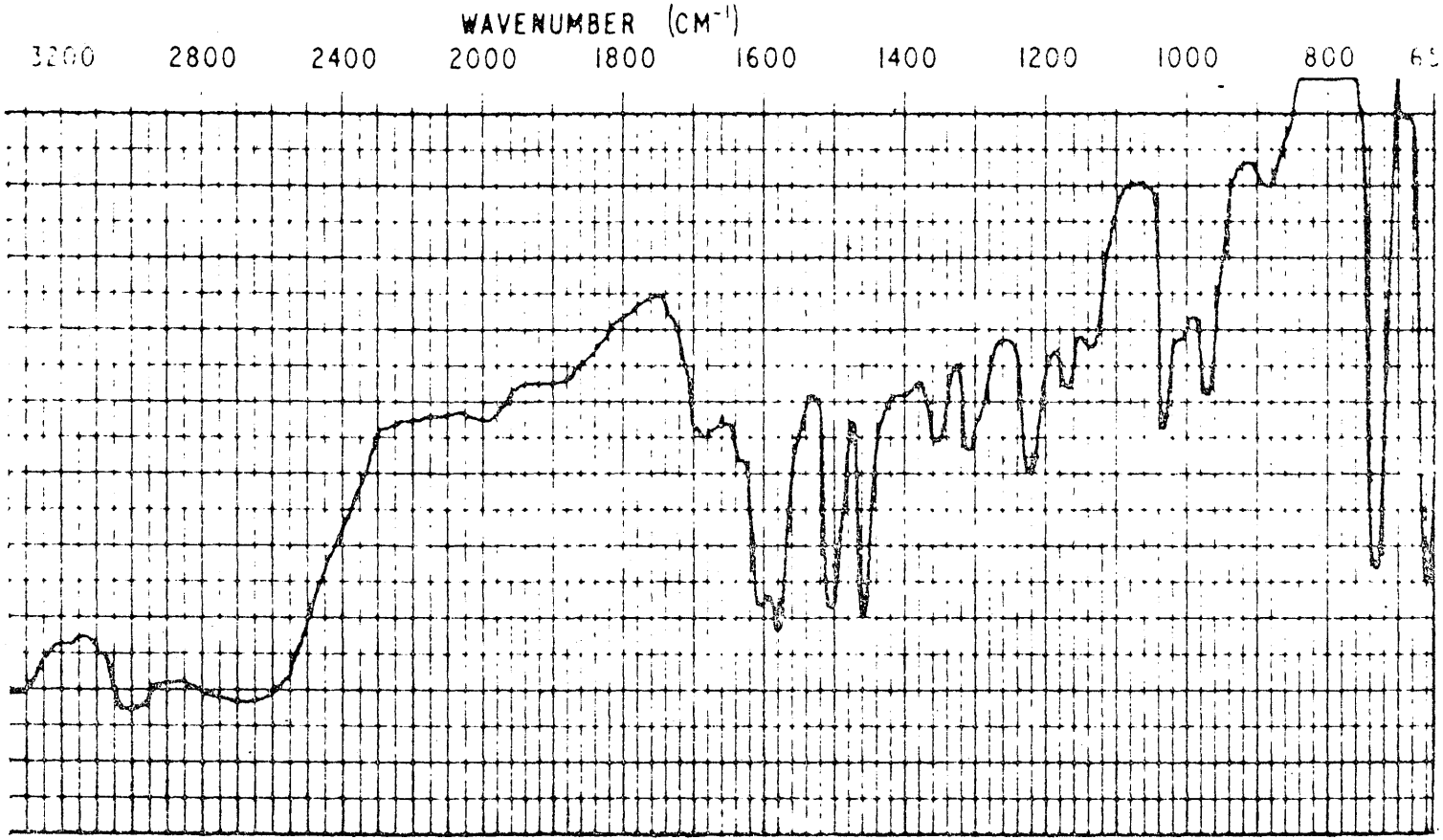
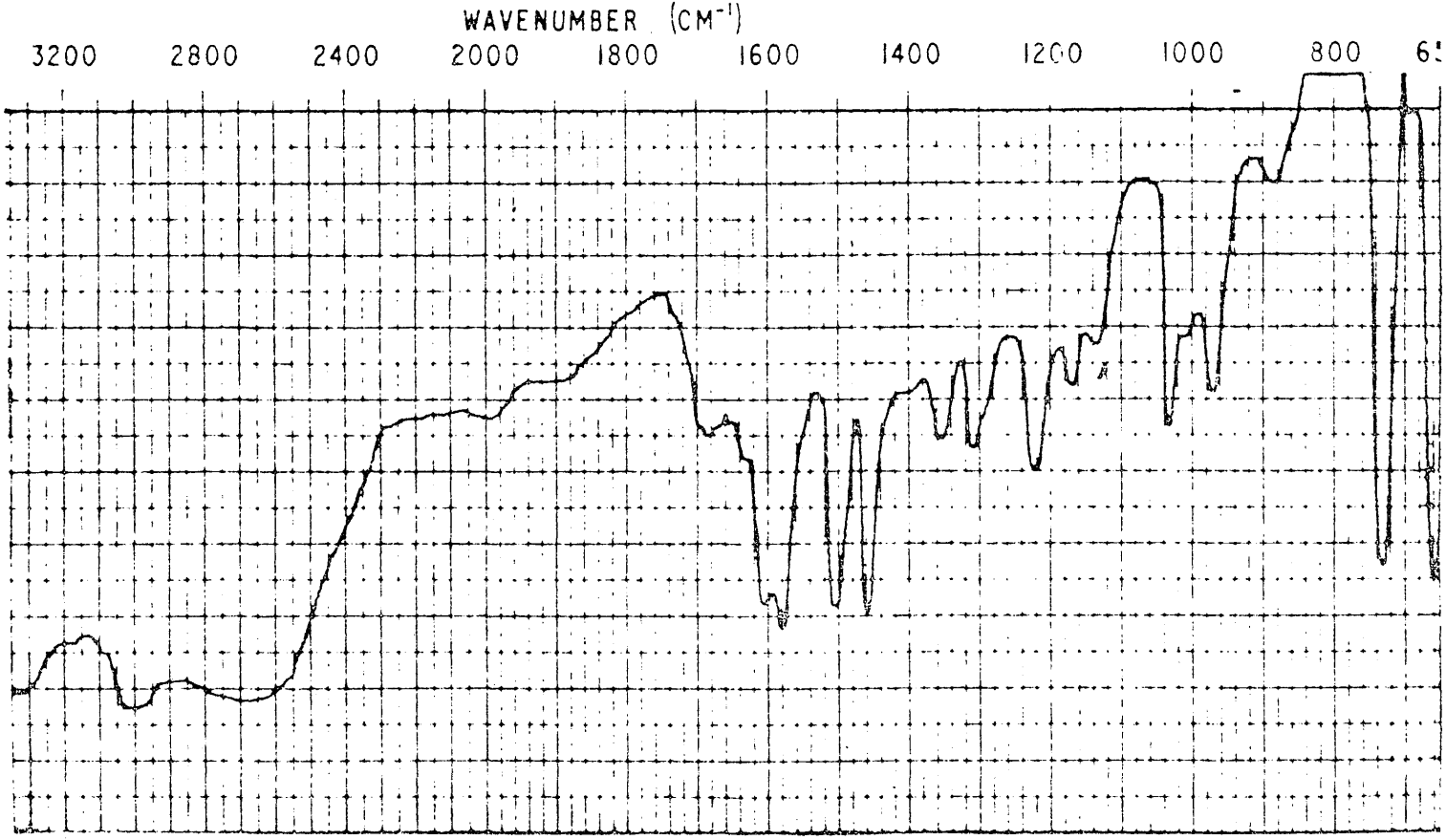


FIGURE 4b

Infra-Red Spectrum of Acetyl Chloride Reaction Product



gave the infra-red spectrum shown in figure 4(b).

Discussion

The production of a red colouration from pyridine and acetyl chloride is not, in itself, proof that the Fujiwara reaction proceeds in this manner. The products, although both red, may be totally different.

There is some indication that they are similar. Both compounds lose their red colouration in acidic solution and become red again on the addition of base. In addition, both are formed by reaction of a compound with pyridine when heated.

If the product is the Fujiwara reaction product then the results indicate that after addition of the acid chloride to the pyridine ring a further molecule of pyridine is involved. This was suggested by Redford-Ellis and Kench (106). Alternatively, there may be sufficient water in the reagents to open the pyridine ring on heating.

However, for definite proof that this product is the same as the Fujiwara reaction product, it is necessary to isolate some of the Fujiwara reaction product, and use anhydrous reagents.

Conclusion

Pyridine and acetyl chloride react to form a red

substance which may be similar to the Fujiwara reaction product. The results obtained suggest that, after the formation of the N-acetyl pyridinium chloride, a further molecule of pyridine is necessary, and not water. However, the reaction may have proceeded due to water in the reagents.

Use of Anhydrous Reagents

Reagents

The acetyl chloride is dry, as it would react with water to form acetic acid. The pyridine was dried over anhydrous sodium sulphate.

Procedure

A little acetyl chloride was reacted with anhydrous pyridine, and gave a white solid. Portions of this solid were treated as follows:-

i) Heated with an excess of anhydrous pyridine. No visible reaction occurred.

ii) Heated with 10% aqueous sodium hydroxide. A deep red-brown colouration resulted.

iii) Heated with non-dried pyridine. This produced a deep red colouration.

iv) Exposed to the atmosphere. The white solid changed rapidly through orange-red to black.

Discussion and Conclusions.

These results indicate that, after the formation of the pyridinium salt, water is necessary. It would also appear that further pyridine is not essential.

Isolation of the Fujiwara Reaction Product

(i) Thin Layer Chromatography

Introduction

Moss and Rylance (95) succeeded in chromatographing the Fujiwara reaction product on Kieselgel G254 using a solvent consisting of ethyl acetate saturated with water. The reaction product was located by re-developing the pink colour by spraying with aqueous sodium hydroxide solution.

Two millilitres of pyridine containing about 9 mg. of chlorbutanol were heated on a boiling water bath with 2 ml. of 10% aqueous sodium hydroxide solution for 10 minutes. Part of the pyridine was spotted on a thin layer chromatography plate and run in ethyl acetate saturated with water. On spraying with 10% aqueous sodium hydroxide solution red spots developed at about Rf 0.6. This is as found by Moss and Rylance (95).

Discussion

This is probably a feasible technique for isolating the Fujiwara reaction product. The use of eluates from thin layer chromatography plates for further analysis is a well established technique. However, past experience using infra-red spectrometry following

separation by thin layer chromatography has not been an unmitigated success. The sample size is always small, and may be contaminated with silica gel, affecting the infra-red spectrum obtained.

(ii) Low Temperature Distillation

Introduction

The Fujiwara reaction product is known to be unstable, thus any attempt to distil off the pyridine using heat may be expected to fail. If, however, the distillation is carried out at room temperature, there may be less decomposition of the reaction product.

This may be achieved at reduced pressure with a receiver cooled in a mixture of solid carbon dioxide and methanol with the boiler maintained at room temperature.

Procedure

The Fujiwara reaction product was prepared by heating 20 ml. of pyridine containing about 90 mg. of chlorbutanol with 10 ml. of 10% aqueous sodium hydroxide on a boiling water bath for 6 hours. The aqueous phase was then separated and discarded.

The pyridine layer was placed in a round bottomed flask connected to a receiver cooled by a solid carbon dioxide - methanol mixture. The apparatus was

evacuated by means of a rotary vacuum pump attached to the receiver. The pyridine vapour condensed and froze in the receiver, this continuing until all the pyridine was removed from the boiler. The residue was a deep red sticky mass.

With this technique, any sodium hydroxide in the pyridine would remain in the residue. To remove organic material, short path distillation at 50°C was used.

Results

The first fraction was a colourless liquid, which in the presence of pyridine and sodium hydroxide gave a pink colouration. The infra-red spectrum of this material is shown in figure 5(a).

The second fraction was a solid on the cold-finger condenser but a colourless liquid at room temperature. This did not give a pink colouration on addition of sodium hydroxide and pyridine. The infra-red spectrum is identical to that of the first fraction shown in figure 5(a).

The residue was a brown/black solid, which gave the infra-red spectrum shown in figure 5(b).

Discussion

The use of low temperature distillation was successful in isolating the reaction product from the

FIGURE 5a

Infra-Red Spectrum of Chlorbutanol Reaction Product. Distilation 2nd Fraction

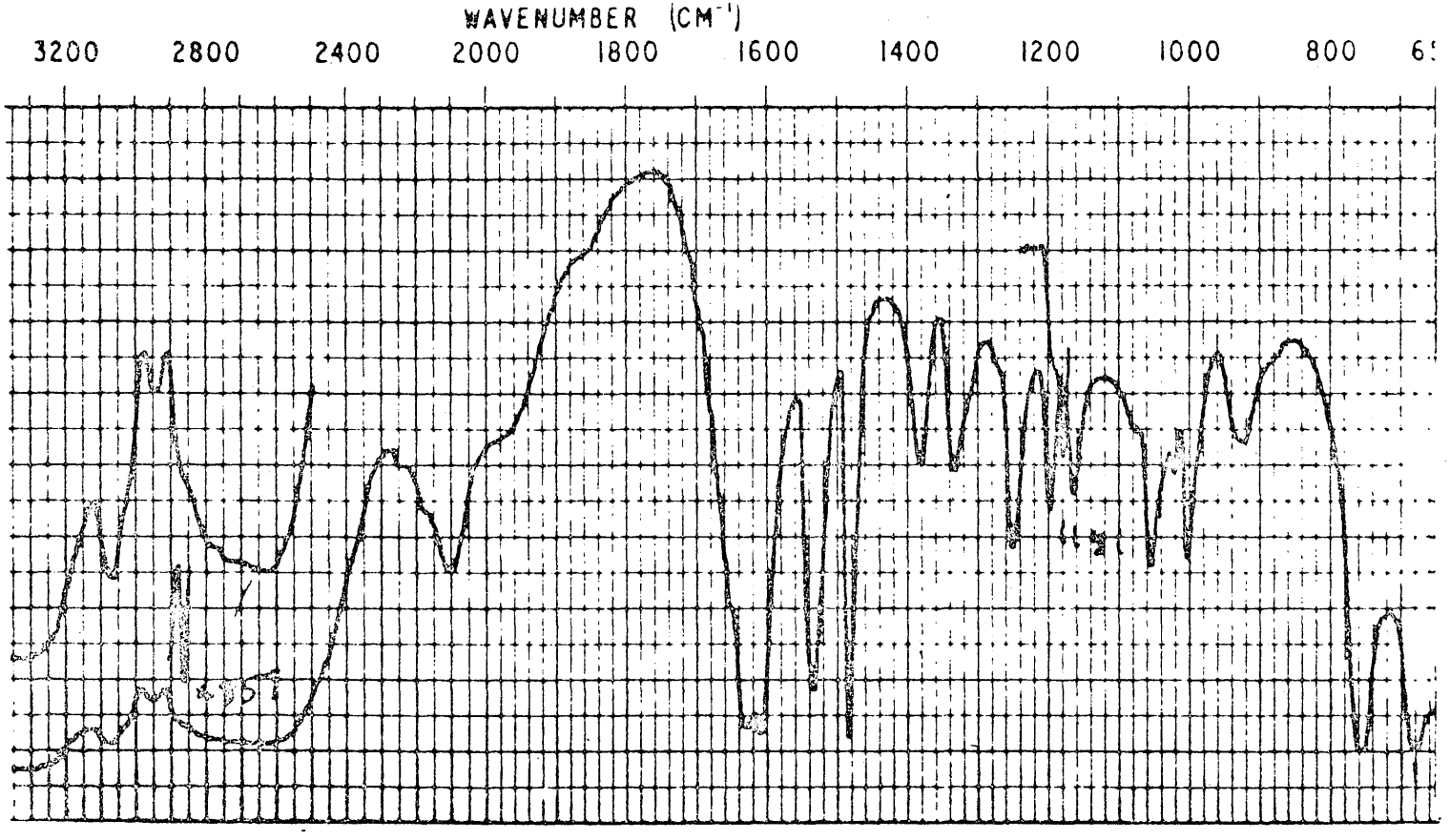


FIGURE 5b

Infra-Red Spectrum of Chlorbutanol Reaction Product. Distilation Residue.

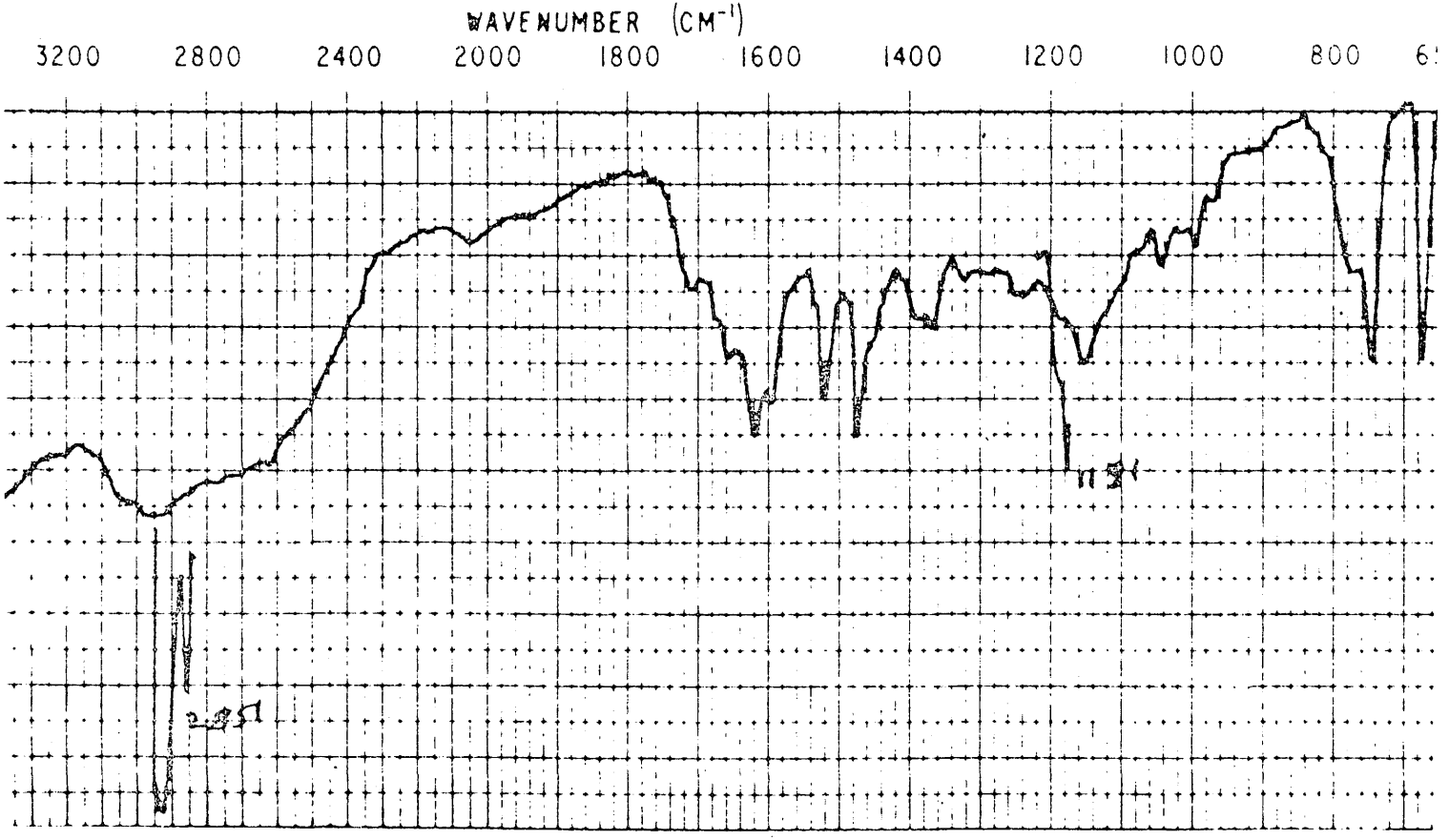


FIGURE 6a

Infra-Red Spectrum of Pyridine

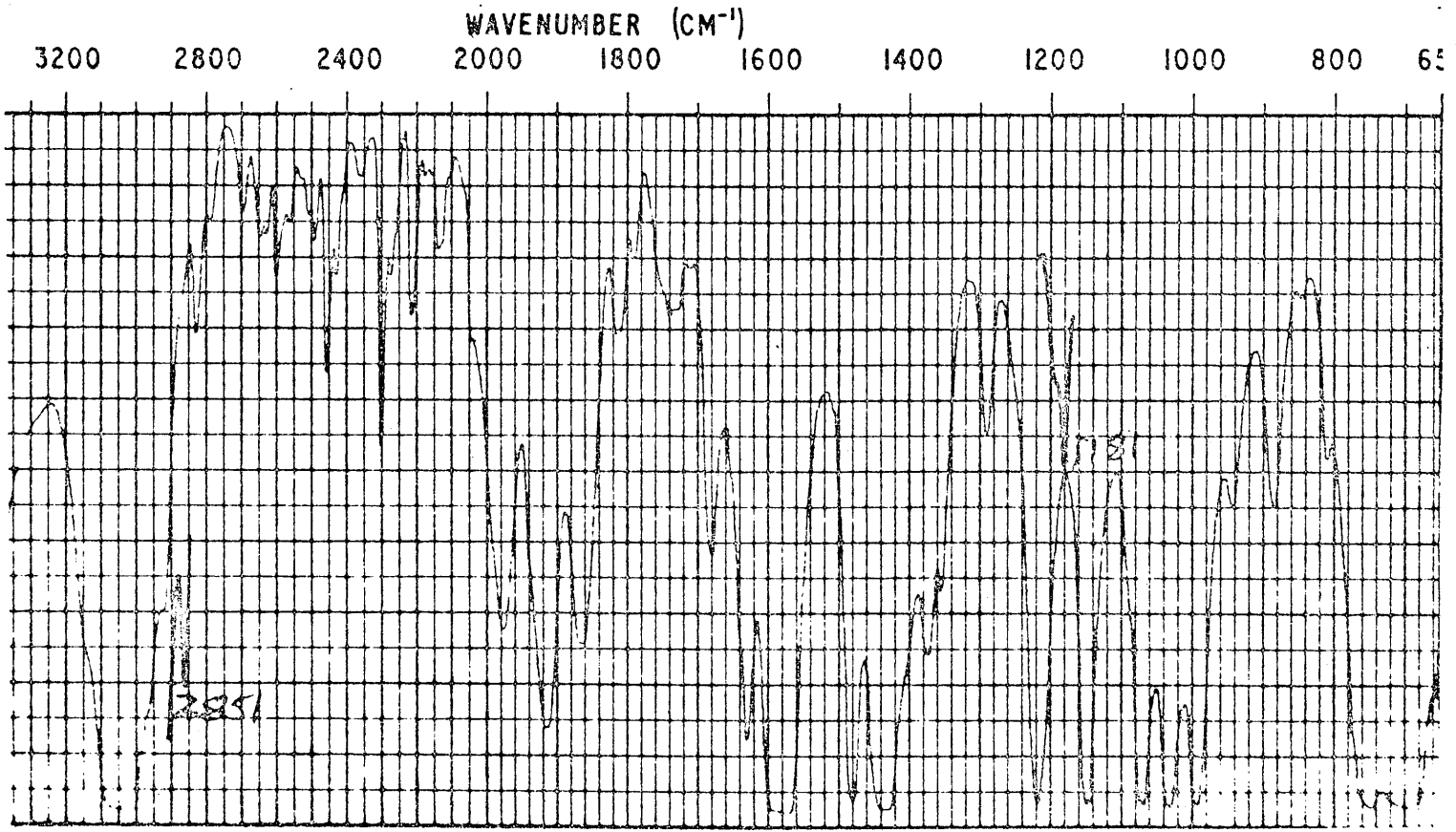
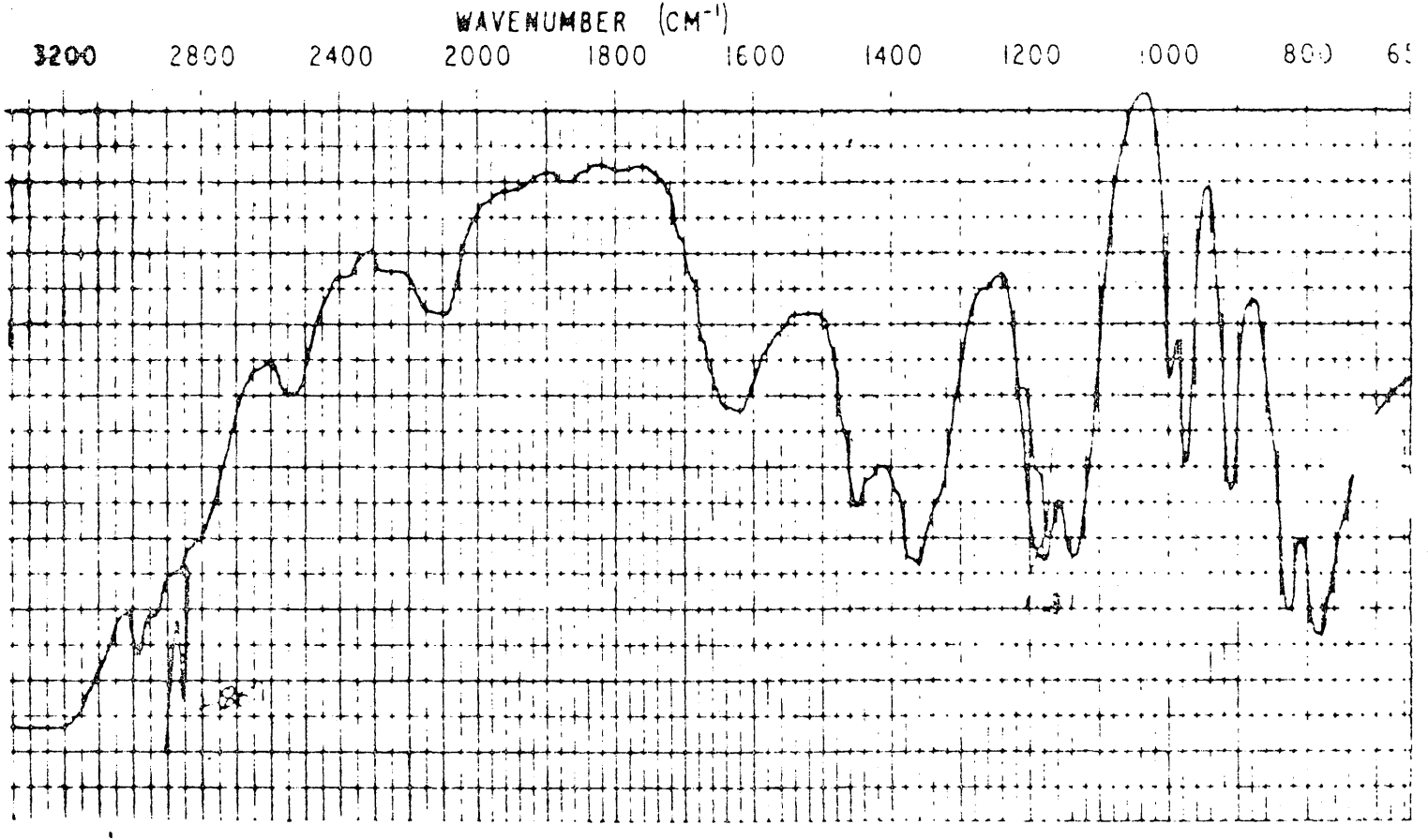


FIGURE 6b

Intra-Red Spectrum of Chlorbutanol



reaction mixture. However, it would appear that the products for which infra-red spectra were obtained was not the pink coloured complex but the final product.

In keeping with the findings of Moss and Rylance (95), the addition of sodium hydroxide to one fraction gave a pink colour. The infra-red spectrum for this fraction was identical to that of the other fraction which did not give a pink colour with sodium hydroxide. This suggests that the pink compound was only present in trace amounts in a liquid largely composed of the final product.

One very interesting factor is that the infra-red spectrum of the Fujiwara reaction product is very similar to the reaction product given by acetyl chloride and pyridine. The only difference is that the peak at 2100 cm^{-1} in the Fujiwara reaction product is shifted to 2000 cm^{-1} with the acetyl chloride product and there is a peak at 1680 cm^{-1} in the acetyl chloride product which does not occur in the Fujiwara product. This latter peak is probably due to a decomposition product. The regions between 650 cm^{-1} and 2000 cm^{-1} which includes the "fingerprint region" are so similar as to suggest very strongly that the two products are the same. This adds further weight to the suggestion that the reaction with chlorbutanol proceeds by way of

the acid chloride.

The most likely contaminants are pyridine and chlorbutanol. These can be excluded from the infra-red spectrum. Figure 6(a) shows the infra-red spectrum for pyridine, and figure 6(b) the spectrum for chlorbutanol.

Conclusions

The knowledge to date suggests that the first stage of the Fujiwara reaction is a reaction between the sodium hydroxide and chlorbutanol. The reaction proceeds to give an intensely pink compound, eliminating all the chloride in the process. This pink compound further decays to give an almost colourless compound for which the infra-red spectra are as shown.

The reaction between acetyl chloride and pyridine yields a red compound which in turn gives an infra-red spectrum virtually identical to that of the Fujiwara reaction product. This suggests two things. Firstly, that this red compound was in fact largely the colourless reaction end product containing a small quantity of the intensely coloured species. Secondly, that the Fujiwara reaction may proceed by way of the acid chloride.

The postulated mechanism involving an acid chloride intermediate fits the available data. This reaction

involves the sequence:-

$CCl_3.C(CH_3)_2.OH \xrightarrow{\bar{O}H} HO.CCl_2.C(CH_3)_2.\ddot{O}H \longrightarrow$

$COCl.C(CH_3)_2.OH$

Use of Substituted Pyridines

Introduction

By using substituted pyridines, two pieces of information may be obtained. Firstly, if the reaction does not proceed when a particular ring position is substituted then it can be said that that particular position is closely involved in the reaction. Secondly, if the coloured reaction product is formed from the pyridine ring residue, then the absorption maximum will differ for different substitution patterns.

There are three types of substituted pyridines. These are the picolenes which are mono-methyl derivatives and exist in three isomers, α , β and γ . Secondly, there are the lutidenes, the commonest being 2, 6 dimethyl pyridine. Thirdly, there is collidine which is 2, 4, 6 trimethyl pyridine. In addition, there is the related material, quinolene.

Initial trials were carried out using trichloroacetic acid. This gives a positive Fujiwara reaction, and a comparison with the products obtained when chlorbutanol is used may assist in elucidating the reaction mechanism.

Procedure

For the trials, 2 ml. of 10% aqueous sodium hydroxide and 2 ml. of the substituted pyridine were

placed in a test tube and a little trichloroacetic acid added. The tubes were heated in a boiling water bath until a colour developed or for 30 minutes, whichever was the shorter.

Picolines

α Produced an orange-red colour, eventually turning brown.

β Produced a red colouration

γ Produced a purple colour, fading to green

2, 6 Lutidene and Quinoline

A red colour was obtained from both these reagents

2, 4, 6 Collidene. No reaction was observed.

Discussion

The results from the picolines indicate that the γ position is not closely involved, and that both α or both β positions are not involved.

The reaction of 2,6 Lutidene would suggest that either the β or γ positions are involved, but since γ picoline reacts the reactive site must be the β position. That only one of the β positions is involved is substantiated by the reaction of quinoline.

However, 2, 4, 6 collidine does not react. This molecule has the positions, other than the β positions, substituted. Thus there is conflicting evidence.

The only satisfactory deduction is that the hindrance to the reaction is not solely steric. The reaction appears to depend upon the opening of the pyridine ring, promoted by an electro-negative group attached to the ring nitrogen. The methyl groups attached to the pyridine ring act as inductive groups, counteracting the effect of the group attached to the nitrogen atom. In 2, 4, 6 collidine the combined inductive effect is sufficient to inhibit the ring opening reaction, but the inductive effect of less than three methyl groups is insufficient to inhibit the reaction completely.

Conclusion

The inhibition of the reaction is due to inductive effects and not due to steric hindrance. These qualitative results give no indication of whether the coloured product is due to the pyridine ring residue, and if so where the ring opens.

Spectrophotometric Comparison

Introduction

This was carried out to determine whether the coloured product contains the pyridine ring residue, and if it is possible to determine where the pyridine ring is opened.

Procedure

A series of tests was carried out using the picolines and both trichloroacetic acid and chlorbutanol. The reaction was carried out as described earlier and spectral measurements made upon a Unicam SP800 spectrophotometer. The organic phases were placed in 1 mm. silica cells, and measurements made against reagent blanks.

Results

The results are given in Table 17.

TABLE 17

Absorption maxima using substituted pyridenes (mμ)

	Chlorbutanol	Trichloroacetic Acid
Pyridine	365, 530	365 530
α -Picoline	381, 520	381, 520
β -Picoline	370, 550	370, 550
γ-Picoline	398, 624	-

Discussion

The reaction with trichloroacetic acid and chlorbutanol appears to yield the same chromophore. The products may not be identical, but are closely related.

The different absorption maxima resulting from the different substituted pyridines do, however, suggest that the chromophore is formed from the pyridine ring residue. The different positions of

the methyl group on the chromophore producing wavelength shifts. This explains the observation of Redford-Ellis and Kench (106) that the absorbance at 365 mμ decreased when using substituted pyridines. This is the maximum for pyridine only. With substituted pyridines, the measurements were being made on the side of the absorption peak.

If the α , β and γ positions of the pyridine ring are all blocked then the Fujiwara reaction does not occur. However, if any one, or more, of these positions is vacant then a colour is produced. This cannot be due to steric hindrance, though this may reduce the rate of reaction. If it were steric hindrance, then blocking the reactive site, or sites, would inhibit the reaction completely. The effect is more likely a combination of the steric hindrance reducing the reaction rate, and the inductive effect of the methyl groups counteracting the electronegative group and further reducing, if not stopping, the rate of ring cleavage.

Conclusions

The Fujiwara chromophore certainly includes either the pyridine ring, or a residue of this ring if cleavage occurs.

The results suggest that the product from trichloroacetic acid and chlorbutanol are the same. This may be only the same chromophore with different side chains, or an identical molecule.

Interpretation of the Infra-Red Spectrum

The infra-red spectrum of even simple compounds cannot be interpreted to show the molecular structure with absolute certainty. Following interpretation a spectrum of the supposed compound should be compared with the spectrum obtained from the sample. The "fingerprint" region of an infra-red spectrum is unique to a particular compound.

Comparison of the spectra obtained during this work showed three things. Firstly, that neither pyridine nor chlorbutanol were present in the samples in significant quantities. Secondly, that the products obtained from the Fujiwara reaction and from the reaction between acetyl chloride and pyridine are almost certainly the same compound. Thirdly, that the spectra were not those of absolutely pure compounds, this hinders interpretation of the spectra, but such an interpretation may give some usable information.

Bands Considered

1610 1630 These are probably two similar groups in different molecular environments. The groups may be:-

$-NH_3^+$

conjugated cyclic $\rangle C = N -$

conjugated $\rangle C = C \langle$ and $\rangle C = O$

Aromatic $\diagdown C = C \diagup$

$-O-NO_2$

$\diagdown N-NO_2$

$C-N=O$

$-O-N=O$

$-CO_2$

cis $R-CH=CH-R_1$

Primary Amide

Exclusions

$-NH_3^+$ The bands in the range 1,610 - 1,485 cm.$^{-1}$ are of low intensity for this group. For the sample they are strong.

Aromatic $\diagdown C = C \diagup$ These can be excluded as the strong bands associated with this group in the regions 1,070 - 960 cm.$^{-1}$ and 900 - 730 cm.$^{-1}$ are absent.

$\diagdown N-NO_2$ The strong bands in the regions 1300 - 1260 cm.$^{-1}$ and 790 - 770 cm.$^{-1}$ normally associated with this group are absent.

$-C-N=O$ This group only occurs in the region of 1600 cm.$^{-1}$ if it is in the form of an α - halogen aliphatic compound. No halide is present in this molecule.

$-O-N=O$ The bands at 845 cm.$^{-1}$ and 680 cm.$^{-1}$ would indicate the cis form of this group. However, the expected band in the region

3,360 - 3,220 cm.$^{-1}$ is absent.

Primary Amide. These give a medium band in the region 1,420 - 1,400 cm.$^{-1}$ which is absent. The two peaks are also rather closer than the normal amide I and II bands.

680 cm.$^{-1}$ and 755 cm.$^{-1}$

These bands can only be associated with a molecule incorporating the group R - CH = CH - R_1, in the cis configuration.

3060 cm.$^{-1}$

This can be associated with the groups -CH = CH-, R_1R_2C = CHR_3, R_1R_2C = CH_2, aromatic C - H, amino acids, and amido acids. The groups, other than -CH = CH - are excluded due to the absence of other bands which have already been considered.

2100 cm.$^{-1}$

This can be associated with -C ≡ CH, isonitriles or cyanide ion. If the band at 2,000 cm.$^{-1}$ in the acetyl chloride is taken as corresponding to this band then the group must be $^{-}C \equiv N$. The other groups do not give bands as low as 2,000 cm.$^{-1}$ The frequency shift may be due to changes of pH.

Discussion

The group -CH = CH- in the cis configuration is undoubtedly present in the molecule. This can be

expected if the pyridine ring is opened. Since this is so, it is very likely that the bands at 1610 $cm.^{-1}$ and 1630 $cm.^{-1}$ are due to the conjugation of such a group with a carbonyl group. The other possible groups - $C(=O)O$, - O - NO_2, and conjugated cyclic $\diagdown C = N \diagup$ cannot be excluded from study of the spectrum. Covalent nitrates are unlikely to be formed under the reaction conditions, but the other two groups may be.

Conclusion.

The molecule contains one, or more, groups of the type -CH = CH- conjugated with carbonyl groups. This is in agreement with the fact that the Fujiwara reaction product reacts with primary amines such as benzidine. The two conjugated carbonyl groups must be in slightly different environments to produce the two bands around 1620 $cm.^{-1}$. The samples would also appear to contain cyanide ion which may be a by-product of the reaction.

i) All three chlorine atoms in the chlorbutanol are eliminated in the formation of the pink colouration.
ii) The initial reaction is between chlorbutanol and sodium hydroxide, probably forming the acid chloride.
iii) The acid chloride then reacts with pyridine, probably forming the N-substituted salt. This then reacts with water.

iv) The final reaction product contains two carbonyl groups conjugated with a cis alkene. Cyanide is a possible reaction by-product.

v) A positive Fujiwara test can be obtained from a variety of materials including 2, 4 dinitrochlorobenzene, benzotrichloride, and chloroform, but not from trichloroethanol.

vi) The ultraviolet/visible spectral absorption for the trichloroethylene differs from that for chlorbutanol or chloroform.

vii) Although trichloroethanol does not give a visible colour in the Fujiwara test after acidification and the addition of benzidine, it yields the same product as the Fujiwara positive materials.

Reaction Mechanism

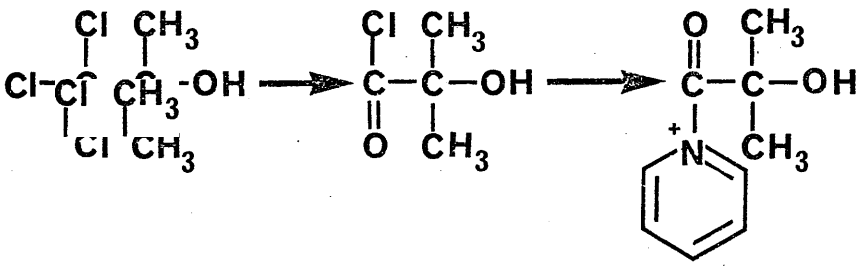
This must occur in two basic steps:-

i) The formation of a group which attaches to the nitrogen atom and has strongly electronegative properties.

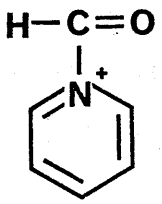
ii) The splitting of the pyridine ring to form a compound, the colour of which is pH dependent.

i) The Initial Reaction.

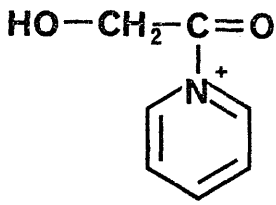
This has been outlined for chlorbutanol as shown:-



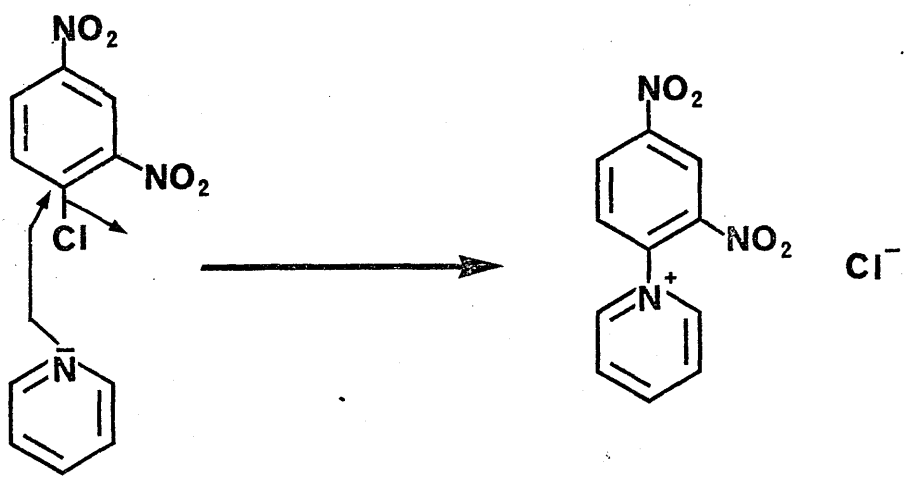
and by a similar mechanism chloroform would give the salt:-



and trichloroethanol the salt

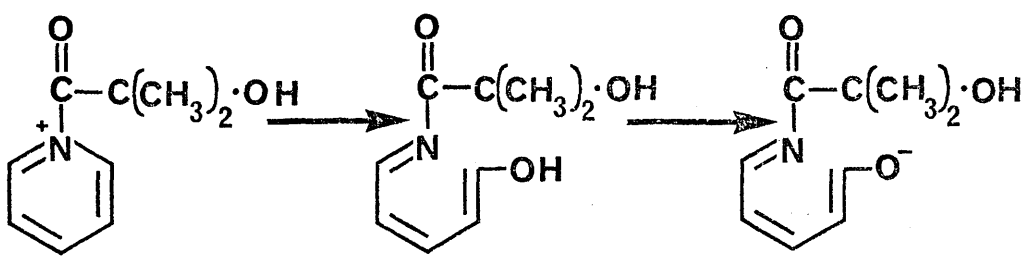


For 2, 4 Dinitrochlorobenzene, this cannot be the mechanism. In this case, there is a direct replacement of the chlorine atom to give 2, 4 Dinitrophenylpyridinium chloride:-

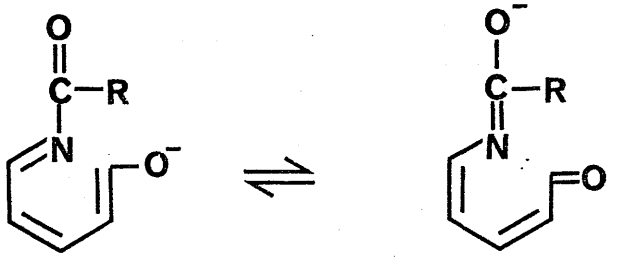


ii) The Formation of the Coloured Complex

This must be by the attack of hydroxide ion at the α position of the pyridine ring:-



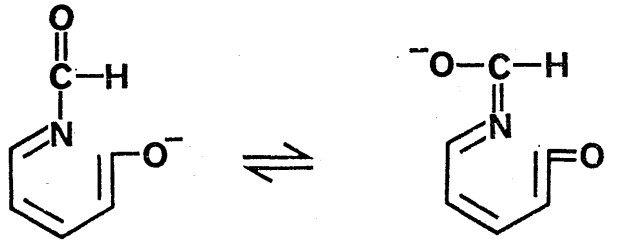
The ionic form is present as the reaction is carried out in the presence of a strong base. Due to the long conjugation, two tautomers will be present:-



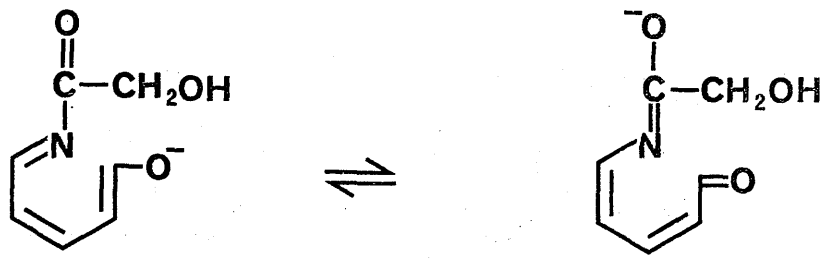
Where $R = C(CH_3)_2 \cdot OH$

The conjugation increases the acidity of the proton attached to either of the systems oxygen atoms as the resultant negative charge can be delocalised throughout the conjugated system. The proton attached to the third oxygen atom is, however, far less acidic as the charge could not be dispersed to anything like the same extent.

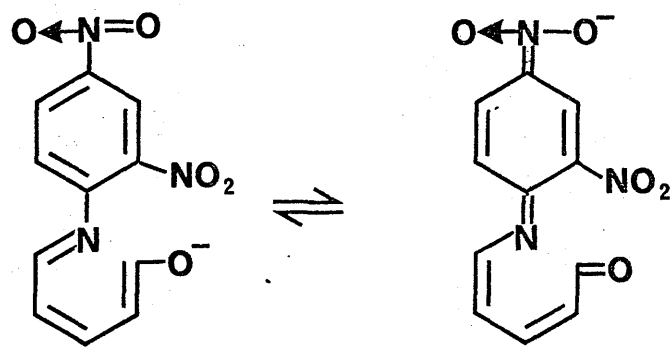
For chloroform the tautomers would be:-



and for trichloroethanol:-

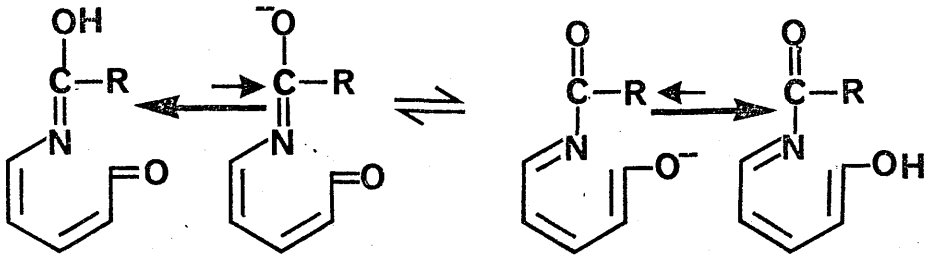


For 2, 4, dinitrochlorobenzene, the charge is even more delocalised:-



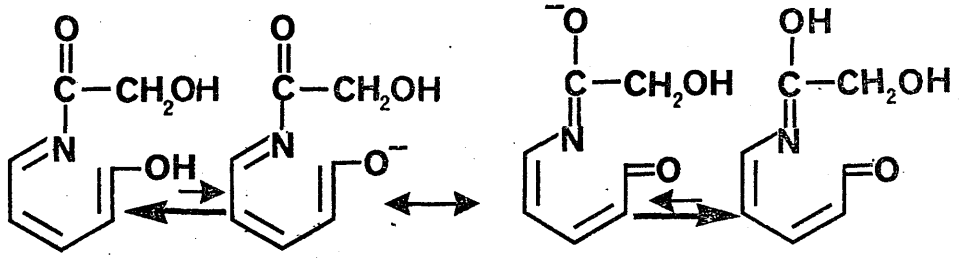
This so far does not explain the absence of colour when the reactant is trichloroethanol. The tautomers have been drawn in the ionised form. The compounds must, however, be weak acids with a little of the ionised species in equilibrium with much more of the undissociated form. Thus, for chlorbutanol, a more

correct representation would be:-

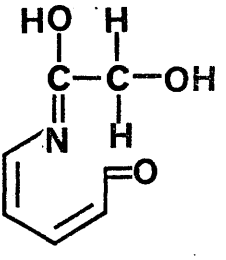


R= $\cdot C(CH_3)_2 OH$

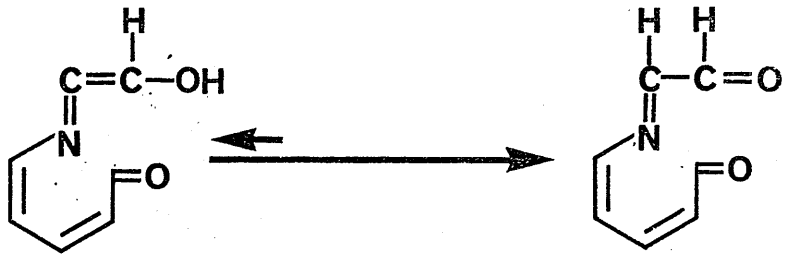
and for trichloroethanol:-



In the species



elimination of water can occur by the mechanism:-

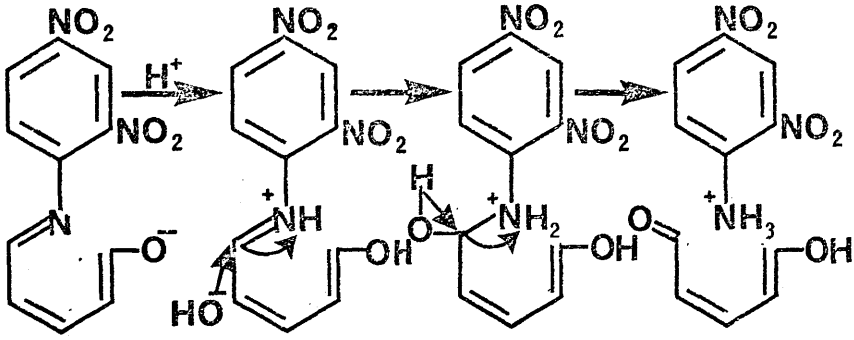


Neither of these gives rise to a charged species which would be highly coloured. In addition, one of the reaction products is removed so the reaction above proceeds to the right until there is none of the coloured species remaining.

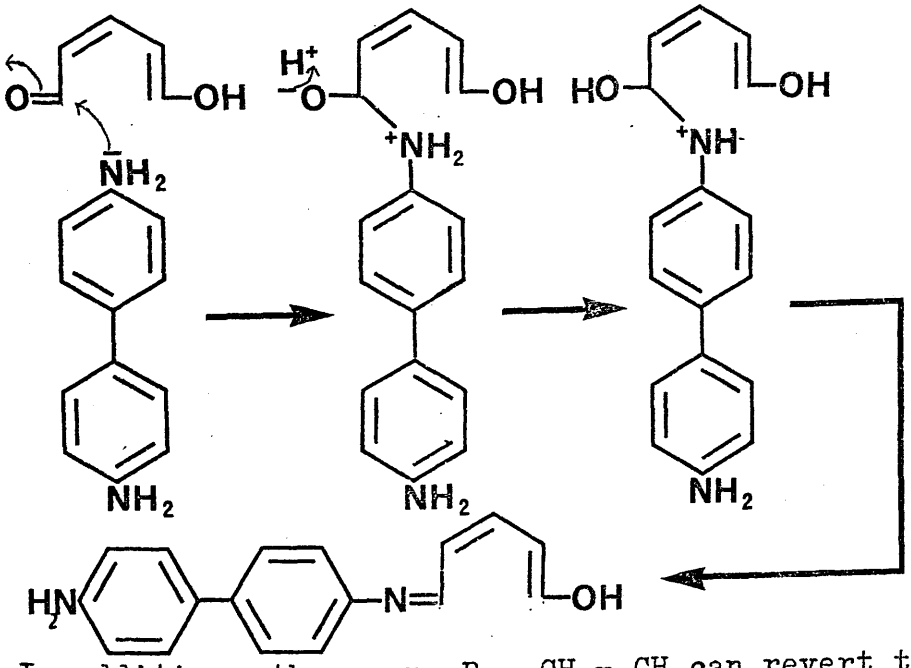
iii) The addition of benzidine in Formic Acid.

This is known to give the same product whether the starting material was chlorbutanol, trichloroacetic acid, trichloroethanol, chloroform, or any of a number of other materials.

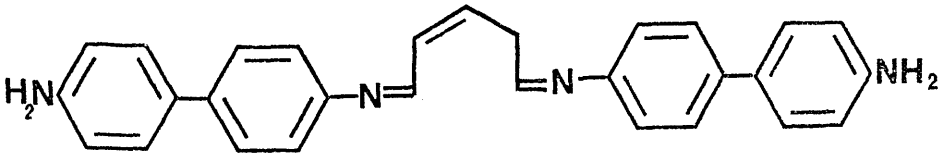
With the product formed from 2, 4 dinitrochlorobenzene, there is only one possible reaction pathway:-



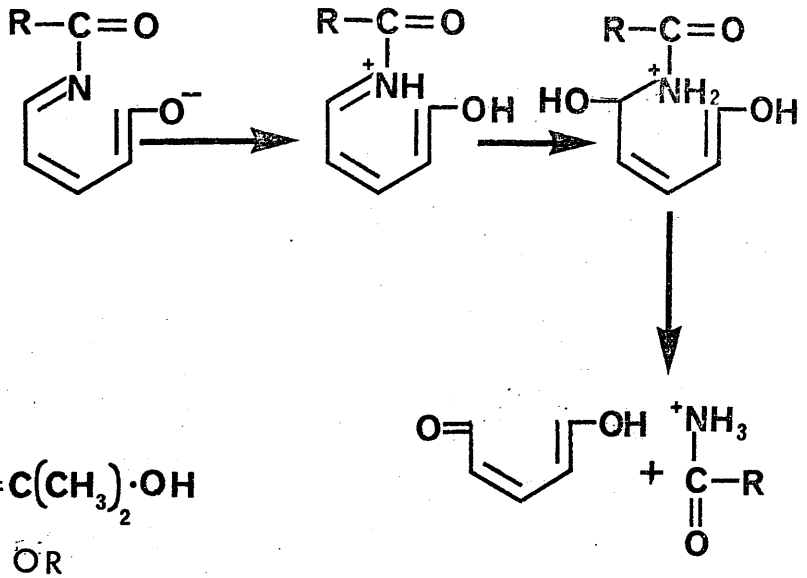
The reaction with the benzidine then occurs:-

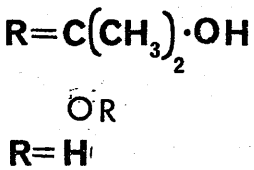


In addition, the group R - CH = CH can revert to the carbonyl form R - CH_2 - C - H which reacts in a similar manner with benzidine to give eventually the compound:-

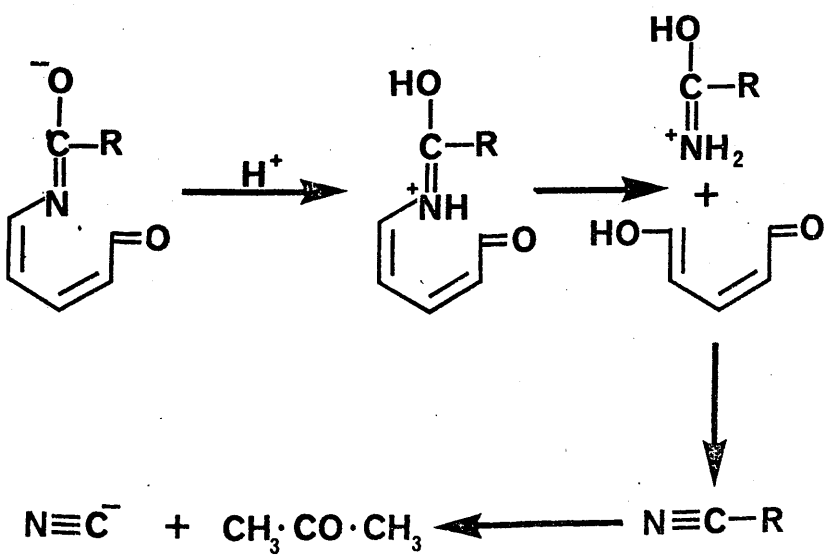


For the products from chlorbutanol and chloroform there are two pathways:-

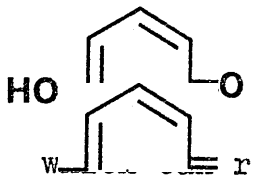




or alternatively:-



As can be seen, both routes give the compound



react with benzidine, as did the product from 2, 4 dinitrochlorobenzene.

The nitrile, decomposing to give cyanide ion, accounts for the bands observed in the infra-red spectra at 2000 cm.$^{-1}$ and 2100 cm.$^{-1}$.

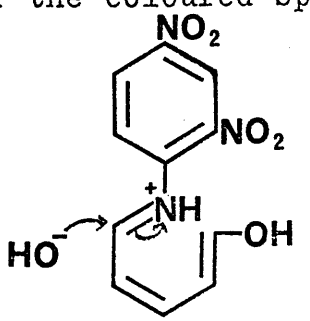
In practice, both routes are probably followed simultaneously, which would account for the acetone found by Moss and Rylance (95).

For chloroform, the products will be 

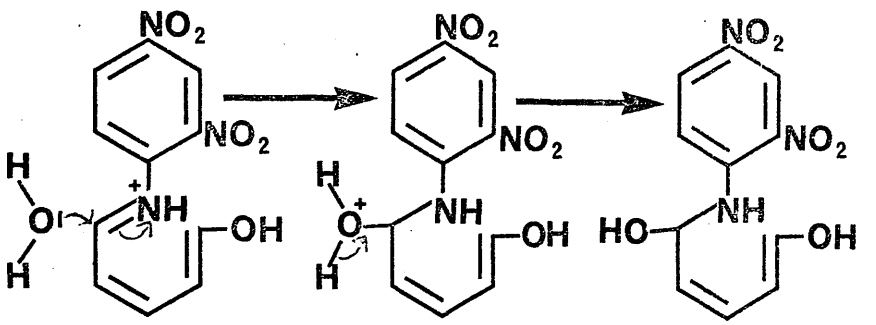
and $H.CO.NH_2$ and $\bar{C} \equiv N$, again the product reacting with the benzidine is as for 2, 4 dinitrochlorobenzene and chlorbutanol.

Discussion

Certain of the steps in the reaction mechanism have been drawn for simplicity. For example, the decomposition of the coloured species has been shown as:-

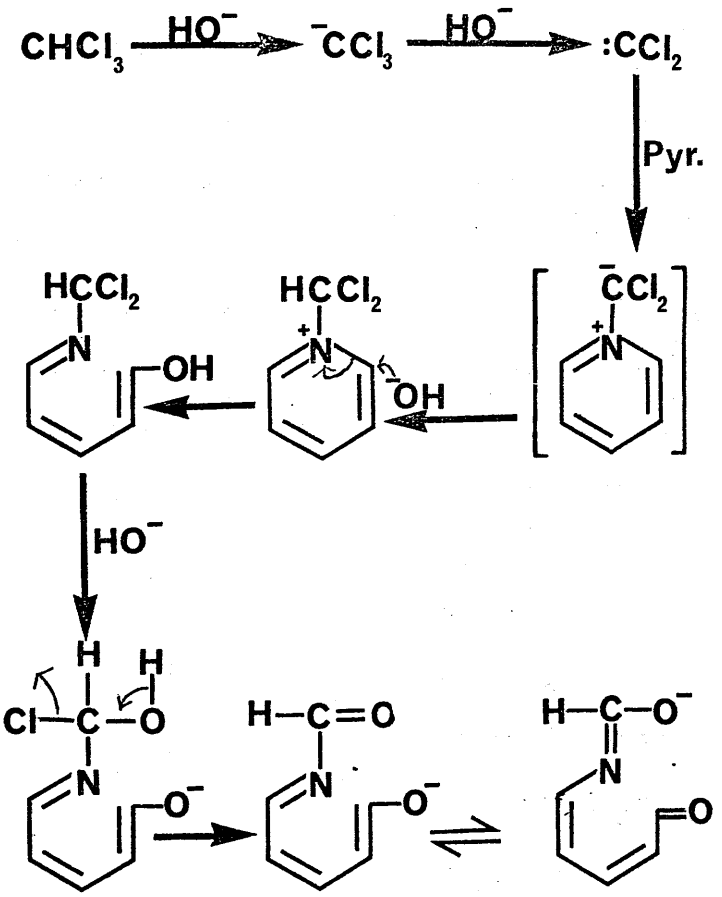


When the reaction will, in fact, involve the lone pair of an oxygen atom in a water molecule and can be more correctly represented as:-



The simpler representation is easier to follow, and is effectively what happens, though the actual steps involved are somewhat more circuitous.

The mechanism outlined does not involve dichlorocarbene. This is very readily formed from chloroform by the action of aqueous sodium hydroxide. Such a mechanism would involve the following steps:-


CHCl3
HO⁻
⁻CCl3
HO⁻
:CCl2
Pyr.
HCCl2
N
OH
HCCl2
N
OH
⁻CCl2
N
HO⁻
H
H
Cl
C
O
N
O⁻
H—C=O
N
O⁻
H—C—O⁻
N
O

This does give a similar reaction product to that obtained using the acid chloride pathway. The coloured product is a charged species with the charge delocalised over a conjugated system involving a carbonyl group.

If such a mechanism is involved when using chlorbutanol, then acetone must be liberated before the formation of the pink colour. None was detected.

Such a mechanism would assist in explaining why there is no coloured product formed by trichloroethanol. Chlorbutanol, chloroform and trichloroacetic acid can all yield dichlorocarbene, trichloroethanol cannot. If the thin layer chromatography results obtained by Moss and Rylance (95) are correct, then there is some evidence that the pink compound is identical for chlorbutanol, chloroform and trichloroacetic acid. This can only be so if the reaction proceeds via dichlorocarbene.

If dichlorocarbene is the sole pathway, however, it is difficult to explain how Liebmann and Hindeman (86) obtained a molar absorbance for chlorbutanol greater than that for chloroform. If there is, in fact, two pathways, one involving dichlorocarbene and one involving the acid chloride then such results may be explained by the linking of two dichlorocarbene

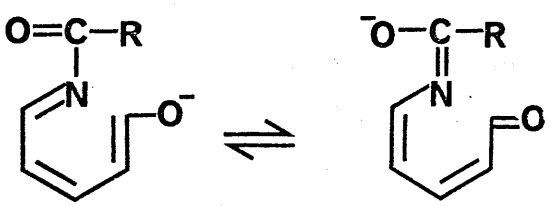
groups to give tetrachloroethylene:-

$$2{:}CCl_2 \longrightarrow Cl_2C{=}CCl_2$$

Such a reaction will be dependent upon the concentration of dichlorocarbene. If the reaction proceeds solely by way of dichlorocarbene for chloroform, and by way of dichlorocarbene and the acid chloride for chlorbutanol, such combinations of dichlorocarbene will be greater for chloroform than for chlorbutanol. This would mean that the molar absorbance would be greater for chlorbutanol as there is less loss due to a side reaction. If the reaction for chlorbutanol involves only the acid chloride then this effect is even more pronounced.

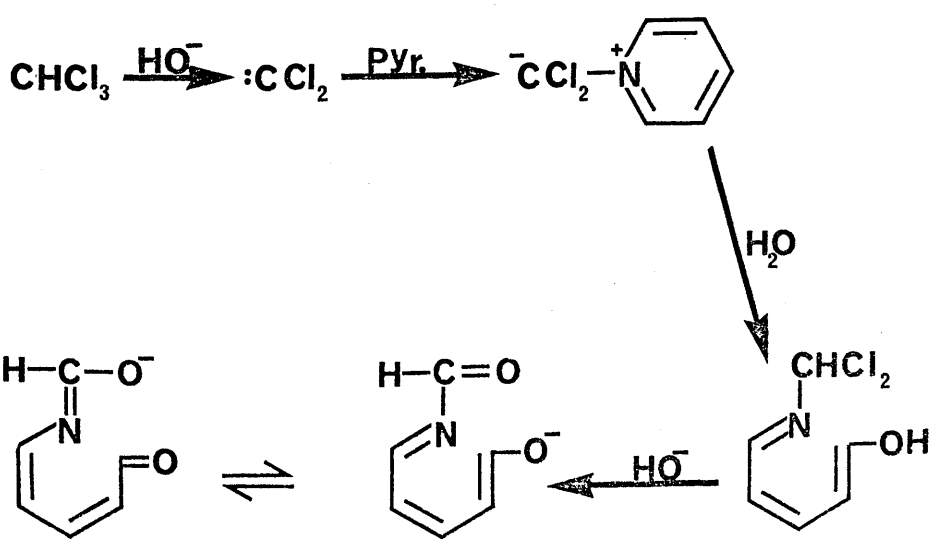
Conclusions

The Fujiwara reaction involves the addition of an electronegative group to the nitrogen atom of the pyridine ring. The ring is opened, and the coloured product is a charged species of the form:-



where R is a substituent group, including hydrogen.

For chloroform, the reaction probably proceeds by way of dichlorocarbene:-



For chlorbutanol, two paths may be involved:-

$CCl_3 - C(CH_3)_2 - OH \longrightarrow \bar{C}Cl_3 + CH_3 - CO - CH_3$

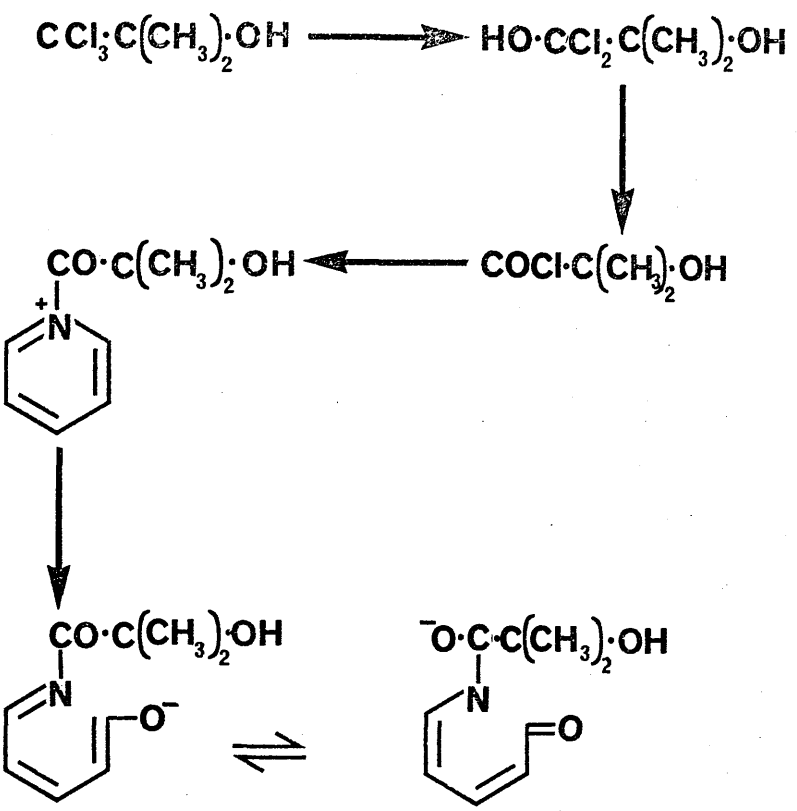
The dichlorocarbene reacting as it does when chloroform is the substrate. The other, major pathway involving the acid chloride is as outlined in the text.

For trichloroethanol, the reaction must be by way of the acid chloride. This would give a product which, after treatment with acid, would react with benzidine. However, the charged species giving the Fujiwara colour would only be briefly formed before removal as outlined in the text. If the concentration of trichloroethanol is high, a brief pink colour may be observed during the course of the reaction.
A positive reaction using trichloroethanol has been obtained by some authors (92).

The reaction cannot proceed solely by way of dichlorocarbene as such a group is not possible from 2, 4 dinitrochlorobenzene.

The Fujiwara reaction must therefore be one in which an electronegative group is attached to the nitrogen atom of the pyridine ring which, in the presence of water, splits to give a charged, stable species.

The work above indicates that for chlorbutanol the reaction must be:-



The charged species A and B above give rise to the pink colour observed when carrying out this reaction.

GAS CHROMATOGRAPHY - REVIEW

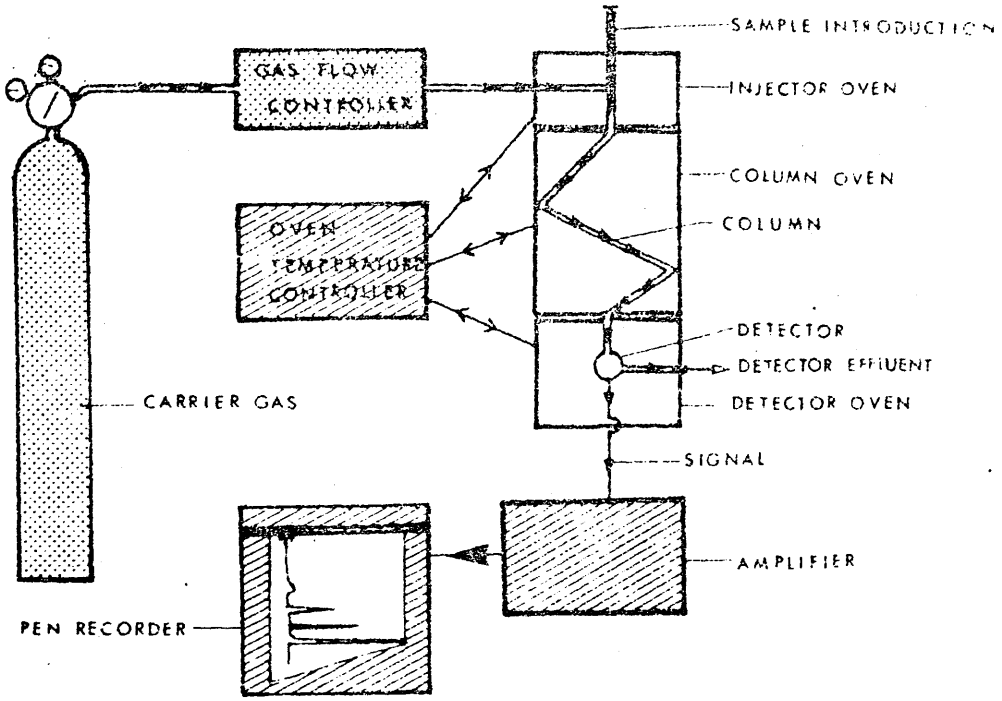
Introduction

Gas chromatography is one member of a family of techniques used to separate mixtures of materials. The original method was described by Twsett in 1903 (128). He used a chromatographic technique to separate coloured materials. The common feature of the techniques is the use of two phases, one stationary and one mobile. Separation depends upon the relative movement of the two phases. In the case of gas - liquid chromatography, the liquid is the stationary phase and the gas the mobile phase.

Gas - liquid chromatography was predicted by Martin and Synge in 1941 (91) as a result of work these authors conducted in the field of liquid - liquid chromatography. The technique of gas - liquid chromatography was proposed by James and Martin in 1951 (71) as a better means of separating distillable compounds than liquid - liquid chromatography. This was because the ratio of the vapour pressures of two members of an homologous series is usually greater than the ratio of their partition coefficients in liquid - liquid chromatography. In 1952 these authors were able to describe the technique of gas - liquid partition chromatography as applied to aliphatic acids (72).

Since then gas - liquid chromatography has been

Fig 7. Gas Chromatograph Layout (Schematic)



used in many fields for the separation of complex volatile mixtures. The technique is capable of giving quantitative as well as qualitative information and requires only small samples. The chief disadvantage of the technique is the cost of the equipment required.

Equipment

A diagram of the equipment is shown in Figure 7. The apparatus can be divided into four main sections:- the equipment for providing a controlled flow of carrier gas, the column and its temperature control system, the means of introducing the sample and the means of detecting and recording the components in the column effluent.

Carrier Gas Supply

The most frequently used carrier gases are nitrogen, helium and a mixture of argon and methane (9 : 1). These gases are available commercially in a high state of purity. Since the gases are delivered in cylinders at pressures of up to 2,500 lbs/sq. in., it is necessary to have pressure reducing valves. These are available commercially.

Since the separation of components by gas liquid chromatography does not intrinsically require a precise control of the flow of gas, the pressure reducing valve

is adequate for simple apparatus. Usually when using gas - liquid chromatography it is desired to identify the components eluted. The commonest way of doing this is to compare the retention times of the components with the retention times of the materials thought to be present. To do this reproducibly, precise control of carrier gas flow is required. Commercial pressure gauges are often sufficient, though they may be used in conjunction with, or after calibration against, a mercury manometer.

The flow of gas is conveniently measured by using a rotameter. These, however, are somewhat less accurate than is required in gas-liquid chromatography. When it is necessary to know flow rates precisely a bubble flow meter is used. These are not continuously indicating and inconvenient to use, but can be used to calibrate rotameters which can then be used to monitor the carrier gas flow.

Column Packings

The column is the heart of the gas - liquid chromatograph, and may be of two types, packed or capillary.

Columns

The packed column may be of stainless steel,

copper or glass tubing of 1/8" or 1/4" external diameter wound in a spiral of 6" to 8" diameter, and the length of tubing from about 3' to 20'.

The bore of the tube is filled with the solid support coated with the liquid, or stationary, phase.

(a) Solid Support

The solid supports commonly used are diatomaceous earths, such as celite, or powdered firebrick such as Johns-Manville C.22. These are both used in the form of powders of closely controlled particle size. Both materials are capable of absorbing large amounts of liquid without becoming noticeably sticky. Since microscopic examination of both materials shows that they have porous surfaces, it is thought that the stationary phases form minute lenses of liquid in the pores. Thus, a given weight of solid support is capable of absorbing a large weight of stationary phase and distributing it over a very large surface area. Measurements made by Ettre (45) show that crushed firebrick had a surface area of 4.14 sq.m/grm, chromosorb W 1.41 sq.m/grm and celite 1.14 sq.m/grm.

Since the stationary phase seems to be distributed in the pores of the solid support, the actual structure of the solid support may be only thinly covered, or not covered at all. This raises the possibility of

catalytic effects, which are usually less with celite than with firebrick. Acid washing diminishes such effects with both materials, possibly by the removal of iron. Tailing is more likely to occur on firebrick perhaps caused by its more polar character and larger surface area.

The finer the solid support is ground, the larger the surface area per gram. However, very fine powder readily clogs the column, restricting the gas flow. The usual powders have particle sizes in the range 30 to 120 mesh B.S.S., though narrow fractions such as 80 to 100 mesh B.S.S. give much more even packing of the column than do wide ranges of particle sizes.

(b) Liquid Phases

There is a wide range of liquid stationary phases available. A selection of the more popular materials together with their maximum operating temperatures and uses is given in Table 18.

The liquid chosen depends upon the materials to be separated and upon the column temperature to be used. If very sensitive detectors are used, for example ionization detectors, the maximum usable column temperature may be lower than given in Table 18 due to bleed from the column affecting the detectors.

The quantity of liquid phase used varies in the

TABLE 18

Selection of Stationary Phases

Substance	Max. Temp.	Used for Separating
Squalene	140	Hydrocarbons
Apiezon L	300	Esters, Boranes
Tritolyl phosphate	125	Aromatics, chlorinated hydrocarbons
Polyethylene Glycol 400	125	Alcohols, ketones
SE 30	375	High temperature general applications
Dinonylphthalate	175	Esters, Phenols
Carbowax 20M	250	Aldehydes, ethers, sulphur compounds.
FFAP	250	Esters, alcohols, ketones, acids, halogenated materials.

range 1 to 30% by weight of the coated support, and is not critical. For the lower quantities of liquid phase the retention time of any given material is usually shorter than is the case when higher quantities are used.

The coated supports are prepared by dissolving the stationary phase in a volatile solvent such as chloroform or acetone and adding the required amount of solid support. The solvent is then evaporated off slowly and carefully with thorough stirring to ensure that an even coating is produced. The final drying may take place under vacuum. Before use, packed columns are usually conditioned at temperatures above those used for the analysis with a slow stream of carrier gas passing through the column. This removes the last traces of the solvent in addition to any volatile materials present in the stationary phase.

(c) Packing

After the packing has been dried, it is ready for use in columns. Stainless steel and copper columns are best prepared from straight pieces of tubing of the required length. A funnel is attached to one end and the packing poured in and the column tapped to compact the packing. This process is continued until no more packing will go in, no matter how long the

tapping is continued. Following this, the ends of the column are plugged with silica wool or sintered metal discs made for this purpose, such as those sold by Perkin-Elmer Ltd., and then the column is bent round a mandrel to form the required spiral.

Glass columns are more difficult. These are obtained ready made in the required spiral to fit the column oven. The same packing technique is used, but it is more difficult to obtain an even packing and to avoid small gaps which drastically reduce column efficiency.

Temperature Control

After packing, the column is ready to be connected into the oven and conditioned as described earlier. For good separation it is not necessary that the column temperature be closely controlled. However, retention times are affected by temperature. So for identification by comparison of retention times close control of temperature is required. Additionally, if quantitative results are required, close control of temperatures are required, particularly if peak height is the parameter chosen for calibration.

Most ovens are asbestos insulated metal jackets through which air is circulated by a fan. The air can be heated to maintain the oven at the required

temperature by an electric element controlled by a thermocouple. Alternatively, the column may be jacketed by a glass tube through which passes the vapour of a liquid which boils at the required temperature.

It is worth noting that metal columns are superior to glass for heat transfer and constancy of temperature. However the bare metal walls are more prone to activity, specifically catalytic activity, than glass walls. Copper tubing is particularly undesirable in this respect.

Capillary Columns

Capillary columns are made of the same materials as the packed column. These are lengths of tubing of 25 ft. to 150 ft. or more in length and internal diameter of about 0.010". The liquid phase is coated on to the walls of the capillary, and no other solid support is used. A description of the preparation and use of capillary columns is given by Golay (58).

The advantages of the capillary column as opposed to the packed column are that the capillary column gives better resolution of peaks and a shorter analysis time. However, capillary columns are not so readily prepared as packed columns, and the quantity of sample which can be used in a capillary column is extremely small. The

sample load is smaller than can be readily introduced into the column, and in practice a stream splitter is used so that only about 1% of the sample introduced passes through the column, the rest being vented to atmosphere.

Sample Introduction

How the sample is introduced into the gas liquid chromatograph depends upon the physical state of the sample, and has a pronounced effect upon the shape of the eluted peaks. The eluted peaks should be as narrow as possible for maximum resolution and accuracy where quantitative data is being sought.

When a sample is introduced it is either as a vapour, or the sample is rapidly vapourised in the chromatograph. The vapour occupies a definite volume, however small this may be. Theoretically the sample may reach the start of the column in one of two limiting ways. If no dilution of the vapourized sample by the carrier gas occurs, the sample arrives with sharp interfaces between the carrier and the sample. This is called "plug" flow. Alternatively rapid dilution of the sample by the carrier gas may occur. The first trace of vapourized sample to reach the column packing does so without dilution. After this the concentration falls off exponentially with time to zero. This is

called "exponential" flow. Both "plug" and "exponential" flow are illustrated diagramatically in Figure 8.

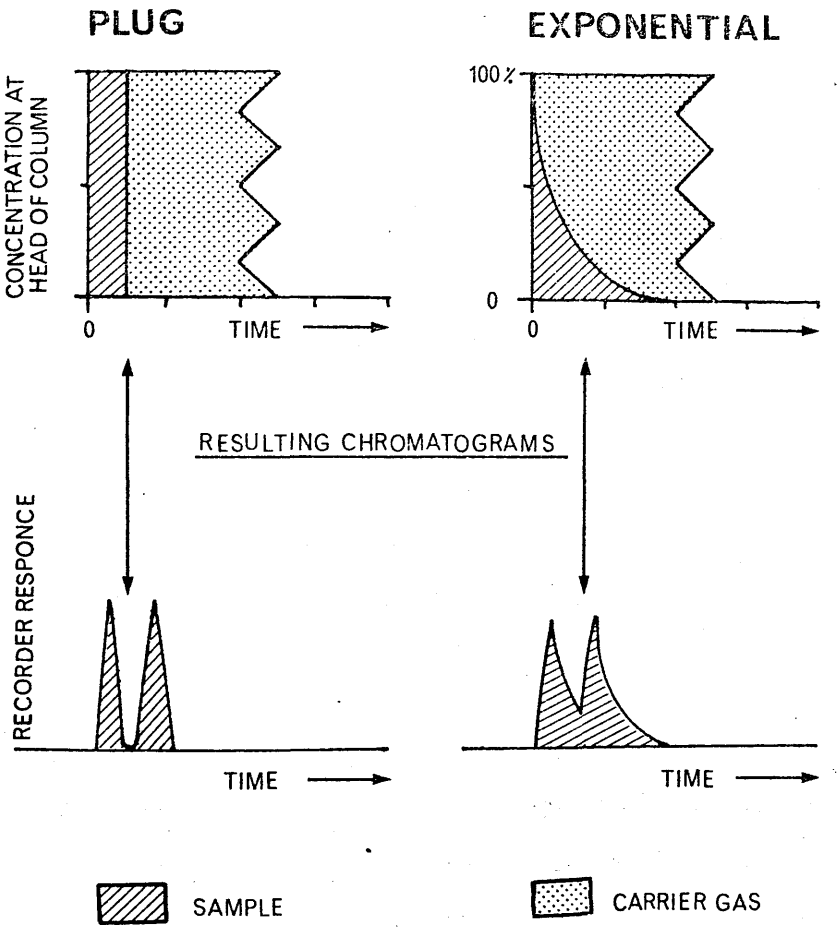
Theoretical and practical studies by Porter, Deal and Stross (101) and Deemter, Zuiderweg and Klinkenberg (41) indicated that "plug" flow gives the narrowest eluted peaks, and therefore the sharpest separations.

Gaseous samples may be introduced by trapping a known volume of sample between two stopcocks and then sweeping the sample on to the column using carrier gas. Care is needed to ensure that there is no interruption of the flow of carrier gas through the column whilst diverting the carrier gas through the sample loop. Alternatively, a syringe may be used to inject an aliquot of the gas through a silicone rubber septum at the beginning of the column. If there is very little space at the column before the start of the packing, the flow may be very nearly "plug" flow.

Microsyringes are a very commonly used method of introducing liquid samples into gas chromatographs. If the tip of the needle is at the top of the column then the sample will dissolve in the liquid phase as it is ejected from the syringe. This approaches very closely the ideal "plug" flow.

The use of a syringe has a disadvantage in that

Fig 8 Schematic Representation of PLUG and EXPONENTIAL Sampling.



the absolute quantity of sample injected is not known accurately. However, this is usually not essential, and a syringe can give good reproducibility when carefully handled.

When it is necessary to know the exact quantity of sample introduced, or when handling solid samples, the samples may be enclosed in sealed glass bulbs. These can be weighed to determine the quantity of sample, and are then introduced to the beginning of the column and crushed to liberate the sample. This technique is capable of giving good results, but is a much more laborious technique than using a syringe.

Detectors

Once a sample has been introduced into a gas chromatograph and separated in the column, it remains to detect the components as they emerge in the column effluent.

The detector may employ any parameter which varies as the amount of eluate in the column effluent varies. It has to be capable of rapid response to changes in concentration of eluate, and also capable of detecting very low concentrations in the effluent. The detectors may be one of two basic types, an integral detector or a differential detector.

Integral Detectors

Integral detectors give a signal proportional to the total amount of materials eluted. The parameters employed by these detectors include titration, conductivity of solutions, and pressure or volume increments of volatile components after absorption of the carrier gas.

Differential Detectors

Parameters used for differential detectors include thermal conductivity, flame ionisation, β-ionisation and flame temperature.

(a) Katherometers

Thermal conductivity detectors, generally referred to as katherometers, are one of the more widely used types of detectors. The underlying principle is that the rate of loss of heat from a heated wire depends upon the thermal conductivity of the surrounding gas. This in turn depends upon the composition of the gas. Thus, in a katherometer there are four resistors arranged in a wheatstone bridge circuit. Two of these are heated by passage of an electric current, and are in thermostatted cells. Through one cell passes pure carrier gas; through the other passes the column effluent. The presence of an eluted material in the

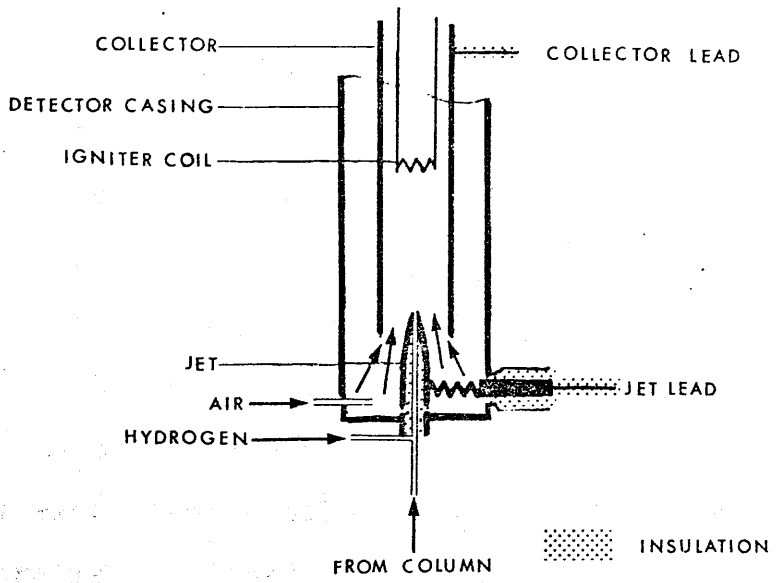
column is detected by a change in resistance of the heated filament. One reason for the popularity of katherometers is that they respond to all materials, including nitrogen, oxygen, carbon dioxide, which are not detected by some other detectors. However, katherometers are less sensitive than ionisation detectors. While it is possible to amplify a detector signal by any required amount, this is of no value unless the detector is free from long term signal variations (drift) and short term signal variations (noise) both of which could mask the signal due to an eluted component.

(b) Flame Ionisation Detector

Ionisation detectors are generally free from signal variations other than those caused by eluted components from the chromatographic column. In general, therefore, they are more sensitive than non-ionisation detectors such as katherometers.

Probably the most widely used of these detectors is the flame ionisation detector. This is shown diagrammatically in Figure 9. In this detector, the column effluent is mixed with a stream of hydrogen and burnt in air. A pair of electrodes are situated near this flame. In fact frequently the jet at which combustion occurs is used as the cathode.

Fig 9 Flame Ionisation Detector (Schematic)



When the flame is merely formed by the combustion of hydrogen diluted by carrier gas very little current flows between the electrodes, but burning an organic material causes a large increase in the ions produced in the flame, and hence a large increase in the current flowing between the electrodes. This detector is not only sensitive, detecting a sample flow in the carrier gas of about 10^{-12} grms/sec., the response of the detector is linearly related to the concentration of material in the eluate over a range of about 1 : 10^6 which is wider than most, if not all, other detectors.

There is one disadvantage of using a flame ionisation detector - all substances are not detected. Those substances not detected include hydrogen, oxygen, ammonia, carbon dioxide and water. The only organic substance not detected is formic acid (122). That water is not detected may be an advantage in that it permits small quantities of organic materials to be determined in the presence of large quantities of water, such as occurs in the analysis of body fluids.

(c) Electron Capture Detector

The electron capture detector is one of the detectors developed with the deliberate aim of achieving a selective response. The detector responds only to molecules which capture electrons. This includes

halogenated, oxygenated and certain unsaturated compounds. Responding to oxygenated materials, the detector is very sensitive to water.

Description

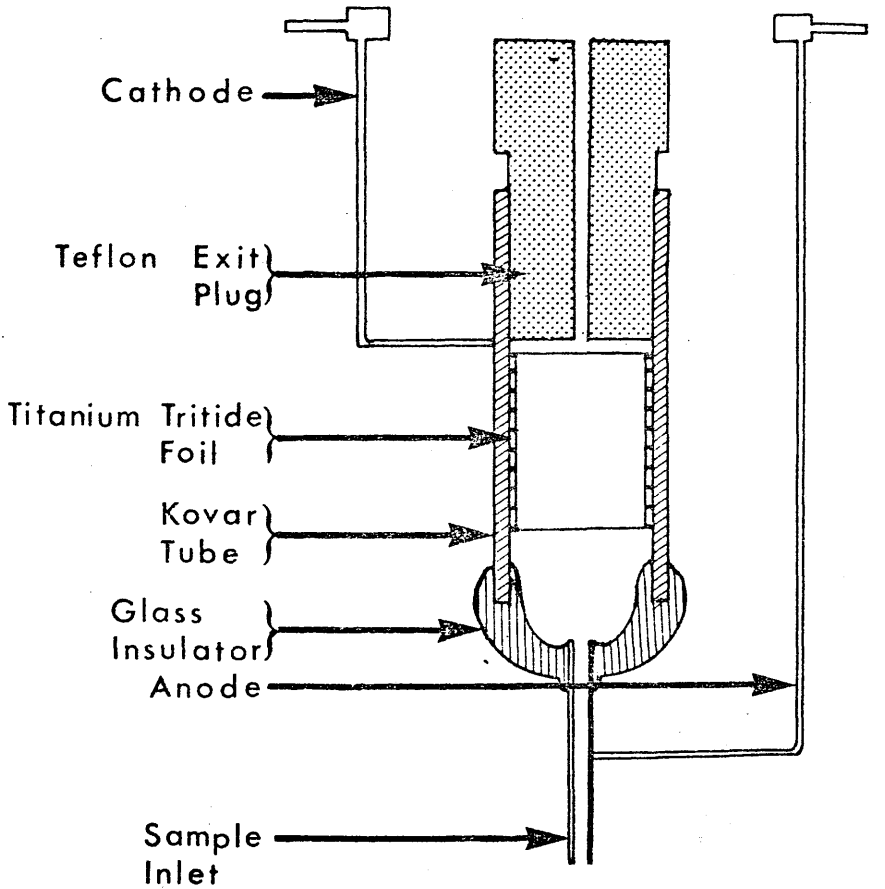
A diagram of an electron capture detector is given in Figure 10. The electrons in this detector are produced by the radioactive decay of tritium or of nickel 63. The tritium is occluded in a Titanium foil, the nickel being incorporated in the alloy used to make the foil.

When the carrier gas only is passing through the detector cell ionisation occurs due to collision between molecules of carrier gas and electrons:- $\beta + C \rightarrow C^+ + \beta + e$ where C is molecule of carrier gas, β the β-ray electron, C^+ the ionised carrier gas, and e the electron detached from the carrier gas molecule. With a steady applied voltage the electrons drift to the anode giving rise to a steady current, the standing current.

If the carrier gas includes an eluate, E, of high electron affinity, however, the process $E + e \rightarrow E^-$ occurs. As the velocity of E^- towards the anode is much lower than that of e, the process $E^- + C^+ \rightarrow E + C$ occurs, being more likely than $e + C^+ \rightarrow C$ because of this difference in drift velocity. Thus in the presence

FIGURE 10

Aerograph Concentric Tube Electron Capture Detector



of a material with a high electron affinity a fall in the standing current occurs (84).

Operation

The fall in standing current depends upon two factors, the energy of the electrons and the ability of the molecules to capture the electrons. At low electron energies the reaction outlined above predominates, but at high electron energies the eluate may dissociate:- $e + AB \rightarrow A^{\cdot} + B^{-}$.

Operating an electron capture detector with a continuous direct current voltage leads to the production of electrons having a wide range of energies. Since it is only the lower energy electrons which are required, the efficiency of the detector may be improved by quenching the higher energy electrons. This can be done by using the carrier gas argon to which has been added 10% methane. Alternatively, the accelerating electric field may be applied for only 1 μ.sec. in about 100 μ.sec. (89). This leads to production of electrons having a narrow range of energies, generally in the thermal region.

Sensitivity

The sensitivity of the detector also depends upon molecular structure, for example chrysene gives a much lower response in an electron capture detector than

TABLE 19

Sensitivity of Electron Capture Detector to Different Compounds (11)

Compound or Class	Adsorbtion coefficient
Hydrocarbons Alcohols Carbonyl groups	0.1
Mono to trifluoro Mono chlor	0.1 to 10
Mono bromo Dichloro	10 to 100
Mono iodo Dibromo Trichloro Polyfluoro	100 to 1000
Mono nitro Metal alkyls Benzopnenone	100 to 1000
Di nitro Di iodo Tribromo Tetrachloro	100 to 1000
Quinones Pyruvate Dihydro pyridine	1000 to 10,000

The detector is sensitive to 5×10^{-14} grms/sec. of CCl_4 in the carrier gas.

does 1, 2, Benzanthracene (28). In general, however, the detector is most sensitive to halogenated materials, less so to oxygenated and phosphorous containing materials, and virtually insensitive to other hydrocarbons, though certain unsaturated materials produce a response. The sensitivity of the detector to various compounds is illustrated in Table 19 and a study of halogenated compounds is given by Clemons and Altshuller (31).

Operating Parameters

Other variables which affect the response of the electron capture detector are temperature and possibly carrier gas flow rate.

As the temperature of the carrier gas increases the standing current decreases and so does the sensitivity of the detector.

There is some dispute as to the effect of carrier gas flow rate. Devaux and Guiochan (42) maintain that the detector is flow sensitive, though to what extent depends upon the cell geometry and the operating mode. Alternatively, Scolnick (116) found that for a concentric cell electron capture detector operated in D. C. mode, the response depended upon the pressure of the gas in the detector cell, but was independent of the carrier flow rate for applied potentials greater than

6 volts.

The greatest disadvantage of the electron capture detector is that the response is only linearly related to concentration for small reductions of standing current. For pulsed operation the response is non-linear if more than 1% of the standing current is absorbed, whilst for the varian aerograph concentric cell design operating in the D. C. mode the manufacturers suggest that reasonably accurate results can be obtained when up to 30% of the standing current is absorbed. Since the response varies with molecular structure, it is necessary to calibrate the detector with each material to be estimated. Above this an increase in concentration of the material produces little or no increase in detector response. This linear dynamic range is in the region of 200:1 for tritium foil detectors and 50:1 for the nickel 63 detectors.

It was thought that the response from these detectors obeyed a Beer-type law of electron absorption (42). However, this has been found not to be the case. Fenimore, Zlatkis and Wentworth (49) showed that the function $(i_b - i_e)/i_e$ is linearly related to eluate concentration. i_b is the standing current when only carrier gas is flowing and i_e the current in the presence of the eluate. The detector response is

equivalent to $(i_b - i_e)$ so an analogue converter must be used to obtain the linear function. Using one the authors found the linear range of a tritium foiled electron capture detector to be 10^4 for o-dichlorobenzene.

One further disadvantage of these detectors when applied to analysis of body fluids is their sensitivity to water. However, if materials of interest can be extracted into a hydrocarbon solvent, which is dried before injection into the chromatograph, the response of the detector will be to the materials of interest only.

Theory of Separation

Gas chromatography is frequently compared with distillation. The gas chromatographic column is considered to consist of a number of short sections. In each section equilibrium between the vapour and dissolved phases is considered to occur before the vapour passes to the next section. Each such section is called a theoretical plate.

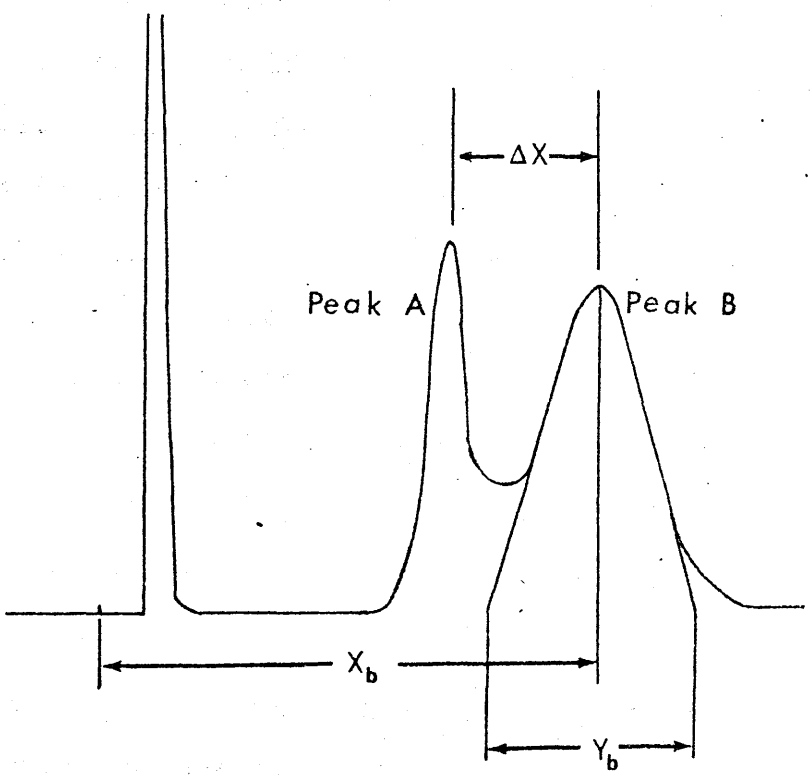
The number of theoretical plates which would give the same separation as a column can be calculated from the traces produced if gas flow through the column and recorder chart speed are constant. The number of theoretical plates is given by $n = 16\, x^2/y^2$ where x, y are as shown in diagram 11 (78).

The number of theoretical plates is a measure of the efficiency of the column. For comparison purposes it is more convenient to consider the Height Equivalent to a Theoretical Plate (H.E.T.P.) which can be calculated by simply dividing the length of the column by the number of theoretical plates. Thus the smaller the value of H.E.T.P. the more efficient the column.

The whole purpose of gas chromatography is separation of volatile materials. The actual resolution achieved can be calculated from the recorder chart. The peak resolution, α, is given by $2\Delta x/y_a + y_b$ as shown in

Figure 11

Gas Chromatogram of a Two Component Mixture



the diagram.

Given the peak resolution it is possible to determine the number of theoretical plates required to achieve a separation with a certain fractional impurity in each band, when the two components are present on a given molar ratio. The fractional band impurity can be calculated from $n(m_a^2 + m_b^2)/2m_am_b$ where n = required fractional band impurity, m_a molar ratio component a, and m_b molar ratio of component B. If the H.E.T.P. is known, the column length can be determined (78).

The preceding factors are all of some interest to a person who merely uses gas chromatography as an analytical tool. However, the theory has been developed to the extent that gas-chromatography may be used for the determination of heats of solution, partition coefficients, and activity coefficients.

Discussion

In applying gas chromatography, there is a choice of detector and of liquid phase used. Having selected these the operating conditions are controlled by the nature of the materials in the sample.

It is almost essential to use an ionisation detector to achieve the necessary sensitivity for the detection of drugs in biological materials. This,

in effect, is a choice between the flame ionisation detector and the electron capture detector.

The advantages of the flame ionisation detector are that it is insensitive to water, is sensitive to nearly all organic compounds, and generally the response is proportional to concentration of material over a wide range of concentrations. The electron capture detector, however, is extremely sensitive to water, and the response of the detector and concentration of material are only linearly related over a narrow range of concentrations. The great advantage of the electron capture detector is its selectivity. It is very sensitive to trichloro materials such as chloroform, chlorbutanol and trichloroacetic acid. The detector gives little response to carbon, hydrogen, nitrogen and oxygen compounds such as are present in urine samples.

Since it is reported that at high concentrations of halogenated volatile compounds the flame ionisation detector does not give a linear response (32) it was decided to exploit the selectivity of the electron capture detector.

The equipment in use was a Varian Aerograph 1522 Series A Gas Chromatograph. The electron capture detector is of the concentric electrode design as illustrated in Figure 10. This particular design has

the advantage that it does not require a purge gas in addition to the carrier gas as the response of the detector is independent of flow rate through the detector cell.

Selection of Stationary Phase - Review

Having selected the detector it was necessary to choose a stationary phase for use in the column. The materials under investigation all have halogen atoms in the molecule and are also alcohols or acids. Thus the commercial stationary phases recommended for these groups would probably be most satisfactory, though trial and error was the only way of choosing the best of these materials.

Examination of the Varian Aerograph catalogue showed that lower acids are determined using Poropak T or FFAP, the lower alcohols by using Poropak Q, Hallcomid m-180L, Carbowax 600 or 1540, FFAP, and halogenated materials using Diisodecyl phthalate, DC550 QF-1, and FFAP. It was interesting to note that the liquid phase FFAP (Free Fatty Acid Phase) is the only one used for all these groups of compounds. In addition, there are reports in the literature of other stationary phases which may be useful. Curry and others (36) used Polyethylene glycol 400 for the determination of ethanol in blood using a flame ionisation detector. Moss and Kenyon (94) used a column of 0.6% Apiezon L on glass beads in conjunction with a β -ionisation detector to determine chlorbutanol, trichloroacetic acid and chloral hydrate. Garret and

Lambert (56) used carbowax 20M in conjunction with an electron capture detector for the same purpose, but could not resolve a peak for trichloroacetic acid. This column was used in conjunction with a flame ionisation detector by Sedivek and Flek (118) for the determination of trichloroethanol. The authors used nitrobenzene as an internal standard. When determining chloral hydrate and trichloroacetic acid in blood and other biological materials Jain and others (70, 73) used a column of 15% FFAP on chromosorb W support in conjunction with an electron capture detector. The internal standard they used for this work was chlorbutanol. Interestingly these authors were able to use direct injections of blood even though the detector is extremely sensitive to water.

Internal Standard - Review

There is no indication of a material which could be used as an internal standard. Such a standard has to be usable with chlorbutanol, trichloroacetic acid and trichloroethanol. Since an electron capture detector was to be used the material must be halogenated, or contain oxygen atoms in the molecule.

The internal standard may be used as a primary standard, or merely to relate concentrations in samples to concentrations in primary standards of the material being estimated. The use of the internal standard as a primary standard is convenient in that the standard is added to the sample, the material chromatographed, and then the ratio of the peak areas for sample material to internal standard is referred to a calibration graph. This does not take into account small variations of the response of the detector to the internal standard and sample material which occur from day to day. If these may be significant it is preferable to analyse a solution containing a known quantity of sample material simultaneous with the analysis of the samples. This solution is used as a primary standard, the internal standard being used to relate sample concentrations to this and to

compensate for variations in the amount of sample introduced into the gas chromatograph.

General Conclusions

This study is concerned with small concentrations of chlorinated materials in a complex organic matrix. For this the electron capture detector was selected, as it has a selective response.

A number of stationary phases require consideration, including phases found to be useful for similar applications, for example the 15% FFAP used by Jain and others (70, 73). Since the compounds to be studied are alcohols or related compounds, stationary phases used for the determination of alcohols must also be considered.

There is no indication of a suitable internal standard. Since an electron capture detector is to be employed, the choice is restricted to halogenated materials and oxygenated compounds.

GAS CHROMATOGRAPHY - EXPERIMENTAL

Introduction

This work was carried out to provide a technique for the identification and estimation of chlorbutanol, chloral hydrate and related materials such as trichloroacetic acid and trichloroethanol. It was essential that this technique should be as simple and speedy as possible whilst still fulfilling the above objectives.

The simplest possible technique would be to add a known quantity of internal standard to a known volume of urine and inject an aliquot of this mixture into the gas chromatograph. This was not found to be possible, but a simple solvent extraction technique was developed.

Equipment and Reagents

The gas chromatograph used was a Varian Aerograph model 1522 Series A machine fitted with an electron capture detector.

The columns were 5' x 1/8" glass installed using PTFE ferrules to ensure gas tight joints. All column packing was carried out by pouring in the packing and tapping until no more could be added. Before use the columns were conditioned at their maximum recommended temperature for 24 hours while a stream of nitrogen was flowing through the columns.

Column packings were generally prepared by dissolving the required weight of liquid phase in a volatile solvent, adding the required weight of solid support (Chromosorb W AW DMCS 60-8 mesh, Perkin-Elmer Ltd.) and mixing. Most of the solvent was evaporated off on a hot plate whilst stirring. The last traces of solvent were removed in a vacuum dessicator. The 15% FFAP was purchased ready prepared from Varian Aerograph (U.K.) Ltd. and the chromosorb 101 packing from Perkin-Elmer Ltd.

Sample introduction was by means of a Hamilton 7101N one microlitre syringe, and the results shown on a Leeds and Northrup "Speedomax W" recorder fitted with

a disc integrator. For some of the work a Kent "Chromalog 1" digital integrator was also used.

Determination of Operating Characteristics

Introduction

The electron capture detector is sensitive to a number of parameters such as temperature and carrier gas flow rate. The effect of such parameters can be largely judged by the detector standing current. The absolute sensitivity to a compound decreases as carrier gas flow increases due to dilution in the detector cell. The following chapters deal with the effects of the various operating conditions.

Carrier Gas Flow Rate

The gas chromatography unit was set up using an empty three foot length of 1/8" stainless steel tubing to connect the injector and detector. The injector temperature was adjusted to 140°C, the column oven to 125°C and the detector temperature to 140°C.

The standing current can be measured by means of a 1 mV recorder, the response of which is proportional to the current flowing.

To measure the effect of varying flow rates, the flow was adjusted as required using a bubble flow meter with the cell voltage switched on. The recorder was then adjusted to zero and the cell voltage switched off. The attenuator is adjusted so that the resultant recorder response is less than full scale deflection. This

deflection is proportional to the standing current and was measured at a variety of carrier gas flow rates. The following results were obtained:-

TABLE 20

Effect of Carrier Gas Flow Rate upon Detector Standing Current

Flow Rate (ml./min.)	Recorder Deflection (mV at 1 x 32 attenuation)
10	0.65
20	0.66
40	0.66
60	0.68
80	0.68
90	0.68

Conclusion

With flow rates of 10 ml./minute to 90 ml./minute very small changes of standing current occur. As the absolute sensitivity to any compound is inversely proportional to the carrier flow rate the preferred flow rate is the lowest usable within the range above. At lower flow rates odd variations may occur.

Effect of Injector Temperature

The equipment used was that described for determining the effect of the carrier gas flow rate. The detector temperature was fixed at 140°C and the column

oven at 125°C, and the carrier gas (nitrogen) flow rate at 40 ml./minute. Varying the injector temperature produced the results shown in Table 21.

TABLE 21

Effect of Varying the Injector Oven Temperature

Injector Temperature (°C)	Recorder Deflection (mV, 1 x 32 Attenuation)
140	0.66
160	0.67
180	0.67
200	0.66
220	0.67

Conclusion

The injector temperature has no effect upon the detector performance. It can therefore be adjusted to give optimum column performance in terms of peak separation and sharpness.

Effect of Column Oven Temperature

For this determination a 5' x 1/8" glass column packed with 15% FFAP on Chromosorb W was installed. The detector standing current was measured with a detector temperature of 150°C, injector temperature 150°C, and a carrier gas (nitrogen) flow rate of 40 ml./minute. The results are shown in Table 22.

TABLE 22

Effect of Variation of Column Oven Temperature

Column Oven Temperature (°C)	Attenuation	Recorder Deflection (mV)
125	1 x 32	0.53
135	1 x 32	0.49
145	1 x 16	0.93
160	1 x 16	0.76
170	1 x 16	0.57
180	1 x 16	0.38

Discussion

Whilst these results were being obtained no variation of carrier gas flow rate detector temperature were observed. As the detector oven is controlled these results imply that the carrier gas does not equilibrate to detector temperature before passing through the detector cell.

Conclusion

The detector operates most effectively with the lowest possible column oven temperature. This must be borne in mind when choosing the column packing.

Equilibration of the column eluate to detector temperature will be facilitated with the smallest possible temperature difference between the column and detector ovens. However, column bleed would then

condense on to the detector foil. A detector temperature well above the column temperature would prevent this and may be necessary.

Effect of Detector Temperature Variations

The equipment was as for the determination of the effects of column temperature variations. Injector temperature was 160°C, column oven 125°C and nitrogen carrier gas flow rate 40 ml./min. The results are shown in Table 23.

TABLE 23

Effect of Detector Temperature Changes

Temperature (°C)	Recorder Deflection (mV 1 x 32 Attenuation)
125	0.73
180	0.57
190	0.50
200	0.41
210	0.36

Conclusion

The performance of the detector is markedly affected by detector temperature. When carrying out quantitative analyses it will be necessary to use either very closely standardised conditions or to run a comparison standard with the samples. Since the detector performance is dependent upon both the column

oven and detector temperatures, results for varying column temperatures were obtained at a series of detector temperatures. These are shown in Table 24.

TABLE 24

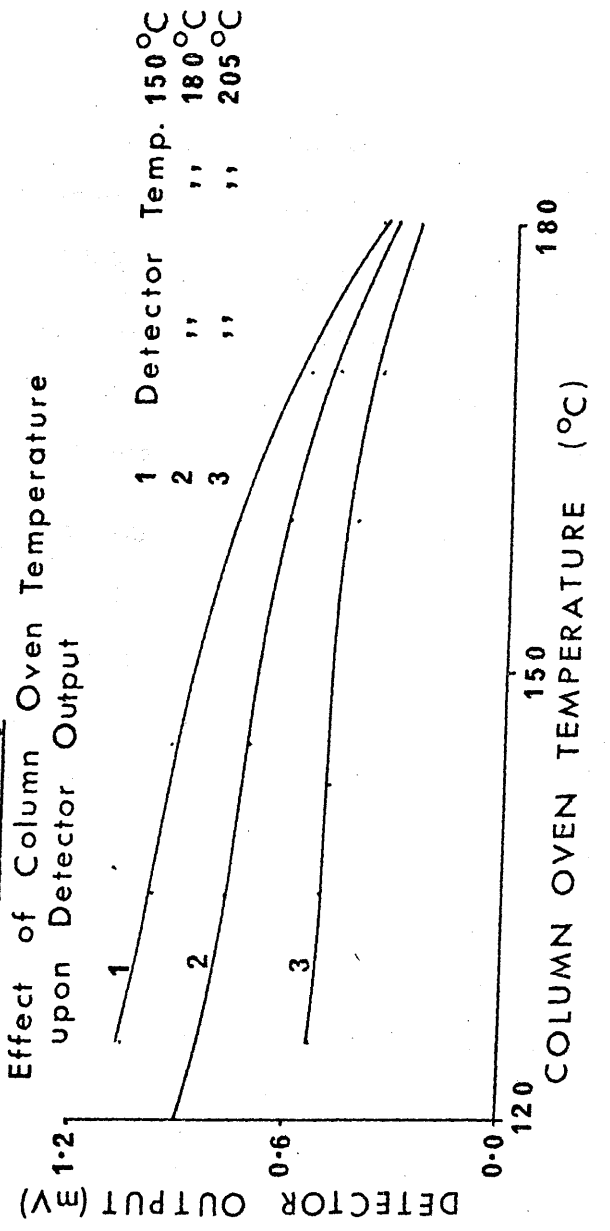
Effect of Column Oven Temperature at various Detector Temperatures

Column Temperature	Recorder Deflection (mV, attenuation 1x16)	
	Detector 180°C	Detector 205°C
120	0.90	0.56
135	0.78	0.52
145	0.72	0.50
160	0.62	0.43
170	0.49	0.37
180	0.34	0.27

These results are shown plotted in Graph 9.

Discussion

These results indicate that the detector is more affected by column oven temperature than by detector oven temperature. If the detector oven temperature is changed from 150°C to 180°C with the column oven at 180°C, the detector standing current decreases by 0.04 mV at an attenuation of 1 x 16. However, if the column oven temperature is raised from 150°C to 180°C with the detector temperature at 180°C, the change in standing current is 0.36 mV at 1 x 16 attenuation.


GRAPH 9
Effect of Column Oven Temperature upon Detector Output
1 Detector Temp. 150°C
2 " 180°C
3 " 205°C
DETECTOR OUTPUT (mV)
1·2
0·6
0·0
120
150
180
COLUMN OVEN TEMPERATURE (°C)

Conclusions

While it is desirable to operate with a low column temperature, a higher detector temperature is acceptable. This means that the foil will be virtually at the detector oven temperature vapourising any condensation of column bleed. The carrier gas, however, will be closer to the column oven temperature, and the cell current will be that of a temperature only slightly above column temperature.

Effect of Carrier Gas Composition

As the detector utilises secondary ionisation of the carrier gas the composition can be expected to affect the detector performance.

The effect of three carrier gases was tried using a column temperature of 95°C, injector temperature 190°C, and a detector temperature of 175°C. The carrier flow rate was 20 ml./minute through a 5' x 1/8" stainless steel column packed with 60 - 80 mesh Chromosorb 101. The results are shown in Table 25.

TABLE 25

Effect of Carrier Gas Composition

Carrier Gas	Standing Current (mV, attenuation 1x32)
Nitrogen	0.72
Helium	0.52
10% Methane/90% Argon	0.84

Conclusion

The argon/methane mixture can be expected to give the best detector performance. Theoretically, helium will give the best peak separations, but the detector performance is unacceptably low if adequate separation can be achieved using nitrogen or argon/methane carrier gas. Nitrogen can be used for convenience, as it is generally available for gas chromatographic work, but if high sensitivity is necessary the argon/methane mixture must be used.

Effect of Applied cell voltage

Normally electron capture detectors are operated at maximum possible voltage, in this case 90 volts. The standing current decreases proportionally to the applied cell voltage as shown in Table 26.

TABLE 26

Effect of Cell Voltage upon Standing Current

Applied Voltage (V)	Recorder Deflection (mV, 1x32 attenuation)
90	0.84
60	0.57
30	0.28

For this the carrier was argon/methane, chromosorb 101 column packing at 95°C with a detector temperature of 175°C.

Conclusion

The only possible advantage of using a low cell voltage is that the response to certain compounds decreases more than to others. This enhances the detector selectivity, but the quantitative response is very sensitive to applied voltage. The selectivity is better achieved by choice of column packing and operating conditions, permitting the use of a higher cell voltage, and a more stable detector response.

General Conclusions

The conditions for optimum detector performance are an argon/methane carrier gas flow rate of about 10 ml./minute, maximum cell voltage, and the lowest possible column oven and detector temperatures. The injector temperature does not affect the detector performance.

In practice, nearly as good results can be achieved using a nitrogen carrier gas. In addition, there will be little loss of performance if the detector is kept slightly above column temperature. This has the advantage of preventing condensation of column bleed upon the foil and the resulting loss of detector sensitivity.

Selection of Column Packing

Introduction

The column packing must separate chlorbutanol, chloral hydrate, trichloroacetic acid and trichloroethanol. These compounds must be separated from water since it is desired to analyse biological samples, particularly urine, by direct injection.

For optimum detector performance this separation should occur with a low column temperature, and a carrier gas flow rate in the range 10 ml. to 90 ml./minute.

To minimise column bleed affecting the detector the maximum working temperature of the stationary phase should be considerably higher than the operating temperature chosen.

In the review, a number of column packings were mentioned. These included Apiezon L used by Moss (93), Carbowax 20M used by Garrett and Lambert (56) and Free Fatty Acid Phase used by Jain and others (70, 73). In addition, there is the possibility of using a column suitable for the analysis of the lower alcohols such as PEG 400 used by Curry and others (36). Neopentyl glycol adipate (81) and a new packing, chromosorb 102, were also investigated.

Preparation of Packing Materials

Apiezon L. (0.6% on glass beads, 5% on Chromosorb W).

This was purchased from local laboratory suppliers, and was tried on glass beads and on Chromosorb W.

For the use on glass beads, 0.6 grms. of Apiezon L. were dissolved in chloroform and 99.4 grms. 60/80 glass beads (Varian Aerograph Ltd.) added. Most of the solvent was removed on a hot plate, and final traces under vacuum. The packing was used in a 5' x 1/8" glass column, and conditioned at 150°C for 24 hours before use.

For use on Chromosorb W, 2 grams of Apiezon L. were dissolved in chloroform and 40 grms. of Chromosorb W Acid Washed DMCS treated (Perkin-Elmer Ltd.). The solvent was removed as previously described. The packing was used in a 5' x 1/8" glass column, and conditioned at 150°C for 24 hours before use.

Carbowax 20M (2.5% on Chromosorb W).

1 grm. of Carbowax 20M (Varian Aerograph Ltd.) was dissolved in acetone and 40 grms. of Chromosorb W (A W DMCS) added. The acetone was removed using heat and reduced pressure. In use the packing was placed in a 5' x 1/8" glass column and conditioned at 200°C for 24 hours before use.

PEG 400 (10% on Chromosorb W).

This is polyethylene glycol 400, and was purchased in the form of a 10% coating on Chromosorb W (A W DMCS) from Perkin-Elmer Ltd. It was used in a 5' x 1/8" glass column, and conditioned at 90°C for 24 hours before use.

Free Fatty Acid Phase (F.F.A.P.) (15% on Chromosorb W)

This was purchased as a 15% coating on Chromosorb W (A W DMCS) from Varian Aerograph (U.K.) Ltd. It was used in 5' x 1/8" glass columns, and conditioned for 24 hours at 225° before use.

Neopentyl Glycol Adipate (10% on Chromosorb W)

This was prepared by dissolving 3.0 grms. of Neopentyl glycol adipate (Phase-Sep. Ltd.) in benzene and adding 27 grms. of Chromosorb W (A W DMCS). The benzene was removed using heat and reduced pressure. Before use in 5' x 1/8" glass columns the packing was conditioned at 200°C for 24 hours.

Chromosorb 102

This was purchased in a prepacked 5' x 1/8" stainless steel column from Perkin-Elmer Ltd. Before use it was conditioned at 225°C for 24 hours.

Reagents

Solutions of chlorbutanol, chloral hydrate, trichloroethanol and trichloroacetic acid were prepared in

50% aqueous ethanol and in amyl alcohol. The amyl alcohol solutions were for use if interference was found due to water.

The qualitative solutions were prepared so that a 1 µl. injection gave a recorder response of 40% to 80% with an amplifier attenuation of 1 x 4.

Procedure

1 µl. injections of the above solutions were made and the retention times of the components noted. The column temperature and carrier gas flow rates were adjusted to give best separations and sharpest possible peaks.

Results

Apiezon L. on glass beads

At 75°C with an argon/methane carrier gas flow of 40 ml./minute a single large peak was observed with chlorbutanol solution. This returned to base line after 1 minute, and there were no other peaks up to 30 minutes.

Apiezon L. on Chromosorb W.

Optimum conditions were a column temperature of 85°C and an argon/methane carrier gas flow of 40 ml./minute. The water produced a broad, tailed peak which could not be separated from chlorbutanol. The chlor-

butanol had a retention time of 4.5 minutes.

Carbowax 20M on Chromosorb W.

Temperatures from 100°C up to 180°C were used with a nitrogen carrier gas flow rate of 50 ml./minute. No peaks were observed for chlorbutanol, although sharp peaks were obtained for water and ethanol.

PEG 400 on Chromosorb W.

Optimum temperature for the column was found to be 90°C with a nitrogen carrier gas flow of 45 ml./minute. This is the maximum recommended temperature for this packing. The retention times found are shown in Table 27.

TABLE 27

Retention Times of Trichloro Sedatives using PEG 400 Column

Compound	Retention Time (minutes)
Chloral hydrate	1.8
Trichloroacetic Acid	1.9
2,2, 2, Trichloroethanol	3.7
Chlorbutanol	>30 minutes

F.F.A.P. on Chromosorb W.

At a temperature of 105°C with a nitrogen carrier gas flow of 40 ml./minute chlorbutanol was just separated from the water peak. Chloral hydrate and trichloroacetic acid were completely masked by the

water peak. The amyl alcohol solutions were injected, and satisfactory conditions for these were a temperature of 120°C and a carrier flow rate of 40 ml./minute. The retention times found at 120°C are shown in Table 28.

TABLE 28

Retention of Trichloro materials on F.F.A.P. Column

Compound	Retention Time (minutes)
Trichloroacetic Acid	0.75
Chloral hydrate	1.50
Chlorbutanol	4.00
Trichloroethanol	5.50
Water	0.60

Neopentyl Glycol Adipate on Chromosorb W.

The most suitable conditions were found to be a column temperature of 95° and a nitrogen carrier gas flow of 40 ml./minute. Chlorbutanol and trichloroethanol were both found to have a retention time of 5.5 minutes.

Chromosorb 102

Initial operating conditions were a column temperature of 90°C with a nitrogen carrier gas flow of 20 ml./minute. Injections of the chlorbutanol solution produced a water peak after 2 minutes and a very broad

chlorbutanol peak after 20 minutes. Increasing the temperature to, eventually, 160°C failed to give a sharp chlorbutanol peak with a reasonable retention time. This was attributed to the fall in carrier gas flow, which was very low at 160°C and could not be increased. This may have arisen due to the packing material softening and blocking the column.

Discussion

The Apiezon L on glass beads, Carbowax 20M on Chromosorb W, PEG 400 on Chromosorb W and Chromosorb 102 column packings are unsuitable as chlorbutanol either is not eluted off the column at all, or only with a very long retention time, producing a broad peak.

The neopentyl glycol adipate on Chromosorb W fails to separate chlorbutanol and trichloroethanol. This separation is required, and so this packing is not suitable.

Both the remaining packings, Apiezon L. and F.F.A.P. on Chromosorb W do separate chlorbutanol from water. However, the water peak is broad, and certainly in the case of F.F.A.P., may mask peaks due to chloral hydrate and trichloroacetic acid.

Since the water cannot be separated from the drugs sought, a solvent extraction procedure will have to be used to isolate the drugs from urine samples.

The F.F.A.P. column gave a slightly sharper water peak. This means that for small quantities of water the resolution of water from the drugs may be complete, and therefore the solvent extract not require drying. With Apiezon L. on Chromosorb W drying may be necessary.

Conclusion

None of the column packing materials considered is suitable for use with aqueous solutions. This means that a solvent extraction technique will have to be used to isolate the drugs.

Two of the packing materials are suitable for use with such a technique, 15% F.F.A.P. on Chromosorb W and 5% Apiezon L on Chromosorb W. The former is preferred due to its slightly better separation of water from the drugs, which should obviate the need to dry the solvent before injection.

Selection of an Internal Standard

Introduction

The internal standard must be a compound detectable when using an electron capture detector. This suggests a halogenated or oxygenated compound.

In addition, under the gas chromatography conditions employed the compound must be completely separated from the solvent and the trichloro materials. Preferably, it must also be soluble in water, but completely extracted by amyl alcohol. This would enable the standard to be added to the sample, extracted with amyl alcohol, and an aliquot of the amyl alcohol injected with no need to ensure quantitative recovery of the organic solvent.

Finally, the internal standard must produce only one peak when chromatographed. Absolute purity is not essential as the estimation is to be by means of a comparison with an aqueous solution of the trichloro material.

Reagents

Qualitative solutions of the materials shown in the results section were prepared in amyl alcohol. The solution strengths were adjusted to give a reasonable recorder response at an amplifier attenuation of 1 x 4, if this was possible.

Procedure

The gas chromatograph was fitted with a 5' x 1/8" glass column packed with15% F.F.A.P. on Chromosorb W. The packing was conditioned at 225°C for 24 hours before use.

The operating conditions were injector temperature 140°C, column temperature 125°C and detector temperature 160°C. The nitrogen carrier gas was flowing at 40 ml./minute.

1 µl. injections of the solutions were made.

Results

These are given in Tables 29, 30 and 31. Table 29 deals with halogenated materials, Table 30 with oxygenated materials and Table 31 with miscellaneous compounds.

TABLE 29

Halogenated materials for Internal Standard

Compound	Retention Time (mins)	Comments
Chloroacetone	about 1.00	Impure. Main peak
2 Chloroethanol	about 1.75	
Chloroacetic acid	about 1.25	
3 Bromopropionic Acid	about 1.25	
2 Bromopropionic Acid	2.00	Impure. Main peak
Ethchlorvynol	6.00	Impure. Main peak
Trichloroethyphosphate	5.50	Degradation to trichloroethanol?
2, 4 Dichlorophenoxy Acetic Acid	8.50	
2, 2, 2, Dichloro-propanol	9.50	Impure. Main peak
2, 2, 2, Trichloro-ethyl chloroformate	9.00	Impure. Main peak
Chloroform	all < 1.00	Mixed with solvent peaks
Carbon tetrachloride		
Dichloroethane (sym)		
Trichloroethylene		
Bromoform		Broad peak, R.T. not readily determined. Very, very tailed.

TABLE 30

Oxygenated materials for Internal Standard

Compound	Retention Time (mins)	Comments
Formaldehyde		Very impure
Phenyl acetaldehyde		
Acetone	0.50	Eluted with solvent peaks
Methyl Ethyl Ketone	0.60	
Dimethyl formamide	0.70	
Dioxan	0.65	
Benzaldehyde	3.00	
iso-Butyric Acid	4.75	
n-Butyric Acid	5.5	
Salicaldoxime	5.5	
Phenyl acetamide	8.0	

Also, the following materials were examined and no response observed:- Anisaldehyde, Benzoyl Peroxide, n-Butanol, Hydroxylamine, Methyl pentynol, p-nitro-aniline, oxalic acid and n-propanol. This lack of response could be due to either the compounds not passing through the column, or not being detected by the electron capture detector.

TABLE 31

Miscellaneous materials for Internal Standard

Compound	Retention Time (mins)	Comments
Amphetamine	7.0	Very broad peak
Piperidine	13.0	
Aniline	21.0	

No response: Benzoyl acetone, Ethyl carbamate, phenacetin, β -phenethylamine, thiopentone, thiourea.

Discussion

The only halogenated material which had a retention time near that required was 2-Bromopropionic acid. Unfortunately, it was only available in impure form. Whilst it could be purified, it is preferable than an internal standard for routine analysis is readily available in usable form.

Although there were no suitable halogenated compounds, there were two possible oxygenated compounds, benzaldehyde and phenylacetamide. The remaining compounds of this group were eluted with unsuitable retention times. Of the two, benzaldehyde is the material of choice. With a retention time of 3 minutes,

it is eluted between the chloral hydrate and chlorbutanol peaks. Phenyl acetamide, however, is eluted after trichloroethanol, and would give a longer analysis time.

The non-oxygenated materials are unsuitable mainly because either they are not eluted under these conditions, or alternatively are not detected. The retention times for piperidine and aniline are too long for use in this technique, whilst amphetamine with a more usable retention time produces a broad peak of a shape not entirely suitable for quantitative work.

Conclusions

Of the materials examined benzaldehyde is the most suitable for use as an internal standard. The operating conditions may require minor adjustment to ensure complete separation of the benzaldehyde.

Conditions for Use of Benzaldehyde as Internal Standard

Introduction

When selecting benzaldehyde as the internal standard a column temperature of 125°C was used. This gives almost, but not complete, separation of benzaldehyde from chlorbutanol. For quantitative work this separation must be complete.

Once the separation from chlorbutanol was achieved a selection of halogenated drugs was injected to ensure that this same complete separation was achieved for all these compounds.

Reagents

Qualitative solutions of the materials in amyl alcohol containing benzaldehyde were prepared. The solution strengths were adjusted so that at an amplifier attenuation of 1 x 4 a reasonable recorder response was obtained.

Procedure

1 µl. injections of the solution of chlorbutanol and benzaldehyde in amyl alcohol were made. The column temperature was lowered until satisfactory separation was achieved.

1 µl. injections of the other solutions were then made and retention times noted.

Results

Benzaldehyde and chlorbutanol were just separated using a column temperature of 105°C. A more satisfactory separation was achieved using a column temperature of 95°C.

The retention times of a number of chlorinated drugs and their metabolites under these conditions are

given in Table 32.

Ethychlorvynol was considered to be worth including as it may be extracted under the same conditions as chlorbutanol. Gas chromatographic and extraction techniques used by other authors (110, 124) suggest that the drug is detectable in urine samples.

TABLE 32

Relative Retention time of Drugs at 95°C

Compound	Relative Retention Time
Benzaldehyde	1.00 (5.5 minutes)
1 Chloral Betaine	0.40
Chloral Hydrate	0.40
Chlorbutanol	1.40
Ethchlorvynol	0.40, 0.65 1.20 (main peak) 3.43
Trichloroacetic acid	0.24
Trichloroethanol	2.36
2 Trichloroethyl phosphate	2.36
Water	0.21

1 Chloral betaine would appear to decompose, giving chloral hydrate, which is detected.

2 Trichloroethyl phosphate would appear to decompose to give trichloroethanol which is detected.

Conclusion

Benzaldehyde can be used as an internal standard. The operating conditions required are a 5' x 1/8" glass column packed with 15% F.F.A.P. on chromosorb W maintained at 95°C with nitrogen carrier gas flowing at 40 ml./minute. Suitable injector and detector temperatures are 140°C and 155°C respectively.

Linear Dynamic Range of the Electron Capture Detector

Introduction

This is the range of concentrations which produce a response linearly, or nearly linearly, related to the concentration. As the electron capture detector has a very narrow linear range this is very important. Further, as the detector responds differently to different compounds the range has to be determined for every compound it is wished to measure.

To carry out the determination, a solution of the compound is prepared. Five injections of 1 µl. are made, the area of the compound peak measured, and the electrometer attenuation noted. A number of other solutions, of decreasing concentration, are prepared from the original solution and five injections of each made.

The true peak area is the product of the measured peak area and the attenuation. The values for the true peak area and the weight of compound injected are plotted on logarithmic graph paper. The linear part of the resulting plot is the linear dynamic range. If the weight of compound injected does not exceed the weight corresponding to the top of the linear range, satisfactory measurements will be obtainable.

(a) Determination of the Linear Dynamic Range for Benzaldehyde

Introduction.

The operating conditions were as defined earlier. A glass column 5' x 1/8" packed with 15% F.F.A.P. at 95°C with a nitrogen carrier gas flow of 40 ml./minute.

Reagents

The initial solution was prepared from 4.3365 grms. of "Analar" benzaldehyde made up in 50 ml. of amyl alcohol. The dilutions were prepared as shown in Table 33.

TABLE 33

Dilutions of Benzaldehyde for determination of the LDR

Solution No.	Using ml.	Of Solution	gives mg%.	weight/ 10µl inject.
1	-	-	8.6730×10^3	8.6730×10^5
2	10	1	1.7346×10^3	1.7346×10^5
3	10	2	3.4692×10^2	3.4692×10^4
4	10	3	6.936×10	6.936×10^3
5	25	4	3.468×10	3.468×10^3
6	10	4	1.387×10	1.387×10^3
7	10	5	6.936	6.936×10^2
8	10	6	2.774	2.774×10^2

Procedure

Each solution was injected five times, using 10 µl. injections. As an alternative to using so many

dilutions the injection size could have been varied, but the error in injecting one or even two µl. from a 10 µl. syringe is greater than in the dilutions.

The results obtained for this are shown in Table 34 and are plotted to give Graph 10. The areas were measured using a disc integrator and are in arbitrary units.

GRAPH 10

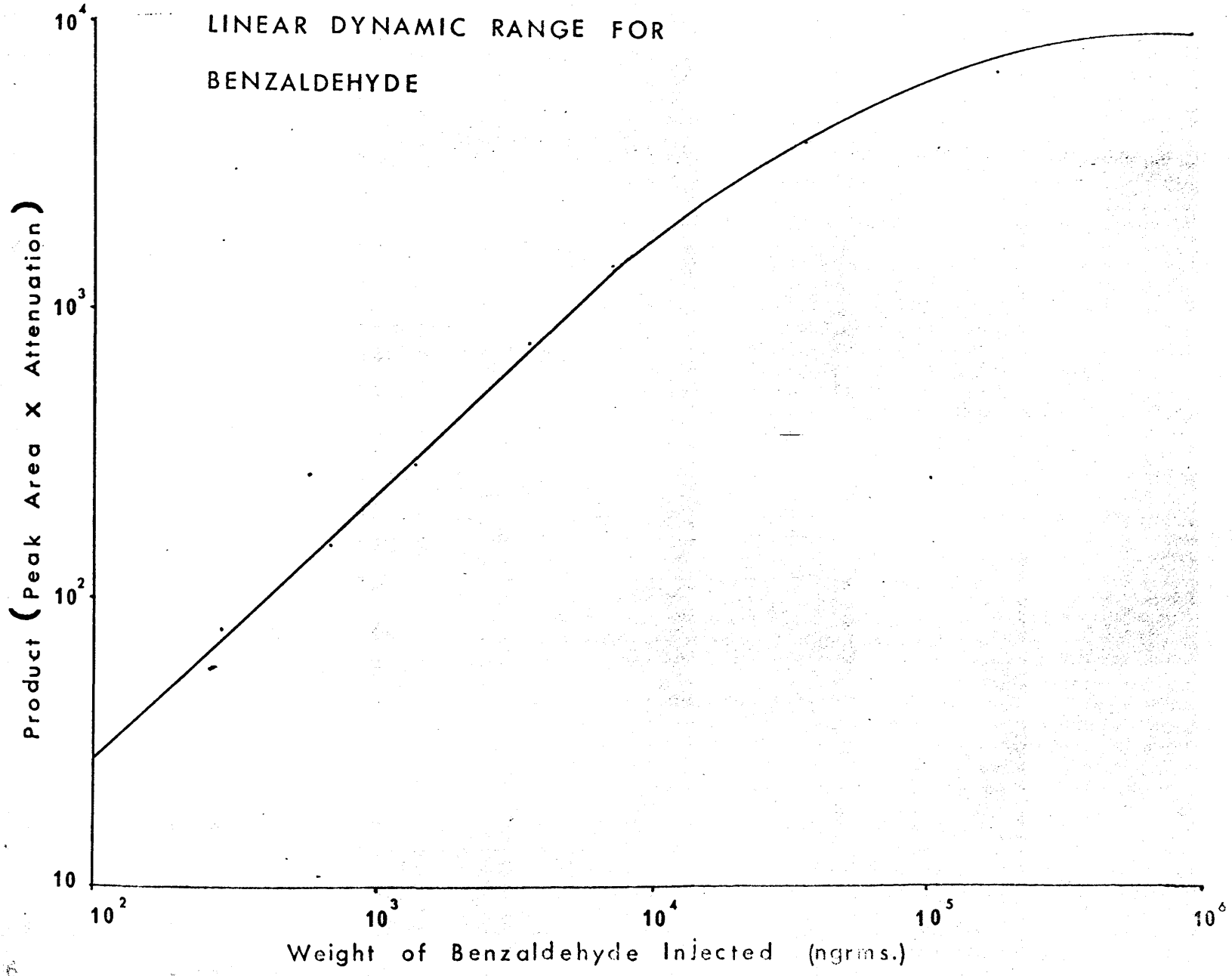

LINEAR DYNAMIC RANGE FOR
BENZALDEHYDE
Product (Peak Area X Attenuation)
10^4
10^3
10^2
10
10^2
10^3
10^4
10^5
10^6
Weight of Benzaldehyde Injected (ngrms.)

TABLE 34

Solution No.	Wt. Injected (µg.)	Attenuation	Area	Product	Mid Value of Product
1	8.673×10^5	x 32	306.5	9.81×10^3	
			320.5	10.25×10^3	
			283.5	9.06×10^3	1.003×10^4
			314.0	10.04×10^3	
2	1.735×10^5	x 32	223.5	7.14×10^3	
			156.0	4.99×10^3	
			238.5	7.64×10^3	7.14×10^3
			240.0	7.67×10^3	
			211.0	6.75×10^3	
3	3.469×10^4	x 32	127.5	4.07×10^3	
			113.0	3.62×10^3	
			121.0	3.87×10^3	4.00×10^3
			128.0	4.09×10^3	
			125.0	4.00×10^3	
4	6.936×10^3	x 32	44.0	1.41×10^3	
		x 16	91.0	1.46×10^3	
			89.0	1.42×10^3	1.46×10^3
			102.0	1.63×10^3	
			95.5	1.53×10^3	
5	3.468×10^3	x 8	98.0	7.84×10^2	
			97.5	7.80×10^2	7.80×10^2
			97.5	7.80×10^2	
			87.5	7.00×10^2	
6	1.387×10^3	x 4	89.0	3.56×10^2	
			80.5	3.22×10^2	
			74.0	2.96×10^2	2.96×10^2
			65.5	2.62×10^2	
			69.0	2.76×10^2	

Table 34 (continued)

Solution No.	Wt.Injected (µg.)	Attenuation	Area	Product	Mid Value of Product
7	6.936×10^2	x 2	58.0	1.16×10^2	
			68.0	1.36×10^2	
			82.0	1.64×10^2	1.55×10^2
			82.5	1.65×10^2	
			77.5	1.55×10^2	
8	2.774×10^2	x 1	68.0	68.0	
			79.5	79.5	
			79.0	79.0	79.0
			82.0	82.0	
			70.0	70.0	

Conclusion

These results indicate that a linear response can be obtained with injections up to 8 µg. of benzaldehyde. Using 1 µl. injections this corresponds to a solution of 800 mg./100 ml.

(b) Linear Dynamic Range for Chlorbutanol

Introduction

The operating conditions were the same as those used for the determination of the linear dynamic range for benzaldehyde.

Reagents

The initial solution was prepared by dissolving 59.1 mg. of chlorbutanol ($C_4H_7OCl_3.\frac{1}{2}H_2O$) in amyl alcohol

and making up to 50 ml. Dilutions were prepared as shown in Table 35, making solutions of 50 ml. final volume.

Procedure

Five 1 µl. injections of each solution were made, and the chlorbutanol peak area measured using a disc integrator.

Results

These are given in Table 36, and plotted in Graph 11.

TABLE 35

Dilutions for Linear Dynamic range for Chlorbutanol

Solution No.	Using ml.	Of Solution	gives mg%	Weight(ng.)/ 1 µl.injection
1	-	-	1.18×10^{2}	1.182×10^{3}
2	10	1	2.36×10	2.36×10^{2}
3	10	2	4.73	4.73×10
4	10	3	9.4×10^{-1}	9.46
5	5	3	4.73×10^{-1}	4.73
6	10	4	1.89×10^{-1}	1.89
7	10	5	9.46×10^{-2}	9.46×10^{-1}
8	10	6	3.78×10^{-2}	3.78×10^{-1}
9	10	7	1.89×10^{-2}	1.89×10^{-1}
10	10	8	7.56×10^{-3}	7.56×10^{-2}

The results obtained from five injections of each solution are shown in Table 36. Again the peak areas

are in disc integrator units.

TABLE 36

Solution No.	Wt. Injected (ng)	Attenuation	Area	Mid Value of Product
1	1.18×10^3	x 32	uncountably large	
2	2.36×10^2	x 32	uncountably large	
3	4.73×10	x 32	296	
			300	
			278	9.06×10^3
			262.5	
			283	
4	9.46	x 32	90	
			111	
			144	3.55×10^3
			108	
			129	
5	4.73	x 16	176.5	2.82×10^3
			180	
			176	
			172	
			176	
6	1.89	x 16	73.5	
		x 8	146	
			150.5	1.18×10^3
			158.5	
			135.0	
7	9.46×10^{-1}	x 4	125.5	
			140.5	
			144	5.54×10^2
			127	
			138.5	

Table 36 (continued)

Solution No.	Wt.Injected (ng)	Attenuation	Area	Mid Value of Product
8	3.78×10^{-1}	x 2	113.5	
			109.5	
			106.5	2.13×10^{2}
			132	
			105	
9	No Injections			
10	7.56×10^{-2}	x 1	41.0	
			50	
			53	47.5
			45	

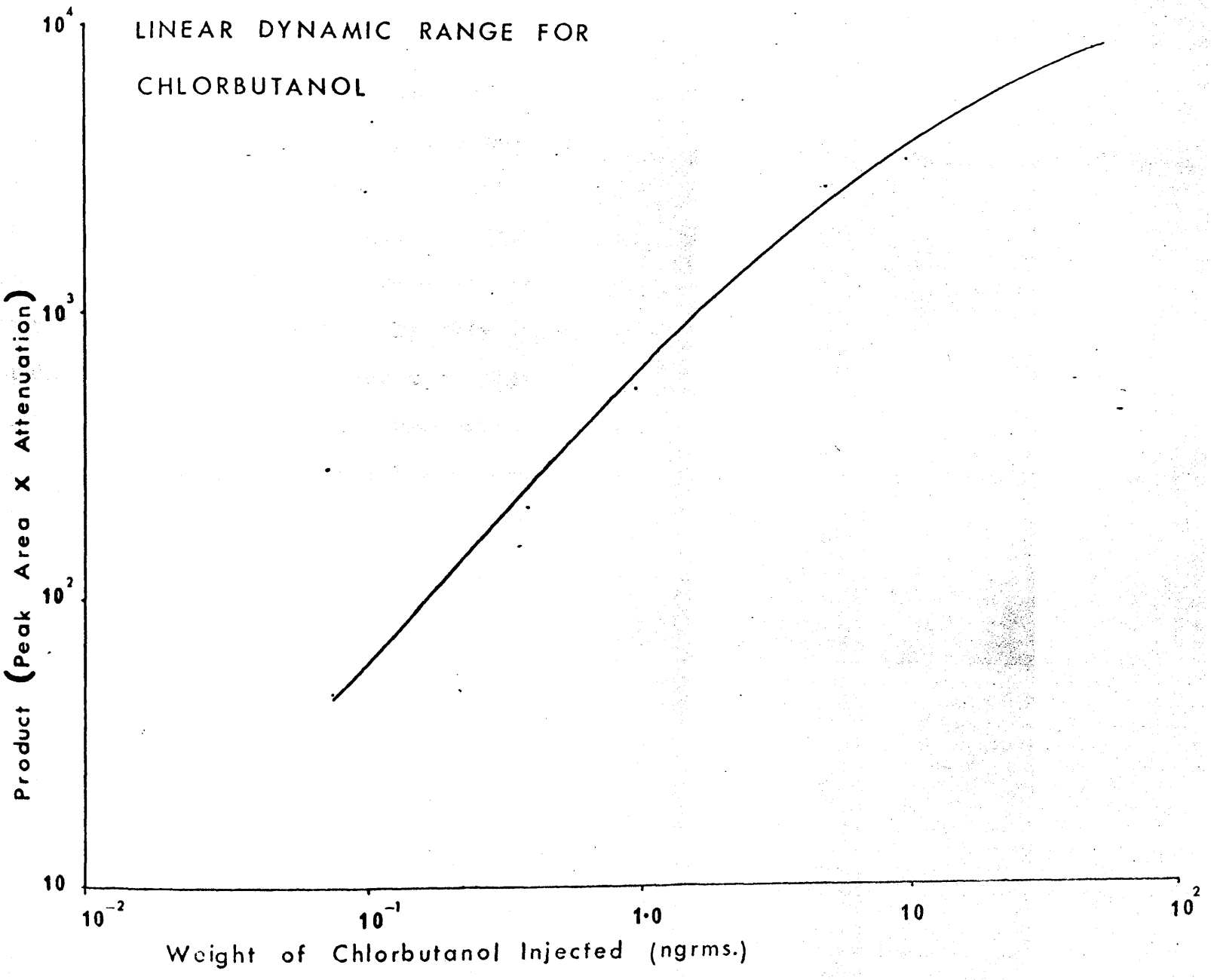
The results are shown plotted on Graph 11.

Conclusions

A linear response can be obtained with injections of up to 2 ng. of chlorbutanol. Using 1 µl. injections this corresponds to a solution strength of 0.2 mg./100 ml.

The nearest solution to 0.2 mg./100 ml. was 0.189 mg./100 ml., and to analyse this an attenuation of x 8 was required and gave a peak height of about 50% f.s.d. To ensure that the linear dynamic range is not exceeded, all analyses were carried out at attenuations of x 4, or less. If the chlorbutanol

GRAPH 11


LINEAR DYNAMIC RANGE FOR
CHLORBUTANOL
Product (Peak Area X Attenuation)
10⁴
10³
10²
10
10⁻²
10⁻¹
1·0
10
10²
Weight of Chlorbutanol Injected (ngrms.)

peak is "on scale" then the weight of chlorbutanol injected lies within the linear range.

The benzaldehyde standard concentration was chosen so that it, too, was "on scale" with an attenuation of x 4. In fact, a concentration was chosen so that the peak would be just "on scale" at an attenuation of x 2. This gives a peak of just less than 50% f.s.d. at x 4 attenuation, large enough for accurate area measurement. By this means, one standard solution could cover two ranges. The benzaldehyde concentration corresponding to this is 300 mg./100 ml., or slightly less, when using 1 µl. injections.

Preparation of a Calibration Graph

Introduction

This graph was not prepared for reference when analysing future samples. It was intended to estimate the chlorbutanol concentration by reference to a chlorbutanol standard solution analysed with the samples. This is to compensate for any deterioration of the internal standard solution, such as the Cannizzaro reaction, and to compensate for any slight change in response of the detector to chlorbutanol and benzaldehyde.

The graph is necessary to demonstrate that, for a fixed benzaldehyde concentration, there is a linear relationship between the chlorbutanol concentration and the ratio of the peak areas of chlorbutanol and benzaldehyde.

However, to do this satisfactorily, the technique must be reproducible.

(a) Reproducibility of the Gas Chromatograph

Introduction

This is necessary to determine whether or not repeated injections of the same solution give the same ratio of peak areas.

Reagents and Equipment

The operating conditions were as defined earlier.

A solution containing 0.121 mg./100 ml. of chlorbutanol and 300 mg./100 ml. of benzaldehyde in amyl alcohol was prepared.

One slight change in equipment was the use of a Kent "Chromalog" Mk. I digital integrator in place of the disc integrator.

Procedure

Ten injections were made, each of between 0.8 µl. and 1 µl. No attempt was made to inject the same volume of solution as the peak area ratio ought to be independent of peak size.

Results

The ten values obtained for the peak area ratios were 0.724, 0.752, 0.719, 0.775, 0.737, 0.760, 0.724, 0.760, 0.803, 0.757.

Conclusion

The average value is 0.760 and the range 0.719 to 0.803. This indicates that the error is approximately ± 6%.

(b) Calibration Graph

Procedure and Solutions

To prepare the calibration graph, eight solutions were prepared. Each contained 300 mg./100 ml. of benzaldehyde, and the series covered a range of chlor-

butanol concentrations from 0.020 mg./100 ml. to 0.161 mg./100 ml. Each solution was injected twice and the areas of the benzaldehyde and chlorbutanol peaks measured. The peak area ratio was calculated and a graph of the peak area ratios against chlorbutanol concentration plotted. The results are given in Table 37, and shown plotted in Graph 12.

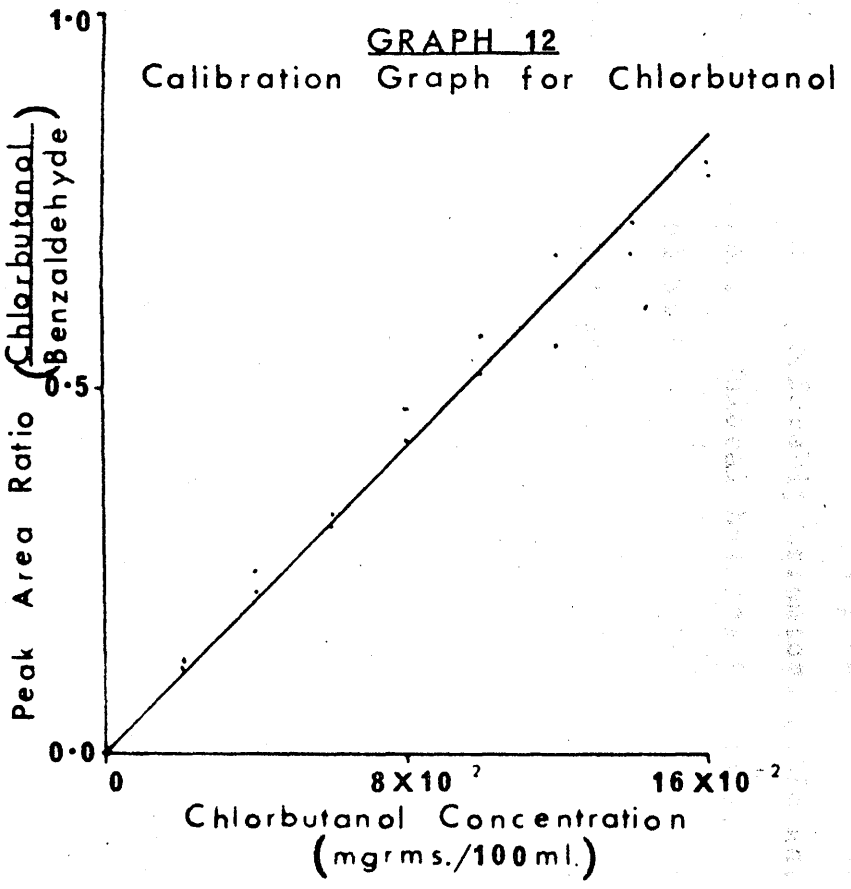
Results

TABLE 37

Concentration of Chlorbutanol (mg./100 ml.)	Peak area Ratio Run I	Run II
0.020	0.119	0.125
0.040	0.223	0.251
0.060	0.324	0.325
0.080	0.428	0.470
0.101	0.521	0.570
0.121	0.560	0.676
0.141	0.683	0.718
0.161	0.779	0.798

Conclusion

The results indicate an accuracy of ± 6%. To within this limitation a linear relationship exists between chlorbutanol concentration and peak area ratio.


GRAPH 12
Calibration Graph for Chlorbutanol
1·0
0·5
0·0
Peak Area Ratio (Chlorbutanol / Benzaldehyde)
0
8X10 -2
16X10 -2
Chlorbutanol Concentration
(mgrms./100ml.)

Selection of Extracting Solvent

Introduction

Since it is not possible to gas chromatograph aqueous solutions, a solvent extraction procedure must be used. The solvent must be capable of extracting chlorbutanol, and preferably trichloroacetic acid and trichloroethanol. Chloral hydrate is rapidly metabolised and will not be found in urine samples.

Comparison of solvents

Introduction

As a means of comparing different solvents, aliquots of an aqueous solution of chlorbutanol were repeatedly extracted with the solvents under consideration. Each extract was examined for chlorbutanol using the gas chromatograph.

The most suitable solvent is that which requires the smallest number of extractions to remove all the chlorbutanol from the aqueous phase.

Reagents

Chlorbutanol solution was prepared by dissolving 2 mg. of chlorbutanol in 1.0 ml. of ethanol and making up to 100 ml. with water. The solvents examined were all "analar" grade.

Procedure

For each solvent a 5.0 ml. aliquot was extracted four times with 2.0 ml. aliquots of the solvent. After separation from the aqueous phase, 1 µl. aliquots of each solvent aliquot were injected into the gas chromatograph.

Results

These are shown in Table 38.

TABLE 38

Solvent	Aliquot			
	1	2	3	4
Diethyl Ether	Positive	Positive (weak)	Negative	Negative
Toluene	Positive	Positive	Positive	Positive (weak)
Pet.Ether 80-100	Positive	Positive	Positive	Positive
Di-isopropyl Ether	Positive	Positive	Positive (weak)	Negative
n-Butyl Acetate	Positive	Positive	Positive (weak)	Negative
Amyl Alcohol	Positive	Positive (weak)	Negative	Negative

Discussion

In Table 38, positive indicates that chlorbutanol was detected in the extract.

The two best solvents are diethyl ether and amyl alcohol. This is not surprising as chlorbutanol is

somewhat polar, and will be solvated, and therefore extracted, better using a slightly polar solvent.

Of the two solvents, amyl alcohol was chosen, as the volatility of diethyl ether may present handling problems when carrying out quantitative work.

Conclusion

The most suitable solvent for the extraction of chlorbutanol from aqueous solution is amyl alcohol.

Extraction of Trichloroethanol

Introduction

To date the only drug of this type found in greyhound urine samples is chlorbutanol. One other possible drug is chloral hydrate, the principal metabolite of which is trichloroethanol. The ability of amyl alcohol to extract trichloroethanol from aqueous solution was investigated.

Reagents

A 2 mg./100 ml. aqueous solution of trichloroethanol was prepared.

Procedure

A 5 ml. aliquot of the trichloroethanol solution was extracted with four 2.0 ml. aliquots of amyl alcohol. 1 µl. of each amyl alcohol extract was injected into the gas chromatograph.

Results

Extracts 1 and 2 contained appreciable quantities of trichloroethanol, extract 3 a trace, and none was detectable in extract 4.

Conclusion

Amyl alcohol is suitable for the extraction of trichloroethanol from aqueous solution, although the recovery will be lower than when chlorbutanol is being extracted.

Recovery of Chlorbutanol

Introduction

The earlier work has shown that the most suitable solvent for extracting chlorbutanol from aqueous solution is amyl alcohol. This work did not indicate, however, how much of the chlorbutanol is recovered.

This section investigates how long the amyl alcohol and aqueous chlorbutanol need shaking for maximum recovery, and what percentage of the chlorbutanol is recovered.

1. Effect of Shaking Time

Reagents

A urine sample known to contain chlorbutanol was employed. A standard solution of 374 mg./100 ml. of benzaldehyde in amyl alcohol was used as internal

standard.

Procedure

A number of flasks were prepared containing 2.0 ml. of the urine sample and 2.0 ml. of amyl alcohol. They were shaken vigorously on a "Griffin" Flask Shaker for various times, and the two phases separated by centrifuging. 1.0 ml. of the amyl alcohol extract was added to 5.0 ml. of the benzaldehyde standard solution, and 1 µl. aliquots of this injected into the gas chromatograph. This procedure was repeated with a different urine sample.

Results

The results are shown in Table 39.

TABLE 39

Shaking Time (minutes)	Urine 1 Peak Area Ratio	Urine 2 Peak Area Ratio
½	4.07	3.06
1	4.38	3.33
2	4.02	3.49
4	4.33	3.52
60	4.45	-

Discussion and Conclusion

The peak area ratio is the ratio of the area of the chlorbutanol peak to the area of the benzaldehyde peak, measured using a disc integrator. Since the

benzaldehyde concentration is the same in all the samples, the ratio is proportional to the concentration of the chlorbutanol in the amyl alcohol extract.

Recoveries within experimental error of the maximum are obtained with 2 minutes shaking. Since the shaker efficiency may vary slightly, 5 minutes shaking was selected for future use.

2. Estimation of Recoveries

Reagents

A 0.34 mg./100 ml. aqueous solution of chlorbutanol and a 374 mg./100 ml. solution of benzaldehyde in amyl alcohol were used.

Procedure

A series of flasks were prepared containing 4.0 ml. of chlorbutanol solution and 4.0 ml. of amyl alcohol. The flasks were shaken vigorously on the "Griffin" Flask shaker for 5 minutes. 2.0 ml. of the amyl alcohol phase was added to 2.0 ml. of the benzaldehyde solution, and 1 µl. injections of the mixture made into the gas chromatograph. The ratio of the areas of the chlorbutanol and benzaldehyde peaks was calculated.

The aqueous phase was extracted with a fresh 4.0 ml. aliquot of amyl alcohol by shaking, 2.0 ml. of the

extract being mixed with 2.0 ml. of benzaldehyde solution, and 1 µl. of the mixture injected into the gas chromatograph. The peak area ratio was calculated.

Calculation Theory

It is known that two extractions with amyl alcohol remove all the chlorbutanol from the aqueous phase. The peak area ratios are proportional to the chlorbutanol concentration in each extract since the benzaldehyde concentration is fixed.

Thus, if A is the chlorbutanol to benzaldehyde peak area ratio for the first extract and B this ratio for the second extract then:-

$\frac{A}{B}$ = ratio of chlorbutanol in the two extracts.

and $\dfrac{\frac{A}{B}}{\frac{A}{B} + 1} \times 100$ = % recovery in the first extract.

$\equiv \dfrac{A \times 100}{A + B}$

Results

For five extractions the average ratio of chlorbutanol in the two extracts was 33.9 : 1, and the worst 20.1 : 1. This gives an average recovery of 97.3%, and the worst 95.2%.

Discussion

It is probable that these results are lower than

the true values since complete separation of the two phases is almost impossible without loss of aqueous phase. If a little amyl alcohol from the first extraction was carried over to the second, the chlorbutanol in this amyl alcohol would seriously affect the ratio of chlorbutanol in the two extracts.

Conclusion

The recovery of chlorbutanol is certainly better than 95% and the average recovery to be expected is 97.3%.

Extraction with Benzaldehyde Standard Solution

Introduction

The procedure used to estimate the recovery of chlorbutanol involved the extraction of the aqueous phase with amyl alcohol, and then adding an aliquot of the amyl alcohol extract to an aliquot of benzaldehyde standard solution. This ensures a constant concentration of benzaldehyde in the final solution.

A more convenient technique for routine analysis would be to extract the aqueous layer with the standard solution of benzaldehyde in amyl alcohol. The results are only valid if the loss of benzaldehyde to the aqueous phase is insignificant.

To investigate this, two techniques were used. The first was to estimate the partition coefficient of benzaldehyde between the aqueous and amyl alcohol phases. The second to compare the results obtained by extraction with the standard benzaldehyde solution with those obtained by extracting with amyl alcohol and subsequently adding the standard benzaldehyde solution.

1. Estimation of Partition Coefficient

Introduction

The partition coefficient is a measure of the concentration of a compound between two immiscible

solvents when the system is in equilibrium.

It is defined as $K = {}^{a}1/a_2$ where K is the partition coefficient, and a_1, a_2 the activity of the compound in the two phases.

At low concentrations, the concentrations in the two phases can be used instead of activities.

Reagents

A 374 mg./100 ml. solution of benzaldehyde in amyl alcohol was prepared.

Procedure

A series of flasks were prepared containing bemzaldehyde, details of which are shown in the results. 1 µl. injections of each solution were made, and the initial peak area for benzaldehyde determined.

10 ml. of water was added to the flasks, and they were then incubated at 20°C in a shaking incubator overnight. Fresh 1 µl. injections of the organic phase were then made to determine the final benzaldehyde peak area.

Theory of Calculations

The benzaldehyde peak areas are proportional to the concentration of benzaldehyde in the organic phase.

If A is the volume of organic phase in ml.

B is the volume of aqueous phase in ml.

C is the initial benzaldehyde concentration in the organic phase

D is the final benzaldehyde concentration in the organic phase.

The quantity of benzaldehyde lost from the organic phase is given by:- $\frac{(C - D)A}{100}$

and is the quantity absorbed by B ml. of water.

Therefore the concentration in the aqueous phase is:-

$$\frac{(C - D)A}{100} \times \frac{100}{B}$$

Hence $K = \frac{D \ B}{A \ (C - D)}$

In this case, A = 5, B = 10.

Therefore, $K = \frac{2D}{(C - D)}$

Results

These are shown in Table 40.

TABLE 40

Partition coefficient for Benzaldehyde

ml. Benzaldehyde standard used	ml. amyl alcohol	Initial Peak Area	Final Peak Area	K
5.0	-	527	500	37.4
5.0	-	527	480	20.0
5.0	-	527	528	
4.0	1.0	422	410	64.5
4.0	1.0	422	324	6.5
4.0	1.0	422	352	10.0
3.0	2.0	316	332	
3.0	2.0	316	281	15.9
3.0	2.0	316	306	62.0
2.0	3.0	211	225	
2.0	3.0	211	208	14.7

Conclusion

No reliable estimate of the partition coefficient can be made. The wide range of values probably arises from the technique not being accurate enough to estimate a small difference between two large numbers. This, in itself, indicates that the loss is small, and therefore unlikely to be significant.

2. Recoveries using Benzaldehyde for Extraction

Introduction

The attempt to estimate the partition coefficient for benzaldehyde between amyl alcohol and water was largely unsuccessful. This is due to the partition coefficient being very much in favour of the amyl alcohol phase.

The alternative approach is to extract an aqueous solution of chlorbutanol with amyl alcohol and with benzaldehyde solution. Following extraction, benzaldehyde solution is added to the amyl alcohol extract, and an equivalent volume of amyl alcohol to the extract using benzaldehyde solution. 1 µl. of each is injected into the gas chromatograph and the peak areas for chlorbutanol and benzaldehyde determined.

If there is significant loss of benzaldehyde to the aqueous phase then the ratio of the peak area for chlorbutanol to benzaldehyde will be greater for the samples extracted with the benzaldehyde standard.

Reagents

A urine sample known to contain chlorbutanol was employed, and a 374 mg./100 ml. solution of benzaldehyde in amyl alcohol.

Procedure

A series of flasks were prepared containing 5.0 ml. of the urine sample. To seven of the flasks was added 5.0 ml. of amyl alcohol, and to the remaining eight 5.0 ml. of the benzaldehyde solution. The flasks were shaken on the "Griffin" shaker for 5 minutes and the phases separated by centrifuging.

From the first seven extracts, 2.0 ml. of the organic phase was removed and 2.0 ml. of the benzaldehyde solution added. From the remaining eight extracts, 2.0 ml. of organic phase was removed and 2.0 ml. of amyl alcohol added.

1 µl. injections of each solution were made into the gas chromatograph, and the peak areas for chlorbutanol and benzaldehyde determined using a disc integrator.

Results

These were obtained during the comparison of extraction with the benzaldehyde solution, and extraction with amyl alcohol and subsequent addition of the standard solution.

Extraction with amyl alcohol.

Peak area ratios:- 4.06, 3.33, 3.62, 3.70, 3.52, 3.21, 3.66.

Average: 3.58, median 3.62

Extraction with standard solution.

Peak area ratios:- 3.49, 3.86, 3.76, 3.46, 3.58, 3.80, 3.54, 3.61.

Average: 3.62, median 3.59.

Conclusion

The loss of benzaldehyde is so small that it does not significantly affect the results. Determinations can be carried out using the standard solution of benzaldehyde in amyl alcohol. Careful selection of volumes will eliminate the dilutions frequently required after extraction.

Hydrolysis of Samples

Introduction

Chlorbutanol is reported to be excreted, in part at least, as the conjugate with glucuronic acid (24, 94). It is possible that the extraction with amyl alcohol removes only the unbound drug from the aqueous phase.

The chlorbutanol can be liberated from the conjugate by hydrolysis with β -glucuronidase or with dilute acid. Three samples of urine were examined to see if acid hydrolysis affected the recovery of chlorbutanol.

Reagents

Three samples of urine known to contain chlorbutanol were used, and a 374 mg./100 ml. solution of benzaldehyde in amyl alcohol.

Procedure

For each urine sample three flasks were prepared containing 2.0 ml. of urine. To one flask was added 0.2 ml. of concentrated hydrochloric acid and all the flasks incubated at 37°C overnight.

Before extraction, 0.2 ml. of hydrochloric acid was added to a second flask, and 0.2 ml. of water to the third.

All the flasks were extracted with 2.0 ml. of

benzaldehyde solution by shaking for 5 minutes.

1 µl. of each organic phase was injected into the gas chromatograph and the peak areas determined for chlorbutanol and benzaldehyde.

The chlorbutanol levels were estimated by comparison of the peak areas with those obtained from 0.2 mg./100 ml. standard aqueous solution of chlorbutanol.

Results

These are given in Table 41, the levels of chlorbutanol being given as mg./100 ml.

TABLE 41

	Chlorbutanol concentrations		
	Sample 1	Sample 2	Sample 3
With Acid before incubation	0.190	0.248	0.158
With Acid after incubation	0.176	0.268	0.235
With water	0.185	0.261	0.230

Conclusions

One result for sample 3 appears to be anomalous. The remainder indicate that hydrolysis does not increase the recovery of chlorbutanol, and that the recovery is independent of pH.

General Discussion

Using the electron capture detector it was not possible to make injections of aqueous solutions into the gas chromatograph. However, the solvent extraction system evolved is speedy, simple, gives a good recovery of chlorbutanol and is fairly accurate. The system also obviates the need for hydrolysis when analysing urine samples.

Urine samples may vary widely in their chlorbutanol concentrations, and by varying the volumes of organic phase and aqueous phase, it is possible to compensate for these variations within wide limits. This gives large peaks for accurate area measurements but operates within the linear range of the detector.

By using an aqueous chlorbutanol standard problems such as deterioration of the benzaldehyde standard and changes in the relative response of the detector to chlorbutanol and benzaldehyde are overcome. Such response changes may arise due to small changes in operating conditions from day to day.

There is also some compensation for incomplete recovery during the extraction process. The recovery is high (97.3%) but by comparison with a chlorbutanol standard extracted simultaneously with the samples small variations in shaker efficiency are overcome.

Conclusion

The final technique is as follows:-

One volume of urine is extracted with an appropriate volume of a 300 mg./100 ml. solution of benzaldehyde in amyl alcohol by shaking vigorously for 5 minutes. A standard solution of chlorbutanol (about 0.2 mg./100 ml.) in water is simultaneously extracted.

1 µl. of each organic phase is injected into the gas chromatograph and the peak areas estimated for chlorbutanol and benzaldehyde.

The gas chromatographic conditions are a 5' x 1/8" glass column packed with 15% F.F.A.P. on Chromosorb W (AW DMCS treated) conditioned for 24 hours at 225°C before use. It is operated at 95°C with a nitrogen or argon/methane 10 : 1 carrier gas flowing at 40 ml./minute. The injector temperature is 125°C and the electron capture detector maintained at 150°C.

This technique provides a ready means of estimating chlorbutanol, detects, and can estimate the related compounds considered.

APPLICATIONS

Introduction

This section is concerned with the applications of the gas chromatographic technique for the detection and estimation of chlorbutanol, and of the Fujiwara test.

The gas chromatographic technique was used to examine the efficiency of the solvent extraction procedure used for the Fujiwara test and to examine the loss of chlorbutanol from samples under varying storage conditions. It was also used as originally intended for the identification of the material in urine samples giving a positive Fujiwara test. This technique showed that chlorbutanol was the only drug of this type employed.

The Fujiwara test was used as a supplement to the gas chromatography technique, providing an independent means of demonstrating the presence of a drug. This test was also used in investigations into the excretion of chlorbutanol which are described in another section.

Methods

1. Gas Chromatography

The technique used was that developed in the preceding section.

For quantitative analysis, 2.0 ml. of urine was extracted with 4.0 ml. of a solution of benzaldehyde in amyl alcohol. This had a concentration of about 300 mg./100 ml. A simultaneous extraction of a 0.2 mg./100 ml. aqueous solution of chlorbutanol was carried out.

1 µl. of the organic phase was injected into the gas chromatograph fitted with a 5' x 1/8" glass column packed with 15% F.F.A.P. on Chromosorb W, and maintained at 95°C.

By comparison of the peak area ratio of chlorbutanol to benzaldehyde in the sample to the standard the chlorbutanol concentration was calculated.

For qualitative analysis about 0.5 ml. of urine was shaken with about 0.2 ml. of amyl alcohol, and 1 µl. of the organic phase injected into the gas chromatograph operating as described above.

2. Fujiwara Test

10 ml. of urine was extracted with 2.0 ml. of toluene and discarded. The toluene was added to a test-tube containing 3.0 ml. of pyridine and 2.0 ml. of 10% aqueous sodium hydroxide. The test tube was placed in a boiling water bath for 15 minutes and observed continuously during this time.

1. Gas Chromatography

(a) To Fujiwara Reaction

Introduction

This reaction relies upon the recovery of chlorbutanol from aqueous samples by extraction with toluene. The conditions for carrying out the reaction have been adjusted to the optimum for the technique, but the actual recoveries by the toluene have not been investigated. The investigation can be conveniently carried out using the gas chromatographic technique developed for the analysis of urine samples.

(i) Maximum Recovery

Procedure

This is an estimate of the best possible recovery using a single extraction with toluene. This was achieved by shaking 4.0 ml. of a 0.34 mg./100 ml. solution of chlorbutanol in water with 4.0 ml. of toluene on a "Griffin" shaker for 15 minutes. The toluene phase was then separated and discarded. The aqueous phase, and a fresh 4.0 ml. aliquot of the chlorbutanol were then extracted using amyl alcohol for estimation of the chlorbutanol remaining. The ratio of the concentration of chlorbutanol in the standard to that in the sample is a measure of the

extraction efficiency. This ratio is conveniently calculated from the ratio of the peak areas of chlorbutanol to benzaldehyde in the samples and standard solutions.

Results

These are given in Table 42.

TABLE 42

Extraction of Chlorbutanol using Toluene

Run	Ratio Chlorbutanol Peak Areas / Benzaldehyde	Ratio standard / sample
1	0.306	7.1
2	0.276	7.9
3	0.110	19.8
4	0.216	10.0
Std.	2.17	

Discussion

These results show a maximum recovery of 95% and a minimum of 87.5% using toluene. The spread of results is probably due to incomplete separation of the toluene from the aqueous phase. The solutions were centrifuged, but with such high recoveries a little toluene may produce a marked difference in the apparent chlorbutanol concentration in the aqueous phase.

Conclusion

These recoveries represent the best that can be achieved in the course of the normal Fujiwara test. In fact the recoveries may be less as the solvent : sample ratio is less than the 1 : 1 used here, and the shaking is not so vigorous or prolonged.

(ii) Recoveries for the Fujiwara Test

Introduction

These were measured using conditions closely resembling those used routinely for the Fujiwara test. In addition to estimating the recoveries using toluene, the recoveries using anisole were also measured. Anisole is a possible alternative to toluene in the Fujiwara test which may give a superior recovery.

Procedure

Ten separating funnels were prepared each containing 50 ml. of 0.34 mg./100 ml. aqueous chlorbutanol solution. To five of the funnels was added 2.0 ml. of toluene, and to the remainder 2.0 ml. of anisole. The extraction was then carried out using repeated inversions for one minute. Repeated inversion is used to avoid the emulsions which may form if vigorous shaking is used when extracting urine samples.

After separating the organic phase, 4.0 ml. of

each aqueous phase was extracted with amyl alcohol to measure the remaining chlorbutanol. For comparison purposes, a further 4.0 ml. of the chlorbutanol solution was also extracted, and all the extracts were used for gas chromatographic analysis.

Results

These are given in Table 43.

TABLE 43

Extractions using Toluene and Anisole

Sample	Toluene Ratios		Anisole Ratios	
	Chlorbutanol / Benzaldehyde	Standard Sample	Chlorbutanol / Benzaldehyde	Standard Sample
1	0.851	2.16	0.525	4.18
2	1.052	1.75	0.658	3.34
3	0.984	1.87	0.792	2.78
4	1.003	1.83	0.525	4.18
5	0.800	2.30	-	-
Std.	1.837	-	2.19	-

Discussion

These results show that the recovery achieved using toluene is 66.4% and using anisole 78.4%. This is an advantage in favour of anisole of 18%, somewhat less than was expected. The recovery using toluene was considerably better than had been feared when using

a low solvent to sample ratio and extraction by inversion.

Conclusions

The toluene recovery is better than expected. The recovery using anisole is better, but this gain in sensitivity would be reduced by the lower sensitivity of the Fujiwara test when using anisole instead of toluene. The test as employed is adequately sensitive, so there is no purpose in using the more expensive anisole in preference to toluene for a marginal gain.

(b) Sample storage

This investigation arose due to a discrepancy in findings between a pre-race laboratory and the Department of Forensic Medicine at Glasgow University. A sample tested in the pre-race laboratory gave a positive result in the Fujiwara Test, and in the Forensic Medicine Department a negative result. Subsequent investigation revealed that the sample had been left in an open bowl for some time.

(i) Loss from Open Bowls

In a preliminary trial, a urine sample containing chlorbutanol was divided into two parts. One part was placed in a bottle sealed with a well-fitting rubber bung and the other placed in an open bowl. After standing overnight at room temperature, both

portions of the sample were analysed using the gas chromatography technique developed.

Results

The results found were as follows.

Initial chlorbutanol concentration	0.36 mg./100 ml.
Concentration in sample in bottle	0.39 mg./100 ml.
Concentration in sample in bowl	0.06 mg./100 ml.

Conclusion

These results show clearly that an appreciable fall of chlorbutanol concentration may occur in samples stored in an open bowl.

In a further experiment a urine sample was placed in an open bowl kept at room temperature. Aliquots of the sample were removed at intervals, and the chlorbutanol concentration determined.

Results

Loss of chlorbutanol from urine sample

Time from Start (hours)	Chlorbutanol concentration (mg./100 ml.)
0	0.52
1½	0.46
3	0.50
4½	0.44
6	0.39
24	0.24
96	0.04

Discussion

Clearly chlorbutanol can be lost from samples stored in open bowls. This is the normal procedure at race-tracks for pre-race testing. The time between sampling and completion of the testing is less than 3 hours. During this time no appreciable loss of chlorbutanol will occur.

If the sample is to be forwarded for further analysis, however, the sample should not be left in an open bowl longer than is necessary.

(ii) Loss from Bottles

Introduction

Samples are normally forwarded from race-tracks in bottles. Previous work has shown that appreciable loss of chlorbutanol can occur from samples stored in open bowls. The possibility of loss from bottles during transit was investigated.

Procedure

Three urine samples were used. The initial chlorbutanol concentrations were determined. One sample was placed in a glass bottle which was then closed with a cork, and the remaining samples were placed in screw capped polythene bottles. The bottles were left at room temperature overnight, and the

chlorbutanol concentrations re-determined.

Results

The results found were as follows.

Glass Bottle. Initial chlorbutanol concentration
0.64 mg./100 ml.
Final chlorbutanol concentration
0.34 mg./100 ml.

Polythene Bottles. Initial chlorbutanol concentrations
0.56, 0.53 mg./100 ml.
Final chlorbutanol concentrations
0.57, 0.51 mg./100 ml.

Discussion

Clearly, if a sample is to be stored, even for a few hours, it must be in a well closed container. Leakage may occur past a cork, but a rubber bung would appear to be satisfactory. The normal containers used are screw capped polythene bottles and these are satisfactory. Care will be necessary to ensure that the screw-caps fit tightly.

Conclusions

Chlorbutanol can be lost quite rapidly from samples stored in open vessels. The rate of loss will not affect normal pre-race test results, but may affect subsequent analysis elsewhere.

If the samples are to be stored for any length of time, screw-capped polythene bottles are to be preferred. Bottles closed with corks must be avoided if possible, as loss past the cork may occur.

(c) To Routine Analysis

(i) Qualitative Analysis

Introduction

Prior to the establishment of the gas chromatographic technique detection of chlorbutanol was based solely upon the Fujiwara test. There are a large number of compounds which give a positive reaction in this test, and they cannot be identified by it. The gas chromatograph enabled the drug present in routine samples to be identified. It was this technique which showed that the only drug used is chlorbutanol. A few illustrative examples are given below.

Examples

A pre-race laboratory found a number of samples to give a positive Fujiwara test. A total of 29 samples were forwarded of which 18 were found to give a positive Fujiwara test. Examination of all the samples by gas chromatography showed a peak corresponding to chlorbutanol in the 18 samples and the remaining samples to be negative.

In a similar incident, 77 samples were forwarded for analysis. Of these 17 were found to be positive by both the Fujiwara test and by gas chromatography. The remaining 60 samples were found to be negative using both tests.

Discussion

These results, and the results from other incidents, confirmed the reliability of the gas chromatographic technique. All the samples found to be positive by the Fujiwara test gave a positive result by gas chromatography. Similarly, there was complete agreement in the negative results. There was no interfering substance found in any of the samples examined.

In addition, the gas chromatographic technique showed that the only drug of this type used was chlorbutanol.

(ii) Quantitative Analysis

This was carried out with two objectives in mind. Firstly, to establish the concentrations found following illicit drugging incidents. This may enable a distinction to be drawn between illicit drugging and therapeutic use to prevent motion sickness. Secondly, to see if the time of administration and dosage can be

determined.

The average concentration found to date is 0.75 mg./100 ml. the range being from 0.19 mg./100 ml. to 2.67 mg./100 ml. This is based, however, upon a limited number of samples, and this work is continuing as, and when, samples become available.

One interesting finding was made. Following an incident 31 samples of urine were forwarded, of which 6 were found to give a positive Fujiwara reaction. Analysis by gas chromatography showed that these samples contained between 0.19 mg./100 ml. and 0.56 mg./100 ml. of chlorbutanol. However, the gas chromatograph also showed one further sample to contain what appeared to be 0.01 mg./100 ml. of chlorbutanol. A positive Fujiwara test could not be obtained; the probable explanation was that the material was chlorbutanol resulting from cross-contamination during sampling.

Discussion

The results obtained so far indicate that it is unlikely that it will be possible to differentiate between drugging and therapeutic dosage or determine when the drug was given. The values found to date cover a wide range of concentrations, and under these circumstances it is impossible to make a definitive

statement regarding any particular incident other than certain samples contained such-and-such a concentration of chlorbutanol.

Conclusion

The analysis of the samples to date has shown that chlorbutanol can be identified and measured using this technique. No sample was found to contain a material which would interfere with the analysis.

The sample which was probably cross-contaminated does show that, as expected, the gas chromatographic technique is more sensitive than the Fujiwara test as normally used.

The quantitative estimation of chlorbutanol has not given any useful information to date. The results indicate that it is unlikely to do so, but a definitive answer cannot be given until more samples are analysed as, and when, they become available.

2. Fujiwara Test

(a) To Routine Analysis

This test is a useful one in conjunction with the gas chromatographic technique. Identification cannot be said to be absolute using only one gas chromatographic column. If more than one column is used, this involves time and inconvenience in changing columns during the analysis.

The Fujiwara test has been found to be sufficiently sensitive to detect chlorbutanol in the concentrations normally encountered. It is a simple, speedy essential supplement to the gas chromatographic technique.

The section dealing with the analysis of routine samples by gas chromatography gives a few examples of how the technique has been employed.

(b) Trial Samples

These samples were obtained with a view to investigating the time period over which chlorbutanol is detectable. The samples were also used to gain some knowledge regarding the reliability of the test as used.

Details of this work are given in another section. The test was found to be reliable in the sense that there were few occasions where one sample was found to be negative, but later ones positive. Such

occurrences were all a long time after administration when the drug concentration would be varying around the limit of detection. Certainly for routine use in pre-race laboratories the test is sufficiently reliable as the drug concentration is normally well above the limit of detection. The only failure encountered in the department was the sample thought to have been contaminated.

General Conclusions

The investigations into the recoveries achieved during the solvent extraction for the Fujiwara test were interesting in that the recoveries were higher than was expected. This work also showed that an improved recovery could be obtained by extracting with anisole instead of toluene.

The investigation into the loss of chlorbutanol from samples showed the need to ensure that samples are stored in open containers for as short a time as possible. A procedure followed at some race tracks is to obtain samples for the pre-race laboratory in open bowls. If further analysis is required, the samples are placed in bottles the following morning and despatched by post. This leads to a loss of chlorbutanol and should be avoided. This work also showed that the screw capped polythene bottles, normally used for posting samples, are superior to the glass bottles closed with a cork which are occasionally used.

The Fujiwara test provides the vital confirmatory test when analysing these urine samples, and was also extremely useful in the work relating to the excretion of chlorbutanol.

THE ACTION OF DRUGS ON RACING GREYHOUNDS

Introduction

Following the improvements in the Fujiwara test it was necessary to assess the effectiveness of the test in practice. The toxicological information required could be obtained by administering chlorbutanol to a number of greyhounds and sampling at specified intervals. However, although dogs are used in experimental work with drugs, little is known about the effect of drugs upon the performance of a racing greyhound. The trials of the Fujiwara test were designed to obtain as much knowledge as possible relating to the effect of chlorbutanol on racing greyhounds, and the detection of chlorbutanol in urine samples.

Objectives of the Trials

The objectives of the trials can be summarised as follows:-

1) To ascertain the length of time after administration during which the drug is detectable in urine samples.

2) To ascertain the effectiveness of the analysis under pre-race laboratory conditions.

3) To ascertain the effect of the drug on the performance of the greyhound.

4) To form an overall picture of a drugged greyhound as described by toxicologists, veterinary surgeons, racing managers and kennel staff.

A certain amount of knowledge had been gained from routine analyses, but it was intended that the trials would provide more precise information.

Establishment of the Trials

Six greyhounds were available for these trials. To obtain information relating to the effect on performance they were carefully selected for their consistent performances. If this had not been done it would have been difficult to decide whether a poor performance was due to the drug or normally erratic behaviour. Throughout the trials, the dogs were kept under normal racing conditions and at their normal racing weight and fitness.

Trial Races, Dosage and Sampling

For these experiments, the six dogs were divided into three pairs. One pair was not drugged and used as controls. The other dogs were given the same weight related dose of chlorbutanol. For security reasons, the dose used cannot be stated.

For control purposes the start time is most conveniently fixed at 1½ hours before the first pair were drugged.

To establish the standards of performance of the greyhounds trial races were held 14 and 7 days prior to

the start time.

At the start time, urine samples were obtained from all the dogs, and one and a half hours later one pair of greyhounds were dosed.

The other pair of greyhounds were dosed 25½ hours after start time, and the first trial race held 29 hours after start time. A second trial was held on day 7.

Veterinary examinations were carried out for the first two days, after this only routine veterinary inspections were made.

The first samples were obtained at the start time, and sampling continued for some eighteen days.

Full details of sample times, trial races and the times of the veterinary inspection are given in the results.

Analysis of Samples

Introduction

The samples were packed in polythene screw-cap bottles and despatched by post to the Department of Forensic Medicine at Glasgow University. They were analysed using the Fujiwara test and qualitative gas chromatography.

When using the Fujiwara test, the heating time

was extended to 20 minutes. Previous work indicated that maximum colour development occurred after 12 minutes heating. To check this, the heating time was extended, and a note made of the time taken for the colour to appear.

At the time the trials were carried out, the gas chromatographic technique was in the development stage. A procedure had been evolved which enabled chlorbutanol to be separated from toluene, which was used to extract the samples. Previous analysis of a small number of samples indicated that those samples which gave a positive Fujiwara reaction gave a peak corresponding to chlorbutanol, and the negative samples no peaks at all. The samples from these trials were used to check the technique, particularly for interference from normal urinary constituents.

Procedure

1. Fujiwara Test

10 ml. of sample was extracted with 2 ml. of toluene. The urine was discarded, and the toluene added to a test tube containing 2 ml. of 10% aqueous sodium hydroxide and 3 ml. of pyridine. The test tube was placed in a boiling water bath for 20 minutes.

2. Gas Chromatography

0.5 ml. of urine was extracted with 0.2 ml. of

toluene by shaking for 15 seconds. 1 µl. injections of the toluene were made. The column was 5' x 1/8" glass packed with 15% F.F.A.P. on Chromosorb W maintained at 95°C. The injector temperature was 125°C, and the electron capture detector was maintained at 150°C. Nitrogen carrier gas was used, flowing at 40 ml./minute.

Results

1. Analytical Results

The analytical results are given in Table 44, in this table a + sign indicates a positive result and a - sign a negative result.

2. Veterinary Surgeons Results

(a) At start + 1 hour - all normal.

(b) At start + 3½ hours - Dog 5 quiet, unsteady on feet. Dog 6 quiet, unsteady on feet. Others normal.

(c) At start + 8 hours. Dog 5 quiet, virtually normal. Dog 6 quiet, recovered somewhat though still unsteady on feet.

(d) At start + 25 hours. All normal.

(e) At start + 27 hours. Dogs 2 and 4 had a slightly unsteady trot, remainder normal.

(f) At start + 29 hours (after trial race) - all normal.

(g) At start + 32 hours - all normal.

TABLE 44

Chlorbutanol Trials - Analytical Results for Urine Samples

Time from Start (Hrs)	DOG 1 (Control)		DOG 2		DOG 3 (Control)		DOG 4		DOG 5		DOG 6		Time for colour to appear (L - R for positives)
	Fujiwara	G.C.	Fujiwara	G.C.	Fujiwara	G.C.	Fujiwara	G.C.	Fujiwara	G.C.	Fujiwara	G.C.	
0	-	-	-	-	-	-	-	-	-	-	-	-	
6	-	-	-	-	-	-	-	-	+	+	+	+	5, 5 mins.
24	-	-	-	-	-	-	-	-	+	+	+	+	15, 15 mins.
27	-	-	+	+	+	+	+	+	+	+	+	+	5,7,5,7,5 mins.
31	-	-	-	+	-	-	+	+	+	+	+	+	8,8,8 mins.
48	-	-	+	+	-	-	+	+	-	+	+	+	4,15,9 mins.
72	-	-	+	+	-	-	+	+	+	+	+	+	4,12,15,5 mins.
96	-	-	+	+	-	-	+	+	+	+	+	+	4,6,13,8 mins.
102	-	-	+	+	-	-	+	+	+	+	+	+	5, 5, 5, 5 mins.
120	-	-			-	-							
126	-	-	+	+	+	+	+	+	+	+	+	+	5,13,5,15,5 mins.
168	-	-	+	+	-	-	+	+	+	+	+	+	9,3,3,15 mins.
171	-	-	+	+	-	-	+	+	+	+	+	+	5,5,5,5 mins.
175	-	-	+	+	-	-	+	+	+	+	+	+	3,3,3,3, mins.
192	-	-	+	+	-	-	+	+	-	+	-	+	10, 10 mins.
198	-	-	-	+	+	+	+	+	+	+	+	+	8,5,7,5 mins.
216	-	-	-	+	-	-	+	+	+	+	+	+	10, 8, 8 mins.

TABLE 44 (continued)

Chlorbutanol Trials - Analytical Results for Urine Samples

Time from Start (Hrs)	DOG 1 (Control)		DOG 2		DOG 3 (Control)		DOG 4		DOG 5		DOG 6		Time for colour to appear (L - R for positives)
	Fujiwara	G.C.	Fujiwara	G.C.	Fujiwara	G.C.	Fujiwara	G.C.	Fujiwara	G.C.	Fujiwara	G.C.	
240	-	-	-	+	-	-	+	+	+	+	+	+	7, 5, 10 mins.
246	-	-	+	+	-	-	+	+	+	+	+	+	8, 5, 5, 8 mins.
264	-	-	-	+	-	-	-	+	-	+	-	+	
270			+	+			-	+	+	+	+	+	7, 15, 7 mins.
288	-	-	-	+	-	-	+	+	-	+	-	+	7 mins.
318	-	-	-	+	-	-	+	+	-	+	-	+	13 mins.
336	-	-	-	+	-	-	+	+	-	+	-	+	11 mins.
360	-	-	-	+	-	-	-	+	-	+	-	+	
384	-	-	-	+	-	-	+	+	-	+	-	+	10 mins.
408			-	+	-	-					-	+	
432			-		-	-	-	+	-	+	-	+	

(h) In routine examinations during the remainder of the trial period the dogs were normal.

These can be summarised as follows:-

Dog 2 - Definite, but not pronounced, symptoms of drugging 3 hours after administration.

Dog 4 - Some symptoms of drugging 3 hours after administration.

Dog 5 - Some symptoms of drugging 2 hours after administration, and persisting 5½ hours after administration.

Dog 6 - Definite, but not pronounced symptoms, 2 hours after administration, slight symptoms 5½ hours after administration.

3. Racing Managers Results

(a) Trial held at start + 28 hours.

Dog 2 - Poor time compared with earlier trials, swerved during race.

Dog 4 - Poor time compared with earlier trials, swerved during race.

Dog 6 - Possibly a marginal improvement in performance.

Remainder - normal.

(b) Trials held at start + 340 hours.

All the greyhounds performed normally.

Discussion

1. Analytical Results

The results indicate that at the dosage employed chlorbutanol is readily detectable from 1½ hours after administration until at least 250 hours after administration by means of the Fujiwara test. These time limits are not absolute. Variation can be expected due to different dogs and different doses. They do provide an indication of the period of time during which chlorbutanol can be detected.

The test is not infallible. However in the first 150 hours or so after administration it does appear to be fairly reliable. This is related to drug concentration, which decreases with time after administration.

The boiling time of 15 minutes for the Fujiwara reaction appears to be adequate. Continual observation is necessary, however, as a sample which gives a pink colour after 5 minutes boiling may appear colourless after 15 minutes boiling.

The gas chromatographic results were interesting. The negative samples obtained from the control dogs and prior to dosing showed no peaks other than solvent and water. The samples found to be positive by the Fujiwara reaction showed one additional peak only. This peak had a retention time corresponding to that of

chlorbutanol. Together these two facts mean that chlorbutanol can be reliably detected by this method, and there is no interference due to other compounds.

The comparison of the results obtained by the two techniques are also of interest. Using the gas chromatograph, chlorbutanol can be detected up to at least 432 hours after administration. In addition, the test is more reliable than using the Fujiwara reaction. Both these observations can be reasonably attributed to the gas chromatographic technique being more sensitive than the Fujiwara test.

2. Veterinary Surgeons and Racing Managers Results

These are conveniently grouped together as they are closely related. The veterinary surgeon observed symptoms of drugging between 2 hours and 5½ hours after administration, and during this time the racing manager regarded the dogs' performances as having been adversely affected.

The veterinary surgeon regarded the dose as having been too low, as few symptoms were present. The observed unsteadiness agrees with reports by race tracks following incidents, but at tracks the symptoms are occasionally so extreme that the greyhound's behaviour is reported as "drunken". Against this it can be said that in the majority of incidents no symptoms are

observed, and the dose administered agrees roughly with that in capsules found by tracks.

The effect on performance was in agreement with the clinical findings. During the time when the symptoms were observable, the performance was adversely affected. However, after 24 hours the performance was not adversely affected; indeed, by this time, the drug may have a quieting effect upon a nervous greyhound and so improve its performance.

3. Conclusions

There are three aspects to the drugging of a racing greyhound with chlorbutanol.

Firstly, in the period up to about 8 to 10 hours after administration the greyhound exhibits symptoms varying between unsteadiness when negotiating obstacles to "drunkenness". This effect usually develops within 2 hours of administration. The drug is readily detectable in the urine when the clinical symptoms develop. During this time the performance of the greyhound is probably adversely affected.

Secondly, in the period between this and not more than 7 days after administration, the dog exhibits no symptoms, but the drug is readily detectable in the urine. The greyhound's performance is not adversely affected; indeed, the performance of a nervous dog

may improve. This period may last considerably less than seven days, but the experiment could not reveal this.

The third period is from 7 days onwards. The drug is detectable in the urine for some time after this, but no symptoms are observed and there is no effect upon the performance of the greyhound.

The greatest problem arising from these results is that chlorbutanol is detectable for so long after administration, making interpretation of pre-race results difficult.

Trials involving Phenobarbitone

Introduction

The previous work discussed the effect of chlorbutanol upon the racing greyhound. While the organisation and greyhounds were available, a similar series of trials were carried out using phenobarbitone. This is commonly used to drug racing greyhounds.

Objectives

These were similar to those for the trials using chlorbutanol.

Establishment

The same general arrangement was employed. Eight greyhounds were available, two of which were used as controls, and the remaining six given phenobarbitone.

In addition to the analysis at the Forensic Medicine Department of Glasgow University, analysis of the samples was carried out by a pre-race laboratory. This was intended to give a comparison of the results obtained under pre-race conditions with those obtained following a more detailed analysis.

Trial Races, Doses and Sampling

The six greyhounds were given a weight related dose of phenobarbitone. This cannot be stated for security reasons. Urine samples were obtained three times a day

and trial races held weekly. The most convenient datum time for these trials is the time of administration of the drug.

Analysis of Samples

1. By Pre-Race Laboratory

A preliminary analysis was carried out by a pre-race laboratory using their standard technique.

Procedure

20 ml. of urine were acidified with hydrochloric acid, extracted by shaking with 50 ml. of ethyl acetate, and discarded. The ethyl acetate was washed with 5 ml. of 5% aqueous sodium bicarbonate, dried over anhydrous sodium sulphate, and evaporated to dryness. The residue was taken up in chloroform, spotted on a thin layer chromatography plate which was developed in chloroform/acetone 3 : 1, and sprayed with saturated aqueous mercurous nitrate. The phenobarbitone and its metabolites show as a black spot on a white background.

2. At the Forensic Medicine Department

Two techniques were employed, thin layer chromatography and ultraviolet spectrophotometry.

a) Thin Layer Chromatography

The extraction procedure was the same as that used by the pre-race laboratory. After spotting the thin

layer chromatography plate was developed in chloroform/ acetone 9 : 1, and then sprayed with saturated mercurous nitrate solution.

b) Ultra-violet spectrophotometry

The technique used was based upon that of Broughton (18) and described by Bogan and Smith (10).

This involved the acidification of 20 ml. of urine with hydrochloric acid and extraction by shaking with 50 ml. of chloroform. The urine was then discarded. The chloroform was washed with 5 ml. of 2.5% aqueous sodium bicarbonate, extracted with 5 ml. of 0.45N aqueous sodium hydroxide and discarded.

1.5 ml. of the sodium hydroxide extract was mixed with 1.5 ml. of 0.45N aqueous sodium hydroxide, giving a solution of pH 14, and the ultra-violet spectrum plotted.

A further 1.5 ml. of the sodium hydroxide extract was mixed with 1.5 ml. of 0.6M aqueous Boric acid buffer, giving a solution of pH 10 and the ultra-violet spectrum plotted.

The difference in absorbance at 260 mμ between the two spectra is proportional to the concentration of phenobarbitone in the extract.

Results

a) Analytical Results

These are given in Table 45, the symbols having the following meaning:-

Pre-race:- The finding of the pre-race laboratory using thin layer chromatography.

Pheno:- The detection of phenobarbitone at the Forensic Medicine Department at Glasgow University using thin layer chromatography.

Metab:- Refers to the detection of a phenobarbitone metabolite at the Forensic Medicine Department using thin layer chromatography.

In all the above columns a + sign means that the material was detected, a - sign that it was not detected, and a blank that the test was not carried out.

The column headed "U.V." refers to the concentration of phenobarbitone in the sample expressed as mg./100 ml., a blank indicating that the test was not carried out.

b) Veterinary Results

Details of the findings are lacking for this trial. The summary of the findings was that clinical symptoms may appear before the phenobarbitone or its metabolites are detectable in the urine. In addition, the dogs

TABLE 45

Phenobarbitone Trials (1) - Analytical Results

Time	DOG 1				DOG 2				DOG 3				DOG 4			
Hours	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.
1½	+	+	+	0.43	+	+	+	0.34	-				-	+	+	
4½	+	+	+	0.49	+	+	+	1.28	+	+	-	0.79	-	+	+	0.79
9½	+	+	+	0.44	+	-	-		+	+	+	0.95	+	+	+	0.95
26	+	+	-		+	+	-	0.60	+	-	-		+	+	-	0.35
29	+	+	+	0.44	+	+	-	0.60	+	+	+	0.16	+	+	-	0.10
32	+	+	+	1.3	+	+	-		+	+	-	0.23				
49	+	+	+		+	+	+	0.32	+	+	-	0.29	+	+	+	0.58
52½	+	-	-		+	+	-	0.11								
74	+	+	+	0.32	+	-	+		+	-	-		+	-	-	
77	+	+	+		+	+	-	0.36	+	+	-	0.35	+	+	-	0.43
80	-	-	-		+	+	+		+	+	+		+	-	+	
98	+	+	+	0.21	+	+	+	0.29	+	+	+		+	+	+	0.36
102	+	+	+	0.38	+	+	-	0.34	+	+	+	0.22	+	+	-	
123	+	-	+		+	+	+	0.18	+	+	+	0.25	+	+	+	0.05
128	+	+	+	0.35	+	+	+		+	+	+		+	+	+	0.29
146	+	+	+	0.21	-	-	+		+	-	+		+	-	-	

TABLE 45 (continued)

Phenobarbitone Trials (1) - Analytical Results

Time	DOG 1				DOG 2				DOG 3				DOG 4			
Hours	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.
170	-	-	-										+	+	+	0.50
173	+	+	+	0.36	+	-	-		+	+	+	0.34	+	+	+	
194	+	+	+		+	+	+	0.09	+	+	+	0.10	+	+	+	0.13
200	+	+	+		+	+	+		+	+	-		+	+	+	
218	+	+	+		+	+	+		+	+	+		+	-	+	
242	+	-	+		+	+	+		+	+	+	0.06	+	+	+	
266	-	+	-	0.38					+	+	+		-	+	+	1.28
290	-	-	+		-	+	+	0.44	-	+	+	0.76	+	+	+	0.32
314	-	-	+		+	+	+	0.60	+	+	+	0.10	+	+	+	0.16
338	+	+	+	0.38	+	+	+		+	+	-	0.23	-	+	+	
362	-	+	-		+	+	+		+	+	+		-	+	-	0.36
386	-	+	+	0.49	+	+	-	0.16	+	+	+		+	+	+	
410	-															

TABLE 45 (continued)

Phenobarbitone Trials (1) - Analytical Results

Time	DOG 5				DOG 6				DOG 7				DOG 8			
Hours	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.
1½	-	-	+		-	+	+		-	+	+		-	-	-	
4½	-	+	+		+	+	-	0.38	-	-	-		-	-	-	
9½	+	+	-	0.25	+	+	-	0.76	+	+	-		-	-	+	
26	+	+	-	0.32	+	+	-	0.15	-	-	+		-	+	-	
29	+	-	-		+	+	-	0.11	-	-	+		-	-	-	
32	+	+	-	0.38	+	+	-	0.30	-	+	+		-	+	-	
49	+	+	+	0.32	+	+	-	0.30	-	-	+		-	-	-	
52½	+	+	-	0.85	+	+	+						-	-	-	
74	+	+	+	0.18	+	+	+		-	-	-		-	-	-	
77	+	+	+	0.44					-	+	+		-	-	-	
80	+	+	+	0.49	+	+	+	0.26	-	+	+		-	-	+	
98	+	+	+	0.33	+	+	+		-	-	+		-	-	+	
102	+	+	+	1.0					-	-	-		-	-	+	
123	-	+	+		+	+	+		-	-	+		-	-	+	
128	+	+	+	0.84	+	+	+	0.17	-	-	-		-	+	+	
146	-	+	+	0.21					+	-	-		+	-	+	

TABLE 45 (continued)

Phenobarbitone Trials (1) - Analytical Results

Time	DOG 5				DOG 6				DOG 7				DOG 8			
Hours	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.	Pre-Race	Pheno.	Metab.	U.V.
170	-	-	-		+	+	+	0.38	-	+	+	0.80	+	-	-	
173					+	+	+	0.43	-	+	+	0.44	+	+	+	
194	+	+	-	0.14	+	+	+		+	-	+		-	-	+	
200	+	+	+		+	+	+		-	-	+		-	-	+	
218	+	+	+		+	+	+		-	-	+		-	-	+	
242	+	+	+		+	+	+		-	-	+		-	-	+	
266	-	-	-		-	+	-	0.08	-	+	+	0.49	-			
290	-				-	+	-	0.95	-	+	-					
314	-	+	-	0.15	-	+	-		-	+	+	0.60	-	-	-	
338	+	+	-	0.30	+	+	+	0.32	+	+	+		-	+	-	
362	+	+	-	0.30	+	+	+		-	-	-		+	+	+	0.44
386	+	+	-	0.35	+	+	-	0.43	-	-	-		-	-	+	
410	+	-	-		-	-	-		-	+	-	0.79				

may appear clinically normal on the day following dosing but the drug may still be detectable in urine samples.

The veterinary surgeon had the pre-race laboratory results available when forming these conclusions.

Discussion

The results are, on the whole, disappointing for a number of reasons.

Firstly, phenobarbitone and metabolites were detected in the urine of the control dogs. This applies particularly to the analysis carried out at Glasgow University, but much less so to the pre-race laboratory analysis. That on rare occasions a trace of the drug or its metabolites was detected could be reasonably ascribed to cross contamination during sampling. However, 0.80 mg./100 ml. of urine cannot be so explained.

Secondly, quite a number of samples were duplicated. As the pre-race laboratory was carrying out analyses the samples were forwarded in batches some time after they were obtained. Consequently, the trial was well advanced before it was noticed that there were different samples bearing the same numbers. This could pass unnoticed if the pre-race laboratory was relying on verbal information and receiving the

samples in sequence and not batches.

Thirdly, the lack of trial race performances, and the limited veterinary data prevent any information, other than toxicological, from being obtained.

Conclusions

No definite conclusions are possible due to the confusion of the samples. From the results it would appear that phenobarbitone can be detected in the urine within 4½ hours of administration, and that it can be detected for some time, up to 14 days, after administration. Further, the technique used by the pre-race laboratories does satisfactorily detect the drug in urine samples.

The trial was, however, most unsatisfactory. To obtain more reliable data the trial was repeated.

Second Phenobarbitone Trials

Introduction

These trials had the same objectives as the chlorbutanol trials and the earlier phenobarbitone trial. The general pattern of dosing followed by the collection of urine samples and trial races was followed. However, the analysis of the urine samples was carried out at the Forensic Medicine Department at Glasgow University only. This was to reduce the volume of sample required, and avoid a complicated procedure which tended to lead to confusion.

Dosage and Sampling

Seven greyhounds were used in this trial, six being given a weight related dose of phenobarbitone and the seventh being used as a control. Details of the findings are given in the results section.

The start time for this trial is the time of collection of the first samples. The drug was administered at varying times and the first trial held 7 hours after start time.

Results

a) Analytical Results

These are given in Table 46. The symbols +, - and U.V. are as used for the previous phenobarbitone

TABLE 46

Phenobarbitone Trials (11) - Analytical Results

Time Hours	DOG 1				DOG 2				DOG 3				DOG 4			
	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom
0		-	-	-		-	-	-		-	-	-		-	-	-
3	0.55	+	+	+	0.54	+	-	-	0.16	+	-	-		-	-	-
6¼	1.09	+	+	+		+	-	-	0.91	+	-	+	0.13	+	+	+
9	2.5	+	-	-	1.1	+	-	-	1.08	+	-	-	2.1	+	-	-
24	0.39	+	-	-	0.405	+	-	-	0.17	+	-	-	0.46	+	-	-
27	0.68	+	-	-	0.265	+	-	-	0.22	+	-	-	0.27	+	-	-
30¼	0.12	+	-	-	0.38	+	-	-	0.50	+	-	-	0.40	+	-	-
48	0.37	+	-	+	0.06	+	-	+	0.70	+	+	+	0.20	+	-	-
51	0.66	+	-	+	0.37	+	-	+	0.64	+	-	+	0.15	+	+	+
55	0.50	+	+	+	0.22	+	-	+	0.39	+	-	+	0.35	+	-	-
72	0.47	+	-	+	0.26	+	-	+	0.06	+	+	+	0.23	+	-	-
79	0.26	+	-	-	0.23	+	-	-	0.44	+	-	-	0.16	+	-	-
96	0.24	+	-	-		+	-	-	0.24	+	-	-	0.15	+	-	-
103	0.21	+	-	-	0.09	+	-	-	0.44	+	-	+	0.22	+	-	-
120	0.23	+	-	-	0.13	+	-	-	0.41	+	-	-	0.23	+	-	+
168		+	+	+	0.06	+	+	+	0.09	+	-	-	0.05	+	-	+
192	0.06	+	-	-		+	-	-	0.12	+	-	+	0.06	+	-	+

TABLE 46 (continued)

Phenobarbitone Trials (11) - Analytical Results

Time Hours	DOG 1				DOG 2				DOG 3				DOG 4			
	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom
216		+	+	+		-	-	-		+	-	+		+	+	+
240		+	+	+		+	-	-		+	-	+		+	+	+
264		-	-	-		-	-	+		+	+	+		+	+	+
288		-	-	+		-	-	+		+	+	+		+	+	+
336		-	-	+		-	-	+		+	-	+		+	+	+
360		+	+	+		-	+	+		+	+	+		-	+	+
384		+	+	+		-	+	+		+	+	+		+	+	+
408		+	+	+		-	+	+		+	+	+		+	+	+
432		+	+	+						-	-	+		+	+	+
456		-	-	+		-	+	+		+	+	+		-	-	+
504		-	-	+		-	+	+		+	+	+		-	+	+
528		-	-	+		-	+	+		-	+	+		-	+	+
552		+	+	+		-	-	-		-	-	-				
576		-	-	-		-	-	+		-	-	-		+	-	+
600		+	+	+		-	-	-		-	-	+		+	-	+
624		-	-	-		-	-	-		+	+	+		-	-	-
696		-	+	+		-	-	+		+	+	+		-	+	+
1008		-	-	+		-	-	+		+	-	+		+	+	+

TABLE 46 (continued)

Phenobarbitone Trials (11) - Analytical Results

Time Hours	DOG 1				DOG 2				DOG 3				DOG 4			
	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom
1032		-	-	+		-	-	-		+	-	-		-	-	-
1056		-	+	+		-	-	-		-	-	+		-	-	+
1080		-	-	+		-	-	-		-	-	-		+	+	+
1224		-	-	+		-	-	-		-	-	-		-	+	+
1248		-	-	-		-	-	-		-	-	-		-	-	-

TABLE 46

Phenobarbitone Trials (11) - Analytical Results

Time Hours	DOG 5 U.V.	DOG 5 Top	DOG 5 Centre	DOG 5 Bottom	DOG 6 U.V.	DOG 6 Top	DOG 6 Centre	DOG 6 Bottom	DOG 7 U.V.	DOG 7 Top	DOG 7 Centre	DOG 7 Bottom
0		-	-	-		-	-	-		-	-	-
3		-	-	-		-	-	-		-	-	-
6¼	1.36	+	+	-	0.08	+	+	-		-	+	-
9	2.70	+	+	+	1.30	+	-	-		-	+	-
24	0.55	+	-	-	0.74	+	-	-		-	+	-
27	0.34	+	-	-	0.45	+	-	-		-	+	-
30¼		-	-	+	0.32	+	-	+		-	+	-
48	0.44	+	-	+	0.49	+	-	+		-	+	+
51	0.41	+	-	+	0.36	+	+	+		-	-	-
55	0.41	+	-	-	0.35	+	+	+		-	-	-
72	0.15	+	-	-	0.28	+	+	+		-	-	-
79	0.22	+	-	-		+	-	-		-	-	-
96	0.14	+	-	-	0.32	+	-	+		-	-	-
103	0.09	+	-	-	0.23	+	-	+		-	-	-
120		+	-	-	0.16	+	-	-		-	-	+
168		-	+	+	0.12	+	+	+		-	-	-
192		+	+	+	0.11	+	-	+		-	-	-

TABLE 46 (continued)

Phenobarbitone Trials (11) - Analytical Results

Time Hours	DOG 5 U.V.	DOG 5 Top	DOG 5 Centre	DOG 5 Bottom	DOG 6 U.V.	DOG 6 Top	DOG 6 Centre	DOG 6 Bottom	DOG 7 U.V.	DOG 7 Top	DOG 7 Centre	DOG 7 Bottom
216		+	-	+		-	+	+		-	-	-
240		+	-	-		-	-	-		-	-	-
264		+	-	+		-	-	+		-	-	-
288		-	-	-		-	-	+		-	-	-
336		+	+	+		+	+	+		-	-	-
360		+	+	+		+	+	+		-	-	-
384		+	+	+		+	+	+		-	-	-
408		+	+	+		+	+	+		-	+	+
432		+	+	+		+	+	+		+	+	+
456		-	+	+		-	-	-		-	-	-
504		-	+	+		+	+	+		-	+	+
528		+	+	+		-	+	+		-	+	+
552		-	+	-		+	+	-		+	-	-
576		+	+	-		+	-	+		-	-	-
600		-	-	-		+	-	+		+	-	-
624		-	-	-		-	-	-		-	-	-
696		-	+	+		+	+	+		-	-	-
1008		+	+	+		+	+	+		-	-	+

TABLE 46 (continued)

Phenobarbitone Trials (11) - Analytical Results

Time Hours	DOG 5				DOG 6				DOG 7			
	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom	U.V.	Top	Centre	Bottom
1032		-	-	+		+	+	+		-	-	-
1056		-	+	+		-	-	-		-	+	+
1080		-	+	+		-	-	-		-	-	-
1224		-	+	+		-	-	-		-	-	-
1248		-	-	-		-	-	-		-	-	-

trial.

b) Veterinary Surgeon's Results

i) At start. All normal.

ii) Start + 2½ hours.

Dog 1 - Slightly quiet, very deliberate movements.

Dog 2 - Slightly quieter, fell, very deliberate movements.

Remainder - Normal.

iii) Start + 4½ hours.

Dog 1 - Nervous, unsteady, deliberate movements.

Dog 2 - More lively than at (ii), but tripped over obstacle.

Dog 3 - Quieter, unsteady, tripped over obstacles.

Dog 4 - Perhaps slightly quieter.

Rest - Normal.

iv) Start + 6¼ hours.

Dog 1 - Recovered, but still slightly unsteady.

Dog 2 - Quiet, stumbled slightly over obstacle.

Dog 3 - Quiet, unsteady, tripped over obstacle.

Dog 4 - Quiet, slight stumbling over an obstacle.

Dog 5 - Quiet, tripped over obstacles.

Dog 6 - Quiet, tripped over obstacles.

Dog 7 - Normal. Seemed markedly alert after examining above six dogs.

v) Start + 9 hours.

Dog 3 - Stumbled badly.

Rest - Apparently normal. Good appetite.

c) Racing Managers Results

i) Trials at start + 7 hours.

Dog 3 - Ran badly, obviously affected.

Dogs 5 and 6. - Appeared to improve slightly

Dogs 1, 2 and 4 - Slightly reduced performances

Dog 7 - Normal.

ii) Trials at start + 105 hours.

All dogs performed as expected.

Discussion

a) Analytical Results

Phenobarbitone, and possibly its metabolites, may be detected in urine samples within two hours of administration. In fact, in one case, the drug was detectable within about 15 minutes of administration.

The drug itself persists in the urine up to about 600 hours after administration, and the metabolites up to 1,230 hours.

The concentration of phenobarbitone in the urine can be related to time to produce a fairly smooth rise and a gradual fall curve. The maximum rate of excretion took place about six hours after administration, but there is wide variation between dogs for both the time and magnitude of the maximum rate of excretion.

This means that it is not possible to deduce the time of administration of the drug from the analysis of a single sample.

The appearance and disappearance of the metabolites is random over the first 100 hours, but there is no pattern here, or later, which may be of assistance in deducing the time of administration of the drug.

b) Veterinary Surgeons and Racing Managers Results

The phenobarbitone produces clinical symptoms within 2 hours of administration, these persist for 4 hours to 8 hours after administration. These symptoms seem to be mainly quietness, unsteadiness and a tendency to trip over obstacles. The veterinary surgeon did report that there is a distinction between a drugged and an undrugged dog, but it is impossible to define this precisely.

The racing manager's findings are remarkable. Even though the dogs exhibited clinical symptoms of drugging, only the performance of one dog was markedly reduced. In fact, two dogs exhibited a slight improvement, while the remaining three exhibited a slight reduction in performance. This was in trials held two to six hours after administration.

The performance of dogs drugged with phenobarbitone obviously varies widely, and the result of such

a drugging is difficult to predict.

Conclusions

These are based upon both trials using phenobarbitone.

The drug rapidly appears in the urine after administration; this may be as soon as 15 minutes after administration, and certainly within four hours.

The drug and its metabolites persist for far longer than was previously thought. The drug is detectable up to about 600 hours after administration, and the metabolites up to 1,230 hours.

Neither the rate of excretion nor the appearance of the metabolites gives any precise indication as to when the drug was administered. Only within the limits above can any comments be passed upon the analytical results obtained from a single sample.

The greyhounds exhibited a marked variation in their response to the drug, an improvement in performance may be observed even though the greyhound has clinical symptoms of drugging.

It is difficult to comment upon the performance of the pre-race testing technique due to the confusion of samples in the first phenobarbitone trial. It does appear, however, that phenobarbitone is detected satisfactorily.

Discussion relating to All Trials

The most noticeable results of all three trials is the length of time after administration during which the two drugs can be detected in urine samples. For both drugs this time period is considerably longer than that suggested by previous experience. These results emphasise the need for caution when interpreting analytical results for a possible connection with any particular race meeting.

It is also apparent that, after administration in normal gelatin capsules, the drugs are soon detectable in the urine and clinical symptoms are evident. This means that to affect the result of a race the drug has to be administered shortly prior to the sampling for pre-race testing. After this, the greyhounds are normally under high security conditions. If the drug is given before the greyhounds are placed in high security kennels the pre-race testing will detect the drug.

The results also show the narrow time interval during which the drugs may have an appreciable effect upon the result of a race. Using chlorbutanol, the effect is reasonably predictable, unless the race occurs some 24 hours after administration. With phenobarbitone, however, the effect is not predictable,

and it would appear that the performance may not be seriously affected even if the greyhound exhibits clinical symptoms of drugging.

The symptoms of drugging with both chlorbutanol and phenobarbitone include quietness. This is not surprising as the former is a hypnotic and the latter a sedative. Other symptoms include unsteadiness and a tendency to stumble over obstacles.

The analysis in all three trials included techniques normally used by pre-race laboratories. These seemed to perform satisfactorily and as expected. The chlorbutanol trials also provided useful data during the development of the gas chromatographic technique.

Unfortunately, the only trial in which a pre-race laboratory was involved was marred by confusion of samples. The phenobarbitone appears to have been satisfactorily detected, but on the data available it is impossible to draw definite conclusions.

Conclusion relating to All Trials

The drugs in question can be readily detected in urine samples a short time after administration. The tests employed in the pre-race laboratories seem to be satisfactory for this task.

The techniques, however, can detect the drugs

in urine samples obtained a considerable time after administration. Thus, it is difficult to say whether the drug is from a recent administration, in which case the performance of the greyhound may be affected, or from an earlier incident.

While it is not possible to give a precise description of a drugged greyhound, there is a definite difference apparent to a veterinary surgeon familiar with the greyhounds.

CAPSULES

Capsule Treatments

Introduction

In the trials carried out using chlorbutanol and phenobarbitone, drugs were detected in urine samples a short time after administration. The administration was in the form of the powdered drug contained in a normal gelatin capsule, a seemingly popular form of administration.

However, as was explained in the trials section, the greyhounds are kept in high security kennels from about two hours before the first race until the end of the meeting. The samples for pre-race testing are obtained when the dogs are placed in the high security kennels. Thus, the drug has to be administered at least 2 hours before the race. If the drug can be administered while the urine samples are being collected, then the drugging has a fair chance of being undetected until after the meeting. If not then, using normal capsules, the drugging will probably be detected.

This can be circumvented by placing the drug in a capsule which will not disintegrate for several hours. Then the drug can be administered while the greyhounds are under low security conditions, but not released until after the urine samples are obtained.

Review

There has been considerable interest in treated capsules since 1915 when Scoville (117) reported that treating gelatin capsules with formalin reduced their rate of disintegration in acid solution.

The interest has had a two-fold purpose; firstly, to develop a means of passing drugs through the stomach to the duodenum without loss or damage in acidic solution; secondly, and more recently, to provide a slow steady release of drug over a period of time.

A vast assortment of fats, oils, cellulose derivatives and silicones have been employed for these purposes.

White (132) used cellulose nitrates, as did Wrubble (133) who also used ammoniated shellac. Shellac was another frequently used material being used by Bukey and Rhodes (20), Mills (93) who used a shellac-cetyl alcohol mixture, and Goorley and Lee (62) who used a shellac-castor oil coating.

All these coatings had one common feature, they rendered the gelatin capsule impervious to the acidic conditions in the stomach, but not to the alkaline conditions found in the duodenum.

In more recent times, shellac has been used to

waterproof capsules (80) in combination with alcohol and castor oil, as has a combination of polyethylene glycol 6000 and cellulose acetate phthalate in acetone (1). Waterproofing is one other way of delaying the release of capsule contents into the body after administration. This waterproofing was developed to produce a film coating whose rate of disintegration could be controlled, giving timed disintegration (6).

In addition to these delayed release capsules, there is the sustained release capsules based upon the use of ion exchange resins (88).

Discussion

For circumventing the security precautions at greyhound race tracks, delayed release is of more interest than sustained release.

In all the techniques described above, the capsules are coated with a layer of virtually insoluble material. This generally requires complex equipment, particularly to produce reliably even coatings. This is generally beyond the means of criminals.

However, treatment of gelatin capsules with formaldehyde in ethanol is a simple means of producing an enteric coating. This is within the means of most people, and would enable greyhounds to be drugged any time after a midday meal to have effect during the

evening meeting. If the capsule is given earlier, it would, of course, be carried through into the duodenum with the meal and dissolve prematurely.

Experimental

Introduction

A brief investigation was carried out into the effect of treating gelatin capsules with formalin in ethanol. This investigation took two forms, laboratory testing and in vivo experiments.

The laboratory testing was used to assess the effect of different lengths of treatment, and was assessed using reagents simulating the pH, enzymatic and temperature conditions of the stomach and duodenum.

Laboratory experiments cannot simulate exactly the conditions existing in the intestines of a greyhound. To see if the results obtained in the laboratory were valid, a drug was administered to greyhounds and urine samples collected. The drug used, chlorbutanol, appears in the urine shortly after administration if untreated capsules are used. By collecting samples and analysing them, it was possible to determine whether the treated capsules delayed the appearance of the drug in the urine. If the appearance was delayed, then it could be said that the treatment of the capsules delayed the release of the drug.

1. Laboratory Testing

(a) Test Procedure

This was based upon the procedure laid down in the British Pharmacopoeia (16) using the apparatus shown in figure 12.

A perspex tube, 80 mm. long by 27 mm. internal diameter, was closed at one end by a copper gauze of about 1.5 mm. mesh. A weight was attached at this end. This was attached to an electric motor so that it could be raised and lowered through 75 mm. The solutions used to test the capsules were incubated at 37°C. At the top of the stroke the gauze just broke the surface of the liquid, and at no time did the upper end of the tube become immersed.

Reagents

Two test solutions were used:-

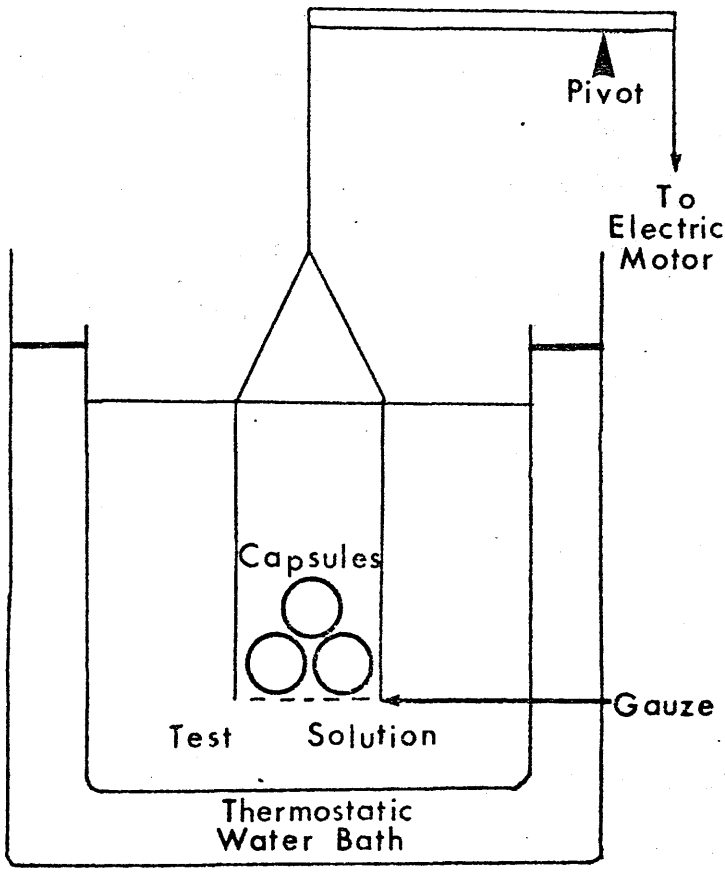
i) Acid pepsin consisting of 3 grms. of pepsin and 6 ml. of concentrated hydrochloric acid made up to 1 litre with water and

ii) Alkaline Pancreatin consisting of 3 grms. of pancreatin, 15 grms. of sodium bicarbonate and 5 grms. of sodium tauroglycocholate made up to 1 litre with water.

These solutions are not intended to reproduce the exact conditions found in the stomach and duodenum.

FIGURE 12

Capsule Testing Apparatus



They do give a means of comparison of different capsule treatments under roughly similar conditions of pH and enzymatic activity.

(b) Treatment of Capsules

The capsules were treated with a 25% solution of Formalin in ethanol. This was done by immersing the capsules in the solution and then air drying them. The capsules were then tested by the procedure described.

Results

The results for different periods of immersion were obtained using both the pepsin and pancreatin solution

i) No Treatment.

In Acid Pepsin. Collapsed within 1½ minutes and the tube contained no gelatin in 5 minutes.

In Alkaline Pancreatin. The capsules collapsed in 3 minutes and all the gelatin had left the tube in 9 minutes.

ii) 2½ minutes Immersion.

In Acid Pepsin. After 30 minutes the capsules started to collapse, after 1½ hours two had disappeared, and after 4 hours the remaining three were reduced to small blobs of gelatin.

In Alkaline Pancreatin. The capsules showed some signs of collapse after 11 minutes, but did not dissolve completely until 4 hours after the start.

iii) 5 minutes Immersion.

In Acid Pepsin. The capsules started to collapse after 1 hour, but complete disintegration took 4 hours.

In Alkaline Pancreatin. The capsules started to collapse after 7 minutes, but complete disintegration took 3 hours.

iv) 10 minutes Immersion.

In Acid Pepsin. Collapse commenced after 40 minutes, but disintegration took about 3 hours.

In Alkaline Pancreatin. Collapse commenced after 15 minutes and by 30 minutes was well advanced.

Discussion

Such experiments do not give a precise indication regarding the behaviour of treated capsules in vivo. In the stomach, the capsules are treated by mechanical action as well as by acid and pepsin. The results, however, do give some indication as to the effect of treatment.

The most obvious fact is that treatment drastically retards the rate of dissolution of the gelatin in both acid pepsin and alkaline pancreatin. The effect is

most marked under acidic conditions, but is noticeable under the alkaline conditions.

The length of time for which the capsule is immersed in the formalin/alcohol mixture does have some effect upon the rate of dissolution. With only 2½ minutes treatment the capsules collapsed in 30 minutes in acidic conditions, but with more than five minutes treatment collapse did not occur for 40 - 60 minutes. Under alkaline conditions, the collapse was much more rapid, 7 - 15 minutes, but noticeably slower than for untreated capsules, 3 minutes.

One other noticeable point is that it is difficult to treat all capsules to the same extent. Within a batch one or more capsules may disintegrate more rapidly than the majority.

Conclusions

Treating gelatin capsules with formalin/ethanol does reduce their rate of collapse, particularly under acidic conditions. More than five minutes treatment does not appear to affect the rate of collapse, but with shorter treatment more rapid disintegration may occur. It is difficult to ensure that all capsules are effectively treated.

2. In vivo Experiments

Introduction

The earlier work investigated in the laboratory the effect of treating gelatin capsules with formalin in ethanol. The laboratory experiments do not indicate exactly how the capsules will behave when used in vivo.

To investigate this, greyhounds were given treated capsules containing chlorbutanol. This drug was chosen as it appears in the urine shortly after administration, and techniques were readily available for the analysis of the urine samples.

The capsule treatment was extended to 10 minutes, rather than the minimum time of 5 minutes, to try to ensure reliable performance.

Preparation and Administration of Capsules

Gelatin capsules were filled with chlorbutanol; the dose cannot be stated for security reasons. The capsules were immersed in formalin/ethanol (1 : 3) for 10 minutes and air dried. They were then administered to the greyhounds.

Samples and Analysis

Urine samples were obtained at the intervals as shown in the results.

The analysis was by means of the Fujiwara test and by gas chromatography. The procedures for these analyses has been given earlier in this work.

Results

These are shown in Table 47.

TABLE 47

Effect of Capsule Treatment

Time after Administration (hours)	Dog A		Dog B		Dog C	
	Fuji.	G.C.	Fuji.	G.C.	Fuji.	G.C.
2			+ve.	0.17		
4½	-ve.	Trace	+ve.	0.45	-ve.	0.08
7			+ve.	0.51		
8½	+ve.	0.86			+ve.	0.34
11½	+ve.	0.58			+ve.	0.43
18½			+ve.	0.51		
23	+ve.	0.56			+ve.	0.35

In this table, a +ve indicates a positive result from the Fujiwara test, and the figures are the concentration of chlorbutanol in the urine samples in mg./100 ml.

Discussion

The drug was readily detectable in the first sample obtained from one of the greyhounds. Therefore the results for that greyhound are of little value. They

do, however, indicate, as was found in the laboratory experiments, that this is not a reliable method of treating capsules.

The results for the samples from the other two greyhounds do show that coating can be effective. The drug is readily detectable, using the Fujiwara test, in about 30 minutes using untreated gelatin capsules. For these two sets of samples, the drug was not detectable by the Fujiwara test 4½ hours after administration. This suggests that the capsules were only then in the process of disintegration and the drug beginning to take effect.

Conclusions

Treatment of gelatin capsules with formalin/ethanol is a feasible way of delaying the release of a drug from the capsule. The process is simple to perform and requires only readily available reagents. The capsules can then be administered, and the drug will be released some time later. The exact delay is difficult to predict as this depends upon the time taken to pass through the stomach. This in turn depends partly upon whether the capsule was administered prior to, or just after a meal.

However, this is a feasible, if somewhat unreliable, means of circumventing the combination of

high security kennels and pre-race testing. The treatment would appear to be effective more frequently than it fails.

TOBACCO MATERIALS

VOLATILE MATERIALS

Introduction

One of the problems of using the Fujiwara reaction is the contamination of the reagents by commonly occurring laboratory reagents, particularly chloroform. If a reagent is contaminated then the only solution is to dispose of it, but the identity of the contaminant may give some indication of where the contamination arose.

The most likely contaminants were thought to be chloroform and trichloroethylene but a whole series of volatile materials was examined.

Ethyl alcohol was one of the solvents investigated and due to the wide separation from other materials it was considered that the gas chromatographic technique used would be a good substitute for the standard technique. This was investigated in conjunction with an investigation of the effect of orally administered fructose upon blood and urine ethanol levels.

Chlorinated Hydrocarbons

Introduction

Various volatile materials have been identified using distillation, or short path distillation. However, a commonly used technique today is gas chromatography (14, 60, 83, 114). This has the advantages of ease of analysis, identification of volatile components, and can supply quantitative data.

The volatile chlorinated materials have been analysed using a variety of column packings. Goldbaum and others (60) used a 6' column which was packed with a mixture of Flexol 8N8, diisodecylphthalate and polyethylene glycol 600 on C.22 firebrick at 95°C. Using this they separated chloroform, carbon tetrachloride, ethylene chloride, trichloroethylene, and tetrachloroethylene.

Bonnichsen and Maehly (14) recommended the use of two columns to separate the commonly encountered halogenated hydrocarbons. The first was a 2.5 metre aluminium column packed with 25% carbowax 1540 on ST 116 Diaport W. and used at 135°C. The second was 20% Apiezon L on chromosorb Q in a 1.8 metre stainless steel column maintained at 125°C. These columns were used to identify methylene chloride, chloroform, carbon tetrachloride, dichloroethane, trichloroethylene

and perchloroethylene.

In "The Isolation and Identification of Drugs" edited by E. G. C. Clarke, H. Leach (83) suggested conditions for the analysis for these materials as a 6' column packed with "Poropak Q" maintained at 160°C.

Discussion

There is no doubt that the most convenient technique for the identification of chlorinated hydrocarbons is gas chromatography. All the authors cited in the review employed gas chromatographs fitted with a flame ionisation detector. Since this detector is not sensitive to water, it is appropriate for the identification of these compounds in biological samples.

For the identification of these compounds in organic solvents, however, it is less suitable. Direct injection of the organic solvent will produce a massive peak, which may mask smaller peaks resulting from the chlorinated impurities.

Experimental

Introduction

Two aspects of the identification of chlorinated hydrocarbons were investigated. The first was the use of an electron capture detector in conjunction with a 5' x 1/8" glass column packed with 15% F.F.A.P. on chromosorb W as described earlier.

The second aspect was the use of other column packings. These investigated were 10% PEG 400 on chromosorb W, as used in the standard analysis technique for ethanol, and chromosorb 102.

1. Using the Electron Capture Detector

This was fitted to a Varian 1522A gas chromatograph and used in conjunction with the 5' x 1/8" glass column packed with 15% F.F.A.P. on chromosorb W.

At the normal operating temperature of 95°C chloroform and carbon tetrachloride were rapidly eluted and not resolved from the solvent, toluene. By progressively lowering the temperature, these compounds were separated. The final operating conditions were a column temperature of 55°C, an injector temperature of 125°C and a detector temperature of 155°C. The carrier gas was nitrogen flowing at 40 ml. per minute.

The chlorinated hydrocarbons were dissolved in toluene and one microlitre injections made.

Results

The retention times for a number of these compounds are given in Table 48.

TABLE 48

Chlorinated Hydrocarbons on 15% F.F.A.P. column

Compound	Relative Retention Time	
Methylene Chloride	0.45	minor peak at 0.59
Chloroform	1.00	
Carbon Tetrachloride	0.41	
Trichloroethylene	0.86	minor peak at 0.45
1,1 Dichloroethane	0.47	
1,2 Dichloroethane	0.45	

All retention times are relative to chloroform, which had a retention time of 2.0 minutes.

Discussion

The column is capable of separating these compounds to a certain extent. Methylene chloride and 1,2 dichloroethane are not resolved and 1,1 dichloroethane is rather near to these two compounds. If contaminated toluene is examined, the contaminant will be detected and a possible identity established. Further analysis under different conditions is necessary to establish the identity unequivocally.

2. Using a Flame Ionisation Detector

(i) This was fitted to a Pye 104 gas chromatograph using 5' x ¼" glass columns. The column packing normally used for ethanol analysis was investigated. This is 10% PEG 400 on chromosorb W, the column being maintained at 90°C, with the nitrogen carrier gas flowing at 60 ml./minute. The chlorinated hydrocarbons were dissolved in toluene and one microlitre injections of the solutions were made.

Results

The relative retention times of these compounds are given in Table 49, calculated relative to chloroform.

TABLE 49

Retention Times on 10% PEG 400

Compound	Relative Retention Time
Methylene chloride	0.69
Chloroform	1.00
Carbon Tetrachloride	0.54
Trichloroethylene	0.92
1,1 Dichloroethane	0.63
1, 2 Dichloroethane	1.42
Toluene	1.20

Discussion.

All the materials under investigation were satisfactorily separated for qualitative analysis using this

column under these operating conditions. However, the toluene and 1,2 dichloroethane were not completely resolved, and so this could not be used for quantitative analysis if required.

Using the 15% F.F.A.P. and 10% PEG 400 columns the identity of any one of the compounds can be established. However, it is better to use two columns which both separate all the materials. It was found that a chromosorb 102 column, although not suitable for chlorbutanol, did appear to give lengthy retention times which might be suitable for separating the chlorinated hydrocarbons.

(ii) Use of Chromosorb 102

When used in 1/8" stainless steel columns, difficulty was experienced in obtaining an adequate carrier gas flow through the column. To overcome this, the Pye 104 gas chromatograph was employed, fitted with a 5' x ¼" glass column packed with chromosorb 102 and fitted with a flame ionisation detector.

At the operating temperature of 140°C, a carrier gas flow rate of 60 ml. per minute was easily obtained. Under these conditions, the following retention times were obtained.

Relative Retention Times on Chromosorb 102

Compound	Relative Retention Time
Methylene Chloride	0.37
Chloroform	1.00
Carbon tetrachloride	1.46
Ethylene dichloride	1.39
Toluene	3.30

All these retention times are relative to chloroform.

Discussion

This column can separate the chlorinated hydrocarbons. The toluene is eluted appreciably after these compounds, rather than between members of this group, which is an advantage when analysing toluene samples.

Conclusions

By using the three columns the likely contaminants in the Fujiwara reagents can be identified. The use of the electron capture detector does facilitate their detection, and enable a probable identity to be established. By using the chromosorb 102 column this identity can be confirmed, and the PEG 400 column need only be used if further proof is necessary.

Applications

1. Toluene Sample

Introduction

A pre-race laboratory suspected that a number of positive Fujiwara results were due to a contaminated bottle of toluene. This was forwarded for analysis.

Analysis

(a) Fujiwara Test

2 ml. of the suspect toluene, 3 ml. of pyridine and 2 ml. of 10% aqueous sodium hydroxide were placed in a test tube and heated on a boiling water bath for 15 minutes.

A positive result was obtained.

(b) Gas Chromatography

1 µl. of the sample toluene was injected into the gas chromatography fitted with the 15% F.F.A.P. column operating at 55°C and using the electron capture detector.

A peak was obtained at a retention time of 1.00 relative to chloroform.

A 1 µl. injection of toluene was also made into a gas chromatograph fitted with a 10% PEG 400 packed column, operating at 90°C and fitted with a flame ionisation detector.

A peak was obtained with a retention time of 1.00 relative to chloroform.

Conclusion

The toluene sample was contaminated with chloroform. This could arise at the pre-race laboratory, or at the manufacturers'.

2. Post Mortem Sample

Introduction

A sample of blood was received for analysis. This was forwarded as there was no adequate cause of death.

Analysis

(a) Qualitative

The initial screening for volatile materials was by injection of 1 µl. of blood into a gas chromatograph fitted with the 10% PEG 400 packed column, operating at 90°C and fitted with a flame ionisation detector.

A peak with a retention time of 0.90 relative to chloroform was observed.

A further 1 µl. of blood was injected into the gas chromatograph fitted with the 15% F.F.A.P. packed column, operating at 55°C and fitted with an electron capture detector.

A peak with a retention time of 0.85 relative to chloroform was observed.

Conclusion

These two retention times identified the material present as trichloroethylene.

(b) Quantitative

This was based upon the technique outlined by Curry (35).

3.0 ml. of blood were extracted with 3.0 ml. of n-hexane. After drying, 1.5 ml. of the extract was diluted to 10 ml. with n-hexane.

1 µl. injections of this solution were made into the gas chromatograph fitted with the 15% F.F.A.P. column and electron capture detector.

A reference standard of 0.133 mg./100 ml. of trichloroethylene in n-hexane was prepared, and 1 µl. injections of this were made into the same gas chromatograph.

The peak areas were measured using a disc integrator.

Results

The trichloroethylene concentration in the blood was found to be 4.3 mg./100 ml.

Discussion

When carrying out quantitative analysis by gas chromatography, it is preferable to use an internal standard, and when using an electron capture detector

to determine the linear dynamic range. Unfortunately in this case there was insufficient time to do either.

The electron capture detector was chosen for this analysis for its selectivity. When using the flame ionisation detector in conjunction with the PEG 400 column the trichloroethylene peak was close to other peaks associated with blood. When using the electron capture detector with the F.F.A.P. column, only a peak corresponding to trichloroethylene was observed.

The dilution step in the extraction ensured that the concentration was well within the linear range. The response of these detectors in very nearly linear until the concentration is sufficient to produce a 30% drop in the cell current. The dilutions used ensured that the fall in cell current was much less than this, and thus the detector response was acceptably linear.

The value of 4.3 mg./100 ml. of trichloroethylene in blood is similar to the findings of Bonnicksen and Maehly (14) in post mortem cases. The values they found in 14 cases ranged from 0.1 mg./100 ml. of blood to 4.1 mg./100 ml.

Conclusion

The value of 4.3 mg. of trichloroethylene in 100 ml. of blood is an adequate cause of death.

Ethanol

Introduction

Ethanol is the most common of all volatile materials met in toxicology. In consequence, a great many techniques exist for its determination, and much research has been carried out into the occurrence of ethanol. All work in connection with ethanol became of greater significance following the introduction of the Road Safety Act of 1967.

Review

The older techniques all employ an oxidising agent such as dichromate in strong acid as in the Cavett method of Kent-Jones and Taylor (77) or Nickolls (97), or alternatively use acidic potassium permanganate solution (67).

All these techniques suffer from the disadvantages of lack of specificity, insensitivity, and lack of accuracy. In addition, the analyses are somewhat time consuming.

An increase in specificity was obtained by the use of an enzymatic technique employing alcohol dehydrogenase, and also an increase in sensitivity. This technique, however, is not absolutely specific for ethanol, and is subject to interference by various compounds including heavy metal ions (103) and some drugs (123). The technique is described by Brink,

Bonnichsen and Theorell (15) and a review of the oxidative and enzymatic techniques in practice is given by Bonnichsen and Lundgren (13).

Since the introduction of the Road Safety Act of 1967, however, the most commonly used technique has been that of gas chromatography. A vast number of variations of column packings and internal standards exist.

Parker, Fontan, Yee and Kirk (99) used a 10' column packed with 40% castor wax on chromosorb W at 120°C. The internal standard they used was ethyl acetate. Bonnichsen and Linturi (12) used a column of 10 grms. of 10% Carbowax 1500 on Teflon followed by 5 grms. of 20% polypropylene glycol on chromosorb at 100°C. They did not use an internal standard. Davis (40) used a Beckmann blood alcohol column at 105°C. The proteins were precipitated and the ethanol extracted using dioxan. For an internal standard Davis employed ethoxyethanol.

A comprehensive review of ethanol determination and other factors is given in "Progress in Chemical Toxicology", Volumes 1 and 3 by Harger and Forney (66) and the gas chromatographic techniques are reviewed by Anders and Mannering (5) in Volume 3 of this book.

The most commonly used gas chromatographic technique is not mentioned however. This is the technique of Curry, Walker and Simpson (36) using a 5' column packed with 10% polyethylene glycol 400 on celite at a temperature of 85°C. These authors used n-propanol as an internal standard. As far as possible the authors used automatic equipment to reduce error such as occurs during dilution and measurement of gas chromatographic peak areas. By these means the authors were able to analyse 20 µl. of blood for ethanol and the results had a standard deviation of 0.00580.

This has become the basis of the standard technique for determining the blood and urine levels for the purposes of the Road Safety Act of 1967 in this country. The only variations are different column packings to confirm the identity of the peaks resulting from a sample.

Experimental

Introduction

There were two main objectives in carrying out this work. Firstly, to find a suitable column packing to supplement the 10% PEG 400 used for the ethanol analyses. Secondly, to examine the behaviour of some volatile materials on both columns.

In view of the ability of the chromosorb 102 packing to separate volatile chlorinated hydrocarbons this was examined as a possible supplement to the PEG 400 packing.

Procedure

The packings were used in 5' x ¼" glass columns fitted in a Pye 104 gas chromatograph equipped with a flame ionisation detector.

The operating temperature for the 10% PEG 400 on chromosorb W packing was 90°C with a nitrogen carrier gas flow of 60 ml./min.

For the Chromosorb 102 packing, the operating temperature was 140°C with nitrogen carrier gas flow of 60 ml./minute.

The volatile materials were dissolved or suspended in 20 mg./100 ml. n-propanol solution and 1 µl. injections made.

Results

TABLE 50

Relative Retention Times of some Volatile Materials

Material	PEG 400	Chromosorb 102
n-Hexane	0.18	0.50
Pet. Ether 40-60	0.19	0.50, 1.17
Pet. Ether 60-80	0.19, 0.51	0.50, 1.17, 3.41
Hexane Fraction	0.20	0.51, 1.57, 3.49
Diethyl Ether	0.21	0.54
Di-isopropyl ether	0.21	1.82
n-Heptane	0.23	4.24
Cyclohexane	0.24	2.86
Carbon Tetrachloride	0.35	3.12
Acetone	0.35	0.54
Xylene	0.36, 0.79, 1.24, 1.37	-
1, 1, Dichloroethane	0.40	-
Ethyl acetate	0.42	1.71
Methylene chloride	0.44	0.80
Ethyl Methyl Ketone	0.47	1.63
Benzene	0.53	3.47
Trichloroethylene	0.59	-
Methanol	0.59	0.26
i-Propanol	0.60	0.53
Ethanol	0.62	0.50
Chloroform	0.64	2.14
Chloral hydrate	0.75	-
Trichloroacetic Acid	0.77	-
Toluene	0.77	7.06
1, 2 Dichloroethane	0.91	-
n-Butyl acetate	0.94	9.67

Table 50 (continued)

Material	PEG 400	Chromosorb 102
Dioxan	1.00	4.68
2, 2, 2 Trichloro-ethanol	1.35	-
Acetic Acid	1.63	1.31
n-Butanol	1.70	2.80
Pyridine	2.30	6.61
Amyl alcohol	2.36	5.56
i-Butyric Acid	2.49	5.78
Ethchlorvynol	4.5	-
Amyl acetate	3.17	-
n-Butyric Acid	not eluted	8.09

All the retention times are relative to n-propanol.

Discussion

On both columns the peaks were symmetrical and sharp.

By using both columns, the identity of each of these materials can be established. One important factor in favour of using the chromosorb 102 packing for routine ethanol determinations is that it separates i-propanol from ethanol. The PEG 400 packing does not do this.

Conclusion

For routine ethanol determinations, chromosorb 102 is superior to PEG 400. By using both column packings the identity of a range of volatile materials can be

established.

Application

Both are used routinely for the analysis of samples taken in connection with the Road Safety Act and either may be used for the quantitative estimation of the ethanol. The importance of using two columns is illustrated in the following example.

A blood sample was forwarded for analysis, and gave a high value for what appeared to be ethanol when analysed on the PEG 400 column. On checking on the chromosorb 102 column two peaks were observed. The final values obtained were 12 mg./100 ml. of ethanol and 160 mg./100 ml. of iso-propanol.

Discussion

The chromosorb 102 column is an entirely satisfactory column packing material to complement the use of PEG 400. The necessity for this is demonstrated in the example cited; if the chromosorb 102 column had not been used, the ethanol concentration reported would have been above that permitted in the Road Safety Act.

The column also satisfactorily separates a range of volatile compounds. Using both column packings, these volatile materials can be identified. Acetone

has been found in a number of post mortem cases.

Ethanol Determination

Introduction

A column giving adequate separation of the materials under study may not be suitable for routine use however. It may be found that after a short while the separation and peak shapes deteriorate due to an accumulation of non-volatile residues on the column. This does not happen with a PEG 400 column until a large number of injections have been made, and when it does occur is readily corrected.

The endurance of the column was investigated concurrently with an investigation into the oral administration of fructose.

Fructose

Review

Investigations by Tygstrup, Winkler and Lundquist (129) showed that intravenous infusion of fructose and ethanol increased the blood flow to the liver. This resulted in an increased rate of disappearance of ethanol from the blood. When fructose or ethanol were administered alone no increase in liver blood flow was observed. The inference of this work is that fructose will lower the blood alcohol level.

This work was the basis for several firms producing fructose to be taken orally, during or after drinking to avoid contravening the Road Safety Act. An investigation by Camps and Robinson (27) concludes that this was not likely to affect blood alcohol levels significantly though certain of their results indicated some effect. In view of the conflicting evidence an experiment was carried out to provide more information on the subject.

Establishment

A number of volunteers were to consume 6 fluid ounces of 70° Proof spirit as rapidly as possible within 15 minutes on each of two successive days. On one day, 20 grms. of fructose dissolved in a little water were to be consumed 2 hours after the start of the experiment. Blood samples were obtained by capillary puncture at 15 minute intervals, starting after 1 hour, and urine samples at 30 minute intervals also starting after 1 hour. Thus, each volunteer would act as his own control. If the fructose did have an effect, this would be shown by a dip in the blood and urine level decline curves.

Analysis

Approximately 20 µl. of each sample were diluted with about 200 µl. of 10 mg./100 ml. aqueous

n-propanol solution using a "Griffin Diluspence". One microlitre of the resulting solution was then injected into a Pye 104 gas chromatograph fitted with a 5' x ¼" glass column packed with chromosorb 102 and maintained at 140°C. The estimation of concentrations was by comparison with a standard ethanol solution by means of peak areas as measured by a Kent "Chromalog 1" digital integrator.

Results

The results are shown plotted on Graphs 13 to 18. As can be seen from these results, on occasions urine samples were not available.

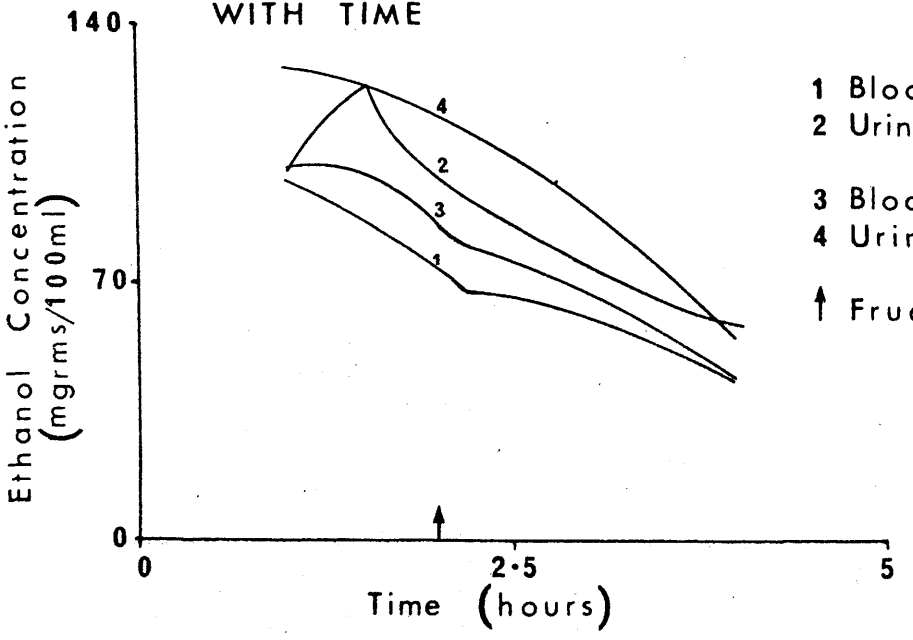
Discussion

In no case is there a pronounced deviation from the steady decline curves for the ethanol concentration. This leads to the conclusion that oral fructose does not affect the metabolism of ethanol. In conclusion, the observation of Camps and Robinson that fructose "is unlikely to be of value to the apparently healthy social drinker who wishes to drive home after an evening's entertainment without infringing the law" is the only reasonable one.

This investigation involved the injection of about 1000 samples into the chromosorb 102 column over

GRAPH 13

CHANGE OF ETHANOL CONCENTRATION WITH TIME



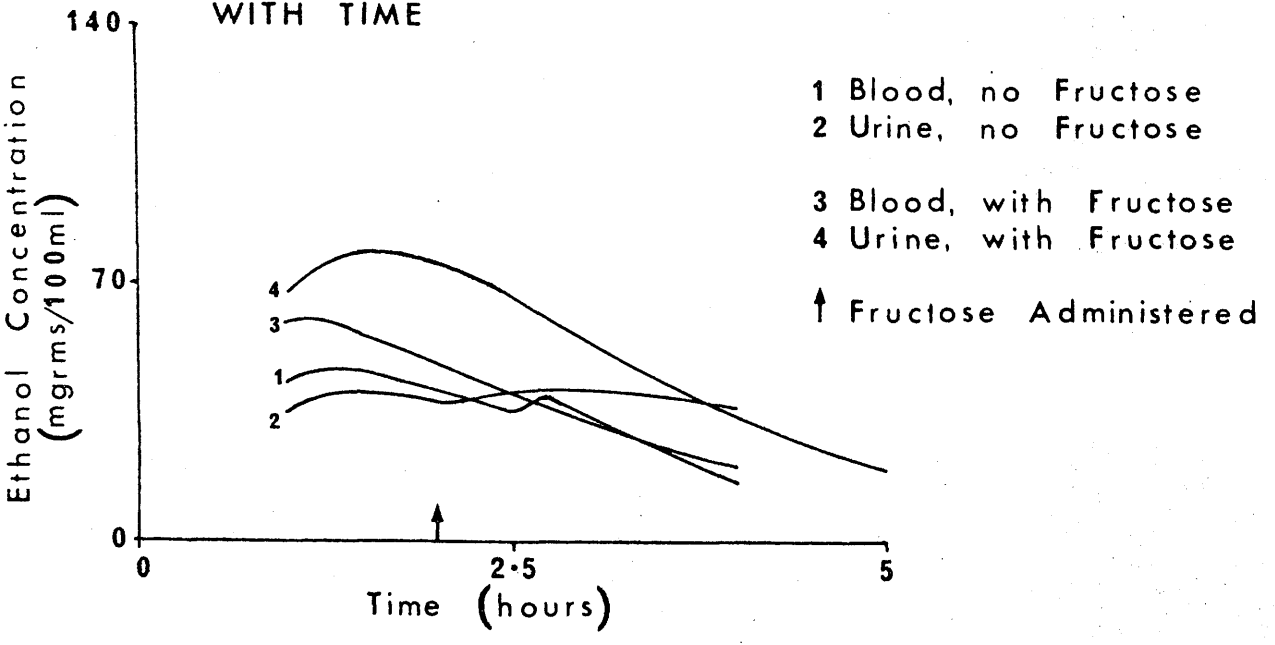
1 Blood, no Fructose
2 Urine, no Fructose

3 Blood, with Fructose
4 Urine, with Fructose

↑ Fructose Administered

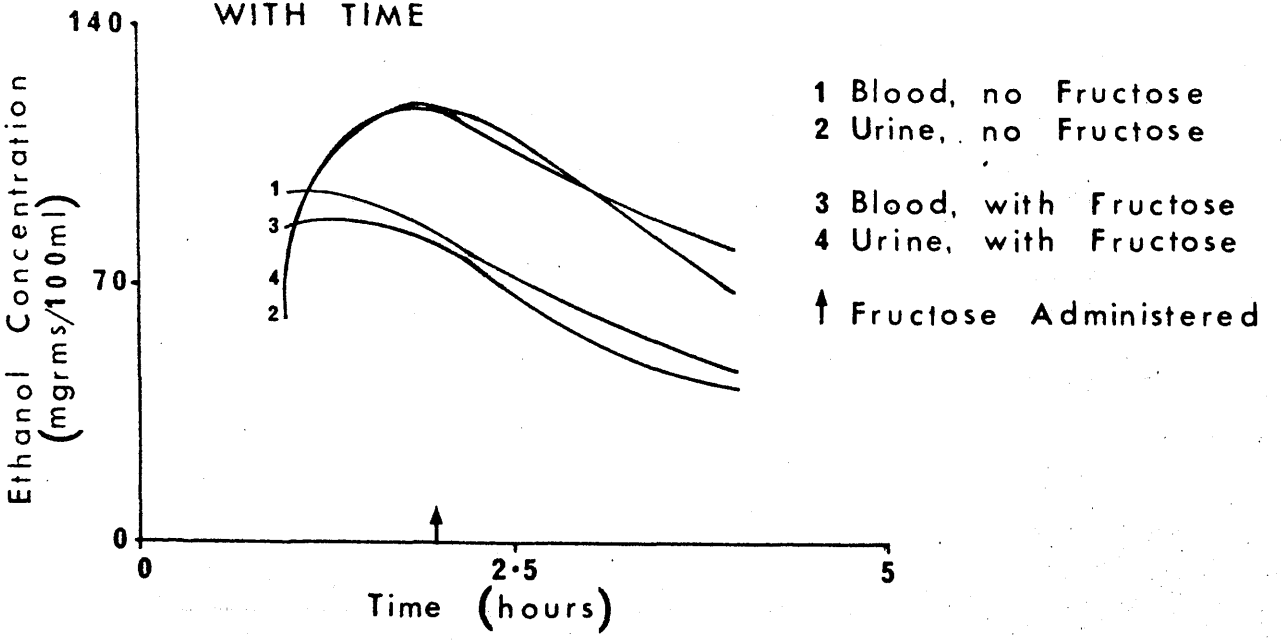
GRAPH 14

CHANGE OF ETHANOL CONCENTRATION WITH TIME



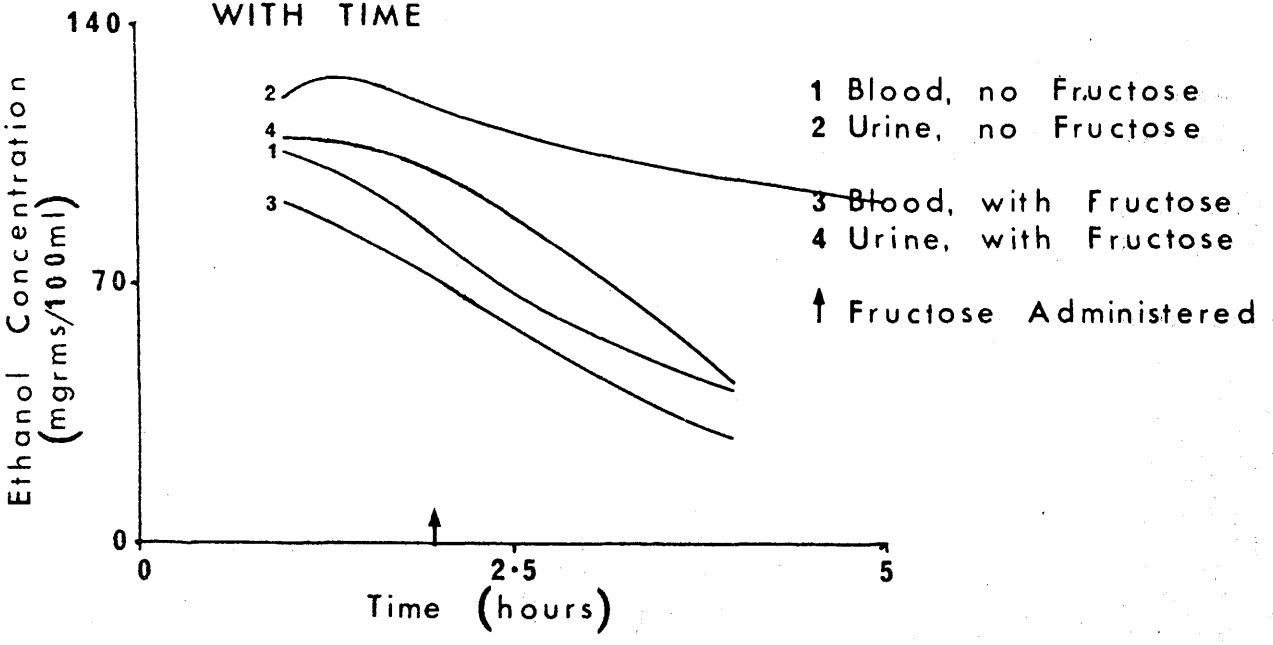
GRAPH 15

CHANGE OF ETHANOL CONCENTRATION WITH TIME



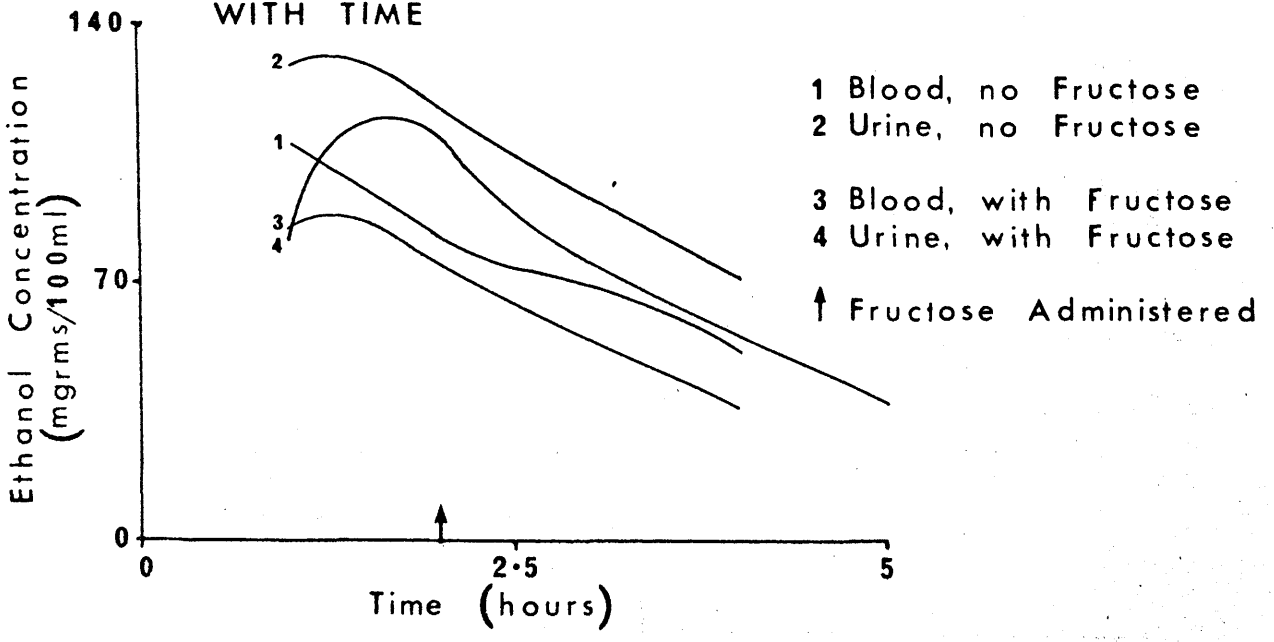
GRAPH 16

CHANGE OF ETHANOL CONCENTRATION WITH TIME

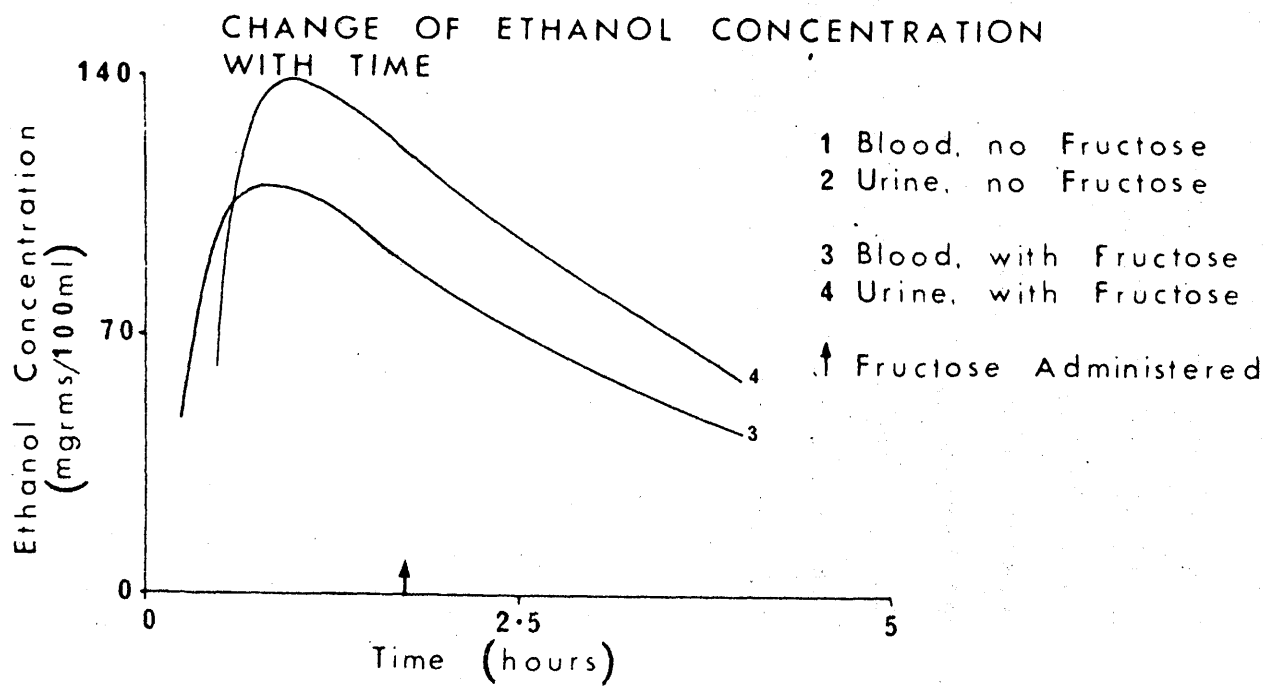


GRAPH 17

CHANGE OF ETHANOL CONCENTRATION WITH TIME



GRAPH 18


CHANGE OF ETHANOL CONCENTRATION WITH TIME
140
70
0
Ethanol Concentration (mgrms/100ml)
0
2·5
5
Time (hours)
4
3
1 Blood, no Fructose
2 Urine, no Fructose
3 Blood, with Fructose
4 Urine, with Fructose
Fructose Administered

a period of 10 working days. There was no deterioration in column performance during this period.

Following this work the chromosorb 102 column was used routinely for analysis of samples for ethanol and other volatile materials. During routine use the first few inches of the column had to be repacked after about 2,500 injections. The column packing is thus as long lived as the PEG 400 column packing and provides a better separation of most of the volatile materials. However, the analysis for ethanol does take somewhat longer.

General Conclusions

Using Chromosorb 102 and 10% PEG 400 on Chromosorb W packed columns, the identity of a number of volatile materials can be established. This includes volatile chlorinated hydrocarbons, which can also be separated by using 15% F.F.A.P. on Chromosorb W. When the chlorinated hydrocarbon was in an organic solvent the use of the F.F.A.P. column in conjunction with an electron capture detector was found to be very useful.

For routine determination of ethanol, the Chromosorb 102 packing was found to be satisfactory. It is superior to the PEG 400 packing in that it will separate i-propanol from ethanol.

MISCELLANEOUS DRUGS

(1) PHENYL BUTAZONE

(2) STEROIDS

Introduction

The earlier work in this thesis was concerned mainly with the detection of chlorinated drugs, and to a lesser extent with phenobarbitone. These are not the only drugs encountered in connection with racing greyhounds.

A number of other drugs such as methaqualone and chlorpromazine have been detected in urine samples from time to time. However, work in connection with these drugs has been confined to their detection and identification in the samples provided.

Quite a number of other drugs have a possible application to the drugging of racing greyhounds, but have never been detected. This may be because they are not used, or because they are not detected with the procedures used.

Preliminary investigations into the detection of two types of drug were carried out. These were phenylbutazone and contraceptive steroids.

Phenyl Butazone (Butazolidin)

Review

This is a drug acting upon the central nervous system, having an anti-inflammatory action.

After administration of the drug to man, two metabolites are rapidly formed, oxyphenbutazone and hydroxyphenbutazone (63). The plasma levels of phenyl butazone and of oxyphenbutazone fall only slowly while the level of hydroxphenbutazone falls much more rapidly. Less than 1% of the drug is excreted unchanged in the urine after administration to man (64). The drug and the metabolites are protein bound, and unless the proteins are denatured, solvent extraction only gives a recovery of 60% (22).

The extraction procedure is generally similar to that used for the barbiturate drugs. This involves solvent extraction from acidic solution, washing the organic phase with dilute sodium bicarbonate solution, and extraction into sodium hydroxide solution (30). The bicarbonate wash removes strong acids, such as salicylates, but Curry (35a) reports that the hydroxyphenbutazone distributes equally between the aqueous and organic phases.

The sodium hydroxide solution of the drug can be used for examination by ultra-violet spectrophotometry.

Phenyl butazone has an absorption maximum at 265 mμ which may interfere in the determination of barbiturates (23).

According to Curry (35a) the metabolites have an absorption maximum at 253 mμ.

Phenylbutazone can also be detected using thin layer chromatography. The plates are coated with silica gel G and developed in a solvent consisting of 3% methanol in chloroform. The spots are developed by spraying with dilute household bleach, phenol solution, and starch/potassium iodide solution (109).

Experimental

Introduction

The drug itself can be readily extracted from acidic solution using an organic solvent. The concentration of pure drug in the urine is very low. The metabolites are also extracted under these conditions, but loss of some of the p-hydroxy metabolite may occur if a sodium bicarbonate wash is included in the extraction procedure.

The ultraviolet spectrum is readily determined. The only complication that can arise is if simultaneous determination of a barbiturate drug is required. The method of Burns et al. uses 2.5N sodium hydroxide; if possible, this could be more conveniently replaced by

0.45N sodium hydroxide. The extraction procedure would then be the same as the standard technique for barbiturate drugs.

The chromatographic procedure is less satisfactory. The solvent is not unusual, but the spray reagent is rather complex. An alternative spray, potassium permanganate, is recommended by E. G. C. Clarke (30). This spray reacts with a number of materials normally occurring in biological samples and may therefore give a false positive.

Since phenylbutazone and its metabolites are required to be included in the pre-race analysis techniques, it was decided to find out if they could be included in the present scheme of analysis. The following experiments were made with this aim in view.

Assessment of the frequently used spray reagents in the detection of Phenylbutazone

Introduction

Two spray reagents are in frequent use, saturated aqueous mercurous nitrate, for barbiturate drugs, and acidified iodoplatinate for the detection of alkaloid drugs. The preparation of these reagents is given below. These were tested for possible application to the detection of phenylbutazone and its metabolites.

Reagents

Mercurous Nitrate

A saturated aqueous solution is prepared in the spray bottle and crystals of mercurous nitrate are present in the bottle.

Iodoplatinate Spray

This is prepared by dissolving 1 grm. of chloroplatinic acid in 10 ml. of water. To this is added a solution of 10 grms. of potassium iodide in 250 ml., and making up to 500 ml. with a solution of 144 ml. of hydrochloric acid in 96 ml. of water.

Procedure

Spots of phenylbutazone and of phenobarbitone dissolved in chloroform were placed on a thin layer chromatography plate and sprayed with each of these reagents.

Results

Mercurous Nitrate Spray

Both phenylbutazone and phenobarbitone gave a black spot on a white background.

Acidified Iodoplatinate Spray

Phenylbutazone gave a brown spot on a pinkish background. Phenobarbitone did not react.

Conclusion

Phenylbutazone can be detected using either of

these spray reagents. If the drug runs on the plate with the solvent used for the barbiturate drugs it can be detected simultaneously.

Assessment of present Thin Layer Chromatography techniques in the analysis of Phenylbutazone

Introduction

Two solvents are in common use, ethyl acetate and chloroform/Acetone 9 : 1. The ethyl acetate does not separate the different barbiturates, but is fast running and separates the drugs from their metabolites. It is suitable for rapid screening such as that carried out in the pre-race laboratories. The chloroform/ acetone solvent separates the different barbiturates giving a guide to their identity but is much slower running. These solvents were tested for possible application to the detection of phenylbutazone and its metabolites.

Procedure

The plates were prepared by spreading a 0.25 mm. layer of MN Keiselgel G (30 grms.) in water (60 ml.) on glass plates. The plates were air dried for 15 minutes and activated at 110^{o}C for 30 minutes, and then stored over silica gel in a desiccator until use.

The solvents were placed in cylindrical tanks

lined for half their circumference with filter paper, and left for 45 minutes before use to equilibrate.

Spots of a dilute solution of the drugs in chloroform were transferred to the plates using a piece of capillary tubing.

Results

Ethylacetate

Phenobarbitone gave a black spot Rf 0.70

Phenylbutazone gave a black spot Rf 0.70

Chloroform/Acetone

Phenobarbitone gave a black spot Rf 0.40

Phenylbutazone gave a black spot Rf 0.75

Conclusion

If enough pure drug is excreted, the existing techniques in both the pre-race and departmental laboratories will detect it. The lack of separation from barbiturate drugs using the ethyl acetate as a solvent is an advantage. The operators merely have to regard a black spot about Rf 0.70 as a positive reaction. This places no onus upon them to interpret the results. Using the chloroform/acetone solvent the separation from barbiturates is sufficient to give an indication of the identity of the drug present.

Practical application of the tests to urine from dogs treated with phenylbutazone

Introduction

These tests involved the administration of phenylbutazone to a greyhound, a total dose of 400 mg. being administered. Samples were obtained after 3 hours, 6½ hours and 17½ hours.

Thin Layer Chromatography Procedure

The extraction procedure was that used in the pre-race laboratories. 10 ml. of sample were acidified with dilute hydrochloric acid and extracted with 40 ml. of ethyl acetate. The urine was discarded, the ethyl acetate washed with 5 ml. of 2.5% aqueous sodium bicarbonate, and then extracted with 5 ml. of 10% aqueous sodium hydroxide. The ethyl acetate was discarded, and the aqueous phase acidified and extracted with 40 ml. of ethyl acetate. The ethyl acetate was dried and evaporated, and the residue spotted on a thin layer chromatography plate. The plates were run in the ethyl acetate and the chloroform/acetone (9 : 1) solvents and sprayed with saturated mercurous nitrate solution.

Results

The results are shown in Table 51.

TABLE 51.

Analysis of Urine Samples
using Thin Layer Chromatography

Time sample taken	Ethyl Acetate solvent	Chloroform/Acetone solvent
3 hrs	negative	negative
6½ hrs	Black spot Rf 0.35	Dark spot Rf 0.15
17½ hrs	Black spot Rf 0.35	Black spot Rf 0.15
Phenylbutazone	0.70	0.75
Phenobarbitone	0.70	-

Conclusions

No phenylbutazone was detected in the urine samples, but spots appeared, probably due to metabolites. These were not detectable until at least 3 hours after administration of the drug. The Rf values in the two solvents give an indication that the spots are due to phenylbutazone metabolites and are distinct from barbiturate drugs.

Ultra-violet Spectrophotometry investigation of urine sample from dogs treated with phenylbutazone

Introduction

These experiments were carried out to see if the metabolites could be characterised using ultra-violet spectrophotometry in the concentrations found, and to

see if the standard quantitative barbiturate procedure is suitable.

Procedure

(a) 5 ml. of urine were acidified with hydrochloric acid and extracted with 40 ml. of chloroform. The urine was discarded and the chloroform washed with 5 ml. of 2.5% aqueous sodium bicarbonate solution. The chloroform was then extracted with 5 ml. of aqueous 0.45N sodium hydroxide solution, and then discarded.

The aqueous phase was then run on the ultra-violet spectrophotometer as for quantitative barbiturates, that is in 0.45N sodium hydroxide at pH 14 and in a mixture of borate buffer and sodium hydroxide at pH 10.

Results

Sample 1 showed a small peak at about 265 mμ at pH 10 and pH 14.

Sample 2 showed a peak, absorbance 1.24 at 263 mμ. There was no shift of the maximum with pH change.

Sample 3 showed a peak, absorbance 0.95 after a five times dilution, at 263 mμ. There was no shift of the maximum with pH change.

Discussion

As was expected from the thin layer chromatography results samples 2 and 3 contained more of the drug or its metabolites than sample 1. The absorptions found indicate that the samples contain a great deal of the drug or its metabolites, and therefore they should be readily detectable.

There is no indication of a peak around 253 mμ, which corresponds to the p-hydroxy metabolite, oxyphenbutazone. As there is no pure drug detectable by thin layer chromatography, the absorption must be due to the w-hydroxymetabolite. Hydroxylation of the butyl side chain would not affect, appreciably, the absorption maximum, so it is probable that in the greyhound the principle metabolite is w-hydroxy phenylbutazone.

Conclusion

Appreciable amounts of the metabolite are extracted from the chloroform into the sodium hydroxide solution, but as there was some doubt as to whether this was complete, the following experiments were made.

The extraction of Phenylbutazone and metabolites from urine

Procedure

A chloroform extract of 10 ml. of urine was

prepared as described above. After the sodium bicarbonate wash, the chloroform was evaporated and the residue dissolved in 5 ml. of 0.45N sodium hydroxide solution and the ultraviolet spectra prepared as described earlier.

Results

Sample 2 gave an absorption of 1.42 at 263 mμ after dilution by 50%.

Sample 3 gave an absorption of 1.84 at 263 mμ after dilution by eight times.

Discussion

After correction for the different volume of sample taken and the different dilutions required , these results indicate that the extraction of the chloroform with 0.45N sodium hydroxide solution recovers 85% of the w-hydroxy phenylbutazone present.

Conclusions

In the greyhound, phenylbutazone gives rise to two metabolites, the principle one appears to be w-1-hydroxy phenylbutazone. On extraction using the quantitative barbiturate technique, 85% of this metabolite is extracted from the chloroform into the sodium hydroxide phase. The metabolite gives an absorption at 263 mμ which is independent of pH.

General Conclusions

The drug and its metabolites can be detected by means of thin layer chromatography using either saturated mercurous nitrate solution or acidified iodoplatinate as spray reagents. No pure drug is detectable in the urine samples, but the metabolites can be differentiated from barbiturates using the chloroform/acetone solvent system.

When carrying out measurements using ultra-violet spectrophotometry, the extraction of the chloroform with 0.45N aqueous sodium hydroxide does not recover all the metabolite present. The only absorption maximum observed was at 263, indicating that the principal metabolite is w-hydroxy phenylbutazone.

Steroids

Introduction

Due to the possible clinical implications of the blood plasma levels a great deal of work has been carried out in connection with steroids, and a large number of such compounds identified. This work is not concerned with normally occurring steroids, merely those used for contraceptive purposes. These are of interest as they may be used to alter the normal biological rhythms of the female greyhound which affect the greyhound's performance.

Any test for these hormones must be capable of being incorporated in the pre-race testing procedure if the results are to be of any value. This imposes a severe restriction upon the choice of techniques because they must be speedy and employ only simple equipment. In addition, the tests must give unambiguous results free from interference by normal body products.

Review

The vast interest in steroids has produced a proportionately large literature. The techniques which have been employed include colourimetry (126), column and paper chromatography (39), thin layer (108,

120) and gas chromatography (17).

The most popular technique is gas chromatography, using either the steroids (7, 87, 96) or derivatives (52, 82, 125). This is understandable as gas chromatographic techniques are sensitive, have good resolution of components and can be quantitative. However, it involves expensive equipment, and generally rather lengthy sample preparation procedures.

The colourimetric techniques lack specificity, and so the choice of techniques is limited to paper or thin layer chromatography. The spray reagents used are generally corrosive (104) and so thin layer chromatography is to be preferred, particularly as it is the faster running of the two techniques.

Thin layer chromatography has been used to separate steroid fractions for subsequent analysis by other techniques (52, 82, 125) and to a lesser extent as an analytical technique in its own right. Kay (74) used one dimensional thin layer chromatography to examine plasma extracts, as did Noiret (98) but more commonly two dimensional techniques are employed, improving resolution (85, 108, 115, 120).

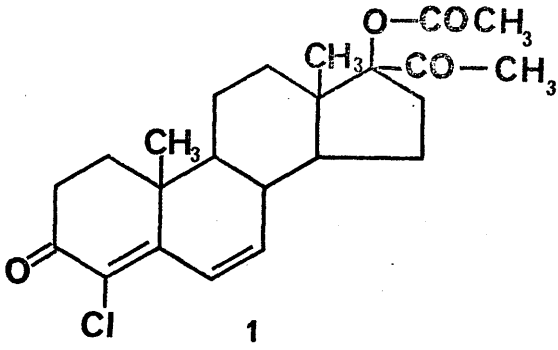
The solvents used in these thin layer chromatographic systems are mixtures of common organic solvent

such as benzene, cyclohexane, ethyl acetate and chloroform. An excellent summary of some of these solvents employed in one dimensional systems is given by Randerath (104) and no doubt could be applied to two dimensional systems also. Keay (76), who was working on the separation of gestogens in oral contraceptives employed a one dimensional system using as solvent a 1 : 1 mixture of cyclohexane and ethyl acetate. The steroids were located by use of an antimony trichloride spray and by means of ultra-violet light.

All the above techniques require prior sample preparation. This is normally by means of extraction with an immiscible organic solvent, diethyl ether being a popular choice (for example 120, 125), but other solvents have been employed such as pyridinium sulphates (74).

FIGURE 13

1. Chlormadinone Acetate
2. Cholesterol
3. Dehydroepiandrosterone
4. Dimethisterone
5. Ethinyloestradiol
6. Ethisterone
7. Ethynodiol Diacetate
8. Lynoestrenol
9. Medroxyprogesterone Acetate
10. Megestrol Acetate
11. Mestanolone
12. Mestranol
13. Methyl Testosterone
14. Norethisterone
15. Norethynodrel
16. Oestradiol
17. Oestradiol Benzoate
18. Oestriol
19. Oestrone
20. Prednisolone
21. Pregnenolone
22. Progesterone



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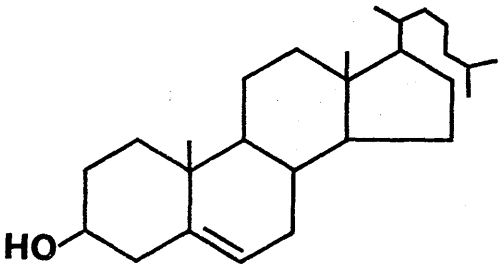

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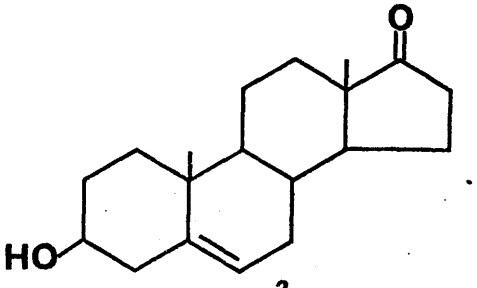

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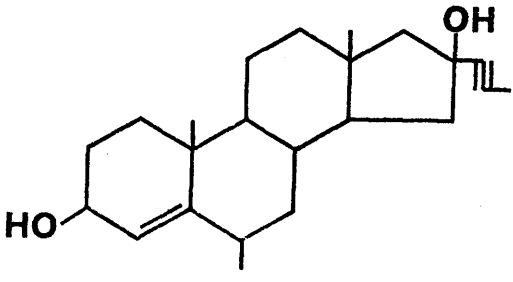

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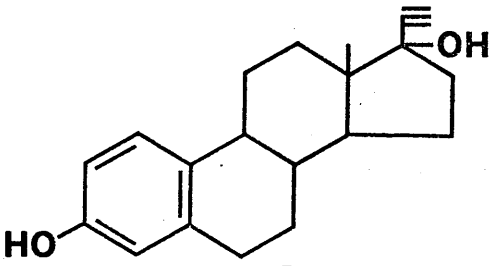

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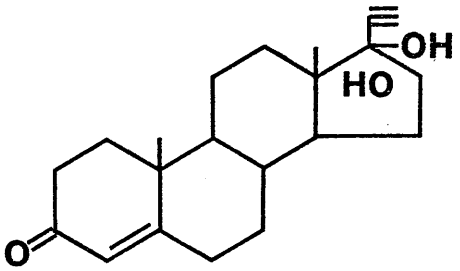

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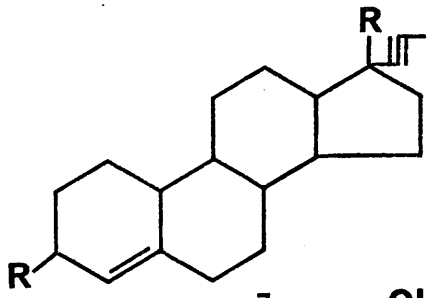

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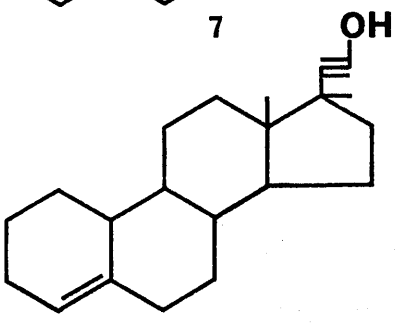
$R = O \cdot CO \cdot CH_3$



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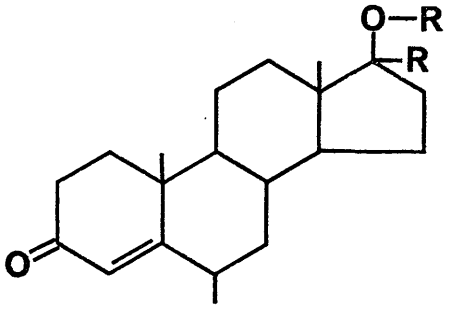

R═ CH_3·CO—

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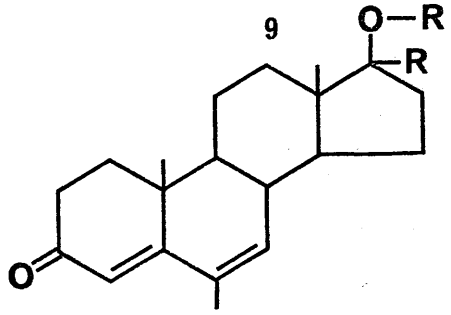

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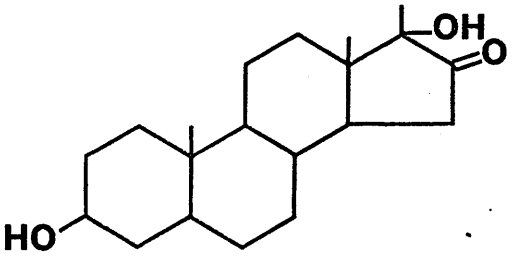

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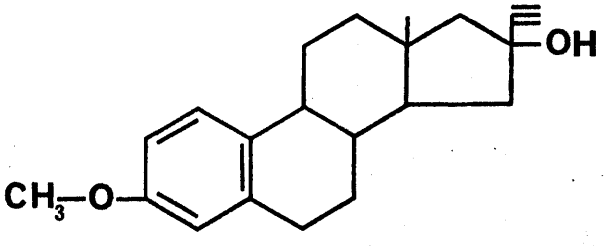

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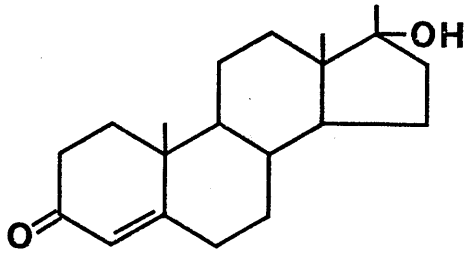

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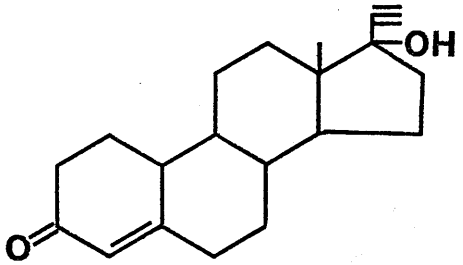

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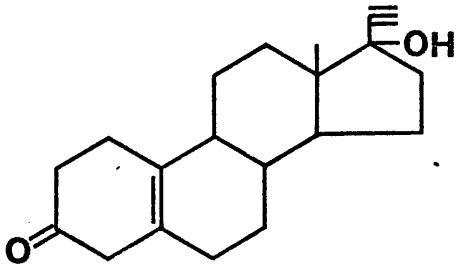

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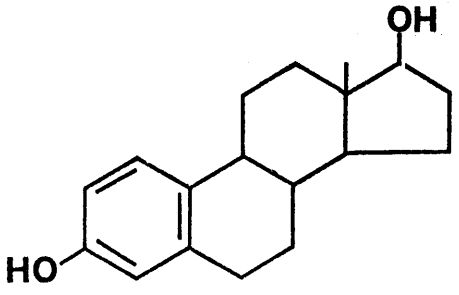

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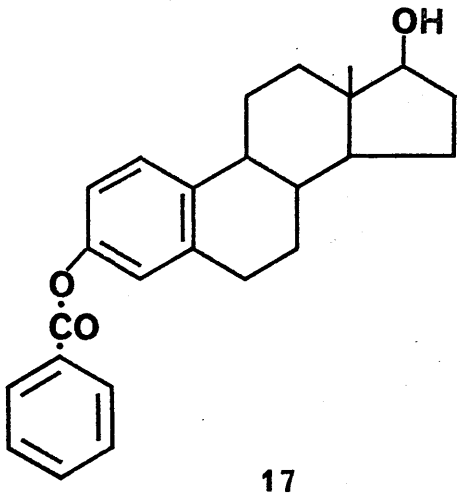

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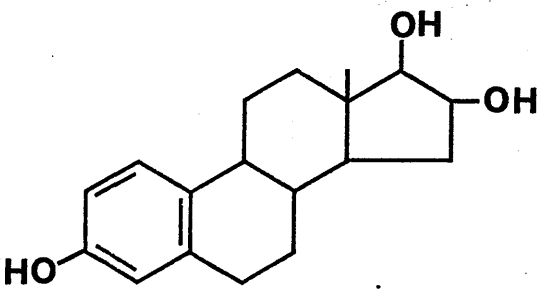

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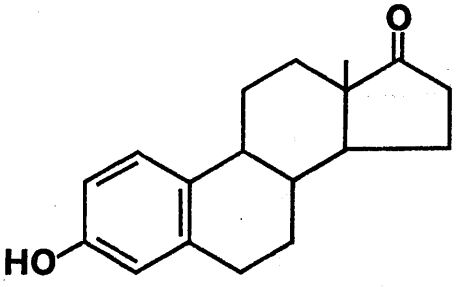

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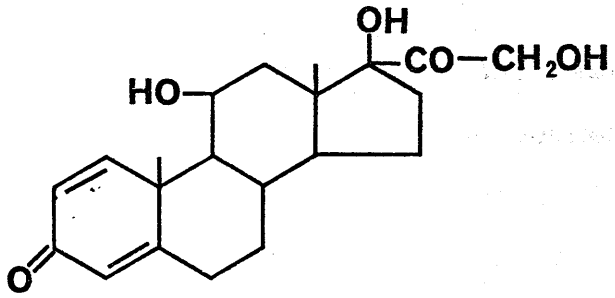

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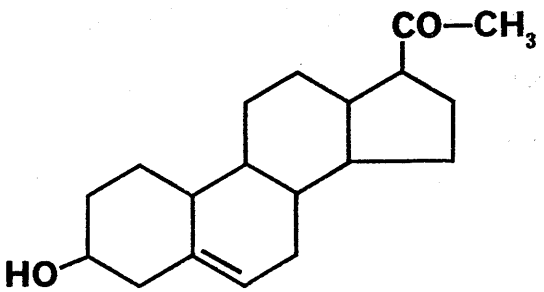

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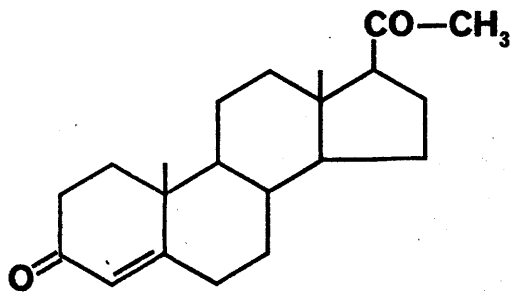


22

Experimental

Introduction

The preparation of the sample by means of solvent extraction is a technique commonly used in the pre-race laboratories, and so presents little difficulty. The use of diethyl ether, however, is undesirable due to the fire hazard.

Due to the limitations of equipment and time the analysis must be by means of one dimensional thin layer chromatography. For this a suitable solvent will have to be selected and also a suitable spray reagent to locate the steroids on the plates.

Materials

These were samples of the nineteen commonly used steroids, supplied by various manufacturers. The basic steroid structure and the modifications of it used in these hormones are given in Figure 13.

Location Reagents

The spray reagents used were those described by Randerath (104), who does not list the reactions with all the steroids under consideration. The reagents are prepared as follows.

(a) Antimony Trichloride

This consisted of 10 grms. of antimony trichloride

dissolved in 30 grms. of chloroform. In use the plates were inspected immediately after spraying, after heating at 120°C for 10 minutes, and under ultra-violet light after the heating.

(b) Antimony Pentachloride

This was prepared by dissolving 10 grams of antimony pentachloride in 40 grams of carbon tetra-chloride. The plates were inspected immediately after spraying, and after heating at 120°C for 20 minutes.

(c) Anisaldehyde

This was prepared by adding 0.5 ml. of anisaldehyde to 50 ml. of glacial acetic acid and then adding 1 ml. of concentrated sulphuric acid. The plates were sprayed and then inspected after heating at 120°C for 5 minutes.

(d) Morin

This was prepared by dissolving 0.01 grams of morin in 100 ml. of methanol. The plates were sprayed, and observed under ultra-violet light, after standing for 5 minutes at room temperature.

(e) Perchloric Acid

This was a 2% aqueous solution. After spraying the plates were heated at 120°C for 15 minutes and then inspected.

(f) Phosphoric Acid

This was a 50% aqueous solution. The plates were inspected immediately after spraying and again after heating at 120°C for 15 minutes.

(g) Sulphuric Acid

The plates were sprayed with concentrated sulphuric acid and heated at 120°C for 10 minutes. They were then inspected under daylight and under ultra-violet light.

(h) 2,3,5, Triphenyltetrazolium chloride (T.T.C.Reagent)

This was prepared as a 4% solution in methanol. Immediately before use equal volumes of this solution and of 4% methanolic sodium hydroxide solution were mixed. The plates were sprayed and heated at 80°C for 30 minutes and inspected under daylight.

(i) Vanillin

This was prepared as a 1% solution in 50% phosphoric acid. After spraying the plates were heated at 120°C for 15 minutes and then inspected by daylight.

Procedure

Dilute solutions (about 0.5% w/v) of each of the drugs in chloroform were prepared. Each solution was spotted five times on a thin layer chromatography plate consisting of a 0.25 mm. layer of MN-Keiselgel G on

glass. The plates were sprayed with the reagent and inspected and treated as described earlier.

Results

These are given in Table 52. In this table, "N.D." means that the material was not detected. When using the T.T.C. reagent, the plates required heating for about 30 minutes; the colours were slow to develop.

Discussion

To be useful for this application, the spray reagent must detect all the compounds under consideration. Therefore the reagents, morin, perchloric acid, T.T.C. and vanillin are unsuitable.

For convenience, it is also better that the spot location does not require the use of ultra-violet light, and that the heating time should be as short as possible. If ultra-violet is not to be used then the antimony trichloride spray reagent is unsuitable.

This restricts the choice of spray reagents to anisaldehyde, phosphoric acid or sulphuric acid. The anisaldehyde requires the shortest heating time, but sulphuric acid is a more widely available reagent. Phosphoric acid is usable, but the other two reagents

TABLE 52

Key to Colours

Y	-	Yellow
O	-	Orange
R	-	Red
P	-	Purple
B	-	Blue
Bl	-	Black
Br	-	Brown
Gy	-	Grey
K	-	Khaki
D	-	Dark
G	-	Green
M	-	Mauve
N.D.	-	Not detected
+ve	-	Positive

TABLE 52

Steroid Spray Colour Reactions

Spray Steroid	a_1	a_2	a_3	b_1	b_2	c	d
Cholesterol	B	P	Br	Br	Bl	P	+ve
Chlormadinone Acetate	N.D.	N.D.	D.B.	N.D.	Bl	B	N.D.
Dehydroepiandro-sterone	Y	Br	Br	R	Bl	P	+ve
Dimethisterone	Br.	Gy	Y	Br	Y	Bl	+ve
Ethinyl oestradiol	N.D.	M	D	Bl	P	P	+ve
Ethisterone	N.D.	K	O	Br	P	P	+ve
Ethynodiol diacetate	R	Bl	D	P	P	Bl	+ve
Lynestranol	N.D.	P	R	Bl	Bl	Bl	+ve
Medroxy Progesterone acetate	N.D.	N.D.	B	N.D.	Br	Bl	+ve
Megesterol Acetate	Y	O	B	N.D.	Bl	O	N.D.
Mestanolone	N.D.	N.D.	P	P	P	O	+ve
Mestranol	N.D.	M	R	Bl	B	P	+ve
Methyl Testosterone	N.D.	N.D.	Y	P	Br	O	+ve
Norethynodrel	N.D.	P	R	G	Bl	P	+ve
Oestriol	N.D.	N.D.	B	N.D.	P	P	N.D.
Oestrone	N.D.	R	P	O	P	P	+ve
Prednisolone	N.D.	N.D.	D	N.D.	P	P	N.D.
Pregnenolone	B	Gy	B	P	P	P	+ve
Progesterone	N.D.	Y	B	N.D.	Br.	O	+ve
Oestradiol-mono benzoate	N.D.	N.D.	G	O	R	Bl	+ve

Column headings refer to spray reagents previously described on pages 385, 386, 387.

TABLE 52 (continued)

Steroid Spray Colour Reactions

Spray Steroids	e	f_1	f_2	g_1	g_2	h	i
Cholesterol	P	Br	Br	R	O	N.D.	Br
Chlormadinone Acetate	B	B	B	B	D	Y	B
Dehydroepiandro-sterone	Bl	M	Bl	Bl	D	O	P
Dimethisterone	Gy	Br	Bl	P	D	R	Bl
Ethinyl oestradiol	P	Br	R	R	D	N.D.	R
Ethisterone	Bl	Br	Gy	Bl	D	R	Br
Ethynodiol diacetate	Br	G	Br	Br	D	R	Br
Lynestranol	P	O	Br	Br	O	R	P
Medroxy Progesterone acetate	Bl	N.D.	B	Br	D	O	Bl
Megesterol Acetate	R	Y	Y	Bl	D	R	Gy
Mestanolone	O	G	Y	Br	Br	N.D.	Y
Mestranol	R	Y	R	R	R	N.D.	R
Methyl Testosterone	O	G	K	Br	O	R	K
Norethynodrel	P	Br	R	P	Br	O	Y
Oestriol	N.D.	P	R	R	D	N.D.	N.D.
Oestrone	O	Y	O	R	D	R	R
Prednisolone	P	G	P	Br	D	R	Bl
Pregnenolone	P	B	Bl	Bl	D	N.D.	Br
Progesterone	Br	G	G	Br	O	R	Y
Oestradiol-mono benzoate	O	Y	Y	R	O	N.D.	R

Column headings refer to spray reagents previously described on pages 385, 386 and 387.

are preferable on grounds of availability and heating time.

Conclusions

The plates can be sprayed with the anisaldehyde reagent, sulphuric acid, or phosphoric acid. Due to its more widespread availability sulphuric acid is the reagent which would be used in the pre-race laboratories.

The other spray reagents may be of use when an identification is necessary as the different colours developed by the different sprays are indicative of identity.

Thin Layer Chromatography Solvents

Introduction

The preceding work has established suitable reagents for detecting the steroids on thin layer chromatography plates.

One further requirement of a thin layer chromatography system is a tank solvent. If the system is required to identify the particular steroid in use then the solvent must separate the steroids from each other and from naturally occurring materials detected using this system. If the solvent is to be used in the pre-race laboratories, the solvent must separate the

contraceptive steroids from naturally occurring materials.

A number of solvent systems were examined for their ability to separate the contraceptive steroids. One commonly occurring steroid, cholesterol was included in this work.

Tank Solvents

Equipment

The chromatography plates were glass, coated with a 0.25 mm. layer of MN-Keiselgel G, air dried for 15 minutes and conditioned at 110°C for 30 minutes, and allowed to cool in a desiccator before use.

The solvents were mixed, by volume, and placed in cylindrical glass tanks lined for half their diameter with Whatmann No. 1 filter paper. The solvent systems were allowed to stand for 45 minutes before use to equilibrate.

Spots of the steroid solutions were placed on the plates 1 cm. above the lower edge, and the solvent allowed to run to about 11 cms. from the lower edge. The Rf values for each spot were then determined.

Solvents Used

(a) Diethyl Ether/Diethylformamide

These were mixed in the proportions of 99 : 1.

This solvent is suggested in Randerath (104) and is the one giving the greatest range of Rf values.

(b) Benzene/Ethanol

These solvents differ markedly in their elutive powers. By using various proportions, solvents with a range of elutive powers were prepared. The ratios used were 80 : 20, 88 : 12 and 95 : 5.

(c) Methylene Chloride/Ethanol

Methylene chloride is a slightly stronger eluting solvent than benzene. By using these two solvents in varying proportions, slightly stronger eluting solvents than (b) were prepared. The ratios used were 95 : 5, 97 : 3 and 98 : 2.

(d) Ethyl acetate/cyclohexane/Acetone

These solvents differ in their elutive power, and by using varying proportions, a much finer adjustment of the elutive power of the final solvent is possible than when using a two component mixture. The combinations used were 5 : 12 : 2, 5 : 10 : 5, 6 : 10 : 4 and 7 : 11 : 4.

Results

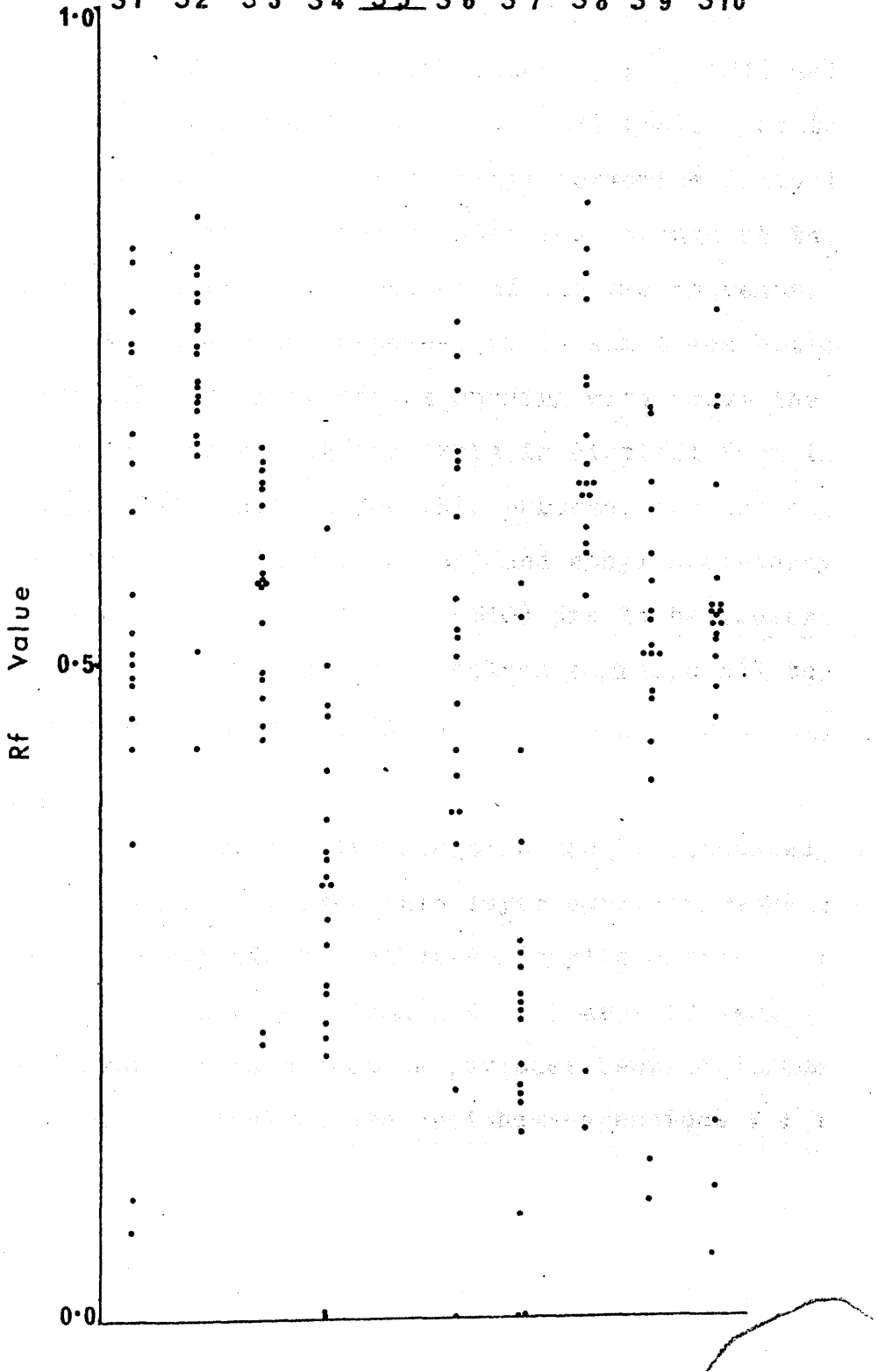
These are shown in Figure 14.

Discussion

For identification purposes, the solvent giving

FIGURE 14

Thin-Layer Chromatography of Steroids



the greatest separation of the steroids is desirable. The two solvents most suitable for this purpose are diethyl ether/dimethylformamide (99 : 1) (S1) and methylene chloride/ethanol (97 : 3) (S6). Of these two, the diethyl ether/dimethyl formamide gives the most even distribution of steroids throughout the range and is therefore the better of the two solvents.

For pre-race purposes, it is sometimes better to have all the drugs sought running with about the same Rf value, providing that this is distinct from interfering materials. For this purpose, the solvents benzene/ethanol 88 : 12 (S3) and ethyl acetate/cyclohexane/acetone 7 : 11 : 4 (S10) are to be preferred. However, neither of these solvents groups all the steroids closely together.

Conclusions

The contraceptive steroids can be separated on MN-Keiselgel G coated thin layer chromatography plates using either diethyl ether/dimethylformamide 99 : 1 or methylene chloride/ethanol 97 : 3 as solvents.

For pre-race testing purposes benzene/ethanol 88 : 12 or ethylacetate/cyclohexane/acetone 7 : 11 : 4 is to be preferred.

Extraction of Steroids

The whole procedure must be rapid, and so precludes hydrolysis of the samples using β -Glucuronidase. Rapid screening procedures are employed in early pregnancy testing. For trials using greyhound urine samples, the Waldi and Munter procedure, outlined by Randerath (104) was used.

Procedure

20 ml. of filtered urine were added to 2 ml. of concentrated hydrochloric acid, and heated on a boiling water bath for 15 minutes. After cooling, the sample was extracted with 3 x 25 ml. portions of cyclohexane and then discarded. The combined cyclohexane extracts were washed twice with 20 ml. of 1N sodium hydroxide and twice with 30 ml. of water. The cyclohexane was then dried over anhydrous sodium sulphate, and evaporated to dryness. The residue was spotted on a thin layer chromatography plate, run in the diethylether/dimethylformamide solvent, sprayed with concentrated sulphuric acid and heated at 120°C for 10 minutes.

Results

Two faint brown streaks were observed at Rfs 0.3 to 0.5 and 0.7 to 0.95 indicating that the test lacks sensitivity.

Conclusion

This procedure may be used for the detection of the elevated pregnanediol concentration occurring in pregnancy. The procedure does not have sufficient sensitivity to detect the normal concentrations of steroids in greyhound's urine. This, together with the time taken, makes this test procedure unsuitable for use in the pre-race testing laboratories.

Such a procedure may be of use if greater volumes of sample are available, and sufficient time to carry out the analysis.

General Conclusion

Spray reagents and solvents have been found which enable contraceptive steroids to be separated and detected. These provide a technique which is satisfactory when working with the pure drugs. Possible interference by naturally occurring steroids has not been investigated, as this procedure cannot be used for the purpose intended.

The procedure lacks the sensitivity required, and is too time consuming. Trials with normal urine samples virtually failed to detect normally occurring steroids, yet the procedure is required to detect steroids used in very small doses. The largest dose of a contraceptive steroid is medroxyprogesterone acetate given as 50 mg. daily, whilst the most commonly used contraceptive steroid, Ethinyl oestradiol is given in doses of 0.002 mg. to 1.0 mg. per day. Whilst medroxyprogesterone acetate may be detected, it would be impossible to detect ethinyl oestradiol by this technique.

The volume of sample, like the time available, is limited in a pre-race laboratory, and so there is no possibility of detecting these materials by increasing the sample size.

CONCLUSION

This thesis was based on the application of chemical toxicology to the detection of illicit drugging of racing greyhounds. The drugs which were of greatest interest were chlorinated hypnotics, principally chlorbutanol and chloral hydrate.

Before this work the commonest technique used was the Fujiwara test as applied to the detection of the metabolites of chloral hydrate. There was no system for identifying the actual drug present.

The Fujiwara reaction was investigated in detail, particularly with respect to the detection of chlorbutanol. The test is sensitive to the reaction conditions, particularly when chlorbutanol is the test substrate. The conditions were carefully investigated and a method devised which gives optimum performance. The resulting test is reliable and sensitive.

The possibility of using this test for identification purposes was investigated using ultra-violet and visible spectrophotometry. This was found to be impossible as a number of compounds gave the same chromophore.

A quantitative application of the technique was developed and proved to be simple and sensitive. It was used for the investigations carried out into the reaction conditions.

The reaction mechanism of the Fujiwara test was not known. This made it difficult to predict the effect of changes in the procedure. An investigation was carried out which elucidated the mechanism for chlorbutanol.

The result of this work is that the test procedure is reliable and sensitive when used either qualitatively or quantitatively.

The problem of identification of the materials giving the Fujiwara reaction was solved by using gas chromatography. The sensitivity and selectivity of electron capture detectors was exploited as fully as possible by careful choice of conditions. This resulted in a simple and reliable technique for the identification of the materials studied. This test was also developed to give quantitative information which was invaluable in the study of the Fujiwara test, and also in the analysis of routine urine samples.

These methods were used successfully in a series of trial experiments designed to investigate the effect of chlorbutanol and phenobarbitone upon greyhounds under racing conditions. In addition to demonstrating the reliability of the test procedures these trials also showed that these drugs and the metabolites of phenobarbitone, could be detected in urine samples

much longer after administration than had previously been thought. For a comparatively short period of time after administration the greyhounds exhibited clinical symptoms of drugging, but the effect upon the performance of the greyhound was surprisingly small. Not only was the effect small, the exact effect was also very varied. Both drugs could be expected to reduce the performance, but in certain instances an improvement was found. As a result, it is now known that any attempted drugging of racing greyhounds using the two most popular drug forms in effective doses will result in detection when a pre-race analysis unit is operating.

One other aspect of the administration of drugs to greyhounds which was investigated was the effect of capsule treatment. The results showed that while the existing organisation is adequate when untreated capsules are used, the security precautions could be circumvented by a very simple capsule treatment procedure. Fortunately, the time of absorption is not easy to judge and is very variable from animal to animal.

Other drugs investigated included phenyl butazone and steroids. The work showed that phenyl butazone metabolites can be detected and characterised within the existing test procedures. For steroids, a revised

procedure was envisaged, but the results showed conclusively that these steroids cannot be detected with the techniques at present employed.

The remaining section deals with volatile materials. This was initially intended to identify contaminants in the reagents used for the Fujiwara test. A suitable technique using gas chromatography was developed. Ethanol was one of the solvents included in the investigation and it was noted that a good separation of this from other solvents was obtained. This was investigated further and a reliable quantitative technique for the estimation in biological materials established. The new method has the advantage of distinguishing between ethanol and iso-propanol. The investigation of the effect of fructose on the levels of alcohol in blood was used to establish that the gas chromatographic technique could stand up to a large work load.

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