

STUDIES IN THE METABOLISM OF
DINITRO-o-CRESOL AND ALKYL BENZENES

by
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A thesis
presented in accordance with
the regulations governing the award of
the degree of Doctor of Philosophy
in the University of
London.

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(University of London),
June, 1953.
St. Mary's Hospital Medical School,
Paddington, W.2.

FOREWORD

These studies have been carried out over the period, November 1950 - May, 1953, in the Biochemistry Department of St. Mary's Hospital Medical School, with the assistance of a Studentship from the Medical Research Council.

In Part I, the results of an investigation of the metabolites of 3:5-dinitro-o-cresol, in the rabbit, have been described. This compound is used in agriculture, as a pesticide and herbicide, and its toxicity to man has caused concern. It is intended that a contribution be made to an understanding of the metabolism of the compound and of nitrophenols in general.

The mechanism of biological oxidation of alkyl-benzenes, described in Part II, is an extension of studies in the detoxication of aromatic substances, which have been made in this Department. A knowledge of the means whereby aliphatic side chains, in a molecule, are eliminated, is of practical application to the development of drug chemistry.

Preliminary discussions of the results have been read as papers to the Biochemical Society (Proc. of

Biochem. Soc., Biochem.J. (1952) 50, xxxvii and (1953) 53, iv), and a fuller account of the urinary products of dinitro-o-cresol metabolism has been published (Biochem.J. (1953) 54, 225).

I am deeply grateful to Professor R.T. Williams, director of this research, for his encouragement and constant supervision, for making available the crystalline glucuronides employed in the studies described in Appendix II and IV, and for permission to include the results of an investigation of cumene metabolism prior to its publication elsewhere. Dr. J.N. Smith has, by his patience and advice, greatly contributed to the understanding of the methods employed and of the results of the work. Dr. Smith prepared the resolved forms of methylphenylcarbinol used in animal experiments.

The figures for glucuronide and ethereal sulphate excretion of amylbenzenes were contributed by Miss E. Smalley.

R. H. Smith

June, 1953.

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PART I

THE METABOLISM OF DINITRO-o-CRESOL

CHAPTER I.

Introduction

The use of the letters DNOC to denote 4:6-dinitro-o-cresol (in the usage of the American Chemical Society), 3:5-dinitro-o-cresol (in the usage of the Chemical Society), or systematically, 2-methyl-4:6-dinitrophenol, has become acceptable in the industrial and academic fields and will be adhered to in this thesis.

The purposes for which this substance has been applied, during the time of its ready availability to medicine and industry, have varied as the potential value of aromatic nitro-compounds has been realized.

The properties as a dye were first exploited. The potassium or ammonium salts of DNOC and 2:6-dinitro-p-cresol, together were named Victoria Orange, Saffron Surrogate, Aniline Orange or gold yellow, and used as colouring agents for foods, but the toxic qualities led to their being forbidden and the next use for the mixture was as an insecticide under the name of Antinonnin (Rosenthaler, 1923). Alone, or with p-cresol, it was found more effective against cockroaches than sodium fluoride or pyrethrum (Graham, 1942), and it is employed with great efficiency against all insects and their larvae. As a spray of 2½%

DNOC in oil or as a dust, vast quantities of DNOC have helped to control the locust menace in Africa. (Gunn, 1945). It is an effective fungicide and ovicide, being sprayed on fruit trees in the dormant season to remove scale and scab. In the last few years, DNOC has assumed importance in British agriculture owing to its selective herbicidal properties. The weak aqueous solution of the salt is sprayed on fruit trees in the spring to thin out blossom, and in the early summer a stronger solution is applied to rid cornfields of mayweed, poppies and other weeds.

With its recognition as a stimulator of metabolism (Dodds & Pope, 1933) DNOC became a slimming agent and caused several fatal poisonings before its use therapeutically was abandoned.

The Toxicity of DNOC.

The selection of DNOC from amongst the many related aromatic substances available, cresols, nitrobenzenes and nitrophenols, has depended in part on the peculiar toxicity to so many forms of life, of its particular arrangement of substituted groups. In Table I the toxicities to laboratory animals of some of these substances are compared. The facts concerning phenolic compounds were taken from von Oettingen (1949).

TABLE I

The Toxicities of some Aromatic Substances

Substance	Fatal Dose (mg./kg.)	Animal	Method of administration	Reference
Benzene	656	guinea pig	intraperit.	from Ellinger (1923)
Toluene	441	guinea pig	intraperit.	Heffter <u>1</u> (37)
Phenol	{ 420-620	rabbit	oral	Deichmann & Witherup (1944)
	{ 620	rabbit	subcut.	" " "
	{ 266	dog	subcut.	Duplay & Cazin (1891)
<u>o</u> -Cresol	{ 940	rabbit	oral	Deichmann & Witherup (1944)
	{ 450-500	rabbit	subcut.	Meili (1891)
<u>m</u> -Cresol	{ 1400	rabbit	oral	Deichmann & Witherup (1944)
	{ 500-600	rabbit	subcut.	Meili (1891)
<u>p</u> -Cresol	{ 620	rabbit	oral	Deichmann & Witherup (1944)
	{ 300-400	rabbit	subcut.	Meili (1891)
Nitrobenzene	750-1000	dog	oral	Gibbs & Reichert (1894)
<u>m</u>-Dinitrobenzene				
<u>o</u> -Nitrophenol	100	dog	intrav.	Gibbs & Hare (1889, 1894)
<u>m</u> -Nitrophenol	83	dog	intrav.	" " " "
<u>p</u> -Nitrophenol	10	dog	intrav.	" " " "
2:4-DNP	{ 200	rabbit	oral	Magne <u>et al</u> (1932 <u>a</u>)
	{ 80	rabbit	subcut.	Walko (1901)
DNOC	{ 15	dog	subcut.	Heymans & Casier (1935)
	{ 20	rabbit	subcut.	" " "
4:6-Dinitro-2 sec- butylphenol	60	rat	oral	Spencer <u>et al</u> (1948)
2:4-Dinitro-6-cyclo- hexylphenol	180	rat	oral	" " " "
Dinitrotoluene	500	cat		White <u>et al</u> (1902)
2:4:6-Trinitro- phenol	{ 700-1000	rabbit	oral	Cheron (1880)
	{ 400-500	rabbit	subcut.	" "
	{ 200-300	rabbit	subcut.	Ilzhofer (1918)

From this table, may be deduced several facts concerning the effect on toxicity to animals of substitution of the alkyl radicals and nitro- and phenolic groupings into the benzene nucleus.

(i) The introduction of a methyl radical brings about various changes. Toluene is more toxic than benzene, although here the effect is reversed in cumulative poisoning, and DNOC is more toxic than 2:4-dinitrophenol. On the other hand, all the cresols are less toxic than phenol, and nitrotoluenes less toxic than nitrobenzenes. An increase in the molecular weight of the alkyl side chain decreases the toxicity to animals but, as will be shown later, this effect does not always parallel their effectiveness as insecticides and fungicides.

(ii) A phenolic grouping in the molecule increases the toxicity. Dinitrophenol is ten times as poisonous as m-dinitrobenzene and an even greater change is obvious when a hydroxyl adjacent to the alkyl radical is introduced into dinitrotoluene.

(iii) A second nitro group increases toxicity. Both nitrobenzene and the nitrophenols illustrate this effect. Dinitrophenols are lethal at oral doses of 40-80 mg./kg. which is between one-half and one-third of the amount of

mononitrophenol required. A third nitro grouping may cause the toxicity to fall again.

(iv) The toxic properties of these substances depend on the mode of administration. An oral dose appears always to have less effect than a subcutaneous, intravenous or intra-peritoneal injection.

No work on the actual metabolic pathway of DNOC has been conducted using invertebrates but numerous toxicological studies have been made on all forms of insect life, with DNOC and related compounds. A summary of the insects which have been shown susceptible to DNOC has been given by Graham (1942). The extent of ionization in the molecule appears to affect the lethal dose. Brian (1945) demonstrated that the fungicidal properties were decreased by as much as three-quarters by changes in pH. The toxicities of various dinitrophenates were compared (Kagy, 1936) using various species of insects, corn ear worms, army worms, cabbage worms and red-legged grasshoppers (American terminology). It was found that 2:4-dinitro-6-cyclohexylphenol and its calcium, magnesium, lead and copper salts (particularly the calcium salt) were extremely toxic. The 2:6-dinitro-4-phenylphenates, calcium 2:6-dinitro-4-cyclohexylphenate and the lead salt of DNOC had little or no toxicity although the parent compounds had strong effects. In the cyclohexyl derivatives the

position of the hydroxyl group greatly determined the toxicity of the substance.

In another communication, Kagy (1941) compared the relative efficiencies of some 2:4-dinitro-6-R-phenols where R was a hydrogen atom, alkyl or alicyclic group. With silk worms he found an increase in length of the chain up to six or seven carbons decreased the lethal dosage. Thus, hexyl and heptyl derivatives possessed twelve times the toxicity of DNOC. The alicyclic phenols were slightly less poisonous than their straight chain analogues. Thus, 2:4-dinitro-6-cyclohexylphenol possessed seven times the toxicity of DNOC.

Tainter, Bergstrom & Cutting (1935) found the effectiveness of the cyclohexyl derivatives greatly varied in different animals but agreed that they had not been shown to stimulate human metabolism.

Similarly, 4:6-dinitro-2-sec-butylphenol, effective as a dust against chinch bugs (Decker, 1943) required a lethal dosage of 60 mg./kg. in rats (Spencer, Rowe, Adams & Irish, 1948).

The herbicidal properties declined in the order 2-sec-butyl, 2-isopropyl, 2-ethyl, 2-methyl (Crafts, 1945) that is, with shortening of the side chains.

Kagy (1941) came to the opinion that the order of toxicity of these phenols in insects and mites is reversed in man.

An aromatic ring inserted into the 6-position produced a substance of inferior metabolic effect in rats and pigeons (Heymans & Casier, 1935; Tainter et al, 1935).

The toxicity on codling moth larvae, after replacing the phenolic hydrogen of DNOC by an acetyl or methyl group, was lowered somewhat (Smith, Siegler & Munger, 1935). 4:6-Dinitrocresol acetate and 2-methyl-4:6-dinitroanisole possessed 90 and 75% respectively of the power of the uncombined material.

The Metabolic Fate of Related Substances

While the metabolism, in the animal body, of the particular combination of alkyl, phenolic and nitro-groupings such as occurs in DNOC has not been investigated before, the fate of aromatic substances containing these substituents alone or in simpler combinations has been frequently studied.

Since 1877 (Baumann & Herter) it has been known that cresols are excreted as their ethereal sulphates and in 1911 (Neuberg & Kretscher) the conjugation of *p*-cresol with glucuronic acid in the dog was demonstrated. Oxidation of

the methyl radical of p-cresol was found by Preusse (1881) who also isolated 2:5-dihydroxytoluene from the urine of dogs fed o-cresol. Bray, Thorpe & White (1950) prepared a balance sheet of the products of o-, m- and p-cresol metabolism in the rabbit. The results for o-cresol were as follows :-

1%	excreted free
15%	" as the ethereal sulphate
72%	" as an ether glucuronide
less than 3%	" as 2:5-dihydroxytoluene.

Nitro groupings may be excreted unchanged or be reduced to amino groups in the animal body and the pathway by which nitrobenzene, as the simplest member of the series, is reduced has been a point of controversy for forty years. Meyer (1905) found p-aminophenol, probably conjugated as the ethereal sulphate or glucuronide, a major metabolite and although aniline itself was not isolated from animals, after dosing, its place as an intermediate had been postulated by Letheby (1863). In fermenting yeast nitrobenzene, nitrosobenzene and phenylhydroxylamine were reduced to aniline (Neuberg & Wilde, 1914). Heubner (1914) postulated nitrosophenol as an intermediate, which would rearrange to form the quinoneimine before further reduction to p-aminophenol

took place. In the latest study on nitrobenzene metabolism in rabbits, Robinson, Smith & Williams (1951) confirmed the presence of free nitrobenzene in urine and showed that the excretion occurred of all three nitrophenols and 4-nitrocatechol. Aniline and o-, m- and p-aminophenols were present. 35% of the dose was estimated as p-aminophenol. In their scheme of the metabolic pathways they did not find it necessary to postulate the formation of p-nitrosophenol or quinoneimine.

All three isomeric dinitrobenzenes were reduced by frog muscle; in the order of decreasing ease, o > m > p (Lipschitz & Osterroth, 1924). Their ability to cause methaemoglobinaemia was in the sequence p > m > o, in the order of decreasing efficiency (Heubner & Lo-Sing, 1938).

The nitrotoluenes were studied by Jaffe (1874, 1878). The p-compound produced p-nitrobenzoic acid, excreted as the hippurate; o-nitrotoluene in dogs produced a mixture of the glucuronides of o-nitrobenzyl alcohol and o-nitrobenzoic acid in the proportion 9:1.

The probability that a methyl radical adjacent to a nitro-grouping might be oxidised in dinitro and trinitro compounds, was suggested by Channon et al (1944) when the detoxication of 2:4:6-trinitrotoluene by rabbits was under

investigation. In this case an oxidation to the alcohol, excreted as the glucuronide, was postulated. These workers also showed that a p-nitro group was more easily oxidised than the o-nitro group (by comparison of the relative amounts of the reduction products in the urine) and they isolated 2:6-dinitro-4-hydroxylaminotoluene from the urine. In rats the methyl group may be completely removed (Lemberg & Callaghan, 1944).

Ease of reduction of nitrophenols has been proved by Gibbs & Hare (1889, 1894) to increase in the order o < m < p (compare with 2:4:6-trinitrotoluene above) and the p compound possessed ten times the toxicity of the o-nitrophenol. They postulated that, as the toxicities of these substances are in the order of ease of reduction, the metabolic products of nitrophenol metabolism are themselves responsible for the toxic effect; for example, by the production of methaemoglobinaemia. This suggestion cannot be correlated with the previously discussed metabolism of dinitrobenzenes and certainly cannot explain the pronounced effects of DNOC and 2:4-dinitrophenol.

The substance closest to DNOC in both chemical and physiological properties is 2:4-dinitrophenol (DNP). It has been used in the dye and explosive industry and, like

DNOC, was employed therapeutically as a reducing agent (von Oettingen, 1949). It was first suggested (Lutz & Baume, 1917) that DNP was excreted unchanged, but in a more intensive study Magne, Mayer & Plantefol (1932) and Guerbet & Mayer (1932) showed that after administration to men and dogs, DNP was excreted unchanged, as 2-amino-4-nitro-o-phenol and 4-amino-2-nitro-o-phenol, and there was a probability that the 2:4-diamino-o-phenol was formed. Conjugated substances were detected. The main product was 2-amino-4-nitro-o-phenol. DNP and reduced forms were identified in the brain, blood and cerebrospinal fluid of dogs after death (Guerbet & Mayer, 1932). Georgescu (1932) working with dogs, precipitated metabolites of DNP with basic lead acetate and digested the solid with 20% sulphuric acid. The solution gave a strong naphthoresorcinol reaction, showing the presence of a glucuronide.

In a bacterial succinic dehydrogenase system, 2-amino-4-nitro-o-phenol was the main reduction product from DNP (Grenville & Stern, 1935).

Results of examination of urine and tissues of patients suffering from toxic symptoms, following the therapeutic use of DNP, have been less definite (Perkins, 1919; Lutz & Baume, 1917; Woodward, 1946). There was no significant

increase in ethereal sulphate excretion (Tainter, Cutting & Hines, 1935).

Parker (1952) demonstrated that there was a very great dissimilarity between the products of reduction formed in vitro with rat tissue homogenates, and the substances appearing in urine. His experiments proved that DNP is affected by an enzymic process. The possible amino derivatives were 4-amino-2-nitro, 2-amino-4-nitro-o-phenol (both ether soluble) and 2:4-diamino-o-phenol which is water soluble. The products were separated into ether soluble and aqueous fractions and after chromatography of the former fraction, using an alumina column followed by development on a paper strip of the bands, were finally identified spectroscopically. He demonstrated that 90% of the total ether-soluble amine was 4-amino-2-nitro-o-phenol. This amine was more rapidly destroyed in homogenates than its isomer, and during the time of its destruction, there was only a slight increase in the water-soluble amine concentration. On whole rats, by paper chromatography of urine extracts, he demonstrated the excretion of 2-amino-4-nitro-o-phenol only and concluded that the excretion products represent the balance of production and destruction of both amino-nitrophenols.

Parker showed that the product of DNOC reduction in rat tissue homogenates was a diazotisable amine which he did not identify.

Absorption and Elimination of DNOC and DNP.

DNOC may enter the body by absorption through the skin, lungs and gut. Spencer et al (1948) demonstrated ready absorption through the skin of guinea pigs and rabbits and it has been confirmed on man (Harvey, Bidstrup & Bonnell, 1951). These workers showed that application of heat increased the concentration of DNOC in the blood after the skin had been painted with a solution, but not after oral administration. Schwartz (1936) proved that absorption via the lungs could lead to severe toxic effects.

The rapidity with which the removal of DNOC can be effected depends on the species. King & Harvey (1953 a) suggested the efficiency in eliminating DNOC is in the order rabbit > rat > man. Parker, Barnes & Denz (1951) have shown that DNOC disappears rapidly from the blood stream of the rabbit. Man can accumulate it (Harvey et al, 1951), blood levels of 15-20 $\mu\text{g./g.}$ resulting from daily doses of 1 mg./kg. Man excretes 0.8% of the dose within 24 hours after a dose of 1 mg./kg. The rabbit, after administration of 25 mg./kg. excretes 5-9% within the same period of time.

Excretion of DNOC may be continuing in man 4-5 weeks after dosing. Storage in the tissues seems to be of a temporary nature, despite the evidence for cumulative poisoning in man. The liver, spleen, kidney, intrascapular fat, muscle and brain of the rat rapidly destroyed DNP and DNOC in vitro (Parker, 1952). The tissues of a man after a fatal case of poisoning contained less than 5 $\mu\text{g./g.}$ DNOC except for the synovial fluid where a concentration of 44 $\mu\text{g./g.}$ was estimated (Steer, 1951). After addition of DNOC to human blood plasma 95% of the material was located in the albumin fraction (King & Harvey, 1953). Therefore such storage as may exist seemed likely to be in the extracellular fluids of the body.

In these studies DNOC was estimated by Parker's method (1949), the limitations of which will be discussed later.

Effect of DNOC and DNP on the Body Tissues.

The two substances are considered together because, qualitatively, their activities are almost identical. DNOC can replace DNP in in vitro experiments with tissue slices and homogenates, although the latter substance has, more often, been used and DNP was replaced by DNOC as a reducing agent only because, as a side action, it may cause agranulocytosis and bilateral cataract (Dodds & Pope, 1933).

The two compounds are among the aromatic nitro-compounds which do not give rise to methaemoglobin and Heinz bodies (Magne et al, 1932 d) but possess at certain concentrations a stimulating action on cell metabolism. The relative activities of some nitrophenols were measured by Heymans & Casier (1934).

2- <u>cyclohexyl</u> -4:6-dinitrophenol	455.1
DNOC	207.5
Dinitrothymol	178.2
2:4-DNP	100
2:4-Dinitronaphthol	99.3
2:4-Dinitro-6-aminophenol	16.4

On whole animals this was revealed by a hyperthermic action, demonstrated on dogs, cats, pigeons; weak or absent in guinea pigs, rabbits, rats and mice (Heymans & Casier, 1935). The validity of these findings has been questioned by Tainter et al (1935).

In man, the onset of poisoning by DNOC is marked by fatigue, sweating, thirst, loss of weight and an "anxiety complex". Soon there is hyperpnoea, tachycardia and hyperthermia and a relapse into coma precedes death (Bidstrup & Payne, 1951).

The inhibition of the Pasteur effect by DNOC was shown (Dickens, 1939) in brain preparations where it increased

respiration and aerobic glycolysis. DNP inhibited the Pasteur effect in bull spermatozoa (Melrose, 1951). A résumé of the results of experiments in vitro by other workers, was published by Loomis & Lipman (1948) the effects including blocking of synthetic processes and nitrogen assimilation, inhibition of growth differentiation, and the formation of adaptive enzymes and they proved that DNP was a reversible inhibitor of the coupling between phosphorylation and oxidation of rabbit kidney homogenates.

DNP did not affect certain enzyme systems, e.g. respiration of α -keto-glutarate, succinate and β -hydroxybutyrate (Judah & Williams-Ashman, 1951) unless they were deficient in inorganic phosphate or adenine nucleotide, in which case, oxidation was stimulated. The apparent liberation of inorganic phosphate has been explained by Tepley (1949) as a discharge from unstable esters in the gel of the system. Ageing of the gel (with discharge of gel phosphate) led to a reduction in the DNP effect, but this postulate has been disproved by Judah (1951), who replaced it by the more acceptable hypothesis that, since DNP inhibited phosphate uptake in respiratory systems from pyridine nucleotide upwards, the substance prevents a transphosphorylation between unstable esters and adenosine-

5-phosphate, at the three successive steps from pyridine nucleotide, through flavoprotein, to the cytochrome system. Turner (1951) holds the same opinion, based on work concerning the stimulation by DNP of lactating mammary tissue. The effect on brain tissue slices was quantitatively equivalent to maximal electrical stimulation and the response was additive except when each was near maximal (Peiss & Field, 1948; McIlwain & Gore, 1951).

This elucidation of the highly specific action of DNP on tissues has been of value for the diagnosis of the mode of action of certain enzyme systems. Thus Turner (1951) suggested that a common mechanism for the Pasteur effect and oxidative phosphorylation was likely, since DNP inhibited them both.

The mode of assimilation of glutamic acid by Staphylococcus aureus has been studied using DNP (Gale & Paine, 1950) as, in its presence, the accumulation of free acid and protein synthesis can be separated from fermentation, in the cell.

Fantl, Lincoln & Nelson (1951) found that DNP increased respiration of rat liver slices metabolising octanoate and pyruvate at a concentration when phosphorylation of fructose was inhibited, and therefore concluded that oxidative phosphorylation is not involved in the intracellular breakdown of these substrates.

DNOC and physiologically significant Substances.

The obvious possible connection between DNOC activity and that of thyroxine was discounted by Dodds & Robertson (1933) who, while determining the optimum therapeutic dosage of DNOC, noted that the increased metabolism occurred without proportional stimulation of the cardio-vascular system. They found that DNOC would not alleviate the symptoms of myxodoema. However, rabbits and rats fed thyroid extracts had a lowered resistance to both DNP and DNOC (Glaubach & Pick, 1934; Barker, 1946 b). Thiouracil-treated rats showed a decreased response to DNOC and Credner & Siedek (1949) recommended 10 ml. of 2.5% sodium methyl thiouracil by intravenous injection as an antidote to DNOC poisoning. They claimed that it reduced the rise in metabolic rate. The common action of DNOC and of the antimalarial drug mepacrine in reversibly uncoupling phosphorylation from oxidation has been noted by Loomis & Lipman (1948). Mepacrine inhibits choline esterase and enzymes containing riboflavin as a prosthetic group, e.g. cytochrome reductase, D-amino-acid oxidase (Sexton, 1949). It has been suggested that, in this case, there is a competition for the protein moiety of the enzyme, between inhibitor and prosthetic group. No reference to a connection between DNOC effect and Vitamin B deficiency has been encountered, although the

aggravation of DNOC toxicity in alcoholics (Koopman, 1937) may be relevant.

While Vitamin C deficiency has been very inconclusively shown to affect the toxicity of benzene a definite linkage has been established between the toxicity of aromatic nitro compounds and Vitamin C levels (Shils & Goldwater, 1949). Therapeutic doses of DNOC and DNP diminish the reducing constituents of urine and DNP may cause cataract in the lens of the eye, which contains comparatively strong concentrations of Vitamin C (von Ottingen, 1949).

The Present Work

The toxicity of DNOC rendered its use as a slimming agent inadvisable, but the large scale use of the chemical as a herbicide, insecticide, larvicide and fungicide has resulted, despite precautions, in the death of several workers. Thus, during the years 1945-1951 nine persons in England, of whom eight were agricultural workers were fatally injured (Bidstrup & Payne, 1951). Treatment with non-specific methods - intramuscular sedatives, rest, oxygen, cold spraying and fluids - have failed in cases where the poisoning was sufficiently advanced to cause obvious symptoms. Anti-thyroid drugs (Barker, 1946;

Credner & Siedek, 1949) have been of doubtful efficacy and it has therefore been desirable to obtain some understanding of the metabolic processes which the substance affects, and by which its own fate in the animal body is determined. Using the closely related substance DNP, the enzyme systems affected have been identified (Judah, 1951), but the estimation of DNOC in the tissues or urine of workers and animals has always been by a method which did not allow for certain structural changes in the DNOC molecule (Parker, 1949). This present work was designed to lead to an identification and estimation of the metabolic products occurring in the urine after the administration of DNOC to rabbits.

CHAPTER II

Materials and Methods

While no comprehensive study of the products of DNOC metabolism in animals has been reported, certain probable pathways of detoxication were deduced from the results obtained when the fate of simpler aromatic substances, containing substituents in the same relative positions as DNOC itself, had been investigated. Compounds containing aromatic nitro groups are eliminated free and as their reduced derivatives. It was likely further that an amino group formed in the body would be partially acetylated before excretion (Williams, 1947).

A methyl radical may be partially oxidised to the alcohol and carboxylic acid and conjugation of the oxidation product is probable.

Detoxication of the phenolic group occurs by conjugation as the glucuronic acid or ethereal sulphate (Williams, 1947).

Interest lay in the question of whether one or both of the nitro groups of DNOC would be reduced. The ease of reduction (judged from the relative proportions of isolated products) of mononitrophenols had been shown by Gibbs & Hare (1889, 1894) to increase in the order $o < m < p$. But picric acid (2:4:6-trinitrophenol) was partially reduced to picramic

acid (4:6-dinitro-2-aminophenol) (Walko, 1901).

DNP, which is so closely related to DNOC principally underwent reduction of the o-amino group in the intact animal (Magne et al, 1932; Parker, 1952) but in rat tissue, Parker (1952) identified 4-amino-2-nitrophenol as the main product although he observed that its rapid destruction followed.

In this investigation, it was thought necessary to prepare as reference compounds for the identification of the metabolites of DNOC, 4:6- and 6:4-aminonitro-o-cresol and 4:6-diamino-o-cresol. 3:5-Dinitro-, 3-amino-5-nitro- and 3:5-diaminosalicylic acids were also made. Corresponding substances in which amino groups have been acetylated were prepared.

The efficiency of elimination of DNOC by various animals had been determined by King & Harvey (1953 a) to follow the order rabbits > rats > man (in the sequence of decreasing ease). Rabbits were, therefore, most suitable animals on which to perform detoxication experiments, excreting about 7% of a dose of 40 mg. in the 24 hours after feeding.

Reference Compounds

The reference compounds were characterised by m.p.'s (uncorrected), colour reactions and their U.V. absorption spectra. They were chromatographically uniform.

DNOC (Pest Control Ltd.) after recrystallisation from 95% ethanol possessed m.p. 86° ; in 0.1N-NaOH, $\lambda\lambda_{\max}$ 265, 370 $m\mu$, $\epsilon_{\max}^{1 \text{ cm.}}$ 5000, 12800 and inflexion λ 408-410 $m\mu$, $\epsilon_{\max}^{1 \text{ cm.}}$ ca 10000; in 0.1N-HCl, $\lambda_{\max}$ 268 $m\mu$, $\epsilon_{\max}$ 12600.

6-Amino-4-nitro-o-cresol (Cazaneuve, 1897). Recrystallisation was from aqueous ethanol as orange needles, m.p. 178° ; in 0.1N-NaOH $\lambda\lambda_{\max}$ 272, 460 $m\mu$, $\epsilon_{\max}^{1 \text{ cm.}}$ 6100, 14600; in 0.1N-HCl, $\lambda_{\max}$ 225 $m\mu$, $\epsilon_{\max}^{1 \text{ cm.}}$ 8600.

6-Acetamido-4-nitro-o-cresol (Friedländer, 1931). Purification was by charcoaling in aqueous ethanol and the very pale yellow crystals, from ethanol, possessed m.p. 209° ; in 0.1N-NaOH, $\lambda\lambda_{\max}$ 285, 425 $m\mu$, $\epsilon_{\max}^{1 \text{ cm.}}$ 6700, 13900; in 0.1N-HCl, $\lambda_{\max}$ 280 $m\mu$, $\epsilon_{\max}^{1 \text{ cm.}}$ 6400.

4-Acetamido-6-nitro-o-cresol (Nietzki & Ruppert, 1890).

Orange needles crystallised from ethanol with m.p. 223° ;

in 0.1N-NaOH, $\lambda\lambda_{\max}$ 252, 445 $m\mu$, $\epsilon_{\max}^{1 \text{ cm.}}$ 14400, 3900.

4-Amino-6-nitro-o-cresol (Nietzki & Ruppert, 1890) prepared as dark red needles, m.p. 122° ; in 0.1N-NaOH, $\lambda\lambda_{\max}$ 237.5, 495 $m\mu$, $\epsilon_{\max}^{1 \text{ cm.}}$ 14000, 4450.

4:6-Diamino-o-cresol dihydrochloride. An equivalent of tin

in HCl accomplished the reduction of both nitro groups of DNOC (Cazaneuve, 1897). For isolation of the product the reaction mixture was neutralized with sodium acetate and acetylated. After dilution and acidification a slow precipitation of 2-acetoxy-3:5-diacetamidotoluene occurred. Hydrolysis was effected by refluxing a suspension of the reprecipitated crystals in concentrated HCl for 3 hours when an off white solid separated out - the dihydrochloride of the base.

3:5-Dinitrosalicylic acid (Meldola, Foster & Brightman, 1917).

After recrystallisation from chloroform the pale yellow product possessed m.p. 172° .

3-Amino-5-nitrosalicylic acid (Meldola et al, 1917). The orange crystals, from ethanol, had m.p. 229° ; in 0.1N-NaOH, $\lambda_{\max} 292.5, 457.5 \text{ m}\mu, \epsilon_{\max}^{1 \text{ cm}} 5750, 16000$.

3-Acetamido-5-nitrosalicylic acid (Meldola et al, 1917), m.p. 246° .

3:5-Diaminosalicylic acid dihydrochloride was prepared by the same method as was used for the corresponding cresol. White crystals were obtained with no m.p.

Colour Reactions.

The following reagents were used as aids in the characterisation of the reference compounds and for the location of substances developed on paper chromatograms. The individual reactions are given in Table 2.

2N-NaOH and 2N-HCl solutions.

Ammoniacal AgNO₃ was prepared from 10 ml. 0.1N-AgNO₃ and 1 drop 40% NaOH with enough dilute ammonia to clear the solution.

Diazo reagents (i) A spray of 1% NaNO₂ in dilute acetic acid was followed a few minutes later by a spray of 1% β -naphthol in N-NaOH.

(ii) With urine the test was carried out by acidifying with dilute HCl, adding a drop of 0.1% NaNO₂, followed by 1% ammonium sulphamate, then coupling with 1% ethanolic dimethyl α -naphthylamine.

10% NaCN. After treating with this solution, the solution in a test tube or on paper was heated to 110° for 10 mins. m-Dinitro compounds, i.e. DNOC and 3:5-dinitrosalicylic acid gave pink colours.

1% FeCl₃, a phenol reagent, gave undecisive results in both test tube and paper tests using cresols.

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April 1953.

"Studies in the Metabolism of Dinitro-o-cresol and Alkylbenzenes".

An abstract of the thesis being presented for the degree of
Doctor of Philosophy in the University of London.

4:6-Dinitro-o-cresol (DNOC) has assumed an importance in agriculture as a selective weed killer and pesticide. These properties, outlined in the introduction to Part I of the thesis are peculiar to the particular arrangement of substituted groups in the molecule. Compounds containing phenolic and nitro groups or alkyl radicals attached to the benzene ring in simpler combinations are toxic and have similar metabolic behaviour in the animal body.

The experiments, in the present work, were conducted on rabbits. After feeding, the urinary products of DNOC breakdown were identified by paper chromatography and their excretion, in most cases, was estimated by U.V. spectroscopy. The extreme toxicity of DNOC and the low recovery of metabolites from the urine, less than 20% of the dose, made normal isolatory procedures impracticable.

The interpretation of results gained by less specific methods of study has been aided.

The introduction to Part II of the thesis is concerned with the chemical and biological mechanisms by which alkyl radicals attached to benzenoid and other structures are oxidised. The stereochemical relationships of the carbinols derived from alkylbenzenes are outlined. The products in rabbit urine of methyl-, ethyl-, propyl-, butyl- and two amylbenzenes have been investigated and of some ketones and carbinols related to them. Glucuronide and ethereal sulphate conjugation was estimated and the glucuronides of the carbinols, formed by oxidation of the alkyl chain, were isolated as the diastereoisomers of the triacetyl methyl esters.

Stereochemical evidence discounts the placing of styrene and acetophenone as intermediates in ethylbenzene metabolism. Alkylaryl ketones form only one diastereoisomer of the carbinols, which can be related to D-glyceraldehyde.

R.M. Smithies.

TABLE 2

Colour Reactions of DNOC and related Compounds

Compound	Colour given in aqueous solution with :-				
	2N-HCl	2N-NaOH	amm. AgNO ₃	Diazo reagents	1% FeCl ₃
DNOC	decolourized	yellow	-	-	-
6-Acetamido-4-nitro- <u>o</u> -cresol	decolourized	orange-yellow	-	yellow with HNO ₂ no coupling	darkening
6-Amino-4-nitro- <u>o</u> -cresol	pale pink	red	slow reduction	yellow with HNO ₂ no coupling	-
4-Amino-6-nitro- <u>o</u> -cresol	pale pink	pink	reduction	slow red-violet	-
4-Acetamido-6-nitro- <u>o</u> -cresol	-	orange	-	-	-
4:6-Diamino- <u>o</u> -cresol dihydrochloride	-	slow decomp. to pink subst.	reduction	orange with HNO ₂ violet coupling	scarlet
3:5-Dinitrosalicylic acid	decolourized	yellow	-	-	red
3-Amino-5-nitrosalicylic acid	decolourized	orange-red		yellow with HNO ₂ violet coupling ²	red-brown
3-Acetamido-5-nitrosalicylic acid	-	orange	-	-	red-brown
3:5-Diaminosalicylic acid dihydrochloride	-	-	reduction	orange with HNO ₂ red-purple coupling	dark red

- denotes no change on adding reagent

The Estimation of DNOC.

In the simplest procedure DNOC is extracted from the material (tree bark) in petrol ether and the yellow colouration in 0.1% NaHCO_3 solution is determined colorimetrically against a standard (Avens, Chapman & Pearce, 1948).

Among toxicologists, the method widely used today and designed for routine estimation of DNOC in blood, tissues and urine is the salting out of the cresol into methylethylketone in which it possesses an intense yellow colour. Solutions of 5 ml. methylethylketone containing 1-10 μg . DNOC are employed as standards and the optical densities are measured at 430 $\text{m}\mu$ (Parker, 1949). It was inferred that the aminonitrocresols would produce a negligible effect since the corresponding derivatives of DNP possess no absorption in the solvent at this wavelength.

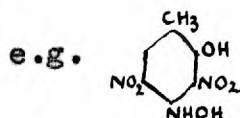
Preliminary separation from washes by steam distillation has been employed (Batt, Martin & Wain, 1944); DNOC is slightly volatile in steam (Gibson, 1925).

It has been estimated, when in relatively large quantities, by an iodine titration (of the acidic phenolic group) or gravimetrically by the formation of molecular compounds with hydrocarbons and aromatic bases (Wain, 1942).

After reduction under certain conditions in acid and

alkaline solution a rose colouration occurs and this observation has been adapted for quantitative estimations (Hovorka, 1943). The colouration of the reduction compound of DNOC with aqueous ferric chloride has been used for solutions containing 5-20 mg. DNOC/40 ml. (Norvall & Kenyon, 1948).

A specific reagent for m-dinitro compounds is cyanide with which hydroxylamines or purpurates are formed.



As employed by Fischer (1938) this method was capable of estimating 0.2-1.5 mg. DNOC in an alkaline extract of a plant insecticide.

It was important for our purpose that very small amounts of unconjugated DNOC and its metabolites should be satisfactorily identified and estimated and thus non-specific methods - by iodine titration and via the reduction products - were inadequate. However, the use of mild agents, e.g. reducing sugars was investigated as a basis for the colorimetric estimation of DNOC, since m-dinitrobenzene has been employed for the estimation of sugars (Poe & Edson, 1931). Glucose reduced the amino and acet-

amidonitro-o-cresols but the colour was unstable. Hodgson & Ward (1945) found that sodium sulphide in boiling alkaline solution reduced DNOC with the formation of 4-nitro-6-amino-o-cresol in a quantitative yield. This substance possesses a rich red colour in alkali and it was shown that the colour intensity, measured in a Spekker photoelectric colorimeter with an Ilford 603 filter (blue-green) was directly proportional to the initial concentration of DNOC over a range of 0-30 $\mu\text{g./ml.}$ A solution containing 1% Na_2S and 5% NaOH was used and the tubes were heated in a boiling water bath for 45 mins. This scheme seemed to have some potential value as the 6-amino- and 6-acetamido-4-nitro-o-cresol were unaffected by the reagent. It was not, however, further investigated.

Colorimetric methods based on the formation of the deeply coloured purpurate (Fischer, 1938) or of the molecular compounds formed with aromatic bases (Wain, 1942) were specific to DNOC but insufficiently sensitive.

Parker (1949) had produced evidence suggestive that the aminonitrocresols did not interfere with the determination of DNOC by its colour in methylethylketone. This was confirmed but when the U.V. spectra of DNOC and 6-acetamido-4-nitro-o-cresol in methylethylketone were plotted the two substances

were observed to possess approximately the same absorption at 430 $m\mu$, the wavelength at which the measurements are taken and the acetamido compound was salted out from aqueous solutions under the conditions advised. Thus, this method of estimation was of reduced value where both substances occurred together and this must be allowed for in the results obtained by those workers who used Parker's method.

To suit the conditions of this study, it was found necessary to prepare ethereal extracts of urine after feeding DNOC to rabbits. Separation of the detoxication products was achieved by paper chromatography of the extracts. Colour tests identified the spots developed and confirmation of the identity of substances and their estimation, was by U.V. spectroscopy of solutions obtained by eluting the spots (cf. Robinson, et al, 1951).

Paper Chromatography.

Downward paper chromatograms were run in the usual way at room temperature, in rectangular glass tanks 20" x 12" x 12", closed at the top with plate glass sheet. No special precautions were taken to make the tanks completely airtight or to equilibrate paper with the solvent. Under these conditions, the ratio R_F between

the distance moved by the spot and the distance moved by the front was not exactly constant in different tanks or over a period of time in any one tank, but the general pattern and order of separation were reproducible. All identification of compounds by their R_F values was by comparison with an authentic sample run on the same paper at the same time.

For most purposes Whatman No. 1 or 4 paper was used. Barcham Green No. 401 paper produced a cloudy solution after elution which caused increased absorption at all wavelengths of the ultraviolet. Whatman No. 3 MM paper was found to be more suitable than the thinner grades when larger amounts of substances were being chromatographed.

Solvents were prepared by shaking the components in the proportions by volume shown in Table 3. The top layer was used for development and the bottom layer was poured into the base of the tanks. Values of R_F obtained with the different solvents are quoted in Table 3, to illustrate the degree of separation achieved.

TABLE 3

Paper chromatography of DNOC and its possible metabolites;

R_F values with Whatman No. 1 paper.

	Benzene/acetic acid/water 1:1:2	<u>n</u> -Butanol/acetic acid/water 4:1:5	Benzene/ <u>n</u> -butanol ammonia(s.g.0.88) 2:5:2
	R _F	R _F	R _F
DNOC	0.96	0.95	0.50
6-Acetamido-4-nitro- <u>o</u> -cresol	0.84	0.95	0.44
6-Amino-4-nitro- <u>o</u> -cresol	0.80	0.95	0.38
4-Acetamido-6-nitro- <u>o</u> -cresol	0.56	0.95	0.20
4-Amino-6-nitro- <u>o</u> -cresol	0.88	0.95	0.22
4:6-Diamino- <u>o</u> -cresol	0	0	0
3-Amino-5-nitrosalicylic acid	0	0.64	0.13
3-Acetamido-5-nitrosalicylic acid	0	0.56	0.07

U.V. Spectroscopy.

To complete the identification of the substances obtained by chromatography of the urine extracts it was necessary to elute spots and then measure their ultraviolet absorption in acid or alkaline solution.

The spectra of reference compounds were determined in the Unicam S.P. 500 Spectrophotometer over the range 215-600 $m\mu$.

When however, strips of chromatography paper containing spots of these substances were eluted, and the U.V. absorption measured, there was distortion of the curve at wavelengths below about 350 $m\mu$. At first, 0.1N-NaOH was employed as the eluting agent but the elution was slow and, despite the precautions taken to exclude the atmosphere from the apparatus in which the operation was carried out, there was formation of carbonate in the solution and consequent interference in the absorption. Ethanol was substituted as the eluting agent and later methanol in which DNOC is almost twice as soluble (Wain, 1942).

Irrelevant absorption persisted and it was not appreciably reduced by washing the paper either in chromatographic solvents or ethanol. It was, therefore, due to the slow

release from the paper in both alkaline and alcoholic solution, of some substance with absorption below 350 m μ and examination of eluates of paper confirmed this.

The effect of this irrelevant material on the absorption curves in alkali was to accentuate the peak in the lower wavelengths, making its extinction comparable with that of the other peak. This was minimised by using larger quantities of material in the spot (which made the volume greater in which the absorption was measured) and by eluting with the same volume of eluent a strip cut from the paper alongside the spot under investigation, and of the same size as that containing the spot, and using this in the compensating cell in the spectrophotometer.

Amounts of the order of 100-200 μ g. in a spot were found necessary to give satisfactory spectra after elution. From the extinction of the peak at the higher wavelengths, the amounts of compounds present on a spot were estimated. Recovery experiments showed that DNOC and 6-amino-4-nitro-o-cresol were recovered with an accuracy of $\pm 10\%$. The recovery of 6-acetamido-4-nitro-o-cresol was more than 70% of the amount deposited on the chromatogram.

CHAPTER III

Animal Experiments

The Metabolites of DNOC

Animals and Dosing.

The rabbits used in this study were Chinchilla does of between 2.5 and 3.5 kg. weight. During the period of excretion, a diet of 70 g. rat cubes (Associated London Flour Millers, Diet 41) and 100 ml. water was allowed per day. This diet had been found to be more suitable during detoxication experiments than green stuffs. The doses were, unless otherwise stated, administered orally, as a suspension in water, by stomach tube. In all, some 40 rabbits were given 20-30 mg./kg. DNOC and survived. Two rabbits which had received 31 and 32 mg./kg. DNOC died within 24 hours of dosing. Heymans & Casier (1935) reported that rabbits tolerated subcutaneous injections of 10-20 mg./kg. Harvey & King (1953) fed daily doses of 25 mg./kg. DNOC orally to rabbits.

Examination of Urine.

The urine excreted in the first 24 hours was found to possess the following reactions. In subsequent periods of time these reactions were very much reduced. The appearance of the urine was normal and the pH neutral or slightly acid.

- (1) In alkali a slight intensification of the yellow colour.
- (2) On acid hydrolysis, there was darkening and no obvious change in colour when the hydrolysate was made alkaline.
- (3) With sodium nitrite in acid solution, an intensification of the yellow colour resulted and there was a slow, faint, purple, diazo colouration on coupling with dimethyl- α -naphthylamine. This reaction was still positive with third and fourth day urines.
- (4) The naphthoresorcinol reaction for glucuronides was unchanged from normal.
- (5) The urine did not possess reducing properties with either Benedict's or ammoniacal silver nitrate solutions.

The ethereal extracts of the urine were made using peroxide-free ether in a continuous extractor. Four extracts were commonly employed, each taken to completion, viz. A Normal urine, neutralized when necessary.

B Urine acidified to approximately pH 3 with a few drops dilute H_2SO_4 .

C The residual urine from one or both these extractions, made 2N with H_2SO_4 , boiled for 3 hours, and then cooled. This extract contained compounds liberated from conjugates by hydrolysis and extractable from acid solution.

D The neutralized residual hydrolysate, containing compounds liberated from conjugates on hydrolysis and extractable at neutral pH.

Each extract was reduced to small volume on a water bath, taken up in ethanol and (unless otherwise stated) small quantities of this solution were evaporated on to the paper as spots, 15-20 mm. in diameter, for investigation by chromatography and U.V. spectroscopy.

Detection and Determination of Metabolites.

DNOC

Unchanged DNOC in 1st day urine was separated by chromatography of extracts A or B in benzene/acetic acid/water, n-butanol/acetic acid/ammonia and benzene/n-butanol/ammonia (Table 3) and identified with a reference spot of the authentic compound. Its presence was confirmed by the reaction with NaCN, by the intensification of the yellow colour with 2N-NaOH and by the absorption spectrum after elution with ethanol (cf. Fig. 1 and Table 4).

By calculation from the extinction of the absorption band in 0.1N-NaOH at 370 $m\mu$ it was found, in two feeding experiments that 3.5 and 4% of the dose was eliminated on the first day.

A further 1.5% of the dose was excreted unchanged on

the second day.

DNOC was also found to be present, by the same method, in extract A, that is, it existed in the urine as a conjugate. In a quantitative experiment 0.7% of the dose was accounted for, presumably as glucuronide or ethereal sulphate.

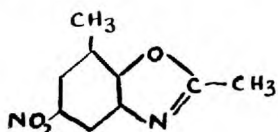
6-Acetamido-4-nitro-o-cresol

Spots corresponding in reaction to 2N-NaOH and with the same R_F value as this substance were developed in chromatograms of extracts A or B, using all three solvents but the first eluates taken from papers run in benzene/n-butanol/ammonia gave a very distorted spectrum in which a large band of absorption occurred with a peak at 335 $m\mu$. When the spot was eluted in methanol, hydrolysed in N-NaOH and the concentration of alkali made 0.1N before the absorption was measured, a maximum at 460 $m\mu$, i.e. at the absorption peak of 6-amino-4-nitro-o-cresol, was obtained. The authentic 6-acetamido-4-nitro-o-cresol applied to paper was found to behave similarly and it was first thought that some substance closely related to the acetamido compound in structure was present in the extract.

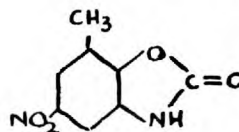
Jaffé & Hilbert (1886) isolated 4-methyl-benzoxazol-2-one as a metabolite of acetanilide in the dog. Williams

(1947) and Bray, James & Thorpe (1949) have suggested that it was formed as an artefact from the o-aminophenol, itself a product of deacetylation, during evaporation of the urine.

2:7-Dimethyl-5-nitrobenzoxazole and 7-methyl-5-nitrobenzoxazol-2-one were the likely substances to be present as artefacts in this extract and they were synthesized (see Appendix I). Their chromatographic behaviour was similar to that of 6-acetamido-4-nitro-o-cresol in all solvents. The alkaline colouration was poor.



2:7-dimethyl-5-nitrobenzoxazole



7-methyl-5-nitrobenzoxazol-2-one

Ultraviolet absorption data :-

7-Methyl-5-nitrobenzoxazol-2-one : in 0.1N-NaOH, $\lambda_{\max}$ 255, 315 $m\mu$, $\epsilon_{\max}$ 20000, 5800; in 0.1N-HCl, $\lambda_{\max}$ 237, 300 $m\mu$, $\epsilon_{\max}$ 15100, 7000.

2:7-Dimethyl-5-nitrobenzoxazole : in 0.1N-NaOH, $\epsilon_{\max}$ 1 cm. at 285 $m\mu$ and 430 $m\mu$, and a high peak between 240-260 $m\mu$.

This peak gradually declined in the alkaline solution while the absorption of the peak at 430 $m\mu$ increased. Thus there was gradual decomposition into 6-acetamido-4-nitro-o-cresol.

It was presumed unlikely that these substances were present and were contributing to the absorption of the

yellow spot with R_F similar to that of 6-acetamido-4-nitro-o-cresol.

When spots of the urinary material taken from papers run in benzene/acetic acid/water were eluted, the absorption curve was observed to be closely similar to that of the authentic substance. Consequently, the anomalous behaviour of the corresponding spot in benzene/n-butanol/ammonia was due to some colourless material present in the extract with an R_F equal to that of 6-acetamido-4-nitro-o-cresol and eluted under the same conditions.

To obtain the curve illustrated in Fig. 2, 5 ml. of the ethereal extract was evaporated to dryness under reduced pressure and again from ethanol. The gum was repeatedly digested with hot benzene and the benzene extract evaporated down to about 5 ml. This solution was passed through an alumina column (1" x 0.5") and the column washed with more benzene. A separation of a yellow band from the irrelevant material occurred and the eluate was evaporated to 3 ml. volume and chromatographed on paper in benzene/acetic acid/water. From the extinction at 425 $m\mu$ it was calculated that 1.5% of the dose was eliminated in 2 days after feeding.

6-Acetamido-4-nitro-o-cresol, conjugated at the HO

group, could not be estimated as the acetyl group was removed during the hydrolysis of the urine.

6-Amino-4-nitro-o-cresol

In extract A, of neutral urine, a very small amount of 6-amino-4-nitro-o-cresol appeared to be present, identified by its R_F in benzene/acetic acid/water and benzene/n-butanol/ammonia. The spot gave an orange colour with 2N-NaOH and a brown colouration with acid NaNO_2 . Attempts to obtain an estimation of the amount of this metabolite, existing free in the urine, were unsuccessful as the effects of irrelevant absorption obscured the U.V. spectrum.

In extract D, this compound was the main metabolite, identified by the R_F , colour reactions and the U.V. absorption spectrum in 0.1N-NaOH and 0.1N-HCl after elution with ethanol. 9.0 and 10.6% of the dose was calculated to be present, from the extinction of the peak at 400 $m\mu$ in alkali in two feeding experiments during the two days after dosing (cf. Fig. 3 and Table 4).

4-Amino-6-nitro-o-cresol

This metabolite in which the nitro group para to the HO has been reduced was present in chromatograms of extract

D with an R_F identical with that of the authentic material in benzene/n-butanol/ammonia and benzene/acetic acid/water. The spot gave a violet colour with $2N$ -NaOH, reduced ammoniacal $AgNO_3$ and gave a red-violet colour in the diazo reaction. There was not sufficient of the material present for its identification to be confirmed by spectroscopy.

3-Amino-5-nitrosalicylic acid

The use of the solvents benzene/acetic acid/water and n-butanol/acetic acid/water, by which substituted cresols and salicylic acids could be separated and distinguished, showed the presence of 3-amino-5-nitrosalicylic acid in extracts C and D. The spot obtained, chromatographically identical with the authentic material, gave a deep orange colour with $2N$ -NaOH, a purple-brown $FeCl_3$ reaction, a pink colour with acidified $NaNO_2$ and an acid reaction to a spray of 1% aqueous bromocresol purple.

Two dimensional chromatograms in n-butanol/acetic acid/water and benzene/acetic acid/water led to a clear separation of this substance from the cresol, which gives some similar colour reactions.

In a chromatogram using benzene/n-butanol/ammonia its presence was masked by the relatively large proportion of 6-amino-4-nitro-o-cresol in the extract having a similar R_F .

TABLE 4

Absorption spectra of metabolites of DNOC in urine of rabbits,
and those of authentic compounds.

Compound or extract		$\lambda_{\max}$ m μ	Absorption spectra $\epsilon_{\max}$ or l cm. $E_{\max}$	$\lambda_{\max}$ m μ	$\epsilon_{\max}$ or l cm. $E_{\max}$
DNOC	authentic	265	5000	(370 ~408-410	12800 ca 10000
	extract A	265	0.36	(370 ~408-410	0.61 0.48
	extract C			370	0.26
6-Acetamido-4-nitro- <u>o</u> -cresol	authentic	285	6700	425	13900
	extract A	285	0.35	425	0.39
6-Amino-4-nitro- <u>o</u> -cresol	authentic	(272	6100	460	14600
		(225*	8600	310	8500
	extract C	(272 (225*	0.31 0.37	460 310	0.64 0.35
3-Amino-5-nitrosalicylic acid	authentic	292.5	5750	457.5	16000
	extract C	~290		455	

~ denotes an inflexion in the curve
 * in 0.1N-HCl; all other determinations in 0.1N-NaOH.

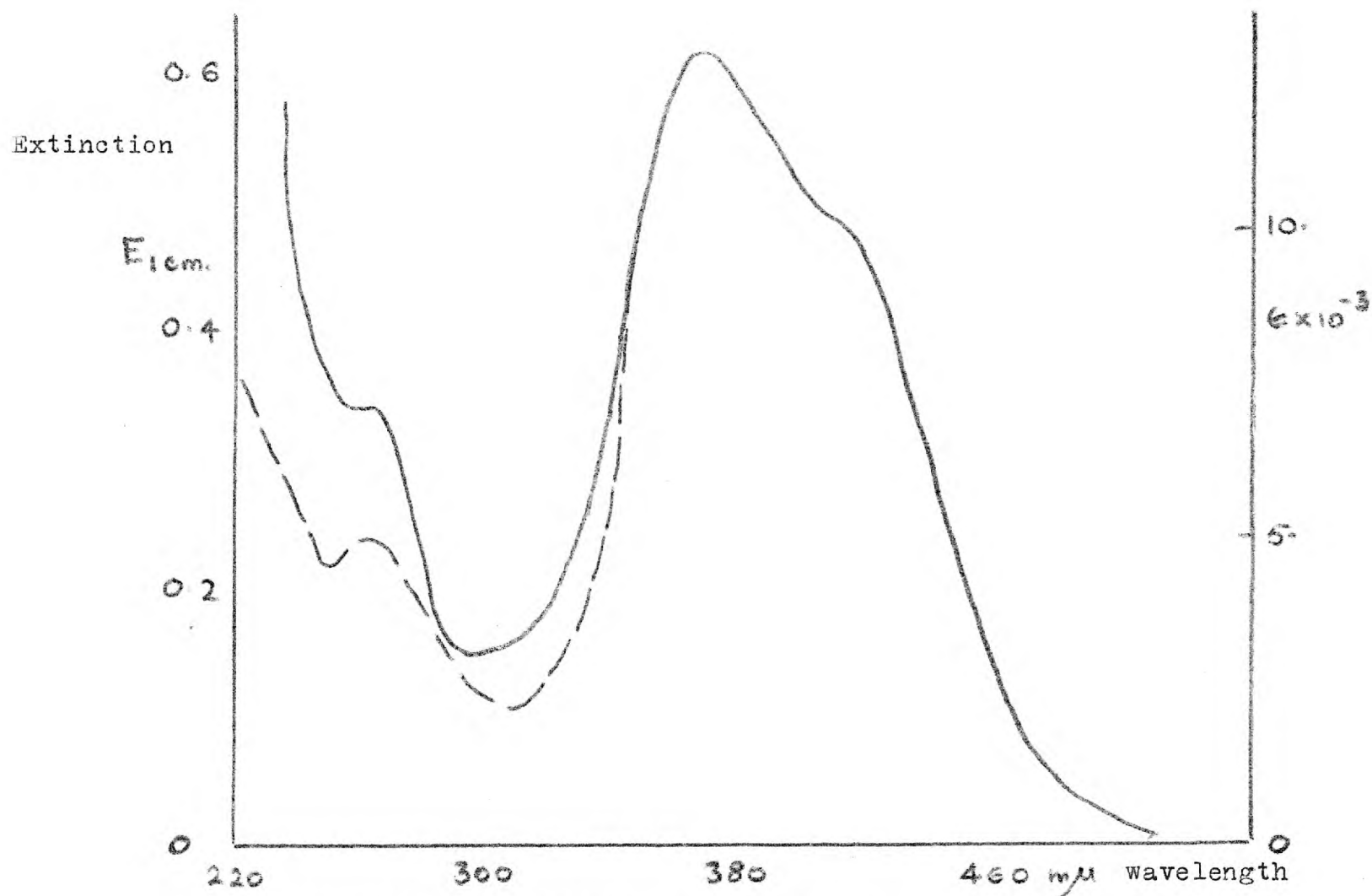


Fig. 1. Absorption spectra of authentic DNOC in 0.1N-NaOH (broken line; ordinate scale ϵ) and of DNOC in extract A (free line; ordinate scale $E_{1 \text{ cm.}}$)

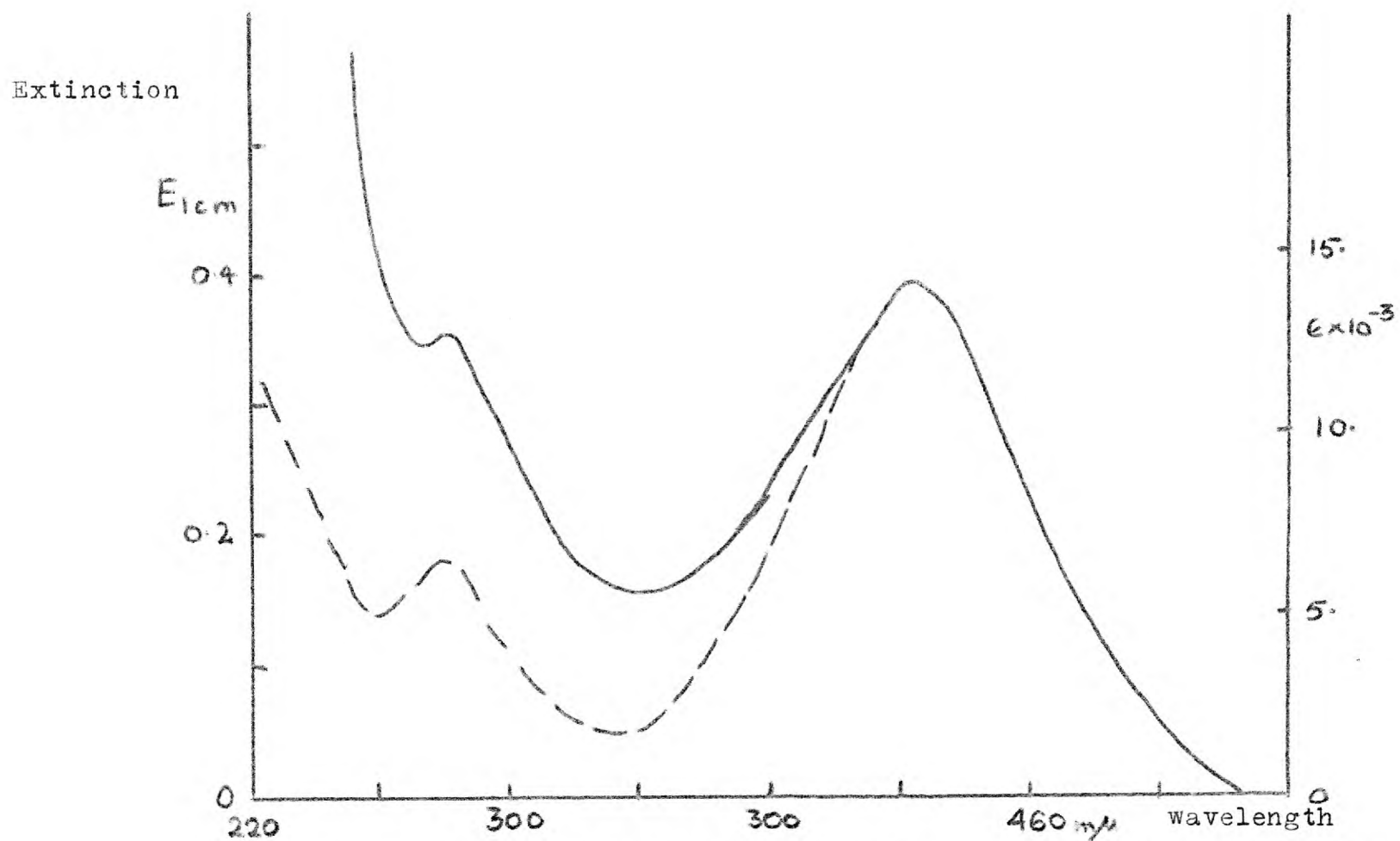


Fig. 2. Absorption spectra of authentic 6-acetamido-4-nitro-o-cresol in 0.1N-NaOH (broken line; ordinate scale $\leftarrow$) and of compound in extract A (free line; ordinate scale E_{1 cm}.)

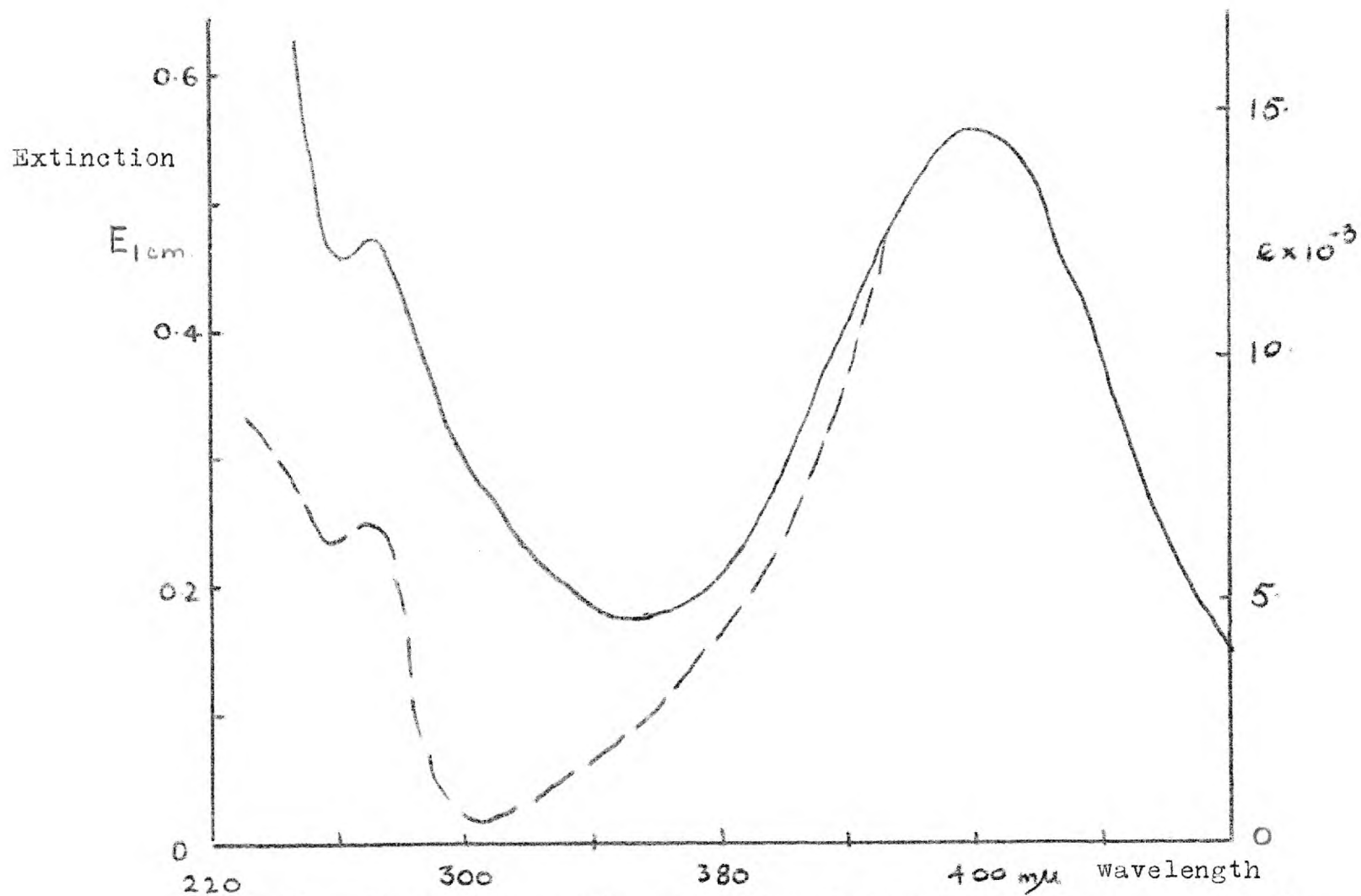


Fig. 3. Absorption spectra of authentic 6-amino-4-nitro-o-cresol in 0.1N-NaOH (broken line; ordinate scale ϵ) and of compound in extract D (free line; ordinate scale $E_{1 \text{ cm}}$.)

Generally, there was insufficient material present for spectroscopic examination to be possible, but in one case a spot of extract C from a two dimensional chromatogram was eluted in methanol and the spectrum in 0.1N-NaOH determined. The curve had a maximum at 455 $m\mu$ and an inflexion at 295 $m\mu$. This compared fairly closely to that of the authentic material (cf. Table 4) but the amount of substance present was small and the spectrum was considerably distorted by irrelevant absorption, which made any quantitative calculations impossible.

Examination of Animal Tissues.

One rabbit (2.8 kg.) which appeared to possess resistance to the effects of DNOC was injected with a total of 120 mg. over two days. At each injection, 20 mg. DNOC was dissolved in the least amount of saturated NaHCO_3 and introduced into one of the veins of the ears. The 48 hr. urine (330 ml.) contained 9% of the dose as DNOC estimated by the method of Parker (1949). On the following day 40 mg. DNOC was injected and the animal died one day later, four hours after 100 mg. DNOC had been administered by stomach tube. On dissection, only the stomach was found to be stained and samples of perinephric and peritoneal fat,

lumbar muscle, liver and brain, the entire duodenum and lower gut and the kidneys were removed. After grinding up the tissues they were steam distilled. The distillates and ether extracts of the distillates were colourless and gave no reaction with $2N$ -NaOH. Thus there was no evidence for storage of DNOC in these tissues.

The Metabolites of 6-acetamido-4-nitro-o-cresol

A chinchilla rabbit (3.1 kg.) was kept under the same conditions as have been previously described. After feeding 2 g. 6-acetamido-4-nitro-o-cresol, the 24 hr. urine was normal in appearance and pH and gave a strong naphthoresorcinol reaction. Benedict and diazo reactions were negative. Paper chromatography of 0.01-0.05 ml. urine showed only two spots, one corresponding to the unchanged compound and the other with the following R_F values :-

Benzene/acetic acid/water	R_F	0
<u>n</u> -Butanol/acetic acid/water		0.68
Benzene/ <u>n</u> -butanol/ammonia		0.07

This spot was detected by its strong quenching of the fluorescence of U.V. light. It gave no immediate reaction with $2N$ -NaOH but a yellow colour developed several hours after spraying the paper.

The glucuronic acid fraction of the urine was prepared through the basic lead salt and was obtained as a yellow syrup. This was neutralized with solid NaHCO_3 , a little absolute ethanol was added and the mixture warmed. On cooling, the hydrated sodium salt of 6-acetamido-4-nitro-o-cresylglucuronide crystallised. It was recrystallised from ethanol/water to give 300 mg. of pale yellow needles decomposing at $160-170^\circ$ and showing $[\alpha]_D^{20} -29.2^\circ$ ($c = 2$ in H_2O).

Analysis:

Found : C, 38.4	$\text{C}_{15}\text{H}_{17}\text{O}_{10}\text{N}_2\text{Na} \cdot 3.5\text{H}_2\text{O}$ requires C, 38.2
H, 5.8	5.94
N, 5.8	5.9
Na, 4.9	4.9

Light absorption : in 0.1N-HCl λ_{max} 240 $\text{m}\mu$, ϵ_{max} 8900 and λ_{max} 290 $\text{m}\mu$, ϵ_{max} 5500; in 0.1N-NaOH , λ_{max} 245 $\text{m}\mu$, ϵ_{max} 9200 and λ_{max} 290 $\text{m}\mu$, ϵ_{max} 5500.

The chromatographic behaviour of the glucuronide was found to be identical with that of the spot found in chromatograms of urine, and the same response to alkali.

The Metabolites of 6-amino-4-nitro-o-cresol

6-Amino-4-nitro-o-cresol was fed by stomach tube as a solution in dilute NaHCO_3 on six occasions to rabbits of

3-4 kg. weight. Two doses of 400 mg./kg. produced no after effects, three doses of 650-700 mg./kg. caused loss of appetite and of weight and one dose of 680 mg./kg. proved fatal. Examination of the rabbit after death showed that extensive haemorrhage was present in the lungs. The stomach and lower gut were heavily stained red but there was no staining of perinephric and peritoneal fat.

Examination of Urine

The urine of the first 24 hours after a dose of 400 mg./kg. was clouded and of a strong red colour. The following reactions were observed :-

- (1) Acidification caused the urine to become coloured yellow.
- (2) Acidified NaNO_2 gave a more intense yellow colouration and on coupling, a blue diazo reaction.
- (3) With reducing agents, Benedict's solution and ammoniacal AgNO_3 , the results were negative.
- (4) The naphthoresorcinol reaction was strong.
- (5) The urine was hydrolysed with $2\text{N-H}_2\text{SO}_4$ and extracted with ether. No FeCl_3 reaction was obtained with this ether extract.

In the next 24 hours the urine possessed the same properties but the diazo colouration was more pronounced. The colour reactions declined in the following 48 hours.

Suitable small volumes (0.01-0.05 ml.) of the daily urines of two rabbits were chromatographed directly on paper without any previous extraction. In benzene/acetic acid/water, n-butanol/acetic acid/water and benzene/n-butanol/ammonia separation of three substances occurred.

Unconjugated 6-amino-4-nitro-o-cresol was identified in the urine of the first three days after dosing by its chromatographic behaviour, its reaction to alkali and by its spectrum after elution in 0.1N-NaOH. From the extinction at 460 m μ it was calculated that in the two rabbits 7 and 18% of the dose was excreted unchanged in this time (cf. Table 5).

6-Acetamido-4-nitro-o-cresol was identified in the first day urine by its chromatographic behaviour, reaction to alkali and its spectrum in 0.1N-NaOH after elution with methanol from benzene/acetic acid/water chromatograms. In the two experiments 1.1 and 6.7% of the dose were excreted as 6-acetamido-4-nitro-o-cresol on the first day (cf. Table 5). There was insufficient material in the urine for spectroscopic examination of the spot in the succeeding days' urine.

TABLE 5

The daily excretion of unchanged and acetylated material
after feeding 6-amino-4-nitro-o-cresol.

Metabolite	Rabbit No.	Weight kg.	Dose mg./kg.	% Dose excreted			Total
				1st day	2nd day	3rd day	
6-Amino-4-nitro- <u>o</u> -cresol	(81	3.2	655	6.1	0.7	0.3	7.1
	(5	3.0	700	10.3	5.9	1.7	17.9
6-Acetamido-4-nitro- <u>o</u> -cresol	(81	3.2	655	1.1	-	-	1.1
	(5	3.0	700	6.7	-	-	6.7

- denotes that insufficient material was on the chromatograms for quantitative estimation by spectroscopy to be possible.

The papers also showed a large spot giving an immediate yellow colouration with $2N$ -NaOH. The chromatographic behaviour of the substance was related to that of 6-acet-amido-4-nitro-o-cresylglucuronide, which however does not give an immediate yellow colouration with NaOH. This spot was assumed to be 6-amino-4-nitro-o-cresylglucuronide. It was eluted with $0.1N$ -NaOH from a paper run in benzene/n-butanol/ammonia and the absorption curves determined. In $0.1N$ -NaOH there was $E_{\text{max}}^{1 \text{ cm.}}$ at 295 $m\mu$; in $0.1N$ -HCl there was $E_{\text{max}}^{1 \text{ cm.}}$ same value at 260 $m\mu$.

Since direct application of the urine to chromatography paper was employed, the presence of 4:6-diamino-o-cresol or diaminosalicylic acid would have been detected if they were present, since although these substances are not developed by the solvents employed, their reactions to $FeCl_3$ and diazo reagents would have been obvious on the residual spot. Moreover, the authentic 4:6-diaminocresol tended to decompose in the alkaline mixture, benzene/n-butanol/ammonia and two pink spots with small R_f separated. These became yellow in alkali and possessed a red-violet fluorescence in U.V. light. Fresh urine of animals dosed with 6-amino-4-nitro-o-cresol when chromatographed in benzene/n-butanol/ammonia, did not give these spots.

CHAPTER IV

Discussion

Detection and Estimation of DNOC Metabolites.

A method has been developed for identification of DNOC and related compounds in ether extracts of urine. Separation is by paper chromatography and the spots are detected by spraying with 2N-NaOH and by their specific colour reactions. Quantitative studies of metabolites can, where sufficient material is present in the extract, be made by elution of the spot and measurement of the extinction of the main peaks of the solution in 0.1N-NaOH using the Unicam S.P. 500 spectrophotometer over the ultraviolet range. Precautions are taken to eliminate the effects of irrelevant absorption.

This method of estimation is not intended to replace the customary procedure in toxicological studies (Parker, 1949) but has been of value in demonstrating that 6-acetamido-4-nitro-o-cresol is excreted by the rabbit. Since this substance has been proved to interfere with the estimation of DNOC by its colour in methylethylketone, allowance for the presence of this detoxication product must be made in the interpretation of results obtained using Parker's method.

Absorption and Elimination of DNOC and Metabolites.

Rabbits were found to survive up to 30 mg./kg. DNOC given by stomach tube. Heymans & Casier (1935) found that subcutaneous injections of 10-20 mg./kg. were tolerated and King & Harvey (1953 a) have fed 25 mg./kg. oral daily doses to rabbits over a period of eight days without fatal injury resulting. Since repetitive dosing can be maintained at a level so near to that of the fatal dose, it indicates that accumulation of DNOC does not occur in the rabbit. The observations of King & Harvey (1953 b), that no significant storage of DNOC occurs in fat, muscle or the upper gut have been confirmed by steam distillation of the tissues of rabbit which had been heavily dosed with it.

From the results obtained in animal experiments it has been established that the aromatic compounds identified and estimated in urine after feeding DNOC form only 17.2% of the dose administered (cf. Table 6).

TABLE 6

Average amounts of major metabolites

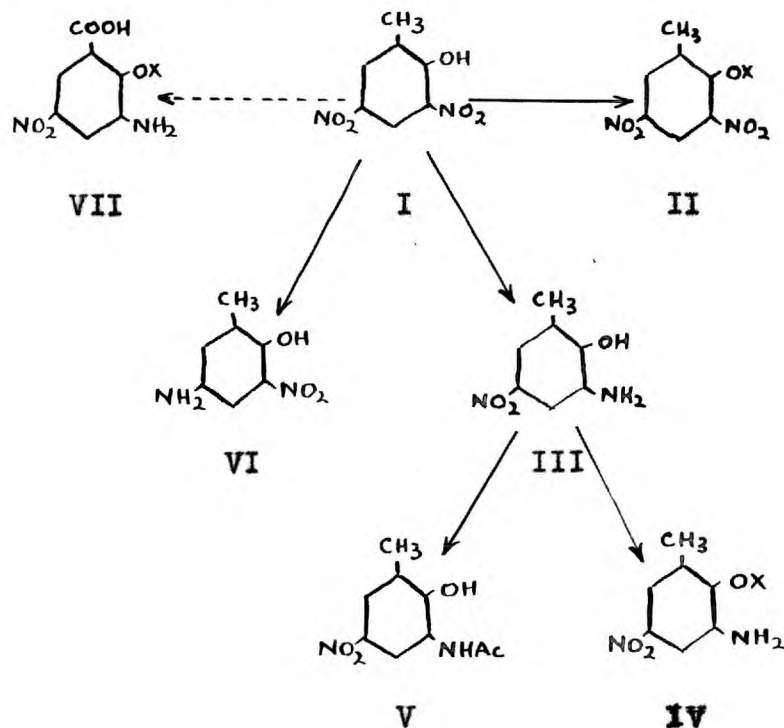
estimated in urine after feeding DNOC. (Expressed as % dose).

Substance	1st day		2nd day	
	free	conjugated	free	conjugated
DNOC	3.75	0.7 ¹	1.5	
6-Acetamido-4-nitro- <u>o</u> -cresol	1.15		0.3	
6-Amino-4-nitro- <u>o</u> -cresol	π	9.8 ¹		

π denotes that the substance was present in insufficient amounts for a quantitative estimation to be possible.

¹ denotes that the extracts of 1st and 2nd days' urine were pooled before chromatography.

The formation of the excretion products of DNOC in the rabbit may be represented by the following scheme :-



- I. DNOC.
- II. DNOC, O-conjugated.
- III. 6-Amino-4-nitro-o-cresol.
- IV. 6-Amino-4-nitro-o-cresol, O-conjugated.
- V. 6-Acetamido-4-nitro-o-cresol.
- VI. 4-Amino-6-nitro-o-cresol.
- VII. 3-Amino-5-nitrosalicylic acid, O-conjugated.

The amount of free phenolic material excreted in the first 48 hours has been found to be 6.7% of the dose. After this time insufficient material was present in the extracts for exact identification, by chromatography and spectroscopy, to be made. King & Harvey (1953 b) in measurements of the DNOC concentration in the blood have demonstrated that in the rabbit the values decline to zero in two days and have estimated 5-8% of the intake in the urine during the first 5 hours after feeding with a further 1% excreted over the following days. Parker et al (1951) measured 4-10% of the dose excreted in rabbits and dogs.

By separation of DNOC from its acetylated reduction product it has been shown that, of the free substances excreted, DNOC forms 5.25% of the dose and 6-acetamido-4-nitro-o-cresol is present and accounts for 1.5% of the dose in two days' urine.

DNOC is also conjugated at the phenolic group before excretion and 0.7% of the dose was accounted for in two days' urine. Thus, DNOC itself is not readily O-conjugated. Presumably, excretion takes place as the glucuronide and ethereal sulphate.

9.8% of the dose was excreted as a derivative of 6-amino-4-nitro-o-cresol. This substance may have been

acetylated in the amino group before O-conjugation. In deciding this, the results of the feeding experiments using 6-amino-4-nitro-o-cresol may be relevant and will be discussed.

3-Amino-5-nitrosalicylic acid has been identified on the chromatograms in insufficient quantity for definite spectroscopic identification. No trace of spots corresponding to other salicylic acids have been observed. Oxidation of the methyl radical is a feature of p-cresol metabolism (Preusse, 1881; confirmed by Bray et al, 1950) and of the o- and p-nitrotoluenes (Jaffé, 1874, 1878).

The presence of only a small amount of 4-amino-6-nitro-o-cresol is a significant finding. The violet colour with alkali on paper is quite distinct from that of the other cresols but confirmation of its identity by determination of the spectrum was not achieved. This failure can be attributed to insufficient material and to the actual absorption spectrum itself. In 0.1N-NaOH there are $\lambda\lambda_{\max}$ 237.5, 495 $m\mu$; $\epsilon_{\max}$ 14000, 4450, which allows for gross distortion by irrelevant material obscuring the peak at the lower wavelength by which identification might have been made. Certainly the results proved that very little of this compound was excreted. Parker (1952) has confirmed that the excretion of reduction products

of DNP is almost entirely as 2-amino-4-nitrophenol, although he demonstrated that tissues rapidly reduced the nitro group in the 4-position. With DNOC his preliminary experiments showed that a diazotisable amine was produced in vitro probably, therefore, 4-amino-6-nitro-o-cresol, since 6-amino-4-nitro-o-cresol does not couple. The product of DNP metabolism in tissues, reduced p- to the phenolic group, was rapidly destroyed and thus the experiments recorded in the present study, whereby the very low percentage of DNOC metabolites in rabbit urine has been established, are best explained by the hypothesis that DNOC is rapidly reduced at the 4-nitro- position and this compound is itself destroyed in the tissues. A small percentage of the reduction occurs in the 6-nitro- group and, as with DNP, this stable compound is excreted and in fact, forms the main urinary detoxication product.

Parker observed that water-soluble amine (i.e. 2:4-diaminophenol) did not appear to be the main end product of DNP metabolism in tissues. No suggestion of the fate of the 83% of a dose of DNOC unaccounted for in the present study can be made. The results of feeding 6-amino-4-nitro-o-cresol and 6-acetamido-4-nitro-o-cresol do not suggest that the destruction of the molecule is preceded by reduction of the

6-nitro group.

6-Acetamido-4-nitro-o-cresol was present in the urine of rabbits after dosing with this substance, both free and conjugated as the glucuronide in amounts sufficient for their identity to be established by chromatography of the urine direct on paper. The glucuronide has been isolated as the sodium salt.

Metabolites of 6-amino-4-nitro-o-cresol were present in the urine for three days after feeding in sufficient quantity for their identification and estimation to be made by direct paper chromatography of urine. Of the phenolic compounds the acetylated derivative was present in only 1/3 to 1/6 of the amount of unchanged substance and no spot exactly corresponding in chromatographic behaviour in benzene/acetic acid/water, benzene/n-butanol/ammonia and n-butanol/acetic acid/water or in alkaline colour to the 6-acetamido-4-nitro-o-cresyl-glucuronide was observed. However, a spot of somewhat similar chromatographic behaviour was present, rapidly becoming yellow in alkali, and the spectrum of this substance after elution has suggested that it might be the glucuronide of 6-amino-4-nitro-o-cresol. Therefore, when 6-amino-4-nitro-o-cresol is fed, acetylation of the amino group is not general before excretion. If the same condition applied

to the metabolism of DNOC, then of the O-conjugated material excreted, the greater part is unacetylated.

No evidence for the production of 4:6-diamino-o-cresol as a reduction product of the mononitrophenol has been secured. The urine gave an intense diazo colouration but this may have been by the presence of 6-amino-4-nitro-o-cresylglucuronide. Bray, Lake, Neale, Thorpe & Wood (1948) noted that o-aminophenylglucuronide readily diazotises and couples while the unconjugated phenol does not.

The urinary products of DNOC metabolism have been shown to consist largely of derivatives of 6-amino-4-nitro-o-cresol. This is a true detoxication process. The 6-amino- and 6-acetamido-4-nitro-o-cresol both possess less than 1/20th of the toxicity of DNOC itself.

PART II

THE METABOLISM OF ALKYL BENZENES

CHAPTER I.

Introduction

The chemistry of drug action has been elaborated in the last ten years and the need arisen to adapt known drugs to suit particular requirements. Detoxication of these compounds depends on the readiness with which they can be transformed into easily eliminated products. The time of duration of action, the efficiency and the toxicity to the animal body are therefore determined by the nature and position in the molecule of reactive groupings. Thus, there have been various pharmacological studies of physiologically significant compounds, in which alkyl chains have been substituted. N-Alkyl sulphonamides and sulphones have been synthesized but were found to lack bacteriostatic activity. (from Northey, 1948).

The substitution of aliphatic groups into barbituric acid and the antimalarial drugs, 2-hydroxy 1:4-naphthoquinones, produced active derivatives. Thus, Sandberg (1952) has shown the introduction of various types of radicals in the 1-position had a dominant influence on the pharmacological properties of the barbiturate molecule.

However, the selection of compounds to be synthesized

with a view to testing their physiological activity has been based on very tenuous considerations. The present work was commenced with the intention of making a contribution to the knowledge of the mechanisms by which alkyl side chains are oxidised in vivo and eliminated from the animal body.

While there is no doubt that the mode of oxidation is influenced by the total structural configuration of the molecule, the study has been restricted to substituted benzenes.

Chemical Oxidation of alkylbenzenes and Related Compounds

The oxidation of alkyl side chains has been assumed to follow the course of successive hydroxylation at the terminal C atom, by the evidence of isolation of intermediates - alcohols and aldehydes.

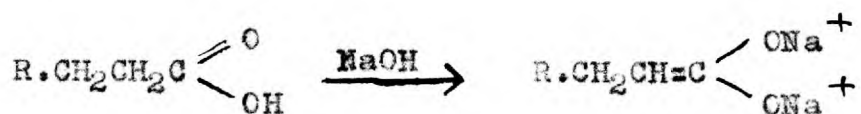
The presence in the molecule of a centre such as a phenyl group or unsaturated bond enhances the activity of the α -methylenic group. This is often, therefore, a preferential point of free radical attack resulting in hydrogen abstraction. The reactivity of α -methylenic groups towards free radicals has been studied by Kooyman

(1951), on the basis of their retarding action on the chain reaction, initiated by benzoyl peroxide, for addition of carbon tetrachloride to olefines. Compounds containing no such groups were inactive, e.g. benzene and tert-butylbenzene, and the activity of other compounds was clearly dependent on the presence of the group. Thus, mesitylene and the xylenes possessed respectively 3 x and 2 x the activity of toluene. Substitution at the α -methylenic group increased the efficiency of retardation. All the compounds, ethylbenzene, cumene, diphenylmethane and triphenylmethane were more active than toluene.

The ease of formation of the free radical, with abstraction of a proton, can be related to the dissociation energies of a C-H bond. $D(\text{CH}_4\text{-H}) \approx 101$ kcal/mole (as in toluene) and $D(\text{CH}_3\text{-H}) \approx 80$ kcal/mole (as in triphenylmethane) (Szwarc, 1951).

α -Activation may be responsible for the biological β -oxidation of fatty acids. β -Oxidation can be reproduced in vitro in a strongly alkaline medium. Mosher (1949) has postulated that enolization, as a first stage, with production of an unsaturated linkage, activates the C atom α to the double bond but at the β -position in regard

to the carboxyl group, i.e.



The capacity of compounds of the type $\text{R}' \text{---} \text{CH} \begin{array}{l} \nearrow \text{R}'' \\ \searrow \text{R}''' \end{array}$

to autoxidise has been studied by Hock and Lang (1943). Free radicals formed by loss of a proton, were stabilised by conjugation with maleic anhydride. Ethylbenzene was more readily attacked than toluene, xylenes and cymenes (in agreement with Kooyman's results). Tert-butylbenzene was unaffected. Autoxidation was promoted by U.V. irradiation and 3.3'diphenylbutane (a dimer) was isolated from the reaction mixture. Only those compounds with a free H atom on the α -C group were attacked by KMnO_4 , suggestive of a free radical mechanism, and Waters (1946) has reached the same conclusion, concerning the oxidation of alkylbenzenes with CrO_3 .

The liquid phase air oxidation of ethylbenzene at 140°C and under pressure proceeds by a free radical mechanism. Emerson, Heyd, Lucas, Cook, Lyness & Stevenson (1948) isolated 3.3'diphenylbutane from the reaction and have postulated the formation of α -Phenylethylhydro-

peroxide, $\text{Ph} \cdot \underset{\text{OOH}}{\underset{|}{\text{CH}}} \cdot \text{CH}_3$, and α -phenylethoxy radicals,

$\text{Ph} \cdot \underset{\text{O} \cdot}{\underset{|}{\text{CH}}} \cdot \text{CH}_3$, as intermediates. By doing so they account

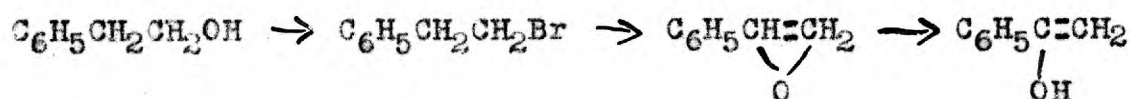
for the proportion of methylphenyl carbinol and acetophenone in the final product.

When ethylbenzene was oxidised by air in the presence of HBr (Rust & Vaughan, 1946) the products of oxidation of the side chain were acetophenone (8%) and phenylacetic acid (12%). Hydrogen peroxide oxidation of ethylbenzene yielded some styrene (Iwai, 1945).

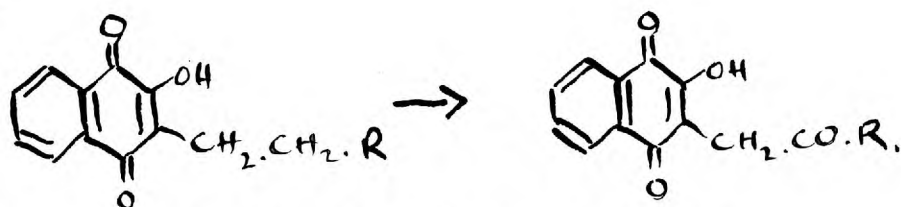
The α -C atom of styrene was found susceptible to oxidation by HNO_3 in the presence of acetic acid, methylphenylcarbinyl acetate, $\text{C}_6\text{H}_5(\text{CH}_3)\text{CHOOC} \cdot \text{CH}_3$, being the end product (Wilkinson, 1949). Methylphenylcarbinol was formed from styrene epoxide by reduction with LiAlH_4 and this reaction has been considered to be ionic (Trevoy & Brown, 1949).

Methylphenylcarbinol and acetophenone are easily interconvertible, carbinols and ketones having intrinsic energies of the same order (cf. Parks & Haffman, 1932). β -Phenylethanol, the primary alcohol, may also be con-

verted to acetophenone, by oxidation with NaOBr and Grignard reagents (Palfray, Metayer & Panouse, 1947). The course of this reaction has been represented as,



Cases of β -oxidation of a side chain exist in the photochemical oxidation of hydroxynaphthoquinone (Ettlinger, 1950).

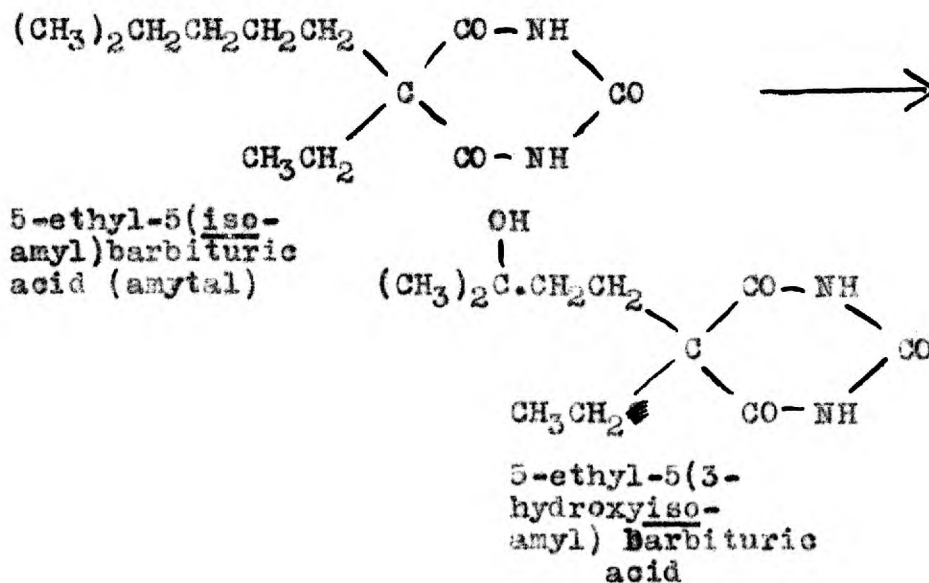


where $\text{R} = \text{CH}_3, \text{C}_2\text{H}_5, \text{CH}(\text{CH}_3)_2, \text{C}_6\text{H}_5, \text{CH}_2\cdot\text{C}_6\text{H}_{11}$.

A free radical mechanism involving the formation of hydroperoxides has been suggested.

This mechanism must be quite distinct from the γ -oxidation of hydrolapachol ($\text{R} = \text{CH}(\text{CH}_3)_2$) by chromic acid for which hydroxyislapachol ($\text{R} = \text{COH}(\text{CH}_3)_2$) has been isolated (Fieser, 1948). It might be more correct to refer to this reaction as penultimate oxidation. Maynert (1952) has oxidised an alkyl derivative of

barbituric acid with CrO_3 in acetic acid at the penultimate, and tertiary, C atom, i.e.



Metabolism of some Alkylbenzenes and Related Compounds

Toluene

In rabbits up to 70% of the dose of injected toluene has been accounted for as benzoic acid (Epstein & Braunstein, 1931); in dogs 80-90% of the dose as hippuric acid (Knoop & Gehrke, 1925). It has appeared probable that the toluene unaccounted for in these experiments was eliminated from the lungs. Srbova & Teisinger (1952) exposed persons to toluene in gas chambers and afterwards during the desaturation time, measured an average excretion of 16.3% of the dose in the expired air. No ethereal sulphate excretion has been observed (Baumann & Herter, 1877) or glucuronide conjugation (Bray, Thorpe & White, 1951).

Ethylbenzene and its oxidation products

The metabolism of ethylbenzene has been quite extensively studied by various workers. Nencki & Giacosa (1880) fed ethylbenzene to dogs and isolated 1/6 of the dose as hippuric acid. They showed there was no excretion of ethereal sulphate. As ethylbenzene was oxidised with CrO_3 to acetophenone and this substance also formed hippuric acid in the body they postulated that acetophenone was an intermediate in the metabolism of ethylbenzene, i.e.

initial oxidation occurred at the α -C atom of the chain. Neubauer (1901) found evidence for the presence of glucuronic acid conjugates in ethylbenzene urine by colour reactions and observed a laevorotation of the urine. Acetophenone gave similar results, styrene did not, and he postulated that both ethylbenzene and acetophenone were eliminated as methylphenylcarbinylglucuronic acid, and that styrene was not an intermediate.

Thierfelder & Daiber (1923) fed ethylbenzene and acetophenone by subcutaneous injection to rabbits and observed a similarity in their metabolism.

	Acetophenone	Ethylbenzene
Glucuronide	35.7% $[\alpha]_D -124.27^\circ$	$[\alpha]_D -122.37^\circ$
Benzoic acid	24.3%	
Mandelic acid	1.8% inactive	1.9% laevorotatory
Ethereal sulphate	slight rise	
Free acetophenone	trace	

The glucuronide was isolated as the monohydrate of the potassium salt and mandelic acid as the cadmium salt. Optical rotations were measured in aqueous solution. The free carbinol obtained by acid hydrolysis of the

glucuronide was slightly dextrorotatory.

When dl-methylphenylcarbinol was injected into rabbits (Thierfelder & Klenk, 1924), 35.6% of the dose was obtained as benzoic acid, 0.4% as mandelic acid and 25% as the glucuronide, $\alpha_D -117.4^\circ$. They suggested that, as the glucuronide was presumably ($\pm$)methylphenylcarbinyl-glucuronide, the metabolisms of ethylbenzene and acetophenone proceeded by asymmetric oxidation and reduction, respectively.

It has been judged unlikely that mandelic acid is an intermediate in the formation of benzoic acid from ethylbenzene, acetophenone or methylphenylcarbinol, as it is mainly excreted unchanged when administered to an animal (Williams, 1947). The small laevorotation, observed by Thierfelder & Daiber (1923) of the cadmium mandelate isolated from ethylbenzene urine may be accounted for by the greater ease with which the (+)isomeride is oxidised to benzoylformic acid (Williams, 1947).

Styrene fed by stomach tube caused a rise in the neutral sulphur excretion of rabbits and rats, accounting for 5-15% of a dose as mercapturic acid (Spencer, Irish, Adams & Rowe, 1942). 50-90% was excreted as hippuric acid

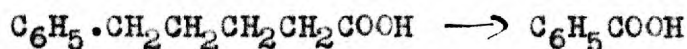
(isolated as benzoic acid). Neubauer's experiments (1901) suggested that only a small fraction of the styrene administered to dogs was excreted as glucuronide.

No evidence has been reported for the presence of phenylacetic acid as a metabolite of ethylbenzene and its oxidation products.

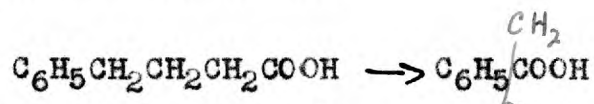
Compounds containing long aliphatic chains

Normal propyl-, butyl and amylbenzenes were fed to rabbits by Thierfelder and Klenk (1924) and the nature of the acidic products (benzoic or phenylacetic acid) suggested that β -oxidation of the alkyl chain was occurring, corresponding to the β -oxidation of the corresponding acid, observed by Knoop (1905) (cf. Breusch, 1948).

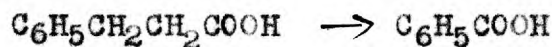
Knoop fed the corresponding acids to dogs and isolated benzoic or phenylacetic acid, free and as the glycine conjugate with the following results.



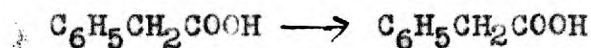
ω -phenylvaleric acid



ω -phenylbutanoic acid



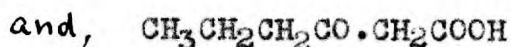
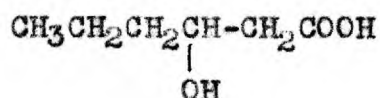
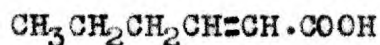
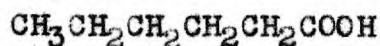
ω -phenylpropionic acid



These results were confirmed by Raper & Wayne (1928) and extended to the higher homologues. Schroenheimer &

Rittenberg (1937) using deuterium labelled stearic acid demonstrated its conversion into palmitic acid in the animal body.

From the 2C fragment liberated by β -oxidation, ketone bodies are produced and Dakin (1923) showed that acetone was produced in amounts of the same order by liver perfusion experiments using caproic acid and its oxidative products, i.e.



Mazza (1935) (from Breusch, 1948) isolated phenylcrotonic acid ($\text{C}_6\text{H}_5\text{CH}_2\text{CH}=\text{CH}.\text{COOH}$) from liver extracts incubated with ω -phenylbutanoic acid.

More evidence that $\alpha\beta$ -unsaturated acids are intermediates in β -oxidation has been furnished by Quick (1928) who measured the proportion of benzoic and phenylacetic acids excreted as glucuronides and glycine conjugates

after feeding saturated and unsaturated acids to dogs. Thus for both cinnamic and ω -phenylpropionic acid the ratio glucuronide/glycine conjugate was 3:1; for -phenylbutyric acid and ω -phenylisocrotonic acid the ratio was 2:1.

Dakin provided chemical confirmation of the theory by demonstrating that protons located at the β -C atom can be easily replaced by oxygen, e.g. with H_2O_2 . $\alpha\beta$ -Unsaturated acids behaved similarly (Wieland, 1924, 1925) and H_2O_2 reacted with palmitic acid to form β, γ and δ keto acids (Clutterbuck & Raper, 1925). A close relationship between a biological process and a free radical reaction has therefore been established.

In a study of branched chain fatty acids, Wick (1941) demonstrated that an unsubstituted α -C atom and one free H on the β -C atom are required for ketogenic activity.

If the interpretation of results gained by the study of free and substituted fatty acids can be applied to alkylbenzene oxidation, as the work of Thierfelder & Klenk might support, their ω -oxidation of the chain occurs as an initial reaction and this is followed by β -oxidation. $\alpha\beta$ -Unsaturated, β -hydroxy, and possibly β -keto acids are intermediates.

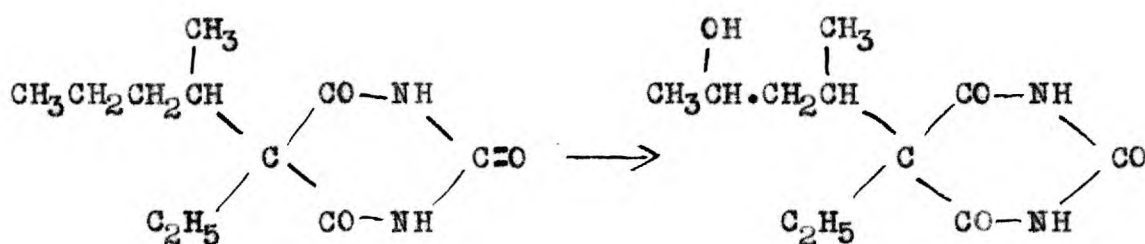
ω -Oxidation has been established as a minor pathway

for fatty acids of medium chain length, although of no significance in the normal metabolic breakdown of fatty acids in the body (Breusch, 1948). Other cases of ω -oxidation have been reviewed by Lincke (1945).

Recent work on the fate in the body of compounds containing alkyl side chains attached to a ureide or naphthoquinone moiety has demonstrated the occurrence of other forms of oxidation.

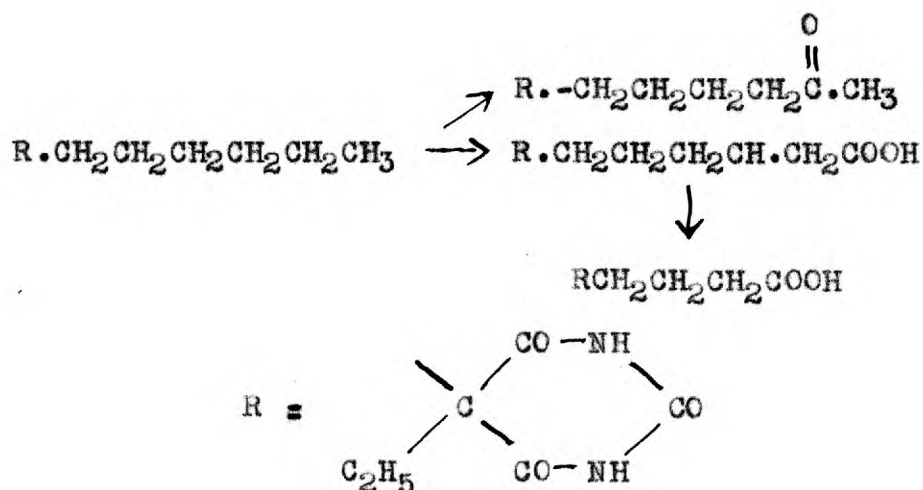
Maynert (1952) identified the major metabolite, in dogs, of amytal, with the penultimate oxidation products already considered in the preceding section.

Oxidation of the sec-amyl chain in pentobarbital occurred at the penultimate or γ -C atom.



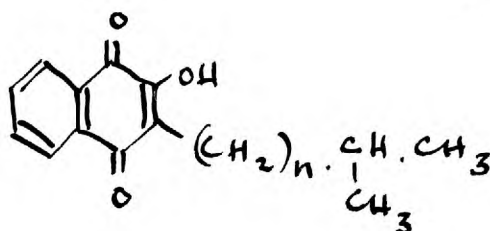
That it was, indeed, penultimate oxidation, has been proved by the investigation of the metabolites of ortal, (5n-hexyl,5-ethylbarbituric acid). Two of the compounds were acids. One of these was the ω -oxidation product;

the other, the corresponding β -oxidation product. The third metabolite identified was the methyl ketone formed by penultimate oxidation, i.e.

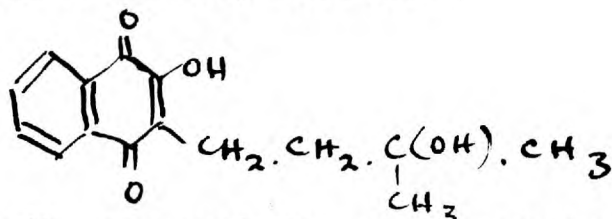


Brodie, Mark, Papper, Lief, Bernstein & Rovenstein (1950) demonstrated that the ω -carboxylic acid derivative of thiopental (in which an O of the ring of pentobarbital has been replaced by S) was excreted in amounts of 10-20% of the dose in man. Maynert has concluded that penultimate oxidation is less general than ω -oxidation.

Results, obtained by Fieser, Chang, Dauber, Heidelberger, Heymann & Seligman with 2-hydroxy-3-alkyl-1:4-naphthoquinones, support this view. Some of the compounds investigated had the general formula,



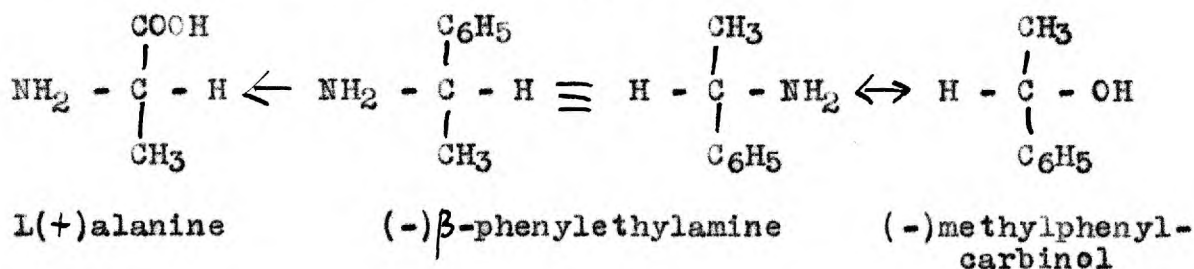
ω -Oxidation occurred, with formation of a carboxylic acid for the substances $6 > n > 2$. But hydrolapachol ($n = 2$) produced a tertiary carbinol



identical with the compound formed by chromic acid oxidation (Fieser et al, 1948).

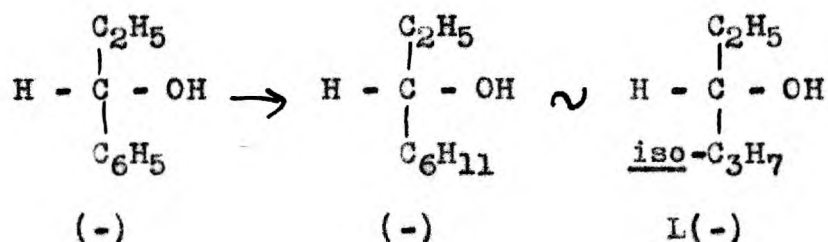
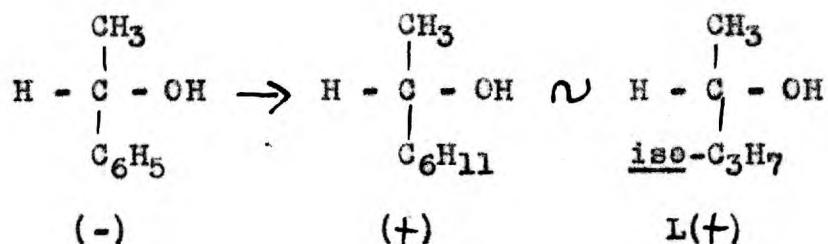
The Stereochemical Relationships of Alkyl-aryl carbinols

(-)-Methylphenylcarbinol has been related, configurationally, to (-)- β -phenylethylamine (Brewster, Hiron, Hughes, Ingold & Rao, 1950) by kinetic studies of the deamination of the latter compound. And (-)- β -phenylethylamine has been oxidised to L(+)-alanine, therefore (-)-methylphenylcarbinol has been shown to be of the L(-) structure.



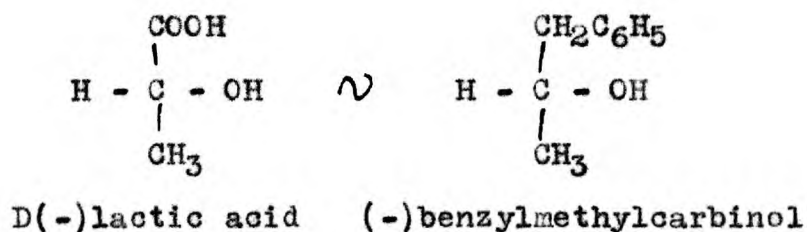
Homologues of methylphenylcarbinol have the same configuration, although reduction with hydrogen in the presence of platinum oxide of methylphenylcarbinol and the higher alkylphenylcarbinols yields alkylcyclohexyl-carbinols of opposite rotations, (+) and (-) respectively (Levene & Stevens, 1930). The cyclohexyl compounds have been shown to be related to each other in the same manner as alkylisopropylcarbinols. The configuration of these aliphatic carbinols has been established (Freudenberg, 1933).

The steps in the argument, for methylphenylcarbinol and ethylphenylcarbinol, are as follows :-

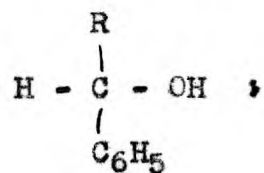


The sign $\sim$ denotes that the substances are configurationally similar.

Benzylmethylcarbinol has been chemically correlated with aliphatic carbinols which can be oxidised to lactic acid (Levene & Walti, 1931). Thus (-)benzylphenylcarbinol was found to be of the same configuration as (-)lactic acid, i.e. possesses a D(-) structure.

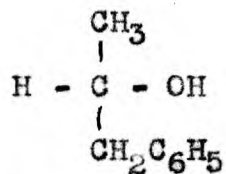


The molecular rotations have been recorded by
Freudenberg (1933) and are as follows, for the structure



R = methyl	$[\text{M}]_{\text{D}}$	-52
ethyl	"	-75.5
propyl	"	-85.8

For benzylmethylcarbinol



$$[\text{M}]_{\text{D}} = +38.5$$

CHAPTER IIOxidation of Ethylbenzene and Related CompoundsMaterials

All the compounds, except styrene epoxide, were purchased and purified by distillation (cf. Table 1). Styrene epoxide was prepared by the method of Hibbert & Burt (1925) (from Organic Syntheses, 1925).

TABLE I

		b.p. (°C)
Ethylbenzene	L. Light & Co. Ltd.	135
(±)Methylphenylcarbinol	B.D.H. Ltd.	205
Acetophenone	Hopkin & Williams, Ltd.	202
Styrene	Hopkin & Williams, Ltd.	146
Styrene epoxide		188-192
β-Phenylethanol	L. Light & Co. Ltd.	220

They were administered with water by stomach tube to Chinchilla doe rabbits of 2-4 kg. weight at a dose level (unless otherwise stated) of 3.8 mg./moles/kg. Preliminary to and during experimental periods, the rabbits were kept on a constant diet of 70 g. rat cubes (Diet 41, Assoc. London Flour Millers) and 100 ml. water per day.

Reactions of Urine

Colour tests were carried out on the urine excreted during the days succeeding dosing. The reactions given in Table 2 for first day's urine were very much reduced in intensity on the second day after feeding.

Reactions

The urine possessed a slightly acid reaction.

Conjugation with glucuronic acid was detected by the naphthoresorcinol reaction. A few drops of the urine reacted on boiling with 0.5 ml. conc. HCl and 5 mg. of the solid reagent to form a blue precipitate, soluble in ether.

Reducing properties were investigated using Benedict's and Fehling's reagents. No reducing substances were detected in the urines.

The absence of phenolic derivatives of the substances in the urine was demonstrated in tests with aqueous 1% FeCl_3 and alcoholic dichloroquinone-chloroimide solutions. No colours resulted when the solutions were made alkaline with NaHCO_3 or Na_2CO_3 . The test was repeated on ether extracts of slightly acidified urine with the same negative results. Ketones were tested for with aqueous and alcoholic Brady's reagent (2:4-dinitrophenylhydrazine).

TABLE 2

Excretion of metabolites after dosing with ethylbenzene and related compounds

Substance	Qualitative reactions on ether extract of acidified urine		Quantitative results expressed as average excretion (% dose)		
	Glucuronides	Ketones	No. fed	Glucuronides	Ethereal sulphates
Ethylbenzene	+	-	3	32	0.4
(+)Methylphenylcarbinol	+	-	3	50	nil
(-)Methylphenylcarbinol	+	-	1	47	nil
(+)Methylphenylcarbinol	+	-	2	52	-
Acetophenone	+	slight	3	47	2.2
Styrene		+	1	16	1.0
Styrene epoxide	-	++	2	15	nil
β -Phenylethanol		-	3	7	nil

Glucuronide and Ethereal Sulphate Formation.

The increases in excretion of glucuronides and ethereal sulphates after dosing were measured, respectively, by the method of Paul (1952, cf. Appendix II) and Sperber (1948, cf. Appendix III). Protocols of the animal experiments are included in Appendix III.

The results of these quantitative experiments are recorded in Table 2.

Isolatory Procedures

Details of experiments in this section are given in Table 3 at the end of the chapter.

Measurement of the optical rotatory power of glucuronide derivatives was made on a 1% solution in chloroform using a Bellingham & Stanley polarimeter, Model A.

Melting points are corrected.

Ethylbenzene

1 g. ethylbenzene was fed to each of two rabbits and the 24 hr. urine and washings (300 ml.) were acidified to pH 3 with H_2SO_4 and extracted with ether in a continuous extractor for 6 hours. The residue from the ether was extracted with petrol ether (to remove any trace of ketone) and then with a few ml. water (to remove the

glucuronide) and hippuric acid crystals remained with m.p. 189° after one recrystallisation from water; mixed m.p. 189° (yield 200 mg.).

Ten rabbits were fed a total of 1.7 g. ethylbenzene and the 24 hr. urine (1700 ml.) treated by the lead acetate procedure for the isolation of a glucuronide (Kamil, Smith & Williams, 1951). The acid non-reducing glucuronide gum (soluble in ethanol) was dissolved in the least amount of methanol and methylated with ethereal diazomethane, and this derivative was acetylated overnight in solution with 5 equivalents acetic anhydride and of pyridine. Dilution of the reaction mixture with water gave the triacetyl methyl ester of the glucuronide (10.6 g.) as an amorphous solid which was well washed and powdered; $[\alpha]_D^{20} -50.2^{\circ}$.

Seven recrystallisations from ethanol and 95% ethanol, in the course of which the melting range and rotation steadily rose, produced 2.74 g. white needles, m.p. 117° , $[\alpha]_D^{19} -83^{\circ}$. This substance proved to possess, on further recrystallisations, a constant m.p. and optical rotation.

Analysis :

Found : C, 57.8	$C_{21}H_{26}O_{10}$ requires C, 57.51%
H, 6.26	H, 5.98%

Later work identified this compound with the triacetyl methyl ester of (-)methylphenylcarbinyglucuronide. There was no depression of m.p. with the ester obtained when (-)methylphenylcarbinol was fed to rabbits.

From the liquors, material with $[\alpha]_D^{19} -35^\circ$ was recovered and this was recrystallised from methanol. A small amount of crystals with a sharper and a higher melting range separated and after six recrystallisations, 0.11 g. of white needles was obtained, m.p. above 130° , $[\alpha]_D^{22} -10^\circ$.

Analysis :

Found : C, 57.8	$C_{21}H_{26}O_{10}$ requires C, 57.51%
H, 6.25	H, 5.98%

Only one spot developed on a paper chromatogram of the glucuronic acid gum in butanol/acetic acid/water (4:1:5 by vol.).

(±)Methylphenylcarbinol

At the dose level of 0.5 g./kg., at which these experiments were carried out, the rabbit lost consciousness within a few minutes of feeding. There was a pronounced reduction in the reflexive response to stimuli. The coma continued for periods up to one hour.

A total of 24 g. was fed to 12 rabbits and the 24 hr. urine was treated by the lead acetate technique to obtain an acid glucuronide gum, soluble in ether/ethanol. After methylation and acetylation, 18.84 g. of the crude triacetyl methyl ester was produced, $[\alpha]_D^{20} -51^\circ$.

This material was a mixture of equal parts of the diastereoisomers, and - triacetylmethyl esters of methylphenylcarbinylglucuronide as was shown by separation of the substances by fractional crystallisation using methanol and ethanol according to Fig. 1.

The diastereoisomers possessed the following characteristics :-

(-) ester, m.p. 117° , $[\alpha]_D^{20} -83^\circ$

Analysis :

Found : C, 57.31	$C_{21}H_{26}O_{10}$	requires C, 57.51%
H, 5.62		H, 5.98%

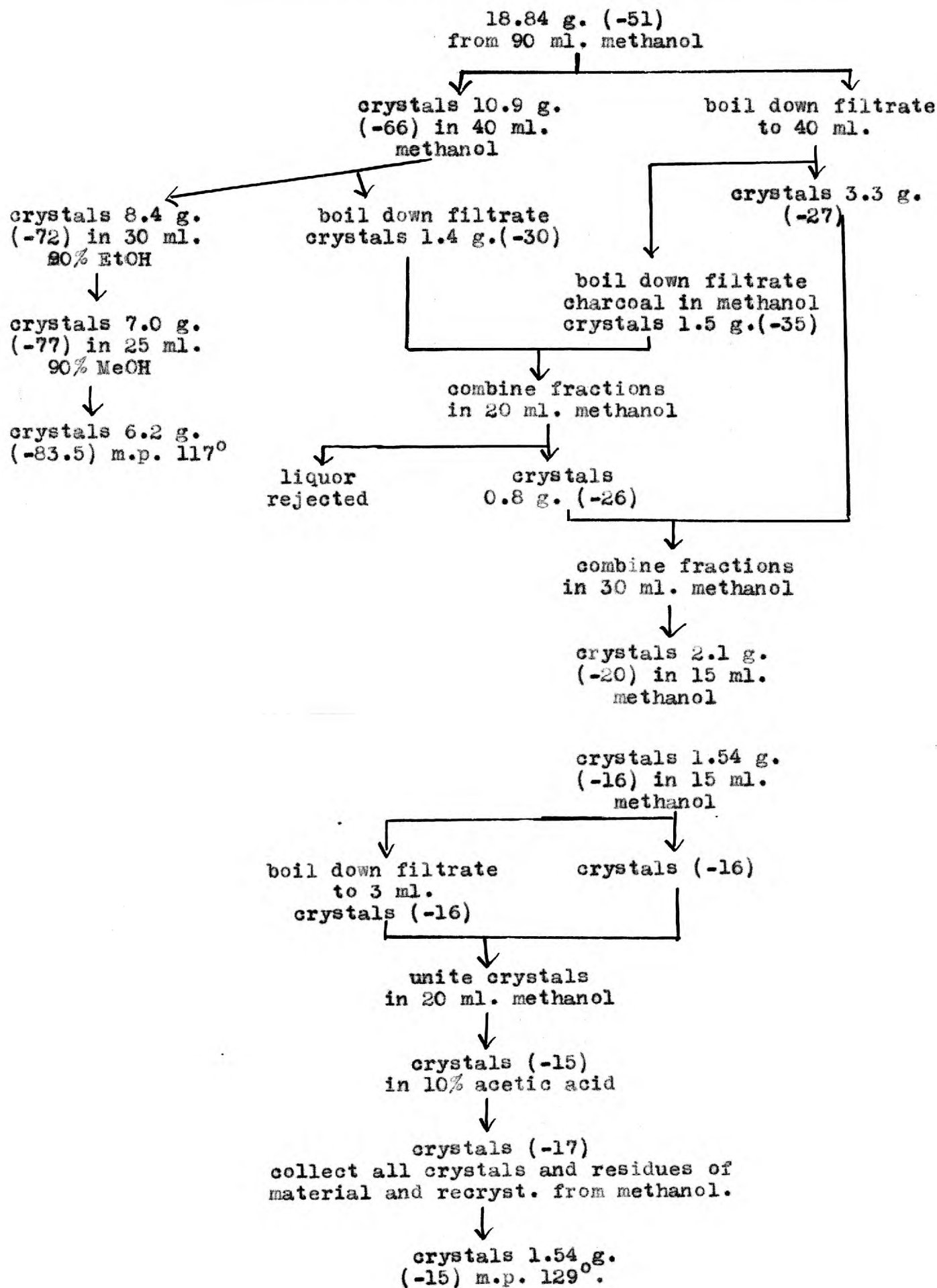
(+) ester, m.p. 129° , $[\alpha]_D^{19} -15^\circ$

Analysis :

Found : C, 57.53	$C_{21}H_{26}O_{10}$	requires C, 57.51%
H, 5.83		H, 5.98%

No change in the melting point or rotation of these two substances occurred when they were crystallised from pure and aqueous methanol and ethanol, dilute acetic acid or n-hexane.

Fig. 1. The separation of the diastereoisomers of the triacetylmethyl ester of methylphenylcarbinylglucuronic acid by fractional crystallisation



The figures in brackets refer to the specific rotation of the material.

(-)-Methylphenylcarbinol

The pure isomer, b.p. 204°C , $[\alpha]_{\text{D}}^{20} -47^{\circ}$ ($c = 1$ in ethanol) was fed to a single rabbit, 2.8 kg. weight. From a 2 g. dose, 400 mg. acid glucuronide gum was obtained and by methylation and acetylation, 160 mg. of the crude ester was formed as an off-white material $[\alpha]_{\text{D}}^{15} -82^{\circ}$. One crystallisation from the least amount of 90% aqueous ethanol (2 ml.) gave 90 mg. white crystals, m.p. 117° , $[\alpha]_{\text{D}}^{15} -82.5^{\circ}$.

Analysis :

Found : C, 57.88	$\text{C}_{21}\text{H}_{26}\text{O}_{10}$	requires C, 57.51%
H, 6.13		H, 5.98%

A mixed m.p. determination proved that this material was identical with the pure (-)-diastereoisomer obtained from (+)-methylphenylcarbinol urine, and from ethylbenzene urine (see above).

(+)-Methylphenylcarbinol

In one experiment a rabbit (3.5 kg. weight) was fed 1.83 g. material, $[\alpha]_{\text{D}}^{20} 40.14^{\circ}$ (cf. Houssa & Kenyon, 1930, obtained (+)-carbinol with $[\alpha]_{\text{D}} 42.88^{\circ}$).

By the customary procedure, 65 mg. crude triacetyl

methyl ester of the glucuronide was obtained as a white powder with $[\alpha]_D^{19} -30^\circ$. When crystallised from 70% aqueous methanol, the crystals possessed $[\alpha]_D^{19} -21^\circ$. Thus there was a mixture of the two diastereoisomers (90% of + form) in the product but insufficient material was available for either one to be prepared pure.

In other experiments corresponding results were obtained, i.e.

Material fed	Material isolated
(c = 1 in EtOH)	
3.0 g. $[\alpha]_D 36^\circ$ (90% (+))	0.9 g. $[\alpha]_D -37^\circ$ (70% (+))
1.1 g. $[\alpha]_D 35.5^\circ$ (88% (+))	0.103 g. $[\alpha]_D -29^\circ$ (79% (+))

Acetophenone

In one experiment a total of 9.6 g. acetophenone was fed to six rabbits and the 24 hr. urine was treated by the lead acetate precipitation method for the isolation of the glucuronide. 4.0 g. gum was obtained, soluble in ethanol, and small amounts were used in attempts to form the p-toluidine, benzylamine and potassium salts but no satisfactory substance was isolated.

Four rabbits were fed a total of 8.0 g. acetophenone

and the crude triacetyl methyl ester of the glucuronide was obtained as amorphous orange material (5.0 g.) with $[\alpha]_D^{20} -79.2^\circ$. One crystallisation from the minimum amount of ethanol gave 4.4 g. white needles, m.p. 117° , $[\alpha]_D^{20} -83.5^\circ$.

Analysis :

Found :	C, 57.58	$C_{21}H_{26}O_{10}$	requires	C, 57.51%
	H, 6.11			H, 5.98%

There was no depression in a mixed m.p. with the ester of (-)methylphenylcarbinyglucuronide already characterised.

Styrene and styrene epoxide

Sixteen rabbits were fed a total of 30 g. styrene and from the 24 hr. urine, 6.8 g. acid glucuronide gum was obtained. The triacetyl methyl ester of the glucuronide would not solidify, but a measurement of the specific rotation of the gum was made; $[\alpha]_D^{19} 41.5^\circ$ ($c = 1$ in $CHCl_3$). The gum would not crystallise from ethanol, methanol or n-hexane.

The excretion of ether soluble acid was followed over the experimental period on the urine of one rabbit,

fed 1.68 g., using the method of Bray, Neale & Thorpe (1946). A doubling of the normal values occurred on the day after feeding, equivalent to an excretion of 39% of the dose. This result may have been affected by the loss in appetite for food and water, which occurred in rabbits after feeding styrene at this dose level (3.8 mg.moles/kg.). 60-90% of styrene fed to rats, rabbits and guinea pigs has been accounted for as hippuric acid in the urine (Spencer, Irish, Adams & Rowe, 1942).

2.9 g. styrene epoxide was fed to two rabbits (4 mg. moles/kg.) and a measurement of the optical activity of the first 24 hr. urine was made on one of the urines (250 ml.) after a few drops HCl had been added and the urine charcoaled; α (at 22°C) + 0.28° (2 dec. tube). This positive rotation would suggest that both styrene and its epoxide are excreted as the same glucuronide, which is distinct from the glucuronide occurring in urine when the saturated compounds, ethylbenzene, the methylphenylcarbinols and acetophenone are fed.

The amount of ketone in styrene epoxide urine was estimated gravimetrically on each rabbit.

The ketone, in 25 ml. urine, (total volume, 250 ml.)

was precipitated by adding 50 ml. aq. Brady's reagent. After 24 hours standing the material was collected in a sintered glass crucible and dried.

For the purpose of the calculation, the ketone was assumed to be acetophenone, but insufficient material was isolated for this to be confirmed.

Rabbit No.	30	25
Dose (g.)	1.54	1.33
Dinitrophenylhydrazone isolated (mg.)	34.3	34.2
% dose excreted as ketone (for acetophenone)	8.46	9.77
Average excretion	9.1% of dose	

Mercapturic acid excretion was also estimated on the urines of rabbits fed styrene epoxide since Spencer et al (1942) have observed a rise in neutral sulphur excretion after dosing rats and rabbits with styrene. Mercapturic acids were estimated by the iodine titration method of Stekol (Parke & Williams, 1951). The excretion was doubled on the experimental day, equivalent to an elimination of 0.9 and 0.7% of the dose (average excretion, 0.8%).

β -Phenylethanol

The 24 hr. urine of three rabbits fed a total of 3.2 g. β -phenylethanol was made acid, pH 3, with H_2SO_4 and extracted continuously with ether for 12 hours. On evaporation, the ether extract deposited crystals and gum. The material was recrystallised from 80% ethanol as purple tinted needles, (0.5 g.), unlike hippuric acid in form, m.p. 143° .

Mixed m.p. with phenaceturic acid, 143° .

Estimation of ether soluble acid excretion (Bray, Neale & Thorpe, 1946) by two rabbits (fed 1.3 and 1.8 g.) over the experimental periods demonstrated a doubling in the urinary acids during the two days after feeding, equivalent to an excretion of 60.5 and 76% of the dose (average excretion 69%).

TABLE 3

Isolation of Glucuronides

Substance	Amt. fed (g.)	As Glucuronide gum (g.)	As ester (g.)	$[\alpha]_D$ ester (crude)	$[\alpha]_D$ isomers isolated
Ethylbenzene	17	18.5	10.6	-50.2°	{ -83° -10°
(+)-Methylphenylcarbinol	12	14.6	8.6	-54.0°	{ -83.5° -15.0°
(-)-Methylphenylcarbinol	2.0	0.4	0.16	-82.0°	-82.5°
95% (+)-Methylphenylcarbinol	1.83	0.9	0.65	-30	-
Acetophenone	8	7.5	5.0	-79.2°	-83°
Styrene	27	6.8	1.7	+ 41.5°	-

CHAPTER IIIOxidation of Propyl-, Butyl- and Amylbenzenes
and Related CompoundsMaterials

When available, substances required were purchased and purified by distillation before use (Table 4).

TABLE 4

		b.p.(°C)
<u>n</u> -Propylbenzene	Eastman Organic Chemicals	159
Propiophenone	B.D.H. Ltd.	218
Benzylmethylketone	B.D.H. Ltd.	216
<u>Isopropylbenzene</u> (cumene)	B.D.H. Ltd.	152
<u>n</u> -Butylbenzene	B.D.H. Ltd.	180
Butyrophenone	Eastman Organic Chemicals	232
<u>sec</u> -Butylbenzene $C_6H_5CH(CH_3)C_2H_5$	B.D.H. Ltd.	173
<u>tert</u> -Butylbenzene $C_6H_5C(CH_3)_3$	B.D.H. Ltd.	167
<u>sec</u> -Amylbenzene $C_6H_5CH(CH_3)C_3H_7$	Eastman Organic Chemicals	189
<u>tert</u> -Amylbenzene $C_6H_5C(CH_3)_2C_2H_5$	B.D.H. Ltd.	189

All the carbinols required and methylphenethylketone were prepared in the laboratory according to the following procedures :-

Ethylphenylcarbinol, $C_6H_5CHOH.C_2H_5$, was prepared by the reduction of propiophenone with $LiAlH_4$ according to Brown (1951). An ethereal solution of the carbinol was dried over anhydrous Na_2SO_4 and then $MgSO_4$. The material distilling at $107^\circ C$ (15 mm.Hg) was collected in 85% yield.

Benzylmethylcarbinol, $C_6H_5CH_2CHOH.CH_3$, was prepared by an identical method, collecting the distillate at $105^\circ C$ (15 mm.Hg) in 80% yield; b.p. 228° at 779.3 mm.Hg.

Phenylpropylcarbinol, $C_6H_5CHOH.C_3H_7$, was prepared from butyrophenone by reduction with an excess of sodium in ethanol solution. The reactants were heated on a water bath to complete the reduction and after addition of water, the carbinol (free from ketone) separated as an oil. This was washed with water, dried over Na_2SO_4 and distilled under reduced pressure, 20 mm.Hg, at $120-125^\circ C$ in a yield of 25%.

Methylphenethylcarbinol, $C_6H_5CH_2CH_2CHOH.CH_3$, by the reduction of benzal acetone ($C_6H_5CH=CH.CO.CH_3$) with $LiAlH_4$ (cf. Brown, 1951), was distilled under reduced pressure in a 70% yield.

Methylphenethylketone, $C_6H_5CH_2CH_2CO.CH_3$, was also prepared from benzal acetone, using sodium amalgam (made by the method of Vogel, 1948) as the reducing agent. The ketone was separated from starting material and the carbinol by forming a bisulphite compound. After decomposition of this derivative in 10% H_2SO_4 , the oil was washed with water, dried over Na_2SO_4 and distilled. The fraction distilling at $236^{\circ}C$ was collected in a yield of 80%.

The detoxication of certain of the compounds, chosen for study in this section has been investigated before, with the following results.

n-Propylbenzene (Thierfelder & Klenk, 1924) - Benzoic acid was the only acid isolated from the urine of dogs after dosing and it was not found possible to isolate the glucuronide.

Propiophenone (Thierfelder & Daiber, 1926) - Subcutaneous injections into rabbits of daily doses of 2 g. produced only hippuric acid and a glucuronide, which analysed as the potassium salt of ethylphenylcarbinylglucuronide;

$[\alpha]_D^{15} -126.1^{\circ}$.

Benzylmethylketone (Dakin, 1908/9) - In the dog, hippuric acid was formed after subcutaneous injection of the ketone.

Cumene (Thierfelder & Klenk, 1924) - In dogs and rabbits, cumene produced no hippuric acid or any other acid. It caused an increase in the excretion of ethereal sulphate (Nencki & Giacosa, 1880).

Butylbenzenes (Thierfelder & Klenk, 1924) - These compounds gave no rise in the hippuric acid excretions of dogs and no other organic acid was detected but in rabbits phenacetic acid was isolated after feeding n-butylbenzene. There was no discernible rise in ethereal sulphate excretion after injection into dogs of n- and sec-butylbenzenes.

Butyrophenone (Thierfelder & Daiber, 1921) - From rabbit urine, after subcutaneous injection of the ketone, propylphenylcarbinyglucuronic acid was isolated as its cadmium salt; $[\alpha]_D^{13} -108.7^{\circ}$. Hippuric acid was the only organic acid isolated.

The compounds were administered under the conditions described in Chapter II.

Reactions of Urine

Iodoform and Rothera tests, when applicable, were made on urines after acid hydrolysis, in addition to the tests defined in Chapter II.

Rothera reaction - Drops of freshly prepared aqueous sodium nitroprusside and of .880 ammonia added to 5 ml. test solution saturated with $(\text{NH}_4)_2\text{SO}_4$ gave a violet colouration when substances containing a CH_3CO grouping were present.

Iodoform reaction - The reaction of penultimate keto and carbinol groups with hypiodite in dioxane solution was conducted according to the directions of Fuson & Tullock (1934) (from Higginbottom, 1948).

No colour reactions with phenolic reagents and Brady's reagent were observed in the urines and the ether extracts of slightly hydrolysed urines. Glucuronides were present in all cases and these were ether soluble. Further reactions of the urines and the quantitative figures for glucuronide, ethereal sulphate and mercapturic acid excretion are given in Tables 5 and 6.

TABLE 5

Reactions of urines after feeding Propyl-, Butyl-
and Amyl-Benzenes

Substance	Reducing	Tests Iodoform	Rothera
<u>n</u> -Propylbenzene	-	+	-
Ethylphenylcarbinol	-	-	-
Propiophenone	-	-	-
Benzylmethylcarbinol	-	+	-
Benzylmethylketone	-	+	-
Cumene	+	-	-
<u>n</u> -Butylbenzene	-	-	-
Butyrophenone	-	-	-
Methylphenethylketone	-	-	-
<u>sec</u> -Butylbenzene	-	+	-
<u>tert</u> -Butylbenzene	-	-	-
<u>sec</u> -Amylbenzene	-	+	-
<u>tert</u> -Amylbenzene	+	-	-

TABLE 6

Excretion of metabolites after feeding Propyl-, Butyl- and Amyl-benzenes and

Related Compounds

Substance	No. fed	Average excretion (% dose)		
		Glucuronides	Ethereal sulphate	Mercapturic acids
<u>n</u> -Propylbenzene	3	21	2.0	-
Ethylphenylcarbinol	3	54	nil	-
Benzylmethylcarbinol	3	43	1.7	-
Cumene	2	69	1.1 [Ⓜ]	-
<u>n</u> -Butylbenzene	3	43	5.1	1.3
<u>sec</u> -Butylbenzene	3	66	11.3 [Ⓜ]	nil
<u>tert</u> -Butylbenzene	2	67	3.3	nil
<u>sec</u> -Amylbenzene ¹	3	51	nil [*]	-
<u>tert</u> -Amylbenzene ¹	3	48	2.6	-

[Ⓜ] denotes an oil formed in the hydrolysate

¹ from the results of Miss E. Smalley

Isolatory Procedures

The specific rotations of glucuronide esters were measured using 1% solutions in chloroform. Melting points are corrected.

n-Propylbenzene

The urine of two rabbits fed a total of 3.0 g. n-propylbenzene was acidified to pH 3 with 20% H_2SO_4 and extracted continuously with ether for 10 hours. On evaporation of the extract, 0.5 g. hippuric acid crystallised out and after one recrystallisation from water possessed m.p. 184° (mixed m.p. 184°). The liquor was further evaporated to a gum containing a non-reducing glucuronide which failed to crystallise free or as the potassium, benzylamine or toluidine derivative.

Ethylphenylcarbinol

In an attempt to isolate the glucuronide, 510 ml. urine, excreted within 24 hours of feeding a total dose of 4.3 g. to three rabbits was treated by the lead acetate precipitation method. 2 g. acid gum (soluble in ethanol/ether) was obtained, from which 1.4 g. triacetyl methyl ester was prepared as a liquid gum, which would not crystallise from ethanol, methanol or n-hexane.

Propiophenone

From a total feed of 8 g. propiophenone to four rabbits, the 24 hr. urine (500 ml.) yielded a glucuronide gum which was neutral. Inorganic material was removed by precipitation with ethanol/ether and the gum acidified with a few drops 20% H_2SO_4 . The excess of inorganic acid was precipitated by titration with $\text{Ba}(\text{OH})_2$ using sodium rhodizionate as an indicator for Ba ions. 1.0 g. acid gum was obtained. This was methylated and acetylated, yielding 0.36 g. amorphous ester, $[\alpha]_{\text{D}}^{18} -69.5^\circ$. Two crystallisations from the least amount of 80% aqueous ethanol produced the pure compound; 0.16 g., m.p. 126° , $[\alpha]_{\text{D}}^{18} -80^\circ$.

Analysis :

Found : C, 58.47	$\text{C}_{22}\text{H}_{28}\text{O}_{10}$	requires C, 58.34%
H, 6.258		H, 6.232%

Further crystallisations from aqueous ethanol and methanol did not affect the physical constants and thus propiophenone was excreted as a single diastereoisomer of ethylphenyl-carbinylglucuronide.

Benzylmethylcarbinol

4.5 g. Benzylmethylcarbinol was fed to three rabbits

and the 24 hr. urine (540 ml.) was worked up to 2 g. non-reducing acidic glucuronide gum, soluble in ethanol/ether. Attempts to isolate the potassium, barium, p-toluidine and benzylamine derivatives failed and the triacetyl methyl ester of the glucuronide was obtained as a liquid gum which did not crystallise from the usual solvents.

Benzylmethylketone

Two rabbits were fed a total of 4 g. benzylmethylketone and from the 24 hr. urine (250 ml.) 1.0 g. clear, non-reducing acidic glucuronide gum was prepared. 0.5 g. was used in attempts to prepare salts without any satisfactory products being isolated and the remainder was methylated and acetylated. The triacetylmethyl ester (0.35 g.), $[\alpha]_D^{18} -23^\circ$, was unaffected in specific rotation by crystallisations from 70% aqueous ethanol and 60% aqueous methanol. The final product was isolated as long white needles, m.p. 124° , $[\alpha]_D^{18} -24^\circ$.

Analysis :

Found : C, 58.35% $C_{22}H_{28}O_{10}$ requires C, 58.34%

H, 6.43%

H, 6.232%

Thus, a single diastereoisomer of benzylmethylcarbinyl-glucuronide had been excreted by rabbits fed the ketone.

n-Butylbenzene

On two occasions, the urines of rabbits fed n-butylbenzene (total doses 12 and 5 g.) were treated by the basic lead acetate precipitation method for the isolation of the glucuronide. Small amounts of glucuronide gum (1.5 and 0.6 g.) were obtained but the triacetylmethyl ester remained liquid and did not crystallise from the usual solvents. Attempts to isolate the aglycones were hampered by the rapid dehydrogenation of the alcohols in acid solution during hydrolysis. This was observed even when the concentration of acid (H_2SO_4) was reduced to 0.5N and the acidified material steam distilled without preliminary refluxing. The instability of these carbinols has been referred to by Wheland (1949) and their tendency to rearrange to form branched chain compounds noted. The unsaturated derivatives isolated by extraction with ether, or by steam distillation of the hydrolysate, were isolated in two fractions.

Thus from a feed of 10 g. (four rabbits) there was collected 0.8 ml. oil; b.p. 90-100°C at 14 mm.Hg (201°C at 759.7 mm.Hg); $\eta = 1.5330$; $\lambda_{max} 250 m\mu$, $E_{1\%}^{1cm} 55$ in ethanol; slowly reducing a solution of $KMnO_4$ in acetone;

and 0.8 ml. oil; b.p. 120-130° at 14 mm.Hg (247°C at 751.7 mm.Hg); $\eta = 1.5178$; λ_{max} 280 $m\mu$, $E_{1\text{ cm.}}^{1\%}$ 300 in ethanol; possessing a rapid reducing action.

Neither of these compounds can be identified by b.p. or refractive index with the characterised unsaturated butylbenzene derivatives.

Butyrophenone

The 24 hr. urine from a feed of 12 g. butyropheneone to six rabbits was treated by the lead acetate precipitation method and 12.2 g. non-reducing acid glucuronide gum was obtained soluble in ether/ethanol mixtures. From 7 g. the triacetylmethyl ester of the glucuronide was prepared as a solid gum, 5.75 g., $[\alpha]_D^{20} -63^\circ$. It failed to form well defined crystals from ethanol or methanol but from 150 ml. 50% acetic acid solution, by slow evaporation, 1.84 g. needles were deposited, m.p. 98-100°, $[\alpha]_D^{20} -78.8^\circ$. The rotation remained constant with further crystallisations from ethanol/water mixtures. Final m.p. 104°.

Analysis :

Found : C, 58.99% $C_{23}H_{30}O_{10}$ requires C, 59.2%
H, 6.685% H, 6.482%

No crystalline material was isolated from the residues

and it has therefore been concluded that butyrophenone formed only one diastereoisomer of phenylpropylcarbinol which is excreted as the glucuronide.

Methylphenethylketone

8.0 g. acid glucuronide gum, isolated from the urine of six rabbits fed a total of 12 g. methylphenethylketone was shown to contain only one glucuronide by paper chromatography in a butanol/acetic acid/water mixture (4:1:5 by vol.). A spray of naphthoresorcinol dissolved in aqueous trichloroacetic acid gave a blue colour with the glucuronide spot after heating the paper at 110° for 5 mins.

5 g. of the gum was methylated and acetylated but the ester gum remained liquid and did not crystallise from ethanol, methanol or 50% acetic acid.

CHAPTER IV

The Excretion of Alkylbenzenes in the expired air of rabbits.

Introduction

A balance sheet for the metabolism of benzene in the rabbit has been prepared by Parke & Williams (1952). 39% of a dose of benzene administered orally was recovered from the expired air. Various halogenobenzenes have been investigated by Azouz, Parke & Williams (1952). Unchanged material has been identified and estimated in the expired air.

The average amount of toluene exhaled by nine persons in twelve experiments exposed in gas chambers to doses of 271-1177 γ /l. was found to be 16.3% of the dose (Srbová & Teisinger, 1952).

Method

Rowe, Atchison, Luce & Adams (1943) have determined mixtures of styrene and alkylbenzene in air spectrophotometrically by passing the vapour through bead-packed absorbers containing EtOH, cooled in solid CO₂.

The apparatus employed in the following experiments has been described by Parke & Williams (1950) (cf. Azouz,

Parke & Williams, 1952). The rabbit was placed immediately after oral feeding in a perspex tank, 24" x 13" x 10". The absorption train generally consisted, in order, of a bottle containing anhydrous CaCl_2 , two bottles containing anhydrous magnesium perchlorate (Anhydrone) and then two bottles containing glass beads and 12 ml. absolute ethanol to absorb the vapours. The last two bottles were immersed in a freezing solution at -70°C .

The first ethanol bottle was renewed periodically throughout the time of the experiment. The concentration of hydrocarbon in the ethanol solution was determined spectrophotometrically. During each estimation, the whole absorption curve was determined.

U.V. absorption data :-

Toluene, b.p. 110° ; λ_{max} 262 $\text{m}\mu$; ϵ_{max} 228.

Ethylbenzene, b.p. 135° ; λ_{max} 261 $\text{m}\mu$; ϵ_{max} 217.

n-Propylbenzene, b.p. 157° ; λ_{max} 261 $\text{m}\mu$; ϵ_{max} 197.

(Magat & Maier (1943) record $\log \epsilon = 2.32$ at 261 $\text{m}\mu$).

The recovery of the vapour from the tank was checked by exposing known amounts of toluene and ethylbenzene in it. Thus when 266 mg. freshly distilled toluene was exposed, 257.6 mg. was recovered in the first bottle and 10.2 mg.

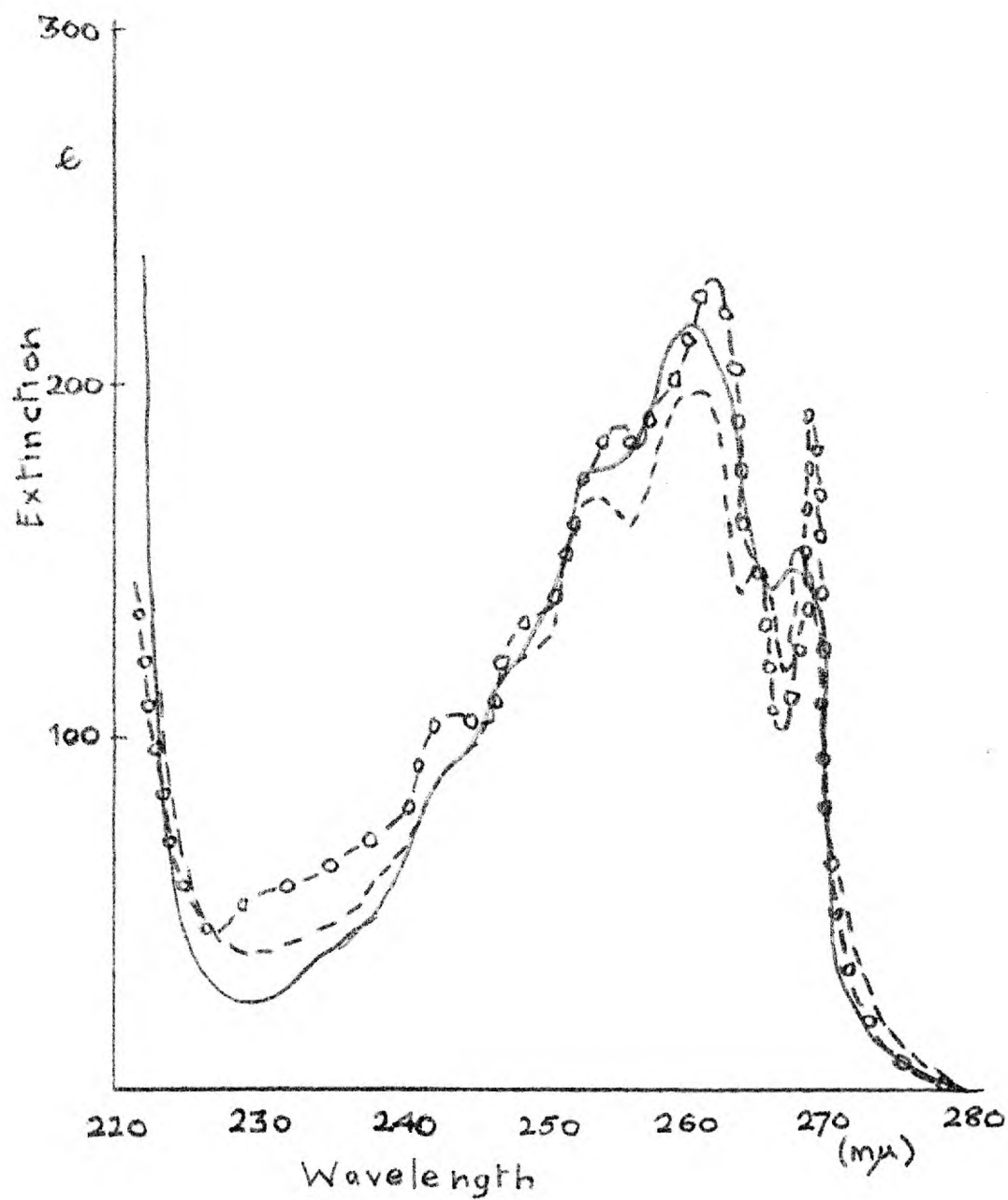
Fig. 2. U.V. Absorption spectra of alkylbenzenes in ethanol.

-o-o- Toluene

— Ethylbenzene

Slit width : 1.0 mm.

----- n-Propylbenzene



in the second (total, 267.8 mg. or 100.6%) after drawing a current of air from outside the laboratory through the tank for 7.75 hours. When 441 mg. ethylbenzene was exposed in the tank, a recovery of 82.2% was achieved in 6 hours and of 103.8% in 17 hours. Each experiment was continued for further periods of time but insufficient amounts of material were collected for quantitative measurements to be made.

Experimental

Toluene

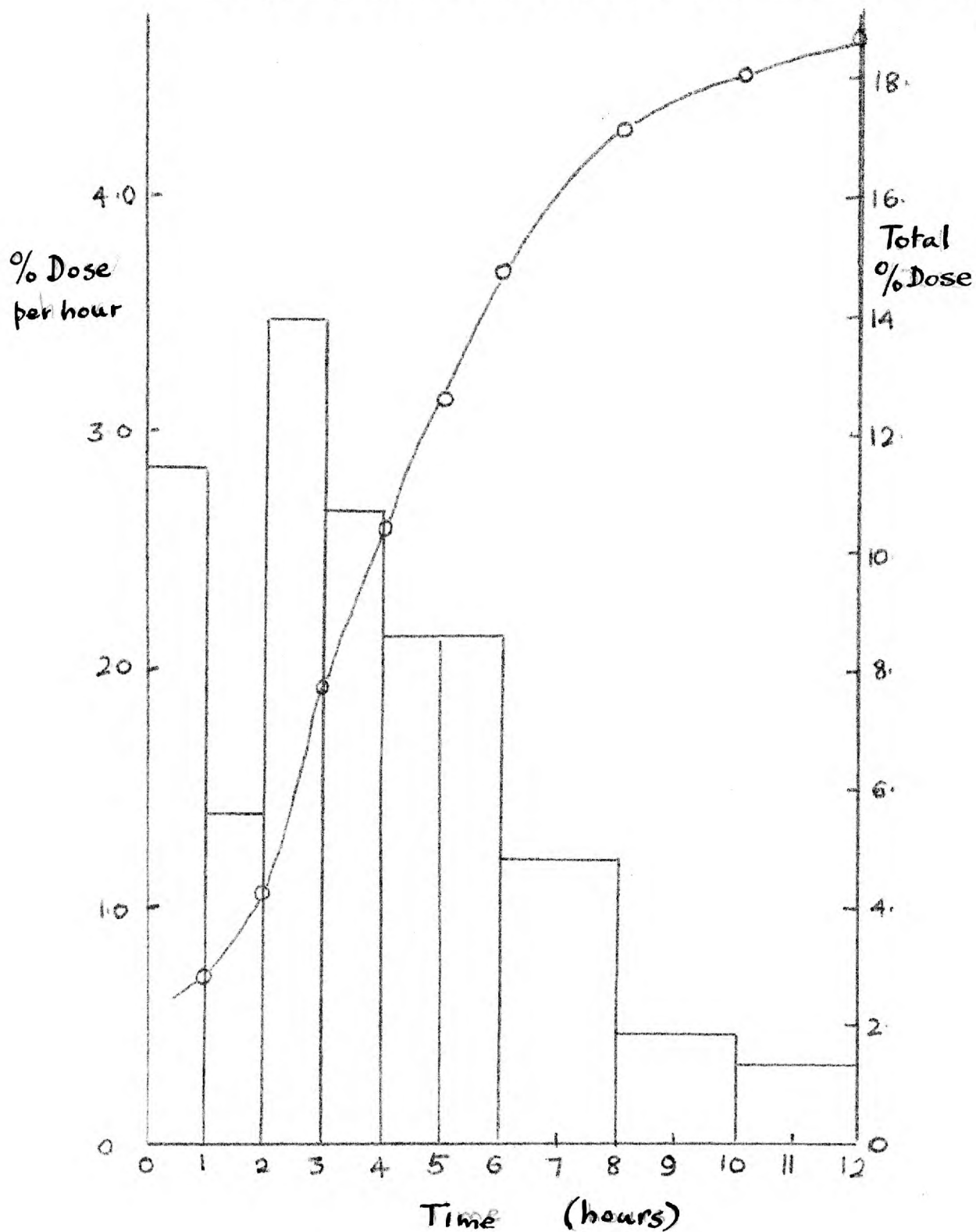
Rabbit No. 70, 3.6 kg., was fed 1.5 ml. toluene (3.8 mg.moles/kg.) and placed immediately in the metabolism tank. A bottle containing 150 ml. Brady's reagent (a saturated solution of 2:4-dinitrophenylhydrazine in dilute HCl) was inserted between the absorption train and the tank. In the first 12 hours after feeding, 15.8% of the dose was accounted for and a further 1.6% was measured in the first ethanol bottle in the 8 hours following. No hydrazone formed in the Brady's reagent showing that if benzaldehyde was formed from toluene, it did not appear in the expired air.

In another experiment, rabbit No. 79 (3.6 kg.) was fed 1.45 ml. toluene (3.8 mg.moles/kg.) and the rate of excretion, followed by regular estimation of the absorption in the ethanol bottles. The experiment was continued for 12 hours and the results are recorded graphically in Fig. 3.

Fig. 3. Excretion of toluene in the expired air.

Histogram - % Dose excreted/hour.

Curve - Total % dose excreted at time of measurement.



The total excretion of toluene was 18.75% of the dose. No urine was passed by the rabbit during the time of the experiment.

For these two animal experiments, the average elimination of toluene via the lungs equalled 18.1% of the dose.

Ethylbenzene

A preliminary 6 hr. experiment in which 1.5 g. ethylbenzene was fed to a rabbit, showed that the excreted vapour did not have a simple composition. The absorption spectra in ethanol of styrene and dl-methylphenylcarbinol were determined since they were possible constituents of a mixture.

U.V. absorption data :-

Styrene, dried over CaCl_2 and distilled at reduced pressure,

λ_{max} 245 $\text{m}\mu$, ϵ_{max} 14100 in ethanol, $\log \epsilon = 4.15$

(Magat & Maier (1943) record $\log \epsilon = 4.0$ at 246 $\text{m}\mu$)

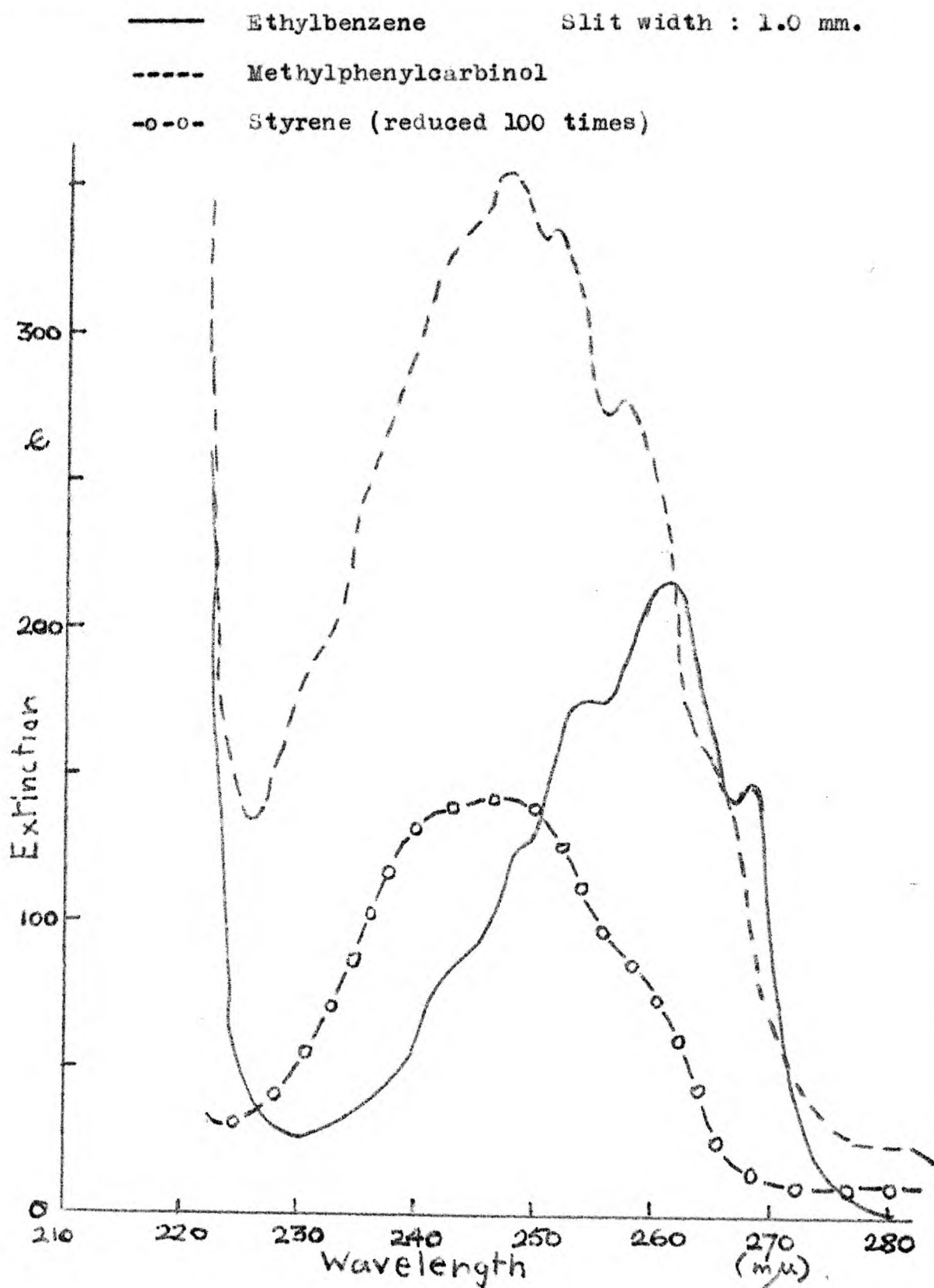
dl-Methylphenylcarbinol, b.p. 205°C ,

λ_{max} 247 $\text{m}\mu$, ϵ_{max} 356 in ethanol.

The curves for ethylbenzene, styrene and dl-methylphenylcarbinol are compared in Fig. 4.

1.5 g. ethylbenzene was fed to a rabbit (2.85 kg. weight) (5.2 mg.moles/kg.) and the expired air passed

Fig. 4. U.V. Absorption spectra in ethanol.



through a bottle containing 150 ml. aqueous Brady's solution between the tank and the absorption train. During the time of the experiment, no hydrazone crystallised from this solution. At the first change, 3 hr. after feeding, the spectrum of the ethanol solution was a smooth curve with two peaks at 250 and 260 $m\mu$. As the experiment continued, the peak at the lower wavelength assumed prominence and moved down the spectrum until in the period of 20-27 hours after feeding, there was a single peak, flattened like styrene and with maximal absorption at 245 $m\mu$. In this period of time, only 0.3 mg. styrene (0.0002% of the dose) was required to produce the extinction observed.

In another experiment, 1.5 g. ethylbenzene was fed (5 mg./moles/kg.) and the expired air passed through a U tube immersed in the freezing mixture before being led directly to the bottles containing ethanol. Thus Anhydrone, which absorbs alcohols, was excluded from the absorption train. A peak at 260 $m\mu$ (presumably due to ethylbenzene) remained prominent as the experiment continued. A mixture of oil and ice collected in the U tube, from which 60 mg. of the oil was separated, with maximal absorption between 260-270 $m\mu$. Methylphenylcarbinol (m.p. 20°C and $\epsilon_{\max}$ at 247 $m\mu$) did not therefore, appear to be present in the expired vapour.

n-Propylbenzene

A rabbit, 3.2 kg. weight, was fed 1.7 ml. n-propylbenzene (3.8 mg.moles/kg.) and placed immediately in the tank. A bottle containing 150 ml. aqueous Brady's solution was inserted between the tank and the absorption train. No formation of hydrazone was observed during the time of the experiment. The total spectrum was measured at each estimation and only in the first curve obtained 2 hours after feeding was there evidence that the amount determined from the extinction at 261 $m\mu$ differed grossly from the propylbenzene present. In the 13 hours of the experiment, 7.2% of the dose was collected in the first absorbing bottle. The second bottle contained too small an amount for it to be estimated.

The interfering substance excreted in the first 2 hours appeared to have a maximal extinction at 258-9 $m\mu$. This value compared well with the maximal peaks of the spectra of ethylphenylcarbinol and of benzylmethylcarbinol, illustrated in Fig. 5.

U.V. absorption data :-

Ethylphenylcarbinol (b.p. 107° at 15 mm.Hg)

$\lambda_{\max}$ 258 $m\mu$; $\epsilon_{\max}$ 173.

Benzylmethylcarbinol (b.p. at 779.3 mm.Hg)

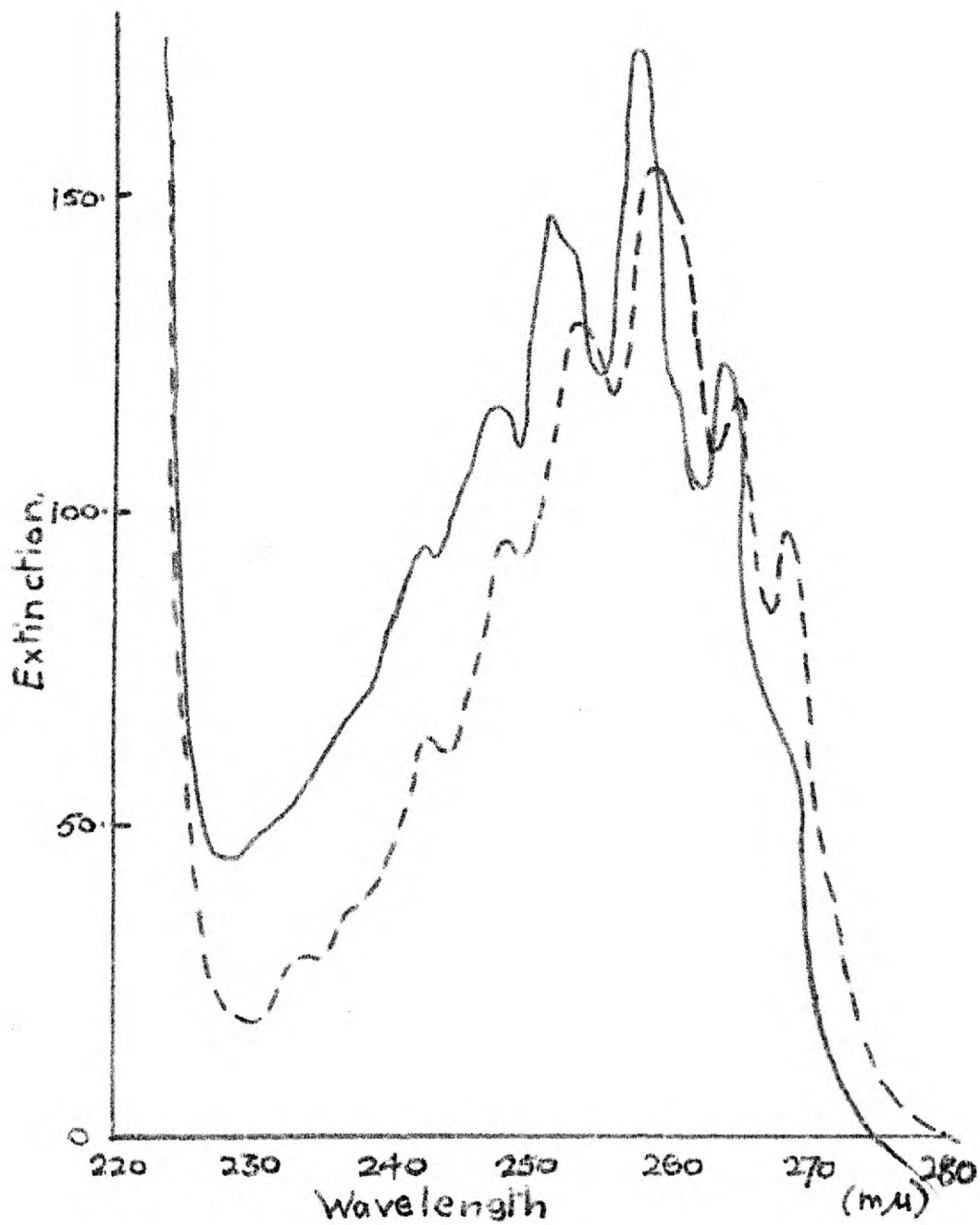
$\lambda_{\max}$ 258.5 $m\mu$; $\epsilon_{\max}$ 192.

At least one of these substances is an intermediate in the metabolism of n-propylbenzene (cf. Chapter III).

Fig. 5. U.V. Absorption spectra in ethanol.

— Ethylphenylcarbinol
----- Benzylmethylcarbinol

Slit width : 1.0 mm.



CHAPTER V.

Discussion

Measurements of the amount of unchanged alkylbenzene present in the expired air of rabbits after dosing, by the methods detailed in Chapter IV, has proved that 18.1% of toluene and 7.2% of n-propylbenzene are lost in this way. A small amount of n-propylbenzene was exhaled in a metabolised form - probably as ethylphenyl- or benzyl-methylcarbinol, during the first two hours.

No figures were obtained from ethylbenzene feeding experiments because of the complex nature of the expired vapour.

Ketones were not eliminated via the lungs.

It is unlikely that higher alkylbenzenes are lost from the animal, by respiration, in significant amounts.

Thus, 81.9% of toluene ingested, an indefinite proportion of ethylbenzene and 92.8% of n-propylbenzene were metabolised. The fate in the body of alkylbenzenes, and their oxidation products, is considered in this discussion of results.

The Metabolism of Ethylbenzene and its Oxidation Products

Formation of glucuronides

Results of previous workers, which demonstrated that a major pathway for ethylbenzene metabolism was via oxidation of the α -C atom of the side chain have been confirmed. ($\pm$)Methylphenylcarbinol has been shown to yield the ($\pm$)methylphenylcarbinyglucuronide in rabbits, by isolation of the separate diastereoisomers of the triacetylmethyl esters of the glucuronides from the urine. Confirmation of this fact has been obtained by feeding the resolved carbinols. Thierfelder & Klenk (1924) assumed that the ($\pm$)methylphenylcarbinol produced a mixture of the diastereoisomers, but their experimental results are in conflict with the findings of the present study. They suggested (in accordance with the earlier work of Thierfelder & Daiber, 1923) that the material they isolated was the potassium salt of ($\pm$)methylphenylcarbinyglucuronide, but the high rotation quoted ($[\alpha]_D -127^\circ$, in water) is more suggestive of a resolved form, not a pseudo-racemic mixture. The potassium salts of the glucuronides from ethylbenzene and acetophenone possessed rotations of the same order, ($[\alpha]_D -122^\circ$ and -124° respectively).

Hydrolysis of the material obtained from acetophenone urine led to isolation of the carbinol as a slightly dextrorotatory compound. But the conditions of isolation of the glucuronide as a potassium salt, which is a difficult procedure, not accomplished with any of the compounds in this study, made it very possible that selective crystallisation of one isomer should occur and racemization of the glucuronide from acetophenone may have happened during the acid hydrolysis. In the present experiments, these faults have been avoided by measuring rotations on the crude amorphous triacetylmethyl esters of the glucuronides. The isolation of the separate diastereoisomers of these derivatives by fractional crystallisation, provided a means of estimating the relative proportions of the diastereoisomers in the crude material, without hydrolysis to the aglycone. Thus, since the diastereoisomers of the triacetylmethyl esters of methylphenylcarbinyl glucuronide possessed rotations of $[\alpha]_D -15^\circ$ and -83° (average -49°), the material obtained from both ethylbenzene and (+)methylphenylcarbinol urine was, with $[\alpha]_D -50^\circ$, an approximately equimolecular mixture. Recrystallisation of the ester from ethylbenzene urine led to the isolation of material

with a slow melting range and very low rotation ($[\alpha]_D -10^\circ$). The evidence suggests that this was the (+) isomer, adulterated, but the material was lost before the physical properties could be checked and the work will be repeated.

The results of paper chromatography of the glucuronide gum from ethylbenzene urine, suggest that only methylphenylcarbinyglucuronides were present.

From acetophenone, only one isomer was formed, (-)methylphenylcarbinyglucuronide. The effect on the optical rotation of formation of a triacetylmethyl ester from a glucuronide has been studied with the menthols (cf. Appendix IV). The ratios

$$\frac{[M]_D \text{ glucuronide (in water)}}{[M]_D \text{ triacetylmethyl ester of glucuronide (in CHCl}_3\text{)}}$$

given below suggest that the material isolated by Thierfelder & Daiber (1923) from acetophenone was in fact the potassium salt of (-)methylphenylcarbinyglucuronide.

$$\begin{array}{rcl} \frac{[M]_D \text{ (-)menthylglucuronide}}{[M]_D \text{ triacetylmethyl ester}} & = & 1.11 \\ \frac{[M]_D \text{ (+)isomenthylglucuronide}}{[M]_D \text{ triacetylmethyl ester}} & = & 1.02 \\ \frac{[M]_D \text{ glucuronide from acetophenone}}{[M]_D \text{ (-)triacetylmethyl ester}} & = & 1.01 \end{array}$$

($[M]_D$ = molecular rotation)

Measurements of glucuronide output of rabbits fed styrene, styrene epoxide and β -phenylethanol, suggest, because of the small percentage of the dose excreted in this way, that these substances are not obligatory intermediates in the oxidation of ethylbenzene to methylphenylcarbinol. Moreover, the glucuronides from styrene and styrene epoxide are dextrorotatory.

Formation of acids

Hippuric acid was isolated from ethylbenzene urine on the first day after feeding, in a yield of 20% of the dose. There was no indication of the presence of phenaceturic acid as a metabolite. Ethylbenzene is not, therefore, detoxicated via terminal oxidation products.

70% of a dose of β -phenylethanol was accounted for as ether soluble acid in the two days after feeding. Only phenaceturic acid was isolated (15% of the dose).

Excretion as ethereal sulphate

Ethylbenzene and its oxidation products produced no very significant rises in ethereal sulphate excretion (i.e. excretion as phenols).

On the other hand, some 60% of a dose of benzene

administered to rabbits can be accounted for by oxidation to phenols (Porteous & Williams, 1949 a and b). Substituted benzenes - nitro-, amino-, halogeno- and cyano-benzenes are all partially detoxicated as phenols. (Smith, 1950).

The extent of hydroxylation in the ring cannot, satisfactorily, be related to the ease or difficulty of detoxication by other pathways. Thus, Thierfelder & Daiber (1923) found ethylbenzene was retained in the body for several days and acetophenone was eliminated within a few hours of feeding. Yet, the excretion of acetophenone as ethereal sulphate is five times that of ethylbenzene (Table 2).

The enzyme systems concerned in hydroxylation of the ring are probably quite distinct from those promoting chain oxidation. β -Diethylaminoethyldiphenylpropyl acetate hydrochloride (SKF 525 A) potentiates the action of various drugs, including the dialkylbarbiturates and the alkyl substituted 2-hydroxy 1:4-naphthoquinones by its blocking of side chain oxidation ^{Axelrod.} (Reichenthal, [^]quinn & Brodie, 1953), but has no action on ring hydroxylation (private communication, Brodie).

The Metabolism of Higher Alkylbenzenes

Formation of glucuronides

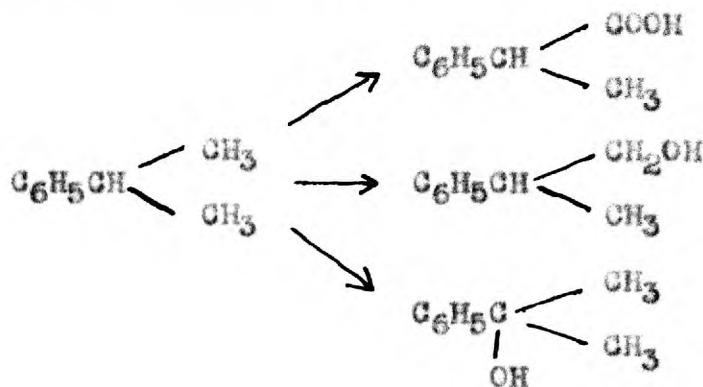
No glucuronides were isolated from the urines of rabbits fed *n*-propyl or butylbenzenes. The carbinols derived from *n*-propylbenzene, ethylphenylcarbinol and benzylmethylcarbinol were similar in that from them were produced gums of the triacetylmethyl esters, which did not crystallise. All attempts to isolate a crystalline triacetylmethyl ester of the glucuronide formed from the aryl-alkyl ketones were successful with the exception of the gum from phenethylmethylketone urine. It would appear that the formation of mixtures of the diastereoisomerides from the alkylbenzenes and carbinols prevented crystallisation. Ketones produced a single isomer which was therefore more easily crystallised from the gum. The lowering in m.p. of the esters, accompanying the increase in the size of the alkyl group, may be partly responsible for the failure of mixtures to crystallise.

Thierfelder & Daiber (1923) reported the isolation of glucuronides from propiophenone and butyrophenone and in each case, the high optical rotation of the metal salt suggests that only one isomer was obtained.

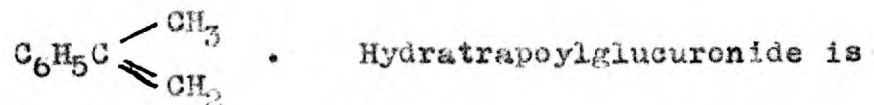
Qualitative reactions of urines

Qualitative tests on the urines of animals fed alkylbenzenes and their oxidative products showed that in all cases the glucuronide was soluble in ether. No ketones were detected in the urines by the Rothera or dinitrophenylhydrazine reagents, but n-propyl, sec-butyl and sec-amyl radicals are probably oxidised at the penultimate C atom, as positive iodoform reactions were obtained on hydrolysates of the urines. Methylphenethylketone gives a very poorly defined iodoform reaction and none was observed on the urines of rabbits dosed with it.

Reducing metabolites (probably ester glucuronides) were detected in the urines of rabbits fed cumene and tert-amylbenzene. The metabolism of cumene has been further studied (Robinson, Smith & Williams, unpublished results) and three glucuronides have been identified in the urine; of hydratropic acid, hydratropic alcohol and dimethylphenylcarbinol, i.e.



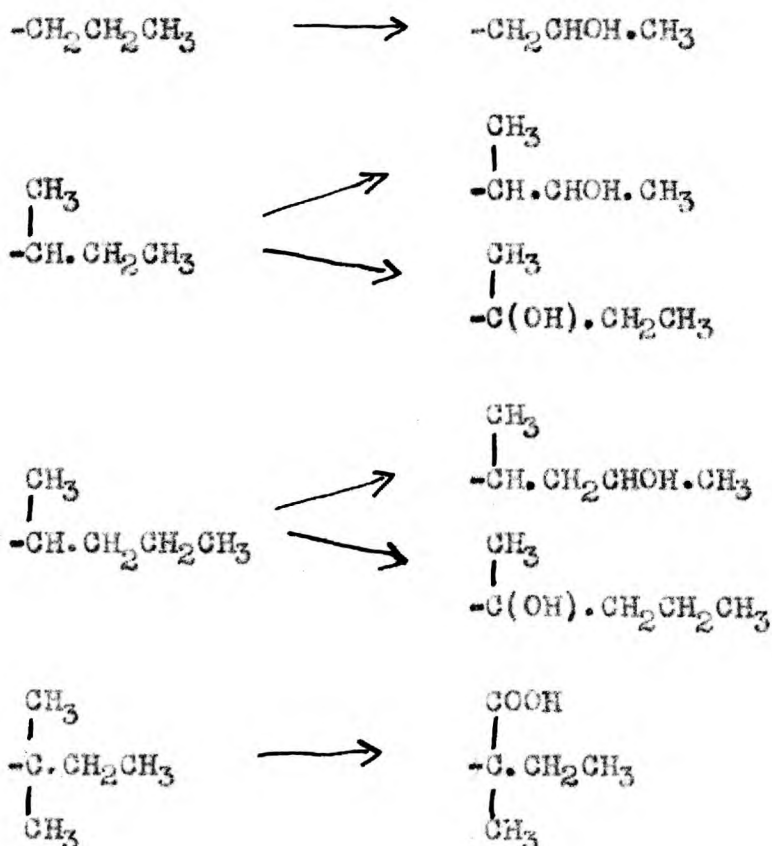
The stability of these glucuronides to acid hydrolysis decreases in the order acid glucuronide > primary alcohol glucuronide > tertiary carbonylglucuronide. Dimethylphenyl-carbonylglucuronide is hydrolysed, with extreme ease, and the free alcohol is rapidly dehydrogenated to form α -methylstyrene



strongly reducing to Benedict's and Fehling's solutions.

The proof of the formation of a tertiary carbonylglucuronide from cumene was foreshadowed by the ease with which hydrolysis of cumene urine occurred. Thus, oily droplets appeared during the hydrolysis in 0.2N-HCl during the estimation of ethereal sulphates (cf. Table 6). A similar turbidity occurred with sec-butyl and sec-amylbenzene urines. It would appear probable that tertiary carbonylglucuronides (conjugated at the α -C atom) are formed from these substances as well.

The conclusions concerning the products of side chain oxidation of alkyl chains attached to the benzene nucleus, suggested by these qualitative reactions are summarized below.



Hippuric acid was isolated from n-propylbenzene urine in a yield of 1/6 of the dose. The acid is the product of terminal oxidation followed by β -oxidation. Its isolation has been recorded by Thierfelder & Klenk (1924).

Excretion as ethereal sulphate

No generalisations concerning the factors affecting hydroxylation of the ring of higher alkylbenzenes can be drawn from the figures for ethereal sulphate conjugation.

Qualitative tests for phenols in hydrolysed urines were negative. Small rises in the ethereal sulphate excretion can be neglected as rises of this order can be caused by ingestion of water alone. n-Propylbenzene, benzylmethylcarbinol, sec- and tert-butylbenzenes and tert-amylbenzene were excreted in significant amounts as ethereal sulphates (cf. Table 6).

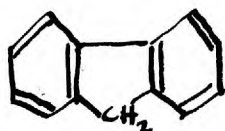
Mode of Oxidation of Alkylbenzenes

A homolytic fission in a molecule to form free radicals requires less expenditure of energy than heterolytic or ionic fission. In a medium of high dielectric constant the position is reversed and in the polar systems of the animal body either type of breakdown is permissible (Evans, 1946). Ethylbenzene possesses a reactive α -C atom and although free radical mechanisms have generally been postulated for its oxidation by purely chemical means (Chapter I), loss of H from the α -C atom in the body may result in the formation of an α -phenylethyl radical, $\text{C}_6\text{H}_5\dot{\text{C}}\text{H}\cdot\text{CH}_3$ or a carbonium ion, $\text{C}_6\text{H}_5\text{CH}^+\text{CH}_3$ according to the environment. In either case, the ease of formation of the initial oxidation product,

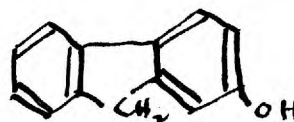
radical or ion, is determined by the energy required for rupture of a C-H bond. The bond dissociation energy decreases with substitution of the C atom by alkyl radicals (Sware, 1951). In conjunction with this fact, the figures for glucuronide conjugation suggest that formation of a free radical or carbonium ion is the first step in oxidation. The branched alkylbenzenes of the form $C_6H_5CH.R_1R_2$ (R_1 and R_2 = alkyl groups) consistently produced higher figures for glucuronide conjugation than the straight chain analogues. The results of the study of cumene metabolism and the qualitative reaction to mild hydrolysis of sec-butyl and sec-amylbenzenes suggest that the tert-carbinyglucuronides form a large proportion of the total amount of glucuronide excreted.

However, there does not appear to be a direct relationship between the presence in a molecule of a reactive α -methylene group and the extent of glucuronide conjugation. Kooyman (1951) found fluorene to have a particularly high reactivity towards free radicals (thirtyfive times that of ethylbenzene) but the extent of conjugation as glucuronide is only moderate in the rabbit (16% of a dose; Smith, unpublished results) and Neish (1948) has produced evidence

that fluorene is excreted as 2-hydroxyfluorene, and that this phenol is partially conjugated as a glucuronide.



fluorene



2-hydroxyfluorene

Moreover, organic acids were not isolated by Thierfelder & Klenk (1924) after feeding cumene and the branched butylbenzenes to rabbits. Thus, the high glucuronide conjugation, spread over 2-3 days, of these substances may be the result of the limited means by which they can be excreted (Appendix III).

Ketones cannot be considered as intermediates in the formation of carbinols from alkylbenzenes because of their asymmetric hydrogenation (Chapters I and III).

Styrene and styrene epoxide are not obligatory intermediates in ethylbenzene metabolism as they produce only small rises in glucuronide output (equivalent to 15-16% of the dose). Neubauer (1901) found stereochemical evidence that styrene was not an intermediate in ethylbenzene metabolism. The equivalent of 39% of a dose of styrene was excreted as ether soluble acid.

Mercapturic acid excretion was raised following the

administration of styrene by 50-250% (Spencer et al, 1942). In this study, mercapturic acids were estimated after feeding styrene epoxide. Less than 1% of the dose was accounted for, but this is equivalent to a rise in the daily output of mercapturic acid of 200-250%. Styrene and its epoxide were the only two compounds studied which produced detectable amounts of ketone in the urine. Kawabata (1945) fed allylbenzene to dogs and showed that the detoxication products were quite distinct from those formed from saturated alkylbenzenes. He proved that 70% of the dose was excreted as benzyl alcohol, 20% as *p*-hydroxyphenylacetic acid, 5% as cinnamic acid and a small amount as α -hydroxy β -phenylpropionic acid.

Qualitative reactions suggest that penultimate oxidation is a feature of alkylbenzene metabolism but no products have been isolated. Penultimate oxidation is a major pathway in dialkyl-barbiturate metabolism (Maynert, 1952), i.e. of amytal (5-sec-amyl-5-ethylbarbituric acid) and of ortal (5-n-hexyl-5-ethylbarbituric acid).

The Asymmetric Hydrogenation of Aryl-alkyl Ketones

Four ketones were fed, acetophenone, propiophenone,

benzylmethylketone, butyrophenone and methylphenethylketone, and from all but one, the triacetylmethyl esters of the glucuronides were obtained, crystalline, as single diastereoisomerides. As the chain lengthened, it became increasingly difficult to isolate a crystalline form and the ester gum from methylphenethylketone did not crystallise.

The formation of a single isomeric form need not to have been entirely unexpected. The phytochemical reduction of ketones always yields optically active carbinols (Neuberg, 1949). Ketones have been suggested as intermediates in the biological synthesis of L-amino acids (from Williams, 1947) and naturally occurring lactic acid, formed from pyruvic acid, is of the L(+) configuration.

In the case of acetophenone, the diastereoisomer has been definitely identified as the ester of (-)methylphenylcarbinylglucuronide, formed when (-)methylphenylcarbinol is fed. By the similarity in the specific rotations it can be assumed that propiophenone and butyrophenone produced carbinols which were also laevorotatory.

Ketone	$[\text{M}]_{\text{D}}$ of (-)carbinol	Glucuronide ester	
		$[\alpha]_{\text{D}}$ in CHCl_3	$[\text{M}]_{\text{D}}$
Acetophenone	-52°	-83°	-364
Propiophenone	-75.5°	-80°	-362
Butyrophenone	-85.8°	-79°	-368

However, benzylmethylketone produced a triacetylmethyl ester with $[\alpha]_D -24^\circ$ and $[M]_D -108$. The carbinol corresponding to benzylmethylketone has a $[M]_D +38.5^\circ$ and the change in rotation of the carbinol, after conjugation, corresponds closely with that of (+)isomenthol (Appendix IV).

	(+) <u>isomenthol</u>	(+) <u>benzylmethylcarbinol</u>
$[M]_D$ of alcohol	+ 27°	+38.5°
$[\alpha]_D$ of glucuronide (in water)	-43°	-
$[\alpha]_D$ of ester (in chloroform)	-29°	-24°

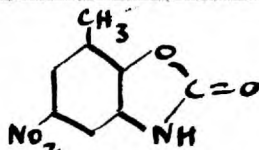
Therefore it is probable that benzylmethylketone is excreted as (+)benzylmethylcarbinyglucuronide in the rabbit.

In Chapter I, the absolute configurations of these carbinols were discussed and (-)methylphenyl-, (-)ethylphenyl, (-)propyl-, phenyl- and (+)benzylmethylcarbinol were all shown to be configuratively similar, i.e. corresponded to L(-)glyceraldehyde. Thus, each of the aryl-alkyl carbinols studied is asymmetrically hydrogenated, in the rabbit, to the L-carbinol. And the carbinols are of the same configuration as lactic acid formed from pyruvic acid by enzymic hydrogenation with the lactic dehydrogenase of animal tissues.

APPENDICES

Appendix I

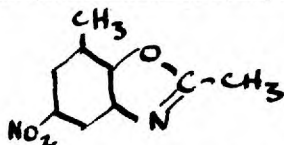
At one time it was considered possible that 6-acet-amido-4-nitro-o-cresol was being transformed into the corresponding benzoxazolone or benzoxazole during the concentration of extracts of urines from DNOC, and these derivatives were therefore prepared.

(a) 7-Methyl-5-nitrobenzoxazol-2-one

10 g., 6-amino-4-nitro-o-cresol was ground up with 3.53 g. (1.1 equiv.) urea and heated to between 150-160°C, until the evolution of ammonia slackened, after one hour. The tar was dissolved in 200 ml. hot N-HCl and the solution filtered. On cooling, crystals separated of the benzoxazolone which were collected in a small yield (0.5 g.). These were recrystallised from absolute ethanol and from a large volume of water as yellow needles, m.p. 239°.

Analysis :

Found : C, 49.80	$C_8H_6O_4N_2$ requires	C, 49.51%
H, 3.056		H, 3.02%
N, 14.1		N, 14.44%

(b) 2:7-Dimethyl-5-nitrobenzoxazole

0.5 g. 6-acetamido-4-nitro-p-cresol was melted at 200-250°C in a metal bath. The resulting tar was dissolved in hot ethanol, filtered and diluted with an equal volume of water. The benzoxazole was deposited as pale buff-yellow needles, m.p. 155°, yield 85 mg.

Analysis :

Found : C, 56.28	$C_9H_8O_3N_2$ requires	C, 56.25%
H, 4.14		H, 4.2%
N, 14.3		N, 14.58%

Appendix IIA comparison of two methods for the estimation of glucuronides, by the naphthoresorcinol reaction

Recently Paul (1952) has suggested a modification of the colorimetric method for estimation of glucuronides devised by Hanson, Mills & Williams (1944).

The two procedures employed may be summarised as follows :-

Method A (Hanson et al, 1944).

2 ml. glucuronide solution (containing 10-80 μ g.),
2 ml. 0.25% aqueous naphthoresorcinol solution
(filtered before use and made up fresh each day),
3 ml. concentrated HCl (Technical grade),
are mixed by inversion in 6" x $\frac{5}{8}$ " pyrex tubes (washed before use with chromic acid and distilled water and dried at 110°). The solution is heated for exactly 2 hours in a boiling water bath, and then cooled for 10 minutes in iced water. The precipitate is extracted with 7 ml. amyl alcohol in a 25 ml. separating funnel which is washed out after the extraction with sufficient ethanol to make the final volume of 10 ml. alcohol solution, in which the colour is estimated.

Estimation is carried out with a Spekker Absorptiometer using an Ilford No. 607 filter (orange) against an alcohol blank.

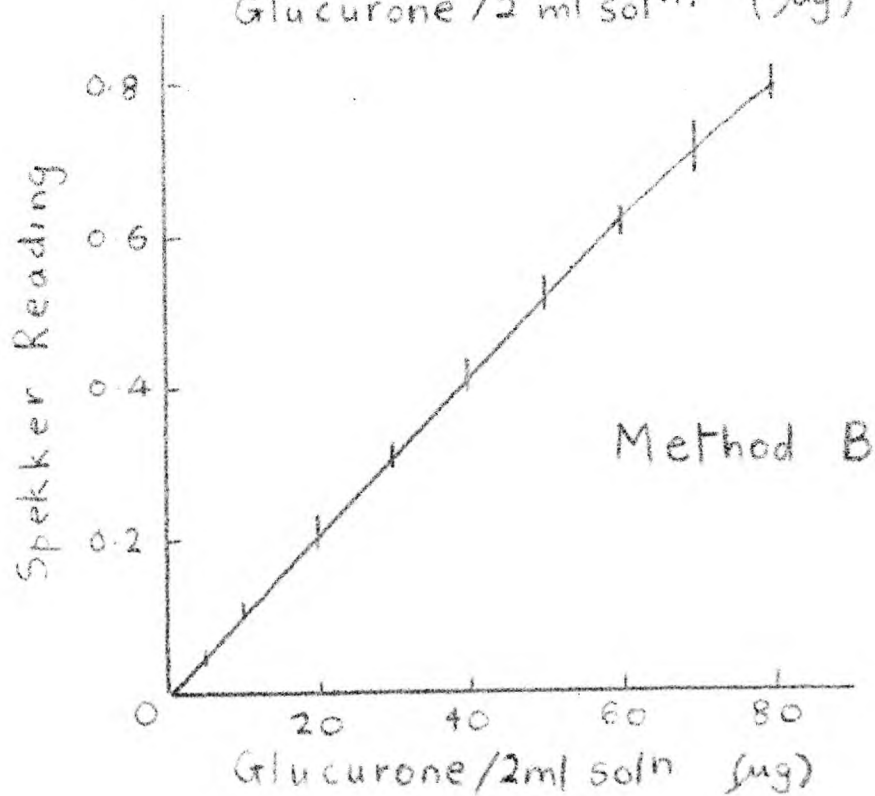
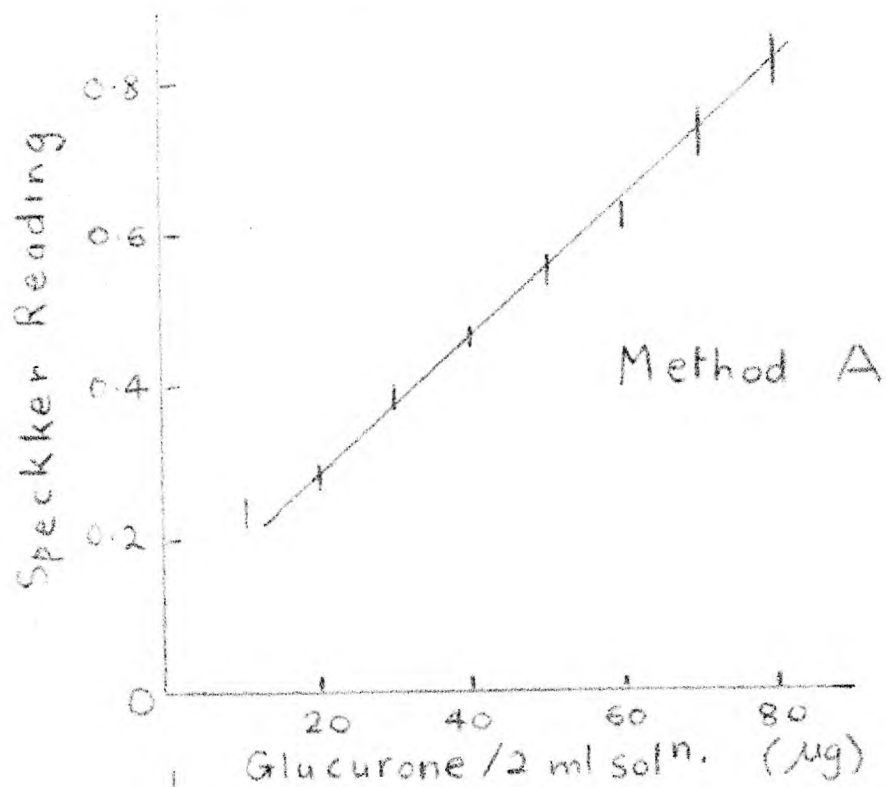
Method B (Paul, 1952)

2 ml. glucuronide solution (containing 5-70 $\mu\text{g.}$),
2 ml. 0.35% naphthoresorcinol in 0.1N- H_2SO_4
(filtered before use and made up fresh each day),
3 ml. 15N- H_2SO_4 ,
0.2 ml. 1% aqueous chloramine T,
react under the conditions detailed in Method A but
extraction is with 8 ml. ethyl acetate, the separating
funnels are washed out with ethyl acetate and the final
volume made up to 10 ml. with the same solvent. The
solutions are estimated with an Ilford No. 605 filter
(yellow green) against a reagent blank.

The standard curves were determined using 2 ml. of
solution containing 0-80 $\mu\text{g.}$ glucurone by dilution of
a solution of 40 $\mu\text{g.}$ glucurone/ml. They are illustrated
in Figs. 6 and 7.

Aqueous solutions of crystalline glucuronides were
made up, each at a concentration equivalent to about 40 $\mu\text{g.}$
glucurone/ml., and these solutions were used in recovery

Fig. 6. Glucuronide Estimation
Standard Curves



experiments to compare the two methods. The results, each the average of duplicate estimations, are recorded in Table 7.

Discussion

Standard curves

For a glucurone solution containing $40 \mu\text{g.}/2 \text{ ml.}$ the maximal error by each method was about the same, $\pm 4 \mu\text{g.}$ but the extraction by amyl alcohol resulted, sometimes, in clouded solutions for which readings could not be recorded.

This occurred, principally, when concentrations of glucurone of less than $20 \mu\text{g.}/2 \text{ ml.}$ solutions were used. Thus a reagent blank was not included. Variations in the purity of the naphthoresorcinol used affected the colour intensity and pure HCl could not be substituted for the technical grade without substantially reducing the slope of the curve. Method A gave reliable and reproducible results for concentrations of glucurone between 20 and $80 \mu\text{g.}/2 \text{ ml.}$ Using Method B, solutions containing $5\text{-}70 \mu\text{g.}/2 \text{ ml.}$ could be estimated.

Recovery experiments

A higher recovery of the glucuronide was obtained using Method A than by Method B, generally between 100 and 110%. This may be caused by the more complete hydrolysis in the presence of the stronger and more concentrated acid.

The low values obtained, in certain cases, using Method B may have been due to incomplete hydrolysis, destruction of the pigment or to incomplete extraction of the pigment. Both methods gave good results with the aliphatic glucuronides.

Method B, in which the ethyl acetate is a specific solvent for the coloured substance formed in the naphtho-resorcinol reaction, possesses an advantage over Method A for the estimation of glucuronides in the presence of interfering substances, i.e. in biological material. Thus, rabbits fed on 70 g. diet 41 (Assoc. Brit. Flour Mills) and 100 ml. water excrete 150-200 mg. glucuronide per day, according to estimations by Method A. By Method B only about 70 mg. glucuronide are estimated and small rises in the glucuronide output can be satisfactorily recognised and measured.

TABLE 7Estimation of glucuronides in solution: a comparison of recoveries by two methods

A (Hanson et al, 1944)

B (Paul, 1952)

Glucuronide	Amount (μ g./2 ml.)	Percentage Recovery	
		A	B
p-chlorophenyl-	73.4	115	99.5
p-bromophenyl-	87.8	103.8	99.0
p-iodophenyl-	96.5	110.0	104.5
p-aminophenyl-	69.0	101.3	92.5
p-cyanophenyl-	71.2	-	91.0
<u>l</u> -menthyl-	79.5	111.6	107.0
<u>d</u> -menthyl-	79.5	104.6	98.8
<u>d-isomenthyl</u> -	79.6	108.1	90.5
<u>d-neomenthyl</u> -	79.5	106.3	112.0
amm. <u>dl-isomenthyl</u> -	85.7	104.7	104.5
p-amino- <u>o</u> -sulphonamido-	94.4	90.2	78.0
<u>o</u> -amino-p-sulphonamido-	95.2	72.2	39.0
K.2-ethylhexanoyl-	85.5	106.2	106.0
K diisopropylmethyl-	83.5	97.2	97.7

Appendix III

The estimation of glucuronides, ethereal sulphates,
ether soluble acids and mercapturic acids excreted
in the urines of rabbits

Glucuronides were estimated by an adaptation by Paul (1952) of the naphthoresorcinol method of Hanson et al (1944). (cf. Appendix II).

The direct turbidimetric method for determining ethereal sulphate in urine was applied according to Sperber (1948). In certain of the estimations with cumene, sec-butylbenzene and sec-amylbenzene an oil formed in the acid hydrolysate of the urines. This was due to the instability of the glucuronide present and before completing the procedure the acid solutions were extracted with ether.

Ether soluble acid in the excreted materials was measured by the titration with 0.1N-NaOH (Bray et al, 1946). For consistent results it was found necessary to remove acid constituents in the ether with aqueous Na_2CO_3 before commencing the extraction.

Mercapturic acids were measured by titration with 0.01N- I_2 solution (Stekol - adapted for rabbit urine by Parke & Williams (1951)).

Protocols of the animal experiments are included overleaf.

EthylbenzeneExcretion as glucuronide and ethereal sulphate

Days	Dose g.	Glucuronides as (mg. glucurone/day)	Ethereal sulphates as mg. SO ₃ /day
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Rabbit No. 80; weight 2.62 kg.

1	1.13	73	16.8
2		555	25.6
3		127.5	17.6
4		74	16.8
Rise (over 2 days)		322	11.3 (over 1 day)
Excretion as % dose		28.4	1.0

Rabbit No. 79; weight 2.75 kg.

1	1.20	73	15.2
2		720	21.2
3		40	13.2
4		63	19.6
Rise over 1 day		397	6.9
Excretion as % dose		33.2	0.06

Rabbit No. 67; weight 2.78 kg.

1	1.21	78	13.2
2		765	20.4
3		95	14.8
4		97	16.0
Rise over 1 day		406	7.81
Excretion as % dose		33.5	0.06

Average excretion 32.0 0.4

(+)-MethylphenylcarbinolExcretion as glucuronide and ethereal sulphate

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day
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Rabbit No. 80; weight 2.82 kg.

1	1.30	65.0	17
2		1112.5	22
3		73.8	20
4		79.0	-

Rise over 1 day		720	-
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Excretion as % dose		55.4	0
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Rabbit No. 79; weight 2.88 kg.

1	1.32	78.8	15.5
2		1100.0	23.0
3		73.8	21
4		66.0	-

Rise over 1 day		712.0	-
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Excretion as % dose		53.8	0
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Rabbit No. 67; weight 2.89 kg.

1	1.35	49.0	14.5
2		837.5	15.5
3		90.0	16.5
4		73.0	-

Rise over 1 day		532.0	-
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Excretion as % dose		39.5	0
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Average excretion		50.0	0
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(-)-MethylphenylcarbinolExcretion as glucuronide and ethereal sulphate

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day
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Rabbit No. 94; weight 2.75 kg.

1		40	16.5
2	1.0	74	29.9
3		762	29.9
4		138	29.2
5		126	29.9
Rise over 1 day		668	-
Excretion as % dose		47.0	0

(+)-Methylphenylcarbinol $[\alpha]_D = +36^\circ$ (c = 1 in EtOH)Excretion of glucuronide on combined urines of two rabbits

Days	Dose g.	Glucuronides as mg. glucurone/day
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Rabbits No. 55; weight 2.6 kg. and No. 57; weight 2.6 kg.

1	3.0	162
2		2522
3		166
4		158.5
Rise over 1 day		2360
Average excretion as % dose		52.0

AcetophenoneExcretion as glucuronide and ethereal sulphate

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day
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Rabbit No. 80; weight 2.72 kg.

1	1.23	81	12
2		1019	40.5
3		65	28.5
4		42.5	22
Rise over 1 day		648	20.5
Excretion as % dose		52.7	2.5

Rabbit No. 79; weight 2.8 kg.

1	1.26	71	16
2		1044	45
3		68.5	27
4		42.5	22.5
Rise over 1 day		657	22
Excretion as % dose		52.2	2.6

Rabbit No. 67; weight 2.85 kg.

1	1.35	60	16
2		894	30.5
3		78.8	17.5
4		67.5	20
Rise over 1 day		495	12.7
Excretion as % dose		36.5	1.4

Average excretion		47.0	2.2
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StyreneExcretion as glucuronide, ethereal sulphate and
ether-soluble acid

Days	Dose	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day	Ether-sol. acids ≡ ml. 0.1N-NaOH/ day
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Rabbit No. 65; weight 4.3 kg.

1	1.68	142.5	15.0	36.6
2		421	27.2	96.5
3		281	16.0	40.0
4		110	14.0	20.0
Rise		266	15.9	63.5
Excretion as % dose	15.9		1.0	39.3

Styrene epoxideExcretion as glucuronide, ethereal sulphate and
mercapturic acid

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day	Mercapturic acids as ml. 0.01N-I ₂ /day
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Rabbit No. 25; weight 2.62 kg.

1	1.33	51	11.3	4.62
2		392	11.1	14.35
3		81.3	16.7	6.51
4		61.5	12.3	2.13

Rise over 1 day	327.4	-	10.0
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Excretion as % dose	16.9	0	0.9
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Rabbit No. 30; weight 3.0 kg.

1	1.54	124.6	34.7	3.50
2		413	30	12.57
3		92.5	27.5	4.76
4		109	21	4.52

Rise	304.3	-	8.4
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Excretion as % dose	13.6	0	0.71
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Average excretion	15.3	0	0.8
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β -PhenylethanolExcretion as glucuronide, ethereal sulphate andether-soluble acid

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day	Ether-sol. acids = ml. 0.1N-NaOH/ day
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Rabbit No. 79; weight 3.3 kg.

1	1.33	112	11.6	50
2		225	12.3	110
3		115.5	13.6	56
4		78.8	13.0	28
Rise over 1 day		83.5	-	64
Excretion as % dose		6.3	0	60.5

Rabbit No. 2; weight 2.5 kg.

1	1.07	66	7.6
2		88	11
3		188	14.5
4		96.8	14.5
Rise over 2 days		122.2	-
Excretion as % dose		8.0	0

Rabbit No. 65; weight 4.3 kg.

1	1.84	121	15.6	45
2		265	12.0	98
3		186	19.2	100
4		127.5	17.0	34
Rise over 2 days		141	-	114
Excretion as % dose		7.7	0	76
Average excretion		7.3	0	68.3

n-PropylbenzeneExcretion as glucuronide and ethereal sulphate

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day
Rabbit No. 41; weight 2.75 kg.			
1	1.33	94	11.0
2		64	15.1
3		840	37.9
4		144	15.8
5		82	14.1
Rise over 2 days		824	23.9 (over 1 day)
Excretion as % dose		43.3	2.3
Rabbit No. 63; weight 3.3 kg.			
1	1.55	92	6.1
2		68	8.9
3		424	28.6
4		146	20.6
5		74	29.5 (over 1 day)
Rise over 2 days		414	15.1
Excretion as % dose		18.2	1.3
Rabbit No. 64; weight 3.15 kg.			
1	1.5	86	9.9
2		540	40.0
3		104	15.8
4		60	21.3
Rise over 2 days		571	28.3 (over 1 day)
Excretion as % dose		29.3	2.5
Average excretion		30	2.0

EthylphenylcarbinolExcretion as glucuronide and ethereal sulphate

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day
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Rabbit No. 71; weight 3.1 kg.

1	1.51	106	29.9
2		1240	46.5
3		122	55.1
4		94	62.3
Rise over 1 day		1123	-
Excretion as % dose		57	0

Rabbit No. 72; weight 3.1 kg.

1	1.51	90	29.9
2		1240	34.4
3		82	21.0
4		62	62.3
Rise over 1 day		1160	-
Excretion as % dose		59	0

Rabbit No. 75; weight 2.6 kg.

1	1.31	114	29.9
2		900	36.5
3		176	43.0
4		132	61.0
Rise over 1 day		764	-
Excretion as % dose		45	0

Average excretion	53.7	0
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BenzylmethylecarbinolExcretion as glucuronide and ethereal sulphate

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day
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Rabbit No. 79; weight 2.96 kg.

1	1.51	110	20.9
2		1100	52.9
3		122	51.6
4		122	29.9
Rise over 1 day		981	53.7
Excretion as % dose		50	6.1

Rabbit No. 80; weight 2.7 kg.

1	1.31	54	29.9
2		700	40.8
3		80	31.0
4		100	29.9
Rise over 1 day		576	12.0
Excretion as % dose		35	1.6

Rabbit No. 81; weight 3.35 kg.

1	1.71	148	29.9
2		1160	52.2
3		192	62.4
4		200	29.9
Rise over 1 day		975	
Excretion as % dose		44	5.5

Average excretion 43 4.7

CumeneExcretion as glucuronide and ethereal sulphate

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day
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Rabbit No. 8; weight 3.9 kg.

1	1.72	119	13.4
2		1800	24
3		165	12.5
4		112.5	13.0
Rise over 2 days		1733	11 (over 1 day)
Excretion as % dose		69	1.1

Rabbit No. 25; weight 2.8 kg.

1	1.29	77.6
2		1337.5
3		99
4		78.5
Rise over 2 days		1281
Excretion as % dose		68
Average excretion		68.5

n-ButylbenzeneExcretion as glucuronide, ethereal sulphate and
mercapturic acid

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day	Mercapturic acids as ml. 0.01N-I ₂ /day
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Rabbit No. 8; weight 3.9 kg.

1	1.98	162.5	14.8	6.26
2		1000	28.5	17.5
3		350	25.5	3.5
4		154.1	14.8	3.25
Rise over 2 days		1034	24.4	13.0
Excretion as % dose		39.6	2.7	0.9

Rabbit No. 25; weight 2.8 kg.

1	1.38	79.2	14.8	4.76
2		618.8	50.5	14.0
3		131.3	29.5	3.0
4		133.3	14.8	3.8
Rise over 3 days		643.4	50.4	10.25
Excretion as % dose		32	6.16	1.0 (over 1 day)

Rabbit No. 30; weight 2.7 kg.

1	1.38	56.4	16.3	4.76
2		1018.8	54.5	26.2
3		164.6	31.5	3.75
4		65.6	17.8	4.5
Rise over 2 days		1061.4	52.0	22.0
Excretion as % dose		58.5	5.36	2.1 (over 1 day)
Average excretion		43.4	5.1	1.3

sec-ButylbenzeneExcretion as glucuronide, ethereal sulphate andmercapturic acid

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day	Mercapturic acids as ml. 0.01N-I ₂ /day
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Rabbit No. 8; weight 3.9 kg.

1	1.98	129	15.4	5.1
2		1475	50.5	6.5
3		250	30.5	5.0
4		195	36.1	5.2
5		162.5	16.2	-
Rise over 3 days 1482			69.7	-
Excretion as % dose 57.2			5.9	0

Rabbit No. 25; weight 2.8 kg.

1	1.38	76	11.5	4.8
2		875	88	3.25
3		265	27.5	5.0
4		163	19.5	4.2
5		79.2	10.5	-
Rise over 3 days 1070			102	-
Excretion as % dose 60			12.4	0

Rabbit No. 30; weight 2.7 kg.

1	1.38	57.5	14.7	3.6
2		1287.5	106.5	2.75
3		209	34	2.5
4		114	33.4	2.9
5		56.4	15.2	-
Rise over 3 days 1440			129	-
Excretion as % dose 80			15.7	0

Average excretion	65.7	11.3
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tert-ButylbenzeneExcretion as glucuronide and ethereal sulphate

Days	Dose g.	Glucuronides as mg. glucurone/day	Ethereal sulphates as mg. SO ₃ /day
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Rabbit No. 8; weight 3.9 kg.

1	4.0	117.5	20.25
2		941.7	29.5
3		875	44.5
4		164	21.8
Rise over 2 days		1535	31.0
Excretion as % dose		58.4	2.6

Rabbit No. 25; weight 2.8 kg.

1	1.39	67.7	15
2		958.3	28.5
3		600	35
4		119	18
Rise over 2 days		1372	30.5
Excretion as % dose		75.2	3.8
Average excretion		66.8	3.3

No significant variation was apparent in the titrations on daily urine of mercapturic acid excretion.

Appendix IV

The preparation of triacetylmethyl esters of menthyl-glucuronides

In the course of the study of the stereochemical aspects of alkylbenzene metabolism, it became necessary to ascertain the effect of methylation and acetylation on the specific rotation of a glucuronide. Of the crystalline glucuronides available (-)menthyl and (+)isomenthylglucuronides were chosen since they contained centres of asymmetry within the aglycone moiety of the molecule.

The glucuronides were methylated in ethereal solutions with diazomethane and after removal of the solvent, acetylated overnight with five molecular equivalents of acetic anhydride and pyridine. The esters crystallised out of solution on addition of water and after washing, they were recrystallised from 90% ethanol.

(-)Menthylglucuronide

The ammonium salt of the glucuronide ($[\alpha]_D -110.7^\circ$ in water) was dissolved in 2N-H₂SO₄ and the free gluc-

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