THE METABOLISM OF SOME DERIVATIVES OF BENZIMIDAZOLE

by

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ABSTRACT.

Utilising a ^{14}C label, the metabolic fates of two isomeric benzimidazoles, 4,5-dichloro- and 5,6-dichloro-2-trifluoromethyl benzimidazole, have been studied in rats and rabbits.

Following an oral dose, the 4,5-dichloro compound is excreted rapidly by both species, all of the eliminated ^{14}C is found in the urine.

The 5,6-dichloro compound is excreted much more slowly by both species, the excretion by the rabbit is slower than the rat. In the latter species eliminated ^{14}C appears in both urine and faeces. There is a marked biliary excretion of both compounds in rats, and strong evidence for the existence of an enterohepatic circulation. The nature and location of the retained radioactivity in rats and rabbits was determined following dosage of the 5,6-dichloro compound. This revealed a marked species difference, in that in rabbits some 90% of the compound in the liver remained unmetabolised after 5 days, while in the rat this figure was only 10% at 2 days. These figures are mirrored by the half-lives of this compound in the blood which are 14 hours and 70 hours for rats and rabbits respectively.

The metabolism of both compounds proceeds via hydroxylation and conjugation, the conjugating moiety being mainly glucosiduronic acid. Evidence has been obtained for the presence of N-sugar derivatives of the parent compounds, the

conjugating moiety is again glucosiduronic acid. A small proportion of the urinary metabolites were found to be dechlorinated or rearranged phenols and to be consistent with the operation of an "NIH" shift.

The results may be partially explained in terms of high protein binding ability, LD_{50} and other factors. A computer programme has been used to calculate electron densities and other molecular parameters, and the findings of this work are compared with the biological results.

CONTENTS.

CHAPTER ONE

SECTION A

GENERAL INTRODUCTION

The use of chlorinated hydrocarbons in agriculture and in disease vector control has been one of the success stories of this century. Land productivity has been vastly increased, spoilage of stored crops has been reduced and the incidence of insect-borne diseases has been dramatically cut, and in some areas eliminated. Thus, the benefits to mankind have been great.

However, it appears that many of the target pests of these chemicals have become quite resistant to them. For example, Rudd (1964) states that over 30 species of major insect pests have begun to show or are already resistant to DDT. To combat this resistance, other insecticides have been synthesized and applied, but often the insects show signs of tolerance after a period of time. There have also been highly publicised effects of pesticide residues or misapplications of pesticides on non-target species, often mammals or birds.

With a rare element of timing the issue of pesticide usage was placed before the public by Rachel Carson, in her book "Silent Spring." Public reaction forced government agencies to act, and in the U.S.A. for example, a President's Science Advisory Committee in 1963 indicated that several areas pertaining to pesticides ought to be investigated. Among these areas, one goal that the Committee desired was the production of safer and more specific pest control. To attain this it was recommended that the research effort be extended to cover the following items:

1). The mode of action of pesticides.

2). Comparative toxicity of new and existing products.

3). The metabolism in insects, plants and higher animals of the compounds, and the processes of chemical degradation and inactivation of them in Nature.

4). Reproductive effects of pesticides on mammals.

5). Chronic effects on the organs of mature and immature animals.

6). Possible synergism and potentiation of the effects of commonly used pesticides with several types of commonly used drugs.

Information on some or all of these points has to be presented to the Food and Drug Authority when an application is made to market a new compound. By this process it is hoped that knowledge will be gained to achieve the Committee's goal of safer and more specific pesticides.

The work reported below was performed in connection with the commercial introduction of new benzimidazole pesticides (Burton et al., 1965), and provides essential background information on their mammalian metabolism.

CHAPTER ONE

SECTION B

THE BIOCHEMISTRY AND PHARMACOLOGY OF BENZIMIDAZOLE AND ITS DERIVATIVES

The literature on benzimidazole and its derivatives has proved difficult to review. There is a large volume of seemingly unrelated facts, and often much of the earlier literature presents conflicting results.

In order to present an overall picture, the possible modes of biological action of these compounds have been considered under the following headings:

1). Uncoupling activity
   a). mitochondrial
   b). chloroplasts
2). "Interference" with Vitamin B_{12}
   a). micro-organisms
   b). other systems
3). "Interference" with RNA or DNA synthesis
4). Miscellaneous

In some cases these headings may not convey a complete picture and the observed biological effects might be partially explained by some other factor.

1). Uncoupling activity

a). Oxidative Phosphorylation in Mitochondria

The acute mammalian toxicities of the 2-trifluoromethyl-benzimidazoles studied in this work have been attributed to the uncoupling ability of these compounds (Jones and Watson, 1965, 1967; Beechey et al., 1965; Beechey, 1966). The effects are those associated with an increased metabolic rate, i.e. panting, salivation, sweating, prostration and coma leading to

death; rigor mortis is accelerated. In these respects they act like 2,4-dinitrophenol (Jones and Watson, 1967; Beechey et al., 1965; Beechey, 1966). That is to say they stimulate ATPase and cause respiration of mitochondria in the absence of a phosphate acceptor.

The uncoupling activity has been attributed to the ionised N-H group of the imidazole ring (see Table 1), and in general this activity rises with a decrease in the pK_a of this group (Jones and Watson, loc. cit.; Beechey, loc. cit.). This activity can be removed by the formation of the N-methyl or N-ethyl derivatives (Buchel et al., 1965; Jones and Watson, 1965). The uncoupling effect is most marked in a series of substituted compounds. For instance, with halogenated derivatives this activity rises to a maximum with the fully substituted compound. Thus tetrachloro-2-trifluoromethyl-benzimidazole (pK_a 5.0) is one of the most potent uncouplers known (Jones, loc. cit.; Beechey, loc. cit.). However, nitro substituted derivatives of lower pK_a are much less active than predicted. This effect is insufficiently explained by the lower lipid to water partition coefficients of these compounds as compared to the halogenated compounds (Jones and Watson, 1967). Beechey (1966) has suggested that this reduced activity may be explained by the formation of a structure of the type:

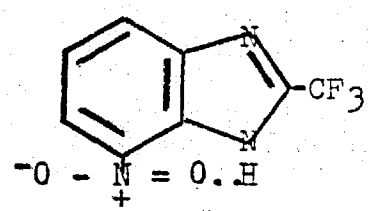


Table 1. UNCOUPLING ACTIVITIES OF SOME 2-TRIFLUOROMETHYLBENZIMIDAZOLES ON OXIDATIVE PHOSPHORYLATION OF RAT-LIVER MITOCHONDRIA.

COMPOUND	pKa	Conc^{n} REQUIRED FOR 50% UNCOUPLING WITH SUCCINATE AS SUBSTRATE (μM)	CYCLOHEXANE-0.1N-HCl PARTITION COEFFICIENT
2-trifluoromethylbenzimidazole	8.79	305	0.11
5-chloro-2-trifluoromethylbenzimidazole	7.97	10.4	0.4
5-methyl-2-trifluoromethylbenzimidazole	8.90	22.0	0.33
4,5-dichloro-2-trifluoromethylbenzimidazole	6.96	0.52	2.0
5,6-dichloro-2-trifluoromethylbenzimidazole	7.40	0.80	*
4,5,6-trichloro-2-trifluoromethylbenzimidazole	6.18	0.29	3.7
4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole	5.04	0.079	8.7
4,5,6-trichloro-1-ethyl-2-trifluoromethylbenzimidazole	-	Inactive at 1.0	n.d.
4-nitro-2-trifluoromethylbenzimidazole	6.80	9.2	0.80
5-amino-2-trifluoromethylbenzimidazole	4.5	Inactive at 1000	*
5-carboxy-2-trifluoromethylbenzimidazole	8.4	Inactive at 1000	*

* Too low for accurate determination.

n.d. Not determined.

Data from Jones and Watson (1967).

The introduction of water soluble groups, such as carboxyl or amino, into the benzene ring also decreases uncoupling activity, possibly because the increased water solubility gives a decreased partition effect (Jones and Watson, 1967).

b). Chloroplasts

Herbicidal activity is also ascribed to the ionising abilities of these compounds (Buchel et al., 1965). Interference with the photosynthetic electron transport mechanism occurs, but at lower concentrations photophosphorylation was inhibited in isolated spinach chloroplasts without any significant effect on electron transport (Buchel et al., 1965; Black and Myers, 1967). Uncoupling activity again rises as the pK_a falls and the 4,5,6,7-tetrachloro-2-trifluoromethyl derivative is one of the most potent uncouplers of photophosphorylation (Buchel et al., loc. cit.). However this activity is possessed only in vitro by some of these uncouplers. The herbicidal activity of these compounds thus cannot be adequately explained on the basis of their uncoupling activity.

2). Interference with Vitamin B12

Interference with vitamin B_{12} activity can be seen to be of two types. Either the benzimidazole derivative can be incorporated into a vitamin analogue which is then inactive, or the compound may physically replace the vitamin at the site of action. Which course is taken by a given compound is uncertain and little can be gleaned from the literature.

Fig. 1

THE STRUCTURE OF VITAMIN B_{12} (CYANOCOBALAMIN)

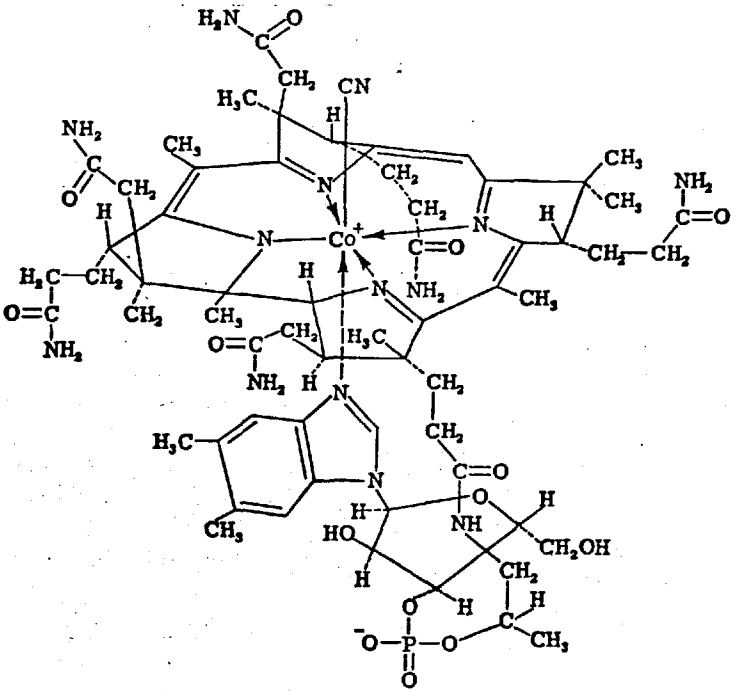
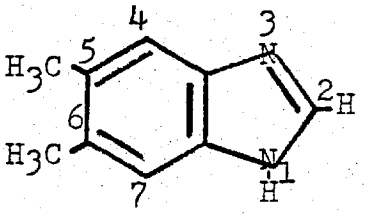


Fig. 2

THE STRUCTURE OF 5,6-DIMETHYLBENZIMIDAZOLE AND ITS NUMBERING SYSTEM



a). Micro-organisms

Vitamin B_{12} was shown to be a benzimidazole derivative by Brink and co-workers (Brink and Folkers, 1949; Brink et al., 1950; Brink and Folkers, 1950). The structure is shown in Fig. 1. It can be seen to contain 5,6-dimethylbenzimidazole (Fig. 2) linked both to the central cobalt ion and to a sugar via an α linkage. This benzimidazole portion can be replaced by other bases, e.g. adenine, 2-methyladenine or guanine, to give naturally occurring analogues of vitamin B_{12}. Many other analogues can be isolated from micro-organisms grown on the desired base and factor B, a B_{12} analogue not containing a nucleotide (Barbieri et al., 1962; Ford, 1955; Goodwin, 1963). Emerson and his co-workers (1950) showed that rats maintained on a diet devoid of animal protein grew successfully when supplied with 5,6-dimethylbenzimidazole at milligram levels as a vitamin B_{12} replacement. The analogue 5-methylbenzimidazole showed very high activity in this respect, but 2,5-dimethylbenzimidazole actually inhibited growth. These workers suggested that the toxicity to vitamin B_{12}-dependent micro-organisms (in the gut of the treated animals) was increased by substituting an alkyl group onto C_2. They also showed that 2-butylbenzimidazole was the most active compound of this series against Lactobacillus lactis, but the activity could not be reversed by supplying vitamin B_{12} in the culture medium. Hendlin and Soars (1951) also suggested that by acting against vitamin B_{12}-dependent micro-organisms, these compounds enabled

the vitamin produced by other organisms to be released to the host animal. This rather interesting speculation does not appear to have been pursued further.

McNair-Scott et al. (1958) working with bacterial mutants showed that L. casei grown on either limiting folic acid or limiting riboflavin media had growth further restricted by the addition of C_2-alkylbenzimidazoles. Chlorinated benzimidazoles inhibited purine, vitamin B_{12} or methionine requiring mutants, but this inhibition could be partially lifted by the provision of methionine, thymine or riboflavin (with purine-dependent E. coli). The inhibition was intensified by the addition of folic acid. Timmis and Epstein (1959), however, found that the compound 4,5,6-trichloro-1-β-D-ribosyl-benzimidazole was a competitive antagonist for vitamin B_{12} in E. gracilis, a vitamin B_{12}-dependent flagellated green alga.

Bishop et al. (1964) screened trifluoromethyl and other perfluoroalkylbenzimidazoles for their antibacterial activity. They found that C_2-heptafluoropropyl derivatives were more potent than the trifluoromethyl ones and that water soluble groups (e.g. $-CH_2OH$, $-CO_2H$ or $-NH_2$) decreased the antibacterial activity of these compounds. The substitution of another electron-withdrawing group at C_5 into a 2-trifluoromethylbenzimidazole produced a large increase in activity, the order of effectiveness being $CF_3 > NO_2 > Cl$. The highest overall activity of any compound tested was given by 2,4,5-tris-trifluoromethylbenzimidazole. The mode of action of these

compounds was not obvious, but these workers showed that 4,5-bis-trifluoromethylbenzimidazole was not acting as an anti-metabolite of vitamin B_{12}, adenine, guanine and histidine. However, some degree of specificity was apparent in that the compounds showed more activity against gram-positive organisms (Staphylococcus aureus and Streptomyces pyogenes). In vivo (in mice) no activity could be obtained with either the 2,4- or 2,5-bis-trifluoromethyl or the 2,4,5-tris-trifluoromethyl derivatives of benzimidazole against Staph. aureus.

b). Other systems

Some interesting findings have been obtained by workers studying non-bacterial systems. Thus Vijayaraghavan (1952) showed that vitamin B_{12} was required for the synthesis of erythrocytes in phenylhydrazine treated mice. Neither 5,6-dimethylbenzimidazole nor the 5,6-dimethyl-1-β-D-ribityl derivative could replace vitamin B_{12}. Abbott and Dodson (1954) followed up this work, and found that 2,5-dimethyl- and 5,6-dimethylbenzimidazole prevented incorporation of ^{15}N-glycine into the haem of chicken erythrocytes.

3). Interference with RNA or DNA synthesis

The necessity to control diseases caused by viruses has meant that many benzimidazole derivatives have been among the thousands of compounds screened for anti-viral activity. Among the first reports of benzimidazole compounds showing such activity were those of Thompson and his co-workers (1947, 1950). They showed that benzimidazole had an inhibitory effect

on the multiplication of vaccinia virus in chick embryonic tissue. This inhibition could not be removed by adenine, guanine or yeast RNA. However, benzimidazole failed to give any protection to mice infected by vaccinia.

Morgan (1952) incubated psittacosis virus (a DNA virus) in embryonated eggs, and in cell cultures (chick embryo tissue), with benzimidazole at the maximum tolerated dosage to the host cells. A decrease in virus titre was obtained without noticeable cytotoxicity to the host cells.

Tamm et al. (1952) showed that 2,5-dimethylbenzimidazole inhibited the multiplication of influenza A virus cultured on chorioallantoic membranes. Again the host tissue appeared to be unaffected.

In a later series of papers (1953a, b), the same authors showed that the inhibition appeared to be in the biosynthetic processes of viral reproduction, and it prolonged the latent period of infection. The inhibition could be removed by washing out the inhibitor. In a further paper (1953c), various alkyl derivatives of benzimidazole were screened for inhibitory activity. Methyl groups at positions 4- and 5- increased the inhibitory effect, but methyl at 2- or 6- did not. 2,5-Dimethylbenzimidazole and 2,4,6-trimethylbenzimidazole however showed an increase in inhibitory activity and the substitution of methyl into all benzene ring positions plus C_2 gave a highly active compound. Substitution at N^1 removed most of the activity (leaving approximately half the activity of benzimida-

zole itself). These workers found that the inhibition produced by these compounds could not be reversed by adenosine, guanosine, deoxyguanosine, uridine, thymidine or mixtures of these. However other workers, e.g. Woolley (1944), have claimed to reverse the inhibitory action of benzimidazole on cell division with purines, but only with micro-organisms. Tamm and Tyrell (1955) suggested that the effect of 2,5-dimethylbenzimidazole was to confront the virus with host cells unable to carry out biosynthetic pathways.

Papers by Tamm et al. (1954a and b) investigated the more potent halogenated derivatives of benzimidazole. For instance, 5,6-dichloro-1-β-D-ribosylbenzimidazole (DRB) was shown to have 3-5 times more inhibitory activity than 2,5-dimethylbenzimidazole on the in vitro influenza virus production on chorioallantoic membranes. It was noted that the inhibition produced by DRB prevented the release of virus and the authors postulated that the compounds were inhibiting the biosynthesis of nucleic acid. However, the inhibitory activity was not reversed by guanosine, adenosine, guanylic acid or adenylic acid or mixtures of these. The sugar moiety was found to be quite specific, since by changing ribofuranose to ribopyranose the activity was reduced 6-fold when 5,6-dichlorobenzimidazole was the base. Substitution with methyl at C_2 reduced the inhibitory activity considerably, but substitution of alkyl groups into the benzene ring did not significantly alter the activity. This is interesting when compared with the non-

halogenated derivatives discussed earlier. At viral inhibitory concentrations, DRB had no observed effect on host cells (2,5-dimethylbenzimidazole had a marked restrictive effect on cell growth at this level), but at more than 8 times the inhibitory concentration there was a reduction in oxygen uptake. This effect could be removed by washing off the compound, as could the viral inhibition at the lower concentrations. In vivo DRB caused an inhibition of the biosynthesis of influenza B virus in chicken embryos and in mouse lung. No ill effects on healthy mice were observed at a dose level of 4 mg. of DRB per animal. Tamm (1956) examined 4,5,6-trichloro-1-β-D-ribosyl-benzimizidazole (TRB) for antiviral activity and found it to be as active as DRB. Again the sugar moiety was shown to be quite specific. However, macroscopic damage to the cells of the membrane was caused at only 2 times the concentration required for inhibition of virus growth.

Tamm et al. (1956a, b) reviewing previous results concluded that the chloro derivatives of benzimidazole offered a selective anti-viral activity coupled with a low host cell toxicity at the concentrations of drug employed. Specificity was afforded by the sugar moiety attached to the N^1 atom. Later, Tamm and Overmann (1957a and b), found that the chlorobenzimidazole derivatives did not have their inhibitory activity towards vaccinia (a DNA virus) altered by conversion into N-β-D-ribosides. Both 2,5-dimethylbenzimidazole and DRB appeared to affect some stage of biosynthesis, but Tamm and

Nemes (1957) showed that with influenza B virus (containing RNA), the DRB inhibition of RNA biosynthesis could be reversed by adenine when the host was a chorioallantoic membrane. Under these conditions, the order of inhibitory activity against influenza virus was shown to be benzimidazole < DRB < TRB. Tamm et al. (1960) confirmed the earlier findings of Tamm and Nemes (1957) and further showed that DRB while retarding the uptake of adenosine into the RNA of uninfected host cells did not affect alanine uptake or the level of oxygen consumption. Adenovirus (a DNA containing virus) was similarly affected by DRB.

A recent paper by Diwan and co-workers (1968) examined the anti-viral activity of halogenated benzimidazoles with a sugar derivative at position N^1 as before, but with 2'-deoxyribose substituted for ribose. They showed that 5,6-dichloro-1-(2'-deoxy-α-D-ribofuranosyl)- and 5,6-dimethyl-1-(2'-deoxy-β-D-ribofuranosyl)benzimidazole were cytotoxic to green monkey and mouse kidney cells but 5,6-dichloro-1-(2'-deoxy-β-D-ribofuranosyl)benzimidazole (dDRB) was not cytotoxic and also inhibited Herpes simplex and polyoma viruses. A lag of 24 hours occurred before virus replication took place when treatment with dDRB was given at the time of infection with H. simplex. There was still a marked inhibition of the replication process when the compound was administered 15 hours after infection, i.e. the inhibition was occurring within the cell and was not preventing the entrance of the virus into the

host cell. This type of inhibition could not be reversed by deoxyribonucleosides. The same authors also showed that dDRB inhibited the biosynthesis of RNA, DNA and protein in green monkey kidney cells at the concentration required for inhibition of the virus. RNA synthesis was found to be the process most affected. These authors concluded that the inhibition was caused by free 5,6-dichlorobenzimidazole interfering in some key step of virus replication, a conclusion similar to that reached earlier by Tamm and Tyrell (1955). Diwan et al. (1968) also concluded from their findings that the riboside was broken down during the period when the inhibitory effect was being exerted, since the pronounced cytotoxic effects of the free base were not shown by either DRB or dDRB. The authors give no reason why they consider such a step likely, and do not consider that either compound might be incorporated into some other molecule in the form of the glycoside.

Earlier Sirlin and Jacob (1964) had investigated the mechanics of the inhibitory activity of DRB and TRB. These workers examined the incorporation of tritiated uridine into the RNA of the nucleoli and chromosomes of salivary glands of the dipteran Smittia parthenogenetica. It was found that the kinetics of inhibition in nucleoli and chromosomes were different for DRB and TRB, but not for unsubstituted benzimidazole. TRB inhibited nucleolar synthesis most effectively and synthesis was in general restored by removal of the inhibitor. Cell damage became a dominant factor after prolonged periods

of incubation (90 minutes), thus preventing RNA synthesis. Conversely, at shorter periods the presence of DRB often stimulated synthesis, especially in the nucleolus. No interpretation of these results was offered.

Interest in the anti-viral properties of trifluoromethyl derivatives of benzimidazole was initiated by Cochran and Fara (1963), who screened several compounds for activity against vaccinia virus in mice and monkeys. They found that while 4,5,6-trichloro- and 4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazole were potent in vitro inhibitors, only the trichloro derivative had any marked in vivo activity in mice, and even then it did not prevent lung lesions.

The high potency of benzimidazole derivatives against viruses has led to a great deal of investigation into their mode of action. However, host cell cytotoxicity and conflicting and ill-explained results have led Tamm and Eggers (1965) to affirm that many of the earlier results arise from faulty experimental technique. Little work is now being carried out with these compounds. It is interesting to note that the two compounds which Diwan et al. (1968) found to be cytotoxic, 5,6-dichloro-1-(2'-deoxy-α-D-ribofuranosyl)benzimidazole and 5,6-dimethyl-1-(2'-deoxy-β-D-ribofuranosyl)benzimidazole, could possibly be antimetabolites of vitamin B_{12}.

4). Miscellaneous

Auverman (1918) reported that benzimidazole caused anaesthesia in mice, but it was left to Goodman et al. (1943)

to characterise the effect and to extend the work to other species. These workers reported that parasthaesia occurred in mice, rats, cats and monkeys at dosages of 200-300 mg/kg. Respiration was only affected at the toxic dosage, this being the cause of death. The compound was thus believed to exert a highly selective depressant action on an unknown locus of the cerebrospinal axis. Domino et al. (1951) showed that benzimidazole acted against the convulsant action of strychnine in mice. The same workers, in a later paper (1952), examined the convulsant action of a number of benzimidazole derivatives in mice. Thus, while 5-methylbenzimidazole induces paraesthesia, 5-nitrobenzimidazole induces convulsions. The authors refrained from drawing any structure-activity correlations and merely concluded that benzimidazole derivative-induced paralysis was due to a selective depression of the central nervous system.

Goodman and Hart (1944) found that oral doses of 200 mg/kg. of benzimidazole in rats caused polydypsia and polyuria, by specifically inhibiting the renal tubular absorption of water. Despite the polyuria young rats continued to grow normally, and there were no organ changes. Bywater and his co-workers (1945a, b) showed that benzimidazole substituted by sulphhydryl at C_2 was goitrogenic in rats. Incorporation of a halogen at position 5 increased this activity, the order of effectiveness being Cl>Br>I. Methylation of the imidazole nitrogen atom destroyed the activity.

Apart from the uncoupling activity and attendant mortality

(LD_{50}'s are presented in Table 1 of the Discussion, p. 113), no marked effects of the 2-trifluoromethylbenzimidazoles have been observed in rats undergoing chronic dosage trials (Dr. K. H. Jones, personal communication).

CHAPTER ONE

SECTION C

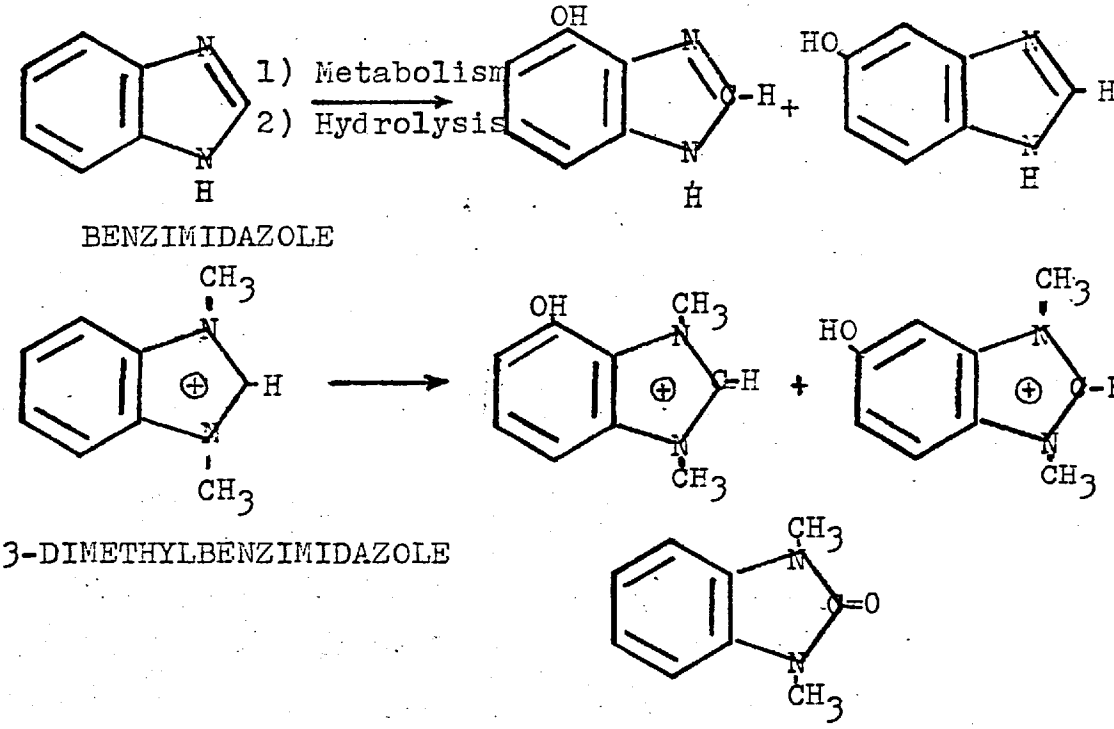
POTENTIAL METABOLIC FATE OF THE COMPOUNDS STUDIED

Detoxication.

Foreign compounds, frequently ingested with the food, are acted upon mainly by the liver after absorption from the gastrointestinal tract. The products of metabolism are eliminated from the body, either via the bile into the faeces, or through the kidneys and into the urine.

The use of 4,5-dichloro- and 5,6-dichloro-2-trifluoromethylbenzimidazole as a herbicide and an acaricide respectively, has made it opportune to study the detoxication of substituted benzimidazoles in a range of living organisms. Accordingly, a joint study has been made, the work reported here being the mammalian metabolism (in rat and rabbit), in which species a large difference in toxicities was observed (see Table 1). Other workers (Bowker and Casida, 1969; Bond, unpublished work) have studied the metabolism for one or more of these compounds in mice, flies and apples. Brief details of these results are reported in the Discussion.

The first paper to report on the mammalian metabolism of benzimidazole was by Ito et al. (1961). These workers injected benzimidazole into rabbits and characterised 4- and 5-hydroxybenzimidazole in the hydrolysed urine. No unchanged benzimidazole could be detected and no quantitative data were given. The same workers also studied the metabolism of injected 1,3-dimethylbenzimidazole in the rabbit (Ito et al., loc. cit.) and showed that it gave rise to the 4- and 5-hydroxy derivatives and also the benzimidazolone in hydrolysed urine. These results may be summarized as follows:



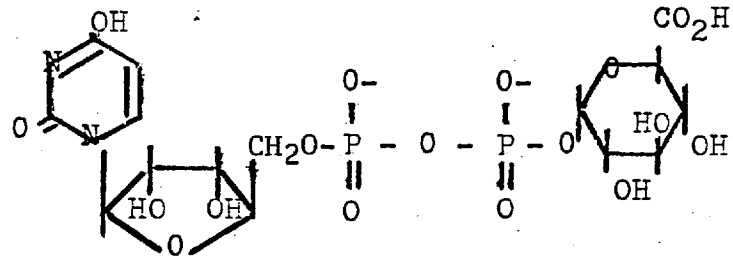
Thus 2-trifluoromethylbenzimidazoles will probably be metabolised mainly by hydroxylation of the benzene ring followed by conjugation reactions (page 34). The following reactions of the amino N-H group are also likely to occur:

1). Conjugation with sugars and sugar derivatives
   a). glucuronic acid
   b). sugars
2). Sulphamic acid formation
3). Methylation
4). Acetylation
5). N-oxide formation
6). Cleavage of the imidazole ring

1a). Conjugation with glucuronic acid

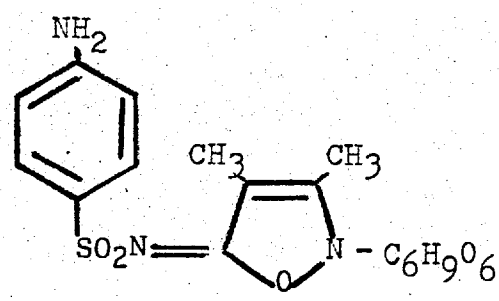
This reaction can occur to varying extents in all mammals and many vertebrates. The formation of a glucuronide requires a coenzyme donor, UDPGA, and enzymes, UDP transglucuronylases.

UDPGA

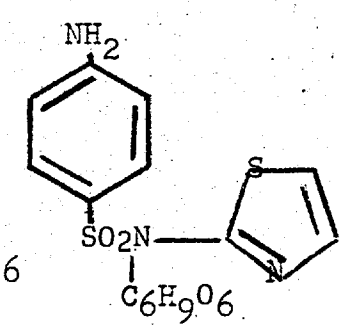


Glucuronide formation occurs mainly in the liver, but it may also occur at other sites in the body, e.g. the kidney. In liver, there appear to be many UDP-transglucuronylases, and indeed there may be more than one enzyme for different types of acceptor (Williams, 1967b). These enzymes also often show species differences in substrate specificity (Parke, 1968).

Several types of N-glucuronides are known. Those of aromatic and aliphatic amino groups are labile and reduce Benedict's reagent. They appear to be artifacts formed spontaneously from glucuronic acid and the free amine (Parke, 1968). The N^{ring}-glucuronide of sulphisoxazole (Uno and Kono, 1962) and the N^{1}-glucuronides of sulphathiazole (Uno and Ueda, 1963) and sulphadimethoxine (Bridges, Kibby and Williams, 1965) are much more stable.



Sulphisoxazole-$N^{2'}$-glucuronide

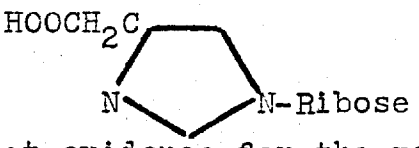


sulphathiazole-N^{1}-glucuronide

An $\underline{N}^1$-glucuronide of a benzimidazole would by analogy be stable. $\underline{N}^1$-glucuronide formation from sulphonamides has been demonstrated in several species including rat, rabbit, monkey and man (Uno and Ueda, 1963; Ueda and Kuribayashi, 1964).

b). sugars

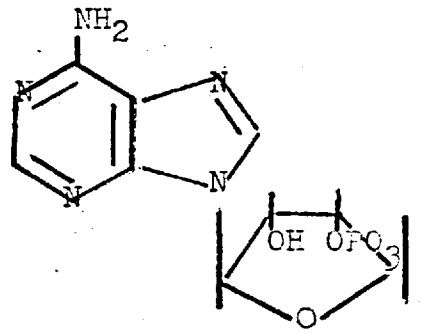
The imino N-H may also be conjugated with ribose. A degradation product of histamine and histidine, namely imidazole-acetic acid, is excreted to varying extents as the riboside in the urine of the rabbit, rat and mouse (Tabor and Hayaishi, 1955; Karjala, 1955).



Indirect evidence for the possibility of $\underline{N}$-glycoside formation in plants comes from the work of Kapoor and Weygood (1965a, b, c), in which benzimidazole was incubated with the detached first leaves of Khapli wheat, and was found to be incorporated into the "nucleoside" fraction.

2). Sulphamic acid formation

Sulphate esters are formed by the transfer of sulphate from adenosine-3'-phosphate-5'-phosphosulphate (PAPS) to the acceptor (alcohol, phenol or amine) by a transferase enzyme (a sulphokinase). PAPS is formed in the liver from ATP. Several sulphokinases are known, and arylsulphamate formation requires an arylamine sulphokinase.



3'-Phosphoadenosine-5'-phosphosulphate (PAPS)

The formation of sulphamates from primary aromatic amines has been demonstrated for aniline and 1- and 2-naphthylamine, rabbit and guinea pig being more effective at this than the rat, which could not conjugate aniline in a soluble liver preparation (Roy, 1958). There is no evidence for secondary amines forming sulphamates.

3). Methylation

Methylation requires a coenzyme, S-adenosylmethionine, which supplies the methyl group, and an enzyme (a methyl transferase) which stereospecifically transfers this to an acceptor. Several different enzymes are known which catalyse the N-methylation of natural and foreign amines. The acceptor may be an amine, phenol or thiol.

N-Heterocyclic methylation has been observed with pyridine acetate (His, 1887) in dogs, when about 4% of the dose was excreted as the N-methyl pyridinium ion. N-Methylation also occurs with the secondary amine histamine, and with the imidazole N-H of imidazoleacetic acid (Karjala, 1956; Schayer, 1956). Methylation of the imidazole N-H of a benzimidazole can be accomplished chemically with diazomethane, and so these derivatives might be expected to arise biochemically during the metabolism of the 2-trifluoromethylbenzimidazoles.

4). Acetylation

According to Williams (1963), the acetylation of foreign compounds only occurs with primary groups, and no evidence exists for the acetylation of a ring secondary amino group. The reaction is hence unlikely to occur. The acetyl compound is possibly very unstable as it could not be obtained under a variety of conditions.

5). Formation of an N-oxide

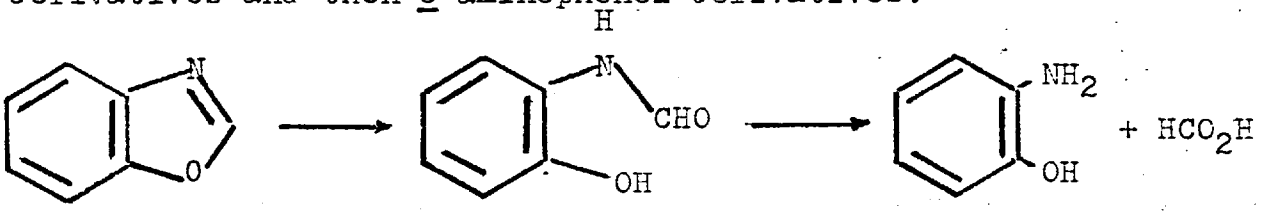
The microsomal system of vertebrates is able to produce trimethylamine N-oxide in the presence of NADPH and oxygen (Baker, 1963). N-Oxide formation has been postulated in the N-dealkylation of drugs by liver microsomes (Brodie et al., 1958).

This is a reaction in which the nitrogen atom becomes tetracovalent; for this reason alone the possibility of N-oxide, and N-hydroxide formation is remote with a heterocyclic compound containing such electron withdrawing groups as trifluoromethyl and chlorine. In support of this, all attempts at chemical synthesis failed.

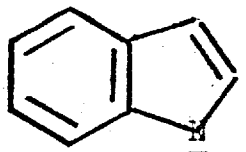
6). Cleavage of the Imidazole ring

This reaction might be expected to occur, but mammalian degradation of imidazole rings has not been observed. Benzoxazole and 2-methylbenzoxazole are metabolised in the rabbit

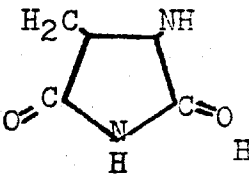
by scission of the oxazole ring to give o-formamidophenol derivatives and then o-aminophenol derivatives:



Similarly indole and hydantoin (in the dog) are also subject to ring scission, the latter probably via the diketone isatin (King, Parke and Williams, 1966).

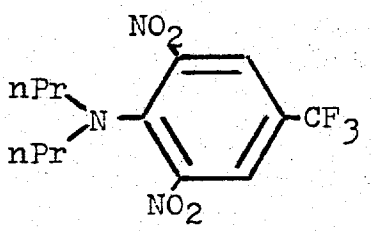


INDOLE



HYDANTOIN

In the case of the 2-trifluoromethylbenzimidazole compounds, the reported chemical stability is such that opening of the ring is improbable. The conversion of a trifluoromethyl group to a carboxyl group and then carbon dioxide has been reported for the herbicide Trifluralin, when carrots were grown in soil treated with the trifluoro-[^{14}C]-methyl compound (Golab et al., 1967). However, the possibility that the reaction was due to micro-organisms and not to the plant itself was not excluded.

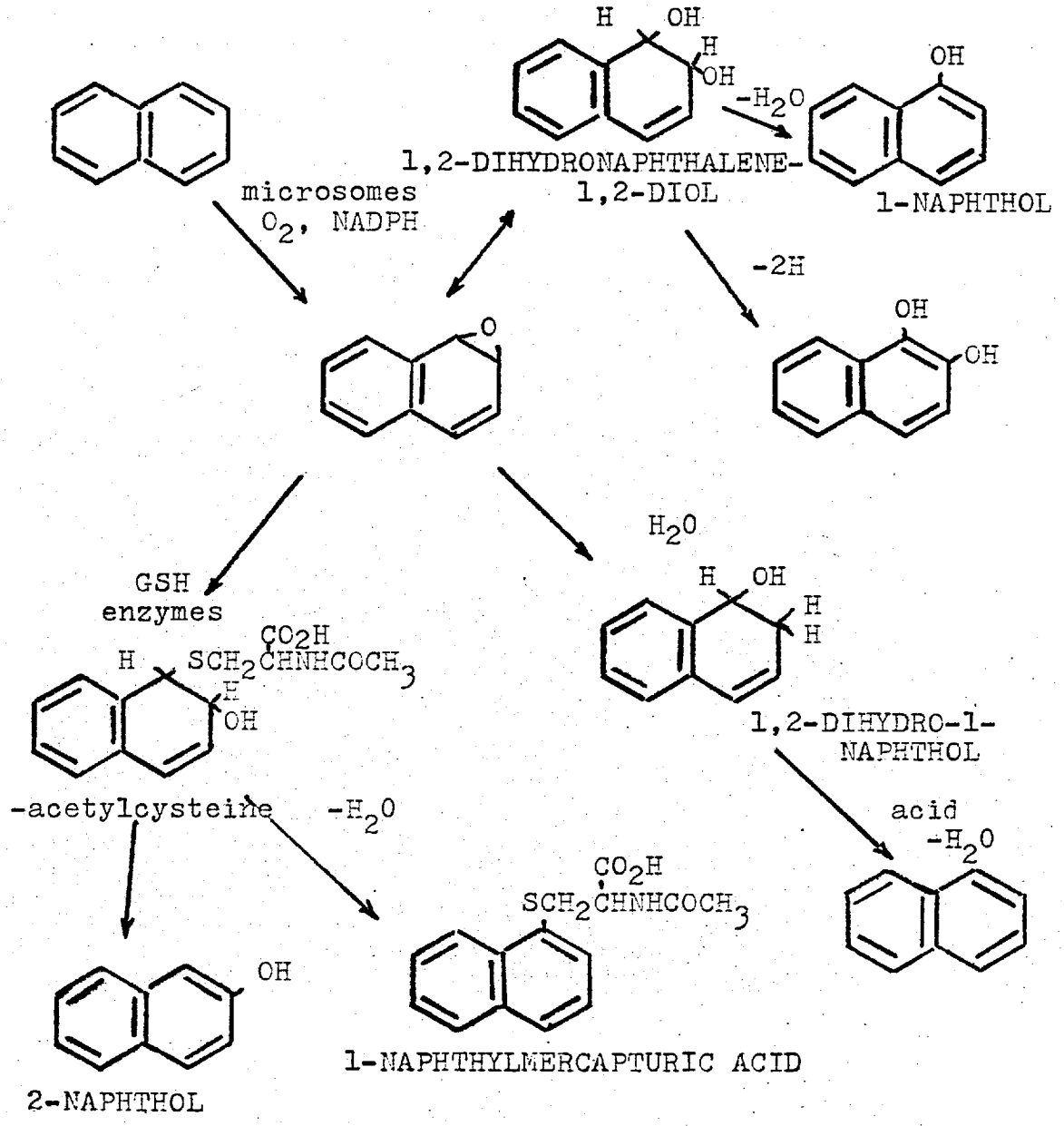


2,6-Dinitro-N,N-di-n-propyl-4-trifluoromethylaniline (Trifluralin)

Reactions of the benzene ring.

Many aromatic compounds are metabolised by hydroxylation by the liver microsomal enzymes. Two mechanisms have been defined, one giving only phenols, and involving hydroxylation

by a free radical, or epoxide formation followed by intramolecular rearrangement to form a phenol. The other, more complex, mechanism involves the formation of 1,2 dihydroarene - 1,2-diols, 1,2 dihydroarenemono-ols and S-(1,2-dihydrohydroxyaryl) - cysteines, which probably have an arene-1,2-epoxide as a common intermediate, and which are converted into phenols, catechols, hydrocarbons and mercapturic acids. Further details of these reactions are included in the Discussion. The reaction scheme for naphthalene according to Parke (1968) is shown below:



Conjugation reactions of the benzene ring and its derivatives.

1). Glucuronide formation

This reaction occurs with primary, secondary and tertiary alcohols and phenols to give an ether type glucuronide. These compounds are stable to alkalis and do not reduce Benedict's reagent.

2). Sulphate formation

This reaction is of very widespread occurrence and is believed to be a primitive method of detoxication. It was discovered by Baumann (1876) who administered phenol to a patient and isolated potassium phenyl sulphate from the urine and showed it to be less toxic than phenol. Phenol sulphokinase is the enzyme catalysing the formation of phenol sulphate. It is relatively non-specific, and is located in the soluble fraction of mammalian liver and kidney homogenates.

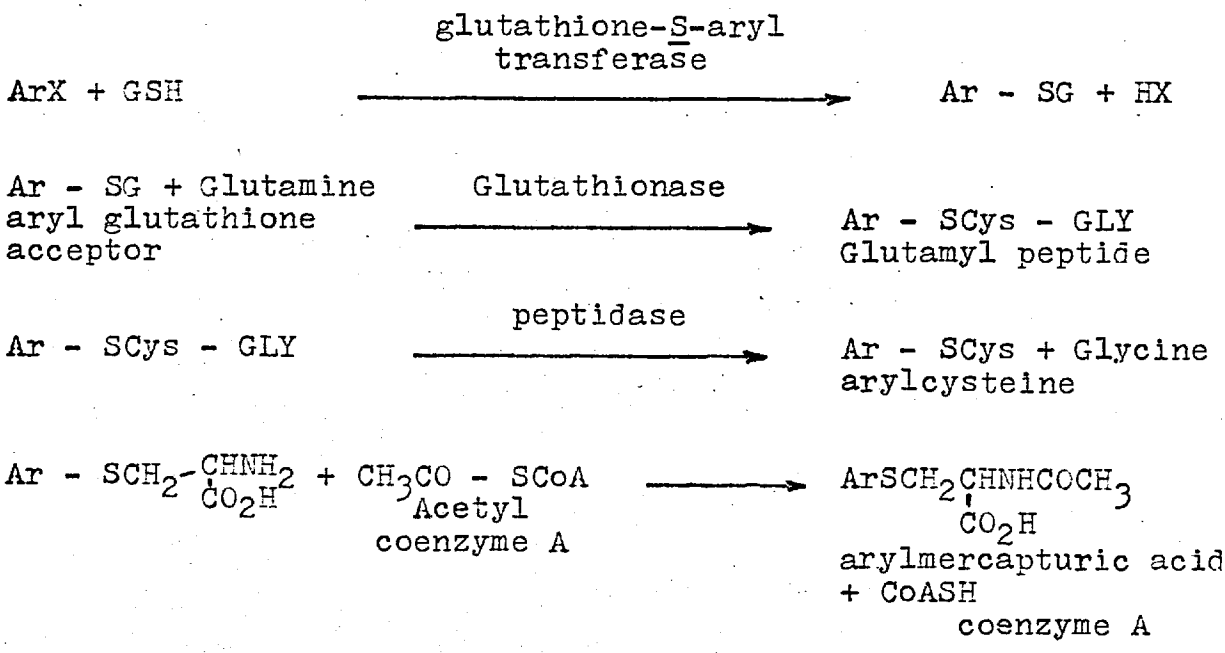
3). Methylation

O-methylation is usually confined to polyhydric phenols containing vicinal hydroxyl groups (catechol o-methyltransferase), and only one hydroxyl group is methylated. 3,5-Diiodo-4-hydroxybenzoic acid is methylated by man (Maclagan and Wilkinson, 1954) but this enzyme seems specific for iodo phenols.

4). Mercapturic acid formation

Mercapturic acids can be formed by the replacement of a labile halogen atom by L-acetylcysteine. The enzyme catalysing the first step is glutathione-S-aryltransferase which is found

in the soluble fraction of liver homogenates. The other enzymes required for mercapturic acid formation, glutathionases, peptidases and acetylases are found in liver and kidney. A schematic diagram of the formation of a mercapturic acid from a halogenated aromatic compound is shown below:



CHAPTER TWO

Materials and Methods.

CHEMICALS.

The following 2-trifluoromethylbenzimidazole derivatives were used in the study:

Non-radioactive compounds	m.p. (uncorrected)
4,5-dichloro-2-trifluoromethylbenzimidazole	214° C
4,5-dichloro-6-hydroxy-2-trifluoromethyl-benzimidazole	232 - 233° C
4,5-dichloro-7-hydroxy-2-trifluoromethyl-benzimidazole	175 - 178° C
4,5-dichloro-6,7-dioxo-2-trifluoromethyl-benzimidazole	194 - 196° C
4,5-dichloro-1-methyl-2-trifluoromethyl-benzimidazole	118° C
5,6-dichloro-2-trifluoromethylbenzimidazole	244 - 245° C
5,6-dichloro-4-hydroxy-2-trifluoromethyl-benzimidazole	199 - 201° C
5,6-dichloro-4,7-dioxo-2-trifluoromethyl-benzimidazole	206 - 208° C
5,6-dichloro-1-(β-D-glucopyranuronic acid)-2-trifluoromethylbenzimidazole	208° C (decomp.)
5,6-dichloro-1-(β-D-glucopyranoside)-2-trifluoromethylbenzimidazole	241 - 243° C
5,6-dichloro-1-methyl-2-trifluoromethyl-benzimidazole	161 - 163° C
4,6-dichloro-5-hydroxy-2-trifluoromethyl-benzimidazole	155 - 157° C

5-chloro-4-hydroxy-2-trifluoromethyl-benzimidazole	177 - 178° C
6-chloro-5-hydroxy-2-trifluoromethyl-benzimidazole	216 - 217° C

Radioactive compounds (specific activities in brackets).

4,5-Dichloro-2-(trifluoromethyl-[^{14}C]-)benzimidazole (3.54 μCi/mg); 5,6-dichloro-2-(trifluoromethyl-[^{14}C]-)benzimidazole (3.50 μCi/mg); 5,6-dichloro-4-hydroxy-2-(trifluoromethyl-[^{14}C]-)benzimidazole (1.93 μCi/mg).

The benzimidazole derivatives were synthesised and supplied by Fison's Agrochemicals Ltd., Chesterford Park Research Station, Saffron Walden, Essex.

Enzyme Preparations.

β-Glucuronidase (Ketodase; Warner-Chilcott, Eastleigh, Hants.) and sulphatase (type H2 from Helix pomatia; Sigma, London) were purchased.

All other chemicals were of the purest grade available commercially.

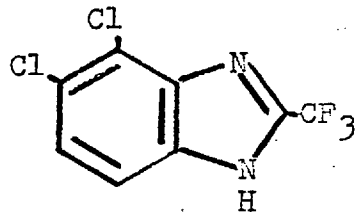
ANIMALS.

Female Wistar albino rats (body weight 200 ± 25 g.) and New Zealand White doe rabbits (body weight 3.5 ± 0.5 kg.) were used. Animals were maintained on diet 41B (J. Rank Ltd.)

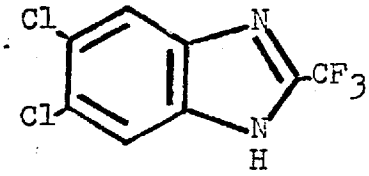
ADMINISTRATION OF COMPOUNDS.

Compounds were administered orally by stomach tube in a 70/30 v/v propyleneglycol-water mixture. For biliary cannulated

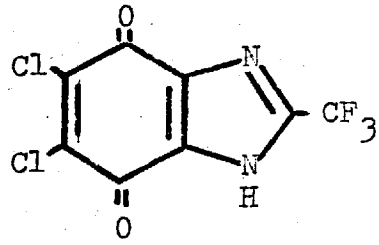
STRUCTURES OF SOME 2-TRIFLUOROMETHYLBENZIMIDAZOLES



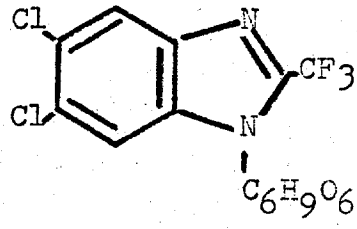
4,5-dichloro-2-trifluoromethylbenzimidazole



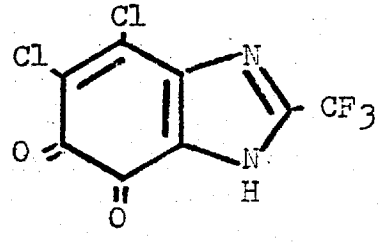
5,6-dichloro-2-trifluoromethylbenzimidazole



5,6-dichloro-4,7-dioxo-2-trifluoromethylbenzimidazole



5,6-dichloro-1-(β-D-glucopyranuronic acid)-2-trifluoromethylbenzimidazole



4,5-dichloro-6,7-dioxo-2-trifluoromethylbenzimidazole

rats, the dose was given by intraperitoneal injections, and for the determination of plasma half lives it was given by intravenous injection. The injections were in the ear vein for rabbits and in the tail vein for rats.

COLLECTION OF BIOLOGICAL SAMPLES.

The animals were kept in metabolism cages with free access to food and water. Urine and faeces were collected daily for periods up to 5 or 7 days after dosing. Biliary fistulae were established in rats by the method of Abou-el-Makarem et al. (1967), and bile was collected for 24 hours. Rabbits dosed with 5,6-dichloro-2-trifluoro-[^{14}C]-methylbenzimidazole were kept in a metabolism cage which allowed collection of expired CO_2 and other volatile derivatives.

Blood samples (1 ml. rabbits, 0.2-0.5 ml. rats) were obtained from animals dosed intravenously with radio-labelled compounds, by nicking the ear vein of rabbits or from cannulas inserted into the femoral vein of rats.

RADIOCHEMICAL TECHNIQUES.

^{14}C In urine (1-2 ml.), bile (0.5-1.0 ml.) and faeces (1 ml. of 10% w/w aqueous homogenate) was determined with a Packard Tri-Carb scintillation spectrometer (model 3214) as described by Bridges, Davies and Williams (1967). ^{14}C In blood was determined by digesting aliquots (0.1 ml.) in 10% NaOH (0.1-0.2 ml.) and bleaching with excess 100 vol. hydrogen peroxide to a pale yellow colour. Foaming was minimised by the addition

of iso-octanol (1 drop). The excess peroxide was destroyed by the addition of conc. HCl, and after neutralisation the radioactivity was estimated by scintillation counting in thixotropic gel (Cab-o-Sil, 5%). Efficiencies were as follows: urine, 60-65%; bile, 60-65%; faeces, 50-55%; blood and most tissues, 50-55%, as determined by internal standardisation. Subcellular fractions of tissue samples were treated by digestion in 10% NaOH, neutralisation (with bleaching if required) and the ^{14}C counted after dispersion in thixotropic gel.

In some experiments respired air was bubbled through 10% aqueous NaOH, a drying tower ($CaCl_2$) and through a liquid nitrogen trap. Saturated $BaCl_2$ was added to portions (10 ml.) of the NaOH and the precipitated $BaCO_3$ was washed with 2 x 10 ml. portions of distilled water, dried and assayed for ^{14}C by scintillation counting in thixotropic gel. The contents of the liquid nitrogen trap after evaporation of N_2 were dissolved in ethanol and assayed by scintillation counting.

The amounts of metabolites were determined either by scanning radiochromatograms (of paper or silica thin layer) with a Packard Radiochromatogram Scanner (model 7200), utilising the disc integrator, or by cutting out the defined area on the paper and counting in scintillator fluid. This latter method was employed for all low level activity work and for small peaks.

Estimations of the metabolites in hydrolysed urine and in tissue extracts were performed by cochromatography with

authentic samples on silica gel thin layers, and scraping off the relevant area (Snyder, 1962). The radioactivity was determined by scintillation counting in thixotropic gel. Recoveries of 89-90% were obtained.

REVERSE ISOTOPE DILUTIONS.

a). 4,5-Dichloro-2-trifluoromethylbenzimidazole in urine.

Aliquots (5 ml.) of urine with added carrier 4,5-dichloro-2-trifluoromethylbenzimidazole (0.5 g.) were refluxed for 6 hours with 10N-HCl (5 ml.). The hydrolysate was extracted with ether (4 x 10 ml.) and the bulked extracts were evaporated to dryness. 4,5-Dichloro-2-trifluoromethylbenzimidazole was recrystallised from aqueous ethanol to constant specific activity and m.p. (214°). This gave an estimate of the free and conjugated compound.

For the free compound, aliquots of urine (5 ml.) and added carrier (0.5 g.) were adjusted to pH 4 with acetic acid and ether extracted (4 x 10 ml.) as before.

4,5-Dichloro-2-trifluoromethylbenzimidazole in faeces.

Free 4,5-dichloro-2-trifluoromethylbenzimidazole was estimated by taking aliquots (5 ml.) of homogenised faeces (usually 1 g. in 10 g. water), adding carrier (0.5 g.) and proceeding as for urine samples.

Total compound was estimated by adding carrier to the homogenate and hydrolysing for 6 hours with 10 ml. 10N-HCl, the rest of the procedure being as before.

4,5-Dichloro-2-trifluoromethylbenzimidazole in bile

The method used was that for urine, but 1 ml. samples were employed.

b). 4,5-Dichloro-1-methyl-2-trifluoromethylbenzimidazole

Carrier (0.5 g.) was added to urine (5 ml), bile (1 ml) and faeces (5 ml. of homogenate), dissolved with the minimum of ethanol and then treated as for 4,5-dichloro-2-trifluoromethyl-benzimidazole. The residue after evaporating off the ether was recrystallised from ethanol to constant specific activity, m.p. 118°.

c). 5,6-Dichloro-2-trifluoromethylbenzimidazole

The procedures for urine, bile and faeces were identical with those for 4,5-dichloro-2-trifluoromethylbenzimidazole, the m.p. was 244-245°.

5,6-Dichloro-2-trifluoromethylbenzimidazole in liver and muscle

5-10 ml. of 9:1 v/w homogenate in 0.25 M-sucrose, with added carrier, was hydrolysed with 10 ml. 10N-HCl for 6 hours. The hydrolysate was extracted with 4 x 20 ml. ether, and evaporated to an oil. An aqueous ethanolic solution of $AgNO_3$ (5%) was added and the precipitated silver salt of the benzimidazole was washed several times with ether/ethanol 1/1 v/v. The dry residue was treated with excess dilute HNO_3 and ether extracted (4 x 10 ml). The extracts were combined, evaporated to dryness and the residue recrystallised from aqueous ethanol to constant specific activity, m.p. 244-245°.

d). 5,6-dichloro-1-methyl-2-trifluoromethylbenzimidazole

The procedures were the same for 4,5-dichloro-1-methyl-2-trifluoromethylbenzimidazole; the compound was recrystallised from ethanol, m.p. 163°.

e). Trifluoroacetic acid

Trifluoracetic acid (1g.) (B.D.H., Poole, Dorset) was refluxed with samples of urine (5 ml.) for 6 hours. The hydrolysate was ether extracted (4 x 10 ml.) and the combined extracts evaporated to a syrup. The syrup was carefully neutralised with 10% aq. NaOH (to phenolphthalein) and excess of S-benzylisothiouronium chloride (in 2 ml. water) was added. The precipitated salt was recrystallised from hot water to zero specific activity, m.p. 176-178°.

CHROMATOGRAPHY.

a). Paper and Thin Layer

The Rf values of the compounds used in this work are shown in Table 1. Paper chromatography utilised unlined tanks and Whatman No. 1 paper.

The following solvent systems were employed for paper:

A. Butan-1-ol saturated with 1.5 M-ammonia/ammonium carbonate aqueous buffer (Fewster and Hall, 1951).

B. Pyridine/pentan-1-ol/water 7:7:6 by volume.

The chromatograms were developed for a distance of about 35 cm. from the origin.

Analytical and quantitative thin-layer chromatography was carried out on 0.25 mm. layers of Silica Gel HF_{254} (E. Merck,

A.-G, Darmstadt, Germany) containing a fluorescent indicator.

The following solvent systems were employed:

A. Fewster and Hall system as for paper.

B. Isobutanol saturated with water (Bowker and Casida, 1969).

C. Chloroform - ethanol 19:1 v/v.

D. Toluene - dioxan 25:4 v/v.

E. Dichloromethane.

F. Benzene - acetone 10:1 v/v (Bowker and Casida, 1969).

G. Dichloromethane - chloroform - ethyl acetate - ammonia (0.88) 120:40:10:1 by vol.

The chromatograms were developed for a distance of about 15 cm. from the origin. Rf values in these systems are given in Table 1.

Detection.

All of the substances appeared as dark quenching spots when paper chromatograms were viewed by U.V. light (254 nm.) from a Hanovia Chromatolite lamp (Engelhard Industries Ltd., Slough, Bucks.). Most of the compounds could also be detected as purple/black quenching spots on fluorescent thin layers, but the phenolic compounds gave yellowish or brown colours, and the quinonoid compounds absorbed only slightly. The phenols tended to oxidise in the atmosphere when on a silica gel surface, and the appearance of a strong yellow colour over a period of several hours served as an indicator of their presence.

Diazotised p-nitroaniline.

p-Nitroaniline (0.25 g.) was dissolved in 25 ml. of N-HCl

Table 1. Rf VALUES OF BENZIMIDAZOLE DERIVATIVES ON PAPER AND THIN-LAYER SILICA GELS.

COMPOUND	A	B	C	D
4,5-dichloro-2-trifluoromethylbenzimidazole	0.90-0.95	0.99	0.85-0.90	0.90
4,5-dichloro-6-hydroxy-2-trifluoromethyl-benzimidazole	0.65-0.70	0.75	0.80-0.90	0.00-0.10
4,5-dichloro-7-hydroxy-2-trifluoromethyl-benzimidazole	0.75-0.80	0.75	0.80-0.85	0.25-0.30
4,5-dichloro-6,7-dioxo-2-trifluoromethyl-benzimidazole	0.70-0.75	0.75	0.55-0.60	-
5,6-dichloro-2-trifluoromethylbenzimidazole	0.90-0.95	0.99	0.85-0.90	0.90
5,6-dichloro-4-hydroxy-2-trifluoromethyl-benzimidazole	0.70-0.80	0.75	0.80-0.85	0.50-0.55
5,6-dichloro-4,7-dioxo-2-trifluoromethyl-benzimidazole	0.85-0.90	0.75	0.60-0.65	-
5-chloro-4-hydroxy-2-trifluoromethyl-benzimidazole	-	-	-	0.55-0.60
6-chloro-5-hydroxy-2-trifluoromethyl-benzimidazole	-	-	-	-
4,6-dichloro-5-hydroxy-2-trifluoromethyl-benzimidazole	-	-	-	0.35-0.40
5,6-dichloro-1-(β-D-glucopyranuronic acid)-2-trifluoromethylbenzimidazole	0.45-0.55	0.40-0.50	0.15-0.20	-
5,6-dichloro-1-(β-D-glucopyranoside)-2-trifluoromethylbenzimidazole	0.45-0.50	-	0.35-0.40	-

See text for details of solvent systems.

Continued.

Table 1, continued. Rf VALUES OF BENZIMIDAZOLE DERIVATIVES ON PAPER AND THIN-LAYER SILICA GELS.

COMPOUND	E	F	G	H
4,5-dichloro-2-trifluoromethylbenzimidazole	0.50-0.55	0.10	0.50-0.55	0.55
4,5-dichloro-6-hydroxy-2-trifluoromethyl-benzimidazole	0.15-0.20	0.0	0.10-0.15	-
4,5-dichloro-7-hydroxy-2-trifluoromethyl-benzimidazole	0.25-0.30	0.0	0.25-0.30	-
4,5-dichloro-6,7-dioxo-2-trifluoromethyl-benzimidazole	0.0	-	-	-
5,6-dichloro-2-trifluoromethylbenzimidazole	0.50-0.55	-	0.48-0.52	0.65
5,6-dichloro-4-hydroxy-2-trifluoromethyl-benzimidazole	0.20-0.25	0.0	0.20-0.25	-
5,6-dichloro-4,7-dioxo-2-trifluoromethyl-benzimidazole	0.05-0.15	0.0	-	-
5-chloro-4-hydroxy-2-trifluoromethyl-benzimidazole	0.40-0.45	-	0.40-0.45	-
6-chloro-5-hydroxy-2-trifluoromethyl-benzimidazole	0.25-0.30	-	0.25-0.30	-
4,6-dichloro-5-hydroxy-2-trifluoromethyl-benzimidazole	-	-	0.45-0.48	-
5,6-dichloro-4-hydroxy-1-methyl-2-trifluoro-methylbenzimidazole	-	0.83+0.67	-	-
5,6-dichloro-4-methoxy-1-methyl-2-trifluoro-methylbenzimidazole	-	0.85	-	-

See text for details of solvent systems.

Continued.

Table 1, continued. Rf VALUES OF BENZIMIDAZOLE DERIVATIVES ON PAPER AND THIN-LAYER SILICA GELS.

COMPOUND	E	F	G	H
5,6-dichloro-4-methoxy-2-trifluoromethyl-benzimidazole	-	0.38	-	-
5-chloro-2-trifluoromethylbenzimidazole	-	-	-	0.7
4,6-dichloro-2-trifluoromethylbenzimidazole	-	-	-	0.56
4,7-dichloro-2-trifluoromethylbenzimidazole	-	-	-	0.52

See text for details of solvent systems.

and the solution diluted to 50 ml. with ethanol. To 10 ml. of this stock solution, 0.1 g. of sodium nitrite was added and the chromatogram was sprayed immediately with this solution. After 5 minutes the chromatogram was resprayed with 0.5N-ethanolic NaOH. Amines and phenols gave non-permanent brown coloured spots (Wichstrom & Salverson, 1952).

Naphtharesorcinol Test.

The chromatogram was sprayed with naphtharesorcinol in acetone (1% w/v) to which 10% phosphoric acid (4:1 v/v) was added prior to use. On heating at 120° for 5 minutes, the glucuronides showed up as bright-blue spots on a pink background. The method was limited to thin layers, since paper tended to be destroyed before the spots appeared (Bridges, Kibby and Williams, 1965).

Gibbs Reagent (Elliott, 1959).

Chromatograms were sprayed with 0.05% of 2,6-dichlorobenzoquinone-4-N-chloroimine in ethanol; after 2 minutes they were oversprayed with saturated $NaHCO_3$. The phenols having a free para position gave a blue colour.

Toennies & Kolb Chloroplatinate reagent (as modified by Barnsley, Thomson and Young, 1964).

0.002M Chloroplatinic acid (40 mls), 1M-KI (0.25 mL) and 2N-HCl (0.4 mL) were added to 76 ml. acetone. On spraying the chromatograms, compounds containing organically bound sulphur gave colourless or pale yellow spots on a pink background.

Table 2. GAS-LIQUID CHROMATOGRAPHY.

COMPOUND	RETENTION TIMES (in minutes) COLUMN A	B	C	D
5,6-dichloro-2-trifluoromethylbenzimidazole	12.4	10.0	10.0	3.0
5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole	8.8 , 23.8	7.0	8.0	6.2 , 7.8
5,6-dichloro-4,7-dioxo-2-trifluoromethylbenzimidazole	1.5 , 22.0	6.2	5.0	1.0 , 13.3
5-chloro-4-hydroxy-2-trifluoromethylbenzimidazole	-	-	-	5.2 , 8.6
6-chloro-5-hydroxy-2-trifluoromethylbenzimidazole	-	-	-	2.7 , 5.0
4,6-dichloro-5-hydroxy-2-trifluoromethylbenzimidazole	-	-	-	6.2 , 9.4
4,5-dichloro-2-trifluoromethylbenzimidazole	7.5 , 16.0	7.2	2.2 , 3.5	2.6 , 5.0
4,5-dichloro-6-hydroxy-2-trifluoromethylbenzimidazole	8.8 , 23.5	3.2 , 17.0	-	5.6
4,5-dichloro-7-hydroxy-2-trifluoromethylbenzimidazole	-	5.2 , 6.5 25.0	8.0 , 10.0 30.0	-
4,5-dichloro-6,7-dioxo-2-trifluoromethylbenzimidazole	4.2	12.0	-	-

Continued.

Table 2, continued. GAS-LIQUID CHROMATOGRAPHY.

All samples were methylated according to the method of Vogel (1956).

Solid supports in all cases were of 100-120 mesh Chromosorb G (acid washed and treated with dimethylchlorosilane) - B. D. H., Poole, Dorset. The carrier gas was Argon/Methane 95/5^{v}/v. Inlet pressure 40 lb. The flash heater and the detector were operated 20^{o} C above column temperature.

b). Gas-Liquid Chromatography.

An F & M model 402 gas chromatograph was used with a variety of column packings, for identification of the metabolites.

Columns and operating temperatures were as follows:

A	4'	3.8% SE30 at 130° C.
B	5'	12% sodium dodecyl benzenesulphonate at 185°.
C	5'	3% PEGA at 170°.
D	4'	3.8% SE30 at 150°.

A tritium foil electron capture detector was employed to detect the compounds in the effluent from the columns. In practice quantities as low as 10^{-12}g. could be detected. Retention times are given in Table 2.

To facilitate separation, and to reduce retention times, the compounds were reacted with excess diazomethane in ethereal solution (Vogel, 1956) to convert imidazole -NH and phenolic -OH groups to the methylated derivatives. On methylation, each symmetrical benzimidazole gave one product and each unsymmetrical benzimidazole gave two products as a result of the tautomeric nature of the imidazole nucleus (Forsyth and Pyman, 1925; Hofmann, 1953). This decreased the usefulness of G.L.C. as a quantitative method.

c). Mass Spectroscopy.

This was performed by Dr. B. Millard of the School of Pharmacy (University of London) on an AEI MS 902 mass spectro-

meter. The aglycones all gave good cracking patterns, but in general, the amounts and quality of material recovered from hydrolysed urine samples were insufficient to allow adequate identification. The pattern obtained from 4,5-dichloro-6-hydroxy-2-trifluoromethylbenzimidazole isolated from hydrolysed rat urine by thin layer chromatography is shown in Fig. 2 of Chapter 3.

TISSUE DISTRIBUTION OF 5,6-DICHLORO-2-TRIFLUOROMETHYLBENZIMIDAZOLE.

Whole rats, previously treated with an oral dose of 5,6-dichloro-2-trifluoro-[^{14}C]-methylbenzimidazole, were dissolved in 1 litre of 20% NaOH in ethanol, with the aid of gentle warming. Aliquots (1 ml.) were taken and neutralised with 4N-HCl and counted in thixotropic gel scintillator. Rat and rabbit organs and tissues were dissected out, weighed and dissolved in 20% NaOH in ethanol (5 ml. per g. of wet tissue), aliquots (1-2 ml.) were neutralised and counted as for whole rats above. Liver, lung, heart and spleen samples were also subjected to the peroxide bleaching technique described under plasma half-lives. Internal standards were included in some of the more coloured samples and also in the peroxide bleached ones, to check for colour quenching and chemical effects. The peroxide bleaching did not appear to have any deleterious effect on any of the ^{14}C compounds used in this study. Isotope dilutions were performed on tissue samples (mainly liver). Quantities of tissue (4-5 g.) were homogenised in 9:1 v/w 0.25

M sucrose/tissue, 0.5 g. of carrier 5,6-dichloro-2-trifluoromethylbenzimidazole was added and the homogenate was then acidified and hydrolysed for 5-6 hours with HCl in the manner described for urine samples. The hydrolysate was ether extracted (4 x 20 ml.), the extracts pooled, evaporated to dryness and the residue recrystallised to constant activity from ethanol-water, with charcoal decolourisation performed where necessary. Fat samples required the procedure outlined under Isotope Dilutions, namely preparation of the silver salt, in order to purify the compound and free it from fatty impurities.

SOLVENT EXTRACTION OF CARCASSES

The remainder of each carcase, after tissues and organs had been removed, was repeatedly extracted with acetonitrile/water (9/1 v/v). The extract was rendered alkaline (pH 8) with 2N-NaOH and ether extracted to remove fatty material. After removing the solvent *in vacuo* the residue was neutralised with 2N-HCl, dissolved in ethanol (0.5 ml.) and portions used for analysis by TLC in the usual way. Treatment of freeze-dried urine samples in this manner was shown to leave conjugates intact.

DETERMINATION OF ^{14}C IN BLOOD

Estimations of metabolites in the blood of animals dosed with [^{14}C]5,6-dichloro-2-trifluoromethylbenzimidazole was performed by TLC co-chromatography of acid-hydrolysed aliquots

obtained as described under Collection of Biological Samples.

CENTRIFUGATION STUDIES

Sub-Cellular Fractionation was performed according to the method of Hogeboom (1953) for liver and to that of Kitiyakara (1953) for muscle. These studies were only performed on rats and rabbits dosed with [^{14}C]5,6-dichloro-2-trifluoromethylbenzimidazole.

Liver.

Quantities of liver (9.0-10.0 g.) were rapidly sectioned after removal from the animal and homogenised in 9 ml. of 0.25 M-sucrose per g. wet weight of tissue, in an homogeniser having a loose fitting Teflon pestle. The pestle was motor driven and 2 x 1 min. periods of homogenisation were used, with intermediate chilling in ice.

10 ml. of homogenate were carefully layered over 10 ml. 0.34 M-sucrose and centrifuged for 10 min. at 2,000 r.p.m. (700 g) at 0-4° in an MSE 'High Speed 17' centrifuge. The supernatant was removed with a Pasteur pipette and the sediment was re-suspended in 0.25 M-sucrose by brief homogenisation. This fraction contained the cell-debris, nuclei and intact cells. The supernatant was centrifuged for 10 min. at 5,000 g to give a pellet of crude mitochondria, this was re-suspended in 0.25 M-sucrose by brief homogenisation and centrifuged at 24,000 g for 10 min.

The combined supernatants from the mitochondrial fraction were centrifuged in an MSE 'Super Speed 40' at 105,000 g av. for 100 min. the residue obtained being crude microsomes. This was re-suspended in 0.25 M-sucrose and recentrifuged at 105,000 g for 2 hours.

All fractions were then dissolved in 10% aq. NaOH and made up to known volumes (5, 10 or 20 mL) with distilled water. Aliquots (1 ml.) were withdrawn, neutralised with 2N-HCl and subjected to scintillation counting. In order to obtain higher efficiency, it was necessary to treat the homogenate to peroxide bleaching. Further aliquots (1 ml. - 2 mL) of the fractions dissolved in NaOH were acidified, ether extracted (4 x 5 ml.) and the combined extracts evaporated to dryness and subjected to TLC analysis by cochromatography. Spots corresponding to known major metabolites (5,6-dichloro-2-trifluoromethylbenzimidazole, 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole and 5,6-dichloro-4,7-dioxo-2-trifluoromethylbenzimidazole) were scraped off and the gel counted in thixotropic gel. In all cases, the quantities of ^{14}C and the metabolites were expressed as a percentage of the radioactivity in the weight of liver taken for analysis.

Muscle.

Tissue was fractionated according to the method of Kitiyakara and Harman (1953). 10 g. of dissected muscle was homogenised for 2 periods of 45 seconds, with intermediate cooling in ice, in a precooled stainless Waring Blender in 75

ml. of 0.25 M-sucrose which contained 0.75 ml. of 0.04 M-$NaHCO_3$. A further 75 ml. of sucrose solution was added and the homogenate was strained through fine cotton gauze to remove fibrous tissue. The filtrate was centrifuged at 0-4° C for 2 min. at 125 g and then for 10 min. at 500 g to remove cell debris, connective tissue, etc. The residue was resuspended by brief (10-20 sec.) homogenisation in 0.25 M-sucrose and recentrifuged at 500 g for 10 min.; the residue so obtained contained myofibrils. The combined supernatants were centrifuged at 500 g for 15 min., the residue was again myofibrils and this was added to the previous pellet. The supernatant was subjected to further centrifugation at 2,250 g for 15 min. to obtain sarcosomes. Purification involved resuspension in 0.25 M-sucrose and centrifugation for 15 min. at 2,250 g The fractions thus obtained were treated as for the liver fractions.

ISOLATION OF NUCLEOTIDES.

The procedure used was that of Schmidt and Thannhauser (1945) as modified by LePage and Heidelberger (1951). The weighed tissue was homogenised in a Waring Blendor in cold 4% $HClO_4$ (10 ml. per g. wet weight) and centrifuged. The residue was washed with cold 4% $HClO_4$ (to remove acid-soluble purines and pyrimidines) and twice with ethanol. Lipids were removed by extracting three times with ethanol / ether 3:1 v/v, at 35-40°. The dry extracted tissue was suspended in 1N-NaOH (10 ml/2 g. fresh tissue) and incubated at 34° for 16 hours; everything dissolved and RNA was hydrolysed to nucleotides.

The incubation was cooled in ice and 3.5 ml. of 65% trichloracetic acid were added for every 10 ml. of NaOH used. A protein-DNA complex precipitated and RNA nucleotides remained in solution. The precipitate was washed with cold 5% TCA and the combined supernatants were adjusted to pH8 with $Ba(OH)_2$. On the addition of 4 volumes of ethanol, barium nucleotides were precipitated; cooling in ice proved beneficial to the process. The residue was filtered off and purified by acidification with HCl and a repetition of the barium hydroxide precipitation. The precipitate was suspended in 10 ml. of distilled water and aliquots (2 ml.) subjected to scintillation counting in thixotropic gel.

The protein-DNA complex was washed with 10 volumes of ethanol and suspended in 10 ml. of neutral 0.3 M-$BaClO_4$ solution. The mixture was heated in a boiling water bath for 20 min., cooled to room temperature and 70% $HClO_4$ added to give a final concentration of 2%. The precipitated protein was removed and the filtrate adjusted to pH8 with $Ba(OH)_2$, 4 volumes of ethanol were added and barium nucleotides were precipitated on cooling in ice. The precipitate was treated as for the RNA nucleotides above.

Aliquots (2 mL) of the suspension of the barium nucleotides were evaporated to recover the solid, suspended in a drop of 4N-HCl and applied to thin layer plates which were chromatographed in systems D or E. The spots which cochromatographed with authentic 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole and 5,6-dichloro-2-trifluoromethylbenzimidazole

and the para-quinone were scraped off and counted in thixotropic gel.

DIALYSIS EXPERIMENTS.

Aliquots (0.2-0.5 ml) of heparinised rat or rabbit blood together with 0-1 ml. of 0.9% aqueous NaCl containing the radio-labelled benzimidazole derivative, were dialysed through Visking tubing into 50-100 ml. of 0.1 M-phosphate buffer pH 7.4. After 24 hours, 25 ml. portions of this liquor were evaporated to dryness, and the residue was ether or alcohol extracted. The extract was assayed for ^{14}C by scintillation counting in the usual way. The technique was also applied to human blood.

Dialysis of non-labelled metabolites

The 6- and 7-hydroxy and 6,7-dioxo derivatives of 4,5-dichloro-2-trifluoromethylbenzimidazole and 5,6-dichloro-4,7-dioxo-2-trifluoromethylbenzimidazole were also tested for their binding ability with rat and rabbit blood. As with the labelled compounds, the concentration used was 20-40 µgm/ml. of aqueous solution. The dialysis liquor was evaporated to dryness and the residues were ethanol or ether extracted (4 x 5 ml. portions). The volume of extract was reduced to 0.5 ml. and an ethereal solution of diazomethane was introduced (Vogel, 1956). After 5-10 min. the ether was evaporated off in a stream of dry nitrogen, and the volume of the solution was adjusted to 0.5 ml. with ethanol. One to 5 µl. aliquots were subjected to G.L.C. analysis as described earlier. Because the derivatives of 4,5-dichloro-2-trifluoromethylbenzimidazole gave multiple peaks

(arising from their tautomeric forms), this method was severely limited in its accuracy. The 6- and 7-hydroxy derivatives of 4,5-dichloro-2-trifluoromethylbenzimidazole were successfully assayed by the method of Folin-Ciocalteu (1927). In this technique 5 ml. of Folin's reagent was added to the residue obtained by evaporating the ethanolic extract of the dry salts to dryness (see above). Fifteen ml. of 20% Na_2CO_3 was added, the mixture was diluted to 50 ml. and incubated at 35°C for 20 min. Readings were taken at 760 nm. in a Unicam SP500 spectrophotometer.

ULTRA-FILTRATION.

The technique used was that described by Bridges et al. (1969). 0.2 ml. samples of heparinised blood, 0.1 ml. of the 0-9% NaCl solution containing the radiolabelled compound and 0.1 ml. of 0.1 M-phosphate buffer pH 7.4 were mixed. The sample was placed in Visking tubing which was tightly sealed and laid on the surface of a sintered glass disc. This was in turn placed in a plastic centrifuge tube and centrifuged for 2 hours in an MSE 'High Speed 17' machine at 3,000 g and 20°C. The sinter and Visking tubing were removed from the tube, and the filtrate was washed into scintillation fluid with ethanol (2-3 ml.). The sample was then counted in the usual way.

CHAPTER THREE

THE METABOLISM OF 4,5-DICHLORO- AND 5,6-DICHLORO-2-TRIFLUOROMETHYLBENZIMIDAZOLE IN THE RAT AND THE RABBIT AND 5,6-DICHLORO-4-HYDROXY-2-TRIFLUOROMETHYLBENZIMIDAZOLE IN THE RAT.

RESULTS.

4,5-Dichloro-2-trifluoromethylbenzimidazole.

Excretion of radioactivity by the rat and the rabbit.

1. Urine.

The excretion figures (presented in Table 1) show that most of the dose is excreted over a period of 3 days in the urine. Chromatography of urine samples (system A on paper or TLC, system C on TLC) revealed the presence of 5 peaks. Radiochromatogram scans of 24 hour urines chromatographed in system A on paper are presented in Fig. 1. Acid hydrolysis of urine samples followed by chromatography of the hydrolysate (system A or C, paper or TLC) revealed only one major peak.

Metabolites.

Quantitation of metabolites was performed by cochromatography of pooled 72 hour urines on thin layers as outlined in Methods. The most important metabolite was 4,5-dichloro-6-hydroxy-2-trifluoromethylbenzimidazole (70% of the dose in rats, 19% in rabbits), together with smaller amounts of the 7-hydroxy isomer (6% and 7% respectively). One rat gave approximately equal amounts of these two compounds, but all others gave these compounds in a 9/1 ratio. (This result could not be repeated and is unexplained.) Only minor quantities of free phenolic compounds could be detected in fresh urine samples, but acid hydrolysis or incubation with β-glucuronidase (as detailed in Methods) released most of the ^{14}C as the 6- and 7-hydroxy compounds together with 4,5-

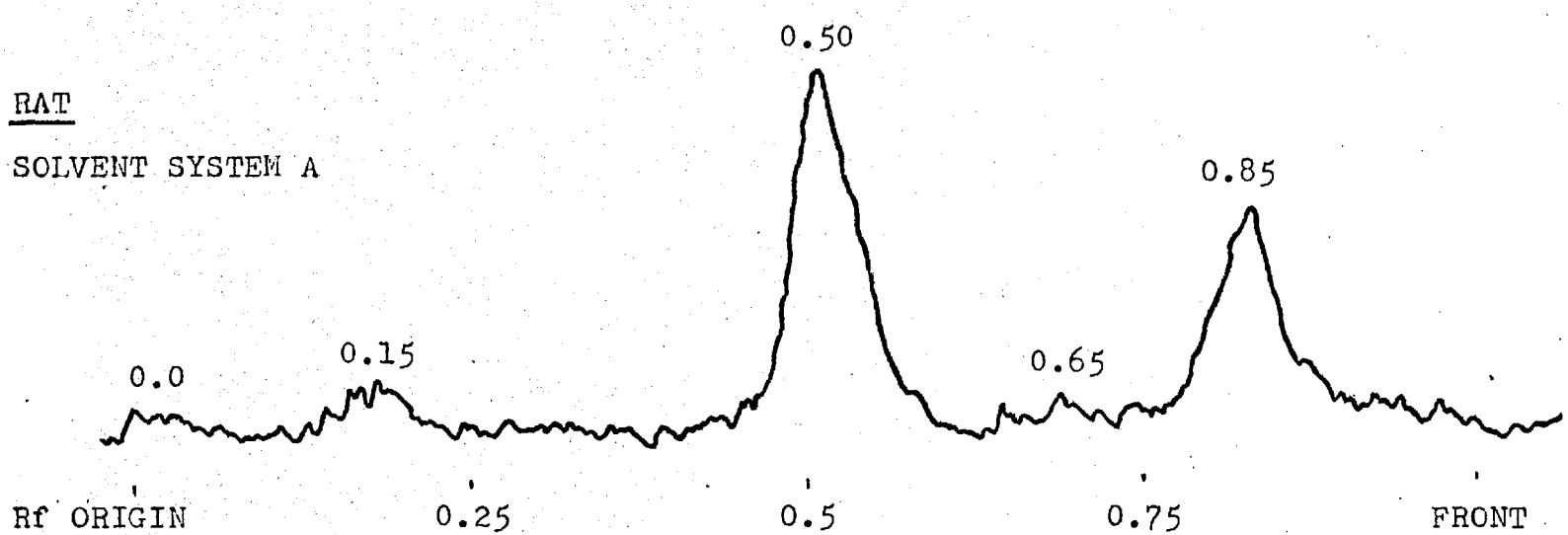
dichloro-6,7-dioxo-2-trifluoromethylbenzimidazole (4% for the rat and 6% for the rabbit). However, peaks at Rf 0.0 (a minor peak in system A on paper or TLC) and Rf 0.5 (a large peak in system A) were more resistant to β-glucuronidase or sulphatase attack. Acid hydrolysis followed by extraction and TLC analysis showed that the Rf 0.5 peak contained both the 6- and 7-hydroxylated derivatives and also 4,6-dichloro-5-hydroxy-2-trifluoromethylbenzimidazole (Rf values in system G. 0.10-0.15, 0.25-0.30, 0.45-0.48 respectively). The Rf 0.0 peak gave a positive Toennies and Kolb test, indicating the presence of organically bound sulphur. The quantities were too small to analyse successfully by the standard methods, but the nature of the metabolite(s) was partially resolved as follows.
A rat was given a dose of 10 μCi of $^{35}SO_4^{2-}$ by intraperitoneal injection, together with an oral dose of 2 mg. of 4,5-dichloro-6-hydroxy-2-trifluoromethylbenzimidazole. A second rat received the $^{35}SO_4^{2-}$ by injection and an oral dose of 2 mg. of 4,5-dichloro-2-trifluoromethylbenzimidazole. The 24 hour urines were freed of inorganic $^{35}SO_4$ by $BaCl_2$ precipitation and were chromatographed as before in system A on paper. Radiochromatogram scanning showed the residual radioactivity to be located at Rf 0.0 and Rf 0.04. The peak at Rf 0.0 was cut out, eluted into acid and hydrolysed. TLC and GLC analysis showed it to contain 4,5-dichloro-6-hydroxy-2-trifluoromethylbenzimidazole and a small amount of 5-chloro-4-hydroxy-2-trifluoromethylbenzimidazole.

Table 1. THE EXCRETION OF ^{14}C BY THE RAT AND RABBIT DOSED WITH 4,5-DICHLORO-2-(TRIFLUORO-[^{14}C]-METHYL)BENZIMIDAZOLE.

		RAT	RAT (BILIARY FISTULA)	RABBIT
Dose	mg/kg.	50 (oral)	52 (intraperitoneal)	6.1 (oral)
	μCi	3.75	4.2	4.5
% Dose in urine	Day 1	70.0(45-88)	-	20(0-59)
	Day 2	11(3.5-20.0)	-	45(32-58)
	Day 3	1.4(0.2-0.3)	-	13(6-22)
	Day 4 and 5	-	-	4(0-14)
Faeces	Day 1-5	< 1.0	-	< 1.0
Bile	Day 1	-	21.0(16.5-26.0)	-
Total excretion		82.4	21.0	82.0

Values are means for 3 animals with ranges shown in parentheses.

Fig. 1. RADIOCHROMATOGRAM SCANS OF 0-24 HOUR URINE OF RATS AND RABBITS DOSED ORALLY WITH 4,5-DICHLORO-2-(TRIFLUORO-[^{14}C]-METHYL)BENZIMIDAZOLE.



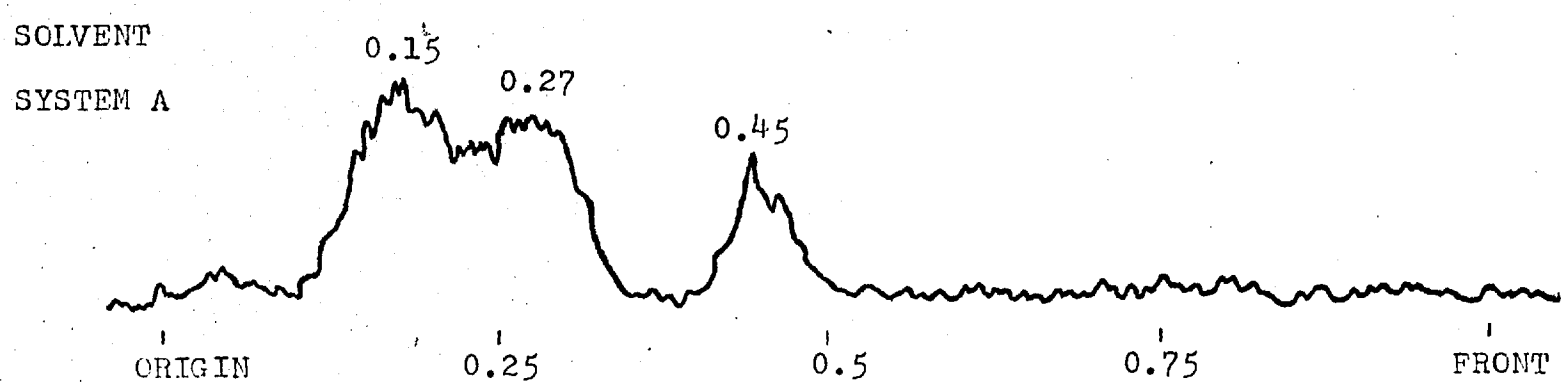


Table 2. METABOLITES OF 4,5-DICHLORO-2-(TRIFLUORO [^{14}C] METHYL)BENZIMIDAZOLE IN THE RAT AND THE RABBIT.

COMPOUND	% DOSE EXCRETED IN RAT URINE**	RAT BILE*	RABBIT URINE+
4,5-Dichloro-2-trifluoromethyl-benzimidazole (free)	-	-	-
4,5-Dichloro-2-trifluoromethyl-benzimidazole (combined)	-	-	4.1(2.9- 6.4)
4,5-Dichloro-6-hydroxy-2-trifluoro-methylbenzimidazole (free)	2.0(0.0- 3.5)	0.9(0.5- 1.6)	1.0(0.4- 1.7)
4,5-Dichloro-6-hydroxy-2-trifluoro-methylbenzimidazole (combined)	68 (12.1-79.5)	8.9(6.1-12.9)	18.0(10.3-24.1)
4,5-Dichloro-7-hydroxy-2-trifluoro-methylbenzimidazole (free)	0.3(0.1- 0.8)	< 0.5(0.0- 0.6)	-
4,5-Dichloro-7-hydroxy-2-trifluoro-methylbenzimidazole (combined)	6.0(3.1-12.1)	7.0(5.4- 7.9)	6.9(6.0- 7.5)
4,6-Dichloro-5-hydroxy-2-trifluoro-methylbenzimidazole	1.5(0.5- 2.8)	2.0(1.8- 2.5)	8.0(6.3- 9.9)
4,5-Dichloro-6,7-dioxo-2-trifluoro-methylbenzimidazole	4.3(0.8- 6.3)	2.2(1.7- 3.1)	6.0(4.8- 7.3)
Unknown metabolites	1.4(1.0- 2.1)	< 0.5(0.1- 0.7)	7.5(6.0- 9.5)
Total	83.5	21.0	51.5
% Dose excreted	82	21	52

* Results for 24 hour samples.
\+ Results for 48 hour samples.
**Results for 72 hour samples.
Values are means for 3 or more animals with ranges in parentheses.
Dose levels and routes are as given in Table 1.

Neither animal excreted free 4,5-dichloro-2-trifluoromethylbenzimidazole, 4,5-dichloro-1-methyl-2-trifluoromethylbenzimidazole or trifluoroacetic acid, as determined by reverse isotope dilution (see Methods). The rabbit, however, excreted a compound (Rf 0.45) which yielded free 4,5-dichloro-2-trifluoromethylbenzimidazole (4.1% of dose) on acid hydrolysis or incubation with β-glucuronidase. Comparison with the authentic $\underline{N}^1$-glucuronide of 5,6-dichloro-2-trifluoromethylbenzimidazole in several solvent systems (A, B or C) on thin layers suggested that the compound could be the $\underline{N}^1$-glucuronide of 4,5-dichloro-2-trifluoromethylbenzimidazole. The metabolite also gave a positive naphtharesorcinol test on TLC plates, tending to confirm this hypothesis.

Six female rats were each orally dosed with 1 mg. of unlabelled 4,5-dichloro-2-trifluoromethylbenzimidazole in 1 ml. of aqueous propane-1,2-diol and the 24 hour urines were collected. The pooled urines were refluxed for 6 hours with 10$\underline{N}$-HCl (50 ml.), evaporated under vacuo at 30-35° to small volume (15 ml.) and applied to 1 mm. thick layers of silica HF_{254}. The plates were developed in system A and the area of Rf 0.60-0.70 was scraped off. The metabolites were eluted off the silica gel with methanol, the extracts were evaporated to dryness and taken up in 1 ml. of water. This solution was applied to a 1 mm. layer of silica gel HF_{254} and the plate was developed in system D. The area at Rf 0.00-0.10 was scraped off and eluted with methanol. The extracts were evaporated to dryness and the

residue was recrystallised from aqueous ethanol to constant melting point. A sample was then characterised by mass spectroscopy and shown by comparison with an authentic sample to be 4,5-dichloro-6-hydroxy-2-trifluoromethylbenzimidazole (Fig. 2).

2. Bile.

Some 21% of the radioactivity from an intraperitoneal dose of 4,5-dichloro-2-trifluoromethylbenzimidazole appeared in the bile in 24 hours in biliary cannulated rats (see Table 1).

A radiochromatogram scan of bile eluted on paper in system A is presented in Fig. 4. Investigation of the three metabolites as described earlier revealed them to be identical to the major urinary metabolites of the rat.

Incubation of aliquots of bile with caecal contents at 37° for 24 hours resulted in complete degradation of the conjugates to the phenolic and quinonoid aglycones, as determined by TLC of ether extracts, or by methylation of the extracts and analysis by GLC.

3. Faeces.

Less than 1% of an oral dose of 4,5-dichloro-2-trifluoro-[^{14}C]-methylbenzimidazole appeared in the faeces of either species over 5 days.

Fig. 2. MASS SPECTRUM OF 4,5-DICHLORO-6-HYDROXY-2-TRIFLUOROMETHYLBENZIMIDAZOLE.

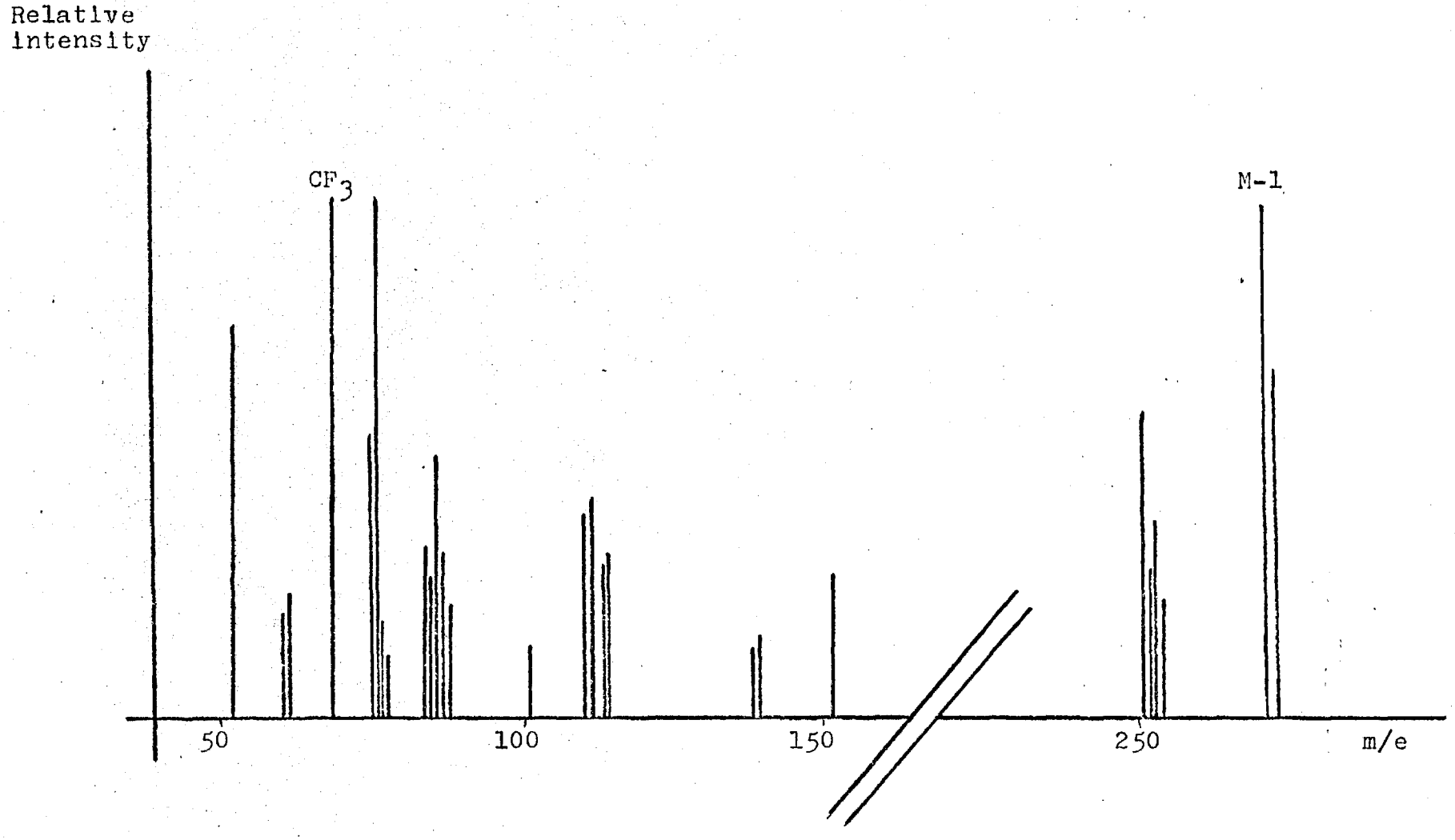


Table 3. EXCRETION OF RADIOACTIVITY BY RATS AND RABBITS DOSED WITH 5,6-DICHLORO-2-(TRIFLUORO[^{14}C] METHYL)BENZIMIDAZOLE.

		RAT	RABBIT	RAT (BILIARY FISTULA)
Dose	mg/kg	33.5 (oral)	3.3 (oral)	33.0 (intraperitoneal)
	µCi/kg	14.5	0.8	9.7
% Dose found in urine	Day 1	17.1(16.1-18.0)	3.7(0-7.0)	–
	Day 2	12.3(9.0-15.1)	5.8(5.2-6.2)	–
	Day 3	3.0(2.9-4.1)	*14.7(14.3-15.4)	–
Bile	Day 1	-	-	29.0 †
Faeces	0-5 Days	28.0(27.3-29.0)	0.1	–
Total		60.4	25.2	29.0

* Value for 3-6 days inclusive.

† Two of the rats died during the experiment.

Results are means with ranges in parentheses.

Table 4. METABOLITES OF 5,6-DICHLORO-2-(TRIFLUORO[^{14}C]METHYL)BENZIMIDAZOLE IN THE RAT AND THE RABBIT.

COMPOUND	RAT			RABBIT
	URINE	BILE	FAECES +	URINE +
5,6-Dichloro-2-trifluoromethyl-benzimidazole (free)	-	3.0	16.5(16.1-17.0)	0.1(0.0-0.2)
5,6-Dichloro-2-trifluoromethyl-benzimidazole (total)	5.5(4.1-7.0)	8.0	16.5(16.1-17.0)	9.2(8.0-10.2)
5,6-Dichloro-4-hydroxy-2-tri-fluoromethylbenzimidazole (free)	1.0(0.8-1.3)	0.9	12.0(10.4-12.6)	0.5(0.2-0.9)
5,6-Dichloro-4-hydroxy-2-tri-fluoromethylbenzimidazole (total)	8.3(7.2-9.4)	6.1	12.0(10.4-12.6)	2.6(2.1-2.9)
4,6-Dichloro-5-hydroxy-2-trifluoro-methylbenzimidazole	0.4(0.3-0.8)	2.1	0.5(0.1- 0.7)	1.6(1.0-2.3)
5-Chloro-4-hydroxy-2-trifluoro-methylbenzimidazole	1.8(1.1-2.2)	5.8		5.0(3.8-6.4)
5-Chloro-6-hydroxy-2-trifluoromethyl-benzimidazole	0.3(0.1-0.6)	2.0		1.5(0.4-2.8)
5,6-Dichloro-4,7-dioxo-2-trifluoro-methylbenzimidazole	1.3(0.9-2.1)	4.6		3.2(0.6-4.5)
Total	17.6	28.5	29.0	23.1
% Dose excreted	17.0(16-18)	29(-)	28.0(27-29)	24.2(19.5-28.6)

+ Urine for rabbits and faeces for rats collected over 5 days and pooled.
Results are the mean values for the determinations on at least 3 animals, except for rat bile which is on only one sample.

5,6-Dichloro-2-trifluoromethylbenzimidazole.

Excretion of radioactivity by the rat and the rabbit.

1. Urine.

The urinary excretion figures (given in Table 3) show that rats excreted 32% of an oral dose in 3 days, and rabbits excreted 20% over a 5 day period. Chromatography of urine samples revealed the presence of 5 metabolite peaks in both cases. Radiochromatogram scans of 24 hour urine samples eluted in system A (on paper) are shown in Fig. 3. Quantitation was performed on urine voided within 24 hours of dosing for rats, and for pooled 5 day samples for rabbits.

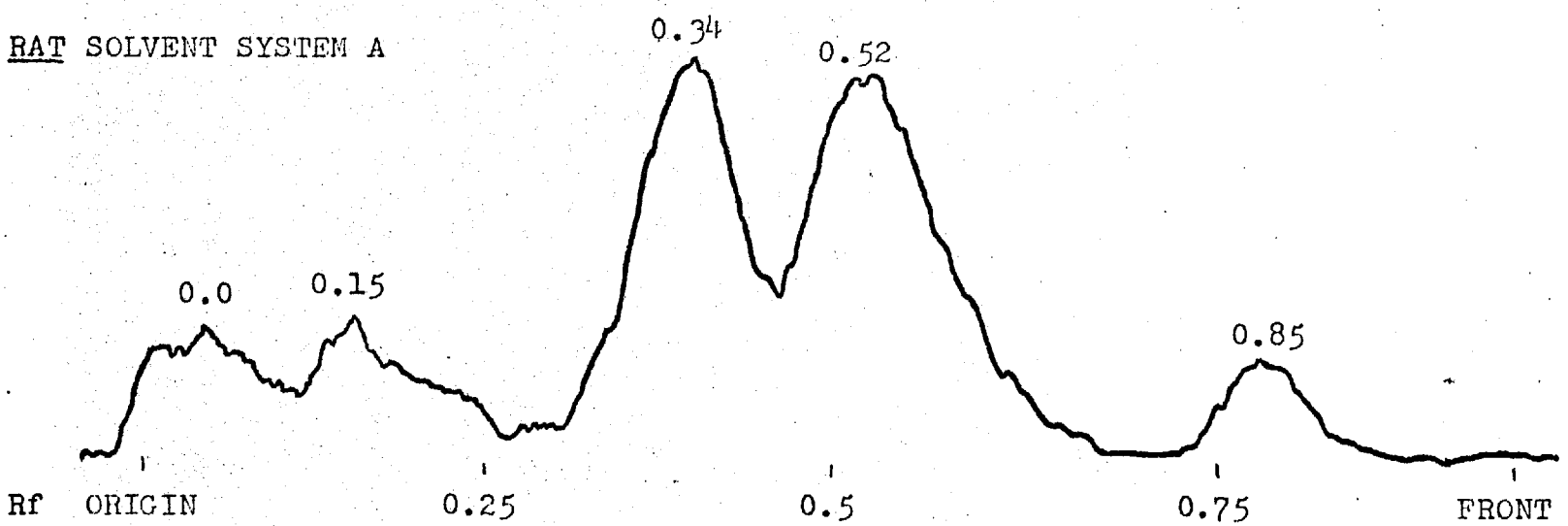
Metabolites.

Only small amounts of unconjugated material were present in fresh urine. Incubation of urine with β-glucuronidase or refluxing with 10N-HCl followed by TLC analysis showed the presence of unchanged drug, 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole, 5,6-dichloro-4,7-dioxo-2-trifluoromethylbenzimidazole plus other phenolic compounds in small amounts, as determined by TLC (system A) or GLC following methylation. The major metabolite in rat urine was shown to be the 4-hydroxylated derivative (8.5% of the dose). In rabbit urine, however, the major metabolite (Rf 0.53-0.60, System A) was the N-glucuronide of 5,6-dichloro-2-trifluoromethylbenzimidazole (9% of the dose) as determined by reverse isotope dilution following incubation of urine samples with β-glucuronidase or after acid hydrolysis. Rat urine contained some 5.5% of this

compound. Reverse isotope dilution experiments failed to detect any 5,6-dichloro-1-methyl-2-trifluoromethylbenzimidazole or trifluoroacetic acid in the urine of either species. Extracts of hydrolysed urine were also shown to contain 5,6-dichloro-4,7-dioxo-2-trifluoromethylbenzimidazole (1.5% in the rat, 3.0% in the rabbit). Confirmation of the presence of this compound was obtained by methylation and gas-liquid chromatography (Rt6.2, column B).

Most of the peaks eluted from chromatograms were found to contain mixtures of hydroxylated derivatives of 2-trifluoromethylbenzimidazole together with glucuronic acid (as shown by a positive naphtharesorcinol test). To pinpoint the site of glucuronic acid conjugation, the following technique was applied to several peaks cut out from paper or thin-layer chromatograms of rat and rabbit urine and rat bile. A methanolic extract was made and reduced to small volume (1 ml.) under vacuo. Following methylation with excess diazomethane, the solution was evaporated to dryness and hydrolysed by refluxing with 2 ml. of 10N-HCl. An ether extract (2 x 5 ml.) was made and subjected to TLC in system F. The spots corresponding to the following compounds were scraped off and assayed for ^{14}C in thixotropic gel: 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole, 5,6-dichloro-4-methoxy-2-trifluoromethylbenzimidazole, 5,6-dichloro-4-hydroxy-1-methyl-2-trifluoromethylbenzimidazole, and 5,6-dichloro-4-methoxy-1-methyl-2-trifluoromethylbenzimidazole. For example the peak at Rf 0.45

Fig. 3. RADIOCHROMATOGRAM SCANS OF 24 HOUR RAT AND RABBIT URINES AFTER AN ORAL DOSE OF 5,6-DICHLORO-2-(TRIFLUORO [^{14}C] METHYL)BENZIMIDAZOLE.



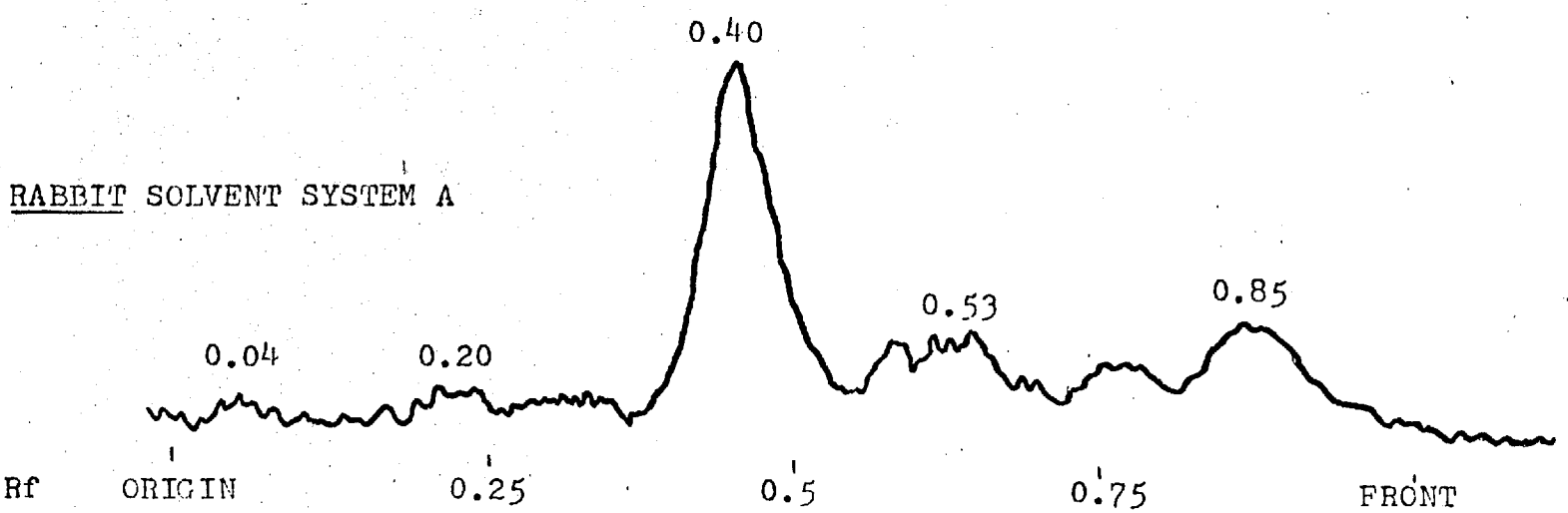
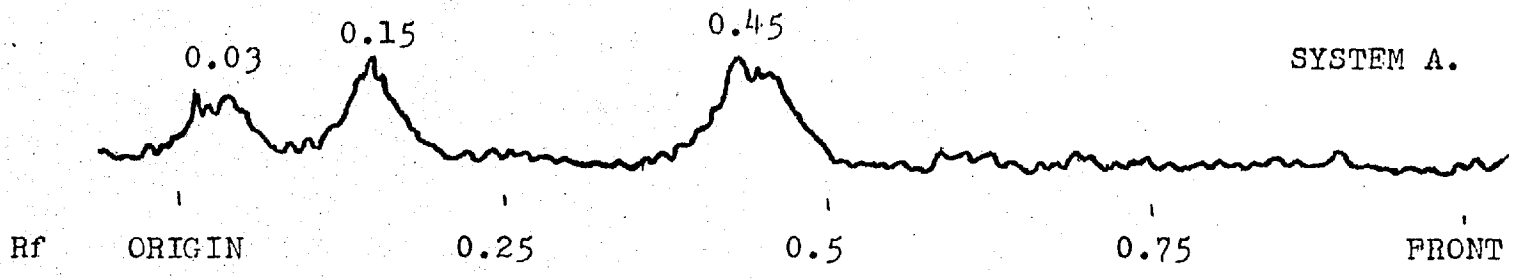
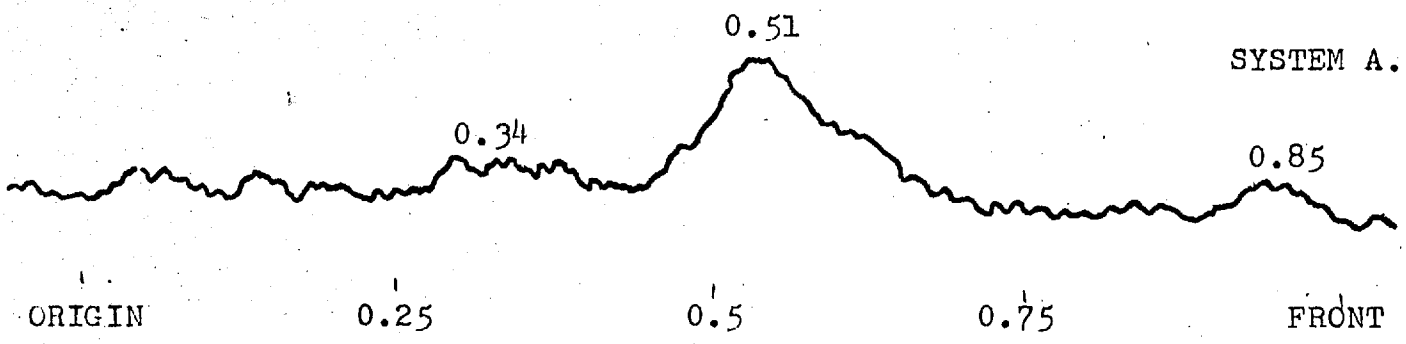


Fig. 4. RADIOCHROMATOGRAM SCANS OF BILE SAMPLE FROM RATS DOSED INTRAPERITONEALLY WITH 4,5-DICHLORO-2-(TRIFLUORO [^{14}C] METHYL)BENZIMIDAZOLE.



RADIOCHROMATOGRAM SCAN OF BILE SAMPLE FROM RATS DOSED INTRAPERITONEALLY WITH 5,6-DICHLORO-2-(TRIFLUORO [^{14}C] METHYL)BENZIMIDAZOLE.



(system A) from rat urine proved to be a mixture of 5,6-dichloro-4-hydroxy-1-glucopyranuronic acid-2-trifluoromethylbenzimidazole (yielding 5,6-dichloro-4-methoxy-2-trifluoromethylbenzimidazole on treatment) and 5,6-dichloro-4-glucopyranuronic acid-2-trifluoromethylbenzimidazole (yielding 5,6-dichloro-4-hydroxy-1-methyl-2-trifluoromethylbenzimidazole). Much smaller quantities of 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole and a possible diconjugate were also present, but these compounds may have resulted from degradation and incomplete methylation respectively.

2. Bile.

Some 29% of an intraperitoneal dose of [^{14}C] 5,6-dichloro-2-trifluoromethylbenzimidazole was obtained in the 24 hour bile of a biliary cannulated rat. Chromatography showed 3 metabolite peaks of the same Rf as urinary metabolites (in system A). A radiochromatogram scan of bile eluted in system A is presented in Fig. 4.

Incubation of bile with caecal contents as described earlier resulted in degradation of all the conjugates to the phenols, the unchanged drug and 5,6-dichloro-4,7-dioxo-2-trifluoromethylbenzimidazole. Reverse isotope dilution of acid hydrolysed or β-glucuronidase treated bile identified 8% of the dose as 5,6-dichloro-2-trifluoromethylbenzimidazole. Cochromatography with authentic material (systems A and C) identified the precursor as the N^1-glucuronide of 5,6-dichloro-2-trifluoromethylbenzimidazole. Reverse isotope

dilutions on fresh urine gave a recovery of 3% of free 5,6-dichloro-2-trifluoromethylbenzimidazole.

3. Faeces.

Some 28% of the oral dose appeared in rat faeces over 5 days, while less than 1% of the dose appeared in rabbit faeces over this period.

Metabolites.

Faeces were treated as described in Methods and centrifuged. The debris were ether extracted and the pooled extracts evaporated to dryness. The residue was taken up in 1-2 ml. of water and added to the supernatant. Less than 1% of the dose remained in the debris after this treatment. Reverse isotope dilutions for 5,6-dichloro-2-trifluoromethylbenzimidazole were performed on hydrolysed and non-hydrolysed extracts, but no difference was noted between the results obtained. Some 16.5% of the dose was excreted as free drug in a 5 day period. Essentially all of the remainder of the ^{14}C was 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole, but some traces of other phenolic compounds were also present. These were identified by methylation and gas chromatography (column D) as 4,6-dichloro-5-hydroxy-, 5-chloro-4-hydroxy- and 6-chloro-5-hydroxy-2-trifluoromethylbenzimidazole. These latter compounds could also be found in hydrolysed urine samples from both species.

4. Respired air.

This experiment was performed with rats and rabbits dosed

orally with [^{14}C] 5,6-dichloro-2-trifluoromethylbenzimidazole as described in Methods. No activity could be found in either trap in any of these experiments.

5,6-Dichloro-4-hydroxy-2-trifluoromethylbenzimidazole.

Excretion of radioactivity by the rat.

1. Urine.

Urinary excretion figures (presented in Table 5) show that some 40% of an oral dose of this compound is excreted in 2 days. A radiochromatogram scan of 24 hour urine eluted in system A (paper) is presented in Fig. 5.

Metabolites.

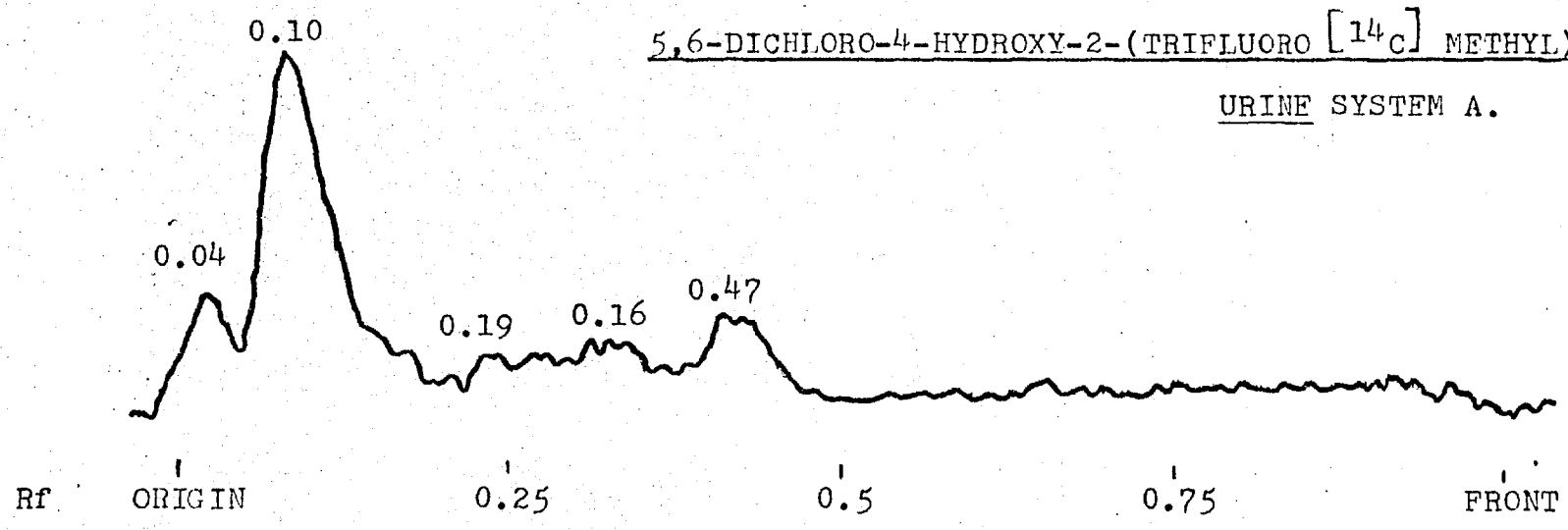
At least 4 metabolites were present in urine. Hydrolytic and chromatographic techniques characterised and identified these as being identical with the hydroxylated metabolites present in the urine of rats dosed with 5,6-dichloro-2-trifluoromethylbenzimidazole. Reverse isotope dilution failed to detect any 5,6-dichloro-2-trifluoromethylbenzimidazole or trifluoracetic acid in the urine. The major peak (Rf 0.10, system A) on radiochromatogram scans proved to be a mixture of glucuronide conjugates of the hydroxylated derivatives of 5,6-dichloro-2-trifluoromethylbenzimidazole, as shown by TLC and GLC techniques, following enzyme hydrolysis with β-glucuronidase of aqueous extracts of this peak. Some 5-6% of the dose was excreted (in 24 hours) as conjugates of rearranged or dechlorinated hydroxylated derivatives. These phenols were identified by methylation and gas chromatography (column D). The quantitative results are presented in Table 6.

Table 5. THE EXCRETION OF ^{14}C BY RATS GIVEN 5,6-DICHLORO-4-HYDROXY-2-(TRIFLUORO [^{14}C] METHYL)BENZIMIDAZOLE.

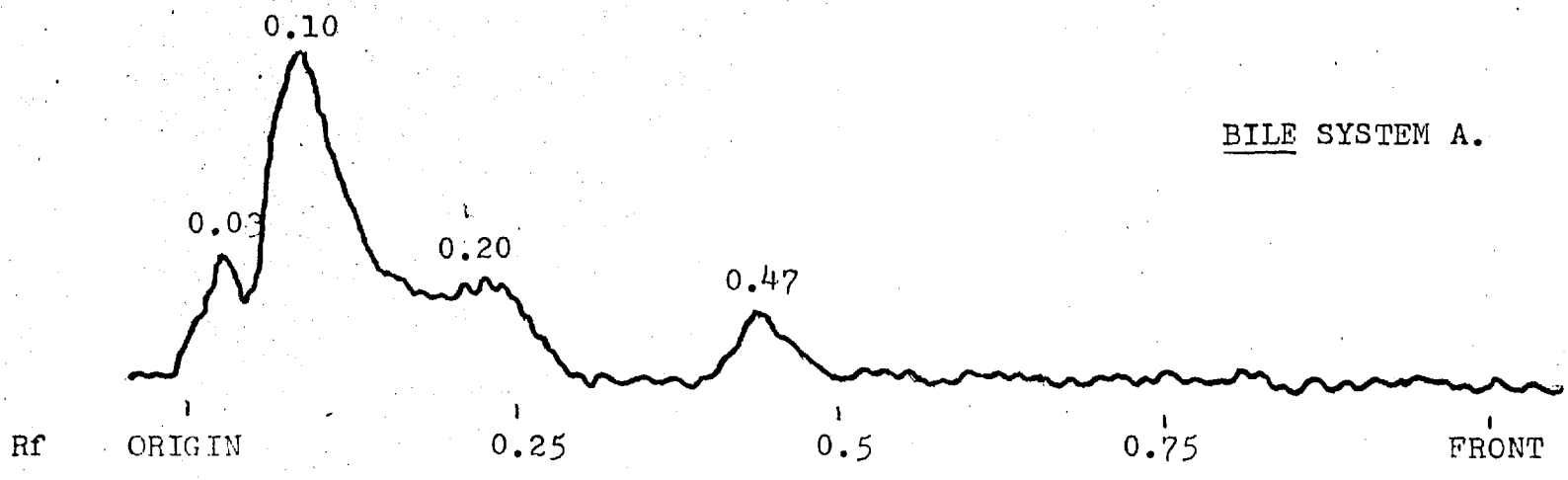
		% DOSE ^{14}C EXCRETED	
		INTACT RATS	BILIARY FISTULAE RATS
In urine	Day 1	32.0(30.5-35)	15.0(12.7-17.4)
	Day 2	8.0(4.5-11.5)	-
	Day 3 and 4	2.0(1.5- 2.0)	-
	Day 5 and 6	0.1(0.1)	-
In bile	Day 1	-	58.0(56.8-60.8)
In faeces	Day 1 and 2	7.0(1.0-16.5)	-
	Day 3 and 4	1.0(0.3- 2.3)	-
	Day 5 and 6	0.8(0.8)	-
Total		50.9	73.0

Results are mean values with ranges in parentheses.

Fig. 5. RADIOCHROMATOGRAM SCANS OF URINE AND BILE SAMPLES FROM RATS DOSED WITH 5,6-DICHLORO-4-HYDROXY-2-(TRIFLUORO [^{14}C] METHYL)BENZIMIDAZOLE.



URINE SYSTEM A.



BILE SYSTEM A.

Table 6. METABOLITES OF 5,6-DICHLORO-4-HYDROXY-2-TRIFLUORO [^{14}C] METHYLBENZIMIDAZOLE IN RAT URINE, BILE AND FAECES.

COMPOUND	URINE	BILE	FAECES
5,6-Dichloro-4-hydroxy-2-trifluoromethyl-benzimidazole (free)	3.1(2.7- 3.4)	4.2(2.1- 5.6)	3.5(0.5-8.5)
5,6-Dichloro-4-hydroxy-2-trifluoromethyl-benzimidazole (total)	25.2(24.1-26.0)	46.1(40.1-51.1)	-
4,6-Dichloro-5-hydroxy-2-trifluoromethyl-benzimidazole (total)	0.8(0.1- 2.1)	1.9(0.4- 2.9)	-
5-Chloro-4-hydroxy-2-trifluoromethyl-benzimidazole (total)	3.5(1.2- 5.5)	5.1(4.0- 6.0)	-
5-Chloro-6-hydroxy-2-trifluoromethyl-benzimidazole (total)	1.5(0.4- 2.4)	1.7(0.3- 2.6)	1.5(0.5-5.0)
4,7-Dioxo-5,6-dichloro-2-trifluoro-methylbenzimidazole (total)	1.1(0.6- 2.0)	2.8(2.0- 3.3)	-
Total	32.1	57.6	5.0

For details of dosing and collection of excreta see Table 5. Metabolites were determined in 24 hour urine and bile samples. Results are mean values with ranges in parentheses.

2. Bile.

Some 58% of an intraperitoneal dose of 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole was excreted in the bile of cannulated rats over 24 hours. A radiochromatogram scan of bile eluted in system A (paper) is shown in Fig. 5.

Metabolites.

Aliquots of bile (0.1-0.5 ml.) were refluxed with 10 N-HCl and ether extracts chromatographed in systems A or C on silica thin layers. Cochromatography in these systems allowed the identification of the administered compound, 6-chloro-5-hydroxy-, 4,6-dichloro-5-hydroxy-, and 5,6-dichloro-4,7-dioxo-2-trifluoromethylbenzimidazole. Gas chromatography also revealed the presence of 5-chloro-4-hydroxy-2-trifluoromethyl-benzimidazole which tends to chromatograph with 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole in the TLC systems. Retention times on column D were 6.2, 7.8 and 5.2, 8.6 minutes respectively. Reverse isotope dilutions failed to detect any 5,6-dichloro-2-trifluoromethylbenzimidazole or trifluoracetic acid. Quantitative results are presented in Table 6.

Small aliquots of bile (0.1 ml.) were incubated with rat and rabbit caecal contents as previously described. All conjugates were shown by paper chromatography (system A) to be degraded to the free phenols. β-Glucuronidase incubations and the naphtharesorcinol test showed that the conjugates were glucuronides of these hydroxylated compounds.

3. Faeces.

Some 8.8% of the dose was excreted in the faeces over 6 days.

Metabolites.

Extraction of the ^{14}C in the manner described for 5,6-dichloro-2-trifluoromethylbenzimidazole (Methods), followed by thin-layer chromatography of portions of this extract (system E) showed that all but 1% of the material present was 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole. The remainder was a mixture of 5,6-dichloro-4,7-dioxo-2-trifluoromethylbenzimidazole and other phenolic compounds. No evidence could be obtained for the presence of 5,6-dichloro-2-trifluoromethylbenzimidazole or trifluoracetic acid by reverse isotope dilutions. The quantitative results are presented in Table 6.

CHAPTER FOUR.

CELL AND TISSUE FACTORS RELATED TO THE METABOLISM OF 5,6-DICHLORO-2-TRIFLUOROMETHYLBENZIMIDAZOLE.

The slow excretion of radioactivity by both the rat and the rabbit given an oral dose of [^{14}C] 5,6-dichloro-2-trifluoromethylbenzimidazole meant that after 6 days the rat still contained some 40% and the rabbit 75% of the dose. Experiments were performed to locate this radioactivity and to determine its chemical identity.

1. Tissue Distribution.

Rats and rabbits were killed by cervical dislocation and one of each species was dissolved in ethanolic NaOH. Portions of the solution (1 ml.) were counted as described in Methods. A total of 95-97% of the administered dose was accounted for in this manner.

The two remaining animals of each species were dissected for various tissues and organs, i.e. brain, heart, spleen, lungs, liver, ovaries, muscles, skin/fur and depot fat. Quantities of these tissues were dissolved in 5 ml. of ethanolic NaOH/gm.weight of tissue and assayed for radioactivity as previously described (Methods). Results are shown in Table 1. In the case of liver and muscle, isotope dilutions were performed for 5,6-dichloro-2-trifluoromethylbenzimidazole as described (Methods). Portions of the homogenates prepared for this purpose were solvent extracted (by ether, benzene or dichloromethane) and the extracts examined for metabolites by TLC. Results are presented in Table 2.

Samples of bone were cleaned of tissue by scraping, defatted with dichloromethane and dissolved in 10N-HCl.

Table 1. TISSUE DISTRIBUTION OF RADIOACTIVITY IN THE RAT AND RABBIT FOLLOWING THE ORAL ADMINISTRATION OF 5,6-DICHLORO-2-(TRIFLUORO[^{14}C]METHYL)BENZIMIDAZOLE.

	RABBIT		RAT	
	% DOSE IN TISSUE	MEAN SPECIFIC ACTIVITY μCi/g	% DOSE IN TISSUE	MEAN SPECIFIC ACTIVITY μCi/g
Fat	12.3(9.4-14.2)	0.002	0.21(0.15-0.31)	0.005
Liver	0.4(0.4-0.4)	0.0002	1.25(0.9-1.51)	0.024
Heart	0.5(0.4-0.5)	0.001	n.d.	-
Kidneys	0.6(0.3-0.9)	0.001	0.3(0.20-0.41)	0.015
Lungs	0.1(0.1-0.1)	0.002	n.d.	-
Ovaries, uterus and fat	2.5(2.0-3.2)	0.002	n.d.	-
Skeletal muscle	34.0(31.8-36.0)	0.001	0.05(0.03-0.10)	0.0004
Spleen	0.4(0.3-0.5)	0.006	1.0(0.4-1.6)	0.012
Gastrointestinal tract	1.7(0.7-3.4)	0.004	7.5(5.0-9.0)	0.115
Skin and fur	13.1(7.5-19.8)	0.001	0.8(0.4-1.5)	0.003
Blood	n.d.	-	3.5(2.5-6.5)	0.055
Total in tissues	65.6		14.7	

Continued.

Table 1, continued. TISSUE DISTRIBUTION OF RADIOACTIVITY IN THE RAT AND RABBIT FOLLOWING THE ORAL ADMINISTRATION OF 5,6-DICHLORO-2-(TRIFLUORO[^{14}C]METHYL)BENZIMIDAZOLE.

	RABBIT		RAT	
	% DOSE IN TISSUE	MEAN SPECIFIC ACTIVITY μCi/g.	% DOSE IN TISSUE	MEAN SPECIFIC ACTIVITY μCi/g.
% dose in 48 hr urine	19.0(14.8-23.2)		35.9(32.0-39.4)	
% dose in 48 hr faeces	0.1(0.1-0.1)		23.6(22.1-26.0)	
% dose recovered	84.7		74.2	
Remainder of carcass	10.1(8.0-12.4)		17.4(15.1-19.9)	
Total dose recovered	94.8		91.6	

5,6-Dichloro-2-(trifluoro[^{14}C]-methylbenzimidazole) was administered orally in 70/30 v/v propane -1,2-diol/water. Rabbits received 7 mg./kg. (1.4 μCi/kg.) and rats 10.1 mg./kg. (23.0 μCi/kg.).

Values are means for 3 animals with ranges in parentheses.

n.d. = not determined.

Table 2. INTRACELLULAR DISTRIBUTION IN LIVER AND MUSCLE OF ^{14}C FOLLOWING THE ORAL ADMINISTRATION OF 5,6-DICHLORO-2-(TRIFLUORO[^{14}C]METHYL)BENZIMIDAZOLE.

RAT	LIVER		MUSCLE
soluble	11.5(10.6-12.1)	soluble	2(1.0-2.8)
microsomes	48(46.3-49.2)	sarcosomes	1(0.6-1.4)
mitochondria	18(17.0-19.6)	myofibrils	1(0.7-1.8)
cell debris/nuclei	21(20.1-21.7)	connective tissue/fat etc.	90(89.2-91.8)
RABBIT			
soluble	48.5(46.8-49.7)	soluble	1(0.05-1.6)
microsomes	16(15.1-17.6)	sarcosomes	1(0.28-1.8)
mitochondria	11.5(10.0-12.6)	myofibrils	1(0.85-1.45)
cell debris/nuclei	23.5(22.1-24.6)	connective tissue/fat etc.	92(90.1-94.2)

Results are the mean of the values for two determinations on two animals and are expressed as a percentage of radioactivity in the fraction taken.

See Table 1 for details of the dose and Methods chapter for details of the technique.

Table 3. NATURE OF METABOLITES IN LIVERS OF RATS AND RABBITS FOLLOWING ORAL ADMINISTRATION OF 5,6-DICHLORO-2-(TRIFLUORO[^{14}C]METHYL)BENZIMIDAZOLE.

COMPOUND	CELL FRACTION			
	CELL NUCLEI/ DEBRIS	MITOCHONDRIA	MICROSOMES	SOLUBLE
RAT				
5,6-dichloro-2-trifluoromethylbenzimidazole	5.1(4.8-5.5)	2.2(2.0-2.6)	4.1(3.6-4.9)	-
5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole	89.7(89.1-90.6)	90.1(89.1-91.9)	85.8(84.9-86.2)	95.1(94.8-95.5)
Other	3.5(3.3-3.8)	8.7(8.3-9.1)	10.6(10.1-11.2)	5.3(5.0-6.1)
RABBIT				
5,6-dichloro-2-trifluoromethylbenzimidazole	75.7(75.2-76.1)	85.1(84.1-86.3)	96.3(94.5-97.2)	83.1(82.2-84.2)
5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole	12.8(12.3-13.4)	8.3(8.0-9.0)	3.8(3.3-4.8)	15.2(14.3-16.5)
Other	11.1(10.4-11.8)	8.1(7.4-8.3)	-	2.0(1.5-3.2)

Values are means for duplicate experiments on two animals, ranges in parentheses.

Results are expressed as a percentage of radioactivity in the sample taken. See Table 1 for details of the dosage.

Aliquots (0.5 mL) were neutralised and counted in thixotropic gel. In neither species was any activity recorded above background levels.

The remainder of each carcass was extracted with acetonitrile and treated as described in Methods. No evidence for the presence of conjugates in the extracts could be found when they were subjected to thin layer or paper chromatography (systems A and C).

2. Sub-cellular Fractionation of Liver and Muscle.

Fractionation of rat and rabbit liver samples was carried out according to the method of Hogeboom (1953) as described in Methods (page 57). The fractions obtained: cell debris/nuclei, mitochondria, microsomes and soluble, were dissolved in 10% aqueous NaOH and adjusted to 10 or 20 mL with distilled water. Aliquots (1 mL) were subjected to scintillation counting following neutralisation with acid. Results are shown in Table 2. Other quantities (1-2 mL) were solvent extracted as previously described and the extracts subjected to quantitation by TLC analysis (systems D and E). Results are given in Table 3.

Muscle was similarly treated, the method being due to Kitiyakara and Harman (1953). The technique is described in Methods (page 57). Results are given in Table 2. Quantitation of metabolites was performed by TLC of ether extracts in systems D and E. Results are shown in Table 3.

3. Incorporation into Nucleotides.

The liver homogenate prepared in Section 1 was used to

isolate RNA and DNA nucleotides, as described in Methods. The solutions of barium salts obtained were assayed for radioactivity. Low levels of activity (2 X background) were recorded, but all attempts to characterise it by TLC and GLC methods were only partially successful. 5,6-Dichloro-2-trifluoromethylbenzimidazole accounted for about 25% of the material extracted and was identified by GLC. It is possible that the activity was present as an impurity.

4. Binding of 5,6-dichloro-2-(trifluoro[^{14}C]methyl)benzimidazole to blood.

Samples of blood from rats and rabbits given intravenous doses of 5,6-dichloro-2-trifluoro[^{14}C]methylbenzimidazole were assayed for radioactivity as described. The experiment was continued for 24 hours on rats and 7 days on rabbits. Results are presented in Table 4 and shown graphically in Figs. 1 and 2. Graphs of the logarithm of concentration of radioactivity against time for both species show that after a linear rate of decline the curve levels out. This may be due to metabolites of 5,6-dichloro-2-trifluoromethylbenzimidazole finding their way back to the blood from their site of formation. The initial pronounced rise for ^{14}C concentration in rat blood, and for the lower dose in rabbit, may be an artifact due to the highly insoluble nature of the administered compound in aqueous media. That is, precipitation may have occurred when the compound was injected, followed by gradual solution. Thus the maximum would not be observed for several hours.

The obvious difference between the rat and the rabbit is that the decay curve has a faster rate of decline for the former species (Figs. 1 and 2). No definite half-life could be obtained, however, because the curves become asymptotic. The difference can be expressed in terms of the time taken for half of the initial dose to disappear, this being 14 hours for the rat and 70 hours for the rabbit. At the close of the experiment rat blood contained some 3.0-3.5% of the dose (48 hours) and rabbit blood contained 25-20% at 150 hours.

Aliquots (0.1-0.2 ml.) of blood were hydrolysed by refluxing with 1 ml. 10N-HCl and ether extracted (4 x 2 ml.). The metabolites were characterised and quantified by TLC in the usual way. Results are shown in Table 5.

Since the significant retention of radioactivity could possibly be explained in terms of binding to blood proteins, quantitation of this factor was performed by dialysis. The experiments were performed for [^{14}C] 4,5-dichloro-, [^{14}C] 5,6-dichloro- and [^{14}C]5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole as described in Methods (page 61). Blood was obtained from rats, rabbits and humans. Results are shown in Table 6.

The faster technique of ultra-filtration (Bridges, Walker and Williams, 1969) was also employed (see Methods, page 62), and applied to the same samples as above. The results obtained by this method agree very well with those obtained by dialysis. They are also presented in Table 6.

Fig. 1. CONCENTRATION OF ^{14}C IN THE BLOOD OF RATS AND RABBITS DOSED INTRAVENEOUSLY WITH 5,6-DICHLORO-2-(TRIFLUORO-[^{14}C]METHYL)BENZIMIDAZOLE.

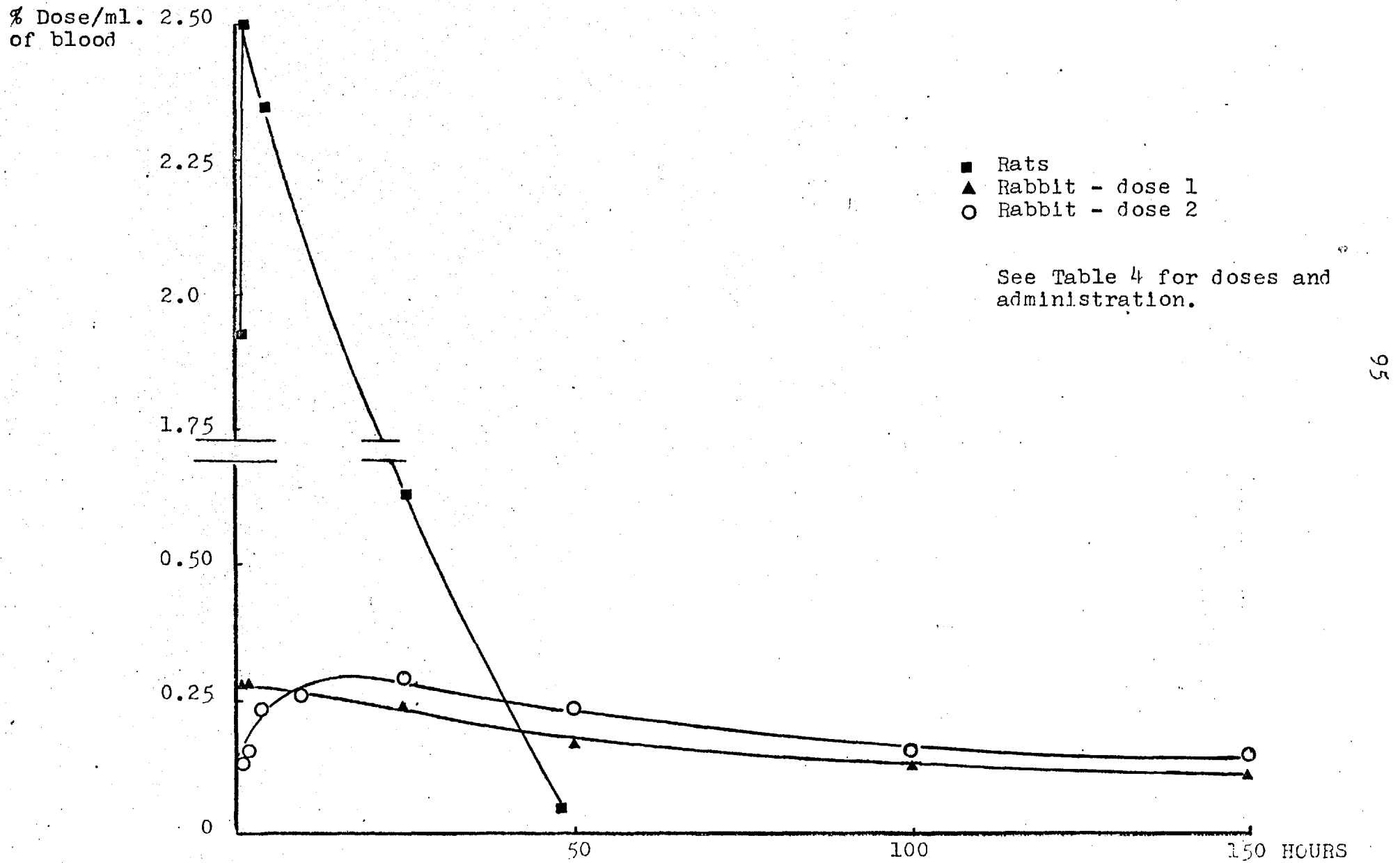


Fig. 2. ^{14}C CONCENTRATION IN RAT AND RABBIT BLOOD AFTER DOSING 5,6-DICHLORO-2-(TRIFLUORO-[^{14}C]-METHYL)BENZIMIDAZOLE

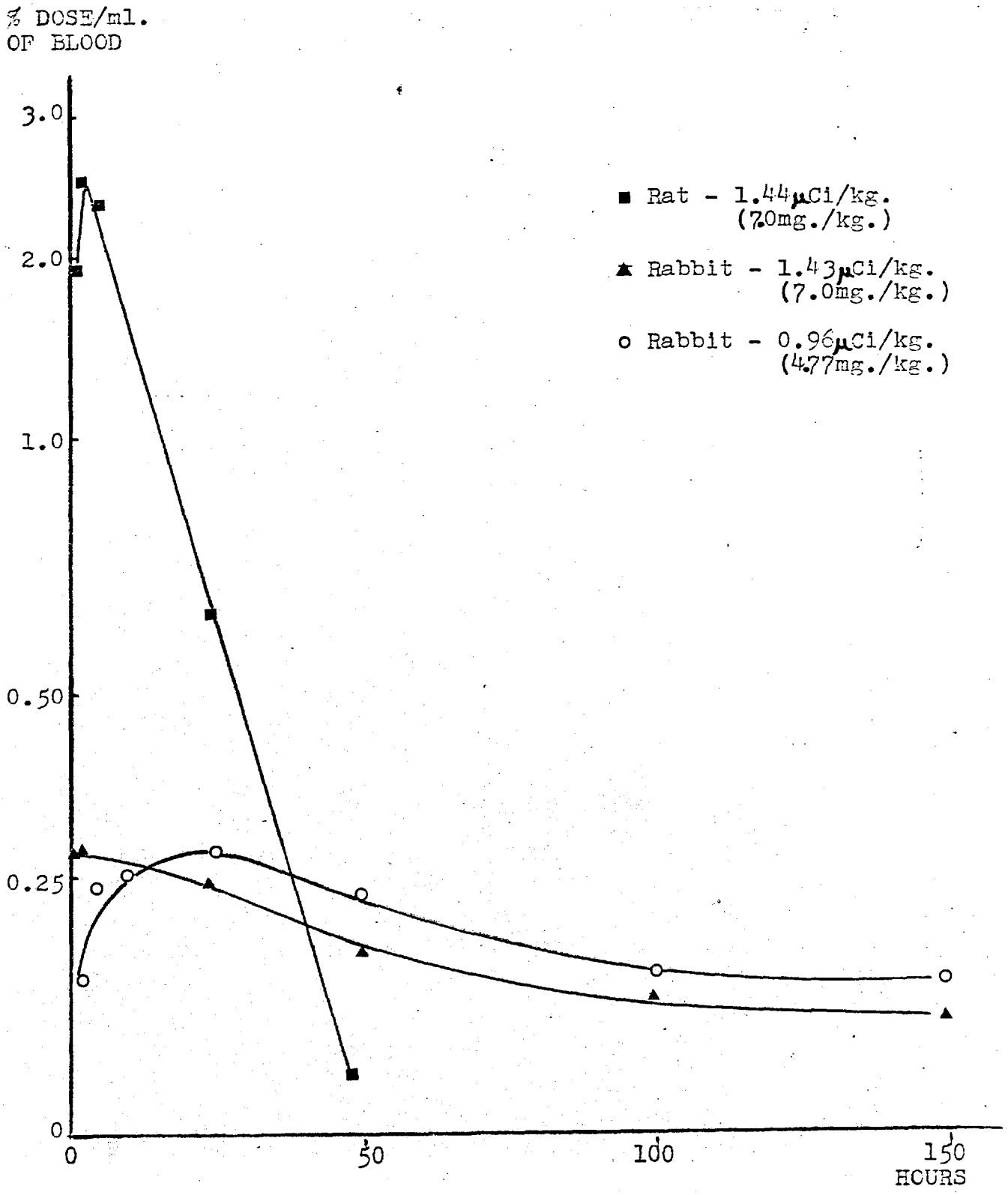


Table 4. CONCENTRATION OF ^{14}C IN THE BLOOD OF RATS AND RABBITS DOSED INTRAVENOUSLY WITH 5,6-DICHLORO-2-TRIFLUORO[^{14}C]METHYLBENZIMIDAZOLE.

TIME IN HOURS AFTER DOSAGE	RABBIT % DOSE /ml OF BLOOD 1	RABBIT % DOSE /ml OF BLOOD 2	RAT
1	0.28	0.14	1.92
2	0.27	0.16	2.50
4	-	0.235	-
5	-	-	2.35
10	-	0.255	-
24	-	-	0.63
25	0.24	0.29	-
48	-	-	0.05
50	0.17	0.23	-
100	0.125	0.15	-
150	0.105	0.14	-

Dosage was by intravenous injection (marginal ear vein for rabbits, tail vein for rats).

Doses for rabbits were 1) 1.43 μCi/kg. (7.0 mg/kg.) and 2) 0.96 μCi/kg. (4.77 mg/kg.) and for rats 1.44 μCi/kg. (70 mg/kg.). The dose was dissolved in 1 ml. of propane-1,2-diol/water 70/30 v/v in all cases. Results are determined from at least two estimations on samples from two or three animals.

Table 5. NATURE OF ^{14}C IN BLOOD OF RATS AND RABBITS FOLLOWING THE INTRAVENOUS INJECTION OF 5,6-DICHLORO-2-(TRIFLUORO[^{14}C]METHYL)BENZIMIDAZOLE.

ANIMAL	TIME (HOUR)	% DOSE /ml BLOOD	% BLOOD ^{14}C AS 5,6-DICHLORO-2-TRIFLUORO-METHYLBENZIMIDAZOLE	% BLOOD ^{14}C AS 5,6-DICHLORO-4-HYDROXY-2-TRIFLUORO-METHYLBENZIMIDAZOLE
Rabbit	1	0.28	99.9(99.0-101.0)	0.0(0.0)
	50	0.17	98.1(97.4-99.1)	2.1(1.4-2.8)
	150	0.105	79.7(78.1-81.4)	20.4(20.1-20.8)
Rat	1	1.92	99.9(98.8-101.2)	0.03(0.0-0.06)
	24	0.63	66.2(65.4-67.3)	33.4(32.1-34.8)
	48	0.05	60.1(58.0-62.1)	40.2(37.9-42.1)

Results are the mean values of at least two determinations on samples from two or three animals. Ranges are given in parentheses.

See Table 4 for details of dosing.

Table 6. THE BINDING OF SOME TRIFLUORO[^{14}C]METHYLBENZIMIDAZOLE DERIVATIVES TO BLOOD.

COMPOUND	% ^{14}C DIFFUSING OFF BLOOD SAMPLE, DETERMINED BY: DIALYSIS			ULTRAFILTRATION		
	RAT	RABBIT	HUMAN	RAT	RABBIT	HUMAN
4,5-dichloro-2-trifluoromethyl-benzimidazole	6.1(5.8-6.4)	2.7(2.4-2.9)	5.4(5.0-6.1)	6.3(6.0-6.7)	2.5(2.1-2.9)	5.5(5.0-5.9)
5,6-dichloro-2-trifluoromethyl-benzimidazole	1.0(0.8-1.5)	0.75(0.45-0.95)	7.1(6.6-7.7)	1.0(0.7-1.6)	0.9(0.5-1.4)	7.0(6.4-7.6)
5,6-dichloro-4-hydroxy-2-trifluoromethyl-benzimidazole	7.0(6.1-8.1)	4.0(3.1-4.9)	0.9(0.5-1.4)	6.9(6.0-7.8)	4.2(3.0-5.4)	0.8(0.4-1.3)

Results are mean values of at least three determinations. Ranges are given in parentheses.

Compounds were used at a level of 20 µg/ml. of blood, and were dissolved in the minimum quantity of ethanol (1-2 µl.) before application to the blood sample.

Temperature of dialysis 10°C, duration 24 hours.

Temperature of ultrafiltration 20°C, duration 2 hours at 3,000 g.

See Methods chapter (pages 61-62) for experimental details.

Extension of these methods to cover the other metabolites, which were not radiolabelled, utilised GLC, TLC and colourimetric methods in order to detect the material diffusing off the blood samples. Details are presented in the relevant section of Methods.

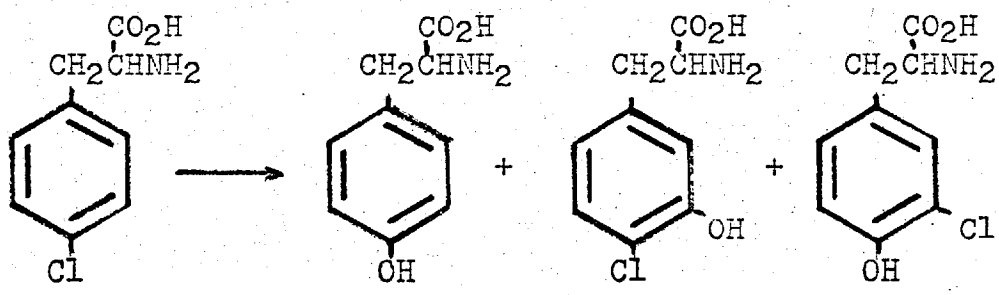
The results showed that the two phenolic metabolites of 4,5-dichloro-2-trifluoromethylbenzimidazole, i.e. 4,5-dichloro-6-hydroxy- and 4,5-dichloro-7-hydroxy-2-trifluoromethylbenzimidazole bound to the extent of about 90% at a level of 20 µg/ml. of blood. Attempts to assay the ortho and para-quinones were abandoned because of the difficulties of quantitation and/or extraction of these compounds.

CHAPTER FIVE

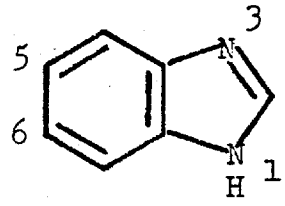
DISCUSSION.

1) Metabolism.

The results presented in Chapter 3 show that the metabolism of both compounds follows the pathways predicted in the Introduction (Section C). Hydroxylation of the benzene ring, to give monohydroxylated derivatives which are conjugated mainly with glucuronic acid, is the major reaction with both compounds. In all cases further metabolism to give quinonoid and rearranged products occurs to a minor extent. These rearranged products have been identified by GLC and TLC as 4-chloro-5-hydroxy-, 5-chloro-4-hydroxy- and 4,6-dichloro-5-hydroxy-2-trifluoromethylbenzimidazole. They seem to be formed in both species from 4,5-dichloro- and also 5,6-dichloro-2-trifluoromethylbenzimidazole. The production of 4,6-dichloro-5-hydroxy-2-trifluoromethylbenzimidazole (and possibly the other compounds) can be explained by the "NIH-shift" reaction, first documented by Guroff et al. (1967). In this microsomal reaction (Guroff et al., 1967; Daly et al., 1968) the enzymic replacement of F, Cl and Br (and of ^{2}H or ^{3}H in some cases) by OH in aromatic compounds is accompanied by the formation of a compound containing the replaced atom ortho to its original position. Thus 4-chlorophenylalanine gives rise to tyrosine, 3-chlorotyrosine and 4-chloro-m-tyrosine:



The replacement of a halogen by hydroxyl has been termed hydroxy-dechlorination by Betts *et al.* (1957). The formation of these metabolites of the benzimidazoles is thus not unprecedented, but in general the shifts reported so far have been of a *para* halogen in substituted anilines, acetanilides, aromatic aminoacids and thiazoleacetic acids (Foulkes, 1969). The analogy may still be applicable, however, since position 5 of the benzimidazole may be considered as *para* to N^1 thus:



Similarly position 6 is *para* to N^3 (equivalent to N^1 by tautomerism). Attempts to produce these compounds with peroxyacetic acid, peroxytrifluoracetic acid or the ascorbate oxidation system of Udenfriend *et al.* (1954) acting on 4,5-dichloro- or 5,6-dichloro-2-trifluoromethylbenzimidazole were not successful. Only the monohydroxylated compounds, 4,5-dichloro-6-hydroxy-, 4,5-dichloro-7-hydroxy- and 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole were formed, and no correlation could be obtained with their yields and the biochemical yield from metabolism studies. The 6- and 7-hydroxy derivatives of 4,5-dichloro-2-trifluoromethylbenzimidazole were produced (in low yield, 5-10%) in almost equal amounts by the ascorbate treatment for 48 hours at 25°. This is in agreement with Diner (1964), who stresses that the similarities

in isomer yield between chemical (radical) and biochemical hydroxylations is not complete. No dehalogenated or rearranged products were obtained by chemical oxidation of 4,5-dichloro-2-trifluoromethylbenzimidazole, although they were formed (together with the 4,7-dioxo compound) by similar treatment of the 6-hydroxy- and 7-hydroxy- compounds.

The postulated courses of the microsomal oxidation of 4,5-dichloro- and 5,6-dichloro-2-trifluoromethylbenzimidazole are given in Figs. 1 and 2 respectively, based on the work of Guroff et al. (1967). This scheme is interesting in that it does not explain the formation of 4,5-dichloro-7-hydroxy-2-trifluoromethylbenzimidazole.

Bowker and Casida (1969) found N^1-conjugation to be a major feature of the metabolism of 5,6-dichloro-2-trifluoromethylbenzimidazole by the mouse, but of lesser importance in the rat. With the 4,5-dichloro-analogue, however, the findings reported in Chapter 3 show that the rabbit excretes some 4% of a dose of this compound as the N^1-glucuronide, while the rat did not excrete any detectable quantity of this compound. Both rat and rabbit were found to excrete the N^1-glucuronide of the 5,6-dichloro compound in this study. Bowker and Casida (loc. cit.) also report the formation of the N^1-riboside of 5,6-dichloro-2-trifluoromethylbenzimidazole by the rat, but little evidence for the formation of this compound could be found in this study. These workers' findings, however, may imply that the compound can proceed some way along the nucleotide bio-

Fig. 1. THE POSTULATED COURSE OF MICROSOMAL OXIDATION OF 4,5-DICHLORO-2-TRIFLUOROMETHYLBENZIMIDAZOLE.

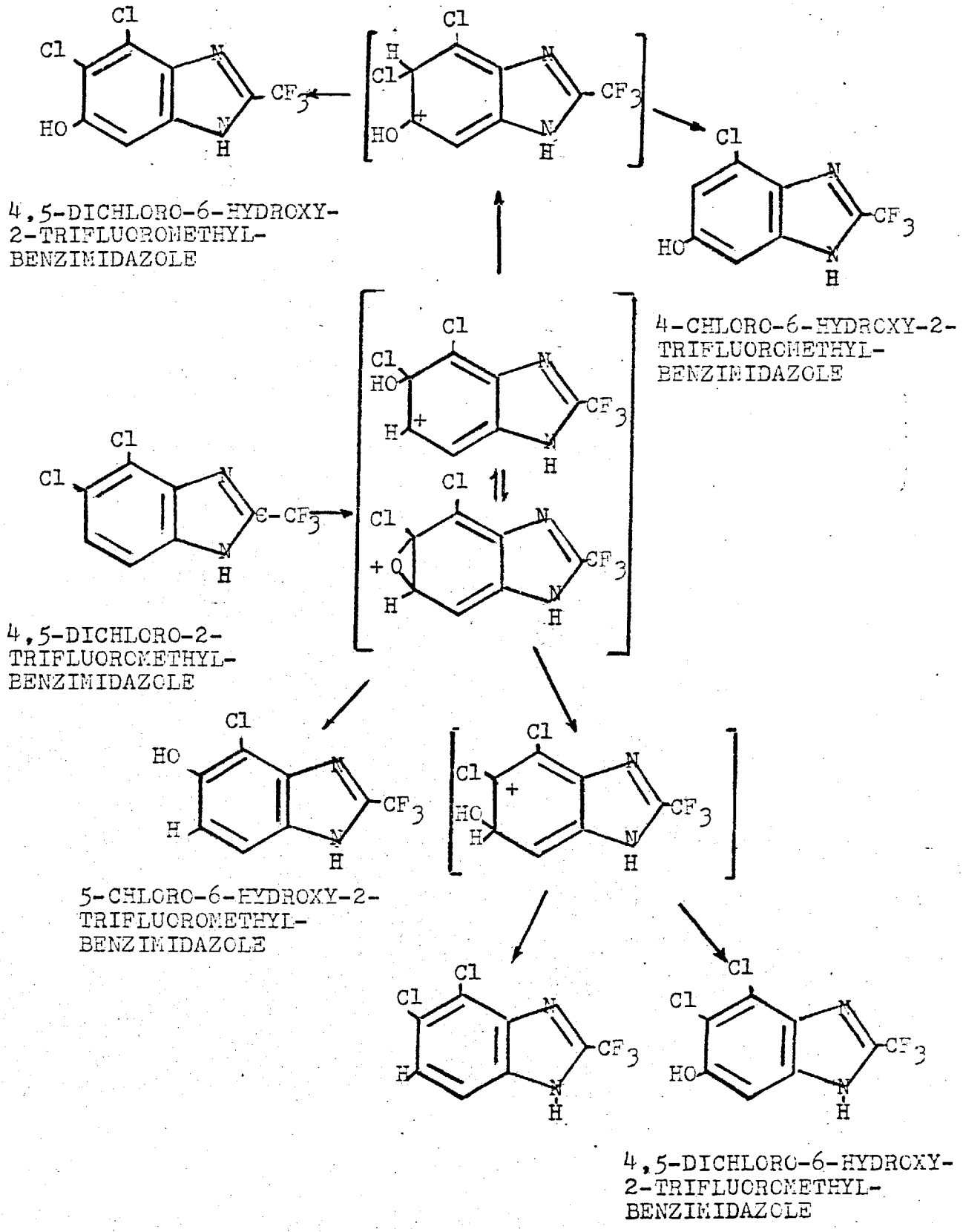
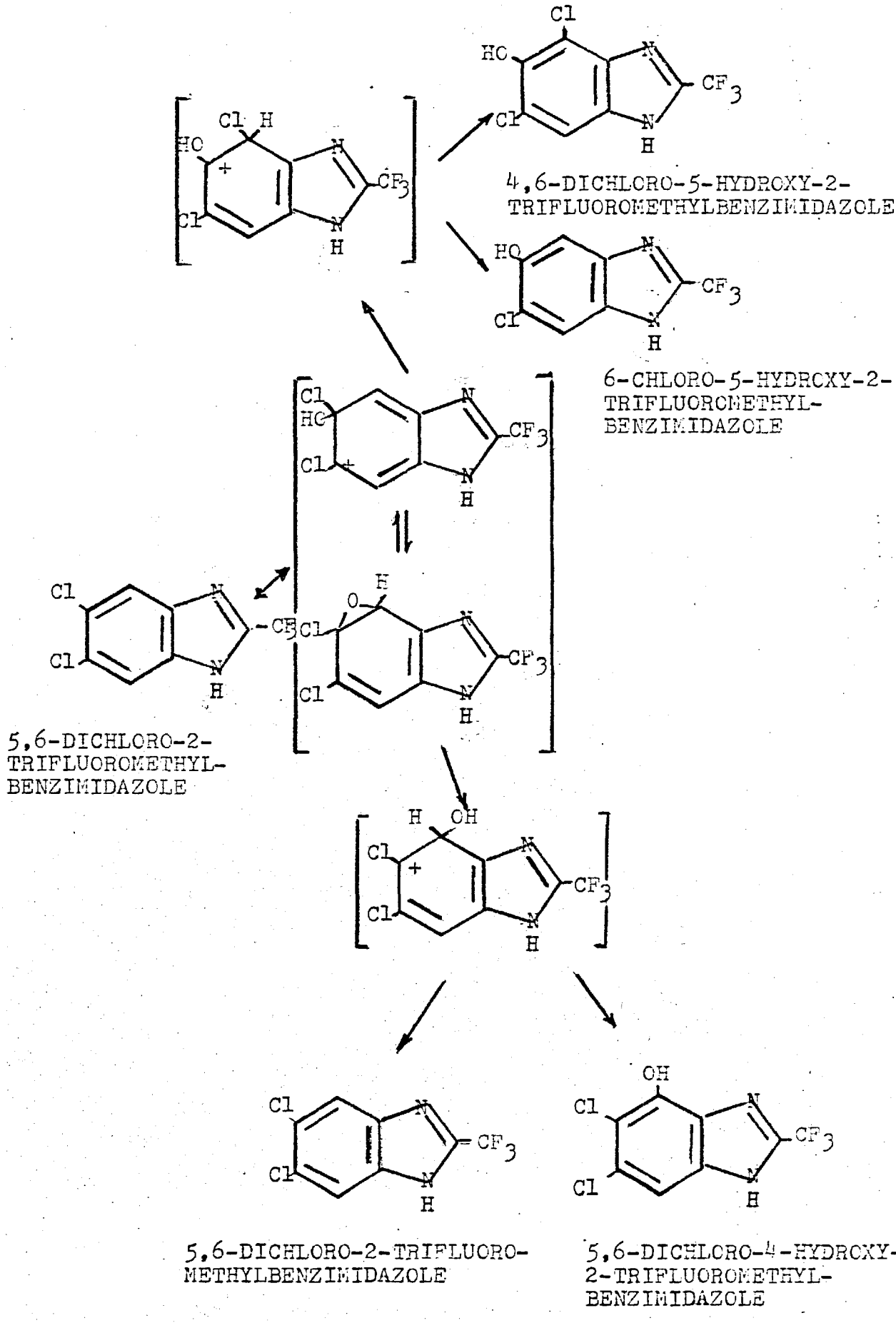


Fig. 2. THE POSTULATED COURSE OF MICROSOMAL OXIDATION OF 5,6-DICHLORO-2-TRIFLUOROMETHYLBENZIMIDAZOLE.



synthesis pathway. However, only very small quantities of ^{14}C could be found associated with RNA or DNA nucleotides from rats and rabbits dosed with [^{14}C]5,6-dichloro-2-trifluoromethylbenzimidazole. Pullman and Pullman (1963) reviewing the biosynthesis of N-ribosides, consider that the action of purine antimetabolites (6-mercaptopurine, 8-azaguanine, etc.) is initiated by the formation of the riboside of the base, using the normal enzymes (Carter, 1959; Leukens and Hemington, 1957; Way and Parks, 1958; Paterson, 1959). Alternatively, the N-riboside may have been formed in order to be incorporated into a Vitamin B_{12} analogue (see Introduction, Section B), presumably by the rat gut flora. The chances of this happening are increased by the large enterohepatic circulation of this compound, which thus has an increased exposure to the flora.

The use of methylation and TLC procedures, as outlined in the Metabolism chapter (page 75), demonstrated that most of the metabolites are mono conjugates of glucuronic acid on either hydroxyl or N^1.

The formation of small quantities of sulphate esters of the phenolic groups has been demonstrated in rat and rabbit by the use of an S^{35} label. In general this is a common reaction of the phenol group in all mammals examined, and has been postulated by Smith (1968) to be the primitive method of conjugation.

The possibility that the minor metabolites have been formed from mercapturic acid derivatives, rather than by the

NIH shift, has not been clarified. It did not prove possible to identify unequivocally any compound containing organically bound sulphur. If present, mercapturic acids account for less than 1% of the excreted dose.

The benzene ring is the most likely point of attack for any ring opening which might occur in benzimidazole metabolism, since the imidazole ring is fairly inert. Reverse isotope dilutions for trifluoracetic acid in urine bile and faeces of treated animals failed to detect any of this compound ($< 0.01\%$ of the ^{14}C present), which should be liberated if the imidazole ring were opened. However, apples appear to contain some trifluoracetic acid after treatment with [^{14}C] 5,6-dichloro-2-trifluoromethylbenzimidazole (Dr. C. P. Bond, personal communication), but whether this is a reaction of the apple or of bacteria has not been proved. The metabolism of Trifluralin by carrots (or a micro-organism thereon) to give a small amount of $^{14}CO_2$ from a $^{14}CF_3$ group has been cited in Chapter 1 (page 34), but trifluoracetic acid was not detected. No evidence could be found for scission of the benzene ring, which would yield an imidazole dicarboxylic acid. Such a compound would possibly occur among the "minor metabolites" found at Rf 0.0 in most chromatography systems employed.

The postulated schemes for the metabolism of 4,5-dichloro- and 5,6-dichloro-2-trifluoromethylbenzimidazole are given in Figs. 3 and 4 respectively.

Fig. 3. THE PATHWAYS OF METABOLISM OF 4,5-DICHLORO-2-TRIFLUOROMETHYLBENZIMIDAZOLE.

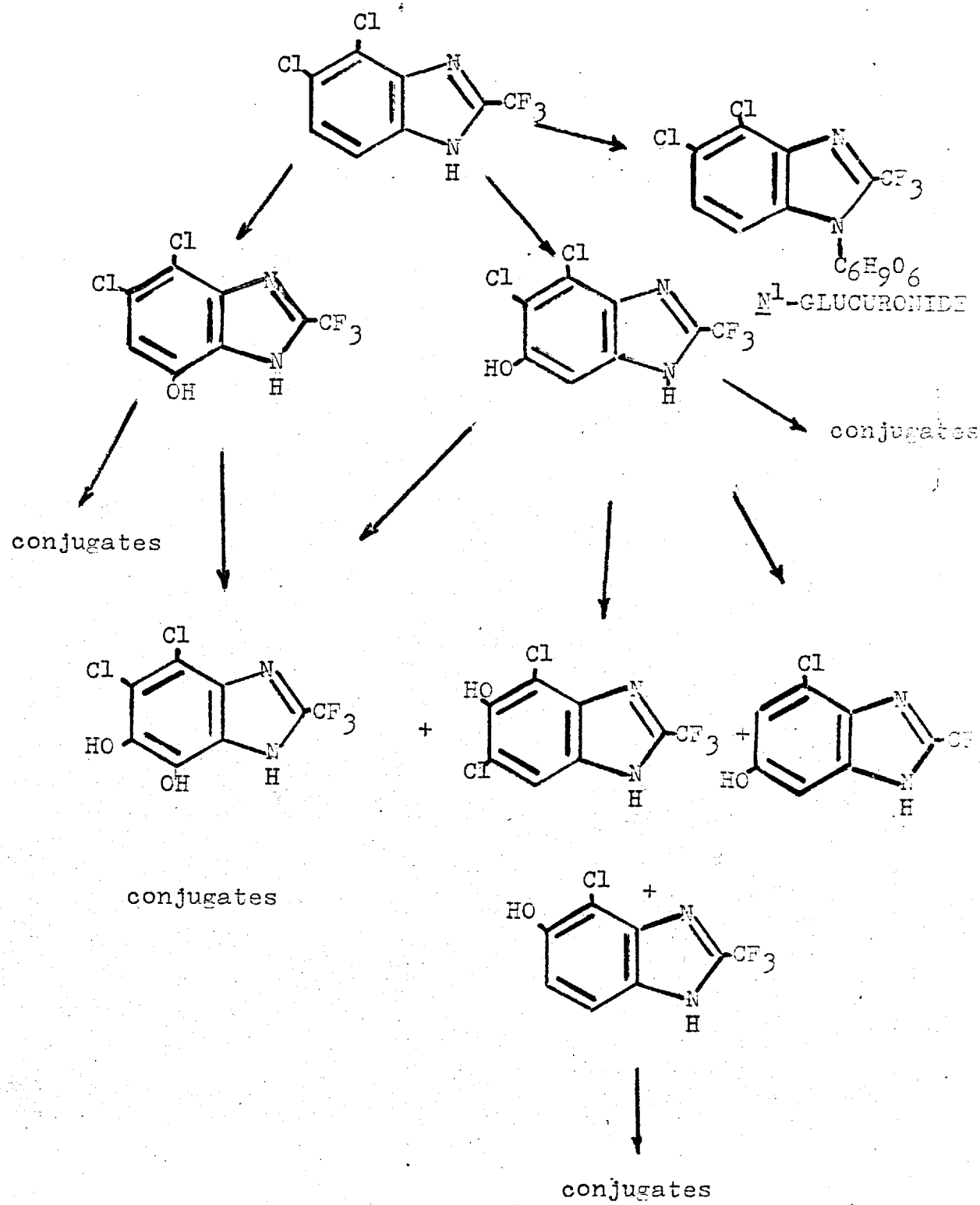
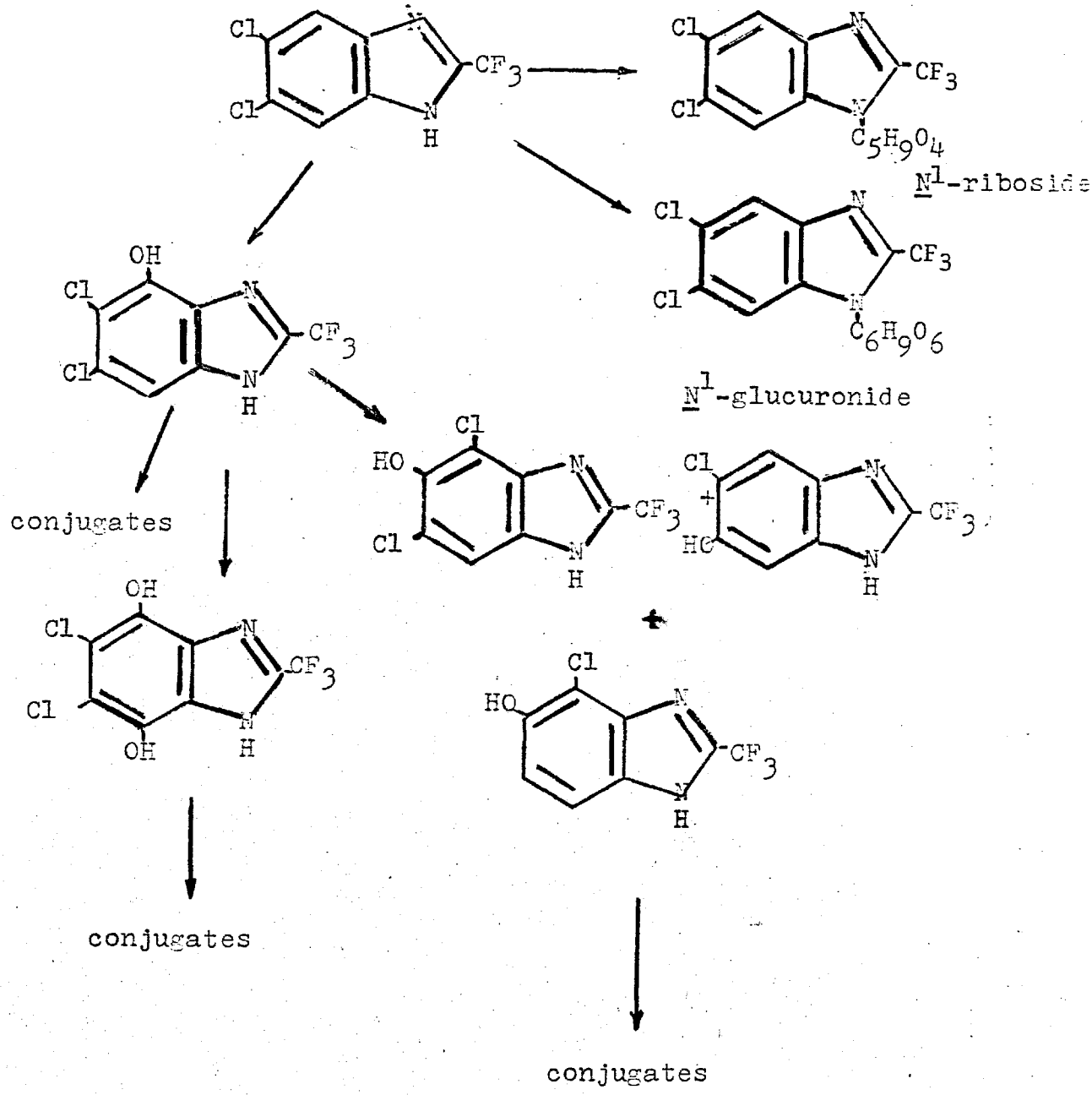


Fig. 4. THE PATHWAYS OF METABOLISM OF 5,6-DICHLORO-2-TRIFLUOROMETHYLBENZIMIDAZOLE.



2) Species differences with reference to metabolism and toxicity.

The results from the metabolism work show that the species difference in LD_{50} is mirrored by a marked difference in the excretion and metabolism of both compounds.

In both species, the majority of the orally administered dose of [^{14}C] 4,5-dichloro-2-trifluoromethylbenzimidazole was eliminated, within 72 hours, mainly in the urine. Rats excreted 95% and rabbits 78% of the dose. The rate of excretion of [^{14}C] 5,6-dichloro-2-trifluoromethylbenzimidazole in the urine was slower for both species than that of the 4,5-dichloro analogue, 32% in 3 days for the rat and 20% in 5 days for the rabbit.

A marked species difference is apparent, in that the rat also excreted about 28% of the radioactive dose in the faeces, whereas the rabbit excreted it solely in the urine.

Both the 4,5-dichloro- and 5,6-dichloro- compounds are more toxic to the rabbit than to the rat when administered orally. However, when the compounds are administered intraperitoneally to rats, the LD_{50} falls to the value for the oral dose to rabbits. This species difference, at least for 5,6-dichloro-2-trifluoromethylbenzimidazole is mirrored by the retention of radioactivity in the blood, following an intravenous dose of the [^{14}C]-labelled compound. Whereas the concentration in rat blood falls off quite rapidly after dosing, that in rabbit falls only slowly, and a significant proportion of the dose is still present after 7 days.

The intracellular distribution and the nature of the metabolites in blood all confirm that the rat has the ability to metabolise the 5,6-dichloro-compound (and presumably the 4,5-dichloro-analogues) reasonably quickly, whereas the rabbit does not have this ability. Both compounds (and also 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole) appear to be well absorbed into both species, possibly because of their high lipid solubilities.

The acute toxicities reported are presumably due to the uncoupling activities of the 2-trifluoromethylbenzimidazole compounds (Table 1). When the graphs of concentration causing 50% uncoupling of liver mitochondria vs pK_a and LD_{50} (oral to rats) vs pK_a are plotted (Figs. 5 and 6), they are both seen to have the same curve, thus lending support to the theories concerning the toxic mode of action of these compounds.

In the rat the high biliary excretions of the 3 compounds studied, together with low or non-existent faecal excretion in the intact animal, suggests that the majority of the material excreted in the bile is resorbed in some form from the intestine. Incubations with caecal contents degraded the conjugates present in bile, lending credence to this hypothesis. Thus an enterohepatic circulation in the rat could tie up large quantities of the administered toxicant and thus effectively lower the amount present at any moment in the liver or other sensitive site. Much evidence exists for enterohepatic circulation (or caecal degradation) of glucuronides, e.g. phenyl glucuronide in the rabbit (Garton, 1949), p-hydroxy-benzoic acid glucuronide in man (Quick, 1932), salicylic acid

Table 1. pK_a AND TOXICITY DATA.

COMPOUND	pK_a	LD_{50} in mg./kg. FEMALE WISTAR RAT		MOUSE	RABBIT
		Oral	i.p.	Oral	Oral
2-trifluoromethylbenzimidazole	8.79	800	-	600	-
4-chloro-2-trifluoromethyl-benzimidazole	7.57	400	-	-	-
5-chloro-2-trifluoromethyl-benzimidazole	7.97	140	-	-	200-250
5-methyl-2-trifluoromethyl-benzimidazole	8.90	1600	-	-	-
4,5-dichloro-2-trifluoromethyl-benzimidazole	6.96	300-400	18	84	10
4,5-dichloro-2-trifluoromethyl-benzimidazole (Na salt)	-	191	50-100	95	6-10
5,6-dichloro-2-trifluoromethyl-benzimidazole	7.4	120	20	200	20
4,5,6-trichloro-2-trifluoro-methylbenzimidazole	6.18	18	-	-	-
4,5,6,7-tetrachloro-2-trifluoro-methylbenzimidazole	5.04	1.56	-	-	7
4,5,6,7-tetramethyl-2-trifluoro-methylbenzimidazole	9.26	800	-	-	-

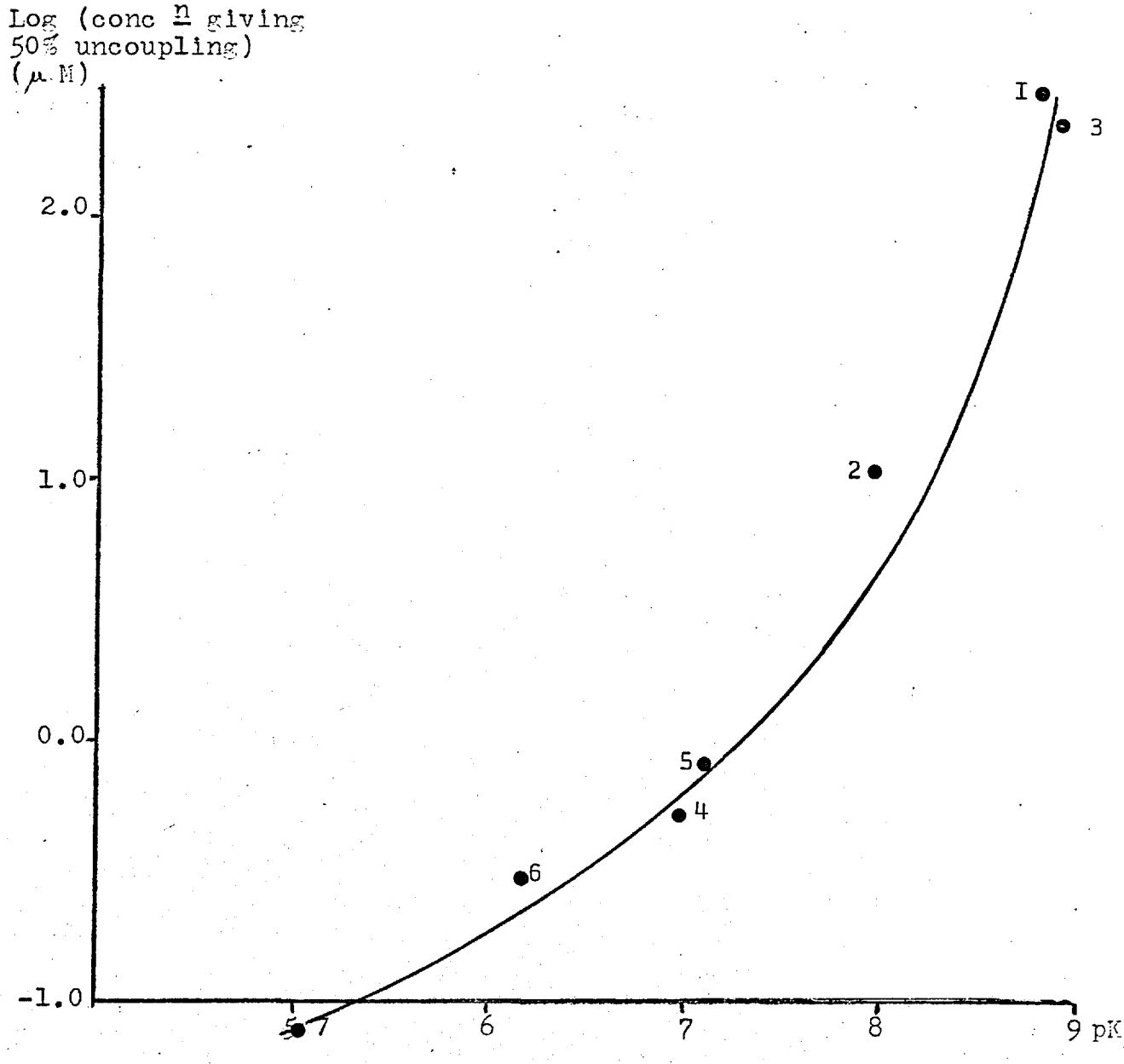
Continued.

Table 1, continued. pK_a AND TOXICITY DATA.

LD_{50} data were supplied by Fisons' Agrochemicals. Apparent pk_a values are from Burton et al. (1965) and from Fisons' Agrochemicals.

i.p. = intraperitoneal injection. The compounds were administered in either glycerol formal or methyl naphthalene to small groups of animals.

Fig. 5. LOG_{10} (CONCENTRATION GIVING 50% UNCOUPLING) VERSUS pK_a

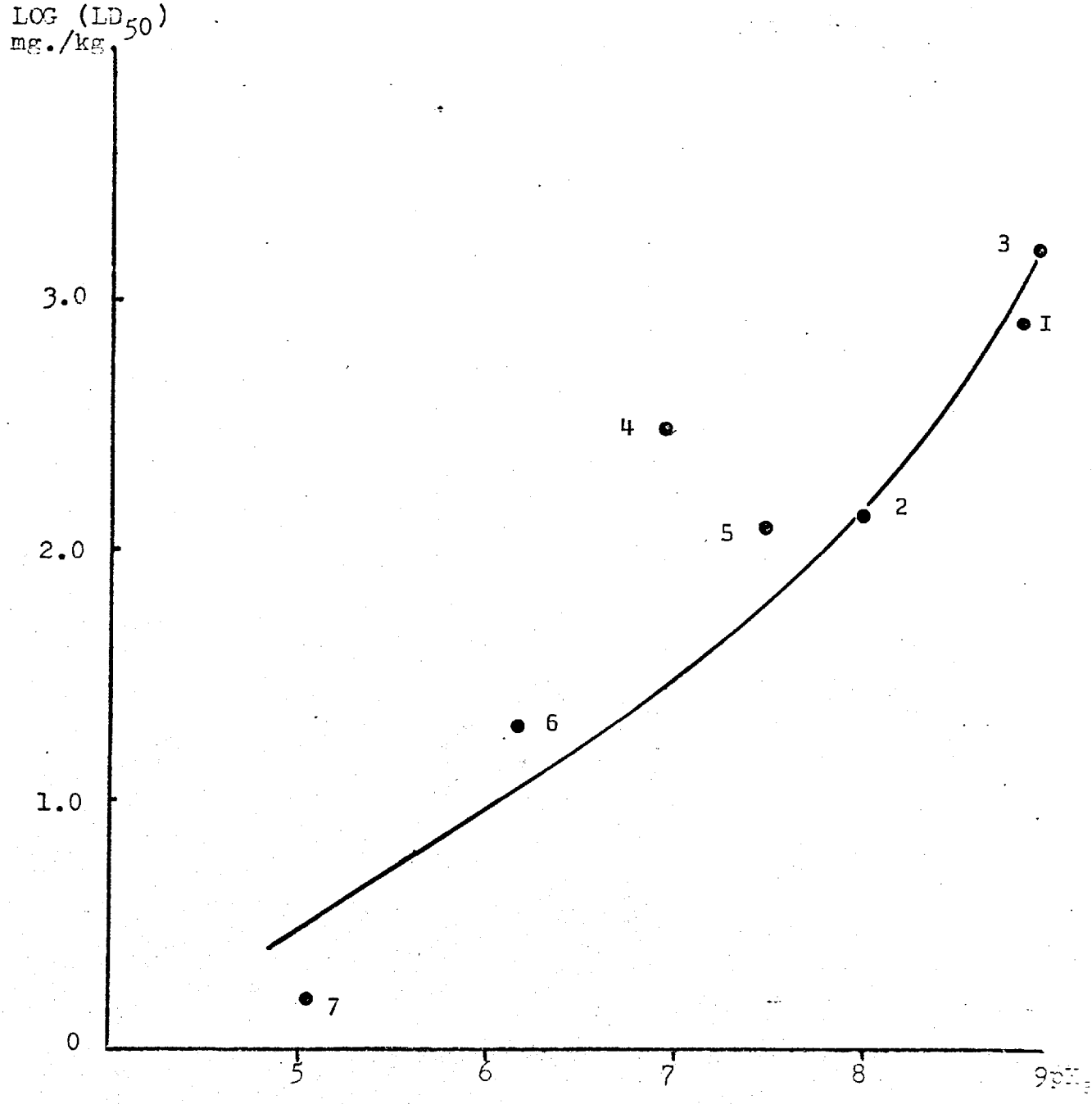


COMPOUNDS

1) 2-trifluoromethylbenzimidazole
2) 5-chloro-2-trifluoromethylbenzimidazole
3) 5-methyl-2-trifluoromethylbenzimidazole
4) 4,5-dichloro-2-trifluoromethylbenzimidazole
5) 5,6-dichloro-2-trifluoromethylbenzimidazole
6) 4,5,6-trichloro-2-trifluoromethylbenzimidazole
7) 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole

See Table 1 (Introduction, Section B) for uncoupling concentrations and pK_a values.

Fig. 6. LOG_{10} LD_{50} IN mg./kg. VERSUS pK_a



COMPOUNDS

1) 2-trifluoromethylbenzimidazole
2) 5-chloro-2-trifluoromethylbenzimidazole
3) 5-methyl-2-trifluoromethylbenzimidazole
4) 4,5-dichloro-2-trifluoromethylbenzimidazole
5) 5,6-dichloro-2-trifluoromethylbenzimidazole
6) 4,5,6-trichloro-2-trifluoromethylbenzimidazole
7) 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole

See Table 1 (page 113) for LD_{50} and pK_a values.

ether glucuronide in rat (Levy, 1966) and the monoglucuronide of diethylstilboestrol in rat (Millburn et al., 1967). However the data available does not indicate whether the toxicity of these compounds is lower in the species having such a circulation compared to those which do not. Such data must await more work in this field. Scheline (1968) believes that a microorganism high in β-glucuronidase activity, such as E. coli which is found in the caecum, is responsible for the cleavage of the glucuronides excreted in the bile.

General Biological Effects.

The high protein-binding ability demonstrated by these halogenated benzimidazoles might mean that they demonstrate other pharmacological effects, arising from their long residence time in the blood. Thus, 5,6-dichlorobenzimidazole was reported by Diwan et al. (1968) to be cytotoxic (Introduction, Section B). However, the much lower water solubility of the 2-trifluoromethyl-benzimidazoles may modify this effect, if the compounds possess it.

The in vitro anti-viral activity of the halogenated benzimidazoles has been discussed in Section B of the Introduction (pages 18-24). However, none of the compounds examined in this work have been screened for their anti-viral activity.

Incorporation into nucleosides, and or interference with their biosynthesis, may also be indicated by the formation of an N-riboside. However, as already reported, very little activity was associated with nucleotides isolated from treated rats and rabbits.

APPENDIX ONE.

PRELIMINARY STUDIES ON THE METABOLISM OF 6-CHLORO-2-TRIFLUOROMETHYLPYRIDOIMIDAZOLE.

As with all other compounds, the ^{14}C labelled compound was given as an oral dose to 200 g. Wistar female rats. Because of its low solubility in organic solvents, the material was given in 1 ml. of 1 M-sodium carbonate, i.e. as the sodium salt. Excretion data on one pair of rats are given in the first table.

As can be seen from the results, the excretion of this compound is divided between urine and faeces, and the total excretion of the compound is lower than for 5,6-dichloro-2-trifluoromethylbenzimidazole at 2 days. A significant difference between the 2 compounds is that the pyridoimidazole does continue to "trickle out" of the animal, since even at 14 days there is a measurable excretion.

Tissue distribution showed that most of the activity was associated with the blood or blood-containing tissues. An analysis of the radioactivity showed that 65% of the ^{14}C in the blood was present as unchanged 6-chloro-2-trifluoromethyl-pyridoimidazole after 14 days.

Metabolites.

The urine and faeces were tested as for the benzimidazoles and subjected to TLC and paper chromatography. The following solvent systems were found to be of particular value for the silica gel thin layer chromatography of acid-hydrolysed urine.

a) 4:1 v/v chloroform : isopropanol.

b) Ethyl acetate.

Chromatography, cochromatography and isotope dilutions confirmed the presence of the free parent compound in urine

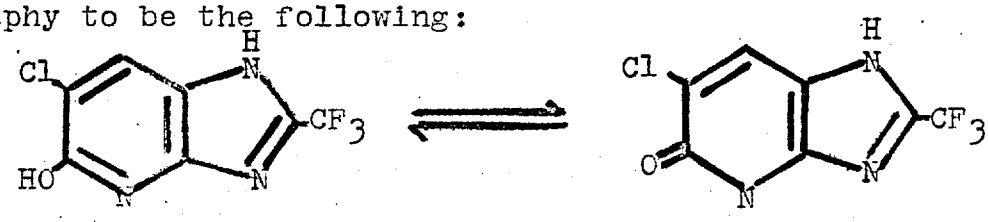
Table 1. THE METABOLISM OF 6-CHLORO-2-TRIFLUOROMETHYL-PYRIDOIMIDAZOLE IN RATS.

^{14}C EXCRETION		% OF DOSE AVERAGE	(RANGE)
In urine	Day 1	7.1	(6.9-7.3)
	Day 2	6.7	(5.8-7.6)
	Day 3-4	8.1	(7.3-9.0)
	Day 5-7	7.8	(7.6-8.0)
	Day 8-14	8.8	(8.3-9.3)
In faeces	Day 1	0.5	(0.2-0.7)
	Day 2	4.8	(4.6-4.9)
	Day 3-4	4.7	(4.6-4.8)
	Day 5-7	4.3	(4.3-4.4)
	Day 8-14	9.7	(9.4-10.0)

Two animals received an oral dose of 15 mg/kg. (11.9 μCi) dissolved in 1 ml. 1M-sodium carbonate.

(10% of the dose in 4 day urine) but no significant amounts of combined compound. Five metabolite peaks were observed on radiochromatogram scans of urine samples using solvent system A (paper or thin layer, developed with iso-butanol saturated with water). One of these peaks was removed by sulphatase preparations and by acid hydrolysis, and may be a sulphate of an (unknown) aglycone.

One acid hydrolysis product has been shown by cochromatography to be the following:



6-chloro-5-hydroxy-2-trifluoropyridoimidazole

Thus the metabolism of 6-chloro-2-trifluoromethyl-pyridoimidazole is even slower than that of other benzimidazole derivatives. The results show that N-conjugation of the parent compound is an insignificant reaction this time, and that at least one of the metabolites is formed by ring hydroxylation. Dr. Bond (personal communication) reported the presence of trifluoracetic acid in rats dosed with this compound.

APPENDIX TWO.

ELECTRON DENSITY CALCULATIONS.

COMPUTER PROGRAMME.

In order to determine the electron densities, free valences and other parameters affecting the reactivity of various positions of the substituted benzimidazoles, the computer programme of Krüger-Thiemer and Hansen (1966) was rewritten for use on the London University Atlas machine. In this programme (Fig. 1) the Huckel molecular orbital (HMO) approximation has been employed. This states the following approximations (Huckel, 1931 a & b, 1932, 1933, and 1937).

a) All of the overlap integrals (S) are dropped, i.e. it is assumed that the atomic orbitals on different atoms are orthogonal, and hence there is no overlap of π -orbitals. Chalvet, Daudel and Peradejordi (1964) note that this is wrong but that it appears to give the right results.

b) Second order effects due to remote atoms are ignored.

Because of the localised nature of the σ bonds, all of the electrons of the molecule other than the p electrons are considered as forming a fixed frame of localised bonds. Each mobile or π electron is considered as occupying a molecular orbital ϕ extending over the whole molecule (in an aromatic system). This orbital is approximately given by a Linear Combination of Atomic p Orbitals (LCAO).

1) $\phi = \Sigma_r C_r X_r$ (Lennard-Jones, 1929)

where X_r is the orbital providing the p electrons on atom r.

Fig. 1.

```
begin   comment HMO-CALCULATION I.R.FLOCKHART;
        integer PROG,ME,HMO,CO,EV,I,J,JJ,M,MZ,N,NB,NY,R,S,T,U,NO;
        real AA,BB,DEN,MOL,MMAX,RHO,V,W,X,Y;

        procedure VERT(V,W);    real V,W;
        begin       real H;
                    H:=V;       V:=W;                   W:=H;
        end ;

        procedure JACOBI(A,C,N,RHO);
        value N,RHO;            integer N;              real RHO;
        begin   real NORM1,NORM2,THR,MU,OMEGA,SINT,COST,INTI,V1,V2,V3
                integer I,J,P,Q,IND;

for I:=1 step 1 until N do
for J:=1 step 1 until I do
begin ifI=J then C[I,J]:= 1 else C[J,I]:=C[I,J]:=0
end;INT1:=0;
for I:=2 step 1 until N do
for J:=1 step 1 until I-1 do

INT1:=INT1+2*A[I,J]exp2;
NORM1:=sqrt(INT1);NORM2:-(RHO/N)*NORM1;
THR:=NORM1;IND:=0;
MAIN:THR:=THR/N;
MAIN1:for Q:=2 step 1 until N do
for P:=1 step 1 until Q-1 do
```

```
begin if abs(A[P,Q])>THR then
begin IND:=1;V1:=A[P,P];V2:=A[P,Q];V3:=A[Q,Q];
MU:=(V1-V3)/2; OMEGA:=(ifMU=0 then 1 else sign (MU))*(-V2)/sqrt
        (V2*V2+MU*MU);
SINT:=OMEGA/sqrt(2*(1+sqrt(1-OMEGA*OMEGA)));
COST:=sqrt(1-SINT*SINT);
for I:=1 step 1 until N do

begin INT1:=A[I,P]*COST-A[I,Q]*SINT:
A[I,Q]:=A[I,P]*SINT+A[I,Q]*COST;
A[I,P]:=INT1;INT1:=C[I,P]*COST-C[I,Q]*SINT;
C[I,Q]:=C[I,P]*SINT+C[I,Q]*COST;C[I,P]:=INT1;
end;
for I:= 1 step 1 until N do
begin A[P,I]:=A[I,P]; A[Q,I]:=A[I,Q]; end;
A[P,P]:=V1*COST*COST+V3*SINT*SINT-2*V2*SINT*COST;
A[Q,Q]:=V1*SINT*SINT+V3*COST*COST+2*V2*SINT*COST;
A[P,Q]:=A[Q,P]:=(V1-V3)*SINT*COST+V2*(COST*COST-SINT*SINT);
end;
end;

if IND=1 then
begin IND:=0; go to MAIN1
end else if THR>NORM2 then goto MAIN;
end JACOBI;
```

```
          for J:=1 step 1 until NB do
begin     P[J]:=0; R:=RJ[J]; S:=SJ[J];
          for I:=1 step 1 until NY do

          P[J]:=P[J]+2*C[R,I]*C[S,I];
end       BOND ORDERS;
          for R:= 1 step 1 until N do

begin     NMAX:=if AT[R]=12 then sqrt(3) else
                if AT[R]=14 then sqrt(2) else
                if AT[R]=16 then 1 else 0;

          AA:=0; for J:=1 step 1 until NB do
begin     if RJ[J]=R or SJ[J]=R then AA:=AA+P[J];
end;      F[R]:=NMAX-AA;
end       FREE VALENCIES;
          AA:=2*NY; BB:=0; for I:=1 step 1 until NY do
          BB:=BB+2*EPS[I];
comment   TOTAL ENERGY OF THE PI-SYSTEM IN GROUND STATE;

          V:=AA; W:=BB; for J:=1 step 1 until NB do
begin     I:=0; if FB[J]>2 then
begin     R:=RJ[J]; S:=SJ[J]; if AT[R]=12 then
begin     if AT[S]=12 then
begin     V:=V-2; W:=W-2; end else I:=S;
end       else I:=R; if I=0 then

begin     V:=V-2; W:=W-H[I]-2*sqrt(K[J]* K[J]+H[I]*H[I]/4);
end;
```

```
end;
end;     for R:=1 step 1 until N do

begin    if QPI[R]≥2 then
         begin V:=V-2; W:=W-2*H[R]; end;
end      DELOCALISATION ENERGY;
         for R:=1 step 1 until N do
begin    PI[R]:=0; for I:= 1 step 1 until NY do
         begin for J:=NY+1 step 1 until N do
             PI[R]:=PI[R]+4*(C[R,I]*C[R,J])exp2/(EPS[I]-EPS[J]);
         end;
end      SELF ATOM POLARISABILITIES;

         for R:=1 step 1 until N do

begin    SS[R]:=0; for I:=1 step 1 until NY do
         SS[R]:=SS[R]+2*C[R,I]*C[R,I]/EPS[I];
end      SUPERDELOCALISABILITIES;
         newline(1);
writetext (←NUMBER/s OF/s CALCULATED/sATOM/s N:= →); print (N,2,0);
         write text (←WITHOUT/s SINGLE /sAND/s AMINO/sGROUP/s
            HYDROGENS→); newline(1);space(13);
select input(1);
NO:=read;
for NE:=1 step 1 until NO do
begin                              RHO:=read;N:=read;
begin    integer array AT[1:N],QPI[1:N];
array EPS[1:N],F[1:N]H[1:N],PI[1:N],Q[1:N],QQ[1:N],SS[1:N],
```

```
   A[1:N,1:N],C[1:N, 1:N];
for R:=1 step 1 until N do
begin I:=read;AT[I]:=read;QPI[I]:=read; H[I]:=read;
end;
NB:=read;
begin integer array RJ[1:NB],SJ[1:NB],FB[1:NB];
real array K[1:NB],P[1:NB];
for J:=1 step 1 until NB do
begin RJ[J]:=read; SJ[J]:=read;FB[J]:=read;K[J]:=read;
end;
newline(1); writetext ('CHEMICAL NAME');

newline(1);writetext( 'RHO=   ');print (RHO,0,10);
for R:=1 step 1 until N do
for S:=1 step 1 until N do
A[R,S]:=0;
for R:=1 step 1 until N do A[R,R] :=H[R];
for J:=1 step 1 until NB do
begin R:=RJ[J];  S:-SJ[J]; A[R,S]:=A[S,R]:=K[J]; end;

JACOBI(A,C,N,RHO);

        for I:=1 step 1 until N do EPS[I]:=A[I,I];

        comment ENERGIES OF MOLECULAR ORBITALS EPS[I] :=
             NEGATIVE EIGENVALUES;

        L1: I:=J:=1;
        L2: if EPS[I+1]>EPS[I] then
```

```
begin   VERT(EPS[I+1],EPS[I]);
        for R:=1 step 1 until N do VERT(C[R,I+1],C[R,I]);
        I:=I+1; if I=N then goto L1 else go to L2;

end     else
begin   I:=I+1; J:=J+1; if I<N then goto L2 else if J=I then goto L1;
end;
        for I:=1 step 1 until N-1 do
begin   if EPS[I]>0 and EPS[I+1]<0 then NY:=I;
end;
        for R:=1 step 1 until N do
begin   Q[R]:=0; for I:=1 step 1 until NY do
        Q[R]:=Q[R]+2*C[R,I]* C[R,I];
end     ELECTRONIC CHARGES;

        for R:=1 step 1 until N do QQ[R]:=QPI[R]-Q[R];
        comment NET CHARGES;
        writetext( ←ELECTR.ß NETß ßßß FREEßßßß SELF-ATOMßß SUPER→);
           space (10);
        writetext(←ENERGYßOF→); newline(1);
        space(12);
        writetext( ←CHARGE ßß CHARGE ßVALENCE ßPOLARIZßßß.
           DELOCALßßßßßß MOL,ORBITAL→);
newline(1);writetext
        (←R ßß ATß QPIßßH[R]ßßQ[R] ßßßß QQ[R]ßßßF[R] ßßßß
           PI[R,R] ßßßßßßßS[R]→);space(12)
writetext(←EPS[I]→);
newline(1);
```

```
        DEN:=EPS[NY]-EPS[NY+1];
        for R:=1 step 1 until N do
begin   newline(1);
print(R,2,0); JJ:=AT[R];
        if JJ=12 then writetext (‡C†) else
        if JJ=14 then writetext (‡N†) else
        if JJ=16 then writetext (‡O†) else
        if JJ=2  then writetext (‡H2†) else
        if JJ=3  then writetext (‡H3†) else
        if JJ=19 then writetext (‡F†) else
        if JJ=35 then writetext (‡CL†) else
        if JJ=32 then writetext (‡S†) else
        if JJ=80 then writetext (‡BR†) else
        if JJ=31 then writetext (‡P†) else
        if JJ=23 then writetext (‡NA†) else
        if JJ=39 then writetext (‡K†) else
        print (AT[R],2,0);print (QPI[R],2,0);print(H[R],1,2);
           print(Q[R],1,4);
        print(QQ[R],1,4);print(F[R],1,4); print(PI[R],2,4);
           print(SS[R],2,4);

        if R=NY then writetext( ‡H.F.ORB.† ) else
        if R=NY+1 then writetext (‡L.E.ORB†) else
                                space(11);print(EPS[R],2,4);
end;
        newline(1);
                              writetext(‡DELENY:=  †);print(DEN,2,4);
```

```
        newline(1);
space(56);writetext(←1/DELENY:= →);print(1/DEN,2,4);newline(1);

        writetext(←NUMBEP/S OF/ BONDS /SNB:= →);print(NB,2,0);
           newline(1);
        space(25);writetext(←BOND/S/S/S/S/S/S TOTAL /SPI-ENERGY /SIN/SGROUND
           /STATE→); newline(1);
        space(25);writetext(←ORDER /S/S/S/S/SEPI:=  →);print(AA,3,4);
           writetext(←  *ALPHA→);
        print(BB,3,4);writetext(←  *BETA→);newline(1);
        writetext(←R/SS/S/SFB/SK[R,S]/SP[R,S]→);
        newline(1);space(35);
              writetext(←DELOCAL,ENERGY/SED:= →);print(W,3,4);
                 writetext(←  *BETA→);
        for J:=1 step 1 until NB do
begin   newline(1);print(RJ[J],2,0);print(SJ[J],2,0);print(FB[J],2,0);
           print(N[J],2,2);
        print(P[J],2,4);
        if J=1 and abs(V)>10-6 then
begin   space(26); print (V,3,4); writetext (←*ALPHA→);
end;
end;
end;
end;
end;
end;
end;
***Z
```

It can be shown that a set of simultaneous equations of the type:

2) $\sum C_j(H_{ij}-ES_{ij})=0$ can be derived with one equation for each value of i.

H_{ij} is the Hamiltonian operator = $\int X_i H_j dv$

E is the Energy of the system as given by the Schrodinger wave equation:

3) $$\left[\frac{-h^2}{8\pi^2 m}\sum_i \nabla_i^2 + V\right]\psi = E\psi$$

4) and S_{ij} the overlap integral = $\int X_i X_j dv$

This system of homogeneous linear equations (2) is often called a system of secular equations. Solutions to C_j can be found by putting

$$\left|H_{ij}-ES_{ij}\right| = 0 \qquad (5)$$

This condition is an equation in E whose degree is equal to the number of functions X_j. The lowest root of the equation gives the best energy value, i.e. the ground state of the system.

If in the function (1) there are 2n atomic orbitals r, the determinant (5) is of the 2nth order and hence there are 2n possible values E_i of the mono-electronic energies. For each F_i there is a molecular orbital ϕ_i whose 2n coefficients Cri are the solutions of the 2n simultaneous equations.

$$\sum C_{si}(H_{rs}-E_i S_{rs}) = 0 \qquad r = 1,2,3.....2n$$

It can be shown that the individual energy E_i of an electron in a molecular orbital ϕ_i is

$$E_i = \int \phi_i H \phi_i \, dv$$

$$= \sum_r (C^2_{ri} H_{rr} + \sum_{s>r} 2C_{ri}C_{si}H_{rs})$$

and the sum total π-electron energy of the system can be written as

$$\varepsilon = 2\sum_i E_i = 2 \sum_i \sum_r (C^2_{ri} H_{rr} + \sum_{s>r} 2C_{ri}C_{si}H_{rs})$$

since all occupied orbitals are doubly occupied, when even numbers of p electrons are present. The method can be extended to cover an odd number of p electrons and to heterocycles or substituted compounds with little difficulty.

The following functions are now defined:

$\alpha_r = \int X_r H X_r dv$ (7) - the Coulomb integral

and $\beta_{rs} = \int X_r H X_s dv$ (8) - the exchange (resonance) integral.

If we now make the assumption of the Huckel approximation, equation (6) reduces to:

$$C_{ri}(\alpha - E) + \sum_{\substack{S \text{ adj.} \\ \text{to } r}} \beta C_{si} = 0 \qquad r = 1,2,3.....2n$$

α-E becomes the diagonal element of the matrix and β the non-diagonal elements which are not equal to zero.

Considering now the case of a heterocyclic molecule, α_r the Coulomb integral is given the same value for all carbon atoms but a different value α_r is given to each non-carbon atom, and for convenience this for an atom X is written as:

$$\alpha_X = \alpha + \delta_X \beta \qquad (10)$$

Similarly the resonance integral Hrs is assumed to be of a fixed value β for carbon atoms, but of different values for C -X bonds.

$$\beta_{C-X} = \eta_{C-X} \beta \qquad (11)$$

The diagonal elements of the matrix can now be written as:

$$\alpha_r - E = \alpha - E + \delta_r$$

and the non-diagonal elements (not equal to zero) become:

$$\beta_{rs} = \eta_{rs}\beta$$

All of the elements of the matrix can now be divided by β and the result $\frac{\alpha - E}{\beta}$ put equal to y.

The set of equations for a system of n atomic orbitals can now be written as

$$C_{ri}(y + \delta_r) + \sum_{\substack{s \text{ adj} \\ \text{to } r}} \eta_{rs} C_{si} = 0 \qquad r = 1,2,3.....2n \qquad (12)$$

<u>π-electron contributions</u>.

The number of π-electrons contributed by a heteroatom or substituent will determine the number of atomic orbitals participating in the molecular orbital, and is thus of great importance.

a) Contributors of one π-electron : = heteroatoms bound by a double bond to another heteroatom or to carbon, e.g. pyridine type N, O in C=O, S in C=S and P in P=O.

b) Contributors of 2 π-electrons : - heteroatoms with lone pairs bound only by single bonds to adjacent atoms, e.g. pyrrole or aniline-type N, furan and phenol O, thiophene or sulphhydryl S, and halogens.

c) Hyper-conjugated groups. CH_2 or CH_3 these can be treated as pseudo $C{=}H_2$ double bonds (one π orbital) and CH_3 treble bond (2 π orbitals), their degree of delocalisation is somewhat smaller than that of the usual π orbitals.

Values of the parameters.

α and β are defined in terms of an Hamiltonian which cannot be written explicitly, and hence the terms α and β are carried through the calculations. This is at first sight a very grave drawback to the whole method, but usually absolute values are not required and more often a scale of relative parameters is of more interest.

For the Coulomb integral for an atom X contributing one p electron and one orbital, the Hamiltonian is the sum of a potential and a kinetic term

$$H = V + T$$

and V is the sum of terms of all of the neighboroughing atoms.

$$V = V_1 + V_2 \ldots\ldots\ldots + V_x + V_n$$

V_x roughly represents the attraction by the atom X for its p electron and is thus proportional to the ionisation potential. It has also been proposed (Mulliken, 1949) that $2x$ varies with the electro-negativity of the atom. The values computed from either source are in good agreement.

The resonance integral β (C=C) has been defined by Lennard-Jones (1937) as half the difference between the energies of double and single bonds.

$$\beta_{C=C} = \tfrac{1}{2} (E_d - E_s)$$

and hence the ratio of the resonance integral $\beta_{C=X} / \beta_{C=C}$ should be

$$\frac{E_{C=X} - E_{C-X}}{E_{C=C} - E_{C-C}}$$

This however is not strictly valid and it can be shown that a more accurate expression is:

$$\sqrt{\eta^2 + \frac{\delta^2}{4}} = E_{C=X} - E_{C-X}$$

The second term under the square root is ignored to give the first expression.

Values of η and δ calculated in this manner or from other sources such as spectra, diamagnetic anisotropy etc. are presented below:

Bond	η	δ
C = N-	I	0.4
C –N– (N with H)	0.9	1
C = $\overset{+}{N}$– (N with H)	1	2
C = O	2	1.2
C –O–	0.9	2
Carom-Caliph	+0.7	-
Carom-	-	-0.1
Caliph-	-	-0.2
C ≡ H_3	2	-0.2
C = H_2	2	3.0
C - F	0.7	3.0
C - Cl	0.5	3.0
C - Br	0.3	0.9

Using this data, the determinant for a molecule may be written out, e.g. for benzimidazole

All of the atoms involved in π-bonding are numbered consecutively as shown.

$$
\begin{array}{c|ccccccccc|}
 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 \\
1 & y+1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \\
2 & 1 & y & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
3 & 0 & 1 & y+0.4 & 1 & 0 & 0 & 0 & 0 & 0 \\
4 & 0 & 0 & 1 & y & 1 & 0 & 0 & 0 & 0 \\
5 & 0 & 0 & 0 & 1 & y & 1 & 0 & 0 & 0 \\
6 & 0 & 0 & 0 & 0 & 1 & y & 1 & 0 & 0 \\
7 & 0 & 0 & 0 & 0 & 0 & 1 & y & 1 & 0 \\
8 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & y & 1 \\
9 & 1 & 0 & 0 & 1 & 0 & 0 & 0 & 1 & y \\
\end{array} = 0
$$

the corresponding set of equations is:

$$
\begin{aligned}
C_1 (y + 1) + C_2 + C_9 &= 0 \\
C_1 + (y)C_2 + C_3 &= 0 \\
C_2 + (y+0.4)\, C_3 + C_4 &= 0 \\
C_3 + yC_4 + C_5 + C_9 &= 0 \\
C_4 + C_5 y + C_6 &= 0 \\
C_5 + C_6 y + C_7 &= 0 \\
C_6 + C_7 y + C_8 &= 0 \\
C_7 + C_8 y + C_9 &= 0 \\
C_1 + C_8 + C_9 y &= 0
\end{aligned}
$$

These are the equations which are solved by the computer programme and the parameters which are printed out by the computer are the following:

a) Resonance energy - defined as the difference between the total energy of the π-system, and the energy the system would have if all of the double bonds were localised.

e.g. For benzimidazole

the energy is $3E_{C=C} + E_{C=N} + \beta$ (lone pair on N_1)

and in terms of α and β

$$6\alpha + 6\beta + (2\alpha + 2.4396\beta) + 2\alpha + 2\beta$$

$$= 10\alpha + 10.4396\beta$$

the result from the programme gives $10\alpha + 13.803\beta$

so the resonance energy is 3.363β.

b) Net charges - this represents the difference between the charge an atom would carry in the absence of delocalisation and its actual calculated charge, and thus represents the loss or gain of charge brought about by delocalisation. The sign "+" means lack and "-" means gain of electrons.

c) Free valence is defined as

$$Fr = Nmax - \sum_{\substack{s \text{ adj.} \\ \text{to } r}} p_{rs}$$

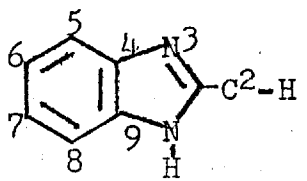
where Nmax is the maximum possible binding power for the atom being considered. Hence F_r is a measure of the amount of potential binding power which has not been used for π-bonding.

For Carbon Nmax = 1.732

Nitrogen Nmax = 1.414

Oxygen Nmax = 1.0

Table 1. DATA INPUT FOR ELECTRON DENSITY CALCULATIONS ON BENZIMIDAZOLE.



10^{-7} (=RHO); 9 (=N)

R	AT	QPI	H(=δ)
1	14	2	1.0
2	12	1	0.0
3	14	2	0.4
4	12	1	0.0
5	12	1	0.0
6	12	1	0.0
7	12	1	0.0
8	12	1	0.0
9	12	1	0.0

10 (=NB)

R	S	FB	K
1	2	1	0.9
1	9	1	0.9
2	3	2	1.0
3	4	1	0.9
4	5	1	1.0
4	9	2	1.0
5	6	2	1.0
6	7	1	1.0
7	8	2	1.0
8	9	1	1.0

KEY

R = no. of atom
S = no. of neighbouring atom
AT = atomic weight
QPI = no. of electrons donated to system

H = δ
N = no. of atoms
NB = no. of bonds
FB = bond character (double, single, etc.)
K = η

RESULTS FROM PROGRAMME.

Results for the calculations on some benzimidazoles are presented in Table 2. Net charge is measured in electrons, and a positive value of X means that X amount of charge has been drained away from the atom, i.e. for a carbon atom donating one π electron, 1-X electrons remain. It is thus a convenient static index of nucleophilic attack. Free valence can be defined as the "residual bonding power" of the atom and is a convenient measure of the ability of this atom to undergo free-radical attack.

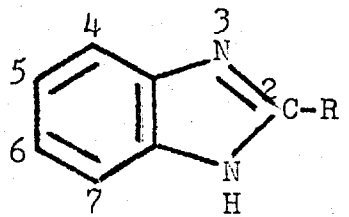
Considering the benzene ring first.

Positions 4(7) are slightly more reactive to free radical attack, as measured by free valence, than 5(6). Predicted hydroxylation should occur at position 2 on both charge and free valence evidence. Since the metabolic evidence is against this, then some reason must be sought, and perhaps the answer may be that the 2-hydroxy compound is unstable. Ito et al. (1961) were unable to find any evidence for the 2-hydroxy compound after benzimidazole was administered to rabbits, but N^1, N^3-dimethyl-benzimidazole gave the benzimidazolone derivative, presumably arising from C_2 attack by hydroxyl.

Substitution by trifluoromethyl at position 2 does, of course, remove this option, and the only positions left for consideration are those of the benzene ring and the nitrogen atoms.

2-Trifluoromethylbenzimidazole should be hydroxylated at

Table 2. ELECTRON DENSITIES AND FREE VALENCES OF BENZIMIDAZOLE DERIVATIVES.



COMPOUND	R	1	2	3	4	5	6	7
Benzimidazole	H	0.395 0.429	0.107 0.430	-0.333 0.207	-0.023 0.439	-0.034 0.402	-0.025 0.404	-0.036 0.435
"	CF_3	0.424 0.454	0.030 0.109	-0.152 0.227	0.005 0.450	-0.223 0.405	0.012 0.411	-0.036 0.440
4-Cl "	"	0.421 0.457	0.027 0.109	-0.147 0.225	0.028 0.313	-0.044 0.411	0.014 0.410	-0.050 0.441
5-Cl "	"	0.425 0.454	0.029 0.109	-0.156 0.229	-0.017 0.456	-0.003 0.275	0.002 0.417	-0.034 0.439

Net charge given first.

Free valence given second and underlined.

4(7) according to both charge and free valence data, although 5(6) will also be attacked, since the free valence figures are very close to those for the 4(5) position (see Table 3).

Chlorine at position 4 removes one position for hydroxylation and enhances the free valence at 7, but on charge considerations, position 6 would seem to be reactive to nucleophilic attack.

Position 5 chlorine enhances 4 hydroxylation, i.e. in the ortho position, but again 7 is almost as reactive. Position 6 is slightly activated to nucleophilic attack.

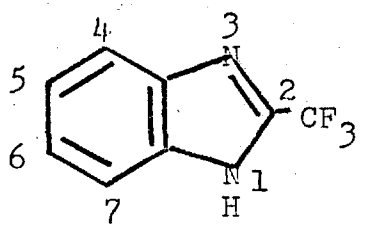
Since the metabolic experiments with the above compounds have not yet been performed, the statements are purely speculative, and a mixture of the possible hydroxylations will probably occur.

Turning to compounds whose metabolism has been studied, the following is found:

For 4,5-dichloro-2-trifluoromethylbenzimidazole free valence predicts a preponderance of the 7-hydroxy derivative, but metabolic results gives a ratio of 6/7 hydroxylation of 9/1 in the rat. The charge results accord with position 6 being more reactive to nucleophiles. The ascorbate oxidation system of Udenfriend, and the peroxyacid oxidations, previously described, produce free radicals (Diner, 1964), and yield equal quantities of 6-OH and 7-OH derivatives of 4,5-dichloro-2-trifluoromethylbenzimidazole in vitro.

For the 5,6-dichloro compound, free radical attack at 4

Table 3. ELECTRON DENSITIES AND FREE VALENCES OF 2-TRIFLUOROMETHYLBENZIMIDAZOLE DERIVATIVES.



SUBSTITUENT ON POSITION NUMBER												
4	5	6	7	1	2	3	4	5	6	7
Cl	Cl	-	-	0.421 0.458	0.026 0.109	-0.151 0.227	0.007 0.322	-0.024 0.284	0.004 0.416	-0.049 0.441
-	Cl	Cl	-	0.424 0.443	0.026 0.124	-0.173 0.207	-0.020 0.463	-0.013 0.280	0.018 0.285	-0.054 0.444
OH	Cl	Cl	-	0.410 0.470	0.018 0.110	-0.140 0.219	0.059 0.226	-0.081 0.314	0.028 0.280	-0.102 0.469
O=	Cl	Cl	O=	0.518 0.341	-0.001 0.104	-0.129 0.178	0.219 0.261	0.032 0.384	0.029 0.382	0.219 0.268
Cl	Cl	OH	-	0.408 0.471	0.017 0.110	-0.147 0.223	0.012 0.318	-0.054 0.308	0.067 0.191	-0.115 0.464

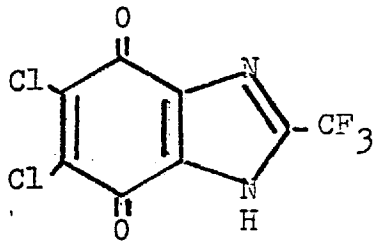
Net charge given first.

Free valence given second and underlined.

or 7 is predicted, but negative values for nucleophilic attack at these positions predict that hydroxylation will be slow, if it occurs by this method. However a positive value of net charges at 6(5) indicates that the chlorine atom may be labile to a nucleophilic attack. This figure might be taken as evidence in favour of the NIH shift mechanism proposed in the Discussion.

When the monohydroxy derivatives of 4,5- and 5,6-dichloro-2-trifluoromethylbenzimidazole are considered with further hydroxylation or dehalogenation in mind, the following features are observed.

For the 5,6-dichloro-4-hydroxy compound, nucleophilic attack should occur at position 6 and free radical attack at position 7. At neither of these two positions do both parameters have high values simultaneously, which is one of Diner's criteria for effective metabolic hydroxylation. The 4,7-dioxo compound, which is possibly the product of further hydroxylation of the phenol, shows some interesting properties which may be explicable in terms of these parameters. Thus positions 5 and 6 (those which are chlorinated) have a positive charge and are thus amenable to nucleophilic attack, they also have a fairly high free valence which will enhance the reactivity of these positions. In accordance with this it is interesting to note that in caustic alkali solutions, the quinone acquires a purple colouration which has been shown polarographically and by synthesis (D. Hughes, Fison's, personal communication) to be the following:



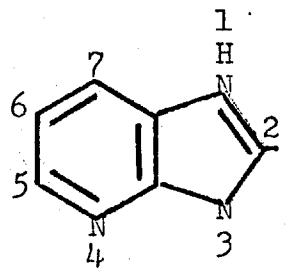
As with 5,6-dichloro-4-hydroxy-2-trifluoromethylbenzimidazole, 4,5-dichloro-6-hydroxy-2-trifluoromethylbenzimidazole shows a positive charge at one of the chlorinated positions, in this case position 4. Also, position 7 has a high free valence and thus will be amenable to hydroxylation by free radical attack; thus the formation of the orthoquinone may be explicable in terms of this finding.

In many cases tautomeric forms are possible, but these have been neglected here for the sake of simplicity.

A series of nitrogen containing compounds, some of which occur naturally, e.g. purine, were also studied. The purpose of this was to endeavour to obtain data on the nitrogen atom which conjugates with sugar derivatives. Thus the purines conjugate with ribose, and the benzimidazoles conjugate with glucuronic acid and/or ribose (Bowker and Casida, 1969). However no concrete facts emerged from this study, and conclusions must await further data or the use of some other parameter.

It is of considerable interest to examine the electron densities and free valences of 6-chloro-2-trifluoromethylpyridoimidazole and related compounds and these are summarized in Table 4. It can be seen that positions 5 and 7 of this

Table 4. ELECTRON DENSITIES AND FREE VALENCES ON PYRIDOIMIDAZOLE DERIVATIVES.



SUBSTITUENTS ON POSITION NUMBER 2	5	6	7	1	2	3	4	5	6	7
-	-	-	-	0.403 0.409	0.105 0.433	-0.329 0.204	-0.212 0.157	0.055 0.405	-0.042 0.404	0.030 0.442
CF_3	-	-	-	0.430 0.434	0.028 0.111	-0.144 0.225	-0.212 0.162	0.094 0.414	-0.030 0.406	0.058 0.454
CF_3	-	Cl	-	0.431 0.434	0.027 0.110	-0.149 0.226	-0.210 0.161	0.085 0.421	-0.010 0.277	0.037 0.460
CF_3	-	OH	-	--- ---	0.039 0.107	--- ---	--- ---	0.162 0.479	0.050 0.191	0.034 0.473
CF_3	OH	Cl	-	0.430 0.441	0.017 0.113	-0.147 0.223	-0.281 0.200	0.138 0.183	-0.036 0.303	0.036 0.454
CF_3	=0	Cl	-	0.414 0.435	-0.019 0.118	-0.158 0.213	-0.327 0.556	0.205 0.182	-0.056 0.407	0.035 0.436

Electron densities given first.

Free valences given second and underlined.

compound have positive net charges (i.e. they have enhanced reactivity to nucleophiles such as OH^-), 5 being more reactive than 7 (0.085ε cf. 0.037ε). The two possible forms of the 6-chloro-5-hydroxy derivative show that:

a) in the phenolic form, position 7 is still open to nucleophilic attack.

b) in the pyridone form position 7 is still reactive but the chlorine atom now has a large free valence of 0.407 and is thus susceptible to free radical attack.

It has been experimentally verified (by polarography) that the chlorine atom is labile under some conditions (personal communication from Dr. Corbett).

The use of the Computer programme has thus thrown some light on the hydroxylation reactions which occur in the benzimidazole series of compounds, but more questions have been posed than have been answered, and a more comprehensive study of the metabolic hydroxylations of these compounds will be a rewarding exercise for the future.

At the present state of the art, prediction of position of hydroxylation is only reasonably certain for some well-defined groups of aromatic compounds. With these limitations in mind, a vast field is presented in which many unrelated facts may be explicable in terms of the simple approach of this programme. Such things as bacterial and mammalian ring scission might be characterised in this way, or a more rigorous

prediction of the possible site of entry of a hydroxyl group, or which conjugate will be preferentially formed on metabolism. It may be that in the near future the data on a compound will be fed into a computer even before the compound is first administered to an animal.

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