

THE METABOLISM OF SOME ARYLARSONIC ACIDS IN THE HEN

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Abstract of Thesis

(1) The fate of 4-nitrophenylarsonic acid, p-arsanilic acid, 4-acetamidophenylarsonic acid and 4-hydroxy 3-nitrophenylarsonic acid in the hen has been investigated.

(2) These compounds undergo few metabolic changes in the hen and are mainly excreted with the arsono substituent intact. Any change in the molecule largely occurs in the second aromatic substituent.

(3) p-Arsanilic acid, administered to hens by mouth or by injection, is excreted unchanged. 4-Acetamidophenylarsonic acid when given orally, is mainly excreted unchanged but after intramuscular administration one third of the dose is eliminated as p-arsanilic acid.

(4) 4-Nitrophenylarsonic acid and 4-hydroxy 3-nitrophenylarsonic acid, given to hens by mouth, are partly reduced to the corresponding aminoarylarsonic acids. A small fraction of the dose is excreted firmly attached to the faecal solids, probably as arsenoxides bound to faecal residues by thiol groups.

(5) The intestinal flora of the hen is capable of reducing nitro and arsono substituents of nitro arylarsonic acids. Evidence is presented suggesting that the in vivo activity of the crop flora can account for the greater proportion of excreted aminoarylarsonic acids after the nitro compounds have been given by mouth.

(6) The contribution of these findings towards an objective assessment of the health risks involved in their agricultural use and towards a better understanding of their growth stimulating action is discussed.

Preface

The work described was carried out between January 1960 and January 1963 in the Department of Biochemistry at St. Mary's Hospital Medical School under the supervision of Professor R.T. Williams.

Since the Second World War there has been a revival of interest in organic arsenicals arising largely from their successful application in animal husbandry as growth stimulants and antiparasitic agents. The subsequent commercial development of these compounds, however, has raised the serious issue as to whether their large scale agricultural application constitutes a serious health risk to the population from the presence of trace quantities of arsenical residues in meat. Although some of the general principles governing the toxicity of arsenicals have been known for many years, the metabolism of these compounds in the body is but poorly understood. As the chemical fate of these compounds and their toxic action are inextricably linked a thorough investigation of the former is essential for any objective assessment of the possible dangers arising from their use.

The first chapter reviews the toxic and parasitocidal effects of arylarsonic acids and summarises present knowledge of their fate in the animal body. A section on the growth stimulating effects has also been included since information on the fate of these compounds may throw light on their mode of action. The following chapters deal with the metabolism of these compounds separately and these findings are brought together in the final chapter for comparison.

It is a pleasure to record my sincere thanks to Professor Williams and the staff for all their help and encouragement during my stay at St. Mary's, to Mr. Audas and the technical staff for their practical assistance and to Dr. Salsbury's Laboratories for the supporting grant and much valuable information. I also wish to express my gratitude to Dr. Robinson and Dr. Todrick for their helpful advice and criticism of the manuscript and to Miss M. Sutherland for her skilful preparation of the typescript.

J. P. Moody

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CHAPTER 1

Introduction.

Compounds of arsenic have been known to mankind for centuries. The naturally occurring sulphide ores were used by the Babylonians as pigments for decorative purposes (Thompson, 1936) and in the times of Hippocrates and Galen, the same compounds were used medicinally for the treatment of ulcers. Fowler (1786) described the therapeutic properties of potassium arsenite in the 18th century and for a time this compound was used medicinally before falling into disrepute. Small quantities of arsenic may have some general tonic properties and beneficial effects on the growth of bone and marrow have been reported by Gies (1877) when inorganic arsenic was fed in small doses to rabbits, chickens and pigs. Moreover, it is interesting to note that in some mountain regions such as Styria (Austria) quantities of arsenic are regularly consumed for its reputed tonic effects.

The parasitocidal properties of inorganic arsenic preparations have been reported by Bruce (1895) and Lingard (1899) but these preparations in therapeutic quantities were too toxic for general medicinal use. This work, however, initiated the developments in arsenical therapy which occurred in the 20th century.

The first organic arsenical which was successfully used against human trypanosomiasis was atoxyl (Thomas and Breinl, 1905), a

compound which had been previously reported inactive against trypanosomiasis in vitro (Ehrlich and Shiga, 1904.). In 1909, Ehrlich and Roehl resolved the difference between the in vivo and in vitro action of atoxyl and suggested that the compound was reduced in the body to the highly active 4-aminophenylarsenoxide. Ehrlich's researches over the next ten years laid the foundation for arsenical therapy and resulted in the development of salvarsan and neosalvarsan, the first successful drugs to be widely used against spirochaete and trypanosome infections. By 1937 the organic chemistry of arsenic had been intensively developed and 8,000 different compounds of arsenic were known.

There have been a number of attempts to apply the known chemotherapeutic properties of arsenicals to the treatment of internal parasitic diseases in animals. Blount (1938) and Bolin and Vardiman (1941) have successfully controlled blackhead in turkeys with intramuscular injections of mapharsen (4-hydroxy 3-aminophenylarsenoxide) and Marcos and co-workers (1946) have treated spirochaetosis in hens with atoxyl and neosalvarsan. Although the results obtained with these compounds were promising, their large scale application was beset with practical difficulties. Trivalent arsenicals of the mapharsen type are often unstable in aqueous solution and unsuitable for use in the food and drinking water of birds. The development of compounds which could be easily and effectively used both therapeutically and prophylactically was therefore desirable.

The modern use of organic arsenicals in animal husbandry stems from the early report of Morehouse and Mayfield (1946). 4-Hydroxy 3-nitrophenylarsonic acid was found to possess coccidiostatic and growth promoting properties when added to the drinking water of turkeys. This was later confirmed by Bird and co-workers (1949) and Morehouse and McKay (1951) in chickens. Subsequently several other compounds were shown to be active growth promoters and antiparasitic agents. p-Arsanilic acid, though only mildly coccidiostatic was also found to possess growth stimulating properties in chickens (Combs, Romero and Bishop, 1954) while 4-nitrophenylarsonic acid prevented or cured blackhead when added to turkey rations (Morehouse and McGuire, 1953). Today, these three compounds account for the greater proportion of organic arsenicals used in animal husbandry.

The commercial use of these compounds has expanded as a result of favourable reports on the effects of arylarsonic acids administered throughout the complete life cycle of birds. In 1954, Morehouse published the results of a field trial in which 4-hydroxy 3-nitrophenylarsonic acid was fed to chickens for one year. Birds receiving this compound in the diet showed greater weight gains and matured earlier when compared with birds receiving no supplement. Lower feed requirements, reduced mortality and improved egg production were also reported. This paper was shortly followed by a report by Libby and co-workers (1955) on the effects of administering p-arsanilic

acid throughout the life cycle of the hen. p-Arsanilic acid was also found to stimulate early growth, improve feed efficiency and increase egg production while mortality was reduced. These observations have since been confirmed by other workers (Price et al., 1956; Thornton and Moreng, 1958).

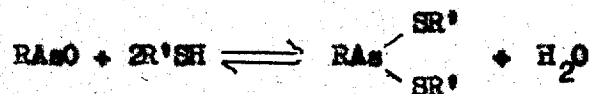
The application of arylarsonic acids to animal husbandry has raised serious problems concerning their safety to persons engaged in their manufacture, handling and use and to the consumer from arsenical residues present in meat. An assessment of the health hazards involved is particularly important since some of the possible metabolites are cumulative and highly toxic. Such an assessment must be firmly based on comprehensive studies of the toxicity and metabolism of these compounds in a number of animals so that the serious risks may be more clearly defined for further investigation. The following sections contain a summary of much of the present day knowledge of the toxicity and metabolism of these compounds and some of the principles governing their parasitocidal action. The metabolism of arylarsonic acids in the body is still not known in detail despite several early investigations and information on the chemical fate of these compounds in the hen is almost non-existent. The object of these studies was to determine the fate of arylarsonic acids in the hen in order to contribute towards an objective assessment of the health hazards involved in their use and towards a better understanding of their action as growth stimulants and antiparasitic agents.

The chemical basis of arsenical therapy.

The chemical basis of the toxic and parasitocidal action of arsenicals has been thoroughly investigated over many years and there are several excellent reviews on the subject (Voegtlin, 1925; Work and Work, 1948; Eagle and Doak, 1951).

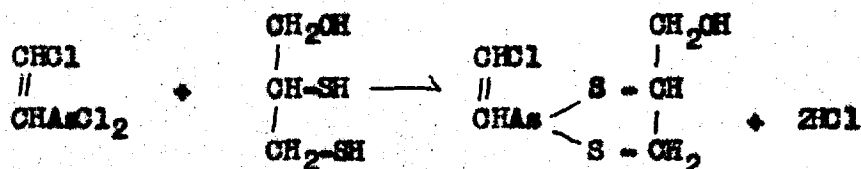
Arsenoxides were suggested by Ehrlich (1908) to be the therapeutically active form of arylarsenicals on the grounds of the greater toxicity of these compounds towards both host and parasite compared with arylarsonic acids and arsenobenzene and the differences between their in vitro and in vivo activity. This hypothesis has been amply supported by the pharmacological and toxicological findings of later workers and there is little evidence to support any alternatives. The conversion of an arylarsonic acid into a toxic arsenoxide in an animal was finally demonstrated by Crawford and Levy in 1947.

The parasitocidal action of arsenoxides has been attributed to their affinity for essential thiol groups with which they react to form arylthioarsenites.



The arsenoxides were originally considered to deprive parasites of essential thiol intermediates such as glutathione (Voegtlin, 1925) but later work has shown that their interaction with the thiol groups of macromolecules is more important. These compounds inhibit many enzyme systems but in many cases the activity may be

restored by monothiols (Barron and Singer, 1943; 1945). The oxidation of pyruvate, however, is inhibited by lewisite and the arsenoxides and in this case the inhibition is only reversed by dithiols. Monothiols are ineffective (Stocken and Thompson, 1946; Barron et al., 1947). The superiority of dithiols over monothiols in restoring pyruvic oxidase activity has been attributed to the formation of highly stable ring compounds of low toxicity (Whittaker, 1947).



As α lipoic acid is an essential co-factor for the enzymes responsible for the oxidation of pyruvate and α keto glutarate, the toxic effects of lewisite and the arsenoxides may be due to their interaction with this co-factor to form arylthioarsenites with a stable six membered ring structure, effectively blocking the tri-carboxylic acid cycle.

Selective toxicity.

Although the basic mechanism for arsenical toxicity may be the same in both host and parasite, the selective effects of these compounds in therapeutic doses requires some explanation. Eagle and co-workers have studied the toxicity of a large number of

arsenoxides in animals and parasites. The dual toxicity of these compounds show wide variations which cannot be attributed to differences in the chemical behaviour of the arsenoso substituent (Eagle et al., 1940). These effects seem to be due to the second aromatic substituents. Doak and Eagle, (1954) have classified these substituents into four groups.

(1) "Inert" substituents ($-\text{Cl}$, $-\text{NO}_2$, $-\text{OH}$, $-\text{CH}_3$, $-\text{F}$.) which have little effect on the dual toxicity of phenylarsenoxide.

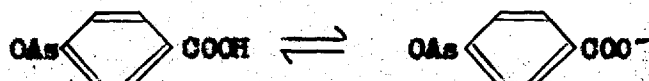
(2) Acidic substituents ($-\text{COOH}$, $-\text{SO}_3\text{H}$.) which reduce the dual toxicity of phenylarsenoxide but the parasitocidal activities are affected to a greater degree.

(3) Amide and ester substituents are generally less toxic than their acid substituted counterparts and have higher parasitocidal activities. The activity/toxicity ratios of these compounds may be several times those of phenylarsenoxide.

(4) Some other substituents ($-\text{NHCOCH}_3$, $-\text{OCOCH}_3$, $-\text{RNHCOCH}_3$, $-\text{RNH}_2$.) do not fall into any of the above groups and may have varying effects on the dual toxicity of phenylarsenoxide.

Secondary substituents may determine the ability of a compound to penetrate trypanosome membranes. There appears to be a general relationship between the activity of arsenoxides and the extent to which they are bound by trypanosomes. Some of the more active compounds may accumulate in trypanosomes in concentrations up to 200 times that of the surrounding medium (Eagle, 1945). Acid substituted

phenylarsenoxides, however, have low parasitocidal activities owing to the low permeability of parasite membranes to these compounds in the ionic state at physiological pH values.



The trypanocidal activity of these compounds increases as the pH of the medium is decreased. This is reflected in the amounts of arsenic bound by the parasites at different pH values. Compounds which do not ionise at physiological pH values do not exhibit these pH dependent effects. Thus, the corresponding ester and amide derivatives have trypanocidal activities which are independent of the pH value of the medium.

Among the acid substituted phenylarsenoxides, phenylbutyric acid-4-arsenoxide and some related compounds appear to be exceptions in their behaviour towards trypanosomes. These compounds are considered to penetrate trypanosome membranes in a different manner to the apparently passive mechanisms encountered with most drugs.

The toxicity of arsenicals.

Information on the toxicity of arsenicals is essential for any assessment of the therapeutic effectiveness of a compound in medicine and agriculture. The differences between the doses or dietary levels which have the greatest therapeutic effect and those which are acutely or chronically toxic give some indication of the margin of safety to

the recipient afforded by these compounds. Moreover, information on the toxicity and tolerance of arsenicals combined with metabolic studies can greatly contribute towards an evaluation of the health hazards involved in their use.

The acute toxicity of organic arsenicals is largely governed by the nature of the arsenic grouping. Secondary aromatic substituents also partly determine the toxicity of a compound but usually to a much lesser degree. The most toxic group of organic arsenicals are the trivalent arsenoxides whose toxicity can be directly related to their action on enzymes in intermediary metabolism. The corresponding arylarsonic acids and arsenobenzenes are far less toxic and largely depend on their conversion in the body into arsenoxides for their toxic properties.

The toxicity of arsenicals is affected by the route of administration. These differences in toxicity are most apparent when oral and systemic tolerances are compared. Fulton and Yorke (1943) found that a reduced tryparsamide (N-phenylglycinamide-4-arsenoxide) was tolerated by mice in oral doses of 8 mg. while only 2 mg. was tolerated by injection. Similar results were obtained with tryparsamide (mono sodium N-phenylglycinamide-4-arsenate). 60 Mg. was the upper tolerance limit when given by injection whereas twice that dose could be given by mouth.

Some animals are more susceptible towards arsenicals than others. Frost, Overby and Spruth (1955) observed that orally

administered arsenicals are more toxic to rats than to chickens and ducks. The tolerance pattern of two of the species (rats and chickens) towards six arsenicals, however, remained basically similar. The same phenomenon has been found in mice and rabbits (Doak and Eagle, 1951).

The retention of organic arsenicals in the tissues of the host reflects their toxicity and limits the extent and duration of their parasitocidal effects. Thus, the most toxic compounds, the arsenoxides, are excreted much more slowly than the arsenobenzenes and arsonic acids (Voegtlin and Thompson, 1922). This relationship between toxicity and excretion has also been found in a number of arsenoxides of widely varying toxicity (Hogan and Eagle, 1944). Highly toxic compounds such as phenylarsenoxide (LD_{50} 0.8 mg./Kg.) when administered to rabbits intravenously are excreted very slowly. Only 20% of the dose may be recovered in the excreta in five days. Mapharsen (LD_{50} 13.3 mg./Kg.), on the other hand is much less toxic and is almost quantitatively eliminated in three to four days.

The toxicity of arsenicals is also related to their uptake by tissues and organs. Hogan and Eagle (1944) found that a number of arsenicals were bound by red blood corpuscles in vitro and in vivo in proportion to their systemic toxicity. Arsenoxides were rapidly taken up by red cells in vitro whilst arsenobenzenes and arsonic acids were only bound to a minor extent. The relationship between tissue binding and toxicity was illustrated in vivo by administering three

compounds (phenylarsenoxide, phenylarsonic acid and tryparsamide) to rabbits at their LD₅₀'s and after 24 hours measuring the tissue arsenic concentrations. Despite a 500 fold difference in the dosage level, these compounds gave comparable levels of arsenic in the tissues. A fourth compound, 4-carboxylphenylarsenoxide, was a noticeable exception and when administered intravenously to rabbits at its LD₅₀, this compound gave rise to much lower arsenic concentrations. 4-Carboxylphenylarsenoxide differs from other arsenoxides in several respects. It is bound by red cells in vitro to only a minor degree and after administration to the rabbit, it is initially excreted very rapidly. Eagle has suggested that it is metabolised in the body to more toxic compounds, accumulating in the kidney causing damage to the organ. This is partly supported by observations of the sharp decrease in the initial rate of excretion of the compound and on the delayed time of death, compared with other arsenoxides, when administered in lethal doses. The metabolism of this compound in animals, however, still remains to be investigated.

For most arsenoxides, there is a direct link between their toxicities and the extent to which they are bound by the tissues. These residues are probably bound in the trivalent form to tissue proteins through thiol groups and may be in equilibrium with free arsenoxides. Such an equilibrium would control the release of these compounds in the body and could determine their rate of excretion. As the more toxic compounds are generally those which are most firmly

bound by the tissues, they would appear to hold most dangers to fattening stock and to man through possible cumulative effects from chronic administration.

The chemical fate of arylarsonic acids in the body.

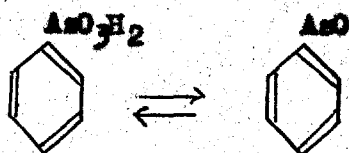
The fate of organic arsenicals in the animal body is not extensively known despite numerous investigations and precise information on the chemical changes undergone by these compounds is meagre. The metabolism of these compounds has already been reviewed by Williams. (1959).

The arsonic acids when injected into the rabbit rapidly disappear from the blood stream and enter the major tissues and organs. After the intravenous administration of tryparsamide, Hogan and Eagle (1944) found high concentrations of arsenic in the tissues one hour after dosing. Liver, kidney, bone and muscle contained the highest arsenic concentrations and significant amounts of arsenic were also found in the brain. Tryparsamide was not retained in the tissues to any significant degree and most of the administered dose was quickly eliminated in the urine. Four hours after the administration of the compound, the distribution of arsenic in the tissues was radically altered and the highest arsenic levels were located in the kidneys. After 24 hours, only traces of arsenic were present in the major tissues. Similar results have been reported when phenylarsonic acid and 4-hydroxy-3-aminophenylarsonic acid were administered intravenously to rabbits

(Chance, Crawford and Levvy, 1946). Though these compounds were rapidly excreted, moderate quantities of arsenic were present in the liver and kidney, one hour after dosing. However, after 24 hours, these tissues contained only traces of arsenic.

Little is known concerning the chemical fate of arylarsonic acids in the human body. The early workers considered these compounds to be degraded to inorganic arsenic but lately, most authors have subscribed to the view that these compounds are largely excreted unchanged.

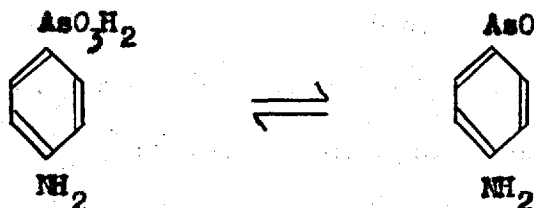
Phenylarsonic acid, a compound with no secondary aryl substituents, was ideally suited for investigating the chemical changes undergone by the arsono group in the body. This compound when administered intravenously to rabbits at dose levels of 1 - 5 mg.As/Kg. was fairly rapidly eliminated (Chance, Crawford and Levvy, 1946). 68 - 83% of the dose was excreted in the urine in 24 hours and only 0.2 - 2.5% in the faeces. Most of the administered dose was excreted unchanged and only small quantities of phenylarsenoxide (less than 10%) were present (Crawford and Levvy, 1947). Furthermore, no inorganic arsenic could be detected in the excreta. This, in fact, was the first chemical evidence of the reduction of arsonic acids to arsenoxides in the body.



The metabolite, phenylarsenoxide, administered to rabbits at 0.45 mg.As./Kg. was excreted very slowly and only 25% of the dose was recovered in the excreta in seven days. Most of the excreted dose was found in the urine but approximately one fifth occurred in the faeces. In three days 6 - 16% of the dose was recovered, half of which was in the form of phenylarsonic acid. No inorganic arsenic was present.

Phenylarsonic acid and phenylarsenoxide, therefore, undergo interconversion in the rabbit but neither compound is degraded to inorganic arsenic. Both these compounds are more toxic than most other arsonic acids and arsenoxides and this is probably due to the relative ease with which phenylarsenoxide penetrates cell membranes (Eagle, 1945) and to the prolonged retention of the compound by the tissues. The slow excretion of phenylarsenoxide and hence its high toxicity, has been attributed to its slow oxidation to the more readily excreted phenylarsonic acid (Chance, Crawford and Levy, 1945) but in view of the considerations outlined in the previous section it would appear more likely that the retention of this compound in the tissues controls its rate of metabolism.

The sodium salt of p-arsanilic acid (atoxyl) and its acetylated derivative, mono sodium 4-acetamidophenylarsonate (arsacetin) were two of the earliest drugs to be used in the treatment of trypanosomiasis. The activity of these compounds is due to their conversion to arsenoxides in the body.



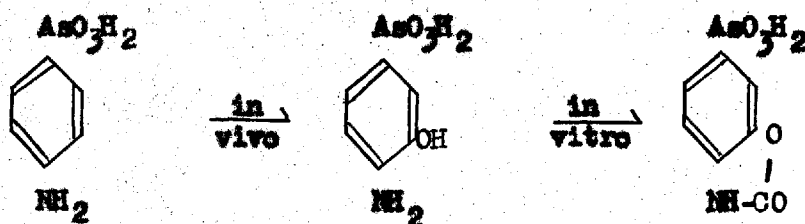
This has been demonstrated in vitro by Yamanouchi (1910) and Terry (1915). Atoxyl incubated anaerobically with red blood cells or liver tissue was converted into a therostable and alcohol soluble trypanocidal substance while fresh tissue alone possessed no trypanocidal activity.

Voegtlin and Thompson (1922) found that atoxyl and arsacetin administered intravenously to rats were almost quantitatively excreted in the urine in 24 hours and a ten fold increase in the dose level had little effect on the rate of elimination. The faecal excretion of these compounds was small and only 5% of the dose was eliminated by this route. This pattern of rapid urinary elimination was repeated when 50 mg. doses of atoxyl were administered to rabbits and to man. (Ingersheimer and Rothmann, 1909).

The chemical form in which atoxyl is excreted, has received a considerable amount of attention. Nierenstein (1909) has isolated three compounds (p-arsanilic acid, 4-hydroxyphenylarsonic acid and 2-ombenzoxazole-6-arsonic acid) and appreciable quantities of inorganic arsenic from the urine of horses which had received twice weekly injections of 1 - 2 gm. atoxyl. These results must be treated with some caution in view of the questionable purity of the starting material

and the harsh isolation procedures used. The inorganic arsenic in the urine probably originated from the breakdown of organic arsenicals during the isolation procedure and not from the biological oxidation of atoxyl since the expected aniline metabolites, p-acetamidophenol and p-acetamidophenyl sulphate, have not been detected in the urine (Blumenthal and Herschmann, 1909). 4-Hydroxyphenylarsonic acid is unlikely to have been a metabolite of atoxyl and may have been an impurity in the administered compound. The benzoxazolonearsonic acid is probably an artifact arising from 4-amino 3-hydroxyphenylarsonic acid in the urine during the isolation procedure.

It is possible, therefore, in the horse that atoxyl is hydroxylated in the meta position to form 4-amino 3-hydroxyphenylarsonic acid.



p-Arsanilic acid appears to be excreted unchanged in man and the hen (Lookmann and Pauke, 1908; Overby and Fredrickson, 1963).

The salts of 4-N-glycolylarsanilic acid are used in the treatment of amoebiasis and are among the least toxic of the arylarsonic acids. The bismuthoxy salt (Milibis) administered by mouth to man or the rat, is mainly excreted in the faeces and only small

quantities of the administered arsenic and bismuth are found in the urine (McChesney and Hoppe, 1950). The sodium salt is also poorly absorbed from the gut and is excreted unchanged in the rat, cat or man. Only 5 - 10% of an oral dose was eliminated in the urine by the rat and less than 5% by man in three days. This compound, however, was rapidly eliminated in the urine of the rat when administered parenterally (McChesney, Hoppe, McMuliff and Banks, 1962).

Monosodium N-phenylglycineamide-4-arsenate (Tryparsamide) was once extensively used in the treatment of sleeping sickness and neuro syphilis because of its supposed superior ability to pass the blood-brain barrier. Later workers have tended to doubt the efficacy of this compound in the treatment of these diseases (Hinrichsen, 1939). Like many other arsenic acids, tryparsamide is rapidly excreted in the urine when administered to the rabbit or man by injection and the dose is almost quantitatively recovered in the urine in 24 hours (Voegtlin and Thompson, 1922; Young and Muehlberger, 1924). The detailed metabolism of tryparsamide is not known but Young and Hamilton (1924) have isolated a substantial quantity of the unchanged material from the urine of men who had received the compound intravenously.

The fate of 4-hydroxy 3-aminophenylarsonic acid in the body is not known but the administered dose of arsenic is rapidly excreted in the urine after the compound is administered by injection to rats and rabbits (Voegtlin and Thompson, 1922; Chance, Crawford and Levy, 1946).

The corresponding arsenoxide, mapharsen, is excreted much more slowly after injection and greater proportions of the excreted dose of arsenic are found in the faeces. Both the dog and man also excrete mapharsen slowly. Over half of the injected dose is eliminated in one week but the dog excretes the administered dose of arsenic predominantly in the faeces whereas man excretes the arsenic predominantly in the urine (Gruhsit, et al., 1936; Foerster, et al., 1935). The fate of mapharsen is not known in detail. In the dog, the compound appears to be metabolised to other compounds and the available evidence suggests that the arsenoso substituent is cleaved from the aromatic nucleus (Maren, 1949).

4-Hydroxy 3-acetamidophenylarsonic acid (acetarsons) and 4-ureidophenylarsonic acid (carbarsone) are two arsenicals which were once used in the treatment of amoebiasis. Chen, Anderson and Leake (1930) found that both these compounds are poorly absorbed from the gut. Only 7 - 13% of the dose could be recovered in the urine in two days when carbarsone was given to man orally in 0.5 gm. doses. Acetarsons was absorbed from the gut to a greater extent and under the same experimental conditions 8 - 20% of the dose was excreted in the urine in 24 hours. The poor alimentary absorption of acetarsons in man has been confirmed by other workers (Tomita, 1939; Ferrari, 1945). When this compound was administered intramuscularly in doses of 1 - 2 gm. it was quickly excreted in the urine. 90% of the dose of arsenic was excreted in six hours and 96% in two days (Cavies, 1946).

In contrast to the arylarsonic acids the arsenoxides have a great affinity for the major body tissues and organs and may be retained by the body long after the compound has been administered. Wright and co-workers (1937) found considerable quantities of arsenic in liver, kidney and muscle shortly after giving intravenous doses of mapharsen to the rat and these tissue concentrations persisted for at least two hours. The highest arsenic levels were found in the liver and kidney but the muscle though containing lower arsenic concentrations, was the largest reservoir of arsenic.

Little is known concerning the chemical fate of arsenoxides in the body. Maren (1949) has administered 4-carbamyl 3-aminophenyl-arsenoxide and mapharsen to the dog. The former compound appears to be relatively stable in the blood stream up to 12 hours after administration while the latter is apparently degraded to inorganic arsenic and other products. The slow excretion of some arsenoxides such as phenylarsenoxide may be limited by their rate of oxidation to the more easily excreted arsenic acids, but their retention by tissue proteins is probably the more important factor. Other compounds such as the carboxyl substituted phenylarsenoxides are initially rapidly excreted but exert a delayed toxic effect. The evidence of Hogan and Eagle (1944) suggests that these compounds may be metabolised to more toxic derivatives and that they exert different toxic effects from the other arsenoxides. The time relationships between toxicity, rate of excretion and metabolism of these compounds must await more detailed investigations.

Some general conclusions can be drawn from the limited information available from studies on the metabolism of arylarsonic acids. These compounds have certain physico-chemical properties in common with some of the "biochemically inert" compounds described by Williams (1954). They are strong electrolytes and are poorly soluble in non-polar solvents and could be expected to be poorly absorbed from the gut when given by mouth and rapidly eliminated in the urine when given by injection (see Chapter 6). Table 1. shows that for the compounds investigated, this in fact occurs. These compounds seem to undergo few metabolic changes in the body and are mainly excreted unchanged. A small part of the dose, however, may be reversibly converted into arsenoxides which may accumulate in the tissues but this has only been confirmed for phenylarsonic acid. As such small scale metabolic changes may result in the formation of compounds of greatly increased toxicity and parasitocidal activity, their significance should never be underestimated.

The action of arsenicals in stimulating growth.

The biological effects of arylarsonic acids on farm animals has stimulated considerable interest in their possible mode of action and several theories have been advanced to explain these effects. Most theories have tried to relate the action of arsenicals to their known parasitocidal and bactericidal properties either through (a)

Table 1

The rate of excretion of arylarsonic acids

Compound	% Urinary excretion after oral administration	% Urinary excretion after injection
Phenylarsonic acid (3 - 15 mg./Kg. rabbits) ¹		25 - 80 (24 hrs.)
Tryparsamide (2 gm., man) ²		88 - 95 (24 hrs.)
4-N-glycolylarsanilic acid (rats) ³	5 - 10	75
Garbarsone (0.5 gm., man) ⁴	8 - 13 (42 - 52 hrs.)	
Acetarsonsone { (0.5 gm., man) ⁴ (1 - 2 gm., man) ⁵	7 - 20 (22 - 24 hrs.)	90 (6 hrs.)

1. Crawford and Levy (1947).

3. McChesney et al. (1962).

2. Young and Muehlberger (1924).

4. Chen, Anderson and Leake (1930).

5. Cavier (1946).

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improved nutrition resulting from changes in the populations of the gut flora or through (b) the reduction of subclinical disease levels from the suppression of pathogenic micro-organisms. Both ideas are supported by some experimental evidence.

Arylarsonic acids, however, are not directly bactericidal but must first be converted into arsenoxides (Albert, Falk and Rubbo, 1944) and if either of the above theories is correct, these compounds must be formed in the tissues of the animal or in the lumen of the gut.

Experiments on the effects of arylarsonic acids on the nutritional requirements of animals lend some support to the nutrition theory. The effects of arsonic acids on vitamin requirements have been examined by several workers (Table 2). Only thiamine has been shown to be spared by these compounds. Other vitamin requirements remain unaffected or may even be increased. Protein requirements and utilization are affected by arsenicals in the diet. p-Arsanilic acid seems to spare the protein requirements of laying hens (Carlson 1957; Pope and Schaible 1959) improving egg production when the diets contain suboptimal quantities of protein. 4-Hydroxy 3-nitrophenylarsonic acid, on the other hand, does not spare the protein requirements of chickens but improved utilization at the higher dietary protein levels have been recorded (Day, West and Hill, 1958).

Differences in rearing conditions also affect the growth

Table 2

The effects of arylarsonic acids on the vitamin requirements of poultry

Compound	Vitamin B ₁₂	Thiamine	Pantothenic acid	Riboflavin	Vitamin K
p-Arsanilic acid	no effect ¹	spared ³	no effect ³		increased ⁵
4-Hydroxy 3 nitro phenylarsonic acid	no effect ²			increased ⁴	increased ⁵

1. Frost and Spruth (1953).

3. Abbot, Bird and Cravens (1954).

2. Bird, Groschke and Rubin (1948).

4. Slinger, Pepper and Evans (1955).

5. Griminger (1953).

responses of poultry to arsenic acids. As the environment may significantly affect the incidence of disease in poultry flocks, growth stimulation caused by these compounds in conditions with a large disease potential may be greater than comparative growth stimulation in a more favourable environment.

Pepper and Slinger (1955) report smaller responses to 4-hydroxy 3-nitrophenylarsonic acid from turkey poults reared on slotted porches than from birds reared in deep litter but as the weight gains of the deep litter controls were greater than those of the controls raised on slotted porches, the disease load in deep litter does not seem to be a major factor affecting development. In chickens, however, the growth stimulation caused by arsenicals is greater for birds raised in batteries than for floor raised birds (Libby et al., 1955).

Morrison, Hunsaker and Aitken (1954) have observed environment dependent differences in the growth responses of chickens to 4-hydroxy 3-nitrophenylarsonic acid which suggest that subclinical disease levels may affect the rate of gain. Highly significant growth responses were obtained in an "old" environment used continuously for the rearing of chickens while the responses obtained in a "new" environment which had been empty for six months and disinfected, were considerably less.

The continuous use of antibiotics and arsenicals in the same environment may reduce the disease load over several years.

Libby and Schaible (1955) compared the growth responses of chickens to these compounds over several years and observed a progressive diminution while the average rate of gain of control birds increased. Furthermore, over the same period of time the mortality rate was significantly reduced. Heth and Bird (1962), however, failed to confirm these findings.

Antibiotics and arsenicals in feeds cause changes in the populations of the gut microflora and attempts have been made to relate these changes to growth responses. Decreases in the faecal Clostridia counts of chickens and pigs have been recorded which may be associated with weight gains produced by growth stimulants (Elam, et al., 1953; Larson and Carpenter, 1952).

Anderson, Cunningham and Slinger (1952) have related growth stimulation in turkey poults to decreases in the pH of the caecal contents and increases in the caecal Lactobacilli populations. Other bacterial populations showed no consistent changes associated with weight gains.

These results have been criticised by Eysen, De Prins and De Somer (1962). Many growth stimulants are absorbed or inactivated in the middle regions of the gastrointestinal tract and cannot directly influence the microflora populations in the hind gut. On administering virginiamycin as a growth stimulant to chickens, these workers observed decreases in the Lactobacilli populations of the crop which they associate with growth responses.

Arsenic acids and antibiotics produce morphological changes in the small intestine of chickens. Coates, Davies and Kon (1955) found that p-arsanilic acid and antibiotics reduce the thickness of the intestinal wall of chickens to proportions similar to those of germ free birds. These workers have suggested that the reduction in the thickness of the gut wall may improve food absorption, thereby increasing feed efficiency and stimulating growth.

There is still insufficient information to define with any precision a mode of action of arsenicals and antibiotics in stimulating growth. Considerations of the relationships between dietary and environmental factors and growth responses suggest that these compounds improve the nutrition of animals and reduce their susceptibility to disease but the biological targets of action still remain to be clarified. Most workers agree that the growth responses elicited by these compounds are related to changes in the microflora populations but opinions differ as to which organisms are specifically affected. It appears that progress in this field must still await their conclusive identification.

Health hazards.

The introduction of organic compounds in industry, medicine, agriculture or the household are always accompanied by some degree of risk to health which can never be predicted with complete certainty. In the toxicological field, there is always a possibility that a given compound may have untoward effects in man which are absent in other

animals, arising from man's greater susceptibility to a compound or from man's diminished ability to render a compound harmless. Dangers from adverse effects on fertility or from the teratogenic, mutagenic or carcinogenic action of compounds are particularly severe since in man, these symptoms of toxicity may only occur after several months or years of exposure. Nevertheless, the toxic action of chemical compounds are more commonly shared by several species and a thorough investigation of their toxic properties in a wide range of animals can be expected to reveal most hazards.

The widespread use of organic arsenicals in agriculture raises serious problems concerning their safety to persons involved in their manufacture and handling and to the population from the consumption of arsenic residues in meat. Dangers could arise from the accumulation of metabolites bound to the tissues which may be far more toxic to man than to other animals, or from the possible degradation of organic arsenicals in the body to inorganic arsenic, a cumulative poison and a suspected carcinogen (Crispens, 1961). Both these issues must be carefully considered in any objective assessment of the health risks arising from the agricultural application of these compounds.

Arsenic acids differ widely in their acute and chronic toxicities (Table 3). For example, p-arsanilic acid (LD_{50} oral, 800 mg./Kg.) is well tolerated by the chicken in feed concentrations up to 1000 ppm. while 4-hydroxy 3-nitrophenylarsonic acid (LD_{50} oral,

Table 3

Acute and chronic toxicity of p-arsanilic acid
and 4-hydroxy 3-nitrophenylarsonic acid

Compound	Animal	LD ₅₀ (oral)	Dietary level producing symptoms of toxicity	Dietary level producing some mortality
p-Arsanilic acid (100 ppm. in feeds)	Hen	800 mg./Kg.	1500 ppm. ¹	2000 ppm. ¹
	Turkey		500 " ²	1000 " ²
	Pig		1500 " ³	
	Dog		400 " ⁴	
4-Hydroxy 3-nitro- phenylarsonic acid (50 ppm. in feeds)	Hen	100 - 120 mg./Kg.	200 " ⁵	400 " ⁵
	Pig		100 " ⁶	
	Dog	50 mg./Kg.	100 " ⁴	
	Rat	155 mg./Kg.		400 " ⁵

1. Abbot, Bird and Cravens (1954).

2. Frost and Spruth (1953).

3. Oliver and Roe (1957).

4. Frost, Overby and Spruth (1955).

5. Kerr, Cavett and Thompson (1963).

6. Carpenter (1954).

120 mg./Kg.) is much more toxic and some symptoms of toxicity may occur when 200 ppm. are included in the diet. 4-Nitrophenylarsonic acid (MTD oral, > 100 mg./Kg.) seems to be of intermediate toxicity and is well tolerated in feed concentrations of 240 ppm. Very little appears to be known about interspecies differences in chronic toxicity, but high levels of p-arsanilic acid are tolerated better by chickens than by turkeys (Frost, Overby and Spruth, 1955). Similarly high dietary levels of 4-hydroxy 3-nitrophenylarsonic acid are slightly better tolerated by chickens than by rats (Kerr, Cavett and Thompson, 1963).

In agriculture, however, p-arsanilic acid and 4-hydroxy 3-nitrophenylarsonic acid are added to feeds in concentrations of 100 and 50 ppm. respectively. These levels are well below those which are chronically toxic and afford reasonable margins of safety to poultry when either compound is used commercially.

The accumulation of arsenic in the tissues and organs of animals receiving arsenicals in the diet has been investigated by several workers. Frost (1953) found that arsenic deposition in the tissues of cockerels was proportional to the feed concentration of p-arsanilic acid. After receiving the compound in the diet for ten weeks, the highest arsenic concentrations were found in the liver and the lowest in leg and breast muscle. On removing the supplement from the diet, liver arsenic levels decreased rapidly over a five day period. Muscle levels also declined but to a lesser extent.

Abbot and co-workers (1955) (1956) have fed p-arsanilic acid to hens at feed concentrations up to 2,250 ppm. and even at these levels, the arsenic concentrations in the muscle were within the limits legally permitted for arsenic residues in foods in the United States. Penicillin given as an additional supplement increased the deposition of arsenic in the tissues but the effect seems to be slight.

The carry over of arsenic to eggs from laying hens given p-arsanilic acid in the feed has been examined by Evans and co-workers (1953). Initially, very little arsenic was deposited in eggs and on continuing the diet for 14 weeks, the arsenic content of eggs declined to even lower amounts ($< .001$ ppm. As.)

Similar results have been reported for 4-hydroxy 3-nitrophenyl-arsonic acid. When this compound was included in the diet of chickens for nine weeks at 50 ppm. small amounts of arsenic were found in liver and muscle and these declined to lower concentrations after the supplement had been withdrawn for five days (Milligan, et al., 1955). Reports from Dr. Salsbury's Laboratories support these findings and also confirm there is very little carry over of arsenic from the hen to the egg.

The distribution of arsenic in the tissues of pigs reared on diets containing 33 - 100 ppm. p-arsanilic acid has been determined by Hanson and co-workers (1955). Only small amounts of arsenic were retained in the tissues, the highest concentrations occurring in the

liver (3.4 ppm. As_2O_3) and lesser concentrations in kidney and muscle. Seven days after the supplement had been withdrawn, liver arsenic concentrations had fallen to 1.4 ppm. As_2O_3 and other tissue levels to 0.1 - 0.2 ppm. Similar arsenic concentrations have been observed when low levels of 4-hydroxy 3-nitrophenylarsonic acid were included in the diet of pigs and these also declined after the supplement had been withdrawn (Hanson, Hill and Ferrin, 1956).

Superficially, the hazard to public health arising from the carry over of arsenic from eggs and carcasses does not appear to be very great. When arylarsonic acids are administered to animals at the feed concentrations recommended by the manufacturers these tissue arsenic levels after several weeks supplementation are usually below 3.5 ppm. As_2O_3 , the maximum permitted level for arsenic residues in fruit in the United States. If the supplement is withdrawn according to the manufacturer's recommendations, five days before slaughter, the tissue arsenic concentrations are usually below 0.3 ppm. for muscle and 1.5 ppm. for liver. Thus a single meal of 100 gm. of liver containing these tissue arsenic concentrations would only increase the arsenic load to 150 μg . above the normal arsenic intake. It is interesting to note that many ocean fish are regularly marketed with tissue arsenic concentrations in excess of 1 ppm. and that some shellfish and crustacea contain up to 174 ppm. arsenic in the edible tissues (Frost, 1953).

It is unlikely that inorganic arsenic spray residues on fruit,

arsenic residues in shellfish and organic arsenical residues in meat constitute identical toxicological hazards. Consequently the use of a single permissible limit to include all arsenic residues in food can only serve as a rough guide as to the quantities of arsenic it is safe to consume.

To define the health risks more precisely, it is necessary to identify the chemical forms in which tissue bound arsenical residues occur and to determine their acute, chronic and subchronic toxicities. As a first step, a study of the hitherto unknown chemical fate of arylarsonic acids in poultry is highly desirable. The hen possesses several oxidative reductive and synthetic mechanisms for dealing with foreign compounds (Baldwin, Robinson and Williams, 1959; Crowle and Sherwin, 1925) and arylarsonic acids may undergo a variety of conversions. Some of these changes (e.g. reduction of the arsono substituent) may result in the formation of compounds with greatly increased toxicity. Even minor metabolic pathways may therefore be of toxicological significance. The object of the following studies was to determine the major and minor pathways of metabolism for arylarsonic acids in the hen in order to clarify some of the public health issues and to contribute towards a better understanding of their biological action.

CHAPTER 2

Methods.

Arsenicals.

4-Hydroxy 3-nitro-, 4-nitro- and $[1 - ^{14}\text{C}]$ 4-nitrophenyl-
arsonic acid (0.1 mCi/gm.) were supplied by Dr. Salsbury's Laboratories.

Phenylarsonic acid and p-arsanilic acid were purchased from
B.D.H. Ltd., and recrystallised from hot water until chromatograph-
ically pure.

4-Acetamidophenylarsonic acid was prepared from p-arsanilic
acid by acetylation and was purified by recrystallization from water
and finally from ethanol.

The following compounds were prepared according to the
literature:-

4-Hydroxyphenylarsonic acid (Barrowcliff, Pyman and Reafry,
1908), 4-hydroxy 3-aminophenylarsonic acid (Jacobs, Heidelberger
and Rolf, 1948), 4-hydroxy 3-acetamidophenylarsonic acid (Raiziss
and Gavron, 1924), 4-nitrophenylarsenoxide (Bart, 1910), 4-amino-
phenylarsenoxide (Ehrlich and Bertheim, 1910), 4-acetamidophenyl-
arsenoxide, m.p. $275 - 284^{\circ}\text{C}$ (Cohen, King and Strangeways, 1934),
4-hydroxy 3 nitrophenylarsenoxide (Bart, 1911), 4-hydroxy 3-amino-
phenylarsenoxide (Cassella, 1927), 4-hydroxy 3-acetamidophenyl-
arsenoxide (Cassella, 1927).

Hens.

Hens used were thoroughbred Brown Leghorn pullets (1.1 - 1.8 Kg.) obtained when six months old from the A.R.C. Poultry Research Centre, Edinburgh. For work on 4-hydroxy 3-nitrophenylarsonic acid, Rhode Island Red x Light Sussex laying hens (2.2 - 3.6 Kg.) were obtained commercially.

All hens were housed in wire floored batteries and maintained on a diet of 100 - 150 gm./day "Range Layers Mash" (R. & W. Paul, Ltd., London) with the addition of 50 gm. of a mixture of equal parts of bran, rolled oats and maize. The birds had free access to water and oyster shell grit.

Administration of compounds.

(1) Arsonic acids.

For oral dosing, weighed amounts of arsonic acids were dissolved in 25 ml. 0.1 N. Na_2CO_3 and administered by crop tube. The container was rinsed with 10 ml. of water and the washings were also fed to the hen.

When the compounds were administered intramuscularly, a given weight of the arsonic acid was brought into solution with the addition of solid sodium bicarbonate. The pH was adjusted to 7.4 and the solution was diluted to give the desired final concentration (18.9 - 50 mg./ml.). The solutions were injected into the breast muscle of birds using a 2 ml. graduated syringe.

(2) Aureomycin.

Aureomycin hydrochloride (Lederle Laboratories) was given orally in 50 mg. pellets. For intramuscular administration, a 50 mg. pellet was powdered in 2 ml. distilled water and injected into the breast muscle using a No. 15 needle.

(3) Polyethylene glycol.

Known weights of polyethylene glycol, M.Wt. 4,000, (L. Light and Co. Ltd.) were dissolved in 25 ml. 0.1 N. Na_2CO_3 containing 4-hydroxy 3-nitro- or 4-nitrophenylarsonic acid and administered by crop tube.

Collection of excreta.

For experimental purposes, hens were placed in 23 in. x 16 in. x 16 in. metabolism cages with 0.6 in. mesh wire floors. The excreta was collected in metal funnels leading into 250 ml. wide necked flasks. 5 ml. $2\text{N. H}_2\text{SO}_4$ and 2 ml. chloroform were added to each collecting flask to prevent bacterial growth and to inhibit the oxidation of any arsenoxides which may be present (Crawford and Levvy, 1947). These conditions reduced any further chemical changes of the arsonic acids in the excreta to a minimum.

Total urine and faeces collections were made daily for 24 hours before administration of the compound and at 12 or 24 hour intervals after dosing. To obtain as quantitative a recovery of material as possible, the cage floor and funnel were washed with

water and the washings added to the collecting flask. Combined excreta and washings were homogenized for 2 minutes in the M.S.E. Atomix and centrifuged in 250 ml. bottles (1,800 r.p.m. for 10 minutes). The supernatant was decanted and the residue washed with water and recentrifuged. The supernatant and washings were combined for analysis and the solid residue was dried with acetone and analysed separately.

The excreta of hens consists of a semisolid mixture of urine and faeces. In order to reduce the solid matter in the excreta, food was withheld from the birds for 24 hours prior to dosing and then, in the case of 4-nitrophenylarsonic acid, for the duration of the experiment up to 3 days. (Baldwin, Robinson and Williams, 1959). If an experiment lasted longer than 3 days, access to food was allowed after this period. Under these conditions 70 - 100 ml. of liquid urine per day were obtained with a dry solid content of 0.5 - 3.9 gm.

In the cases of p-arsanilic acid, 4-acetamide - and 4-hydroxy 3-nitrophenylarsonic acid, access to small quantities (about 50 gm.) of poultry mash was allowed in most experiments after the initial 24 hour starvation. Any deviations from these practices are described in Chapters 3, 4 and 5.

The excreta thus obtained was usually coloured green owing to the presence of bile pigments. After oral administration of

arsonic acids, the output of these pigments was greatly increased.

Glass cannulas (Hart and Essex, 1942) were used in one experiment to collect urine samples for short intervals. After administration of the compound, the glass bulb of the cannula was greased with vaseline and inserted into the cloaca. The bulb blocked the rectum but an opening lay directly underneath where the ureters entered the cloaca. Urine samples were collected directly from the cannula into 3 in. x $\frac{1}{2}$ in. sample tubes. Since the birds tended to expel the cannula after 5 - 10 minutes, the method could only be used qualitatively.

Radiochemical techniques.

(1) Counting.

Hen urine and faeces homogenates and supernatants were counted on 2.5 cm. inverted nickel plated iron planchets at infinite thickness in a Geiger-Müller end window counter. Routine determinations of background, standard perspex reference (1 μ C/gm. Radiochemical Centre, Amersham), and of samples were determined in turn throughout the period of use, in order to correct for any changes in the sensitivity of the instrument. Counts were corrected for dead time and after subtraction of the background, samples were standardized against the perspex source.

Solutions of $[1 - ^{14}\text{C}]$ 4-nitrophenylarsonic acid consistently counted lower than theoretical ones for solid dilutions. Consequently

all solutions assays were multiplied by 1.034 to correct for this.

Dried solid faecal residues were ground in glass mortars with acetone and plated out on 2.5 cm. planchets. These samples were dried in air and finally under an infrared lamp and counted against the same standard.

Dry isotope dilution samples were ground in agate mortars with petroleum ether (b.p. $60^{\circ} - 80^{\circ} \text{C}$) and plated out on 1 cm. concave nickel plated iron planchets. These samples were dried in air and finally under an infrared lamp. All samples were standardized against a $1 \mu\text{C/gm.}$ perspex reference of the same dimensions.

For most determinations the standard error was $\pm 5\%$ but for samples of low specific activity this would have entailed counting for very long periods. In these cases the samples were counted for as long as was practicable.

(2) Isotope dilution methods.

(a) 4-Nitrophenylarsonic acid 1.5 - 3 gm. of carrier 4-nitrophenylarsonic acid in 5 ml. 2N. NaOH were dispersed in 10 - 30 gm. of the excreta supernatant made alkaline with 1 ml. 40% NaOH.

After standing for 15 minutes the mixture was centrifuged (2,000 r.p.m. for 20 minutes) and the supernatant neutralized with conc. HCl., charcoaled and acidified to precipitate the nitroarsonic acid which was filtered. This was recrystallized from water and

ethanol to constant specific activity and finally converted into the monobenzylamine salt.

Monobenzylamine 4-nitrophenyl-arsenate was prepared by adding an equivalent amount of benzylamine to a solution of 4-nitrophenyl-arsonic acid in hot ethanol. The salt separated on keeping for a short time and was recrystallized from hot water. It formed cream coloured platelets, m.p. 231°C , insoluble in ethanol but soluble in hot water.

(Found:- As, 20.85%; $\text{NO}_2\text{C}_6\text{H}_4\text{AsO}_3\text{H}_2$, 69.8%; N, 7.6%.
 $\text{C}_{15}\text{H}_{15}\text{O}_5\text{N}_2\text{As}$ requires:- As, 21.15%; $\text{NO}_2\text{C}_6\text{H}_4\text{AsO}_3\text{H}_2$, 69.7%;
 N, 7.9%.)

(b) p-Arsanilic acid. This acid was added to the excreta supernatant as described above for the nitro compound. After filtration from charcoal, the filtrate was adjusted to pH 3 - 4 with conc. HCl. Arsanilic acid separated and was recrystallized to constant specific activity from water and from ethanol. The compound was then converted into N-(phenyl-4-arsonic acid) glycine diethylamide (Jacobs and Heidelberger, 1949). This was recrystallized from water to constant specific activity. (M.p. and mixed m.p. $199 - 201^{\circ}\text{C}$.)

A sample isotope dilution is given in Table 1A.

(3) Autoradiography.

Paper chromatograms of the excreta of hens fed [1 - ^{14}C] 4-nitrophenylarsonic acid, developed in five different solvent systems were air dried and placed in light tight cassettes in contact with X-ray film for 24 days. The spots revealed on development of the film were identified by markers and confirmed by colour reactions on paper.

Chemical determinations.

(1) Arsenic estimations.

The microestimation of total arsenic in biological materials is subject to a number of errors and practical difficulties. The method of Oliver and Funnell (1959) based on a HCl digestion to decompose organic arsenicals was tried and discarded since it did not give quantitative recoveries. Finally it was necessary to degrade biological samples by dry or wet oxidative procedures.

Two methods for the estimation of total arsenic were used. One involved the separation of arsenic from inorganic phosphate by distillation as arsenic trichloride and the other involved volatilization as arsine. Arsenic was estimated colorimetrically as the reduced molybdiarsenic acid complex at 820 m μ with the Unicam SP.600 spectrophotometer.

The major materials used in the following methods were either "Analar", "Low in arsenic" or "Arsenic free" grades.

(a) Distillation method. (Sultzberger, 1943).

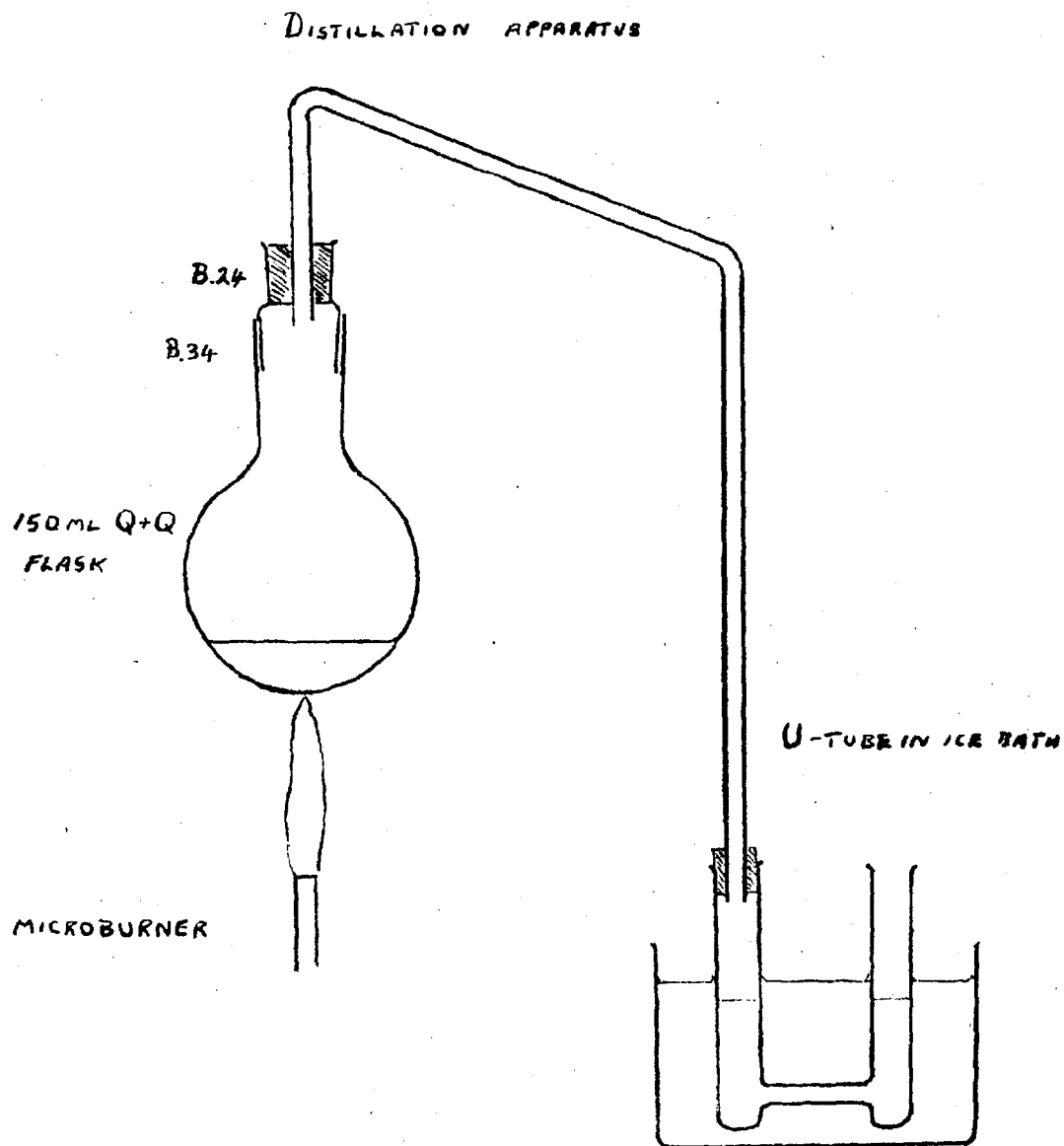
Urine samples containing up to 70 μ g. arsenic were digested with conc. H_2SO_4 (5 ml.) and conc. HNO_3 (2 - 5 ml.) in 30 ml. Kjeldahl flasks until white fumes of SO_3 were evolved. On cooling 10 ml. distilled water were added and heating was continued to boil off all traces of nitric acid. (When digestion was complete the necks of the flasks were cool enough to handle.)

Digest and aqueous washings (5 ml.) were transferred to the distillation apparatus (Figure 1) and after cooling a powdered mixture of K.Br. (0.5 gm.); $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$ (1 gm.) and KCl. (4 gm.) were added. The mixture was gently heated and the evolving gases were passed through an ice cooled U-tube containing 2 ml. conc. HNO_3 and 10 ml. distilled water at a rate of one bubble per second. Heating was continued until all gas had been evolved and the first drop of distilled liquid fell into the U-tube.

The contents of the U-tube and 2 ml. washings were transferred to a 25 ml. Erlenmeyer flask. Two anti-bumping granules were added and nitric acid was boiled off at "low" heat on an electrical hotplate. When only 1 ml. of absorbent remained, the flask was heated to dryness in an electric oven at $110^\circ C$.

10 ml. 1% ammonium molybdate in 5 NH_4SO_4 were added to 10 ml. 0.15% hydrazine sulphate and the mixture was diluted to 100 ml. This solution was prepared fresh from the stock solutions prior to each series of estimations. 10 ml. of this mixture were added to

Figure 1



were added to each dried flask and heated in a boiling water bath for 10 minutes. On cooling the coloured solutions were diluted to 25 ml. and estimated in the spectrophotometer.

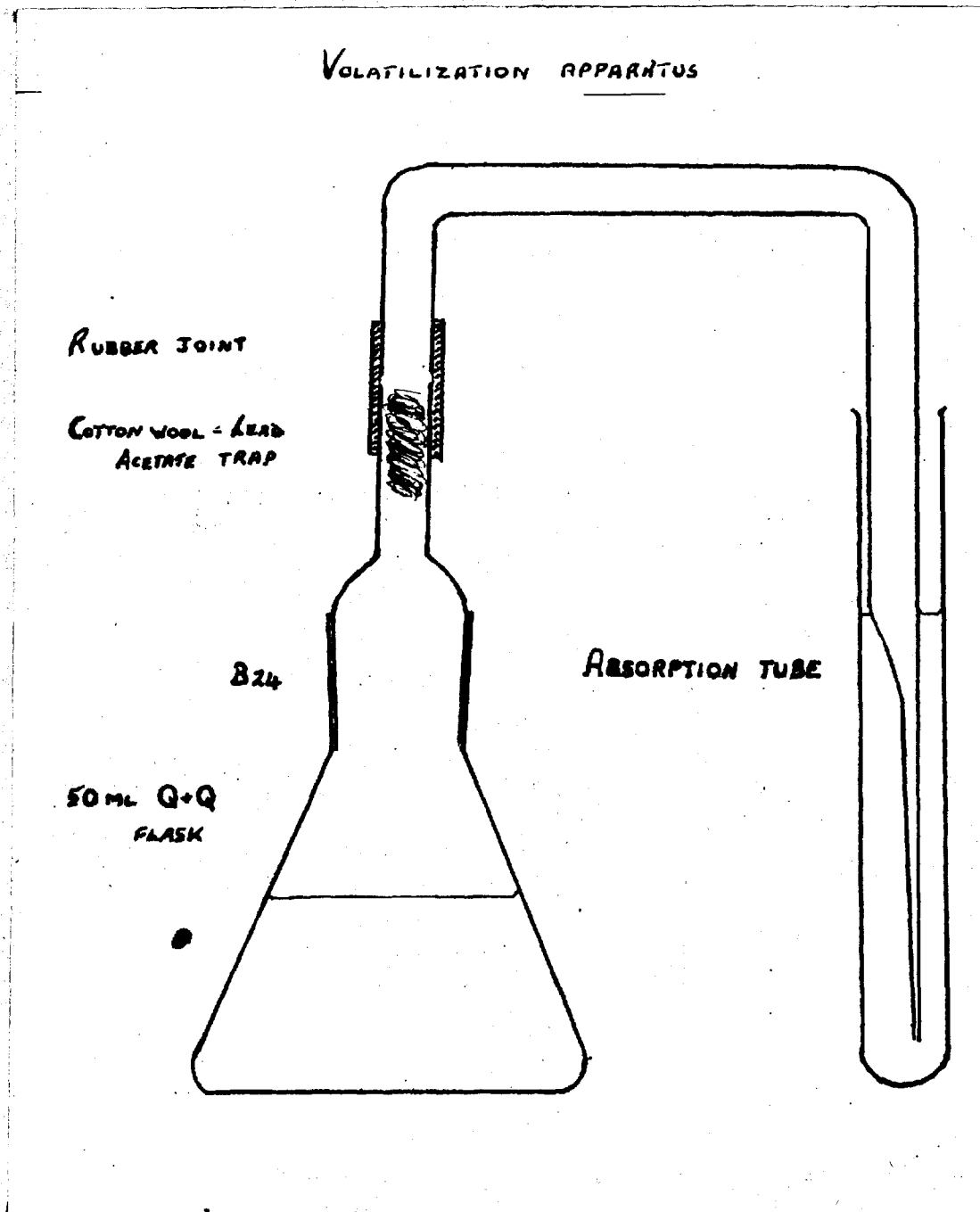
This method gave $100 \pm 3\%$ recovery for 18 - 70 μg . arsenic.

(b) Volatilization method. (Beers and Klug, 1956).

Samples containing up to 35 μg . arsenic were added to 4.8 cm. diameter Sillax porcelain crucibles lined with cones of Whatman No.1 filter paper. 6 ml. arsenic free saturated $\text{Mg}(\text{NO}_3)_2$ solution were added and the mixture was evaporated to complete dryness on an electrical hotplate. Lids were fitted to the crucibles which were then placed in a cold muffle furnace. The temperature was gradually increased to 600°C and ashing was continued overnight.

On cooling the ash was wetted with 5 ml. distilled water, dissolved in 5 ml. $12\text{N.H}_2\text{SO}_4$ and the solution was transferred to a 50 ml. Erlenmeyer flask with a B.24 ground glass neck. The crucible was washed with a further 5 ml. $12\text{N.H}_2\text{SO}_4$ and 15 ml. distilled water and the washings were added to the flask. 0.5 ml. fresh 40% SnCl_2 were added and 2 gm. arsenic free zinc shot (20 - 30 mesh). The stopper, lubricated with conc. H_2SO_4 , and lead tube (Figure 2) was quickly replaced and hydrogen evolved was passed through cotton wool moistened with saturated lead acetate solution and bubbled from a capillary through a receiving tube containing 7 ml. 0.002 N. I_2 and 0.5 ml. 4.2% NaHCO_3 . Volatilization was continued for one hour after which the apparatus was dismantled

Figure 2



and the iodine solution decolorized with one drop of fresh 5% sodium metabisulphite solution.

10 ml. 1% ammonium molybdate in $5\text{NH}_2\text{SO}_4$ and 10 ml. 0.15% hydrazine sulphate were diluted to 50 ml. and 2.5 ml. aliquots of this solution were added to each tube. The colours developed on heating for 10 minutes in a boiling water bath. On cooling the volumes were adjusted to 10 ml. and the colours were estimated in the spectrophotometer.

This method gave a mean recovery of $89 \pm 5\%$ for 4 - 35 μg . arsenic.

(e) Inorganic arsenic estimations. (Sandell, 1959).

25 ml. distilled water, 5 ml. conc. HCl . (sp.g. 1.18), 2 ml. 15% KI . and 0.5 ml. 40% SnCl_2 were added to 2 ml. hen excreta in a 50 ml. Erlenmeyer flask with one drop of octyl alcohol to prevent foaming. 2 Gm. arsenic free zinc shot (20 - 30 mesh) were added and the stopper quickly replaced. Hydrogen evolved was passed through a H_2S trap and bubbled from a capillary into an absorption tube containing 3 ml. 0.05 NI_2 and 0.6 ml. 4.2% NaHCO_3 .

After one hour the colour was developed and estimated as in the previous method using the arsenomolybdate reagent.

Recoveries for 19.6 μg . inorganic arsenic in the presence of 400 μg . p-arsanilic acid were $94 \pm 5\%$.

This method was useful for measuring inorganic arsenic in the presence of monosubstituted arsonic acids. However, it was not

applicable to the disubstituted arsonic acids because of the very high blank values obtained.

(2) p-Arsanilic acid and 4-acetamidophenylarsonic acid.

p-Arsanilic acid and 4-acetamidophenylarsonic acid were estimated using methods based on those of Bratton and Marshall (1939). An aliquot of excreta supernatant containing up to $3.5 \mu\text{M}$ p-arsanilic acid in up to 20 ml. was diluted to 30 ml. with water. 10 ml. 15% TCA were added and the mixture was centrifuged (3,000 r.p.m. for 10 minutes). A similar aliquot was made up to 20 ml. with water and 10 ml. of standard p-arsanilic acid (10^{-7}M) added as an internal standard. This was similarly treated with TCA and centrifuged.

5 - 10 ml. aliquots of supernatant were pipetted directly into 25 ml. graduated tubes for estimations of free arsanilic acid.

For estimations of total arsanilic acid, 5 ml. aliquots of supernatant were hydrolysed with 0.5 ml. 4 N.HCl. for 30 minutes at 100°C in 10 ml. centrifuge tubes. The hydrolysate was cooled, centrifuged and transferred to a 25 ml. graduated tube. The centrifuge tube was washed with 2 ml. water and the washings added to the original.

Solutions in the graduated tubes were diazotized with 2 ml. 0.1% NaNO_2 . After two minutes 2 ml. 0.5% ammonium sulphamate were added followed by 2 ml. 0.1% N(1-naphthyl)ethylene diamine dihydrochloride after another three minutes. Colours were developed for 30 minutes, diluted to 25 ml. and estimated in the Unicam S.P.600

spectrophotometer at 540 m μ against a similar solution to which all reagents had been added except the coupling agent.

4-Acetamidophenylarsonic acid was determined by the difference between the results for free and total p-arsanilic acid.

In experiments involving 4-nitrophenylarsonic acid administration, p-arsanilic acid was determined directly from a calibration curve in normal excreta supernatant. When p-arsanilic acid itself or 4-acetamidophenylarsonic acid were administered, an internal standard was used (see 4-hydroxy 3-aminophenylarsonic acid) and results were calculated from the increase in optical density that this gave over that of the excreta supernatant alone.

The recovery of p-arsanilic acid alone or 4-acetamidophenylarsonic acid (10 - 100 μ M) added to normal hen excreta (25 gm.) was $99 \pm 6\%$ using an internal standard and $94 \pm 5\%$ using a calibration curve.

(3) 4-Nitrophenylarsonic acid.

Several methods have been tried for the quantitative reduction of 4-nitrophenylarsonic acid to p-arsanilic acid for colorimetric estimation. Zinc shot and HCl., titanous chloride and sodium hydro-sulphite reduction gave poor recoveries and inconsistent results but the following method based on reduction with ferrous hydroxide at pH 14 was found to be swift simple and reliable.

3 Ml. excreta supernatant containing 0.25 - 2 μ M 4-nitro-

phenylarsonic acid were mixed with 6 ml. 10% NaOH and 6 ml. fresh 2% $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ in a 15 - 16 ml. stoppered tube. After standing at room temperature for 10 minutes, tubes were placed in a water bath at 85 - 95°C for 10 minutes with occasional shaking. On cooling the solutions were filtered through Whatman No.1 filter paper. 3 ml. of filtrate were acidified with 5 ml. N.HCl.. diazotized, coupled and diluted to 25 ml. as for p-arsanilic acid. The colours were estimated in the Unicam S.P. 600 spectrophotometer at 540 m μ .

In a sample containing both 4-nitrophenylarsonic acid and p-arsanilic acid, measurements before and after reduction gave estimates of the respective amino and nitro compounds. Recoveries were 98% $\pm$ 5% for 20 - 100 μM added to 25 gm. hen faeces.

(4) 4-Hydroxy 3-nitrophenylarsonic acid.

The method used for the estimation of 4-hydroxy 3-nitrophenylarsonic acid in excreta supernatant was based on that of Cavett (1956).

5 - 20 ml. of excreta supernatant containing up to 14 μM 4-hydroxy 3-nitrophenylarsonic acid was diluted to 20 ml. and 5 ml. 15% TCA added. The mixture was centrifuged (3,000 r.p.m. for 10 minutes) and 20 ml. of supernatant treated with 4 ml. N.NaOH and adjusted to pH 9 - 10 (indicator paper). Above pH 10 a yellow pigment from the urine interfered with the estimation and below pH 8 recoveries of 4-hydroxy 3-nitrophenylarsonic acid were incomplete. The volume was made up to 25 ml. the solution centrifuged and the

supernatant was treated with 0.5 gm. activated charcoal for 30 minutes. After a further centrifugation the supernatant was filtered through Whatman No.1 filter paper. The solution was divided into two 10 ml. portions. 3 ml. NH_4Cl were added to one portion and 2.5 ml. N.NaOH to the other. Volumes were made up to 25 ml. with water and both solutions were estimated at 410 m μ in the Unicam S.P. 600 spectrophotometer using the acid solution as a blank.

The recovery for 50 - 200 μM 4-hydroxy 3-nitrophenylarsonic acid added to 25 gm. hen faeces was $96\% \pm 4\%$.

(5) 4-Hydroxy 3-aminophenylarsonic acid.

4-Hydroxy 3-aminophenylarsonic acid in excreta supernatant was estimated according to the colorimetric method of Hareu (1949) for 4-hydroxy 3-aminophenylarsonoxide. An internal standard was used for all estimations on excreta supernatants instead of a calibration curve.

20 ml. of excreta supernatant containing up to 2.75 μM 4-hydroxy 3-aminophenylarsonic acid was diluted to 50 ml. and 10 ml. 15% TCA added.

0.78 μM 4-hydroxy 3-aminophenylarsonic acid in 10 ml. water were added to a second 20 ml. aliquot, diluted to 50 ml. and another 10 ml. 15% TCA added.

The solutions were centrifuged (3,000 r.p.m. for 10 minutes) and 20 ml. aliquots diazotized with 1 ml. 0.5% NaNO_2 . After five

minutes 15 ml. 15% Na_2CO_3 and 1 ml. 0.1% n-hexyl resorcinol in 50% ethanol were added. The colour was developed for 30 minutes and estimated in a Unicam S.P. 600 spectrophotometer at 490 m μ . against a similar blank in which 1 ml. water was substituted for the coupling reagent.

Readings for excreta supernatant alone were subtracted from those of excreta supernatant and standard and values were calculated as follows:-

	<u>Optical Density</u>
20 ml. excreta supernatant	a
20 ml. excreta supernatant + 0.78 μM 3-amino -	b
μM 4-hydroxy 3-aminophenylarsonic acid	
	/ 20 ml. excreta supernatant
$= \frac{a}{b - a} \times 0.78$	

(6) Glucuronide.

Total glucuronide was estimated according to the colorimetric method of Paul (1951). The method used for hen excreta is described by Baldwin (1961).

(7) Ethereal sulphate.

Total ethereal sulphate in hen excreta was estimated according to the gravimetric method of Folin (1905-6). This method is also described by Baldwin (1961).

(8) Polyethylene glycol.

The procedure of Shaffer and Critchfield (1947) was slightly modified for the colorimetric estimation of polyethylene glycol (M.Wt. 4,000). 4-Hydroxy 3-nitrophenylarsonic acid did not interfere.

20 - 50 ml. of excreta supernatant containing up to 200 mg. polyethylene glycol were mixed with 10 ml. 25% ∇/v HCl. and 10 ml. 10% $BaCl_2$ in 100 ml. volumetric flasks and diluted to 100 ml. The solutions were centrifuged (3,000 r.p.m. for 15 minutes) and 10 ml. supernatant added to 15 ml. centrifuge tubes containing 1 ml. 25% ∇/v HCl, 1 ml. 10% $BaCl_2$ and 1 ml. 10% phosphomolybdic acid. A green precipitate of the polyethylene glycol - phosphomolybdic acid complex settled out. After standing for one hour the tubes were centrifuged (2,500 r.p.m. for 10 minutes) and the supernatant carefully removed by suction through a capillary. The precipitates were fragmented with a glass rod, washed with 10 ml. 0.1 N.HCl. and recentrifuged. This treatment was repeated and the precipitates were finally washed into 30 ml. Kjeldahl flasks and digested with 2.5 ml. conc. H_2SO_4 and 5 ml. conc. HNO_3 until white fumes of SO_3 were evolved. On cooling, 10 ml. water were added and heating was continued to boil off any remaining traces of nitric acid.

10 ml. water were added to the cold digest and 9.2 ml. 40% NaOH. After cooling, four drops of phenol phthalein were added and the solution was made slightly acid with 2 ml. H_2SO_4 and diluted to 100 ml. The solutions were left overnight to allow any precipitate

to settle. 2 ml. 1% ammonium molybdate in 5 NH_2SO_4 and 0.5 ml. fresh 1-amino 2-naphthol 4-sulphonic acid solution were added to 20 ml. aliquots of the diluted digest and the volumes were made up to 25 ml. Colours were developed for four hours at room temperature and estimated in the Unicam SP.600 spectrophotometer at 720 m μ against a reagent blank.

Mean recoveries from excreta supernatant were $105 \pm 5\%$ for 40 - 160 mg. polyethylene glycol.

Physical techniques.

(1) Paper chromatography.

Paper chromatograms of the excreta of hens were developed in six different solvent systems. Ascending paper chromatography in screw top glass jars was used for the nitrile solvents and descending paper chromatography in glass tanks with ground glass lids was used for the other solvents. Temperature conditions were not rigidly controlled so authentic material was always run at the same time as the unknowns.

Chromatograms were generally developed on Whatman No. 1 paper but Whatman 3 MM paper was occasionally used for locating the presence of minor metabolites since it would tolerate much heavier loading.

The Rf. values of arsenicals and their behaviour to locating agents are summarised in Tables 2A, 3A and 4A.

(2) Melting points.

Melting points were determined in glass capillaries in an electrically heated metal block. All values are uncorrected.

(3) pKa determinations.

80 - 150 Mg. of the arsenic acid were dissolved in 50 - 150 ml. hot water and cooled to room temperature. The solutions were titrated against $\frac{N}{20}$ NaOH or $\frac{N}{20}$ HCl from a microburette using a Pye or a Cambridge Instruments pH meter.

pKa's were determined as the pH values of the solutions at the half equivalent point according to the Henderson and Hasselbach equation (Table 5A).

(4) U.V. absorption spectra.

The absorption spectra of arsenicals in the visible and ultraviolet region were determined in a Unicam S.P. 500 spectrophotometer using 1 cm. silica cells (Tables 6A, 7A and 8A.)

CHAPTER 3

The metabolism of 4-nitrophenylarsonic acid.

The rate of elimination of 4-nitrophenylarsonic acid.

Six Brown Leghorn hens were given single oral doses of 50 mg. $[1 - {}^{14}\text{C}]$ 4-nitrophenylarsonic acid. ${}^{14}\text{C}$ in the homogenised excreta from these birds was determined daily for 4 - 6 days after dosing or until no further radioactivity could be detected. The average rate of elimination of ${}^{14}\text{C}$ is shown in Table 4.

The amounts of ${}^{14}\text{C}$ in the daily excreta usually decreased as the experiment progressed until only traces were detectable. In the later stages, the specific activity of the excreta was very low and estimates of ${}^{14}\text{C}$ in these samples were subject to greater counting errors. After oral administration of $[1 - {}^{14}\text{C}]$ 4-nitrophenylarsonic acid, wide variations were found in the rate of excretion of ${}^{14}\text{C}$ by individual birds. In the first 24 hours 15 - 82% of the dose was recovered. By the third day, 60 - 90% of the dose had been excreted and thereafter small amounts of ${}^{14}\text{C}$ were detectable in the excreta up to 8 - 11 days after dosing. The total recovery of ${}^{14}\text{C}$ was 83 - 100% in 8 - 10 days.

These results are comparable with those of Table 12A, where unlabelled 4-nitrophenylarsonic acid was fed to hens at similar dose levels and the metabolites estimated colorimetrically.

Table 4

The excretion of ^{14}C by hens receiving 50 mg.[¶] of
1 - ^{14}C 4-nitrophenylarsonic acid orally

Day after dosing	% of dose of ^{14}C excreted [†]
1	50 (15 - 82) ⁶
2	68 (36 - 89) ⁶
3	77 (63 - 90) ⁶
4	80 (66 - 92) ⁶
5 - 6	85 (76 - 95) ⁵
7	91 (81 - 99) ³
8	92 (82 - 101) ³
9	93 (83 - 101) ³

¶ About 36.7 mg./Kg.

† The figure given is the average for 3 - 6 hens. Ranges are given in parenthesis and the superscript gives the number of hens.

^{Table}
 The values in this ~~figure~~ are derived from Table 9A.

Intramuscularly administered 4-nitrophenylarsonic acid, however, was excreted much more rapidly (Table 12A). 91.5% of the dose was recovered in the excreta in 24 hours and 97% in three days.

The comparative excretion of ^{14}C , total and inorganic arsenic.

In order to determine whether 4-nitrophenylarsonic acid is degraded to inorganic arsenic in the hen, three birds were given single oral doses of $[1 - ^{14}\text{C}]$ 4-nitrophenylarsonic acid and the daily output of ^{14}C , total and inorganic arsenic measured for five consecutive days. Any cleavage of arsenic from the aromatic nucleus should be revealed by the appearance of inorganic arsenic in the excreta or differences in the output of ^{14}C and total arsenic.

The results (Table 10A) show that the excretions of ^{14}C and total arsenic are equivalent. In the two cases where inorganic arsenic was measured, the output was less than 0.2 mg. in five days. This is hardly greater than the daily output of background arsenic (0.15 mg./day) and is within the experimental error.

It appears, therefore, that the C - As bond of 4-nitrophenylarsonic acid remains intact and arsenic is not cleaved from the aromatic nucleus.

Identification of the metabolites of 4-nitrophenylarsonic acid in the excreta.

Qualitative examination of the excreta of hens fed 4-nitrophenylarsonic acid revealed the presence of a diazotizable arylamine.

The excreta gave only faintly positive reactions with ammoniacal silver nitrate and Fehling's solution, no colour with ferric chloride solution and no evidence for glucuronides with the naphtho-resorcinol test.

The excreta of three hens receiving 50 mg. 4-nitrophenylarsonic acid was chromatographed on paper in five different solvent systems (Table 3A). Only two spots were found by colour reactions and these were identified as 4-nitrophenylarsonic acid and p-arsanilic acid. No spots corresponding to 4-acetamidophenylarsonic acid, or to 4-nitro, 4-amino and 4 acetamidophenylarsenoxide were found and there was no evidence for the presence of p-nitrophenol or p-amino-phenol.

This was substantiated when $[1 - ^{14}\text{C}]$ 4-nitrophenylarsonic acid was fed to the hen. Autoradiography of chromatograms of the excreta showed that only two radioactive components were present. These components could be separated in the nitrile solvents but not in n-butanol; acetic a. : water. The radioactive spots did not move from the origin in benzene: acetic a.: water or di-isopropyl-ether: water. Colour reactions and markers confirmed these components as unchanged 4-nitrophenylarsonic acid and p-arsanilic acid.

Quantitative determination of the metabolites of 4-nitrophenylarsonic acid.

The metabolites of 4-nitrophenylarsonic acid appearing in the

excreta of hens given single doses of the compound were measured by isotope dilution and colorimetric procedures. Oral dosage levels varied over the range 34.7 - 43.4 mg./Kg. when $[1 - {}^{14}\text{O}]$ 4-nitrophenylarsonic acid was given while unlabelled 4-nitrophenylarsonic acid was administered orally or intramuscularly at 33 mg./Kg.

Hens were fasted for 24 hours prior to oral or intramuscular administration of 4-nitrophenylarsonic acid. Food was withheld from the birds throughout the course of the experiment but unlimited access to water was allowed.

Control experiments showed that when 10 mg. 4-nitrophenylarsonic acid were added to 100 mg. normal hen excreta and kept at room temperature for 24 hours without the addition of any preservatives, up to 3 mg. was reduced to p-arsanilic acid. Addition of chloroform (2 ml.) and 2 N.H₂SO₄ (5 ml.) to hen excreta under the same conditions reduced these changes to insignificant proportions (< 0.1 mg.).

1. Oral administration.

The excreta from hens can be separated into two simple components, an aqueous extract of hen excreta (excreta supernatant) and the solid residue, consisting of undigested food particles, bacteria and uric acid. When the excreta from hens fed $[1 - {}^{14}\text{O}]$ 4-nitrophenylarsonic acid was fractionated in this manner and counted, most of the radioactivity was found in the soluble fraction.

However, when the distribution between the soluble and solid fractions was followed for three days after dosing, the radioactive content of the soluble fraction declined whereas that of the solid residue remained constant (Table 11A).

The nature of these arsenic residues attached to the faecal solids is discussed in a later section.

Radiochemical assay of the excreta of hens fed $[1 - {}^{14}\text{C}]$ 4-nitrophenylarsonic acid at dosage levels of 35 - 43 mg./Kg. have shown that 44 - 67% of the administered dose was excreted in three days, 4 - 8% of the dose was bound to the faecal solids and 40 - 60% was in the excreta supernatant. Isotope dilutions have shown that most of the activity (31 - 43%) was present as unchanged 4-nitrophenylarsonic acid and 7 - 12% as p-arsanilic acid. (Table 11A). 2 - 6% of the administered dose of ${}^{14}\text{C}$ found in the excreta supernatant remained unidentified.

For comparison unlabelled 4-nitrophenylarsonic acid was fed to hens at 33 mg./Kg. and the metabolites in the excreta supernatant were estimated colorimetrically. In these cases 38 - 69% of the dose was excreted unchanged in three days and 14 - 32% as p-arsanilic acid (Table 12A). In view of the relatively large variations in the output of p-arsanilic acid no significance can be attached to the apparently greater output of p-arsanilic acid recorded in these results compared with those for ${}^{14}\text{C}$ labelled 4-nitrophenylarsonic acid.

2. Intramuscular administration.

When 4-nitrophenylarsonic acid was administered to hens intramuscularly at the same dose level 90 - 94% of the dose was excreted in 24 hours and 95 - 101% in three days. Only 4.9 - 10.6% of the dose was excreted as p-arsanilic acid in this period and the rest occurred as unchanged 4-nitrophenylarsonic acid.

3. Urinary output of 4-nitrophenylarsonic acid and p-arsanilic acid.

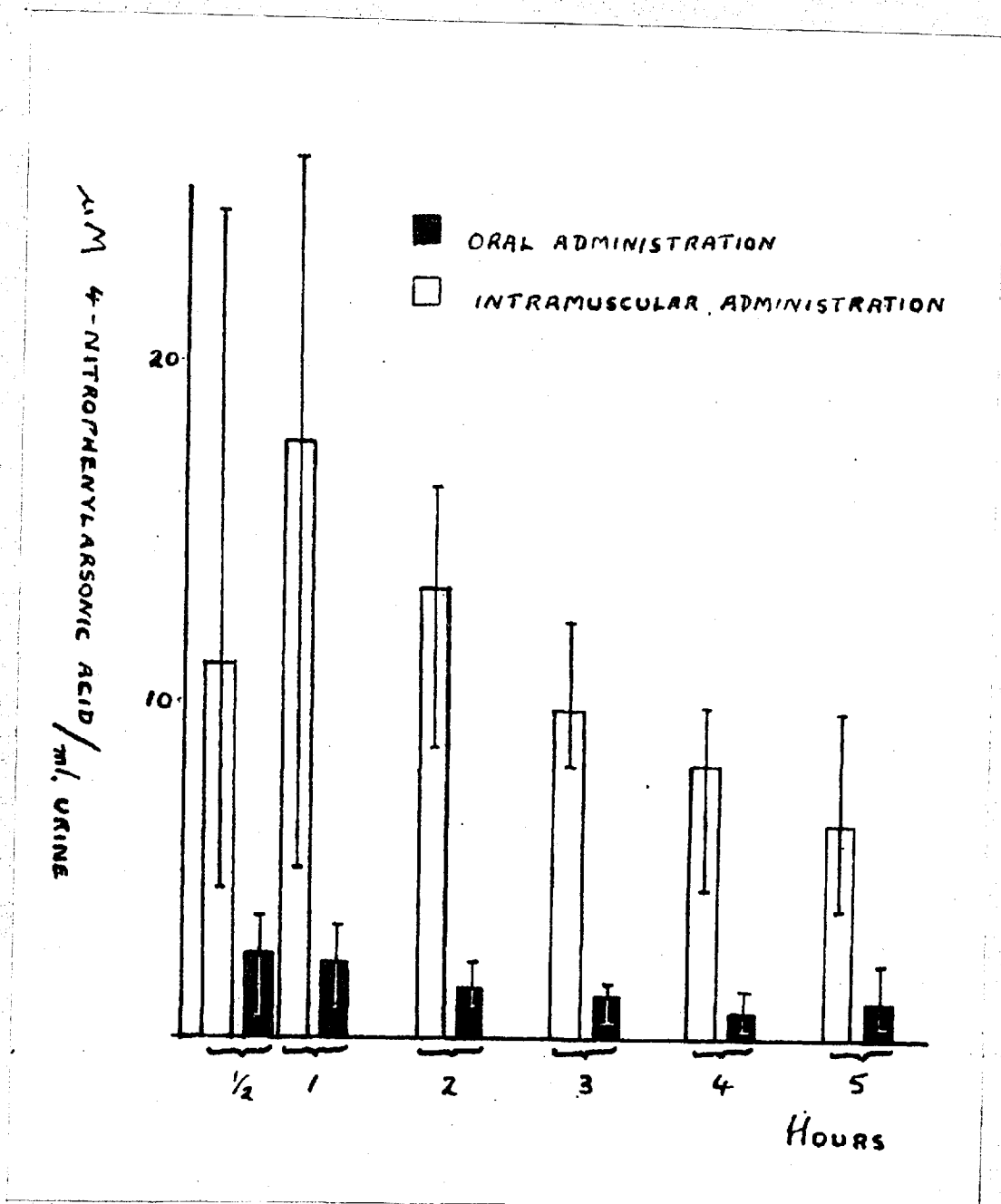
This fundamental difference in the rate of excretion and extent of reduction of 4-nitrophenylarsonic acid when administered orally and intramuscularly was also reflected when the concentration of these compounds in the cannulated urine was examined.

Two groups of three hens were given oral or intramuscular doses of 4-nitrophenylarsonic acid at 33 mg./Kg. and samples of urine were taken with a Hart and Essex cannula at hourly intervals for 5 - 6 hours. Urine samples were diluted forty-fold and analysed for 4-nitrophenylarsonic acid and p-arsanilic acid colorimetrically.

The results obtained are shown in Figure 3. After intramuscular injection the concentration of 4-nitrophenylarsonic acid in hen urine increased to a maximum of 8 - 26 $\mu\text{M}/\text{ml}$. in 1 - 2 hours and steadily declined over the next 3 - 4 hours to 4 - 10 $\mu\text{M}/\text{ml}$. Only small amounts (up to 0.16 $\mu\text{M}/\text{ml}$.) of p-arsanilic acid were detected.

Orally administered 4-nitrophenylarsonic acid was slowly excreted in the urine reaching a maximum concentration of only

Fig. 3. Urinary elimination of 4-nitrophenylarsonic acid



1 - 4 $\mu\text{M}/\text{ml}$. in 30 minutes - 1 hour after dosing followed by a slight decline over the next four hours to 0.2 - 2.4 $\mu\text{M}/\text{ml}$. As in the previous case only small amounts of p-arsanilic acid were detected but the proportion of total metabolites excreted as p-arsanilic acid was greater than when the compound was given by injection.

Paper chromatographic examination of hen urine confirmed that the excreted metabolites are the same using both routes of administration of 4-nitrophenylarsonic acid.

Factors influencing the reduction of 4-nitrophenylarsonic acid to p-arsanilic acid in the hen.

The excretion of p-arsanilic acid in hens given oral doses of 4-nitrophenylarsonic acid showed considerable variation. When 4-nitrophenylarsonic acid was given intramuscularly there was a diminished output of p-arsanilic acid although the rate of excretion of total metabolites was considerably enhanced. Both hen tissues and the gut flora are known to contain active nitroreductase enzymes but the relative contribution each system made to the reduction of 4-nitrophenylarsonic acid in vivo remained unknown.

1. The effect of aureomycin on the reduction of 4-nitrophenylarsonic acid in vivo.

Aureomycin is known to inhibit bacterial nitroreductase but there is little information concerning the inhibition of hen liver

nitroreductase by aureomycin. In order to assess the relative importance of bacterial and hen liver nitroreductase in the metabolism of 4-nitrophenylarsonic acid in vivo a series of experiments were conducted to determine the influence of oral and intramuscular doses of aureomycin on the metabolism of 4-nitrophenylarsonic acid.

In one experiment, aureomycin was administered orally to six birds in the form of 50 mg. pellets. Initially the birds were given 100 mg. aureomycin by mouth and after an interval of 4 - 5 hours a further 100 mg. dose was administered. 24 Hours after the first dose a final oral dose of 100 mg. aureomycin was given followed by the administration of 4-nitrophenylarsonic acid orally or intramuscularly.

In another experiment 50 mg. aureomycin was injected into the breast muscle of six birds at the same time as the administration of 4-nitrophenylarsonic acid. 50 Mg. doses were also given by injection on the two days following the administration of 4-nitrophenylarsonic acid.

The results obtained in these experiments are shown in Table 12A.

When 4-nitrophenylarsonic acid was administered intramuscularly 5 - 11% of the dose was recovered in the excreta in three days as p-arsanilic acid. However, when aureomycin was administered simultaneously by either route less than 1% of the dose

was recoverable as p-arsanilic acid in the same length of time. These results suggested that aureomycin administered by either route was capable of inhibiting the enzymes responsible for the reduction of 4-nitrophenylarsonic acid to p-arsanilic acid in hen tissues in vivo.

After oral administration of 4-nitrophenylarsonic acid 14 - 33% of the dose was excreted as p-arsanilic acid in three days. Pretreatment with aureomycin orally reduced the amount of p-arsanilic acid present in the excreta to 4 - 14% and pretreatment with aureomycin intramuscularly reduced the output to 7 - 19% over the same period of time.

Orally administered aureomycin inhibited both nitroreductase systems and caused the greatest fall in the excretion of p-arsanilic acid from an average of 22% to 9% of the dose in three days. Intramuscularly administered aureomycin was largely inhibitory to the enzymes of hen tissues and caused a lesser fall in output from 22% to 14%.

2. The reduction of 4-nitrophenylarsonic acid in the crop of the hen.

The experiments with aureomycin suggested that the gut flora was partly responsible for the reduction of 4-nitrophenylarsonic acid to p-arsanilic acid in vivo. The distribution of arsenic in hens after dosing with 4-nitrophenylarsonic acid, reported in a later section, showed that most of the arsenic present in the alimentary

canal of birds 24 hours after dosing, remained in the crop contents. The crop was, therefore, a potential site for nitroreductase activity.

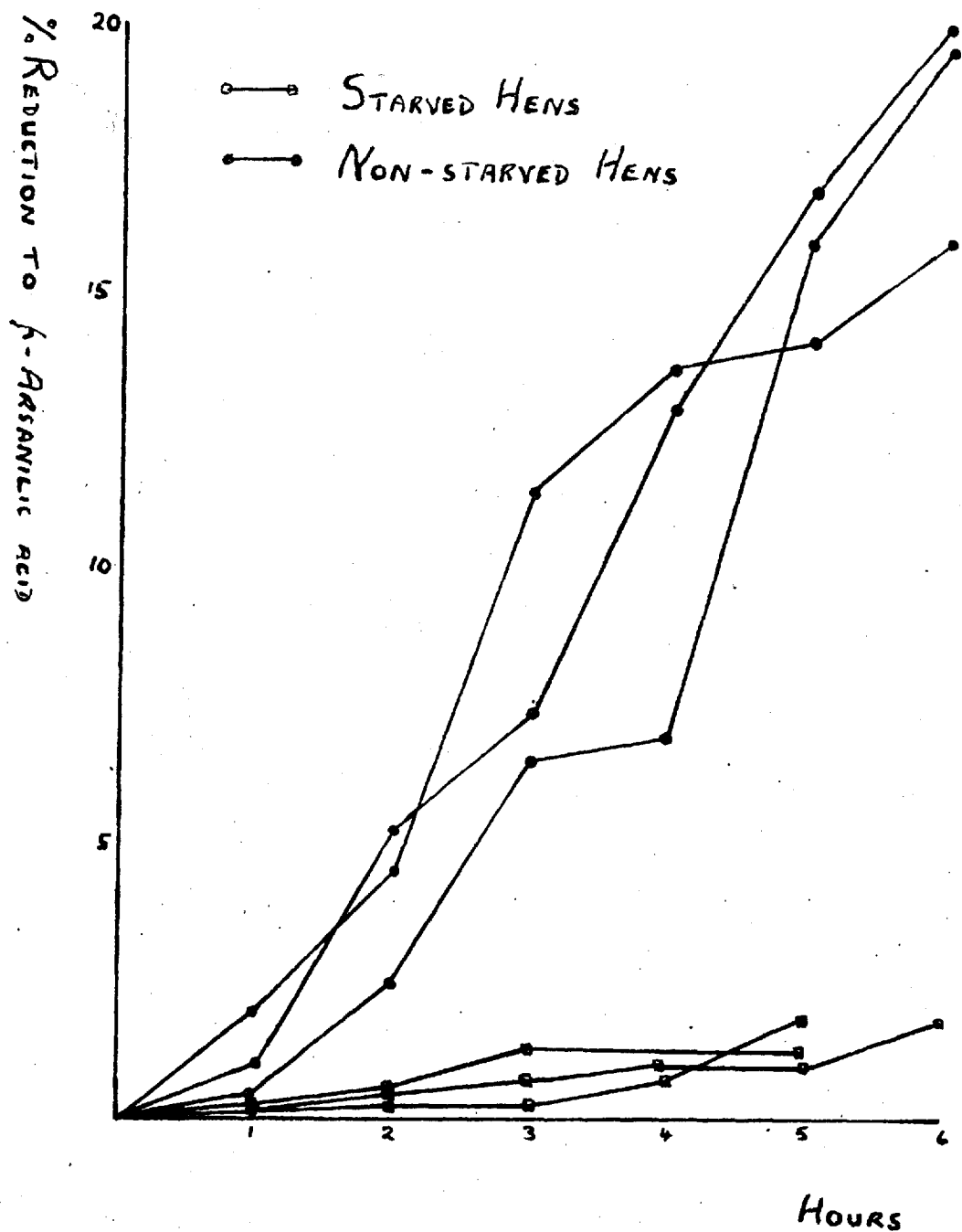
Three hens which had not been previously fasted for 24 hours, were given oral doses of 4-nitrophenylarsonic acid at 33 mg./Kg. 1 - 2 Ml. samples of the crop contents were taken at hourly intervals for six hours using a polythene crop tube attached to a 2 ml. syringe. The samples obtained were centrifuged and the supernatant analysed for p-arsanilic acid and 4-nitrophenylarsonic acid. The results obtained are shown in Figure 4.

With non starved birds there was a progressive reduction of 4-nitrophenylarsonic acid in the crop and after 6 hours up to 20% of the compound had been converted into p-arsanilic acid. In contrast when these experiments were repeated with starved birds, nitroreductase activity in the crop had been reduced to minor proportions. After six hours only 2 - 3% reduction to p-arsanilic acid occurred.

Poultry mash alone did not possess nitroreductase activity. When 100 mg. 4-nitrophenylarsonic acid was incubated with 20 gm. poultry mash in 100 ml. 0.1 M citrate buffer pH 5.0 (pH of crop contents) at 39°C for 6 hours, no reduction to p-arsanilic acid occurred.

Because of the nutritional requirements for nitroreductase activity in the crop, it was inferred that this was of micro biological origin.

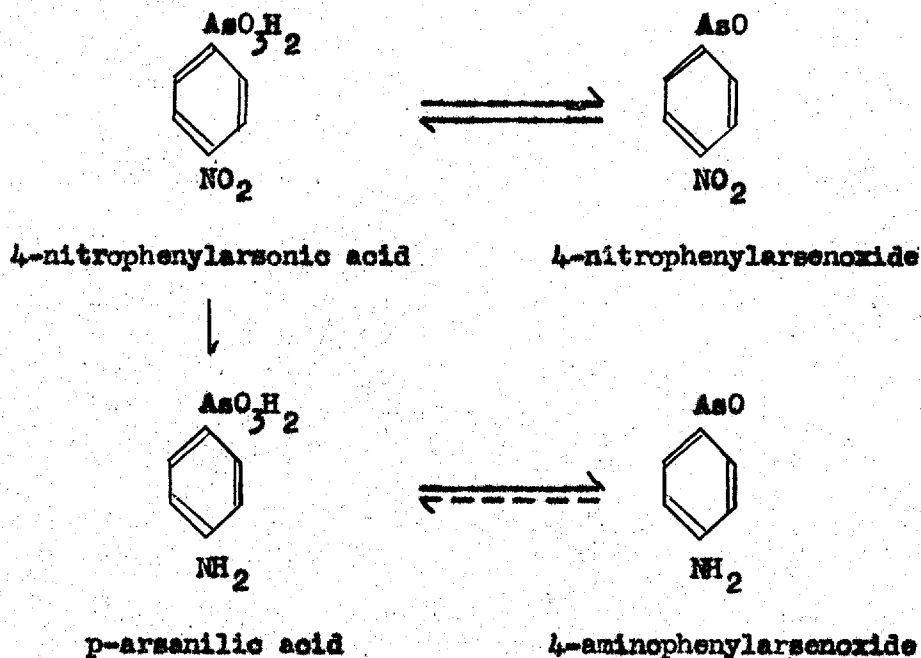
Fig. 4. Nitro reductase activity in the crop



3. The reduction of 4-nitrophenylarsonic acid by hen faeces.

After 4-nitrophenylarsonic acid (100 μ M) was incubated anaerobically for 24 hours with an extract of hen faeces in 0.05 M phosphate buffer pH 7.4 (40 ml.), p-arsanilic acid, 4-nitrophenylarsenoxide and 4-aminophenylarsenoxide were detected chromatographically in the medium. p-Arsanilic acid incubated under the same conditions was reduced to 4-aminophenylarsenoxide but when 4-nitrophenylarsenoxide was incubated with the extract, 4-nitrophenylarsonic acid and p-arsanilic acid were detected but not 4-aminophenylarsenoxide.

Under anaerobic conditions the following reactions may take place in hen faeces.



Arsenic residues attached to the solid fraction of hen excreta.

1. Properties and identity of arsenic residues.

Arsenoxides were not detected in the solid fraction of hen excreta by paper chromatography using colour reactions and autoradiography and there was no evidence that they were formed *in vivo*. Some evidence was obtained, however, which indicated that small amounts of these compounds occurred firmly bound to the faecal residue.

Isotope dilution experiments have shown that 4 - 8% of an administered dose of 4-nitrophenylarsonic acid may be excreted attached to the solid fraction of the faeces in three days. The daily output of these residues was fairly constant and did not decrease in the same manner as the metabolites present in the soluble fraction of the excreta.

The binding of these arsenic residues to the faecal solids was quite stable. ^{14}C and arsenic could not be removed by dialysis or extraction with hot methanol, cold N.HCl , cold 2% NaHCO_3 or 0.1 M EDTA. The residues could be removed by hot or cold N.NaOH and partly removed by hot N.HCl , 0.02 NI_2 and 0.1 M Diethylammonium diethyl dithiocarbamate (Table 13A). These results suggested that part of the arsenic residues attached to the faecal solids were attached as aryl thioarsenites through SH groups.

Paper chromatography of hot acid extracts have shown the presence of 4-nitrophenylarsonic acid, p-arsanilic acid, 4-nitrophenyl-

arsenoxide (solvents D and E) and there is some evidence for the presence of 4-aminophenylarsenoxide (solvent B). Furthermore, chromatograms of iodine extracts of faecal solids only showed the presence of 4-nitrophenylarsonic acid and p-arsanilic acid.

2. The binding of arsenicals onto the solid fraction of hen excreta.

When 4-nitrophenylarsenoxide (100 μ M) or 4-aminophenylarsenoxide was added to hen faeces (50 - 60 ml. homogenate) and kept for 24 hours at room temperature, 5% of the nitro compound and 7% of the amino compound became bound to the faecal solids. However, 4-nitrophenylarsonic acid was not bound under the same conditions and p-arsanilic acid was only bound to the extent of 2%.

The absorption and tissue distribution of 4-nitrophenylarsonic acid.

1. The distribution of arsenic residues in the tissues.

The distribution of arsenic in the tissues of hens, 24 hours after receiving an oral dose of 4-nitrophenylarsonic acid was determined for three birds. Blood was obtained from the dead birds by severing the neck and bile from the opened carcass using a syringe. Other tissues and organs were dissected and minced or homogenized according to ease. Skin and feathers and the residual carcass were dissolved in warm 40% NaOH.

Excreta, skin and feathers, fat and the residual carcass were analysed for arsenic by the wet combustion method of Sultzbberger (1943).

Other samples were subjected to dry combustion with magnesium nitrate and total arsenic estimated according to Beers and Klug (1956).

The average results for three hens are shown in Table 14A. Over 70% of the administered dose was found in the excreta (56%) or the contents of the alimentary canal (16%). The tissues of the bird contained 18% of the administered dose but this value is probably high because the residual carcass and skin and feathers may have been contaminated with excreta and the gut contents. The edible tissues (liver, muscle, heart and fat) contained only 1.5% of the administered dose i.e. 0.35 mg. arsenic. Muscle tissues contained only 0.5% of the dose (0.3 $\mu\text{g. As./gm. tissue}$).

When the contents of the alimentary canal were compared the crop and gizzard contained most of the arsenic residues (12%) and the lower regions of the gut (small intestine, caeca and large intestine) only contained 4% of the dose. This distribution indicated that the crop and gizzard may control the rate of elimination of orally administered 4-nitrophenylarsonic acid.

2. The comparative excretion of orally administered 4-nitrophenylarsonic acid and polyethylene glycol.

Polyethylene glycol (M.Wt. 4,000) is an inert, water soluble polymer which is not absorbed from the gut and which has been used as a marker to trace the passage of food through the alimentary canal in farm animals (Corbett, et al. 1959). The simultaneous oral admin-

istration of polyethylene glycol and 4-nitrophenylarsonic acid gave a good comparison between the rate of elimination of 4-nitrophenylarsonic acid and p-arsanilic acid and a compound known to be excreted in the faeces.

Three Brown Leghorn hens (Wts. 1.2 - 1.6 Kg.) were fasted for 48 hours in order to minimize the polyethylene glycol blanks. The birds were then fed 4-nitrophenylarsonic acid at 40 mg./Kg. and 4 gm. polyethylene glycol. Collections of total excreta were made at 12, 24, 48 and 72 hours after dosing and were analysed for polyethylene glycol and total nitro and aminophenylarsonic acids. The results are shown in Table 15A.

The total excretion of polyethylene glycol and the arsonic acids over the period of time studied are remarkably similar. Although this does not directly prove that 4-nitrophenylarsonic acid is poorly absorbed in the small intestine it does suggest that the factors regulating the elimination of both compounds are essentially the same.

CHAPTER 4

The metabolism of p-arsanilic acid and 4-acetamidophenylarsonic acid.

The metabolism of p-arsanilic acid and 4-acetamidophenylarsonic acid was studied using Brown Leghorn hens under the experimental conditions outlined in Chapter 2. However, in contrast to the investigations on 4-nitrophenylarsonic acid, the hens were maintained throughout the experimental period on 30 gm. of poultry mash per day.

No changes occurred to p-arsanilic acid and 4-acetamidophenylarsonic acid under the standard conditions for collecting hen excreta.

Identification of the metabolites of p-arsanilic acid and 4-acetamidophenylarsonic acid in the excreta.

The excreta supernatant of hens dosed with p-arsanilic acid or 4-acetamidophenylarsonic acid was chromatographed on paper in the five solvent systems described in Table 3A.

When p-arsanilic acid was administered orally or intramuscularly, only one spot was found on paper chromatograms (Solvents A, B and C) and this was identified as unchanged p-arsanilic acid. No spots corresponding to 4-acetamidophenylarsonic acid could be detected neither were there any indications that arsenoxides were present (Solvents D and E).

Two spots were found on chromatograms of the excreta of hens given oral or intramuscular doses of 4-acetamidophenylarsonic acid. One corresponded to p-arsanilic acid and the other to 4-acetamidophenylarsonic acid. There was no evidence that arsenoxides were present in the excreta.

Quantitative determination of p-arsanilic acid.

The quantitative results for the excretion of orally or intramuscularly administered p-arsanilic acid are shown in Table 16A. Analyses for p-arsanilic acid and total arsenic gave equivalent results when the compound was administered by either route suggesting that no significant metabolic changes occurred and that it was excreted unchanged. Comparing the results for both routes of administration at the same dose level, p-arsanilic acid, like 4-nitrophenylarsonic acid, was excreted more rapidly after intramuscular injection than after oral dosage.

Quantitative determination of the metabolites of 4-acetamidophenylarsonic acid.

The results for the metabolism of 4-acetamidophenylarsonic acid are shown in Table 17A. As in the previous case, analyses for total arsanilic acid and total arsenic gave equivalent results when the compound was administered by either route.

There were, however, two components present in the excreta supernatant, namely 4-acetamidophenylarsonic acid and p-arsanilic acid

and there was a quantitative difference in the extent of deacetylation depending on whether the compound was given orally or intramuscularly. After oral dosing, only 5% of the dose was deacetylated in three days but after injection 36% of the dose occurred in the excreta as p-arsanilic acid. This suggests that only a small proportion of orally administered 4-acetamidophenylarsonic acid was absorbed from the gut and from the extent of deacetylation of an intramuscular dose it may be estimated that 14% of an oral dose is absorbed from the small intestine.

The deacetylation of 4-acetamidophenylarsonic acid.

Some experiments have been performed to determine the site of deacetylation of 4-acetamidophenylarsonic acid in the hen.

When this compound was incubated with hen excreta in 0.05 M phosphate buffer (pH 7.4) at 37°C for 24 hours, practically no p-arsanilic acid was formed and it was concluded that the gut flora was not responsible for any appreciable deacetylation.

Several hen tissues were examined for deacetylase activity. Each tissue was homogenized in four volumes of cold isotonic KCl. 1 ml. of homogenate was mixed with 4 ml. 0.05 M tris buffer (pH 7.4) containing 2 μ M 4-acetamidophenylarsonic acid. The mixture was incubated for one hour at 37°C and the reaction stopped by 5 ml. 15% trichloroacetic acid. After centrifuging for 5 minutes, p-arsanilic acid was determined colorimetrically on 2 ml. of the

supernatant. Blanks were set up using tissue homogenate boiled for 5 minutes on a water bath.

The results in Table 18A show that the hen kidney was the only significant site of deacetylase activity when 4-acetamidophenyl-arsonic acid was used as substrate. Moreover, no significant acetylation of p-arsanilic acid occurred under the same experimental conditions.

The deacetylase activity of hen kidney homogenate was proportional to the time of incubation up to 15 minutes and to the amount of homogenate in the reaction mixture. The optimum pH was found to be 7.4 in 0.05 M tris buffer and 6.6 in 0.1 M phosphate buffer. The preparation, however, was 40% more active at pH 6.6 in phosphate buffer than at pH 7.4 in tris buffer.

The cellular location of hen kidney deacetylase was determined by fractional centrifugation. The kidneys of one bird were homogenized in 3.5 vols. cold 0.25 M sucrose using a Potter-^{Elvehjem}~~Elvehjem~~ homogeniser with a plastic pestle and diluted to 7 vols. with sucrose. Another bird's kidneys were homogenized for 60 seconds in 7 vols. cold sucrose in a Waring blender. The homogenates were centrifuged at 3°C to give the following fractions,

- (a) washed nuclei and debris at 450 g. for 10 mins.
- (b) washed mitochondria at 19,900 g. for 10 mins.
- (c) microsomes + supernatant.
- (d) washed microsomes at 105,000 g. for 60 mins.
- (e) supernatant from (d).

Each fraction was examined for deacetylase activity towards 4-acetamidophenylarsonic acid and the results are shown in Table 19A. Deacetylase activity was located mainly in the mitochondrial fraction of the hen kidney and though some activity was present in the other particulate fractions, this is probably due to contamination with mitochondria. No activity was found in the soluble fraction of hen kidney.

CHAPTER 5

The metabolism of 4-hydroxy 3-nitrophenylarsonic acid.

The fate of 4-hydroxy 3-nitrophenylarsonic acid in laying hens was investigated under experimental conditions similar to those outlined in Chapter 4 for p-arsanilic acid and 4-acetamidophenylarsonic acid.

Hens were fasted for 24 hours prior to the administration of the compound but throughout the rest of the experimental period the birds were given 50 gm. poultry mash per day. Any variations in these practices are described in the following sections.

Rhode Island Red x Light Sussex laying hens were generally used for these studies but four Brown Leghorn hens were used in experiments involving the total recovery of arsenic in the excreta over a period of 10 - 11 days after oral administration of the compound.

The rate of elimination of 4-hydroxy 3-nitrophenylarsonic acid.

Four Brown Leghorn hens were given oral doses of this compound at 75 mg./Kg. to determine the rate of elimination of total arsenic in the excreta. Collections of total urine and faeces were made daily until no more arsenic could be detected in the excreta. Each collection was homogenized, diluted to a known volume and estimations of total arsenic were performed on suitable aliquots using the method of Sultzeberger (1943). The results

obtained are shown in Table 5.

4-Hydroxy 3-nitrophenylarsonic acid was excreted slowly when given by mouth. Only 25 - 33% of the dose was excreted in 24 hours and only 45 - 76% in four days. After the fourth day, progressively smaller quantities of arsenic were eliminated until 88 - 102% of the original dose had been recovered in 10 - 11 days.

The breed of hen used in these experiments had little effect on the rate of elimination of 4-hydroxy 3-nitrophenylarsonic acid. If the results shown in Table 5 for Brown Leghorn hens are compared with those for Rhode Island Red x Light Sussex hens at the same dosage level, (Table 23A) the differences in the rates of excretion of arsenic by the two groups of birds over the first three days, are insignificant. In three days Brown Leghorn hens excreted 55% of the administered dose whereas Rhode Island Red x Light Sussex hens excreted 61% of the dose.

The dosage level used did not greatly influence the clearance of orally administered 4-hydroxy 3-nitrophenylarsonic acid from the hen. At dosage levels of 75, 37.5 and 18.9 mg./Kg., Rhode Island Red x Light Sussex hens excreted on average between 61 and 65% of the administered dose in three days (Table 23A).

When 4-hydroxy 3-nitrophenylarsonic acid was administered to hens intramuscularly at 37.5 mg./Kg. all the birds died 25 - 30 hours after dosing but in this length of time 48 - 67% of the dose was recovered (Table 24A). In contrast, orally administered 4-hydroxy-

Table 5

The excretion of total arsenic by hens receiving
oral doses of 4-hydroxy 3-nitrophenylarsonic acid
at 75 mg./Kg.

Day after dosing	% of dose excreted
1	29 (25 - 33)
2	45 (31 - 53)
3 - 4	64 (45 - 76)
5	73 (58 - 82)
6	80 (65 - 87)
7 - 8	89 (85 - 94)
9	93 (88 - 100)
10 - 11	95 (88 - 102)

The figure given is the average for 4 hens. Ranges
are given in parenthesis.

These values are derived from Table 20a.

3-nitrophenylarsonic acid at the same dosage level was excreted more slowly. 25 - 43% of the dose was excreted as arsenic in 24 hours. At the lower dosage level of 18.9 mg./Kg. these differences between the clearance of the compound when given by mouth and by injection became more marked. On intramuscular administration 90 - 103% of the dose was excreted in three days whereas on oral administration 60 - 71% of the dose was recovered in the same length of time.

Identification of the metabolites of 4-hydroxy 3-nitrophenylarsonic acid present in the excreta.

Three Brown Leghorn hens were given oral doses of 100 mg. 4-hydroxy 3-nitrophenylarsonic acid. The 24 hour excreta was homogenized, centrifuged and chromatographed on Whatman 3 MM paper in the four solvent systems described in Table 4A. Colour reactions showed the presence of unchanged 4-hydroxy 3-nitrophenylarsonic acid, a minor impurity of 4-hydroxy 3-nitrophenylarsonic acid and 4-hydroxy 3-aminophenylarsonic acid (Solvents A, B and C). There was no indication that these compounds were converted to their respective arsenoxides (Solvent F) or that the 3-acetamido derivatives were formed. Because of adverse salting effects, these spots were eluted with water from chromatograms developed in solvent C and rechromatographed in solvents A and B in order to confirm their identity.

Arsenic analyses on a strip chromatogram developed in solvent C showed the presence of three major components corresponding

to the original compound, its amino derivative and the impurity (Figure 5).

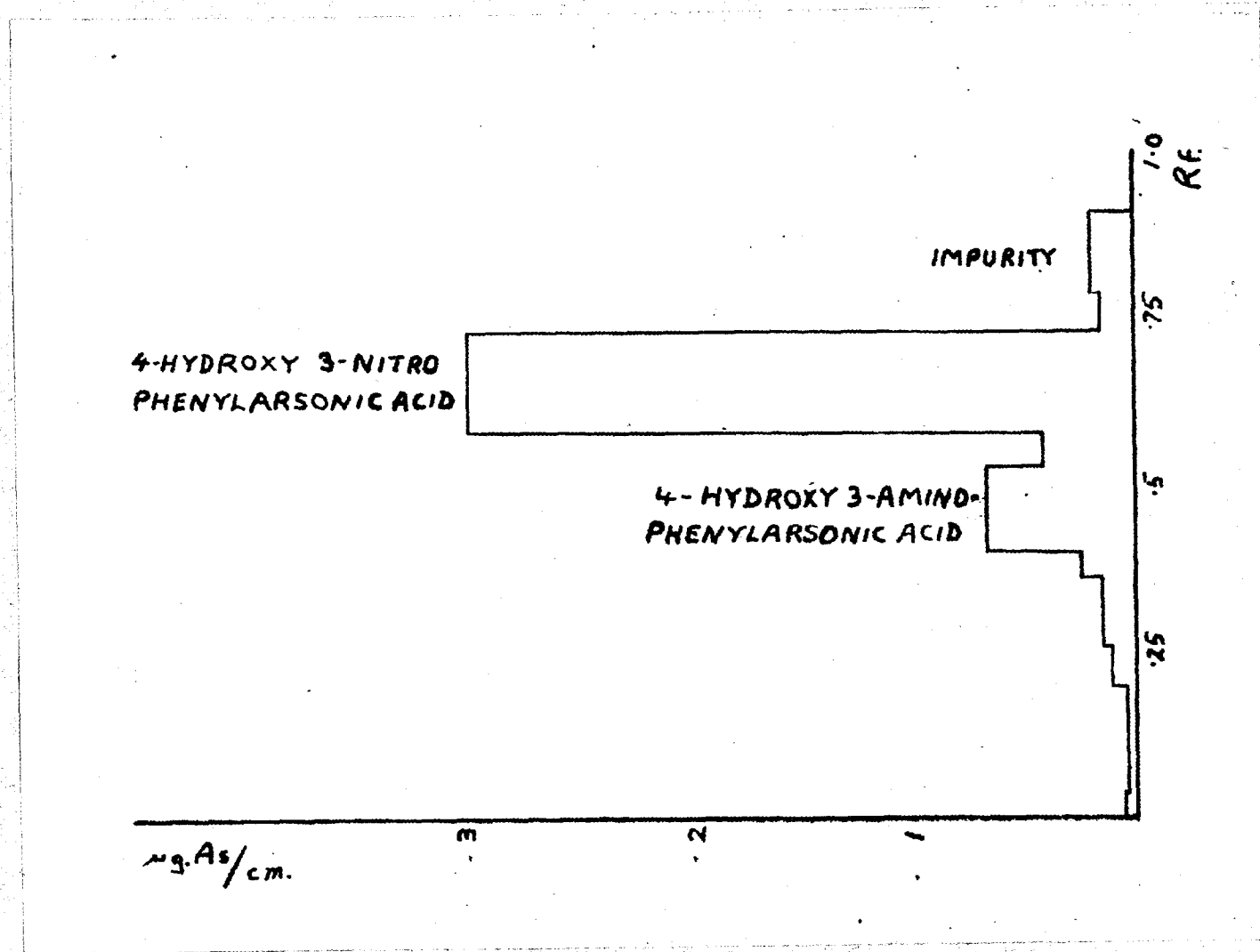
No increased excretion of ethereal sulphates was recorded when 4-hydroxy 3-nitrophenylarsonic acid was administered orally to hens (Table 21A). There was an increase in the output of glucuronic acid but this was largely due to the increased excretion of bile pigment which interfered with the method of estimation (Table 21A). There was no chromatographic evidence for the presence of glucuronide derivatives of metabolites found in the excreta.

Quantitative determination of the metabolites of 4-hydroxy 3-nitrophenylarsonic acid.

Investigations involving the quantitative determination of metabolites present in the excreta of hens given oral or intramuscular doses of 4-hydroxy 3-nitrophenylarsonic acid were carried out under similar experimental conditions to those used for p-arsanilic acid and 4-acetamidophenylarsonic acid. Three hens were used in each three day experiment and all experiments were repeated at one or more different dosage levels. The common dosage levels used were 75.6, 37.9 and 18.9 mg./Kg. for oral administration and 37.9 and 18.9 mg./Kg. for intramuscular administration. No changes occurred to the administered compound under the standard conditions for collecting hen excreta.

When the compound was given by mouth at 37.9 mg./Kg. under

Fig. 5. Chromatogram of the urinary metabolites of 4-hydroxy-3-nitrophenylarsonic acid (Solvent G.)



starvation conditions and the distribution of arsenic between the soluble and solid fractions of the excreta determined, most of the excreted dose was recovered in the soluble fraction and only 3% was found attached to the faecal residues (Table 22A). The excreta supernatant was therefore used for all quantitative estimations of the metabolites and the faecal solids were investigated separately.

1. Oral administration.

The results for the quantitative determination of 4-hydroxy 3-nitrophenylarsonic acid, its amino derivative and total arsenic in the excreta supernatant after oral administration, are shown in Table 23A.

The dosage level had little effect on the rate of excretion of orally administered 4-hydroxy 3-nitrophenylarsonic acid. 57 - 72% of the dose was excreted in three days irrespective of dosage level variations of 19 - 75 mg./Kg.

Throughout the course of the experiments the sum of the estimates for 4-hydroxy 3-nitrophenylarsonic acid and its amino derivative were comparable with the amounts of total arsenic found in the excreta. This confirms these compounds as the major excretory products.

The extent to which orally administered 4-hydroxy 3-nitrophenylarsonic acid was reduced to its amino derivative varied considerably. When the compound was given by mouth at 75.6 mg./Kg.

about half of the administered dose was excreted unchanged in three days and 7 - 20% (average 12%) as 4-hydroxy 3-aminophenylarsonic acid in the same length of time. There was a tendency for the proportion of the administered compound excreted as the 3-amino derivative to increase as the dosage level was reduced.

2. Intramuscular administration.

When the compound was administered to hens intramuscularly at 37.9 mg./Kg., all birds died 25 - 30 hours after dosing. During that period, however, 48 - 70% of the dose was recovered mainly unchanged. At a lower dosage level of 18.9 mg./Kg. 77 - 84% of the dose was excreted in 24 hours and 90 - 104% in three days. Most of the compound was excreted unchanged and only 2 - 6% as 4-hydroxy 3-aminophenylarsonic acid (Table 24A).

There are several important differences between the results outlined above and those obtained from hens given oral doses of 4-hydroxy 3-nitrophenylarsonic acid. The compound was excreted more rapidly, though metabolized to a lesser extent, when given by injection than when given by mouth. Furthermore, despite the ease of elimination of intramuscularly administered 4-hydroxy 3-nitrophenylarsonic acid, the compound was more toxic to birds when administered by this route. These factors suggest that oral and intramuscularly administered 4-hydroxy 3-nitrophenylarsonic acid may undergo dissimilar physiological fates in the hen.

Arsenic residues attached to the solid fraction of hen excreta.

Table 25A shows that 1% of an orally administered dose of 4-hydroxy 3-nitrophenylarsonic acid was eliminated daily in the solid fraction of hen excreta. This amounted to 0.8 - 1.7 μ M arsenic firmly bound to each gm. of dry faecal residue. 30 - 40% of the bound arsenic could be removed by dialysis or extraction with hot HCl., cold NaHCO₃ or EDTA. NaOH (80%) Iodine (85%) and diethylammonium diethyl dithiocarbamate (63%) were the most successful extraction reagents which suggests that part of the arsenic attached to the faecal solids was in the trivalent form. However, paper chromatograms of hot HCl, cold NaOH and iodine extracts of faecal solids only showed the presence of 4-hydroxy 3-nitrophenylarsonic acid.

The reduction of 4-hydroxy 3-nitrophenylarsonic acid by hen faeces.

The reduction of 4-hydroxy 3-nitrophenylarsonic acid to its 3-amino derivative and possibly to arsenoxides attached to the faecal solids, may be of microbiological origin. When the compound (100 μ M) was incubated anaerobically with an extract of hen faeces in 0.05 M phosphate buffer pH 7.4 (40 ml.) for 24 hours at 37^o, 4-hydroxy 3-aminophenylarsonic acid and small quantities of 4-hydroxy-3-nitrophenylarsenoxide were detected chromatographically in the medium (Solvents A, B and C). A sterilized extract did not reduce the compound to these two derivatives under the same conditions.

Factors affecting the excretion of orally administered 4-hydroxy 3-nitrophenylarsonic acid.

1. Diet.

The effect of diet on the metabolism and rate of excretion of 4-hydroxy 3-nitrophenylarsonic acid was examined using starved birds and also birds receiving two different diets. Three hens were starved for 24 hours prior to dosing and for the duration of the three day experiment while other birds were maintained throughout the course of the experiment on a diet of 100 gm. Range Layers Mash/day (Diet 1.) or 100 gm. of a diet in mash form which was observed to be more acceptable to the birds (Diet 2. 1 pt. bran, 1 pt. corn and oats, $\frac{1}{10}$ th pt. S.G. 1 pellets).

The results obtained are recorded in Table 26A. The diet of hens had little or no effect on the rate of excretion of orally administered 4-hydroxy 3-nitrophenylarsonic acid but some effects on the output of 4-hydroxy 3-aminophenylarsonic acid were apparent. Starved birds excreted on average 3.8% of the dose as 4-hydroxy 3-aminophenylarsonic acid over three days whereas birds receiving Diet 1 excreted on average 5.6% as the 3-amino derivative and birds receiving Diet 2 excreted 10.9% of the dose as the 3-amino compound in the same length of time.

2. The distribution of drug residues in the gut.

The distribution of drug residues in the gut contents of

hens receiving oral doses of 4-hydroxy 3-nitrophenylarsonic acid at 37.5 mg./Kg. was followed for five birds which had been killed 6, 12, 24, 48 and 72 hours after dosing. Gut contents and organ washings obtained from the birds were homogenized and centrifuged. The supernatant was analysed for 4-hydroxy 3-nitro and 4-hydroxy 3-aminophenylarsonic acid and the residue was analysed for total arsenic.

Table 27A shows that throughout the period of investigation most of the administered compound (49 - 94%) was found in the crop or the excreta. Smaller amounts were found in the gizzard and only trace quantities were present in other digestive organs. The crop, therefore, appears to be the major organ regulating the excretion of the compound.

The crop was also the major site for the reduction of the administered compound to its 3-amino derivative. The extent to which reduction occurred varied considerably but there was a tendency for the NO_2/NH_2 ratio to fall as the time of death after dosing increased (Table 28A).

Large amounts of the administered dose (0.6 - 16.7%) were found attached to the solid fraction of the crop contents but the properties and identity of these residues have not been investigated.

CHAPTER 6

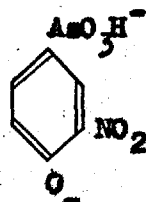
Discussion

The widespread use of arylarsonic acids as growth promoters and medicants in animal husbandry has raised serious problems concerning possible hazards to the health of persons involved in the manufacture, handling and use of these compounds and to the population from the consumption of arsenical residues in meat. Although the general principles governing the toxicity of arsenicals have been known in outline for 40 years and in some detail for 20 years, knowledge of the fate of these compounds in the body is either sketchy or non-existent. Information on the metabolism of these compounds in the hen should be very useful in improving any assessment of the safety of these compounds, explaining their parasitocidal and growth stimulating effects and in designing better and safer compounds for future agricultural use.

The arylarsonic acids used in these studies were all fairly strong dibasic acids with pK_a 's for the first ionisable hydrogen of the order 3 - 4. Thus at physiological pH values, 4-nitrophenylarsonic acid, p-arsanilic acid and 4-acetamidophenylarsonic acid occur largely in the monoionised form.

The 4-hydroxy 3-nitrophenylarsonic acid molecule contains two acidic substituents in the aromatic ring, a dibasic arsono grouping and a monobasic phenolic grouping to which three pK_a values can be

attributed by titration. Spectrophotometric data based on the appearance of the 410 mμ absorption peak characteristic of ionised O^- - nitrophenols suggests the second hydrogen ion dissociation (pK_a 5.8) occurs at the phenolic grouping (Figure 6.) and this has been confirmed by extinction /pH measurements. This compound is therefore found mainly in the following di-ionised form at pH 7.

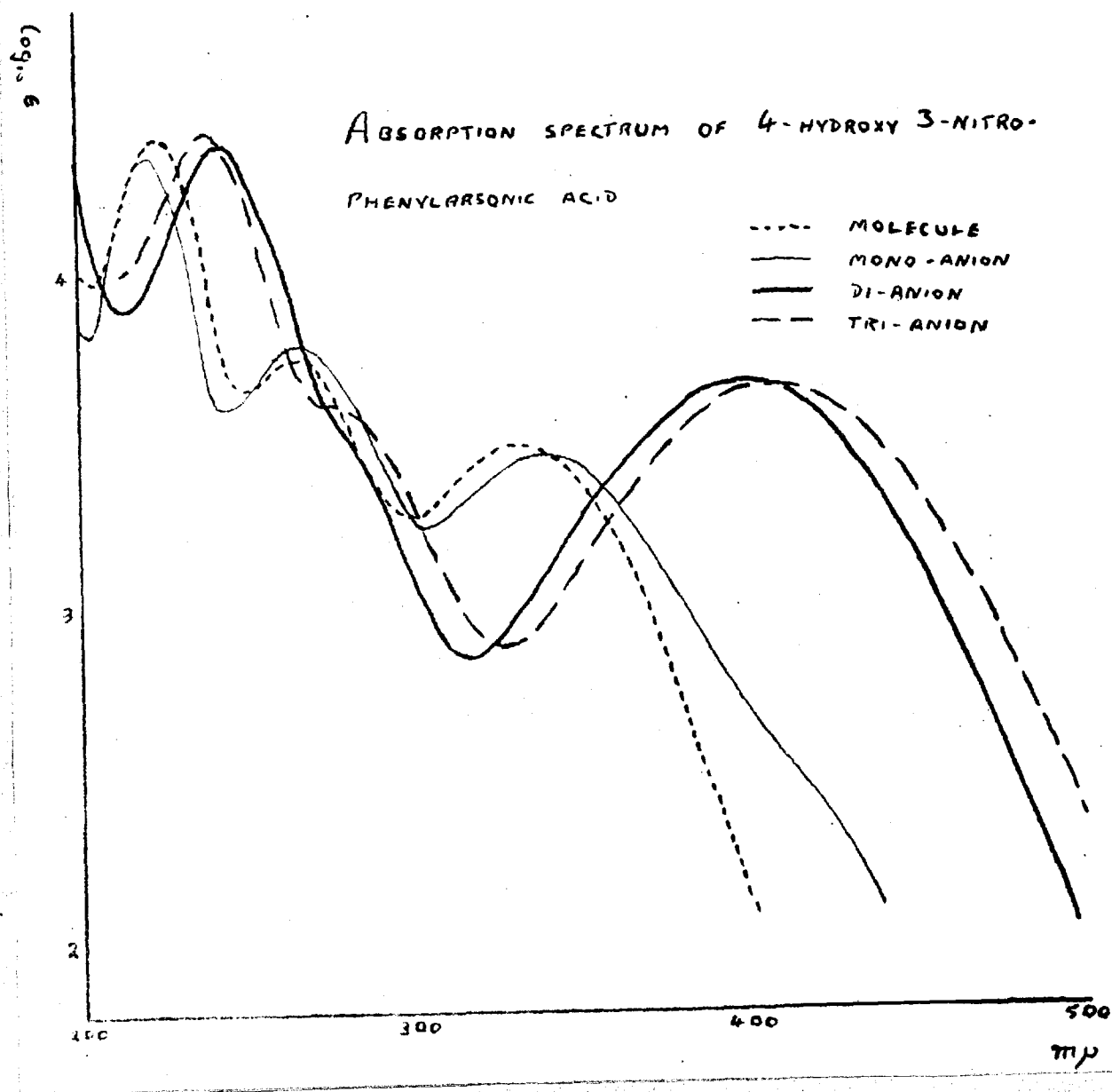


These four arsonic acids are strong electrolytes and are poorly soluble in non-polar solvents (Friedheim, 1938) which suggests they would be rapidly eliminated by the kidney when administered by injection and poorly absorbed from the gut when given by mouth (Brodie and Hogben, 1957).

The absorption of arylarsonic acids.

Optimum alimentary absorption of arylarsonic acids would be expected to occur in the simple mammalian stomach where, under the prevailing acidic conditions, the absorption of these compounds would be dependent on the lipid solubility of the free acid. From the evidence summarised in Chapter 1, it would appear that many arylarsonic acids are poorly absorbed from the mammalian gut. Nevertheless, a small amount of any orally administered arsenical is

Figure 6



excreted in the urine. (Table 1).

The alimentary canal of the hen is morphologically different from that of the mammal but the same physico-chemical considerations governing drug absorption should apply. Thus, the most likely sites for the absorption of acidic compounds are the proventriculus (pH 3 - 4.5) and the gizzard (pH 2 - 3.5).

Hens possess a common cloaca and excrete urine and faeces in a mixed form. Consequently, it is difficult to estimate the extent to which arylarsonic acids are absorbed from the alimentary canal. However, 4-acetamidophenylarsonic acid undergoes two metabolic fates in the hen. The orally administered compound is largely excreted unchanged whereas the intramuscularly administered compound is extensively deacetylated. As the responsible deacetylase enzyme is solely located in the hen kidney, this strongly suggests that 4-acetamidophenylarsonic acid is poorly absorbed from the alimentary canal. By analogy, the poor alimentary absorption of p-arsanilic acid and 4-nitrophenylarsonic acid would be expected since they possess similar physico-chemical properties. This has not been confirmed for the latter compound. Up to 18% of the dose may be present in the tissues of hens, 24 hours after receiving 4-nitrophenylarsonic acid by mouth, which would suggest that the total absorption from the gut is substantial as this compound is rapidly eliminated by the kidney. However, this figure is probably high for reasons already given. Only low concentrations of

4-nitrophenylarsonic acid are present in the cannulated urine of hens given oral doses of the compound. The absorption of 4-nitrophenylarsonic acid from the gut seems to be slow but it is uncertain whether this is due to poor absorption from the proventriculus and gizzard or to the slow passage of food from the crop to these organs. The comparative excretion of an inert polymer, polyethylene glycol, which is not absorbed from the gut, and 4-nitrophenylarsonic acid also suggests that the latter compound is poorly absorbed, but as the passage of food from the crop to the small intestine appears to control the rate of excretion, no definite conclusions can be reached.

The extent to which 4-nitrophenylarsonic acid is absorbed from the gut is, therefore, undecided and it is difficult to see how the absorption of this compound can be measured without recourse to surgically modified hens whereby urine and faeces can be collected separately. Perhaps the development of techniques such as these will prove rewarding in the future when similar problems are encountered.

The total excretion of arylarsonic acids.

Each compound administered orally to hens was subject to wide variations in the rate of elimination which were independent of age, weight of hens and the dosage level used. Because of these variations and the small number of experimental birds, the differences

in the average excretion rates when different experiments are compared are not significant. In order to establish statistical significance between the different rates of excretion a large number of experimental birds would have been required and it was considered that the possible information to be obtained did not justify the effort involved. Experiments using a small number of hens suggest that neither dosage level, nutritional state or breed have much effect on the rate of excretion of orally administered 4-hydroxy-3-nitrophenylarsonic acid and this may hold true for the other compounds.

Of the four arylarsonic acids examined, 4-nitrophenylarsonic acid, p-arsanilic acid 4-hydroxy 3-nitrophenylarsonic acid were excreted relatively slowly after oral dosing averaging 50, 51 and 29% elimination in 24 hours. The fourth compound, 4-acetamidophenylarsonic acid, was excreted more rapidly averaging 70% elimination in the same length of time. When the nitroarylsonic acids were administered to hens, the excretion rate progressively diminished till after four or five days only traces of ^{14}C or arsenic could be detected in the excreta. Complete elimination of the administered dose required 9 - 11 days.

The excretion of orally administered arylarsonic acids may be retarded by the entero-hepatic circulation of the compounds. The elimination of trivalent arsenicals in the faeces after intravenous administration is well established (Voegtlin and Thompson, 1922;

Gruhzit, et al., 1936) and some authors have suggested that appreciable quantities of orally ingested 4-hydroxy 3-nitrophenylarsonic acid and p-arsanilic acid are excreted through the bile (Kerr, Cavett and Thompson, 1963; Overby and Fredrickson, 1963).

Although this may be true when the daily intake of these compounds is low (3 - 5 mg./bird/day) there is no evidence that proportionately greater quantities are eliminated in the bile when relatively larger doses are used. The poor alimentary absorption of arylarsonic acids and the small faecal excretion when these compounds are given by injection suggests that the entero-hepatic circulation of these compounds is of diminished importance at higher dosage levels (Chen, Anderson and Leake, 1930; Cavier, 1946; McChesney, Hoppe, McAuliff and Banks, 1962).

The individual variations in the rates of elimination of orally administered arylarsonic acids may be attributed to the peculiar structure of the hen foregut. The anterior end of the alimentary canal includes a blind food storage organ, the crop, in which food may be retained for an indefinite length of time. (Marshall, 1960). The available evidence from the distribution of drug residues through the alimentary canal suggests these compounds are also retained in the crop. Thus the intermittent and variable muscular activity of the crop and gizzard may account for the wide variations found in the individual excretion rates. In support of this hypothesis it has been shown that the factors governing the rates

of excretion of 4-nitrophenylarsonic acid and an inert polymer, polyethylene glycol, which is not absorbed from the gut, are very similar. The slow excretion of 4-nitrophenylarsonic acid is therefore due to physical factors governing the rate of passage of food through the alimentary canal and not the absorption of the compounds or the entero-hepatic circulation.

Arylarsonic acids, in common with many other strong organic acids, are retained by the tissues to a minor extent. Most of the compound is rapidly eliminated in the urine. The sodium salts of p-arsanilic acid and 4-acetamidophenylarsonic acid are almost quantitatively recovered in the urine in a few hours when injected into rabbits and man and very little arsenic is recovered in the faeces (Voegtlin and Thompson, 1922; Ingerscheimer and Rothmann, 1909).

The sodium salts of 4-nitrophenylarsonic acid, p-arsanilic acid, 4-acetamidophenylarsonic acid and 4-hydroxy 3-nitrophenylarsonic acid are also rapidly eliminated when injected into the breast muscle of hens. 80 - 90% of the dose is recovered in the excreta in 24 hours and nearly 100% in three days. The excretion of 4-hydroxy 3-nitrophenylarsonic acid administered at 37.5 mg./Kg. is a notable exception but this dosage level is fatal to the hen and at the present time the experiment was performed the intraperitoneal LD₅₀ of the compound (34 mg./Kg.) was not known.

It is impossible to decide from these results, the extent to which these compounds are excreted by the kidney. From the limited

experimental evidence available of the concentrations of 4-nitro-phenylarsonic acid in the cannulated urine of hens 30 minutes after receiving intramuscular doses of the compound, it appears that the hen kidney does not differ significantly from the mammalian kidney in its ability to eliminate these compounds.

The removal of arsenic from the aromatic nucleus.

Some early workers claim to have isolated or detected significant amounts of inorganic arsenic from the urine of animals given intramuscular doses of aromatic arsenicals (Sieberg, 1916; Nierenstein, 1909). The preparations administered often lacked a satisfactory criterion of purity and the techniques used for the isolation of urinary metabolites have been criticised since they may result in the further degradation of the compounds (Voegtlin 1925). More recently, Maren (1949) has produced some evidence suggesting that inorganic arsenic is released from mapharsen in the dog but Crawford and Levvy (1947) could find no inorganic arsenic in the excreta of rabbits receiving phenylarsonic acid or phenylarsenoxide. As inorganic arsenic is an accumulative poison and a suspected carcinogen, the possible cleavage of arsenic from the aromatic ring and its retention in the tissues are important public health issues concerning the safety of arylarsonic acids in poultry feeds.

Should such a release of arsenic occur it would be expected that both ^{moieties} ~~metabolites~~ would suffer dissimilar biochemical fates in

the hen which would be revealed by their relative excretion rates. The recent findings of Overby and Fredrickson (1963) suggests that no such cleavage occurs when p-arsanilic acid is administered to hens in the feed. This is in agreement with the results for some experiments conducted with 4-nitrophenylarsonic acid. When ^{14}C labelled 4-nitrophenylarsonic acid is given to hens by mouth, the total estimates of inorganic arsenic are less than 2% of the dose in five days and are below the lower limits for the method of estimation. Estimates for ^{14}C and total arsenic in the excreta are in good agreement which suggests that the arsenic remains firmly attached to the aromatic nucleus. Moreover, when p-arsanilic acid, 4-acetamidophenylarsonic acid and 4-hydroxy 3-nitrophenylarsonic acid are administered to hens, colorimetric estimates of total metabolites and total arsenic are also in agreement and it appears that the arsono substituent of these compounds are stable.

The tissue distribution of 4-nitrophenylarsonic acid.

The distribution of arsenic in the tissues of rabbits receiving intravenous doses of arylarsonic acids or arsenoxides has been investigated by Hogan and Eagle (1944) and Chance, Crawford and Levy (1946). These workers found that whereas the arsenoxides were extensively retained by the tissues, the arsonic acids were only bound by the tissues to a minor extent and most of the dose was rapidly eliminated in the urine.

It is generally assumed that the toxicity of arylarsonic acids

is due to their partial conversion to arsenoxides, compounds with known affinities for protoplasmic thiol groups and that this is a predominant factor governing the binding of arsenicals to the tissues. Thus when arylarsonic acids are administered to animals, the tissue arsenic levels found are likely to be a function of both these changes and their reversibility.

The presence of arsenic in the tissues of hens receiving arylarsonic acids in the diet for several weeks has been determined by a number of workers. The extent to which arsenic accumulates in the organs depends largely on the original feed concentration (Abbot, et al., 1955; 1956). At the levels recommended for their agricultural use, these are usually less than 3 ppm. As_2O_3 . The findings of these workers give no indication of the absolute amounts of arsenic present in each organ or whether orally administered arylarsonic acids are extensively absorbed from the gut. Furthermore, it is not known whether the absorbed arsenic is metabolized in the same way as the intravenously administered compound.

In order to obtain more information on some of these points three hens were given single oral doses of 4-nitrophenylarsonic acid and killed after 24 hours for the tissue arsenic levels to be determined. As orally administered 4-nitrophenylarsonic acid is only slowly excreted, it was hoped that this period of time would be sufficient for arsenic equilibration between the tissues to occur. Arsenic is ubiquitously distributed in nature and estimates of total

arsenic in the tissues representing administered 4-nitrophenylarsonic acid are subject to some errors owing to residual background arsenic. However, judging from the satisfactory total recoveries, this does not affect the gross distribution of the compound in the tissues.

24 Hours after the administration of the compound, 18% of the dose was found in the tissues. This figure may be misleading owing to the contamination of some tissues with arsenic from other sources and cannot be regarded as a reliable index of absorption. The remainder of the dose was either present in the alimentary canal or the excreta. The crop and gizzard still contained relatively large amounts of the dose 24 hours after the administration of the compound. Moreover, a similar distribution of drug residues has been found throughout the alimentary canal of hens given oral doses of 4-hydroxy 3-nitrophenylarsonic acid. The retention of these compounds in the crop and gizzard suggests that these are the organs which ultimately regulate their rate of elimination.

High arsenic concentrations are present throughout the whole of the alimentary canal but the concentrations in the small intestine are substantially lower than those in the crop and gizzard. Though the absorption of arylarsonic acids from the small intestine cannot be completely eliminated, it is more probable that this phenomenon reflects the dilution of the compound by the secretions of the pancreas, gall bladder and small intestine.

After single oral doses of 4-nitrophenylarsonic acid, only

small amounts of arsenic are found in the tissues. The viscera, skin and feathers, liver and kidney contain the highest arsenic concentrations (14 - 3 $\mu\text{g. As./gm.}$). Other organs possess lower concentrations of arsenic and only traces are detectable in the fat and muscle (0.5 - 0.3 $\mu\text{g. As./gm.}$). These results are in general agreement with the findings of Cavett (1960) for the tissue distribution of ^{14}C labelled 4-nitrophenylarsonic acid in birds receiving the compound for six days in the rations at 250 ppm. However, only traces of arsenic were found in the bile of birds given single oral doses of 4-nitrophenylarsonic acid whereas Cavett reports high concentrations of ^{14}C in the bile of hens receiving the labelled compound in the diet. This probably reflects a difference between the fates of free and tissue bound 4-nitrophenylarsonic acid. Judging from the results of Cavett, tissue bound 4-nitrophenylarsonic acid appears to be only slowly metabolised and the arsenic residues found in the bile may result from this source.

The presence of arsenic in the tissues of animals receiving arylarsonic acids in the diet may constitute a hazard to the health of the consumer and knowledge of the metabolism of these compounds in the hen is, therefore, important in assessing the health risk. The major chemical changes occurring to nitroarylsonic acids are appreciably less toxic. Since these metabolites are readily eliminated by the kidney, this pathway does not constitute a serious toxicological problem.

4-Nitrophenylarsonic acid → p-Arsanilic acid
MTD (rats orally) 75 mg./Kg. MTD (rats orally) 400 mg./Kg.

4-Hydroxy 3-nitrophenylarsonic acid → 4-Hydroxy 3-aminophenylarsonic acid.
LD₅₀ (rats i.p.) 66 mg./Kg. LD₅₀ (mice i.p.) 775 mg./Kg.
MTD (rats i.p.) 500 mg./Kg.

The formation of arsenoxides in the hen from the reduction of arylarsonic acids is far more dangerous as such a chemical change may increase the toxicity by a factor of 30 to 250. Since these compounds are tightly bound by the tissues and only slowly excreted, their possible accumulation in toxic proportions would appear to be a serious health hazard. However, little arsenic was found in the tissues of hens 24 hours after receiving oral doses of 4-nitrophenylarsonic acid. Furthermore, no accumulation of these compounds or their metabolites has been reported after chronic administration of p-arsanilic acid or 4-hydroxy 3-nitrophenylarsonic acid (Frost, Overby and Spruth, 1955; Kerr, Cavett and Thompson 1963) nor have there been any manifestations of toxicity under similar experimental conditions (Prier, Nees and Derse, 1963). The toxic accumulation of arsenoxides and their derivatives in tissues does not seem to occur at the recommended feed concentrations.

The carry over of arsenic from the tissues to the consumer has recently received experimental attention (Overby and Frost, 1962). When tissue bound p-arsanilic acid was included in the diet of rats, the secondary accumulation of arsenic in the tissues of the recipient was found to be slight. It appears, therefore, that the agricultural

use of arylarsonic acids constitutes little danger to the consumer but perhaps experiments on the chronic toxicity of these compounds and their carry over should be extended to a much wider range of animals.

The metabolism of arylarsonic acids.

The arylarsonic acids used in these studies undergo few metabolic changes in the hen and are mainly excreted unchanged. In this respect they show certain similarities with arylsulphonic acids (Sammons, Shelswell and Williams, 1941; Smith and Williams, 1948) with which they share certain physico-chemical properties. However, as minor changes to the molecule may have profound biological effects, any comparison with the biochemically inert compounds must not be drawn too closely.

Arylarsonic acids do not undergo oxidative and synthetic changes in the body neither are they degraded to inorganic arsenic. They are subject to two types of chemical change:-

- (1) Changes to the second aromatic substituent.
- (2) Reduction of the arsono substituent.

Of these, only the first has been examined in any detail. The second route of metabolism undoubtedly occurs in the rabbit but could not be confirmed in the hen with present analytical techniques. This does not necessarily mean that arsenoxides are not formed by the hen as the known affinity of these compounds for the tissues, their

slow rate of excretion and their possible reconversion to arylarsonic acids would make any detection of arsenoxides in the excreta difficult. Moreover, it is convenient to assume that conversion to arsenoxides do take place to account for the toxicity of arylarsonic acids.

Nitroarylarsonic acids.

Mechanisms for the reduction of nitro compounds to arylamines in the hen are known to occur in vivo (Crowdle and Sherwin, 1923) and in vitro (Westerfield, Reichert and Higgins, 1957) and the reduction of nitroarylarsonic acids to their amino derivatives could have been predicted with some confidence.

There is a quantitative difference in the output of the amino metabolite according to the route of administration of the original nitro compounds. After intramuscular administration less than 5% of the dose is excreted as the amino derivative whereas after oral administration a much greater proportion of the dose is reduced. Bray, Hybs, James and Thorpe (1953) have shown that the intestinal flora may contribute towards the reduction of orally administered nitro compounds and this may be particularly important when the compounds are only slowly absorbed from the gut. Hen faeces possess considerable nitroreductase activity and bufferised extracts when incubated with nitroarylarsonic acids will reduce these compounds to the corresponding aminoarylarsonic acids and to their arsenoxides. This activity appears to be of microbiological origin since it is

abolished by the sterilization of the extract.

The intestinal microflora of the rat has been successfully suppressed by administering sulphonamides and tetracyclines orally (Dodgson, Powell, Rose and Tudball 1961; Lloyd, 1961) and it was hoped that similar techniques could be used in the hen thus affording an opportunity to determine the contribution of the tissues towards the reduction of orally administered 4-nitrophenylarsonic acid. As sulphonamides interfere with the analytical method for estimating p-arsanilic acid, the use of this group of compounds as gut sterilants had to be discarded. Aureomycin is a known inhibitor of the nitro-reductase system of *E. coli* (Saz and Slie 1954) with definite antimicrobial activity towards the intestinal microflora (Jacob, Schweinberg and Rutenberg, 1951; Brisou and Ardeson 1952) and was therefore used to demonstrate the contribution of the gut microflora towards the reduction of orally administered 4-nitrophenylarsonic acid.

Aureomycin administered by either route appears to inhibit avian nitroreductase in vivo as determined by intramuscular 4-nitrophenylarsonic acid and consequently its effects must be interpreted with caution. As the reduction of oral 4-nitrophenylarsonic acid is inhibited to a greater extent by oral pretreatment with aureomycin than after intramuscular pretreatment, the positive contribution of the intestinal microflora seems to be indicated. However, these effects are not statistically significant at the $P = 0.05$ level.

The alimentary canal of the hen is known to possess a varied

microflora throughout the whole of its length and all the organs are possible sites for microbiological nitroreductase activity. The distribution of drug residues throughout the alimentary canal show that the crop may retain large quantities of orally administered nitroarylarsonic acids for long periods of time during which they may be reduced to the amino derivatives. An analysis of the crop contents 6 - 48 hours after birds had received oral doses of 4-hydroxy-3-nitrophenylarsonic acid revealed considerable amounts of 4-hydroxy-3-aminophenylarsonic acid present. Experiments with 4-nitrophenylarsonic acid have demonstrated high nitroreductase activity in the crop in vivo which appears to be linked to the nutritional state of the bird. As the activity in hens receiving rations was ten times that of starved birds it is possible that the effects of aureomycin on the reduction of orally administered 4-nitrophenylarsonic acid would have been more pronounced had the hens received rations throughout the course of the experiment.

Though the activity of the gut may account for the greater proportion of the excreted aminoarylarsonic acids when the nitro compounds are given by mouth, nitroreductase activity also occurs in the tissues. Westerfield has described two chicken liver preparations with nitroreductase activity. One enzyme system present in the particulate fraction reduced 4-nitrophenylsulphonamide to sulphanilamide and specifically required NADH_2 as co-substrate. Another system associated with xanthine dehydrogenase activity reduced 2,4-dinitro-

phenol to 2-nitro 4-aminophenol but had no activity towards 4-nitro-phenylsulphonamide.

Dr. Robinson has demonstrated nitroreductase activity in hen liver homogenates which, under optimum conditions, can account for the output of p-arsenilic acid in vivo when 4-nitrophenylarsonic acid is administered intramuscularly. The enzyme is located in the microsome and supernatant fraction and possesses a broad pH optimum between 7.2 and 8.0. Both NADH_2 and NADPH_2 can function as electron donors but in contrast to many other reported nitroreductase preparations, the system is unaffected by oxygen. There appears to be a general requirement for a divalent metal ion. The system is inhibited by EDTA while magnesium and manganese ions added in equivalent concentrations restore enzyme activity. The nitroreductase system of hen liver has a broad co-substrate specificity and a number of different reducing systems may be utilized. Of the systems tested in dialysed preparations glucose -6-phosphate and NADP is the most effective but malate and succinate and NAD also possess considerable activity.

It is generally recognised that several nitroreductases may be present in the mammalian liver. Thus the two NADH dependent systems described by Müller (1958) which catalyze the reduction of β -(2-nitrobenzoyl)alanine can be readily distinguished from the NADPH dependent system of Fouts and Brodie (1957) for the reduction of chloramphenicol. Whether the microsomal system present in hen

liver, responsible for the reduction of 4-nitrophenylarsonic acid is a single entity (or complex) or whether it represents a number of different systems with the common ability to reduce nitroarylarsonic compounds, must remain a matter of some conjecture. However, the lack of donor specificity when compared with the partly purified system described by Westerfield for the reduction of 4-nitrophenyl sulphonamide suggests that more than one nitroreductase system may be present.

When nitroarylarsonic acids are administered to hens by mouth, a small but fairly constant proportion of the dose is always associated with the solid fraction of the faeces. Normal hen excreta does not bind arylarsonic acids to any significant extent at room temperature whereas these metabolites are firmly attached to the faecal solids and are not removed by dialysis, treatment with EDTA or with mild acidic and basic reagents. These bound metabolites appear to be in a completely different chemical form to those present in free solution in the excreta supernatant. The experimental evidence suggests that these metabolites are in part bound in the trivalent form, possibly as arylthioarsenites. Thus, reagents which oxidise or hydrolyse arylthioarsenites release appreciable amounts of arsenic from the faecal solids. Furthermore, hot acid extracts of the faecal solids contain small but definite traces of 4-nitrophenylarsenoxide and 4-aminophenylarsenoxide as shown by paper chromatograms.

It is not known whether the metabolites in the faecal solids

arise from the tissues of the hen or from the activity of the gut microflora. The micro-organisms of the gut and of hen faeces, however, possess powerful reducing properties and in the case of the latter, reduction of arylarsonic acids to arsenoxides has been demonstrated. This permits a certain degree of speculation as to the mode of action of arylarsonic acids in stimulating the growth of hens and in decreasing the caecal coccidia populations.

The supplementation of hen or turkey rations with p-arsanilic acid or 4-hydroxy 3-nitrophenylarsonic acid results in a definite decrease in the faecal and caecal anaerobe populations and the Clostridia and Coliforms are particularly affected (Anderson, Cunningham and Slinger, 1952; Elam, et al., 1953). The powerful reducing properties of these organisms may result in the lethal synthesis of arsenoxides from arylarsonic acids thus effecting their own inhibition. Such a phenomenon is not beyond the bounds of possibility as the sensitivity of these organisms to trivalent arsenicals has already been established (Albert, Falk and Rubbo, 1944). Should the use of these compounds decrease the total bacterial populations, the reduced competition between the gut and gut flora would make more ingested food available to the hen and improvements in feed efficiency would be observed. Alternatively, any selective suppression of the pathogenic bacteria of the gut may improve weight gains and feed efficiency since the birds would be less susceptible to diseases. Furthermore, beneficial effects on vitamin requirements

may be noted if these compounds reduced the populations of bacteria requiring vitamins for growth or if induced changes in the gut microflora resulted in a numerical increase in the vitamin synthesizing bacteria. In view of the powerful nitroreductase activity of the crop, the microflora of this organ and its relationship to the nutrition of the hen would appear to warrant much greater attention than has hitherto been given.

It is more difficult to explain the inhibition of the caecal coccidia by arylarsonic acids. This may be due to the release of arsenoxides from anaerobes by autolytic changes but the possibility of a more direct interaction must not be ruled out.

In conclusion, it appears that a better understanding of the coccidiostatic properties and growth promoting action of arylarsonic acids in the hen would benefit from a more critical examination of the metabolism of these compounds by isolated strains of bacteria, the toxic effects of the metabolites towards the gut flora and the changes of the gut flora populations during dietary supplementation with arsenicals.

p-Arsenilic acid and 4-acetamidophenylarsonic acid.

Atoxyl was the first aromatic arsenical to find medicinal application but the fate of this compound has never been satisfactorily resolved (see Chapter 1.). In theory, several chemical changes may occur to the compound in the body. The arsono substituent may be reduced, inorganic arsenic may be released from the compound, oxidative

and synthetic changes may occur in the aromatic ring or the arylamino group may be acetylated. In practice none of these changes have been detected by chemical methods. Recently, Overby and Fredrickson (1963) have shown that p-arsanilic acid administered to hens is excreted unchanged. However, small quantities of the administered compound are bound by the tissues and apparently occur in two chemical forms of unknown identity which can be distinguished from p-arsanilic acid or 4-aminophenylarsenoxide. These residues seem to revert back to p-arsanilic acid on excretion.

The results for p-arsanilic acid partly confirm the findings of these workers. No 4-acetamidophenylarsonic acid or 4-aminophenylarsenoxide could be detected in the excreta after oral or intramuscular administration of p-arsanilic acid at 50 or 100 mg./Kg., nor was there any chromatographic evidence for the presence of other metabolites. Moreover, in vitro experiments have shown that no significant acetylation of this compound occurs in hen liver homogenates.

The antiparasitic action of arylarsonic acids in animals is generally attributed to their reduction to arsenoxides. Apart from phenylarsenoxide, the trivalent form of these compounds have never been detected in the excreta. This may be due to the ease with which arsenoxides in solution are oxidised back to the parent arsonic acids. From a qualitative observation, 4-acetamidophenylarsenoxide appears to be one of the most stable of these compounds in solution and it was hoped that by administering 4-acetamidophenylarsonic acid to the hen,

the reduction of the arsono substituent in vivo would be successfully demonstrated.

When this compound was given to hens by mouth or by injection, no 4-acetamidophenylarsenoxide was detected in the excreta. It appears, therefore, that arsenoxide formation in the hen is a very minor metabolic pathway. So far no suitable micro and ultramicro chemical techniques have been devised to investigate this phenomenon.

4-Acetamidophenylarsonic acid is deacetylated in the hen and partly excreted as p-arsanilic acid but the extent to which deacetylation takes place, depends on the route of administration. Intramuscularly administered 4-acetamidophenylarsonic acid is extensively deacetylated whereas the orally administered compound is mainly excreted unchanged.

The fate of orally administered 4-acetamidophenylarsonic acid in the hen differs from that of the acetylated sulphonamides which are mainly excreted in the N_4 deacetylated form when given by mouth. (Shaffer and Beiter, 1950). The metabolism of sulphonamides in the hen is complicated by the presence of enzymes in the liver capable of N-acetylation and enzymes in the kidney with strong β -deacetylase activity.

The fates of p-arsanilic acid and 4-acetamidophenylarsonic acid are much simpler. Under the experimental conditions used, no acetylation of p-arsanilic acid occurred in hen liver homogenates and as far as can be determined, the reversible acetylation of this compound

does not occur in vivo. It is not known whether this lack of acetylation in vitro is due to the inability of p-arsanilic acid to penetrate liver microsomes where the transfer of acetyl groups from CoA to arylamines normally takes place (Fraser, 1958) or to the substrate specificity of the enzyme. This point will probably only be resolved after hen liver aryl-N-acetylase has been purified.

The aryl-N-deacylase in hen kidneys described by Shaffer and Beiter (1950) is probably identical to the system described in Chapter 4 and to the mitochondrial system of Nimmo Smith (1960). This enzyme has a broad specificity towards substrates with optimum activity for para substituted N-acetylated arylamines and probably contains thiol groups at the active centre. Differences in the metabolism of orally administered acetylated sulphonamides and 4-acetamidophenylarsonic acid may be explained by the different extents to which these compounds are absorbed from the gut. Thus the N₄ acetylated sulphonamides are readily absorbed from the gut and undergo deacetylation in the hen kidney prior to elimination. The same enzyme system can hydrolyse 4-acetamidophenylarsonic acid in vitro and in vivo but since little deacetylation occurs after oral administration, it appears that alimentary absorption is poor.

Conclusions.

It is evident from these experiments that arylarsonic acids undergo few metabolic transformations in the hen and are mainly excreted unchanged. The compounds examined, however, are subject to two types

of chemical change as illustrated in Figure 7.

These are changes to the second aromatic substituent and reduction of the arsono substituent to form arsenoxides. The former are the most extensive and have been examined in some detail. Investigation of the latter pathways has been hampered by the practical difficulties already outlined.

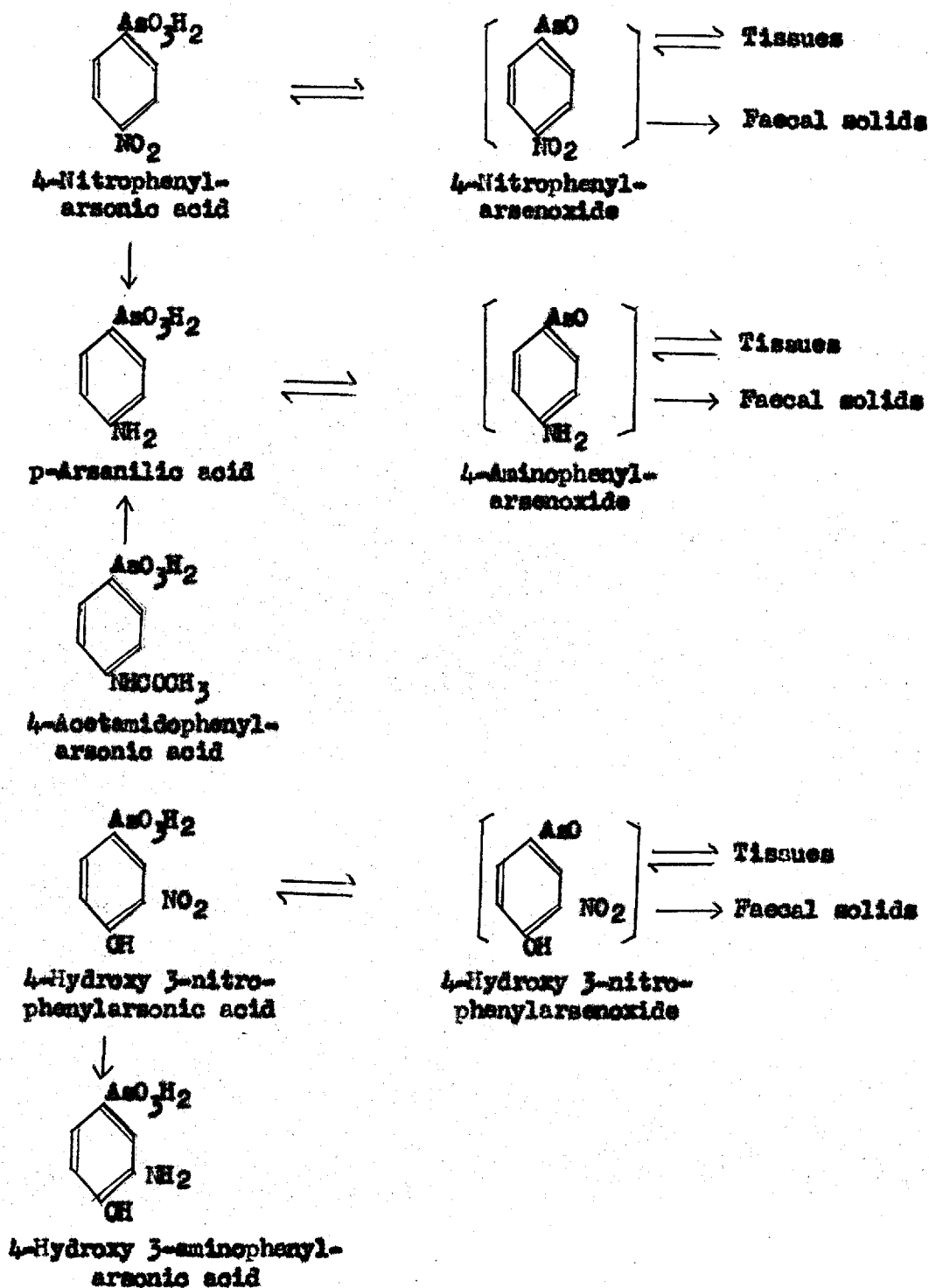
As far as can be determined from the experimental data the fate of these compounds in the hen is largely governed by their physico-chemical properties in accordance with well established principles. These compounds, which are non lipid soluble strong acids should be readily eliminated by the kidney when given by injection and poorly absorbed from the gut when given by mouth. The rapid elimination of intramuscularly administered arylarsonic acids has been demonstrated but the extent to which these compounds are absorbed from the gut has not been accurately determined. Indirect evidence does suggest that 4-acetamidophenylarsonic acid and 4-nitrophenylarsonic acid are only slowly absorbed.

Analytical techniques so far developed are insufficiently sensitive to follow the dynamics of the chemical fate of these compounds in the tissues but useful observations have been obtained from the tissue arsenic levels. The half life of these compounds in the tissues is relatively short and soon after dosing the arsenic levels of most organs decline as these compounds are eliminated in the urine.

It is generally recognised that anions penetrate the lipo-

Figure 7

The fate of 4-nitrophenylarsonic acid, p-arsanilic acid, 4-acetamidophenylarsonic acid and 4-hydroxy 3-nitrophenylarsonic acid in the hen



protein membrane of the endoplasmic reticulum only with difficulty (Brodie, Gillette and La Du, 1958). Consequently enzyme catalysed transformations to these compounds in this cellular compartment may occur only to a slight extent (as in the case of nitroarylarsonic acids) or may be absent (as in the case of p-arsanilic acid and aryl-N-acetylase).

Mitochondrial membranes appear to be much more permeable to organic acids and the extensive deacetylation occurring to intramuscularly administered 4-acetamidophenylarsonic acid may be partly attributed to the location of hen kidney aryl-N-deacylase in the more permeable cell compartment. In view of the apparently different permeability mechanisms controlling the penetration of these two cell components by organic acids, it may be significant that the enzymic mechanisms for hippuric acid synthesis are also located in the mitochondria (Kielley and Schneider, 1950; Leuthardt and Nielson, 1954).

The importance of the gut microflora of the hen in the metabolism of orally administered foreign compounds should never be overlooked even when the compounds are readily absorbed since microbial activity may be substantial in the anterior regions of the gut before the normal sites of drug absorption. Some of the chemical changes occurring to orally administered arylarsonic acids arise from the activity of the gut microflora and not from the tissues. The powerful reducing activity of these organisms is capable of

transforming nitro compounds into arylamines and arylarsonic acids into toxic arsenoxides. Arsenoxides thus formed may have a selective bactericidal effect which may be of benefit to the hen should the following alterations to the microflora occur

- (a) A reduction of the total bacterial populations.
- (b) Suppression of pathogenic bacteria.
- (c) An increase in the populations of vitamin synthesizing bacteria.
- (d) A decrease in the populations of bacteria requiring vitamins.

These effects may be difficult to establish under field conditions. However, the bactericidal action of arylarsonic acids on cultures of the gut microflora in vitro ought to be examined more thoroughly.

As far as can be determined from these studies and from the work of others, arylarsonic acids in agriculture do not appear to be a serious public health risk. In the hen, these compounds are not degraded to inorganic arsenic, a possible carcinogen, but are largely excreted with the arsono substituent intact. Organic arsenicals do not appear to be carcinogenic. No evidence of carcinogenicity was found in subchronic toxicity studies on 4-hydroxy-3-nitrophenylarsonic acid in mice (Prier, Nees and Derse, 1963) nor was there any evidence that p-arsanilic acid potentiates the carcinogenic action of 9, 10 dimethylbenzanthracene (Bantwell, 1963).

There seems to be little danger from the direct toxic effects of arylarsonic acids. In the hen these compounds are largely excreted unchanged or metabolised to compounds of lesser toxicity. If these compounds and their excretory metabolites were present in tissue residues, their repeated ingestion would not lead to any cumulative effects and they would not constitute a serious health risk. The possibility remains that the tissue bound arsenic may be in chemical forms which are completely different from the excretory metabolites and these may be an unknown toxicological hazard. Overby and Frost (1962), however, could find no evidence that these residues were retained in the tissues when administered to rats.

These experiments have been interpreted on the assumption that the toxicological and biochemical findings in animals are directly applicable to man but this assumption may not be valid. Though animals may possess broadly similar mechanisms whereby organic compounds penetrate to receptor sites and exert their toxic and therapeutic actions, wide variations are encountered in the ability of animals to metabolize foreign compounds both to active and to inactive derivatives (Williams, 1959). Moreover, foreign compounds may undergo different metabolic fates in man and animals. Brodie (1964) has pointed out that interspecies differences in the fate of organic compounds are more likely to occur if they are lipid soluble. Compounds of low lipid solubility seem to be subject to less

interspecies variations in their metabolic fate and experimental findings in animals can be extrapolated to man with a greater degree of confidence.

Minor metabolic pathways appear to be responsible for the activation of arylarsonic acids to compounds of greatly increased toxicity. Thus, small variations in the abilities of two species to reduce these compounds to arsenoxides may result in large differences in their ability to tolerate these compounds. Perhaps in the final analysis it is advisable to extrapolate the toxicological findings in animals to man with some caution.

APPENDIX

Table 1A

Example of isotope dilution analysis.

In the excreta supernatant, let X gm. of material be present with an original specific activity of x. For isotope dilution, let Y gm. of material be added and let the isolated material have a specific activity of y.

It follows that

$$\frac{X}{Y + X} = \frac{y}{x}$$

By rearrangement

$$X = \frac{Y}{\left(\frac{x}{y} - 1\right)} \quad \text{Equation (1)}$$

$[1 - {}^{14}\text{C}]$ 4-Nitrophenylarsonic acid had specific activities of 105.5 $\mu\text{C/gm.}$ (2.5 cm. reference) and 124.4 $\mu\text{C/gm.}$ (1 cm. reference).

Hen 13 (1.55 Kg.) was fed 55.4 mg. $[1 - {}^{14}\text{C}]$ 4-nitrophenylarsonic acid containing 5.85 μC (Worked on the basis of the 2.5 cm. reference). After 24 hours, the excreta supernatant contained 1.91 μC (32.7% of the dose).

1.068 gm. 4-nitrophenylarsonic acid (M.Wt. 247) were added to a quarter of the excreta supernatant. On re-isolation and purification, it had a specific activity of 0.436 $\mu\text{C/gm.}$ (1 cm. reference) and after conversion to the benzylamine salt (M.Wt. 354) the specific activity fell to 0.303 $\mu\text{C/gm.}$

The specific activity of pure 4-nitrophenylarsonic acid

$$= 0.303 \times \frac{354}{247} = 0.434$$

Using equation (1),

Wt. 4-nitrophenylarsonic acid present in one quarter of the excreta supernatant is :-

$$\frac{1.068}{\left(\frac{124.4}{0.434} - 1 \right)} = 0.00374$$

∴ % Dose excreted as 4-nitrophenylarsonic acid

$$= \frac{0.00374 \times 4 \times 100}{0.0554} = 27.0\%$$

Table 2A

Paper chromatography of arsenicals

Solvent systems:

A, Acetonitrile: ammonia solution (sp.g. 0.88): water (13: 1: 6) for 1.5 hours. B, Acetonitrile: conc. nitric acid: water (39: 1: 10) for 1.5 hours. C, n-Butanol: acetic acid: water (4 : 1: 2) for 16 hours. D, Benzene: acetic acid: water (1 : 1: 2) for 5 hours. E, Diisopropylether saturated with water, for 5 hours. F, n-Butanol: ammonia solution (sp.g. 0.88): water (4 : 1: 3) for 16 hours.

All ratios are by volume. Solvents A and B were developed by ascending technique until the solvent front had travelled approximately 20 cm. from the origin. The other solvents were run using the descending technique. For these solvents, the front was normally about 35 cm. from the origin.

Rf. values are quoted for Whatman No. 1 paper.

Locating agents:

(a) 0.1% ^w/_v p-Dimethylaminocinnamaldehyde (DMO) in 2 N acetic acid revealed amino compounds as red spots. For acetamido compounds the red colour developed slowly, but the reaction could be accelerated by light spraying with 2 N.HCl. and heating at 100°C for 5 minutes before using this reagent. (b) 0.8% Titanous chloride in 0.3 N.HCl. was used to reduce nitro compounds prior to spraying

with the DMC reagent. (c) 0.25% Diazotized sulphanilic acid in 5% Na_2CO_3 used as a dipping reagent revealed 4-hydroxy-phenyl arsenicals. (d) Ammonia fumes showed up 4-hydroxy 3-nitro compounds as yellow spots. (e) 0.1% Starch solution followed by 0.002 N. iodine was selective for arsenoxides. (f) All compounds showed up as dark spots on illumination with ultraviolet light (270 m μ) from a Hanovia chromatolite.

Table 3A

Rf. values and colour reactions of 4-nitrophenylarsonic acid and its possible metabolites

Compound	Rf. values in solvents					Colour reactions		
	A	B	C	D	E	DMS	DMS + TiCl_3	U.V. (270 mμ)
4-Nitrophenyl- arsonic acid	0.45	0.88	0.67	0.0	0.0	-	red-brown	
4-Nitrophenyl- arsenoxide	0.95	0.97	0.84	0.13	0.47	-	red	all compounds give dark spots on paper
p-Arsanilic acid	0.27	0.76	0.60	0.0	0.0	red	red	
4-Aminophenyl- arsenoxide	0.98	0.99	0.82	0.18	0.65	red	red	
4-Acetanidophenyl- arsonic acid	0.34	0.84	0.70	0.0	0.0	red slowly	red slowly	
4-Acetanidophenyl- arsenoxide	0.83	0.86	0.90	0.0	0.0	red slowly	red slowly	

Table 4A

Rf. values and colour reactions of 4-hydroxy 3-nitrophenylarsonic acid and its possible metabolites

Compound	Rf. values in solvents				Colour reactions					
	A	B	C	F	DMC	DMC + TlCl ₃	Diazotised sulphanilic acid	NH ₃	Starch + I ₂	U.V. (270 mμ.)
4-Hydroxy 3-nitro- phenylarsonic acid	0.08	0.71	0.70	0.0	-	red	green- yellow	yellow	-	
4-Hydroxy 3-nitro- phenylarsenoxide	0.44	0.82	0.85	0.10	-	red	green- yellow	yellow	white on blue background	
4-Hydroxy 3-amino- phenylarsonic acid	0.06	0.23	0.45	0.0	red	red	olive brown	-	-	all compounds give dark spots on paper
4-Hydroxy 3-amino- phenylarsenoxide	0.33	0.23	0.50	0.38	red	red	olive brown	-	White on blue background	
4-Hydroxy 3-acetamide- phenylarsonic acid	0.10	0.61	0.62	0.0	red slowly	red slowly	orange	-	-	
4-Hydroxy 3-acetamide- phenylarsenoxide	0.33	0.60	0.76	0.35	red slowly	red slowly	orange	-	white on blue background	

Table 5A
pKa values of arsonic acids

Compound	Cation	Mono-anion	Di-anion	Tri-anion
Phenylarsonic acid	-	3.5 ^m	8.5 ^m	-
4-Nitrophenylarsonic acid	-	3.1	7.8	-
p-Arsanilic acid	2.6	4.1	8.9	-
4-Acetamidophenylarsonic acid	-	3.6	8.6	-
4-Hydroxyphenylarsonic acid	-	3.9 ^m	8.4 ^m (phenol)	10.0 ^m
4-Hydroxy 3-nitrophenylarsonic acid	-	3.2	5.8 (phenol)	8.9
4-Hydroxy 3-aminophenylarsonic acid	-	4.4	-	-
4-Hydroxy 3-acetamidophenylarsonic acid	-	3.5	-	-

^m Figures taken from Kortüm, Vogel and Andrussov (1961)

Table 6A

The ultraviolet absorption spectra of phenylarsonic acids

The ultraviolet absorption spectra of p-substituted phenylarsonic acids can be related to that of benzene according to the deductions of Doub and Vandenbelt (1947).

These workers have shown that a single substituent in the aromatic nucleus causes a definite displacement of the primary band (203.5 mμ) of benzene. p-Disubstituted benzenes cause a displacement of the primary band depending upon the nature of the substituents. If both groupings are of the same nature (electron attracting or electron donating) then the displacement is similar or less than that of the most displaced monosubstituted benzene. If the substituents are different, the displacement may be greater than that of the appropriate monosubstituted benzenes.

Phenylarsonic acid.

Phenylarsonic acid in the molecular form has a primary band at 215 mμ and a secondary band at 262 mμ. The arsonic acid grouping in the aromatic ring is weakly electron attracting and causes an initial displacement of the primary band of benzene of 11.5 mμ. Ionisation has little further effect on the positions of the peaks but the second ionisation causes a drop of 20% in the extinction of both bands.

4-Nitrophenylarsonic acid.

Both the nitro grouping (displacement 65 m μ) and the arsonic acid grouping (displacement 11.5 m μ) of 4-nitrophenylarsonic acid are electron attracting and this compound has an initial displacement of 47 m μ for the primary band of benzene. Ionisation weakens the electron attracting properties of the arsonic acid grouping and causes a shift of the primary band to longer wavelengths. This shift is 8 m μ for the first ionisation and 10 m μ for the second.

p-Arsanilic acid.

This compound in the molecular form contains two groupings of different nature. The amino substituent is electron donating while the arsonic acid substituent is electron attracting. The displacement of the primary band of benzene in p-arsanilic acid is 53.5 m μ which is greater than that of the amino substituent alone (26.5 m μ) or the arsonic acid substituent alone (11.5 m μ). Ionisation reduces the electron attracting properties of the arsonic acid grouping and causes a progressive shift of the primary band of p-arsanilic acid to shorter wavelengths of 7 and 6 m μ for the mono and di-anion.

Charging of the amino grouping at low pH values abolishes the electron attracting properties of the substituent and the absorption spectrum is similar to that of phenylarsonic acid.

p-Arsanilic acid cation has absorption maxima at 214 and 262 m μ with molecular extinctions of 6,590 and 808. Those for

phenylarsonic acid are 215 and 262.5 mμ with molecular extinctions of 9,210 and 447.

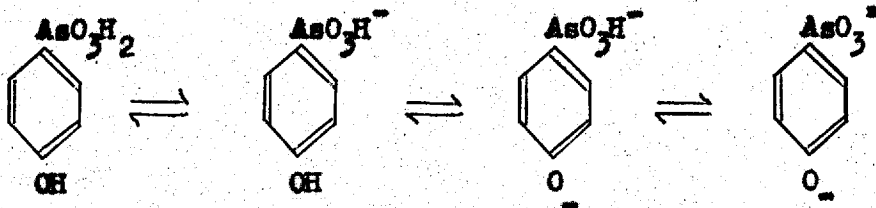
4-Acetamidophenylarsonic acid.

4-Acetamidophenylarsonic acid also contains two groups of opposite nature but the acetamido grouping is a much weaker electron donor than the amino substituent. The displacement of the primary band of benzene is 53.5 mμ similar to p-arsanilic acid. On ionisation, shifts in the primary band are not as marked as in p-arsanilic acid but are towards shorter wavelengths. These shifts are 5 mμ for the mono-anion and 3 mμ for the di-anion.

The molecular extinctions of 4-acetamidophenylarsonic acid are much greater than those of p-arsanilic acid which is consistent with the known ability of N-acetylated aromatic amines to absorb more intensely than the free amines.

4-Hydroxyphenylarsonic acid.

According to the pKa values recorded, 4-hydroxyphenylarsonic acid ionizes in three stages:-



The hydroxyl substituent is weakly electron donating whereas the arsonic acid grouping is electron attracting. The

primary band displacement for 4-hydroxyphenylarsonic acid is 30.5 m μ compared with 7 m μ for phenol and 11.5 m μ for phenylarsonic acid. The first ionisation of this compound causes the primary band to shift 4 m μ to shorter wavelengths with little change in the secondary band. The second ionisation of the molecule occurs in the phenolic grouping and increases its electron donating properties and the shift in the primary band is 21.5 m μ towards longer wavelengths. The third ionisation further decreases the electron attracting properties of the arsonic acid grouping and causes a further shift towards shorter wavelengths of 2.5 m μ while the extinction is greatly increased to 18,900.

Table 7A

Absorption spectra of mono substituted phenylarsonic acids

The spectra are for the peaks between 210 and 300 m μ . $\epsilon_{\max}$ is given in thousands

p-substituent	<u>Cation</u>		<u>Molecule</u>		<u>Mono-anion</u>		<u>Di-anion</u>	
	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$
H	{ -	-	215	9.2	214.5	9.4	216.5	7.1
	{ -	-	262.5	0.45	262	0.6	261.5	0.4
			pH 1.7		pH 6.4		pH 12.2	
NO ₂	{ -	-	-	-	-	-	215.5	5.1
	{ -	-	251.5	11.9	259.5	11.4	269.5	11.0
			pH 1.7		pH 6.1		pH 12.7	
NH ₂	{ 214	6.6	-	-	-	-	-	-
	{ 262	0.8	257	11.8	250	13.4	244	12.9
			pH 0.6		pH 3.3		pH 6.3	
			pH 12.3					
CH ₃ CONH	{ -	-	-	-	-	-	-	-
	{ -	-	257	20.75	252	19.0	249	17.3
			pH 1.2		pH 6.0		pH 12.3	

Table 8A

Absorption spectra of 3-substituted 4-hydroxyphenylarsonic acids

The spectra are for the peaks between 210 and 410 mμ $\epsilon_{\max}$ is given in thousands

Compound	<u>Cation</u>		<u>Molecule</u>		<u>Mono-anion</u>		<u>Di-anion</u>		<u>Tri-anion</u>					
	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$	$\lambda_{\max}$	$\epsilon_{\max}$				
4-Hydroxyphenyl- arsonic acid	{ -	-	234	13.9	} pH 1.5	230	13.8	} pH 6.1	251.5	12.5	} pH 9.4	249	18.9	} pH 12.5
	{ -	-	~ 265	1.1	}	265	0.9	}	235	7.3	} pH 7.35	285	2.5	}
									-	-				
4-Hydroxy 3-nitro- phenylarsonic acid	{ -	-	225	25.9	} pH 1.5	223	22.25	} pH 4.4	244	23.7	} pH 7.35	240	26.4	} pH 12.7
	{ -	-	266	5.4	}	267	5.7	}	285	2.5	}	277.5	4.0	}
	{ -	-	331	2.9	}	339	2.7	}	398	4.5	}	409	4.45	}
4-Hydroxy 3-amino- phenylarsonic acid	{ 234	14.0	214	16.0	} pH 0.8	211	41.0	} pH 7.0	211	40.8	} pH 9.1	218	36.8	} pH 12.8
	{ ~ 265	1.6	~ 232	10.4	} pH 3.0	242	6.8	}	243	5.6	}	256	10.9	}
			292.5	1.5	}	290.5	3.8	}	292.5	4.4	}	298	5.8	}

Table 9A

The recovery of ^{14}C in hens given 50 mg. of $[1 - ^{14}\text{O}]$ 4-nitrophenylarsonic acid orally

Hen No.	F.530	F.544	46	3	6	8								
Wt. of hen (Kg.)	1.3	1.6	1.4	1.1	1.6	1.3								
Dose (mg.)	53.5	49.95	49.95	47.46	48.78	49.74								
Dose (mg./Kg.wt.)	41.2	31.2	35.7	43.1	30.5	38.3								
Total % ^{14}C excreted	75.9 (4)	75.6 (6)	85.2 (6)	83.1 (10)	95.8 (11)	101.2 (9)								
% excreted														
Day	Daily	Total	Daily	Total	Daily	Total	Daily	Total	Daily	Total	Daily	Total		
1	20.8	20.8	14.9	14.9	40.7	40.7	57.5	57.5	77.8	77.8	82.5	82.5		
2	31.9	52.7	20.8	35.7	27.7	68.4	14.4	71.9	8.1	85.9	6.3	88.8		
3	19.5	72.2	27.2	62.9	7.4	75.8	3.5	75.4	1.8	87.7	1.1	89.9		
4	3.7	75.9	2.6	65.5	3.4	79.2	1.3	76.7	1.6	89.3	2.3	92.2		
5			10.1	75.6	6.0	85.2	1.1	77.8	3.7	93.0	2.9	95.1		
6														
7														
8							2.7	80.5	1.2	94.2	4.6	99.7		
9							1.4	81.9	0.6	94.8	1.5	101.2		
10							1.2	83.1	0.1	94.9	0.0	101.2		
11							0.0	83.1	0.9	95.8				
									0.0	95.8				

Table 10A

The excretion of arsenic and ^{14}C after administration of [1 - ^{14}C] 4-nitrophenylarsonic acid to hens

Day after dosing		1	2	3	4	5	Total excretion
<u>% of dose excreted</u>							
Hen 7	(Inorganic As.	0.1	0.1	0.5	0.2	0.2	1.1 = 0.2 mg.
	(Total As.	26.0	15.6	35.2	12.4	4.6	93.8
	(^{14}C	25.6	14.8	34.2	11.6	3.7	89.9
Hen 8	(Inorganic As.	0.3	0.0	0.0	0.9	0.7	1.9 = 0.21 mg.
	(Total As.	40.8	13.3	5.9	9.3	4.9	74.2
	(^{14}C	43.3	13.8	5.7	8.6	5.2	76.6
Hen 6	(Total As.	87.2	7.1	1.2	2.5	5.0	103.0
	(^{14}C	82.5	6.4	1.1	2.3	2.9	95.2

Doses: Hen 7: wt. 1.35 Kg: dose 60 mg. 4-nitrophenylarsonic acid
 Hen 8: wt. 1.35 Kg: dose 46.7 mg.
 Hen 6: wt. 1.30 Kg: dose 49.7 mg.

Table 11A

The metabolites of orally administered [1 - ^{14}C] 4-nitro-phenylarsonic acid in hen excreta determined by isotope dilution

Hen 2. 1.5 Kg. 43.4 mg./Kg.

	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Total</u>
4-Nitrophenylarsonic acid	26.3	14.9	1.8	43.0
p-Arsanilic acid	6.8	3.5	2.1	12.4
Faecal residue	2.6	1.9	1.8	6.3
Total	35.7	20.3	5.7	61.7
Total ^{14}C in the excreta	37.0	20.5	9.2	66.7

Hen 13. 1.55 Kg. 35.8 mg./Kg.

	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Total</u>
4-Nitrophenylarsonic acid	26.9	9.5	4.7	41.1
p-Arsanilic acid	4.0	2.8	3.0	9.8
Faecal residue	2.5	2.5	3.2	8.2
Total	33.4	14.8	10.9	59.1
Total ^{14}C in the excreta	35.2	15.8	15.5	66.5

Hen 15. 1.6 Kg. 34.7 mg./Kg.

	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Total</u>
4-Nitrophenylarsonic acid	26.8	2.5	1.3	30.6
p-Arsanilic acid	2.6	1.5	2.4	6.5
Faecal residue	1.1	1.1	1.5	3.7
Total	30.5	5.1	5.2	40.8
Total ^{14}C in the excreta	30.9	5.5	7.3	43.7

Table 12A

The effect of aureomycin on the excretion of metabolites of 4-nitrophenylarsonic acid by hens after injection or oral administration

Three hens (wt., 1.1 - 1.6 Kg.) were used in each experiment. The dose of 4-nitrophenylarsonic acid was 33 mg./Kg. The acid was dissolved in sodium bicarbonate solution (pH 7). 4-Nitrophenylarsonic acid and p-arsanilic acid in the excreta were determined colorimetrically. The results are expressed as the average of three hens with ranges in parenthesis.

Metabolites excreted in % of dose

Day after dosing	p-Arsanilic acid	4-Nitrophenylarsonic acid	Total
<u>(a) oral administration</u>			
1	7.6 (5.7 - 8.6)	41.8 (25.6 - 51.5)	49.4
2	7.2 (5.0 - 11.1)	9.4 (7.2 - 12.7)	16.8
3	7.2 (5.2 - 12.8)	4.3 (2.9 - 5.2)	11.5
Total	22.0 (14.1 - 32.5)	55.5 (19.3 - 69.3)	77.5

(b) oral administration after pretreatment with aureomycin orally

1	5.8 (3.5 - 8.5)	50.8 (28.2 - 72.8)	56.6
2	3.2 (0.3 - 5.3)	8.6 (7.1 - 10.0)	11.8
3	0.2 (0.0 - 0.6)	4.1 (1.7 - 6.6)	4.3
Total	9.2 (3.8 - 13.8)	63.5 (44.8 - 81.6)	72.7

(c) oral administration after pretreatment with aureomycin intramuscularly

1	7.6 (6.1 - 9.0)	36.1 (25.1 - 56.0)	43.7
2	2.8 (0.7 - 4.4)	8.0 (1.6 - 15.7)	10.8
3	3.6 (0.2 - 7.9)	3.9 (0.2 - 6.3)	7.5
Total	14.0 (6.9 - 18.7)	48.0 (36.8 - 57.8)	62.0

Metabolites excreted in % of dose

Day after dosing	p-Arsanilic acid	4-Nitrophenylarsonic acid	Total
<u>(d) intramuscular injection</u>			
1	5.3 (3.6 - 10.3)	86.2 (83.2 - 89.2)	91.5
2	0.9 (0.4 - 1.2)	3.5 (2.4 - 4.4)	4.4
3	0.0 (0.0 - 0.0)	1.3 (0.4 - 2.1)	1.3
Total	6.2 (4.9 - 10.6)	91.0 (87.1 - 95.8)	97.2

(e) intramuscular injection after pretreatment with aureomycin orally

1	0.8 (0.6 - 1.0)	83.7 (83.2 - 84.5)	84.5
2	0.0 (0.0 - 0.0)	3.2 (2.0 - 5.5)	3.2
3	0.0 (0.0 - 0.0)	0.5 (0.2 - 0.7)	0.5
Total	0.8 (0.6 - 1.0)	87.4 (86.0 - 89.2)	88.2

(f) intramuscular injection after pretreatment with aureomycin intramuscularly

1	0.8 (0.6 - 1.0)	94.0 (84.9 - 102.6)	94.8
2	0.1 (0.0 - 0.3)	3.0 (1.0 - 6.2)	3.1
3	0.0 (0.0 - 0.0)	0.5 (0.3 - 0.7)	0.5
Total	0.9 (0.8 - 1.0)	97.5 (91.8 - 104.1)	98.4

Table 13A

The removal of drug residues attached to the faecal solids

The faecal solids contained 0.6 - 0.9 μ M As./gm.

Treatment	% Drug residues removed
Dialysis/distilled water (16 hrs.)	11
N.HCl. at room temp. ($2\frac{1}{2}$ hrs.)	5
N.HCl. at 100°C (1 hr.)	42
2 % NaHCO ₃ at room temp. ($2\frac{1}{2}$ hrs.)	23
N.NaOH at room temp. ($2\frac{1}{2}$ hrs.)	89
N.NaOH at 100°C (1 hr.)	95
0.02 N. Iodine ($2\frac{1}{2}$ hrs.)	55
0.1 M Diethylam. diethyldithiocarbamate at room temp. ($2\frac{1}{2}$ hrs.)	50
0.1 M EDTA at room temp. ($2\frac{1}{2}$ hrs.)	0

Table 14A

The distribution of total arsenic in the tissues and excreta of three hens 24 hours after receiving 4-nitrophenylarsonic acid orally

Dose of arsenic acid 76.9 mg. (75.6 - 79.2 mg.) i.e. 23.4 mg. As. Average weight of hens 1.69 Kg. (1.60 - 1.75 Kg.) The figures are the average for three hens with ranges in parenthesis.

(a) Tissues and tissue fluids

<u>Tissue</u>	<u>Arsenic content</u>	
	<u>% of dose</u>	<u>MG./G.</u>
Blood	0.15 (0.1, 0.2)	1.5 (1, 2)
Bile	0.05 (0.01, 0.09)	2.0 (1, 3)
Heart	0.05 (0.01 - 0.1)	1.0 (0.5 - 2)
Lung	0.08 (0.04 - 0.14)	2.0 (1 - 3)
Liver	0.6 (0.3 - 0.7)	4.0 (2 - 5)
Spleen	0.01 (0.0 - 0.01)	2.0 (0.5 - 3)
Skin and feathers	4.0 (2 - 5)	4.0 (2 - 6)
Kidney	0.2 (0.08 - 0.2)	3.0 (2 - 4)
Ovary and oviducts	0.15 (0.01, 0.3)	1.0 (0.2, 2.0)
Muscle	0.5 (0.2 - 0.7)	0.3 (0.1 - 0.4)
Fat	0.3 (0.1 - 0.6)	0.5 (0.1 - 1)
Bone and residual tissue	8.0 (5 - 11)	4.0 (2 - 5)

(b) Tissues of alimentary canal and contents of alimentary canal.

<u>Tissue</u>	<u>Arsenic content</u>		<u>contents of</u>	<u>% of dose</u>
	<u>% of dose</u>	<u>MG./G.</u>		
Crop	1.0 (0.3 - 2.0)	13 (4 - 25)	Crop	10 (1 - 25)
Gizzard	2.0 (1.0 - 3.0)	14 (9 - 27)	Gizzard	2 (0.7 - 3)
Small intestine	0.3 (0.2 - 0.5)	5 (2 - 8)	Small intestine	2 (0.7 - 3)
Caeca	0.1 (0.02 - 0.1)	4 (1 - 6)	Caeca	0.2 (0.06 - 0.5)
Large intestine	0.1 (0.06 - 0.2)	7 (2 - 12)	Large intestine	2.0 (0.05 - 5)

(c) Distribution of arsenic

	<u>% of dose</u>
In tissues (a) and (b)	18
In contents of alimentary canal	16
In excreta	56
Total As. accounted for	90 (87 - 98)

Table 15A

The comparative excretion of orally administered 4-nitrophenylarsonic acid and polyethylene glycol

Three hens (wts. 1.2 - 1.6 Kg.) were given oral doses of 4-nitrophenylarsonic acid at 40 mg./Kg. and 4 gm. polyethylene glycol simultaneously.

Time after dosing	Hen 66		Hen 143		Hen 149	
	% 4-nitro and 4-amino	% polyethylene glycol	% 4-nitro and 4-amino	% polyethylene glycol	% 4-nitro and 4-amino	% polyethylene glycol
12 hours	10.6	14.1	53.5	50.8	27.1	33.6
24 "	8.0	9.0	18.8	21.3	16.5	17.6
48 "	12.9	15.0	10.8	12.3	19.4	25.0
72 "	12.3	17.0	2.3	2.0	9.2	15.0
Total	43.8	55.1	85.4	86.4	72.2	91.2

Table 16A

The excretion of p-arsanilic acid by hens

Three or six hens (wt. 1.1 - 1.8 Kg.) were used in each experiment at dosage levels of 50 or 100 mg. p-arsanilic acid/Kg.wt. The results are expressed as averages with the ranges in parenthesis.

% of dose excreted as

<u>Day after dosing</u>	<u>p-Arsanilic acid</u>	<u>Total arsenic</u>
<u>(a) oral administration at 100 mg./Kg.</u>		
1	53 (42 - 59) ³	51 (38 - 59) ³
2	21 (15 - 28) ³	20 (16 - 28) ³
3	5 (3 - 9) ³	5 (3 - 8) ³
Total	79 (77 - 81) ³	76 (74 - 80) ³
<u>(b) oral administration at 50 mg./Kg.</u>		
1	64 (47 - 83) ³	
2	7 (2 - 13) ³	
3	3 (0 - 8) ³	
Total	74 (67 - 84) ³	
<u>(c) intramuscular administration at 50 mg./Kg.</u>		
1	83 (79 - 86) ⁶	85 (83 - 88) ⁶
2	2 (2 - 4) ⁶	3 (2 - 4) ⁶
3	1 (0 - 1) ⁶	1 (0 - 1) ⁶
Total	86 (80 - 89) ⁶	88 (85 - 92) ⁶

Table 17A

The excretion of 4-acetamidophenylarsonic acid by hens

Three or four hens (wt. 1.1 - 1.8 Kg.) were used in each experiment at dosage levels of 50 or 100 mg./Kg.wt. The values for 4-acetamidophenylarsonic acid are the differences between "free" and "total" p-arsanilic acid. Results are expressed as averages with the ranges in parenthesis.

% of dose excreted as

<u>Day after dosing</u>	<u>free</u>	<u>p-Arsanilic acid</u> <u>total</u>	<u>Total arsenic</u>
<u>(a) oral administration at 100 mg./Kg.</u>			
1	5 (4 - 6) ⁴	81 (72 - 100) ⁴	80 (74 - 96) ⁴
2	0.5 (0 - 0.8) ⁴	3 (2.6 - 3.7) ⁴	4 (2.7 - 8.8) ⁴
3	0.1 (0 - 0.3) ⁴	1 (0.2 - 2.6) ⁴	0.5 (0.3 - 2.2) ⁴
Total	5.6 (4 - 6.5) ⁴	85 (76 - 103) ⁴	84.5 (77 - 99) ⁴
<u>(b) oral administration at 50 mg./Kg.</u>			
1	3.7 (2.4 - 5.6) ³	60 (57 - 67) ³	
2	0.6 (0.5 - 0.7) ³	8 (3 - 12) ³	
3	0.5 (0 - 1) ³	2 (1 - 2.7) ³	
Total	4.8 (3.1 - 7.1) ³	70 (68 - 71) ³	
<u>(c) intramuscular administration at 50 mg./Kg.</u>			
1	31 (30 - 38) ³	80 (75 - 89) ³	81 (75 - 85) ³
2	4 (3.7 - 4.3) ³	8 (6.7 - 8.2) ³	10 (6 - 13) ³
3	1 (0.9 - 1.8) ³	2 (0.9 - 3.7) ³	2 (0.9 - 3.5) ³
Total	36 (34 - 43) ³	90 (86 - 96) ³	93 (90 - 99) ³

Table 18A

The deacetylation of 4-acetamidophenylarsonic acid
by hen tissues

<u>Tissue</u>	<u>% deacetylation in 1 hour</u>	
	<u>Hen 1</u>	<u>Hen 2</u>
Kidney	74	63
Liver	0.3	0.0
Heart	0.0	0.2
Breast muscle	0.8	0.0
Brain	0.3	0.0
Gut	0.0	0.2

Table 19A

The cellular location of deacetylase activity in hen kidney

<u>Fraction</u>	Deacetylation μ moles/g. tissue/hr.	
	^{Elvehjem} <u>Potter Blending</u>	<u>Waring Blender</u>
Nuclei + debris	3.24	1.96
Mitochondrial fraction	6.60	10.18
Microsomes + supernatant	1.56	1.44
Microsomes	0.80	1.28
Supernatant	0.0	0.0
Whole homogenate	8.91	14.89

Table 20A

The recovery of total arsenic from hens given oral doses of 4-hydroxy 3-nitrophenylarsonic acid at 75 mg./Kg.

Hen No.	17	18	66	138				
Wt. of hen (Kg.)	1.6	1.4	1.5	1.6				
Dose (mg.)	119.8	105.2	113.0	116.2				
(mg./Kg.wt.)	74.9	75.2	75.3	72.7				
% Total As.	88.9 (10)	88.2 (10)	100.1 (11)	102.1 (11)				
% excreted								
Day	Daily	Total	Daily	Total	Daily	Total	Daily	Total
1	28.8	28.8	29.5	29.5	25.1	25.1	33.4	33.4
2	21.4	50.2	23.1	52.6	6.2	31.3	12.7	46.1
3	17.4	67.6	18.0	70.6	13.6	44.9	11.9	58.0
4	8.0	75.6	5.9	76.5				
5	6.0	81.6	4.0	80.5	13.2	58.1	14.7	72.7
6	5.7	87.3	5.6	86.1	6.5	64.6	9.4	82.1
7	1.2	88.5	1.1	87.2	9.4	74.0	7.3	89.4
8					10.6	84.6	5.0	94.4
9	0.3	88.8	0.8	88.0	12.4	97.0	5.5	99.9
10	0.1	88.9	0.2	88.2	3.1	100.1	2.2	102.1
11								

Table 21A

The excretion of glucuronides and ethereal sulphates by hens receiving oral doses of 4-hydroxy 3-nitrophenylarsonic acid

Six Rhode Island Red x Light Sussex hens were used in the experiment. After 48 hours three birds received oral doses of the compound at 75 mg./Kg. Estimations of total glucuronide and ethereal sulphate were made on the excreta supernatant.

Glucuronides (mg.)

<u>Day</u>	<u>Dosed Hens</u>	<u>Controls</u>
1	11.2 (7.7 - 13.0)	13.2 (9.8 - 17.2)
2	14.7 (13.5 - 15.6)	16.2 (12.6 - 18.3)
3	15.5 (12.8 - 20.2)	15.8 (14.8 - 16.6)
4	25.1 (14.9 - 32.8)	11.4 (9.1 - 13.4)
5	22.4 (15.6 - 31.3)	18.6 (17.8 - 19.5)

Ethereal sulphates (mg.)

<u>Day</u>	<u>Dosed Hens</u>	<u>Controls</u>
1	28.1 (21.4 - 36.8)	27.4 (20.8 - 34.0)
2	13.7 (4.1 - 23.2)	16.7 (2.1 - 30.4)
3	14.4 (10.6 - 17.5)	14.1 (5.7 - 23.0)
4	18.0 (9.6 - 22.2)	27.6 (17.3 - 35.6)
5	14.8 (13.7 - 15.7)	16.5 (13.7 - 21.2)

Table 22A

The distribution of total arsenic in the excreta of hens
receiving 4-hydroxy 3-nitrophenylarsonic acid orally

Three hens (wts. 2.4 - 2.8 Kg.) were given oral doses of the compound at 37.9 mg./Kg. Food was withheld from the birds for 24 hours prior to dosing and throughout the course of the experiment.

The figures are the average for three hens with ranges in parenthesis.

% Dose excreted as As.

<u>Day</u>	<u>Excreta supernatant</u>	<u>Faecal solids</u>	<u>Total</u>
1	34.7 (24.4 - 43.1)	1.3 (1.1 - 1.5)	36.0 (25.5 - 44.3)
2	25.3 (23.4 - 27.7)	0.8 (0.5 - 1.1)	26.1 (24.5 - 28.2)
3	12.8 (11.8 - 14.2)	1.0 (0.8 - 1.2)	13.8 (12.6 - 15.3)
Total	72.8 (62.0 - 79.8)	3.1 (2.7 - 3.3)	75.9 (65.3 - 82.5)

Table 23A

The metabolites of orally administered 4-hydroxy 3-nitrophenylarsonic acid in the excreta of hens

Three hens (wt. 2.2 - 3.6 Kg.) were used in each experiment. The compound was given orally in sodium carbonate solution at the dosage levels specified and the excreta was analysed for three days for nitro and amino hydroxyphenylarsonic acids and total arsenic.

Dosage level 75.6 mg./Kg.

Day	% 3 - nitro	% 3 - amino	% 3 - nitro + 3 - amino	Total As.
1	25.5 (21.2, 29.7)	1.6 (1.6, 1.7)	27.1 (22.8, 31.4)	27.4 (24.8, 30.0)
2	17.3 (9.7 - 24.4)	5.5 (3.0 - 8.9)	22.8 (12.7 - 29.1)	24.0 (16.2 - 29.8)
3	5.4 (2.8 - 10.4)	4.9 (1.2 - 9.9)	10.3 (4.2 - 14.1)	10.6 (5.3 - 14.1)
Total	48.2 (41.9 - 57.1)	12.0 (7.6 - 20.4)	60.2 (45.4 - 64.7)	62.0 (58.5 - 65.1)

Dosage level 37.9 mg./Kg.

Day	% 3 - nitro	% 3 - amino	% 3 - nitro + 3 - amino	Total As.
1	31.5 (24.2 - 36.1)	5.1 (0.6 - 8.3)	36.6 (24.8 - 42.6)	36.9 (25.4 - 43.0)
2	9.1 (6.2 - 11.0)	4.8 (2.0 - 7.2)	13.9 (8.2 - 17.4)	16.4 (12.6 - 19.6)
3	2.5 (1.3 - 3.2)	2.6 (1.1 - 4.1)	5.1 (4.0 - 7.1)	7.7 (5.2 - 12.2)
Total	43.1 (37.4 - 50.3)	12.5 (11.9 - 13.0)	55.6 (49.6 - 63.1)	61.0 (57.2 - 65.3)

Dosage level 18.9 mg./Kg.

Day	% 3 - nitro	% 3 - amino	% 3 - nitro + 3 - amino	Total As.
1	34.0 (30.3 - 38.0)	14.6 (10.4 - 20.9)	48.6 (42.8 - 54.6)	46.9 (45.0 - 50.2)
2	7.4 (5.3 - 11.3)	4.1 (2.0 - 6.5)	11.5 (7.3 - 17.8)	15.3 (10.4 - 21.7)
3	0.6 (0.0 - 1.9)	0.2 (0.0 - 0.5)	0.8 (0.2 - 1.9)	2.7 (0.3 - 4.9)
Total	42.0 (35.9 - 51.2)	18.9 (16.7 - 23.1)	60.9 (52.6 - 68.1)	64.9 (59.9 - 71.6)

Table 24A

The metabolites of intramuscularly administered 4-hydroxy 3-nitrophenylarsonic acid in the excreta of hens

Three hens (wt. 2.2 - 3.6 Kg.) were used in each experiment. The compound was administered intramuscularly in sodium bicarbonate solution (pH 7) at the dosage levels specified and the excreta were analysed for three days for nitro and amino hydroxyphenylarsonic acids and total arsenic.

Dosage level 37.9 mg./Kg.

Day	% 3 - nitro	% 3 - amino	% 3 - nitro + 3 - amino	Total As.
1	59.6 (47.6 - 69.8)	0.6 (0.0 - 1.6)	60.2 (47.6 - 70.0)	58.0 (47.6 - 66.7)
2	0.3 (0.0 - 0.6)	0.2 (0.0 - 0.3)	0.5 (0.3 - 0.6)	1.0 (0.5 - 1.6)
Total	59.9 (47.6 - 69.8)	0.8 (0.2 - 1.6)	60.7 (47.9 - 70.0)	59.0 (48.1 - 66.7)

N.B. All hens died 25 - 30 hours after dosing.

Dosage level 18.9 mg./Kg.

Day	% 3 - nitro	% 3 - amino	% 3 - nitro + 3 - amino	Total As.
1	79.4 (77.2 - 82.9)	2.4 (0.7 - 4.6)	81.8 (78.7 - 87.5)	79.8 (76.8 - 84.3)
2	9.8 (5.4 - 15.0)	1.5 (0.0 - 4.5)	11.3 (9.1 - 15.0)	11.9 (10.7 - 13.7)
3	2.7 (1.6 - 4.0)	0.4 (0.0 - 0.9)	3.1 (2.5 - 4.0)	3.6 (2.5 - 5.7)
Total	91.9 (85.0 - 101.9)	4.3 (2.1 - 6.1)	96.2 (90.3 - 106.5)	95.3 (90.0 - 103.7)

Table 25A

The removal of drug residues attached to the faecal solids

The faecal solids contained 0.8 - 1.7 μ M As./gm.

Treatment	% Drug residues removed
Dialysis/distilled water (24 hrs.)	39
N.HCl. at room temp. ($2\frac{1}{2}$ hrs.)	14
N.HCl. at 100°C (1 hr.)	40
2 % NaHCO ₃ at room temp. ($2\frac{1}{2}$ hrs.)	28
0.2 N.NaOH at room temp. ($2\frac{1}{2}$ hrs.)	79
0.2 N.NaOH at 100°C (1 hr.)	94
0.02 N. Iodine at room temp. ($2\frac{1}{2}$ hrs.)	85
0.1 M Diethylam. diethyldithiocarbamate at room temp. ($2\frac{1}{2}$ hrs.)	63
0.1 M EDTA at room temp. ($2\frac{1}{2}$ hrs.)	16

Table 26A

The effect of diet on the metabolism of orally administered
4-hydroxy 3-nitrophenylarsonic acid

Three hens (wts. 2.2 - 3.4 Kg.) were used in each experiment. The compound was administered orally at 37.5 mg./Kg. and the excreta supernatants were analysed for nitro and amino hydroxyphenylarsonic acids.

Starved birds.

Day	% 3 - nitro	% 3 - amino	Total
1	26.5 { 9.4 - 39.6 }	2.2 { 0.0 - 5.9 }	28.7 { 9.4 - 45.5 }
2	14.1 { 8.2 - 18.4 }	0.3 { 0.0 - 1.0 }	14.4 { 9.2 - 18.4 }
3	5.8 { 4.2 - 6.8 }	1.3 { 0.0 - 3.8 }	7.1 { 4.2 - 10.6 }
Total	46.4 (34.1 - 59.4)	3.8 (0.0 - 5.9)	50.2 (34.1 - 65.3)

Birds receiving Diet 1.

Day	% 3 - nitro	% 3 - amino	Total
1	24.0 { 12.7 - 40.2 }	2.1 { 0.0 - 6.3 }	26.1 { 12.7 - 46.5 }
2	9.2 { 6.3 - 13.8 }	1.4 { 0.0 - 3.2 }	10.6 { 7.3 - 13.8 }
3	5.2 { 1.1 - 9.5 }	2.1 { 0.0 - 3.4 }	7.3 { 1.1 - 12.4 }
Total	38.4 (24.0 - 63.5)	5.6 (3.2 - 9.2)	44.0 (28.4 - 72.7)

Birds receiving Diet 2.

Day	% 3 - nitro	% 3 - amino	Total
1	31.2 { 30.7 - 32.9 }	1.3 { 1.3 - 1.4 }	32.5 { 32.1 - 34.2 }
2	10.1 { 9.4 - 10.5 }	6.3 { 1.1 - 12.6 }	16.4 { 10.5 - 23.1 }
3	6.2 { 5.3 - 7.2 }	3.3 { 2.4 - 5.1 }	9.5 { 7.7 - 12.3 }
Total	47.5 (46.4 - 50.6)	10.9 (4.8 - 16.4)	58.4 (51.2 - 62.9)

Table 27A

The distribution of drug residues in the contents of the
alimentary canal of hens receiving 4-hydroxy 3-nitrophenyl-
arsonic acid orally

Five hens (wts. 2.6 - 3.6 Kg.) were given oral doses of the compound at 37.5 mg./Kg. The birds were killed 6 - 72 hours after dosing and the contents of the alimentary canal analysed for 4-hydroxy 3-nitro and 4-hydroxy 3-aminophenylarsonic acid in the supernatant fraction and total arsenic in the solid fraction.

(a) % Dose in the contents of the alimentary canal.

<u>Organ</u>	<u>6 hrs.</u>	<u>12 hrs.</u>	<u>24 hrs.</u>	<u>48 hrs.</u>	<u>72 hrs.</u>
Crop	46.8	48.7	76.6	24.0	5.0
Gizzard	7.4	8.1	9.7	2.7	3.5
Small intestine	2.9	1.6	1.9	0.8	0.7
Caeca	3.1	1.9	2.3	1.0	1.1
Large intestine	0.3	0.9	0.6	0.6	6.8
Excreta	21.3	34.1	17.3	53.1	44.1
Total	81.8	95.3	108.4	82.2	61.2

Table 28A

(b) The occurrence of 4-hydroxy 3-nitrophenylarsonic acid, 4-hydroxy 3-aminophenylarsonic acid and of drug residues attached to the solid fraction of the crop contents

<u>Time of death after dosing</u>	<u>% 4 - hydroxy- 3 - nitro</u>	<u>% 4 - hydroxy- 3 - amino</u>	<u>% attached to solid fraction</u>	<u>Total</u>
6 hours	31.0	3.5	12.3	46.8
12 "	18.3	21.2	9.2	48.7
24 "	53.6	6.3	16.7	76.6
48 "	10.8	12.6	0.6	24.0
72 "	0.5	2.9	1.6	5.0

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