ASPECT OF THE METABOLISM OF CYANIDE IN THE RAT

By

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Ph.D. THESIS SEPTEMBER, 1978

ASPECTS OF THE METABOLISM OF CYNIDE

THE RAT

A Thesis

Submitted in accordance with the

requirements of the

UNIVERSITY OF LIVERPOOL

for the degree of

DOCTOR IN PHILOSOPHY

by

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September, 1978

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ACKNOWLEDGEMENT

The work described in this thesis was carried out in the Biochemistry Department of the University of Liverpool between July 1975 and September 1978.

I am most grateful to Dr. G.A.J. Pitt for his constant guidance and valuable assistance in the preparation of this thesis and for his concern for my welfare throughout the course of the research work.

I sincerely thank Mr. T. Howard who prepared the special diets and carried out the routine work associated with the welfare of the experimental animals.

Special thanks to my wife for her help and encouragement, and to Chief A.O. Okoh, Messrs, D.O. Osemeha and R.I. Okoh and all other members of my family for their support throughout the course of my education.

Acknowledgement is gratefully extended to the following people: Misses C. Baker and C. Wynne for their assistance and to all other members of the teaching and technical staff of this department, who were helpful to me at one stage or the other during the course of this work.

Finally, I would like to express my deep appreciation to Professor T.W. Goodwin for giving me the opportunity of working in this department, to Professor J. Glover for all the letters written on my behalf in connection with my sponsorship and to the Ahmadu Bello University, Zaria, Nigeria for granting me the study leave.

Patrick Nwanze Okoh.

ABSTRACT

The use of inorganic cyanide for cyanide metabolism studies in animals has the advantage that the cyanide administered is fully available as opposed to cyanogenic glycosides in which the cyanide radical is not completely liberated in the animal body.

However, when KCN was added to the diet which had been moistened with water, it was lost by transformation to other products, and perhaps also as volatile HCN. A preliminary study revealed that formate is one of the products formed. A method was developed for the addition of KCN to the normal rat diet, in which no significant loss or transformation of the added substance occurred.By this method it was possible to compare tho relative toxicities of KCN and vitamins A and D when mixed in the diet, as measured by weight gain in growing rats. KCN given to groups of rats at a dose level of 77 umoles/rat/day in the diet did not diminish weight gain whereas vitamins A (38•5 umoles/rat/day) or D, (0.55 umoles/rat/day) significantly diminished weight gain in rowing rats.

A study of tho metabolism of " C-labelled cyanide was carried out in the rat exposed to semi-continuous intake of KCN in the diet for throe weeks. The pattern of excretion and tissue levels of" C activity was similar after subcutaneous or intraperitoneal injections and oral administration of $Na^{1k}CN$. After intraperitoneal and subcutaneous injections of Na"CN, *2k* of dosed activity was excreted in the urine within 9h of which 71% was in the form of thiocyanate. The corresponding value after 9h in the faeces was 0.5.• About 4.3 of the injected activity appeared in the respiratory air of which 85 was present as CO and only 15 as cyanide.

All tissues contained radioactivity 9h after the Na"CN administration but very high amounts were found in the gastro-intestinal tract, particularly in the stomach, which accounted for 18% of the total **dosed** activity. Moat of this was in the contents of the stomach, of which over 80% **was** in the form of thiocyanate.

When a small amount of $KS^{11}CN$ was given by mouth to the rat with elevated plasma ScN level, most of the activity waa excreted in the urine (mainly as SCN) and only small amounts were found in the faeces. This indicated that the thiooyanate secreted into the stomach contents of the rat was largely reabsorbed by the intestine into the body fluid, to be partly excreted in the urine and partly resecreted into the gastric juice.

The time of initial secretion of thiocyanate into the rat stomach contents after subcutaneous injection of cyanide was calculated to bo 7 min, while the rate of secretion was estimated to be 0.31 of the administered dose per minute.

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CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

1. Occurrence of cyanide

Traces of cyanide can be demonstrated in almost all plants. According to Montgomery (1969) it is found mainly in form of cyanogenic glycosides.

Cyanogenic glycosides are compounds which liberate HCN, one or more molecules of sugar, and, with one exception, an aldehyde or ketone on treatment with dilute acid or the appropriate hydrolytic enzymes (Conn, 1969).

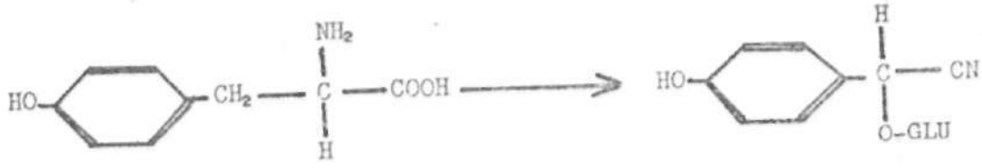
Research in several laboratories has shown that four of the common cyanogenic glycosides are derived from closely related amino acids (Fig. I.1). Tyrosine is known to be effectively converted into dhurrin; phenylalanine into prunasin; valine into linamarin and isoleucine into lotaustralin (Conn, 1969). In each of these biosyntheses, there is loss of the carboxyl carbon of the amino acid and oxidation of the α-carbon and nitrogen atom to the level of nitrile.

Table I.1 shows a compilation of plants with extremely high cyanide content (present as cyanogenic glycosides). Among the known sources of cyanogenic glycosides, cassava (Manihot sp.) represents the most important food crop. It is the principal source of carbohydrate for about 300 million people in the developing countries of the tropics. Linamarin accounts for about 90% of the total cyanogenic glycoside in cassava.

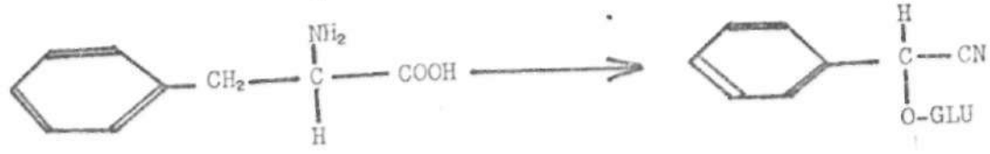
However, most of the cyanide in cassava is lost during its processing. Thus, although the cyanide contents of the pulp of some

FIGURE I.1

Precursors of some cyanogenic glycosides.



L-Tyrosine — Dhurrin



L-Phenylalanine — Prunasin



L-Valine — Linamarin



L-Isoleucine — Lotaustralin

TABLE I.1

Cyanide content of various food plants (taken from Baumeister *et al.* 1975).

Plant	HCN content mg/100 g
Bitter almond	250
Bitter cassava	
bark of root (dried)	245
stem	113
root (total)	55-90
Sorghum	
fresh leaves	0-15
Bamboo	
tops of unripe sprouts	800
stem, unripe	300
Lima bean (Phaseolus lunatus)	
several variations	312
Java, coloured	300
Puerto Rico, black	300
Burma, white	17
Arizona, coloured	10
America, white	
White clover	20-30

cassava varieties vary between 163 and 698 mg per kilogram wet weight (Table I.2), that of some major cassava products (like its flour or purupuru paste prepared from the pulp), vary between 20 and 60 mg per kilogram wet weight (Table I.2). This extensive loss of cyanide during cassava preparation obviously makes cassava products less harmful to animals than the unprocessed material.

In tobacco smoke, cyanide occurs in considerable amounts, in concentrations which differ from one sort of tobacco to the next (Osborne, 1956; Wynder, 1967; Stedman, 1968). In the tobacco smoke of cigarettes without filter, amounts between 150 and 300 µg per cigarette have been reported (Newsome et al., 1965; Baumeister and Schievelbein, 1971). In cigarettes with a cellulose filter, only a negligible retention by the filter was observed (Baumeister and Schievelbein, 1971). Suitable absorbent filters on the other hand reduce the original cyanide content by 20 to 80%.

Another source of cyanide is nitroprusside. Nitroprusside is used as an antihypertensive drug. Recent evidence indicates that nitroprusside can be broken down rapidly by free and intracellular haemoglobin by a non-enzymic process (Smith and Kruszyna, 1976). The reaction sequence is shown in Fig.I.2. However, with controlled administration of the drug by a physician, acute poisoning from nitroprusside will not occur.

Finally, inhalation of HCN compounds in the chemical and galvanic industry as well as during gold extraction is another possibility for humans to take considerable amounts of cyanide.

2. Metabolism of cyanide

Several ways of metabolism of cyanide have been described. These ways have been reviewed by Williams (1959), Oke (1969) and

TABLE I.2

Cyanide content of cassava and some cassava products (mg/kg)

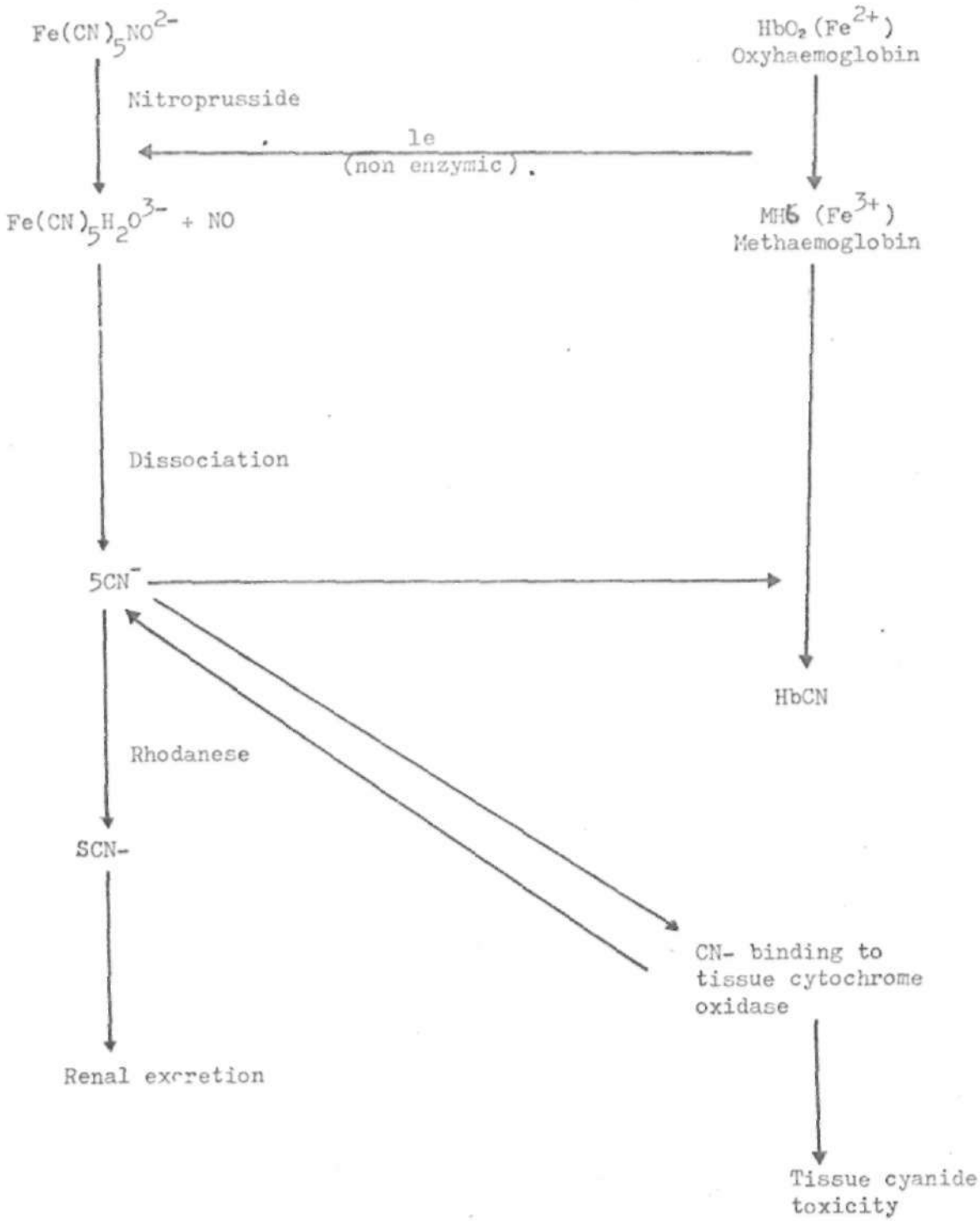
(Taken from Van Der Velden and Kinthaert, 1973).

	Pulp	Integument
Fresh roots (various species)	163	227
	180	284
	454	446
	698	963
Flour	30	-
*Purupuru paste	20-60	-

*From Osuntokun (1970).

FIGURE I.2

Metabolism of sodium nitroprusside (taken from Smith and Kruszyna, 1976).



more recently by Baumeister (1975).

Following injection of $Na^{14}CN$ in dogs ^{14}C appears in urine, in free cyanide, as a constituent of cyanocobalamin and in thiocyanate. A part of the cyanide and thiocyanate carbon is oxidized directly to form CO_2, which is achieved, according to Boxer and Rickards (1952), probably via cyanate.

The carbon of cyanide appears in the carbon of the methyl groups of choline and methionine and in the urea carbon of allantoin as well. The formic acid isolated in these experiments contains a high amount of ^{14}C. Based on this fact formic acid could be considered as a starting material for the introduction of the cyanide carbon into one-carbon metabolism (Boxer and Rickards, 1952).

The best known mechanism for the detoxification of cyanide is concerned with the conversion of cyanide to relatively non-toxic thiocyanate. Two metabolic pathways are involved in this conversion. One reaction which has been known for a longer time is catalysed by the enzyme thiosulphate - sulphurtransferase (EC 2.8.1.1) named rhodanese by Lang (1933a). Thiosulphate and cyanide form thiocyanate and sulphite in this reaction. This enzyme is further discussed in Chapter VI. In the second pathway, the enzyme cyanide sulphur-transferase (EC 2.8.1.2) (Fiedler and Wodd, 1956) utilizes 3-mercaptopyruvate as the sulphur donor and thiocyanate and pyruvate are formed as products.

Another independent way in which detoxification of cyanide can take place is the reaction with cystine to form cysteine and β-thiocyanoalanine. The reaction takes place spontaneously *in vitro* (Schobert and Hann, 1948). Voegtlin *et al.* (1926) were the first to observe that a dose of cystine injected immediately before injection

of cyanide protected animals from a dose that would otherwise be lethal. Wood and Cooley (1956) isolated 2-iminothiazolidine-4-carboxylic acid from the urine of rats after subcutaneous injection of sodium cyanide. In this case cyanide is thought to react with cystine forming cysteine and β-thiocyanoalanine. The latter is tautomerized forming 2-amino-thiazoline-4-carboxylic acid or the equivalent 2-iminothiazolidine-4-carboxylic acid (Schöbert et al. 1951). The second compound proved to be metabolically inert in rats (Wood and Cooley, 1956). Subsequent investigators however, have been unable to identify 2-iminothiazolidine-4-carboxylic acid in the urine of either normal rats or rats exposed to cyanide doses for a prolonged period of weeks (Smith and Foulkers, 1966; Mehta and McGinity, 1977).

One compound which has been implicated in the disposal of cyanide is cobalamin (vitamin B_{12}). Two forms of the vitamin have been known for a long time, in which the central cobalt atom is connected with either a hydroxyl or a cyanide group. When the central cobalt atom of the vitamin is connected with a hydroxyl group, hydroxocobalamin (or aquocobalamin) is formed, while connection of the cobalt atom with a cyanide group gives rise to cyanocobalamin (Brink et al. 1950). When solutions of cyanocobalamin are exposed to light the coordinately bound cyanide is split off. On the other hand, in neutral solutions hydroxocobalamin can react with KCN to form cyanocobalamin. In addition to the coordinately bound cyanide, cyanocobalamin can react with two more moles of cyanide to form complexes (Beavan, 1950; Conn et al., 1951). Dietary deficiency of vitamin B_{12} leads to increased thiocyanate excretion and injection of sub-lethal doses of cyanide to rats causes a significant depletion of the liver store of vitamin B_{12},

indicating that this is an important detoxifying agent during cyanide poisoning (Smith, 1961). However, even assuming that all the B_{12} existing in the liver was converted to cyanocobalamin, the total amount would be less than 1000 µg (Drouet et al., 1953) and the amount of cyanide contained would be very small.

The different pathways of metabolism are utilized to various degrees in the organism. Balance investigations which could give an insight into the utilization of the different pathways have not been published. But it can be assumed that detoxification to thiocyanate accounts for the main part, since after injection of sodium cyanide into rats 80% of the injected cyanide was found in urine as thiocyanate (Wood and Cooley, 1956). The therapeutic effect of thiosulphate in cyanide intoxication could be a hint that the detoxification of cyanide by means of thiosulphate-sulphurtransferase is of great importance.

A hypothesis for cyanide utilization has been proposed by Wokes and Pikard (1955). They proposed that the liver contains some B_{12} (not combined with cyanide) together with a lot of rhodanese. When cyanide is ingested, both B_{12} and rhodanese compete for it; some cyanide ions are detoxified by rhodanese with the help of sulphur donors, such as sulphur-containing amino acids or their products of metabolism, to thiocyanate ions which are all excreted in the urine with very little in the faeces (Meister and Fries, 1949). Some of the cyanide ions combine with vitamin B_{12} to form cyanocobalamin. Cyanocobalamin can lose some of the cyanide to supply one carbon fragments for the synthesis of important compounds such as choline and other substances containing labile methyl groups, e.g. the conversion of homocysteine to methionine (Kratzer, 1953; Smith, 1954). Some of the cyanide is lost as carbon dioxide in the breath. That

vitamin B_{12} may be participating in the detoxification of cyanide is supported by the fact that vegans who are short of vitamin B_{12} excrete excess thiocyanate and therefore may be detoxifying more of their cyanide by the rhodanese pathway (Oke, 1969).

3. Cyanide intoxication

There are two aspects of cyanide intoxication: acute intoxication and chronic intoxication.

Acute cyanide intoxication results from an intake of a single large dose of cyanide. Most of the symptoms of acute cyanide poisoning can be explained on the basis of the affinity of cyanide for metal ions such as iron and copper. For example, it combines with the copper of cytochrome oxidase and thereby inhibits the latter's function in electron transport. It also combines with methaemoglobin to form cyanmethaemoglobin which is not an oxygen carrier.

The occurrence of acute intoxication symptoms depends upon the velocity of the increase of cyanide concentration in tissues. This decisive factor is determined by the kind of cyanide compound, by the route of intake, by the ingested dose and by the ability of the organism to detoxify cyanide.

The minimum lethal dose of cyanide taken by mouth has been estimated in man at between 0.5 mg and 3.5 mg cyanide ion per kilogram body weight (Chen et al., 1934b; Gettler and Baine, 1938; Halstrom and Moller, 1945). Upon uptake of higher doses death can occur within a few minutes, on medium doses survival times up to three hours have been reported. Human survivors have described initial symptoms of peripheral numbness and lightheadedness. This is followed by mental confusion and stupor, twitching and convulsions with terminal coma

(Montgomery,1969). Small non-fatal doses may produce headache, a sensation of tightness in the throat and chest, palpitations and muscle weakness. In experimental animals, the principal manifestations of toxicity are stupor and convulsions (Montgomery, 1969).

The actual cause of death in acute intoxication is the cessation of respiration, which will take place some minutes before cardiac arrest (Baumeister et al., 1975).

The chronic intake of cyanide in small doses, which as single doses do not cause clinical symptoms, has been implicated in the pathogenesis of a number of diseases. A diminution of some ways of cyanide metabolism is probably responsible in some cases (Schievelbein et al., 1969). A review of the clinical states that result from chronic cyanide intoxication has recently been given by Baumeister et al. (1975). The clinical states described to date are: tobacco amblyopia, retrobulbar neuritis with pernicious anaemia, Leber's optic atrophy, tropical ataxic neuropathy, sterility in women who are heavy smokers and goitre.

(a) Tobacco amblyopia

The cyanide in cigarette and tobacco gas smoke has been suggested as the cause of the visual failure in tobacco amblyopia. In patients with tobacco amblyopia, vascular changes and degeneration of ganglion cells in the retina and of nerve fibres of the optic nerve were described (Heaton et al., 1958; Duke-Elder, 1954). The pathological anatomic findings show a striking parallel to the observations in animal experiments on cyanide administration. Repeated small doses of cyanide cause a demyelinization in the central nervous system in rats and monkeys (Hurst, 1942; Lumsden, 1950). Leishman (1951) thought he observed a correlation between amblyopia and deficiency of gastric acid and therefore assumed a connection in the aetiology of

amblyopia and pernicious anaemia.

The good therapeutic effect of vitamin B_{12} in tobacco amblyopia suggests also deficiency of vitamin B_{12} as a cause. This can develop either by decreased uptake of vitamin B_{12} or by transformation of 'functional' hydroxocobalamin into 'non-functional' cyanocobalamin (Victor, 1963; Knox, 1970). Several authors have examined the metabolism of vitamin B_{12} in smokers. Kommerell and Castrillon-Oberndorfer (see Baumeister et al., 1975) found a severe alteration of B_{12} absorption in two out of five patients with tobacco amblyopia. On the other hand, Watson et al. (1969) did not find a decrease of the absorption in 15 patients with tobacco amblyopia. But if the patients smoked during the experiment the absorption of B_{12} decreased. According to investigations of Foulds et al. (1968), 40% of patients with tobacco amblyopia showed decreased levels of serum B_{12}. In 45% the absorption of vitamin B_{12} was disturbed. Foulds et al. (1969) could demonstrate in patients with tobacco amblyopia a direct correlation between the consumption of tobacco, the vitamin B_{12} absorption, and the B_{12} level in serum. If amblyopia occurred in a patient with a small tobacco consumption, the absorption of vitamin B_{12} was insufficient, or the serum B_{12} level was low. If however, the serum levels were high, the disease became manifest only with extremely high tobacco consumption. Foulds et al. (1968) also investigated the concentration of serum thiocyanate in heavy smokers with tobacco amblyopia and in smokers without tobacco amblyopia, as well as in non-smokers. They found a significantly increased thiocyanate level in serum of smokers compared with the values in non-smokers. In contrast to these findings the thiocyanate concentration in patients with tobacco amblyopia was significantly

decreased compared with healthy smokers. Although the patients with tobacco amblyopia did smoke more than the healthy smokers, Baumeister et al. (1975) suggested that these findings might be due to a possible enzyme defect, a hereditary deficiency of enzymes which are able to form thiocyanate.

(b) Retrobulbar neuritis in pernicious anaemia

The optical changes which occur in this condition are accompanied by pernicious anaemia (Cohen, 1936). There is now evidence that retrobulbar neuritis in pernicious anaemia and tobacco amblyopia may be one and the same condition (Freeman and Heaton, 1961). The histological changes of the optic nerve in retrobulbar neuritis proved to be similar to those found in tobacco amblyopia (Duke-Elder, 1954).

(c) Leber's optic atrophy

Leber (1871) is credited with the first description of this condition which is relatively rare and is said to be hereditary. Leber's optic atrophy is characterized by acute or subacute failure of central vision and the development of bilateral central scotomas. The condition is found mainly in young males in their late 'teens' or in early twenties, though the age distribution varies over a wide range. In some patients other neurological involvement occurs together with the ophthalmologic condition (Enghoff, 1963; Wilson, 1963; Lees et al., 1964).

Wilson (1963) concluded that the disease is caused by an enzymatic impairment, the manifestations of which are triggered by exogenous factors. Smoking was suggested as an exogenous factor for the manifestation of the visual disorder. In healthy smokers the blood level and the excretion in urine of thiocyanate are increased

compared with non-smokers. But in smokers with Leber's optic atrophy the increase of the thiocyanate level in blood is less pronounced or even absent. The question of an enzymatic impairment could not be answered by measuring the activity of the rhodanese. In liver tissue from needle biopsy a decreased activity of rhodanese was found for one patient compared with a control person. The liver of one patient showed at postmortem examination a somewhat smaller enzymatic activity compared with two control livers. The enzymatic content of the brain sample was however, somewhat higher in the patient with Leber's optic atrophy compared with the control person.

Patients with Leber's optic atrophy, smokers as well as non-smokers, show a significantly higher proportion of cyanocobalamin in the total B_{12} content in serum compared with healthy persons (Wilson et al., 1971). Smith (1964) described infection as a source of cyanide. Wilson (1967) observed that the impairment of vision in two children from families with Leber's optic atrophy occurred in connection with an infection of the urinary tract, and suggested that this might be a contributory factor in Leber's disease occurring in non-smokers. This is supported by the finding that E.coli is capable of forming cyanide in the urinary tract.

(d) Tropical or Nutritional ataxic neuropathy

Tropical or Nutritional ataxic neuropathy, also known as tropical amblyopia occurs in parts of the tropics where the staple diet consists of cassava or its derivatives (Money, 1959; Osuntokun, 1968). The tropical amblyopia probably represents a less severe form of the fully developed syndrome, which has been extensively studied in Nigeria and hence called Nigerian nutritional ataxic neuropathy (Osuntokun et al., 1968; Williams and Osuntokun, 1969;

Osuntokun and Osuntokun, 1971).

Tropical ataxic neuropathy as seen in Nigerian patients is characterized by bilateral optic atrophy, bilateral nerve deafness and predominantly posterior column myelopathy with or without peripheral neuropathy (Williams and Osuntokun, 1969). There is evidence that chronic cyanide intoxication from cassava may be an aetiologic factor (Monekosso and Wilson, 1966; Osuntokun, 1968). Williams and Osuntokun (1969) studied the pathologic changes in the peripheral nerves of patients with the syndrome, employing light and electron microscopy, and demonstrated evidence of demyelination.

A comparison between the concentration of thiocyanate and the level of vitamin B_{12} in plasma of Nigerian patients who suffered from degenerative neurologic symptoms and in plasma of healthy persons has been made (Monekosso and Wilson, 1966). As expected, the thiocyanate concentration was significantly higher in patients than in healthy persons. Since the consumption of cassava is high in Nigeria, it was suggested as the main source of cyanide and hence thiocyanate. On the other hand, no differences were found between patients and healthy persons with regard to total vitamin B_{12} content. However, the plasma content of hydroxocobalamin was smaller in patients than in healthy persons. Osuntokun (1968) also found high plasma thiocyanate levels in patients suffering from the ataxic neuropathy and concluded that this represented detoxification of cyanide. The levels fell when the patients were kept in hospital on a cassava-free diet (Osuntokun, 1968).

(e) Sterility in female heavy smokers

According to the report of Heller (see Baumeister et al., 1975) the thiocyanate content of the cervix mucus is between 0.2 and 1.0 mg/

100 ml. In two women with sterility which could not otherwise be explained, thiocyanate concentrations were between 1.0 and 2.3 mg/ 100 ml. Heller (see earlier) thus suggested that values about 1.0 mg can damage the spermatozoa and hence explain the sterility af some female heavy smokers.

(f) Goitre

The goitrogenic action of cassava was first demonstrated by Ekpechi (1967) in experimental studies with rats. Since then, several papers have appeared confirming this finding and implicating thiocyanate as the main cause (see Van Der Velden and Kinthaert, 1973). The action of thiocyanate on the thyroid gland has been described by Wolff (1964). Thiocyanate causes a drastic depletion of thyroid iodine content and a decrease in the plasma-bound iodine, thus explaining its goitrogenic effect.

4. Treatment of cyanide intoxication

(a) Treatment of acute cyanide intoxication

Several reviews on the methods for the treatment of acute cyanide intoxication have been made (Oke, 1969; Baumeister et al., 1975). Generally, the therapeutic aim is the lowering of the cyanide concentration in blood and tissues.

Sulphur-containing substances have been shown to be good antidotes especially when injected before the cyanide dose. An outstanding compound in this regard is sodium thiosulphate, which acts as the sulphur donor for the enzymatic conversion of cyanide to thiocyanate.

$$CN^- + Na_2S_2O_3 + O \longrightarrow NaSCN + Na_2SO_4$$

Its disadvantage however, is the relatively slow rate of action (Friedberg, 1968). A combined administration of sodium thiosulphate

with the detoxicating enzyme, rhodanese, was described by Clemedson et al. (1954). Another sulphur-containing antidote is sodium tetrathionate (Draize, 1933), which was shown by Chen et al. (1934b) to be as effective as sodium thiosulphate.

Compounds capable of forming methaemoglobin could serve as antidote in acute cyanide poisoning (Kiese, 1974). Geiger (1933b) reported successful treatments with methylene blue. Methylene blue is known to react with haemoglobin to form non-toxic cyanmethaemoglobin. Other compounds which are capable of forming methaemoglobin include sodium nitrite for injection and amylnitrite for inhalation (Geiger, 1933a; Chen et al., 1933a, 1933b). According to Chen and Rose (1952) the combination of nitrites and thiosulphate has proved therapeutically successful in animal experiments and in human cases of poisoning. More recently, aminophenols have been recommended as good methaemoglobin producers (Kiese and Weger, 1965a, 1965b) for detoxification of cyanide. One of these (4-dimethylaminophenol) exerts the quickest action in human blood compared with other tested aminophenols (Kiese and Weger, 1969). Also, with respect to its action on respiration and circulation, 4-dimethylaminophenol is superior to nitrite. In man, 30% of the haemoglobin could be oxidized with doses of 4-dimethylaminophenol which had little effect on blood pressure and pulse. Doses of sodium nitrite which are recommended for therapy and have a comparable effect produce collapse. The formation of methaemoglobin only reaches a maximum of 6% within 30 minutes (Kiese et al. 1968).

The mechanism whereby these chemicals are able to produce methaemoglobin is not known. It has been suggested however, that the formation of intracellular H_2O_2 which rapidly converts haemoglobin

to methaemoglobin may be responsible (Lehmann and Huntsman, 1974).

Some authors have recommended the application of cobalt compounds: aquocobalamin (hydroxocobalamin), cobalt EDTA and cobalt histidine (Mushett et al., 1952; Weber et al., 1962; Friedberg et al., 1966; Friedberg and Schwarzkopf, 1969). These compounds act by binding cyanide in circulating blood, and thus rapidly reducing its concentration in the extracellular fluids. Schwarzkopf and Friedberg (1971) investigated the antidotal action of cobalt EDTA and cobalt-histidine in guinea pigs and cats poisoned with a continuous infusion of NaCN solution. They concluded that these substances have a high rate of action but rather a low detoxification capacity. Administration of higher doses of these compounds to increase the detoxification capacity is not advisable, due to the danger of cobalt poisoning. On the contrary hydroxocobalamin can be applied in quantities of 300 mg/kg body weight and more without fear of intoxication (Friedberg and Shukla, 1975). Furthermore, these workers have shown that the combination of hydroxocobalamin (100 mg/kg) and thiosulphate (500 mg/kg) proved to possess a high rate of reaction and a large detoxification capacity in guinea pigs. Similar results were obtained in cats with antidote doses of 200 mg aquocobalamin/kg combined with 500 mg thiosulphate/kg. It is important to note that when combining aquocobalamin with thiosulphate as a means of therapy, one must avoid mixing these substances before injection or injecting them together. When mixed in solution, they form a thiosulphate-cobalamin compound that can no longer bind cyanide (Evans, 1964). This undesirable effect can be averted by observing a one-minute time interval between the two injections (Friedberg and Shukla, 1975).

(b) Treatment of chronic cyanide intoxication

Although chronic intake of cyanide does not lead to death, a number of visual defects of varying severity (already discussed in this chapter) is associated with prolonged cyanide intake. It is believed that most of these patients who are exposed to an abnormal intake of cyanide have reduced ability to convert cyanide to less toxic thiocyanate, and that this biochemical lesion is aggravated by vitamin B_{12} deficiency states (for literature, see Pettigrew and Fell, 1972).

The medical treatment consists of administration of doses of hydroxocobalamin (Wokes, 1958; Chisholm et al., 1967; Pettigrew and Fell, 1972). This treatment caused increased concentration of thiocyanate in plasma and urine, and these biochemical changes were accompanied by significant clinical improvement in vision (Pettigrew and Fell, 1972).

The treatment with hydroxocobalamin has been demonstrated to be superior to the use of cyanocobalamin (Smith and Duckett, 1965; Chisholm et al., 1967). Chisholm et al. (1967) divided patients with tobacco amblyopia into two therapeutic groups. One was treated with intramuscular injections of hydroxocobalamin, the other with cyanocobalamin. The improvement of visual acuity in the group treated with hydroxocobalamin was significantly higher compared with the group treated with cyanocobalamin. In patients who did not stop smoking, total healing could not be achieved in a single case with cyanocobalamin, but healing could be achieved with hydroxocobalamin.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

Materials and Methods

1. Materials

a) Equipment

Spectrophotometers: Unicam models SP600, SP800 and SP1800 were used during these studies.

Scintillation counter: The Intertechnique SL40 liquid scintillation counter was used throughout these studies.

b) Solvents

Chloroform and Methanol: Reagent grade.

Diethyl ether: Dried over sodium-lead alloy and redistilled with reduced iron present. This solvent was prepared fresh when required.

Toluene: ANALAR grade. This was used in scintillation fluid.

Dioxane: Refluxed for 2 h with sodium metal present and then redistilled. This solvent was used in scintillation fluid.

Light petroleum (60-80°): Dried over sodium-lead alloy and redistilled.

Pyridine: ANALAR grade.

NCS solubilizer: This is a product of Amersham/Searle Corporation and was used in tissue solubilization.

c) Special Chemicals

Bispyrozolone: BDH Chemicals Ltd.

Pyrozolone: BDH Chemicals Ltd.

2. Methods

2(a) Experiments with animals

The only animals used during the course of this work were albino rats from the stock colony of the Department of Biochemistry, University of Liverpool, which was derived from the CFHB strain of Carworth Europe (now Anglia Laboratory Animals), Alconbury, Huntingdon.

Food and water were given ad libitum.

2(b) Diets

Experimental rats were given either a basal diet with extracted soyabean meal as the main protein source, which contained retinoic acid but no retinol, or the standard rat cubes, commercial diet 41B. In each experiment, the particular diet employed is specified.

Extracted soyabean meal diet	Per cent
Sucrose	48.3
Soyabean meal (extracted)	40.0
Arachis oil	3.0
Methionine	0.5
Choline chloride	0.2
Minerals	6.0
B vitamins in glucose	1.0
Fat soluble vitamins in oil	1.0

The minerals were salts N of Fox and Briggs (1960) with the following additions to each 60 kg:

$NaMoO_4 . 2H_2O$	5 g
Na_2SeO_3	219 mg
NaF	500 mg
$CoCl_2 . 6H_2O$	2 g

H_3BO_3	10 g
$K_2Al_2(SO_4)_4.24H_2O$	50 g
$Na_2SiO_3.9H_2O$	50 g

The solution of fat-soluble vitamins in arachis oil contained (per kg arachis oil):

Crystalline vitamin D_3	1 mg
α-Tocopheryl acetate	15 g
Vitamin K_1 (phylloquinone)	500 mg

Retinoic acid was added in the arachis oil to give a final concentration of 3 µg/g diet.

The B vitamin mix in glucose contained (g/kg glucose):

Thiamine hydrochloride	12 g
Inositol	50 g
Calcium pantothenate	10 g
Nicotinic acid	50 g
p-Aminobenzoic acid	4 g
Riboflavin	8 g
Pyridoxine hydrochloride	4 g
Folic acid	2 g
Cyanocobalamin	0.015 g
Biotin	0.15 g

All diets were mixed freshly every fortnight and stored. They were moistened with arachis oil before being given to the rats in order to increase acceptability and minimize waste.

This basal diet was used in many feeding experiments and also for investigating the stability of cyanide when added to the diet. It is referred to in the thesis as the 'basal soyabean meal diet'.

In all experiments where this diet was used, retinyl acetate was added once weekly to provide 140 µg (426 n moles) per rat per day, as it contained retinoic acid and no retinol.

2(c) Administration of supplements

All supplements of vitamin A were either administered orally as solutions in arachis oil or added in arachis oil to the diet (5 g arachis oil to 95 g diet). In either case, the tocopherols present in arachis oil acted as antioxidants. When the vitamin A supplement was administered orally, the appropriate dose was contained in 0.2 ml oil and administered by means of a 1 ml syringe. The rat was gripped firmly at the back of the neck with the thumb and forefinger and its mouth forced open by drawing the facial skin tight. A rat held in this position swallowed the supplement with a minimum of discomfort.

Administration of cyanide as potassium cyanide was carried out by addition of a known quantity of the salt to the diet mixed with arachis oil (see Chapter III) instead of moistening with water (5 g arachis oil to 95 g diet). The cyanide was added to about 10 g diet which was given to the rat in the evening. 10 g was about the amount of diet which each rat could consume overnight. When this had all been eaten (by the morning), the rat was given the soyabean meal diet (containing no cyanide) ad libitum. The cyanide was relatively stable in such a diet and the loss from samples kept overnight was always less than 3%. The problems of adding potassium cyanide to rat diet when moistened with water are discussed in the next chapter. In certain experiments, cyanide was also given as dilute aqueous solutions of its potassium salt in rat-water bottles where losses were found to be around 1% when kept overnight (Chapter III).

2(d) Examination of rat skeleton

As much tissue as possible was dissected off the carcass which was then placed in boiling water to soften the remaining tissues and facilitate their removal. The bones were carefully cleaned with forceps and a needle, and bleached overnight in hydrogen peroxide (20 vol). They were then passed through absolute alcohol and chloroform and allowed to dry before examination.

2(e) Histology of the kidney

Kidneys (cut into two, longitudinally) were fixed in phosphate buffer, pH 7.0, with 4% v/v formaldehyde. The period of fixation was 48 hours. Dehydration was effected by immersion in alcohol of increasing strength (50%, 70%, 80%, 90% and absolute), followed by clearing in chloroform. All kidneys were embedded in paraffin (m.p. 58°) and sectioned at 4 µm using a Cambridge rocking microtome.

For routine examination, sections were mounted on albuminized slides, dried in an oven at about 60°, and stained by either the haematoxylin eosin technique or by the Von Kossa technique. Xylol was used throughout as the dewaxing agent before staining.

i) Haematoxylin and Eosin staining

Dewaxed slides were transferred from water to haematoxylin solution, where they were left for five minutes. After draining off excess haematoxylin, slides were rinsed in water and differentiated by immersing in 1% HCl. They were then rinsed again in distilled water and 'blued' in tap water. Finally slides were rinsed in water and counterstained in 1% Eosin solution. The counterstained slides were dehydrated in absolute alcohol and cleared in xylol (two changes) and then mounted.

ii) Von Kossa staining

The deposition of calcium in kidney cells was confirmed by staining sections by the Von Kossa technique. Here, dewaxed slides were taken out of distilled water, placed in a jar of freshly prepared 1.5% silver nitrate solution, and allowed to stand in the dark for two minutes. The sections were then rinsed in distilled water and reduced in freshly prepared 1% hydroquinone in 10% sodium sulphite until calcium turned black (as checked with a control section). Sections were counter stained in Van Gieson solution for ten minutes, cleared in xylol and mounted.

Complete details of the dehydrating, clearing, staining and mounting technique may be obtained from Culling (1963).

The entire histological work was carried out by the author in the Department of Pathology, University of Liverpool. Mr. John Massey and other members of the technical staff of the above Department offered me valuable assistance and supervision during the course of the work, for which I am most grateful.

3. Experimental methods

3(a) Estimation of Vitamin A

The use of trifluoroacetic acid to estimate retinol and its esters has been described by Neeld and Pearson (1963). They showed that the trifluoroacetic acid (TFA) method retained the sensitivity and specificity of the antimony trichloride test (Carr and Price, 1926) but did not exhibit the turbidity and film-forming properties of the latter reagent in the presence of moisture. Furthermore, the blue colour of the TFA reagent does not fade as quickly as the Carr-Price test. The method was calibrated with retinyl acetate. The amount of retinyl acetate in each tube was dissolved in a mixture

of 1 ml cyclohexane (spectroscopically pure) and 0.5 ml chloroform. 1.5 ml trifluoroacetic acid solution (33 ml trifluoroacetic acid made up to 100 ml with chloroform) was added to the spectrophotometer cell containing the vitamin by using a rapid delivery syringe. Each sample was read against a blank tube containing 1 ml cyclohexane, 0.5 ml of chloroform and 1.5 ml trifluoroacetic acid solution.

i) Extraction and estimation of retinol in rat plasma

The chemical methods for estimating the vitamin A concentration in blood plasma depend for their accuracy on complete removal of the retinol from its carrier protein. In order to achieve this, the protein component is precipitated and the free vitamin A extracted. Kahan (1966) described a method of quantitative extraction of vitamin A based on studies of the partition of the vitamin in various two-phase solvent systems. Usually, ethanol (99.5%) is used to precipitate the protein and to dissolve the vitamin initially; a second solvent, e.g. cyclohexane, light petroleum or chloroform is added to the mixture and shaken and the whole mixture centrifuged, the top layer (of cyclohexane for example) then contains the vitamin and is removed. Kahan (1966) claimed recoveries of over 95% for added retinol using this procedure on a small volume of human plasma.

A modification of this technique given by McLean (1970) was adopted. 2 to 10 ml plasma was mixed with ethanol (10 ml) in a separatory funnel and 20-30 ml light petroleum was added and the mixture shaken. The solution was allowed to settle and the protein layer was run off; the petrol layer was collected. The protein solution was re-extracted with 20 ml light petroleum and the petrol extracts were combined and blown down under nitrogen. The residue was taken up in a mixture of 1 ml cyclohexane and 0.5 ml chloroform,

1.5 ml TFA solution was added and the absorbance was read at 620 nm in an SP600 spectrophotometer (at a fixed time following addition of TFA - 30 seconds). A standard curve (Fig.II.1) was prepared using known amounts of retinyl acetate dissolved in chloroform. At amounts higher than about 4.5 µg retinyl acetate, the absorbance becomes non-linear.

By adding 100 µg retinol to 20 ml plasma (from rats which had been maintained on a vitamin A deficient diet supplemented with retinoic acid) and taking the blood through the extraction procedure as described, McLean (1970) obtained recoveries near 100 per cent.

ii) Extraction and estimation of retinyl acetate added to diets

Retinyl acetate added to diet was extracted three times with 50 ml portions of freshly redistilled ether. 5 ml of the ether extract was diluted to 100 ml with ethanol and the absorbance at 326 nm measured in a spectrophotometer (SP 800) against a blank extraction using the same diet with no added vitamin A. The amount of retinyl acetate was calculated from the absorbance at 326 nm using the molar extinction coefficient, 50,800, without further correction. The procedure was checked using the TFA method and the results obtained by the two independent methods were found to agree very closely (difference was below 5%).

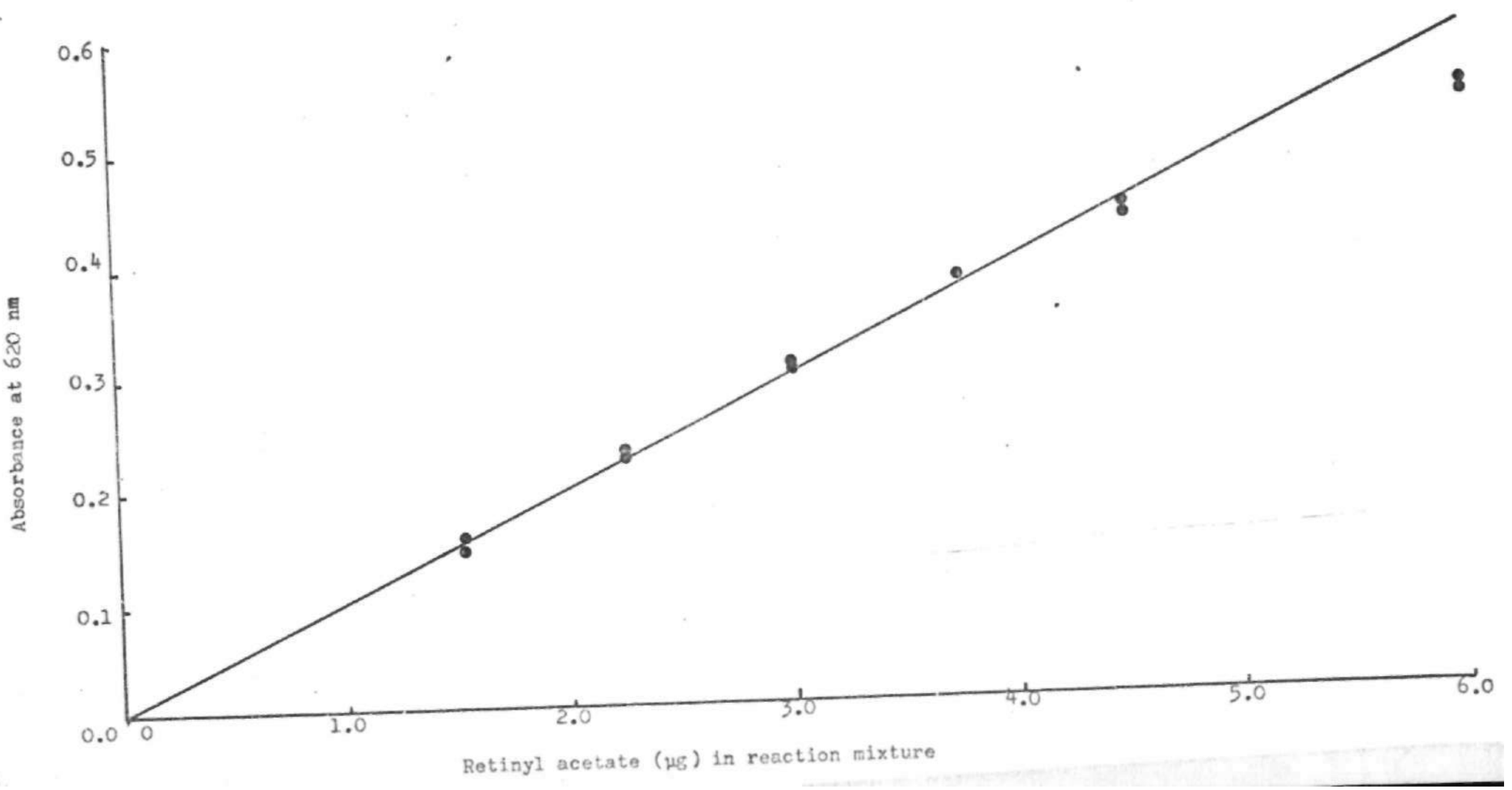
iii) Estimation of vitamin A in liver

Saponification of tissue and extraction of non-saponifiable lipid.

The method used was that described by Coward (1966). Homogenized liver (2-5 g) was saponified by refluxing with a mixture of 10.7 M aqueous KOH (0.5 ml per gm tissue) and 0.07 M pyrogallol in ethanol (1 ml per gm tissue) for 35 minutes. The saponification mixture was then diluted with an equal volume of water and extracted

FIGURE II.1

Standard curve for the estimation of vitamin A using the Trifluoroacetic acid (TFA) method.



three times with freshly distilled dry peroxide-free ether. The ether extract was washed with water until the washings were neutral, dried over anhydrous sodium sulphate and evaporated to dryness.

The unsaponifiable residue was redissolved in cyclohexane and its U.V. spectrum drawn. In some cases where the retinol content was high, it was possible to estimate the amount of retinol directly from this spectrum without using a correction procedure of the type described by Cama et al. (1951). Otherwise, the retinol was estimated chemically by the trifluoroacetic acid method already described (see estimation of retinol in plasma).

The amount of retinol as estimated directly from the U.V. spectrum was found to agree closely with that obtained by the TFA method when the liver retinol store was so high that the irrelevant absorption was relatively negligible.

3(b) Tissue protein estimation

Protein was estimated by the method of Lowry et al. (1951) according to Campbell and Sargent (1967).

To the sample of tissue (usually a homogenate suspended in 1 ml water) were added 4 ml of a mixture (50 parts 0.2M Na_2CO_3 in 0.1M NaOH and 1 part 0.04M $CuSO_4$ in 0.07M sodium potassium tartrate) and the solution was allowed to stand 10 min. 0.5 ml Folin-Ciocalteu reagent (diluted 100 to 236 with distilled water) was added with vigorous mixing, and the entire mixture diluted to 20 ml. The extinction at 750 nm was read after 30 min. A standard calibration curve was prepared using bovine serum albumin. The calibration curve shows two linear portions; the slope at higher protein concentrations being less than that at lower concentrations (also observed by

Campbell and Sargent, 1967). This limitation was overcome by working at protein concentrations where the slope was linear. The typical calibration curve obtained is shown (Fig. II.2).

3(c) Spectrophotometric determination of methaemoglobin and total haemoglobin.

i) Methaemoglobin by the cyanide method

Methaemoglobin reacts with cyanide (even at low concentration) to form cyanmethaemoglobin (ferrihaemoglobin cyanide) but ferrohaemoglobin does not react with cyanide at low concentrations. Furthermore, methaemoglobin shows a strong absorption at 630 nm which disappears on the addition of cyanide due to the formation of cyanmethaemoglobin. The decrease in absorption of methaemoglobin at 630 nm which is observed by the addition of cyanide is used in the determination of the former in the presence of ferrohaemoglobin.

1 ml heparinized blood was lysed in a solution with 30 mls detergent (Nonidet P40 diluted 1-100) and 20 mls 0.1M phosphate buffer pH 7.0 and centrifuged to remove stroma. The supernatant was used for the estimation of methaemoglobin content of the blood.

A portion of the supernatant (designated D) was read at 630 nm = D1.

1 drop of KCN (50 g/l) was added and the absorbance at 630 nm read again = D2.

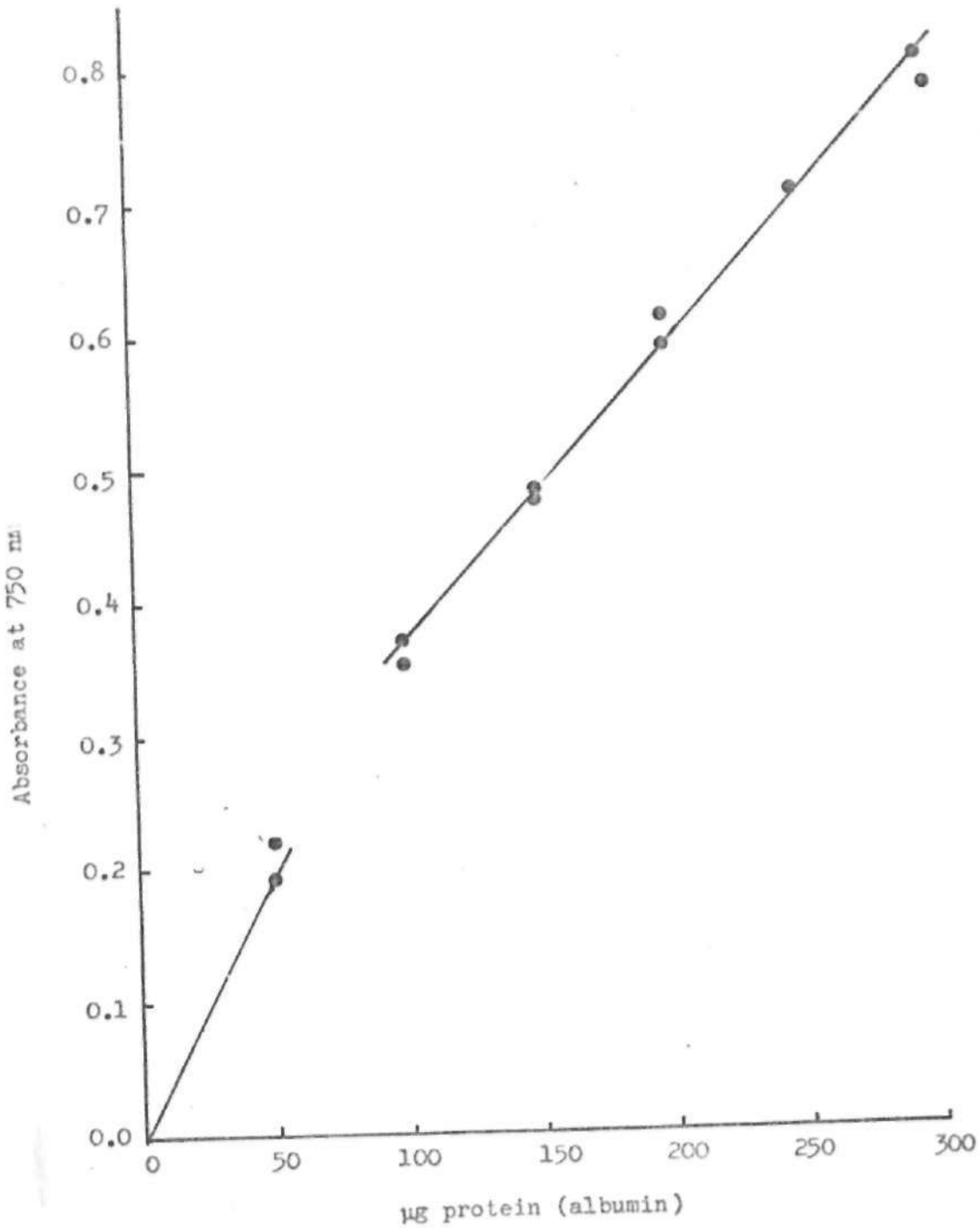
To a second portion of the supernatant (designated E) was added 1 drop of ferricyanide (50 g/l) and absorbance read at 630 nm = E1.

1 drop of KCN (50 g/l) was added and the absorbance read at the same wavelength = E2.

The percentage methaemoglobin was calculated from the

FIGURE II.2

Calibration curve for the estimation of protein by the method of Lowry et al. (1951).



following equation:

$$\% \text{ methaemoglobin} = \frac{D1 - D2}{E1 - E2} \times 100$$

ii) Total haemoglobin by the methaemoglobin method

Total haemoglobin was calculated from E1-E2 (section 3 c.i) using the calibration curve obtained by the method below.

The calibration curve for the measurement of total haemoglobin by conversion to methaemoglobin was prepared using heparinized blood from a male rat (methaemoglobin is unstable and hence standard samples are not available).

Stroma free supernatant was prepared as already described (section 3 c.i) and various dilutions were made with a mixture of phosphate buffer and detergent (ratio 2:3). Each was treated with a drop of potassium ferricyanide followed by a drop of potassium cyanide and the reading taken at 630 nm (E630).

The concentration of haemoglobin in the blood was determined by the cyanmethaemoglobin method described below (section 3 c.iii). From the values obtained, the concentrations of haemoglobin in the various dilutions of the supernatant were calculated and these were plotted against the corresponding E630 readings (Fig.II.3).

iii) Total haemoglobin by the cyanmethaemoglobin method

Total haemoglobin may be determined by oxidation with potassium ferricyanide in the presence of KCN to form cyanmethaemoglobin with an absorption maximum at 540 nm.

A calibration curve (Fig.II.4) was prepared for the measurement of haemoglobin by its converaion to cyanmethaemoglobin using a sample of standard cyanmethaemoglobin.

The stroma-free supernatant (section 3 c.i) was diluted with phosphate buffer: detergent (2:3) to give a 200 times dilution of

FIGURE II.3

Calibration curve for the estimation of haemoglobin by the conversion to methaemoglobin.

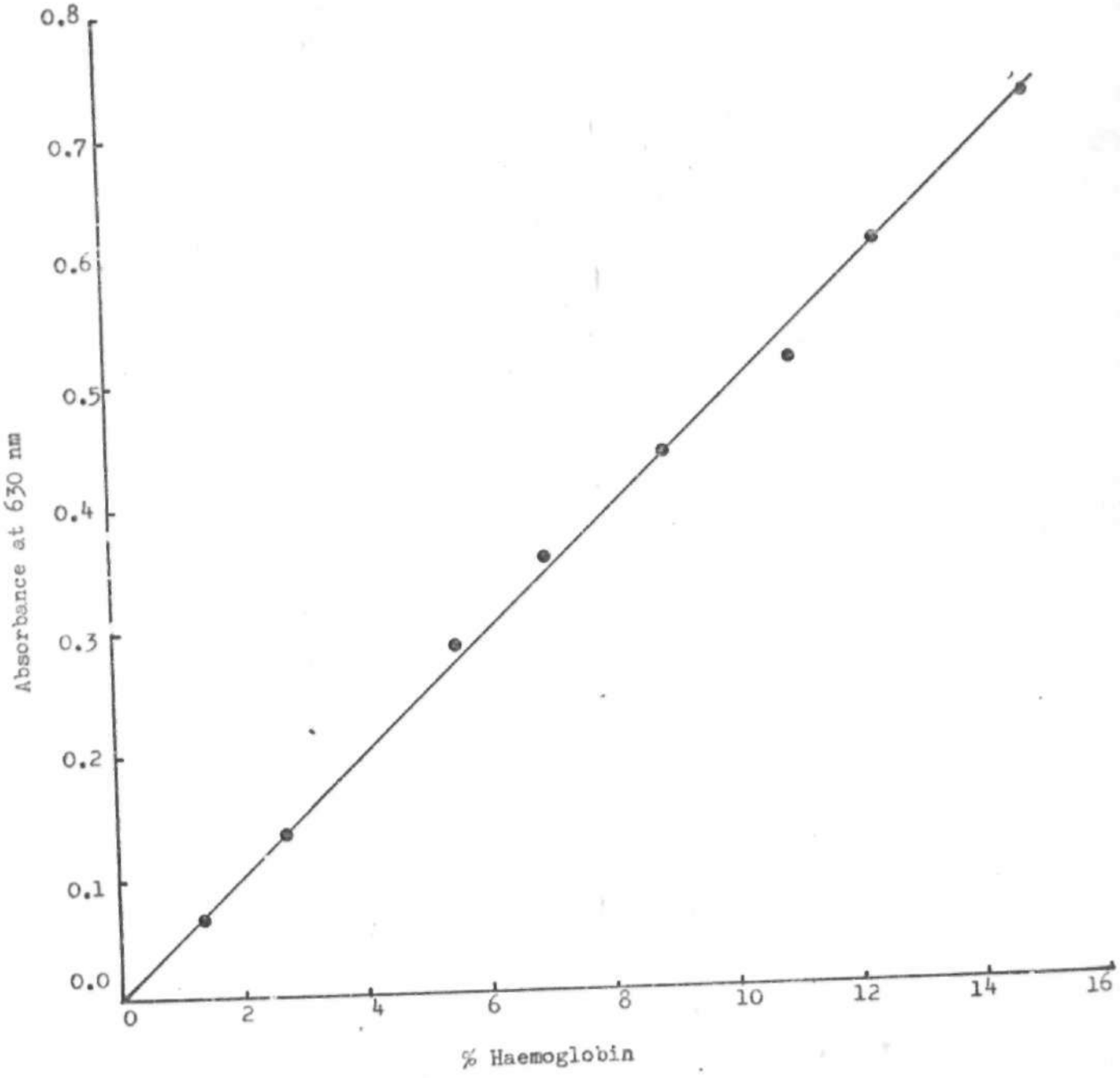
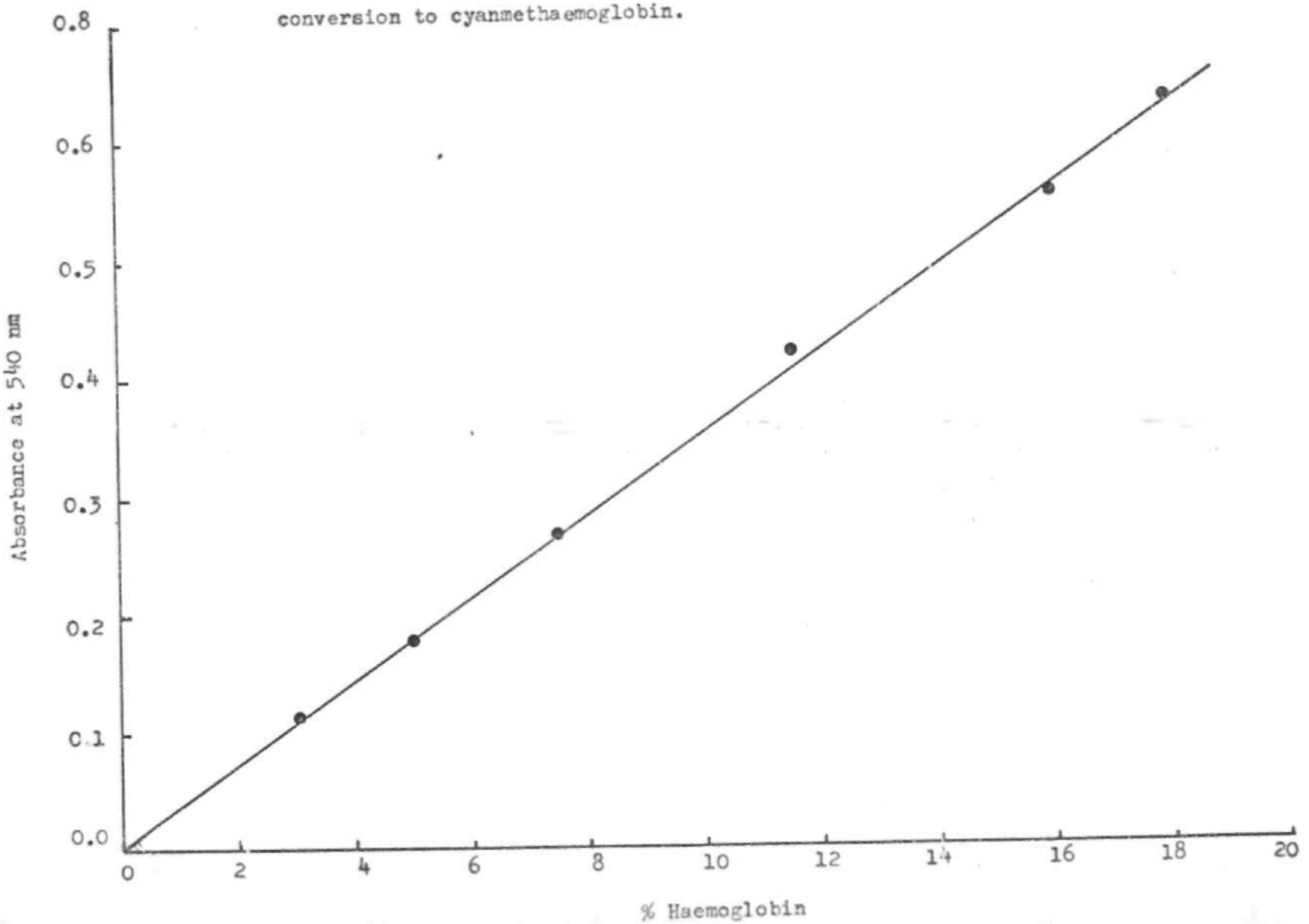


FIGURE II.4

Calibration curve for the estimation of haemoglobin by the conversion to cyanmethaemoglobin.



original blood. A drop of potassium ferricyanide was added followed by a drop of KCN and the absorbance was read at 540 nm. The reading was used with the calibration curve (Fig.II.4) to obtain the concentration of haemoglobin in grams per 100 mls blood.

iv) Methaemoglobin by the carbon monoxide method

Ferrohaemoglobin combines with carbon monoxide to form a carbon monoxy derivative (HbCO, a particularly stable derivative). Methaemoglobin does not react. However, methaemoglobin can be reduced to ferrohaemoglobin by the addition of sodium dithionite.

The method of Nichol and Morell (1968), which was used here for the estimation of methaemoglobin, depends upon the increase in extinction at 569 nm after adding dithionite to a suitable diluted blood sample gassed with carbon monoxide. Before addition of dithionite, the reading at 569 nm - absorption maximum of HbCO - includes some absorption due to methaemoglobin. A correction is applied after reduction by adding the decrease in absorption at 638 nm (where absorption for methaemoglobin is the same as at 569 nm) to the value of A569 nm.

The assay was performed as follows: 1.0 ml of oxalated blood was diluted to 10 ml with distilled water and then centrifuged (3000 g x 10 min) to remove stroma. To 1 ml of the supernatant was added 9.0 ml of pH 6.0 phosphate buffer (prepared by mixing 6.15 ml 0.1M Na_2HPO_4 with 43.85 ml 0.1M NaH_2PO_4 and diluting to 100 ml with distilled water) and a moderate stream of carbon monoxide was passed through the solution for 3 minutes in the form of large bubbles to avoid denaturation of proteins (Nichol and Morell, 1968). The absorbance was measured immediately at 569 nm and 638 nm using an SP1800 spectrophotometer (delay beyond 10 mins at this pH may permit

autoxidation of haemoglobin giving falsely high values). A few crystals (about 2 mg) of fresh sodium dithionite were added to the solution which was then mixed by repeated gentle inversion and the absorbance was measured again at 569 nm and 638 nm.

The methaemoglobin concentration expressed as grams per 100 ml whole blood is calculated from the formula:

$$\text{Methaemoglobin (g/100 ml blood)} = \frac{A569 + A638 + 10 \times 16.1}{15.5}$$

where 15.5 = mM for carbon monoxyhaemoglobin, 10 = dilution factor for blood sample and 16.1 is derived from the internationally recognized molecular weight per haem of haemoglobin which is 16,115 (Nichol and Morell, 1968).

v) Total haemoglobin by the dipyridine method

The following procedure was used (Nichol and Morell, 1968):

To 1 ml of the 1-10 diluted centrifuged blood (section 3 c.iv) were added 1M NaOH and a few crystals of sodium dithionite. The mixture was diluted to 25 ml with aqueous pyridine (diluted with an equal volume of distilled water). Total haemoglobin was calculated from the following formula (Nichol and Morell, 1968):

$$\text{Total haemoglobin (g/100 ml blood)} = \frac{A557 \text{ nm} \times 25 \times 16.1}{30.9}$$

where 30.9 = mM for dipyridine ferroprotohaemochrome. 16.1 is derived from the molecular weight of a one haem-containing subunit of haemoglobin.

3(d) Packed cell volume

The packed cell volume of heparinized whole blood was determined by the microhaematocrit method. Haematocrit capillary tubes were used. The blood samples were thoroughly mixed by inversion, and allowed to enter the tubes by capillarity, leaving about 15 mm unfilled.

The tubes were then sealed by means of a plastic seal and centrifuged for five minutes using the microhaematocrit centrifuge. The packed cell volume (PCV) was measured directly from a reading device provided with the instrument.

3(e) Estimation of cyanide added to diets

Between 0.5 and 2.0 g of the diet was placed in a Markham distillation apparatus and the sample washed down with about 5 ml distilled water. The apparatus was assembled so that the tip of the condenser dipped below the surface of 50 ml 0.6M NaOH in the receiver. About 8 ml 2.5M HCl was added to the sample in the distillation flask (care being taken to ensure that no HCN escaped), the mixture was steam distilled, and about 150 ml distillate was trapped in the receiver. The distillate (which now contains alkali cyanide) was diluted to 250 ml with distilled water, 8 ml 6M NH_4OH and 2 ml KI solution (50 g/L) were added and the sample was titrated with 0.02M $AgNO_3$ using a microburette. The endpoint was indicated by a faint but permanent turbidity and was easily recognized especially against a black background.

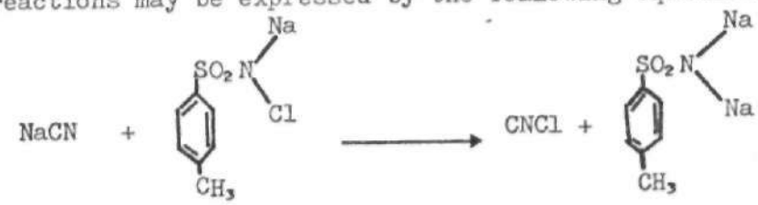
1 ml 0.02M $AgNO_3$ = 1.08 mg HCN

The distillation procedure was checked by distilling known volumes of standard KCN solution. Recoveries were always near 100% (97%-101%), when the amount of potassium cyanide was 5 mg or greater. Below 5 mg KCN there was a progressive increase in over-estimation (128% recovery was obtained for 2.5 mg KCN). This technique for estimating cyanide is discussed in "Official methods of analysis of Association of Official Agricultural Chemists" (1970).

3(f) Estimation of microquantities of cyanide and thiocyanate in plasma or serum.

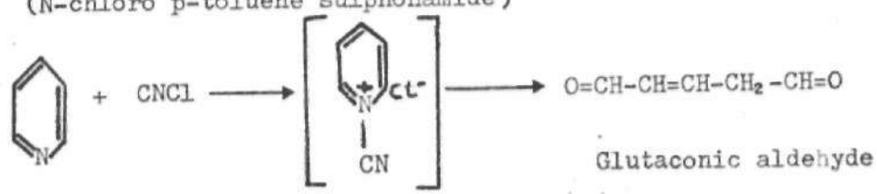
i) Cyanide

Cyanide in serum or plasma was measured by separating it from the sample, absorbing into sodium hydroxide, and estimating the CN in the alkaline extract. Separation was achieved by the nitrogen aeration of Boxer and Rickards (1952). The aeration vessel contained 1 ml sample of serum or plasma, 1 ml 0.1M sulphuric acid, 1 ml 8% aqueous silicone antifoaming agent and 2 ml distilled water. Oxygen-free nitrogen was swept through the aeration vessel for 45 mins at a flow rate of about 800 ml/minute and the volatile HCN absorbed into 2 ml 0.05M sodium hydroxide. Cyanide in the alkaline extract was estimated by the technique of Epstein (1947). In this method, cyanide is converted to cyanogen chloride by chlorination with chloramine T solution. On the addition of a mixture of pyrazolone and pyridine, pyrazolone reacts with excess chloroamine T while cyanogen chloride preferentially reacts with the pyridine to form an intermediate aldehyde (glutaconic aldehyde). The aldehyde then combines with the excess pyrazolone to form a blue coloured dyestuff. The reactions may be expressed by the following equations:

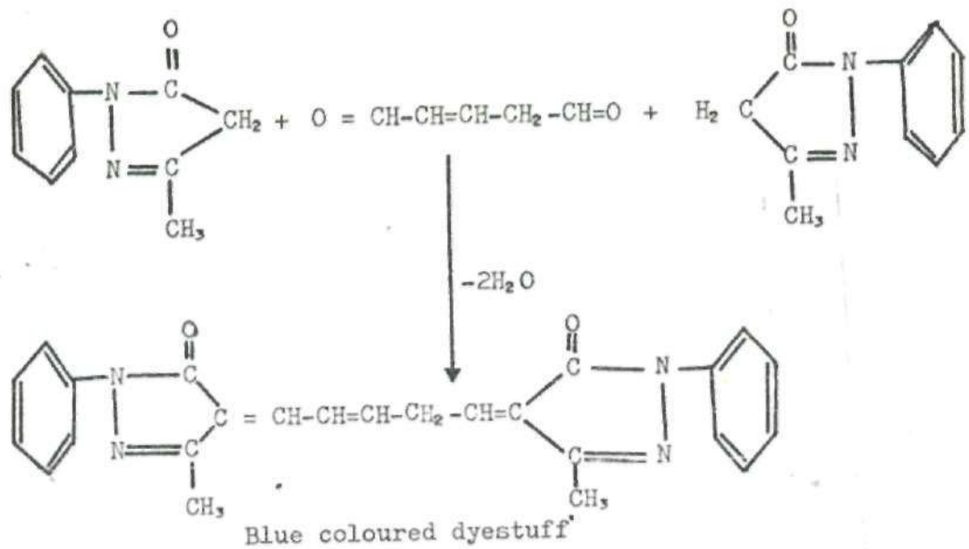


Chloramine T

(N-chloro p-toluene sulphonamide)



Glutaconic aldehyde



Blue coloured dyestuff

Thus to 1 ml cyanide (absorbed in 0.05M sodium hydroxide), in an ice cooled test tube, was added 0.2 ml chloramine-T reagent (Boxer and Rickards, 1951). The tube was stoppered, mixed thoroughly and replaced in ice bath. After 2 min, 3 ml pyrazolone reagent was added and the mixture allowed to stand for 1 hr at room temperature. Absorbance of the blue-coloured solution was read at 630 nm against blank reagents. A calibration curve (Fig.II.5) was prepared using 1 ml of known concentrations of cyanide in 0.05M sodium hydroxide.

Details of the method for preparing and ensuring the stability of solutions employed, and conditions for obtaining maximum colour development have been given by Boxer and Rickards (1951) and Ballentyne et al. (1972).

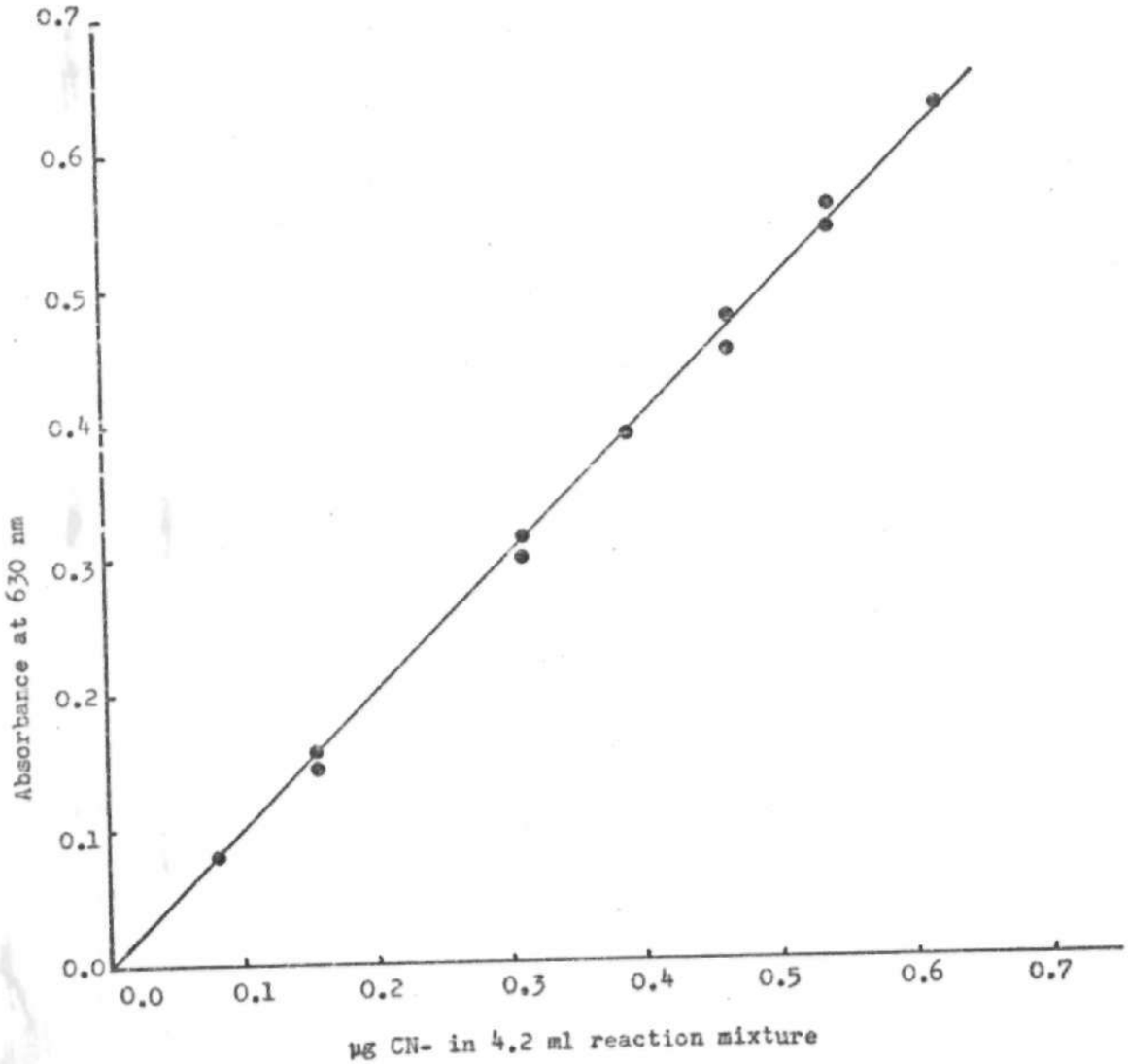
ii) Thiocyanate

Thiocyanate was measured in the same sample of plasma used to determine cyanide, by oxidizing it to cyanide with permanganate and measuring the cyanide so formed.

To the previously aerated fluid was added 3 ml 20% trichloroacetic acid to precipitate the proteins. After centrifugation, 4 ml of supernatant was transferred to a fresh aeration tube and 1 ml 0.01M

FIGURE II.5

Calibration curve for the estimation of microquantities of cyanide by the method of Epstein (1947).



potassium permanganate and 1 ml 0.05M sulphuric acid were added. After allowing 3 minutes for full oxidation, 1 ml 2% stannous chloride was added to reduce excess permanganate and aeration for separation of volatile hydrogen cyanide was begun. The separation and estimation of cyanide was as described above.

3(g) Estimation of cyanate formed by tissues from cyanide

Two independent methods were used to estimate cyanate: the method of Shaw and Bordeaux (1955) and that of Chung and Wood (1971).

i) The Shaw and Bordeaux method

This method employs ion-exchange removal of interfering cations, conversion of cyanate ions to ammonium ions by dilute acid, separation of ammonium ions by ion-exchange, and photoelectric colorimetric analysis of ammonium ions with Nessler's reagent. The following steps were followed:

(1) To 3 ml acetate buffer pH 5.0 was added 1 ml diluted blood (containing 0.1 - 0.2 ml heparinized blood).

(2) The mixture was allowed to equilibrate in a water bath at 37^{o} for 4 minutes.

(3) The reaction was started by the addition of 20 µmoles KCN (in 0.1 ml solution), and the mixture incubated for 20 minutes (assay tubes were stoppered to minimise loss of HCN by evaporation).

(4) After incubation, the tubes were cooled rapidly in ice and centrifuged at 3,000 g for 10 minutes.

(5) The supernatant from (4) was adjusted to pH 6-7.6 using bromothymol blue indicator, and quantitatively transferred to an ion-exchange column (11 ml Dowex 50, 20 to 50 mesh in a 15 mm by 7 cm column joined to a 35 mm by 200 mm reservoir).

(6) The solution was passed through the column at a rate of 1 drop every three seconds and the effluent retained in a 100 ml volumetric flask (this effluent contained the cyanate while interfering cations were retained).

(7) The column was washed with water and washings retained in the same volumetric flask used in step 6.

(8) To wash out interfering cations from the column, the column reservoir was filled with about 90 ml water and 2 ml 1M NaOH added. This solution was allowed to flow at a full flow rate and the column was then rinsed thoroughly with water.

(9) To the effluent containing cyanate (steps 6 and 7) was added 10 ml 0.5M H_2SO_4. The mixture was allowed to stand for 10 minutes (cyanate ion is converted to NH_4+).

(10) The solution was titrated to a light green end point (using bromothymol blue) with dilute sodium hydroxide solution (proper pH is essential for quantitative adsorption on column) and quantitatively transferred to the column.

(11) Steps 6 and 7 were repeated except that the effluent which contained the interfering anions were discarded in this case.

(12) The column was washed with water until indicator free and 20 ml water containing 0.5 ml 1M NaOH was used to elute the ammonium ions at a flow rate of 1 drop every two seconds.

(13) 1 ml Nesslers reagent was added and the mixture diluted to 25 ml.

(14) The solution was allowed to stand for 15 minutes and absorbance measured at 420 nm in an SP600.

(15) The blank consisted of assay systems treated similarly but containing either no enzyme or no KCN (a boiled enzyme blank was also tested).

Using a standard cyanate solution, recoveries ranged between 83.0 to 88.0% (mean 86.6%) for four determinations.

A standard calibration curve (see Fig.II.6) was prepared by reacting known concentrations of ammonia with Nessler's reagent.

ii) The Chung and Wood method

The Chung and Wood procedure for estimation of cyanate was employed with modifications.

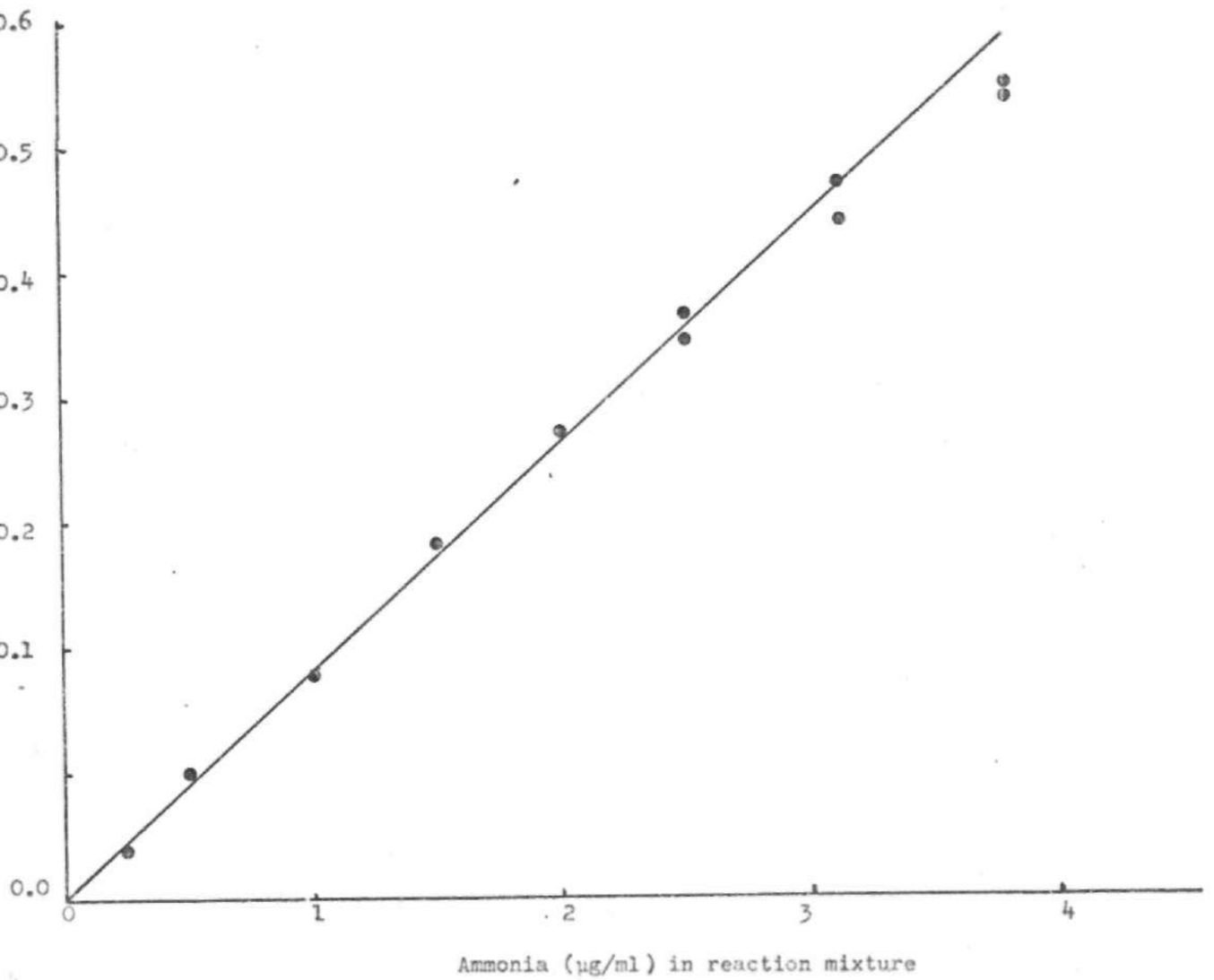
After incubation of assay tubes, they were rapidly cooled and centrifuged at 3,000 g for 10 minutes (as already described under Shaw and Bordeaux method). The supernatant was transferred into a 25 x 150 mm test tube. 1 ml 0.5M H_2SO_4 was added, mixed, the tube was stoppered and allowed to stand for 10 minutes (cyanate is converted to ammonium ion in this step). Two drops of phenolphthalein indicator were added and the pH of solution adjusted to about 10 by dropwise addition of MNaOH. 1 ml 8% silicon antifoaming agent was added and the tube was closed with a two-hole rubber stopper fitted with gas inlet and outlet tubes. Two traps were connected in series: (1) a 25 x 150 mm test tube containing 5 ml 0.1M HCl and (2) a tube of the same size but containing 0.1M NaOH. The first trap served to remove ammonia while the second removed HCN and presumably other volatile acidic gases. Complete transfer of ammonia was achieved over a 20 minute period by aeration with nitrogen (it was found necessary to raise the temperature to 40° to ensure quantitative removal of ammonia from the assay tube).

After aeration 1 ml of Nessler's reagent was added to the tube containing HCl; the mixture was diluted to 25 ml and colour developed as already described (see Shaw and Bordeaux method).

The amount of cyanate determined in this way was corrected for ammonia in the incubation mixture using either tissue homogenate

FIGURE II.6

Standard curve for the estimation of ammonia by Nessler's reagent.



in the absence of cyanide or boiled tissue in the presence of cyanide (both blank types gave the same quantity of ammonia).

Using standard ammonia or cyanate solutions, recoveries ranged between 90-94% (mean 92.1%) for six determinations.

3(h) Estimation of inorganic phosphate in plasma

Plasma inorganic phosphate was estimated by the method of Fiske and Subbarow (1925).

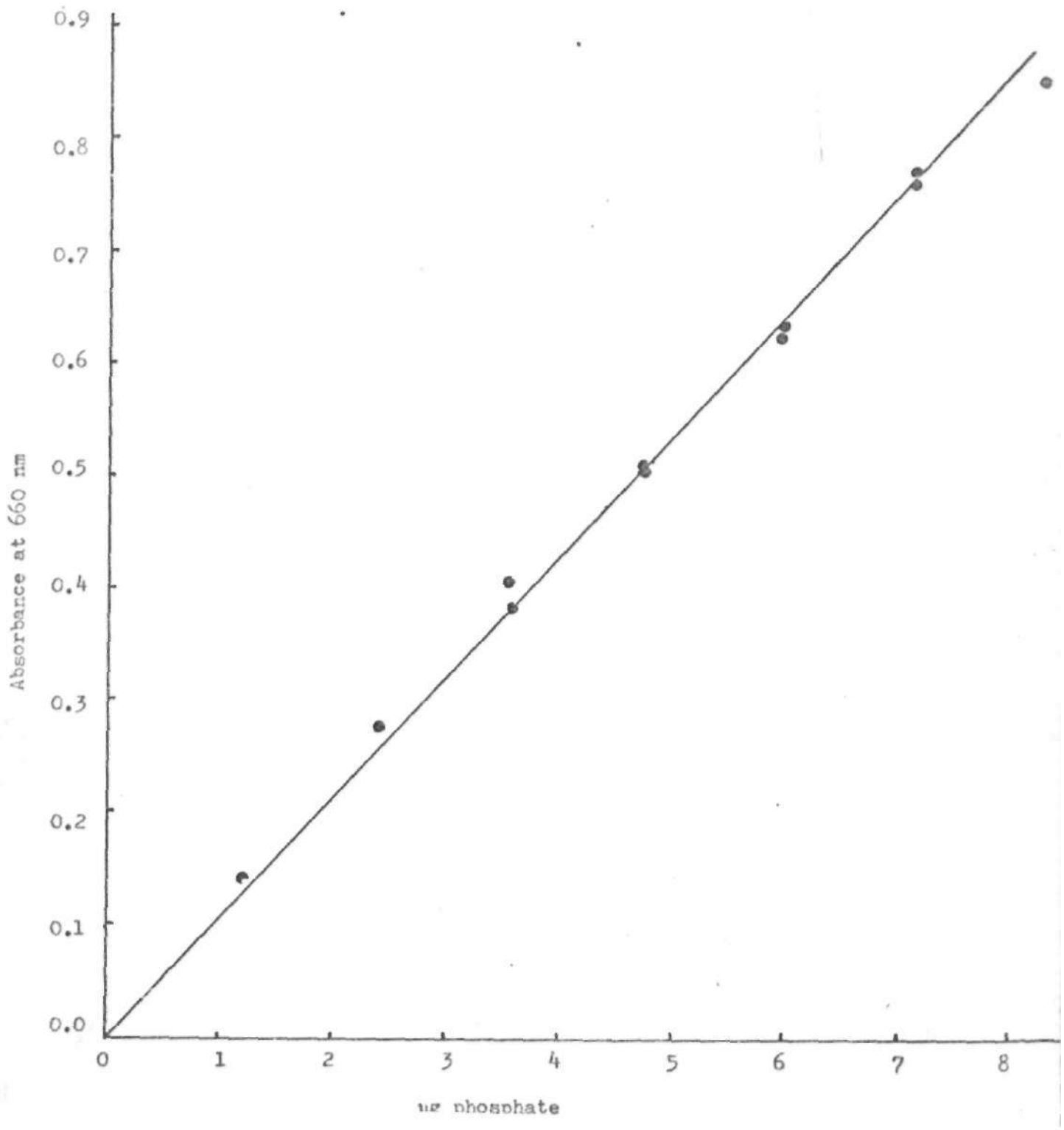
To 8 ml 10% trichloroacetic acid in a centrifuge tube was added 2 ml plasma drop by drop with constant mixing (in certain cases 1 ml plasma was added to 4 ml trichloroacetic acid). The tube was shaken well and allowed to stand for two minutes, and the clear supernatant stored for estimation of plasma calcium and inorganic phosphate content.

1.8 ml clear supernatant was mixed with 5 ml 2 mM ammonium molybdate solution and 0.2 ml Fiske-Subbarow reducer solution (10 mM 1-amino-2-naphthol-4-sulphonic acid with 0.56M sodium sulphite). Colour was allowed to develop for exactly 15 minutes at room temperature and absorbance was measured at 660 nm in a spectrophotometer zeroed with the tube prepared in the same way but containing 1.8 ml 8% trichloroacetic acid in place of plasma supernatant.

A calibration curve (Fig.II.7) was prepared using various concentrations of sodium dihydrogen orthophosphate in 8% trichloroacetic acid. Readings were reproducible and formed a straight line between 2.5 to 15 µg phosphate per assay tube.

FIGURE II.7

Calibration curve for the estimation of phosphate by the method of Fiske and Subbarow (1925).



3(i) Plasma calcium estimation

Calcium in plasma was measured by the method of Clark and Collip (1925). In this method, calcium is precipitated as calcium oxalate and the oxalic acid liberated when sulphuric acid is added to this precipitate is titrated with potassium permanganate.

To 4 ml clear supernatant (see precipitation of plasma proteins in method used for determination of plasma inorganic phosphate) in a 15 ml centrifuge tube was added 1 ml 0.25M ammonium oxalate solution. Ammonia solution (diluted 1:1 with water) was added dropwise until methyl red indicator changed from red to yellow. The tube was allowed to stand overnight and then centrifuged until the precipitate was well packed in the bottom of the tube. The supernatant liquid was carefully poured off, and the tube was clamped for 5 minutes at an angle of 45° to drain. The precipitate was stirred up and the sides of the tube washed with 3 ml dilute ammonia water (2 ml concentrated ammonia [31.5% w/w] and 98 ml distilled water). The suspension was centrifuged and the supernatant liquid drained again as just described; then 2 ml 0.5M sulphuric acid was added. The tube and contents were placed in a hot water bath maintained at 70-75°, shaken properly to dissolve the precipitate and the oxalic acid liberated was titrated with 0.002M potassium permanganate, using a microburette.

1 ml 0.002M $KMnO_4$ $\equiv$ 0.2 mg calcium.

Using standard calcium carbonate solution (dissolved in presence of HCl) in place of clear supernatant as standard, recoveries were found to range between 94% to 99% (average 96.3%) in three separate determinations.

4. Isotopic material and counting technique

[^{14}C] Potassium cyanide of specific activity 57.0 mCi/mmole was obtained from the Radiochemical Centre, Amersham. The K^{14}CN as obtained was dissolved in 0.1M NaOH, stored in the frozen state, and purified as needed.

4(a) Purification of K^{14}CN

K^{14}CN was purified by the method of Boxer and Rickards (1952), using a Warburg vessel.

A concentrated solution of the radioactive potassium cyanide was introduced into the outer compartment of a Warburg vessel, a small calculated excess of alkali was introduced into the centre well, and an excess of sulphuric acid into the side arm. After evacuation of the vessel, the acid was tipped into the outer compartment and the closed evacuated vessel left at room temperature for 1 hr. The contents of the centre well were quantitatively removed, the cyanide concentration determined, and the appropriate dilutions prepared. This procedure is known to separate hydrogen cyanide quantitatively from cyanate or thiocyanate and formate (Boxer and Rickards, 1952).

A check on the recovery of cyanide by the above purification method was carried out using non-radioactive cyanide, and a mean of 99 ± 3.8 (SD) was obtained in six separate purifications. Recoveries of ^{14}CN following purification by same procedure were also 100%. This clearly shows that the radioactive K^{14}CN as supplied was 100% pure.

4(b) Solubilization of tissues for liquid scintillation counting

Homogenates of one part of tissue in two parts of water were dispensed by pipette; 0.2 ml-0.5 ml samples of each tissue were

digested with 3 ml NCS solubilizer, overnight at room temperature and then at 50° until digestion appeared to cease. Heating times varied from less than 1 hr to a maximum of 6 hrs. Sample digestions were carried out in glass counting vials, of which the foil-lined caps were provided with insert liners of teflon.

Background samples were prepared for labelled tissue samples by performing identical procedures on unlabelled tissues from control animals. All samples were prepared in duplicate.

Digests were examined for completeness of dissolution and diluted samples for evidence of precipitation by holding vials against a dark background. Digestion was usually continued until no further change in appearance was observed.

A detailed account of solubilization procedures for liquid scintillation counting of biological materials has been given by Hansen and Bush (1967).

4(c) Decolourization

Only whole blood was found to be so highly coloured as to require bleaching. In this case, benzoyl peroxide was used as bleaching agent. A solution of benzoyl peroxide was prepared freshly by heating 1.0 g of the reagent in 5 ml toluene at 60°. The solution was cooled to room temperature and filtered. To decolourize a sample, 0.3 ml of the filtered solution was added per 1 ml of NCS used and the sample further digested until no further decolourization was observed.

4(d) Scintillation counting

Two types of scintillator solutions were used throughout these studies: (1) a dioxane scintillator solution and (2) a toluene

scintillator solution.

For each type of scintillator solution, quench correction curves were prepared by the external standard method. This permitted correct computation of absolute activity in samples. Typical quench correction curves based on the Intertechnique SL40 (refrigerated) used throughout these studies are given in Figures II.8 and II.9. Vials were usually left in the dark for a few hours before counting.

i) Solubilized tissues

A toluene scintillator fluid was used for solubilized tissues. The solubilized tissues were cooled before addition of scintillator solution to give a final volume of approximately 20 ml. The concentration of fluor in the scintillator solution was such that it yielded 6 g/L PPO (2,5-Diphenyloxazole) and 0.075 g/L POPOP (1,4-Di-[2-(5-phenyloxazolyl)]-benzene) in the final counting solution.

ii) Aqueous inorganic solutions

For aqueous inorganic solutions, NCS was mixed with the toluene scintillator fluid before addition of the aqueous samples.

In certain cases, dioxane scintillator solution was used for aqueous inorganic solutions. The scintillator cocktail used in this case was: 1 litre dioxane (purified by refluxing for 2 hr with sodium metal and then distilling).

7 g PPO

0.3 g POPOP

100 g naphthalene (scintillation grade)

In this case, NCS was not required and 0.1ml-2ml aqueous solution was counted in 10 ml of dioxane scintillator solution.

iii) Trichloroacetic acid precipitates (TCA precipitates)

About 0.1g-0.4g TCA precipitate was solubilized in 3-5ml NCS and counted in toluene scintillator fluid as already described for solubilized tissues.

FIGURE II.8

^{14}C quench curve from toluene scintillation fluid (10 ml per vial) containing increasing volumes of CCl_4 (10 µl to 175 µl). Data based on SL40.

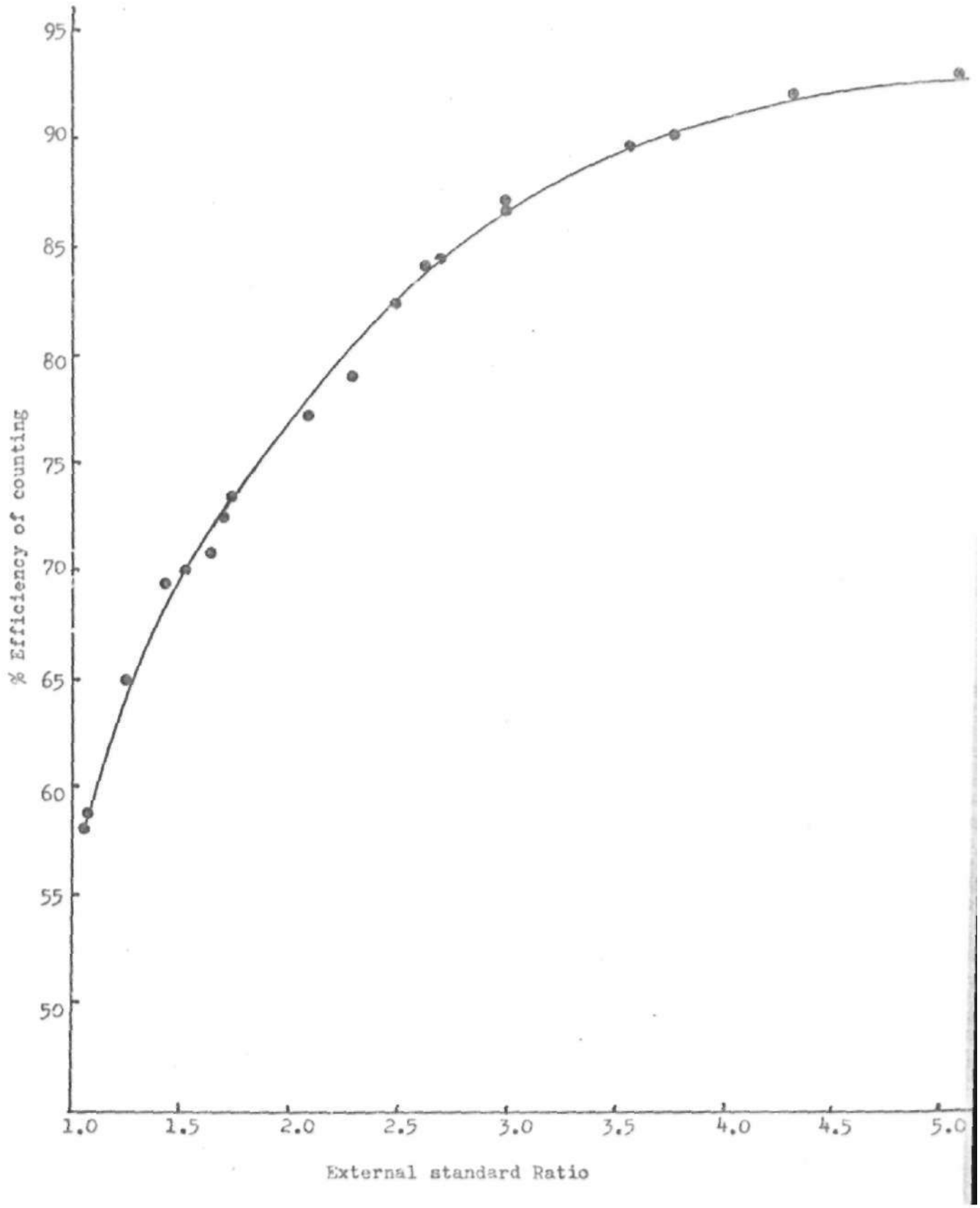
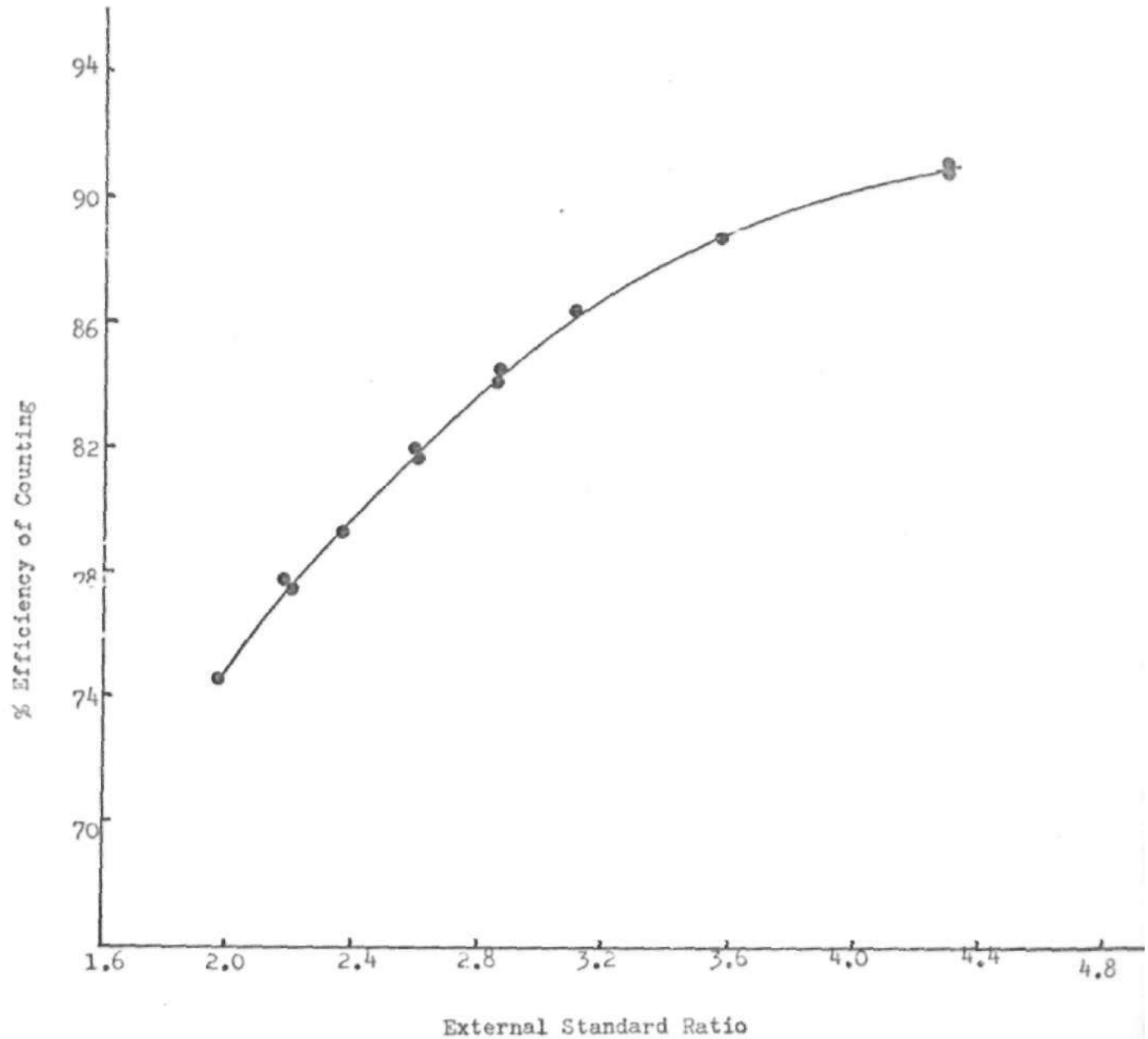


FIGURE II.9

^{14}C quench curve from dioxane scintillation fluid (10 ml per vial) containing increasing volumes of CCl_4 (16 µl to 112 µl). Data based on SL40.



CHAPTER III

CHAPTER III

The availability of potassium cyanide added to the diets or drinking water of the rat and the tolerance of cyanide by the rat.

1. Introduction

Potassium (and sodium) cyanide in aqueous solution is hydrolysed with comparative ease. Hydrolysis proceeds even at room temperature, though slowly.

$$KCN + H_2O \rightleftharpoons KOH + HCN$$

The solution is thus strongly alkaline and smells of HCN.

Moreover, hydrocyanic acid is a very weak acid which quickly reacts with water when in solution and more rapidly in the presence of inorganic acids to form first of all formamide and then ammonium formate (Oke, 1969).

$$HCN \xrightarrow{H_2O} HCONH_2 \xrightarrow{H_2O} HCOONH_4$$

In view of the proven instability of cyanide, most investigators have evaluated the toxicity and metabolism of cyanide present in foodstuffs largely by feeding diets containing cyanogenic glycosides to experimental animals (Blakley and Coop, 1949; Osuntokun, 1970; Van Der Valden and Kinthaert, 1973). When sodium or potassium cyanide has been given to experimental animals over a prolonged period of days, it has been administered, in dilute solution by repeated injections (Smith and Foulkes, 1966; Chander and McGinity, 1977). Estimation of cyanide toxicity by the administration of cyanogenic glycosides to experimental animals may not provide a good index, as the actual amount of cyanide absorbed will depend on the mode of metabolism of the glycosides - whether they are converted to

free cyanide or not. On the other hand, injection of inorganic cyanide to experimental animals is physiologically unrelated to the normal mode of ingestion. In a few cases however, potassium cyanide has been administered to experimental animals either in their diet or in their drinking water (Clark, 1936, 1939).

So far as is known, no report has been presented on the stability of inorganic cyanide when added to diets of experimental animals. However, studies on the stability of inorganic cyanide when added either to saline or to phosphate buffer (pH 7.0) at room temperature have been reported (Pettigrew and Fell, 1972; Ballantyne, 1973). In neither solution was there extensive loss of HCN, but the experiments lasted only for one hour. It cannot be concluded that cyanide would be stable when left in the diet over a period of many hours.

Recently, a number of papers have reported on the stability of cyanide added to blood or plasma of animals or on the blood or plasma removed from cyanide-poisoned animals (Ballantyne *et al*. 1970, 1972, 1973); Balantyne, 1976, 1977). They emphasized that in the context of both forensic and clinical toxicology, it is clearly necessary to be aware of the various factors that may lead to alterations in cyanide concentrations in blood or plasma after its withdrawal from the body. When there has been delay between removal of blood originally containing high concentrations of cyanide and the analysis of such blood, transformation of cyanide may present problems (Ballantyne, 1974, 1975). Transformation or loss of cyanide could give unreliable analytical results.

In studies on the metabolism and chronic toxicity of cyanide, potassium cyanide has been added to diets of animals (Clark, 1936,

1939). However, to date, no paper has reported on the stability of inorganic cyanide added to animal diets. Experiments described in this chapter are designed to establish the availability of cyanide added to diets of experimental animals. A method is presented for administering potassium cyanide in the diets of experimental animals (normally consumed over a long period) in which no significant loss of cyanide occurred.

2. Experimental procedure

The stability of potassium cyanide in the diets of experimental animals was investigated using three types of diet. Two were semi-synthetic - one based on extracted soyabean meal, another based on skimmed milk and a third was obtained by grinding up cubes of the commercial diet 41B.

10 ml of KCN solution (10 mg/ml) was added to 0.5-1 g of diet and then mixed properly. The mixture was left overnight in an open 100 ml beaker for about 18 hours. Estimation of cyanide in each sample was carried out by distilling off the cyanide into standard sodium hydroxide solution using the Markham apparatus, followed by titration with standard $AgNO_3$ solution (see Chapter II).

3. Results

3(a) Stability of potassium cyanide added to diets and drinking water

Clark (1936, 1939) described experiments in which KCN and HCN were given to rats in drinking water. No report was given on the stability of the cyanide solution during the period of administration. The stability of KCN solution when stored at room temperature in an open beaker or in a rat water bottle (corked and inverted in a rat cage, in the same manner in which water is normally administered to

experimental rats) is presented in Table III.1. Loss of KCN from the water bottles (as HCN) was only about 2% over a period of 18 hrs. In contrast, KCN solution kept in an open beaker overnight lost about 40% of KCN. This indicates that KCN in an open beaker is lost largely as HCN by diffusion.

In preliminary experiments on the stability of potassium cyanide when added to rat diet, 0.5-1.0 ml KCN solution (100 mg/ml) was added to 10 g of diet, mixed thoroughly and left in an open beaker overnight (about 18 hours). No cyanide was detectable in 1-2 g of such diets by the assay method already described in Chapter II. KCN added to such a diet was thus completely lost during storage in an open beaker. The loss of KCN from such diet was confirmed by the failure of 10 g of such diet (originally containing 100mg of KCN) to produce toxic effects in rats weighing about 200 g each. Rats on such a diet ate well and grew like controls.

Because of the practical difficulties of detecting small amounts of cyanide in diet, a different procedure was adopted in subsequent experiments - the effect of diet on the loss of cyanide from an aqueous solution of KCN was studied (see experimental procedure described in section 2 of this chapter). The results are shown in Tables III.2 and 3. It can be seen that about 50% of cyanide was lost when 0.5 g of diet was added to 10 ml of KCN solution containing 10 mg/ml (Table III.2). When this was compared with the loss of cyanide from KCN solution (alone) in an open beaker (Table III.1) it was seen that the addition of soyabean and groundup commercial diets resulted in an increased loss of cyanide from the KCN solution by about 10%. In contrast, the loss of cyanide from the KCN solution when skimmed milk was added is much the same as that lost from an open beaker. When the amount of each diet added to the KCN solution

TABLE III.1

Recovery of cyanide from KCN solution after storage.

Type of storage	Storage time (hrs)	% of KCN recovered (mean ± S.E.M., n=5)
Open beaker	18.0	64.6 ± 6.3
Rat water bottle	18.0	98.4 ± 1.0
Control	0.0	99.6 ± 1.1

TABLE III.2

Recovery of cyanide from KCN solution added to 0.5 g diet.

Type of diet	Storage Time (h)	% of added KCN recovered (mean ± SEM, n=4)
Semi-synthetic diet based on soya bean meal	0	95.5 ± 0.9
	18	48.5 ± 1.9
Semi-synthetic diet based on skimmed milk	0	94.3 ± 1.4
	18	63.0 ± 1.4
Ground up commercial diet 41B	0	94.0 ± 1.6
	18	52.5 ± 0.9

TABLE III.3

Recovery of cyanide from KCN solution added to 1g diet.

Type of diet	Storage time (h)	% of added KCN recovered (mean ± SEM, n = 4)
Semi-synthetic diet based on soya bean meal	18	29.8 ± 1.0
Semi-synthetic diet based on skimmed milk	18	19.0 ± 1.4
Ground up commercial diet 41B	18	31.3 ± 2.2

was increased to 1 g, greater loss of KCN (about 70% in the case of skimmed milk or soya bean meal diet and about 80% in the case of commercial diet 41B, Table III.3) occurred. The causes of this loss are investigated later in Chapter VII.

It is clear therefore that the toxic level of cyanide cannot be correctly estimated by addition of potassium cyanide to diets of animals to which water is usually added. Furthermore, estimation of cyanide toxicity by feeding diets containing cyanogenic glycosides to experimental animals may not provide a useful index, as the actual amount of cyanide absorbed will depend on whether the glycoside can be converted to free cyanide ions or not (Oke, 1969). Thus addition of inorganic cyanide to the diet of animals may represent a more reliable method for evaluating the toxic level of cyanide. However, if inorganic cyanide is to be mixed thoroughly in the diet, the latter must be powdered. But powdered diet needs addition of a little water to avoid undue spilling by the rats and to improve acceptability. As has been shown here, water, which is usually employed for this purpose, causes substantial loss of cyanide. A procedure for administering potassium cyanide in diets to rats, using arachis oil, was described in ch. II. The stability of KCN in these conditions was investigated using a diet based on extracted soyabean meal (basal soyabean meal diet).

To 20 g of the diet was added about 0.4 g KCN. The diet was mixed thoroughly with the KCN and stored in an open 100 ml beaker for about 18 hours. Estimation of KCN was carried out in approximately 1 g samples using the method already described in Chapter II. The recovery of KCN from the oil diet is much the same as the recovery from the diet before storage (Table III.4, $P = 0.3$). The KCN is therefore stable when mixed in an 'oil' diet. Moreover, the diet mixed with arachis oil was found to be acceptable to the rats.

TABLE III.4

Recovery of cyanide from semi-synthetic diet based on soya bean meal (mixed with arachis oil) to which KCN was added.

Storage time (h)	% of added KCN recovered (mean[a] ± SEM, n = 4)
0	95.5 ± 0.9
18	93.0 ± 1.8

[a]The difference is not significant statistically.

Although cassava, which contains the cyanogenic glycoside linamarin, has been used extensively in the study of cyanide toxicity and metabolism (Clark, 1936, 1939; Osuntokun, 1970; Van Der Valden and Kinthaert, 1973; Tewe et al. 1977), only recently (Barrett et al. 1977) has a paper been presented on the fate in the rat of purified linamarin given orally in the absence of the active linamarase which in cassava liberates cyanide from linamarin. The report showed that no intact linamarin was present in the faeces of dosed animals. Over 16% of the dosed linamarin was excreted unchanged in urine, indicating that a substantial amount of ingested linamarin is absorbed intact from the intestinal tract.

Thus, the addition of KCN to semi-synthetic diet mixed with 5% arachis oil represents a most effective way of ensuring that the administered cyanide is taken by the animal in its fully available form. It is also a method of administration which is physiologically related to the normal mode of ingestion.

3(b) Plasma thiocyanate content of rats given potassium cyanide in the diet.

Eight male rats were divided into two groups of four rats each (groups DPA and DPB). The rats, weighing between 200 g and 230 g, were transferred from commercial diet 41B to the soyabean meal diet. After one week on this diet, a supplement of 5.0 mg (77.0 µmoles) KCN was added to the diet of group DPB rats. The cyanide was added to the amount of diet which each rat would consume overnight (about 10 g) after which they were fed ad libitum (see Chapter II). No KCN was added to the diet of group DPA rats.

At the end of three weeks on the cyanide supplement, one rat at a time from each group was anaesthetised with halothane and blood was

removed by heart puncture, chilled and centrifuged as rapidly as possible (at 3,000 g for 10 min) to obtain plasma. Cyanide and thiocyanate contents of each plasma were estimated by the method already described in Chapter II.

The thiocyanate content of the plasma of rats on cyanide was significantly higher than those of control rats ($P < 0.01$, Table III.5). A number of investigations on the relationship between the concentration of thiocyanate in the plasma and the cyanide intake have been published (Osuntokun, 1968, 1970; Osuntokun et al., 1968; Van Der Velden and Kinthaert, 1973; Tewe et al., 1977). These workers confirmed that considerably higher amounts of thiocyanate are found in the plasma of animals ingesting cyanide in the diet compared with animals not ingesting this ion.

Furthermore, animals ingesting cyanide are known to excrete more thiocyanate in the urine compared with control animals (Wokes, 1958; Mehta and McGinity, 1977). It has also been shown (Chapter VIII) that the addition of potassium cyanide to the diets of rats by the method described in this chapter, led to an increase in thiocyanate excretion in the urine. Clearly therefore, the cyanide added to the diet mixed with arachis oil (as described in this Chapter) is not only stable in the diet, but also gets into the body of the animals ingesting the diet.

3(c) The tolerance of cyanide by rats

Eighteen albino rats weighing between 70 and 120 g, used for the experiments described here, were maintained on diet 41B. They were divided into three groups of six rats each (D, E and F). Group D rats served as controls, E were given drinking water containing 0.1 mg KCN/ml while F rats had drinking water containing 0.25 mg KCN/ml. The

TABLE III.5

The plasma thiocyanate concentration of rats given cyanide in the diet.

Group	Substance added to diet µmoles/rat/day	Thiocyanate concentration µg/100 ml	µ moles/100 ml
DPA	None	99	1.7
		180	3.1
		226	3.9
		157	2.7
Mean		166	2.9
S.E.M.		±26	±0.5
DPB	Potassium cyanide (77.0)	1,189	20.5
		858	14.8
		1,015	17.5
		429	7.4
Mean		873	15.1
S.E.M.		±163	±2.8

actual amount of cyanide taken by each rat per day was calculated from the concentration of cyanide in drinking water and the volume of water taken. The experiment lasted for 48 days. The drinking water containing the cyanide was provided in rat water bottles.

The addition of cyanide to the drinking water of rats of groups E and F resulted in a reduction of water intake (Table III.6, Fig. III.1) relative to control group ($P < 0.025$). Rats of groups E and F nevertheless drank adequate amounts of water (for the last 20 days, around 30 ml/day). In a preliminary investigation it had been found that rats drank less water when KCN was added to it, possibly because of the high alkalinity of the cyanide solution.

The rats given cyanide in the drinking water gained slightly less weight than did the controls (Table III.7 and Fig. III.2) but the differences were not statistically significant, even at the high level of dosing in group F ($P > 0.1$).

The average amount of KCN taken by each rat ranged from 2.0 to 8.9 mg/day over a period of 48 days; the total amounts of cyanide taken by each rat during the same period varied between 97 and 425 mg.

3(d) Haemoglobin and Methaemoglobin content of rats on cyanide

The haemoglobin content of the blood of rats given potassium cyanide in the drinking water (group F) was determined by the dipyridine method (Chapter II) while that of another group of rats given cyanide in the diet (group DB) was determined by the methaemoglobin method (Chapter II). The results are given in Tables III.8 and III.9. The haemoglobin content of the rats in group F did not show any difference when compared with control rats (group D). Rats in group DB however, showed lower levels of haemoglobin compared with controls, although this was not statistically significant ($P > 0.05$).

TABLE III.6

The amount of water and KCN taken by rats when potassium cyanide was added to the drinking water for 48 days.

Group	Concentration of KCN in the drinking water mg/ml	Average volume of water taken per day	Total KCN taken for 48 days
D	0.0	33.0	0.0
	0.0	36.4	0.0
	0.0	30.5	0.0
	0.0	37.6	0.0
	0.0	28.9	0.0
	0.0	30.0	0.0
	0.0	32.7	
Mean		±1.5	
S.E.M.			
E	0.1	20.3	97.4
	0.1	25.4	121.9
	0.1	24.0	115.2
	0.1	28.4	136.3
	0.1	31.3	150.2
	0.1	27.5	132.0
	0.1	26.2	125.5
Mean		±1.6	±7.5
S.E.M.			
F	0.25	29.0	348.0
	0.25	22.6	283.2
	0.25	22.0	264.0
	0.25	24.3	292.8
	0.25	21.3	255.8
	0.25	35.5	427.2
	0.25	25.8	311.8
Mean		± 2.2	±26.6
S.E.M.			

FIGURE III.1

Daily water intake of groups of rats given cyanide in drinking water.

(Bars are ± S.E.).

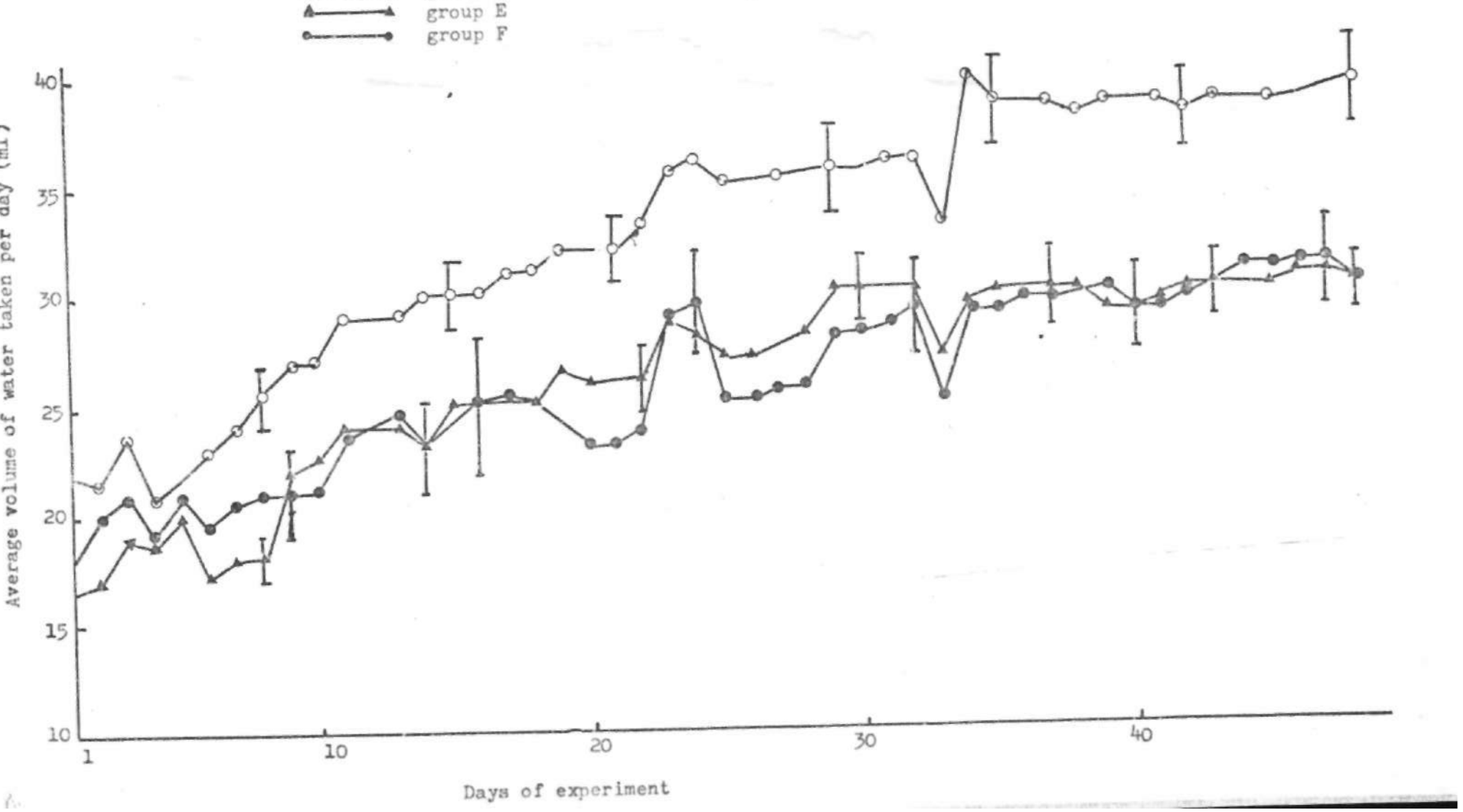


TABLE III.7

Growth of rats given cyanide in the drinking water for 48 days.

Group	Initial weight of rats (g)	Final weight of rats (g)	Gain in weight (g)
D	97	286	189
	77	305	228
	84	320	236
	86	287	201
	89	308	219
	97	318	221
Mean	88	304	216
S.E.M.	±3	±6	±7
E	68	230	162
	73	246	173
	70	275	205
	62	282	220
	109	322	213
	85	285	200
Mean	78	273	196
S.E.M.	±7	±13	±9
F	115	325	210
	94	255	161
	77	262	185
	84	280	196
	106	240	134
	140	380	240
Mean	103	290	188
S.E.M.	±9	±22	±15

The differences in weight gain between groups D and E, and D and F were not statistically significant ($0.1 < P < 0.2$).

FIGURE III.2

Weight gain of rats given cyanide in drinking water.

D = Controls

E = On KCN^- water - 0.1 mg/ml, Took 2.03-3.81 mg KCN per day.

F = On KCN water - 0.25 mg/ml, Took 5.33-8.8 mg KCN per day.

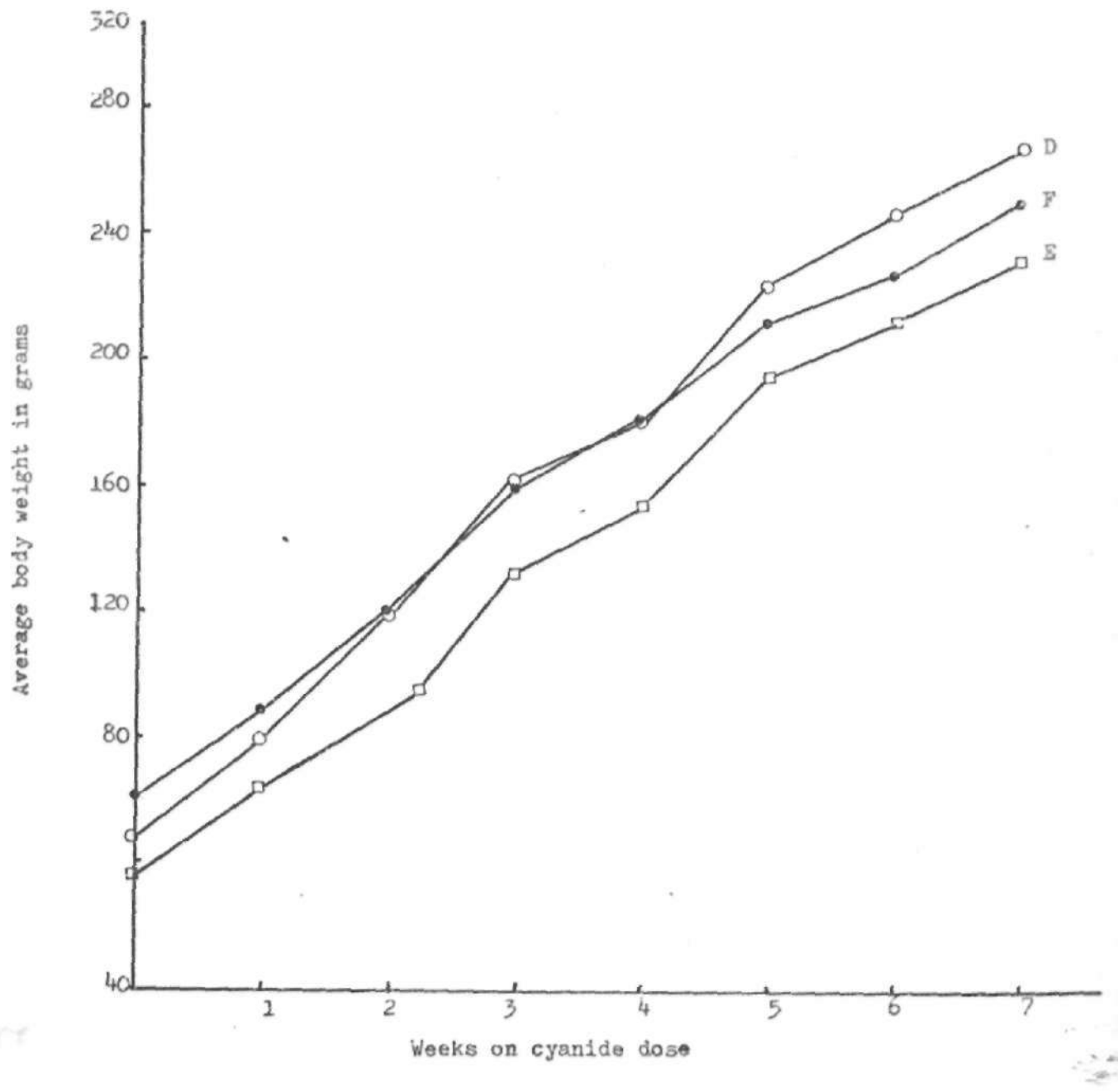


TABLE III.8

The packed cell volume, haemoglobin concentration (measured by dipyridine method) and methaemoglobin concentration (measured by CO method) of rats given cyanide in drinking water for 48 days.

Group	Supplement mg KCN per day	Packed cell volume %	Total haemoglobin g/100 ml blood	Methaemoglobin g/100 ml blood
D	0.0	39.5	11.5	0.9
	0.0	40.0	13.1	1.0
	0.0	35.5	12.5	0.4
	0.0	39.0	13.9	0.9
	0.0	32.5	13.9	0.9
	0.0	33.0	14.5	0.8
Mean		36.0	13.2	0.8
S.E.M.		±1.5	±0.5	±0.1
F	7.3	36.5	11.8	0.3
	5.9	39.5	12.1	0.6
	5.5	42.5	12.5	0.9
	6.1	30.0	13.5	0.4
	5.3	32.5	14.1	1.0
	8.9	39.0	14.2	1.0
Mean		36.7	13.0	0.7
S.E.M.		±1.9	±0.4	±0.1

None of the differences between groups D and F was statistically significant.

TABLE III.9

The methaemoglobin concentration (measured by cyanide method) and haemoglobin concentration (measured by methaemoglobin method) of rats on a diet containing potassium cyanide (38.5 µmole/rat/day) for three weeks.

Group	Substance added to diet	Methaemoglobin g/100 ml blood	Total haemoglobin g/100 ml blood
DA	None	0.8	15.0
		0.6	15.3
		0.6	15.3
		0.9	14.0
		0.5	13.6
		0.6	14.0
Mean		0.7	14.5
S.E.M.		±0.1	±0.3
DB	Potassium cyanide	0.6	10.9
		0.9	14.0
		0.4	13.8
		0.7	13.8
		0.8	14.3
		0.8	13.0
Mean		0.7	13.4
S.E.M.		±0.1	±0.5

Differences between groups DA and DB were not statistically significant.

The methaemoglobin content of the blood of rats in groups F (plus controls) and DB (plus controls) was measured by the carbon monoxide method and the cyanide method respectively (Tables III.8 and III.9). In both cases, the methaemoglobin content of the rats on cyanide was found to be similar to those of control groups.

It would appear therefore that methaemoglobin does not increase in the blood of rats given either a diet containing 2.5 mg potassium cyanide per rat per day or drinking water containing KCN in which each rat took a mean of 6.5 mg per day.

As ferrohaemoglobin autoxidizes slowly, and methaemoglobin in erythrocytes is enzymically reduced to ferrohaemoglobin, a low steady state concentration of methaemoglobin in normal blood is to be expected. By chemical means, methaemoglobin can be transformed in vitro, to the ferric state (Fe^{3+}). This will not complex with oxygen, but can bind cyanide to form cyanmethaemoglobin.

Smith (1973) has reported that when mice were injected intraperitoneally with sodium thiocyanate, the haemoglobin levels were found to be lower, and the methaemoglobin levels higher than in the blood of control mice. Also, the sum of these pigments was lower, by approximately 10%, than in the blood of control animals, measured two hours after the injection. He proposed that this 10% might be accounted for by the formation of cyanmethaemoglobin. An attempt to repeat this experiment in this laboratory was made by Cordwell (1978). She found no apparent increase in the methaemoglobin content of mice injected with thiocyanate compared with controls. Also the same worker did not observe any difference in the methaemoglobin concentrations of rats fed potassium cyanide (5 mg/rat/day) in the diet for three weeks and that of control rats.

4. Discussion

The minimal lethal dose of cyanide taken orally by mouth in a single dose has been estimated at between 0.5 mg and 3.5 mg per kg body weight (Chen et al., 1934b; Gettler and Bain, 1938; Halstrøm and Møller, 1945). In the work to be described in Chapter IV it was observed that rats could survive and grow apparently normally on a dose of 5 mg of KCN per rat per day (equivalent to 7-16 mg of cyanide/kg body weight) provided the dose of cyanide was added to the diet and taken over a period of about 16 hours each day.

Clark (1936) produced toxic symptoms and then death in rats by administering KCN in drinking water and in diets. None of these effects was observed in the experimental rats given 5.3-8.9 mg KCN/rat/day in drinking water, rather the rats grew well and were perfectly healthy over the seven weeks period. Possibly, Clark was able to produce toxic effects mainly because his rats were much younger especially as it has been observed in this laboratory (Chapter VI) that kidney rhodanese activity appears to increase with age.

Recovery of potassium cyanide added to the diet moistened with water was found to be very poor. However, when potassium cyanide was added to the diet mixed with arachis oil instead of water, no significant loss of the added substance occurred. This method will therefore be useful in the study of the effects of regular intake of small amounts of cyanide in the diet.

Chronic ingestion of cyanide is known to result in elevated plasma thiocyanate level as well as leading to increased excretion of thiocyanate in the urine (Tewe et al., 1977; Mehta and McGinity, 1977). Since the rats given potassium cyanide in the diet had an elevated thiocyanate concentration in plasma and urine (Chapter VIII)

compared with control rats, it is clear that the administered cyanide was not only stable in the diet but was also taken into the animal body.

Although potassium cyanide was found to be stable in both drinking water and diet (mixed with arachis oil), this substance will be best administered to experimental rats in the diet. This is because rats on KCN diet ate adequate amounts of the diet like controls (Chapter V) while the addition of KCN to drinking water caused a reduction of water intake in the rats compared with controls.

CHAPTER IV

CHAPTER IV

The relative toxicities of Vitamin A and Potassium Cyanide

1. Introduction

Cyanide is notorious among the general public as a very poisonous substance. As has been mentioned in Chapter I, it is ingested by man and animals, and estimates suggest that the amounts taken in the diet are sometimes considerable. Indeed they seem larger than the amounts of vitamin A which are known to produce signs of vitamin A poisoning. It was therefore decided to compare the toxicity of cyanide and vitamin A when given in the diet of experimental animals.

1(a) Vitamin A Nomenclature

The Commission on the Nomenclature of Biological Chemistry of the International Union of Pure and Applied Chemistry (1960) advised that vitamin A alcohol, vitamin A esters and vitamin A acid should be called retinol, retinyl esters and retinoic acid. Since that time this body (1965) has approved the use of the name 'retinaldehyde' for vitamin A aldehyde. This terminology will be used throughout this thesis. The term 'vitamin A' will be sometimes used in a more general sense to cover all substances with vitamin A activity.

1(b) The mode of action of vitamin A

Vitamin A is required by animals for reproduction and growth and for normal vision. Its role in the visual process is now largely understood, but its more general function in metabolic processes in the body is still not clear.

Retinol will satisfy all the known functions of vitamin A in animals. It will prevent keratinization of certain epithelial tissues, changes in bone structure and cerebrospinal fluid pressure, and the loss of appetite in vitamin A-deficient animals. It will also maintain normal reproductive performance in male and female animals. Retinoic acid will replace retinol in all its other functions, but cannot maintain vision and normal reproductive performance in animals because it cannot be converted to retinol or retinaldehyde. It may be the functional form in growth.

For vision, the retina requires retinol, which it converts to retinaldehyde, or more strictly the 11-cis isomer of retinaldehyde, which participates in the visual system. The role of retinaldehyde in vision has been reviewed by Wald (1960), Bridges (1967) and Ebrey and Honig (1975).

1(c) Hypervitaminosis A

Large oral doses of vitamin A can be toxic. For calves when more than 3.2 mg retinol per kilogram live weight was fed, cerebrospinal fluid pressure was reduced below normal levels (Hazzard et al., 1964), while bone lesions were discernable when 1.33 mg retinol per kilogram live weight was given. Thus in calves, it appears that bone lessions are a more sensitive measure of retinol excess (Thompson, 1975). When much higher doses are given to rats, bones become resorbed and then fracture.

In young chicks, the most frequently observed clinical signs of hypervitaminosis A include loss of appetite, decreased growth rate, diarrhoea, encrustation around the mouth, and reddening of the eyelids (Pudelkiewicz et al., 1964; Taylor et al., 1968).

In pigs serum albumin is lowered not only in retinol deficiency but also when too much retinol is given (Hurt et al., 1966).

The occurrence of hypervitaminosis A in humans is also well documented (Gerber et al., 1954). The condition generally results from prolonged administration of large quantities of the vitamin used as a therapeutic agent or from misguided consumption of excessive amounts of pharmaceutical vitamin preparations in the belief that this will increase the benefits obtained. In the later case, young children are especially vulnerable.

In female rats massive doses of vitamin A administered at early pregnancy caused resorption of foetuses (Cohlan, 1953, 1954).

When retinoic acid was tested for its ability to produce the characteristic lesions of hypervitaminosis A, it was found that, compared with retinyl acetate or high potency fish oils, a much smaller dose of the acid would produce more severe symptoms in a shorter time (Thompson and Pitt, 1960, 1961).

Hypervitaminosis A has been studied on the assumption that the symptoms observed are exaggerations of its normal mode of action. Furthermore, work on hypervitaminosis A was stimulated when Fell and Mellanby (1952) showed that the action of vitamin A on skeletal tissue is direct and is not mediated through any other organ, for instance the parathyroid. Using the limb bones from 6-day embryos and near-term-foetal mice grown in tissue culture, they found that excess vitamin A added to the medium caused rapid resorption of foetal bone and dissolution of cartilage matrix, accompanied by loss of metachromatic materials.

Two separate hypotheses have been put forward to explain the mechanism of the action of large doses of vitamin A on bones.

The internal and external surfaces of developing bones are populated, in normal circumstances, with either osteoblasts, which lay down bone, or osteoclasts which erode it. Growth of the bone occurs

by a remodelling sequence involving both erosion and deposition of bone brought about by correct organisation of the osteoblasts and osteoclasts. In one hypothesis put forward by Wolbach (1947) it was suggested that during hypervitaminosis A the osteoclastic activity normally involved in the remodelling still occurred but was accelerated relative to the other processes involved in bone development. He claimed that in the long bones calcification of new bone does not keep pace with the accelerated resorption, resulting in defectively calcified bone which is structurally weak and which leads to the characteristic fractures seen in hypervitaminosis A.

In another hypothesis, the action of large doses of vitamin A on bones is said to be mediated through its action on membranes. From the experiments of a number of workers (for example, Dingle, 1961; Fell and Dingle, 1963), it has been found that vitamin A induces the release of proteolytic enzymes from lysosomes in vitro, and that the crude protease preparation derived from rat liver lysosomes effectively degrades the intracellular material of cartilage. On the basis of this finding, it was concluded that by altering the composition or stability of the membranes of cells and subcellular particles vitamin A can release enzymes. These enzymes are in turn responsible for the degradative changes seen in the matrix of limb-bone rudiments grown in the presence of excess vitamin A.

The question then was: could these in vitro findings be extended to the in vivo conditions of hypervitaminosis A? Dingle, Sharman and Moore (1963, 1966) have reported that free proteolytic activity of rat liver increases in hypervitaminosis A. Similar increases in free activity during hypervitaminosis A have been found in the livers of guinea-pigs (Weissman, Uhr and Thomas, 1963). In addition, lysosomes isolated from these animals were more fragile when incubated in vitro

at 37° or subjected to ultraviolet irradiation.

Thus there is a lot of evidence to indicate that the sites of action of excessive amounts of vitamin A are the lipoprotein membranes of cells and their organelles. This view is strengthened by the fact that retinol has been found to penetrate lipid monolayers (Bangham, Dingle and Lucy, 1964; Dingle, 1964).

Roels et al.(1969) have described work on the influence of vitamin A on the structure of simple lipid model systems which indicates that the vitamin could affect lipid-protein interactions in certain areas of biological membranes and thus make the membranes unstable. Possible roles for vitamin A in the structure of membranes have been discussed by Lucy (1969), but as yet evidence available to support such hypothetical models is not great.

From work on a-retinol (cited by Pitt, 1969), it was thought that hypervitaminosis A may not represent an exaggeration of the normal mode of action of the vitamin. This was because effects of excess a-retinol were comparable to those of excess retinol yet a-retinol, though it could be stored in the liver, did not have growth promoting activity. This deduction is now not acceptable since it has recently been suggested (Willetts et al., 1976) that the poor growth-promoting activity of a-retinol is due to its inability to complex with "retinol binding Protein", which stabilizes retinol in plasma and ensures its release from the liver and subsequent transport to target tissues (Muhilal and Glover, 1975).

Aim of Investigation

The subject of naturally occurring toxicants in food has been briefly reviewed by several authors (Tanner and Tanner, 1953; Liener,

1966; Strong, 1966). Most of the naturally occurring toxic substances are of plant origin and this has provided immense problems in the effective utilization of plants as a useful source of dietary protein. In spite of this, foods of plant origin still account for 70% of the world's supply of protein (Abbott, 1966).

The loose terms toxic factor or toxicant are commonly used when referring to those substances found in foods which produce a deleterious effect when ingested by man or animals. Toxicologists frequently assess the toxicity of a substance in terms of its LD_{50}, that is the dose which causes the death of 50% of the animals tested. Although there are some plants which are known to produce a violent expression of poisoning, it is more common to observe much more subtle effects produced only by prolonged ingestion of a given plant. Such effects might include an inhibition of growth, liver damage, pancreatic hypertrophy etc. The term 'toxic', as used in this text, should therefore be given a more liberal interpretation to mean nothing more or less than an adverse physiological response produced in man or animals by a particular food or food substances derived therefrom.

It must be mentioned however, that the evidence that a particular food or food substances constitutes a hazard to health of man is frequently overemphasized. As might be expected, much of the research relating to the toxicity of foodstuffs has been done with animals. Under these conditions the animals consume large quantities of a particular plant over a long period of time, a situation quite foreign to the normal eating pattern of man. Thus a toxic substance, if present, might produce symptoms of poisoning which might not otherwise be apparent. If the isolation of the causative factor has been achieved, its toxicity is frequently evaluated by using a

route of administration, such as intraperitoneal or subcutaneous injection, which is physiologically unrelated to the normal mode of ingestion.

Cyanide is universally recognized as a toxic substance. If members of the public were asked to name half a dozen powerful poisons, it is probable that most would mention cyanide.

In contrast, vitamin A would almost certainly be considered a wholly beneficial substance, for its toxicity is not widely known outside a narrow circle of doctors, nutritionists and biochemists.

The suggestion that vitamin A might have the same order of toxicity as cyanide would be heard with incredulity by most people - even, one suspects, by many scientists. A study of the literature however indicates that vitamin A might be as toxic as cyanide, when both are taken in a number of regular small doses.

The experiments described in this chapter were designed to test the relative toxicities of vitamin A and cyanide when ingested regularly in the diet.

Most investigators have evaluated the toxicity of cyanide present in foodstuffs either by feeding cyanogenic glycosides to experimental animals or by intraperitoneal or subcutaneous injection of sodium or potassium cyanide to animals in amounts equivalent to those assumed to be present in the food (Blakley and Coop, 1949; Smith and Foulkes, 1966; Osuntokun, 1970; Van Der Valden and Kinthaert, 1973; Mehta and McGinity, 1977). Its toxicity has been evaluated here by addition of potassium cyanide to the normal diet. Estimation of cyanide toxicity by administration of cyanogenic glycosides to experimental animals may not provide a good index, as the actual amount of cyanide absorbed will depend on the mode of metabolism of the glycoside , whether it could be converted to free

cyanide or not. On the other hand, injection of sodium or potassium cyanide to experimental animals represents an administration which is physiologically unrelated to the normal mode of ingestion.

So far as is known, the present experiments are the first in which potassium cyanide has been administered to experimental animals mixed into their diet to provide a semi-continuous supply of known amounts which do not cause death.

Experimental

Twelve male albino rats of the same age were weaned at three weeks of age on to a commercial diet, 41B. At five weeks of age they were transferred to the basal soyabean meal diet (Chapters II and III). These rats of approximately equal weights were divided into three groups of four rats each (A, B, C). Group A rats were maintained on soyabean diet plus a supplement of 140 µg (426 n moles) retinyl acetate per week, as no retinol was present in the diet (Chapter II). Group B rats were maintained on the basal diet (soyabean diet) plus 12.65 mg (38.5 µ moles) retinyl acetate per day given orally (Chapter II) while group C rats were given the basal diet plus 2.5 mg (38.5 µ moles) KCN per day, and 140 µg (426 n moles) retinyl acetate per week. The cyanide was given to the rat by the method described in Chapter II. The experiment was carried out for three weeks during which period the daily weights of the animals were recorded.

This experiment was carried out twice (groups A1 and A2, B1 and B2, C1 and C2). In the first experiment (A1, B1, C1) the retinyl acetate was administered orally. In the second experiment (A2, B2, C2) it was mixed in the diet in the same way as the cyanide. In the

second experiment (A2, B2, C2) the daily KCN dose of 2.5 mg (38.5 μ moles) was increased to 5.0 mg (77 μmoles).

At the end of three weeks, each rat was anaesthetised with chloroform, the neck chopped off by means of a guillotine, and blood collected in a heparinised 50 ml beaker. The blood of rats in each group was pooled, chilled and a sample used to estimate packed cell volume and total haemoglobin content (Chapter II). The remaining blood sample was centrifuged at 1300 g for 10 minutes and plasma removed by means of a pipette. The vitamin A content of this plasma was estimated as described in Chapter II. The pair of kidneys and the liver of each rat were dissected out, chilled, minced roughly with scissors, weighed and the liver divided into two portions. Rhodanese contents of the kidneys and a portion of the liver were determined and the results are presented in Chapter VI, while the remaining portion of the liver was stored at -40° for the estimation of total vitamin A content (Chapter II).

Results and Discussion

Comparisons were made of the harmful effects of vitamin A and potassium cyanide when mixed in the diet, as measured by weight gain in growing rats.

The growth rate of rats on a high level of retinyl acetate (groups B1 and B2) was found to be significantly lower than that of control rats in groups A or rats on cyanide in groups C (Table IV.1 and IV.2). Most of the rats in group B showed loss of appetite, a most frequently observed sign of hypervitaminosis A, by the second week of the experiment. In addition, the decline in growth rate of B2 rats became sharper by the end of the second week (Fig.IV.2). No rat however, showed other signs of hypervitaminosis A, such as

TABLE IV.1

The change in body weight of groups of rats given equal molar amounts of cyanide or retinyl acetate.

Group	Substance administered µmoles/rat/day	Body weight at start of experiment (g)	Body weight at end of experiment (g)	Gain in weight (g)	Mean weight gain	Standard error
		168	235	67		
A1	None	130	188	58	71	6
		178	252	74		
		177	265	85		
		174	220	46		
B1	Retinyl acetate (38.5)	177	199	22	40	6
		176	226	50		
		154	196	42		
		152	257	105		
C1	Potassium cyanide (38.5)	167	236	69	95	9
		149	253	104		
		163	266	103		

Statistical significance of differences between groups.

Groups A and B $0.01 < P < 0.02$

B and C $0.001 < P < 0.005$

A and C $0.05 < P < 0.1$

TABLE IV.2

The change in body weight of groups of rats given retinyl acetate or cyanide of twice the molar amount of retinyl acetate.

Group	Substance administered (μmoles/rat/day)	Body weight at start of experiment (g)	Body weight at end of experiment (g)	Gain in weight	Mean weight gain	Standard error
		138	238	100		
A2	None	146	253	107	100	6
		157	241	84		
		136	245	109		
		123	138	15		
B2	Retinyl acetate (38.5)	131	176	45	42	9
		154	206	52		
		160	216	56		
		140	242	102		
C2	Potassium cyanide (77.0)	154	243	89	101	4
		123	229	106		
		170	275	105		

Statistical significance of differences between groups

Groups A2 and B2 $0.001 < P < 0.005$

B2 and C2 $0.001 < P < 0.005$

A2 and C2 $0.90 < P < 0.95$

encrustation around the mouth, reddening of the eye lids and impaired movement. A few rats showed slight signs of diarrhoea.

The average weight change with time for each group for the two experiments carried out can be seen in Fig.IV.1 and IV.2. All rats in all groups gained weight during the experimental period, but the growth rate of rats in group B was approximately half that of groups A and C. In the second experiment (B2) the growth rate appeared to be progressively reduced as dosing progressed. In contrast, giving potassium cyanide did not reduce the growth of the rats relative to that of the control group.

The most characteristic effect of hypervitaminosis A is on the skeleton. In early experiments high potency fish oils and vitamin A concentrates were shown to produce bone fragility in rats (Collazo and Rodriguez, 1933; Bomskov and Seeman, 1933; Davies and Moore, 1933). It was Moore and Wang (1945) who proved conclusively, using crystalline retinyl acetate, that vitamin A was the active constituent in causing these bone lesions. Much of the work on rarefaction of bones has been done with the rat (Moore and Wang, 1945; Wolbach, 1947; Thompson and Pitt, 1960), but lesions have been described in other species including guinea-pigs (Wolbach, 1947), calves (Grey et al., 1965) and chicks (Wolbach and Hegsted, 1952; Baker et al., 1967).

Examination of the long bones (tibia, fibula and humerus) of the rats in the three groups (A, B and C) did not reveal any damage. The bones in group B rats were similar to those of controls (group A) and to those of rats on cyanide (group C) as judged by weight and by visual inspection.

Analysis of the vitamin A content of the livers showed that, as expected, rats of groups B1 and B2 had large, and in some cases

FIGURE IV.1

Body weight changes of male rats on cyanide or a high level of vitamin A.

A1 = controls

B1 = Rats on high level of retinyl acetate (38.5 µmoles/rat/day)

C1 = Rats on cyanide (38.5 µmoles KCN/rat/day).

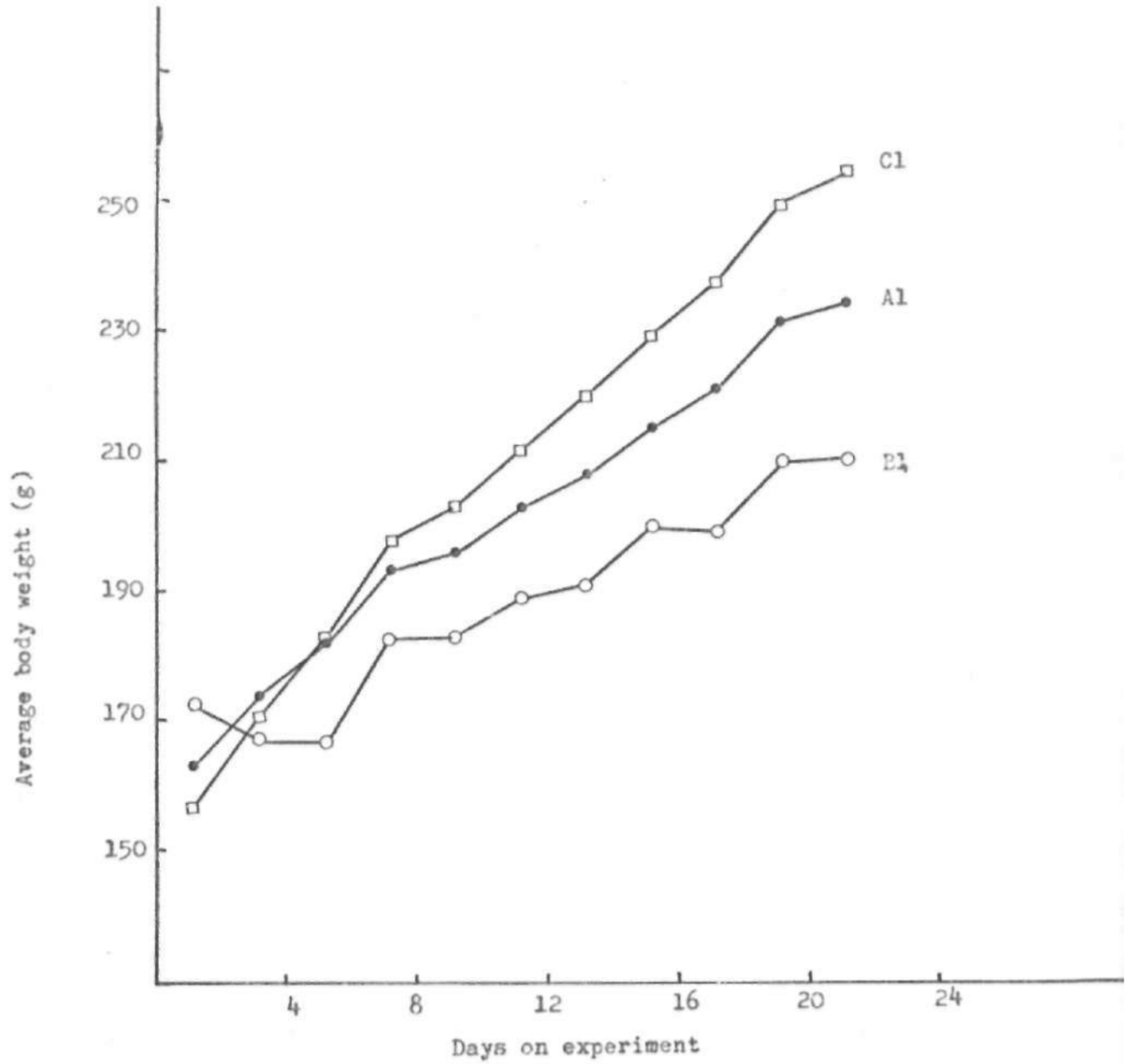
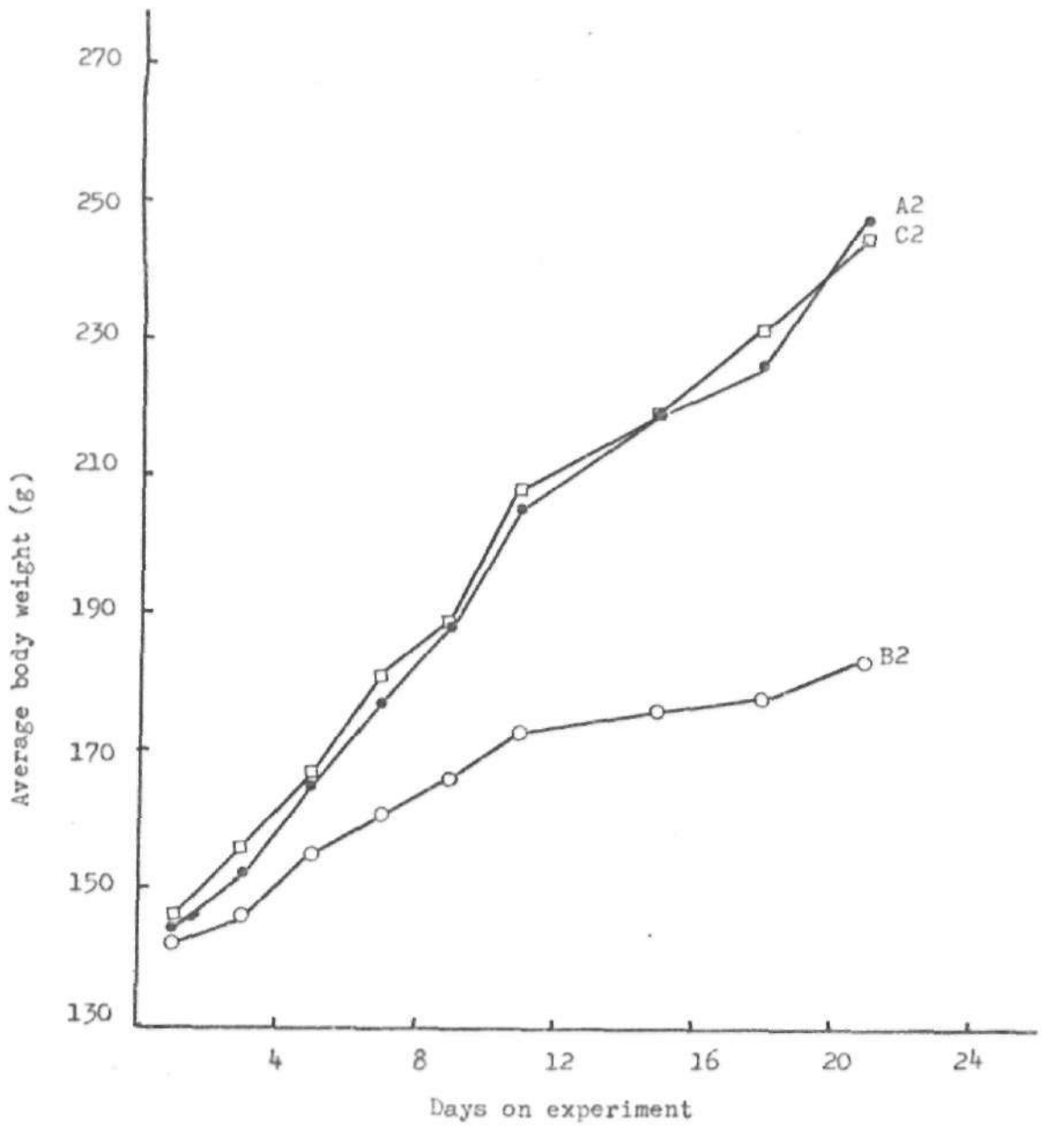


FIGURE IV.2

Body weight changes of male rats on cyanide or a high level of vitamin A.



extremely large, stores ranging up to 42 mg per liver. There was no significant difference between the vitamin A content of the livers of groups A1 and C1 or A2 and C2. Administration of cyanide in amounts up to 77.0 μ moles (5 mg KCN) per day had not affected the liver stores of vitamin A (Tables IV.3 and IV.4).

4(a) Plasma level of vitamin A

It is now well established that vitamin A circulates in the plasma mainly as retinol, associated with a transport protein of hydrated density greater than 1.21, known as retinol-binding protein (RBP) (Goodman et al., 1965; Glover, 1973). Knowledge of the level of the vitamin circulating in the bloodstream is important in the determination of the nutritional status of a subject with respect to this essential nutrient. Glover (1973) has shown that apo-RBP (i.e. RBP not complexed with retinol) accumulates in the liver of rats deficient in retinol and that once retinol is available the holo-RBP (i.e. RBP complexed with retinol) is formed and released immediately into the blood stream. Thus retinol appears to be involved in triggering the release of the holoprotein from the liver. Furthermore, retinol is stored in the liver mainly as retinyl palmitate but it is usually liberated into the plasma only as retinol complexed with RBP. Free retinol is unstable but is greatly stabilized when complexed with RBP. Thus RBP appears to stabilize retinol and ensure its transport to target tissues.

The plasma samples from three groups of rats (A2, B2 and C2) were tested for their vitamin A content by the trifluoroacetic acid method. While the levels of vitamin A in control rats (group A2) and cyanide rats (group C2) were normal (30 and 34 μg retinol/100 ml respectively), that of group B2 rats had a considerably elevated

TABLE IV.3

The liver vitamin A content of male rats given cyanide or high doses of retinyl acetate.

Group	Substance administered (µmoles/rat/day)	Weight of liver (g)	Vitamin A expressed as mg retinol per g liver	Vitamin A expressed as mg retinol per liver	Mean	Standard error
A1	None	10.5	34.0	0.4	0.8	0.3
		8.4	47.5	0.4		
		11.9	105.0	1.3		
		13.6	97.3	1.3		
B1	Retinyl acetate (38.5)	11.8	507.0	6.0	13.1	4.1
		9.3	880.0	8.2		
		11.0	1245.0	13.7		
		10.7	2287.0	24.7		
C1	Potassium cyanide (38.5)	12.0	11.8	0.1	0.6	0.2
		11.7	55.9	0.7		
		11.6	55.0	0.6		
		13.6	60.4	0.8		

There was no significant difference in the liver vitamin A content of groups A1 and C1 ($0.4 < P < 0.5$).

TABLE IV.4

The liver vitamin A content of rats given high doses of retinyl acetate or cyanide of twice the molar amount of retinyl acetate.

Group	Substance administered (μmoles/rat/day)	Weight of liver (g)	Vitamin A expressed as mg retinol per g liver	Vitamin A expressed as mg retinol per liver	Mean	Standard error
A2	None	12.1	23.2	0.3	1.0	0.6
		12.9	22.6	0.3		
		13.6	208.5	2.8		
		16.2	27.0	0.4		
B2	Retinyl acetate (38.5)	8.3	4,362.2	36.0	29.5	8.0
		10.0	3,306.0	33.1		
		11.9	3,599.2	42.7		
		13.3	484.0	6.4		
C2	Potassium cyanide (77.0)	14.0	28.9	0.4	0.4	0.0
		12.6	28.3	0.4		
		13.3	24.2	0.3		
		15.1	34.3	0.5		

There was no significant difference in the liver vitamin A content of groups A1 and C1 ($0.4 < P < 0.5$).

level of vitamin A (168 µg retinol equivalents/100 ml, Table IV.5). Thus while rats on cyanide retained the ability to maintain a normal level of vitamin A in plasma, those given a high supplement of retinyl acetate appeared to fail to maintain a normal level of vitamin A in plasma once the liver storage capacity was exceeded. Presumably, a high proportion of the retinol in this elevated state, is circulating in the plasma uncomplexed with RBP and in the ester form (Mallia et al., 1975). It is tempting to speculate that certain signs of hypervitaminosis A might be produced by non-specific interaction of vitamin A with target organs when uncomplexed with RBP. Most of the signs of hypervitaminosis A presumably develop only after prolonged exposure of target organs to excessive amounts of retinyl esters, as the rats in this experiment showed no sign of hypervitaminosis A other than poor appetite and retarded growth.

4(b) Other changes in blood composition

Poumeau-Delille (1943) and Walker, Eylenberg and Moore (1947) recorded a marked reduction in the number of red blood cells in hypervitaminotic rats. Baker, Howell and Thompson (1967) however, did not observe these haematological findings in their experiments with hypervitaminotic chicks. The blood from rats given large doses of retinyl acetate (group B2) was tested for packed cell volume and total haemoglobin content (Table IV.5). The packed cell volume as determined by the microhaematocrit method (Chapter II) and the total haemoglobin content as determined by the cyanmethaemoglobin method (Chapter II) did not differ from those of control rats (group A2) and cyanide rats (group C2), rather the values obtained were within the normal range. Thus, at this stage of hypervitaminosis A, the rats did not show any abnormality in either the total packed cell volume or the total haemoglobin content.

CHAPTER V

The relative toxicities of vitamin D and potassium cyanide

1. Introduction

The results in the previous chapter show that vitamin A is more harmful to growing rats than is cyanide, when mixed in the diet. As hypervitaminosis D can be produced with doses of vitamin D lower than those of vitamin A necessary to induce hypervitaminosis A, one can predict that vitamin D will be very much more toxic than cyanide. An experiment was designed to test this prediction.

1(a) Vitamin D Nomenclature

The International Union of Nutritional Sciences (1969) and the American Institute of Nutrition (1970) have advised that the term vitamin D be used as the generic descriptor for all steroids exhibiting qualtitatively the biological activity of cholecalciferol. Thus phrases such as 'vitamin D activity' and 'vitamin D deficiency' represent preferred usage. These bodies also advised that the terms 'vitamin D_3' and 'vitamin D_2' be used specifically for cholecalciferol and ergocalciferol (calciferol) respectively. This terminology will be used in this thesis.

1(b) Functions and metabolism of vitamin D

Vitamin D promotes calcification by stimulating elevated levels of plasma calcium and phosphate to supersaturating levels in the plasma and extracellular fluid. A deficiency of vitamin D results in a subnormal level of either plasma calcium or phosphate or both (Howland and Kramer, 1921; Newman, 1958). It has been demonstrated

that a major function of vitamin D in causing an elevated calcium level in plasma is due to its ability to stimulate intestinal calcium absorption (Howland and Kramer, 1921). This process is an active one requiring metabolic energy to transport calcium from the lumen of intestine to the serosal fluid.

Another important function of vitamin D is to stimulate the mobilization of calcium from bone. This was first demonstrated in 1952 by Carlson and was later shown by Harrison et al. (1958) and Rasmussen et al. (1963) to require the participation of both vitamin D and parathyroid hormone. This system serves as another mechanism whereby vitamin D increases serum calcium and phosphorus concentrations.

In 1941, Harrison and Harrison suggested that vitamin D increases renal tubular reabsorption of phosphorus. Subsequently, adequate reasons have been provided to support this view (Holick and Deluca, 1974). It seems likely that these three basic functions of vitamin D in intestine, bone and kidney contribute to elevating plasma calcium and phosphorus concentrations to levels sufficient for normal mineralization to occur.

It has been recognized that vitamin D must be converted to the active form which operates at the genome of the target organ to induce synthesis of specific proteins. With isotopically labelled cholecalciferol, it became possible to identify the nature and the sites of transformation (Norman and Deluca, 1963; Neville and Deluca, 1966; Lund and Deluca, 1966).

Two hydroxylation processes occur on the vitamin D molecule to convert it to the form participating in calcium translocation. Both processes are catalyzed by specific hydroxylases. The first hydroxylation occurs at position 25, and the reaction is catalyzed

by a microsomal enzyme system that occurs in the liver. The product of this reaction is 25-hydroxyl cholecalciferol. The second hydroxylation occurs in the kidney and the reaction is catalyzed by 1-α-hydroxylase. The product of this reaction is 1,25-dihydroxy-cholecalciferol. This hydroxylation is sensitive to actinomycin D and is stimulated by parathyroid hormone. 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) is fully effective in initiating both intestinal calcium transport and bone calcium mobilization in anephric animals (Holick et al., 1972; Wong, et al., 1972). Thus, it has been concluded that $1,25(OH)_2D_3$ or its further metabolite is the metabolically active form of vitamin D.

Both the D_3-25-hydroxylase and the D_3-1,25-hydroxylase are subject to some regulation. The 25-hydroxylase is inhibited by increasing levels of vitamin D_3 (Bhattacharyga and Deluca, 1973). The enzyme is highest in vitamin D-deficient animals and hence is probably not inducible. Administration to animals of radioactive or nonradioactive vitamin D_3 results within 3 hrs in a marked depression of the 25-hydroxylase activity of liver homogenates and isolated microsomes. The degree and length of this depression of 25-hydroxylase activity is related to the dose of the vitamin given. The larger the dose, the more rapid the reduction in the hydroxylase activity, the greater the extent, and the greater the length of time before the hydroxylase activity recovers.

The realization that $1,25(OH)_2D_3$ is a metabolically active form of the vitamin in both intestine and bone gave birth to the idea that dietary and serum calcium might regulate its synthesis (Boyle et al. 1971). Animals given vitamin D show a marked ability to adapt to dietary calcium (Nicolaysen et al., 1953; Rottensten, 1938;

Fairbanks and Mitchell, 1936). Those on a low calcium diet adapt by showing a markedly elevated intestinal calcium absorption while those on a high calcium diet show a markedly reduced efficiency of calcium absorption.

1(c) Storage of vitamin D

Most of the early studies of tissue distribution of vitamin D were carried out on animals given a single, usually very large, dose of the vitamin (Vollmer, 1939; Warkany et al., 1942; Cruickshank et al., 1954). Results of such experiments showed vitamin D to be distributed rather uniformly among a variety of tissues. In a more recent experiment (Rosenstreich et al., 1971), rats with established vitamin D deficiency were replenished with ^{14}C ring-labeled vitamin D_3 and the tissue levels of radioactivity were measured during repletion and also during a subsequent period of vitamin D deprivation. The results obtained showed adipose tissue as the main depot of large capacity which accumulates vitamin D in proportion to its concentration in plasma and releases it at a much slower rate proportionate to its concentration in fat. These features of accumulation and release would protect against toxicity during short periods of vitamin overdosage, would tend to maintain a relatively stable concentration in blood under natural conditions where absorption and production of the vitamin could vary significantly and would provide continued maintenance of vitamin D in plasma for hepatic conversion to 25-hydroxy-cholecalciferol during a relatively prolonged period of low intake.

1(d) Hypervitaminosis D

Excessive quantities of cholecalciferol or ergocalciferol are toxic. Large daily doses of either cholecalciferol or ergocalciferol have been shown to stop or retard growth of rats (Ham and Lewis, 1934; Hartles and Leaver, 1961, 1962). Pigs given

250,000 i.u. (6.3 mg) vitamin D per day showed signs of intoxication, and pathological calcification was found in several organs; however, there was evidence of recovery as the blood content of the vitamin declined to normal (Quarterman et al., 1964). In hypervitaminotic animals, the serum alkaline phosphatase levels fell and the pH of blood rose to about 7.8 (Follis, 1955).

The chief symptoms of toxicity due to overdosage of vitamin D are anorexia and polyuria. Calcification of soft tissues, and particularly of the renal arterioles and the aorta, is observed in advanced stages. Vitamin D overdosage is indicated by a rise in serum calcium.

Since it has been shown that 25-OH-D_3, but not D_3, has a direct effect on mobilization of calcium from bone (Trummel, 1969) it has been suggested (Holick and Deluca, 1974) that at least part of the toxicity due to vitamin D may be due to the elevated levels of 25(OH)D_3 found in plasma under conditions of hypervitaminosis D. Vitamin D toxicity can be experienced in completely nephrectomized animals (Pavlovitch et al., 1973) which would suggest that the 1,25$(OH)_2D_3$ which is made exclusively in the kidney is not necessary for toxicity.

2. Design of experiment

Eighteen male rats were weaned at three weeks of age on to diet 41B. At 5 weeks of age, they were transferred to the basal soyabean meal diet. These rats of approximately equal weights were divided into three groups of six rats each (GB, HB, IB). Group GB rats were maintained on the basal diet. Group HB rats were maintained on the basal diet plus a dose of 0.210 mg (0.55 μmoles) vitamin D_3 per day given in diet, while group IB rats were given the basal diet

plus 5.0 mg (77.0 μmoles)KCN per day. The vitamin D_3 and cyanide were added to the amount of diet which each rat consumed overnight (10 g diet), after which they were then fed ad libitum the next morning before the next dose of supplement was administered. Group GB rats (controls) were also given 10 g diet overnight and then fed ad libitum. The amount of diet consumed by each rat per day was recorded. The dosing of rats with the supplements lasted for a period of three weeks, at the end of which each rat was anaesthetised with chloroform, blood taken by heart puncture, and the liver and kidneys dissected out. One of each pair of kidneys was cut into two longitudinal halves and fixed in phosphate buffer, pH 7.0, with 4% (v/v) formaldehyde for histological examination (Chapter II). The liver and the remaining kidney were homogenized and their rhodanese content determined (Chapter VI).

3. Results and Discussion

The rats on cyanide (group IB) grew like control rats (group GB) and appeared healthy. The body weight curves for the groups are shown in Fig.V.1. A t-test applied to the body weight gain of the groups showed that the slight differences in the body weight gain between GB and IB rats were not significant (Table V.1). On the other hand, rats on vitamin D_3 (group HB) showed significantly smaller weight gain ($P < 0.005$) when compared with either the control rats (group GB) or the cyanide rats (group IB). However, the amount of diet consumed by the rats on vitamin D_3 was smaller than that consumed by rats in groups GB and IB ($P < 0.01$). Furthermore, rats in group HB tended to spill their diet more than did rats of the other groups, and in such circumstances it is not easy to determine accurately the food intake, so the figures given in Table V.1 are

FIGURE V.1

Body weight of rats on cyanide (77 µmols/rat/day) or vitamin D, (0.55 µmoles/rat/day) doses.

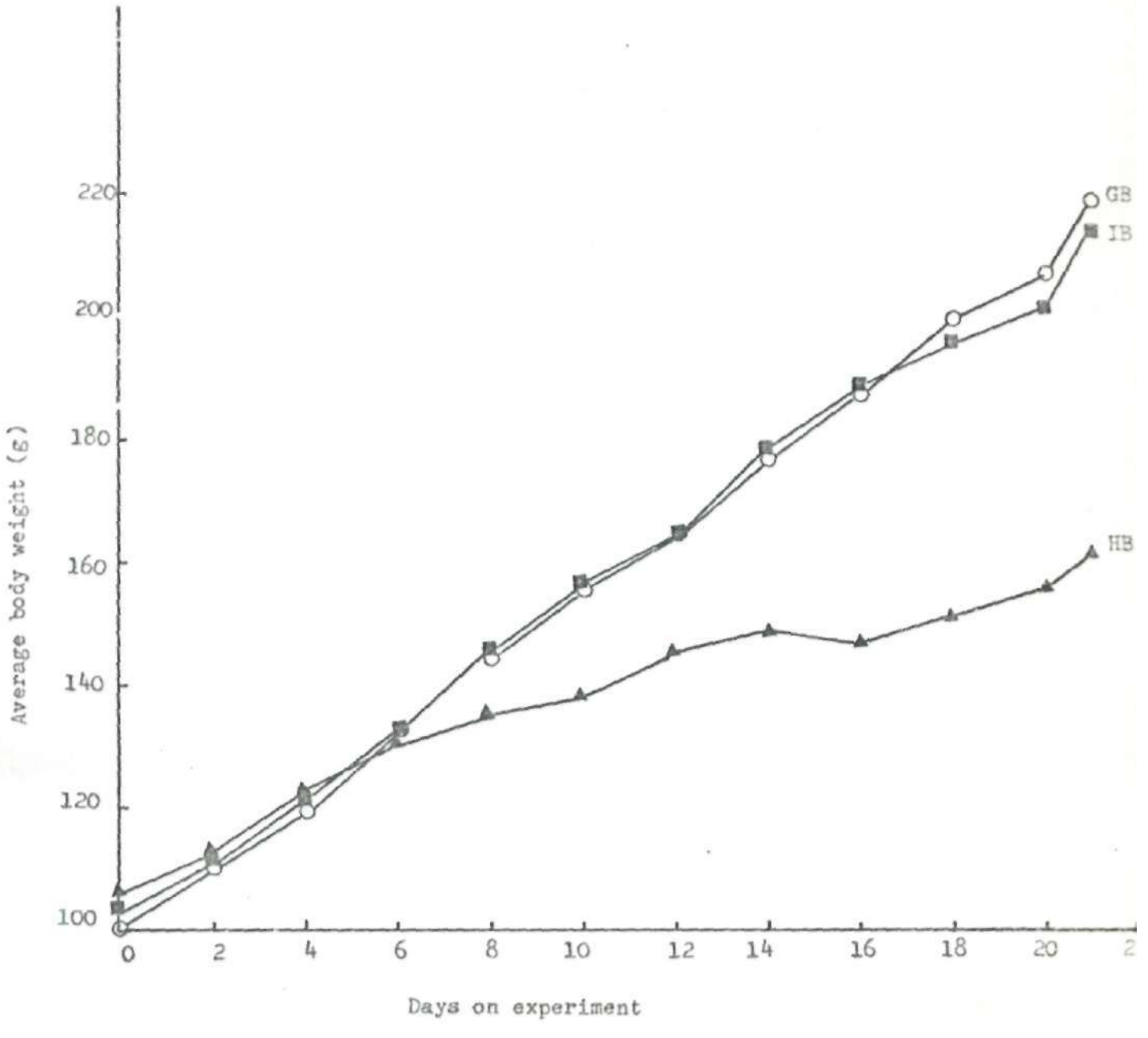


TABLE V.1

The change in body weight of groups of rats given cyanide (77 μmoles/rat/day) or vitamin D_3 (0.55 μmoles/rat/day) and the average amount of diet consumed per rat per day.

Group	Substance administered	Body weight at start of experiment (g)	Body weight at end of experiment (g)	Gain in weight (g)	Mean ± S.E.M.	Average diet consumed per rat per day (g)	Mean ± S.E.M.
GB	None	80	165	85	119±10	11.1	13.6±0.9
		104	226	122		13.0	
		80	219	139		13.5	
		126	259	133		16.7	
		123	262	139		15.6	
		86	179	93		11.4	
HB	Vitamin D_3	85	125	40	55±10	7.4	10.9±0.8
		113	194	81		12.1	
		109	184	75		12.8	
		109	182	73		12.1	
		110	130	20		10.0	
		109	152	43		11.1	
IB	Potassium cyanide	80	191	111	111±15	13.1	14.0±0.6
		130	253	128		16.7	
		87	204	117		13.6	
		102	194	92		12.8	
		120	223	103		13.7	
		95	207	112		13.9	

Statistical significance of differences between groups:

Weight gain - Groups GB and HB $0.005 < P$
GB and IB $0.4 < P < 0.5$
HB and IB $0.005 < P$

Diet - Groups GB plus IB and HB $0.005 < P < 0.01$

likely to overestimate slightly the amount of diet consumed by rats in group HB.

Apart from the reduced growth rate and loss of appetite, the only other observed external sign of vitamin D_{Δ}^{excess} was diarrhoea.

Large daily doses of either cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2) have been shown to retard or even stop growth of rats (Ham and Lewis, 1934; Hartles and Leaver, 1961, 1962). Indeed, administration of 60,000 i.u. (1.5 mg) of vitamin D_3 per rat per day has been shown to cause death of rats within one to three weeks (Clark and Basset, 1962). In a preliminary experiment to determine the suitable dose of vitamin D to be administered to rats without causing death within a few weeks, it was found that a massive dose of 14.8 mg (592,000 i.u) of vitamin D_3 given to each rat for only one day, resulted in loss of appetite only after about two days but death of half of the dosed rats by the third day. Finally, only one out of six rats survived the dose.

It is interesting to recall (see Introduction) that vitamin D_3 is converted to 25(OH)D_3 in the liver and that the latter metabolite is thought to be responsible for toxicity (Blunt *et al.*, 1968). Since loss of appetite and toxic signs were observed only after about 48 hours following the administration of the large dose of vitamin D_3, it seems most likely that the observed lag phase might represent the minimum period required by the liver to produce toxic level of 25(OH)D_3 from administered vitamin D_3. Moreover, the level of 25(OH)D_3 is known to be regulated (Blunt *et al.*, 1968; Holick and Deluca, 1974). The rate of appearance of 25(OH)D_3 in the blood from D_3 is not linear. Instead, there appears to be a plateau at relatively low doses of vitamin D_3 beyond which there is only a 2-3 fold increase of blood 25(OH)D_3 with every 1000fold increase in

vitamin D_3 dosage. This regulation is overcome however, by greatly increasing the dosage of vitamin D_3 (Blunt et al., 1968). Possibly, a single large dose of vitamin D_3 also overcomes the regulation of 25(OH)D_3 formation and hence results in the observed toxic effects after 48 hours.

3(a) Plasma levels of calcium and inorganic phosphorus

The intestinal absorption of calcium and phosphorus is increased in rats given a moderate overdose of vitamin D, and high concentrations of these substances are found in blood (Follis, 1955; Stewart, 1975). Taylor et al. (1968) using chicks, agreed that plasma calcium was increased but found inorganic phosphorus lower than in control chicks. Harris (1956) thought that hypercalcaemia and hyperphosphataemia could be prolonged if sufficient calcium and phosphorus were absorbed by the animal. In this investigation, the plasma of rats given 0.55 µmoles of vitamin D_3 per rat per day had a significantly higher calcium concentration than that of either the control rats or the rats on cyanide (Table V.2). On the other hand, the plasma phosphate concentration appeared to be slightly lower in rats on D_3 dose but the differences were not significant (HB VS GB, $P \simeq 0.1$).

The Fiske and Subbarow molybdate method used to estimate phosphate was found to give a more intense colour in the presence of trichloroacetic acid (in the estimation of plasma phosphate, proteins are first precipitated with trichloroacetic acid). To avoid over-estimation, trichloroacetic acid was added to standard phosphate solutions in the same concentration as was present in plasma supernatants (Chapter II).

Rats on small daily doses of cyanide in the diet showed levels

TABLE V.2

The plasma calcium and inorganic phosphorus concentrations of groups of rats given cyanide (77 µmoles/rat/day) or vitamin D_3 (0.55 µmoles/rat/day).

Group	Substance added to diet	Plasma calcium mg/100 ml	Mean ± S.E.M.	Plasma inorganic phosphate mg/100 ml	Mean ± S.E.M.
GB	None	10.5	10.6±0.6	3.1	2.8±0.2
		13.0		2.8	
		11.2		3.0	
		9.0		2.3	
		9.3		-	
		10.5		-	
HB	Vitamin D_3	12.5	13.8 ±0.3	2.2	2.3±0.2
		13.8		2.3	
		13.5		2.0	
		14.5		2.8	
		14.2		-	
		14.2		-	
IB	Potassium cyanide	11.0	11.3±0.3	3.2	2.6±0.2
		12.3		2.2	
		11.8		2.9	
		11.5		2.2	
		10.5		2.1	
		10.5		3.1	

"-" means not determined.

Plasma Ca^{2+} concentrations were statistically significantly higher in group HB than in groups GB or IB ($P < 0.001$). Differences in plasma phosphate concentrations were not statistically significant.

of calcium and phosphorus in plasma not significantly different from those of control rats (Table V.2).

3(b) Kidney histology

It has been established that the principal pathological changes attributable to excessive doses of vitamin D in man or animals were in the form of abnormal deposits of calcium in many organs and tissues (Bauer and Freyberg, 1946; Christensen et al., 1951; Hass, 1956). The extent and distribution of abnormal deposits of calcium in tissues vary with the duration and severity of the disease and are less conspicuous in the kidney than in the aorta. Clark and Bassett (1962) observed extensive cortical calcification in kidneys of rats (weighing 150 g each) dosed with 1.5 mg (60,000 i.u) vitamin D_2 per rat per day. Furthermore, rats given 0.45 mg (18,000 i.u.) of D_2 had greater deposition of calcium in the medulla than the cortex. A similar observation has been made by Hass et al. (1958). Clark and Bassett (1962) suggested that in rats given 0.45 mg (18,000 i.u) of vitamin D_2, the rate of bone resorption must have been less and consequently the proximal tubules of the kidney were not affected severely. They pointed out that since the collecting tubules of the kidney were responsible for reabsorption of water, it is not unreasonable to expect that the solubility products of calcium and phosphate salts might be exceeded, and hence these structures would become calcified at the lower dose of vitamin D.

Clark (1936) claimed a number of histological changes in livers and kidneys of rats associated with chronic cyanide ingestion. He claimed that with small doses, on which the general health of the animal is not affected, the earlier sign is vacuolation, probably fatty, of the hepatic cells in the periphery of the lobules. This spreads until the condition is general throughout the lobules. The

sinusoids are congested and there is some distortion of the normal structure in places. He also claimed that the kidneys showed some amorphous material visible in the glomeruli within Bowman's capsule, while there was fatty degeneration in the epithelium of the secretory tubules, with some degeneration of the nuclei.

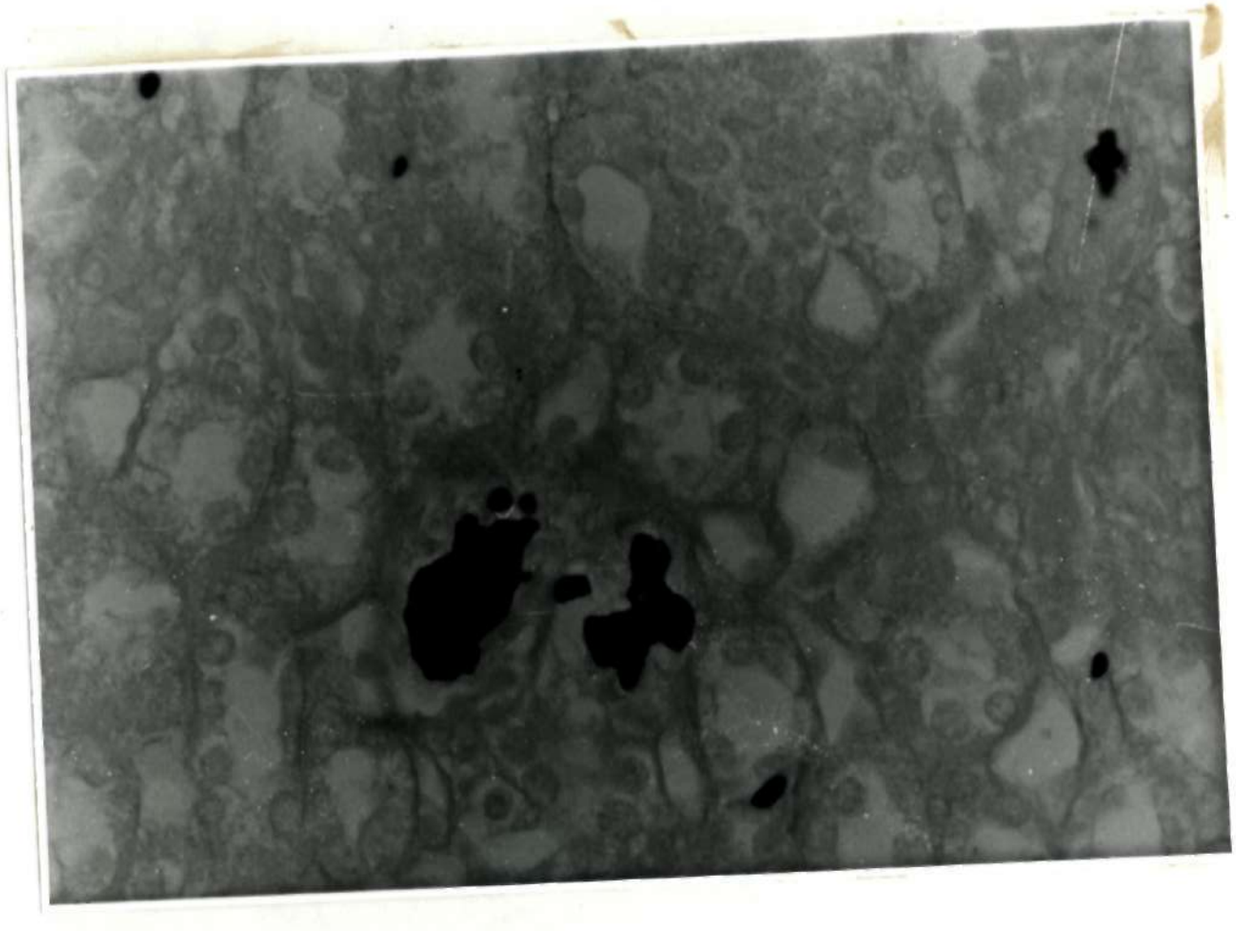
Ballantyne *et al.* (1972) on the other hand have shown that most rabbits killed by acute cyanide poisoning do not present any specific histological features. They observed however, subpleural haemorrhages in the lungs of a few rabbits killed by injection of KCN or HCN. The lungs and other organs from the majority of the rabbits appeared normal.

The differences in the findings of Clark and those of Ballantyne *et al.* may be species differences or may be due to the fact that the former studied animals on prolonged small doses of cyanide in the diet while the later examined animals killed by a single muscular injection of a large dose of cyanide.

Histological examination of 12 kidneys from six rats on a diet containing cyanide (77 µmoles/rat/day, group IB) was carried out after staining by the haematoxylin/eosin and the Von Kossa techniques. The kidneys of rats on a high level of vitamin D (0.55 µmoles/rat/day, group HB) and those of control rats (group GB), stained similarly, formed useful comparative groups especially as vitamin D overdosage results in several pathological changes of the kidney tissue (see introduction to this Chapter). The kidneys of three rats in group HB showed pathological deposition of calcium salts in the medulla, but the deposits were not extensive (Plate V.1). There was also only slight disorganization of medulla cells in areas where calcium deposits occurred. This clearly shows that the vitamin D overdosage

PLATE V.1

Histology of the kidney from a rat maintained on the basal soya bean meal diet containing vitamin D_3 (0.55 µmoles/rat/day). The black spots are calcium deposits. Von Kossa x 160.



was not very advanced (Clark and Bassett, 1962). The kidneys of rats on cyanide (group IB) on the other hand showed no pathological changes and were normal like those of control rats (Plates V.2 and V.3). The only tissue where serious pathological changes associated with chronic cyanide ingestion have been established is nervous tissue. Here, repeated small doses of cyanide cause a demyelinization in the central nervous system in rats and monkeys (Baumeister et al., 1975). Chronic cyanide intoxication is now known to be responsible for a number of disorders in the eye and nervous tissues (Chapter I).

PLATE V.2

Histology of the kidney of a control rat maintained on the basal soya bean meal diet. No calcium deposits. Van Kossa x 160.

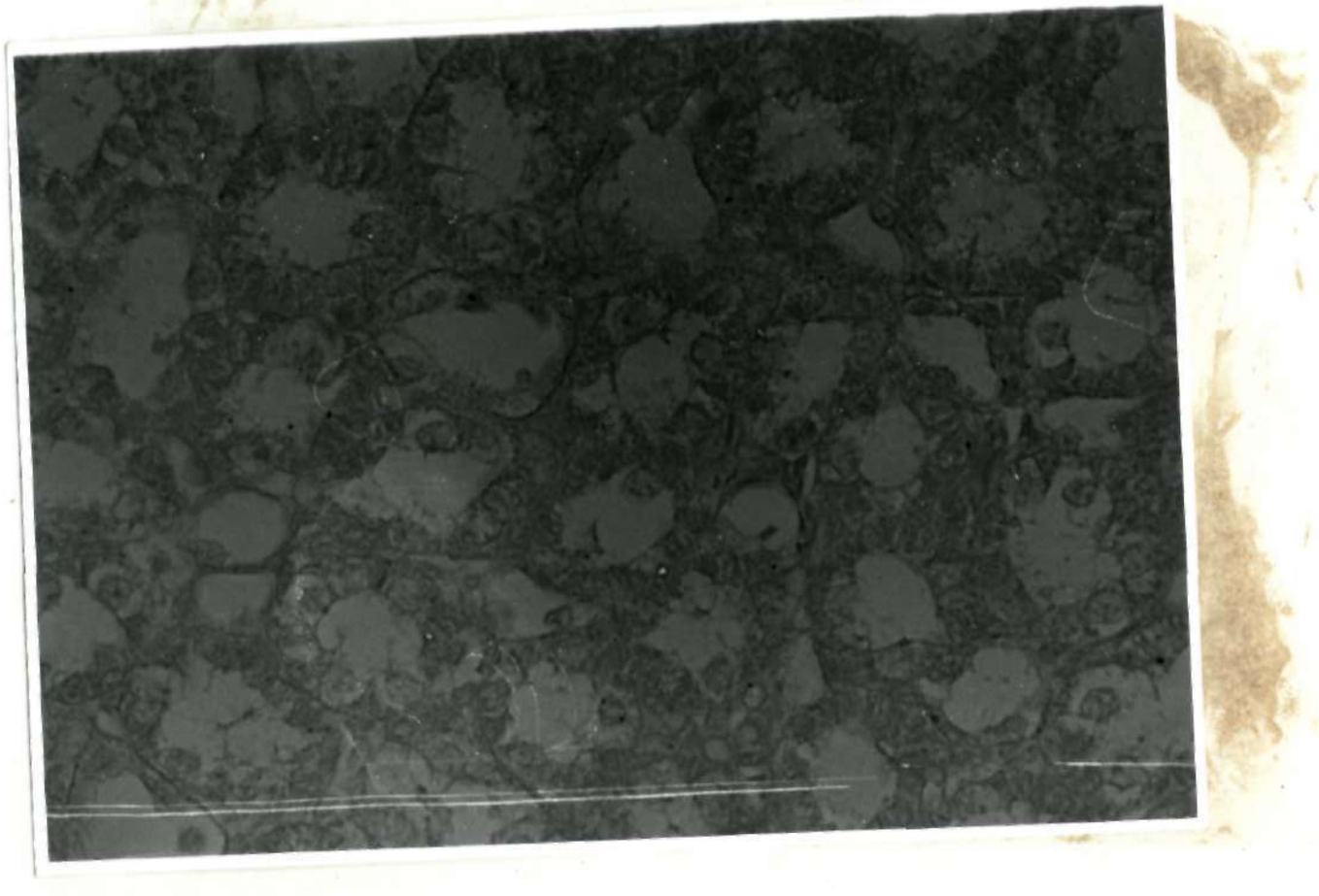
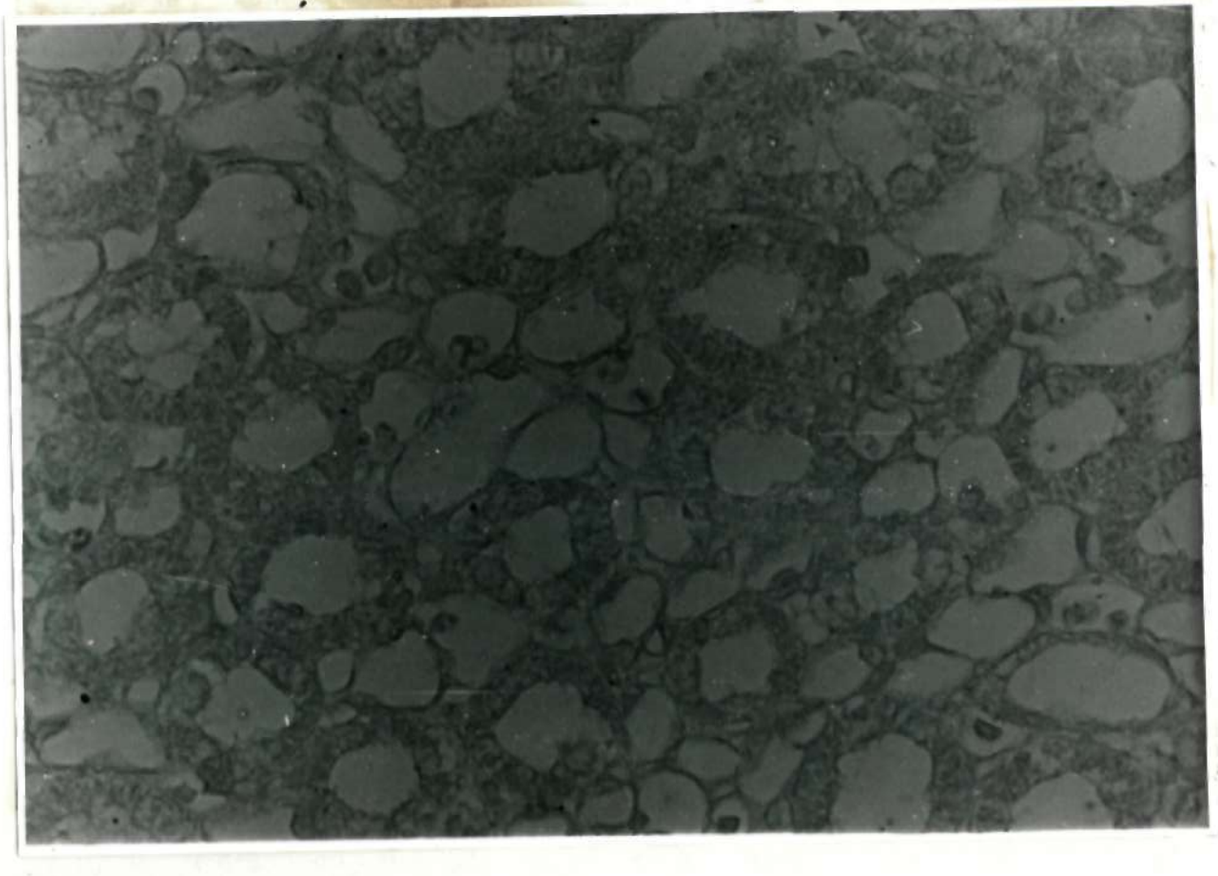


PLATE V.3

Histology of the kidney of a rat maintained on the basal soya bean meal diet containing potassium cyanide (77 µmoles/rat/day). No calcium deposits. Von Kossa x 160.



CHAPTER VI

CHAPTER VI

The estimation of rhodanese activity in rat tissues

1. Introduction

In Chapters IV and V, it was observed that death did not occur when an otherwise lethal dose of cyanide was taken by rats over a prolonged period of hours in the diet. This is because, given time, the animals were able to convert cyanide, mainly, to relatively non-toxic thiocyanate, by means of the action of rhodanese enzyme in tissues. It was therefore of interest to determine the rhodanese content of rat tissues.

Iron (III) ions react with thiocyanate ions in acid solution to yield a red colour. This reaction has been used extensively for the estimation of rhodanese activity in tissues by photometric measurement of the thiocyanate formed from cyanide by the enzyme (Lang, 1933a; Himwich and Saunders, 1948; Sorbo, 1953). With thiosulphate as sulphur donor, the procedure has been adopted as a spot test for locating rhodanese in effluent fractions from column chromatography, and for locating the enzyme on electrophoresis strips (Westley, 1973). The basic method has some problems and it has been undergoing modifications in order to make it quantitative. One of the problems resulted from the fact that excess thiosulphate is decomposed by acid in the ferric reagent, precipitating sulphur. Rosenthal *et al.* (1950) circumvented this by the addition of formaldehyde after stopping the reaction. In addition ferric ions form a blue coloured complex with thiosulphate and Sorbo (1953) avoided this difficulty by terminating rhodanese-catalysed reactions with formaldehyde which inactivates the enzyme and at the same time prevents formation of the blue ferric-thiosulphate complex and removes excess

cyanide. When organic thiosulphates are used as donor substrates, the ferric thiocyanate colour fades rapidly in a photocatalysed reaction. Mintel and Westley (1966) found that this effect can be prevented by addition of inorganic thiosulphate to reaction mixtures after the formaldehyde but before the ferric reagent.

1(a) Distribution of rhodanese in tissues

Lang (1933a; 1933b) described an enzyme that in the presence of a suitable sulphur donor was able to convert cyanide to thiocyanate. This enzyme which was named rhodanese has been purified (Sorbo, 1951; 1953) and the primary, secondary and tertiary structure characterized (Ploegman et al., 1978).

Several tissues of a variety of animals have been tested for rhodanese activity. Lang (1933a) found rhodanese activity in all tissues of a dog except blood and muscle, being highest in adrenals and liver. In the liver of other animals, the activity was highest in frogs followed by rabbits and cattle. Human beings, chickens, cats and dogs had lower levels.

Further work with animal rhodanese was carried out in the late 1940s after the publication of the assay procedure of Cosby and Summer (1945). Himwich and Saunders (1948) and Benard et al. (1948, 1949) undertook studies on the distribution of rhodanese in mammalian tissues. The results of the two groups of workers were in general agreement with Lang's earlier findings of rhodanese ubiquity, but great species to species and organ to organ variability was evident. Muscle, stomach, intestine and blood of most species were reported to have rhodanese activity.

Subsequent studies on the distribution of animal rhodanese activity have included Rosenthal's (1955) finding that hepatomas contain only about 20% as much as normal liver or half the level of

regenerating liver of protein-depleted rats. Coltorti and Giusti (1956) have reported on the rhodanese activity of the erythrocyte. In contrast to Lang's result, they claimed that red blood cells have about 2½% as much activity as liver. Since the activity decreases steadily on dialysis of the haemolysate, however, there may be grounds to wonder whether the small thiocyanate formation observed resulted from genuine rhodanese activity in this case.

Most recently, Schievelbein et al. (1969) determined the rhodanese content of various tissues of a broad phylogenetic span of species. Whereas most land animals had this activity in liver and kidneys, marine animals tended to have larger amounts in the gills.

There are disturbing features of these reports; for example, a potential source declared free of rhodanese (blood) in one publication (Lang, 1933a) is later the subject of a paper on its rhodanese activity (Coltorti and Giusti, 1956). Westley (1973) has suggested that part of this discrepancy is probably the result of real biological variability; much of it however, may relate to the extreme sensitivity of the thiocyanate-based assay methods and the ubiquitous occurrence of non-specific catalysts for the thiosulphate-cyanide reaction.

2. Experimental procedure

The experimental method used to assay rhodanese activity is that described by Himwich and Saunders (1948). In a preliminary study of this assay method, it was observed that a high concentration of thiocyanate is formed by a non-enzymic reaction of thiosulphate with cyanide. Furthermore, the colour of the iron-thiocyanate complex formed was usually too intense with amounts greater than a few micrograms. Himwich and Saunders (1948) circumvented the later problem by diluting

tissue homogenates with a high level of activity to a suitable concentration before assay. However, blanks and calibration curves prepared by adding known amounts of thiocyanate to the tissue system in the absence of cyanide (Himwich and Saunders, 1948) do not correct for the non-enzymic synthesis of thiocyanate from KCN and thiosulphate in the enzyme assay. In order to minimize interference from the non-enzymic reaction and at the same time correct for it in a blank, it was usually necessary to avoid a very low enzyme concentration, so that the enzymatic synthesis will be detectably higher than the inevitable non-enzymic formation of thiocyanate from cyanide and thiosulphate. The ferric thiocyanate colour obtained under such condition normally required further dilution before its absorbance could be read.

Dilution of the red-coloured ferric thiocyanate solution with water usually resulted in an unstable colour, possibly due to the following reaction:

$$Fe^{3+} + 3H_2O \rightleftharpoons Fe(OH)_3 + 3H^+$$

The rate of decay of the ferric thiocyanate colour when diluted with water (1 -- 20) is shown in Fig.1. When the ferric thiocyanate colour was diluted not with water but with ferric nitrate reagent (ferric nitrate in nitric acid, see earlier), the resulting diluted solution was found to be stable for up to one hour (Fig.VI.1); but it decays rapidly on further standing. It must be mentioned however, that the decay could occur in less than 1 h with prolonged exposure to light. The standard curve for the determination of thiocyanate (in the presence of the other components of the rhodanese assay system) in which the final dilution was made with the ferric nitrate reagent is shown in Fig.VI.2. Beer's law is perfectly obeyed over the concentration range employed (0.02 µmoles to 0.18 µmoles of thiocyanate

FIGURE VI.1

The rate of decay of ferric thiocyanate colour on dilution with water or ferric nitrate reagent (mean ± S.D. n=4). The ferric thiocyanate colour was developed in the presence of other components of the rhodanese assay system; total amount of SCN^- in each system was 150 µmoles; absorbance was measured in a 1cm cell in each case (o-o, dilution with ferric nitrate reagent giving a final conc. of 0.06 µmoles/ml; e-e, dilution with water giving a final conc. of 0.30 µmoles/ml).

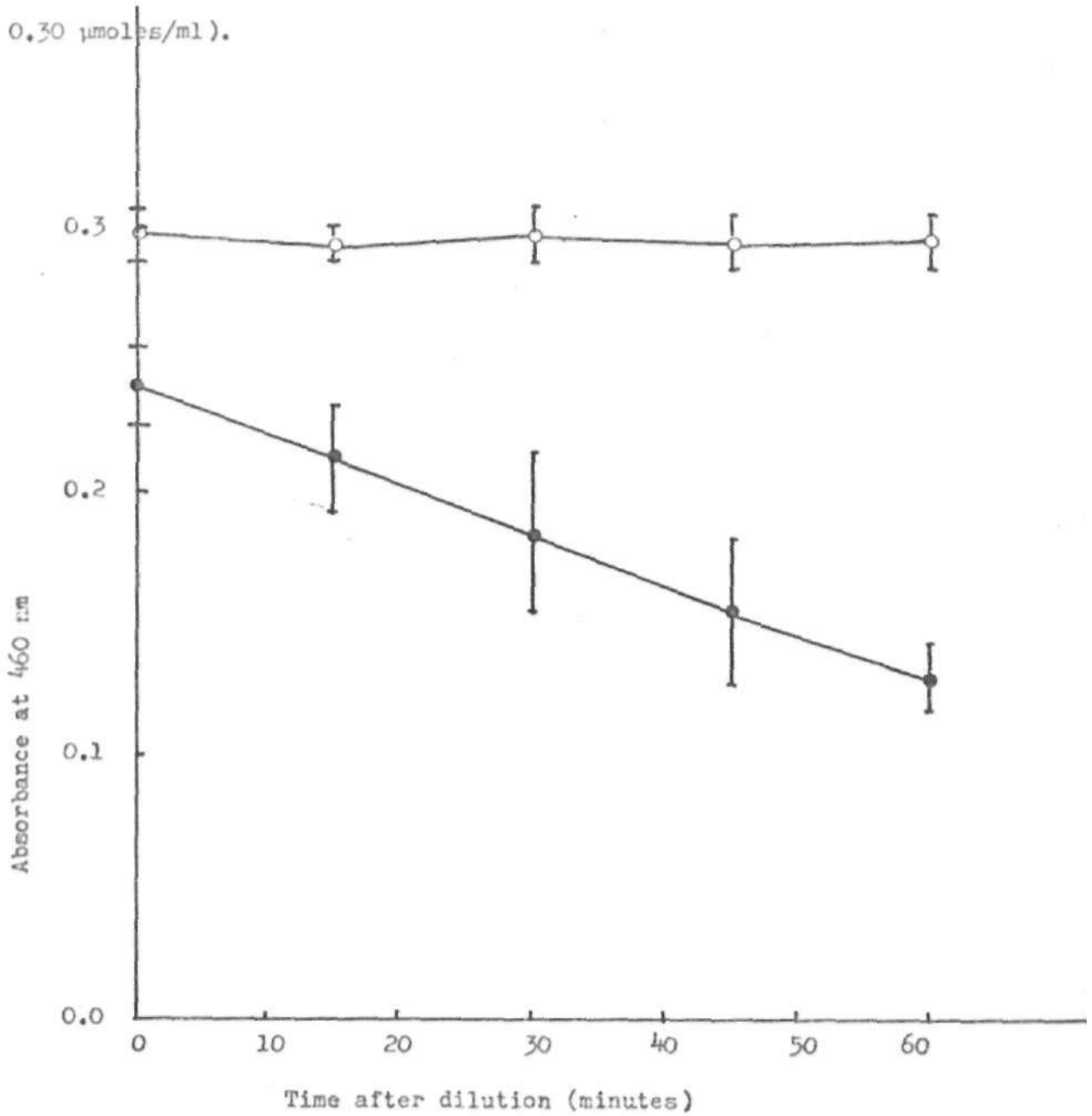
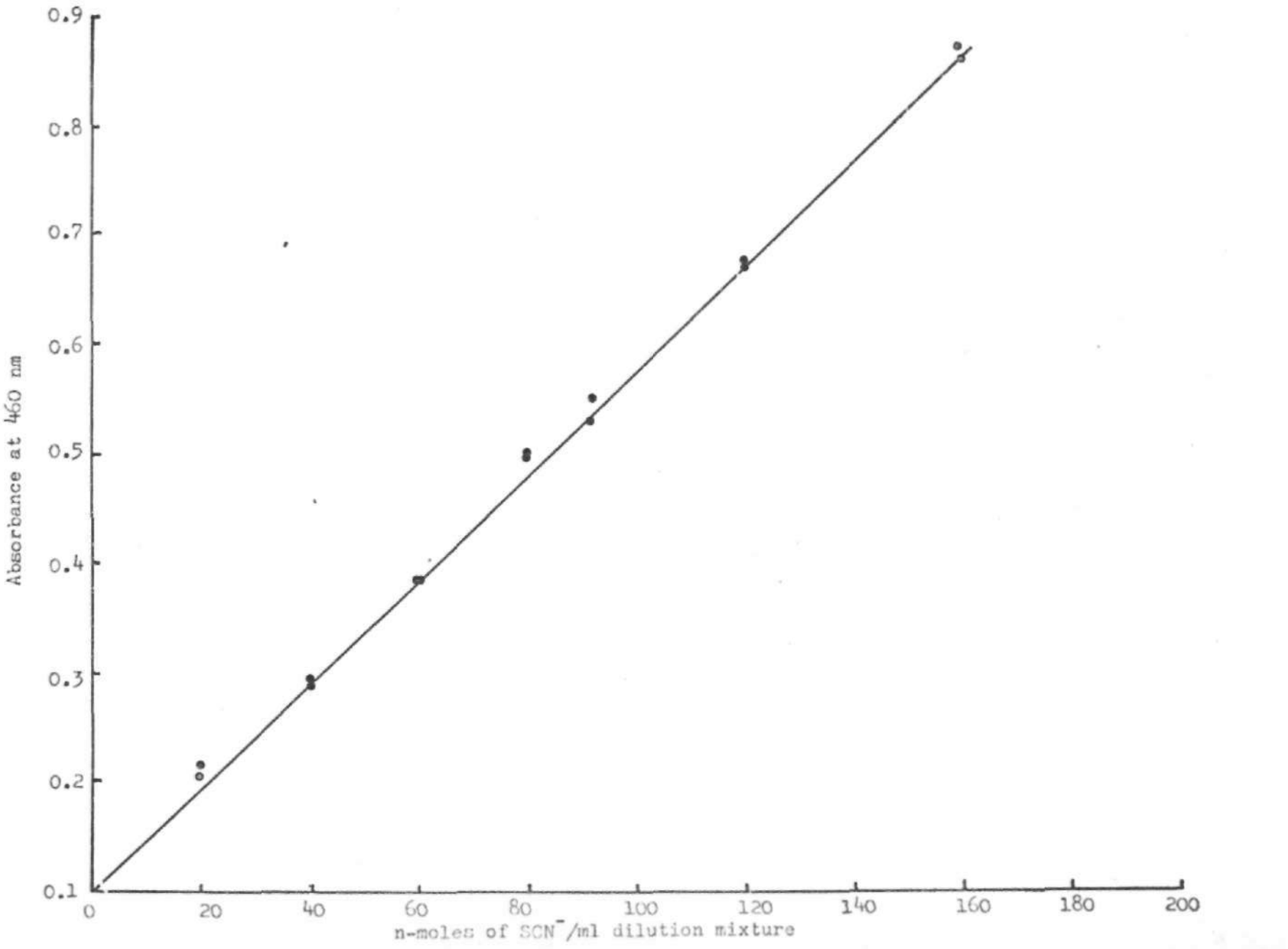


FIGURE VI.2

Standard curve for the estimation of thiocyanate by ferric nitrate reagent.



per ml final dilution mixture, even when dilutions as high as 50 times were made.

All available evidence indicates that interaction between Fe^{3+} and SCN^- consists of the following series of equilibria (Lewin and Wagner, 1953):

$$Fe^{3+} + SCN^- \rightleftharpoons FeSCN^{2+}$$

$$FeSCN^{2+} + SCN^- \rightleftharpoons Fe(SCN)_2^+$$

$$Fe(SCN)_2^+ + SCN^- \rightleftharpoons Fe(SCN)_3$$

$$Fe(SCN)_3 + SCN^- \rightleftharpoons Fe(SCN)_4^-$$

$$Fe(SCN)_4^- + SCN^- \rightleftharpoons Fe(SCN)_5^{2-}$$

$$Fe(SCN)_5^{2-} + SCN^- \rightleftharpoons Fe(SCN)_6^{3-}$$

Thus more than one product can be formed when thiocyanate reacts with ferric ion, depending upon the thiocyanate concentration. From the work of Van Valkenburgh and Schlesinger (1931), Von Kiss, Abraham and Hegedus (1940) showed that with excess SCN^- the principal absorbing species is $Fe(SCN)_6^{3-}$ but that the molecule $Fe(SCN)_3$ predominates in the absence of excess SCN^-. When Fe^{3+} is in excess, the only absorbing species is $FeSCN^{2+}$ (Bent and French, 1941; Edmonds and Birnbaum, 1941). Dilutions of strongly coloured solution with ferric nitrate reagent most probably contain essentially $FeSCN^{2+}$ ions, which are stable in acid solution. The actual procedure developed which allowed for the above mentioned problems is given below.

The rhodanese activity was assayed by the method of Himwich and Saunders (1948) with modification. Activity was expressed in terms of µmoles thiocyanate produced from cyanide per gram protein in tissue or per gram wet weight of tissue.

The rats were killed by a blow at the back of the neck (when blood samples were required, they were killed by first anaesthetizing with chloroform and then chopping off their heads in a guillotine).

Tissues were removed from animals as rapidly as possible and homogenates prepared in distilled water (10 ml of water per gram of tissue). The homogenates were filtered through two thicknesses of cheese cloth and stored in a cold room at about 4°.

The enzyme was assayed in a 35 ml test tube with the following test systems: 3.7 ml 0.2M phosphate buffer, pH 7.4 (prepared by mixing 40.5 ml 0.2M Na_2HPO_4 with 9.5 ml 0.2M NaH_2PO_4), 2 ml 2.1M sodium thiosulphate, 0.05-0.3 ml tissue homogenate (boiled in case of blank) and 2 ml 0.7M KCN, all in a total volume of 10 ml (the difference being made up with distilled water). The reaction was usually started by the addition of the KCN solution. The test system then contained: sodium thiosulphate, 0.42M, phosphate 0.074M and cyanide, 0.14M. The enzyme activity was stopped after 15 minutes at 37.5° by adding 0.5 ml formaldehyde solution (385 g/l water, Sorbo 1953), whereupon 10 ml of ferric nitrate reagent (containing 100 g $Fe(NO_3)_3.9H_2O$, 200 ml HNO_3, S.G.1.40, and distilled water to 1000 ml) was added. The reaction mixture was then diluted to 25 ml with distilled water, shaken, centrifuged and the absorbance at 460 nm in a 1 cm cuvette measured in the SP600 spectrophotometer after appropriate dilution (see below).

Thiocyanate was determined by means of a standard curve prepared by adding a known amount of thiocyanate to the assay system in the presence of cyanide but in the absence of enzyme (Fig.VI.2). The thiocyanate solution was standardized argentometrically.

Since the colour of the iron-thiocyanate complex was too intense with amounts greater than a few micrograms, it was necessary to dilute with ferric nitrate reagent. The validity of such dilutions has been discussed earlier.

It must be emphasised that several of the solutions and reagents used in the assay are chemically unstable, and hence the following precautions are recommended in order to ensure accuracy of results:

(1) The sodium thiosulphate solution must be stored in a dark reagent bottle at 4^{o} as it forms colloidal sulphur and sulphonic acid under the influence of proton catalysis.

(2) The ferric nitrate reagent should also be stored in a dark reagent bottle as it decomposes, by an unknown mechanism, in the light.

(3) To ensure stability of the ferric thiocyanate colour formed, the assay tube should be exposed to as little light as possible.

3. Results

3(a) Protein content

The protein content of homogenized kidney and liver was estimated by the Lowry method (based on the Folin colour reaction, Chapter II). Lowry et al. (1951) have shown that their method when used on whole tissues will be in error by about 3 to 6 percent. Tables VI.1 and VI.2 give the protein content of kidney and liver (respectively) as determined by the colour reaction of Lowry et al. (1951).

The levels of protein in the kidney did not show any marked difference between treatment means (Table VI.1). The mean protein concentration of the livers from control rats was however, slightly lower than that of rats on vitamin D_3 or potassium cyanide. This difference was not however, statistically significant ($P > 0.05$).

On the whole, the figures obtained for the mean protein concentrations of the kidneys or the livers were lower than those reported by other workers for rat tissues. The tissue homogenates used for protein estimation (by the Lowry method) were stored for a

TABLE VI.1

Protein concentration of kidneys of groups of male rats given potassium cyanide (77 μmoles/rat/day) or vitamin D_3 (0.55 μmoles/rat/day) in the diet.

Group	Substance added to diet	Weight of kidney (g)	% Protein based on Lowry et al. Colour	Mean ± S.E.M.
		1.64	10.0	
		2.05	9.0	
GB	None	1.75	8.6	10.9±1.0
		2.21	10.7	
		2.30	11.7	
		1.54	15.3	
		1.30	9.2	
		1.76	10.6	
HB	Vitamin D_3	1.83	10.4	10.7±0.4
		1.80	11.7	
		1.45	11.3	
		1.70	10.7	
		1.74	10.6	
		2.78	9.7	
IB	Potassium cyanide	1.74	11.7	11.1±0.4
		1.70	12.1	
		2.05	11.6	
		1.90	10.7	

None of the differences in protein concentration between groups were statistically significant ($P > 0.4$).

TABLE VI.2

Protein concentration of livers of groups of rats given potassium cyanide (77 µmoles/rat/day) or vitamin D_3 (0.55 µmoles/rat/day) in the diet.

Group	Substance added to diet	Weight of liver (g)	% Protein based on Lowry et al. Colour	Mean ± S.E.M.
		8.76	15.7	
		12.28	16.6	
GB	None	12.28	15.5	16.1±0.6
		13.37	15.0	
		12.67	15.0	
		8.91	18.0	
		6.24	16.3	
		9.04	17.5	
HB	Vitamin D_3	7.87	17.3	17.5±0.4
		8.74	18.5	
		6.10	16.8	
		6.77	18.4	
		10.08	17.2	
		14.34	15.6	
IB	Potassium cyanide	10.17	18.2	17.4±0.4
		9.14	18.0	
		11.90	16.9	
		10.37	18.3	

None of the differences between groups in protein concentration were stastically significant ($P > 0.05$).

period of about three weeks at 4°. Storage of kidney homogenates at such temperature and for such length of time was found to give a reduction of colour by between 20 and 30%. Thus the lower levels of protein found in kidneys and livers (particularly the kidney) might be a result of the storage conditions. It was hoped however that the results obtained would be useful for comparative purposes since tissues of all groups were stored under the same conditions and for the same length of time.

3(b) Rhodanese activity

The improved method (described in this Chapter) was used to estimate the rhodanese activity in the liver, kidney, blood, muscle, stomach and small intestine of the following groups of male rats: rats on a chronic dose of cyanide and rats on large doses of vitamins A or D (Chapters IV and V).

In all the groups of rats studied, the stomach, small intestine, blood and muscle did not show any detectable rhodanese activity. Thus, either these tissues are devoid of rhodanese activity, or the level of activity present was not detectable by the assay method employed. Himwich and Saunders (1948) reported low levels of rhodanese activity in the blood, muscle and intestine of a number of animal species. Their finding should be viewed with caution since their assay system for the preparation of a standard curve (and possibly blank) for thiocyanate estimation, did not contain KCN, and hence did not correct for the considerable amount of thiocyanate that is formed by nonenzymic reaction between KCN and thiosulphate in the assay tube. As has been pointed out earlier in this chapter, the procedure used here was modified and shown to correct for such errors. The discrepancies that exist in the published reports on both tissue

and species distribution of rhodanese (Westley, 1973) may be due (at least in part) to failure to correct for the non-enzymic catalysis of the thiosulphate-cyanide reaction.

The liver and the kidney are known to contain large amounts of rhodanese enzyme (Himwich and Saunders, 1948). The rhodanese activity of livers and kidneys of rats on cyanide and vitamins A and D are given in Tables VI.3, VI.4 and VI.5. The rats on cyanide did not show a level of rhodanese activity significantly different from that of control rats in either the liver or the kidney. This indicates that dosing rats with cyanide up to the concentration of 77 µmoles per rat per day, did not affect the turnover rate of this detoxicating enzyme. This is in agreement with the report of Tewe et al. (1977) who demonstrated that rats on a diet based on cassava (which contains cyanide as cyanogenic glycoside) maintained a level of rhodanese enzyme similar to that of control rats.

Although rats on high doses of vitamin A were very severely affected, their liver concentration of rhodanese on a tissue wet weight basis was not different from the liver concentrations in control rats or those given cyanide (Table VI.3). It was unfortunate that the protein content of the liver homogenates was not determined in this experiment, for in the later experiment in which rats were given high doses of vitamin D, an odd feature was observed. The liver rhodanese concentration, expressed on a tissue wet weight basis, was not different in rats given high doses of vitamin D compared with controls or rats given cyanide ($P > 0.8$). When, however, rhodanese activity was expressed relative to protein, there was a significant lowering of activity in the vitamin D group relative to controls and rats given cyanide ($P < 0.025$, Table VI.4). The concentration of

TABLE VI.3

Rhodanese activity of livers of groups of rats given potassium cyanide (77 µmoles/rat/day) or vitamin A (38.5 µmoles/rat/day) in the diet.

Group	Substance added to diet	µmoles SCN^- formed per g liver per min.	Mean ± S.E.M.	M moles SCN^- formed per liver per min.	Mean ± S.E.M.
		558		6.74	
A2	None	540	492±33	6.94	6.65
		446		6.04	
		425		6.87	
		498		4.11	
B2	Retinyl acetate	510	488±10	5.10	5.27
		466		5.53	
		474		6.35	
		496		6.95	
C2	Potassium cyanide	560	491±27	7.08	6.66
		475		6.32	
		433		6.52	

There was no significant difference between groups ($P > 0.8$) in rhodanese activity expressed in terms of tissue level weight.

TABLE VI.4

Rhodanese activity of livers of groups of rats given potassium cyanide (77.0 μmoles/rat/day) or vitamin D_3 (0.55 μmoles/rat/day).

Group	Substance added to diet	μmoles SCN^- formed per g liver per min.	m-moles SCN^- formed per liver per min.	Moles SCN^- formed per liver per 24 h.	M-Moles SCN^- formed per g protein per min.	Mean ± S.E.M.
GB	None	435	3.81	5.50	2.77	2.95±0.03
		480	5.90	8.50	2.89	
		469	5.76	8.29	3.03	
		435	5.82	8.40	2.90	
		438	5.55	8.00	2.92	
		540	4.81	6.93	2.87	
HB	Vitamin D_3	512	3.20	4.61	3.14	2.67±0.10
		492	4.45	6.41	2.45	
		478	3.76	5.41	2.76	
		485	4.24	6.11	2.62	
		425	2.60	3.74	2.53	
		459	3.10	4.46	2.50	
IB	Potassium cyanide	509	5.13	7.39	2.96	2.83±0.03
		442	6.33	9.12	2.83	
		499	5.10	7.34	2.74	
		508	4.65	6.70	2.82	
		480	5.71	8.22	2.84	
		515	5.34	7.69	2.81	

There were no significant differences between groups in rhodanese activities expressed in terms of wet tissue weight, but the activity in group HB was lower than in groups GB and IB ($P < 0.025$) when expressed relative to protein.

TABLE VI.5

Rhodanese activity of kidnyes of groups of rats given potassium cyanide (77 μmoles/rat/day) or vitamin D_3 (0.55 μmoles/rat/day).

Group	Substance added to diet	μmoles SCN^- formed per g kidney per min.	m-moles SCN^- formed per kidney per min.	Moles SCN^- formed per kidney (pair) per 24 h.	m-moles SCN^- formed per g protein per min.	Mean ± S.E.M.
GB	None	165	0.27	0.39	1.64	1.65±0.11
		143	0.29	0.42	1.60	
		187	0.33	0.48	2.17	
		165	0.37	0.53	1.56	
		177	0.41	0.59	1.51	
		218	0.36	0.52	1.42	
HB	Vitamin D_3	163	0.21	0.30	1.77	1.46±0.08
		132	0.23	0.33	1.23	
		159	0.30	0.43	1.53	
		150	0.27	0.39	1.28	
		162	0.24	0.35	1.43	
		161	0.27	0.39	1.50	
IB	Potassium cyanide	169	0.29	0.42	1.59	1.73± 0.07
		160	0.45	0.65	1.65	
		210	0.37	0.53	2.02	
		186	0.32	0.46	1.59	
		193	0.40	0.58	1.71	
		194	0.37	0.53	1.81	

Rhodanese activity was lower in group HB than in groups GB and IB when expressed in terms of tissue wet weight ($P<0.02$) or protein(P 0.05).

rhodanese in the kidneys of rats on high doses of vitamin D was significantly lower than in controls or rats given cyanide whether calculated on a wet weight ($P < 0.02$) or protein ($P < 0.05$) basis.

4. Discussion

The activity of the enzyme rhodanese is affected by several factors including the time of storage of the tissue homogenate (Saunders and Himwich, 1950; Dinan, 1976). In general, there is a decrease in rhodanese activity with prolonged storage at 4° and this is more marked in a highly diluted homogenate (Saunders and Himwich, 1950). Saunders and Himwich (1950) found that the instability of greatly diluted homogenates could be prevented by the addition of thiosulphate or cysteine.

Furthermore, it has been shown in the rat kidneys that the activity of the rhodanese present does significantly vary with age (Holcroft, 1977). However, when the liver was examined under similar conditions it failed to exhibit any significant variation in activity.

In view of the above observations and the ubiquitous occurrence of non-specific catalysts for the thiosulphate-cyanide reaction, it is clear that comparison of the levels of rhodanese activity reported by different investigators under varying conditions will be difficult. However, in the cases where tissue levels of this enzyme were obtained for groups of animals of the same age under the same experimental conditions (as reported here) useful comparisons are possible.

In a number of animal species, including the rat, rhodanese activity has been demonstrated in almost every tissue (Mendel et al., 1946; Himwich and Saunders, 1948). Unlike the findings of these workers, rhodanese activity could not be demonstrated in the muscle, stomach, blood and small intestine of the rat by the assay method

already described. The probable reasons for the discrepancy have been presented.

The kidneys and the livers (in particular) of all groups of rats examined had large catalytic capacities for the detoxification of cyanide, as assayed by its enzymic conversion to thiocyanate. Calculated from *in vitro* results (using control rats in group GB, for example) the amounts of cyanide which the liver and kidney of a rat can theoretically convert to thiocyanate are enormous. The rat liver weighing 11.4 gm could detoxify 198 gm cyanide per day and the pair of kidneys (wt 1.92 gm) of the same rat, 12.7 gm per day. Thus the total catalytic capacity for the detoxification of cyanide is greatly in excess of that which the animal would normally expect to encounter by way of dietary intake of cyanide. It is not clear why this apparent anomaly should exist, but it has been suggested that *in vivo*, the reaction may be limited by other factors including the availability of sulphur donors (Himwich and Saunders, 1948). This is supported by the fact the injection of thiosulphate to animals increases the LD_{50} as much as three to four times (Ohen *et al.*, 1934a).

CHAPTER VII

CHAPTER VII

The in vitro transformation of cyanide in rat plasma

1. Introduction

It has been observed in animals poisoned with cyanide, that delays in removing tissues, or in their chemical analysis, may result in low recoveries, because of binding or transformation of cyanide (Curry, 1963). More recently Ballantyne *et al.* (1973) have measured the rate at which decreases in measurable cyanide occur in rabbit whole blood, serum and urine to which cyanide was added *in vitro*. They observed that when potassium cyanide was added to human or rabbit serum, the amount recoverable decreased rapidly with time. Using whole blood, the rate of disappearance of added cyanide was less marked than with serum; this was confirmed in whole blood removed from cyanide-poisoned animals. Losses of cyanide as hydrogen cyanide by simple evaporation were probably minimal because of the pH of serum and since stoppered vessels were used. This suggestion is supported by the finding of very small decreases in measurable cyanide in phosphate buffers (Ballantyne *et al.*, 1973; Pettigrew and Fell, 1972). The possible reasons suggested by Ballantyne *et al.* (1973) for the decreases in measurable cyanide include slow conversion to ammonium formate and aminomalonitrile (Guatelli, 1964; Ioanid and Bors, 1961; Oke, 1969). Pettigrew and Fell (1972) have also shown that cyanide added to plasma *in vitro* is only briefly stable at room temperature.

Following injection of ^{14}CN to rats, Boxer and Rickards (1952) have shown that cyanide (and thiocyanate) carbon is converted *in vivo*

to carbon dioxide in the rat. Although the mechanism of CO_2 formation from cyanide is not known, Boxer and Rickards (1952) suggested that CO_2 formation might result from the hydrolysis of cyanate, since Ellinger and Lenzberg (1926) claimed that muscle brei oxidized cyanide to cyanate. To date, no work has been reported confirming cyanate as source of CO_2 following cyanide administration to animals.

Chung and Wood (1971) have shown that thiocyanate reacts with bovine haemoglobin *in vitro*, in the presence of hydrogen peroxide to yield sulphate and cyanide. No other substance catalyzing this reaction was found in haemolysates of bovine blood. Moreover, oxyhaemoglobin was a more active catalyst than methaemoglobin but was rapidly converted to the latter in the incubation system. The mechanism of oxidation of thiocyanate by hydrogen peroxide has been given by Chung and Wood (1970, 1971):

$$SCN^- + H_2O_2 \longrightarrow OSCN^- + H_2O \quad (1)$$

$$OSCN^- + H_2O_2 \longrightarrow OOSCN^- + H_2O \quad (2)$$

$$OOSCN^- + H_2O_2 \longrightarrow OOOSCN^- + H_2O \quad (3)$$

$$OOOSCN^- + H_2O \longrightarrow HSO_4 + HCN \quad (4)$$

The sum of the reaction steps is thus given by:

$$3H_2O_2 + SCN^- \longrightarrow HSO_4^- + HCN + 2H_2O \quad (5)$$

The cyanide produced from thiocyanate was converted to cyanate by a non-enzymic reaction:

$$OOSCN^- + H_2O_2 \longrightarrow HSO_3 + HOSCN \quad (6)$$

$$HOSCN + HCN \longrightarrow HOCN + HSCN \quad (7)$$

The latter reaction may involve the intermediate formation of sulphur dicyanide:

$$HOSCN + HCN \longrightarrow S(CN)_2 + H_2O \quad (8)$$

Sulphur dicyanide rapidly hydrolyses to cyanate and SCN^-

$$S(CN)_2 + H_2O \longrightarrow HOCN + HSCN \quad (9)$$

If the above pathway is responsible for the formation of cyanate in the intact animal, then hydrolysis of cyanate would yield carbon dioxide. However, it seems unlikely that hydrogen peroxide exists *in vivo* in animal blood in concentrations similar to those used to demonstrate the reaction *in vitro*. Moreover, it is only in the mouse that methaemoglobin has been reported to increase following administration of thiocyanate (Smith, 1973).

The results obtained from a study of the transformation of cyanide added to rat serum or plasma *in vitro* are presented in this chapter. The results are compared with those obtained by Ballantyne *et al.* (1973) for rabbit serum.

2. Experimental procedure

Blood was obtained from rats by first anaesthetising them with chloroform and chopping off their heads in a guillotine. Blood was allowed to run into a chilled 50 ml beaker, transferred to centrifuge tubes and centrifuged at 3,000 g for 10 minutes to obtain serum. Plasma was obtained in a similar way except that heparin was added to the collecting beaker.

To 5 ml serum in a test tube was added 0.1 ml of an aqueous solution containing 0.255 µmoles KCN. The tube was stoppered with a rubber bung, incubated at 25° and sampled periodically (5 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr) for measurement of cyanide and thiocyanate content. One ml of the mixture was transferred to an aeration vessel and the separation and quantitative determination of cyanide and thiocyanate was carried out as already described in Chapter II.

The transformation of cyanide in serum was also studied using $Na^{14}CN$ and the experimental procedure in this case is presented under results with $Na^{14}CN$.

3(a) Results with potassium cyanide

The *in vitro* rate of transformation of cyanide added to serum is shown in Fig.VII.1 and Table VII.1. Measurements of thiocyanate content and cyanide content were made on all samples of serum before addition of cyanide so that absolute changes were calculated. No cyanide could be detected in normal serum by the assay method employed, and hence the level present must be insignificant compared with the concentration of cyanide added, or recovered after the addition of cyanide. The thiocyanate concentrations measured in all serum samples before addition of potassium cyanide are given in Table VII.2.

One hour after the addition of potassium cyanide to rat serum, a mean of 46% was still present as measurable cyanide. Of the 0.028 μmoles/ml of non recoverable cyanide in rat serum at one hour, only 0.011 μmoles/ml (39%) could be accounted for as thiocyanate. The rate of decrease of measurable cyanide was less marked after one hour. Thus four hours after addition of potassium cyanide to serum, measurable cyanide was still as high as 42% (compared with 46% at 1 hour). Furthermore the thiocyanate formed at four hours did not show further increase, rather it showed a slight decrease, accounting for 32% of the non-recoverable cyanide.

When rat plasma was used in place of the serum, it was observed that transformation in the former occurs in a similar manner as that reported here for the serum.

As a control, potassium cyanide was added to 0.2M phosphate buffer pH 7.0 to give a final concentration of 0.051 μmoles/ml in the mixture. The mixture was incubated at 25° and sampled periodically for measurement of cyanide content in the same manner as that described for the serum (see earlier). No detectable changes in measurable cyanide occurred in the phosphate buffer for the period of four hours studied (Table VII.3 and Fig. VII.2).

FIGURE VII.1

The rate of decrease in concentration of measurable cyanide and appearance of thiocyanate in rat blood serum.

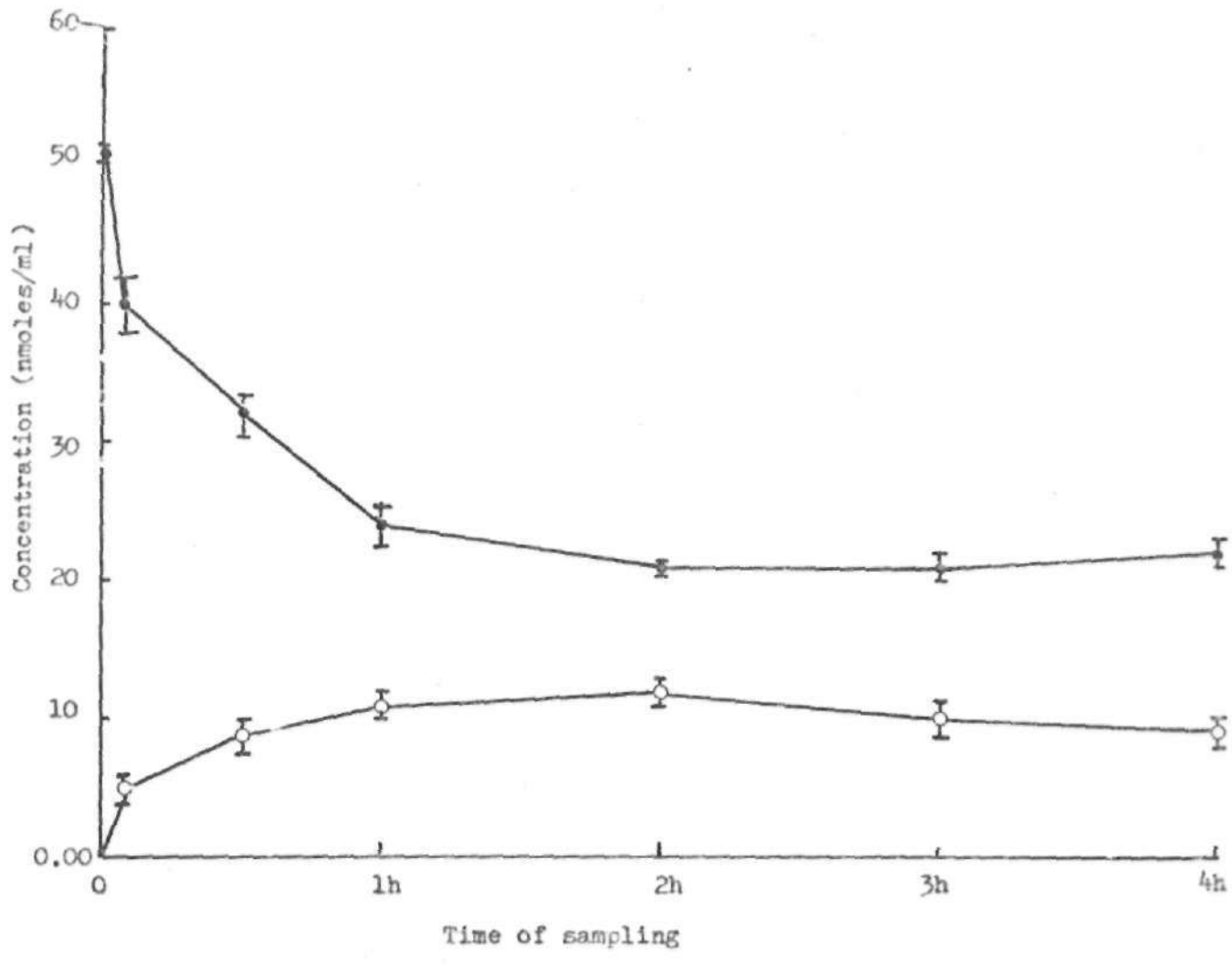


TABLE VII.1

Decreases in measurable cyanide levels and increase in measurable thiocyanate in rat serum[a] (mean ± S.E.M., n = 4).

	Recoverable CN^- and measurable SCN^- with time					
	5 min.	0.5 h	1 h	2 h	3 h	4 h
Recoverable CN^- n moles/ml	40.0	32.0	24.0	21.0	21.0	22.0
S.E.M.	2.3	1.5	1.3	0.5	1.0	1.0
Measurable SCN^- n moles/ml	3.0	9.0	11.0	12.0	10.0	9.0
S.E.M.	0.8	1.4	1.0	0.7	1.4	1.2

[a] Mean concentration of KCN added per ml serum was 51 n moles ± 1 (S.E.M.)

TABLE VII.2

Thiocyanate concentration in normal rat[a] serum.

	Thiocyanate concentration	
	as μmoles/100 ml	as μg/100 ml
	0.90	52.2
Range	0.8 - 1.2	46.4 - 69.6
S.E.M.	0.1	2.9
No. of samples	8	8

[a]Female rats were used.

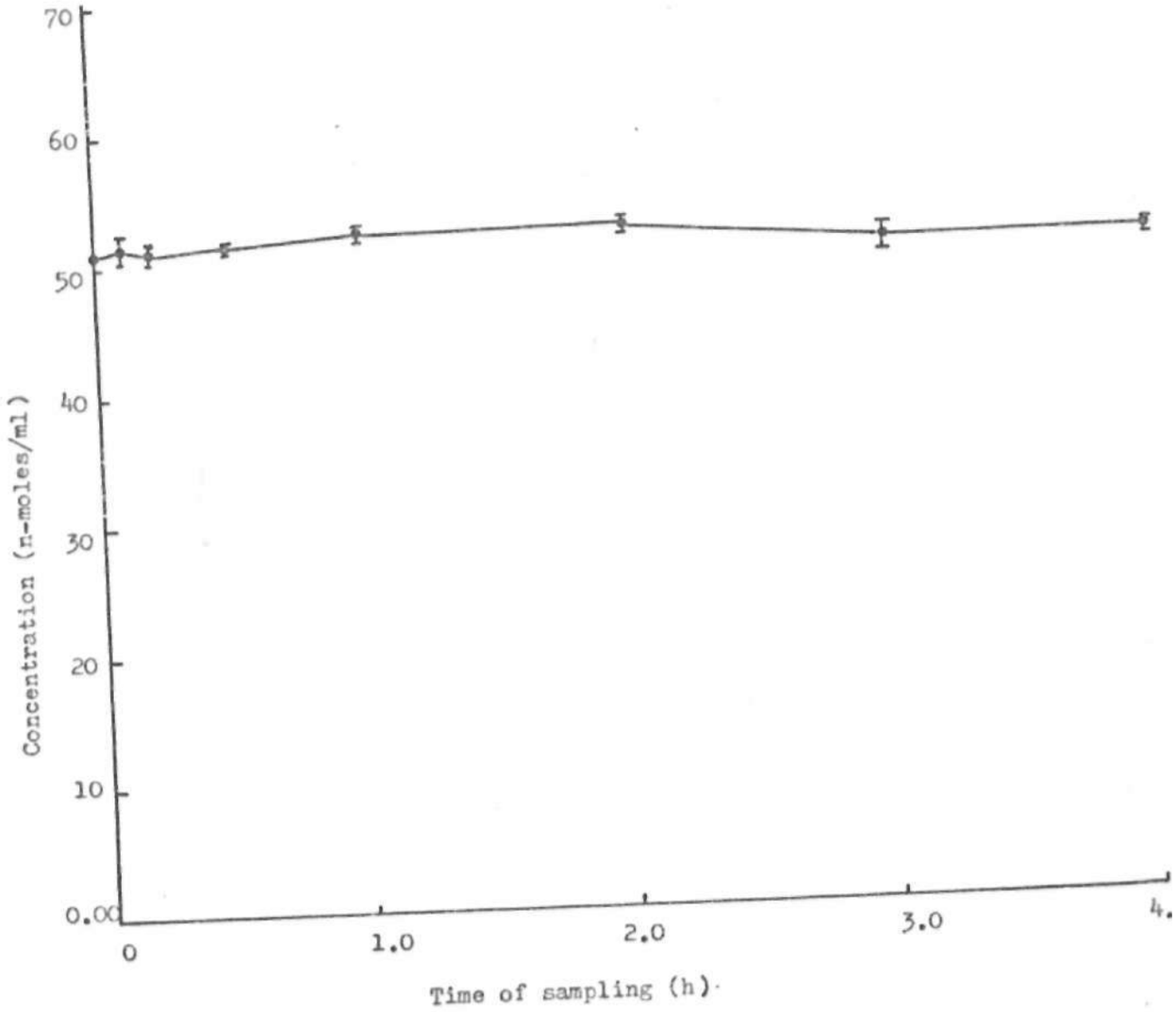
TABLE VII.3

Measurable cyanide levels in 0.2M phosphate buffer at pH 7.0 on standing at room temperature.

Period of incubation at 25°	n moles of CN^- recovered mean ± S.E.M., n = 7
0 min	51.1 ± 0.4
6 min	51.7 ± 1.0
12 min	51.0 ± 0.7
30 min	51.4 ± 0.3
1 h	51.8 ± 0.6
2 h	52.1 ± 0.7
3 h	50.5 ± 1.1
4 h	50.7 ± 0.5

FIGURE VII.2

Concentration of measurable cyanide in 0.2M phosphate buffer pH 7.0 on standing at room temperature.



3(b) Results with $Na^{14}CN$

A study of the transformation of cyanide in normal and heat-treated sera was carried out with $Na^{14}CN$. The radioactive cyanide was purified just before use by the method already described in Chapter II. Denaturation of the serum was accomplished by heating for one minute in a boiling water bath.

0.1 ml $Na^{14}CN$ solution (0.052 µmoles/0.1 ml), activity 28,449 dpm per 0.1 ml, was added to 1 ml serum and the mixture was incubated for a period of 1 hr at 25°. At the end of 1 hr, the activity in the serum was separated into the following fractions: (1) CYANIDE FRACTION, (2) THIOCYANATE FRACTION, (3) CARBON DIOXIDE FRACTION, (4) TRICHLOROACETIC ACID FRACTION and (5) SUPERNATANT FRACTION, by a similar procedure to that described in Chapter IX. The amounts of radioactivity present in the various fractions are given in Table VII.4.

The mean cyanide recovered from the normal serum after one hour of incubation was 40% of the amount added. In heat-treated serum, the recovery was higher, accounting for about 59% of total cyanide added.

Although thiocyanate was formed in both normal and heat-treated sera, a significantly lower amount was found in the heated serum ($P \ll 0.001$). This indicates that part of the thiocyanate formed in serum from cyanide was made by enzymic action.

The activity in the carbon dioxide fraction is variable but appear to be more or less the same in heat-treated and normal sera ($P > 0.60$). The variability may be due to losses of CO_2 and partly to the presence of variable amounts of acidic materials like formate in this fraction. Precipitation of the carbonate by means of excess $Ba(OH)_2$ solution revealed that between 70-86% was associated with the precipitated $BaCO_3$.

TABLE VII.4

Distribution of radioactivity in various fractions after incubation of serum with $Na^{14}CN$. 0.1 ml of 0.52 mM $Na^{14}CN$; activity 28,449 dpm/0.1 ml was added to 1 ml serum.

Sample	Radioactivity in fractions as dpm				
	CN^- fraction	SCN^- fraction	CO_2 fraction	TCA precipitate fraction	Supernatant fraction
Serum (normal)	12,432	7,612	621	3,170	5,716
	10,956	7,082	636	5,860	3,460
	11,504	7,222	421	4,679	3,512
	11,226	5,561	135	3,998	2,952
Mean	11,530	6,619	628	4,427	3,911
S.E.M.	321	346	253	569	615
Serum (heat treated)	16,928	1,306	760	3,721	3,077
	17,402	1,501	606	3,473	3,009
	15,972	1,652	360	3,789	3,413
	16,458	1,340	364	3,447	3,799
Mean	16,689	1,450	521	3,608	3,325
S.E.M.	307	80	98	86	181

The activity in the TCA precipitate fraction of normal serum was somewhat higher than that present in the same amount of heat-treated serum, although this was not statistically significant ($P > 0.2$).

The mean total radioactivity recovered in normal serum was 95% while that recovered in heat-treated serum was 90%, but the difference was not statistically significant ($P > 0.2$). Since 90-95% of radioactivity added to serum could be accounted for, it is clear that the loss of cyanide added to serum was not due to its conversion to volatile HCN.

The radioactivity remaining in the supernatant fraction after removal of the thiocyanate was found to be variable especially in the normal serum. However, the amounts found in normal and denatured serum were similar. Although a study of the chemical nature of the transformation products of cyanide present in the supernatant fraction was not carried out, it is likely that formate and aminomalonitrile are major components (Guatelli, 1964; Ioanid and Bors, 1961; Oke, 1969).

In Chapter III, it was observed that potassium cyanide added to the diet moistened with water could not be recovered. In order to check if transformation of cyanide also occurred in the diet, a solution of $Na^{14}CN$ (purified by the method already described in Chapter II) of known specific activity was added to known amounts of the basal soyabean meal diet in a test tube. The tube was stoppered and left overnight at room temperature (about 18 hr). Analysis of the cyanide present by a similar procedure (nitrogen aeration) as that described for the serum revealed that less than 50% of the cyanide added could be recovered (Table VII.5). Extensive loss of the added

TABLE VII.5

Recovery of ^{14}CN added to 2g basal soya bean meal diet.

µg CN^- added	Total activity dpm	% of added CN^- recovered	Mean ± S.E.M.
12.5	52,550	7.6	7.8 ± 0.1
12.5	52,550	7.7	
12.5	52,550	7.9	
12.5	52,550	7.9	
20,007	52,550	38.1	38.5 ± 0.3
20,007	52,550	38.8	
20,007	52,550	38.0	
20,007	52,550	39.0	
40,001	52,550	43.5	43.5 ± 1.1
40,001	52,550	45.5	
40,001	52,550	42.8	
40,001	52,550	40.3	
20,007	3.0×10^6	40.6	40.6

cyanide as HCN from the mixture was unlikely since 98% of the activity that could not be recovered was found to be present in the aeration vessel after removal of the HCN by nitrogen aeration. This meant that the non-recoverable cyanide activity may have been transformed into substances that could not be liberated under the conditions of nitrogen aeration.

In the system in which cyanide of total activity 3.0×10^6 dpm was added to 2 g diet (Table VII.5), the mixture in the aeration vessel after the removal of the recoverable cyanide (by nitrogen aeration) was centrifuged to precipitate large food particles.

Thin-layer chromatography (T.L.C.) was performed on the diet supernatant fraction. The adsorbent used was silica gel G, type 60 (Merck Ltd.) while the solvent system was ethanol:ammonia:water (80:4:16). This system is known to separate formic acid from other higher fatty acids (Prey et al., 1962). The results of the T.L.C. are shown in Figures VII.3. The radioactivity was distributed over the plate but large amounts co-chromatographed with marker and carrier formic acid. Location of the formic acid on the chromatogram was achieved by spraying with alcoholic methyl red indicator (Lynes, 1964). In this system formic acid had an Rf value of 0.57.

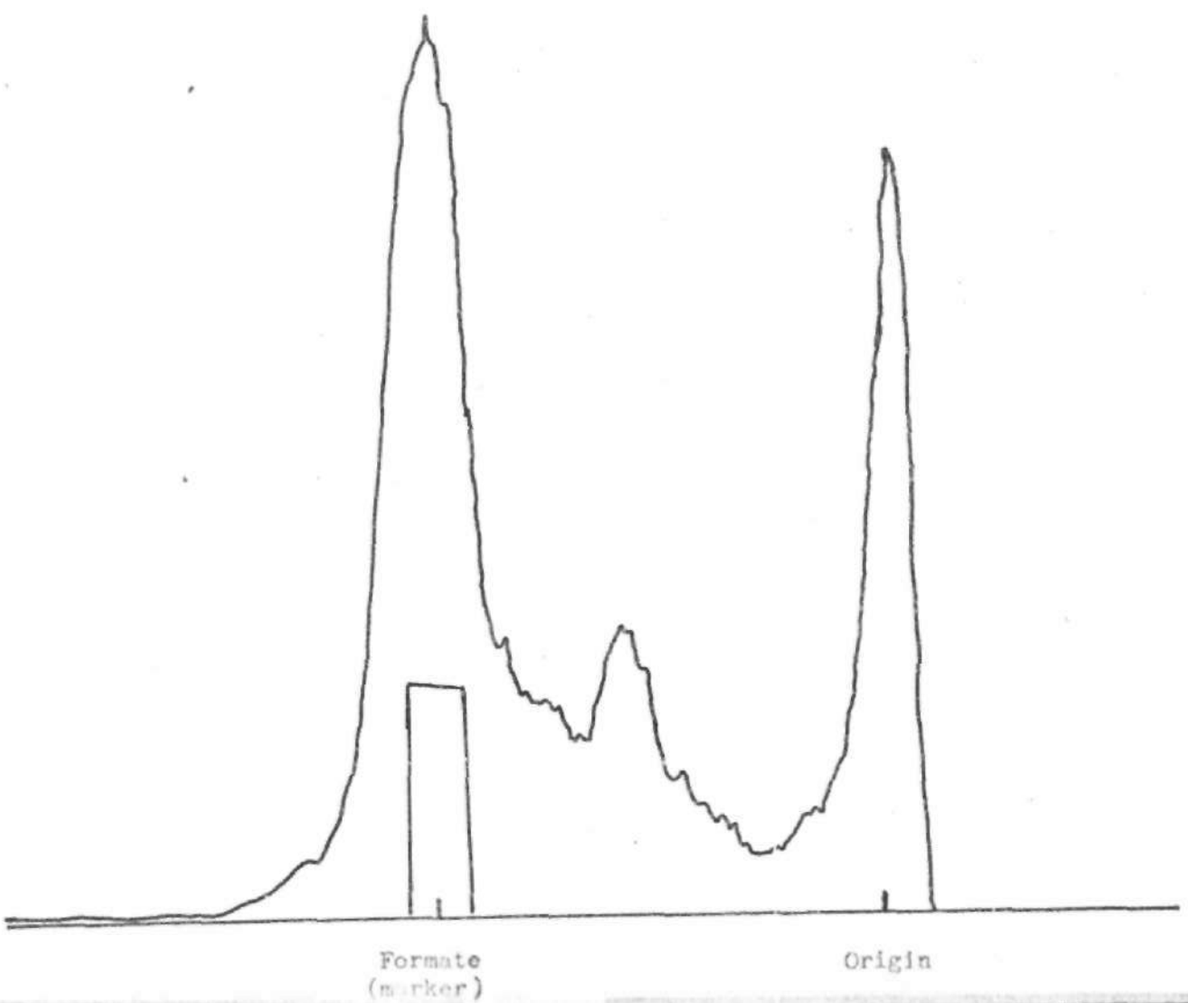
Although further purification and identification of the formate was not carried out, this preliminary study by means of T.L.C. has revealed that formate is most likely the major product formed by the transformation of potassium cyanide in the diet.

Discussion

The experiment reported in this chapter has clearly shown that when small amounts of potassium cyanide were added to rat serum, there was a fairly rapid decrease in the amount that could be

FIGURE VII.3

The scan of the thin-layer chromatography of the radioactivity present in diet supernatant after removal of recoverable cyanide.



subsequently recovered. The most rapid losses occurred during the first 30 minutes, and a further, but slower, decrease continued for a total period of about 1 h. Thereafter, the amounts of recoverable cyanide appeared fairly constant, even for a period of four hours. By one hour after the addition of a known amount of cyanide, only 40-54% could be detected as measurable cyanide. Furthermore only 29-46% of the non-recoverable cyanide could be accounted for as thiocyanate. It was concluded that losses of cyanide as hydrogen cyanide were minimal partly because no decreases in measurable cyanide in phosphate buffer were observed using identical analytical techniques and partly because studies with radioactive cyanide revealed that a large proportion of the non-recoverable cyanide was associated with the TCA precipitate and the supernatant.

It was of interest to compare the results reported here for the rat serum with the observation of Ballantyne et al. (1973) for rabbit serum. These workers found that 31-44% of added cyanide could be detected as measurable cyanide, and that measurable thiocyanate accounted for only 26-33% of non-recoverable cyanide.

It has been suggested by Ballantyne et al. (1973) that decreases in measurable cyanide could be accounted for in part by slow hydrolysis to ammonium formate and conversion to aminomalonitrile.

Thiocyanate was found in both normal and heat-treated serum incubated with cyanide. This means that the formation of this radical from cyanide may not be entirely due to enzymic activity.

Lang (1933a) had earlier reported that colloidal sulphur would serve as a donor for rhodanese (in liver homogenate). This was shown to be incorrect with a highly purified enzyme (Sorbo, 1953). It was at first presumed that a second enzyme called "rhodanese s",

was responsible for the activity with colloidal sulphur (Sorbo, 1953). However, Sorbo later indicated that this was a non-enzymic reaction and that the 'rhodanese s' activity, in serum at least, could be attributed primarily to a catalytic action of serum albumin which did not, however, involve its sulphydryl or disulphide groups (Sorbo, 1955). However, it is not known if free sulphur is present in the serum or blood of animals.

It has been reported (Davis, 1962; Sorbo, 1957) that metal ions catalyse a non-enzymic thiosulphate-cyanide reaction.

Whatever the mechanism for the formation of thiocyanate, it is clear that this radical is formed in the serum and that the denaturation of the serum protein reduced the amount of measurable thiocyanate formed.

As a result of the work of Chung and Wood (1971) it is known that thiocyanate reacts with bovine haemoglobin *in vitro*, in the presence of H_2O_2 to yield sulphate and cyanide. In this system, the cyanide is converted to cyanate by a non-enzymic reaction (see earlier). It has been argued, however, that hydrogen peroxide may not exist *in vivo* in animal blood in concentrations similar to those used to demonstrate the reaction *in vitro* (Smith and Kruszyna, 1976). Using the methods desdribed in Chapter II, the formation of cyanate from cyanide could not be demonstrated in the rat blood in the absence of hydrogen peroxide.

CHAPTER VIII

CHAPTER VIII

The excretion of cyanide and thiocyanate carbon in the rat

1. Introduction

Cyanogenic glycosides have long been known to be present in certain plant foods and to release cyanide into the body when ingested by animals (Chapter I). Furthermore ^{14}C-labelled potassium cyanide is an important precursor used at high specific activity in the synthesis of many labelled organic compounds, and hence has long been available. However, only a limited amount of work has been done on the fate of cyanide in the animal body.

The pioneering work was by Boxer and Rickards (1952) who injected six rats each with five doses of 215 µg of ^{14}CN, specific activity 2.16×10^9 dpm/m mole, at hourly intervals and the animals were killed 2 hr after the last injection. Respiratory air was collected at hourly intervals over the experimental period and cyanide and carbon dioxide were separated for analysis. Their results showed that 1.7% of the injected carbon appeared in the respiratory air, of which 90% was present as carbon dioxide and only 10% as cyanide.

Since the report of Boxer and Rickards (1952) a few investigators have studied the respiratory elimination of cyanide carbon under varying conditions and in various species (Friedberg and Schwarzkopf, 1969; Tolbert and Hughes, 1959; Burrows et al., 1976). However, comparison has been difficult because of the variations in experimental conditions employed. It has been pointed out that one difficulty concerned variations in the cyanide dosage. Burrows et al. (1976) argued that the response of the animal changes markedly as the

dose of cyanide varies, and that tissue respiratory stimulation rather than depression is more likely when cyanide is administered at less than 1 mg per kg body weight. They indicated that a dose level of less than 2.65 mg cyanide/kg of body weight (100 µmoles/kg) would yield very different results than those where higher doses of cyanide are employed, since oxygen utilization and body metabolism are markedly different. Such differences, it is believed, would alter various physiological processes, such as the rate of biotransformation and the rate of urinary excretion.

Oxygen has been shown to potentiate markedly the effectiveness of the classical sodium nitrite-sodium thiosulphate combination in the treatment of acute cyanide poisoning (Way et al., 1966). Burrows et al. (1976) have investigated the effect of air or oxygen on the respiratory excretion of cyanide carbon. Air or oxygen used either alone or with various combinations of $NaNO_2$ (100 mg/kg, injected subcutaneously) and $Na_2S_2O_3$ (1 g/kg, injected intraperitoneally) were administered to mice prior to the administration of $Na^{14}CN$ (injected subcutaneously). The respiratory gases were collected in a trapping solution of ethanol-ethanolamine and analysed for radioactivity. Treatment with $Na_2S_2O_3$ prior to injection of $Na^{14}CN$ accelerated the rate of excretion of ^{14}C for either air or oxygen, but not when combined with $NaNO_2$. However, the total respiratory ^{14}C excretion, in the 3½ hrs of the experiment, was not increased with $Na_2S_2O_3$ for either air or oxygen groups. Based on the above finding, it was suggested that if the rate of respiratory excretion of radioactivity is dependent upon blood levels of free cyanide; then those animals which received $Na_2S_2O_3$ had a more rapid rate of respiratory excretion than those receiving only air or oxygen,

because the product of cyanide detoxification, namely thiocyanate, is extensively bound with plasma proteins and haemoglobin (Ponde and McMenamy, 1970; Pollay et al., 1966). Cyanide and thiosulphate are also bound, but to a lesser extent (Uchida et al., 1970). Thus thiosulphate administration by virtue of enhanced thiocyanate production may compete for cyanide-binding sites causing an increase in cyanide availability for respiratory excretion. However, since in this report, the respiratory excretion was not resolved into its various radioactive components - namely cyanide and carbon dioxide - it is not certain whether the rapid rate of respiratory excretion observed was due to cyanide itself or to carbon dioxide.

Smith and Foulkes (1966) have shown that urinary excretion of thiocyanate is decreased in the rat, following chronic administration of cyanide over a prolonged period of time. They administered, by subcutaneous injection, 1 mg potassium cyanide once weekly for 20 weeks to adult albino Wistar rats. A basal urine was collected prior to the initiation of the experiment and thereafter urine samples were collected at monthly intervals for the 24 hrs immediately following the injections. The estimation of urinary thiocyanate was carried out by the method of Aldridge (1945). The result of this experiment showed that seven of the eight rats subjected to the procedure gradually adapted their metabolic pathways to the injected cyanide so that thiocyanate excretion in the urine decreased as compared with the thiocyanate found after the initial injection. It was shown that delayed excretion was not responsible for the decrease in thiocyanate excretion. It was thought that the

detoxification pathway which involves formation of 2-iminothiazolidine-4-carboxylic acid from cystine and cyanide (Wood and Cooley, 1956) might have superseded the rhodanese/thiocyanate mechanism. However, 2-iminothiazolidine-4-carboxylic acid could not be identified in the urine of a rat which was excreting less cyanide as thiocyanate. Smith and Foulkers (1966) thus concluded that one-carbon compounds other than thiocyanate must have been playing the major important parts in cyanide metabolism.

In an attempt to confirm this observation of altered cyanide metabolism made by Smith and Foulkes (1966), Mehta and McGinity (1977) have examined the urinary elimination of thiocyanate in male and female rats following chronic administration of potassium cyanide. Twenty four female rats were divided into three groups of eight. Group one received subcutaneous injections of 5 mg KCN/Kg body weight once a week; group two received 5 mg KCN/kg body weight, twice weekly, and group three received subcutaneous injection of an equivalent volume of saline. In the same experiment, sixteen male rats were divided into two groups of eight. One group received by subcutaneous injection 5 mg KCN/kg body weight twice weekly while the second group received an equivalent volume of saline by subcutaneous injection. For a period of 24 hrs immediately following the last KCN injection or saline injection (each week), the urine sample from each rat was collected in dry ice-cooled containers. Analysis of thiocyanate in urine was also carried out by the method of Aldridge (1945). Unlike the finding of Smith and Foulkers (1966), female rats dosed at the level of 5 mg KCN/kg, either once a week or twice a week, displayed no significant difference in the excretion of thiocyanate in urine after periods of up to eight weeks of study.

Similarly male rats that were given 5 mg KCN/kg twice weekly showed no significant difference in the amount of thiocyanate excreted. Furthermore, the elimination patterns of thiocyanate in all rats showed no significant differences. The results suggest that a substrate saturation phenomenon is not operative with cyanide metabolism at the dose level of potassium cyanide employed in this study. This is therefore contrary to the observation made by Smith and Foulkers (1966) who reported a decrease in urinary excretion of thiocyanate after chronic cyanide administration to rats.

Aims of the investigation

To date, only one detailed report on the respiratory and urinary excretion of cyanide carbon in the rat has been given (Boxer and Rickards, 1952). Recently, however, Crawley and Goddard (1977), have studied the elimination of cyanide carbon in breath and urine. Unlike the report by the first group of workers, these latter workers did not investigate the proportion of the major metabolites of cyanide (such as carbon dioxide in respiratory air or thiocyanate in urine) in the total excretion. Accordingly, comparison is difficult. It was therefore of interest to study the urinary and respiratory elimination of cyanide and its main metabolites in the rat, following administration of ^{14}C-labelled cyanide, and to compare the findings with those reported by Boxer and Rickards (1952).

Furthermore, while one group of workers (Smith and Foulkers, 1966) have reported that chronic ingestion of cyanide results in decreased urinary excretion of thiocyanate, another group (Mehta and McGinity, 1977) have failed to confirm this finding by the same chemical technique. By using rats exposed to chronic intake of

cyanide for three weeks prior to injection of ^{14}C-labelled cyanide, in an excretion study, it was hoped that any effect chronic intake of cyanide had on mode of elimination of cyanide carbon would readily be observed by the more sensitive radiochemical technique.

Experimental procedure

The metabolism apparatus used for the collection of urine, and for carbon dioxide and cyanide in respiratory air is illustrated in Fig.VIII.1. Rubber tubing was used to connect the members of the assembly.

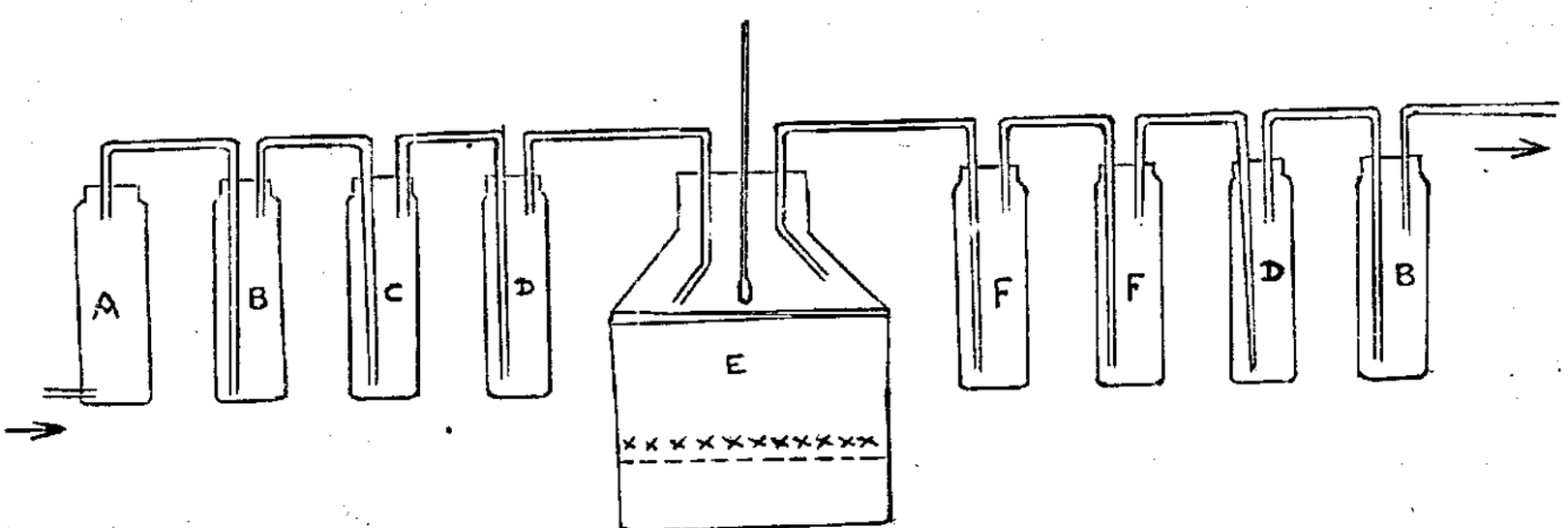
Following injection or oral administration of purified radioactive cyanide (Chapter II) each rat was placed immediately in the desiccator (metabolism jar, E). The direction of airflow when the pump was opened was from A, through E, to B. The desiccator was equipped with a coarse screen support for the rat, and a fine screen for collection of faeces. The urine was collected at the bottom of the desiccator.

The apparatus was checked for leaks by running it for several hours without a rat and with water replacing the alkali in the NaOH absorbers (F). If leaks had been present, the terminal $Ba(OH)_2$ solution would have become cloudy. During the experiment, complete recovery of expired CO_2 was also indicated by the absence of cloudiness in the terminal $Ba(OH)_2$ solution.

The internal temperature of the metabolism chamber, with a rat present, was generally 24° with fluctuations not greater than 1°. Outward leaks from the apparatus were prevented by the slight negative pressure in the metabolism chamber and the more negative pressure in the NaOH absorbers.

FIGURE VIII.1

Metabolism apparatus.



A = NaOH pellets to absorb CO_2

B = $Ba(OH)_2$ Soln. to check efficiency of CO_2 trap (A)

C = Dilute NaCl soln. for humidity regulation

D = Empty trap

E = Metabolism jar (dessicator)

F = 100 ml 2.5M NaOH to absorb expired CO_2 and cyanide.

At the end of each experiment, the contents of the sodium hydroxide absorbers (F) were stored at -20°C, till required for analysis. Faeces collected was homogenized in water (1:3) and stored at -20°. The urine in the bottom of the desiccator was rinsed out with water and the total urine and washings diluted to a known volume and stored at -20°.

From an aliquot of the alkali removed from the NaOH absorbers or an aliquot of the urine sample, quantitative separation and collection of carbon dioxide, cyanide and thiocyanate were carried out by the method described in Chapter IX.

In experiments where only urine and faeces samples were collected over a period of one day or more, metabolic cages equipped for the collection of urine and faeces (but not respiratory air) were used. The method used to collect urine and faeces is specified in the presentation of each result.

The estimation of radioactivity was carried out by the method described in Chapter II.

4. Results

4(a) Urinary excretion of thiocyanate by rats exposed to chronic ingestion of cyanide in the diet.

Four male rats were weaned onto diet 41B and maintained on this diet until they weighed about 140 g each. They were then put separately in metabolic cages equipped for the collection of urine and faeces only (not including respiratory air). The 24 hr urine of each rat was collected (after adapting the rats to the cages for three days) while they were still maintained on diet 41B. The diet 41B was then replaced by the basal soya bean meal diet (see Chapter

II) which was used throughout the rest of this experiment. Another 24 hr urine of each rat was collected on the 7th day following the transfer of the rats to soya bean diet.

Potassium cyanide was then added to the basal soya bean meal diet of each rat to provide 77 µmoles per rat per day. The addition of potassium cyanide to the diet began on the 8th day following starting on the basal soya bean meal diet. This 8th day will now be referred to as day 1, and subsequent days designated 2, 3, 4 etc. Urine from each rat was collected separately for the following days: day 7, 15, 16, 17, 18, 19, 20 and 21.

All urine samples were stored at 4^{o} in screw-capped bottles and the cyanide and thiocyanate content estimated within one week of collection.

Each 24 hr urine was diluted 10-fold (in the case of samples collected before administration of cyanide in the diet) and 100-fold (in the case of samples collected during cyanide administration). Thiocyanate cyanide was obtained by mild oxidation with permanganate at room temperature, followed by nitrogen aeration and estimation by the method already described in Chapter II for rat serum.

The amounts of thiocyanate excreted in the urine in 24 hr by four male rats on the two separate diets (diet 41B and basal soya bean meal) are given in Table VIII.1. The mean thiocyanate content of the 24 hr urine sample collected while the rats were on the basal soya bean meal diet was slightly lower than that obtained for the same group of rats while on diet 41B. However, the differences are not statistically significant ($P > 0.4$).

When potassium cyanide was added to the diet of each rat to provide 77 µmoles per rat per day, the mean daily excretion of

TABLE VIII.1

Urinary excretion of thiocyanate by four male rats on two separate diets without added KCN supplement.

Rat No.	Diet	Period of urine collection	Thiocyanate content of 24 h urine (µmoles)	Mean ± S.E.M.
U-1			1.94	
U-2	Diet 41B	Last day on diet	1.35	1.69±0.19
U-3			1.40	
U-4			2.10	
U-1			1.67	
U-2	Diet based on extracted soya bean	7th day on diet	1.56	1.53±0.06
U-3			1.37	
U-4			1.54	

thiocyanate for the four rats increased 18-30 fold (Table VIII.2). The daily thiocyanate excretion in the urine of each rat was variable with a range of 21-61 µmoles per rat per 24 hr. However, the pattern of daily excretion of thiocyanate in the urine of each rat does not appear to indicate that any increase or decrease occurred with prolonged cyanide ingestion.

On the assumption that all the cyanide added to the diet of each rat was quantitatively taken and absorbed then 36-60% (mean 50%) of the daily dose of cyanide was excreted as thiocyanate per rat per day. Thus of a total of 42 mg cyanide ion ingested over a period of three weeks only 21 mg was excreted as thiocyanate in the urine.

4(b) Excretion of radioactivity by rats after oral administration of $KS^{14}CN$

To check on the fate of thiocyanate, the four rats on a daily dose of cyanide in the diet (see earlier) were given radioactive potassium thiocyanate by mouth. The thiocyanate (3.4 µCi, specific activity 59 µCi/µmole) was administered in 0.1 ml solution of a mixture of water and glycerol (ratio 1:1) by means of a 1 ml syringe. The method of administration of supplements by mouth has been described in Chapter II. After administration of the thiocyanate, each rat was replaced in the metabolic cage, equipped for the collection of urine and faeces only. The daily urine and faeces samples were collected separately for a period of one week.

The separation of thiocyanate in the urine samples was achieved by the method described in Chapter IX, and radioactivity in all samples estimated as already described in Chapter II.

Large amounts of radioactivity were excreted in the urine by each rat in the first two days, with the largest amounts in the first

TABLE VIII.2

Urinary excretion of thiocyanate by four male rats exposed to chronic ingestion of KCN (77 μmoles/rat/day) in diet.

Rat No.	Thiocyanate content (μmoles) of 24 h urine collected on the specified days following daily addition of KCN to diet.							
	day 7	day 15	day 16	day 17	day 18	day 19	day 20	day 21
U-1	36.06	44.71	61.06	26.44	37.50	45.67	37.98	50.00
U-2	33.08	32.21	46.64	35.58	46.15	43.27	25.10	32.21
U-3	24.04	43.27	32.21	28.36	32.21	47.11	32.21	37.50
U-4	30.03	47.60	36.06	20.67	32.69	47.60	32.69	37.50
Mean	30.80	41.95	43.99	27.76	37.14	45.91	34.50	39.30
S.E.M.	2.57	3.37	6.46	3.07	3.23	0.97	1.32	3.78

24 hr samples in most cases (Table VIII.3). This was followed by a sharp decline in the radioactivity excreted on the 3rd day. A mean of 53% of the total dose given by mouth was excreted in the urine on the first day and 25% on the second day. On the third day less than 7% was excreted, and this ddclined to less than 0.1% by the 7th day. The total urinary radioactivity excreted in seven days accounted for about 90% of the dose administered.

The amounts of thiocyanate in the daily urine samples are given in Table VIII.4. For each of the four rats, thiocyanate was found to account for a large proportion of the total urinary excretion. The mean thiocyanate excreted in the urine by the four rats over five day period amounted to 82% of total activity given by mouth. Thiocyanate thus accounted for about 91% of total urinary activity. This means that thiocyanate given by mouth is largely absorbed into the body fluid and excreted, to a large measure unchanged, in the urine.

The faecal excretion of radioactivity was very small compared to that present in the urine (Table VIII.5). For this reason, the thiocyanate fraction of the total activity in the faeces was not determined. In two rats where very little faeces were excreted during the first 24 hr, only a trace of radioactivity was present in the first day sample. The mean faecal excretion of radioactivity accounted for only 2.4% of the dose administered, and part of this must be due to contamination by the urine, which was impossible to prevent in the metabolic cages used.

TABLE VIII.3

Urinary excretion of radioactivity by four rats after oral administration of $KS^{14}CN$. Each rat was given 3.4 µCi of $KS^{14}CN$; specific activity 59 µCi/µmole.

Rat No.	Radioactivity excreted per day after administration (µ Ci)							
	day 1	day 2	day 3	day 4	day 5	day 6	day 7	Total
U-1	1.060	1.179	0.160	0.086	0.029	0.024	0.010	2.55
U-2	2.330	0.437	0.170	0.067	0.023	0.006	0.001	3.04
U-3	2.030	0.788	0.329	0.120	0.067	0.001	0.001	3.34
U-4	1.726	1.020	0.258	0.189	0.059	0.002	0.001	3.26
Mean	1.787	0.856	0.229	0.116	0.045	0.008	0.003	3.05
S.E.M.	0.270	0.160	0.040	0.026	0.011	0.005	0.002	

TABLE VIII.4

Radioactivity in the thiocyanate fraction of urine after oral administration of $K^{35}CN$ to four rats. Each rat was given 3.4 μCi of $K^{14}CN$; specific activity 59 μCi/μmole.

Rat No.	Radioactivity in thiocyanate fraction per day (μCi)					
	day 1	day 2	day 3	day 4	day 5	Total
U-1	1.039	1.140	0.146	0.079	0.028	2.432
U-2	2.149	0.392	0.015	0.065	0.022	2.643
U-3	1.930	0.680	0.290	0.110	0.066	3.076
U-4	1.550	0.910	0.256	0.175	0.057	2.948
Mean	1.668	0.781	0.177	0.107	0.043	2.780
S.E.M.	0.240	0.160	0.060	0.024	0.011	

TABLE VIII.5

Faecal excretion of radioactivity by four rats after oral administration of $KS^{14}CN$. Each rat was given 3.4 µCi of $KS^{14}CN$; specific activity, 59 µCi/µmole.

Rat No.	Radioactivity excreted per day after administration (µCi)					
	day 1	day 2	day 3	day 4	day 5	Total
U-1	Trace	0.026	0.013	0.010	0.002	0.051
U-2	0.014	0.020	0.011	0.009	0.006	0.060
U-3	Trace	0.040	0.018	0.012	0.004	0.027
U-4	0.040	0.027	0.014	0.011	0.001	0.093
Mean	0.014	0.028	0.014	0.011	0.003	0.083
S.E.M.	0.009	0.004	0.001	0.001	0.001	

4(c) Excretion of radioactivity in the cyanide fractions of the the urine and respiratory air

The excretion of radioactivity in the urine, faeces, and respiratory air was studied in the male rats used for tissue distribution studies (Chapter IX). Each rat was given 16.3 µmoles $Na^{14}CN$ (specific activity 1.15 µCi/µmole) by one of the following routes: two doses of 8.3 µmoles CN^- given with a 30 min interval by (1) subcutaneous injection or (2) intraperitoneal injection. The quantitative collection of urine, respiratory activity and faeces was achieved by means of the metabolic apparatus described in Fig. VIII.1.

Respiratory activity was collected for a period of 9 hr, and cyanide and CO_2 were separated for analysis by nitrogen aeration (Chapter IX). The total activities excreted in respiratory air are summarized in Table VIII.6. About 4.3% of the injected carbon appeared in the respiratory air of which 86% was present as CO_2 and only 14% as cyanide.

Urine was voided by each rat during injection and at the time of killing and this was collected in a stainless steel tray. This urine was combined with that excreted in the metabolism apparatus. Urinary excretion of radioactivity by the rats over a period of 9 hr was found to be relatively constant with a mean of 24.0% $\pm$ 3.3 ($\pm$ S.D.). The quantitative distribution of the injected cyanide carbon in the various fractions of the urine is illustrated in Table VIII.7. The urinary cyanide includes the B12 cyanide. Neither cyanide (including B12 cyanide) nor carbonate plays a major role as an excretory pathway of injected cyanide carbon. The largest amounts of urinary activity were found in the thiocyanate fraction of the urine from each rat. Only small amounts of radioactivity were found in the faeces excreted during the 9 hr period (Table VIII.7).

TABLE VIII.6

Respiratory excretion of radioactivity by five rats after injections (intraperitoneally in four rats and subcutaneously in one rat) of $Na^{14}CN$. Each rat injected with two doses of 8.3 μmoles CN^- with a 30 min interval; specific activity of injected material 1.15 μCi/μmole; animals killed 8½ hr after last injection.

Rat No.	Radioactivity as % of dose injected		
	Total respiratory excretion	Respiratory carbon dioxide	Respiratory cyanide (by difference)
RU1	3.12	2.80	0.32
RU2	5.34	4.39	0.95
RU3	3.50	2.98	0.52
RU4	4.04	3.58	0.46
RU5	5.62	4.65	0.97
Mean	4.32	3.68	0.64
S.D.		0.64	0.30

TABLE VIII.7

Urinary and faecal excretion of radioactivity by five rats after injections (intraperitoneally in four rats and subcutaneously in one rat) of $Na^{14}CN$. Each rat injected with two doses of 8.3 μmoles of CN^- with a 30 min interval; specific activity of injected material 1.15 μCi/μmole; animals killed 8½ h after last injection.

Rat No.	Total urinary excretion % of dose	Urinary thiocyanate as % of total urinary excretion	Urinary cyanide as % of total urinary excretion	Urinary carbon dioxide (carbonate) as % of total urinary excretion	Total faecal excretion
RU1	25.02	77.40	1.50	6.40	0.01
RU2	22.24	71.40	1.30	5.20	2.26
RU3	27.68	70.60	1.30	4.30	0.02
RU4	25.70	66.50	0.78	5.30	0.06
RU5	19.20	68.40	0.75	5.50	0.06
Mean	23.97	70.86	1.13	5.34	0.48
SD	3.30	4.13	0.34	0.75	1.12

4(d) Rate of excretion of cyanide carbon in urine and respiratory air

The rate of excretion of radioactivity in the urine and respiratory air was studied using the groups of rats (groups S_1, S_2, S_3, S_4) also employed in the studies on rate of secretion of thiocyanate into the stomach (Chapter IX). Collection of the urine and respiratory radioactivity was achieved by means of the metabolism apparatus described in this chapter.

The elimination of the ^{14}C activity in urine and respiratory air up to one hour after subcutaneous injection of $Na^{14}CN$ is summarized in Table VIII.8. At the end of a study period, any urine in the bladder was forced out of each rat into a stainless steel tray by gripping the rat firmly at the back of the neck with the thumb and forefingers and then stretching the animal by a slight pull at the tail. A rat held in this way emptied all the contents of the bladder.

No radioactivity was detected in the urine of rats 10 minutes after injection of the $Na^{14}CN$. However, urine collected by 20 minutes contained detectable activity in all rats. In contrast, excretion of radioactivity by the respiratory air was detected 10 minutes after injection of the dose (Table VIII.8). The urinary excretion of radioactivity was more variable than respiratory excretion. When urinary cyanide and thiocyanate were separated from the total urinary excretions, it was observed that the latter radical was the major excretory component at each period (20, 30 and 60 minutes).

5. Discussion

In Chapter III a method was presented for the addition of potassium cyanide to the diet of rats in which there was no significant loss of this volatile substance. It was also demonstrated (Chapter III) that rats on a diet containing potassium cyanide (to

TABLE VIII.8

Rate of appearance of radioactivity in the respiratory air, urinary cyanide and thiocyanate after subcutaneous injection of $Na^{14}CN$ to the rat. Each rat was injected with 4 μmoles of cyanide; specific activity of injected material 1.15 μCi/μmole.

Time after injection (min.)	Radioactivity as % of injected dose ± S.E.M., n = 3			
	Total respiratory air	Total urinary excretion	Urinary thiocyanate	Urinary cyanide
10.0	0.19±0.03	0.00	0.00	0.00
20.0	0.33±0.04	0.49±0.21	0.33±0.16	0.06±0.02
30.0	1.21±0.05	0.71±0.02	0.38±0.04	0.09±0.02
60.0	2.55±0.29	3.46±2.01	2.57±1.67	0.27±0.02

provide 38.5 µmol/rat/day) for three weeks had thiocyanate in plasma significantly elevated compared to control rats.

In this chapter, it has been demonstrated that rats on a diet containing potassium cyanide to provide 77 µmoles/rat/day excreted large amounts of thiocyanate in the urine. It is well known in animals, that elevated plasma thiocyanate or increased urinary excretion of thiocyanate or both are indications of the exposure to chronic cyanide intake (Pettigrew and Fell, 1972; Tewe et al., 1977). All rats used in the excretion studies described in this chapter, had been placed on a diet containing potassium cyanide (to provide 77 µmol/rat/day) for at least three weeks. The results presented, therefore represent the pattern of excretion of cyanide carbon under this condition of chronic cyanide intake.

Smith and Foulkes (1966) claimed that urinary excretion of thiocyanate is decreased in the rat, following chronic administration of cyanide over a prolonged period of weeks. The data presented in this chapter (Table VIII.2) do not indicate any decrease in thiocyanate excretion as a result of daily intake of cyanide in the diet for three weeks. This is in agreement with the recent report by Mehta and McGinity (1977) who demonstrated that groups of rats given 5 mg KCN/kg body weight either once or twice weekly for 8 weeks did not show any decrease in the urinary excretion of thiocyanate. It is therefore unlikely that a substrate saturation phenomenon is operative in the rat after chronic cyanide administration as reported by Smith and Foulkers (1966).

The amounts of ^{14}C in urine, faeces or breath after administration of $Na^{14}CN$ or $KS^{14}CN$ to rats after chronic ingestion of potassium cyanide for three weeks have been presented. The available data on the elimination of cyanide carbon confirm that

urinary excretion is the major route of elimination, e.g. 80% in 48 hr in the rabbit (Baumann et al., 1933); 92% in the dog (Boxer and Rickards, 1952); 74% in the rat (Crawley and Goddard, 1977). The data reported here confirm that urinary excretion is the main route of elimination of cyanide carbon, accounting for 83% of cyanide carbon excreted (in urine, faeces and breath) 9 hr after administration. When $KS^{14}CN$ was given by mouth to rats exposed to chronic cyanide ingestion, 90% of the dose administered was excreted in urine in 7 days and only 2.4% in the faeces. Of the total activity excreted in urine, 78% was excreted in the first 2 days. It has been reported by Funderburk and Middleswort (1971) that rats given carrier doses of thiocyanate (5 mg) along with a tracer dose of $^{35}SCN^-$ showed a faster rate of disappearance of SCN^- from plasma than those given only the tracer amount of SCN^-. This was interpreted to be due to (1) the saturation of SCN^- concentrating compartments and (2) the inability of the kidney tubule to reabsorb large amounts of filtered thiocyanate. It is important to recall that rats used in this investigation had been exposed to chronic cyanide intake in the diet and hence had a large pool of thiocyanate in the body. This probably explains why the tracer dose of thiocyanate (less than 0.06 μmol) given here was excreted at a very rapid rate.

Thiocyanate in the urine accounted for 91% of total urinary activity. Since the tracer dose of thiocyanate was given by mouth, this means that this radical was largely absorbed into the body to be excreted (unchanged) largely in the urine.

The respiratory elimination of cyanide carbon has been examined by a number of investigators under carying conditions and

in various species (Boxer and Rickards, 1952; Friedberg and Schwarzkopf, 1969; Tolbert and Hughes, 1959 and Crawley and Goddard, 1977). However, comparisons are difficult because of the variations in experimental conditions employed. One major study on elimination of cyanide carbon in breath after subcutaneous injection of ^{14}C-labelled sodium cyanide in rats showed that the amount exhaled was 1.7% of initial activity of which 90% was ^{14}C-labelled CO_2 and 10% unchanged cyanide (Boxer and Rickards, 1952). In the experiments reported in this chapter, 4.3% of the injected cyanide carbon appeared in the respiratory air (9 hr after the dose) of which 86% was ^{14}C-labelled carbon dioxide and only 14% was unchanged cyanide. Although a dose comparison cannot be made because of differences in experimental conditions (e.g. dose of cyanide; duration of experiment etc.), it is clear that carbon dioxide is the major product in the respiratory air after administration of cyanide to the rat.

CHAPTER IX

CHAPTER IX

The tissue distribution of cyanide carbon in the rat

1. Introduction

A number of investigators have studied the distribution of cyanide ion in human tissues following intake of acute doses of cyanide (Halstrøm and Møller, 1945; Curry, 1963; Sunshinie and Finkle, 1964; Bonnichsen and Maehly, 1966).

Among the earliest work on the content of cyanide in tissues and organs of humans poisoned with cyanide is the report by Halstrøm amd Møller (1945). Tissues from individuals killed by acute cyanide poisoning were removed as quickly as possible and stored at 0°. Cyanide in the macerated tissues was liberated into 2M NaOH by steam distillation after acidification with sulphuric acid. The resulting cyanide was estimated chemically by a method involving oxidation with bromine to bromine cyanide, reacting the bromine cyanide with potassium iodide and titrating the resulting iodine set free with standard thiosulphate solution. In contrast to some reports that it is not always possible to demonstrate hydrocyanic acid in the organs of cyanide-poisoned animals (see Halstrøm and Møller, 1945), these workers found cyanide in several organs and tissues investigated. The liver, kidneys and brain contained approximately the same concentrations in the majority of cases ranging from about 0.2 to 2 mg, calculated as HCN per 100 g tissue. The concentration in the brain seemed to vary a great deal more than that in the liver and the kidneys. The hydrocyanic acid in the muscles was constantly lower than that of the liver and kidneys. In contrast

to other poisons, it was found that in cyanide poisoning, the blood contains essentially more of the poison than the other tissues.

Subsequently, a number of other analytical methods have been employed to investigate the content of cyanide ion in tissues and organs of humans poisoned with cyanide (Curry, 1963; Sunshinie and Finkle, 1964; Bonnichsen and Moehly, 1966; Curry *et al.*, 1967). Their results also confirmed that cyanide could be demonstrated in several tissues and organs following acute cyanide poisoning.

More recently, Ballantyne *et al.* (1972) have studied the content of cyanide in the tissues of rabbits killed with intramuscular injections of potassium cyanide or hydrogen cyanide. The greatest concentrations of cyanide were found in liver and brain. There were significant differences in the concentrations of cyanide in blood from animals killed with HCN and those killed with KCN. However, there were generally no such differences in concentration in various tissues removed from HCN-killed animals compared with similar tissues from KCN-killed animals. The concentration of cyanide in saline-perfused muscle, kidney and liver was significantly lower than the concentrations of the radical in similar, but blood-containing tissues, indicating that a significant proportion of the cyanide content of skeletal muscle, kidney and liver removed from animals poisoned with cyanide is probably in the blood, rather than in the parenchyma cells of these tissues. However, the fact that perfusion with saline did not cause a drop in the concentration of cyanide in central nervous tissue suggests that a selective uptake of the radical may occur in this tissue. Studies on distribution of cyanide radical in tissues have now been carried out in sheep killed by intramuscular injection of potassium cyanide (Ballantyne, 1975).

The distribution of cyanide in the tissues of both the rabbit and the sheep were found to be in agreement.

It must be emphasised that the results obtained in such experiments are complicated by the problem of postmortem production of cyanide occurring between death and the analysis of tissues (Ballantyne, 1974). Postmortem production of cyanide may occur in tissues such as blood, liver, kidney, uterus, and stomach and intestinal contents (Curry, 1963, 1974; Curry et al., 1967). The production of cyanide in blood has been observed to occur in samples stored at -20° (Ballantyne, 1977).

It is well established that chronic intake of cyanide or diets containing cyanogenic glycosides results in damage to the central nervous system (Rose et al., 1954; Williams and Osuntokun, 1969; Osuntokun and Osuntokun, 1971). Furthermore chronic intake of cyanide in diets and tobacco smoke has been implicated as the cause of a number of clinical states observed in humans (Baumeister et al., 1975). However, it is not clear if the observed clinical states in chronic cyanide intoxication are due to cyanide per se or to products of cyanide metabolism.

Cyanide is rapidly metabolised in animals to thiocyanate which is excreted largely in the urine (Boxer and Rickards, 1952). Boxer and Rickards (1952) have demonstrated, however, the presence of a small metabolically active pool of cyanide in the body by means of radioactive tracers. Furthermore they have shown that cyanide carbon is oxidized to carbon dioxide and formate, and therefore enters into the metabolism of one carbon compounds.

Rose et al. (1954) have carried out experiments designed to establish whether cyanide per se or its major detoxification product,

the thiocyanate ion was responsible for any deleterious effect on the central nervous system in chronic cyanide intoxication. They administered KCN (3 mg per kg) daily to one group of rats by subcutaneous injections. The dose was gradually increased until all rats but one died after 118 days. The final dose level was 14 mg/kg. A second series comprised rats given daily intraperitoneal injections of KSCN, starting with 50 mg/kg. As in the first series, the dose was gradually increased to 850 mg/kg on the 103rd day. In the former group on cyanide doses, 6 out of 93 rats showed brain damage at death, while in the latter group on thiocyanate doses, three out of 39 rats showed brain damage. It is not possible to draw a reliable conclusion from the above results in view of the known interconversion of cyanide and thiocyanate in animals (Boxer and Rickards, 1952; Pines and Crymble, 1952).

2. Aim of Work

The studies on the distribution of cyanide in the tissues of animals have been concerned with the distribution of cyanide radical per se (Halstrøm and Møller, 1945; Curry, 1963; Sunshinie and Finkle, 1964; Ballantyne et al., 1972; Ballantyne, 1975), rather than with the distribution of cyanide carbon and hence its metabolites. Furthermore, these studies have only involved use of animals killed by acute cyanide doses. Such studies may thus be of interest only to the Forensic Toxicologists since the large single dose of cyanide administered in these experiments is not in any sense 'physiological'. In normal animals exposed to chronic cyanide ingestion, it is unlikely that the cyanide radical could be demonstrated in most of the tissues by known chemical methods. This is because cyanide is rapidly

metabolised in animals, largely to thiocyanate, and as Boxer and Rickards (1952) pointed out, the ratio of cyanide to thiocyanate in the body fluids is about 1:1000. However, by means of radioactive tracers, it has been possible to demonstrate the presence of a small metabolically active pool of cyanide in the animal body (Boxer and Rickards, 1952).

It was therefore thought that a study of the distribution of cyanide carbon (using ^{14}C-cyanide) in rats previously exposed to chronic intake of cyanide in the diet might prove interesting.

3. Experimental

The rats used for the experiments on distribution of cyanide carbon were male albinos. They were transferred from diet 41B to the basal soya bean meal diet. Before each rat was used for distribution studies, KCN was added to the diet for a period of three weeks, to provide an intake of 38.5 µmoles per rat per day.

3(a) Administration of $Na^{14}CN$

$Na^{14}CN$ was administered to the rats about one week after the KCN supplement was discontinued. Three independent routes were used for the administration of $Na^{14}CN$ to the rats, and the method utilized in each case is described with the presentation of each result. Each rat was placed in a metabolism apparatus (see Chapter VIII) equipped for the collection of respiratory air, urine and faeces after administration of the radioactive cyanide.

3(b) Removal of tissues

Each rat was anaesthetised with Halothane and blood removed by heart puncture. Between 10 and 15 ml of blood was removed from each rat by the above technique. Tissues to be analysed were carefully

dissected out, as quickly as possible, rinsed thoroughly with water, blotted dry and weighed. Blood was stored with all other tissues in screw capped bottles at -20° until required for analysis. Solubilization and counting of radioactivity in each tissue was carried out as described in Chapter II.

4. Results

4(a) Tissue distribution of cyanide carbon after intraperitoneal injection of $Na^{14}CN$

Four male rats, mean weight 365 g, were each given two doses of 8.3 µmoles $Na^{14}CN$ (by intraperitoneal injections) with a 30 minutes interval. The specific activity of injected material was 1.15 µCi/µmole. Each rat was killed 8½ hr after the last injection. Blood and tissues were removed from each rat as already described.

The ^{14}C levels in tissues examined are given in Table IX.1. The total radioactivity in muscle and blood were calculated on the basis that the former accounted for 40% of total body weight while the latter accounted for 64.1 ml per one kilogram body weight (Altman and Dittmer, 1964).

All tissues examined contained radioactivity, but very high amounts were found in the stomach plus contents which accounted for about 18% of the total injected radioactivity. This uptake of radioactivity by the stomach was variable with a range of between 14 and 22% of the total injected radioactivity.

A very high proportion of the injected radioactivity is present in the blood, accounting for 11% of the total dose. A similar amount of the injected radioactivity was also found in the muscle because of its large mass, and substantial radioactivity was found in livers.

TABLE IX.1

Amounts of ^{14}C in tissues after intraperitoneal injection of $Na^{14}CN$ to rats. Four rats, mean weight 365 g, each injected with two doses of 8.3 µmoles of CN^- with a 30 min interval; specific activity of injected material 1.15 µCi/µmole; animals killed 8½h after last injection.

Tissue	Wet Weight[a] (g)	% injected activity per gram	% injected[a] activity per tissue
Blood[b]	23.26±0.76(ml)	0.48	11.19±2.72
Heart	1.21±0.04	0.12	0.14±0.01
Spleen	0.42±0.03	0.24	0.10±0.01
Kidney	2.23±0.21	0.33	0.74±0.06
Lung	1.71±0.11	0.28	0.48±0.05
Liver	13.70±1.29	0.23	3.19±0.86
Muscle[b]	146.00±6.08	0.08	11.32±0.95
Brain	2.57±0.35	0.04	0.09±0.02
Large intestine plus contents	6.32±0.78	0.16	1.01±0.18
Small intestine plus contents	8.39±0.57	0.23	1.93±0.41
Stomach plus contents	2.35±0.64	8.36	18.33±3.63
Adipose tissue	-	0.02	-
Testis	3.32±0.13	0.22	0.72±0.06
Eye	0.30±0.03	0.10	0.03±0.01
Total activity in tissues			50.03
Total excretion			29.75
Total			79.78

[a] mean value with the standard deviation

[b] Total radioactivity was calculated from body weight of rats (Altman and Dittmer, 1964).

"-" means not determined

Expressed on a tissue weight basis, there was an extremely high concentration of radioactivity in the stomach and contents. Blood had a higher concentration of radioactivity than in most tissues. Although blood was removed from each animal by heart puncture, and tissues were washed thoroughly when removed, a small part of the radioactivity in the very vascular tissues like liver, kidney and heart etc. may be due to the presence of a little blood. The radioactivity in the relatively non-vascular tissue of the testis is however, surprisingly comparable to that per unit weight of a more vascular tissue like the liver. The lowest level of radioactivity per unit wet weight of tissue was found in the adipose tissue, followed by the brain and muscle.

The total amounts of ^{14}C in tissues, excluding bone and fat, amounted to about 50% of total dose injected. Total urinary and respiratory activity for the 9 hr period of the experiment was about 30% (Chapter VIII), giving a total recovery of about 80% (excluding radioactivity in bone and fat).

4(b) Tissue distribution of cyanide carbon after subcutaneous injection of $Na^{14}CN$

One male rat weighing 364 g was given two doses of 8.3 µmoles $Na^{14}CN$ (by subcutaneous injection in the region of the thigh muscle) with 30 minutes interval. The specific activity of injected cyanide was 1.15 µCi/µmole. Blood and tissues were removed from the rat, 8½ hr after last injection as already described.

The results obtained (Table IX.2) show that the amounts of radioactivity in tissues after subcutaneous injection of $Na^{14}CN$ are similar to those obtained after intraperitoneal injection. The stomach and contents also contained the highest level of radioactivity, accounting for about 22% of total injected dose. This meant that

TABLE IX.2

Amounts of ^{14}C in tissues after subcutaneous injection of $Na^{14}CN$ to rats. One rat, weight 364 g, injected with two doses of 8.3 μ moles of CN^- with a 30 min interval; specific activity of injected material, 1.15 μCi/μmole; animal was killed 8½h after last injection.

Tissue	Wet Weight (g)	% injected activity per g tissue	% injected activity per tissue
Blood[b]	23.08 (ml)	0.38	8.82
Heart	1.25	0.10	0.13
Spleen	0.42	0.26	0.11
Kidney	2.21	0.32	0.70
Lung	1.69	0.32	0.54
Liver	13.51	0.16	2.15
Muscle[b]	144.00	0.10	13.60
Brain	2.77	0.04	0.11
Large intestine plus contents	7.54	0.26	1.99
Small intestine plus contents	8.79	0.33	2.93
Stomach plus contents	2.63	8.47	22.27
Adipose tissue	-	0.01	-
Testis	4.03	0.16	0.65
Eye	0.26	0.09	0.02
Salivary gland	1.85	0.15	0.28
Total in tissues			54.31
Total excretion			24.96
Total			79.27

[b]Total radioactivity was calculated from body weight of rat (Altman and Dittmer, 1964).

"-" means not determined.

the high level of radioactivity in stomach plus contents after intraperitoneal injection could not be a consequence of the site of injection. Furthermore, the total recovery of radioactivity (excluding that in the bone and fat) is about 79% of total injected dose.

The stomach was dissected and the contents separated from the wall. Investigation of the radioactivity in these two fractions revealed that a greater part of the radioactivity in the stomach plus contents is associated with the contents (accounting for 59% of total stomach plus content radioactivity, Table IX.3). The radioactivity in the salivary glands of this rat was examined and found to be similar to the levels in tissues like the liver, heart or testes (when expressed per gm wet weight).

4(c) Tissue distribution of cyanide-carbon after oral dose of $Na^{14}CN$

One male rat weighing 350 g was given 16.6 µmoles of $Na^{14}CN$ (specific activity 1.15 µci/µmole) orally. The appropriate dose in 0.2 ml aqueous solution was mixed with 0.1 ml glycerol to improve acceptability. The mixture (in 0.3 ml) was administered by a syringe using the procedure described in Chapter II. Traces of the mixture were lost during the administration.

Blood and tissues were removed from the rat 9 hr after the administration, and the radioactivity in each tissue determined by the same procedure as that described earlier for those rats given an intraperitoneal injection of $Na^{14}CN$. In this case, however, the stomach, small intestine and large intestine were separated into wall and contents.

Table IX.4 gives the distribution of radioactivity in tissues examined. The pattern of distribution of radioactivity in most of the tissues was comparable to those observed after either subcutaneous

TABLE IX.3

The amounts of ^{14}C in the stomach wall and stomach contents of one rat after subcutaneous injection of $Na^{14}CN$.

Tissue	Weight	% of dose in tissue or content
Stomach wall	2.05	9.11
Content	0.58	13.16

TABLE IX.4

Amounts of ^{14}C in tissues after oral dose of $Na^{14}CN$ to rat. One rat, weighing 350 g was given 16.6 μmoles of CN^- orally; specific activity of dosed material 1.15 μCi/μmole; animal was killed 9 h after administration.

Tissue	Wet Weight (g)	% dosed activity per g tissue	% dosed activity per tissue
Blood[b]	22.30 (ml)	0.61	13.65
Heart	1.22	0.12	0.15
Spleen	0.40	0.15	0.06
Kidney	2.27	0.33	0.74
Lung	1.72	0.30	0.51
Liver	12.69	0.28	3.59
Muscle[b]	139.00	0.07	9.35
Brain	2.80	0.03	0.09
Large intestine plus contents	5.77	0.13	0.73
Small intestine plus contents	8.20	0.35	2.86
Stomach and contents	2.82	5.66	15.95
Adipose tissue	-	0.02	-
Testis	3.40	0.20	0.68
Eye	0.29	0.10	0.03
Salivary gland	1.92	0.15	0.28
Total activity in tissues			48.69
Total excretion			20.32
Total			68.98

[b]Total radioactivity was calculated from body weight of rat (Altman and Dittmer, 1964).

"-" means not determined.

or intraperitoneal injections. Once again, the gastrointestinal tract contained a high proportion of the dosed radioactivity. A detailed investigation into the distribution of radioactivity between wall and contents of the three regions of the gastrointestinal tract revealed that most of the radioactivity was associated with the contents (Table IX.5). The percentages of radioactivity in contents for stomach, small intestine and large intestine were 67%, 61% and 59% respectively.

The total radioactivity in the urine and faeces during the 9 hr period of the experiment was 20.31%. Total radioactivity in tissues (excluding bone and fat) was 48.69%, giving a total recovery of 68.98%. The recovery of radioactivity in this case is about 10% less than that recovered after intraperitoneal injection. This may be attributed to losses of traces of the material during oral administration.

4(d) Distribution of radioactivity in various cyanide metabolite fractions

Tissues from rats killed after intraperitoneal injection of two doses of 8.3 μmoles $Na^{14}CN$ were used for these studies. All homogenized tissues were stored at -20° until required for analysis.

A known volume of the tissue homogenate was put into a tube with a rubber stopper equipped with gas inlet and outlet tubes. One ml of 8% silicone antifoaming agent was added and the mixture acidified to pH 3-4 by dropwise addition of 0.5M H_2SO_4. Four traps were connected in series: (1) the first two containing each 6 ml 0.02M Ag_2SO_4 in 0.05M H_2SO_4; (2) the last two containing each 5 ml 0.1M NaOH. Cyanide and carbon dioxide were transferred over a period of 45 min by aerating with oxygen-free nitrogen at a flow rate of between

TABLE IX.5

Distribution of radioactivity in various regions of the gastrointestinal tract after oral administration of $Na^{14}CN$.

Tissue	Weight	% of dose in tissue or contents
Stomach wall	1.79	5.26
Contents	1.03	10.69
Small intestine wall	6.10	1.11
Contents	2.10	1.75
Large intestine wall	3.07	0.30
Contents	2.70	0.43

700 and 800 ml per minute. The silver sulphate traps removed the cyanide to give what was called the CYANIDE FRACTION, while carbon dioxide liberated was trapped in the NaOH absorbers and called the CARBON DIOXIDE FRACTION.

Trichloroacetic acid (20% solution) was added to the same sample of tissue, from which cyanide and carbon dioxide had been removed, to give a final concentration of about 7%. The protein precipitated was centrifuged, precipitate washed with 5 ml distilled water, and centrifuged again. The precipitate was weighed and called the TCA PRECIPITATE FRACTION (TRICHLOROACETIC ACID PRECIPITATE FRACTION).

The combined supernatant and washing were transferred to the same aeration tube and 1 ml 0.01M potassium permanganate (or twice this volume in some cases) and 1 ml 0.1M H_2SO_4 were added. After allowing 3 minutes for full oxidation of thiocyanate to cyanide, 1 ml of 2% stannous chloride was added to reduce excess permanganate. The hydrogen cyanide formed was aerated into 5 ml 0.1M NaOH by sweeping with nitrogen as already described. The radioactivity in the NaOH trap in this case was called the THIOCYANATE FRACTION, while that remaining in the aeration vessel was called the SUPERNATANT FRACTION.

The radioactivity in each sodium hydroxide trap and in the trichloroacetic acid precipitate were determined by solubilization in NCS as already described (Chapter II).

However, the radioactivity in the silver sulphate traps could not be determined directly by solubilization in NCS since the silver was found to cause serious quenching. Accordingly, 5 ml 11M H_2SO_4 were added to 10 ml of the contents of the silver sulphate trap in a test tube which was then connected to an aeration train consisting

of a tube containing 10 ml 0.05M H_2SO_4 and the usual collection tube containing 5 ml 0.1M NaOH. The hydrogen cyanide was aerated into the alkali by sweeping with nitrogen at a flow rate of between 700 and 800 ml/min for 1½ hr. Amounts of radioactivity in the NaOH trap were determined as already described in Chapter II.

The distribution of radioactivity in the various cyanide metabolite fractions of rat tissues is given in Table IX.6. The largest proportion of cyanide carbon in each tissue was present in the thiocyanate fraction. The 'TCA precipitate fraction' also contained large amounts of radioactivity, and part of this could be due to extensive binding of thiocyanate ion to protein. Although the radioactivity in the cyanide fraction in each tissue was generally very much lower than that present in the thiocyanate fraction, relatively higher proportions were found in heart, muscle, brain, testes, large intestine and salivary glands compared with other tissues like liver, kidney etc. The activity in the supernatant fraction represents that incorporated into other tissue metabolites like formate, amino acids etc. (Boxer and Rickards, 1952).

The amounts of radioactivity in the cyanide and thiocyanate fractions (of each tissue) expressed as percentages of total dose injected are given in Table IX.7. The highest amount of thiocyanate is present in the stomach plus contents where its concentration was about 470 times that of cyanide. The total amount of radioactivity in all tissues examined expressed as a percentage of total injected dose is about 50% (Table IX.1). Therefore, the total thiocyanate in these tissues accounted for about 70% of the amount present. Cyanide on the other hand accounted for only about 2% of total activity in tissues examined.

The proportion of radioactivity in the thiocyanate fraction of stomach contents was investigated in the rat killed 9 hr after

TABLE IX.6

Distribution of radioactivity in various fractions after injection of $Na^{14}CN$ (mean values ± SD, N = 3).

Tissue	Fractions, as % of total activity in each tissue				
	CN^-	SCN^-	CO_2	TCA precipitate	Supernatant
Blood[b]	3.43±0.26	54.46±12.24	2.34±0.29	34.34±11.68	3.48±1.62
Heart	15.64±2.87	55.38±4.18	2.33±0.22	21.98±8.60	5.56±0.26
Kidney	1.71±0.38	79.69±2.22	0.41±0.18	14.84±2.81	8.92±0.19
Lung	2.37±0.13	73.97±1.22	0.16±0.06	14.98±0.76	5.52±0.33
Liver	1.98±0.81	59.35±7.40	4.75±0.62	19.02±3.19	9.29±0.88
Muscle[b]	10.26±0.55	69.43±3.17	0.36±0.06	12.56±1.29	8.13±0.43
Brain	9.15±1.49	78.38±1.10	2.88±0.04	6.03±1.10	6.43±1.16
Stomach and contents	0.59±0.24	84.07±5.78	0.90±0.15	5.73±0.73	6.27±3.91
Small intestine and contents	3.57±4.29	52.80±4.19	2.99±3.27	29.86±3.54	8.57±5.08
Large intestine and contents	8.86±12.62	57.53±11.79	1.32±2.21	26.03±8.27	10.10±9.80
Testis	14.41±8.05	67.07±1.14	5.38±1.38	4.22±3.64	0.49±0.85
Eye	2.83±0.52	64.94±10.34	Trace	32.24±9.83	Trace
Salivary gland*	24.12±5.25	58.40±2.76	6.58±0.95	15.41±0.85	0.49±0.69

[b]Total radioactivity was calculated from body weight of rat (Altman and Dittmer, 1964).

* n = 2

TABLE IX.7

The radioactivity present in cyanide and thiocyanate fractions of the rat tissues after intraperitoneal injections of $Na^{14}CN$.

Tissue	Fractions, as % of total injected radioactivity		Ratio of SCN^- to CN^-
	Cyanide	Thiocyanate	
Blood[b]	0.38	6.09	16.0
Heart	0.02	0.08	4.0
Kidney	0.01	0.59	59.0
Lung	0.01	0.36	36.0
Liver	0.06	1.89	31.5
Muscle[b]	1.16	7.86	6.8
Brain	0.08	0.67	8.4
Stomach plus contents	0.11	15.41	467.4
Small intestine plus contents	0.07	1.02	14.6
Large intestine plus contents	0.09	0.58	6.4
Testis	0.10	0.48	4.8
Eye	0.002	0.07	
Salivary gland*	0.07	0.16	2.3

[b]Total radioactivity was calculated from body weight of rat (Altman and Dittmer, 1964).

* From two rats, after subcutaneous and oral administration of $Na^{14}CN$ respectively.

administration of cyanide by mouth, and was found to account for about 79% of total activity in contents (Table IX.8). This value is only slightly lower than the 83% obtained for the stomach contents of the rat given $Na^{14}CN$ subcutaneously (Table IX.9).

4(e) Rate of secretion of thiocyanate into the rat stomach

Twelve male rats were used in these studies. They were divided into four groups of three (S_1, S_2, S_3 and S_4). Each rat had been reared on diet 41B until about five weeks old when each was transferred to the basal soya bean meal diet. After one week, potassium cyanide was added to the diet of all groups to provide 77.0 µmoles/rat/day. The method of administration of potassium cyanide in the diet without loss of added material has been presented in Chapter III. Each rat in every group was on the potassium cyanide supplement for at least 21 days, after which each received 4 µmoles of $Na^{14}CN$ (specific activity 1.15 µCi/µmole) by subcutaneous injection. Immediately after injection, the time was recorded and the rat placed in a metabolism unit (described in Chapter VIII). The radioactive dose of cyanide was given to one rat at a time. Animals in group S_1 were killed 10 minutes after the dose of $Na^{14}CN$ while those in groups S_2, S_3 and S_4 were killed after 20, 30 and 60 minutes respectively. Blood was removed by heart puncture (while rats were anaesthetised with Halothane) into a heparinized syringe, and the stomach of each rat carefully dissected out, rinsed, blotted dry and weighed. The blood was immediately chilled with ice and then centrifuged. Plasma was removed and analysis of cyanide and thiocyanate content started within 5 mins. The methods used for the analyses of cyanide and thiocyanate were as already described in this chapter. The stomach was dissected open

TABLE IX.8

Proportion of radioactivity in the cyanide metabolite fractions of the rat stomach contents after oral dose of $Na^{14}CN$.

Fraction	Radioactivity as % of total in contents
Cyanide	3.9
Thiocyanate	78.7
Carbon dioxide	0.6
Remaining activity	13.1

TABLE IX.9

Proportion of radioactivity in the cyanide metabilite fractions of the rat stomach contents after subcutaneous injection of $Na^{14}CN$.

Fraction	Radioactivity as % of total in contents
Cyanide	Trace
Thiocyanate	82.9
Carbon dioxide	0.6
Remaining activity	17.0

and the contents rinsed out thoroughly into a clean beaker. The weight of the stomach wall was recorded and that of the contents determined by difference (by substracting weight of stomach wall from weight of stomach plus contents). The stomach contents of each rat were diluted to a known volume and stored with stomach wall in screw capped bottles at -20°. Analyses of the thiocyanate content of the stomach wall and stomach contents were carried out within one week of removal.

As shown in Table IX.10, ^{14}C-thiocyanate was secreted into the stomach contents at a very rapid rate. One hour after injection of Na^{14}CN, approximately 16% of the dose injected was present in the stomach contents as thiocyanate. This amount of thiocyanate accounted for about 88% of total radioactivity present in the contents.

Although an increase in thiocyanate content was observed in the stomach wall (Table IX.11) with increase in the time between injection and death of the rat, this was generally much lower than that present in the contents at the same time after injection.

By 10 mins after the injection of Na^{14}CN to the rats, 5% of the radioactivity was found in the plasma (calculated from body weight of rats, Table IX.12), of this about 80% was present in the thiocyanate fraction while less than 1% was present in the cyanide fraction.

The total thiocyanate fraction of the plasma appeared to reach a peak at 20 mins after the injection of Na^{14}CN. There was only a small fall in the total thiocyanate content of plasma by 60 mins, but it was statistically significant (P = 0.01).

5. Discussion

The distribution of a compound in tissues can be influenced by several factors, including the age and sex of animal and the dose of the compound administered. If the method of administration is an

TABLE IX.10

Radioactivity in the thiocyanate fraction of stomach contents of the rat after subcutaneous injection of $Na^{14}CN$. Each rat was injected with 4 μmoles of CN^-; specific activity 1.15 μCi/μmole.

Time after injection (min.)	Radioactivity as % of injected dose ± S.E.M., n = 3	
	Total in Stomach contents	Thiocyanate fraction
10	1.3 ± 0.1	1.2 ± 0.1
20	3.9 ± 0.4	3.4 ± 0.4
30	9.1 ± 2.3	7.4 ± 1.7
60	18.5 ± 2.2	16.2 ± 1.9

TABLE IX.11

Radioactivity in the thiocyanate fraction of stomach wall of the rat after subcutaneous injection of $Na^{14}CN$. Each rat was injected with 4 μmoles of CN^-; specific activity of injected material 1.15 μCi/μmole.

Time after injection (min.)	Radioactivity as % of injected dose ± S.E.M., n = 3	
	Total in Stomach Wall	Thiocyanate fraction
10	0.82 ± 0.09	0.64 ± 0.06
20	1.06 ± 0.06	0.85 ± 0.06
30	1.64 ± 0.32	1.36 ± 0.26
60	2.12 ± 0.13	1.77 ± 0.16

TABLE IX.12

Radioactivity in plasma cyanide and thiocyanate fractions after subcutaneous injection of $Na^{14}CN$ to the rat. Each rat was injected with 4 µmoles of CN^-; specific activity of injected material 1.15 µCi/µmole.

Time after injection (min)	Radioactivity as % of injected dose ± S.E.M., n = 3			
	Total per ml plasma	Thiocyanate fraction (per ml plasma)	Cyanide fraction (per ml plasma)	Total plasma thiocyanate[b]
10	0.72±0.09	0.57±0.09	0.05±0.02	5.42±0.66
20	0.96±0.05	0.81±0.01	0.02±0.00	7.06±0.26
30	0.86±0.12	0.65±0.07	0.03±0.01	6.69±0.50
60	0.83±0.06	0.58±0.03	0.02±0.00	5.76±0.11

[b]Calculated from body weight of rats (Altman and Dittmer, 1964).

unnatural one, such as intraperitoneal or intravenous injection, this too can influence the distribution pattern obtained.

The ^{14}C levels in all tissues examined after the administration of $Na^{14}CN$ to male rats by the three independent routes (oral administration and subcutaneous or intraperitoneal injections) have been presented. There was a good deal of similarity in the pattern of distribution of radioactivity 9 hr after administration of $Na^{14}CN$ by the three separate routes.

Recently, Crawley and Goddard (1977) have worked on the distribution of cyanide carbon in tissues and on the retention and excretion kinetics. They administered $K^{14}CN$ to female rats by three independent routes (intravenous injection and pulmonary or gastric incubation). Their data confirm that the distribution of cyanide carbon is independent of the route of administration (a few differences in tissue distribution of ^{14}C-cyanide were observed however, after skin absorption). Although the dose levels employed by these workers were at least 450 times less than those employed here, the patterns of distribution of radioactivity in tissues were very similar. It is therefore unlikely that the distribution pattern of cyanide carbon will be influenced by any non-acute dose of cyanide.

The highest uptake of radioactivity was observed in the gastrointestinal tract particularly the stomach. The ratio of radioactivity in tissue plus contents at 9 hr for stomach: small intestine:large intestine was 18:2:1. Since the radioactivity in the gastrointestinal tract is largely associated with the contents, it is very likely that the initial concentration of this activity occurs in the stomach and that activity in the lower region of the gut is due to the movement of the stomach contents down the gut.

It has been observed in animals killed by an acute dose of cyanide that a selective uptake of this radical occurs in the brain (Ballantyne et al., 1972; Ballantyne, 1975). The cyanide radical present in all tissues examined 9 hr after administration of a non-acute dose of $Na^{14}CN$ was generally around 0.1% of dose or less and that in the brain was only 0.08% of dose injected (Table IX.7). Thus, it is clear that any selective uptake of cyanide radical that may have occurred in the brain was extensively removed and detoxified to thiocyanate over the 9 hr period.

The discovery that dimethylnitrosamine was acutely hepatotoxic to a number of laboratory animal species (Barnes and Magee, 1954) led to the subsequent investigation of its possible carcinogenic action (Magee and Barnes, 1956). Magee and Barnes (1956) observed that rats on a diet containing 50 ppm dimethylnitrosamine developed malignant liver tumors within less than a year. Since this observation a number of papers have appeared on the carcinogenic activity of not only dimethylnitrosamine but also a wide range of nitrosocompounds. Excellent reviews on the carcinogenic action of different nitroso-compounds have been published (Druckery et al., 1967; Magee and Barnes, 1967).

Nitrosamines are formed by the chemical reaction between nitrous acid and the respective alkylamino compounds.



Since secondary amines and nitrite are present in a number of food sources (Lijinsky and Epstein, 1970) there has been increasing interest in the possibility that nitrosamines can be formed in vivo and so give rise to tumours in individuals not apparently exposed to

nitrosamines *per se*.

Mirvish (1970) has studied the nitrosation of dimethylamine in buffered aqueous solution using tritium-labelled amine. In an aqueous solution containing 0.02M $(CH_3)_2NH.HCL$ 0.1M $NaNO_2$ and 0.05M NaCl, maximum yield of nitrosamine occurred at pH 3.4. It is therefore not surprising that most of the work on the *in vivo* formation of nitrosamine has concentrated on the stomach as a possible site of synthesis (Sen *et al.*, 1969; Sander *et al.*, 1968). Indeed, there is now evidence that nitrosamines are formed in the stomach of rodents (Sander and Bürkle, 1969; Mirvish *et al.*, 1972 and Lijinsky *et al.*, 1973).

It is now known that the nitrosation reaction is catalyzed by some anions, particularly thiocyanate (Ridd, 1961; Boyland *et al.*, 1971). Boyland and Walker (1974) have shown that at pH 1.5, 1.0 mM thiocyanate increases the rate of reaction about 550 times. Under these acid conditions (below pH 2.0) the nitrosation reaction rate is proportional to the concentration of (1) nitrite; (2) thiocyanate; and (3) amine. At pH 3.5 and greater, thiocyanate has less catalytic action.

It has long been known (Crandall and Anderson, 1934; Manson *et al.*, 1951; Logothetopoulos and Myant, 1956) that thiocyanate is concentrated into the gastric juice of animals. It is also well known that thiocyanate is the main detoxification product of cyanide in animals (Lang, 1894) and that is largely excreted in the urine (Boxer and Rickards, 1952). So far as is known, the work described in this chapter is the only study carried out, to date, to establish if thiocyanate is concentrated in the stomach of animals following administration of cyanide. Although Crawley and Goddard (1977) observed the concentration of radioactivity in the stomach of female

rats after administration of $K^{14}CN$, these workers did not identify the chemical nature of the concentrated material. The results reported here confirm that the radioactivity concentrated into the stomach contents following administration of $Na^{14}CN$ is mainly thiocyanate.

The secretion of thiocyanate by the stomach was found to occur very quickly after the subcutaneous injection of $Na^{14}CN$ and to proceed at a very rapid rate. To the experimental points (Fig.IX.1) for the rate of secretion of thiocyanate into the stomach, a straight line was fitted by the method of least squares (Fix.IX.2). This manipulation is not wholly justified in that it ignores any reduction of thiocyanate in the stomach contents through passage on to the intestine, but the reasonably good fit of the points in Fix.IX.2 indicates that the basic assumption is not very wrong. The rate of secretion of thiocyanate from the slope of the straight line was 0.31% of cyanide dose per minute, and the time of initial secretion of thiocyanate not more than 7 mins.

It is important to recall that the rats used in these experiments had been exposed to chronic ingestion of cyanide in the diet and hence had elevated thiocyanate in plasma (Chapter III). Furthermore, it has been demonstrated in Chapter VIII, that when rats exposed to chronic cyanide intake in the diet were each given 0.06 μ moles of $KS^{14}CN$ (less than 1/1000 of daily cyanide ingested), by mouth they excreted large amounts of the radioactivity in the urine and only small amounts in the faeces. This indicates that the thiocyanate secreted into the stomach must be reabsorbed in the lower part of the gut into the body fluid, to be partly excreted in the urine and partly resecreted in the gastric juice. Thus in the rat, there is a gastrointestinal circulation of thiocyanate linked with the body thiocyanate pool.

FIGURE IX.1

The rate of increase of thiocyanate in the rat stomach after subcutaneous injection of $Na^{14}CN$. (Bars indicate S.E.M.).

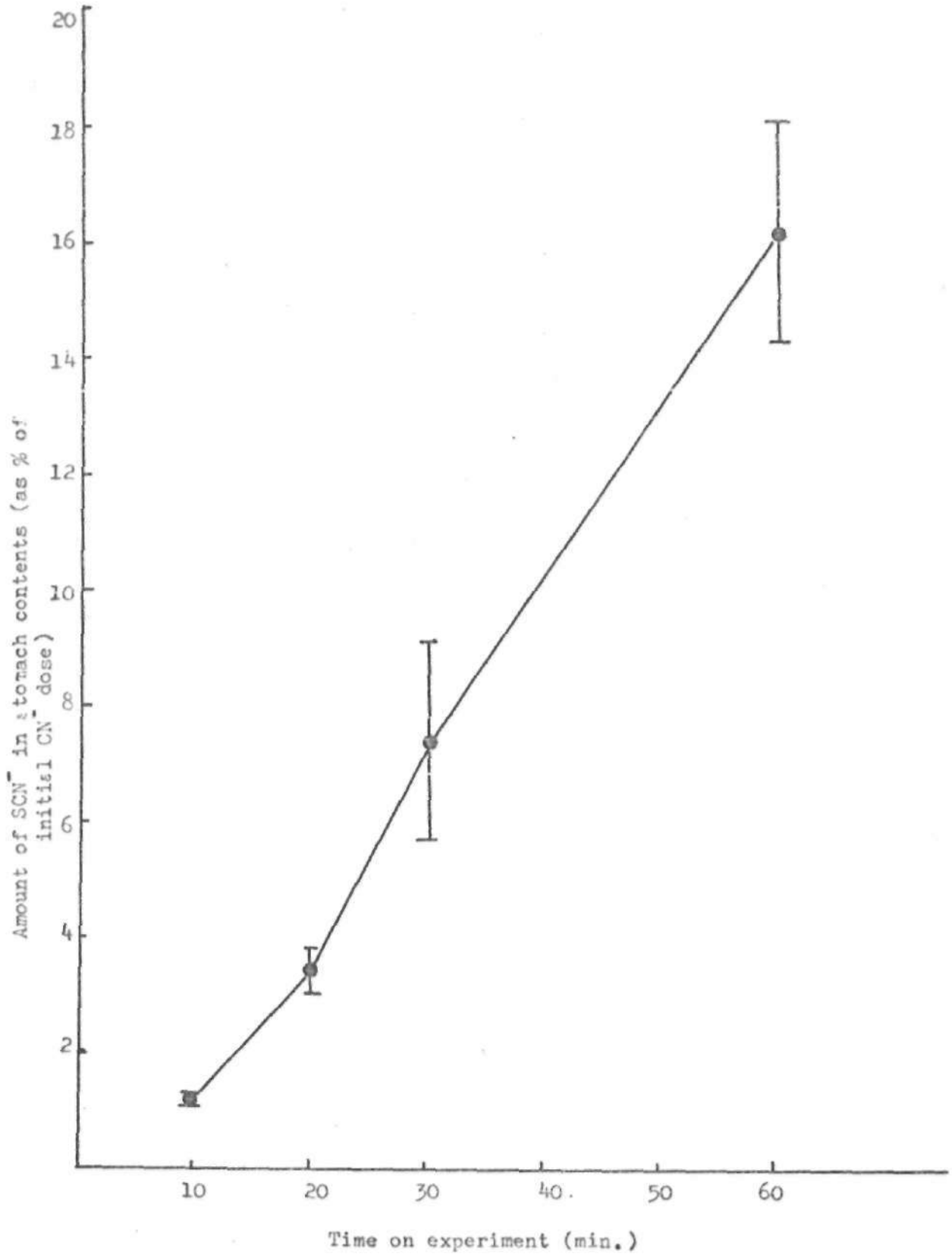
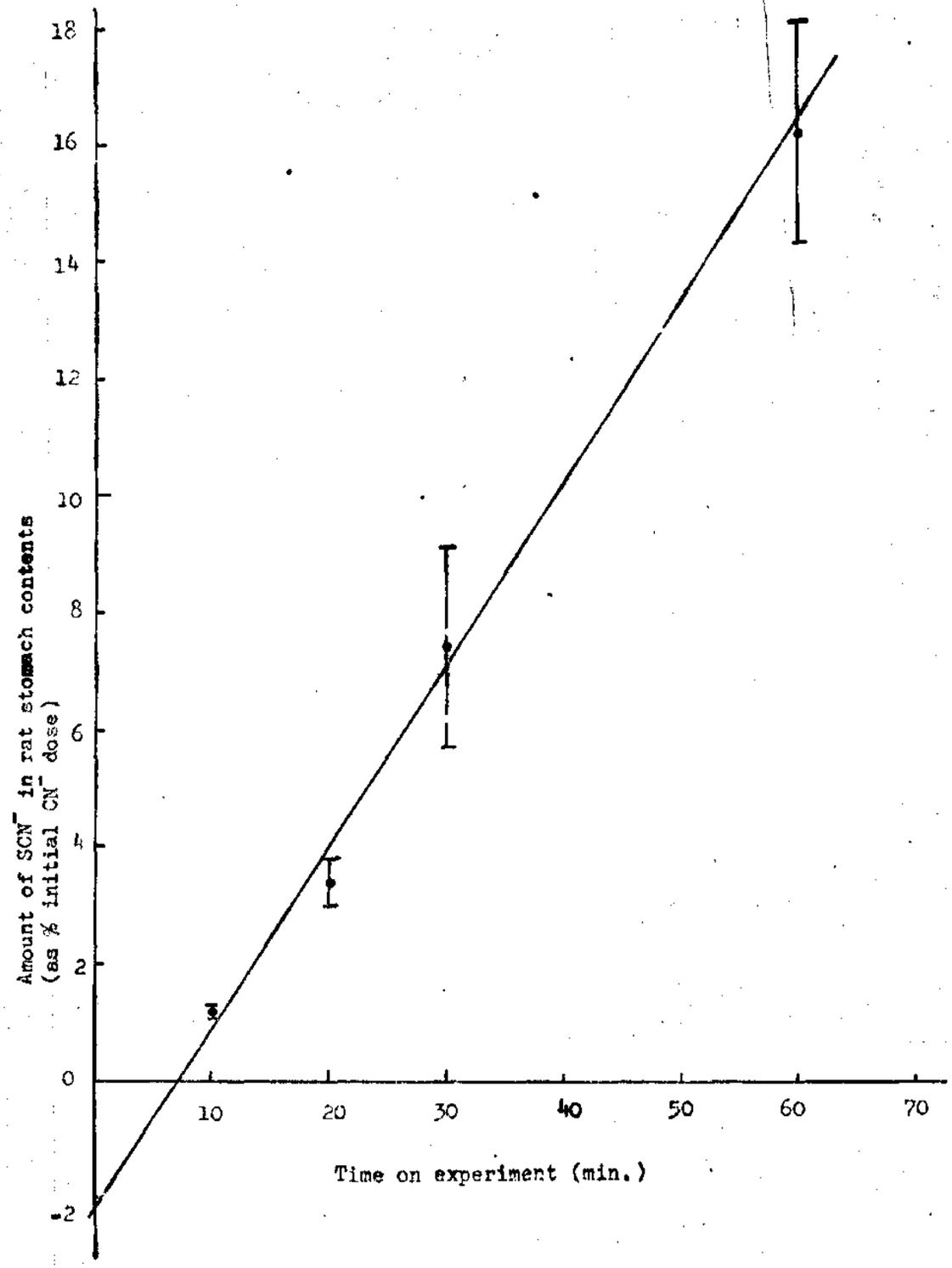


FIGURE IX.2

Rate of secretion of thiocyanate into rat stomach (assuming that a linear regression analysis is applicable). Bars indicate S.E.M.



The salivary gland of many animals is known to concentrate and secrete thiocyanate. The rat salivary gland is different since it was observed not to concentrate this radical. A similar observation was made by Logothetopoulos and Myant (1956). Thus the thiocyanate concentrated in the stomach of rats cannot be due to swallowed saliva.

A number of investigators (Boyland and Walker, 1974; Ruddell et al., 1977) have observed that thiocyanate was present in the stomach of humans, and that larger amounts are found in smokers compared with non-smokers. Ruddell et al. concluded that the thiocyanate in the gastric juice was exogenous, and probably salivary in origin.

As Logothetopoulos and Myant (1956) found that other species besides the rat can secrete thiocyanate into the gastric juice, it seems probable that part of the thiocyanate found in the stomach of humans may be due to gastrointestinal circulation of this radical.

CHAPTER X

CHAPTER X

General Discussion

The results of the investigations described in this thesis have already been discussed in detail in the relevant chapters. The purpose of the general discussion is therefore to stress the main findings of the work described and to make suggestions for future research.

Much of the research on the metabolism of cyanide in animals has been centred on the evaluation of antidotes for cyanide poisoning. Only very limited work has been done on the metabolism of small amounts of cyanide and on the nature of chronic cyanide intoxication in animals.

One of the main reasons why we know little about the metabolism of cyanide is because it is generally accepted as a very lethal substance. This view is not wholly correct since it is well known that the intake of small amounts of cyanide over a prolonged period will not cause death. When a dose of cyanide which will normally cause death when ingested in a single dose is taken over a prolonged period of hours, death may not occur. This is because, given time, the animal is able to convert cyanide to relatively non-toxic thiocyanate and other substances. Thus, although cyanide is regarded as a highly toxic substance, it is much less harmful when given semicontinuously in the diet than vitamins A and D, which are generally regarded as beneficial.

Most investigators have evaluated the toxicity and metabolism of cyanide present in foodstuffs largely by feeding diets containing cyanogenic glycosides to experimental animals. When inorganic cyanide

has been given to experimental animals over a period of days, it has been administered in dilute solution by repeated injections. The use of inorganic cyanide for cyanide metabolism studies has the advantage that the cyanide administered is fully available as opposed to cyanogenic glycosides in which the cyanide is not completely liberated in the animal body.

Other workers have administered inorganic cyanide to experimental animals by injection - a method which is physiologically different from the normal mode of ingestion. The only published work in which potassium cyanide was given in the diet is that of Clark (1936), and he seemed to be unaware of the loss of cyanide from the diet. The work described in this thesis has established that potassium cyanide cannot be recovered when added to the diet of experimental rats which has been moistened with water as is customary when powdered diets are fed. Investigation revealed that the cyanide was lost by transformation to other products, and perhaps also to volatile HCN. A preliminary study revealed that a number of products may be formed, including formate, which could be a major one. Further studies on the nature of cyanide transformation in diet to which water has been added, may prove interesting. Knowledge acquired from such studies might help to improve the method of cassava processing with a view to reducing its cyanide content.

When inorganic cyanide was added to the diet mixed with arachis oil instead of water, no significant loss or transformation of the added substance occurred. This method will therefore be very useful in the study of the effects of regular intake of small amounts of cyanide in the diet (a mode of ingestion related to the normal mode) especially as the administered cyanide is fully available to the animals.

Previous studies on the distribution of cyanide in the tissues of animals have been concerned with the distribution of cyanide radical *per se*, rather than on the distribution of cyanide carbon and hence its metabolites. Furthermore, these earlier studies only involved use of animals killed by acute cyanide doses.

Under conditions where cyanide is taken semi-continuously over a prolonged period, no cyanide could be demonstrated in tissues by known chemical methods. This is because cyanide is rapidly metabolised in animals largely to thiocyanate. A study of the distribution pattern of ^{14}C activity, 9 hr after $Na^{14}CN$ injection, was carried out in the rat exposed to semi-continuous intake of potassium cyanide in the diet for three weeks. Analysis of the activity in the respiratory air during the 9 hr period revealed that a large proportion of the expired radioactivity was associated with the carbon dioxide fraction (86%) and only a small amount was found in the free cyanide fraction (14%). The only previous major study on the elimination of cyanide carbon in breath (Boxer and Rickards, 1952) after subcutaneous injection of ^{14}C-labelled sodium cyanide in rats showed that 90% was ^{14}C labelled CO_2 and 10% unchanged cyanide.

All tissues examined 9 hr after the injection of $Na^{14}CN$ contained radioactivity, but a high proportion of the cyanide carbon was found to be secreted into the stomach contents as thiocyanate. When a small amount of [^{14}C] thiocyanate of high specific activity was given to the rat (ingesting 77 µmoles of KCN daily for three weeks), the radical was largely excreted in the urine and only very small amounts were found in the faeces. Since the rats used in these experiments had elevated plasma thiocyanate levels (as they had been on a diet containing cyanide) it was clear that the thiocyanate in the stomach contents must be reabsorbed in the intestine into the

body to be partly excreted in the urine and partly resecreted into the gastric contents. Thus, these studies have established that there is a gastrointestinal circulation of thiocyanate in the rat and that this is linked with the body thiocyanate pool.

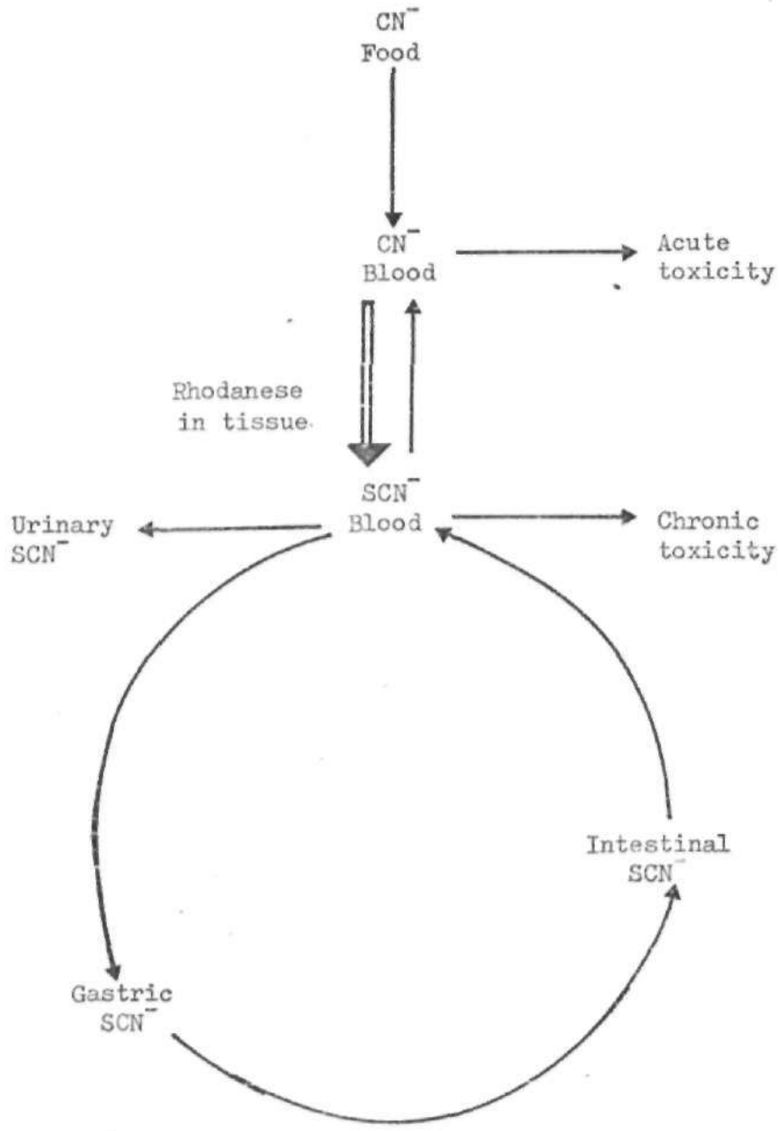
A study of the proportion of cyanide and thiocyanate in the tissues of the rat 9 hr after administration of cyanide revealed that the largest amount of cyanide carbon in every tissue examined was in the form of thiocyanate. Furthermore, since the rate of secretion of this radical (thiocyanate) into the stomach contents was very rapid, it was clear that detoxification of cyanide was occurring at a fairly rapid rate *in vivo*. As the body pool of cyanide was found to be very small under conditions of chronic ingestion of this substance, it would appear likely that chronic cyanide toxicity is due to thiocyanate rather than cyanide.

Smith and Foulkes (1966) claimed that urinary excretion of thiocyanate is decreased in the rat, following chronic administration of cyanide over a prolonged period of weeks. They therefore suggested that a substrate saturation phenomenon was operative in the rat after chronic cyanide administration. In Chapter VIII, it was shown that groups of rats on 77 µmoles of cyanide per rat per day did not reveal any decrease in thiocyanate excretion as a result of daily intake of this radical for three weeks. Furthermore, thiocyanate was the major metabolite of cyanide in all tissues of the rat (previously exposed to chronic cyanide ingestion for three weeks) examined 9 hr after administration of [^{14}C] cyanide.

On the basis of the observations made to date, the following hypothesis on the metabolism and toxicity of cyanide seems plausible (Fig. X.1). Ingestion of cyanide leads to an initial high level of this radical in the blood and tissues. The occurrence of acute

FIGURE X.1

Gastrointestinal circulation of thiocyanate in the rat.



toxicity (commonly leading to death) depends on the level of cyanide ingested. With a non lethal dose of cyanide, respiratory arrest does not occur and the animal is able to form relatively large amounts of thiocyanate by the action of rhodanese in tissues, mainly liver and kidney. Thiocyanate in itself is toxic at high concentrations, but it is secreted in large amounts into the stomach contents. The secreted thiocyanate is carried down into the intestine along with the gastric contents. In the intestine, the thiocyanate is largely reabsorbed into the body fluid to be partly excreted in the urine and partly resecreted into the gastric contents. The biological importance of this gastrointestinal circulation of thiocyanate in the rat is not clear. Logothetopoulos and Myant (1956) have reported, however, that the concentration of thiocyanate into the gastric juice probably begins in foetal life. It is tempting to speculate that animals may have evolved the gastrointestinal circulation of thiocyanate as a means of removing this radical from tissues where it might be more toxic, while allowing time for its elimination in the urine. However, the possibility exists that this gastrointestinal circulation of thiocyanate may have a physiological role in the animal especially as it is known to inhibit gastric HCl secretion.

The mechanism of secretion of thiocyanate into the stomach contents is not clear. Originally, it was suggested that thiocyanate decreased gastric HCl secretion by virtue of its action on carbonic anhydrase (Sakshaug et al., 1965). However, subsequent work rendered this explanation untenable (Sakshaug et al., 1965; Sander et al., 1968). LèFevre et al. (1964) proposed that the inhibitory action is dependent on the presence of a nitrogen with a pair of unshared electrons, which complexes with a metal associated with an enzyme essential for H^+ secretion. Recently, Sanders et al. (1978) have suggested that

thiocyanate inhibits H^+ secretion by virtue of its action on the molecular machinery.

Regardless of the mechanism of action of thiocyanate as a gastric acid inhibitor, it is clear that this radical is secreted in large amounts into the stomach contents and that this could act as a catalyst for the nitrosation of amines in the stomach.

A thiocyanate concentration of 1 mM reduces the optimal pH for the in vitro nitrosation of N-methylaniline from pH 3 to 1.5 and accelerates the reaction by 550 times (Boyland and Walker, 1974). It is difficult to decide what would be the effective concentration of thiocyanate in vivo, but it seems reasonable to conclude that if nitrosation is occurring, it willbe greatly accelerated in the stomach of animals or humans consuming cyanide.

The amount of nitrosamine formed in the stomach is proportional to the concentrations of (1) nitrite; (2) thiocyanate and (3) amine. Before deciding whether thiocyanate is of importance in human carcinogenesis, in those parts of the tropics where diets containing cyanogenic glycosides are consumed in large amounts, a detailed study of the nitrite and secondary amine content of foods consumed in these areas is clearly necessary.

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