# Biochemical Effects Of Chronic Cyanide Exposure In The Chicken And Their Relevance To The Mechanism By Which Cyanide Alleviates Selenium Toxicity.

by

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## ABSTRACT

Chronic cyanide exposure is more widespread than acute, but less well characterised biochemically. Selenosis is becoming increasingly important.

Cyanide ameliorates selenium toxicity, but the mechanism of this interaction has not yet been adequately explained. Selenium and cyanide metabolism and toxicity have been reviewed. The biochemical effects of chronic cyanide exposure in chickens and their relevance to the mechanism by which cyanide alleviates selenosis have been investigated.

Sodium nitroprusside proved a suitable experimental source of dietary cyanide.

Hepatic glycogen concentration was reduced in chronic cyanide exposure and dietary supplementation with gluconeogenic precursors, L-alanine, L-lactate and L-serine, exacerbated cyanide toxicity, indicating the importance of anaerobic glucose catabolism.

Cyanide caused little alteration in the redox state of hepatic pyridine nucleotides, but decreased total glutathione and GSH, increased GSSG and reduced the GSH:GSSG ratio.

Selenium produced changes consistent with an increased demand for GSH and NADPH. Liver NADH and NADPH concentrations were decreased and $NADP^+$ increased resulting in elevated $NAD^+$:NADH and $NADP^+$:NADPH ratios. Hepatic total glutathione and GSH were increased. Cyanide reversed these effects.

Dimethylselenide exhalation by chicks consuming high selenium diets was decreased by cyanide.

Cyanide reduced liver total selenium, decreased the proportion present as selenite and increased the percentage of selenoamino acids. An adequate supply of methionine or cysteine was required for the latter two effects and for alleviation of selenosis.

Cyanide exerted greater influence in the cytoplasm than in whole liver or the mitochondrion.

Thiocyanate ions were not responsible for the amelioration of selenosis by cyanide.

The mechanism of the alleviation of selenosis appears to involve cyanide-mediated alteration of the form of selenium in tissues. No evidence has been obtained to show that cyanide achieves its effects through changes in cellular redox state.

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## ABBREVIATIONS

| | |
|---|---|
| Acetyl Co-A | acetyl coenzyme A |
| ADH | alcohol dehydrogenase |
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| Cys | cysteine |
| d | days |
| DAN | 2-3 diaminonaphthalene |
| DmSe | dimethylselenide |
| DNA | deoxyribonucleic acid |
| DTNB | 5-5'-dithio-bis-(2-nitrobenzoic acid) |
| $\epsilon$ | molarabsorptivity (molar extinction coefficient) |
| E.C. | enzyme commission |
| EDTA | ethylenediaminetetraacetic acid |
| EMP | Embden-Myerhof-Parnas |
| gfw | per gram fresh weight |
| GlDH | glutamate dehydrogenase |
| gluconate-6-P | gluconate-6-phosphate |
| glucose-6-P | glucose-6-phosphate |
| Y-GLU-CYS | Y-glutamyl cysteine |
| GR | glutathione reductase |
| GSCN | cyanoglutathione |
| GSH | reduced glutathione |
| GSHPx | glutathione peroxidase |
| GSSeH | selenopersulphide |
| GSSeSG | glutathione selenotrisulphide |
| HPLC | high performance liquid chromatography |
| I.U. | international units |
| Km | Michaelis-Menten constant |
| KU | kilounits (of enzyme activity) |
| LD | lethal dose |
| LDH | lactate dehydrogenase |
| LOM | linseed oil meal |
| LSD | least significant difference |
| Met | methionine |
| $NAD^+$ | oxidised nicotinamide adenine dinucleotide |
| NADH | reduced nicotinamide adenine dinucleotide |
| $NADP^+$ | oxidised nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| NMR | nuclear magnetic resonance |
| NS | no significant difference |
| P | probability |
| Pi | inorganic phosphate |
| RNA | ribonucleic acid |
| RSSeSR | selenotrisulphide |
| RSSR | disulphide |
| SCN | thiocyanate |
| SeCN | selenocyanate |
| SeGSHPx | selenium-dependent glutathione peroxidase |
| SEM | standard error of the mean |
| SNP | sodium nitroprusside |
| TCA | tricarboxylic acid |
| TEA | triethanolamine |
| TmSe | trimethylselenonium |
| tRNA | transfer ribonucleic acid |
| u | units (of enzyme activity) |
| v/v | volume/volume |
| w/v | weight/volume |

This thesis is dedicated to my father.

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I am grateful to my family and friends, especially my husband Michael, for all their support and encouragement.

# CHAPTER 1

## INTRODUCTION

The majority of the interest in cyanide has focused on its acute toxicity, the biochemical effects of which are well documented (Section 2.3). However, its most widely distributed toxicological problems result from chronic exposure, through the habitual ingestion of cyanogenic plants, by man and livestock in many developing countries (Section 2.1). The assumption has been that the metabolic bases of acute and chronic cyanide intoxication are similar, but differ in magnitude, leading to different overall effects. Whilst there is some evidence in support of such a hypothesis, this has been obtained either by studying the effects of cyanide as a pollutant on freshwater fish (Kovacs & Leduc, 1982; Raymond *et al.*, 1986) or by attempting to mimic chronic oral exposure by repeated injections of the toxin (Padmaja & Panikkar, 1989). In addition, these investigations have been concerned only with cyanide-mediated changes in glucose catabolism. The need remains for a detailed study of the biochemical effects of chronic exposure to dietary cyanide in commercial livestock.

Selenium toxicity has, until recently, presented problems only in certain areas of the world. Selenosis in humans and livestock has been reported in parts of China, North and South America, Australia, South Africa and Ireland (Yang, 1987; Ancizar-Sordo, 1947; Knott & McCray, 1959; Brown & DeWit, 1967; Fleming, 1962; Rosenfeld & Beath, 1964).

With our increased understanding of the biological roles and essentiality of selenium, the supplementation of animal feed, with this element, has become common practice. A relatively narrow margin exists between the levels of selenium that are nutritionally important and those that may be toxic. Selenosis has become more widespread as a result of the improper use of selenium-containing preparations.

Several dietary factors, of which cyanide is one, have proved beneficial against selenium toxicity (see Levander, 1987 and Martin, 1973 for reviews). Dietary modification by the inclusion of

substances that alleviate selenosis may have potential for preventing or treating selenium poisoning provided that the nature of this interaction is well understood.

The only mechanism so far proposed to account for the alleviation, by cyanide, of selenosis, is that selenocyanate may be formed, preventing selenium from forming more toxic compounds (Palmer & Olson, 1979). Whilst there is some evidence suggesting that selenocyanate production is possible, the significance of the rate of its synthesis *in vivo* remains questionable (Ganther *et al.*, 1977; Cannella *et al.*, 1975; Prohaska *et al.*, 1977; Kraus & Ganther, 1980; Beilstein & Whanger, 1984). In addition, selenocyanate has a toxicity comparable with that of selenite and is metabolised to yield similar products (Vadhanavikit *et al.*, 1987).

Formation of selenocyanate as the mechanism for the Se-CN interaction appears to be inadequate and other possibilities should be investigated. Levander *et al.* (1970) proposed, based on dialysis studies, together with the observation that linseed oil meal increased the amount of selenium in tissues, that cyanide might alter the form in which selenium exists in the body, in favour of less toxic compounds. Alternatively, if inhibition of cytochrome c oxidase and impaired electron transport were features of chronic, as well as acute, cyanide intoxication, stimulation of selenium metabolism, as a result of an increased availability of reducing equivalents, might provide an explanation for the Se-CN interaction.

The experiments described in this thesis were designed in an attempt to determine both the metabolic effects of chronic cyanide intoxication, and the mechanism by which cyanide alleviates selenosis.

SNP was chosen as a stable, cheap source of dietary cyanide for these investigations. Initial studies were therefore required to confirm the rapid breakdown of this potentially hypotensive agent, prior to absorption. The experimental animal was the chicken. This species is commercially important in many seleniferous or cyanide prevalent areas; it is also suitable for controlled nutritional investigations.

Liver glycogen, lactate and pyruvate levels were monitored, together with blood glucose concentrations, as a measure of the importance of anaerobic glucose catabolism, during chronic exposure to dietary cyanide and/or selenium.

The possibility that gluconeogenic precursors, such as L-alanine, L-lactate, L-serine and L-cystine, might be capable of modifying the severity of cyanide toxicity was also investigated.

The redox state of the hepatic pyridine nucleotide system was determined during chronic intoxication with cyanide and/or selenium, as a measure of respiratory function. Glutathione redox state was also investigated as both NADPH and GSH are required for selenium metabolism. Hepatic glutathione reductase activity was monitored because this enzyme is responsible for linking the GSSG/GSH couple to the pyridine nucleotide system.

The effect of cyanide on the subcellular distribution and redox state of selenium in liver was studied. The influence of dietary L-cysteine and DL-methionine on these processes was determined and related to the effects of sulphur amino acids supply both on the toxicities of selenium or cyanide, and on the Se-CN interaction. Cyanide effects on the exhalation of selenium in volatile forms were investigated.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Sources of Cyanide

Cyanide and its precursors are ubiquitous in the environment. Man and animals may be exposed to this toxin from a variety of sources.

Cyanide ingestion occurs principally through the consumption of plants containing cyanogenic glycosides, including cassava, sorghum, linseed, sweet potatoes, maize, millet and some legumes. Cassava (*Manihot esculenta*) is the most important of the foodstuffs in which the content of cyanide creates nutritional problems since it forms the staple diet of people and livestock in several regions of Africa, Asia and South America (Cooke and Coursey, 1982). The maximum yield of cyanide from some cyanogenic plants can be as high as 100-300 mg per 100 g of tissue (Montgomery, 1969). It has been estimated that a human adult in the tropics may consume at least 35 mg HCN daily (Nartey, 1981) which represents half the lethal dose of this toxin (Williams & Langford, 1967).

Other sources of cyanide include industrial processes, pesticides, smoke inhalation and some therapeutic drugs (Homan, 1987).

## 2.2 Cyanide Toxicology

### 2.2.1 Chronic Exposure

Evidence suggests that chronic cyanide exposure is a major contributing factor in various human diseases, producing symptoms associated with lesions in the central nervous system. Much of the available data is epidemiological or is based on field studies without controlled experimental conditions, and, in practice it is difficult to ascribe the effects specifically to cyanide. Even so, numerous correlations implicate chronic cyanide exposure in specific diseases. These include: tropical ataxic neuropathy (Osuntokun, 1971), Leber's

optical atrophy, pernicious anaemia, tobacco amblyopia, cretinism, lathyrism, diabetes and goitre (Ekpechi *et al.*, 1966).

### 2.2.2 Acute Exposure

Signs of acute cyanide toxicity vary according to dose, route and source of administration, and range from headaches and dizziness, to seizures, coma and death. These symptoms are caused by the effects of cyanide on the respiratory, cardiovascular and central nervous systems and result primarily from hypoxia (see Holland & Kozlowski [1986] for a review).

## 2.3 Metabolic Effects of Acute Cyanide Toxicity

Consideration will be given to the metabolic effects of acute cyanide toxicity. It is generally assumed that similar effects occur during both chronic and acute exposure, but that these differ in magnitude, leading to the different overall symptoms described above.

### 2.3.1 Enzyme Inhibition

Cyanide is a potent inhibitor of a large number of enzyme systems (Solomonson, 1981). Mechanisms for enzyme inactivation include: the formation of cyanohydrins with carbonyl compounds required for enzyme activity (pyridoxal phosphate dependent enzymes) e.g. glutamate decarboxylase (Tursky & Sajter, 1962); cyanide addition to Schiff's bases (Hansen & Dekker, 1976); scission of essential disulphide links; elimination of sulphur as thiocyanate e.g. xanthine oxidase (Massey & Edmondron, 1970) and combination with functionally essential metal ions e.g. succinate dehydrogenase (Zanetti *et al.*, 1973), superoxide dismutase (Rotilio *et al.*, 1972) and cytochrome c oxidase (Antonini *et al.*, 1971).

Cytochrome c oxidase, the terminal oxidase of the respiratory chain, is the primary target for the action of cyanide, which interacts with the ferric iron in cytochrome $aa_3$. The inhibition of

this enzyme is a two-stage process, involving the initial weak binding of cyanide to the protein, followed by its reaction with haem. Cyanide binds to both the oxidised and reduced forms of cytochrome oxidase, although it has a higher affinity for the oxidised enzyme (Van Buuren *et al.*, 1972). However, the rate of this reaction is two orders of magnitude slower than that between cyanide and the reduced form. Cyanide probably reacts with the reduced enzyme which is subsequently converted to an oxidised enzyme - cyanide complex (Way, 1984). This complex is relatively stable, but in the presence of reducing equivalents, cyanide can dissociate from the enzyme-inhibitor complex to reactivate the enzyme (Solomonson, 1981). The potentially reversible nature of the inhibition of cytochrome oxidase provides the basis for antidotal treatments which deplete intracellular cyanide. These include: nitrites which may act either by promoting methaemoglobin formation (cyanide can interact with the ferric iron in methaemoglobin to form cyanmethaemoglobin) or by virtue of their vasodilatory action; cobalt-containing compounds such as dicobalt EDTA and hydroxocobalamin (cobalt forms a stable metal complex with cyanide); carbonyl compounds including sodium pyruvate and α-ketoglutarate which react with cyanide to form cyanohydrin derivatives, and sulphur donors such as thiosulphate which aid the conversion of cyanide to thiocyanate (see Way *et al.*, 1984 and Way *et al.*, 1988 for reviews).

Studies into the effect of cyanide on cytochrome oxidase *in vitro* have been extensive, however, there remains a paucity of information regarding the actual importance of this inhibition during cyanide exposure *in vivo*. Several workers have demonstrated cytochrome oxidase inhibition in tissues of animals subjected to acute cyanide intoxication (Albaum *et al.*, 1946; Piantadosi *et al.*, 1983; Isom *et al.*, 1975, 1976 & 1982) with brain and heart being the major target sites. However, some studies have suggested that inhibition of cytochrome oxidase may not be the ultimate biochemical lesion in cyanide-induced lethality. Petterson and Cohen (1985) found an equivalent degree of inhibition of this enzyme in brain and heart from mice following subcutaneous injection of 4 or 20 mg KCN $kg^{-1}$; these doses caused 0% and 100% lethality respectively.

### 2.3.2 Carbohydrate Metabolism

The major consequences of the inhibition of cytochrome oxidase and resultant impairment of oxidative phosphorylation are a decreased mitochondrial $NAD^+$:NADH ratio (Baxter & Hensley, 1969; Sahlin & Katz, 1986) and depletion of cellular ATP levels (Albaum *et al.*, 1946; Estler, 1965; Katsumata *et al.*, 1983; Hattori, 1986 and Benabid *et al.*, 1987) both of which bring about feedback inhibition of the TCA cycle. Isom *et al.* (1975) demonstrated a reduction in the involvement of the TCA pathway in $^{14}C$-glucose catabolism in the cyanide treated mouse. Hoyer *et al.* (1984) showed that the levels of the TCA cycle intermediates succinate, fumarate and malate increased in rats receiving an intravenous infusion of 4 mg CN $kg^{-1}$ $hr^{-1}$.

The impairment of mitochondrial function in acute cyanide toxicity results in increased emphasis being placed on anaerobic metabolism to supply the energy requirements of the cells. Many studies have demonstrated cyanide induced decreases in tissue glycogen, ATP, glucose and phosphocreatine concentrations with concomitant increases in ADP, inorganic phosphate, blood and tissue lactate, and blood glucose levels (Albaum *et al.*, 1946; Olsen & Klein, 1947; Estler, 1965; Detwiler, 1972 and Katsumata *et al.*, 1983). Similar effects have been observed in the chicken. Elzubeir (1986) reported increases in blood lactate and pyruvate levels together with an increased lactate: pyruvate ratio in birds during acute cyanide exposure.

The traditional interpretation of such data has been that decreased ATP:ADP ratios stimulate glycolysis resulting in overproduction of pyruvate, which is subsequently converted to lactate with concomitant oxidation of NADH to $NAD^+$. However, this widely accepted view may well be an oversimplification of events occurring during acute cyanide intoxication.

Baxter and Hensley (1969) demonstrated an increase in hepatocyte cytoplasmic $NAD^+$:NADH ratio after administration of a sublethal dose of KCN by stomach tube to rats. However, they also recorded a decrease in liver lactate concentration accompanied by an increase in pyruvate levels. The lactate:pyruvate ratio was halved and there was a significant reduction in the sum of the concentrations of these two

metabolites in the livers of cyanide fed animals. The discrepancies between these results and those previously mentioned cannot be explained in terms of dose or the time-course of the investigation. Baxter and Hensley accounted for their findings by postulating the existence of an alternative pathway of triose phosphate metabolism (Figure 1).

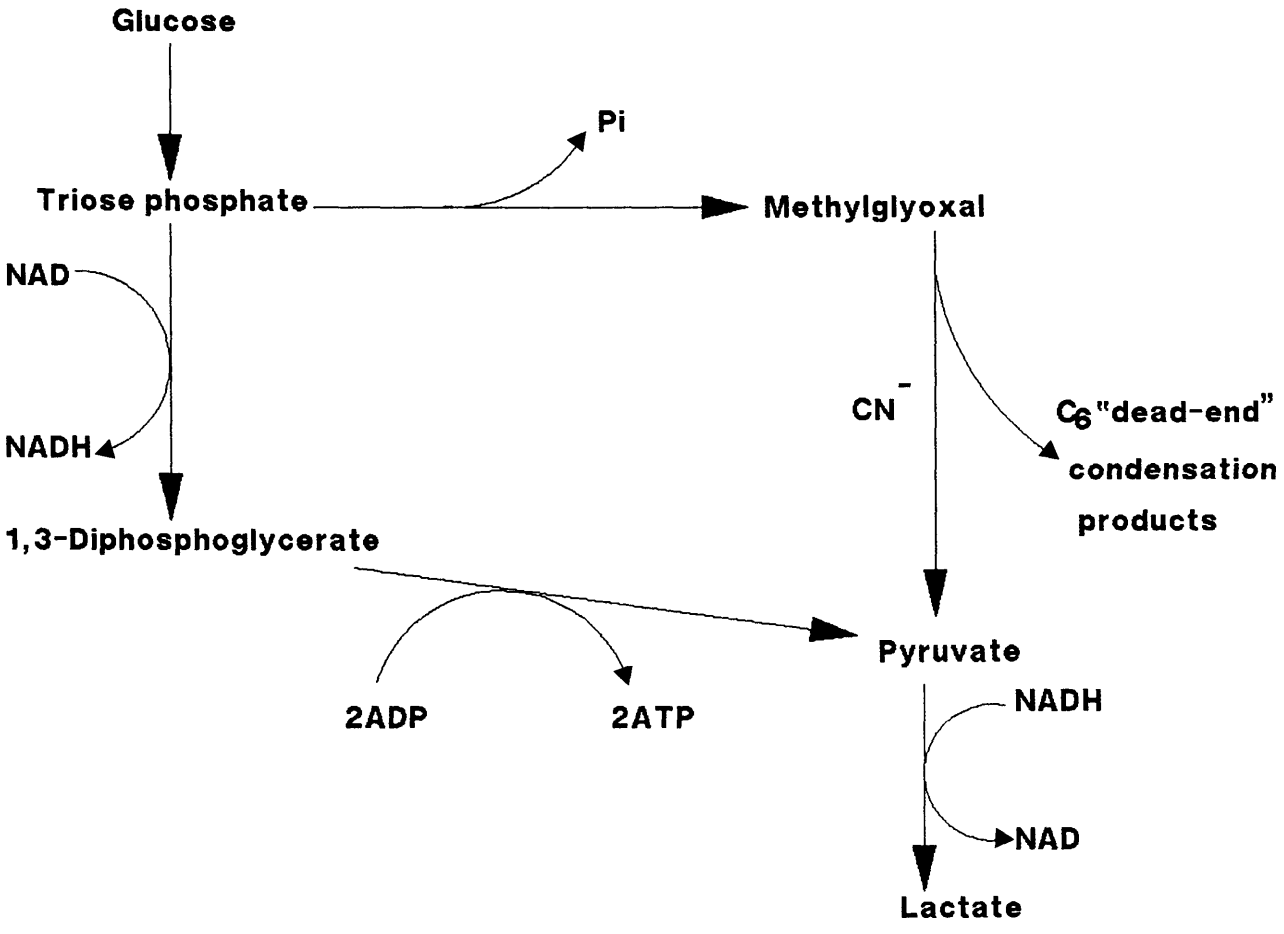



**Figure 1. An alternative pathway for carbohydrate metabolism in the presence of cyanide.**

They based their proposal on *in vitro* experiments which had previously demonstrated, at physiological pH, and in the presence of cyanide, the conversion of a mixture of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate via methylglyoxal ($CH_3COCHO$) to pyruvate (Pette & Ruge, 1963). The first step, the dephosphorylation of triose phosphate to form methylglyoxal, appears to be non-enzymic and independent of cyanide. Cyanide then catalyses the non-enzymic conversion of methylglyoxal to pyruvate, in the proportion 0.5 mole pyruvate formed per mole methylglyoxal. The remaining 3-carbon skeletons are reduced and condensed to unidentified 6-carbon compounds (Smythe, 1933). Since 50% of the methylglyoxal formed is converted to "dead-end" products, lactate formation would be decreased, together with the total lactate and pyruvate concentrations.

Isom *et al.* (1975) investigated the effects of sublethal doses of cyanide on glucose catabolism in mice using radiorespirometric techniques. They concluded that cyanide actually caused an approximate 50% decrease in the catabolism of glucose via the glycolytic pathway as well as decreasing utilisation of the TCA cycle and the glucuronate pathway. This was accompanied by a 100% stimulation of the pentose phosphate pathway, thereby, allowing continued anaerobic degradation of glucose. In an attempt to reconcile these results with the increased lactate levels demonstrated by many previous workers, Isom and colleagues invoked the involvement of the methylglyoxal pathway as a mechanism for increased pyruvate (and therefore lactate) production in the face of a decreased rate of glycolysis. However, it seems unlikely that this pathway would operate sufficiently rapidly to account for such results, especially since only 50% of the total triose phosphate available can be converted to pyruvate in this manner. In fact, since Isom and co-workers used yields from C-3(4) (given off as $CO_2$ when pyruvate is oxidatively decarboxylated to yield acetyl Co-A) as a direct measure of the EMP participation in glucose catabolism, their results might well be explained in terms of the operation of the methylglyoxal pathway, rather than by decreased glycolysis.

There remains some doubt therefore whether the increased anaerobic catabolism of glucose during acute cyanide intoxication is mediated via the glycolytic or the pentose phosphate pathway. Isom *et al.*

(1975) suggested that increased operation of the pentose phosphate pathway and the accompanying increase in NADPH may reflect a compensating mechanism to maintain a balanced redox state since NADPH can reduce $NAD^+$ by means of a transhydrogenase enzyme.

Isom's results raise questions regarding the effect of cyanide on glucose catabolism in the chicken, which has no functional pentose phosphate pathway (see Pearce, 1977 for a review).

Although the precise pathways of glucose metabolism in the cyanide treated animal remain uncertain, there seems little doubt that they are fuelled by glycogenolysis. The well established cyanide induced decreases in liver and brain glycogen (Estler, 1965; Shaffi & Prasad, 1979 and Albaum *et al.*, 1946) are the result of increased glycogen breakdown, rather than decreased synthesis. Jakob and Diem (1974) showed that glucose production and phosphorylase activity in isolated perfused rat livers were increased by cyanide. These findings were confirmed by Detwiler *et al.* (1972) and by Conaglen *et al.* (1984).

## 2.4 Metabolic Effects of Chronic Cyanide Toxicity

There is a dearth of information regarding the metabolic consequences of chronic cyanide exposure. The majority of interest in this field has centred around the effects of cyanide, as a water pollutant, on freshwater fish. Kovacs and Leduc (1982) monitored liver metabolite levels over a period of 20 days, in trout exposed to 0.015 mg $L^{-1}$ HCN. They observed an initial decrease in liver glycogen levels with a concomitant increase in lactate, indicative of a shift towards anaerobic respiration. This was followed by a return to aerobic metabolism after 5-10 days.

Raymond *et al.* (1986) studied cytochrome c oxidase activity and glycogen levels in the livers of rainbow trout exposed to 0.01, 0.02 and 0.03 mg $L^{-1}$ HCN for 20 days. Cytochrome c oxidase activity was inhibited by 60-80% within the first 24 hours of exposure. This level of inhibition remained constant over the experimental period for each of the three concentrations tested. Liver glycogen levels were

depressed, particularly at 0.03 mg $L^{-1}$ HCN. At the lower levels of exposure, glycogen concentrations returned to normal after 7-10 days.

Recently, Padmaja and Panikkar (1989) attempted to mimic the chronic cyanide exposure experienced when cassava forms the staple diet, by intraperitoneal injection of linamarin or cyanide into rabbits, twice weekly, for six months. They demonstrated a considerable accumulation of both lactate and pyruvate in the liver, heart and brain of treated rabbits. Only in skeletal muscle (a tissue with a large capacity for anaerobic respiration) did the expected decrease in pyruvate content occur. These workers also reported decreased liver and brain glycogen levels, together with increased blood glucose concentrations as a result of linamarin or cyanide treatment. However, the relevance of this study to situations in which cyanide intake is daily and by the oral route remains questionable.

Taken together though, the available evidence relating to the metabolic effects of chronic cyanide exposure does suggest that, despite the existence of efficient detoxification mechanisms, prolonged intake of sublethal doses of cyanide may result in its concentration in the tissues reaching a level sufficient to inhibit respiration.

## 2.5 Cyanide Metabolism

It has been known for some time that the major pathway for detoxification of cyanide administered to mammals involves its transulphuration to thiocyanate, which is then excreted in the urine (Boxer & Rikards, 1952). Oh *et al.* (1987) confirmed that similar mechanisms operate in the chicken. The detoxifying reactions are primarily enzymic and may be related to the activity of rhodanese (thiosulphate : cyanide sulphurtransferase, E.C. 2.8.1.1), mercaptopyruvate sulphurtransferase (E.C. 2.8.1.2) and serum albumin, all of which catalyse the transfer of a sulphane sulphur atom (an ionised sulphur bonded only to another sulphur).

Rhodanese, a mitochondrial enzyme, (Sörbo, 1951) which is particularly active in liver and kidney (Himwich & Saunders, 1948) was originally thought to provide the primary mechanism for detoxification of cyanide, through catalysis of the direct reaction between cyanide and a sulphur donor, such as thiosulphate (Lang, 1933). However, several observations suggest that this conventional view may be inadequate. Liver samples from patients with hereditary optic atrophy, who are unable to detoxify cyanide by converting it to thiocyanate, have been shown to possess normal rhodanese activity (Wilson, 1965). Similarly, Rutkowski *et al.* (1985) demonstrated a lack of correlation between hepatic rhodanese activity and acute cyanide lethality in mice. Also, despite the efficacy of thiosulphate as an antidote in cyanide poisoning, it seems unlikely that much of this anion would gain access to the thiosulphate transferase enzyme located in the mitochondrial matrix (Cardozo & Edelman, 1952; Way *et al.*, 1984).

Recently, a broader view of detoxification of cyanide by conversion to thiocyanate has been developed by Westley (1981). He proposes that the supply of sulphane sulphur for the reaction with cyanide is from a rapidly equilibrating pool of potential donors, which include per- and poly-sulphides, thiosulphanates, polythionates, inorganic thiosulphate and protein associated elemental sulphur. The sulphurtransferases catalyse the formation and interconversions of the sulphane sulphur containing compounds. Ultimately, sulphane sulphur may be derived from cysteine, via mercaptopyruvate and the reactions of mercaptopyruvate sulphurtransferase. This enzyme is found in blood, liver and kidney and catalyses the cleavage of the carbon-sulphur bond of its substrate. When the acceptor substrate is sulphite, thiosulphate is the product. Whereas when the acceptor is a thiol, the product is a persulphide (see Sörbo, 1975 for a review of this enzyme and Jarabak & Westley, 1980 for more recent details of reaction mechanism). Rhodanese in the presence of thiols can catalyse the interconversions of all of the physiological forms of sulphane sulphur. The sulphane carrier is thought to be plasma albumin; the sulphane sulphur-albumin complex then reacts with cyanide (Figure 2).

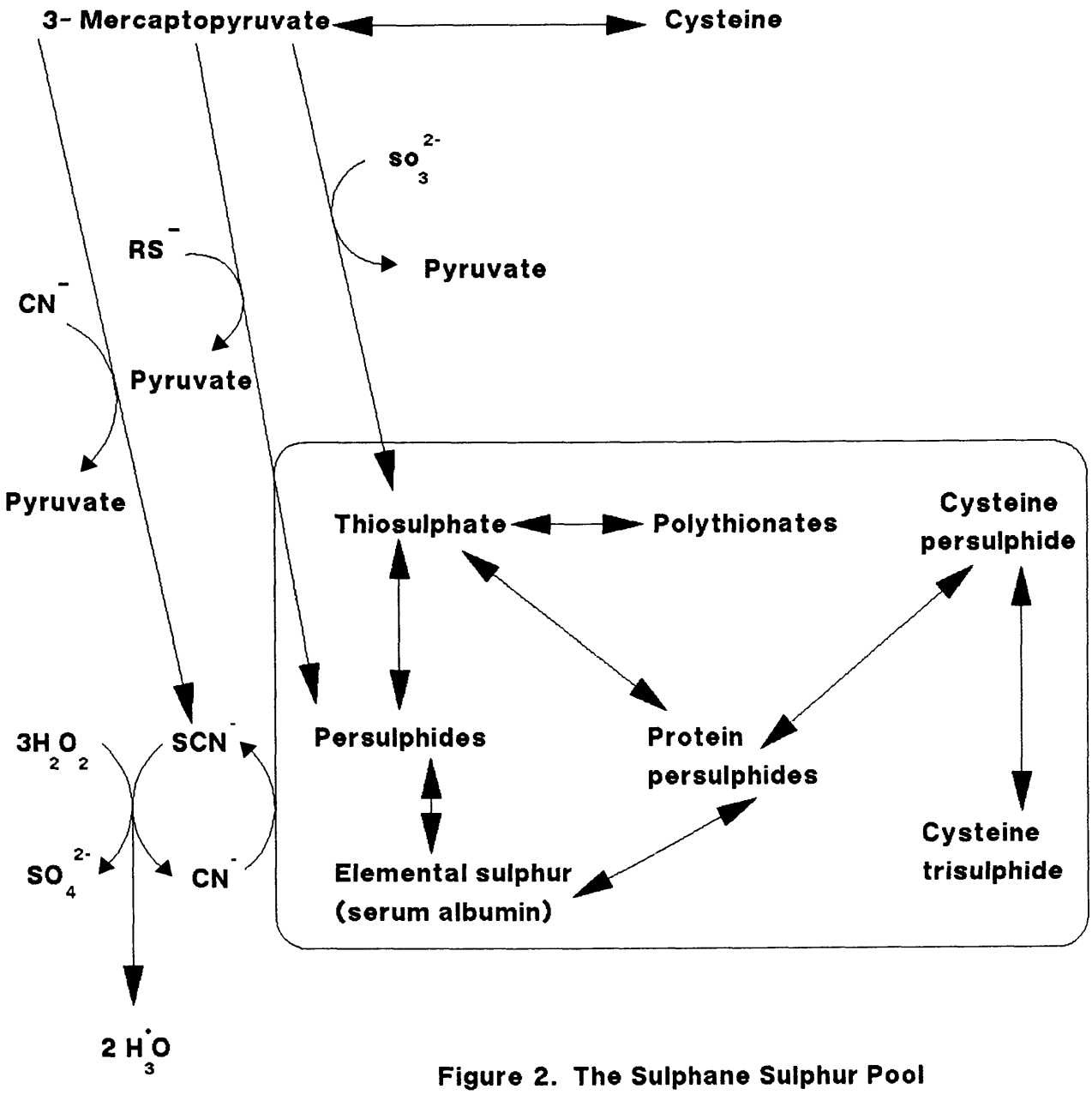



**Figure 2. The Sulphane Sulphur Pool**

Some evidence is beginning to emerge in support of such a hypothesis, however, contradictory evidence also exists, leaving the precise mechanisms of cyanide detoxification somewhat unclear.

*In vivo* pharmacokinetic studies suggest that the conversion of cyanide to thiocyanate occurs predominantly in the central compartment, with a volume of distribution approximating to the blood volume (Sylvester *et al.*, 1983; Way, 1984). Schneider and Westley discovered as long ago as 1963 that serum albumin rapidly becomes

labelled with cyanide-reactive $^{35}S$-sulphane sulphur *in vivo*. More recently, *in vitro* studies of Jarabak and Westley (1986) have shown that some form of sulphur present at very low concentrations in a colloidal suspension of elemental sulphur, is rapidly bound and very slowly released by albumin. Cyanide present at millimolar concentrations reacts rapidly with the complex, forming thiocyanate.

Conflicting evidence has been presented by Piantadosi and Sylvia (1984) who showed that sodium thiosulphate efficiently antagonises cyanide in rats from which the blood has been removed by exchange transfusion. Similarly, Devlin *et al.* (1989) demonstrated the thiosulphate-mediated metabolism of cyanide to thiocyanate in isolated, perfused rat liver and skeletal muscle, in the absence of albumin.

Westley (1988) has recently developed a method for quantitatively estimating the size of the physiologically available pool of sulphane sulphur by monitoring cyanide depletion, under controlled conditions, in the presence of a sulphurtransferase. Measured by this method, the sulphane sulphur concentration in mammalian liver tissue was only about 0.3 mmol $kg^{-1}$ with even less in the bloodstream. The author suggested that, despite the low levels involved, the specific binding of sulphane sulphur by serum albumin, and the rapid reaction of cyanide with the complex, might still allow for a significant role for albumin in cyanide detoxification. However, *in vitro* experiments, including those of Jarabak and Westley (1986) in which the sulphur-albumin complex has been shown to catalyse the conversion of cyanide to thiocyanate, have been carried out at pH 9.5. The significance of the rate of this reaction at physiological pH remains questionable.

Other minor detoxification pathways for cyanide are as follows:

(1) Exhalation in breath as HCN and as $CO_2$ from oxidative metabolism (Boxer & Rickards, 1952; Johnson & Isom, 1985).

(2) Spontaneous reaction with cystine to produce 2-iminothiazolidine-4-carboxylic acid, which tautomerises to 2-imino-4-thiazolidine carboxylic acid (Wood & Cooley, 1956).

(3) Combination with hydroxocobalamin to form cyanocobalamin which is excreted in urine and bile (Herbert, 1975).

The majority of investigations into the metabolism of cyanide have been carried out during acute intoxication. However, when Okoh (1983) monitored the excretion of $^{14}C$-labelled cyanide in rats exposed to chronic cyanide intakes, he was able to demonstrate that urinary excretion of thiocyanate was also the predominant mode of elimination under these conditions.

## 2.6 Cyanide Metabolism and Sulphur Supply

Consideration of the pathways involved in cyanide detoxification has led to suggestions that sulphur supply may limit this process. Westley (1980) went so far as to propose that depletion of the sulphane sulphur pool might be responsible for some of the secondary effects of chronic cyanide toxicity.

Since the ultimate sources of endogenous sulphur are the dietary sulphur amino acids, numerous attempts have been made to assess the efficacy of these nutrients in the alleviation of chronic cyanide toxicity. Several workers have reported an improved growth performance in poultry, pigs, rats and rabbits, when methionine is added to cassava-based diets (Enriquez & Ross, 1967; Ross & Enriquez, 1969; Olsen *et al.*, 1969; Maner & Gómez, 1973 and Adegbola, 1977). In contrast, Gómez *et al.* (1984) failed to demonstrate any beneficial effect of methionine supplementation on growth when cassava-based diets were fed to pigs. Similar results were obtained by Elzubeir (1986) using the chicken as an experimental animal, although in this study, cystine was beneficial. Dietary methionine supplementation has been shown to increase thiocyanate excretion when cyanide is fed to rats (Barrett *et al.*, 1978; Maner & Gómez, 1973), however, Elzubeir (1986) found no effect of dietary methionine on plasma thiocyanate levels in cyanide treated chickens.

The results of dietary sulphur amino acid supplementation studies have, therefore, been both contradictory and inconclusive. Alternative approaches have focused around attempts to demonstrate

depletion of endogenous sulphur in cyanide intoxication. Osuntokun (1973) reported a drastic reduction in plasma sulphur amino acid levels in patients suffering from ataxic neuropathy associated with high cassava diets. By contrast, Vis *et al.* (1982) found no difference in serum methionine level between euthyroid patients from cassava eating areas of Africa and a control group from Brussels. Since cassava containing diets are generally deficient in sulphur amino acids, any correlation between their levels in serum and consumption of this foodstuff does not imply a causal relationship between these factors.

Oh *et al.* (1987) demonstrated a decrease in the excretion of thiosulphate in chickens following injection of 30 µmoles of cyanide intramuscularly, which may be indicative of increased utilisation of sulphane sulphur. However, simultaneous administration of either thiosulphate or sulphur amino acids decreased thiocyanate excretion. This result was explained in terms of increased *in vivo* production of sulphite which competed with cyanide for transferable sulphur.

On the basis of the available evidence to date, therefore, it is impossible to conclude whether endogenous sulphur is a limiting factor in cyanide detoxification.

## 2.7 Sodium Nitroprusside as a Source of Cyanide in Experimental Diets

The cyanide content of cassava, both before and after processing, is highly variable, making it unsuitable for controlled experimental investigation of chronic cyanide toxicity. The high cost of individual cyanoglycosides restricts their use as sources of dietary cyanide. Consequently, the majority of studies have made use of cyanide salts of alkali metals (Maner & Gómez, 1973; Hill, 1977 and Philbrick *et al.*, 1979). However, these salts are highly unstable and yield poor recoveries from standard diets (Okoh, 1978; Carew, 1986 and Palmer & Olsen, 1979). Sodium nitroprusside ($Na_2[Fe(CN)_5NO].2H_2O$) which has been employed clinically as a hypotensive agent (Page *et al.*, 1955), was used as a stable, alternative source of dietary cyanide in the investigations reported in this thesis.

There is ample evidence to suggest that SNP breaks down in the body with the release of cyanide. Vesey *et al.* (1974) detected cyanide in the blood and tissues after administration of SNP and *in vitro* studies have demonstrated the release of cyanide from this drug on contact with animal tissues (Page *et al.*, 1955 and Nakamuro *et al.*, 1977).

Recently, Butler *et al.* (1988) have challenged these early studies. They reported on NMR spectroscopic studies which demonstrated that the inorganic products formed on incubation of SNP with intact erythrocytes were nitric oxide and hexacyanoferrate (11), $[Fe(CN)_6]^{4-}$. At no stage was free cyanide liberated. They dismissed the results of previous workers as being artefactual, proposing that exposure of SNP to light, either during administration or at the assay stage, resulted in its conversion to aquapentacyanoferrate ion $[Fe(CN)_5.H_2O]^{2-}$, which, being unstable at the low pH used to analyse cyanide, then decomposed to release cyanide. However, this hypothesis is incompatible with the following contemporary observations:

(1) Norris and Hume (1987) demonstrated that mice brain cytochrome c oxidase activity was sensitive to the *in vitro* addition of KCN, but was unaffected by SNP. However, enzyme activity was inhibited in brains removed from mice after injection of the animals with an $LD_{80}$ dose of either KCN or SNP. In KCN-treated animals cytochrome c oxidase activity was inhibited by 60% 1 minute after injection, however, 3 minutes were required after SNP administration before the same level of inhibition was obtained. The design of this study ensured that the production of aquapentacyanoferrate ions would have been negligible. These results confirm not only that cyanide is released from SNP *in vivo*, but that a time interval is necessary for this release to occur.

(2) SNP administration results in raised blood thiocyanate levels (Page *et al.*, 1951; Vesey *et al.*, 1974 and Elzubeir & Davis, 1988b) as well as an increase in expired HCN (Vesey, 1987).

(3) Cyanide antidotes are effective against SNP poisoning (Vesey, 1987).

(4) The alterations in carbohydrate metabolism following SNP injection parallel those occurring in cyanide intoxication (Rommel & Hoyer, 1984).

There is an abundance of evidence, therefore, supporting the conclusion that cyanide is released from SNP *in vivo*. The NMR results provided by Butler's group may well be artefactual, caused by the very high concentrations of SNP employed in their investigations.

The decomposition of SNP involves its interaction with sulphydryl compounds (Page *et al.*, 1955) of the hydrogen donor type (Nakamura *et al.*, 1977). *In vitro* studies have suggested that the erythrocytes may be important in cyanide release from SNP (Page *et al.*, 1955 and Smith & Krusyna, 1974). Butler *et al.* (1988) demonstrated that incubation of intact erythrocytes with SNP resulted in oxidation of cellular glutathione to diglutathione. The reaction of nitroprusside with glutathione occurred after the ion had crossed the erythrocyte membrane. However, several studies have demonstrated that the nitroprusside ion, when present in clinical concentrations, does not penetrate the red cell membrane (Mishra & Passow, 1969; Smith & Krusyna, 1974; Vesey *et al.*, 1983; Rodkey & Collison, 1977 and Vesey *et al.*, 1987). Schulz (1984) has proposed that injected SNP reacts with sulphydryl compounds, and/or reducing agents derived from the red blood cell and arterial walls.

With regard to the use of orally administered SNP as a source of dietary cyanide, Page *et al.* (1955) demonstrated that chronic oral administration of the drug (30-60 mg, 4 times a day) did not result in hypotension. It may be concluded that no appreciable amounts of unchanged sodium nitroprusside are absorbed from the gastrointestinal tract.

Considering all of the available evidence, SNP does appear to be a suitable source of dietary cyanide for nutritional biochemical studies. In addition, it will be shown in Section 4.1 of this thesis, both that cyanide is released from dietary SNP in the gastrointestinal tract of the chicken, and that ingested SNP is not absorbed intact into the bloodstream.

## 2.8 Natural Occurrence of Selenium

Selenium, an element possessing both metallic and non-metallic properties, is widely distributed throughout the environment, being present in rocks, soils, water and air. This element enters the food chain when taken up by plants, and occurs in foods and feedstuffs almost exclusively in organic compounds, primarily selenomethionine, Se-methylselenomethionine, selenocysteine and selenocystine (Shrift, 1969 and Olson *et al.*, 1970). Selenium is recognised as an essential trace nutrient as well as a highly toxic substance. Since the selenium content of plants depends on the concentration and availability of soil selenium, intakes of this nutrient in man and animals vary considerably in different areas of the world, with deficiency being far more widespread than toxicity (Yang, 1987; Liu *et al.*, 1987; Combs & Combs, 1986). The supplementation of animal feeds with selenium in the form of inorganic sodium selenite has become common practice.

## 2.9 Essentiality of Selenium

The nutritional importance of selenium was first recognised by Schwartz and Folz (1957) who demonstrated its ability to prevent necrotic degeneration of the liver in vitamin E deficient rats. This observation was immediately confirmed in the chicken by Schwartz *et al.* (1957) and by Patterson *et al.* (1957), who showed that selenium prevented the vitamin E deficiency disease known as exudative diathesis, a condition characterised by leakage of plasma through the capillaries into the subcutaneous spaces. Subsequently, Thompson and Scott (1969), using low selenium diets to which graded levels of vitamin E were added, obtained evidence that selenium is essential for growth, independent of, or additional to its function as a substitute for vitamin E. Dietary selenium levels of only 0.1-0.2 mg $kg^{-1}$ are adequate to satisfy the nutritional requirements of most animals, and in some cases vitamin E may partially replace selenium (Combs & Combs, 1986).

## 2.10 Functions of Selenium

The close association between selenium and vitamin E, a known antioxidant, lead to proposals that both might fulfil similar metabolic roles. These suggestions were verified in 1973 when Rotruck *et al.* discovered that selenium is an integral component of the enzyme glutathione peroxidase (glutathione:$H_2O_2$ oxidoreductase, E.C. 1.11.1.9). Selenium-dependent GSHPx functions as one of the cellular antioxidant defence mechanisms, which include vitamin E, catalase and superoxide dismutase. Such components protect the cell from the adverse effects of reactive oxygen and of free radical initiators, such as hydrogen peroxide (Chow, 1979). If unchecked, these reactive species would initiate lipid peroxidation (an autocatalytic process resulting in membrane damage) together with structural alteration of proteins, including critical enzymes. Glutathione peroxidase utilises reducing equivalents from GSH in the reduction of $H_2O_2$ (Mills, 1959), free fatty acid hydroperoxides (Christopherson, 1969) or sterol hydroperoxides (Little, 1972), according to the following general reaction:

$$ROOH + 2GSH \xrightarrow{SeGSHPx} ROH + H_2O + GSSG$$

It is not yet clear whether SeGSHPx can use esterified fatty acid hydroperoxides as substrates *in vivo* (MaCay *et al.*, 1976 and Chow, 1979). The function of SeGSHPx is complementary to that of vitamin E. The former is located in the cytosol and mitochondrial matrix (Green & O'Brien, 1970), whilst vitamin E, owing to its lipophilic nature is confined within membranes. The substrate specificity and compartmentation of SeGSHPx, therefore, lend credence to the view that its function, *in vivo*, is to reduce hydrogen peroxide rather than lipid hydroperoxides.

SeGSHPx is composed of four identical sub-units and most authors report a stoichiometry of about 4 g-atoms of selenium per mole of enzyme (Combs & Combs, 1986). Wendel *et al.* (1975) showed that selenium participates at the active site of the enzyme, and Forstrom *et al.* (1978) demonstrated its presence as selenocysteine. The catalytic mechanism of SeGSHPx has not been fully elucidated, but it is thought to be of the double displacement type and involve changes

in the oxidation state of the enzyme-bound selenium (see Combs & Combs, 1986, for a review).

SeGSHPx activity has been used as a measure of selenium status in both man and animals (Omaye & Tappel, 1974; Gabrielsen & Opstvedt, 1980). Although this approach can be extremely useful, it should be remembered that the relationship between these factors may become alinear in some situations (Lane *et al.*, 1979; Elzubeir, 1986; McMurray *et al.*, 1987; Whanger & Butler, 1988).

SeGSHPx is not the only enzyme with peroxidase activity that utilises glutathione as substrate. There is a non-selenium-dependent GSHPx, which acts on lipid hydroperoxides, but shows very little affinity for $H_2O_2$ (Lawrence & Burk, 1976). This activity has been attributed to the glutathione S-transferases (Lawrence & Burk, 1978, and Pierce & Tappel, 1978).

Selenium may have functions independent of its role in SeGSHPx. Several other selenoproteins have been identified in mammalian tissues (Tappel *et al.*, 1984; Tappel, 1987; Beilstein *et al.*, 1981; McConnell *et al.*, 1979; Motsenbocker & Tappel, 1982a; Behne *et al.*, 1989; Whanger *et al.*, 1989). No biological function has been ascribed to any of these proteins, except for a plasma protein, designated selenoprotein P, which is thought to function as a transport protein for selenium (Motsenbocker & Tappel, 1982b).

Selenium may also have roles in drug metabolism (Burk & Correia, 1981) and haem homeostasis (Correia & Burk, 1983).

## 2.11 Absorption of Selenium

The majority of selenium compounds are efficiently absorbed from the gastrointestinal tract, with estimates of absorption ranging from 80-95% of administered dose for most species (Thom son & Stewart, 1973; Brown *et al.*, 1972 and Cary *et al.*, 1973). Uptake does not appear to be regulated since the selenium status of the animal does not influence absorption (Brown *et al.*, 1972). The major site of uptake appears to be the small intestine (Whanger *et al.*, 1976; Pesti

& Combs, 1976, and Wolffram *et al.*,1985). The mechanism of selenium absorption is dependent on its form. The majority of available evidence suggests that, whilst L-selenomethionine and selenate can be actively transported against a concentration gradient, selenocysteine and selenite are absorbed by passive diffusion (McConnell & Cho, 1965; Ardüser *et al.*, 1985, and Wolffram *et al.*, 1985). Contrasting data were provided by Anundi *et al.* (1984a), who reported that intestinal cells can concentrate selenite, and suggested that reduced glutathione and Y-glutamyltransferase may play a role in this process.

## 2.12 Plasma Transport of Selenium

Absorbed selenium is transported around the body bound to plasma proteins (Hirooka & Galambos, 1966a; Jenkins *et al.*, 1969; Burk, 1973 and Porter *et al.*, 1979). *In vitro* studies have suggested that this binding may require the initial uptake and metabolism of selenium by erythrocytes (Lee *et al.*, 1969; Jenkins & Hidiroglou, 1972; Gasiewicz & Smith, 1977; Mas & Sakar, 1989). However, the significance of such reactions *in vivo* remains questionable since dietary selenium is likely to have been altered by the intestinal cells prior to reaching the blood. The relative distribution of selenium among plasma proteins shows species, time and dose dependent variations (Jenkins *et al.*, 1969; Sternberg & Imbach, 1967; Hirooka & Galambos, 1966a; Burk, 1973; Burk, 1974 and Herrman 1977). Protein synthesis may be required for the binding of selenium to plasma proteins (Burk, 1973 and Herrman, 1977) which, dialysis studies suggest, may involve a selenium-sulphur bond (Burk, 1973 & 1974; Herrman, 1977). The mechanisms by which selenium is ultimately released from the plasma proteins and taken up by tissues remain unclear, however, Gómez and Tappel (1989) have recently demonstrated the presence of a selenoprotein P receptor in the rat.

## 2.13 Tissue Distribution and Forms of Selenium

The tissue distribution of selenium is influenced by selenium status (Burk *et al.*, 1972 and Hopkins *et al.*, 1966), dose (Hirooka & Galambos, 1966a), the form of selenium administered (Sternberg &

Imbach, 1967) and the time after dosing (Bopp *et al.*, 1982). In animals consuming selenium at levels close to their nutritional requirements, the highest concentrations are found in the liver and kidney, with lower levels in muscle and glandular tissue (Schoental, 1968 and Thom son & Stewart, 1973).

A similar pattern of distribution has been observed in both acute and chronic selenosis. Detoxifying organs tend to accumulate the greatest amounts of selenium. The highest concentrations are found in the liver, followed in decreasing order by the kidneys, spleen and lungs (Millar & Williams, 1940, McConnell, 1941; Rhian & Moxon, 1943; Rosenfeld & Beath, 1945). Myocardium, skeletal muscle and brain contain only small amounts and fat has virtually no selenium (Martin, 1973).

Other investigators have observed that in selenium toxicity, the kidney accumulates higher levels of the element than does the liver (Levander & Argrett, 1969; Levander *et al.*, 1970; Levander & Morris, 1970).

Selenium is present in tissues in at least three oxidation states: acid volatile selenium, zinc-hydrochloric acid-reducible selenium assumed to be selenite and higher oxidation states such as selenate and organic selenium derivatives, with the latter group accounting for around 70% of the total selenium (Diplock *et al.*, 1971). Tappel (1987) has recently suggested that over 80% of the total selenium in rat tissues is associated with protein in the form of selenocysteine. The chemical forms of selenium present in tissues are influenced by the chemical forms of selenium administered (Beilstein & Whanger, 1988; Whanger, 1986; Millar *et al.*, 1973; Whanger & Butler, 1988) as well as by other factors including vitamin E and arsenic (Diplock *et al.*, 1971).

## 2.14 Selenium Metabolism

Selenium metabolism in animals is generally considered to be related to the metabolism of selenite, which may well be an oversimplified view. The retention and utilisation of selenium does

vary according to its source (Cary *et al.*, 1973; Whanger, 1986, and Whanger & Butler, 1988).

Selenium ingested in organic forms ultimately becomes incorporated into the same metabolic pool and yields the same products as selenium from selenite (Millar *et al.*, 1973; Nahapetian *et al.*, 1983; Thom son *et al.*, 1975a and b; Richold *et al.*, 1977; Sunde, 1984, and Foster *et al.*, 1986), although the initial metabolism of selenoamino acids does differ from that of selenite (Thom son *et al.*, 1975a). This might be explained in terms of the widely accepted view that these compounds first enter the sulphur amino acids pool (Esaki *et al.*, 1981). However, recent evidence suggests that some of the pathways of selenoamino acid metabolism may differ from those of their sulphur-containing analogues (Esaki *et al.*, 1982 and Soda *et al.*, 1987).

Selenate is probably metabolised as selenite following an initial reduction step (McConnell & Portman, 1952; Hirooka & Galambos, 1966b, and Nakamuro *et al.*, 1977).

Two major excretory end products of selenium metabolism in whole animals have been identified as dimethylselenide in expired air (McConnell & Portman, 1952; Nakamuro *et al.*, 1977) and trimethylselenonium ion in urine (Byard, 1969; Palmer *et al.*, 1969 & 1970; Kiker & Burk, 1974). However, the pathways which might be involved in the reduction and methylation of selenite to yield such products, have generally been investigated *in vitro*. A scheme for selenite metabolism proposed in 1974 by Ganther and Hsieh, based on such data, has become widely accepted, although its relevance to processes occurring *in vivo*, has yet to be firmly established (Figure 3).

In 1966 Ganther demonstrated the conversion of sodium selenite into dimethylselenide in mammalian tissue slices and homogenates. Further investigation of this reaction in cell-free liver preparations lead to the following observations:

(1) The process was enzyme catalysed.
(2) Anaerobic conditions were necessary for optimal activity.
(3) There was an absolute requirement for glutathione.

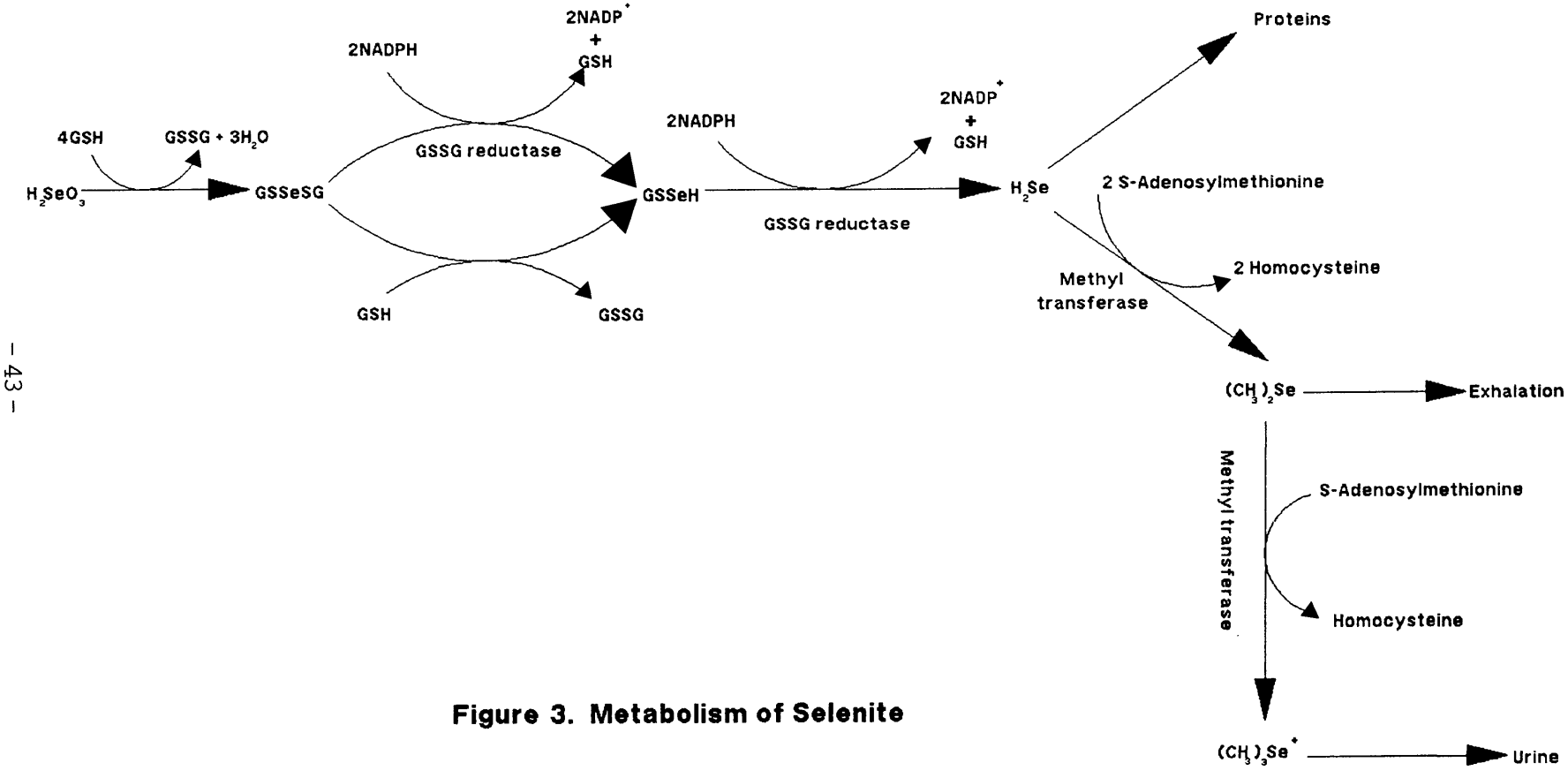



**Figure 3. Metabolism of Selenite**

(4) NADPH stimulated the reaction.
(5) S-adenosylmethionine was the methyl donor.

Ganther (1968) then showed that selenious acid ($H_2SeO_3$) reacts spontaneously with various thiols to yield the disulphide (RSSR) and selenotrisulphide (RSSeSR), both of which are relatively unstable. By lowering the pH to 1.3 and increasing the concentration of reactants to 16.7 mM, Ganther was able to obtain a stoichiometry of 4:1, conforming to the reaction originally proposed by Painter in 1941.

$$4RSH + H_2SeO_3 \longrightarrow RSSeSR + RSSR + 3H_2O$$

However, the significance of this reaction *in vivo* must be questioned. Under physiological conditions, with a GSH:Se ratio greatly in excess of 4:1 ($10^{-6}$M selenious acid, 4 mM GSH, pH 7.0 and 25°C), the major product formed was selenopersulphide (GSSeH) (Ganther, 1971).

Ganther (1971) went on to examine the activity of GSSeSG as a substrate for glutathione reductase (E.C.1.6.4.2), a possibility suggested by its close structural similarity to GSSG. He was able to demonstrate the formation of GSSeH by the reaction of glutathione reductase on GSSeSG, in the presence of NADPH, under physiological conditions. Therefore, if GSSeSG is formed, *in vivo*, there may be two pathways for selenopersulphide formation in animals.

Selenopersulphide is unstable and may decompose to GSH and elemental selenium (Ganther, 1971). However, under anaerobic conditions, GSSeH can be converted to acid-volatile selenium, presumably $H_2Se$, either enzymatically, by glutathione reductase in the presence of NADPH, or non-enzymatically with excess GSH (Hsieh & Ganther, 1975). Studies of Diplock *et al.* (1973) suggest that such reactions might be important *in vivo*. These workers demonstrated the presence of acid-volatile selenium, which behaved in a manner indistinguishable from $H_2Se$, in homogenates or microsomal fractions from livers of rats given sodium selenite.

Hsieh and Ganther (1977) provided information on the methylation reactions of selenite metabolism. They studied the synthesis of

dimethylselenide (DmSe) from sodium selenite by cell-free preparations derived from rat liver and kidney under anaerobic conditions, in the presence of GSH, an NADPH generating system, S-adenosylmethionine and coenzyme A. Fractionation of the soluble portion of liver or kidney, produced only one fraction (fraction C) which possessed the ability to synthesise DmSe. Further purification of this fraction yielded one protein (30,000 molecular weight) which retained this activity. The addition of another fraction (A) stimulated DmSe synthesis by fraction C. This activity was found to be associated with glutathione reductase and another NADPH-dependent disulphide reductase. The authors concluded that fraction C contained a methyltransferase (arsenite insensitive) acting on small amounts of $H_2Se$ produced in the presence of excess GSH and that stimulation by fraction A resulted from the production of additional $H_2Se$ catalysed by glutathione reductase. Washed liver microsomes also synthesised dimethylselenide and addition of the soluble fraction again stimulated activity. This microsomal activity was attributed to another methyl-transferase (arsenite sensitive) which methylates $H_2Se$ produced in the soluble fraction. Therefore, the pathways for reduction of selenite to $H_2Se$ occur in the cytosolic fraction of the cell, whilst methylation of the $H_2Se$, so produced, occurs in both cytosolic and microsomal fractions. It is not yet known whether these methyltransferases are specific for $H_2Se$ or whether they belong to a group of such enzymes known to be active in methylating compounds containing sulphur and oxygen. The microsomal methylation system can be induced by alterations in the diet, or by exposure to xenobiotics (El-Bergeammi *et al.*, 1980; Ganther *et al.*, 1966 and Hsieh & Ganther, 1976).

Challenger (1951 & 1959) proposed an alternative pathway for the formation of DmSe based on work with fungi, in which selenite is first methylated then reduced (Figure 4). However, no evidence has been presented for the existence of such a pathway in animals.

It is generally assumed that the other major product of selenite metabolism, urinary trimethylselenonium, is synthesised from dimethylselenide by the addition of a third methyl group. Mozier *et al.* (1988) have identified and purified an enzyme from the cytosol of mouse lung and liver capable of catalysing this reaction, in the presence of S-adenosylmethionine. The enzyme, designated S-adenosyl-

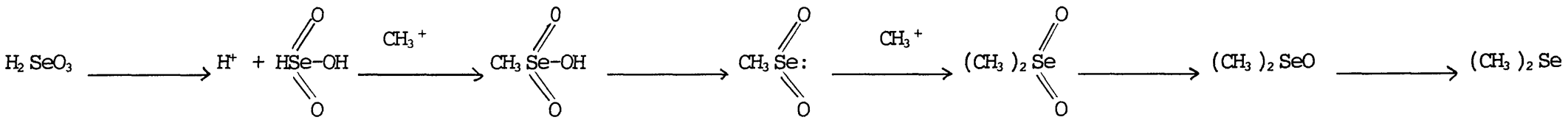





Figure 4. Alternative Pathway For Dimethylselenide Formation As Proposed By Challenger.

L-methionine: thioether S-methyltransferase, which has a low $K_m$ for DmSe and is subject to product inhibition by S-adenosylhomocysteine, appears to be solely responsible for TmSe synthesis in this species. However, earlier work suggested that the metabolic relationship between DmSe and TmSe can be more complex than this in some situations. Palmer *et al.* (1969) administered arsenic to rats on the assumption that this element, which lowers DmSe formation, would likewise, inhibit the production of TmSe. Although arsenic did reduce TmSe excretion slightly, the results failed to achieve significance, indicating that DmSe may not be an obligatory intermediate in synthesis of the urinary metabolite. Obermeyer *et al.* (1971) reported that less than 0.5% of the $^{75}Se$ dose from intraperitoneally administered DmSe was excreted in the urine of rats within 24 hours. Conversely, following the injection of $^{75}Se$-TmSe, 3-9% of the dose was excreted in expired air in 6 hours. No attempt was made to identify the volatile selenium compound, but based on a "garlic-like" odour of the breath it was presumed to be DmSe, suggesting that TmSe can be demethylated to yield DmSe.

Hydrogen selenide produced by the reduction of administered selenite, therefore, either undergoes methylation and is subsequently excreted, or it becomes bound to tissue proteins (McConnell, 1948). Whether this occurs through direct reaction with protein sulphydryl groups, or requires prior synthesis of selenoamino acids, remains controversial (McConnell & Wabnitz, 1957; Fuss & Godwin, 1975; Swartz & Sweeney, 1964). Most authors do agree that selenocysteine synthesis from administered selenite is possible, but that selenomethionine is not formed in this manner (Olson & Palmer, 1976).

The incorporation of selenium into proteins may involve either non-specific substitution (Hoffman *et al.*, 1970; Olson & Palmer, 1976; Ganther, 1975; Favier, 1989; Mukai, 1974), or a specific process, such as occurs in the incorporation of selenocysteine into SeGSHPx. The mechanism of selenocysteine insertion has yet to be fully elucidated, but it probably occurs cotranslationally by modification of a serinyl tRNA (Hawkes *et al.*, 1979; Sunde & Everson, 1987; Stadtman, 1987; Leinfelder *et al.*, 1988).

## 2.15 Selenium Excretion and Homeostasis

Selenium can be eliminated from the body by the lungs, the urinary tract, the intestinal tract and in sweat. The amount of selenium excreted by each route is dependent upon the level of selenium intake (Hopkins *et al.*, 1966; Burk *et al.*, 1972 & 1973; McConnell & Roth, 1966; Hirooka & Galambos, 1966b), the form of selenium administered (Thomson *et al.*, 1975 a & b; Richold *et al.*, 1977), previous exposure to selenium (Halverson *et al.*, 1962; Magos *et al.*, 1987), dietary composition (Hopkins *et al.*, 1966; Ganther *et al.*, 1966) and the presence of certain toxins such as arsenic and cyanide (Kamstra & Bonhorst, 1953; Palmer *et al.*, 1970; Ganther & Baumann, 1962; Levander & Baumann, 1966a; Palmer & Olson, 1979).

At physiological levels of selenium intake, urine and faeces are the predominant routes of excretion, accounting for approximately 60% and 35% respectively, of administered dose, with expired air, saliva and sweat each eliminating a further 1% (Stewart *et al.*, 1978; Patterson *et al.*, 1987). Selenium homeostasis under such conditions is achieved largely by regulation of urinary excretion, with faecal elimination accounting for a relatively constant fraction of the total selenium output over a range of dietary intakes (Burk *et al.*, 1972). Burk *et al.* (1973) showed that, in rats, there is a dietary threshold of approximately 0.05-0.08 mg $Se^{-1}$ $kg^{-1}$ below which the percentage of dose excreted in the urine remains constant. The existence of such a threshold probably represents a mechanism for selenium conservation during periods of low intake.

At high or toxic levels of selenium intake the pulmonary route of excretion assumes far greater importance. McConnell & Roth (1966) reported that male rats receiving 0.005 mg $Se^{-1}$ $kg^{-1}$ subcutaneously, excreted only 0.2% of the selenium in expired air. Increasing the dose to 0.9 mg $Se^{-1}$ $kg^{-1}$ increased respiratory elimination to 10.6%. With 2-3 $mg^{-1}$ $kg^{-1}$ doses about 40% was excreted by the lungs and with 4-5 $mg^{-1}$ $kg^{-1}$ this figure rose to 50-60%. The percentage of selenium excreted in the urine decreased concomitantly. Of the selenium exhaled within 24 hours, 70% was lost in the first 6 hours. With toxic doses of selenium, therefore, the normal pathways for TmSe synthesis appear to be saturated and DmSe is formed, providing a route

for the rapid elimination of selenium under these conditions. (As previously mentioned S-adenosyl-L-methionine: thioether S-methyltransferase is known to have a low $K_m$ for DmSe).

Trimethylselenonium is the only well-characterised urinary metabolite of selenium, although several others have been isolated. Following its identification in the urine of rats given selenite (Byard, 1969; Palmer *et al.*, 1969), subsequent work demonstrated that, in this species, TmSe is an important excretory product of selenium from most sources, accounting for 20-50% of the total urinary selenium, whilst a second metabolite, an unidentified organic compound, designated U-2 contributed 11-28% (Palmer *et al.*, 1970). Kiker and Burk (1974) identified the same two metabolites in rat urine, together with an inorganic form and two other compounds, designated U-3 and U-4, all of which remain uncharacterised. Ganther (1987) recently demonstrated that over 50% of the selenium excreted in human urine was present in cationic forms of which only 11% was TmSe. The remaining 40% of selenium metabolites were anionic.

The relative proportions of the various urinary metabolites of selenium appear to be affected both by the selenium status of the animal and by dose (Byard & Baumann, 1967; Kiker & Burk, 1974). In fact, the quantitative significance of TmSe at low doses of selenium has been questioned (Nahapetian *et al.*, 1983). Recently, Ostadalova *et al.* (1988) suggested that the primary selenium-containing excretory products in the urine of young rats injected with a toxic dose of [$^{75}$Se] selenite were glutathione selenotrisulphide (GSSeSG) and an unidentified neutral substance, whilst adult rats excreted predominantly TmSe. The authors evoked ontogenetic differences in selenium metabolism as an explanation for their results. Further investigation is therefore required in order to identify the forms of selenium excreted in urine under different conditions.

At least two volatile selenium compounds have been identified in expired air, dimethylselenide (McConnell & Portman, 1952) and dimethyldiselenide (Jiang *et al.*, 1983). The nature of the selenium compounds excreted is dependent upon the form in which the element is administered. Jiang *et al.* (1983) analysed selenium metabolites in the breath of mice after administration of DL-selenomethionine, DL-

selenocystine and selenite. Selenocystine and selenite addition to drinking water resulted in the production of dimethylselenide together with some dimethyldiselenide, while for selenomethionine the main compounds were dimethyldiselenide and another unidentified selenium metabolite.

## 2.16 Selenium Toxicity

Three clinical types of selenium toxicosis have been recognised: acute selenosis, sub-acute selenosis causing neurological symptoms and respiratory distress (blind staggers), and chronic selenosis resulting in dermatitic lesions and emaciation (alkali disease). The latter two conditions are most frequently observed in livestock grazing in seleniferous areas. A relatively narrow range separates the levels of selenium that are nutritionally essential from those that may be toxic during chronic exposure (Fitzhugh *et al.*, 1944). Data relating to the chronic toxicity of the various selenium compounds are difficult to collate (see Combs & Combs, 1986, for a review of published studies). However, certain broad generalisations are possible. The soluble inorganic salts, selenite and selenate are relatively toxic. The selenoamino acids, such as selenomethionine, selenocysteine, selenocystine and glutathione selenotrisulphide have relatively moderate toxicities, whilst the insoluble forms, such as elemental selenium, are the least toxic.

### 2.16.1 Biochemical Lesions

The primary biochemical sites at which selenium exerts its toxic effects remain unclear, however, they may involve the oxidation of, and/or binding to, sulphydryl groups essential for normal cellular function.

Selenium intoxication, whether acute or chronic in nature, appears to be associated with increases in the hepatic concentrations of GSH, GSSG and/or other non-protein sulphydryls, together with a shift in the GSSG:GSH ratio towards a more oxidised state (Martin & Spallholz, 1970; Chung & Maines, 1981; Le Boeuf & Hoekstra, 1983 and Hoffman *et*

*al.*, 1989). Chung and Maines actually demonstrated an initial depletion of GSH together with the elevation of GSSG, prior to the rise in GSH levels. The primary lesion may, therefore, involve the conversion of GSH to GSSG which accompanies selenium metabolism. GSH levels may rise later in an attempt to maintain a normal GSSG:GSH ratio. These workers also recorded selenium-mediated increases in the activities of Y-glutamylcysteine synthase (E.C. 6.3.2.2.) and GSSG-reductase (E.C.1.6.4.2.) which were again considered to be secondary adaptive changes.

More detailed information concerning the importance of such redox changes in selenium toxicity has been provided by *in vitro* studies of Anundi *et al.* (1984) and Ståhl *et al.* (1984). Incubation of isolated hepatocytes with selenite at concentrations known to cause cell damage and lysis resulted, initially, in selenium accumulation accompanied by an increase in oxygen uptake (confirmed by Garberg & Högberg, 1987), together with glutathione oxidation and resultant shift in the $NADP^+$:NADPH ratio towards the more oxidised state. After about 1 hour volatilisation of selenium commenced, resulting in partial loss of accumulated selenium. The authors proposed that selenium-glutathione complexes, produced as a result of the action of glutathione reductase, accumulated during the lag phase. They went on to demonstrate, using modifiers of selenium toxicity and metabolism, that the observed redox changes could be explained in terms of redox cycling of these autooxidisable metabolites and that this process was important for selenite-mediated cellular lysis. Anundi *et al.* (1984) suggested that depletion of NADPH rather than of GSH resulted in the observed cytotoxicity of selenite.

These findings might also provide some explanation as to how the anaerobic conditions known to be necessary for the conversion of selenite to DmSe by tissue preparations (Ganther, 1966; Hsieh & Ganther, 1975 & 1977) might be attained *in vivo*.

Further indication that selenite metabolites rather than selenite per se exert toxic effects, is the finding that weanling rats, which metabolise selenite slowly (Ostadalova *et al.*, 1982), are more resistant to acute selenite toxicity than adult rats (Ostadalova *et al.*, 1979).

Other possible mechanisms of toxicity discussed in the literature include: the general replacement of sulphur by selenium in cellular metabolism (Stadtman, 1974); inhibition of essential sulphydryl groups in enzymes such as methionine adenosyltransferase (Hoffman, 1977); general inhibition of several dehydrogenating enzymes including succinate dehydrogenase (Tsen & Collier, 1959; Klug *et al.*, 1950b; Ray & Ray, 1975; Ignesti *et al.*, 1986; Nebbia, 1990); depletion of S-adenosylmethionine (Hoffman, 1977); inhibition of protein synthesis (Vernie *et al.*, 1974; Safer *et al.*, 1980); inhibition of RNA and DNA polymerases (Frenkel *et al.*, 1986; Frenkel & Falvey, 1989); chromosomal alterations (Lo *et al.*, 1978; Sirianni & Huang, 1983; Whiting *et al.*, 1980) and DNA fragmentation (Shamberger, 1985; Garberg, 1988; Högberg *et al.*, 1989).

### 2.16.2 Factors Affecting Selenium Toxicity

The toxicity of selenium is affected by several factors related to diet and previous exposure to the element.

There is some evidence to suggest that animals adapt to high selenium intakes. Animals previously exposed to selenium are less susceptible to selenosis, possibly as a result of increased excretion of DmSe and TmSe, which decreases whole body retention of the element (Hopkins *et al.*, 1966; Burk *et al.*, 1972; Magos *et al.*, 1987).

A high protein diet can reduce selenium toxicity with casein being particularly effective in this respect. Some protection can also be provided by lactalbumin, but edestin and gelatin exert no positive action (Gortner, 1940). The beneficial effect of casein is not clearly understood, however, a true detoxification occurs since all symptoms of selenosis are equally reduced (Rosenfeld & Beath, 1964).

Methionine protects against selenium toxicity provided that sufficient vitamin E or fat-soluble antioxidants are present in the diet (McConnell, 1952; Witting & Horwitt, 1964; Levander & Morris, 1970). Levander and Morris demonstrated that the combined effect of methionine and vitamin E is the reduction of liver selenium levels. The beneficial action of methionine may be related to the production

of S-adenosylmethionine. Analogy with studies carried out using physiological levels of selenium (Hove & Hardin, 1952; Sukharevskaya & Shtutman, 1968) lead the authors to propose that vitamin E may increase the availability of the methyl group of methionine. In this investigation which used rats as the experimental animal and peanut meal as the selenium source, the interaction appeared to be specific since cysteine and the methyl group donor, betaine, failed to alleviate selenosis. Contrasting data were provided recently by Lowry and Baker (1989) who demonstrated the ability of cysteine to ameliorate selenite-induced growth depression in chickens. This discrepancy is probably a function of selenium source since these workers found that cysteine failed to counteract selenosis caused by selenomethionine supplementation.

Other factors capable of moderating selenium toxicity include sulphate (Halverson *et al.*, 1960 and 1962; Ganther & Baumann, 1962) and certain heavy metals and trace elements, such as arsenic, cadmium, copper, mercury and silver.

The protective effect of arsenic against selenosis was first discovered by Moxon in 1938. Arsenite and arsenate have been found effective in preventing the toxic action of selenium from seleniferous wheat, selenite and selenocysteine in several species including the chicken (Rhian & Moxon, 1943; Dubois *et al.*, 1940; Moxon *et al.*, 1944; Moxon *et al.*, 1945; Carlson *et al.*, 1954; Thapar *et al.*, 1969). The basis for this protective effect has been shown to reside in the ability of arsenic to increase the biliary excretion of selenium (Ganther & Baumann, 1962; Levander & Baumann, 1966 a & b). In contrast, arsenic actually reduces the exhalation of volatile selenium compounds in animals injected with selenite (Kamstra & Bonhorst, 1953; Ganther & Baumann, 1962). Later Ganther (1966) and Hsieh and Ganther (1977) were able to demonstrate that arsenic inhibits the methylation of selenium *in vitro*. Current literature is contradictory regarding the effects of arsenic on tissue selenium levels. Some researchers have been unable to detect any alteration in the distribution of selenium after arsenic treatment ( Peterson *et al.*, 1950; Arnold *et al.*, 1973), while others have reported that arsenic decreases the amount of selenium deposited in the liver of experimental animals (Levander & Argrett, 1969; Moxon *et al.*, 1941; Moxon & Dubois, 1939;

Palmer & Bornhorst, 1957; Ganther & Baumann, 1966 a & b; Palmer *et al.*, 1983).

The inclusion of mercuric chloride, cupric sulphate or cadmium sulphate in the diet of chicks has been shown, by Hill (1974), to partially overcome the growth depression and mortality caused by selenium. Evidence was obtained suggesting that these metals exert their protective effects by reacting with selenium, probably within the intestinal tract, to form compounds of lower toxicity. Similar effects were reported for copper, by Jensen (1975), and for mercuric mercury, by Levander and Argrett (1969) and Magos and Webb (1980). These workers also demonstrated that mercury increases whole-body retention of selenium and reduces the formation of methylated selenium metabolites. Elevated tissue selenium levels were reported by Ganther and Baumann (1966b) following cadmium treatment.

Silver ions can also protect against selenium toxicity (Jensen, 1975) and lower the total amount of selenium in the liver (Diplock *et al.*, 1971). Jensen obtained evidence that silver modifies selenium toxicity by interfering with the absorption of this element as well as by complexing with it.

The mechanisms by which heavy metals influence selenium toxicity are not therefore universal but depend on the element concerned. In addition, there appears to be a lack of correlation between the ability of a particular metal to alleviate selenosis and its effect on tissue selenium levels.

### 2.16.3 The Interaction Between Cyanide and Selenium.

A protein source which has proven particularly effective in protecting against selenosis is linseed oil meal (LOM) (Olson & Halverson, 1954; Halverson *et al.*, 1955). The active factor was demonstrated, by fractionation studies, to be non-proteinacious (Halverson *et al.*, 1955; Jensen & Chang, 1976) and to contain the cyanoglucosides linustatin and neolinustatin (Palmer *et al.*, 1980; Smith *et al.*, 1980). The observation of Palmer & Olson (1979), that the inclusion of cyanide in the drinking water partially prevented

chronic selenosis in rats, supported the notion that cyanide released from the linseed cyanoglucosides was responsible for the protective effect of the meal.

Addition of cyanide to the diets of animals consuming toxic amounts of selenium results in improved weight gains and feed conversion efficiencies, decreased tissue selenium levels, increased SeGSHPx activities and restoration of relative liver size (increased by selenium) to normal. At normal or low selenium intakes, cyanide decreases SeGSHPx activities, whilst still decreasing tissue selenium levels and may be capable of inducing deficiency in animals consuming diets only marginally adequate in selenium (Elzubeir, 1986; Elzubeir & Davis, 1988b; Elzubeir & Davis, 1990). All of these effects were observed following administration of 0.1 g SNP per kg feed, except for the reduction in tissue selenium content, which was only apparent after treatment with 0.3 - 0.4 g of the toxin.

The effects of linseed oil meal on tissue selenium levels are less consistent. Addition of this protein to low selenium diets failed to alter the amount of selenium in rat liver (Halverson & Palmer, 1975). However, Levander *et al.*, (1970) recorded tissue selenium levels in rats fed LOM plus 10 mg Se $kg^{-1}$ which were 2-4 fold higher than the levels in animals fed casein plus selenium.

The only mechanism so far proposed to account for the cyanide-selenium interaction is formation of selenocyanate, leading to a reduction in the level of selenium metabolites, which may themselves be toxic, or which may be converted to more toxic compounds such as $H_2Se$ (Palmer & Olson, 1979).

There is some evidence supporting the notion of selenocyanate formation, however, the significance of the rate of its synthesis *in vivo* remains questionable. This compound can be formed *in vitro* by the non-enzymic reaction between glutathione selenotrisulphide (GSSeSG) and cyanide (Ganther *et al.*, 1977). Purified beef liver rhodanese forms selenocyanate from selenosulphate (Cannella *et al.*, 1975), but the conditions best suited to this seleno transfer would not occur *in vivo*. Selenocyanate can also be a product when highly purified ovine erythrocyte glutathione peroxidase is incubated with

excess cyanide (Prohaska *et al.*, 1977). However, impure preparations of the enzyme are not sensitive to cyanide unless the enzyme is first converted to an oxidised state and high concentrations of cyanide are used at high pH. Furthermore, selenocyanate may be formed from later reactions (Ganther *et al.*. 1977) since cyanoglutathione (GSCN) and selenol are the initial products (Kraus & Ganther, 1980). Beilstein & Whanger (1984) reported that a selenium compound that co-chromatographed with selenocyanate was a minor metabolite in the urine of rats treated, either with selenium plus cyanide, or with selenium alone.

Even if selenocyanate were formed to a significant extent *in vivo*, it is difficult to see how this might lead to alleviation of selenosis. This compound cannot simply be viewed as a non-toxic, inert form, in which selenium can be trapped. The chronic toxicity of KSeCN is comparable with that of selenite and when injected into rats, selenocyanate is extensively metabolised, with much of the selenium being excreted in methylated forms (Vadhanavikit *et al.*, 1987). It remains unclear whether this methylation occurs prior to, or following, scission of the C-Se bond (Figure 5). The alternative possibility, that selenocyanate formation might somehow lead to increased elimination of selenium from the body, also seems unlikely, especially if the reactive seleno-compound is GSSeSG, a normal intermediate of selenium metabolism.

Selenocyanate formation does not appear to provide an adequate explanation for the cyanide-selenium interaction and other mechanisms should be considered. One possibility is that cyanide might alter the form in which selenium exists in the tissues. Levander *et al.* (1970) demonstrated that EDTA increased the amount of selenium that could be dialysed from liver homogenates prepared from rats fed a casein-based diet, but had no effect on those from rats fed a casein-linseed oil meal ration.

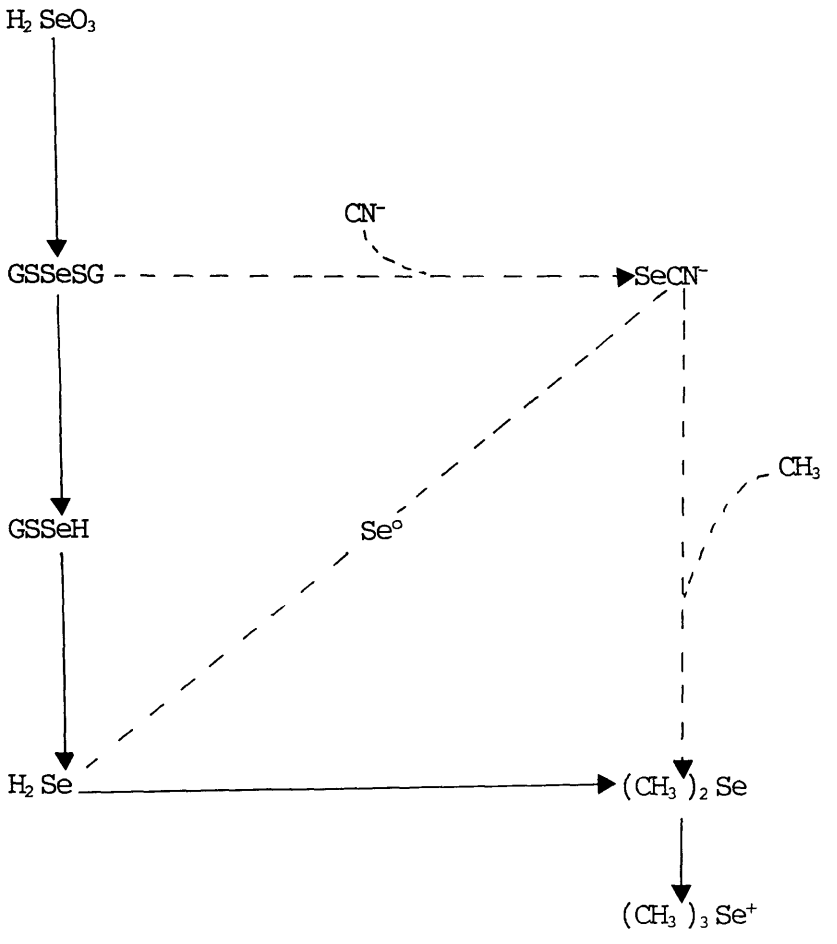



Figure 5. Hypothetical pathways of selenium metabolism in the presence of cyanide.

## 2.17 Glutathione Metabolism.

Glutathione (Y-glutamylcysteinylglycine) is the most abundant of the nonprotein thiols, being present in millimolar concentrations in many plant and animal tissues (see Meister & Anderson, 1983; and Kaplowitz *et al.*, 1985, for reviews on glutathione). This tripeptide plays a critical role in detoxification reactions. In addition to its function in SeGSHPx, GSH also participates in the GSH S-transferase mediated detoxification of certain xenobiotics (Kaplowitz, 1980). The ratio of the two forms of glutathione (GSH : GSSG) may affect the redox state of protein thiols, and in turn modulate many cellular processes (Sies & Moss, 1978; Gilbert, 1982).

Glutathione synthesis occurs in virtually all cells, but the liver is particularly important in this respect. This organ plays a

critical role in GSH homeostasis, exporting GSH synthesised in hepatic cytoplasm mainly into the plasma, but also into the bile (Kaplowitz *et al.*, 1985). Synthesis of GSH from its constituent amino acids involves two ATP-requiring steps. An amide bond is formed between glutamate and cysteine and then between the resulting dipeptide and glycine. The first reaction, catalysed by Y-glutamylcysteine synthase (E.C. 6.3.2.2.), is rate limiting and is subject to feedback inhibition by GSH (Davis *et al.*, 1973; Richman & Meister, 1975). The enzyme is also regulated by the availability of cysteine (Viña *et al.*, 1978). The second enzyme in the pathway, GSH synthase (E.C. 6.3.2.3.) has been less well characterised, but is known to be unaffected by GSH levels (Oppenheimer *et al.*, 1979).

GSH released from the liver is acted upon by Y-glutamyl transferase (or transpeptidase) (E.C. 2.3.2.2.) located in the call membrane of another tissue. The glutamyl group is transferred to an acceptor amino acid. The resultant y-glutamyl amino acid, and the cysteinylglycine fragment of GSH, are both released into the cell interior (Griffith & Meister, 1979). Y-glutamyl cyclotransferase (E.C. 2.3.2.4.) releases the original amino acid with the concomitant formation of 2-pyrrolidone-5-carboxylate (5-oxoproline). This lactam is hydrolysed back to glutamate completing what is known as the Y-glutamyl cycle (Figure 6). In actual fact, different parts of the "cycle" assume different importance depending upon location. Synthesis is high in the liver, whilst transpeptidase activity is high in the kidney. Glutamate released in the kidney is unlikely to make a significant contribution to GSH synthesis in the liver.

Since cystine is one of the better substrates for the transpeptidase, another function of glutathione may be to transport cyst(e)ine (Thompson & Meister, 1977). Cyst(e)ine in the plasma exists largely in the form of cystine which is poorly transported into the liver (Reed & Orrenius, 1977). The active site of the enzyme also shows preferential affinity for glutamate and methionine.

The redox state of glutathione is affected by the actions of SeGSHPx (E.C. 1.11.1.9.) and GSSG reductase (E.C. 1.6.4.2.) (Figure 7). Maintenance of the GSH:GSSG ratio (at around 250:1) requires GSSG

reductase and a supply of NADPH, to offset the effects of the peroxidase and other systems that oxidise GSH.

Another enzyme responsible for GSH-GSSG interchange is thiol transferase (E.C. 2.5.1.18) which mediates thiol-disulphide exchange. This transferase may be important in the synthesis, structure, functioning and degradation of proteins as a result of its ability to insert disulphide bridges into macromolecules (Mannervik & Axelsson, 1980; López-Barea & Bárcena, 1988; Ziegler, 1985).

Cysteine for GSH synthesis can be supplied from the diet or through protein breakdown. In addition, the liver possesses the unique ability to generate cysteine from methionine via transsulphuration catalysed by cystathionase (E.C. 4.4.1.1.) (Reed & Orrenius, 1977; Beatty & Reed, 1977). This enables the liver to maintain GSH export even if the supply of dietary precursors is limited.

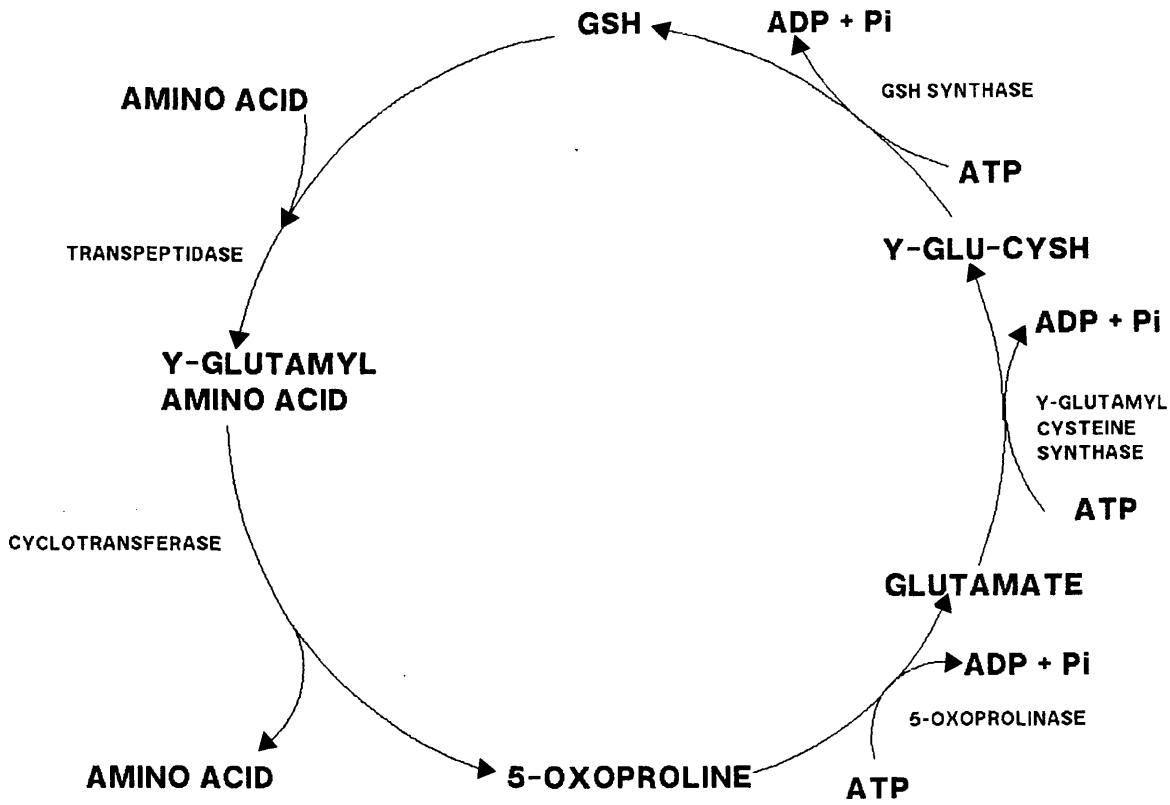



**Figure 6. The Y-Glutamyl "Cycle".**

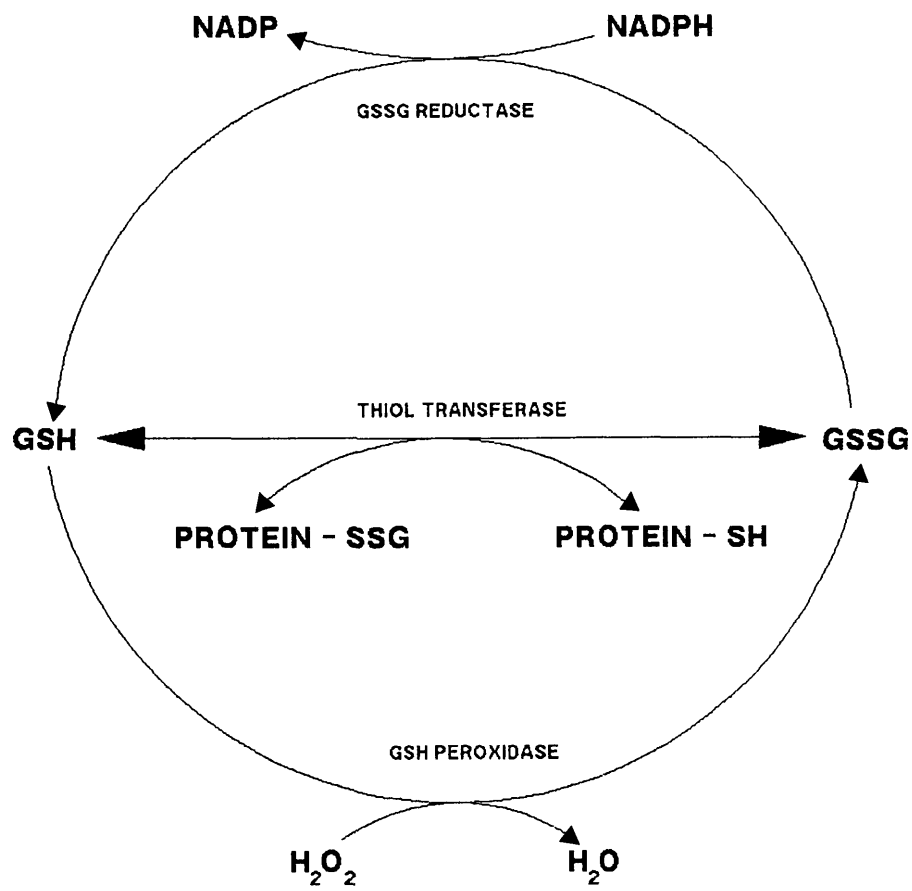



**Figure 7. The GSH - GSSG Redox System.**

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials.

### 3.1.1 Animals and Diets.

Male chicks of a commercial laying strain (Hisex or ISA Brown) were used throughout these investigations. Hisex chicks were supplied by Joyce and Hill (Hisex) Ltd., Fakenham, whereas ISA Brown birds were purchased from ISA Poultry Services Ltd., Peterborough.

Chicks were obtained at one day old and reared on cereal-based diets, the composition of which are shown in Tables 1.1 and 1.2. The components of these diets were supplied by T. Denne and Sons Ltd., Wye, Kent, except for the vitamin and mineral supplement (Beta 130) and methionine which were obtained from B.P. Nutrition UK Ltd.

Experimental diets were derived from a single batch of feed, mixed at the start of each investigation. All additions were made at the time of mixing, with the exception of SNP which was added to stock diets weekly. All experimental rations were stored in the dark and in a cool place.

### 3.1.2 Chemicals and Biochemicals.

Chemicals of AnalaR grade were used whenever possible and, unless otherwise stated, were obtained from Sigma Chemical Co., Poole, England, or from B.D.H. Chemicals Ltd., Poole, England.

### 3.1.3 Spectrophotometry and Fluorimetry.

An SP8-100 UV/vis spectrophotometer (Pye Unicam Ltd.) was used for all spectrophotometric determinations. Fluorimetric measurements were carried out with a Baird Nova 2 spectrofluorimeter (Baird Atomic Ltd.).

3.1.4 Homogenisation and Centrifugation.

Homogenisation was achieved with an Ultra-turrax apparatus (Janke and Kunkel KG, Stauffen i. Breisgau) set at medium speed (5), unless otherwise stated. An M.S.E. Hi-Spin 21 refrigerated centrifuge, fitted with an angle-head rotor, was used for all spins.

**Table 1.1 Composition of Chick Mash.**

| Ingredients | g $kg^{-1}$ |
|---|---|
| Maize | 251 |
| Wheat | 410 |
| Barley | 105 |
| Extracted toasted soya (44%) | 113 |
| Fish meal | 80 |
| Meat and bone meal | 28 |
| Limestone | 8 |
| Supplement Beta 130 | 5.0 |

**Nutrient Content[1]:**

| | |
|---|---|
| Protein (g $kg^{-1}$) | 192.0 |
| Metabolisable energy (MJ $kg^{-1}$) | 10.4 |
| Fat (g $kg^{-1}$) | 23.5 |

[1] Calculated from data provided by Bolton and Blair (1974).

Table 1.2 Composition of Cassava - Soyabean Based Diet

| Ingredients | g $kg^{-1}$ |
|---|---|
| Cassava | 463.1 |
| Extracted toasted soya (44%) | 400.0 |
| Corn Oil | 100.0 |
| Dicalcium phosphate | 25.5 |
| Supplement Beta 130 | 5.0 |
| Limestone | 3.8 |
| Sodium chloride | 2.5 |

**Nutrient Content[1]:**

| | |
|---|---|
| Protein (g $kg^{-1}$) | 180.0 |
| Metabolisable energy (MJ $kg^{-1}$) | 13.8 |
| Fat (g $kg^{-1}$) | 106.3 |

[1] Calculated from data provided by Bolton and Blair (1974).

Beta 130 provided the following (per kg diet):

| | | | |
|---|---|---|---|
| Vitamin A | 10,000 i.u. | Choline | 75 mg |
| Vitamin $D_3$ | 3,000 i.u. | Antioxidant | 20 mg |
| Vitamin E | 8 mg | Folic acid | 1 mg |
| Riboflavin | 5 mg | Fe | 10 mg |
| Vitamin K | 2 mg | Co | 0.25 mg |
| Nicotinic acid | 20 mg | Mn | 80 mg |
| Pantothenic acid | 10 mg | Cu | 5 mg |
| Thiamine | 1 mg | Zn | 60 mg |
| Pyridoxine | 1 mg | I | 1 mg |
| Biotin | 25 $\mu$g | Se | 0.15 mg |
| Vitamin $B_{12}$ | 8 $\mu$g | | |

## 3.2 Animal Husbandry.

Birds were reared on conventional chick mash in a tier brooder for 10 days from 1 day old. They were then transferred to battery cages and allowed to acclimatise before being considered for experimentation at 14 days of age. Selection was on the basis of body weight, a median group of about 100 chicks being chosen from an initial group of 150. These birds were then wing-banded and allocated randomly to experimental diets. Chicks were caged singly and allowed free access to feed and water. Lighting was 14 hours per day (06.00 - 20.00) and temperature was maintained at 24°C. Feed intake and weight gain were monitored twice weekly for approximately 21 days.

## 3.3 Specimen Collection.

### 3.3.1 Livers For Storage As Whole Tissue.

Anaesthesia was established by exposure to diethyl ether, and judged to be adequate when a comb pinch failed to elicit any reflex response. At this stage, but prior to cessation of the heartbeat, the bird was dissected to expose the liver. One lobe was removed and immediately freeze-clamped using tongs which had been pre-cooled in liquid nitrogen. The samples were stored at -70°C until required.

### 3.3.2 Blood or Plasma Samples.

Blood was collected, into heparinised tubes, from birds anaesthetised with ether. The samples were obtained by cardiac puncture, prior to cessation of the heartbeat.

## 3.4 Preparation of Reagents.

### 3.4.1 Acid mixture:

- containing concentrated nitric acid (70% v/v, 15.7 mol $L^{-1}$) and concentrated perchloric acid (70% v/v, 11.6 mol $L^{-1}$) in the ratio 4:1.

3.4.2 Alcohol dehydrogenase, 300 KU $L^{-1}$ (Alcohol:NAD oxidoreductase, E.C. 1.1.1.1.):

- prepared every few days by dilution from a stock suspension (from yeast, 9,000 KU $L^{-1}$, Boehringer Mannheim) with ammonium sulphate solution (3.2 mol $L^{-1}$).

3.4.3 Alcoholic potassium hydroxide (0.5 mol $L^{-1}$):

- the required amount of potassium hydroxide was dissolved in ethanol (99.8% v/v)/water (50:50).

3.4.4 Amyloglucosidase, 10 KU $L^{-1}$ (exo-1,4-(α)-D-glucosidase E.C. 3.2.1.3.):

- prepared freshly as required, by reconstituting 20 mg of lyophilised enzyme (from *Aspergillus niger*, $\geq$10 U $mg^{-1}$, B.D.H.) with 20.0 ml acetate buffer (0.2 mol $L^{-1}$, pH 4.8).

3.4.5 Chloramine-T-phosphate:

- one volume of 0.25% chloramine-T was mixed, immediately before use, with three volumes of sodium dihydrogen orthophosphate ($NaH_2PO_4$) solution (0.1 mol $L^{-1}$) and kept in an ice bath.

3.4.6 Copper sulphate reagent:

- prepared daily by mixing 50.0 ml of a solution containing 2% sodium carbonate in sodium hydroxide (0.1 mol $L^{-1}$) with 1.0 ml of 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% sodium/potassium tartrate.

3.4.7 Cysteine solution:

- 50 mg L-cysteine-HCl-$H_2O$ were dissolved in 10.0 ml Tris chloride buffer (0.2 mol $L^{-1}$, pH 7.6) and the pH was adjusted to 7.6 with sodium hydroxide solution (0.5 mol $L^{-1}$). The cysteine solution was prepared immediately before use.

3.4.8 2-3 Diaminonaphthalene (DAN) solution (0.1%):

- 0.1g DAN (97% purity, Aldrich Chemical Co.) was weighed into a conical flask and dissolved in 100.0 ml hydrochloric acid (0.1 mol $L^{-1}$). The flask was placed in a water bath at 60°C for 10-15 minutes and agitated occasionally to ensure complete dissolution of the DAN, then cooled under running water. The DAN solution was transferred to a separating funnel and extracted with 25.0 ml cyclohexane. This procedure was repeated three times using a clean separating funnel for each wash. The DAN reagent was prepared daily.

3.4.9 5-5'-Dithio-bis (2-nitrobenzoic acid) (DTNB) solution, 6 mmol $L^{-1}$:

- 23.8 mg DTNB were dissolved in 10.0 ml phosphate/EDTA buffer (Section 3.4.17). The resulting solution was stored in the dark at 0-4°C and renewed weekly.

3.4.10 Diphosphate buffer (0.1 mol $L^{-1}$, pH 8.8):

- containing diphosphate ($Na_4P_2O_7.10H_2O$) (0.1 mol $L^{-1}$) and semicarbazide hydrochloride (45 mmol $L^{-1}$) in deionised water. This buffer was stable for several weeks.

3.4.11 Folin-Ciocalteu phenol reagent:

- prepared immediately before use by dilution of a stock solution (B.D.H.) to 1.0 mol $L^{-1}$ with water.

3.4.12 Glucose-6-phosphate dehydrogenase, 14 KU $L^{-1}$ (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase, E.C. 1.1.1.49.):

- prepared every two or three days by dilution from a stock suspension (from yeast, 350 KU $L^{-1}$, grade 1, Boehringer Mannheim) with ammonium sulphate solution (3.3 mol $L^{-1}$).

3.4.13 Glutamate dehydrogenase, 24 KU $L^{-1}$ (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating), E.C. 1.4.1.3.):

- the required activity was obtained by diluting a stock suspension (from liver, 2,400 KU $L^{-1}$, Boehringer Mannheim) with ammonium sulphate solution (2.0 mol $L^{-1}$).

3.4.14 Glutathione reductase, 200 KU $L^{-1}$ (NAD(P)H: oxidised glutathione oxidoreductase, E.C. 1.6.4.2):

- prepared weekly by diluting a stock enzyme (yeast glutathione reductase in ammonium sulphate solution (3.2 mol $L^{-1}$), 200 KU $L^{-1}$, Sigma) with phosphate/EDTA buffer (Section 3.4.17).

3.4.15 Lactate dehydrogenase, 50 KU $L^{-1}$ (L-lactate: $NAD^+$ oxidoreductase, E.C. 1.1.1.27):

- a stock suspension (from beef heart 5,000 KU $L^{-1}$, Sigma) was diluted with ammonium sulphate solution (2.2 mol $L^{-1}$) to form the working reagent. Once diluted, the suspension was used within a few days.

3.4.16 NADPH/buffer solution:

- containing NADPH (0.3 mmol $L^{-1}$) dissolved in phosphate, EDTA buffer (Section 3.4.17). This reagent was prepared every two weeks and stored at 4°C.

3.4.17 Phosphate/EDTA buffer:

- a sodium phosphate buffer (0.125 mol $L^{-1}$, pH 7.5) containing ethylenediaminetetraacetic acid (EDTA, 6.3 mmol $L^{-1}$).

3.4.18 Pyrazalone-pyridine reagent:

- prepared by mixing five volumes of saturated aqueous 1-phenyl-3-methyl-5-pyrazalone (Eastman Kodak Co.) with one volume of a 0.1% solution in pyridine of bis-(1-phenyl-3-methyl-5-pyrazalone) (Sigma Chemical Co.). The saturated aqueous pyrazalone was stable for many months kept in the dark, but the bis-pyrazalone solution and the final pyrazalone-pyridine reagent were prepared freshly, when required.

3.4.19 Substrate mixture (for NADPH assay):

- comprising 2-oxoglutarate (0.1 mol $L^{-1}$) and ammonium chloride (0.2 mol $L^{-1}$). 0.116 g 2-oxoglutarate-$Na_2$.$2H_2O$ and 53 mg $NH_4Cl$ were dissolved in 4.5 ml water.

3.4.20 Triethanolamine (TEA)/phosphate mixture:

- composed of TEA (0.5 mol $L^{-1}$), $KH_2PO_4$ (0.4 mol $L^{-1}$) and $K_2HPO_4$ (0.1 mol $L^{-1}$).

3.4.21 Tris chloride buffer (0.2 mol $L^{-1}$, pH 7.6):

- prepared by dissolving 3.02 g tris (hydroxymethyl) aminomethane and 11.80 g tris (hydroxymethyl) aminomethane hydrochloride in 500 ml water.

## 3.5 Methods

### 3.5.1 Estimation of Cyanide in Biological Fluids.

Cyanide was quantified using the procedure of Epstein (1947), based on the reaction of cyanogen halides to cleave pyridine, and modified for microdiffusion analysis by Feldstein & Klendshoj (1954).

2.0 mls of sodium hydroxide solution (0.1 mol $L^{-1}$) were pipetted into the central well of a Conway dish. 2.0 ml of the sample to be analysed and 0.5 ml sulphuric acid (10% v/v) were placed at opposite sides of the outer chamber. The dish was supported, inclined at a slight angle, to prevent sample and acid from mixing at this stage. The ground glass cover was then spread with a thin layer of vacuum grease and placed on top of the microdiffusion cell. The dish was carefully rotated to mix the solutions in the outer chamber. This mixing process was repeated every 30 minutes and a diffusion time of 2 hours at room temperature was employed to allow for the absorption of hydrogen cyanide into the sodium hydroxide in the centre chamber. Each sample was analysed in duplicate and a water blank was prepared for each batch of assays.

At the end of the incubation period, a 1.0 aliquot of sodium hydroxide was removed from the centre well of each dish and pipetted into a test-tube (pre-cooled in an ice bath). 0.2 ml of cold chloramine-T-phosphate was added and the samples were allowed to stand for 2-3 minutes in ice. This was followed by the addition of 3.0 ml pyrazalone-pyridine reagent. The tubes were then agitated and incubated for 60 minutes at room temperature.

The absorbance, at 630 nm, for each sample was determined against the water blank and converted to cyanide ion concentration by means of a calibration graph. This graph, constructed using standard solutions of potassium cyanide dissolved in sodium hydroxide (0.1 mol $L^{-1}$) was linear up to 1.0 µg CN $ml^{-1}$ (0.038 mmols $L^{-1}$) and passed through the origin ($\epsilon$ = $2.34 \times 10^4$ L $mol^{-1}$ $cm^{-1}$).

### 3.5.2 Estimation of Cyanide in Chick Gut Contents

Anaesthetised chicks were dissected to expose the intestinal tract. Sections of gut were removed, cut open and their contents washed out with 5.0 ml sodium hydroxide solution (0.05 mol $L^{-1}$), into a pre-weighed boiling tube. This washing procedure was repeated two more times. The tube was then re-weighed and the amount of material obtained from each section of gut recorded. The final volume of solution was adjusted to 20.0 ml with sodium hydroxide and 2.0 ml aliquots were then assayed for cyanide (Section 3.5.1).

### 3.5.3 Analysis of Plasma Glucose Concentration.

Heparinised blood samples were centrifuged immediately after collection at 3,000 *g* and 4°C for 10 minutes. The supernatants were assayed for glucose using a diagnostic test kit (Boehringer Mannheim, GOD-Perid method).

### 3.5.4 Glutathione and Glutathione disulphide Assays.

The glutathione content of blood and liver was determined using the method of Griffith (1980). This assay was based on the non-enzymatic reaction of GSH with DTNB, in the presence of glutathione reductase (to reduce GSSG to GSH).

A 2.0 ml sample of heparinised blood was pipetted, immediately after collection, into a centrifuge tube containing 0.4 ml acetic acid (6% v/v). The tube was vortexed and 1.6 ml sulphosalicylic acid (10% w/v) were added to the haemolysate. The sample was vortexed again, then centrifuged at 3,000 *g* for 3 minutes. The supernatant was stored at -70°C. When required, samples were thawed in ice-water and 3 µl portions were used in the assay.

Livers removed from anaesthetised chicks were rinsed briefly with ice-cold saline (0.9% NaCl w/v) and blotted dry. The tissue was immediately weighed and homogenised for 20 seconds with five volumes (w/v) of picric acid (1% w/v).

The homogenate was then centrifuged for 10 minutes at 10,000 *g* and 4°C. The supernatant was decanted and stored at -70°C. When required, the samples were thawed in ice-water and 3 µl portions were assayed for glutathione.

The method described below allowed the determination of total glutathione and oxidised glutathione in the acidic protein-free supernatants. Reduced glutathione was then calculated by difference. The assay was made specific for GSSG by pre-treating the samples with 2-vinylpyridine (Aldrich). The GSH was derivatised leaving only GSSG available for detection. For liver samples, this was accomplished by mixing 100 µl of the supernatant with 2 µl of 2-vinylpyridine and leaving to stand for 60 minutes at room temperature.

In the case of blood samples, 100 µl of supernatant was pipetted into an Eppendorf tube and 5 µl of tri-ethanolamine was placed as a droplet on the inside of the tube, above the liquid level. The tube was then vortexed, 2 µl 2-vinylpyridine was added, the mixing process was repeated and the reaction allowed to proceed for 60 minutes at room temperature.

For measurement of glutathione, the following reactants were pipetted into a 1.0 ml micro-cuvette:

(1) 0.7 ml NADPH/buffer solution
(2) 0.1 ml DTNB solution
(3) 0.2 ml sample (or standard) plus water.

The cuvette was stoppered, mixed thoroughly and placed in a temperature-controlled cuvette holder at 30°C for 5 minutes. Glutathione reductase solution was added (5 µl for total glutathione, 20 µl for GSSG). The cuvette was mixed again and immediately placed in a spectrophotometer. The change in absorbance with time was monitored at 412 nm.

The values of $\Delta A/\Delta t$ obtained were converted into glutathione concentrations using calibration curves. The reaction mixtures used to obtain these curves contained protein denaturant, triethanolamine

and 2-vinylpyridine in amounts identical to those in experimental samples.

The calibration curve for total glutathione measurements was constructed over the range 0.0-6.0 µmol GSH $L^{-1}$ and gave a straight line passing through 0.03 absorbance units per minute and with a slope of 0.17.

For GSSG determinations, a calibration curve was prepared using 0.0-1.0 µmol GSH $L^{-1}$ and also yielded a straight line (C = 0.15, m = 0.60).

Liver and blood total glutathione and GSSG contents were calculated by assuming that the water content of these tissues is 80% and 85% respectively (Griffiths, 1979). The appropriate corrections were made for dilution of the sample during preparation, and in the case of GSSG, for the volumes of 2-vinylpyridine and triethanolamine.

### 3.5.5 Measurement of Glutathione Reductase Activity in Liver.

Liver glutathione reductase activity was determined by the method of Goldberg & Spooner (1979).

A 1.0 g piece of deep frozen liver tissue was homogenised for 20 seconds in 5.0 ml ice-cold phosphate buffer (0.12 mol $L^{-1}$, pH 7.2). The homogenate was centrifuged at 3,000 *g* and 4°C for 5 minutes, and the resultant supernatant was decanted and used in the assay.

The following reactants were pipetted successively into the cuvette:

(1) 2.7 ml phosphate buffer (0.12 mol $L^{-1}$, pH 7.2)
(2) 0.1 ml EDTA (15 mmol $L^{-1}$)
(3) 0.1 ml GSSG (65.3 mmol $L^{-1}$, prepared fresh)
(4) 0.1 ml sample.

The cuvette was stoppered, thoroughly mixed, then allowed to equilibrate to 37°C in a thermostatically controlled cuvette holder.

After 5 minutes, 0.05 ml NADPH solution (12 mmol $L^{-1}$ in 1% w/v $NaHCO_3$) was added, the cuvette contents were mixed again and the change in absorbance with time ($\Delta A/\Delta t$) monitored at 339 nm and 37°C. Each sample was assayed in duplicate and glutathione reductase activities were calculated from the initial reaction rate using the following formula (Bergmeyer, 1979):

catalytic activity concentration in sample =

$$\frac{\Delta A \times 1000}{\epsilon \times d \times \Delta t \times \phi} \quad U\ L^{-1}$$

where:

d = light path (mm)
$\phi$ = volume fraction of sample in assay mixture (sample volume/final volume)
$\epsilon$ = millimolar absorption coefficient for NADPH (0.63 L $mmol^{-1}$ $mm^{-1}$).

### 3.5.6 Determination of Liver Glycogen Concentration.

Liver glycogen concentration was estimated using the method of Keppler and Decker (1979).

A 1.0 g piece of deep frozen liver tissue was homogenised for 1 minute with 5.0 ml ice-cold perchloric acid (0.6 mol $L^{-1}$). A 0.2 ml aliquot of the resulting suspension was immediately pipetted into a 25 ml conical flask, covered and kept in ice, together with the remainder of the homogenate, until required.

When several samples had been prepared, 0.1 ml potassium hydrogen carbonate solution (1.0 mol $L^{-1}$) and 2.0 ml amyloglucosidase solution were pipetted into each flask. The flasks were stoppered and incubated at 40°C in a water bath, with shaking, for 2 hours to allow complete hydrolysis of the glycogen. The reaction was then stopped by the addition,with thorough mixing, of 1.0 ml perchloric acid (0.6 mol $L^{-1}$) and the hydrolysates were then centrifuged at 3,000 *g* for 10

minutes. The resulting supernatants were analysed for glucose content using a commercially prepared diagnostic kit (Boehringer Mannheim GOD-Perid Method).

The remainder of each homogenate was also centrifuged and the supernatant assayed for glucose. This allowed the glucose content of the homogenate to be corrected for the free glucose present before hydrolysis. The glycogen content of the original liver was then calculated, taking into account the dilution factor of the tissue in the homogenate (the molecular weight of the glucosyl moiety in glycogen is 162).

### 3.5.7 Measurement of Liver L-Lactate and L-Pyruvate Concentrations.

Liver pyruvate concentration was determined using Sigma diagnostic kit No. 726, and Boehringer Mannheim kit No. 139 084 was used in the estimation of liver lactate level. Both methods utilise lactate dehydrogenase and monitor changes in the absorbance of NADH at 340 nm.

All operations were carried out rapidly at 0-4°C to prevent post-mortem alteration in the levels of pyruvate and/or lactate.

Minor modifications in the Sigma procedure for sample preparation allowed this method, designed for use with whole blood, to be applied to liver tissue. A 2.5 g piece of deep-frozen liver was homogenised for 1 minute in 10.0 ml of perchloric acid (1.0 ml $L^{-1}$). The homogenate was then centrifuged at 1,500 *g* and 4°C for 10 minutes. A 2.5-fold dilution of the supernatant, in water, was used in the assay.

For the estimation of liver lactate concentration, samples were prepared according to the schedule given for the determination of L-lactate in meat products. The assay protocol suggested that samples should be read against a reagent blank prepared by substituting water for the sample solution. However, interference resulting from the non-enzymic reduction of $NAD^+$ by substances in the sample, necessitated the use of a blank containing sample but no lactate dehydrogenase.

### 3.5.8 Estimation of Total Protein Concentration in Biological Samples.

The Folin-phenol method of Lowry *et al.*, (1951), was used in the quantitative determination of protein.

A 1.0 ml protein sample, diluted if necessary, was added to 5.0 ml copper sulphate reagent in a test tube, mixed and left to stand for 10 minutes at room temperature. 0.5 ml Folin-Ciocalteu phenol reagent was pipetted into the tube which was immediately vortexed, then allowed to stand again. After 30 minutes, the absorbance of the sample was read at 750 nm against a reagent blank. All samples were analysed in duplicate. The results were converted into protein concentrations by means of a calibration curve constructed using standard solutions containing 0.1 - 0.5 mg $ml^{-1}$ bovine serum albumin dissolved in the appropriate sample buffer. The graph was linear over this range, passed through 0.44 at 0.1 mg $ml^{-1}$ and had a slope of 1.82.

### 3.5.9 Analysis of Pyridine Nucleotides by HPLC.

The determination of $NAD(P)^+$ and NAD(P)H in liver was initially attempted using the isocratic reverse-phase high-performance liquid chromatographic (HPLC) method of Kalhorn *et al.*, (1985). The major problem with this procedure was a lack of reproducibility in the results. Samples for analysis of reduced pyridine nucleotide content were maintained at high pH to maximise the stability of these species. However, repeated injection of these samples dissolved silica in the top of the column. Reproducibility improved if a new column was fitted after 30-50 applications. However, cost soon proved prohibitive and an alternative method was sought.

### 3.5.10 Fluorimetric Analysis of Pyridine Nucleotides.

A fluorimetric method for the determination of pyridine nucleotides was developed based on procedures described by Williamson & Corkey (1969) and by Klingenberg, (1979).

The assays described below made use of the fact that the reduced forms of pyridine nucleotides absorb light at 340 nm and emit a fluorescent band at a longer wavelength which has a peak at 465 nm. About a 100-fold gain in sensitivity was achieved by measuring the fluorescence rather than absorption changes of NADH and NADPH in enzyme catalysed reactions.

Sample Preparation.

Sample preparation was completed as quickly as possible at 0-4°C.

For estimation of liver $NAD^+$ and $NADP^+$ content a 2.0 g (approx.) piece of deep-frozen tissue was rapidly weighed and homogenised, for 45 seconds, in five volumes (w/v) of ice-cold perchloric acid (0.6 mol $L^{-1}$). A 5.0 ml aliquot of the homogenate, the internal standard, was injected with 10.0 µl each of solutions containing $NAD^+$ (50.0 mmol $L^{-1}$) and $NADP^+$ (9.15 mmol $L^{-1}$) in perchloric acid (0.6 mol $L^{-1}$), to give final concentrations for these metabolites of 100 µmol $L^{-1}$ and 18.3 µmol $L^{-1}$ respectively. These values represented the expected concentration of the nucleotides in the homogenate, based on reference ranges published for rat liver (Bergmeyer, 1979). Both the internal standard and the sample were then centrifuged for 5 minutes at 5,000 *g* and 4°C. The supernatants were carefully removed and their volumes recorded. Dipotassium hydrogen orthophosphate solution (1 mol $L^{-1}$) was added at 0.2 ml per ml supernatant, followed by potassium hydroxide solution (3.0 mol $L^{-1}$), with vigorous stirring (magnetic stirrer), in an ice-bath, until the pH was 7.2 - 7.4. The final volume was recorded and potassium perchlorate was allowed to sediment for 2-3 minutes before the supernatants were assayed for $NAD^+$ and $NADP^+$.

For estimation of liver NADH and NADPH content a 0.5 - 1.0 g piece of deep-frozen tissue was rapidly weighed and homogenised, for 1 minute, in ten volumes of alcoholic potassium hydroxide solution (pre-cooled to -20°C). A 3.0 - 5.0 ml portion of the homogenate was supplemented with NADH and NADPH dissolved in alcoholic potassium hydroxide (not more than 10.0 µl), to form an internal standard, with final concentrations for these nucleotides of 16.7 and 53.3 µmol $L^{-1}$ respectively. Both the sample and standard were incubated for 5

minutes at 65°C, with shaking, then rapidly replaced in an ice-bath. Once cool, they were neutralised to pH 7.8 by the slow addition of a recorded volume of triethanolamine/phosphate reagent, with cooling and vigorous stirring (magnetic stirrer). The samples were then left to stand for 10 minutes to allow the flocculation of denatured protein before being centrifuged, for 10 minutes, at 40,000 *g* and 4°C. Portions of the supernatant were assayed immediately for NADH and NADPH.

Assays.

These methods were developed by systematically selecting:

(1) a sample dilution that yielded a fluorescence change within the linear region of the appropriate calibration curve and minimised quenching whilst retaining sufficient sensitivity.
(2) an excess concentration of substrate
(3) the highest possible dilution of enzyme compatible with a rapid reaction.

Determination of NAD.

NAD concentration was calculated by monitoring the production of NADH in a reaction catalysed by alcohol dehydrogenase.

$$\text{Ethanol} + \text{NAD}^+ \xrightleftharpoons{\text{ADH}} \text{acetaldehyde} + \text{NADH} + \text{H}^+$$

The following reactants were pipetted into a low fluorescence quartz cuvette:

(1) 1.5 ml diphosphate buffer
(2) 1.5 ml sample, diluted as appropriate with $HClO_4$ / $K_2HPO_4$ / KOH, pH 7.2 - 7.4 (filtered before use) to give a final concentration in the assay of no more than 2.0 µmol $NAD^+$ $L^{-1}$.
(3) 0.015 ml absolute ethanol (17.2 mol $L^{-1}$, 99.8% v/v).

The contents of the cuvette were mixed thoroughly and fluorescence was monitored at excitation wavelength 340 nm, emission wavelength 465 nm, and emission slit width 10 nm, continuously for about 5-10 minutes, until a constant reading ($F_1$) was obtained. The reaction was started by the addition, with mixing, of 0.015 ml alcohol dehydrogenase suspension. The change in fluorescence with time was then followed for a further 3-5 minutes, until a final constant value ($F_2$) was reached. A second addition of 0.015 ml alcohol dehydrogenase allowed the fluorescence attributable to the enzyme to be determined and the final increase in fluorescence ( $\Delta F = F_2 - F_1$ ) to be corrected accordingly. Values for $\Delta F$ were converted into $NAD^+$ concentrations using a calibration curve which was linear up to 2.0 µmol $L^{-1}$ (final concentration in the assay) and passed through the origin.

The results were adjusted to take into account the recoveries achieved for the internal standards. This helped to minimise variation resulting from losses during sample preparation or from the underestimation of nucleotide content due to quenching by substances in the assay.

Correction for dilution of the sample, both during preparation and in the assay, enabled the $NAD^+$ content of the original liver tissue to be calculated.

Determination of $NADP^+$

$NADP^+$ concentration was measured by monitoring the reaction catalysed by glucose-6-phosphate dehydrogenase.

$$\text{Glucose-6-P} + NADP^+ \underset{}{\overset{\text{G6PDH}}{\rightleftharpoons}} \text{gluconate-6-P} + NADPH + H^+$$

The following reactants were pipetted into a fluorimeter cuvette:

(1) 3.0 ml sample containing 10-50 µmol $NADP^+$ $L^{-1}$
(2) 0.015 ml magnesium sulphate solution (0.2 mol $L^{-1}$)
(3) 0.075 ml glucose-6-phosphate suspension.

After thoroughly mixing, the fluorescence of the reaction mixture at an excitation wavelength of 340 nm and an emission wavelength of 465 nm was monitored for about 10-15 minutes until a constant value was obtained ($F_1$). The reaction was started by the addition, with mixing, of 0.015 ml glucose-6-phosphate dehydrogenase suspension and followed over the next 20-30 minutes until completion ($F_2$). The fluorescence change, after correction for the fluorescence attributable to glucose-6-phosphate dehydrogenase, was used in the calculation of liver $NADP^+$ concentration using a calibration curve which was linear over the range indicated. The appropriate adjustments were again made for the recoveries obtained for the internal standards.

Determination of NADH and NADPH

NADH and NADPH were determined in the same alkaline extract. The oxidation of NADH was carried out using lactate dehydrogenase followed by the determination of NADPH with glutamate dehydrogenase.

$$\text{NADH} + \text{H}^+ + \text{pyruvate} \xrightleftharpoons{\text{LDH}} \text{NAD}^+ + \text{L-Lactate}$$

$$\text{2-Oxoglutarate} + \text{NH}_4^+ + \text{NADPH} \xrightleftharpoons{\text{GlDH}} \text{glutamate} + \text{H}_2\text{O} + \text{NADP}^+$$

Glutamate dehydrogenase reacts at roughly equal rates with NADPH and NADH. Therefore, if NADH is present it must be oxidised prior to the determination of NADPH. Lactate dehydrogenase reacts not only with NADH, but also to a small extent with NADPH. However, at pH 7.8 the reaction with NADPH occurs about 2,000 times more slowly than that with NADH. Therefore, at this pH, and using only a small quantity of LDH it was possible to determine NADH quantitatively.

For estimation of NADH and NADPH baseline fluorescence was determined 10-15 minutes after mixing the following reactants together in a cuvette:

(1) 3.0 ml, sample diluted as appropriate with alcoholic potassium hydroxide solution (neutralised to pH 7.8 with triethanolamine/ phosphate reagent), containing no more than 3.0 µmol $L^{-1}$ of either NADH or NADPH.

(2) 0.015 ml pyruvate (0.3 mol $L^{-1}$) neutralised to pH 7.5 with sodium hydrogen carbonate solution (1.0 mol $L^{-1}$).

(3) 0.075 ml substrate mixture containing 2-oxoglutarate (0.1 mol $L^{-1}$) and ammonium chloride (0.2 mol $L^{-1}$).

The reaction was started by the addition, with mixing, of 0.015 ml lactate dehydrogenase suspension and was monitored over the next 10-12 minutes until completion. This was followed by the addition of 0.015 ml glutamate dehydrogenase suspension. The resulting fluorescence change was again monitored until the reaction reached completion. Further addition of LDH and GlDH were then made in order that the results might be corrected for the fluorescence of the enzymes themselves. The decrease in fluorescence resulting from the action of LDH was used to calculate the NADH concentration in the sample. Similarly, the fluorescence change observed after the addition of GlDH enabled the NADPH content of the sample to be estimated. The appropriate corrections were made, as before, for the recoveries obtained for the internal standards.

A calibration curve constructed using standard solutions of NADH and NADPH was linear over the range indicated and passed through the origin.

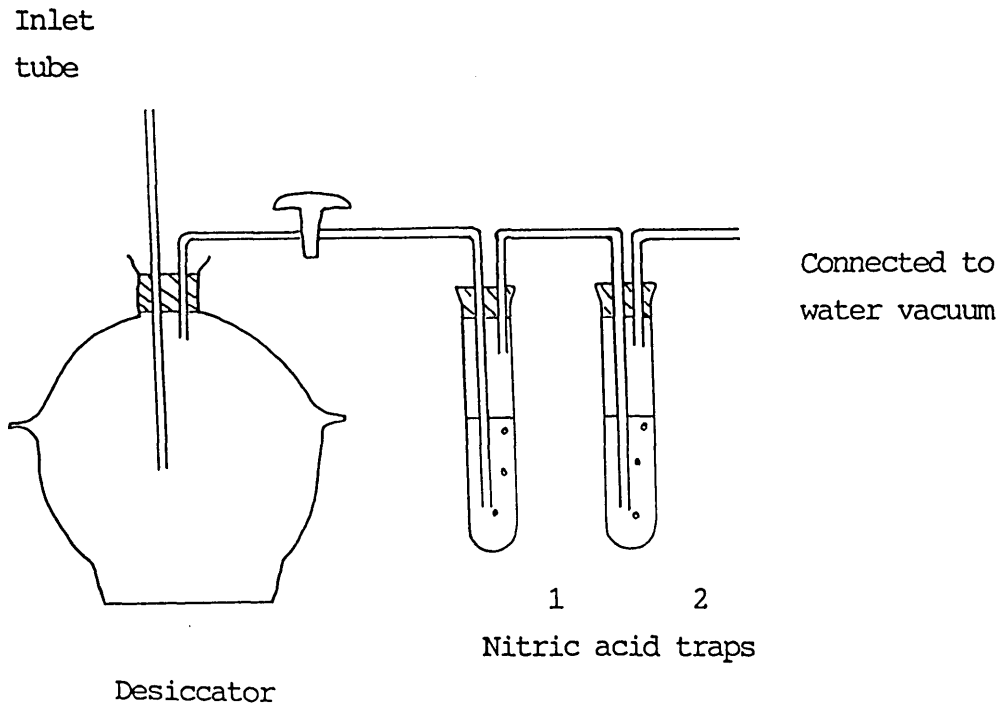
### 3.5.11 Selenium Estimation: Glassware Decontamination.

All glassware for use in selenium estimation was washed thoroughly, then soaked overnight in nitric acid (1.6 mol $L^{-1}$) before being rinsed several times in distilled water and dried in a 100°C oven.

3.5.12 Collection of Selenium in Expired Air.

The apparatus shown in Figure 8 was used to collect volatile selenium compounds present in the expired air of chicks. Four desiccators were connected to water vacuum pumps (two per pump). One or two chicks, depending on age and therefore size, were selected at random from each experimental diet and placed in a desiccator. Air was drawn through the system and expired selenium was collected in traps containing 10.0 ml concentrated nitric acid (70% w/w, 15.7 mol $L^{-1}$). After two hours, the traps were removed, sealed and stored at 4°C pending analysis of selenium content (Section 3.5.15). Treatments were circulated between desiccators to minimise any variation inherent in the system.

FIGURE 8 Apparatus Used For Collecting Volatile Selenium.



### 3.5.13 Determination of The Oxidation State of Selenium in Liver: Sample Preparation.

Diplock *et al.* (1971 and 1973) described a method for determining the oxidation state of tissue selenium using a radio-isotope of this element. However, the high cost of $Na_2{}^{75}SeO_3$ and lack of availability of a Y-isotope counter prohibited the use of this procedure in these investigations. Consequently, an alternative method (based on the work of Diplock *et al.*) was developed for use with standard fluorimetric procedures of selenium analysis. Whereas Diplock *et al.* were able to measure directly the [$^{75}$Se] hydrogen selenide produced by treating [$^{75}$Se] sodium selenite with zinc dust and concentrated hydrochloric acid, the method described below relied on calculation by difference.

A 1.0 g piece of deep-frozen liver tissue was gently homogenised (Ultra-turrax speed 2) for 15 seconds in 9.0 ml Tris-HCl buffer (0.05 mol $L^{-1}$, pH 7.4) containing DL-α-tocopherol acetate (110 mg $L^{-1}$) as antioxidant. The α-tocopherol acetate was emulsified in a small portion of the buffer by homogenisation and subsequently diluted with the remainder to give the appropriate concentration. This buffer was prepared freshly as required from a stock solution of Tris-HCl.

Two 2.5 ml aliquots of the homogenate, diluted as necessary to contain no more than 5.0 μmol Se $L^{-1}$, were pipetted into two Pyrex culture tubes (180 x 18 mm) containing 4.0 ml acid mixture. These duplicate samples were used in the estimation of total liver selenium content. A further four 2.5 ml aliquots were pipetted into test tubes, two of which contained 150 mg zinc dust each. (Diplock *et al.* used 350 mg zinc dust, but this was found to give a high background fluorescence in subsequent selenium estimations resulting in a decrease in the sensitivity of the method. Treatment of standard solutions of sodium selenite with zinc dust and hydrochloric acid demonstrated that 150 mg of zinc was sufficient to volatilise all of the selenium over the range of concentration present in the samples). Four drops of octanol were then added to each tube to prevent frothing. Oxygen-free nitrogen was then bubbled through the samples at a steady rate. After 5 minutes, 1.0 ml concentrated hydrochloric acid was added, rapidly, to each tube, without stopping the flow of

nitrogen, which was continued for a further 10 minutes. The samples were then transferred, using a Pasteur pipette, into culture tubes containing 4.0 ml acid mixture. The test tubes were thoroughly rinsed with approximately 1.0 ml water and the washings were also pipetted into the culture tubes. The samples were then capped and left to stand overnight prior to analysis for selenium.

The difference between the selenium content of untreated samples and that of hydrochloric acid treated samples was attributed to acid-labile volatile selenide. The difference between the results for acid treated samples, and those for samples also exposed to zinc dust was attributed to selenite. The remaining non-volatile selenium, presumably at a higher oxidation state, was considered to consist largely of selenium in organic compounds, together with some selenate.

### 3.5.14 Selenium Estimation: Assay.

The method of Koh & Benson (1983) was used for the determination of selenium in samples prepared, as above, from liver tissue or expired air. In the latter case, a 10.0 ml aliquot of each sample in concentrated nitric acid was pipetted into a culture tube containing 0.8 ml concentrated perchloric acid (70% v/v, 11.6 mol $L^{-1}$). The liver samples required no further preparation.

Two or three antibumping granules were added to each tube and the samples were heated in an aluminium block until the digests were free of nitric acid and fumes of perchloric acid were just visible. Koh & Benson claimed that digestion could be started with the temperature set to reach 210°C. However, a three-stage heating process was employed with these samples to prevent bumping and the resultant loss of selenium from the digests. Heating was commenced at 150°C for two hours, followed by one and a half hours at 180°C and finally four hours at 210°C. To facilitate even digestion, an aluminium foil shield was placed around the tubes, which were rotated once every hour.

Once digestion was complete, the samples were allowed to cool before 0.5 ml concentrated hydrochloric acid was pipetted into each

tube. They were then heated for a further 30-40 minutes at 140°C to reduce Se VI to Se IV.

After cooling, 16.0 ml EDTA (0.0025 mol $L^{-1}$) were added to each tube, followed by 1.0 ml DAN solution and 5.0 ml cyclohexane (AnalaR, B.D.H.). Teflon-lined caps were tightly screwed onto the culture tubes, which were then shaken vigorously, by hand, for 30 seconds and incubated in a water bath at 60° for 40 minutes. The tubes were removed from the water bath, shaken again for 30 seconds to solvent extract the Se-DAN complex, then left to stand for 5 minutes to allow phase separation.

The fluorescence of the cyclohexane layer was then recorded at an excitation wavelength of 364 nm and an emission wavelength of 523 nm, with an emission slit width of 10 nm, and an integration time of 4 seconds. The fluorimeter was zeroed using a reagent blank prepared by incubating 1.0 ml water with EDTA, DAN solution and cyclohexane.

The readings were converted into selenium concentrations by means of a calibration curve constructed with standard solutions containing sodium selenite in hydrochloric acid (0.1 mol $L^{-1}$). The graph was linear up to 5.0 µmol Se $L^{-1}$ and passed through the origin.

For determination of the redox state of selenium in liver, it was necessary to correct the fluorescence reading obtained for the background fluorescence of the chemicals used during sample preparation. Accordingly, aliquots of homogenisation medium were subjected to this procedure, yielding three blanks, one for total selenium estimation, one for samples treated with hydrochloric acid and the third for use with zinc/hydrochloric acid treatments.

### 3.5.15 Measurement of Sodium Nitroprusside (SNP) Concentration in Blood Plasma.

Plasma sodium nitroprusside concentration was determined using a modification of the method of Rodkey & Collison (1977). Samples were analysed both for free cyanide content and for total cyanide available

after degradation of any SNP present. This allowed the amount of cyanide derived from SNP to be calculated by difference.

10.0 ml (approx.) portions of blood were collected into heparinised, aluminium foil covered syringes and transferred into centrifuge tubes standing in ice, in the dark. The samples were immediately centrifuged at 3,000 *g* and 4°C for 10 minutes. The plasma was used in the assays.

For estimation of total cyanide, 1.0 ml plasma was pipetted into the outer chamber of a Conway dish. 1.0 ml cysteine solution and 0.5 ml methaemoglobin solution were placed at the opposite side of the chamber. The centre well contained 2.0 ml sodium hydroxide solution (0.1 mol $L^{-1}$). The microdiffusion cells were sealed, rotated to mix the contents of their outer chambers, and incubated at room temperature, in the dark, for 1 hour.

Simultaneously, for determination of free cyanide content, 1.0 ml portions of plasma were incubated as above but with 1.5 ml water replacing methaemoglobin and cysteine solutions.

At the end of this pre-incubation period, 0.5 ml sulphuric acid (10% v/v) was rapidly added to each dish and the procedure continued as described in Section 3.5.1 for estimation of cyanide in biological fluids. The only difference being that two reagent blanks were required in this case. All samples were analysed in duplicate as before.

Absorbance readings were converted into cyanide ion concentrations using calibration curves. The standard curve prepared for use in the estimation of free cyanide, constructed using aqueous solutions of potassium cyanide, was linear over the range 0.0-1.0 µg CN $ml^{-1}$ (0.0-0.04 mmols $L^{-1}$) and passed through the origin ($\epsilon$ = 2.50 x $10^5$ L $mol^{-1}$ $cm^{-1}$). A calibration curve for "total" cyanide estimation, prepared by incubating aqueous solutions of potassium cyanide with methaemoglobin and cysteine also passed through the origin and was linear between 0.0-4.0 µg CN $ml^{-1}$ (0.00 - 0.15 mmols $L^{-1}$) ($\epsilon$ = 8.97 x $10^3$ L $mol^{-1}$ $cm^{-1}$).

Incubation of standard solutions containing 0.1 - 20.0 µg SNP per 1.0 ml aliquot in the absence of methaemoglobin and cysteine yielded no free cyanide.

The yield of free cyanide in the presence of these reagents corresponded to the release of 4 moles of cyanide per mole of nitroprusside, rather than the 5 moles which would be expected on the basis of its chemical formula, $Na_2$ $[Fe(CN)_5NO].2H_2O$. However, evidence suggests that the "biological" yield of cyanide from SNP injected into animals is also 4 moles per mole (Smith & Krusyna, 1974).

### 3.6 Statistical Methods.

Data were assessed by analysis of variance using GENSTAT, a computer programme developed at Rothamsted Experimental Station, copyright 1980.

### 3.7 Calculation of Ratios

Lactate:Pyruvate, GSH:GSSG, pyridine nucleotide and feed conversion ratios for each dietary treatment were calculated by taking the mean of the values obtained for individual birds.

# CHAPTER 4

## RESULTS AND DISCUSSION

### 4.1 Sodium Nitroprusside as a Source of Cyanide in Metabolic Studies (Experiments 1-3).

For investigation of the metabolic effects of chronic cyanide exposure, it was essential that any changes observed be attributable to cyanide alone, rather than to any hypotensive or sulphydryl-consuming properties of SNP. Whilst the majority of the available evidence (reviewed in Section 2.7) suggests that cyanide is released from SNP *in vivo*, a dissenting view has been expressed (Butler *et al.*, 1988). In addition, the site of SNP breakdown has not yet been established. Consequently, experiments 1-3 were designed to determine whether SNP is degraded in the intestinal tract of the chicken or absorbed unaltered into the blood stream.

#### 4.1.1 Intestinal Cyanide Content in Chicks Consuming SNP (Experiment 1).

Birds maintained on chick mash containing 0.3 g SNP $kg^{-1}$ were denied access to food for 16 hours, overnight. Chicks were sacrificed, in pairs, 0.5, 1 and 2 hours after refeeding and their gut contents analysed for cyanide. Food consumption prior to slaughter was recorded for each bird.

**Results.**

Cyanide was present in the guts of chicks 0.5, 1 and 2 hours after the birds were allowed access to SNP-containing rations. The highest concentrations were detected in the crop (Table 2.1), however the yields were low in comparison to the amounts consumed as SNP with the feed. Assuming that 4 moles of cyanide were produced from each mole of SNP (Section 3.5.16), then complete degradation would have resulted in the release of 105 µg cyanide per gramme of food consumed.

Table 2.1 Intestinal Cyanide Content in Chicks Consuming SNP

| Intestinal Section | Bird Number | Time After Feeding | Weight of Feed Consumed g | Weight of Material Collected g | Cyanide Content (µg) |
|---|---|---|---|---|---|
| Crop | 1 | 0.5 | 6.2 | 6.6 | 10.1 |
| | 2 | 0.5 | 5.0 | 4.7 | 7.5 |
| | 3 | 1.0 | 5.0 | 4.2 | 6.9 |
| | 4 | 1.0 | 7.1 | 6.2 | 8.8 |
| | 5 | 2.0 | 9.4 | 4.6 | 13.8 |
| | 6 | 2.0 | 10.0 | 6.4 | 4.2 |
| Gizzard and Proventriculum | 1 | 0.5 | 6.2 | 4.5 | 1.1 |
| | 2 | 0.5 | 5.0 | 5.3 | 1.9 |
| | 3 | 1.0 | 5.0 | 2.8 | 1.1 |
| | 4 | 1.0 | 7.1 | 3.0 | 1.3 |
| | 5 | 2.0 | 9.4 | 4.9 | 3.9 |
| | 6 | 2.0 | 10.0 | 5.8 | 0.9 |
| Small and Large Intestine | 1 | 0.5 | 6.2 | 0.7 | 0.9 |
| | 2 | 0.5 | 5.0 | 0.9 | 0.7 |
| | 3 | 1.0 | 5.0 | 2.7 | 0.8 |
| | 4 | 1.0 | 7.1 | 2.7 | 0.8 |
| | 5 | 2.0 | 9.4 | 2.7 | 2.8 |
| | 6 | 2.0 | 10.0 | 4.1 | 1.6 |

### 4.1.2 Cyanide Content of Blood and Alimentary Tract in Chicks Fed SNP (Experiment 2).

Although the crop appeared, from the results of experiment 1, to represent the major site for SNP breakdown in the gut, it was possible that the 2 hour collection period employed was insufficient to allow ingested feed to reach the lower regions of the intestine. In addition it was necessary to establish that liberated cyanide entered the bloodstream. Experiment 2 was designed to address these problems.

Birds maintained on chick mash containing 0.3 g SNP $kg^{-1}$ were slaughtered, in pairs, 30 minutes before daily illumination commenced, then at regular intervals throughout the day. Blood samples were collected at each stage, whilst gut contents were sampled only at the end points.

**Results.**

Cyanide was present in the blood of chicks consuming SNP-containing rations. It was not detected in blood collected following the overnight fasting period or in samples obtained from control birds (Table 2.2).

The crop represented a major site for the release of cyanide from ingested SNP, six hours into the feeding period (Table 2.3). However, this longer time course study also established the presence of cyanide in the large intestine.

**Table 2.2 Blood Cyanide Content After Feeding SNP.**

| Added SNP ($g\ kg^{-1}$) | Time Relative to Start of Feeding (hours) | | | |
|---|---|---|---|---|
| | -0.5 | +1.0 | +4.0 | +6.0 |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 0.3 | 0.0 | 11.5 | 15.4 | 26.9 |
| 0.3 | 0.0 | 13.5 | 13.5 | 16.5 |

Figures are blood cyanide concentrations ($\mu mol\ L^{-1}$).
Two birds were analysed at each time point.

**Table 2.3 Intestinal Cyanide Content in Chicks Fed SNP**

| Time Relative to Start of Feeding (hrs) | Intestinal Section | Bird Number | Weight of Material Collected g | Cyanide Content (µg) |
|---|---|---|---|---|
| -0.5 | Crop | 1 | None | None |
| | | 2 | None | None |
| | Small Intestine | 1 | 2.09 | 0.44 |
| | | 2 | 3.56 | 0.35 |
| | Large Intestine | 1 | 7.74 | 1.66 |
| | | 2 | 6.50 | 1.42 |
| +6.0 | Crop | 1 | 1.96 | 3.51 |
| | | 2 | 4.04 | 9.26 |
| | Small Intestine | 1 | 5.24 | 0.76 |
| | | 2 | 4.63 | 0.99 |
| | Large Intestine | 1 | 2.95 | 0.77 |
| | | 2 | 3.07 | 0.81 |

### 4.1.3 SNP Concentration in The Plasma of Chicks Given Diets Containing This Compound (Experiment 3).

Although the results of experiment 1 clearly demonstrated the release of cyanide from dietary SNP in the alimentary tract of the chicken, the yields obtained were poor. It remained possible, therefore, that a large proportion of the ingested SNP had survived intact and had been absorbed, unaltered, into the bloodstream. Consequently, in experiment 3, plasma SNP concentration was estimated in birds fed either chick mash containing 0.3 g SNP $kg^{-1}$ or chick mash alone.

**Results**

There was no significant difference ($P>0.05$) between the amount of cyanide released from the plasma of chicks fed 0.3 g $kg^{-1}$ SNP and the amount released in control animals (Table 2.4).

Table 2.4 SNP Concentration in Plasma From Chicks Given This Compound in The Diet.

| Added SNP g $kg^{-1}$ | "Bound Cyanide" µg $ml^{-1}$ |
|---|---|
| 0.0 | 0.386 |
| 0.3 | 0.435 |
| ± S.E.M. | 0.001 |
| LSD (P=0.05) | N.S. |

Each value is a mean of three chicks.
N.S. = No Significant Difference

### 4.1.4 Discussion of Experiments 1-3.

Cyanide is released from dietary SNP in the crop and, to a lesser extent, in the large intestine of the chicken. The presence of cyanide in whole blood following SNP treatment is consistent with the findings of numerous other workers (see Schulz, 1984 and Vesey, 1987 for reviews) using the intravenous route for administration of the drug. These observations, together with the failure to detect any intact SNP in plasma from chicks fed this compound, suggest that SNP is degraded prior to absorption and does not enter the blood stream in its unaltered state. SNP was therefore considered to be a suitable source of dietary cyanide for investigation into the metabolic effects of chronic cyanide exposure.

A point worth checking, especially in view of the low yields of cyanide obtained on analysis of gut contents from chicks fed SNP, is whether any of the drug survived intact to be excreted in the faeces. The amount of cyanide reaching the bloodstream may be lower than that expected based on the dose of SNP administered.

## 4.2 The Effect of Dietary Potassium Thiocyanate on the Chronic Toxicity of SNP (Experiment 4).

In common with other animals, chickens possess increased blood thiocyanate levels after administration of cyanide, either as a salt (Oh *et al.*, 1987) or as SNP (Elzubeir & Davis, 1988b). It was therefore necessary, if the mechanism of the selenium-cyanide interaction were to be elucidated, to determine whether this effect was attributable to cyanide itself, or to thiocyanate derived from it by transulphuration.

Four experimental diets were formulated based on conventional chick mash supplemented with 3 g DL Met. $kg^{-1}$.

Four birds (ISA Brown) were assigned to each of the following treatments:

| Treatment | Added Selenium | Added KSCN |
|---|---|---|
| | mg $kg^{-1}$ | g $kg^{-1}$ |
| A | 0.0 | 0.0 |
| B | 0.0 | 0.49 |
| C | 10.0 | 0.0 |
| D | 10.0 | 0.49 |

The rate of inclusion of KSCN was chosen to correspond, in terms of cyanide ion concentration, to the addition of 0.3 g SNP $kg^{-1}$ assuming that the yield of cyanide from SNP is 5 moles of CN per mole of drug, although this may be an overestimation (Section 3.5.15).

The diets were randomly dispersed between two equivalent growth rooms with the constraint that two replicates of each treatment appeared in each room. Body weight gains and feed intakes were monitored over a period of 19 days.

**Results.**

See Table 3.1 overleaf.

There was no interaction between selenium and thiocyanate for growth performance, as monitored by body weight gain, feed intake and the efficiency of feed utilisation. Selenium treatment resulted in highly significant ($P<0.001$) reductions in all three variables, whilst thiocyanate did not affect growth.

No growth room effects were observed in this trial.

### 4.2.1 Discussion of Experiment 4.

These results clearly demonstrate that the thiocyanate ion is not responsible for the alleviation of chronic selenium toxicity by dietary sources of cyanide.

Table 3.1 The Effect of Dietary Potassium Thiocyanate on The Growth Performance of Chicks Consuming Toxic Quantities of Selenium.

| Treatment | Added Selenium (mg $kg^{-1}$) | Added KSCN (g $kg^{-1}$) | Body Weight Gain (g) | Feed Intake (g) | Feed Conversion Ratio |
|---|---|---|---|---|---|
| A | 0.0 | 0.0 | 322[a] | 669[a] | 2.08[a] |
| B | 0.0 | 0.49 | 325[a] | 676[a] | 2.09[a] |
| C | 10.0 | 0.0 | 192[b] | 519[b] | 2.73[b] |
| D | 10.0 | 0.49 | 211[b] | 573[b] | 2.77[b] |
| | | ± SEM | 17.2 | 20.1 | 0.12 |
| | | LSD (P=0.05) | 52.9 | 61.9 | 0.37 |
| Individual Factors | | | | | |
| | 0.0 | | 324[a] | 673[a] | 2.08[a] |
| | 10.0 | | 202[b] | 546[b] | 2.75[b] |
| | | 0.0 | 257[c] | 594[c] | 2.41[c] |
| | | 0.49 | 268[c] | 625[c] | 2.43[c] |
| | | ± SEM | 12.1 | 14.2 | 0.09 |
| | | LSD (P=0.05) | 37.4 | 43.8 | 0.26 |

Each value is a mean of 4 chicks.

Values within a column sharing the same superscript are not significantly different at $P<0.05$.

## 4.3 The Effects of Chronic Exposure to Dietary SNP and/or Selenium on Carbohydrate Metabolism in the Chicken.

The increased anaerobic catabolism of glucose in the face of impaired mitochondrial function during acute cyanide exposure is well documented (Albaum *et al.*, 1946; Olsen & Klein, 1947; Estler, 1965; Isom *et al.*, 1975). However, the question of whether chronic exposure to cyanide can result in its presence in the tissues, in quantities sufficient to inhibit electron transport, has received little attention and has not been addressed using the chicken.

Similarly, almost nothing is known about the effects of selenium toxicity on carbohydrate metabolism and the possibility that cyanide and selenium might interact at this level has not previously been considered.

In the experiments described below, growth performance, liver glycogen, lactate and pyruvate levels as well as plasma glucose concentrations were measured to assess the effects of chronic intoxication by cyanide and/or selenium on carbohydrate metabolism in the chicken. The possibility that some adaptation may occur with increasing length of exposure was also investigated.

### 4.3.1 The Effect of Short-term Dietary Exposure to Sub-lethal Doses of SNP and/or Selenium on Liver Glycogen and Plasma Glucose Concentrations (Experiment 5).

A cassava-soya bean diet formed the basis for the following four treatments in this 2 X 2 factorial experiment:

| Treatment | Added Selenium | Added SNP |
|---|---|---|
| | mg $kg^{-1}$ | g $kg^{-1}$ |
| A | 0.0 | 0.0 |
| B | 0.0 | 0.5 |
| C | 10.0 | 0.0 |
| D | 10.0 | 0.5 |

Cassava was chosen because it is the most widespread cyanogenic feed used in poultry diets. The supply available contained less than 5 mg cyanide $kg^{-1}$, negligible in comparison to the amounts added (Elzubeir & Davis, 1988). This diet was inherently deficient in sulphur amino acids, which may be important in cyanide detoxification. The feed was therefore supplemented with DL-methionine at the level of 6 g $kg^{-1}$ to ensure adequate supply both of methionine and of total sulphur amino acids (based on figures supplied by Bolton & Blair, 1974). Twenty chicks (Hisex) were allocated to each of the experimental treatments which were dispersed completely randomly within two equivalent growth rooms. At 1, 2, 3 and 7 days after transfer to the experimental diets, chicks were slaughtered, four per diet at each time point, for analysis of liver glycogen and plasma glucose concentration.

**Results**

No growth room effects were apparent on any of the variables recorded in this experiment.

There were significant interactions between selenium and SNP in their effects on weight gain on all days apart from the second. Addition of SNP to the basic diet resulted in a highly significant reduction ($P<0.001$) in body weight gain on each of the first three days of exposure. By day 7, this effect was less pronounced, though still significant ($P<0.01$) (Table 4.1 and Figure 9.1). Inclusion of selenium in the basic diet also reduced body weight gain, however, this effect, in contrast to that of SNP, became more obvious with time. Following an initial decrease in weight gain ($P<0.01$) on day 1, selenium failed to affect ($P>0.05$) this variable on day 2, then caused significant reductions in gain on days 3 and 7 ($P<0.01$ and $P<0.001$ respectively).

No interaction was observed between selenium and SNP for effects on feed intake, and selenium alone generally did not affect this variable. The exception was day 3 when the interaction achieved significance ($P<0.01$) and selenium depressed feed intake ($P<0.01$). Addition of SNP to the diet resulted in a highly significant ($P<0.001$)

reduction in feed intake in the first three days of exposure. By day 7, however, this effect was no longer significant ($P>0.05$) (Table 4.1 and Figure 9.2).

Feed conversion ratio showed little response to any of the dietary treatments in this short-term trial. However, SNP did cause a significant decrease in the efficiency of feed utilisation on days 2 and 3 ($P<0.01$ and $P<0.05$ respectively) (Table 4.1).

Liver weight was not significantly altered by diet except on day 2 when SNP decreased this variable ($P<0.001$), but only in the presence of selenium.

There was no interaction between selenium and SNP for effects on liver glycogen concentration until day 7 when significance was achieved ($P<0.05$). Exposure to dietary selenium did not alter the glycogen concentration in chick liver ($P>0.05$). In contrast, dietary intake of a sub-lethal dose of SNP resulted in a significant reduction in liver glycogen concentration, particularly during the first few days of exposure ($P<0.01$ for day 1 and $P<0.05$ for day 3) (Table 4.2 and Figure 9.3).

There was no significant difference in plasma glucose concentration between chicks fed control and experimental diets.

### 4.3.2 Effect of Long-Term Dietary Exposure to Sub-lethal Doses of SNP and/or Selenium on Liver Glycogen Concentration (Experiment 6).

In an attempt to decrease the large variation, seen in experiment 5, between individual birds within a particular treatment, conventional chick mash was used as the basic diet for this longer term study, and the rate of inclusion of SNP was reduced to 0.3 g $kg^{-1}$ (Cassava, even when sieved, is heterogeneous allowing a high degree of sorting of the feed by the birds). The chick mash required supplementation with 2 g DL-methionine per kg to ensure adequacy both of this nutrient and of total sulphur amino acids. Nine chicks (ISA Brown) were allocated to each of the following four treatments,

**Table 4.1** **The Effect of Short-Term Exposure to Dietary SNP and/or Selenium, on Growth Performance in Chicks.**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain (g) Day | | | | Feed Intake (g) Day | | | | Feed Conversion Ratio Day | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 7 | 1 | 2 | 3 | 7 | 1 | 2 | 3 | 7 |
| A | 0.0 | 0.0 | $12.3^{a}$ | $23.5^{a}$ | $39.2^{a}$ | $124.6^{a}$ | $27.0^{a}$ | $53.7^{a}$ | $88.7^{a}$ | 266.7 | 2.9 | $2.4^{b}$ | 2.5 | 2.2 |
| B | 0.0 | 0.5 | $0.2^{b}$ | $10.1^{b}$ | $23.9^{b}$ | $100.6^{b}$ | $20.7^{b\,c}$ | $40.0^{b}$ | $73.2^{b}$ | 243.8 | 0.6 | $5.4^{a\,b}$ | 3.3 | 2.5 |
| C | 10.0 | 0.0 | $4.7^{c}$ | $20.1^{a}$ | $30.2^{c}$ | $91.4^{b}$ | $25.3^{a\,c}$ | $48.9^{a}$ | $79.9^{c}$ | 254.4 | 3.4 | $3.6^{b}$ | 2.7 | 2.9 |
| D | 10.0 | 0.5 | $3.4^{b\,c}$ | $9.3^{b}$ | $25.5^{b\,c}$ | $104.4^{b}$ | $22.2^{c}$ | $42.6^{b}$ | $77.0^{b\,c}$ | 245.5 | 4.6 | $6.9^{a}$ | 3.2 | 2.5 |
| | | ± SEM | 1.5 | 1.6 | 1.9 | 5.6 | 1.3 | 2.1 | 2.3 | 6.5 | 1.5 | 1.2 | 0.2 | 0.2 |
| | | LSD (P = 0.05) | 4.2 | 4.6 | 5.3 | 16.1 | 3.5 | 6.0 | 6.5 | NS | NS | 3.3 | NS | NS |

Each value is a mean of 20 chicks for day 1, 16 chicks for day 2, 12 chicks for day 3 and 8 chicks for day 7.

NS = No significant differences.

Values within a column sharing the same superscript are not significantly different at $P<0.05$.

**Table 4.2** **Effect of Short-Term Exposure to Dietary SNP and/or Selenium, on Liver Weight, Liver Glycogen Content and Plasma Glucose Concentration in Chicks**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | Liver Weight (g) Day | | | | Liver Glycogen (g $kg^{-1}$) Day | | | | Plasma Glucose mmol $L^{-1}$ Day | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 7 | 1 | 2 | 3 | 7 | 1 | 2 | 3 | 7 |
| A | 0.0 | 0.0 | 6.8 | $7.2^{a\,b}$ | 7.2 | 9.8 | $9.0^{a}$ | 14.3 | $12.2^{a}$ | 24.7 | 15.6 | 19.2 | 16.6 | 18.0 |
| B | 0.0 | 0.5 | 6.3 | $6.8^{b\,c}$ | 6.9 | 9.2 | $3.4^{b}$ | 5.4 | $7.1^{b}$ | 14.1 | 16.5 | 18.6 | 17.1 | 17.7 |
| C | 10.0 | 0.0 | 7.2 | $7.8^{a}$ | 6.9 | 7.9 | $8.0^{a}$ | 9.9 | $9.2^{a}$ | 10.8 | 15.5 | 18.0 | 17.7 | 17.4 |
| D | 10.0 | 0.5 | 6.7 | $6.2^{c}$ | 6.7 | 9.6 | $5.7^{b}$ | 6.8 | $7.3^{b}$ | 17.6 | 19.8 | 18.5 | 17.9 | 19.2 |
| | | ± SEM | 0.3 | 0.2 | 0.3 | 0.6 | 0.9 | 4.3 | 1.3 | 3.9 | 1.4 | 1.1 | 0.6 | 1.1 |
| | | LSD (P = 0.05) | NS | 0.7 | NS | NS | 2.9 | NS | 4.0 | NS | NS | NS | NS | NS |

Each value is a mean of 4 chicks.

NS = No significant differences.

Values within a column sharing the same superscript are not significantly different at $P<0.05$.

**Figure 9.1**

**Effect of Dietary Selenium and/or SNP on Weight Gain.**

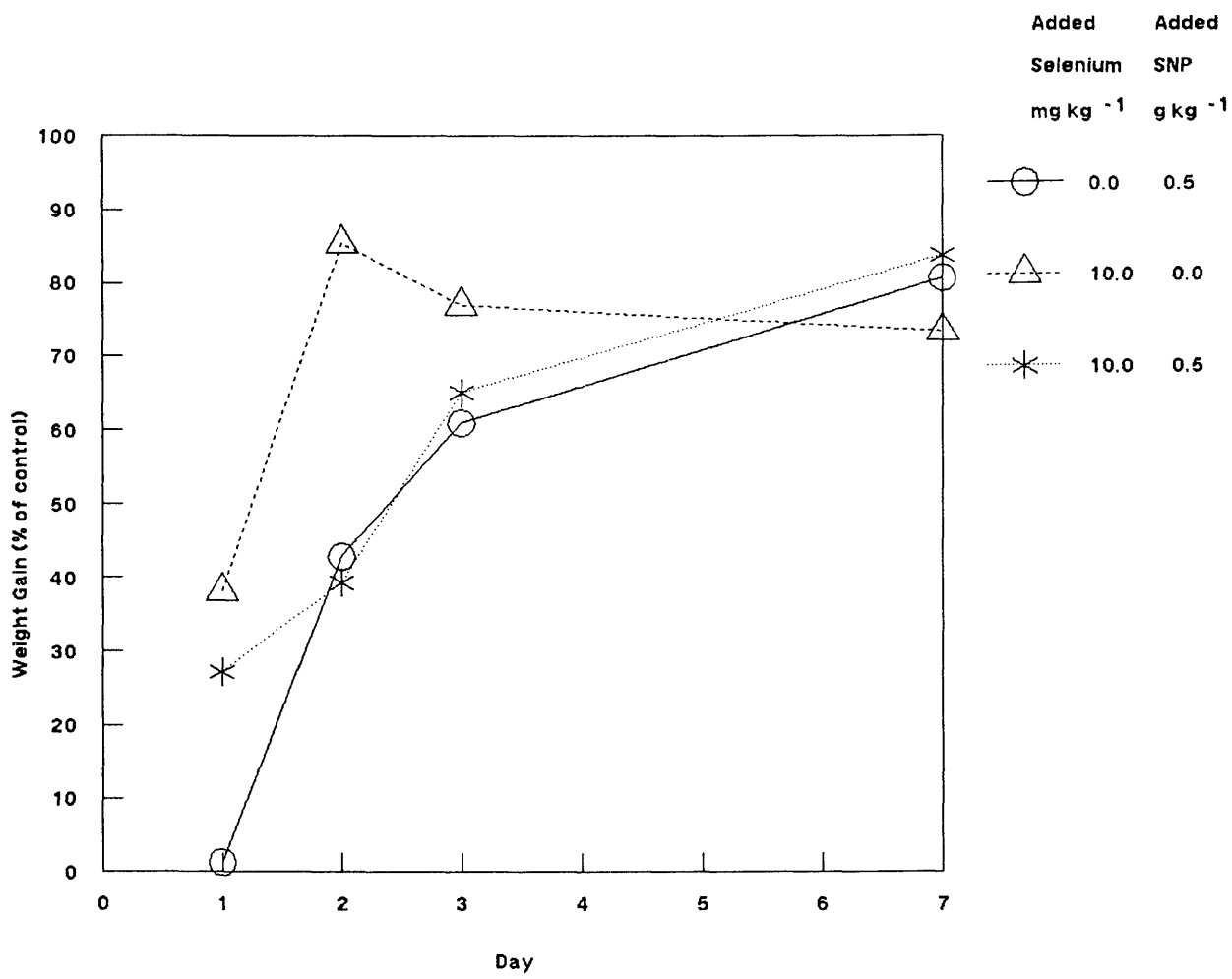


**Figure 9.2**

**Effect of Dietary Selenium and/or SNP on Feed Intake.**

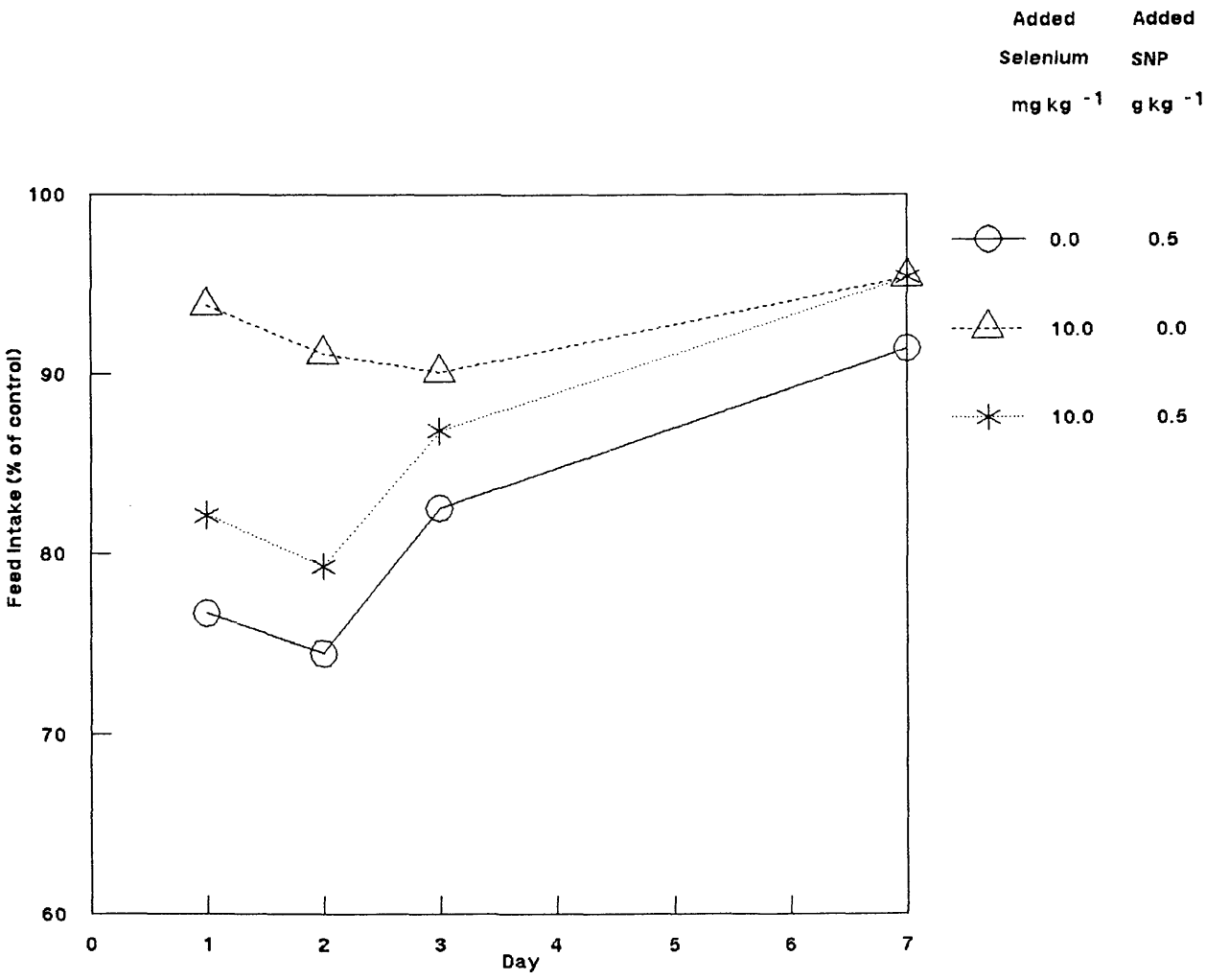
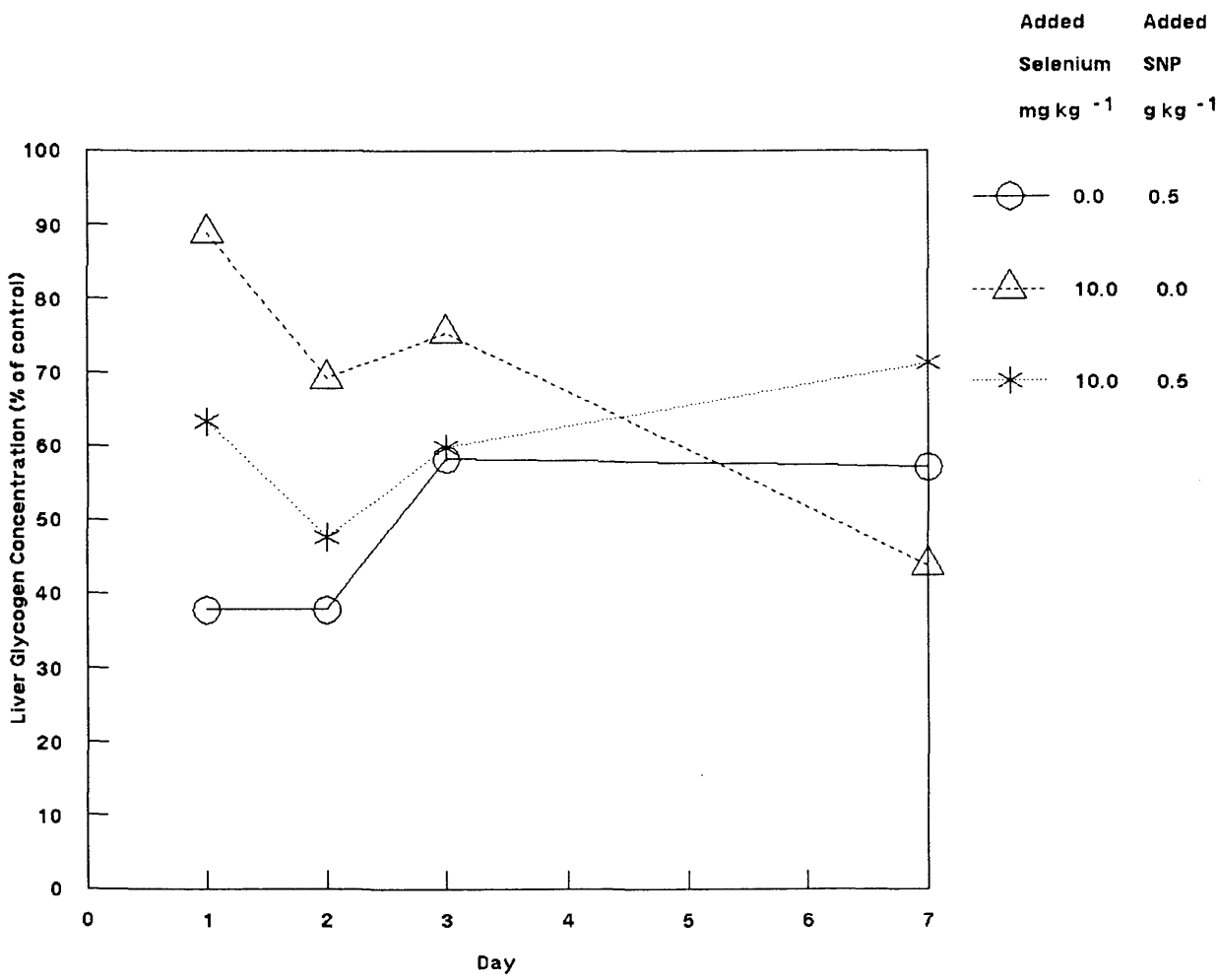


**Figure 9.3**

**Effect of Dietary Selenium and/or SNP on Liver Glycogen Concentration.**



organised in a totally randomised manner within a single growth room:

| Treatment | Added Selenium | Added SNP |
|---|---|---|
| | mg $kg^{-1}$ | g $kg^{-1}$ |
| A | 0.0 | 0.0 |
| B | 0.0 | 0.3 |
| C | 10.0 | 0.0 |
| D | 10.0 | 0.3 |

Twenty-one days after transfer to the experimental diets, the chicks were slaughtered for analysis of liver glycogen concentration.

**Results**

(See Table 4.3).

There were significant interactions between selenium and cyanide in their effects on body weight gain, feed intake and the efficiency of feed utilisation. Addition of SNP to the basic diet significantly decreased weight gain ($P<0.01$) and feed intake ($P<0.05$), but did not affect feed conversion ratio ($P>0.05$). Selenium inclusion caused highly significant reductions in these three variables. However, selenosis was partially alleviated by addition of SNP to the high selenium diet which resulted in a marked improvement in weight gain ($P<0.001$).

An interaction ($P<0.05$) was also observed between selenium and cyanide for effects on liver weight. SNP significantly ($P<0.01$) decreased liver weight when added to the control diet, but did not alter this variable in the presence of selenium.

In contrast, there was no interaction between selenium and SNP for effects on hepatic glycogen concentration. Treatment with SNP resulted in a highly significant ($P<0.001$) decrease in liver glycogen concentration. Selenium did not affect ($P>0.05$) this variable.

**Table 4.3** The Effect of Long-Term Dietary Exposure to SNP and/or Selenium on Growth Performance and Liver Glycogen Concentration in Chicks.

| Treatment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain g $bird^{-1}$ $21d^{-1}$ | Feed Intake g $bird^{-1}$ $21d^{-1}$ | Feed Conversion Ratio | Liver Weight g | Liver Glycogen g $kg^{-1}$ |
|---|---|---|---|---|---|---|---|
| A | 0.0 | 0.0 | $362.7^{a}$ | $765.7^{a}$ | $2.1^{a}$ | $14.16^{a}$ | $17.63^{a}$ |
| B | 0.0 | 0.3 | $310.7^{b}$ | $696.1^{b\,c}$ | $2.2^{a}$ | $12.18^{b}$ | $9.97^{b}$ |
| C | 10.0 | 0.0 | $237.7^{c}$ | $641.2^{c}$ | $2.8^{b}$ | $12.24^{b}$ | $16.79^{a}$ |
| D | 10.0 | 0.3 | $315.6^{b}$ | $704.4^{b}$ | $2.2^{a}$ | $12.26^{b}$ | $8.50^{b}$ |
| | | ± SEM | 11.7 | 19.2 | 0.1 | 0.5 | 1.7 |
| | | LSD (P = 0.05) | 33.8 | 55.3 | 0.2 | 1.47 | 4.90 |

Each value is a mean of 9 chicks.

Values within a column sharing the same superscript are not significantly different at $P<0.05$.

NS = No significant differences.

### 4.3.3 Effect of Dietary Exposure to Sub-lethal Doses of SNP and/or Selenium on Liver Lactate and Pyruvate Levels in Chicks (Experiment 7).

A cassava-soya bean diet supplemented with DL-methionine at a level of 6 g $kg^{-1}$ provided the basis for the following four treatments in this 2 X 2 factorial experiment:

| Treatment | Added Selenium | Added SNP |
|---|---|---|
| | mg $kg^{-1}$ | g $kg^{-1}$ |
| A | 0.0 | 0.0 |
| B | 0.0 | 0.5 |
| C | 10.0 | 0.0 |
| D | 10.0 | 0.5 |

Chronologically, experiment 7 was carried out before experiment 6, when the cassava-based diet and the higher rate of SNP inclusion were in use. Six chicks (Hisex) were allocated to each of the treatments, which were dispersed in a totally randomised manner between two growth rooms. Eighteen days after transfer to the experimental diets the chicks were sacrificed for analysis of liver lactate and pyruvate concentrations.

**Results**

(See Table 4.4).

Addition of SNP to the basic diet resulted in a significant ($P<0.01$) reduction in body weight gain. Growth rate was also significantly ($P<0.05$) decreased by dietary exposure to selenium. No interaction was observed ($P>0.05$) between selenium and SNP for effects on growth. SNP, at the high inclusion rate used in this study, failed to alleviate selenosis. Liver weight was not altered by any of the dietary treatments.

Chronic dietary exposure to SNP resulted in a significant ($P<0.01$) reduction in liver lactate concentration. Treatment with selenium however, had no effect ($P>0.05$) on the level of this metabolite.

**Table 4.4** **The Effect of Dietary Exposure to Sub-lethal Doses of SNP and/or Selenium on Body Weight Gain and Liver Lactate and Pyruvate Concentration.**

| Treatment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain g $bird^{-1}$ $18d^{-1}$ | Liver Weight g | Liver Lactate nmols $g^{-1}$ | Liver Pyruvate nmols $g^{-1}$ | Lactate: Pyruvate Ratio |
|---|---|---|---|---|---|---|---|
| A | 0.0 | 0.0 | $304.2^{a}$ | 13.35 | $3.26^{a}$ | $0.23^{a}$ | $14.62^{a}$ |
| B | 0.0 | 0.5 | $219.7^{b}$ | 12.39 | $2.06^{b}$ | $0.29^{a}$ | $7.03^{b}$ |
| C | 10.0 | 0.0 | $249.5^{b}$ | 13.35 | $3.23^{a}$ | $0.42^{c}$ | $7.89^{b}$ |
| D | 10.0 | 0.5 | $215.3^{b}$ | 11.54 | $2.39^{b}$ | $0.27^{a}$ | $8.79^{b}$ |
| | | $\pm$ SEM | 18.0 | 0.68 | 0.27 | 0.03 | 0.97 |
| | | LSD (P = 0.05) | 53.1 | NS | 0.81 | 0.09 | 3.12 |

Each value is a mean of 6 chicks.

Values within a column sharing the same superscript are not significantly different at $P<0.05$.

NS = No significant differences.

There was no significant ($P>0.05$) interaction between selenium and SNP although addition of SNP to the diet produced a greater effect in the absence of selenium (significant at $P<0.01$) than in its presence (significant at $P<0.05$).

In contrast, a significant ($P<0.05$) interaction was observed between selenium and SNP for effects on liver pyruvate concentration. Exposure to selenium significantly ($P<0.05$) increased liver pyruvate concentration, but only in the absence of SNP. Treatment with SNP alone had no effect ($P>0.05$) on this variable.

There was a highly significant ($P<0.001$) interaction between selenium and SNP for effects on liver lactate:pyruvate ratio. Inclusion of SNP in the basic diet resulted in a highly significant ($P<0.001$) decrease in liver lactate:pyruvate ratio. However, when this toxin was added to the high selenium rations lactate:pyruvate ratio was unaltered, and remained significantly ($P<0.001$) lower than the value observed in birds consuming the control diets. Similarly, hepatic lactate:pyruvate ratio was reduced ($P<0.01$) by inclusion of selenium in the basic diet. Simultaneous treatment with SNP did not alter this ratio.

Again, there were no significant effects of growth room on any of the variables measured.

### 4.3.4 Discussion of Experiments 5-7.

Chronic exposure of chicks to dietary selenium produced obvious signs of toxicity, manifested by impaired growth performance. These effects became more pronounced with time, toxicity being firmly established seven days into the study.

Liver glycogen and lactate concentrations were unaffected by chronic exposure to selenium, suggesting that this toxin did not cause major lesions in the aerobic pathways of glucose catabolism. However, liver pyruvate concentration was elevated in selenium toxicity and the liver lactate:pyruvate ratio was commensurately decreased.

Although selenium effects on carbohydrate metabolism have received little attention to date, similar trends have been noted by other workers. Caravaggi (1971) observed that chronic selenium toxicity had little effect on the oxygen uptake of rat liver and kidney minces, with or without added glucose, but that the aerobic production of $^{14}CO_2$ from D-$^{14}$C-glucose by these minces was markedly diminished. The author proposed selenium-mediated stimulation of fatty acid degradation as a possible explanation for this latter observation. Shearer (1973) was unable to demonstrate any significant change in the concentrations of several glycolytic and TCA cycle intermediates in livers and kidneys from rats exposed to toxic doses of dietary selenium. However, in this study, which used only 4 mg Se per kg feed, there was a tendency for pyruvate concentration to be increased, whilst lactate level was largely unaltered.

Although the selenium-mediated increase in hepatic pyruvate content seen in experiment 7 may be related to alterations in the pathways of gluconeogenesis or fat metabolism, minor pertubations in glucose catabolism, particularly at the levels of enzymes involved in pyruvate metabolism remain a possibility. In fact, several workers have demonstrated the *in vivo* inhibition of certain enzymes of the TCA cycle after selenium treatment,including α-ketoglutarate dehydrogenase (Ignesti *et al.*, 1986), succinate dehydrogenase (Klug *et al.*, 1950b; Ray & Ray, 1975) and isocitrate dehydrogenase (Nebbia *et al.*, 1990). The latter two are key regulatory enzymes in this cycle. Nebbia's group also reported a selenium-mediated decrease in liver lactate dehydrogenase activity. These changes would be expected to lead to a slowing of the TCA cycle and a build up of pyruvate, and might therefore explain the observations of experiment 7. Recently, Davis & Frear (unpublished) have demonstrated that selenium not only decreases the activity of certain mitochondrial enzymes (the Fe-S containing proteins, succinate dehydrogenase and NADH: coenzyme Q reductase), but also reduces the acid-labile sulphide content of these organelles. This observation reinforces the view that the selenium-induced increase in hepatic pyruvate concentration results from restricted mitochondrial oxidation.

Chronic exposure to dietary SNP produced signs of toxicity in the chicks, revealed by impaired growth performance. In contrast to the

pattern observed in the development of selenosis, SNP produced its greatest effects in the first few days of exposure, a fact probably related to the marked reduction in voluntary feed intake occurring at this time. This finding is in agreement with results reported by Elzubeir (1986).

Liver glycogen concentration was markedly reduced by SNP treatment, as was lactate level. Hepatic pyruvate content was increased, though not significantly, resulting in a decreased lactate:pyruvate ratio. A similar decrease in liver glycogen concentration during chronic cyanide intoxication was also demonstrated by Shaffi & Prasad (1979), Kovacs & Leduc (1982) and by Raymond *et al.* (1986) in their studies on freshwater fish, and by Padmaja & Panikkar (1989) using rabbits. In only two of these studies (Kovacs & Leduc, 1982; Padmaja & Panikkar, 1989) were liver lactate levels measured, but in both cases the concentration of this metabolite was increased by cyanide exposure. However, owing to differences in both mode of administration (Padmaja and Panikkar gave an intraperitoneal injection of cyanide twice weekly) and species used, the relevance of these studies to events occurring during chronic exposure to dietary cyanide in chickens remains questionable. In addition, both investigations employed much lower levels of cyanide than were used in experiments 5-7. Even so, if the SNP induced reduction in hepatic glycogen concentration is taken as indicative of increased anaerobic glucose catabolism, the accompanying decrease in lactate content requires some explanation.

As discussed in detail in Section 2.3.2, similarly conflicting results have been obtained from investigation of the mechanisms of acute cyanide toxicity. Although the majority of studies have demonstrated cyanide-induced decreases in tissue glycogen levels with concomitant increases in tissue lactate concentration, Baxter & Hensley (1969) reported a reduction in the amount of lactate in the livers of cyanide treated rats. The lactate:pyruvate ratio was halved and the sum of the concentrations of these two metabolites was decreased, findings which are all in agreement with the results of experiments 5-7. They explained these results by invoking the operation of a cyanide catalysed alternative route for carbohydrate metabolism, the methylglyoxal pathway (Figure 1). It is conceivable,

particularly given the high dose of SNP used in experiment 7, that this pathway assumed sufficient importance to produce the results observed in this trial.

Plasma glucose data failed to reflect increased glycogenolysis. However, this may not be surprising in the chicken because the control of blood glucose in this species differs from that of mammals (Pearce, 1977 and 1983).

In conclusion, it seems probable, based on the liver glycogen data, that chronic cyanide exposure does result in impaired mitochondrial function leading to increased anaerobic catabolism of glucose; (whether glucose breakdown is fuelled by increased glycolysis remains questionable in view of the observed decrease in liver lactate content after SNP treatment). However, a direct effect of the toxin on the enzymes involved in glycogen synthesis or degradation cannot be excluded on the basis of the results of experiments 5-7.

Liver glycogen levels showed no adaptation to SNP with increasing time of exposure. This observation is in agreement with the findings of Raymond *et al.* (1986) who demonstrated that glycogen levels in the livers of trout *(Salmo gairdneri)* gradually returned to normal after 7-10 days of exposure to 0.01 and 0.02 mg HCN $L^{-1}$, but remained depressed when a higher concentration of the toxin (0.03 mg HCN $L^{-1}$) was used.

The interaction between selenium and cyanide, whereby cyanide alleviates selenium toxicity, does not appear to occur at the level of anaerobic glucose metabolism. No interaction was observed between these toxins for effects on liver glycogen concentration. There were interactions between cyanide and selenium for liver pyruvate concentration and lactate:pyruvate ratio. However, these effects were probably secondary. In the case of pyruvate levels, the interaction arose only because selenium was without effect on this variable when cyanide was present in the diet. Both toxins, when given alone, decreased the hepatic lactate:pyruvate ratio, but for different reasons. As previously mentioned, cyanide decreased liver lactate content, whilst selenium increased pyruvate. When both toxins were present in the diet, the selenium effects were nullified, cyanide

effects remained and lactate:pyruvate ratio appeared unaltered when compared with the cyanide only, or selenium only control, leading to a statistically significant interaction.

## 4.4 The Effect of Pyruvate Donors on The Chronic Toxicity of Cyanide.

If the mechanism of chronic cyanide poisoning involves inhibition of cytochrome oxidase and resultant stimulation of the anaerobic catabolism of glucose, pyruvate donors might be expected to modify the degree of toxicity. Experiments 8 and 9 were designed to examine this possibility.

### 4.4.1 The Effect of Dietary Cystine, Alanine and Lactate on the Chronic Toxicity of SNP (Experiment 8).

Conventional chick mash supplemented with 2.5 g DL-methionine per kg formed the basis for the following treatments:

| Treatment | Added L-Cystine g $kg^{-1}$ | Added L-Alanine g $kg^{-1}$ | Added L-Lactate g $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|---|---|
| A | 0.00 | 0.00 | 0.00 | 0.00 |
| B | 0.00 | 0.00 | 0.00 | 0.30 |
| C | 3.00 | 0.00 | 0.00 | 0.00 |
| D | 3.00 | 0.00 | 0.00 | 0.30 |
| E | 6.00 | 0.00 | 0.00 | 0.00 |
| F | 6.00 | 0.00 | 0.00 | 0.30 |
| G | 0.00 | 2.25 | 0.00 | 0.00 |
| H | 0.00 | 2.25 | 0.00 | 0.30 |
| I | 0.00 | 4.50 | 0.00 | 0.00 |
| J | 0.00 | 4.50 | 0.00 | 0.30 |
| K | 0.00 | 0.00 | 4.50 | 0.00 |
| L | 0.00 | 0.00 | 4.50 | 0.30 |

L-cystine and L-alanine were added at two comparable levels, whilst L-lactate was included only at the higher rate.

Six chicks (ISA Brown) were allocated to each of the treatments, which were dispersed in a totally randomised manner within two equivalent growth rooms. Weight gains and feed intakes were monitored over a period of 21 days.

**Results**

(see Table 5.1)

No growth room effects were apparent in this trial. Addition of L-cystine, L-lactate or the lower level of L-alanine to the diet of chicks did not significantly ($P>0.05$) affect growth rate. However, a significant ($P<0.05$) increase in body weight gain was observed in birds fed the higher level of dietary L-alanine.

SNP failed to alter the growth of chicks consuming either the control diet or the L-cystine supplemented diets. In contrast, addition of SNP to diets containing the low level of L-alanine or a high concentration of L-lactate resulted in a significant ($P<0.01$) decrease in body weight gain. At the high level of L-alanine, SNP inclusion caused a highly significant ($P<0.001$) reduction in growth rate.

### 4.4.2 The Effect of Dietary Alanine and Serine on the Chronic Toxicity of SNP (Experiment 9).

This experiment was designed to determine whether the unexpected and detrimental effect of L-alanine in chronic cyanide intoxication, observed in experiment 8, was reproducible. The effect of another pyruvate precursor, L-serine, was also investigated.

**Table 5.1** **The Effect of Dietary L-Cystine, L-Alanine and L-Lactate on the Growth Performance of Chicks Fed SNP.**

| Treatment | Added L-Cystine g $kg^{-1}$ | Added L-Alanine g $kg^{-1}$ | Added L-Lactate g $kg^{-1}$ | Added SNP g $kg^{-1}$ | Weight Gain g $bird^{-1}$ $21d^{-1}$ | Feed Intake g $bird^{-1}$ $21d^{-1}$ | Feed Conversion Ratio |
|---|---|---|---|---|---|---|---|
| A | 0.00 | 0.00 | 0.00 | 0.00 | $377.3^{bc}$ | 649.8 | 1.72 |
| B | 0.00 | 0.00 | 0.00 | 0.30 | $359.0^{cd}$ | 615.9 | 1.72 |
| C | 0.30 | 0.00 | 0.00 | 0.00 | $384.7^{ab}$ | 679.7 | 1.77 |
| D | 0.30 | 0.00 | 0.00 | 0.30 | $384.3^{ab}$ | 660.9 | 1.72 |
| E | 0.60 | 0.00 | 0.00 | 0.00 | $395.2^{ab}$ | 695.6 | 1.76 |
| F | 0.60 | 0.00 | 0.00 | 0.30 | $375.3^{bc}$ | 628.8 | 1.68 |
| G | 0.00 | 2.25 | 0.00 | 0.00 | $380.5^{abc}$ | 698.0 | 1.83 |
| H | 0.00 | 2.25 | 0.00 | 0.30 | $339.2^{d}$ | 614.5 | 1.81 |
| I | 0.00 | 4.50 | 0.00 | 0.00 | $405.0^{a}$ | 706.1 | 1.74 |
| J | 0.00 | 4.50 | 0.00 | 0.30 | $346.2^{d}$ | 646.0 | 1.87 |
| K | 0.00 | 0.00 | 7.25 | 0.00 | $372.2^{bc}$ | 682.6 | 1.84 |
| L | 0.00 | 0.00 | 7.25 | 0.30 | $335.8^{d}$ | 638.2 | 1.88 |
| | | | | ± SEM | 8.7 | 23.7 | 0.05 |
| | | | | LSD (P = 0.05) | 24.7 | NS | NS |

Each value is a mean of 6 chicks.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.
NS = No significant differences.

Chick mash supplemented with 2.5 g DL-methionine per kg feed provided the basis for the following treatments:

| Treatment | Added L-Alanine g $kg^{-1}$ | Added L-Serine g $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|---|
| A | 0.00 | 0.00 | 0.00 |
| B | 0.00 | 0.00 | 0.30 |
| C | 4.00 | 0.00 | 0.00 |
| D | 4.00 | 0.00 | 0.30 |
| E | 0.00 | 4.72 | 0.00 |
| F | 0.00 | 4.72 | 0.30 |

Four birds (ISA Brown) were allocated to each of the treatments, which were dispersed in a totally randomised manner within a single growth room.

**Results**

(See Table 5.2)

Inclusion of L-alanine or L-serine in the diet did not significantly ($P>0.05$) affect the growth performance of chicks, as measured by body weight gain, feed intake and feed conversion ratio. Both amino acids exacerbated SNP toxicity. Addition of SNP to the basic diet caused significant ($P<0.01$) reductions in both weight gain and feed intake, leaving the feed conversion ratio unaltered. When SNP was added to the L-alanine supplemented diet its effects were more pronounced, resulting in highly significant ($P<0.001$) decreases in weight gain and feed intake, together with a significant ($P<0.05$) increase in feed conversion ratio. Similarly, SNP in the presence of L-serine produced a highly significant ($P<0.001$) reduction in weight gain, together with significant ($P<0.01$) decreases in feed intake and the efficiency of feed utilisation.

**Table 5.2** **The Effect of Dietary L-Alanine and L-Serine on the Growth Performance of Chicks Fed SNP.**

| Treatment | Added L-Alanine g $kg^{-1}$ | Added L-Serine g $kg^{-1}$ | Added SNP g $kg^{-1}$ | Weight Gain g $bird^{-1}$ $21d^{-1}$ | Feed Intake g $bird^{-1}$ $21d^{-1}$ | Feed Conversion Ratio |
|---|---|---|---|---|---|---|
| A | 0.00 | 0.00 | 0.00 | $349.6^{a}$ | $798.4^{a}$ | $2.30^{a}$ |
| B | 0.00 | 0.00 | 0.30 | $272.2^{b}$ | $656.9^{b}$ | $2.43^{a}$ |
| C | 4.00 | 0.00 | 0.00 | $380.7^{a}$ | $857.4^{a}$ | $2.25^{a}$ |
| D | 4.00 | 0.00 | 0.30 | $250.1^{b}$ | $618.3^{b}$ | $2.47^{b}$ |
| E | 0.00 | 4.72 | 0.00 | $369.9^{a}$ | $815.6^{a}$ | $2.21^{a}$ |
| F | 0.00 | 4.72 | 0.30 | $259.2^{b}$ | $654.8^{b}$ | $2.53^{b}$ |
| | | | $\pm$ SEM | 16.1 | 30.1 | 0.07 |
| | | | LSD (P = 0.05) | 47.7 | 89.7 | 0.20 |

Each value is a mean of 4 chicks.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.

### 4.4.3 Discussion of Experiments 8 and 9.

Dietary L-alanine. L-Lactate and L-serine all exacerbated the chronic toxicity of SNP.

Under normal conditions, the opposing pathways of glycolysis and gluconeogenesis are controlled in response to fluctuations in the fuel supply and energy level in the cells. Assuming that impaired mitochondrial function and increased glycolysis are features of chronic cyanide toxicity, one would expect the flux through the gluconeogenic pathways to be commensurately reduced. Newsholme & Underwood (1966) demonstrated, in an acute study using kidney cortex slices, that gluconeogenesis from glycerol or dihydroxyacetone was very rapidly inhibited by the addition of cyanide.

Any treatment which favours gluconeogenesis over glycolysis during cyanide exposure might be expected to increase the energy deficit and exacerbate the toxicity of this poison. This may provide a possible explanation for the effects of L-alanine, L-serine and L-lactate. The main precursors for glucose formation in the liver are glycerol, amino acids and lactate. Experiments with perfused rat liver have suggested that an increase in the plasma concentration of any of these precursors could result in a stimulation of gluconeogenesis (Exton *et al.*, 1970).

The mechanism of this effect has not been well investigated, however, Start (1969) has shown that administration of lactate to an intact animal raises the concentration of pyruvate in the liver which should stimulate pyruvate carboxylase (E.C. 6.4.1.1). Start also demonstrated that the administration of lactate increases the hepatic content of citrate which would be expected to inhibit phosphofructokinase (E.C. 2.7.1.11). In addition, liver pyruvate kinase (E.C. 2.7.1.40) is inhibited by L-alanine (Seubert & Schoner, 1971; Eigenbrodt & Schoner, 1977).

Investigation of the effects of modifiers of gluconeogenesis such as acetoacetate (stimulator) and p-tert-butylbenzoic acid (inhibitor) on the growth performance of chicks consuming SNP alone or SNP plus alanine, serine or lactate might provide useful information on the

mechanism by which these compounds exacerbate chronic cyanide toxicity.

Dietary supplementation with L-cystine did not exacerbate cyanide toxicity. This may be attributable to its involvement, either directly or indirectly, in cyanide detoxification.

## 4.5 The Effect of Dietary Supplementation with L-Cystine on the Chronic Toxicity of SNP (Experiment 10).

In experiment 8, L-cystine appeared to have a very different effect on the severity of chronic cyanide intoxication from that of the other pyruvate donors alanine, lactate and serine. Voegtlin *et al.* discovered as long ago as 1926 that cystine, cysteine and GSSG protected against acute cyanide intoxication in rats. More recently Elzubeir (1986) demonstrated a beneficial effect of dietary L-cystine on the growth performance of chicks consuming SNP. In addition, Benz *et al.* (1990) evaluated the ability of the optical isomers of cysteine and N-acetylcysteine to act as antidotes against acrylonitrile toxicity in the rat and concluded that these compounds were detoxifying cyanide produced during the metabolism of this compound.

Since the impairment of growth performance normally observed on addition of SNP to the basic diet failed to achieve significance in experiment 8, it was not possible to determine whether L-cystine was actually beneficial under the conditions of these experiments. Experiment 10 was designed to clarify this point.

Chick mash, supplemented with DL-methionine at 1 g $kg^{-1}$, provided the base for the following four treatments in this 2 X 2 factorial experiment:

| Treatment | Added L-Cystine g $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|
| A | 0.0 | 0.0 |
| B | 0.0 | 0.3 |
| C | 6.0 | 0.0 |
| D | 6.0 | 0.3 |

Four birds (ISA Brown) were allocated to each of the treatments, which were dispersed in a totally randomised manner within a single growth room. Body weight gains and feed intakes were monitored over a period of 14 days.

**Results**

(See Table 6.1).

Significant ($P<0.01$) interactions were observed between cystine and SNP in their effects on body weight gains and feed conversion ratios. L-cystine supplementation did not significantly ($P>0.05$) affect growth performance. Inclusion of SNP in the basic diet caused highly significant ($P<0.001$) reductions in body weight gain, feed intakes and the efficiency of feed utilisation. However, SNP had much less effect when added to the cystine supplemented diet. Weight gains and feed intakes were still decreased but to a lesser extent (with probabilities of 0.01 and 0.05 respectively), whilst feed conversion ratios were unaffected. In fact, birds consuming both cystine and SNP performed as well as those fed the control rations.

### 4.5.1 Discussion of Experiment 10

Supplemental L-cystine alleviated the detrimental effects of chronic exposure to dietary SNP on growth performance. Thus, when SNP is toxic, L-cystine does have a beneficial effect under the conditions of these experiments. The lack of effect of SNP in experiment 8 can only be attributed to natural variation in feed constituents which is a common problem in nutritional studies.

Table 6.1 The Effect of Dietary L-Cystine on the Chronic Toxicity of SNP.

| Treatment | Added L-Cystine g $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body weight gain g $bird^{-1}$ $14d^{-1}$ | Feed Intake g $bird^{-1}$ $14d^{-1}$ | Feed Conversion Ratio |
|---|---|---|---|---|---|
| A | 0.0 | 0.0 | $213^{ac}$ | $437.4^{ac}$ | $2.06^{a}$ |
| B | 0.0 | 0.3 | $131^{b}$ | $345.6^{b}$ | $2.66^{b}$ |
| C | 6.0 | 0.0 | $232^{a}$ | $470.1^{a}$ | $2.03^{a}$ |
| D | 6.0 | 0.3 | $200^{c}$ | $415.7^{c}$ | $2.08^{a}$ |
| | | ± SEM | 7.3 | 14.8 | 0.09 |
| | | LSD(P=0.05) | 22.5 | 45.6 | 0.27 |

Each value is a mean of 4 chicks.

Values within a column sharing the same superscript are not significantly different at $P<0.05$.

## 4.6 The Effect of Dietary Selenium and/or SNP on the Redox State of Pyridine Nucleotides in Chicken Liver (Experiment 11).

Variables such as hepatic concentrations of glycogen, lactate and pyruvate provide only indirect measures of cyanide action. In the experiment described below the redox level of the pyridine nucleotide couples was determined as a direct index of the state of the electron transport system during chronic cyanide intoxication. If the toxin is present in quantities sufficient to inhibit cytochrome C oxidase then one might expect the primary effect of chronic cyanide exposure to be a lowering of the mitochondrial $NAD^+$:NADH ratio. A secondary result

might be the accumulation of NADH and NADPH in the cytoplasm which, in turn, might facilitate selenium metabolism.

It is generally considered that the disturbances occurring during normal fractionation procedures for subcellular compartments will alter the redox state of pyridine nucleotides (Williamson *et al.*, 1967). Consequently, whole liver nucleotide concentrations were estimated in this investigation. The alternative technique, using redox reactions to calculate mitochondrial and cytosolic NAD(P):NAD(P)H ratios, demands assumptions which are questionable (Akerboom *et al.*, 1979). However, since the amounts of pyridine nucleotides are higher in the mitochondria than in the cytosol, changes in whole tissue NAD(P)/NAD(P)H levels primarily reflect alterations in the mitochondrial content of these metabolites (Tischler *et al.*, 1977; Sahlin & Katz, 1986; Henriksson *et al.*, 1986).

The effects of chronic exposure to selenium and/or cyanide on the pyridine nucleotide system have not previously been reported.

Chick mash supplemented with DL-methionine at 2 g $kg^{-1}$ provided the basis for the four treatments shown below in this 2 X 2 factorial experiment:

| Treatment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|
| A | 0.0 | 0.0 |
| B | 0.0 | 0.3 |
| C | 10.0 | 0.0 |
| D | 10.0 | 0.3 |

Nine chicks (ISA Brown) were allocated to each of the treatments, which were dispersed in a totally randomised manner within a single growth room. Weight gains and feed intakes were monitored at regular intervals over a period of 26 days, after which the chicks were

slaughtered for estimation of liver pyridine nucleotide concentrations.

**Results**

(See Table 7.1).

Highly significant interactions ($P<0.001$) were observed between the effects of selenium and SNP on growth performance as measured by body weight gains, feed intakes and the efficiency of feed utilisation.

Selenium alone caused a highly significant ($P<0.001$) decrease in body weight gain, but failed to affect ($P>0.05$) this variable in chicks consuming SNP. Inclusion of SNP in the basic diet resulted in a highly significant ($P<0.001$) reduction in body weight gain, however, when added to the high selenium diet, SNP dramatically improved growth rate ($P<0.001$), i.e. SNP alleviated selenium toxicity.

Addition of SNP to the basic diet caused a highly significant ($P<0.001$) reduction in feed intake, but when added to the high selenium diet, SNP did not affect ($P>0.05$) this variable. In contrast, although selenium alone also decreased ($P<0.001$) feed intake, when this element was added to SNP-containing diets, feed intake was significantly ($P<0.001$) increased. That is, selenium partially overcame the toxic effects of SNP on feed consumption.

Both SNP and selenium given alone significantly increased feed conversion ratio with probabilities greater than 0.05 and 0.01 respectively. However, addition of SNP to the high selenium diets dramatically reduced ($P<0.001$) feed conversion ratio, such that the efficiency of feed utilisation in chicks on treatment D was not significantly ($P>0.05$) different from that of control birds.

**Table 7.1** **The Effect of Dietary Selenium and/or SNP on Growth Performance and the Redox State of Hepatic Pyridine Nucleotides in Chickens**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain g $bird^{-1}$ $26d^{-1}$ | Feed Intake g $bird$ $26d^{-1}$ | Feed Conver-sion Ratio | Liver Weight g | Liver NAD µmols gfw* | Liver NADH µmols gfw* | Liver $NAD^+$ : NADH | Liver $NADP^+$ µmols gfw* | Liver NADPH µmols gfw* | Liver $NADP^+$ : NADPH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0.0 | 0.0 | $544.9^{a}$ | $1228.6^{a}$ | $2.26^{a}$ | $15.84^{a}$ | 0.696 | $0.211^{a}$ | $3.56^{a}$ | $0.136^{a}$ | $0.428^{a}$ | $0.437^{a}$ |
| B | 0.0 | 0.3 | $406.1^{b}$ | $1021.8^{b}$ | $2.52^{b}$ | $12.84^{b\,c}$ | 0.667 | $0.184^{a}$ | $4.99^{a\,b}$ | $0.166^{a\,b}$ | $0.314^{b}$ | $0.653^{a\,b}$ |
| C | 10.0 | 0.0 | $238.0^{c}$ | $812.3^{c}$ | $3.47^{c}$ | $11.63^{c}$ | 0.769 | $0.125^{b}$ | $5.97^{b}$ | $0.179^{b}$ | $0.252^{b}$ | $1.050^{b}$ |
| D | 10.0 | 0.3 | $443.3^{b}$ | $1073.6^{b}$ | $2.43^{a\,b}$ | $13.93^{b}$ | 0.713 | $0.168^{a\,b}$ | $4.72^{a\,b}$ | $0.182^{b}$ | $0.298^{b}$ | $0.777^{a\,b}$ |
| ± SEM | | | 13.6 | 36.0 | 0.07 | 0.64 | 0.048 | 0.017 | 0.61 | 0.011 | 0.037 | 0.168 |
| LSD (P = 0.05) | | | 39.3 | 103.9 | 0.20 | 1.84 | NS | 0.051 | 1.77 | 0.032 | 0.108 | 0.489 |

* = Per gram fresh weight.

Each value is a mean of 9 chicks.

NS = No significant differences.

Values within a column sharing the same superscript are not significantly different at $P<0.05$.

A highly significant ($P<0.001$) interaction was observed between selenium and SNP for effects on liver weight. Selenium decreased ($P<0.001$) liver weight in the absence, but not in the presence, of SNP. SNP also reduced liver weight ($P<0.01$) when included in the basic diet, but increased this variable when added to the high selenium diet, partially overcoming the toxic effects of this element.

Pyridine nucleotide data were analysed in a randomised blocked manner, taking into account variation introduced as a result of differences in the length of storage prior to determination of these unstable metabolites. In order to minimise such variation, all analyses were completed within two weeks of slaughter.

No significant ($P>0.05$) effects of SNP and/or selenium on liver $NAD^+$ concentration were observed in this investigation.

An effect of blocks was evident in the data for NADH content, but treatment differences still attained significance. Addition of selenium to the standard chick diet decreased the hepatic concentration of this metabolite ($P<0.01$). However, the element had no effect ($P>0.05$)on liver NADH content in chicks consuming SNP-containing diets, although the interaction did not quite achieve significance. SNP treatment did not alter liver NADH levels ($P>0.05$).

An effect of blocks was also apparent in the data on $NADP^+$ concentrations. Inclusion of selenium in the basic diet resulted in a significant ($P<0.05$) increase in hepatic $NADP^+$ concentration, however, the element did not alter the levels of this nucleotide when added to SNP-containing diets. There was no interaction between selenium and cyanide for effects on this variable. SNP treatment did not alter liver $NADP^+$ levels.

An effect of blocks was again evident in the data for NADPH concentrations. A significant ($P<0.05$) interaction was observed between the effects of selenium and SNP on hepatic NADPH content. Selenium, when added to the standard diet caused a significant ($P<0.01$) reduction in liver NADPH concentration, but did not affect this metabolite ($P>0.05$) when added to the SNP-containing diet.

Similarly, SNP also decreased NADPH content in the absence of selenium ($P<0.05$), but not in its presence.

A significant interaction ($P<0.05$) was also observed between the effects of selenium and SNP on hepatic $NAD^+$:NADH ratio. Selenium increased the ratio when included in the basic diet ($P<0.01$), but failed to alter this variable when added to SNP-containing diets ($P>0.05$). SNP treatment did not affect liver $NAD^+$:NADH ratio.

Selenium significantly ($P<0.05$) increased hepatic $NADP^+$:NADPH ratio, when added to the standard chick mash, but did not alter this variable when included in SNP-containing diets. SNP treatment did not affect liver $NADP^+$:NADPH ratio ($P>0.05$).

### 4.6.1 Discussion of Experiment 11.

The decreased total liver NADH and NADPH concentrations together with the increased $NADP^+$ content and elevated $NAD^+$:NADH and $NADP^+$:NADPH ratios observed in selenium toxicity are probably best explained in terms of an increased demand for reducing equivalents. Selenium metabolism generates $NADP^+$ at the expense of NADPH in glutathione reductase catalysed reactions (Ganther & Hsieh, 1974; Ganther, 1966; Hsieh & Ganther, 1975). The resultant displacement in the $NADP^+$:NADPH ratio in the cytosol would be expected to cause readjustments throughout the pyridine nucleotide system in both cytosol and mitochondria.

SNP treatment had little effect on the redox state of the pyridine nucleotide system. The only significant alteration was a decrease in total liver NADPH concentration. In fact, the trend, though not significant, was for both redox couples to become more oxidised in the presence of SNP. Such changes might be expected to slow rather than facilitate selenium metabolism. The effect of SNP on NADPH may simply reflect this general trend or may result from the direct action of cyanide on an enzyme catalysing an NADPH-generating reaction. Although chronic cyanide exposure does not increase the reduction level of pyridine nucleotides, it is still possible that some inhibition of cytochrome C oxidase may be occurring since the results

provide no information on the flux through the electron transport chain. If the rate of reduction of $NAD^+$ to NADH by the TCA cycle and the rate of NADH oxidation were both decreased to the same extent, the $NAD^+$:NADH ratio would remain constant despite the decrease in flux through the pathway. One can envisage an adaptation to chronic cyanide exposure leading to an altered steady state in the pathways of energy production.

The only information currently available concerning the redox state of pyridine nucleotides during cyanide exposure has been obtained during acute studies. In general, total or mitochondrial $NAD^+$:NADH ratios are decreased in cyanide intoxication (Baxter & Hensley, 1969; Hattori *et al.*, 1986; Sahlin & Katz, 1986). However, anomalies have been observed even in these acute investigations. Hattori's group actually observed a slight increase in redox state at low concentrations of cyanide (0.5 µg ml $blood^{-1}$). In addition, Baxter & Hensley demonstrated a cyanide-induced increase in cytoplasmic $NAD^+$:NADH ratio which they explained in terms of the operation of the methylglyoxal pathway (Figure 1).

When selenium was added to the SNP-containing diet it failed to have any effect on pyridine nucleotide concentrations or ratios, reinforcing the idea that cyanide might indeed act by slowing selenium metabolism.

Similarly, when SNP was added to the high selenium diet, NADPH levels were unaffected.

It should be pointed out that extraction of the liver with potassium hydroxide or perchloric acid disturbs any non-covalent bonding of metabolites to proteins and other cell constituents so that all pyridine nucleotides, whether free or bound, were measured in this investigation, although only free nucleotides would be available for direct participation in metabolic reactions.

## 4.7 The Effect of Dietary Selenium and/or SNP on the Redox State of the Glutathione System (Experiments 12-14).

Since glutathione is essential for selenium metabolism and many other cellular processes it seemed possible that the GSSG/GSH couple might play a role in the selenium-cyanide interaction. To test this hypothesis, hepatic and blood GSH and GSSG concentrations were determined in chicks following chronic exposure to selenium and/or SNP. Hepatic glutathione reductase activity was also monitored because this enzyme is responsible for linking the GSSG/GSH couple to the pyridine nucleotide system in addition to its function in the maintenance of the redox state of glutathione.

### 4.7.1 The Effect of Dietary Selenium and/or SNP on the Concentration and Redox State of Hepatic Glutathione (Experiment 12).

The liver was considered the most suitable tissue in which to study the effects of selenium and cyanide on the glutathione system because it is the major site both of selenium deposition and glutathione homeostasis.

Chick mash, supplemented with 3.0 g DL-methionine per kg feed, provided the basis for the following four treatments in this 2 X 2 factorial experiment:

| Treatment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|
| A | 0.0 | 0.0 |
| B | 0.0 | 0.3 |
| C | 10.0 | 0.0 |
| D | 10.0 | 0.3 |

Eight birds (ISA Brown) were allocated to each of the treatments, which were dispersed in a randomised blocked design between two equivalent growth rooms. Body weight gains were monitored over a period of 21 days, after which birds were slaughtered for determination of liver glutathione concentrations.

**Results**

(See Table 8.1).

No growth room effects were apparent in this trial. A highly significant ($P<0.001$) interaction was observed between selenium and SNP in their effects on growth. Inclusion of selenium in the basic diet resulted in a highly significant ($P<0.001$) reduction in body weight gain. SNP, when added to the basic chick mash did not affect growth, however, when included in the high selenium diet, this toxin caused a highly significant ($P<0.001$) increase in body weight gain. The alleviation of selenium toxicity by SNP was complete, at least in terms of effects on growth since chicks consuming the diet containing both toxins grew as well as those fed the control diet.

Chronic exposure to dietary selenium increased ($P<0.05$) hepatic total and reduced glutathione concentrations. Consideration of the individual factors in this experiment revealed that inclusion of SNP in the feed resulted in a highly significant ($P<0.001$) decrease in these variables. Such effects failed to achieve significance when SNP was included in the basic chick mash, however, addition of this compound to the high selenium diet decreased ($P<0.001$) both liver total and reduced glutathione. The levels of these metabolites in chicks consuming selenium with SNP were not significantly ($P>0.05$) different from those observed in control birds. However, there was no significant interaction between the effects of these two toxins on either hepatic total or reduced glutathione.

There was no significant effect of any of the dietary treatments on hepatic oxidised glutathione, or on the GSH:GSSG ratio. In the latter case, variation was particularly high because it represented the combined variation in both variables. However, there was a tendency for selenium to decrease GSSG and increase the ratio.

A significant interaction ($P<0.01$) was observed between selenium and SNP in their effects on glutathione reductase activity. Addition of selenium to the basic diet reduced ($P<0.001$) the activity of this enzyme. SNP alone tended to decrease glutathione reductase activity although this effect failed to achieve significance. However, when

**Table 8.1** **The Effects of Dietary Selenium and/or SNP on Growth Performance and Hepatic Glutathione Concentration in Chicks.**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain[1] g $bird^{-1}$ $21d^{-1}$ | Liver Weight g | Liver Total Glutathione µmols gfw* | Liver GSH µmols gfw* | Liver GSSG µmols gfw* | Liver GSH:GSSG Ratio | Liver GR Activity[1] I.U. gfw* |
|---|---|---|---|---|---|---|---|---|---|
| A | 0.0 | 0.0 | 334[a] | 11.46 | 4.95[a] | 4.62[a] | 0.16 | 33.1 | 4.66[a] |
| B | 0.0 | 0.3 | 341[a] | 11.84 | 4.41[a] | 4.09[a] | 0.16 | 32.0 | 4.26[a] |
| C | 10.0 | 0.0 | 232[b] | 11.15 | 5.85[b] | 5.51[b] | 0.14 | 47.3 | 3.72[b] |
| D | 10.0 | 0.3 | 335[a] | 11.74 | 4.56[a] | 4.20[a] | 0.13 | 35.6 | 4.31[a] |
| ± SEM | | | 10.9 | 0.35 | 0.23 | 0.24 | 0.02 | 7.2 | 0.15 |
| LSD (P = 0.05) | | | 31.8 | NS | 0.68 | 0.71 | NS | NS | 0.44 |
| Individual Factors: | | | | | | | | | |
| | 0.0 | | | 11.65 | 4.68[a] | 4.36[a] | 0.16 | 32.6 | |
| | 10.0 | | | 11.44 | 5.20[b] | 4.86[b] | 0.13 | 41.4 | |
| | | 0.0 | | 11.30 | 5.40[b] | 5.07[b] | 0.15 | 40.2 | |
| | | 0.3 | | 11.79 | 4.49[a] | 4.15[a] | 0.14 | 33.8 | |
| ± SEM | | | | 0.24 | 0.17 | 0.17 | 0.01 | 5.1 | |
| LSD (P = 0.05) | | | | NS | 0.48 | 0.50 | NS | NS | |

Each value is a mean of 8 chicks. * = Per gram fresh weight.

[1] Individual factors invalid because a significant interaction was observed between their effects on this variable.

Values within a column sharing the same superscript are not significantly different at $P<0.05$.

NS = No significant differences.

SNP was added to the high selenium diet enzyme activity was significantly increased ($P<0.05$) and returned to the control level.

There was no effect with any of the dietary treatments on liver weight so the observed changes in metabolite concentrations cannot be explained in terms of altered liver size.

### 4.7.2 The Effect of Dietary Selenium and/or SNP on the Concentration and Redox State of Blood Glutathione (Experiment 13).

No significant effects on hepatic glutathione were observed in experiment 12 when SNP alone was added to the basic diet. Since cyanide released from SNP is absorbed from the alimentary canal into the bloodstream it was considered that the concentration and therefore effect of this toxin would be greater in blood than in liver. Also, since glutathione exists in a more oxidised state in the blood than it does in the liver it was hoped that changes in GSSG concentration might be more easily monitored in the former tissue.

Conventional chick mash, supplemented with 2 g DL-methionine per kg feed provided the basis for the usual four treatments in a 2 X 2 factorial experiment.

| Treatment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|
| A | 0.0 | 0.0 |
| B | 0.0 | 0.3 |
| C | 10.0 | 0.0 |
| D | 10.0 | 0.3 |

Nine chicks (ISA Brown) were allocated to each of the treatments which were dispersed in a randomised, blocked design within a single growth room. Body weight gains and feed intakes were monitored over a period of 21 days after which the birds were slaughtered for determination of blood GSSG and GSH concentrations.

**Results**

(See Table 8.2).

Significant interactions were observed between the effects of selenium and SNP on body weight gains ($P<0.001$), feed intakes ($P<0.01$) and the efficiency of feed utilisation ($P<0.001$). Addition of selenium to the basic diet significantly ($P<0.001$) impaired growth performance as measured by these three variables. Inclusion of SNP in the chick mash also impaired growth performance, although less dramatically. Weight gain was decreased ($P<0.01$) as was feed intake ($P<0.05$), but feed conversion ratio remained unaltered. However, addition of SNP to the high selenium diet alleviated the toxic effects of this element on growth. Weight gain and the efficiency of feed utilisation were increased ($P<0.001$) to values not significantly different ($P>0.05$) from those observed in control birds. Feed intake was also increased ($P<0.05$) but not returned to the control level.

No interactions were observed between selenium and SNP in their effects on blood glutathione. Dietary exposure to selenium did not affect blood glutathione concentration or redox state. Consideration of the effects of the individual factors in this 2 X 2 factorial experiment demonstrated a SNP-induced decrease in blood total and reduced glutathione ($P<0.05$ and $P<0.001$ respectively), together with an increase in oxidised glutathione concentration ($P<0.001$) and the resultant reduction in the GSH:GSSG ratio ($P<0.001$). When treatment effects were considered it became apparent that the SNP-mediated changes in total and reduced glutathione were significant only in the presence of selenium, whilst effects on oxidised glutathione were significant only in the absence of this toxin. However, the GSH:GSSG ratio was similarly altered by SNP treatment in birds consuming both the normal and the high selenium diet.

**Table 8.2** **The Effects of Dietary Selenium and/or SNP on Growth Performance and Blood Glutathione Concentration in Chicks.**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain[1] g $bird^{-1}$ $21d^{-1}$ | Feed Intake[1] g $bird^{-1}$ $21d^{-1}$ | Feed Conversion Ratio[1] | Blood Total Glutathione mmol $L^{-1}$ | Blood GSH mmol $L^{-1}$ | Blood GSSG mmol $L^{-1}$ | Blood GSH:GSSG Ratio |
|---|---|---|---|---|---|---|---|---|---|
| A | 0.0 | 0.0 | $363^{a}$ | $766^{a}$ | $2.11^{a}$ | $1.52^{a\,b}$ | $1.08^{a\,b}$ | $0.220^{a}$ | $5.07^{a}$ |
| B | 0.0 | 0.3 | $311^{b}$ | $696^{b\,c}$ | $2.24^{a}$ | $1.42^{a\,b}$ | $0.84^{b\,c}$ | $0.312^{b}$ | $2.74^{b}$ |
| C | 10.0 | 0.0 | $238^{c}$ | $641^{c}$ | $2.77^{b}$ | $1.72^{b}$ | $1.17^{a}$ | $0.270^{a\,b}$ | $4.41^{a}$ |
| D | 10.0 | 0.3 | $316^{b}$ | $704^{b}$ | $2.24^{a}$ | $1.23^{a}$ | $0.63^{c}$ | $0.302^{b}$ | $2.04^{b}$ |
| ± SEM | | | 11.8 | 19.2 | 0.07 | 0.12 | 0.09 | 0.018 | 0.37 |
| LSD (P = 0.05) | | | 34.1 | 55.4 | 0.21 | 0.35 | 0.27 | 0.053 | 1.06 |
| Individual Factors: | | | | | | | | | |
| | 0.0 | | | | | $1.47^{a\,b}$ | $0.96^{a\,b}$ | $0.266^{a\,c}$ | $3.91^{a}$ |
| | 10.0 | | | | | $1.47^{a\,b}$ | $0.90^{b\,c}$ | $0.286^{a\,b}$ | $3.22^{a}$ |
| | | 0.0 | | | | $1.62^{a}$ | $1.13^{a}$ | $0.245^{c}$ | $4.74^{b}$ |
| | | 0.3 | | | | $1.33^{b}$ | $0.74^{c}$ | $0.307^{b}$ | $2.39^{c}$ |
| | | | ± SEM | | | 0.08 | 0.07 | 0.013 | 0.26 |
| | | | LSD (P = 0.05) | | | 0.24 | 0.19 | 0.037 | 0.75 |

[1] Each value is a mean of 9 chicks.
Individual factors invalid because a significant interaction was observed between their effects on this variable.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.

### 4.7.3 The Effect of Dietary L-Cystine and/or SNP on the Concentration and Redox State of Hepatic Glutathione (Experiment 14).

In experiments 12 and 13, SNP treatment decreased total and reduced glutathione, whilst increasing GSSG and thereby decreasing the GSH:GSSG ratio. It seemed possible therefore, that the glutathione system might play a role in the mechanism of chronic cyanide intoxication.

If this were the case, then the protective action of L-cystine against this toxin, observed in experiment 10, may have been related to its function as a precursor for glutathione biosynthesis, rather than to its involvement, either directly, or indirectly in cyanide detoxification. Consequently, livers from birds fed the control and high cystine diets in experiment 8 were analysed for GSSG and GSH.

To recapitulate, conventional chick mash supplemented with 2.5 g DL-methionine per kg feed formed the basis for the following four treatments:

| Treatment | Added L-Cystine $g\ kg^{-1}$ | Added SNP $g\ kg^{-1}$ |
|---|---|---|
| A | 0.0 | 0.0 |
| B | 0.0 | 0.3 |
| C | 6.0 | 0.0 |
| D | 6.0 | 0.3 |

Six birds (ISA Brown) were allocated to each of the treatments which were dispersed in a totally randomised manner throughout two equivalent growth rooms, with the constraint that each diet appeared three times in each room. Weight gains and feed intakes were monitored at regular intervals over a period of 21 days.

**Results**

(See Table 8.3).

**Table 8.3** **The Effects of Dietary L-Cystine and/or SNP on Growth Performance and Hepatic Glutathione Concentration in Chicks.**

| Treat-ment | Added L-Cystine g $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain g $bird^{-1}$ $21d^{-1}$ | Feed Intake g $bird^{-1}$ $21d^{-1}$ | Feed Conversion Ratio | Liver Weight g | Liver Total Glutathione µmols gfw* | Liver GSH µmols gfw* | Liver GSSG µmols gfw* | Liver GSH:GSSG Ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| A | 0.0 | 0.0 | $377^{ab}$ | $649.8^{ab}$ | 1.72 | $13.52^{a}$ | $4.81^{ac}$ | $4.69^{ac}$ | 0.063 | 75.42 |
| B | 0.0 | 0.3 | $359^{a}$ | $615.9^{a}$ | 1.72 | $12.14^{b}$ | $4.02^{b}$ | $3.88^{b}$ | 0.070 | 58.30 |
| C | 6.0 | 0.0 | $395^{b}$ | $695.6^{b}$ | 1.76 | $13.74^{a}$ | $5.01^{a}$ | $4.87^{a}$ | 0.073 | 67.69 |
| D | 6.0 | 0.3 | $375^{ab}$ | $628.8^{a}$ | 1.68 | $12.19^{b}$ | $4.31^{bc}$ | $4.18^{bc}$ | 0.063 | 68.87 |
| ± SEM | | | 7.2 | 18.2 | 0.03 | 0.41 | 0.22 | 0.21 | 0.006 | 5.78 |
| LSD (P = 0.05) | | | 22.4 | 53.7 | NS | 1.22 | 0.65 | 0.64 | NS | NS |
| Individual Factors: | | | | | | | | | | |
| | 0.0 | | $368^{ac}$ | $632.9^{ac}$ | 1.72 | $12.83^{ab}$ | $4.42^{ac}$ | $4.28^{ac}$ | 0.067 | 66.86 |
| | 6.0 | | $385^{ab}$ | $662.3^{ab}$ | 1.72 | $12.96^{ab}$ | $4.66^{ab}$ | $4.52^{ab}$ | 0.068 | 68.28 |
| | | 0.0 | $386^{b}$ | $672.7^{b}$ | 1.74 | $13.63^{b}$ | $4.91^{b}$ | $4.78^{b}$ | 0.068 | 71.55 |
| | | 0.3 | $367^{c}$ | $622.4^{c}$ | 1.70 | $12.16^{a}$ | $4.16^{c}$ | $4.03^{c}$ | 0.067 | 63.58 |
| ± SEM | | | 5.1 | 12.8 | 0.02 | 0.29 | 0.15 | 0.15 | 0.004 | 4.09 |
| (P = 0.05) | | | 15.0 | 37.9 | NS | 0.86 | 0.46 | 0.46 | NS | NS |

* gfw = per gram fresh weight.
Each value is a mean of 6 chicks.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.
NS = No significant differences.

No growth room effects were observed in this trial and there were no significant ($P>0.05$) interactions between dietary L-cystine and SNP for any of the variables measured.

Consideration of the individual factors in this 2 X 2 experiment demonstrated a SNP-induced decrease in body weight gain and feed intake ($P<0.05$), whilst L-cystine treatment significantly ($P<0.05$) increased weight gain. There were no effects of either factor on the efficiency of feed utilisation.

L-cystine did not affect liver glutathione concentration or redox state. In contrast, SNP significantly decreased hepatic total and reduced glutathione concentrations both in terms of factors effects ($P<0.01$) and treatment effects ($P<0.05$). However, hepatic GSSG content and the GSH:GSSG ratio were unaltered by SNP. The SNP-mediated decrease in GSH concentration is not explicable in terms of changes in liver size because this toxin actually reduced liver weight with probabilities of $P<0.01$ and $P<0.05$ for factors and treatment effects respectively.

### 4.7.4 Discussion of Experiments 12-14.

Selenium-mediated changes in the glutathione system were evident in liver but not in blood from chicks fed a high selenium diet, which presumably reflects the expected higher concentration of this element in the former tissue. The increases in hepatic total and reduced glutathione observed during chronic selenium intoxication are in agreement with the findings of LeBoeuf & Hoekstra (1983), LeBoeuf *et al.* (1985) and Hoffman *et al.* (1989). A similar pattern of glutathione change has been observed following a single subacute injection of sodium selenite (Chung & Maines, 1981; LeBoeuf & Hoekstra, 1983). In these acute studies, as well as in the multiple time-point investigation of LeBoeuf *et al.*, (1985), the rise in GSH was preceded by an increase in GSSG concentration and was therefore considered to be a secondary adaptive change initiated in an attempt to maintain a normal GSH:GSSG ratio. In support of this contention, Chung & Maines (1981) also demonstrated a selenite-induced increase in the activity of Y-glutamylcysteine synthase. In addition, glutathione

reductase activity was increased by selenium treatment in all the aforementioned investigations. This is in direct contrast to the results of experiment 12 in which selenium decreased hepatic glutathione reductase activity. This discrepancy is unlikely to be explicable in terms of species differences since Hoffman *et al.* (1989) also used an avian species, the Mallard duck (*Anas platyrhynchos*), but may well be a function of dose. LeBoeuf's group used a maximum of 6 mg selenium per kg feed and whilst Hoffman *et al.* demonstrated an increase in glutathione reductase activity after treatment of the ducklings with selenium at 10 mg $kg^{-1}$, this effect was not significant at higher doses. Mallard ducks are unusually resistant to selenite; growth was unaffected by exposure to 10 mg selenium per kg feed.

As expected, the effects of SNP on the glutathione system were more pronounced in blood than in liver tissue. SNP decreased total and reduced glutathione, whilst increasing GSSG and thereby decreasing the GSH:GSSG ratio.

The role of glutathione in the mechanism of chronic cyanide toxicity has not previously been investigated, however, evidence suggests that glutathione depletion may be an important factor in acute cyanide poisoning. Peroxidation of cellular membrane lipids, which may be related to altered regulation of calcium homeostasis, is known to be a feature of acute toxicity (Johnson *et al.*, 1987; Isom *et al.*, 1988; Younes & Strubelt, 1988). Misra *et al.* (1988) demonstrated HCN-mediated decreases in the total and non-protein sulphydryl contents of rat liver. Hatch *et al.* (1990) showed that GSH and GSSG protected against KCN lethality in mice. In addition, they reported that depletion of tissue glutathione by ethyl maleate greatly enhanced KCN toxicity. Similarly Younes & Strubelt (1990) demonstrated that addition of exogenous glutathione and resultant augmentation of cellular glutathione content protected isolated perfused rat liver against damage induced by cyanide, as evidenced by leakage of lactate dehydrogenase and hepatic calcium accumulation. Ardelt *et al.* (1989) reported KCN-induced decreases in the activities of catalase, glutathione reductase and GSHPx in the brains of mice and correlated these changes with a reduction in GSH content and increased lipid peroxidation.

From the results of experiments 12-14 it appears that glutathione depletion is also a feature of chronic cyanide intoxication. Utilisation of GSH with the concomitant generation of GSSG, during GSHPx catalysed reduction of hydroperoxides, could well account for the observed changes in glutathione status and redox state. However, it is also possible that glutathione depletion is simply related to the increased utilisation of sulphur compounds for cyanide detoxification.

With regard to the selenium-cyanide interaction, the alleviation of selenium toxicity by SNP was apparent in growth effects, although the interaction failed to achieve significance for changes in the glutathione system. However, the effects of these two toxins were opposite and, in fact, the perturbations in the glutathione system observed in chicks fed the high selenium diet were completely reversed by the simultaneous administration of SNP. It remains possible therefore that glutathione may play a role in the mechanism by which cyanide alleviates selenium toxicity.

Dietary supplementation with L-cystine did not increase total hepatic glutathione concentration in this investigation. This result contrasts with the findings of Klug *et al.*, (1950a); Suberville *et al.*, (1987) and Garcin *et al.*, (1989). However, these studies used low protein diets which were inherently deficient in sulphur amino acids. This lack of effect of L-cystine on glutathione levels, together with the observation that SNP decreased total and reduced glutathione, both in the presence and absence of this amino acid, suggests that the beneficial effect of L-cystine in chronic cyanide intoxication is not mediated through provision of glutathione.

## 4.8 The Effect of Dietary Supplementation With DL-methionine on the Chronic Toxicity of Selenium and/or SNP, and on the Interaction Between these Toxins (Experiments 15 and 16).

As previously mentioned (Sections 2.6 and 2.16.2) methionine has, in some cases, been beneficial against chronic intoxication by selenium (McConnell, 1952; Witting & Horwitt, 1964; Levander & Morris, 1970) and by cyanide (Enriquez & Ross, 1967; Ross & Enriquez, 1969;

Olsen *et al.*, 1969; Maner & Gómez, 1973; Adegbola, 1977). It was considered possible, therefore, that dietary supplementation with this amino acid might influence the selenium-cyanide interaction, particularly in view of the importance of methyl groups in selenium metabolism. Experiments 15 and 16 were designed to determine the effect of methionine on the ability of SNP to alleviate selenium toxicity, thereby possibly gaining further information on the mechanism of this interaction.

### 4.8.1 The Effect of Dietary Supplementation With DL-methionine on the Selenium-Cyanide Interaction (Experiment 15).

The cassava-based diet was selected for this experiment in an attempt to establish methionine deficiency. This diet inherently supplies only 2.56 g Met $kg^{-1}$, whilst conventional chick mash provides 3.82 g Met $kg^{-1}$. The unsupplemented cassava ration would be deficient in both methionine and total sulphur amino acids by 2.04 g $kg^{-1}$ and by 3.34 g $kg^{-1}$ respectively (calculated from data provided by Bolton & Blair (1974)). This feeding trial was carried out relatively early in these investigations when the inclusion rate for SNP of 0.5 g $kg^{-1}$ was still in operation.

Six experimental diets were formulated, as follows :

| Treatment | Added L-Met g $kg^{-1}$ | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|---|
| A | 0.0 | 0.0 | 0.0 |
| B | 0.0 | 10.0 | 0.0 |
| C | 0.0 | 10.0 | 0.5 |
| D | 6.0 | 0.0 | 0.0 |
| E | 6.0 | 10.0 | 0.0 |
| F | 6.0 | 10.0 | 0.5 |

Four chicks (Hisex) were allocated to each of the treatments, which were distributed in a totally randomised manner within a single

growth room. Body weight gains and feed intakes were monitored over a period of 14 days.

**Results**

(See Table 9.1).

Dietary supplementation with 6 g DL-methionine $kg^{-1}$ feed did not significantly ($P>0.05$) improve growth performance as measured by body weight gains, feed intakes and feed conversion ratios. Selenium, when added to the methionine deficient diet, did not affect growth performance. However, body weight gain and feed intake were significantly ($P<0.05$) decreased by the inclusion of this toxin in the high methionine diet. Simultaneous exposure of the chicks to selenium and SNP in the absence of supplemental methionine proved highly detrimental, causing significant decreases in body weight gain ($P<0.01$), feed intake ($P<0.001$) and the efficiency of feed utilisation ($P<0.001$). In contrast, when SNP was added to the high selenium diet in the presence of methionine, although weight gain and feed intake were again reduced, the effects were much less pronounced ($P<0.05$ in both cases), whilst feed conversion ratios remained unaltered. In fact, there was some improvement in growth performance over the selenium only control although the alleviation of toxicity failed to achieve significance.

### 4.8.2 The Effect of Dietary DL-Methionine on the Growth Performance of Chicks Fed Selenium and/or SNP (Experiment 16).

As the results of experiment 15 had demonstrated a requirement for dietary methionine in the alleviation of selenium toxicity by SNP it was considered necessary to investigate this phenomenon further. In experiment 16, graded levels of DL-methionine were used, a SNP only control was included and the rate of inclusion of this toxin was reduced to 0.3 g $kg^{-1}$.

**Table 9.1** **The Effect of Dietary DL-Methionine on the Chronic Toxicity of SNP and on the SNP-Selenium Interaction.**

| Treatment | Added DL-Met g $kg^{-1}$ | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain g $bird^{-1}$ $14d^{-1}$ | Feed Intake g $bird^{-1}$ $14d^{-1}$ | Feed Conversion Ratio |
|---|---|---|---|---|---|---|
| A | 0.0 | 0.0 | 0.0 | $204^{a\,b}$ | $552^{a}$ | $2.71^{b}$ |
| B | 0.0 | 10.0 | 0.0 | $156^{b\,c}$ | $516^{a\,b}$ | $3.35^{b}$ |
| C | 0.0 | 10.0 | 0.5 | $22^{d}$ | $321^{c}$ | $12.73^{a}$ |
| D | 6.0 | 0.0 | 0.0 | $244^{a}$ | $533^{a}$ | $2.70^{b}$ |
| E | 6.0 | 10.0 | 0.0 | $142^{c}$ | $410^{b\,c}$ | $2.97^{b}$ |
| F | 6.0 | 10.0 | 0.5 | $181^{b\,c}$ | $501^{a\,b}$ | $2.76^{b}$ |
| | | | $\pm$ SEM | 18.3 | 39.0 | 1.61 |
| | | | LSD (P = 0.05) | 54.7 | 116.5 | 4.82 |

Each value is a mean of 4 chicks.

Values within a column sharing the same superscript are not significantly different at $P<0.05$.

The cassava-based diet was used to prepare the following experimental rations in this 2 X 4 X 2 factorial study:

| Treatment | Added Selenium mg $kg^{-1}$ | Added DL-Met g $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|---|
| A | 0.0 | 0.0 | 0.0 |
| B | 0.0 | 0.0 | 0.3 |
| C | 0.0 | 1.0 | 0.0 |
| D | 0.0 | 1.0 | 0.3 |
| E | 0.0 | 2.0 | 0.0 |
| F | 0.0 | 2.0 | 0.3 |
| G | 0.0 | 4.0 | 0.0 |
| H | 0.0 | 4.0 | 0.3 |
| I | 10.0 | 0.0 | 0.0 |
| J | 10.0 | 0.0 | 0.3 |
| K | 10.0 | 1.0 | 0.0 |
| L | 10.0 | 1.0 | 0.3 |
| M | 10.0 | 2.0 | 0.0 |
| N | 10.0 | 2.0 | 0.3 |
| O | 10.0 | 4.0 | 0.0 |
| P | 10.0 | 4.0 | 0.3 |

Four birds (ISA Brown) were allocated to each of the diets which were distributed in a random manner between the two equivalent growth rooms. The only constraint imposed was that each treatment should appear twice in each room. Body weight gains and feed intakes were monitored over a period of 21 days.

**Results**

(See Table 9.2).

There was no significant ($P>0.05$) effect of growth room on any of the variables studied.

No significant interactions were observed between selenium and methionine, SNP and methionine or selenium, SNP and methionine in

**Table 9.2** **The Effect of Dietary DL-Methionine on the Growth Performance of Chicks fed Selenium and/or SNP.**

| Treatment | Added Selenium mg $kg^{-1}$ | Added DL-Met g $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain g $bird^{-1}$ $21d^{-1}$ | Feed Intake g $bird^{-1}$ $21d^{-1}$ | Feed Conversion Ratio |
|---|---|---|---|---|---|---|
| A | 0.0 | 0.0 | 0.0 | $261^{cde}$ | $814.0^{a}$ | $3.18^{bcde}$ |
| B | 0.0 | 0.0 | 0.3 | $163^{gh}$ | $565.7^{ef}$ | $3.52^{abcd}$ |
| C | 0.0 | 1.0 | 0.0 | $319^{bc}$ | $793.0^{ab}$ | $2.50^{efg}$ |
| D | 0.0 | 1.0 | 0.3 | $212^{efgh}$ | $628.0^{cdef}$ | $2.98^{def}$ |
| E | 0.0 | 2.0 | 0.0 | $356^{ab}$ | $817.5^{a}$ | $2.35^{fg}$ |
| F | 0.0 | 2.0 | 0.3 | $250^{def}$ | $639.0^{cde}$ | $2.64^{efg}$ |
| G | 0.0 | 4.0 | 0.0 | $397^{a}$ | $793.0^{ab}$ | $2.01^{g}$ |
| H | 0.0 | 4.0 | 0.3 | $300^{bcd}$ | $690.5^{bcd}$ | $2.31^{fg}$ |
| I | 10.0 | 0.0 | 0.0 | $192^{fgh}$ | $613.5^{def}$ | $3.26^{bcde}$ |
| J | 10.0 | 0.0 | 0.3 | $146^{h}$ | $565.5^{ef}$ | $3.92^{ab}$ |
| K | 10.0 | 1.0 | 0.0 | $236^{def}$ | $653.0^{cde}$ | $2.80^{defg}$ |
| L | 10.0 | 1.0 | 0.3 | $227^{efg}$ | $660.0^{cde}$ | $3.73^{abc}$ |
| M | 10.0 | 2.0 | 0.0 | $148^{h}$ | $536.7^{f}$ | $4.14^{ab}$ |
| N | 10.0 | 2.0 | 0.3 | $265^{cde}$ | $686.0^{cde}$ | $2.63^{efg}$ |
| O | 10.0 | 4.0 | 0.0 | $246^{def}$ | $666.2^{cde}$ | $2.75^{defg}$ |
| P | 10.0 | 4.0 | 0.3 | $319^{bc}$ | $728.5^{abc}$ | $2.29^{fg}$ |
| | | | $\pm$ SEM | 23.0 | 36.1 | 0.29 |
| | | | LSD (P = 0.05) | 66.0 | 103.1 | 0.83 |

Each value is a mean of 4 chicks.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.

their effects on growth performance. However, the selenium, SNP interaction was highly significant ($P<0.001$) for all three variables.

Dietary supplementation with DL-methionine at 1.0, 2.0 and 4.0 g $kg^{-1}$ significantly ($P<0.001$) increased body weight gain; feed intake was also increased but only at the highest level of methionine inclusion ($P<0.01$), whilst feed conversion ratio was decreased ($P<0.05$ for 1.0 and 2.0 g $kg^{-1}$ and $P<0.001$ for 4.0 g $kg^{-1}$).

Selenium significantly reduced weight gain ($P<0.001$), feed intake ($P<0.001$) and the efficiency of feed utilisation ($P<0.01$).

SNP treatment also caused significant reductions in body weight gain ($P<0.01$), feed intake ($P<0.001$) and the efficiency of feed utilisation ($P<0.01$).

Alleviation of selenium toxicity by SNP was significant, in terms of growth performance, only at the two higher levels of methionine supplementation (2.0 and 4.0 g $kg^{-1}$).

### 4.8.3 Discussion of Experiments 15 and 16.

Growth performance was improved by dietary supplementation with DL-methionine suggesting that deficiency had, in fact, been established in birds consuming the basic diet. That this effect did not achieve significance in experiment 15 is probably attributable to the short duration of this particular trial (the investigation was curtailed owing to the poor health of birds consuming diet C).

Methionine failed to ameliorate the toxic effects of selenium on growth performance. This finding is in agreement with the results of other workers in the field who have demonstrated beneficial effects of this amino acid only in the presence of relatively high levels of vitamin E or fat-soluble antioxidants (McConnell, 1952; Witting & Horwitt, 1964; Levander & Morris, 1970). In fact, in experiment 15, selenium toxicity was observed in the presence, but not in the absence of methionine. The rate of inclusion of this amino acid in experiment 15, chosen to ensure adequacy, was rather high and may, in combination

with excess selenium, have proved slightly toxic. In addition, in experiment 16, although there was no significant interaction between methionine and selenium, the toxicity of the latter was more pronounced, in terms of growth performance, in chicks also consuming methionine at 2.0 or 4.0 g per kg feed.

Methionine also failed to ameliorate the detrimental effects of SNP on growth performance. Although this result contradicts those mentioned in Section 4.8 it is supported by more recent studies of Gómez *et al.* (1984) and of Elzubeir (1986). There is at present no single obvious explanation for these discrepancies.

An adequate supply of methionine does seem to be essential for the alleviation of selenium toxicity by SNP. In experiment 16, although the methionine, selenium, SNP interaction failed to achieve significance, growth rate was markedly improved by addition of SNP to high selenium diets in the presence of 2.0 and 4.0 g Met $kg^{-1}$, but not with 0.0 and 1.0 g $kg^{-1}$. This effect may be specific for methionine since diets supplemented with 2.0 g Met $kg^{-1}$, though adequate for this amino acid, were still deficient in total sulphur amino acids to the extent of 1.34 g $kg^{-1}$. The lack of significant alleviation in experiment 15 in the presence of 6.0 g Met $kg^{-1}$, together with the highly detrimental effect of simultaneous addition of Se and SNP to the control diet are probably due to the high level of SNP used in this trial.

The requirement for methionine in the alleviation of selenium toxicity by SNP may be related either to its function as a sulphur donor for cyanide detoxification or, to the provision of methyl groups for the synthesis of the methylated forms of selenium and the elimination of this toxin from the body. The latter explanation may be the more likely because alleviation was observed in the presence of methionine even when the total sulphur amino acid supply was limited. Selenium volatilisation in the rat can be increased two or three-fold by dietary supplementation with methionine (Ganther *et al.*, 1966). Addition of graded levels of methionine to diets containing an alternative sulphur donor such as cysteine might clarify this point.

## 4.9 The Effect of Dietary SNP on The Exhalation of Volatile Selenium Compounds (Experiment 17).

The apparent requirement for methionine in the alleviation of selenium toxicity by SNP, demonstrated in the previous experiments, suggested that the function of cyanide might be to enhance selenium elimination. However, the SNP-mediated decreases in NADPH and GSH observed in experiments 11 and 12-14 respectively might have been expected to have the opposite effect by slowing the reduction phase of selenium metabolism. Data relating to the effects of cyanide on the excretion of selenium in expired air, urine and faeces are contradictory (Ganther *et al.*, 1966; Palmer & Olsen, 1979; Beilstein & Whanger, 1984; Elzubeir, 1986). It was therefore considered necessary to ascertain the effect of SNP on selenium elimination under the conditions of these investigations. The exhalation of volatile selenium compounds was studied because this is generally considered to represent the major route for excretion of the element at high or toxic levels of intake (McConnell & Roth, 1966).

Conventional chick mash supplemented with 2 g DL-Met $kg^{-1}$ provided the basis for the following four treatments in this 2 X 2 factorial experiment:

| Treatment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|
| A | 0.0 | 0.0 |
| B | 0.0 | 0.3 |
| C | 10.0 | 0.0 |
| D | 10.0 | 0.3 |

Eight birds (ISA Brown) were allocated to each of the treatments which were dispersed at random between two equivalent growth rooms with the constraint that each diet appeared four times in each room. Weight gains and feed intakes were monitored over a period of 21 days and birds were removed weekly for collection of expired air. Unfortunately, owing to the limited availability of desiccators, collections were spread over a period of 2 days, with birds from

individual growth rooms being analysed on separate days. In weeks one and two the birds were of a sufficient size that two chicks from each treatment could be placed in each collection apparatus, thereby minimising individual variation and allowing eight chicks to be sampled over the two day period. However, after three weeks the number of birds had to be reduced to one per desiccator.

**Results**

(See Table 10.1).

In week one there were no effects of dietary treatment on growth performance. The only significant difference in body weight gain and feed intake was the result of a growth room effect ($P<0.001$). In practice this can probably be attributed to the fact that chicks from room 1 were sampled on day 3 of the trial whilst those from room 2 were sampled on day 4.

By the second week, even though growth room effects were still significant ($P<0.01$ and $P<0.001$ for weight gains and for feed intakes respectively), treatment-induced differences were becoming apparent. A significant ($P<0.05$) interaction was observed between selenium and SNP for effects on weight gain. Addition of SNP to the basic diet resulted in significant ($P<0.01$) decreases in both measures of growth performance. Selenium exposure also reduced body weight gain ($P<0.001$) and feed intake ($P<0.05$). At this stage of the trial addition of SNP to the high selenium diet failed to alleviate the toxic effects of this element on growth.

Three weeks into the study growth room effects were absent. A highly significant ($P<0.001$) interaction was observed between selenium and SNP for effects on body weight gain and feed intake. Addition of SNP to the basic diet decreased both variables ($P<0.01$) as did selenium exposure ($P<0.001$). Inclusion of SNP in the high selenium diet significantly ($P<0.01$) improved body weight gain although the alleviation of toxicity was not complete since growth rate did not reach the control value.

**Table 10.1** **The Effects of Dietary Selenium and/or SNP on Growth Performance and Selenium Exhalation in Chicks.**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | WEEK ONE | | | WEEK TWO | | | WEEK THREE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Body Weight Gain[1] g | Feed Intake[1] g | nmols Selenium Exhaled[1] $kg^{-1}$ $hr^{-1}$ | Body Weight Gain[1] g | Feed Intake[1] g | nmols Selenium Exhaled[1] $kg^{-1}$ $hr^{-1}$ | Body Weight Gain[2] g | Feed Intake[2] g | nmols Selenium Exhaled[2] $kg^{-1}$ $hr^{-1}$ |
| A | 0.0 | 0.0 | 31 | 67.4 | $1.51^{a}$ | $145^{a}$ | $285.0^{a}$ | $1.30^{a}$ | $360^{a}$ | $770^{a}$ | $3.52^{a}$ |
| B | 0.0 | 0.3 | 27 | 63.4 | $1.78^{a}$ | $121^{b}$ | $260.0^{b}$ | $1.67^{a}$ | $295^{b}$ | $666^{b}$ | $4.08^{a}$ |
| C | 10.0 | 0.0 | 29 | 66.5 | $10.77^{b}$ | $117^{b}$ | $266.6^{b}$ | $9.24^{b}$ | $245^{c}$ | $651^{b}$ | $17.36^{b}$ |
| D | 10.0 | 0.3 | 28 | 63.3 | $2.46^{a}$ | $120^{b}$ | $260.7^{b}$ | $2.19^{a}$ | $305^{b}$ | $693^{b}$ | $4.09^{a}$ |
| ± SEM | | | 1.5 | 1.6 | 0.96 | 5.1 | 6.1 | 0.38 | 13.1 | 21.0 | 2.77 |
| LSD (P = 0.05) | | | NS | NS | 2.99 | 14.8 | 17.5 | 1.15 | 38.1 | 60.9 | 8.61 |

[1] Each value is a mean of 8 chicks.
[2] Each value is a mean of 4 chicks.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.
NS = No significant differences.

Although treatment effects on growth performance did not become apparent until the second week of the trial, whilst alleviation of selenium toxicity by SNP was not observed until week three, the selenium exhalation data were remarkably consistent throughout the experimental period. In contrast to the results for growth performance, there was no effect of growth room on selenium exhalation. In the first and second weeks a highly significant ($P<0.001$) interaction was observed between selenium and SNP for effects on the exhalation of volatile selenium compounds. Selenium exposure resulted in a highly significant ($P<0.001$) increase in the amount of selenium excreted by the pulmonary route. Addition of SNP to the basic diet did not affect selenium exhalation. However, when SNP was included in the high selenium diet the amount of selenium eliminated through the lungs fell dramatically ($P<0.001$) to a value not significantly ($P<0.05$) different from that observed in control birds. Similar results were obtained after three weeks of exposure to the experimental diets, however, owing to the increased variation resulting from a decrease in the number of replicates per treatment, probabilities were reduced to 0.01.

### 4.9.1 Discussion of Experiment 17.

The results of this investigation clearly demonstrate that the amount of selenium exhaled by chicks consuming a high selenium diet can be dramatically reduced by the simultaneous administration of dietary SNP. These findings, which have since been verified by other workers in the same laboratory (Mitchell, Dowell & Davis, [unpublished]), agree with those obtained in an acute study by Palmer & Olsen (1979), who observed a delay in exhalation of selenium after rats had been injected with both cyanide and selenite compared with selenite alone. Contradictory results were reported, in 1986, by Elzubeir, who demonstrated increased volatilisation of selenium following cyanide treatment. Elzubeir did establish chronic selenosis, but this study differed from experiment 17 in several important details. Elzubeir used a cassava-based diet supplemented with 6 g DL-Met $kg^{-1}$ and implemented an equalised regime of feed administration. This level of methionine supplementation was

excessive and appeared, in experiments 15 and 16, to result in a slight exacerbation of selenium toxicity.

A possible explanation for these conflicting results might be provided by suggesting that cyanide effects on selenium exhalation are related to other dietary factors, which may, or may not include methionine content. Certainly selenium volatilisation can be controlled by diet. Ganther *et al.* (1966) reported that rats injected with a single subacute dose of selenite exhaled selenium in amounts that depended upon the diet fed previously. Volatilisation could be increased by increasing the selenium, protein or methionine content of the basal diet, or by the inclusion of certain unpurified feedstuffs. The activity of the latter did not appear to reside solely in their selenium, protein or methionine contents. In this particular study, linseed oil meal was without effect on selenium exhalation. Although it is tempting to speculate that cyanide can only increase volatilisation in the presence of excess methionine, other factors may well be involved.

One conclusion which can be drawn, however, is that cyanide effects on selenium exhalation are not crucial to the alleviation of selenium toxicity by this poison. Alleviation was apparent in Elzubeir's investigation, although the effects of SNP on DmSe exhalation were opposite to those observed in experiment 17.

The identity of the volatile selenium compound or compounds exhaled cannot be determined by the analytical methods employed in this study. However, they probably include dimethylselenide which is readily trapped by nitric acid (Ganther & Baumann, 1962; Ganther, 1966; Diplock *et al.*, 1973).

## 4.10 The Effects of Dietary DL-Methionine, L-Cysteine and SNP on the Toxicity of Selenium and on the Subcellular Distribution and Redox State of this Element in the Liver (Experiment 18).

The mechanism by which SNP alleviates selenium toxicity cannot be fully described from the data presented so far in this thesis. SNP does not appear to enhance selenium metabolism and elimination.

However, the possibility remains that cyanide might alter the form in which selenium exists in the tissues. Levander *et al.* (1970) demonstrated that EDTA increased the amount of selenium that could be dialysed from liver homogenates prepared from rats fed a casein-based diet, but had no effect on those from rats fed a casein-linseed oil meal ration.

Diplock *et al.* (1971), in their studies on vitamin E, pioneered a method for separating several forms of tissue selenium. They demonstrated that the selenium found in rat liver was present in at least three oxidation states. The acid-volatile portion of this selenium was considered to be selenide, an assumption which was validated by Diplock *et al.* in 1973 when they demonstrated that this acid-labile selenium behaved in a manner quite unlike $(CH_3)_2Se$, but indistinguishable from $H_2Se$. However, complete characterisation of this fraction proved impossible on practical grounds. A zinc-hydrochloric acid-reducible portion was assumed to be selenite. However, Ganther (1987) pointed out that selenium linked to proteins by S-Se bonding, e.g. selenotrisulphides, would also have been included in this fraction. The portion of selenium not released by reduction with Zn and HCl was thought to comprise selenoamino acids and higher oxidation states of the element, such as selenate. In practice, one might expect selenoamino acids to have accounted for the majority of this selenium.

A modified version of Diplock's method was used in the experiment described below in an attempt to determine the effect of cyanide on the subcellular distribution and redox state of hepatic selenium. Since methionine had proved important for the alleviation of selenium toxicity by SNP (experiments 15 and 16) both methionine deficient and methionine adequate controls were included. L-cysteine containing rations were also formulated in an attempt to determine the specificity of the methionine effect.

Conventional chick mash provided the basis for the following twelve treatments:

| Treatment | Added DL-Met g $kg^{-1}$ | Added L-Cys g $kg^{-1}$ | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ |
|---|---|---|---|---|
| A | 0.0 | 0.0 | 0.0 | 0.0 |
| B | 0.0 | 0.0 | 0.0 | 0.3 |
| C | 0.0 | 0.0 | 10.0 | 0.0 |
| D | 0.0 | 0.0 | 10.0 | 0.3 |
| E | 3.0 | 0.0 | 0.0 | 0.0 |
| F | 3.0 | 0.0 | 0.0 | 0.3 |
| G | 3.0 | 0.0 | 10.0 | 0.0 |
| H | 3.0 | 0.0 | 10.0 | 0.3 |
| I | 0.0 | 2.44 | 0.0 | 0.0 |
| J | 0.0 | 2.44 | 0.0 | 0.3 |
| K | 0.0 | 2.44 | 10.0 | 0.0 |
| L | 0.0 | 2.44 | 10.0 | 0.3 |

Six birds (ISA Brown) were allocated to each of the diets in the main trial (A-H), leaving four chicks per treatment for investigation of cysteine effects (I-L). Experimental treatments were randomly distributed throughout two equivalent growth rooms. Body weight gains and feed intakes were monitored over a period of 19 days after which birds were slaughtered for determination of the redox state of hepatic selenium.

**Results**

No significant effects of growth room were observed.

Growth performance was unaffected by dietary supplementation with methionine or cysteine (Table 11.1).

In the absence of added sulphur amino acids, treatment with either SNP or selenium resulted in a highly significant ($P<0.001$) reduction in body weight gain and feed intake. The efficiency of feed utilisation was also decreased ($P<0.01$) in both cases. When the two poisons were administered simultaneously, SNP failed to alleviate the toxic effects of selenium on growth performance.

**Table 11.1** **The Effect of Added DL-Methionine or L-Cysteine on the Chronic Toxicity of Selenium and/or SNP as Monitored by Changes in Growth Performance and Liver Weight.**

| Treat-ment | Added DL-Met g $kg^{-1}$ | Added L-Cys g $kg^{-1}$ | Added Selenium mg $kg^{-1}$ | Added SNP g $kg^{-1}$ | Body Weight Gain g $bird^{-1}$ $19d^{-1}$ | Feed Intake g $bird^{-1}$ $19 d^{-1}$ | Feed Conversion Ratio | Liver Weight g |
|---|---|---|---|---|---|---|---|---|
| A[1] | 0.0 | 0.0 | 0.0 | 0.0 | $309.2^{ab}$ | $688.0^{a}$ | $2.23^{cd}$ | $12.39^{ab}$ |
| B[1] | 0.0 | 0.0 | 0.0 | 0.3 | $147.4^{g}$ | $392.9^{g}$ | $2.69^{ab}$ | $8.65^{e}$ |
| C[1] | 0.0 | 0.0 | 10.0 | 0.0 | $203.4^{ef}$ | $534.8^{ef}$ | $2.64^{ab}$ | $11.01^{abcd}$ |
| D[1] | 0.0 | 0.0 | 10.0 | 0.3 | $139.7^{g}$ | $391.5^{g}$ | $2.87^{a}$ | $7.73^{e}$ |
| E[1] | 3.0 | 0.0 | 0.0 | 0.0 | $304.3^{ab}$ | $669.3^{a}$ | $2.24^{cd}$ | $13.03^{a}$ |
| F[1] | 3.0 | 0.0 | 0.0 | 0.3 | $273.7^{acd}$ | $562.8^{cde}$ | $2.06^{d}$ | $11.35^{abc}$ |
| G[1] | 3.0 | 0.0 | 10.0 | 0.0 | $196.5^{f}$ | $495.3^{f}$ | $2.59^{ab}$ | $9.54^{de}$ |
| H[1] | 3.0 | 0.0 | 10.0 | 0.3 | $270.7^{cd}$ | $567.2^{cde}$ | $2.10^{d}$ | $10.88^{bcd}$ |
| I[2] | 0.0 | 2.44 | 0.0 | 0.0 | $310.9^{a}$ | $651.3^{ab}$ | $2.10^{d}$ | $12.56^{ab}$ |
| J[2] | 0.0 | 2.44 | 0.0 | 0.3 | $301.9^{abc}$ | $598.1^{bcd}$ | $1.99^{d}$ | $11.76^{abc}$ |
| K[2] | 0.0 | 2.44 | 10.0 | 0.0 | $242.3^{de}$ | $607.1^{bc}$ | $2.53^{bc}$ | $12.00^{abc}$ |
| L[2] | 0.0 | 2.44 | 10.0 | 0.3 | $272.9^{acd}$ | $547.0^{def}$ | $2.00^{d}$ | $11.97^{abc}$ |
| | | | | ± SEM[3] | 11.5 | 16.7 | 0.10 | 0.74 |
| | | | | LSD (P = 0.05)[3] | 32.8 | 47.5 | 0.29 | 2.11 |
| | | | | ± SEM[4] | 14.1 | 20.5 | 0.12 | 0.91 |
| | | | | LSD (P = 0.05)[4] | 40.1 | 58.2 | 0.35 | 2.58 |

[1] Each value in this row is a mean of 6 chicks.
[2] Each value in this row is a mean of 4 chicks.
[3] For comparison of treatments A-H.
[4] For comparisons involving treatments I-L
Values within a column sharing the same superscript are not significantly different at $P<0.05$.

Methionine was beneficial against SNP intoxication. SNP, when included in the methionine supplemented rations, did not significantly ($P>0.05$) alter growth or feed conversion ratio, although feed intake was still reduced ($P<0.001$). In contrast, methionine proved ineffective against selenosis. When included in the methionine-containing diets, selenium again caused highly significant ($P<0.001$) decreases in body weight gain and feed intake accompanied by a reduced ($P<0.05$) efficiency of feed utilisation. In this instance, SNP did alleviate the toxic effects of selenium on growth. Addition of SNP to the high selenium diet resulted in a highly significant ($P<0.001$) improvement in body weight gain together with significant ($P<0.01$) increases in feed intake and the efficiency of feed utilisation, although only in the latter case did birds consuming both toxins perform as well as those fed the control diet.

Supplemental cysteine also proved beneficial against SNP intoxication. The cyanide source failed to alter growth performance when added to cysteine-containing rations. In addition, the effects of selenium treatment were also less marked than those observed when this element was included in the unsupplemented or methionine adequate diets. Body weight gain and the efficiency of feed utilisation were again reduced by selenium treatment but these effects only achieved significance at $P<0.01$ and $P<0.05$ respectively. Selenium did not alter feed intake in birds consuming cysteine supplemented diets. Concerning the alleviation of selenosis by SNP, the improvement in weight gain and feed intake observed when this compound was added to high selenium diets containing cysteine did not achieve significance although the efficiency of feed utilisation was increased. However, since cysteine partially ameliorated selenium toxicity, one might expect any beneficial effects of SNP to be less marked. In fact, birds fed both toxins performed as well as those fed the control diets in terms of weight gain and feed conversion ratios.

The effects of SNP and selenium on liver weight were also modified by methionine or cysteine supplementation (Table 11.1). In the absence of supplemental sulphur amino acids, SNP caused a significant decrease in liver weight when added to the diet of chicks consuming both normal and high selenium rations ($P<0.001$ and $P<0.01$ respectively). Selenium inclusion, however, was without effect on

liver weight. In contrast, SNP did not alter liver weight when added to methionine adequate diets, whilst selenium caused a significant ($P<0.01$) decrease in this variable. Neither SNP nor selenium had any effect on liver weight in the presence of supplemental cysteine.

Tables 11.2 - 11.5 show the effects of dietary methionine, cysteine and SNP on the concentration, subcellular distribution and redox state of hepatic selenium. These investigations were performed only in chicks which had been fed high selenium rations.

SNP significantly ($P<0.01$) reduced whole liver selenium content in chicks consuming control or methionine-containing diets (Table 11.2). This effect was more pronounced in birds receiving cysteine supplemented rations when the differences observed achieved significance at the highest level ($P<0.001$). The SNP-induced reduction in liver selenium concentration was accompanied by a decrease in hepatic selenite ($P<0.01$) and selenoamino acids/selenate ($P<0.05$). These effects of SNP were influenced by the availability of sulphur amino acids. Supplemental methionine potentiated the SNP-induced decrease in liver selenite content but nullified the action of this compound on the selenoamino acids/selenate fraction. In contrast, cysteine enhanced the ability of SNP to reduce the amount of selenoamino acids in liver but did not modify its action on selenite. This apparent lack of effect of cysteine could simply result from the tendency for this amino acid to decrease the amount of selenite in liver anyway.

Liver selenide content was highly variable and was unaffected by any of the dietary treatments.

In addition to its effects on hepatic total selenium, selenite and selenoamino acids content, it became apparent when the results were expressed in terms of the total liver selenium, that SNP also altered the relative proportions of the various selenium compounds. SNP reduced the percentage of selenium present as selenite ($P<0.05$), whilst increasing the proportion of selenoamino acids/selenate, although this latter effect did not achieve significance in birds consuming the unsupplemented rations. Both sulphur amino acids

**Table 11.2** **The Effects of Dietary DL-Methionine, L-Cysteine and SNP on Hepatic Selenium Content and Redox State in Chicks Fed High Selenium Diets.**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added DL-Met g $kg^{-1}$ | Added L-Cys g $kg^{-1}$ | Added SNP g $kg^{-1}$ | pmols mg $protein^{-1}$ | | | | % of total Selenium | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Total Se | Selenide | Selenite | Seleno-amino acids/ Selenate | Selenide | Selenite | Seleno-amino acids/ Selenate |
| C | 10.0 | 0.0 | 0.0 | 0.0 | $524.2^{a}$ | 32.0 | $205.7^{a}$ | $286.6^{a}$ | 6.4 | $38.4^{a\,c}$ | $55.3^{a}$ |
| D | 10.0 | 0.0 | 0.0 | 0.3 | $277.3^{b}$ | 32.0 | $80.1^{b\,c}$ | $165.4^{b\,c}$ | 8.8 | $26.8^{b}$ | $64.4^{a}$ |
| G | 10.0 | 3.0 | 0.0 | 0.0 | $459.2^{a}$ | 13.1 | $210.0^{a}$ | $236.1^{a\,b}$ | 3.0 | $45.6^{c}$ | $51.5^{a}$ |
| H | 10.0 | 3.0 | 0.0 | 0.3 | $171.8^{b}$ | 13.3 | $11.7^{c}$ | $146.8^{b\,c}$ | 7.7 | $6.5^{d}$ | $85.8^{b}$ |
| K | 10.0 | 0.0 | 2.44 | 0.0 | $488.6^{a}$ | 16.0 | $160.7^{a\,b}$ | $311.7^{a}$ | 3.3 | $32.7^{a\,b}$ | $64.0^{a}$ |
| L | 10.0 | 0.0 | 2.44 | 0.3 | $126.1^{b}$ | 3.6 | $12.6^{c}$ | $110.0^{c}$ | 2.9 | $10.0^{d}$ | $87.2^{b}$ |
| | | | | ± SEM | 51.6 | 12.6 | 28.0 | 29.8 | 2.7 | 3.5 | 4.7 |
| | | | | LSD (P = 0.05) | 158.3 | NS | 85.9 | 91.4 | NS | 10.9 | 14.4 |

Each value is a mean of 4 chicks.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.
NS = No significant differences.

potentiated the action of SNP. In the presence of either methionine or cysteine the SNP mediated decrease in selenite content was highly significant ($P<0.001$). Cysteine appeared to be less effective than methionine in promoting the increase in the proportion of selenium present as selenoamino acids after SNP treatment. Addition of the toxin to the methionine-containing rations caused a highly significant ($P<0.001$) increase in this variable, whilst in the presence of cysteine the observed difference achieved significance at ($P<0.01$). However, this may simply result from the fact that cysteine treatment alone tended to increase the relative proportion of this form of selenium. The percentage of total selenium present as selenide was not altered by any of the dietary treatments.

SNP also exerted a profound effect on the selenium content of the post-mitochondrial supernatant from liver (Table 11.3). Treatment with this toxin significantly reduced total selenium ($P<0.001$), selenite ($P<0.05$) and selenoamino acids/selenate ($P<0.001$) concentration. However, in contrast to the situation occurring in whole liver, sulphur amino acid availability had relatively little effect on the SNP action. Methionine potentiated the SNP-induced reduction in selenite concentration in the post-mitochondrial supernatant, but diminished the effect of this toxin on selenoamino acid content. Cysteine supplementation did not modify the action of SNP on selenoamino acids/selenate and actually nullified its effect on selenite. Again, selenide content was highly variable and demonstrated no significant response to any of the dietary treatments. In addition, the relative proportions of the various forms of selenium were unaltered by diet.

Selenium contained in the mitochondria was less susceptible to the action of SNP than was that present in whole liver or in the post-mitochondrial supernatant (Table 11.4). Mitochondrial total selenium content was reduced by SNP treatment, but this effect achieved significance only in birds consuming the unsupplemented diets. SNP also decreased mitochondrial selenite concentration ($P<0.05$). This effect was amplified by methionine, but not by cysteine supplementation because cysteine treatment alone significantly ($P<0.001$) reduced the amount of selenite in this subcellular compartment. Mitochondrial selenide and selenoamino acids/selenate

concentrations were unaltered by SNP treatment. The relative proportions of the various forms of mitochondrial selenium were largely unaffected by SNP. The percentage of selenium present as selenite was reduced but only in birds consuming the sulphur amino acid supplemented rations.

SNP treatment also altered the distribution of liver total selenium between the various subcellular fractions (Table 11.5), but only in the presence of supplemental sulphur amino acids. When included in the methionine or cysteine-containing diets SNP increased the proportion of the total selenium that was contained in the post-mitochondrial supernatant ($P<0.05$) at the expense of that present in the cell debris fraction, comprising nuclei and unbroken cells.

### 4.10.1 Discussion of Experiment 18.

One feature that stands out in these results is that, in contrast to the findings of experiment 16, methionine supplementation ameliorated the detrimental effects of chronic cyanide exposure on growth performance. As mentioned in Sections 2.6 and 4.8.3, current literature is highly contradictory regarding the possible benefits of supplemental methionine in cyanide intoxication. A possible explanation for such anomalies could be provided by the suggestion that certain, as yet unidentified, dietary factors might influence the methionine-cyanide interaction. In fact, conventional chick mash provided the basis for experimental treatments in experiment 18 whilst a cassava ration was used for experiment 16. These two diets differ markedly in their inherent vitamin and mineral content. However, the important ingredient(s) are not readily identifiable since any major deficiencies would have been corrected by the β-130 supplement.

As expected, given the findings of experiment 10 using supplemental L-cystine, cysteine proved beneficial against cyanide intoxication. In addition, selenium toxicity was also reduced, in terms of growth performance, when cysteine was included in the feed. This latter observation agrees with results reported by Lowry & Baker (1989) who demonstrated a 28% improvement in the weight

**Table 11.3** **The Effects of Dietary DL-Methionine, L-Cysteine and SNP on Selenium Content and Redox State in the Post-mitochondrial Supernatant from Livers of Chicks Consuming High Selenium Diets.**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added DL-Met g $kg^{-1}$ | Added L-Cys g $kg^{-1}$ | Added SNP g $kg^{-1}$ | pmols mg $protein^{-1}$ | | | | % of total Selenium | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Total Se | Selenide | Selenite | Seleno-amino acids/ Selenate | Selenide | Selenite | Seleno-amino acids/ Selenate |
| C | 10.0 | 0.0 | 0.0 | 0.0 | $302.6^{a}$ | 19.8 | $43.0^{a\,c}$ | $239.7^{a}$ | 6.0 | 14.7 | 79.3 |
| D | 10.0 | 0.0 | 0.0 | 0.3 | $143.0^{b}$ | 4.0 | $21.7^{b}$ | $117.5^{b}$ | 2.9 | 15.2 | 82.0 |
| G | 10.0 | 3.0 | 0.0 | 0.0 | $280.0^{a}$ | 8.5 | $56.2^{a}$ | $214.9^{a}$ | 3.2 | 20.4 | 76.3 |
| H | 10.0 | 3.0 | 0.0 | 0.3 | $166.3^{b}$ | 2.2 | $22.8^{b}$ | $141.4^{b}$ | 1.3 | 13.9 | 84.8 |
| K | 10.0 | 0.0 | 2.44 | 0.0 | $285.8^{a}$ | 5.9 | $27.4^{b\,c}$ | $252.5^{a}$ | 2.2 | 10.0 | 87.8 |
| L | 10.0 | 0.0 | 2.44 | 0.3 | $140.7^{b}$ | 7.0 | $13.0^{b}$ | $128.0^{b}$ | 4.9 | 9.4 | 85.7 |
| | | | | ± SEM | 20.3 | 4.3 | 5.6 | 19.4 | 1.9 | 2.5 | 3.3 |
| | | | | LSD (P = 0.05) | 62.2 | NS | 17.2 | 59.7 | NS | NS | NS |

Each value is a mean of 4 chicks.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.
NS = No significant differences.

**Table 11.4** **The Effects of Dietary DL-Methionine, L-Cysteine and SNP on Mitochondrial Selenium Content and Redox State in the Livers of Chicks Consuming High Selenium Diets.**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added DL-Met g $kg^{-1}$ | Added L-Cys g $kg^{-1}$ | Added SNP g $kg^{-1}$ | pmols mg $protein^{-1}$ | | | | % of total Selenium | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Total Se | Selenide | Selenite | Seleno-amino acids/ Selenate | Selenide | Selenite | Seleno-amino acids/ Selenate |
| C | 10.0 | 0.0 | 0.0 | 0.0 | $612.1^{a}$ | 75.1 | $159.0^{a}$ | 378.0 | 11.9 | $25.6^{a\,b}$ | $62.5^{a\,b}$ |
| D | 10.0 | 0.0 | 0.0 | 0.3 | $371.9^{b\,c}$ | 63.4 | $101.8^{a\,b}$ | 206.7 | 15.2 | $26.1^{a\,b}$ | $58.7^{b}$ |
| G | 10.0 | 3.0 | 0.0 | 0.0 | $498.7^{a\,b}$ | 58.8 | $161.4^{a}$ | 299.5 | 11.8 | $30.6^{a}$ | $58.6^{b}$ |
| H | 10.0 | 3.0 | 0.0 | 0.3 | $298.5^{b\,c}$ | 25.4 | $41.1^{b\,c}$ | 232.1 | 9.6 | $14.8^{b\,c}$ | $75.6^{a\,c}$ |
| K | 10.0 | 0.0 | 2.44 | 0.0 | $409.4^{a\,b\,c}$ | 13.8 | $86.2^{b}$ | 309.4 | 3.3 | $21.3^{a\,b}$ | $75.4^{a\,c}$ |
| L | 10.0 | 0.0 | 2.44 | 0.3 | $242.5^{c}$ | 28.4 | $11.2^{c}$ | 203.2 | 13.7 | $4.4^{c}$ | $80.9^{c}$ |
| ± SEM | | | | | 70.5 | 19.0 | 20.1 | 55.4 | 3.4 | 3.9 | 5.4 |
| LSD (P = 0.05) | | | | | 216.3 | NS | 61.8 | NS | NS | 12.0 | 16.7 |

Each value is a mean of 4 chicks.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.
NS = No significant differences.

**Table 11.5** **The Effects of Dietary SNP, DL-Methionine and L-Cysteine on the Amount and Subcellular Distribution of Hepatic Selenium.**

| Treat-ment | Added Selenium mg $kg^{-1}$ | Added DL-Met g $kg^{-1}$ | Added L-Cys g $kg^{-1}$ | Added SNP g $kg^{-1}$ | Liver Total Selenium nmols Whole $Liver^{-1}$ | Mean % of Total Liver Selenium in: | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Post-mitochondrial Supernatant | Mitochondrial fraction | Debris fraction |
| C | 10.0 | 0.0 | 0.0 | 0.0 | $799.2^{a}$ | $29.4^{a\,b}$ | 13.8 | $56.8^{a\,b}$ |
| D | 10.0 | 0.0 | 0.0 | 0.3 | $344.6^{b}$ | $32.0^{a\,b}$ | 22.5 | $45.6^{a\,b\,c}$ |
| G | 10.0 | 3.0 | 0.0 | 0.0 | $650.2^{a}$ | $25.8^{a}$ | 14.6 | $59.6^{a\,b}$ |
| H | 10.0 | 3.0 | 0.0 | 0.3 | $265.3^{b}$ | $44.1^{b\,c}$ | 17.1 | $38.8^{b\,c}$ |
| K | 10.0 | 0.0 | 2.44 | 0.0 | $830.7^{a}$ | $27.8^{a}$ | 7.6 | $64.7^{a}$ |
| L | 10.0 | 0.0 | 2.44 | 0.3 | $231.8^{b}$ | $48.4^{c}$ | 17.1 | $34.5^{c}$ |
| | | | | ± SEM | 75.6 | 5.4 | 5.0 | 6.7 |
| | | | | LSD (P = 0.05) | 222.9 | 16.1 | NS | 19.9 |

Each value is a mean of 4 birds.
Values within a column sharing the same superscript are not significantly different at $P<0.05$.
NS = No significant differences.

gains achieved by young chicks when cysteine was added to high selenium rations. Inclusion of cysteine in the basal diet did not affect growth. Mechanisms proposed in the literature to account for the effectiveness of L-cysteine in ameliorating selenium-induced growth depression include provision of GSH for metabolism of the element (Combs & Combs, 1986) and the formation of a selenium-cysteine chelate, similar to that occurring between cysteine and either copper or cobalt (Baker & Czarnecki-Maulden, 1987).

In common with the findings of experiments 15 and 16, methionine supplementation promoted the alleviation of selenium toxicity by SNP. Cysteine appeared to be less effective than methionine in supporting this interaction. However, selenium was less toxic in the presence of cysteine so any improvements in weight gain demonstrated by SNP treated birds would have been less marked.

SNP decreased hepatic total selenium content, an effect which was potentiated by supplemental methionine or cysteine. Examination of the current literature reveals a lack of correlation between the protective effects of cyanide sources (linseed oil meal in particular) against selenium intoxication and effects on the tissue levels of this element. Palmer *et al.* (1980) reported a LOM-induced decrease in kidney selenium content. Purified cyanoglycosides tended to have a similar effect although significance was not achieved. Both substances gave protection against chronic selenium intoxication. In addition, Beilstein & Whanger (1984) showed that administration of cyanide to rats in their drinking water reduced blood, kidney, liver and muscle selenium content. Evidence also suggests that cyanide may be capable of inducing deficiency in animals consuming diets only marginally adequate in selenium (Palmer & Olsen, 1981; Elzubeir, 1986; Elzubeir & Davis, 1990).

In contrast, some reports in the literature have described increased tissue selenium concentrations in the presence of LOM (Olsen & Palmer, 1955; Levander *et al.*, 1970). LOM again exerted a protective effect against chronic selenosis. These observations, together with the dialysis studies mentioned in the introduction to experiment 18, lead Levander *et al.* to propose that the action of LOM might be explained in terms of this protein, or the metabolites derived

thereof, fixing selenium and preventing it from reacting with sensitive cellular sulphydryl sites.

In other cases amelioration of selenosis, by cyanide, has been observed in the absence of any significant alteration in tissue selenium content (Palmer & Olsen, 1979). Elzubeir (1986) demonstrated that SNP, when included in the feed of chicks, either at 0.1 or 0.3 g $kg^{-1}$, alleviated selenium toxicity, however, only at the higher rate of inclusion were tissue selenium levels reduced.

Such conflicting data suggest that lowering of tissue selenium levels may not be crucial to the mechanism of the alleviation, by cyanide, of chronic selenosis. The observation that tissue selenium concentration does not always correlate with the severity of toxicosis lends some support to this argument. For example, selenite is twice as growth depressing as selenomethionine, but selenomethionine elevates liver selenium twofold over that occurring with selenite supplementation (Lowry & Baker, 1989; Palmer *et al.*, 1983; Deagen *et al.*, 1987; Whanger & Butler, 1988; Beilstein & Whanger, 1988). In addition, there was no evidence from the results of experiment 18 that the beneficial effect of L-cysteine against selenium toxicity was accompanied by any lowering of the tissue levels of this poison.

It seems possible that as yet unidentified nutritional factors in the relatively unpurified diets used in animal feeding trials might prove capable of modifying the effect of cyanide or LOM on tissue selenium levels. An indication that such an interaction may exist was provided by the work of Halverson & Palmer (1975). The effects observed were small and failed to achieve significance, however, there was a tendency for LOM to decrease liver selenium when added to vitamin E deficient diets, but increase it when included in vitamin E adequate rations.

The observation that SNP decreased the relative proportion of hepatic selenium which was present as selenite, whilst increasing the percentage of selenoamino acids/selenate provides a possible mechanism for the protective action of SNP against selenosis. As previously mentioned, selenoamino acids are less toxic than selenite. Studies on the relative toxicities of selenite and selenate have revealed, either

that their toxicities are comparable, or that selenate is slightly less toxic than selenite (see Combs & Combs, 1986, for a review of published data). For example, Franke & Moxon (1936) determined that the minimum lethal dose of selenite in rats was 3.25-3.5 mg Se kg body weight$^{-1}$, while that of selenate was 5.25-5.75. The minimum lethal doses of selenocystine and selenomethionine in rats were reported by Klug *et al.*, (1949) to be 4.0 and 4.25 mg Se kg body weight$^{-1}$ respectively.

The effect of sulphur amino acid supplementation either alone, or in conjunction with SNP, on the form in which selenium was present in the liver emphasised the importance of the cyanide action. Cysteine, which as previously mentioned was beneficial against selenosis, did not alter hepatic selenium content, agreeing with the findings of Lowry & Baker (1989), but had a tendency to decrease both the concentration and proportion of selenite, whilst increasing selenoamino acids. These changes were particularly pronounced in the mitochondrion, where the effect on selenite concentration achieved significance. Conversely, methionine supplementation, which may actually exacerbate selenium toxicity, tended to increase selenite and reduce selenoamino acid content. More importantly, SNP did not alleviate selenosis in chicks consuming the unsupplemented diets despite its ability to bring about a dramatic reduction in hepatic selenium content. However, in the presence of methionine or cysteine, both of which allowed SNP to alter the form in which selenium was present in the liver, alleviation of selenosis became apparent.

The mechanism by which SNP alters the relative proportions of the various forms of hepatic selenium remains unclear. The requirement for sulphur amino acids provides limited information, because there are several points at which methionine or cysteine supply might affect the interaction. The sulphur amino acids, and cysteine in particular, are the ultimate sources of sulphane sulphur for cyanide detoxification, but it is difficult to envisage how inactivation of this toxin might enhance its ability to alleviate selenosis. Cysteine could provide GSH necessary for the metabolism of selenite to hydrogen selenide and the ultimate incorporation of selenium into proteins. Methionine may be important for the continued supply either of GSH or methyl groups. Alternatively, a cysteine source may be required for

synthesis of selenocysteine and the incorporation of selenium into proteins. That both amino acids were effective in potentiating the SNP-induced alteration in the relative proportions of selenite and selenoamino acids suggests that a supply of GSH or cysteine rather than of methyl groups may be important for the SNP action.

It is possible that methionine and cysteine may exert their greatest influence at separate sites. However, most of the apparent differences between the relative abilities of these amino acids to modify the interaction can be related to their inherent capacity for altering selenium concentration and redox state. One observation which cannot be explained in this manner is the greater effectiveness of cysteine in potentiating the SNP-induced decrease in hepatic total selenium and selenoamino acid concentrations. Tappel *et al.* (1984) reported that over 80% of the total selenium in the tissues of rats fed [$^{75}$Se] selenite was present, in protein, in the form of selenocysteine. In addition, most workers agree that selenocysteine can be synthesised from dietary selenite, whereas selenomethionine is not formed in this manner. It is therefore tempting to speculate that selenocysteine formation might be important in this process.

Interestingly, SNP exerted a proportionately greater effect on selenium content and redox state in the post-mitochondrial supernatant, i.e. the site of selenium metabolism, than it did in whole liver or the mitochondrion, which is traditionally regarded as the primary target for cyanide action.

A notable feature of experiment 18 was the variability of the hepatic selenide concentration data. Diplock *et al.* (1971) using mercaptoethanol and DL- α-tocopherol acetate as antioxidants in the homogenising medium obtained values that were both higher, particularly for the post-mitochondrial fractions, and more consistent. Mercaptoethanol reacts with selenite to form selenotrisulphides (Ganther, 1968). Even though any selenium present as selenotrisulphides should be released by treatment with Zn and HCl (and therefore be included in the selenite fraction), it was considered safer, particularly in view of the high concentration of selenite used, to omit mercaptoethanol from the homogenising medium of

experiment 18. This might explain the variability of the selenide results. Diplock *et al.* (1973) stated that when only α-tocopherol acetate was present as an antioxidant in the homogenisation medium a similar proportion of the selenium was found to be present in the selenide form as when α-tocopherol and mercaptoethanol were both present. However, this does not always appear to be the case. In 1971, they concluded that either mercaptoethanol or α-tocopherol used separately was not as effective in protecting the selenide *in vitro* as a mixture of the two antioxidants. An inevitable consequence of the calculation method used in experiment 18 to determine liver selenite concentration was that these results were also slightly more variable than those reported by Diplock's group. However, the other data presented in tables 11.1 to 11.5 are in general agreement with the findings of Diplock *et al.* (1971), including the observation that selenide is particularly associated with the mitochondrial fractions.

# CHAPTER 5

## FINAL DISCUSSION

In the previous chapter the results of each experiment were discussed separately. The purpose of this final discussion is to integrate the results of the various trials, in so far as this is possible, given that "between experiment" comparisons are not strictly valid in nutritional research. An attempt will also be made to draw some overall conclusions and to suggest areas for further investigation.

### 5.1 The Effect of Chronic Cyanide Exposure on Glucose Catabolism and Cellular Redox State.

Chronic exposure to a sublethal dose of dietary cyanide reduced hepatic glycogen concentration, which agreed with results obtained by Kovacs and Leduc (1982), Raymond *et al.* (1986), and by Padmaja and Panikkar (1989). Glycogen levels were decreased one day after the start of the treatment and remained depressed three weeks into the investigation (experiments 5 & 6). The reduction in liver glycogen content could not be accounted for by decreased food consumption. Cyanide-mediated depression of food intake was most pronounced during the first few days of exposure to this toxin, after which food consumption stabilised at a level approximately 90% of that seen in control birds (Figure 9.2).

The reduction in hepatic glycogen content was not accompanied by the increases in liver lactate and blood glucose concentration, observed during acute investigations (Albaum *et al.*, 1946; Olsen & Klein, 1947; Estler, 1965; Detwiler, 1972; Elzubeir, 1986) and taken as indicative of enhanced anaerobic catabolism of glucose. In fact, lactate level was actually reduced by SNP treatment resulting in a lowered lactate:pyruvate ratio (experiment 7). However, it should be remembered that glycogen levels and lactate:pyruvate ratios were not measured in the same experiment. It is possible that the unexpected liver lactate data may have resulted from significant operation of the methylglyoxal pathway (Section 2.3.2 and Figure 1) in the presence of

the relatively high concentration of cyanide (0.5 g SNP per kg feed) employed in this particular trial. The observation that the lactate:pyruvate ratio was halved, and the sum of the concentrations of these two metabolites was reduced, in livers from cyanide treated birds, provided evidence in support of this explanation of the results. Alternatively, the depletion of glycogen, observed during chronic cyanide intoxication, may have a different basis. The results may reflect a direct action of cyanide on the enzymes involved in glycogenolysis or glycogenesis, rather than a stimulation of the anaerobic catabolism of glucose.

Dietary supplementation with the gluconeogenic precursors, L-alanine, L-lactate and L-serine, exacerbated cyanide toxicity (experiment 8). This effect could be indicative of the importance of the opposing, catabolic, pathways during chronic cyanide exposure, however, other explanations should be considered in light of the lactate, pyruvate and blood glucose results. Exacerbation of cyanide toxicity by pyruvate precursors may suggest that pyruvate removal is restricted during chronic exposure to this toxin. Pyruvate concentration was increased by SNP treatment, although the difference failed to achieve significance. Certainly, if conversion of pyruvate to glycogen were inhibited this would account for the reduction in liver glycogen concentration. However, acute studies have demonstrated an effect of cyanide on glycogen phosphorylase, but not on the synthase (Detwiler *et al.*, 1972; Jakob & Diem, 1974; Conaglen *et al.*, 1984). Alternatively, cyanide might inhibit the removal of pyruvate through acetyl-CoA and the TCA cycle. Isom *et al.* (1975) demonstrated that utilisation of the TCA cycle was reduced during acute cyanide toxicity.

Chronic exposure to dietary cyanide produced little alteration in the redox state of whole liver pyridine nucleotides (experiment 11). The only significant effect was a cyanide-mediated decrease in hepatic NADPH concentration, although the trend was for both redox couples to become more oxidised in the presence of cyanide. These changes are opposite to those generally obtained during studies of acute cyanide poisoning (Hattori *et al.*, 1986; Sahlin & Katz, 1986). Since the lactate:pyruvate ratio is largely determined by the redox state of the pyridine nucleotides this finding may well provide an explanation for

the increased oxidation state of this cytoplasmic couple during chronic cyanide intoxication. The lack of effect of cyanide on the redox state of hepatic pyridine nucleotides can not be taken as conclusive evidence that inhibition of cytochrome c oxidase assumes little importance during chronic exposure to this toxin. The results provided no information on flux through the TCA cycle and electron transport chain.

Cyanide decreased total and reduced glutathione concentrations in liver and blood, increased blood GSSG and reduced the GSH:GSSG ratio. Whilst these findings may also be related to the aforementioned changes in the redox state of the pyridine nucleotide and lactate/pyruvate couples, there are other points at which cyanide might affect the GSH/GSSG pair. The cyanide induced alteration in glutathione status and redox state may result from the SeGSHPx catalysed reduction of hydroperoxides or may reflect an increased demand for sulphur compounds during cyanide detoxification. Whatever its cause, glutathione depletion appears to be a feature both of acute (Misra *et al.*, 1988; Hatch *et al.*, 1990; Younes & Strubelt, 1990; Ardelt *et al.*, 1989) and of chronic cyanide intoxication.

It has been suggested that many metabolic reactions might be regulated by the reversible oxidation and reduction of enzyme thiols/disulphides (Sies & Moss, 1978; Gilbert 1982 & 1984; Ziegler, 1985). In this scheme the redox state of protein thiols would be dependent upon the thiol/disulphide status of the surrounding environment (in particular the GSH:GSSG ratio) and coupled to the redox potential of the cell. Such a mechanism might well explain some of the effects of chronic cyanide intoxication on carbohydrate metabolism. For example, the catalytic subunit of rabbit liver glycogen phosphorylase phosphatase is inactivated by GSSG (Shimazu *et al.*, 1978). In addition, Ernest and Kim (1973 & 1974) have described the inactivation of rat liver glycogen synthase D by thiol:disulphide exchange with GSSG. However, the ratios of GSH:GSSG required to produce significant changes in enzyme activity are well outside the range normally encountered physiologically. The lack of specifity in the oxidation of protein thiols by disulphides also remains a major problem with regard to the physiological significance of these reactions. In practice *in vivo* evidence in favour of such a

hypothesis is currently impossible to obtain since techniques for assessing the redox state of protein thiols are inadequate.

## 5.2 The Effects of Chronic Selenium Exposure on Glucose Catabolism and Cellular Redox State.

Liver glycogen and lactate concentrations were unaltered by selenium exposure indicating that this toxin does not cause major lesions in the pathways of glucose breakdown. Liver pyruvate concentration was, however, elevated in chronic selenosis and the lactate:pyruvate ratio was commensurately reduced. Similar observations have been made by other workers investigating acute selenium toxicity (Caravaggi, 1971; Shearer, 1973). Information gained from acute studies has provided a possible explanation for the selenium-induced increase in liver pyruvate content. The toxin has been shown to inhibit certain enzymes involved in glucose metabolism including α-ketoglutarate dehydrogenase, succinate dehydrogenase and lactate dehydrogenase (Ignesti *et al.*, 1986; Klug *et al.*, 1950; Ray & Ray, 1975; Nebbia *et al.*, 1990). That similar mechanisms may operate during chronic selenium poisoning has been suggested recently by Davis and Frear (unpublished) who demonstrated that long-term exposure to this toxin decreases the activity of succinate dehydrogenase and NADH:coenzyme Q reductase and reduces mitochondrial acid-labile sulphide content.

Selenium produced changes in the pyridine nucleotide and glutathione redox couples consistent with an increased demand for GSH and NADPH in the metabolism of this element. Selenium decreased whole liver NADH and NADPH concentrations, increased $NADP^+$ content and elevated the $NAD^+$:NADH and $NADP^+$:NADPH ratios. Selenium also increased hepatic total and reduced glutathione. These effects on the glutathione system are consistent with results from both chronic and acute studies (LeBoeuf & Hoekstra, 1983; LeBoeuf *et al.*, 1985; Hoffman *et al.*, 1989; Chung & Maines, 1981) and were considered to be secondary adaptive changes initiated in an attempt to maintain a normal GSH:GSSG ratio. The effects of selenium toxicity on the redox state of pyridine nucleotides have not previously been reported.

## 5.3 The Importance of Effects on Glucose Catabolism and Cellular Redox State in the Mechanism by which Cyanide Alleviates Selenosis.

The selenium-cyanide interaction does not appear to occur at the level of glucose catabolism or through changes in cellular redox state. There were no interactions between these toxins for effects on hepatic glycogen, $NAD^+$, $NADP^+$ or NADH levels, $NADP^+$:NADPH ratio, or glutathione concentration and redox state. Interactions were observed between selenium and cyanide for pyruvate and NADPH contents as well as for lactate:pyruvate and $NAD^+$:NADH ratios. However, these were probably secondary effects, resulting simply from the fact that selenium-induced changes were nullified in the presence of cyanide.

One of the starting hypotheses of this work was that the beneficial effect of cyanide against selenosis might result from cyanide-induced alteration in cellular redox state. It was considered possible that inhibition of cytochrome c oxidase following ingestion of cyanide, might increase reducing potential for selenium metabolism through suppression of the mitochondrial electron transport chain. Although the glycogen results and the effects of dietary supplementation with gluconeogenic amino acids may be suggestive of an increased emphasis on anaerobic glucose catabolism resulting from impaired mitochondrial function, the lactate/pyruvate and blood glucose data do not support this conclusion and other interpretations are possible. In addition, cyanide produced little alteration in the redox state of the hepatic pyridine nucleotides. In fact, the observed effects and, in particular, the cyanide-mediated decreases in cellular NADPH and GSH levels might be expected to slow, rather than facilitate, selenium metabolism.

## 5.4 The Effects of Dietary Supplementation with Sulphur Amino Acids on the Growth Performance of Chicks Consuming Selenium and/or SNP.

Dietary supplementation with L-cystine or L-cysteine ameliorated cyanide toxicity, whilst DL-methionine proved beneficial in some trials, but not in others. The greater efficacy of L-cyst(e)ine over DL-methionine may suggest that factors other than provision of sulphane sulphur are involved in the action of this amino acid. That

L-cystine does not exert its effects through provision of glutathione was demonstrated in experiment 14. Spontaneous reaction between cyanide and cystine leading to the formation of 2-imino-4-thiazolidine carboxylic acid (Wood & Cooley, 1956), may well explain this observation. The variability of the response to dietary DL-methionine supplementation lead to the proposal that certain, as yet unidentified, dietary factors might be capable of influencing the methionine-cyanide interaction.

L-cysteine supplementation also proved beneficial against selenosis. The mechanism of this action was not investigated, however, some evidence was obtained to suggest that L-cysteine treatment decreases mitochondrial selenite content (Section 5.8). Mechanisms proposed in the literature to explain the effectiveness of L-cysteine in ameliorating selenium-induced growth depression include provision of GSH (Combs & Combs, 1986) and the formation of a selenium-cysteine chelate that is less efficiently absorbed from the gut or deposited in the tissues (Baker & Czarnecki-Maulden, 1987). However, no evidence was obtained in experiment 18 to suggest that the beneficial effect of L-cysteine was accompanied by any lowering of tissue selenium levels. Provision of GSH also seems an unlikely explanation because, in trials 16 and 18, methionine actually appeared to exacerbate selenium toxicity as measured by effects on growth performance and liver weight. It is known that when DL-methionine increases in the diet, the amount of L-cysteine produced inevitably increases (Tateishi *et al.*, 1981). In addition, most workers agree that both methionine and cysteine can contribute to hepatic glutathione (Leaf & Neuberger, 1947; Reed & Orrenius, 1977; Tateishi *et al.*, 1981; Suberville *et al.*, 1987). The reason for the slight exacerbation of selenosis by DL-methionine remains unclear, but may also involve changes in the form in which selenium is present in the tissues (Section 5.8).

An adequate supply of either DL-methionine or L-cysteine was essential to allow cyanide to overcome the growth depressing effects of selenium exposure. Methionine appeared to be more effective than cysteine in supporting the interaction. However, since selenium was less toxic in the presence of cysteine any improvement in weight gain as a result of cyanide treatment would have been less marked.

There are several points at which the sulphur amino acids might influence the selenium-cyanide interaction. Both may provide sulphane sulphur, although it is difficult to see how detoxification of cyanide might enhance its action. The results of experiment 4 clearly demonstrated that thiocyanate is not capable of alleviating selenosis. The degree of cyanide toxicity may be important, a possibility also suggested by data reported by Elzubeir and Davis (1988a). Graded levels of SNP (0.0-0.4 g kg feed$^{-1}$) were fed to chicks consuming selenite at 10 mg kg feed$^{-1}$. Alleviation of selenosis, as measured by growth performance, was most pronounced at 0.1 g SNP and declined as concentration increased. However, total liver selenium decreased progressively with increasing dose, whilst plasma GSHPx activity improved.

Alternatively, the sulphur amino acids could provide GSH or a cysteine source (for synthesis of selenocysteine). Another possible site of interaction is through the supply of methyl groups. This explanation seems less likely since both methionine and cysteine were effective in permitting the operation of the selenium-cyanide interaction, however, some evidence was obtained to suggest that these amino acids may exert their greatest effects at different sites.

The mechanism by which methionine and cysteine permit the alleviation, by cyanide, of selenosis remains unclear. It is tempting to speculate that these amino acids provide reactants necessary for the enhanced metabolism of selenium, thereby allowing cyanide to decrease selenite whilst increasing protein-bound, or methylated forms. However, the possibility that cyanide and the sulphur amino acids have independent, additive, effects can not be excluded. Certainly, methionine and cysteine did appear to have a particular effect on mitochondrial selenium, whilst cyanide exerted its greatest influence in the post-mitochondrial fraction.

## 5.5 The Effects of Cyanide on DmSe Exhalation and Tissue Selenium levels.

Cyanide decreased hepatic total selenium content, an effect which was potentiated by supplemental cysteine. As mentioned in Section

4.10.1 examination of the current literature reveals a lack of correlation between the effects of cyanide sources on tissue selenium levels and their ability to alleviate selenosis. This observation was substantiated by the results of experiment 18. Cyanide failed to ameliorate the growth-depressing effects of selenium in chicks consuming the amino acid unsupplemented diets even though liver selenium levels were markedly reduced. Similarly anomalous results have been obtained from studies into the effects of certain heavy metals on tissue selenium (Section 2.16.2).

In the experiments described in this thesis, SNP was administered at a level of at least 0.3 g kg feed$^{-1}$. As previously mentioned, Elzubeir (1986) and Elzubeir and Davis (1988a) demonstrated that selenosis could be overcome by a lower dose of SNP (0.1 g per kg feed) in the absence of any alteration in liver selenium content. It is not impossible that two mechanisms may have been in operation during these investigations, one by which cyanide alleviates selenium toxicity and another by which an excess of this toxin reduces tissue selenium levels. The same could also be true for heavy metals.

In experiment 17, cyanide decreased the amount of selenium exhaled as volatile forms, by chicks consuming conventional mash supplemented with 2 g DL-Met kg$^{-1}$ and 10.0 mg Se kg$^{-1}$, to levels not significantly different from those observed in the control birds. This effect of cyanide is probably secondary, resulting either from the reduction in tissue selenium levels or from detoxification of this element by other mechanisms. Cyanide-mediated changes in the amount of selenium excreted by the lungs do not appear to be crucial for alleviation of the growth-depressing action of selenite. The effects of cyanide on the exhalation of volatile selenium compounds are highly variable. However, whether cyanide increases (Elzubeir, 1986) or decreases (Palmer & Olson, 1979; Mitchell, Dowell & Davis, unpublished; experiment 18) pulmonary excretion of DmSe, it still ameliorates selenosis. The effects of cyanide on selenium exhalation can probably be influenced by dietary factors which may, or may not, include methionine.

That cyanide decreases both liver selenium levels and DmSe exhalation suggests that this toxin may increase the amount of

selenium excreted through other routes, such as urine and faeces, and/or it may decrease tissue uptake and deposition of the element. Interestingly, Bopp *et al.* (1982) stated that the main route of selenium excretion under conditions of chronic administration of relatively high levels of selenium would be the urine. They based this conclusion on the work of Halverson *et al.* (1962) who demonstrated that, in rats given diets containing 5 mg selenium (as selenate) per kg feed, the amount of selenium in urine rose with increasing time of exposure. The same appears to be true for selenium given as selenite (Burk, 1976). In addition, Beilstein and Whanger (1984) demonstrated a small increase, compared with controls, in urinary excretion of $^{75}Se$ after its injection as selenite into rats that had received 150 p.p.m. cyanide in their drinking water for the previous two weeks.

The only selenium balance study carried out to date was reported by Elzubeir in 1986. He demonstrated a SNP-induced decrease in the selenium content of liver, kidney and carcass from chicks consuming high selenium diets. The amount of selenium excreted in urine and faeces, taken together, remained unaltered, comprising about 65% of administered dose, leading Elzubeir to propose that SNP increases selenium exhalation. He went on to prove this hypothesis in a separate experiment. However, the conditions employed in Elzubeir's investigations differed both from the "norm" and from experiments 17 and 18 in several important ways. Firstly, he employed an equalised regime of feed administration with the result that control birds, and those consuming selenium with cyanide, would have been short of food prior to refeeding (once every two days). These birds may, therefore, have become deficient in certain important nutrients. In addition, Elzubeir used a cassava-based diet supplemented with 6 g DL-Met. per kg. This level of inclusion of methionine was far in excess of requirements (Section 4.8.3). There is no doubt that volatilisation of selenium can be increased by raising dietary methionine intake (Ganther *et al.*, 1966). It is tempting to speculate that cyanide increases selenium exhalation, only in the presence of an excess of dietary methionine, however, other factors may well be involved.

## 5.6 The Effect of Cyanide on the Form in which Selenium is Present in the Tissues.

Cyanide altered the relative proportions of the various forms of selenium in liver tissue. Although cyanide decreased hepatic selenite and selenoamino acids content together with total selenium concentration, these fractions were affected to different extents. Consequently, cyanide reduced the proportion of total selenium present in the form of selenite, whilst increasing the percentage of selenoamino acids/selenate. An adequate supply of either methionine or cysteine was required for cyanide to achieve these effects.

The observation that cyanide alters the oxidation state of hepatic selenium provides a possible explanation for the alleviation, by cyanide, of selenosis. The probable effect of cyanide is to reduce the amount of a particularly damaging form of selenium. Certainly, selenoamino acids are less toxic than selenite (Sections 2.16 and 4.10.1). However, since current methods permit only a rather broad fractionation of the total tissue selenium other forms may be involved. For example, the selenite fraction would have included glutathione selenotrisulphide (GSSeSG) which has been shown to inhibit protein synthesis *in vitro* (Vernie *et al.*, 1974) and might well represent the damaging species. Another alternative is selenide. This fraction proved highly variable in experiment 18 and requires further investigation particularly in view of the suggestion by Diplock *et al.* (1971) that the active form of selenium in the tissues may be selenide.

Three observations support the notion that cyanide-mediated changes in the form in which selenium exists in the tissues might indeed be important for the amelioration of growth depression caused by selenite. Firstly, cyanide did not alleviate selenosis in chicks consuming the sulphur amino acid unsupplemented diets. Secondly, cysteine which proved beneficial against selenium toxicity had a tendency to bring about similar changes in the oxidation state of this element. Thirdly, methionine supplementation, which may actually exacerbate selenosis, tended to increase the proportion of liver selenium present as selenite, whilst decreasing the selenoamino acids fraction.

The mechanism by which cyanide alters the oxidation state of hepatic selenium remains unclear, however, it seems likely that cyanide-mediated changes in the metabolism of selenium will prove to be involved.

Although the scheme for selenite metabolism, proposed in 1974 by Ganther and Hsieh (Figure 3), based on studies *in vitro*, has become widely accepted, the processes operating *in vivo* may actually be more complex, since several alternative excretory end products have been identified (Palmer *et al.*, 1969; Obermeyer *et al.*, 1971; Ostadalova *et al.*, 1988; Jiang *et al.*, 1983). Cyanide may well interact with pathways which have not yet been identified.

Only hepatic selenium content and oxidation state were determined in these investigations. Differences may well exist between tissues. Elzubeir (1986) demonstrated a large increase in the selenium content of skin and feathers in response to selenite supplementation which was unresponsive to SNP treatment. In addition, Behne *et al.* (1989) have demonstrated that, in selenium deficiency, this element is directed to, and retained by, certain priority tissues.

## 5.7 SNP as a Source of Dietary Cyanide in Nutritional and Biochemical Studies

Cyanide is released from ingested SNP in the intestinal tract of the chicken. The crop appears to be the major site for SNP degradation (experiments 1 & 2). Analysis of blood obtained from chicks consuming SNP revealed the presence of cyanide, but not of unaltered SNP. It was concluded that this potentially hypotensive agent is rapidly degraded in the chick gut and is not absorbed, intact, into the bloodstream. The observed decreases in blood and hepatic total glutathione and GSH content and the accompanying increase in blood GSSG level in birds consuming SNP are, therefore, related to cyanide itself rather than to the consumption of GSH or other sulphydryl compounds during SNP degradation. Certainly, glutathione derived from the gut walls may play a part in the breakdown of SNP, but the crop is unlikely to contain sulphydryl compounds in quantities sufficient to account for the observed

results. In addition, Page *et al.* (1955) demonstrated that cysteine was particularly effective in releasing cyanide from SNP, however, in experiment 14, in which chicks were fed an excess of dietary L-cystine, hepatic total glutathione and GSH were still decreased in SNP treated birds.

The other, indirect, evidence advocating the use of SNP as a source of dietary cyanide was that it alleviated selenium toxicity in the vast majority of the experiments described. SNP also increased plasma thiocyanate concentration (results not included in this thesis).

## 5.8 Suggestions for Further Study.

The experiments designed to determine the effects of chronic exposure to dietary cyanide on glucose catabolism and redox state yielded several surprising or anomalous results which might be clarified using the following approaches:

(a) It would be interesting to measure liver glycogen concentrations and lactate:pyruvate ratios in birds from a single feeding trial in which SNP was administered at a level of 0.3 g kg feed$^{-1}$ (or lower).

(b) Whether glycolysis is, in fact, stimulated during chronic cyanide intoxication might be investigated using radiorespirometric techniques.

(c) A point worth checking is whether similar changes in the oxidation state of the lactate/pyruvate, $NAD^+$/NADH, $NADP^+$/NADPH and GSSG/GSH couples occur after feeding dietary KCN. Care would have to be taken to maximise the stability of the cyanide salt in the feed. This approach should serve to dispel any remaining concerns that such changes may have been related to the utilisation of GSH in the degradation of SNP.

Questions concerning the flux through the electron transport and tricarboxylic acid pathways, and the compartmentation of pyridine

nucleotides, might prove more difficult to investigate during chronic cyanide intoxication. Cell fractionation would be required resulting in alteration of the metabolic steady state.

Further information concerning the effects of cyanide on the retention and forms of selenium in tissues might be obtained by pursuing the following lines of investigation:

(a) A selenium balance trial could be carried out, using conventional chick mash, in which the selenium content of liver, kidney, carcass, skin, feathers, urine, faeces and expired air were estimated within a single experiment.

(b) Much work is needed to gain more information about the forms in which selenium exists in the tissues and, in particular, the composition of the broad selenoamino acids/selenate fraction. Data relating to the proportions of selenomethionine and selenocysteine might provide an explanation for the differences observed after feeding DL-methionine or L-cysteine. Derivatisation of selenium-containing species with Sanger's reagent, followed by HPLC (Ganther, 1987) may prove useful in identifying the forms of selenium present. This procedure has been used, not only to achieve the separation of selenoamino acids, but has also been found suitable for the unequivocal identification of hydrogen selenide (Ganther & Kraus, 1984). This approach may also yield information about the mechanism by which cyanide alters the forms of selenium in tissues as well as helping to separate and identify several, as yet uncharacterised, products of selenium metabolism. However, in practice, the primary site of the selenium-cyanide interaction may well prove difficult to establish since any investigation would inevitably be based on a somewhat random search.

## 5.9 Concluding Remarks

Although some of the biochemical changes associated with chronic cyanide intoxication are similar to those observed in acute poisoning,

important differences do occur, suggesting that the underlying metabolic bases of these conditions may also differ.

No evidence was obtained to suggest that cyanide exerts its beneficial effects against selenosis at the level of carbohydrate metabolism or through changes in intracellular redox potential.

Cyanide alters the form in which selenium is present in the tissues in favour of less toxic species. These effects do appear to be important for the alleviation of selenium toxicity, but, at present, their mechanism remains unclear.

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