THE METABOLISM OF CYANIDE AND SULPHUR

IN THE TOXIC OPTIC NEUROPATHIES

Thesis presented for the degree of M.Sc.

by

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SUMMARY.

This work is an attempt to provide a biochemical rationale for any clinical changes which may occur during the treatment of patients suffering from the toxic amblyopias, particularly tobacco amblyopia and Leber's hereditary optic atrophy.

Cyanide is presumed to be an important cause of the visual failure found in these conditions, and the patients may have a defect in the mechanism for the detoxification of cyanide. The major metabolite of cyanide is thiocyanate, and measurements of plasma and urinary thiocyanate therefore provide a convenient guide to the patients' metabolism of cyanide.

The previously unsatisfactory colorimetric procedure for thiocyanate has been improved following the suggestions of Bark & Higson (1964). The method for cyanide estimation is still not completely satisfactory, but is now greatly inproved by the introduction of a microdiffusion technique, using the Bark & Higson colour reaction.

Measurements were made of plasma thiocyanate levels in a group of 20 normal smokers, and were significantly higher than those in a group of 10 normal non-smokers ($P < 0.025$).

The levels of plasma thiocyanate in 18 untreated tobacco amblyopia patients and in 12 untreated Leber's hereditary optic atrophy patients were found to be significantly lower than those found in the

normal smokers ($P < 0.001$). An impairment of thiocyanate excretion was also observed in these patients.

After 4 months intramuscular hydroxocobalamin therapy, the plasma thiocyanate levels of these patients rose significantly and approached those of the normal smokers. There were also increases in urinary thiocyanate, improvement in thiocyanate excretion and improvements in vision.

Seven tobacco amblyopia patients were treated with an oral preparation of hydroxocobalamin. After 4 months, 3 of the group showed increases in plasma and urinary thiocyanate similar to that obtained with the intramuscular therapy.

Six tobacco amblyopia patients were treated with an oral cystine preparation at a dose of 4.0 gm. per day. After 4 months treatment, there were similar increases in plasma and urinary thiocyanate, and satisfactory visual improvement in 3 of the group.

Using a fluorometric procedure, red cell glutathione levels were measured in 32 tobacco amblyopia patients and were found to be significantly lower than those found in 34 normal (non-amblyopic) subjects of similar ages ($P < 0.01$). Sequential measurements of red cell glutathione and plasma thiocyanate were made in patients receiving treatment, and the results suggest a possible relationship between these two factors.

Acknowledgments.

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SECTION ONE.

GENERAL INTRODUCTION

BIOCHEMICAL ASPECTS OF THE STUDIES

Cyanide ions are chemically and biologically very active, and are normally quickly metabolised to less toxic compounds. The measurement of cyanide in plasma is difficult, both in the technique required, and in the meaningful interpretation of the results. Studies of the metabolism of cyanide are therefore best carried out indirectly, by examining the more stable products of its detoxification.

Although the ultimate metabolite of cyanide is carbon dioxide in expired air, the major proportion of exogenous or endogenous cyanide is excreted in the urine as thiocyanate, formed by combination with some sulphur source within the body. Both these substances may also enter the body from certain exogenous sources (e.g. diet, tobacco smoke, etc.). Cyanide may also be produced from certain bacterial growth within closed cavities, such as urinary diverticula, and intestinal blind loops, (see Fig. 1.).

The conversion of cyanide to thiocyanate is accomplished enzymatically, by thiosulphate : cyanide sulphurtransferase (E.C.2.8.1.1.), also known as rhodanese (Lang,K.,1933), and by 3-mercaptopyruvate : cyanide sulphurtransferase (E.C.2.8.1.2.)

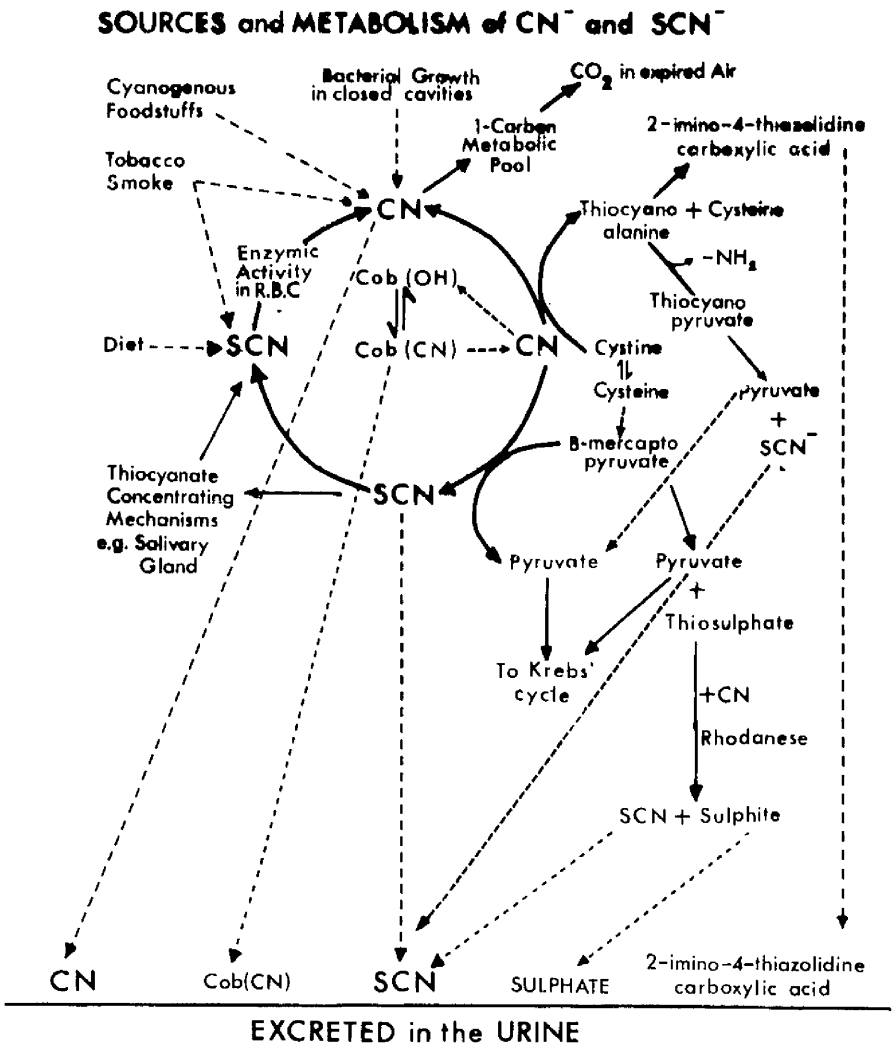


Fig. 1-1. The Sources and Main Metabolic Routes for Cyanide and Thiocyanate.

(Fiedler & Wood,1956). This conversion has been shown to take place in the mucosa of the alimentary tract, in the liver, in the kidneys as well as in other tissues (Clemedson,Sorbo, & Ullberg, 1960) (see Figs. 1-1. & 1-2.).

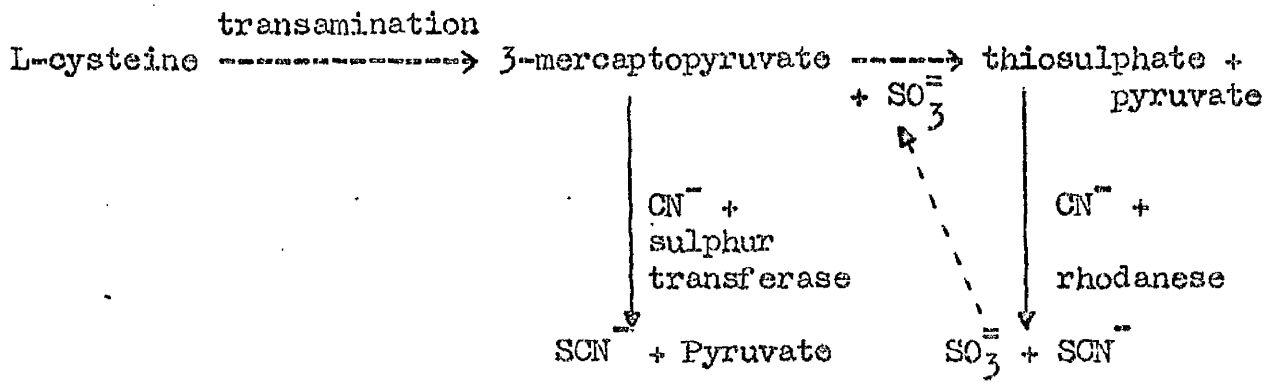


Fig. 1-2. - Enzymatic Conversion of Cyanide to Thiocyanate.

Another independent way in which cyanide may be detoxified is the reaction with cystine to yield cysteine and β-thiocyano-alanine (Voigtlin et al.,1926). Later workers showed that the latter tautomerises to 2-aminothiazolidine-4-carboxylic acid (Schoberl,Kawohl, & Hamm,1951), or to the equivalent 2-imino-4-thiazolidine carboxylic acid (Wood & Cooley,1956). The B-thiocyano-alanine may also undergo deamination to form thiocyanopyruvate, which can be degraded to pyruvate. (see Figs. 1-1. & 1-3.). The cystine may be derived from other sulphur-containing amino acids, such as methionine, homocysteine, cystathionine, and cysteine, or from cystine disulphide residues in proteins (Catsimpoolas & Wood,1964).

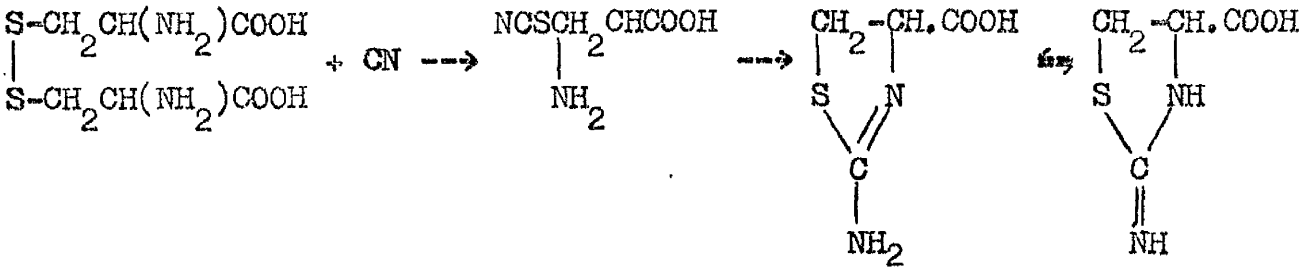


Fig. 1-3. - Detoxification of Cyanide with Cystine.

Cyanide may also be incorporated into the 1-carbon metabolic pool, perhaps via combination with hydroxocobalamin (Boxer & Rickards,1952; Wokes & Picard,1955). (see Fig. 1-1.)

Detoxification of Cyanide with Rhodanese

In 1932, Lang discovered an enzyme which catalysed the conversion of cyanide to thiocyanate in the presence of thiosulphate (Lang,K.,1933). He named the enzyme "rhodanese", the ending "ese" implying that the conversion is a synthetic, irreversible process, thus :-

$$HCN + Na_2S_2O_3 \longrightarrow HSCN + NA_2SO_3$$

This enzyme is particularly active in mammalian liver and kidney, but has also been detected in most mammalian tissues (Himwich & Saunders,1948;Benard et al.,1948;Saunders & Himwich, 1950). Rhodanese activity has also been reported in plants, and in a number of bacteria. In mammalian tissues, rhodanese is mainly associated with the mitochondria (Ludewig & Chanutin,1950;Sorbo, 1951a).

The enzyme is inhibited by cyanide and sulphite, if they are added to the enzyme prior to thiosulphate (Lang,K.,1949; Saunders & Himwich,1950;Sorbo,1951a); and inhibition by these compounds is prevented by cysteine (Sorbo,1951a). Saunders & Himwich proposed that the enzyme formed a loose combination with thiosulphate which then breaks down to yield sulphur in a form acceptable to the cyanide ion. They explained that the inhibitory

effect of certain sulphur-containing compounds such as sodium sulphide, dithiobiuret and cysteine could be due to the blocking of the enzyme so that it can not combine with thiosulphate.

Sorbo (1951b) suggested that rhodanese contains an active disulphide group (rather than sulphhydryl groups) which could account for the fact that cyanide and sulphite both inhibit rhodanese when added before thiosulphate, reacting with the enzyme to form inactive complexes.

Szczepkowski (1961a) suggested that a double displacement mechanism could explain the various reactions catalysed by rhodanese. This was confirmed by Green & Westley (1961), who showed polarographically that one mole of rhodanese reacts with sulphite or cyanide to form two moles of thiosulphate or thiocyanate. They concluded that the crystalline enzyme is a complex (rhodanese-S_2), which contains two atoms of labile sulphur per mole, and which is formed from thiosulphate and rhodanese, the overall reaction being described thus -

$$\text{Rhodanese} + 2SSO_3^{=} \longrightarrow \text{Rhodanese-}S_2 + 2SO_3^{=}$$

$$\text{Rhodanese-}S_2 + 2CN^{-} \longrightarrow \text{Rhodanese} + 2SCN^{-}$$

Although Green & Westley write the second equation above as a reversible reaction, Sorbo (1953c) has confirmed the essential irreversibility of the reaction. Westley & Nakamoto (1962) confirmed

the existence of labile sulphur in rhodanese by demonstrating the transfer of $[^{35}S]$ from radioactive thiosulphate to the enzyme. Treatment of the labelled enzyme with cyanide, sulphite, trichloroacetic acid or heat released the $[^{35}S]$; in the case of cyanide and sulphite, $[^{35}S]$-labelled thiocyanate and thiosulphate respectively were formed. From a kinetic analysis of thiocyanate formation by rhodanese, Mintel & Westley (1966a) were able to deduce that the scission of the sulphur-sulphur bond is the rate-limiting step in overall cyanolytic reaction.

Lang,K. (1933) suggested that rhodanese may function in mammalian tissues in cyanide detoxification, and this view has been expressed by a number of workers from time to time. Bénard et al. (1948) demonstrated that the toxic action of cyanide on yeast respiration is reversed by liver extracts, in the presence of thiosulphate, and a similar re-activation of cyanide-inhibited mammalian cytochrome oxidase by rhodanese was reported by Sorbo (1957c). In T. denitrificans, rhodanese activity is increased several fold by the inclusion of cyanide in the growth medium, indicating a possible role for this enzyme in cyanide detoxification (Bowen et al.,1965). There is no direct evidence, however, that the primary function of rhodanese is in cyanide detoxification, and, moreover, the high activities in some mammalian tissues appear to be inconsistant with the extremely low levels of cyanide to which the organisms are normally exposed.

Thiocyanate Oxidase.

Following upon the work of Lang,K. (1933), Goldstein & Rieders (1953) were able to show that the erythrocytes of man, dog, rabbit and rat contain an enzyme capable of producing cyanide from thiocyanate, the reverse of rhodanese action. They showed that the new enzyme was not identical with rhodanese, borne out by the fact that rhodanese preparations did not catalyse the formation of cyanide from thiocyanate, and that the optimum temperature for the new enzyme of 56°C., completely inactivated rhodanese. The enzyme described by Goldstein & Rieders is able to form about 1 % cyanide from a given amount of thiocyanate rather slowly, even under optimal conditions, while rhodanese is able to convert cyanide to thiocyanate almost quantitatively and with great rapidity.

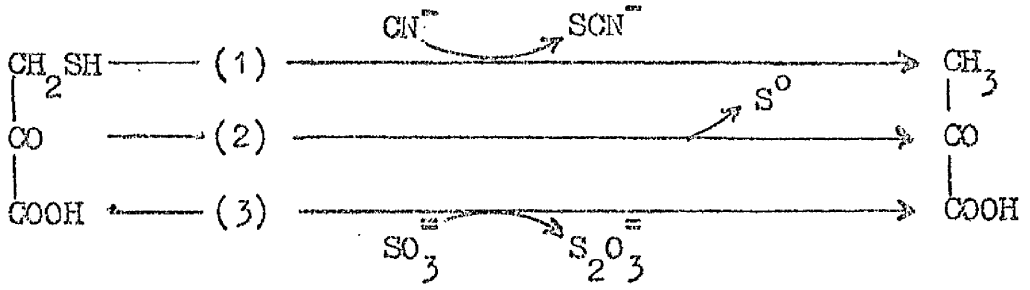
Bénard et al. (1948) showed that rhodanese is present, admittedly in negligible amounts, in erythrocytes, which Goldstein and Rieders suggested were the sole source of the new enzyme.

Since the new enzyme appeared to oxidise thiocyanate to cyanide, Goldstein & Rieders decided that it might properly be named thiocyanate oxidase.

Recently, however, this enzyme has been shown to be merely due to peroxidase activity of haemoglobin (Chung & Wood, 1971) (see also Fig. 1-1.)

3-mercaptopyruvate sulphurtransferase.

Wood & Fiedler (1953) reported that 3-mercaptopyruvate reacts with cyanide to form thiocyanate in the presence of crude extracts of acetone powders of pig liver (reaction 1.). They attributed this activity to rhodanese, but later work showed that it is due to a seperate and specific enzyme (Sorbo,1954;Fiedler & Wood,1956). Miester et al. (1954) showed that rat liver and other tissues convert 3-mercaptopyruvate to pyruvate and elemental sulphur (reaction 2.). Sorbo (1957b) demonstrated the transfer of sulphur from 3-mercaptopyruvate to sulphite or sulphinates by rat tissues to form thiosulphate and thiosulphonates respectively (reaction 3.) These three desulphuration and transsulphuration activities have since been shown to be catalysed by a single enzyme, 3-mercaptopyruvate sulphurtransferase (Kun & Fanshier, 1958,1959a,1959b) and may be represented thus - (Fig. 1-4.).



3-mercaptopyruvate pyruvate

The enzyme is present in many animal tissues and in bacteria and appears to be specific for 3-mercaptopyruvate.

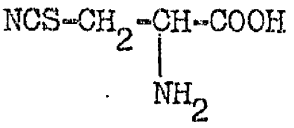
Kun & Fanshier (1959b) and Fanshier & Kun (1962) proposed a mechanism for 3-mercaptopyruvate sulphurtransferase involving the formation of an enzyme-trisulphide or persulphide. The latter reacted either with an acceptor (CN^-, $SO_3^=$), or decomposed to enzyme and elemental sulphur. Sorbo (1957b) had earlier suggested that the formation of a persulphide intermediate and drew attention to the fact that all acceptors for this reaction have strong S-nucleophilic properties. Hylin & Wood (1959) showed that 3-mercaptopyruvate sulphurtransferase reacts with 3-mercapto-pyruvate to form a non-dialysable sulphur-enzyme complex, which can subsequently react with cyanide to form thiocyanate. On the basis of these results, a double displacement mechanism similar to that proposed for rhodanese appears to be indicated for 3-mercapto pyruvate sulphurtransferase, thus -

$$\underset{\text{mercaptopyruvate}}{R\text{--}SH} + E \longrightarrow E\text{-}S + \underset{\text{pyruvate}}{R\text{-}H}$$

$$E\text{-}S + \text{acceptor} \longrightarrow \underset{\text{enzyme}}{S\text{-acceptor} + E}$$

Detoxification of Cyanide with Cystine.

Cyanide may also be detoxified be reacting with cystine to yield cysteine and β-thiocyanoalanine (compound 1.) (Voigtlin et al.,1926). Later workers were able to show that the latter tautomerises to 2-aminothiazolidine-4-carboxylic acid (compound 2.) (Schoberl,Kawohl, & Hamm,1951), or to the equivalent 2-imino-4-thiazolidine carboxylic acid (compound 3.)



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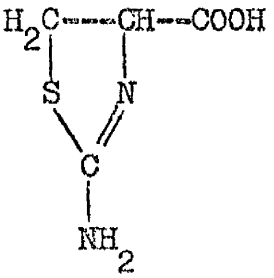
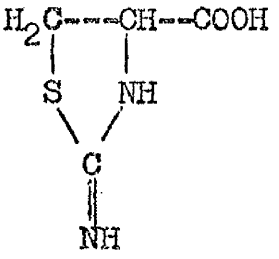


(2.)



(3.)

The chemical properties of this cyanide-cystine reaction product have been studied in great detail by earlier workers (Schoberl & Hamm,1948;Aldridge,1951;Behringer & Zillikens,1951). Later studies on the reaction product supported formulation of the structure as 2-imino-4-thiazolidine carboxylic acid (Wood & Cooley,1956). The compound was found to be metabolically inactive when fed to rats or when injected, but, on treatment with acid, a small amount of thiocyanate was produced. Wood & Cooley were further able to prove that in rats, 80-90 % of injected cyanide

could be isolated in the urine as thiocyanate, and up to 15 % as iminothiazolidine carboxylic acid, supporting earlier work (Voigtlin et a 1926) showing that cystine, injected into the bloodstream of rats, immediately before a sub-cutaneous injection of cyanide, was able to protect the animals from minimal lethal doses of cyanide. When [^{35}S]-labelled cystine was injected into the rat immediately before the cyanide, Wood & Cooley were able to show that the specific activity of the 2-imino-4-thiazolidine carboxylic acid was about 13 times that of the thiocyanate. Twenty three per cent of the label was in the iminothiazolidine and 2 % was in the thiocyanate. Since cystine does not serve as a substrate for rhodanese directly (see Fig. 1-1.), the labelled thiocyanate must have been produced subsequent to the metabolic alteration of the injected cystine. When the sulphur pool was labelled by feeding [^{35}S]-labelled methionine, the most radioactive product of cyanide detoxification was thiocyanate.

Thiocyanate may also be produced from β-thiocyanoalanine formed by the reaction of cyanide and cystine. This compound may undergo deamination to form thiocyanopyruvate, which may then be further degraded to pyruvate and thiocyanate (see Fig. 1-1.).

Detoxification of Cyanide with Vitamin B 12.

The fact that conditions such as menstruation, pregnancy. and lactation, where there is an increased requirement for vitamin B 12, cause an increase in thiocyanate excretion, makes it likely that vitamin B 12 may be involved, directly or indirectly in the formation of thiocyanate in the body. This was confirmed by the finding that dietary deficiency of vitamin B 12 leads to increased thiocyanate excretion (Wokes et al.,1955), and that injections of sub-lethal doses of cyanide caused a significant depletion of the rat liver stores of vitamin B 12, indicating that it might therefore be an important detoxifying agent in cyanide poisoning (Smith, 1961).

Study of the chemistry of vitamin B 12 and its analogues has revealed the presence of cobalt in an organic co-ordination complex, by means of which cyanide can be taken up. Spectrophotometric studies on the effect of light on cyanocobalamin revealed a system thus -

$$\text{Cyanocobalamin} \underset{CN^-}{\overset{\text{light}}{\rightleftharpoons}} \text{Hydroxocobalamin}$$

in which the cyanide is readily taken up and liberated according to the experimental conditions, including pH and exposure to light (Veer et al., 1950;Wokes et al., 1953). The proposal by

Mushett et al. (1952), that vitamin B 12 is an effective antidote to cyanide poisoning in mice, even up to 8 times the normal minimal lethal dose, does assume that the vitamin occurs in the hydroxo- form, which can be replaced by the cyano- form. Indeed, Wokes et al. (1953) have shown that ampoules of cyanocobalamin, purporting to hold 100 mg. of cyanocobalamin, contained varying percentages of the hydroxo- form.

Undoubtedly, some of the vitamin B 12 in the human liver exists as hydroxocobalamin, but, even assuming that all of the vitamin B 12 occurs in this form, the total amount would still be less than 1000 µg. (Drouet et al.,1953), and the amount of cyanide which could be detoxified would be equivalent to about 25 µg. Thus, the detoxification of ingested amounts of cyanide is presumably effected in the liver, mainly by the enzyme rhodanese. It is not coloured, and presumably, does not contain cobalt, which, in vitamin B 12, takes up cyanide. Both rhodanese and vitamin B 12 occur together in many organs and organisms, and their functions are therefore probably closely interrelated.

Wokes & Picard (1955) suggested a hypothetical cycle to explain the interactions of cyanide, rhodanese and vitamin B 12 (see Fig. 1-5.)

In the liver, hydroxocobalamin (CobOH) and rhodanese are assumed to compete for cyanide, and some will be taken up by the hydroxocobalamin, converting it to cyanocobalamin (CobCN). The

latter can then carry out its various metabolic functions, one of which may be the supply of essential one-carbon fragments for the synthesis of labile methyl groups.

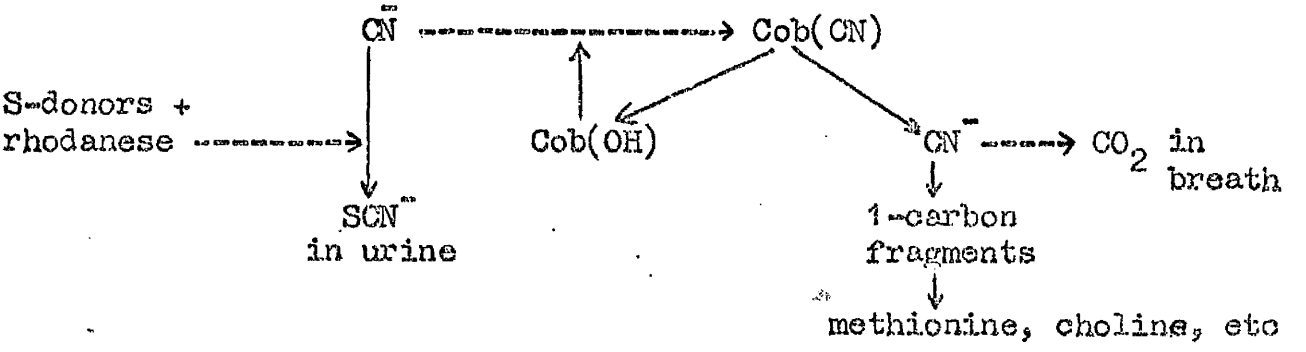


Fig. 1-5. - Scheme of Cyanide Utilisation (after Wokes & Picard, 1955)

The metabolic activity of the carbon of cyanide was reported by Stekol & Weiss (1950), and later by Boxer & Rickards (1952) who reported that when [^{14}C] CN was given to dogs, the carbon could be quickly detected in the exhaled carbon dioxide, in choline, in the ureido carbon of allantoin, as well as in cyanocobalamin. The cycle is completed when some of the cyanide is liberated from the cyanocobalamin, thereby regenerating the hydroxocobalamin. The cyanide in the liver not taken up by the hydroxocobalamin will be converted to thiocyanate by rhodanese, with the help of sulphur donors such as sulphur amino acids, or the products of the metabolism of such amino acids. Koister & Fries (1949) showed that the thiocyanate is mainly excreted in the

urine, with very little in the faeces.

This suggested mechanism could explain the increased thiocyanate excretion observed in subjects suffering form a dietary deficiency of vitamin B 12, who, because of their shortage of the vitamin, would not be able to utilise as much cyanide as in the above cycle, and would therefore have to exorete more cyanide as thiocyanate. The hypothesis put forward by Wokes & Picard also offers a possible explanation of the involvement of vitamin B 12 in the metabolism of certain sulphur amino acids. The cobalamin cycle makes available cyanide radicals for the production of labile methyl groups or other one-carbon fragments, for example, for the conversion of homocysteine to methionine, a function of vitamin B 12 now widely accepted. In vitamin B 12 deficiency, more cyanide would have to be converted to thiocyanate by the rhodanese system, thereby increasing the need for sulphur donors. If methionine itself were the donor, any increase in detoxification by rhodanese would diminish the liver reserves of methionine. Hartman (1949a,b), studying the excretion of thiocyanate in patients with liver disease, was able to show that methionine can act as sulphur donor in the detoxification of cyanide compounds by rhodanese, If, on the other hand, cysteine or cystine were the donor, this would also lead to methionine deficiency, on the assumption that, in the presence of vitamin B 12, both cysteine and cystine can act as precursors of methionine, as has been shown in Neurospora and

other microorganisms. In the system (see Fig. 1-6.), the sulphur of cysteine is transferred to homoserine, forming cystathionine, which undergoes cleavage to homocysteine, the precursor of methionine. The system is a reversal of the normal metabolic pathway from methionine to cysteine, and is believed not to occur in higher animals.

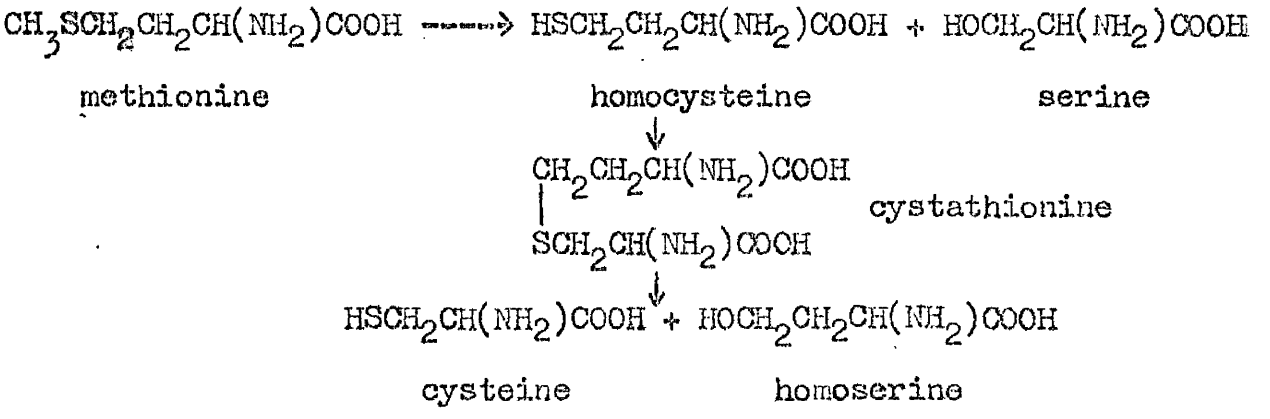


Fig. 1-6. - Mechanism of Sulphur transfer between Methionine & Cystein

Wokes & Picard further observed that the extra amount of thiocyanate sulphur excreted in vitamin B 12 deficiency appeared to be too small to appreciably affect the liver stores of sulphur amino acids. In vitro experiments have shown that the efficiency of these acids as sulphur donors is very low, compared with that of sodium thiosulphate (Himwich & Saunders,1948;Sorbo,1953), but that it still remains uncertain whether naturally occurring substances other than these amino acids, or related substances such as

glutathione can serve as sulphur donors. In addition to the findings of Hartman (1949a,b) that methionine or cysteine are able to act as sulphur donors, work on sheep by Blakely & Coop (1949) has shown that cystine as well as thiosulphate can act as a sulphur donor in the conversion of cyanide to thiocyanate by rhodanese. It may be that the sulphur amino acids do not act directly, but only after oxidation to one of the intermediate stages in their metabolism to sulphite, sulphate, and thiosulphate (see Fig. 1-1.). This low efficiency of the sulphur amino acids led Wokes & Picard to suggest that the rhodanese system in the body may require a large excess of these in order to function at an effective rate.

The observation by Boxer & Rickards (1952) that cyanide can be shown to contribute only a minor fraction of the required one-carbon metabolites, led them to put forward an alternative hypothesis to deal with the quantitative aspects of the study.

Boxer & Rickards showed that body tissues contain much more thiocyanate than cyanide, and, from the results of in vitro experiments, they suggested that, in the presence of this large excess, hydroxocobalamin may take up thiocyanate to form thiocyanatocobalamin (CobSCN)(see Fig. 1-7.). This form of vitamin B 12 has been found to be as effective as cyano- and hydroxocobalamin in the therapy of pernicious anaemia and in growth promotion of various microorganisms used to measure vitamin B 12 activity (Buhs et al.,1951)

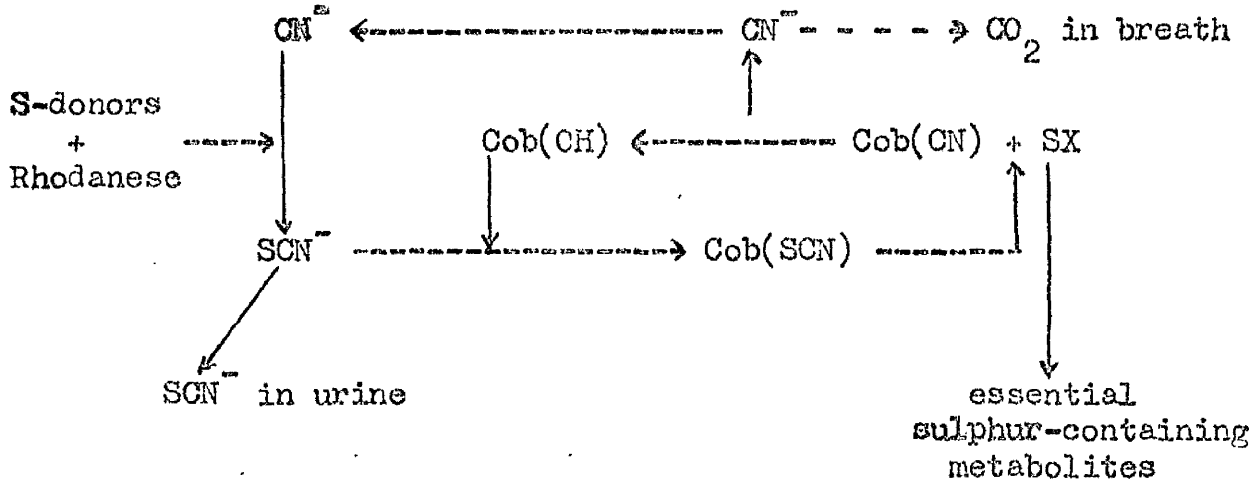


Fig. 1-7. - Hypothetical Sulphur Transfer Cycle (after Wokes & Picard)

They suggested that thiocyanatocobalamin under physiological conditions reverts to cyanocobalamin, giving up its sulphur to an active intermediate, "X", to form "SX", which could be a precursor of biologically important sulphur-containing compounds, such as sulphur amino acids, and glutathione. The cyanocobalamin then completes the cycle by regenerating hydroxocobalamin, and the cyanide thus liberated will be converted by rhodanese to thiocyanate and again be available for the cycle. If, as they suggest, this mechanism operates in the formation of methionine, it may involve a reaction between homoserine and "SX" to give homocysteine which methylated to methionine. This mechanism is analogous to that in Neurospora (see Fig. 1-6.), with "SX" taking the place of cysteine in supplying the sulphur to homoserine. Alternatively, if methionine were the sulphur donor, this mechanism would demonstrate how

vitamin B 12 may catalyse the normal transulphuration reaction, methionine -> homocysteine -> cystathionine -> cysteine.

The cycle suggests a way in which sulphur may be made available by vitamin B 12 for the synthesis of sulphur-containing amino acids and related compounds. It also shows, as already pointed out, that if cysteine or cystine were the sulphur donors, how sulphur may be transferred from these donors via thiocyanate to homocysteine and hence to methionine.

Thus, the findings of Wokes & Picard support the existence of a cycle in which minute quantities of cyanide, in conjunction with vitamin B 12, can act as carriers of sulphur in relatively large amounts. The proposed cycle is not put forward as an alternative to mechanisms involving the synthesis and utilisation of labile methyl groups and hence, among other things, the last stage of methionine synthesis, viz. homocysteine -> methionine. There is much evidence which strongly suggests that this is one of the functions of vitamin B 12 (Kratzer,1953;Stekol et al.,1953;Smith, 1954). Closely related to this, is the evidence that vitamin B 12 is involved in the conversion of glycine to serine which is important as a precursor of cysteine, and hence of glutathione (Arnstein & Neuberger,1953).

Other workers have shown that vitamin B 12 is involved in the reduction of disulphide linkages to, and the maintenance of, sulphydryl groups, for example in homocysteine and glutathione,

both in vitro and in vivo (Dubnoff,1950a,b,1951;Ling & Chow,1953).

Thus, vitamin B 12 may play an important part in the general metabolism of sulphur, and as a result, be involved in the detoxification of cyanide.

Reaction of Cyanide with Protein.

It is well known that cyanide can react with protein in vitro (Pascheles,1894). Following up this work, Catsimpoolas & Wood (1964) studied the reaction of cyanide with bovine serum albumin (BSA) to assess the factors affecting the release of thiocyanate from protein. They showed that the reaction yields thiocyanate, only under alkaline conditions. The formation of thiocyanate may arise from the decomposition of the thiocyanoalanine moiety formed from cleavage of cystine disulphide bonds with cyanide, and also by cyanolysis of the persulphide resulting from the alkaline degradation of the protein. The persulphide is probably a trisulphide which is formed from the initial degradation product. The increases in thiocyanate yield with pH, suggests that all disulphide bonds in the intact protein are not readily accessible to attack by cyanide. Cyanide produced 4 to 5 moles of thiocyanate per mole of BSA at pH 8.0, 13 to 14 at pH 10.0, and 18 at pH 12.0. At pH 7.0, where no alkaline degradation effect is exerted, only 4 bonds are broken in the native protein, no thiocyanate is formed, but thiazolidine groups are formed instead of thiocyanoalanine. They showed from model studies, that formation of the iminothiazolidine ring is often accompanied by cleavage of an acyl group from cystine derivatives. Similarly, formation of the iminothiazolidine ring on proteins caused cleavage of a peptide bond at the cystine amino group.

Tobacco Smoke as a Possible Source of Cyanide.

There have been reports suggesting that the measurement of thiocyanate in body fluids might be used as an index of a physiologically significant exposure to cigarette smoke. Smokers exhibited higher thiocyanate levels in body fluids than did non-smokers.(Lawton et al.,1943;Trasoff & Schneeberg,1944;Maliszewski & Bass,1955;Stoa,1957;Wilson & Matthews,1966). This is thought to be the result of detoxification of cyanide entering the body via inhaled tobacco smoke, which is known to contain cyanide in significant concentrations (Osborne et al.,1956;Johnstone & Plimmer, 1959; U.S. Surgeon General's report,1964). The number of cigarettes bought or used may not be perfectly correlated with actual exposure to the combustion products, since individuals vary greatly in their habits of usage, such as length of cigarette smoked, or depth of inhalation.

Maliszewski & Bass (1955) studied the levels of thiocyanate in plasma, saliva, urine and sweat of smokers and non-smokers. They showed that the higher value of thiocyanate in smokers (3-6 fold) is probably associated with smoking directly, as cessation of smoking was accompanied by a gradual but marked decrease in the level of thiocyanate in all body fluids. The plasma thiocyanate concentration fell to about 39 % of the levels for smoking controls by the tenth day, salivary levels dropped to approximately 50 % of

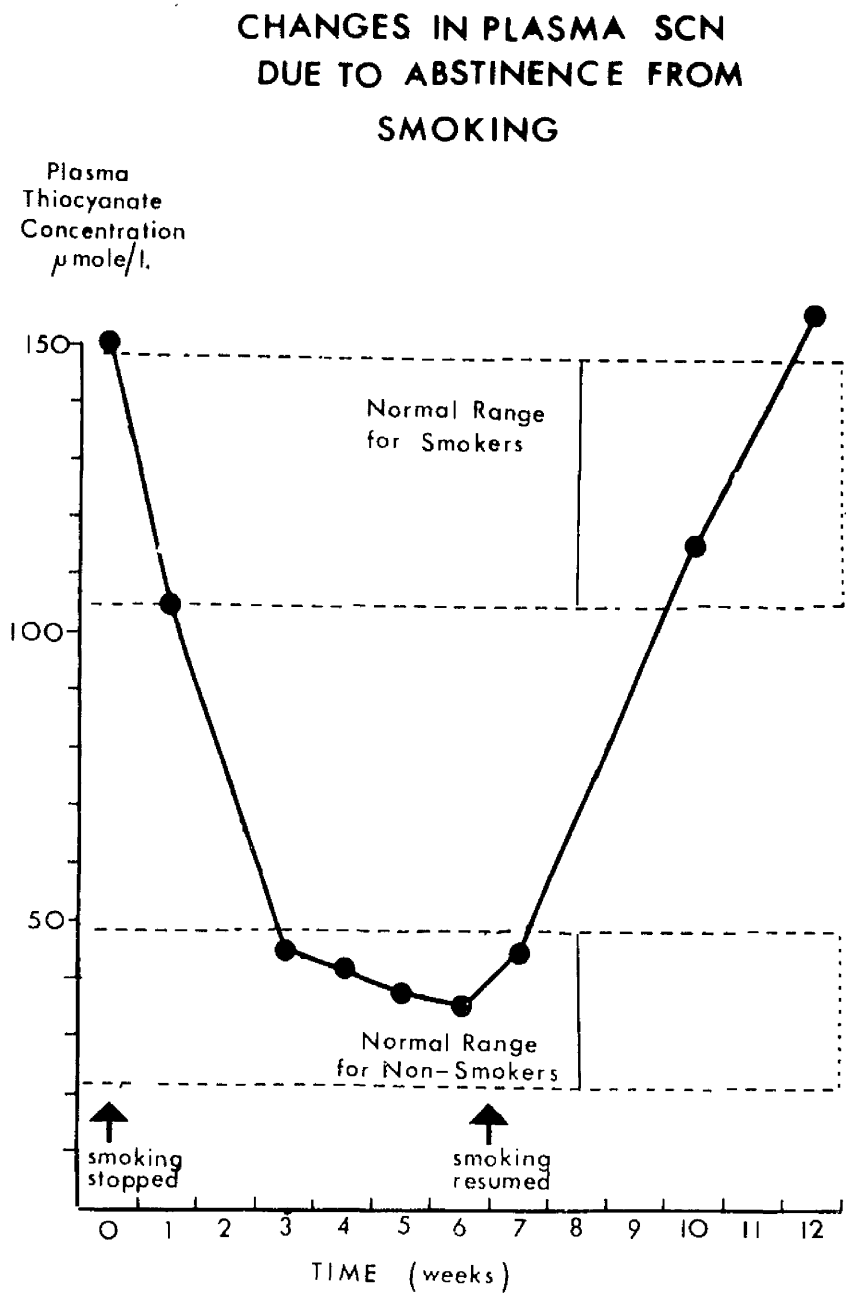


Fig. 1-8. Changes in Plasma Thiocyanate Levels in a Normal Subject due to Abstinence followed by Resumption of Cigarette Smoking.

of controls, and urinary excretion dropped more than 50 %. The recovery, after smoking was resumed, was found to occur at a slower rate than the fall during non-smoking.

The work of Maliszewski & Bass was studied more closely in this work, when a volunteer subject stopped smoking, and aggreed to weekly blood samples being taken off for thiocyanate estimation. Fig. 1-8. shows the normal ranges of thiocyanate in plasma for both normal smokers and normal non-smokers, and follows the changes in plasma thiocyanate in the normal subject who stopped smoking for 7 weeks, and then resumed. The level of thiocyanate fell rapidly after cessation of smoking, thenequilibrated within the normal range for non-smokers. After resumption of smoking, the value of thiocyanate rose at a slower rate than that of the fall during abstinence, aggreeing with the findings of Maliszewski & Bass.

As a control, thiocyanate estimations were also carried out on plasma from other subjects, both smokers and non-smokers. The results were found to aggree with the accepted values of plasma thiocyanate for smokers and non-smokers.

CLINICAL ASPECTS OF THE STUDIES.

This work has been directed towards providing a biochemical rationale for any clinical changes occurring during the treatment of patients suffering from the toxic amblyopias, particularly tobacco amblyopia and Leber's hereditary optic atrophy. The optic neuropathy of diabetes, the rarer optic neuropathy of pernicious anaemia, and the various nutritional optic atrophies, although not mentioned in any great detail here, also belong to this group of conditions.

Many workers have suggested that cyanide is important in these conditions, coming from tobacco smoke, or certain foods, e.g. tropical pulses (Clark,1936;Montgomery,1964), or may be the result of infection by certain cyanide-producing organisms (Smith, 1964). These conditions are associated with a probable failure by the patient to detoxify the cyanide load to which they find themselves subjected. This failure may result from an inherited defect in the process necessary for the metabolism of cyanide, as has been suggested for Leber's hereditary optic atrophy, or from an absolute depletion of body vitamin B 12, as in pernicious anaemia, nutritional deficiency and malabsorption states.

Tobacco Amblyopia.

Tobacco has been associated with amblyopia since MacKenzie's observation in 1854, that abstention from tobacco

allowed visual improvement. Although cyanide has long been recognised as a constituent of tobacco smoke (Lehmann & Gunderman,1912), Leishman (1951) was one of the first to suggest that the agent responsible for tobacco amblyopia may be other than nicotine.

The cyanide in tobacco smoke has been suggested as a cause of the visual failure in tobacco amblyopia and in Leber's hereditary optic atrophy (Wokes,1958;Heaton et al.,1958;Smith,1961;Wilson,1963, 1965). It was Wokes who focussed attention on cyanide, and so initiated research into the complexities of cyanide and vitamin B 12 interrelationships in these conditions.

As mentioned earlier, smokers show higher plasma thiocyanate levels than do non-smokers, even as high as 3-6 fold. The mean plasma thiocyanate concentration of 20 non-amblyopic smokers was found in this laboratory to be 65 $\pm$ 33.0 µmole/l. (Chisholm & Pettigrew,1970). This was significantly higher than the mean concentration of 32 $\pm$ 16.0 µmole/l. found in 18 non-smokers ($P < 0.001$). The mean plasma thiocyanate concentration in 26 untreated tobacco amblyopia patients was found to be 28 $\pm$ 12.0 µmole/l., which is significantly lower than the concentration found in the non-amblyopic smokers ($P < 0.001$), but was no different from the concentration found in the non-smokers.($P > 0.01$). This was surprising, since the tobacco amblyopia patients in general, smoked more heavily than did the non-amblyopic smokers, and would therefore be expected to exhibit higher plasma thiocyanate

levels. This would suggest that the tobacco amblyopia patients have a reduced ability to convert cyanide to thiocyanate, and must, therefore, have increased amounts of detectable cyanide free in the body fluids. Wilson & Matthews (1966) were able to show a negative relationship between plasma cyanide and total serum vitamin B 12. Other workers have shown reduced concentrations of vitamin B 12 in the serum of tobacco amblyopia patients (Heaton et al.,1958;Foulds et al.,1969a), which would suggest that there may be raised levels of cyanide in the plasma of such patients. The mean concentration of cyanide in 15 untreated tobacco amblyopia patients of 0.61 µmole/l., as found by Chisholm & Pettigrew (1970), was higher than the concentration of 0.22 µmole/l. found by Wilson & Matthews (1966) in healthy smokers. The now apparent difficulties in estimation of cyanide in plasma at the low levels encountered, must make meaningful interpretation of these results extremely difficult. Comparison of these plasma cyanide results with total serum vitamin B 12 did, however, show a negative relationship, which supported the premise that elevated concentrations of cyanide in the presence of reduced serum vitamin B 12 levels may lead to neurological changes (Smith,Duckett, & Waters,1963; Wilson & Matthews,1966).

Only a very small proportion of all smokers contract the disease. Cigars and cigarettes, especially the latter, are only rarely causally associated, the smoking of strong tobacco in pipes

being the typical finding. The reason for the preponderance of pipe smokers is not certain. It has been suggested that one factor may be that the increase in the proportion of pipe smokers that occurs in middle age, together with the known decrease in serum vitamin B 12 levels, makes the development of amblyopia more likely in elderly, pipe-smoking subjects. It may also be that pipe smoking, unlike that of cigarettes, causes increased salivation, and the habitual "sucking" of the pipe, thereby allows the ingestion of additional, more concentrated toxins from the mouthpiece via the swallowed saliva. It has been shown that the amount of tobacco consumed by those who develop amblyopia varies considerably, but the usual history is of heavy smoking over a

Leber's Hereditary Optic Atrophy.

Leber's Hereditary Optic Atrophy.

Close comparisons have been drawn between tobacco amblyopia and Leber's hereditary optic atrophy, both in its clinical features, and in its mode of presentation (Schepens,1946;Foulds, 1969). Wilson (1963,65) suggested that the disease might be caused by an inherited defect in the mechanism necessary for the detoxification of cyanide.

It is a fact that many of the patients developing the symptoms of Leber's optic atrophy do smoke, and a lower than expected thiocyanate level in the plasma of such patients has been demonstrated (Wilson,1965). The mean plasma thiocyanate conc-

centration of 38 ± 32 µmole/l., found in 8 Leber's optic atrophy patients who smoked (Chisholm & Pettigrew,1970) was no different from the concentration found in untreated tobacco amblyopia patients, but tended to be lower than that of the normal smokers. The results from the smokers in particular, do suggest that these patients reveal a reduced ability to convert cyanide to thiocyanate. There are, of course, other sources of cyanide than tobacco smoke, which might account for the fact that this disease can occur in non-smoking subjects.

Optic Neuropathy of Pernicious Anaemia.

The defect in vision which accompanies the optic neuropathy of pernicious anaemia is similar to that found in tobacco amblyopia, and, it has been suggested that the condition is identical to tobacco amblyopia (Freeman & Heaton,1961). The original diagnosis depends upon whether the patient presents to an ophthalmologist or to a haematologist. Foulds et al. (1969b) have stated that of a group of 63 patients with tobacco amblyopia, 11 were found to have pernicious anaemia. Two of these patients were diagnosed initially as pernicious anaemia and the ocular defect associated at a later date, while the other patients were found to have pernicious anaemia during their treatment for tobacco amblyopia. The optic neuropathy of pernicious anaemia has also been reported in non-smoking subjects (Adams et al.,1967).

Optic Neuropathy of Diabetes.

The optic neuropathy of diabetes as such, is rare, but, as Foulds et al. (1969b) suggested, it may be a commoner cause of visual failure in patients suffering from diabetes than is generally realised. The visual defect in such patients therefore, may not be peculiarly associated with diabetes, but, in fact, may merely be due to tobacco amblyopia.

Nutritional Optic Atrophy.

Interest in thiocyanate has been further stimulated by the finding in patients suffering from a chronic degenerative neuropathy (nutritional ataxic neuropathy), which is common in parts of Nigeria, of an increased concentration of thiocyanate in plasma and in urine (Osuntokun,1968;Osuntokun et al.,1968). An acute retrobulbar neuritis which is probably a manifestation of this same condition in children, is accompanied by similar changes in the levels of thiocyanate. Both these diseases are associated with the long-standing consumption of large amounts of cassava (manioc tuber), which contains large amounts of the cyanogenic glycoside, linamarin. Hospitalisation of these patients, with the removal of cassava from their diet, is accompanied by a drop in the plasma thiocyanate concentration from the high value of 85 µmole/l. towards normal levels, but this trend is reversed when a return is made to the normal diet. Osuntokun et al. (1968) have shown that this high level of thiocyanate in plasma is associated with a very low level

of cysteine + cystine in the plasma, and they suggested that this was due to the utilisation of these sulphur amino acids for the detoxification of cyanide derived from the cassava, Such a detoxification could take place through the formation of 2-imino-4-thiazolidine carboxylic acid from cystine (see Fig. 1-3.). Osuntokun et al. (1968) have therefore proposed to use the sulphur amino acids in the therapy of these neurological conditions which could be the result of chronic intoxication with cyanide derived from the cassava.

However, it must be stressed that the daily intake of cyanide by these patients, in particular, is in the order of 8 mg. (0.3 m.mole) per day, so that the amount of cyst(e)ine required for detoxification must be negligible compared with the amounts oxidised daily to produce the 25 m.moles of sulphate excreted every day. It is therefore hard to believe that the daily intake of 0.3 m.mole of cyanide would cause a significant drop in the level of cyst(e)ine in the plasma, unless a severe protein deficiency were also present.

Methods of Treatment.

As stated earlier, the major metabolite of cyanide is thiocyanate, formed by combination with sulphur within the body. The treatment of these conditions, which may be associated with chronic cyanide toxicity, must therefore involve increasing the patients' ability to detoxify cyanide, by providing an increased source of sulphur. This has been accomplished in two ways - by therapy with hydroxocobalamin, or by administration of oral cystine.

1. Hydroxocobalamin Therapy.

The use of hydroxocobalamin as an effective antidote for cyanide poisoning, is well known from the work of Mushett et al. (1952), on cyanide poisoning in mice. The hypothetical cycles of cyanide utilisation and sulphur transfer (see Figs. 1-5. & 1-7.), as suggested by Wokes & Picard (1955), do indicate the possible role of hydroxocobalamin in the therapy of cyanide intoxication. Previously the vitamin B 12 has been administered intramuscularly with considerable success, but now, in addittion, an oral preparation of hydroxocobalamin is being used as a possible alternative. Results from both forms of therapy are reported in these studies.

2. Oral Cystine therapy.

The use of oral preparations of cystine as a source of additional sulphur is reasonable, in view of the results of the experiments of Voiatlin et al. (1926), showing the protective action of injected cystine against minimal lethal doses of cyanide. The

work of Osuntokun et al. (1968) also suggests the use of sulphur amino acids, such as cystine, in the therapy of the neurological conditions, believed to be due to chronic cyanide intoxication as a result of a high consumption of cassava.

The Aims of The Studies.

If cyanide is to be considered an important cause of the symptoms found in these conditions, the treatment must be concerned with increasing the patients' ability to detoxify cyanide. This would cause increased levels of thiocyanate in plasma and in urine. The measurement of these two parameters in the patients in these studies, thereby provides a convenient means of assessing the treatment, with regard to their detoxification of cyanide. If these changes in thiocyanate can be related to changes in the clinical condition of the patient, with particular regard to improvement in their vision, it would provide good evidence for the suggested importance of cyanide in these conditions.

SECTION TWO.

MATERIALS AND METHODS.

THIOCYANATE AND CYANIDE ESTIMATIONS.

The standard colorimetric methods for the estimation of thiocyanate and cyanide are based on either the method developed by Aldridge (1944,45), or on that developed by Epstein (1947), which are themselves both based on the Konig synthesis of pyridine dyestuffs (Konig,1904,05), by the reaction of pyridine and an aromatic amine. Aldridge's method involves reacting cyanogen bromide with a pyridine -benzidine mixture, whereas Epstein uses cyanogen chloride reacting with a mixture of pyridine and 1-phenyl-3-methyl-5-pyrazalone with a small quantity of bis(1-phenyl-3-methyl-5-pyrazalone).

The method described here is a modification of the Aldridge method, with the carcinogenic amine, benzidine replaced by p-phenylene diamine, as suggested by Bark & Higson (1964).

Since both cyanide and thiocyanate are converted to cyanogen bromide, this method can be used to estimate both. The colorimetric reaction does not distinguish cyanide from thiocyanate, and so the minute amounts of cyanide in plasma require to be separated and concentrated for estimation. This was previously done by the method of Boxer & Rickards (1950), by aeration into a small volume (1 ml.)

of 0.2 N NaOH, using a stream of cyanide-free nitrogen. The cyanide content of this concentrated sample was then determined colorimetrically. Difficulties in aeration and concentration led to the investigation of microdiffusion methods as a possible alternative. It has been found that the method of Feldstein & Klendshoj (1954a,1954b), using Conway No. I units, although requiring a longer time, is more efficient to use for the concentration of cyanide from plasma.

REACTION

$$HCN + Br_2 \longrightarrow CNBr + HBr$$

$$KSCN + 4Br_2 + 4H_2O \longrightarrow CNBr + 6HBr + 6H_2SO_4 + KBr$$

$$CNBr + \text{pyridine/p-phenylene diamine reagent} \longrightarrow \text{pyridine dyestuff}$$

REAGENTS

Bromine water - saturated

Arsenious oxide - 2% W/V As_2O_3 in weak NaOH titrated to neutral

p-phenylene diamine - 0.2 % W/V in 0.5 M HCl

Pyridine reagent - pyridine : conc. HCl : de-ionised water

6 : 1 : 4 V/V

Pyridine/p-phenylene diamine reagent - pyridine : p-phenylene diamine

3 : 1 V/V

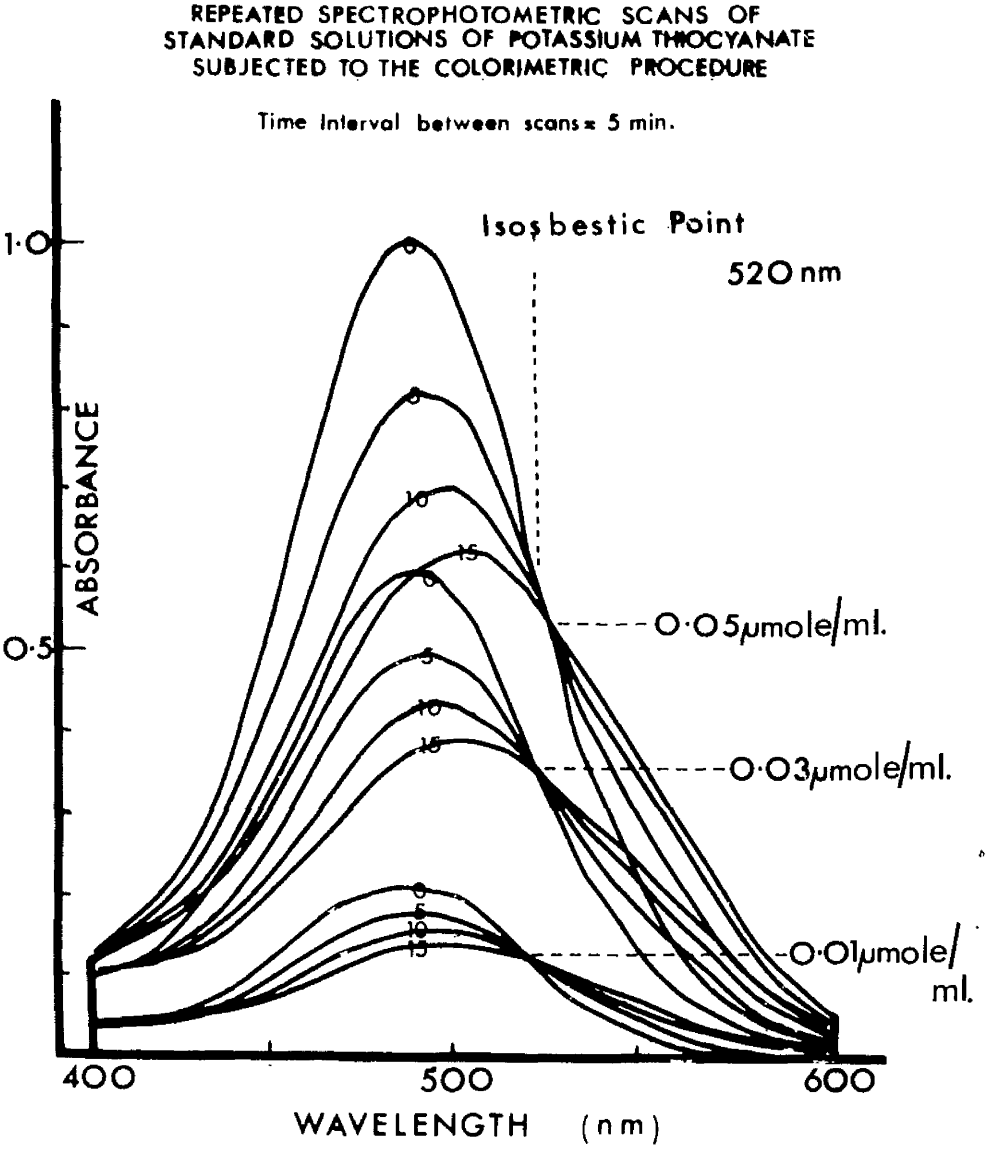


Fig. 2-1. Determination of the Isosbestic Point for the Colorimetric Procedure.

Procedure.

To 1 ml. of test solution made acid by the addition of 0.5 ml. of N HCl, add 2 drops of saturated bromine water, followed, after mixing, by 3 drops of 2 % arsenious oxide solution to remove any excess bromine present. Any further bromine is then removed under vacuum. To this is added 1.8 ml. of the pyridine/pephenylene diamine mixture, and the resultant reddish-pink colour read against a reagent blank, within 10 minutes at the isosbestic point for this particular colour reaction. The wavelength of this isosbestic point was found to vary between 505 nm. and 520 nm., apparently being dependent upon the freshness of the reagents, particularly the pyridine. The isosbestic point has therefore to be determined for each new batch of reagents, by examining the colour reaction in a recording spectrophotometer. From such observations, the isosbestic point is generally found at 520 nm., and all subsequent absorbance readings have been made at this wavelength (see Fig. 2-1.).

Standard curves for cyanide and thiocyanate are constructed similarly, by subjecting 1 ml. aliquots of varying concentrations of standard solutions of potassium cyanide and potassium thiocyanate to the above colorimetric procedure.

The specificity of this colour reaction, and its advantages over the previous colorimetric procedure, can be demonstrated by comparison of the results obtained from two series of recovery experiments, where varying concentrations of potassium thiocyanate

or cyanide were added to plasma and to urine (see Table 2-1.).

A system of quality control, using aliquots of pools of deep-frozen plasmas was introduced for thiocyanate, to maintain a check on the precision of the estimation procedure; an aliquot of the deep-frozen plasma being estimated along with each batch of thiocyanate estimations.

The improvement in the precision, brought about of the new colorimetric procedure can be seen in Table 2-2.

Table 2-1. - Recovery of Added Thiocyanate or Cyanide.

1. - THIOCYANATE

(a) Previous pyridine/benzidine method.

Plasma	μ.moles of KSCN		
Added	0.005	0.010	0.020
Recovery %	75.3	88.2	88.6
S.E.M.	3.40	1.40	3.70
No. of samples	6	5	6

(b) Present pyridine/p-phenylene diamine method.

Plasma	μ.moles of KSCN			
Added	0.005	0.0125	0.025	0.050
Recovery %	96.4	99.7	100.1	99.9
S.E.M.	0.88	2.15	1.46	1.20
No. of samples	8	8	11	14

Urine	μ.moles of KSCN		
Added	0.025	0.050	0.100
Recovery %	88.4	88.2	99.2
S.E.M.	2.54	2.72	1.17
No. of samples	5	6	6

2. - CYANIDE

(a) Previous aeration method

Plasma	μ.moles of KCN			
Added	0.010	0.020	0.030	0.050
Recovery %	47.5	70.5	59.0	69.3
S.E.M.	9.10	5.50	4.50	9.10
No. of samples	8	6	8	8

(b) Present Conway microdiffusion method.

Plasma	μ.moles of KCN	
Added	0.010	0.020
Recovery %	82.7	78.4
S.E.M.	4.40	3.40
No. of samples	6	6

Table 2-2. - Comparison of Quality Control Results for Thiocyanate

(a) Previous method				
Mean SCN concn. (μ.moles/l.)	26.9	35.0	33.2	
S.E.M.	3.0	0.73	1.26	
No. of aliquots taken from pool	10	45	20	
(b) New method				
Mean SCN concn. (μ.moles/l.)	56.0	56.3	36.0	36.2
S.E.M.	0.34	0.49	0.29	0.41
No. of aliquots taken from the pool	40	24	45	30

APPLICATION OF THE METHOD TO BIOLOGICAL MATERIALS.

The method as described here, can be easily adapted for the estimation of thiocyanate and cyanide in such materials as blood, urine, pancreatic juice and other protein-containing fluids, after suitable de-proteinisation.

1. - Thiocyanate. - (a) - in plasma - 1 ml. of heparinised plasma is de-proteinised by the addition of 9 ml. of 10 % trichloroacetic acid. 1 ml. aliquots of the clear protein-free supernatant after centrifugation are then analysed for thiocyanate and cyanide as described above.

(b) - in urine - An aliquot of a 24-hour collection of urine is diluted 1 to 10 with de-ionised water, and 1 ml. aliquots of this dilution are analysed for thiocyanate as before.

2.- Cyanide - (a) - in plasma - The minute quantities of cyanide present in plasma require to be separated and concentrated for estimation. This was previously done by the aeration method of Boxer & Rickards (1950), but it has now been found to be more efficiently carried out by the microdiffusion method of Feldstein & Klendshoj (1954a,b), as described by Conway (1962). After two hours incubation, 1 ml. portions of the alkaline solution in the centre well of the Conway No. I units are subjected to the colorimetric estimation procedure as described before. A standard curve for cyanide may be similarly prepared by placing 2 ml. aliquots of a standard solution of potassium cyanide in the outer chamber, instead of the plasma.

Although, apparently much improved by the use of the microdiffusion technique, the estimation of cyanide, per se, in plasma, is still not completely satisfactory. Difficulties in the recovery of cyanide added to plasma, led to the investigation into the apparent disappearance of cyanide added to plasma in vitro. A known concentration of cyanide was added to plasma, and portions of this mixture were removed at intervals and estimated for cyanide. A control experiment, where the cyanide was added to normal saline, instead of plasma, was also undertaken.

The rate of disappearance of cyanide added to plasma was found to be very rapid, falling to approximately 50 % of the original concentration after only twenty minutes. In saline, however, the loss was almost negligible, the concentration

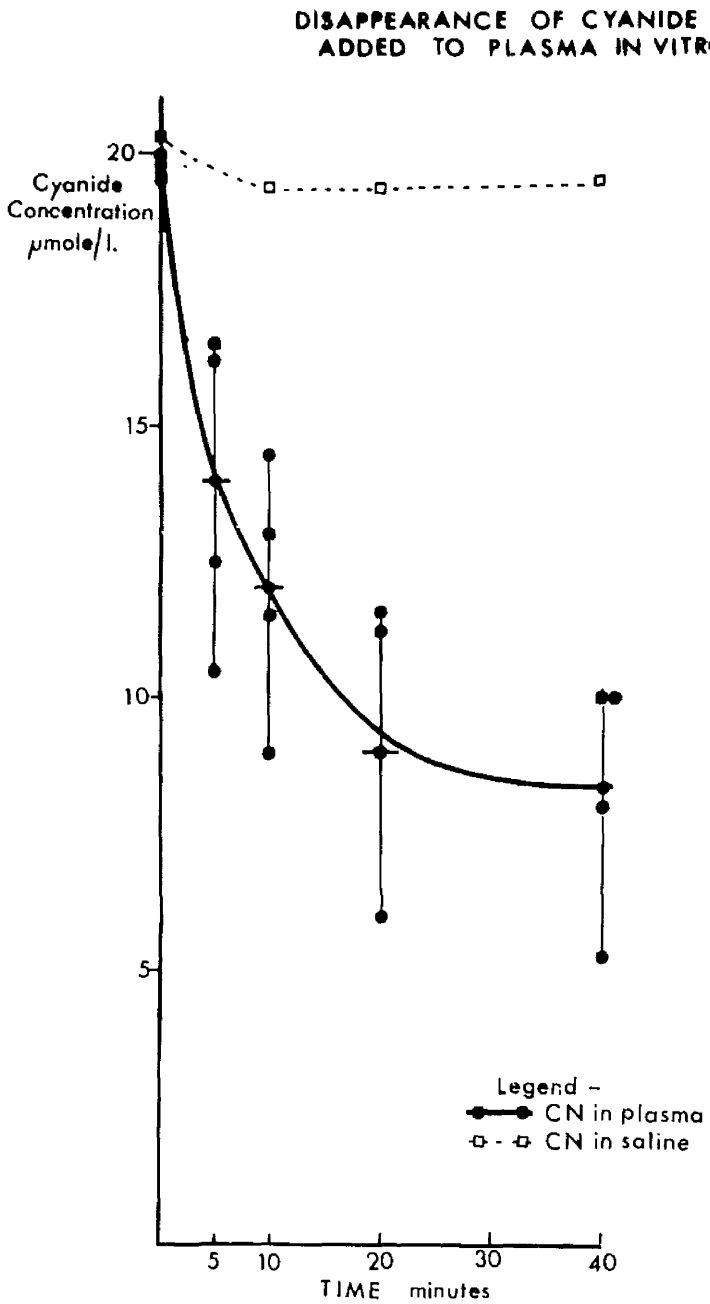


Fig. 2-2. Disappearance of Cyanide added to Plasma in vitro

(represents results from 4 experiments)

dropping only very slightly, even after as long as forty minutes (see Fig. 2-2).

The most likely explanation would be that protein, possibly albumin, or, more particularly, some sulphur-containing grouping (e.g. cystine disulphide bonds) had reacted with cyanide, as shown from the work of Pascheles (1894) and of Catsimpoolas & Wood (1964).

This apparent rapid disappearance of cyanide added to plasma in vitro, must raise doubts as to the validity of the estimation of cyanide in plasma, at the normally low levels encountered. These technical difficulties in estimation, therefore, make meaningful interpretation of plasma results very difficult. Accordingly, more emphasis should be placed upon the measurement of thiocyanate in plasma and urine, as a guide to cyanide detoxification.

THE ESTIMATION OF CYSTINE IN URINE.

Cystine in the urine is usually estimated quantitatively by the use of nitroprusside (Brand et al.,1930;Fischl et al., 1961), phosphotungstic acid (Folin & Looney,1922;Folin & Morenzi, 1929;Shinohara & Padis,1935), iodometric titration (Virtue & Lewis,1936), or by the Sullivan colorimetric reaction (Sullivan, 1924;Sullivan & Hess,1936,37). These methods however, have the disadvantage of being non-specific for cystine in the presence of other urine components. The Sullivan reaction using sodium β-naphthoquinone-4-sulphonate, with a modification by Csonka et al. (1944), was found to be adequate for the quantitative testing for cystine in the urine.

The method described here is a modification by McDonald & Fellers (1968), of the basic Sullivan reaction, using the further improvements suggested by Curnow & Lynch (1969).

REAGENTS

Sodium Hydroxide - 5.0 N - 100 gm. NaOH dissolved in H_2O to a final volume of 500 ml.

Sodium Hydroxide - 0.5 N - Dilute 10 ml. of 5.0 N NaOH to a final volume of 100 ml.

Sodium Cyanide - 1.0 M in 0.5 N NaOH - 5.0 gm. NaCN dissolved in 0.5 N NaOH to 100 ml.

Naphthoquinone - 0.2 % W/V - 0.2 gm. sodium β-naphthoquinone in water to a final volume of 100ml

Sodium Sulphite - 0.8 M - 20 gm. sodium sulphite dissolved

in 0.5 N NaOH to a final volume of 200 ml.

Sodium Dithionite - 0.115 N in 0.5 N NaOH - 1.0 gm. of $Na_2S_2O_4$ dissolved in 0.5 N NaOH to a final volume of 50 ml.

SAMPLE PREPARATION

A 25 ml. aliquot of a 24-hour volume of urine is made strongly alkaline by the addition of 0.3 gm. of NaOH pellets. (A single NaOH pellet weighs approximately 0.1 gm.). The final pH must be greater than 10.0. After the NaOH has completely dissolved and mixed, the solution is allowed to stand for 30 mins. Excess phosphates will be precipitated out and can be removed by filtration. 10 ml. of the solution is diluted to 50 ml. with water for the estimation.

STANDARD SOLUTION

A control non-cystine-containing urine sample of 100 ml. with an approximate specific gravity of 1.02 is similarly prepared by the addition of 1.0 gm. of NaOH and filtration; this is solution A.

The standard cystine solution is prepared by the addition of 50 mg. of L-cystine to 50 ml. of urine (solution A). This makes a solution with a concentration of 1000 mg./L of cystine; this is solution B.

ASSAY PROCEDURE

Working cystine standards are prepared as follows; 20 ml. of solution A are diluted to 100 ml.; 10 ml. of solution B are diluted to 50 ml. These 1 to 5 dilutions are made in de-ionised

water. The volumes used for the dilutions for the working standards are as shown in Table 2-3.

Test urines - 10 ml. in duplicate of the diluted test urine are transferred to individual flasks for the estimation.

Table 2-3. - Volumes used for the working standards.

Cystine concentration (mg./L)	Diluted solution A (ml.)	Diluted solution B (ml.)
0	10	0
100	9	1
200	8	2
300	7	3
400	6	4
500	5	5

REACTION

To each flask, add 2.0 ml. of 5 % NaCN. Mix thoroughly and allows to stand for 10 min.

Add 1.0 ml. of freshly prepared 0.2 % naphthoquinone reagent, followed by the addition of 5.0 ml. of freshly prepared 10 % $Na_2S_2O_4$. The immediate sequential addition of these two reagents is necessary. Mix thoroughly. Allow to stand for 25 mins. Varying colours may be noted at this stage.

Add 2.0 ml. of 5.0 N NaOH followed immediately by the

addition of 1.0 ml. of freshly prepared 2 % $Na_2S_2O_4$. Mix thoroughly. The final colour is salmon pink.

Read at 505 nm. within 5 min. with the diluted blank urine set at 100 % transmission.

THE FLUOROMETRIC ASSAY OF GLUTATHIONE IN RED BLOOD CELLS.

The sulphur-containing peptide, glutathione, is a well-known and important source of non-protein sulphydryl (SH) groups. The assay procedure is based on a modification by Fell & Tilstone (1969) of the method described by Cohn & Lyle (1966), which itself is a modification of the reaction procedure with o-phthalaldehyde reported by Shore, Burkhalter & Cohn (1966). At pH 8.0, reduced glutathione (GSH) condenses with o-phthalaldehyde to form a stable and extremely specific fluorescent product, with an excitation wavelength of 365 nm. and a fluorescence emission at 420 nm.

REAGENTS

Glutathione - Boehringer

Metaphosphoric acid - 3 % W/V in mM EDTA

Tris buffer - 0.2 M

o-phthalaldehyde - 1.0 % W/V in methanol (AR) prepared each day.

Because of the variable quality of metaphosphoric acid, it is necessary to titrate aliquots of the 3 % solution in EDTA with the tris buffer in order to calculate the amount of buffer necessary to bring the mixture to pH 8.0 $\pm$ 0.1.

A Perkin-Elmer Model 203 fluorescence spectrophotometer was used for all the fluorometric determinations.

STANDARD SOLUTIONS

Stock standard - 100 μg/ml. in de-ionised water.

Working standards - 10, 5.0, 2.5, 0.625 µg/ml. GSH were prepared daily from the stock solution by dilution with metaphosphoric acid.

CONDENSATION REACTION

The calculated amount of Tris buffer is added to an aliquot (0.5 or 1.0 ml.) of the standard GSH solution to give a final pH of 8.0 ± 0.1, the volume made up to 4.0 ml. with water and 0.1 ml. of the o-phthalaldehyde reagent added. The mixture is kept in the dark for 15 min. to allow formation of the fluorescent conjugate.

DAILY CALIBRATION OF THE P.E. 203.

The instrument is set for full (100%) scale deflection (FSD) with the most concentrated standard solution in the sample cell (10 µg/ml.); and for zero deflection with the 3 % metaphosphoric acid in the sample cell.

The standard GSH solutions are then read in ascending order and the percent F.S.D. at 420 nm. is noted and a standard curve for GSH prepared.

SAMPLE PREPARATION.

Fresh heparinised blood is centrifuged and the plasma separated. The red blood cells are then washed 3 times with 2 vols. of isotonic saline. 2.0 ml. of the washed red cells is then diluted to 20 ml. with mM EDTA to haemolyse the cells. This haemolysate can then be stored deep-frozen until the estimation is performed. 1.0 ml of the EDTA haemolysate is then de-proteinised with 0.5 ml. of

ice-cold 3 % metaphosphoric acid, centrifuged, and then aliquots of the clear protein-free supernatant taken for the fluorometric assay as described before.

SECTION THREE.

RESULTS AND DISCUSSION.

The major metabolite of cyanide is thiocyanate, formed by combination with sulphur. The treatment of these toxic amblyopias, which are associated with cyanide toxicity, must, first and foremost, involve enhancing the patients' ability to detoxify cyanide to thiocyanate. This may be accomplished by directly administering an additional source of sulphur or some other agent which will detoxify the cyanide either by direct combination, or indirectly, by making sulphur available in a form acceptable for combination with the cyanide. Technical problems encountered in the estimation procedure (see Materials & Methods section) made meaningful interpretation of plasma cyanide results difficult, and therefore, more emphasis has been placed upon the measurement of thiocyanate, rather than cyanide in plasma and in urine. Measurement of these two parameters therefore provides a convenient and satisfactory assessment of the patients' detoxification of cyanide.

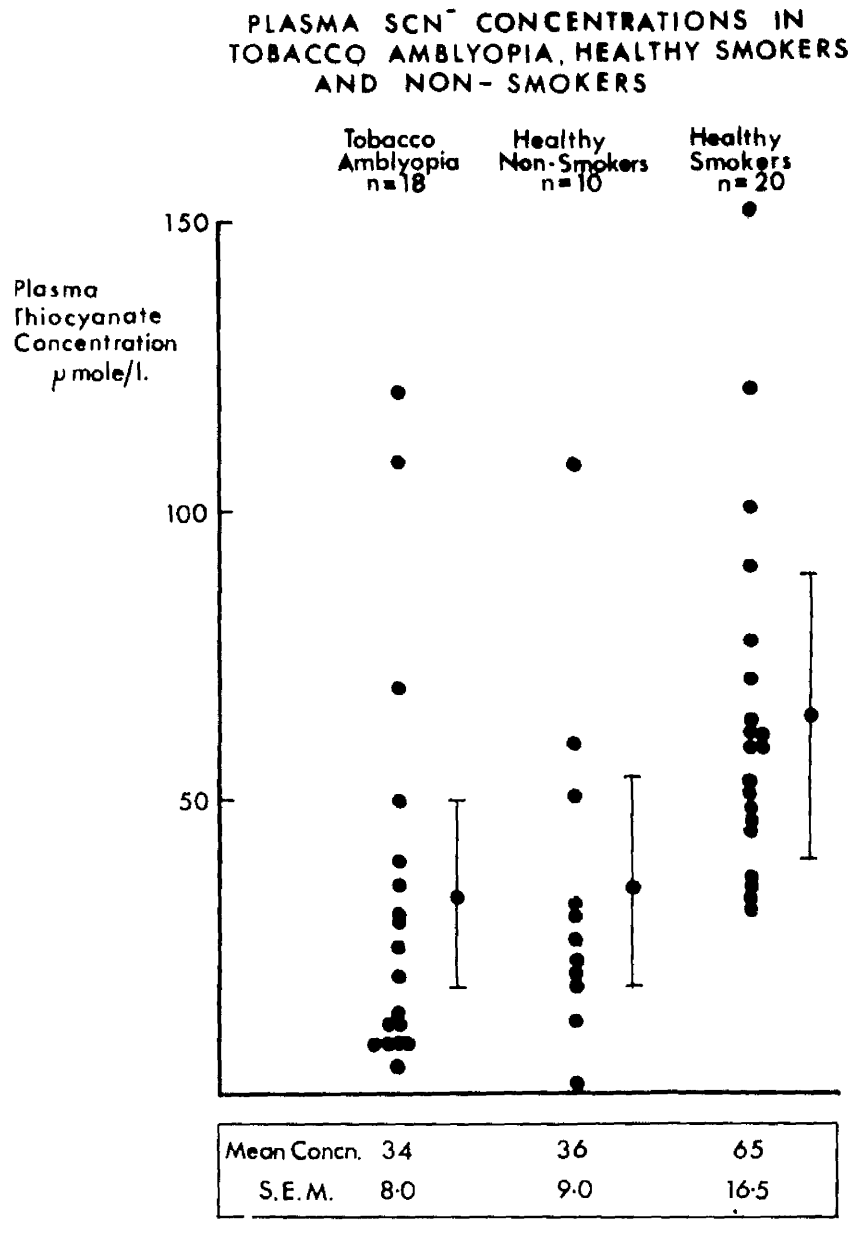


Fig. 3-1. Plasma Thiocyanate Concentrations in Tobacco Amblyopia, Healthy Smokers and Non-smokers (Mean ± 2 S.E.M.).

Smoking, Cyanide, Thiocyanate and Tobacco Amblyopia.

Healthy smokers exhibit higher thiocyanate levels in body fluids than do non-smokers (Lawton et al.,1943;Trasoff & Schneeberg,1944;Maliszewski & Bass,1955;Stoa,1957;Matthews et al., 1965;Wilson & Matthews,1965). This is believed to result from detoxification of cyanide, which is present in significant concentrations in tobacco smoke (Osborne,Adamek, & Hobbs,1956;Johnstone & Plimmer,1959;U.S.Surg.Gen.report,1964). Fig. 3-1. shows the plasma thiocyanate concentrations which were found in tobacco amblyopia patients, normal smokers and non-smokers. The mean concentration for 20 non-amblyopic smokers was found to be 65 $\pm$ 33 μmole /l., which was significantly higher than the mean concentration of 36 $\pm$ 18 μmole/l. found in 10 non-smokers ($0.025 > P > 0.01$). The level of significance has been taken at $P < 0.05$, and this convention has been adhered to throughout these studies. The mean plasma thiocyanate concentration in 18 untreated tobacco amblyopia patients was 34 $\pm$ 16 μmole/l., which was significantly different from that found in the non-amblyopic smokers ($0.01 > P > 0.005$), but was not statistically significantly different from the concentration found in the non-smokers. The tobacco amblyopia patients in general, smoked more heavily than did the non-amblyopic smokers, and would therefore be expected to exhibit higher thiocyanate levels. The fact that they did not, suggests that, if cyanide is to be considered an important cause of tobacco amblyopia, these patients reveal a

a reduced ability to convert cyanide to thiocyanate.

Wilson & Matthews (1966) showed a positive correlation between plasma cyanide and thiocyanate concentrations, which might have been expected from the equilibrium between cyanide detoxification and cyanide formed endogenously by the action of a red cell enzyme, originally named thiocyanate oxidase (Goldstein & Rieders,1953), but recently shown to be due to peroxidase activity of haemoglobin (Chung & Wood,1971).

They also found that the proportion of cyanocobalamin was raised in smokers, and that hydroxocobalamin was inversely related to plasma thiocyanate levels. This supported the view of Smith (1961) who claimed that, in very heavy smokers, the additional cyanide inactivated some of the serum hydroxocobalamin by conversion to the cyano- form, and that the neuro-retinal toxic effect of cyanide in vitamin B 12 deficiency could be explained by this conversion.

Recent work by Wilson,Linnell & Matthews (1971) has demonstrated that plasma cyanocobalamin levels are significantly raised in tobacco amblyopia, Leber's hereditary optic atrophy, and in other types of optic atrophy. They suggested that this may be evidence of inborn errors of cyanide metabolism. In vary heavy smokers with signs of amblyopia, the cyanocobalamin concentrations might be expected to reflect the degree of cyanide exposure from tobacco smoke, but the amounts found were often in excess of those found even in the heaviest of normal smokers, suggesting a metabolic defect.

In Leber's hereditary optic atrophy, clinical and metabolic evidence suggests that, in many patients, the onset and severity of the disease may be related to smoking (Wilson,1963,65).

Wilson & Matthews (1966) also demonstrated a negative relationship between plasma cyanide and total serum vitamin B 12, which they suggested might be the result of vitamin B 12 depletion due to the high intake of cyanide associated with smoking. This supports the suggestions that vitamin B 12 contributes to the detoxification of exogenous cyanide (Wokes,1955;1958;Smith,1961), and that chronic administration of cyanide depletes the liver stores of vitamin B 12 (Braekkan,Njaa, & Utne,1957). Alternatively, Wilson & Matthews (1966) suggest that relatively low (though physiological) levels of vitamin B 12 may reduce the ability to detoxify cyanide, resulting in relatively high concentrations of it in the blood.

It is known that patients suffering from tobacco amblyopia show reduced serum vitamin B 12 levels (Heaton et al.,1958;Foulds et al.,1969a), and that raised cyanide concentrations in the plasma of such patients might reasonably be expected. The mean concentration of cyanide in 15 untreated tobacco amblyopia patients of 0.61 μmole /l. (Chisholm & Pettigrew,1970) is higher than the concentration of 0.22 μmole/l. found by Wilson & Matthews (1966) in normal smokers. We were able to show an inverse relationship between plasma cyanide and serum vitamin B 12 in these patients, although it failed to be statistically significant ($P > 0.05$).

Summary.

Cyanide is presumed to come from tobacco smoke, and elevated thiocyanate concentrations have been found in the plasma and urine of healthy smokers, when compared to non-smokers. The plasma thiocyanate concentration in untreated tobacco amblyopia patients was lower than that of the normal smokers, which suggested that, although their tobacco, and presumably cyanide intake was greater, they were less able to convert cyanide to thiocyanate by the normal processes. The inverse relationship found between plasma cyanide and total serum vitamin B 12 (Chisholm & Pettigrew, 1970) supported the earlier findings that, at low serum vitamin B 12 levels, the plasma cyanide concentration might be sufficiently elevated to produce the chronic neuropathological changes associated with tobacco amblyopia (Heaton,McCormack, & Freeman,1958;Smith,1961).

HYDROXOCOBALAMIN THERAPY.

(a) Intramuscular Therapy in Tobacco Amblyopia.

The use of vitamin B 12 in the therapy of tobacco amblyopia is now well established from the work of Heaton et al. (1958), showing that some patients with tobacco amblyopia may improve when given vitamin B 12 parenterally, even when smoking is continued. Smith (1961) suggested that hydroxocobalamin is the physiologically active fraction, and its conversion to cyanocobalamin provides an alternative route for cyanide detoxification. The superiority of hydroxocobalamin over cyanocobalamin in the treatment of this disease, demonstrated by Chisholm et al. (1967) does support this view.

Foulds et al. (1970) have stressed that the vitamin B 12 status of patients with toxic amblyopias must be fully investigated before therapy is commenced, for those patients with an obvious or latent pernicious anaemia will require therapy permanently, while those without evidence of such disease, will only require therapy until visual recovery is complete. They also showed a relationship between tobacco consumption and the serum level of vitamin B 12, suggesting that tobacco intake is the major factor affecting therapy with hydroxocobalamin, visual recovery being best in those patients smoking least and receiving high doses of hydroxocobalamin.

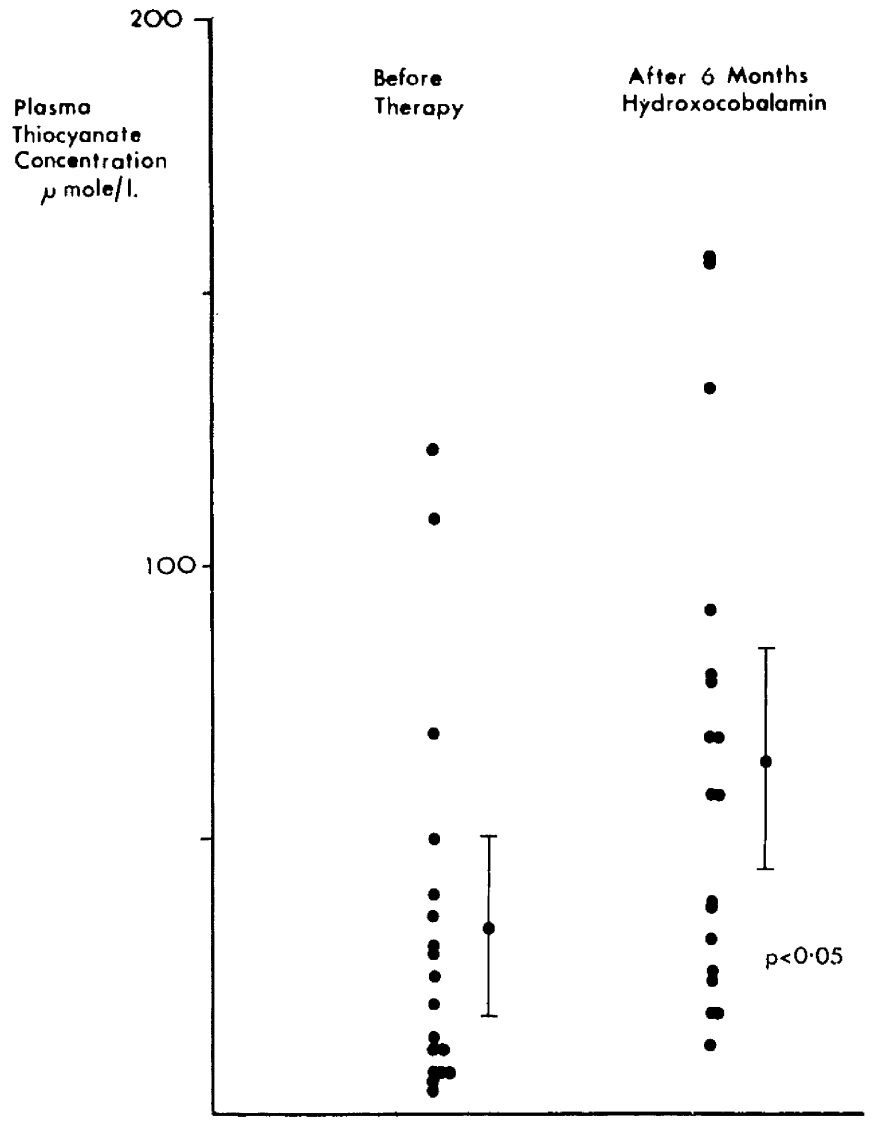


Fig. 3-2. Changes in Plasma Thiocyanate Concentrations in 18 Tobacco Amblyopia Patients after 6 months Intramuscular Hydroxocobalamin Therapy (Means ± 2 S.E.M.).

To assess the optimum dose of hydroxocobalamin in the treatment, Foulds et al. (1970) estimated the rate of visual recovery in patients who stopped smoking and received no other therapy, and, from the results, suggested that to obtain similar recovery in patients continuing smoking, sufficient hydroxocobalamin must be given to keep the ratio of hydroxocobalamin to tobacco consumption at a calculated value. Allowing for the loading dose of hydroxocobalamin (5 mg./day for 2 weeks, then 1 mg./day for 2 weeks), they suggested that a patient smoking 2 oz. of tobacco per week will require 1 mg. twice weekly, while a patient smoking 5 or 6 oz. of tobacco would require a much larger dose, and that in general, if tobacco consumption is kept at or below 3 oz. per week, satisfactory visual improvement occurs with 1 mg. of hydroxocobalamin thrice weekly.

Fig. 3-2. shows the alteration in plasma thiocyanate concentration after 6 months intramuscular therapy with hydroxocobalamin in 18 tobacco amblyopia patients. The mean plasma thiocyanate concentration rose from 34 $\pm$ 16 µmole/l. to 64 $\pm$ 20 µmole/l. This is a significant change ($0.05 > P > 0.025$) and the mean concentration in the treated patients was close to that of the group of healthy (non-amblyopic) smokers as shown in Fig. 3-1. This rise in plasma thiocyanate levels is accompanied by an increase in urine thiocyanate excretion. Fig. 3-3. shows the mean percentage alteration in the urinary thiocyanate concentration and the urine volume in a

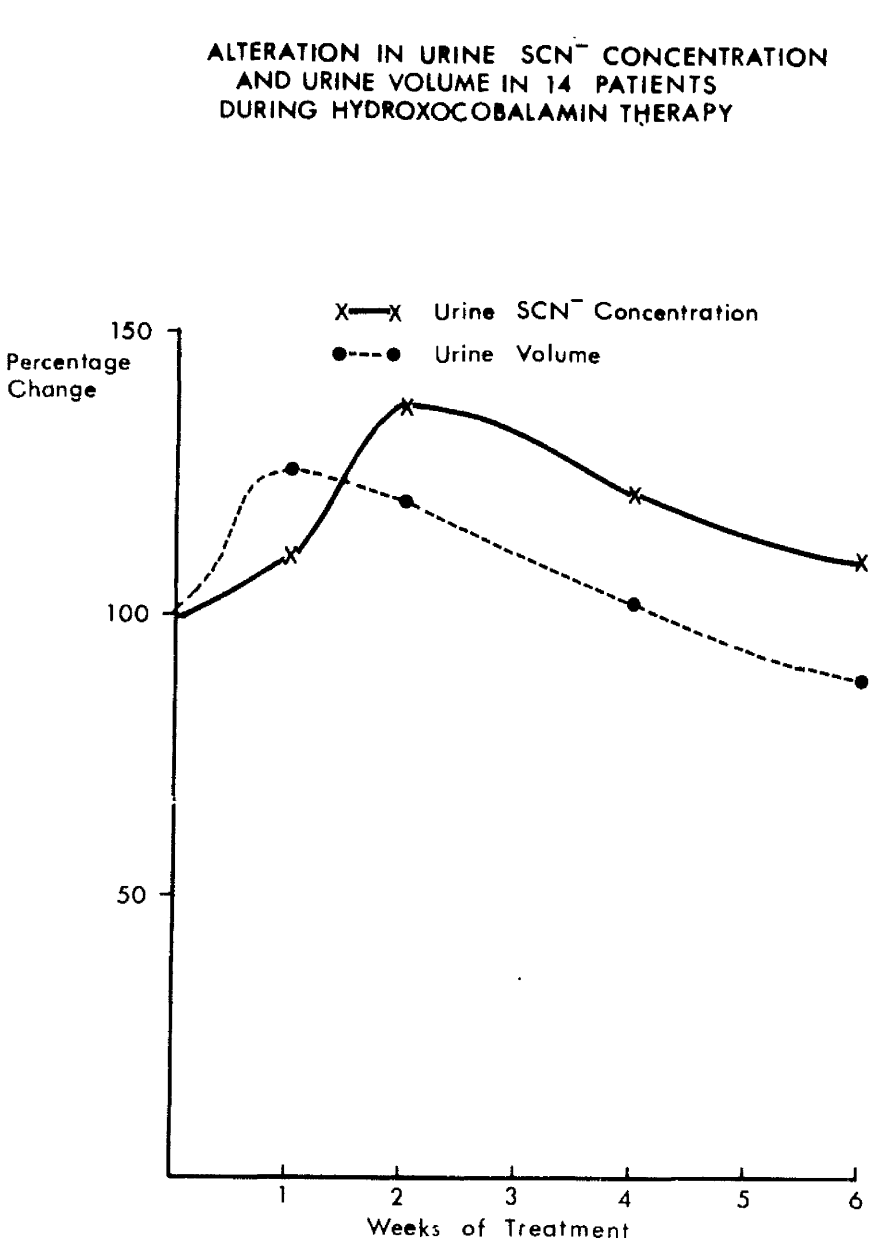


Fig. 3-3. Mean Alteration in Urinary Thiocyanate Concentration in 14 Tobacco Amblyopia Patients during Treatment with Intramuscular Hydroxocobalamin.

group of 14 tobacco amblyopia patients placed on treatment with intramuscular hydroxocobalamin. There is a rapid rise which reaches a peak around the end of the second week, followed by a gradual decline. Wright-Thomson (1902) noticed such an increase in the toxicity of the urine and a similar diuresis in tobacco amblyopia patients who were treated by abstinence from tobacco.

Thiocyanate ion behaves like chloride and is distributed in the extracellular water in a similar manner. Thomas (1949) showed that its renal excretion was directly related to chloride excretion and to urine volume. Wokes & Ellis (1966) demonstrated a diurnal rhythm in renal clearance which roughly parallelled the urinary excretion of total nitrogen. They showed that the ratio of thiocyanate to total nitrogen in the urine was more or less constant, but was lower in teetotal non-smokers on normal diets, than in normal subjects but who had lower intakes of vitamin B 12.

In a group of untreated tobacco amblyopia patients a significant inverse relationship was shown to exist between plasma thiocyanate concentration and its renal clearance. After 3 months treatment with intramuscular hydroxocobalamin, however, this relationship became positive, the mean clearance rising from 0.59 ± 0.40 ml./min. to 1.62 ± 1.62 ml./min. These findings are contrary to the report by Stoa (1957) showing that there was no relationship between these two factors in normal, healthy adults.

Assessment of the patients' response to treatment may be made by measurement of the levels of thiocyanate in plasma and in urine, which provides a guide to the detoxification of cyanide, the presumed toxic factor in these conditions. Clinical assessment can be made by measurements of the patient's visual acuity, where the visual acuity on the Snellen test type at 6 metres is expressed as a percentage, taking 6/6 as 100 % (Ridley,1959). Visual improvement may then be expressed in arbitrary units, with each step representing a doubling or halving of the previously recorded visual acuity.

Foulds et al. (1970) compared the improvement in vision over a period of treatment with a fraction representing the total amount of hydroxocobalamin in mg. divided by the total tobacco consumption in oz. during the period of treatment, finding a significant positive correlation. They also suggested that tobacco intake was the major factor affecting response to therapy and compared visual improvement after hydroxocobalamin treatment with the total intake of tobacco alone, this time finding a significant inverse correlation. From these results, they suggested that visual improvement was best in those patients smoking least and receiving high doses of hydroxocobalamin, but, that to obtain satisfactory improvement in very heavy smokers, it may be necessary to curtail smoking during treatment with intramuscular hydroxocobalamin.

Summary.

Hydroxocobalamin, by intramuscular injection, has been used successfully for some years to treat tobacco amblyopia. The observed rises in plasma and urinary thiocyanate and in renal clearance of thiocyanate suggest that hydroxocobalamin is enhancing detoxification of cyanide by conversion to thiocyanate and facilitating excretion of thiocyanate in the urine. The visual improvement obtained on treatment with hydroxocobalamin is dependent upon tobacco (and hence cyanide) intake, and supports the dual aetiology proposed for tobacco amblyopia (Heaton et al.,1958;Smith, 1961).

(b) Intramuscular Therapy in Leber's Hereditary Optic Atrophy.

Close comparisons have been drawn between Leber's hereditary optic atrophy and tobacco amblyopia, both in its clinical picture and in its mode of presentation (Schepens,1946;Foulds,1969). An inherited defect in the mechanism for the detoxification of cyanide has been suggested as a possible cause of the disease (Wilson,1963, 65). Indeed, similarities in the thiocyanate levels in body fluids found in tobacco amblyopia and in Leber's optic atrophy, in both untreated and treated state, tend to suggest a common aetiology.

The mean plasma thiocyanate concentration in 12 Leber's optic atrophy smokers of 38 $\pm$ 32 µmole/l. (Chisholm & Pettigrew,1970) is no different from the mean concentration of 34 $\pm$ 16 µmole/l. found in 18 untreated tobacco amblyopia patients ($P > 0.05$), but is much lower than the mean concentration of 65 $\pm$ 33 µmole/l. in a group of non-amblyopic smokers (see Fig. 3-1.), this difference being highly statistically significant ($P < 0.01$). This supports the findings of Wilson (1965) that Leber's optic atrophy patients who smoked showed lower mean plasma thiocyanate concentration than the mean concentration found in normal smokers.

As with tobacco amblyopia, Leber's optic atrophy patients are placed on intramuscular hydroxocobalamin therapy. Fig. 3-4. shows the changes in plasma thiocyanate, urine thiocyanate and renal clearance in 10 such patients over 12 months treatment .

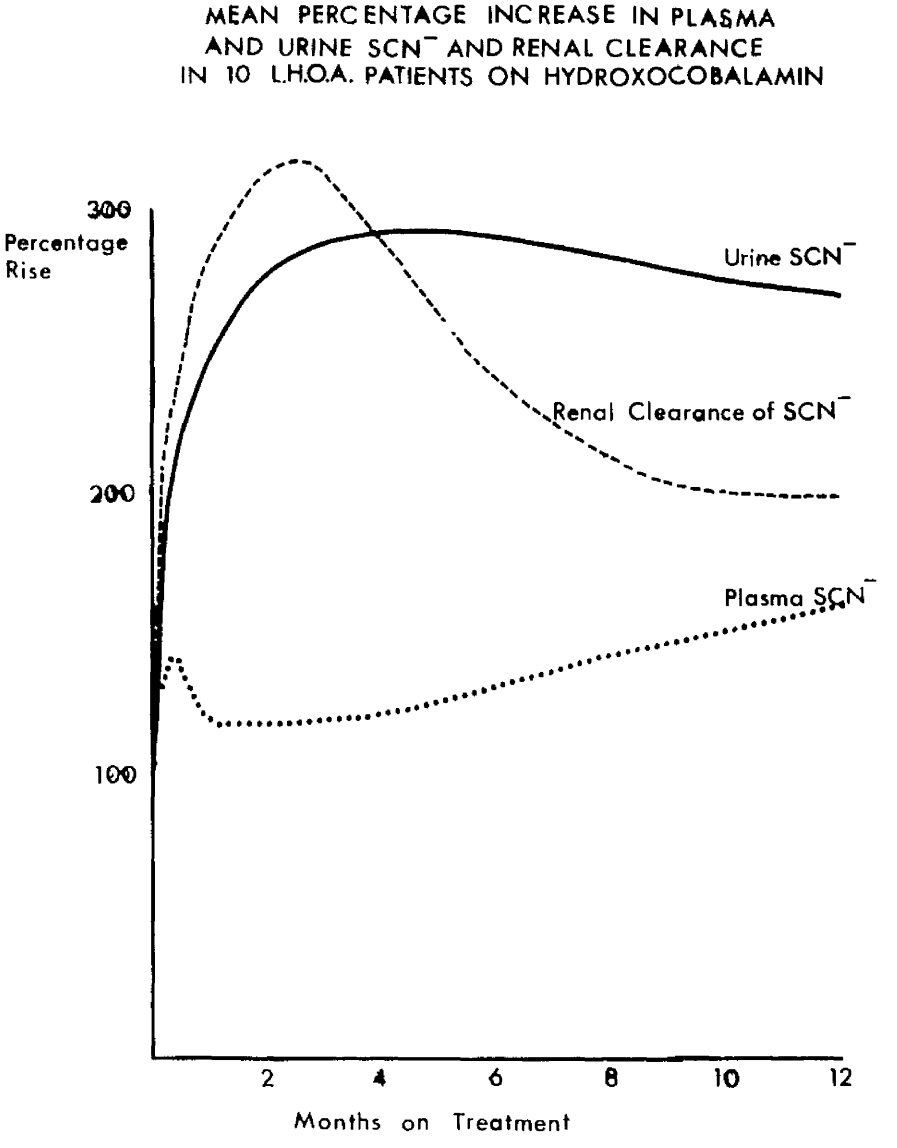


Fig. 3-4. Mean Alteration in Plasma and Urinary Thiocyanate and Renal Clearance of Thiocyanate in 10 Leber's Hereditary Optic Atrophy Patients during treatment with Hydroxocobalamin.

These patients exhibit a rapid rise in urinary thiocyanate concentration with a slow, progressive rise in plasma thiocyanate and a very rapid rise in renal clearance.

The negative relationship between thiocyanate renal clearance and plasma thiocyanate concentration, found in untreated tobacco amblyopia, can also be observed in Leber's optic atrophy patients who smoke. After 12 months treatment, this relationship did, however, remain negative but at a statistically non-significant level (Chisholm & Pettigrew, 1970).

Summary.

Although similar in many aspects to tobacco amblyopia, Foulds et. al. (1969b) have shown that there is no corresponding vitamin B 12 deficiency in Leber's hereditary optic atrophy. The response to therapeutic hydroxocobalamin of Leber's optic atrophy patients, in terms of thiocyanate is similar to tobacco amblyopia. This supports the suggestion of Wilson (1963,1965) that this disease may be due to an inherited defect in the mechanism necessary for the detoxification of cyanide. Visual improvement in these patients is not as pronounced as in tobacco amblyopia patients receiving the same treatment, but the increases in urinary thiocyanate and in the renal clearance of thiocyanate would suggest that cyanide is being satisfactorily converted to thiocyanate and excreted in the urine.

Patient	Plasma SCN (µm./l.)			Visual Acuity (%)			Total Tobacco Consumption (oz.)	B 12 absorbed (mg.) / Total Tobacco Consumed in 4 months.
	Before	After	Change	Before	After	Change in arbitrary units		
A.C.	110	122	+ 12	2	48	+ $4\frac{1}{2}$	56	84/56 = 1.5
A.H.	37.5	17.5	- 20	5	16	+ $1\frac{1}{2}$	64	84/64 = 1.31
J.I.	90	124	+ 34	4	8	+ 1	32	84/32 = 2.62
D.M.	16	26	+ 10	5	16	+ $1\frac{1}{2}$	32	84/32 = 2.62
J.M.	26			32	48	+ $\frac{1}{2}$	stopped smoking	
F.P.	124	96	- 28	16	20	+ $\frac{1}{2}$	48	84/48 = 1.75
E.S.	36	8	- 28	16	32	+ 1	96	84/96 = 0.875
Mean	62.8	65.6		11.4	26.8	+ $1\frac{1}{2}$		
S.E.M.	16.6	22.1		4.06	6.1			
S.D.	43.9	54.2		10.7	16.1			

Table 3-1. Plasma Thiocyanate and Visual Acuity results from 7 Tobacco Amblyopia Patients receiving Oral Hydroxocobalamin at a dose of 100 mg./day for a period of 4 months. Visual improvement is expressed in arbitrary units, with each unit representing a doubling or halving of the previously recorded Visual Acuity (see also p. 56.).

(c) Oral Hydroxocobalamin Therapy in Tobacco Amblyopia.

Hydroxocobalamin therapy by intramuscular injection has the advantage of ensuring that the patient is receiving treatment. There are, however, many obvious disadvantages to repeated injections, and, at present, several tobacco amblyopia patients are receiving an oral preparation of hydroxocobalamin, which may also be used in conjunction with the parenteral therapy.

Treatment has recently been commenced on a group of 7 patients suffering from tobacco amblyopia with an oral preparation of hydroxocobalamin, at a dose of 1 tablet of 100mg. per day. This removes the inconvenience of repeated injections, but has several disadvantages in that it has been suggested that only 1 % of the preparation is absorbed into the body, and only 70 % of this 1 % is available as hydroxocobalamin. This means that, of the original 100 mg. tablet, possibly only 700 µg. are available as hydroxocobalamin, the effective therapeutic form.

After 4 months on this treatment, 3 of the group have shown increases in plasma thiocyanate concentrations similar to that observed in patients treated with intramuscular hydroxocobalamin. The mean plasma thiocyanate concentration of the group rose from 62.8 µmole/l. to 65.6 µmole/l., although there were considerable variations between individuals (see Table 3-1).

The mean renal clearance of thiocyanate of the group rose from 1.1 ml./min. to 2.2 ml./min. which is a significant change ($p < 0.01$).

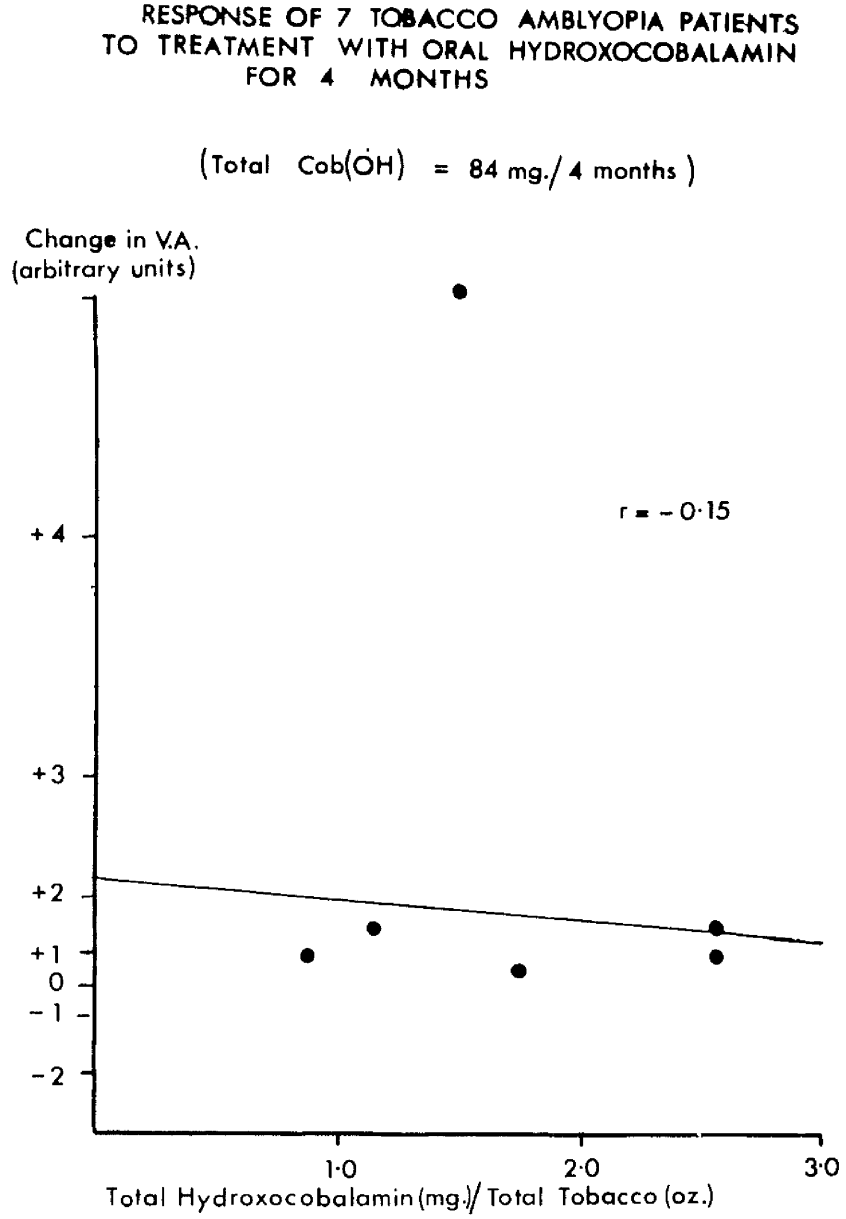


Fig. 3-5. Relationship between change in Visual Acuity in 7 Tobacco Amblyopia Patients and a fraction representing the total dose of Hydroxocobalamin and the total Tobacco Intake during the 4 months of treatment.

Although the changes in plasma and urinary thiocyanate concentrations are not large, there has been visual improvement in all the patients in the group. Visual acuity measurements refer to the right eye only, and this convention has been adhered to in the remainder of this text. In the 7 patients receiving oral hydroxocobalamin therapy, the mean rate of recovery of vision was 1.5 units in 4 months, or the equivalent of a rise in visual acuity from 6/60 to better than 6/24 on the Snellen test type. As with the thiocyanate results, the variation between individuals was considerable, with only one patient (A.C.) showing satisfactory visual improvement of $4\frac{1}{2}$ units after the period of treatment.

As with the intramuscular therapy, it seemed that the consumption of tobacco and the dose of hydroxocobalamin would influence the response to treatment. Visual improvement over a period of 4 months of treatment was compared with a fraction representing the total amount of hydroxocobalamin in mg. (84 mg. over the 4 months period) divided by the total consumption of tobacco in oz. during the same period. The results are shown in Fig. 3-5., where a negative correlation is evident ($r = -0.15$). Foulds et al. (1970) found a positive relationship between these two factors, in patients receiving intramuscular hydroxocobalamin treatment, which suggested that the visual improvement was best in those receiving high doses of hydroxocobalamin and smoking least.

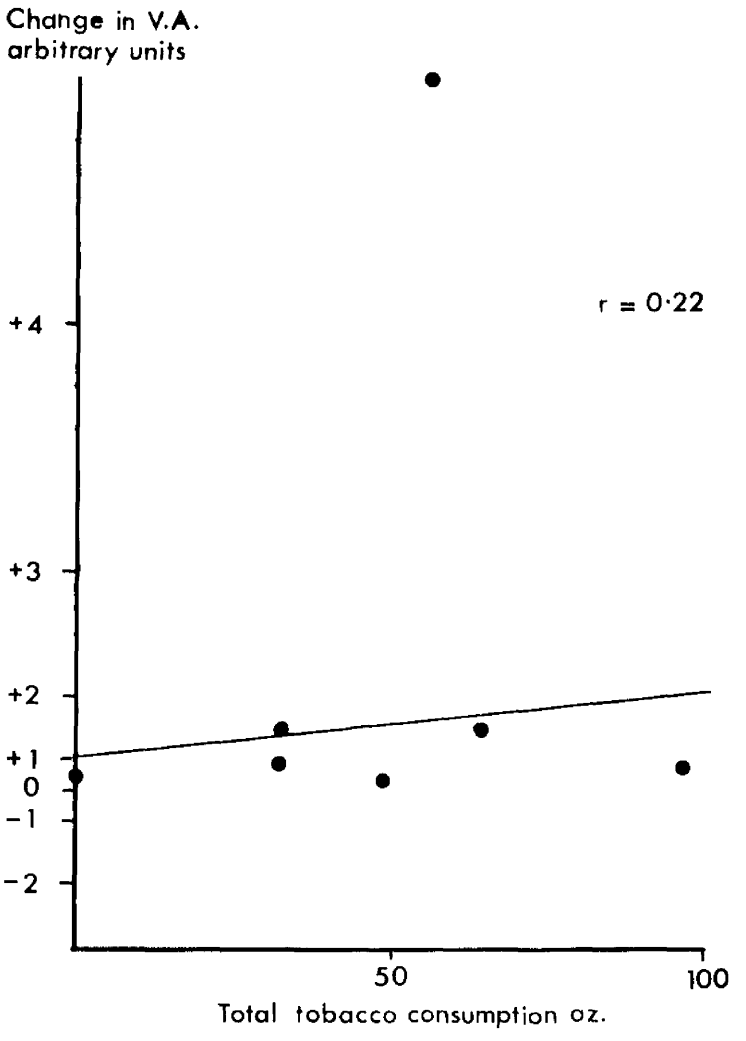


Fig. 3-6. Relationship between the change in Visual Acuity in 7 Tobacco Amblyopia Patients and the total Tobacco Intake during the 4 months of treatment.

Anomalous results were also obtained when the visual improvement after 4 months of treatment was compared with the total tobacco intake over this period. Fig. 3-6. shows that a positive relationship was obtained between these two factors. This is contrary to the findings of Foulds et al. (1970), who found an inverse relationship between these two factors.

Summary.

The changes in plasma thiocyanate concentration and in renal clearance of thiocyanate reported in this study, suggest that, as with the intramuscular treatment, the oral hydroxocobalamin is improving the conversion of cyanide to thiocyanate and its removal in the urine.

The clinical picture is unclear, with visual improvement being observed in all the patients, as shown by the mean rate of recovery of the group of 1.5 units, but with variations between the responses of individuals in the group.

The results shown in Fig. 3-5. and Fig. 3-6. could be explained by various factors.

1. The patients may be absorbing only very small quantities of the preparation.

2. The patients may be absorbing only a small proportion of the preparation as hydroxocobalamin, perhaps even less than the 700 µg. suggested previously.

3. They may be absorbing all the preparation, but the dosage is insufficient to produce significant improvement.

4. The number of patients studied is small, producing atypical responses.

Hydroxocobalamin and its Effect on Cyanide Metabolism.

To further the study of the effect of hydroxocobalamin on cyanide and thiocyanate metabolism, a number of non-amblyopic subjects, both smokers and non-smokers, were given a course of hydroxocobalamin. The subjects (in-patients being treated for cataract) were given 5 mg. per day for each of 3 days. Blood and urine collections were made, before and after the treatment. Measurements of plasma and urinary thiocyanate levels were undertaken, for the smokers and non-smokers separately, and for the group as a whole. The smokers and non-smokers, both male and female were of comparable ages.

(a) Smokers - The renal clearance of thiocyanate rose from 0.73 ml./min. to 1.08 ml./min., while the mean plasma thiocyanate concentration appeared to fall from 54 $\pm$ 25 μmole/l. to 35 $\pm$ 11 μmole/l. The mean 24-hour urinary thiocyanate concentration of the smokers did not alter significantly.

(b) Non-smokers - The mean renal clearance of thiocyanate showed a significant rise from 0.86 ml./min. to 1.86 ml./min. ($0.02 > p > 0.01$). The mean plasma thiocyanate concentration appeared to fall from 36 $\pm$ 18 μmole/l. to 28 $\pm$ 9 μmole/l., although not significantly. The mean 24-hour urinary thiocyanate concentration of the non-smokers rose from 55 μmole/24 hr. to 82 μmole/24 hr.

There was therefore, no evidence of a significant difference between the effect of hydroxocobalamin on either the smokers or the non-smokers taken separately. When these two groups are combined,

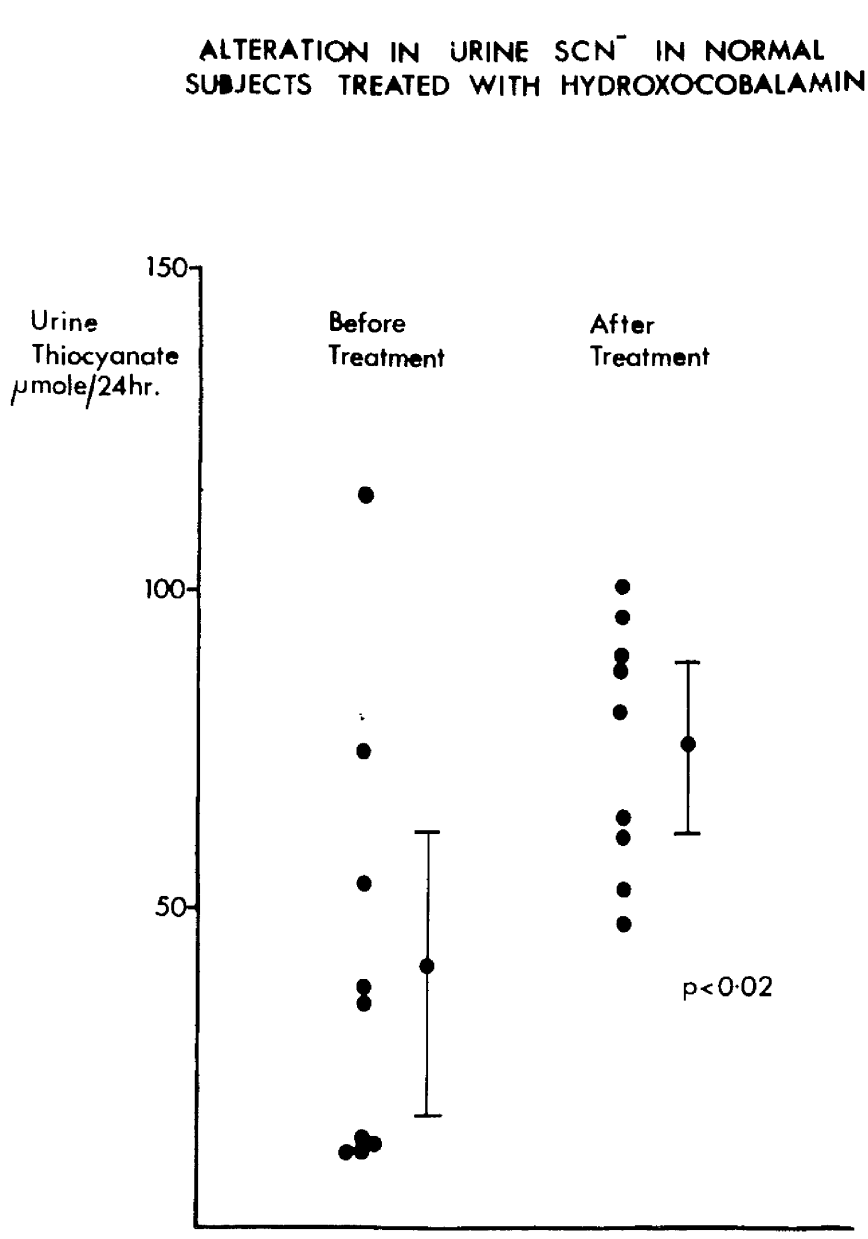


Fig. 3-7. Alteration in Urinary Thiocyanate Concentration in 9 Normal Subjects treated with Hydroxocobalamin (Mean ± 2 S.E.M.).

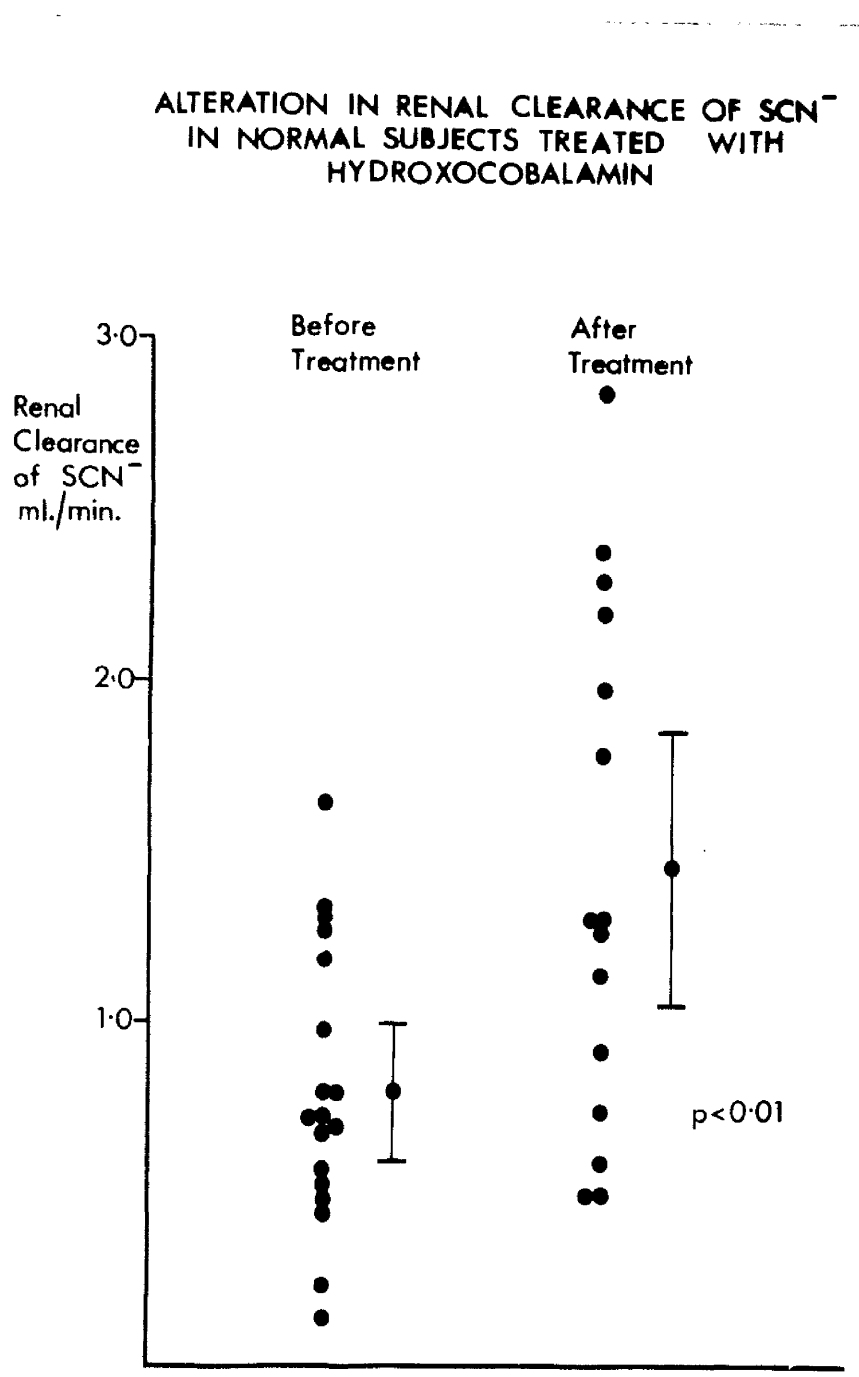


Fig. 3-8. Alteration in Renal Clearance of Thiocyanate in 18 Normal Subjects treated with Hydroxocobalamin (Mean± 2 S.E.M.)

several interesting relationships emerge. Although the mean plasma thiocyanate concentration did not alter significantly, there was a significant rise in the mean urinary thiocyanate level from 41 ± 22 μmole/24 hr. to 75 ± 11 μmole/24 hr. ($0.02 > P > 0.01$). (see Fig. 3-7.). This significant rise in urinary thiocyanate level was accompanied by a significant rise in renal clearance from 0.81 ml./min. to 1.5 ml./min. ($0.01 > P > 0.005$), but not by any increase in the 24-hour urine volume. (Fig. 3-8).

Summary.

In this study, hydroxocobalamin treatment caused rises in the renal clearance of thiocyanate in both the smokers and non-smokers, when taken separately, or together, and rises in the urinary thiocyanate concentration of the group as a whole, without a significant rise in urinary volume. These changes support the previous findings that hydroxocobalamin acts by aiding in the conversion of cyanide to thiocyanate, and by enhancing its excretion in the urine.

Possible Mode of Action of Therapeutic Hydroxocobalamin.

Hydroxocobalamin has been successful in the treatment of a number of toxic amblyopias believed to be associated with cyanide intoxication. Such therapy has allowed visual improvement in tobacco amblyopia and in Leber's optic atrophy patients, even when smoking, and therefore cyanide intake, is maintained. The mechanism of its action is not clear

It may combine with cyanide directly to form cyanocobalamin (Wokes & Picard, 1955) Smith (1961) proposed that the neuro-retinal toxic effect of cyanide in vitamin B 12 deficiency could be explained by this conversion, and suggested that in very heavy smokers, with a presumed increased cyanide intake, a much higher proportion of plasma vitamin B 12 was in the form of cyano-cobalamin than in non-smokers. Wilson et al. (1971) have substantiated this proposal by showing significantly elevated plasma cyanocobalamin levels in tobacco amblyopia, Leber's hereditary optic atrophy and in other toxic neuropathies, and proposed that this was evidence of errors or failures in cyanide detoxification.

Boxer & Rickards (1952) showed that body tissues contain more thiocyanate than cyanide, and suggested that, in the presence of such an excess, hydroxocobalamin could combine with thiocyanate to form thiocyanatocobalamin as well as combining with cyanide to form cyanocobalamin.

In a later report (Wokes & Picard,1955), they suggested that this thiocyanatocobalamin reverted to cyanocobalamin, giving up its sulphur to an active intermediate, to form a possible precursor of sulphur-containing compounds such as sulphur amino acids. The cyanocobalamin would then lose its cyanide to regenerate hydroxocobalamin, and the cyanide thus liberated, could be converted to thiocyanate by rhodanese, thereby completing the cycle (see Fig. 1-7.)

Vitamin B 12 and Sulphur Amino Acids.

Cysteine is derived from methionine, an essential amino acid, in steps thus -

Methionine → Homocysteine → Cystathionine → Cysteine + Homoserine

Serine

The net effect of the above reaction is an exchange of the sulphhydryl group of homocysteine with the hydroxyl group of serine in a process termed transsulphuration. Thus, in the biosynthesis of cysteine, the carbon chain arises from serine, whereas the sulphur is derived from methionine. The interconversion of cysteine to cystine occurs readily in the body.

The biosynthesis of methionine is a reversal of the above pathway, with the final step being the methylation of homocysteine believed to require the presence of a vitamin B 12 co-enzyme.

Vitamin B 12 is not active as a co-enzyme, for any enzymatic

reaction. Rather a group of related substances, the vitamin B 12 co-enzymes. function in this capacity. The B 12 co-enzymes were initially isolated by Barker et al. (1958), who showed that they had the cyanide replaced as a cobalt ligand, by the 5-deoxyadenosyl group. The enzymatic conversion of vitamin B 12 to the coenzyme B 12 requires FAD, NADH, ATP and glutathione as cofactors. This deoxyadenosyl coenzyme B 12 is important in the transmethylation of of homocysteine to methionine - the final step in the biosynthesis of methionine from cysteine. This reaction is complicated, requiring NADH, FAD, S-adenosyl methionine, N^5-methyl-tetrahydrofolate and the vitamin B 12 coenzyme.

N^5-methyl tetrahydrofolate + Homocysteine

↓ NADH, FAD, S-adenosyl methionine, coenzyme B 12

Methionine + Tetrahydrofolate

The details of the reaction are far from clear. It has been established that the methyl group of S-adenosyl methionine is not transferred to homocysteine in this reaction, and that this substance acts catalytically. Recent experiments have shown that a new form of cobalamin coenzyme may participate in methionine biosynthesis. Methylation of fully reduced hydroxocobalamin yields methylcobalamin, which transfers its methyl group, both non-enzymatically, and, at an increased rate, enzymatically in the

presence of the B 12 coenzyme. This finding strongly suggests that the coenzyme is an intermediate in methionine biosynthesis.

In mammalian systems, N^5-methyl tetrahydrofolate serves as a methyl group donor for methionine biosynthesis in a reaction requiring catalytic amounts of S-adenosyl methionine.

The increases in plasma and urinary thiocyanate levels and in renal clearance of thiocyanate brought about by treatment with hydroxocobalamin, suggest that its mode of action is concerned with increasing the availability of sulphur in a form acceptable to the cyanide in the conversion to thiocyanate. Therapeutic hydroxocobalamin may then act directly by combining with cyanide to form cyanocobalamin, which is excreted in the urine. It may also, as the thiocyanatocobalamin proposed by Wokes & Picard (1955), act as a carrier of sulphur in relatively high amounts as shown by the ratio of free cyanide to thiocyanate in body fluids which is in the order of 1 : 1000. Hydroxocobalamin may also act by replenishing the body stores of adenosyl coenzyme B 12 and could, therefore act through methionine and the other sulphur amino acids to provide the necessary substrates for the conversion of cyanide to thiocyanate.

Sulphur Amino Acids in the Treatment of Cyanide Intoxication.

ORAL CYSTINE THERAPY.

The failure of a patient to detoxify cyanide may be due to a deficiency of vitamin B 12, the importance of which has been discussed, or, of some sulphur donor necessary for the conversion of cyanide to thiocyanate. One such compound is the sulphur amino acid, cystine, well known to react with cyanide to yield cysteine and β-thiocyanoalanine (Voigtlin et al.,1926), thereby providing a possible route for the detoxification of cyanide. Later workers showed that the latter tautomerises to 2-imino-4-thiazolidine carboxylic acid, the structure of which was proved by Wood & Cooley (1956). They showed that up to 15 % of cyanide entering the body could be recovered in the urine as this compound, with the remaining 85 % excreted in the urine as thiocyanate.

The use of cystine in the treatment of conditions associated with cyanide intoxication has been suggested by Osuntokun et al. (1968) in a study of tropical ataxic neuropathy, thought to be caused by consumption of the cyanide-containing foodstuff, cassava. This condition was accompanied by elevated levels of thiocyanate in plasma and in the urine, and by reduced plasma levels of sulphur amino acids, including cystine. They suggested that there may be a conditioned abnormality of cyanide metabolism arising from the

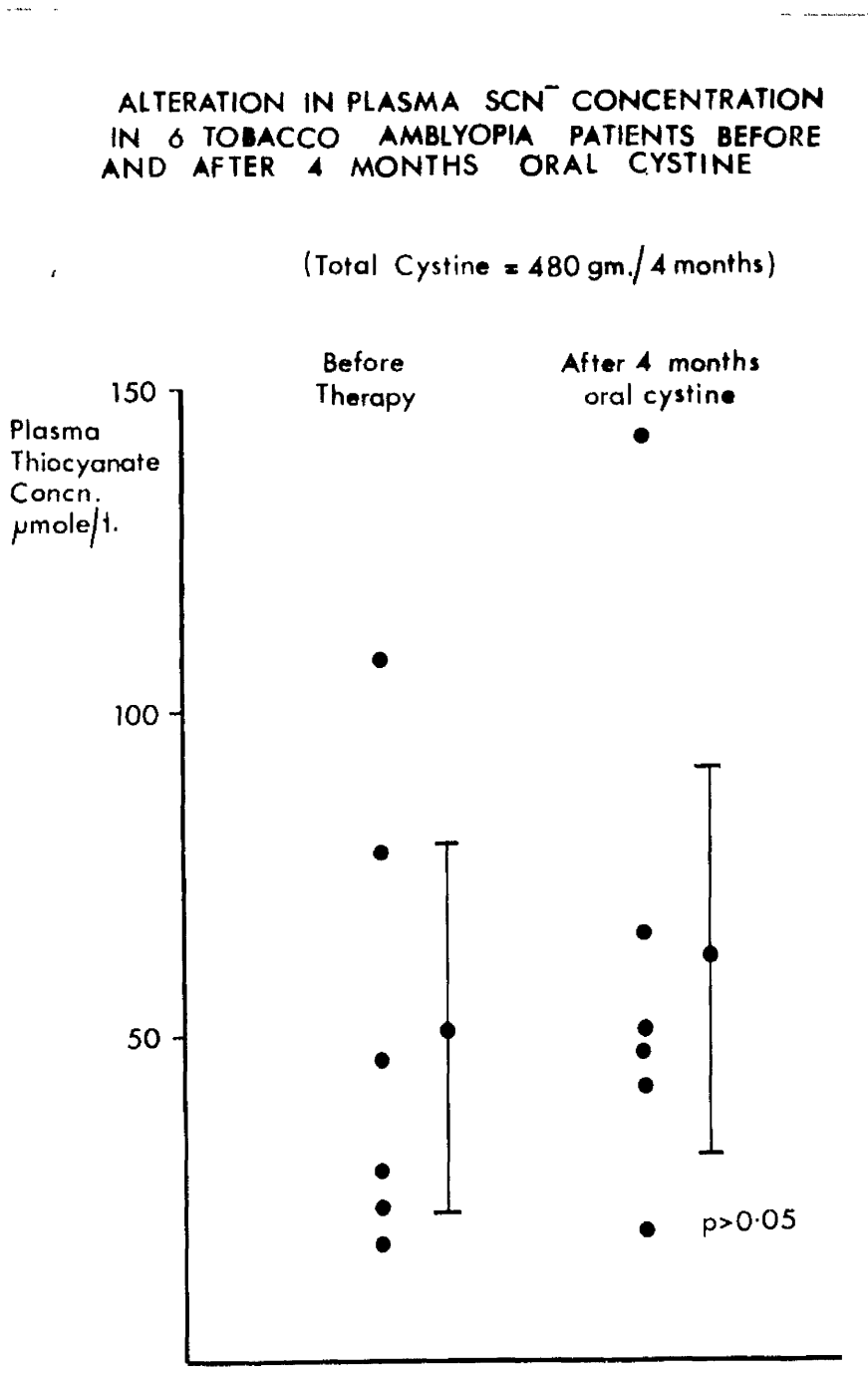


Fig. 3-9. Alteration in Plasma Thiocyanate Concentration in 6 Tobacco Amblyopia Patients treated with Oral Cystine at a dose of 4.0 gm. per day for 4 months. (Mean ± 2 S.E.M.)

Patient	Plasma SCN (um./l.)			Visual Acuity (%)			Total Tobacco Consumption (oz.)
	Before	After	Change	Before	After	Change in arbitrary units	
F.C.	46.5	51	+ 4.5	100	100	0	stopped smoking
R.D.	78	143	+ 65	5	5	0	64
G.H.	108	66	- 42	4	64	4	16
J.L.	29	42	+ 13	25	64	$1\frac{1}{2}$	48
P.M.	23.5	47	+23.5	8	64	3	42
F.M.	18	19.4	+ 1.4	7	10	$\frac{1}{2}$	56
Mean	50.5	61.4		24.8	51.2	$1\frac{1}{2}$	
S.E.M.	14.5	17.4		15.3	14.9		
S.D.	35.6	42.7		37.6	36.6		

Table 3-2. Plasma Thiocyanate and Visual Acuity results from 6 Tobacco Amblyopia Patients receiving Oral Cystine (4 gm./day for 4 months. Visual improvement is expressed in arbitrary units, with each unit representing a doubling or halving of the previously recorded Visual Acuity.(see also p. 56.).

low protein intake and subsequent lack of sulphur amino acids to act as substrate for cyanide detoxification. They therefore proposed theuse of cysteine, which is interconvertible with cystine in the body, in the treatment of this condition which can obviously be related to other neurological conditions similarly associated with cyanide toxicity.

Treatment was commenced on a group of 6 tobacco amblyopia patients with a preparation of oral cystine at a dose of 4.0 gm. per day. After one month on this treatment, 3 of the group were shown to have increases in plasma and urinary thiocyanate levels similar to that previously obtained in patients treated with intramuscular hydroxocobalamin. The mean plasma thiocyanate concentration of the group rose from 31 µmole/l. to 62 µmole/l., and the renal clearance of thiocyanate from 0.6 ml./min. to 3.6 ml./min., both of these changes being statistically significant (Chisholm & Pettigrew, (1970).

After 4 months on this treatment, the mean plasma thiocyanate concentration of the group rose from 50.5 $\pm$ 29 µmole/l to 61.4 $\pm$ 34.8 µmole/l., which fails to be significant ($P > 0.05$). These results are shown in Fig. 3-9. and in more detail in Table 3-2.

The mean rate of visual improvement of the group was 1.5 units in the 4 months of treatment, which represents an improvement in visual acuity of from 6/60 to better than 6/24 Snellen. To assess the patients' response to the therapy, the improvement in vision

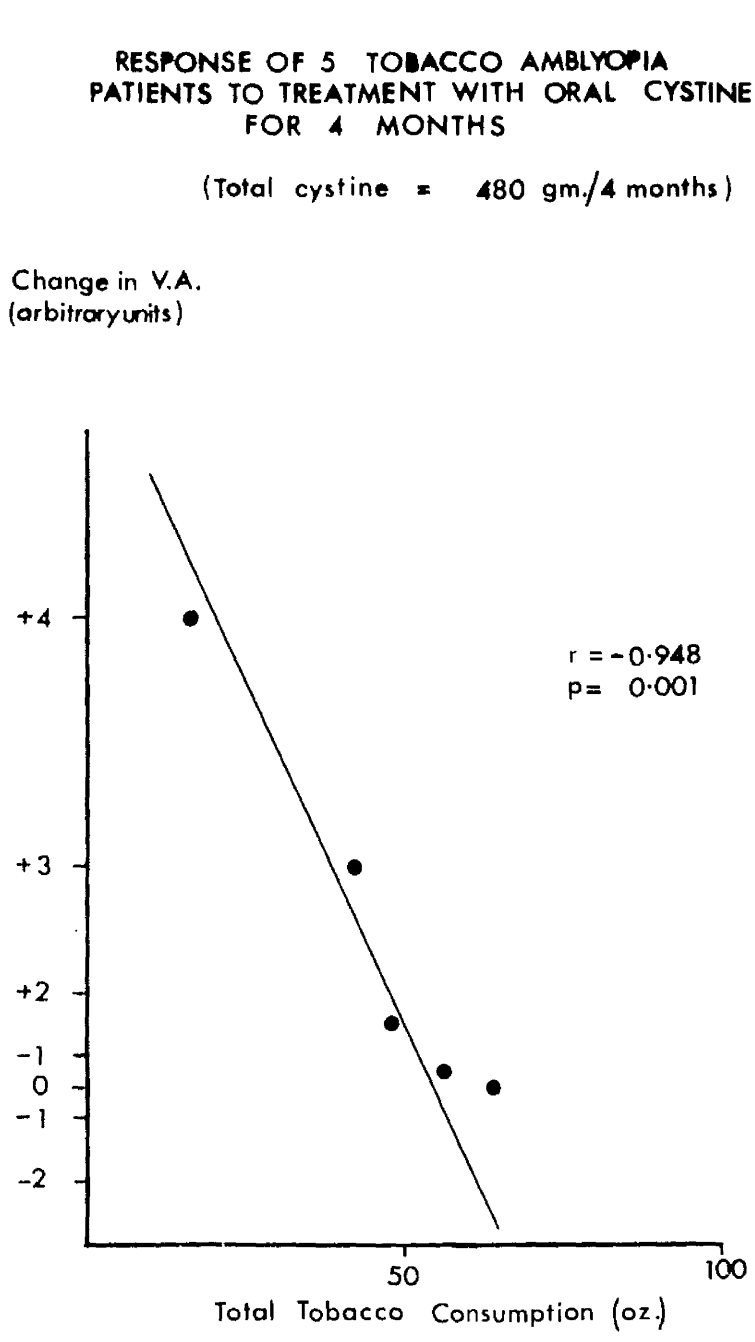


Fig. 3-10. Relationship between the change in Visual Acuity in 5 Tobacco Amblyopia Patients and the total Tobacco Intake during the 4 months of treatment with Oral Cystine. There is a significant correlation ($r = -0.948$, $P = 0.001$).

over the period of treatment was compared with the total intake of tobacco alone. The results are shown in Fig. 3-10., where a significant inverse relationship is evident ($r = -0.948, P = 0.001$). As with the patients on intramuscular hydroxocobalamin therapy, visual improvement is best in those with a low intake of tobacco.

The large dose of 4.0 gm. of cystine per day is used in an attempt to ensure that an adequate quantity of this rather insoluble substance is absorbed, and that a sufficiently high level of cystine is maintained in the tissues to aid in the detoxification of cyanide.

In an effort to ascertain the percentage of the oral preparation of cystine absorbed, estimations of the levels of cystine in the urine of patients receiving this treatment were undertaken. The results indicated that the levels obtained in normal and in thes urines were not statistically significantly different, and that they fell within the published ranges for cystine in the urine.

Summary.

In this study, the response to oral cystine therapy, in terms of thiocyanate, is similar to the previous findings with intramuscular hydroxocobalamin. The increases in plasma thiocyanate and in renal clearance of thiocyanate in patients receiving oral cystine suggest that the cystine is possibly providing sulphur in a form acceptable for the conversion of cyanide to thiocyanate. The sulphur coming from cystine and hence cysteine , could, via methionine, stimulate the synthesis of other sulphur amino acids to provide sulphur in a form acceptable for combination with the cyanide. Some of the cystine may also detoxify cyanide by combining directly with it to form 2-imino-4-thiazolidine carboxylic acid, which can then be excreted in the urine.

The response to therapy, as shown by a mean rate of recovery of vision of the group of 1.5 units, was encouraging, and was apparently dependent upon tobacco intake, aggreeing with findings in the patients treated with intramuscular hydroxocobalamin.

Other Sources of Sulphur for Cyanide Detoxification.

GLUTATHIONE IN RED BLOOD CELLS.

The sulphur-containing peptide, glutathione, is believed to be an important source of reduced sulphhydryl groups in the blood. Ling & Chow (1953) showed that changes in soluble sulphhydryl content in red blood cells reflected primarily changes in glutathione levels.

Vitamin B 12 and Glutathione Levels.

Ling & Chow (1953) studied levels of glutathione in the red cells of vitamin B 12 deficient rats and their vitamin B 12 - injected and normal controls, and in the blood cells of patients with pernicious anaemia in relapse, before and after treatment with vitamin B 12. They concluded that vitamin B 12 deficiency in rats caused a significant reduction in soluble sulphhydryl compounds in the blood (as measured by red cell glutathione). A similar abnormality was also observed in patients with pernicious anaemia in relapse. Administration of vitamin B 12 to either deficient rats or pernicious anaemia patients, was followed by a significant rise in the levels of soluble sulphhydryl compounds in the blood, which eventually became stabilised at a level comparable to those in normal, healthy individuals.

A later report (Ling & Chow, 1954) showed that the blood

levels of glutathione in normal rats could be lowered by the feeding of a high carbohydrate - low fat diet coupled with glucose injections. This was accompanied by a rise in blood sugar levels which persisted long after the cessation of glucose injections. Since vitamin B 12 deficiency likewise causes the lowering of blood glutathione, they suggested that the high carbohydrate intake brought about a rapid depletion of vitamin B 12 stores in the body, owing to increased requirement for the vitamin in carbohydrate metabolism. Administration of glutathione or vitamin B 12 lowered the blood sugar and they concluded that the effect of this vitamin on blood glutathione may be of significance in its role in metabolism. The rapidity with which glutathione acts to lower the blood sugar after its injection, suggests that it may be directly involved in the utilisation of carbohydrate. This supposition is substantiated by the observations of Cavallini (1951) on the role of glutathione in the coupled oxidative decarboxylation of pyruvate, and of Racker (1951) on the mechanism of glyoxalase function and by the discovery by Krimsky & Racker (1952) that glutathione is the prosthetic group of 3-phosphoglyceraldehyde dehydrogenase.

Red Cell Glutathione in Tobacco Amblyopia.

From these findings of Ling & Chow (1953,54), it seemed reasonable to expect reduced levels of glutathione in the red cells of patients suffering from tobacco amblyopia, which most readily

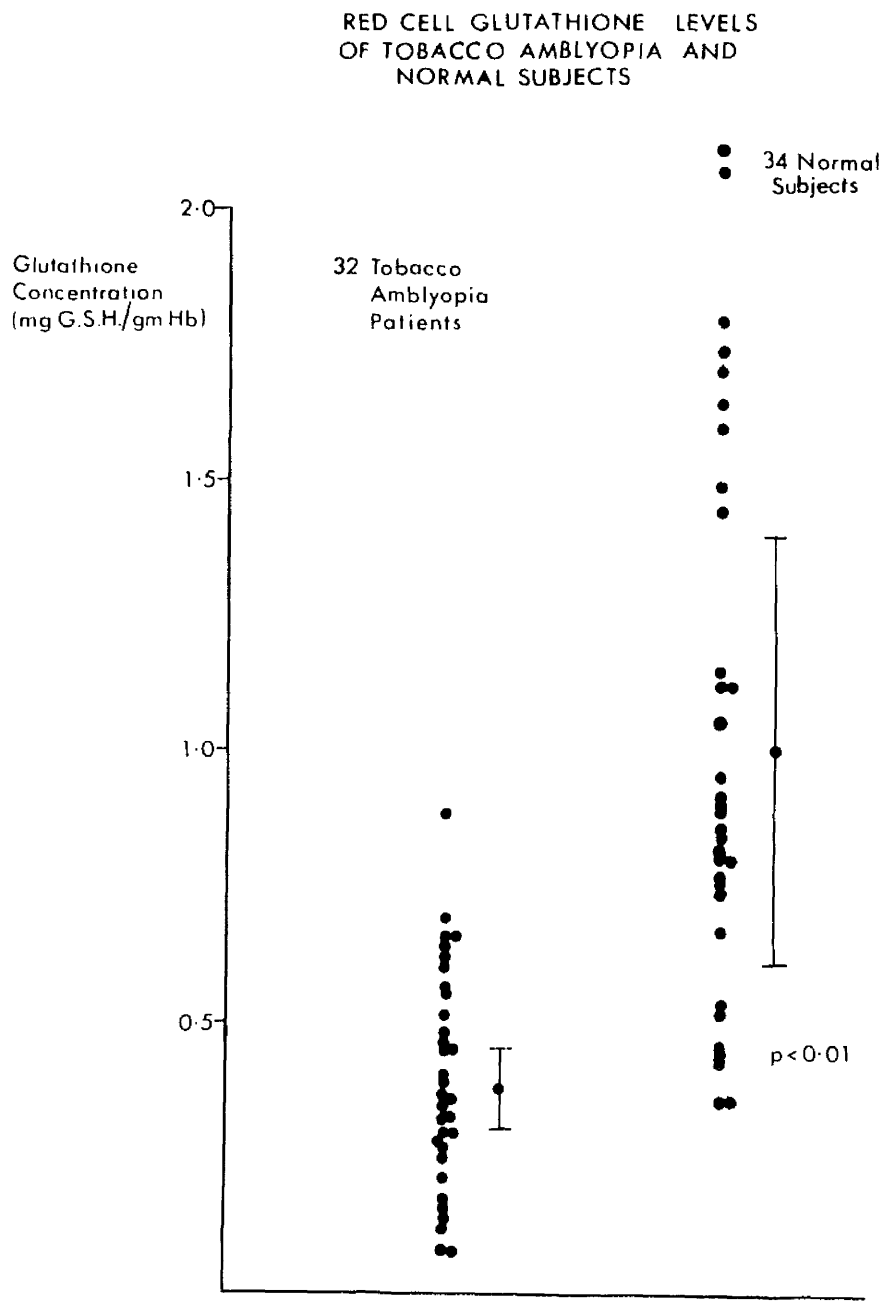


Fig. 3-11. Levels of Red Cell Glutathione in 32 Tobacco Amblyopia Patients and in 34 Normal Subjects. There is a significant difference ($P < 0.005$).

develops in patients whose dietary vitamin B 12 or absorption of vitamin B 12, is, for some reason, defective. Foulds et al. (1969) reported that in a group of 65 tobacco amblyopia patients, 40 % showed reduced serum levels of vitamin B 12 and 45 % had defective vitamin B 12 absorption, and that the levels of serum vitamin B 12 in tobacco amblyopia was significantly lower than in normal, non-amblyopic subjects.

The levels of glutathione in the red cells of 32 tobacco amblyopia patients were measured. Care was taken to exclude from the study any such patients who also had the accompanying condition of pernicious anaemia, while the remainder were presumed to ahve a normal vitamin B 12 status.

Fig. 3-11. shows the concentration of glutathione in the red cells of 32 tobacco amblyopia patients compared with that of 34 normal, non-amblyopic subjects. The normal subjects were those used by a colleague in the Royal Infirmary as controls, anf were, therefore not expected to have either pernicious anaemia or tobacco amblyopia. The ages in both groups were comparable and the estimation conditions were identical. Glutathione concentrations were expressed as mg. of glutathione (G.S.H.) per gm. of haemoglobin (Hb).

Ling & Chow (1953) reported that the level of glutathione in normal healthy individuals ranged from 220 - 270 /whole/100 ml. of cells, which, assuming an average value of 15 gm. of Hb per 100 ml. of whole blood converts to 2.03 to 2.5 mg./gm. of Hb. These results

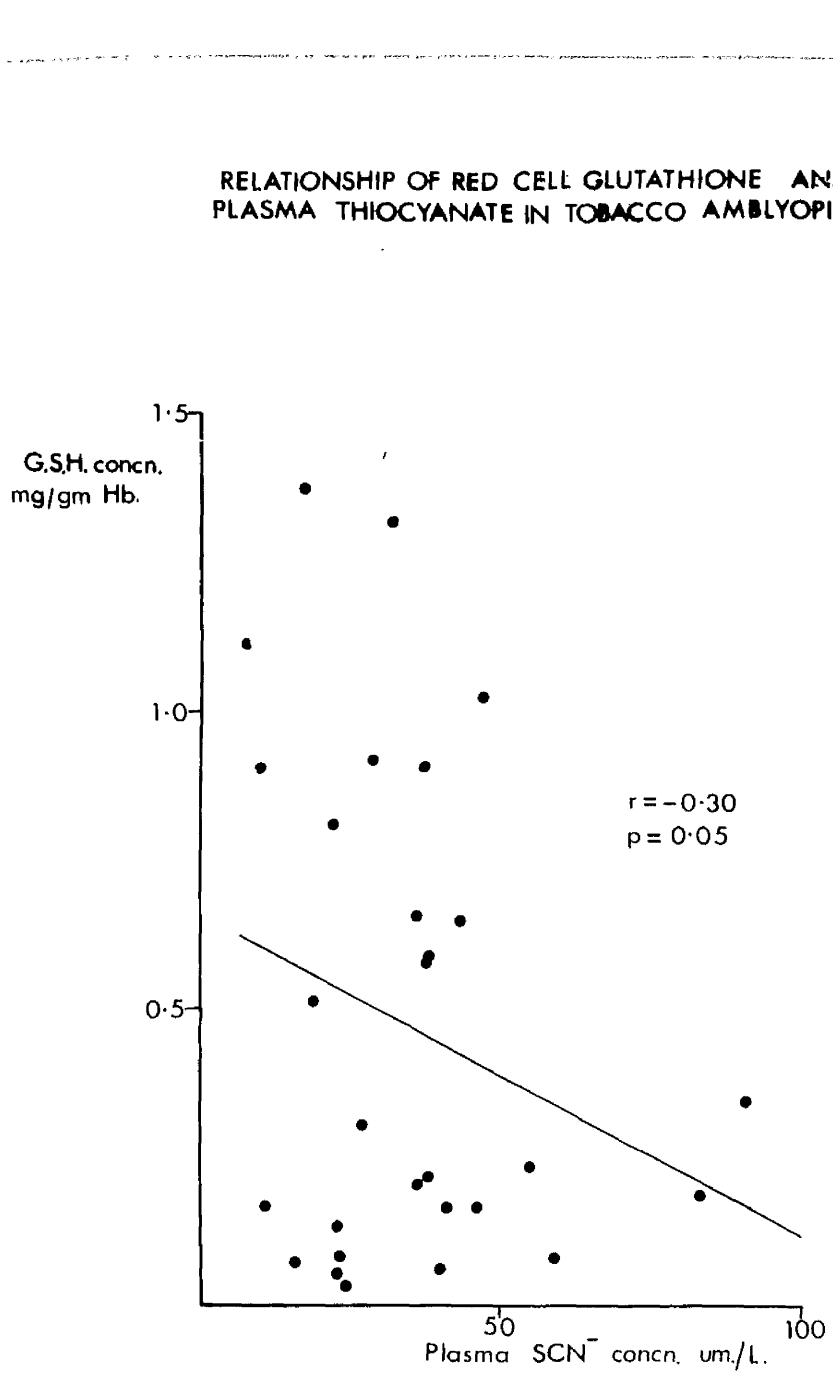


Fig. 3-12. Relationship of Red Cell Glutathione and Plasma Thiocyanate in Tobacco Amblyopia Patients during treatment.

were in accord with earlier findings of Benedict & Gottschall (1932) that the normal glutathione content in human blood averaged 114 umole /100 ml. of whole blood, or 2.3 mg./gm. of Hb. upon conversion. The mean level of glutathione in the normal subjects of 2.01 $\pm$ 0.60 mg. /gm. of Hb., was therefore in complete aggreement with the published range, while the value of 0.786 $\pm$ 0.13 mg./gm. of Hb., found in the tobacco amblyopia patients, was significantly lower ($0.01 > P > 0.005$), and well outside the normal range.

Sequential measurements of red cell glutathione and plasma thiocyanate were made in patients receiving treatment. The results are shown in Fig. 3-12., which suggests that there is a possible inverse relationship between these two factors ($r=-0.30, P=0.05$).

Summary.

In this study, the levels of glutathione in the red cells of patients suffering from tobacco amblyopia were found to be significantly lower than the levels in the normal subjects. This was not unexpected, from the proven vitamin B 12 deficiency in tobacco amblyopia (Heaton et al.,1958;Foulds et al.,1969), and from the reduction in glutathione levels demonstrated in vitamin B 12 deficiency (Ling & Chow,1953), but it is nonetheless surprising, since care had been taken to exclude tobacco amblyopia patients with known pernicious amaemia, and the tobacco amblyopes examined were presumed to have normal vitamin B 12 status.

The possible inverse relationship between red cell glutathione and plasma thiocyanate, makes it likely that glutathione may be involved, either directly or indirectly, in the production of sulphur for the conversion of cyanide to thiocyanate.

SECTION FOUR.

CONCLUSIONS.

These studies support previous suggestions that cyanide is of considerable importance in the development of the toxic optic neuropathies described here, and that its metabolism is very closely linked with both vitamin B 12 and sulphur amino acid metabolism.

In particular, the results suggest the following -

Tobacco smoke contains cyanide - and evidence of its conversion to thiocyanate is the decrease in plasma thiocyanate observed during abstinence, followed by the rise after resumption of smoking. A heavy tobacco consumption could therefore allow a high intake of cyanide.

Measurements of plasma thiocyanate in patients with tobacco amblyopia and in those with Leber's hereditary optic atrophy who smoke, revealed lower levels than those found in normal (non-amblyopic) smokers. This was surprising, since the tobacco, and presumably cyanide intake of these patients tended to be greater than that of the normal smokers, and does suggest that if cyanide derived from tobacco smoke, is to be considered important in the development of these conditions, these patients reveal a reduced ability to convert cyanide to thiocyanate, which could also explain the elevated levels of cyanide found in the plasmas of such patients.

Tobacco amblyopia and Leber's hereditary optic atrophy

patients show an apparent impairment in their excretion of thiocyanate in the untreated state.

The increases in plasma and urinary thiocyanate and in its excretion during the treatment of both tobacco amblyopia and Leber's hereditary optic atrophy patients with intramuscular hydroxocobalamin suggest that it is acting by making more sulphur available for the conversion of cyanide to thiocyanate, and by facilitating its excretion, perhaps by replenishing the body stores of Adenosyl coenzyme B 12, necessary for the biosynthesis of methionine and other sulphur amino acids.

There is no corresponding vitamin B 12 deficiency in Leber's hereditary optic atrophy, yet the response to therapeutic hydroxocobalamin, in terms of thiocyanate, suggests that cyanide is being detoxified in much the same way. The inheritable factor in Leber's hereditary optic atrophy may be a defect in the preparation of a suitable sulphur donor, either before or after the point at which coenzyme B 12 is required.

These increases in the conversion of cyanide to thiocyanate are accompanied by visual improvement. Treatment may be required for longer periods in Leber's hereditary optic atrophy patients where visual improvement is poor, and is continued, provided satisfactory excretion of thiocyanate is maintained.

Although removing the inconvenience of repeated injections, the use of oral hydroxocobalamin has not been as successful as the

intramuscular hydroxocobalamin treatment. The generally poor visual improvement observed in patients receiving oral hydroxocobalamin may not be a typical response, but may be due to the small number of patients studied and/or problems in absorption of sufficient quantities of the preparation. A fuller evaluation of the oral hydroxocobalamin preparation as a replacement or supplement to the intramuscular hydroxocobalamin therapy must therefore use larger doses and a larger number of patients.

The supply of additional sulphur directly, in the form of oral cystine, has been successful in the conversion of cyanide to thiocyanate as shown by the increases in both plasma and urinary thiocyanate levels similar to those previously obtained in patients treated with intramuscular hydroxocobalamin. As before, these changes have also been accompanied by improvement in the patients' vision.

The search for other possible sources of sulphur, led to the tripeptide, glutathione, known to be an important source of reduced sulphhydryl groups in the blood. Measurements of red cell glutathione in patients with tobacco amblyopia revealed lower levels than in normal subjects, even when care had been taken to exclude those tobacco amblyopes who also had known pernicious anaemia. Glutathione is important in the maintenance of the activity of several enzymes, and a deficiency of it may therefore also affect the metabolism of many other compounds, and not particularly those containing sulphur,

necessary for the conversion of cyanide to thiocyanate.

The success of the oral cystine preparation in the treatment of tobacco amblyopia, together with of the finding of reduced levels of glutathione in the red cells of such patients, suggests that future investigation should include a more complete study of the general sulphur metabolism of the patients, with particular reference to those sulphur compounds and their metabolites closely involved in the detoxification of cyanide, which appears to be the important factor in the development of the toxic optic neuropathies dealt with in these studies.

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