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SYMPOSIUM ON 'IMPORTANCE OF ASSESSMENT OF VITAMIN STATUS IN MAN AND ANIMALS'

The level of vitamin C reserves required in man: towards a solution to the controversy

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Many years have passed since vitamin C (ascorbic acid) was first isolated and shown to be the substance in fruit juice that would prevent scurvy (Szent-Györgi, 1928). It is considerably longer since scurvy was a common condition. The introduction of root crops into Europe in the middle ages and the demonstration that fruit juice could be used to treat scurvy (Wilson, 1975) have contributed to making a disease familiar to the ancients, a rare clinical finding today. Yet interest in the vitamin persists. Controversy rages about the dietary requirement for vitamin C in both health and disease. Some researchers believe that daily intakes of up to 10 g are required in some conditions (Lewin, 1976), whilst in contrast it is clear that individuals are able to survive on intakes that are only a fraction of this (Eddy, 1968). The actual role of the vitamin in intermediary metabolism is also in doubt. One essential function has been elucidated, the hydroxylation of proline and lysine during collagen synthesis (Barnes, 1975), but many others have been suggested (Table 1) and although these findings as yet remain inconclusive, many indicate fundamental roles for the vitamin in maintaining health.

Table 1. *Possible functions of vitamin C in man*

Drug metabolism	Beattie & Sherlock, 1976; Zannoni & Sato, 1975.
Folate metabolism	Stokes *et al.* 1975.
Leucocyte function	Anderson & Theron, 1979; Rebora *et al.* 1980.
Cyclic nucleotide metabolism	Lewin, 1976.
Interferon production	Seigel, 1974.
Amine metabolism	Lewin, 1976; Tolbert *et al.* 1979.
Glucose metabolism	Losert *et al.* 1980.
Cholesterol catabolism	Ginter, 1979.
Iron metabolism	Prasad, 1975.
Carnitine synthesis	Hulse *et al.* 1978.

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An accurate assessment of vitamin C reserves will be essential in helping to provide answers to these questions. Studies during the last few years have revealed some surprising findings, but more accurate and specific measurements will be required to refute or substantiate some of the more improbable claims that have been made for vitamin C in recent years (Pauling, 1970; Bjorksten, 1979; Cameron *et al.* 1979). Before considering what estimations could be applied to the problems outlined it will be appropriate to consider what techniques are currently available for the estimation of vitamin C reserves and what their application has revealed.

Assay of vitamin C

Techniques for assaying vitamin C are numerous but the majority are based on two procedures, the formation of an osazone between dehydroascorbic acid and 2,4-dinitrophenylhydrazine (Roe & Kuether, 1943), or the reducing potential of ascorbic acid which can be measured with a number of indicators, most commonly 2,6-dichlorophenol indophenol (Bessey, 1938). There are, however, problems with both procedures. The hydrazine technique is relatively specific (Roe & Kuether, 1943), but estimates not only the biologically active components of vitamin C, ascorbic acid and dehydroascorbic acid, but also the biologically inactive oxidation product of L-dehydroascorbic acid, diketogulonic acid (Schaffert & Kingsley, 1955). On the other hand indophenol is only reduced by ascorbic acid and not by the more oxidized metabolites of the vitamin, but it is susceptible to other reducing agents. Several workers have tried to increase the specificity of the indophenol reaction (Hughes, 1964; Howard & Constable, 1966). Whilst these modifications have increased specificity, the reagent which offers most potential is probably ascorbate oxidase (Avigliano *et al.* 1978) an enzyme which in theory could be utilized in a similar manner to the way glucose oxidase has been used to increase the specificity of the glucose estimation (Morley *et al.* 1968).

A more difficult problem than the development of a specific assay technique is the selection of the tissue or biological fluid in which vitamin C should be estimated. Plasma is readily available, but its vitamin C concentration is rapidly affected by changes in oral intake (Sauberlich, 1975) and in times of poor intake becomes depleted when tissue reserves remain adequate (Bartley *et al.* 1953). Tissue reserves reflect more adequately whole body vitamin stores, but variations from tissue to tissue (Hornig, 1975) can make interpretation of tissue levels difficult. In addition, organ biopsy is unlikely to be used in population studies. Tissue vitamin C levels have been assessed using leucocytes which contain a high concentration of vitamin C. Changes in leucocyte levels also seem to reflect changes in other tissues in the human in the few studies where comparisons have been made (Sauberlich *et al.* 1973; Gerson, 1975). Unfortunately, leucocytes isolated following dextran sedementation of the red cells, the most frequently used technique (Denson & Bowers, 1961), are contaminated with platelets and more strictly should be described as a buffy coat preparation (Attwood *et al.* 1974). Such contamination leads to error in the estimation when different ratios of leucocytes and platelets are obtained and could contribute to the inverse relationship seen

between leucocyte numbers and leucocyte vitamin C concentration (Schorah *et al.* 1978).

Separation of individual cell factions is becoming easier (Attwood *et al.* 1974) and should be used when leucocyte vitamin C measurements are to be made in individuals where leucocyte and platelet numbers may vary greatly, such as the seriously ill. Even then extrapolation of whole-body reserves from white cell measurements in these populations may be difficult as changes occur rapidly in leucocyte vitamin C in acute disease (Hume *et al.* 1972; Hume & Weyers, 1973) and may not necessarily reflect changes in whole-body reserves. However, the study of such changes is of interest as it leads to a better understanding of fluctuations in vitamin C metabolism during disease.

As might be expected, a relationship exists between plasma and leucocyte vitamin C (Loh & Wilson, 1971). The regression is not linear, levels in leucocytes reaching saturation whilst the levels in plasma still continue to rise, or alternatively plasma concentrations falling to levels which are too low to be measured whilst leucocyte reserves remain measurable (Lowry *et al.* 1946; Bartley *et al.* 1953). However, in terms of population studies it is possible to say with hindsight that plasma vitamin C reflects leucocyte (tissue) reserves sufficiently well for it to have been used to assess body vitamin C status, and buffy layer measurements have probably contributed little additional information.

The ideal measurement for any substance which is capable of influencing enzymic activity is a measure of its biological activity. This has long been realized in endocrinology where immunological measurements of hormones may not reflect biological activity (i.e. insulin in the maturity-onset diabetic). As vitamins are often metabolized to co-factors which can directly affect enzymic activity, an estimation of total vitamin concentration may not reflect the ability of that vitamin to carry out its biological function. The biological activity of vitamins can be assessed by measuring the activity of the enzymes for which they act as co-factors, and such techniques are best illustrated by the B vitamins where the activity of a red cell enzyme is commonly used to assess status (Bayoumi & Rosalki, 1976). The functional activity of vitamin C in the hydroxylation of lysine and proline has been measured indirectly by assessing the excretion of these amino acids and their hydroxylated derivatives in urine (Windsor & Williams, 1970; Bates, 1977), but this has been considered an insensitive assessment of vitamin C status (Bates *et al.* 1979). More recently, muscle carnitine has been suggested as a sensitive measure of tissue vitamin C activity, low levels occurring in vitamin C depletion. This finding might explain the lassitude and fatigue which is said to precede clinical scurvy in man (Hughes *et al.* 1980; Hulse *et al.* 1978).

Although the assessment of the biological activity of vitamin C may be difficult and inappropriate in population surveys or day to day diagnostic services, the use of such reference measurements will be required if we are to be able to assess the biological and medical significance of various grades of vitamin C depletion as assessed by simpler techniques, such as plasma estimations.

The estimation of the biological turnover of vitamin C also has a place, although

again essentially as a research procedure. The technique involves the study of the distribution and excretion of isotopically labelled vitamin C given during tissue depletion or repletion of the vitamin (Baker *et al.* 1971) or during steady state conditions where vitamin C intake and reserves are held constant (Kallner *et al.* 1979). Although calculations based on such studies inevitably involve a number of theoretical assumptions, the techniques allow an assessment of the total body vitamin C pool size and an approximation of the vitamin distribution throughout the various compartments of the body. Such studies will be invaluable in assessing changes in vitamin C metabolism which appear to occur in disease (Hume *et al.* 1972; Davies *et al.* 1979) and during therapy (Rivers & Devine, 1975).

Where isotope studies are impractical or unethical an estimation of the degree of vitamin C deficiency and altered rates of ascorbate metabolism can be made by assessing the quantity of vitamin C that has to be taken orally before at least 10% of the dose (11 mg/kg) is excreted unchanged in a 2 h urine sample collected 4 h after the oral dose (Harris & Abbasy, 1937).

Vitamin C reserves in man

In spite of the practical problems in achieving an adequate assessment of vitamin C reserves, the last 20 years have seen many investigations which have helped to illuminate our understanding of vitamin C metabolism in the human.

There are now several publications which indicate that plasma and leucocyte vitamin C concentrations in a large proportion of elderly people are less than the range found in blood donors or younger volunteers (Bowers & Kubik, 1965; Andrews *et al.* 1966; Burr *et al.* 1974). The proportion who have very low values (equivalent to those found in clinical scurvy) increases in the elderly who are in hospital for long periods (Table 2) (Kataria & Rao, 1965; Andrews *et al.* 1966; Schorah, 1979; Schorah *et al.* 1979) and such levels can also be found in younger institutionalized patients (Schorah, 1979). It would seem that low vitamin C intake is in part to blame for such poor reserves in the hospitalized (Eddy, 1968) but

Table 2. *Vitamin C reserves in different populations**

			Vitamin C					
			Plasma (mg/l)			Leucocytes (μg/10^8)		
Subjects	Sex	Age range (years)	Mean	95% range	*n*	Mean	95% range	*n*
Blood donors	♂/♀	19–55	10·3	(3·1–21·0)	31	31·5	(16·2–54·0)	46
Elderly: domiciliary	♀	65–75	4·8	(1·2–13·5)	27	24·1	(7·2–45·0)	15
Elderly: institutionalized	♀	65–75	1·3	(0·6– 3·3)	16	8·2	(2·5–13·5)	7
Cancer of bronchus	♂/♀	47–74	2·5	(0·6– 8·1)	26	9·5	(4·8–13·7)	25
Patients with scurvy†	—	—	0·8	(0·0– 1·5)	34	4·0	(0·0– 8·0)	30

*All samples were collected between January and the first week of May (except from patients with scurvy.

†See Schorah, 1979 for source.

another contributory factor could be disease which depletes vitamin C reserves (Table 2) possibly by increasing its rate of metabolism (Davies *et al.* 1979) in the acutely ill and in the chronic sick (Hume *et al.* 1972; Wilson *et al.* 1973; Kelleher, 1979; Linaker, 1979; Olusi *et al.* 1979). Poor intake, of course, may still be an important factor in precipitating low levels in those with chronic disease.

Two questions arise from these findings. Firstly, why does clinical scurvy remain rare in the elderly, the chronic sick and the institutionalized (Table 2) where vitamin C levels can be as low as those seen in patients with scurvy? The answer may, in part, be the seasonal variation which occurs in ascorbic acid intake (Schorah *et al.* 1978), late winter and early spring being periods of low intake probably due to both the expense of fresh fruit and vegetables and the consumption of old potatoes, low in vitamin C at this time of the year (McCance & Widdowson, 1960). Those who are well (Schorah *et al.* 1978) and those who are in hospital (Table 3) show a fall in vitamin C reserves at this time of the year, but whilst the former remain adequately supplied, a large proportion of those in hospital, who have more marginal reserves, become depleted (Table 3). Frank clinical scurvy takes about 80–120 d to develop under experimental conditions of zero intake (Bartley *et al.* 1953; Hodges *et al.* 1971). It would seem possible that the 4–5 months of reasonable intake during the summer and autumn may well help to prevent the precipitation of frank clinical scurvy in winter and early spring in these at risk populations.

The second question arises from the concern that low levels of vitamin C may compromise the health of these subjects in ways that do not manifest as classical scurvy. The answer to this is not yet available, but its solution could be approached in two ways. Are there symptoms in subjects with low levels that could be attributed to lack of the vitamin, and does their health improve when vitamin C reserves are repleted? There are reports of low vitamin C reserves that do not lead to clinical scurvy, slowing wound healing (Crandon *et al.* 1961; Taylor *et al.* 1974) and affecting collagen metabolism (Windsor & Williams, 1970), but are there more

Table 3. *Seasonal changes in the vitamin C reserves of people in hospital for long periods*

	Vitamin C (mg/l plasma)									
	June				July–September			March–May		
Institution	Mean	SD	*n*	%<1·5*	Mean	SD	*n*	%<1·5	Mean	SD	*n*	%<1·5
Psychiatric†	—		—	—	3·5	±1·7	11	9	1·4	±1·1	11	73
Geriatric	2·5	±0·9	21	10	5·0	±3·9	6	0	1·1	±0·4	21	71

Values for March and May were significantly lower than July–September for both the psychiatric and the geriatric patients ($P<0·005$ and $P<0·05$ respectively) and were lower than June in the geriatric patients ($P<0·001$) (paired t test).

*Upper limit of plasma levels found in patients with scurvy (see Table 2).

†Both summer and spring samples from these patients were assayed in the same analysis batch. Samples were stored at −40° and no effect of storage on vitamin C values was found.

widespread effects? Vitamin C has not been shown to be essential for any of the proposed roles listed in Table 1, but this does not mean that the vitamin could not add important lubrication to these metabolic processes and thus make malfunctions less likely to occur. There is some evidence that this might be the case.

In the absence of clinical scurvy, low vitamin C reserves have been found to be associated with behavioural changes (Milner, 1963; Kinsman & Hood, 1971) poor recovery from surgical procedures (Hill *et al.* 1977), the presence of sublingual petechiae (Taylor, 1966; Andrews & Brook, 1966), weight loss (Bates *et al.* 1979) and poor immune function (Thomas & Holt, 1978). Supplementation with vitamin C in such groups has led to an improvement in some of these parameters (Milner, 1963; Kinsman & Hood, 1971; Schorah *et al.* 1979; Anderson & Theron, 1979; Rebora *et al.* 1980). Such findings suggest that low vitamin C reserves may have a deleterious effect on health in addition to the accepted symptoms of scurvy. This may be especially significant in the sick who not only have an increased frequency of low vitamin C values but in whom it is important to maintain immune function, protein reserves and avoid psychological deterioration.

In addition to the arguments about the need to prevent marginal vitamin C depletion, there has been support for the idea that pharmacological intakes of vitamin C (>500 g/d) could be effective in treating a number of conditions such as respiratory tract infections (Bjorksten, 1979), malignancy (Cameron *et al.* 1979) and hypercholesterolaemia (Ginter, 1979). Trials of the vitamin in these conditions have, however, failed to lend support to this idea (Chalmers, 1975; Creagan *et al.* 1979) with the exception of hypercholesterolaemia where the vitamin seems to reduce plasma cholesterol by increasing the conversion of cholesterol to bile acids (Ginter, 1979). It remains to be seen whether such treatment will be effective in reducing the incidence of ischaemic heart disease.

Future studies

It is clear that much remains to be done. Initially, the ideal level of body vitamin C reserve must be established by identifying clinical, physiological and biochemical signs of marginal deficiency and measuring the tissue vitamin C concentrations at which these occur. This will require an accurate technique for vitamin C determination which distinguishes ascorbic acid from the more oxidized dehydroascorbic acid and diketogulonic acid. This assessment of tissue vitamin reserve must then be referred to a simple procedure which must be rapid and robust enough to be used routinely to identify vitamin C deficiency. It seems unlikely that any technique will be ideal here, but a description of its limitations will reduce error of interpretation.

We must then investigate what dietary intake will maintain ideal vitamin C reserves. This may vary in different conditions so an estimate of the rate of the metabolism and excretion of the vitamin and its metabolites will be required in both health and disease. Iatrogenic effects on vitamin C must also be examined for there is evidence that drugs and vitamin C mutually affect each others metabolism (Rivers & Devine, 1975; Zannoni & Sato, 1975).

Such analytical efforts may seem excessive. It may be that we shall find that vitamin C has little contribution to make to maintaining health provided that the minimum intake needed to prevent clinical scurvy (10 mg/d) is maintained. I suspect, however, from evidence available, that although vitamin C may not be essential in concentrations above this minimum, higher levels will be a bonus, possibly helping to reduce organ deterioration and making us less susceptible to disease. However, we lack evidence for this, but considering how widespread low tissue reserves are in the UK and that a significant proportion of those with chronic disease or who are institutionalized have levels equivalent to those seen in scurvy, the need for a thorough investigation of vitamin C status becomes obvious.

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