SELENIUM AND SULPHUR IN HUMAN TISSUES BY ACTIVATION ANALYSIS

THE DETERMINATION OF SELENIUM AND SULPHUR IN HUMAN TISSUES BY MEANS OF RADIOACTIVATION ANALYSIS

BY

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SCOPE AND CONTENTS:

A procedure was established for instrumental radioactivation analysis of selenium in biological materials using Se^{77m}, and used to determine selenium bound to plasma and cells of blood from 254 normal individuals. Variations with time, age, sex and hematocrit were investigated. Analysis of subfractions of plasma and cells indicated the highest selenium concentration in the plasma alpha and beta globulins. The selenium content of other tissues was determined for two individuals.

A method was devised for instrumental radioactivation analysis of sulphur in dialyzed biological material using S^{37}. The bound sulphur content of samples of plasma and cells from normal human blood was determined. The method would appear to have application in the instrumental activation analysis for other elements in various matrices.

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CHAPTER 1

GENERAL INTRODUCTION

The number of elements recognized as being normally present in living tissues has increased enormously within the last few years. Many elements, such as carbon, hydrogen, oxygen, nitrogen, phosphorus and calcium are known to be necessary for the existence of life by virtue not only of their participation in the macroscopic chemistry of life, but also their role in the structure of protein and bone. However, most plants and higher organisms also are found to contain small amounts of elements, a few of which have a known function, but many whose purpose, if any, is as yet unknown. In fact, a human, being a sort of garbage disposal unit which consumes quantities of soil along with a wide variety of plant and animal material, is expected to contain nearly every element at low levels. It has been the analytical difficulties associated with measuring these low levels, which has led to the term "trace element", since until recent years analyses could only indicate "traces" of these elements, not exact amounts. The development of sensitive analytical techniques has stimulated workers to establish the function of the minor elemental constituents of biological systems. "Essential" trace elements, those which are necessary for existence of an organism by virtue of their participation in life-sustaining biochemical reactions, are being found in virtually every living system. Most,

such as zinc in carbonic anhydrase, and manganese in arginase, activate catalytic enzymes.

General awareness of trace element importance has derived from the widespread public knowledge of systems such as protein-bound iodine, and its participation in thyroid function. These bound, or non-dialyzable elements, including the iron in hemoglobin, and the magnesium in chlorophyll, are of particular interest since they commonly play a direct part in protein function. Inorganic, or non-bound elements maintain the ionic strength of biological fluids, and serve as a pool from which an organism can draw amounts necessary for metabolism. The amount of research devoted to the study of low level elemental constituents of tissue is increasing rapidly.

In view of the importance of trace elements in metabolism, the present research programme was begun by a survey of the elements present in human blood. Because of the sensitivity of the nuclear technique known as radioactivation analysis, and the availability of a reactor, this technique was chosen to carry out the survey. Blood was selected for analysis not only because of its fundamental participation in the transport of nutrients, enzymes, and other reagents to all parts of the body, but also because its suggested removal from normal healthy persons encountered less opposition than other tissues.

Although many of the elements reported in the literature as existing in human blood, such as zinc and manganese, were found in the survey, the only element found, which had not previously

been reported to be bound to non-dialyzable components of normal human blood, was selenium. Since selenium is a member of the important oxygen-sulphur family of the periodic table of the elements, it was considered worthwhile to make a more detailed study of its occurrence in human tissue. It was hoped to establish whether selenium appeared at random in the population or was distributed in a significant fashion. It was also hoped to determine the chemical form of selenium in tissue in order to shed light on its role in human metabolism. It was conceivable that specific selenoproteins, each with a particular function, exist in tissue.

Study of the conditions of analysis of selenium by means of instrumental methods, following radioactivation, revealed an interference due to the presence of oxygen which limited the sensitivity and accuracy of the procedure. A correction method was devised, based on the amount of oxygen present, which enabled instrumental activation analysis of selenium in dialyzed tissue with a sensitivity and accuracy allowing significant intercomparison of results. The introduction of other trace elements was avoided in sample preparation, in order that samples may be used in further studies.

Using the technique developed, analyses were carried out for the selenium bound to the plasma and cell fractions of the blood of a population of 254 normal individuals. All blood samples were found to contain selenium, and when results were analyzed statistically, a gaussian type of distribution was found for the

population, similar to that of other essential trace elements. Variations in blood selenium with time, age, sex, hematocrit, and living conditions, were investigated.

Analysis of protein subfractions of blood plasma and cells, revealed that the major percentage of plasma selenium was concentrated in the alpha and beta globulins. Attempts to pinpoint further, the chemical nature of bound selenium, by hydrolysis and degradation of tissue, did not lead to conclusive results, but suggested further studies which may shed light on the problem.

During the course of the research, it became evident that the selenium to sulphur ratio varied considerably in the protein from various blood fractions. With the object of determining the accurate sulphur content of prepared samples, a study was begun of the possibility of carrying out direct instrumental activation analysis for sulphur using the isotope S^{37}. Such a procedure had not previously been recorded in the literature. However, a new correction technique was devised which permitted analysis of the sulphur bound to the non-dialyzable portions of tissue. Using this technique, several samples of the plasma and cell fractions of normal human blood were analyzed. The method would appear to have wider applicability in the instrumental activation analysis for other elements.

CHAPTER 2

HISTORICAL INTRODUCTION

A. The Biological Significance of Selenium

(a) Early history

Since early in the history of modern man, the physiological impact of selenium on plants and animals, although unrecognized as such until recently, has been documented in numerous sources. In fact, the historical aspects of the disease syndromes produced by selenium are as interesting as they are unique in the way in which they shed light on the biological significance of the element.

The writings of Marco Polo, circa 1295 A.D., refer to the poisoning of his pack animals near the Tibetan border of western China, as a result of eating certain plants now known to have a high selenium content (1). Such plants, called "selenium indicators" because their growth and distribution is restricted to seleniferous formations and soils, have been shown to have the ability to convert inorganic selenium into an available form (2). Two classic and picturesque disease states are produced in animals consuming selenium-rich plant material. The "blind staggers", or acute selenium poisoning results from the ingestion of quantities of species of the selenium indicators such as Astragalus and Stanleya, whereas "alkali disease", or chronic selenium poisoning results from the ingestion of seleniferous grains and grasses (3).

Acute poisoning is characterized by blindness, paralysis, abdominal pain, salivation, and grating of the teeth. The symptoms of chronic poisoning are lethargy, emaciation, stiffness, lameness, soreness and sloughing of hoofs, loss of hair, and atrophy of the heart and liver. Death often results from starvation and thirst because in addition to loss of appetite, the lameness and pain from the condition of the hoofs are so severe that the animals are unwilling to move about to secure food and water (3).

The earliest recorded disease syndromes in humans, resulting from selenium poisoning, were chronic selenoses resembling alkali disease, recorded in Colombia by Father Pedro Simon in 1560, and in the Irapuato district of Mexico. However, it was selenium poisoning and subsequent losses in the southwestern United States of domestic animals, such as cavalry horses at Fort Randall in Nebraska in 1857, and horses, cattle and sheep in the Wyoming and Nebraska areas from 1893 on, which stimulated early investigations of the origin of the disease. During the summers of 1907 and 1908, more than 15,000 sheep died in Wyoming of selenium poisoning. It was gradually recognized that vegetation in certain restricted areas was responsible, but not until 1931 was it suggested that selenium was the active ingredient. Subsequent studies of vegetation in the United States and Canada have revealed concentrations of several thousand parts per million in the indicator plants which flourish on seleniferous soils (4).

(b) Recent studies of selenium toxicology

The administration of selenium compounds to livestock or laboratory animals produces toxic symptoms, but does not produce

typical alkali disease or blind staggers. The selenium compounds in plants which can produce different disease syndromes are not known. A great many studies have been made in the last twenty years, on the effects of oral and injected selenium compounds, both inorganic and organic, on every conceivable type of organism ranging from the protozoa, through fish, and chickens, to the higher animals such as rats, dogs, cattle and sheep (5, 6). Subacute, acute, and chronic symptoms induced by selenium administration, as well as effects on reproduction and congenital malformations, and distribution of selenium in the tissues of animals treated, are dealt with at great length in the literature. Rosenfeld and Beath have compiled this information in detail, and also summarized attempts to combat selenium poisoning by hastening its excretion, or by administering metabolic antagonists to selenium compounds, such as arsenic and sulphur (7).

Attempts to associate certain clinical groupings of humans living in seleniferous areas of the United States, with the selenium levels of their food and drinking water, have yielded inconclusive results (8). However, chronic selenosis is known in humans from Colombia, South America, and results in malformed children and extensive loss of hair and nails (9). Selenium is suspected to increase susceptibility to dental decay in children drinking water from certain seleniferous areas (10). Specific cases of poisoning or irritation due to industrial exposure to selenium, have been noted (11), as well as a few cases due to the ingestion of seleniferous foods (12). Chronic selenium poisoning

leading to hepatic cirrhosis in Egyptian children has been established (13). Maximum permissible selenium levels in foods and drinking water have been tentatively set (5).

(c) Selenium as an essential nutritional requirement

Disease syndromes in animals are known to result from both an excess and a lack of selenium in the diet. The former have an extensive history and were discussed above in sections (a) and (b). However, diseases produced by a dietary selenium deficiency, whose investigation has provided the chief evidence for the essential nature of selenium, have been recognized and studied only relatively recently. The steadily increasing numbers of conferences and symposia devoted either partially or entirely to the nutritional significance of selenium reflect the growing interest in the problem (14, 15, 16, 17, 18). The first International Symposium on Selenium in Biomedicine is being held at Oregon State University, September 6-8, 1966, and will review all known aspects of the metabolism of selenium.

After the discovery of vitamin E (α-tocopherol) in animal tissues, several vitamin E-deficiency diseases and conditions in animals were found. Among these are included muscular dystrophy in rabbits, guinea pigs and lambs, fatal exudative diathesis and encephalomalacia in chicks, and necrotic liver degeneration in the rat, pig and other species. However, in 1951, Schwarz discovered that a substance isolated from tissues and plants, which he called Factor 3, protected rats from experimental liver necrosis (19). The effective agent in Factor 3 was established in 1957, to be

selenium (20). It was subsequently shown that inorganic selenium as selenite or selenate could substitute for Factor 3 in the treatment or prevention of necrosis. The remarkable prophylactic feature of selenium was its effectiveness in extremely minute doses. As little as 4 micrograms of selenium as selenite per 100 grams of diet, was sufficient to prevent hepatic necrosis (20). In this respect selenium was 500 times more active than vitamin E, and 250,000 times more active than selenium-free cystine.

Nearly all naturally-occurring or laboratory-induced disease states which have been shown to be responsive to vitamin E therapy, are also responsive to selenium. For example, in vitamin E- and selenium-depleted weanling rats, either selenium or α-tocopherol will protect against hepatic necrosis, and in similarly-depleted lambs, selenium and to a lesser extent α-tocopherol, will protect against white muscle disease (18). The action of selenium in producing growth response and preventing white muscle disease in lambs, suggests that selenium and vitamin E are interrelated in their metabolic functions, but that vitamin E cannot completely replace the need for selenium in livestock nutrition (21).

Although it has been shown that selenium is an essential nutrient for certain plants and animals (22), and a selenium cycle in nature has been proposed analogous to those known for carbon, nitrogen and sulphur (23), little is known of the nutritional

requirements for selenium in humans. In view of the similarities between certain selenium-responsive disease states in animals, and corresponding states in humans, knowledge of these requirements would seem to be extremely important. The disease "kwashiorkor" found in Jamaican children, and formerly attributed to protein malnutrition, may be associated with selenium deficiency (24). Supplementation of the diet of two affected individuals with traces of selenium, gave immediate response in weight gain and increase in food intake.

An area of selenium prophylaxis, commonly ignored or bypassed by investigators, concerns the treatment of certain abnormal skin conditions in humans. Napoleon is known to have sent soldiers with skin problems to bathe in the selenium-containing mineral waters of Roche-Posay in France, and even planned to set up a hospital in the area (25). The beneficial effects of selenium with regard to skin diseases were recognized recently, when attempts were made to use selenium compounds for the treatment of dandruff, on the basis of the effectiveness of analogous sulphur compounds. Selenium sulphide in detergent suspension has been found to be effective in controlling seborrheic dermatitis (severe dandruff), blepharitis marginalis (granulation of the eyelids) and 85% to 95% of all forms of mild common dandruff (26). Although patents in the commercial development of selenium-containing preparations have assumed that, similar to sulphur, selenium is a potent fungicide, recent studies have indicated that seborrheic dermatitis is not necessarily caused by microorganisms,

and that in fact, very dilute solutions of selenite may be as effective as gross amounts of suspended SeS_2 in the treatment of this condition (27). It is possible that selenium reduces the large number of free-SH groups present, and thus repairs abnormalities of the keratin structure of diseased skin (28, 29).

From the knowledge of diseases of animals and humans, which are both caused by and preventable or treatable by selenium, it may be concluded that further studies of the detailed biochemical behaviour of, and metabolic pathways followed by selenium, are of the utmost importance.

(d) Biochemistry and metabolism of selenium

Although a great deal of work has been carried out in attempts to elucidate the behaviour of selenium at a molecular level, and its function in metabolic processes, little agreement has been reached in the literature, and few definitive conclusions have been drawn. The most recent symposium called to bring together workers involved in studies in this area, was run almost in the manner of a debate (18).

Basically, there have been two schools of thought on the function of selenium in living organisms. The first, whose foremost protagonist is Dr. K. Schwarz, holds that selenium compounds play a specific physiological role. The second, championed by Dr. A. L. Tappel, believes that active forms of selenium behave as antioxidants, and that this feature explains and reconciles the interrelationship of selenium and vitamin E.

After the isolation of Factor 3 selenium by Schwarz, many experiments were carried out which involved the administration of

small amounts of radioisotopically-labelled selenium compounds to a variety of organisms. The localization of activity in various metabolic end products such as tissue protein, has been used as evidence for the postulation of metabolic pathways and function of selenium. Animals have shown the ability to reduce inorganic selenium compounds and incorporate the selenium into the amino acids selenocystine and selenomethionine (30). Lower forms of life such as Escherichia coli have also shown this ability (31). Radioselenium has been found after injection into dogs, to concentrate first in the albumin fraction of blood serum, and then to be transferred into the alpha and beta globulins (32). Se^{75}-selenomethionine has been shown to be incorporated into animal tissues (33, 34). However, biochemical differences in the metabolism of selenium and sulphur have been found for species of the plant Astragalus which are selenium accumulators and non-accumulators (35). A distinct difference between selenium and sulphur metabolism in higher organisms is suggested by Schwarz, who concluded that it is most likely that selenium in normal tissues is not found in the form of sulphur amino acid analogues (36).

Tappel and co-workers have demonstrated the radioprotective and antioxidant properties of selenium compounds. Vitamin E has been postulated as a tissue antioxidant which prevents damaging lipid peroxidation reactions (37). Selenium has been shown to have similar lipid antioxidant behaviour (38). In addition, selenium compounds have been shown to be effective in decomposing tissue

peroxides (39) and scavenging free radicals (37, 40). Although these results have led Tappel to conclude that a primary function of selenium in tissue is to participate in the inhibition of free-radical peroxidation damage to the lipid moiety of lipoproteins (42), it has been pointed out that his experiments were conducted using levels of seleno amino acids which were many-fold in excess of the amount of selenium compounds present in normal tissue (43). This point might well be emphasized for the majority of the research which has been carried out on the biochemistry and metabolism of selenium. Additional evidence suggesting that selenium may not function in tissue primarily as an antioxidant, comes from nutritional studies. Three groups of diseases related to selenium and vitamin E deficiency are recognized in animals: those which are strictly vitamin E-responsive and are not affected by selenium supplements, those responsive to selenium or vitamin E, and those which are selenium responsive and not influenced by vitamin E. Into the last category fall white muscle disease in sheep from certain parts of the world, and the failure of reproduction of rats on selenium-deficient basal diets to which ample amounts of vitamin E have been added (43). It is this group which provides the strongest arguments for the hypothesis that selenium has a specific physiological role, and does not merely act as an antioxidant.

Caldwell and Tappel have proposed an involvement for selenium compounds in the biologically important oxidation-reduction reactions of thiols in tissues (44). Selenocystine, but not cystine, was shown by these workers to accelerate oxidation of

cysteine, glutathione and homocysteine, and yet to protect the sensitive sulfhydryl groups of sulfhydryl enzymes such as creatine kinase and alcohol dehydrogenase, which must remain intact for the proper functioning of these enzymes. On the basis of this evidence, the authors suggest that the biochemical activity of selenium results from its combined reactions in a variety of redox systems, rather than from a specific and limited metabolic role (44).

The recent work of Desai and Scott proposes an explanation for the apparent interrelationship and interdependency of selenium and vitamin E (45). Studies involving the feeding of labelled selenium compounds and labelled α-tocopherol to chicks, indicated that vitamin E may, in fact, be carried by a selenolipoprotein fraction associated with plasma gamma globulin. It was concluded that at least one biological role of selenium appears to lie in a selenium-containing compound which acts as a carrier of vitamin E, and which may function in absorption, retention, prevention of destruction and perhaps transfer across cell membranes, of d-α-tocopherol, thereby enhancing its biological activity in the blood and throughout the body (45). This theory would ascribe great importance to the selenium bound to certain blood proteins.

It is entirely possible that selenium compounds take part in several or all of the biochemical functions which have been suggested. The number of suspected metabolic pathways in which selenium is active, is rapidly increasing, and reflects the probable essential nature of this trace element in many phases of the chemistry of life. In fact it has even been suggested that the

high selenium content observed in the retina of the eyes of certain animals, may be related to the visual acuity of these species (46).

B. The Chemistry and Analysis of Selenium

Selenium is an element belonging to Group VI A of the periodic table, which includes oxygen, sulphur, selenium, tellurium and polonium. Like sulphur, selenium exhibits allotropy, but has more metallic character and a higher electronegativity. Selenium shows oxidation states of -2, -1, 0, +2, +4 and +6, and forms compounds analogous to, but more unstable than, most known inorganic and organic sulphur compounds (47, 48). The chemistry of selenium has a history of interest comparable to that of its nutritional aspects. The most interesting industrial uses of selenium are those depending on its remarkable photoelectric properties (49). Selenium generates a flow of electrons when it is exposed to light, and thus has been employed in a wide variety of light-sensing devices which require no external source of current. It is interesting to note that a selenium photocell has nearly the same spectral sensitivity as the human eye. Other commercial uses include the construction of rectifiers, the pigmentation of glass to form the ruby-red colour used in automobile tail lights, inclusion in rubber to increase its resiliency, and in inks and oils to prevent oxidation. The selenium sulphide suspension marketed under the name "Selsun", and used in the treatment of skin ailments, has previously been mentioned. It is undoubtedly the industrial potential of selenium compounds which has generated the vast amount

of research which has been carried out in the field of selenium chemistry. Details of this chemistry may be found in various texts and monographs devoted partially or exclusively to selenium (50, 51, 52). Of particular interest with regard to the biological behaviour of selenium compounds, is the fact that the selenium analogues of cystine and methionine differ sufficiently in chemistry from the corresponding sulphur amino acids, to exhibit different chromatographic behaviour (53, 54).

It has been stated that "the importance of establishing accurate and sensitive methods for the analysis of selenium cannot be overemphasized. It is extremely important to know normal as well as abnormal values of selenium in tissues, before the nutritional significance of this trace element may be evaluated in humans" (55). Because of the commercial applications of selenium, methods for analysis at relatively macroscopic levels have been available for many years. Methods of dissolving samples may be classified into three groups: wet processing, fusion in a sealed tube, and combustion methods. In the case of organic selenium-containing compounds, the so-called Schöniger method of combustion has been widely used and involves burning the sample in a flask filled with oxygen (56). Digestion with a mixture of sulphuric and nitric acids has also been used extensively in many laboratories (57). Problems associated with these "classical" techniques have been discussed, and modifications rendering the Schöniger method more simple, rapid and accurate, have been developed (58).

Errors in analysis, such as contamination, or losses of selenium by volatilization during ashing, have been found not to

be a problem in the determination of quantities of selenium above about 0.01 milligrams using the methods described above. However, in the determination of smaller amounts, of the order of a microgram or less, the possibility of introducing trace quantities of selenium by contamination, and of incurring other errors, has led to the refinement of the classical methods and use of many "modern" techniques in analysis. The importance of detecting and measuring trace amounts of selenium was made clear by the recognition of the function of minute amounts of selenium in various disease syndromes (59).

Including radioactivation analysis, which will be discussed in the next section, nearly every analytical tool known to modern science has been used in the analysis for trace quantities of selenium. In most of the methods for the determination of microgram quantities in biological material, which has been the matrix of greatest interest, the sample is ashed in a manner similar to that used in the classical techniques (60). Unfortunately the extensive chemical manipulations involved in ashing are very susceptible to the introduction of amounts of selenium comparable to those already present, and to the loss of selenium by volatilization (61).

After ashing, the most common analytical procedure has been to introduce a substance which reacts with selenium to develop a colour, or form a fluorescent compound. The concentration of the selenium is thus measured by colorimetry, spectrophotometry or fluorometry. Diamino compounds such as 4,5-diamino-6-

thiopyrimidine (62), substituted phenylenediamines (63), 2,3-diaminonaphthalene (64, 65) and 3,3-diaminobenzidine (61, 66, 67, 68, 69) have most commonly been used in the fluorometric method and give a sensitivity of about 0.1 micrograms of selenium. The popularity of the most extensively-used reagent, 3,3-diaminobenzidine would be expected to wane in light of its now suspected carcinogenic behaviour (70).

Selenium has been analyzed in biological materials (after ashing where necessary) by reduction to elemental selenium, the colour of which is used as a measure of its concentration (71), by the ring oven technique (72) and with the aid of millipore filters (73). In the list of more recent and novel methods may be mentioned the use of a catalytic reaction (74), isotope dilution (75), and atomic absorption spectroscopy (76). The sensitivity of this latter technique is of the order of 1 part per million. Finally, selenium has been determined polarographically in urine and other biological materials with a sensitivity of about 0.2 micrograms (77, 78), and by means of X-ray fluorescence spectroscopy (79). All of the methods mentioned are subject to their own particular sources of error, interference and lack of precision of results. The accuracy of results are commonly checked by comparison with those of neutron activation analysis (64, 69).

Very few analyses for selenium in normal human tissues have been reported in the literature. Of these, only one or two have been performed using the techniques described above

(i.e. apart from activation analysis). The greatest disadvantage of most of the methods is the length of time required to carry them out. Gofman and co-workers found samples of whole human blood serum to contain from 0.05 to 0.15 parts per million of selenium (79).

C. Radioactivation Analysis

(a) General

Perhaps no other analytical technique in the history of chemistry has become so "fashionable" and widely used in such a variety of applications in such a short length of time, as radioactivation analysis. In 1965, de Hevesy introduced the Proceedings of the Symposium on Radiochemical Methods of Analysis in Salzburg, Austria, and included an account of how he himself first conceived the principles underlying activation analysis, and carried out the first analysis by means of the technique in 1938 (80). It is really only in the last ten years, however, that extensive use has been made of activation analysis. In 1955, papers were presented by A. A. Smales and W. W. Meinke at a symposium on trace analysis, outlining the procedures involved in, and sensitivity of activation analysis compared to other methods (81). It is to these two men which must be given the credit for "popularizing" the technique in its early days.

The extensive proliferation of the number of papers in every conceivable field of study over the last few years, is an indication of how widely activation analysis has come to be used, and in what esteem it is held. It would be futile to attempt to

discuss even a small percentage of the literature dealing with activation analysis. Excellent textbooks have appeared recently, which treat all aspects of the method, its applications and sources of error in great detail (82, 83, 84, 85, 86, 87). In addition, various conferences and symposia have been held, which were devoted partially or entirely to applications and new developments in activation analysis (88, 89, 90, 91, 92). Several bibliographical compilations of activation analysis literature have been made (93, 94, 95), and an excellent card index file has been produced and is being kept up to date and distributed free of charge (96).

The basic principle of activation analysis is that an isotope of an element, when irradiated by neutrons or charged particles, can undergo a nuclear transformation to produce a radioactive nuclide. After the radionuclide is formed, and its emanations have been characterized by radiation detection equipment, qualitative and quantitative inferences can be made of the elemental composition of the original sample before irradiation. Radioactivation differs from most other methods of chemical analysis in that it is based on the properties of nuclei and not on the behaviour of the outer electrons. Thus it is a method of elemental analysis only, and can be used only indirectly in a few cases, to analyze for molecules. The fact that it can be used to differentiate between different isotopes of a single element, while of no importance in most applications due to the constancy of isotopic composition of most elements, is useful in certain geochemical problems.

Radioactivation analysis perhaps comes closest to satisfying the criteria of the ideal analytical technique of all those known to modern science. These criteria are accuracy, precision, specificity, nondestructivity, speed, economy and universal applicability.

When an element is exposed to a flux of neutrons or charged particles, the rate of change of the number of target atoms N of the isotope of the element undergoing the nuclear reaction in question, is given by the expression

$$\frac{dN}{dt} = -f \sigma N$$

where f is the flux of bombarding particles in units of particles per square centimeter per second, and σ is the isotopic cross section for the nuclear reaction in units of square centimeters per target atom (and might therefore be considered as the "effective" area of the atom for the nuclear reaction). Integration of this simple first order expression yields the equation

$$N = N_o e^{-f \sigma t}$$

where N_o is the number of target atoms at the start of irradiation. Since $f \sigma t$ is generally very small, it may be assumed that the number of target atoms is essentially unchanged, and that $N_o = N$ throughout irradiation.

The rate of growth of the number of radioactive atoms N', is given for practical purposes, therefore, by the expression

$$\frac{dN'}{dt} = f \sigma N - \lambda N'$$

where λ is the radioactive decay constant for the radionuclide

formed, being connected with the half-life T of the nuclide, by the relation $\lambda = 0.693/T$. Integration of this expression over the period of irradiation t, yields

$$N' = f \sigma N (1 - e^{-\lambda t})/\lambda$$

The amount of radioactivity A_t, in disintegrations per second, exhibited by the atoms N' produced up to a time t, is given by the expression

$$A_t = \lambda N' = f \sigma N (1 - e^{-\lambda t}) = f \sigma N (1 - e^{-0.693t/T})$$

where t and T are expressed in the same units.

If a weight W grams of an element (including all isotopes) of atomic weight M is irradiated, then

$$A_t = 6 \times 10^{23} f \sigma \theta W (1-e^{-0.693t/T})/M$$

where θ is the abundance of the particular isotope under consideration. After the irradiation is stopped, the activity formed will decay with its characteristic half-life, so that at a time d (in the same units as t and T) after cessation of the irradiation, the activity becomes (97)

$$A = 6 \times 10^{23} f \sigma \theta W (1 - e^{-0.693t/T}) (e^{-0.693d/T})/M$$

It can be seen from this relationship that for a given time of irradiation, high activity (and therefore high sensitivity) is obtained from a given mass of an element if the flux and activation cross section are large, and the decay constant λ is large (short half-life). Other things being equal, sensitivity is greater for lower-atomic-weight elements and for those with a high relative abundance of the isotope concerned. The half-life of the radionuclide does not control the inherent sensitivity of

the method except insofar as it becomes a practical limitation, either for long-lived species on the time of irradiation required, or for short-lived species on the time between the irradiation and the actual measurement of radioactivity. The character of the radiations emitted by the radionuclide formed also affects the sensitivity of the method, in view of the difficulties associated, say, with the counting of beta particles as opposed to gamma rays.

Using the above expression, it appears that determination of the absolute disintegration rate should enable calculation to be made of the absolute mass of the constituent being determined. Unfortunately, accurate knowledge of the flux and cross section is not usually available, and accurate determination of the disintegration rate is not possible. In practice, therefore, a comparative procedure is used wherein samples and standards are irradiated simultaneously or under identical conditions. After irradiation, the sample and standard are dissolved, a known weight of the element to be determined is added as carrier and the solutions are treated in such a way that the element is isolated free from all other radionuclides. The chemical yield of the chemical step is determined, and the radioactivities of sample and standard are compared under identical counting conditions. Then the mass of X, the constituent to be determined, is obtained as follows:

$$\frac{\text{Mass of X in unknown}}{\text{Mass of X in standard}} = \frac{\text{Total count from element X in unknown}}{\text{Total count from element X in standard}}$$

In certain favorable cases chemical manipulations of sample and standard are not required after irradiation, and gamma ray

spectroscopy using a multi channel analyzer may permit "instrumental" analysis by direct comparison of the activity from the element being analyzed, in the sample and standard respectively (98).

Since high fluxes are suited to highest sensitivity, the most common irradiation source used in activation analysis has been the nuclear reactor. However in some applications the flux given by a neutron generator is sufficient. Other sources which lead to nuclear reactions differing from the usual (n,γ) reaction, such as high intensity gamma sources and accelerators, are gaining in popularity as facilities become available.

Radioactivation analysis has many advantages and disadvantages as an analytical technique. It is both qualitative and quantitative, specific, sensitive, often simple and rapid, and non-destructive. On the other hand, flux variations over short distances, and opacity of the sample to the flux can lead to significant errors. Other elements in the sample may, by other nuclear reactions, lead to the radionuclide being measured. Contamination of samples prior to analysis is very easy in view of the sensitivity of the method. Practical limitations of activation analysis include cost, the fact that states of chemical combination are not differentiable, and problems encountered if other elements in the matrix of the sample require difficult radiochemical separations for which time is not available. Certain elements cannot be determined for various reasons, depending on the source of irradiation which is available. For example, hydrogen and lead have very low cross sections, nitrogen and oxygen lead to isotopes

of very short half-life, and carbon and beryllium lead to isotopes of very long half-life. Thus time may not be sufficient to allow separation after analysis, or may not be available to irradiate for a long enough period.

The applications of radioactivation analysis have been innumerable, and have been discussed in the various textbooks and conferences mentioned above. Among the most interesting of these, are forensic applications (99), archaeological applications (100), and such esoteric applications as identification of the sources of illicit narcotics (101) and "moonshine" (102). Of more immediate interest, however, is the use which has been made of activation analysis in the determination of trace elements in biological materials.

Activation analysis has been widely used in the fields of biochemistry and medicine. Its application in these fields has been discussed in the literature in review articles (103, 104, 105), in conference proceedings (106), in monographs (107) and in a large number of papers dealing with one or more specific elements (i.e. 108, 109, 110). Results of activation analyses of a wide variety of human and animal tissues have been reported for virtually every element to which the technique has been applied.

(b) Selenium

Activation analysis can be carried out for selenium by making use of the production of a radionuclide from one of several stable isotopes. Only three of the possible nuclides can be made

in high specific activities (111). The nuclear reactions occurring upon bombardment of selenium with thermal neutrons, which lead to these, are:

Se^{74} (n, γ) Se^{75} T 1/2 = 120 days

Se^{76} (n, γ) Se^{77m} T 1/2 = 18.8 seconds (112)

Se^{80} (n, γ) Se^{81} T 1/2 = 18.6 minutes

Most workers have used Se^{75} to determine the element by activation analysis (113, 114). This nuclide has a convenient gamma-ray for counting, and its half-life is more than adequate to allow complete chemical separation from other activities. Its main disadvantage is the long activation time required. Most analysts have activated their samples for only 7 to 14 days, which gives them only 5 to 10 percent of the specific activity which could be produced. This results in a corresponding loss of sensitivity (111). Se^{81} has been used in conjunction with rapid radiochemical separations, to determine the selenium content of human blood and other biological material (111). The chief disadvantage of this technique is the fact that Se^{81} is almost a pure beta emitter and is therefore more difficult to count than a nuclide emitting gamma rays.

Analysis for selenium utilizing the very short-lived species Se^{77m} has been carried out successfully on materials such as raw sulphur, where little interfering radioactivity was produced (115, 116). However, the short length of time available after irradiation does not readily permit separations to be carried out, and attempts to perform selenium analyses on biological

material using Se^{77m}, have shown that the production of O^{19} activity limits the accuracy and sensitivity of the technique (117, 118). This is unfortunate in that instrumental activation analysis, utilizing such a short-lived species, is very rapid.

(c) Sulphur

Although classical techniques have been well-established, and used for many years to determine the sulphur content of biological materials, very few analyses of the non-dialyzable sulphur content of human blood proteins have been reported. A great deal of work has been carried out on the dialyzable sulphur compounds in blood, and a few values for the total sulphur content of blood have been published (79, 119, 120, 122). Especially in the light of work indicating the importance of the sulphur amino acids in the rate of wound healing (123), it would appear that the bound sulphur levels in blood could be of great significance.

Various "modern" methods have been used to determine sulphur in biological materials. Spark source mass spectometry (122), gravimetric precipitation using Ba^{139} (124), X-ray fluorescence (125) and atomic absorption spectrometry (126), have recently been employed.

Radioactivation analysis for sulphur has been used with increasing frequency in the last few years. The nuclear reactions undergone by stable isotopes of sulphur, which have been utilized for analysis, are shown below.

S^{32} (d, α) P^{30} T 1/2 = 2.6 minutes

S^{32} (n, p) P^{32} T 1/2 = 14.3 days

S^{34} (n, γ) S^{35} T 1/2 = 87.1 days

S^{36} (n, γ) S^{37} T 1/2 = 5 minutes

Sue and Albert used deuteron irradiation to produce P^{30} for counting and analysis for sulphur, however sensitivity is low, and the beta-emitting P^{30} has too short a half-life to permit simple radiochemical separations (127, 128). S^{35} has been used in analysis, but has the disadvantage of being a very weak beta emitter (129, 130). More extensively employed, has been the production of P^{32}, by means of the (n, p) reaction (131, 132, 133, 134, 135, 136, 137). In fact, the sulphur content of the hair of persons accidentally irradiated with a fatal dose of neutrons was determined by means of this reaction (137). The chief disadvantage of the production of P^{32} for sulphur analysis of biological material is the presence of phosphorus in most such material, which can also lead to P^{32} by the (n, γ) reaction. The production of S^{37} for purpose of activation analysis of sulphur has not been reported in the literature, probably due to the relatively lower sensitivity obtainable, and the difficulty in carrying out radiochemical separations due to the short half-life of the species (131).

CHAPTER 3

EXPERIMENTAL METHODS

A. Preparation of Samples for Selenium Analysis

All reagents used were analytical grade and where possible were chosen to avoid addition of elements which either might be of interest in further trace element studies, or would become highly radioactive and thus necessitate removal prior to or after irradiation of the sample.

(a) Plasma and cell fractions of blood

(i) Separation of fractions

All blood samples were obtained from subjects who had had no food or drink in the previous twelve hours and who were in a state of rest. Approximately 18 ml of whole blood was drawn from the antecubital vein of the forearm, through a disposable 20 G, 1 1/2" stainless steel needle, into a 165 x 16 mm evacuated glass tube. The latter was a specially prepared "Vacutainer" (Becton, Dickinson and Company, Toronto, Canada) containing 37.5 mg of ammonium oxalate anticoagulant. The blood was refrigerated at 4°C, until two to three hours after withdrawal, when it was gently but thoroughly mixed, and 15 ml was pipetted into a 20 ml Lusteroid centrifuge tube, using a clean, dry pipette. An additional 1 ml sample was removed at the same time, and the percentage packed

cell volume, or "hematocrit", was determined by centrifugation in a capillary tube.

The 15 ml sample was centrifuged for 30 minutes in a high-speed multiplace angled head, fitted to an International Clinical Centrifuge, at approximately 6000 rpm, or 5000 G. The clear yellow supernatant plasma was decanted as completely as possible, using a glass rod, into a 100 ml beaker. The Lusteroid tube was then filled to its original level with isotonic ammonium citrate solution. This solution, containing 28 grams of $(NH_4)_2HC_6H_5O_7$, and 10 ml of concentrated NH_4OH in one litre of distilled water, was found to be isotonic by microscopic examination of red blood cells in a series of solutions of different concentrations.

The contents of the tube were stirred with a glass rod, and recentrifuged as above. The supernatant was decanted as before, into the beaker containing plasma. Isotonic ammonium citrate solution was again added, the contents of the tube stirred, recentrifuged, and the supernatant added to the beaker of plasma. The cell fraction of the blood, including both red blood cells, and the grey "buffy coat" of white blood cells layered on the surface of the red cells, was washed with distilled water into a second 100 ml beaker.

(ii) Dialysis

Prior to separation of the blood fractions, 22" lengths of seamless cellulose dialysis tubing, size 27/32, 1 20/64" flat width, (Union Carbide Canada Ltd., Visking Division, Lindsay, Ontario, Canada) were cut, soaked for several hours, rinsed

thoroughly, and then stored in distilled water. One end of a length of tubing was twisted tight, and knotted twice, 1/2" apart, and 1"-2" from the end. A slit polyethylene disc was numbered to enable sample identification, slipped onto the dialysis tubing between the two knots, and fastened securely by pressing the edges of the slit together.

The open end of the sac thus produced, was slipped over the stem of a long-stem 7 cm glass funnel supported in a clamp, and the contents of one of the beakers containing a blood fraction was rinsed into the sac with distilled water. About 1/2" of air space was left above the liquid level in the sac, and the open end was twisted and knotted twice. Excess tubing was cut off and discarded. The sac was inverted several times to mix the contents, and was placed in a polyethylene pail containing 10 litres of distilled water, which in turn was placed in a cold room at $4^{\circ}C$. A maximum of 9 dialysis sacs were placed in each pail. The distilled water was changed three times every twenty-four hours, for 4 days or until a flame test for sodium, performed with a platinum wire loop on the dialysis bath before a water change, was negative. At the time of each water change, the dialysis sacs were kneaded to accelerate dialysis by dispersing precipitate deposited on the inner walls.

(iii) Drying of samples

250 ml polyethylene beakers were cleaned and dried and then each was lined with a 14" x 14" sheet of 25 gauge (0.00025" thick) Type C Mylar film (DuPont of Canada Ltd., Montreal, Quebec) by

forcing the film into the beaker with the body of an appropriately-sized clean polyethylene bottle. The protruding film was pinned to the outside of the beaker with a washed elastic band, placed as low as possible on the film.

A sac containing a dialyzed blood sample was kneaded thoroughly to free any material adhering to inner walls of the tubing, and then rinsed with distilled water. The sac was folded double, and the fold inserted into a lined beaker. The sac was carefully punctured between the folded halves with a stainless steel needle. This permitted release of gas pressure built up within the tube during dialysis in such a way as to prevent loss of the contents through splashing.

After pressure was released, one end of the sac was cut off as close as possible to the knot, using stainless steel scissors. The tube was drained, and then the other end was cut off in a similar manner. The tube was rinsed thoroughly into the beaker.

After each sample was quantitatively transferred in the above manner, each of the numbered, lined beakers was placed on a glass shelf in a drying oven, at 80° - 90°C. Drying was allowed to proceed until plasma samples were completely dry, and until cell samples had reached the consistency of cork. At this point the film containing a cell sample was slipped from the beaker, kneaded to fragment the drying material, and then returned to the beaker. Drying of the cell samples was then continued until no further moisture could be driven off at the temperature of the

oven. If fragmentation of the cell material was not carried out, it formed a glass-like solid which cut the Mylar film during attempts to insert it into a container.

Each film, containing a sample, was removed from its beaker, and trimmed with scissors to remove the portion which had protruded beyond the rim of the beaker. The sample was wrapped tightly in the remaining film, and inserted into a clean, numbered polyethylene capsule (Nalgene container with cover, size # 25, 8 ml capacity; The Nalge Co. Inc., Rochester, New York, U. S. A.). Capsules were spot-welded shut with the tip of a hot glass rod, and were placed in a multicompartment plastic box for storage until irradiated for analysis.

(b) Subfractions of blood cells and plasma

(i) Cells

Several litres of outdated ACD transfusion blood (stored longer than twenty-one days after donation) were obtained from the blood bank of St. Joseph's Hospital, Hamilton, Ontario, Canada; the blood was centrifuged in 100 ml polycarbonate centrifuge tubes at $4^{\circ}C$, in an International Portable Refrigerated Centrifuge Model PR-2, for 30 minutes at 3000 rpm (approximately 1500 G). The supernatant plasma and the layer of white blood cells and white blood cell debris, were removed using a polyethylene dropper and rubber bulb. The red blood cells were washed twice by stirring with isotonic ammonium citrate solution, centrifuging, and decanting the supernatant. They were then transferred to dialysis tubing (3" flat width) and set to dialyze in distilled water at $4^{\circ}C$.

The white blood cells and debris, collected from two or three litres of centrifuged blood, were pooled, resuspended in isotonic ammonium citrate solution, stirred, centrifuged as above, and transferred to dialysis tubing for dialysis.

The sacs were kneaded, and the water in the dialysis bath was changed three times a day until the water in the bath gave a negative flame test for sodium. The completely hemolyzed red blood cells were removed from their sacs, and centrifuged as above for eight hours at $4^{\circ}C$. The supernatant overlying the creamy-white layer of red blood cell stroma was drawn off using a polyethylene dropper. The stroma were stirred with distilled water, and recentrifuged. The supernatant was again drawn off, and the stroma material transferred to a Mylar-lined beaker and dried at 80° - $90^{\circ}C$.

The supernatant from the first centrifugation of the dialyzed red blood cells was transferred to a Mylar-lined beaker and dried as above. The white blood cells and white blood cell debris were also dried in a Mylar-lined beaker.

After thorough drying, a quantity of the material from each of the three fractions: red blood cell stroma, red blood cell supernatant, and white blood cells, was weighed and encapsulated for analysis.

(ii) Plasma

The plasma obtained after centrifugation in (i) above, was dialyzed at $4^{\circ}C$. The euglobulin material which precipitated during the course of dialysis, was centrifuged from the fully-dialyzed

mixture, and dried separately from the supernatant thus obtained. A quantity of the dried material from each of these fractions was weighed and encapsulated for analysis.

(c) Cohn fractions of blood plasma

A weighed amount of each of the following Cohn fractions (138) of human blood plasma was dialyzed, dried, and encapsulated for analysis: Albumin, crystallized; albumin fraction V; alpha globulin fraction IV-4; alpha globulin fraction IV-1; beta lipoprotein globulin fraction III-0; beta globulin fraction III, gamma globulin fraction II. (Nutritional Biochemicals Corporation, Cleveland, Ohio, U. S. A.).

(d) Electrophoretic fractions of blood plasma

A J. K. M. - Stubbings continuous preparative electrophoresis apparatus (J. K. M. Instrument Company, Durham, Pennsylvania, U. S. A.) employing a bed of packed glass microspheres, and barbital buffer eluent containing 1.4 grams of sodium barbital and 0.265 grams of barbital per litre was used to fractionate plasma proteins. Water chilled to $4^{\circ}C$ at input was used to cool the separation chamber.

Outdated ACD transfusion blood plasma was dialyzed at $4^{\circ}C$ against the electrophoretic buffer with changes of the dialysis bath until the conductivity of the plasma solution was identical to that of the buffer. The dialyzed plasma was injected by means of a syringe and constant rate syringe drive, into the second injection port from the cathode side of the separation chamber. The separated protein fractions were collected in a series of bottles beneath the packed bed.

Samples of two different blood plasmas were separated under the following operating conditions: 43 ml of plasma A was injected at a rate of 14.4 ml per hour, under an applied potential of 450 volts (current varied from 143 to 190 milliamperes during the run) with a buffer flow rate of 1200 ml per hour. 57 ml of plasma B was injected at a rate of 14.2 ml per hour under an applied potential of 450 volts (current varied from 120 to 168 milliamperes during the run) with a buffer flow rate of 1100 ml per hour.

In each case, half of the solution collected in each fraction bottle was freeze-dried under vacuum, and the resultant material redissolved in a few ml of water for analysis by cellulose strip electrophoresis to determine the proteins present. The other half was dialyzed at 4°C against distilled water, dried in Mylar-lined polyethylene beakers, and encapsulated for analysis. In each case, also, an amount of the whole plasma equal to that injected was dialyzed, dried, and encapsulated for analysis.

(e) Human tissues other than blood

Specimens of a series of tissues were obtained at the time of autopsy, from the bodies of two individuals, a male aged 1 1/2 years (death due to pneumonia) and a male aged 52 years (death due to a ruptured aortic aneurysm). From 0.1 to 1.0 grams of whole tissue was removed using stainless steel instruments, transferred to polyethylene capsules and frozen for transportation. In addition, autopsy samples of liver, muscle and skin, were obtained from ten individuals in a similar manner.

After thawing, the tissue was weighed, minced with a stainless steel scalpel, dialyzed in distilled water at 4°C, dried and encapsulated as in the procedure described for blood samples.

(f) Miscellaneous materials

(i) Urine

700 ml of human urine was dialyzed in distilled water at 4°C, and dried in a Mylar-lined beaker. The residue was encapsulated for analysis.

(ii) Egg protein

Samples of the "white" (albumin), the yolk, and the egg shell membrane from a chicken egg, were dialyzed, dried and encapsulated for analysis.

(iii) Milk

75 ml of homogenized cow's milk was dialyzed, dried, and encapsulated for analysis.

(g) Selenium standards

6.439 grams of desiccated powdered selenium (99-100%) was dissolved in 200 ml of 1:2 H_2O-concentrated HNO_3, and diluted to one litre in a volumetric flask. This solution was further diluted by means of pipette and volumetric flasks to obtain a series of standard solutions.

A set of 4 selenium standards was prepared as follows. Into each of four polyethylene capsules (Nalgene # 25, 8 ml capacity) was pipetted a quantity of a standard solution. The contents of one of the capsules was evaporated to dryness at 60° - 70°C in the drying oven. To the remaining three were added varying amounts of distilled water to approximate totals of 1, 3, and 5 ml of solution. All four capsules were spot-welded shut.

A total of six sets of standards, each set containing a fixed amount of selenium but varying amounts of water, was prepared

in this manner, by pipetting quantities of different standard selenium solutions for each set.

(h) Acid hydrolysis of protein (139)

The plasma and cell fractions of the outdated ACD transfusion blood from several individuals, were pooled respectively, dialyzed at 4°C against distilled water, and dried in Teflon-coated trays at 70° - 80° C.

100 gram quantities of the dried protein were hydrolyzed by refluxing in a 1 litre flask with 500 ml of 20% HCl in 50% formic acid solution. Using an Allihn condenser, heating mantle, and thermix magnetic stirrer, reflux was maintained on different samples, for periods varying from 6 to 60 hours. The hydrolysate was filtered hot by suction through a pressed glass fibre filter, reduced to a thick syrup by vacuum distillation, and then diluted to twice its volume with distilled water. This solution was adjusted to pH 4.6 with a hot 50% solution of sodium acetate, and allowed to cool and remain at room temperature for periods up to two weeks.

The mixture was filtered by suction, on filter paper, and the precipitate obtained was dried at 70° C. KBr plates were prepared for a specimen of the material, and the infrared spectrum was observed from 2 to 16 microns. The precipitate was dissolved in 100 ml of 5N NH_4OH, the pH was adjusted to 9 with concentrated acetic acid and the solution was chilled for 4-5 hours at 4°C. The mixture was filtered on paper, the filtrate was adjusted to pH 5.3 with concentrated acetic acid, kept for 6 days at room

temperature and refiltered. Precipitates obtained in the latter two filtrations, were dried at 70°C, and KBr plates prepared for infrared analysis. The above procedure was also carried out on 100 grams of clean, dry human hair.

(i) Hydrazinolysis of protein (140)

Several litres of outdated ACD transfusion blood were obtained and separated into plasma and cell fractions. The material was dialyzed thoroughly in distilled water at 4°C, dried on Teflon-coated trays at 70° - 80° C, and powdered in a mortar and pestle.

10 grams of powdered protein was placed in a one litre three-necked round-bottomed flask (ground glass joints) along with a Teflon-coated stir bar, 200 ml of 85% hydrazine hydrate, and 10-15 drops of Dow Corning Silicone 200 Fluid antifoamant (Dow Corning Corporation, Midland, Michigan, U. S. A.). The flask was fitted with two stoppers, an Allihn condenser, a heating mantle with Variac control, and was supported above a thermix magnetic stirring unit.

The mixture was stirred and reluxed (118° - 120° C) for nine hours. After the apparatus was cooled, the condenser was rinsed into the flask with distilled water, and removed.

(i) Acid evolution of H_2S

The flask was fitted with a glass inlet tube, projecting to the bottom, an outlet tube above the surface of the flask contents, and a separatory funnel containing 200 ml of 20 N H_2SO_4. The outlet tube was led by means of Tygon tubing into a gas-washing

trap containing distilled water acidified to pH < 1 with H_2SO_4 and thence into a fritted glass bubbler in a trap containing 200 ml of lead acetate solution (10 grams of $Pb(Ac)_2.3\ H_2O$ and 100 ml concentrated acetic acid in 1 litre of solution. A trap containing ammoniacal $CdSO_4$ solution (100 grams of $3CdSO_4.8\ H_2O$ dissolved in 500 ml of distilled water and 500 ml of concentrated NH_4OH) was placed at the end of the gas evolution train in order to check the completeness of precipitation of the H_2S in the $Pb(Ac)_2$ trap.

Nitrogen gas from a cylinder was bubbled into the reaction flask through the inlet tube, and when bubbling in all traps indicated that the gas flow had reached a steady state the 20 N H_2SO_4 was added dropwise with stirring. Air pressure was applied to the top of the separatory funnel to prevent gas from bubbling up. During acid addition, the contents of the flask heated up to 80° - 90° C which obviated the application of external heat to accelerate evolution of H_2S. After completion of acid addition, the pH was approximately 2 to 3, in which range essentially all H_2S would be in the molecular, non-ionized state. Nitrogen flow was continued for one hour after acid addition.

The precipitate obtained in the lead acetate trap, was filtered by suction using a Büchner funnel, through two layers of 4.25 cm No 1 grade Whatman filter paper. The precipitate was washed thoroughly with distilled water, and dried at 40° - 50° C in the drying oven. The dry solid was weighed and sealed into a polyethylene capsule for analysis.

(ii) Zn (Hg) evolution of H_2S

The procedure utilized in evolution of H_2S from the products of hydrazinolysis, was identical to that in section (i) except that 70 grams of zinc amalgam was added to the flask, and 30 grams to the washing trap, prior to the start of the N_2 flow and acid addition. The Zn (Hg) was prepared by stirring 100 grams of 20 mesh zinc metal with 100 ml of a 3% solution of $HgCl_2$, containing 1 ml of concentrated HNO_3, for ten minutes. The amalgam was thoroughly washed by stirring with, and decantation of, distilled water, and was stored in distilled water until used.

(iii) Control experiments involving Se^{75}-selenocystine and Se^{75}-selenomethionine

Se^{75}-selenomethionine was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England, and Se^{75}-selenocystine was produced by irradiating 0.08 grams of selenocystine (Calbiochem, Los Angeles, California, U. S. A.) in a flux of 10^{10} neutrons/cm^2/sec, at 30° - 40° C, in the thermal column of the McMaster reactor, for 7 days (141).

Mixtures containing 0.5 grams methionine, 0.5 grams cystine, and 0.3 mg Se^{75}-selenomethionine having an activity of 0.25 millicuries, were hydrazinolyzed, and treated by methods (i) and (ii) above, respectively.

Mixtures containing 0.5 grams methionine, 0.5 grams cystine, and 0.04 grams of Se^{75}-selenocystine having an activity of 0.03 millicuries, were hydrazinolyzed, and treated by methods (i) and (ii) above, respectively.

B. Preparation of Samples for Sulphur Analysis

As in preparation of samples for selenium analysis, all reagents used were analytical grade, and were chosen to avoid introduction of elements either of possible interest in further trace element studies, or which would become highly radioactive on irradiation.

(a) Plasma and cell fractions of blood

Outdated ACD transfusion blood from a number of different individuals was obtained, and the plasma and cell fractions were separated by centrifugation and washing with isotonic ammonium citrate solution as described in part A.

Samples of different sizes of both the cell and plasma fractions were pipetted, dialyzed in distilled water at $4^{o}C$, dried in Mylar-lined beakers, and encapsulated as in part A. For each sample, two identical portions were pipetted, and to one, prior to drying, was added a measured quantity of standard sulphur solution.

(b) Sulphur standards

7.062 grams of dried, desiccated hydrazine sulphate ($NH_2NH_2.H_2SO_4$) was weighed, dissolved, and diluted to one litre in a volumetric flask. This solution thus contained 1.74 grams of sulphur per litre.

Portions of this standard solution were pipetted both for addition to dialyzed blood prior to drying, and for separate drying in Mylar-lined beakers and encapsulation for use as comparator standards.

C. Radioactivation Analysis for Selenium and Sulphur

(a) Irradiation and counting facilities

Samples were irradiated in a flux of approximately 10^{13} neutrons/cm^2/second using the 2 Megawatt McMaster University light water moderated reactor. The rapid transit pneumatic tube "rabbit" system enabled irradiations of short duration, and minimal delay time between the end of irradiation, and delivery for counting.

Because of the short half life of isotopes utilized for analysis, and the resultant short time available for handling of samples between irradiation and counting, a quick-opening polyethylene rabbit was developed (Figure 1) which held each sample in a fixed position concentric with the rabbit body and the irradiation tube, and provided reproducible irradiation geometry.

After removal from the rabbit after irradiation, samples were placed in a 50 ml, 29 x 105 mm Lusteroid centrifuge tube, which in turn was dropped into a glass tube 67 cm long (I.D. 34 mm, O.D. 38 mm) with bottom rounded to the curvature of the centrifuge tube. This glass tube was suspended into a shielding cave constructed of high-density barium concrete blocks, (each 12" x 8" x 4") in which was located the detector used for sample counting. The cave provided a minimum of 8" of barium concrete shielding in any direction.

The end of the glass sample-holding tube was positioned in the centre of the face of a 3" x 3" NaI (Tl) scintillation

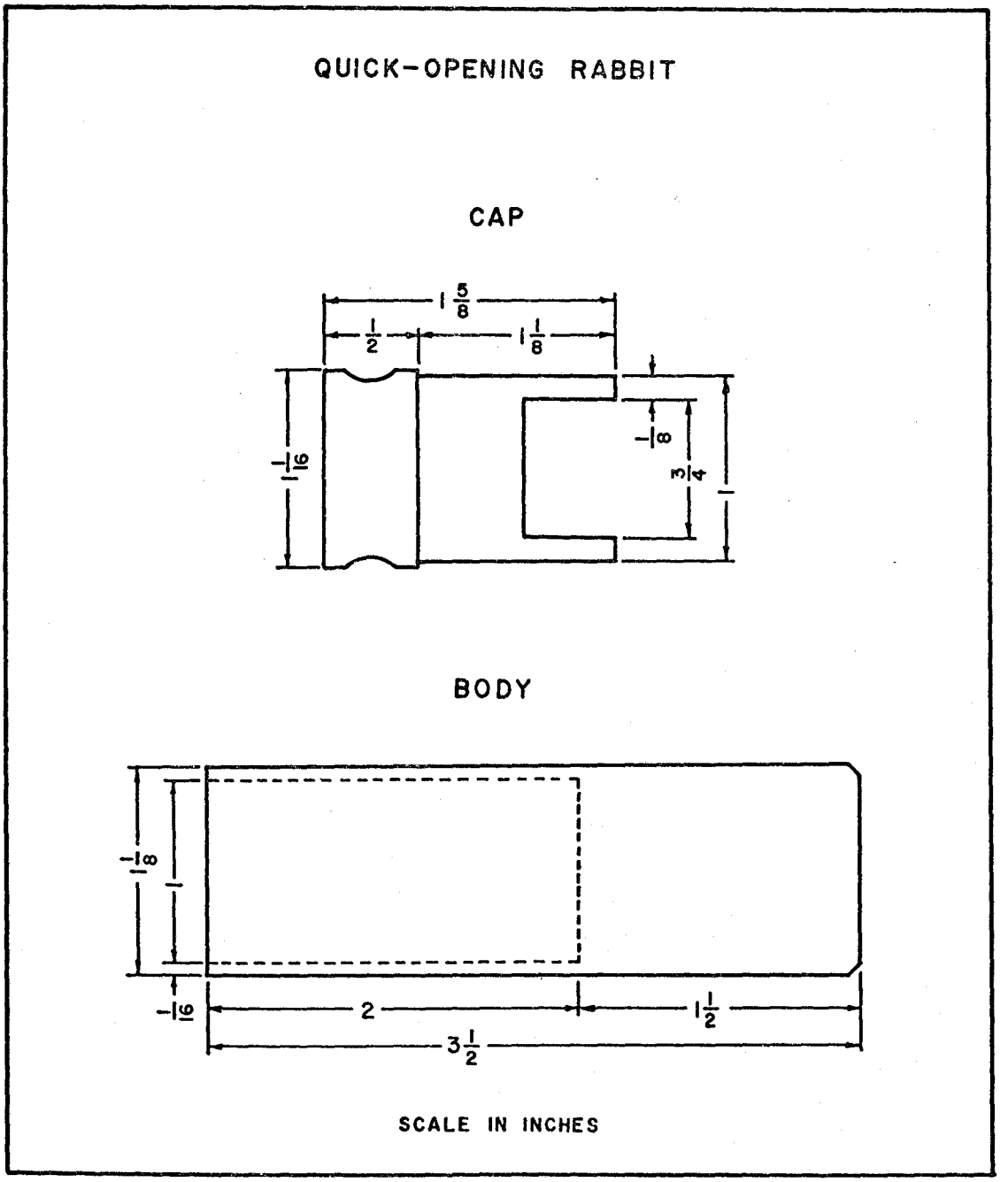


Figure 1

Quick-Opening Rabbit

crystal, which was supported in the centre of the 29" x 26" x 25" cavity within the cave. The detector was used in conjunction with various multichannel gamma-ray spectrometers during the course of analyses performed, including an RCL 256 channel analyzer, and a Victoreen Pip 400 channel analyzer.

(b) Conditions of irradiation and counting for selenium analysis

Each series of samples analyzed was irradiated twice, or three times if the results of the first two irradiations differed by greater than 4%. A sample was irradiated in the rabbit system for 20 seconds. Thirty seconds after the end of irradiation, a 30 second live time count was begun, using one half of the channels available in the analyzer used. The gain of the analyzer was adjusted prior to the start of irradiations, in order that the peak from the 0.16 Mev gamma of Se^{77m}, and the peak from the 0.19 Mev gamma of O^{19} fall in the centre of the spectrum obtained.

After irradiation and counting of two samples, the spectrum was printed out. Standards containing selenium only, and standards containing the same amount of selenium plus a quantity of water, were irradiated before, during, and after the series of sample irradiations. A series of irradiations was performed only after the reactor had reached full flux stability (4 or 5 days after startup), in order that no shimming be required during the course of irradiations. The slight drift in flux always encountered over the period of time required to carry out a set of irradiations (approximately 8 hours) was monitored by irradiating standards throughout the set. An estimate of reproducibility of irradiation

geometry was also obtained from analyses of these standards. Stop-watch timing of the delay between irradiation and counting, was facilitated by a warning light, whose appearance marked the reversal of the pneumatic tube air flow, and the start of rabbit return.

The multichannel analyzer used to count gamma radiation emanating from irradiated samples, stored in a memory location, or "channel", the number of counts corresponding to each energy of gamma ray falling within the energy range of interest. At the end of the counting period, the analyzer both printed out the total number of counts accumulated at each energy, and also displayed on an oscilloscope, a plot of these totals, versus energy, as a continuous spectrum. It was the printed totals, corresponding to "peaks" in this spectrum, which were used in calculations for the purpose of analysis.

An estimate of the counts beneath the selenium and oxygen peaks in the gamma spectrum was made as follows. Counts in the top five channels in each peak were summed, and from this sum was subtracted 2.5 times the sum of the counts in the first channel on each side of the peak, which was lower than either of its neighbours (the background counts).

For each series of analyses, a plot was made of the logarithm of the selenium count versus the oxygen count for a set of selenium-water standards and the slope of the straight line obtained was determined.

After irradiation of a tissue sample, a point representing the selenium and oxygen counts obtained, was plotted on the

correction graph above, and a line drawn through the point, parallel to that of the selenium-oxygen standards, was extrapolated to zero oxygen. This gave the "true" or corrected selenium count for the sample, which was related to that from a standard containing selenium only, and the selenium in the sample was calculated. Using the correction procedure, a series of identical samples of blood plasma and cells dried for different lengths of time, and thus containing various amounts of oxygen as water, were analyzed for selenium content.

In practice, the extrapolation technique, and other calculations required, were performed by an IBM 7040 computer, using a Fortran programme given in Appendix I. In the case of blood samples analyzed, the hematocrit of the blood, and other available data such as age and sex of the subject, permitted further computer analysis of a large number of results.

A second Fortran programme, given in Appendix II, was used to calculate the mean, standard deviation, standard error and normal distribution of results for the population studied.

(c) Conditions of irradiation and counting for sulphur analysis

Each sample was irradiated in the rabbit system for 4 minutes. Two and one half minutes after the end of irradiation, a 10 minute clock time count was begun, into the full channel capability of the analyzer used. The gain was adjusted in order that the peak from the 3.1 Mev gamma of S^{37} appear well within the limits of the energy spectrum observed. After each irradiation, the spectrum was printed out.

As described for selenium analyses, an estimate of the count in the S^{37} photopeak was obtained by summing the top five channel counts and subtracting a background consisting of 2.5 times the sum of the first channel count on each side of the peak, lower than either of its neighbours.

If the count in the S^{37} photopeak from a standard containing A grams of sulphur was C_A, the count from the sample was C_S, and the count from the same blood sample with A grams of sulphur added was C_T, then the "true" or corrected count corresponding to sulphur in the blood sample itself, was calculated as follows:

$$\text{Corrected Count } Cc = \frac{C_A}{C_T - C_S} \times C_S$$

This count was then directly compared with C_A, to obtain the amount of sulphur in the blood sample.

CHAPTER 4

EXPERIMENTAL RESULTS

A. Selenium Analyses

(a) Experimental method

With the exception of those used in the preparation of primary comparator standards, no reagent or material used in the course of analysis was found to contain a quantity of selenium detectable with the sensitivity of the method used. These reagents included the distilled water used in dialysis (a quantity of which comparable to that used throughout the entire dialysis procedure, was evaporated and analyzed), and the Mylar film and polethylene capsules used in sample encapsulation.

In Table I are shown the results of the analysis of a series of identical blood samples, which were dried as completely as possible, and then heated at the drying temperature for prolonged periods of time.

In Table II, are shown the results of analysis for selenium in series of identical samples of cells and plasma respectively, using the correction procedure. Each series included samples containing amounts of oxygen, as water, which varied over the entire range of oxygen content permitted for analysis using linear correction graphs.

In Table III are shown the results of analysis for selenium in a series of identical standards, evaporated to

dryness and heated for a prolonged period of time at the drying temperature.

TABLE I

Counts from selenium and oxygen in a series of identical blood samples dried for a prolonged period of time

Sample number	Hours of drying at 80°-90°C	Counts in Se^{77m} photopeak	Counts in O^{19} photopeak
Cells 1	20	4800	3500
Cells 2	23	4900	4900
Cells 3	25	4900	4600
Cells 4	27	4700	5400
Cells 5	30	4800	5200
Plasma 1	12	3500	1200
Plasma 2	14	3500	1100
Plasma 3	16	3600	740
Plasma 4	18	3500	1100
Plasma 5	20	3400	1000
Plasma 6	22	3600	1000

TABLE II

Selenium analysis of identical blood samples containing different amounts of oxygen (water) using the correction procedure

Sample	Micrograms of selenium in cells	Micrograms of selenium in plasma
1	1.42	0.95
2	1.38	1.02
3	1.39	0.94
4	1.46	0.96
5	1.39	0.97
6	1.46	0.96
7	1.46	0.98
8	1.39	0.91
9	1.45	0.97
10	1.48	
Mean	1.43	0.96
Standard deviation	0.04	0.03
Standard deviation (as percent of mean)	2.8%	3.1%

TABLE III

Counts from selenium in a series of identical standards, dried and heated for a prolonged period of time, at 65°C

Sample number	Hours heated after reaching dryness	Analysis	Counts in Se^{77m} photopeak
1	0	1	11,100
		2	10,800
2	0	1	10,100
		2	10,700
3	51	1	9,500
		2	10,100
4	24	1	9,000
		2	9,700
5	51	1	11,000
		2	8,900
6	51	1	8.900
		2	10,400
	Mean		10,000
	Standard deviation		800
	Standard deviation (as percent of mean)		8%

In Table IV are shown the results of analyses of selenium standards performed over a period of time comparable to that required for analysis of a series of blood samples.

TABLE IV

Counts in Se^{77m} photopeak from selenium standards irradiated at different times on a given day

Standard number	Time (hours)	Counts in Se^{77m} photopeak		
		Day A	Day B	Day C
1	0	23,600	25,900	29,300
2	0	25,100	25,800	28,500
1	0.5	23,500	24,600	28,800
2	0.5	25,200	26,800	26,700
1	4	21,200	23,700	27,100
2	4	23,000	25,100	25,600
1	4.5	22,700	23,800	25,800
2	4.5	22,300	24,300	26,400
1	8	21,000	21,600	24,600
2	8	20,700	22,800	26,600
1	8.5	21,400	23,700	27,200
2	8.5	20,600	23,400	26,100
Mean		22,500	24,300	26,900
Standard deviation		1,600	1,450	1,400
Standard deviation (as percent of mean)		7.1%	6.0%	5.4%

In Table V are shown the results of analysis for selenium added to series of identical samples of cells and plasma respectively.

TABLE V

Analysis for selenium added to identical blood samples

Sample Number	Analysis	Selenium standard added (micrograms)	Total selenium determined (micrograms)	Added selenium determined (micrograms)
Cells 1	Mean of ten samples	____	1.43	____
Cells 2	1	6.44	7.96	6.53
	2	6.44	7.92	6.49
Cells 3	1	6.44	7.89	6.46
	2	6.44	7.77	6.34
Cells 4	1	6.44	7.83	6.40
	2	6.44	7.75	6.32
Plasma 1	Mean of nine samples	____	0.96	____
Plasma 2	1	6.44	7.29	6.33
	2	6.44	7.47	6.51
Plasma 3	1	6.44	7.34	6.38
	2	6.44	7.32	6.36
Plasma 4	1	6.44	7.25	6.39
	2	6.44	7.30	6.34
			Mean	6.40
			Standard deviation	0.08
			Standard deviation (as percent of mean)	1.3%

Figure 2 shows the results of plotting the logarithm of the selenium count, versus the oxygen count, for a series of standards containing a fixed amount of selenium and varying amounts of oxygen (as water). Each line represents such a series, containing a different amount of selenium.

Figure 3 shows a series of four spectra corresponding to a series prepared as above, with the Se^{77m} photopeak at 0.16 Mev, and the O^{19} photopeak at 0.19 Mev in each spectrum.

Figure 4 shows a plot of the counts in the photopeaks of the dry selenium standards constituting the "zero-oxygen" point in the lines in Figure 2, versus the actual selenium content of the standards.

Figure 5 shows a plot of selenium-oxygen standards, and of the series of identical samples of blood cells and plasma which contain various amounts of oxygen (as water). After correction procedures described in Section C (b) of Chapter 3 these plots yielded the data given in Table II.

Figure 6 shows the same plot as Figure 5, on an expanded scale.

(b) Plasma and cell fractions of blood

The plasma and cell fractions of a 15 ml sample of whole blood was analyzed for a total of 254 individuals who were arbitrarily considered normal with respect to blood composition. Using the hematocrit value for each blood, the amounts of selenium in 100 ml of plasma, and 100 ml of cells were also calculated. The complete list of individual results, and breakdown into age,

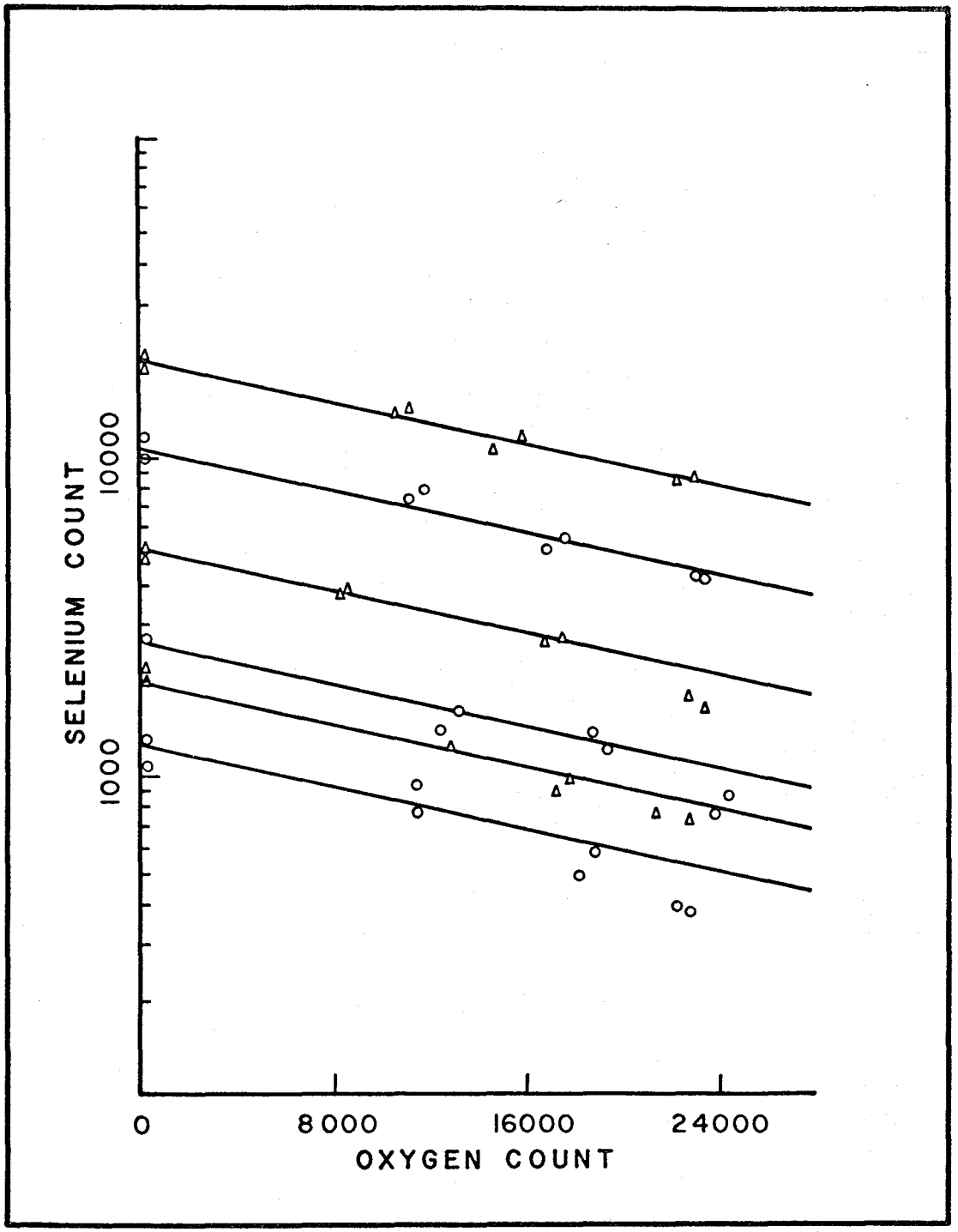


Figure 2

Logarithm of the Selenium Count Versus the Oxygen Count for Series of Standards each Containing a Fixed Amount of Selenium and Varying Amounts of Oxygen

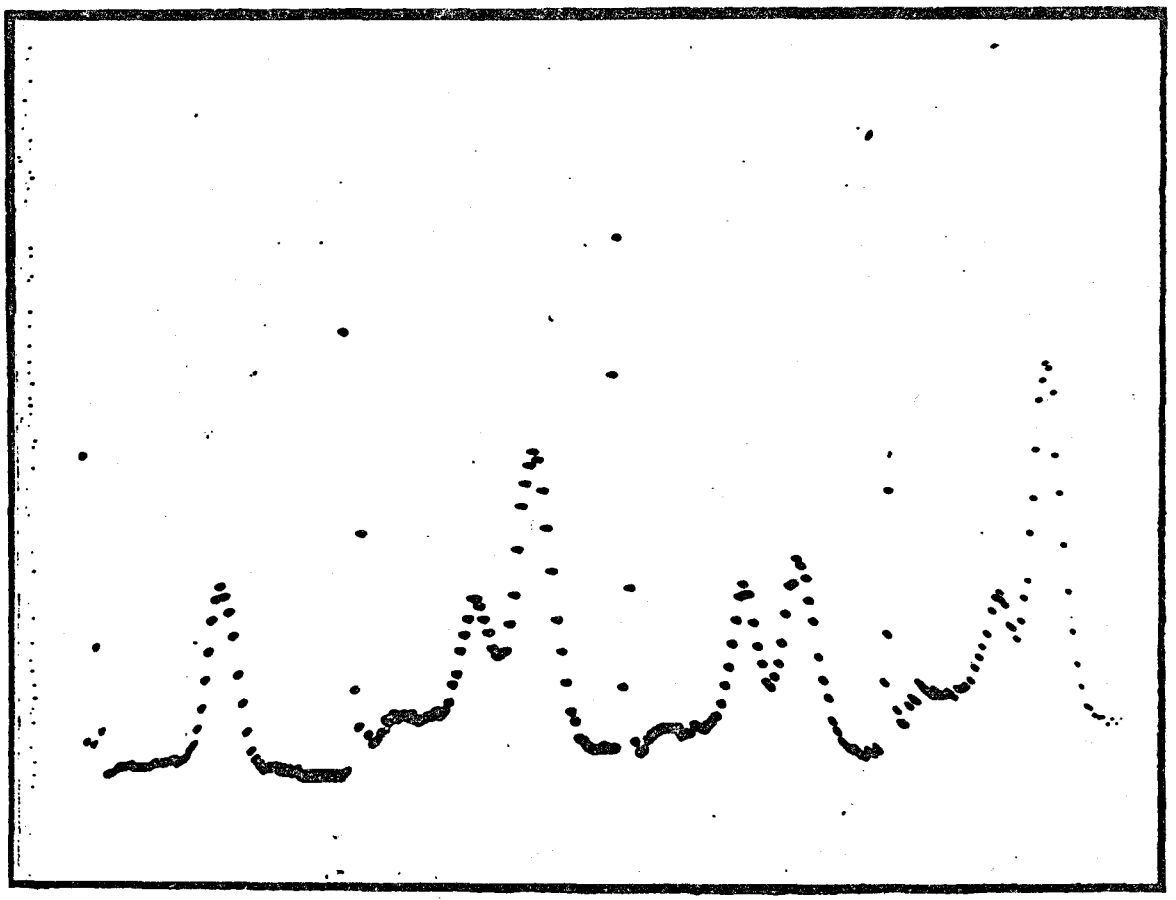


Figure 3

Four Spectra Corresponding to Four Standards Containing the Same Amount of Selenium and Varying Amounts of Oxygen

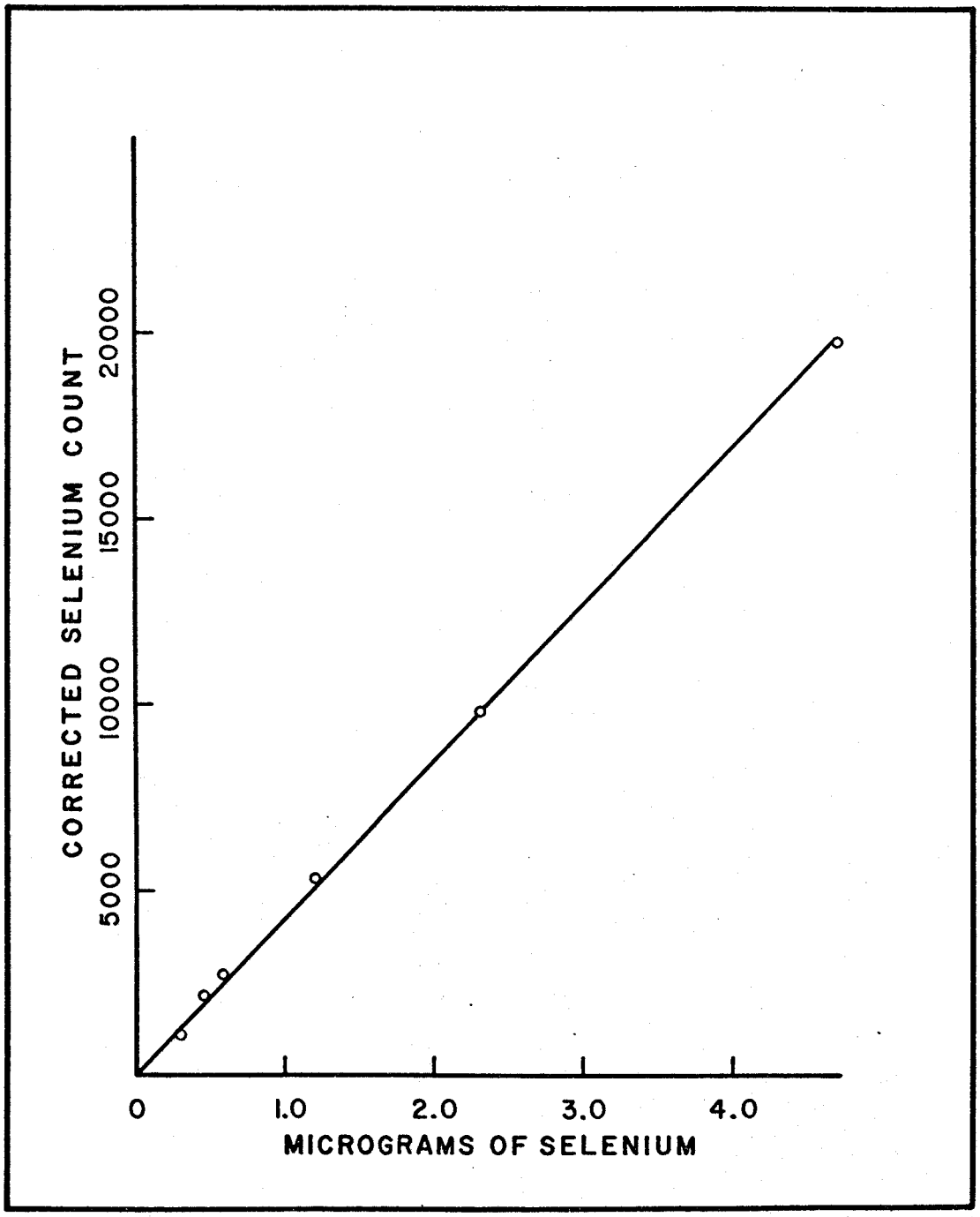


Figure 4

The "Zero-Oxygen" Selenium Count From the Lines in Figure 2, Versus the Selenium Content of the Standards

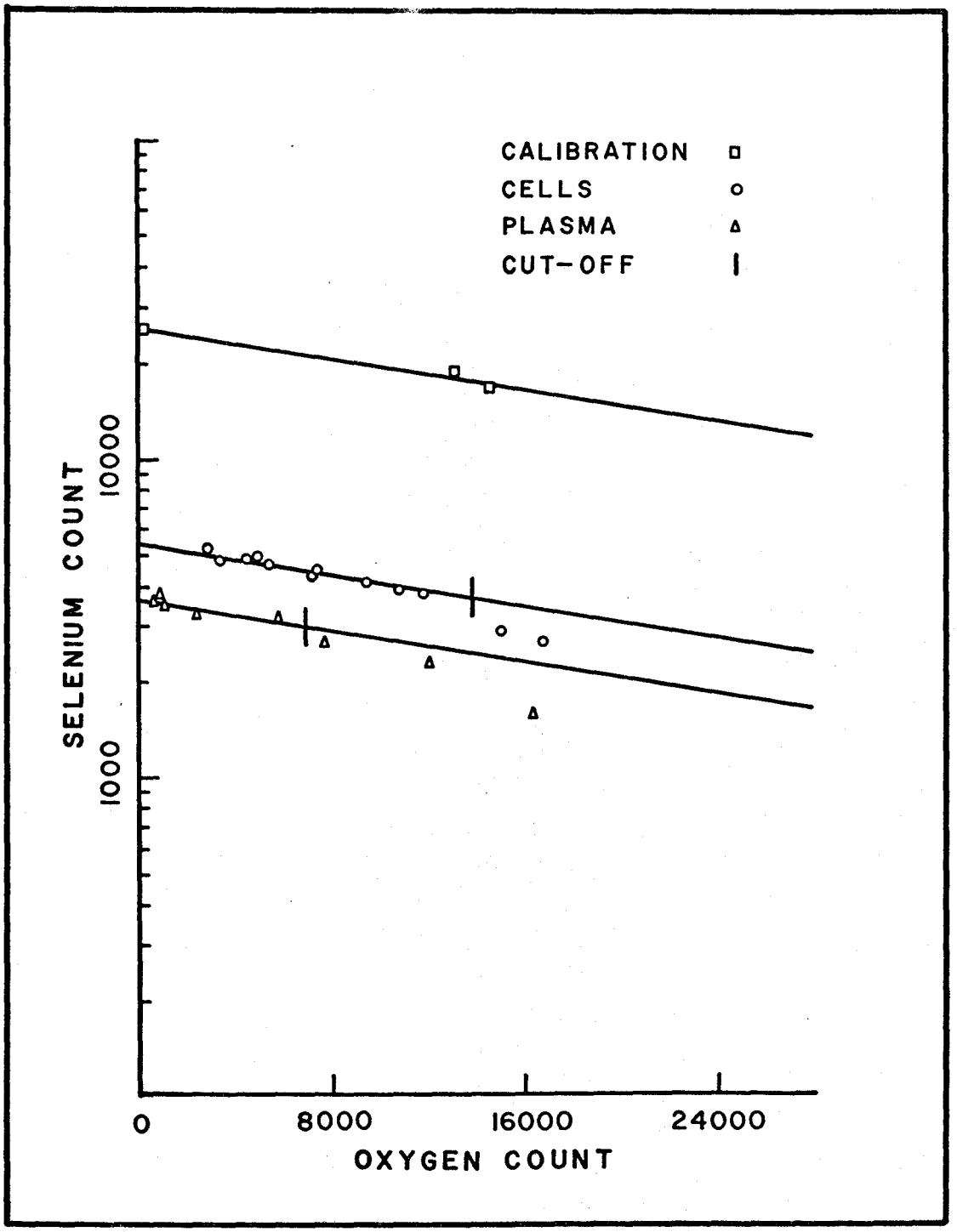


Figure 5

Logarithm of the Selenium Count Versus the Oxygen Count for Series of Identical Samples of Blood Cells and Plasma Containing Various Amounts of Oxygen

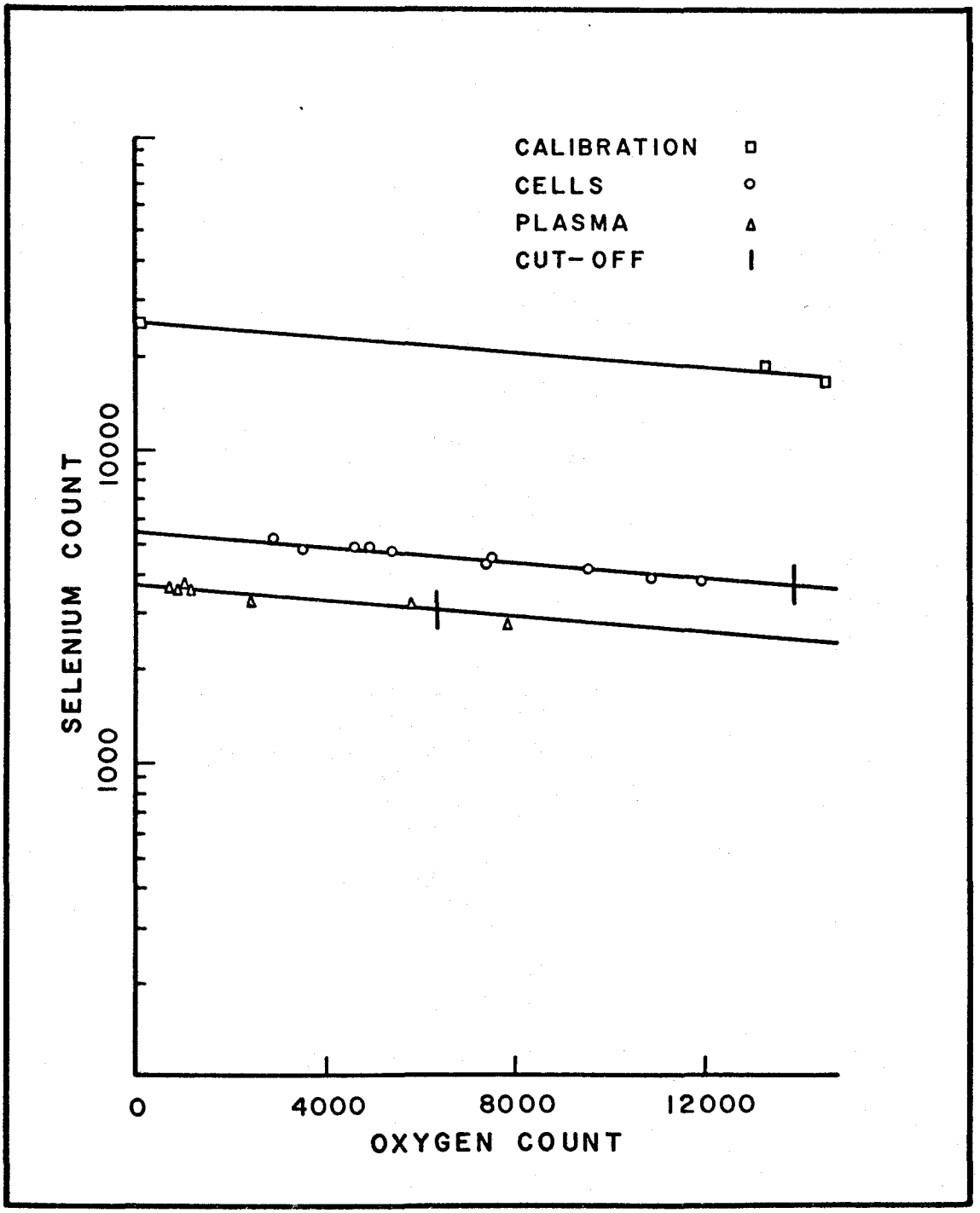


Figure 6

Logarithm of the Selenium Count Versus the Oxygen Count of Series of Identical Samples of Blood Cells and Plasma Containing Various Amounts of Oxygen

sex, and selenium level groupings, is provided in Appendix III.

A statistical evaluation of each of the six fractions: cells, plasma, whole blood, 100 ml cells, 100 ml plasma, 100 ml cells and 100 ml plasma, was carried out. Using the computer programme in Appendix II, the mean, standard deviation, and the standard population (defined as all values lying within three times the standard deviation from the mean), were calculated for each fraction. The results are shown in Table VI.

Histograms showing the incidence of a given selenium level, as a function of the specific level, are given in Figures 7, 8, 9, 10, 11 and 12. A scatter plot showing the variation in selenium content of the plasma fraction of blood, as a function of the selenium content of the cell fraction of the same blood sample, is shown in Figure 13.

Figure 14 shows the variation of cell and plasma selenium with hematocrit. Figure 15 shows the variation with age, of the average selenium content of the cells and plasma from 15 ml of whole blood, for the entire population studied. Table VII lists the data used in Figure 15.

In the case of 23 individuals, the selenium level in the fractions of their blood, was followed over a period of time. Table VIII shows the results obtained.

Using the home addresses of the individuals whose blood was analyzed, the population studied was broken into two groups, one composed of those residing within the city limits of Hamilton, Canada, an industrial city whose water supply is Lake Ontario,

TABLE VI

Selenium analyses of blood plasma and cells for 254 individuals

Blood fraction	Number of individuals in standard population	Sex	Mean selenium content (micrograms)	Standard deviation (micrograms)
Cells (from 15 ml whole blood)	113	Male	1.49	0.36
Plasma (from 15 ml whole blood)	113	Male	1.16	0.22
Whole blood (15 ml)	113	Male	2.65	0.51
100 ml cells	112	Male	22.0	5.26
100 ml plasma	115	Male	14.4	2.83
100 ml cells & 100 ml plasma	113	Male	36.4	7.13
Cells (from 15 ml whole blood)	138	Female	1.56	0.39
Plasma (from 15 ml whole blood)	139	Female	1.25	0.25
Whole blood (15 ml)	138	Female	2.80	0.60
100 ml cells	137	Female	24.7	6.02
100 ml plasma	139	Female	14.5	2.99
100 ml cells & 100 ml plasma	137	Female	39.1	8.20
Cells (from 15 ml whole blood)	250	Both	1.52	0.37
Plasma (from 15 ml whole blood)	253	Both	1.21	0.24
Whole blood (15 ml)	250	Both	2.73	0.55
100 ml cells	251	Both	23.6	6.00
100 ml plasma	253	Both	14.4	2.86
100 ml cells & 100 ml plasma	250	Both	37.9	7.84

TABLE VII

Age variation of average selenium content of cells and plasma

Age group	Number of individuals	Mean selenium content of cells (micrograms)	Standard deviation	Standard error	Mean selenium content of plasma (micrograms)	Standard deviation	Standard error
0-19	25	1.69	0.46	0.09	1.32	0.24	0.05
20-29	37	1.72	0.46	0.08	1.34	0.34	0.06
30-39	44	1.61	0.49	0.07	1.22	0.30	0.05
40-49	31	1.59	0.40	0.07	1.20	0.24	0.04
50-59	46	1.43	0.29	0.04	1.15	0.23	0.04
60-69	33	1.41	0.41	0.07	1.14	0.24	0.04
70-79	27	1.42	0.32	0.06	1.18	0.23	0.04
80-	8	1.34	0.34	0.12	1.15	0.26	0.09

TABLE VIII

Variation with time of the selenium level of the blood of individuals

Individual	Blood fraction (cells or plasma)	Elapsed time (days)	Selenium content in 15 ml whole blood (micrograms)
A	C	0, 14, 28	1.2, 1.3, 1.4
	P		0.9, 0.9, 1.0
B	C	0, 14, 28	1.1, 1.1, 1.2
	P		0.9, 0.9, 1.0
C	C	0, 15, 28	0.6, 1.0, 1.2
	P		0.5, 1.1, 1.3
D	C	0, 14, 22	1.3, 1.4, 1.7
	P		1.1, 1.2, 1.4
E	C	0, 15, 22	0.9, 1.2, 1.2
	P		0.9, 1.4, 1.0
F	C	0, 2, 17	1.4, 1.4, 1.6
	P		1.2, 1.1, 1.3
G	C	0, 3, 10	1.3, 1.2, 1.4
	P		1.2, 1.0, 1.2
H	C	0, 28	1.2, 1.3
	P		1.3, 1.0
I	C	0, 21	1.1, 0.9
	P		1.1, 0.9
J	C	0, 14	1.4, 1.7
	P		1.9, 0.9
K	C	0, 10	1.1, 1.2
	P		0.9, 1.0

TABLE VIII continued

Variation with time of the selenium level of the blood of individuals

Individual	Blood fraction (cells or plasma)	Elapsed time (days)	Selenium content in 15 ml whole blood (micrograms)
L	C	0, 7	1.2, 1.4
	P		0.8, 1.4
M	C	0, 7	1.3, 1.2
	P		1.4, 1.1
N	C	0, 7	2.0, 1.9
	P		1.6, 1.3
O	C	0, 7	1.3, 1.1
	P		1.1, 1.0
P	C	0, 7	1.5, 1.4
	P		0.9, 1.0
Q	C	0, 7	1.5, 1.5
	P		1.1, 1.1
R	C	0, 7	1.7, 1.6
	P		1.0, 1.1
S	C	0, 7	2.0, 2.0
	P		1.3, 1.6
T	C	0, 7	1.3, 1.5
	P		1.3, 1.1
U	C	0, 5	1.2, 1.3
	P		1.1, 1.4
V	C	0, 2	1.3, 1.7
	P		1.2, 1.4
W	C	0, 2	2.0, 1.7
	P		1.4, 1.4

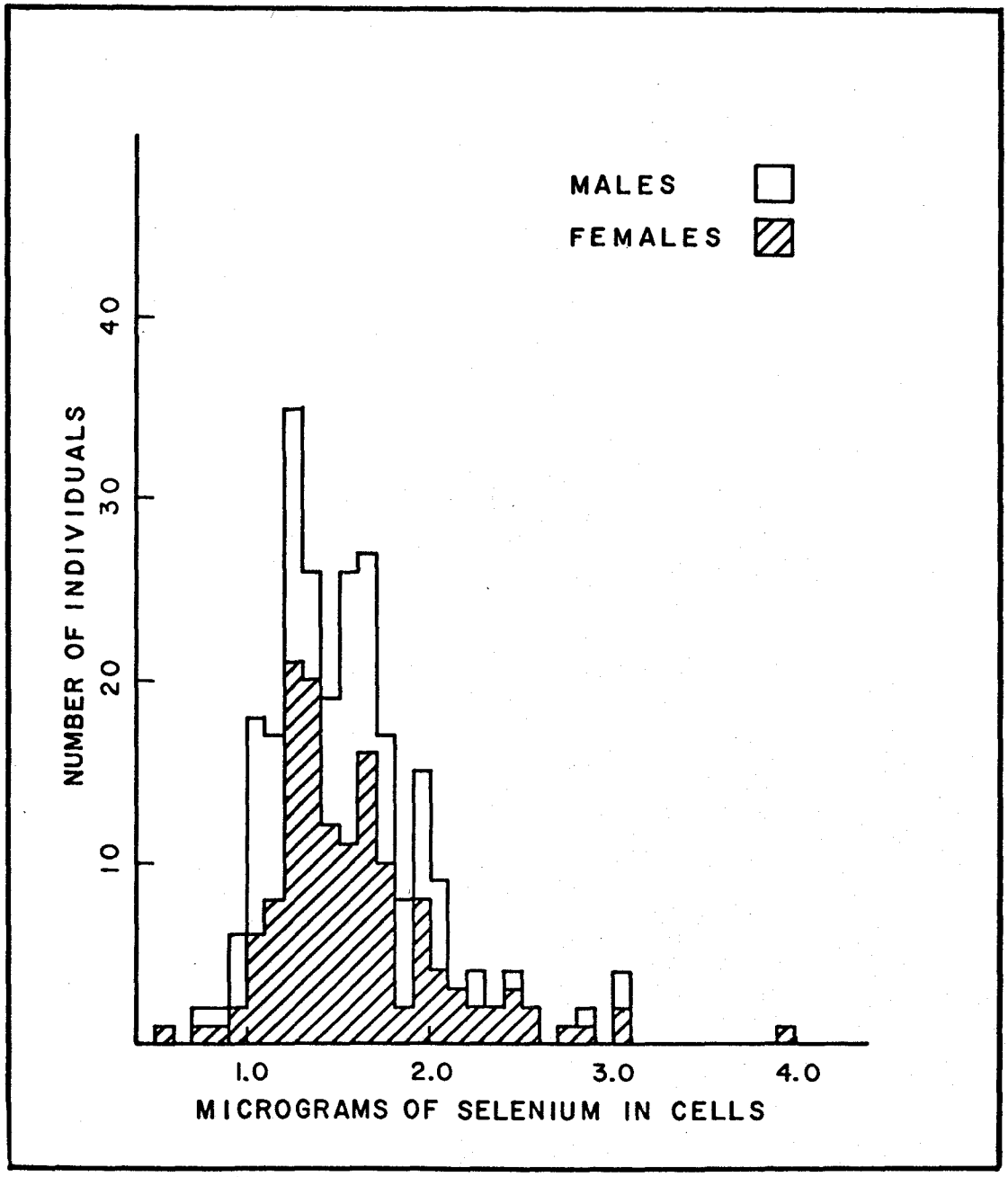


Figure 7

Incidence of a Given Selenium Content in the Cells from 15 ml of Whole Blood as a Function of Content, for the Entire Population Analyzed

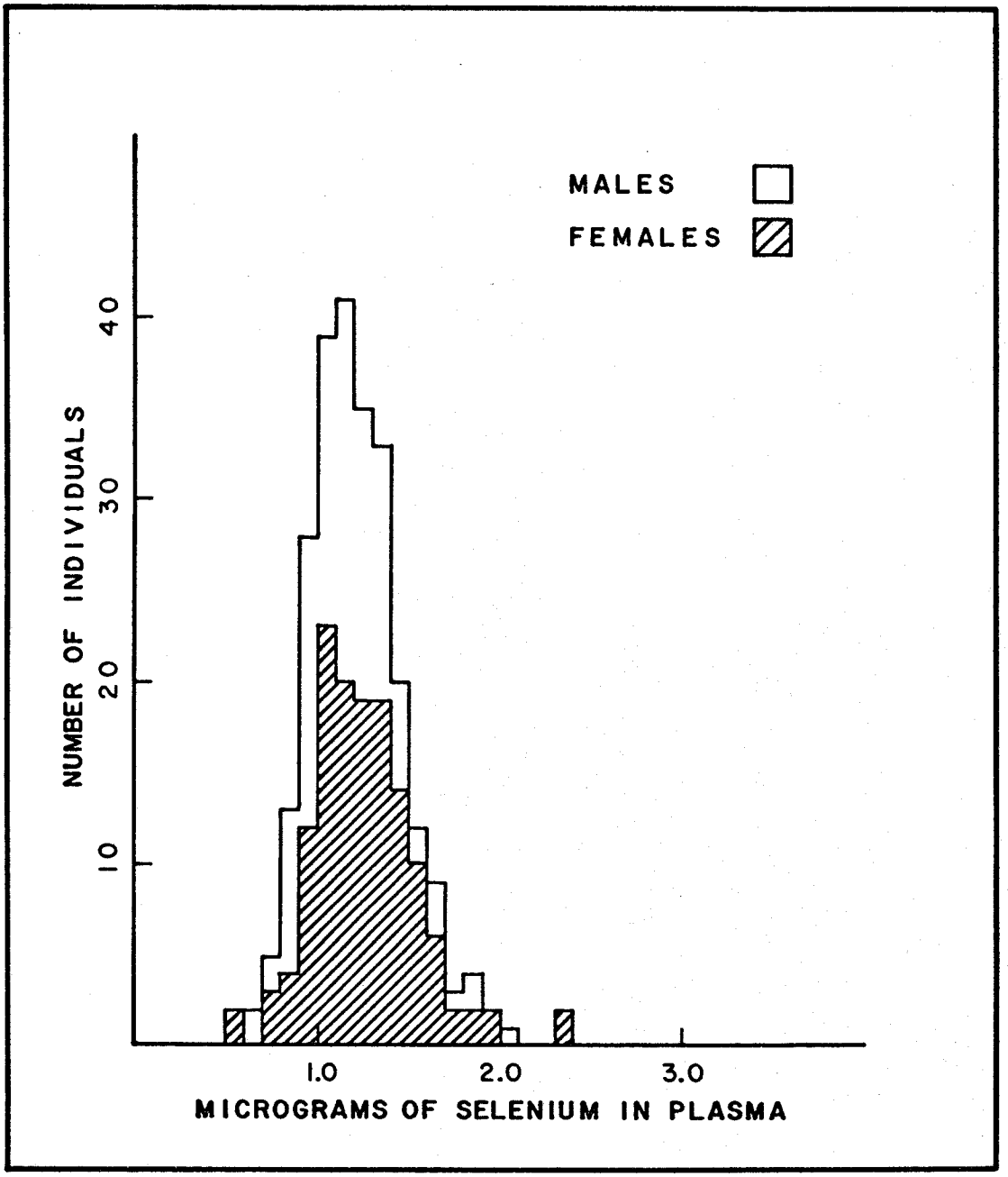


Figure 8

Incidence of a Given Selenium Content in the Plasma from 15 ml of Whole Blood as a Function of Content, for the Entire Population Analyzed

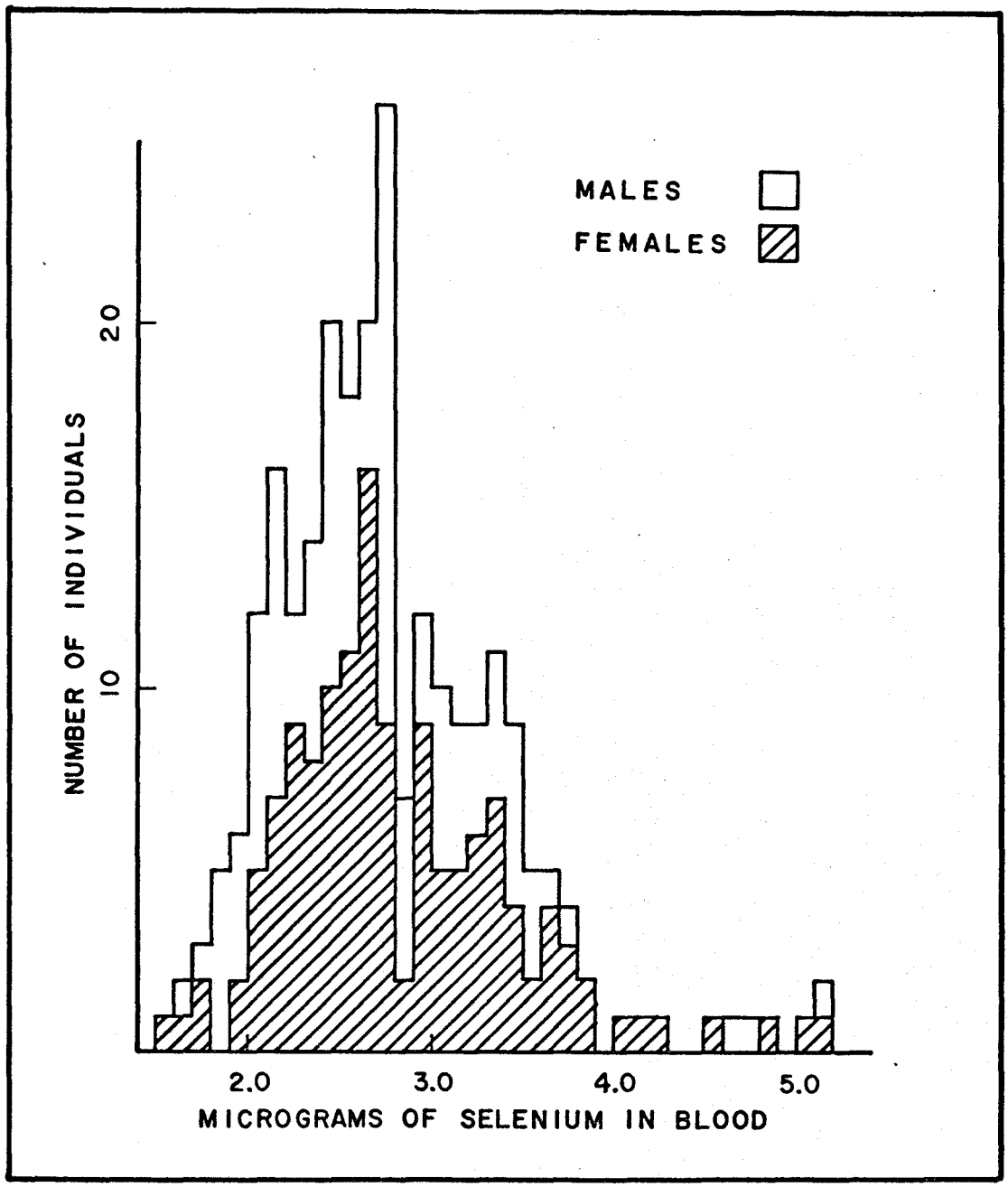


Figure 9

Incidence of a Given Selenium Content in 15 ml of Whole Blood as a Function of Content, for the Entire Population Analyzed

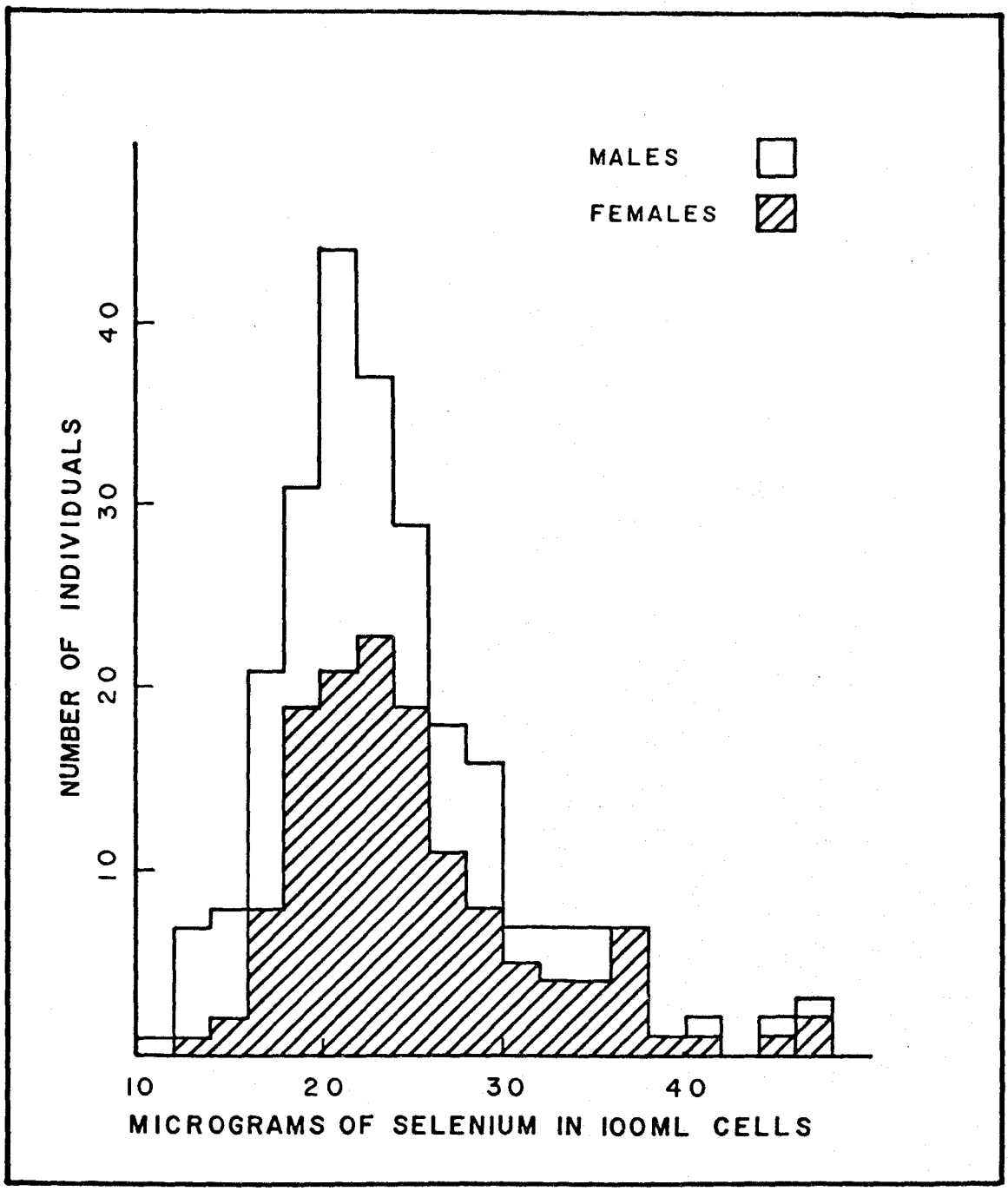


Figure 10

Incidence of a Given Selenium Content in 100 ml of Cells as a Function of Content, for the Entire Population Analyzed

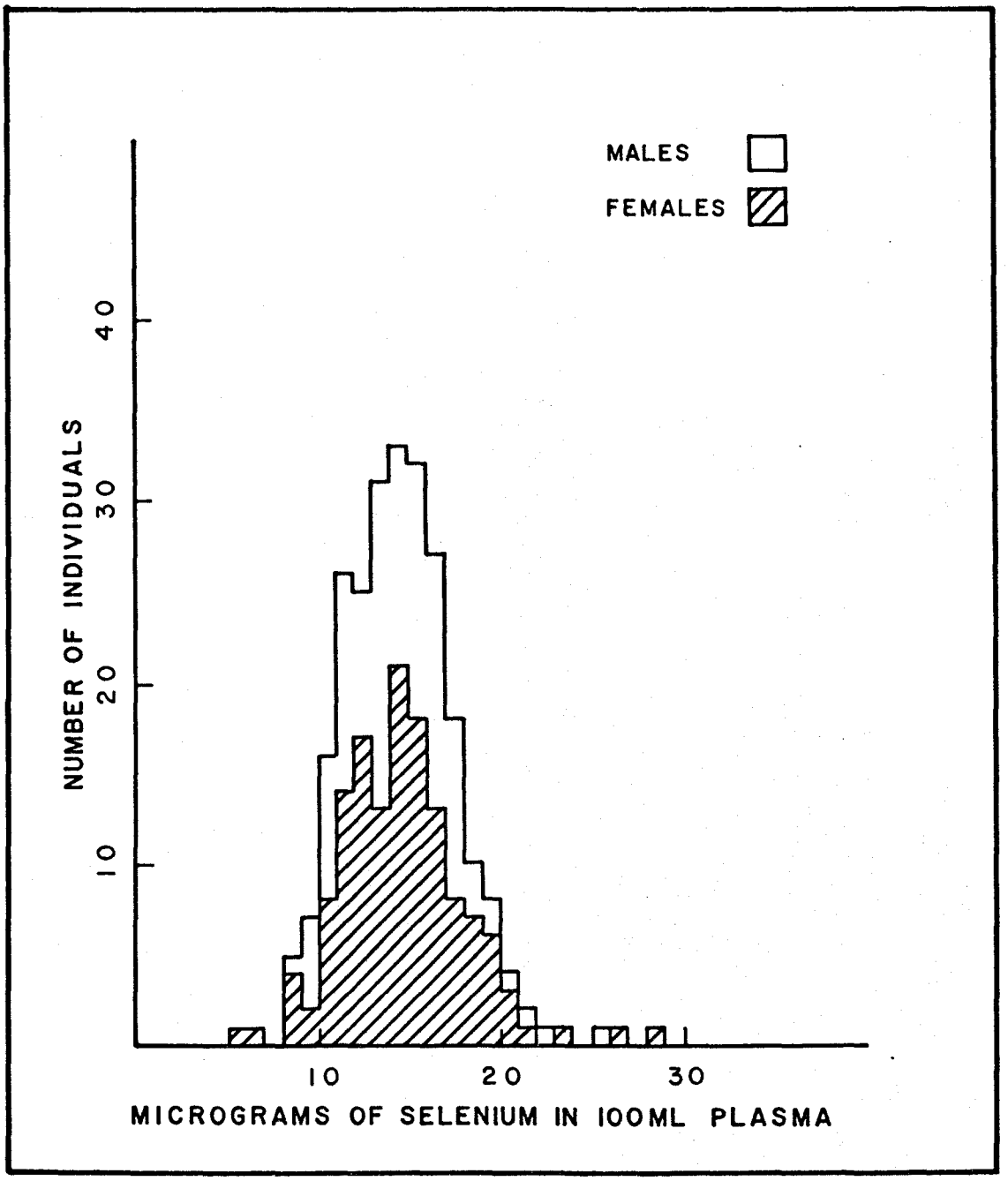


Figure 11

Incidence of a Given Selenium Content in 100 ml of Plasma as a Function of Content, for the Entire Population Analyzed

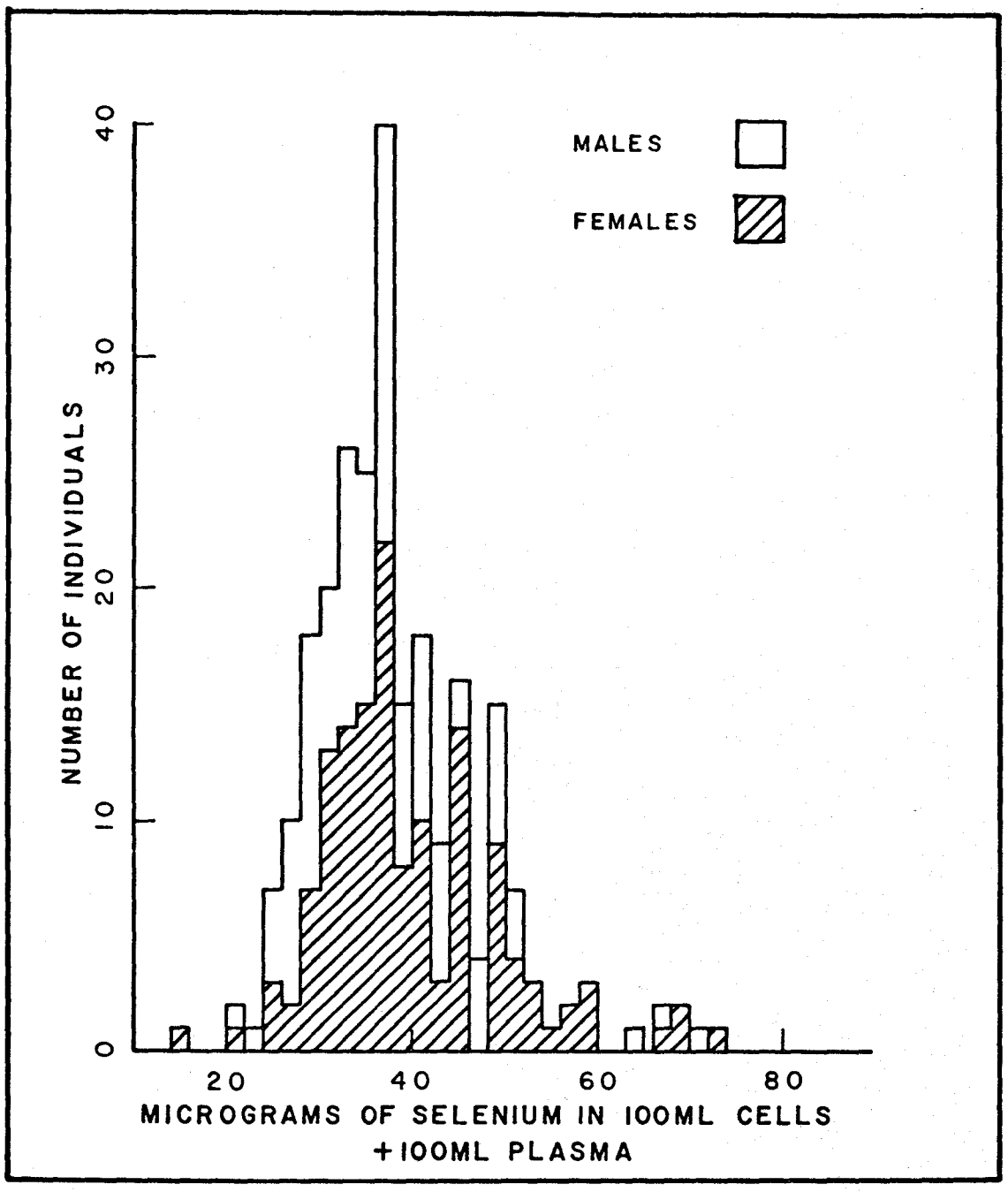


Figure 12

Incidence of a Given Selenium Content in 100 ml of Cells Plus 100 ml of Plasma as a Function of Content, for the Entire Population Analyzed

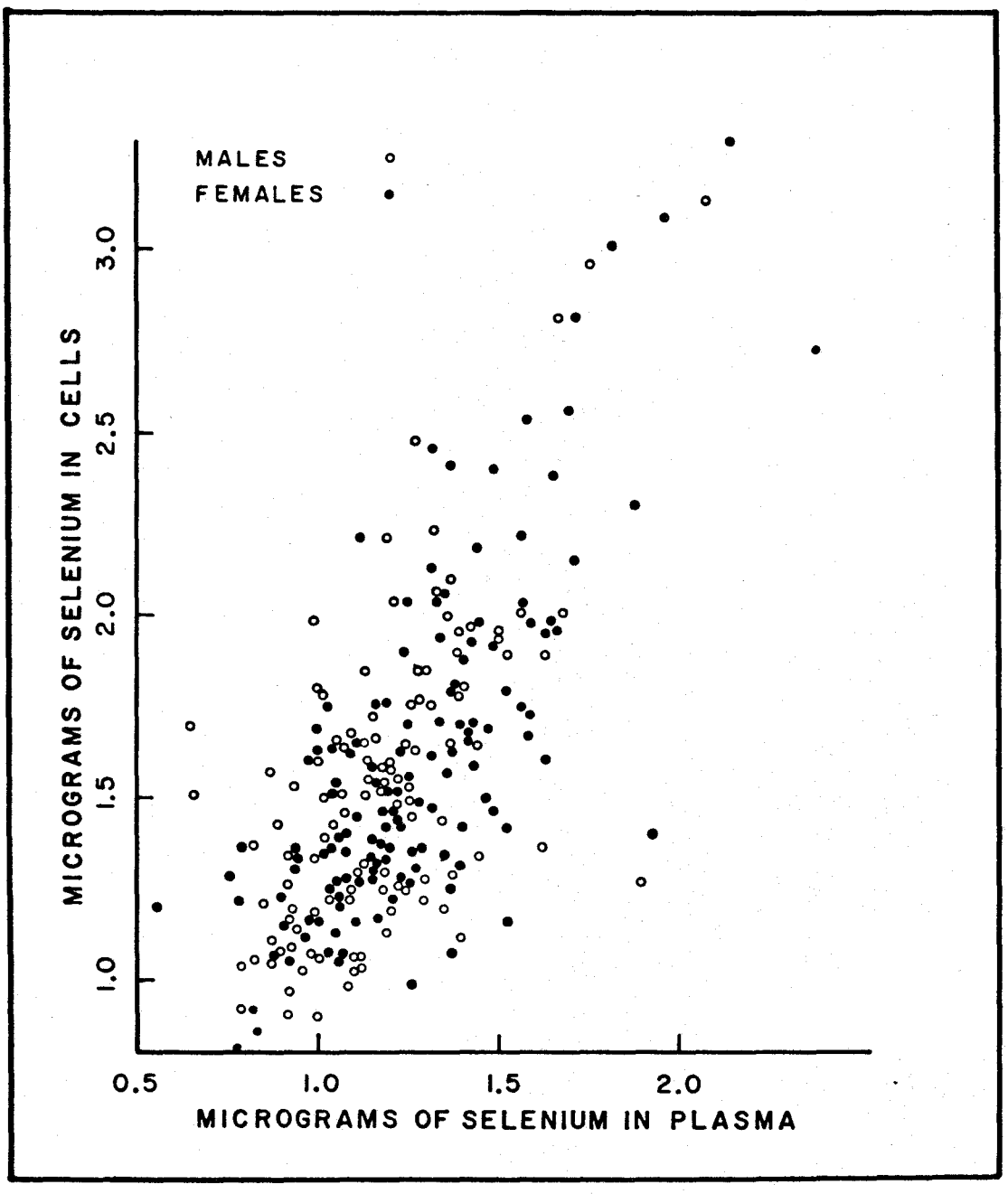


Figure 13

Selenium Content of the Plasma Fraction of the Blood of an Individual as a Function of the Selenium Content of the Cell Fraction of the Same Individual

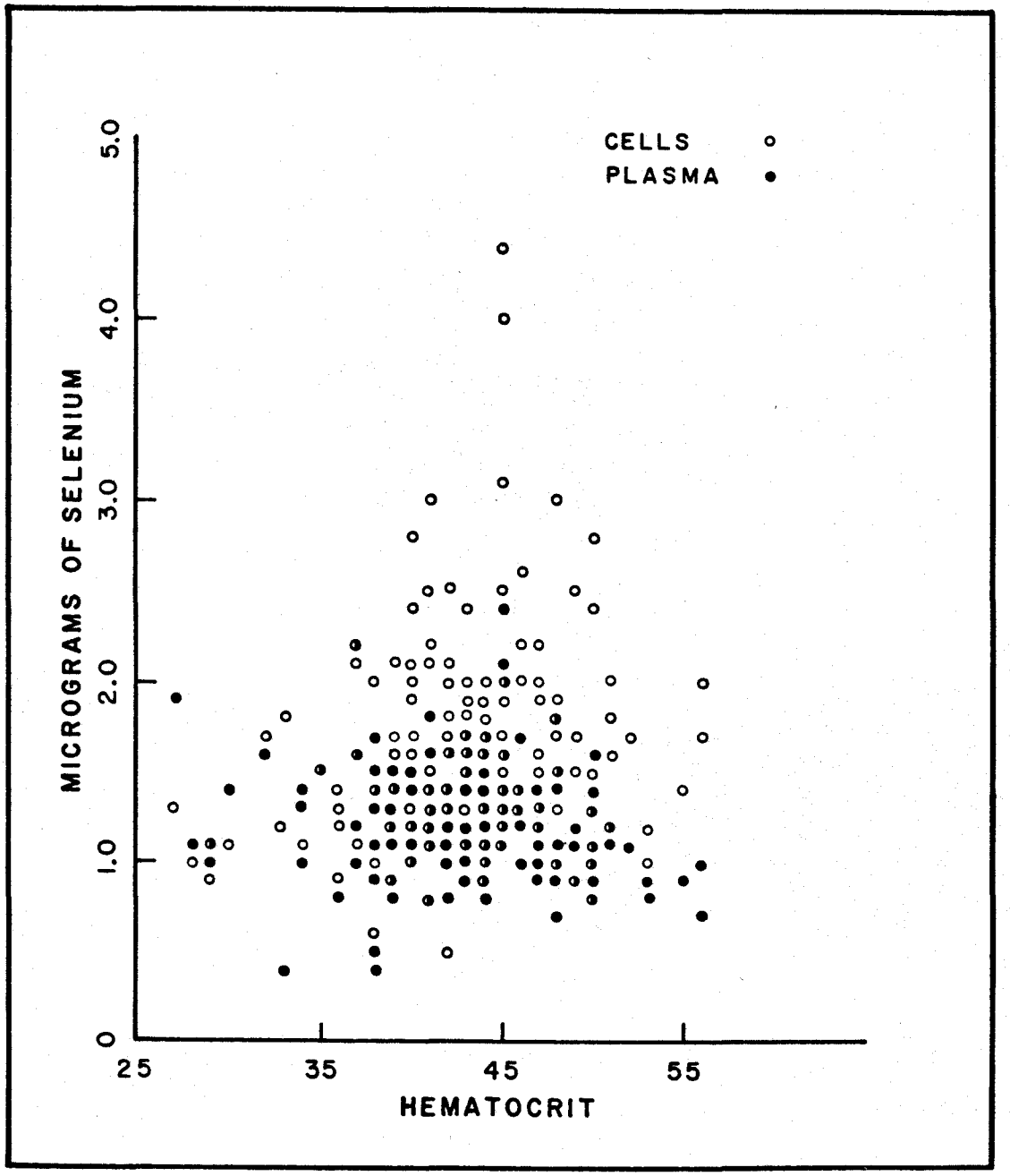


Figure 14

Variation of Cell and Plasma Selenium with Hematocrit

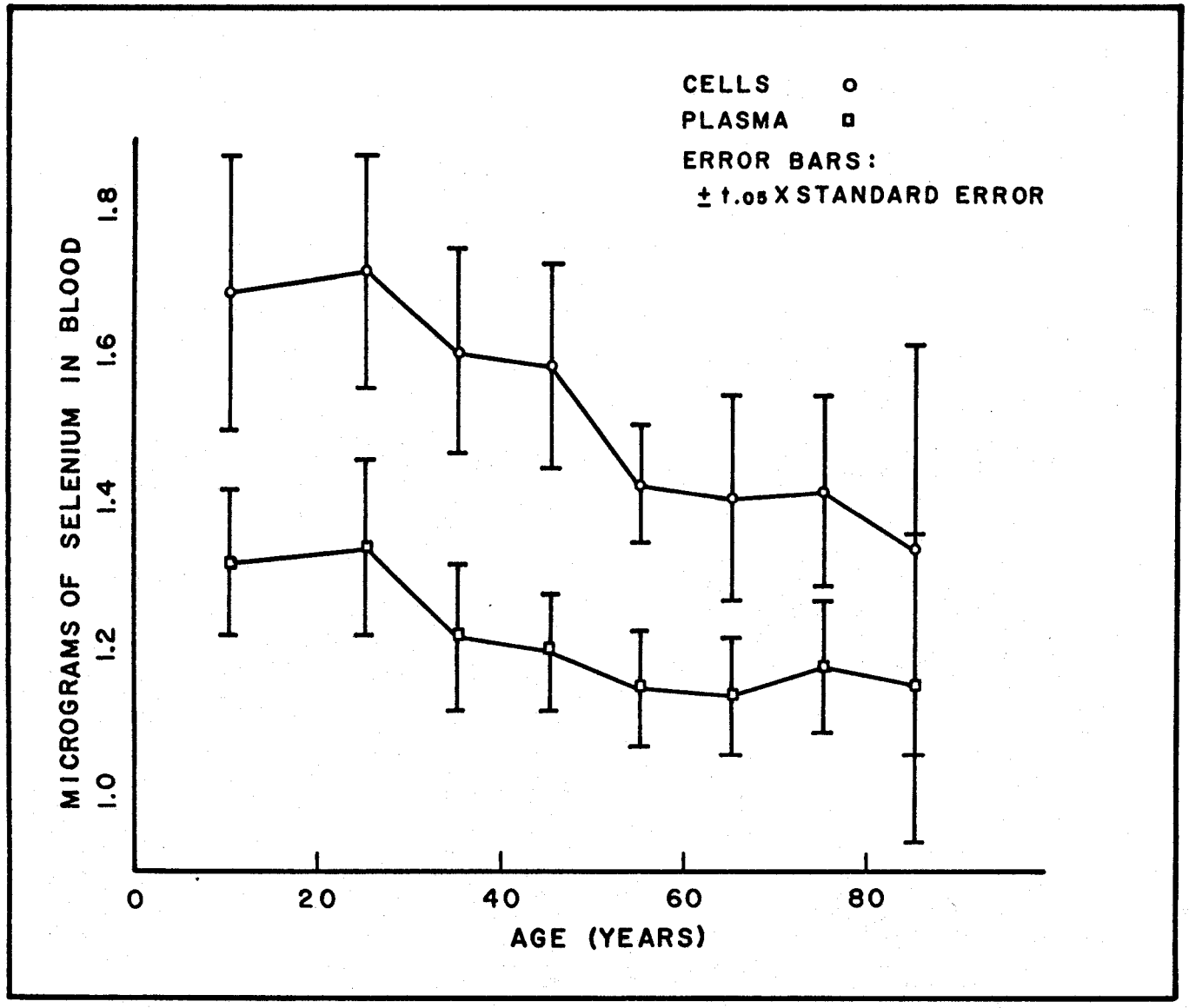


Figure 15

Age Variation of the Average Selenium Content of the Cell and Plasma Fractions of Whole Blood, for the Entire Population Studied

and one composed of those residing in suburban and rural areas whose water supply is from wells. The selenium content of whole blood was averaged for each group, and the results are shown in Table IX.

TABLE IX

Selenium content of whole blood as a function of water supply

Number of individuals	Living conditions	Mean selenium content in 15 ml whole blood (micrograms)	Standard deviation	Standard error
193	urban, water supply from Lake Ontario	2.74	0.57	0.04
57	suburban, water supply from wells	2.70	0.50	0.07

(c) Subfractions of blood plasma and cells

Table X shows the results of selenium analyses for the subfractions indicated.

TABLE X

Selenium content of subfractions of blood plasma and cells

Sample	Weight of dry protein (grams)	Selenium content (micrograms)	Selenium content per gram (micrograms)
Red blood cell stroma	1.8	1.23	0.68
Stroma supernatant	4.3	0.98	0.23
White blood cells and debris	1.3	1.56	1.20
Plasma euglobulins	2.4	3.29	1.35
Water soluble plasma proteins	2.5	0.24	0.10

(d) Cohn fractions

Samples of the Cohn fractions of blood plasma were analyzed, and the results are shown in Table XI.

TABLE XI

Selenium content of the Cohn fractions of blood plasma

Cohn fraction	Sample weight (grams)	Selenium content (micrograms)	Selenium content per gram (micrograms)
Albumin (crystallized)	0.51	0.11	0.22
Albumin, fraction V	0.63	0.13	0.22
Alpha globulin fraction IV-4	0.40	0.55	1.38
Alpha globulin fraction IV-1	0.41	0.74	1.85
Beta globulin fraction III-0	0.43	1.43	3.58
Beta globulin fraction III	0.42	0.65	1.63
Gamma globulin fraction II	0.50	0.22	0.44

(e) Electrophoretic fractions

The selenium content of the electrophoretic fractions of the plasma of two individuals is shown in Table XII. The percentage of the total amount in the plasma, of a given protein appearing in the fraction shown, is indicated in parentheses after the protein in question.

TABLE XII

Selenium content of electrophoretic fractions of plasma

Plasma sample	Fraction	Proteins in fraction	Selenium content (micrograms)
A	1	γ globulin (100%)	0.06
	2	fibrinogen (100%), β globulin (33%)	0.12
	3	β globulin (66%), α_2 globulin (33%)	0.26
	4	α_2 globulin (66%), α_1 globulin (100%), albumin (75%)	0.41
	5	albumin (25%)	0.09
Whole plasma A	-	All of above	0.96
B	1	γ globulin (22%)	not detectable
	2	γ globulin (78%) fibrinogen (100%)	0.10
	3	β globulin (70%)	0.25
	4	β globulin (30%) α_2 globulin (100%), α_1 globulin (100%), albumin (50%)	0.43
	5	albumin (50%)	0.14
Whole plasma B	-	All of above	0.91

(f) Human tissues other than blood

The results of selenium analyses of autopsy tissue specimens are given in Table XIII for the infant, and adult males, and in Table XIV for the series of ten individuals.

TABLE XIII

Selenium content of tissues* other than blood

Tissue	Selenium content per gram of whole tissue (micrograms)	
	Infant	Adult
Stomach	0.19	0.17
Liver	0.34	0.39
Pancreas	0.05	0.13
Spleen	0.37	0.27
Kidney	0.92	0.63
Intestine	0.31	0.22
Heart	0.55	0.22
Lung	0.17	0.21
Artery	0.27	0.27
Muscle	0.31	0.40
Fat	0.09	0.12
Trachea	0.14	0.24
Gonad	0.46	0.47
Thyroid gland	0.64	1.24
Brain	0.16	0.27
Adrenal gland	0.21	0.36
Lymph node	0.26	0.10

* These values refer to single analyses of tissue samples from one child and one adult. They should therefore be interpreted only to indicate the presence of selenium in the tissues.

TABLE XIV

Selenium content of three tissues for ten individuals

Individual (sex and age)	Selenium content per gram of whole tissue (micrograms)		
	Liver	Skin	Muscle
F69	0.44	0.30	0.32
F77	0.42	0.30	0.25
M61	0.18	0.19	0.26
M65	0.66	0.12	0.40
F62	0.53	0.62	0.32
F63	0.48	0.17	0.44
M72	0.44	0.37	0.48
M45	0.43	0.29	0.59
M63	0.30	0.22	0.28
M29	0.45	0.12	0.35

(g) Miscellaneous materials

(i) Urine

The non-dialyzable residue from 700 ml of human urine was found to contain 1.01 micrograms of selenium.

(ii) Egg protein

Table XV shows the results of selenium analysis of samples of egg protein.

(iii) Milk

75 ml of homogenized milk was found to contain 0.29 micrograms of non-dialyzable selenium.

(h) Results of acid hydrolysis of protein

Under no conditions of hydrolysis or precipitation, were

TABLE XV

Selenium in egg protein (distribution in a single egg)

	White	Yolk	Egg shell membrane
Weight of whole tissue in one 50 gram egg	35 grams	15 grams	0.2 grams
Selenium content (micrograms)	1.26	2.33	0.25
Sulphur content of protein (grams) (142)	0.05	0.03	0.008
Selenium/Sulphur ratio ($\times 10^{-6}$)	25	78	31

detectable quantities of cystine isolated from blood protein. Small amounts of tyrosine (0.1 to 0.5 grams) were isolated from both cell and plasma protein.

10 grams of cystine was obtained and purified by reprecipitation after the hydrolysis of 100 grams of human hair.

(i) Hydrazinolysis

The results of selenium analyses of sulphur liberated by hydrazinolysis from 10 gram samples of dry blood plasma and cells, are given in Table XVI.

It was found that hydrazinolysis of the control mixtures containing Se^{75}-selenomethionine, followed by evolution of H_2S using either acid alone, or acid in the presence of zinc amalgam, resulted in no liberation of selenium from the reaction flask. All Se^{75} activity was retained in the flask.

Following hydrazinolysis of the mixtures containing Se^{75}-selenocystine, it was found that evolution of H_2S by acid alone resulted in no liberation of selenium from the reaction flask.

TABLE XVI

Sulphur liberated from blood protein by hydrazinolysis

Protein	Selenium per 10 grams of dry intact protein (micrograms)	Weight of PbS precipitated (gms)	Selenium content of PbS (micrograms)
Cells	7.6	0.089	0.12
Cells	7.6	0.094	less than 0.05
Cells	7.6	0.069	less than 0.05
Plasma	7.9	0.62	0.06
Plasma	7.9	0.54	less than 0.05

However, in the presence of zinc amalgam, a portion of the Se^{75} activity was evolved. 50% of the activity was retained in the reaction flask, 20% was deposited on the inner walls of the tubing leading from the reaction flask to the first trap, and 30% appeared in the precipitate of PbS and PbSe (which are isomorphous) in the lead acetate trap. No activity was found in the first trap, or in the tubing leading from the first to the second trap.

B. Sulphur Analysis

(a) Experimental method

In Table XVII are shown the results of irradiations carried out on blood samples and on sulphur standards.

It was found that addition of sulphur standards to blood samples did not materially affect the live time of the counting system, and that the counts in the photopeak of S^{37} from a series

TABLE XVII

Counts in S^{37} photopeak from blood samples and standards

Sample	Sulphur added (grams)	Counts in S^{37} photopeak
Cells	———	6,800
	1.74×10^{-2}	24,200
Plasma	———	22,300
	1.74×10^{-2}	33,600
Sulphur standard	1.74×10^{-2}	19,700

of sulphur standards varied linearly as the amount of sulphur present, over the range of sulphur contained in blood, as shown in Figure 16.

In Table XVIII are given the results of analyses for sulphur in identical samples of blood plasma and cells. Standards used for comparison were counted after the same delay time as the corresponding samples.

(b) Plasma and cell fractions of blood

In Table XIX are given the results of analyses for sulphur in the blood of different individuals.

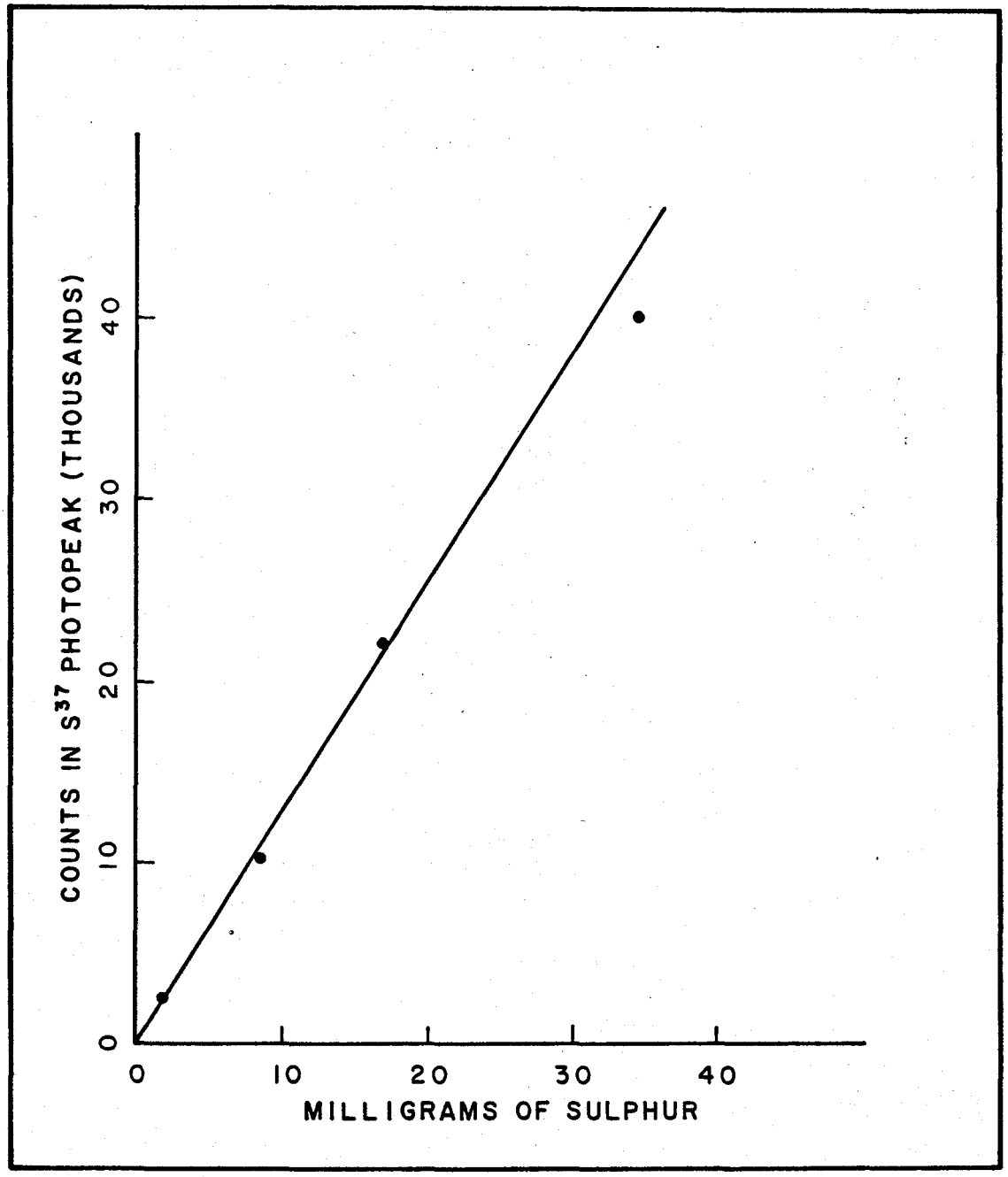


Figure 16

Counts in S^{37} Photopeak from Series of Sulphur Standards, Versus the Sulphur Content

TABLE XVIII

Analysis of sulphur in identical samples

Sample	Sulphur added to duplicate sample (grams)	Delay time (minutes)	Sulphur content of sample (grams)
25 ml plasma A	1.74×10^{-2}	2 1/2	2.95×10^{-2}
25 ml plasma A	3.48×10^{-2}	3 1/2	3.50×10^{-2}
50 ml plasma A	3.48×10^{-2}	2 1/2	7.57×10^{-2}
5 ml cells A	1.74×10^{-2}	2 1/2	0.85×10^{-2}
5 ml cells A	3.48×10^{-2}	3 1/2	0.79×10^{-2}
10 ml cells A	3.48×10^{-2}	2 1/2	1.58×10^{-2}

	Plasma (25ml)	Cells (5ml)
Mean sulphur content (grams x 10^{-2})	3.41	0.81
Standard deviation	0.43	0.03
Standard deviation (as percent of mean)	12.6%	3.7%

TABLE XIX

Sulphur in the blood of different individuals

Sample	Sulphur added to duplicate sample (grams)	Delay time (minutes)	Sulphur content of sample (grams)
25 ml plasma B	1.74×10^{-2}	2 1/2	3.44×10^{-2}
25 ml plasma C	1.74×10^{-2}	2 1/2	3.25×10^{-2}
50 ml plasma D	1.74×10^{-2}	2 1/2	3.10×10^{-2}
5 ml cells B	1.74×10^{-2}	2 1/2	0.68×10^{-2}
5 ml cells C	1.74×10^{-2}	2 1/2	0.68×10^{-2}
10 ml cells D	3.48×10^{-2}	2 1/2	1.71×10^{-2}
10 ml cells E	3.48×10^{-2}	2 1/2	1.94×10^{-2}

CHAPTER 5

DISCUSSION OF RESULTS AND CONCLUSIONS

A. Experimental Methods

(a) Analysis for selenium

A procedure was devised, whereby dialyzed samples of tissue could be analyzed for selenium, in a manner which did not require chemical separations after irradiation. The chief need for dialysis of tissue samples prior to irradiation, arose from the large amounts of sodium chloride present. Without removal, the sodium chloride led to very high activity, which obscured the contributions from minor constituents. However, dialysis not only made radioactivation analysis of the trace elements feasible, but incurred the additional benefit of removing inorganic and dialyzable components of the sample, and allowed the determination of "bound" elements incorporated into a nondialyzable complex. These bound elements, such as protein-bound iodine in blood plasma, are often more directly involved in metabolism than inorganic constituents of tissue which preserve the ionic strength of the tissue medium and form a metabolic pool. Since no foreign elements were introduced during sample preparation, the same samples may be used for further studies of trace elements in human tissues.

While initially, drying of tissue samples prior to irradiation was carried out for the practical purpose of reducing

the physical size of the samples, the extent of drying proved to be a vital and essential part of the overall method of analysis, especially inasmuch as all samples contained a residuum of water, even when dried as thoroughly as possible at the temperatures used.

The situation leading to the necessity of using a correction procedure in the analysis for selenium, was encountered purely empirically when it was found that apparently identical samples did not yield the same count in the photopeak from Se^{77m}. The presence of the O^{19} photopeak, which not only was immediately adjacent to the Se^{77m} photopeak but was also the only other main peak in the spectrum after a 20 second irradiation, suggested a possible interference. This interference was noted by Wainerdi and coworkers (117). The necessity for correction apparently derives from the fact that the live time of counting equipment used, varies appreciably over the counting period, which itself extends over more than a half-life of both O^{19} and Se^{77m} which are the main contributors to dead time. The dead time of the analyzer prevented the processing of selenium counts to a degree which varied as the number of oxygen counts also present. The correction was necessary for all analyzers and scintillation crystals used.

It was determined empirically, using a series of standards containing a fixed amount of selenium and various amounts of oxygen (as water), and a series of identical blood samples containing various residual quantities of water, that the logarithm of the selenium count varied linearly with the oxygen count

within a certain range (Figures 2 and 6). This plot was found to be parallel to others representing different amounts of selenium (Figure 2). The slope was constant for a series of irradiations performed on the same day, but varied by a few percent from day to day, apparently because of flux variations, and thus was redetermined during each set of analyses carried out. Attempts to derive the form of the correction mathematically, resulted in an integral which was not reducible to a form which could be related to the desired expression, and which was soluble only by numerical methods. The numerical solution was not obtained since the form of the correction expression was known from experiment. No solution to the encountered problem was found in the literature, although as mentioned, the difficulty was encountered by Wainerdi (117).

Longer irradiations to produce selenium isotopes of greater half-life which permit subsequent radiochemical treatment, can be used to attain greater sensitivity than achieved by the method utilized in the present work. However, such chemical manipulations often lead to errors through loss of a portion of the element sought. The correction procedure developed, is both rapid and simple, and avoids errors due to chemical procedures both before and after irradiation. The average time for analysis of prepared samples was less than five minutes per sample, including irradiation, counting, and computation of results using the computer programme.

The problems presented by oxygen interference, which had limited the sensitivity and accuracy of the direct instrumental radioactivation analysis for selenium (117), were overcome to an extent which allowed analysis of tissue samples and a meaningful intercomparison of results.

The overall reproducibility or precision of the method developed, based on the standard deviation from the mean of analysis of a series of identical selenium standards, was found to be $\pm$ 7% (Table IV). This reproducibility included random errors due to timing of irradiation and delay periods, irradiation geometry, counting geometry, and variations in flux during a series of analyses. The reproducibility of selenium analysis in blood samples, based on the standard deviation from the mean for a series of identical samples, was found to be $\pm$ 2.8% for cells, and $\pm$ 3.1% for plasma (Table II).

The accuracy of the method was estimated from analysis of blood samples to which a known quantity of selenium was added (Table V). Based on the standard deviation from the mean, the accuracy was found to be $\pm$ 1.3%. This is comparable to the level of accuracy achieved by the majority of activation analysis procedures.

The correction plots utilized, were found to be linear only within a certain range of selenium and oxygen content (Figures 5 and 6). All samples analyzed fell within the linear region, with redrying carried out if necessary to ensure this condition. Although it was not absolutely necessary in order to

obtain selenium analyses, arbitrary cut off limits were defined (Figure 5) in order that computer calculation of results could be carried out.

The sensitivity of the method was estimated from the range over which correction plots remained linear, to be approximately 0.1 micrograms of selenium, with the precision and accuracy indicated above, for samples of dialyzed blood. This was a significant improvement over the previous limit of 0.5 ppm established by Guinn for instrumental activation analysis for selenium using Se^{77m} and a flux comparable to that used in the present work (143). In most of the work reported in this thesis, samples analyzed were found to contain more than 0.1 micrograms. Based on the observed height of the Se^{77m} photopeak encountered in gamma spectra observed, it may be assumed that amounts as small as 0.001 micrograms could be detected. This limit is determined largely by the extent to which the background of the spectrum in the region in which the selenium peak appears, obscures the counts in the selenium photopeak, due to the presence of other elements in the sample. The background in this region is extremely favorable for samples of dialyzed biological material, and is further reduced by the short irradiation time used.

Se^{77m} was produced for analysis by the reaction Se^{76} (n, γ) Se^{77m}. Other possible modes of production of this isotope might include Br^{77} (n, p) Se^{77m} or Kr^{80} (n, α) Se^{77m}. Br^{77} is radioactive and could lead to Se^{77m} only through a secondary reaction of low probability. Kr^{80} is not only of low abundance

in samples, but the cross section for (n, α) reactions with nuclides in this mass region, are very small.

As indicated under experimental methods, instead of integrating the area under a spectral peak, an estimate of the counts in a photopeak representing Se^{77m}, or O^{19}, was made by summing the top five channel counts, and subtracting from this, a background consisting of 2.5 times the sum of one background channel on each side of the peak. This procedure led to smaller errors than are introduced through variables such as flux, timing, and geometries of irradiation and counting, and made calculations much simpler and more rapid.

Selenium standards were prepared by successive dilutions in glass volumetric flasks. Subsequent analyses of several of the diluted solutions showed that the number of counts in the selenium photopeak varied linearly with the theoretical amount of selenium present (Figure 4). Thus it may be assumed that no errors in standard preparation resulted from adsorption of selenium on the walls of the volumetric flasks.

Analysis of samples prepared by evaporating a quantity of standard selenium solution to dryness, and then heating for prolonged periods of time (Table III) showed that little selenium, if any, was lost by volatilization. Standards used for comparison in analysis, were taken only to dryness, and were not heated further, in any case.

The results given in Table I indicate that heating of dried blood samples for a prolonged period of time did not result

in loss of selenium by volatilization. It may be noted that the amount of oxygen (as water) retained in samples dried as completely as possible at the temperature used, varied considerably.

(b) Analysis for sulphur

Radioactivation analysis for sulphur utilizing the production of S^{37} has not been reported in the literature. The principal difficulty has been the fact that S^{37}, having a 5 minute half-life, is too short-lived to permit simple radiochemical separations after irradiation (131) particularly when a tissue matrix is involved. As a result, either the production of S^{35}, which is a weak beta-emitter and presents problems in counting, or of P^{32} with subsequent chemical separations, have been used for analysis.

Although sulphur could not be termed a "trace" element at the level at which it appears in human blood, and although other classical wet chemical methods are available for the analysis of sulphur in any given matrix, radioactivation analysis was explored with the aim of analyzing tissue samples previously prepared, in order to determine the selenium to sulphur ratio present.

Initial attempts to use the production of S^{37} for sulphur analysis, by direct comparison of the counts in the S^{37} photopeak given by samples and standards, resulted in data such as those in Table XVII and indicated that instrumental analysis would not be possible. The live time of counting tissue samples was lower than that for sulphur standards, because of activity generated by

other elements present in these samples, during irradiation. Live time counts for samples and standards were therefore not directly comparable, since the differences in live time remained large over the time period in which all S^{37} activity decayed.

However, it was found experimentally, that addition of small amounts of sulphur to a given tissue sample, resulted in the same live time of count after irradiation as for a duplicate sample without added sulphur, irradiated under the same conditions. It was assumed, therefore, that the same percentage loss of counts would result for sulphur both added and already present in the sample. Thus the theoretical "true", or corrected number of counts which would result from the sulphur inherent in the sample if the dead time were zero, was calculated from the known percentage loss of counts arising from added sulphur standard. This corrected count was then compared with the number of counts given by sulphur standards alone, which did not incur the large live time losses due to the presence of other elements. It was observed that the number of counts in the S^{37} photopeak varied linearly with the amount of sulphur present, over the range of sulphur content investigated in this work (Figure 16).

Using the correction procedure described in experimental methods, therefore, a rapid, instrumental technique was developed for sulphur analysis by radioactivation. Furthermore, the method would be assumed to be applicable to any element in any matrix,

which, on irradiation gives rise to a photopeak whose area may be estimated, and whose addition as a standard to the sample investigated, does not materially affect the live time for the sample alone. Under these conditions, instrumental analysis for many elements, without the necessity of radiochemical separations, should be feasible. Sensitivity is limited only by the statistics afforded by the number of counts in the photopeak used for analysis.

In the present work, since the photopeak due to the 3.1 Mev gamma of S^{37} was well resolved and separated from adjacent peaks at the high energy end of the spectrum, analysis of sulphur in a tissue matrix was facilitated. The sensitivity of the method, based on the observed height and resolution of the S^{37} photopeak in samples encountered, was estimated to be approximately 0.5 milligrams of sulphur.

Overall reproducibility, or precision of the correction procedure developed, was determined from the standard deviation from the mean, of analyses performed on duplicate blood samples, to be $\pm$ 3.7% for cells, and $\pm$ 12.6% for plasma. Inhomogeneity of reactor flux observed during the course of the plasma analyses, as reflected in variations of the photopeak count from sulphur standards during these irradiations, would suggest that increased precision should be obtainable.

The preparation of a standard solution of hydrazine sulphate as used for sulphur standards, is a well-established classical procedure. Since the solutions contained a relatively

macroscopic quantity of sulphur, no errors due to losses by adsorption on the walls of volumetric flasks used, were expected.

Sulphur^{36} was demonstrated to be the only source of the nuclide S^{37} produced on irradiation. Although Cl^{37} could lead to S^{37} by an (n, p) reaction, and A^{40} could lead to S^{37} by an (n, α) reaction, irradiation of chloride in quantities comparable to those present in tissue, did not lead to production of a S^{37} photopeak, and there is insufficient argon in tissue to cause interference.

B. Selenium Analyses

(a) Plasma and cell fractions of blood

The range of the amounts of selenium bound to the non-dialyzable portion of human blood plasma and cells was established for the population of 254 normal individuals studied. Analyses performed by other investigators have been carried out on only a few samples of undialyzed blood (66, 79, 111). The mean selenium content of 15 ml of undialyzed whole human blood, calculated from the results of the eight analyses performed by Bowen (111) was 4.7 micrograms. The mean selenium content of the undialyzed serum from 15 ml of whole human blood, calculated from the results of the twenty-one analyses performed by Gofman (79) was 0.9 micrograms. These results differ considerably from those obtained in the present study where the average selenium content of 15 ml whole blood (dialyzed) was found to be 2.73 micrograms, and the average content of the plasma (dialyzed) from 15 ml whole blood was found to be 1.21 micrograms. Such variations may reflect

differences in dietary selenium levels, or in the case of the analyses of Bowen, the presence of a relatively large amount of non-bound selenium.

All subjects from whom blood samples were drawn for selenium analysis, were patients in St. Joseph's Hospital, Hamilton, Canada, who were considered by the hematology department to be "normal" with respect to blood composition. These included persons having fractured limbs, sprains, injured backs, lacerations etcetera. The chief reasons for the utilization of hospital patients were the high density of available, unsuspecting donors, and the ease of control of rest and fasting conditions.

The gaussian type of distribution found for selenium in the various fractions of blood, throughout the population studied (Figures 7, 8, 9, 10, 11 and 12), as opposed to a more random distribution, is similar in form to that of other trace elements such as iodine. The relative narrowness of the distribution of selenium in plasma, suggests a greater biological significance for selenium in plasma protein than in cell protein since the body seems to have imposed tighter restrictions on the allowed range.

As seen in Figure 13, the plasma selenium content of an individual was observed to parallel roughly, the cell selenium content for the same individual. It is possible that selenium is supplied to these two tissues at least, and perhaps all human tissues, through a common metabolic pathway at some stage in their synthesis.

The apparent "peaking" of the scatter plot of the cell and plasma selenium contents versus blood hematocrit, about a normal hematocrit level, as shown in Figure 14, would appear to refute suggestions that a relationship may exist between the concentration of selenium in red cells, and the degree of anemia (144). The significance of the "peaking" is not known.

It can be seen from Figure 15 and Table VII that the average selenium content of the blood apparently decreases with increasing age. Although this trend may indeed be valid, there are insufficient numbers of individuals in each age group to give definite statistical significance to such a conclusion. The same may be said for the indication in Table VI, that the selenium content of all blood fractions is higher for females than for males.

The variations observed in the selenium content of the cells and plasma of the blood of individuals, sampled after various time periods, indicated, as shown in Table VIII, that relatively large changes can occur within periods of time as short as a few days. Changes in cell content tended to parallel changes in plasma content, and such changes appeared to be constant trends, rather than random, perhaps reflecting dietary changes while in hospital. The most dramatic illustration of the possible effects of diet, was the change in blood selenium of an individual (sample numbers 12, 71, 125) who had resided in Mexico prior to arriving in Canada. Such large changes would indicate that the mean selenium content of blood tissues for a

given population may reflect the dietary sources available to the population.

As indicated in Table IX, essentially no difference in the mean selenium content of whole blood, was observed for persons whose homes were normally located in an urban area, as opposed to those normally residing in a rural area. However, adjustments in tissue selenium could have taken place while the individuals were in hospital.

The selenium to sulphur ratio in blood protein was found to be higher than that in terrestrial sources such as sea water, or some sulphur deposits. The ratios for various terrestrial materials are shown in Table XX.

TABLE XX

Selenium to sulphur ratios in various materials

Material	Reference	Selenium content (micrograms per gram)	Sulphur content (micrograms per gram)	Se/S ratio
Sea water	(147)	0.004	880	4.5×10^{-6}
Soils	(147)	0.01	850	1.2×10^{-5}
Terrestrial rocks	(147)	0.09	520	1.7×10^{-4}
Sulphur (Japan)	(115)	68		6.8×10^{-5}
Sulphur (Texas)	(113)	0.06		6×10^{-8}
Sulphur (Japan)	(116)	100		10^{-4}

While sulphur from deposits in the southwestern United States (113) has been shown to be of biological origin (145) Japanese sulphur is primarily of volcanic origin (146), and contains larger quantities of selenium due to high temperature sublimation processes.

It would appear that the method of formation and deposition of elemental sulphur is indicated by its selenium content.

The following selenium and sulphur ratios, for blood plasma and cells, were calculated, assuming where necessary, that ACD blood plasma is 44% whole plasma, and an hematocrit of 47. Data was taken from Tables VI and XIX.

It is observed that selenium ratios, sulphur ratios, and selenium to sulphur ratios for plasma and cells, differ by a factor of about three in all cases. This would suggest that selenium does not simply parallel sulphur in its overall metabolism and incorporation into blood tissues.

(b) Subfractions of blood plasma and cells.

Since all subfractions analyzed, were prepared from blood pooled from several individuals, their analysis constitutes a form of "instant mean". Pooling was used, because large amounts of blood were required to yield desired quantities of such minor constituents as the red cell stroma, and white blood cells.

It was found that per gram of dried protein, red blood cell stroma contained more selenium than did the protein in solution within the cells (Table X). This was to be expected, since the selenium content of tissues has been seen to parallel the sulphur content, and hemoglobin, the main protein component of the contents of red blood cells, has a low sulphur content (148).

(a) $$\frac{\text{Selenium per gram of dry cell protein}}{\text{Selenium per gram of dry plasma protein}} = \frac{0.76 \times 10^{-6}}{0.83 \times 10^{-6}} = 0.92$$

(b) $$\frac{\text{Sulphur per gram of dry cell protein}}{\text{Sulphur per gram of dry plasma protein}} = \frac{0.70 \times 10^{-2}}{2.6 \times 10^{-2}} = 0.27$$

(c) $$\frac{\text{Selenium per gram of dry cell protein}}{\text{Sulphur per gram of dry cell protein}} = \frac{0.76 \times 10^{-6}}{0.70 \times 10^{-2}} = 1.1 \times 10^{-4}$$

(d) $$\frac{\text{Selenium per gram of dry plasma protein}}{\text{Sulphur per gram of dry plasma protein}} = \frac{0.83 \times 10^{-6}}{2.6 \times 10^{-2}} = 0.32 \times 10^{-4}$$

(e) $$\frac{\text{Selenium in the plasma from 15 ml whole blood}}{\text{Selenium in the cells from 15 ml whole blood}} = \frac{1.2 \times 10^{-6}}{1.5 \times 10^{-6}} = 0.80$$

(f) $$\frac{\text{Sulphur in the plasma from 15 ml whole blood}}{\text{Sulphur in the cells from 15 ml whole blood}} = \frac{2.5 \times 10^{-2}}{1.1 \times 10^{-2}} = 2.3$$

(g) $$\frac{\text{Selenium in the plasma from 15 ml whole blood}}{\text{Sulphur in the plasma from 15 ml whole blood}} = \frac{1.2 \times 10^{-6}}{2.5 \times 10^{-2}} = 0.48 \times 10^{-4}$$

(h) $$\frac{\text{Selenium in the cells from 15 ml whole blood}}{\text{Sulphur in the cells from 15 ml whole blood}} = \frac{1.5 \times 10^{-6}}{1.1 \times 10^{-2}} = 1.4 \times 10^{-4}$$

The white blood cells and debris thereof, were found to contain more selenium per gram of dried protein than the red blood cells (Table X). This again reflects the low sulphur content of hemoglobin, the chief protein component of red cells. In a sample of whole blood, however, the amount of selenium contributed to the total by the small quantity of white blood cells present, is negligible. It should be noted that due to the decomposition of white blood cells, which starts between four and six hours after sampling, even under refrigeration, the material analyzed as "white blood cells and white blood cell debris" was a mixture of indeterminate composition. The transfusion blood from which the material was derived was more than twenty-one days old with respect to the date of donation. However, analysis of the debris would be assumed to give a reasonable approximation to the actual selenium content.

The proteins in blood plasma, which are insoluble when placed in a salt-free environment, as during dialysis, were found to contain a factor of 14 times more selenium per gram of dried protein, than the corresponding distilled water soluble proteins (Table X). These insoluble proteins, the so-called "euglobulins", are comprised of a number of different individual proteins, chief among which are the lipid rich β euglobulin, and to a lesser extent, various γ euglobulins (138, 149).

(c) Cohn and electrophoretic fractions

These fractions of plasma proteins should be distinguished due to the fact that, whereas the Cohn fractions are isolated from

blood pooled from a number of individuals, the electrophoretic fractions were isolated from the plasma of single individuals.

It appears from the results given in Table XI that the amount of selenium per gram of certain plasma fractions, exceeds that of other fractions by more than a factor of 10.

Cohn (138) shows the protein components of the fractions bearing his name, to be as follows:

II	γ globulin
III	β globulin, water soluble
III-O	β euglobulin, lipid-rich, water insoluble
IV-1	α globulin, lipid-rich, water soluble
IV-4	α and β globulins, lipid-poor, water soluble
V	albumin

Fraction III-O containing the water-insoluble lipid-rich β euglobulin was observed to have the highest selenium content (Table XI). These data correlate with the results of the analysis of the euglobulins of plasma (section (b)), which proved to contain far more selenium than the water-soluble proteins.

The results of the electrophoretic analyses in Table XII indicate the same results as discussed above, that it is the α and β globulins which contain a greater proportion of selenium than the other proteins of blood plasma.

Since Heimburger and coworkers have shown (150) that albumin, which constitutes about 60% of all plasma protein, as opposed to β globulin which represents only 10%, is the richest in sulphur-containing amino acids of the Cohn and electrophoretic

fractions analyzed, it would appear that the selenium to sulphur ratio is far greater in certain of the α and β globulins, than in albumin. The highest selenium to sulphur ratio appears to be in the lipid-rich β euglobulin of Cohn fraction III-O. The present work would indicate that a higher percentage of selenium bound in human plasma protein is in lipoprotein, than was found by McConnell after injection of $H_2Se^{75}O_3$ into dogs (151). Desai and Scott have postulated that the interrelationship and abilities of selenium and vitamin E to spare one another, result from the presence of selenolipids in tissue. Selenolipoproteins, proposed as carriers of vitamin E, may function in absorption, retention, prevention of destruction and perhaps transfer of d-α-tocopherol, thereby enhancing its biological activity (45). Although these workers postulate that selenolipoproteins associated with the gamma globulin fraction of blood plasma, may be the specific site of retention of tocopherols in blood, the data in Tables XI and XII indicate that only a very small portion of the total selenium bound to blood proteins of the human, appears in the gamma globulin. It is possible that the lipid-rich beta euglobulin found to be highest in selenium content, is the vitamin E carrier (45).

McConnell found that 24 hours after administration of $Se^{75}Cl_4$ subcutaneously to dogs, 60% of the total radioactivity was distributed equally between the alpha-2, and beta-1 globulin fractions, with smaller amounts in the other fractions (32). The present work would suggest a similar distribution in normal human blood plasma

proteins, with a larger proportion of the selenium in the β globulins (Tables XI and XII). Although these results indicate a similarity in mammalian metabolism of selenium, it is difficult to estimate to what extent the excretion pathways for injected selenium, parallel those of normal metabolism. The finding of McConnell that 80% of Se^{75} injected into rats localized in the red blood cells (32) for example, differs markedly from the percentage of the whole blood selenium found in the cell portion of normal human blood, in the present work (Table VI).

Selenium is low in albumin (Table XI) which has a high cystine content (150), and is high in the alpha and beta globulins amongstwhich are the only fractions having a methionine to cystine mole ratio greater than one (150). If selenium is incorporated into protein in the form of selenoamino acids, it may be speculated that selenium metabolism is more closely associated with that of methionine, than of cystine.

(d) Human tissues other than blood

All tissues analyzed were found to contain selenium. The concentration of selenium in the tissues analyzed was found to vary by as much as a factor of ten. In the infant, the kidney, heart and thyroid gland, were notably high (Table XIII).

The administration of inorganic and organic selenium compounds labelled with Se^{75} has indicated localization of highest

activity in the kidney, liver, pancreas, spleen, adrenal, pituitary and thyroid (153, 154). Analysis of the tissues of animals poisoned accidentally and experimentally with toxic amounts of various selenium compounds, has shown highest concentrations to be in the liver, blood, kidney, lungs, spleen, heart, muscle, and brain (155). Differences in distribution in both cases, were observed to depend on the animal investigated, the form of the selenium administered and the method of administration. It is probable that differences in the distribution of selenium in tissues between that found after administration of amounts in excess of those normally encountered by the animal and that in normal tissues as determined in the present work reflect the different metabolic pathways followed by the relatively large amounts of administered selenium. Analysis of the liver, muscle and skin of ten individuals showed considerable variability as to the tissue of highest selenium content (Table XIV).

(e) Miscellaneous materials

(i) Urine

Although only one urine sample was analyzed (Section g (i) of results) the result is significant in that no previous reference to the existence of selenium bound to non-dialyzable components of normal human urine has been found in the literature.

(ii) Egg protein

The selenium to sulphur ratio was observed to be higher in the yolk, than in the white, or egg shell membrane of the chicken egg (Table XV). This difference could relect a difference in the level of selenium required in the embryonic development of certain tissues. Egg protein would appear to be a significant dietary source of selenium.

(iii) Milk

It was found that appreciable amounts of non-dialyzable selenium occur in cow's milk (Section g (iii) of results). Milk, like egg, appears to be a dietary source of selenium.

(f) Acid hydrolysis

Hydrolyses of blood protein were carried out, with the intention of isolating cystine and measuring its selenium content. This would indicate the percentage of bound selenium incorporated into the protein as the selenium analogue of cystine, and the percentage bound in other ways, such as in the selenium analogue of methionine.

The conditions of hydrolysis were chosen as being suitable for the recovery of cystine (139). Although cystine was obtained in good yield from human hair, no cystine was recovered after

hydrolysis of blood tissues. Since cystine should accompany the tyrosine which was obtained, the cystine was apparently destroyed by an unknown mechanism. This suggestion was supported by experimental attempts to precipitate cystine as the cuprous salt after hydrolysis, which also yielded no product.

(g) Hydrazinolysis

Hydrazine was chosen as the agent for carrying out hydrolysis and reduction of protein, because of its ability to quantitatively reduce the cystine-cysteine sulphur of these amino acids to H_2S, without releasing the sulphur of methionine. The procedure was adopted when attempts to isolate cystine failed, and it was hoped that similar reductive behaviour of hydrazine with the corresponding selenoamino acids would permit differentiation between the amounts of selenium incorporated into protein as the selenium analogues of cystine and methionine.

Although it was recognized from the oxidation potentials listed below, that selenium in the-2 oxidation state could be oxidized by H_3O^+ ion in acidic solution, it was hoped that reaction rates would allow liberation and sweeping of H_2Se from solution before an appreciable amount had been converted into elemental selenium.

In basic solution:	Oxidation potential (volts)
$N_2H_4 = N_2+4H_2O+4e^-$	1.15
$Se^{-2} = Se + 2e^-$	0.78
$S^{-2} = S+2e^-$	0.51
In acidic solution:	
$Zn = Zn^{+2}+2e^-$	0.76
$H_2Se(aq) = Se+2H^++2e^-$	0.36
$H_2S(aq) = S+2H^++2e^-$	-0.14

Experiments carried out concurrently on protein samples and control mixtures, indicated that the sulphur and selenium in cystine and selenocystine but not in methionine or selenomethionine were reduced respectively in basic solution to the-2 oxidation state. However, attempts to acidify and sweep H_2Se from solution along with H_2S which was recovered as PbS in quantitative yield, resulted in oxidation to elemental selenium. Only very small amounts of selenium were observed to reach the lead acetate trap.

Zinc amalgam, added to the hydrazinolysis solution prior to acidification, was found not to release sulphur or selenium from methionine or selenomethionine, but still enabled only partial evolution of the H_2Se formed from selenocystine. Conditions for quantitative evolution of H_2Se might well be resolved by further studies.

C. Sulphur Analyses

(a) Plasma and cell fractions of blood

From the samples analyzed, it appears that there are greater variations in the total bound sulphur content of blood plasma, than of cells (Table XIX).

This perhaps reflects the greater variability in the protein composition of plasma, than in the cells, whose chief protein component is hemoglobin. No literature sources were found for the bound, non-dialyzable or protein sulphur content of blood tissues with which to compare the present results.

SUMMARY OF RESULTS AND CONCLUSIONS

1. Trace quantities of the element selenium were found to be bound to non-dialyzable components of normal human blood and other tissues.

2. A correction procedure was established whereby the limits of the sensitivity and accuracy of the instrumental activation analysis of selenium in biological materials by means of the isotope Se^{77m}, were extended.

3. The range of selenium bound to the plasma and cells of the blood of a population of 254 individuals was determined. Variations in the selenium content of these fractions with time, sex, hematocrit and age were investigated.

4. Analysis of protein subfractions of normal blood plasma and cells indicated that selenium appears in highest concentration in alpha and beta globulins of the plasma.

5. The selenium content of human tissues other than blood, was determined on single autopsy samples taken from selected organs of two individuals.

6. A correction procedure was devised, by means of which instrumental activation analysis for sulphur using the isotope S^{37} could be carried out on dialyzed tissue samples. This procedure would appear to have wider application in the instrumental activation analysis for other elements in a variety of matrices.

7. The bound sulphur content of the plasma and cell fractions of human blood was determined for several samples.

APPENDIX I

COMPUTER PROGRAMME USED IN SELENIUM ANALYSES

The following pages contain the Fortran programme which was used in conjunction with an IBM 7040 computer, to process data obtained by irradiating blood samples and standards for selenium analysis. The programme performed the correction necessitated by the presence of oxygen in the samples, calculated the amount of selenium present in the various fractions of the blood, and carried out a statistical breakdown of results by sex, age, and selenium content.

For each blood sample, four data cards are required, representing the data for duplicate irradiations of both cell and plasma fractions. On each card, columns 7-31 contain the five peak channel counts for oxygen, ending in columns 11, 16, 21, 26 and 31 respectively. Columns 32-39 contain the two background channel counts for oxygen, ending in columns 35 and 39 respectively. Columns 40-64 contain the five peak channel counts for selenium, ending in columns 44, 49, 54, 59 and 64 respectively. Columns 65-72 contain the two background channel counts for selenium, ending in columns 69 and 72 respectively.

The four data cards described above form a set which must be maintained in a fixed order during computation. The first card contains one of the sets of irradiation data for the

cell fraction of a blood sample, and the sample number itself in columns 1-3, ending in column 3, and the letter C in column 4. Columns 5 and 6 contain the hematocrit of the blood sample, expressed as a percent.

The second card contains the other set of irradiation data for the cell fraction, along with the sample number and letter C in columns 1-4 as described for card one. Columns 5 and 6 are left blank.

The third card contains one set of irradiation data for the plasma fraction of the sample, the sample number in columns 1-3, the letter P in column 4, and an integer from 1 to 8 in column 5, depending on the age of the individual (see table below). Column 6 is left blank.

Age code for column 5 of third card

Age range	Under 20	20-29	30-39	40-49	50-59	60-69	70-80	Over 80
Number	1	2	3	4	5	6	7	8

The fourth card in the set contains the other set of plasma irradiation data, the sample number in columns 1-3, a + sign in column 4, column 5 blank, and the number 1 in column 6 if the individual is a male. Otherwise column 6 contains the number 2.

The two calibration parameters necessary for the correction and calculation of results from raw data, were designated in the programme as the SLOPE, and the constant CORR.

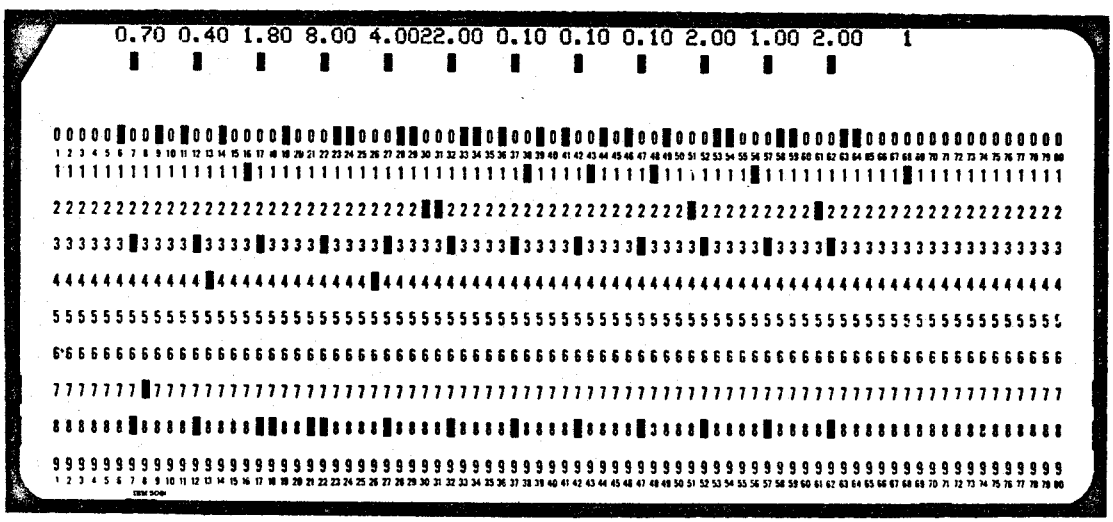
SLOPE, is the slope of the plot of the logarithm of the selenium count versus the oxygen count, for a series of standards

containing the same amount of selenium and different amounts of oxygen, during the period of irradiation of the samples for which cards are being punched.

CORR is obtained by dividing 6.439, the number of micrograms of selenium in the standards irradiated, by the mean number of counts observed in the Se^{77m} photopeak for the standards containing selenium only.

SLOPE is punched on the first and third card of each set of four, in columns 73-79. CORR is punched on the second and fourth cards, in the same columns. Both constants were punched in the form 2222E-4, which is read by the programme as 2.222×10^{-4}.

Prior to the sets of data cards representing a number of blood samples, is placed a parameter card containing the number of samples (i.e. sets of four cards) in columns 1-4, ending in column 4. The remainder of the data on the parameter card gives low limits, and channel increments for statistics. These numbers are shown on the sample parameter card below.



```
      C     ACTIVATIØN ANALYSIS PRØGRAM INCLUDING STATISTICS,  RC DICKSØN,1964 $$
1           INTEGER RES1,RES2,HØLD                                              $$
2           BACK(T,U,V,W,X,Y,Z)=(T+U+V+W+X)-2.5*(Y+Z)                           $$
3           DIMENSIØN XLØ(6),ADD(6),XDAT(4,7),YDAT(4,7),AMT(4),A(6),S(4),       $$
           1RES1(24,8,6),RES2(24,8,6),          HØLD(27)                        $$
4           DØ 1 KCH=1,24                                                       $$
5           DØ 1 KAGE=1,8                                                       $$
6           DØ 1 KFRAC=1,6                                                      $$
7           RES1(KCH,KAGE,KFRAC)=0                                              $$
10        1 RES2(KCH,KAGE,KFRAC)=0                                              $$
11          READ 2,N,XLØ,ADD,M                                                  $$
12        2 FØRMAT(I4,12F5.2,I4)                                                $$
13          PRINT 3                                                             $$
14        3 FØRMAT(20X,80HACTIVATIØN ANALYSIS FØR UNITS SUBSTANCE X IN SIX FRA  $$
           2CTIØNS ØF HUMAN BLØØD          /20X,30HUNITS ARE MICRØGRAMS        $$
           3   /20X,13HFRACTIØNS ARE 10X ,20HF1  RED BLØØD CELLS  /43X,10HF2   $$
           4PLASMA/43X,30HF3  PLASMA + RED BLØØD CELLS   )                      $$
15          PRINT 9                                                             $$
16        9 FØRMAT (43X,30HF4  100 ML. RED BLØØD CELLS   /43X,20HF5  100 ML. P  $$
           1LASMA   / 43X,50HF6  100 ML. PLASMA + 100 ML. RED BLØØD CELLS       $$
           2        /20X,30HSUBSTANCE X IS SELENIUM                  /20X,27HSAM $$
           3PLE 15.0 ML. WHØLE BLØØD          )                                $$
17          PRINT 5                                                             $$
20        5 FØRMAT(1H-/1H-/1H-,38X,6HSAMPLE,3X,6(7HUNITS X,5X)/                 $$
           129X,3HSEX,2X,3HAGE,2X,6HNUMBER,5X                                   $$
           1,5HIN F1,7X,5HIN F2,7X,5HIN F3,7X,5HIN F4,7X,5HIN F5,7X,5HIN F6)    $$
21          DØ 150 I=1,N                                                        $$
22          READ 6,NØ,HEM,(YDAT(1,KK),KK=1,7),(XDAT(1,KK),KK=1,7), SLØPE1,      $$
           1              (YDAT(2,KK),KK=1,7),(XDAT(2,KK),KK=1,7),  CØR1,      $$
           2KAGE,         (YDAT(3,KK),KK=1,7),(XDAT(3,KK),KK=1,7), SLØPE2,     $$
           3KSEX,         (YDAT(4,KK),KK=1,7),(XDAT(4,KK),KK=1,7),  CØR2       $$
23        6 FØRMAT(I3,1X, F2.0,    5F5.0,2F4.0,5F5.0,2F4.0,  E7.3/              $$
           16X,                    5F5.0,2F4.0,5F5.0,2F4.0,  E7.3/              $$
           24X,I1,1X,              5F5.0,2F4.0,5F5.0,2F4.0,  E7.3/              $$
           35X,I1,                 5F5.0,2F4.0,5F5.0,2F4.0,  E7.3               )$$
24          S(1)=SLØPE1                                                         $$
25          S(2)=SLØPE1                                                         $$
26          S(3)=SLØPE2                                                         $$
27          S(4)=SLØPE2                                                         $$
30          DØ 7 JJ=1,4                                                         $$
31        7 AMT(JJ)=BACK(XDAT(JJ,1),XDAT(JJ,2),XDAT(JJ,3),XDAT(JJ,4),XDAT(JJ,5 $$
           1),XDAT(JJ,6),XDAT(JJ,7))*EXP(2.303*S(JJ)*BACK(YDAT(JJ,1),YDAT(JJ,2 $$
           2),YDAT(JJ,3),YDAT(JJ,4),YDAT(JJ,5),YDAT(JJ,6),YDAT(JJ,7)))         $$
32          A(1)=CØR1*(AMT(1)+AMT(2))/2.                                        $$
33          A(2)=CØR2*(AMT(3)+AMT(4))/2.                                        $$
34          A(3)=A(1)+A(2)                                                      $$
35          CML=15.*HEM/100.                                                    $$
36          PML=15.-CML                                                         $$
37          A(4)=100.*A(1)/CML                                                  $$
40          A(5)=100.*A(2)/PML                                                  $$
41          A(6)=A(4)+A(5)                                                      $$
42          PRINT 10,KSEX,KAGE,NØ,A                                             $$
```

```
43          10 FØRMAT(20X,3I5,6(2X,F10.3))                                                  $$
44             IF(M.NE.1)  GØ TØ 150                                                        $$
45             DØ 100 KFRAC=1,6                                                             $$
46             DØ 99 KCH=1,23                                                               $$
47             CH=KCH-1                                                                     $$
50             IF(A(KFRAC)-XLØ(KFRAC)-(ADD(KFRAC)*CH))31,32,99                              $$
51          31 GØ TØ (11,21),KSEX                                                           $$
52          32 GØ TØ(12,22),KSEX                                                            $$
53          11 RES1(KCH,KAGE,KFRAC)=RES1(KCH,KAGE,KFRAC)+1                                  $$
54             GØ TØ 100                                                                    $$
55          21 RES2(KCH,KAGE,KFRAC)=RES2(KCH,KAGE,KFRAC)+1                                  $$
56             GØ TØ 100                                                                    $$
57          12 RES1( KCH+1 ,KAGE,KFRAC)=RES1( KCH+1 ,KAGE,KFRAC)+1                          $$
60             GØ TØ 100                                                                    $$
61          22 RES2( KCH+1 ,KAGE,KFRAC)=RES2( KCH 1 ,KAGE,KFRAC)+1                          $$
62             GØ TØ 100                                                                    $$
63          99 CØNTINUE                                                                     $$
64             GØ TØ (13,23),KSEX                                                           $$
65          13 RES1(24,KAGE,KFRAC)=RES1(24,KAGE,KFRAC)+1                                    $$
66             GØ TØ 100                                                                    $$
67          23 RES2(24,KAGE,KFRAC)=RES2(24,KAGE,KFRAC)+1                                    $$
70         100 CØNTINUE                                                                     $$
71         150 CØNTINUE                                                                     $$
72             IF(M.NE.1) GØ TØ 999                                                         $$
73             PRINT 101                                                                    $$
74         101 FØRMAT(132H1STATISTICAL DISTRIBUTIØN WITH RESPECT TØ PARAMETERS P           $$
              1AND Q          PARAMETER P IS AGE                PARAMETER Q IS SEX         $$
              2          / 70X,11HP1 UNDER 20,20X,8HQ1 MALE /70X,11HP2 20-29               $$
              32CX,10HQ2 FEMALE ,/70X,11HP3 30-39    /70X,11HP4 40-49    /70X,            $$
              48HP5 50-59/70X,8HP6 60-69/70X,8HP7 70-79/70X, 12H80 AND ØVER  )            $$
75             PRINT 102                                                                    $$
76         102 FØRMAT (1H-,100HUNITS X VALUE GIVEN IN FIRST CØLUMN IS LØWER LIMIT          $$
              1 ØF CHANNEL. UPPER LIMIT IS NEXT VALUE GIVEN.                          )    $$
77             DØ 200 KFRAC=1,6                                                             $$
100            PRINT 104,KFRAC                                                              $$
101        104 FØRMAT(1H- 5X,11HUNITS X IN ,15X,12HPARAMETER Q1 ,24X,12HPARAMETER          $$
              1 Q2 ,24X,12HSUM ØF Q1+Q2    /6X,10HFRACTIØN F 11,3(6X,30H P1 P2 P3          $$
              2P4 P5 P6 P7 P8 ALL P  ))                                                    $$
102            DØ 200 KCH=1,24                                                              $$
103            IF (KCH-1)105,105,106                                                        $$
104        105 CHAN=0.00                                                                    $$
105            GØ TØ 107                                                                    $$
106        106 CH=KCH-2                                                                     $$
107            CHAN        =XLØ(KFRAC)+(CH*ADD(KFRAC))                                      $$
110        107 DØ 108 KHØLD=1,27                                                            $$
111        108 HØLD(KHØLD)=0                                                                $$
112            DØ 110 KAGE=1,8                                                              $$
113            HØLD(KAGE)=RES1(KCH,KAGE,KFRAC)                                              $$
114            HØLD(9)=HØLD(9)+HØLD(KAGE)                                                   $$
115            HØLD(KAGE+9)=RES2(KCH,KAGE,KFRAC)                                            $$
116            HØLD(18)=HØLD(18)+HØLD(KAGE+9)                                               $$
117            HØLD(KAGE+18)=HØLD(KAGE)+HØLD(KAGE+9)                                        $$
120            HØLD(27)=HØLD(27)+HØLD(KAGE+18)                                              $$
121        110 CØNTINUE                                                                     $$
122            PRINT 109,CHAN,HØLD                                                          $$
123        109 FØRMAT ( 8X,F6.2,3X,3(6X,8I3,2X,I4))                                         $$
124        200 CØNTINUE                                                                     $$
125        999 CØNTINUE                                                                     $$
126            STØP                                                                         $$
127            END                                                                          $$
       $ENTRY
```

APPENDIX II

COMPUTER PROGRAMME USED IN STATISTICAL TREATMENT OF DATA

The following pages contain the Fortran programme which was used in conjunction with an IBM 7040 computer, in order to obtain for a given set of results, the mean, standard deviation and standard error, of the values in the set lying within an arbitrarily chosen deviation from the mean.

Any number of sets of results, each containing any number of values, may be processed consecutively, with the card preceding the data cards for each set indicating the number of values in that respective set. The comments included in the programme indicate the conventions adopted regarding the punching of data, and the position of decimal points.

```
C                          CALCULATIØNS     RØBERT C DICKSØN                    $$
C     CALCULATIØN ØF MEAN, STANDARD DEVIATIØN, AND STANDARD ERRØR FØR A        $$
C     NUMBER ØF RESULTS                                                        $$
C     THE FIRST DATA CARD CØNTAINS ØNLY THE NUMBER ØF VALUES N UPØN WHIC $$ H
C     STATISTICS ARE BEING CARRIED ØUT, AND THE NUMBER 1. CØLUMNS 1 TØ 4 $$
C    .CØNTAIN THE NUMBER N ENDING IN CØLUMN 4 AND CØLUMN 8 CØNTAINS THE $$ NUMBER 1
C     DATA IS READ FRØM SUCCESSIVE GRØUPS ØF 7 CØLUMNS ØN EACH DATA CARD $$  AFTER
C     THE FIRST (SEE ABØVE) ØNLY CØLUMNS 1 TØ 77 ARE USED ØN EACH CARD.  $$ UP TØ 11
C     DATA VALUES MAY BE PUNCHED PER CARD. NØ DECIMAL PØINT IS PUNCHED I $$ N DATA
C     VALUES, BUT IS UNDERST00D BY THE CØMPUTER TØ BE AFTER THE THIRD CØ $$ LUMN IN
C     EACH SET ØF SEVEN. ALLØWANCE IS THUS MADE FØR 3 NUMBERS BEFØRE AND $$  4
C     NUMBERS AFTER THE DECIMAL PØINT IN DATA VALUES                      $$
C     EACH ØF THE DATA PØSITIØNS ØN EACH CARD EXCEPT THE LAST MUST BE ØC $$ CUPIED
C     SINCE THE CØMPUTER READS THE NUMBER N VALUES FRØM THE FIRST N DATA $$
C     PØSITIØNS THAT IT ENCØUNTERS IN A RØW                               $$
C     IF A PARTIALLY-FILLED DATA CARD IS ENCØUNTERED IN THE MIDST ØF A S $$ ET ØF
C     DATA THE CØMPUTER WILL MERELY TREAT THE VACANT DATA PØSITIØNS AS D $$ ATA
C     VALUES ØF ZERØ                                                      $$
1          DIMENSIØN C(1000,1)                                            $$
2        2 READ 11,M,N,NUM                                                $$
3          READ 12, ((C(I,J),J=1,N),I=1,M)                                $$
4       15 FØRMAT(10(4X,F8.4))                                            $$
5        1 M1=0                                                           $$
6          WRITE(6,15)((C(I,J),J=1,N),I=1,M)                              $$
7          SIG=0.                                                         $$
10         NUM=NUM+1                                                      $$
11         DØ 21 I=1,M                                                    $$
12         DØ 21 J=1,N                                                    $$
13         IF (C(I,J).GT.0.)M1=M1+1                                       $$
14      21 SIG=SIG + C(I,J)                                               $$
15         AMEAN = SIG/FLØAT(M1)                                          $$
16         PRINT 13,M1,AMEAN,NUM                                          $$
17         SUM = 0.                                                       $$
20         DØ 31 I=1,M                                                    $$
21         DØ 31 J=1,N                                                    $$
22      31 SUM = SUM + (C(I,J))**2                                        $$
23         SDEV = SQRT((SUM-SIG**2/FLØAT(M1))/FLØAT(M1-1))                $$
24         PRINT  14, SDEV,NUM                                            $$
25         T2=FLØAT(M1)                                                   $$
26         SERR=SDEV/SQRT(T2)                                             $$
27         PRINT17,SERR,NUM                                               $$
30         I1=0                                                           $$
31         DØ 41 I=1,M                                                    $$
32         DØ 41 J=1,N                                                    $$
33         IF(C(I,J).EQ.0.) GØ TØ 41                                      $$
C     ØN THE TWØ CARDS FØLLØWING THIS SERIES ØF CØMMENT CARDS, THE NUMBE $$ R 3
C     SIGNIFIES THAT THE CRITERIØN FØR REJECTIØN ØF DATA VALUES AFTER     $$
C     CALCULATIØN ØF THE MEAN AND STANDARD DEVIATIØN, IS THAT ØF BEING G $$ REATER
C     ØR LESS THAN (THE MEAN, PLUS ØR MINUS 3 TIMES THE STANDARD DEVIATI $$ ØN).
C     THE NUMBER 3. MAY BE REPLACED BY ANY ØTHER FLØATING PØINT NUMBER S $$ UCH AS
C     2. ØR 5. ØR 1.736 TØ CHANGE THE REJECTIØN CRITERIØN. THE REST ØF T $$ HE CARD
C     REMAINS THE SAME.                                                   $$
```

```
34            IF(ABS(C(I,J)-AMEAN).GT.  3.*SDEV) I1=I1+1                                    $$
35            IF(ABS(C(I,J)-AMEAN).GT.  3.*SDEV) C(I,J)=0.                                  $$
36         41 CØNTINUE                                                                      $$
37            IF (I1.GT.0)  GØ TØ 1                                                         $$
40         11 FØRMAT (3I4)                                                                  $$
41         12 FØRMAT (11 F 7.4)                                                             $$
42         13 FØRMAT(1HO,13H THE MEAN ØF ,I3,10HVALUES IS ,F10.5,9H FØR RUN ,I4)            $$
43         14 FØRMAT(1HO,26H THE STANDARD DEVIATIØN IS ,   F10.5,9H FØR RUN ,I4)            $$
44         17 FØRMAT(1HO,22H THE STANDARD ERRØR IS ,F14.5,9H FØR RUN ,I4)                   $$
45            WRITE (6,16)                                                                  $$
46         16 FØRMAT (1H1)                                                                  $$
47            GØ TØ 2                                                                       $$
50            END                                                                           $$
      $ENTRY
```

APPENDIX III

INDIVIDUAL AND GROUPED RESULTS OF ANALYSES FOR SELENIUM IN BLOOD SAMPLES

The following pages contain the complete set of individual and statistically treated results of the selenium analysis of the blood fractions of 254 normal individuals. The results are given in the form in which they were provided in the computer output.

EXECUTIØN
ACTIVATIØN ANALYSIS FØR UNITS SUBSTANCE X IN SIX FRACTIØNS ØF HUMAN BLØØD
UNITS ARE MICRØGRAMS
FRACTIØNS ARE
F1 RED BLØØD CELLS
F2 PLASMA
F3 PLASMA + RED BLØØD CELLS
F4 100 ML. RED BLØØD CELLS
F5 100 ML. PLASMA
F6 100 ML. PLASMA + 100 ML. RED BLØØD CELLS
SUBSTANCE X IS SELENIUM
SAMPLE 15.0 ML. WHØLE BLØØD

SEX	AGE	SAMPLE NUMBER	UNITS X IN F1	UNITS X IN F2	UNITS X IN F3	UNITS X IN F4	UNITS X IN F5	UNITS X IN F6
1	5	1	1.575	1.199	2.774	26.253	13.326	39.579
1	2	2	1.200	1.343	2.543	19.993	14.925	34.918
1	4	3	1.762	1.261	3.022	26.692	15.009	41.701
1	7	4	2.206	1.190	3.396	34.202	13.919	48.121
1	4	5	1.129	1.185	2.313	17.498	13.858	31.356
1	5	6	1.217	1.080	2.297	20.796	11.804	32.600
1	2	7	1.571	0.874	2.445	25.547	9.871	35.418
1	5	9	2.228	1.320	3.548	32.284	16.296	48.580
2	2	12	1.373	1.042	2.416	23.475	11.392	34.867
2	5	13	1.513	1.052	2.564	28.011	10.956	38.967
2	7	14	1.417	1.234	2.650	22.487	14.179	36.666
2	5	15	1.204	0.562	1.766	18.247	6.689	24.936
2	1	16	1.166	0.976	2.142	18.500	11.222	29.723
2	5	17	1.225	0.902	2.127	19.920	10.197	30.117
2	7	18	1.163	1.102	2.265	22.152	11.307	33.459
2	4	19	1.685	0.996	2.681	24.421	12.293	36.714
2	1	20	2.182	1.438	3.619	30.946	18.083	49.028
2	2	21	1.485	1.283	2.769	24.757	14.258	39.015
2	6	22	1.691	1.466	3.158	27.502	16.569	44.071
2	2	23	2.029	1.574	3.603	36.554	16.654	53.208
2	5	24	1.461	1.213	2.674	21.642	14.706	36.349
2	4	25	1.387	1.064	2.451	19.670	13.384	33.054
2	4	26	1.275	1.228	2.503	25.004	12.406	37.411
2	4	27	1.754	1.030	2.784	24.360	13.207	37.567
2	5	30	1.353	1.261	2.614	23.129	13.778	36.907
2	3	31	2.066	1.337	3.403	34.436	14.857	49.292
2	3	32	2.039	1.328	3.367	33.155	15.002	48.157
2	3	33	1.340	1.145	2.485	22.913	12.512	35.426
2	6	34	2.037	1.249	3.286	30.861	14.870	45.731
2	1	35	1.215	1.213	2.428	17.604	14.976	32.580
2	7	36	1.593	1.146	2.738	23.594	13.885	37.479
2	1	37	1.253	1.368	2.621	20.370	15.457	35.827
2	5	38	1.109	0.972	2.081	18.034	10.987	29.021
2	7	39	1.130	1.047	2.177	19.822	11.257	31.079
1	1	40	1.902	1.385	3.287	25.881	18.101	43.982
1	7	41	1.186	0.930	2.116	20.272	10.162	30.433
1	5	42	1.077	0.896	1.973	16.693	10.484	27.178
1	4	43	1.224	1.294	2.518	21.477	13.915	35.392

1	6	44	1.590	1.197	2.787	23.045	14.774	37.819
1	3	45	1.533	1.248	2.782	21.745	15.704	37.449
2	6	46	1.648	1.121	2.769	22.891	14.374	37.266
2	4	47	1.395	1.078	2.473	21.135	12.832	33.967
2	1	48	1.344	0.940	2.285	20.370	11.195	31.565
2	5	49	2.207	1.115	3.322	26.746	16.525	43.271
2	7	50	1.542	1.158	2.699	23.357	13.783	37.141
2	3	51	2.300	1.878	4.179	37.403	21.223	58.627
2	6	53	1.148	0.905	2.052	18.216	10.401	28.617
2	3	54	2.148	1.705	3.853	37.684	18.328	56.013
2	1	55	1.817	1.394	3.211	27.533	16.595	44.128
2	5	56	1.473	1.324	2.797	19.639	17.653	37.292
2	7	57	1.308	1.389	2.697	23.568	14.694	38.262
2	2	58	1.394	1.918	3.312	25.811	19.983	45.794
2	7	59	1.757	1.163	2.920	23.431	15.505	38.936
2	3	60	1.751	1.563	3.314	27.153	18.276	45.428
2	6	62	1.355	1.294	2.649	20.536	15.405	35.940
2	2	63	1.696	1.253	2.949	23.561	16.060	39.621
1	5	64	1.284	1.148	2.432	17.472	15.002	32.474
1	6	68	1.762	1.313	3.075	23.037	17.864	40.901
1	3	70	1.218	1.027	2.245	15.619	14.258	29.877
1	4	72	1.297	1.181	2.477	18.010	15.135	33.145
1	5	73	1.602	0.997	2.599	20.540	13.850	34.390
1	1	74	1.813	1.404	3.218	25.184	18.004	43.188
1	5	75	1.962	1.394	3.356	27.245	17.873	45.119
1	2	76	1.648	1.236	2.884	22.416	16.156	38.573
1	3	77	2.824	1.865	4.689	45.911	21.079	66.990
1	3	78	1.725	1.152	2.877	23.475	15.053	38.529
1	3	79	1.767	1.284	3.051	23.556	17.120	40.676
1	2	80	1.849	1.301	3.151	24.176	17.705	41.881
1	4	81	1.137	0.938	2.075	14.578	13.021	27.599
1	3	83	1.997	1.364	3.361	28.938	16.840	45.778
1	4	85	1.064	0.815	1.879	13.643	11.322	24.965
2	7	86	1.514	1.201	2.715	21.024	15.402	36.426
2	3	87	1.054	0.921	1.975	16.733	10.582	27.315
2	5	88	1.299	0.936	2.234	18.037	11.996	30.034
2	5	91	1.272	1.238	2.510	16.956	16.511	33.467
2	5	92	1.334	1.194	2.528	20.212	14.217	34.429
2	4	93	1.602	0.992	2.594	26.056	11.205	37.261
2	7	94	1.300	1.269	2.569	21.136	14.337	35.472
2	2	95	1.922	1.483	3.405	32.848	16.209	49.057
2	1	96	2.403	1.492	3.895	31.413	20.302	51.715
1	3	99	1.630	1.269	2.899	20.903	17.622	38.525
1	5	101	1.509	1.132	2.641	18.982	16.063	35.044
1	2	103	2.041	1.208	3.249	26.678	16.432	43.109
1	1	104	1.276	1.287	2.564	17.723	16.506	34.229
1	4	105	1.506	1.064	2.570	19.303	14.777	34.079
1	3	106	1.187	0.991	2.178	18.409	11.587	29.997
1	4	107	1.365	0.816	2.181	19.776	10.077	29.853
1	2	108	1.248	1.237	2.485	15.405	17.923	33.329
1	2	109	1.635	1.074	2.709	22.702	13.774	36.476
1	3	110	1.598	1.138	2.736	20.485	15.804	36.289
1	1	111	2.960	1.748	4.708	41.117	22.405	63.523
1	3	112	1.420	1.037	2.457	18.210	14.403	32.613
1	1	113	1.503	1.022	2.524	20.873	13.096	33.969
1	3	114	1.550	1.143	2.694	21.533	14.656	36.189
2	6	2	0.792	0.783	1.574	12.872	8.846	21.718
2	3	5	1.074	1.073	2.147	24.688	10.076	34.763

2	2	7	1.339	1.009	2.348	22.312	11.210	33.522
1	6	8	1.648	1.369	3.017	24.413	16.600	41.012
2	6	10	0.975	1.257	2.232	19.127	12.692	31.820
1	6	11	0.762	0.860	1.621	10.158	11.462	21.620
2	3	12	0.558	0.524	1.082	9.785	5.636	15.421
1	3	13	0.923	0.794	1.717	13.984	9.455	23.439
1	5	14	0.965	0.918	1.884	13.405	11.773	25.179
1	2	15	1.040	0.790	1.829	12.838	11.443	24.281
2	3	16	2.382	1.649	4.031	36.935	19.286	56.220
2	3	17	1.346	0.943	2.289	20.865	11.033	31.898
1	2	18	1.170	0.915	2.085	16.590	11.512	28.102
2	3	19	1.322	1.126	2.448	18.748	14.163	32.911
2	2	20	1.787	1.515	3.303	24.824	19.426	44.250
1	5	21	1.514	0.655	2.169	21.031	8.398	29.429
2	4	22	1.952	1.636	3.588	29.581	19.474	49.055
1	6	23	2.011	1.684	3.695	30.470	20.047	50.517
1	4	24	1.061	1.096	2.156	15.712	13.280	28.992
1	4	25	1.212	0.853	2.065	15.242	12.103	27.344
2	5	26	1.072	1.057	2.130	16.246	12.588	28.834
2	3	27	1.411	1.190	2.601	20.900	14.428	35.328
2	3	28	1.337	1.352	2.690	21.746	15.281	37.027
2	6	29	1.068	1.365	2.433	20.946	13.787	34.734
2	6	30	1.636	1.371	3.007	25.965	15.760	41.725
2	2	31	1.441	1.111	2.551	22.339	12.990	35.328
2	3	32	1.230	1.062	2.292	16.080	14.443	30.523
2	4	33	1.937	1.336	3.273	29.356	15.904	45.260
2	1	34	1.354	1.077	2.431	21.486	12.380	33.867
2	2	35	1.581	1.434	3.015	22.419	18.044	40.463
1	6	36	0.908	0.910	1.818	12.358	11.898	24.255
1	6	37	1.112	0.866	1.978	17.239	10.129	27.368
2	6	38	2.457	1.323	3.780	39.008	15.207	54.215
1	7	39	1.392	1.020	2.412	22.099	11.724	33.823
1	4	40	1.028	0.956	1.983	13.702	12.741	26.444
2	3	41	1.456	1.222	2.677	22.571	14.288	36.859
2	6	42	1.755	1.181	2.936	22.937	16.067	39.004
2	6	43	1.216	0.791	2.007	19.770	8.936	28.706
1	7	45	1.094	0.934	2.029	16.582	11.121	27.703
2	5	46	1.297	1.162	2.459	19.645	13.834	33.479
1	5	47	1.971	1.420	3.391	29.205	17.209	46.415
2	5	48	1.393	1.147	2.539	20.182	14.157	34.339
1	5	50	1.525	0.928	2.453	20.749	12.130	32.879
1	8	51	1.288	1.367	2.656	22.605	14.700	37.305
2	4	52	1.553	1.246	2.799	22.025	15.676	37.701
1	6	54	0.896	1.001	1.897	20.602	9.396	29.998
1	3	55	1.255	0.924	2.179	17.436	11.844	29.280
2	7	58	1.795	1.383	3.178	28.499	15.893	44.392
2	2	60	1.208	1.071	2.279	20.656	11.704	32.359
2	8	61	0.920	0.821	1.740	15.721	8.969	24.690
2	3	62	1.625	1.043	2.668	25.194	12.198	37.392
1	6	63	1.696	0.654	2.350	20.191	9.914	30.105
1	2	64	1.647	1.128	2.774	21.110	15.666	36.775
1	6	65	1.336	1.000	2.336	17.813	13.327	31.140
1	3	66	1.655	1.047	2.702	22.519	13.686	36.205
1	5	67	1.187	1.198	2.385	19.787	13.308	33.096
1	2	68	1.449	1.065	2.514	20.126	13.655	33.782
1	4	69	1.672	1.160	2.833	22.753	15.167	37.920
1	5	70	0.977	1.078	2.054	14.797	12.831	27.628
2	6	72	1.250	1.044	2.294	19.376	12.215	31.591

2	6	73	1.892	1.398	3.290	26.275	17.929	44.203
1	5	74	1.554	1.218	2.773	28.006	12.892	40.898
2	7	75	1.421	1.524	2.946	24.938	16.390	41.328
1	7	76	1.993	0.993	2.986	23.724	15.049	38.773
1	8	77	1.846	1.281	3.128	27.355	15.530	42.885
2	4	78	1.705	1.336	3.041	25.257	16.192	41.449
2	5	80	1.597	1.630	3.227	28.015	17.529	45.544
2	8	81	1.370	0.803	2.173	22.273	9.072	31.344
2	7	82	1.301	1.167	2.468	20.650	13.417	34.067
2	2	83	1.976	1.586	3.562	31.369	18.225	49.594
2	7	84	1.287	0.760	2.046	19.493	9.042	28.535
2	5	85	1.276	1.084	2.360	22.383	11.661	34.044
1	7	86	1.027	1.112	2.139	17.116	12.360	29.477
1	8	87	1.057	0.996	2.053	20.731	10.058	30.789
2	3	89	1.168	1.156	2.324	17.308	14.015	31.323
2	7	91	1.069	0.880	1.949	16.192	10.476	26.667
2	7	92	1.071	1.029	2.100	21.001	10.393	31.394
1	8	93	1.111	1.386	2.496	24.678	13.200	37.878
2	3	94	1.622	1.094	2.715	24.024	13.259	37.282
1	3	95	1.338	0.915	2.253	22.867	10.003	32.870
2	5	96	1.504	1.462	2.966	28.639	14.998	43.638
2	5	98	1.287	1.106	2.393	20.427	12.711	33.139
2	5	99	1.385	1.155	2.540	21.991	13.271	35.262
1	6	100	1.073	0.984	2.057	19.337	10.413	29.750
2	3	102	1.237	1.085	2.322	21.139	11.861	33.001
1	5	103	1.250	1.181	2.431	18.516	14.318	32.833
2	8	104	1.219	1.080	2.299	19.345	12.418	31.763
1	6	105	1.291	1.108	2.399	17.213	14.768	31.980
2	6	106	1.192	0.985	2.177	16.913	12.387	29.300
2	3	107	1.797	1.238	3.034	36.294	12.317	48.611
2	2	108	1.644	1.232	2.877	27.408	13.691	41.099
2	5	110	1.411	1.402	2.813	24.751	15.077	39.828
2	2	111	1.510	1.210	2.720	24.556	13.675	38.231
1	7	112	1.940	1.497	3.437	32.328	16.637	48.965
1	4	114	1.796	1.001	2.798	27.852	11.710	39.561
1	8	115	1.890	1.524	3.414	28.632	18.148	46.780
1	7	116	1.271	1.885	3.156	31.391	17.216	48.607
1	7	118	1.777	1.389	3.166	27.548	16.245	43.794
2	5	119	3.086	1.964	5.050	45.723	23.808	69.531
1	3	120	3.131	2.074	5.204	46.378	25.138	71.516
2	4	121	2.406	1.373	3.779	32.073	18.309	50.383
1	1	122	2.068	1.333	3.402	35.358	14.573	49.931
2	3	126	1.259	1.090	2.350	19.081	12.981	32.063
2	5	127	2.564	1.696	4.260	37.159	20.932	58.092
2	4	128	1.473	1.481	2.955	22.844	17.325	40.169
2	4	129	2.211	1.563	3.774	35.956	17.657	53.613
2	2	130	2.731	2.389	5.120	46.682	26.105	72.787
2	5	131	1.162	1.519	2.681	19.856	16.603	36.459
1	6	132	1.447	1.259	2.706	20.521	15.836	36.357
1	5	134	1.542	1.179	2.721	21.868	14.835	36.704
1	4	135	2.010	1.564	3.574	28.504	19.676	48.179
1	5	137	1.415	0.890	2.305	17.148	13.182	30.330
1	1	138	1.893	1.632	3.525	28.039	19.786	47.824
2	1	140	0.862	0.830	1.693	15.970	8.651	24.620
2	2	141	4.035	2.377	6.412	59.778	28.817	88.595
2	2	143	1.648	1.320	2.968	26.161	15.171	41.331
2	1	145	1.458	1.202	2.660	23.700	13.583	37.283
2	2	146	1.699	1.432	3.131	28.315	15.914	44.229

2	4	147	1.257	1.097	2.354	22.050	11.799	33.849
2	7	148	1.573	1.363	2.936	26.216	15.148	41.364
1	5	150	1.479	1.221	2.700	20.977	15.364	36.341
1	7	151	1.015	1.097	2.112	24.163	10.160	34.323
1	6	152	1.433	1.337	2.770	20.766	16.511	37.278
2	2	153	2.130	1.323	3.452	34.633	14.944	49.577
2	3	154	1.666	1.586	3.252	25.835	18.547	44.381
2	3	155	1.256	1.052	2.308	19.479	12.300	31.779
2	1	156	1.978	1.646	3.624	34.706	17.695	52.401
2	3	157	1.537	1.052	2.589	20.491	14.033	34.524
1	1	158	1.578	1.193	2.771	20.622	16.237	36.859
1	1	159	1.370	1.622	2.992	23.418	17.728	41.146
2	7	160	1.496	1.195	2.692	22.168	14.490	36.658
1	6	161	1.069	1.110	2.179	17.379	12.540	29.919
1	5	162	1.792	1.012	2.804	24.888	12.978	37.866
1	5	163	1.256	1.228	2.484	21.465	13.425	34.890
2	4	164	1.970	1.673	3.643	30.538	19.571	50.110
2	2	165	1.356	1.175	2.532	25.119	12.240	37.360
2	3	166	1.613	1.312	2.925	27.570	14.342	41.912
1	1	167	1.637	1.438	3.075	25.978	16.534	42.512
2	1	168	1.976	1.440	3.416	32.934	15.997	48.930
1	1	169	2.098	1.367	3.465	34.973	15.185	50.158
1	1	170	1.337	1.440	2.777	24.755	14.998	39.753
1	1	171	1.847	1.132	2.979	28.634	13.240	41.874
1	2	172	2.480	1.274	3.754	33.742	16.651	50.393
1	2	173	1.517	1.169	2.686	20.640	15.284	35.924
1	3	174	1.682	1.087	2.769	23.361	13.939	37.299
2	5	175	1.627	0.999	2.626	24.645	11.894	36.539
1	6	176	1.037	0.866	1.903	18.193	9.311	27.504
2	3	177	2.819	1.720	4.539	46.980	19.114	66.094
2	5	178	1.732	1.590	3.322	36.073	15.589	51.662
1	6	179	1.488	1.260	2.747	19.836	16.795	36.631
2	4	181	2.536	1.577	4.113	41.233	17.815	59.049
2	2	185	1.659	1.405	3.064	25.142	16.725	41.868
2	2	188	1.934	1.427	3.361	27.439	17.948	45.386
2	2	190	3.007	1.812	4.819	48.894	20.479	69.373
2	2	196	1.694	1.415	3.109	28.956	15.469	44.426
2	4	197	1.703	1.403	3.105	25.797	16.698	42.496
1	6	199	1.937	1.495	3.432	29.350	17.793	47.144

STATISTICAL DISTRIBUTIØN WITH RESPECT TØ PARAMETERS P AND Q

PARAMETER P IS AGE
P1 UNDER 20
P2 20-29
P3 30-39
P4 40-49
P5 50-59
P6 60-69
P7 70-79
80 AND ØVER

PARAMETER Q IS SEX
Q1 MALE
Q2 FEMALE

UNITS X VALUE GIVEN IN FIRST CØLUMN IS LØWER LIMIT ØF CHANNEL. UPPER LIMIT IS NEXT VALUE GIVEN.

UNITS X IN FRACTIØN F1	PARAMETER Q1 P1	P2	P3	P4	P5	P6	P7	P8	ALL P	PARAMETER Q2 P1	P2	P3	P4	P5	P6	P7	P8	ALL P	SUM ØF Q1+Q2 P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1
0.70	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	2	0	0	2
0.80	0	U	0	U	0	1	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	2
0.90	U	n	1	0	2	1	0	0	4	0	0	0	0	0	1	0	1	2	0	0	1	0	2	2	0	1	6
1.00	0	1	0	3	1	3	3	1	12	0	0	2	0	1	1	2	0	6	0	1	2	3	2	4	5	1	18
1.10	0	2	1	2	1	1	1	1	9	1	0	1	0	2	2	2	0	8	1	2	2	2	3	3	3	1	17
1.20	1	1	2	3	4	1	1	1	14	2	1	4	2	7	2	2	1	21	3	2	6	5	11	3	3	2	35
1.30	2	0	1	1	0	1	1	0	6	2	4	4	2	4	1	2	1	20	4	4	5	3	4	2	3	1	26
1.40	0	1	1	0	2	3	0	0	7	1	2	2	1	3	0	3	0	12	1	3	3	1	5	3	3	0	19
1.50	2	2	3	1	6	1	0	0	15	0	2	1	1	3	0	4	0	11	2	4	4	2	9	1	4	0	26
1.60	1	3	3	1	1	2	0	0	11	0	6	4	2	1	3	0	0	16	1	9	7	3	2	5	0	0	27
1.70	0	0	2	2	1	1	1	0	7	0	1	2	3	1	1	2	0	10	0	1	4	5	2	2	3	0	17
1.80	3	1	0	0	U	0	0	2	6	1	0	0	0	0	1	0	0	2	4	1	0	0	0	1	0	2	8
1.90	1	0	1	0	2	1	2	0	7	2	3	U	3	0	0	0	0	8	3	3	1	3	2	1	2	0	15
2.00	2	1	0	1	0	1	0	0	5	0	1	2	0	0	1	0	0	4	2	2	2	1	0	2	0	0	9
2.10	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	3	1	1	1	0	0	0	0	0	3
2.20	0	0	0	0	1	0	1	0	2	0	0	0	1	1	0	0	0	2	0	0	0	1	2	0	1	0	4
2.30	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	0	2	0	0	0	0	0	2
2.40	0	1	0	0	U	0	0	0	1	1	0	0	1	0	1	0	0	3	1	1	0	1	0	1	0	0	4
2.50	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	2	0	0	0	1	1	0	0	0	2
2.60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.70	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1
2.80	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	2	0	0	0	0	0	2
2.90	1	0	1	0	0	0	0	0	2	0	2	0	0	1	0	0	0	3	1	2	1	0	1	0	0	0	5

UNITS X IN FRACTIØN F2	PARAMETER Q1 P1	P2	P3	P4	P5	P6	P7	P8	ALL P	PARAMETER Q2 P1	P2	P3	P4	P5	P6	P7	P8	ALL P	SUM ØF Q1+Q2 P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.50	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	2	0	0	1	0	1	0	0	0	2
0.60	0	0	0	0	1	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	2
0.70	0	1	1	0	0	0	0	0	2	0	0	0	0	U	2	1	0	3	0	1	1	0	0	2	1	0	5
0.80	0	1	0	3	2	3	0	0	9	1	0	0	0	0	0	1	2	4	1	1	0	3	2	3	1	2	13
0.90	0	1	3	2	3	3	3	1	16	2	0	2	2	4	2	0	0	12	2	1	5	4	7	5	3	1	28
1.00	1	2	4	3	3	1	2	0	16	1	3	8	4	3	1	2	1	23	2	5	12	7	6	2	4	1	39
1.10	2	2	3	3	6	3	2	0	21	0	2	4	0	6	2	6	0	20	2	4	7	3	12	5	8	0	41
1.20	1	4	3	2	3	2	0	1	16	2	4	2	2	3	3	3	0	19	3	8	5	4	6	5	3	1	35
1.30	3	2	1	0	2	3	1	2	14	2	2	4	3	1	4	3	0	19	5	4	5	3	3	7	4	2	33
1.40	3	0	0	0	1	1	1	0	6	3	6	0	2	2	1	0	0	14	6	6	0	2	3	2	1	0	20
1.50	0	0	0	1	0	0	0	1	2	0	3	2	2	2	0	1	0	10	0	3	2	3	2	0	1	1	12
1.60	2	0	0	0	0	1	0	0	3	1	0	1	2	2	0	0	0	6	3	0	1	2	2	1	0	0	9
1.70	1	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	0	2	1	0	2	0	0	0	0	0	3

	PARAMETER Q1									PARAMETER Q2									SUM ØF Q1+Q2								
	P1	P2	P3	P4	P5	P6	P7	P8	ALL P	P1	P2	P3	P4	P5	P6	P7	P8	ALL P	P1	P2	P3	P4	P5	P6	P7	P8	ALL P
1.80	0	0	1	0	0	0	1	0	2	0	1	1	0	0	0	0	0	2	0	1	2	0	0	0	1	0	4
1.90	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	2	0	1	0	0	1	0	0	0	2
2.00	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
2.10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.30	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	2
2.40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

UNITS X IN	PARAMETER Q1									PARAMETER Q2									SUM ØF Q1+Q2								
FRACTIØN F3	P1	P2	P3	P4	P5	P6	P7	P8	ALL P	P1	P2	P3	P4	P5	P6	P7	P8	ALL P	P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	1	0	0	1	0	0	2	1	0	1	0	1	1	0	1	5	1	0	2	0	1	2	0	1	7
1.80	0	1	0	1	1	2	0	0	5	0	0	0	0	0	0	0	0	0	0	1	0	1	1	2	0	0	5
1.90	0	0	0	1	1	2	0	0	4	0	0	1	0	0	0	1	0	2	0	0	1	1	1	2	1	0	6
2.00	0	1	0	2	1	1	1	1	7	0	0	0	0	1	2	2	0	5	0	1	0	2	2	3	3	1	12
2.10	0	0	2	2	1	1	3	0	9	1	0	1	0	2	1	1	1	7	1	0	3	2	3	2	4	1	16
2.20	0	0	2	0	1	0	0	0	3	1	1	2	0	1	2	1	1	9	1	1	4	0	2	2	1	1	12
2.30	0	0	0	1	2	3	0	0	6	0	1	4	1	2	0	0	0	8	0	1	4	2	4	3	0	0	14
2.40	0	2	1	1	4	0	1	1	10	2	1	2	2	1	1	1	0	10	2	3	3	3	5	1	2	1	20
2.50	2	2	0	2	1	0	0	0	7	0	2	1	2	5	0	1	0	11	2	4	1	4	6	0	1	0	18
2.60	0	1	1	0	1	0	0	1	4	2	0	4	1	4	1	4	0	16	2	1	5	1	5	1	4	1	20
2.70	2	2	4	1	4	4	0	0	17	0	2	1	2	1	1	2	0	9	2	4	5	3	5	5	2	0	26
2.80	0	1	2	1	1	0	0	0	5	0	1	0	0	1	0	0	0	2	0	2	2	1	2	0	0	0	7
2.90	2	0	0	0	0	0	1	0	3	0	2	1	1	1	1	3	0	9	2	2	1	1	1	1	4	0	12
3.00	1	0	1	1	0	2	0	0	5	0	2	1	1	0	1	0	0	5	1	2	2	2	0	3	0	0	10
3.10	0	1	0	0	0	0	2	1	4	0	2	0	1	0	1	1	0	5	0	3	0	1	0	1	3	1	9
3.20	2	1	0	0	0	0	0	0	3	1	0	1	1	1	2	0	0	6	3	1	1	1	1	2	0	0	9
3.30	0	0	1	0	2	0	1	0	4	0	3	2	0	2	0	0	0	7	0	3	3	0	4	0	1	0	11
3.40	2	0	0	0	0	1	1	1	5	1	2	1	0	0	0	0	0	4	3	2	1	0	0	1	1	1	9
3.50	1	0	0	1	1	0	0	0	3	0	1	0	1	0	0	0	0	2	1	1	0	2	1	0	0	0	5
3.60	0	0	0	0	0	1	0	0	1	2	1	0	1	0	0	0	0	4	2	1	0	1	0	1	0	0	5
3.70	0	1	0	0	0	0	0	0	1	0	0	0	2	0	1	0	0	3	0	1	0	2	0	1	0	0	4
3.80	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	2	1	0	1	0	0	0	0	0	2
3.90	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.00	1	0	2	0	0	0	0	0	3	0	3	3	1	2	0	0	0	9	1	3	5	1	2	0	0	0	12

UNITS X IN	PARAMETER Q1									PARAMETER Q2									SUM ØF Q1+Q2								
FRACTIØN F4	P1	P2	P3	P4	P5	P6	P7	P8	ALL P	P1	P2	P3	P4	P5	P6	P7	P8	ALL P	P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8.00	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1
10.00	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
12.00	0	1	1	2	1	1	0	0	6	0	0	0	0	0	1	0	0	1	0	1	1	2	1	2	0	0	7
14.00	0	1	1	3	1	0	0	0	6	1	0	0	0	0	0	0	1	2	1	1	1	3	1	0	0	1	8
16.00	1	1	1	1	3	4	2	0	13	1	0	3	0	2	1	1	0	8	2	1	4	1	5	5	3	0	21
18.00	0	1	2	3	3	3	0	0	12	1	0	3	1	7	4	2	1	19	1	1	5	4	10	7	2	1	31
20.00	2	3	4	1	7	4	1	1	23	3	1	5	1	5	2	4	0	21	5	4	9	2	12	6	5	1	44
22.00	1	2	5	1	0	2	2	1	14	1	5	2	3	2	2	7	1	23	2	7	7	4	2	4	9	2	37
24.00	4	2	0	0	1	1	1	1	10	0	6	4	5	2	1	1	0	19	4	8	4	5	3	2	2	1	29
26.00	0	1	0	2	2	0	1	1	7	1	3	2	1	1	2	1	0	11	1	4	2	3	3	2	2	1	18
28.00	2	0	1	1	2	1	0	1	8	0	2	0	2	3	0	1	0	8	2	2	1	3	5	1	1	1	16
30.00	0	0	0	0	0	1	1	0	2	2	1	0	1	0	1	0	0	5	2	1	0	1	0	2	1	0	7
32.00	0	1	0	0	1	0	1	0	3	1	1	1	1	0	0	0	0	4	1	2	1	1	1	0	1	0	7
34.00	2	0	0	0	0	0	1	0	3	1	1	1	1	0	0	0	0	4	3	1	1	1	0	0	1	0	7
36.00	0	0	0	0	0	0	0	0	0	0	1	4	0	2	0	0	0	7	0	1	4	0	2	0	0	0	7
38.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1

40.00	1	0	0	0	0	0	0	0	1
42.00	0	0	0	0	0	0	0	0	0
44.00	0	0	1	0	0	0	0	0	1
46.00	0	0	1	0	0	0	0	0	1
48.00	0	0	0	0	0	0	0	0	0
50.00	0	0	0	0	0	0	0	0	0
52.00	0	C	0	0	0	0	0	0	0

40.00	0	0	0	1	0	0	0	0	1
42.00	0	0	0	0	0	0	0	0	0
44.00	0	0	0	0	1	0	0	0	1
46.00	0	1	1	0	0	0	0	0	2
48.00	0	1	0	0	0	0	0	0	1
50.00	0	0	0	0	0	0	0	0	0
52.00	0	1	0	0	0	0	0	0	1

40.00	1	0	0	1	0	0	0	0	2
42.00	0	0	0	0	0	0	0	0	0
44.00	0	0	1	0	1	0	0	0	2
46.00	0	1	2	0	0	0	0	0	3
48.00	0	1	0	0	0	0	0	0	1
50.00	0	0	0	0	0	0	0	0	0
52.00	0	1	0	0	0	0	0	0	1

PARAMETER Q1

UNITS X IN FRACTIØN F5	P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	0	0	0	0	0	0	0
4.00	0	0	0	0	0	0	0	0	0
5.00	0	0	0	0	0	0	0	0	0
6.00	0	0	0	0	0	0	0	0	0
7.00	0	0	0	0	0	0	0	0	0
8.00	0	0	0	0	1	0	0	0	1
9.00	0	1	1	0	0	3	0	0	5
10.00	0	0	1	1	1	2	2	1	8
11.00	0	2	2	2	2	2	2	0	12
12.00	0	0	0	2	4	1	1	0	8
13.00	2	2	2	4	5	1	1	1	18
14.00	2	1	3	1	2	2	0	1	12
15.00	1	2	3	3	2	1	1	1	14
16.00	3	3	1	0	2	3	2	0	14
17.00	1	2	2	0	2	2	1	0	10
18.00	2	0	0	0	0	0	0	1	3
19.00	1	0	0	1	0	0	0	0	2
20.00	0	0	0	0	0	1	0	0	1
21.00	0	0	1	0	0	0	0	0	1
22.00	1	0	0	0	0	0	0	0	1
23.00	0	0	0	0	0	0	0	0	0
24.00	0	0	0	0	0	0	0	0	0
25.00	0	0	1	0	0	0	0	0	1
26.00	0	0	0	0	0	0	0	0	0

PARAMETER Q2

UNITS X IN FRACTIØN F5	P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	0	0	0	0	0	0	0
4.00	0	0	0	0	0	0	0	0	0
5.00	0	0	1	0	0	0	0	0	1
6.00	0	0	0	0	1	0	0	0	1
7.00	0	0	0	0	0	0	0	0	0
8.00	1	0	0	0	0	2	0	1	4
9.00	0	0	0	0	0	0	1	1	2
10.00	0	0	2	0	3	1	2	0	8
11.00	2	3	2	2	3	0	2	0	14
12.00	1	2	5	3	2	3	0	1	17
13.00	1	2	1	2	3	1	3	0	13
14.00	1	2	8	0	4	2	4	0	21
15.00	2	3	2	2	2	3	4	0	18
16.00	1	4	0	2	3	2	1	0	13
17.00	1	1	0	3	2	1	0	0	8
18.00	1	2	3	1	0	0	0	0	7
19.00	0	2	2	2	0	0	0	0	6
20.00	1	1	0	0	1	0	0	0	3
21.00	0	0	1	0	0	0	0	0	1
22.00	0	0	0	0	0	0	0	0	0
23.00	0	0	0	0	1	0	0	0	1
24.00	0	0	0	0	0	0	0	0	0
25.00	0	0	0	0	0	0	0	0	0
26.00	0	2	0	0	0	0	0	0	2

SUM ØF Q1+Q2

UNITS X IN FRACTIØN F5	P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	0	0	0	0	0	0	0
4.00	0	0	0	0	0	0	0	0	0
5.00	0	0	1	0	0	0	0	0	1
6.00	0	0	0	0	1	0	0	0	1
7.00	0	0	0	0	0	0	0	0	0
8.00	1	0	0	0	1	2	0	1	5
9.00	0	1	1	0	0	3	1	1	7
10.00	0	0	3	1	4	3	4	1	16
11.00	2	5	4	4	5	2	4	0	26
12.00	1	2	5	5	6	4	1	1	25
13.00	3	4	3	6	8	2	4	1	31
14.00	3	3	11	1	6	4	4	1	33
15.00	3	5	5	5	4	4	5	1	32
16.00	4	7	1	2	5	5	3	0	27
17.00	2	3	2	3	4	3	1	0	18
18.00	3	2	3	1	0	0	0	1	10
19.00	1	2	2	3	0	0	0	0	8
20.00	1	1	0	0	1	1	0	0	4
21.00	0	0	2	0	0	0	0	0	2
22.00	1	0	0	0	0	0	0	0	1
23.00	0	0	0	0	1	0	0	0	1
24.00	0	0	0	0	0	0	0	0	0
25.00	0	0	1	0	0	0	0	0	1
26.00	0	2	0	0	0	0	0	0	2

PARAMETER Q1

UNITS X IN FRACTIØN F6	P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	0	0	0	1	0	0	1
22.00	0	0	1	0	0	0	0	0	1
24.00	0	1	0	1	1	1	0	0	4
26.00	0	0	0	3	2	2	1	0	8
28.00	0	1	3	2	1	3	1	0	11
30.00	0	0	0	1	1	3	1	1	7
32.00	1	2	2	1	5	0	1	0	12
34.00	1	3	0	2	3	0	1	0	10
36.00	1	2	5	1	3	4	0	2	18
38.00	1	1	2	1	1	0	1	0	7
40.00	2	1	1	1	1	2	0	0	8
42.00	3	1	0	0	0	0	1	1	6
44.00	0	0	1	0	1	0	0	0	2
46.00	1	0	0	0	1	1	0	1	4
48.00	1	0	0	1	1	0	3	0	6
50.00	1	1	0	0	0	1	0	0	3
52.00	0	0	0	0	0	0	0	0	0
54.00	0	0	0	0	0	0	0	0	0
56.00	0	0	0	0	0	0	0	0	0
58.00	0	0	0	0	0	0	0	0	0
60.00	0	0	0	0	0	0	0	0	0
62.00	1	0	0	0	0	0	0	0	1
64.00	0	0	0	0	0	0	0	0	0
66.00	0	0	2	0	0	0	0	0	2

PARAMETER Q2

UNITS X IN FRACTIØN F6	P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	1	0	0	1	0	0	2
22.00	0	0	0	0	0	0	0	0	0
24.00	1	0	0	0	1	0	0	1	3
26.00	0	0	1	0	0	0	1	0	2
28.00	1	0	0	0	2	3	1	0	7
30.00	1	0	4	0	2	2	2	2	13
32.00	2	2	3	3	3	0	1	0	14
34.00	1	2	4	0	4	2	2	0	15
36.00	1	1	4	5	5	1	5	0	22
38.00	0	3	0	0	2	1	2	0	8
40.00	0	4	1	2	0	1	2	0	10
42.00	0	0	0	1	2	0	0	0	3
44.00	1	5	2	1	1	3	1	0	14
46.00	0	0	0	0	0	0	0	0	0
48.00	2	3	3	1	0	0	0	0	9
50.00	1	0	0	2	1	0	0	0	4
52.00	1	1	0	1	0	0	0	0	3
54.00	0	0	0	0	0	1	0	0	1
56.00	0	0	2	0	0	0	0	0	2
58.00	0	0	1	1	1	0	0	0	3
60.00	0	0	0	0	0	0	0	0	0
62.00	0	0	0	0	0	0	0	0	0
64.00	0	0	0	0	0	0	0	0	0
66.00	0	3	1	0	1	0	0	0	5

SUM ØF Q1+Q2

UNITS X IN FRACTIØN F6	P1	P2	P3	P4	P5	P6	P7	P8	ALL P
-0.00	0	0	1	0	0	2	0	0	3
22.00	0	0	1	0	0	0	0	0	1
24.00	1	1	0	1	2	1	0	1	7
26.00	0	0	1	3	2	2	2	0	10
28.00	1	1	3	2	3	6	2	0	18
30.00	1	0	4	1	3	5	3	3	20
32.00	3	4	5	4	8	0	2	0	26
34.00	2	5	4	2	7	2	3	0	25
36.00	2	3	9	6	8	5	5	2	40
38.00	1	4	2	1	3	1	3	0	15
40.00	2	5	2	3	1	3	2	0	18
42.00	3	1	0	1	2	0	1	1	9
44.00	1	5	3	1	2	3	1	0	16
46.00	1	0	0	0	1	1	0	1	4
48.00	3	3	3	2	1	0	3	0	15
50.00	2	1	0	2	1	1	0	0	7
52.00	1	1	0	1	0	0	0	0	3
54.00	0	0	0	0	0	1	0	0	1
56.00	0	0	2	0	0	0	0	0	2
58.00	0	0	1	1	1	0	0	0	3
60.00	0	0	0	0	0	0	0	0	0
62.00	1	0	0	0	0	0	0	0	1
64.00	0	0	0	0	0	0	0	0	0
66.00	0	3	3	0	1	0	0	0	7

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