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The Concentrations of Cobalt, Copper, Iron and Zinc in some Normal Human Tissues as Determined by Neutron-Activation Analysis

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In recent years there had been much interest in the concentrations of trace metals occurring in human and animal tissues and in the manner in which these concentrations may alter in malignant and other diseases. For most trace metals there are well-established chemical methods of analysis of suitably high sensitivity. However, some metals, e.g. cobalt, occur in such low concentrations in biological materials that their determination by conventional methods of analysis is difficult and uncertain, and consequently relatively little is

known about their concentrations in normal and pathological tissues.

Thermal-neutron-activation analysis, which has been shown to be a useful technique in the general field of trace-element studies (Atkins & Smales, 1959; Bowen & Cawse, 1959), is potentially a very valuable technique for the determination of cobalt at the concentrations existing in human tissues. Even without the use of the highest available neutron fluxes, activation analysis affords for cobalt a far greater sensitivity than that

of any other current technique (Meinke, 1955). Further, it allows many of the problems that arise from sample and reagent contamination to be minimized since these are generally important only during the pre-irradiation period that need involve no chemical treatment of the sample. Additionally, high specificity may be obtained, since interfering nuclear reactions are usually unimportant, and the induced radioactivity can in most cases be identified unambiguously by γ -ray spectrometry and half-life determinations, after chemical separations have been carried out.

Of the several methods that have been described for the determination of cobalt in biological materials by activation analysis (Koch, Smith, Shimp & Connor, 1956; Haerdi, Vogel, Monnier & Wenger, 1960; Kaiser & Meinke, 1960; Pijck, Gillis & Hoste, 1961), none possesses the sensitivity and specificity necessary for its estimation at the concentrations existing naturally in most tissues, particularly when only small samples are available for analysis.

A more sensitive procedure has therefore been developed by us for the determination of cobalt, and this procedure also permits the simultaneous determination of three other metals, namely copper, iron and zinc.

The method, which is described in detail in the present paper, permits the concurrent determination of cobalt, copper, iron and zinc in a sample consisting of less than 1 g. of tissue. The concentrations of these metals in a number of normal human tissues are also reported for comparison with the results of similar measurements on pathological tissues obtained by Hunt, Parr, Taylor & Trott (1963).

METHODS

Sample preparation. 'Normal' tissue samples were obtained at autopsy from apparently healthy individuals who had died suddenly as the result of accidents. The samples were dried and prepared for activation by the methods described by Parr & Taylor (1963), great care being taken to minimize the risk of contamination by the use of very carefully cleaned apparatus.

The samples of blood serum were collected from healthy volunteers. Blood was withdrawn from a median basilic vein through a no. 2 gauge platinum-iridium needle, mounted in the end of a 30 cm. length of 1 mm.-bore polyethylene tubing, and run directly into a 15 ml. polyethylene tube. After clotting the blood was centrifuged and the serum removed. Serum samples were dried and prepared for activation in a similar way to the tissue samples.

For irradiation, samples were placed in small polyethylene tubes each containing approx. 0.1 g. of dried tissue or 0.3 g. of dried serum. Up to 14 tubes containing dried tissue, or seven containing dried serum, were packed into type A irradiation cans (Atomic Energy Research Establishment, Harwell, Berks.) together with tubes containing standards and a 'blank' tube.

Standards. The calculation of the results was based on a comparison of the radioactivities of the samples with those of individual external standards of cobalt, copper, iron and zinc, irradiated at the same time and under precisely the same conditions. Standard solutions, containing about 50 μ g. of cobalt/ml., 100 μ g. of copper/ml., 1 mg. of zinc/ml. or 10 mg. of iron/ml., were prepared from spectroscopically pure samples of the metals (Johnson Matthey and Co. Ltd., London) dissolved in double-distilled hydrochloric acid, and diluted as appropriate with demineralized water. Then 0.1 g. samples were weighed out into individual polyethylene 'activation tubes' and evaporated to dryness in a heated desiccator.

Activation. The samples and standards were activated simultaneously by thermal neutron irradiation in BEPO (Atomic Energy Research Establishment, Harwell) at a flux of 10^{12} neutrons/cm.²/sec. for about 7 days. A 'cooling period' of 1 day was allowed before the next stage of the analysis.

Chemical procedure. After irradiation the chemical separation procedure consisted of a selective adsorption of the elements of interest on to a column of anion-exchange resin from a solution in 10N-hydrochloric acid. Unwanted elements were removed by washing the column with a further quantity of 10N-hydrochloric acid, and the cobalt, copper and iron were eluted with dilute acid, the zinc remaining bound to the resin.

This procedure provided sources for assay that were sufficiently free from ²⁴Na and ³²P (and other nuclides that comprised the major part of the induced radioactivity in the samples), and of sufficient radiochemical purity for the ⁶⁰Co, ⁶⁴Cu, ⁵⁹Fe and ⁶⁵Zn radioactivities to be measured by the use of a single-channel γ -ray spectrometer. Complete and quantitative recovery of each of the desired elements was attainable, with the consequence that separate determinations of chemical yield were not required. The entire contents of an irradiation can (usually 20 samples including the standards and blanks) could be processed easily in about 8 hr.

The detailed procedure was as follows. After activation each sample was transferred to a 300 ml. Erlenmeyer flask, and 100 μ g. each of cobalt, iron and zinc and 5 mg. of copper were added as carriers. The samples were then wet-washed with the minimum quantity of nitric acid and sulphuric acid and the resultant solution was evaporated to dryness. The residue was dissolved in about 5 ml. of 10N-hydrochloric acid and loaded on to a column (1 cm. diam. $\times$ 15 cm. long) of De-Acidite FF (100-200 mesh) (The Permutit Co. Ltd.) previously washed with 10N-hydrochloric acid.

After transfer of the radioactive solution to the ion-exchange resin the column was washed with 50 ml. of 10N-hydrochloric acid and the washings, containing the bulk of the induced radioactivity, were discarded. A siphoning device, constructed of 1 mm. bore polyethylene tubing, was used to transfer the washing and eluent solutions to the column in such a way as to keep the volume of solution above the resin to a minimum. Unless this were done, satisfactory removal of the unwanted radioactivity could not be achieved without the use of such large volumes of 10N-hydrochloric acid that some of the ⁶⁴Cu would be lost in the washings.

Cobalt, copper and iron were completely eluted from the column with 40 ml. of N-hydrochloric acid, and the eluate

was evaporated to a small volume, transferred to a 20 ml. stoppered polyethylene tube and diluted to a standard volume with distilled water. The tube was shaken to ensure thorough mixing and briefly centrifuged to remove the drops of liquid adhering to the sides and lid. The cobalt, copper and iron radioactivities were determined by measurements on this solution.

The ion-exchange resin was washed into a 50 ml. glass tube with water; the resin was stirred with a glass rod to ensure a homogeneous distribution of the absorbed radioactivity and allowed to settle. The zinc radioactivity was determined by measurement of the tube and resin.

Tissue samples containing excessive quantities of iron, as for example from a patient with haemochromatosis,

Table 1. *Test of the chemical procedure and the recovery of added tracers*

Experimental details are given in the text. The 4*N*-hydrochloric acid eluate was obtained in the second separation to remove excess of iron. The results are given as means $\pm$ S.D. where applicable.

Tracer	No. of expts.	Percentage recovery in Co-Cu-Fe fraction	Percentage recovery in Zn fraction	Percentage recovery in 4 <i>N</i> -HCl eluate
⁵⁸ Co	4	99.3 $\pm$ 0.2	0.05	98.9 $\pm$ 0.7
⁶⁴ Cu	4	100.0 $\pm$ 0.3	0.05	52 $\pm$ 3
⁵⁹ Fe	6	99.2 $\pm$ 0.3	0.5	3.9 $\pm$ 1.5
⁶⁵ Zn	4	0.05	98.5 $\pm$ 0.8	—

Table 2. *Isotopes utilized in the analysis*

Element	Activation product	Half-life	Principal γ -rays (Mev)	Sensitivity (μ g.)
Cobalt	⁶⁰ Co	5.3 years	1.17, 1.33	0.0002
Copper	⁶⁴ Cu	12.8 hr.	0.51	0.001
Iron	⁵⁹ Fe	45 days	1.10, 1.29	0.1
Zinc	⁶⁵ Zn	245 days	1.12	0.01

Fig. 1. γ -ray spectra of the cobalt-copper-iron fraction from neutron-activated human liver. (a) After 2 days' decay measured with samples in the well of the counter. (b) After 2 days' decay measured with samples on top of counter showing relatively greater prominence of the ⁶⁴Cu radioactivity peak at 0.5 Mev. (c) After 4 weeks' decay when the ⁶⁴Cu radioactivity has decayed leaving only ⁵⁹Fe and ⁶⁰Co radioactivities.

required a further chemical separation to decrease the interference in the measurement of the ^{60}Co radioactivity due to the high radioactivity of ^{59}Fe . An adequate removal of iron was achieved by a further ion-exchange separation (carried out after the ^{59}Fe radioactivity had been measured) in which the cobalt-copper-iron solution in 10N-hydrochloric acid was loaded on to a fresh column of resin, and the cobalt was eluted with 40 ml. of 4N-hydrochloric acid. This provided a solution containing approx. 99% of the original ^{60}Co radioactivity, but only about 4% of the original ^{59}Fe radioactivity.

The full chemical procedure was tested by adding tracer amounts of radioactive ^{60}Co , ^{64}Cu , ^{59}Fe or ^{65}Zn to non-radioactive rat liver which was then processed as above. The recoveries of tracer in the various chemical fractions are recorded in Table 1, which shows that no appreciable errors would be introduced by assuming 100% recovery of each element in the particular fraction prepared for assaying its induced radioactivity. For this reason, no separate determinations of chemical yield were made. However, a simple method was available, and was occasionally used, for determining losses due to splashing or to accidental breakage of a flask during the wet-ashing process. The relatively large amount of copper carrier (5 mg.) made possible a very simple colorimetric determination of the chemical yield of this element by using the diethyldithiocarbamate reaction (Snell & Snell, 1949). Except for losses occurring during some of the final stages of the separation

(e.g. the preparation of the zinc fraction for counting), the recovery of copper could also be taken as a measure of the recoveries of cobalt, iron and zinc.

Measurement of the induced radioactivities. The radioactive isotopes of cobalt, copper, iron and zinc utilized in this procedure are listed in Table 2 together with their relevant physical properties and the sensitivities attained. All the radioactivity measurements were made by γ -ray spectrometry, by using a 5 in. $\times$ 6 in. well-type sodium iodide-crystal detector. Initially, the results were calculated from results recorded by a 100-channel pulse-height analyser; typical examples of the spectra obtained with this instrument are shown in Figs. 1 and 2. However, it was found that a single-channel analyser and scaler was no less accurate, and most of the actual measurements were made in this way. Nevertheless, the multichannel analyser was always used for checking qualitatively the radiochemical purity of each sample before assay.

The ^{64}Cu radioactivity was determined by measurements in an energy band containing its 0.51 Mev photopeak. Decay curves for this part of the spectrum (Fig. 3) may be analysed into two components, a short-lived component (the ^{64}Cu radioactivity, half-life 12.8 hr.) and a component which is essentially constant on this time-scale, the ^{59}Fe and ^{60}Co radioactivities. The contribution from ^{64}Cu radioactivity in this energy band was determined by making two radioactivity measurements, the first immediately after the chemical processing and the second

Fig. 2. γ -ray spectrum of the zinc fraction (0.4 $\mu\text{g.}$ of zinc) from (a) neutron-activated human serum compared with (b) a pure solution of ^{65}Zn . The vertical bars in (a) indicate s.d.

Fig. 3. Decay curves for the ^{64}Cu radioactivity in the cobalt-copper-iron fraction. $\circ$, Human tissue samples; $\bullet$, ^{64}Cu standard.

coincident annihilation photons produced by the ^{64}Cu positrons made the 0.51 Mev peak less prominent relative to the ^{60}Co and ^{59}Fe radioactivity contributions at this energy (Fig. 1).

The remaining radioactivities were determined about 1 week later, when the ^{64}Cu radioactivity could no longer be detected in the samples. The ^{60}Co radioactivity was assayed by means of the 2.50 Mev sum peak obtained with the sample placed inside the well of the crystal; and the ^{59}Fe radioactivity, which does not give a sum peak since its γ -rays are not emitted in coincidence, was assayed by means of the double photopeak (1.10 and 1.28 Mev) appearing in the same spectrum. The counting rate in the 1.00–1.40 Mev band was corrected for the small contribution from the ^{60}Co radioactivity to yield a value for the true ^{59}Fe radioactivity.

The ^{65}Zn radioactivity was assayed by means of the photopeak at 1.12 Mev obtained with the resin sample placed inside the well of the crystal.

Assay of ^{60}Co radioactivity in samples containing a high ^{59}Fe radioactivity. Although the ^{59}Fe and ^{60}Co radioactivities can be completely resolved by the sum-peak effect (which produces the 2.50 Mev peak in the ^{60}Co radioactivity spectrum), 'random summations' can invalidate the method if the total ^{59}Fe radioactivity is too high.

This effect is illustrated in Fig. 4, which shows, superimposed, two ^{59}Fe radioactivity spectra both normalized with respect to the heights of the photopeaks. One of these spectra was obtained with a ^{59}Fe source of relatively low radioactivity (approx. 10^3 disintegrations/min.) and the other with a source of relatively high radioactivity (4×10^5 disintegrations/min.). With a source of high radioactivity, random summations of pulses within the apparatus cause the spectrum to be distorted so that it extends upwards into the ^{60}Co radioactivity sum-peak region. As might be expected, the counting rate at the energy due to the ^{59}Fe radioactivity is determined approximately by the square of the ^{59}Fe radioactivity (Fig. 4), and it is not difficult to apply a correction for the effect. In practice, for all samples requiring more than a small correction, a further ion-exchange separation was always employed to remove most of the iron, and so diminish the effect.

RESULTS

The concentrations of cobalt, copper, iron and zinc found in some 'normal' human tissues are listed in Table 3 and similar results for young children are given in Table 4. Consideration of the possible sources of error affecting trace-element determinations by activation analysis (Plumb & Lewis, 1955) suggests that our measurements were not subject to systematic or random errors greater than about $\pm 5\%$. In no cases were the radioactivities recorded in the blank samples sufficient to indicate that any appreciable contamination had occurred.

DISCUSSION

The problem of obtaining samples of human tissue that may be regarded as truly normal has been discussed by Koch *et al.* (1956) and Tipton (1960), and it is considered that the most suitable samples are obtained from apparently healthy individuals who had suffered an instantaneous accidental death. Such cases are relatively infrequent and for this reason the results presented are based on a small number of samples.

Cobalt. Cobalt was found to be present in all the tissues examined and the concentrations (Table 5) were in general much lower than those reported by other workers who used less sensitive methods. There is little that can be said at present about the significance of these values. However, by comparison with the reported concentrations in human tissues of vitamin B_{12} (which contains approx. 4% of cobalt), estimates can be made of the proportion of the total cobalt which may exist in this form.

Swenseid, Hvolboll, Schick & Halsted (1957) found that normal adult human liver contains an average of 0.72 μg . of vitamin B_{12} /g. wet wt. of

Table 4. Concentrations of cobalt, copper, iron and zinc in the tissues of young children

Experimental details are given in the text. The results are expressed as μg /g. wet wt. of tissue.

Tissue	Age	Sex	Concn. of cobalt	Concn. of copper	Concn. of iron	Concn. of zinc
Liver	4 hr.	Female	0.014	58	244	228
	6 hr.	Female	0.006	30	490	105
	13 days	Male	0.008	29	276	172
	2 years	Male	0.053	9.5	111	80
Kidney	2 years	Male	0.0115	2.21	44	33
	6 hr.	Female	0.0022	1.40	71	28
	13 days	Male	0.0026	1.30	29	21
Spleen	6 hr.	Female	0.0035	0.86	512	15
	13 days	Male	0.0019	1.20	490	12
	2 years	Male	0.0042	1.00	137	24
Pancreas	6 hr.	Female	0.0021	2.5	77	58
Thymus	6 hr.	Female	0.00073	0.48	47	16.7
Brain	6 hr.	Female	0.00013	1.39	16	7.2
Adrenal gland	6 hr.	Female	0.0031	0.91	48	10.7

Table 5. *Reported values of cobalt concentrations in some human tissues*

In some of the original papers, cobalt concentrations were quoted as $\mu\text{g./g.}$ dry wt. or ash wt. Conversion into the wet wt. was made by using the conversion factors reported by Tipton (1960). The methods of analysis were: A, activation analysis; C, colorimetry; S, spectrography.

Tissue	Method of analysis	Concn. of cobalt ($\mu\text{g./g.}$ wet wt. of tissue)	Reference
Liver	C	1.26	Butt, Nusbaum, Gilmour & Didio (1960)
	A	0.30	Leddicotte (1959)
	C	0.16	Gul'ko (1961)
	A	0.16	Koch <i>et al.</i> (1956)
	C	0.12	Heyrovsky (1952)
	C	0.029–0.075	Zizum (1957)
	S	0.050	Tipton (1960)
	A	0.043–0.074	This paper
Kidney	A	0.60	Koch <i>et al.</i> (1956)
	C	0.51	Butt <i>et al.</i> (1960)
	C	0.25	Bertrand & Macheboeuf (1925)
	A	0.20	Leddicotte (1959)
	S	0.05	Tipton (1960)
	A	0.013	This paper
Spleen	C	0.83	Butt <i>et al.</i> (1960)
	C	0.47	Bertrand & Macheboeuf (1925)
	A	0.10	Leddicotte (1959)
	A	0.03	Koch <i>et al.</i> (1956)
	S	0.05	Tipton (1960)
	A	0.0039–0.0065	This paper
Blood serum	A	0.012	Koch <i>et al.</i> (1956)
	C	0.0085	Heyrovsky (1952)
	C	0.005–0.008	Babin (1953)
	C	0.00006–0.00008	Thiers, Williams & Yoe (1955)
	A	0.00008–0.00058	This paper

tissue. This value suggests that about 50 % of the cobalt present in human liver may be present as the vitamin. Similarly, a comparison with reported concentrations of vitamin B₁₂ in serum (Smith, 1960; Barakat & Ekins, 1961) suggests that only about 5–10 % of serum cobalt normally exists in this form.

Trace-element patterns in newborn infants are commonly different from those in adults (Underwood, 1956), and the measurements reported above suggest that the cobalt concentrations found in the liver of newborn infants are significantly lower than in adults ($P < 0.05$).

Copper, iron and zinc. The observed concentrations of copper, iron and zinc in all the adult tissues analysed are in substantial agreement with the values reported by other workers (Underwood, 1956; Tipton, 1960; Tipton & Cook, 1963).

The tissues from newborn infants showed several variations from the adult pattern. In particular, significantly elevated concentrations of copper ($P < 0.05$) and zinc ($P < 0.05$) were observed in the liver. For copper, this is a common finding in almost all species for which adequate data are available (Underwood, 1956). However, for zinc, Widdowson & Spray (1951) found no evidence of increased storage in the liver of newborn infants.

SUMMARY

1. A method is described for determining cobalt, copper, iron and zinc in biological materials by neutron-activation analysis. The sensitivities attainable are 0.0002, 0.001, 0.1 and 0.01 $\mu\text{g.}$ respectively.

2. The 'normal' concentrations of cobalt, copper, iron and zinc have been determined in liver, spleen and other tissues taken from healthy human subjects, both infants and adults, who were victims of sudden accidental death. The elements were also determined in normal human serum.

3. Cobalt concentrations in adult tissues ranged from a mean value of 0.0003 $\mu\text{g./g.}$ in serum, to 0.07 $\mu\text{g./g.}$ wet wt. of tissue in liver. The liver of newborn infants contained much lower concentrations than adults.

4. Copper, iron and zinc were detected at concentrations that were in general agreement with values reported by workers using other methods of analysis. Significantly elevated concentrations of both copper and zinc were observed in the liver of newborn infants.

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Hydrolysis of Polyamino Acids by an Extracellular Protease from *Penicillium cyaneo-fulvum*

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Penicillium cyaneo-fulvum, a member of the *P. chrysogenum* group, produces an extracellular protease when grown in an ox-heart infusion-peptone-glucose medium (Singh & Martin, 1960). Highly purified preparations of the enzyme did not appear to have peptidase activity, being unable to hydrolyse a wide variety of short-chain peptides (Martin, Singh, Ankel & Khan, 1962) or various substituted amino acids and peptides, such as have been used to characterize the better-known proteases. However, the enzyme was able to cleave the isolated peptide chains of bovine insulin (Martin *et al.* 1962). Examination of the reaction products suggested that the enzyme was an endo-peptidase capable of cleaving a wide range of inner peptide bonds.

To investigate the mode of action of this protease we have studied the hydrolysis of several synthetic water-soluble polyamino acids. This paper reports on properties of polyaspartic acid,

polyglutamic acid, polylysine and polyproline as substrates, the pH optima, evidence for the random nature of cleavage and some aspects of the kinetics of the hydrolysis. Also undertaken was a more detailed study of the hydrolysis of polylysine: the final products of the reaction were identified, the accumulation and subsequent disappearance of intermediate peptides was observed and the rates of hydrolysis of some of these intermediates were estimated.

EXPERIMENTAL

Materials

Diethylaminoethylcellulose, type 40 (DEAE-cellulose), was obtained from Carl Schleicher and Schull Co., Keene, N.H., U.S.A., and Sephadex G-25 from Pharmacia Co., Uppsala, Sweden. Di-isopropyl phosphorofluoridate (DFP) was from Aldrich Chemical Co., Milwaukee, Wis., U.S.A., ethylenediaminetetra-acetic acid (disodium salt) from