

It was intended to collect the materials and data listed from the 301 individuals included in the original sample. However, five subjects of the original sample died during the year. The overall recovery of subjects was 92 per cent. The tests were carried out in the order listed in the preceding paragraphs.

3.2.4.1. Saliva collection for chemical analyses.

Diurnal variations in the excretion of major and trace elements along various pathways⁽¹⁰²⁾⁽¹⁰³⁾⁽¹⁰⁴⁾⁽¹⁰⁵⁾⁽¹⁰⁶⁾⁽¹⁰⁷⁾⁽¹⁰⁸⁾⁽¹⁰⁹⁾⁽¹¹⁰⁾⁽¹¹¹⁾⁽¹¹²⁾ may affect some elements and not others. They are also subject to individual differences as well as to controversy. Therefore, the desirability of collecting saliva specimens from all subjects at the same time of the day was realized, but this aim was impossible to achieve under the field conditions. However, the specimens were collected not sooner than two hours after the last meal, and the time of day and the time elapsed since the last meal were recorded in respect of each sample.

Saliva collection followed a brief rinse with the usual drinking water of the subjects. Stimulated whole saliva was obtained from subjects chewing soft vinyl tubing ('Portex' 8HE, The Boots Company (Aust.) Pty. Ltd., Sydney, N.S.W.), of 25 mm length and 7.0 mm external diameter, for ten minutes. The saliva was collected in pre-identified 12 cm long 4 cm diameter polyethylene test tubes, held by the subjects within sight of the author. The specimen was transferred into one or more specially cleaned, pre-identified, 25 ml capacity, snap-topped polypropylene containers (25-P, Disposable Products, Adelaide, S.A.) and the volume of each sample was estimated to the nearest 0.5 ml by

comparing the level to graduations marked on an otherwise identical container. The specimens were sterilized by autoclaving in a pressure cooker (Model 7, Namco, Overseas Corporation of Australia Ltd., Sydney, N.S.W.) at 14 p.s.i. pressure for 10 minutes. On cooling, the samples were stored in specially constructed polyester racks at ambient temperature (30 - 36°C) in the field, and transported by air to the laboratory.

On arrival it was found that because of damage in transit nearly 30 per cent of the samples had decreased in volume. The samples were stored at -25°C until examination of the damage, and laboratory processing described in sub-section 3.3.2.2. was carried out.

3.2.4.2. Collection of pooled plaque. Sample (2).

The primary reason for collecting this sample was to provide material for method development for plaque trace element analyses, which are outside the scope of this report. However, the material was collected by a uniform procedure and therefore the quantity obtained should be representative of the amount of plaque individuals had on this occasion.

The sample consisted of all plaque that could be removed from the following permanent tooth surfaces: the buccal surface of the upper right first molar, the labial surface of the upper right central incisor and the lingual surface of the lower left first molar.

No plaque was collected where a carious cavity occupied more than 25 per cent of the surface area. For these, as well

as for missing surfaces, contralateral teeth were substituted as first preference. Where collection from the latter was also not practical, corresponding second molars and the right or left upper canines were substituted for first molars and central incisors respectively. Where no substitution was possible, adjustment was made to the plaque quantity on the basis that, as an approximation, 40 and 20 per cent of the total plaque obtained was associated with the molar and incisor surfaces respectively.

Otherwise the details of collection procedure were the same as described under 3.2.3.5. The procedure used to establish the weight of this sample (Variable 203) is given in Laboratory methods, sub-section 3.3.2.3.

3.2.4.3. Measurement of mesio-distal crown diameter of upper anterior teeth.

Rejection of cusp height and cusp angle data collected previously, caused mainly by the presence of cusp wear in a large proportion of the sample and to an extent by imperfect reproduction of models, made it desirable to select other means of monitoring morphological variations in the sample.

As no suggestive trends of anatomical differences were observed clinically, the following points were considered in the choice of parameters:

Difficulties associated with the study of dental variation in human populations, ⁽¹¹³⁾ particularly when minute details are studied.

In the past, associations based on major characteristics

were detected,⁽¹⁴⁾ although on that occasion clinical impressions helped in selecting the types of measurements.

In view of the field conditions and past experiences, in-vivo measurements were considered preferable to the use of impressions and models, despite the fact that in practice this limited the choice to anterior teeth.

The greatest mesio-distal diameter of the crowns of the upper right permanent canine, lateral and central incisor teeth was measured in the stated order, using dial calipers calibrated to read in 0.05 mm (No. 505-633, Mitutoyo, Tokyo, Japan). The jaws of the calipers were fitted with stainless steel plates of 0.1 mm thickness tapering to a fine edge, which projected 8 mm beyond the jaws at an angle of 90° to the plane of the instrument.

For measurements, the ends of the plates were fitted into the labial aspects of the interproximal embrasure and the maximum value was recorded to the nearest estimated 0.01 mm. (At a later date the figures were rounded to the nearest 0.05 mm.) When a tooth was missing, or when the interproximal outline was altered by a carious lesion, the contralateral tooth was measured. When both teeth were involved no data were recorded.

The reproducibility of measurements was thought to vary with the shape, alignment and positional relationship of teeth. The precision of the method was tested by taking ten blind replicate measurements of three teeth chosen to represent ideal, average and unfavourable cases. The means and standard deviations in increasing order of difficulty were: $\bar{x}$ (mm) = 7.37, S.D. = ± 0.09 ;

$\bar{x}$ (mm) = 4.91, S.D. = ± 0.11 ; $\bar{x}$ (mm) = 7.41, S.D. = ± 0.21 .

The measurements taken of the central and lateral incisors and the canine correspond to Variables 205, 206 and 207 respectively.

3.2.4.4. Recording of the frequency of betel-chewing.

The chewing of Areca nut, the fruit of the betel palm (Areca catechu) with 'pepper' (Piper betle) and with lime, is a habit widespread in lowland communities in Papua New Guinea, often acquired in early childhood.

The practice produces an intoxicating effect and is said to depress hunger, help to sustain physical exertion and cause a feeling of well-being. Mastication of the Areca nut - pepper - lime mixture appears to increase the flow of saliva which is dyed bright red, as are the lips, teeth and oral soft tissues.

Although little evidence could be found concerning possible relationships between betel-chewing and caries,⁽¹¹⁴⁾⁽¹¹⁵⁾ the evidence available to the author suggested that possible associations between the prevalence of the habit and caries should be investigated.

All subjects were extensively questioned about the number of Areca nuts consumed each day and whether the conventional Areca nut - pepper - lime or other combinations were preferred.

Because of unfamiliarity with numbers, bashfulness of some subjects to disclose information relating to a habit discouraged by missionaries and Department of Health personnel, and simple boasting by others, the data obtained are considered exploratory.

Two aspects, the number of Areca nuts used per day and the time elapsed since the last episode of betel-chewing at the time of saliva collection, were included in the analyses as Variables 208 and 209 respectively.

3.2.5. July, 1973, Field Visit.

This visit was conducted to measure the viscosity, buffering capacity and bicarbonate content of saliva. The occasion was used to replace saliva specimens damaged in transit after the April field visit. Rather than limiting the collection to subjects whose samples were damaged, the whole range of samples were re-collected.

The principles and general field routine described for previous visits were followed. The overall recovery of subjects was 91 per cent.

3.2.5.1. Collection of saliva.

Stimulated whole saliva was obtained by the method previously described in sub-section 3.2.4.1. The saliva was collected in specially cleaned, pre-identified, 25 ml capacity, snap-topped polypropylene containers (25-P, Disposable Products, Adelaide, S.A.), held by the subjects, within sight of the examiners. If froth or saliva level approached the top of the container, the subject was given a new container. The corresponding specimens were pooled before colour estimation and chemical analyses in the laboratory.

The volume of each saliva sample was estimated at the end of each day to the nearest 0.5 ml by comparing the level of

saliva to graduations marked on an otherwise identical container.

Saliva specimens were sterilized on the day of collection by autoclaving in a pressure cooker (Model 7, Namco Overseas Corporation of Australia, Ltd., Sydney, N.S.W.), at 14 p.s.i. pressure for 10 minutes. On cooling, the specimens were stored in specially constructed polyester racks at ambient temperature ($30 - 36^{\circ}\text{C}$) in the field, followed by air transport to the laboratory and storage at -25°C until required for analysis.

Analysis of these samples, on which Variables 239 (calcium ppm), 240 (calcium per cent of solids) and 243 (fluoride ppb) were based, are described in Laboratory methods, sub-section 3.3.2.2.

3.2.5.2. Viscosity of saliva.

Marked variation in the viscosity of saliva observed in various populations in Papua New Guinea and in the present sample, suggested the examination of this parameter.

The viscosity of the saliva samples was measured immediately after collection. The measurements were made using a modification of Hess' method⁽¹¹⁶⁾ which was also adopted by Ericsson and Stjernström.⁽¹¹⁷⁾

The viscometer was constructed from two 0.1 ml pipettes graduated in 0.01 ml with internal bore diameters of approximately 0.7 mm (Kimax-51 No. 37022, Owens-Illinois Inc., Toledo, Ohio, U.S.A.) aligned in parallel, and connected by a T piece to an 80 cm long flexible vinyl tube of 3.0 mm internal diameter equipped with a mouthpiece. A polyethylene tube tapering from 3.6 mm to 0.3 mm internal diameter was inserted into each end

of the vinyl tubing to reduce airflow (Figure 7, page 87).

When the tips of the pipettes were immersed in water at the same level and negative pressure was applied to the mouth-piece, the water rose at a uniform rate in both tubes. The viscosity of saliva in relation to that of water was measured by immersing one pipette in saliva, the other in water and applying negative pressure until the water column reached the top graduation of the pipette, marked 0. At this point, the height of the saliva column was read to the nearest half division. As the numerical values marked on the pipettes increased with decreasing height, direct readings gave arbitrary viscosity values which increased with increasing viscosity of the samples.

Before each measurement, the sediment in the saliva specimen was re-suspended and the water and saliva levels were aligned. As a routine the pipettes were immersed to a depth of approximately 10 mm. When the flow rate of the saliva sample was not uniform because of obstruction of the pipette by particulate matter or exceptionally viscous mucin clot, the measurement was repeated until a uniform flow rate was achieved. Between samples, the pipette used for saliva was rinsed with glass-distilled, deionized water, and frequent checks were carried out to ensure that the flow rate of water remained uniform in both tubes. As a rule, 2 - 3 measurements were carried out on each specimen and the average value was recorded. (Variable 214.)

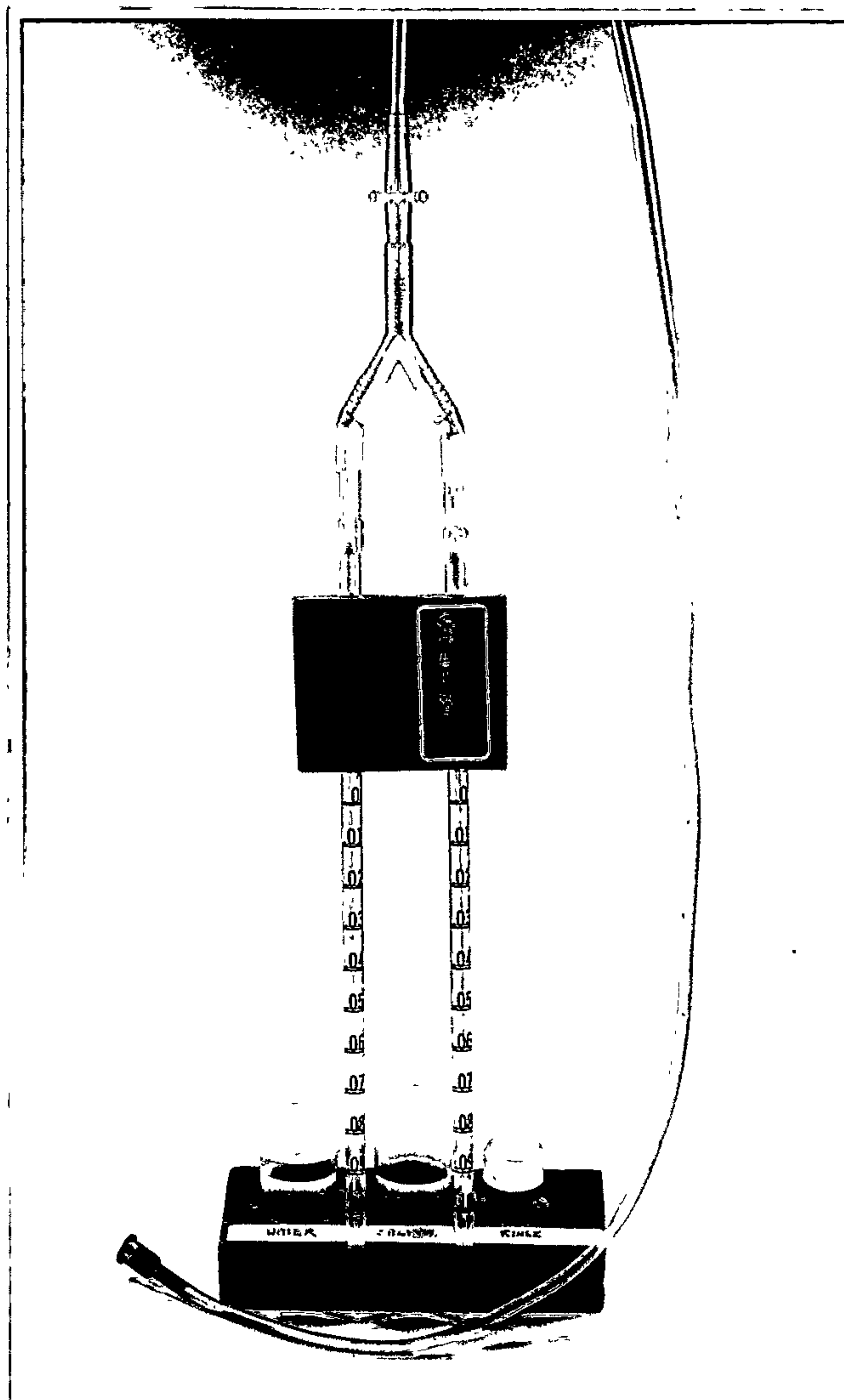
3.2.5.3. Buffering capacity and bicarbonate content of saliva.

In his investigation of buffering systems in saliva, Lilienthal⁽¹¹⁸⁾ has shown that variation in bicarbonate content

was responsible for individual differences in salivary buffering capacity and concluded that "activated saliva owes its buffering capacity largely to the presence of bicarbonate." A combined test based on the method of Segal⁽⁴⁶⁾ was developed for field use and employed for the estimation of the buffering capacity and the bicarbonate content of saliva.

FIGURE 7

KIT FOR MEASUREMENT OF VISCOSITY OF
SALIVA



of saliva.

(a) Buffering capacity.

A quantity of stimulated whole saliva, slightly in excess of 1 ml, was obtained immediately after collection of saliva specimens for chemical analyses. The method of collection was basically as described for samples collected for chemical analyses, except that the specimen was collected into a 17 mm diameter polypropylene container (5-P, Disposable Products, Adelaide, S.A.), under an approximately 3 mm thick layer of paraffin oil (B.P. Liquid Paraffin, Government Stores, Sydney, N.S.W.).

A 1.0 ml aliquot of this saliva was immediately transferred into another plastic container of 5.3 cm² surface area (25-P, Disposable Products, Adelaide, S.A.) for testing. The wide diameter container was used to facilitate evolution of carbon dioxide by having a large surface/volume ratio. Following measurement of the initial pH of the sample (Variable 217), using a battery operated portable pH meter (Phillip Harris, Ltd., Birmingham, Staffs., U.K.), 0.5 ml of 0.02 N HCl (A.R. Grade, Ajax Chemicals, Sydney, N.S.W.) was added. The sample was then vigorously agitated for a minimum of ten minutes, to promote the evolution of carbon dioxide, after which the pH was again recorded to give the first chosen parameter of buffering capacity (Variable 218).

The test was continued by stepwise addition to the manually agitated sample of 0.02 N HCl in aliquots of 0.1 ml, decreasing to 0.05 ml on nearing the selected end point of pH 4.0. This end point was selected because previous in-situ measurements of

plaque pH in the same group of subjects indicated that this was the approximate limit to which pH would be lowered at the enamel surface under biological conditions.

The amount of acid needed to lower the pH of the sample to pH 4.0 provided the second measure of buffering capacity. (Variable 219).

(b) Bicarbonate content.

The test consisted of back-titrating the saliva samples used for buffering capacity determinations.

After the buffering capacity test the samples were set aside until back-titration could commence, usually after 2 - 8 hours, at the end of the day's field work. (Laboratory tests have shown that standing for various periods did not affect the bicarbonate content of the acidified samples.)

Before back-titration commenced, the pH of the samples was again measured to test for possible change during standing. Where warranted, further acid was added until the pH was adjusted to 4.0. This was necessary in approximately one in four of the samples. The average amount of HCl needed per sample was 0.1 ml.

The samples, which were now at pH 4.0 or below, were back-titrated to the initial pH by adding 0.01 N NaOH (diluted from 'Volucon' 1.0 N NaOH, May and Baker Ltd., Dagenham, Essex, U.K.), from a 1.0 ml pipette graduated at 0.01 ml, to the continuously agitated sample. The difference between the milli-equivalents of HCl and NaOH added represented the milli-equivalents of HCO_3^- ions lost.

Following a comparable procedure, Lilienthal⁽¹¹⁸⁾ found that the milli-equivalents of HCO_3^- lost, when the pH was lowered below 5.0, and thus accounted for, represented practically all of the HCO_3^- originally present.

The precision of the method was tested by performing the test on 5 aliquots of the same saliva sample. The mean bicarbonate content was 0.0147 milli-equivalents/ml. (S.D. = ± 0.00024).

Additions of known amounts of bicarbonate to six saliva samples were recovered within ± 2 per cent of the expected values.

Variable 215 represents the bicarbonate content of saliva, expressed in milli-equivalents per ml.

3.3. Laboratory methods.

3.3.1. General comments on methods employed.

3.3.1.1. Selection and basic principles of methods used for the determination of inorganic constituents of surface enamel and activated whole saliva.

The major limitation in the choice of analytical procedures was that very small quantities of enamel were available for analysis and that the expected concentration levels of some of the elements were extremely low. The amount of saliva available was not so limiting, but the concentrations of some of the elements were even lower than in enamel.

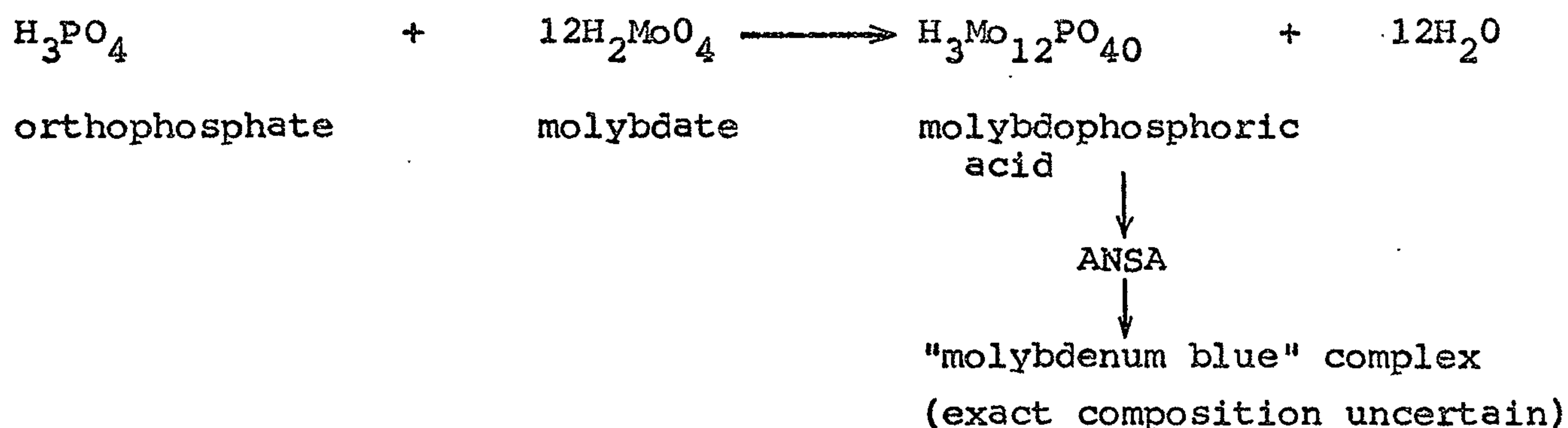
A. Non-metallic elements.

a) Phosphorus.

The amount of enamel present in each biopsy specimen was calculated from the calcium content of the sample. However, the phosphorus content of the specimens was also determined for comparison with the calcium values, partly as a possible measure of accuracy and partly to examine the controversial issue of calcium: phosphorus ratio in sound surface enamel.

Several colorimetric methods were available for phosphorus estimations. The well-proven procedure described by Fiske and Subbarow⁽¹¹⁹⁾⁽¹²⁰⁾ and modified by Lazarus and Chou,⁽¹²¹⁾ appeared to be adaptable to the enamel samples.

The method is based on the absorption of light by the blue complex formed when molybdophosphoric acid is reduced by an aminonaphthosulphonic acid - sulphite mixture (ANSA).



The "molybdenum blue" complex has a broad absorption peak between 600 and 850 nm wavelengths, depending on the conditions of formation.

b) Fluorine.

The specific ion activity electrode developed by Frant and Ross⁽¹²²⁾ is now routinely used for fluoride estimations. The method is based on the development of a potential difference across

the lanthanum fluoride crystal membrane of the electrode, which varies with the concentration of ionic fluoride in the solution in contact with the surface of the crystal. Given that the pH of the solution is between 5 and 7, no interfering complexing agents are active and the ionic strength is constant, the relationship between the base 10 log of the fluoride concentration and the potential difference is linear down to 5×10^{-6} M fluoride and nearly so to 1×10^{-6} M fluoride. Details of the method have been documented in the Orion Instruction Manual.⁽¹²³⁾

The author has had considerable experience in determining the fluoride content of small samples of enamel.⁽²¹⁾ Review of the literature suggested that one of the preparatory procedures used by Birkeland,⁽¹²⁴⁾ Singer and Armstrong,⁽¹²⁵⁾ Edgar,⁽¹²⁶⁾⁽¹²⁷⁾ McCann,⁽¹²⁸⁾ Grøn, McCann and Brudevold⁽¹²⁹⁾ or Bäumler,⁽¹³⁰⁾ followed by measurements using the specific ion activity electrode, could be adapted to estimations of the fluoride content of saliva. Eventually a modification of the method of Singer and Armstrong⁽¹²⁵⁾ was employed.⁽¹³¹⁾

B. Metallic elements.

With regard to metals at high concentration levels such as calcium and magnesium, conventional flame atomic absorption spectroscopy⁽¹³²⁾ was considered the method of choice. For the estimation of the remaining metals at low or trace levels, barium, potassium, strontium and zinc, flameless atomic absorption spectroscopy had the promise of sufficient sensitivity and the potential of reaching adequate levels of precision to show individual differences using micro-quantities of materials. Atomization

using heated graphite furnaces instead of flame has been successfully tested by L'vov,⁽¹³³⁾⁽¹³⁴⁾ Massman⁽¹³⁵⁾ and West and Williams⁽¹³⁶⁾ and has been followed by recent commercial production of equipment. Varian Techtron Pty. Ltd. were the first firm to market a flameless atomizer (Model A61 in 1971, replaced within a few months by Model A63). In view of the fact that expertise outside commercial developmental laboratories was virtually nonexistent, the manufacturers were requested, as a condition of purchasing the equipment, to carry out trial analyses on enamel and saliva samples. While in some respects this proved to be an asset, in many instances, the claims made and results reported were not substantiated by our findings.

Atomic absorption spectroscopy is based on the phenomenon that atoms in their ground state absorb radiation at a limited number of wavelengths which correspond to their specific resonance lines. In conventional atomic absorption spectroscopy, a solution containing a compound of the analyte is converted into a fine vapour which is introduced into a flame positioned in the path of light of the appropriate wavelength and constant intensity produced by a hollow cathode lamp. The heat of the flame provides energy required to partially dissociate the compound into its atoms. As the atoms of the analyte travel through the flame, they cause absorption of the light, which is proportional to the concentration of the analyte in the solution. The absorption is measured by a spectrophotometer, as a change of intensity of light reaching the instrument when a solution is aspirated into the flame. Solutions containing known amounts of the analyte and a background similar to that of the sample serve as standards.

The energy needed to overcome the molecular bond energy and to dissociate a compound into atoms, can be provided by means other than a flame. In the present case, the sample is atomized in a hollow graphite cylinder (tube-furnace), which is heated in a programmed manner by passing electric current through it, until atomization and corresponding absorption of the light directed through the cylinder occur.

Under these conditions a high density population of ground state atoms is produced within a confined space, in contrast to the low density population produced by the flame which is subject to a fast rate of flow through the flame and through the light axis. The higher efficiency of the flameless atomization process results in an increase in sensitivity which may be several orders higher than the sensitivity of the conventional technique, depending on the relative volatilities of the analyte element, matrix components and decomposition products.

In atomic absorption, sensitivity is commonly defined as the amount of analyte that causes 1 per cent absorption⁽¹³⁷⁾ and is expressed in concentration units (ppm) for the flame method and as absolute weight for flameless atomization. In order to compare "typical sensitivities" reported,⁽¹³⁸⁾⁽¹³⁹⁾ the ppm values quoted for the flame method were converted to weight figures, assuming that 1 ml of solution is required for a stable reading. A comparison of sensitivities for the elements dealt with in this report, are shown below. It should be realized, however, that sensitivities vary with instrument characteristics and experimental conditions and should not be regarded as absolute quantities.

<u>Analyte</u>	<u>Sensitivity using flame atomization (ng in 1 ml volume)</u>	<u>Sensitivity using graphite tube furnace atomization (ng)</u>
Ca	21	.0003
Ba	200	.03
K	10	.0009
Mg	3	.00006
Sr	44	.005
Zn	9	.00008

The sample volume required is 1 - 5 μ l per estimation for the graphite tube atomizer as opposed to 1 - 2 ml using the flame technique. Further advantages are that, in many cases aqueous standards can be used and that chemical interferences inherent in the flame method are eliminated.

Different types of interferences, both spectral and chemical, are present, however. The two main types of spectral interferences are caused by non-atomic absorption and by the light emitted by the incandescent graphite tube. Non-atomic absorption is the result of absorption or scattering of light at the same wavelength as the analyte resonance line by molecular species, matrix components, carbon particles or other material extraneous to the element of interest, during atomization. It can be identified and measured with a hydrogen continuum lamp in the 190 - 400 nm region of the spectrum and by the use of a nearby non-absorbing line at higher wavelengths. The incandescent graphite furnace emits light in the visible region which can cause an artificial peak, through photo-multiplier fatigue, during atomization. The effect can be eliminated or minimized by reducing temperature and heating time, consistent with complete atomization of the analyte, by masking the furnace, reducing slit opening and using a high lamp current/photo-multiplier gain ratio.

Chemical interferences may result in depression of vaporization of the analyte, mostly caused by the presence of excess cations or refractory sulphides or phosphates in the matrix, or by the formation of an analyte carbide which is stable at the temperature attained during the atomization stage. Furthermore, halides may form volatile compounds with the analyte, causing early vaporization and depletion of the number of free atoms available.

With the instrument used for the analyses, the atomization process consisted of three consecutive heating stages which were regulated by voltage/time settings adjusted according to the nature of the analyte and the matrix. The optimum settings for an analyte in a given matrix were established during trial analyses, on the basis of absorbance tracings produced during the drying, ashing and atomizing stages, monitored on a potentiometric (Y-time) recorder. Examples of some optimum and non-optimum conditions are illustrated in Figures 8, 9 and 10, pages 97 - 102.

1. Drying stage.

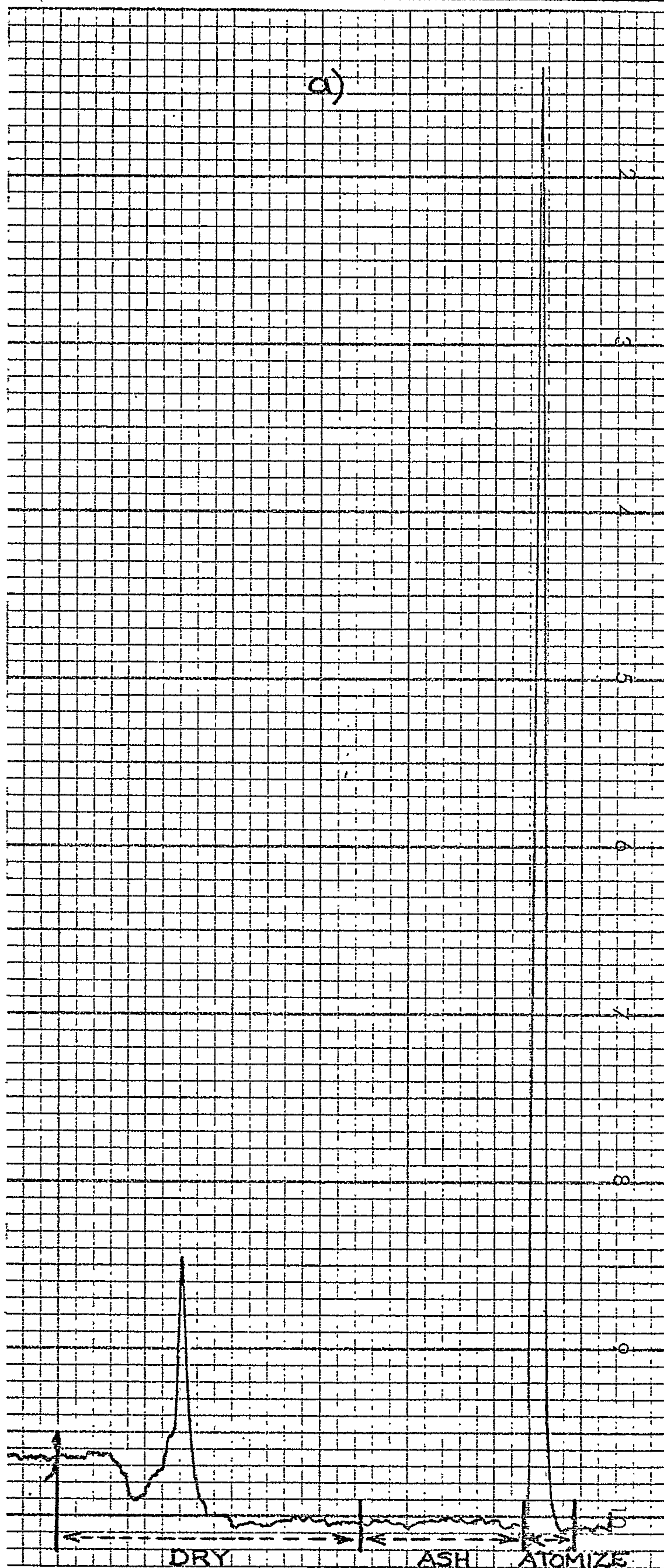
The settings are adjusted to evaporate the solvent completely without vigorous boiling or sputtering; otherwise some of the sample may be lost.

2. Ashing stage.

The dry residue is heated to eliminate organic matrix components or inorganic constituents that are more volatile than the analyte element, to eliminate or reduce intracrystalline water in the case of aqueous solutions or inorganic substances, and to pre-heat the

Figure 8 . a) b) and c).

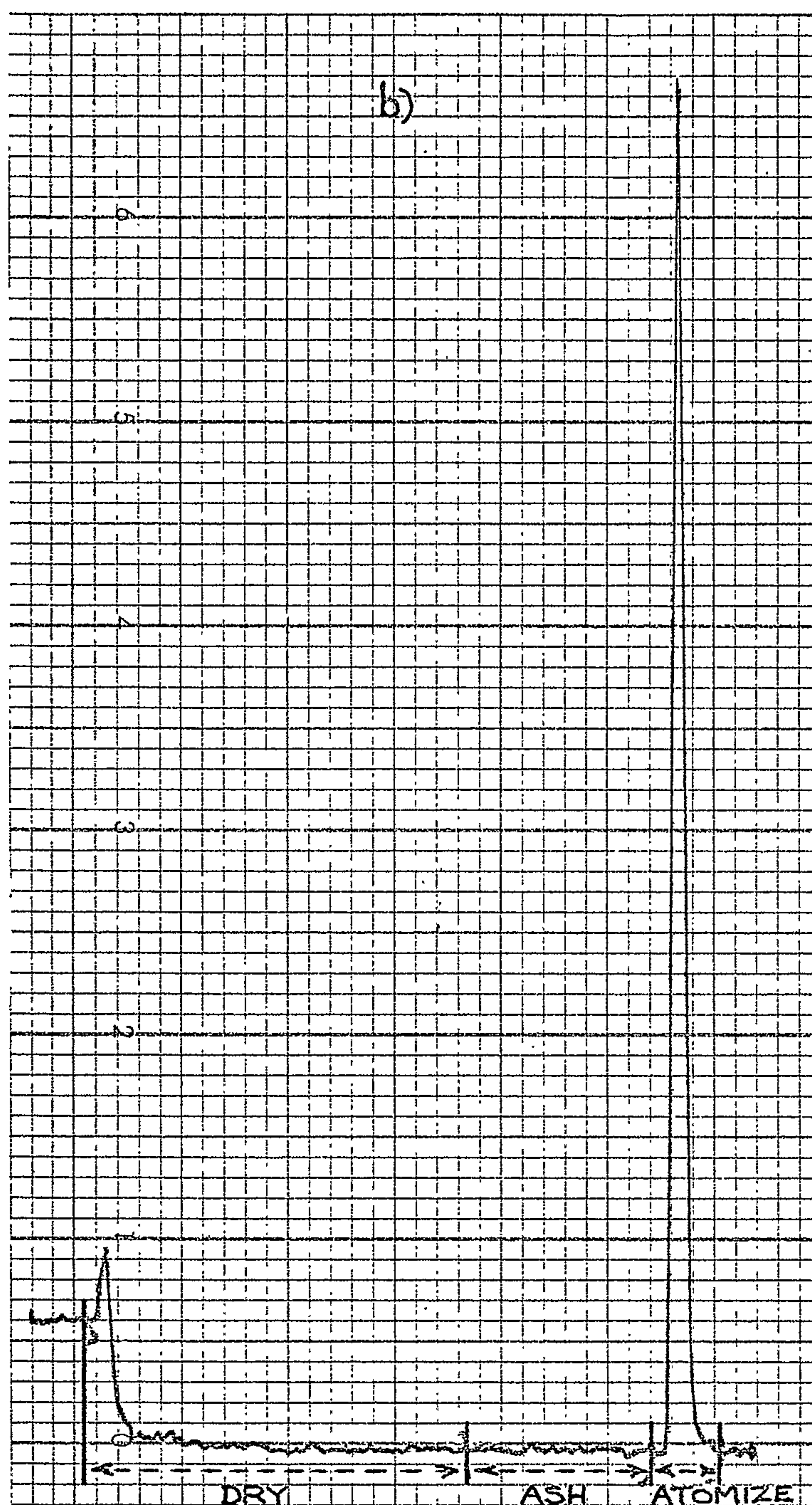
OPTIMUM AND NON-OPTIMUM 'DRY' CONDITIONS. AQUEOUS STANDARD, 0.03 ppm ZINC.



a) Optimum dry setting (zinc lamp)

Figure 8 . a) b) and c).

OPTIMUM AND NON-OPTIMUM 'DRY' CONDITIONS. AQUEOUS STANDARD, 0.03 ppm ZINC.

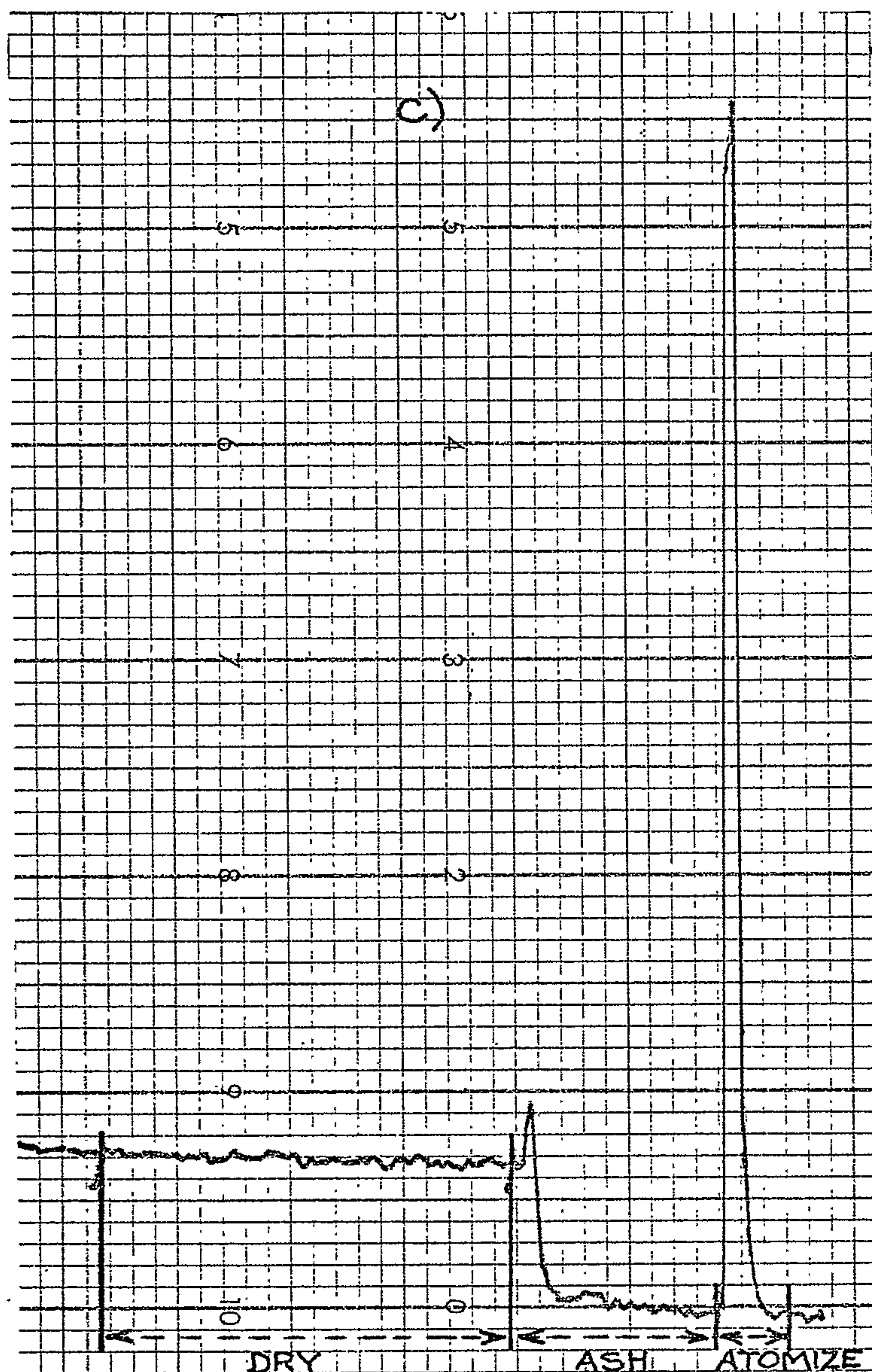


b) Dry setting too high (zinc lamp)

The early peak in the dry stage indicates sputtering and consequent loss of sample giving a lower peak in the atomize stage.

Figure 8 . a) b) and c).

OPTIMUM AND NON-OPTIMUM 'DRY' CONDITIONS. AQUEOUS STANDARD, 0.03 ppm ZINC.

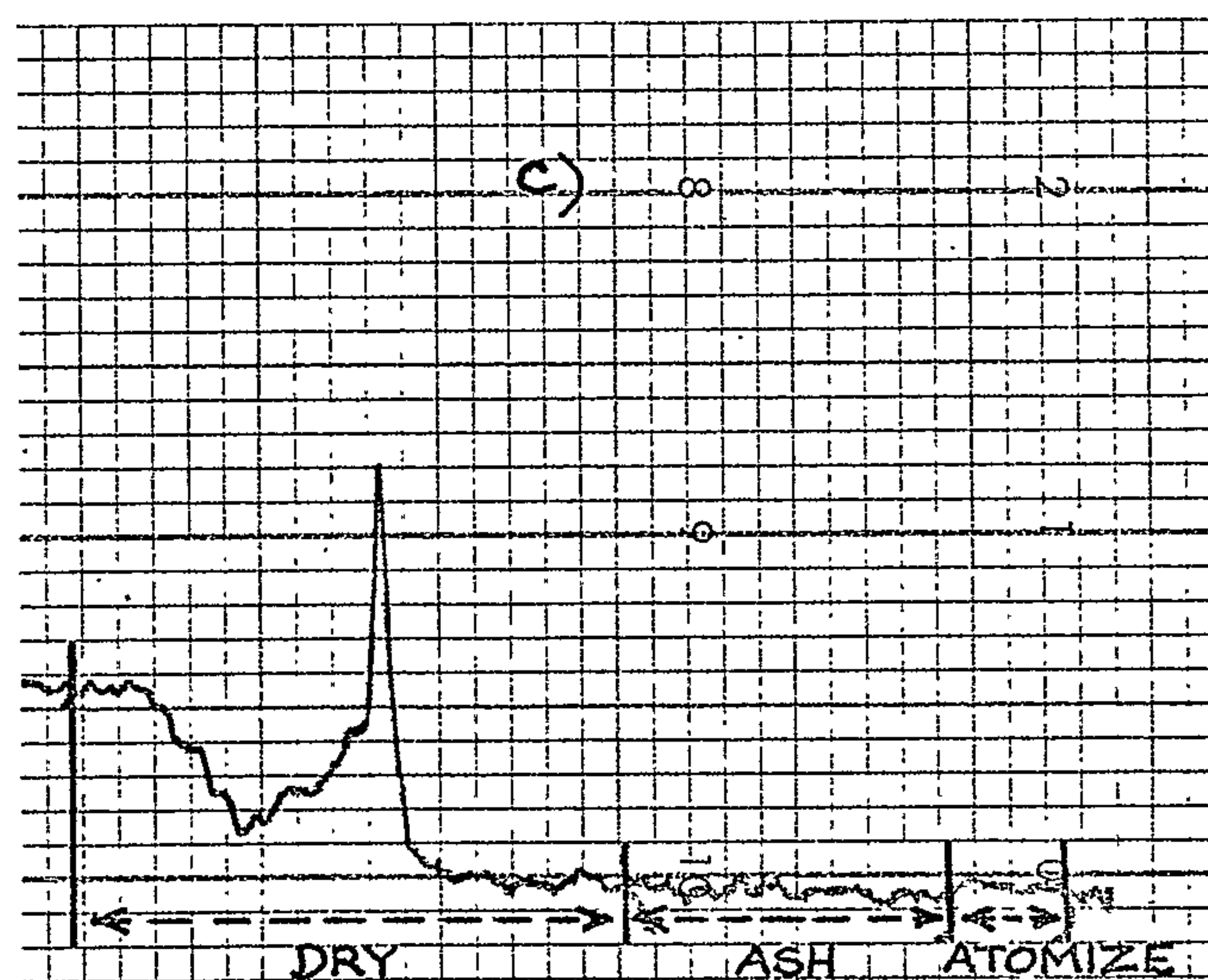
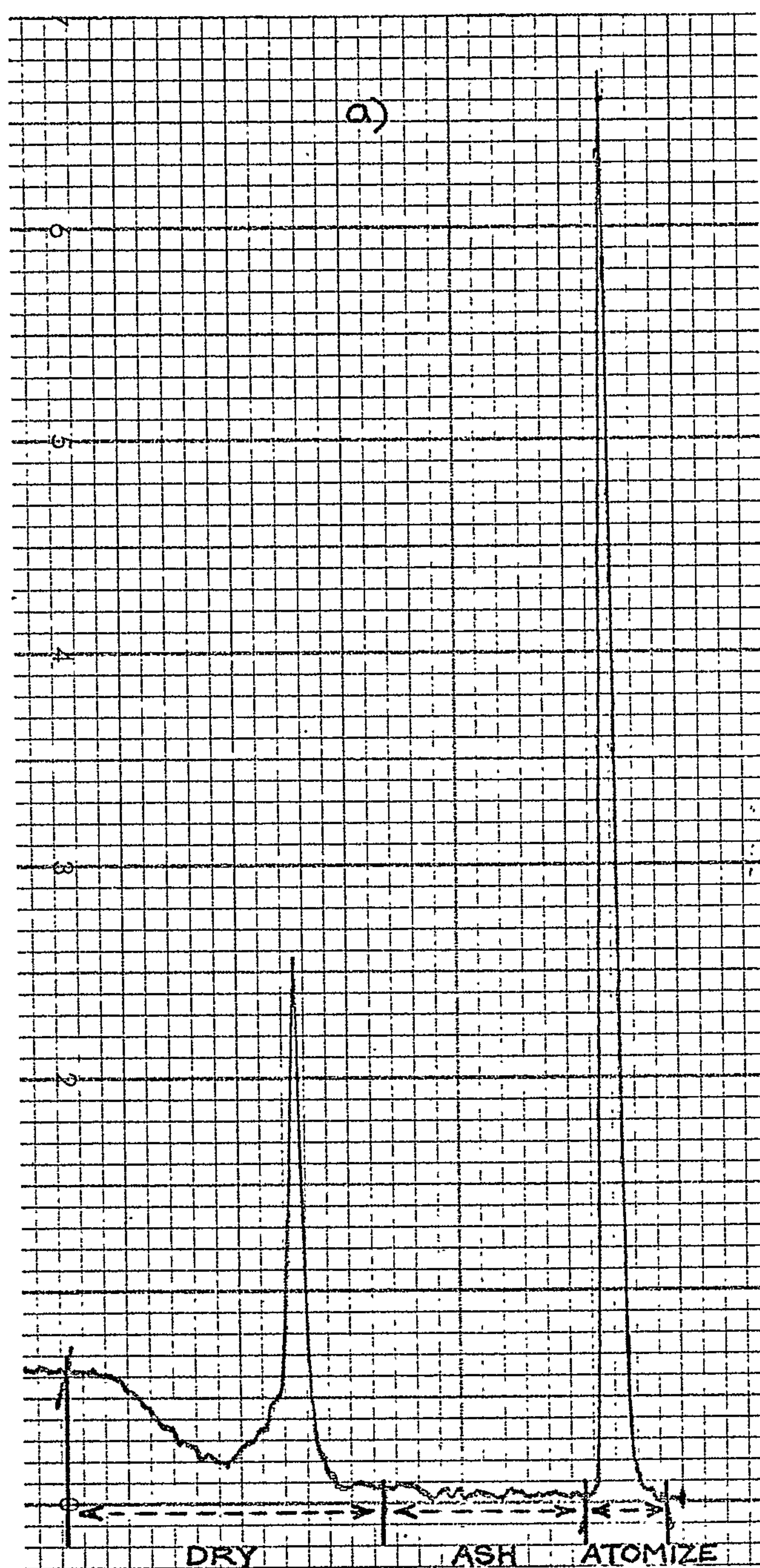


c) Dry setting too low (zinc lamp)

The trace in the dry stage does not return to the base-line indicating no drying of the sample; the peak at the beginning of the ash stage is caused by sputtering and consequent loss of sample, giving a lower peak in the atomize stage.

Figure 9 . a) b) and c).

OPTIMUM AND NON-OPTIMUM 'ASH' CONDITIONS. AQUEOUS STANDARD, 0.02 ppm ZINC.

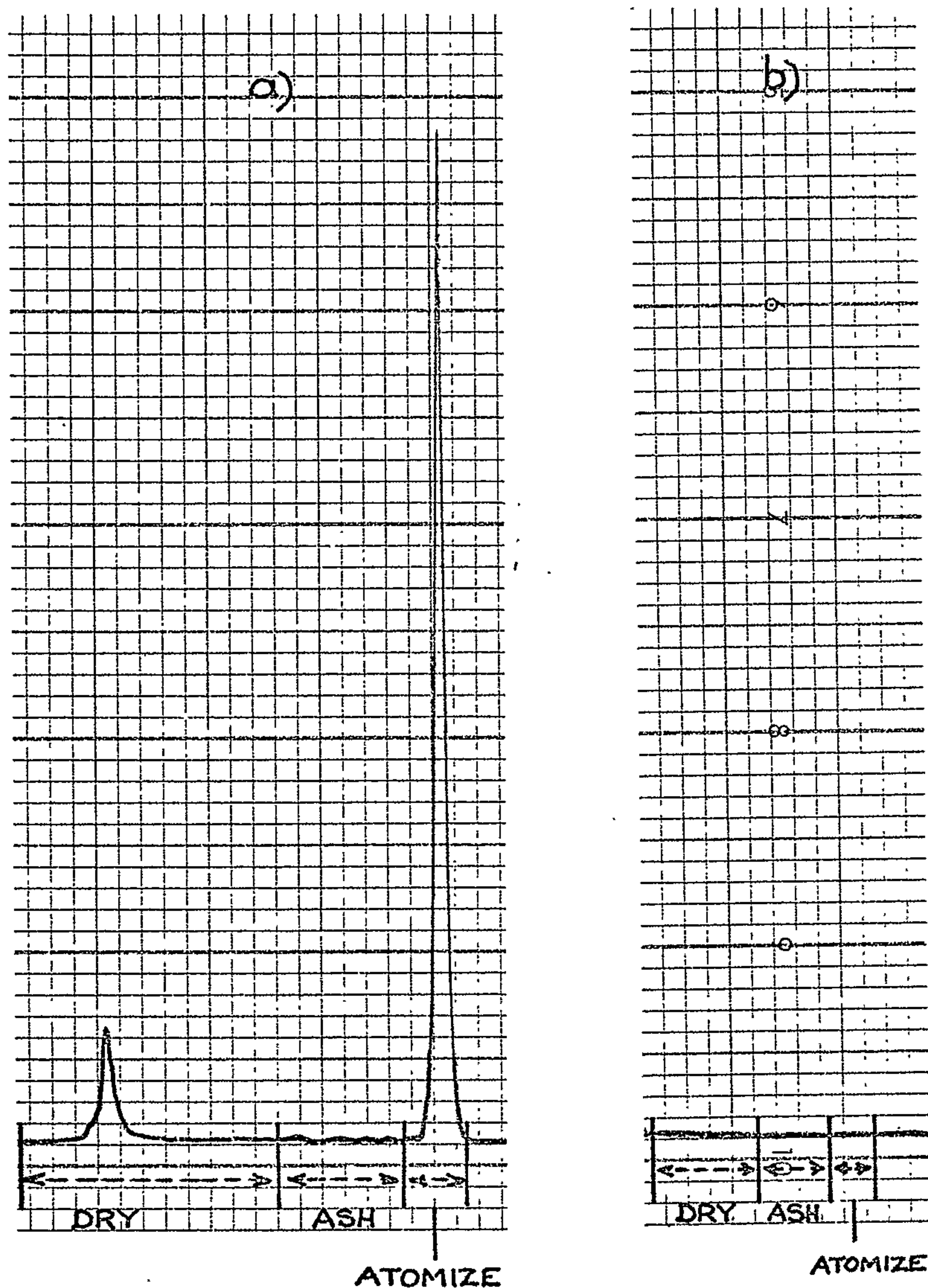


- a) Optimum ash setting (zinc lamp)
- b) Ash setting too high (zinc lamp)
- c) Ash setting too high (hydrogen continuum lamp)

No peaks in the ash or atomize stages in c) demonstrate that the multiple peaks in b) are zinc atomic absorption peaks, indicating that the reduction of peak height in the atomize stage is caused by loss of zinc in the ash stage.

Figure 10. a) - g)

OPTIMUM AND NON-OPTIMUM 'ATOMIZE' SETTINGS. AQUEOUS STANDARD, 0.01 ppm ZINC.



- a) Optimum atomize setting (zinc lamp)
- b) Firing following a) at the optimum atomize setting without a sample.

The lack of any peak in the atomize stage indicates that all the zinc was volatilized in a).

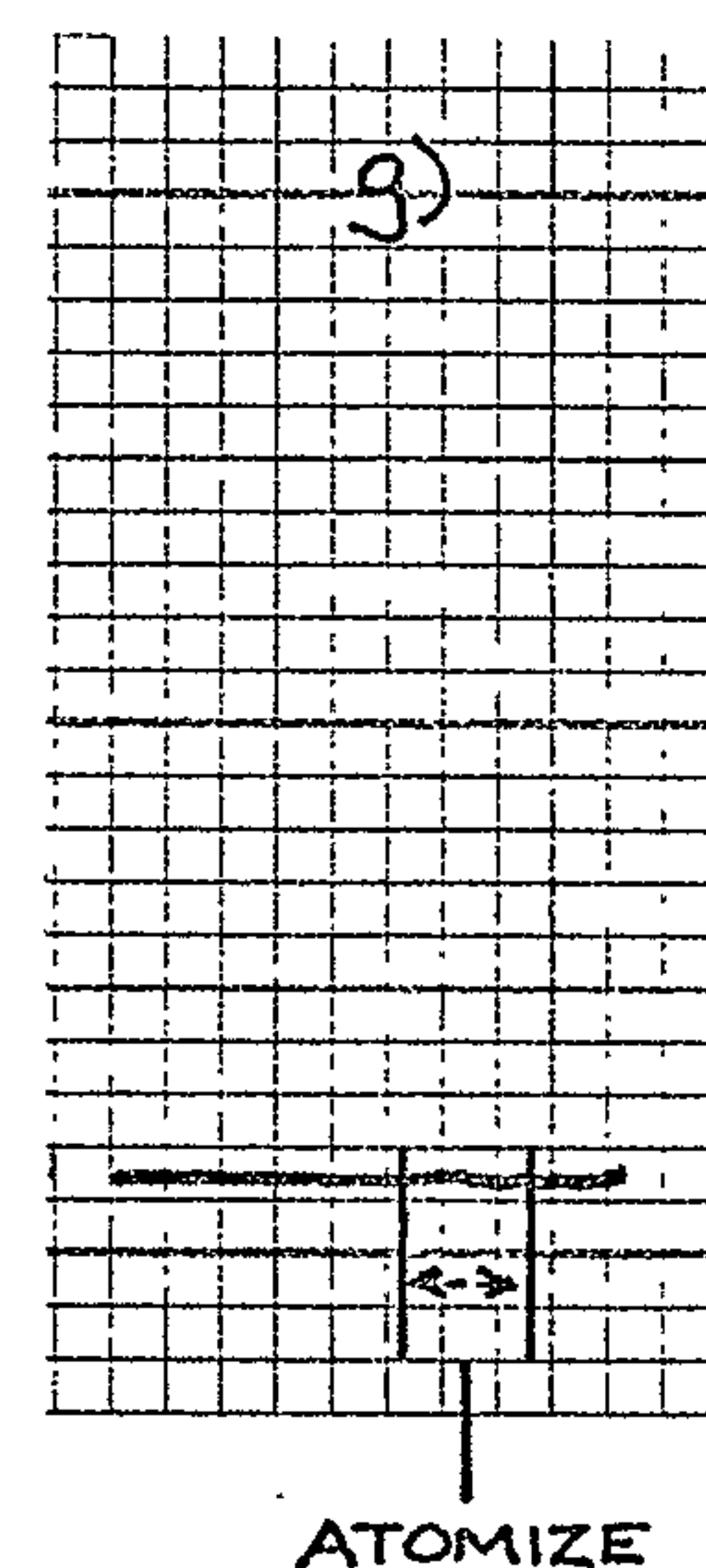
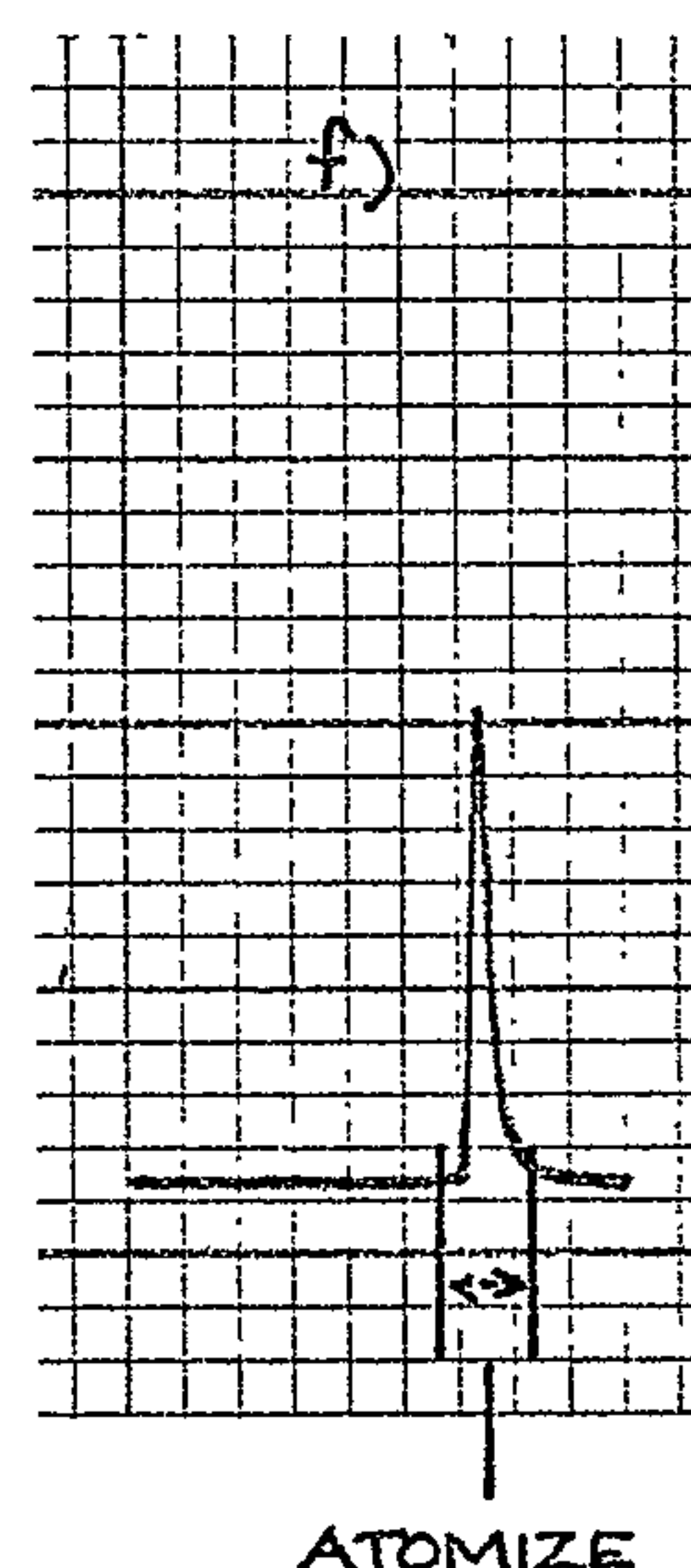
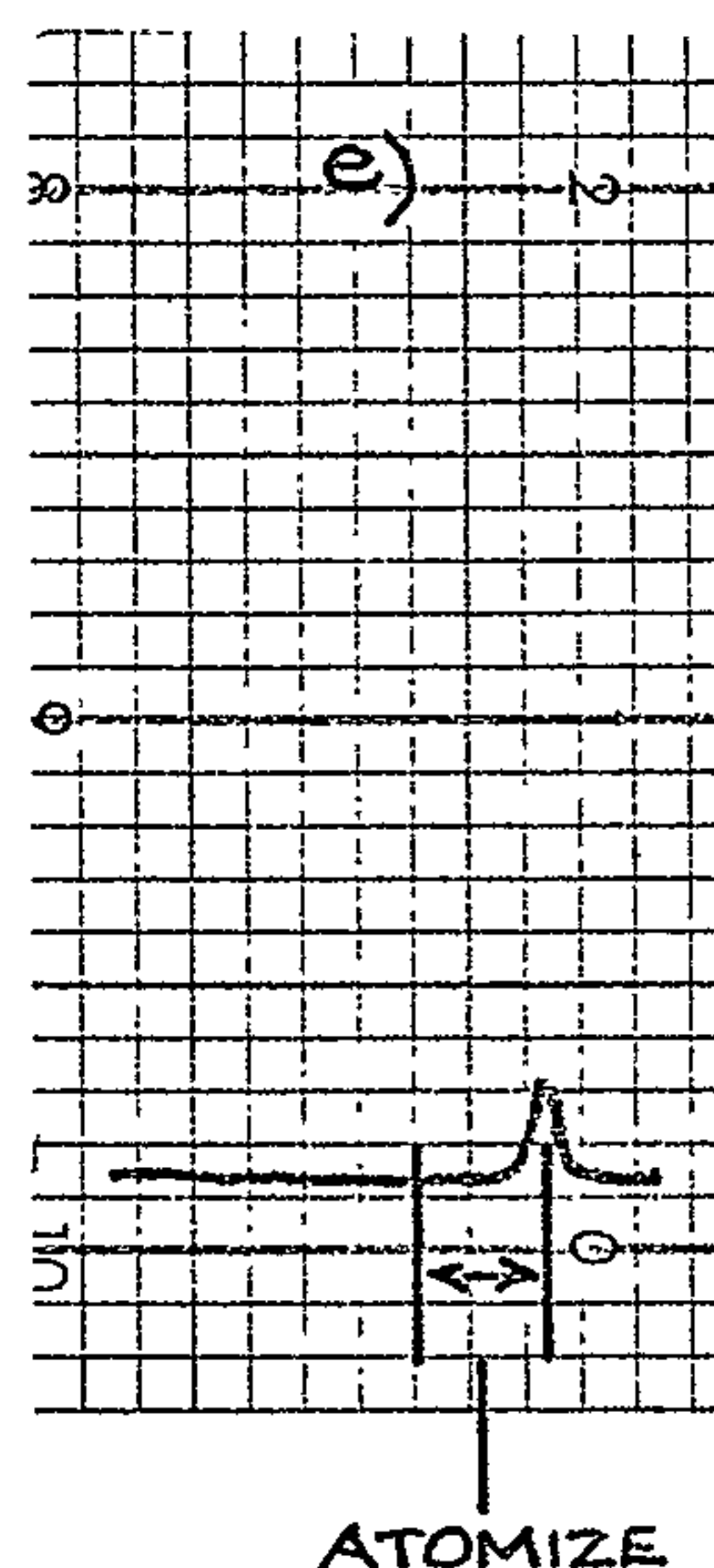
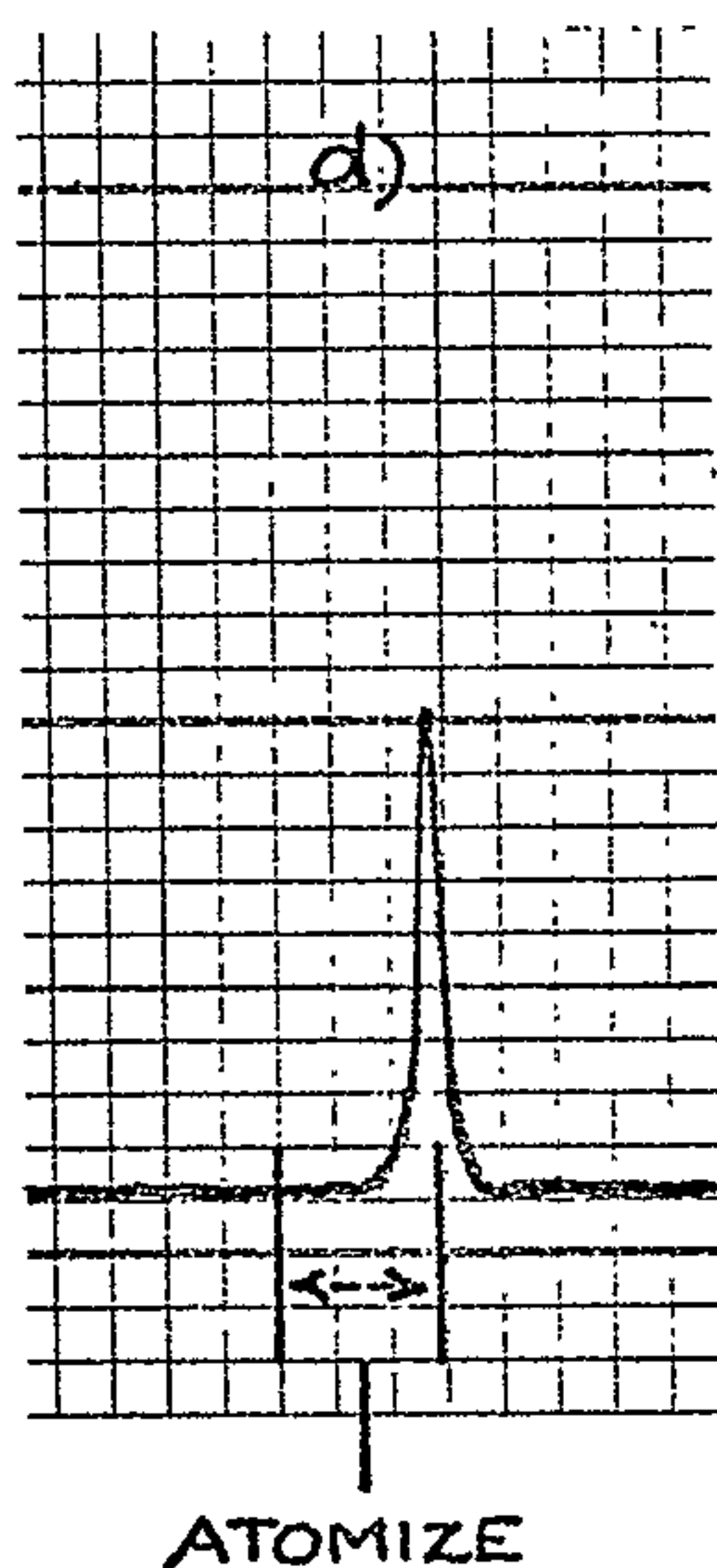
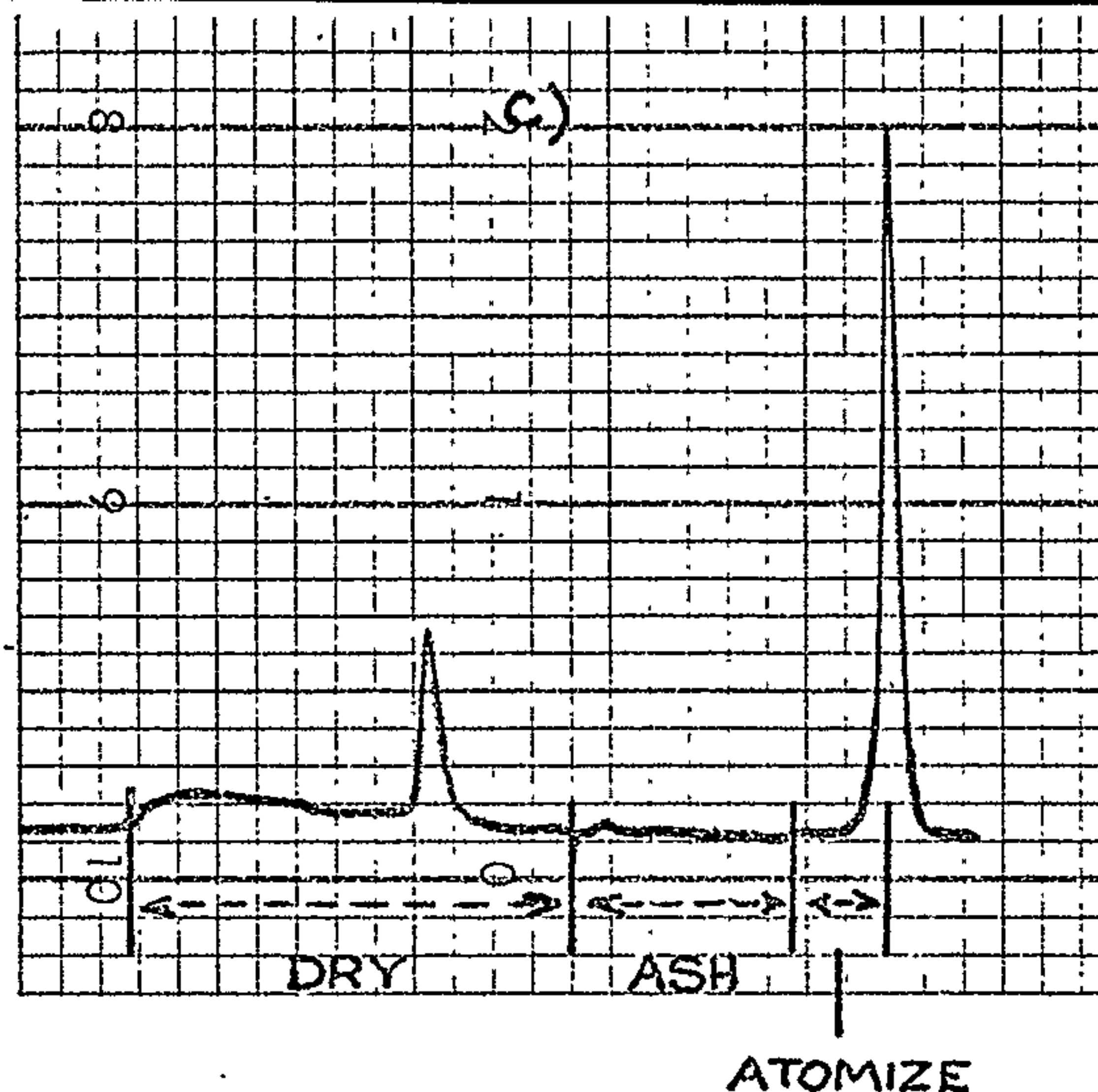


Figure 10. a) - g) ctd.

OPTIMUM AND NON-OPTIMUM ATOMIZE SETTINGS. AQUEOUS STANDARD, 0.01 ppm ZINC.

c) Atomize setting too low. The decreased peak in the atomize stage compared with a) indicates incomplete volatilization of zinc.

d) and e) Consecutive firings at the same atomize setting as c), without addition of sample. The peak in the atomize stage decreases as the residual zinc is gradually volatilized.

f) Firing at the optimum atomize setting without further addition of sample. The presence of a substantial peak in the atomize stage indicates that zinc still remained in the atomizer tube.

g) The absence of a peak in the atomize stage on a further firing at the optimum atomize setting without addition of sample indicates that all zinc was volatilized in f).

sample for the atomization stage. Too high a temperature results in volatilization of the analyte, whereas too low settings lead to the retention of matrix components. In this stage it is not possible to eliminate components of higher volatility than the analyte.

3. Atomizing stage.

The temperature of the furnace is raised to the level required for complete volatilization of the analyte. Too low voltage or time settings result in incomplete volatilization of the analyte. On subsequent firing without introducing a sample, a peak representing a carry-over memory effect appears. Too high settings may result in photomultiplier fatigue, rapid decline in furnace performance and in consequent loss of precision. There are two modes of atomization available. In the step-atomizing mode, the temperature is suddenly raised by a pulse of current, while in the ramp-atomizing mode a gradual rise of temperature is produced by increase of voltage to the chosen cut-off point at a pre-selected rate. The latter mode makes possible the separation of atomic and non-atomic peaks and the elimination of matrix components which are less volatile than the analyte. The analyte is atomized in an inert atmosphere provided by laminar flow of nitrogen surrounding the atomizer - electrode assembly. Reducing conditions can be created by a hydrogen flame during the ashing and atomizing stages. A water-cooling system reduces waiting time between analyses.

The range of settings is shown below:

	<u>Voltage control settings *</u>	<u>Actual voltage</u>	<u>Time control settings (seconds)</u>
Dry	0 - 10	0 - 1.8	0 - 40
Ash	0 - 10	0.4 - 4.0	0 - 30
Atomize - step	0 - 10	2.0 - 9.0	0 - 10
- ramp	0 - 10 **		0 - 10 ***

* --Continuously variable

** Cut-off point

*** Ramp rate; volt/second

According to the manufacturers, 'dry' and 'ash' settings are governed by the nature of the matrix and once optimum settings are established these are suitable for any analyte in the same matrix. Conversely, the 'atomize' settings were said to depend on the analyte and that the same settings could be used for that analyte regardless of the matrix. In practice, many exceptions were found to these statements. Variation in dilution resulted in variation in the total amount of material present per estimation and in changes in the viscosity of the material. The type and degree of interaction between the analyte and the matrix, manifested by the volatilization characteristics of the analyte, as well as the analyte : matrix ratio, were different for each element. These variables made it mandatory to establish individual 'dry', 'ash' and 'atomize' settings for all elements and to vary these settings according to the condition of the graphite tube atomizer when material changes in its performance were observed with usage.

Unfortunately, no published information is available concerning the temperatures reached by the furnace at various voltage-time settings and repeated attempts to obtain this information from the manufacturers were unsuccessful. To permit a more meaningful interpretation of the analytical conditions used, the temperatures attained during the 'dry' and 'ash' stages were repeatedly measured using a bimetal thermocouple (chromel-alumel, custom-made) placed into the graphite furnace in the position normally occupied by the sample. The thermocouple was calibrated against ice-water mixture, boiling water and a mercury thermometer in an oil bath at 250°C.

Temperatures reached during the atomization stage were measured in the Department of Analytical Chemistry, University of New South Wales, using an optical pyrometer positioned to measure the incandescence emitted from the area of the graphite furnace where the sample is placed, through the hole used for introducing the sample. The temperature readings could be reproduced to $\pm 10^{\circ}\text{C}$ and for 5 readings in the range where measurements by both methods were possible (650-1160°C), the differences between the values obtained by the two instruments were within 4 per cent of the smaller value. However, as it was not intended to make a detailed study of this aspect of the work, the temperatures given under atomization conditions in sub-sections 3.3.2.1.4. to 3.3.2.1.7., are considered approximations and are intended as a guide only. The maximum attainable temperature is believed to be approximately 3000°C.

Before use, contamination present on or within the pyrolytic graphite coating of new atomizer tubes was removed routinely by

firing the tube without a sample at voltage-time settings known to completely atomize the analyte element. New tubes were usually decontaminated by 2-3 firings. Air-borne contamination, measurable after relatively short pauses in estimations, was removed by a single firing.

Analytical techniques were developed over a period of 14 months with particular attention to flameless atomization procedures. Trial analyses were carried out using enamel samples taken by the biopsy technique used in the field, from some 100 extracted canine teeth of Australian origin.

The concentration levels of the elements of interest found during the trial analyses were in general agreement with values obtained through first-hand information from workers active in related fields⁽¹⁴⁰⁾⁽¹⁴¹⁾ and from the literature,⁽⁶⁶⁾⁽⁶⁷⁾⁽⁷⁰⁾⁽⁸⁶⁾⁽¹⁴²⁾⁽¹⁴³⁾⁽¹⁴⁴⁾⁽¹⁴⁵⁾⁽¹⁴⁶⁾⁽¹⁴⁷⁾⁽¹⁴⁸⁾⁽¹⁴⁹⁾⁽¹⁵⁰⁾⁽¹⁵¹⁾⁽¹⁵²⁾⁽¹⁵³⁾⁽¹⁵⁴⁾⁽¹⁵⁵⁾⁽¹⁵⁶⁾⁽¹⁵⁷⁾⁽¹⁵⁸⁾⁽¹⁵⁹⁾ although very wide variations in values were noted.

The regimen developed for sample preparation (sub-section 3.3.2.1.1.) was based on the concentration level found for each element in the trial analyses, and on the sensitivity of the chosen analytical method.

3.3.1.2. Microbiological methods.

The only practical means of approximating the total number of streptococci against a background of vast numbers and large variety of other microorganisms found in plaque, was to employ a

cultural method using a selective medium followed by plate counts. It was recognized that these conditions would limit the scope to the consideration of streptococci which remain viable through collection, dispersion and preservation procedures, and that these may be depressed to some extent by the selective medium. It was considered, however, that provided the procedures were rigidly standardized and the limitations of the method were fully recognized in the evaluation and interpretation of the results, the information obtained would make intercomparisons within the sample possible and valid. Personal consultations with workers active in this field, a review of methods used in epidemiological studies and allied projects by Gibbons and Loesche,⁽¹⁶⁰⁾ Loesche and Henry,⁽¹⁶¹⁾ Krasse, Jordan, Edwardsson, Svensson and Trelle,⁽¹⁶²⁾ Jordan, Krasse and Möller,⁽¹⁶³⁾ de Stoppelaar, van Houte and Backer Dirks,⁽¹⁶⁴⁾ Jordan, Englander and Lim,⁽¹⁶⁵⁾ Rogers,⁽¹⁶⁶⁾⁽¹⁶⁷⁾ van Houte, Backer Dirks, de Stoppelaar and Jansen,⁽¹⁶⁸⁾ Littleton, Kakehashi and Fitzgerald,⁽¹⁶⁹⁾ Thomson,⁽¹⁷⁰⁾ Woods,⁽¹⁷¹⁾ Shklair, Keene and Simonson,⁽¹⁷²⁾ and previous experiences by the author and associates,⁽¹²⁾⁽²⁰⁾ supported this approach.

Much consideration was given to the selection or establishment of a system for estimating relative proportions of the streptococci isolated. Having completed primary isolation, in principle at least, recognized methods of classification were available on the basis of colony morphology, biochemical and physiological tests and immunological characteristics. In practice, however, in spite of extensive work carried out recently in this area by Rogers,⁽¹⁶⁶⁾ Thomson,⁽¹⁷⁰⁾ Carlsson,⁽¹⁷³⁾ Edwardsson⁽¹⁷⁴⁾ and Guggenheim,⁽¹⁷⁵⁾ the unequivocal identification

and categorization of all streptococcal isolates on the basis of biochemical characteristics, is not possible. Immunological methods for identification of oral strains in common use are either too broad or are limited to S. mutans.^{(176) (177) (178)} Furthermore, as yet no reliable differentiation between certain serotypes, such as Bratthall's serotypes a and d, is possible using fluorescent antibody techniques.⁽¹⁷⁹⁾ More sophisticated methods such as bacteriocin typing reported by Kelstrup and Gibbons⁽¹⁸⁰⁾ or examination of DNA base content^{(181) (182)} or DNA-DNA re-association⁽¹⁸³⁾ or ultrastructural patterns⁽¹⁸⁴⁾ would have been impractical to use, because of the training or expertise required, the limitation of available techniques to certain species and the volume of the work. Further consideration was given to the time-cost-benefit aspects of the problem and it soon became clear that in terms of best distribution of resources and effort, it would be unjustified to conduct full scale biochemical and immunological tests.

The best alternative appeared to be, to establish a system of classification at the colony morphology level, and if justified on the basis of the results obtained, to follow up as a separate project with a more detailed study of the isolates, which are shown to be of aetiological interest.

3.3.1.3. Determination of selected inorganic constituents of water samples.

The use of atomic absorption and of the fluoride ion electrode are routine techniques employed for the determination of metals and fluoride, respectively, in water samples. These

techniques were adopted for the water analyses.

3.3.2. Details of methods.

3.3.2.1. Determination of selected inorganic constituents and dissolution rate of surface enamel.

Aliquots of samples obtained by the enamel biopsy, described in sub-section 3.2.3.12., were used for the determination of calcium, magnesium, strontium, potassium, barium, zinc and phosphorus content of the surface biopsy specimens.

The specimens obtained by the enamel dissolution rate tests, described in sub-section 3.2.3.10., were used for fluoride determinations.

3.3.2.1.1. Dispensing and storage of sample dilutions for Ca, Mg, Sr, K, Ba, Zn and P determinations.

(a) General routine and precautions.

Extreme care in sample-handling is necessary to prevent contamination in trace element analysis.⁽¹⁸⁵⁾ Fluctuating contamination is especially liable to lead to spurious results.⁽¹⁸⁶⁾ Chemicals and reagents are obvious sources of possible contamination and, as such, can be monitored with relative ease. Contamination arising from contacts with the environment and with containers is much more insidious and difficult to control or to trace. Dust may contain extremely high concentrations of potential contaminants. Levels of 5000 ppm lead and 1000-1500 ppm barium and zinc attributed to deteriorating paintwork were reported by Scott.⁽¹⁸⁷⁾ Plastics are suspect because of the fillers, stabilizers and colouring materials⁽¹⁸⁸⁾ they contain. As complete isolation of

samples from the laboratory environment was clearly not possible, we endeavoured to keep conditions constant, allowed minimum contact between open samples and the environment and arranged handling sequences so that the effect of fluctuating contamination would be minimized.

The laboratory where the samples were to be dispensed was thoroughly cleaned three days before opening the samples. As complete air filtering was not possible, window seams were sealed with tape to reduce the amount of dust entering and the filter of the air conditioner was renewed. Smoking was banned in the laboratory and entry of personnel not directly concerned with the work was discouraged for the duration of this work.

Trial analyses indicated that the risk of contamination was greatest in respect of zinc and potassium, because of a combination of high environmental levels of these elements and the extreme sensitivities of the respective analytical methods, requiring high dilution of the samples.

Because the aliquots dispensed for some of the analyses were very small, (50 - 100 μ l) the possibility of evaporation during storage and analysis presented a considerable additional risk. It was decided to heat-seal aliquots of 100 μ l or less into previously tested surgical quality colourless polyethylene tubing ('Portex' PP 280 and PP 355, Boots Pure Drug Co., Hythe, Kent, U.K.) and to dispense aliquots of 2 ml or more into air-tight polypropylene vials (5-P, Disposable Products, Adelaide, S.A.). Only new tubing or vials were used, following an appropriate decontamination process.

The procedure of container decontamination varied with the type of estimation that was to follow. Polypropylene vials for samples for calcium and magnesium estimations were soaked in a 5 per cent solution of combined anionic - cationic detergent (Detex 11, Borer Chemie, Solothurn, Switzerland) for four hours, followed by rinsing in distilled, deionized water until no trace of the detergent remained. Vials used for zinc, potassium and phosphorus analyses were soaked in the same detergent and rinsed as above, followed by rinses in approximately 0.1 M hydrochloric acid (A.R. Grade, Ajax Chemicals, Sydney, N.S.W.) and glass-distilled, double-deionized water. Polyethylene tubing was soaked in 4.0 M hydrochloric acid overnight and rinsed in a large excess of glass-distilled, double-deionized water.

The washing and rinsing were performed in clear polyethylene bags and the containers were dried in a stainless steel-lined drying oven ("Qualtex", Watson Victor Ltd., Sydney, N.S.W.) at 55°C within the same bags which were left slightly open. After drying, the tubing was cut into suitable lengths and heat-sealed at one end with artery forceps over a fine flame. The washed vials and tubing were always handled with plastic gloves and were kept in airtight storage until used. Pipette tips, water and acid containers and beakers were all cleaned in the same manner.

The effectiveness of the washing procedure was tested by contaminating containers with high concentrations of the analytes and subjecting them to the decontamination procedure, followed by analyses of container blanks, which proved to be satisfactory.

The storage of aqueous solutions containing low concentrations of metals involves the risk of losing some or all of the analyte through adsorption to container walls. Although total adsorption losses were stated to be small,⁽¹⁸⁹⁾ even minute losses can be critical at the concentration levels used with flameless atomic absorption techniques. Struempier⁽¹⁹⁰⁾ reported that adsorption varied with concentration, pH, time of contact with the container, composition of dissolved salts, type of container and complexing agents. It is generally recognized, however, that acidification to pH 2 or less reduces or eliminates adsorption in most instances. As the enamel biopsy specimens were collected, stored and dispensed at low pH values, no complications were expected in this regard.

This was confirmed by a control procedure wherein periodic re-analyses of trial samples, made up using the same routine and dilution ranges as employed for the New Guinea samples and stored for 4-5 months, did not show reductions in the concentration of analytes.

Dispensing of the aliquots was carried out over an uninterrupted period of eight working days by the author and two graduate assistants.

After stocktaking, the samples were laid out on a bench and a number corresponding to one day's work and representing a full cross-section of the human sample, was removed for dispensing. Representative subsamples were dispensed each day to avoid possible bias that may have arisen from minor day to day variations in procedure, such as change of a repeating pipette used, or from

transient contamination. Randomly selected blank controls were dispensed with each daily lot.

A register was kept of the samples dispensed each day and records were made of changes in instrumentation and daily events. Samples containing less than four biopsies were listed to allow for adjustment of the final basic sample volume. (Page 114).

(b) Preparation of basic sample dilution.

The sample tubes were centrifuged for 5 minutes at approximately $5000 \times g$ to move moisture and disc fragments to one end. One corner of the opposite end of the tube was cut open with a stainless steel scalpel and 300 μ l of 0.016M nitric acid (Aristar Grade, B.D.H. Chemicals Ltd., Poole, Dorset, U.K., diluted in glass-distilled, double-deionized water) was introduced into the tube with repeating micro-pipettes (Eppendorf Gerätebau Netheler G.m.b.H., Hamburg, West Germany).

The accuracy and precision of the delivery of the pipettes was tested by weighing. The mean weight of 20 aliquots of 300 μ l of water was 299.52 mg, S.D. $\pm$ 1.08 mg. This result indicated that it was possible to attain a higher level of precision using repeating pipettes than indicated by the (approximately) 1 per cent coefficient of variation reported by Joyce and Tyler. (191)

The open end of the sample tube was sealed with a film of fine adhesive plastic ("Gladwrap", Union Carbide (Aust.) Pty. Ltd., Sydney, N.S.W.) and the sample was thoroughly shaken to dissolve and mix any solid residue adhering to the top part of the tube. The solution was then moved into the closed end of the tube by a "thermometer shakedown" action. The sample was kept in an upright

position for at least 10 minutes, to allow foam particles to settle before dispensing the aliquots.

In the majority of cases this basic dilution contained four pooled biopsies in a volume of 320 μ l of approximately 0.27 M nitric acid.

The volumes of six samples that contained three biopsies and eight samples that contained two biopsies were 315 and 310 micro-litres respectively.

The aliquots for the estimation of various elements were removed in the order listed below, after which the tubes containing the basic dilutions were heat-sealed and stored at room temperature in an airtight container.

(c) Dispensing of aliquots for analyses.

Aliquots for calcium determinations.

One microlitre of each basic sample was removed with a 3.0 μ l total capacity syringe (Type 3A-RN-GP, Scientific Glass Engineering Pty. Ltd., Melbourne, Vic.), fitted with a repeating adapter set at 1.0 μ l and a custom-made polyethylene tip, which held the whole volume, and added to 2.00 ml of lanthanum nitrate base solution (10,000 ppm La), previously dispensed (E-mil dispenser, James A. Jobling & Co., Stone, Staffs, U.K.) into a polypropylene container (5-P, Disposable Products, Adelaide, S.A.) and stored frozen until 1 - 2 hours before use.

The final dilution of the basic enamel sample was 1 in 2001.

The accuracy and precision of delivery of 1.00 μ l volumes

were tested in advance by weighing water delivered into pre-weighed polyethylene capillary tubes on a torsion balance (Model 420-A, Sauter A.G., Ebingen, West Germany). The mean net weight of 10 aliquots of 1.00 μ l volumes was 0.9982 mg, S.D. $\pm$ 0.0086 mg. The accuracy and precision of dispensing 2.00 ml volumes were tested by weighing on an electric balance (Model H4, E. Mettler, Zürich, Switzerland). The mean weight of ten aliquots of water dispensed at the setting used was 1.997 g, S.D. $\pm$ 0.010 g.

To prepare the 10,000 ppm lanthanum background solution, 29.32 g of oven-dried lanthanum oxide, La_2O_3 (Univar grade, Ajax Chemicals, Sydney, N.S.W.), dissolved in 500 ml of 1.58 M nitric acid (50,000 ppm La stock), was diluted fivefold with distilled, deionized water.

Aliquots for barium determinations.

Fifty microlitres of the basic sample dilution was transferred with a repeating pipette (Eppendorf) fitted with a custom-made, fine polyethylene tip into polyethylene tubing ('Portex' PP 280) and heat-sealed. Between samples, the pipette tip was rinsed five times in 0.016 M nitric acid. The samples were stored at room temperature in an airtight container.

Aliquots for strontium determinations.

Five microlitres of the basic sample was added to 100 μ l of 0.016 M nitric acid, previously dispensed into polyethylene tubing ('Portex' PP 355) using repeating pipettes (Eppendorf) fitted with custom-made, fine polyethylene tips. On delivering the sample, the pipette tip was rinsed in the diluent twice and between samples

it was rinsed four times in 0.016 M nitric acid. The diluted samples were stored at room temperature in an airtight container. The dilution of the basic sample was 1 in 21.

Aliquots for potassium, zinc and phosphorus determinations.

Twenty microlitres of the basic sample was added to 2.00 ml of 0.158 M nitric acid in a polypropylene vial (5-P) using a repeating pipette (Eppendorf). The pipette tip was rinsed in the diluent twice and in 0.016 M nitric acid three times between samples. The diluted samples were stored in airtight containers at -25°C between analyses or further dilutions, which at a later stage were necessary for zinc and phosphorus estimations. The dilution of the basic sample was 1 in 101.

Aliquots for magnesium determinations.

Twenty microlitres of the basic sample was added to 2.00 ml of one per cent lanthanum base solution in 0.32 M nitric acid in a polypropylene vial (5-P) using a repeating pipette. The pipette tip was rinsed into the diluent three times after delivering the sample and five times in 0.016 M nitric acid between samples. The rinsings were discarded in each case to prevent carry-over of the lanthanum solution into the basic sample. The diluted samples were stored in airtight containers at -25°C until required for analyses. The dilution of the basic sample was 1 in 101.

3.3.2.1.2. Calcium determinations.

The determinations were carried out by atomic absorption spectroscopy (Model AA-5, Varian Techtron Pty. Ltd., Springvale, Vic.) using 2 ml aliquots of 1 in 2001 dilutions of the basic

sample in one per cent lanthanum background solution. The absorbance was recorded on a strip chart recorder (Model A25, Varian Techtron).

Batches of approximately 50 samples, representing a cross-section of the total sample and two blanks were analysed each day.

Instrument parameters:

Wavelength: 422.67 nm

Lamp current: 4.0 mA

Spectral band width: 0.33 nm Slit height: 10 mm

Photomultiplier gain: Low gain 3

Scale expansion: 1x Damping: B

Flame: Nitrous oxide-acetylene

Gas flow rates: Nitrous oxide: 10.0 l/minute, 14.7 p.s.i.a., 70°F

Acetylene: 4.0 l/minute, 14.7 p.s.i.a., 70°F

Burner: 5.0 cm, 1 slot

Standards.

The calcium stock solution of 1000 ppm was prepared by slowly dissolving 2.4974 g of oven-dried calcium carbonate (Univar grade, Ajax Chemicals, Sydney, N.S.W.), in 100 ml of 1.0 M nitric acid (Aristar) and making up to 1000 ml with distilled, deionized water.

Working standards containing 1, 2, 3 and 4 ppm calcium were prepared by the dilution of 100, 200, 300 and 400 µl of the stock solution to 100 ml in 1 per cent lanthanum background solution. The same background solution, dispensed in an identical manner,

was used as 0 ppm calcium standard. Standards were interposed between groups of approximately 15 samples. Typical mean absorbance x 100 values were:

0 ppm	1 ppm	2 ppm	3 ppm	4 ppm
1.2	17.9	34.2	50.8	66.9

A typical calibration graph is shown in Figure 11, page 119. Ninety per cent of the samples fell within the range of 27 - 40 units of absorbance x 100.

Blank values.

The blank values were very close to the 0 ppm standards, indicating that most of the value was derived from the lanthanum background solution. The mean value was 0.098 ppm, S.D. = ± 0.018 ppm, n = 12.

Replicate analyses.

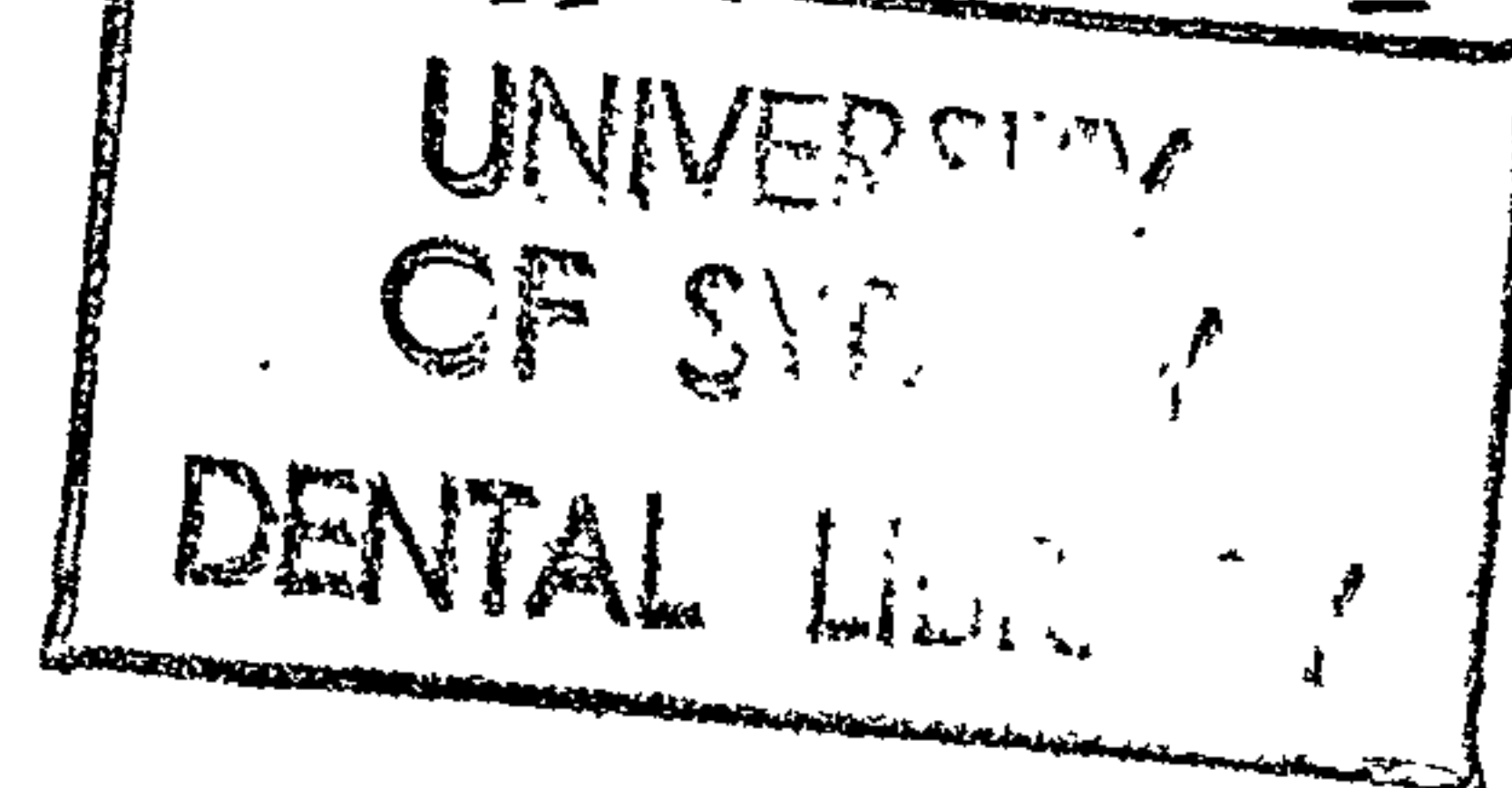
All calcium analyses were made in duplicates. For the second series of analyses, the order of samples was reversed.

The estimate of precision was based on the differences, d, between values obtained for the individual members of duplicate pairs, in ppm. The standard deviation, s, of a single determination was calculated from n = 299 duplicate values using the formula

$$s = \sqrt{\frac{\sum d_i^2}{2n}} \quad \text{and was found to be } \pm 0.067 \text{ ppm.}$$

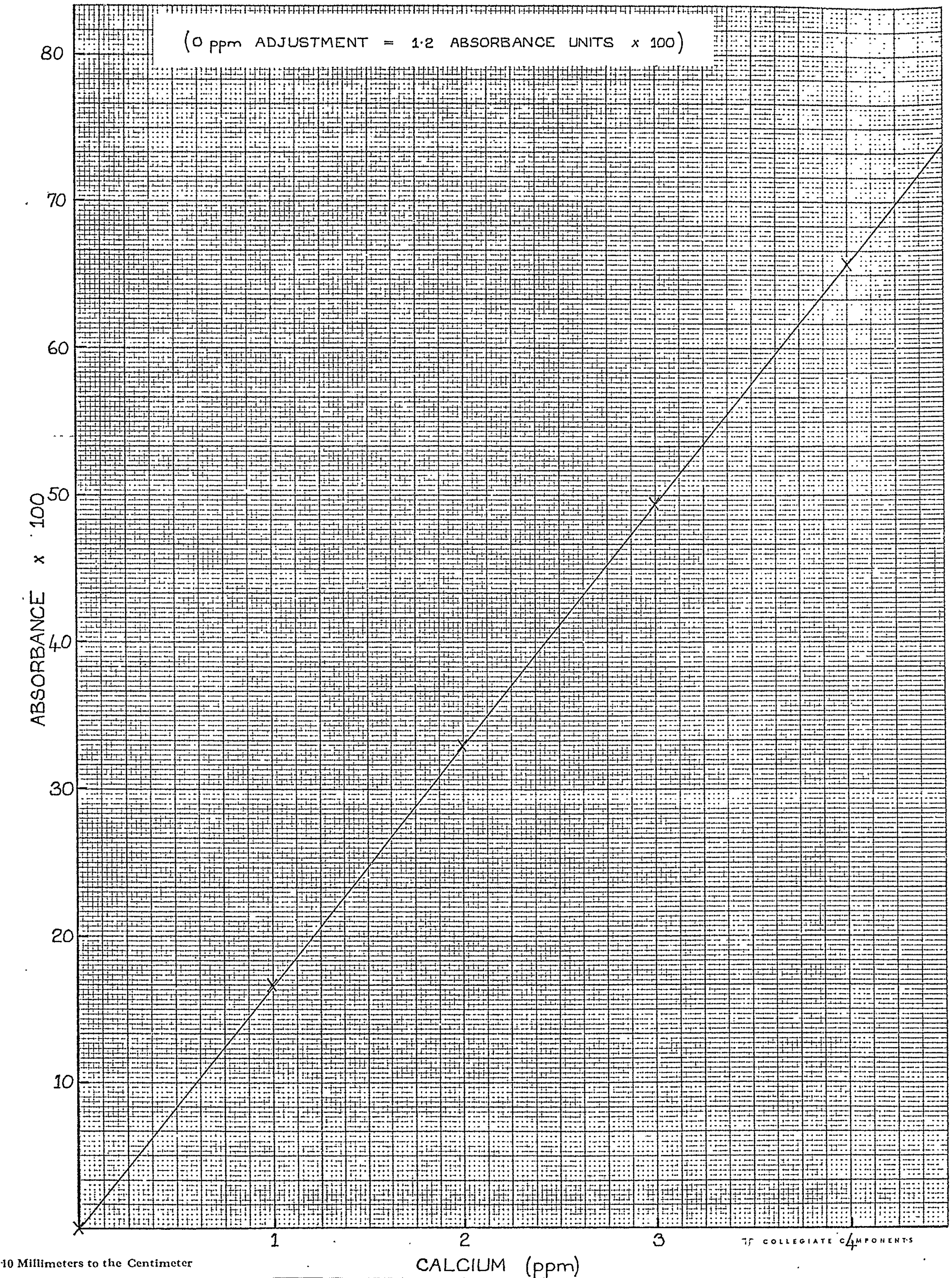
Recovery of known amounts of calcium added to the samples.

The test material was prepared by adding 0.5 ml of 2 ppm



TYPICAL CALIBRATION GRAPH FOR CALCIUM DETERMINATION IN ENAMEL

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standard to 1.2 ml sample solution made up by pooling 0.2 - 0.4 ml aliquots from 3 - 6 samples. The estimations were carried out on five separate occasions, in duplicates. The mean recovery was 101 per cent, S.D. = ± 1.76 per cent, $n = 10$.

Calculation of results.

Following deduction of the blank value, the calcium content of the biopsy specimen (Variable 220), was calculated from the ppm value determined, Ca (ppm), the dilution factor, D, and the basic sample volume in ml, B.

$$\text{Ca (mg)} = \frac{\text{Ca (ppm)} \times D \times B}{10^3}$$

Calculation of the enamel content of the specimen was based on the amount of calcium found, assuming the calcium content of enamel to be 37.0 per cent.

The biopsy depth was estimated using the formula given in 3.2.3.12.

3.3.2.1.3. Magnesium determinations.

The determinations were carried out by atomic absorption spectroscopy using the same instrumentation and a similar working routine as was described for calcium estimations. Two millilitre aliquots of 1 in 101 dilutions of the basic samples in 1 per cent lanthanum background solution were used.

Instrument parameters:

Wavelength: 285.21 nm

Lamp current: 3 mA

Spectral band width: 0.165 nm, Slit height: 10 mm.

Photomultiplier gain: Low gain 2

Scale expansion: 2x Damping: B

Flame: air-acetylene

Gas flow rates. Air: 12.5 l/minute, 14.7 p.s.i.a., 70°F

Acetylene: 1.0 l/minute, 14.7 p.s.i.a., 70°F

Burner: 10.2 cm, 1 slot

Standards.

To prepare the 1000 ppm magnesium stock solution, 0.500 g of magnesium turnings (Laboratory reagent grade, B.D.H. Pty. Ltd., Hythe, Kent, U.K.), which were cleaned by pickling in dilute nitric acid and washed and dried before weighing, were dissolved in 3.95 M nitric acid (Aristar) and made up to 500 ml with distilled, deionized water.

Working standards containing 0.1, 0.15, 0.2 and 0.3 ppm magnesium were prepared by the dilution of 10, 15, 20 and 30 μ l respectively of the 1000 ppm stock solution to 100 ml in 1 per cent lanthanum background solution. The same background solution, dispensed in an identical manner, was used as the 0 ppm magnesium standard.

The standards, estimated before, during and after sample analyses, gave the following typical mean absorbance x 100 values:

0 ppm	0.1 ppm	0.15 ppm	0.2 ppm	0.3 ppm
5.1	25.1	35.2	45.8	65.5

A typical calibration graph is shown in Figure 12, page 123. Ninety per cent of the samples fell within the range of 20-38 units of absorbance x 100.

Blank values.

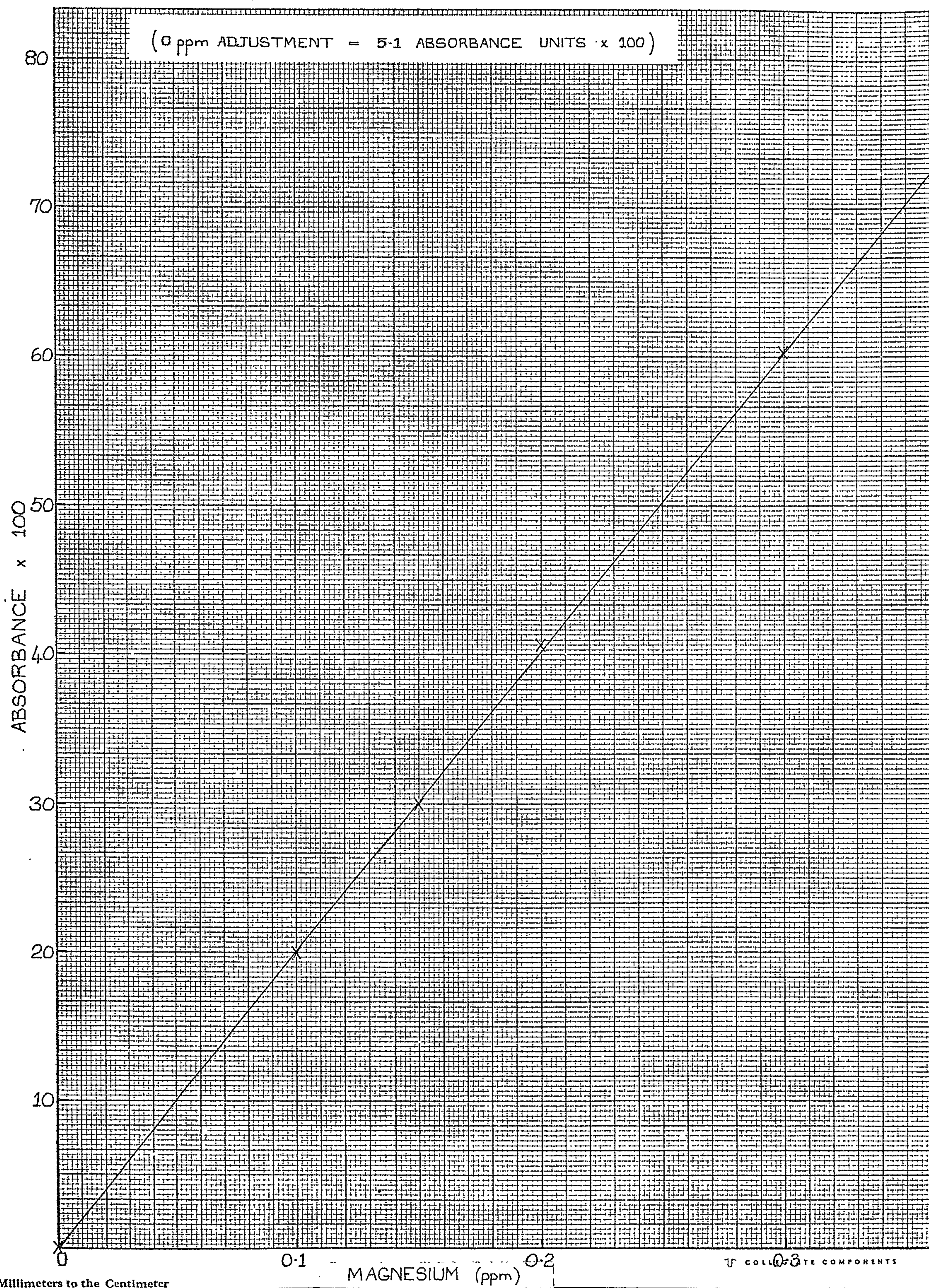
Because of relatively high blank values, particular care was taken in evaluating variation within the 13 blanks prepared in the field. Because of slight day to day fluctuations in sensitivity, the absorbance readings of blanks used in different series were adjusted to a standard level of sensitivity where the absorbance of the 0.1 ppm standard, uncorrected by the 0 ppm value, equalled 25.0 units of absorbance x 100, before taking the average value. Individual adjustments were made to the 14 samples which contained less than four biopsies originally, to compensate for a lesser contribution of the biopsy procedure to the total blank value. For example, the mean blank value for the series in which sample 80 was estimated was 0.045 ppm, made up by the contributions of the lanthanum base diluent (0 ppm) and the biopsy materials, 0.025 and 0.020 ppm respectively. As sample 80 had only three biopsies pooled, the contribution of the latter was reduced by 25 per cent to 0.015 ppm giving a corrected blank value of 0.040 ppm.

The mean magnesium content of the 13 blanks was 0.048 ppm, S.D. = ± 0.004 ppm.

Replicate analyses.

The analyses were made in duplicates estimated on different days. For the second series of determinations, the order of samples was reversed. The estimate of precision was based on the

TYPICAL CALIBRATION GRAPH FOR MAGNESIUM DETERMINATION IN ENAMEL



differences between 299 pairs of duplicate values using the method described under calcium determinations. The standard deviation of a single determination was found to be ± 0.0033 ppm.

Recovery of known amounts of magnesium added to the samples.

Additions of 0.5 ml of the 0.2 ppm standard were made to 10 composite samples of 1.2 ml each, made up by pooling 400 μ l of each of three individual samples.

Recovery estimations were made on five different occasions. The mean recovery was 102 per cent of the expected value, S.D. = ± 1.94 per cent, $n = 10$.

Calculation of results.

The magnesium content of the biopsy specimen was calculated in the same way as the calcium content, sub-section 3.3.2.1.2.

The magnesium content of surface enamel, Variable 230, was calculated from the respective weights.

3.3.2.1.4. Strontium determinations.

The analyses were carried out by flameless atomic absorption spectroscopy. The output was traced on a high-speed strip chart recorder (Model 63 atomizer, Model A25 recorder, Varian).

Repeating micro-pipettes and/or glass micro-syringes (Eppendorf and S.G.E.) fitted with custom-made polyethylene tips were used to introduce the samples into the atomizer tube. The whole sample volume was always held in the polyethylene pipette-tips.

The determinations were made over a continuous period. The samples processed each day were selected to represent a cross-section of the human sample. Before opening the samples, the seal of each vial was inspected. Samples which showed high strontium levels were tested for correct volume by drawing-up the sample solution into graduated polyethylene tubing attached to a micro-pipette.

Sample parameters;

Dilution of basic sample: 1 in 21

Volume used per estimation: 5 μ l

Number of replicate estimations: 2 - 4

Instrument parameters:

Wavelength: 460.7 nm

Spectral band width: 0.165 nm Slit height: 4 mm

Lamp current: 6.7 mA

Photomultiplier setting: High gain 3.6

Scale expansion: 1x

Atomization conditions:

Stage	Voltage Setting	Actual Voltage (V)	Time (Seconds)	Temperature* (°C)
Dry	3	0.5	24	116
Ash	7	2.9	5	480
Step-atomize	9.0	8.3	3	2,520

* Average highest temperature reached at the end of the stage at the area where the samples were deposited (for all elements).

This is not equivalent to the temperature at which the analyte element is atomized.

Standards.

As the strontium absorption signal was not depressed in the presence of the matrix, aqueous standards were used.

The 1000 ppm strontium primary stock solution was prepared by dissolving 1.208 g of oven-dried strontium nitrate (Univar grade, Ajax Chemicals, Sydney, N.S.W.) in 500 ml of 0.158 M nitric acid (Aristar). An intermediate stock solution of 10 ppm strontium was prepared by diluting 1.00 ml of the primary stock to 100 ml with 0.158 M nitric acid. Working standards containing 0.03, 0.06 and 0.09 ppm strontium were prepared by diluting 30, 60 and 90 μ l of the intermediate stock solution to 10 ml with 0.158 M nitric acid. Ten millilitres of the same nitric acid was dispensed in an identical manner for use as the 0 ppm standard.

The sensitivity of the method is illustrated by the relationship between the weight of analyte in 5 μ l of the standards used and the mean absorbance x 100 values recorded under optimum conditions:

Standard (ppm)	0	0.03	0.06	0.09
Sr ($\text{g} \times 10^{-10}$)	0.1	1.6	3.1	4.6
Mean absorbance x 100	2	33	64	95

Repetitive firings caused rather rapid deterioration of the graphite tube furnace, manifested by a somewhat irregular decrease in sensitivity. As the rate of deterioration varied from tube to tube, standards were interposed between groups of three sample estimations. After 30 - 40 firings, the sensitivity decreased by about 50 per cent and although the standards remained

linear, precision became poor and a new atomizer-electrode assembly was fitted. Typical calibration graphs representing the performances of new and old assemblies are shown in Figure 13, page 128. Ninety per cent of the samples fell within the range of 0.033 - 0.080 ppm in the dilution estimated.

Tests for non-atomic absorption.

The absence of non-atomic absorption was tested using a lithium lamp which emits a non-absorbing resonance line at the wavelength of 460.3 nm. When the lamp current was set at 17 mA, the intensity of the signal equalled the intensity of the strontium lamp signal and no change in photomultiplier gain setting or slit opening was required. A sample of milk was used to confirm that non-atomic absorption would be detected under these conditions. Atomization of the milk sample with strontium settings, produced peaks in the 'dry', 'ash' and 'atomize' stages, the first two being non-atomic peaks, due to light scattering and molecular absorption caused by the evaporation of water and ashing of organic matter, while the third one was an atomic absorption peak due to the strontium content of the milk. When the same sample was atomized using lithium settings, the 'dry' and 'ash' stage (non-atomic) peaks were reproduced but there was no absorption in the 'atomize' stage.

No non-atomic absorption was detected when seven randomly selected enamel samples were tested under these conditions.

Replicate analyses.

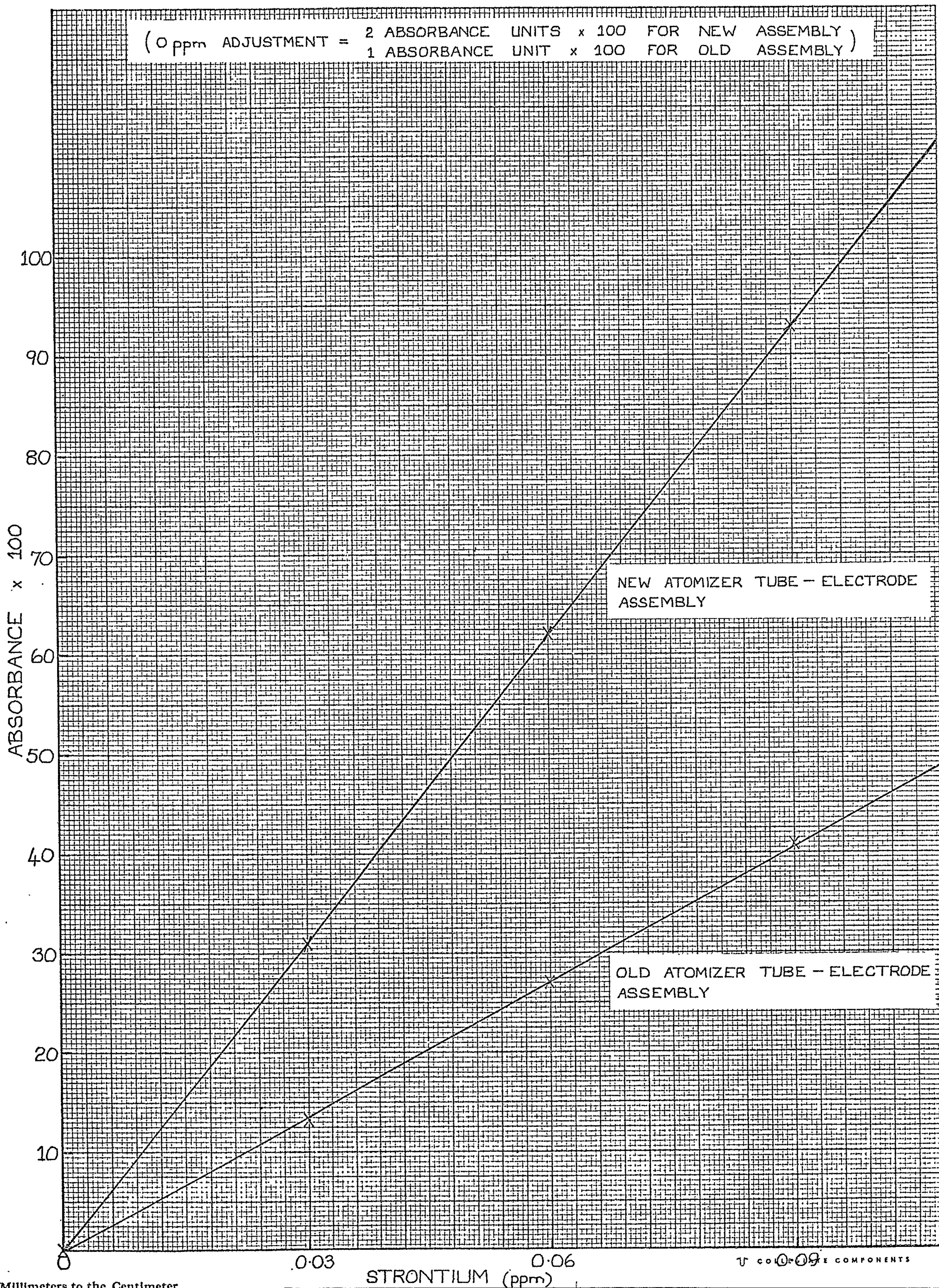
At least two determinations were made for each sample.

FIGURE 13:

TYPICAL CALIBRATION GRAPH FOR STRONTIUM DETERMINATION IN ENAMEL

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(0 ppm ADJUSTMENT = 2 ABSORBANCE UNITS x 100 FOR NEW ASSEMBLY)
 1 ABSORBANCE UNIT x 100 FOR OLD ASSEMBLY)



Having completed the first estimation for a series, usually consisting of 20 samples, duplicate estimations were made of the same series using a new atomizer-electrode assembly. For the second estimation, the order of samples within the series was reversed to minimize variation arising from possible fluctuation in the performance of the atomizer tube with increasing usage. If duplicates differed by more than 10 per cent of the smaller value, a third estimation was made (18.7 per cent of samples) and if the third value fell outside the first two values, a fourth estimation was carried out (4.0 per cent of samples). Results were based on the mean values calculated after rejecting obviously outlying figures. A result was rejected if the value fell outside ± 2 S.D. of the mean of all estimations made for that sample. This was the case for 1.8 per cent of values.

Recovery of known amounts of strontium added to the samples.

Standard recoveries were tested in two ways. The first technique consisted of adding to a sample a small volume of concentrated standard of known concentration and atomizing aliquots of the mixture. The second method involved pre-drying 5 ul of a standard in the atomizer tube, followed by adding a sample of known concentration and atomizing the standard and the sample together. Care was taken to make the amount added similar to the amount of analyte contained in the sample. Although the second method was more convenient, it was somewhat less reliable than the first one. Using the first addition technique, the mean percentage recovery was 99.4, S.D. = ± 3.84 , $n = 10$. When the second method was used the mean recovery was 98.5 per cent, S.D. = ± 4.80 per cent, $n = 10$.

Precision of the method.

Two types of estimates of precision were made: the first test, a) was based on the standard deviation of the mean value obtained on repeated determinations of the strontium content of the same sample. The second test, b) was based on all replicate determinations made for each sample. A test comparable to a) is to use the S value calculated from the 'within-sample mean square', S^2 , in an analysis of variance for data classified in one way.

a) Because of the gradual deterioration of tube performance with use and corresponding decrease in sensitivity, the relative standard deviation of 12 estimations of the same sample (#282) was calculated on the basis of ppm values obtained against standards interposed between each of the 12 determinations. The mean concentration of strontium was 0.0347 ppm, S.D. = ± 0.0015 ppm.

b) The S value obtained was 0.0024 ppm.

Comparison of the two results is of interest. The S.D. under a) is a measure of precision when a limited number of determinations were made within a short period of time, in the full cognizance that they constituted a test. The S value represents the precision level of some 700 determinants made over several weeks. Clearly, precision 'attainable' under optimum conditions a), is not necessarily a true reflection of precision attained in the general daily routine b), however much care is exercised.

Blank values.

Corresponding dilutions of the 13 blanks made up in the field were estimated with each batch of the samples. The values

were generally low and uniform with a mean value of .003 ppm, S.D. = $\pm$.00022 ppm, n = 13.

Decontamination - memory effect.

A constant peak of 1.0 per cent absorption decreasing to 0.5 per cent absorption with continued use of the tube, corresponding to approximately 0.001 ppm strontium, was observed when the tubes were fired between samples. The peak did not disappear after decontamination firings at higher voltage-time settings than required to volatilize strontium, did not increase in size after standards containing up to 1.0 ng strontium were atomized and did not appear when the lithium lamp at 430.3 nm was used. Thus, it was considered to be atomic absorption, possibly caused by the presence of strontium in the tube itself, rather than by a memory effect. Appropriate adjustments were made to the sample values.

Calculation of results.

The strontium content of surface enamel (Variable 234) was calculated along the lines described under calcium and magnesium, sub-sections 3.3.2.1.2. and 3.3.2.1.3.

3.3.2.1.5. Potassium determinations.

The analyses were carried out by flameless atomic absorption spectroscopy, using the instrumentation and general working routine described under strontium determinations.

Sample parameters;

Dilution of basic sample: 1 in 101

Volume used per estimation: 2.5 μ l

Number of replicate estimations: 2 - 3

Instrument parameters:

Wavelength: 769.9 nm

Spectral band width: 0.66 nm Slit height: 3.5 mm

Lamp current: 10.0 mA

Photomultiplier setting: High gain 4.15

Scale expansion: 1x

Atomization conditions:

Stage	Voltage Setting	Actual Voltage (V)	Time (Seconds)	Temperature (°C)
Dry	3	0.5	20	112
Ash	5	2.2	5	298
Step-atomize	7	6.9	3	2,125

Standards.

As the absorption signal for potassium was not depressed by the presence of the matrix, aqueous standards were used.

The 1000 ppm potassium primary stock solution was prepared by dissolving 1.2928 g of oven-dried potassium nitrate (Pro analysi, E. Merck G.m.b.H., Darmstadt, Germany) in 500 ml of 0.158 M nitric acid (Aristar). An intermediate stock solution of 10 ppm potassium was prepared by diluting 1.0 ml of the primary stock solution to 100 ml with 0.158 M nitric acid. Working standards containing 0.025, 0.050, 0.075 and 0.100 ppm potassium were prepared by diluting 25, 50, 75 and 100 µl of the intermediate stock solution to 10 ml with 0.158 M nitric acid. Ten ml of the same nitric acid was dispensed in an identical manner for use as the 0 ppm standard.

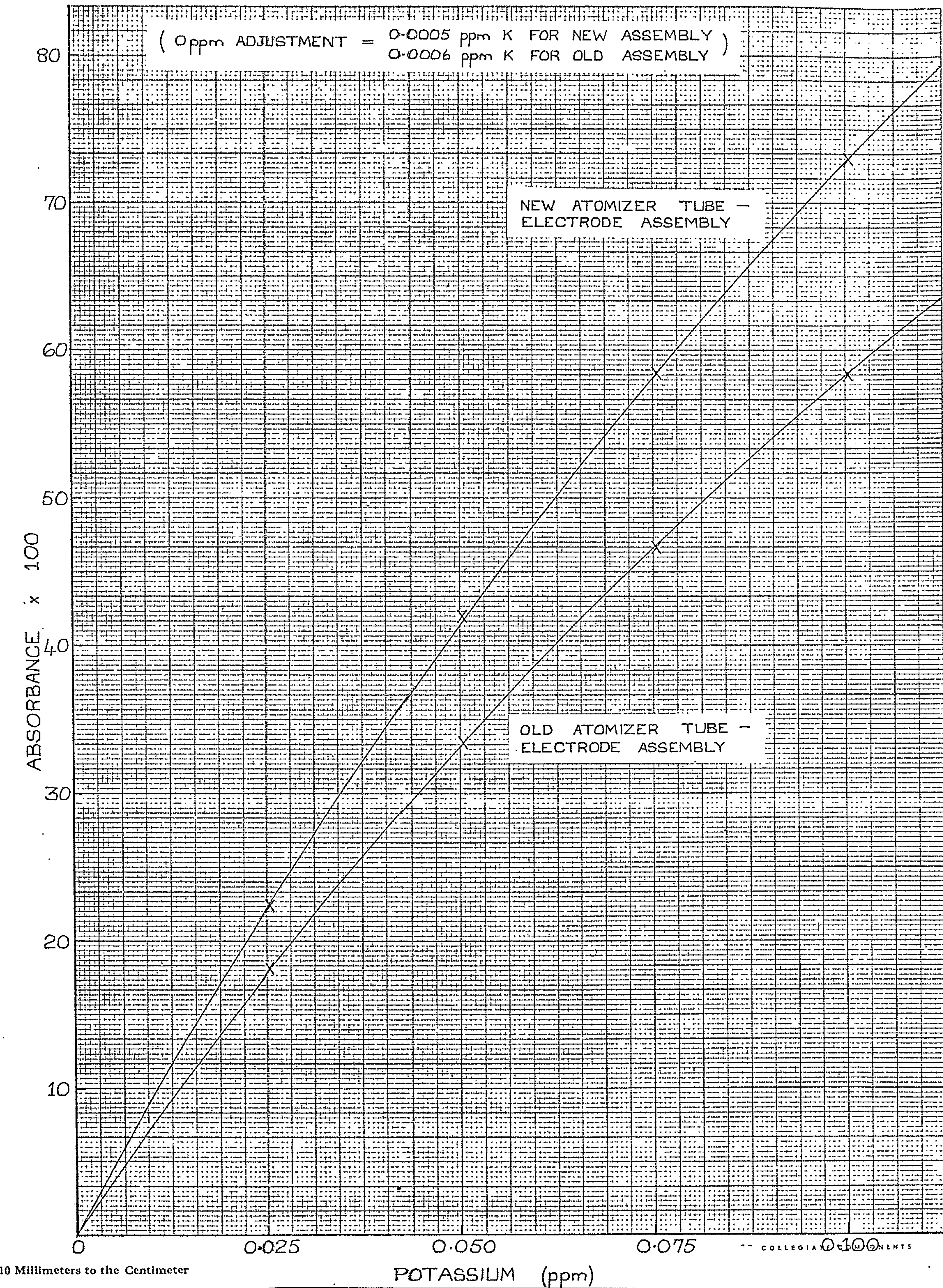
The relationship between the weight of analyte in 2.5 μ l of the standards and the absorbance x 100 values obtained under optimum conditions is shown below:

Standard	0	0.025	0.050	0.075	0.100
K ($\text{g} \times 10^{-10}$)	0.012	0.637	1.262	1.887	2.512
Mean absorbance x 100	0.5	22.5	42.0	58.5	73.0

With repeated firings, the atomizer tube performance showed a very slow and regular deterioration in performance manifested by a decline in sensitivity. On the average, however, the total decrease in sensitivity was no more than about 20 per cent over 100 - 150 firings, after which the atomizer tube - electrode assembly was replaced. Under these conditions, the precision and repeatability invariably remained good. Standards were interposed between each 10 - 15 samples. Typical calibration graphs representative of the performance of a new atomizer tube - electrode assembly and of the same assembly before discarding it are shown in Figure 14, page 134. Ninety-one per cent of the samples fell within the range of 0.035 - 0.069 ppm in the dilution estimated.

Tests for non-atomic absorption.

The appropriate tests were carried out following the considerations described under strontium. The nearby non-absorbing resonance line was provided by a magnesium lamp at 766.5 nm. At a lamp current setting of 3.0 mA, the intensity of signal was the same as that emitted by the potassium lamp and no change in photomultiplier gain or in slit opening was required. The peaks obtained on ashing and atomizing small pieces of paper showed



that, under these conditions, non-atomic absorption would be detected. However, no non-atomic component was detected in the atomize peak on random testing of eight enamel samples.

Replicate analyses.

For the great majority of samples (89.6 per cent), the determinations were carried out in duplicates, with the second determination immediately following the first one. Consideration was given to carrying out the second estimation for each sample at a different time and in a sequence opposite to that of the first estimations, as discussed under strontium. However, in view of the steady performance of the atomizer tube, the high degree of uniformity of estimations exhibited from day to day and the risk of contamination, which increased with the amount of handling of the samples, the procedure adopted was thought to suit potassium estimations better. A third estimation was made for the occasional sample as an additional test of reproducibility and for all samples where the first two estimations differed by more than 10 per cent of the smaller of the two values. The calculation of the results was based on the mean value of replicate estimations.

Recovery of known amounts of potassium added to the samples.

Standard recoveries were tested by depositing and pre-drying 2.5 μ l of a standard in the atomizer tube followed by adding 2.5 μ l of a sample of known concentration and atomizing the standard and the sample together. A standard with comparable potassium content to that of the sample was chosen for recovery tests.

The mean percentage recovery for 12 tests was 100.6, S.D. = ± 1.45 per cent.

Precision of the method.

The tests described under strontium determinations were used:

a) The standard deviation of 10 consecutive determinations of the same sample with a mean concentration of 0.0556 ppm was ± 0.00085 ppm. This level of precision, attained using 1.39×10^{-10} g of analyte per estimation, matches the level of precision expected with conventional atomic absorption spectroscopy for which about 2.0×10^{-6} - 6.0×10^{-6} g analyte would be used per determination under comparable conditions. (The latter calculation was based on aspirating 1.0 ml solution containing 2.0 - 6.0 ppm potassium, using air-propane flame and 769.9 nm wavelength setting.)

b) The S value was 0.00465, indicating a similar relationship between the two estimations to that noted under strontium.

Blank values.

Appropriate dilutions of each of the 13 blanks made up in the field, were estimated with the samples. The blank results were uniform with a mean value of 0.015 ppm, S.D. = ± 0.0007 ppm, $n = 13$.

Decontamination - memory effect.

Initial contamination was readily removed by two firings of the tube using the atomize settings previously given. Possible retention of some of the analyte was tested by repeating the atomize step after atomization of standards and samples with high potassium content. No memory effect was detected, indicating complete volatilization of potassium at the settings employed.

Calculation of results.

The potassium content of surface enamel, (Variable 231), was calculated along the lines previously described. (3.3.2.1.2.)

3.3.2.1.6. Barium determinations.

The analyses were carried out by flameless atomic absorption spectroscopy using the instrumentation described under strontium estimations. The determinations were made over a continuous period, but because of the experimental difficulties outlined below, only 20 - 25 determinations could be carried out each day. The daily arrangement of samples added up to progressive cross-sections of the whole sample.

The experimental difficulties associated with barium determinations fell into the following categories:

1. The new atomizer tubes supplied by the manufacturers were contaminated with barium. As the contamination was internal, apparently associated with the spectrographic graphite from which the tubes were machined, it could not be eliminated completely by the usual decontamination procedure. In some batches, purchased initially, the contamination level was relatively low and it could be reduced to an acceptable level, ranging from 2 - 10 per cent absorption, usually by 4 - 6 firings at very high voltage and time settings (10 V for 5 sec.). In batches manufactured and purchased at a later date, the level of contamination was so high that decontamination was not possible and they

were not usable for barium estimations. When the difficulty became apparent, all batches of atomizer tubes were tested and 90 or so, which could be decontaminated, were reserved for barium estimations. In spite of this practice, the number of replicate analyses was severely limited. The situation is being investigated by the manufacturers, but at this stage there is no evidence that remedy is near.

2. The performance of atomizer tubes and electrodes showed rapid and rather irregular decline with use, without any period of stability. The rate of decline varied from tube to tube.

3. Of the order of 1 : 100,000, the analyte : matrix ratio was the lowest of all elements estimated, giving rise to rapid accumulation of residual matrix components.

4. The high temperature - time settings required to atomize barium, and presumably the high quantity of residual matrix components, limited the useful life of the tubes and electrodes to 15 - 30 firings. Again, the length of useful life varied from tube to tube.

5. The barium signal was depressed by about 20 per cent in the presence of the matrix.

Sample parameters:

Dilution of basic sample: 1 in 2

Volume used per estimation: 5 μ l

Number of replicate estimations: 1 - 4

Instrument parameters:

Wavelength: 553.55 nm

Spectral band width: 0.165 nm Slit height: 2.0 mm

Lamp current: 10.0 mA

Photomultiplier setting: High gain 6.3

Scale expansion: 3x

Atomization conditions:

Stage	Voltage Setting	Actual Voltage (V)	Time (Seconds)	Temperature (°C)
Dry	3.0-2.25	0.5	24-60	116-105
Ash	6.25	2.7	15	585
Step-atomize	9.75	8.85	3.5	2,780

Preliminary work indicated that for barium determinations some degree of scale expansion would be necessary, and that the most acceptable sensitivity : background-noise ratio was obtained at approximately 3x scale expansion. During the initial period of analysing the New Guinea material, it became apparent that, to bring the majority of samples into a desirable working range at a 3x scale expansion setting, a 1 in 2 dilution of the samples or a reduction of sample volume from 5 to 2.5 μ l was desirable. Because of the higher precision level attained in delivering 5 μ l volumes with the pipette at our disposal at that time, and the relatively low contamination risk with regard to barium, the former alternative was chosen. The samples were diluted individually before the estimation, by adding an equal volume of 0.158 M nitric acid to 5 μ l of sample and mixing by drawing up into polyethylene tubing attached to a repeating pipette (Eppendorf).

The drying settings were varied because, after some use of the atomizer tube and increasing accumulation of retained matrix components, the sample tended to "creep" up the wall of the tube and if it reached the hottest area opposite the electrode terminals, sputtering, with consequent irregular loss of sample took place. This phenomenon, considered to have been caused by capillarity or by the hygroscopic nature of the retained matrix components, was countered by gradual reductions of voltage and increase of time during the drying phase.

Standards.

Aqueous standards were needed for developmental work, for the establishment of the barium content of an internal enamel standard and for standard recovery tests.

The 1000 ppm barium primary stock solution was prepared by dissolving 0.7185 g of oven-dried barium carbonate (Analytical reagent grade, B.D.H.) by the slow addition of 50 ml of approximately 1 M nitric acid (Aristar). The solution was made up to 500 ml with glass-distilled, deionized water. An intermediate stock solution of 10 ppm barium was made up by diluting 1.0 ml of the primary stock solution to 100 ml with 0.158 M nitric acid. Working standards containing 0.05, 0.10, 0.15 and 0.20 ppm barium were prepared by diluting 50, 100, 150 and 200 μ l of the intermediate stock solution to 10 ml with 0.158 M nitric acid. Ten millilitres of the same nitric acid were dispensed in an identical manner for use as the 0 ppm standard.

Because of interference with the barium signal by the matrix and variability of atomizer tube performance, an internal

standard of enamel was used for the actual estimations. The bulk standard was prepared by pooling and diluting enamel obtained by 40 biopsies made on upper canine teeth extracted in Sydney, using the same procedures as for the New Guinea samples. The calcium content of the pooled standard was determined by flame atomic absorption spectroscopy before completing the dilution procedure. The final dilution was made to approximate the calcium concentration of the standard to the mean concentration of calcium in the New Guinea enamel samples (3940 ppm).

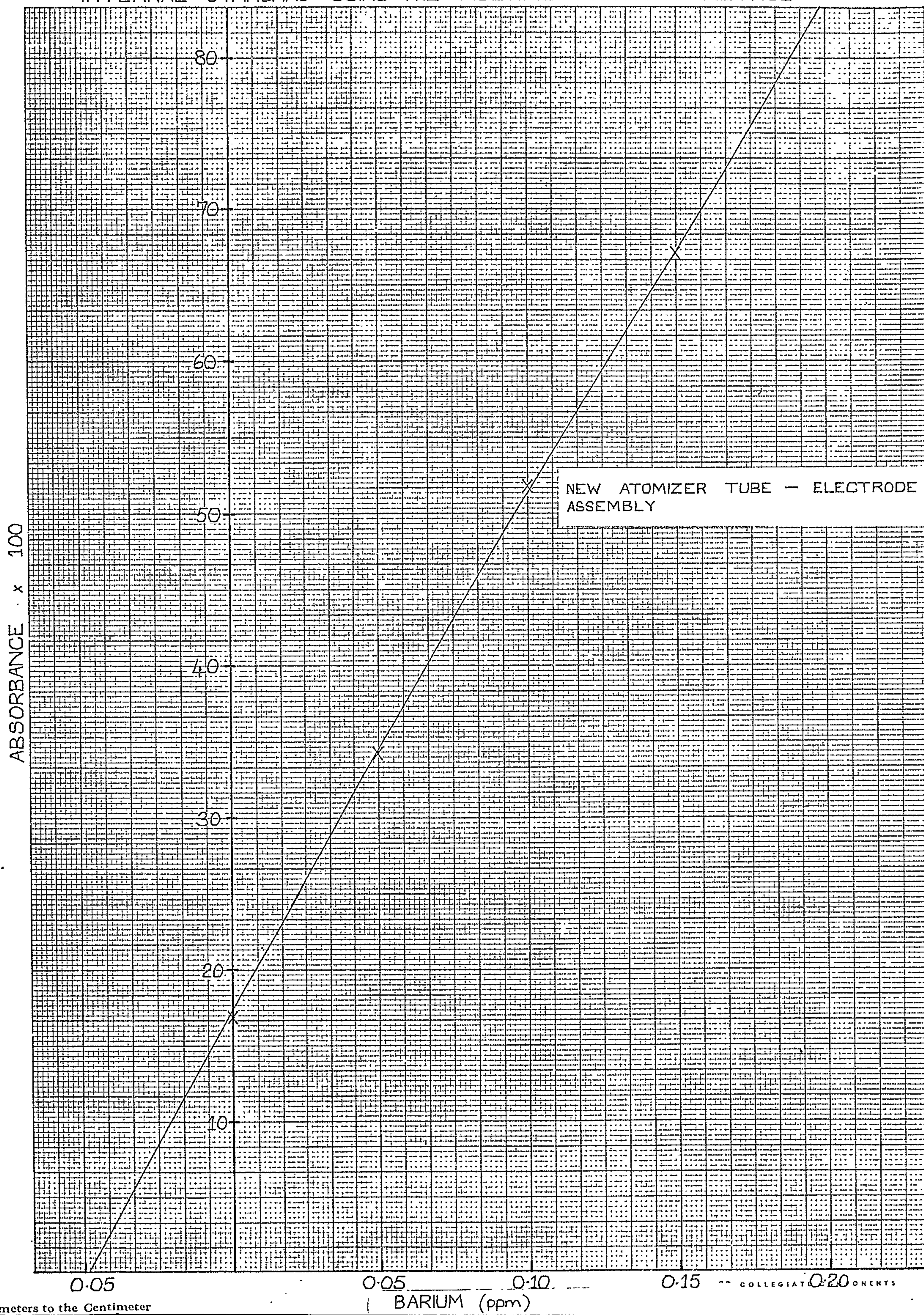
The barium concentration of the enamel standard was determined by the technique of multiple additions of known amounts of barium to the standard.

To eliminate the effect of deterioration of atomizer tube performance with use, in practice 6 - 8 additions of 0 ppm, 0.05 ppm, 0.10 ppm and 0.15 ppm barium were made to the enamel standard, using a new atomizer tube - electrode assembly for each set of additions. The combined peak height was expressed as a proportion of the peak height obtained on atomizing aqueous standards interposed between firings. The mean results are shown in Figure 15, page 142, indicating that the barium content of the enamel internal standard was 0.048 ppm.

The relationship between the weight of analyte in 5 μ l of the standards and the mean absorbance x 100 values when a new atomizer tube - electrode assembly and 3x scale expansion were used, is shown below:

ESTABLISHMENT OF THE BARIUM CONTENT OF THE ENAMEL
INTERNAL STANDARD USING THE MULTIPLE ADDITIONS METHOD

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	A	E	A	A	A	A
Standard (ppm)	0	0.048	0.050	0.100	0.150	0.200
Ba (g x 10 ⁻¹⁰)	0	2.4	2.5	5.0	7.5	10.0
Mean absorbance x 100	0	16.8	22.0	44.0	64.0	83.0

A = aqueous standard

E = enamel internal standard

For the estimations, enamel internal standards were interposed between each two sample firings, so that each sample could be related directly to an adjacent standard. The assembly was changed when the sensitivity decreased by 60 - 65 per cent.

Approximately 14 per cent of the samples had concentrations of barium higher than 0.160 ppm and these samples had to be estimated using an extension of the calibration graph.

Tests for non-atomic absorption.

The tests were made along the lines described under strontium and potassium, using a magnesium lamp at 552.8 nm and at 555.5 nm at 20 mA lamp current without change in photomultiplier gain settings or in slit opening. Paper pulp impregnated with the 0.1 ppm magnesium standard solution was used to confirm the validity of the test. No non-atomic absorption was detected on frequent tests performed on the enamel samples.

Replicate analyses.

The level of precision attained in barium estimations was the lowest amongst all elements analysed and therefore it would have been desirable to carry out the highest number of replicate

determinations. However, because of the contamination of the atomizer tubes discussed previously, even duplicate determinations could not be made for a number of samples. The greatest need for replication was decided on the basis of multiple daily analytical variations, some of which were inexplicable and therefore impossible to include in a system based on rigid statistical rules. The samples were distributed between the number of analyses carried out per sample as follows:

Number of analyses	1	2	3	4
Number of samples	104	162	21	5

Recovery of known amounts of barium added to the samples.

Standard recovery tests carried out during development work indicated that the barium signal was depressed by approximately 15 - 25 per cent in the presence of the matrix. The amount of depression was of the same order within the range of enamel contained in the New Guinea samples and appeared to be independent of the concentration of barium within the expected range. Results of standard additions made to New Guinea samples selected to represent low and high levels of matrix concentration, indicated a similar reduction of recovery. Once again the number of atomizer tubes suitable for barium estimations limited the number of recovery tests that could be carried out.

The mean recovery of seven standard additions made apart from those carried out to establish the concentration of barium in the enamel internal standard was 80.7 per cent, S.D. = $\pm$ 9.06 per cent.

Precision of the method.

Because of the shortage of suitable atomizer tubes only test b) was carried out.

The high co-efficient of variation of 51.2 per cent, calculated from the S value of 0.0425 and the mean of 0.083 ppm, indicate an unusually low level of precision. For this reason, data relating to the barium content of enamel are regarded as semi-quantitative.

Blank values.

The mean of 12 blank estimations was 0.008 ppm, S.D. = $\pm$ 0.0026 ppm.

Decontamination - memory effect.

As stated earlier, barium contamination could not be completely eliminated from the atomizer tubes even at very high voltage-time settings (10 V for 5 sec.), nor did prolonged acid treatment have the desired effect. Higher temperatures could have been attained during decontamination firings by increasing the time settings in the step-atomize mode up to 10 seconds, or by using a high voltage setting at a slow ramp rate. However, when these procedures were tested, virtually no useful tube life remained after the test firings. Therefore, it was decided to begin sample estimations once the peaks obtained on successive decontamination firings at 10 V - 5 sec. settings had decreased to a constant level, provided that the peak height was not higher than 15 absorbance x 100 units. In most tubes that were usable

at all, a decontamination level corresponding to 2 - 6 absorbance x 100 units was achieved after 4 - 5 firings. The height of decontamination peaks was monitored throughout the estimations by following each sample and standard estimation with a decontamination (without sample) firing. Although the decontamination peak heights for a given atomizer tube varied by approximately $\pm 10 - 12$ per cent of the mean, lack of association between the peak height of samples or standards and the immediately following decontamination peak height indicated that no true memory effect was present.

The mean decontamination peak height calculated for each atomizer tube was deducted from each sample and standard peak obtained using that tube. Following this, each sample peak height was expressed as the percentage of the peak height of the adjoining enamel internal standard and the barium content of the sample was calculated from this figure.

Photomultiplier fatigue.

This phenomenon, manifested by a very fast peak at the end of the atomize period was noted during barium estimations, no doubt as a consequence of light emitted by the furnace, due to the high voltage-time settings, when using a wavelength in the visible range.

The peak appeared after the recorder, tracing the absorption due to barium, had returned to the baseline, and could be clearly distinguished from the barium peak.

Calculation of results.

Because of the relatively low precision of the method, barium determinations in surface enamel, (Variable 233), are regarded as semi-quantitative. The results were calculated as for elements previously reported.

3.3.2.1.7. Zinc determinations.

The analyses were carried out by flameless atomic absorption spectroscopy, using the instrumentation and general working routine described under strontium estimations.

Sample parameters:

Dilution of basic sample: 1 in 1111

Volume used per estimation: 3.0 μ l

Number of replicate estimations: 2 - 4

Instrument parameters:

Wavelength: 213.86 nm

Spectral band width: 0.33 nm Slit height: 4.0 mm

Lamp current: 5.0 mA

Photomultiplier setting: High gain 4.3

Scale expansion: 1x

Atomization conditions:

Stage	Voltage Setting	Actual Voltage (V)	Time (Seconds)	Temperature ($^{\circ}$ C)
Dry	3.5	0.6	18	126
Ash	4.5	1.85	5	270
Ramp-atomize	8.2	7.8 (cut-off point)	6.0 (volts/sec.)	1,950

To meet the combined requirements imposed by the zinc levels found in the New Guinea samples, the sensitivity of the system at the only suitable resonance line, (213.86 nm) and the use of a practical sample volume, 5.0 μ l of the aliquot prepared for zinc determinations was diluted to 55 μ l with 0.158 M nitric acid (Aristar), giving a final dilution of the basic sample of 1 in 1111, before commencing the main body of determinations. For zinc determinations, the ramp-atomization mode gave results of higher precision than the step-atomization mode. The cutoff voltage and ramp rate settings used, resulted in a considerably higher final temperature than was needed to atomize zinc. It is probable that, the higher precision was the result of eliminating matrix components, which accumulated and interfered with the atomization of zinc at lower temperature settings.

Standards.

As the zinc absorption signal was not depressed by the matrix, aqueous standards were used.

The 1000 ppm zinc basic stock solution was prepared by dissolving 0.500 g of zinc powder (Analar grade, B.D.H.) by slowly adding 100 ml of 5.3 M nitric acid (Aristar) and making up the solution to 500 ml with double-deionized, glass-distilled water. An intermediate stock solution of 10 ppm zinc was prepared by diluting 1.0 ml of the basic stock to 100 ml with 0.158 M nitric acid (Aristar) in double-deionized, glass-distilled water. Working standards containing 0.005, 0.01 and 0.02 ppm zinc were prepared by diluting 5, 10 and 20 μ l of the intermediate stock solution to 10 ml with 0.158 M nitric acid as above. Ten millilitres of the

same nitric acid, dispensed in an identical manner, were used for the 0 ppm zinc standard.

The relationship between the weight of the analyte in the standards and the absorbance x 100 values recorded under optimum conditions is shown below:

Standard (ppm)	0	0.005	0.010	0.015	0.020
Zn ($\text{g} \times 10^{-11}$)	0.07	1.57	3.07	4.57	6.07
Mean absorbance x 100	1.5	25.4	44.0	58.0	67.8

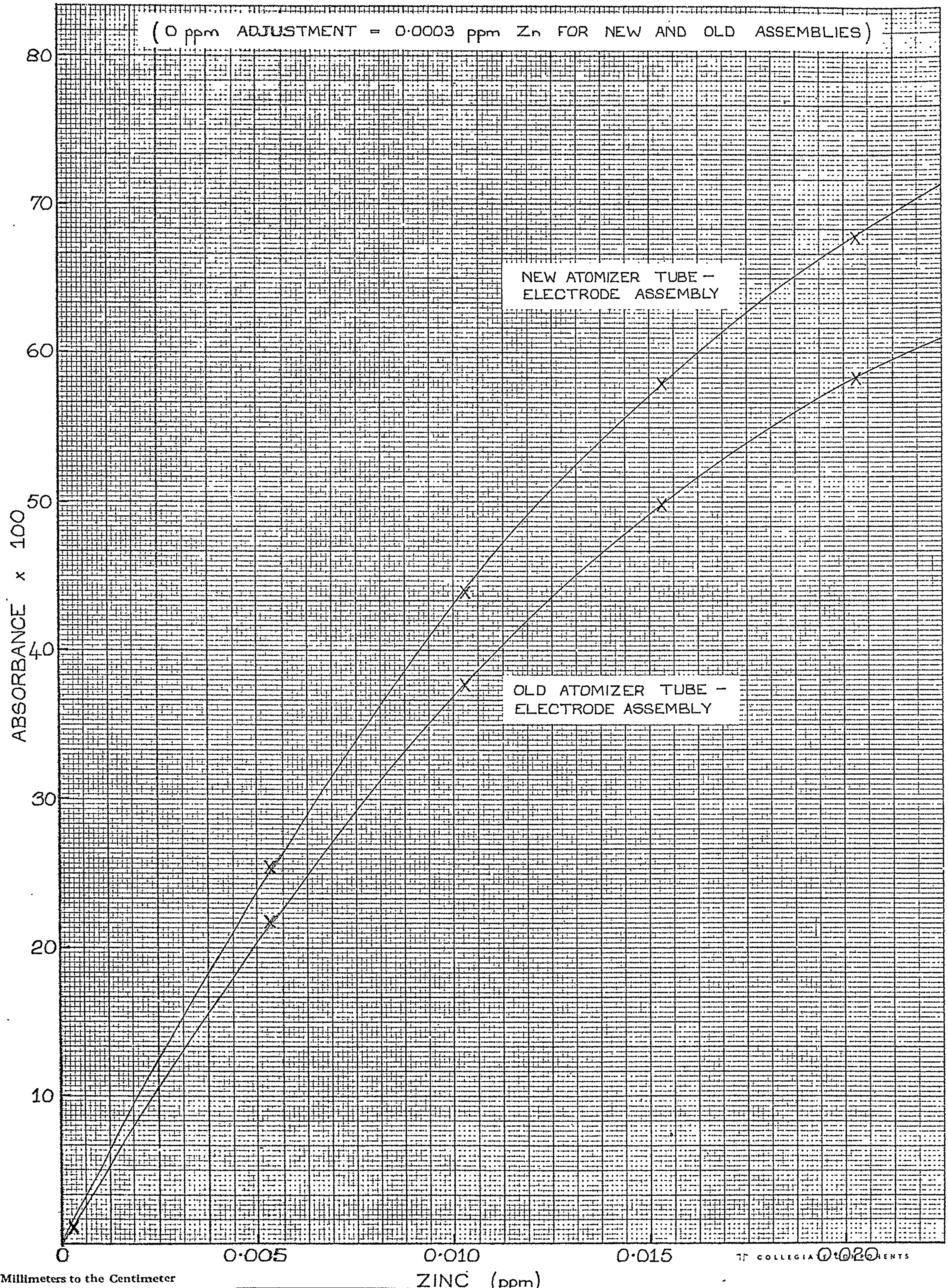
The decrease in sensitivity observed with increasing usage of the atomizer tube - electrode assembly was slow and regular. For the average atomizer tube, the total decrease in sensitivity was between 10 - 18 per cent over 200 - 250 firings, after which the assembly was replaced. Standards were interposed between each 12 - 15 samples. Calibration graphs, illustrating the performance of new and old atomizer tube - electrode assemblies, are shown in Figure 16, page 150. Ninety per cent of the samples fell within the range of 0.0055 - 0.0141 ppm in the dilution estimated.

Tests for non-atomic absorption.

Non-atomic absorption was tested using a hydrogen continuum lamp at 12.5 mA lamp current without change in photomultiplier gain setting or in slit opening. The validity of the test using these conditions has been shown by Koirttyohann and Pickett.⁽¹⁹²⁾ No non-atomic absorption was detected on testing six enamel samples.

TYPICAL CALIBRATION GRAPH FOR ZINC DETERMINATION IN ENAMEL

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Replicate analyses.

At least two determinations were made for each sample. For 21.7 per cent of the samples, where the duplicates differed by more than 10 per cent of the smaller value, a third determination was made. If the third replicate result fell outside the first two values, a fourth determination was carried out (1.0 per cent of samples). Because the performance of the atomizer tube was even and the risk of contamination with environmental zinc was very high, it was desirable to minimize handling of the samples and replicate estimations were made consecutively.

The results were based on the mean of all determinations.

Recovery of known amounts of zinc added to the samples.

Standard recoveries were tested by pre-drying a standard in the atomizer tube, followed by adding a sample containing a known concentration of zinc and atomizing the standard and the sample together. The mean recovery of 11 standard additions tested was 99.9 per cent, S.D. = ± 6.62 per cent.

Precision of the method.

- (a) The first type of estimate of precision was based on the variation within ten consecutive estimations of the same sample. The mean concentration of zinc in the dilution estimated was 0.0090 ppm, S.D. = ± 0.00049 ppm.
- (b) The standard deviation of a single determination based on differences between replicate determinations of the same sample was 0.00055 ppm.

Blank values.

Some of the 13 blanks made up in the field were estimated together with each batch of the samples. The mean zinc content of the blanks was 0.0016 ppm, S.D. = ± 0.00039 ppm, $n = 13$.

Decontamination - memory effect.

No difficulties were encountered with the initial decontamination of new atomizer tubes and no memory effect was noted at the settings used.

Calculation of results.

The zinc content of surface enamel, expressed in ppm, (Variable 235) was calculated as previously described for other elements.

3.3.2.1.8. Phosphorus determinations.

The total phosphorus content of enamel was determined as orthophosphate by the colorimetric method of Fiske and Subbarow, (119) (120) modified by Lazarus and Chou, (121) using a double-beam spectrophotometer (Model 124, Perkin-Elmer, Maywood, Ill. U.S.A.) with a micro-cell attachment (Model 124-0042, Hitachi Ltd., Tokyo, Japan).

Standards and reagents.

The 1000 ppm phosphorus stock solution was prepared by dissolving 0.439 g of oven-dried potassium dihydrogen orthophosphate, KH_2PO_4 (Analar grade, B.D.H.), in 100 ml of distilled, deionized water.

Working standards containing 1.00, 2.00, 3.00, 4.00 and 5.00 ppm phosphorus in 0.32 M nitric acid (Aristar) were prepared by diluting 125, 250, 375, 500 and 625 μ l respectively, of the 1000 ppm phosphorus stock solution to 100 ml in 0.04 M nitric acid and adding, before the estimation, 0.5 ml of colour developing reagents to 2.0 ml of standards (see below). Aliquots of 2.0 ml of 0.04 M nitric acid, dispensed in an identical manner, were used as the 0 ppm phosphorus standards.

The molybdate reagent was prepared by dissolving 2.5 g of ammonium molybdate tetrahydrate (Analar grade, B.D.H.) in 100 ml of 1.5 M sulphuric acid (Analar grade, B.D.H.).

The ANSA reagent was newly prepared at least every two weeks, as follows: 27.4 g of sodium metabisulphite (Laboratory reagent grade, B.D.H.) was dissolved in 200 ml of distilled, deionized water. To 195 ml of this 15 per cent sodium bisulphite solution were added 0.5 g of 1-amino-2-naphthol-4 sulphonic acid (Laboratory reagent grade, B.D.H.), and 5 ml of a 20 per cent sodium sulphite solution (A.R. grade, Drug Houses of Australia Ltd., Sydney, N.S.W.), followed by warming. The reagent was stored at 4°C in the dark.

Sample preparation and analyses.

Calculations showed that an approximately 1 in 500 dilution of the basic enamel sample was necessary to bring the samples into the optimum analytical range. Therefore, 0.5 ml of the initially prepared 1 in 101 dilution in 0.158 M nitric acid (3.3.2.1.1.) was made up to 2.0 ml with distilled, deionized water, immediately prior to the estimations.

To the samples and standards were added 0.25 ml of the molybdate reagent and, after 60 seconds, 0.25 ml of ANSA solution. The preparations, which were tested individually at two-minute intervals, were then left standing at ambient temperature (20 - 24°C) for exactly 10 minutes, to allow full development of colour.

The absorbances of the solutions were measured at 725 nm wavelength and 2 nm spectral band width settings, against a distilled water reference.

As a routine, batches of 30 samples, representing a cross-section of the human sample, and one or two blanks were analysed at a time. A calibration graph constructed from the average values of sets of standards estimated before and after each batch, was used to establish the phosphorus content of the samples. A typical calibration graph is shown in Figure 17, page 155.

Replicate analyses.

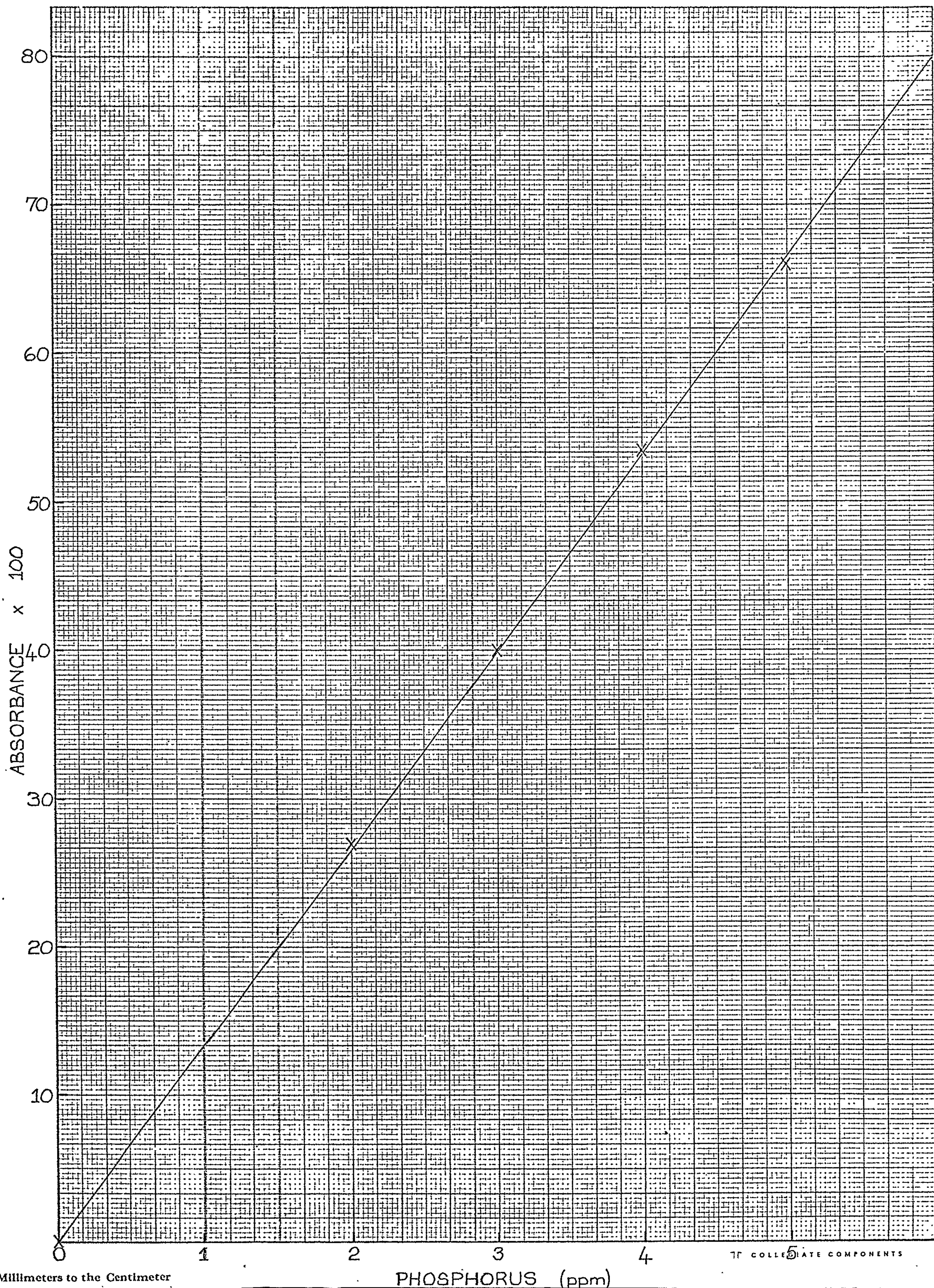
All samples were analysed in duplicates, estimated on different days. For 0.5 per cent of the samples, where duplicates differed by more than 5 per cent of the smaller value, a third estimation of the same sample was carried out.

Blank values.

The mean phosphorus content of 12 blanks was 0.04 ppm, S.D. = $\pm$ 0.02 ppm.

Recovery of known amounts of phosphorus added to the samples.

It has been shown over decades of practical use, that the Fiske-Subbarow method is practically free of interferences under



the present experimental conditions. This fact was confirmed by the quantitative recovery of standards added to two pooled enamel samples. The amounts of phosphorus recovered were 103.0 and 101.0 per cent of the expected amounts.

Estimation of the accuracy and precision of the method.

A concentrated solution of calcium phosphate ($\text{Ca}_3 (\text{PO}_4)_2$) (Pro analysi, E. Merck G.m.b.H., Darmstadt, Germany) was analysed for calcium and phosphorus by atomic absorption spectroscopy and by the Fiske-Subbarow method respectively. The findings of 3420 ppm calcium and 1740 ppm phosphorus gave an established molar Ca : P ratio of 1.52 against an expected theoretical value of 1.50.

The estimate of precision was based on the differences between replicate determinations of the same sample. The standard deviation of a simple determination, S , calculated from the 'within-sample mean square' of an analysis of variance, using data classified in one way, was 0.06 ppm.

Calculation of results.

As for other elements, the phosphorus content of enamel was calculated on the basis of the weight of the enamel biopsy specimen which in turn was obtained from the amount of calcium found in the sample (sub-section 3.3.2.1.2.). The phosphorus content of surface enamel (Variable 232) was expressed as per cent of dry weight.

3.3.2.1.9. Determination of dissolution rate and fluoride content of surface enamel.

The collection and preservation of the enamel surface

biopsy specimens used for fluoride determinations have been described in sub-section 3.2.3.10.

On opening the sample containers, the volume of each sample was verified by drawing up the sample solution into a fine bore, graduated polyethylene tube, attached to a micropipette. No loss of volume was detected in any of the samples.

Of the 201 μ l total volume, 50 μ l was transferred into 2.00 ml of one per cent lanthanum background solution. This aliquot was used for the determination of the calcium content of the sample, which was carried out by atomic absorption spectroscopy using the same instrumentation and similar experimental conditions to those described in sub-section 3.3.2.1.2.

The dissolution rate of surface enamel (Variable 22) was expressed as micrograms of calcium contained in the standard etch.

The biopsy depth was estimated using appropriate modification of the formula given in 3.2.3.12.

The ionized fluoride content of the samples was measured using a fluoride ion activity electrode (Model 94-09, Orion Research Inc., Cambridge, Mass., U.S.A.) and a custom-made double-junction, saturated calomel reference electrode, in conjunction with a solid state electrometer (Model 602, Keithley Instruments Inc., Cleveland, Ohio, U.S.A.) and a digital readout volt-ohm meter (Model 9000, Systron Donner Corp., Concord, Calif., U.S.A.). To reduce the sample volume required, an inverted electrode arrangement similar to that described by Durst and Taylor⁽¹⁹³⁾ was devised.

Standards.

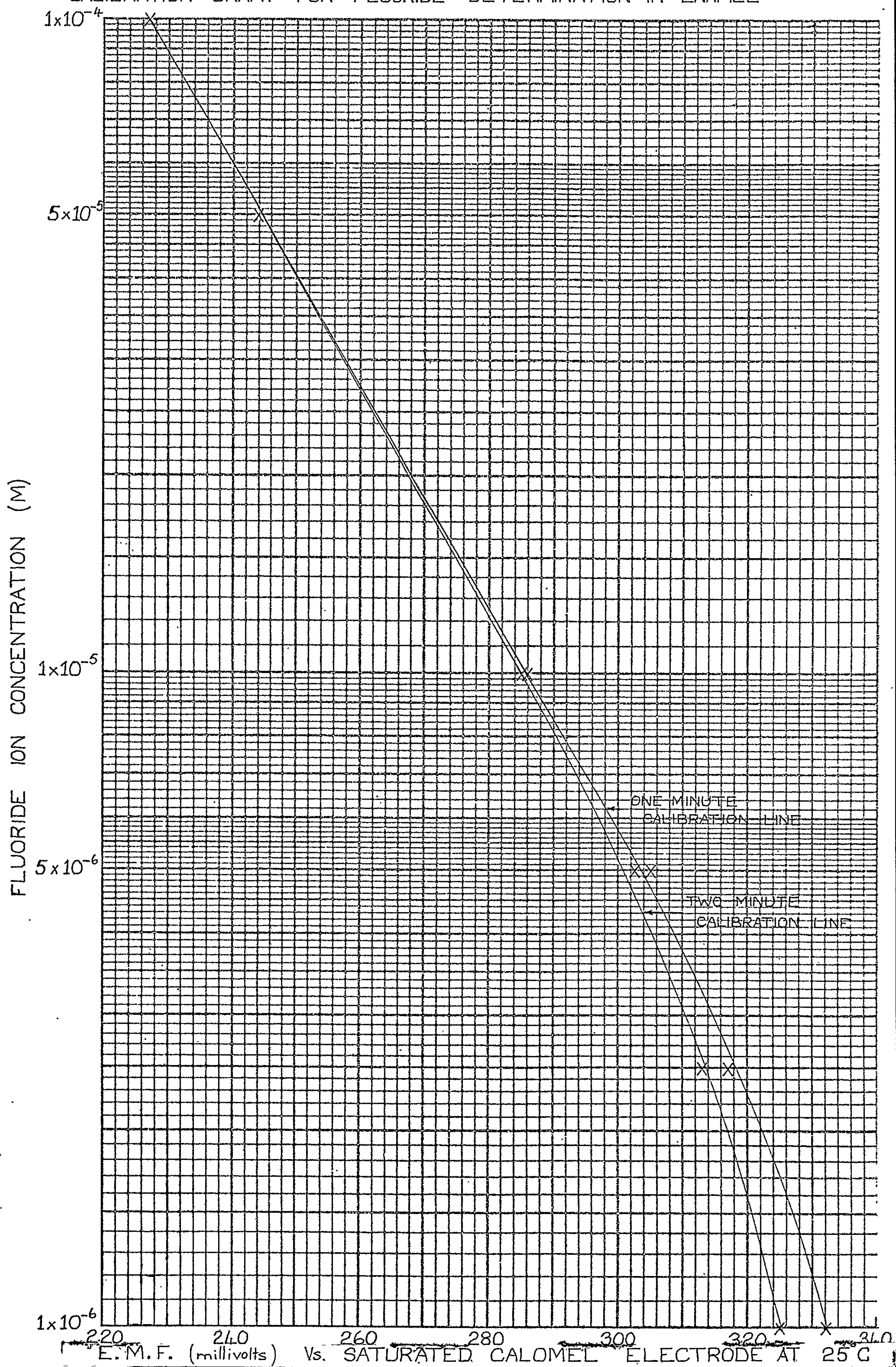
The electrodes were calibrated with standards containing 1.0×10^{-4} M, 5.0×10^{-5} M, 1.0×10^{-5} M, 5.0×10^{-6} M, 2.5×10^{-6} M and 1.0×10^{-6} M fluoride ion made up from oven-dried sodium fluoride (laboratory reagent grade, May and Baker Ltd., Dagenham, Essex, U.K.) in a background solution of 0.23 M sodium formate buffer at pH 4.6. The buffer was made up from analytical reagent grade formic acid (B.D.H. Chemicals Ltd., Poole, Dorset, U.K.) and "Volucon" sodium hydroxide (May and Baker Ltd.), in distilled, deionized water.

Before calibration, the electrode crystal and the reference salt bridge were washed thoroughly with distilled, deionized water and dried. For calibration 20 μ l of the most dilute standard solution was placed on the central area of the crystal face of the inverted electrode. The liquid was not permitted to contact the edge of the crystal. The voltage (E.M.F.) vs. saturated calomel electrode (S.C.E.) reading was taken after exactly one minute. The electrode was washed again and the procedure was repeated using the standard containing the next highest concentration of fluoride. The E.M.F. values obtained in this way were used to produce a calibration graph illustrated in Figure 18, page 159. The temperature of the laboratory was kept within narrow limits ($25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) throughout the fluoride determinations.

Samples.

Approximately 25-35 enamel samples were estimated each day. To avoid bias associated with undetected changes in experimental

CALIBRATION GRAPH FOR FLUORIDE DETERMINATION IN ENAMEL



conditions, the daily batches were selected so that each batch represented consecutive cross-sections of the human sample. The estimations were made in the same manner as described for the standards. However, after the E.M.F. reading was taken for the sample, a standard addition of 19 ng of fluoride in 20 μ l of sodium formate buffer was made to each sample. The E.M.F. was again read 1 minute after the addition.

Although the standard calibration curve usually remained unchanged over extended periods of time, standards were interposed between each 5-6 samples.

Recovery of known amounts of fluoride added to the samples.

The percentage recovery of the 19 ng of fluoride added to each sample, was calculated from the observed molarity and the sample volume after the addition. The mean recovery of 298 standard additions was 95.0 per cent, S.D. = $\pm$ 7.9 per cent. When known amounts of fluoride were added to 40 aliquots of the same sample, the mean recovery was 98.5 per cent, S.D. = $\pm$ 4.9 per cent.

Precision of the method.

The precision of the method was tested by repeated estimations of two enamel samples pooled from field material, chosen to represent the lower end and the middle of the concentration range respectively, of fluoride found in the New Guinea specimens.

The mean fluoride content of the first sample was found to be 0.44 ng, S.D. = $\pm$ 0.09 ng, $n = 10$, corresponding to 1.16×10^{-6} M

fluoride ion in the dilution estimated.

Blank values.

All blank values were below the detectable fluoride level.

Calculation of results.

The fluoride content of the enamel biopsy specimen was expressed in nanograms of fluoride:

$$F(\text{ng}) = \frac{M \times \text{At. wt.} \times V}{10^{-3}} \times 10.05$$

M = observed molarity, At. wt. = 19, V = 20 = volume estimated in μl .

The calculation of the fluoride content of surface enamel, expressed in ppm, was based on the amount of calcium found in the biopsy specimen, assuming that the calcium content of enamel was 37.0 per cent.

$$\text{Fluoride content of surface enamel (ppm)} = \frac{F^-(\text{ng}) \times 10^3}{\text{Ca}(\mu\text{g}) \times 2.7}$$

The depth of the enamel biopsy was calculated assuming uniform etching from an area of 3.14 mm^2 , and taking 2.95 as the density of enamel. (87)

$$\text{Depth } (\mu\text{m}) = \frac{\text{Weight } (\mu\text{g})}{\text{Area } (\text{mm}^2) \times \text{density}}$$

3.3.2.2. Preparation and analyses of saliva specimens.

3.3.2.2.1. Volume estimation and colour grading.

It was reported in sub-section 3.2.4.1., that a proportion of the samples collected during the March - April, 1973 field

visit was damaged in transit between the field and the laboratory.

On arrival from the field, the saliva samples were stored at -25°C . After thawing out, the volume of each specimen was again determined to the nearest 0.5 ml, by comparing the level with graduations marked on an otherwise identical container.

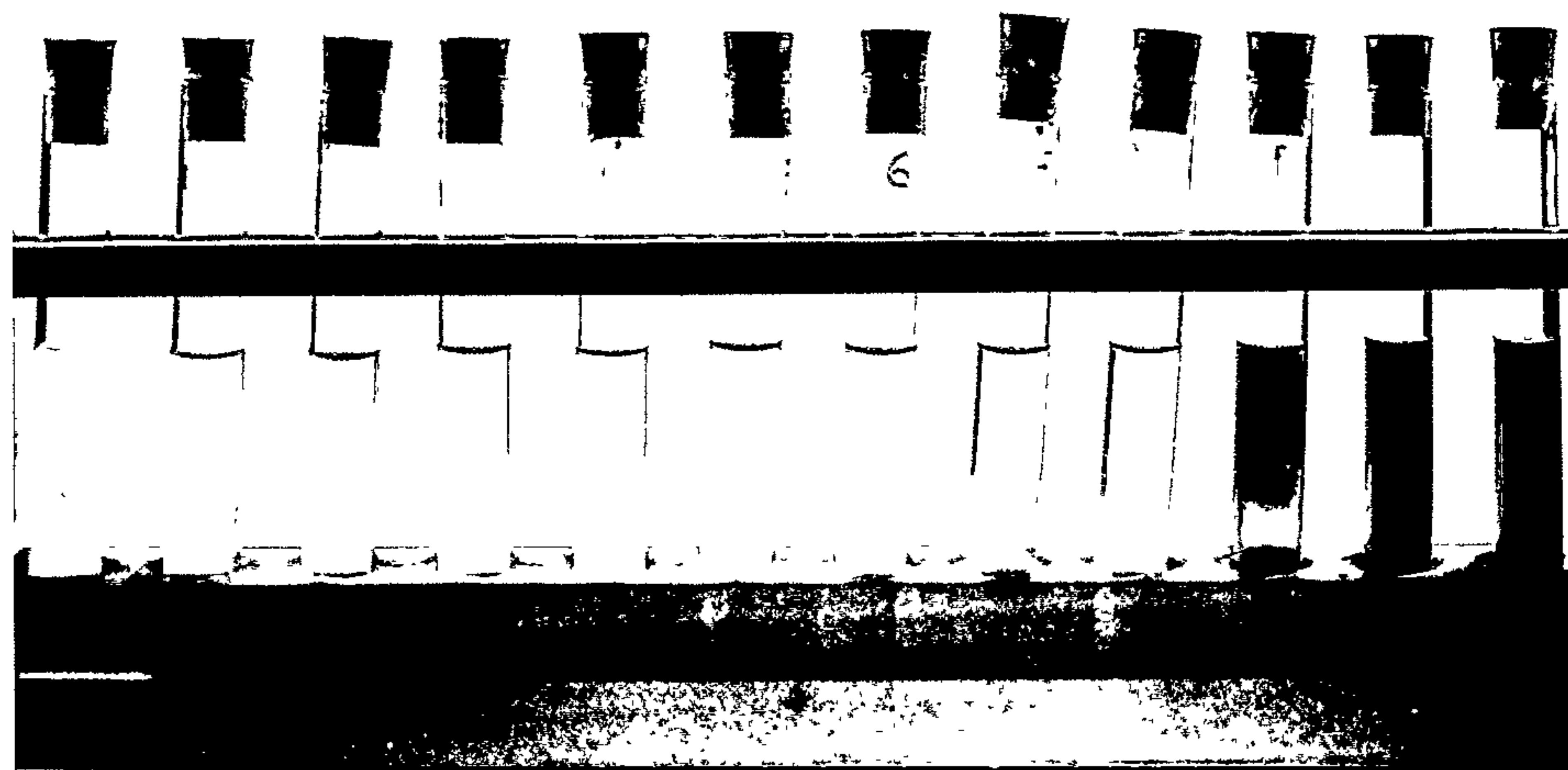
Saliva samples, which had an unchanged volume or had lost less than 10 per cent of the volume estimated in the field, were pooled with the specimens collected from the same individual in July, 1973. Samples which had lost 10 per cent or more of the original volume were rejected.

The pink to deep red discolouration of saliva samples, a visible manifestation of recent betel-chewing, was graded by matching, visually, a sample to the nearest of a series of 12 samples selected as standards, to represent increasing colour intensity on an arbitrary scale of 0 - 11, (Figure 19, page 163). Scores 1 and 2 were given to samples classified as "doubtful-unlikely" and "doubtful but probable" respectively, with regard to the presence of light pink colour.

Before colour grading and at 10 minute intervals during the grading process, sedimented material was re-suspended both in the standards and in the samples, which were then allowed to stand for two minutes, to permit coarse particles to settle. The gradings were carried out in obliquely transmitted natural daylight, against a white background. Saliva colour is represented by Variable 213.

FIGURE 19

SALIVA COLOUR STANDARDS



3.3.2.2.2. Fluoride determinations.

After colour grading and before acidification of the samples, the saliva specimens were thoroughly suspended and representative aliquots were transferred into polypropylene containers (5-P) for fluoride determinations. The amount of sample used for fluoride analysis depended on the total quantity of saliva available; 0.5 ml, 1.0 ml or 2.0 ml aliquots were dispensed, when the total sample volumes were less than 5.0 ml, 5.0 - 8.0 ml or more than 8.0 ml respectively.

The samples were prepared for analysis by the addition of one-quarter volume of 0.2 M sodium acetate buffer at pH 5.2, containing 1.0 per cent cyclohexylene di-nitrilo tetra-acetic acid (acetic acid: analytical reagent grade, Byproducts and Chemicals Ltd., Sydney, N.S.W.; sodium hydroxide: "Volucon", May and Baker Ltd.; C.D.T.A.: "Titriplex IV", E. Merck, Darmstadt, Germany). The pH of the buffered saliva samples was tested using a pH meter and standard electrodes ("Expandomatic", Beckman Instruments Inc., South Pasadena, Calif., U.S.A.). Where necessary, the pH was adjusted to 5.2 by dropwise addition of 0.1 M acetic acid.

The ionized fluoride concentration of the samples was measured using a combination fluoride electrode (Model 96-09, Orion Research Inc., Cambridge, Mass., U.S.A.) or a fluoride ion activity electrode (Model 94-09, Orion) in conjunction with a custom-made, saturated calomel reference electrode, fitted with a junction containing 0.05 M acetic acid. Otherwise, the instrumentation used was identical to that described in sub-section 3.3.2.1.9.

Standards.

The electrodes were calibrated with standards containing 1.0×10^{-4} M, 1.0×10^{-5} M, 5.0×10^{-6} M, 1.0×10^{-6} M, 7.5×10^{-7} M, 5.0×10^{-7} M, 2.5×10^{-7} M and 1.0×10^{-7} M fluoride ion made up from oven-dried sodium fluoride (Analar grade, B.D.H. Pty. Ltd., Poole, Dorset, U.K.), in a background solution of 0.05 M sodium acetate buffer (pH 5.2), containing 0.04 M sodium chloride (Analar grade, B.D.H.), and 0.2 per cent C.D.T.A. ("Titriplex IV", Merck). Before and between readings, the electrodes were washed in distilled, deionized water and dried. A thin film of silicone oil (Product 94-00-03, Orion) was applied to the dry electrode crystal. For calibrations, the electrodes were immersed in standard solutions in order of increasing concentration. During readings, the samples were agitated by bubbling a fine stream of fluoride-free air through the solution.

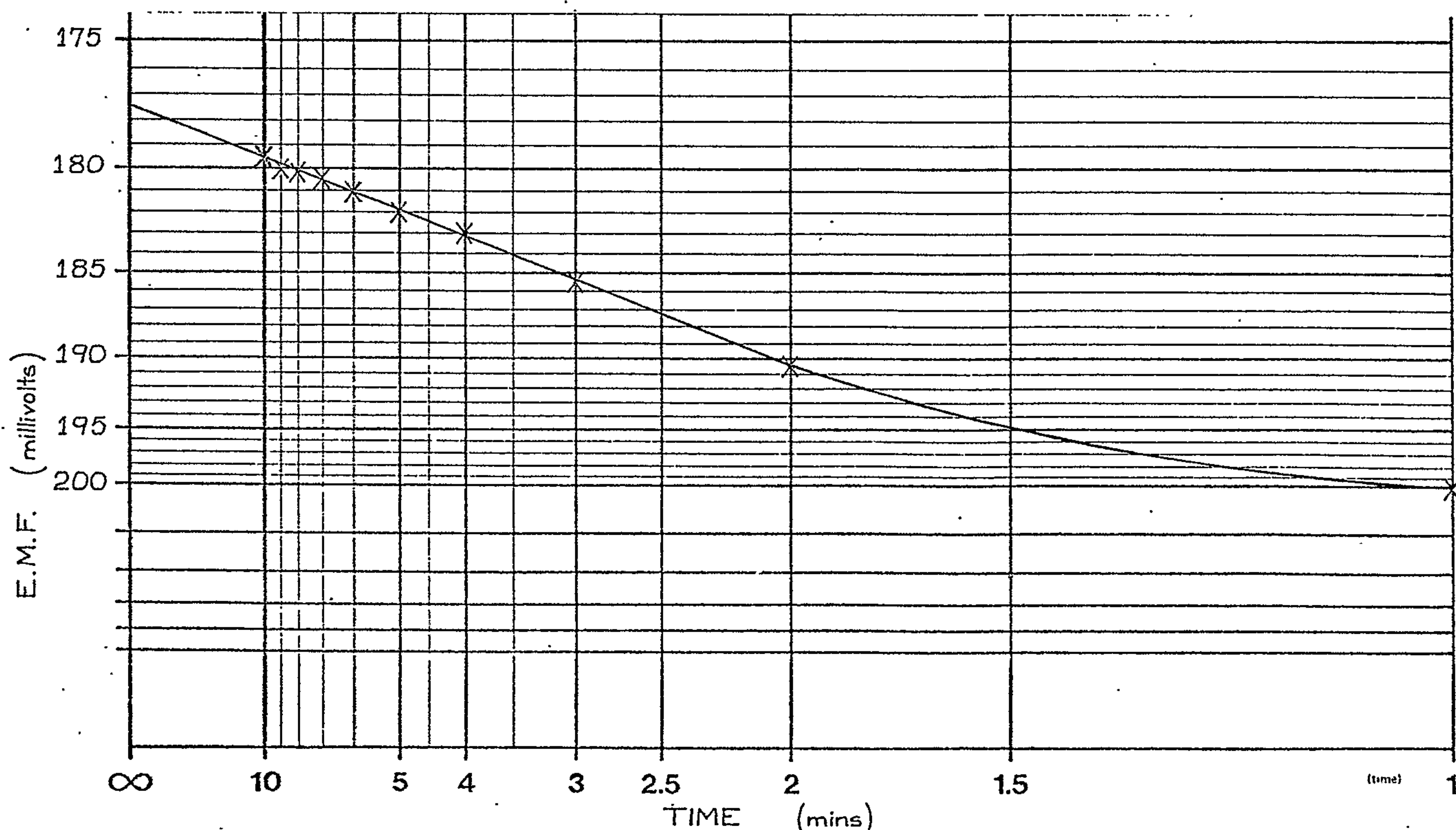
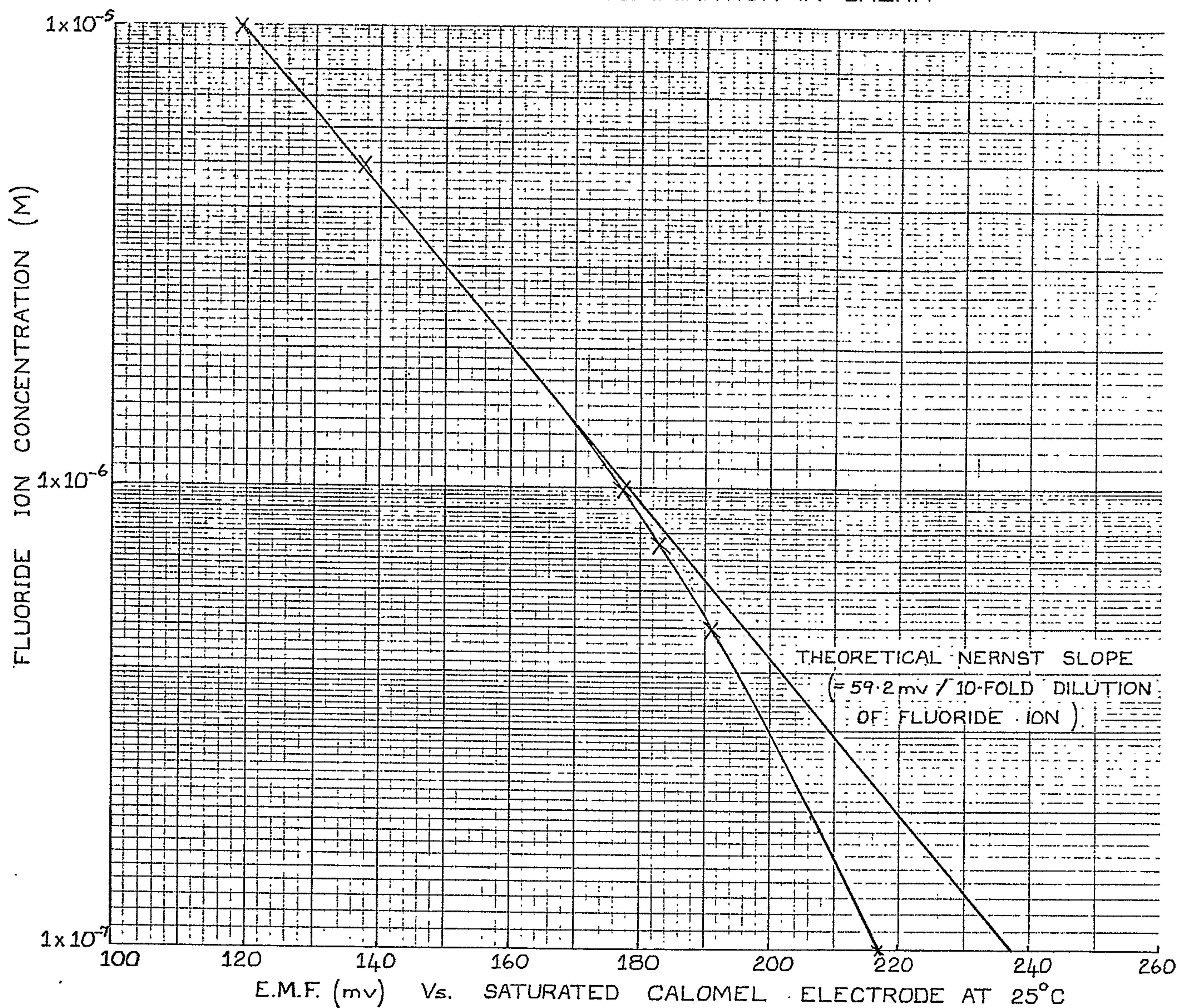
The electrode potential at infinite time was estimated by taking readings at one-minute intervals over a ten-minute period, and plotting the electrode potential in millivolts against time, on a time-response chart (Product 90-00-91, Orion). A typical calibration graph and a time-response plot are shown in Figure 20, page 166.

Samples.

Approximately 20 - 30 samples were estimated each day, in the manner described for the standards. Daily batches represented cumulative cross-sections of the human sample. Standards were interposed between each 5 - 6 samples. Ninety per cent of the samples fell within the range of 5×10^{-7} - 2.5×10^{-6} M fluoride.

FIGURE 20:
CALIBRATION GRAPH AND TIME RESPONSE PLOT FOR
FLUORIDE DETERMINATION IN SALIVA

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Recovery of known amounts of fluoride added to the samples.

The mean percentage recovery of 19 ng of fluoride ion added to 17 saliva samples in the form of 100 μ l of 1.0×10^{-5} M standard, was found to be 99.6 per cent, S.D. = ± 6.7 per cent, $n = 17$.

Precision of the method.

The precision of the method was estimated at two levels of fluoride ion concentration, because the error increased with decreasing molarity. Ten replicate analyses were carried out for each of two saliva samples, a) and b). The mean molarity of test solution a) was 1.8×10^{-6} M fluoride, corresponding to 43.5 ppb fluoride in saliva, S.D. = ± 0.4 ppb, $n = 10$. The mean molarity of test solution b) was 3.3×10^{-7} M fluoride, corresponding to 8.0 ppb fluoride in saliva, S.D. = ± 2.9 ppb, $n = 10$. It is pertinent to point out again that 90 per cent of the samples contained fluoride at a higher level than 5.0×10^{-7} M.

Blank values.

The background material contained a mean of 1.35×10^{-7} M fluoride ion, S.D. = $\pm 0.31 \times 10^{-7}$ M, $n = 50$. The blank values were within the range of the background readings.

Calculation of results.

The fluoride content was expressed as part per billion ($\text{g}/1 \times 10^9 \text{ ml}$) of saliva (Variable 243).

$F^-(\text{ppb}) = \mu\text{M} \times 1.25 \times 19$, where μM is the molarity of fluoride $\times 10^{-6}$ of the buffered saliva.

3.3.2.2.3. Calcium determinations.

The precautions taken to minimize contamination during sample handling were similar to those described in sub-section 3.3.2.1.1.

Following removal of an aliquot for fluoride determinations, the saliva samples were acidified to less than pH 3.0 by the addition of 50 - 100 μ l of 15.8 M nitric acid (Aristar). After standing for 1 hour, the samples were thoroughly suspended and a maximum of 12.0 ml of the suspension was transferred into pre-weighed, 15 x 130 mm, new borosilicate glass test tubes (H.B. Selby and Co. Pty. Ltd., North Ryde, N.S.W.). When the volume was less than 12.0 ml, all the available amount, measured to the nearest 0.5 ml was transferred. The samples were evaporated in a stainless steel-lined oven ("Qualtex", Watson Victor) at 100 - 120°C. On cooling, the test tubes were re-weighed and the weight of solids in each sample was obtained by subtraction.

The weighings were performed using an electric balance (Model H4, E. Mettler, Zürich, Switzerland) to the nearest 0.1 mg. The repeatability of weighings was tested in two ways: a) Ten repeated weighings of the same empty test tube gave a mean value of 16.33399 g, S.D. = ± 0.00006 g. b) The standard deviation of a single weighing calculated from the differences between 14 pairs of duplicate weighings, d , using the formula $S.D. = \sqrt{\frac{\sum d_i^2}{2n}}$, was found to be 0.00032 g. The mean weight of the dried saliva residues was 0.112 g.

In preparation for wet ashing, 15.8 M nitric acid (Aristar) was added to the samples according to their dry weight category:

0	-	40	mg dry wt	2 ml
41	-	80	" " "	3 ml
81	-	160	" " "	4 ml
161	-	200	" " "	5 ml

The test tubes were covered with borosilicate glass condensers, placed in an aluminium heating block and digested at 100 - 120°C for approximately 24 hours, or until the solutions appeared colourless. After wet ashing was completed, the acid was evaporated and the residual white solid was dissolved in 4.2 ml of 0.8 M nitric acid (Aristar). After heating at 80°C for 1 hour, the samples were transferred, warm, into polypropylene vials (5-P) and stored at -25°C until required for analysis.

The calcium content of the samples was determined by atomic absorption spectroscopy, using the same instrumentation and similar experimental conditions as described for enamel in sub-section 3.3.2.1.2. Pertinent differences are pointed out below:

In preparation for the analyses, 20 µl aliquots of the ashed samples were diluted in the ratio of 1 in 101 in a background solution containing 1000 ppm caesium. The background solution was prepared by dissolving 1.267 g of oven-dried caesium chloride (Univar grade, Ajax Chemicals, Sydney, N.S.W.) in 1 litre of 0.16 M nitric acid (Aristar).

Standards.

Working standards were prepared in 1000 ppm caesium background. Typical mean absorbance x 100 values were:

0 ppm	1 ppm	2 ppm	3 ppm	4 ppm
1.5	20.0	38.1	46.8	75.6

A typical calibration graph is shown in Figure 21, page 171. Ninety per cent of the samples fell within the range of 23.4 - 62.9 units of absorbance x 100. Further fourfold dilutions were prepared for six samples, which had calcium values outside the range of the standards.

Blank values.

The mean blank value was 0.082 ppm, S.D. = ± 0.012 ppm, n = 6.

Replicate analyses.

All analyses were made in duplicates. The estimate of precision was based on the differences between duplicate pairs, as described in 3.3.2.1.2. The standard deviation of a single determination was found to be ± 0.04 ppm.

Recovery of known amounts of calcium added to the samples.

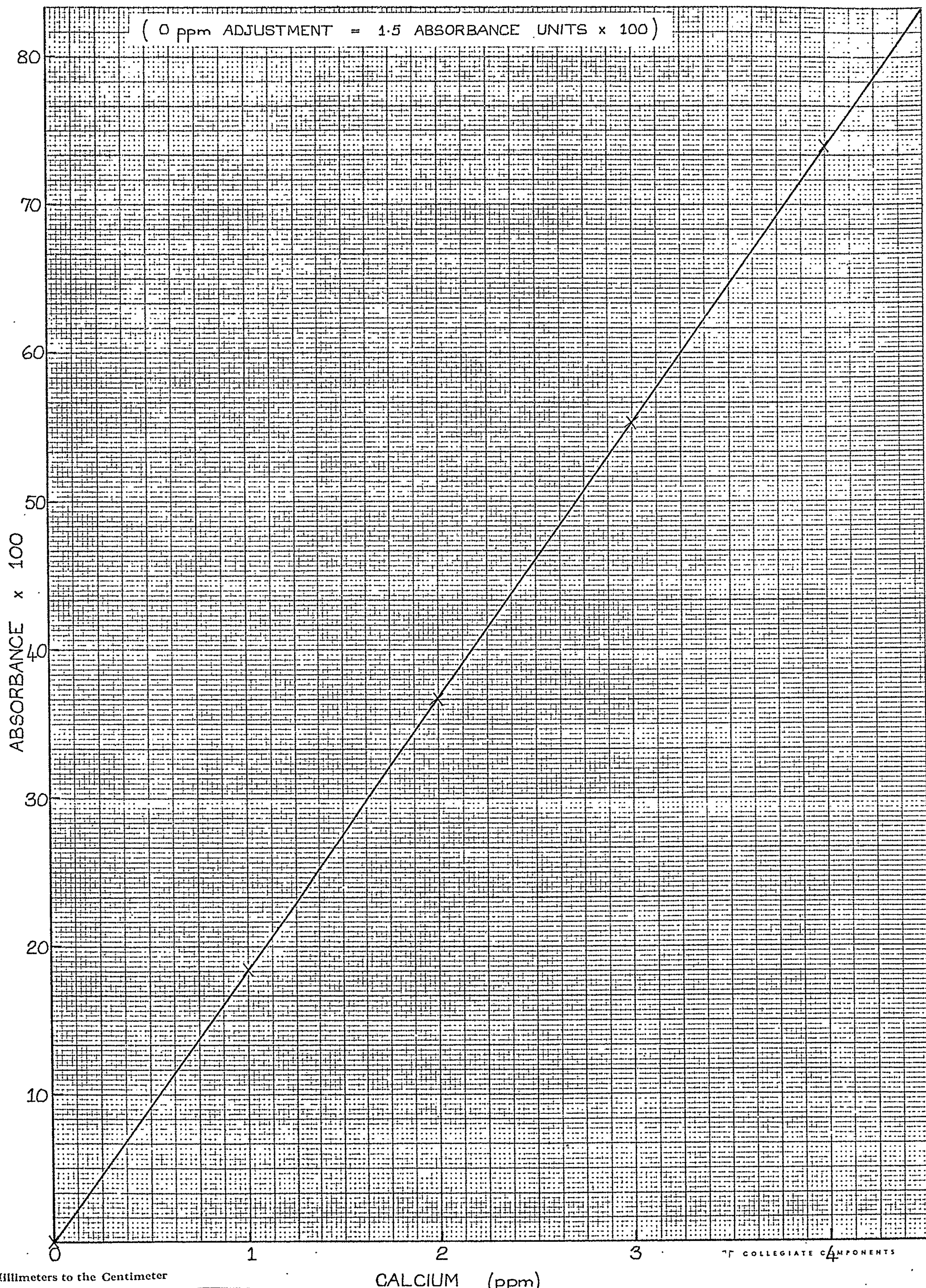
The test material was prepared by adding 0.5 ml of 2.0 ppm standard to 1.5 ml of sample solution. The mean recovery was 100.0 per cent, S.D. = ± 1.7 per cent, n = 12.

Calculation of results.

The calcium content of saliva was expressed as ppm (Variable 239) and also as per cent of solids on a dry weight basis (Variable 240).

TYPICAL CALIBRATION GRAPH FOR CALCIUM DETERMINATION IN SALIVA

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$$\text{Ca in saliva (ppm)} = \frac{\text{Ca in ashed sample } (\mu\text{g})}{\text{Volume of saliva analyzed (ml)}}$$

$$\text{Ca in saliva (per cent of solids)} = \frac{[\text{Ca in ashed sample } (\mu\text{g})] \times 100}{[\text{Wt. of saliva solids (mg)} \times 10^3]}$$

3.3.2.3. Determination of the dry weight of pooled plaque samples (1) and (2).

The time needed for drying samples of plaque to a constant weight was tested using eighteen specimens collected for this purpose, ranging in dry weight from 0.80 mg to 17.82 mg. These samples were weighed after having been dried for 2 hr, 4 hr, 6 hr, 24 hr and 72 hr periods at 102°C. No reduction in weight, beyond the error of the method, was observed after 4 hours of drying.

The field samples were dried to a constant weight in batches, over periods of 8 - 16 hours in a stainless steel-lined oven ("Qualtex", Watson Victor) at 101 - 103°C in the container lids used for collection. After drying, the samples were allowed to cool to ambient temperature (19 - 21°C) in an airtight container in the presence of dry silica gel. The plaque material was then separated from the container wall and was transferred into a celluloid trough and weighed immediately to the nearest 0.01 mg on an electric balance (Model H4, E. Mettler).

The same procedure was used for samples (1) and (2).

The estimate of precision of the method was based on the standard deviation of a single weighing, calculated from the differences, d , between 15 pairs of duplicate weighings using the formula $\text{S.D.} = \sqrt{\frac{\sum d_i^2}{2n}}$. The standard deviation was found to be ± 0.049 mg.

The mean weights of samples (1) and (2), representing Variable 16 and Variable 203, were 5.30 mg and 1.73 mg respectively.

3.3.2.4. Determination of the total number and the proportions of predominating colony types of cultivable Streptococci in dental plaque.

Procedures involved in the collection, dispersion and storage of two plaque specimens (the pooled and UL2 samples), were described in sub-sections 3.2.3.5. and 3.2.3.7. Field methods.

The laboratory procedures followed in processing the plaque specimens were identical for the pooled and UL2 samples.

As far as possible, culturing and classification of streptococci were carried out in a continuous process, bringing small batches of 5-10 samples into the flow routine as progress permitted. Although it was not possible to process the samples in a pre-determined numerical order, because of the danger of inadvertently thawing out samples during selection, it was assured that each batch represented subjects from each of the major caries prevalence strata. This precaution was taken to avoid bias which could have arisen if batches of samples from subjects with a uniform or similar caries prevalence had been exposed to undetected variations in the procedure.

The samples coming into the work routine were transferred from storage at approximately -196°C in liquid nitrogen (Vivostat CPV-13, Cryoproducts Division, The British Oxygen Co., Ltd., London, U.K.), into a water bath at 40°C . After thawing out, the samples were vibrated on a vortex mixer (Model VM, Rota-Line, Bio-Tech

Pty. Ltd., Guildford, N.S.W.) for 10 seconds to suspend the material, opened with a sterile, stainless steel scalpel and emptied into a sterile 3/8 x 2.5" glass test tube. After further vortex mixing for 20 seconds, 0.1 ml of the suspension was transferred into the first of a series of dilution tubes containing 3.9 ml of sterile peptone water, using a 0.1 ml glass pipette graduated in 0.005 ml (Type G5081 E-mil, Jobling Laboratory Division, Stone, Staffs, U.K.). The pipette was twice rinsed into the peptone water suspension after delivery. After vortex mixing this suspension for 5 seconds, further serial dilutions were prepared in peptone water following the same routine, but using new pipettes for each transfer. The extent of dilution of a given sample was based on the wet weight of the material collected. The dilution sequences used are shown in Table 2, page 175.

After completing the procedure for a sample, the three highest dilutions were re-suspended and duplicate 0.1 ml aliquots of each suspension were transferred to and spread out on the surface of Mitis-salivarius agar plates (No. 11428 BBL, Cockeysville, Md., U.S.A.) which had been prepared in the following manner: after re-hydrating the medium according to the manufacturers' instructions, it was autoclaved at 15 p.s.i. for 15 minutes and allowed to cool to 50°C when 1 ml of 1.0 per cent potassium tellurite (B.D.H., Poole, Dorset, U.K.) per litre was added slowly while spinning the flask holding the medium in a water bath at 50°C. The medium was then poured into 9 cm diameter sterile disposable Petri dishes ("Hospak", Ramsay Surgical (N.S.W.) Pty. Ltd.) to form culture plates of 5 mm average thickness. After visual inspection, the occasional plate, which appeared to vary by more than ± 25 per cent from the average thickness, was discarded. The plates were

TABLE 2.

Dilution sequences used for UL2 and pooled plaque cultures.

Plaque Wet wt (mg)*	Serial Tube Dilutions									
3.0	40**	400	2 x 10 ³	10 ⁴	5 x 10 ⁴	2.5 x 10 ⁵	1.25 x 10 ⁶	6.25 x 10 ⁶		
	***0.1 + 3.9	0.5 + 4.5	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4	
1.5	40	400	4 x 10 ³	2 x 10 ⁴	10 ⁵	5 x 10 ⁵	2.5 x 10 ⁶			
	0.1 + 3.9	0.5 + 4.5	0.5 + 4.5	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4		
1.0	40	400	2 x 10 ³	10 ⁴	5 x 10 ⁴	2.5 x 10 ⁵	1.25 x 10 ⁶			
	0.1 + 3.9	0.5 + 4.5	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4		
0.5	40	200	10 ³	5 x 10 ³	2.5 x 10 ⁴	1.25 x 10 ⁵	6.25 x 10 ⁵			
	0.1 + 3.9	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4		
0.3	40	400	2 x 10 ³	10 ⁴	5 x 10 ⁴	2.5 x 10 ⁵				
	0.1 + 3.9	0.5 + 4.5	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4			
0.1	40	200	10 ³	5 x 10 ³	2.5 x 10 ⁴	1.25 x 10 ⁵				
	0.1 + 3.9	1 + 4	1 + 4	1 + 4	1 + 4	1 + 4				
0.05	40	200	10 ³	5 x 10 ³	2.5 x 10 ⁴					
	0.1 + 3.9	1 + 4	1 + 4	1 + 4	1 + 4					

* Plaque samples nearest to the weight indicated were diluted according to the sequence shown.

** Dilution factor.

*** Sequential transfer in peptone water (ml).

allowed to desiccate under cover overnight. Before preparation of the cultures, the plates were placed in an oven at 40°C to evaporate free surface moisture.

The six culture plates prepared from each sample were processed according to the method described by Jordan, Krasse and Möller,⁽¹⁶³⁾ modified by concluding the incubation regime with an additional 48 hour anaerobic incubation period to facilitate recognition and classification of colony types. Reduced oxygen tension was created by the 'candle-jar' technique. After incubation, plates containing 30-300 colonies were counted without magnification. On rare occasions, when less than two plates were found within these limits, the dilution regime was modified for the specimen and the culture was repeated.

The total number of colony forming units of streptococci per mg wet weight of plaque was calculated using the formula:

Total no. colony forming units of streptococci/mg wet wt.

$$(\text{pooled or UL2}) \text{ plaque} = C \times 25D \times \frac{1}{X}$$

Where C = the mean number of colonies at a given dilution, obtained from the average of usually four platecounts after adjustment to a common dilution level.

D = common dilution level of plates (Table 2, page 175).

X = wet weight of plaque (mg).

The total number of colony forming units of streptococci assumed to be present in each specimen collected, was obtained by multiplying the above result by the wet weight of the corresponding plaque sample.