sequestrum, being non-vascular and inaccessible to the osteoclasts has remained unchanged. In clinical radiography judgements are based on relative rather than on absolute image densities.

Our figures show a slight and statistically non-significant increase in the calcium content of sequestra (group E) as compared with normal compact bone (group A). It may be argued that this is suggestive ($p = 0{\cdot}1$) and that a small increase in calcium content would account for an increased radiographic density. We have investigated this point by taking X-rays of a series of slices of femur cortex of different thicknesses, and have found that under the conditions of clinical radiography it would require at least a 50% increase in calcium content to give any clearly visible difference in radiographic density. Nothing of this sort occurs in sequestra.

SUMMARY

1. An estimation has been made of the calcium and nitrogen content of a series of samples of human bone tissue cleaned as thoroughly as possible under a dissecting microscope, defatted and dried.

2. Tables are given showing calcium and nitrogen content of groups of samples of normal bone from (A) compact cortical bone of adult femur, (B) cancellous bone of adult femur, (C) adult rib, (D) infant rib, and of abnormal bone (E) in the form of sequestra composed of dead compact bone.

3. No statistically significant differences were found between groups A and B or between groups A and E. There was a significant difference between groups C and D, infantile rib (D) showing a lower calcium and a higher nitrogen content than adult rib (C). This could be explained by the greater amount of osteoid tissue seen on histological examination in the rapidly growing bones of infants. There was a significantly greater amount of nitrogen in adult rib (C) as compared with adult femur (A and B); no explanation of this can be offered at present.

4. Taking the femur (average of compact and cancellous bone) of six individuals, the figures range from 25·01 to 25·73% calcium and from 4·15 to 4·59% nitrogen, suggesting a fairly constant composition.

5. The degree of calcification of the collagenous (protein) bone matrix may be expressed by the calcium/nitrogen or by the calcium salt/protein ratio. The calcium salt/protein ratio averaged 2·4 for compact bone of femur cortex, 2·3 for cancellous bone of femur, 2·1 for cancellous bone of adult rib, 1·9 for cancellous bone of infant rib and 2·5 for compact bone of sequestra.

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The Cerimetric Determination of Glucuronic Acid, using the Conway Burette

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As a preliminary to a study of β-glucuronidase, it was necessary to be able to estimate small amounts of glucuronic acid in the presence of excess unhydrolyzed glucuronides. This can be done by determining reducing sugar by one of the methods for blood glucose. Thus Masamune (1934) used the Hagedorn-Jensen technique, while Fishman (1939) employed a modification of the cerimetric method of Miller & Van Slyke (1936). None of the methods used by previous workers, however, was sufficiently

sensitive to determine the amounts of free glucuronic acid liberated in the enzyme experiments planned. The possibility was therefore examined of applying some other method for the determination of reducing sugar. Cerimetric procedures appeared most promising.

The method of Miller & Van Slyke (1936) is suitable for the determination of 0·1–0·8 mg. glucose. The instability of ceric sulphate solutions in the concentration used (0·003N) is a disadvantage in this method. Giragossintz, Davidson & Kirk (1936) have described a procedure, based on earlier work by Whitmoyer (1934), which is similar in many respects to Miller & Van Slyke's. Extension to the determination of 1–12 μg. blood glucose by the drop-scale technique was carried out by Heck, Brown & Kirk (1937). To determine amounts of blood glucose of the order of 10 μg. without resorting to Kirk's specialized technique, Humoller (1943) titrated with 0·0004N-$Ce(SO_4)_2$, using a potentiometric method to detect the end-point. At such dilutions, ceric sulphate solutions are extremely unstable.

It was considered that the use of the microburette developed by Conway (1939) should offer many advantages in the cerimetric determination of reducing sugars, and a satisfactory procedure for glucuronic acid was worked out on this basis.

It was found possible to estimate 10–300 μg. glucuronic acid, using a concentration of ceric sulphate which is perfectly stable. Somogyi's copper tungstate technique, as modified by Giragossintz *et al.* (1936), proved satisfactory for the removal of protein. Further modification was necessary for protein precipitation at the acid pH required for glucuronidase studies. The addition of solid barium carbonate, as recommended by Miller & Van Slyke (1936) for cadmium hydroxide precipitation, was found to prevent interference by copper during reduction of ferricyanide when the initial pH was as low as 4·25. Since the use of barium carbonate made conditions in the precipitation of protein much less critical, with consequent improvement in end-points, its use in neutral solution was found to be of advantage. The procedure worked out for glucuronic acid was found to be applicable to the determination of blood glucose without any important alteration.

EXPERIMENTAL

Reagents

Unless otherwise stated, reagents were of analytical grade.

Copper sulphate. 25 g. $CuSO_4.5H_2O$ dissolved in water and made to 1 l.

Alkaline sodium tungstate. 25 g. $Na_2WO_4.2H_2O$ and 5 g. anhydrous Na_2CO_3 made to 1 l.

Barium carbonate, powdered.

Alkaline potassium ferricyanide. Prepared daily by diluting 1 ml. 5% (w/v) $K_3Fe(CN)_6$ to 10 ml. with 11% (w/v) Na_2CO_3. The $K_3Fe(CN)_6$ should be tested for ferrocyanide as described by Folin (1928).

Sulphuric acid. (*a*) 11N-H_2SO_4. Tested for reducing substances (Miller & Van Slyke, 1936). (*b*) N-H_2SO_4. Prepared from 11N.

Indicator. 0·03% setopaline C.

Ceric sulphate. (*a*) approximately 0·3N-$Ce(SO_4)_2$. Prepared from the solid (British Drug Houses Ltd, 'low in other rare earths') according to the directions of Miller & Van Slyke (1936). (*b*) approximately 0·012N-$Ce(SO_4)_2$. 10 ml. 0·3N diluted to 250 ml. after addition of 23 ml. 11N-H_2SO_4.

Ferrous ammonium sulphate. (*a*) 0·1N-$Fe(NH_4)_2(SO_4)_2.6H_2O$. 9·8035 g. solid dissolved in 60 ml. water and, after addition of 25 ml. 11N-H_2SO_4, diluted to 250 ml. (*b*) 0·002N-$Fe(NH_4)_2(SO_4)_2.6H_2O$. 2 ml. 0·1N diluted to 100 ml. after adding 10 ml. 11N-H_2SO_4, just before use.

d-*Glucurone.* Standard solutions prepared from solid (La Roche) immediately before use; m.p. 175–177° (Stacey, 1939, gives m.p. as 176–178°).

Procedure

To 0·8 ml. of a solution containing not more than 300 μg. glucuronic acid, in a 10 ml. centrifuge tube, were added 0·6 ml. $CuSO_4$ solution and, after mixing, 0·6 ml. Na_2WO_4 in Na_2CO_3 solution. The tube was closed with a clean rubber stopper and vigorously shaken. A pinch (about 10 mg.) of $BaCO_3$ was dropped in and, after being allowed to stand for 10 min., the tube was again shaken. The stopper was removed and the tube centrifuged for 5 min.

With a dry blow-out pipette, 1 ml. supernatant was transferred to a fresh 10 ml. centrifuge tube. After addition of 0·25 ml. $K_3Fe(CN)_6$ in Na_2CO_3 solution, a glass bulb was placed in the mouth of the tube, which was inserted in a metal stand and placed in vigorously boiling water for 15 min. Stand and tubes were then transferred to cold, running water for 3 min.

Immediately before titrating, 0·2–0·25 ml. 11N-H_2SO_4 was added to the contents of the tube, followed by 1 or 2 drops of indicator after shaking. The tube was slipped over the jet of the Conway burette and held in place by sliding the titration platform under it. Air stirring by means of a capillary tube attached to the burette jet was commenced. When vigorous CO_2 evolution had ceased, 0·012N-$Ce(SO_4)_2$ solution was run in till an end-point stable for 30 sec. was reached. The colour change was from yellow or greenish yellow to red-brown.

The microburette. The microburette had a ground-glass joint in place of the rubber stopper in the standard model described by Conway (1939), and the reservoir was shielded from light. No change in the titre of the $Ce(SO_4)_2$ in the reservoir was observed in a year under these conditions. In

using the burette with the strongly acid solution it was found necessary to grind in the stopcocks till only a trace of lubricant was required. At the beginning of each day, the glass tubing in the burette was washed out with fresh $Ce(SO_4)_2$ solution from the reservoir, and the jet and air capillary were rinsed with distilled water between titrations. A daylight lamp was found to be of considerable assistance in observing the end-point.

Reagent controls. These were carried out concurrently with all estimations, and titration figures were corrected for the value of the blank. The reagent blank in pure solution is due largely to the reduction of ferricyanide on heating with alkali, the total, including excess $Ce(SO_4)_2$ required to change the colour of the indicator, varying from 0·009 to 0·015 ml. 0·012N-$Ce(SO_4)_2$. The amount of ferrocyanide initially present in the alkaline ferricyanide could be reduced to negligible proportions by the use of freshly prepared, pure reagents, while the protein precipitants added nothing to the blank. Efforts to decrease the 'latent' ferricyanide blank led to a corresponding fall in the amount of reagent reduced by a given quantity of glucuronic acid.

Standardization. The $Ce(SO_4)_2$ solution may be standardized before or after dilution of the 0·3N-stock solution, using the appropriate concentration of $Fe(NH_4)_2(SO_4)_2$. In standardizing the solution in the Conway burette, two different quantities of 0·002N-$Fe(NH_4)_2(SO_4)_2$ were measured into centrifuge tubes and the volume made up to about 1·5 ml. with N-H_2SO_4 prior to adding indicator. If the ml. of $Fe(NH_4)_2(SO_4)_2$ were plotted against ml. of $Ce(SO_4)_2$, the straight line joining the two points cut the $Ce(SO_4)_2$ axis at the volume causing a colour change in 1·5 ml. N-H_2SO_4 (0·001–0·003 ml.), while its slope gave the normality of the $Ce(SO_4)_2$ solution.

Determination of the glucuronic acid equivalent. The reaction between $K_3Fe(CN)_6$ and glucuronic acid (or glucose; cf. Nimmo-Smith, 1946), although following a linear relationship, was not stoiochiometric, and it was necessary to determine the glucuronic acid equivalent of the $Ce(SO_4)_2$ solution under the conditions specified. Varying amounts of *d*-glucurone in 1 ml. water were treated with ferricyanide and the ferrocyanide formed titrated as described above. The factor was found to remain unchanged over the period examined, 1 μg. glucuronic acid being equivalent to 0·002 ml. 0·0124N-$Ce(SO_4)_2$. This factor was calculated from the relationship found with $Ce(SO_4)_2$ solution of slightly different concentration. No change in the factor was produced by reducing the concentration of Na_2CO_3 in the alkaline ferricyanide from 10 to 1%.

The use of *d*-glucurone as a standard in determining glucuronic acid rests on the assumption that these substances in equivalent amounts have the same reducing power under the conditions of the estimation. Goebel & Babers (1933) found that while glucuronic acid and its lactone had the same reducing power by Bertrand's method, they differed slightly in this respect when the method of Shaffer & Hartmann was used. No direct evidence is available as to the relative reducing values of the two substances for potassium ferricyanide in concentrated sodium carbonate, but it seems unlikely that the difference between them, if such exists, will be appreciable compared with the experimental error in glucuronidase studies.

Since only half the glucuronic acid in the sample was taken for treatment with ferricyanide, μl. 0·0124N-$Ce(SO_4)_2$ in the final titration corresponded to μg. glucuronic acid in the protein-containing solution. More than 1 ml. supernatant could be taken after protein precipitation provided that the procedure was suitably modified. Under the conditions described, 0·25 ml. alkaline ferricyanide was completely reduced by 153 μg. glucuronic acid.

RESULTS

Samples were prepared containing 0·05N-acetate buffer, varying amounts of glucuronic acid and 0·2 ml. of a glucuronidase preparation in a total volume of 0·8 ml. The enzyme was taken at different stages of purity during its preparation from beef spleen. Since, prior to using barium carbonate during the protein precipitation, false results were sometimes obtained when no protein was present, determinations were also made in which the enzyme in the mixture described was replaced by water. Recoveries obtained at varying initial pH are shown in Table 1.

All results have been corrected for reducing substances originally present in the enzyme preparation. The total blank from this and other sources was always less than 0·03 ml. 0·012N-$Ce(SO_4)_2$. It can be seen from the table that the error tended to increase with falling pH. In spite of all precautions, it was found impossible to carry out determinations in absence of protein at pH 4, and even when enzyme was present the results were unsatisfactory.

In a series of 28 determinations in which 99·5 μg. glucuronic acid were added to enzyme buffered to pH 5, the mean recovery was 95·0 μg. and the standard deviation of a single observation from the mean was 2·4 μg.

DISCUSSION

While not so sensitive as the method of Heck *et al.* (1937) the procedure described in the present paper for the cerimetric determination of reducing sugars has many advantages, not the least of which is the stability of the ceric sulphate solution used. The simplicity in construction and convenience in operation of the Conway microburette make it very suitable for routine determinations.

The efficiency of copper tungstate precipitation in retaining reducing material derived from animal tissues, other than glucose, has been discussed by Giragossintz *et al.* (1936). These workers recommended the use of sodium carbonate to precipitate

Table 1. *Recovery of glucuronic acid at varying initial* pH *in presence and absence of glucuronidase*

	Enzyme present				Enzyme absent			
	Glucuronic acid		Error		Glucuronic acid		Error	
pH	Added (μg.)	Recovered (μg.)	(μg.)	(%)	Added (μg.)	Recovered (μg.)	(μg.)	(%)
7	187	181	−6	3	156	158	+2	1
	125	118	−7	6	94	96	+2	2
	62	61	−1	2	37	35	−2	5
	37	40	+3	8	31	32	+1	3
	31	31	0	0	19	19	0	0
	19	18	−1	5	—	—	—	—
	12	9	−3	25	—	—	—	—
5	185	175	−10	5	185	182	−3	2
	155	147	−8	5	93	93	0	0
	124	118	−6	5	37	39	+2	5
	37	34	−3	8	31	31	0	0
	37	40	+3	8	19	18	−1	5
	31	28	−3	10	—	—	—	—
	19	17	−2	11	—	—	—	—
	12	11	−1	8	—	—	—	—
4·25	187	173	−14	7	94	91	−3	3
	125	116	−9	7	37	36	−1	3
	62	58	−4	6	31	35	+4	13
	37	36	−1	3	19	18	−1	5
	31	35	+4	13	—	—	—	—
	19	16	−3	16	—	—	—	—
	12	10	−2	17	—	—	—	—
4	187	196	+9	5	—	—	—	—
	125	134	+9	7	—	—	—	—
	62	65	+3	5	—	—	—	—
	37	31	−6	16	—	—	—	—
	31	31	0	0	—	—	—	—
	19	9	−10	53	—	—	—	—
	12	5	−7	58	—	—	—	—

last traces of Cu^{++}. At acid pH, however, this step did not prevent Cu^{++} passing the precipitate, and increasing the amount of sodium tungstate or sodium carbonate was of no assistance since it raised the solubility of the copper tungstate. Within the pH range 4·25–7, addition of barium carbonate prevented interference by Cu^{++}. Indeed, with the quantities of copper sulphate and sodium tungstate specified above, sodium carbonate could be entirely replaced by barium carbonate if sufficient time were allowed to elapse for complete reaction. Outside the pH range 4·25–7, it would be necessary to bring the solution to neutrality prior to precipitation of protein by the present technique. The concentration of the reagents added for protein precipitation could not be much increased beyond the figures given since the concentration of glucuronic acid (or glucose) in the supernatant was then found to be no longer representative of the total concentration in the contents of the centrifuge tube.

The use of trichloroacetic acid for removal of protein in determining glucuronic acid, as in the procedure of Fishman (1939), cannot be recommended. Trichloroacetic acid, unlike copper tungstate, appreciably increased the reagent blank, and it allowed rather a large amount of reducing material, other than glucuronic acid, to escape the precipitation. A more serious error in Fishman's technique arose from the use of phenolphthalein as indicator in neutralizing the protein-free filtrate. Phenolphthalein, although itself invisible, made the end-point in the ceric sulphate titration very difficult to observe and greatly increased the blank.

The same factor for the glucuronic acid equivalent was obtained when the concentration of sodium carbonate present during the reduction of the ferricyanide was decreased to one-tenth. The use of concentrated sodium carbonate was, however, preferred in order to decrease the interference caused by sodium bicarbonate (cf. Giragossintz *et al.* 1936). Acetate ion, as such, did not interfere in the reduction, but the possibility of bicarbonate formation through reaction between acetic acid in the buffer and carbonate added in the protein precipitation had to be taken into account in applying the method to glucuronidase studies. Altering the ferricyanide concentration to any considerable extent led to a change in the glucuronic acid equivalent.

The volume of oxidizing agent necessary for a colour change in the volume of solution titrated corresponded to 1 μg. glucuronic acid. In practice, however, the lower limit of the method was largely determined by the amount of ferricyanide reduced

in the absence of glucuronic acid. This corresponded to about 5 μg. glucuronic acid. Obviously, by using more dilute ceric sulphate in the burette, with consequent sacrifice of stability, and less ferricyanide, the lower range of the method could be extended.

The error in recovery for any given initial pH appeared to be constant in amount over most of the glucuronic acid range studied. When the initial pH was 5, the standard deviation of a single observation from the mean was 2·4 μg.

SUMMARY

1. A method is described for the cerimetric determination of 10–300 μg. glucuronic acid in tissue preparations, using the Conway microburette. Protein is removed with copper tungstate and barium carbonate.

2. The error appeared to be constant in amount over most of the range of glucuronic acid values studied. Of 99·5 μg. glucuronic acid added, the mean recovery was 95·0 μg., the standard deviation of a single observation from the mean being 2·4 μg.

3. Various factors in the determination of reducing sugars were examined.

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The Recognition of Material Present in Horse Muscle Affecting the Formation of α-Toxin by a Strain of *Clostridium welchii*

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The α-toxin of *Cl. welchii* type A (*Cl. perfringens*) was shown to be a lecithinase by Macfarlane & Knight (1941); fairly good yields of α-toxin/ml. of culture filtrate were obtained by growing strain S 107 of the organism in a medium composed essentially of peptone (Evans Sons, Lescher and Webb Ltd.), glucose, and a protein-free muscle extract (Deutsch, Eggleton & Eggleton, 1938). The presence of the muscle extract appeared to increase α-toxin formation/ml. of culture filtrate. Various published observations had already indicated that the incorporation of muscle (as a solid) in culture media favoured the production of high potency α-toxin by *Cl. welchii* type A, although the function of the muscle remained undetermined. In order to investigate the factors which influence toxin formation by an organism it is necessary to take into account the alterations in the potency of culture filtrates/unit volume, due simply to variations in the mass of bacterial growth which may have occurred. Hence, in order to find whether substances which really enhance α-toxin formation (henceforth called toxigenic factors, or T.F.) were present in the muscle extracts, apart from substances directly affecting nutrition and growth, the amount of lecithinase (α-toxin) produced/ml. of medium should be related to the amount of bacterial growth which had occurred.

This paper records the examination of various materials for the presence of so-called toxigenic factors, i.e. material which increases the yield of α-toxin/unit weight of bacterial matter formed, and the attempt to characterize and purify such material from protein-free muscle extracts.

During the course of this work we freely interchanged information with Dr A. M. Pappenheimer, Jr., who for a time studied the same problem. Such liaison between scientists in the U.S.A. and Great

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