From the sedimentation and diffusion constant values obtained in a solution containing 1 g./100 ml. H substance, and using the determined value of the partial specific volume, the molecular weight was calculated from the usual equation (Svedberg & Pedersen, 1940) giving a value of 320 000. From the same data the frictional ratio f/f_o obtained was 4·2. This corresponds to an axial ratio of > 100, or may be interpreted as indicating a high degree of hydration.

DISCUSSION

The data indicate that electrophoretically the substance is essentially homogeneous, which means that the nitrogen in the preparation is not attributable to a protein contaminant but is an integral part of the molecule.

For a substance of molecular size 320 000 associated with the observed large frictional ratio which is reflected in the concentration dependence of the sedimentation constant, a rather sharper boundary would be expected to be maintained throughout sedimentation if the substance was strictly monodisperse. The molecular size appears to be rather higher than that previously reported for blood-group A substance (Kekwick, 1950) and Le^a substance (Kekwick, 1952), and there is evidence of a higher degree of molecular asymmetry.

The conclusion must probably be drawn that the substance is moderately polydisperse, but the data are insufficient to give a precise expression of the range and distribution of molecular size in the preparation.

SUMMARY

1. The blood-group H substance has been found essentially homogeneous electrophoretically.

2. The mean molecular weight of the substance calculated from sedimentation and diffusion data is 320 000 and the frictional ratio 4·2.

3. The substance is probably moderately polydisperse and the molecular shape highly asymmetrical.

I wish to acknowledge the technical assistance of Mr H. Murray.

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Citric Acid and Bone Metabolism

By T. F. DIXON and H. R. PERKINS
Institute of Orthopaedics, London, W. 1

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Since Dickens (1941) showed that 90 % or more of the citric acid of the body was located in the skeleton, and Class & Smith (1943) demonstrated its presence in various body concretions, evidence has been accumulating that this substance plays an important part in the metabolism of bone. A connexion between calcium and citric acid was established when Pincus, Peterson & Kramer (1926) found that these substances formed a soluble complex which is assumed to account for the non-ionizable part of the diffusible fraction of serum calcium. Gomori & Gulyas (1944) showed that citrate injections in dogs made serum calcium more ultrafiltrable so that a rapid urinary excretion of calcium took place, and similar results were obtained in dogs by Chang & Freeman (1950). A rise in serum calcium caused by parathormone was shown by Alwall (1944) to be accompanied by a rise in serum citrate, and in Gomori's experiments, in which dogs were injected with citrate, microscopic examination of the bone showed a picture similar to that observed after toxic doses of parathormone. The connexion between serum calcium and citrate was further established by Freeman & Chang (1950). These authors found that high doses of vitamin D administered to dogs raised not only the serum calcium but also the serum citrate, and suggested that this was due to increased liberation of these substances from the bone reservoir. A possible explanation for the presence of citrate in bone was indicated by the work of Kuyper (1938), who showed that the precipitate of calcium, phosphate and citrate formed *in vitro* in the presence of suitable concentrations of these substances is actually a complex such as may exist in bone. He also found that the solubility of the precipitate was increased

by the presence of citrate and magnesium in the solution.

The facts make it seem likely that calcification mechanisms may be influenced by the amount of citrate present in the circulating fluids and at the site of calcification. A study in bone of the enzyme systems concerned with the well known citric acid cycle seemed of interest in elucidating the role of citric acid in this tissue. Thus the enzymes concerned with the formation and further conversion of citric acid, namely citrogenase, aconitase and *iso*citric dehydrogenase, were studied in different bone regions. The effect of citric acid on the *in vitro* calcification of tibia slices from rachitic rats was also investigated.

EXPERIMENTAL

Calcification of bone slices from rachitic rats. The young white rats used were maintained on the 'Glaxo' rachitogenic diet for 14 days and then sacrificed. Immediately after death the tibiae were dissected out and scraped free of adhering tissue. Thin sagittal sections were then cut and incubated in the solution containing calcium and phosphate, with or without the addition of Mg or citrate. The basal solution contained NaCl, 0·075 M; $NaHCO_3$, 0·022 M; KCl, 0·005 M; $CaCl_2$, 0·002 M; sodium phosphate buffer, 0·0017 M, pH 7·4. This gives concentrations of Ca = 8 mg./100 ml. and P = 5 mg./100 ml. After incubation at 37° for the desired period, the slices were removed, washed in distilled water, and then stained with 1% (w/v) $AgNO_3$, washed, and allowed to blacken under the influence of light. With each pair of experimental sections two control sections were run, one incubated in the basal medium alone, and one in the same solution without Ca or P.

Preparation of tissues for enzyme experiments. For aconitase estimation the tissues were ground in a mortar with washed silver sand and 10 ml./g. of 0·05 M-phosphate, pH 7·4, and the centrifuged extract was used for determination of the enzyme content. In agreement with the results of Johnson (1939), the aqueous extract was found to contain practically all the activity of the whole tissue. Although there was a possibility that phosphate might, by its affinity for calcium, tend to prevent extraction of the enzyme from the bone, phosphate buffer and 0·9% NaCl solution were, in fact, found to extract the same amount of aconitase. It was convenient to use extracts, since they contained only small amounts of citric acid compared with the whole bone, and thus did not tend to obscure the enzymic formation from *cis*-aconitate.

When citrogenase or *iso*citric dehydrogenase were being estimated, the kidney and liver were finely minced, and the bone was chopped as uniformly and finely as possible. *iso*-Citric dehydrogenase was demonstrated qualitatively by the use of sliced bone, but attempts to show the enzyme in bone brei or extracts were completely unsuccessful. It was therefore concluded that excessive destruction of the tissue led to breakdown or inactivation of the small quantities of enzyme present in bone. For this reason, in subsequent quantitative experiments, chopped bone was employed, since the physical properties of the rabbit bones used prevented the cutting of slices. Experiments on the optimal conditions for the demonstration of the citrogenase content of the tissues also led to the conclusion that the minimum of destruction gave the best results. For example, in an experiment with rat liver, the following results were obtained: mg. citric acid formed/g. tissue (*a*) finely minced, 7·5; (*b*) whole homogenate, 5·0; (*c*) supernatant after centrifugation of homogenate, 0·7; (*d*) residue resuspended, 1·1. Minced tissue was used rather than slices so that a comparison could be made with the activity of bone.

Rabbit tissues were obtained from animals weighing about 600–700 g. The tissues studied were the kidneys, liver, red marrow, newly calcified metaphysis with the bony trabeculae, epiphyseal line cartilage and diaphyseal cortex of the tibiae and femora. After dissection, histological examination of the parts used showed that the portion described as metaphysis consisted mainly of bony trabeculae, which showed some cartilage inclusions, and a slight contamination with the cartilage of the epiphyseal plate. The epiphyseal cartilage portion consisted of the remainder of the epiphyseal plate, with a few bony trabeculae attached to the part opposite the metaphysis. The marrow and cortex specimens were uncontaminated by other tissue.

Estimation of citrogenase activity of tissues. The method used for estimation of the enzyme system which forms citrate from oxaloacetate and acetate was based upon the technique of Stern & Ochoa (1951). These authors point out that the reaction involves the conversion of acetate to acetyl phosphate, the transfer of the acetyl group to form acetyl-coenzyme A, and the final condensation of the acetyl group with oxaloacetic acid to form citric acid. In the present investigation the system as a whole was studied, and is referred to for convenience as 'citrogenase'.

The animals were killed by stunning and bleeding, and samples of 0·100 g. of finely minced kidney, liver and marrow, and about the same weight of chopped bone, were used for the experiments. The incubation medium consisted of 2 ml. of a solution containing sodium acetate, 0·01 M; KCl, 0·1 M; $MgSO_4$, 0·003 M; phosphate, pH 7·4, 0·005 M; cysteine, 0·01 M; adenosinetriphosphate (ATP), 0·002 M; oxaloacetate, 0·01 M. The final pH of the reaction mixture was brought to 7·4. In the control experiments the oxaloacetate was replaced by 0·01 M-acetate, so that when bone was used extraction of the tissue citrate during the incubation took place to the same extent in the control as in the experimental tubes. The weighed tissue was added to the medium and incubated at 28° for 1 hr. with constant agitation. It was shown experimentally that even with an active tissue like kidney the rate of formation was essentially constant during the first hour. The reaction was stopped by the addition of 1 ml. of 25% (w/v) trichloroacetic acid (TCA), and the supernatant after centrifugation was used for citrate determination. In the case of the bone, in order to demonstrate the formation of relatively small amounts of citrate in the presence of the large amounts contained in the tissue, after incubation the tubes were cooled in ice and centrifuged. The supernatant, together with water with which the residue had been further washed, was then treated with TCA as above. Duplicate experiments showed that this method gave a sufficiently uniform and small extraction of the bone citrate to enable the formation of citric acid in the supernatant by the enzyme reaction to be estimated. It was also found that when bone was first boiled to inactivate its enzymes, no citrate formation was observed by this method. In view of the low bone citrogenase activity observed, it was thought possible that the system might be lacking in coenzyme A (see Stern & Ochoa, 1951). Therefore, in one series of experiments, coenzyme A powder prepared from

pigeon liver, and shown by activation of sulphanilamide acetylation to have an activity of 1·4 units/mg. (Kaplan & Lipmann, 1948), was added to the medium. However, little if any increased citrate formation was observed and therefore coenzyme A was not employed in subsequent experiments.

Estimation of aconitase activity of tissues. The method of estimating aconitase activity was based upon that of Johnson (1939). *cis*-Aconitic anhydride was prepared by the method of Anschütz & Bertram (1904) and neutralized with $NaHCO_3$ in the cold immediately before use. The sample thus prepared contained no citric acid. For aconitase estimation, 0·5 ml. of the centrifuged tissue extract was incubated at 37° with 0·5 ml. of a solution containing the equivalent of 1 mg. of *cis*-aconitic anhydride. The period chosen was such that not more than 50 % of the *cis*-aconitate had been converted to citrate, in the case of the bone, 1 hr., and for the soft tissues, 10 min. After incubation, the reaction was stopped by the addition of 0·5 ml. of 25 % (w/v) TCA and the resulting solution was used for citric acid determination.

Estimation of iso*citric dehydrogenase activity of tissues.* Our first successful qualitative demonstrations of *iso*citric dehydrogenase activity in bone were carried out according to the technique of Follis & Berthrong (1949). Thin free-hand sections of freshly dissected rat bone (humerus was found to be the most suitable because of the strong trabeculae in the epiphysis) were cut to include the epiphysis, epiphyseal line and the metaphysis. The sections were incubated anaerobically on a microscope slide at 37° with 0·1 M substrates dissolved in physiological saline solution (Robinson, 1949). Dehydrogenase activity was observed in a low-power microscope by the reduction of either methylene blue, with which the sections had been previously treated, or triphenyltetrazolium chloride contained in the solution (1 mg./ml.), the latter forming a red insoluble compound on reduction.

Triphenyltetrazolium was then used for the quantitative determination of the enzyme according to the method of Kun & Abood (1949). Although the substrate for the enzyme is *iso*citric acid, the substance added to the medium in these experiments was citric acid. Since the tissues have been shown in other experiments (see below) to contain sufficient aconitase to ensure the establishment of the equilibrium

$$\text{citrate} \rightleftharpoons \textit{cis}\text{-aconitate} \rightleftharpoons \textit{iso}\text{citrate},$$

this procedure was considered to be justified. To the weighed tissue (about 0·1 g.) were added 1·5 ml. of 0·05 M-phosphate, pH 7·4, 0·5 ml. of water or 0·2 M-sodium citrate and 0·5 ml. of 0·2 % (w/v) triphenyltetrazolium chloride. After mincing, the tubes were incubated at 37° with intermittent shaking for a suitable period, which was found to be 10 min. for the kidney and liver, and 2 hr. for the bone. After incubation the tubes were cooled in ice and 7 ml. of acetone were added to each and shaken. The mixture was centrifuged and the supernatant, after filtration if necessary, was used for comparison of colour intensity against an arbitrary standard. The amount of reduced dye present was then read from a standard curve prepared from known amounts of triphenyltetrazolium, reduced under standard conditions by $Na_2S_2O_4 . 2H_2O$. By this method, a reproducible straight-line relationship between colour intensity and concentration was obtained.

Oxidative removal of citrate by minced tissues. In the experiments on the oxidative removal of added citrate the following technique was employed. To weighed samples of minced kidney or chopped bone 7·2 ml./g. of a physiological salt solution, pH 7·4 (Krebs & Henseleit, 1932), containing 7 mg./ml. of citric acid, were added. The gaseous phase used was 5 % (v/v) CO_2 in O_2 at atmospheric pressure, and the flasks were incubated at 37° with constant agitation for the desired period. The reaction was stopped by the addition of 1 ml. of 25 % (w/v) TCA, and after centrifugation the supernatant was used for citric acid determination. In the bone experiments the same technique of centrifugation before the addition of TCA was used as that described in the method for citrogenase.

Estimation of the alkaline phosphatase content of tissues. The tissue was treated with 20 ml./g. of $CHCl_3$-saturated water, ground with washed silver sand and allowed to autolyse overnight at 3°. The centrifuged extract was used for alkaline phosphatase determination by a method essentially similar to that of Shinowara, Jones & Reinhart (1942).

Determination of citric acid. Citric acid was determined in the TCA extracts by the colorimetric method of Weil-Malherbe & Bone (1949). A standard curve was employed, and a standard was included with each determination.

RESULTS

Influence of citrate on the calcification of rachitic cartilage. Following the technique of Robison (1932) the influence of magnesium and citrate on the *in vitro* calcification of bone slices from rachitic rats was studied. The concentrations used were only a little higher than those normally occurring in blood. A number of experiments was performed, and typical results are given in Table 1. From these it

Table 1. *Inhibition of* in vitro *calcification of bone slices from rachitic rats by citrate and magnesium*

(Degree of calcification was graded from 0 (no calcification) to + + + + (complete calcification).)

	Calcification after	
	8 hr.	17 hr.
Basal medium	+ +	+ + + +
Basal medium + 5×10^{-4} M-citrate	0	+
Basal medium + 5×10^{-4} M-citrate + 1×10^{-3} M-magnesium	0	+
Basal medium + 1×10^{-3} M-magnesium	+ +	+ + +

can be seen that, although magnesium (as sulphate) at a level of 1×10^{-3} M produced only a slight inhibitory effect on calcification, sodium citrate at 5×10^{-4} M produced a marked inhibition. This amount of citrate, which is slightly greater than the normal level in blood, would be sufficient to form a complex with about 3 mg./100 ml. of the ionized calcium. Shipley, Kramer & Howland (1926) had previously shown that calcification is inhibited if calcium is supplied as calcium citrate. Here it is shown that the addition of citrate equivalent to a part only of the calcium has the same effect.

Citrogenase. Rat or rabbit kidney and liver were found to form citrate very readily under the conditions used, and, in one experiment with rat kidney, 32 % of the oxaloacetate was converted into citrate, a figure higher than those usually reported in the literature (e.g. Green, Loomis & Auerbach (1948), 18 %). Much lower citrogenase activities were found in bone, and the comparison between these results and those for kidney and liver is given below.

Table 2. *Aconitase activities of rib parts of young rats*

Part of rib	Aconitase activity (mg. citric acid formed/g. tissue/hr.)
Bone	10·2
Costochondral junction	18·3
Cartilage	9·4

Aconitase. Of the three enzymes of the citric acid cycle studied, aconitase was found in greatest amount in bone. In addition to the results obtained for kidney, liver and various parts of the bones of rabbits given below, it is of interest to mention that the ribs of young rats, and the costochondral junctions in particular, were also shown to have a high aconitase activity (Table 2). Here, as with the rabbit bone, the actively calcifying region had a higher enzyme activity than either the bony or cartilaginous rib.

iso*Citric dehydrogenase.* Bone slices produced considerable reduction of hydrogen-accepting dye when either succinate or citrate was present as a substrate. Typical results obtained with the humerus of a young rat are given in Table 3. The region in which reduction of dye first appeared was that in the immediate vicinity of the centre of the epiphyseal-line cartilage. As the experiment proceeded the area of reduction extended to include a roughly circular area centred on the region where a change was first observed. The reduced form of triphenyltetrazolium chloride did not reoxidize in air as did that of methylene blue, and the former dye was therefore used in the quantitative experiments.

Comparison of activities of citrogenase, aconitase, isocitric dehydrogenase, alkaline phosphatase and of content of citric acid in rabbit tissue. Enzyme activities and citric acid contents of the various tissues are given in Table 4. Alkaline phosphatase is included for comparison. Citric acid contents of the kidney, liver and marrow were very low indeed, whereas considerable amounts were found in each of the bone regions studied, hard fully calcified cortex having the highest content. The citrogenase, aconitase, *iso*citric dehydrogenase and phosphatase activities of the metaphysis and epiphyseal cartilage are much greater than those of the cortex. In order to facilitate comparison, the enzyme activity of the more active parts of the bone are given in Table 5 as fractions of the activity of the kidney and liver respectively. From this it can be seen that the citrogenase activities of the metaphysis and epiphyseal plate are about one-quarter and one-sixth respectively of the activity of the kidney, whereas the

Table 3. *Dehydrogenase activities of rat humerus slices*

(Dye reduction observed under a low-power microscope. Dehydrogenase activities graded from 0 (no activity) to + + + + (maximum activity).)

Added substrate	Condition	Reduction of dye: Methylene blue	Reduction of dye: Triphenyltetrazolium
None	Aerobic	0	+
None	Anaerobic	+	+
Acetate	Anaerobic	+	+
Citrate	Anaerobic	+ + + +	+ + + +
Succinate	Anaerobic	+ + +	+ + +

Table 4. *Citric acid content and enzymic activities of rabbit tissues*

(Citrogenase and aconitase activities expressed as mg. citric acid produced/g. tissue/hr. *iso*Citric dehydrogenase activity expressed as mg. *iso*citric acid decomposed/g. tissue/hr. calculated from dye reduction. Alkaline phosphatase activity expressed as mg. P liberated/g. tissue/hr. Number of determinations given in brackets after each figure.)

Tissue	Citrogenase	Aconitase	*iso*Citric dehydrogenase	Alkaline phosphatase	Citric acid (mg./g.)
Kidney	2·12 (4)	133 (3)	2·05 (4)	3·78 (2)	—
Liver	1·27 (4)	54 (3)	1·37 (4)	0·62 (2)	—
Marrow	0·26 (4)	8·6 (2)	0·014 (3)	0·99 (2)	—
Metaphysis	0·51 (3)	11·9 (2)	0·041 (4)	5·49 (2)	6·7 (4)
Epiphyseal line cartilage	0·31 (4)	12·1 (3)	0·038 (3)	3·15 (2)	7·1 (4)
Bone cortex	0·04 (3)	0·61 (3)	0 (4)	0·41 (2)	9·5 (4)

Table 5. *Ratios of enzyme activities in rabbit bone in kidney and liver*

(Number of determinations given in brackets after each figure.)

Ratio	Citrogenase	Aconitase	*iso*Citric dehydrogenase
Metaphysis/kidney	0·25 (3)	0·096 (2)	0·023 (4)
Epiphyseal line cartilage/kidney	0·16 (4)	0·096 (2)	0·017 (3)
Metaphysis/liver	0·45 (3)	0·222 (2)	0·043 (4)
Epiphyseal line cartilage/liver	0·28 (4)	0·210 (2)	0·025 (3)

*iso*citric dehydrogenase activities of the same tissues are only about one-fortieth and one-sixtieth respectively of the kidney value. The ratios for the aconitase lie somewhere between. It thus appears that in the bony tissues there is a relative excess of the citric acid-forming enzyme over the citric acid-removing enzyme, since the aconitase merely serves to establish the equilibrium

citrate (89·5 %) ⇌ aconitate (4·3 %) ⇌ *iso*citrate (6·2 %)

(Krebs & Eggleston, 1943).

Oxidative removal of citrate. The very low level of *iso*citric dehydrogenase present in bone was confirmed in experiments on the removal of added citrate. Whereas the kidney showed considerable activity by the method used (disappearance of added citrate after 1 hr., 29 %; after 2 hr., 43 %; after 4 hr., 61 %) no continued removal by bone could be demonstrated (disappearance of added citrate: after 1 hr., 10 %; after 2 hr., 10 %; after 4 hr., 10 %). The small initial disappearance of citrate was evidently due to the activity of the aconitase present, which converted a small proportion of the citrate into aconitate and *iso*citrate. This hypothesis was confirmed by use of a kidney extract containing aconitase but no dehydrogenase, which had the same effect of causing the disappearance of a constant small fraction of the added citrate.

Table 6. *Removal by minced rat kidney of citrate added either as bone powder or as sodium citrate*

(Approx. 7 mg. citric acid added per g. kidney in each case.)

Exp. no.	Form of added citrate	Incubation (hr.)	Citrate found (% of initial)
1	Acetone-dried rat bone powder	0	100
		1	87
		3	81
	Sodium citrate	0	100
		1	39
		3	12
2	Ox-bone cortex powder (300 mesh)	0	100
		1	97
		3	96
	Sodium citrate	0	100
		1	58
		3	14

The availability of bone citrate for enzymic removal. The active aconitase and *iso*citric dehydrogenase of rat kidney were used in an attempt to metabolize further the citrate present in bone. The bone used was either a fine powder made from acetone-dried rat bone, or powdered ox-bone cortex (300-mesh). The activity of the minced kidney used was shown by a parallel experiment in which citrate was added in the form of sodium citrate. The results are given in Table 6, from which it can be seen that the citrate present in the rat bone was removed to a small extent by the active kidney enzymes, whilst in the more highly calcified ox-bone cortex only a slight amount of the citrate was removed.

DISCUSSION

It appears that all the parts of bone examined have, by comparison with other tissues, e.g. kidney or liver, citrogenase and aconitase activities much greater than those of *iso*citric dehydrogenase. The mechanism for the production of a local high concentration of citric acid therefore exists in bone. Such citric acid may be co-precipitated with calcium phosphate during its deposition, in which case it may influence the amount of deposition by holding up calcium in a complex form. A high citrate concentration could even reverse the process of calcification and solubilize bone salt already laid down. Excess of citrogenase over *iso*citric dehydrogenase in marrow does not lead to citrate deposition since no bone salt is laid down anyway.

SUMMARY

1. It has been shown that the 'citrogenase' and aconitase activities of various rabbit bone regions are relatively greater than that of *iso*citric dehydrogenase compared with kidney and liver.

2. In extension of earlier observations increasing concentrations of citrate inhibit the calcification of hypertrophic rat cartilage *in vitro*.

3. Enzyme levels in bone indicate the relatively greater metabolic activities of metaphysis and epiphysis over that of cortex.

4. Amounts of citrate in bone regions are in inverse ratio to their metabolic activities.

5. It is suggested that the higher 'citrogenase' and lower *iso*citric dehydrogenase levels in bone regions may produce local increased concentrations of citric acid which may then become co-precipitated during deposition of bone salt.

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The Determination of Glucosamine by Alkaline Decomposition

By M. V. TRACEY
Rothamsted Experimental Station, Harpenden, Hertfordshire

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Estimation of free glucosamine is usually done by the method of Elson & Morgan (1933) or by modifications of this method. Some workers have found difficulty in getting reproducible results unless conditions are most rigorously standardized (Ogston & Stanier, 1950; Johnston, Ogston & Stanier, 1951; Schloss, 1951). This may be explicable on the grounds that both the initial reactants, the amino sugar and acetylacetone, are unstable under the conditions of reaction and that several products are formed, including a number of chromogens (Schloss, 1951). The specificity of the method is not complete; acetylglucosamine gives a similar colour and so do mixtures of amino-acids and sugars (Sideris, Young & Krauss, 1938; Horowitz, Ikawa & Fling, 1950; Immers & Vasseur, 1950). Urea and glyceraldehyde have also been reported as interfering (Lutwak-Mann, 1941). Recently a new colorimetric method in which deamination of the amino sugar precedes its estimation by heating with indole and hydrochloric acid has been proposed by Dische & Borenfreund (1950). This has been reported as giving high results in the presence of other sugars (McCrea, 1951). It has been found that an essentially similar method which had been developed using orcinol for colour development after deamination also gave high results in the presence of other sugars.

The method presented here which has been communicated in a preliminary form (Tracey, 1951) is based on the observation by Morgan (1936) that glucosamine and galactosamine 'yield practically the whole of their nitrogen in the form of ammonia on heating for a few minutes with normal alkali at 100°'. Conditions under which the yield of ammonia is quantitative and in which interference by other substances is minimal are described.

EXPERIMENTAL

Reagents

Phosphate-borate mixture. Saturated Na_3PO_4 is saturated with $Na_2B_4O_7.10H_2O$ at room temperature (at 14°, 1 l. of saturated Na_3PO_4 will dissolve about 42 g. of borate).

Nessler's reagent. This was prepared after Vanselow (1940). 45·5 g. HgI_2 and 34·9 g. KI are dissolved in as little water as possible, 112 g. KOH added and the solution diluted to 1 l.

Procedure

The sample, which should be 2 ml. or less in volume and not strongly acid, is introduced into a Markham (1942) still. The sample is washed in with a total of 5 ml. of the phosphate-borate mixture. Distillation is then carried out slowly (about 3 ml. distillate/min.), the distillate being collected in a test tube matched for use in a photoelectric colorimeter and graduated at 10 ml. Before use 1 ml. of 2% (w/v) boric acid is placed in each tube. Distillation is stopped when the distillate reaches the 10 ml. mark on the receiving tube. Blanks are distilled at the beginning and end of a set of distillations, and also solutions of $(NH_4)_2SO_4$