inorganic ions obtained using samples representing any considerable volume of bone. Many of the earlier results for the rate of exchange of the inorganic constituents of the skeleton represent only mean values for a series of areas in different states of equilibrium with the plasma.

SUMMARY

1. The rate of exchange of phosphate in the cortical bone of cats is very much slower than in this type of bone in rabbits and rats. This difference does not extend to the trabeculae of the cancellous metaphyseal bones.

2. The distribution of the tracer within the

femoral and tibial cortical bone of rabbits shows a high degree of heterogeneity. The layer next to the marrow is always very active compared with the rest of the cortex. The outer layers (sub-periosteal) have a relatively low activity but are slightly higher than those from the depth of the bone.

3. The significance of this heterogeneity of distribution is discussed.

We take pleasure in acknowledging the help given to us by the Medical Research Council, who provided grants for two of us (S.M.W. and H.G.J.) and in thanking Dr Dawson and other members of the Biochemistry Department of the University of Leeds, for helpful discussion. Mr R. Lofthouse gave much technical assistance.

REFERENCES

- Amprino, R. (1952). *Experientia*, **8**, 20.
 Burns, C. N. & Henderson, N. (1936). *Biochem. J.* **30**, 1207.
 Dallemagne, M.-J. (1943). *La Nature de la substance minérale osseuse*. University of Liège.
 Engfeldt, B., Engström, A. & Zetterström, R. (1952). *Biochim. biophys. Acta*, **8**, 375.
 Hammett, F. S. (1925). *J. biol. Chem.* **64**, 409.
 Hevesy, G. (1948). *Radioactive Indicators*. 1st ed. New York: Interscience.
 Hevesy, G., Levi, H. B. & Rebbe, D. H. (1940). *Biochem. J.* **34**, 532.
 Lacroix, A., Devis, R. & Schicks, E. (1952). *Experientia*, **8**, 113.
 Logan, M. A. (1935). *J. biol. Chem.* **110**, 375.
 Manly, M. & Bale, W. F. (1939). *J. biol. Chem.* **129**, 125.
 Manly, R. S., Hodge, H. C. & Manly, M. (1940). *J. biol. Chem.* **134**, 293.
 Neuman, W. F. & Mulryan, B. J. (1950). *J. biol. Chem.* **185**, 705.
 Neuman, W. F. & Riley, R. F. (1947). *J. biol. Chem.* **168**, 545.
 Perrottet, E. & Duckert, R. (1951). *Experientia*, **7**, 419.
 Rogers, H. J. (1949). *Nature, Lond.*, **164**, 625.
 Rogers, H. J., Weidmann, S. M. & Parkinson, A. (1952). *Biochem. J.* **50**, 537.
 Rutishauser, E. & Majno, G. (1951). *Bull. Hosp. Jt Dis.* **12**, 468.
 Strobino, L. J. & Farr, L. E. (1949). *J. biol. Chem.* **178**, 599.
 Volker, J. F. (1942). *J. dent. Res.* **21**, 339.
 Volker, J. F. & Ginn, J. T. (1942). *J. dent. Res.* **21**, 339.
 Weidmann, S. M. & Rogers, H. J. (1950). *Biochem. J.* **47**, 493.
 Zetterström, R. (1952). *Biochim. biophys. Acta*, **8**, 283.

Bromide Determination in Body Fluids

By G. HUNTER

*Electro-medical Research Unit, Medical Research Council,
 Stoke Mandeville Hospital, Aylesbury, Bucks*

(Received 20 November 1952)

In a research involving the distribution of administered bromide between blood plasma and cerebrospinal fluid (c.s.f.), a method applicable to samples of about 1 ml. fluid containing from about 5 to 50 mg. bromine/100 ml. was required. The long-known colour reaction between bromide and auric chloride, used by Walter (1929), Wuth (1927) and others, was regarded as insufficiently accurate for our purposes. A number of methods depending on other principles seemed to be too elaborate or insufficiently proved.

A method based on the Van der Meulen (1931) reaction: $\text{Br}^- + 3\text{OCl}^- \rightarrow \text{BrO}_3^- + 3\text{Cl}^-$ has proved to

be suitable in most cases, and a preliminary report of this has already been given (Hunter, 1952). This principle was used by Dixon (1934) to determine bromide in normal blood, using 10 ml. The reaction was notably improved by D'Ans & Höfer (1934) who used phosphate instead of borate as buffer, and formate, instead of hydrogen peroxide, to destroy excess of hypochlorite. The reaction was further studied by Kolthoff & Yutzy (1937), and Friedman (1942) has used it to determine bromide in trichloroacetic acid filtrates from blood.

The present communication is based on the above principles, but the procedure has been modified and

the pretreatment of the material to be analysed has been described, with a view to giving increased speed and accuracy to the method.

EXPERIMENTAL

Reagents. 40% (w/v) $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; sodium hypochlorite, 1.0 N in 0.15 N-NaOH; sodium formate, 50% (w/v); sulphuric acid, 6N; potassium iodide, 20% (w/v); starch, 0.2% (w/v); sodium thiosulphate, 0.005 N; potassium hydroxide, 2.0 N; potassium carbonate, 2.0 N; ethanol, absolute.

No great precautions are necessary to avoid the presence of bromide in most reagents; very little seems to be present in most laboratory materials, except HCl and halides.

Method for simple solutions

The following has been found suitable for 50–350 μg . Br. To a solution of bromide (about 6 ml.) in a 100 ml. Erlenmeyer flask, are added 5.0 ml. phosphate buffer, followed by 3.0 ml. hypochlorite solution. The flask is then immersed in a boiling-water bath for 10 min. The flask is removed and 1.0 ml. formate solution is added. The flask is well shaken, and immersed again in the boiling-water bath for 5 min. The flask is then cooled in water and 5.0 ml. 6N- H_2SO_4 are added, followed by iodide solution (1.0 ml.). The solution is immediately titrated with 0.005 N- $\text{Na}_2\text{S}_2\text{O}_3$ using six drops of 0.2% starch solution as indicator. A blank determination is carried out at the same time, omitting the addition of bromide:

1 ml. 0.005 N- $\text{Na}_2\text{S}_2\text{O}_3 \equiv 66.7 \mu\text{g}$. Br.

Notes

(1) The aqueous volume at start should not exceed about 10 ml. When the volume was 25 ml., only about 80% of the Br present was recovered.

(2) Addition of up to 5 ml. 2N-NaOH has no effect on the results.

(3) Increasing the phosphate to 11.0 ml. has no effect on the results.

(4) One ml. of the hypochlorite solution is insufficient; with this amount less than 80% Br present is accounted for. If 6.0 ml. hypochlorite solution are used correct values are obtained, but the blank value is thus unnecessarily increased. A blank titration is indispensable in this method as a small amount of ClO_3^- is always present in the reagent. A rather lower blank has been found with a reagent made from fresh specimens of a commercial bleach called Parozone (6 vol. + 4 vol. water) than with the reagent as usually made by passing Cl_2 into cold NaOH solution. The blank in the present procedure, using diluted Parozone, is usually less than 0.20 ml. of 0.005 N-thiosulphate. This hypochlorite solution is stable for many months if stored in a refrigerator.

(5) One ml. formate solution is ample to destroy the excess hypochlorite.

(6) Five minutes' heating is insufficient to convert quantitatively the bromide to bromate.

(7) A carrier to hold nine flasks which can be immersed in a 26 cm. water bath has been found convenient.

Results

With this method bromide present can be quite accurately accounted for, as shown in Table 1.

Table 1. *Determination of bromide in simple solution*

Present	Br (μg)	
	Found	
320	319.6, 319.0, 319.0	
160	161.4, 159.0, 160.5	
50	50.6, 50.8, 49.7	
10	10.6, 10.2, 10.0	

Chlorides, even in considerable amount, have little effect on the method, and this is one of its great advantages. Indeed, Van der Meulen added NaCl to complete the reaction, but Kolthoff & Yutzy showed this to be unnecessary. This subject has been exhaustively studied by Haslam & Moses (1950) who used the method to determine Br in brines. The effect of varying concentrations of bromide-free NaCl on the blank values and with Br levels at 10 and 50 μg . was tested as shown in Table 2.

Table 2. *Effect of NaCl on blank titrations and on values using small amounts of bromide*

NaCl added (mg.)	Blank (ml. 0.005 N)	Br (μg)	
		Present	Found
0.0	0.18	10.0	10.0
		50.0	50.6
30.0	0.19	10.0	10.6
		50.0	50.6
60.0	0.18	10.0	10.0
		50.0	50.0
90.0	0.18	10.0	10.0
		50.0	49.2
500.0	0.16	50.0	39.3
1000.0	0.16	50.0	30.7
2000.0	0.17	50.0	29.3

On the other hand, iodide titrates quantitatively with the method. A commercial specimen of KI, which contained a small amount of Br, showed the following values: 103.6, 212.4, 314.0 and 419.6. (Present 100, 200, 300 and 400 μg . as I respectively.)

Application of method to body fluids

Some workers have used protein-free filtrates of blood directly for bromide estimation by this method, although one might expect interference through the presence of urea, phenols and other organic substances. Trichloroacetic acid filtrates of blood have been tried here and sometimes found to give nearly correct values, but gross inaccuracies have also been encountered. Results with ashed and unashed urine, shown in Table 3, illustrate this point. Accordingly, all bromide determinations on body fluids have been preceded by ashing.

The ashing of more than 0.1–0.2 ml. of blood or serum is tedious and if none of the bromide in blood is in organic combination a filtrate may be used. It should be such that it may be readily dried and ashed. Neither tungstic acid nor trichloroacetic acid seemed suitable for this, and as a strictly protein-free filtrate is not necessary, a procedure similar to that used by Conway & Flood (1936) has been adopted. The use of absolute ethanol, instead of 95% methanol, permits less dilution of sera. The procedure for deproteinization and ashing of the filtrate is as follows.

Table 3. *Effect of ashing on the determination of Br in urine*

(1 ml. of urine taken in each determination.)

Urine no.	Br added ($\mu\text{g./ml.}$)	Ashed		Not ashed	
		Br found ($\mu\text{g./ml.}$)	Error (%)	Br found ($\mu\text{g./ml.}$)	Error (%)
1	0	3	—	5	—
	100	102	-1	93	-12
	300	306	+1	278	-9
2	0	4	—	82	—
	100	100	-4	88	-52
	300	292	-4	81	-79
3	0	5	—	16	—
	100	102	-3	59	-49
	300	298	-2	166	-47

Table 4. *Recovery of bromide added to serum and to serum ethanol filtrates*

(To 3.0 ml. normal serum 2.4 mg. Br were added in 0.24 ml. solution. In other experiments, to the ethanol filtrate from 0.5 ml. original serum, 360 $\mu\text{g.}$ Br were added. In all cases 0.5 ml. solution was taken. All filtrates were dried and ignited in nickel crucibles and determinations completed as described in text. Values given are $\mu\text{g.}$ Br in 0.5 ml.)

Material	No. of detmsns.	Br added	Br present	Br found		
				Mean	s.d.	Range
Serum	1	0	2.9	—	—	—
Serum + bromide	6	370.3	373.2	370.9	2.2	367.1-373.8
Serum filtrate + bromide	6	360.0	362.9	360.5	1.8	359.0-363.7

To 4.0 ml. absolute ethanol in a centrifuge tube is added dropwise 1.0 ml. serum. (If less than 1 ml. is available the difference is made up with ethanol.) The contents are thoroughly mixed by rapid rotation of the tube between the hands. The contents are centrifuged, and 4.0 ml. of the supernatant fluid are transferred to a 30 ml. nickel crucible to which has been added 1 drop each of 2.0N-KOH and 2.0N- K_2CO_3 . The crucible is placed in an oven at 110° to dry.

With c.s.f. and urine the preliminary treatment with ethanol is usually not required, the necessary amounts of fluid being taken directly into the crucible with the small amount of fusion mixture.

The ashing is carried out with a half-ventilated ordinary bunsen flame, with the crucible on a pipe-clay triangle. The flame is directed downwards around the outside of the crucible successively at the corners of the triangle until patches of dull red appear. It is then directed symmetrically around the bottom of the crucible. The contents melt slightly as the crucible begins to redden. When a dull red has spread all over the bottom, the flame is removed and the crucible is transferred to a metal plate to cool. The whole procedure occupies little over a minute. There is no appreciable loss of bromide by this procedure; the presence of a little carbon has no harmful effect.

The contents of the crucible are now transferred to a 100 ml. Erlenmeyer flask as follows. With a pipette about 3 ml. water are run down the sides of the crucible. The material is stirred with a small glass rod and transferred to the flask. The process is repeated with about 2 ml. water, then 1 ml. water. To the flask are now added 5 ml. phosphate solution, and 3 ml. hypochlorite reagent and the procedure continued as described for simple bromide solutions.

Bromide added to blood sera is adequately accounted for as shown in Table 4.

DISCUSSION

The presence of large amounts of sodium chloride has a tendency to lower the blank value, but the effect is slight. The addition of up to 90 mg. has no appreciable effect on bromine values of 10 $\mu\text{g.}$ but the addition of 500 mg. sodium chloride or more lowers the values seriously even at the 50 $\mu\text{g.}$ level. With biological material the sodium chloride originally present could exceed 90 mg. only when relatively large amounts of material like blood and urine, were taken. Indeed, any chloride present in biological material is likely to be rather irrelevant where the hypochlorite reagent contains at least 1 % sodium chloride, and where Cl^- is a product of the main reaction. Nevertheless, when bromide values are low and the sodium chloride present exceeds 100 mg., a calibration curve showing the effect of chloride on the bromide levels in question should be constructed.

The fact that iodide is determined by this method does not invalidate it for present purposes, as the amount of iodide normally present in blood is negligible. However, in the use of the method with material from hospital patients, one should be aware of interference that may arise from iodide medication. Relatively large amounts of iodide are found not uncommonly in urine.

Determination of bromide by this method is accurate to about $\pm 1\%$, when the bromine present is greater than about 10 mg./100 ml. and when

0.5–1.0 ml. is taken. With bromine values from 3 to 10 mg./100 ml. the error is within $\pm 5\%$. A minimum of about 50 μg . bromine is desirable with the method, although with special care, fair estimates may be made with considerably less. Many hundreds of determinations have been performed with this method and it has been found very reliable and trouble-free. It is well adapted to the determination of bromine in many samples in a short time.

The method has also been used with whole blood and erythrocytes. In the latter case, haemolysis with an equal volume or 2 vol. of water has preceded precipitation. In the presence of large amounts of protein, methanol yields a more discrete precipitate and may be substituted for ethanol. If whole erythrocytes or haemoglobin solutions are ashed, the potential interference of ferric ions in freeing iodine from iodide should be borne in mind.

However, the concentration of phosphate present under the conditions described adequately suppresses this action.

SUMMARY

1. Details are given for the application of the Van der Meulen reaction—the oxidation of bromide to bromate by hypochlorite—to the determination of bromide in blood, cerebrospinal fluid and urine.

2. The method is not affected by the presence of chloride in moderate concentration, but iodide reacts quantitatively like bromide.

3. This semi-micro method is well adapted to the determination of bromide in body fluids following bromide administration.

4. The accuracy is about $\pm 1\%$ for samples containing 50 μg . bromide in 0.5 ml. fluid.

I am indebted to Mr A. A. Goldspink for technical assistance.

REFERENCES

- Conway, E. J. & Flood, J. C. (1936). *Biochem. J.* **30**, 716.
D'Ans, J. & Höfer, P. (1934). *Angew. Chem.* **47**, 71.
Dixon, T. F. (1934). *Biochem. J.* **28**, 48.
Friedman, M. M. (1942). *J. biol. Chem.* **144**, 519.
Haslam, J. & Moses, G. (1950). *Analyst*, **75**, 343.
Hunter, G. (1952). *Abstr. 2nd Int. Congr. Biochem., Paris*, p. 357.
Kolthoff, I. M. & Yutzy, H. (1937). *Industr. Engng Chem. (Anal.)* **9**, 75.
Van der Meulen, J. H. (1931). *Chem. Weekbl.* **28**, 82.
Walter, F. K. (1929). *Die Blut-Liquorschanke*. Leipzig: Thieme.
Wuth, O. (1927). *J. Amer. med. Ass.* **88**, 2013.

A Note on the Hydrolysis of the Urinary Conjugated Adrenocortical Steroids by β -Glucuronidase Preparations

BY R. I. COX AND G. F. MARRIAN

Department of Biochemistry, University of Edinburgh

(Received 10 November 1952)

It was reported by Kinsella, Doisy & Glick (1950) that the yields of 'reducing lipids' extractable from the urine of human subjects by a chloroform-ether mixture could be increased 3- to 5-fold by a preliminary incubation of the urine with an enzyme preparation obtained from *Escherichia coli* which was rich in β -glucuronidase activity. At about the same time Corcoran, Page & Dustan (1950) reported 10- to 20-fold increases in extractable formaldehydrogenic and glycogenic material after incubation of urine with a commercial preparation of β -glucuronidase. Shortly afterwards it was independently observed by Cox & Marrian (1951) that the yield of chloroform-extractable 'formaldehyde-genic steroid' obtainable from urine could be increased about tenfold, compared with that obtained after hydrolysis at pH 1.0 for 24 hr., by

incubating the urine, or butanol-extractable material from urine, with ox-spleen β -glucuronidase preparations. Following these pioneering studies, preliminary treatment of urine with bacterial or animal-tissue β -glucuronidase preparations has been widely used in quantitative studies on the urinary glycogenic, reducing, and formaldehyde-genic steroids, as well as in work directed towards the isolation and characterization of such steroids (cf. Venning, 1951, 1952; Kinsella, Baggett & Glick, 1951; Daughaday, Farr & Houghton, 1951; Corcoran, Dustan & Page, 1951; Cohen, 1951; Baggett, Glick & Kinsella, 1952; Schneider, 1952; Romanoff, Wolf & Pincus, 1952; Wilson, 1952; Bayliss, 1952). Cox (1952) has also recently shown that the yield of acetaldehyde-genic material, determined by periodate oxidation, which can be