The Separation and Determination of ^{24}Na and ^{32}P in Animal Tissues

By A. H. ENNOR and H. ROSENBERG
Department of Biochemistry, John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T. Australia

(*Received* 5 *May* 1952)

In experiments employing either ^{24}Na or ^{32}P, the determination of the radioactivity of each isotope in tissues or tissue extracts presents no difficulty, although inaccuracies may arise because of the lack of specificity of methods employed, especially if it is desired to measure the activity of ^{32}P when this is present as inorganic phosphate (cf. Ennor & Rosenberg, 1952*a*). Problems do arise, however, when both isotopes are simultaneously employed, and it is essential to assess the contribution of each to the total activity. For such a differentiation, Hevesy & Rebbe (1940) have taken advantage of the difference in the rates of decay of these isotopes. Thus, since ^{24}Na and ^{32}P have half-lives of 14·8 hr. and 14·3 days respectively, an initial measurement of the radioactivity of a mixture containing both these isotopes, followed by a measurement some days later, will permit the calculation of the contribution which each isotope made to the initial activity of the mixture, but the accuracy of such a procedure will depend upon the proportion in which each isotope was initially present. If the mixture contained roughly equal proportions of each, or an excess of ^{24}Na, such a procedure would be expected to give accurate results. On the other hand, if the radioactivity of ^{32}P present greatly exceeded that of ^{24}Na an error would almost certainly be introduced.

Experiments to be described later (Ennor & Rosenberg, 1952*b*) have employed amounts of ^{24}Na and ^{32}P such that the radioactivity of ^{32}P exceeded that of ^{24}Na by a factor of 5–10 and the present communication is concerned with a method for measuring each isotope accurately.

METHODS AND RESULTS

Principle of the separation. The method to be described depends on the separation of ^{32}P and ^{24}Na by preferential extraction of ^{32}P as a molybdate complex from an aqueous phase into *iso*butanol. ^{24}Na remains in the aqueous phase.

Measurement of radioactivity. In all cases radioactivity was determined as described by Ennor & Rosenberg (1952*a*).

Development of procedure and results

An initial experiment was carried out to determine the maximum amount of inorganic P which could be extracted from a 10 ml. volume without appreciable loss. For this purpose, a sample (10·0 g.) of skeletal muscle was taken from a rabbit which had received intravenously 1 mc. ^{32}P as $NaH_2{}^{32}PO_4$ 24 hr. before. The muscle sample was ashed in a Pt crucible in an electric muffle at about 800°. Complete oxidation of the material to clear inorganic salts was achieved in about 45 min. After cooling, the salts were dissolved in about 0·5 ml. of 10 N-H_2SO_4 and the solution transferred to a 25 ml. graduated flask. The crucible was repeatedly washed with small volumes of N-H_2SO_4 and the washings added to the original solution. When the combined washings reached about 15 ml. (fifteen washes) the radioactivity remaining in the crucible was checked by means of a conventional end-window counter. The counts remaining did not exceed the background count by more than 20 counts/min.

The combined washings were then allowed to stand in a water bath at 100° for 30 min. to hydrolyse pyrophosphate. After cooling to room temperature the volume was adjusted to 25 ml. with N-H_2SO_4. The inorganic P content and the total radioactivity were measured on a 1 : 100 dilution of this solution.

The precise procedure followed was varied somewhat throughout the three experiments, the results of which are given in Table 1.

In Exp. 1 a 1·0 ml. sample of the undiluted solution of inorganic salts was pipetted into a separating funnel, together with 2·0 ml. of 5% (w/v) NaCl, 0·4 ml. 10 N-H_2SO_4 and 4·1 ml. of water. Then 2·5 ml. of 5% (w/v) ammonium molybdate and 20 ml. of *iso*butanol (saturated with water) were injected simultaneously and the flask, after stoppering, was vigorously shaken for 10 sec. The separated aqueous layer was carefully drawn off into a second separating funnel containing 20 ml. of *iso*butanol, which had been previously saturated with water. This flask was then shaken for 10 sec.

Table 1. *Extraction of* ^{32}P *from aqueous phase with* isobutanol

	Exp. 1	Exp. 2	Exp. 3
Volume of sample (ml.)	1·0	5·0	5·0
Total radioactivity added (counts/min.)	5830	29 150	29 150
Inorganic P added (mg.)	0·725	3·62	3·62
Volume and concentration of ammonium molybdate added	2·5 ml. of 5% (w/v)	2·5 ml. of 5% (w/v)	2·5 ml. of 20% (w/v)
Total radioactivity (counts /min.) found in aqueous phase after extraction with *iso*butanol	8	15 590	40

Table 2. *The insolubility of* ^{24}Na *in* isobutanol

Radioactivity of aqueous phase (counts/min.)				Radioactivity in *iso*butanol (counts/min.)	
Aqueous layer*	Wash 1†	Wash 2†	Total	In presence of 2·5 ml. 20% (w/v) ammonium molybdate	In absence of ammonium molybdate
1750	125	5	1880	122	0

* Aqueous layer refers to the initial aqueous solution after extraction with *iso*butanol.

† Wash 1 and wash 2 refer respectively to the first and second volumes of N-H_2SO_4 with which the *iso*butanol was washed after the initial aqueous solution had been extracted and run off. All phases were made to 25 ml. before counting For further details see text.

and the aqueous layer collected in a 25 ml. volumetric flask. The first *iso*butanol extract was then shaken with 10 ml. of N-H_2SO_4 and this, after separation, was run into the second funnel where the aqueous layer was washed with *iso*butanol. This washed aqueous layer was then combined with the first and made to 25 ml. by the addition of water previously saturated with *iso*butanol. The solution, after mixing, was finally filtered through Whatman filter paper no. 1, and its activity measured. The use of *iso*butanol saturated with water was adopted to prevent the diminution in volume of the aqueous phase, due to the solubility of water in the solvent. Water, saturated with *iso*butanol, was used to adjust volumes to 25 ml. in order to avoid solution of the very fine drops of *iso*butanol which are commonly present in the aqueous phase. A final filtration through paper removes all such contamination.

Exps. 2 and 3 followed similar lines, except that 5·0 ml. samples were taken and in Exp. 3 the use of 20% (w/v) ammonium molybdate was adopted in place of the 5% (w/v) solution which had been used before. The volume of the aqueous phase was 10 ml.

The results (Table 1) show that in Exp. 1, in the presence of 0·725 mg. of inorganic P, the efficiency of the extraction procedure was such that only 8 counts/min. out of a total of 5830 counts/min. were not extracted and thus appeared in the aqueous phase. In Exp. 2, when a 5·0 ml. sample was taken, the amount of inorganic P present (3·62 mg.) was in excess of that which could be combined with molybdic acid to form phosphomolybdic acid and, as a result, 15 590 counts/min. were found in the aqueous phase out of a total of 29 150 counts/min., which were originally present. That this is the correct explanation is proven by Exp. 3, where the amount of ammonium molybdate present was increased by a factor of 4. In this experiment only 40 counts/min. were found in the aqueous phase.

It is thus clear that, if the procedure adopted in Exp. 3 is followed, when relatively large amounts of inorganic P are present, the efficiency of the extraction procedure is such that it may be anticipated that 99·90% of the inorganic P will be extracted into the *iso*butanol.

The number of counts (40) found in the aqueous phase of Exp. 3 is that which would be expected on stoicheiometric grounds (cf. 8 counts/min. in Exp. 1). This led to the belief that these counts may have been due to the presence of a minute amount of inorganic pyrophosphate which had escaped hydrolysis and which would not react with molybdic acid and therefore would not be extracted by the *iso*butanol.

As a test of this hypothesis the aqueous layer from Exp. 3 was subjected to further hydrolysis for a period of 30 min. at 100° and re-extracted with *iso*butanol. After this treatment no radioactivity could be detected in the aqueous layer. It may, therefore, be concluded that a completely quantitative separation is readily achieved.

The successful application of this method to experiments in which ^{32}P and ^{24}Na are simultaneously employed depends also upon the retention of ^{24}Na in the aqueous phase. To test the possibility of ^{24}Na entering the alcohol phase, additional experiments have been carried out using similar techniques as were used in Exp. 3 above. In these experiments a sample containing ^{24}Na as $^{24}NaCl$ was pipetted into the separating funnels together with the usual reagents. In one case ammonium molybdate was present and in the other it was omitted, but in no case was inorganic P added.

In these experiments, since it was necessary to determine the radioactivity in each aqueous phase after the washing of the *iso*butanol layer, neither the aqueous layer nor the two washes were combined. Each was made to 25 ml. and, together with the *iso*butanol phase, checked for radioactivity.

As far as the aqueous phase is concerned (Table 2), it is seen that the bulk of the ^{24}Na is removed when the original solution is run off after extraction. Of the radioactivity which remains in the separating funnel, 125 counts/min. are removed in the first wash and 5 in the second. It will also be noted that 122 counts/min. were detected in the *iso*butanol when extraction was carried out in the presence of molybdate. When, however, molybdate was omitted no radioactivity could be detected. This finding is suggestive of a contaminant in the $^{24}NaCl$ which reacts with ammonium molybdate to form a complex which is preferentially soluble in *iso*butanol, but which, in the absence of molybdate, is retained in the aqueous layer.

Table 3. *Decay of radioactivity in* isobutanol *layer*

(For details see text.)

Date of counting	Time of counting	Interval	Counts/min.
5. iii. 52	14.30 hr.	0	104
5. iii. 52	16.50 hr.	2 hr.	103
6. iii. 52	06.50 hr.	16 hr.	103
7. iii. 52	12.10 hr.	46 hr.	91
18. iii. 52	16.30 hr.	13 days 2 hr.	52

The chemical behaviour of this contaminant, together with the decay rate (Table 3), show that it is ^{32}P and that $^{24}NaCl$, as supplied by the Atomic Energy Research Establishment, Harwell, England, is contaminated with ^{32}P, which is present as inorganic P. It is, in fact, formed from the Cl of the NaCl by the following reaction: ^{35}Cl (n, α) ^{32}P.

The results of a final experiment in which the two isotopes were quantitatively separated from a mixture of each in plasma and muscle is of interest (Table 4).

Table 4. *Separation of* ^{24}Na *and* ^{32}P *from samples of plasma and muscle*

(For treatment of animal, see text. All results are expressed as radioactivity counts/min./ml. of plasma or/g. of muscle.)

		Aqueous layer containing ^{24}Na		
	*iso*Butanol layer containing ^{32}P	Observed		Calculated*
		0 time	0+108 hr.	0+108 hr.
Plasma	139 700	1750	11	11
Muscle	9 450	1010	9	7

* Based on the theoretical counts/min. expected, assuming that the counts/min. observed at 0 time were due entirely to ^{24}Na.

In this experiment a well fed rabbit (2·4 kg.) was injected intravenously with approximately 15 μc. of ^{24}Na as $^{24}NaCl$ and, 25 min. later, with approximately 1 mc. ^{32}P as $NaH_2{}^{32}PO_4$. The animal was killed 5 min. later and blood collected for separation of plasma. A sample (1 ml.) of plasma was ashed in a Pt crucible at 800° as was done with the muscle samples described above. The inorganic residue was treated in the same way and made to the same volume. The muscle sample was treated as before. Samples from the solutions of inorganic salts from plasma and muscle were then subjected to the extraction procedure, as outlined in Exp. 3, and the radioactivity present in the aqueous phase and in the *iso*butanol determined. It is clear that excellent separation of the two isotopes had been achieved in both cases.

DISCUSSION

The foregoing experimental results indicate that the extraction procedure outlined in Exp. 3 is sufficiently well designed to permit the quantitative measurement and separation of ^{24}Na and ^{32}P from a mixture of each, even though the radioactivity of the ^{32}P present considerably exceeds that of ^{24}Na. The accuracy with which such a separation may be achieved depends upon several factors, the most important of which are: (1) the completeness of the conversion of inorganic pyrophosphate to inorganic orthophosphate. To ensure this, it is apparently necessary to keep the acid solutions of the ashed inorganic salts at 100° for 1 hr.; (2) the maintenance of a sufficient excess of ammonium molybdate to allow the complete transformation of inorganic P to phosphomolybdic acid in which form the ^{32}P may be extracted into *iso*butanol; (3) the speed with which the first-formed phosphomolybdic acid is extracted by the *iso*butanol. In this connexion it should be remembered that when several mg. of inorganic P are present it is essential that the ammonium molybdate and the *iso*butanol be added simultaneously. If any delay in the addition of the *iso*butanol occurs then the phosphomolybdic acid, which forms very rapidly, is formed in such amounts as to exceed the solubility product of the complex in the aqueous phase and precipitation occurs. In the wet solid state the compound is insoluble in *iso*butanol.

Whilst it is clearly advantageous to eliminate all ^{32}P in the aqueous phase and to count only ^{24}Na in this phase, it should be remembered that if ^{32}P does appear it can only constitute a small fraction of the total and in any event may be readily corrected for on the basis of results obtained by determination of the residual radioactivity some days later.

The ^{32}P contaminant which was detected in the sample of ^{24}Na was of such an order that when the experiments were commenced the radioactivity due to ^{32}P represented about 7% of the total. Clearly such a contamination could lead to erroneous interpretation of results obtained in *in vivo* experiments because of the different distribution of P and Na throughout the body fluids and tissues. However, it should be remembered that the sample of ^{24}Na employed in the present experiments was used 5 days after leaving Harwell, so that, on the basis of the half lives of both ^{32}P and ^{24}Na, this contamination 5 days earlier would have amounted to about 0·03% of the total. Such a contaminant is negligible at the source of the material, but clearly may be sufficiently large to vitiate results if undetected and used some distance away from the source, e.g. in Australia. It can be avoided by obtaining ^{24}Na from the irradiation of $NaHCO_3$ instead of NaCl.

SUMMARY

A method for the quantitative estimation and separation of ^{32}P and ^{24}Na from a mixture of each in body fluids and tissues is described. ^{32}P is extracted as a molybdate complex into *iso*butanol, ^{24}Na remains in the aqueous phase.

It is a pleasure to express our thanks to Dr C. E. Eddy, Director, Commonwealth X-ray and Radium Laboratory, Melbourne, for the organization and supply of the isotopes used in this study.

Our grateful thanks are also due to Mr G. P. N. Watt, Chairman of the Australian National Airlines Commission, and to Mr C. O. Turner, General Manager, Qantas Empire Airways, for their generosity in transporting freight-free the ^{24}Na used.

We are also grateful to Dr F. G. Morgan, Director of the Commonwealth Serum Laboratories and to the Director General of Health, for a generous provision of laboratory space pending the establishment of this Department in Canberra, and to Miss J. D'Alton for skilled technical assistance.

REFERENCES

Ennor, A. H. & Rosenberg, H. (1952*a*). *Biochem. J.* **50**, 524.
Ennor, A. H. & Rosenberg, H. (1952*b*). (In preparation.)
Hevesy, G. & Rebbe, O. (1940). *Acta physiol. scand.* **1**, 191.