The use of tritium, of antipyrene and of N-acetyl-4-amino-antipyrene in the measurement of body water in living rabbits

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In biological and medical research, especially that into nutritional problems, it is frequently necessary to know the total amount of water in the body, or 'water space' of living animals. From such knowledge it is possible to calculate the composition and calorific value of the bodies of cattle (Reid, Wellington & Dunn, 1955) and pigs (Clawson, Sheffy & Reid, 1955). During the past decade much attention has been given, therefore, to methods of determining total body water.

Use of the dilution technique in the measurement of body water in living animals was first suggested by observations on the rate of turnover of water in the body with deuterium as a reference substance (Hevesy & Hofer, 1934; McDougall, Verzár, Erlenmeyer & Gaertner, 1934). Subsequently, other reference materials such as tritium (Pace, Kline, Schachman & Harfenist, 1947), antipyrene (AP) (Soberman, Brodie, Levy, Axelrod, Hollander & Steele, 1949), *N*-acetyl-4-aminoantipyrene (NAAP) (Berger, Brodie, Axelrod, Dunning, Porosowska & Steele, 1950) and ^{131}I-labelled 4-iodoantipyrene (Talso, Lahr, Spafford, Ferenzi & Jackson, 1955), were proposed for this purpose. A method for determining the total body water with urea and the extracellular space with thiocyanate and the subsequent calculation of the content of fat and cell mass was described by McCance & Widdowson (1951*a*, *b*).

Although some of these methods are being used extensively, the body-water values derived from their use have seldom been compared with those determined directly by desiccation or distillation. Generally, where comparisons have been made, the range in body-water contents was narrow. Satisfactory estimates were obtained by the use of deuterium in nine rabbits ranging in water content from 67 to 77% (Moore, 1946) and by the use of tritium in two rabbits containing 55·5 and 55·9% of water (Pace *et al.* 1947). When compared with desiccation the antipyrene method gave accurate estimates of body water in five monkeys (66·4–71·6% body water), in four dogs (68·1–72·5%) and in four rabbits (69·6–77·0%) (Soberman, 1950). The water values derived by the use of AP agreed well with those determined by toluene distillation in studies with twenty-nine pigs containing 37·9–59·3% water (Clawson *et al.* 1955) and with twenty cattle containing 56·3–78·5% water (Wellington, Reid, Bratzler & Miller, 1956).

The validity of the indirect methods has been tested most frequently against other indirect methods. Antipyrene gave values similar to those obtained with the specific-gravity method (Soberman *et al.* 1949; Messenger & Steele, 1949; Osserman, Pitts,

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Welham & Behnke, 1950; Kraybill, Hankins & Bitter, 1951; Kraybill, Goode, Robertson & Sloane, 1953), the deuterium method (Soberman *et al.* 1949; Friis-Hansen, Holiday, Stapleton & Wallace, 1951; Farber & Soberman, 1956) and the tritium method (Prentice, Siri, Berlin, Hyde, Parsons, Joiner & Lawrence, 1952). The validity of values obtained with NAAP can be assessed from their agreement with those obtained with AP (Brodie, Berger, Axelrod, Dunning, Porosowska & Steele, 1951; Talso *et al.* 1955; Pawan, 1956) and the uniform distribution of NAAP in body tissues (Brodie *et al.* 1951). The volume of body water into which ^{131}I-labelled 4-iodo-antipyrene became distributed in sixteen human subjects was similar to the water spaces for AP and NAAP (Talso *et al.* 1955).

In view of some of the limitations of previous studies, it seemed desirable to compare in living animals (rabbits), of a wide range of fatness, the water contents, estimated concurrently by three commonly used indirect methods (AP, NAAP, and tritium), with the water contents determined by subsequent desiccation of the same animals.

EXPERIMENTAL

Outline of experiment

For 3–6 months before the beginning of the experiment the amount of food offered to nineteen rabbits was controlled so as to produce rabbits with a wide range of body water.

The general procedure consisted of: (1) the injection into an ear vein of 8·9–11·3 ml. of a solution containing AP (48·0 mg/ml.), NAAP (31·7 mg/ml.) and water labelled with tritium (TOH) and giving $6{\cdot}15 \times 10^7$ counts/ml.; (2) the withdrawal by cardiac puncture of 4–5 ml. blood from each rabbit at about hourly intervals during a period of 3–4 h after injection; and (3) the slaughter of the rabbits from 5 min to 2 h after the last blood sample was taken. Two of the rabbits received no TOH. At slaughter the contents of the bladder and of the gastro-intestinal tract were weighed and sampled for analysis. The total body and the remaining contents of the viscera were then dried at 100° to constant weight, normally 5 days. Preliminary experiments showed that AP and NAAP were compatible in solution and, when injected intravenously in the rabbit, the presence of the one drug did not affect the chemical measurement of the other in the body fluids.

Protein-free filtrates of blood serum, urine and gut contents were used for the determination of the reference substances. Tritium was determined by the method of Glascock (1951, 1954) which depends on the reaction of 10 mg samples of water with butyl magnesium bromide and the counting of the resultant butane in a gas counter. Antipyrene and NAAP were determined by the methods outlined by Brodie, Axelrod, Soberman & Levy (1949) and Brodie *et al.* (1951), respectively. Antipyrine was determined colorimetrically as 4-nitrosoantipyrine at 350 mμ in a Unicam 5 P 500 spectrophotometer. After the hydrolysis of NAAP by hot HCl, and the diazotization of the resultant 4-aminoantipyrine with HNO_2, NAAP was determined colorimetrically at 490 mμ as a diazo salt coupled with α-naphthol. Separate samples of the deproteinized solutions were used to determine the blank values of the re-

agents. An additional rabbit receiving the same diet and which had received no injection was used for the determination of 'blank' values for AP and NAAP in the digesta.

Calculation of body water from dilution of reference substance in blood

By extrapolation. The amounts of the three reference substances and of water were determined in the serum or plasma separated from the blood. The logarithms of the concentrations in serum water of AP and NAAP, determined at various intervals after uniform distribution had occurred, were extrapolated to determine the concentration which would have been found at the time of injection had uniform distribution occurred immediately. Since a preliminary experiment on one rabbit showed that the tritium content of the blood water remained constant from 65 to 253 min after injection, only one blood sample taken 1 or 2 h after injection was normally used for the determination of blood tritium concentration during the main experiments. The body water content was computed as follows:

$$\text{Body water (ml.)} = \frac{\text{dose injected (mg of AP or NAAP; counts/min of TOH)}}{\text{theoretical initial concentration (mg of AP or NAAP; counts/min of TOH)/ml. serum water}}.$$

By recovery of urine. With NAAP a second method was also used for calculating body water (Brodie *et al.* 1951). It necessitates only one blood sample and one representative sample of all the urine secreted from the time of injection to the time of sampling. The expression used in this method was

$$\text{Body water (ml.)} = \frac{\text{(NAAP (mg) injected)} - \text{(NAAP (mg) in urine)}}{\text{NAAP concentration (mg/ml.) in blood water}}.$$

RESULTS

Comparison of values obtained by desiccation with those obtained by use of tritium, AP and NAAP

The estimates of water content obtained with AP and NAAP in the 'extrapolation' technique, with NAAP by recovery of urine and the values obtained by desiccation are summarized in Table 1. The water content of the rabbits as measured by desiccation ranged from 48 to 70% in the total body and from 44 to 69% in the empty (digesta-free) body. The coefficients of correlation between the water content of the whole body determined by desiccation and that determined with tritium, AP and NAAP were 0·98, 0·99 and 0·99, respectively, and the corresponding regression coefficients were 0·946, 0·986 and 1·018. For the relationship between the water content determined by desiccation of the digesta-free body, and that estimated by the use of NAAP, the coefficients of correlation and regression were 0·99 and 0·915, respectively. These various values demonstrate the close relationship between the water content of the body as determined by desiccation and by each of the three reference substances. Although related the values were not, however, identical. Examination of the results in Table 1 showed that there was a highly significant difference attributable to methods ($P < 0{\cdot}001$). Although the mean water content (by

desiccation) of the whole body was significantly different ($0{\cdot}01 > P > 0{\cdot}001$) from the values determined both by desiccation of the empty body and by the use of NAAP, it was not significantly different from the mean water content determined by the use either of tritium ($P > 0{\cdot}1$) or of AP ($P > 0{\cdot}1$). Further, the mean water space for tritium was not significantly different from that for AP ($P > 0{\cdot}1$) although the values with both these substances were significantly different ($0{\cdot}01 > P > 0{\cdot}001$) from that with NAAP. The water space with NAAP was not different ($P > 0{\cdot}1$) from the water content measured by desiccation of the empty body.

Table 1. *Comparison of water contents of rabbits determined by desiccation with those estimated in vivo by the use of indirect methods*

Rabbit no.	Water content (g) by desiccation		Water content (g) by indirect methods		
	Whole body	Empty body	Tritium	Antipyrene*	*N*-acetyl-4-amino-antipyrene*
A	1509	1283	—	1513	1314
B	1390	1192	—	1394	1232
1	1218	1016	1222	1250	1029
2	1236	1067	1229	1202	1094
3	1160	971	1197	—	(990)†
4	1003	822	1047	1005	841
5	1290	1066	1258	1312	1075
6	1456	1243	1441	1460	1262
7	992	863	937	993	891
8	1182	1005	1217	1175	1019
9	1137	957	1108	1115	964
10	869	718	893	914	686
11	1061	901	1019	1052	852
13	1043	877	1066	1016	890
14	1218	1005	1263	1217	1023
15	1375	1159	1410	1336	1178
16	1249	1072	1287	1248	1113
17	1255	1065	1263	1321	1082
18	1019	816	1014	1004	821

* Values computed by 'extrapolation' procedure (see p. 45).

† Value computed from dosage corrected for NAAP in urine in digesta and concentration of NAAP in water of one blood sample; since AP is metabolized, the corresponding value for AP could not be determined.

Compared with desiccation, the tritium and AP methods overestimated total body water by 0·5% (range −5·5 to +4·5%) and 0·2% (range −2·8 to +5·3%), respectively. If suspect data for rabbits nos. 10 and 11 are disregarded, the mean overestimate of the empty body water by NAAP was 1·9% (range +0·6 to +3·9%).

Entry of AP into gut contents

The concentration of AP was determined in the gut contents of thirteen rabbits, but full confidence in the results is lacking because of several apparently spurious values determined for the reagent–filtrate blank (see p. 44). In general the concentration of AP in the water of the gut contents tended to increase with time elapsing after injection.

After the first six rabbits had been examined, it became clear that estimates of the water space obtained with NAAP differed from those obtained with AP and tritium. Accordingly, in the remaining thirteen rabbits samples of gut contents were analysed for NAAP. In twelve rabbits the concentration of NAAP in the water of the gut contents increased from about 20% to about 180% of the computed simultaneous concentration in the blood during periods of 216–379 min after injection (Fig. 1). The thirteenth rabbit was killed 73 min after injection and the concentration of NAAP in the gut water was then only 10% of that in the blood. The surprisingly small amount of NAAP entering the gut during the period required for application of the extrapolation technique (240 min) explains why the water space obtained with NAAP was a slight overestimate of the water content of the empty body.

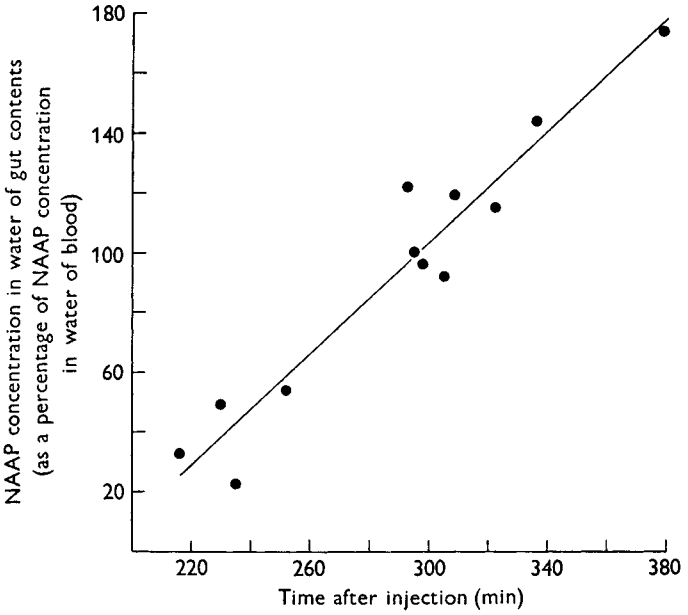

Fig. 1. Relationship between the time elapsing after injection and the concentration of *N*-acetyl-4-aminoantipyrene in the water of the gut contents of twelve rabbits. ($Y=0{\cdot}931X-175{\cdot}7$; $Sy.x=13{\cdot}3$, when $Y=100$, $X=296$ min).

Fate of NAAP in the body

Of the NAAP injected, $100{\cdot}7 \pm 3{\cdot}01$% was recovered at slaughter (Table 2) (mean time, 272 min after injection) .Of the total recovered, 60·4% was in the water of the body, 31·0% in the water of the urine excreted during the interval and found in the bladder at slaughter, and 8·6% in the water of the gut. These findings indicate that NAAP was not metabolized by rabbits during periods of up to 379 min. In cattle $99{\cdot}5 \pm 1{\cdot}79$% of injected NAAP was recovered in urine collected during a period of 50 h (Reid, Balch, Head & Stroud, 1957).

Use of NAAP for determination of total body water

The findings (i) that NAAP was not metabolized, (ii) that very little of it entered the gut water in less than 200 min after injection, but (iii) that it was uniformly distributed in all the body water, including the gut contents, about 296 min after injection, suggested that NAAP might be used to determine at any one time the total body water, in addition to its use (see above) for determining the empty body water. To find total body water (see p. 45) it was necessary to recover the urine secreted between injection and blood sampling, and to correct the dose of NAAP for the amount of NAAP found in the urine. Only a few of the rabbits (nos. 1, 2, 4, 5 and 6) were killed at times reasonably close to 296 min after injection. As shown in Table 2, this method gave estimates of the water content of the whole body of these rabbits in reasonably close agreement with the values obtained by desiccation.

Table 2. *Recovery of injected* N-*acetyl*-4-*aminoantipyrene from the body fluids of rabbits and effect of time elapsing after injection upon the water volume estimated by the use of NAAP in the 'corrected dosage' procedure*

Rabbit no.	Time after injection (min)	Recovery* of NAAP (%)	Water content of empty body (g)		Water content of whole body (g)	
			NAAP	Desiccation	NAAP	Desiccation
1	293	99·9	1018	1016	1266	1218
2	295	99·6	1076	1067	1246	1236
4	298	104·3	771	822	944	1003
5	305	100·0	1065	1066	1272	1290
6	309	99·5	1261	1243	1514	1456
3†	73	(98·2)	(990)	971	(1067)	1160
10	216	109·2	653	718	703	869
18	230	99·0	804	816	925	1019
11	235	98·0	923	901	958	1061
17	252	101·8	1042	1065	1144	1255
7	321	99·4	875	863	1024	992
8	335	100·2	1000	1005	1255	1182
16	379	100·2	1066	1072	1378	1249

* NAAP recovered from empty body, urine and digesta as a percentage of the amount injected.

† Animal died soon after one blood sample was taken. In order to determine the NAAP concentration of the blood at the time corresponding to that when urine and digesta were sampled, it was assumed that the blood level of NAAP declined with the same slope as that for blood level of another animal of similar size and having about the same volume of water; the values which were derived from this assumption are in parentheses.

As would be expected in view of the relationship shown in Fig. 1, estimates of the total body water content based on the NAAP method, for those rabbits (nos. 10, 11, 17 and 18) killed considerably earlier than 296 min after injection, were somewhat lower than the values measured by desiccation. For the rabbits (nos. 7, 8 and 16) killed at various times later than 296 min after injection the total body water contents were correspondingly overestimated by the NAAP method.

DISCUSSION

Accuracy of indirect methods of estimating body water

It has been suggested that in the indirect method using deuterium the exchange of deuterium with the hydrogen of compounds other than water causes an overestimate of body water equivalent to 0·5–2·0% of the body-weight (Hevesy & Jacobsen, 1940; Schoenheimer, 1942; Moore, 1946; Schloerb, Friis-Hansen, Edelman, Solomon & Moore, 1950). In the present experiment, however, the value for the mean water content of the rabbits was greater with tritium than that obtained by desiccation by only 0·5% and this difference was not significant. Elsewhere it has also been reported that the water content by desiccation was not appreciably different from that derived from the use of tritium in two rabbits (Pace *et al.* 1947) or of deuterium in nine rabbits (Moore, 1946).

In fifty-seven human subjects, some normal and some with ascites, Prentice *et al.* (1952) found water spaces for tritium to be 2–4% higher than those for AP; the difference was attributed to the exchange of tritium for protein-bound hydrogen.

The water spaces for deuterium and AP were found to be of the same relative size in man (Soberman *et al.* 1949), infants (Friis-Hansen *et al.* 1951) and patients with oedema (Farber & Soberman, 1956), whereas in oedema Hurst, Schemm & Vogel (1952) observed consistently lower water spaces for AP than for deuterium and suggested that the difference was proportional to the degree of oedema.

In our experiments the concentration of AP in blood has not been adjusted to compensate for the possibility of AP becoming bound to protein, but the AP method gave satisfactory values ranging from 97 to 105% of those obtained by desiccation. In similar experiments with cattle it was found that the total water content was slightly underestimated by the AP method because of slow equilibration with the water of the gut contents (Reid *et al.* 1957). Soberman (1950) had earlier suggested that, because of protein binding, it should be assumed that the observed plasma concentration of AP is 93% of the true concentration. With rabbits and cattle this correction appears to be unnecessary. It seems probable that if AP became bound to protein or other body constituents the slow entry of AP into the water of the gut contents had a compensatory effect, as discussed below.

The accuracy of methods for determining both the total and empty body water by means of NAAP has already been discussed.

Effect of the gut water

No special attention appears to have been given to the fact that the water space appears to be larger for AP than for NAAP, although it is evident from data given by Brodie *et al.* (1951) and was confirmed by Reid *et al.* (1957) in experiments with cattle. Similar water spaces for AP and NAAP were found in human subjects in a postabsorptive state (Talso *et al.* 1955), and at least one report (Pawan, 1956) records a water space for NAAP in man larger than that for AP. A larger proportion of the total body water is in the gut contents of herbivorous animals, and especially of ruminants, than in the gut contents of other animals (Cizek, 1954; Mäkelä, 1956). The

reduction in gut contents brought about by deprivation of food and water for 24 h is also least marked in herbivores (Cizek, 1954). For these reasons the simultaneous use of NAAP with either tritium or AP appears to offer special advantages for experiments with herbivorous animals. Reid *et al.* (1957) have shown that this two-marker technique promises to provide a means of determining the amount of water in the gut contents of cattle. For non-herbivorous animals in the postabsorptive state the advantage from use of two markers would be very small.

Advantages of the tritium method

In the present experiment the total body water content was estimated with a high degree of accuracy by the indirect method using tritium, despite the fact that in each rabbit, with few exceptions, only a single determination of the tritium content was made on the water from only one blood sample. Where laboratories are equipped for tritium determinations by methods of accuracy comparable to ours, the tritium method for determining total body water would appear to be the best of the three indirect methods tested.

SUMMARY

1. A comparison was made of tritium, antipyrene (AP) and *N*-acetyl-4-amino-antipyrene (NAAP) as injected reference substances for the in vivo estimation of the water content of nineteen rabbits by dilution techniques. The three indirect methods were used simultaneously and the body-water estimates so derived were compared with the water contents determined by desiccation of the total body and of the body after removal of gut contents (empty body).

2. The tritium and AP methods gave accurate estimates of the total body water.

3. The values for the body water, or water space, by the use of NAAP were similar to those found by desiccation of the empty body. Only small amounts of NAAP entered the water of the gut contents during the estimation, but these amounts later increased steadily, and at about 296 min after injection the concentrations of NAAP in the gut water and in the blood water were equal.

4. In thirteen rabbits NAAP was not metabolized during periods as long as 379 min after injection and $100{\cdot}7 \pm 3{\cdot}01$ % of the injected NAAP was recovered in the water of the empty body, urine and gut contents.

5. Since NAAP was not metabolized by rabbits it was used in a second procedure which, when applied at different intervals after the injection of NAAP, gave estimates of the water content of both the total and empty body.

6. It is suggested that NAAP, with either tritium or AP, could be used simultaneously in many nutritional experiments, especially with herbivorous animals, since the techniques offer a means of determining the extent of changes in body composition during experiments, and also the water in the whole body, empty body and gastro-intestinal contents.

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