

SEPARATION OF UNBOUND IODIDE IN

¹²⁵I-LABELED ANTIPYRINE

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Iodine-125-labeled antipyrine (¹²⁵I-Ap) (4-iodo-1,5-dimethyl-2-phenyl-3-pyrazolone) has been used in many laboratories as a label for organ or total-body water and for the estimation of organ blood flow using uptake or washout techniques. In our own use (1) we thought it imperative that the isotopic radical be firmly attached to the parent compound to prevent erroneous interpretation of the results.

Munck and Andersen (2) observed that up to 26% of the ¹²⁵I label became detached from ¹²⁵I-Ap after dilution in saline. Previously we had not been concerned that this might be a significant problem because ¹²⁵I-Ap washout curves from isolated heart preparations showed no evidence of a diffusion limitation to washout, all of the curves being superimposable when plotted on an abscissa of the product of actual flow and time (3). Since tissue permeability to halides is high compared, for example, with that of sodium and potassium, those experiments did not provide a critical test for the presence of ¹²⁵I⁻; and because we wished to use ¹²⁵I-Ap in future experiments and to compare its movement with that of labeled water, we needed to establish that the unbound fraction was small. Also we needed to choose a method that would be routine for us to use as well as one in which we could have confidence.

MATERIALS AND METHODS

Iodine-125-antipyrine dissolved in distilled water was obtained from the Radiochemical Centre (Amersham, England); it had an initial activity of about 1 mCi/ml. Seven batches were tested.

At different time intervals before an in vitro test, the ¹²⁵I-Ap, kept in a glass container, was further diluted in triple-distilled water to an activity of about 100 μ Ci/ml (this is the solution which is ordinarily used in our animal experiments). Subsequently, this was further diluted with distilled water or isotonic saline, usually in a ratio greater than 20:1, and all in vitro tests were run on these solu-

tions. The number of days in storage from receipt of the compound to the time of second dilution was designated T_{store}. The number of days from the second dilution to the test run was designated T_{dil}. After the second dilution the solutions were kept in either glass or plastic* test tubes at room temperature.

Three methods of separating the bound from the unbound isotope were used in the initial tests. The three methods were:

1. Descending and ascending paper chromatography (PC) on Whatman 3 MM using *n*-butanol-acetic acid-water (120:30:50). About 25–50 μ l of the ¹²⁵I-Ap solution was placed at the origin. Development was for 6 hr at room temperature, resulting in a solvent front movement of 14–15 cm.
2. Thin-layer chromatography (TLC) on plates† coated with silica gel or cellulose containing a fluorescent indicator. The solvent was chloroform-methanol-15 M ammonium hydroxide (100:30:5). A 1-hr run resulted in a solvent front movement of 10 cm.
3. High-voltage electrophoresis (HVE) on Whatman 3 MM paper wetted with 0.075 M barbital buffer‡ at pH 8.6. The electrophoresis was run at 10–15°C on a flat plate|| at either 3,000 volts for 20 min or 2,000 volts for 30 min, resulting in I⁻ migration of 14–15 cm.

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* Butyrex, cellulose acetate butyrate, Lusteroid Container Co., Inc., Maplewood, N.J.

† Precoated by E. Merck A. G., Darmstadt, Germany.

‡ Buffer B-2, Beckman Instruments, Inc., Palo Alto, Calif.

|| Model FP-30A, Savant Instruments, Inc., Hicksville, N.Y.

At the completion of each run, the paper or the plate was dried in an oven for 20 min at 70–80°C. The paper was cut across its width into parallel strips, 1 or 2 cm wide, and the strips were placed in plastic test tubes. The plates were divided into narrow strips parallel to the line of origin. Each small segment was then scraped off, and the material was placed in plastic test tubes. The plastic test tubes were then capped, and the gamma emissions were counted in an automatic well counter*.

The positions of the unbound iodide and the iodinated compound on the strips were checked each time by using simultaneous parallel samples of ^{127}I -NaI and sometimes by using antipyrine. The $^{127}\text{I}^-$ position was detected by spraying the strip with palladium chloride. The position of the antipyrine was detected by its native fluorescence or by the quenching of an added fluorescent indicator under ultraviolet light (366 or 254 nm).

To ensure that the results of the separation techniques were not biased by loss of either free or bound ^{125}I , the following preliminary tests were made.

1. Adsorption by the container. Six glass and six plastic test tubes were washed and dried and then counted for radioactivity. Five milliliters of either diluted ^{125}I -NaI or diluted ^{125}I -Ap was placed in the tubes, and the radioactivity was counted. After 60 min, the tubes were emptied, rinsed with water, soaked for 1 hr in running water at about 30°C, and dried with a towel and an air jet; then the residual radioactivity was counted. The percentage of residual activity was 12.7% for ^{125}I -Ap on glass, 0.5% for ^{125}I -Ap on plastic, 0.02% for ^{125}I -NaI on glass, and 0.00% for ^{125}I -NaI on plastic.
2. Container effect on percentage of $^{125}\text{I}^-$. With paired samples after various times in storage and for various periods after dilution and storage in either glass or plastic test tubes, the paired differences in percentage $^{125}\text{I}^-$ were examined. Twenty pairs of samples were examined. There was always more $^{125}\text{I}^-$ from samples kept in plastic test tubes than in glass (0.20–24.5% more), and the ratio of the percentage in plastic to that in glass averaged 1.50 (s.d. 0.55; the t-test for the difference from ratio of 1 gave a p value of less than 0.001).
3. Transformation of iodide to iodine. To test whether free iodide was being lost to the air

after oxidative conversion to iodine, TLC separation was performed in air and in a nitrogen atmosphere. In four runs each in both groups, the fraction of $^{125}\text{I}^-$ was 2.5 to 3.8%; the ratios of the percentage free in nitrogen to that free in air averaged 1.13 (s.d. 0.16; $p = 0.3$).

4. Iodide loss with HVE. The total activity of ^{131}I -NaI after HVE was compared with that of an equal volume of the same solution absorbed by filter paper. There was no difference detected in the total ^{131}I activity.
5. Iodide loss during oven drying. Equal volumes of ^{131}I -NaI and ^{125}I -NaI were placed at previously marked spots on Whatman paper prewetted in barbital solution. Then the papers were dried at 80°C for 10, 20, 30, 40, and 50 min. Each test was done in triplicate. The counts after drying were no different from those obtained from undried, paired control samples.
6. Preferential loss from iodide or iodoantipyrine. The dried strips of paper from two HVE runs with ^{125}I -Ap were kept in capped plastic test

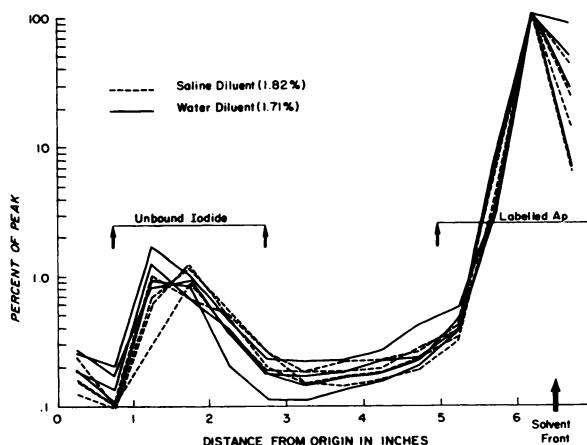


FIG. 1. Counting rate on strips from paper chromatography of ^{125}I -Ap with either water or saline as diluent.

tubes and counted at 1, 8, and 40 days. The percentages of $^{125}\text{I}^-$, estimated from the areas under the curves, at these times were 7.5, 7.6, and 7.4% for one run and 7.3, 7.2, and 7.2% for the other run.

7. Reproducibility of estimates by HVE. Three separations were made on each of six differently prepared specimens of ^{125}I -Ap diluted in saline or water. The means (\pm s.d.) of the percentage $^{125}\text{I}^-$ for each specimen were: 7.0% \pm 0.23, 7.17% \pm 0.06, 7.44% \pm 0.08, 7.49% \pm 0.59, 7.94% \pm 0.69, and 26.84% \pm 0.53%.

* Autowell II, Picker Corp., Med. Product Division, North Haven, Conn.

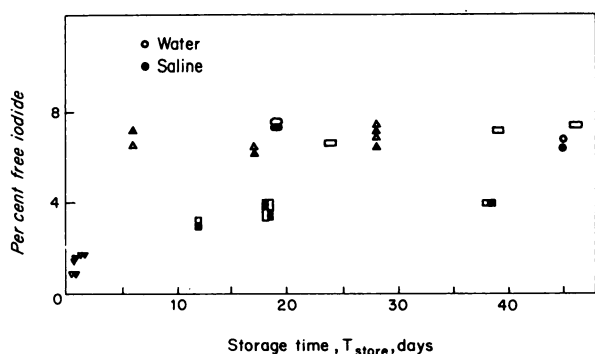


FIG. 2. Effect of brief dilution in saline (solid symbols) or distilled water (open symbols) on amount of free iodide present in batches of ^{125}I -Ap solutions. Rediluted samples were kept in plastic test tubes. T_{store} is time of storage of ^{125}I -Ap in distilled water kept in glass containers at room temperature. Each pair represents similar method of separation—HVE, TLC, or PC. Unpaired open rectangles give result from batch not rediluted. No influence of diluents was observed nor was there much more free iodide at longer storage times.

From these auxiliary tests it was concluded that the procedures were reproducible and did not result in preferential loss of iodide. Both the free label and the labeled compound seemed to adhere to glass when in dilute solution.

RESULTS

The plots of counting rate versus distance from origin (Fig. 1) showed clear separation of the bound and free label with all three methods. The results are expressed as percentage of $^{125}\text{I}^-$ in the total mixture, $D_i 100/(D_i + D_c)$ in which D_i is the area under the portion of the curve representing $^{125}\text{I}^-$ and D_c is the area under the portion of the curve representing ^{125}I -Ap.

Comparison of the different methods of separation. Simultaneous separations were done using the three methods on the same amounts of ^{125}I -Ap taken from the same container. A total of 10 runs was done by each method on three successive days. The percentages of unbound iodide were: by HVE, 9.21% (s.d. 0.25); by TLC, 8.32% (s.d. 0.405); and by PC, 7.45% (s.d. 0.47). The differences between the means of each method are significant ($p < 0.001$). It is interesting to note as well that the total isotopic activity—that is, the sum of the bound and unbound iodides—showed no differences after separation by HVE or PC, but this value was significantly less after TLC.

Effect of storage time and brief dilution in saline or water. Solutions were stored after receipt and initial dilution in distilled water. At the end of this period (T_{store} days), samples were further diluted to 5–10 $\mu\text{Ci/ml}$ in either saline or distilled water and were electrophoresed within 24 hr. Plots of

percentage $^{125}\text{I}^-$ as a function of T_{store} are shown in Fig. 2 for seven batches of ^{125}I -Ap. The diluent used just before separation made no difference; the ratio of $^{125}\text{I}^-$ after brief saline dilution to that after water dilution was 0.99 (s.d. = 0.62; $n = 13$ pairs; $p = 0.60$). The graph also shows that even fairly lengthy storage in distilled water did not cause much degradation of the ^{125}I -Ap.

Effect of prolonged dilution in saline or water. Paired dilutions in saline and water were made on four batches of ^{125}I -Ap after storage for various intervals (T_{store} days) (Fig. 3). The amount of $^{125}\text{I}^-$ was determined by HVE for each batch at several times after the final dilution (T_{dil}) in either saline or distilled water. Both glass and plastic tubes were used for one batch, but only plastic was used for the earlier batches. The $^{125}\text{I}^-$ increased substantially with time after saline dilution, particularly when plastic tubes were used. Seven of the 17 samples stored in saline in plastic tubes for more than 10 days showed greater than 20% $^{125}\text{I}^-$, and an additional

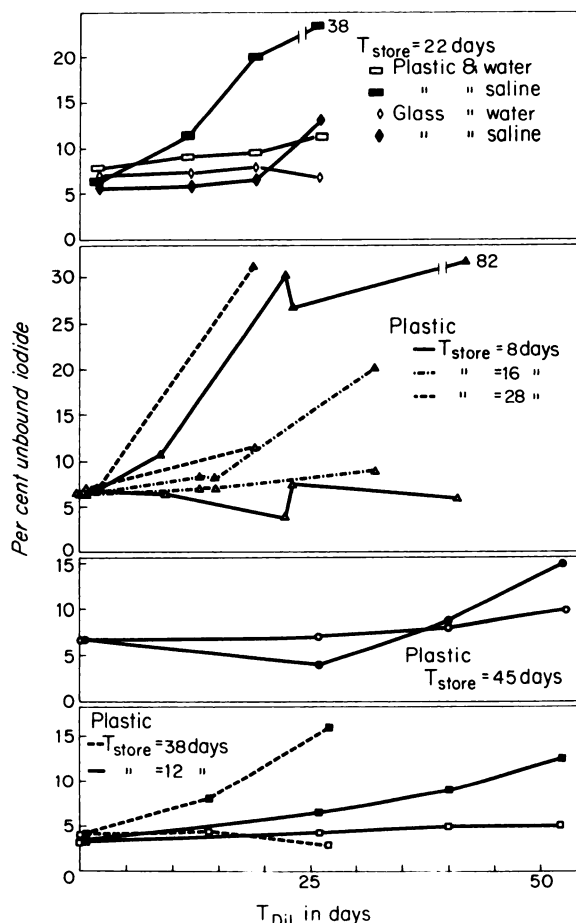


FIG. 3. Effect of storage after dilution to 5–20 $\mu\text{Ci/ml}$ in water (open symbols) or saline (solid symbols). Each panel represents each batch of ^{125}I -Ap tested by HVE. Symbols refer to same batches of ^{125}I -Ap as in Fig. 2. Degradation is fastest with combination of plastic test tubes and saline dilution.

five showed more than 10%, indicating the importance of avoiding prolonged storage in this fashion.

DISCUSSION

Høye (4) reviewed some of the observations on decomposition of iodoamino acids and, in his own work on L-thyroxine, showed that the extent of the decomposition is related to both the specific activity and the time in storage. In our study we have not examined the effects of different initial specific activities.

Munck and Andersen (2), using PC for the separation, found values of 1.4–5.4% for unbound iodide in the compound obtained from the Radiochemical Centre; there was no statistically significant change with further dilution in water. Our values for the water-diluted samples were up to 2% higher, and a still higher value was obtained when T_{dil} was increased. But, when T_{dil} was less than 24 hr, prolonging the T_{store} (even in saline) before final dilution did not appreciably affect the degradation. This is not surprising if one considers that the samples were always kept in glass containers during the T_{store} ; our data show that the amount of unbound iodide is less with glass containers than with plastic ones.

However, when T_{dil} was varied, a rapid increase of unbound iodide occurred with saline dilution and use of plastic test tubes; these test tubes had not

shown marked absorption or adsorption of $^{125}I^-$ or $^{125}I-Ap$. In Munck and Andersen's report, when T_{dil} was varied from only 1 to 4 days in the presence of saline, the unbound iodide increased from an average of 4.2% to 14.4%; the type of container was not mentioned.

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