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PAPER CHROMATOGRAPHY IN SYNTHETIC ORGANIC CHEMISTRY. MICROGRAM SCALE SYNTHESES OF LABELED MONOIODOTYROSINE, DIIODOTYROSINE AND THYROXINE

R. M. Lemmon, Winifred Tarpey and Kenneth G. Scott

July 8, 1949

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ABSTRACT
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ABSTRACT

July 8, 1949

Syntheses of I^{131}-labeled monoiodotyrosine, diiodotyrosine and thyroxine of high specific activity have been carried out on a microgram scale. The products have been separated from their reaction mixtures and identified through the use of paper chromatography. A semi-quantitative estimation of the products has been made by spectrophotometry.

* The work described in this paper was sponsored by the Atomic Energy Commission. It was supported by a grant from the Henry, Laura and Irene B. Dernham Fund of the American Cancer Society and the Christine Breon Fund.

PAPER CHROMATOGRAPHY IN SYNTHETIC ORGANIC CHEMISTRY. MICROGRAM SCALE SYNTHESIS OF LABELED MONOIODOTYROSINE, DIIODOTYROSINE AND THYROXINE (1)

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The availability of labeled organic iodide of high specific activity is very desirable for tracer studies of thyroid metabolism. Investigations into some of the differences in the thyroid activity of normal and tumor-bearing animals are now underway at the University of California Medical School and, in connectionwith these studies, we have carried out in vitro syntheses of I^{131}-labeled monoiodotyrosine, diiodotyrosine and thyroxine on a microgram scale. In order to isolate and identify the products of these syntheses we have made use of the technique of paper chromatography (2). This technique, which has already proven so valuable in amino acid work, provided us with a method by which our products could be isolated from their respective reaction mixtures without the addition of any inactive carrier. The positions of the amino acids on the chromatograms were located by preparing radioautographs of the chromatograms. The identity of thematerial in a given spot was established by preparing mixed chromatograms of the eluted material with a known sample of monoiodotyrosine, diiodotyrosine or thyroxine. Although a number of procedures for the

(1) The work described in this paper was sponsored by the Atomic Energy Commission. It was supported by a grant from the Henry, Laura and Irene B. Dernham Fund of the American Cancer Society and the Christine Breon Fund.

(2) R. Consden, A.H. Gordon and A.J.P. Martin, Biochem J., 38, 224 (1944).

quantitative estimation of amino acids on paper chromatograms have been published (3,4,5,6,7) we were not able to use any of them as they all involve a chemical reaction, usually the ninhydrin reaction, which leads to the destruction of the amino acid. Instead, we were able to make semi-quantitative estimations of our yields by spectrophotometry of the pure products. This technique is not feasible for most amino acids but is very satisfactory for the tyrosine derivatives due to the presence of the aromatic group. In alkaline solution the phenolate ions of these compounds have comparatively high molar extinction coefficients at their absorption maxima.

The presence of monoiodotyrosine in the thyroid has been reported by Fink and Fink (8). The only in vitro synthesis of this compound (other than the hydrolysis of iodinated protein material) which hitherto has been reported is that of Harington and Rivers (9). The present work shows that this compound may be synthesized easily, together with diiodotyrosine, by means of the direct iodination of tyrosine.

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(3) H.B. Bull, J.W. Hahn and V.H. Baptist, J. Am. Chem. Soc., 71, 550 (1949).

(4) A.J. Landua and J. Awapara, Science, 109, 385 (1949).

(5) A.J. Woiwod, Nature, 161, 169 (1948).

(6) R.B. Fisher, D.S. Parsons and G.A. Morrison, Nature, 161, 764 (1948).

(7) L. Naftalin, Nature, 161, 763 (1948).

(8) K. Fink and R. M. Fink, Science, 108, 358 (1948).

(9) C.R. Harington and R.V.P. Rivers, Biochem J., 38, 320 (1944).

EXPERIMENTAL PART

Preparation of 3,5-Diiodo^{131}-L-tyrosine and Monoiodo^{131}-L-tyrosine. - The method of Harington (10) for the iodination of tyrosine was adapted to a micro-scale preparation. Radioactive iodide was oxidized to iodine with potassium iodate by the method of Horeau and Sue" (11). Micropipets were used to add aliquot portions of the reactants to the 0.2 ml. centrifuge tube used as the reaction vessel.

In a typical experiment, 3050 μc. (0.028 μg.) of carrier-free NaI^{131} in 1.0 ml. of a sodium bisulfite buffered solution was added gradually to 288 μg. (1.43 μmole) of potassium iodide in 30 μl. of water. The solution was concentrated to approximately 10 μl. by means of a heat lamp and a stream of nitrogen blown over the surface of the liquid.

After the iodide solution was cooled in an ice bath, 120 μg. (0.56 μmole) of potassium iodate in 20 μl. of water and 11 μl. of glacial acetic acid were added, giving 1.72 μeq. of iodine. Ninety micrograms (0.497 μmole) of recrystallized L-tyrosine dissolved in 20 μl. of concentrated ammonium hydroxide was added to the iodine solution with stirring; the iodine color slowly disappeared during this addition. To complete the reaction, 84 μg. (0.66 μeq.) of iodine in 20 μl. of 0.174 N potassium iodide solution was added and the reaction mixture was allowed to stand for six hours with occasional stirring. The entire solution was then transferred to the corner of a large filter paper and chromatographed (details below).

The monoiodotyrosine obtained after elution contained 79 μc. or 2.6% of the starting activity and the diiodotyrosine contained 355 μc. or 11.6%

(10) C. R. Harington, Biochem J., 22, 1429 (1928).

(11) A. Horeau and P. Sue", Bull. soc. chim. biol., 27, 483 (1945).

of the starting I^{131} (allowing for the radioactive decay of the iodine during the reaction). That the starting tyrosine was completely reacted was shown by spraying the paper with ninhydrin. No tyrosine could be detected (sensitivity limit about 5 μg.). From the known specific activity of the iodine used in the reaction (3050 μc./5.86 μeq. = 520 μc./μeq.) the specific activities of the mono- and diiodotyrosine products are known to be 520 and 1040 μc./μmole, respectively. From these values and the total activities given above, the yields of the two products were calculated to be 47 and 148 μg., respectively. These yields are 31% and 68% of the starting tyrosine; therefore, the yields, as well as the results of the ninhydrin test, indicate a quantitative utilization of the tyrosine. A semi-quantitative determination by spectrophotometry (as described below) gave a value of 136 μg. for the yield of diiodotyrosine.

A decrease in the starting ratio of iodine to tyrosine led to a decreased quantity of diiodotyrosine. For example, in a preparation with a starting ratio of iodine : tyrosine of 2.1, the ratio of diiodotyrosine : monoiodotyrosine was 2.2. When the starting ratio was 1.9, the ratio for the products was 1.4. A reaction period shorter than six hours was found to give incomplete iodination of the tyrosine.

Chemical analyses were obtained for an inactive iodination reaction run on a 100 mg. scale under the same conditions as the micro preparation described. In this case, the 3,5-diiodo-L-tyrosine was precipitated from the reaction mixture by removing the excess ammonia *in vacuo* and adjusting the solution to a pH of 4. (The monoiodo-L-tyrosine was not recovered from the filtrates.) The product which was purified by charcoal treatment and recrystallized from dilute alcohol melted at 202° (dec.)

Anal. Calcd. for $C_9H_9O_3NI_2$: C, 24.96; H, 2.09; I, 58.62. Found: C, 24.92; H, 2.06; I, 58.40.

Chromatography and Spectrophotometry of Mono- and Diiodotyrosine. - The chromatography of the mono- and diiodotyrosine reaction mixture was carried out on Schleicher and Schuell filter paper No. 589 (23" x 23"). Satisfactory separations were obtained by developing the paper in both directions with a butanol-acetic acid-water (74:19:51 parts by volume) solvent. The chromatography was accomplished in the usual fashion (12) by allowing the paper to hang down from a glass trough containing the developing solvant in which one edge of the paper is immersed; the operation was carried out in a closed cabinet which was placed in a room where the temperature was kept at 22-25° C. The developed papers were allowed to dry in a hood at room temperature. A radioautograph of the chromatogram was prepared by placing a sheet of X-ray film (Eastman "No-Screen", 14" x 17") directly in contact with the chromatogram for about one minute in a photographic dark romm and then developing the film in the usual way. The radioautograph showed three distinct, well-separated spots. Two of these spots (together containing about 15% of the activity on the paper) were near each other in the far corner of the paper; their average R_F values (13) were 0.60 and 0.76. The third spot, which contained most of the activity, was not developed nearly as far (R_F value approximately 0.32) and contained unused sodium iodide. The radioautographs from some of the syntheses also showed a small fourth area of activity which had an R_F value approaching unity. This area contained free iodine.

The two spots which contained radioactive organic iodine were cut out of the paper and eluted separately overnight with approximately 3 ml.

(12) For details of the chromatographic procedures see R. Consden, Nature, 162, 359 (1948).

(13) The R_F value is the ratio of the distance which a substance has moved on a chromatogram to the distance which the solvent moved on the same chromatogram.

of 50% alcohol. The elution was effected by cutting the paper so that it had a tapered end, suspending the paper downwards from a glass trough containing the eluting solvent and allowing the tapered end to rest against the side of a sloping test tube - the operation was carried out under a glass jar. The elution was followed by observing the decrease of radioactivity on the paper strip as the solvent was drawn down the paper by capillary action.

A semi-quantitative estimation of the amount of diiodotyrosine prepared was made by adding one drop of 0.1 N sodium hydroxide to the eluate and observing the optical density of the solution at 315 mμ, the wave length of themain absorption maximum, as determined with a Beckman spectrophotometer (Model DU). The molar extinction coefficient for diiodotyrosine in basic alcoholic solution at this wave length was found to be 6.58×10^3. In order to carry out the spectrophotometry it was necessary to pre-wash the paper and to use redistilled solvents for both the chromatography and the elution. The paper was pre-washed by allowing the redistilled butanol-acetic acid developer to move all the way down the empty paper and drip from the paper for about one day; the pre-washing otherwise was carried out in the same manner as the chromatography itself. A blank on the spectrophotometry was obtained by eluting an empty area of the chromatographic paper which was adjacent to the diiodotyrosine spot and equal to it in area; the volume of the eluting solvent was equal to that used for the diiodotyrosine spot. The optical density of this solution was subtracted from that obtained for the diiodotyrosine eluate in order to determine the optical density due to the amino acid alone. In this way it was found to be convenient to measure the optical density of solutions of 50-150 μg. of the amino acid in 2-3 ml.

of solution. With 150 μg. of diiodotyrosine the estimated accuracy of the method is within 5%.

The identity of the monoiodityrosine was established by re-chromatographing the radioactive material from the lower (R_F value 0.66) of the two adjacent spots with a sample of known monoiodotyrosine which was prepared by C.R. Harington and R.V.P. Rivers (9). The radioactivity coincided exactly with the position of the amino acid as shown by spraying the paper with the ninhydrin reagent (0.5% in absolute alcohol) and by preparing a radioautograph of the chromatogram. The chromatographic identity was established using not only the butanol-acetic acid solvent but also when water-saturated 2,4-lutidine was used as the chromatographic developer. The identity of the upper spot (R_F value 0.76) was diiodotyrosine was established in exactly the same way (for the two different developers). Two different samples of known diiodotyrosine were used for the mixed chromatograms with the radioactive product. One was a recrystallized sample of the Eastman white label product; the other was a sample of the product which was prepared on a macro-scale by the iodination of tyrosine. The chemical analysis has been given above.

The re-chromatographing of the pure mono- and diiodotyrosine products always showed that a small fraction of the labeled iodide was detached from the organic molecule during the elution and re-chromatographing. This fraction varied from 2-4% for both products. No monoiodotyrosine spot could be observed when the diiodotyrosine was re-chromatographed.

Preparation of I^{131}-labeled Thyroxine. - Labeled thyroxine was prepared by means of the exchange reaction with inorganic iodide described by

Frieden, et.al. (14). The preparation was carried out several times and the quantitative data presented below are from a single representative experiment. One hundred and forty (±5) μg. of recrystallized thyroxine and 1.7 mc. of I^{131} (as carrier-free sodium iodide) were dissolved in 2 ml. of a 9:1 butanol-water mixture; hydrochloric acid was added in very small amount to bring the solution to a pH of 5-6 and to aid in dissolving the thyroxine. The solution was refluxed for 12 hours, cooled and evaporated under reduced pressure to about 0.5 ml. The residual solution was transferred to the corner of the chromatographic paper; a small amount of 0.1 M ammonium hydroxide was used to effect the transfer. Elution of the thyroxine from the developed paper followed by spectrophotometry of the eluate (as described below) showed that 120 μg. (83-89%) of the thyroxine was recovered. This thyroxine contained 11% of the labeled iodide used in the reaction (allowing for the radioactive decay of the iodine during the reaction). The specific activity of the thyroxine was 0.75 μc./μg.

Chromatography and Spectrophotometry of Thyroxine. - The chromatography of the thyroxine reaction mixture was accomplished in a fashion similar to that described for the mono- and diiodotyrosine except that in later work it was found to be unnecessary to run the chromatogram in the second direction. In addition, a 7:1 butanol-water solution was used instead of the butanol-acetic acid developer as the R_F value for thyroxine (0.82) is lower in the former solvent and a more satisfactory separation from free iodine can be obtained.

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(14) E. Frieden, N.B. Lipsett and R.J. Winzler, Science, 107, 353 (1948).

The position of the labeled thyroxine was established by preparing a radioautograph of the chromatogram in the manner described above for mono- and diiodotyrosine. The radioautographs from the thyroxine preparations always showed three spots: the lowest spot (R_F approximately 0.20) was sodium iodide, the middle spot (R_F 0.82) was thyroxine and the uppermost spot (R_F approximately 0.95) was free iodine. The thyroxine spot was, for a given weight of amino acid, more diffuse than the mono- and diiodotyrosine spots, and, in general, showed some streaking. This streaking indicated that some iodine (principally as iodide) was detached from the thyroxine during chromatography.

The thyroxine spot was cut out of the paper and eluted overnight with approximately 3 ml. of 50% alcohol which was made alkaline with 1% of ammonium hydroxide. One drop of 0.1 N sodium hydroxide was added to the eluate, the solution was transferred to the cell of a Beckman spectrophotometer and the optical density was determined at 326 mμ. Thyroxine in alkaline solution shows an absorption maximum at this wave length with a molar extinction coefficient of 6.05×10^3. A blank on the spectrophotometry was obtained in the same manner as described above for mono- and diiodotyrosine.

The identity of the thyroxine was established by re-chromatographing a small fraction of the radioactive product together with about 50 μg. of recrystallized thyroxine (Roche-Organon, Inc. product). The developed chromatogram was sprayed with the ninhydrin solution in order to establish the position of the thyroxine spot and a radioautograph was prepared in order to locate the radioactivity. The positions of the ninhydrin color and of the activity coincided exactly.

The re-chromatographing of the thyroxine showed that a considerable quantity of the iodine in the thyroxine reappeared as inorganic iodide. The fraction of I^{131} which reappeared as inorganic iodide and free iodine during the processes of elution, evaporation and re-chromatographing (all carried out at room temperature) varied from 22-28%. The paper chromatography seems to indicate considerable instability of the thyroxine molecule with respect to its keeping iodine in organic combination.

Acknowledgment. - We are indebted to Dr. A. A. Benson who suggested the use of paper chromatography for this work and to Dr. Choh Hao Li who provided us with some of the sample of monoiodotyrosine which was given to him by Prof. C. R. Harington. We are also grateful to Professors Melvin Calvin and Joseph G. Hamilton for making available to us the facilities of their laboratories.

SUMMARY

Syntheses of I^{131}-labeled monoiodotyrosine, diiodotyrosine and thyroxine of high specific activity have been carried out on a microgram scale. The products have been separated from their reaction mixtures and identified through the use of paper chromatography. A semi-quantitative estimation of the products has been made by spectrophotometry.