series. This is unusually large reduction for the ethyl Grignard reagent.[4]

Experimental

Preparation of Grignard Reagent.—A large supply of the reagent was prepared and diluted to a strength of 1.35 *M*. This was filtered and stored under nitrogen. Periodic checks on molarity were made.

Reaction of Ketones and Grignard Reagent.—A solution of 0.27 mole of ethylmagnesium bromide (200 ml.) was placed in a 3-neck flask equipped with dropping funnel, stirrer and ice-water condenser. The apparatus was previously flushed and filled with dry nitrogen. The solution was heated on a water-bath to reflux temperature and 0.25 mole of ketone in 125 ml. of dry ether was added. Reflux temperature and stirring was maintained during and for 30 minutes after addition. Any gas evolved was conveyed from the top of the condenser to the mouth of an inverted carboy filled with water. Samples were transferred from this to an Orsat apparatus and analyzed for unsaturated gas.[5] Suitable corrections for volume of added liquid, water vapor, etc., were made. After 30 minutes of heating gas evolution had ceased. Water was then added through the dropping funnel until the stirrer was stopped by crystallization of magnesium salts. The gas evolved during this addition was collected in a separate reservoir. In all cases this gas corresponded quite closely (±50 ml.) to the 0.02 mole excess of Grignard used. The saturated gas was taken as a measure of enolization; the unsaturated gas as a measure of reduction. Spot checks on these results indicated a reproducibility of ±50 ml. (±1%).

Isolation of Liquid Products.—The clear ether solution resulting from the above procedure was decanted from the crystalline magnesium salts. The salts were triturated with dry ether and the two ether solutions combined in a Claisen flask and distilled without further treatment. No evidence of water was found in the subsequent distillation. After the ether was removed by distillation on a water-bath, the residue was distilled at reduced pressure. The pressure used was usually between 5 and 10 mm. Three-ml. fractions were taken. Fractions with the same refractive index and b.p. were combined and carbon and hydrogen analyses run. For the first 15 ketones, the index and b.p. were essentially constant after the first two fractions. For the last two ketones a constant boiling, constant index distillate was recovered in the first fractions. These crystallized to give secondary alcohol, m.p. 54–55°. The last two fractions collected in each case were discolored and gave unacceptable carbon and hydrogen values for tertiary alcohol.

(4) M. S. Kharasch and S. Weinhouse, *J. Org. Chem.*, **1**, 209 (1937), and H. S. Mosher and E. LaCombe, THIS JOURNAL, **72**, 3994 (1950), contain most of the pertinent references related to reduction.

(5) F. C. Garrett, "Allen's Commercial Organic Analysis," Fourth Ed., Vol. III, Blakiston's Son and Co., Philadelphia, Penna., 1920, p. 4.

DEPARTMENT OF CHEMISTRY
EMORY UNIVERSITY, GEORGIA

Synthesis of 2-Methylaminofluorene-N-methyl-C^{14}[1]

BY JUANITA N. LITTLE AND FRANCIS E. RAY

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The synthesis of 2-methylaminofluorene-N-methyl-C^{14} was undertaken in a continuation of the study of derivatives of the carcinogen 2-aminofluorene.[2] Weisburger and Quinlin[3] prepared 2-dimethylaminofluorene in 22% yield, but a synthesis of the secondary amine has not been reported in the literature. In our preparation of this latter compound it was desirable to design a method which would be applicable to the introduction of C^{14} in the methyl carbon in maximum yield. For isotopic dilution experiments the compound should be free from the dimethyl and unmethylated amines. For this purpose the sodium salt of 2-tosylaminofluorene (2-*p*-toluenesulfonamidofluorene) was treated with methyl iodide and the resulting compound was hydrolyzed with hydrochloric acid.

Experimental

2-Sodium Tosylaminofluorene.—Fifty-three grams (0.15 mole) of tosylaminofluorene, m.p. 161°,[4] was dissolved with stirring and heating in 250 ml. of xylene dried over sodium hydride for 3 days. Sodium hydride, 4.2 g. (0.175 mole), was added and heating with stirring was continued for 15 minutes. The white sodium salt came out of solution. Excess sodium hydride was destroyed with 150 ml. of absolute ethanol and stirring. The precipitate became stiffer and whiter as the sodium ethylate reacted with more tosylaminofluorene until all the solvent was absorbed. The mixture was cooled, filtered with suction and washed with acetone until free of color. The yield of 2-sodium tosylaminofluorene was 55 g. or 98% of theoretical. This compound can be recrystallized from water containing a small amount of sodium hydroxide if necessary. The sodium salt should be freshly prepared for the next step.

2-Methyltosylaminofluorene.—2-Sodium tosylaminofluorene, 55 g. (0.15 mole), was dissolved in 800 ml. of 50% ethanol in a 2-liter flask and cooled. Ten ml. (0.16 mole) of methyl iodide was added and the flask was immediately connected to a long reflux condenser. The solution was warmed gently with occasional shaking for 1 hour and cooled. The white crystals of 2-methyltosylaminofluorene were collected with suction and dried. The yield was 48.4 g. (99.6%), m.p. 134–136°.

Anal. Calcd. for $C_{21}H_{19}O_2NS$: S, 9.73. Found: S, 9.54.

2-Methylaminofluorene.[5]—Seven-tenths gram of 2-methyltosylaminofluorene was placed in an 85-ml. Carius tube with 20 ml. of 50% hydrochloric acid. The tube was sealed and heated at 165° for 1 hour. It was found that placing the tube in a horizontal position and subjecting it to occasional agitation not only increased the yield but lowered the time necessary for hydrolysis. When cool, the tube was opened and the contents removed with warm water. The 2-methylaminofluorene hydrochloride was recrystallized once with charcoal from ethanol–water to remove a small amount of tar present.

Anal. Calcd. for $C_{14}H_{14}NCl$: Cl, 15.3. Found: Cl, 15.2.

The purified filtrate of the hydrochloride was converted to the free amine by cooling and neutralizing with 50% ammonium hydroxide. 2-Methylaminofluorene comes down as a white, flocculent precipitate which darkens on standing. It should be filtered with suction at once, washed several times with water, dried in a desiccator and stored in the dark; m.p. 73–75. Recrystallization from ethanol–water (charcoal) gave white plates, m.p. 76–77°. The yield was 0.34 g. (87%).

Anal. Calcd. for $C_{14}H_{13}N$: C, 86.16; H, 6.66; N, 7.17. Found: C, 86.41, 86.26; H, 6.30, 6.56; N (by difference), 7.29, 7.18.

2-Methylaminofluorene-N-methyl-C^{14}.—One millimole, 0.1419 g. (specific activity 1 millicurie per millimole) of methyl-C^{14} iodide[6] was treated in a vacuum line with two millimoles of 2-sodium tosylaminofluorene, 0.715 g., in acetone for 4 hours. Then the reaction was completed with

(1) This investigation was supported by Research Grant C-1066 from the National Cancer Institute of the National Institutes of Health, Public Health Service. Presented before the Meeting in Miniature of the Florida Section, A.C.S., Jacksonville, May 3, 1952.

(2) H. P. Morris, C. S. Dubnik and J. M. Johnson, *J. Nat. Cancer Inst.*, **10**, 1201 (1950); F. E. Ray and M. F. Argus, *Cancer Research*, **11**, 783 (1951); *cf. ibid.*, p. 423.

(3) E. K. Weisburger and P. M. Quinlin, THIS JOURNAL, **70**, 3964 (1948).

(4) N. Cambpell, W. Anderson and J. Gilmore, *J. Chem. Soc.*, 446 (1940).

(5) NOTE ADDED IN PROOF.—Since this work was completed a synthesis of 2-methylaminofluorene has been published by F. Bielschowsky (*Brit. J. Cancer*, **6**, 89 (1952)) who treated the sodium derivative of 2-acetylaminofluorene with methyl iodide and obtained a compound of m.p. 74° in unspecified yield.

(6) Obtained from Tracerlab on allocation of the Atomic Energy Commission.

0.25 ml. (4 millimoles) of base methyl iodide for 4 hours. The product was precipitated with water, centrifuged and washed. Hydrolysis was carried out as previously described. The yield was 0.35 g. or 90% of theoretical. The melting point was 68–70°. One recrystallization would bring the melting point up to the desired 73–75°.

The specific activity as determined with an external counter (2.1 mg./cm.^{2}) on a Raychronometer was 2.54 microcuries/mg.; this was 99.4% of theoretical. The overall radioactive yield based on the number of milligrams of compound obtained was 89.5%.

CANCER RESEARCH LABORATORY
UNIVERSITY OF FLORIDA
GAINESVILLE, FLORIDA

Countercurrent Distribution of Sheep Adrenocorticotropic Protein Preparations

BY GEORGE P. HESS, FREDERICK H. CARPENTER AND CHOH HAO LI

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Although the adrenocorticotropic hormone (ACTH) preparations isolated from sheep and pig pituitary glands by the method of Li, *et al.*, and Sayers, *et al.*,[1] have been found to behave as homogeneous proteins in electrophoretic, ultracentrifugal and solubility studies, considerable evidence[2] has accumulated which indicates that the ACTH activity is not restricted to a protein with the properties described by Li, *et al.*, and Sayers, *et al.*[1] When submitted to partial pepsin and acid hydrolysis, the protein preparations retained biological activity.[2a–e] These results could be interpreted to mean that only a portion of the protein molecule was necessary for biological activity. However, material with an ascorbic acid depleting activity[3] up to 100 times greater than the activity of the protein preparations, has been prepared from the extracts of the pituitary gland.[2f,g,h] In addition, Dixon, *et al.*,[4] obtained a separation of the adrenal weight-increasing activity from the adrenal ascorbic acid-depleting activity in a pig ACTH protein preparation, using ion exchange chromatography. These observations may be explained in part by the assumption that the protein preparations were not homogeneous.

In an attempt to answer the question of homogeneity, we have subjected the protein preparations to other separation techniques. In a recent communication from this Laboratory[5] it was reported that the ACTH protein preparation could be separated into two fractions by electrodialysis. One fraction, the cathode material, possessed nearly all the biological activity of the original protein preparation as measured by the adrenal ascorbic acid depleting method.[3] Further evidence for the inhomogeneity of the ACTH protein preparations has now been obtained from investigations which have made use of the countercurrent distribution method of Craig.[6]

The ACTH protein preparations were submitted to countercurrent distribution between 2,4,6-collidine and water, a solvent system which has been described previously[7] for the investigations of partial peptic hydrolysates of the ACTH protein.[2a] The results of a typical experiment in which 47 mg. of ACTH protein preparation was subjected to a 25-transfer distribution are shown in Table I and Fig. 1. At least three components were revealed. The main component (Tubes 0-14) contained

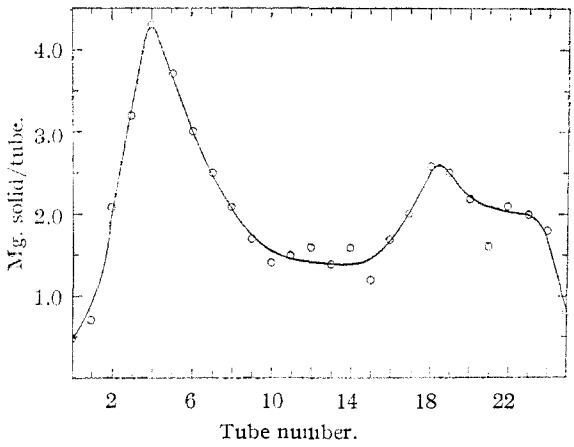

Fig. 1.—Twenty-four transfer distribution of sheep ACTH protein between 2,4,6-collidine and water.

TABLE I

DISTRIBUTION OF SOLIDS AND BIOLOGIC ACTIVITY IN VARIOUS FRACTIONS OBTAINED BY A 25-TRANSFER DISTRIBUTION OF A SHEEP ACTH PROTEIN PREPARATION BETWEEN COLLIDINE AND WATER

Fraction	Amount, mg.	Dose, μg.	Depletion of ascorbic acid, mg./100 g. adrenal	ACTH potency, I.U./mg.
Starting material	47	2	−140, −118, −143, −117, −78, −99, −112, −142, −101	2
Tubes[a] 0–14	31	5	−29, −62, −64, −57, +28, +32	0.04
Tubes[a] 15–20	12	1	−68, −75, −76, −49, −59, −52, −120, −91, −41, −76, −44, −48, −49, −44	1
Tubes[a] 21–25	8	0.2	−69, −78, −78, −107, −81, −142, −89, −74, −85, −63, −116	10

[a] See Fig. 1.

(1) (a) C. H. Li, H. M. Evans and M. E. Simpson, *J. Biol. Chem.*, **149**, 413 (1943); (b) G. Sayers, A. White and C. N. H. Long, *ibid.*, **149**, 425 (1943).

(2) (a) C. H. Li, *Trans. Macy Conf. on Metabolic Aspects of Convalescence*, **17**, 114 (1948); (b) N. G. Brink, M. A. P. Meisinger and K. Folkers, THIS JOURNAL, **72**, 1040 (1950); (c) J. B. Lesh, J. D. Fisher, I. M. Bunding, J. J. Koesis, L. S. Walaszek, W. F. White and E. E. Hays, *Science*, **112**, 43 (1950); (d) C. H. Li, THIS JOURNAL, **73**, 4146 (1951); (e) N. G. Brink, F. A. Kuehl, Jr., M. A. P. Meisinger, M. N. Bishop and K. Folkers, *ibid.*, **74**, 480 (1952); (f) R. W. Payne, M. S. Raben and E. B. Astwood, *J. Biol. Chem.*, **187**, 719 (1950); (g) B. Cortis-Jones, A. C. Crooke, A. A. Henly, P. Morris and C. J. O. R. Morris, *Biochem. J.*, **46**, 173 (1950); (h) H. B. F. Dixon, S. Moore, M. P. Stack-Dunne, and F. G. Young, *Nature*, **168**, 1044 (1951).

(3) M. Sayers, G. Sayers and L. A. Woodbury, *Endocrinology*, **42**, 379 (1948).

(4) H. B. F. Dixon, M. P. Stack-Dunne, F. G. Young and D. B. Cater, *Nature*, **168**, 1084 (1951).

(5) G. P. Hess, J. I. Harris, F. H. Carpenter and C. H. Li, THIS JOURNAL, **73**, 5918 (1951).

(6) (a) L. C. Craig and D. Craig in A. Weissberger, "Techniques of Organic Chemistry," Interscience Publ., New York, N. Y., Vol. III, 1950, Chap. 4; (b) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **73**, 877 (1951).

(7) F. H. Carpenter, "XIIth International Congr. of Pure and Applied Chem.," Abstracts of Papers, pp. 69 (1951).