precipitate was decomposed by the pyridine procedure.[9] The digitonin precipitable fraction, **epiandrosterone (III)**, weighed 168 mg., m.p. 169–175°, raised to 174–175° after one recrystallization from acetone, $\lambda_{max}^{CHCl_3}$ 5.76 and 9.62 μ. From the original digitonin filtrate after evaporation to dryness, there was isolated 240 mg. of **androsterone (II)**, which after one recrystallization from acetone exhibited m.p. 184–186°, undepressed upon admixture with authentic androsterone (m.p. 184–185°, $[\alpha]_D$ +94.5° (EtOH)), $[\alpha]^{20}D$ +97° (EtOH), $\lambda_{max}^{CHCl_3}$ 5.76 and 10.0 μ. **Androsterone acetate** showed m.p. 165–166°, $[\alpha]^{20}D$ +87° (EtOH).

Hydrogen-free W-2 Raney nickel catalyst[5] gave essentially the same proportion of epiandrosterone (III) (34%) and androsterone (II) (42%). Androstan-17-one was not reduced by non-pyrophoric Raney nickel, but when androstane-3,17-dione (I) was treated under the above conditions with fresh (pyrophoric) W-2 Raney nickel catalyst, infrared examination demonstrated that both carbonyl groups had been reduced completely.

(9) *Cf.* G. C. Butler and G. F. Marrian, *J. Biol. Chem.*, **124**, 237 (1938).

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The Course of the Acylation of 2,6-Lutidine in the Presence of Phenyllithium

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In earlier reports from other[1] and these laboratories[2,3] the following scheme has been suggested for the base-effected acylation of the methylated tar bases, 2-picoline, 4-picoline, quinaldine and 2,6-lutidine, with esters.

$$CH_3Z + MR \longrightarrow RH + MCH_2Z \quad (1)$$

$$MCH_2Z + RCO_2R' \longrightarrow R'OM + RCOCH_2Z \quad (2)$$

$$RCOCH_2Z + MCH_2Z \longrightarrow (RCOCHZ)^-M^+ + CH_3Z \quad (3)$$

or

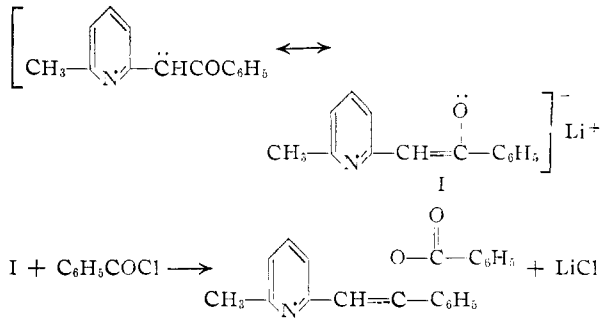
$$RCOCH_2Z + MR \longrightarrow (RCOCHZ)^-M^+ + RH \quad (4)$$

Z = 2-pyridyl, 4-pyridyl, 2-quinolyl or 6-methyl-2-pyridyl radical

MR = $NaNH_2$, KNH_2 or C_6H_5Li

In support of this scheme it has been shown[2] that the interaction of molar equivalents of phenyllithium, 2-picoline and ethyl benzoate gave a 58% yield of 2-phenacylpyridine (based on the assumption that the third step indicated above occurs). When this reaction was repeated except that a 2:2:1 molar ratio of reactants was used, the yield of ketone was increased to 80%.

More convincing evidence for the existence of an anion in step three would be available if it were possible to isolate a diacylated tar base by adding an extremely reactive acylating agent, *e.g.*, an acid chloride, to a mixture of the tar base, phenyllithium and ester which had been allowed to react for the customary reaction time. Therefore, benzoyl chloride was added to a mixture of phenyllithium, 2,6-lutidine and methyl benzoate. From this reaction there was isolated a mixture of 2-phenacyl-6-methylpyridine (67%), the lithium salt of 2-phenacyl-6-methylpyridine (18%) and the enol benzoate of 2-phenacyl-6-methylpyridine (11%). The isolation of both the lithium salt of 2-phenacyl-6-methylpyridine and the enol ester of 2-phenacyl-6-methylpyridine indicates that 2-phenacyl-6-methylpyridine exists as an anion in the reaction mixture.



It is interesting to note that when the last reaction was effected in the absence of benzoyl chloride a 94.5% yield of 2-phenacyl-6-methylpyridine[3] and none of the enol benzoate were obtained. It should be noted also that Wibaut and co-workers[4,5] have treated 2,6-lutidyllithium (prepared from 2,6-lutidine and phenyllithium) with benzoic anhydride and obtained only the enol benzoate. These results indicate that the anion of 2-phenacyl-6-methylpyridine is a stronger base than the chloride and benzoate ions but a weaker base than methoxide ion.

It was also of interest to determine whether the reaction of phenyllithium with 2-phenacyl-6-methylpyridine would give the corresponding tertiary alcohol. However, carbinol formation did not occur. Instead, because of the apparent high acidity of the ketone, an acid–base reaction occurred in preference to addition to the carbonyl group to give the lithium derivative of the ketone, which on treatment with benzoyl chloride gave a mixture of 2-phenacyl-6-methylpyridine (5–6%), the lithium salt of 2-phenacyl-6-methylpyridine (46–46.5%) and the enol benzoate of 2-phenacyl-6-methylpyridine (45–47%).

Experimental

Reaction of 2,6-Lutidine with Phenyllithium and Methyl Benzoate Followed by the Addition of Benzoyl Chloride.—2,6-Lutidine (21.4 g., 0.2 mole) was added to an ether solution of phenyllithium (0.2 mole) and this was followed by the addition of methyl benzoate (0.1 mole, 13.6 g.). The mixture was refluxed for 30 minutes. Then benzoyl chloride (14.1 g., 0.1 mole), dissolved in 50 ml. of anhydrous ether, was added and the mixture refluxed for two hours and then processed as described in the following experiment to give 14.1 g. (66.8%) of 2-phenacyl-6-methylpyridine, b.p. 143–145° at 1.0 mm.; picrate,[3] m.p. 180–181°; 3.4 g. (10.8%) of the enol benzoate of 2-phenacyl-6-methylpyridine, m.p. 137–138°, and 3.9 g. (18.0%) of the lithium salt of 2-phenacyl-6-methylpyridine.

When the reaction was repeated except that the mixture was refluxed for 16 hours after the benzoyl chloride was added, there were obtained 5.3–6.8 g. (25.1–32.8%) of 2-phenacyl-6-methylpyridine, 11.8–12.8 g. (37.5–40.6%) of the enol benzoate and 3.4–4.6 g. (13.8–21.2%) of the lithium salt.

Reaction of 2-Phenacyl-6-methylpyridine, Phenyllithium and Benzoyl Chloride.—2-Phenacyl-6-methylpyridine (21.1 g., 0.1 mole), dissolved in 50 ml. of anhydrous ether, was added to an ether solution of phenyllithium (0.1 mole) prepared as described earlier.[2] Then benzoyl chloride (14.1 g., 0.1 mole), dissolved in 50 ml. of anhydrous ether, was added dropwise. The mixture was refluxed for two hours and then poured onto 300 g. of crushed ice and 100 ml. of water. A yellow solid precipitated from the basic solution and was

(1) M. J. Weiss and C. R. Hauser, THIS JOURNAL, **71**, 2023 (1949).

(2) N. N. Goldberg, L. B. Barkley and R. Levine, *ibid.*, **73**, 4301 (1951).

(3) N. N. Goldberg and R. Levine, *ibid.*, **74**, 5217 (1952).

(4) C. C. Kloppenburg and J. P. Wibaut, *Rec. trav. chim.*, **65**, 393 (1946).

(5) J. I. de Jong and J. P. Wibaut, *ibid.*, **70**, 962 (1951).

filtered. This solid consisted of a mixture of the enol benzoate of 2-phenacyl-6-methylpyridine and the lithium salt of 2-phenacyl-6-methylpyridine. Soxhlet extraction with 60–70° petroleum ether separated the mixture into its components, the enol ester being soluble and the lithium salt being insoluble in petroleum ether. The original filtrate was extracted with several portions of ether, the combined ether phases dried over sodium sulfate, the ether removed at atmospheric pressure and the residue distilled in vacuum. In this manner, from several experiments, there were obtained 14.1–14.7 g. (44.8–46.7%) of the enol benzoate of 2-phenacyl-6-methylpyridine, m.p. 137.5–138.5°[4,5]; 10.0–10.1 g. (46.1–46.5%) of the lithium salt of 2-phenacyl-6-methylpyridine and 1.1–1.3 g. (5.2–6.2%) of 2-phenacyl-6-methylpyridine, b.p. 152–154° at 1.7 mm., m.p. 77–78°[3] (from 60–70° petroleum ether).

Structure of the Enol Benzoate of 2-Phenacyl-6-methylpyridine.—A sample (0.4 g.) of the enol benzoate was warmed for one minute with 25 ml. of 25% hydrochloric acid. On cooling the solution, a white solid precipitated and was filtered. The solid was shown to be benzoic acid (0.15 g., 97%), m.p. 120.6–121.4° alone and when mixed with an authentic sample. The filtrate was made basic with 5% sodium bicarbonate solution, extracted with ether and the ether removed to give 0.25 g. (93%) of 2-phenacyl-6-methylpyridine, m.p. 76.5–77.8°; picrate, m.p. 180–181° dec.[3]

The Identity of the Lithium Salt of 2-Phenacyl-6-methylpyridine.—When a sample of this material was placed in a flame, the color of the flame became bright red, indicative of the presence of lithium ions, after the sample carbonized. The identity of the salt was established further by dissolving it in hydrochloric acid and neutralizing the solution with 5% sodium carbonate solution. The mixture was cooled in an ice-bath and the solid which precipitated then was separated by filtration. The solid was air-dried and recrystallized from 60–70° petroleum ether. It melted at 76.5–78° and gave a picrate, m.p. 180–181° dec. A mixed melting point between this picrate and that prepared from an authentic sample of 2-phenacyl-6-methylpyridine showed no depression.

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The Reduction of the Disulfide Bonds of Insulin

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Pierce[1] has recently reported the separation of the A and B chains of oxidized insulin by countercurrent procedures and shown that these two components almost certainly account for the whole of the insulin molecule. A similar conclusion has been reached in these laboratories by use of paper electrophoretic techniques on reduced insulin. Reduction offers theoretical advantages in that it is more specific and easily controlled than oxidation, but in the case of insulin suffers from the disadvantage that the products are insoluble in aqueous solutions except at extremes of *p*H. This difficulty has been overcome in the present work by the use of 8 *M* urea solutions. If insulin is reduced by 0.1 *M* lithium thioglycolate at *p*H 5 in the presence of 8 *M* urea and electrophoresis on paper is carried out with the same solution as supporting electrolyte, two components can be detected. By elution from the paper followed by hydrolysis and paper chromatography it was shown that the amino acid composition of the anodic component agreed qualitatively with that given by Sanger for the A chain whilst the cathodic component showed similar qualitative agreement with Sanger's B chain.

Evidence has been given by other workers that in the absence of urea approximately one-third of the cystine disulfide bonds of insulin are reduced at *p*H 5.[2,3] This was confirmed in the present work for specific reducing conditions used. This could be interpreted to imply either a limited reduction of all the disulfide bonds or alternatively a specific reduction of one sulfur bond of the three dissimilar bonds in the insulin molecule. There is considerable experimental evidence in the analogous case of wool to suggest that the latter alternative is more probable.[4]

Specific evidence for this view was obtained by reducing insulin at *p*H 5 with lithium thioglycolate and coupling the thiol groups so formed with iodoacetamide to give combined carbamyl-S-methylcysteine residues in the insulin molecule. Reduction of this product by lithium thioglycolate at *p*H 5 in the presence of 8 *M* urea and subsequent electrophoresis caused separation into two components which were isolated separately. By hydrolysis and paper chromatography it was shown that a strong spot corresponding in position to that expected for carboxy-S-methylcysteine occurred in the hydrolysate of the anodic fraction whereas the cathodic component gave only a very weak spot. This provides strong evidence that the major reaction which occurs during the reduction of insulin by thioglycolate solutions at *p*H 5 is fission of the intrachain disulfide bond of the A chain.[5]

The structure of insulin proposed by Lindley and Rollett[6] provides possible alternative explanations for the enhanced reactivity of this particular disulfide bond. In this proposed structure, the amino acid sequences of the A and B chains as determined by Sanger and collaborators[5,7,8] have been used and the polypeptide chains have been given essentially the configuration of an α helix.[9] In the particular arrangement of helices which has been proposed,[6] the intrachain disulfide bond of the A chain is more accessible to reagents than either of the interchain disulfide bonds. Alternatively, an unbonded NH group is present in the proposed structure in the vicinity of the intrachain disulfide bond and could create enhanced reactivity by stabilizing an intermediate stage in a single electron transfer mechanism of reduction. If this latter suggestion is correct then reduction at *p*H 5 may provide a simple chemical means of estimating intrachain disulfide bonds in which the two halves of the cystine residue are separated by compara-

(1) J. G. Pierce, THIS JOURNAL, **77**, 184 (1955).

(2) J. Fraenkel-Conrat and H. Fraenkel-Conrat, *Biochem. Biophys. Acta*, **5**, 89 (1950).

(3) H. Lindley, *Biochem. J.*, **42**, 481 (1948).

(4) For a general review of this work see H. Phillips, "Fibrous Proteins," Symposium of Soc. Dyers Colourists, England, 1946, p. 39.

(5) F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **58**, vi (1954).

(6) H. Lindley and J. S. Rollett, *Biochim. Biophys. Acta*, in press.

(7) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951).

(8) F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 353, 366 (1953).

(9) L. Pauling, R. B. Corey and H. R. Branson, *Proc. Nat. Acad. Sci.*, **37**, 205 (1951).