Adler (1) in 1934 demonstrated that a physiologically inactive extract of human male urine can be obtained with butyl alcohol and that, by subsequent heating with strong acid, the inactive extract can be converted into a potent material which promotes the growth of the combs of capons. Peterson, Gallagher, and Koch (2) in 1937 confirmed the acid hydrolysis of the inactive form and have determined the optimum time of hydrolysis by boiling urine acidified with one-tenth by volume of commercial hydrochloric acid. It is well known that the active forms of the comb growth-promoting substances are water-insoluble and lipid-soluble. Dingemanse, Borchardt, and Laqueur (3) in 1937 demonstrated that the inactive form is water-soluble and lipid-insoluble. They have also discovered the important facts that the inactive form is not made lipid-soluble by even prolonged boiling with strong aqueous or alcoholic potassium hydroxide and that the latent activity is not destroyed by such treatment, for, if followed by acid hydrolysis, the active form is completely recoverable, as shown in tests made by injection into capons. Dingemanse and Laqueur (4) have studied methods of avoiding destruction of androgens while activating the inactive form with hydrochloric acid. Recently, Peterson, Hoskins, Coffman, and Koch (5) have found that normal butyl alcohol extracts all the androgenic material from male urine and that this material is practically all in an inactive form. At the same time McCullagh, Osborn, and Osgard (6) made a similar preliminary report of the experiments discussed in this paper.

Experiments designed to throw further light on the properties of the free and inactive androgens in urine have been carried out
in this laboratory by use of the rat assay method of Korenchevsky (7), the capon injection assay method of Gallagher and Koch (8), and also the capon comb inunction assay method of Fussgänger (9). McCullagh and McLin (10) showed that quantitative extraction of free androgens from urine can readily be made by the use of dibutyl ether. The method of separation of free and inactive androgens was based upon that work combined with the fact established in this paper that there is no appreciable extraction of inactive urinary androgens by the use of this solvent.

McCullagh, McCullagh, and Hicken (11) were the first to point out the existence of androgens in human blood. The hormone content of blood was obtained by ether extraction of the solid mass resulting from the desiccation of the blood with anhydrous sodium sulfate. The possible existence of an inactive androgen in blood was not suspected at that time but definite evidence is presented in this paper to prove the existence of such a compound, and to show it to be insoluble in dibutyl ether.

EXPERIMENTAL

In preliminary experiments, urine from unemployed men who were being fed and housed by the city of Cleveland was examined for androgens and for substances which become androgenic following boiling with acid. In these experiments extraction with dibutyl ether showed that this urine contained little if any active androgenic material. The urine was extraordinarily low in total androgenic material, containing only 7 international units per liter after boiling for 15 minutes with 5 per cent sulfuric acid. The assays were made by the methods of Korenchevsky (7) and of Gallagher and Koch (8). These methods necessitated the use of rather large samples of urine and hence an extended period of extraction. There was indication that some activation occurred during this time.

Therefore, freshly excreted urine from four normal men working in this institution was studied. In order to make the extractions more rapid, small quantities of urine were used and the extracts were assayed according to the inunction method on the combs of capons. In each case, 150 cc. of freshly voided urine were acidified by the addition of 7.5 cc. of concentrated sulfuric acid and extracted twice by shaking vigorously for 10 minutes with an
equal volume of cold dibutyl ether. The two dibutyl ether extracts were combined, washed with water, and evaporated to dryness by steam distillation in a partial vacuum. The oily residue was dissolved in 6 cc. of sesame oil. 60 cc. of the extracted urine were then boiled for 15 minutes, cooled under running water, and reextracted in a similar fashion. Five birds were used to assay each preparation; 0.1 cc. of oil was applied to each side of the comb daily for 5 days. The combs were measured at the beginning of the experiment and again on the 7th day. Table I gives the results of this study. In another publication (12) the dose-response curve of the capon comb following the administration of androsterone is given. The experiments show that contrary to the opinions expressed by earlier workers the specimens of fresh urine examined contained no measurable androgenic material. The same urine boiled for 15 minutes with 5 per cent sulfuric acid by volume contained an average of 44 international units per liter of dibutyl ether-soluble androgen. It seems probable that the more drastic methods of extraction employed by other workers caused partial activation of the hormone during the process of extraction.

Further similar tests show that the inactive hormone is insoluble in benzene, chloroform, and dibutyl ether. It is quite soluble in butanol, slightly soluble in ethyl acetate, and can to a large extent be adsorbed on charcoal.

**Table I**

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Androgens per liter before boiling with acid</th>
<th>Androgens per liter after boiling with acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>46</td>
</tr>
</tbody>
</table>

Androgens in Blood

Various methods have been used in this laboratory for the extraction of androgenic material from blood. The most effective is the following simple procedure.
30 cc. of oxalated blood are laked with 270 cc. of water and 15 cc. of concentrated sulfuric acid. The mixture is boiled for 15 minutes and thoroughly extracted twice with 150 cc. quantities of dibutyl ether. Emulsions which may form are broken down by the addition of sodium taurocholate. The dibutyl ether is washed twice with 50 cc. of 10 per cent sodium hydroxide and twice with 50 cc. of water and evaporated to dryness by steam distillation in a partial vacuum. The residue is removed from the flask to a small beaker with ethyl ether; 5 cc. of sesame oil are added and the ether removed on a water bath. The oil is applied evenly over the combs of five birds over a period of 5 days; i.e., each bird receives 0.1 cc. of oil on each side of the comb each day. The increase in comb size is measured 24 hours after the final inunction. The results can be expressed in international units after interpretation according to the dose-response curve.

Twenty assays carried out on a total of 100 capons have already been made. It has been found that the extracts of 50 cc. of fresh human male blood which has not been boiled caused no comb growth in five birds. Mixed samples standing at room temperature for some hours contain traces of free androgenic material. The greatest yield was obtained after boiling for 15 minutes, although boiling for shorter periods causes some activation. Continued boiling causes a rapid decrease in androgenic content. Boiling for 30 minutes decreases the yield about 50 per cent. Some androgenic activity remains after boiling for 5 hours. With the method suggested above, about 4 international units are obtained from 100 cc. of blood from normal young men.

**SUMMARY**

1. All the androgenic material in freshly voided male human urine is in an inactive form; androgens are present after boiling with acid.
2. The inactive androgenic substance is soluble in butanol, less soluble in ethyl acetate, and insoluble in benzene, chloroform, and dibutyl ether. It is adsorbed by charcoal.
3. A method is described for the extraction and assay of androgens in blood.
4. All the androgenic material in normal male human blood is inactive and can be activated by boiling with acid.
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