THE PREPARATION OF EXTRACTS CONTAINING THE ADRENAL CORTICAL HORMONE

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Since the report by Rogoff and Stewart (1) that the life span of adrenalectomized dogs could be prolonged by the administration of adrenal cortical extracts, a number of methods for preparing active extracts have been published (2-7). Many of these procedures are well adapted for preparing potent extracts on a small laboratory scale. However, in attempting to apply them on a larger basis for the preparation of sufficient quantities of adrenal cortical hormone for clinical use and for fractionation studies, we very early found that many of the steps were difficult to apply and were often accompanied by considerable losses of hormone.

One of the chief difficulties in preparing adrenal cortical extracts from whole adrenal glands has been the removal of toxic impurities, particularly epinephrine and its decomposition products. The permutit step as employed by Swingle and Pfiffner (3) is capable of removing large quantities of epinephrine, especially if repeated several times. However, this operation is somewhat difficult to adapt to the filtration of large volumes of extract, and the alternative procedures, as suggested by Kendall and coworkers (8) and Grollman and Firor (6), involving extraction with dilute acids or alkalies, have, in our experience, resulted in a considerable loss of cortical hormone.

The present study was undertaken to develop a more simplified procedure for making active extracts sufficiently purified for clinical use and readily adaptable to large scale manipulation.

EXPERIMENTAL

Assay of Extracts—Adrenalectomized rats and dogs have been used; a comparative study of these two methods of assay constitutes a separate report from this laboratory. The rat unit is
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defined as the minimum daily dose of extract which, administered by single subcutaneous injection to 4 week-old rats weighing 50 to 60 gm., over a period of 20 days, is sufficient to maintain life in 80 per cent of the animals and permit an average growth of 20 gm. per rat for the 20 day period. The average survival of uninjected controls is 6 to 7 days, and the animals injected with sustaining amounts of hormone for 20 days have been observed to die in an average of 7 to 8 days after cessation of injections. Out of 675 adrenalectomized rats used in these studies only nineteen have shown indefinite survivals without extract and in most of these the presence of residual cortical tissue could be demonstrated upon autopsy. The rat method is somewhat less time-consuming than the dog method and has routinely been used for following the potency of the various intermediate fractions. A fraction was called inactive if, injected in daily doses representing 12 gm. of gland, it failed to lengthen the survival period in a group of at least five rats as compared to that of five uninjected controls.

The final extracts have also been assayed by the dog method according to the procedure of Harrop, Pfiffner, Weinstein, and Swingle (9). Either of these methods is capable of giving satisfactory results, although the hormone requirement of immature growing rats is relatively much greater on a per kilo basis than that of adult adrenalectomized dogs.

Starting Material—Whole beef adrenal glands were used in all of these studies. The glands were frozen at the packing house and shipped to the laboratory packed in dry ice. In every case the glands were finely chopped while frozen and transferred immediately to the initial solvent.

Preparation of Extracts—Preliminary experiments were concerned with studying the various published methods for preparing adrenal cortical extracts; 3 to 4 kilo quantities of gland were used and the end-fractions assayed by the rat method. These experiments indicated that we were able to obtain the best yields of hormone by the procedure of Swingle and Pfiffner (3). Consequently, this method was used as a basis of comparison in our attempts to develop a more simple and effective procedure for obtaining a product of maximum purity in a minimum number of steps.

For initial extraction of the glands a water-miscible solvent
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offers definite advantages over a non-miscible solvent such as ether, employed by Hartman and coworkers (2). Swingle and Pfiffner (3) have used alcohol as an initial solvent. A comparison of acid acetone as recommended by Kendall and McKenzie (7) with neutral acetone as employed by Kutz (5), Grollman and Firor (6), and others showed that acid acetone extraction gave poorer yields of hormone and a less pure product.

Neutral acetone was next compared with neutral alcohol in parallel extractions of 3 kilo aliquots of the same lot of glands. Alcohol yielded 44.7 gm. of extracted solids per kilo of fresh gland, whereas acetone yielded 31.6 gm. of extracted solids per kilo of gland. When these two lots were finished by the method of Swingle and Pfiffner (3), the yields of hormone by the rat method were found to be equivalent. The acetone extract contained much less phospholipid than was obtained in an alcohol extract. Consequently acetone is a more effective solvent than alcohol in that it yields a first extract of greater purity without sacrificing hormone yields.

The acetone extract was next concentrated in vacuo below 45° to remove acetone. The aqueous residue so obtained contains considerable quantities of inert fatty material. Solvents such as benzene or ether will remove this fatty material but also will extract the cortical hormone almost quantitatively. Consequently, the use of such a solvent at this step yields an active hormone extract from which the inert lipid contaminants can be removed only with considerable difficulty. However, it was found that extraction of the aqueous residue with petroleum ether would remove large quantities of fat, leaving all of the cortical hormone in the aqueous phase. Thus, the use of petroleum ether here accomplishes the separation of a large amount of impurities, making subsequent extraction of the hormone from the aqueous phase much easier.

The next step was to choose a solvent which would extract the cortical hormone quantitatively from the aqueous solution and remove only a minimum quantity of extraneous substances. Benzene is a good solvent for the hormone but it also removes considerable quantities of phospholipids and epinephrine. If benzene is used at this point, two additional steps must be introduced for the removal, respectively, of phospholipids and epi-
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nephrine. However, it was found that ethylene dichloride is a highly selective solvent, since it extracts from the aqueous solution all of the cortical hormone but only a minimum of inert solids and no significant amount of phospholipids or epinephrine. The specificity of ethylene dichloride with respect to the exclusion of these undesirable substances is shown by the fact that the hormone-free aqueous fraction remaining after ethylene dichloride extraction will yield to subsequent benzene extraction considerable quantities of phospholipids and epinephrine. The biologically active ethylene dichloride fraction, on the other hand, contains no phospholipid which can be precipitated by cold acetone and the epinephrine content is so low that no further steps are required for its removal. Thus, the simple expedient of using ethylene dichloride for extraction of the cortical hormone from the petroleum ether-washed aqueous residue has made possible the elimination of the specific steps for the removal of phospholipids and epinephrine which have been so troublesome in previously described procedures.

The ethylene dichloride, although it separates quickly from the aqueous solution, carries with it a very small amount of water and water-soluble substances which can be removed by chilling to \(-15^\circ\) and filtering from the separated ice. The small amount of cholesterol and neutral fat still present in the ethylene dichloride solution can be removed by partitioning between aqueous alcohol and petroleum ether. This can best be done by using the fractional method as described by Butenandt (10) who used this step in the purification of theelin fractions from pregnancy urine. The ethylene dichloride solution from 100 kilos of glands is concentrated \(\textit{in vacuo}\) to remove the solvent and the residue is dissolved in 200 cc. of ethyl alcohol. An equal volume of petroleum ether, b.p. 30-70°, is added and mixed. Sufficient water is then added to make the alcohol 90 per cent, which causes the separation of a part of the petroleum ether, which is removed. The addition of water sufficient to reduce the alcohol concentration from 90 to 80 per cent causes the separation of a second portion of the petroleum ether. Finally, the alcohol phase is reduced to 70 per cent alcohol by the addition of water and extracted thoroughly with petroleum ether. When the distribution was done in this way, little trouble was encountered with emulsions.
The 70 per cent alcohol solution containing the cortical hormone is concentrated in vacuo below 45°, leaving an aqueous colloidal solution. This solution upon cooling and after addition of NaCl to make 0.9 per cent deposits an insoluble tarry substance which is inactive. It was found that a concentration of 10 mg. of extractive solids per cc. was optimum for the precipitation of this substance. The pH varied from 4.5 to 5.0. After the removal of this inactive precipitate by centrifuging, the clear supernatant solution is decanted and diluted with 0.9 per cent saline, so that each cc. represents 40 gm. of fresh whole adrenal glands. The addition of 10 per cent alcohol makes the extract more stable and does not interfere with its parenteral use. The solution is sterilized by Berkefeld filtration.

The complete process for 100 kilos of gland, giving a typical distribution of extractive solids and hormone activity, is outlined in the accompanying diagram. The final solution contains less than 1 part in 400,000 of epinephrine as measured by the dog blood pressure method. Most of the extracts assayed between 1:

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**Flow Sheet of Fractionation Procedure for Preparation of Adrenal Cortical Hormone**

<table>
<thead>
<tr>
<th>Step</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract with acetone:</td>
<td>250 liters, 99%; 200 liters, 80%</td>
</tr>
<tr>
<td>Acetone extract, 3500 gm.</td>
<td>Concentrate to 80 liters and extract with petroleum ether: 20 liters</td>
</tr>
<tr>
<td>Aqueous fraction, 2000 gm.</td>
<td>Extract with ethylene dichloride: 40 liters, 40 liters, 40 liters</td>
</tr>
<tr>
<td>Ethylene dichloride-soluble, 4.8 gm.</td>
<td>Solvent removed and fraction partitioned between 70% alcohol and petroleum ether</td>
</tr>
<tr>
<td>70% alcohol-soluble, 3.7 gm.</td>
<td>Alcohol removed and NaCl added to 0.9%</td>
</tr>
<tr>
<td>Aqueous solution.</td>
<td>Make volume up to 2500 cc. adding NaCl to make 0.9% and alcohol to make 10%</td>
</tr>
<tr>
<td>Precipitate, 1.9 gm.</td>
<td>Sterilize by Berkefeld filtration.</td>
</tr>
</tbody>
</table>

Total gland extractives, 1.8 gm. Assay, 250,000 dog units
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800,000 and 1:1,000,000 of epinephrine. The extractive solids per cc. of solution representing 40 gm. of fresh glands have varied between 0.6 and 1.0 mg. Nitrogen determination by micro-Kjeldahl procedure indicates approximately 3.5 per cent nitrogen in the extracted solids.

No toxic effects have been observed following the injection of doses of extract amounting to 5 to 15 cc. per kilo subcutaneously or intravenously in guinea pigs and rabbits. The extract has been found to be suitable for human clinical study without danger of toxic reactions.

The aqueous solution containing 10 per cent alcohol is stable for at least 1 year in the refrigerator at about 4°. Assays after

<table>
<thead>
<tr>
<th>Extract No.</th>
<th>Extractives per kilo fresh gland</th>
<th>Dog assay</th>
<th>Rat assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>Per kilo fresh gland</td>
<td>Per mg. extractives</td>
</tr>
<tr>
<td>102-MHK-2</td>
<td>10.0</td>
<td>1500</td>
<td>150</td>
</tr>
<tr>
<td>136-MHK-2</td>
<td>19.2</td>
<td>2500</td>
<td>130</td>
</tr>
<tr>
<td>185-MHK-2</td>
<td>24.5</td>
<td>2550</td>
<td>127</td>
</tr>
<tr>
<td>230-MHK-2</td>
<td>20.0</td>
<td>3380</td>
<td>134</td>
</tr>
<tr>
<td>245-MHK-2</td>
<td>25.3</td>
<td>1800</td>
<td>106</td>
</tr>
<tr>
<td>292-MHK-2</td>
<td>17.0</td>
<td>2400</td>
<td>144</td>
</tr>
<tr>
<td>14-MHK-5</td>
<td>16.7</td>
<td>2300</td>
<td>97</td>
</tr>
<tr>
<td>51-MHK-5</td>
<td>23.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 year at room temperature indicate about 75 per cent of original activity by both rat and dog methods.

In Table I we have given the results of rat and dog assays on some typical extracts prepared by the method described. The final hormone solution contains approximately 100 dog units or approximately 4 rat units per cc., representing 40 gm. of fresh gland. This corresponds to approximately 2500 dog units per kilo of gland, which is a yield equal to those reported by Pfiffner and coworkers (11) for their better extracts. Since the extracted solids usually run slightly less than 1 mg. per cc., this gives a potency of 100 dog units per mg., which is a degree of purity greater than we have been able to obtain by the application of
the Swingle and Päßner method (3). The extract prepared by our abbreviated method would appear to offer excellent starting material for further attempts at purification of the cortical hormone. This work is being continued.

We wish to express our appreciation to Dr. David Klein and The Wilson Laboratories for their assistance in supplying the adrenal glands for these studies.

SUMMARY

A method is described for preparing adrenal extracts containing the cortical hormone. The procedure consists essentially in extracting adrenal glands with neutral acetone, removing the acetone in vacuo, which leaves an aqueous residue, extracting the aqueous residue with petroleum ether to remove inert fatty substances, and extracting the cortical hormone from the aqueous residue with ethylene dichloride. The residue remaining after removal of ethylene dichloride is partitioned between dilute alcohol and petroleum ether to remove a small amount of residual cholesterol. To the aqueous solution remaining after concentration of the dilute alcohol phase is added sodium chloride to make 0.9 per cent, an inactive precipitate removed by centrifuging, and the resultant aqueous solution sterilized by Berkefeld filtration.

By this comparatively simple procedure, extracts assaying 2500 dog units per kilo of fresh gland extracted and 100 dog units per mg. of extracted solids can be easily obtained. The extracts so obtained are substantially free of epinephrine and suitable for clinical study.

BIBLIOGRAPHY

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