THE CHEMISTRY OF CRYS TALLINE SUBSTANCES
ISOLATED FROM THE SUPRARENAL GLAND

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An investigation of the chemistry of the suprarenal cortex has been under way in this laboratory for the past few years. In December, 1933, a crystalline organic substance was first obtained from an extract of the gland. This substance was used in place of the whole extract of the suprarenal cortex without any other change in experimental conditions (3). The results indicated that the crystalline material possessed physiologic activity similar to that of the extract from which it had been separated. Subsequent investigation has shown that the problem is much more complex than at first realized. We have found several substances which can be separated in crystalline form, all of which appeared to be closely related. It has also been shown that suprarenalectomized dogs and patients with Addison's disease can be maintained in a normal condition without any extract of the suprarenal gland, provided the diet contains adequate amounts of sodium chloride and sodium bicarbonate or citrate (1, 2). A discussion of the physiologic activity of the many crystalline fractions will be deferred until a later publication, but it is now apparent that the crystals first separated do not influence the level of urea in the blood.

Recently Wintersteiner and Pfiffner (7) and Reichstein (6) have also described the separation and properties of several crystalline fractions from the suprarenal gland. Previous reports on our work have been given at various meetings. We are now presenting in detail the results of our chemical studies which have been carried further than those of Wintersteiner and Pfiffner and of Reichstein. A comparison of their results with ours is given in the discussion.
Although the details of the method for the preparation of the extract have been changed from time to time, the essential points in the procedure of isolation are briefly as follows: (1) extraction of the minced glands with acetone in the cold, (2) concentration of the extract at low temperature and pressure and extraction of the aqueous concentrate with an organic solvent, (3) fractionation of the material contained in the organic solvent.

It has been recognized for some time that this original crude crystalline material was a mixture of several chemical individuals, so closely related in many of their chemical and physical properties that their complete separation has become a major problem in the investigation. Some success in the isolation of individual compounds has been achieved and degradation products have furnished information as to the components of some mixtures which could not be resolved.

A general description of the fractionation procedures is briefly as follows: The original aqueous concentrate was thoroughly extracted with benzene which was found to be the most suitable organic solvent for this step. The benzene solution was reduced to a small volume and extracted four times with twice its volume of water. The combined aqueous extracts were then reduced to a convenient volume and extracted four times with twice the volume of benzene. This water-benzene, benzene-water distribution was repeated from fifteen to twenty times. This is similar to the procedure of Pfiffner, Wintersteiner, and Vars (5), except that distilled water was used instead of solutions of sodium bicarbonate and hydrochloric acid; also, the process was carried much further.

By this means the crude original extract was separated into three main fractions. Fraction I is much more soluble in benzene than in water and therefore accumulates almost entirely in the first three benzene residues. Fraction II, being somewhat more soluble in water than in benzene, is found in the first four or five aqueous residues; whereas Fraction III possesses about the same solubility in benzene as in water and can be transferred almost quantitatively from one solvent to the other, while Fractions I and II are left behind. Consequently, Fraction III is found in the last aqueous extract of the series of extractions.

Before proceeding to a description of the further treatment of these fractions, which are still mixtures at this stage, consideration
of some of the methods used is in order. Molecular weights were determined chiefly by Rast's camphor method. In order to check the validity of this method for the compounds under consideration, the molecular weight of crystals obtained from Fraction II was determined by the method of Menzies, alcohol being used as the solvent. The results obtained by the two methods were in good agreement. Wintersteiner and Pfiffner stated that some difficulty was encountered in the use of the camphor method due to decomposition. We have not had this trouble with most of our compounds and Reichstein did not mention any such difficulty. In the case of Compounds A and E, decomposition was noted if the determination was prolonged. However, at least three readings of the melting point could be taken before there was any evidence of decomposition. Since these readings agreed well, the results are taken with reasonable confidence. The formula of Compound E has been established beyond doubt through the dinitrophenylhydrazone and our confidence in the camphor method is well justified.

Since these compounds contain only carbon, hydrogen, and oxygen, the functions of the oxygen atoms can be determined with the aid of the Grignard reagent. This was done in a "Grignard machine" adapted to quantities of 5 to 15 mg. of the substance to be tested. The apparatus is essentially a small scale reproduction of that described by Kohler and Richtmeyer (4), which permits the simultaneous estimation of the number of carbonyl groups and active hydrogen atoms. The determination of active hydrogen atoms is the same as in the usual Zerewitinoff method. The carbonyl groups are estimated by comparison of the total amount of methane evolved on addition of water after the Grignard reagent has acted on the substance with the amount of methane that would be evolved by addition of water to the same amount of pure Grignard reagent. The reagent which has combined with carbonyl groups will not yield methane. The data are expressed as per cent of hydroxyl or of carbonyl. The maximal absolute error in the determination of active hydrogen atoms is estimated as 0.1 cc., and in the determination of carbonyl groups as 0.2 cc. Sufficient material (12 to 15 mg.) was always used so that at least 1 cc. of gas was measured for each active group. When more than one group was present, the accuracy was entirely satisfactory. Even
though a maximal error of 20 per cent was involved, it was easily possible to distinguish between one and two carbonyl groups. The consistency of our results indicates the suitability of this method for the compounds under discussion. Since it is essential that the substance under examination be completely in solution before addition of the Grignard reagent, and since our compounds are very slightly soluble in isoamyl ether, anisole, and various hydrocarbons usually used as solvents in this procedure, pyridine was employed. However, this latter solvent is somewhat objectionable, since it produces a rather large blank in spite of rigid purification. Finally, a mixture of 0.5 cc. of pyridine and 1.0 cc. of isoamyl ether was found to be satisfactory in regard to blank and complete solution of the substances under discussion, and was used for samples up to 15 mg.

The micromethods of Pregl were used for the tests for methoxyl and ethoxyl groups and for ultimate analysis.

Fraction I

This fraction contains two substances, probably more. One of these has been isolated in what is believed to be a pure state. This was accomplished by crystallization of the crude fraction from water. The product of the first crystallization (215 mg.) was a mixture of prisms and rosettes of needles. Recrystallization from water, by allowing the acetone to evaporate from a dilute acetone solution, again yielded a mixture of large prisms and rosettes. The prisms were sufficiently dense to settle rapidly, while the rosettes tended to float. The two forms were thus separated mechanically.

Compound A—The prisms weighed 121 mg., but still contained a small amount of the other crystalline form. The melting point was indefinite and measurement of optical activity showed \([\alpha]^{26}_{D} = +342^\circ\). Two more recrystallizations yielded first 100 mg. and finally 77 mg. of prisms which were entirely free of the rosettes. The melting point was then 177–179.5° (uncorrected) and \([\alpha]^{26}_{D} = +347^\circ\) (0.23 per cent in benzene). There was no color test for a double bond with tetranoitromethane and no fluorescence in ultraviolet light.

\(\text{C}_{26}\text{H}_{36}\text{O}_{6}\). Calculated. C 72.89, H 8.47, mol. wt. 428
Found. " 73.12, " 8.51, " 410
Oxidation of these prisms produced an acid which had been encountered several times previously on oxidation of various crude non-crystalline fractions. The material (88 mg. of Compound A) was dissolved in 15 cc. of acetone. To this was added a mixture of 25 cc. of a 2.0 N solution of potassium dichromate, 7 cc. of 5 N sulfuric acid, and 18 cc. of water. The oxidation was allowed to proceed 1½ hours at room temperature. After removal of the acetone under reduced pressure the reaction mixture was thoroughly extracted with ether. The acid was extracted from the ether with a solution of sodium carbonate. An insoluble, highly crystalline acid (35 mg.) was precipitated from the aqueous solution upon acidification with hydrochloric acid. There remained in the ether 40 mg. of a non-acid having \([\alpha]_{D}^{247} = +257^\circ\) (0.06 per cent in benzene). This, on retreatment with chromic acid, yielded a second crop of the acid. The acid was recrystallized from hot water. It does not have a true melting point, but decomposes over a considerable range of temperature (245–260°). When it was tested with tetranitromethane, a strong yellow color was produced; \([\alpha]_{D}^{247} = +290^\circ\) (0.1 per cent in 30 per cent alcohol).

\[ \text{C}_{20}\text{H}_{26}\text{O}_{4}. \quad \text{Calculated, } \text{C } 72.68, \text{ H } 7.93; \text{ found, } \text{C } 72.58, \text{ H } 8.07 \]

Previous samples of an acid which decomposed over the range of 240–260° and which were obtained by the oxidation of crude material had much the same properties. The analytical values lay between the values calculated for \(\text{C}_{20}\text{H}_{26}\text{O}_{4}\) and those calculated for \(\text{C}_{20}\text{H}_{28}\text{O}_{4}\). The test with tetranitromethane was usually weak. Repeated crystallizations of a large sample of this kind yielded a product, the analysis of which gave values which agreed well with those calculated for \(\text{C}_{20}\text{H}_{28}\text{O}_{4}\). It gave no color with tetranitromethane; \([\alpha]_{D}^{267} = +267^\circ\) (0.10 per cent in 30 per cent alcohol).

\[ \text{C}_{20}\text{H}_{28}\text{O}_{4}. \quad \text{Calculated. } \text{C } 72.24, \text{ H } 8.49; \text{ found. } \text{C } 72.55, 72.32, \text{ H } 8.38, 8.24 \]

In another case, a sample of this type of acid was obtained by chromic acid oxidation of amorphous material, and decomposed at 248–260°. After several recrystallizations from dilute alcohol the analysis of the acid agreed with the values calculated for \(\text{C}_{20}\text{H}_{28}\text{O}_{4}\). The molecular weight was determined by titration; 27 mg. required 8.2 cc. of 0.01 N sodium hydroxide.
This sample gave a strongly positive test with tetryntrichloro-
methane. It proved to contain 1 active hydrogen atom and two 
carbonyl groups.

C_{20}H_{26}O_{4}. Calculated. C 72.68, H 7.93, mol. wt. 330
Found. C 72.38, 72.54, " 8.07, 7.93, " 329

For future reference we shall designate the acid, C_{20}H_{26}O_{4}, as
Acid 1 and C_{20}H_{28}O_{4} as Acid 2.

Compound B—In most cases, however, in which the oxidation
was carried out on crude amorphous material, the analyses of the
products indicated mixtures of C_{20}H_{26}O_{4} and C_{20}H_{28}O_{4}. Since
Compound A is the precursor of the acid C_{20}H_{26}O_{4}, it is possible
that the substance (Compound B) obtained in this same fraction
and which crystallizes in the form of rosettes of needles possessing
a melting point of 135–139° (uncorrected) is the precursor of the
acid C_{20}H_{28}O_{4}. As yet, not enough of this compound has been
obtained sufficiently pure for an investigation of this possibility.
Analysis of this material showed the following.

C_{20}H_{26}O_{4}. Calculated. C 71.23, H 8.98
Found. C 71.50, " 8.48; found, C 71.55, H 8.76

Both of these C_{20} acids are quite resistant to further oxidation
with chromic acid. On the other hand, they are easily attacked
by alkaline silver solutions.

Fraction II

The crude crystals generally contained some amorphous material
which was best removed by solution in acetone and precipitation
with petroleum ether. The crystals obtained by this procedure
were invariably needles, usually grouped in rosettes. The needle
form could be changed to octagonal plates by crystallization from
dilute alcohol, although sometimes the needle form also separated
from this solvent. The crystals thus obtained varied in composi-
tion. The carbon content varied from 68.0 to 69.5 and the hydro-
gen from 8.8 to 9.5 per cent. The material was subjected to
various schemes of fractionation with only partial success; that is, it was not possible to isolate chemical individuals from the mixture. However, after oxidation with alkaline silver it was possible to isolate in pure form an acid having the composition C_{21}H_{34}O_{6}, which we shall call Acid 3. The yield of this acid and the optical activity of the crude material were used to follow the fractionation. The most successful method involved solution in acetone, addition of a relatively large volume of water, removal of the acetone in vacuo, and finally evaporation of the water in vacuo. When the acetone was all removed a portion of the material separated promptly (Subfraction II, a). As the water was evaporated further separation of crystals occurred (Subfraction II, b). The evaporation was interrupted from time to time and the crystals removed. The volume was finally reduced to about 25 cc. and the portion which remained dissolved was designated as Subfraction II, c. This procedure was repeated with the various fractions until their properties ceased to change.

Subfraction II, a, Compound C—This fraction gave the largest yield of Acid 3 and possessed the highest specific rotation. It was further fractionated from acetone and petroleum ether (1:1) and, although the less soluble portion yielded a larger proportion of acid, the optical activity was not changed appreciably. The maximal yield of Acid 3 was 45 per cent and could not be increased by further fractionation. The precursor of Acid 3 will be designated as Compound C. Although not actually isolated, some of its properties are well characterized and this designation will facilitate discussion.

The melting point of the best product was 220–227° (uncorrected) with decomposition. The optical activity varied greatly with the solvent. In alcohol \([\alpha]_{D}^{25} = +110°\) (0.087 per cent), while in benzene \([\alpha]_{D}^{25} = +56°\) (0.06 per cent). The solubility in water was about 0.025 per cent; in benzene, about 0.06 per cent; it was readily soluble in acetone and alcohol.

Other samples of this fraction varied somewhat in properties. Analyses indicated a mixture of C_{21}H_{34}O_{6} and one or more other compounds having slightly different carbon and hydrogen contents. The molecular weight was determined by Rast's camphor method and by Menzies' method.
Determinations of active hydrogen atoms and carbonyl groups showed the presence of three hydroxyl groups, one carbonyl group, and 1 inactive oxygen atom. The inactive oxygen atom is presumed to be involved in an ether linkage. The test for a methoxyl or ethoxyl group was negative.

\[
\text{C}_7\text{H}_8\text{O}_4. \, \text{Calculated.} \quad 3\text{OH} \, 13.9, \, 1\text{CO} \, 7.65
\]
\[
\text{Sample 1. Found.} \quad \text{OH} \, 13.7, \, \text{CO} \, 6.1
\]
\[
\text{" 2. "} \quad \text{" 13.4, "} \quad \text{" 7.1}
\]

The oxidation of this fraction with alkaline silver was usually carried out as follows: to 100 mg. dissolved in 5 to 10 cc. of alcohol was added a solution of 625 mg. of silver sulfate in 8 cc. of 2 N ammonium hydroxide and 8 cc. of N sodium hydroxide solutions. After a slight lag the oxidation proceeded rapidly and was complete within 45 minutes. The consumption of silver was 2.3 to 2.9 equivalents for each mole, calculated for a molecular weight of 366. The metallic silver was filtered out and washed well with 50 per cent alcohol. The excess silver ions in the filtrate were precipitated with hydrochloric acid. The filtrate was then made alkaline with sodium carbonate and the alcohol was removed in \textit{vacuo}. The aqueous residue was extracted with ether to remove a small amount of non-acid. After acidification to Congo red and saturation of the solution with sodium chloride the acid was extracted with ether. Assuming a molecular weight of 366 the yield was 90 to 100 per cent. The dry product was leached with small portions of cold acetone. Up to 42 per cent of the material (Acid 3) remained undissolved. Addition of several volumes of petroleum ether to the acetone solution precipitated material which, on further treatment with cold acetone, yielded enough Acid 3 to raise the total yield of this acid to 45 per cent.
Acid 3—The acetone-insoluble portion was dissolved in hot ethyl acetate with the aid of a little alcohol. On standing overnight long blades were deposited. For analysis Acid 3 was recrystallized to a constant melting point. It is sparingly soluble in hot acetone, cold dioxane, and petroleum ether; readily soluble in water and alcohol. It decomposes sharply at 240-242° (uncorrected). Under ultra-violet light it fluoresces strongly with an almost white light. It is slowly attacked by alkaline silver and does not give a color with tetryanitromethane. The molecular weight was determined by titration. A sample of 45.6 mg. required 11.75 cc. of 0.01 N NaOH.

\[
\text{C}_2\text{H}_4\text{O}_4. \quad \text{Calculated.} \quad \text{C} \ 65.92, \quad \text{H} \ 8.96, \quad \text{mol. wt.} \ 382 \\
\text{Found.} \quad \text{“} \ 66.03, 65.86, \quad \text{“} \ 9.02, 9.09, \quad \text{“} \ 388
\]

\[
\text{C}_2\text{H}_3\text{O}_3. \quad \text{Calculated.} \quad \text{C} \ 75.41, \quad \text{H} \ 9.50 \\
\text{Found.} \quad \text{“} \ 75.36, 75.42, \quad \text{“} \ 9.52, 9.59
\]

Oxidation of Acid 3, Ketone 1—Acid 3 is readily oxidized by chromic acid to a ketone. The acid (50 mg.) was dissolved in water (10 cc.) as the sodium salt. To this was added a 2 N solution of potassium dichromate (2 cc.) and N sulfuric acid (5.5 cc.). A flocculent non-crystalline precipitate appeared but disappeared as the oxidation proceeded. In this instance 4 equivalents of oxidizing agent were used in 1 hour. 12 mg. of acid and 34 mg. of a non-acid were obtained. The non-acid melted at 159-160° (uncorrected) with slight softening at 156° after recrystallization from water.

Subfraction II, b—In addition to its greater solubility in water, this fraction differs from Subfraction II, a in that, when treated with alkaline silver under the same conditions, it gives not more than a 20 per cent yield of Acid 3.

At first the total acid obtained from the silver oxidation of this fraction was treated with chromic acid under the same condition as was Acid 3. The non-acid recovered from the oxidation crystallized from water as a mixture of two crystalline forms—needles and plates. This mixture melted at 142-145° with decomposition. The analyses of several such preparations were not in very good agreement, indicating a mixture.
Inasmuch as oxidation of Fraction II, b always gave some Acid 3 (10 to 20 per cent), this was removed after oxidation with silver through its insolubility in acetone before proceeding with further study of the acetone-soluble fraction. Apparently this is practically identical with the acid fraction derived from the silver oxidation of Subfraction II, a which is soluble in acetone.

Its properties suggest that it is a mixture. Part of it easily forms an amorphous lactone which is resistant to oxidation with chromic acid under the conditions used for the oxidation of Acid 3. However, the portion which does not close to a lactone can be oxidized to a ketone with chromic acid. Thus far attempts to crystallize this acid or mixture of acids have failed for the most part. In one case, a small amount of crystalline material was obtained from this fraction. It is not certain that this is representative of the portion which does not form a lactone.

C50H22O6. Calculated, C 65.17, H 8.76; found, C 64.98, H 8.76

Oxidation of Acid Fraction Soluble in Acetone from Subfraction II, b, Lactone Formation—Several preparations of the acid fraction soluble in acetone have been oxidized with chromic acid. A typical result is as follows: From 84 mg. of Subfraction II, b ([α] = +104°) there were obtained by oxidation with silver under the conditions previously described, 78 mg. of acid. This yielded only 8 mg. of Acid 3. The remainder was easily soluble in less than 1 cc. of cold acetone. To this acid fraction in 5 cc. of methyl alcohol and 1.6 cc. of water, 1 cc. of 5 N sulfuric acid was added. After standing for 5 minutes the alcohol was removed in vacuo and the aqueous residue was extracted with ether. The ether extract was washed with sodium carbonate solution and then with water. The ether contained 39 mg. of a non-acid and 30 mg. of acid were recovered from the sodium carbonate solution after acidification and extraction with ether. The treatment with mineral acid, therefore, had converted more than half of the acid originally present into a lactone. Each of these fractions was treated separately with chromic acid under the conditions used for the oxidation of Acid 3. The non-acid fraction yielded 25 mg. of material which was apparently unchanged. On heating with 0.1 N sodium hydroxide for a half hour it required 0.7 cc. of 0.1 N base.

Ketone 2—With chromic acid the acid fraction (30 mg.) gave a
nearly quantitative yield of needle-like crystals. This material was non-acid, was not affected with sodium hydroxide, and melted, after two recrystallizations from water, at 159–161° (uncorrected). It forms a crystalline semicarbazone.

\[ \text{C}_{12}\text{H}_{15}\text{O}_3 \]. Calculated, C 74.94, H 9.27; found, C 75.03, H 9.19

Further work is under way to determine the nature of this entire acetone-soluble acid fraction.

**Subfraction II, c, Compound D**—This fraction consists largely of a polyalcohol or possibly a mixture of two such alcohols, which can be separated by heating the mixture with dilute sodium hydroxide. Since the compounds of Subfraction II, b, as well as of Subfraction II, a, are completely converted to acids by this treatment, the polyalcohol, which is stable to sodium hydroxide, can be further separated by extraction of the alkaline solution with ether. It is characterized by its relatively low solubility in cold acetone and high solubility in water. Repeated crystallization from hot acetone gave a product which crystallized in hexagonal plates and melted at 214–216° (uncorrected). This is undoubtedly the same material described by Wintersteiner and Pfiffner as Compound A and by Reichstein as Substance A. Some of the properties and the analyses show considerable discrepancies, however. Our best preparation gave an analysis which agreed very well with the values calculated for \( \text{C}_{20}\text{H}_{35}\text{O}_5 \). Other preparations, which had melting points of 213–215° after preliminary softening at about 170°, had lower carbon contents. It was thought that this was due to the presence of hydrated crystals and that the partial melting at about 170°, also observed by Wintersteiner and Pfiffner and by Reichstein, was caused by the presence of this hydrate which lost water at that temperature and consequently showed the same final melting point as the anhydrous material. The experiment to be described later supports this hypothesis, but the decidedly higher carbon content of Reichstein's product and the agreement in composition of his samples dried at 100° and 170° are contrary to this conclusion. Lack of material has prevented us from settling this point at present.

Our best sample (Compound D) was derived from 379 mg. of crude crystalline material. It was decolorized in hot acetone solution with charcoal and recrystallized to a constant melting
point; \([\alpha_{D}]^{29°} = +29°\) (0.13 per cent in acetone). It crystallized from hot acetone as large hexagonal prisms. This particular sample did not show a preliminary softening at about 170° but melted at 214-216° (uncorrected). It was dried to constant weight at 80° and 0.1 mm. over anhydrous magnesium perchlorate and was then very hygroscopic.

\[
\begin{align*}
C_{25}H_{24}O_{6} & \quad \text{Calculated.} \quad C \ 67.74, \quad H \ 9.67, \quad 4\text{OH} \ 19.2 \\
C_{26}H_{26}O_{6} & \quad \text{Found.} \quad C \ 67.27, \ 67.33, \ H \ 10.25, \ 10.00, \ 19.5 \\
\end{align*}
\]

4 active hydrogen atoms were found; there was no evidence of a carbonyl group in the quantitative estimation, and the material was recovered unchanged after treatment in aqueous solution with a solution of dinitrophenylhydrazine in 2 N hydrochloric acid. The test with tetrinitromethane was negative.

The following experiments create uncertainty as to the number of hydrogen atoms, but support the conclusion that there are 20 carbon atoms.

About 15 mg. of the crystals which were analyzed were dissolved in a few drops of warm alcohol. The solution was diluted to 15 cc. with water and allowed to stand 48 hours. The solution was perfectly clear. Then 1 cc. of concentrated hydrochloric acid was added. In a short time the solution became cloudy and crystals soon appeared. The separation of crystals was rather slow but was complete in 24 hours. These crystals were large blades. After being dried in the air they melted at 145-155° with vigorous evolution of gas. Drying for 3 hours at 80° and 0.1 mm. did not change the melting point, but after the substance was crystallized from acetone the melting point was 165-170° with decomposition. This was repeated with 19.0 mg. of material. The solution in 2 cc. of alcohol was diluted to 21 cc. with water and 1 cc. of concentrated hydrochloric acid was added. After standing 4 days, there were recovered 15.0 mg. of crystals dried to constant weight at 80° and 0.1 mm. over anhydrous magnesium perchlorate. This material was not hygroscopic. It melted at 150-155° (uncorrected).

\[
\begin{align*}
C_{26}H_{34}O_{12} \cdot 2H_2O & \quad \text{Calculated.} \quad C \ 66.07, \quad H \ 9.71 \\
\quad & \quad \text{Found.} \quad C \ 65.99, \ 66.07, \ H \ 9.82, \ 9.86 \\
\end{align*}
\]
When tested with tetranitromethane, a yellow color was produced. After the material had been dried at 170° and 0.1 mm., the test was negative.

In another experiment 50 mg. of the alcohol dissolved in 0.5 N sulfuric acid were heated for 2 hours on the steam bath. The product thus obtained was similar to that just described; m.p. 172–178° (uncorrected).

C₁₈H₃₄O₄·½H₂O. Calculated. C 66.07, H 9.71
C₁₈H₃₄O₄·½H₂O. “ “ 65.70, “ 10.21; found, C 65.86, H 9.72

In the meantime most of the material had been used for other purposes and a settlement of the uncertainties was not possible. For the present we conclude that the substance described possesses the formula C₂₀H₃₄O₂₅ or C₂₀H₃₆O₆, that it forms a very stable hydrate which contains a half molecule of water, and that this can be removed by heating above 155° or by repeated crystallization from hot acetone. There are four hydroxyl groups in the molecule and 1 inactive oxygen atom. Further work on this substance is in progress.

Oxidation of this alcohol with chromic acid yielded Ketone 3. The alcohol (140 mg.) was dissolved in 10 cc. of acetone and 5 cc. of water. To this solution were added 6 cc. of 2 N potassium dichromate solution and 2 cc. of 5 N sulfuric acid. This mixture was allowed to stand for 2½ hours at room temperature. At the end of this time almost 10 equivalents of oxidizing agent had been consumed. A small portion of the product was acidic and was discarded. The crude non-acid portion weighed 100 mg. This was recrystallized from dilute alcohol. The first crop weighed 53 mg. and consisted of blade-like needles which melted at 160–161.5° (uncorrected); [α]D²⁰ = +229° (0.074 per cent in benzene). The second crop of 30 mg. melted at a lower temperature and was obviously impure. The first fraction was dried for 2 hours at 80° and 0.1 mm. over anhydrous magnesium perchlorate. Analysis speaks for the composition C₁₈H₂₉O₃ (Ketone 3).

C₁₈H₂₉O₃. Calculated. C 74.94, H 8.40
C₁₈H₂₉O₃. “ “ 74.48, “ 9.03
Found. “ “ 74.92, 75.06, “ 8.57, 8.84
The determination of hydroxyl and carbonyl groups showed the presence of one of each. The third oxygen atom was inactive.

\[ \text{C}_{13}\text{H}_{11}\text{O}_8 \]. Calculated, OH 5.90, CO 9.7; found, OH 6.41, CO 11.3

The ketone did not give an iodoform test. 25 mg. yielded 15 mg. of an oxime which crystallized in rosettes of needles and melted with final decomposition over the range 205–232°. Difficulties encountered in the attempt to purify the oxime prevented a more definite characterization of this derivative.

**Fraction III**

*Compound E*—On concentration of the aqueous solution containing Fraction III several crops of crystals were obtained. The least soluble portion of the crude material usually had a specific rotation of about +200° in benzene. The specific rotation was raised as high as +257° by repeated crystallizations from hot benzene but could not be raised further. It was found that the material still contained some of Compound D which had been carried through in the partition procedure. The specific rotation was raised to a maximum by repeated crystallization from water. The material was dissolved in acetone and this solution diluted with water. As the acetone evaporated, crystals separated. Three crystallizations were sufficient to yield a single substance. A fourth treatment caused no change in optical activity; \([\alpha]^2_{D} = +269^\circ \) (0.125 per cent in benzene). The melting point was 201–208° (uncorrected) with decomposition. The analysis and the molecular weight determination were in excellent agreement with the formula \(\text{C}_{21}\text{H}_{30}\text{O}_8\). This was confirmed by analysis of the dinitrophenylhydrazone. The substance was dried 2 hours at 110° and 0.1 mm. over anhydrous magnesium perchlorate.

\[ \text{C}_{21}\text{H}_{30}\text{O}_8 \]. Calculated. C 69.57, H 8.35, mol. wt. 362

Found. \( 69.82, 69.60, " 8.37, 8.72, " 364 \)

The 2,4-dinitrophenylhydrazone proved to be a well characterized derivative which easily could be obtained analytically pure. It is so easily separated and recognized that it has been used for the determination of Compound E in certain mixtures. For its preparation 25.0 mg. of Compound E were dissolved in 4 cc. of alcohol. To this solution were added 10 cc. of a saturated solution
of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid. Almost immediately a red gelatinous precipitate appeared. After standing several hours the precipitate was centrifuged out and washed once with 25 per cent alcohol. It was dissolved in a hot mixture of 6 cc. of ethyl acetate and 10 cc. of alcohol; 5 cc. of water were added while hot and the solution allowed to stand overnight. The red-orange crystals weighed 29 mg. and after recrystallization from ethyl acetate melted at 255–256° (uncorrected) with decomposition.

C_{17}H_{15}O_{5}N_{4}. Calculated. C 59.75, H 6.32, N 10.33

The dinitrophenylhydrazone is sparingly soluble in cold alcohol and ethyl acetate. By crystallization from a large volume of hot ethyl acetate it can be separated readily from impurities.

Compound E shows selective absorption with a maximum at 2370 Å. The molecular extinction coefficient at 2370 Å was 16,150.1 Absorption in this region by their active hormone fraction has been noted by Pfiffner, Wintersteiner, and Vars. Reichstein observed that his Substance C showed a band with maximal absorption at about 2350 Å. As has been pointed out by these workers, such an absorption band is indicative of an \( \alpha,\beta \)-unsaturated ketone grouping. The nature of this compound is under investigation.

DISCUSSION

The properties of the compounds are summarized in Table I. The similarities of Compounds C, D, and E are obvious. Compound C, although not isolated, deserves some further comment, since the information gained from its oxidation permits a few conclusions as to its structure. The oxidation to an acid with alkaline silver speaks for the presence of an aldehyde group. We are not unaware of the possibility of a rearrangement in the alkaline medium but this seems unlikely. When the crystals of Subfractions II, a and II, b are heated with 0.05 N sodium hydroxide,

1 We are indebted to Professors Koch and Hogness of the University of Chicago for the determination of the absorption spectrum and to the Rockefeller Foundation which is supporting spectroscopic biological investigation at the University of Chicago.
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<thead>
<tr>
<th>Compound</th>
<th>Acid</th>
<th>Ketone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>M.p. (uncorrected)</td>
<td>[α]_D^20</td>
</tr>
<tr>
<td>A</td>
<td>C_{12}H_{26}O_5</td>
<td>177–179.5°C</td>
</tr>
<tr>
<td>B</td>
<td>C_{12}H_{26}O_5</td>
<td>135–139</td>
</tr>
<tr>
<td>C</td>
<td>C_{12}H_{26}O_5</td>
<td>Separate as mixed crystals</td>
</tr>
<tr>
<td>D</td>
<td>C_{12}H_{26}O_5</td>
<td>214–216</td>
</tr>
<tr>
<td>E</td>
<td>C_{12}H_{26}O_5</td>
<td>201–208</td>
</tr>
</tbody>
</table>

* Neither of these two acids has a definite melting point or decomposition point. They begin to shrivel and turn brown at 245°C and finally evolve gas at 260°C when the temperature is raised at the rate of 5°C a minute.
† The relation of Acid 2 to Compound B is a matter of speculation at present. They are grouped together for convenience.
‡ It is not known whether Ketone 2 is derived from Acid 4. They are placed on the same line since both are derived from the substance or substances which accompany Compound C.
they are quantitatively converted to a mixture of acids among
which there is none of the acid (Acid 3) produced by the oxidation
of Compound C with alkaline silver. This conversion to acid with
alkali alone does not occur to an appreciable extent in the cold.
The degradation of the acid, C_{21}H_{34}O_{6}, to the ketone, C_{20}H_{30}O_{3},
leads to the conclusion that there is a tertiary alcohol group
adjacent to the aldehyde group. Since only 1 carbon atom but 3
oxygen atoms were lost, a molecule of water must have been
eliminated with formation of a double bond. This was confirmed
by a positive, though not very strong, test with tetranitromethane.
Assuming that the inactive oxygen atom is an ether oxygen, we
may write the following partial formulas for Compound C and its
oxidation products.

\[
\begin{align*}
\text{H} & \quad \text{H} \\
({\text{HO}})_{2} & [O < \text{C}_{19}{\text{H}}_{30}] > \text{C} & - & \text{C} = & \text{O} & \rightarrow & ({\text{HO}})_{2} & [O < \text{C}_{19}{\text{H}}_{30}] > \text{C} & - & \text{COOH} & \rightarrow \\
\text{OH} & & & & & \text{OH} \\
\text{HO} & [O < \text{C}_{19}{\text{H}}_{30}] > \text{C} & - & \text{O}
\end{align*}
\]

This interpretation is somewhat different from the one quoted
by Reichstein. Our previous interpretation was based on the
preparation of a ketone, C_{19}H_{30}O_{3}, and its semicarbazone. The
ketone was thought to be derived from the acid C_{21}H_{34}O_{6} before
the nature of the mixture from which this acid came was fully
understood. It is now evident that the side chain which is elimi-
nated in the ketone consists of 1 carbon atom instead of 2.

A comparison of our compounds with those of Wintersteiner
and Pfiffner and of Reichstein reveals agreement only in the case
of our Compound D. The other substances which we have iso-
lated have no counterpart in those described by the other workers.
Several of our preparations have had compositions identical with
that of a compound, C_{21}H_{34}O_{6}, as given by Reichstein for his
Substances C, D, and E, but the oxidation with alkaline silver
revealed them as mixtures. His Substance G has an absorption
band and a high specific rotation in common with our Compound
E, but the melting point and composition are not at all similar.
We have also had many crystalline preparations with the properties
and composition of the Compound B of Wintersteiner and Pfiffner
but they have invariably proved to be mixtures.

These other workers have recognized the difficulties involved in
the separation of individuals from the mixture of compounds present in the extracts of the suprarenal gland and the possibility that some of the "compounds" may in fact be mixtures. Portions of their analytical data suggest that that is the case.

Our experience in heating crystalline preparations with 2,4-dinitrophenylhydrazine in various media was similar to that of Wintersteiner and Pfiffner; no definite products could be isolated. However, treatment in the cold has yielded crystalline dinitrophenylhydrazones from which several individuals have been isolated. This derivative of Compound E has been described.

The test for a double bond with tetranitromethane has been used with some success, but there was one case of a false positive test which should be pointed out. When Compound D was crystallized from approximately N hydrochloric acid, it gave a strongly positive test which at first was interpreted to mean that a double bond had been produced through loss of a molecule of water. The analysis, however, was not compatible with this interpretation. When the material was dried at 170° and 0.1 mm., the residue did not give a test with tetranitromethane. Evidently the water of hydration caused the development of the yellow color.

We have not seen any previous mention of such a false indication of the presence of a double bond, although negative tests have been reported when a double bond was undoubtedly present.

SUMMARY

The fractionation of an extract of the suprarenal gland has been described briefly. Four compounds have been isolated and a fifth has been identified through its products of oxidation. These compounds, which contain only carbon, hydrogen, and oxygen, are closely related in physical and chemical properties and are accompanied by other closely allied compounds which have not yet been isolated in a pure state. Their general nature is that of polyhydroxycarbonyl compounds. Some of the details of their structures have been determined. A possible error in the application of the tetranitromethane test for double bonds has been discussed.

BIBLIOGRAPHY

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Harold L. Mason, Charles S. Myers and Edward C. Kendall


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