J. Physiol. (1967), **191**, *pp.* 691–697
With 4 text-figures
Printed in Great Britain

EVIDENCE FOR THE PRESENCE OF 16α-HYDROXYPREGN-4-ENE-3,20-DIONE IN ADRENAL VENOUS BLOOD OF YOUNG PIGS

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(*Received* 6 *April* 1967)

SUMMARY

1. Evidence was obtained for the presence of 16α-hydroxypregn-4-ene-3,20-dione in each adrenal venous blood sample collected from eight young pigs under conditions of operative stress.

2. The chemical identity of this steroid was tested on μg amounts by paper and gas chromatographic methods before and after the formation of derivatives.

INTRODUCTION

During studies of the adrenal steroids which are secreted by domestic animals, small amounts of a compound with chromatographic properties similar to those of 16α-hydroxypregn-4-ene-3,20-dione (16α-OH-progesterone) were found in the adrenal vein blood from a young pig (Heap, Holzbauer & Newport, 1966).

This compound has now been detected in fifteen adrenal blood samples collected from another eight pigs and further evidence for its identity with 16α-OH-progesterone has been obtained.

METHODS

Female Landrace or Large White pigs, 8–12 weeks old, were premedicated with Sernylan (Parke, Davis and Co., 1-(phenyl-cyclohexyl) piperidine HCl, 1 mg/kg body weight, subcutaneous) and when sedated they were anaesthetized with halothane followed by chloralose (Merck, 40 mg/kg body weight, 0·7 % solution in 0·9 % NaCl, I.V.). An abdominal mid line incision was made and the viscera and both kidneys were removed. The region of the inferior vena cava in the proximity of the adrenal glands was freed from surrounding tissue. Venous tributaries not coming from the adrenals were tied. The abdominal aorta and cava were ligated below the renal vessels and a cannula was inserted into the cava above this ligature. The vena cava was occluded at its entry into the liver and the venous blood from both adrenals was collected in a cooled glass cylinder. Collection periods were either 5 or 10 min. Both adrenals were removed from the animal immediately after the collection of the blood.

The blood samples and the adrenals were extracted and the extracts purified as described previously (Holzbauer, 1957, 1964). All samples were further purified and analysed by paper and gas-liquid chromatography. The paper used was Whatman no. 50. It was first treated with 2N-NaOH followed by distilled water (Sharman, 1963) and then washed with the mixture used to elute the chromatograms (ethyl acetate:methanol, 2:1) and the mobile phase of Bush's system B_3 (Bush, 1952). This purification procedure greatly reduced non-specific peaks ('paper blanks') which otherwise occurred on the gas chromatographic record when eluates from papers were applied. Furthermore, steroid spots on chromatograms developed on NaOH-pretreated Whatman no. 50 paper showed much less tailing than on untreated Whatman no. 2 paper.

Gas-liquid chromatography was carried out on a Model 402 F & M gas chromatograph using a 4 ft. (122 cm) 3·8 % SE-30 column at 230° C, a flame ionization detector and argon as carrier gas. The retention times of the steroids were expressed in relation to that of cholestane which averaged 20 min, and they are referred to as relative retention times. The dried eluates from paper chromatograms were dissolved in 40 μl. of ethanol and volumes of 1–2 μl. applied to the gas-liquid chromatograph. All solvents used were specially purified (Heap *et al.* 1966).

RESULTS

The purified blood extracts were first chromatographed for 4 hr in the E_2B system of Eberlein & Bongiovanni (1955) to separate off the major glucocorticoids. In this system, 16α-OH-progesterone has an R_F value of 0·85. The eluates of these regions were rechromatographed for 3 hr in the B_3 system to separate 16α-OH-progesterone from other C_{21} and C_{19} steroid.

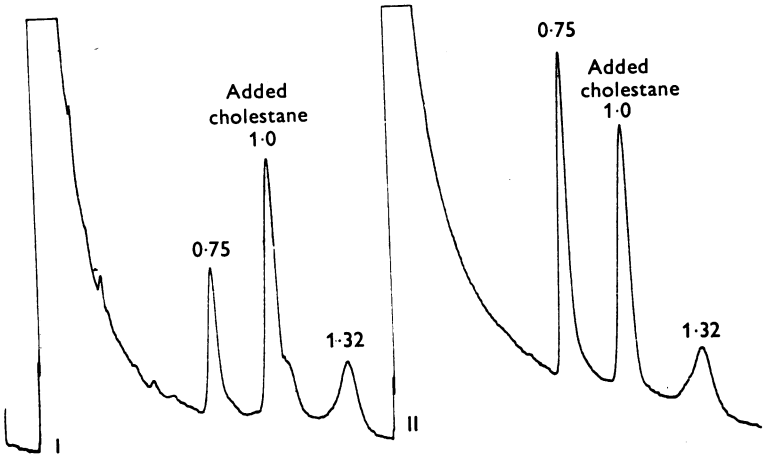

Fig. 1. Gas chromatograph tracings. I: purified pig adrenal vein blood extract, rechromatographed in B_3 (24 hr). U.V. absorbing spot with R_F value equal to that of authentic 16α-OH-progesterone was eluted, dried, dissolved in 40 μl. ethanol, 1 μl. applied to the gas-liquid chromatograph (range 1, attenuation 8). (Shoulder on cholestane peak due to 'paper blank', relative retention time 1·04.) II: authentic 16α-OH-progesterone. The figures above each peak give the retention times relative to that of cholestane.

Provisional evidence was obtained by gas-liquid chromatography for the presence of 16α-OH-progesterone in each individual sample.

For further identification the eluates of eight blood samples were combined and then divided into three equal parts.

One-third was rechromatographed in the B_3 system for 24 hr to separate 16α-OH-progesterone from 17α,21-dihydroxypregn-4-ene-3,20-dione (compound *S*). Two U.V. absorbing spots were visible with R_F values corresponding to those of authentic 16α-OH-progesterone and compound *S*. The 16α-OH-progesterone region was eluted.

When authentic 16α-OH-progesterone was applied to the gas chromatograph two peaks were consistently observed with relative retention times of 0·75 and 1·32 (Fig. 1, II). The first peak was always larger than the second one. The peak size ratio varied which makes it unlikely that one of the peaks is due to an impurity. It is more likely that the steroid is split

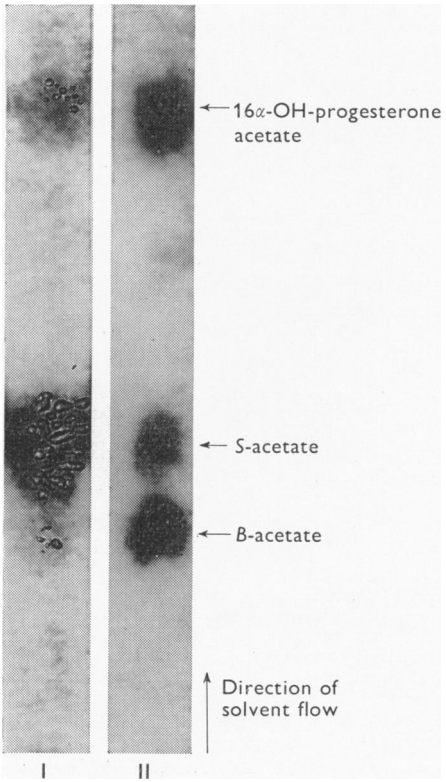

Fig. 2. Contact photograph of U.V. absorbing spots on a paper chromatogram developed for 3½ hr in B_3. I: partially purified 16α-OH-progesterone fraction from pig adrenal vein blood, acetylated. II: authentic steroid acetates.

into these two compounds on the column of the gas chromatograph. The eluate of the 16α-OH-progesterone region of the paper chromatogram produced on the gas-chromatographic records two peaks with the same relative retention times as the authentic steroid (Fig. 1, I). Cholestane was injected simultaneously to enable precise assessment of the relative retention times.

The second part of the combined sample was evaporated to dryness, acetylated (Holzbauer & Vogt, 1961) and chromatographed in the B_3 system for 3½ hr. Authentic 16α-OH-progesterone (20 μg) and compound *S* (20 μg) were treated in the same way. The lane to which the acetylated blood extract was applied showed two U.V. absorbing spots in positions corresponding to those of compound *S* acetate and 16α-OH-progesterone acetate on the control lane (Fig. 2). Both the authentic 16α-OH-progesterone acetate and the corresponding region of the chromatogram of the blood extract were eluted and subjected to gas-liquid chromatography. Each eluate produced two peaks with relative retention times of 0·72 and 1·75 and a small peak at 1·04 corresponding to the 'paper blank' (Fig. 3). Like the free steroid, the acetate produced two peaks on the gas chromatograph tracing. In this case the compound which was retained longer was the

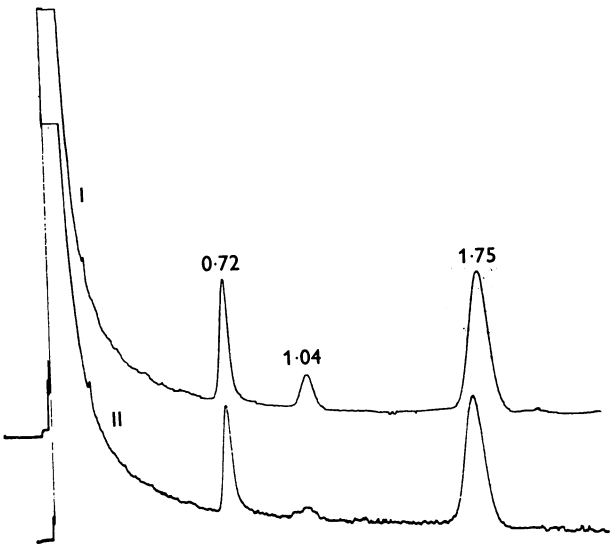

Fig. 3. Gas chromatograph tracings. I: eluate of U.V. absorbing spot with R_F equal to that of 16α-OH-progesterone acetate from lane I, Fig. 2. II: eluate of authentic 16α-OH-progesterone acetate, lane II, Fig. 2. Relative retention time 1·04: 'paper blank'. Both eluates dried, dissolved in 40 μl. ethanol, 1 μl. portions applied to the gas-liquid chromatograph (range 1, attenuation 8). The figures above each peak give the retention times relative to that of cholestane.

dominant one, but again the peak size ratio varied from run to run, whereas the relative retention times remained precisely reproduceable. The first peak was not due to 16α-OH-progesterone which had escaped acetylation as this would not have occurred in the same region of the paper chromatogram as the acetate. It is possible, however, that the breakdown product formed on the column in the gas chromatograph with a relative retention time of 0·72 was similar to that compound which produced a peak with a relative retention time of 0·75 when the free steroid was applied.

Further evidence for the identity of the substance in question with 16α-OH-progesterone was obtained by its conversion into 16α,20β-dihydroxypregn-4-en-3one diacetate. For this purpose the last third of the combined sample was incubated with the enzyme 20β-hydroxysteroid dehydrogenase (Boehringer, Mannheim) under conditions in which it reduces a 20-keto group to a 20β-hydroxyl group (Henning & Zander, 1962; Heap, 1964). Authentic 16α-OH-progesterone (20 μg) was treated in the same way. Subsequently the purified extracts of the incubation media were acetylated and chromatographed for 6 hr in the E_1 system of

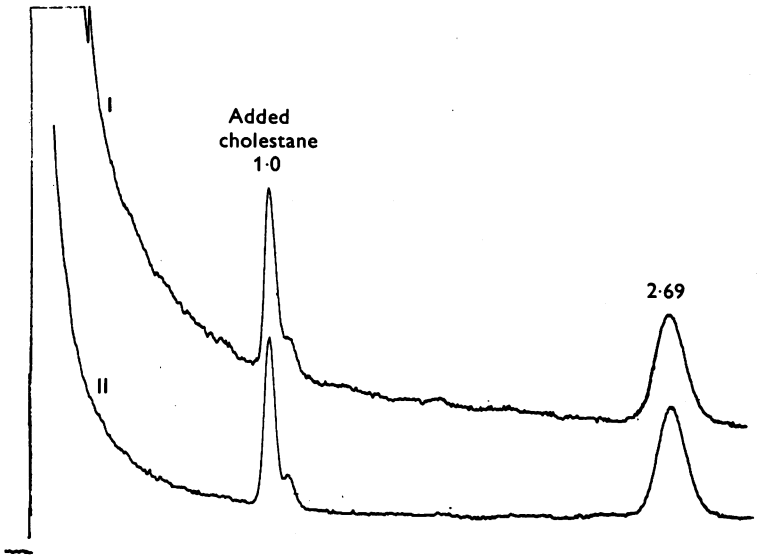

Fig. 4. Gas chromatograph tracings. I: partially purified 16α-OH-progesterone fraction from pig adrenal vein blood after incubation with 20β-hydroxysteroid dehydrogenase, acetylation and chromatography in E_1, 6 hr. Eluate of U.V. absorbing spot with R_F equal to that of authentic 16α,20β-dihydroxypregn-4-en-3one diacetate. II: authentic 16α-OH-progesterone after incubation with 20β-hydroxysteroid dehydrogenase, acetylation and chromatography in E_1, 6 hr. (Shoulder on cholestane peak due to 'paper blank'.) Both eluates dried, dissolved in 40 μl. ethanol, 1 μl. portions applied to the gas-liquid chromatograph (range 1, attenutation 8). The figures above each peak give the retention times relative to that of cholestane.

Eberlein & Bongiovanni (1955). Both the incubated authentic steroid and the blood extract showed a U.V. absorbing spot at R_F 0·5. These regions were eluted and the eluates subjected to gas-liquid chromatography (Fig. 4). Each eluate produced only one peak with a relative retention time of 2·69 in addition to the small 'paper blank' peak (relative retention time 1·04).

The combined extracts of eight pig adrenals were tested in an equal fashion for the presence of 16α-OH-progesterone and the results were the same as those obtained with the blood extracts.

DISCUSSION

The results of the present experiments are evidence for the presence of 16α-OH-progesterone in adrenal venous blood which was collected from 8 to 12 week old pigs under conditions of operative stress. It is unlikely that this steroid could have arisen from any source other than the adrenal cortex since no venous blood from organs which are capable of producing C_{21} steroids could return to the circulation. Extracts from the adrenal glands of the same animals also contained 16α-OH-progesterone.

The amounts available were not sufficient for identification by infra-red spectroscopy or by crystallization and estimation of the melting point. However, formation of derivatives and their analysis by gas-liquid chromatography after repeated paper chromatography in different solvent systems leaves little doubt of the chemical identity of the compound with 16α-OH-progesterone. Its rate of secretion was of the order of 10–50 μg/g adrenal/hr and the amount extracted from 10 g adrenal tissue was about 20 μg. Accurate quantitative estimation of 16α-OH-progesterone by gas-liquid chromatography, complicated by its splitting into two compounds, might be achieved by forming the 20β-hydroxy derivative, which, in its free form, has a relative retention time of 1·51 and appears to be stable on gas-liquid chromatography.

Wettstein, Neher & Urech (1959) isolated a small amount of 16α-OH-progesterone from pig adrenals. The ability of the human adrenal gland to effect 16α-hydroxylation of progesterone *in vivo* was demonstrated by Bird Wiqvist, Diczfalusy & Solomon (1966) who infused [^{14}C]progesterone into intact human foetuses and found 16α-OH-progesterone in the blood of the umbilical artery. This steroid was no longer formed when the foetuses were adrenalectomized (Wilson, Bird, Wiqvist, Solomon & Diczfalusy, 1966).

On the other hand, no 16α-OH-progesterone was found by us (to be published) in the adrenal venous blood collected under similar conditions from three 10-week-old puppies of both sexes; neither was it detected in the adrenal tissue of these animals.

The biological significance of the secretion of 16α-OH-progesterone by the adrenal of young pigs under stress conditions remains at present obscure.

Our thanks are due to Professor W. Klyne (M.R.C. Steroid Reference Collection) for a sample of crystalline 16α-OH-progesterone, to Dr R. B. Heap for his help with the enzyme incubation and to Mr R. Hughes for his technical assistance.

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