Since adrenal output in presence of ACh is approximately 1/5 that achieved with ACTH and since AChase does not inhibit ACTH action, it would appear that ACTH action is not mediated *via* an ACh controlled mechanism.

Summary. The *in vitro* effect of acetylcholine on adrenocortical secretion of rat adrenals was investigated by direct methods of analysis. ACh increases rat adrenal output of BT reducing substances without corresponding increase in substances measurable by UV regardless of strain of rat or type of ACh derivative used. A relationship between dose and response was observed with ACh doses between 0.01 to 1000 μg/100 mg adrenal. Output achieved with a maximally effective dose of 1000 μg/100 mg adrenal was approximately 1/5 that observed following maximal ACTH stimulation. Specificity of the ACh effect was demonstrated by inhibition with acetylcholinesterase.

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Enzymic Hydrolysis of Stilbestrol Diphosphate *in vitro* and *in vivo*. (26326)

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Stilbestrol diphosphate is an inactive "transport" form of stilbestrol currently employed in treatment of prostatic cancer. By virtue of the high level of phosphatase activity in malignant prostatic tissue(1) active stilbestrol is presumed to be preferentially released within the tumor and its metastases(2, 3). The absence of gynecomastia and other feminizing effects in patients treated with stilbestrol diphosphate is adduced as further evidence for target specificity of the substance. Recent studies with C^{14}-labelled stilbestrol diphosphate in prostatic carcinoma patients have shown the occurrence of considerable localization of free stilbestrol in the prostate; no localization occurred when stilbestrol was administered(4). However, in view of the abundant distribution of acid and alkaline phosphatases in animal tissues it might be expected that the hydrolysis of stilbestrol diphosphate would not be restricted to prostatic tissues, malignant or otherwise. In fact, it has been reported(5), without supporting data, that the compound is dephosphorylated by various rat tissues and fresh human prostatic tissue. In the present study, the phosphatase activity of several rat tissues towards stilbestrol diphosphate has been examined. Also, an attempt has been made to ascertain the extent of this activity *in vivo* by measurement of stilbestrol plasma levels following administration of the disphosphate ester.

Materials and methods. Tissue preparations. Homogenates of rat spleen, prostate, kidney, liver and of brain were prepared by grinding the tissues in a Potter-Elvehjem type homogenizer with 0.154 M KCl in the proportion of 1:20. To 1.0 ml of homogenate was added 0.5 ml of 0.2 M buffer solution and the mixture placed in a water bath at 37° for 5 minutes; 0.5 ml of 4×10^{-3} M stilbestrol diphosphate (disodium salt), dissolved in the same buffer, was then added to start the reaction. Tris (hydroxymethyl) aminomethane buffer was used to provide a pH of 7.4; acetate for pH 5.0, and borate for pH 9.0. A tissue blank was run with each experimental vessel. After the desired incubation time, which varied from 5 minutes to 2 hours, 5 ml of 6% trichloracetic acid was

added to terminate the reaction, and the sample centrifuged. The orthophosphate content of the clear supernatant was determined by the method of Fiske and Subbarow(6). Phosphate content of the appropriate tissue blank in each case was subtracted from that of the experimental sample. The stilbestrol diphosphate stock solution was assayed and found to contain no determinable free phosphate or free stilbestrol.

Determination of stilbestrol in plasma. Dryer(7) has described a method for assay of stilbestrol in tissue extracts. Modifications were required for application of the method to blood plasma. Dichloromethane replaced the acetone-ether of Dryer's method for extraction of stilbestrol from blood plasma. The advantages of dichloromethane are that it yields recoveries of 98 ± 2.1%, provides solutions free of turbidity, and gives zero blank values.

Procedure. Shake the plasma sample (usually 2 ml) with 10 ml of dichloromethane in a separatory funnel for 3 minutes. Transfer the organic phase to a second funnel and extract with 2 ml of 2.5N sodium hydroxide to remove the free stilbestrol. Separate the 2 phases, and acidify the aqueous phase with 2 ml of 5N sulfuric acid and extract with 10 ml of dichloromethane as before. Transfer the latter phase to a 20 × 170 mm Pyrex test tube and evaporate to dryness over a steam bath. Dissolve the dried residue in 1 ml of glacial acetic acid, add 0.1 ml of bromine reagent (1% v/v in glacial acetic acid) and place the tube in boiling water for exactly 2 minutes. Cool the tube, add 5 ml of 40% ethanol, mix, and read the optical density after 1 min at 500 mμ (Bausch & Lomb Spectronic 20) against a blank prepared by carrying a stilbestrol-free plasma sample through the whole procedure. The optical density measured at 500 mμ is a linear function of the plasma stilbestrol content within the range 0-25 μg. Reproducibility was found to be 1.2% at the 25 μg level and 6.5% at the 2 μg level.

Materials. Stilbestrol diphosphate (Honvol, Horner), a sterile aqueous solution of the disodium salt, was used for intravenous administration to rabbits. N-Acetyl-p-aminophenol (Atasol, Horner) 250 mg/kg body weight, was given by stomach tube as a suspension in tap water.

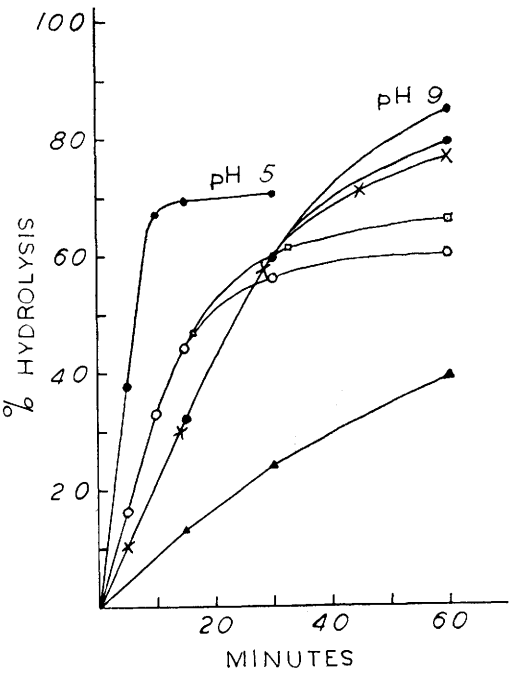

FIG. 1. Hydrolysis of stilbestrol diphosphate by rat tissue homogenates; pH 7.4, unless otherwise indicated. ● prostate, ○ liver, □ kidney, × spleen, ▲ brain.

Results and discussion. The hydrolysis of stilbestrol diphosphate by homogenates of several different rat tissues was measured at pH 7.4; hydrolytic activity of prostate tissue was studied further at pH 5 and 9. Results are presented in Fig. 1. It can be seen that the ester is readily dephosphorylated by prostate, spleen, kidney, liver and brain tissues. At pH 7.4 liver and kidney show the same rate of phosphatase activity, which is slightly greater than that of prostate and spleen. At might be expected, the rate of hydrolysis by prostatic phosphatase was greatly increased by lowering the pH to 5.0, while increasing the pH to 9.0 failed to influence the rate. The results also indicate that hydrolysis proceeds beyond the monophosphate stage but not to complete dephosphorylation; this can be clearly seen if the incubation is continued to 2 hours.

Erythrocytes of several animal species, in-

TABLE I. Plasma Levels of Stilbestrol in Rabbits Following a Single Intravenous Injection of Stilbestrol Diphosphate (92 mg/kg Body wt).

Min. after dosage	Plasma stilbestrol, μg/ml*
15	16.3
30	9.1
45	3.9
60	2.5
90	2.1
120	1.2

* Avg values from 3 rabbits, except for 60 and 120 min. intervals which are avg values from 6 rabbits.

cluding the human(8) show appreciable acid phosphatase activity. It was of interest, therefore, to test the behavior of red cell phosphatase towards stilbestrol diphosphate. The latter at 10^{-3} M concentration was incubated with whole rabbit blood at 37°C. After 10, 20 and 30 minutes of incubation it was found that 12.6, 17.3 and 22.4%, respectively, of the ester was hydrolyzed. When the same amount of stilbestrol diphosphate was incubated with plasma for 30 minutes, only 5% was hydrolyzed. Thus the red cells, and probably to some extent the leukocytes, account for most of the phosphatase activity of whole rabbit blood toward stilbestrol diphosphate.

As an indication of hydrolysis of the ester *in vivo,* stilbestrol diphosphate (92 mg/kg body wt) was injected intravenously into rabbits over a period of 2-3 minutes, and blood plasma was assayed chemically for free stilbestrol. It was found that free stilbestrol appeared in the blood stream as early as 5 minutes after termination of injection, and within 15 minutes had reached a concentration of 16.3 μg/ml of plasma (Table I). This was followed by a rapid decline in concentration, only 1.2 μg/ml remaining after 2 hours. Plasma levels of free stilbestrol were greatly increased by oral administration of a competitive glucuronylation inhibitor, N-acetyl-p-aminophenol (250 mg/kg) one hour prior to injection of stilbestrol diphosphate (unpublished results). Since glucuronylation occurs predominantly in the liver, the marked effect of the glucuronylation inhibitor probably indicates that a considerable amount of the stilbestrol diphosphate hydrolyzed in the liver was returned to the plasma as stilbestrol glucuronide. In this connection, stilbestrol monoglucuronide has been isolated from rabbit urine following subcutaneous injection of stilbestrol diphosphate(9). Studies in human subjects have shown the localization of stilbestrol in the prostate, liver, kidney, bone, and other tisues when stilbestrol diphosphate was administered(4,10,11). Hydrolysis of stilbestrol diphosphate by various tissues of the rabbit probably accounts for the presence of stilbestrol in the blood stream. Undoubtedly the acid phosphatases of the erythrocytes(8) also make a contribution.

Comment. The adult human prostate has up to 1000 times the acid phosphatase activity of that of other human tissues(12,13,14). However, total phosphatase activity of other body tissues combined, including blood, far outweigh that of the prostate and its malignancies. Although the localization of stilbestrol in prostatic tissue following intravenous administration of stilbestrol diphosphate has been demonstrated to occur(4,11), it is obvious that the ester would be hydrolyzed by other tissues as well. The high plasma levels of free stilbestrol observed in rabbits following injection of stilbestrol diphosphate tend to bear this out. It is probable therefore that stilbestrol diphosphate therapy of prostatic cancer allows for indirect effects of stilbestrol, for example, through the pituitary by inhibition of gonadotrophin secretion(15) as well as a direct cytotoxic action on prostatic tissue.

A notable feature of stilbestrol diphosphate therapy is the absence of gynecomastia(16), which has not been adequately explained. Considering the size of the individual doses employed in therapy (usually 500-1000 mg)(11,17), it is conceivable that the absence of gynecomastia may be due to presence of high concentrations of stilbestrol, which would tend to suppress the estrogenic stimulation of mammary gland growth. This biphasic action of estrogens is a well known phenomenon. Gardner(18) demonstrated that small doses of estrogen stimulated, while large doses inhibited mammary gland growth in mice and monkeys. Huggins *et al.*(19)

have shown that small amounts of estrogens stimulate growth of mammary tumors in ovariectomized rats, while large quantities block their growth.

Summary. Stilbestrol diphosphate is rapidly hydrolyzed by the phosphatases present in homogenates of rat prostate, liver, kidney and spleen, and to a lesser extent by brain tissue. A convenient chemical procedure is described for assay of free stilbestrol in plasma. Following administration of stilbestrol diphosphate to rabbits, appreciable amounts of free stilbestrol appear in the blood.

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Control of Plasma Non-Esterified Fatty Acids in Pregnancy and the Puerperium.* (26327)

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As term approaches the concentration of non-esterified fatty acids (NEFA) in peripheral blood plasma is significantly increased in normal human pregnancy. Prompt return toward normal non-pregnant values occurs within 48-72 hours after delivery. The NEFA fraction thus participates in the hyperlipidemia of pregnancy, although the physiological basis for the gestational increase in blood lipids remains unknown. We have suggested, however, that the lipid change may reflect altered carbohydrate utilization as another aspect of the diabetogenic influence of pregnancy(1).

The studies of Dole(2-3) and of Gordon (4-5) have related the plasma concentration of NEFA to carbohydrate utilization. Increase in plasma non-esterified fatty acids is observed when carbohydrate is either unavailable or cannot be utilized because of metabolic error. In either case, as Gordon has suggested, "caloric homeostasis" tends to be preserved due to the extremely rapid rate of NEFA utilization even at low plasma levels (5). Although in general NEFA release from fat depots to plasma reflects the prevailing level of carbohydrate utilization, the following observations relative to experimental modification of plasma NEFA in pregnancy and the puerperium suggest that control of fatty acid concentration in blood may be quite complex.

This complexity was first suspected when it was observed that degree of change in plasma NEFA after insulin treatment did not

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