because of the large doses it was necessary to use; the infected mice receiving both compounds died more quickly than those receiving 3-hydroxy-4-aminobenzoic acid alone. One of a group of control uninfected mice died after receiving the same treatment.

The above results suggest an explanation of the *in vivo* therapeutic action of 4-nitrobenzoic acid upon pneumococci which has been reported (Mayer & Oechslin, 1939). It is known that 4-nitrobenzoic acid is reduced in the animal body (Flynn & Kohl, 1941). It is possible that 4-hydroxyl-aminobenzoic acid arises during this process and may be rearranged to the active 3-hydroxy-4-aminobenzoic acid.

SUMMARY

1. Thirty-three substances related to 4-aminobenzoic acid have been examined for inhibitory action on the growth of streptococci.

2. Three of them, 3-hydroxy- and 3-chloro-4-aminobenzoic acid and 3:4-diaminobenzoic acid, were found to be inhibitory. The action of the first substance was approximately one-third to one-ninth that of sulphanilamide *in vitro*. This substance exhibited a slight therapeutic effect in mice infected with streptococci or pneumococci.

3. Five of the compounds examined antagonized the bacteriostatic effect of sulphanilamide on streptococci *in vitro*.

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The Colorimetric Estimation of Diethylstilboestrol, Hexoestrol and Dienoestrol

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The extensive use of synthetic oestrogens in both human and veterinary medicine has made the chemical assay of these substances a matter of great importance. Methods have already been described for the estimation of diethylstilboestrol (4:4'-dihydroxy- $\alpha\beta$ -diethylstilbene) by absorption spectrophotometry (Elvidge, 1939), by volumetric analysis (Sondern & Burson, 1942), and by colorimetry (Dingemanse, 1940; Dechene, 1941; Tubis & Bloom, 1942; Huf & Widmann, 1942; Cocking, 1943; Dracass & Foster, 1943). Elvidge (1939) has also proposed absorption spectrophotometry as a suitable method for the estimation of hexoestrol (4:4'-dihydroxy- $\gamma\delta$ -diphenyl-*n*-hexane), but otherwise no chemical methods are available for the assay of this substance, or of dienoestrol (4:4'-dihydroxy- $\gamma\delta$ -diphenyl- $\Delta^{\delta\delta}$ -hexadiene).

The methods described here differ from one another only in their detail, and are based on the recommendations of Stoughton (1936), who advised the use of acetic acid as a solvent in the estimation of non-water-soluble phenolic compounds and de-

scribed the development of a yellow colour after treating such solutions with nitric acid and their subsequent neutralization.

METHODS

Standard solutions. Standard oestrogen solutions are prepared by dissolving the appropriate oestrogen in glacial acetic acid to give a final strength of 0.1% (w/v). It is necessary to store these solutions in the dark in order to prevent the formation of a yellow colour (diethylstilboestrol, hexoestrol) or to retard its development (dienoestrol). No loss of chemical activity could be detected in these solutions after 1 month's (diethylstilboestrol) or 6 months' (hexoestrol, dienoestrol) storing and the presence of a yellow colour in dienoestrol solutions after such a period was not correlated with any loss in activity as determined by chemical assay. Possible effects of storing on the biological activity of the oestrogens were not investigated.

Unknown solutions. For all estimations the oestrogen is required in solution in glacial acetic acid (minimum strength approx. 0.01% (w/v)). The procedure used for this purpose will depend upon the nature of the preparation to be investigated. Ether extraction of certain aqueous suspensions,

followed by evaporation of the ethereal solution to dryness and re-solution in acetic acid, has proved satisfactory in some cases (e.g. rumen liquor) while Dechene (1941), Sondern & Burson (1942) and Dracass & Foster (1943) have suggested extractions from oily solutions which could easily be adapted to give the final acetic acid solution needed for these estimations. For non-oily preparations having a high solids content, drying in a steam oven followed by direct extraction of the residue with hot acetic acid has been used. Loss of the oestrogen occurs when acetic acid solutions of dien-oestrol are heated, and it is necessary to extract this substance at room temperature.

Estimation of diethylstilboestrol. A volume of acetic acid solution containing 0.5–2 mg. diethylstilboestrol is pipetted into a 50 ml. standard flask, and diluted further with glacial acetic acid to give a final volume of 6 ml. Conc. H_2SO_4 (0.1 ml.) is added, followed by 0.04 ml. conc. HNO_3 . The solution is shaken and heated on the steam-bath for $1\frac{1}{2}$ min. with gentle shaking at 15 sec. intervals. It is cooled immediately by the addition of 10 ml. distilled water, and made alkaline with 15 ml. conc. NH_3 solution (sp.gr. 0.88). A fine orange colour develops at this stage in the presence of diethylstilboestrol, and the solution is left to cool to room temperature before making to volume with distilled water. The final colour is compared with that given by 1 ml. of standard diethylstilboestrol solution treated in the same way.

Estimation of hexoestrol. The same procedure is used as for the estimation of diethylstilboestrol up to the addition of conc. HNO_3 . The solutions are then made alkaline, with no prior heating, by the addition of 25 ml. 25% NaOH . When cool they are made to volume (50 ml.) and the colour compared with that given by 1 ml. of standard hexoestrol solution.

Estimation of dien-oestrol. The method for diethylstilboestrol is again followed. After the addition of conc. HNO_3 , however, the solutions are not heated, but left to stand for 1 hr. at room temperature. They are then diluted with 10 ml. distilled water, made alkaline by the addition of 15 ml. conc. NH_3 solution (sp.gr. 0.88), allowed to cool and diluted to 50 ml. The final colour is compared with that given by 1 ml. of standard dien-oestrol solution.

Colorimetry. Measurements were made with a Hellige drum-scale colorimeter, using a spectrum green colour filter (Ilford 604, $520\text{m}\mu$). A photo-electric spectrophotometer was used for the tests of colour permanence.

EXPERIMENTAL

Addition of H_2SO_4 . The addition of H_2SO_4 is essential for colour development only in the case of dien-oestrol solutions. Although it is unnecessary in the estimation of diethylstilboestrol its omission leads to the formation of a precipitate in solutions

having an oestrogen content of 2 mg. or more, whereas no precipitate is formed when H_2SO_4 is added, even in solutions with a content of 5 mg. With hexoestrol solutions also the use of H_2SO_4 permits a slightly higher oestrogen concentration to be estimated before the appearance of precipitates. For diethylstilboestrol solutions the time of heating for maximum colour development (*vide infra*) is also influenced by H_2SO_4 and is materially reduced in its presence. In the diethylstilboestrol and dien-oestrol methods an increase in the number of drops of H_2SO_4 added leads to a progressive decrease in the colour strength produced and it is essential that this addition should be well controlled. No corresponding effect on the final colour was found for hexoestrol solutions.

Addition of HNO_3 . No effect on the final colour in diethylstilboestrol or hexoestrol estimations was produced by varying the HNO_3 addition from 0.02 to 0.06 ml. With dien-oestrol solutions 0.02 ml. was insufficient to give the fullest colour development.

Time of heating. Fig. 1 illustrates the effect of varying the time of heating, after the addition of HNO_3 , on the final colour development in diethylstilboestrol solutions.

Fig. 1. Relationship between the time of heating and colour development in standard solutions of diethylstilboestrol. (Duboscq type colorimeter, green light filter, $520\text{m}\mu$.)

In the case of hexoestrol solutions an intensification of the final colour is also caused by heating, but utilization of this fact in the method is made inadvisable by the danger of further chemical change occurring in the solution, characterized by the evolution of nitrous fumes and the formation of cloudy precipitates in the final solution. Neutralization with conc. NH_3 solution was found to give precipitates in the final solutions both when they had been previously heated and also when this treatment had been withheld.

Heating after the addition of HNO_3 similarly causes a greater colour development in dienooestrol solutions, the optimum heating period being $1\frac{1}{2}$ min., but a like effect, though retarded, is also observed when the solutions are allowed to stand at room temperature, and since, if heating is used in dienooestrol estimations, a confusion of these two processes leads to variable values when series of estimations are run, and when HNO_3 is added to all the solutions at the same time, it was thought more convenient to eliminate heating altogether from the method, maximum colour development at room temperature being given by dilution and neutralization after 1 hr. standing.

Permanence of the final colours. An absolute measure of the rate of fading in solution was obtained for the oestrogens by using a photoelectric

loss (diethylstilboestrol, dienooestrol) is a function of time for the period studied, while steady values are maintained in hexooestrol solutions. The loss of optical density in the first 3 hr. is in all cases approximately 5%. The use of standard oestrogen solutions prepared at the same time as the unknown solutions, for the colorimetric comparison, automatically compensates for any loss due to fading, but the use of stable dyes for comparison is clearly inadmissible.

Application of Beer's law. In all cases the law is obeyed for the oestrogen concentrations (0.5–2 mg.) most useful for visual colorimetry. Results of typical experiments, in which different amounts of standard oestrogen solutions were used, are given in Table 1.

Outside this most sensitive range it has been verified that the linear relationship between concentration and colour intensity persists for diethylstilboestrol and dienooestrol solutions up to a concentration of 4.0 mg. oestrogen in the test solution; for hexooestrol the application is limited by the appearance of precipitates in solutions of higher oestrogen content.

Preparation of dinitrohexooestrol compound. The precipitate formed in solutions of hexooestrol containing more than 2 mg. oestrogen was isolated. It was also prepared by stopping neutralization, following HNO_3 treatment, at the point of maximum precipitation and, while the solution was still acid, washing the precipitate free of salts with water and recrystallizing from benzene. The yellow prisms dissolved in alkali to give an orange solution and had the composition of a dinitrohexooestrol compound (m.p. $229\text{--}230^\circ$; N, 7.70%, theoretical, 7.78%; NO_2 , 24.96%, theoretical, 25.56%).

Fig. 2. Relationship between time and the rate of fading of colours given by standard solutions of synthetic oestrogens. (Photoelectric spectrophotometer.)

● Diethylstilboestrol; ○ dienooestrol; ● hexooestrol.

spectrophotometer (Thompson, 1942) adjusted to give a broad green band of the spectrum, which was then passed through the filter normally used for colorimetry. Extinction curves for standard solutions are shown in Fig. 2. In all cases the colour fades most rapidly over the first 10 hr.; subsequent

APPLICATION OF THE METHOD

The estimation of synthetic oestrogens in the presence of some common tablet excipients was made, using test solutions in which known amounts of stearic acid, lactose or cholesterol in glacial acetic acid solution were added to 1 ml. of standard

Table 1. Application of Beer's law to colour development in oestrogen solutions of different concentrations

Final oestrogen concentration (mg./50 ml.)	Diethylstilboestrol		Hexooestrol		Dienooestrol	
	Colorimeter reading* (mm.)	Recovery (% theoretical)	Colorimeter reading* (mm.)	Recovery (% theoretical)	Colorimeter reading* (mm.)	Recovery (% theoretical)
0.50	49.3	101.4	51.3	97.5	49.0	102.1
0.75	33.4	99.7	35.3	94.3	32.9	101.2
1.00	24.8	100.8	25.3	98.8	25.0	100.0
1.25	20.0	100.0	20.9	95.7	19.8	101.0
1.50	16.6	100.6	17.5	95.4	16.8	99.4
1.75	14.3	100.0	14.9	96.0	14.5	98.6
2.00	12.4	100.8	12.9	96.9	12.8	97.7

* Standard solution for comparison (1 mg./50 ml.) set at 25.0 mm.

oestrogen solution. The insolubility of starch in glacial acetic acid permits the simple estimation of oestrogens when mixed with this substance, while experiments in which the oestrogens were estimated in the presence of methyl testosterone proved the value of the methods for assessing the relative absorption of these substances from mixed oestrogen-androgen tablets in implantation experiments (Table 2).

groups in the molecule, and the isolation of a dinitro hexoestrol derivative, prepared under the conditions used in the quantitative procedure, confirms the supposition for this oestrogen. It contradicts the view of Stoughton (1936), who considered it very unlikely that nitration would follow the use of such small amounts of acid, and postulated the formation of nitroso derivatives to account for the colours produced by phenol and related substances under

Table 2. *Recovery of oestrogens in the presence of added substances*

(1 mg. of the oestrogen and 1 mg. of added substance present in each determination.)

Added substance	Diethylstilboestrol		Hexoestrol		Dienoestrol	
	Colorimeter reading (mm.)	Recovery (% theoretical)	Colorimeter reading (mm.)	Recovery (% theoretical)	Colorimeter reading (mm.)	Recovery (% theoretical)
—	25.0	—	24.8	—	25.2	—
Stearic acid	25.7	97.3	26.1	95.0	26.2	96.2
Lactose	24.5	102.1	25.3	98.0	25.5	98.8
Cholesterol	25.0	100.0	24.7	100.4	24.1	104.6
Methyl testosterone	24.9*	100.4	25.0	99.2	24.1	104.6

* Modified method.

For this last estimation the heating process was eliminated from the normal method for diethylstilboestrol assay, since, with heating, methyl testosterone also gives a pale yellow colour.

DISCUSSION

The methods described here for the estimation of synthetic oestrogens are non-specific, and similar colours may be developed by a variety of substances—both phenolic and non-phenolic (Stoughton, 1936)—which might be present in biological materials. A successful application of the method for diethylstilboestrol to the estimation of this oestrogen after its admixture with the rumen contents of a bovine, suggests, however, that the method may prove to have great usefulness even where quite complex biological substances are involved.

The chromogen in all cases shows properties normally associated with the presence of nitro

similar conditions. Analogy and the observed properties of the chromogens in solution would suggest strongly that the nitration of hexoestrol is indeed typical of the reactions occurring when other phenolic substances, including diethylstilboestrol and dienioestrol, are similarly treated.

SUMMARY

1. Methods are described for the colorimetric estimation of three synthetic oestrogens: diethylstilboestrol, hexoestrol and dienioestrol.
2. The experimental conditions and the chemical nature of the reactions involved are discussed.

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