REFERENCES

Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
Haber, E. & Anfinsen, C. B. (1961). *J. biol. Chem.* **236**, 422.
Ikawa, M. & Snell, E. E. (1960). *J. biol. Chem.* **235**, 1376.
Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
Lederberg, J. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 574.
Mandelstam, J. (1958). *Biochem. J.* **69**, 103.
Mandelstam, J. (1961). *Nature, Lond.*, **189**, 855.
Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Perkins, H. R. & Rogers, H. J. (1959). *Biochem. J.* **72**, 647.
Primosigh, J., Pelzer, H., Maass, D. & Weidel, W. (1961). *Biochim. biophys. Acta*, **46**, 68.
Repaske, R. (1958). *Biochim. biophys. Acta*, **30**, 225.
Rogers, H. J. & Perkins, H. R. (1959). *Nature, Lond.*, **184**, 520.
Salton, M. R. J. (1953). *Biochim. biophys. Acta*, **10**, 512.
Salton, M. R. J. (1958). *J. gen. Microbiol.* **18**, 481.
Salton, M. R. J. & Pavlik, J. G. (1960). *Biochim. biophys. Acta*, **39**, 398.
Wright, H. D. (1933). *J. Path. Bact.* **37**, 257.
Zinder, N. D. & Arndt, W. F. (1958). *Proc. nat. Acad. Sci., Wash.*, **42**, 586.

Biochem. J. (1962) **84**, 299

Inhibition of Cell-Wall-Mucopeptide Formation in *Escherichia coli* by Benzylpenicillin and 6-[D(−)-α-Aminophenylacetamido]penicillanic Acid (Ampicillin)

BY H. J. ROGERS AND J. MANDELSTAM
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 14 *February* 1962)

Duguid (1946) suggested, on the basis of cytological observations, that penicillin might interfere with cell-wall synthesis. Lederberg (1956) noted that, when *Escherichia coli* was treated with penicillin, the bacteria were converted into osmotically fragile spheroplasts, and suggested that the drug acted by inhibiting the formation of cell wall. However, most of the evidence for the mode of action of penicillin depends on work done with staphylococci. Park (1952) showed that penicillin caused accumulation of uridine-linked peptides which are believed to be precursors of cell-wall mucopeptide (Park & Strominger, 1957). Inhibition of mucopeptide synthesis by penicillin in washed staphylococci has been shown by Park (1958), Mandelstam & Rogers (1959) and Rogers & Jeljaszewicz (1961).

However, work with Gram-negative bacteria has produced divergent views on penicillin action. Trucco & Pardee (1958) found no inhibition of [^{14}C]glucose incorporation into the wall of *E. coli*; Meadow (1960), also working with *E. coli*, reported that penicillin did not inhibit incorporation of αε-diamino[^{14}C]pimelic acid into the cell wall; Hugo & Russell (1960*a*, *b*, 1961) showed that loss of viability in Gram-negative organisms treated with penicillin differed from the incidence of spheroplast formation, and concluded that the lethal effect of the drug was not a consequence of fragility. The original suggestion by Lederberg (1956) was, however, supported by Nathenson & Strominger (1961), who showed that penicillin inhibited the incorporation of tritiated diaminopimelic acid by about 50–75%.

The present work, reported in a preliminary form by Rogers & Mandelstam (1961), was undertaken to obtain further evidence on the mode of action of penicillin in *E. coli*. Penicillin was found to inhibit the incorporation of carbon from [^{14}C]-glucose into bound diaminopimelic acid. So far as is known, bound diaminopimelic acid occurs only in the cell-wall mucopeptides, and these experiments therefore support the contention that penicillin has the same action in *E. coli* as in staphylococci.

METHODS

Organism. E. coli K12 was grown at 35°, with shaking, in a mineral-salts medium (Mandelstam, 1962*a*) with glucose (10 g./l.) as carbon source, and harvested when the culture had reached a density of about 0·5 mg. dry wt. of bacteria/ml.

Incorporation of carbon from [^{14}C]*glucose.* The bacteria were washed twice with mineral-salts medium (no glucose) and suspended at 1·0 mg. dry wt./ml. in 100 ml. of the same mineral-salts medium containing [^{14}C]glucose [10–20 μc; final concn., 0·2% (w/v)]. Sucrose (20%, w/v) was present to prevent lysis of any spheroplasts that might be formed. The flasks (500 ml.) were shaken at 35° for a suitable period, usually 60 min., and incorporation was stopped by adding trichloroacetic acid to give a final concentration of 5% (w/v). A zero-time sample was obtained by adding trichloroacetic acid immediately after the cells

had been suspended in the incubation medium. The precipitates were washed twice with 15 ml. of trichloroacetic acid (5%, w/v) containing [^{12}C]glucose (1 mg./ml.), extracted with trichloroacetic acid at 90° and then with lipid solvents, and dried (Mandelstam, 1958*a*).

Isolation of diaminopimelic acid. This was based on the easy separation of the amino acid from all the amino acids known to occur in trichloroacetic acid precipitates, except cystine, by paper chromatography with butanol–acetic acid–water (63:10:27, by vol.). To separate diaminopimelic acid from cystine, the precipitate was first treated as follows with performic acid to oxidize cystine to cysteic acid. Hydrogen peroxide (1 ml. of '100 vol.' soln.) was added to 9 ml. of formic acid (90%, v/v), left for 1 hr., cooled to 2°, added to the precipitate from 100 mg. dry wt. of bacteria, and left overnight in the cold. The solution was dried *in vacuo* over NaOH and H_2SO_4. The residue (about 70 mg.) was hydrolysed in a sealed tube with 6N-HCl (5 ml.) at 100° for 24 hr. The hydrolysate was dried *in vacuo* and repeatedly evaporated with small amounts of water to remove HCl. The residue was dissolved in 0·5 ml. of water and applied to two sheets of Whatman no. 3 as 30 cm. bands across the width of the paper. Glycine and diaminopimelic acid markers (50 μg. of each) were placed at both ends of the sheets. The chromatograms were developed for 3 days in butanol–acetic acid–water (see above), and the marker region of the papers was streaked with ninhydrin (0·1%). The area containing diaminopimelic acid and cysteic acid was cut out of each sheet and extracted with water. The combined solution (about 20 ml.) was acidified with HCl (final concn. 0·1N) and put on a column (2 cm.^{2} cross-section) containing 10 ml. of Zeo-Karb 225 resin (H^+ form). Under these conditions diaminopimelic acid is retained on the resin whereas cysteic acid runs through. The column was washed with 50 ml. of HCl (0·1N), and the diaminopimelic acid was eluted with 6N-HCl (40 ml.). The effluent was dried *in vacuo* and the residue, dissolved in water, was applied to Whatman no. 3 paper as a band 10–15 cm. long, and chromatographed as before. Diaminopimelic acid was eluted from the paper with water and the volume made up to 2·0 ml. Samples (0·4 ml.) were used for measurement of specific radioactivity (Mandelstam, 1958*a*).

Isolation of glycine–serine fraction from trichloroacetic acid precipitates. To check the effect of benzylpenicillin on amino acids not concerned in mucopeptide synthesis, glycine and serine were isolated from the hydrolysates. Glycine is not present in mucopeptide from Gram-negative organisms at all (Mandelstam, 1962*b*), and serine is present in such small amount that it is a negligible portion (about 3%) of the total serine of the trichloroacetic acid precipitate.

The same chromatograms were used as those from which the diaminopimelic acid–cysteic acid area had been removed. The region containing glycine and serine was extracted with water and the solution applied to columns of Zeo-Karb 225 (H^+ form) (dimensions as above), and eluted with aq. 2N-NH_3 (Mandelstam, 1958*b*). The effluent was evaporated to about 0·3 ml. at 100° and transferred as a 15 cm. band to Whatman no. 3 paper. The sheet was chromatographed for 2 days with butanol–acetic acid–water and the glycine–serine area was eluted with water. The volume was made up to 2·0 ml., and samples (0·2 ml.) were taken for determination of specific radioactivity (Mandelstam, 1958*a*).

In later experiments in which only diaminopimelic acid was isolated, a simpler procedure was adopted. The trichloroacetic acid precipitate was oxidized, hydrolysed and evaporated to dryness as above. The residue was dissolved in 0·1N-HCl (20 ml.), applied directly to a column of Zeo-Karb 225 (H^+ form), and eluted with 6N-HCl (as above). The effluent was evaporated to dryness *in vacuo*, and the residue applied to Whatman no. 3 paper as a 30 cm. band. The chromatograms were developed with butanol–acetic acid–water for 3 days to separate the diaminopimelic acid from lysine and arginine. The diaminopimelic acid was then extracted from the paper and the specific radioactivity determined as described.

RESULTS

Effect of penicillin and of chloramphenicol on the incorporation of carbon from [^{14}C]glucose into bound diaminopimelic acid and into bound glycine and serine. E. coli was incubated in mineral-salts medium containing sucrose (20%, w/v) and [^{14}C]-glucose, as described in the Methods section. Flasks contained the following further additions: (*a*) no antibiotics (control); (*b*) benzylpenicillin (100 μg./ml.); (*c*) chloramphenicol (50 μg./ml.); (*d*) benzylpenicillin (100 μg./ml.) and chloramphenicol (50 μg./ml.). For the zero-time sample a fifth flask, containing both antibiotics, was treated with trichloroacetic acid immediately. The other flasks were shaken for 1 hr. at 35°, diaminopimelic acid and the glycine–serine fraction were isolated from the precipitate and their specific radioactivities were measured.

Benzylpenicillin reduced incorporation into diaminopimelic acid from 335 to 59 counts/min./100 μg., i.e. by 82% (see Table 1). The mean inhibition in four experiments was 70% (range 56–82%). Chloramphenicol alone also caused a decrease of about 35% in incorporation. In the absence of chloramphenicol the bacterial mass in-

Table 1. *Effect of benzylpenicillin and of chloramphenicol on incorporation of carbon from [^{14}C]glucose into bound diaminopimelic acid of* Escherichia coli

Bacteria were incubated in sucrose–mineral-salts medium containing [^{14}C]glucose for 60 min. Diaminopimelic acid was isolated from the trichloroacetic acid precipitate and its specific radioacticity was measured. Full experimental details are given in the text.

	Diaminopimelic acid	
	Amount isolated (μg.)	Specific activity (counts/min./100 μg.)
Zero-time control	81	1
No antibiotics	94	335
Penicillin (100 μg./ml.)	94	59
Chloramphenicol (50 μg./ml.)	107	210
Penicillin (100 μg./ml.) + chloramphenicol (50 μg./ml.)	74	7

creased by about 30 %, so that the observed inhibition is what would be expected if chloramphenicol prevented the formation of new protein and the rate of mucopeptide synthesis were limited by the enzymes present before the chloramphenicol was added. The addition of penicillin as well as chloramphenicol reduced incorporation to 7 counts/min./100 μg. of diaminopimelic acid (Table 1).

The effect of both antibiotics on protein synthesis, as measured by incorporation into bound glycine and serine, is shown in Table 2. Benzylpenicillin had no effect, and chloramphenicol inhibited incorporation by over 95 %.

All the remaining experiments described in this paper were done in the presence of chloramphenicol (50 μg./ml.).

Time-course of incorporation of [^{14}C]glucose into bound diaminopimelic acid in the presence and absence of penicillin. The time-course of glucose incorporation into diaminopimelic acid was examined to ensure that it was not misleading to take a single measurement of inhibition at the end of 1 hr. Bacteria were incubated as described above with [^{14}C]glucose in the presence of benzylpenicillin (100 μg./ml.). Samples (100 ml.) were taken at 0, 20 and 60 min. and treated with trichloroacetic acid. A control culture without penicillin was treated similarly. The specific activity of diaminopimelic acid isolated from the precipitates was determined.

In the presence of benzylpenicillin, incorporation was roughly linear (see Table 3). In the control there seems to have been either a lag of 5–10 min. or an acceleration in the rate of mucopeptide synthesis. To establish this more definitely, a larger number of points would have had to be taken and, since it was not relevant to the main issue, the question has not been pursued, especially as a similar experiment in the absence of penicillin gave a linear rate of incorporation from the start.

Table 2. *Effect of benzylpenicillin and of chloramphenicol on incorporation of carbon from [^{14}C]glucose into bound glycine and serine of* Escherichia coli

Details were as described in Table 1. Glycine and serine were isolated from the trichloroacetic acid precipitate. Results are given as glycine equivalents.

	Glycine + serine	
	Amount isolated (μg.)	Specific activity (counts/min./100 μg.)
Zero-time control	178	1
No antibiotics	172	487
Penicillin (100 μg./ml.)	268	488
Chloramphenicol (100 μg./ml.)	191	17
Penicillin (100 μg./ml.) + chloramphenicol (50 μg./ml.)	143	15

Comparison of inhibitory effects of benzylpenicillin and 6-[D(−)-α-aminophenylacetamido]penicillanic acid (ampicillin) on incorporation of [^{14}C]glucose into bound diaminopimelic acid. A new penicillin, ampicillin, described as being effective against *E. coli* and other Gram-negative bacteria (Rolinson & Stevens, 1961), was kindly supplied by Dr W. Brumfitt. The activity of this compound in inhibiting the incorporation of glucose into diaminopimelic

Table 3. *Time-course of incorporation of carbon from [^{14}C]glucose into bound diaminopimelic acid*

Bacteria were incubated in sucrose–mineral-salts medium containing [^{14}C]glucose and chloramphenicol (50 μg./ml.). Samples were taken at 20 and 60 min. Diaminopimelic acid was isolated from the trichloroacetic acid precipitate and its specific activity was measured.

	Diaminopimelic acid	
	Amount isolated (μg.)	Specific activity (counts/min./100 μg.)
Zero-time control	320	2
20 min., no penicillin	342	35
60 min., no penicillin	300	150
20 min., with penicillin (100 μg./ml.)	258	37
60 min., with penicillin (100 μg./ml.)	214	67

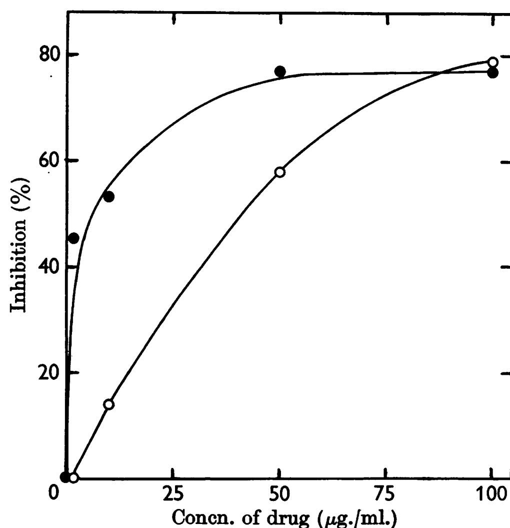

Fig. 1. Effect of benzylpenicillin and of ampicillin on incorporation of carbon from [^{14}C]glucose into bound diaminopimelic acid. Bacteria were incubated for 60 min. in sucrose–mineral-salts medium containing [^{14}C]glucose, chloramphenicol and various concentrations of either benzylpenicillin (○) or ampicillin (●). Diaminopimelic acid was isolated and its specific activity was measured. In the absence of either drug the specific activity was 132 counts/min./100 μg. of diaminopimelic acid.

acid was compared with that of benzylpenicillin as follows. *E. coli* was incubated for 1 hr. with [^{14}C]-glucose in sucrose–mineral-salts medium at drug concentrations of 0, 2, 10, 50 and 100 μg./ml. Chloramphenicol was present at 50 μg./ml. The specific radioactivity of isolated diaminopimelic acid was measured.

The inhibition curve obtained with increasing concentrations of either penicillin has the general form of an adsorption isotherm, but the ampicillin was far more potent (Fig. 1). Inhibition of about 50% was obtained at 4 μg. of ampicillin/ml., whereas 40–50 μg. of benzylpenicillin/ml. was required to give a comparable effect.

DISCUSSION

From Tables 1 and 2 it is apparent that benzylpenicillin inhibits incorporation into the diaminopimelic acid of mucopeptide, whereas incorporation into amino acid components of protein is unaffected. The action of the penicillins in *E. coli* is thus very like that reported in *S. aureus* (Mandelstam & Rogers, 1959); even the degree of inhibition (about 70%) is very similar. The main difference is that, in sensitive staphylococci, 50% inhibition of incorporation into mucopeptide is found at 0·1–0·2 μg. of benzylpenicillin/ml. (Rogers & Jeljaszewicz, 1961), whereas in *E. coli* the concentration has to be increased about 500 times to achieve a similar effect.

Our results thus agree with those of Nathenson & Strominger (1961), and it will be useful to consider possible reasons for the apparent disagreement between these findings and those of some other workers (see above). The findings of Trucco & Pardee (1958) can be understood in the light of what is now known about the cell walls of Gram-negative bacteria. These authors examined the effect of penicillin on incorporation of [^{14}C]glucose into the whole wall of *E. coli*. However, the substance whose synthesis is affected by benzylpenicillin is the mucopeptide, and in *E. coli* this constitutes less than 5% of the wall (Mandelstam, 1962*b*), the rest being lipid, polysaccharide and protein (Salton, 1953). Since penicillin would not be expected to affect the synthesis of these substances, its effect on glucose incorporation into the whole wall would be extremely difficult to detect.

Hugo & Russell (1961) noted that, in *Aerobacter cloacae* treated with penicillin, the loss of viability exceeded the incidence of spheroplast formation and concluded that the lethal action was not necessarily a consequence of osmotic fragility. However, their results show that the discrepancy between viability and osmotic fragility is most marked at high concentrations of penicillin. Thus, at 20 000 units of benzylpenicillin/ml. [i.e. more than 1% (w/v)], no spheroplasts were produced, although most of the cells were killed (i.e. failed to give rise to colonies). At 5000 units/ml. a few spheroplasts were formed; at 1000 units/ml. about half of the cells formed spheroplasts, and at this concentration there was fairly good agreement between spheroplast formation and viable count. The experiments suggest that, at concentrations of about 1%, penicillin may well have some other lethal effect on the cells, but that as the concentration approaches more customary antibiotic levels the discrepancy between osmotic fragility and killing of cells begins to disappear.

Finally there remain the experiments of Meadow (1960). These were done in the absence of sucrose. However (P. Meadow, personal communication), when the experiment was repeated with sucrose in the medium, the incorporation of labelled diaminopimelic acid into mucopeptide was inhibited as reported by Nathenson & Strominger (1961).

The evidence at present thus shows that in *E. coli*, as in staphylococci, penicillin inhibits the synthesis of mucopeptide. Further, the relative activities of ampicillin and benzylpenicillin in this respect are correlated with their antibiotic properties. A correlation of this type has been reported for a series of penicillins tested in sensitive staphylococci (Rogers & Jeljaszewicz, 1961). The present results provide further support for the contention that penicillin exerts its lethal action by inhibiting the synthesis of mucopeptide.

SUMMARY

1. Benzylpenicillin inhibits cell-wall-mucopeptide synthesis as measured by the incorporation of [^{14}C]glucose into bound $\alpha\epsilon$-diaminopimelic acid.

2. A new penicillin, 6-[D(−)-α-aminophenylacetamido]penicillanic acid (ampicillin) also has the same effect, 50% inhibition being found at 4 μg./ml. About 40 μg. of benzylpenicillin/ml. is needed to produce a comparable inhibition.

3. Divergent views on the action of penicillin in Gram-negative bacteria are discussed; it is concluded that penicillin has the same action in Gram-negative and Gram-positive bacteria.

We are much indebted to Miss R. Coyle and to Mr I. Mathison for technical assistance.

REFERENCES

Duguid, J. P. (1946). *Edinb. med. J.* **53**, 401.
Hugo, W. B. & Russell, A. D. (1960*a*). *Nature, Lond.*, **188**, 875.
Hugo, W. B. & Russell, A. D. (1960*b*). *J. Bact.* **80**, 436.
Hugo, W. B. & Russell, A. D. (1961). *J. Bact.* **82**, 411.
Lederberg, J. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 574.
Mandelstam, J. (1958*a*). *Biochem. J.* **69**, 110.

Mandelstam, J. (1958*b*). *Biochem. J.* **69**, 103.
Mandelstam, J. (1962*a*). *Biochem. J.* **82**, 489.
Mandelstam, J. (1962*b*). *Biochem. J.* **84**, 294.
Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Meadow, P. (1960). *Biochem. J.* **76**, 8P.
Nathenson, S. G. & Strominger, J. L. (1961). *J. Pharmacol.* **131**, 1.
Park, J. T. (1952). *J. biol. Chem.* **194**, 897.
Park, J. T. (1958). *Biochem. J.* **70**, 2P.
Park, J. T. & Strominger, J. L. (1957). *Science*, **125**, 99.
Rogers, H. J. & Jeljaszewicz, J. (1961). *Biochem. J.* **81**, 576.
Rogers, H. J. & Mandelstam, J. (1961). *Biochem. J.* **81**, 43P.
Rolinson, G. N. & Stevens, S. (1961). *Brit. med. J.* ii, 191.
Salton, M. R. J. (1953). *Biochim. biophys. Acta*, **10**, 512.
Trucco, R. E. & Pardee, A. B. (1958). *J. biol. Chem.* **230**, 435.

Biochem. J. (1962) **84**, 303

The Structure of Urinary Oestriol Monoglucuronide

BY J. G. D. CARPENTER* AND A. E. KELLIE
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

(*Received* 25 *January* 1962)

A conjugate of oestriol [oestra-1,3,5(10)-triene-3,16α,17β-triol] was first isolated from human-pregnancy urine by Cohen & Marrian (1936). The compound is probably a monoglucuronide in which the phenolic hydroxyl group at C-3 in the steroid is free (Cohen, Marrian & Odell, 1936; Grant & Marrian, 1948, 1950). It has not been established, however, whether glucuronic acid is linked to oestriol at the C-16 or C-17 hydroxyl group.

The present work concerns the synthesis of derivatives of the two possible ring D monoglucuronides of oestriol, and the comparison of these compounds with corresponding derivatives of oestriol glucuronide isolated from late-pregnancy urine. A preliminary account has been presented (Carpenter & Kellie, 1961).

EXPERIMENTAL

Materials

Solvents, except ethanol, were distilled in an all-glass apparatus before use. Celite 535 (Johns Manville and Co. Ltd.) was covered with hot conc. HCl for 24 hr., filtered, washed thoroughly with water until free from chloride, and dried at 120°. The neutral alumina (Merck) was sieved to exclude particles < 200 mesh. β-Glucuronidase was prepared as 'powder B', of activity 10^6 units/g., from the visceral hump of the limpet (*Patella vulgata*) according to Dodgson & Spencer (1953). Diazomethane was obtained from *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide (Diazald; Aldrich Chemical Co. Inc.) by the method of de Boer & Backer (1954). Methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate (bromo ester) was prepared according to Pelzer (1959), and stored under vacuum over P_2O_5 in the dark at 5°.

Methods

Paper chromatograms were run on unwashed Whatman no. 2 paper by the descending method (Bush, 1952). Oestriol and its derivatives were detected by a modification of the Kober reaction (Ittrich, 1958). Melting points were determined on the Kofler hot-stage and are corrected. Infrared-absorption spectra were measured on a Perkin–Elmer Model 21 recording spectrophotometer with sodium chloride optics. Optical rotations were determined in ethanol. Elementary analyses were carried out by Weiler and Strauss, Oxford.

Synthesis of the two isomeric derivatives of oestriol glucuronide

Methyl [16α-acetoxy-3-methoxyoestra-1,3,5(10)-trien-17β-yl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]uronate (VII) and methyl [17β-acetoxy-3-methoxyoestra-1,3,5(10)-trien-16α-yl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]uronate (X) were synthesized from oestrone methyl ether [3-methoxyoestra-1,3,5(10)-trien-17-one] (I) by the route outlined in Scheme 1. The enol acetate (II), the epoxide (III) and the α-ketol (IV) were synthesized by methods similar to those described in the preparation of the analogous C-3 acetates by Leeds, Fukushima & Gallagher (1954). The enol acetate (II) has been described by Johnson & Johns (1957); the preparation of the α-ketol (IV) has been reported by Mueller & Johns (1961).

3-*Methoxyoestra*-1,3,5(10)-*trien*-17-*one* (I). Dimethyl sulphate (15 ml.) was added during 15 min. to a stirred boiling solution of oestrone (2·54 g.) in methanol (45 ml.) and aq. 10% (w/v) NaOH (90 ml.). The mixture was refluxed for 1·5 hr., and then treated with further quantities of 10% (w/v) NaOH (30 ml.) and dimethyl sulphate (15 ml.) during 15 min. The suspension was refluxed for 1 hr., cooled, diluted to 300 ml. with water and filtered. The yield of oestrone methyl ether (m.p. 169·5–170°) was 2·6 g. (98%).

* Present address: Department of Obstetrics and Gynaecology, Columbia University, College of Physicians and Surgeons, New York, 32 N.Y., U.S.A.