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Deacetylcephalosporin C

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Structure I ($R = CH_3 \cdot CO \cdot O$) has been proposed for cephalosporin C (Abraham & Newton, 1961; Hodgkin & Maslen, 1961). This paper describes the formation and some of the properties of deacetylcephalosporin C (I; $R = \cdot OH$). Deacetylcephalosporin C appeared to be one of the products formed when cephalosporin C was treated with dilute alkali at room temperature. However, under these conditions hydrolysis of the acetoxyl group was accompanied, to some extent, by opening of the β -lactam ring and by other changes in the molecule. The conversion of cephalosporin C into deacetylcephalosporin C in neutral aqueous solution was accomplished, in good yield, by use of an acetyl esterase. Enzymes which hydrolyse esters of acetic acid, but which differ from acetylcholinesterases in that they are not inhibited by low concentrations of eserine, have been found to occur in plants, fungi and animal tissues (Hofstee, 1960). The acetyl esterase used here was that from citrus fruits described by Jansen, Jang & Macdonnell (1947). They reported that acetyl esters were hydrolysed by this enzyme much more readily than esters of other acids, but that *N*-acetyl groups were not hydrolysed.

METHODS AND EXPERIMENTAL

Elementary analyses were by Weiler and Strauss. Infrared spectra were measured with a Perkin-Elmer double-beam photometer model 21.

Electrophoresis and chromatography on paper. Electrophoresis on Whatman no. 1 paper (14 v/cm. for 2.5 hr.) was carried out as described by Newton & Abraham (1954) in collidine-acetate buffer (0.05M to acetate), pH 7.0, in pyridine-acetate buffer (0.05M to acetate), pH 4.5, and in 10% (v/v) acetic acid, pH 2.2. The apparatus used was similar to that of Flynn & de Mayo (1951). Paper chromatograms were run on Whatman no. 1 paper in butan-1-ol-acetic acid-water (4:1:4, by vol.) (Woiwod, 1949), and in propan-1-ol-water (7:3, v/v). Samples of 50–200 μ g. were applied to the paper. After chromatography, or after electrophoresis in 10% (v/v) acetic acid, the paper was placed between the viewer and a source of ultraviolet light (230–400 m μ ; Corning 9863 filter). Compounds with the cephalosporin C chromophore appeared as dark, light-absorbing, spots.

Bioautographs. After paper electrophoresis or chromatography the paper was dried in air and applied to the surface of a plate of nutrient agar seeded with *Staphylococcus aureus* (Oxford strain, NCTC 6571) or with *Salmonella typhi*, strain 'Mrs S' (Felix & Pitt, 1935). After 20 min. the paper was peeled off and the plate incubated at 37° overnight. Clear zones with no bacterial growth marked the positions of substances active against the test organisms.

Antibacterial activities. These were measured by the hole-plate method, cephalosporin C being used as a standard (Abraham, Newton & Hale, 1954). *Staph. aureus* (NCTC 6571) and *Salm. typhi*, strain 'Mrs S' (Felix & Pitt, 1935) were the test organisms. One unit of activity is contained in 0.1 mg. of cephalosporin C sodium salt dihydrate.

Electrometric titrations. These were carried out at 20° in the manner described by Newton & Abraham (1953).

Citrus acetyl esterase. Partially purified preparations of acetyl esterase were prepared from orange peel by the method of Jansen *et al.* (1947), except that $Na_2S_2O_4$ (0.5 mg./ml.) was added to the press juice, before precipitation with $(NH_4)_2SO_4$, to minimize oxidation. The precipitate obtained with $(NH_4)_2SO_4$ (Jansen *et al.* 1947) was mixed with 0.1M-sodium oxalate to form a slurry and the latter dialysed against a large volume of 0.1M-sodium oxalate (adjusted to pH 5.5) at 2° for 60 hr. Most of the solid went into solution and the remaining insoluble material was removed by centrifuging.

About 200 ml. of stock solution of enzyme was thus prepared from the press juice obtained from the peel (1.5 kg.) of 30 Israeli Jaffa oranges. This is referred to as stock solution *A*. About 100 ml. of enzyme solution was also prepared in a similar manner from the press juice obtained from the peel (1.0 kg.) of 40 South African Outspan oranges. This is referred to as stock solution *B*. The stock solutions were stored at 2° and retained their activity for many weeks.

Manometric determination of enzymic hydrolysis. The rates of hydrolysis of cephalosporin C and of triacetin in the presence of citrus acetyl esterase were determined manometrically at pH 7.0 in a Warburg respirometer under conditions similar to those used by Pollock (1952) for determining the activity of penicillinase. The vessels were gassed with $\text{CO}_2 + \text{N}_2$ (5:95, v/v) and measurements were made at 30°.

Triacetin as substrate. The main compartment of each Warburg vessel contained 2 ml. of an aqueous solution of triacetin and 0.5 ml. of 0.043M- NaHCO_3 . The side bulb contained 0.1 ml. of 0.043M- NaHCO_3 and 0.4 ml. of stock solution *A* of acetyl esterase or of a solution made by diluting the latter. Reaction rates were determined with five different concentrations of substrate, ranging from 8 to 130 mM.

Cephalosporin C as substrate. The main compartment of each vessel contained 1.7 ml. of water, 0.3 ml. of stock esterase solution and 0.5 ml. of 0.043M- NaHCO_3 . The side bulb contained 0.1 ml. of 0.043M- NaHCO_3 and 0.4 ml. of a solution of cephalosporin C sodium salt. Reaction rates were determined with five different concentrations of substrate, ranging from 1.7 to 28 mM.

Preparative scale hydrolysis of cephalosporin C by acetyl esterase. The enzymic hydrolysis was followed by titration of the acetic acid liberated. In a typical experiment, a preparation of cephalosporin C sodium salt dihydrate (2.18 g., estimated from its antibacterial activity to be about 80% pure) was dissolved in water (5 ml.) and the solution placed in a Pyrex glass vessel fitted with a water jacket kept at 30°. A solution of citrus acetyl esterase (10 ml. of stock solution *A* whose pH had been adjusted to 6.6 with N-NaOH) was warmed to 30° and added. The mixture was stirred with a magnetic stirrer while 0.97N-NaOH was added from a burette at such a rate that the pH of the solution (glass electrode) remained in the region of 6.6. After 5 hr., when the rate of addition of NaOH had fallen almost to zero, the experiment was stopped. The acid liberated was then equivalent to 0.8 equiv./475 g. of the cephalosporin C preparation used (mol.wt. of cephalosporin C sodium salt dihydrate, 475). The pH of the solution was adjusted to 5.6 with acetic acid and the solution freeze-dried.

Isolation of deacetylcephalosporin C. The freeze-dried product from the enzymic reaction was dissolved in 5 ml. of pyridine-acetate buffer, pH 5.0 (0.3M to acetate), and applied to a column (30 cm. $\times$ 2.9 cm.) of Amberlite XE-58 (120–200 mesh; acetate form). Elution was effected with the same pyridine-acetate buffer, pH 5.0. Fractions (10 ml.) were collected every 10 min. and the ninhydrin colour densities given by 0.1 ml. samples of the fractions were measured by the method of Moore & Stein (1948). A small band of ninhydrin-positive material emerged in fractions 7–15 and a major band was eluted between fractions 80 and 122. Fractions 85–114 were combined and freeze-dried. The freeze-dried powder was dissolved in a

minimum amount of water, precipitated with acetone (about 60 ml.), ground to a fine powder under acetone, and washed twice with a further quantity (about 50 ml.) of dry acetone. After having been freed from acetone the powder was dissolved in water (20 ml.), the pH of the solution adjusted to 8.0 by addition of 0.2N-NaOH, and the solution freeze-dried to yield amorphous deacetylcephalosporin C sodium salt (1.24 g.). The amorphous product was crystallized from aqueous ethanol (Found: C, 37.0; H, 5.5; N, 9.2; S, 7.0; loss of wt. on drying *in vacuo* at 20°, 13.4%. $\text{C}_{14}\text{H}_{18}\text{O}_7\text{N}_3\text{SNa}\cdot 3.5\text{H}_2\text{O}$ requires C, 36.7; H, 5.5; N, 9.4; S, 7.0%; loss of 3.5H₂O, requires 13.7%). It showed $[\alpha]_D^{25} + 107^\circ$ in water (c, 1.4).

Characterization of acetic acid liberated on enzymic hydrolysis. A solution of cephalosporin C sodium salt (317 mg.) in water (2 ml.) was added to 5 ml. of acetyl esterase (stock solution *A* adjusted to pH 6.5). The mixture was stirred at 30° and 0.2N-NaOH added at such a rate that the pH remained close to 6.5. After 1.5 hr. the pH remained virtually constant without further addition of NaOH, although the solution showed strong acetyl-esterase activity when tested by the method of Byrde & Fielding (1955). [About 10 μl . of the solution was spotted on Whatman no. 1 paper and the paper sprayed with diacetylfluorescein (0.01%) in aqueous 50% (v/v) acetone. After keeping the paper for 15 min. at room temperature the spot appeared as a bright green fluorescent zone when viewed in ultraviolet light.] The solution was cooled and its pH adjusted to 2.9 by addition of 2N- H_2SO_4 . It was then freeze-dried and the distillate collected. Electrometric titration of the distillate with 0.2N-NaOH indicated the presence of an acid with pK_a approx. 4.7. The titre (at pH 8) corresponded to 0.55 equiv./mole of cephalosporin C. This low value was probably due to the retention of some of the acetic acid on freeze-drying. The neutralized distillate was concentrated in a rotary evaporator to about 3 ml., a solution of *p*-bromophenacyl bromide (101 mg.) in ethanol (2 ml.) was added, and the mixture heated under reflux for 70 min. The product which separated on cooling was recrystallized from ethanol-water. It had m.p. 82–84°. When mixed with a sample of authentic *p*-bromophenacyl acetate (m.p. 84°) the m.p. was not depressed.

Alkaline hydrolysis of cephalosporin C. This was carried out at constant pH values by use of a pH Stat (type SBR 2/SBU 1 Titrigraph in conjunction with type TTT 1 automatic titrator, made by Radiometer, Copenhagen). A solution of cephalosporin C sodium salt (20 mg.) in 4 ml. of water was placed in a water-jacketed Pyrex glass vessel. The latter was covered with a polythene lid through holes in which passed a glass electrode, the KCl bridge of a calomel reference electrode, a glass tube carrying a stream of N_2 (which was used to stir the solution and exclude CO_2), and a fine glass tube from a syringe containing 0.1N-NaOH. After 0.1N-NaOH had been added from a fine pipette to adjust the pH of the solution to a predetermined value, further 0.1N-NaOH was added automatically from the syringe at such a rate that the pH remained constant and acid groups liberated by hydrolysis of cephalosporin C were neutralized. The rate of addition of NaOH was recorded. Experiments were carried out at pH 11 and 18° and at pH 9.5 and 38°. After 1.0 or 1.5 equiv. of NaOH/mole of cephalosporin C had been added, Dowex 50X8 (H form) was added to the stirred solution until the pH fell to 6. The solution was concentrated to about 1 ml. and 5 μl . samples

of the concentrate were analysed by paper chromatography in butan-1-ol-acetic acid-water and in propan-1-ol-water.

N-Benzoylation of deacetylcephalosporin C. This was carried out with benzoyl chloride (1 mol.prop.) in a mixture of acetone and aqueous NaHCO_3 at 0° , as described by Abraham & Newton (1956) for the benzylation of cephalosporin C. After removal of the acetone *in vacuo*, the pH of the mixture was adjusted to 2.5. The *N*-benzoyl derivative was extracted with butan-1-ol cooled to -10° (three times with 1 vol.) and immediately re-extracted into water, as the sodium salt, at pH 8.0. The solution of the sodium salt was freeze-dried.

RESULTS

Action of citrus acetyl esterase on cephalosporin C and other compounds

The manometric results obtained with triacetin and cephalosporin C as substrates for the acetyl esterase were analysed by a plot of v against v/s as recommended by Eadie (1942) and by Hofstee (1959). Since the Michaelis-Menten equation can be written in the form:

$$v = V_{\max} - K_m(v/s)$$

this plot gives a straight line with slope $-K_m$ and intercept $V_{\max}$ with the ordinate axis. The plot of v against v/s with cephalosporin C as substrate and stock enzyme solution B is shown in Fig. 1. Values found for Michaelis constants (K_m) with triacetin and cephalosporin C respectively, and relative values of the maximum velocity in the presence of excess of substrate ($V_{\max}$) are shown in Table 1.

No reaction occurred, as judged by manometric experiments, when the preparations of citrus

acetyl esterase were mixed with benzylpenicillin, cephalosporin C_o (deacetylcephalosporin C lactone) (Abraham & Newton, 1961), or cephalosporin C_A (pyridine) (I; $R = -C_6H_5N^+$) (Hale, Newton & Abraham, 1961).

Properties of deacetylcephalosporin C

The elementary composition of the product formed by the action of citrus acetyl esterase on cephalosporin C corresponded with that of a hydrated sodium salt of deacetylcephalosporin C (I; $R = OH$). Its ultraviolet-absorption spectrum ($\lambda_{\max}$ 261, $\log \epsilon$ 3.93), which is shown in Fig. 2, was similar to that of cephalosporin C. Its infrared-absorption spectrum (in Nujol) showed a strong band at 5.77μ , but, unlike that of cephalosporin C, no band at 5.62μ or 9.7μ . Electrometric titration in water indicated the presence of ionizable groups with pK_A values of < 2.5 , 3.0 and 9.7 respectively.

Deacetylcephalosporin C sodium salt showed an activity of 2.3 units/mg. against *Staph. aureus* and 1.8 units/mg. against *Salm. typhi*, i.e. about 20% of that of the sodium salt of cephalosporin C. Highly purified preparations of penicillinase from *Bacillus cereus* 569/H and 5/B (Pollock, 1960) had no detectable effect on deacetylcephalosporin C, as measured by the manometric method of Pollock (1952), when used in a concentration 10^4 times that which was sufficient to hydrolyse 2 mg. of benzylpenicillin in about 30 min.

On electrophoresis at pH 7.0 and pH 4.5, deacetylcephalosporin C migrated slightly further towards the anode than cephalosporin C. At pH 2.3 cephalosporin C behaved as though it had no net charge, whereas deacetylcephalosporin C migrated slightly towards the cathode (Table 2). On paper chromatography in propan-1-ol-water or butan-1-ol-acetic acid-water the R_F of deacetylcephalosporin C was significantly less than that of cephalosporin C and the two compounds were well resolved when run together (Table 2). In butan-1-ol-acetic acid-water, however, deacetylcephalosporin C appeared to undergo some degree of change as judged from the appearance of the chromatogram after coloration with ninhydrin.

N-Benzoyldeacetylcephalosporin C showed an activity of about 13 units/mg. against *Staph. aureus* and 0.3 unit/mg. against *Salm. typhi*. Its ultraviolet-absorption spectrum showed $\lambda_{\max}$ at $228 m\mu$ ($\log \epsilon$ 4.1) and an inflexion at $265 m\mu$. Its infrared spectrum (in Nujol) showed a strong band at 5.62 . It migrated nearly twice as far as deacetylcephalosporin C towards the anode on electrophoresis at pH 7.0.

In acid solution at room temperature deacetylcephalosporin C was converted into a compound which was indistinguishable by paper electrophoresis and paper chromatography (Table 2) from

Fig. 1. Plot of v against v/s , where v is the rate of hydrolysis of cephalosporin C sodium salt in the presence of citrus acetyl esterase (μ l. of CO_2 evolved/min. from NaHCO_3) and s is the concentration of cephalosporin C (mm).

Table 1. *Comparison of the behaviour of triacetin and cephalosporin C as substrates for citrus acetyl esterase*

Acetyl esterase from	K_m (mm) for		V_{max} (μ equiv./min./ml. of stock enzyme soln.) for	
	Triacetin	Cephalosporin C	Triacetin	Cephalosporin C
Israeli oranges (stock solution A)	32 $\pm$ 2	4.7 $\pm$ 0.2	2.70 $\pm$ 0.2	0.79 $\pm$ 0.03
South African oranges (stock solution B)	—	4.7 $\pm$ 0.2	—	—
Californian oranges	30*	—	—	—

* Value reported by Jansen, Jang & Macdonell (1947).

Table 2. *Behaviour of deacetylcephalosporin C, cephalosporin C, cephalosporin C_o and cephalosporin C_A (pyridine) on paper electrophoresis and chromatography*

The positions of the cephalosporin compounds on paper were detected by coloration with ninhydrin, by viewing the paper in ultraviolet light and by bioautographs (for details see experimental section). Migration towards the anode is denoted by + and towards the cathode by -. In the apparatus used for electrophoresis, buffer was able to evaporate from the paper. At a given potential gradient, more evaporation occurred with collidine-acetate, pH 7.0, than with pyridine-acetate, pH 4.5, because the higher conductivity of the former buffer resulted in a greater production of heat. Differences in the resulting flow of buffer on the paper may have been responsible for the fact that strongly acidic compounds migrated further towards the anode at pH 4.5 than at pH 7.0.

Compound	Migration (cm.) at			$R_{glycine}$ in	
	pH 7.0	pH 4.5	pH 2.3	Butan-1-ol-acetic acid-water	Propan-1-ol-water
Deacetylcephalosporin C	+4.3	+7.2	-1.2	0.57	0.60
Cephalosporin C	+4.2	+6.8	-0.6	0.78	0.77
Cephalosporin C _o	-1.0	-1.7	-4.4	0.85	0.98
Cephalosporin C _A (pyridine)	-1.0	-1.7	—	—	—
Glucose	-1.0	-1.7	-0.6	—	—

deacetylcephalosporin C lactone, cephalosporin C_o. The latter had been previously obtained from cephalosporin C itself by Abraham & Newton (1961). The product from deacetylcephalosporin C was also indistinguishable from cephalosporin C_o with respect to its ultraviolet-absorption spectrum (λ_{max} , 257 m μ) and to the change in this spectrum which occurred when the solution was made weakly alkaline (Fig. 2). Its identity with cephalosporin C_o was proved by isolation, the procedure used being the same as that described by Abraham & Newton (1961) for obtaining cephalosporin C_o from cephalosporin C except that lactonization was brought about by treatment with N-HCl for 20 min. The crystalline product from deacetylcephalosporin C showed an infrared-absorption spectrum which was identical with that of authentic cephalosporin C_o, and the two products were reported by Professor Dorothy Hodgkin and Mr R. Diamond to give identical X-ray powder diagrams.

Analysis of reaction mixtures by paper electrophoresis showed that the formation of cephalosporin C_o from deacetylcephalosporin C occurred rapidly at pH values less than 1.0. When deacetylcephalosporin C sodium salt was dissolved in 20 equiv. of N-HCl the reaction was complete in

10 min. Under these conditions very little cephalosporin C_o was formed from cephalosporin C.

Deacetylcephalosporin C was treated with aqueous pyridine under the conditions used by Hale, Newton & Abraham (1961) to demonstrate the formation, on a small scale, of cephalosporin C_A (pyridine) from cephalosporin C. Deacetylcephalosporin C and authentic cephalosporin C_A (pyridine) were easily distinguished by paper electrophoresis at pH 4.5 (Table 2). A bioautograph, made after electrophoresis of the reaction mixture, failed to reveal the presence of any cephalosporin C_A (pyridine), although the formation of the latter in more than 2% yield should have been detected under the conditions used.

Formation of deacetylcephalosporin C by alkaline hydrolysis of cephalosporin C

When an aqueous solution of cephalosporin C sodium salt (13.5 mg./ml.) was kept at 18° and at pH 11.0, 1 equiv. of acid was liberated by hydrolysis in 3.6 hr. Analysis of the reaction mixture by paper chromatography at this stage showed that it contained approximately equal amounts of compounds with R_f values corresponding to cephalosporin C and deacetylcephalosporin C respectively,

Fig. 2. Ultraviolet-absorption spectra of deacetylcephalosporin C and of products formed from it by treatment with acid and alkali. $\times$, Deacetylcephalosporin C sodium salt in water; $\bullet$, solution obtained by keeping deacetylcephalosporin C sodium salt in 0.25N-HCl for 30 min. at 20°; $\circ$, solution obtained by keeping deacetylcephalosporin C sodium salt in 0.25N-HCl for 60 min. and then at pH 10.5 for 35 min. Extinctions are for 0.05 mg. of deacetylcephalosporin C sodium salt (or products formed from 0.05 mg. of the latter)/ml.

together with uncharacterized ninhydrin-positive material. A similar mixture of products was obtained when hydrolysis was carried out at 38° and pH 9.5 for 3.0 hr. From these results it appeared unlikely that deacetylcephalosporin C could be prepared in good yield by simple alkaline hydrolysis of cephalosporin C.

DISCUSSION

The enzymic hydrolysis of a number of different esters ($R \cdot CO_2X$) appears to involve the preliminary formation of an enzyme-substrate complex ($R \cdot CO_2X-E$), followed by acylation of the enzyme (E) to yield $R \cdot CO-E$ and subsequent hydrolysis of the acylated enzyme (Gutfreund & Hammond, 1959; Hofstee, 1960).

If this were so in the hydrolysis of cephalosporin C and triacetin by citrus acetyl esterase the Michaelis constants, K_m (determined, for example,

from a plot of v against v/s), should be related to the different velocity constants by the expression of Gutfreund & Sturtevant (1956):

$$K_m = (K_m)_1 \frac{k_3}{k_2 + k_3}$$

where

$$(K_m)_1 = \frac{k_{-1} + k_2}{k_1}$$

and the overall velocity constant, k_0 , is given by

$$1/k_0 = 1/k_2 + 1/k_3$$

For the hydrolysis of two acetyl esters k_3 should be the same. The determined Michaelis constant, K_m , with triacetin as substrate is about seven times that with cephalosporin C. According to the above scheme the value assigned to $(K_m)_1$ with triacetin would thus be $7(k_2 + k_3)/(k'_2 + k_3)$ times as great as with cephalosporin C, where k_2 and k'_2 are corresponding velocity constants for triacetin and cephalosporin C respectively. The finding that V_{max} (i.e. $k_0[E]$) is about 3.4 times as great for triacetin as for cephalosporin C would imply that $k_2 > k'_2$. Hence $(K_m)_1$ with triacetin would be at least seven times that with cephalosporin C but not significantly greater than seven times if acylation were the rate-determining process in each case, that is, if $k_2 \ll k_3 \gg k'_2$.

A number of properties of deacetylcephalosporin C, including its ultraviolet-absorption spectrum and its ready conversion into cephalosporin C, indicated that the enzymic removal of the acetyl group from cephalosporin C had been brought about without other changes having occurred in the molecule. The failure of deacetylcephalosporin C to form cephalosporin C_A (pyridine) was to be expected if the formation of the latter from cephalosporin C involved displacement of the acetoxyl group, as suggested by Hale *et al.* (1961). The presence of a band at 5.77 μ and the absence of a band at 5.62 μ in the infrared spectrum of deacetylcephalosporin C sodium salt, however, were unexpected. In the infrared spectrum of cephalosporin C a band at 5.62 μ is attributed to the C=O of the β -lactam ring and a band at 5.77 μ to the C=O of the acetoxyl group. The displacement of the lactam C=O band from its normal position in deacetylcephalosporin C sodium salt may be due to effects which occur in the solid state. This view is consistent with the finding that the infrared spectrum of *N*-benzoyldeacetylcephalosporin C showed a strong band in the expected position at 5.62 μ and no band at 5.77 μ .

SUMMARY

1. Citrus acetyl esterase catalyses the hydrolysis of the *O*-acetyl group in cephalosporin C. The Michaelis constant for the reaction was found to be 4.7 mM.

2. Deacetylcephalosporin C has been isolated as a crystalline sodium salt. It resembles cephalosporin C in a number of its properties, including its ultraviolet-absorption spectrum, but is more readily converted into the lactone, cephalosporin C₆, in acid solution.

3. Deacetylcephalosporin C shows about 20 % of the activity of cephalosporin C against *Staphylococcus aureus* (Oxford strain) and *Salmonella typhi*. It is highly resistant to hydrolysis by purified preparations of penicillinase from *Bacillus cereus* strains 569/H and 5/B.

4. Deacetylcephalosporin C and cephalosporin C may be distinguished by paper chromatography.

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Studies on Human Urinary and Pituitary Gonadotrophins

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The interest in human gonadotrophic hormones derives from the necessity for their accurate measurement in body fluids and from the prospect of their therapeutic usefulness. The separation of these hormones from urine and from pituitary glands and the comparison of certain of their biological, physical and immunological properties form the subjects of this paper.

METHODS

Preparation of gonadotrophins

From urine. Urinary gonadotrophins have been prepared by two different methods.

(i) Pooled urine of postmenopausal women was extracted by the benzoic acid-tungstic acid method described by

Butt (1958). Material soluble in water was adsorbed on diethylaminoethylcellulose and was then eluted in 0.5M-ammonium acetate. After precipitation from 3 vol. of ethanol the material was redissolved in water and fractionated by chromatography on a column of diethylaminoethylcellulose. Material eluted in 0.2M-ammonium acetate was collected and reprecipitated from 3 vol. of ethanol, washed with ethanol and ether and dried (Butt, Crooke & Cunningham, 1959).

(ii) A generous supply of urinary gonadotrophin from postmenopausal women (HMG-J5) was given to us by Dr S. G. Johnsen (Statens Seruminstitut, Copenhagen). It had been prepared by Johnsen's (1955) method and was further fractionated by chromatography on a column of calcium phosphate by the procedure described by Butt, Ingram & Round (1956), except that the calcium phosphate was prepared by a method described by Main, Wilkins & Cole (1959). Calcium phosphate was precipitated by