256. *Tautomeric Forms of Streptomycin.*

By Donald P. Young.

The salts, presumed to be of a tautomeric form of streptomycin, prepared by Heuser, Dolliver, and Stiller [1] are designated β-streptomycin salts. It is now shown that their difference from ordinary or α-streptomycin salts is a labile, chemical one. The β-streptomycin cation can exist in solution for a limited time, and the various β-salts are interconvertible by metathesis; but they revert completely to the α-form when their solutions are kept. This change can be followed polarimetrically. The only known way of forming β-streptomycin is by keeping (" ageing ") the 4-ethyl-1-3′-ethylpentyloctyl sulphate (tergitate), or a closely related salt, in the solid state. The α ⇌ β changes in both directions are accelerated by bases, and retarded by acids. β-Streptomycin tergitate reacts with carbonyl reagents much more slowly than the α-form; hence it is thought that in β-streptomycin the aldehyde group is bound by cyclisation, possibly with the methylamino-group. β-Streptomycin salts are characteristically crystalline, but they are probably all solvated in that state.

Heuser, Dolliver, and Stiller [1] prepared a series of streptomycin salts which they thought to be tautomeric with the normal salts. Their supposition now appears to be correct; it is therefore proposed to designate compounds obtained by their method as β-streptomycin salts, using α-streptomycin to denote the " ordinary " form of the antibiotic.

Heuser *et al.* precipitated streptomycin as the tergitate (4-ethyl-1-3′-ethylpentyloctyl sulphate; Tergitol-7 is the sodium salt). This when kept for a few days (" aged ") became crystalline. It could then be converted into a sulphate, hydrochloride, etc., which unlike the corresponding α-streptomycin salts were crystalline. The most striking difference was shown by β-streptomycin sulphate, which was very sparingly soluble in water, whereas the α-sulphate is amorphous and extremely soluble. Formation of the β-sulphate in this way has been used commercially as a means of purifying the antibiotic. Heuser *et al.*

[1] Heuser, Dolliver, and Stiller, *J. Amer. Chem. Soc.*, 1953, **75**, 4013.

showed that their β-streptomycin salts further differed from the α-salts in distribution coefficient and the shape of the polarogram. By making use of these they demonstrated, in a roughly quantitative manner, that β-streptomycin salts reverted to the α-form in solution, and that this change was retarded by the presence of acids.

The clearest evidence [2] that the phenomenon was not merely one of allotropy or solvation is that solutions of β-streptomycin hydrochloride gave a precipitate of the β-sulphate on addition of sulphate ion, so that the β-streptomycin cation preserved its identity in solution.

The observations of Heuser *et al.* have now been confirmed and extended. β-Streptomycin hydrochloride was converted, by precipitation of its freshly made aqueous solution with the appropriate sodium salt, into the biphenyl-4-sulphonate, 5-cyclohexyl-2-hydroxy-3-methylbenzoate, and 2-hydroxy-5-isobutyl-3-methylbenzoate. In the case of the first two of these, the α-streptomycin salts are also crystalline,[3,4] but the pairs of salts had

FIG. 1. *Mutarotation of β-streptomycin tergitate (c, 5).*

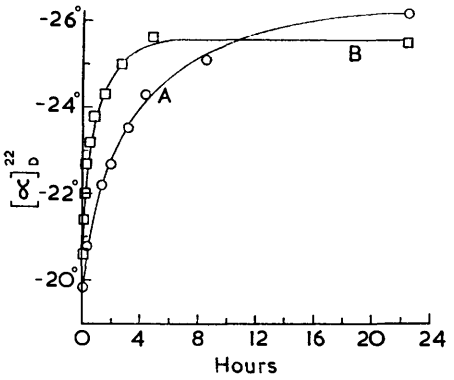
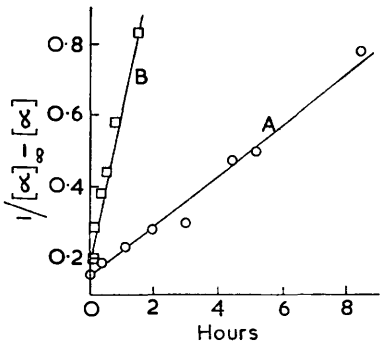


FIG. 2. *Second-order plot for mutarotation of β-streptomycin tergitate.*



(A) *In neutral methanol.* (B) *In* 0·01N-*methanolic sodium hydroxide.*

different *X*-ray diffraction patterns. These three new salts were then converted into β-streptomycin sulphate.

The reversion of β- to α-streptomycin salts in solution can be followed polarimetrically, most easily with the tergitate in methanol (50 mg./ml.), which showed a change of $[\alpha]_D^{20}$ from −19·8° to −26·4°. As ordinarily prepared, the β-tergitate reverted in this way to the α-form in a few days. In 0·01N-methanolic sodium hydroxide, the mutarotation was more rapid (Fig. 1); but in 0·01N-methanolic sulphuric acid there was no measurable alteration in $[\alpha]_D$ during 18 hr., and in 0·1N-methanolic acetic acid a change of only 0·2° in 24 hr., corresponding to *ca.* 3% conversion into the α-form. Further, if the β-tergitate was recrystallised from methanol–water containing a trace of sulphuric acid, it became stable in methanol solution, and its stability was not destroyed by subsequent recrystallisation from unacidified methanol. The " stabilised " tergitate did nevertheless revert rapidly to the α-form if a trace of sodium hydroxide was added to its solution. The change, surprisingly, was of the second order in all cases.

β-Streptomycin 5-cyclohexyl-2-hydroxy-3-methylbenzoate showed a similar, very rapid change in rotation in methanol solution as it reverted to the α-form. For the hydrochloride in aqueous solution the change was too small and too fast for accurate observation: $[\alpha]_D^{22}$ was −82·3° ten minutes after preparation (50 mg./ml.), falling to a final value of −79·1° (24 hr.) (at least three-quarters complete in 50 min.). Gravimetric estimation of the β-tautomer by precipitation as sulphate led to the same conclusion. The change

[2] Heuser, U.S.P. 2,663,464.
[3] Ziegler, U.S.P. 2,857,376.
[4] Ziegler, U.S.P. 2,857,375.

was appreciably retarded by 0·001N-concentration of hydrochloric acid, and in 0·01N-acid it occupied many hours; here again the reaction was approximately of the second-order (Fig. 3); the rate, however, varied with different batches of β-salt. In 5% aqueous toluene-*p*-sulphonic acid, the β-hydrochloride initially showed $[\alpha]_D^{18}$ −70·8° changing, in the opposite sense, to −78·5° (final: 24 hr.).

Probably all crystalline β-streptomycin salts are solvated. The sulphate lost the equivalent of $7H_2O$ when dried; the dried salt dissolved in water, but the crystalline hydrate was soon precipitated. *X*-Ray examination of the dried material revealed a partly orientated structure. The hydrochloride crystallised from methanol with 2 mol. of methanol, which was readily driven off, leaving an amorphous substance. Recrystallised β-streptomycin tergitate is hydrated.[1]

The " ageing " of streptomycin tergitate involves a spontaneous change from the α- to the β-form, accompanied by crystallisation. Precipitated α-streptomycin tergitate is soap-like and difficult to purify, but samples were conveniently obtained by keeping a methanolic solution of the β-tergitate for a few days and then removing the solvent. This left the α-tergitate as a glass, stable when dry. Only in presence of water did it change into the β-form. Like the β ⟶ α change, this was strongly accelerated by a trace of alkali, and inhibited by aqueous acid.

So far, no other streptomycin salt except the related 4-ethyl-1-isobutyloctyl sulphate [2] (from Tergitol-4) has been found which changes from the α- into the β-form. In all streptomycin salts examined, there was no detectable amount of β-form in equilibrium with the α-tautomer in solution; solutions of α-streptomycin showed no measurable mutarotation, and it has proved impossible to obtain the β-sulphate from them by adding sulphate ion. It is therefore clear that a change towards the β-form could not occur in a solid phase in contact with a solvent containing appreciable amounts of dissolved streptomycin. Although the presence of water is necessary for the formation of β-streptomycin tergitate—presumably because the driving force is crystallisation of the hydrate—the tergitate is very insoluble in water; this combination of properties may be almost unique amongst streptomycin salts. Heuser *et al.*[1] thought that some α-tergitate was present at equilibrium, which may indeed arise from back-reaction in the aqueous phase.

It has been established that the β-streptomycin cation has an independent existence, and that its salts have the same composition as the corresponding α-streptomycin ones. The equilibrium between the two is sufficiently mobile to justify the term tautomerism, although, in the absence of molecular-weight determinations, reversible dimerisation cannot be excluded.

Mannosidostreptomycin shows the same phenomenon,[2] but dihydrostreptomycin and streptomycin oxime do not, which implicates the aldehyde group as the seat of the tautomerism. This was confirmed when it was found that β-streptomycin tergitate, in a metastable condition in methanolic 0·1N-acetic acid, reacted much more slowly than the α-salt with hydroxylamine and 4-phenylsemicarbazide (reactions followed polarimetrically) (cf. Fig. 4). Evidently, in β-streptomycin the aldehyde group is combined in such a way that it has become unreactive.

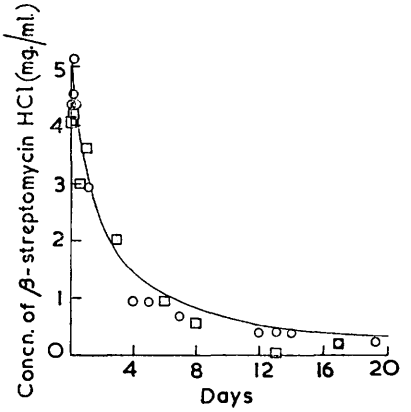
Heuser *et al.*[1] interpreted their polarographic results (which we have confirmed) in the opposite sense, namely, that it was the α-tautomer that had a bound (unreactive) aldehyde group. The extra polarographic wave on the more positive side with the α-form could, however, equally be interpreted as arising from an easily reducible group, *i.e.*, a free aldehyde group, in that tautomer.

The pronounced effect of bases on the α ⇌ β changes is manifested at the neutralisation point of the third basicity of the streptomycin cation. This suggests that the weakest basic group, *i.e.*, the NHMe-group, may also be concerned in the tautomerism. Possibly it combines with the aldehyde group to form a ·CH(OH)·NMe· structure; this would result in a 7-membered ring, which, from atomic models, is sterically possible (dimerisation of the type common amongst α-ketols is sterically unlikely). The fact that the β ⟶ α

reaction, resulting in the freeing of a bound aldehyde group, is of the second order is puzzling, but it need not indicate that the reaction is bimolecular. There is no appreciable difference in base-strength between α- and β-streptomycin.

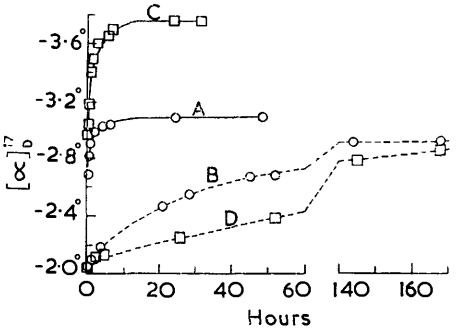
Other authors [5] have obtained evidence, from polarographic and distribution experiments, of tautomerism of streptomycin in solution. Their results might at first sight be

FIG. 3. *Change of β- into α-streptomycin hydrochloride in* 0·01N-*hydrochloric acid at* 17°.



○ *Polarimetric observations.* □ *Gravimetric observations.* —— *Calculated second-order curve,* k = 0·0125 g. ml.$^{-1}$ day^{-1}.

FIG. 4. *Reaction of streptomycin tergitates* (0·6 *mmole*) *with carbonyl reagents in* 0·1N-*acetic acid in methanol* (20 ml.).



(A, C) α-, (B, D) *β-Tergitate.* (A, B) *Hydroxylamine acetate* (0·7 mmole); (C, D) *4-phenylsemicarbazide* (0·6 mmole).

explained by an equilibrium between α- and β-streptomycin. However, it now appears that this equilibrium would be entirely on the side of the α-form; thus there may be more than two desmotropic forms of streptomycin.

EXPERIMENTAL

Samples for analysis and bioassay were dried at 60°/0·1 mm. for 8 hr.; this did not always completely remove water. Nitrogen analyses, whether by the Dumas or the Kjeldahl method, often gave low results for streptomycin derivatives. Bioassays were by *Bacillus subtilis* NTCC 8236. Rotations were measured with 2-dm. tubes.

β-Streptomycin tergitate, m. p. 155—158°, was prepared by precipitation, " ageing," and recrystallisation, as described by Heuser *et al.*[1] Under the polarising microscope it appeared as small flat birefringent needles.

Change of β- into α Streptomycin Tergitate.—A solution of β-tergitate in methanol was kept until the rotation ceased to rise. The apparent pH of the solution remained unchanged (within 0·1 unit). Evaporation under a vacuum left the α-tergitate as a glass, soluble in carbon tetrachloride and acetone in which the β-tergitate is insoluble; such solutions did not crystallise, even with added water. With aqueous guanidine sulphate and butanol it gave no crystalline streptomycin sulphate.

Evaporation of a solution of β-tergitate in 0·01N-methanolic sulphuric acid after 18 hr. left unchanged β-tergitate, which crystallised. This was convertible into β-streptomycin sulphate.

Conversion of α- into β-Streptomycin Tergitate.—(a) *Under neutral conditions.* α-Streptomycin tergitate, obtained as above, remained unchanged for months in a closed vessel. When covered with distilled water, the glass softened to a jelly, then after 3 days became friable and opaque. After 7 days it was dried, and then consisted of birefringent crystals, m. p. 149°. In methanol $[\alpha]_D$ changed from −19·9° to −24·1°. By evaporation of this solution and addition of water, the cycle could be repeated.

(b) *In presence of alkali.* α-Streptomycin tergitate was covered with 0·01N-aqueous sodium

[5] Bricker and Vail, *J. Amer. Chem. Soc.*, 1951, **73**, 585; Titus and Fried, *J. Biol. Chem.*, 1948, **174**, 57.

hydroxide. In ½ hr. it had peptised to a thick colloidal suspension, but after 1 hr. this coagulated to waxy masses, leaving a mobile supernatant liquid. The solid was crystalline, with m. p. ~155°.

(c) *Under acid conditions.* The α-tergitate, after 10 days under 0·01N-hydrochloric acid, was unchanged.

" *Stabilised* " β-*Streptomycin Tergitate.*—Crude β-tergitate (15 g.) was dissolved in methanol (100 ml.) containing sulphuric acid (0·02 ml.) at 30—40°. The solution was cooled and water (10 ml.) added. After some hours at 0° the crystals (11·2 g.) were collected and recrystallised from methanol–water. They then had m. p. 145°, $[\alpha]_D^{28}$ −21·6° in methanol, rising to −22·6° after 12 days.

Reaction of Streptomycin Tergitates with Hydroxylamine.—To streptomycin tergitate (1 g.) in 0·1N-methanolic acetic acid (20 ml.) was added a solution (1 ml.) of hydroxylamine acetate [from hydroxylamine hydrochloride (1·02 g.) and sodium acetate (1·25 g.) in methanol, filtered, and made up to 25 ml.].

When the reaction with the α-tergitate was complete (48 hr.; Fig. 4), 0·9N-aqueous methylamine sulphate (2·2 ml.) was added, and the precipitated *oxime sulphate* (0·38 g.) was collected (Found: C, 32·9; H, 6·4; N, 14·2; S, 6·8. $C_{21}H_{40}N_8O_{12},1\frac{1}{2}H_2SO_4,3H_2O$ requires C, 32·9; H, 6·2; N, 14·1; S, 6·1%). This was biologically almost inactive (potency found, 28 u./mg.).

The reaction with β-tergitate was not complete after 7 days. The product at that point was converted into sulphate, which from its assay (80 u./mg.) was oxime containing an appreciable amount of unchanged streptomycin.

Streptomycin Oxime Tergitate.—To a solution of α-streptomycin sulphate (7·3 g.) and hydroxylamine hydrochloride (1·04 g.) in water (50 ml.), sodium hydroxide was added as necessary to keep the pH at 6. After 3 hr., a slight excess of Tergitol-7 was added. The pasty precipitate was centrifuged off and washed with water. It did not crystallise and was dried to a glass (Found: C, 53·8; H, 9·1; N, 6·4; S, 6·0. $C_{21}H_{40}N_8O_{12},3C_{17}H_{26}O_4S,2H_2O$ requires C, 53·1; H, 8·8; N, 6·9; S, 5·9%). With methylamine sulphate in aqueous methanol, it gave the oxime sulphate (potency 48 u./mg.).

Reaction of Streptomycin Tergitates with 4-Phenylsemicarbazide.—Streptomycin tergitate (1·0 g.) and 4-phenylsemicarbazide (0·10 g.) were dissolved in 0·1N-methanolic acetic acid (total volume, 20 ml.).

The reaction with α-tergitate was complete in 24 hr. (Fig. 4). 0·9N-Aqueous methylamine sulphate (2·2 ml.) was added to this solution to precipitate *streptomycin 4-phenylsemicarbazone sulphate*, which was washed with methanol and dried. It was biologically inactive (Found: potency, 20 u./mg.; C, 36·7; H, 6·2; N, 14·0; S, 5·3. $C_{28}H_{46}N_{10}O_{12},1\frac{1}{2}H_2SO_4,3H_2O$ requires C, 36·7; H, 6·1; N, 15·3; S, 5·3%). The reaction with β-tergitate was still incomplete (cf. Fig. 4) after 4 weeks, when the $[\alpha]_D^{17}$ had reached −35·4°.

β-*Streptomycin Hydrochloride.*—The hydrochloride, prepared in methanol [1] and air-dried, formed birefringent rhombic prisms. On drying at 60°/0·1 mm. for 8 hr., it lost 9% in weight (corresponding to 2MeOH) and became amorphous, although still apparently retaining water (Found: C, 34·8, 35·2; H, 6·7, 6·4; Cl, 15·0, 15·2; N, 13·4, 13·8%; potency, 861 u./mg. Calc. for $C_{21}H_{39}N_7O_{12},3HCl,H_2O$: C, 34·6; H, 6·2; Cl, 15·0; N, 13·8%; potency, 820 u./mg.). The methanol-free material was soluble in methanol; a 5% solution soon deposited crystals of the methanol solvate, which was almost insoluble. The crystalline compound gave a diffuse *X*-ray pattern, but it became amorphous during the exposure.

Conversion of β- *into* α-*Streptomycin Hydrochloride* (Fig. 3).—Solutions of the β-hydrochloride (50 mg./ml.) were observed polarimetrically, and the amount of β-streptomycin was estimated at intervals gravimetrically: an aliquot part was treated with an equal volume of 0·5N-guanidine sulphate (acidified to pH 3) and kept at 0° for 30 min. The precipitated β-sulphate was filtered off (filter-stick), washed with 0·1N-sulphuric acid, ethanol, and acetone, briefly dried *in vacuo*, and weighed.

β-*Streptomycin Tergitate from* β-*Hydrochloride.*—A fresh solution of the β-hydrochloride in water with a trace of hydrochloric acid was treated with Tergitol-7. The tergitate was precipitated immediately in crystalline condition (m. p. 148—149°).

β-*Streptomycin Biphenyl-4-sulphonate.*—A hot solution of sodium biphenyl-4-sulphonate (1·90 g.) in water (50 ml.) was added to a freshly prepared one of the β-hydrochloride (1·60 g.) in water (50 ml.). The β-*streptomycin salt* was precipitated immediately (yield 68%); it had m. p. 187—189° (decomp.) (Found: C, 51·4; H, 5·7; N, 7·5; S, 7·5. $C_{21}H_{39}N_7O_{12},3C_{12}H_{10}O_3S,3H_2O$ requires

C, 51·1; H, 5·6; N, 7·3; S, 7·2%). With the equivalent amount of guanidine sulphate in aqueous methanol, it gave β-streptomycin sulphate (92%), which was crystallised from water; its identity was checked by its infrared spectrum.

In a similar manner were prepared β-*streptomycin 5-cyclohexyl-2-hydroxy-3-methylbenzoate* (85%), m. p. 170—175° (decomp.; rapid heating), $[\alpha]_D^{19}$ −22·0° (5 min.), −26·7° (190 min.), −27·0° (27½ hr.) in MeOH (*c* 5) (Found: C, 57·9, 57·6; H, 7·7, 7·6; N, 7·2, 7·2. $C_{21}H_{39}N_7O_{12},3C_{14}H_{18}O_3$ requires C, 57·7; H, 7·3; N, 7·6%), and the *2-hydroxy-5-isobutyl-3-methylbenzoate* (83%), m. p. 90—95° (Found: C, 55·2; H, 7·4; N, 7·5%; potency, 437 u./mg. $C_{21}H_{39}N_7O_{12},3C_{12}H_{16}O_3,2H_2O$ requires C, 55·1; H, 7·4; N, 7·9%; potency, 469 u./mg.). Both these were converted in good yield into β-streptomycin sulphate.

From α-streptomycin sulphate were obtained α-streptomycin biphenyl-4-sulphonate [3] (85%), m. p. 187·5—189° (charred) (Found: C, 50·2; H, 5·5; N, 6·6; S, 7·4%: potency, 466, 453 u./mg. Calc. for $C_{21}H_{39}N_7O_{12},3C_{12}H_{10}O_3S,4H_2O$: C, 50·4; H, 5·8; N, 7·2; S, 7·1%; potency, 432 u./mg.), and 5-cyclohexyl-2-hydroxy-3-methylbenzoate [4] (99%), m. p. 160—165° (charred), 93° (decomp.; in preheated bath), $[\alpha]_D^{21}$ −27·4° in MeOH (*c* 5) (Found: C, 55·6; H, 7·7; N, 6·3%; potency, 448, 510 u./mg. Calc. for $C_{21}H_{39}N_7O_{12},3C_{14}H_{18}O_3,2H_2O$: C, 56·1; H, 7·4; N, 7·4%; potency, 441 u./mg.). The *X*-ray patterns of these two were similar to, but distinguishable from, those of the corresponding β-salts. α-Streptomycin hydroxyisobutylmethylbenzoate formed a gum.

β-*Streptomycin Sulphate from Hydrochloride.*—β-Streptomycin hydrochloride (5·0 g.) was dissolved in water (100 ml.), and 0·9N-methylamine sulphate (25 ml.) immediately added. The β-sulphate that crystallised was filtered off, washed with a little water and then with ethanol, and air-dried (2·9 g.). When dried at 60°/0·1 mm. for 8 hr., this product lost 15% in weight (corresponding to $7H_2O$), and was then anhydrous (Found: C, 34·8; H, 6·1; N, 12·7; S, 6·2%; potency, 817 u./mg. Calc. for $C_{21}H_{39}N_7O_{12},1\frac{1}{2}H_2SO_4$: C, 34·6; H, 5·8; N, 13·5; S, 6·6%; potency, 798 u./mg.). It then dissolved in water, but the solution rapidly afforded crystals of the hydrate. This recrystallised from 0·01N-sulphuric acid (dissolution at 40°) at 0°. The hydrate gave a sharp *X*-ray pattern; the dried β-sulphate gave diffuse rings, suggesting that part of the crystalline orientation remained. The infrared spectrum (KBr disc) was indistinguishable from that of α-streptomycin sulphate, in which no C=O band is detectable.

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