TABLE V—ESTIMATES OF FIRST-ORDER RATE CONSTANTS[a] FOR THE DISAPPEARANCE OF LINCOMYCIN AND LINCOMYCIN ESTER

	k_1[b], 95% C.L.	k_2,[c] 95% C.L.
Lincomycin-2-propionate	8.32 (2.29–14.35)	0.17 (0.05–0.30)
Lincomycin-2-butyrate	6.06 (0.00–12.4)	0.13 (0.01–0.25)
Lincomycin-2-hexanoate	9.67 (0.00–20.9)	0.06 (0.02–0.11)

[a] Obtained from nonlinear regression analysis. [b] k_1 rate constant for ester. [c] k_2 rate constant for lincomycin.

first-order rate constants for the better absorbed 2-ester are shown in Table V. Even these calculated rate constants must be considered estimates, since this equation does not take into consideration the rate of enzymatic hydrolysis in the intestine. The amounts of lincomycin base and the ester in the intestine are not only affected by the disappearance of each through absorption, but also by the amount of ester being hydrolyzed. However, even with these limitations, this method allows one to estimate k_1 and k_2 when the semilog absorption plots are not linear. Because of the linearity of its semilog absorption plots (Fig. 1), the rate constant for the absorption of lincomycin HCl was calculated by the usual method using the slope obtained by linear regression analysis. This rate constant was 0.21 $hr.^{-1}$ for lincomycin HCl. The estimates for lincomycin HCl are lower in Table V, 0.17 $hr.^{-1}$, 0.13 $hr.^{-1}$, and 0.06 $hr.^{-1}$.

This model and the data presented in this study indicate that the 2-ester and its hydrolysis product, lincomycin HCl, are both absorbed at the early time intervals, while only lincomycin HCl is absorbed in the later time intervals, the ester being completely hydrolyzed.

SUMMARY

The absorption of lincomycin was improved by using derivatives of lincomycin which consisted of esters of short-chain fatty acids (3 and 4 carbons). The absorption efficiencies of the various esters were modified by intestinal hydrolysis. The explanation for absorption of these esters is based on a model which states that the lincomycin ester and lincomycin base both contribute to this absorption.

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Keyphrases

Lincomycin HCl, esters—absorption
Jejunum, rat—ligated loops
Absorption, lincomycin HCl, esters—intestinal loops
Model, intestinal absorption—lincomycin HCl, esters
Intestinal homogenates—lincomycin HCl, esters hydrolysis

Ultraviolet Spectrum Correlations with the Conjugate Acid-Base Species of Acetarsone and Arsthinol

By C. F. HISKEY and F. F. CANTWELL

The ionization constants of acetarsone (pK_1 = 3.73, pK_2 = 7.9, pK_3 = 9.3) and arsthinol (9.5 ± 0.1) have been determined potentiometrically in aqueous solution and the UV spectra of the individual conjugate species have been deduced. Assignment of the second ionization step of acetarsone to the phenolic proton is made. Hydrolytic atmosphere oxidative cleavage of arsthinol in alkaline aqueous solution to produce acetarsone has been demonstrated and spectrophotometric assay methods for these two substances are proposed.

ACETARSONE (3-acetamido-4-hydroxy phenylarsonic acid) and arsthinol (cyclic 3-hydroxypropylene ester of 3-acetamido-4-hydroxy dithiobenzene arsenous acid) have the following structures:

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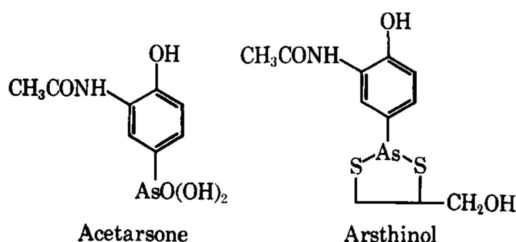


The first of these has been known for many years being Ehrlich's 596. Its synthesis has been described by Raiziss and Fisher (1). The synthesis of arsthinol (by the reaction of 3-acetamido-4-hydroxy phenyl arsenoxide with 2,3-dimercapto-propanol) was reported by Friedheim (2) in 1953.

Some of the chemical properties of this substance and methods of assay for it in the various dosage forms have been briefly described (3). In order to understand more about its acid-base chemistry and its absorption spectra for analytical purposes an experimental study was made of it, of acetarsone, and of some of their surrogates.

An examination of the literature revealed that Gex and Kreimer (4) had done differential titrations of acetarsone and reported four (sic) ionization constants having the following values: $pK_1 = 3.3$; $pK_2 = 4.2$; $pK_3 = 7.6$, and $pK_4 = 9.4$. They appear to have been under the misapprehension that the amide group had amine-like characteristics. They were also troubled by extensive precipitation of the acetarsone as they made their solutions acidic and seem to have made no provision to take this into account. They also present some data on the UV absorption spectrum of acetarsone.

EXPERIMENTAL

The arsthinol used in this study was regular production grade material, corresponding in its characteristics to its description in Tests and Standards of the N.N.R. It was used without further purification.

The acetarsone used in the titration study was dissolved in aqueous alkali solutions, filtered to remove any insoluble matter, and then precipitated with hydrochloric acid. The precipitates were washed repeatedly with distilled water until all traces of chloride were removed, after which they were dried at 105° to constant weight. This produced a fine powder which dissolved rapidly during the titration experiments as described below.

The absolute methanol was analytical grade and the propylene glycol was USP grade material.

The acid-base titrations were performed in a wide-mouth conical flask with magnetic stirring, using a Teflon-coated stirring bar, and employing a model 7 Corning pH meter. About 1.5 mmoles of the compound was suspended in a known volume of carbon dioxide-free water and titrated with approximately 0.1 *N* sodium hydroxide. Throughout the course of the titration a vigorous stream of nitrogen was blown into the solution to prevent carbon dioxide uptake.

A Beckman model DK-2 ratio-recording spectrophotometer was used in obtaining the spectrograms in this study and a Beckman model DU spectrophotometer was used for measuring absorbances at isolated wavelengths. Measurements were made in matched 1.00-cm. quartz cells.

Solutions for spectrophotometric measurements were prepared in the following manner: An amount of pure substance, approximately equal to a quarter of a millimole, was accurately weighed and transferred to a 100-ml. volumetric flask. Solution was effected using either anhydrous methanol in one group of studies or more commonly by the addition of 0.01 *N* sodium hydroxide. The solutions were brought to volume after which an appropriate aliquot was transferred to a second 100-ml. volumetric flask, and there again diluted with appropriate reagents to give the desired solution.

When determining the various spectra associated with the conjugate acid-base species of acetarsone, solutions were buffered so as to have a pH in the region where each species was dominant.

Solutions in approximately 0.1 *N* sodium methoxide were prepared by dissolving about 0.1 mole of metallic sodium in anhydrous methanol previously distilled over sodium. A 5.00-ml. aliquot of a stock solution of arsthinol in anhydrous methanol was transferred to a 100-ml. volumetric flask and the sodium methoxide solution was added to mark. A reference solvent for spectral work was prepared in an identical manner, using 5.00 ml. of methanol in place of the arsthinol stock solution.

Iodometric titrations employed in this study were done as follows: An aliquot was acidified with sulfuric acid, an equal amount of propylene glycol, and then an excess of standard iodine solutions was added. The iodine is back titrated with thiosulfate to a water-white solution. The starch-iodine end point cannot be used in this instance because the high concentration of propylene glycol present precludes its formation.

RESULTS

Titration of Acetarsone—Acetarsone has a very limited solubility in water at room temperature. Consequently it was necessary in the initial stages of the titration to wait after each addition of alkali for the pH meter to drift to its final steady-state values. During this period, more undissociated acetarsone went into solution until both the solubility and the ionization equilibria were satisfied. This situation continued until about 80% of the first equivalent of alkali had been added, at which point all of the undissolved acid was in solution and from then on equilibrium was established quickly after each addition of alkali. Typical of the data obtained are those presented in Fig. 1 where they are plotted as the individual points. The titration curve shown in the figure is that calculated, using the ionization constants determined in this study.

As expected, it was found that acetarsone titrates as a triprotic acid, *i.e.*, two of the protons come from the arsonic moiety and one from the phenolic group *para* to it. The first stage of ionization is separated

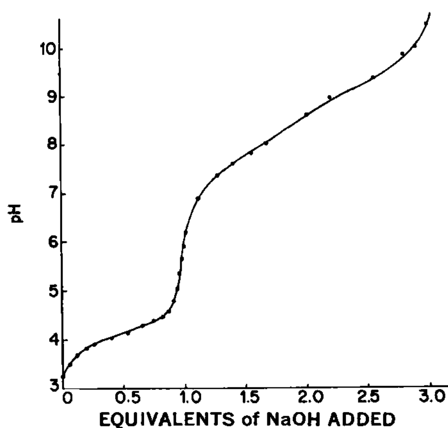


Fig. 1—Potentiometric titration curve of acetarsone with sodium hydroxide. Points shown are experimental results and the line is that calculated from the evaluated constants.

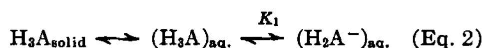
from the second stage by about four orders of magnitude, while the second and third stages of ionization are so close to each other that there is only a barely perceptible break observable in that region of the titration curve.

The evaluation of the successive ionization constants was performed as follows:

First it was assumed that the ionization of this triprotic acid could be represented by the equilibria:

$$H_3A \overset{K_1}{\rightleftharpoons} H_2A^- \overset{K_2}{\rightleftharpoons} HA^= \overset{K_3}{\rightleftharpoons} A^{\equiv} \quad \text{(Eq. 1)}$$

Since the first stage of ionization is so widely separated from the second and third stages, its constant may be evaluated separately according to the equilibria:



$$H_3A_{solid} \rightleftharpoons (H_3A)_{aq.} \overset{K_1}{\rightleftharpoons} (H_2A^-)_{aq.} \quad \text{(Eq. 2)}$$

The first ionization constant is then

$$K_1 = \frac{(H^+)\,(H_2A^-)}{(H_3A)} = \frac{(H^+)\,(H_2A^-)}{S} \quad \text{(Eq. 3)}$$

In this expression S is the solubility of the undissociated acetarsone. The latter term in Eq. 3 applies when dilute slurries of acetarsone are being considered while the middle term applies to its solutions.

The equation for the titration function of this monoprotic acid is as follows:

$$H - OH = \left[H_2A^-\right] C_B \quad \text{(Eq. 4)}$$

In the above expression, since these data are only approximate, only the H and OH terms represent the activities of the H_3O^+ and OH^- in the solution. The other terms refer to the concentration of strong base, C_B, and to the concentration of the conjugate base H_2A^-. Substitutions for the H_2A^- term may be made to treat the situations where acetarsone is (a) partly and (b) completely dissolved. These are, respectively:

$$H - OH = \frac{K_1S}{H} - C_B \text{ and} \quad \text{(Eq. 5}a\text{)}$$

$$H - OH = \frac{mK_1}{H + K_1} - C_B \quad \text{(Eq. 5}b\text{)}$$

where m is the formal concentration of acetarsone.

To use Eq. 5a the solubility of the undissociated acetarsone must be known. It was determined spectrophotometrically in 0.1 N hydrochloric acid (0.478 g./l.; 1.74×10^{-3} M) and in water (0.616 g./l.; 2.24×10^{-3} M).

Since the solubility in water is substantially greater than it is in 0.1 N hydrochloric acid, it is evident that considerable ionization has occurred in the pure water solvent, *i.e.*, 23%. From these data and Eq. 3, K_1 is calculated to be approximately 1.5×10^{-4}; $pK_1 = 3.8$. However, a more accurate value of 3.73 was determined by fitting the potentiometric titration data to Eqs. 5a and 5b.

The evaluation of K_2 and K_3 was performed by a method originally developed by Linderstrom-Lang and subsequently modified by Bjerrum (5). With it the data of Fig. 1 were evaluated; the slope at the second equivalence point was found to be 0.66 which gives a value for the spreading factor of 5.0. From this it follows that pK_2 equals $8.6 - \log 5.0 = 7.9$ and pK_3 equals $8.6 + \log 5.0 = 9.3$.

Thus all three constants for this triprotic acid have been determined and it now becomes possible to calculate the relative abundance of all of the four conjugate species as a function of pH. The results of these calculations are plotted in Fig. 2. These results were used in assigning spectra to the different conjugate species.

Titration of Arsthinol—The only acidic functional group in the arsthinol molecule is the phenolic one. Consequently the initial part of its titration curve prior to the addition of a full equivalent of alkali was consistent with this view and consistent with a pH for that phenolic group of about 9.4 to 9.6. Inasmuch as the phenolic ionization constant is so small, there is little of the compound in solution when a slurry of arsthinol in water is made. The solubility as determined in water at 25° was 0.32 g./l. or 0.00116 M. Less than 1% of the dissolved arsthinol would be ionized. However, somewhat before the first equivalent of alkali was added, all of the arsthinol had dissolved, leaving a water-white solution.

Addition of more than one equivalent of alkali did not cause the pH to change from about 11.2 even though two, three, and four equivalents were added. This indicated that hydrolytic cleavage of the arsenic sulfur bonds was occurring, and that the sulfhydryl groups and the arsenous acid were consuming the alkali. This formulation is consistent with

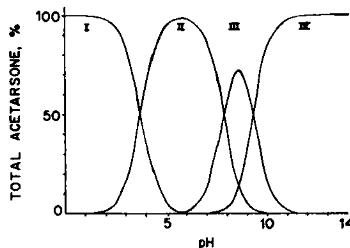
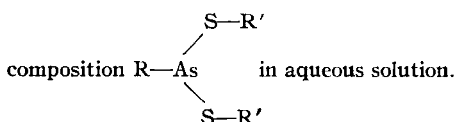


Fig. 2—Relative abundance of the various conjugate acid-base species of acetarsone as a function of pH. Key: I, H_3A; II, H_2A^-; III, $HA^=$; IV, $A^{\equiv}$.

the experimental observations of Cohen *et al.* (6) on the reversible hydrolysis of dithioarsenites of the composition

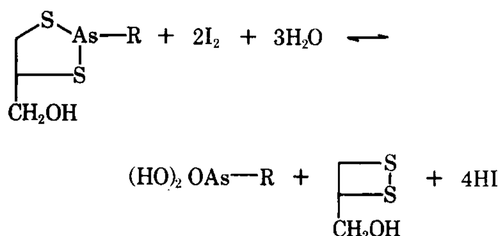


in aqueous solution.

When air was substituted for the nitrogen, the pH began to fall immediately, suggesting that the trivalent arsenic was being oxidized to make acetarsone which, with its greater acid ionization constants, would cause a shift to lower values of the pH. This continued until a pH of about 10.2 was reached and then as the air bubbling continued, the solution became turbid while the pH abruptly shifted to higher values.

The explanation of this phenomenon is also easily understood in terms of the reactions given above. After all, or the bulk of the trivalent arsenical was oxidized to acetarsone, the fully ionized 2,3-dimercaptopropanol is next oxidized to give disulfido linkages producing the insoluble matter observed and simultaneously releasing two equivalents of alkali to the solution, this accounting for the sudden increase in pH. The precipitate that is formed resembles the precipitate formed when 2,3-dimercaptopropanol is oxidized.

Additional evidence for aqueous alkaline air oxidation of arsthinol was obtained from a measurement of the iodometric titer of previously alkaline arsthinol solutions which had been aerated at room temperature for various periods of time. In these studies a 0.029 M arsthinol solution in 0.1 N sodium hydroxide was aerated. At periodic intervals an aliquot was taken and titrated according to the procedure described above. The arsthinol is titrated to acetarsone according to the following reaction:



It is entirely possible that the disulfide compound is polymeric, *i.e.*, $(\text{—S—CH}_2\text{—CH(CH}_2\text{OH)—S—})_x$. The elastic, gummy nature of the precipitate suggests this. The data obtained are listed in Table I and show that with time, continued aeration will eventually oxidize the arsthinol to acetarsone and disulfide.

When a similar type of experiment was done, using anhydrous methanol as the solvent, with sodium methoxide as the alkali, little or no oxidative cleavage of the arsenic-sulfur bonds occurred. This fact made it possible to obtain the phenolate spectrum of arsthinol.

Further supporting evidence for the hydrolytic cleavage of the sulfur arsenic bonds and the oxidation of the sulfhydryl groups in alkaline solution was elicited by means of the nitroprusside test (3). When arsthinol solutions are made alkaline in the presence of nitroprusside, a red-violet color is observed indicating the presence of free sulfhydryl groups. If these solutions are acidified with a bicarbonate buffer to a pH of about 7.0 to 8.0, the nitroprusside test for the mercaptan group fails. However, if additional alkali is again added, cleavage of the linkages occurs and the nitroprusside test is again positive. This is indicative of a reversible hydrolysis of the —S—As bonds of arsthinol. In the presence of oxygen, however, this nitroprusside test weakens in intensity and in due course fails as all of the mercaptan groups are oxidized.

Table I—Air Oxidation of Arsthinol in Alkaline Solution

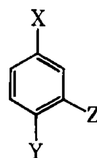
Aeration Time, min.	Molarity of Arsthinol	Initial Molarity, %
0	0.0288	100.0
10	0.0261	90.4
20	0.0234	81.2
30	0.0220	71.3
60	0.0021	7.0

Finally, as will be demonstrated below, the spectrum of $10^{-5}M$ arsthinol in aqueous alkali solution, prepared with air-saturated water, gave only the spectrum of acetarsone. This could be proved easily by acidulating these solutions, whereupon the spectrum which was obtained was that of unionized acetarsone and not that of arsthinol. In order to observe the spectrum of phenolate species of arsthinol it was necessary to use a sodium methoxide solution in anhydrous methanol. This result is exactly what would be anticipated in the light of the failure to oxidize this substance in the experiment cited previously.

Spectrophotometric Studies and Their Interpretations—Some of the spectra determined in the course of this study are presented in Figs. 3–8 and if examined before reading the following will facilitate the understanding of the interpretations.

Spectra of Acetarsone—Considering acetarsone first, it may be noted that the spectra of the H_3A, H_2A^-, and $A^=$ species (Fig. 3) were obtained by scanning solutions at pH's of about 1, 5.16, and about 13, respectively, where it will be seen (Fig. 2) that the particular species accounts for at least 98% of the formal acetarsone concentration. The spectrum of $HA^=$ was deduced from scans made on solutions with pH values in the vicinity of 8.6 by subtracting the fractional contribution of the other conjugate species present from the observed spectrum.

The spectrum of acetarsone, a trisubstituted benzene derivative, is essentially the spectrum of benzene considerably modified by the substituent groups (7).[1] It has been shown that the B and C bands (designated first primary and secondary by Doub and Vandenbelt) of compounds of the type:



[1] The absorption band nomenclature used in this discussion is that proposed by Moser, C., and Kohlenberg, A. I., *J. Chem. Soc.*, 1951, 804.

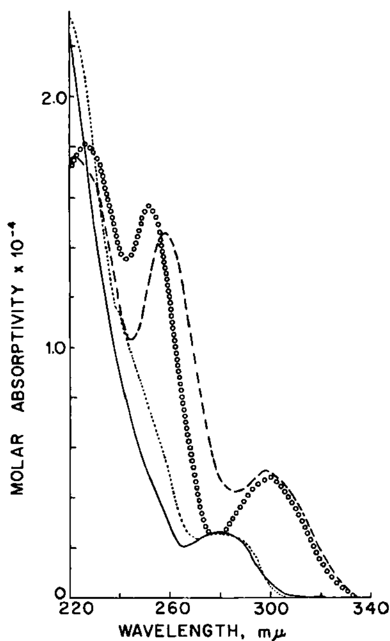


Fig. 3—Spectra of conjugate acid-base species of acetarsone in aqueous solvents. Key: · · ·, H_3A; ——, H_2A^-; - - -, $HA^=$; ooo, $A^≡$.

where Y and Z are electron donating and X is electron attracting often resemble that band which is the most displaced among corresponding bands of the three constituent disubstituted compounds.

The *o-p*-directing properties of the hydroxyl and acetamido groups are well established and their bathochromic effect on benzene bands is evident in the spectra of phenol and acetanilide. The spectrum of phenylarsonic acid (Fig. 4), on the other hand, would indicate little or no *m*- or *o-p*-directing properties for the arsonic group in all three states of protonation since the C band is very similar to that of benzene being shifted only a few mμ and increased in intensity two to five times.

However, the *m*-directing nature of the arsonic group when under the influence of a strongly *o-p*-directing group *para* to it is inferred from the shift in the B band of *p*-hydroxyphenylarsonic acid produced by the loss of the first proton (Fig. 5). The loss of the proton from an electron-attracting group would be expected to diminish its electron-attracting prop-

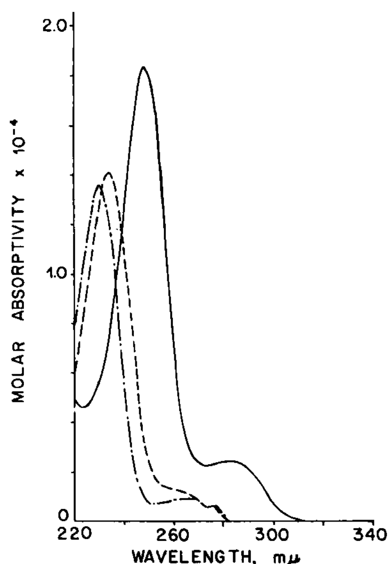


Fig. 5—Spectra of p-*hydroxyphenylarsonic acid in 0.1* N *HCl, 5% MeOH,* - - -; *pH = 6, 5% MeOH,* - · · -; *and in 0.1* N *NaOH, 5% MeOH,* ——.

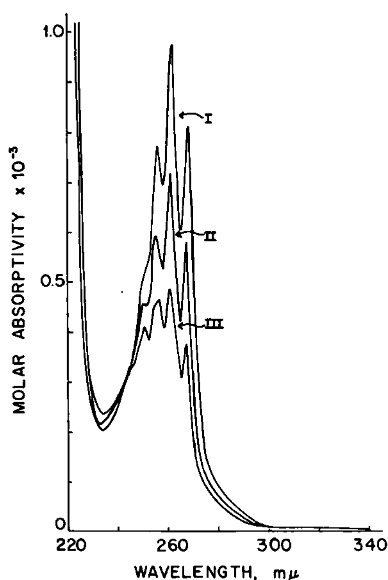


Fig. 4—Spectra of phenylarsonic acid in 0.1 N *HCl, 5% MeOH (I); pH = 6, 5% MeOH (II); and in 0.1* N *NaOH, 5% MeOH (III).*

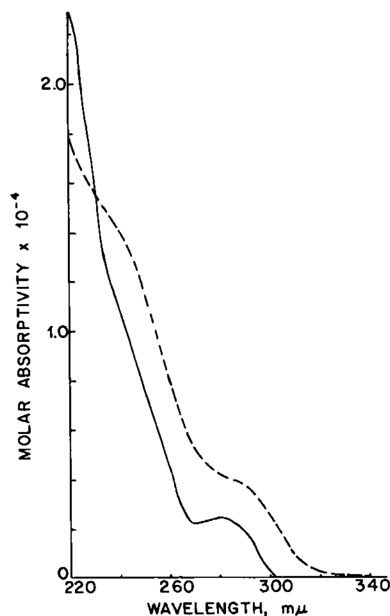


Fig. 6—Spectra of acetarsone, —— *and arsthinol,* - - - *in 0.1* N *HCl, 5% MeOH.*

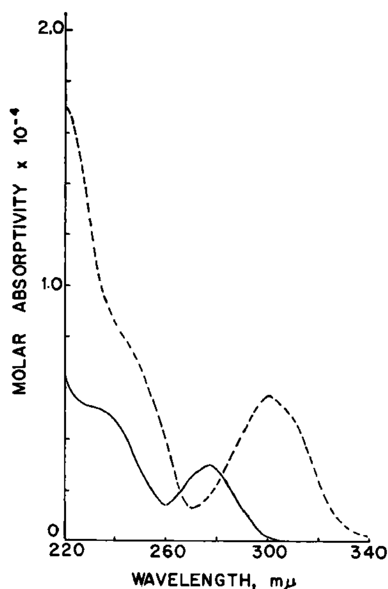


Fig. 7—Spectra of o-*hydroxyacetanilide in 0.1* N *HCl, 5% MeOH, ——; and in 0.1* N *NaOH, 5% MeOH, - - -.*

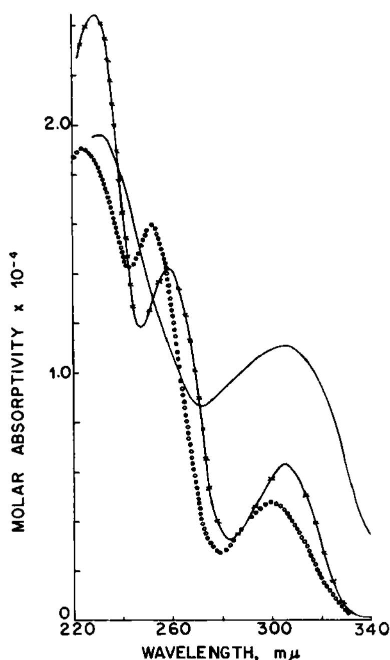


Fig. 8—Spectra of acetarsone in 0.1 N *NaOH, 5% MeOH, I:* ooo; *in 0.1* N *NaOH, 95% MeOH, II:* ×; *and of arsthinol in methanol, 0.1* N *NaOMe, III: ——.*

erties and move the band to shorter wavelengths (7).

An examination of the spectrum of the H_3A species of acetarsone (Fig. 6) reveals a C band at 280 mμ resembling the C band of *o*-hydroxy acetanilide (Fig. 7) at 278 mμ. Loss of the first proton, which undoubtedly comes from the arsonic group, produces little change in the C band but appears to have a hypsochromic effect on the B band below the spectral range investigated. Loss of the second proton produces a marked bathochromic effect on the B and C bands which are now located at 251 mμ and 300 mμ, respectively. The C band now resembles closely the band of the phenolate form of *o*-hydroxyacetanilide. This type of effect is frequently observed upon loss of a proton from an electron-donating substituent group (*i.e.*, phenol). Finally, loss of a third proton from acetarsone produces little effect on the C band but has a hypsochromic effect on the B band; analogous to that observed with the loss of the first (arsonic) proton. It would therefore seem that the second ionization step can only be associated with the phenolic hydroxyl group and the third ionization step with the remaining arsonic proton.

This finding is not completely unanticipated since Pressman and Brown (8) have postulated a similar ionization sequence for *p*-hydroxyphenylarsonic acid. They reasoned that since the analinium ion of aniline has its acidity increased by about 1.6 pK units as a consequence of having a singly ionized arsonic acid group *para* to it in *p*-aminophenylarsonic acid, the same should apply to the phenolic group in *p*-hydroxyphenylarsonic acid (pK_1 = 3.88, pK_2 = 8.37, pK_3 = 10.05) as compared to phenol.

Designation of the peak at 226 mμ in the fully ionized acetarsone spectrum as an A band or as an additional B band is uncertain. The effect of the less polar solvent, methanol, on both the 226-mμ and the 251-mμ bands is to shift them to the red about 6–7 mμ although the absorptivities of the two are affected differently (Fig. 8). This similar bathochromic solvent effect would seem to favor the designation of the higher energy band as an additional B band possibly associated with the *o*-hydroxyacetanilide portion of the acetarsone molecule. The 251-mμ band closely resembles in position and peak form, the B band of the fully ionized *p*-hydroxyphenylarsonic acid and is probably associated with that portion of the acetarsone molecule.

Spectra of Arsthinol—A consideration of the spectra of arsthinol in aqueous acid (Fig. 6) and methanolic alkali (Fig. 8) solvents reveals a greater similarity to *o*-hydroxyacetanilide than to acetarsone. The disappearance of the acetarsone B band at 257 mμ in methanolic alkali upon substitution of the cyclic 3-hydroxypropyldithioarsenous groups for the arsonic acid group indicates the different character of these groups and further supports the association of that band with the *p*-hydroxyphenylarsonic portion of the acetarsone molecule.

Analytical Applications—It is self-evident from these data that the analyst is afforded a wide range of choice in devising spectrophotometric methods of assay for these two pharmaceutical entities. If one uses anhydrous alkaline methanol as the solvent medium, the spectra of arsthinol will be that of the intact molecule. Any oxidative hydrolysis which may have occurred to it as a consequence of aging in its various pharmaceutical formulations can be readily detected by the appearance of a peak at 260 mμ due to acetarsone as well as a substantial reduction in its absorption in the 307-mμ region. Coupling this method with the iodometric titration affords corroborative data.

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Keyphrases

Acetarsone—acid-base chemistry
Arsthinol—acid-base chemistry
Ionization constants—acetarsone, arsthinol
Acid-base titration—acetarsone, arsthinol
Iodometric titration—acetarsone, arsthinol
UV spectrophotometry—analysis

Hypoglycemic Activity and Chemical Structure of the Salicylates

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The results obtained in a study undertaken to clarify both the structural requirements of salicylate hypoglycemic activity in the hope of diminishing the unpredictability associated with it and the mechanism of such activity are presented in an effort to shed further light on the pathophysiology of diabetes mellitus. Results of the structure studies point to the carboxyl group as being essential to the salicylate hypoglycemic activity, the hydroxyl radical in the *ortho* position as being beneficial but not imperative with respect to this activity. Results of the action mechanism studies involve suppression of the release of fatty acids from adipose tissues in salicylate hypoglycemic activity.

The salicylates have been used for nearly a century in the treatment of diabetes mellitus but their usefulness as hypoglycemic agents has been limited by the fact that the doses required to bring about a significant blood glucose lowering response are relatively large and the response is somewhat unpredictable in that a wide variety of effects other than the hypoglycemic effect is elicited concomitantly. The advent of insulin and the oral hypoglycemics signaled a loss of interest in the antidiabetic action of these compounds.

The effectiveness of salicylates in decreasing glycosuria and hyperglycemia in various animal preparations (1–6) and in man (7–12) is, however, well established. The discovery of the cause of their unpredictability and the mechanism of their action has not been achieved.

Salicylates diminish the hyperglycemia of some, not all, human diabetics, both of maturity-onset and juvenile types. Salicylate-induced hypoglycemia can be demonstrated in alloxan or pancreatectomized diabetic animals of different species.

In general, a fairly large dose of salicylates is required to produce significant lowering of blood sugar. In man the maximal hypoglycemic activity is reached at plasma salicylate concentrations of between 20 and 30 mg./100 ml. Salicylate levels as low as 6 to 10 mg./100 ml. have been claimed to reduce hyperglycemia effectively (13–16). The effect of salicylates to reduce hyperglycemia in the rat is not abolished by hypophysectomy (6, 17) or by bilateral adrenalectomy (5, 18).

The blood sugar response to salicylates in humans and animals is related to blood salicylate level and to initial blood glucose concentration. The hypoglycemic activity of salicylates is more

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