Requirements for the Growth of *Aspergillus versicolor* on Atropine Sulfate

By GILBERT C. SCHMIDT, HOWARD W. WALKER*, FRANK R. ROEGNER†, and CARL G. FISCHER‡

Three strains of *Aspergillus versicolor* were isolated from soil, obtained in pure culture, and grown with atropine sulfate as the sole source of carbon and nitrogen. Optimum growth was obtained at 25°, when cultures were aerated by continual shaking, and were buffered to pH 5.5 with 0.2 to 0.4 M phosphate. Optimal media contained 1.25 per cent atropine sulfate and were supplemented with vitamins and minerals. Growth rate was inhibited by higher substrate levels. Peak growth was attained in 5 days, following a 2-day lag period.

ALTHOUGH atropine is toxic for many micro-organisms, microbial growth often is observed in aqueous solutions of the alkaloid. Little is known of the contaminating organisms, of other organisms capable of utilizing this alkaloid, or of the degradative pathways that are involved.

An organism that utilized atropine, hyoscyamine, scopolamine, tropine, or tropinone as sole carbon and nitrogen sources was first reported by Bucherer (1), who classified it as *Corynebacterium belladonnae* (Nov. spec.). Eighteen years later, phenylacetic acid and tropic acid were isolated from the growth medium, and the presence of atropinesterase was reported (2). In the interim, Kaczkowski (3) had reported that *Arthrobacter terregens* produced tropine, nortropine, tropic acid, and atropic acid, when grown with atropine as the sole source of carbon and nitrogen. Niemer (2) also isolated atropic acid during his studies with *C. belladonnae*, but only traces were obtained. He reasoned that atropic acid arose by dehydration during isolation and was an artifact rather than a metabolite of atropine.

Using complete disappearance from the growth medium as the criterion for degradation, Kedzia and co-workers (4) surveyed 744 strains for ability to degrade atropine. Fifty-four strains, one of which was an *Aspergillus*, degraded the alkaloid. In all instances, ability to degrade atropine was an unstable property that was reacquired when the organism was subcultured 7 to 15 times in a meat broth medium that contained atropine.

From a soil sample, the authors have isolated pure cultures of three strains of *Aspergillus* that utilize atropine as a sole source of carbon and nitrogen. The authors intend to use these strains in studies of atropine metabolism and as a source of atropine-degrading enzymes. This report describes the identification of the three strains and the establishment of optimal growth conditions for that strain which grew most rapidly in aqueous solutions of atropine sulfate.

MATERIALS AND METHODS

Isolation of Aspergillus Strains.—Harary's method (5) for obtaining nicotinic acid-dependent organisms was modified by using atropine sulfate, rather than nicotinic acid, as the sole source of carbon and nitrogen. Three strains of *Aspergillus*, a *Penicillium* strain, and one *Sclerotium* species initially grew together in atropine solutions. Standard mycological methods (6) were used to obtain the five organisms in pure culture. The ability of each to grow in atropine solutions was then tested, and only the three *Aspergilli* utilized atropine sulfate as a sole carbon and nitrogen source. These three strains were stored on sterile soil and were repeatedly subcultured on atropine-agar slants before use.

Identification of the Organisms.—For identification of organisms, cultural and morphological characteristics, determined by standard mycological methods (6), were compared with published data (7). Mycophil agar, Czapek's agar, and Czapek's agar containing 20% sucrose[1] were used routinely. Descriptions are based on observations of colonies arising from hyphal transfers, following examination of the colonies at weekly intervals for 2 months.

Measurement of Growth.—For the measurement of growth, distilled water suspensions of spores, diluted to contain 30 mcg. of nitrogen/ml., were used for inoculation. Spore suspensions were prepared from 15-day-old second transfers on

[1] Czapek's agar is available from Difco, Detroit, Mich. Mycophil agar is available from Baltimore Biological Laboratories, Baltimore, Md.

agar slants that contained 1.5% agar, 1% atropine sulfate, and 0.025% $MgSO_4 \cdot 7H_2O$. Triplicate sets of conical flasks, each containing 20 ml. of the appropriate medium, were inoculated with 0.2 ml. aliquots of the standard spore suspension. With stated exceptions, the inoculated flasks were incubated at room temperature as stationary cultures.

At appropriate times, mats were filtered individually through previously dried and tared 8-ml. Pyrex crucibles, having fine porosity disks. Mats were washed with water until free of atropine, dried at 105° to constant weight, and progress curves were obtained by plotting mat weight as a function of duration of incubation. Each point on the progress curves was the average of at least three mat weights.

Maximum growth was read directly from the progress curves. Progress curves were extrapolated to the abscissa to estimate the time that growth was initiated. The lag phase was assumed to be that interval between inoculation and the initiation of growth. Rate of growth was determined from the slope of that tangent to the curve which passed through the time of growth initiation.

Stock Solutions and Buffers.—Reagent grade chemicals were used to prepare all solutions. Vitamin and alkaloid solutions were sterilized by filtration through a Morton bacterial filter, having an ultrafine fritted disk. All other solutions were sterilized by autoclaving for 15 min. at 15 lb. pressure.

Minerals, vitamins, and atropine as concentrated, sterile, stock solutions were added to previously sterilized buffer solutions. Phosphate buffers were used in all experiments and were 0.2 M final concentration, with stated exceptions.

For supplementation with $MgSO_4$, a final concentration of 0.025% of the hydrated salt was used routinely. When complete mineral supplements were used, 1 ml. of stock solution was added to each 100 ml. of culture medium. Each milliliter of the mineral stock solution contained 2 mg. of $FeCl_3 \cdot 6H_2O$, 2 mg. of $ZnSO_4 \cdot 7H_2O$, 2 mg. of $MnSO_4 \cdot H_2O$, and 40 mg. of $MgSO_4 \cdot 7H_2O$. These salts were selected to avoid the introduction of extraneous carbon and nitrogen sources and did not support growth in the absence of atropine or similar carbon and nitrogen source.

One milliliter of stock vitamin solution, which was added to each 100 ml. of medium when vitamins were used, containing 0.4 mcg. of biotin and cyanocobalamin, 40 mcg. of niacin, and 80 mcg. each of riboflavin, thiamine HCl, pyridoxine HCl, and calcium pantothenate. This concentration of vitamins did not support growth in the absence of atropine or other carbon and nitrogen source.

With stated exceptions, a final concentration of 1% atropine sulfate was used routinely. The alkaloid was sterilized as a 25% aqueous solution and an appropriate amount was added to previously sterilized buffer immediately before use.

RESULTS AND DISCUSSION

Identification of Organisms.—Cultural and morphological characteristics indicated that the three strains capable of utilizing atropine as a sole source of carbon and nitrogen were all strains of *A. versicolor*, differing in details of color, texture, and

pigment formation.[2] Two of the three strains were green spored, one was rough textured, and the other was smooth textured. The third strain was an unstable, asporogenous variant that represented about 2% of the total population and quickly "reverted" to one or the other of the more stable forms. Except for data concerned with identification and descriptions, results reported here were obtained with the strain that grew most rapidly on media containing atropine as the sole source of carbon and nitrogen.[2]

Growth on Czapek's agar was very slow, colonies were 25–30 mm. in diameter after 4 weeks, sporulation was delayed, and no hulle cells were produced. The colony was flat, with few radial folds, having some floccose growth in the folds or in the center of the colony. The color changed during growth from an initial white, to light gray, through shades of yellow, orange, and green to a final, dark gray green or olive green. In some variants, the different colors appeared in definite zones, while in others there were irregular patches. Droplets of pink exudate appeared in younger colonies, particularly those with a maroon reverse. The reverse was usually maroon, but it was yellow in some strains. Relatively few well-developed conidial heads occurred, but there were many conidial chains borne on mycelial branches without vesicles.

Growth on Czapek's agar with 20% sucrose was somewhat more rapid than on Czapek's agar. Colonies were 35–45 mm. in diameter after 4 weeks, there was more abundant sporulation, and hulle cells were produced. The colony was flat, with a slightly raised center of floccose mycelium. Conidial heads were almost hemispherical, becoming radiate upon longer incubation. Color of the colony was extremely variable, with different shades of gray, green, yellow, orange, and occasionally brick red. Color occurred in definite, concentric zones or with some variant types in irregularly shaped patches. When the brick-red color occurred, the vesicles and sterigmata usually had the same color as the mycelium. Hulle cells of the *A. nidulans* type were produced abundantly throughout the mycelium, and in pale yellow clusters resembling perithecia.

Growth on Mycophil agar at pH 7.0 was very rapid, and sporulation occurred after only a few days' incubation at room temperature. After 4 weeks, colonies were 45–55 mm. in diameter. Small differences in colony color, colony texture, and degree of sporulation were much more apparent on Mycophil agar than on the other media. The colony types varied from relatively smooth and flat colonies, to raised colonies with considerable radial folding, to types having predominantly flocculose growth. Color ranged through pale green, to yellow green, to the darker shades of green, depending on age of the colony. Hulle cells were abundant throughout the mycelium, but were most abundant in clusters that appeared near the outer edges of the colony. These yellow clusters resembled irregularly shaped perithecia,

TABLE I.—COMPARISON OF MORPHOLOGICAL CHARACTERISTICS[a] OF ONE ATROPINE-DEPENDENT STRAIN[b] WITH PUBLISHED DATA[c] FOR *A. versicolor*

Characteristic	*A. versicolor*	Atropine-Dependent Strain
Heads	Hemispherical to radiate. Up to 100–125 μ diam.	Hemispherical to radiate. 88–111 μ diam. Some chains of conidia borne on mycelial branches without vesicles.
Conidiophores	5–10 μ diam. Smooth	2.7–4.6 μ diam. Smooth
Vesicles	15–20 μ diam.	8.9–13.1 μ diam.
Primary sterigmata	8–9 $\times$ 3 μ	4.2–10.7 $\times$ 1.8–3.2 μ
Secondary sterigmata	5–10 $\times$ 2–2.5 μ	3–7.7 $\times$ 2.1–3 μ
Conidia	Globose, echinulate, 2.5–3 μ. Occasionally 3.5–4 μ	Globose, echinulate, 3.0–4.5 μ
Hulle cells	Up to 25 μ diam. *A. nidulans* type	Globose, thick-walled, 12.5–19.6 μ diam.
Perithecia or sclerotia	Absent	Absent. Clusters. Hulle cells resembling perithecia are present.

[a] Determined on Czapek's agar.   [b] Smooth-textured variant, A. R. S. No. A-12,238.   [c] Thom and Raper (7).

but contained only hulle cells with no evidence of asci or ascospores.

A comparison of the morphological characteristics of the green-spored, smooth-textured, atropine-dependent variant with published descriptions of *A. versicolor* (7) is shown in Table I. From the data, it was concluded that the strain, used to establish optimal growth conditions, is a variant of *A. versicolor*[3] capable of utilizing atropine as a sole source of carbon and nitrogen.

Effects of Hydrogen Ion Concentration on Growth.—Maximal and prolific growth was observed when the test organism was grown on dextrose and ammonium sulfate buffered to pH 6.5 for 5 to 7 days. In contrast, after 20 days' incubation in atropine sulfate solutions, growth of the organism was scanty. On the assumption that a change in substrate might be accompanied by a shift in the pH optimum, the effects of pH on growth rate and maximum growth were studied in an attempt to obtain better growth on atropine.

Stationary cultures were grown in liquid media buffered to different hydrogen ion levels in the pH range 5.0–8.0, flasks were processed for mat weight at 3-day intervals from 1 through 3 weeks, and average mat weights were determined for each pH at each incubation time. Progress curves were plotted, and maximum growth and growth rate were determined from the curves for each pH level. The effects of pH on growth rate and on growth maximum are shown in Table II.

Peak growth and rate of growth were maximal at pH 6.0, and rapidly declined within 0.5 pH unit on either side of the optimum. Maximum growth approximated that earlier seen in stationary cultures on glucose–ammonium sulfate at pH 6.5, but rate of growth on the atropine medium was much less than on the simple carbon and nitrogen source. This difference in growth rates caused the authors to consider vitamin and mineral supplementation.

Effects of Vitamin and Mineral Supplements.—Preliminary experiments disclosed a shift in the pH

TABLE II.—EFFECT OF pH ON MAXIMUM GROWTH AND GROWTH RATE[a]

Initial pH	Max. Growth, mg. Mat Wt.	Growth Rate, mg. Mat/Day
5.0	24.6	4.3
5.5	41.0	6.0
6.0	55.0	8.4
6.5	40.2	4.5
7.0	31.9	3.5
7.5	30.9	3.0
8.0	21.8	2.0

[a] Growth of atropine-utilizing *A. versicolor* at room temperature, in stationary cultures, 0.2 *M* phosphate buffers, 1% atropine sulfate supplemented with 0.025% magnesium sulfate. Data taken from individual growth curves, as described in text.

optimum to pH 5.5, increased growth, and increased growth rate in supplemented media. These observations were confirmed by studying the effects of supplements at both pH 5.5 and pH 6.0.

Growth at both hydrogen ion levels, in solutions of atropine supplemented with magnesium, minerals, vitamins, and vitamins plus minerals was measured over 3 weeks, in the manner previously described. Throughout this period, growth on mineral, vitamin, and vitamin–mineral controls was insignificant (2–3 mg.). From the individual growth curves, maximum mat weights and growth rates were determined for each of the eight test media, and are shown in Table III.

Significant increases in maximum mat weight and the growth rate were observed in media supplemented with minerals or vitamins plus minerals. These increases were accompanied by a shift in the pH optimum from 6.0 to 5.5. Lysis was much more rapid at the higher hydrogen ion level, as is apparent from the two progress curves shown in Fig. 1. In formulating the "optimal" medium, vitamins were arbitrarily included, although their addition did not produce growth increases significantly greater than those produced by mineral supplements alone.

Effects of Temperature on Growth.—In early experiments, cultures were incubated at 25° because this temperature is recommended (8) for the growth of *A. versicolor* on simple carbon and

TABLE III.—EFFECT OF SUPPLEMENTS ON MAXIMUM GROWTH AND GROWTH RATE AT pH 5.5 AND pH 6.0[a]

Supplement Added	Max. Growth, mg. Mat Wt.		Growth Rate, mg. Mat/Day	
	pH 5.5	pH 6.0	pH 5.5	pH 6.0
Magnesium sulfate	40.1	48.8	6.4	8.0
Minerals	56.3	45.9	9.5	7.8
Vitamins	21.6	11.1	1.9	1.6
Vitamins plus minerals	59.4	56.0	10.0	8.0

[a] Growth in stationary cultures, room temperature, 0.2 M phosphate buffers, 1% atropine sulfate solution, supplemented as indicated and as described in the text. Growth rates and maximum growth were determined from individual growth curves.
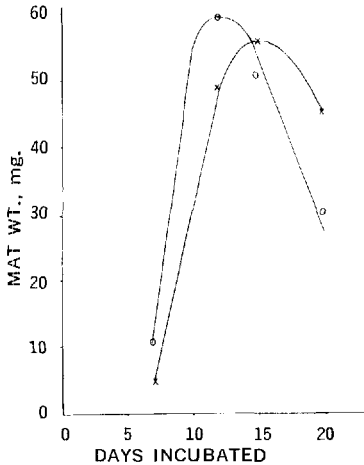

Fig. 1.—Growth of *A. versicolor* on optimal media adjusted to pH 5.5 and pH 6.0. Growth in stationary culture at 25°, 0.2 M phosphate buffers, 1% atropine sulfate supplemented with both minerals and vitamins. Key: O, pH 5.5; and ×, pH 6.0.

nitrogen sources. Since atropine is a complex carbon and nitrogen source, temperature effects on growth of the atropine-dependent strain were studied.

Using stationary cultures and optimal media adjusted to pH 5.5, growth curves were plotted for incubations at 0°, 8°, 20°, 25°, 30°, and 36°. Maximum mat weights, determined from the individual growth curves, were then plotted as a function of incubation temperature, as shown in Fig. 2.

It is apparent from the curve that there was a sharp temperature optimum at 25°, as reported for *A. versicolor* grown on simple carbon and nitrogen sources. Thus, the change from simple carbon and nitrogen sources to atropine did not affect the optimum incubation temperature.

Effects of Aeration.—Because fungi usually require good aeration for maximum growth, the effect of aeration by shaking was investigated. Two sets of flasks, one set incubated without shaking and the other shaken continuously on a rotary shaker, were incubated under otherwise identical conditions. At 2-day intervals or less, flasks were removed from each set in quadruplicate and were processed for mat weights. The results obtained at different time intervals, for shaken and stationary cultures, are compared in Table IV.

Aeration caused a striking increase in both growth

rate and maximum mat weight, the lag phase was reduced from 6 days to 2 days, and maximum mat weights were 50% higher in shaken cultures. These conditions appeared to be nearly optimal because total mat weights, and the incubation period required, were almost identical with those observed in early experiments in which simple carbon and nitrogen sources were supplied for the organism.

Effect of Phosphate Concentration.—Because the *Aspergilli* usually grow best at phosphate levels between 0.01 and 0.05 M, the possible toxic effects of the phosphate concentrations used in development of the optimal medium were considered.

Cultures were grown 4 days, with continuous shaking at pH 5.5 and 25°, on media that were optimal except for varying phosphate concentrations. Four flasks for each of the following phosphate concentrations were employed: 0.00, 0.01, 0.02, 0.05, 0.10, 0.20, 0.30, and 0.40 M. Average mat weight was plotted as a function of phosphate concentration, as shown in Fig. 3.

Growth on atropine was very slight at phosphate levels below 0.15 M and increased with increasing phosphate, through the highest phosphate concentration tested. Since incubation time was constant and had not reached the optimum, maximum mat weights would not necessarily follow the same pat-
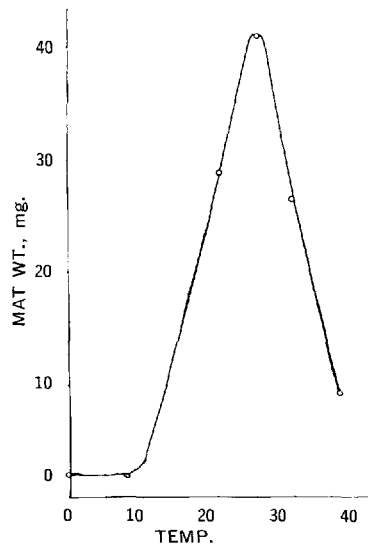

Fig. 2.—Effect of temperature on growth of *A. versicolor*. Growth on optimal medium, stationary cultures, pH 5.5. Maximum mat weight obtained from individual growth curves.

TABLE IV.—COMPARATIVE GROWTH OF *A. versicolor* IN SHAKEN AND STATIONARY CULTURES[a]

Incubation Time, Days	Max. Mat Wt., mg.	
	Shaken Cultures	Stationary Cultures
0	1.2	1.3
2	12.0	2.5
4	39.5	4.8
6	65.0	20.2
7	82.6	30.0
8	79.0	48.7
10	51.7	55.0

[a] Growth on optimal medium at pH 5.5, 0.2 M phosphate buffers. Each point is the average of 4 individually determined mat weights.
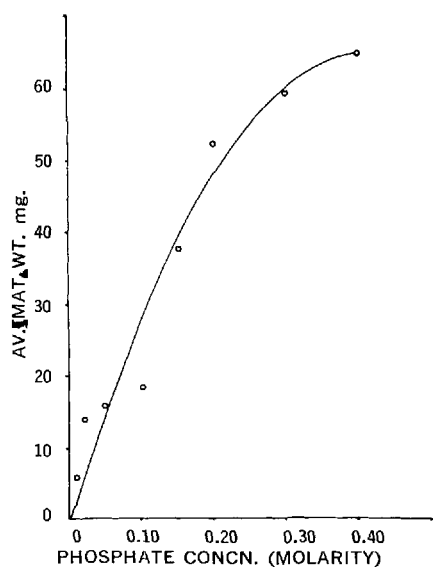

Fig. 3.—Effect of phosphate concentration on growth of *A. versicolor*. Growth for 4 days, continuous shaking, pH 5.5, on media that were optimal except for variations in phosphate levels. Each point is the average of four mat weights.

tern. In more prolonged incubations, the relative rates of growth and lysis would have an important influence on observed mat weights. Lysis was especially apparent in cultures grown in 0.3 and 0.4 M phosphate.

In contrast to the growth of *A. versicolor* on simple carbon and nitrogen sources, phosphate requirements are unusually high when atropine is the sole carbon and nitrogen source. This observation is unexplained, but may be associated with the complexity of the substrate, or with increased requirements for nucleic acid synthesis. More extensive studies would be required to clarify this point.

Growth as a Function of Alkaloid Concentration.— Unequivocal proof of atropine utilization by the strain of *A. versicolor* used in these experiments was obtained by relating growth to concentration of the alkaloid, in the absence of other sources of carbon and nitrogen.

Atropine concentrations were varied from 0.06 to 2.0%, using stationary cultures and otherwise

optimal media and conditions. To obtain growth rate directly from mat weight, cultures were incubated for 12 days, at which time growth was submaximal and was increasing linearly with time. Growth rates, obtained by averaging triplicate mat weights at each atropine level, were plotted as a function of alkaloid concentration, as shown in Fig. 4.

There was a linear increase in growth rate throughout the concentration range 0–0.5% atropine, a maximum at 1.25%, and a sharp decrease beyond this point. In the absence of other carbon and nitrogen sources, the linear relationship between growth rate and atropine level confirms the ability of this strain of *A. versicolor* to utilize atropine as a sole carbon and nitrogen source. That growth rate is inhibited by high substrate levels is indicated by the existence of a maximum.

SUMMARY AND CONCLUSIONS

Three soil fungi, capable of utilizing atropine as a sole source of carbon and nitrogen, were obtained in pure culture, identified, and the identifications then independently confirmed. Although cultural and morphological characteristics differed somewhat from published data, all three were identified as strains of *A. versicolor*, differing in details of color, texture and pigment formation. These differences were far more apparent on the Mycophil agar used than on other media.

Optimal growth conditions, when atropine sulfate was the sole source of carbon and nitrogen, were determined for that strain which initially grew most rapidly in solutions of atropine supplemented with magnesium. For maximal growth, supplementation with vitamins and minerals was required, although the omission of vitamins had little effect. Using the supplemented medium, the organism grew best at 25° and pH 5.5. Aeration during growth greatly increased growth and decreased the induction period. Phosphate requirements were unusually high, good growth occurring at 0.2–0.4 M phosphate, but essentially none occurring below these levels.
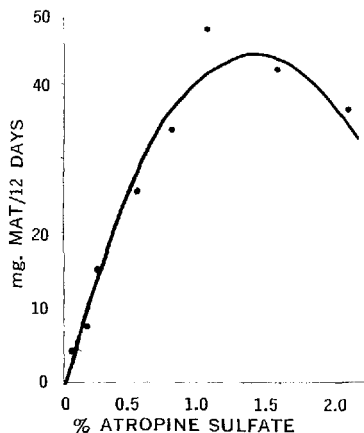

Fig. 4.—Growth of *A. versicolor* as a function of atropine concentration. Optimal media, pH 5.5, 0.2 M phosphate, stationary cultures. Each point is the average of three individually determined mat weights.

The need for high phosphate concentrations was not explained. Growth was optimal at an atropine concentration of 1.25% and was inhibited by higher concentrations of alkaloid.

The details of atropine utilization are being studied and will be reported at a later date, but there is evidence that an atropinesterase is involved. There is also evidence that growth requirements are quite specific for atropine, hyoscyamine, or their hydrolysis products.

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Analysis of Steroids in Mixtures Using the Kinetics of Blue Tetrazolium Reduction

By DAVID E. GUTTMAN

The rate of formation of formazan resulting from the base-catalyzed reduction of blue tetrazolium by certain steroids was studied spectrophotometrically. With cortisone and hydrocortisone, the reaction rate exhibited a first-order dependency on steroid concentration. The rate constant for cortisone-containing systems was significantly larger than that found for hydrocortisone systems. Studies with cortisone acetate showed that hydrolysis of the ester was prerequisite to reaction with the tetrazolium salt. Differences in rates of color development were used to analyze mixtures of cortisone and hydrocortisone and of cortisone and cortisone acetate.

A WIDELY USED colorimetric method for the determination of the purity of corticosteroids and the potency of dosage forms containing such steroids is based on the formation of a colored formazan resulting from the base-catalyzed reduction of blue tetrazolium by the α-ketol side chain of the steroid molecule. The method consists of determining, after a specified time period, the intensity of color in a test preparation and comparing it with that produced under similar conditions in a standard preparation of the steroid under consideration. There have been a number of published studies of the rates of color formation in systems containing reducing steroids and blue tetrazolium and it has been observed that closely related steroids can exhibit significant differences in their rates of reaction with the tetrazolium salt. For example, Chen, Wheeler, and Tewell (1) presented data which suggested that color generation in cortisone-containing systems was much more rapid than in systems containing hydrocortisone. Meyer and Lindberg (6), in their extensive study, showed that the position and configuration of certain keto- and hydroxy-groups in the steroid molecule influenced reducing characteristics. Recknagel and Litteria (7) also demonstrated differences in reaction rate by

their determination of the optimum incubation times for maximum color development for various steroids; *i.e.*, 30 min. for cortisone and 11-deoxycorticosterone as contrasted to 50 min. for corticosterone and hydrocortisone. Similarly, Izzo, Keutmann, and Burton (3) reported that reducing steroids with an 11-keto group developed maximum color faster than those with an 11-hydroxyl group. Martin and Salvador (5) found that acetylation of the 21-hydroxyl group decreased reaction rate relative to the parent alcohol, while Johnson, King, and Vickers (4) reported that with hydrocortisone hemisuccinate, and triamcinolone, color development was unusually slow compared to other steroids.

It has not been generally recognized that, under the conditions usually employed in the assay procedure where the steroid is present at a much lower concentration than that of the catalyst and the tetrazolium reagent, the rate of appearance of formazan exhibits a first-order dependency on the steroid concentration. This behavior is of potential analytical utility because of the lack of direct methods for analyzing mixtures of closely related steroids and because certain steroids which might be found or combined in mixtures do exhibit differences in the rate of this reaction. The present study was conducted to test the feasibility of utilizing such differences as the basis for analytical methods for the determination of steroids in mixtures. Toward this end, this laboratory has studied, under closely