Table IV. Effect of Inhibitor and Substrate Concentration on Hydrolysis of Casein by Trypsin

	A. Inhibitor Absent						B. Inhibitor Present					
Casein concentration, S, %	5	4	3	2	1	0.5	5	4	3	2	1	0.5
Absorbance at 620 mμ after 2 hours at 37° C., V	0.238	0.237	0.201	0.196	0.178	0.123	0.133	0.115	0.102	0.112	0.095	0.068
$1/S$	0.20	0.25	0.33	0.5	1	2	0.20	0.25	0.33	0.5	1	2
$1/V$	4.2	4.2	5.0	5.1	5.6	8.1	7.5	8.7	9.8	9.0	10.5	14.7

indicates that the inhibition was noncompetitive.

Chemical Nature of Inhibitor. Because the active principle was precipitated from water by dilution with a relatively large volume of ethyl alcohol, it was thought that it might be inorganic in nature. However, this was shown not to be true when a portion of the isolated substance was ashed and the residue was found to be noninhibitory.

The inhibitor probably is not a heat-coagulable protein, as coagulable material was eliminated during isolation. However, it could be a polypeptide with a sufficiently low molecular weight not to coagulate with heat. This possibility was investigated by refluxing 5 mg. of the isolated material with 6N hydrochloric acid overnight. The hydrochloric acid was removed by repeated distillation at reduced pressure at 40° C. The final residue was dissolved in 1 ml. of water and 25 μl. were chromatographed on Whatman No. 1 filter paper, using 8 to 2 phenol-water as the developing solvent.

When the paper was sprayed with ninhydrin, several sharply defined spots were observed. A similar chromatogram of unhydrolyzed inhibitor gave no spots with ninhydrin. Hence, the spots obtained with the hydrolyzate were due to amino acids released during hydrolysis.

Although the data suggest the inhibitor is a polypeptide, it is recognized that the amino acids might have arisen from hydrolysis of a polypeptide impurity. Further investigation of its purity will be needed before it can be characterized unequivocally.

Discussion

It is necessary to include adequate blanks when assaying various preparations for inhibitory activity by the method employed in this work. Water extracts of alfalfa contain free amino acids, and a correction for them must be made when enzymic hydrolysis of casein in the presence of an extract is used to measure the degree of inhibition. Furthermore, extracts of dehydrated plant tissue are amber in color. Often the intensity of this color is sufficient to cause appreciable light absorption at the wave length used to measure the blue color of the amino acid–copper complex.

Inspection of Table II will indicate the variability of blanks. Both casein and trypsin are essentially devoid of free amino acids, and a very low absorbance was obtained in the boiled trypsin experiment. When the plant extract was added, a marked increase in absorbance of the blank resulted. The addition of 4 mg. of the isolated inhibitor had essentially no effect on absorbance. Hence, dialysis and ethyl alcohol precipitation eliminated the free amino acids and much of the substances responsible for the amber color.

Isolation of the trypsin inhibitor will permit in vivo studies to determine whether it is involved in chick growth depression. If the inhibitor were altered while in the crop or gizzard, it might no longer inhibit trypsin in the intestinal tract. Crude extracts are not suitable for such tests, because other constituents of alfalfa are known to inhibit growth (*5*).

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MEASUREMENT OF URINARY ANDROGENS

The Effect of Interfering Chromogens in Spectrophotometric Determination of Ruminant Urinary Androgens

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As has been demonstrated, androgenic 17-ketosteroids (17-KS) frequently appear in the urine, originating in most mammals as steroid metabolites of either the testes or adrenal glands (*3*). Of these compounds, androsterone [androstane-3 (α)-ol-17-one] and dehydroepiandrosterone [Δ^5-androstene-3(β)-ol-17-one], or androstenolone, as recently suggested (*4*), are not only produced in the greatest amounts, but are also among those which are the most active biologically.

The quantitative determination by various spectrophotometric methods of these and related compounds, all of which are frequently referred to as "urinary androgens," has classically been interpreted as a measure of testicular and/or adrenal cortical function.

Among the spectrophotometric methods are the Zimmerman (*5*), Pincus (*9*), and Allen (*1*) reactions, each of which produces chromogens having characteristic absorption maxima in the visible wave length range. These reactions have been employed by many workers to study the 17-KS content of urines isolated from a variety of subjects (*3*). However, certain nonsteroid ketones as well as non-17-KS present in biological materials probably confound the interpretation of results obtained as a consequence of using the chemical methods of 17-KS assay.

Only relatively recently has the probable nature of at least one group of these compounds been recognized (*6*) as ionone derivatives. In addition, cow urine has been shown to contain a group of chromogens (*7*) which very probably are ionone derivatives arising from dietary carotenoids. These compounds are ex-

The presence of nonsteroidal ketones and non–17-ketosteroid chromogens in biological materials, particularly urine of ruminants, has been recognized for some time. The possible extent of interference of these materials in the spectrophotometric determination of 17-ketosteroids remains to be defined completely. This investigation indicates that: The effect of these urinary chromogens is to displace toward the shorter wave lengths the absorption maxima of the chromogens produced by treating known 17-ketosteroids according to the Allen, Pincus, and Zimmerman reactions; the effect of the urinary chromogens in each of these reactions is to produce an intensity of color equal to or greater than that produced by similar treatment of known 17-ketosteroid alone; and the absorption maxima produced by urinary chromogens in the presence of known 17-ketosteroid are due to an apparent additive effect and in every case are higher than that produced by an equal amount of known ketosteroid alone.

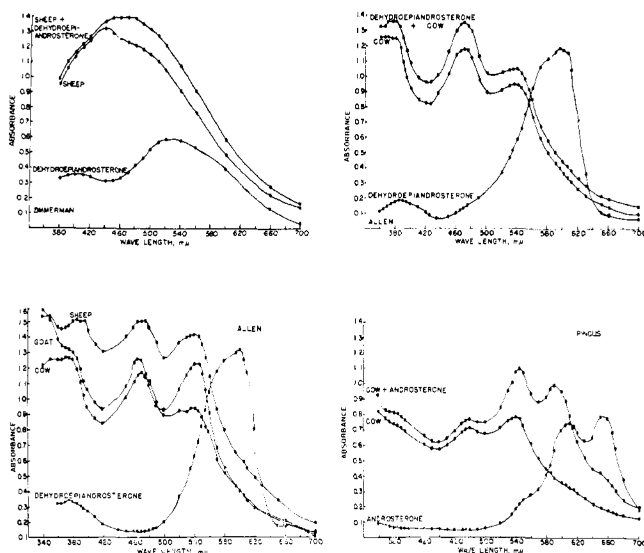

Figure 1. Distinctive and reproducible curves, each containing characteristic maxima, result when each of the three types of reactions (Allen, Pincus, and Zimmerman) is conducted in the presence of neutral extracts of urine plus known amounts of pure 17-KS

tractable by the same procedures as those used for the isolation of urinary androgens, but give different spectra when subjected to the conditions of the Allen, Pincus, and Zimmerman reactions.

This paper presents data which indicate that non–17-KS chromogens not only confound spectrophotometric determinations of urinary androgens, but their presence may actually enhance the response of the very small amounts which may be inherently present, or which are intentionally added.

The experimental procedures and/or results described herein are not claimed to be amenable to practical application. However, the results do have an important bearing on the general problem of recognizing that even standard and well-accepted bench procedures may have certain pitfalls which need to be recognized by workers in a variety of fields.

The mechanism of the effect of crystalline steroid hormones and of certain hormonally active synthetic compounds on endogenously produced hormones and on tissue anabolism needs considerable clarification. The natural products whose level in biological media the methods described in this paper are presumed to measure, play an important role not only in the maintenance of most mammals, including livestock, but also in the production of certain edible products derived from them.

Methods

Neutral extracts of urine were prepared according to the acid hydrolysis method of Mixner (*7*), employing 15% hydrochloric acid (v./v.) and a 10-minute boiling period.

The color-developing reactions employed in this study were those of Zimmerman, as modified by Holtorff and Koch (*5*), Pincus (*9*), and Allen (*1*). Five milliliters of urine equivalent were used in the Zimmerman reaction, 10 ml. in the Pincus reaction, and 5 ml. in the Allen reaction. A Beckman DU spectrophotometer was used for all measurements.

Table I. Chick Comb Bioassay of Androgens

Steroid Treatment, γ	*Comb Weight, Mg. / Body Weight, G.*
Control	0.61
Androsterone, 28	1.17
Androsterone, 56	1.49
Androsterone, 84	1.58
Unknown I	0.60
Unknown II	0.55

Biological assays to determine the androgenic potency of the neutral extracts of urine were performed by employing the chick comb test as modified by Munson (*8*). Reference standards and unknowns were dissolved in absolute alcohol. Three different levels, 4, 8, and 12 γ of the standard (androsterone), were applied daily for a 7-day period. The unknowns were neutral extracts of two different wether urines applied in amounts equivalent to 10 ml. of urine. Absolute ethyl alcohol was used as the control. Each group of day-old chicks contained 10 individuals of approximately equal size.

Ovine urine collection was accomplished by modifying the apparatus of Dick and Mules (*2*). Instead of using a specially manufactured rubber collection bag, a hemisphere made of an ordinary plastic beach ball was employed. Bovine urine collection was accomplished by conventional methods.

Results

The absorption spectrum produced by the Zimmerman reaction (Figure 1, upper left) treatment of dehydroepiandrosterone is characterized by a maximum at approximately 520 mμ. In the presence of neutral extracts of ovine urine, the maximum becomes broadened and undergoes a shift to 440 mμ. When the reaction is conducted in the presence of neutral extracts of urine to which known amounts of pure 17-K S(65

γ) have been added, the maximum is fairly broad and still displaced to 440 to 470 mμ.

The absorption spectrum of the Allen reaction (Figure 1, upper right; lower left) in the presence of dehydroepiandrosterone (65 γ) demonstrates a remarkably distinct and prominent maximum at 600 mμ and a much less prominent absorption at 380 mμ. In the presence of neutral extracts of bovine urine, this reaction demonstrates absorption maximum at 370, 465, and 540 mμ, respectively (Figure 1, upper right, lower left). These same effects are noted when neutral extracts of wether and goat urine are employed (Figure 1, lower left). When the reaction is performed in the presence of a neutral extract of bovine urine to which has been added 50 γ of dehydroepiandrosterone (Figure 1, upper right), the urinary chromogens completely obscure the maxima characteristic of the steroid alone, but are still consistent with the urinary, neutral extract spectra.

The absorption spectrum of the Pincus reaction (Figure 1, lower right) in the presence of androsterone (150 γ) demonstrates a prominent double peak, the first wave of which is at 610 mμ and the second, a slightly higher wave, at 660 mμ. In addition, there is a slight, although definite, indication of absorption occurring at 545 mμ. When this reaction is performed in the presence of a neutral extract of bovine urine, two obvious maxima located at 470 and 540 mμ are discernible. When a mixture of a neutral extract of bovine urine and known 17-KS is subjected to this reaction, three distinct maxima at 470, 545, and 590 mμ, respectively, become obvious. In addition, there is a faint, but again a definite, peak at about 650 mμ.

Biological assays (Table I), employing the chick comb test indicate that neutral extracts of wether urine contain few, if any, androgenic ketones, despite the characteristic, albeit displaced, maxima demonstrable by spectrophotometric methods.

Discussion

In the case of the Zimmerman reaction, interfering materials in the neutral extracts of urine largely displace the absorption maximum characteristic of a known 17-KS (dehydroepiandrosterone) in the presence of this reaction. In the case of the Allen reaction, the prominent maximum occurring at 600 mμ, when pure steroid (dehydroepiandrosterone) is subjected to the reaction conditions, completely disappears when the neutral urine extract containing steroid is treated likewise. In the case of the Pincus reaction, not only is there apparent displacement of the maxima which are characteristic of the pure steroid (androsterone) in the presence of this reaction, but also virtually complete disappearance of the second prominent wave at 660 mμ.

These data indicate that the characteristic absorption spectra may be obtained employing the Allen, Pincus, and Zimmerman reactions in the presence of pure 17-KS, neutral extracts of urine, and neutral extracts of urine to which have been added known amounts of pure 17-KS. In each case, however, the absorption spectra, although characteristic, are different. Moreover, unidentified compounds, normally present in bovine, ovine, and goat urine, not only may be responsible for the shifts in maxima which have been demonstrated, but also may serve to enhance or otherwise confound the effect of small amounts of 17-KS present either normally or as a consequence of intentional addition. Thus, it would appear that small amounts of endogenous 17-KS would not be detectable in neutral extracts of ruminant urine which also contain these interfering substances, when subjected to the reaction conditions of any of these methods.

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FUNGICIDE EVALUATION

Fungicidal Activity of Some New Amino Alcohols Synthesized from Citrus (+)-Limonene

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THE SYNTHESIS of several new amino alcohols from (+)-limonene has been reported (*2*). Partial hydrogenation involving the exocyclic double bond of (+)-limonene followed by oxidation of the endocyclic double bond with peracetic acid afforded *p*-menthane-1,2-epoxide. The epoxide ring was readily cleaved by ammonia to give the mixed trans isomers of 2-amino-1-*p*-menthanol (I). Cleavage of the epoxide by methylamine and dimethylamine afforded the mixed trans isomers of 2-methylamino-1-*p*-menthanol (II) and 2-dimethylamino-1-*p*-menthanol (III), respectively. Since this work was reported, an additional new amino alcohol, 2-dimethylamino-$\Delta^{8(10)}$-*p*-menthen-1-ol (IV), has been prepared by cleavage of $\Delta^{8(10)}$-*p*-menthene-1,-2-epoxide by dimethylamine. This series of amino alcohols was originally prepared for animal testing with the hope that some of the compounds would show physiological activity. No such activity was observed for any member of this series.

A preliminary investigation of these compounds as fungicides, for sanitizing of food processing plants, was investigated after becoming familiar with the properties of another derivative of (+)-limonene, tetrahydrocarvone (*1, 3*). It bore a menthollike odor and, in dilution, the odor resembled other compounds used for sanitizing purposes. The amino alcohols—I, II, III, and IV—although not as pleasant in odor, were also investigated as possible fungicides.

Experimental Procedure

Fungicidal Tests on Gauze Pads. Emulsions of the compounds to be tested were prepared in a 1 to 10 dilution with 2 drops of Tween 80 to ensure