not have been so obvious if the starch analyses had been performed iodometrically, since some of the samples show an increase in amylose. Also, on December 1, when the effects of lateral budding would be most pronounced because of mild temperatures, the drop in total starch as shown in Table II would not have been evident iodometrically for varieties CP 55-30 and L 60-1 since there was a substantial increase in the amylose fraction of the starch.

The iodometric method of starch determination, which has found broad acceptance and application in sugar cane research, is not reliable under the conditions previously cited, especially when analyzing raw juice from sugar cane which was grown domestically. The anthrone method has been found suitable for sugar cane starch work, producing results which are completely independent of any changes in starch composition. Limited preliminary experiments (unpublished) have shown this method to be adaptable to refinery liquors as well as raw juices, and in both applications reproducibility has been excellent.

Acknowledgment

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RESIDUE ANALYSIS

Spectrophotofluorometric Determination of Reserpine Residue in Poultry Tissues

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The fluorescence induced by the reaction of reserpine with nitrous acid has been used for its determination in poultry products. A cleanup procedure eliminates interfering compounds and other biological constituents. The method is sensitive to about 0.2 μg. of reserpine and will determine in the range of parts per billion, utilizing the spectrophotofluorometer. The accumulation of reserpine in poultry tissues and in eggs of birds on a medicated diet containing recommended levels is "relative zero" or nondetectable.

RESERPINE has proved effective as a stress - ameliorating agent for chickens, and is used to control and prevent aortic rupture in turkeys (*1*). It is available commercially as Serpasil Premix 0.08% in a carrier of confectioner's sugar and soybean feed (*2*). Reserpine is usually incorporated in feed at a level of 1 to 2 parts of reserpine per million for layers and broilers, and as little as 0.2 p.p.m. to aid in the prophylaxis and treatment of internal bleeding in turkeys. Considerations of human health made it necessary to determine whether significant amounts of reserpine residue were present in the edible parts and eggs of birds receiving a diet containing reserpine in recommended levels. For this reason, an investigation was undertaken to provide a method for the estimation of reserpine in poultry tissues and eggs in submicro quantities. Because of the low treatment levels involved, extremely sensitive, as well as specific, analytical procedures were required for its determination in biological material.

The extreme sensitivity of fluorescent measurements suggested an investigation of this technique. Sheppard, Wagle, and Plummer (*8*) noted that reserpine produced fluorescence in solutions of sulfuric acid and carboxylic acids, and in solutions of chlorinated hydrocarbons after exposure to ultraviolet light. Glazko and colleagues (*3*) found that solutions of reserpine in ethylene dichloride became strongly fluorescent in the presence of trichloroacetic acid and a small amount of nitroprusside. The latter was utilized by these authors to study the metabolism of reserpine in white rats, dogs, and monkeys. Poet and Kelly (*7*) described a fluorometric procedure suitable for determining reserpine in blood and urine, which is based on heating solutions of reserpine in sulfuric acid containing selenious acid. A modification of this method was utilized by Hess, Shore, and Brodie (*5*) to study the persistence of the drug administered in a large dose to rabbits. The current method (*9*) used by regulatory agencies for the analysis of medicated feeds is based on nitrous acid–induced fluorescence. This paper is a continuation and extension of the nitrite reaction to the determination of reserpine in parts per billion in biological material utilizing the spectrophotofluorometer.

Method Development

Although the nitrite technique was finally adopted for this work, a survey of the fluorescent response of reserpine in various reagents was first undertaken in an effort to ascertain the method of maximum sensitivity.

A stock solution was prepared containing 10 μg. per ml. of reserpine in methanol. Measurements were made at

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a dilution of 1 μg. per ml. in the reagents.

For each sample, the wave length of maximum excitation as well as the fluorescent peak was determined on the Aminco-Bowman spectrophotofluorometer with a quartz cell having a 1-cm. light path using a $^1/_{16}$-inch defining slit (band pass 1 cm.) and an RCA 1P21 multiplier phototube.

Under these conditions, reserpine has an uncorrected excitation maximum at 280 mμ and a corresponding fluorescent maximum at 360 mμ. The excitation and fluorescent scan of a solution of reserpine in aqueous acid is shown in Figure 1. The excitation spectrum is represented by a plot of the activating wave length against the intensity of the fluorescent light at 360 mμ. The fluorescent spectrum represents the relative intensity of the fluorescent light when excited at 280 mμ. The spectra were recorded with a Mosely Model No. 3 flat bed X - Y recorder. Although the spectra in all reagents were essentially the same, the order of magnitude varied as indicated in Table I. A linear response was obtained for standard solutions of reserpine in citric acid in concentrations between 0.5 and 5 μg. per ml. of final solution; however, in tissue-recovery experiments, results were not reproducible from day to day, apparently because of interference by other biological constituents at this wave length.

Previously, it was determined by the authors (*6*) that the fluorogen formed with nitrous acid has a fluorescence peak at 510 mμ when excited at 390 mμ, the strongest absorption band. The activation and fluorescent spectra obtained with trichloroacetic acid containing nitroprusside, and sulfuric acid with selenious acid, are the same as that obtained with nitrous acid. However, the fluorescent emission is only one half as great as that induced with nitrous acid. Nevertheless, the concordant excitation and fluorescent spectra of the fluorochromogenic compounds produced by nitroprusside, selenious acid, and nitrous acid suggest the formation of the same compound. Typical curves appear in Figure 2.

Table I. Fluorescence of Reserpine in Various Reagents

Reagent	Fluorescent Intensity
Acetic anhydride, 2%	100
Citric acid, 2%	98
Acetic acid, 2%	94
Phosphoric acid, 0.1*N*	91
Acetic anhydride, 2% with H_2O_2	90
Sulfuric acid, 0.1*N*	75
Isopropylamine, 2%	65
Perchloric acid, 0.1*N*	60
Trichloroacetic acid, 2%	26

In a prior publication (*4*) it was established that 3-dehydroreserpine has a fluorescence peak at 510 mμ when activated at 390 mμ, whereas the maximal fluorescent emission of another oxidation product, tetradehydroreserpine, was found at 440 mμ when excited at 340 mμ. Thus the fluorophor induced with nitroprusside, selenious acid, or nitrous acid is apparently the result of oxidation to 3-dehydroreserpine. The lower intensity of the fluorescence produced by nitroprusside and selenious acid is possibly due in part to incomplete oxidation. A plot of the fluorescent intensities induced with nitrous acid *vs.* concentrations of reserpine is linear in the range of 0.02 to 0.1 μg. per ml. of final solution, as shown in Figure 3. Although similar values were obtained for different experiments using the same concentration of reserpine, there were significant differences, indicating that a standard must be run with each series. The reaction is enhanced in methanolic solutions of 40% or greater.

Analytical Procedure

The following procedure is a modification of the fluorometric nitrite method for feeds (*9*) utilizing the spectrophotofluorometer for quantitation.

Apparatus. The manufacturer's name is given for some materials solely for identification. All glassware and equipment which come in contact with the sample must be scrupulously clean and should be washed with chloroform and/or methanol, and dried prior to assay.

Complete all assays in one day and avoid exposure of chloroformic solutions of reserpine to light. To prevent absorption of reserpine by glass, coat all separatory funnels with Desicote (Beckman Instruments, Inc., Fullerton, Calif.).

Reagents. All reagents are reagent grade unless specified otherwise.

2,2,4-Trimethylpentane. Purify by shaking with sulfuric acid until the extracts are practically colorless, and wash with water until washings are neutral to litmus paper. Dry the solvent with silica gel and redistill between 98.5° and 100° C. Recently, Merck reagent grade *n*-hexane has been found satisfactory without further purification.

Reserpine Standard Solution (1.0 μg. per ml.). Dissolve 25 mg. of reserpine in chloroform and dilute to 100 ml. (Solution 1). Dilute 4 ml. of Solution 1 to 100 ml. with chloroform (Solution 2). Dilute 10 ml. of Solution 2 to 100 ml. with chloroform (Solution 3). Prepare daily.

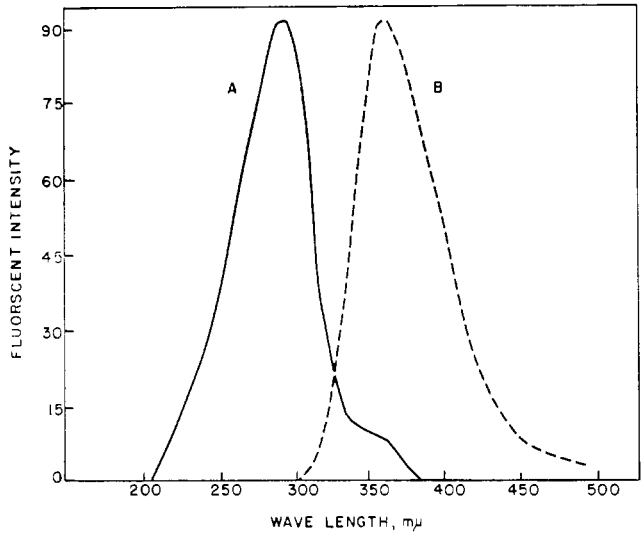

Figure 1. Fluorescent excitation and emission spectra of reserpine in citric acid

Reserpine concentration, 3.2×10^{-6} mole per liter
A. Excitation band for fluorescence measured at emission maximum, 360 mμ
B. Emission band produced at excitation maximum, 280 mμ

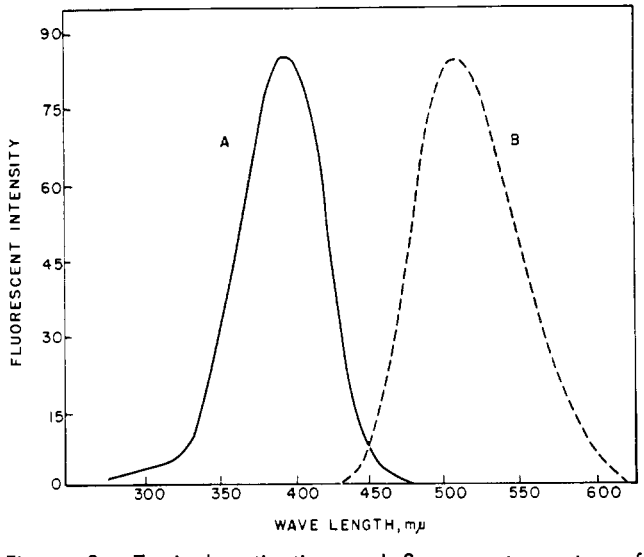

Figure 2. Typical activation and fluorescent spectra of reserpine oxidized by nitroprusside, selenious acid, or nitrous acid

A. Activation scan measured at fluorescent maximum, 510 mμ
B. Fluorescent scan excited at activation maximum, 390 mμ

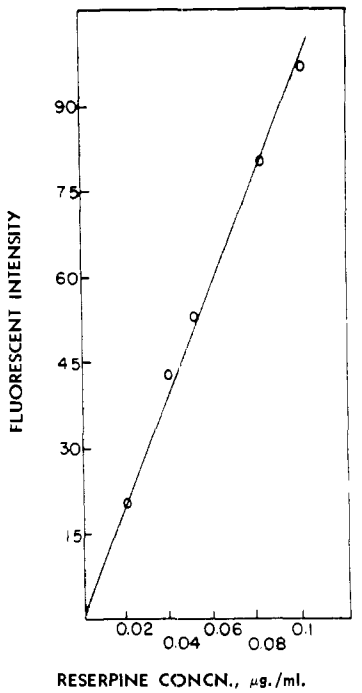

Figure 3. Nitrite-induced fluorescence as a function of final reserpine concentration

Citric Acid Solution, 2%. Dissolve 2.0 grams of citric acid in water and dilute to 100 ml.

Sodium Bicarbonate Solution, 1%. Dissolve 1.0 gram of sodium bicarbonate in water and dilute to 100 ml.

Sodium Nitrite Solution, 0.1%. Dissolve 0.1 gram of sodium nitrite in 50 ml. of water and dilute to 100 ml. with methanol. Prepare daily.

Extraction and Cleanup. Pass the tissue through a meat grinder. Weigh a 100-gram sample and transfer to a suitable high speed blender, such as the Lourdes Multi-Mixer, and homogenize with 150 ml. of chloroform for 5 minutes. For whole eggs, wash with methanol and warm water, and shell prior to extraction. In the case of blood, use a 100-ml. sample and proceed as outlined. Transfer the homogenate to a bottle, and centrifuge until the layers separate. Withdraw the lower chloroform layer through paper into a suitable round-bottomed flask, retaining the solids in the bottle. Repeat the extraction with four additional 100-ml. portions of chloroform. Evaporate the solution to about 25 ml. by means of a rotating vacuum evaporator at a temperature of not more than 60°. Transfer the residual chloroform solution to a 500-ml. separatory funnel with the aid of two 10-ml. portions of chloroform.

Add 400 ml. of trimethylpentane and mix well. To another 500-ml. separatory funnel add 1 ml. of standard reserpine Solution 3, 40 ml. of chloroform, and 400 ml. of trimethylpentane. Treat both the sample and standard similarly and simultaneously. Extract with four 10-ml. portions of citric acid solution by shaking each extract gently for about 5 minutes. If color is present or emulsions develop, wash the citric acid extracts with 10 ml. of trimethylpentane; centrifuge, draw off the trimethylpentane layer, and discard. Filter the combined aqueous extracts through fluted filter paper into a 125-ml. separator and extract with four 10-ml. portions of chloroform by gentle shaking for about 1 minute. Filter the chloroform extracts through paper into another 125-ml. separatory funnel, add 15 ml. of sodium bicarbonate, and shake gently for 1 minute. Allow the layers to separate and withdraw the lower chloroform layer through paper into a suitable round-bottomed flask. Evaporate the chloroform to about 2 ml. by means of a rotating vacuum evaporator at a temperature of not more than 60° C. Transfer the residual solution to a 10-ml. volumetric flask using exactly 4 ml. of methanol and small portions of chloroform to rinse the flask; dilute to volume with chloroform and mix well.

Development and Measurement of Fluorescence. Pipet 5 ml. of the biological preparation and 5 ml. of extracted standard, respectively, into separate 10-ml. volumetric flasks. Use the remaining 5 ml. of each extract to constitute the blanks. To the first set of flasks containing the biological extract and standard add 1.0 ml. of sodium nitrite solution and 6 drops of hydrochloric acid to the other flasks (blanks); add 1.0 ml. of 50% aqueous methanol and 6 drops of hydrochloric acid. Let stand 30 minutes, swirling occasionally, and then dilute to volume with methanol.

Set the activating wave length of the spectrophotofluorometer at 390 mμ and the fluorescent wave length at 510 mμ. Use the standard cuvettes supplied for the instrument in making the fluorescent measurements. Adjust the photomultiplier microphotometer to obtain a meter reading of approximately 80 using an unextracted standard preparation developed as above for the extracted standard and sample. Determine the fluorescent response of the standard preparation, S; tissue preparation, T; and blanks, S_o and T_o. Reserpine ($C_{33}H_{40}N_2O_9$) in μg./100 grams of tissue = $[(T - T_o)/(S - S_o)] \times 0.5 \times 2$. P.p.b. reserpine = $[(T - T_o)/(S - S_o)] \times 10 \times 100/W$, where W is weight of sample in grams.

Application and Discussion

Recovery Experiments. The method described involves separation of reserpine by a series of extractions with chloroform and acid, and oxidation with nitrous acid to form a highly fluorescent product which is estimated spectrophotofluorometrically at 510 mμ with excitation at 390 mμ. It was evaluated by analyzing samples of poultry tissues and eggs containing known amounts of reserpine at four different levels from 2 to 10 p.p.b. Along with these samples, portions of the poultry products containing no additives were analyzed to determine if significant interference is encountered from natural components. The tissues and eggs were obtained from several flocks of chickens which had been fed continuously on a commercial ration. Table II shows the results obtained in carrying through all the procedural steps in the presence of control tissues.

Most of the recoveries fall within 80 to 120% and are considered satisfactory for residue analysis of this type. A slight positive interference is present at less than 5 p.p.b., whereas negative hindrance is present at 10 p.p.b. Artifacts were sometimes present at levels under 2 p.p.b. even when stringent precautions were taken and possibility of contamination was eliminated; therefore, recoveries at the 1-p.p.b. level have not been included here. Possibly, the difficulty is the result of instrumental scatter due to the high sensitivity setting. The method was less successful when applied to pigmented tissues. Low recoveries from liver may be due to the enzymatic destruction of reserpine by the action of this tissue. Although the reactions and processes carried out in the analysis are basically simple, minute attention to detail and meticulous technique are required to obtain consistent and reproducible results.

Analysis of Treated Poultry. Tissue and egg samples were obtained from commercial-type birds which were raised on basic rations to which reserpine was added as a micronized premix. They had free access to the feed. Muscle tissues and eggs were taken from individual birds, while other tissues were combined to obtain sufficient sample for testing. The tissues were collected as the birds were sacrificed, immediately frozen, and maintained in the frozen state until analyzed. Eggs were stored at 5° C. until examined, or rinsed with warm water, shelled, homogenized, frozen immediately, and stored frozen until time of analysis. Samples from treated birds were analyzed in multiples with a corresponding sample from control birds processed with each set. The control was included to indicate the sensitivity of the method and to keep a constant check on reagents. Residue data from treated chickens using the procedure described are tabulated in Table III.

The values observed are insignificant since, in most cases, the sensitivity was larger than the actual values recorded. "Relative zero" results were still found after prolonged oral administration at the recommended level of 2 p.p.b. for 14 months. Even with higher concentrations of reserpine which resulted in excessive tranquilization of the birds, little of the drug was found. Eggs from chickens fed reserpine in concentrations of 2 mg. per kg. of diet are free of the drug or below the sensitivity of the method. No deposition, localization, or accumulation of residues in specific tissues was noted in birds raised on medicated formulations containing up to several-fold the recommended concentration of reserpine. Since tissue and egg residues of the active ingredient do not accu-

Table II. Recovery of Known Amounts of Reserpine from Poultry Products

Tissue	Reserpine, P.P.B. Added	Reserpine, P.P.B. Found[a]	Mean Recovery, %
Blood (pooled)	10	8.9	89
Carcasses (ground)	5	5.2 ± 0.3(6)	104
Eggs (whole)	2	2.5 ± 0.1(2)	125
	3	3.3 ± 0.3(2)	110
	5	5.3 ± 0.6(2)	106
	10	8.3 ± 0.1(2)	83
Eggs (homogenized frozen)	10	8.6 ± 0.1(2)	86
Fat (pooled)	2	2.6 ± 0.1(3)	130
	3	3.4 ± 0.2(3)	113
	5	5.3 ± 0.3(3)	106
	10	8.9 ± 0.6(4)	89
Gizzard (pooled)	10	8.7 ± 0.3(3)	87
Heart (pooled)	10	7.2	72
Liver (pooled)	10	7.8 ± 0.8(3)	78
Muscle (leg + breast)	2	2.0 + 0.2(3)	100
	3	3.2 ± 0.2(3)	107
	5	5.0 ± 0.3(3)	100
	10	9.2 ± 0.8(4)	92

[a] Numbers in parentheses refer to number of determinations involved.

Table III. Reserpine Residues in Chickens and Eggs from Medicated Birds

Tissue	No. Analyzed[a]	Reserpine Feed Schedule: Concn., p.p.m.	Reserpine Feed Schedule: Duration, weeks	Reserpine Found, P.P.B.
Blood (pooled)	2	2	8	<2
	2	4	8	<2
	1	40	2	2
Carcasses	7	4	8	<2
Eggs				
Whole	6	2	8	<2
	6	2	9	<2
Homogenized	7	2	8	<2
Whites	5	2	4	<2
Yolks	5	2	4	<2
Fat (pooled)	1	40	2	4
Heart/gizzard (pooled)	2	2	8	<2
	1	40	2	4
Livers (pooled)	2	2	8	<2
	6	10	4	<2
	2	40	2	2
Muscle				
Breast + leg	3	40	2	<2
Light	3	2	8	<2
Dark	3	2	8	<2
Skin	1	40	2	4

[a] Each sample from a different bird or separate pool of birds.

mulate during the feeding of the formulation, the observance of a withdrawal period is not necessary. This paper demonstrates that no reserpine is present, or is nondetectable, in either poultry tissues or eggs of birds on a medicated diet containing recommended levels of reserpine. Confirmatory investigations by some of our associates with tritium-labeled reserpine indicated no detectable drug in chicken tissues after a diet containing 2.5 mg. per kg. of feed for 9 weeks.

To demonstrate differences, if any, in the way in which reserpine is metabolized in turkeys, similar experiments were initiated with this species. The birds were fed a complete ration containing 2 p.p.m. of reserpine for several months with no withdrawal. The residue studies showed no reserpine present in any tissues and there was no evidence that turkeys differ from chickens.

To broaden the spectrum of coverage, antibiotics were combined with reserpine in commercial feed formulations. This required tissue residue levels for each agent and for each combination. However, it was necessary to restrict the number of combinations, as it would have been impractical to clear all possible combinations of established therapeutic agents. Broad trials were run in the field with reserpine in combination with penicillin and streptomycin, zinc bacitracin, and chlortetracyline. Studies were designed for long-term and short-term feeding of therapeutic levels and prophylactic levels of antibiotics in the feeds of broilers, layers, and turkeys. Tissues and eggs were collected for antibiotic studies as well as for reserpine determinations. Since all antibiotics tested were established feed additives, clearance became a cooperative effort with other companies.

These studies showed no statistically significant residues of any antibiotics in samples of blood, muscle, liver, kidney, heart, gizzard, and eggs. Also, discontinuance of use before slaughter was not indicated. No difference could be demonstrated between birds fed the antibiotic alone and birds fed the antibiotic in combination with reserpine. Likewise, our results indicated no detectable concentration of residual reserpine in any of the tissues and eggs. Thus, the presence of antibiotics in the diet of chickens and turkeys did not effect the retention of reserpine. In summary, the total results are the same for the combination groups as for those treated with one agent.

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Correction

The Determination of the Vapor Pressures of Some Phenoxyacetic Herbicides by Gas-Liquid Chromatography

In this article by David J. Jensen and E. D. Schall [J. AGR. FOOD CHEM. **14,** 123 (1966)], Equations 1, 4, and 5 should be changed by adding a minus sign before each log term on the right side of the equation. These changes also require inversion of Equation 6 to read $\alpha = z_1/z_2 = p_2^0/p_1^0$.

These equations then read:

$$\log V_{21} = -\log (p_1^0/p_2^0) - \log (\gamma_1/\gamma_2) \quad (1)$$

$$\log (z_1/z_2) = -\log (p_1^0/p_2^0) - \log (\gamma_1/\gamma_2) \quad (4)$$

$$\log (z_1/z_2) = -\log (p_1^0/p_2^0) \quad (5)$$

$$\alpha = z_1/z_2 = p_2^0/p_1^0 \quad (6)$$