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Laser Light Scattering Bioassay for Veterinary Drug Residues in Food Producing Animals. 1. Dose-Response Results for Milk, Serum, Urine, and Bile

Philip J. Wyatt,* David T. Phillips, and Edward H. Allen

A rapid, inexpensive, and extremely sensitive bioassay has been developed for the quantitative estimation of veterinary drug residues in food producing animals. The bioassay is based on the measurement of changes in differential light scattering (DLS) patterns from suspensions of drug-susceptible bacteria illuminated by a vertically polarized He–Ne laser. Various dose–response curves are presented for milk, bovine serum, urine, and bile specimens fortified with four representative drugs: penicillin-G, chlorotetracycline, furaltadone, and neomycin. The 2 to 3 h assay protocol yields log–linear dose–response curves for the four drugs over approximate ranges of 0.003–1, 0.03–10, 3–100, and 0.1–3 μg/ml, respectively.

The recent review article by Oehme (1973) has reemphasized the existence of possible health hazards in our food supply resulting from the use (Huber, 1971) of veterinary antibiotic drugs. Such drugs are used for two principal reasons: (1) the stimulation of growth and (2) the treatment of disease.

The Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) have been given the additional tasks of detecting and identifying residual drugs in consumable products and quantitating the amounts present. The FDA is currently examining the possibility of circumventing costly tissue assays by establishing correlations between tissue levels and those in the associated serum and urine.

During the past few years, our laboratory has been developing a completely new bioassay technique using differential light scattering (DLS) methods. The scattering data provided by such measurements depend upon the average cell size, shape, and structure as well as the size distribution and concentration of the bacterial assay culture being examined (Wyatt, 1968, 1969, 1972, 1973, 1975; Wyatt et al., 1972, 1976; Berkman and Wyatt, 1970; Berkman et al., 1970; Mellett and Wyatt, 1975; Mellett et al., 1976). Such methods have been shown to be particularly effective for detecting very low levels of antibiotics and evaluating bacterial antibiotic susceptibility in brief periods of time. Recently, these methods have been extended to the detection and quantitation of antineoplastic drugs in serum, urine, and bile (Wyatt et al., 1976; Mellett and Wyatt, 1975; Mellett et al., 1976). The success of these latter studies has been due in large part to the development of the Differential III, an automated instrument originally designed for the rapid quantitative determination of antibiotic susceptibilities (Wyatt, 1975). (Differential is a registered trademark of Science Spectrum, Incorporated.) New protocols have been developed that are directly applicable for the detection and quantitative determination of antibiotic residues in food producing animals. They are fast (quantitative results are produced in 2 to 3 h), require very small specimens (0.1 ml), and detect quite low levels of most drugs (e.g., 3 ng/ml for penicillin).

This assay technique has been confirmed (Wyatt et al., 1976; Mellett and Wyatt, 1975; Mellett et al., 1976) through extensive experience with antineoplastic drugs using both fortified specimens and specimens taken from animals undergoing drug therapy. The animal results to date have been in excellent agreement with conventional disc assay procedures (cf. Hunt and Pittillo, 1968) and radiometric determinations (Henderson et al., 1965; Bischoff et al., 1971). Similar methodologies have recently been applied successfully to the assay of human serum specimens containing antibiotics (MacLowry, 1975).

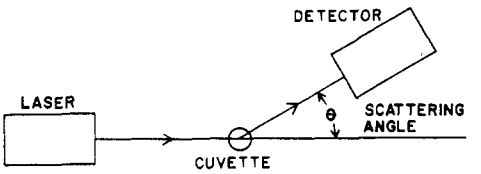
The work presented in this paper is concerned exclusively with the generation of standard dose–response curves for milk, serum, urine, and bile specimens using four representative veterinary drugs (penicillin-G, chlortetracycline, neomycin, and furaltadone). Later papers will specifically address liver, muscle, and kidney tissues, problems associated with drug binding, as well as different types of drugs.

DIFFERENTIAL LIGHT SCATTERING BIOASSAY

Figure 1 illustrates the light scattering measurement that forms the basis for the differential light scattering (DLS) bioassay. A suspension of exponential phase bacteria ($\sim 10^6$/ml) contained in a conical cuvette is placed in a fine laser beam which passes diametrically through the cuvette. A collimated detector rotates about the cuvette and generates a signal proportional to the intensity of the scattered light detected. The variation of scattered light intensity as a function of angle (detector position) is called the differential light scattering pattern (Wyatt, 1973). These patterns are recorded and processed by a small computer.

Figure 2 shows typical DLS patterns obtained from suspensions of penicillin-sensitive *Staphylococcus aureus* cells illuminated with a vertically polarized He–Ne laser (wavelength 632.8 nm). Four similar samples were prepared using suitably fortified milk whey. The control and penicillin-fortified specimens were incubated for 2 h while one sample was refrigerated to preserve the initial conditions. A normally growing culture interacting with whey containing no penicillin is the control. Dotted curves show

Science Spectrum, Inc., Santa Barbara, California 93105 (P.J.W., D.T.P.), and the Food and Drug Administration, Rockville, Maryland 20852 (E.H.A.).



LIGHT SCATTERING MEASUREMENT

Figure 1. Schematic of light scattering measurement.

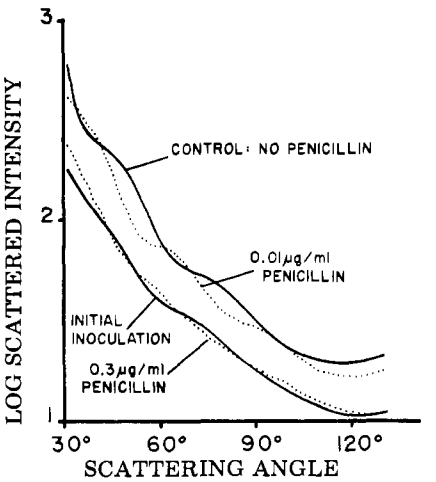


Figure 2. DLS patterns show the response of *Staphylococcus aureus* SS41 to penicillin-fortified whey after 2-h incubation for two levels of penicillin. Results are contrasted with initial inoculum and control samples.

the effect of the penicillin-fortified whey on the bacteria. At 0.01 μg/ml penicillin the bacteria continue to grow, almost up to the control curve. However, the changes in the DLS pattern indicate the cells are undergoing morphological change. At 0.3 μg/ml penicillin growth is stopped at the level of the initial inoculation. Again, morphological changes are observed. Note the pronounced change in the DLS pattern from the *S. aureus* suspension exposed to milk whey containing 0.01 μg/ml penicillin.

The DLS pattern is a sensitive function of the average size, shape, structure, size distribution, and number density of the scattering bacteria. Research at Science Spectrum during the past few years has shown that for susceptible strains, all antibiotics affect some of these features (Berkman et al., 1970; Wyatt et al., 1972; Wyatt, 1973). Within a period of approximately 60–120 min the DLS pattern of a susceptible strain will undergo changes, thereby indicating the effect of the drug. The *degree* of pattern change is a sensitive function of drug concentration. It is this fact that permits the rapid quantitation of most drugs.

The general DLS protocol begins with an exponential phase broth culture of a susceptible bacterial strain at a concentration of about 10^8 organisms per ml (optical density ~0.1 to 0.2 at 633 nm). Approximately 200–300 μl of this culture is combined with an equivalent aliquot of the assay sample (whey, serum, urine, or bile for the present study) and incubated at about 39 °C for 60–180 min.

Following incubation, approximately 300 μl of the mixture is diluted with 15 ml of demineralized water or dilute broth typically 33% Brain–Heart Infusion (BHI, BioQuest), and equilibrated at room temperature for about 30 min. After equilibration the specimen is compared in a Differential III instrument (Wyatt, 1975) with a similarly prepared control sample. The score (0–1000) produced by the Differential III is then interpolated on a standard dose–response curve run earlier and the drug concentration deduced. The exact quantities of bacterial broth and assay sample, as well as incubation times and other protocol details, are variables that are usually optimized for each class of drug and fluid assayed.

The Differential III is a computer-based instrument that can process up to ten specimens (including controls) per tray. The system scans the control and treated specimens sequentially and digitally stores their associated DLS patterns in appropriate memory locations. These patterns are subsequently compared via an algorithm also stored in the computer memory and a "score" is calculated. The score generated is based on two comparisons relative to the control pattern: the change in *shape* of the patterns and the vertical displacement of the specimen pattern relative to the control. Morphological changes in the assay organisms or changes in their size distribution result in the shape change of the DLS pattern. Vertical displacement of the DLS pattern is indicative of a change in the number density of the assay organism.

In assay work over widely varying ranges of drug concentration, we have occasionally found that the morphological effect reaches a maximum at a certain critical level of drug, and then falls back as the bacteriostatic or bactericidal effects are completed. In addition, test organisms exposed to a *sub*-lethal concentration of drug may continue to grow but with distortions in morphology. Thus, there tends to be a maximum morphological effect at a drug concentration below a killing level. In fact, high drug levels which kill rapidly, without extended abnormal growth, may at times produce smaller morphological effects than sublethal doses. This can even lead to the slight reversal of the contributions of the growth and morphology terms to the score at 0.3 μg/ml of chlortetracycline with *S. aureus* SS41.

The DLS assay technique is subject to many of the same advantages and disadvantages of conventional bacterial plate assays. Some bacteria respond to almost any antibiotic while others have highly specific responses to certain drugs. The identification and quantitative measurement of an unknown drug residue may require the use of several test organisms and some variation in sample dilution. The development of such multi-drug analysis systems is now in progress at this laboratory.

PHYSICAL AND MATHEMATICAL DESCRIPTION OF DLS MEASUREMENT

The theoretical calculation of DLS patterns for various bacteria models may be computed using well-established methods (Wyatt, 1968). Such computations agree well with experimental measurements (Wyatt, 1975; Wyatt and Phillips, 1972).

Several essential features of the differential light scattering (DLS) patterns from dilute (water) suspensions of bacteria are worth noting. (1) The scattered intensity at any angle from a suspension of bacteria is proportional to the number of bacteria present if all other parameters (size, structure, etc.) are constant and the number density is not great enough (Wyatt, 1973) to result in multiple scattering. (2) The DLS pattern fluctuates in angle as a function of the mean diameter of the bacteria because of resonance effects. (3) Forward scattering, from 0 to 30°, is dominated by diffraction. Most of this diffraction peak is confined to λ/D radians, where D is the mean diameter of the bacteria and λ the wavelength of the incident light in water. (4) The DLS pattern corresponds roughly to a double slit interference pattern from the front and back of the bacteria. There are approximately $2nD/\lambda$ intensity

peaks, where n is the mean refractive index of the bacteria relative to water. Suspensions with very narrow size distributions show sharp, well-defined features. As the size distribution broadens, these features smooth out. (5) Because of the above, it is clear that a simple turbidimetric measurement or a scattering measurement at a single fixed angle (nephelometer) cannot provide an accurate measure of bacterial number density since cell size and changes influence light scattering in a complex manner.

The scattered intensity (DLS pattern) from a bacterial suspension illuminated by light polarized perpendicularly to the plane of scattering may be represented as:

$$I(\theta) = N\overline{|f(\theta)|^2} \quad (1)$$

where N is the number density of bacteria and $\overline{|f(\theta)|^2}$ represents the scattered intensity averaged over the bacterial size distribution. It is desirable (Wyatt, 1973) to record and store the DLS pattern in a logarithmic mode for subsequent processing and analysis. For normalization purposes in the Differential III system, the logarithm is arbitrarily multiplied by a factor of 300 to yield:

$$L(\theta) = 300 \log I(\theta) = 300 \log N + 300 \log \overline{|f(\theta)|^2} \quad (2)$$

$L(\theta)$ is then recorded digitally at 100 angles from 31 to 130°. We define the displacement D_t of the test data $L_t(\theta)$ relative to the control data $L_c(\theta)$ as:

$$D_t = \frac{1}{\theta_2 - \theta_1} \int_{\theta_1}^{\theta_2} d\theta \, [L_c(\theta) - L_t(\theta)] \quad (3)$$

The morphological index, M_t, is defined by:

$$M_t = \frac{1}{\theta_2 - \theta_1} \int_{\theta_1}^{\theta_2} d\theta \, |L_c(\theta) - L_t(\theta) - D_t| \quad (4)$$

By subtracting the average displacement, D_t, the influence of the bacterial number density, N, is removed, thereby resulting in the term M_t which must reflect morphological changes only.

The Differential III score, S, is then defined by:

$$S = D_t + 3M_t \quad (5)$$

This combination of displacement and morphological terms was established phenomenologically during earlier studies of antibiotic susceptibility testing (Stull, 1973; Wyatt, 1975). If the size distribution and structure are unchanged, D_t becomes a measure of the change in number density of the bacteria, i.e.

$$D_t = 300 \log (N_c/N_t) \quad (6)$$

If, for example, the test culture were fixed at the initial inoculation level by some bacteriostatic agent, while the control tripled in 2 h, then $D_t = 143$.

For certain assays the test culture may have the same average scattering level as the control, suggesting that almost the same number density of bacteria is present, and yet have the light scattering peaks at completely different angles, which implies a change in the size or structure of the cells. Such conditions are illustrated by the data for 0.01 μg/ml penicillin in Figure 2.

While it is possible in principle to compute the characteristic size and size distribution of the bacteria, and even fit structural parameters such as cell wall thickness (Wyatt, 1970), these computations are difficult and costly. For a practical bioassay the phenomenological parameter M_t provides a measure of size and structure changes that has proven effective. If bacterial density is high enough to avoid significant background scattering from the media and sample, and low enough to avoid multiple scattering, the scores are independent of laser power, detector sensitivity, and initial number density of the bacteria.

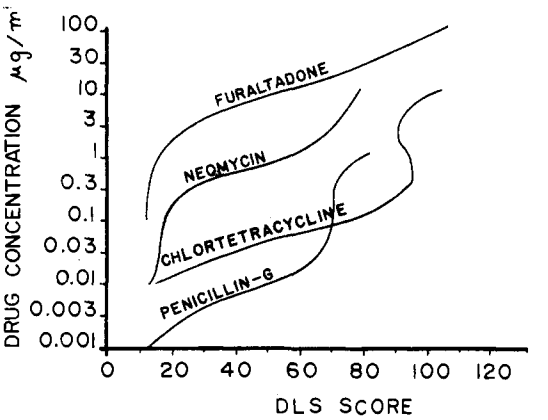


Figure 3. DLS dose-response curves for drug-fortified whey. The assay organism is *S. aureus* SS41 for penicillin-G, chlortetracycline, and furaltadone. *S. aureus* SS329 was used for neomycin.

ASSAY PROTOCOL AND DOSE-RESPONSE DATA

Sample Preparation. Chilled whole milk was allowed to stand for 1 h and the cream was skimmed off. About 0.5 ml of commercial renin (Hannilase) was added to 20 ml of skim milk. This mixture was allowed to stand at 39 °C for 1.5 h, then centrifuged for 5 min at 2000 rpm, decanted, and filtered with a Whatman No. 1 filter. Whole blood was clotted and spun down to produce serum. No filtering or other preparation has been required for serum specimens. Urine, serum, or bile specimens were usually filtered through a Whatman No. 40 filter to remove solids and epithelial cells. If bacterial contamination was suspected, the urine was further filtered through a 0.45-μm Nuclepore filter. Bile specimens were examined without filtration.

Assay bacteria were removed from a recent BHI agar plate and grown overnight in BHI broth at 39 °C. This seed culture was used to inoculate subsequent assay cultures in BHI broth. The assay cultures were initially standardized to 70% transmission at 633 nm using a Spectronic 20 spectrophotometer. The assay cultures were incubated for 45 min at 39 °C before use.

A small assay sample (0.5 ml) was diluted with prewarmed BHI broth (0.5 ml) and incubated at 39 °C for 5 min. Next, this mixture was combined with 0.5 ml of the assay culture and incubated for a period of 2–3 h as required by a particular assay organism (typically, 2 h for *S. aureus* SS41).

After incubation, 0.3 ml of the assay mixture was transferred to a light scattering cuvette and diluted with 15 ml of one-third strength BHI broth at room temperature. The cuvette was swirled to dislodge bubbles and thoroughly mix the sample. After equilibrating for 30 min at room temperature, the samples were read on the Differential III.

The curves shown in Figures 3, 4, 5, and 6 show the Differential III scores for assays of drug-fortified samples of milk whey, serum, and urine, respectively, as a function of drug concentration. These plots show mean values for 10 to 15 runs together with the probable error of the mean values and the root-mean-square deviation of the individual determinations. Similar dose–response curves were obtained for bile using penicillin-G and neomycin.

Preliminary binding studies for neomycin-fortified whole milk have shown that only 10–25% of neomycin remains

Table I. Useful Response Range (μg/ml)

	Milk whey (Fig. 3)	Serum (Fig. 4)	Urine	Bile
Penicillin-G (4J626)				
S. aureus SS41	0.003-0.03	0.003-1	0.002-1	0.003-1
S. aureus ATCC 6538P	NA[a]	NA	0.002-0.03	NA
Chlortetracycline (7670B-95-1)				
S. aureus SS41	0.03-0.3	0.2-10	0.02-0.3	NA
S. aureus ATCC 6538P	NA	NA	0.02-0.3	NA
Furaltadone (S-3144, RN41992)				
S. aureus SS41	3-100	10-100	2-30	NA
S. aureus ATCC 6538P	NA	NA	3-100	NA
Neomycin (5C781)				
S. aureus SS41	NA	0.2-10	0.3-10	NA
S. aureus ATCC 6538P	NA	NA	1.0-10	NA
S. epidermidis SS206	0.3-10	0.1-10	0.1-3	0.1-10
S. epidermidis ATCC 12228	NA	NA	0.1-3	NA

[a] Not assayed.

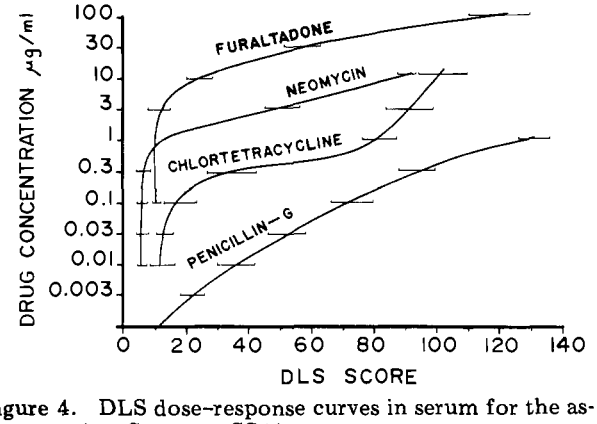


Figure 4. DLS dose-response curves in serum for the assay organism *S. aureus* SS41.

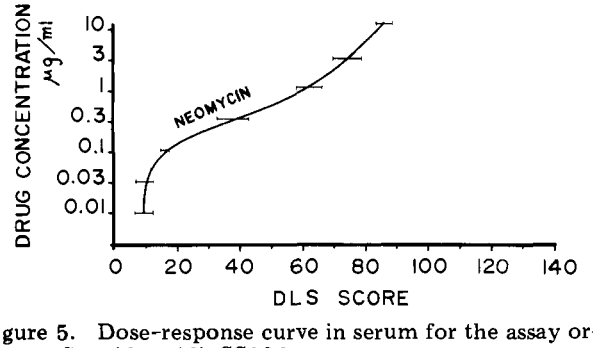


Figure 5. Dose-response curve in serum for the assay organism *S. epidermidis* SS206.

in the whey after separation. Nevertheless, the sensitivities obtained provide an adequate assay.

DISCUSSION

The assay protocol described in this paper provides the highest sensitivity consistent with simple and rapid sample preparation. The sensitivities obtained are sufficiently high so that in many practical applications it is desirable to dilute the samples further, so that the measured response will fall in the linear region of the dose–response curve. Dilution also reduces the effect of sample-to-sample variations in color, turbidity, etc. The accuracy of the rapid DLS assay method is comparable to that of the conventional disc assay method, though the Differential III technique is effective at much lower concentrations in some cases. The observed useful response ranges of the DLS assays for undiluted samples are summarized in Table I. As in any bioassay procedure, the dynamic range of the

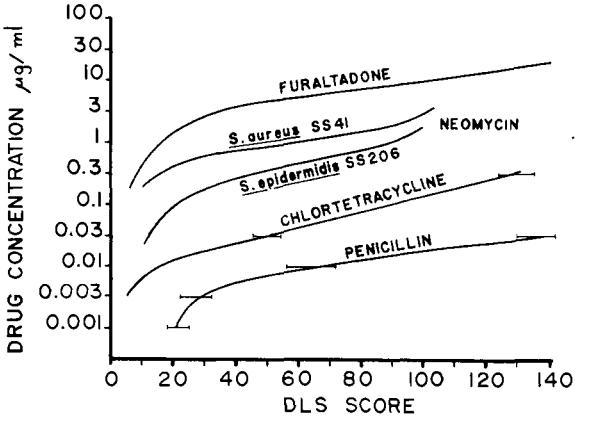


Figure 6. DLS dose-response curves for drug-fortified bovine urine. The assay organism *S. aureus* SS41 was used for all drugs and, in addition, *S. epidermidis* SS206 was used for neomycin.

measurement is limited and several sample dilutions may be required to accurately assay an unknown sample.

Several conflicting requirements must be considered in choosing a standard procedure. One basic motivation is to maintain a high rate of growth in the assay culture so the drug will be absorbed and its effects become noticeable.

Diluting the sample with standard BHI broth tends to make growth uniform. Such dilutions, however, result in a lower drug concentration environment for the assay bacteria. A reasonable compromise has typically been found to consist of a combining 0.5-ml sample, 0.5 ml of BHI broth, and 0.5 ml of assay culture in BHI broth. In applications where reduced sensitivity is desirable, higher initial dilutions are used.

The assay culture is heavily inoculated so that bacterial scattering will dominate sample turbidity after the culture is subsequently diluted for DLS measurement. Approximately 0.3 ml of the assay culture is diluted in 15 ml of isotonic saline or 33% BHI broth for DLS measurement. Maintaining isotonicity reduces sample turbidity by avoiding the precipitation of dissolved proteins.

The differential light scattering assay of milk presents an unusual problem: the whey tends to be turbid, and actively growing bacteria initially *reduce* the turbidity by metabolizing proteins in the whey. Ordinary optical transmission measurements would be completely confounded by this effect since it is exactly opposite to the normal increase in turbidity expected with increases in bacterial density. Because the technique is sensitive to

changes in bacterial morphology, a sensitive and reproducible assay can still be obtained. Note the difference between the milk and the serum dose–response curves for penicillin (Figures 3 and 4). The effect of sample turbidity may be reduced by using a higher dilution ratio.

The four drugs studied may be assayed quite effectively in serum. Assay organisms tend to exhibit initially slow growth in 100% serum. This problem is overcome by the initial 1:1 dilution with BHI broth. Urine samples involve the simplest preparation and also provide the most sensitive DLS assay. However, the correlation of antibiotic residue levels in urine and tissue has not yet been fully studied. Bile samples yield sensitivities similar to those in serum.

An important feature of the DLS method is the simplicity of the various sample preparations. Because of the relatively large final dilution, sample cleanup time is minimized and effectively eliminated. As will be described in later papers, even tissue specimens are easily prepared with negligible sample cleanup labor.

The high speed and high sensitivity obtained with the single test organism *S. aureus* SS41 suggest its immediate use as a negative screening organism to be used routinely on large numbers of samples. The inherent speed and simplicity of the DLS assay will permit the early realization of fully automated systems with throughputs of hundreds of samples per hour. Work now in progress suggests that by using several test organisms with varied sensitivity, a particular drug can be assayed in the presence of other drugs. The basic techniques developed here are applicable to tissue samples and to a wide range of drugs. The potential applications of the new DLS assay method are limited only by the genetic possibilities of bacteria. Some bacterial strains with specific nutritional requirements can be used for vitamin assays, etc., while other organisms exhibit high sensitivity to toxins ranging from pesticides to heavy metals. The DLS assay technique promises to become an important tool for many parts of the food industry.

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Pyrolysis of Some Sulfur-Containing Amino Acids at 850 °C

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The pyrolysis of cysteine, cystine, homocystine, methionine, methionine sulfone, and methionine sulfoxide at 850 °C in a nitrogen atmosphere produces pyrolysates whose composition is qualitatively similar to those obtained from non-sulfur-containing amino acids except for a few organosulfur compounds. Most of the sulfur in the amino acids listed above is converted to carbon disulfide, carbon oxysulfide, or to derivatives of these compounds.

The pyrolysis of protein (Higman et al., 1970; Smith et al., 1974) and amino acids (Higman et al., 1970; Patterson et al., 1969, 1971, 1973) has been shown to give pyrolysates with qualitative similarities but with quantitative differences. These quantitative differences were suggested to arise from variations in reactive intermediate concentrations which in turn are related to the structure of the substance pyrolyzed. Because sulfur or sulfur derivatives are known to combine with carbon species to form stable heterocyclic systems and to function as dehydrogenation agents (Vadekar and Pasternak, 1970; Plattner

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