tramuscularly. The animals were killed at various times after this treatment (9 to 14 days), and complete autopsies were performed. Half of each ovary was fixed in 4 percent buffered glutaraldehyde or in 1 percent buffered osmium tetroxide and embedded in araldite; thin sections were stained with uranyl acetate and lead citrate and examined in a Philips-200 electron microscope. The remaining portion of each ovary was fixed in 10 percent buffered formalin and embedded in paraffin; semiserial sections were stained with hematoxylin–eosin and periodic acid–Schiff reagent. Thick sections of material embedded in aradite were stained with 1 percent toluidine blue.

Under the light microscope there were signs of stimulation in both the follicular apparatus and the oocyte of all treated animals. Dissociation of follicular cells, early formation of the antrum, and hyperplasia of the theca cells were observed. No alterations of the zona pellucida were detected even in mature eggs examined shortly before ovulation.

When studied with the electron microscope, most of the oocytes (in secondary and tertiary follicles) of animals treated with gonadotropin showed various degrees of alteration of the zona pellucida. These changes consisted of a fine vesiculation of the outer parts of the zona, especially in areas where there was an early retraction of the granulosa cell prolongations (Fig. 1). The severity of the vesiculation was closely related to the stage of follicular and oocyte maturation; it spread throughout the zona just before ovulation. The granulosa cells had the appearance of active secretory cells. No alterations of the zona pellucida were observed in the oocytes of the control monkeys (Fig. 2).

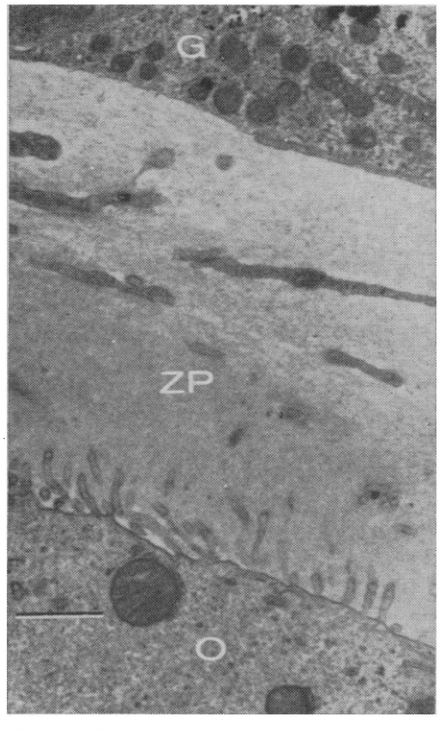

Fig. 2. Electron micrograph of an oocyte (*O*) of untreated animal with normal zona pellucida (*ZP*). *G*, granulosa cell. Line represents 1 μ. ($\times$ 11,520)

The increased response to the gonadotropin stimulation was evident in the ovaries as well as in other reproductive organs of the experimental animals. Signs of hyperstimulation, such as ovarian cysts, and a hyperplastic proliferative endometrium with increased ciliogenesis were common findings. These might have been responses to high concentrations of estrogen induced by the treatment, as the fine vesiculation of the zona pellucida might also be. On the other hand, the influence of estrogens on the loss of the zona pellucida has been demonstrated in the rat (*5*); in this animal the zona persists if the ovaries are removed on day 2 of pregnancy and, unless estrogen is supplied, remains undissolved in the uterine cavity.

Since the rhesus monkeys showed no changes in the zona pellucida observable with the light microscope, even in cases of gonadotropic hyperstimulation, it seems improbable that the "anomalies" reported in the baboon (*3*) are related to gonadotropin treatment. Since it is very difficult to fix the zona pellucida properly, it is possible that the changes in the zona pellucida of the baboon were caused by an acid fixative, especially when one analyzes the modifications present in the ova and granulosa cells of the reported pictures.

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References and Notes

1. D. L. Odor, *Amer. J. Anat.* **116**, 493 (1965).
2. J. Hope, *J. Ultrastruct. Res.* **12**, 592 (1965).
3. A. A. Katzberg and A. G. Hendrickx, *Science* **151**, 1225 (1966).
4. Obtained from Cutter Laboratories, Berkeley, California.
5. A. Psychoyos, *Nature* **211**, 864 (1966).
6. Supported in part by NIH grant FR 00163. Publication No. 255 of the Oregon Regional Primate Research Center. I thank Dr. R. J. Blandau for helpful suggestions.

5 July 1967

Phenotypic Masking and Streptomycin Dependence

Abstract. *In attempting to define the role of ribosomes in the mechanism of streptomycin dependence, a new phenomenon has been discovered. Analysis of this phenomenon—called phenotypic masking—leads to the conclusion that "streptomycin dependent" mutants are actually "drug dependent" because their dependence is equally satisfied by several drugs. These drugs, some of which are totally unrelated chemically, act on the ribosome and induce misreading in vitro and suppression in vivo.*

Streptomycin induces suppression of a number of genetic mutations (*1*, *2*) and causes misreading of the genetic code (that is, ambiguity) in vitro (*3*, *4*). The site of this action is the 30*S* subunit of the ribosome (*3*). Mutations at the streptomycin locus (Sm) may reduce or eliminate ambiguity (*3*, *4*). They may also restrict or abolish the genetic suppression of nonsense mutations (*5*). The molecular basis of induction of ambiguity and of restriction of suppression is not known, but the two phenomena may be ascribed to two alterations of the ribosomal structure with opposite effects, namely that the ribosome in performing its function of translation, may become too flexible in the presence of streptomycin or too rigid upon genetic mutation. Specifically, streptomycin added to ribosomes from streptomycin-sensitive (Sm^S) strains induces misreading of certain codons, thereby introducing ambiguity into the process of translation. Ribosomes from streptomycin resistant (Sm^R) mutants are restricted in comparison to Sm^S in the sense that, if they permit ambiguity at all in the presence of streptomycin, it is only to a very limited extent. Streptomycin dependence (Sm^D), for which ribosomal involvement has been postulated (*6*) and confirmed (*7*), may be explained by the appearance of ribosomes so restricted that they do not allow the translation of certain or any codons at all. Streptomycin may permit or stimulate the normal reading of those codons which cannot be read in the absence of the antibiotic. Functionally this property of streptomycin may be analogous to its capacity to produce ambiguity with Sm^S ribosomes. However, streptomycin need not pro-

duce significant ambiguity with the Sm^D ribosomes as it does with the more flexible Sm^S ribosomes.

We now report the phenomenon of "phenotypic masking" in which growth in the presence of a drug results in a phenotypic ribosomal alteration that masks the actual properties of the cell. When this masking effect was removed, the Sm^D strains analyzed could respond to several misreading agents, thus supporting the hypothesis that drug-induced ambiguity can overcome translational restriction.

To test one of the predictions of the hypothesis that dependence is a requirement for factors introducing translational ambiguity rather than specifically for streptomycin, the isolation and study of mutants dependent on misreading agents other than streptomycin was attempted. The related drug paromomycin was chosen because it causes misreading in vitro and can replace streptomycin for phenotypic suppression of amber mutations. Moreover, spontaneous mutants surviving exposure to paromomycin and those surviving exposure to streptomycin occurred with a similar frequency, in contrast to the extreme infrequency of stable survivors to other drugs, such as kanamycin, neomycin, or gentamicin, that induce misreading. The experiments were performed with *Escherichia coli* strain L1, an arginine auxotroph of strain B which, like the parent, is sensitive to streptomycin and to paromomycin. Strain L1 carries an amber mutation (OTC^{88u}) in the ornithine transcarbamylase structural gene which is responsive to streptomycin phenotypic suppression, and it is derepressed for the arginine pathway (arg R^-) as well (*8*). Thus this strain allows one to test phenotypic suppression by use of different misreading agents. About 10^{10} cells in the log phase of growth were spread on nutrient (L medium) agar plates containing 500 μg of streptomycin or 200 μg of paromomycin per milliliter. About 40 percent of the survivors (frequency 1×10^{-9}) in streptomycin were unconditionally dependent on (or stimulated by) this drug. In the paromomycin selection more than 90 percent of the survivors (frequency 1×10^{-9}) were similarly unconditionally dependent on (or stimulated by) paromomycin. None of the dependent isolates yielded colonies when transferred directly from the selective (L streptomycin or L paromomycin) medium to plates containing the other drug (paromomycin or streptomycin) or a mixture of the two.

At first glance it appeared that streptomycin- and paromomycin-dependent mutants were two distinct classes. That this was not the case was discovered in an attempt to replace either one of the drugs with methyl or ethyl alcohol, which may in certain Sm^D strains satisfy the streptomycin requirement (*9*) and introduce misreading in cell-free systems (*10*). It was first found that dependent mutants selected with either streptomycin or paromomycin grow on L plates containing 2 percent ethanol or 6 percent methanol (propanol is far less effective and polyalcohols, such as ethyleneglycol or glycerol, have no effect). When 20 drug-dependent clones, which arose independently on either streptomycin or paromomycin, were grown on L medium containing ethanol and then were retested by spotting them directly on plates of L medium containing streptomycin, paromomycin or ethanol, any one of the three drugs alone permitted growth. It thus appeared that the two classes were phenotypically identical. Once grown in streptomycin, however, the dependent mutants appear sensitive to paromomycin but dependent on streptomycin or ethanol; conversely, once grown in paromomycin they appear sensitive to streptomycin but dependent on paromomycin or ethanol. But in all cases, the cells are unable to form colonies in the presence of streptomycin and paromomycin together.

Table 1. Effect of prior growth on the phenotype of $Drug^D$ mutants. The growth medium was solid (agar) medium L containing Bacto-Tryptone 2 percent, yeast extract 1 percent, and glucose 0.2 percent. The drugs were used in the following concentrations: streptomycin (Sm) 500 μg/ml; paromomycin (Pm) 200 μg/ml; kanamycin (Km) 100 μg/ml; and ethanol (EtOH) 3 percent. The dependent mutants isolated on L medium containing streptomycin or paromomycin were incubated overnight on either type of plate depending on their origin, and then they were spotted on plates of L medium containing ethanol. Colonies grown on the L-ethanol medium (overnight or after 2 to 3 days depending on whether the prior growth was in the presence of paromomycin or of streptomycin) were then respotted on plates containing the different drugs in the combination shown (at the intersections between columns and rows). Finally, the colonies grown on L streptomycin and L paromomycin were directly spotted again on the same series of plates used for spotting colonies grown on L ethanol. Isolation and retesting of single colonies demonstrated that the cultures were homogeneous, and that there was no selection of different mutants in the different tests since the same phenotypes were repeatedly observed. Symbols: 0 means no growth after several days of incubation at 37°C; ± means growth after 2 days or more; + means normal size growth after 1 day.

Drug present during prior growth	Growth in the presence of the following drugs or mixtures of them				
	→ None	None	Sm	EtOH	Pm
EtOH	None	0			
EtOH	Sm	+			
EtOH	EtOH	+	0		
EtOH	Pm	+	0	+	
EtOH	Km	0	0	0	+
Pm	None	±			
Pm	Sm	0			
Pm	EtOH	+	0		
Pm	Pm	+	0	+	
Pm	Km	0	0	+	+
Sm	None	±			
Sm	Sm	+			
Sm	0tOH	+	0		
Sm	Pm	0	0	0	
Sm	Km	0	0	0	0

This sensitivity to a mixture of the two drugs can account for the changes in phenotype produced by prior growth in the presence of either one of the two antibiotics (but not ethanol), if one assumes that the antibiotic becomes fixed to the cell during growth, or that it induces a modification of its structure. The cells modified in this way cannot grow if directly exposed to the other antibiotic because this is equivalent to exposing them to the mixture streptomycin plus paromomycin, which is toxic. It is no wonder that dependent strains selected on streptomycin plates and kept in the presence of this drug have been generally believed to be dependent solely on streptomycin. However, it has been noted that some Sm^D strains can also grow on paromomycin but are sensitive to streptomycin plus paromomycin in combination (*11*).

Table 1 summarizes these results. The mutants are equally dependent either on ethanol or streptomycin or paromomycin, and sensitive to all drug mixtures in which streptomycin is one component. Moreover, in the presence of paromomycin they appear resistant to kanamycin, another aminoglycoside antibiotic producing misreading (*3*), but their dependence is not satisfied by this drug alone. Furthermore, since strain L1 carries the phenotypically suppressible OTC^{88u} mutation, the drug-dependent derivatives were tested for phenotypic suppression by paromomycin or streptomycin. Either antibiotic permits growth in the absence of arginine. Thus either drug is able to suppress this amber mutation.

Table 2. Phenotype of Drug^D mutants that were reverted to drug independence while retaining drug resistance (partially or totally). Eighty percent of the revertants were sensitive to streptomycin (type I), 20 percent were resistant to streptomycin (type II). Prior growth of type II in the presence of either streptomycin or paromomycin resulted in phenotypic masking. The test of drug resistance was done in L medium containing the drugs in the concentrations given in Table 1. The symbols, 0 and +, mean inability or ability to grow in the presence of the drugs or their mixtures.

Type	Sm	Pm	Km	Sm+Pm	Sm+Km	Pm+Km
I	0	+	+	0	0	+
II	+	+	+	0	0	+

This dependence on several drugs (Drug^D) could be the result of successive mutational steps unintentionally selected for during the repeated isolations of single colonies. However, by transduction mediated by bacteriophage P1, it was found that the entire complex phenotype described above is transferred in one step from a Drug^D donor to a wild-type recipient on selecting for resistance to any one of the drugs to which the donor strain is resistant or dependent. Subsequent analysis of all dependent isolates obtained by either type of selection (40 from streptomycin and over 200 from paromomycin) failed to demonstrate the existence of a different class of dependent mutants. This failure suggests that the Drug^D mutants described above are the most commonly occurring dependent mutants.

The change in the phenotypic response to an antibiotic, depending on the conditions of growth before testing, which we call phenotypic masking, is the result of the combination of two factors: (i) growth in the presence of streptomycin or paromomycin (and possibly of ethanol, but to a far less extent) results in a modification of a cellular element either directly (as by stable adsorption of the drug) or indirectly, and (ii) the toxicity of any drug mixture in which streptomycin is one component. Concerning the first point, amino acid incorporation experiments in vitro (*12*) with ribosomes extracted from Drug^D mutants, show that susceptibility to misreading depends on the drug used for cell growth, and that it is the ribosomal component which is affected since the origin of the soluble components is irrelevant. This indicates that the ribosome is the cellular element responsible for this phenotypic masking.

Concerning the second point, it was established that the mixtures of streptomycin and paromomycin, streptomycin and kanamycin, and streptomycin and ethanol are bactericidal, and that the extent of killing depends on the mixtures. That is, 99, 94, and 50 percent of a cell population of these Drug^D strains growing exponentially in L plus streptomycin (500 μg/ml) were killed 5 minutes after the addition of either paromomycin (200 μg/ml) or kanamycin (100 μg/ml) or ethanol (3 percent), respectively. When the proportions of the drugs were changed, it was found that an amount of streptomycin as low as 5 μg added to 200 μg paromomycin per milliliter or conversely 10 μg of paromomycin added to 500 μg streptomycin per milliliter were equally bactericidal. Notice that 5 μg of streptomycin or 10 μg of paromomycin per milliliter are the minimal amounts of drug which are required to kill the parent strain L1 (streptomycin and paromomycin sensitive). Amino acid incorporation experiments (*12*) in which the amount of streptomycin used (20 μg/ml) was above that saturating the system gave similar indications. With ribosomes extracted from these mutants after growth in L medium containing ethanol, it was found that ethanol, paromomycin, or streptomycin, individually, produce a small amount of misreading or none at all, but when they are tested in any combination containing streptomycin the misreading increases considerably. Furthermore, the efficiency of killing and the extent of misreading are correlated for the different drug combinations. The maximum amount of misreading, obtained with streptomycin plus paromomycin, was comparable to that obtained with wild-type ribosomes. These results indicate that, although a cell dependent on either one of several drugs must be implicitly resistant to each one of them, this resistance is precarious and may be easily nullified if the growth-promoting drugs are used in combination. A similar phenotype is displayed by the Drug^D mutants that were reverted to drug independence while retaining drug resistance (Table 2). The revertants of type II, although resistant to streptomycin, paromomycin, or kanamycin when used alone, are sensitive to any mixture in which streptomycin is one component and exhibit phenotypic masking when previously grown in the presence of any one of the drugs.

It is clear that the nature of this type of resistance is different from that exhibited by drug-resistant mutants selected in one step. In fact it has been possible to independently introduce the Sm^R and Drug^D mutations into the same genome. A classical Sm^R mutation has been transduced into strain L1 and the resulting LlSm^R strain has been exposed to L plates containing paromomycin. The majority of the survivors were stimulated by paromomycin. After growth in ethanol their phenotype appeared to be drug-dependent, like the analogous mutants derived from LlSm^S (as described above). However, in contrast to the LlSm^{S}Drug^D strains, these LlSm^R-Drug^D strains were resistant to the mixture of drugs that included streptomycin and prior growth in either streptomycin or paromomycin did not result in phenotypic masking. This difference in resistance to streptomycin plus paromomycin has been used (*13*) to distinguish Sm^{S}Drug^D from Sm^R-Drug^D transductants in crosses between Sm^{R}Drug^D donors and wild-type recipients (Sm^{S}Drug^S). Transductants selected on plates of medium L containing streptomycin or paromomycin were scored for sensitivity to streptomycin plus paromomycin. That there is 1 percent disjunction between the Sm^R and the Drug^D sites confirms the coexistence of genetically separable Sm^R and Drug^D mutations in the same genome, each contributing to the phenotype. The low frequency of disjunction in this cross is consistent with the low frequency of Sm^S recombinants in crosses between Sm^R and SM^D mutants (*14*). This genetic evidence, together with the fact that the method of selection used here is that commonly used for selecting Sm^D mutants, indicates once again that the Drug^D mutants are typical of the Sm^D mutants described in the literature.

To summarize, all of the dependent strains isolated, regardless of weather they were selected as survivors on streptomycin or paromomycin, appear to comprise only one class of Drug^D mutants. Their phenotype is characterized by dependence on any of the following when added individually to the growth medium: streptomycin, paromomycin, or alcohols. These

substances cause misreading in vitro and phenotypic suppression in vivo (*1, 3, 10*). Furthermore, resistance to these drugs of the $Drug^D$ mutants is converted into sensitivity when combinations of drugs are used, unless the genome carries another mutation to resistance in addition to the $Drug^D$ mutation. Indicative of this is the contrast between $Sm^S Drug^D$ and Sm^R-$Drug^D$ strains, the first being sensitive and the second resistant to the mixture of streptomycin and paromomycin. Thus $Drug^D$ mutants possess an altered ribosome too restricted to be functional per se, but responsive to the antirestrictive action of drugs that introduce ambiguity. This responsiveness is a delicate matter, however, and may result in killing instead of permitting growth, if limits in the amount or quality of the introduced ambiguity are surpassed. This specificity of required ambiguity is further illustrated by the fact that kanamycin, which is able to produce misreading in vitro (*3*) and to which the cells are resistant, is unable to support growth of these $Drug^D$ mutants.

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References and Notes

1. L. Gorini and E. Kataja, *Proc. Nat. Acad. Sci U.S.* **51**, 487 (1964).
2. E. M. Lederberg, L. Cavalli-Sforza, J. Lederberg, *ibid.*, p. 678.
3. J. Davies, W. Gilbert, L. Gorini, *ibid.*, p. 883.
4. S. Pestka, R. Marshall, M. W. Nirenberg, *ibid.* **53**, 639 (1965).
5. M. Couturier, L. Desmet, R. Thomas, *Biochem. Biophys. Res. Commun.* **16**, 244 (1964); T. K. Gartner and E. Orias, *J. Bacteriol.* **91**, 1021 (1966); P. Strigini and L. Gorini, in preparation.
6. C. R. Spotts and R. Y. Stanier, *Nature* **192**, 633 (1961).
7. J. G. Flaks, E. C. Cox, M. L. Witting, J. R. White, *Biochem. Biophys. Res. Commun.* **7**, 390 (1962).
8. L. Gorini, G. A. Jacoby, L. Breckenridge, *Cold Spring Harbor Symp. Quant. Biol.* **31**, 657 (1966).
9. I. Gado and I. Horvath, *Life Sci.* **10**, 741 (1963).
10. A. So and E. W. Davie, *Biochemistry* **3**, 1165 (1964).
11. W. Szybalski and J. Cocito-Vandermeulen, *Bacteriol. Proc.* **1958**, p. 37, (1958).
12. R. Zimmermann and L. Gorini, in preparation.
13. R. Rosset and L. Gorini, in preparation.
14. K. Hashimoto, *Genetics* **45**, 49 (1960).
15. This work was supported by an American Cancer Society grant E-226E and USPHS-National Institute of Allergy and Infectious Diseases grant 5 ROI A102011-10. For skillful technical help we thank Mrs. C. Herbert and Miss C. B. Steinkuller. We thank Dr. H. E. Machamer of the Parke, Davis Co. for paromomycin and Dr. H. L. Dickison of the Bristol Laboratories for kanamycin sulfate.

24 July 1967

Electron Spin Resonance of Gamma-Irradiated Oriented DNA Prepared by Wet Spinning

Abstract. *When moist, oriented DNA is gamma-irradiated and its electron spin resonance spectrum is recorded at 77°K, an unresolved spectrum is obtained, the amplitude of which is strongly dependent on the angle between the direction of the DNA helices and the field. Annealing at 199°K gives an eight-line thymine-like spectrum which also has a marked angular dependence. For dry, oriented DNA, the unresolved spectrum dominates even at room temperature, and the spectra exhibit lower degrees of anisotropy.*

The recent development of a new, wet-spinning method for the preparation of oriented DNA (*1*) has made possible physicochemical studies of oriented DNA with various techniques. Studies on the semiconductivity of dried, oriented DNA (*2*) and the hydration of moist, oriented DNA with nuclear magnetic resonance (*3*) have been made. We now report some results from electron spin resonance (ESR) measurements on oriented calf-thymus DNA irradiated with γ-rays.

Our DNA samples were prepared in a humid atmosphere as follows. A 10-mm-wide film of oriented DNA was folded back and forth (perpendicularly to the molecular orientation) to form a concertina-like pack which was slightly compressed into a package of oriented DNA having a parallelepipedal form with the approximate dimensions 2 by 2 by 10 mm. After the sample was equilibrated with a desired relative humidity, it was instantly frozen in liquid nitrogen and irradiated with cobalt-60 γ-rays to a dose of 0.45 megarad. The sample was thereafter mounted between two parallel, thin Teflon plates of a long forklike holder which was inserted into a 3-mm quartz tube fitting the Dewar flask of the ESR spectrometer. These and subsequent manipulations were performed in liquid nitrogen. The quartz tube was repeatedly evacuated and flushed with nitrogen to remove oxygen from the sample and finally evacuated. The tube was connected to an angular scale, and it could be rotated in the Dewar to obtain any desired angle between the DNA helices and the magnetic field. The ESR spectra (first derivative) were recorded with a Varian, X-band spectrometer having a modulation unit of 100 kc/sec. The microwave power was only about 0.3 mw; nevertheless, it was sufficient to cause some saturation of the absorption studied.

Moist lithium DNA and sodium DNA samples (Worthington, batch 6HE) at 77°K gave an unresolved spectrum, the amplitude of which was strongly dependent on the angle between the direction of the DNA helices and the field. Annealing of the samples at 199°K for 5 minutes and recording at 77°K gave an eight-line thymine radical-like spectrum which also showed a marked angular dependence and indicated an anisotropy in the hyperfine interaction. The *g*-values (spectroscopic splitting factors) of the spectra also varied slightly with angle. Figures 1 and 2 give some results for a lithium DNA sample stored at 66 percent relative humidity before irradiation. The corresponding curves for a sodium DNA sample stored at 75 percent relative humidity indicated a slightly smaller anisotropy. The angular dependence observed is real and not due to experimental artifacts. This was tested by studying a polycrystalline isotropic sample of γ-irradiated sucrose under the same experimental conditions; no angular dependence was observed. The ESR signals from oriented sodium DNA, dried in a vacuum at 70°C for 4 days, exhibited a smaller angular dependence than those of the wet samples, and the annealing transition to a thymine-like spectrum was almost completely inhibited (Fig. 3). Later annealing and recording at room temperature seemed to increase slightly the size of the satellites, but the anisotropy was further diminished. A less extensively dried sample gave a higher portion of satellite structure in the spectra. The shape and transformation of the ESR absorption described is in general agreement with several ESR studies of unoriented DNA (*4–8*), but the angular dependence now observed is a new feature of the absorption.

Little is known about the sites of the unpaired electrons giving the unresolved absorption at 77°K. Comparisons with spectra of the constituents of DNA have not been helpful (*4, 5*). This technique has, however, been more successful for interpreting part of the satellite spectra which was found to exhibit similarities with the spectra of thymine and thymidine (*5–8*). Salovey *et al.* (*6*) suggested the radical $-CH_2-\dot{C}(CH_3)-$ as giving rise to the