*ANTIBIOTIC GLYCOSIDES, IV. STUDIES ON THE MECHANISM OF ERYTHROMYCIN RESISTANCE IN BACILLUS SUBTILIS**

By Sheldon B. Taubman,† Frank E. Young,‡ and John W. Corcoran§

DEPARTMENTS OF BIOCHEMISTRY AND PATHOLOGY, WESTERN RESERVE UNIVERSITY SCHOOL OF MEDICINE

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Three general patterns for the acquisition of antibiotic resistance have been described in microorganisms.[1] These bacterial modifications include: (*a*) the development of a mechanism for the complete, or an increased rate of, destruction of the antibiotic, (*b*) a decreased or complete loss of permeability of the cell for the antibiotic, and (*c*) the acquisition or enhancement of a mechanism for overcoming the antibiotic-inhibited process. Examples of each type of resistance have been reported from studies of microorganisms.[2-10] Only the last of the above-mentioned processes need be related to the mechanism of action of the antibiotic. One or more of these mechanisms, or a hitherto unrecognized type of alteration in cellular physiology, may be operative in the development of antibiotic resistance in a specific microorganism. In the case of erythromycin-A,[11] one of the macrolide group of antibiotic glycosides, there has been no convincing confirmation or rejection of any of the mechanisms of resistance mentioned. Although qualitative recovery of antibiotic activity, after growth of resistant organisms in the presence of erythromycin, has been reported,[12, 13] no attempt was made to demonstrate either the retention of the original chemical structure or the fate of any intracellular antibiotic. Relative to mechanism (*c*), Brock and Brock have described an inhibition of protein synthesis by both chloramphenicol and erythromycin in *Escherichia coli*.[14] However, because this organism is not very sensitive to erythromycin, a very high concentration of the antibiotic (1 mg/ml) was used. No data relative to the effect of such a high antibiotic concentration on protein synthesis in a less sensitive strain of *E. coli* were presented.

In order to extend the previous observations, and to confirm their validity for a microorganism sensitive to therapeutic levels of erythromycin (<1 μg/ml), a study was undertaken to investigate the mechanism of action of erythromycin and to explore the biochemical basis of resistance to erythromycin in *Bacillus subtilis*. Results with an erythromycin-resistant, genetically stable mutant were compared with those obtained with the erythromycin-sensitive parent strain. The data obtained demonstrate that neither degradation of the antibiotic nor change in permeability to the antibiotic play significant roles in resistance. Erythromycin appears to interfere with some aspect of the utilization of amino acids by the sensitive strain of *B. subtilis*. This same process is not significantly inhibited, at comparable antibiotic levels, in the erythromycin-resistant strain.

Experimental.—Bacillus subtilis: Erythromycin-resistant mutants of *B. subtilis* were obtained by plating organisms (1×10^8) of the wild strain (*B. subtilis* 23) and an indole auxotroph (*B. subtilis* 168) on minimal glucose agar[15] containing erythromycin (2 μg/ml, 2.7 μM). The genetic nature of the erythromycin resistance was demonstrated by transformation of the erythromycin-sensitive strain to resistance by preparations of DNA from the erythromycin-resistant strain.[16] An additional genetic marker, adenine auxotrophy, was introduced by transformation of the strains by DNA from a mutant strain of *B. subtilis* 168 (SB 23).[17] Thus, organisms utilized in this in-

TABLE 1

CHARACTERISTICS OF BACTERIAL STRAINS

Strain designation	Genetic traits	Erythromycin ID_{50}* μg/ml	Growth requirements
Erythromycin-resistant	I^-, Ad^-, Ery^R	3.0	Indole, adenine
Erythromycin-sensitive	I^-, Ad^-, Ery^S	0.04	Indole, adenine

* ID_{50} = concentration required to inhibit growth 50% starting with an inoculum of about 10^6 bacteria per ml.

vestigation have the designations, genetic properties, and erythromycin sensitivities shown in Table 1.

Stock cultures of *B. subtilis* were maintained as described previously.[18] Bacteria were first grown in minimal salts medium[16] containing glucose (0.5%), neutralized acid hydrolyzed casein (0.02%), L-tryptophan (50 μg/ml), and adenine hydrochloride (50 μg/ml). All experiments were carried out at 37°C with logarithmically growing cells in the above medium on a New Brunswick rotary shaker, either model WBR at 200 rpm, 1/2″ stroke, or Model G-25 at 300 rpm, 1″ stroke.

Turbidities were determined on a Klett-Summerson Colorimeter (filter number 54) and correlated with viable cell count (one Klett unit is approximately equivalent to 3×10^6 bacteria/ml).

Preparation of H^3-erythromycin: Erythromycin-A was synthesized by *Streptomyces erythreus*, utilizing sodium 2,3-H^3-propionate (Volk Radiochemicals, Inc.) as a precursor. Preliminary isolation of the erythromycin synthesized was as described.[19] The final purification was by partition chromatography at 25°C on a powdered cellulose column (34 × 1.8 cm, 50 *g*) employing the lower layer of a methylcyclohexane:n-butanol:acetone:phosphate buffer (pH 6.85, 0.01 *M*) system (75:15:10:25 v/v) as the stationary phase (2:1 w/v) and the upper layer of the same system as the mobile phase. The effluent fractions containing the bulk of the antibiotic activity and radioactivity were found to correspond exactly. Solute isolated from these fractions gave only a single radioactive spot (corresponding to authentic erythromycin-A) on paper chromatography, using the above solvent system and also using a modification of that system.[20] Both of these systems have been shown capable of separating erythromycin-A from closely related compounds, some of which have antibiotic activity. Radioactive purity of the H^3-erythromycin was further indicated by retention of the activity on dilution with nonradioactive erythromycin, followed by chemical derivatization and purification as erythromycin-2″-O-benzoate.[19] The specific activity of the H^3-erythromycin obtained from this preparation was 2×10^4 cpm per μg.

Recovery of H^3-erythromycin from cultures of B. subtilis: Experiments were carried out in which *B. subtilis* was grown with H^3-erythromycin (20,000 cpm/μg) added to the standard minimal salts medium. After the desired level of growth was attained, each incubation was terminated by cooling to 0°C. In the experiments from which both extra- and intracellular erythromycin were recovered, a large amount of nonradioactive erythromycin (25 mg) was added, after which the bacteria were lysed with lysozyme and the erythromycin fraction was isolated by chloroform extraction at pH 9.5.[19] In the experiment from which only intracellular erythromycin was recovered, the bacteria (approximately 7.5×10^{10} organisms) were first washed (five times, 10 ml each time) with minimal salts medium containing glucose (0.5%) and nonradioactive erythromycin (10 μg/ml). Finally, a large amount of nonradioactive erythromycin (25 mg) was added, after which the bacteria were lysed with lysozyme and the antibiotic was recovered by diethyl ether extraction at neutral pH. In both cases, recovery of H^3-erythromycin was calculated by the isotope dilution method, after the preparation, isolation, and purification of erythromycin-2″-O-benzoate.[19]

Studies of H^3-erythromycin uptake by B. subtilis: The uptake of H^3-erythromycin from the extracellular medium, by erythromycin-sensitive and erythromycin-resistant bacteria, was determined by filtering an aliquot of the bacterial suspension through a membrane filter (Millipore, Type HA, 25 mm). The bacteria collected in this manner (about 5×10^8 organisms) were then quickly washed by repeated suspension in the filtration apparatus (seven times, 2.5 ml each time) in minimal salts medium containing glucose (0.5%) and nonradioactive erythromycin (10 μg/ml). After washing, the bacteria and the membrane filter were dried by gentle heating (<40°C) and then added to a liquid scintillation counting vial containing 10 ml of the toluene-based scintillant described by Davidson.[21] The toluene, besides lysing the bacteria, permeates the pores of the membrane filter and renders it almost completely transparent.

Lysozyme-spheroplasts (osmotically sensitive), prepared according to the method of Landman,[22] were used to determine the role of the intact cell wall of *B. subtilis* in erythromycin uptake. After collection by gentle filtration, the spheroplasts were washed (as described above) with a solution containing sodium succinate (0.5 *M*), $MgSO_4$ (0.01 *M*), and EDTA (0.005 *M*).

Studies of H^3-tryptophan and C^{14}-adenine uptake by B. subtilis: The experimental techniques used were essentially the same as outlined above for the studies of H^3-erythromycin uptake, except that the nonradioactive forms of the compounds being studied were added to the resuspending solution. The C^{14}-adenine hydrochloride (0.02 μC/mg) and the H^3-tryptophan (4.0 μC/mg) were obtained from Calbiochem and Volk Radiochemicals, Inc., respectively.

Results and Discussion.—The initial experiments with the erythromycin-resistant strain of *B. subtilis* showed that 90 per cent of the erythromycin which was added to cultures of the bacteria could be recovered after growth (Table 2, flask 1). From 94–97 per cent of added erythromycin was recovered from two control experiments, one of which employed the erythromycin-sensitive strain of *B. subtilis* (Table 2, flask 2) and the other of which received no bacterial inoculum (Table 2, flask 3). In these experiments the antibiotic was recovered from the combined medium and the lysozyme-lysed bacteria. In a different experiment the resistant bacteria, after growth in the presence of tritiated erythromycin, were washed repeatedly to remove adsorbed H^3-erythromycin and then lysed with lysozyme. Here it was possible to recover 100 per cent of the radioactivity taken up by the bacteria. In this case the external erythromycin concentration was 0.5 μg per ml (2×10^4 cpm/μg) and the uptake of erythromycin was 0.035 μg per 10^{11} bacteria. Collectively, these experiments rule out the possibility that antibiotic degradation plays a significant role in the mechanism by which the erythromycin-resistant mutant strain of *B. subtilis* grows in the presence of normally bacteriostatic concentrations of erythromycin. The results are in agreement with the qualitative findings of both Haight and Finland[12] and Straightoff,[13] who investigated this same problem by means of a procedure similar to that developed by Gots[23] to detect penicillinase activity. Since loss of extracellular biological activity served as the sole criterion of antibiotic destruction in these studies, subtle chemical change would not have been recognized unless it led to marked diminuition of antibiotic activity. Furthermore, these experiments, as well as those reported in Table 2, did not test the possibility that the mutant, resistant bacteria have acquired some mechanism for the intracellular destruction of erythromycin prior to its reaching the normal site of antibacterial action. The fact that gross intracellular destruction of erythromycin does not occur is demonstrated by the result of the experiment in which the erythromycin taken up by the resistant bacteria was quantitatively recovered.

The recovery of unchanged erythromycin from the resistant, mutant forms of *B. subtilis* does not rule out a change in permeability of the bacterial cell for the

TABLE 2

RECOVERY OF H^3-ERYTHROMYCIN FROM CULTURES OF *Bacillus subtilis*

	Flask No. 1	Flask No. 2	Flask No. 3
Bacteria	Erythromycin-resistant	Erythromycin-sensitive	None
Inoculum (cells/ml)	1.1×10^6	1.6×10^6	—
Growth, (Δ Klett-units) (filter no. 54)	122	3	—
Total H^3-erythromycin recovered, (μg)	10.75	11.3	11.6
Recovery as erythromycin-2″-O-benzoate (%)	89.5	94.2	96.8

Incubations were carried out in 300-ml nephelometer flasks with 15 ml of the standard minimal medium containing 0.8 μg H^3-erythromycin per ml.

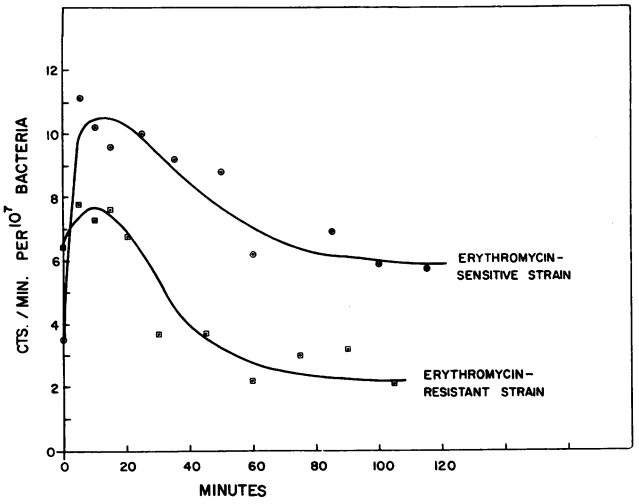

FIG. 1.—Uptake with time of erythromycin by erythromycin-sensitive and erythromycin-resistant *B. subtilis*. At the start of the experiment the bacteria (approximately 5×10^7 per ml) were present in standard minimal medium (20 ml) containing H^3-erythromycin (0.5 μg/ml, 2×10^4 cpm/μg). The incubations were carried out in 300-ml nephelometer flasks, aliquots were removed at varying time intervals, the bacteria were collected and washed, and the uptake of H^3-erythromycin was determined as described in the text.

antibiotic as being an essential part of the resistance mechanism. Accordingly, several experiments were carried out to investigate the possible role of antibiotic exclusion in the resistance to erythromycin observed in *B. subtilis*. Although a difference in uptake of H^3-erythromycin, between the sensitive and resistant bacteria, was consistently observed in these experiments (Figs. 1 and 2) it was much lower than the 100-fold difference in their sensitivity (Table 1). A similar difference in the uptake of antibiotic also was noted in lysozyme-spheroplasts of the sensitive and resistant strains. The data presented graphically in Figure 1 indicate that there is an initial rapid uptake of the antibiotic and then a slower release until an equilibrium level is attained. The amount of antibiotic actually taken up from the extracellular medium is insignificant in terms of lowering the concentration of antibiotic present. We can offer no logical explanation for the complex uptake phenomenon represented in Figure 1.

With increasing extracellular concentrations of the antibiotic, the uptake of H^3-erythromycin appears to increase proportionately in both the sensitive and resistant strains of *B. subtilis* (Fig. 2). This occurs in spite of the fact that cell multiplication ceases in the erythromycin-sensitive strain when an antibiotic level of 7.5 μg per 10^{11} bacteria is reached. Using the data presented in Figure 2, and the approximate size of the bacteria, one can calculate[24] both the internal concentration of the antibiotic (assuming homogeneous dispersion) and the degree of its concentration or dilution as compared with that in the extracellular fluid. The erythromycin-sensitive strain can effect an antibiotic concentration of about 60-fold, while the resistant organism concentrates the drug only 20 times above the extracellular level. At the higher antibiotic concentrations, as represented in Figure 2, the uptake of erythromycin per bacterium for the resistant strain has already surpassed the level which inhibits multiplication in the sensitive strain ($\geqq$7.5 μg erythromycin/10^{11} bacteria). In spite of this there is no effect on the multiplication rate of the resistant bacteria. This provides a further indication that a loss of permeability cannot explain the observed resistance. It is, of course, possible that the two strains of *B. subtilis* differ somewhat in intracellular distribu-

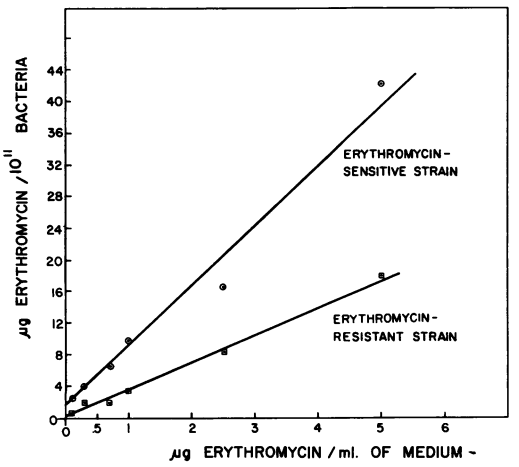

FIG. 2.—Uptake of erythromycin by erythromycin-sensitive and erythromycin-resistant *B. subtilis* as a function of extracellular antibiotic concentration. The bacteria (2–5 × 10^8) were added to standard medium (5 ml) in an Erlenmeyer flask (25 ml) containing varying concentrations of erythromycin. Depending on the concentration, the specific activity of the erythromycin varied from 2 × 10^4 to 10^5 cpm per μg. The cells were incubated at 37°C on a rotary shaker. After 60 min an aliquot was withdrawn, the bacteria were collected and washed, and H^3-erythromycin uptake was determined as described in the text. The remainder of the sample was diluted and used for a viable cell count.

tion of the antibiotic. If so, this, coupled with the difference in uptake which is observed (Fig. 1), might result in a substantial difference in the amount of erythromycin which reaches the sensitive site within the bacterium. Results to be presented below minimize this possibility by showing that some other mechanism for the resistance does in fact exist.

In the case of *B. subtilis* there is a rapid exchange between intra- and extracellular erythromycin. The data showing this, in the case of the sensitive strain, are plotted in Figure 3. Similar results were obtained using the erythromycin-resistant strain, although the accuracy was below that obtained with the sensitive strain because a smaller amount of antibiotic was taken up. The almost complete recovery of intact erythromycin from the combined medium and bacteria of *B. subtilis* cultures (Table 2) is particularly significant in view of this rapid and reversible uptake of the antibiotic by the bacterial cells. It seems likely that much of the H^3-erythromycin reisolated from the medium in these experiments had been "taken up" by the bacteria, only to be released again unchanged. If intracellular destruction could occur, then the extracellular erythromycin should have been gradually diluted with the degradation products, if these could be released by the bacteria. If they could not be released, the extracellular pool of erythromycin should have decreased in size. Since neither of these effects was noted, the recovery of erythromycin from the medium in these experiments is significant in supporting the conclusion that erythromycin is not destroyed intracellularly by the resistant bacteria.

An approximation of the over-all processes of protein and nucleic acid synthesis in the erythromycin-resistant and -sensitive strains of *B. subtilis* was possible because of the requirements of these organisms for exogenous L-tryptophan and adenine. Studies of the utilization of these two substrates were carried out in both the presence and the absence of erythromycin (1 μg/ml). With erythromycin present, the resistant mutant grows at an essentially unaltered rate, while growth of the sensitive strain is reduced to about 20 per cent of the normal rate (as measured turbidimetrically). The data obtained from these studies are presented in Figures 4*A–D*. They were obtained by measuring both the turbidity and bacterial radio-

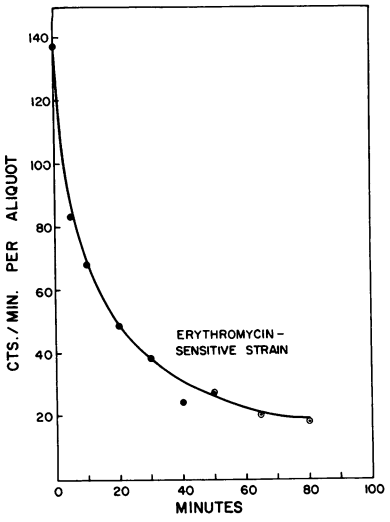

FIG. 3.—Exchange of intracellular and extracellular erythromycin on incubation of *B. subtilis*. The erythromycin-sensitive strain was inoculated into the standard minimal medium containing H^3-erythromycin (0.1 μg/ml, 2×10^4 cpm/μg), and incubated for 60 min at 37°C with shaking. The bacteria were then separated from the medium by centrifugation and resuspended (5×10^8 bacteria/ml) in standard minimal medium containing nonradioactive erythromycin (0.1 μg/ml). Aliquots of the suspension were removed at varying time intervals, after which the bacteria were collected by filtration and washed, and the content of H^3-erythromycin was determined as described in the experimental portion of the text.

activity of aliquots of the suspensions of *B. subtilis* at varying time intervals. These data show that uptake of H^3-tryptophan and C^{14}-adenine was similar in both the erythromycin-resistant and the erythromycin-sensitive strain of *B. subtilis*, as long as erythromycin was absent (Figs. 4*A* and 4*C*, respectively). In both of these cases the ratio of incorporation of adenine relative to tryptophan was about 1.8:1. When erythromycin was present, however, a marked difference between the two strains of *B. subtilis* was observed. The resistant strain grew at almost a normal rate, and both adenine and tryptophan were incorporated in about the same proportions as before (Fig. 4*B*). The erythromycin-sensitive strain grew at a low rate, and a reduced but significant incorporation of adenine occurred. Tryptophan incorporation, however, was disproportionately low (Fig. 4*D*). The ratio of incorporation of adenine relative to tryptophan was increased (3.2:1), and this strongly suggests that some aspect of the mechanism whereby amino acids are utilized for protein synthesis is affected by erythromycin in the sensitive strain of *B. subtilis*. The resistant mutant is able to utilize amino acids at nearly the normal rate in the presence of the antibiotic.

So and Davie,[25] using a cell-free amino acid incorporation system derived from yeast and *E. coli*, have recently studied some of the characteristics of chloramphenicol action. In collaboration with these authors, and using a similar cell-free system derived from the above strains of *B. subtilis*, we have been able to demonstrate that the incorporation of amino acids (lysine, valine, leucine), by extracts from the sensitive cells is markedly inhibited by erythromycin, while the incorporation is only slightly inhibited in extracts from the resistant cells.[26]

The results obtained with the cell-free system, besides demonstrating the existence of a method for further study of the mechanism of erythromycin action, increase the probability that resistance to the antibiotic in *B. subtilis* is related to the effect of erythromycin on amino acid utilization. The possibility that resistance results from a difference in intracellular distribution of the erythromycin (see above) between the resistant and sensitive forms of the *B. subtilis* seems unlikely, since a difference in sensitivity to the antibiotic is maintained in the cell-free state.

The findings presented in this communication provide strong evidence that the mechanism of resistance of a *B. subtilis* mutant to erythromycin is one whereby an increased tolerance to the antibiotic-inhibited process has developed. The

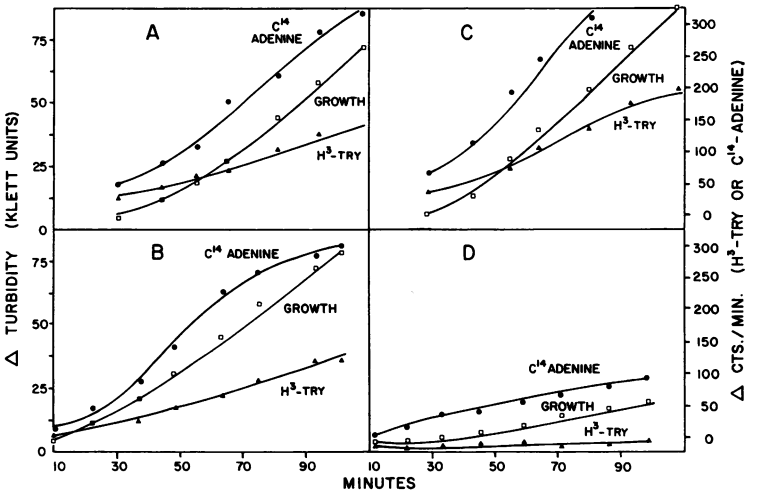

FIG. 4.—The effect of erythromycin on the uptake of C^{14}-adenine and H^3-tryptophan by erythromycin-sensitive and erythromycin-resistant *B. subtilis*. At the start of the incubation the bacteria ($\sim 10^8$/ml) were suspended in standard minimal salts medium containing glucose (0.5%), neutralized acid hydrolyzed casein (0.02%), H^3-L-tryptophan (5 μg/ml, 4 μC/mg), and 8-C^{14}-adenine hydrochloride (50 μg/ml, 0.02 μC/mg). Logarithmically growing cells from the flasks growing without erythromycin were used to inoculate the flasks containing erythromycin. Aliquots of each bacterial incubation were removed at varying time intervals, the bacteria collected by filtration and washed, and the uptake of the radioactive constituents was determined as described in the text. *A*—erythromycin-resistant *B. subtilis*, without erythromycin. *B*—erythromycin-resistant *B. subtilis*, with erythromycin (1 μg/ml). *C*—erythromycin-sensitive *B. subtilis*, without erythromycin. *D*—erythromycin-sensitive *B. subtilis* with erythromycin (1 μg/ml).

erythromycin seems to interfere with some aspect of amino acid utilization in sensitive bacteria.

Summary.—Erythromycin resistance has been studied in a resistant, genetically stable strain of *B. subtilis* and compared with the erythromycin-sensitive parent strain. Destruction of the antibiotic has been ruled out as a significant factor in this resistance, and exclusion of the antibiotic also does not appear to account for the change in erythromycin sensitivity. Using the auxotrophic requirements (L-tryptophan and adenine), it has been demonstrated that the utilization of amino acids is inhibited by erythromycin in the sensitive bacteria and that the resistant cells are able to overcome this inhibition. Thus, the mechanism of resistance seems to be one in which an increased tolerance to the antibiotic-inhibited process has occurred.

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* For paper III in this series see reference 19.

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*DELOCALIZED VERSUS LOCALIZED PICTURES IN RESONANCE ENERGY TRANSFER**

BY ZOLTAN BAY† AND ROBERT M. PEARLSTEIN‡

INSTITUTE FOR MUSCLE RESEARCH, MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS

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1. *Introduction.*—At the conference on Comparative Effects of Radiation the question was discussed whether in excitation transfer a delocalized (collective) or a localized picture should be accepted.[1] The consensus of participants appears to have been that a delocalized picture is appropriate for strong and medium interac-