Do Cellular Slime Molds Form Intercellular Junctions?

Intercellular contact specializations such as gap junctions have been thought to play a role in growth and differentiation in many systems. This idea is based on the observation that small molecules can pass directly between the cytoplasms of adjacent cells and on the strong circumstantial evidence that gap junctions may provide for this intercellular permeability (*1*). Gap junctions are found in a wide variety of organisms, and it is reasonable to ask whether they could be associated with the process of differentiation in the cellular slime mold *Dictyostelium discoideum*. In this organism small molecules, such as adenosine 3′,5′-monophosphate (cyclic AMP), play a role in the cell movements which occur during aggregation. In addition, differentiating cells bear antigenic determinants not found on vegetative cells (*2*), and a particular size of intramembranous particles (IMP's) has been implicated in the differentiation process (*3*). These observations suggest a role for cell surface components. However, several studies carried out over a number of years in our laboratories have failed to provide evidence for the existence of gap junctions or, for that matter, any other junctions (occluding, septate, or adhering) in *D. discoideum*.

In our studies we used a variety of fixation procedures at different stages of cell development. The cells were fixed with glutaraldehyde-formaldehyde or osmium [including osmium vapors, see (*4*)], or both. Both fixative concentration and buffer composition were varied. We investigated the cells and slugs with both thin-section and freeze-fracture techniques. Although small gap junctions (less than 0.1 μm in diameter) would be difficult to identify in sections, those involving as few as five to ten IMP's have been convincingly identified in freeze-fracture replicas of other cell systems. We sought evidence for the presence of intercellular junctions at various times during differentiation, from aggregating streams of amoebas to early slug stages; at no time were junctions observed.

Because we are reporting a negative result, we have explored in detail the possibility that junctions do exist in *D. discoideum* but were not detected. A conceivable argument is that typical gap junctions exist in slime molds but did not withstand the various preparative procedures that were used. This hypothesis is not very likely, as the morphology of gap junctions in other systems has been shown to be fairly insensitive to treatment with hyperosmolar salts, EDTA, or proteases, and even during cell lysis (*5*). Another possibility is that the frequency with which gap junctions occur in slime molds is so low as to have escaped detection. The lowest reported value for the area occupied by gap junctions is that calculated for BHK cells, where it is 0.05 percent for those interfaces that contain junctions (*6*). For the slime molds this would mean about 70 junctions per 1000 μm^2, each 0.1 μm in diameter (containing 75 IMP's). If such a low density of junctional material were found only in specialized regions of the cell surface (at the tips of microvilli or at the anterior and posterior ends of the cells where antigenic factors are found in differentiating slime mold cells), the overall density would be even lower, although still detectable (about seven junctions of 75 IMP's per 1000 μm^2). The detection of junctions is complicated not only by the possibility that the areas where junctions occur may be of limited size, but also by the fact that junctions may form only at very specific times and be short-lived, thus further reducing chances of detection.

We conclude that it is impossible to prove the absence of gap junctions. Electrophysiological attempts have also failed to detect low-resistance junctions, largely due to technical difficulties (*7*). We nevertheless postulate that gap junctions do not play a role in slime mold differentiation. This may not be surprising, as slime molds are commonly classified among the fungi, and gap junctions have not been observed in the plant kingdom. Failure to find gap junctions in *D. discoideum* does not lead one to generalize about the role of gap junctions in differentiation.

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Synergism Between Trimethoprim and Sulfamethoxazole

In a recent report (*1*) Poe suggests that the observed synergism between trimethoprim and sulfonamide occurs as a result of their simultaneous binding to dihydrofolate reductase. While these data direct attention to factors affecting the binding of diaminopyrimidines to the isolated enzyme, this suggestion overlooks important quantitative aspects of the inhibition of the growth of *Escherichia coli* and cannot be considered an adequate explanation of synergistic inhibition of growth by these two drugs.

The concentration of sulfamethoxazole required in this experiment to enhance the binding of trimethoprim is greatly in excess of that needed to produce synergism in experiments with growing organisms. Bushby (*2*) has shown that the concentration of sulfamethoxazole required to obtain strong synergistic effects on the growth of various *E. coli* strains in the presence of trimethoprim varies between 2000 and 61,000 times lower than the inhibition constant, K_i, of sulfamethoxazole for *E. coli* dihydrofolate reductase as measured by Poe (*1*). We know of no evidence which shows that sulfamethoxazole is concentrated by this organism.

Poe (*1*) quotes from Webb (*3*, pp. 498–500) the conclusion that multiple blockade of a linear sequence of irreversible reactions is theoretically incapable of producing greater inhibition than a single agent alone. From this he argues that sequential inhibition of the biosynthesis of dihydrofolate and its subsequent reduction by sulfamethoxazole and trimethoprim, respectively, cannot account for synergism. However, Webb also states that the operation of an isolated monolinear chain in the cell is probably very uncommon. In fact, dihydrofolate reductase is involved in the recycling of the tetrahydrofolate which is oxidized to dihydrofolate by the action of thymidylate synthetase, as well as the synthe-

sis of tetrahydrofolate de novo. Trimethoprim thus inhibits an enzyme of a cyclic pathway, and the overall concentration of intermediates of this pathway can be reduced by sulfonamides. Webb (*3*, p. 501) has noted that in similar cases two such inhibitors might have a combined effect out of all proportion to their individual inhibitions. Such effects could lead to synergism at the inhibitor concentrations where synergism is observed experimentally in growing bacteria.

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Poe's observation (*1*) that sulfonamides weakly inhibit dihydrofolate reductase and influence binding of trimethoprim to the same enzyme led him to propose an alternative explanation for the currently accepted mechanism of synergism between these drugs. While the effects of simultaneous binding to isolated dihydrofolate reductase have to await confirmation, at this laboratory we think that there are several relevant facts which are difficult to reconcile with the mechanism proposed by Poe (*1*).

Several authors have observed that dihydrofolate reductase inhibitors of widely varying structures potentiate sulfonamides to the same extent as does trimethoprim in vitro. The lack of synergism between 2,4-diaminopteroylaspartate and sulfadiazine, which was cited by Poe as being an exception, refers to chemotherapeutic experiments with plasmodia and is readily explained by the fact that this compound, similar to other folate derivatives, does not penetrate bacterial and plasmodial cells, as was also mentioned by Rollo (*2*).

If the data from numerous published isobolograms of trimethoprim-sulfamethoxazole combination are replotted in molar concentrations, one finds that the concentrations needed to produce synergism are in the nanomolar range for trimethoprim and in the micromolar range for sulfamethoxazole (*3*). Hence the sulfonamide concentration is generally four orders of magnitude below that which is needed to obtain any observable synergistic effect in Poe's model. These concentrations are reached in most tissues.

The most difficult fact to reconcile with Poe's proposal is the effect of *p*-aminobenzoic acid (*p*ABA), which in low concentrations completely eliminates any antibacterial activity of the sulfonamide and any synergism with trimethoprim. We observed that the presence of 1 μg of *p*ABA per milliliter ($7.6 \times 10^{-6} M$) completely suppressed potentiation, leaving unimpaired the activity of trimethoprim. This has also been demonstrated in growth-kinetic experiments (*4*). On the other hand, we also found that *p*ABA inhibited by 50 percent the activity of dihydrofolate reductase at a concentration of $1.5 \times 10^{-2} M$ (0.06 m*M* substrate). Hence, if one assumes that Poe's mechanism is correct, micromolar concentrations of *p*ABA would probably have to replace millimolar concentrations of sulfonamide acting on the dihydrofolate reductase. This remains to be proved.

We conclude that in vitro and in vivo the mechanism proposed by Poe (*1*) can hardly be considered as an alternative to what has so far been more adequately explained by the mechanism of sequential blockade.

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As was stated in (*1*), "Very high concentrations of sulfonamide are required to observe this synergism [between trimethoprim and sulfamethoxazole]." Burchall and Then correctly note a number of situations where dihydrofolate reductase inhibition by sulfa drugs could not be of significance since biological effects were noted at micromolar concentrations of the drugs; this includes Then's data for *p*-aminobenzoic acid. Nevertheless, very high concentrations of sulfamethoxazole [up to $2 \times 10^{-3} M$ (*2*)] are attained in normal trimethoprim-sulfamethoxazole clinical regimens. And, the theory of sequential inhibition does not provide an explanation for synergism between sulfa drugs and trimethoprim noted in sulfa-resistant organisms (*3*); in this case high concentrations of sulfa are used.

Then's suggestion that classical antifolates such as methotrexate and 2,4-diaminopteroylaspartate do not potentiate sulfa action in bacteria and plasmodia because of membrane impermeability is well made.

In summary, the available data do not rule out the possibility that the theory of multiple simultaneous inhibition of dihydrofolate reductase accounts for at least some of the trimethoprim-sulfamethoxazole synergism observed in clinical situations.

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Marihuana and Epilepsy

In a recent paper on cannabinoid induction of behavioral seizures in a strain of rabbits (*1*) Martin and Consroe make several statements that should be clarified.

First, they state that in other species electroencephalographic (EEG) patterns of convulsive-like activity and behavioral seizures have only been reported after administration of "lethal or near-lethal doses" of cannabinoids. If accurate, this would suggest that their observations of seizures are perhaps an idiosyncratic strain-specific reponse of their rabbits to cannabinoids, and the relevance to human epilepsy would be unclear. Since they do not report the lethal dose for their strain of rabbits, it is also possible that their epileptogenic dosages were close to the lethal dose (they note the death of one subject). However, we have reported similar results with comparable doses of cannabinoids in two other species; the naturally epileptic beagle dog, and cats with focal epilepsy induced by injection of alumina cream into the motor cortex. In our work (*2*) we have noted activation of temporal lobe seizures and myoclonus in dogs given a single oral dose (5 mg/kg) of Δ^9-tetrahydrocannabinol (Δ^9-THC), a psychoactive ingredient of marihuana. This is not a "near-lethal" dose since we did not observe any fatalities with dosages as high as 20 mg/kg (oral). In the cat we have reported activation of interictal spike discharges in