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## *THE ROLE OF RNA IN REPRESSION OF ENZYME SYNTHESIS**

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It is known that chloramphenicol (CAP) inhibits protein synthesis by interfering with the transfer of amino acids from sRNA to ribosomes.[1] In agreement with the general effect of CAP on protein biosynthesis, the synthesis of numerous individual enzymes is inhibited by this antibiotic.[2] Working with low concentrations of CAP, it was demonstrated that the synthesis of various repressor-controlled enzymes was affected to a greater degree than the formation of total protein or constitutive enzymes.[3, 4] The preferential inhibition by CAP of the synthesis of β-galactosidase, a repressor-controlled enzyme, is attributed to the reduced rate of formation of β-galactosidase messenger RNA.[4] Therefore, CAP preferentially inhibits enzyme synthesis by the same mechanism involved in enzyme repression.

Not only is the CAP-promoted repression a general effect for repressor-controlled enzymes, but it requires a functional regulator gene for the appropriate enzyme. Thus, CAP does not repress β-galactosidase or tryptophan synthetase in strains mutant for the respective regulator genes.[5] Furthermore, the CAP-promoted repression of inducible enzymes is not a manifestation of catabolite repression, nor does the repression of biosynthetic enzymes result from increased pools of biosynthetic end products.[5]

The participation of a functional regulatory system in the CAP-promoted repression of enzyme synthesis indicates that CAP elicits the increased production of specific repressor molecules within the cell. With the goal of obtaining evidence on the nature of the molecules which might be responsible for CAP-promoted repression, some of the metabolic changes which occur during CAP treatment have been examined. The data of this report demonstrate a consistent correlation between high relative rates of RNA synthesis, concomitant with the repression by CAP of β-galactosidase synthesis. These findings support the hypothesis[6] that certain polyribonucleotides are involved as repressors of enzyme synthesis.

*Materials and Methods.—Bacterial cultures and media:* The following strains of *Escherichia coli* were employed: ML30, inducible for β-galactosidase; $15T^{-}U^{-}M^{-}$ (from T. D. Brock), inducible for β-galactosidase and auxotrophic for thymine, uracil, and methionine; K-12: 3000, inducible for β-galactosidase; and K-12: 3300 (from F. Jacob), constitutive for β-galactosidase. Experiments were usually conducted in a mineral salts medium with 0.5% glycerol as carbon source.[4]

Shift-up experiments were conducted by first growing the bacteria in minimal medium supplemented with arginine, leucine, glycine, alanine, valine, threonine, isoleucine, and lysine. To effect the shift, vitamin-free casamino acids (0.1%), L-tryptophan (50 $\mu$g/ml), and the ribonucleosides uridine, cytidine, guanosine, and adenosine (each at 25 $\mu$g/ml) were added to the medium. The final differential rates of $\beta$-galactosidase synthesis were the same in both media.

Shift-down experiments were conducted by first growing the bacteria in glycerol-salts medium supplemented with 200 $\mu$g/ml L-serine, in which $NH_4^+$ and serine provided the nitrogen sources. The bacteria were removed from the medium by filtration, and then resuspended in nitrogen-free salts medium with L-serine as the sole nitrogen source. This procedure resulted in an uninterrupted shift in the growth rate from 0.60 doublings/hour to 0.45 doublings/hour for bacteria growing in the absence of CAP.

*Biochemical and enzymological methods:* $\beta$-galactosidase activity was determined as previously described.[4] Protein was determined by the method of Lowry *et al.*,[7] and RNA was assayed by the orcinol method,[8] using purified yeast RNA as a standard.

*Inhibitor studies:* Experiments with bacteria growing logarithmically were performed as described by Sypherd and DeMoss.[5]

For resting cell studies, the bacteria were depleted of thymine, uracil, or methionine by a 45-min incubation, with vigorous shaking in the absence of the particular nutrient, followed by a similar 45-min incubation in the absence of both the nutrient and glycerol. Following the successive starvation periods, growth could be initiated only by providing the suspension with both the nutrients for which they were starved. Protein synthesis in resting cells was determined from the quantity of $C^{14}$-leucine incorporated into TCA-insoluble material. $\beta$-Galactosidase was induced with methyl-$\beta$-D-thiogalactoside (TMG), at a final concentration of $10^{-3}$ *M*. In some cases, isopropyl-$\beta$-D-thiogalactoside (IPTG) was used, but there was no qualitative difference in the results with either TMG or IPTG. CAP was used in a concentration which would reduce the rate of $C^{14}$-leucine incorporation to 50–60% the control rate (*ca.* 0.8–1.2 $\mu$g/ml).

*Results.*—The addition of CAP (0.8 $\mu$g/ml) to an exponentially growing culture results in an immediate shift to a lower growth rate. Accompanying this shift is a more severe reduction in the rate of $\beta$-galactosidase synthesis (Fig. 1*A* and *B*). Figure 1*B* also shows that the rate of DNA synthesis continues unabated, while RNA synthesis is accelerated by CAP. This latter phenomenon has been reported by others.[9, 10]

In view of the hypothesis that repressor levels are elevated in CAP-treated cells,[5] and the fact that the RNA synthesis is similarly stimulated, several experimental approaches were used to test the possibility that accelerated rates of RNA synthesis

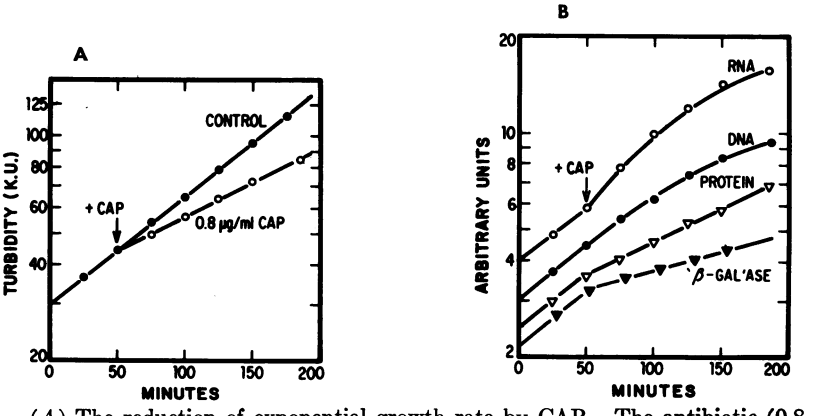


FIG. 1.—(*A*) The reduction of exponential growth rate by CAP. The antibiotic (0.8 $\mu$g/ml) was added to a culture of ML30 growing exponentially in glycerol-salts medium. (*B*) The synthesis of macromolecules in CAP-treated culture shown in (*A*).

were essential for the manifestation of CAP-promoted repression of β-galactosidase formation. The rates of RNA and β-galactosidase synthesis were determined during prolonged growth in CAP. It was shown previously that the CAP-promoted repression of β-galactosidase formation is released after approximately three generations in CAP.[4] This recovery of enzyme synthesis occurs even though the rates of growth and total protein synthesis remain inhibited. Figure 2*A* and *B* shows a close correlation between the repression by CAP of β-galactosidase and the concomitant stimulation of RNA synthesis. It can also be seen that the rate of RNA synthesis declines to the normal, pretreatment rate just prior to the recovery of β-galactosidase formation.

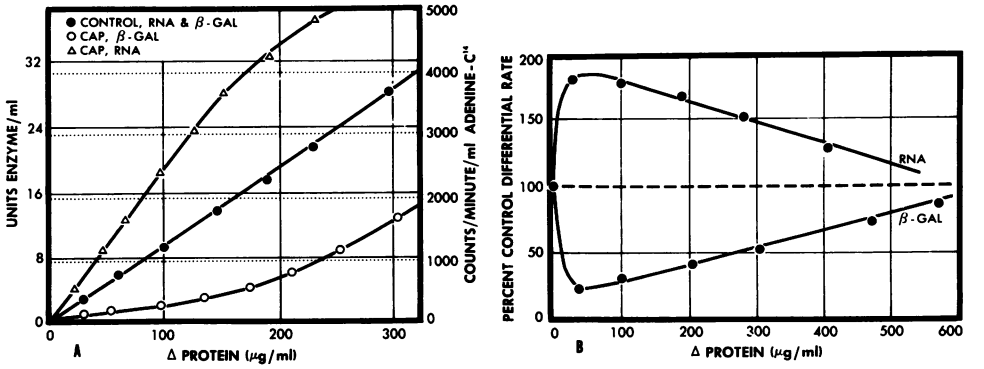


FIG. 2.—(*A*) The synthesis of β-galactosidase and RNA in CAP-treated and nontreated cultures of ML30 growing exponentially in glycerol-salts medium with $10^{-3}$ *M* TMG. Growth proceeded for over three generations after the addition of CAP. (*B*) The values for β-galactosidase and RNA in the CAP-treated culture in (*A*) are plotted as per cents of the respective rates in the nontreated culture.

It was previously shown[4, 5] that CAP does not repress β-galactosidase synthesis in nitrogen-depleted cells, which may indicate that a nitrogenous compound mediates the CAP-promoted repression. The polynucleotide nature of such a compound may be inferred from experiments with a thymine-less, uracil-less auxotroph. A strain of *E. coli* ($15T^-U^-M^-$), which requires thymine, uracil, and methionine for growth, was starved for each of the pyrimidines separately. The depleted cultures were then induced to synthesize β-galactosidase in the presence and absence of CAP. Figures 3 and 4 show that CAP elicited the repression of β-galactosidase synthesis only in the culture which was starved for thymine. Repression did not occur in the uracil-starved culture, even though leucine-$C^{14}$ incorporation was inhibited 45 per cent. It was also found that when a culture of this organism was starved for methionine, CAP did not repress β-galactosidase synthesis. While this result must be interpreted cautiously, it is significant that RNA synthesis ceases when this organism is depleted of its methionine. The conclusion from these experiments is that uracil (and perhaps methionine) deprivation resulted in a loss of the CAP-promoted repression due to the limited RNA synthesis (or turnover) which occurs under these conditions. The experiments also indicate that a uracil-containing class of molecules mediates the CAP-promoted repression of enzyme synthesis.

The results obtained with the starved cultures support those obtained with growing cells: there is a correlation between the CAP-promoted repression of β-

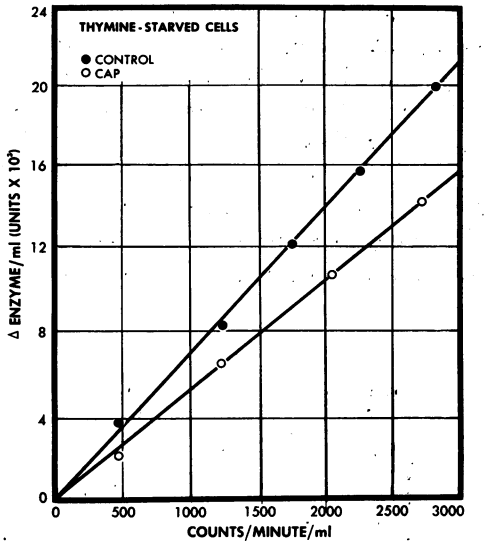


FIG. 3.—The relative rates of synthesis of β-galactosidase after induction with TMG ($10^{-3}$ *M*) in a thymine-glycerol-starved culture of 15T⁻U⁻M⁻. $C^{14}$-Leucine incorporation was inhibited 40% in the CAP-treated culture.

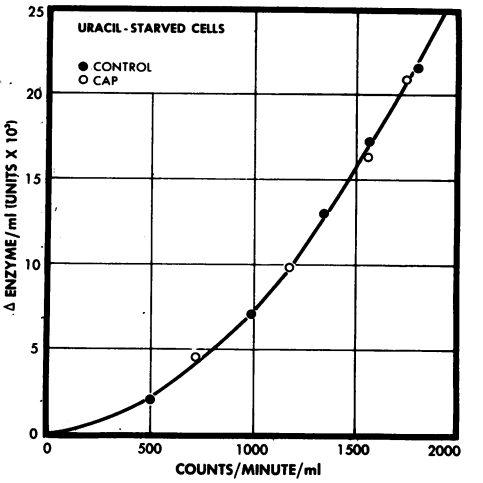


FIG. 4.—The relative rates of synthesis of β-galactosidase after induction with TMG ($10^{-3}$ *M*) in a uracil-glycerol-starved culture of 15T⁻U⁻M⁻. $C^{14}$-Leucine incorporation was inhibited 45% in the CAP-treated culture.

galactosidase synthesis and the stimulation of RNA synthesis which occurs during CAP treatment. All these data suggest that increased rates of RNA synthesis are necessary for the CAP-promoted repression to occur. The obligatory nature of this relationship is suggested by experiments in which RNA synthesis in CAP-treated bacteria is halted. It is known that shifting a culture to a slower growth rate will result in the immediate cessation of RNA synthesis.[11] In such "shift-down" cultures, RNA formation resumes only after the synthesis of sufficient protein to establish the protein/RNA ratio characteristic for the new growth rate. Shift-down conditions (see *Materials and Methods*) similarly result in the cessation of RNA synthesis in CAP-treated cells (Fig. 5*A*). Figure 5*B* shows that following the shift and the subsequent cessation of RNA synthesis, the repression by CAP of β-galactosidase synthesis is released. Under these conditions, when RNA is no longer being made at an accelerated rate, the rate of β-galactosidase formation in the CAP-treated culture returns to the normal, pretreatment rate.

Thus, cessation of the accelerated rate of RNA synthesis in CAP-treated cells results in the release of the CAP-promoted repression of β-galactosidase synthesis. These data are in agreement with previous indications that RNA synthesis must proceed at an accelerated rate to maintain the repression by CAP of β-galactosidase synthesis.

The conclusion derived from the above experiments is that the CAP-promoted repression of β-galactosidase synthesis is the result of RNA synthesis proceeding at a disproportionately greater rate than total protein synthesis. Thus, it might be predicted that any condition which leads to accelerated rates of RNA synthesis will also elicit the repression of β-galactosidase synthesis. It is possible to establish conditions which yield accelerated rates of RNA synthesis in the absence of CAP.

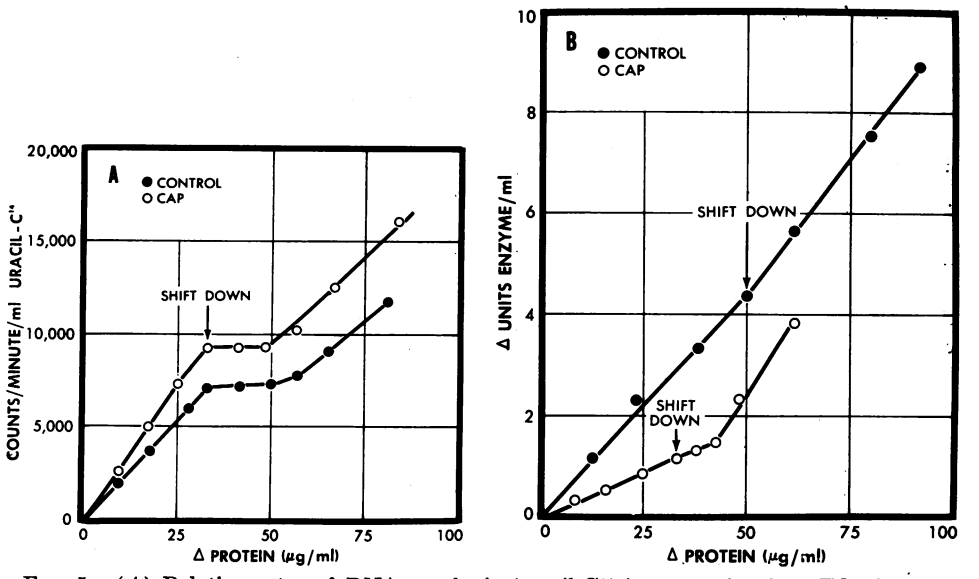


FIG. 5.—(*A*) Relative rates of RNA synthesis (uracil-$C^{14}$ incorporation into TCA-insoluble material), before and after shifts to slower growth rates, of a CAP-treated and nontreated culture of ML30. (*B*) Relative rates of synthesis of β-galactosidase following shifts to slower growth rates of the cultures shown in (*A*).

For example, when the growth rate of a culture is abruptly increased (shifted-up), RNA synthesis will proceed at a greater rate than protein synthesis, until the RNA/protein ratio reaches that which is characteristic of the new growth rate.[11] If an accelerated rate of RNA formation is sufficient to elicit repression of β-galactosidase synthesis, it is predicted that in a culture which undergoes "shift-up," β-galactosidase synthesis will be repressed. This prediction was tested by subjecting a culture of K-12: 3000 (i+) to shift-up conditions (see *Materials and Methods*) in the presence of the inducer TMG. Table 1 shows that following the shift-up, RNA synthesis proceeds at a greater rate than total protein synthesis. In Figure 6, it can be seen that concomitant with the acceleration of RNA synthesis, there is a transient repression of β-galactosidase synthesis. This repression occurs during the time that the RNA/protein ratio is undergoing its greatest change (Table 1).

Before the conclusion could be made that shifting-up produces the same effect on β-galactosidase synthesis as does CAP, a similar experiment was performed with the β-galactosidase-constitutive mutant, K-12: 3300. β-Galactosidase syn-

TABLE 1

GROWTH RATES AND RNA/PROTEIN RATIOS BEFORE AND AFTER A SHIFT-UP IN GROWTH

| Time after shift-up | K-12: 3000 (i+) | | K-12: 3300 (i−) | |
|---|---|---|---|---|
| | *k* | RNA/prot. | *k* | RNA/prot. |
| −60 | 0.47 | 0.275 | 0.48 | 0.260 |
| −30 | 0.47 | 0.273 | 0.48 | 0.260 |
| 0 | 0.47 | 0.275 | 0.48 | 0.260 |
| 15 | 1.0 | 0.355 | 1.1 | 0.342 |
| 30 | 1.1 | 0.485 | 1.2 | 0.477 |
| 60 | 1.1 | 0.580 | 1.2 | 0.560 |
| 80 | 1.1 | 0.575 | 1.2 | 0.568 |

*k* = growth rate in doublings/hour.

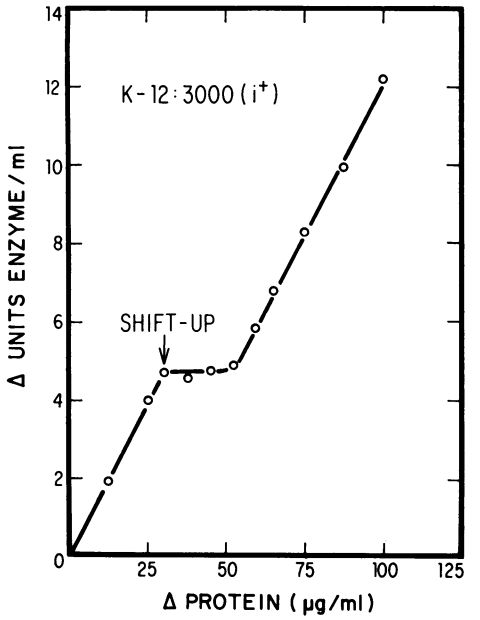


FIG. 6.—The effect on β-galactosidase synthesis of a shift to a higher growth rate (see *Materials and Methods*) of K-12: 3000 (i+).

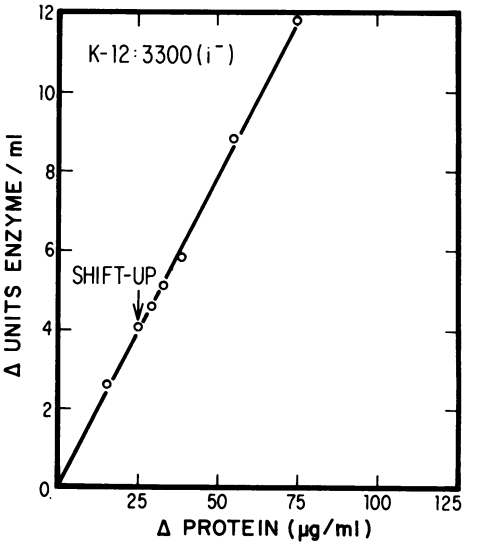


FIG. 7.—The absence of an effect on β-galactosidase of a shift to a higher growth rate of K-12: 3300 (i−).

thesis in this organism is resistant to repression by CAP.[5] If the repression elicited by shifting is similar to that elicited by CAP, shifting-up should not result in the repression of enzyme synthesis in this organism. The experiment was performed in the same manner as with the inducible organism, and the results are shown in Figure 7. Although the shift resulted in the same per cent increase in the growth rate and in RNA synthesis (Table 1), β-galactosidase synthesis in this mutant was not affected by the shift. It is evident, therefore, that the gene change i+ → i− in K-12: 3300 renders β-galactosidase nonrepressible both by CAP[5] and by a shift-up in growth rate. Since β-galactosidase synthesis in the i− mutant is susceptible to catabolite repression,[5] it may be concluded that the effects of a shift-up in growth on enzyme synthesis in the i+ strain (Fig. 6) are not due to catabolite repression. An interpretation of these experiments is that a shift-up in growth, like CAP treatment, produces accelerated rates of RNA synthesis and of the formation of specific repressor molecules. The resulting increase in repressor levels may lead to the repression of induced enzyme synthesis. However, in the constitutive mutant, which presumably makes no repressor, the acceleration of RNA synthesis, either by CAP treatment or shift-up in growth, is without effect on enzyme synthesis. In this way, the imbalance of macromolecule synthesis during shift-up may produce effects on β-galactosidase synthesis similar to those elicited by CAP.

*Discussion.*—The stimulation by CAP of total RNA synthesis has been shown by other workers, and provided much of the rationale for investigating its relationship to the CAP-promoted repression of β-galactosidase. The results from the present study show a clear inverse relationship between the rates of synthesis of RNA and of β-galactosidase in CAP-treated cells. In a previous study we showed that

the repression of β-galactosidase in exponentially growing cells may be elicited by certain antibiotics other than CAP,[4] but not by general growth inhibitors.[4, 5] It has also been found that total RNA synthesis is stimulated by all those antibiotics which elicit the repression of β-galactosidase.[12] We have been led to conclude that the repressive action of CAP is the result of an imbalance in the synthesis of macromolecules following antibiotic treatment, rather than a direct involvement of CAP in the regulatory system of β-galactosidase.

The fact that the CAP-promoted repression of β-galactosidase synthesis acts through the normal regulatory system[5] makes it attractive to postulate that CAP treatment results in a relative enrichment of specific repressor molecules. Furthermore, the finding that the repression of β-galactosidase synthesis is accompanied by the elevation of the internal concentration of RNA leads us to propose that a polyribonucleotide functions as a repressor component. It may be argued that among the polyribonucleotides accumulated during CAP treatment, there exists messenger RNA for a protein repressor. This could then lead to an increased rate of synthesis of a protein-repressor component. However, this possibility makes it necessary to postulate that the synthesis of such a repressor protein is less sensitive to inhibition by CAP than the synthesis of all other proteins. Therefore, the simplest interpretation of the data presented here is that the RNA acts directly.

The conclusion regarding the participation of RNA as a repressor component is necessarily tempered by the fact that a causal relationship between the stimulation by CAP of RNA synthesis and the CAP-promoted repression of β-galactosidase synthesis could not be unequivocally demonstrated. The possibility that specific repressors of enzyme synthesis are polyribonucleotide has been proposed by a number of workers in recent years.[6] However, Jacob *et al.*[13] have presented evidence, involving suppressor mutations, that the repressor of λ phage is polypeptide in nature. Similar evidence has recently been reported by Garen and Garen,[14] suggesting that the products of the regulator genes for alkaline phosphatase are protein. Monod *et al.*[15] have proposed that "allosteric" enzymes function as repressors of enzyme synthesis. Therefore, we suggest that repressor systems involve at least two components. One component, a protein, has specificities for a small molecule "effector"[6] and for a polyribonucleotide. The second component is the polyribonucleotide, with specificity for the operator gene or gene product.[16] One can readily develop models which place repressor function in a system involving both an RNA and a protein component. Thus, it is probable that the exact nature of all the components of specific repressor systems can be elucidated only in *in vitro* enzyme synthesizing systems.

*Summary.*—Several experimental approaches have shown that a close correlation exists between the CAP-promoted repression of β-galactosidase synthesis and the stimulation of RNA synthesis. These findings have led to the conclusion that a polyribonucleotide functions as a component of the regulatory system for β-galactosidase.

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† The data of the report were taken from a thesis submitted by the senior author to the Graduate

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## *PERIODIC BEHAVIOR IN CHARGED MEMBRANES AND ITS PHYSICAL AND BIOLOGICAL IMPLICATIONS**

BY R. H. ARANOW

RIAS, BALTIMORE, MARYLAND

*Communicated by S. Lefschetz, October 25, 1963*

Periodic variations in solvent flow, electrical potential, concentration, and pressure in membranes can be explained as a consequence of the solution to the hydrodynamic equations of motion if all forces and boundary conditions are taken into account.

In the effort to explain the periodic behavior observed in the nerve model system of Teorell[1] where a silica gel membrane separated two stirred compartments containing electrolyte of the same species but of different conductance, Teorell used the equations of electrokinetics but ignored the gradient of chemical potential and the dependence of current flow on pressure. He introduced nonlinearity by deriving a relationship in which the steady-state resistance is a nonlinear function of bulk flow. He then introduced the *ad hoc* assumption that the rate of change of the instantaneous resistance is proportional to the difference of the instantaneous resistance from the steady-state value. With his equations he succeeded in describing quantitatively the results of his experiments.

We have succeeded in deriving all of Teorell's equations from first principles and in the process have shown that the periodic behavior is a consequence of the effect of boundary conditions on hydrodynamic stability.

The implications of the theory to be presented and of Teorell's experiment are