[2] Kaplan, N. O., M. M. Ciotti, M. Hamolsky, and R. E. Bieber, *Science*, **131**, 392 (1960).
[3] Cahn, R. D., N. O. Kaplan, L. Levine, and E. Zwilling, *Science*, **136**, 962 (1962).
[4] Pesce, A., R. H. McKay, F. Stolzenbach, R. D. Cahn, and N. O. Kaplan, in preparation.
[5] Cahn, R. D., N. O. Kaplan, L. Levine, and S. White, *Fed. Proc.*, **21**, 407 (1962).
[6] Brand, L., J. Everse, and N. O. Kaplan, *Biochemistry*, **1**, 423 (1962).
[7] Cahn, R. D., Ph.D. dissertation, Brandeis University (1962).
[8] Wieme, R. J., and J. E. Herpol, *Nature*, **194**, 287 (1962).
[9] Fine, I. H., N. O. Kaplan, and D. Kuftinec, *Biochemistry*, in press.
[10] Fine, I. H., N. O. Kaplan, and S. White, *Fed. Proc.*, **21**, 409 (1962).
[11] Dewey, M. M., and J. L. Conklin, *Proc. Soc. Expt. Biol. Med.*, **105**, 492 (1960).
[12] Dreyfus, J. C., J. Demos, F. Schapira, and G. Schapira, *C. R. Acad. Sci. (Paris)*, **254**, 4384 (1962).
[13] Personal communication from C. A. Markert.

*THE INHIBITION OF A BIOLOGICAL CLOCK BY ACTINOMYCIN D**

BY MARLENE W. KARAKASHIAN† AND J. WOODLAND HASTINGS

BIOCHEMISTRY DIVISION, UNIVERSITY OF ILLINOIS, URBANA

Communicated by H. E. Carter, October 1, 1962

Persistent daily rhythms have been studied in organisms ranging from unicellular to mammalian forms.[1] Characteristically, such rhythms involve a daily variation in some biological function, with a maximum occurring at a particular time of day. In *Gonyaulax polyedra*, the marine dinoflagellate used in the present study, a rhythm of bioluminescence exhibits a maximum late at night, whereas photosynthetic capacity, which is also rhythmic, is greatest during the day.[2]

Evidence from numerous investigations indicates that rhythms are a manifestation of a biological time-measuring system, exerting temporal control over the capacities of specific biochemical processes. Under constant conditions (Fig. 1) diurnal rhythmicity in *Gonyaulax* persists undamped. The period, although approximately equal to the solar day, is actually slightly different from 24 hours.[3] Similar observations have been reported from studies of rhythms in many other organisms.

In spite of the compelling evidence concerning the existence of a "metabolic cellular clock," substantial understanding concerning the chemical pathways involved in its operation and control has been lacking. In fact, some features of rhythms are not altogether characteristic of a metabolically dependent physiological function. First, the frequency is essentially temperature-independent; that is to say, the period of a rhythm is relatively unaffected when organisms are placed in different (but constant) temperatures.[4, 5] Second, rhythms have been reported to be insensitive to a variety of metabolic inhibitors. In the studies reported it was not possible to alter appreciably either the timing (phase) of a rhythmic function or to abolish its expression by various agents tested.[6–8]

These observations concerning temperature and inhibitor effects have been taken by some workers as evidence that biological timing mechanisms must be related to an exogenous "signal."[9] These same facts, however, are consistent with the hypothesis that rhythms relate to a truly functional biological clock, since the utility of a feature such as temperature independence for a timekeeping mechanism is evident.

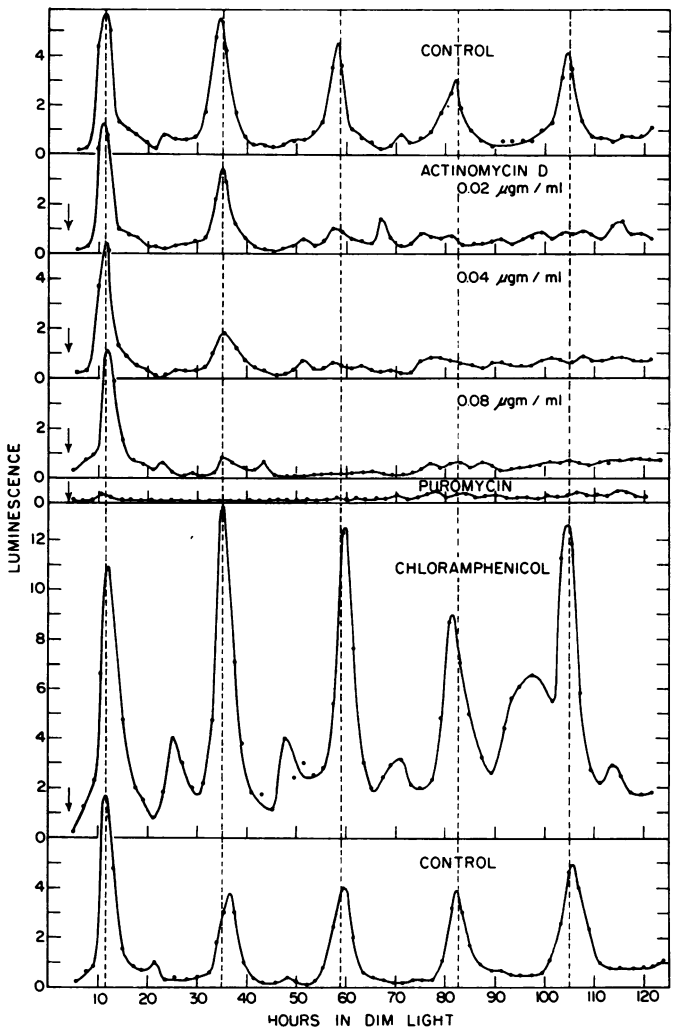

FIG. 1.—Effects of actinomycin D (concentrations as noted on figure), chloramphenicol (3×10^{-4} *M*), and puromycin (10^{-5} *M*) upon the persistent rhythm of bioluminescent glow. Top curve: control for experiments with actinomycin D addition (next three curves). Bottom curve: control for experiments with chloramphenicol and puromycin. Cells were grown in a cycle of alternating light and dark periods of 12 hr each and placed in constant dim light and constant temperature at the end of a light period, zero time on the graph. Inhibitors were added at the time indicated by the arrows. Luminescence in arbitrary units. Vertical grid lines are for guidance only.

In the course of an investigation of the action of agents known to interfere with the synthesis of macromolecules, we have found that actinomycin D (also referred to as C_1), influences these biological rhythms. Actinomycin D is one of a group of antibiotics having two peptide chains attached to a phenoxazone chromophore.[10, 11] Its inhibitory effect is believed to be related to its strong and specific binding to the guanine residue of DNA.[12, 13]

All known effects of low concentrations of actinomycin D upon living cells are consistent with the hypothesis that it acts by inhibiting DNA-dependent RNA syn-

thesis. Kirk, using rapidly growing *Staphylococus aureus* cells, found that 0.2 μg/ml actinomycin D stopped RNA synthesis immediately and protein synthesis shortly thereafter, but the partial inhibition of DNA synthesis did not occur until considerably later.[14] She found no effects upon respiration and glycolysis even at very high concentrations. In mammalian cells in tissue culture Reich and coworkers have shown that 0.1 μg/ml actinomycin D inhibits the synthesis of cellular RNA and the yields of the DNA-containing vaccinia virus. However, in the same host cell, neither cellular DNA synthesis, nor the multiplication and yield of Mengovirus, whose genetic material consists of RNA, is affected.[15]

In a cell-free system, Hurwitz *et al.*[16] have reported the inhibition of purified RNA polymerase by low concentrations of actinomycin, and have shown that the site of inhibition is the DNA primer.

In the studies reported here we have found that actinomycin D alters the expression of the luminescent and photosynthetic rhythms in *Gonyaulax*. This constitutes the first positive evidence concerning the nature of the biochemical pathway involved in a biological clock mechanism.

Experimental.—Many of the experimental techniques used have been described previously.[8, 17] Cultures were grown in liter quantities under conditions of alternating light and dark periods of 12 hours each at 21°C. At a density of about 3,000 cells per ml, cultures were dispensed in 12-ml aliquots into vials suitable for the Packard liquid scintillation counter turntable.[18] A modification of this instrument permitted the repeated measurement and recording (every two hours) of the spontaneous glow of luminescence[8, 19] of the cells in each of the vials. The cells were kept on the turntable in constant dim light (125 foot-candles) and constant temperature (21°C) throughout the experiment. Measurements of photosynthetic capacity were made by determining the light-dependent incorporation of $C^{14}O_2$ using procedures previously described.[20]

The type of luminescent rhythm which occurs is shown in Figure 1. The two control curves (top and bottom) show the persistence of the rhythm under constant conditions and the degree of variation encountered from one experiment to another.

The effects of three different concentrations of actinomycin D added at the times indicated by the arrows are shown. At all concentrations tested (up to 1 μg/ml) the first luminescence cycle after addition of actinomycin is expressed, although its amplitude may be diminished at higher concentrations. At lower concentrations the second cycle is also expressed, although with a somewhat smaller amplitude. Thereafter, no further expression of rhythmicity in bioluminescence is observed.

The other effects of actinomycin upon the cells have been evaluated in several ways. Growth curves for various concentrations of actinomycin D under conditions of alternating light and dark periods are shown in Figure 2. It should be noted that at a concentration of 0.02 μg/ml, where the rhythm is ultimately lost, growth is only partially inhibited. In this way the possibility exists of obtaining a growing culture in which the luminescence rhythm is selectively inhibited. At the higher concentrations shown, growth is stopped completely within 48 hours, although the pattern of growth inhibition is somewhat unusual, in that the occurrence of divisions after the addition of actinomycin D is concentration-dependent.

In spite of the fact that growth has been stopped by actinomycin D, and even after no additional divisions occur (Fig. 2), the cells are not immediately killed.

The cells remain intact morphologically and physiologically, as evidenced by their microscopic appearance and motility, by the continued low level of nonrhythmic luminescence emission (Fig. 1), and by their ability to photosynthesize, measured as light-dependent $C^{14}O_2$ fixation. The cells differ from untreated ones principally in that they are somewhat less motile, significantly larger in size, and more heavily pigmented.

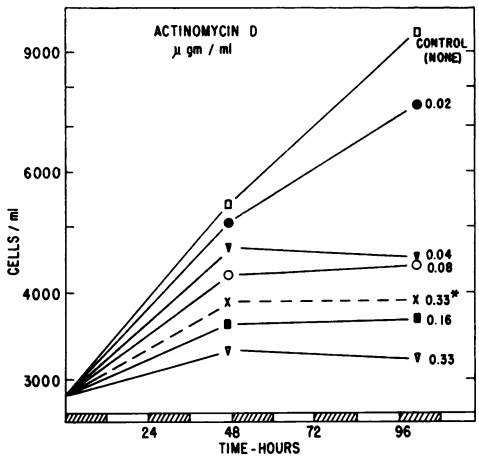

FIG. 2.—Effect of actinomycin D at the concentrations indicated upon cell division in *Gonyaulax*. Ordinate, cell number on a logarithmic scale; abscissa, time. Cultures were maintained on a cycle of alternating light and dark periods of 12 hr each. This graph also illustrates (dashed line with asterisk) the light inactivation of actinomycin D. A solution of actinomycin D was exposed to a bright fluorescent light (1,000 foot-candles) for 48 hr prior to being added to a growing culture. It is less effective than the unirradiated inhibitor, but still retains considerable biological activity.

In addition to the direct inhibitory effect of actinomycin D upon DNA-dependent RNA synthesis, there occurs in many cells an inhibition of protein synthesis by actinomycin.[14] This is due to the dependence of protein synthesis upon the continued production of a short-lived messenger RNA.[21] The inhibition of the rhythmicity by actinomycin could be explained therefore either by its direct effect on RNA synthesis or by its indirect effect on protein synthesis. An attempt to distinguish between these two possibilities was made by testing the effects of inhibitors affecting protein synthesis (Fig. 1).

As previously reported,[8] chloramphenicol penetrates the cell and stops division and growth. As illustrated however, the luminescent rhythm persists. The strikingly increased amplitude may be due to the stimulation of messenger-like RNA production reported to occur in some microbial systems upon the addition of chloramphenicol.[22]

On the other hand, there is a very clear inhibition of rhythmicity subsequent to the addition of puromycin. The compound promptly inhibits growth and luminescence, including the expression of the first cycle. Again, however, although the cells fail to divide, they remain active and motile throughout the period of the experiment shown.

From Figure 1 it is evident that at least the one subsequent luminescent cycle was expressed when actinomycin was added shortly before the actual onset of the rhythmic increase in luminescence. Other experiments revealed that even higher concentrations of actinomycin (1 μg/ml) added at this time did not prevent the appearance of the subsequent luminescent peak. These results suggest that the actinomycin-sensitive step involved in the determination of a given peak occurs at some earlier time in the luminescent cycle. In order to determine the approximate time of this step, high concentrations (1 μg/ml) of inhibitor were added to separate cultures at the times indicated by arrows in Figure 3. The results show that the sensitive period occurs 20–25 hours prior to the maximum of the cycle being

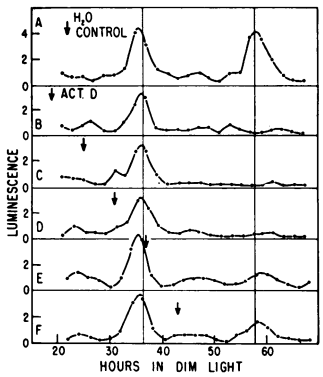

FIG. 3.—This experiment is similar to the ones with actinomycin shown in Figure 1 except that the times of addition were varied in the separate experiments so as to completely scan the cycle. In addition, a higher concentration of actinomycin D was used (1.0 μg/ml, i.e., about 10^{-6} M). These experiments serve to show that the actinomycin-sensitive step involved in the determination of the rhythmic increase in luminescence occurs at least 20 hr prior to that increase.

affected. The slight phase shift which occurs (Curve E) is a characteristic and significant phenomenon which will be described more fully in another paper.

In addition to the rhythm of bioluminescence, *Gonyaulax* exhibits persistent diurnal rhythms of both cell division[23] and photosynthetic capacity.[20] Experiments were carried out to determine if actinomycin was also effective in preventing the expression of the photosynthesis rhythm. Although actinomycin, even at very high concentrations (2 μg/ml), does not immediately decrease the rate of photosynthesis *per se*, it does inhibit the rhythm (Fig. 4). In both these respects its action is similar to the effect upon the rhythm of luminescence.

However, whereas only a single peak of luminescence occurs subsequent to the addition of high concentrations of actinomycin D (Fig. 3), in the case of the rhythm of photosynthetic capacity at least two subsequent peaks occurred. Such a result indicates the possibility that at a certain concentration of actinomycin D one rhythm may be inhibited while the other is not.

Discussion.—Numerous other inhibitory compounds have been previously studied with regard to their action on persistent daily rhythms.[8] Many of these substances clearly inhibit the organisms (for example by stopping growth) without stopping rhythmicity. Other compounds inhibit the expression of a rhythmically occurring function (photosynthesis, for example) by blocking a specific pathway. An inhibitor of this kind, such as dichlorophenyl dimethyl urea (DCMU), completely but reversibly inhibits photosynthesis at a concentration of 10^{-7} M, but has no effect upon the rhythm of bioluminescence.[8, 24]

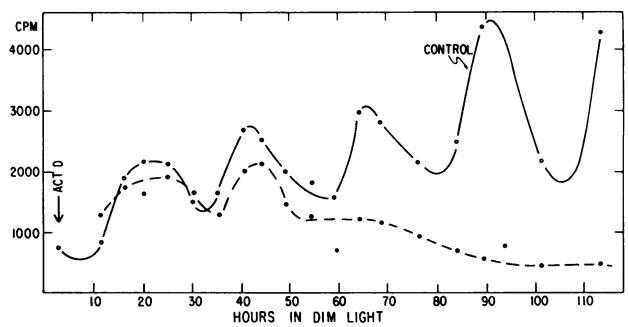

FIG. 4.—This illustrates the inhibition of the rhythm of photosynthetic capacity by actinomycin D. Cells were grown in a cycle of alternating light and dark periods of 12 hr each and placed in constant dim light and constant temperature at the end of a light period, zero time on the graph. Actinomycin D was added 4 hr later (arrow) and the rate of photosynthesis measured as counts of $C^{14}O_2$ incorporated in 20 min at a saturating light intensity (1,000 foot-candles). Temperature 20°C.

In summary, there are effective inhibitors, either inhibiting growth generally or a specific cell function, which do not affect cellular rhythmicity.

From the results presented here it is evident that the addition of actinomycin D results in a very different effect—a rather specific and selective inhibition of cellular rhythmicity without any immediate deleterious effects upon either the cells or upon the mechanism responsible for the immediate expression of the rhythm. Moreover, at a sufficiently low concentration, rhythmicity is inhibited without complete stoppage of growth. This apparent differential sensitivity is especially significant and may be of considerable importance in investigating the chemical events responsible for the rhythm.

At the biochemical level, the effect of actinomycin upon rhythmicity is presumed to be related to its binding to DNA and the consequent inhibition of DNA-dependent RNA synthesis. There are several considerations which are pertinent in connection with this hypothesis. Actinomycin D is effective in inhibiting the luminescence rhythm at very low concentrations (0.02 μg/ml), suggesting a very specific binding, such as is known with DNA. The only known effect of actinomycin D bound to DNA at these low concentrations is upon RNA polymerase. Other reactions involving DNA which have been examined, such as DNA polymerase, are not inhibited to such a marked extent.[16] In addition, the only known mechanism for informational transfer from DNA involves the synthesis of RNA as an intermediate. In normal cells all RNA synthesis is apparently DNA-dependent[25] and actinomycin D-sensitive.[15]

The hypothesis that clock function is dependent upon RNA synthesis presents no difficulties as a consequence of the fact that growth and division (but not rhythmicity) may be stopped by various agents, including at least two inhibitors of DNA synthesis: 5′-fluoro-2′ deoxyuridine (FUDR), and amethopterin.[8, 26] Since these inhibitors would not have a deleterious effect upon the functional integrity of the DNA already present, it is expected that RNA synthesis could continue, and that the rhythm would therefore not be modified.

Although the results indicate clearly that RNA is involved in rhythmicity, the question concerning the participation of RNA-directed protein synthesis remains unanswered. Although both chloramphenicol and puromycin are considered to be quite specific in blocking protein synthesis, a detailed biochemical mechanism is not known for the action of either one. Moreover, the inhibition of protein synthesis in *Gonyaulax* by these compounds has not been measured directly. It is not impossible, of course, that the control of rhythmicity occurs via an RNA-directed pathway not immediately involving protein synthesis. Additional studies are required to investigate these questions.

The conclusion that clock function is related to informational transfer from DNA might appear to conflict with the experimental results of Sweeney and Haxo.[27] They reported that the rhythm of photosynthetic capacity in *Acetabularia* persisted for at least five cycles in enucleated plants, and concluded that the nucleus was not essential for the immediate maintenance of timekeeping in this plant.

At least two explanations can be offered. First, the difference may be related to the stability of the RNA involved in the rhythmic function. Our experiments indicate that the detailed chemical mechanism governing the rise and fall of a rhythmic process in *Gonyaulax* is established considerably prior to the time when that

event occurs. In *Acetabularia*, an even longer-lived "determinant" system might be formed. A second explanation of the results of Sweeney and Haxo could relate to the possible occurrence of cytoplasmic or chloroplast nucleoprotein component which could function in the rhythm-controlling capacity. It will be of considerable importance to determine the effect of actinomycin upon the *Acetabularia* rhythm in both normal and enucleated specimens.

There exists the more general question as to whether the inhibitory action is upon the clock mechanism or upon its expression. The addition of actinomycin D does not inhibit the luminescent reaction in extracts, and *in vivo* the immediate expression of luminescence and photosynthesis remains unaffected (Fig. 1), the action being effective only at a later time. It may be concluded that the pathway inhibited is remote to the observed expression, but a more definitive evaluation of the question must await studies concerned with reversibility and phase shifting.

Finally, it should be noted that the differential effects of actinomycin, as for example upon growth and rhythmicity, or upon different rhythms, may have an explanation in terms of the base composition of the particular region of the DNA concerned with the function in question. At very low concentrations of actinomycin D the luminescence rhythm may be inhibited, while growth (and presumably most RNA synthesis) continues. Since actinomycin binds specifically to guanine, the inhibitory effect upon RNA synthesis might be expected to be greater in regions of the DNA strand where guanine is relatively abundant. Such a differential sensitivity has been found with RNA polymerase as measured by nearest neighbor analysis of the product.[28]

We wish to thank the National Institutes of Health, Cancer Chemotherapy Division, for the actinomycin D.

* This work was supported by grants from the National Science Foundation and the Graduate College of the University of Illinois.

† National Science Foundation Postdoctoral Fellow. Present address, Department of Biology, Reed College, Portland, Oregon.

[1] Pittendrign, C. S., in *Perspectives in Marine Biology*, ed. A. A. Buzzati-Traverso (Los Angeles: University of California Press, 1958).

[2] Hastings, J. W., *Ann. Rev. Microbiology*, **13**, 297 (1959).

[3] Hastings, J. W., and B. M. Sweeney, *Biol. Bull.*, **115**, 440 (1958).

[4] Hastings, J. W., and B. M. Sweeney, these PROCEEDINGS, **43**, 804 (1957).

[5] Sweeney, B. M., and J. W. Hastings, in *Biological Clocks*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 25 (1960), p. 87.

[6] Bühnemann, F., *Biol. Zentralblatt*, **74**, 691 (1955).

[7] Bünning, E., in *Rhythmic and Synthetic Processes in Growth*, ed. Dorthea Rudnick (Princeton: Princeton University Press, 1957).

[8] Hastings, J. W., in *Biological Clocks*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 25 (1960), p. 131.

[9] Brown, F. A., Jr., *Science*, **130**, 1535 (1959).

[10] Waksman, S. A., E. Katz, and L. C. Vining, these PROCEEDINGS, **44**, 602 (1958).

[11] Brockmann, H., *Fortsch. Chem. Org. Naturstoffe*, **18**, 1 (1960).

[12] Rauen, H. M., H. Kersten, and W. Kersten, *Z. Physiol. Chem. Hoppe-Seyler's* **321**, 139 (1960).

[13] Kersten, W., *Biochem. Biophys. Acta*, **47**, 610 (1961).

[14] Kirk, J. M., *Biochem et Biophys. Acta*, **42**, 167 (1960).

[15] Reich, E., and R. M. Franklin, these PROCEEDINGS, **47**, 1212 (1961); Reich, E., R. M. Franklin,

A. J. Shatkin, and E. L. Tatum, *Science*, **134**, 556 (1961); Reich, E., R. M. Franklin, A. J. Shatkin, and E. L. Tatum, these PROCEEDINGS, **48**, 1238 (1962).

[16] Hurwitz, Jerard, J. J. Furth, M. Malamy, and M. Alexander, these PROCEEDINGS, **48**, 1222 (1962).

[17] Sweeney, B. M., and J. W. Hastings, *J. Cell Comp. Physiol.*, **49**, 115 (1957).

[18] Packard Instrument Co., LaGrange, Illinois.

[19] Hastings, J. W., and B. M. Sweeney, in *Photoperiodism and Related Phenomena in Plants and Animals* (Washington, D. C.: American Assn. for the Advancement of Science Press, 1959).

[20] Hastings, J. W., L. Astrachan, and B. M. Sweeney, *J. Gen. Physiol.*, **45**, 69 (1961).

[21] Monod, J., and F. Jacob, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 389.

[22] Spiegelman, S., personal communication; M. Hayashi, Ph.D. thesis, University of Illinois (1962).

[23] Sweeney, B. M., and J. W. Hastings, *J. Protozoology*, **5**, 217 (1958).

[24] Bishop, N. I., *Biochem. Biophys. Acta*, **27**, 205 (1958).

[25] Hall, B. D., and S. Spiegelman, these PROCEEDINGS, **47**, 137 (1961); Yankofsky, S., and S. Spiegelman, these PROCEEDINGS, **48**, 1069 (1962); Yankofsky, S., and S. Spiegelman, these PROCEEDINGS, **48**, 1466 (1962).

[26] Karakashian, M. W., unpublished results.

[27] Sweeney, B. M., and F. Haxo, *Science*, **134**, 1361 (1961).

[28] Strelzoff, E., and J. Hurwitz, personal communication.

CONFORMATIONAL CHANGES IN ENZYME CATALYSIS

BY BERNARD LABOUESSE,* BENT H. HAVSTEEN,† AND GEORGE P. HESS‡

DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY

Communicated by Alfred T. Blomquist, August 30, 1962

More than 30 years ago, Sumner[1] isolated crystalline urease and demonstrated that enzymes are proteins which can function without cofactors. Today, the accepted view is that the enzyme protein is merely a structural entity and necessary for the formation of a small area on its surface which carries out a particular catalytic process.[2, 3] Whether the formation of this "active site" is the only function of the protein in enzyme catalysis is a question of fundamental importance to an understanding of this process.[4] This question still remains unanswered.[4] In this paper, we present *the first* comprehensive evidence that some enzyme-catalyzed reactions are accompanied by conformational changes of the enzyme which appear to be involved in the catalytic reaction.

Two reactions of α-chymotrypsin (CT) are considered in this investigation: the stoichiometric reaction of CT with diisopropylphosphorofluoridate (DFP) to give diisopropylphosphoryl-α-chymotrypsin (DIP-CT) and HF;[5] and the CT catalyzed hydrolysis of p-nitrophenyl acetate.[6] The latter reaction proceeds via an intermediate, monoacetyl-CT, which can be isolated below pH 5.0.[7] Above pH 6.0, monoacetyl-CT, isolated according to the procedure of Marini and Hess,[8] is kinetically the intermediate in the chymotrypsin catalyzed hydrolysis of p-nitrophenyl acetate.[8] It has been demonstrated that in the latter reaction and in the reaction of CT with DFP the same seryl hydroxyl group of CT is substituted.[9] Since the hydrolysis of specific substrates for CT most probably involves the same mechanism as the CT-catalyzed hydrolysis of p-nitrophenyl acetate,[10] the data ob-