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Experiments on the Modification of Myosin Nucleosidetriphosphatase*

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Received April 27, 1964

In the sense that they activate the 25° Ca^{2+}-adenosine-5′-triphosphatase, inhibit the 25° Ca^{2+}-inosine-5′-triphosphatase, and inhibit the 0° activity of both nucleosidetriphosphatases, dioxane and hydrogen peroxide are true "modifiers" of myosin nucleosidetriphosphatase. Modification by peroxide, as by *p*-mercuribenzoate, clearly involves reaction of certain myosin sulfhydryl groups, but no compelling evidence for sulfhydryl involvement was found in modification by dioxane, and there was found some direct evidence against sulfhydryl involvement in modification by 2,4-dinitrophenol. On the other hand, the groups on the myosin molecule *directly* responsible for either activating or inhibiting phases of "modification" are probably the same for all modifiers. This hypothesis, expressed quantitatively in a stochastic model, was affirmatively tested in the case of *p*-mercuribenzoate and 2,4-dinitrophenol. It was found incidentally that histidine protects against modification which involves oxidation because histidine itself is easily oxidized in a reaction involving its amino group.

We are concerned in this paper with interpreting experiments in which the nucleosidetriphosphatase activity of Ca^{2+}-myosin is modified by various reagents. Such an objective presumes that the activity of *un*modified enzyme is understood, and this is not really the case. However, as a result of the work of many laboratories, there is a hypothesis about unmodified and modified myosin catalysis into which many observations seem to fit, and it is useful to state this hypothesis at the outset and in some degree to justify it *a posteriori*.

A Hypothesis about Myosin Nucleosidetriphosphatase.—In most experiments with substrate-saturated and Ca^{2+}-saturated myosin, the substrate is ATP, the temperature is *ca.* 25°, and the *p*H is near neutral. These conditions have come to define the "normal" or reference behavior of the enzyme, and one can discuss consequences of changing conditions; for example, at 25° a partial titration of the sulfhydryl content activates ATPase and inhibits ITPase,[1] but at 0° the same titration inhibits both. We (see Gilmour, 1960; Morales and Hotta, 1960) have felt it helpful to focus on more general properties of the enzyme and of the reagents which affect it. In our terms, the crucial properties of myosin catalysis are as follows (see also Figs. 1 and 2).

The *p*H-dependence of myosin NTPase is sigmoidal (Fig. 1), but at 25° the curve of ATPase has an "aberration," viz., a depression near *p*H 7–8. The rate-modifying substances (e.g., SH reagents) inhibit the NTPase of a "normal" sigmoidal curve. However, if one starts with the "aberrant" 25° ATPase, the modifiers first return its *p*H-dependence to the normal sigmoid shape (this involves an "activation" in the neutral range), and then on further addition they inhibit, just as they inhibit any other "normal" system.

Because near neutrality ATP and ITP have similar ionization behavior, and because the temperature coefficient of disappearance of the ATPase "aberration" is so high, it is natural to assume (as have Koshland, Blum, and Gilmour: Levy, Sharon, and Koshland, 1959; Blum, 1960; Gilmour and Griffith, 1957) that the relatively slow ATPase at 25° and neutral *p*H are due to a conformation of the enzyme (or of the water near the enzyme, Klotz (1963), which disappears toward either *p*H extreme or with lowered temperature. We may call this hypothetical inhibitory conformation α, and call the normal sigmoidal conformation obtaining elsewhere β. Furthermore we must assume that the passage from α to β and on to a state of total inhibition (γ) is a continuum, and that potentially all modifiers, in appropriate increments or in concert, are capable of effecting any portion of the $\alpha \rightarrow \beta \rightarrow \gamma$ transition. Now it has to be assumed that ITP is not only a substrate but also a modifier,[2] so that even at 25° and neutral *p*H, ITP in-

* This work was supported by grant HE-06285 of the U.S.P.H.S., grant 60-CI-8 of the American Heart Association, and grant G-19442 of the National Science Foundation.

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[1] The following abbreviations are used: AET, *S*-β-aminoethylisothiuronium bromide; NTP, nucleosidetriphosphate; NTPase, nucleosidetriphosphatase; ITPase, inosine-5′-triphosphatase; DNP, 2,4-dinitrophenol; GSH, glutathione; IAA, iodoacetamide.

[2] One may alternatively construct a hypothesis in which ITP is "passive" and ATP induces the α form. However, this hypothesis contains two undesirable features: modifiers and ATP now "pull" in opposite directions, and either an ATP-induced rearrangement of reagent (reagent added first, then ATP) or an ATP protection of deactivating sites from reagent (ATP added first, then reagent) must be invoked to explain why 25° ATPase is not inhibited by initial additions of modifier.

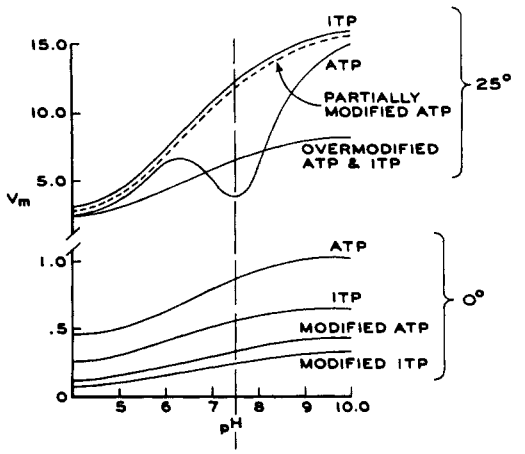

FIG. 1.—Schematic *p*H dependence of myosin NTPase activity in 0.6 M KCl.

duces, and hydrolyzes from, the β form, and further, that the additive effect of ITP and another modifier, in whatever amount, leads to an enzyme inhibited relative to the activity of the β form.

In the foregoing terms the paper to follow will be concerned with how various reagents bring about the transition from control → activation → inhibition. It is already known, and will be re-emphasized here, that the maximum activation is different with different reagents. This characteristic of modification compels the assumption that when it induces the $\alpha \rightarrow \beta$ transition a modifier molecule attaches to the enzyme with a greater affinity constant than when it induces the $\beta \rightarrow \gamma$ transition. Therefore it is reasonable to say that for every modifier there are $\alpha \rightarrow \beta$ sites and $\beta \rightarrow \gamma$ sites. However, this constraint is insufficient to specify a unique model of binding, as illustrated by the "independent-site" and "contingent-site" alternatives of Figure 2. Because calculations are easier with the former, we will use the language of "two kinds of (independent) sites," remembering that as yet we are referring to a mathematical model, not necessarily to physical reality. A further complication is the possible existence of 'irrelevant" sites with the same activity toward a modifier as the sites which influence enzymatic events ("relevant sites"). This can be illustrated in the case of SH groups only some of which are believed to underlie the modification reaction. Amino acid analysis (Kominz *et al.*, 1954) shows that myosin contains about 8.6 moles of cysteyl per 10^5 g of myosin. Since the molecular weight of myosin is at least 4.2×10^5 g, one must assume that there are upwards of 46 moles cysteyl/mole myosin, and it is very unlikely that all of these participate in catalysis (indeed the presence of SH groups in both heavy and light meromyosins and the fact that all the ATPase properties of myosin exist in the heavy fragment show that at least some SH groups are enzymatically irrelevant) even though their reactivities toward reagents may be quite similar (cf. Sekine and Kielley, 1964). A theoretical consequence of these circumstances is that the ordinate of any activity versus amount of reagent plot must be thought of as the *probability* that *particular* SH groups have been reacted; a practical consequence of these circumstances is the recognition that even very careful SH titrations may miss the reaction of a few relevant groups.

EXPERIMENTAL

Usually the enzymes employed in these experiments were "myosins" or "myosin A's," prepared by 5- to 10-minute extractions of finely divided rabbit skeletal muscle in the presence of low [ATP] at *p*H 6.8. After final purification such preparations failed to show any increases in 350-mμ turbidity when ATP was added in a "superprecipitation" medium (0.1 M KCl); this is the most sensitive test for actin contamination of a myosin solution. Occasionally, a "5-hour-extracted myosin B" was used, but in cross checks of the experiments being reported no differences in method of preparation ("A" or "B") were observed. Both types of preparation were extensively purified after extraction, and in 0.6 M KCl, 0.1 M Tris, 1 mM $CaCl_2$, 25°, and *p*H 8.00, all showed activities between 4 and 5 μmoles P_i/g-sec. The various salts employed, including the nucleotides, were of the best grade commercially available, in most instances reagent grade. Dioxane was Matheson, Coleman and Bell Spectroquality reagent. In critical usages (*vide infra*) this reagent was treated by repeated stirring with chunks of fresh metallic sodium for about 2 days (until the fresh sodium surfaces remained shiny after immersion); in some instances it was immediately redistilled using a nitrogen flush. Histidine was Calbiochem, either hydrochloride or, more frequently, free base. In critical usages histidine was analyzed and purified by adsorbing on Bio-Rad cation exchanger AG-50W-X4 from 0.01 M CH_3COOK, *p*H 4.50, then eluted by superimposing a linear [KCl] gradient (to 1.5 M). The eluate showed a single sharp symmetrical peak, and the contents of tubes corresponding to the peak

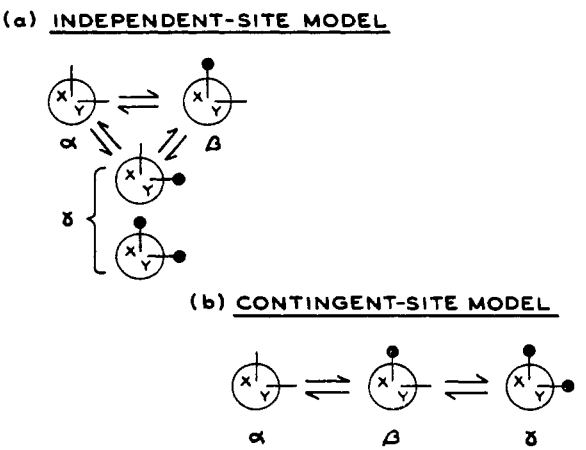

FIG. 2.—Simple models of myosin modification. *X* = activating site; *Y* = deactivating site.

region were used for the experiments. No attempt was made to eliminate the KCl because in separate experiments with nonpurified histidine it was shown that high concentration of KCl did not influence the dioxane effect. SH content was usually measured spectrophotometrically (Boyer, 1954) using readings made after 200 seconds at room temperature or after 24 hours at 4° after mixing *p*-mercuribenzoate with protein. (We noticed some time ago that readings with myosin are unstable in time; this phenomenon has recently been thoroughly investigated [Gilmour and Gellert, 1961]). In the presence of DNP the spectrophotometric method is unsuitable due to the ultraviolet absorbance of DNP, so in that case we used the amperometric method (Benesch and Benesch, 1957).

When effect was to be measured as a function of time during which myosin was exposed to a modifier, means of terminating exposure were necessary. With dioxane and DNP this was dilution to an ineffective concentration followed by dialysis; with H_2O_2, either catalase or SO_3^{2-} (again followed by dialysis) arrested

the modification. The sulfite ion must be rigorously eliminated prior to *p*-mercuribenzoate titrations, since it interacts with *p*-mercuribenzoate and produces errors in the titration.

Results

(1) Modification by Dioxane.—As shown in Table I, increasing concentrations of dioxane at first activate (to a maximum of 2-fold) and then inhibit the 25° ATPase activity of myosin near neutral *p*H (cf. Ebashi and Ebashi, 1959, and Tonomura *et al.*, 1961), but they only inhibit the ITPase activity. Moreover, in experiments not shown, it was found that even 5% dioxane did inhibit the 25° ATPase of myosin previously treated with *S*-β-aminoethylisothiuronium (i.e., the 25° ATPase–modified myosin) by 43% also, 10% dioxane inhibited the 0° ATPase by 26%. It is virtually impossible to rid dioxane of peroxides completely; however, the foregoing results were obtained with our purest preparations of dioxane, and with very short (*ca.* 2 minutes) incubations, and at least with low concentrations of dioxane (e.g., 10%), the activity effects were completely reversible. Therefore, the results establish dioxane as a typical "modifier" of myosin catalysis. The *mechanism* of this dioxane effect is certainly obscure. About the only sure statement possible is that the effect is not due to the change in the *bulk* dielectric constant; in a 5% dioxane solution the change in this quantity is less than 6% whereas the increase in the activity is upwards of 75%; ATPase and ITPase are oppositely affected; finally, the same effect on ATPase is brought about by solvents which lower the bulk dielectric constant (e.g., acetone) as by solvents which raise it (e.g., formamide) (Yasui and Watanabe, 1964).

Table I
Immediate Effect of [Dioxane] on NTPase[a]

Vol % Dioxane	ATPase	ITPase
0	4.87	18.6
2.5	6.49	
5.0	7.75	16.2
7.5	9.40	
10.0	10.90	7.45
15.0	11.80	
17.0	11.20	
20.0	8.70	

[a] Ionic strength, 0.6 M; 0.05 M Tris, *p*H 8.0; 10^{-2} M $CaCl_2$; 0.0207% myosin. Activities in μmoles P_i/g-sec. Incubation time with dioxane, 1–2 minutes.

It was anticipated that the peroxides presumed to contaminate dioxane would complicate the results of longer-term experiments, especially of experiments conducted at higher *p*H, where SH groups of myosin are more ionized and hence more vulnerable to oxidation. Attempts to forestall oxidations with sulfite ion met with limited success because, when dioxane is present, sulfite itself seems to react with myosin, presumably as follows (see, e.g., Swan, 1957):

$$SO_3^{2-} + \text{Prot-S}^- \rightleftharpoons \text{Prot-S-SO}_3^- + 2e^-$$

Table II shows the result of 1-hour incubation followed by elimination of reagents through dilution or dialysis. Although the only reagents added intentionally were dioxane and sulfite, it must be assumed that peroxides accompanied the dioxane and reacted with the myosin to some extent. The conclusion from Table II is that activity and SH titer are not in a 1:1 correspondence; this is certainly true, but no sharp inferences can be drawn. Thus, if we assume that there are many "irrelevant" cysteyls with the same reactivity as the $\alpha \rightarrow \beta$ site(s), and many others with the same reactivity as the $\beta \rightarrow \gamma$ site(s), then the following explanation is likely. During the 1-hour incubation with dioxane, the dioxane bound reversibly to the $\alpha \rightarrow \beta$ and $\beta \rightarrow \gamma$ sites in such a way as to produce a net activation of 2-fold, but the peroxides permanently oxidized many irrelevant sites with the $\alpha \rightarrow \beta$ reactivity. Thus on dilution it was found that the 25° ATPase activity returned virtually to initial levels, but the SH titer reflected the decrease due to oxidation. The addition of sulfite probably had dual effects; as a general antioxidant it largely cancelled the peroxides

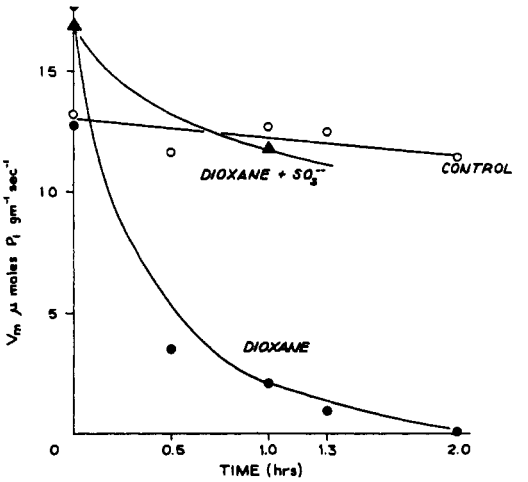

Fig. 3.—Effect of dioxane and dioxane-SO_3^{2-} on ATPase activity as a function of time. Conditions: 0.6 M $(CH_3)_4NCl$, 10^{-2} M $CaCl_2$, 0.05 M alanine, t = 25°, *p*H 9.5. [Myosin] = 0.01%, [dioxane] = 5.00%, [SO_3^{2-}] = 4 mM.

Table II
Changes in SH-Titer and ATPase Activity on Exposure to Dioxane[a]

	Control	10 mM SO_3	5% Dioxane	5% Dioxane + 10 mM SO_3^{2-}
(A)				
V_m	4.16	"4.16"	4.68	2.60
SH	100.00	100.00	64.00	100.00
(B)				
V_m	4.43		5.67	2.43
SH	100.00		64.50	100.00

[a] Ionic strength, 0.6 M KCl; 0.05 M Tris (*p*H 8.0). Incubation with dioxane for 1 hour at room temperature followed by dialysis for 18 hours at 5°. Activities in μmoles P_i/g-sec. SH titers in per cent control titer.

The results of incubation with the same substances at high *p*H are shown in Figure 3. At this *p*H the "aberration" in the 25° ATPase scarcely exists, so little activation by dioxane is to be expected. On the other hand, at *p*H 9.0 both the vulnerability of cysteyl to oxidation and the effectiveness of sulfite as an antioxidant (due to $OH^- + HSO_3^- \rightarrow H_2O + SO_3^{2-}$) are so greatly enhanced, the activity-destroying effect of contaminating dioxane, but with high selectivity the sulfite probably reacted with the $\beta \rightarrow \gamma$ sites. Thus on dilution it was found that the activity reflected the $\beta \rightarrow \gamma$ reaction, but out of the total number of cysteyls the reaction of these particular groups went undetected in the SH titer.

"dioxane" is very powerful as is the protection afforded by sulfite, and these expectations are borne out in the results.

(2) *Modification by* H_2O_2.—The surmise that peroxides existed in dioxane originally led us to investigate effects of H_2O_2. If myosin is exposed to high $[H_2O_2]$ for appreciable lengths of time its ATPase

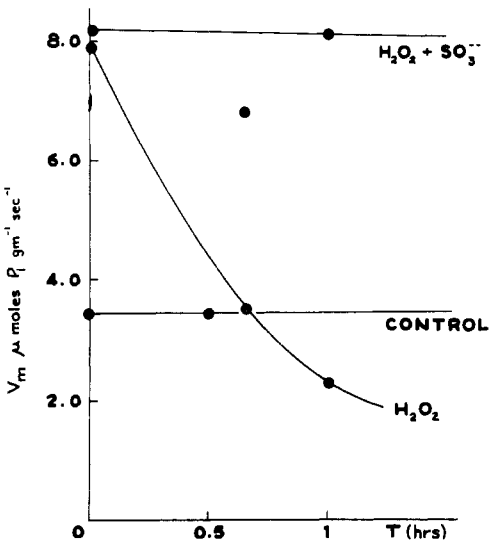

FIG. 4.—ATPase activity on exposure to H_2O_3 and H_2O_2–SO_3^{2-}. Conditions: 0.6 M KCl, 4×10^{-5} M ATP, t = 25°, pH 8.0. [Myosin] = 0.0015%, $[H_2O_2]$ = 0.003%, $[SO_3^{2-}]$ = 0.01%.

activity is certainly destroyed, and it is reasonable to infer that enzymatically critical (e.g., SH) groups have been destroyed by oxidation (Mehl, 1944; Konishi *et al.*, 1958). However, in using limited exposure a familiar pattern is discerned. ATPase activity is increased and ITPase activity is decreased (Fig. 4; Table III). The slow decay (not the initial acceleration) of ATPase activity which follows the initial acceleration can be counteracted by SO_3^{2-} and GSH,

TABLE III
EFFECT OF LIMITED EXPOSURE TO H_2O_2 ON NTPASE ACTIVITY[a]

ATPase	Immediate	40 min	60 min
Control	3.45	3.45	3.45
H_2O_2	7.91	3.50	2.13
$H_2O_2 + SO_3^{2-}$	8.20	6.84	8.10
ITPase	**Immediate**	**3 min**	**5 min**
Control	14.50	14.50	14.50
H_2O_2	10.72	3.62	1.02
H_2O_2 + 0.01 M Hist	11.00		9.70

[a] Ionic strength, 0.6 M; 0.05 M Tris, pH 8.0; 10^{-2} M $CaCl_2$; 0.0015% myosin. When used, $[H_2O_2]$ = 0.0177 M; when used, $[SO_3^{2-}]$ = 0.01 M. Activities in μmoles P_i/g-sec.

and both intermediate and long-term effects of H_2O_2 are accompanied by some loss of SH (Table IV).

(3) *Modification by 2,4-Dinitrophenol and p-Mercuribenzoate.*—It has been established by others (see particularly Grenville and Needham, 1955; Chappel and Perry, 1955) that DNP is a "typical" modifier, at least as regards inhibition of ITPase and activation of 25° ATPase. Insolubility of the compound precludes direct investigation of an effect on $\beta \rightarrow \gamma$ sites, but it would appear that maximum activation by DNP is less than 3-fold, so probably it reacts with both activating and inhibiting sites.

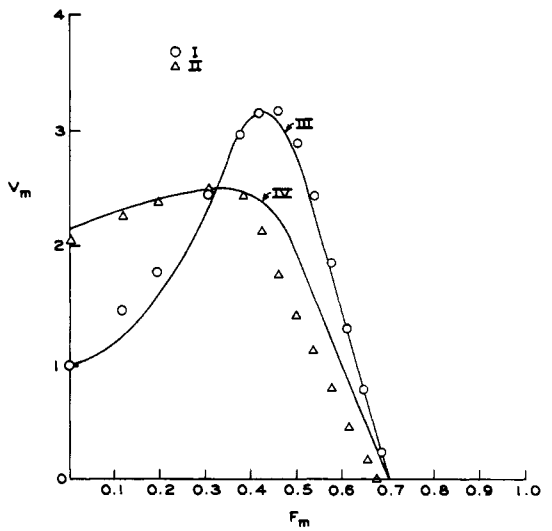

FIG. 5.—Normalized maximum reaction rate, V^{max}, as a function of F, the fraction of the total SH groups which have reacted with p-mercuribenzoate. I. Circles: measurements made after 24–48 hours of incubation with appropriate amounts of p-mercuribenzoate. II. Triangles: Aliquots similar to those of I, but then made 4×10^{-3} M with respect to DNP and measured immediately. III and IV: Curves calculated according to equations (B) and (D).

Our attempts to show that DNP acts on SH-groups have been inconclusive. If myosin is exposed to DNP, and then dialyzed exhaustively, there is a 5% reduction in SH titer over control level. If myosin is SH-titrated amperometrically, in the presence of DNP the titer is also shown to be reduced by a few per cent. Since these effects are marginal, it may be reasonable to infer that DNP is not acting at SH groups; on the other hand in both types of experiments DNP may be easily displaced by the heavy metal. We have also

TABLE IV
LOSS IN SH/10^5 G MYOSIN ON H_2O_2 EXPOSURE[a]

	Immediate	1 hour
Control	7.05	7.05
H_2O_2	4.80	2.60
H_2O_2 + 1 M SO_3^{2-}	6.37	6.20

[a] Ionic strength, 0.6 M; 0.05 M Tris, pH 8.0; $[H_2O_2]$ = 0.0177. Exposure followed by prolonged dialysis.

measured directly the binding of DNP to myosin by equilibrium dialysis. In this case, assuming a single "average" type of site, we deduce[3] that *ca.* 4 moles DNP bind with 10^5 g of myosin (with a small affinity constant, 10^1–10^2 $mole^{-1}$, as might be expected). Since pretreatment of the myosin with excess p-mercuribenzoate does not alter this binding, we may again infer that

[3] The "Klotz plot" of the data shows distinct curvature. We have fitted least squares parabolas to the data and from these calculated the number of sites and the free energies of binding (−1 to −3 kcal/mole) at infinitely high DNP concentration (i.e., from the intercept on the Y-axis).

DNP and *p*-mercuribenzoate do not bind to the same sites. However, a third approach, based on the additivity of DNP and *p*-mercuribenzoate effects on enzymatic activity suggests that if DNP and *p*-mercuribenzoate do not bind to the same sites they nevertheless exert precisely the same influences on the catalytic process. The interpretation of this experiment requires that one first interpret the effects of *p*-mercuribenzoate alone. Since to our knowledge this has not been done we present our analysis in some detail.

Gilmour and Gellert have studied the time dependence of the reaction of myosin with *p*-mercuribenzoate, and have shown that the plot of V_m versus per cent of SH titrated is stable only after incubations of 24 hours at low temperature. Such a plot is shown in Figure 5,

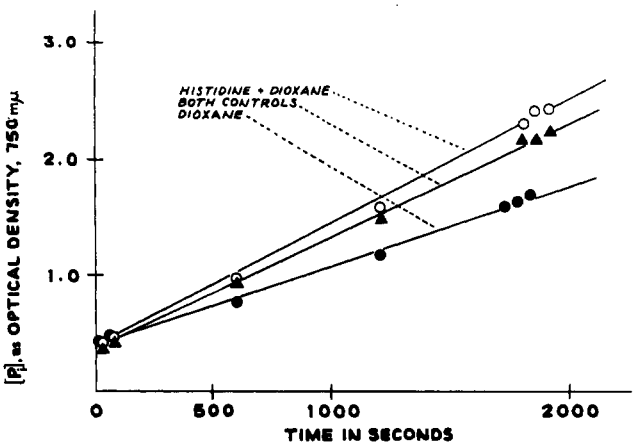

FIG. 6.—Effect of dioxane and dioxane-histidine on P_i liberation as a function of time. Conditions: 0.6 M $(CH_3)_4NCl$, 10^{-2} M $CaCl_2$, 4×10^{-3} M ATP, 0.05 M Tris, *p*H 8.0 $t = 25°$. [Myosin] = 0.0276%, [dioxane] = 1.0%, [histidine] = 0.1 M (ionic strength kept constant by reducing $(CH_3)_4NCl$).

curve I. However, it differs from the results of Gilmour and Gellert (1961) and resembles those of Blum (1962) in that (a) the rising limb is concave upward, and (b) catalytic activity disappears when approximately three-fourths (not all) of the SH groups have been titrated. Curve II of the same figure records, for each SH-titrated sample, the activity immediately upon adding 4×10^{-3} M DNP. These curves are consistent with our introductory hypothesis. Curves III and IV are computed curves (see Appendix) based on the assumption that both *p*-mercuribenzoate and DNP affect the ATPase activity by acting on each of two kinds of sites, viz., the $\alpha \rightarrow \beta$ sites, and all other sites having equal (and high) reactivity for *p*-mercuribenzoate (all "*X*" sites), and the $\beta \rightarrow \gamma$ site and all other sites having equal (and low) reactivity for *p*-mercuribenzoate (all "*Y*" sites). In other words, the titration results are easier to explain if one assumes that *p*-mercuribenzoate and DNP affect the same processes. This is not precisely the same as saying that they attach to the same physical sites (e.g., they could attach to different sites and bring about the same conformational change; this has also been suggested by Sekine and Kielley, 1964). In order to fit the experimental curves closely, some further assumptions must be made. The upward concavity of the rising limb of curve I is better reproduced if one assumes the *two* particular SH sites must be reacted in order to effect the $\alpha \rightarrow \beta$ process. (It is interesting in this connection that Blum and Sanadi, 1964, have found dithiol reagents to be effective in modifying myosin, and that the curve of activity versus amount of dithiol reagent does not show an upward concavity in its rising limb.) The disappearance of activity at about 70% titration (curve I) suggests that the SH groups with which *p*-mercuribenzoate can react fall into a minimum of three classes: a high-reactivity class which includes the two $\alpha \rightarrow \beta$ sites (the *X* class), a medium-reactivity class which includes the $\beta \rightarrow \gamma$ site (the *Y* class), and a low-reactivity class containing only enzymatically irrelevant SH groups (a *Z* class). The computed curve (III) of Figure 5 incorporates these assumptions.

(*4*) *The "Histidine Effect."*—Nagai and his collaborators (Konishi *et al.*, 1958) reported that histidine protected myosin from inactivation by H_2O_2, and concluded from this that histidine was a member of the active site. It is a fact that the activity-*p*H dependence of the β form at high ionic strength strongly resembles

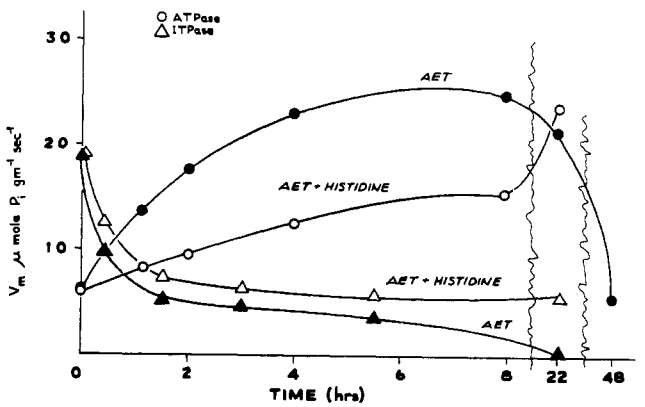

FIG. 7.—Retarding effect of histidine on AET modification of myosin NTPase. Conditions: 0.6 M $(CH_3)_4NCl$, 0.05 M Tris, 10^{-2} M $CaCl_2$, *p*H 8.0, $t = 25°$. [Myosin] = 0.0035%, [AET] = 1 mM, [histidine] = 0.15 M (ionic strength kept constant by reducing $(CH_3)_4NCl$).

an acid-ionization curve with *pK* near 6.5 (Morales and Hotta, 1960), and recently Stracher (1963) has reported that when myosin is inhibited by radioactive IAA one histidyl group is labeled. These various observations alerted us to possible effects of histidine in the foregoing modifications of myosin, and indeed consistent effects were found. For example, not only did histidine appear to protect against both activation and inhibition by H_2O_2, but also it allayed the modifying effects of dioxane (Fig. 6) and most surprisingly, it retarded modification by AET (Fig. 7). The explanation of these histidine effects resulted from the chance discovery that histidine in contact with dioxane develops a strong difference spectrum with a peak at 255 mμ (Fig. 8). We have since shown that under these circumstances there is formed irreversibly a new compound with a spectrally inferred *pK* of 3.8. The kinetics of formation are those of a slow oxidation. The formation is inhibited by sulfite, and by eliminating traces of heavy metals (with 10^{-3} M EDTA). We have not tested peptide-bound histidine, but the formation does not occur with *N*-acetylated histidine, and does occur with COOH-esterified histidine; therefore the amino group is necessary for the process. Thus it appears that histidine serves as a "buffer" against oxidation. Even in this context the effect on AET modification would remain strange. However, at least at *p*H 8 disulfide-exchanging reagents modify myosin more swiftly than do SH-compounds.[4] The retardation of the AET modification by histidine could be because

[4] Personal communication from Dr. David Hartshorne.

the preliminary oxidation of AET to the disulfide is retarded. The protective action of histidine on modification is therefore not evidence that histidine participates at the active site.

Summary and Conclusions

The peroxides which contaminate dioxane do confuse its effect as a modifier of NTPase activity, but the experiments reported here indicate that, contaminations aside, dioxane is a typical modifier, and we shall return presently to the consequences of this observation. Hydrogen peroxide, investigated originally as a model of the peroxides in dioxane, also turns out to be a typical modifier. Histidine does counteract H_2O_2 modification, probably not because it is at the active site (as claimed by Nagai *et al*, 1958) but because it is itself subject to oxidation, in a reaction which has been preliminarily characterized here. Since organic mercurials like *p*-mercuribenzoate, thought to be highly specific for sulfhydryl groups, exhibit clearly the activation and inhibition phases of modification, it is unsurprising that H_2O_2 should do likewise. But, in terms of conventional chemistry, there is no reason why dioxane or DNP should act particularly on SH groups; indeed in the case of DNP we have offered some evidence that it does not bind at the same sites as *p*-mercuribenzoate, even though it carried out both activation and inhibition functions, and is in these effects additive to *p*-mercuribenzoate. From these observations emerges the picture that a reagent may promote the $\alpha \rightarrow \beta$ and $\beta \rightarrow \gamma$ processes *indirectly*. Either process must be some alteration at the active site, but the sites at which the reagent physically binds may be remote, and there is probably some proportionality between degree of occupation at those remote sites and degree of advancement of, say, the $\alpha \rightarrow \beta$ process. (The simple statistical treatment developed here tacitly assumes a constant of proportionality equal to unity.) What is the mechanism of this remote action? The natural, indeed the prevalent, assumption is that there occurs a local change in the conformation of the enzyme structure. An alternative which must be kept in mind, however, is that reagents such as dioxane or DNP disrupt the structure of the water surrounding the enzyme, and that this affects the catalytic events.

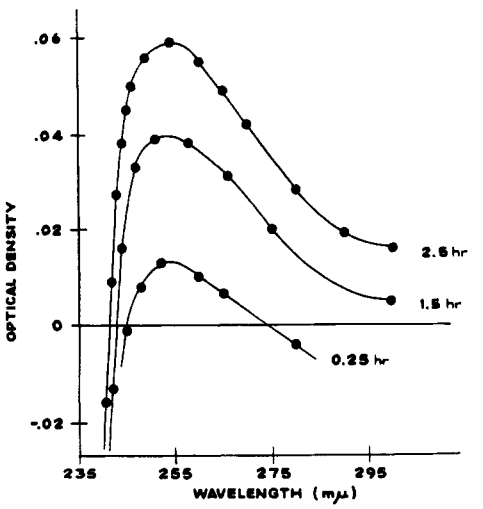

FIG. 8.—Difference spectrum (relative to unmixed substances) of histidine in 10% dioxane-water. Conditions: 0.6 M KCl, 0.1 M Tris, *p*H 8.0. [Histidine] = 0.075 M.

In our opinion, unequivocal physical evidence for the first alternative is not yet at hand, but the alternative is favored by the consideration that ITP and ATP are different in their influence on the $\alpha \rightarrow \beta$ process when their structures are for the most part identical.

Acknowledgments

We are very much indebted to our colleague Dr. Charles Walter who kindly programmed, for digital computation, the calculations indicated in the Appendix, and to Messrs. Dale Mecham and Harvey Sokol of the Western Regional Research Laboratory, U.S.D.A., in whose laboratory the amperometric titrations were performed. Miss Linda Stowring performed, very competently, many of the histidine-dioxane experiments.

Appendix

We shall assume that the many "sites" at which a modifying reagent can combine with myosin fall into reactivity classes, X, Y, and Z. We shall consider reagents of two kinds. Reagents such as *p*-mercuribenzoate will be assumed to react irreversibly, with bimolecular rate constant h for class X, rate constant k for class Y, and rate constant l for class Z. Reagents such as DNP, with a relatively weak affinity for the sites, and existing in solution at a concentration much greater than the site concentration, will be thought to be at equilibrium with the sites, with affinity constant, K_x, for class X, K_y for class Y, and K_z for class Z. We will assume that there are r sites of type X, s sites of type Y, and t sites of type Z, that $r + s + t = N$, that $r/N = f_x$, $s/N = f_y$, and that $t/N = f_z$. The myosin concentration will be called C_o.

p-Mercuribenzoate Alone.—Let the subscript λ indicate a particular site of class X, and let x_λ, indicate the concentration of filled λ sites; similarly, let y_μ, and z_ν, refer to filled sites of classes Y and Z. If P_o is the concentration of *p*-mercuribenzoate, then the simplest assumptions about rate of reaction lead to r equations of the form:

$$\frac{dx_\lambda}{dt} = h(C_o - x_\lambda)(P_o - S_x - S_y - S_z) \qquad (1)$$

s equations of the form:

$$\frac{dy_\mu}{dt} = k(C_o - y_\mu)(P_o - S_x - S_y - S_z) \qquad (2)$$

t equations of the form:

$$\frac{dz_\nu}{dt} = l(C_o - z_\nu)(P_o - S_x - S_y - S_z) \qquad (3)$$

where S_x is the sum of all the x_λ, and correspondingly for S_y and S_z.

By combining the differential equations of any two variables in the same class, and applying the boundary condition that at $t = 0$ all sites are empty, it may be shown that all x_λ are the same, all y_μ are the same, and all z_ν are the same, so subscripts may now be omitted. On the other hand, by combining the equations of x with y, it may be shown that

$$1 - \frac{x}{C_o} = \left(1 - \frac{y}{C_o}\right)^a \qquad (a \equiv h/k) \qquad (4)$$

We may consider x/C_o the probability that an X site is filled (and correspondingly for y and z), and to emphasize this meaning, we may rewrite (4) as

$$1 - p_x = (1 - p_y)^a \qquad (5)$$

and similarly,

$$1 - p_z = (1 - p_y)^b \qquad (b \equiv l/k) \qquad (6)$$

If there are at least as many sites as there are *p*-mercuribenzoate molecules, then a reaction of this sort will stop when, in equations (1)–(3), the last factor on the right-hand side vanishes, i.e., when

$$S_x + S_y + S_z = P_o$$

or

$$rx + sy + tz = P_o \qquad (7)$$

Dividing equation (7) by NC_o we obtain,

$$f_x p_x + f_y p_y + f_z p_z = \frac{P_o}{NC_o} \qquad (8)$$

Now P_o/NC_o is F, the fraction of the total number of sites which has been titrated by P_o. Substituting equations (5) and (6) into (8), we obtain $F(P_y)$, viz.,

$$F = f_x[1 - (1 - p_y)^a] + f_y p_y + f_z [1 - (1 - p_y)^b] \qquad (9)$$

Next we seek an expression for $\bar{V} \equiv V(P_o)/V(0)$, i.e., the ratio of the activity observed in the presence of *p*-mercuribenzoate concentration P_o and the "control" activity. We shall assume that if $k_2^{(m)}$ is the zero-order rate constant for enzyme existing in state (or "phenotype") m at concentration $E_o^{(m)}$, then,

$$V = \sum_{\text{all } m} k_2^{(m)} E_o^{(m)} \qquad (10)$$

In the present case there are only two active states, α and β, and we shall assume that $k_2^{(\beta)} = \kappa k_2^{(\alpha)}$. However, there are molecules of various "genotypes." The probabilities of occurrence of all of these genotypes are given by expanding the generating function,

$$\text{g.f.} = (p_x + q_x)^m (p_y + q_y)^n \qquad (11)$$

where q_x and q_y are conjugate probabilities, m is the number of particular X sites which must be filled in order to effect the $\alpha \rightarrow \beta$ transition, and n is the number of Y sites which must be filled to effect the $\beta \rightarrow \gamma$ transition. If we assume $m = 2$ (to give some concavity to the rising limb $m > 1$), and $n = 1$, then it turns out that,

$$E_o^{(\alpha)} = (2p_x q_x q_y + q_x^2 q_y) E_{\text{tot}}$$

and

$$E_o^{(\beta)} = p_x^2 q_y E_{\text{tot}}$$

Recognizing that $k_2^{(\alpha)} E_{\text{tot}}$ is the "control" activity, we obtain,

$$\bar{V} \equiv \frac{V(P_o)}{k_2^{(\alpha)} E_{\text{tot}}} = 2p_x q_x q_y + q_x^2 q_y + \kappa p_x^2 q_y \qquad (12)$$

The computational procedure is now to assume values for p_y, to calculate F (equation 9) and $\bar{V}$ (equation 12) on a digital computer, and then to plot $\bar{V}(F)$, which is the experimental curve.

To compute the curve in which DNP is superimposed on various degrees of titration, F, some further maneuvers are necessary. It is assumed that the addition of DNP in no way displaces the *p*-mercuribenzoate molecules bound, so that only sites left free after *p*-mercuribenzoate have to be considered. In the following we tabulate, I, the "genotype" after *p*-mercuribenzoate; II, the "operations" exerted by DNP, using P to mean probability of DNP binding and Q to mean probability of nonbinding; III, the "genotype" after both *p*-mercuribenzoate and DNP; and IV, the "phenotype" after both *p*-mercuribenzoate and DNP:

I	II	III	IV
$p_x^2 q_y$	Q_y	$p_x^2 q_y Q_y$	β
$2p_x q_x q_y$	$P_x Q_y$	$2p_x q_x q_y P_x Q_y$	β
	$Q_x Q_y$	$2p_x q_x q_y Q_x Q_y$	α
$q_x^2 q_y$	$2P_x Q_x Q_y$	$2q_x^2 q_y P_x Q_x Q_y$	α
	$Q_x^2 Q_y$	$q_x^2 q_y Q_x^2 Q_y$	α
	$P_x^2 Q_y$	$q_x^2 q_y P_x^2 Q_y$	β

By the same arguments as those leading up to equation (12), one obtains after both *p*-mercuribenzoate and DNP,

$$\bar{V} = 2p_x q_x q_y Q_x Q_y + 2q_x^2 q_y P_x Q_x Q_y + q_x^2 q_y Q_x^2 Q_y + K(p_x^2 q_y Q_y + 2p_x q_x q_y P_x Q_y + q_x^2 q_y P_x^2 Q_y) \qquad (13)$$

The values of the constants used in computing curves III and IV of Figure 5 were chosen in the following way. In order to obtain an *apparent* F-intercept (actually the curves never cross the F axis) of *ca.* 0.7, we required that $f_x + f_y = 0.7$, and took $b = 0.005$ (when b is 10^{-2} or less the curves become insensitive to this parameter). We then required that the curves pass through the experimentally found maxima, i.e., through $V^{(\max)}_{p\text{-mercuribenzoate}}$ at $F^{(\max)}_{p\text{-mercuribenzoate}}$ and through $V^{(\max)}_{p\text{-mercuribenzoate} + \text{DNP}}$ at $F^{(\max)}_{p\text{-mercuribenzoate} + \text{DNP}}$. This was accomplished in each case by first finding the approximate value of q_x when $dV/dp_y = 0$ (dV/dF can be thought of as $[dV/dp_y]/[dF/dp_y]$); in this approximation, q_x^2 was neglected relative to q_x. This value of q_x was then substituted into the expressions for V and F, and the expressions were equated to the experimentally found quantities:

$$0.4(1 - \varphi) + 0.3(1 - \varphi^{1/a}) = F^{(\max)}_{p\text{-mercuribenzoate}} \qquad \text{(A)}$$

$$\varphi^{1/a}[\kappa - 2(\kappa - 1)\varphi] = V^{(\max)}_{p\text{-mercuribenzoate}} \qquad \text{(B)}$$

$$0.4[1 - (\varphi/Q_x)] + 0.3[1 - (\varphi/Q_x)^{1/a}] = F^{(\max)}_{p\text{-mercuribenzoate} + \text{DNP}} \qquad \text{(C)}$$

$$Q_y(\varphi/Q_x)^{1/a}[\kappa - 2(\kappa - 1)\varphi] = V^{(\max)}_{p\text{-mercuribenzoate} + \text{DNP}} \qquad \text{(D)}$$

where $\varphi = \kappa/2a(\kappa - 1)$. Equations (A) and (B) involve κ and a only, so, by graphical means they can be solved simultaneously to fix κ and a. When κ and a are known, equation (C) fixes Q_x, and when κ, a, and Q_x are known, equation (D) fixes Q_y. Since $Q = 1/[1 + (4 \times 10^{-3})K]$, Q_x and Q_y in turn fix K_x and K_y. Undoubtedly, better fitting than that illustrated in Figure 5 is possible, but our present objective is merely to show that a simple stochastic model generates $V(F)$ curves of the right sort, using reasonable values of some independently measurable constants (e.g., $\kappa \cong 4$, apparent $K \cong 10^2$).

The suggestion that the modification of myosin and other enzymes should be treated in the foregoing statistical manner has been made by Ray and Koshland (1961) and in our laboratory by Dr. Hiroshi Asai. A general paper on this subject by Dr. Asai and one of us will appear later.

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Polymerization Reactions Catalyzed by Intracellular Proteinases. IV. Factors Influencing the Polymerization of Dipeptide Amides by Cathepsin C*

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Received May 4, 1964

The polymerization of four dipeptide amides by beef spleen cathepsin C was studied as a function of substrate concentration, enzyme concentration, and *p*H. Between *p*H 7 and 8, in the *p*H range of polymerization, L-alanyl-L-phenylalaninamide is polymerized efficiently to form a hexapeptide amide, whereas the polymerization of glycyl-L-phenylalaninamide and glycyl-L-tyrosinamide is accompanied by extensive hydrolysis, and the products formed are largely octapeptide amides. The polymerization of glycyl-L-tryptophanamide also was examined.

The polymerization of dipeptide amides by cathepsin C (Fruton *et al.*, 1953; Würz *et al.*, 1962; Fruton and Knappenberger, 1962) provides a model system for the study of the enzyme-catalyzed elongation of peptide chains under physiological conditions of temperature and *p*H. In previous papers of this series, observations were reported on the polymerization of L-alanyl-L-phenylalaninamide (Ala.Phe[NH_2]), glycyl-L-phenylalaninamide (Gly.Phe[NH_2]), glycyl-L-tyrosinamide (Gly.Tyr[NH_2]), and glycyl-L-tryptophanamide (Gly.Trp[NH_2]). In the work described in the present communication, the polymerization of these substrates was studied as a function of the concentration of substrate, concentration of enzyme, and *p*H.

Experimental

The cathepsin C preparation (De la Haba *et al.*, 1959) had a specific activity $[C.U.]^{GTA}_{mg\ protein} = 62$.[1] The dipeptide amides were prepared as described previously (Fruton *et al.*, 1953; Theodoropoulos and Fruton, 1962). The enzyme experiments were conducted at 37.5°, with 0.01 M β-mercaptoethylamine as the enzyme activator. Ammonia liberation during the course of enzymic action was measured by the microdiffusion method of Seligson and Seligson (1951) and carboxyl liberation was measured by the method of Grassmann and Heyde (1929).

For the determination of the yield of polymer formed from Gly.Tyr(NH_2), the procedure described in Würz *et al.* (1962) was used; the insoluble polymer was filtered with suction (analytical Celite was used as a filter aid), washed thoroughly with water, and dissolved in alkali (final concentration, 0.01 N NaOH), and the tyrosine content of the solution was determined spectrophotometrically at 294 mμ (ε, 2389). For the determination of the amide-N of the polymer from Gly.Tyr(NH_2), a portion of the alkaline solution was heated to 110° for 15 hours in the presence of 6 N HCl in a sealed tube; after removal of HCl, samples of the acid hydrolysate were taken for NH_3 determination by the microdiffusion method.

Since the polymers derived from Gly.Phe(NH_2) and Ala.Phe(NH_2) were insoluble in alkali, the precipitates were filtered, transferred quantitatively into 6 N HCl, and hydrolyzed at 110° for 15 hours in sealed tubes. Samples of the hydrolysates were taken for separate determination of amide-N (by microdiffusion) and of phenylalanine (by spectrophotometry at 258 mμ; ε, 195).

In the case of the polymer derived from Gly.Trp(NH_2), the hydrolysis was conducted with $Ba(OH)_2$ as described in Würz *et al.* (1962). The tryptophan content of the hydrolysate was determined spectrophotometrically at 280 mμ (ε, 5400). Analytical data on the composition of hydrolysates of the polymers were also obtained by means of automatic amino acid analysis by the method of Spackman *et al.* (1958). To facili-

* This work was aided by grants from the U. S. Public Health Service (RG-6452) and the National Science Foundation (G-7451).

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[1] Cathepsin units per mg protein using glycyl-L-tyrosinamide as substrate.