EFFECTS OF SURFACE ACTIVE AGENTS ON CRYSTALLINE RABBIT MUSCLE PHOSPHORYLASE

by

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Many examples of denaturation of enzymes by detergents or surface active agents (SAA) are known (PUTNAM[14]). Few instances of activation of enzymes by such agents have been reported. ALLEN AND BODINE[1] were able to activate protyrosinase of grasshopper eggs by anionic alkyl sulfates. Cationic alkyl amines were ineffective or inhibitory. On the other hand, KREBS[11] and HUGHES[9] reported that certain cationic SAA increased the activities of bacterial glutamic acid decarboxylase and glutaminase while anionic SAA inhibited these enzymes. Nonionic SAA were without effect in the few cases where tests have been reported.

Cationic, anionic and nonionic SAA in dilute solution display anomalies in certain physicochemical properties, such as viscosity, surface tension, equivalent conductivity, freezing point lowering, *etc.*, with change of concentration. These anomalies are interpreted as arising from the association of molecules of the SAA into aggregates or micelles, MCBAIN[12]. This transition is rather abrupt with change of concentration of the SAA and the concentration at which the anomalies appear is referred to as the critical micelle formation concentration (CMC). These agents exert their most profound effects upon proteins when present in such amounts that micelles exist. Indeed, HUGHES[10] found that the minimum concentrations of cationic SAA required to activate glutamic acid decarboxylase and glutaminase coincide with their CMC values. MATHEWS[13] has shown a correlation between the CMC values of several anionic SAA and the concentrations of these SAA for equivalent inhibition of hyaluronidase.

Crystalline rabbit muscle phosphorylase is activated by nonionic surface active agents. Cationic and anionic SAA inhibit this enzyme. The importance of the micellar structure of the SAA for activation or inhibition is shown by the facts that the concentrations of nonionic SAA for activation and of cationic SAA for inhibition coincide with their concentrations for micelle formation. Further, certain nonionic SAA which do not form micelles fail to activate rabbit muscle phosphorylase.

MATERIALS AND METHODS

Surface active agents were obtained from the following sources. Atlas Powder Company supplied the nonionic agents Tween 80 (polyoxyethylene sorbitan monooleate), Myrj 51 (polyoxyethylene stearate), Brij 35 (polyoxyethylene lauryl alcohol), Span 20 (sorbitan monolaurate), Arlacel C

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(sorbitan sesquioleate), G-1144 (polyoxyethylene sorbitol oleate), and G-2240 (polyoxyethylene sorbitol). Polyethylene glycols (PEG) of various average molecular weights were furnished by Dow Chemical Company. The average molecular weight as reported by the supplier is designated by the sample number, *i.e.*, PEG-400 is a sample of av. mol. wt 400. Triton X-100 is a nonionic alkyl aryl polyethylene glycol supplied by Rohm and Haas Company. Union Carbide and Carbon Corporation furnished the cationic agent, Amine 220. The anionic agent Aerosol O.T. (dioctyl sodium sulfosuccinate) was purchased from Eimer and Amend Company and the cationic agent, cetyldimethylethylammonium bromide (CDEA), from Eastman Kodak Company.

Determinations of critical micelle-formation concentration (CMC) were made by a modification of the method of CORRIN AND HARKINS[4]. This method is based on the color change that an appropriate acid-base indicator will undergo, even in a well-buffered solution, when a SAA is added in amounts equal to or in excess of the CMC. A solution of 0.0015% by weight bromothymolblue (Eimer and Amend Company) in 0.03 *M* imidazole buffer of pH 6.8 was used in the present experiments. A marked color change from the greenish-blue that is characteristic of bromothymolblue at pH 6.8 to a bright yellow occurred when a nonionic or anionic SAA (previously adjusted to pH 6.8 when tested with a glass electrode pH meter) was added above the CMC. Cationic SAA such as CDEA or Amine 220 altered the color to a brilliant blue. A series of samples of constant composition, except for progressive dilution of the SAA, were compared in a Klett-Summerson photoelectric colorimeter (Klett filter No. 56). The concentration of SAA required for half the maximum color transformation was selected as the CMC.

Rabbit muscle phosphorylase was crystallized by the method of GREEN AND CORI[8]. Phosphorylase activity is expressed in the units defined by CORI, CORI AND GREEN[2]. Number of units = $1000 \times K$, where,

$$K = 1/t \log \frac{x_e}{x_e - x} \qquad (1)$$

The percent glucose-1-phosphate (G-1-PO_4) converted to polysaccharide in time, t, is represented by x, while x_e is the percent converted to polysaccharide at equilibrium. Glycogen was present in excess. The measure of activity was the rate of inorganic phosphate liberation during the reaction. The phosphorylase preparation was preincubated at 30° C with 0.015 *M* cysteine at pH 6.8 for fifteen minutes. The preincubated enzyme was added to a solution of glucose-1-phosphate and glycogen in such amounts that the ultimate composition of the assay mixture was enzyme, 0.0075 *M* cysteine, 0.015 *M* G-1-PO_4 and 1% glycogen at pH 6.8. Incubation was continued at 30° and samples were withdrawn at appropriate intervals for determination of inorganic phosphate by the method of FISKE AND SUBBAROW[6]. For determination of total phosphorylase (phosphorylase *a* + phosphorylase *b*), the assay mixture also contained 1×10^{-3} *M* muscle adenylic acid (AMP). Phosphorylase *a*/phosphorylase (*a* + *b*) corresponds to the ratio of activities found in the absence and in the presence of AMP respectively.

RESULTS

Crystalline rabbit muscle phosphorylase could be activated as much as two-fold by the additon of certain nonionic SAA such as Tween 80 during the preincubation period with cysteine. The data of Table I indicate that the activation is a relatively slow process. Cationic and anionic SAA inhibit the enzyme strongly.

Activation of both phosphorylase *a* and phosphorylase *b* by a nonionic SAA could be demonstrated by the experiment of Fig. 1. Phosphorylase *a* is converted to phosphorylase *b* by the PR enzyme isolated from rabbit muscle[3]. Prior to exposure of crystalline phosphorylase *a* to PR enzyme, Tween 80 activated the enzyme 190% in the absence of AMP and 150% with AMP present in the assay mixture. After incubation of the phosphorylase with PR for varying periods, aliquots of the mixture had progressively diminishing activity in the absence of AMP (conversion of phosphorylase *a* to phosphorylase *b*) but the per cent activation by Tween 80 in the presence of AMP in the assay mixture remained at about 150%.

It is unlikely that the nonionic SAA merely stabilizes the enzyme and thereby prevents denaturation during the preincubation and assay periods. Phosphorylase decreases in activity only 10% during as long as three hours preincubation at 30° and the same loss occurred in the presence of Tween 80. Furthermore, the percent activation by

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TABLE I

EFFECT OF TIME OF INCUBATION WITH TWEEN 80 ON THE EXTENT OF ACTIVATION OF CRYSTALLINE RABBIT PHOSPHORYLASE

All enzyme samples were preincubated with 0.015 *M* cysteine at 30° for 15 minutes prior to addition to an equal volume of the G-1-PO_4 and glycogen mixture.

Conditions	*Phosphorylase a activity*		
	Assay sample time	*Units*	*Percent activity*
No Tween 80 added	5 minutes	12.2	
	10 minutes	13.4	100
	15 minutes	14.4	
0.01 % Tween 80 added to the G-1-PO_4 and glycogen mixture 1 min before the end of preincubation	5 minutes	14.8	
	10 minutes	18.1	130
	15 minutes	18.9	
0.01 % Tween 80 added to the G-1-PO_4 and glycogen mixture at the start of 15 min preincubation	5 minutes	16.8	
	10 minutes	18.9	140
	15 minutes	20.7	
0.01 % Tween 80 added to the enzyme and cysteine mixture 5 min before the end of the preincubation	5 minutes	19.6	
	10 minutes	20.7	155
	15 minutes	20.5	
0.01 % Tween 80 added to the enzyme and cysteine mixture at the start of 15 min preincubation	5 minutes	21.0	
	10 minutes	21.6	160
	15 minutes	21.1	

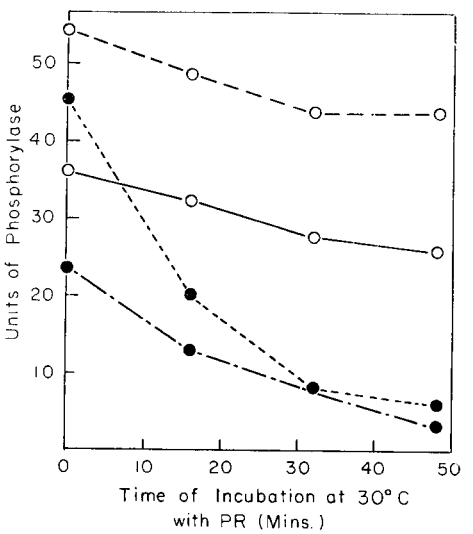

Fig. 1. Action of PR enzyme on phosphorylase *a*: Effect on phosphorylase activation by Tween 80. Samples were assayed for phosphorylase activity after 5′ incubation at 30° with 0.03 *M* cysteine and 0.1 *M* NaF ± 0.02 % Tween 80 and ± $1 \cdot 10^{-3}$ *M* AMP.

	Tween 80	*AMP*
———	—	+
– – – –	+	+
— · —	—	—
· · · · ·	+	—

Tween 80 was the same whether incubation and assay was at 8° or 30°, and was constant over a seven-fold change in phosphorylase concentration. Also excluded is an influence of the SAA on the relation of enzymic activity to the presence or absence of adenylic acid, inosinic acid or cysteine; glycogen concentration (0–2.5%); G-1-P concentration (0.005–0.05*M*); or pH of the reaction mixture Activation also seemed independent of

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the condition of the phosphorylase crystals such as number of recrystallizations, age of the preparation or the ratio of phosphorylase *a*/phosphorylase (*a* + *b*).

Not all of the nonionic SAA increase the activity of crystalline rabbit muscle phosphorylase although the change in surface tension may be of the same order. For example, Tween 80 and polyethylene glycol, PEG-400, both lower the surface tension of aqueous solutions to 35–40 dynes/cm. Yet PEG-400 had no influence on phosphorylase activity even when present in amounts up to 1%. The higher molecular weight PEG-4000, however, produced activation about equivalent to that by Tween 80 (Table II) as though the

TABLE II

COMPARISON OF THE ACTION OF SURFACE ACTIVE AGENTS ON RABBIT MUSCLE PHOSPHORYLASE AND THE ABILITY TO FORM MICELLES

Agent	*Activity of phosphorylase**	*Action on bromothymolblue at pH 6.8*		*Published values*	
		Color change	*CMC*	*CMC*	*Reference*
None	100%				
Tween 80	180	Deep yellow	0.008% (wt)		
Myrj 51	180	Deep yellow	0.006% (wt)		
Brij 35	160	Deep yellow	0.014% (wt)		
Triton X-100	155	Deep yellow	0.017% (wt) ($3 \cdot 10^{-4}$ *M*)	$9 \cdot 10^{-4}$ *M*	7
G-1144	145	Deep yellow	0.011% (wt)		
Span 20	150	Slight yellow**			
Arlacel C	140	Slight yellow**			
PEG-200	100	No change			
PEG-400	100	No change			
PEG-600	115	No change			
PEG-4000	150	No change			
G-2240	85	No change			
Aerosol O.T.	Strong inhibition	Slight yellow	0.008 *M*	0.01 *M*	12
Amine 220	Inhibition	Deep blue	$8 \cdot 10^{-5}$ *M*		
CDEA	Inhibition	Deep blue	$1 \cdot 10^{-4}$ *M*		

* Enzyme incubated with SAA plus cysteine for 15 minutes. Conc. of SAA was 0.04%; except for PEG-400 (1%), and Aerosol, Amine 220 and CDEA (0.0002 *M*).

** Span 20 and Arlacel C were too insoluble for CMC determination.

greater size of the polymer were important. This possibility is of interest in light of the observation of HUGHES[10] that the minimum concentration of cationic SAA for activation of bacterial glutamic acid decarboxylase and glutaminase coincides with the concentration required for creation of micellar structures within the solution by aggregation of the SAA. It may be seen from Table II that those SAA which activated phosphorylase are in all cases, except for the higher molecular weight polyethylene glycols, the same agents that gave evidence of micelle-formation by change in color of bromothymolblue. The data for determination of the CMC of Tween 80 and superposition of data for activation of rabbit muscle phosphorylase by this agent are given in Fig. 2. That the concentration of the cationic agent, CDEA, for inhibition of rabbit muscle phosphorylase also coincides with its CMC is indicated by Fig. 3.

Crystalline rabbit muscle phosphorylase was recrystallized from a solution of 0.07*M* cysteine and 0.03*M* β-glycerol phosphate, pH 6.8, to which was added Tween 80 to 0.02% (an amount in excess of the CMC). The recrystallized preparation was comparable in activity with an identical sample recrystallized in the absence of Tween 80, if the Tween

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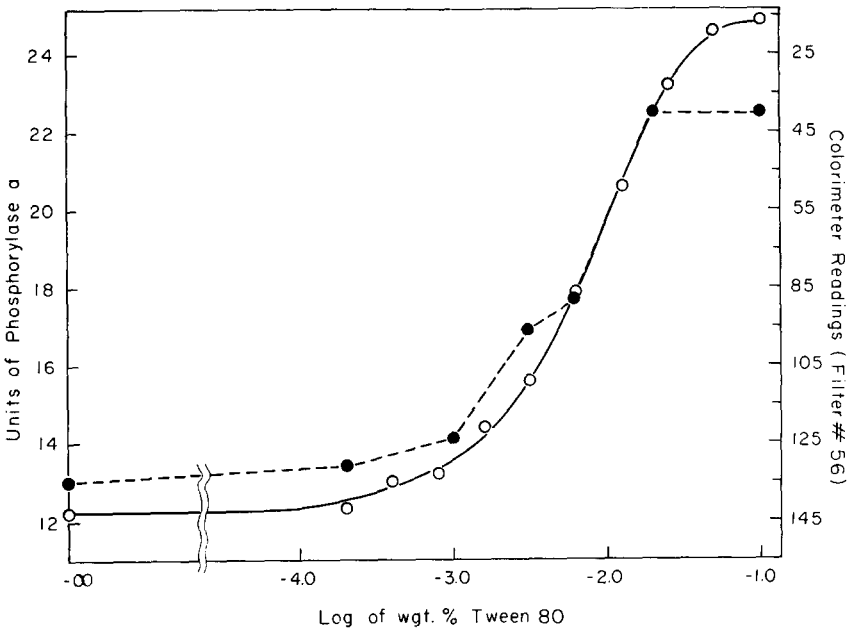

Fig. 2. Correlation of activation of phosphorylase *a* by Tween 80 and change of color of bromothymolblue by Tween 80. Activation of phosphorylase *a* —— —; Change of color of bromothymolblue – – – –.

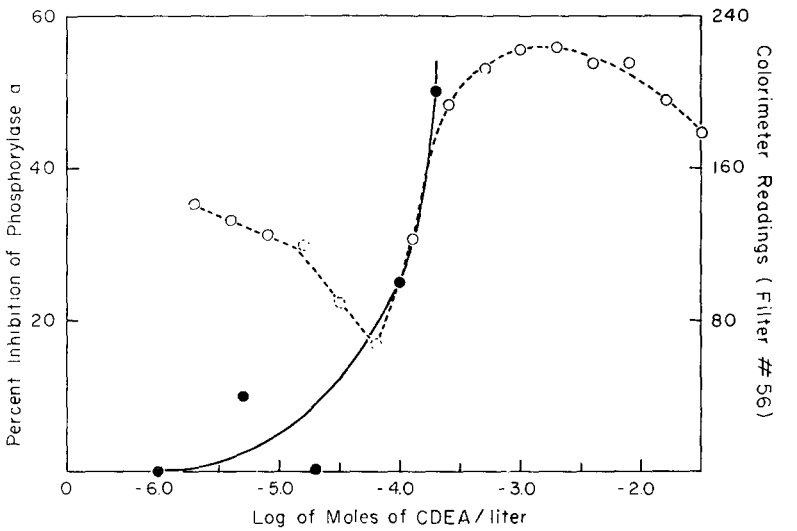

Fig. 3. Correlation of inhibition of phosphorylase *a* by CDEA and change of color of bromothymolblue by CDEA. Inhibition of phosphorylase *a* ———; Change of color of bromothymolblue – – – –. Dotted circles indicate that samples were turbid.

80 was diluted below its CMC value before the assay for activity (Table III). It may be noted that both the enzyme recrystallized in the presence and in the absence of Tween 80 were activated to the same extent by Tween 80 at concentrations above the CMC value in the assay mixture.

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TABLE III

ACTIVITY OF RABBIT PHOSPHORYLASE RECRYSTALLIZED IN THE PRESENCE AND IN THE ABSENCE OF TWEEN 80

*Conditions during recrystallization**	*Assay without Tween 80*			*Assay with 0.02% Tween 80*		
	Phosphorylase units		*Ratio*	*Phosphorylase units*		*Ratio*
	a	*(a + b)*	*a/(a + b)*	*a*	*(a + b)*	*a/(a + b)*
Absence of Tween 80	11.9	15.7	0.76	20.7	24.4	0.85
Presence of 0.02% Tween 80	9.8	11.9	0.82	16.9	21.6	0.78

* See text for details of recrystallization.

DISCUSSION

Results of the experiments described above extend the observations that certain enzymes are activated or inhibited by SAA and that the effects may be related to the formation of micellar structures. If micelle-formation is the critical change that leads to phosphorylase activation, the way by which the micelles accomplish this was not revealed by the present experiments. Activation of protyrosinase (ALLEN AND BODINE[1]) and of glutamic acid decarboxylase (HUGHES[10]) by SAA are believed to involve rearrangement of the protein to expose the enzyme moiety in the first instance and removal of an inhibitor in the second. Neither of these processes is in accord with the results for activation of crystalline rabbit muscle phosphorylase. Another possibility would be that the micelle structure might change the thermodynamic environment of the reaction mixture. For example, the micelles might maintain the relatively huge phosphorylase and glycogen molecules in positions favorable for repeated interaction. The above results, however, give no indication of such a mechanism, *i.e.*, the extent of activation of rabbit muscle phosphorylase by Tween 80 does not vary with change of glycogen concentration of the assay mixture up to concentrations that saturate the enzyme.

The results of Table III indicate that activation of the enzyme is not a permanent change in the enzyme molecule but persists only so long as the SAA is present in micelle-forming concentrations in the assay mixture. This phenomenon stands in contrast to the increased phosphorylase activity of lobster muscle extracts during storage[5]. The latter increase in activity can be accelerated by a variety of agents including surface active agents, both ionic and nonionic. However, the change in crude lobster muscle extracts appears to be due to the conversion of an inactive phosphorylase to phosphorylase *a*. This change appears to require other factors present in the crude extract. Once the level of lobster phosphorylase has risen, that level is maintained on purification and irrespective of the presence or absence of surface active agents in the assay mixture.

SUMMARY

1. Activity of rabbit muscle phosphorylase *a* and phosphorylase *b* could be increased as much as two-fold by the addition of certain nonionic surface active agents. The concentrations of these agents required for activation of rabbit muscle phosphorylase coincide with the concentrations required for aggregation of these agents into micelles.

2. Cationic and anionic surface active agents inhibit rabbit muscle phosphorylase. This inhibition also seems to be connected with the formation of micelles by these agents.

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RÉSUMÉ

1. Les activités de la phosphorylase *a* et de la phosphorylase *b* du muscle de lapin peuvent être accrues jusqu'à deux fois par l'addition de certains agents tensioactifs non ioniques. Les concentrations des détergents nécessaires à l'activation de la phosphorylase du muscle de lapin coïncident avec les concentrations pour lesquelles les détergents s'aggrègent en micelles.

2. Les agents tensioactifs cationiques et anioniques inhibent la phosphorylase du muscle de lapin. Cette inhibition semble également être en rapport avec la formation de micelles par ces agents.

ZUSAMMENFASSUNG

1. Durch Hinzufügen von bestimmten nichtionischen oberflächenaktiven Stoffen konnte die Aktivität von Phosphorylase *a* und Phosphorylase *b* aus Kaninchenmuskeln verdoppelt werden. Die für die Aktivierung von Kaninchenmuskelphosphorylase benötigten Konzentrationen dieser Stoffe stimmen mit den Konzentrationen überein, welche notwendig sind, um diese Stoffe zu Mizellen zusammenzuballen.

2. Kationische und anionische oberflächenaktive Stoffe hemmen Kaninchenmuskelphosphorylase. Diese Hemmung scheint gleichfalls mit der Mizellenbildung dieser Stoffe zusammenzuhängen.

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