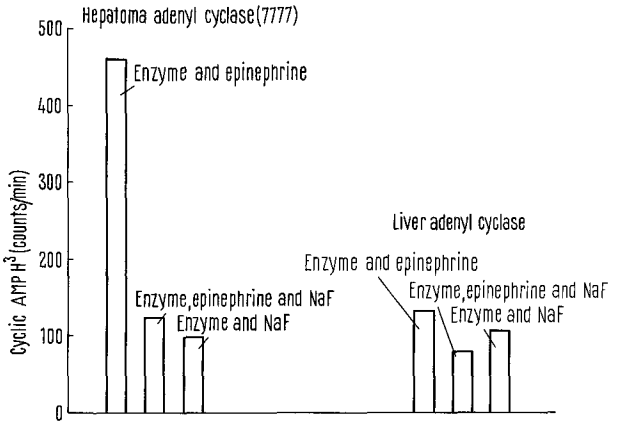
substrate (15.00 mg Na_2ATP, 3.54 mg $MgSO_4$, 2.08 mg NaF, 6.50 mg caffeine, in a 0.05*M Tris* buffer, pH 7.2)[7]. The reaction was allowed to proceed at 37 °C for 15 min, at which time it was stopped by placing the tube containing the reaction mixture into boiling water for 3 min and then into an ice-bath for 10 min. In certain of the experiments, 3 μC of ^3H-Na_2ATP was added to the reaction mixture, and the amount of cyclic AMP formed measured by radio-isotopic method[8]. This procedure involves the removal of the denatured protein material by centrifugation followed by partial separation of the reactants and products on a short Dowex-50 column. After this the fraction containing the partially separated cyclic AMP is treated with barium hydroxide and zinc sulfate which further separates the cyclic AMP by coprecipitating the contaminating materials. After coprecipitation and centrifugation, an aliquot of the supernatant activity is measured by liquid scintillation counting. The amount of cyclic AMP formed is given in terms of counts of tritiated cyclic AMP/min/mg.

Results and discussion. The stimulatory effect of epinephrine ($4.4 \times 10^{-5} M$) reported earlier[9] was not observed when NaF was present in the incubation mixture of enzyme from either normal or hepatoma tissue. We assume, prima facie, that NaF can stimulate adenyl cyclase maximally and that epinephrine has no further stimulatory effect. However, epinephrine does markedly further stimulate activity of hepatoma-derived enzymes in the absence of NaF. Several interpretations of this observation are possible. One thesis is that a structural abnormality of the enzyme molecule occurs in the tumors studied and that is related to the increase in the activity of the enzyme and the difference in response in epinephrine in the presence and absence of NaF. However, the possibility also exists that the variation in response to epinephrine is related to the state of disruption of the enzyme-membrane complex and that the variation in the disruption of this complex is a function of whether or not one has normal tissue or hepatoma tissue[10].



Bar graph relating tissue source and experimental conditions to amount of cyclic AMP formed.

Zusammenfassung. Die in vitro Stimulation der Adenyl-Cyclase durch Epinephrin zur Bildung der zyklischen AMP ist im Hepatomgewebe signifikant höher als in der normalen Rattenleber. Sodiumfluorid hemmt in beiden Fällen die Stimulation.

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[7] E. W. SUTHERLAND, T. W. RALL and T. J. MENON, Biol. Chem. *237*, 1220 (1962).
[8] G. KRISHNA, B. WEISAL and B. BRODIE, J. Pharmac. exp. Ther. 163, 379 (1968).
[9] G. A. ROBINSON, R. W. BUTCHER and E. W. SUTHERLAND, Ann. N.Y. Acad. Sci. *139*, 703 (1967).
[10] This work was supported by the U.S. Public Health Service.

Effect of Proteins on the Reversibility of the Thermal Inactivation of *Bacillus subtilis* α-Amylase

Since the precipitation which occurs upon heat denaturation of protein is probably due to noncovalent inter- and intrachain associations, and since such interactions are believed to be dissociated by urea or guanidine-HCl, attempts were made to regain activity from heat-denatured precipitated enzyme by dissolving it in either guanidine-HCl or urea and then removing the latter by dilution. This was successful with *Escherichia coli* galactosidase[1] and luciferase[2]. It is known that bacterial α-amylase can recover activity after treatment with high concentration of urea[3].

Experiments with urea or guadidine treatment were carried out. Crystalline amylase was dissolved in 8*M* urea or 6*M* guanidine and kept for 6 min at room temperature. The protein solution was then diluted 1000-fold with buffer, as below, and the recovery of activity was very close to 100%. To compare with the dilution experiment, the same sample was dialysed against successive dilutions of denaturing agents; in both cases, recovery was lower than after diluting sample. It is known that amylase once inactivated by urea is not reactivated by dialysis[4]. It was therefore thought interesting to test whether that enzyme, first denatured by heat, could recover its active configuration after being exposed to guanidine-HCl or urea.

Crystalline α-amylase (EC 3.2.1.1) from *Bacillus subtilis* (Sigma Type IIA) was dissolved in phosphate buffer 0.15*M* pH 6.8 with 0.05*M* NaCl and 0.05*M* NaF.

[1] D. PERRIN and J. MONOD, Biochem. biophys. Res. Commun. *12*, 425 (1963).
[2] J. FRIEDLAND and J. W. HASTINGS, Biochemistry *6*, 2893 (1967).
[3] T. TAKAGI and T. ISEMURA, J. Biochem., Tokyo *52*, 314 (1962).
[4] B. HAGIHARA, T. NAKAYAMA, H. MATSUBARA and K. OKUNUKI, J. Biochem., Tokyo *43*, 469 (1956).

Activity was assayed by an amyloclastic method[5]. 0.25 ml samples (1 mg/ml) were denatured by heating in a boiling bath for 3 min. The white precipitate was dispersed and suspended to take samples. Less than 2×10^{-3} of the activity originally present remained. The protein suspensions were then centrifuged and supernatants discarded. The precipitates were dissolved either in 0.25 ml 8 *M* urea or 0.25 ml 6 *M* guanidine-HCl. After 6 min, the preparations were centrifuged, some insoluble material was discarded and clear and colourless supernatants were diluted 1000-fold with buffer. Enzymatic activity was measured after 1 h at room temperature. Experiments illustrating results obtained under such conditions are presented in Table I.

Table I. Recovery of activity (% of initial activity) of heat denatured α-amylase

Treatment	% Recovery
Precipitate suspended in phosphate buffer	0.2
Supernatant (discarded)	1–2
Precipitate dissolved in 8 *M* urea, then diluted in phosphate buffer	15
Precipitate dissolved in 6 *M* guanidine-HCl, then diluted in phosphate buffer	19

In another experiment, one-step dilution of guanidine-HCl with buffer was compared to dilution with buffer containing proteins (Table II). Some acidic and basic proteins were tested at 2 concentrations, 0.01 mg/ml and 0.1 mg/ml; at higher concentrations, the proteins interfered with the amyloclastic method.

Table II. Effect of various proteins on renaturation of heat denatured α-amylase

Protein added			% Recovery
Nature	pHi	Concentration (mg/ml)	
Without protein	–	–	19
Equine acidic	2.3	0.01	27
α-1-glycoprotein	–	0.1	31
Human serumalbumine	4.9	0.01	22
		0.1	34
Lysozyme	10.5	0.01	17
		0.1	15
Protamine	12	0.01	15
		0.1	8

Unfolding agent: guanidine-HCl 6 *M*.

From the above experiments it may be seen that guanidine-HCl and, to a lesser extent, urea could reverse denaturation by heat. The presence of acidic proteins accelerated the rate of reactivation which was found to depend on protein concentration.

The same results were obtained with luciferase, by FRIEDLAND and HASTINGS[2], using bovine serumalbumine. This protein is not an absolute requirement for renaturation, but it enhances the process. YUTANI et al.[6] observed that acidic proteins accelerated the reactivation of urea denatured bacterial amylase; they concluded that BSA interacted reversibly with unfolded polypeptide chain of denatured enzymes and promoted directly the reformation of the native structure. EPSTEIN et al.[7] reported that the renaturation of denatured lactate dehydrogenase was accomplished in renaturation medium containing BSA to prevent physical side-reaction such as adsorption of protein to glass.

To examine this possibility, glass beads were added to the solution of denatured amylase in the course of regeneration. As shown in Table III, the amylase activity that was recovered was increased when HSA was added in the early stage of regeneration.

Table III. Effect of glass surface on recovery of activity of heat denatured α-amylase

Glass surface (cm^2)	25	45	60	96	240
% Recovery after dilution with:					
Phosphate buffer	19	18	17	14	9
Phosphate buffer containing 0.1 mg/ml HSA	34	26	24	21	16

Unfolding agent: guanidine-HCl.

The results of the present study are compatible with the theory that amino-acid sequence determines secondary and tertiary structure of protein. High dilution of amylase is necessary during the first stage of the renaturation. During this process, denatured molecules tend to be adsorbed on the wall of the glass vessel; acidic proteins prevent this adsorption. The inhibitory effect of lysozyme and protamine may be due to their basic character. It presents a similitude with the inhibitory effect of glass surface which could be of ionic nature.

Zusammenfassung. Die Reaktivierung von thermisch desaktivierter α-Amylase aus *Bacillus subtilis* durch Zusatz von Guanidin-HCl oder Harnstoff oder von Guanidin mit Zusatz verschiedener Proteine wurde untersucht.

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[5] M. SOMOGYI, Clin. Chem. *6*, 23 (1960).
[6] K. YUTANI, A. YUTANI and T. ISEMURA, J. Biochem., Tokyo *62*, 576 (1967).
[7] C. J. EPSTEIN, M. M. CARTER and R. F. GOLDBERGER, Biochim. biophys. Acta *92*, 391 (1964).