ly, as shown by the first set of experiments, we would have been led to believe that there was no stimulatory factor present had we not prepared our SS early in the morning.

The time of injection after hepatectomy is also important. Our earlier work (*3*) showed a time lag between injection of SS and the appearance of its effect. The alternative interpretation, assumed by Llanos in his work with mice (*8*), that the absence of an effect is due to the different time of day at which the injection is given, does not appear to operate in these experiments since the same stimulation was produced regardless of the time of day.

Variations in the donor animals must also be carefully controlled. Not only is the time of preparation of the SS from the donor important (as shown in this report) but also, with increasing age of the donor animals, the stimulatory effect disappears (*3*).

The problem of determining what so elegantly controls liver regeneration has been intriguing and elusive. The necessity of using a live animal model produces additional variables, difficult to control and not present in classical cell-free biochemical studies or in work with tissue culture, isolated perfused organs, or isolated cell suspensions. Failure to appreciate and to control adequately some of the readily accessible variables has led to much of the confusion and dispute in this literature (*1, 2*). Diurnal rhythm affects a variety of liver functions (*9*) including endogenous and regenerative growth (*5, 6*) and must be carefully regarded in studies of these phenomena.

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Enhancement of Bovine Pancreatic Ribonuclease Activity by Mercaptoethanol

Abstract. *Incubation of ribonuclease with 0.1*M *mercaptoethanol at* p*H 8.5 can increase the enzyme's hydrolytic activity toward cytidine 2',3'-monophosphate (cyclic CMP) under standard assay conditions. Cation-exchange chromatography of the ribonuclease-thiol reaction mixture revealed seven fractions. The fraction with the highest activity had an approximate tenfold decrease in the apparent Michaelis constant for cyclic CMP with respect to native ribonuclease. The enhanced activity is a metastable property since this fraction reverts back to the control activity and chromatographic behavior of native ribonuclease on standing in solution at room temperature.*

While developing a method for the selective ^{13}C enrichment of the *S*-methyl carbon of methionine-29 in bovine pancreatic ribonuclease for nuclear magnetic resonance studies, we unexpectedly prepared ribonuclease derivatives with enhanced activity toward cytidine 2',3'-monophosphate (cyclic CMP). The labeling reaction requires two steps: (i) the methylation of ribonuclease with $^{13}CH_3I$, which produces *S*-methylmethionine-29 ribonuclease (*1*), and (ii) the regeneration of the native methionyl residue via a nucleophilic displacement of one of the *S*-methyl groups by 2-mercaptoethanol (*2*). This reaction sequence results in a product that incorporates 50 percent of the ^{13}C label (*3*).

Unfortunately, ribonuclease contains four disulfide bonds which potentially could be reduced by the thiol leading to a disruption of the enzyme's tertiary structure. During trial experiments to determine what concentration of mercaptoethanol native ribonuclease would tolerate without a loss in activity, we observed an unexpected increase in ribonuclease activity.

Ribonuclease (Worthington RAF, phosphate free and monophoretic on gel electrophoresis) was incubated with mercaptoethanol (0.025 to 0.200*M*) in 0.2*M* phosphate at *p*H 8.5 and room temperature. The thiol concentration and enzyme activity were measured periodically as described in Fig. 1. With initial thiol concentrations below 0.1*M*, the activity remained at control levels for about 3 days before the increase in activity was observed (Fig. 1A). With greater than 0.1*M* thiol, we found an initial decrease in enzymatic activity which returned to and eventually increased above the control level.

We have repeated similar experiments seven times and, although the maximum activity reached (160 to 220 percent of control), the time to peak enhancement (10 to 19 days), and the residual thiol concentration at the activity maximum (0 to 40 percent of the initial level) varied, the general pattern of activity enhancement was always the same. This enhancement effect and the variability observed appeared to be a function of the degree of exposure of the samples to the air, which, in turn, controlled the rate of thiol loss via autoxidation.

The importance of sample exposure to the air is demonstrated in Fig. 1B and Fig. 2. No increase in activity was found in any of the samples incubated in syringes to exclude air (Fig. 1B). After 19 days of incubation, the thiol concentration decreased from 0.1 to 0.08*M* while the ribonuclease activity simultaneously decreased to 80 percent. In contrast, the 0.1*M* sample incubated for the same length of time in the test tubes (Fig. 1A) had a residual thiol concentration of 0.032*M* and a ribonuclease activity of 178 percent of control. If greater exposure to the air was ensured by incubating the samples in open beakers (Fig. 2), then maximum activity was generated in only 4 to 5 days. These data suggested that the oxidation of mercaptoethanol to diethanoldisulfide may be involved in the enhancement process. However, when diethanoldisulfide (0.05 to 0.50*M*) was included in the reaction mixtures in either the test tubes or the beakers (Fig. 2), the rate of thiol decrease, the time required for the generation of peak activity, and the degree of activity enhancement were much the same as observed in the absence of the disulfide. Apparently, either sufficient amounts of disulfide were generated by mercaptoethanol autoxidation or the decrease in thiol level per se rather than the simultaneous increase in the disulfide concentration was the cause of activity enhancement.

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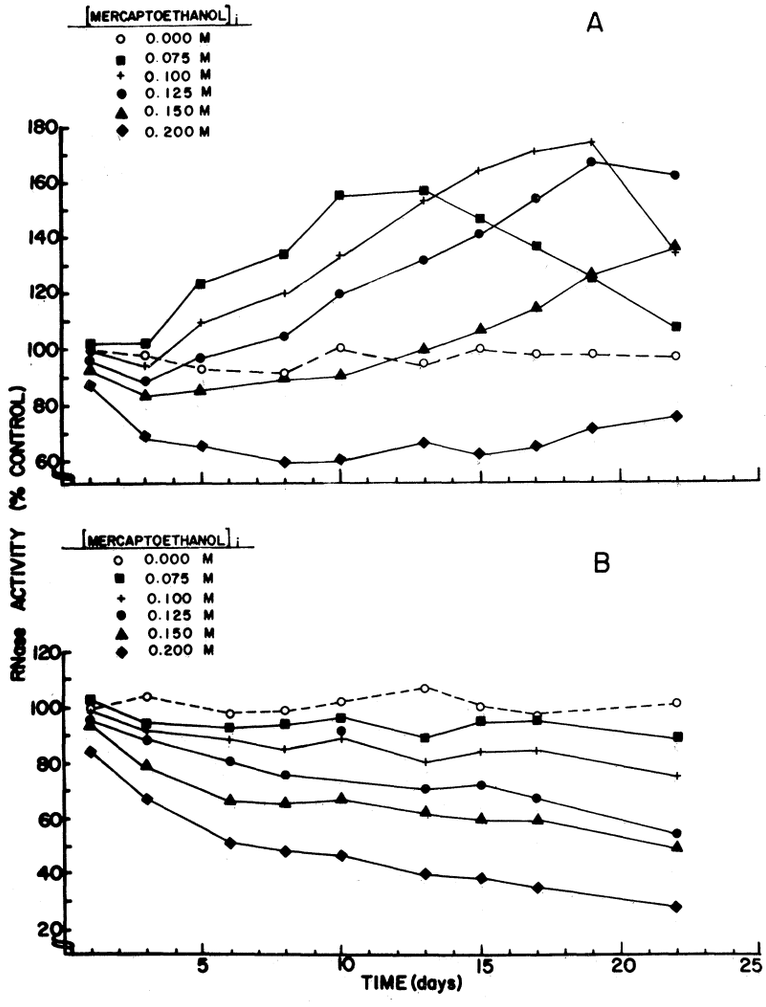

Fig. 1. The effect of various initial mercaptoethanol concentrations on ribonuclease activity as a function of time. Each sample consisted of 20 ml of ribonuclease ($1.8 \times 10^{-5}M$) with the appropriate mercaptoethanol concentration in 0.2M phosphate, pH 8.5, at room temperature. Portions (1 ml) were removed periodically to assay for thiol content and enzyme activity. Thiol concentrations were measured by the method of Ellman (*17*). Ribonuclease activity toward cyclic CMP was determined by the standard assay of Crook *et al.* (*4*) at pH 7.1 and 25°C. The activity is expressed as a percentage of control enzyme activity with no thiol present. (A) These samples were incubated in stoppered 25-ml test tubes. Except for keeping the test tubes capped between assays, we made no additional effort to eliminate contact of the sample with air. The 0.025M thiol sample behaved similarly to the control, and the 0.050M thiol sample showed only a 20 percent increase in activity. They have been eliminated from the figure for clarity. The specific activity (rate of change in absorbance at 284 nm per milligram per milliliter of ribonuclease) corresponding to 100 percent was 2.31. (B) These samples were incubated in capped 20-ml syringes with the plunger adjusted to eliminate the air space over the samples. However, the samples were not degassed. The 0.025 and 0.050M thiol samples behaved similarly to the control and were not included in the figure. The specific activity corresponding to 100 percent was 2.02.

If this increased activity was due to a reaction of the thiol with any of the four disulfide bridges of ribonuclease, then many products could be generated. Figure 3 illustrates the chromatographic separation of the products generated during incubation with mercaptoethanol. At least seven fractions (A to G) were observed. Fraction D had the highest specific activity which was four to five times that of fraction G. Native ribonuclease had the same elution volume and specific activity as fraction G. Therefore, we designated this fraction as unreacted ribonuclease. Fractions A, B, and C had activities two to three times higher, fraction E had slightly higher, and fraction F had lower activity than the native enzyme. When portions were taken on various days of incubation, their chromatographic profiles were similar to the profile in Fig. 3, although the relative amounts of each fraction (A to G) changed.

Since the specific activity of fraction D was the highest found, the tubes with identical activities were pooled, dialyzed, and lyophilized. Fraction G was isolated in a similar manner. Preliminary steady state kinetic experiments on cyclic CMP hydrolysis were carried out on fractions D and G in 0.1M tris-HCl (ionic strength 0.2), pH 7.1, at 25°C by the method of Crook *et al.* (*4*). The substrate concentration was varied from 0.2 to 1.0 mM and the enzyme (E) concentrations of fractions D and G were 50 and 50 nM, respectively. The initial velocities [change in absorbance at 290 nm (ΔA_{290}) per minute] were converted into moles of substrate hydrolyzed per minute with the experimentally determined ΔE_{290} of $1000M^{-1}$ cm^{-1}. The Lineweaver-Burk plots for fractions D and G were analyzed with Cleland's computer program (*5*). The maximum velocity of fraction D was similar to fraction G. However, there was a substantial decrease in the apparent Michaelis constant (K_m) for fraction D (K_m = 1.5 mM) with respect to the apparent K_m of native ribonuclease and fraction G (K_m = 12.5 mM). Since fraction D has a lower apparent K_m and the standard Crook enzyme assay uses substrate concentrations below the K_m, the increased rate of hydrolysis seen

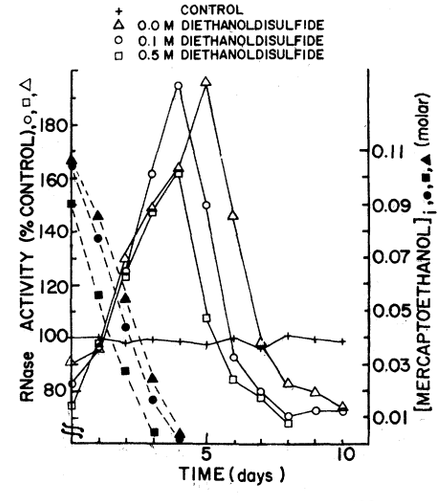

Fig. 2. Mercaptoethanol enhancement of ribonuclease activity in the open beakers. Samples (10 ml) of ribonuclease ($1.8 \times 10^{-5}M$) with 0.1M mercaptoethanol were incubated in 30-ml beakers with 0.0, 0.1, and 0.5M diethanoldisulfide. Care was taken to maintain the proper enzyme concentrations by daily replacement of the water lost by evaporation. Small portions (50 μl) were removed for the determination of thiol concentration and ribonuclease activity. The specific activity corresponding to 100 percent was 3.13.

under standard assay conditions is reasonable.

Moore and co-workers (*6*) have recently reported the preparation of cross-linked dimers of ribonuclease with unusual kinetic properties. We gel-filtered 3 mg of the preparative ribonuclease-mercaptoethanol reaction mixture on Sephadex G-75 (2 × 120 cm) equilibrated in 0.2*M* phosphate, *p*H 6.3, at room temperature (*7*). All of the fractions which separated on the Biorex 70 column (Fig. 3) had an elution volume equivalent to that of native ribonuclease. Thus, all of the fractions with increased activity are monomeric ribonuclease species.

There are considerable data on the interaction of thiols with ribonuclease and on the reoxidation of fully reduced ribonuclease (*8, 9*). These studies demonstrated that mercaptoethanol concentrations of approximately 0.1*M* are insufficient to totally reduce ribonuclease in the absence of denaturants but may reduce up to two of the disulfide bridges. Several workers have shown that various enzymes can be partially reduced without a loss of activity (*10*). Also, thiols can react with protein disulfide bridges to produce mixed disulfides (*11*). In particular, two of the four disulfide bridges of ribonuclease have been selectively ruptured with the incorporation of four phosphorothioate groups (4PS-ribonuclease) (*9*). These groups are covalently linked to the protein as mixed disulfides. 4PS-Ribonuclease had an activity toward cyclic CMP of 220 percent with respect to native ribonuclease under standard assay conditions. Thus, our enhanced activity species could possibly be ribonuclease-mercaptoethanol mixed disulfide analogs of 4PS-ribonuclease.

Neumann *et al.* (*9*) demonstrated that the phosphorothioate groups could be removed from 4PS-ribonuclease within 10 minutes after treatment with a thiol. Thus, we began reversal incubations of fractions A, B, C, and D with a variety of concentrations of dithiothreitol (DTT, 0.01 to 10.0 m*M*) and mixtures of reduced and oxidized glutathione (GSH 0.5 to 50.0 m*M*, and GSSG 1.0 to 10.0 m*M*, respectively) (*12*). However, in all of our reversals the return to native activity required 2 to 4 days. Even in the control samples with no added thiol, a spontaneous decay to native activity was found in the mixtures incubated at room temperature. However, when the fractions were treated as above except that the incubation temperature was 4°C, only a slight decrease in activity was found over a 2-week period even in the presence of thiol.

To investigate the nature of these reversal products, we chromatographed a sample of fraction D which had spontaneously decayed to approximately native activity (Fig. 4). This profile suggested that the reversal of enhanced activity was due to a conversion of fraction D to predominantly fraction G. However, detectable amounts of fractions A, B, and C were also produced. All fractions observed had specific activities equivalent to those illustrated in Fig. 3.

This reversion of fraction D into fraction G (native ribonuclease) indicates that this species with enhanced activity is a metastable structure, possibly a mixed disulfide (*13*). However, we have been unable to demonstrate a thiol-catalyzed reversion of enhanced activity. In addition, recent incubation experiments with native ribonuclease and 20 m*M* DTT, which does not form mixed disulfides (*14*), yielded enhancement of ribonuclease activity. Both of these observations argue against mixed disulfide formation as an explanation for the increased ribonuclease activity. Other possible interpretations include nonnative disulfide bridge formation or altered backbone structures which are trapped as the native bridges in the partially reduced protein reoxidize. Pflumm and Beychok (*15*) have suggested that the positions of the two cysteine residues Cys-72 and Cys-110 are sufficiently close in the ribonuclease molecule to form a nonnative bridge, but no good evidence exists for structurally altered forms of ribonuclease upon reoxidation from the fully reduced state (*8, 12*). Suzuki *et al.* (*16*), however, have explained the twofold increase in ribonuclease activity toward cyclic CMP, which they ob-

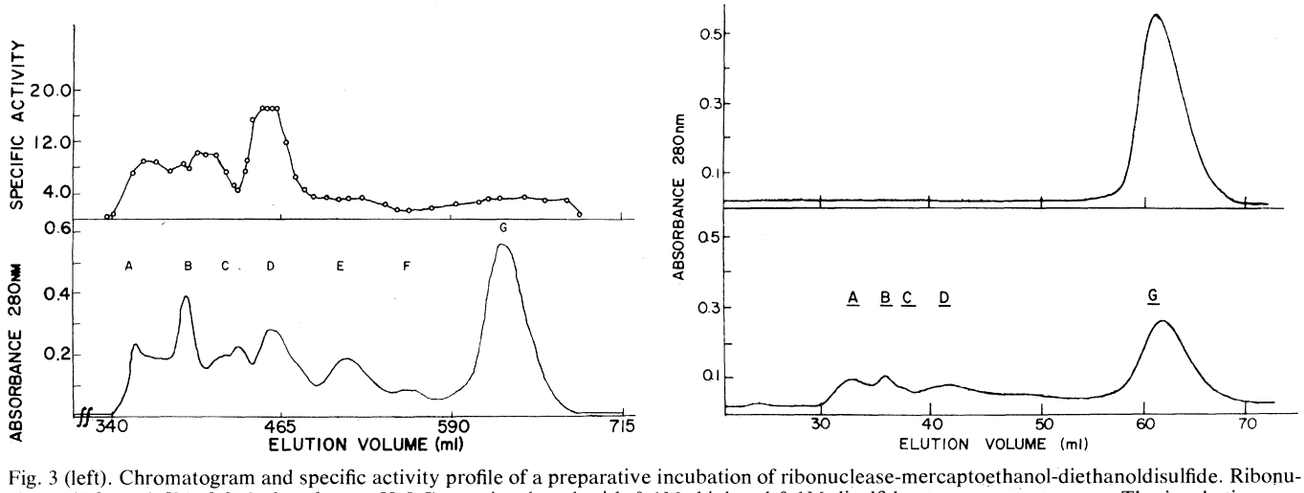

Fig. 3 (left). Chromatogram and specific activity profile of a preparative incubation of ribonuclease-mercaptoethanol-diethanoldisulfide. Ribonuclease (1.8 × 10^{-5}*M*, 0.2*M* phosphate, *p*H 8.5) was incubated with 0.1*M* thiol and 0.1*M* disulfide at room temperature. The incubation was terminated when the reaction mixture had 198 percent of the activity of the control (day 16). The sample was dialyzed in a Biofiber 50 beaker (Bio-Rad) at 4°C against 0.01*M* acetic acid brought to *p*H 5.0 with concentrated NH_4OH until the thiol concentration was below 5 μM. The sample was lyophilized and subsequently chromatographed on Biorex 70 (2.5 × 97 cm) at 4°C. The protein was eluted with 0.2*M* phosphate, *p*H 6.3, at a flow rate of 60 ml/hour. The specific activities for many of the 3-ml fractions were calculated with protein concentrations determined from the absorbance at 280 nm and by the method of Lowry (*18*). Ultraviolet scans of the different fractions from 350 to 240 nm were made to determine if the absorbance at 280 nm would be a reliable indicator of the protein concentration. Except for fraction C, the specific activities calculated from the protein concentrations determined by both methods agree to within 5 percent. The specific activities shown in the figure were calculated with protein concentrations determined by the Lowry method. Fig. 4 (right). Analytical chromatography of fraction D after its reversal to native ribonuclease activity. Five milligrams of fraction D were dissolved in 10 m*M* ammonium bicarbonate, *p*H 7.8, at room temperature. After 2 days, the activity had decayed to approximately native ribonuclease levels and remained constant. On day 6 the sample was lyophilized. Then, the sample was chromatographed at 4°C on an analytical column of Biorex 70 (1 × 47 cm). The protein was eluted with 0.2*M* phosphate, *p*H 6.3, at a flow rate of 4.5 ml/hour. The specific activities of the lettered fractions were similar to the activities shown in Fig. 3. The chromatographic profile of native ribonuclease is shown above the profile for the reversed fraction D.

served when ribonuclease interacted with an activating antibody, as the entrapment of the antigen (ribonuclease) in a conformational form with a higher enzymatic activity. Thus, some evidence for metastable conformational forms of ribonuclease with altered kinetic properties exists.

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Eye Movements of African Chameleons: Spontaneous Saccade Timing

Abstract. *Despite asynchrony, saccades of left and right eyes of African chameleons had similar timing statistics. Prominent qualitative aspects of these statistics did not change if one or both eyes were masked. Evidently, an internal stochastic process regulated chameleon saccade generation.*

Although many animals scan their environments by rapid, jumplike eye movements called saccades, the only saccade patterns yet examined in detail come from humans (*1*). To gain insight into the temporal order of central nervous integration in a nonmammalian vertebrate, the African chameleon, I constructed frequency distributions of interval durations between successive chameleon saccades. The distributions (Fig. 1A) were indistinguishable from distributions taken from human subjects (*2*). Chameleons made most saccades randomly according to a constant-probability process whose rate did not vary despite perceptual restrictions as extreme as occlusion of one or both eyes.

In one obvious respect, chameleon saccade timing is more complex than human saccade timing. Chameleons can move their left and right eyes separately, in different directions, and with different amplitudes. Such independent eye movements are characteristic of the voluntary scanning movements of most vertebrate species (*3*). The independence of chameleons' eye movements is striking because their eyes protrude and are extremely mobile (approximately 200° horizontal freedom, 90° vertical freedom).

Since chameleons generally initiate saccades of left and right eyes asynchronously, one chameleon generates two sequences of saccade initiation times, one sequence for each eye. Such sequences were constructed from electrooculographic records of chameleon eye movements. Ten adult chameleons of three species, *Chameleo dilepis*, *C. jacksoni*, and *C. hohnelii*, provided more than 100,000 saccadic eye movements. (Figure 1C, although based on *C. dilepis* data, shows results typical of all three species.)

Two electrode pairs (1-mm Ag-AgCl disks moistened with conductive paste), placed horizontally on the orbital skin about each eye, measured the potential differences across left and right orbits. The horizontal component of an eye movement caused a change in the potential difference measured by its electrode pair. Saccadic eye movements caused characteristic rapid, steplike changes with amplitudes between 0.1 and 0.6 mV [compared with 0.2 to 0.8 mV for humans (*4*)]. Amplifiers with large time constants (3.4 seconds) amplified the left and right potential differences 1000-fold.

Electrooculograms (EOG's) of these amplified potential differences were made by filming them as traces on an oscilloscope screen, and by tape recording them for playback to a special saccade recognition circuit. This circuit passed only those EOG deflections caused by saccades. The saccade defining rules required that the deflection exceed a preset slope for a preset time (approximately 2 mV/sec for 10 msec). At its output, the circuit made rectangular pulses corresponding to the saccade-caused deflections in the input EOG. A minicomputer timed and tallied these pulses and stored the data for subsequent analysis by a general-purpose computer. The automatic recognition system found all saccades with horizontal amplitudes in excess of 1° and specified saccade initiation times to within 2 msec.

Each of ten recording sessions produced an unbroken pair of saccade time sequences 4.5 hours long. During the sessions, chameleons could move along a pair of dowels horizontally suspended in a large (1 m^3) electrically shielded cage. Because the dowels paralleled the transparent cage door, chameleons usually situated themselves parallel to the door with one eye facing outward, the other eye facing inward. This visual asymmetry concerned me until my data showed that it had no apparent effect on saccade timing. I easily occluded one or both eyes with bits of opaque black tape stuck over the eyes' palpebral fissures. Occluded eyes retained full mobility.

All recorded saccades were spontaneous—they did not compensate or stabilize changes in visual or acceleration fields caused by the chameleons' movements. Chameleons made most saccades while otherwise inactive. Moreover, their occasional movements were unaccompanied by visual tracking of the environment. The EOG's showed no smooth tracking eye movements whatever. The static environment and the typical slothful behavior of these animals ensured that sequences of spontaneous saccades were uncontaminated by ocular stabilizing movements.

As a first description of any stochastic process, the frequency distribution of interval durations between events is crucial (*5*). Intervals between successive chameleon saccades distributed unimodally with positive skew. The distributions had exponential tails (Fig. 1B). Poisson processes (simple stochastic processes by which events occur at constant low probability, independent of previous occurrences) generate intervals

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