The inhibition of muscle phosphorylase by *p*-chloromercuribenzoate*

Muscle phosphorylase does not exhibit maximal activity unless cysteine, glutathione[1] or ethylenediaminetetraacetic acid (Versene) is present. The possibility that the enzyme might require intact thiol groups for normal functioning was tested by determining the effect of *p*-chloromercuribenzoate (PCMB) on the activity of the enzyme. Crystalline phosphorylase *a* was prepared from rabbit muscle by the method of GREEN AND CORI[2]. PCMB, in a buffer consisting of 0.0015 *M* Versene and 0.02 *M* sodium glycerophosphate at pH 6.8, was mixed with the phosphorylase *a* protein in the same buffer. After one hour and thirty minutes at room temperature the enzymic activity was determined by the method of CORI, CORI AND GREEN[1], using Versene in place of the cysteine.

The results, tabulated in Table I, show that as the molar ratio of PCMB to phosphorylase is increased the enzymic activity of the protein decreases until inhibition is complete. This inhibition may be reversed completely by the addition of cysteine. A graph of the data in Table I indicates that there is a direct linear relationship between the extent of enzymic inhibition and the molar ratio of PCMB to protein. Approximately 17 thiol groups per mole of enzyme are titrated by this enzymic method. From the amino acid composition[3] the maximum number of sulfhydryl groups which phosphorylase would be expected to contain is 18.

TABLE I

THE EFFECT OF PCMB ON PHOSPHORYLASE *a* ACTIVITY

The enzyme, at a concentration of $1.2 \cdot 10^{-6}$ molar, was pre-incubated with various concentrations of PCMB for 90 minutes at room temperature before the enzymic activity was determined on aliquots diluted 20 times.

Moles PCMB per mole enzyme	*Percent residual § enzymic activity*
0.5	100
1.0	100
2.1	97.8
3.1	88.6
4.1	84.2
5.2	73.9
6.3	72.9
8.4	57.0
10.4	43.8
12.5	32.2
14.6	18.8
18.0	1.3

§ Means of from two to four values

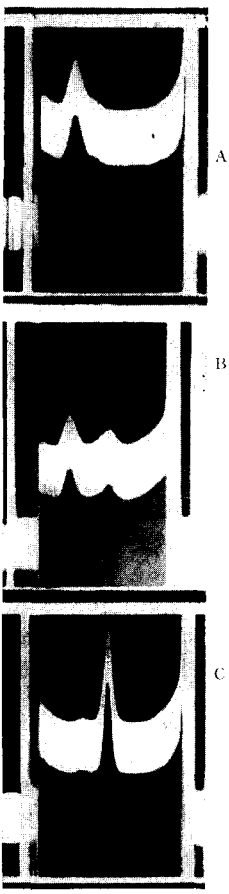

Fig. 1. The effect of PCMB on the sedimentation pattern of phosphorylase *a*, together with the reactivation by cysteine. A. 6.6 mg/ml of phosphorylase *a* ($1.33 \cdot 10^{-5}$ *M*) after treatment with $4.0 \cdot 10^{-4}$ *M* PCMB. 40′ picture. B. 7.0 mg/ml of phosphorylase *a* after treatment with 10 moles of PCMB per mole of protein. 35′ picture. C. 6.0 mg/ml of phosphorylase *a* completely inhibited by $2.5 \cdot 10^{-4}$ *M* PCMB, then reactivated by 0.1 *M* cysteine. 35′ picture.

The thiol groups on the phosphorylase *a* protein were titrated with PCMB by the spectrophotometric method developed by BOYER[4], and a value of 18.5 was obtained, in agreement with the above results. When the rate of reaction of phosphorylase with PCMB was followed by both enzymic and spectrophotometric methods it was found that second order kinetics were obeyed in both cases, although there was an initial anomaly in the latter case. The reaction as measured spectrophotometrically was somewhat faster than that measured enzymically, the rate constants being 51 and 43 liters per mole per second, respectively.

When reaction mixtures of PCMB and phosphorylase *a* were studied in the analytical ultracentrifuge it was found that a new molecular species with a smaller sedimentation constant than

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either phosphorylase *a* or *b* had replaced the original enzyme. This enzymically inactive protein had an $s_{20,w}$ of 5.6, compared to 13.2 for phosphorylase *a* and 8.2 for phosphorylase *b*[5]. The sedimentation pattern of this protein is shown in Picture A of Fig. 1, where an excess of PCMB is present. An experiment conducted by F. R. N. GURD is illustrated in Picture B, in which 10 moles of PCMB have been added per mole of protein, equivalent to 55% of the thiol groups. Area analysis of the sedimentation pattern indicates that 60% of the phosphorylase has been transformed into the lighter component. This experiment, and others of a similar nature, shows that the extent of conversion of phosphorylase *a* to the lighter protein component, like the extent of the enzymic inhibition, is proportional to the molar ratio of PCMB to protein.

When cysteine is used to reactivate the phosphorylase *a* which has been completely inhibited by PCMB, the enzyme has the usual sedimentation constant for phosphorylase *a*, and it crystallizes in the usual manner. The sedimentation pattern of such "reactivated" phosphorylase is shown in Picture C of Fig. 1.

Inactivation of phosphorylase *b* by PCMB is also accompanied by a change to a new molecular species which has a sedimentation constant similar to that of the PCMB inhibited phosphorylase *a*. On reversal with cysteine the new molecular species is converted back to phosphorylase *b* only. It should be noted that the molecular weight of phosphorylase *b* (242,000) is half that of phosphorylase *a* (495,000)[5].

Preliminary estimates of the molecular weight of the new molecular species suggest that when phosphorylase *a* is inhibited by PCMB the protein is split into four parts. The phosphorylase *b* molecule would therefore be expected to be split into two parts. Upon removal of the mercurial by cysteine the parts recombine to form a protein which is similar, if not identical, to the original enzyme.

Further investigations are in progress and it is hoped to publish a more detailed report later.

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CO_2 incorporation by extracts of *Thiobacillus thioparus*

Thiobacillus thioparus, an autotrophic, non-photosynthetic sulfur bacterium, derives its energy from the oxidation of inorganic sulfur compounds, and like photosynthetic organisms synthesizes its protoplasmic constituents from atmospheric CO_2. Chemosynthetic autotrophs present as a major problem the mechanism of CO_2 fixation.

Recent experimental work[1] has established that the first stable product formed during green plant photosynthesis is phosphoglyceric acid (PGA). CALVIN AND MASSINI[2] postulated that the primary mechanism of CO_2 fixation in photosynthesis involved a carboxylation of ribulose diphosphate resulting in the production of PGA. WEISSBACH *et al.*[3] and QUAYLE *et al.*[4], working with spinach and *Chlorella* extracts respectively, have shown that these extracts can synthesize PGA from ribulose diphosphate and CO_2. Thus far this reaction has been found only in photosynthetic systems. The data presented below demonstrate that extracts of *T. thioparus*, like those of photosynthetic organisms, can convert ribulose diphosphate and CO_2 to PGA.

Crude alumina-ground extracts of *T. thioparus* were incubated with various substrates plus radioactive CO_2. At the end of the incubation period the reaction mixture was deproteinized with trichloroacetic acid, and the supernatant fluid analyzed for total radioactivity (see Table I). Of the various substrates tested only ribulose diphosphate stimulated the incorporation of radioactive CO_2*.

To demonstrate the presence of ^{14}C in the formed PGA, this acid was isolated in the following manner. The supernatant solutions of the reaction mixtures were fractionated individually with $Ba(OH)_2$ at pH 8.2 after the addition of carrier PGA. PGA is precipitated by this procedure along with hexose diphosphate and various nucleotides. The precipitates were collected, washed

* Essentially similar results were obtained in an experiment carried out in conjunction with Dr. WILLIAM JACOBY.