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Staveley, L. A. K., & Spice, B. (1958) *J. Chem. Soc.*, 406–414.
Sullivan, K. H., Jain, M. K., & Koch, A. L. (1974) *Biochim. Biophys. Acta 352*, 287–297.
Swenson, R. P., & Oxford, G. S. (1980) *Prog. Anesthesiol. 2*, 7–16.
Tanford, C. (1980) *The Hydrophobic Effect*, Wiley, New York.
Trudell, J. R. (1977) *Biochim. Biophys. Acta 470*, 509–510.
Vikingstad, E. (1978) *J. Colloid Interface Sci. 64*, 510–513.
Vikingstad, E. (1979) *J. Colloid Interface Sci. 72*, 75–80.
White, S. H., King, G. I., & Cain, J. E. (1981) *Nature (London) 290*, 161–163.
Zavoica, G., & Kutchai, H. (1980) *Biochim. Biophys. Acta 600*, 263–269.

Stabilization of the Quaternary Structure of Transcarboxylase by Cobalt(II) Ions†

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ABSTRACT: When dilute solutions of transcarboxylase are incubated at 25 °C in an alkaline 50 mM buffer, the enzyme rapidly loses activity. This loss of activity is accompanied by the dissociation to enzymatically inactive subunits. The inclusion of 2 mM Co^{2+} in the buffer reduces both dissociation and the loss of enzymatic activity. This stabilization does not take place with 2 mM Mg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Ca^{2+}, or Cu^{2+}, but there is a slight protection by Zn^{2+}. At Co^{2+} concentrations of less than 2 mM, the stabilization decreases. The cobalt involved in the stabilization is not that required for catalysis as evidenced by the fact that the "catalytic" cobalt does not exchange with added free Co^{2+} under the conditions that prevent loss of enzymatic activity. The stabilizing effects of Co^{2+} were also observed toward inactivation with guanidinium chloride and by heat. It is proposed that Co^{2+} shifts the equilibrium of the dissociation of transcarboxylase toward the associated form and thus enzymatic activity is retained at alkaline pH.

Transcarboxylase (methylmalonyl-CoA:pyruvate carboxyltransferase, EC 2.1.3.1) is a biotin-containing enzyme found in the propionic acid bacteria [for reviews, see Wood & Zwolinski (1976) and Wood (1979)]. An illustration of the quaternary structure of the enzyme and its dissociation to enzymatically inactive subunits is shown in Figure 1. By use of transcarboxylase isolated from cells grown in ^{65}Zn or ^{60}Co, it has been demonstrated that the outer $5S_E$ subunit contains Co^{2+} and Zn^{2+} (Northrop & Wood, 1969; Ahmad et al., 1972; Fung et al., 1974). These two metals are tightly bound and dissociate only at a very low pH or in the presence of sodium dodecyl sulfate (Ahmad et al., 1972). Even at pH 9, EDTA[1] fails to remove these metals. These metals will be referred to as the "catalytic" metals.

In this report, we show that the presence of 2 mM exogenously added Co^{2+} ions protects transcarboxylase against dissociation and loss of activity at pH 8 and 9. These Co^{2+} ions are distinct from the catalytic metals and the protective effect is specific for Co^{2+}. Co^{2+} also protects against denaturation of the enzyme by guanidinium chloride and treatment at 50 °C.

Materials and Methods

The 26S transcarboxylase was isolated by the method of Wood et al. (1977) and assayed as described by Wood et al. (1969). For the tests of the effects of cobalt on enzymatic activity, the mixtures with and without Co^{2+} were incubated at 25 °C and exposed to air unless otherwise stated, under the conditions described in the text and figure legends. Aliquots were removed at the stated times and assayed. Unless otherwise stated, the Co^{2+} concentration was 2 mM and the buffer was 50 mM Hepes (Na^+), pH 8 or 9. Transcarboxylase containing ^{60}Co was isolated as described by Ahmad et al. (1972) and the $6S_E$ subunit by dissociation of 26S transcarboxylase at neutral pH, followed by glycerol gradient centrifugation (Wood et al., 1977). The $12S_H$ subunit was purified as recently described by Bahler et al. (1981). Reductive methylation was with [^{14}C]formaldehyde and $NaCNBH_4$ as described by Jentoft & Dearborn (1979). ^{60}Co was purchased from New England Nuclear and had a specific activity of 123 Ci/mg. Urea and guanidinium chloride were from K & K Laboratories, Rare and Fine Chemicals, Plainview, NY. All other chemicals were reagent grade or better.

Results

Effect of Co^{2+} on the Activity of Transcarboxylase Incubated at pH 8. The results shown in Figure 2 were obtained when transcarboxylase was diluted into 50 mM Hepes buffer, pH 8, at 25 °C, with and without the addition of 2 mM Co^{2+}, and was assayed with time. The inclusion of 2 mM Co^{2+} in the incubation buffer results in a pronounced protection of the activity of the enzyme. Similar results are obtained when the experiment is done at pH 9. The effect of Co^{2+} is most pronounced at low protein concentrations (≤1 mg/mL). No protective effect was observed with 2 mM Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Ni^{2+}, or Ca^{2+}, but a slight protection was noted in the presence of Zn^{2+} (Figure 2). If Co^{2+} is added to samples that have been incubated without Co^{2+} for 5, 10, or 20 min, there is no reactivation of enzyme. Inactivation of transcarboxylase

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GdmCl, guanidinium chloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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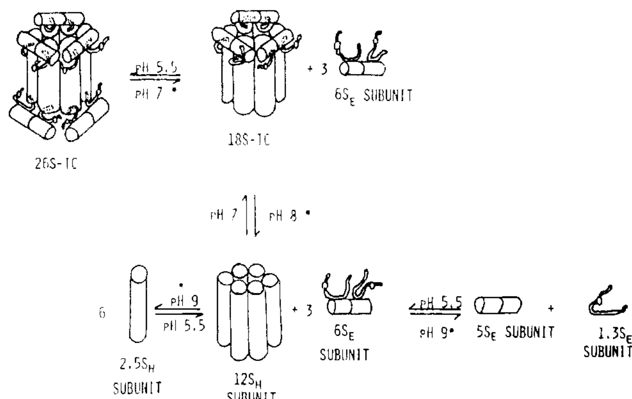

FIGURE 1: Schematic diagram of transcarboxylase and its dissociation to subunits. The undissociated enzyme has a sedimentation coefficient of 26 S and is designated 26S-TC (M_r 1.2 × 10^6). At neutral pH, there is selective dissociation of outer $6S_E$ subunits (M_r 1.44 × 10^5) from one face of the central $12S_H$ subunit, giving rise to the 18S form of the enzyme (M_r 7.9 × 10^5). At a more alkaline pH, further dissociation occurs, and at about pH 8, no $6S_E$ subunits remain attached to the central $12S_H$ subunit. At pH 9, the $6S_E$ subunits dissociate to the $5S_E$ subunit (M_r 1.2 × 10^5) and two $1.3S_E$ biotinyl subunits (M_r 1.2 × 10^4). The central hexameric $12S_H$ subunit also dissociates at pH 9 to six $2.5S_H$ monomeric subunits, but oxygen must be excluded to obtain this reversible dissociation (Bahler et al., 1981). The $12S_H$ and $6S_E$ subunits have no enzymatic activity. The $5S_E$ subunit contains tightly bound Co^{2+} and Zn^{2+}, which is required for catalysis. Secondary Co^{2+} atoms bind at pH 8 and 9 and stabilize the quaternary structure of both transcarboxylase and its subunits (see the text). The steps at which stabilization has been demonstrated are indicated by asterisks.

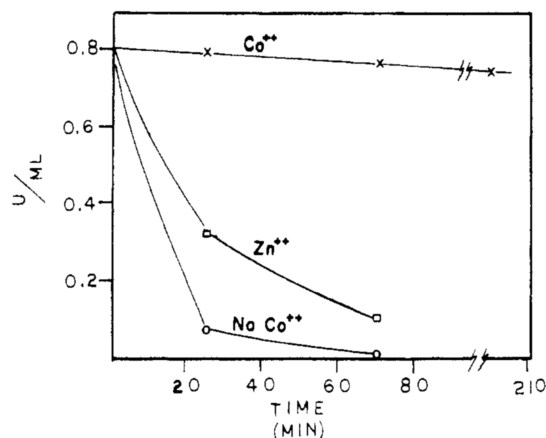

FIGURE 2: Effect of Co^{2+} and Zn^{2+} on enzymatic activity of transcarboxylase at pH 8. Transcarboxylase (0.8 unit/mL) in 50 mM Hepes buffer, pH 8.0, was incubated at 25 °C and assayed with time. (×) With 2 mM Co^{2+}; (□) with 2 mM Zn^{2+}; (○) with no divalent metal.

in the absence of Co^{2+} is much slower at low temperatures (4 °C).

Effect of Co^{2+} on Inactivation at 50 °C and by Guanidinium Chloride. The loss of activity with time at 50 °C with or without Co^{2+} is shown in Figure 3A. With Co^{2+}, the activity decreased rapidly in the first 2 min, but beyond that time there was about 4% residual activity that persisted past 25 min. The sample without Co^{2+} was devoid of activity in 1 min. On the other hand, the sample that was preincubated with Co^{2+} for 20 min at pH 8 at 25 °C and was then warmed to 50 °C lost ~75% of its activity within 4 min, but after that the activity decreased only slowly over a period of 1 h. Preincubation of transcarboxylase with Co^{2+} under conditions where little dissociation occurs (for example, in phosphate buffer, pH 6.3) provides little protection against denaturation at 50 °C at pH 8. Apparently, it is necessary for the conformation of the enzyme to be "opened up" at alkaline pH so that the Co^{2+} can occupy sites that stabilize the enzyme.

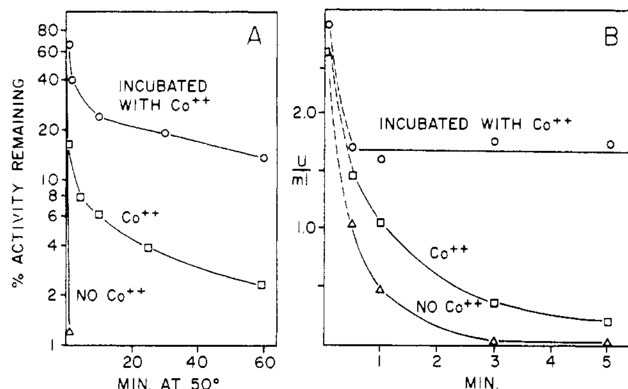

FIGURE 3: Effect of Co^{2+} on inactivation of transcarboxylase at 50 °C or by guanidinium chloride (GdmCl). (A) Transcarboxylase (0.5 mg/mL) (15.2 units/mL) in 150 mM Hepes buffer, pH 8.0, was incubated at 50 °C. (○) Preincubated 20 min at 25 °C in buffer, pH 8, containing 2 mM Co^{2+} prior to heating to 50 °C; (□) 2 mM Co^{2+} without prior preincubation; (△) no Co^{2+}. (B) Transcarboxylase (0.1 mg/mL, 2.75 units/mL) in 50 mM Hepes, pH 9.0, at 25 °C was incubated 30 min with 2 mM Co^{2+} (the activity then was 2.05 units/mL); then (time zero on the graph) 6 M GdmCl was added to give 0.25 M GdmCl (○); GdmCl and 2 mM Co^{2+} were added without preincubation (□); GdmCl was added in the absence of Co^{2+} (△).

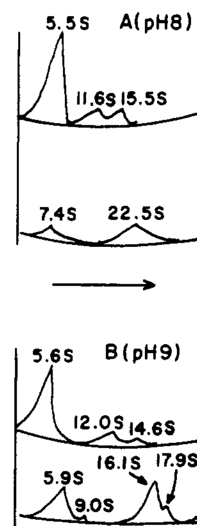

FIGURE 4: Sedimentation velocity profiles of transcarboxylase with and without Co^{2+} at pH 8 and 9. Aliquots of transcarboxylase (4–5 mg/mL) were dialyzed 18 h under N_2 at 5 °C vs. 50 mM Hepes buffer, pH 8 or pH 9, with or without 2 mM Co^{2+}. At the end of this time, the activities at pH 8 were 5.5 units/mg without Co^{2+} and 15.5 units/mg with Co^{2+}, and at pH 9, they were 5.7 units/mg without Co^{2+} and 16.4 units/mg with Co^{2+}. Centrifugation was in a Beckman Model E ultracentrifuge with an AnD rotor at 60000 rpm. (A) pH 8, without Co^{2+} (top) and with Co^{2+} (bottom); (B) pH 9, without Co^{2+} (top) and with Co^{2+} (bottom).

Transcarboxylase is rapidly inactivated in 0.5 M GdmCl. With 0.026 mg/mL (0.85 unit/mL) transcarboxylase in 50 mM Hepes at pH 8, all activity was lost in 3 min. In the presence of 2 mM Co^{2+}, there was loss of activity, but 40–50% of the activity was retained at 1 h. At pH 9, the protection by Co^{2+} against inactivation was not very effective if the GdmCl was added at the same time as the Co^{2+}. However, if the enzyme was preincubated with the Co^{2+} at pH 9 for 30 min at 25 °C and then the GdmCl (0.25 M) was added, a pronounced protection resulted with no further loss of activity even after 40 min (Figure 3B). These results again indicate that it is necessary to introduce the Co^{2+} into the sites prior to subjecting the enzyme to destabilization.

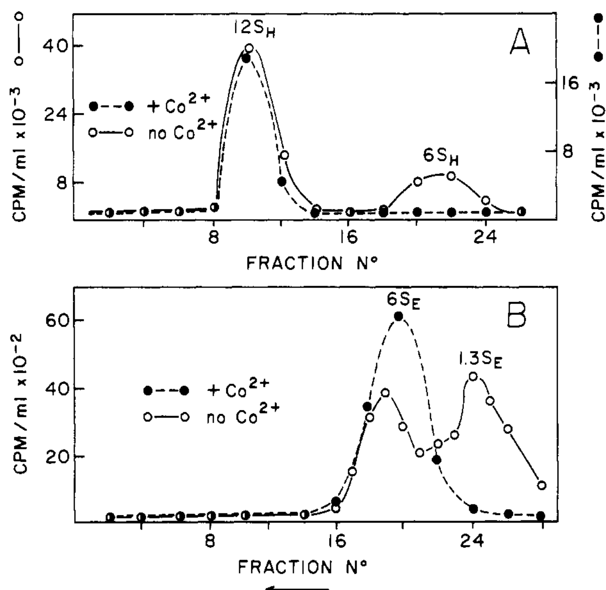

FIGURE 5: Effect of Co^{2+} on dissociation of $12S_H$ and $6S_E$ subunits. (A) $12S_H$ subunit (0.56 mg/mL and 5.9×10^5 cpm/mg) that had been lightly methylated with $^{14}CH_2O$ was incubated in 50 mM Hepes, pH 8.0, without Co^{2+} and with 2 mM Co^{2+} for 2 h at 25 °C and then for 16 h at 4 °C. 300 μL of the sample without Co^{2+} was layered on 11-mL 10–30% glycerol gradients in 50 mM Hepes, pH 8.0, containing no Co^{2+}, and that containing Co^{2+} was layered on a gradient containing 2 mM Co^{2+}. Centrifugation was at 39000 rpm in an SW 41 rotor for 18 h at 4 °C. Fractions of 0.3 mL were collected and counted. Fractions 8–12 contained undissociated $12S_H$ subunit while fractions 18–24 contained $6S_H$ dimer. (B) $6S_E$ subunit (1.3 mg, 13000 cpm in 0.3 mL) containing [^{3}H]biotinyl $1.3S_E$ subunit was treated the same as the $12S_H$ subunit. Fractions 23–28 contained free $1.3S_E$ biotinyl subunit (M_r 1.2×10^4) whereas fractions 17–21 contained undissociated $6S_E$ subunit. A control gradient in 0.3 M phosphate buffer, pH 6.3, gave a profile like that with Co^{2+}.

Effect of Co^{2+} on Quaternary Structure of Transcarboxylase. Sedimentation velocity profiles of transcarboxylase are shown in Figure 4, with (A) being at pH 8 and (B) at pH 9. In the absence of Co^{2+} (top), the majority of the enzyme was converted to 5.6S material, which presumably is a mixture of $5S_E$, $6S_E$, and $6S_H$ subunits (see Figure 1). There was some $12S_H$ subunit present and not much active enzyme, which has a sedimentation coefficient greater than 12 S. No $2.5S_H$ monomer was observed. The dissociation was done under N_2, but mercaptoethanol was not added because at alkaline pH with Co^{2+} a colored precipitate forms. Perhaps even under N_2, mercaptoethanol is necessary to prevent formation of disulfide-linked $6S_H$ dimers as described by Bahler et al. (1981).

In the presence of Co^{2+} (bottom), at pH 8, a peak with an $s_{20,w}$ value of 22.5 S was observed, and at pH 9 there were peaks at $s_{20,w}$ values of 16.1 and 17.9 S. No $12S_H$ subunit was present. Clearly, the Co^{2+} promoted the binding of the $6S_E$ subunits to the $12S_H$ subunit and retarded the dissociation of transcarboxylase into inactive subunits. The dissociation of transcarboxylase is rapidly reversible and the 22.5S peak at pH 8 in the presence of Co^{2+} represents forms of transcarboxylase with an average of about four attached outside subunits (Poto & Wood, 1977).

Effects of Co^{2+} on Dissociation of $12S_H$ and $6S_E$ Subunits. Figure 5A shows the results obtained at pH 8 by glycerol gradient centrifugation of $12S_H$ subunit that had been lightly labeled with [^{14}C]formaldehyde. Undissociated $12S_H$ subunit was in fractions 8–12, and the $6S_H$ subunits were in fractions 18–24. In these experiments, precautions were not taken to keep the solutions anaerobic, and under these conditions, disulfide bonds are formed between the $2.5S_H$ monomers to yield $6S_H$ dimers (Bahler et al., 1981). Clearly, there was dissociation in the absence of Co^{2+} (solid line), but in the presence of Co^{2+}, dissociation did not occur and only the $12S_H$ subunit was observed. A control sample centrifuged in phosphate buffer, pH 6.3, in which $12S_H$ remains intact gave a profile identical with that observed at pH 8 in the presence of Co^{2+}. The $12S_H$ subunit of fraction 10 was tested to determine its competence in forming active enzyme. An excess of $6S_E$ subunit was added to the fraction in 0.5 M phosphate buffer at pH 6.5 at 4 °C. The high phosphate concentration promotes combination of the subunits and formation of active enzyme (Wood et al., 1975). The reconstituted enzyme had a specific activity of 75 units/mg of $12S_H$, which was the same as that obtained with the untreated $12S_H$ subunit. Fraction 22 ($6S_H$ dimer) did not reconstitute to active enzyme with $6S_E$ subunit.

A similar experiment was conducted with the $6S_E$ subunit, which contained [^{3}H]biotin in the $1.3S_E$ subunit (Figure 5B). The undissociated $6S_E$ subunit was in fractions 17–21 and dissociated $1.3S_E$ subunit in fractions 23–28. In the presence of added Co^{2+} (dashed line) there was no dissociation. A control centrifugation in phosphate buffer at pH 6.3 gave results identical with those shown in Figure 5B with Co^{2+}. Clearly, Co^{2+} not only retards the dissociation of $6S_E$ from the $12S_H$ subunit of transcarboxylase but also retards the dissociation of the $12S_H$ to monomers and the $6S_E$ subunit to $5S_E$ and $1.3S_E$ subunits.

Capacity of Co^{2+}-Treated $6S_E$ and $12S_H$ Subunits To Reconstitute to Active Transcarboxylase. The $12S_H$ or $6S_E$ subunit was incubated at pH 8 or pH 9 with and without Co^{2+} for 2 h at 30 °C and then was reconstituted to active enzyme by combination with the other subunit, which had not been treated with Co^{2+} or with base. Aliquots were taken with time and assayed. Figure 6A shows the results when the $6S_E$ subunit was subjected to alkaline conditions and Figure 6B when the $12S_H$ subunit was subjected to this treatment. It is apparent from Figure 6A that Co^{2+} protected the capacity of the $6S_E$ subunit to reconstitute with $12S_H$ subunit. This is most evident at pH 9 where the dissociation of the $6S_E$ subunit to $5S_E$ and $1.3S_E$ subunits is quite complete (Figure 1). Although not shown here, this difference between $6S_E$ subunit treated with and without Co^{2+} persisted even when the $6S_E$ subunit was incubated at pH 8 over a longer period of time. It should be noted that, although the dissociation of the $6S_E$ subunit to $5S_E$ and $1.3S_E$ subunits is reversible, an excess of $1.3S_E$ subunit is required to obtain complete conversion of the $5S_E$ subunit to $6S_E$ subunit (Wood et al., 1975).

When the $12S_H$ subunit was subjected to alkaline treatment, the effect of Co^{2+} was more pronounced (Figure 6B) than with the $6S_E$ subunit. In part, this may be because the experiments were done in solutions exposed to air, and therefore, during dissociation of $12S_H$ to the monomer, conversion to the disulfide-linked $6S_H$ dimer occurred, and this dimer does not reconstitute with the $6S_E$ subunit to form active transcarboxylase (Bahler et al., 1981). The results with both the $6S_E$ and $12S_H$ subunits indicate that Co^{2+} retards dissociation of the subunits, and thus their capacity for reconstitution is retained.

Stability of Co^{2+} Bound to Transcarboxylase and Stoichiometry of Co^{2+} Binding. For these experiments, transcarboxylase was incubated at pH 8 with or without Co^{2+} until the control sample (no Co^{2+}) had 25% of the enzymatic activity of the sample with $^{60}Co^{2+}$. Because of the high protein concentration, several hours at 30 °C were required for the inactivation. Samples were then precipitated with ammonium sulfate, dialyzed against either acetate buffer, pH 5.5, or

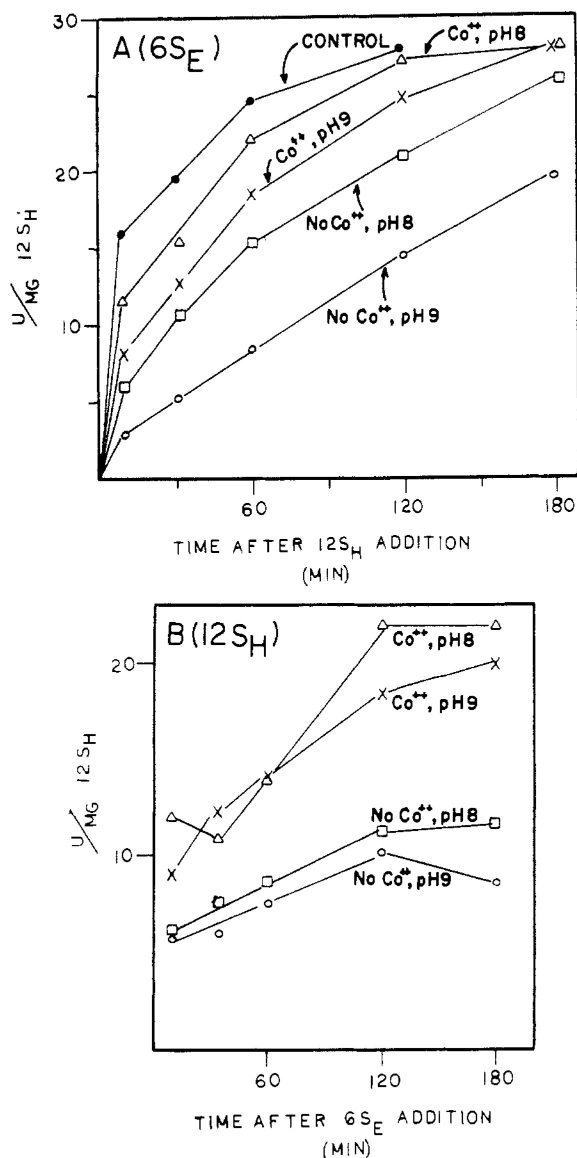

FIGURE 6: Effect of Co^{2+} on capacity of $6S_E$ and $12S_H$ subunits to reconstitute to active enzyme following treatment at pH 8 or pH 9. The subunit, $6S_E$ (10 μg) or $12S_H$ (1.4 μg), each in 150 μL of 50 mM Hepes buffer, pH 8 or 9, was incubated with or without the addition of Co^{2+} for 2 h at 30 °C. Reconstitution to active enzyme was done by adding to the treated $6S_E$ subunit 150 μL of 1.5 M phosphate buffer, pH 6.3, at 4 °C containing 1.4 μg of untreated $12S_H$ subunit or to the treated $12S_H$ subunit 150 μL of the phosphate buffer containing 10 μg of untreated $6S_E$ subunit. The mixtures were then assayed with time for transcarboxylase activity. A control in which neither subunit was treated at pH 8 or pH 9 was also done. (A) Untreated $12S_H$ subunit plus treated $6S_E$ subunit: (O) pH 9, no Co^{2+}; (×) pH 9, plus Co^{2+}; (□) pH 8, no Co^{2+}; (△) pH 8, plus Co^{2+}; (●) control. (B) Untreated $6S_E$ subunit plus treated $12S_H$ subunit: (O) pH 9, no Co^{2+}; (×) pH 9, plus Co^{2+}; (□) pH 8, no Co^{2+}; (△) pH 8, plus Co^{2+}. All results are expressed in units per milligram of $12S_H$ since in the reconstitution the $6S_E$ subunit is in excess.

phosphate buffer, pH 6.8, and then centrifuged in glycerol gradients buffered at pH 5.5 or pH 6.8. At pH 5.5, the 26S form is stable, whereas at pH 6.8, the 18S form, with only three outer $6S_E$ subunits, is predominant (Figure 1). No Co^{2+} was included in the gradient buffers so that only those Co^{2+} ions that remained bound during ammonium sulfate precipitation and dialysis were associated with the enzyme. Figure 7 shows the results of such an experiment. Fractions 21–25 contained the $6S_E$ subunit while fractions 3–12 contained the enzyme. In the sample centrifuged at pH 5.5, some $6S_E$ subunit was present, indicating either that there was some

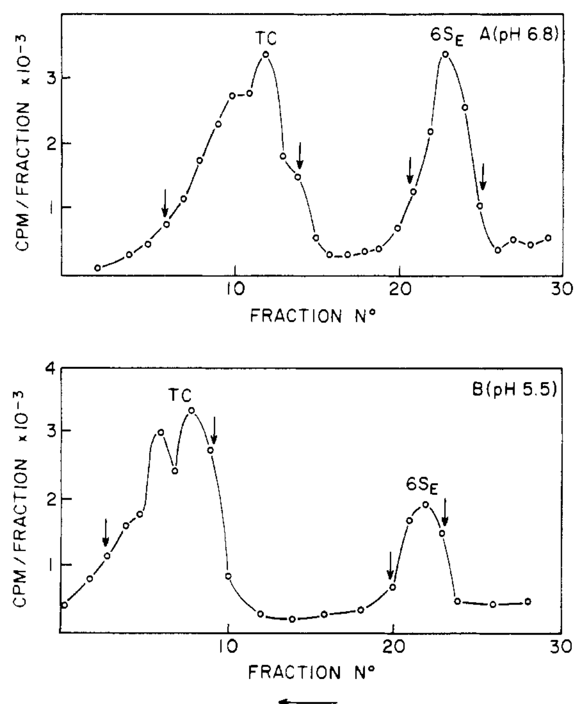

FIGURE 7: Stability and stoichiometry of ^{60}Co bound to transcarboxylase. 26S transcarboxylase was incubated at 25 °C for 5 h in 50 mM Hepes, pH 8, containing 2 mM $^{60}Co^{2+}$ (2330 cpm/nmol). It then contained 9.11 mg/mL and had an enzymatic activity of 30.2 units/mg. A control treated similarly without Co^{2+} contained 9.76 mg/mL and an activity of 7.9 units/mg. It was not used in the remainder of the experiment. The sample with $^{60}Co^{2+}$ was brought to pH 6.7 with 100 μL of 1 M KH_2PO_4 and was precipitated by addition of $(NH_4)_2SO_4$ to 60% saturation. The precipitate was dissolved in cold 100 mM acetate buffer, pH 5.5, and half was dialyzed at 4 °C against this buffer. The other half was dialyzed against 50 mM phosphate buffer, pH 6.8. The resulting sample at pH 5.5 had an enzymatic activity of 42 units/mg and 24 100 cpm/mg and that at pH 6.8 of 23 units/mg and 16 200 cpm/mg. The samples were applied to a 10–30% glycerol gradient buffered with 100 mM acetate buffer, pH 5.5, or 50 mM phosphate buffer, pH 6.8, and centrifuged at 39 000 rpm in an SW 41 rotor for 16 h at 4 °C. Fractions of 0.3 mL were collected, assayed for radioactivity, and pooled (arrows). (A) Profile in pH 6.8 buffer. Fractions 4–12 contained enzyme; fractions 21–25 contained $6S_E$ subunits. (B) Profile in pH 5.5 buffer. Fractions 3–7 contained enzyme; fractions 21–24 contained $6S_E$ subunits. The stoichiometry of $^{60}Co^{2+}$ binding is given in Table I.

dissociation of the 26S enzyme or that after the treatment at pH 8, the reassociation to 26S enzyme was incomplete. Since native 26S enzyme does not dissociate at pH 5.5, we assume the latter was the case.

Pools from the gradients (arrows) were assayed for protein, enzyme activity, and radioactivity, and the results are shown in Table I. The moles of ^{60}Co bound per mole of enzyme and per mole of $6S_E$ subunit were calculated on the basis of the specific radioactivity of the $^{60}Co^{2+}$ and the cpm per milligram of protein observed in the fraction. The values are approximations but they show that ^{60}Co was bound to the $6S_E$ subunit. Since Co^{2+} has a stabilizing effect on the isolated $12S_H$ subunit (Figures 5A and 6B), it is likely a portion of the Co^{2+} is also bound to the $12S_H$ subunit in the transcarboxylase.

The experiment of Figure 8 was done to determine if enzyme into which Co^{2+} was introduced at pH 8 is stable in the absence of exogenous Co^{2+}. For this purpose, the labeled enzyme of fractions 4–12 of Figure 7A was used, and it was found that this enzyme was only slightly more resistant to inactivation at pH 8 than transcarboxylase that had not been treated with Co^{2+}. This result indicates either the Co^{2+} dissociated from the enzyme when incubated at pH 8 or, if it remained attached, it is not responsible for the observed stabilizing influence of

Table I: Cobalt Content of Pooled Fractions from Glycerol Gradients of Figure 7

pooled fractions	[protein] (mg/mL)	sp act. (units/mg)	^{60}Co (cpm[a]/mg)	mol of ^{60}Co/mol of enzyme or subunit
7A (pH 6.8)				
4-12 (TC)	0.40	32.7	15 666	5.3[b]
21-25 ($6S_E$)	0.29	0	20 800	1.3[b]
7B (pH 5.5)				
3-7 (TC)	0.52	33.6	13 212	5.7[b]
21-24 ($6S_E$)	0.14	0	33 646	2.1[b]

[a] ^{60}Co contained 2330 cpm/nmol. [b] For these calculations, it has been assumed that the enzyme obtained at pH 6.8 was 18S transcarboxylase, M_r 7.9 × 10^5. Considerable $6S_E$ subunit was observed in the glycerol gradient at pH 5.5 (Figure 7B). Therefore, it is assumed the enzyme had four outer subunits and an M_r of 9.36 × 10^5. The ^{60}Co content of the 6S material has been calculated on the basis that it was predominantly $6S_E$ subunit of M_r 1.44 × 10^5.

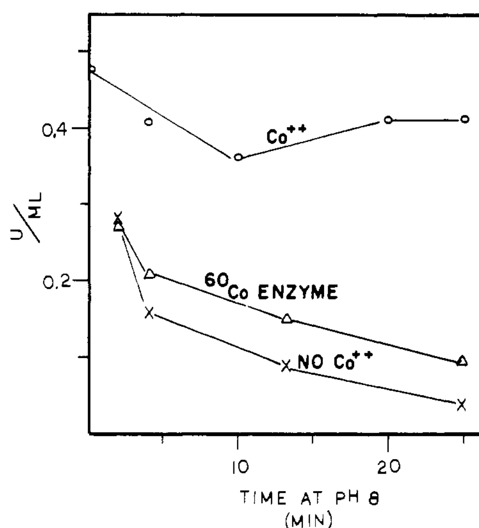

FIGURE 8: Effect of incubation at pH 8 on enzyme in which Co^{2+} had been introduced at pH 8. Aliquots of enzyme were diluted to 0.45 unit/mL into pH 8 buffer (25 °C) and assayed with time. (O) Untreated enzyme diluted in pH 8 buffer containing 2 mM Co^{2+}; (×) untreated enzyme at pH 8, no Co^{2+}; (△) 18S enzyme from fractions 4-12 of Figure 7A containing ^{60}Co.

2 mM Co^{2+}. To investigate this possibility, we dialyzed the labeled enzyme (fractions 4-12 of Figure 7A) against 50 mM Hepes buffer, pH 8.0, for 7 h at room temperature. Following this treatment, 70% of the ^{60}Co remained attached to the enzyme. Passage of the enzyme through a column of Chelex resin at pH 9 likewise failed to remove the radioactivity from the enzyme; half of the ^{60}Co emerged from the column still attached to the dissociated enzyme. Since the tightly bound Co^{2+} did not stabilize the enzyme, it appears that weakly bound Co^{2+} ions are responsible for the stabilizing effect. Presumably, the weakly bound Co^{2+} is in equilibrium with unligated Co^{2+} in solution, and 2 mM Co^{2+} is required to maintain the concentration of bound Co^{2+} sufficiently high to stabilize the enzyme.

Comparison of Catalytic Cobalt and Cobalt Incorporated at pH 8. Native transcarboxylase contains cobalt that is considered to be at the keto acid site on the $5S_E$ subunit (Northrop & Wood, 1969; Ahmad et al., 1972; Fung et al., 1974) and which we have designated catalytic cobalt. An experiment was done with transcarboxylase isolated from cells grown in medium containing ^{60}Co (Ahmad et al., 1972). The specific radioactivity of this transcarboxylase was 12 000 cpm/mg. Aliquots of the ^{60}Co transcarboxylase were incubated at pH 8 with or without added unlabeled Co^{2+} (2 mM). A third sample of the ^{60}Co transcarboxylase was incubated with 2 mM Co^{2+} to which $^{60}Co^{2+}$ was added as a tracer. When the sample without Co^{2+} had 5% of the enzymatic activity of the Co^{2+}-containing samples, all the samples were dialyzed extensively vs. 0.25 mM acetate buffer, pH 5.5, and then assayed for specific radioactivity. The samples of ^{60}Co transcarboxylase that had been incubated in the presence and absence of unlabeled Co^{2+} both remained at a specific radioactivity of ~12 000 cpm/mg. Hence, no loss or exchange with the catalytic cobalt of the enzyme took place. In addition, the sample incubated at pH 8 in the presence of added $^{60}Co^{2+}$ increased in specific radioactivity to 19 000 cpm/mg, indicating that the incubation led to the binding of additional Co^{2+} ions that are distinct from those that are tightly bound in the $5S_E$ subunit.

Effect of Co^{2+} on Titratable Sulfhydryl Residues of Transcarboxylase. It has been shown (Bahler et al., 1981) that the reconstitution of dissociated transcarboxylase is most effective if the dissociation and reconstitution are done under anaerobic conditions, which prevent oxidation of sulfhydryl groups. It seemed possible that Co^{2+} protected the sulfhydryl groups at pH 8. We, therefore, tested the effects of Co^{2+} on the total number of titratable thiol groups in the enzyme. Identical aliquots of 26S transcarboxylase (8 mg/mL) were incubated at 25 °C at pH 8, in the presence or absence of 2.5 mM $^{60}Co^{2+}$ (1542 cpm/nmol of Co^{2+}). After the incubation, the sample without Co^{2+} had a specific enzymatic activity of 19.5 units/mg whereas that incubated with Co^{2+} had an activity of 28 units/mg. Each sample was then desalted on Sephadex G-25 in 50 mM Hepes buffer, pH 8, and the enzyme in the protein fraction was titrated with DTNB in the presence of sodium dodecyl sulfate and EDTA (Habeeb, 1972). The EDTA was included in the reactions to prevent any metal-catalyzed oxidation of thiol groups. The starting transcarboxylase has 102 cysteines by amino acid composition (Wood & Zwolinski, 1976). The transcarboxylase incubated at pH 8 with Co^{2+} contained 62.3 titratable sulfhydryl groups whereas that incubated without Co^{2+} contained 78 titratable groups. Hence, the sample incubated with Co^{2+} possessed 16 fewer titratable sulfhydryl groups than that incubated without Co^{2+}. The specific radioactivity of the transcarboxylase incubated with Co^{2+} was 11 708 cpm/mg. This corresponds to 8.6 mol of ^{60}Co/mol of 26S transcarboxylase. There was substantial dissociation to subunits during the desalting at pH 8, and the specific activity of the enzyme without Co^{2+} decreased to 1.8 units/mg and that with Co^{2+} to 4 units/mg. However, all the subunits of transcarboxylase are present in the void volume of a Sephadex G-25 column; thus the total sulfhydryl group determination on the protein peak is valid. When this experiment was repeated with a different sample of 26S transcarboxylase, similar results were obtained. The starting transcarboxylase had 80 thiol groups, that incubated without Co^{2+} had 73.5, and that incubated with Co^{2+} had 55, a net loss of 18.5 thiol groups. Thus, for each Co^{2+} bound, there were approximately two less titratable thiol groups.

It therefore seemed possible that the Co^{2+} was ligated between pairs of thiol groups. Such ligands should have an absorption band at 340 nm due to charge transfer between cobalt and thiol ligands as observed by Sytkowski & Vallee (1976) using cobalt-labeled alcohol dehydrogenase. To test this possibility, we incubated transcarboxylase (~5 mg/mL) at 25 °C at pH 8 in the presence and absence of 2 mM Co^{2+} until the sample without Co^{2+} lost greater than 80% of its

original activity. The samples were then dialyzed against 50 mM sodium acetate buffer, pH 5.5, at 4 °C for 16 h to remove unbound metal ions, and the difference spectra were recorded. On the basis of the extinction coefficient given by Sytkowski & Vallee (1976), a significant difference would have occurred if cobalt–thiol bonds had been formed. None was observed; thus, it is concluded the cobalt ions are not ligated to the enzyme through cysteine linkages. Perhaps the decrease in titratable thiol groups is due to stimulation of oxidation of sulfhydryl by the Co^{2+} per se.[2]

Discussion

It has been demonstrated that added Co^{2+} (2 mM) protects transcarboxylase from the loss of enzymatic activity that normally occurs at 25 °C in 50 mM Hepes buffer at pH 8. The effect is specific for Co^{2+}. Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Ni^{2+}, and Cu^{2+} are ineffective, and there is only a slight protection by Zn^{2+} (Figure 2). There also is protection at an alkaline pH against denaturation by guanidinium chloride (0.5 M) and by heat (50 °C) (Figure 3). This protection is most effective if the enzyme is first incubated with the Co^{2+} and then the enzyme is subjected to the guanidinium chloride or heat treatment. If the pretreatment with Co^{2+} is in phosphate buffer at pH 6.5, there is little or no protection. Apparently, an alkaline pH is required to "open" the conformation of the enzyme so that the sites that bind the Co^{2+} are exposed. Such exposure may result from the dissociation of transcarboxylase to subunits at pH 8 as illustrated in Figure 1.

Transcarboxylase is a metalloenzyme and contains very firmly bound cobalt and zinc. The proportion of each metal in the enzyme depends on the relative concentration of Co^{2+} and Zn^{2+} in the medium used to grow the bacteria (Ahmad et al., 1972). The cobalt and zinc have been shown to be near the keto acid site in the outer $5S_E$ subunit (Northrop & Wood, 1969; Ahmad et al., 1972; Fung et al., 1974). At pH 8, Co^{2+} did not replace ^{60}Co of labeled enzyme, and when the labeled enzyme was treated at pH 8 with $^{60}Co^{2+}$, there was an increase of radioactivity due to incorporation of additional ^{60}Co. Clearly, the cobalt that is introduced at pH 8 does not enter and replace the cobalt at the catalytic site.

It was shown by ultracentrifugation that in the absence of Co^{2+} at pH 8 or pH 9, the enzyme is largely dissociated to its subunits, whereas in the presence of Co^{2+}, the various forms of the active enzyme still persist (Figure 4). Outer $5S_E$ subunits are bound to the $12S_H$ subunits via the biotinyl subunit (see Figure 1), and it has been shown (Kumar & Beegen, 1981) that the binding to the $12S_H$ subunit involves residues 2–14 of the biotinyl subunit and binding to the $5S_E$ subunit residues 15–26. However, the effect of Co^{2+} on the dissociation of transcarboxylase is not confined to these binding sites, since Co^{2+} retards the dissociation at pH 8 of the $12S_H$ subunit and likewise that of the $6S_E$ subunit to the $5S_E$ subunit and the $1.3S_E$ biotinyl subunit (Figure 5). Thus, the Co^{2+} effects multiple types of binding of the subunits. The effect of Co^{2+} is also evident in maintaining the capability of the $12S_H$ and $5S_E$ subunits to reconstitute to active transcarboxylase (Figure 6).

The evidence available suggests that there are three types of cobalt binding to the enzyme. The one is at the catalytic cobalt. The second type is formed when the enzyme is incubated with exogenous Co^{2+} at pH 8, during which some Co^{2+} becomes tightly bound and is not removed by dialysis, ammonium sulfate precipitation, or treatment at alkaline pH or with a Chelex resin. There are approximately six such cobalts per 26S transcarboxylase molecule. These cobalts do not appear to be bound to the enzyme through thiol linkages and, by themselves, have little influence on the dissociation properties of transcarboxylase. A third class of cobalt apparently is more weakly bound and is in equilibrium with the free Co^{2+}, which must be maintained at about 2 mM in order to stabilize the enzyme at alkaline pH. This weakly bound Co^{2+} effects the equilibrium of the association and dissociation of transcarboxylase. At 25 °C in the presence of 2 mM Co^{2+} at pH 8, the dissociation is not complete and partial activity is retained by the enzyme. It is considered that information concerning the sequence of amino acids in the $12S_H$ and $5S_E$ subunits and perhaps their quaternary structure will be required before an explanation of this stabilizing effect of exogenous cobalt will be forthcoming.[2]

[2] It has been suggested by one of the reviewers that the Co^{2+} may have been oxidized to Co^{3+}, which forms a more stable complex, and that if the complex was treated with dithionite, the Co^{3+} would be reduced to Co^{2+} and should easily be removed by a Chelex column. We have treated transcarboxylase with $^{60}Co^{2+}$ at pH 8, removed the unbound $^{60}Co^{2+}$ by dialysis, and then passed the enzyme with and without treatment with dithionite over Chelex. The treatment with dithionite did not alter the amount of $^{60}Co^{2+}$ that was tightly bound. This provides proof that the tightly bound cobalt is not Co^{3+} and in addition that the Co^{2+} is not ligated between thiols.

References

Ahmad, F., Lygre, D. G., Jacobson, B. E., & Wood, H. G. (1972) *J. Biol. Chem. 247*, 6299–6305.

Bahler, C., Goss, N., Poto, E. M., & Wood, H. G. (1981) *Biochem. Int. 3*, 349–358.

Fung, C.-H., Mildvan, A. S., & Leigh, J. S., Jr. (1974) *Biochemistry 13*, 1160–1169.

Habeeb, F. S. A. (1972) *Methods Enzymol. 25*, 457–464.

Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359–4365.

Kumar, K. G., & Beegen, H. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1767.

Northrop, D. B., & Wood, H. G. (1969) *J. Biol. Chem. 244*, 5801–5807.

Poto, E. M., & Wood, H. G. (1977) *Biochemistry 16*, 1949–1955.

Sytkowski, A. J., & Vallee, B. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 344–348.

Wood, H. G. (1979) *CRC Crit. Rev. Biochem. 7*, 143–160.

Wood, H. G., & Zwolinski, G. K. (1976) *CRC Crit. Rev. Biochem. 4*, 47–122.

Wood, H. G., Jacobson, B. E., Gerwin, B. I., & Northrop, D. B. (1969) *Methods Enzymol. 13*, 215–230.

Wood, H. G., Ahmad, F., Jacobson, B., Chuang, M., & Brattin, W. (1975) *J. Biol. Chem. 250*, 918–926.

Wood, H. G., Chiao, J.-P., & Poto, E. M. (1977) *J. Biol. Chem. 252*, 1490–1497.