Proton Magnetic Resonance, Anion-Exchange, and Polarographic Studies of the Cobalt–Glycylglycine–Oxygen Complex[1]

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Complexes of glycylglycinate with Co and Mn in aqueous solution, before and after oxygenation, have been examined by proton magnetic resonance spectroscopy. The attachment of oxygen to the cobalt–glycylglycinate complex is reversible, whereas it is irreversible when manganese replaces cobalt. Both proton magnetic resonance and polarographic results indicate that an oxygenated cobalt–dipeptide complex is not formed when glycylprolinate is used instead of glycylglycinate. A possible explanation is that glycylproline does not have an amide hydrogen. The net charge of an oxygenated cobalt–glycylglycinate complex has been determined to be −1.

Introduction

The formation of a red complex in alkaline solutions containing cobalt and glycylglycine (GG) was first observed by Smith[2] and the complex was isolated and crystallized by Gilbert, et al.,[3] who obtained analytical data to show that it contained Co, GG, and molecular O_2 in the ratio of 2:4:1. Gilbert, et al.,[3] and Tanford, et al.,[4] have postulated that a possible structure of the oxygenated (red species) cobalt complex of GG is

$$[(GG)_2Co\overset{\overset{\displaystyle H}{\underset{\displaystyle O}{|}}}{\underset{O-O}{<}}\overset{}{>}Co(GG)_2]^{-1}$$

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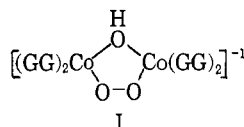
However, experimental data to confirm the net charge for this complex have not been available. This paper describes the use of an anion-exchange method to determine the charge.

In solution, the oxygenation of cobalt–GG complex is achieved by bubbling oxygen into the solution and is reversed by flushing with nitrogen. Several authors[5] have indicated that the reaction is reversible for the cobalt chelate but not for the manganese chelate. Accordingly, we have used the proton magnetic resonance (p.m.r.) method to study the cobalt and manganese chelates of GG before and after passing oxygen, and after flushing with nitrogen. The p.m.r. data serve to indicate changes in paramagnetic susceptibility of the solutions as a result of passing oxygen and nitrogen and also serve to indicate the sites of binding in GG toward cobalt and manganese ions.

Crook and Rabin[6] have reported a colorimetric procedure for the determination of dipeptides based on the formation of oxygenated cobalt complexes of the dipeptides. However, they found that no color is obtained from glycylproline, although this substance forms complexes with Co(II) of stability greater than those of GG. They suggest that a possible explanation of this is that, in the formation of a colored complex, ionization of the amide hydrogen is necessary, and glycylproline does not have an amide hydrogen. In order to investigate this point further we have used the p.m.r. and polarographic methods for the purpose of studying the glycylproline complexes under the same conditions as the glycylglycine complexes.

Experimental

Materials.—Glycylglycine was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; a chromatographically pure sample of glycyl-L-proline was obtained from Mann Research Laboratories, New York, N. Y.; 99.8% D_2O was obtained from Bio-Rad Laboratories and used without further purification.

Preparations of Solutions.—All solutions were prepared in D_2O as solvent. Stock solutions of $CoCl_2$ and $MnSO_4$ were prepared by dissolving the requisite amount of the dehydrated salts in D_2O. A stock solution of 2 M NaOD was prepared by diluting a CO_2-free saturated solution of sodium hydroxide with D_2O and standardizing with potassium hydrogen phthalate. Stock solutions of 1 M glycylglycine and glycyl-L-proline were prepared immediately before use. Finally, 2-ml. samples for p.m.r. measurements were prepared by volumetric dilution of the stock solutions and contained 0.5 M glycylglycine or 0.5 M glycyl-L-proline, 0.5 M NaOD, and 10^{-2} to 10^{-6} M $CoCl_2$ or $MnSO_4$.

P.m.r. Measurements.—Proton magnetic resonance spectra were recorded with a Varian Associates Model A-60 spectrometer. A sweep width scale of 500 c./sec. was used to record the spectra. Solutions in 99.8% D_2O were examined in spinning Wilmad coaxial tubes with benzene in the annulus as an external reference compound. Data are reported in terms of the frequency-independent unit δ

$$\delta = \frac{\nu - \nu_{benzene}}{60} \text{ p.p.m.} \tag{1}$$

with the positive sign given to peaks at higher field side than benzene. The sample temperature was 37 ± 1°.

Polarographic Measurements.—The polarographic half-wave potentials of the cobalt complexes after passing oxygen were measured with a Fisher Elecdropode. The half-wave potentials were determined in the manner described by Li and Chen.[7]

Anion-Exchange Studies.—Adsorbabilities were measured with the strong base anion-exchange resin Dowex-1 (polystyrene divinylbenzene quaternary amine resin, 100–200 mesh). Resin in the glycylglycinate form was obtained by treating resin in the chloride form with 0.1 M sodium glycylglycinate until the effluent, pH about 6.3, gave negligible chloride test with $AgNO_3$. The resin was then washed with water to remove excess glycylglycinate. After resin was air-dried overnight, portions of it were analyzed for moisture by drying in an oven at 110°. All resin weights refer to the oven-dried material.

Oxygen was passed through 70 ml. of a solution containing 0.1 M sodium glycylglycinate and radioactive Co^{60} for 12 hr. for the purpose of obtaining a fully oxygenated cobalt–GG solution. Then varying amounts of this solution and a 1 M sodium glycylglycinate stock solution were mixed in order to have the glycylglycinate concentration in the range of 0.1 to 0.5 M. Finally, 18 ml. of the solutions thus prepared was equilibrated with resin in the glycylglycinate form in 25-ml. ground glass-stoppered flasks for 16 hr. at 25 ± 1°. The solutions were filtered through glass wool rapidly, and 4-ml. aliquots were removed for assay of the γ-activity of Co^{60} with a sodium iodide scintillation counter.

(1) This work was supported by a grant from the National Science Foundation (G-21532).

(2) E. L. Smith, *J. Biol. Chem.*, **173**, 571 (1948).

(3) J. B. Gilbert, N. C. Otey, and V. E. Price, *ibid.*, **190**, 377 (1951).

(4) C. Tanford, D. C. Kirk, Jr., and M. K. Chantooni, Jr., *J. Am. Chem. Soc.*, **76**, 5325 (1954).

(5) L. H. Vogt, Jr., H. M. Faigenbaum, and S. E. Wiberley, *Chem. Rev.*, **63**, 269 (1963).

(6) E. M. Crook and B. R. Rabin, *Biochem. J.*, **68**, 177 (1958).

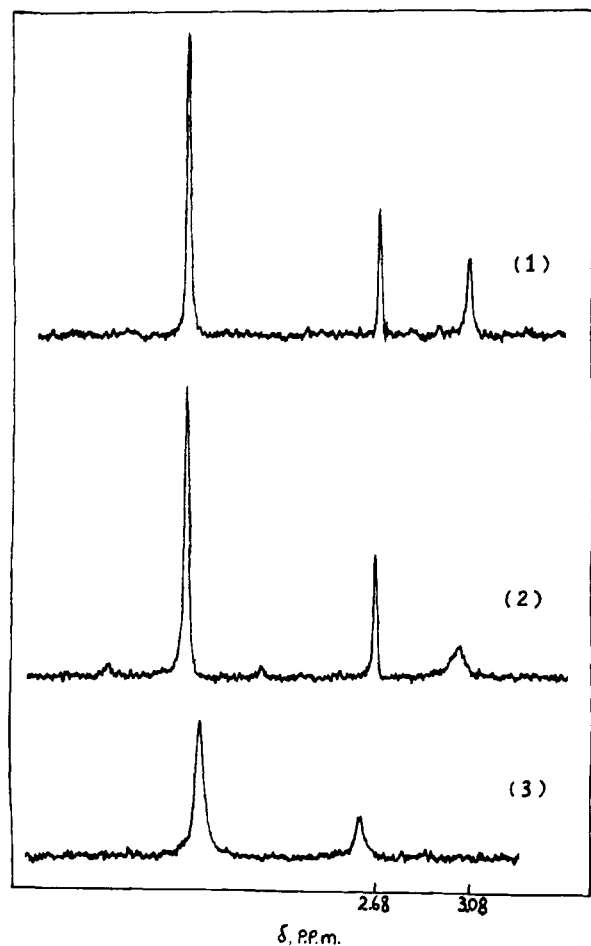(7) N. C. Li and M. C. M. Chen, *J. Am. Chem. Soc.*, **80**, 5678 (1958).

Fig. 1.—Proton chemical shifts in 0.5 M glycylglycinate in the presence of: (1) 10^{-4} M, (2) 10^{-3} M, and (3) 10^{-2} M $CoCl_2$.

Results and Discussion

(A) P.m.r. Measurements.—The p.m.r. spectra of 0.5 M glycylglycinate in the presence of 10^{-4}, 10^{-3}, and 10^{-2} M $CoCl_2$ and in the presence of 10^{-6}, 10^{-4}, and 10^{-3} M $MnSO_4$ are presented in Fig. 1 and 3, respectively. D_2O was used as solvent to reduce the obscuring resonance of H_2O. However, exchange of labile protons of GG with solvent and the H_2O present in 99.8% deuterium oxide introduce sufficient protons to give a sharp HDO proton resonance in the presence of 10^{-4} M $CoCl_2$ and 10^{-6} M $MnSO_4$. Li, et al.,[8] have shown that in the absence of any divalent metal ion, there are two lines in 0.5 M glycylglycinate: the signal at 2.68 p.p.m. comes from the CH_2 adjacent to carboxylate group and the signal at 3.11 p.p.m. comes from the CH_2 which is adjacent to the amino group. Figure 1 (2) shows that in the presence of 10^{-3} M $CoCl_2$, the signal at 2.68 p.p.m. remains fairly sharp while the signal at 3.08 is broadened considerably. In Fig. 1 (3), in the presence of 10^{-2} M $CoCl_2$, the 2.68 signal becomes also broadened and is shifted slightly downfield, while the 3.08 signal becomes so broad that it becomes unobservable. Li, et al.,[8] have shown that paramagnetic metal ions selectively broaden the p.m.r. lines of the nuclei which are adjacent to the sites of binding because the magnetic field of the ion decreases the relaxation time of the nuclei and because the complexed ligand is exchanging rapidly with the free ligand in

(8) N. C. Li, R. L. Scruggs, and E. D. Becker, *J. Am. Chem. Soc.*, **64**, 4650 (1962).
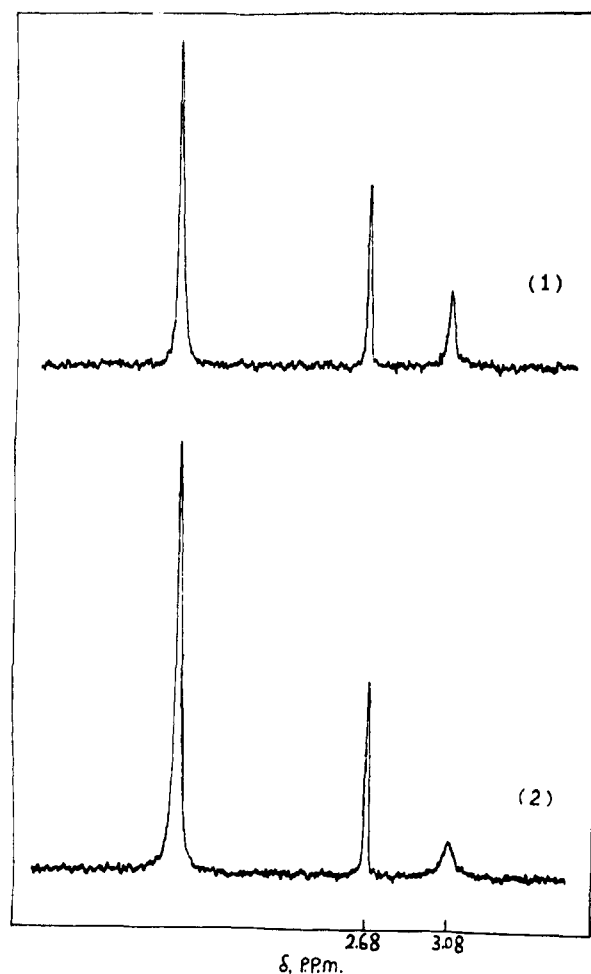

Fig. 2.—Proton chemical shifts in 0.5 M glycylglycinate, 10^{-2} M $CoCl_2$: (1) after passing oxygen 18 hr.; (2) after passing oxygen 18 hr., then passing nitrogen 6 hr.

solution. Since both Fig. 1 and 3 show that Co(II) and Mn(II) affect the 3.08 p.p.m. signal to a greater extent than the 2.68 signal, we have concluded that a preferred binding site in GG toward Co(II) and Mn(II) is the amino group rather than the carboxylate.

Figure 3 (3) shows that in the presence of 10^{-3} M $MnSO_4$, both signals in GG become unobservable. This situation also holds for a solution containing 0.5 M glycylglycinate and 0.5 M $CoCl_2$. At high concentrations of paramagnetic metal ions, therefore, the ability to characterize the signals by virtue of selective broadening is lost.

Figure 2 (1) shows the spectrum of 0.5 M glycylglycinate, 10^{-2} M $CoCl_2$, after passage of oxygen for 18 hr. On comparison of this with Fig. 1 (3), it is obvious that upon oxygenation the paramagnetic susceptibility of the cobalt–GG system has decreased considerably. In fact, Fig. 2 (1) resembles Fig. 1 (1), which, because of the low concentration of $CoCl_2$ present, is almost like the spectrum of glycylglycinate in the absence of a divalent metal ion. Undoubtedly the small broadening of the 3.08 p.p.m. signal observed in Fig. 2 (1) is due to the fact that the oxygenation is not quite 100% and the oxygenated complex itself is diamagnetic. It is especially noteworthy that the oxygen molecule, which in the free state is paramagnetic and therefore broadens p.m.r. signal, becomes diamagnetic when attached to the complex. Since a possible structure of the oxygenated cobalt complex of GG has been
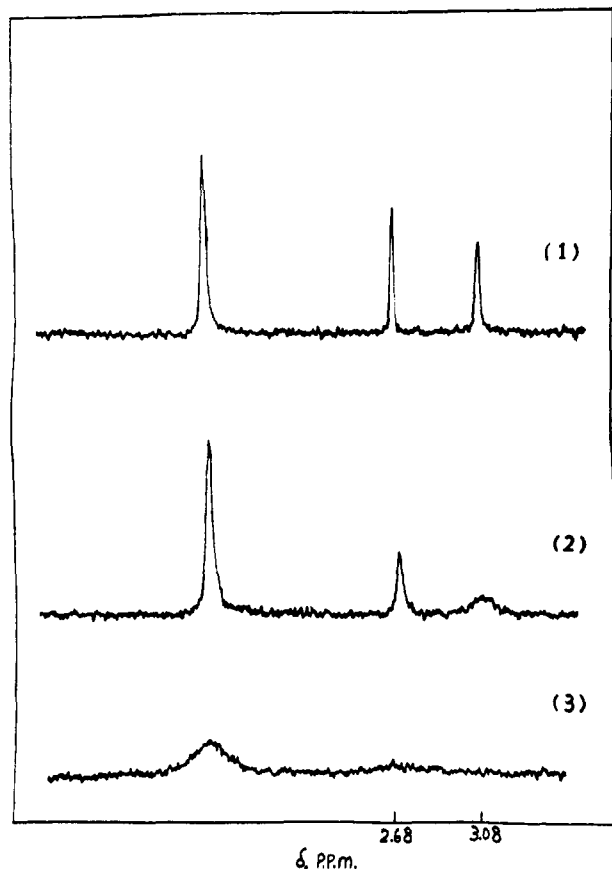
Fig. 3.—Proton chemical shifts in 0.5 M glycylglycinate in the presence of: (1) 10^{-6} M, (2) 10^{-4} M, and (3) 10^{-3} M MnSO$_4$.
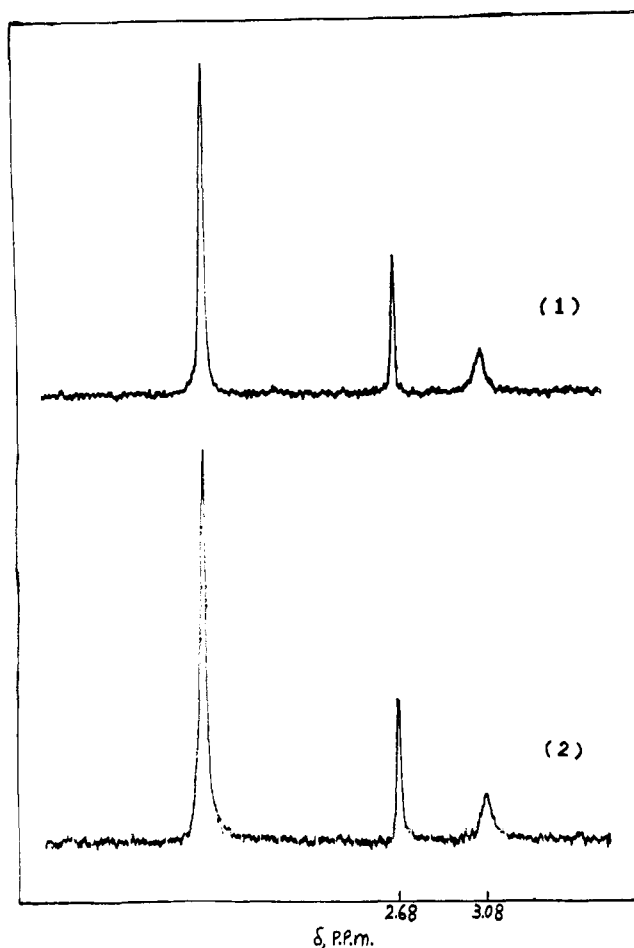

Fig. 4.—Proton chemical shifts in 0.5 M glycylglycinate, 10^{-4} M MnSO$_4$: (1) after passing oxygen 18 hr.; (2) after passing oxygen 18 hr., then passing nitrogen 6 hr.

postulated as I, an orbital picture of the oxygen and hydroxyl coordination with the two cobalt nuclei in the binuclear glycylglycine complex may then be as shown in Fig. 5. The complex would be diamagnetic, in agreement with the present p.m.r. observations and with the results of direct magnetic susceptibility measurements obtained by White, et al.[9]

Upon flushing the oxygenated system with nitrogen, Fig. 2 (2) is obtained. It is seen that now the 3.08 signal has become broadened, indicating that the attachment of oxygen is somewhat reversible. This situation is the same as that found by Michaelis[10] for the reversible oxygenation of the cobalt–histidine system.

Figure 4 (1) shows the spectrum of 0.5 M glycylglycinate and 10^{-4} M MnSO$_4$, after passage of oxygen for 18 hr. On comparison of this with Fig. 3 (2), it is seen that the 3.08 p.p.m. signal has sharpened on oxygenation, so that the paramagnetic susceptibility of the system has decreased. Upon flushing with nitrogen for 6 hr., Fig. 4 (2) is obtained, showing no apparent change from Fig. 4 (1). This is in agreement with the statement that oxygenation of the Mn-glycylglycine complex is not reversible, in contrast with the reversible oxygenation of the Co–GG complex.[10]

(B) Comparison of Glycylproline with GG Complexes.—After passing oxygen through a solution containing 5×10^{-3} M Co(NO$_3$)$_2$, 0.02 M sodium glycylglycinate, and 0.13 M KNO$_3$, the polarographic re-

duction yields two waves with half-wave potentials of -0.56 and -1.21 v. (vs. s.c.e.). When glycylprolinate is used in the above experiment in place of glycylglycinate, only one wave is found, with a half-wave potential of -1.25 v. Since a cobalt wave of around -0.5 v. is indicative of Co(III) → Co(II) and a cobalt wave of around -1.2 is indicative of Co(II) → Co(0),[11] the results of our polarographic experiments indicate that glycylprolinate does not form an oxygenated complex whereas glycylglycinate does.

We have isolated crystals of oxygenated Co–GG complex according to the method of Gilbert, et al.[3] When a p.m.r. spectrum is obtained for a solution of the isolated product dissolved in D$_2$O, there are two poorly resolved peaks at 3.23 and 2.60 p.p.m. and a sharp peak at 2.25 p.p.m. These peaks are indicative of diamagnetic species. Again using the method of Gilbert, et al.,[3] but with glycylproline instead of glycylglycine, we have obtained a solid product, the spectrum of which in D$_2$O shows that all signals are broadened to the extent that they become unobservable. Our p.m.r. experiments therefore confirm the findings from polarographic experiments in that glycylproline, which does not have an amide hydrogen, does not form an oxygenated complex under the same conditions that glycylglycine does. Qualitatively, it can be seen that passage of oxygen through solutions of Co(NO$_3$)$_2$,

(9) J. M. White, T. J. Weisman, and N. C. Li, J. Phys. Chem., 61, 126 (1957).

(10) L. Michaelis, Arch. Biochem., 14, 17 (1947).

(11) I. M. Kolthoff and J. J. Lingane, "Polarography," 2nd Ed., Interscience Publishers, Inc., New York, N. Y., 1952, pp. 481–486.
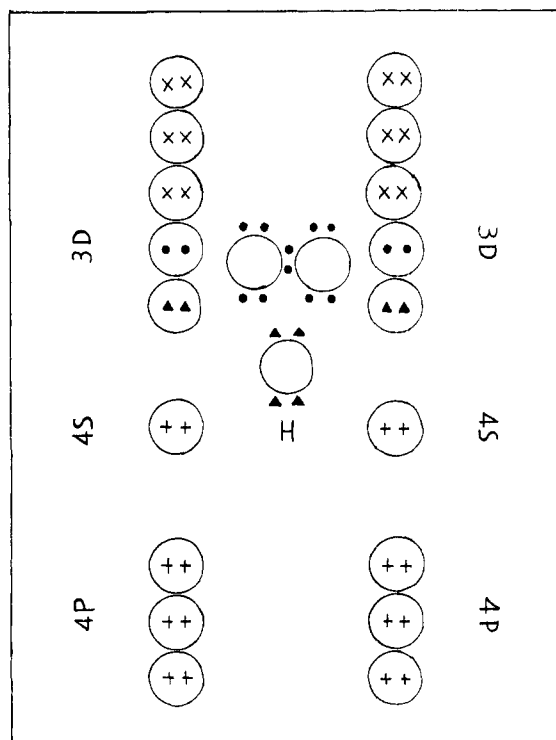
Fig. 5.—Orbital picture of oxygen coordination with two cobalt nuclei: $\times$, electrons from cobalt; $\bullet$, electrons from oxygen; $\blacktriangle$, electrons from OH; $+$, electrons from glycylglycine.

glycylsarcosinate, or glycylprolinate results in no significant change of pH or color, whereas considerable changes in pH and color are produced on oxygenation of dipeptides which do have amide protons. Glycyl-sarcosine, like glycylproline, does not have an amide hydrogen.

(C) **Anion-Exchange Studies.**—In order to determine the charge of an oxygenated cobalt–GG complex, C^{x-}, we have carried out anion-exchange experiments using Dowex-1 resin glycylglycinate. The results are summarized in Fig. 6, which is a log–log plot of the distribution coefficient D vs. molarity of sodium glycylglycinate. Treatment of the data follows that of Nelson and Kraus[12] and Li and White.[13] In glycylglycinate media we may consider the ion-exchange equilibrium

$$aC^{x-} + x(GG^{a-})_r = a(C^{x-})_r + xGG^{a-} \quad (2)$$
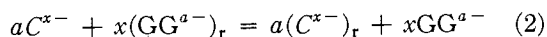
where the subscript r denotes the resin phase. Where no subscript is given, the aqueous phase is implied. The mass action expression for the above equilibrium may be written

$$K = \frac{(C^{x-})_r{}^a(GG^{a-})^x}{(C^{x-})^a(GG^{a-})_r{}^x} \quad (3)$$

where K is the exchange constant. Since tracer amount only of radioactive Co^{60} was used in the presence of

(12) F. Nelson and K. A. Kraus, J. Am. Chem. Soc., **77**, 801 (1955).
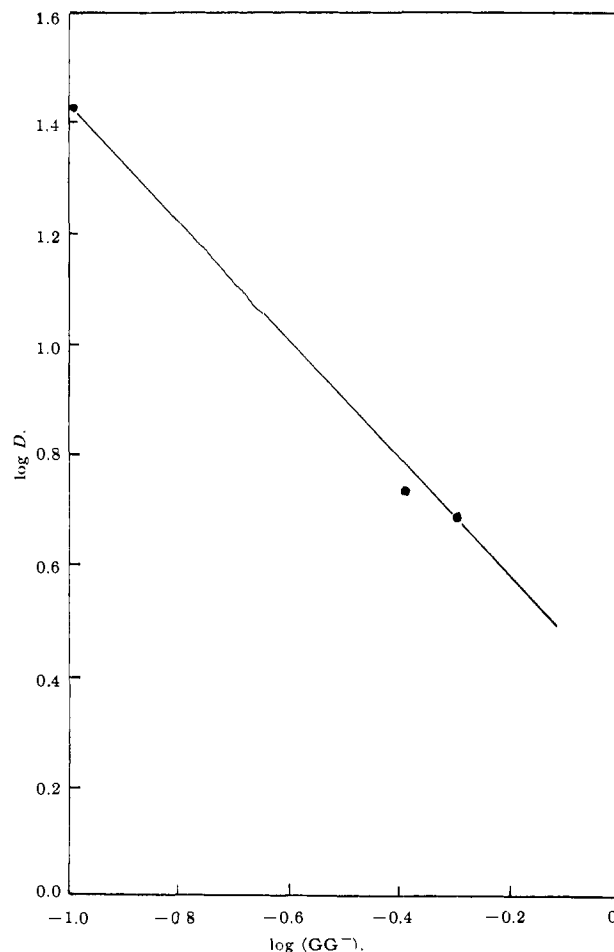(13) N. C. Li and J. M. White, J. Inorg. Nucl. Chem., **16**, 131 (1960).

Fig. 6.—Adsorbability of oxygenated Co^{60} glycylglycinate complex in glycylglycinate solutions by Dowex-1 resin glycyl-glycinate.

0.1 to 0.5 M sodium glycylglycinate and in the presence of 0.66 to 0.82 g. of resin glycylglycinate, the value of $(C)_r$ is small compared with $(GG)_r$. We may assume therefore that $(GG^{a-})_r$ remains constant, so that eq. 3 can be written in the form

$$K(GG^{a-})_r{}^x = \text{constant} = D^a(GG^{a-})^x \quad (4)$$

since

$$D = (C^{x-})_r/(C^{x-}) \quad (5)$$

Differentiation of eq. 4 gives the equation

$$\frac{d \log D}{d \log (GG^{a-})} = -\frac{x}{a} \quad (6)$$

where x is the charge of the oxygenated Co–GG complex ion and a is the charge on the complexing glycyl-glycinate anion. Figure 6 shows that the slope in the log D vs. log (GG^-) plot is -1.1. Since $a = 1$, this means that $x = -1$. Although the cobalt–glycyl-glycinate–oxygen system has been studied extensively, this is the first time that experimental data are presented to show that the net charge is -1.