value for K_O, and the K values of Table I, we obtain the data plotted in Fig. 2. The relation between log $(1/K = 1/K_O)$ and pH is indeed linear with slope -1.0, within experimental error. Since log $(K_H/K_O) \sim 0$ and $K_H \cong K_O$, the pK for the dissociation of the acid group in the reactive site is about 5, which is near that of an isolated carboxyl group. It is to be noted that the effects of acid pH on the anti-OA and anti-BSA systems are quantitatively indistinguishable.[2]

Our results are therefore consistent with the presence of *one carboxyl* group in each Ag–Ab bond which must be ionized in order for the bond to form. Alternative causes of the acid bond dissociation may be postulated, including (a) non-specific electrostatic repulsion between the positively charged Ag and Ab molecules, and (b) reversible configurational changes in either or both the Ag and Ab molecules. The former of these, however, may be eliminated for the anti-OA system by the same arguments which were valid in the anti-BSA system.[2] As for the latter possibility, while BSA does undergo marked reversible configurational changes in acid solution,[11,12] there is no evidence of similar behavior with OA.[11]

We conclude, therefore, that a single ionized carboxyl group is an essential part of the Ag–Ab bond in the rabbit anti-OA, as well as the rabbit anti-BSA, systems. The inference is made that a single positively charged group is present in each complementary site, which is consistent with (a) the dissociation of OA–Ab bonds in sufficiently alkaline as well as acid solutions[5]; and (b) the positive standard entropy change in the Ag–Ab reaction.[3,4]

Although it has long been suspected that salt linkages constitute a part of the mechanism of the reactions between *natural* protein antigens and their antibodies, quantitative and unambiguous evidence has heretofore been lacking. Whether a single critical salt linkage per bond is a feature common to most, or all, such systems requires further investigation.

(11) C. Tanford, *Proc. Iowa Acad. Sci.*, **59**, 206 (1952).
(12) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

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Interactions of Neutral Amino Acids with Human Serum Albumin and γ-Globulin[1]

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In order to discover whether or not neutral amino acids were bound by proteins at neutral pH, human serum albumin and human γ-globulin were dialyzed against a tenfold excess of C^{14}-labeled glycine, leucine or phenylalanine, and radioactivity measurements performed on the protein solution and the dialysate. Under the above conditions no binding of the amino acids by the proteins could be demonstrated. An improved technique has been developed whereby protein solutions containing C^{14}-labeled amino acids could be pipetted onto the planchets, dried and counted.

I. Introduction

In the course of studies on the free amino acids of human plasma, it became of interest to determine whether or not these compounds were bound by the proteins of plasma at pH 7.5. In the equilibrium dialysis experiments described below, investigations were restricted to three neutral amino acids: namely, glycine, leucine and phenylalanine, in order to reduce charge effects to a minimum while varying the hydrocarbon side chain. The plasma proteins employed were human serum albumin and human γ-globulin. The amino acids were labeled with C^{14} and the amount of binding was calculated from a comparison of the counts inside and outside the dialysis bag.

II. Materials and Methods

Human Serum Albumin.—The dried powder used as starting material in these experiments had been prepared by fractionation with alcohol and recrystallized three times.[2] An 8% solution of the powder was passed through a mixed-bed de-ionizing resin[3] and the resultant solution frozen at $-5°$ when not in use.

Human γ-Globulin.—The γ-globulins had been prepared by fractionation with alcohol.[4] A 10% solution was twice dialyzed against 10 volumes $\Gamma/2 = 0.1$ NaCl, $\Gamma/2 = 0.05$ phosphate buffer, pH 7.45. Concentrates of the final dialysate were ninhydrin negative. The resultant protein solution was frozen at $-5°$ when not in use.

The C^{14}-labeled amino acids[5] were obtained as solutions of the following molarity and activity:

glycine	0.005 M	2,310,000 c.p.m./ml.
leucine	.025 M	500,000 c.p.m./ml.
phenylalanine	.025 M	100,000 c.p.m./ml.

These were added to the unlabeled amino acid solutions such that the final count was between 2,500 and 100,000 c.p.m./ml. and the molarity of the solution was 10^{-2} or 5×10^{-3}.

Equilibrium Dialysis Procedure.—Two ml. of 10^{-3} M albumin or 5×10^{-4} M γ-globulin was dialyzed in Visking cellulose tubing ($^1/_4$" diam.) against 10 ml. 10^{-2} or 5×10^{-3} M amino acid. In the experiments at low salt concentrations, the amino acids and the protein were brought to pH 7.5 by the addition of 0.1 N NaOH.[6] In the experiments at a higher salt concentration, the ionic strength was 0.15 ($\Gamma/2 = 0.1$ NaCl, $\Gamma/2 = 0.05$ phosphate) and the final pH 7.45. The dialysis was carried out in glass-stoppered tubes at $+1°$ for 24 hours with continuous rotation.

After this time the dialysis bags were removed from the tubes, wiped dry and emptied into small test-tubes. Opti-

(1) This work has been supported by funds of Harvard University, by grants from the National Institutes of Health, and by contributions from industry.

(2) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(3) H. M. Dintzis, Dissertation for Ph.D. Thesis, Harvard University, 1953.

(4) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., THIS JOURNAL, **71**, 541 (1949).

(5) The authors are indebted to Dr. R. Loftfield of the Massachusetts General Hospital for the C^{14}-labeled amino acids.

(6) In the experiments with γ-globulin at low ionic strength the 2-ml. aliquots of protein were dialyzed against 500 ml. water, the bags wiped dry and transferred to the dialysis tubes. The final pH of the protein solution in these experiments was always slightly low, about 6.9–7.1.

cal density measurements at 280 mμ were routinely performed on a Beckman D.U. spectrophotometer to obtain final protein concentrations and to ascertain that no leakage of protein through the bags had occurred during dialysis.

Preparation of Planchets.—When a protein solution is dried on a planchet it tends to crack and does not adhere to the base of the planchet. The addition of a disc of lens paper to the planchet will not correct the latter drawback. For reproducible results, however, it is essential that the sample be distributed evenly over a known area. Previously, this has been accomplished by precipitation of the protein or by the conversion of the C^{14} to $C^{14}O_2$ followed by precipitation as barium carbonate. This latter procedure is very tedious.

In the experiments described below, the formation of an even film of standard area was achieved by the addition of concentrated sucrose solution to the protein solution in amounts not less than half the final dry weight of the material on the planchet.

This procedure resulted in the formation of a smooth, adhering glass when the material on the planchet was dried.

Precise details of the procedure for the preparation of the planchets are given in the following paragraph.

The sides of the planchets were wiped with a trace of silicone grease, discs of lens paper, approximately the same diameter as the inside diameter of the planchets, placed in the planchets and the planchets weighed.

1.2-ml. protein solution (*ca.* 90 mg.) was added to 0.6 ml. of 20% sucrose solution (120 mg.) and 0.3-ml. aliquots pipetted on the planchets, the final dry weight of material on the planchet thus being about 35 mg.

2.0 ml. of the dialysate (1–10 mg.) was added to 1.7 ml. of 20% sucrose solution (340 mg.) and 0.37-ml. aliquots pipetted on the planchets (approximately 35 mg. dry weight per planchet).

Each planchet thus contained 0.2 ml. of the original solutions.

The planchets were dried at 100 mm. over $CaCl_2$ as it was found that, at atmospheric pressure, drying was slow enough to permit salt crystals to form in the samples at the higher ionic strength. Oven drying at 60° caused the sample to brown and did not give even films. Aluminum planchets, 2.85 cm.^{2} area, were used throughout.[7] Each solution was plated in quintuplet.

The dried planchets were weighed[8] and counted in a Robinson windowless proportional flow counter.[9]

In calculating the concentration of amino acid inside the bag, allowance was made for the volume occupied by the protein. The partial specific volume of albumin was taken as 0.733, that of γ-globulin as 0.739.[10]

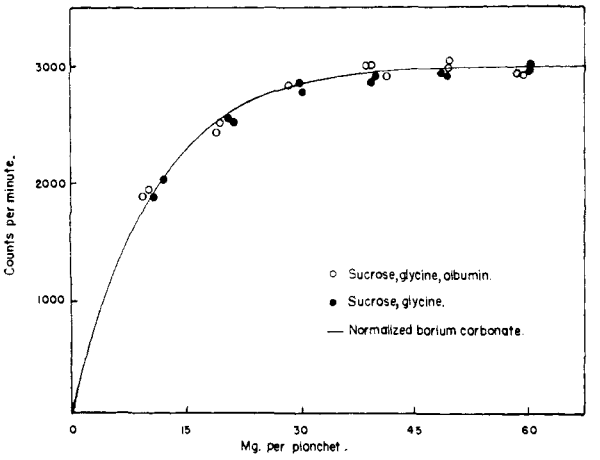

Fig. 1.—Self-adsorption curve for sucrose films and barium carbonate.

(7) L. E. Glendenin and A. K. Solomon, *Science*, **112**, 623 (1950).

(8) Some counts were made at infinite thickness but the accuracy did not seem as good as when thinner films were prepared.

(9) C. V. Robinson, *Science*, **112**, 198 (1950).

(10) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

III. Results

Self-absorption curves for protein–amino acid–sucrose and amino acid–sucrose films were obtained and compared with a barium carbonate curve[11,12] normalized to 2800 c.p.m. at 27 mg. per planchet as shown in Fig. 1. As the three curves were identical, the self-absorption correction factors which had previously been determined for barium carbonate[13] could be applied to the sucrose films. In the studies on amino acids and proteins, the counts were routinely corrected to what they would have been had the sample weighed 10 mg.

The results obtained after equilibrium dialysis of albumin with the three amino acids are shown in Table I. Since variations in quintuplet planchets were not greater than the fluctuations of the standard planchet (less than 5% of the actual count) and since the weights of the films never varied by more than 5% within one set of samples, a mean value was taken for both the weight and the raw count.

The concentration of amino acid inside and outside the dialysis bag after dialysis never varied by more than 5%. As the concentrations of albumin and amino acid were such that had one mole of amino acid been bound per mole of protein, there would have been 10% more amino acid inside the bag than outside, it appeared that no measurable binding of the amino acids by albumin had occurred under the conditions investigated.[14]

Similar results were obtained with γ-globulin and are summarized in Table II.[16] It is of interest to note that, although γ-globulin is stabilized by glycine at relatively high concentrations,[17] there was no evidence for any strong binding which should have been observable at the glycine/γ-globulin ratios employed.

IV. Discussion

In considerations of the synthesis and metabolism of proteins, the existence of both specific and non-specific binding of amino acids by proteins has been postulated. To discover whether or not such non-specific binding was likely to be an important factor in amino acid–protein interactions, binding studies were carried out with two well-characterized proteins which, as far as is known, are not directly involved in protein synthesis or metabolism and where specific amino acid binding of biological importance appeared least likely to occur. A further restriction was imposed in that the amino acid R-groups were all hydrocarbon chains of varying size and degree of saturation. Since the experiments were conducted at *p*H 7.5 the amino acids had no net charge, and any binding therefore would

(11) P. E. Yankwich and J. W. Weigl, *Science*, **107**, 651 (1948).

(12) M. L. Karnovsky, J. M. Foster, L. I. Gidez, D. D. Hagerman, C. V. Robinson, A. K. Solomon and C. A. Villee, *Anal. Chem.*, in press.

(13) A. K. Solomon, R. G. Gould and C. B. Anfinsen, *Phys. Rev.*, **72**, 1097 (1947).

(14) Dr. F. R. N. Gurd, in studies no zinc glycinate–albumin interactions,[15] was unable to detect any binding of glycine by albumin under similar conditions.

(15) F. R. N. Gurd, "Ion Transport Across Membranes," Edited by Hans T. Clarke, Academic Press, Inc., New York, N. Y., 1954, p. 264.

(16) Since the γ-globulin was insoluble in the equilibrium dialysis experiments at low ionic strength, the protein suspension was transferred to a test-tube and 0.1 ml. of 1.0 *M* sodium chloride added to give a clear solution which was then treated as described above.

(17) E. J. Cohn, *Proc. Amer. Phil. Soc.*, **88**, 159 (1944).

TABLE I

EQUILIBRIUM DIALYSIS OF AMINO ACIDS AND HUMAN SERUM ALBUMIN (*ca.* 10^{-3} *M*)

Amino acid	Γ/2	Inside dialysis bag[a] *f*	*a*	*b*	*c*	Outside dialysis bag[a] *f*	*a*	*b*	Ratio inside/outside
Glycine	0.15	2.48	2253 ± 27	5587	5881	2.56	2364 ± 32	6051	0.972
Glycine	.15	2.51	2278 ± 27	5718	6019	2.64	2391 ± 47	6312	0.954
Glycine	.00	1.67	711 ± 6.8	1192	1242	1.97	629 ± 1.8	1238	1.003
Glycine	.00	1.69	716 ± 7.0	1209	1259	1.97	635 ± 1.7	1255	1.003
Leucine	.00	1.80	259 ± 3.0	466	486	2.04	239 ± 4.3	488	0.996
Leucine	.00	1.58	331 ± 3.7	515	537	2.01	266 ± 3.2	536	1.001
Phenylalanine	.00	2.14	991 ± 7.0	2119	2207	2.17	986 ± 26	2143	1.030
Phenylalanine	.00	2.14	799 ± 5.0	1712	1790	2.20	810 ± 16	1784	1.005

[a] *f* = factor to correct the count to what it would be if the same activity were in 10 mg.; *a* = c.p.m. in 0.2 ml. solution; *b* = c.p.m. in 0.2 ml. solution corrected to a 10 mg. weight; *c* = c.p.m. in 0.2 ml. buffer corrected to 10 mg. This was corrected for the volume occupied by the protein.

TABLE II

EQUILIBRIUM DIALYSIS OF AMINO ACIDS AND HUMAN γ-GLOBULIN (*ca.* 5×10^{-4} *M*)

Amino acid	Γ/2	Inside dialysis bag[a] *f*	*a*	*b*	*c*	Outside dialysis bag[a] *f*	*a*	*b*	Ratio inside/outside
Glycine[b]	0.15	2.35	2342 ± 26	5504	5832	2.53	2380 ± 17	6021	0.969
Glycine[b]	.15	2.34	2345 ± 17	5499	5788	2.50	2409 ± 37	6022	0.961
Glycine	.15	2.40	1178 ± 22	2834	2983	2.54	1216 ± 15	3085	0.967
Glycine	.15	2.36	1173 ± 16	2773	2919	2.54	1192 ± 14	3024	0.965
Glycine	.15	2.48	1147 ± 18	2845	2994	2.83	1050 ± 64	2977	1.005
Glycine	.15	2.46	1149 ± 4	2833	2982	2.85	1059 ± 9	3016	0.989
Glycine	.00	2.35	794 ± 16	1871	1978	2.32	828 ± 12	1922	1.029
Glycine	.00	2.30	960 ± 13	2209	2335	2.31	986 ± 23	2276	1.026
Leucine	.00	2.36	905 ± 23	2136	2248	2.31	963 ± 17	2224	1.011
Leucine	.00	2.41	865 ± 16	2082	2192	2.35	951 ± 22	2235	0.981

[a] See footnote *a* of Table I. [b] In these two dialyses, the amino acid/protein ratio was 16:1. In all other cases the ratio was 8:1.

have to be due to the common $NH_3^+CHCOO^-$ groups (partial charge or dipole attractions) or to the hydrocarbon chains (van der Waals forces).

Under these conditions no binding of amino acid by protein was demonstrable, either in the presence or absence of salt. This would indicate that van der Waals or dipole attractive forces in amino acids are too weak to cause any significant interaction between these molecules and proteins at the amino acid/protein ratios studied. Any association constant for their interaction must be less than 10. This situation, however, may not hold for the charged amino acids as albumin, for example, shows considerable affinity for negative ions.[18]

(18) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 535, 540 (1950).

Whether or not binding occurs at higher amino acid/protein ratios is difficult to determine experimentally, but as the amino acid/protein ratios in tissues are lower than those investigated, it appears unlikely that non-specific binding of amino acids due to van der Waals or dipole attractive forces is an important consideration in physiological systems.

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