Ferrihemochrome and Ferrohemochrome Formation with Amino Acids, Amino Acid Esters, Pyridine Derivatives, and Related Compounds*

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The reaction of heme or hematin with a number of nitrogenous ligands to form ferrihemochromes and ferrohemochromes has been studied spectrophotometrically. The following substances formed both ferri- and ferrohemochromes: pyridine, imidazole, histidine, *N*-methylhistidine, histidine ethyl ester, leucine methyl and ethyl esters, lysine, lysine ethyl ester, glycine ethyl and butyl esters, phenylalanine, phenylalanine ethyl ester, tryptophan, and *S*-ethyl cysteine. The following substances formed ferrohemochromes, but not ferrihemochromes: nicotinic acid, nicotinic acid methyl ester, nicotinamide, cysteine ethyl ester, tyrosine, thiohistidine, arginine, leucylleucine, leucylleucylleucine, poly-L-lysine, poly-L-proline, trypsin, casein, ovalbumin, pyrimidine, thiamine. Under the conditions used, neither ferri- nor ferrohemochromes were formed by glycine, glycylglycine, glycylglycylglycine, glycylproline, glycylleucine, alanine, proline, hydroxyproline, *N*-acetyl proline, leucinamide, betaine, creatinine, creatine, glutathione, α-aminobutyric acid, valine, serine, aspartic acid, asparagine, glutamic acid, δ-amino valeric acid, α-aminovaleric acid, citrulline, cysteine, poly-L-glutamic acid, poly-L-aspartic acid, and polysarcosine. A wide difference in the reactivity of the various ligands with heme or hematin was noted. The esters of α-amino and other acids were considerably more reactive than the free acids. Polypeptides of basic amino acids were more reactive than the monomeric residues. Increasing electronegativity of the ligand apparently enhances reactivity, provided steric factors do not prevail. Shifts in absorption maxima during ferrohemochrome formation and indications of a two-step association curve for certain ligands were noted. However, first-order reaction kinetics were observed, with rates of formation of both ferri- and ferrohemochromes being greatly influenced by *p*H and ligand concentration. Variations in *p*H had marked effects on reactivity; ferrihemochromes were formed more readily at neutral *p*H, and ferrohemochromes at alkaline *p*H values.

Ferri- and ferrohemochrome complexes formed by the combination of heme or hematin with nitrogenous ligands have been the object of considerable research. Early work is summarized by Lemberg and Legge (1949); pertinent recent reports not referred to elsewhere in this paper include those of Lauch (1961), Keilin (1949, 1952, 1955), and Tohjo *et al.* (1962). Denatured globin has a high affinity for heme and the ferrohemochrome formed is fairly stable. It is usually assumed that imidazole groups are involved, but there have been no quantitative studies on the reactive groups of the constituent amino acids involved.

The assumed reaction for ferrohemochrome formation is: $Fe + 2\,B = FeB_2$, where Fe is in the protoporphyrin nucleus and B represents the coordinating base. Many investigators, however, have observed that dissociation curves do not conform to this simple reaction, that dimerization of heme confuses the kinetic interpretation, and that a two-step association reaction takes place (Anson and Mirsky, 1928; Davies, 1940; Shack and Clark, 1947; Walter, 1952; Smith, 1959).

Ferrohemochromes can readily be oxidized to give ferrihemochromes, with the rate of the reaction apparently related to the oxidation-reduction potential of the ferro-ferrihemochrome system (Lemberg and Legge, 1949). It has not yet been established whether one or two molecules of ligand are attached to iron in the ferrihemochrome structure, the difficulty being partly because of the tendency of hematin to polymerize. Although the evidence from the analytical composition of solid ferrihemochromes is contradictory, a recent report (Corwin and Reyes, 1956) confirmed by chemical analysis the early work of Langenbeck (1932) and showed that the structure of imidazole ferriprotoporphyrin complex consisted of two molecules of imidazole and one molecule of ferriprotoporphyrin. Cowgill and Clark (1952) also reported a 2:1 coordination for imidazole with ferrimesoporphyrin. The general structures assigned to ferrihemochromes show two moles of coordinating base on the acid side of the *pK*, or one base and one hydroxyl group on the basic side of the *pK* (Lemberg and Legge, 1949).

The purpose of the present study was to examine the effect of varying conditions on the rate of reaction and relative affinity of potential reactive nitrogenous substances for heme and hematin in an effort to explain the differential reactivity of the various ligands.

MATERIALS AND METHODS

Materials were obtained from the following sources. From Mann Research Laboratory: crystalline hemin, L-histidine ethyl ester hydrochloride, L-leucine methyl ester, L-leucine ethyl ester hydrochloride, tyrosine ethyl ester hydrochloride, L-lysine ethyl ester, glycine butyl ester, DL-phenylalanine ethyl ester hydrochloride, L-cysteine ethyl ester hydrochloride, nicotinic acid methyl ester, glycine ethyl ester hydrochloride, *S*-ethyl-L-cysteine, L-leucinamide, DL-proline, L-leucylleucine, L-leucylleucylleucine, *N*-acetyl-DL-proline, pyrimidine, poly-L-lysine hydrobromide, polysarcosine, poly-L-proline, poly-L-aspartic acid, and poly-L-glutamic acid; from Distillation Products Industries: nicotinic acid, imidazole, glycylleucine, nicotinamide, DL-α-amino-*n*-valeric acid, δ-amino-*n*-valeric acid hydrochloride, and thiamine hydrochloride; from Sigma Chemical Company: DL-tyrosine, L-cystine, glycylglycylglycine, and glycylglycine; from Schwartz Laboratories: L-valine, L-cysteine hydrochloride, L-alanine, L-asparagine, L-tryptophan, L-tyrosine, L-phenylalanine.

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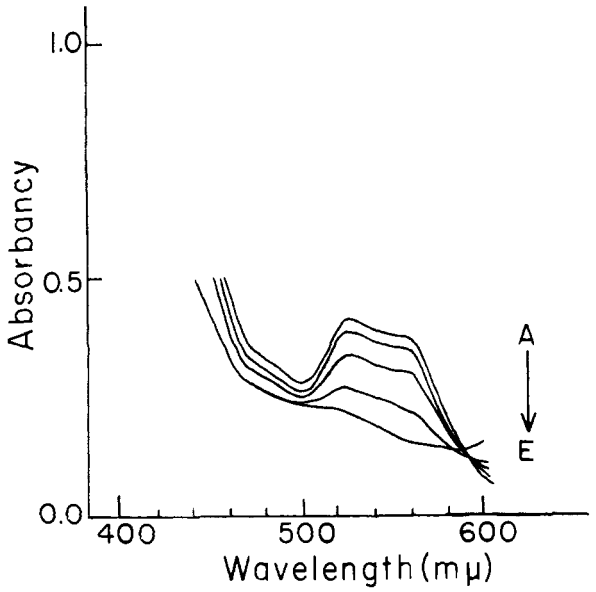

FIG. 1.—Absorption specta of leucine ethyl ester ferrihemochrome in 0.1 M sodium borate buffer, *p*H 8.5. Concentrations of leucine ethyl ester: A, 0.18 M; B, 0.12 M; C, 0.06 M; D, 0.03 M; E, 0.006 M. Initial hematin concentration, 0.04 mM.

L-arginine hydrochloride, L-lysine hydrochloride, L-aspartic acid, L-glutamic acid, and glutathione; from California Corporation for Biochemical Research: DL-serine, L-1-methylhistidine, betaine, hydroxy-L-proline, L-2-thiolhistidine, DL-α-amino-butyric acid, L-citrulline, creatine, and creatinine; from Worthington Biochemical Corporation: trypsin, casein, and ovalbumin; from General Biochemicals: L-histidine hydrochloride; and from Merck: pyridine. All were used without further purification.

The stock solutions of hematin were made up daily by adding 5 ml of 0.1 N NaOH to a 10-ml volumetric flask containing 13.4 mg hemin, and then bringing to volume with water. The hemin solution in 0.05 N NaOH was not stable; the Soret peak had an absorbancy of 2.35 at 385 mμ immediately after mixing the solution; this value dropped to 1.78 after 24 hours. The Soret peak of hemin solutions kept in an ice bath had a constant optical density for 3-4 hours. Fresh heme solutions were made up before every experiment.

The affinity of different compounds for hematin was determined as follows: tubes were made up to contain 0.205 μmole hematin (0.1 ml of the hematin solution described above), increasing concentrations of the potential ligands, and 0.1 N sodium borate buffer, *p*H 8.5, to a final volume of 5 ml. The substances used were dissolved in the same sodium borate buffer, except for some insoluble amino acids that were dissolved first in acid or alkali; in such cases, the *p*H of the amino acid solution was brought to 8.5 by the addition of dilute NaOH or HCl.

Most of the experiments were run in 0.1 N sodium borate buffer, *p*H 8.5; hematin solutions are fairly stable at that *p*H. Barron (1937) suggested that sodium borate buffer combines with hematin affecting the aggregation state of the solution; however Shack and Clark (1947) believe that this does not influence the observations.

Where *p*H was the variable, different buffers (potassium phosphate, sodium borate, or glycine: 0.1 N, *p*H from 6 to 9.5) were used; *p*H was measured with a Beckman glass electrode *p*H meter and was checked in the final system in every case.

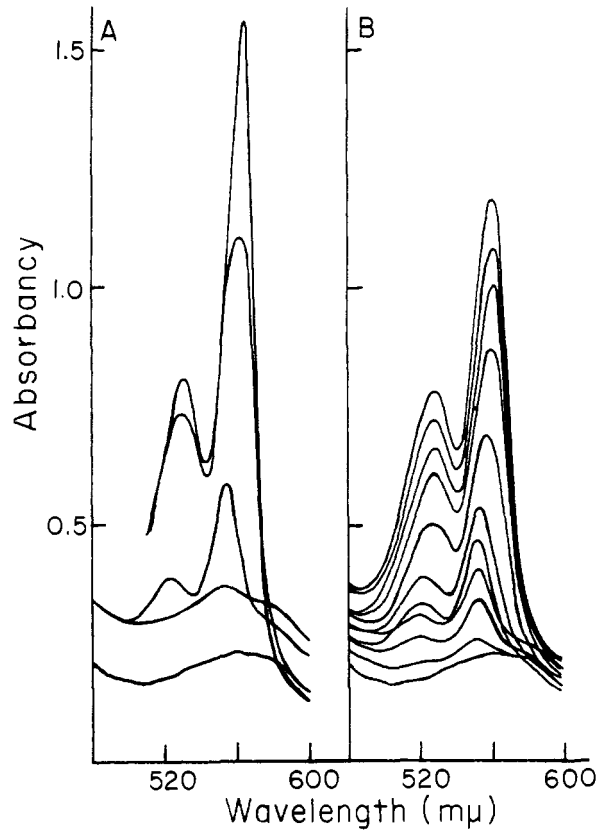

FIG. 2.—Absorption spectra of ferrohemochromes. Curves, top to bottom, are for the following initial concentrations of: A, glycine ethyl ester, 0.53, 0.133, 0.066, and 0.013 M, and none; and B, pyrimidine, 0.0093, 0.0067, 0.0054, 0.0047, 0.0044, 0.0042, 0.0040, 0.0033, 0.0026, and 0.0013 M, and none. Initial heme concentration, 0.04 mM, and *p*H 8.5 in every case.

No effort was made to keep the ionic strength constant. Sodium dithionite ($Na_2S_2O_4$) was used as a reducing agent; because of the fact that it can cause side reactions, only small amounts were used. Following preliminary experiments to determine a safe concentration range, 2–5 mg was added from a spatula tip directly to the solution (usually 3 ml in a cuvet) when required. The spectrum of the test solution was recorded after mixing.

Ferri- or ferrohemochrome formation was ascertained spectrophotometrically in a Cary Model 11 instrument using a 1-cm light path. For kinetic experiments, a specific wavelength, usually that of a pronounced peak in the visible region, was chosen. The initial absorption at that wavelength and the subsequent changes with time were recorded.

RESULTS

Upon the addition to hematin solutions of gradually increasing concentrations of nitrogenous ligands capable of reacting to form ferrihemochromes, the following changes in absorption spectra took place. At low concentrations of ligand little or no change in the hematin spectrum was noted; as the concentration was increased the absorption spectrum changed to that of a ferrihemochrome. Upon continued addition of some ligands there was an increase in absorbancy of the entire hematin spectrum with no change in its shape prior to the appearance of a ferrihemochrome spectrum. If sodium hydrosulfite was then added, the ferrihemochrome was reduced to the ferrohemochrome and a corresponding change in spectrum was observed.

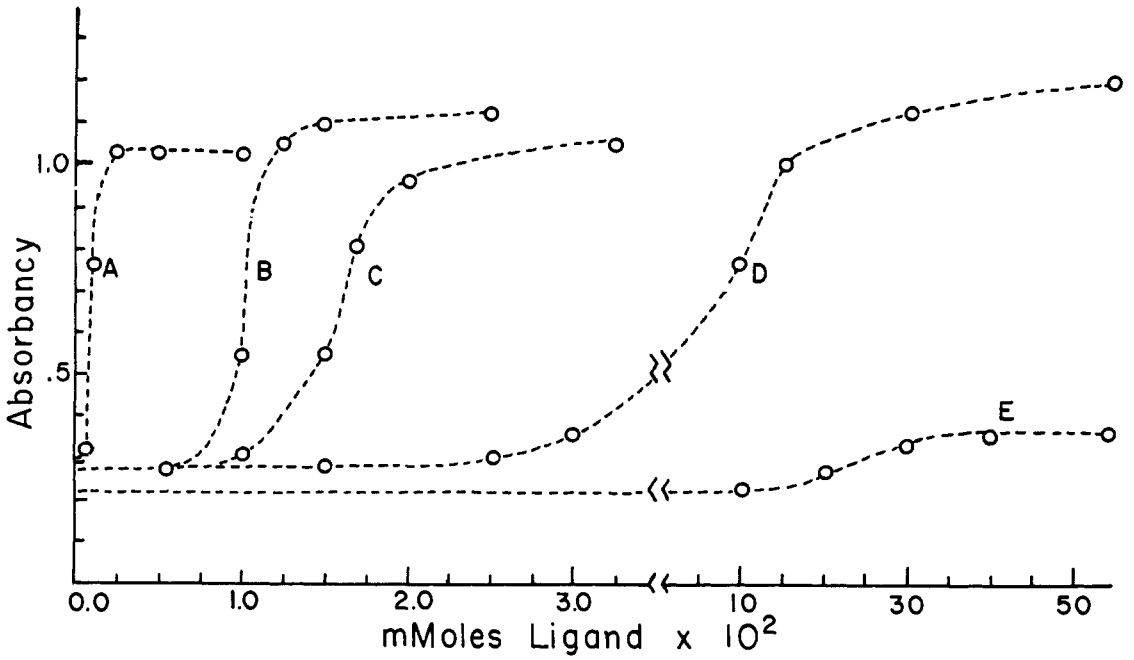

FIG. 3.—Association curves of ferri- and ferrohemochromes, *p*H 8.5; based on absorbancies at alpha peak for ferrohemochromes or beta peak for ferrihemochromes. A, nicotinic acid methyl ester ferrohemochrome (558 mμ); B, pyridine ferrohemochrome (559 mμ); C, phenylalanine ethyl ester ferrohemochrome (558 mμ); D, leucine ethyl ester ferrohemochrome (558 mμ); E, histidine ferrihemochrome (530 mμ). Initial heme or hematin concentration, 0.04 mM.

Ferrohemochromes could also be formed directly by the addition of ligand to heme solutions. After the required concentration of added ligand was reached, and ferri- or ferrohemochrome formation was noted spectrophotometrically, increased concentrations of ligand caused increases in absorbancy, until at some high concentration there occurred no additional increase in absorbancy at a given spectral peak. These changes were measured for a number of nitrogenous ligands added to heme and hematin under various conditions. Figures 1 and 2 show typical examples of ferri- and ferrohemochromes; usually the nitrogenous ligands capable of forming ferrihemochromes yielded ferrohemochromes. In general, a lower concentration of ligand was required for ferro- than for ferrihemochrome formation.

The gradual change of the heme or hematin spectrum to that of the corresponding ferri- or ferrohemochrome with increasing ligand concentration was especially well seen in the Soret region where the maxima shifted from 380–85 mμ to 415–20 mμ. This movement toward longer wavelengths started with an initial splitting of the heme Soret band into two maxima, the second band appearing first as a shoulder on the curve at about 415 mμ and 420 mμ for the ferri- and ferrohemochrome, respectively. The shoulder, indicating incomplete formation of the complex, intensified with additional added ligand to give the Soret maximum of the complex while that of the original heme or hematin disappeared.

The ferrihemochromes had alpha band maxima at 558–62 mμ, beta band maxima at 525–30 mμ, and Soret band maxima at 404–08 mμ. The alpha band appeared as a shoulder except for imidazole ferrihemochrome, where two distinct maxima were seen. The imidazole ferrihemochrome spectrum was otherwise different from that of other ferrihemochromes in that the alpha, beta, and Soret maxima were located at 565, 542, and 435 mμ, respectively. Ferrohemochromes, formed by reduction of ferrihemochromes, showed, at low ligand concentrations, a band at 555 mμ and a shoulder at 580 mμ. With increasing ligand concentration maxima at 555–65, 525–30, and 415–20 mμ were observed; the alpha bands showed higher absorbancy than the beta ones. Leucine methyl ester and leucine ethyl ester ferrohemochromes showed double banded Soret maxima at 440 and 420 mμ.

If absorbancy at a given wavelength of one of the maxima is plotted versus increasing concentration of ligand, an S-shaped association curve is obtained. Such curves were obtained for all reacting ligands; representative examples are shown in Figure 3. The nature of the equilibrium involved is not precisely known, and cannot be interpreted in terms of simple constants (Shack and Clark, 1947). We have used the negative logarithms of the concentration of ligand required for half saturation of heme or hematin as an expression of relative affinity (see Olcott and Lukton, 1961). Table I shows these apparent affinity constants for a number of nitrogenous ligands, together with the minimum concentrations of ligand necessary for complex formation and the maximum concentration of ligand, beyond which there were no further changes in spectra. In most cases the absorbancies of the complexes formed after addition of concentration of ligand shown as highest continued to change very slightly with further addition of ligand. The rate of change however was markedly reduced at some high ligand concentration and this was taken as the end-point. Reproducibility was generally very good; it was poorest for the lowest reactive concentration of ligand. Such differences were not often found and did not significantly affect apparent affinity constants.

Examples of association curves, among which are extremes, are shown in Figure 3. In some cases small differences in concentration of ligand resulted in complete saturation of the heme or hematin with a resulting steep slope of the association curve; in others a considerable amount of ligand was required before the change occurred. In still other cases the association curve indicated a two-step reaction, as is illustrated by

TABLE I
RELATIVE AFFINITIES OF NITROGENOUS LIGANDS FOR HEME AND HEMATIN; ABSORPTION SPECTRA OF RESULTING COMPOUNDS

Ligand	pH	Absorption Spectra: Alpha Band Wavelength (mμ)	Alpha Band Ligand Conc.[a] (M × 10³)	Alpha Band A	Beta Band Wavelength (mμ)	Beta Band Ligand Conc.[a] (M × 10³)	Beta Band A	Soret Band Wavelength (mμ)	Soret Band Ligand Conc.[a] (M × 10³)	Soret Band A	Apparent Affinity Constants: Ligand Conc. Minimum (M × 10³)	Ligand Conc. Maximum (M × 10³)	pA
Ferrihemochromes													
Imidazole	8.0	565	2	0.72	542	2	0.76	435	2	2.5			2.7
	8.5	565	2	0.66	542	2	0.71	435	1	1.75	0.5	5	2.4
	9.0	565	4	0.56	542	4	0.6	435	4	2.25			2.2
1-methylhistidine	8.5	561	40	0.34	530	40	0.41	405	4	3.5	40	80	1.9
Tyrosine ethyl ester	8.5	560	20	0.32	525	20	0.45	408	4	2.58	2	20	1.9
Histidine ethyl ester	8.5	561	60	0.26	530	60	0.32	409	60	3.3	20	80	1.7
Phenylalanine ethyl ester	8.5	560	80	0.37	525	80	0.41	405	4	2.22	8	80	1.7
Tryptophan	8.6	562	40	0.31	530	40	0.36	408	20	3.19	20	80	1.4
Histidine	8.5	562	120	0.29	530	120	0.35	405	40	2.52	20	120	1.3
Leucine ethyl ester	8.5	558	180	0.37	525	180	0.41	405	30	2.9	30	180	1.2
Leucine methyl ester	8.5	560	40	0.25	525	40	0.31	405	40	3.2	20	120	1.1
Phenylalanine	9.3	560	132	0.26	530	132	0.30	409	132	3.18	30	150	1.1
Glycine ethyl ester	8.5	560	150	0.26	525	150	0.31	408	150	3.3	8	200	0.9
Lysine ethyl ester	8.5	560	130	0.26	525	130	0.29	405	130	2.97	60	140	0.9
S-ethylcysteine	8.5	560	160	0.24	525	160	0.30	407	160	2.48	—	—	—
Pyridine	8.6	562	800	0.27	525	800	0.32	404	800	3.12	—	—	—
Ferrohemochromes													
Nicotinic acid methyl ester	8.5	558	0.5	1.03	528	0.5	0.63	415	0.5	3.4	0.1	0.5	3.7
	8.5	563	30	1.17	530	30	0.72	—	—	—	—	—	—
Poly-L-lysine	8.5	559	5	0.65	528	5	0.41	425	5	2.62	0.25	5.0	2.9
Nicotinamide	6.7	560	8	0.6	530	8	0.47	—	—	—	—	—	2.2
	7.5	562	4	0.8	531	4	0.58	—	—	—	—	—	2.5
	8.5	562	4	1.0	530	4	0.65	418	4	2.32	1.4	4.0	2.7
	8.7	565	2	0.95	533	2	0.67	—	—	—	—	—	2.7
	8.9	565	1	0.88	533	1	0.6	—	—	—	—	—	2.9
Pyridine	8.5	559	2.5	1.06	526	2.5	0.64	4.28	2	3.01	5.0	15.0	2.6
		562	80	1.24	530	80	0.74	—	—	—	—	—	—
Tyrosine ethyl ester	8.5	558	20	0.97	525	40	0.46	420	20	2.3	0.2	20.0	2.5
Phenylalanine ethyl ester	8.5	558	2	0.29	—	—	—	—	—	—	2.0	80.0	2.5
		561	80	1.22	531	80	0.54	425	—	—	—	—	—
Pyrimidine	8.5	555	3	0.42	522	3	0.32	—	—	—	1.34	9.0	2.4
		562	9	1.22	530	9	0.82	418	45	2.63	—	—	—
1-methylhistidine	8.5	555	40	1.02	525	40	0.56	420	4	3.1	1.0	40.0	2.3
Cysteine ethyl ester	8.5	555	10	0.95	525	10	0.44	415	4	2.48	1.0	20.0	2.1
Imidazole	8.5	556	20	0.55	528	20	0.36	420	20	2.92	2.5	50.0	2.0
Leucine ethyl ester	8.5	558	180	1.27	525	180	0.61	415	6	2.0	3.0	180.0	1.8
Lysine	8.5	—	—	—	—	—	—	—	—	—	—	—	<1.0
	11.1	555	20	0.36	525	40	0.24	420	20	1.55	4.0	20.0	1.7
Thiamine	8.6	548	60	0.42	—	—	—	416	60	2.33	10.0	80.0	1.6
	12.4	552	6	0.98	522	6	0.47	—	—	—	0.2	6.0	2.7

TABLE I (*continued*)
RELATIVE AFFINITIES OF NITROGENOUS LIGANDS FOR HEME AND HEMATIN; ABSORPTION SPECTRA OF RESULTING COMPOUNDS

Ligand	pH	Absorption Spectra: Alpha Band Wavelength (mμ)	Alpha Band Ligand Conc.[a] (M × 10³)	Alpha Band A	Beta Band Wavelength (mμ)	Beta Band Ligand Conc.[a] (M × 10³)	Beta Band A	Soret Band Wavelength (mμ)	Soret Band Ligand Conc.[a] (M × 10³)	Soret Band A	Apparent Affinity Constants: Ligand Conc. Minimum (M × 10³)	Ligand Conc. Maximum (M × 10³)	pA
Nicotinic acid	8.5	552	40	0.72	521	40	0.43	410	10	1.55	5.0	40.0	1.6
Histidine ethyl ester	8.5	555	60	0.57	526	60	0.34	420	60	2.86	10.0	80.0	1.4
Lysine ethyl ester	8.5	555	66	0.85	525	66	0.42	419	32	3.12	8.0	130.0	1.4
Histidine	8.5	555	16	0.35	526	16	0.25	418	16	1.92	8.0	80.0	1.4
Leucine methyl ester	8.5	556	16	0.60	525	16	0.38	418	16	2.95	3.0	80.0	1.2
		558	40	0.96	529	40	0.6	440	40	2.26	—	—	—
								420	40	2.9	—	—	—
		566	200	1.3	—	—	—	—	—	—	—	—	—
Tryptophan	8.6	556	160	0.6	526	160	0.44	420	80	2.23	40.0	160.0	1.0
Tyrosine	9.5	556	154	0.35	525	154	0.29	420	154	1.62	77.0	—	1.0
Glycine ethyl ester	8.5	555	15	0.37	—	—	—	418	76	3.27	15.0	500.0	0.9
		555	76	0.62	523	76	0.35	—	—	—	—	—	—
		562	114	0.95	528	114	0.57	—	—	—	—	—	—
		565	266	1.55	530	266	0.80	—	—	—	—	—	—
Phenylalanine	9.3	555	400	0.77	526	400	0.47	420	132	1.33	132.0	400.0	0.6
S-ethylcysteine	8.5	555	160	0.3	524	160	0.24	415	160	1.45	—	—	—
Thiolhistidine	8.6	555	36	0.31	525	36	0.24	418	18	1.49	—	—	—
Glycine butyl ester	9.4	555	600	0.27	—	—	—	415	600	1.17	—	—	—
Leucylleucine	8.5	555	84	0.47	525	84	0.36	418	84	2.0	—	—	—
Leucylleucylleucine	8.5	555	80	0.33	525	80	0.21	419	80	1.97	—	—	—
Arginine	12.0	555	40	0.35	524	40	0.24	420	40	1.52	—	—	—
Poly-L-proline	8.5	561	390	1.35	530	390	0.88	420	390	3.23	—	—	—
	12.0	562	390	1.50	531	390	0.95	—	—	—	—	—	—
Trypsin	8.5	557	—[a]	0.57	530	—[a]	0.55	415	—[a]	2.18	—	—	—
	11.8	557	—[a]	1.29	527	—[a]	0.83	—	—	—	—	—	—
Casein	8.5	560	—[a]	1.37	530	—[a]	1.06	—	—	—	—	—	—
Ovalbumin	8.5	560	—[a]	0.4	530	—[a]	0.34	425	—[a]	3.05	—	—	—
	9.2	559	—[a]	0.68	530	—[a]	0.44	420	—[a]	3.5	—	—	—

[a] Concentrations based on monomeric residue weights for polyamino acids; for proteins, 100 mg was used per determination.

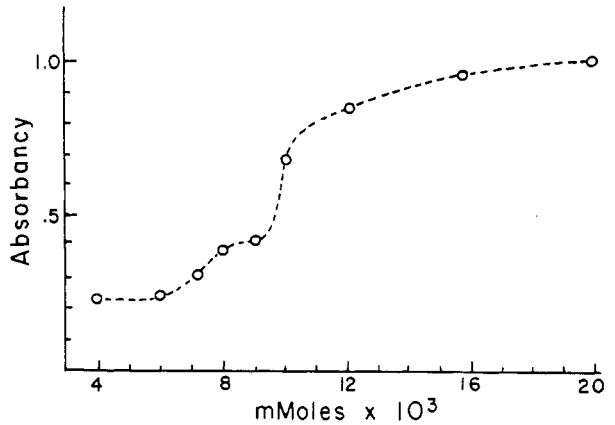

FIG. 4.—Association curve for nicotinamide ferrohemochrome, *p*H 8.5. Based on alpha peak absorbancy. Initial heme concentration, 0.04 mM.

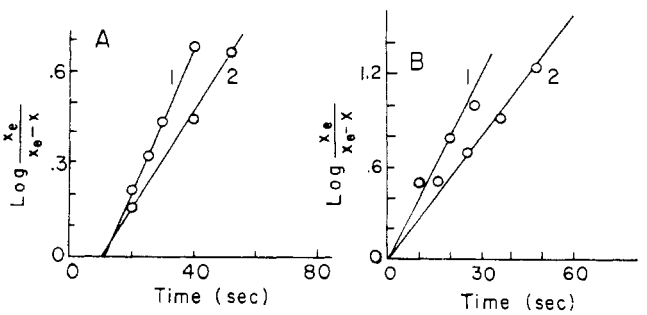

FIG. 5.—Rates of formation of ferri- and ferrohemochromes. Concentration of complex at time t is x; concentration at equilibrium is x_e. A, formation of imidazole ferrihemochrome based on 542 mμ maximum; 1, in 0.1 M potassium phosphate buffer, *p*H 7.4, 0.0016 M imidazole; 2, in 0.1 M sodium borate buffer, *p*H 8.6, 0.005 M imidazole. B, formation of ferrohemochromes based on indicated maxima; 1, 0.002 M nicotinamide, *p*H 8.5, 562 mμ; 2, 0.114 M glycine ethyl ester, *p*H 8.5, 555 mμ. Initial hematin or heme concentration in all cases, 0.04 mM.

the behavior of nicotinamide ferrohemochrome shown in Figure 4. Similar results were seen with glycine ethyl ester, pyrimidine, and nicotinic acid. Absorption spectra data for all the ferri- and ferrohemochromes formed are given in Table I. Since the association curves are not linear, it follows that molar extinction coefficients are meaningful only over narrow ranges, at least for many of these compounds; therefore, specific absorbancies are given for the indicated ligand concentrations. In a few cases, when extremely high concentrations of ligand were added, shifts of alpha and beta peaks of ferrohemochromes towards longer wavelengths were noted. Table I includes data showing these shifts for ferrohemochromes of nicotinic acid methyl ester, pyridine, phenylalanine ethyl ester, pyrimidine, leucine ethyl ester, and glycine ethyl ester. Glycine ethyl ester ferrohemochrome formed at these high ligand concentrations was diluted 1 and 5 times; the absorption maxima were shifted 10–15 mμ toward shorter wavelengths.

Whenever a substance was unreactive at a concentration of 0.2 M (except for proteins) it was considered not to form a ferri- or ferrohemochrome. The possibility exists that at still higher concentrations the complexes may have been formed, and in some cases where solubility and availability permitted higher concentrations were tried. The following compounds were found not to be reactive at concentrations up to 0.2 M: glycine, glycylglycine, glycylglycylglycine, glycylproline, glycylleucine, alanine, proline, hydroxyproline, *N*-acetylproline, leucinamide, betaine, creatinine, creatine, glutathione, α-aminobutyric acid, valine, serine, aspartic acid, asparagine, glutamic acid, δ-aminovaleric acid, α-aminovaleric acid, citrulline, cysteine, and cystine. Poly-L-aspartic acid, poly-L-glutamic acid, and polysarcosine were not reactive at a concentration of 0.2 M, based on monomer content. When some of these compounds were added in high concentration to heme or hematin, a slightly increased general absorbancy in the 500–600 mμ range was noted; however spectra characteristic of ferri- or ferrohemochromes were not seen.

TABLE II
RATES OF FORMATION OF FERROHEMOCHROMES AND FERRIHEMOCHROMES

Ligand	Wavelength (mμ)	*p*H	Ligand Conc. (M × 10^3)	Rate Constant ($k \times 10^3$ sec^{-1})
Imidazole ferrihemochrome	540	7.4	1.6	50
		8.6	1.6	6
		8.6	5.0	35
Pyridine ferrohemochrome	560	8.6	2.0	2
	562	12.0	80.0	26
Nicotinamide ferrohemochrome	562	8.2	2.0	96
		8.5	8.0	156
		12.0	2.0	220
Glycine ethyl ester ferrohemochrome	562	8.5	114.0	57
Leucine ethyl ester ferrohemochrome	555	6.7	60.0	—[a]
		7.5	60.0	—[a]
		9.0	60.0	—[a]

[a] Rates meaningless due to rapid oxidation; net formation greatest at *p*H 7.5.

Poly-L-proline, at a concentration equivalent to 0.39 M (as monomer), formed a ferrohemochrome; at concentrations up to 0.1 M it was not reactive. Poly-L-lysine, casein, ovalbumin, and trypsin did not form ferrihemochromes at concentrations adequate for forming ferrohemochromes. Trypsin, tried at *p*H 11.8 as well as 8.5, was more reactive at the higher *p*H.

Kinetic Studies.—Rates of formation of ferri- and ferrohemochromes varied considerably with nitrogenous ligand. The formation reaction was so rapid for a few ligands (e.g., nicotinamide ferrohemochrome) that it was difficult to measure by ordinary means; 5 seconds approximately were required to obtain an initial spectrophotometric reading in our procedure. At lower ligand concentrations the rates were slower. At high concentrations of some ligands precipitation occurred. Ferrohemochromes that were rapidly oxidized (e.g., those of nicotinic acid, nicotinic acid methyl ester, and leucine) did not yield good kinetic data. At high ligand concentrations, formation reactions appeared to be first-order reversible ones; rate constants were calculated by plotting time (t) versus $x_e/x_e - x$, where x is the concentration of complex formed at time t, and x_e is the concentration at equilibrium. Figure 5 shows representative plots; rate constants calculated from the slopes of such plots are given in Table II. The plot of the line for the formation of imidazole ferrihemochrome did not pass through the origin.

Most of the ligands formed the corresponding ferri- or ferrohemochromes within a few minutes; thus for purposes of studying the final system, reaction times of 1–2 minutes prior to spectrophotometric reading were usually adequate. Pyridine ferrohemochrome formed from 0.002 M pyridine was an exception to this

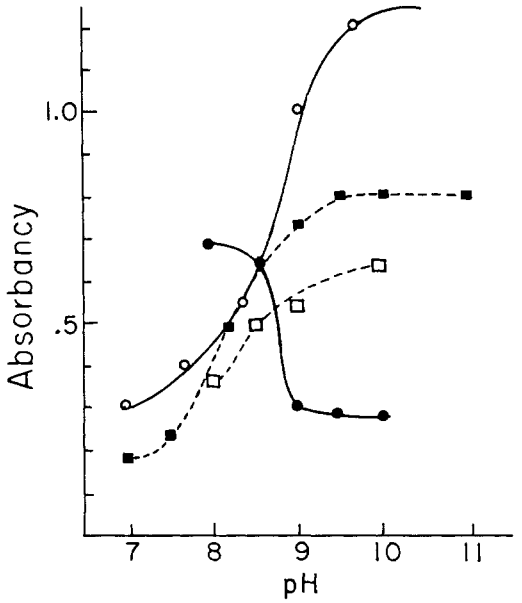

FIG. 6.—Ferri- and ferrohemochrome formation as a function of *p*H, based on indicated maxima; O — O, pyridine (0.08 M) ferrohemochrome (562 mμ); ■ - - - ■, glycine ethyl ester (0.076 M) ferrohemochrome (555 mμ); □ - - - □, imidazole (0.01 M) ferrohemochrome (556 mμ); ● — ●, imidazole (0.05 M) ferrihemochrome (542 mμ).

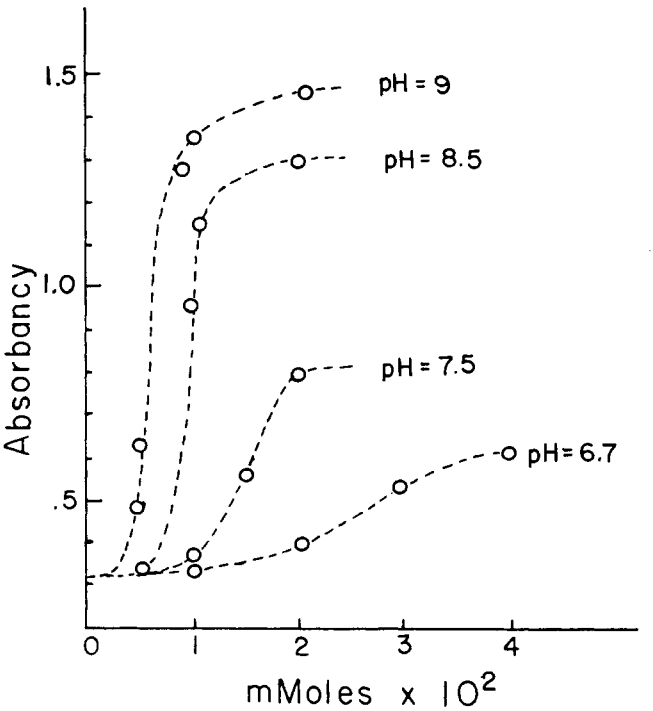

FIG. 7.—Effect of *p*H on nicotinamide ferrohemochrome association curves. Initial heme concentration, 0.04 mM. *p*H 6.7 and 7.5, 0.1 M potassium phosphate buffer; *p*H 8.5 and 9.9, 0.1 M sodium borate buffer. Absorbancy values from 560 mμ peak.

rule; the mixture increased in absorbancy for at least 15 minutes. When the pyridine concentration was 0.08 M, the reaction was complete within 3 minutes.

*p*H *Effects.*—Generally, ferrohemochromes were more readily formed at higher *p*H values, while ferrihemochrome formation was favored by a neutral *p*H. Curves depicting ferri- or ferrohemochrome formation versus *p*H have the appearance of acid-base titration curves; examples are shown in Figure 6. It may be seen from these curves that dissociation constants, expressed as *p*H values at half dissociation of the complex, range from 8.5 to 9.0. Two compounds did not yield this type of curve, but showed optima: leucine ethyl ester ferrihemochrome (*p*H 7.5) and nicotinic acid ferrohemochrome (*p*H 8.5–9.0).

The *pA* values of the association curves as previously described are greatly influenced by *p*H; examples are included in Table I. Figure 7 illustrates the nature of this influence on nicotinamide ferrohemochrome formation.

A general pattern may be described from combinations of the above observations. Ligand concentrations too low for ferrohemochrome formation at *p*H 7 will react at higher *p*H values, while concentrations of ligand unreactive with hematin at *p*H 12 will react to form ferrihemochromes at lower *p*H.

DISCUSSION

Association Behavior.—The esters of amino or other acids had association constants higher (up to 100 times) than those of the corresponding acids, presumably because of their greater electronegativity. For example, glycine ethyl ester readily formed a ferrohemochrome; glycine did not. The *pK* for the dissociation of the amino group of glycine is 9.6 (Greenstein and Winitz, 1961) and therefore at *p*H 8.5, only about 8% of the glycine is in the basic form. Glycine ethyl ester, on the other hand, has a *pK* for the amino group of 7.8 and therefore at *p*H 8.5 the basic form predominates. That glycine butyl ester was less reactive than the ethyl ester was presumably due to steric hindrance.

For the formation of ferrohemochromes of amino acids and amino acid esters a trend was noted for higher affinity constants to be associated with increasing isoelectric points of the ligands. Figure 8 illustrates this point. Many of the unreactive ligands have low isoelectric points; for example, aspartic acid, glutamic acid, and glutathionine. However, arginine and ornithine, with *pI* values of 10.8 and 9.7, respectively, did not form the complexes at *p*H 8.5. Since the *pK* of the guanidine group is 12.5, it follows that at *p*H 8.5 there is little basic form present. Arginine at *p*H 12 readily forms a ferrohemochrome.

Poly-L-lysine has been reported to react with heme to give a ferrihemochrome-type spectrum which is intensified at *p*H 11 (Blauer, 1961). In our experiments, a ferrihemochrome was not formed with concentrations of poly-L-lysine that formed the ferrohemochrome. Poly-L-lysine was ten times as reactive as lysine for ferrohemochrome formation, and poly-L-proline formed a ferrohemochrome while proline did not. It appears therefore that polypeptides are more reactive than their monomeric residues; this was previously suggested by Kaziro and Tsushima (1961). The greater reactivity of polyamino acids compared to monomers may be due to the decreased inductive effect of the carboxylic group with a resulting more electronegative, and hence more reactive, nitrogen. Histidylhistidine is more reactive than histidine (Olcott and Lukton, 1961).

The reactive groups becoming available for ferrohemochrome formation upon denaturation of a protein may be considered on the basis of our data to be, in decreasing binding power: the imidazole ring of histidine, the ε-amino group of lysine, *N*-terminal aromatic amino acids, and the terminal α-amino group of nonaromatic amino acids. It has been shown that in hemoglobin 53% of the total nitrogen-base residues of the globin moiety can bind heme (Kaziro and Tsushima, 1961). Keilin (1960) has estimated that about 62% of all available heme-binding groups in denatured

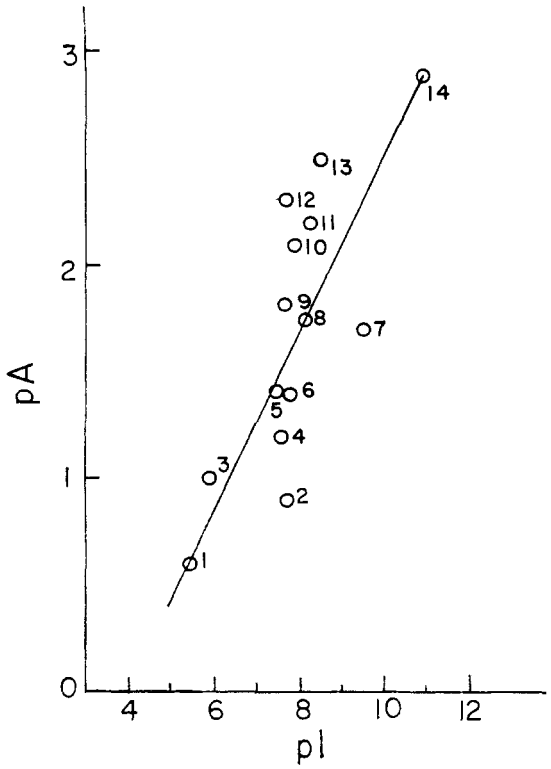

FIG. 8.—Relationship of affinity constants, *pA* to isoelectric points, *pI*, of amino acids and amino acid esters reacting to form ferrohemochromes. 1, phenylalanine; 2, glycine ethyl ester; 3, tryptophan; 4, leucine methyl ester; 5, histidine; 6, histidine ethyl ester; 7, lysine; 8, carnosine; 9, leucine ethyl ester; 10, cysteine ethyl ester; 11, anserine; 12, methyl histidine; 13, tyrosine ethyl ester; 14, polylysine. Literature values for *pI* taken from Cohn and Edsall (1943), Greenstein and Winitz (1961), Greenberg (1951), and Long (1961). *pA* values for carnosine and anserine taken from Olcott and Lukton (1961). For esters, the *pK* of the amino group is used as the indicated *pI* in the plot; for cysteine ethyl ester and tyrosine ethyl ester, *pI* is assumed to be the average of the *pK* of the amino group and the —SH or —OH group, respectively; for polylysine, *pI* is taken as the *pK* of the ε-amino group of lysine.

myoglobin and hemoglobin are involved in ferrohemochrome formation.

Haurowitz (1935) reported no ferrohemochrome formation by histidine, alanine, leucine, glycine, cysteine, or glutathione. Holden and Freeman (1929) found that glycine formed a ferrohemochrome in 5% NaOH. We did not obtain a ferri- or ferrohemochrome with glycine. Histidine, however, was very reactive, in agreement with recent reports (Keilin, 1960; Olcott and Lukton, 1961). Keilin (1960) reported that the following substances did not form ferrohemochromes: urea, guanidine, creatine, creatinine, pyrrole, indole, indolacetate, proline, adenine, guanine, hypoxanthine, xanthine, uric acid, caffeine, theophylline, theobromine, cytosine, thymine, adenosine, and flavine. The conditions used for her experiments were not given.

Absorption Spectra.—The pronounced shift to longer wavelength of the Soret peak of imidazole ferrihemochrome is probably due to more low-spin character in the bond than in those of the other ligands, since ferriporphyrin complexes generally show Soret bands at longer wavelengths the more covalent the bond formed (Brill and Williams, 1961).

It has been established (Shack and Clark, 1947) that the addition of ligands tends to depolymerize heme dimers and higher aggregates; presumably this occurred in our studies at lower concentrations of ligands; upon continued addition, a mass action effect resulted in completion of the ferrohemochrome formation reaction. With still increasing ligand concentrations there occurred aggregation, culminating finally in precipitation.

The anomalous behavior of leucine methyl ester and leucine ethyl ester ferrohemochromes in showing two-banded Soret peaks is likely due to di- or polymerization. Such associations are also indicated by the shift of visible region peaks toward longer wavelengths with high concentrations of glycine ethyl ester, nicotinic acid methyl ester, and pyridine ferrohemochromes. The shift back to shorter wavelengths upon dilution of glycine ethyl ester ferrohemochrome is probably due to depolymerization of the complex since absorption maxima of dimers are shifted to longer wavelengths (Smith, 1959); this supports the conclusion that the shifts we observe at high ligand concentration are due to aggregation of the ferrohemochrome.

Kinetics.—The complexity attendant on the presumed polymerization of heme or hematin solutions makes it unlikely that the ligand association reaction follows a particular order. However at high ligand concentration first-order equilibrium kinetics were noted, presumably because the ligand acted to depolymerize heme. In this connection it should be noted that for the formation of imidazole ferrihemochrome a plot of log per cent complex formed versus time yielded a straight line that did not pass through the origin (Fig. 5). It is likely that the lag period so indicated occurs because the added ligand is depolymerizing the heme. The spectrophotometric techniques used did not allow us to ascertain if there were spectral changes accompanying the presumed depolymerization. The first point measured was at 20 seconds; at this time the spectrum had already changed to that of the ferrihemochrome. Following the depolymerization the imidazole readily coordinates with heme. That this behavior did not occur with any ferrohemochrome is to be expected, since with the ferrohemochromes the zero time point was that of addition of reducing agent to the ligand-heme solution. Presumably depolymerization had already occurred.

*p*H *Conditions.*—Ligand anions compete with hydroxyl ions bound to ferric iron at alkaline *p*H; with increasing acidity and concomitant increasing FeOH dissociation (Shack and Clark, 1947), coordination of ligand with ferric iron is more readily obtained and ferrihemochrome formation results. A condition of low *p*H also tends to prevent hematin aggregation (Anson and Mirsky, 1930; Shack and Clark, 1947).

For ferrohemochrome formation, however, a different situation prevails. Since reduced heme has coordinated water molecules, the competition between hydroxyl ions and ligand for the coordination spheres does not exist. Ligands react better at increasing *p*H where, in the basic form, they can better donate electrons to ferrous iron to form the covalent bond required for the ferrohemochrome.

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Aspartase-Catalyzed Synthesis of *N*-Hydroxyaspartic Acid*

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Certain microorganisms, such as *B. cadaveris*, can grow in the presence of rather high concentrations of hydroxylamine (10^{-3} M). Hydroxylamine disappears from the medium during growth, and also when incubated with resting cell suspensions of these organisms. This phenomenon is due to the presence of the enzyme aspartase. Partially purified aspartase catalyzes the condensation of hydroxylamine with fumaric acid to yield *N*-hydroxyaspartic acid. That this activity is caused by aspartase was shown by the constant ratio of the activities during purification, and by inhibition studies. Hydroxylamine is a competitive inhibitor of aspartase acting upon aspartic acid. Similarly, aspartic acid competitively inhibits the fumarate-hydroxylamine reaction. K_m and K_i values for aspartic acid and hydroxylamine are reported. The product of the condensation reaction, *N*-hydroxyaspartic acid, is very unstable and could not be crystallized, but was identified by its chemical and physical properties. Enzymatically produced *N*-hydroxyaspartic acid was catalytically reduced to give L-aspartic acid, showing that the enzyme retains its stereospecificity in the condensation of fumaric acid with hydroxylamine.

Within the past decade a number of hydroxamic acids have been isolated from microbial fermentations. In contrast to simple hydroxamic acids, which are *N*-acyl derivatives of hydroxylamine, these naturally occurring hydroxamic acids are *N*-acyl derivatives of *N*-hydroxyamino acids. Although free *N*-hydroxyamino acids are unstable at neutral *p*H and have not been demonstrated in living cells, in several instances these compounds have been isolated from the parent hydroxamic acid after acid hydrolysis. Thus, ϵ-*N*-hydroxylysine has been isolated from mycobactin (Snow, 1954) and δ-*N*-hydroxyornithine from the ferrichrome compounds (Emery and Neilands, 1961) as well as the antibiotic albomycin (Turková *et al.*, 1962). α-*N*-Hydroxyamino acids are also known to occur in hydroxamate linkage: *N*-hydroxyisoleucine in aspergillic acid (Dutcher, 1947), *N*-hydroxyleucine in pulcherrimin (Kluyver *et al.*, 1953), *N*-hydroxytyrosine and alanine in mycelianamide (Birch *et al.*, 1956), and *N*-hydroxyglycine in hadacidin (Kaczka *et al.*, 1962). All these compounds, except hadacidin, are either potent microbial growth factors or have antibiotic activity. Hadacidin has been reported to have antitumor activity.

Although nothing is known about the biogenesis of organically bound hydroxylamine, one interesting possibility is the direct addition of hydroxylamine to a double bond. It will be the purpose of this paper to show that the enzyme aspartase can catalyze the addition of hydroxylamine to fumaric acid to form *N*-hydroxyaspartic acid.

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EXPERIMENTAL

Preparation of Aspartase.—*B. cadaveris* (ATCC 9760) was grown in 10 liters of sterile medium containing 1% Bacto yeast extract, 1% Bactopeptone, and 0.5% monopotassium phosphate. After growth for 48 hours at 32°, with gentle aeration, the cells were harvested by centrifugation and frozen overnight at −17°. All subsequent operations were conducted at 0°. The frozen cells (26.4 g) were ground with about 2 g of Alcoa activated alumina for 15 minutes. To the thick paste was added twice the volume of 0.1 M potassium phosphate buffer, *p*H 6.8, and grinding was continued for 5 minutes. The extract was transferred with additional buffer to centrifuge tubes and the aluminum oxide and cell debris were removed by centrifugation. To the clear supernatant solution (43 ml) was added 3.2 ml of 1% protamine sulfate (Nutritional Biochemicals). After removal of the precipitate by centrifugation, the supernatant solution was brought to 60% saturation with solid ammonium sulfate. The precipitate was redissolved in 10 ml of 0.1 M potassium phosphate buffer, *p*H 6.8, and dialyzed overnight against 0.005 M potassium phosphate buffer, *p*H 6.8. Eight ml of this solution, containing 37.6 mg protein, was fractionated by the addition of solid ammonium sulfate (see Table I).

Aspartase Assay.—Aspartase was assayed by a modified method of Racker (1950). The 1-cm quartz cuvet