TABLE VII
HYDROLYSIS OF N-ACETYLDEHYDROALANINE[a,b,c]

pH[d]	$k \times 10^4$ (min^{-1})
7.4	0.1
6.4	0.5
5.6	2.1
5.0	5.8
4.2	31.6

[a] At 78° in 25% ethanol-water. [b] Compound at 2.7×10^{-4} M. [c] Rate measured by decrease of absorbance at 240 mμ. [d] pH determined at 78°. For composition of buffers, see Experimental.

ampoule technique described. Results are given in Table VII.

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Equilibrium Constants for the Synthesis of Hydroxamic Acids*

W. P. JENCKS, M. CAPLOW,† M. GILCHRIST, AND R. G. KALLEN‡

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts§

Received June 19, 1963

Hydroxamic acid formation from hydroxylamine and unactivated carboxylic acids proceeds readily in aqueous solution at elevated temperatures at pH 4–6 or at 25° in dilute acid. The equilibrium constants for the formation of acetohydroxamic, hexanohydroxamic, octanohydroxamic, and *N*-acetyl-L-tyrosine hydroxamic acids have been determined at 25°. At pH 7 the apparent equilibrium constants for the formation of simple hydroxamic acids are near 1, while that for acetyl-L-tyrosine hydroxamic acid is 0.042.

The determination of activated acyl groups by their conversion to hydroxamic acids has been an extremely useful analytical tool because of the specificity of the reaction for different types of acyl groups and the ease with which the ferric complex of hydroxamic acids can be determined spectrophotometrically. The requirements for the formation of a hydroxamic acid from an acyl compound are, first, that the rate of the reaction be appreciable and, second, that the equilibrium of the reaction be favorable under the particular experimental conditions employed. It is sometimes assumed that hydroxylamine will react only with activated "energy-rich" acyl compounds, and that either the rate or the equilibrium constants are unfavorable for reactions of hydroxylamine with "low-energy" acyl compounds, including carboxylate ions.

* This work was supported in part by the National Science Foundation, and by a grant (CA-03975) from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

† This work was carried out during the tenure of a postdoctoral fellowship awarded by the National Institute of Dental Research.

‡ Postdoctoral trainee on a National Institutes of Health training grant (2G-212).

§ Publication No. 243.

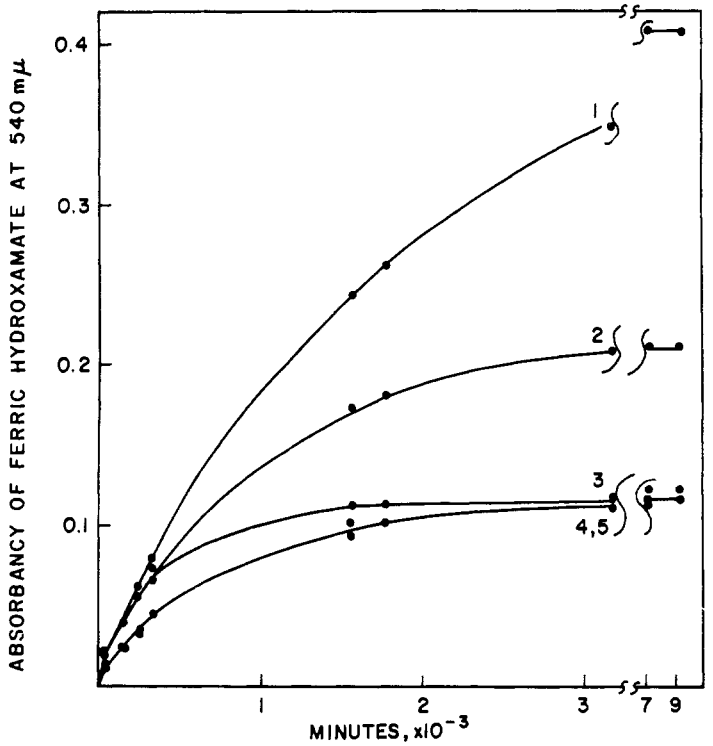

FIG. 1.—Dependence of the rate of acetohydroxamic acid formation on the concentrations of reactants and catalyst at 25°. Curve 1: 0.25 M HCl, 3.46 M CH_3COOH, 1.6 M $NH_2OH \cdot HCl$. Curve 2: Same, 0.5 M HCl. Curve 3: Same, 1.0 M HCl. Curve 4: 0.5 M HCl, 1.73 M CH_3COOH, 1.6 M $NH_2OH \cdot HCl$. Curve 5: 0.5 M HCl, 3.46 M CH_3COOH, 0.8 M $NH_2OH \cdot HCl$.

At neutral or slightly acidic *p*H the free base form of hydroxylamine in aqueous solution reacts readily with energy-rich acyl compounds which have a good leaving group, such as acylimidazoles, acyl phosphates, thiol esters, and phenyl esters (Lipmann and Tuttle, 1950; Noda *et al.*, 1953). At alkaline *p*H hydroxylamine reacts rapidly with esters of aliphatic alcohols in a base-catalyzed reaction (Hestrin, 1949; Chantrenne, 1948; Bergmann and Wurzel, 1953). Esters with a moderately good leaving group or with electron-withdrawing substituents on the acyl portion, such as amino acid esters and the ester group of *N,O*-diacetylserinamide, react at a detectable rate with hydroxylamine near neutral or slightly alkaline *p*H, but do so largely through a base-catalyzed reaction, which can easily be distinguished from the reactions of compounds with a good leaving group, which proceed readily without specific base catalysis even at *p*H 5–6 (Bergmann and Wurzel, 1953; Raacke, 1958; Anderson *et al.*, 1961; Anderson, 1962). Amides react on prolonged incubation at alkaline *p*H (Bergmann, 1952; Goldenberg and Spoerri, 1958), or, generally at elevated temperatures, at slightly acidic *p*H (Lipmann and Tuttle, 1950; Katz *et al.*, 1953; Meister *et al.*, 1955). The reactions of energy-rich compounds at neutral *p*H proceed to a variable extent through the intermediate formation of unstable *O*-acylhydroxylamines, but no such intermediate has been demonstrated for the other reaction conditions (Jencks, 1958).[1]

Relatively little is known about the equilibrium constants for hydroxamic acid formation. Since hydroxamic acids are synthesized from carboxylate ions and concentrated hydroxylamine in the presence of certain hydrolytic enzymes, including pancreatic and liver esterases, acetylcholine esterase, ω-amidase, glutaminase, asparaginase, and an extract from *Proteus vulgaris*, the equilibrium constant must not be strongly in favor of hydrolysis at neutral or slightly acidic *p*H (Lipmann and Tuttle, 1950; Hestrin, 1950; Wilson *et al.*, 1950; Collier and Solvonuk, 1955; Lynn and Perryman, 1960; Meister *et al.*, 1955; Miller and Waelsch, 1953). Bernhard *et al.* (1960) have reported a value for the equilibrium constant for hippuryl hydroxamic acid formation. Meister[2] and Wilson[2] and their coworkers have recently carried out independent measurements of the equilibrium constants for the formation of γ-glutamyl and acetyltyrosine hydroxamic acids and have obtained results in good agreement with those reported in this communication.

The equilibrium constant determinations for hydroxamic acid formation which are reported here were carried out as a part of a program to obtain further

[1] Although *O*-acylhydroxylamines are formed from activated acyl compounds, the formation of *O*-acylhydroxylamines from carboxylic acids has not been demonstrated and would be expected to be energetically unfavorable. In any case, *O*-acylhydroxylamines cannot accumulate at neutral *p*H in the presence of concentrated hydroxylamine, because they are quantitatively converted to hydroxamic acids under these conditions; there is no rapid interconversion of *O*-acylhydroxylamines and hydroxamic acids under the conditions of the ferric chloride assay, as shown by the fact that *O*-acylhydroxylamines do not give a color with ferric chloride, even in the presence of hydroxylammonium ion (Jencks, 1958).

[2] Ehrenfeld *et al.* (1963) have found that the apparent equilibrium constant for the formation of γ-glutamyl hydroxamic acid at *p*H 7.2 and 37° is 0.24–0.43. This gives values, for the conventions used in this paper, of approximately 350 for K_I, 5.6 for K_{II}, 5.6×10^6 for K_{III}, and 0.51 for K'_{pH7}. These values are in good agreement with those reported here and show the expected effect of the acidity of the γ-carboxyl group of glutamate (pK_a 4.25). Epand and Wilson (1963) have obtained a value of 0.09 for the equilibrium constant for acetyltyrosine hydroxamic acid formation at *p*H 6.6. This is in good agreement with the data reported here.

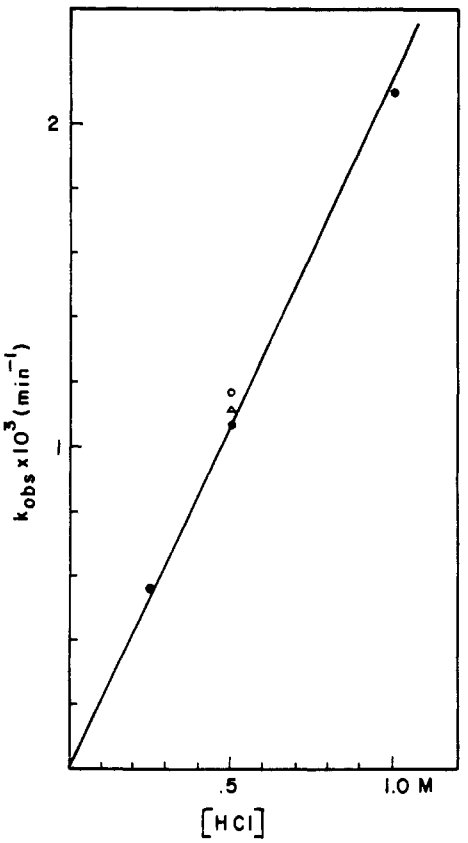

FIG. 2.—Pseudo first-order rate constants for the approach to equilibrium of acetohydroxamic acid formation at 25° as a function of acid concentration. Solid circles, 3.46 M CH_3COOH, 1.6 M $NH_2OH \cdot HCl$; open circle, 1.73 M CH_3COOH, 1.6 M $NH_2OH \cdot HCl$; triangle, 3.46 M CH_3COOH, 0.8 M $NH_2OH \cdot HCl$.

evidence regarding the energy-rich nature, as measured by the free energy of hydrolysis, of different classes of acyl compounds (see Jencks *et al.*, 1960). In addition, it was of interest to obtain further information regarding the experimental conditions under which hydroxamic acids are formed nonenzymatically from carboxylic acids.

EXPERIMENTAL

Hog liver esterase was purified according to Lipmann and Tuttle (1950) through step L-4. The preparation was found to have a specific activity of 5.2 μmoles $\cdot$ min$^{-1} \cdot$ mg^{-1} for the hydrolysis of 0.0036 M ethyl butyrate at 37°, *p*H 7.3. α-Chymotrypsin was obtained from the Worthington Biochemical Corporation and was used without further purification. Hydroxylamine hydrochloride was recrystallized from ethanol-water. Hexanoic and octanoic acids were distilled and the concentrations of aqueous solutions were determined by titration. Hexanohydroxamic acid (mp 64°) and octanohydroxamic acid (mp 78–79°) were synthesized essentially by the procedure of Safir and Williams (1952) and sodium *N*-acetyl-L-tyrosine hydroxamic acid, mp 191–191.5 (decomp), was synthesized according to Hogness and Niemann (1953). Acetohydroxamic acid (mp 89–92.5°) was prepared from methyl acetate and alkaline hydroxylamine in water-methanol at 0°. The alkaline solution was brought to *p*H 4 with HCl and evaporated to dryness under reduced pressure. The product was taken up and recrystallized in absolute ethanol.

Hydroxamic acid concentration was followed by addition of aliquots of the reaction mixtures to ferric chloride in hydrochloric acid solution and spectrophotometric determination of the ferric hydroxamate at 540 mμ (Lipmann and Tuttle, 1945). Hexano- and octanohydroxamic acids were determined by adding the aliquot plus sufficient water to give 2.0 ml to 4.0 ml of a solution containing 10% $FeCl_3 \cdot 6\ H_2O$, 0.66 M hydrochloric acid, and 3.3% trichloroacetic acid. The samples were centrifuged and read within 2 hours. A small correction (5–15 Klett units) was made for color from the enzyme alone. Acetohydroxamic acid was determined by the addition of a 0.5-ml aliquot and 0.5 ml of water to 4.0 ml of 10% $FeCl_3 \cdot 6\ H_2O$ in 0.3 M hydrochloric acid followed by the addition of 1.0 ml of a solution prepared by mixing equal volumes of 4 M hydroxylamine hydrochloride and 3.5 M sodium hydroxide. *N*-Acetyltyrosine hydroxamic acid formation was measured by the addition of 0.2-ml aliquots of the reaction mixture to 4.0 (or 8.0) ml of 20% $FeCl_3 \cdot 6\ H_2O$ in 1.4 M HCl, followed by the addition of 0.1 (or 0.2) ml of 6.1 M trichloroacetic acid, centrifugation, and filtration through glass wool to remove precipitated protein.

The concentrations of carboxylic acids and hydroxylamine were obtained from the amounts added to the reaction mixture after correction for any concentration changes due to hydroxamic acid formation or hydrolysis. The fraction of hydroxylamine present as the free base was determined by titration or from the measured *p*H and the ionization constant, determined at 25° at the same ionic strength as the reaction mixture. Determinations of *p*H were made with a Radiometer PHM 4b *p*H meter with a G200B glass electrode. The concentration of water in a given reaction mixture was corrected for the volume taken up by other reactants, using the convention that the activity of pure water is 1.0; this correction was small in most experiments. Ethylenediaminetetraacetic acid, 2×10^{-4} M, was added in most experiments at neutral *p*H to retard heavy metal-catalyzed decomposition of hydroxylamine.

The pK_a values of the carboxylic acids and hydroxylamine hydrochloride were determined at 25° by titration, using linear plots of the equation $pH = pK' + \log [\text{base}]/[\text{acid}]$. Solutions of 0.010 M octanoic acid were made up in excess base and titrated with acid. Precipitation of octanoic acid occurred after approximately 30% titration so that the pK_a' was determined by extrapolation of the data obtained in the initial third of the titration. The pK_a' values of hexanoic and octanoic acids were found to be 4.56 and 4.60, respectively, at ionic strength 1.0, maintained with sodium chloride. The pK_a' values of *N*-acetyltyrosine and hydroxylammonium chloride in 2.0 M sodium chloride were found to be 3.10 and 5.97, respectively, by titration and the pK_a' of *N*-acetyltyrosine hydroxyamic acid in 2.0 M potassium chloride was found to be 8.67, from measurements of the *p*H values of partially neutralized solutions. The pK_a values of other hydroxamic acids are well above the range of *p*H used in these experiments (Wise and Brandt, 1955). Values of the pK_a' of hydroxylamine hydrochloride at lower salt concentrations were taken from Kurtz and Niemann (1962).

RESULTS

Acetohydroxamic Acid.—It was shown in preliminary experiments that hydroxamic acids are formed rapidly upon heating aqueous solutions containing hydroxylamine and acetic or formic acids. Meister *et al.* (1955) have previously demonstrated the nonenzymatic

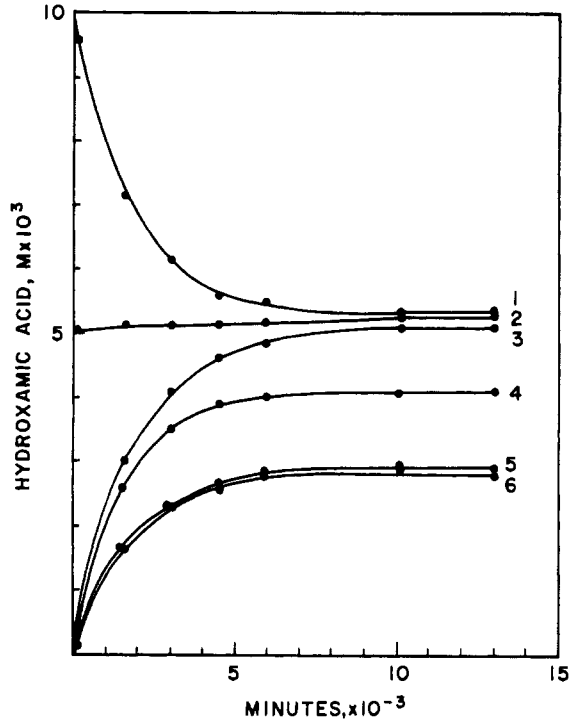

FIG. 3.—Approach to equilibrium for acetohydroxamic acid formation in 0.25 M HCl at 25°. Curves 1–3: 3.46 M CH_3COOH, 1.60 M $NH_2OH \cdot HCl$, $CH_3CONHOH$ as indicated. Curve 4: Same, + 1.0 M KCl. Curve 5: Same as 1–3 except 0.80 M $NH_2OH \cdot HCl$. Curve 6: Same, except 1.73 M CH_3COOH.

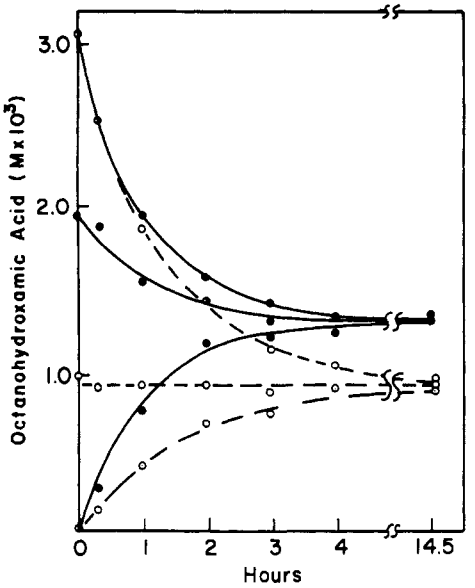

FIG. 4.—Approach to equilibrium of octanoic acid, hydroxylamine, and octanohydroxamic acid at 25.0°, catalyzed by liver esterase, 2.0 mg/ml. Total carboxylate + hydroxamic acid = 3×10^{-3} M; 2×10^{-4} M EDTA. Solid circles, *p*H 7.24 ± 0.02, 0.97 M hydroxylamine; open circles, *p*H 7.22 ± 0.02, 0.49 M hydroxylamine.

formation of hydroxamic acids from dicarboxylic acids in aqueous solution at elevated temperature. The rate of hydroxamic acid formation from acetic acid was found to show a maximum at *p*H 5.0, which suggests that free acetic acid and hydroxylamine are the reactive species. The rate with formic acid was found to be five to ten times faster than that with acetic acid. It was shown that the equilibrium constant for acetohydroxamic acid formation was larger than 15 (based on the concentrations of uncharged reactants and products), but it was not possible to obtain an accurate value for the equilibrium constant because it was not possible to approach equilibrium from both directions. Experiments were therefore carried out at 25° with the help of enzymatic or acid catalysis to approach equilibrium.

Aqueous acetic acid and hydroxylammonium chloride react spontaneously in the presence of a catalytic amount of hydrochloric acid to form acetohydroxyamic acid (Fig. 1). The reactions follow pseudo first-order kinetics for the approach to equilibrium. Both the rate and the extent of hydroxamic acid formation are increased by increasing the acetic acid or hydroxylamine hydrochloride concentrations. Increasing the concentration of hydrochloric acid causes a decrease in the equilibrium concentration of acetohydroxamic acid, but also decreases the time required to reach equilibrium. The pseudo first-order rate constants increase linearly with acid concentration (Fig. 2), although this increase does not reflect an actual increase in the rate (in moles per minute) of hydroxamic acid formation (Fig. 1). Similarly, the pseudo first-order rate constants do not depend on the acetic acid or hydroxylamine hydrochloride concentrations (Fig. 2) although the actual rates of hydroxamic acid formation increase with increasing concentration of the reactants (Fig. 1). This behavior results from the fact that the reactions are going to equilibrium, rather than to completion, so that the observed rates reflect the reaction rate constants in both directions, k_1 and k_{-1}, rather than the forward rate constant k_1, alone. For a first-order reaction, the observed rate constant, k_{obs}, is equal to $k_1 + k_{-1}$. The situation is more complicated here because the reaction in the forward direction is second order, but since at equilibrium the

TABLE I

EQUILIBRIUM CONSTANTS FOR ACETOHYDROXAMIC ACID FORMATION AT 25° IN 0.25 M HCl

	Equilibrium Concentrations					
Run[a]	$CH_3\overset{O}{\overset{\|}{C}}OOH$ M	$NH_2OH \cdot HCl$ M	$CH_3\overset{O}{\overset{\|}{C}}NHOH$ M × 10³	Fraction H_2O	K_A[b] × 10⁴	K_I[c]
1	3.46	1.61	5.40	0.76	1.84	425
2	3.46	1.61	5.30	0.76	1.81	426
3	3.46	1.60	5.11	0.76	1.75	410
4[d]	3.46	1.60	4.13	0.76	1.42	460
5	3.46	0.80	2.90	0.79	2.07	360
6	1.73	1.60	2.80	0.86	2.18	390
			Average (excluding 4)		1.93	402

[a] See Fig. 3. [b] $K_A = [CH_3CONHOH][H^+]$ fr. $H_2O/[NH_3OH^+][CH_3COOH]$. [c] $K_I = [CH_3CONHOH]$ fr. $H_2O/[NH_2OH][CH_3COOH].[NH_2OH]$ estimated from measured *p*H and pK_a' $NH_3OH^+ = 6.00$. [d] 1.0 M KCl added.

TABLE II
EQUILIBRIUM CONSTANTS FOR HEXANOIC AND OCTANOIC HYDROXAMIC ACID FORMATION AT 25° AND IONIC STRENGTH 1.0

Acid	pH	Equilibrium Concentrations			Time to Obtain Equilibrium (hours)	K'[b]	K_I[c]
		NH_2OH[a] (M)	RCOOH[a] (M × 10^3)	RCONHOH (M × 10^3)			
C_8	7.24	0.97	1.70	1.30	14.5	0.76	352
C_8	7.21	0.49	2.10	0.90	14.5	0.85	377
C_8	7.04	1.00	1.30	1.70	10.0	1.26	380
C_8	7.03	0.50	1.82	1.18	10.0	1.28	378
C_6	7.56	0.98	7.00	3.00	10.0	0.42	433
C_6	7.50	0.49	8.16	1.84	10.0	0.45	404
C_6	7.19	1.00	3.06	2.94	21.5	0.93	420
C_6	7.18	0.50	4.02	1.98	21.5	0.97	430

[a] Refers to total concentration of all ionic forms present in solution. [b] Experimental value, at the indicated pH. [c] For uncharged reactants and products.

reaction proceeds only a very short distance toward completion, the observed rates will be dominated by the first-order rate constant for the reverse reaction k_{-1}. The approach to equilibrium from two directions is shown in Figure 3. The equilibrium concentration of hydroxamic acid is decreased by the addition of 1.0 M potassium chloride. The results are summarized in Table I. The equilibrium constants are given in terms of K_A, based on the concentrations of the acidic species of the reactants, and in terms of K_I, based on the concentrations of the uncharged species of the reactants. These equilibrium constants do not provide an exact measure of the expected tendency for aceto-hydroxamic acid formation in dilute aqueous solution, because of the appreciable deviations from ideality in these solutions, which are demonstrated by the effects of variations in potassium chloride, hydroxylamine hydrochloride, and acetic acid concentrations on the equilibrium constants. The data suggest, however, that these activity coefficient effects are not so large as to invalidate the results for most purposes, and this conclusion is supported by the similarity of the equilibrium constants (K_I) for aceto-, hexano-, and octanohydroxamic acid formation (Tables I and II).

Hexano- and Octanohydroxamic Acids.—The approach to equilibrium for octanohydroxamic acid at pH 7.03 and 7.22, catalyzed by liver esterase, is shown in Figure 4. The same equilibrium position is approached from both directions and is maintained in a reaction mixture in which the initial concentrations of reactants were near the expected equilibrium values. These results and the results of similar experiments with hexanohydroxamic acid are summarized in Table II. These equilibrium constants are given in terms of K', which is the apparent equilibrium constant at a given pH value and refers to the total of all ionic species of each reactant, and in terms of K_I, which refers to only the uncharged species of the reactants and products and is independent of pH. As shown in Table II, the values of K' do depend on the pH as expected, while those of K_I are independent of pH, within experimental error, in the range of pH examined.

N-Acetyl-L-Tyrosine Hydroxamic Acid.—The approach to equilibrium of the chymotrypsin-catalyzed reaction of *N*-acetyl-L-tyrosine and hydroxylamine to form the corresponding hydroxamic acid is shown in Figure 5. The same equilibrium position is approached from both directions at each of two pH

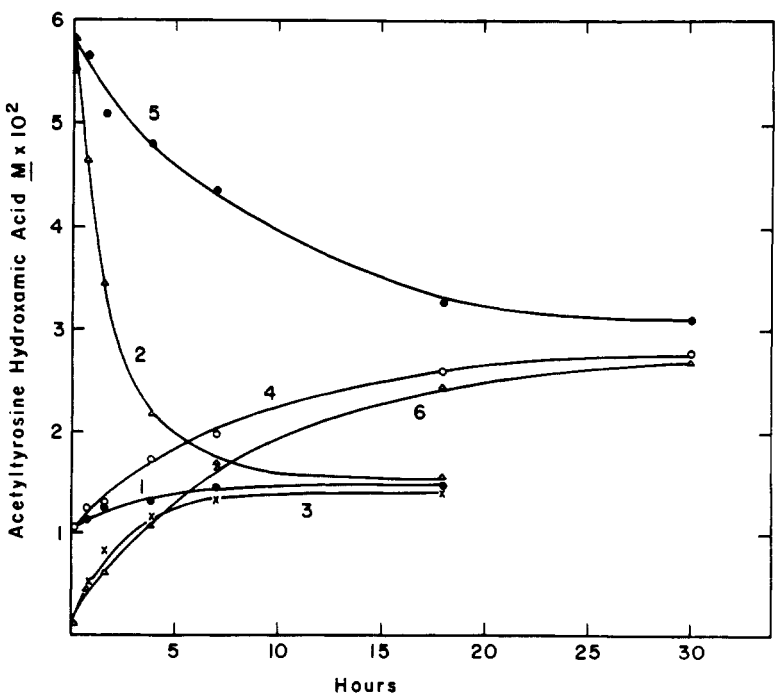

FIG. 5.—Approach to equilibrium of acetyl-L-tyrosine, hydroxylamine, and acetyl-L-tyrosine hydroxamic acid at 25°, catalyzed by chymotrypsin. The numbers refer to Table III, in which the experimental conditions are given.

TABLE III

EQUILIBRIUM CONSTANT FOR THE SYNTHESIS OF *N*-ACETYLTYROSINE HYDROXAMIC ACID AT 25° AND IONIC STRENGTH 2.0[a]

Reaction No.	Equilibrium Concentrations NH_2OH[b] (M)	Equilibrium Concentrations RCOOH[b] (M)	Equilibrium Concentrations RCONHOH (M)	Final *p*H	K'[c]	K_I[d]
1	1.11	0.146	0.0147	6.67	0.087	388
2	1.10	0.141	0.0155	6.59	0.096	366
3	1.10	0.159	0.0140	6.67	0.077	342
4	0.538	0.285	0.0275	6.22	0.177	363
5	0.529	0.281	0.0310	6.14	0.204	373
6	0.533	0.289	0.0268	6.21	0.171	348
					Average:	363

[a] Reaction catalyzed by 2.4×10^{-4} M chymotrypsin. Ionic strength maintained with NaCl. [b] Sum of all ionic species in solution at equilibrium. [c] Experimental value, at the indicated *p*H. [d] For uncharged reactants and products.

TABLE IV

EQUILIBRIUM CONSTANTS FOR HYDROXAMIC ACID FORMATION AT 25° EXPRESSED ACCORDING TO DIFFERENT CONVENTIONS

	Water Activity Taken as	Aceto-hydroxamic	Hexano-hydroxamic	Octano-hydroxamic	*N*-Acetyl-tyrosine Hydroxamic
$K_I = \frac{[RCONHOH][H_2O]}{[RCOOH][NH_2OH]}$	1.0	402	422	372	363
same	55.5	22,300	23,400	20,600	20,200
$K_{II} = \frac{[RCONHOH][H_2O]}{[RCOO^-][NH_3OH^+]}$	1.0	16	15.4	14.7	0.49
$K_{III} = \frac{[RCONHOH][H_2O]}{[RCOO^-][NH_2OH][H^+]}$	1.0	1.60×10^7	1.54×10^7	1.47×10^7	4.6×10^5
$K'_{pH7} = \frac{[RCONHOH][H_2O]}{[RCOO^-][NH_2OH]_{tot}}$	1.0	1.46	1.40	1.34	0.042

values, with minor variations due largely to differences in the *p*H values of the different reaction mixtures. The results are summarized in Table III according to the conventions described.

DISCUSSION

The equilibrium constants for the formation of the hydroxamic acids examined in this study are summarized in Table IV. They are expressed according to several different conventions, each of which is useful for certain purposes. All the values are based on concentrations, rather than activities; i.e., they are not thermodynamic equilibrium constants.

The equilibrium constants for hydroxamic acid synthesis reported here are several orders of magnitude smaller than those reported by Bernhard *et al.* (1960) for the synthesis of hippuryl hydroxamic acid. However, it is not certain from the data presented by these workers that equilibrium was reached in their experiments, and the possibility might be considered that their data reflect the relative rates of chymotrypsin-catalyzed hydrolysis and hydroxylaminolysis of methyl hippurate rather than a true equilibrium.

The equilibrium constant expressed according to convention (I), which utilizes the concentrations of nonionized reactants and products, is useful for theoretical comparison of the affinities of different groups for each other. Carpenter (1960), in particular, has

$$RCOOH + H_2NR' \rightleftharpoons RCONHR' + H_2O$$

$$K_I = \frac{[RCONHR'][H_2O]}{[RCOOH][H_2NR']} \quad (I)$$

advocated the use of a convention of this kind as a standard. In the hydroxamic acid series, it is of interest that the equilibrium constants for hydroxamic acid formation from simple fatty acids and from acetyltyrosine are the same according to this convention, although the amounts of hydroxamic acid in equilibrium with a given amount of hydroxylamine and carboxylic acid are quite different for the different acids under most experimental conditions. *N*-Acetyltyrosine is some 30-fold stronger an acid than the fatty acids, reflecting the electron-withdrawing effect of the acylamino and phenyl groups. The fact that the equilibrium constants for hydroxamic acid formation from these different types of acids are very similar, i.e., that the equilibrium constant is not changed by electron-withdrawing groups, means that the groups —COOH and —CONHOH must have a similar sensitivity to the effects of polar substituents. For example, if the charge distribution in —COOH and —CONHOH were such that carboxylic acids are more destabilized by electron-withdrawing substituents than are hydroxamic acids, one would expect the equilibrium constant for acetyltyrosine hydroxamic acid formation to be larger than that for acetohydroxamic acid formation.

Since the carbonyl group is considerably more electronegative than hydrogen and the resonance

$$-O \quad \quad -O$$
$$| \quad \quad |$$
$$RC{=}\overset{+}{N}R_2' \quad \leftrightarrow \quad RC{=}N R_2'$$

structure $RC{=}\overset{+}{N}R_2'$ is important in amides, it would be expected that reaction (I) would be favored by electron-donating substituents on the amine group. The equilibrium constants for the synthesis of glutamine, propionamide, *N*-methylpropionamide, *N*-dimethylpropionamide, and the peptide bonds of benzoyltyrosylglycylamide and benzoyltyrosylglycylanilide are 272, 1,310, 1.5×10^6, 3.0×10^4, 6,600, and 6,000, respectively, calculated from the data of Benzinger *et al.* (1959), Morawetz and Otaki (1963),

Dobry *et al.* (1952), and Gawron *et al.* (1961).[3] On the other hand, preliminary data obtained by Higuchi and Miki (1961) suggest that the equilibrium constant for anilide formation from polycarboxylic acids is near 1.0.[4] While the relative magnitudes of several of these values cannot be easily accounted for, there is a tendency for the equilibrium constants to increase as the substituents on the amide nitrogen atom become more electron-donating. It is, therefore, of interest that hydroxamic acids, derived from hydroxylamine, fall in the same class as glutamine and propionamide, which are derived from ammonia, in spite of the strong electron-withdrawing properties of the hydroxyl group. This may be another reflection of the fact that hydroxylamine has a greater tendency than most other amines to add to the carbonyl group (Jencks, 1959; Jencks and Carriuolo, 1960).

An estimate of the relative affinity of the carbonyl group for amines and for oxygen-containing compounds may be made if the equilibrium constants are expressed in terms of the actual molar concentration of water, 55.5, rather than according to the convention that water activity is 1.0. This raises the equilibrium constants for hydroxamic acid formation to approximately 20,000 and those for the amides of several more basic amines to even higher values. This means that, on a molar basis, the carbonyl group prefers to form a bond with hydroxylamine over a bond with water by a factor of approximately 20,000, when it is presented with both molecules in aqueous solution. The corresponding value for carboxylic esters is near one, indicating that the carbonyl group has an approximately equal affinity for water and for an alcohol, as would be expected from the chemical similarity of water and alcohols. This difference presumably reflects the low electronegativity of nitrogen and the large ability of the nitrogen atom to donate electrons by resonance.

In spite of its usefulness for some purposes, we do not feel that convention I should be used as a general standard for the following reasons: (a) For many reactions, convention I describes a situation that can never be realized experimentally, because there is no *p*H value at which all the reactants and products of a reaction are in the nonionized form. For example, a nonionized carboxylic acid cannot exist in the presence of most nonionized amines; one or both will be ionized at any attainable *p*H value. (b) In order to determine the value of K_I for a given reaction from experimental measurements, it is necessary to know the dissociation constants of all groups in the reactants and products which are appreciably ionized under the experimental conditions chosen for study, in order that the equilibrium concentrations of the nonionized species may be used to calculate K_I. These dissociation constants are often unknown or very difficult to determine, particularly in the case of compounds which exist as zwitterions. (c) Convention I becomes unwieldy when it is necessary to deal with molecules containing several ionizable groups, not all of which are directly involved in the reaction under consideration. Either all groups on a molecule must be converted to the nonionized form, which is very difficult for molecules containing both acidic and basic groups, such as ATP, coenzyme A, or amino acids; or only the reacting groups are converted to the nonionized form, in which case the molecules are not completely uncharged.

Convention II is a particularly convenient *p*H-independent convention for expressing the equilibrium constant for amide synthesis, because the ionic species

$$RCOO^- + H_3\overset{+}{N}R' \rightleftharpoons RCONHR' + H_2O$$

$$K_{II} = \frac{[RCONHR'][H_2O]}{[RCOO^-][H_3N^+R']} \quad (II)$$

given are those which actually exist in solution over a considerable range of *p*H in the *p*H region often used in experimental work. For hydroxamic acid synthesis it does not correspond directly to an attainable experimental situation, because hydroxylamine is not completely protonated at any *p*H value at which the carboxyl group is fully ionized, but it is still useful for comparative purposes. According to this convention, electron-withdrawing substituents on the acyl group will destabilize the products more than the starting carboxylate ion and will therefore drive the equilibrium position to the left. (We regard this as a more correct way of expressing the situation than to ascribe the same facts to the greater acidity of acids containing electron-withdrawing substituents. The latter procedure implies a particular reaction path, which is not desirable in a thermodynamic comparison.) The expected effect is seen in a comparison of the equilibrium constants, K_{II} (Table IV), for hydroxamic acid synthesis from simple fatty acids (approximately 15) with that for acetyltyrosine hydroxamic acid synthesis (0.49). The electron-withdrawing effect of the side chain in acetyltyrosine is reflected in the 30-fold greater ionization constant of this acid compared to acetic acid.

Electron-withdrawing substituents on the amine will tend to drive equilibrium II to the right, because such substituents will destabilize the positively charged ammonium ion more than the amide. The expected effect is seen in the equilibrium constants for simple amides (0.028–1.4) and glutamine (3.1×10^{-3}), as well as the peptide bonds of benzoyltyrosylglycinamide (0.5) and benzoyltyrosylglycylanilide (0.1), which are smaller than those for the synthesis of simple hydroxamic acids. This convention most clearly expresses the previously mentioned experimental fact that the synthesis of hydroxamic acids is considerably easier at neutral or slightly acid *p*H than is the synthesis of most amides.

Convention III is given because it is the easiest for calculating values of K'_{app} or $\Delta F^{0\prime}$ for hydroxamic acid formation at different *p*H values at neutral or slightly basic *p*H. In the absence of appreciable ionization of hydroxylamine or hydroxamic acid and under

$$RCOO^- + H_2NR' + H^+ \rightleftharpoons RCONHR' + H_2O$$

$$K_{III} = \frac{[RCONHR'][H_2O]}{[RCOO^-][H_2NR'][H^+]} \quad (III)$$

conditions in which the carboxylic acid is completely

[3] In calculating the equilibrium constant for glutamine formation according to this convention, only those groups which are directly involved in the reaction, i.e., ammonia and the γ-carboxyl group of glutamate, were corrected to the uncharged form. The calculations for propionic acid derivatives are based on a pK_a' value of 4.6 for propionic acid. The calculations for *N*-methylpropionamide are based on a corrected value of 4.4 kcal/mole for ΔF^0_{298} (H. Morawetz, personal communication; see Morawetz & Otaki, 1963).

[4] The formation of these anilides was reported to take place readily only with polycarboxylic acids, suggesting that the reaction is facilitated by a carboxyl group other than that which is making a new bond with the amine. The fact that hydroxamic acid formation takes place readily with hydroxylamine and monocarboxylic acids indicates that such facilitation is not necessary for amide formation. A similar conclusion may be reached from the experiments of Morawetz and Otaki (1963) on formation of simple amides from monocarboxylic acids, although these reactions are considerably slower.

ionized it is only necessary to substitute the hydrogen-ion concentration to obtain these constants.

Convention IV, the equilibrium constant based on the total stoichiometric concentrations of all ionic

$$\text{acid} + \text{amine} \rightleftharpoons \text{amide} + H_2O$$

$$K'_{pH_7} = K_{IV} = \frac{[\text{amide}]_{tot}[H_2O]}{[\text{acid}]_{tot}[\text{amine}]_{tot}} \quad \text{(IV)}$$

species of the reactants and products at a given *p*H value, is the simplest convention to deal with experimentally, because it does not require knowledge of the dissociation constants of the reactants and products. It is this constant, K', that is generally obtained from experimental measurements. This apparent equilibrium constant will vary as the *p*H is changed, because it does not take into account the ionization of the reactants and the uptake or release of hydrogen ions. Nevertheless, this convention is very useful for comparisons at a particular *p*H value, because it gives directly the ratio of the concentrations of reactants and products at that *p*H value. Comparisons at *p*H 7.0 are particularly useful, and the values of the free energy of hydrolysis at *p*H 7.0, $\Delta F^{0\prime}{}_{pH\,7}$, are often used to compare the energy-rich nature of acyl and phosphoryl compounds. The value of $K'_{pH\,7}$ near one for fatty acid hydroxamic acids expresses the surprising fact that in the presence of 1 M hydroxylamine at neutrality a carboxylic acid exists approximately one-half as the hydroxamic acid and one-half as the carboxylate ion at equilibrium; i.e., the free energy of hydrolysis ($\Delta F^{0\prime}$) of hydroxamic acids at *p*H 7 is near zero (actually +200 calories/mole). For acetyltyrosine hydroxamic acid, K'_{app} at *p*H 7 is 0.042 and $\Delta F^{0\prime}$ (hydrolysis) is −1870 calories/mole, reflecting the effect of the electron-withdrawing groups on the acyl moiety of this compound.

Although there is only a small thermodynamic barrier to the formation of hydroxamic acids from even the least activated of all acyl groups, the carboxylate ion, there remains a kinetic barrier. It is by making use of this kinetic barrier by careful regulation of the reaction conditions, as indicated in the introduction, that the formation of hydroxamic acids from different classes of activated acyl groups may be used as a specific analytical method for the analysis of such groups.

Acknowledgments

We are grateful to Miss Gloria Deutsch, Mr. Charles Allen, and Mr. Ray Adman for carrying out preliminary experiments. We wish to thank the National Science Foundation and the National Cancer Institute of the National Institutes of Health for financial support.

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