(to remove unchanged ketone which otherwise led to a gummy product). Final removal of iodine was effected by treatment with thiosulfate solution. The resulting product which was now nearly colorless was boiled with water and filtered hot. The residue was treated with concentrated ammonia to liberate the base and then filtered. The product was crystallized from 50% alcohol.

In some cases, the residue after boiling with water was gummy. In such cases, longer contacts (for 7 to 8 hours) with concentrated ammonia or even refluxing with concentrated ammonia on a water-bath was necessary. The gummy products hardened up after such treatment with only two or three exceptions. Thiazole bases which still resisted crystallization were obtained crystalline by treating their picrates with lithium hydroxide and extracting with ether.

Thiazoles derived from carboxyphenylthioureas dissolved in ammonium hydroxide. The bases, in such cases, were liberated by treatment with acetic acid.

Mercuration of Thiazoles.—The thiazole (1 mole) in alcohol–dilute acetic acid solution was treated with an aqueous solution of mercuric acetate (1.3 moles) acidified with acetic acid. There was precipitation after some time. The reaction mixture was kept overnight. The precipitate was filtered and purified by repeated washing with hot water, alcohol and very dilute acetic acid.

The properties and analytical data of the resulting thiazoles are given in Table I and those of mercurated thiazoles in Table II.

The Rideal–Walker Drop Dilution method was used for the comparative antibacterial action. The bactericidal activities change from group to group as follows: chloro > naphthyl > phenyl > tolyl > nitro > carboxy.

TABLE II

2-(ACETOXYMERCURI-ARYLAMINO)-4-METHYL-5-CARBETHOXYTHIAZOLE

H_3CC——N ‖ ‖ H_5C_2OOCC—S—$CNHRHgOCOCH_3$

Compound no.	Nature of substituent, R	M.p., °C.	Yield, %	Hg, % Calcd.	Found
1	Phenyl	241–243	87	38.46	38.29
2	*o*-Tolyl	Above 260	80	37.31	37.12
3	*p*-Tolyl	Dec. 165	83	37.31	37.09
4	*o*-Chlorophenyl	112	78	36.03	35.92
5	*m*-Chlorophenyl	235	70	36.03	35.90
6	*p*-Chlorophenyl	188	80	36.03	35.94
7	*o*-Carboxyphenyl	Above 260	72	39.68	39.15
8	*p*-Carboxyphenyl	Above 260	75	39.68	39.03
9	*m*-Nitrophenyl	178	72	35.39	35.32
10	*p*-Nitrophenyl	165	70	35.39	35.20
11	α-Naphthyl	154–156	72	35.09	34.92
12	β-Naphthyl	172	70	35.09	34.82

The maximum activities noted were 1:4000 in case of unmercurated thiazoles and 1:140,000 in case of mercurated thiazoles. Details of these investigations will be published elsewhere.

ORISSA, INDIA

[CONTRIBUTION FROM THE CLINICAL UNIT, LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE]

Application of Ion Exchange Chromatography to the Enzymatic Resolution of Amino Acids[1]

BY CARL G. BAKER AND HERBERT A. SOBER

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Conditions are described for the isolation of the optical isomers of nine representative racemic amino acids by the application of a cation exchange column separation to the products of an asymmetric enzymatic hydrolysis procedure. This separation procedure is conveniently applicable to the resolution of relatively small amounts of initial racemic substrates equivalent to 0.3 to 1.0 g. of the amino acid enantiomorph.

The enzymatic procedure for the resolution of amino acids developed in this Laboratory[2a,b] depends upon the asymmetric enzymatic hydrolysis of the N-acyl or amide derivative of the racemic amino acid. The liberated free L-amino acid is separated from the unhydrolyzed D-derivative by the addition of ethanol; the latter derivative is subsequently converted into the D-amino acid by acid hydrolysis. However, since certain amino acids, *e.g.*, isovaline, are soluble in alcohol, and hence cannot be separated in this manner, another isolation procedure was devised. This alternative procedure, employing ion-exchange chromatography for the separation of the products of the asymmetric enzymatic hydrolysis, was designed to permit the use of the small amounts of material usually involved in the synthesis of isotopically labeled amino acids. The method also has been used for the large scale preparation of the enantiomorphs of isovaline.[3]

(1) Presented in part before the Division of Biological Chemistry at the 120th Meeting of the American Chemical Society at Atlantic City, N. J., September, 1952.

(2) (a) S. M. Birnbaum, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952); (b) J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *ibid.*, in press.

(3) C. G. Baker, S-C. J. Fu, S. M. Birnbaum, H. A. Sober and J. P. Greenstein, THIS JOURNAL, **74**, 4701 (1952).

If cation-exchange chromatography is applied to the mixture obtained after enzymatic resolution of the N-acyl derivative, the free L-amino acid is retained by the column and the unhydrolyzed D-amino acid derivative is eluted with water. After action of the enzyme upon the amide derivative, however, a weaker cation-exchange resin is used, which permits the free amino acid to pass through the column, while the unhydrolyzed derivative is retained and subsequently removed from the column with weak acid. Cation exchange therefore affords sufficiently mild conditions to avoid hydrolysis of the N-acyl or amide derivative.

Experimental

Preparation of the Ion-exchange Resin Columns.—Dowex-50[4] and Amberlite XE-64[5] in the acid phase were used. The resin as received from the manufacturer was subjected to two cycles of washing with 5 *N* HCl, water, 1 *N* NaOH, water, and followed by a final 5 *N* HCl and water wash. Washing with water in each case was continued to completion as indicated by congo red and phenolphthalein. Resin columns (see Table I for dimensions) were prepared

(4) A strong cation-exchange resin with sulfonic acid functional groups (200–400 mesh) obtained from the Dow Chemical Company, Midland, Mich.

(5) A weak cation-exchange resin with carboxylic acid exchange groups obtained from the Resinous Products Division, Rohm and Haas, Philadelphia, Penna.

TABLE I

EXPERIMENTAL CONDITIONS FOR OBTAINING THE OPTICAL ISOMERS OF AMINO ACIDS (SEE TEXT)

DL-Amino acid derivative	Wt. of derivative, g.	Wt. of added enzyme, mg.	Time of incubation, hr.	Resin used	Column size height × diam., cm.	Hydrochloric acid eluting agent, N	Agent for converting the hydrochloride into the free amino acid
N-Chloroacetylaspartic acid	3.15	Acylase II 90.0	20.5	Dowex-50	32.5 × 2.5	1	Aniline
N-Acetylhistidine	2.77	Acylase I 194.0[b]	40.0	XE-64	55.0 × 2.5	1	LiOH
Proline amide[a]	[a]	[a]	[a]	XE-64	38.0 × 2.5	0.12	Ag_2CO_3
N-Chloroacetylserine	3.45	Acylase I 3.0	6.5	Dowex-50	57.0 × 2.5	2.5	Aniline
N-Chloroacetylphenylalanine	2.94	Carboxypeptidase 33.0	10.0	Dowex-50	35.0 × 2.0	5	Aniline
N,N'-Dichloroacetylornithine	4.32	Acylase I 115.0[c]	42.0	Dowex-50	43.0 × 2.5	5	.....
N-Acetylalanine	2.94	Acylase I 2.2	13.5	Dowex-50	31.3 × 1.1	2.5	Aniline
N-Acetylmethionine	2.56	Acylase I 0.2	13.5	Dowex-50	31.5 × 2.5	5	Aniline
N-Acetylvaline	2.72	Acylase I 5.0	7.0	Dowex-50	36.4 × 2.5	5	Aniline

[a] Non-enzymatic experiment: 0.880 g. of L-proline amide plus 0.887 g. of L-proline (see text). [b] Added in two portions: 179 mg. initially and an additional 15 mg. at 18 hr. [c] Added in two portions: 105 mg. initially and an additional 10 mg. at 18 hr.

from suspensions of the washed resin, allowed to settle in a glass column and then washed with an additional liter of distilled water. Dowex-50 was used for alanine, methionine, valine, aspartic acid, serine, ornithine and isovaline, and XE-64 was used for proline, histidine and arginine.

Substrates and Enzymes.—Many of the amino acid derivatives used were donated by Dr. J. P. Greenstein. All gave melting points in agreement with those in the literature[2] and the theoretical elemental analyses. The purified hog kidney enzymes, acylase I and II,[2] were prepared by Drs. S. M. Birnbaum and K. R. Rao, respectively. Crystalline carboxypeptidase was obtained from the Worthington Biochemical Sales Company.

Incubation with Enzyme.—N-Acyl-DL-amino acid, equivalent in amount to one gram of each enantiomorph, was dissolved in water and the *p*H adjusted to 7.6 (cresol red) with 6 *N* lithium hydroxide. Sufficient acylase was added to produce the theoretical 50% hydrolysis in one to two hours[2] and the *p*H was readjusted to 7.6. The mixture was diluted to a final substrate concentration of 0.1 *M* and incubated at 37°, the progress of the hydrolysis being followed by manometric-ninhydrin determinations. When 50% hydrolysis had been attained, the incubation mixture was deproteinized at the isoelectric point of the enzyme[2] with Norit, and the solution was reduced *in vacuo*[6] to a small volume.

Chromatography and Isolation of the D-Amino Acid.—The concentrated solution from the enzymatic run was added to the top of the appropriate resin column at a rate of about 0.5 ml. per minute and carefully washed into the resin with several 1-ml. aliquots of water. The N-acyl-D-amino acid was then eluted from the resin by water at a rate of about 10 ml./hr. and collected in fractions of 5–15 ml. The D-amino acid can be detected in the fractions most simply by the drop in *p*H which results from the acidic nature of the derivative.[7] Alternatively, aliquots of each fraction may be spotted on filter paper and examined with: (a) ninhydrin for the appearance of a ninhydrin positive color *only after* preliminary hydrolysis at 100° in 2 *N* HCl,[8] (b) various specific amino acid reagents,[9] or (c) the reagent of Rydon and Smith.[10]

The fractions containing the N-acyl-D-amino acid were combined and evaporated to dryness *in vacuo*. The residue was taken up in about 150 ml. of absolute ethanol or dry acetone and filtered to remove any residual protein. The filtrate was evaporated to dryness *in vacuo*, and the residue was hydrolyzed at 100° for 2 to 3 hours in 2 to 2.5 *N* HCl. The solution was then treated with Norit, filtered and taken to dryness *in vacuo* four times to remove excess HCl. The residue of D-amino acid hydrochloride was then converted to the free amino acid by the reagents indicated in Table I. The compounds were recrystallized from water–ethanol, with the exception of proline, in which case absolute ethanol was employed. Occasionally a second recrystallization was required.

Isolation of the L-Amino Acid.—Several hundred ml. of water was passed through the column subsequent to the complete emergence of the N-acyl-D-amino acid derivative from the column. Elution of the free amino acid was accomplished with hydrochloric acid at concentrations depending upon the resin and the particular amino acid involved. The conditions used are given in Table I. Detection of the fractions containing the L-amino acid was accomplished most conveniently by means of the ninhydrin spot test on paper,[11] although specific reagents also may be used.[9]

The fractions containing the L-amino acid were combined and evaporated to dryness *in vacuo*. The residue was taken up in about 150 ml. of concentrated HCl and filtered to remove salts which usually are eluted with some of the later fractions. The filtrate was diluted and after treatment with Norit the filtered solution was taken to dryness *in vacuo* four times to remove excess HCl. The L-amino acid hydrochloride was converted into the free amino acid in the manner used for its enantiomorph.

Determination of Optical Purity.—Optical rotations were all performed in a two-decimeter tube with a Hilger M375 polarimeter with triple field-type polarizer at 27°. Several of the isomers (*cf.* Table II) were examined for their enantiomorphs by the enzymatic procedure of Meister, Levintow, Kingsley and Greenstein[12] which is sensitive to at least one part of enantiomorph in one thousand parts of the assayed material.

The general procedure given above was followed for the individual amino acids and the specific details are given in Table I. In order to test the feasibility of the column separation, as indicated in Table I, a preliminary non-enzymatic experiment was performed wherein a concentrated solution of L-proline and L-proline amide was put directly on the column. Since proline is not retained by the XE-64 column used, it was eluted by the passage of water through

(6) All concentration procedures were performed *in vacuo* below 40°.

(7) Indicator paper is sufficiently sensitive. This test will apply only when the acyl derivative is used, since with the amide derivatives, the free amino acid emerges first from the XE-64 column.

(8) A 0.25% solution of ninhydrin in acetone is used; *cf.* G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951). A positive ninhydrin test in the early non-acidic as well as in the acid fractions, except where the amide derivative has been used, should be taken as evidence that the capacity of the resin column for that particular amino acid has been exceeded.

(9) The Sakaguchi test for arginine, the isatin test for proline and hydroxyproline, the Pauly test for histidine and tyrosine, the platinic iodide test for methionine, and the periodate-Nesslerization procedure for hydroxy amino acids; *cf.*, R. J. Block and D. Bolling, "The Amino Acid Composition of Foods. Analytical Methods and Results," 2nd Edition, C. C. Thomas, Springfield, Ill., 1952. The corresponding colors in the tests for proline amide and for the N-acetyl derivatives of histidine, arginine and methionine develop somewhat more slowly. The N-acetyl derivatives of the hydroxy amino acids do not give NH_3 after treatment with periodate.

(10) Except for methionine, tyrosine, cysteine and cystine, a blue starch–iodine color is given in this test with amino acids, peptides, proteins, amides and N-acyl derivatives previously exposed to chlorine; *cf.* H. N. Rydon and P. W. C. Smith, *Nature*, **169**, 923 (1952). A positive test with this reagent in the absence of a positive ninhydrin reaction, locates the N-acyl amino acids.

(11) Because of the acidity of the effluent fractions, the paper containing the aliquot spots should be exposed to an atmosphere of NH_3 for about 5 minutes and the excess NH_3 blown off before the paper is dipped into the ninhydrin solution.[8]

(12) A. Meister, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **193**, 535 (1951).

TABLE II

YIELDS AND PROPERTIES OF RESOLVED AMINO ACIDS

Amino acid	Isomer	Yield, %	Concn. mg. per 2 ml.	Solvent	$[\alpha]_D$ Found, degrees	Nitrogen, % Calcd.	Nitrogen, % Found
Aspartic acid	D[b]	63	38.3	6 *N* HCl	−24.7	10.5	10.4
	L	92	40.0	6 *N* HCl	+25.3		10.6
Histidine	D	55	37.5	H_2O	+38.5	27.1	27.1
	L	49	33.5	H_2O	−38.4		27.1
Proline	L[a]	54	32.0	H_2O	−84.4	12.2	12.2
	L	47	26.8	H_2O	−85.1		12.1
Serine	D	69	82.7	1 *N* HCl	−14.5	13.3	13.2
	L	48	95.6	1 *N* HCl	+14.4		13.2
Phenylalanine	D[b]	36	32.4	H_2O	+35.1	8.5	8.7
	L[b]	35	31.9	H_2O	−34.5		8.6
Ornithine·2 HCl	D	46	65.4	5 *N* HCl	−18.2[c]	13.7	13.9
	L	60	62.3	5 *N* HCl	+17.9[c]		13.7
Alanine	D	65	55.9	5 *N* HCl	−14.2	15.7	15.9
	L[b]	61	81.7	5 *N* HCl	+14.2		15.8
Methionine	D	66	52.4	6 *N* HCl	−22.7	9.4	9.4
	L	54	40.0	6 *N* HCl	+23.6		9.4
Valine	D[b]	52	35.5	6 *N* HCl	−27.3	11.9	11.9
	L[b]	74	46.0	6 *N* HCl	+28.2		11.9

[a] Derived from the amide (see text). [b] Examined by the enzymatic test[12] and shown to contain less than 0.1% enantiomorph. [c] The only recorded value in 5 *N* HCl is ±18.2°, S-C. J. Fu, K. R. Rao, S. M. Birnbaum and J. P. Greenstein, *J. Biol. Chem.*, 199, 207 (1952).

the column. After an additional several hundred ml. of water had passed through, proline amide was eluted with weak acid. In a similar non-enzymatic experiment with L-arginine and N-acetyl-DL-arginine, the acetyl derivative was eluted from the XE-64 column with water and the free amino acid removed with weak acid.

It should be pointed out that although the di-amino acids would be retained by the weak cation exchanger, XE-64, it was necessary to use the strong cation-exchange resin, Dowex-50, for the column separation in the ornithine resolution reported here. Starting with the N,N′-dichloroacetyl-amino acid, only the α-amino group is liberated. The products of the enzymatic hydrolysis are similar in their behavior on the strong exchange resin to those obtained in the resolution of the monoamino-monocarboxylic amino acids.

Results and Discussion

The results obtained with nine representative amino acids are given in Table II. Amino acid enantiomorphs with rotation values in good agreement with those in the literature, with theoretical nitrogen analyses[13] and, where tested with optical purity[12] of at least 99.9%, were obtained in the yields listed in Table II. These yields were calculated on the basis of the acyl or amide derivatives.

The percentage yields obtained with the small amounts employed in this study are similar to those obtained with larger amounts using the usual procedure.[2] A batch procedure with methionine was investigated with Dowex-50 and was considerably more complicated, involved larger volumes of solution and resulted in somewhat lower yields.

It is thus believed that, in general, the chromatographic procedure is more suitable for small amounts of material. Furthermore, in some instances, namely, proline and histidine, the column procedure is simpler than the usual technique.

The amino acids before recrystallization were, in every case tested, free of enantiomorph (better than 99.9%) when examined by the enzymatic test.[12] However, since strong HCl elution from the Dowex-50 column also carried along salts with free amino acid, the amino acid hydrochlorides obtained *directly* from the column were not *analytically* pure, as shown by low nitrogen analyses and specific rotation values one to two degrees low. The yield of both *optically* and *analytically* pure amino acid could no doubt be increased even beyond the recorded values, either by prechromatographic desalting of the enzymatic resolution mixture or by more selective elution of the free amino acid with weaker HCl at a lower amino acid load per resin column.[14]

(13) Analyses by R. J. Koegel and his staff.

(14) S. Moore and W. H. Stein, *Cold Spring Harbor Symposia*, **14**, 179 (1949).

BETHESDA, MD.

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Synthesis and Properties of Some 4,5,6,7-Tetrahydroisatins[1]

BY LESTER HORWITZ[2]

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When 2-ketocyclohexylglyoxylic acids or esters are condensed with aromatic amines they form N-aryl-4,5,6,7-tetrahydroisatin-3-anils in excellent yields. When strongly basic amines are used in the condensation, N,N′-substituted-oxamides are isolated in addition to N-alkyl-4,5,6,7-tetrahydroisatin-3-imines. In the case of the formation of the anils it appears that the isatins are not intermediates. In the case of the imines, formed with the strongly basic amines, a possible intermediate to the symmetrically substituted oxamides is the isatin. The 3-anils and 3-imines are hydrolyzed by methanolic hydrochloric acid to the isatins. N-Substituted-4,5,6,7-tetrahydroisatins show β-keto, β-enolic, and α,β-diketo properties.

The syntheses of certain 4,5,6,7-tetrahydroindoles have been described.[3,4] In synthetic preparations designed to prepare 4,5,6,7-tetrahydroisatins, cyclohexanone, 3- and 4-methylcyclohexanone and cyclopentanone were condensed with ethyl oxalate according to the methods described by Kötz, *et al.*[4–6] The resulting glyoxylic acids and their esters were then treated with amines; the reaction of ethyl 4-methyl-2-ketocyclohexylglyoxylate with aniline has been described by Kötz and Hesse.[4]

In every case where condensation products were isolated they proved to be N-substituted-4,5,6,7-tetrahydroisatin-3-anils or -3-imines. Ethyl 2-ketocyclopentylglyoxylate even under the mildest

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(3) (a) A. Treibs, *Ann.*, **524**, 285 (1936); (b) A. Treibs and D. Dinelli, *ibid.*, **517**, 152 (1935).

(4) A. Kötz and L. Hesse, *ibid.*, **342**, 306 (1905).

(5) A. Kötz, *ibid.*, **348**, 111 (1906).

(6) A. Kötz and A. Michels, *ibid.*, **350**, 204 (1906).