Enantioselective Solvent Extraction of Neutral DL-Amino Acids in Two-Phase Systems Containing *N*-*n*-Alkyl-L-proline Derivatives and Copper(II) Ion

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Distribution behavior of neutral amino acid enantiomers was examined in the aqueous and organic solvent of a two-phase system containing cupric ion and *N*-*n*-dodecyl-L-proline or *N*-*n*-alkyl-L-hydroxyproline. Significant enantioselectivity was observed when *n*-butyl, *n*-amyl, or *n*-octyl alcohol was used as the organic solvent. Equilibrium constants of ligand exchange reaction for several amino acid enantiomers were estimated for the *n*-butyl alcohol–water system. The enantioselectivity seems to depend primarily on the difference of the stability of mixed ligand complexes in the organic phase.

In the last few years, a number of publications have appeared on the direct resolution of DL-amino acids by ligand exchange chromatography with a chiral mobile phase (*1–4*) or a chiral stationary phase (*5–8*).

Hare and Gil-Av, for instance, separated DL-amino acids by using an aqueous solution of L-proline–copper(II) complex as the mobile phase and cation exchange resin (*1*) or ODS–silica gel (*2*) as the stationary phase and ascribed the resolution to differences in stability and polarity of diastereomeric complexes formed in the system.

However, there are contradictry observations about the difference of stabilities of diastereomeric complexes in aqueous solution. For example, Gillard et al. reported that stabilities of diastereomeric amino acid copper(II) complexes were shown to be identical (*9*, *10*), while Angelici et al. reported that equilibrium constants for the coordination of L-amino acids to (L-valine-*N*-monoacetato)copper(II) complex were higher than those of corresponding D-amino acids (*11*). Davankov et al. also reported the difference of stabilities between the diastereomeric complexes formed from cupric ion, *N*-benzyl-L-proline, and either D- or L-proline (*12*).

For chiral stationary phase systems, Davankov et al. reported various systems in which chiral reagents were covalently bound to stationary phases (*6*, *7*) or dynamically coated on ODS–silica gel (*8*). They considered that the retention of amino acid in these systems would depend primarily on the stability of mixed ligand complexes composed of a metal ion, a fixed ligand, and a mobile ligand. However, they also observed that the nature of the support significantly affected the enantioselectivity. Consequently, in these systems, the mechanism of resolution was explained as a combination of the difference in stability of mixed ligand complexes and the interaction between the stationary phase and the mixed ligand complex.

Recently, we found enantioselective distribution of neutral DL-amino acids in *n*-butyl alcohol–water systems containing cupric ion and *N*-*n*-dodecyl-L-proline and achieved base line resolution of DL-isoleucine by droplet countercurrent chromatography using this system (*13*). In the present paper, to clarify the factors to develop the enantioselectivity in liquid–liquid two-phase systems, we examined the distribution behavior of neutral DL-amino acids in various organic solvent–water systems containing cupric ion and *N*-*n*-dodecyl-L-proline or *N*-*n*-alkyl-L-hydroxyproline and estimate equilibrium constants of ligand exchange reactions in the *n*-butyl alcohol–water system.

EXPERIMENTAL SECTION

Chromatographic System I. The system was assembled from a minimicropump (type KSU-16H, Kyowa Seimitsu, Tokyo, Japan), a loop injection valve (Seishin Pharmaceutical, Tokyo, Japan) and a UV spectrometer (UVIDEC-100-III, Japan Spectroscopic, Tokyo, Japan). Columns, 10 cm × 4 mm i.d. stainless steel tubes, were prepared with Develosil ODS-7 (7 μm, Nomura Chemical, Seto-Shi, Japan) by the slurry packing technique. Eluents were 0.1 M acetate buffer (pH 6.0) containing 1 mM cupric acetate and appropriate proportions of acetonitrile and were run at a flow rate of 0.8 mL/min. The UV spectrometer was operated at 254 nm and monitored the UV absorbance of the copper(II) complex of the ligand. An aliquot of 55 μL of sample solution was injected onto the column.

Chromatographic System II. The detector and the injection valve of system I were replaced by a spectrofluorometer (FP-110, Japan Spectroscopic, Tokyo, Japan) and a syringe injection valve (Seishin Pharmaceutical, Tokyo, Japan). Both eluent and *o*-phthalaldehyde reagent for postcolumn derivatization were prepared as described by Hare and Gil-Av (*1*). Fluorescence intensity was measured at 450 nm with excitation at 340 nm. An aliquot of 5 μL of sample solution was injected onto the column.

Reagents. All reagents were of analytical grade and purchased from commercial sources.

Preparation of *N*-*n*-Dodecyl-L-hydroxyproline (C_{12}-Hyp). *n*-Dodecyl aldehyde (0.2 mol) was dissolved in ethyl alcohol (150 mL). L-Hydroxyproline (0.1 mol) and 5% palladium on carbon catalyst (1.5 g) were added to the solution as suspension. The mixture was stirred with hydrogen under atmospheric pressure at room temperature for a few days until no L-hydroxyproline was detected by TLC. The catalyst was removed by filtration and the filtrate was evaporated under reduced pressure. The residue was washed with ether and recrystallized. Other *N*-*n*-alkyl-L-hydroxyproline derivatives (C_n-Hyp) and *N*-*n*-dodecyl-L-proline (C_{12}-Pro) were prepared from the corresponding straight chain aliphatic aldehyde and L-hydroxyproline or L-proline in a similar manner. Their analytical data, melting points, and the solvents used for recrystallization are listed in Table I.

Preparation of Two-Phase Systems. Equal volumes of 0.1 M acetate buffer (pH 4.8) containing cupric acetate and an organic solvent containing C_{12}-Pro or C_n-Hyp (except Hyp and C_1-Hyp) were equilibrated by shaking for 20 min. Hyp and C_1-Hyp were dissolved in acetate buffer because of their low solubility in organic solvents. An aliquot of 10 mM DL-amino acid aqueous solution was added to the aqueous phase to yield 0.1 mM solution.

Determination of the Concentration of C_n-Hyp and Cupric Ion in Each Phase. The concentration of C_n-Hyp in each phase was determined in the chromatographic system I. The sample solution was prepared by diluting each phase with the eluent to yield approximately 0.02 mM C_n-Hyp solution. The concentration of cupric ion in each phase was determined by chelometric titration. An aliquot of 1 mL of aqueous or organic phase was taken and an approximately 10-fold volume of ethyl alcohol was added to it. This solution was titrated with 0.5 mM EDTA aqueous solution using 0.1% 1-(2-pyridylazo)-2-naphthol ethanolic solution as an indicator.

Determination of the Distribution Ratio of Amino Acid Enantiomers. Equal volumes (2 mL) of aqueous and organic phases were placed in a centrifuge tube, and the mixture was

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Table I. *N*-*n*-Alkyl-L-proline Derivatives

derivative	formula	calcd, % C	calcd, % H	calcd, % N	found, % C	found, % H	found, % N	melting point, °C	solvent
C_{12}-Pro	$C_{17}H_{33}NO_2$	72.04	11.73	4.94	71.84	11.62	5.12	112–113	ether
C_1-Hyp	$C_6H_{11}NO_3$	49.65	7.64	9.65	49.55	7.55	9.65	241–242	methanol–ether
C_3-Hyp	$C_8H_{15}NO_3$	55.47	8.73	8.09	55.47	8.89	8.11	178–180	acetonitrile
C_5-Hyp	$C_{10}H_{19}NO_3$	59.68	9.51	6.96	59.41	9.40	7.03	157–158	acetonitrile
C_6-Hyp	$C_{11}H_{21}NO_3$	61.37	9.83	6.51	61.08	9.51	6.30	174–175	ether
C_8-Hyp	$C_{13}H_{25}NO_3$	64.17	10.35	5.76	64.12	11.16	5.54	165–166	water
C_{12}-Hyp	$C_{17}H_{33}NO_3$	68.19	11.11	4.68	68.13	10.97	4.65	165–167	water

Table II. Distribution Ratios (D) and Separation Factors (α) of Leucine Enantiomers in Organic Solvent–Water Systems Containing Cupric Ion and C_{12}-Pro or C_{12}-Hyp[a]

organic solvent	C_{12}-Pro	D	α	D_{Cu}[b]	C_{12}-Hyp	D	α	D_{Cu}[b]
n-butyl alcohol	D	1.18	1.51	2.08	D	1.73	1.92	4.90
	L	0.78			L	0.90		
n-amyl alcohol	D	1.00	1.49	2.63	D	1.24	1.94	5.47
	L	0.67			L	0.64		
n-octyl alcohol	D	0.77	1.48	3.17	D	0.93	1.86	7.84
	L	0.52			L	0.50		
ethyl acetate	D	0.07	1.00	15.79			[c]	
	L	0.07						
chloroform		<0.01		26.60			[c]	
dichloroethane		<0.01		26.47			[c]	
nitrobenzene		<0.01		15.13			[c]	
toluene		<0.01		51.80			[c]	
diisopropyl ether			[c]				[c]	
n-hexane			[c]				[c]	

[a] Initial concentrations of cupric ion in acetate buffer and C_{12}-Pro or C_{12}-Hyp in organic solvents were 5 mM and 10 mM. [b] Distribution ratio of cupric ion. [c] Solubility of C_{12}-Pro or C_{12}-Hyp was less than 2 mM.

equilibrated by vortex mixing for 2 min. After centrifugation for 5 min at 3000 rpm, the concentration of the enantiomers in the aqueous phase was determined in the chromatographic system II. The distribution ratio is expressed as $D = (C_i - C_{aq})/C_{aq}$, where C_i is the initial concentration of amino acid in the aqueous phase and C_{aq} is the concentration of amino acid in the aqueous phase after equilibration. The separation factor is the ratio of distribution ratios of D-amino acid to L-amino acid.

RESULTS AND DISCUSSION

Effect of Organic Solvents on the Distribution Ratio of Leucine Enantiomers. Dependence of distribution ratios on the nature of organic solvents used in two-phase systems containing cupric ion and C_{12}-Pro or C_{12}-Hyp at a molar ratio of 1:2 was studied with DL-leucine which has a single asymmetric center and a relatively high hydrophobicity among the neutral aliphatic amino acids (Table II).

When alcohols were used as extraction solvents, enantioselective distribution of DL-leucine was observed and higher values of distribution ratio and separation factor were obtained in C_{12}-Hyp than in C_{12}-Pro systems. With the alcohols studied, the distribution ratios decrease as the number of alkyl carbons of alcohol increases; however, the separation factors virtually remain unaffected.

Although C_{12}-Hyp showed higher enantioselectivity than C_{12}-Pro, its low solubility restricted distribution studies to the alcohols. C_{12}-Pro was soluble in various solvents except diisopropyl ether and *n*-hexane. In the system of C_{12}-Pro and ethyl acetate, D- and L-leucine were extracted to a slight extent into the organic phase; however, no appreciable enantioselectivity was observed. When chloroform, dichloroethane, nitrobenzene, and toluene were used, D-and L-leucine were not extracted into the organic phase, while the distribution ratio of cupric ion was higher than that observed in alcohol systems.

In contrast with the above distribution data for DL-leucine, under conditions where the molar ratio of cupric ion to C_{12}-Pro or C_{12}-Hyp was well below 0.5, higher distribution ratios of D- and L-leucine were observed in C_{12}-Pro systems than in C_{12}-Hyp systems. Under these conditions, the concentration of cupric ion in the aqueous phase became negligible. However, in either C_{12}-Pro or C_{12}-Hyp systems under the conditions of Table II, cupric ion was present appreciably in the aqueous phase and higher concentrations of cupric ion were observed in the C_{12}-Pro system than in the C_{12}-Hyp system. Therefore, lower distribution ratios of D- and L-leucine in C_{12}-Pro than in C_{12}-Hyp systems (Table II) can be explained as the result of increased formation of the copper complex of leucine in the aqueous phase.

Effect of Length of *N*-Alkyl Chain of Hydroxyproline on the Enantioselectivity. With *n*-butyl alcohol as the organic solvent of the two-phase system, the effect of the lipophilic nature of C_n-Hyp on the enantioselectivity was studied (Figure 1). When hydroxyproline was used as the chiral reagent, enantioselective distribution of DL-leucine was not observed. Both enantioselectivity and distribution ratios increased with the length of the *N*-alkyl chain of C_n-Hyp and reached an essentially constant value at C_8-Hyp.

Since a linear relationship was observed between the number of alkyl carbons of C_n-Hyp and the logarithm of the distribution ratio of C_n-Hyp and expressed as $\log D = 0.53n - 2.45$ for $n = 3$ to 8, the corresponding values for hydroxyproline, C_1-Hyp, and C_{12}-Hyp can be estimated as 3.5×10^{-3}, 1.2×10^{-2}, and 8.1×10^3. The enantioselectivity is more readily observed when the chiral reagent is present in the organic phase rather than in the aqueous phase. Lengthening the *n*-alkyl chain of C_n-Hyp increases the concentration of C_n-Hyp in the organic phase and the enantioselectivity in the system. However, for C_8-Hyp, essentially most of the chiral reagents are extracted into the organic phase and further increase of the length of carbon chain does not increase the concentration of C_n-Hyp in the organic phase. Thus, both the enantioselectivity and distribution ratios do not increase with the length of *n*-alkyl chain after the *n*-octyl derivative.

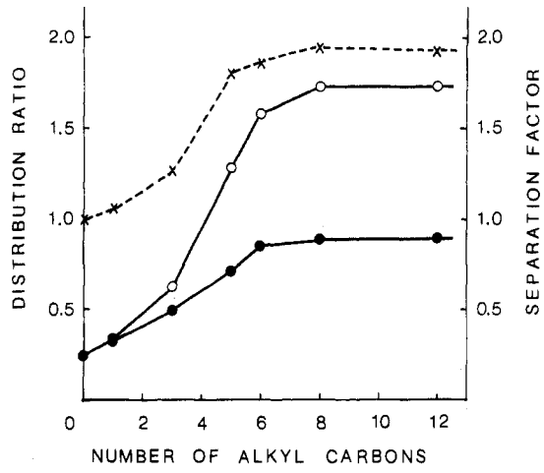

Figure 1. Distribution of leucine enantiomers in *n*-butyl alcohol–water systems containing cupric ion and C_n-Hyp. Initial concentrations of cupric ion in acetate buffer and C_n-Hyp (except Hyp and C_1-Hyp) in *n*-butyl alcohol were 5 mM and 10 mM. Hyp and C_1-Hyp were dissolved in acetate buffer: (—) distribution ratios of D-leucine (O) and L-leucine (●); (---) separation factor.

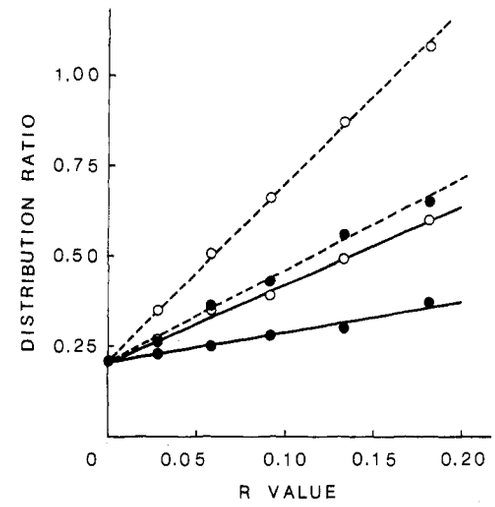

Figure 2. Plots of distribution ratios of D- and L-leucine in the *n*-butyl alcohol–water system against *R* values to determine K_c values. Initial concentration of cupric ion in acetate buffer was in the range of below 5 mM, and that of C_{12}-Pro or C_{12}-Hyp in *n*-butyl alcohol was 40 mM: (—) C_{12}-Hyp system; (---) C_{12}-Pro system; (O) D-leucine; (●) L-leucine.

Stability of Mixed Ligand Complexes in the Organic Phase. In the two-phase system of *n*-butyl alcohol and acetate buffer without cupric ion and C_{12}-Pro or C_{12}-Hyp, the same value of the distribution ratio (0.2) was obtained for both D- and L-leucine. The addition of cupric ion to the system increased them slightly, while the addition of C_{12}-Pro or C_{12}-Hyp did not affect them. When both cupric ion and either C_{12}-Pro or C_{12}-Hyp were added to the system, a significant increase of the distribution ratios was observed for both D- and L-leucine, and enantioselectivity developed. Consequently, D- and L-leucine seem to interact with both cupric ion and C_{12}-Pro or C_{12}-Hyp, to form mixed ligand complexes.

When cupric ion is extracted into the hydrophobic organic phase, it should be present in the form of electrostatically neutral molecular species. If the organic phase contains C_{12}-Pro or C_{12}-Hyp as a ligand (L), cupric ion should take one of three forms of molecular species with respect to the ligand, namely, Cu–L_2, Cu–L, or the ligand free form (Cu). Since $CH_3CO_2^-$, the only other anion present, coordinates weakly with cupric ion, the metal should be primarily present in the form of Cu–L_2, especially when the concentration of the ligand to the cupric ion is in great excess.

Consequently, the ligand exchange reaction which results in the enantioselective distribution of DL-amino acids can be considered as

$$[\text{am}]_{org} + [\text{Cu–L}_2]_{org} \rightleftarrows [\text{am–Cu–L}]_{org} + [\text{L}]_{org} \quad (1)$$

where [am], [L], [Cu–L_2], and [am–Cu–L] are the concentrations of amino acid, free ligand, copper(II) complexes with 2 mol of the ligand, and the mixed ligand complex, respectively. The suffix org means organic phase. The distribution ratio (*D*), which can be experimentally determined, is expressed as

$$D = \frac{[\text{am}]_{org} + [\text{am–Cu–L}]_{org}}{[\text{am}]_{aq}} \quad (2)$$

where $[\text{am}]_{aq}$ is the concentration of amino acid in the aqueous phase. Since essentially all of the ligands are present in the organic phase, $[\text{am–Cu–L}]_{aq}$ is neglected in eq 2. Total concentrations of the ligand and cupric ion in the organic phase can also be determined experimentally and approximated as

$$[\text{L}]_{org,total} = [\text{L}]_{org} + 2[\text{Cu–L}_2]_{org} \quad (3)$$

$$[\text{Cu}]_{org,total} = [\text{Cu–L}_2]_{org} \quad (4)$$

$[\text{Cu–L}]_{org}$ and $[\text{am–Cu–L}]_{org}$ are very low compared to $[\text{L}]_{org}$ and $[\text{Cu–L}_2]_{org}$, and they are neglected in eq 3. Similarly, $[\text{Cu}]_{org}$, $[\text{Cu–L}]_{org}$, and $[\text{am–Cu–L}]_{org}$ are neglected in eq 4. The equilibrium constant of the reaction (K_c) is expressed as

$$K_c = \frac{[\text{am–Cu–L}]_{org}[\text{L}]_{org}}{[\text{am}]_{org}[\text{Cu–L}_2]_{org}} \quad (5)$$

Combining eq 2–5, *D* is expressed as

$$D = D_0(1 + K_cR) \quad (6)$$

where D_0 is the distribution ratio of amino acid in the absence of cupric ion and *R* is the molar ratio of $[\text{Cu–L}_2]_{org}$ to $[\text{L}]_{org}$. They are expressed as

$$D_0 = [\text{am}]_{org}/[\text{am}]_{aq} \quad (7)$$

$$R = \frac{[\text{Cu}]_{org,total}}{[\text{L}]_{org,total} - 2[\text{Cu}]_{org,total}} \quad (8)$$

By use of the 0.1 M acetate buffer (pH 4.8) containing cupric ion at a concentration below 5 mM and *n*-butyl alcohol containing 40 mM C_{12}-Pro or C_{12}-Hyp, a linear relationship was obtained between the distribution ratio of amino acid enantiomers and *R* values (Figure 2). From these plots, equilibrium constants (K_c) of eq 5 were obtained as the quotient of the slopes divided by corresponding D_0 values. The C_{12}-Pro system gave ratios of K_c values of D-amino acid to L-amino acid between 1.8 for leucine and 2.4 for isoleucine, and the C_{12}-Hyp system gave ratios between 2.5 for leucine and 4.5 for isoleucine (Table III). These ratios are larger than the separation factors (Table II), which include the effect of nonselective extraction of amino acid enantiomers in addition to the enantioselectivity directly related to ratios of K_c values. For other DL-amino acids, enantioselectivity can appear, if they distribute adequately into the organic phase.

Enantioselectivity observed in ligand exchange chromatography was higher when L-hydroxyproline derivatives were coated on ODS–silica gel (*8*) but lower when they were covalently bound to either silica gel (*5*, *6*) or organic polymers (*7*). The degree of enantioselectivity observed in the present system was between these systems. Further studies will be required to explain whether the difference of enantioselectivity observed in various systems is due to the difference of equilibrium constants of the ligand exchange reaction in these systems or to the combined effect of other factors, for example, hydrophobic interaction between the mixed ligand complex

Table III. K_c Values of Amino Acid Enantiomers

amino acid		C_{12}-Pro K_c	C_{12}-Pro $K_{c,D}/K_{c,L}$[a]		C_{12}-Hyp K_c	C_{12}-Hyp $K_{c,D}/K_{c,L}$[a]
valine	D	23.0	2.3	D	13.9	3.5
	L	10.1		L	4.0	
norvaline	D	24.2	2.0	D	10.4	3.2
	L	12.2		L	3.3	
leucine	D	24.0	1.8	D	10.1	2.5
	L	13.3		L	4.0	
norleucine	D	24.9	2.1	D	9.8	2.7
	L	11.6		L	3.6	
isoleucine	D	34.1	2.4	D	17.1	4.5
	L	14.1		L	3.8	

[a] Ratio of K_c value of D-amino acid to that of L- amino acid.

and the matrix of a stationary phase like silica gel or organic polymers.

Equilibrium constants were estimated under conditions where the chiral reagent was in great excess with respect to cupric ion and a chiral reagent of high hydrophobicity was used, because the presence of cupric ion and chiral reagent in the aqueous phase decreases the enantioselectivity. Grushka et al. calculated formation constants of diastereomeric mixed ligand complexes in the stationary phase of a reversed-phase system from the retention behavior of amino acid enantiomers (*14*). However, the reduction of enantioselectivity by the increase of concentration of cupric ion or the chiral reagent in the mobile phase was not observed. The discrepancy might be due to the difference in the nature of the chiral reagent used.

In conclusion, distinct enantioselectivity was demonstrated in the liquid–liquid two-phase system studied and expressed as the ratio of the equilibrium constants of pertinent ligand exchange reactions. Because factors which might affect the enantioselectivity are defined more precisely in liquid–liquid distributions compared to systems which involve solid materials like silica gel or organic polymer resins, the enantioselectivity in the ligand exchange reaction is expected to be understood more precisely in the present case.

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Registry No. DL-Valine, 516-06-3; DL-norvaline, 760-78-1; DL-leucine, 328-39-2; DL-norleucine, 616-06-8; DL-isoleucine, 443-79-8; *n*-butyl alcohol, 71-36-3; *n*-amyl alcohol, 71-41-0; *n*-octyl alcohol, 111-87-5.

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Direct Determination of Trace Oxygen in Organic Materials with an Infrared Detector after Conversion to Carbon Monoxide

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A new method to determine oxygen in the 0.01 to 1% range is described. The sample is volatilized and reacted with platinized carbon to convert the oxygen to carbon monoxide which is measured by an infrared detector. By use of hydrogen as a carrier gas, the high inconsistent blank is minimized since the hydrogen reduces any metal oxide impurity in the pyrolysis filling. The plugging problem of the carbon filling is overcome by using a vertically placed combustion tube that contains a combination of graphite chips and platinized carbon powder. The method can be used to determine trace oxygen in liquid samples such as naphthas and gas oils and in solid samples such as polymers and petroleum resids. The relative standard deviation for samples containing less than 0.5% oxygen is about 5%.

Oxygen in organic materials can be determined directly by various modifications of the Schutz method (*1–3*). In this method, the oxygen in the sample is converted to carbon monoxide when the sample is pyrolyzed and passed through carbon at 1100 °C. The carbon monoxide is converted to carbon dioxide by an oxidizer, such as copper oxide, and the carbon dioxide can be determined manometrically, volumetrically, iodometrically, or gravimetrically. In 1954, Oita and Conway (*3*) published a method in which platinized carbon at 900 °C was substituted for carbon at 1100 °C. The lower temperature minimized the reaction of the quartz tube with the carbon filling and resulted in a smaller blank. Also, the lower temperature requires a smaller, less expensive furnace. Because of these advantages, most commercial direct oxygen apparatus on the market today use a platinized carbon or a similar metal carbon filling (*4*).

In 1960, Oita published a method of determining trace oxygen in naphthas (*5*). The chemistry is the same as in the earlier publication, except some modifications were made to the pyrolysis tube and the platinized carbon filling. Also a unique spiral sampling boat was used to handle a relatively

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