for browning and lysine loss even based on initial lysine content. Obviously this variability is probably due to the reaction of other amino acids such as arginine, glutamine, asparagine, histidine, and tyrosine, as well as to structural availability of the amino acids.

This simple study shows that protein substitution in formulated IMF products is a complicated situation with respect to chemical and nutritional stability. The processor might substitute with a protein product of lower lysine content but this does not ensure either increased stability to browning or retention of the lysine content. In addition, this work verifies that supplementation with free lysine in an IMF product which contains reducing sugars cannot be done. Lastly, this work points out that some accelerated test for browning should be used in product development to determine applicability of a protein in an IMF product such as done by Mizrahi et al. (1970).

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Preparation of Optically Active Proline. Optical Resolution of *N*-Acyl-DL-proline by Preferential Crystallization Procedure

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To establish a practical method for the production of optically active proline, the optical resolution of DL-proline by a preferential crystallization procedure was investigated. DL-Proline was easily resolved by converting it to simple *N*-acyl derivatives, such as *N*-acetyl-, *N*-chloroacetyl-, *N*-*n*-butyryl-, and *N*-isobutyrylproline. The optically active acylproline obtained by this resolution method was hydrolyzed to the optically active proline without racemization. The undesired optically active acylproline was readily racemized into the racemic modification and could be reused for the resolution step.

Optically active proline is an important substance in the pharmaceutical and food industries. L-Proline, especially, as well as the essential amino acids, has been proven to be necessary in parenteral nutrition (Dolif and Juergens, 1971) and is widely used as a component of amino acid infusion.

L-Proline has been produced by hydrolysis of natural protein or by fermentation methods. To find a more economical method for the production of optically active proline, we have investigated the optical resolution of synthesized DL-proline. DL-Proline can be synthesized by several chemical methods, for instance, by the method of Albertson and Fillman (1949).

With respect to the optical resolution of DL-proline, chemical and enzymatic procedures have been reported (Greenstein and Winitz, 1961; Kovacs et al., 1957; Vogler and Lanz, 1966). These conventional methods seem to be laborious and unsatisfactory for commercial production.

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On the other hand, the preferential crystallization procedure is considered to be one of the most useful methods for industrial application, since it enables the desired optically active isomer to crystallize preferentially from a supersaturated solution of the racemic modification. So far as proline is concerned, no report has appeared on optical resolution by this simple procedure. Therefore, the optical resolution of DL-proline by the preferential crystallization procedure has been investigated. The advantages of this simple resolution method and the screening methods for resolvable derivatives were described in our previous reports (Yamada et al., 1973a,b; 1975a,b).

DL-Proline itself had no properties suitable for this resolution method. It was reported by Hamer and Greenstein (1951) that the melting points of L isomers of *N*-acetylproline and *N*-chloroacetylproline are much higher than those of the corresponding racemic modifications. This satisfies one of the conditions under which they exist as a racemic mixture (conglomerate). Since the most desirable situation for the preferential crystallization procedure is that the racemic modification crystallizes as a racemic mixture, they might be expected to be resolved by this simple method. Therefore, *N*-acetyl-, *N*-chloro-

Table I. *N*-Acyl Derivatives of Proline

N-Acyl derivative (elemental composition)		Elemental anal., % Calcd	Found DL	Found L
Acetylproline[a]	C	53.49	53.66	53.66
($C_7H_{11}NO_3$)	H	7.06	7.02	7.06
	N	8.91	9.05	9.04
Chloroacetylproline[b]	C	43.88	43.73	43.60
($C_7H_{10}NO_3Cl$)	H	5.26	5.30	5.25
	N	7.31	7.26	7.28
Propionylproline[b]	C	56.13	56.14	56.27
($C_8H_{13}NO_3$)	H	7.65	7.71	7.63
	N	8.18	8.25	8.28
n-Butyrylproline[a]	C	58.36	58.30	58.35
($C_9H_{15}NO_3$)	H	8.16	8.15	8.18
	N	7.56	7.53	7.54
Isobutyrylproline[b]	C	58.36	58.32	58.38
($C_9H_{15}NO_3$)	H	8.16	8.20	8.15
	N	7.56	7.50	7.52
Isovalerylproline[b]	C	60.28	60.40	60.46
($C_{10}H_{17}NO_3$)	H	8.60	8.51	8.75
	N	7.03	7.09	7.11

[a] Recrystallized from water. [b] Recrystallized from acetone.

acetyl-, and furthermore, *N*-propionyl-, *N*-*n*-butyryl-, *N*-isobutyryl-, and *N*-isovalerylproline were prepared. The elemental analyses and the physical properties are shown in Table I and Table II. The properties of these racemic modifications were investigated in order to judge their applicability for the preferential crystallization procedure. The properties to predict whether each racemic modification crystallizes as a racemic mixture or a racemic compound (racemate) are shown in Table III. This suggests that *N*-acetyl-DL-proline monohydrate, *N*-chloroacetyl-DL-proline, and *N*-*n*-butyryl-DL-proline crystallize as a racemic mixture and *N*-propionyl-DL-proline, *N*-isobutyryl-DL-proline, and *N*-isovaleryl-DL-proline crystallize as a racemic compound or racemic solid solution. In fact, *N*-acetyl-DL-proline and *N*-chloroacetyl-DL-proline, and also *N*-*n*-butyryl-DL-proline could be easily resolved by the preferential crystallization procedure in the usual manner described in the Experimental Section.

The racemic modification of *N*-isobutyryl-DL-proline did not show the typical characteristics of a racemic mixture. Namely, the infrared spectrum of the racemic modification was different from that of the optically active isomer and the saturated solution of the racemic modification dissolved the optically active isomer. However, *N*-isobutyryl-DL-proline could be resolved only under the conditions that the crystallization was induced by seeding with optically active isomer and was continued slowly without stirring or scratching. This indicates that resolution is possible in some cases where the racemic modification does not exist as a racemic mixture at equilibrium state in the usual way but its solubility is much higher than that of each of the optically active isomers. On these resolvable derivatives, conditions for the resolution and the analyses for separated crystals are summarized in Table IV. Among these derivatives, *N*-*n*-butyrylproline was most suitable for practical purposes since the crystals had adequate solubility and suitable characteristics for easy handling. Therefore, the detailed studies were made with *N*-*n*-butyrylproline. The results obtained by the successive resolution of *N*-*n*-butyryl-DL-proline are shown in Table V.

The optically active *N*-*n*-butyrylprolines obtained by this resolution method were almost optically pure and could be easily hydrolyzed to the optically active proline without racemization. On the other hand, the undesired

Table II. Properties of *N*-Acylprolines

N-Acylproline	Isomer	Mp, °C	$[\alpha]^{25}$D, deg (*c* 1, water)	Solubility, g/100 ml 20 °C	Solubility, g/100 ml 30 °C	Solvent
Acetylproline monohydrate[a]	DL	63-64		43.4	84.5	Water
	L	82-83	−102.4	15.1	23.7	
Chloroacetylproline	DL	88-89		24.8	40.5	Acetone
	L	118-120	−113.1	10.3	14.0	
Propionylproline	DL	66-68		*b*		Acetone
	L	99-100	−112.9			
n-Butyrylproline	DL	89-90		16.0	25.4	Water
	L	114-116	−105.4	6.0	7.7	
Isobutyrylproline	DL	85-86		32.3	54.5	Acetone
	L	123-124	−107.8	14.5	20.5	
Isovalerylproline	DL	103-105		*c*		Acetone
	L	73-74	−96.4			

[a] Optically active and racemic acetylprolines, respectively, crystallize as a monohydrate. The monohydrates were dried at 25 °C in a chamber of relative humidity 67%. Drying the monohydrates in vacuo over P_2O_5 yielded their anhydrates, mp 105-106 °C (DL), 116-117 °C (L). [b] The solubility of racemic modification was higher than that of the L isomer. [c] The solubility of racemic modification was lower than that of the L isomer.

Table III. Properties of Racemic Modifications of *N*-Acylprolines

N-Acylproline	Comparison with L isomer Mp[a]	Solubility[a]	Ir spect.	Characteristic[b] of solubility	Judgment[c] as racemic mixture
Acetylproline monohydrate	DL < L	DL > L	Identical	O	O
Chloroacetylproline	DL < L	DL > L	Identical	O	O
Propionylproline	DL < L	DL > L	Different	X	X
n-Butyrylproline	DL < L	DL > L	Identical	O	O
Isobutyrylproline	DL < L	DL > L	Different	X	X
Isovalerylproline	DL > L	DL < L	Different	X	X

[a] For instance, DL > L shows that melting point or solubility of racemic modification is much higher than that of the L isomer. [b] O: A saturated solution of the racemic modification no longer dissolved the L isomer. X: A saturated solution of the racemic modification dissolved the L isomer. [c] O: The racemic modification may be judged as a racemic mixture. X: The racemic modification may be judged as a racemic compound or racemic solid solution.

Table IV. Optical Resolutions of *N*-Acylprolines

		Composition of solution				Crystallization		Separated crystals		
N-Acylproline	Run no.	DL form, *B*, g	Active form, *A*, g	Solvent, ml	Seed crystals, *S*, g	Time, min	Temp, °C	Yield, *W*, g	Opt. purity, *P*, %	Resolution rate,[a] *R*, %
Acetylproline monohydrate	1 (L)	24.60	0.70	50 (water)	0.10	120	20	1.58	94.4	5.6
	2 (D)	24.60	0.69	50 (water)	0.10	120	20	1.57	87.8	4.8
Chloroacetylproline	1 (L)	12.15	0	30 (acetone)	0.10	100	20	1.00	100.0	14.8
	2 (D)	12.15	0.90	30 (acetone)	0.10	100	20	1.82	100.0	13.5
n-Butyrylproline	1 (L)	25.50	0	100 (water)	0.20	20	20	2.29	92.0	15.0
	2 (D)	25.50	1.91	100 (water)	0.20	20	20	4.28	97.7	16.2
Isobutyrylproline[b]	1 (L)	10.90	0	20 (acetone)	0.10	16 h	20	0.37	95.3	4.6
	2 (D)	10.90	0.25	20 (acetone)	0.10	16 h	20	0.67	94.7	5.1

[a] $R = 100 \times (W \times P/100 - A - S) \times 2/B$. [b] See text.

Table V. Successive Resolutions of *N*-*n*-Butyryl-DL-proline[a]

		Composition of solution		Separated crystals	
Run no.	Added DL form, g	DL form, g	Active form,[b] g	yield, g	Optical purity, %
1 (L)	25.50	25.50	0	2.29	92.0
2 (D)	3.10	25.50	1.91	4.28	97.7
3 (L)	4.35	25.50	2.07	4.19	98.0
4 (D)	4.22	25.50	1.84	4.72	87.8
5 (L)	4.83	25.50	2.10	4.70	97.5
6 (D)	4.70	25.50	2.28	4.76	97.4

[a] Resolutions were carried out at 20 °C on a 100-ml scale by use of 0.20 g of seed crystals. [b] Values calculated from analysis of separated crystals.

optically active *N*-*n*-butyrylproline was completely racemized by heating at a temperature above its melting point for several minutes in the presence of a catalytic amount of acetic anhydride. The racemized *N*-*n*-butyrylproline could be reused for the resolution step.

In the present work, we cannot reveal a relationship between the kind of acyl group of *N*-acylproline and applicability for preferential crystallization procedure. However, the practical optical resolution of DL-proline could be accomplished by converting it to simple *N*-acyl derivatives. The optical resolution method now presented is very advantageous providing the optically active seed crystals are available because it does not require any optically active resolving agent. Industrial production of optically active proline by this method is considered to be promising if combined with a proper synthetic method for DL-proline.

EXPERIMENTAL SECTION

Materials. Analytical standard grade DL- and L-proline manufactured by our company, Tanabe Seiyaku Co., Ltd., were used. D-Proline used for seed crystals was obtained by the preferential crystallization procedure. All acid chlorides were obtained from Tokyo Kasei Kogyo Co., Ltd. These were used without further purification.

Analyses. All samples were dried overnight in vacuo at room temperature and then at 50 °C unless otherwise noted. Melting points were measured with a Yamato MP-21 melting point apparatus in an unsealed capillary tube and are uncorrected. Infrared spectra of samples were determined in KBr disks using a Shimazu infrared spectrophotometer, Model IR-27G. Optical rotations were measured with a Perkin-Elmer 141 automatic polarimeter. Elemental analyses were performed with a Perkin-Elmer 240 elemental analyzer. Solubility was determined by approaching saturation equilibrium from both sides of undersaturation and supersaturation. Solute concentration was measured with a Karl Zeiss immersion refractometer.

Preparation of *N*-Acylproline. *N*-Acyl derivatives of DL-, D-, and L-proline were prepared as usual by acylation with acid chloride in chilled aqueous alkali. In the case of *N*-*n*-butyryl-DL-proline, *n*-butyryl chloride (114 ml, 1.1 mol) and 7 N NaOH (200 ml, 1.4 mol) were added in small portions into a solution of DL-proline (115.1 g, 1.0 mol) dissolved in 7 N NaOH (143 ml, 1.0 mol). The reaction mixture was always kept slightly alkaline with stirring and ice cooling. After the reaction the mixture was acidified to pH 1.8 with 12 N HCl (ca. 95 ml) and cooled in a refrigerator overnight. The precipitates and further crops obtained by extracting with ethyl acetate were collected, washed with cold water, and dried in vacuo. The total yield was 166.0 g (89.6%). The products were used for resolution without further purification. *N*-*n*-butyryl-D- and -L-proline were prepared in the same way as *N*-*n*-butyryl-DL-proline. The racemic modifications and the optically active isomers of *N*-acetylproline, *N*-chloroacetylproline, *N*-propionylproline, *N*-isobutyrylproline, and *N*-isovalerylproline were prepared similarly as above in high yield (70–90%). The elemental analyses are shown in Table I.

Properties of *N*-Acylproline. The melting points, the optical rotations, and the solubilities of the pure *N*-acyl-DL- and -L-proline are summarized in Table II.

To know whether respective racemic *N*-acylprolines form a racemic mixture or a racemic compound, their infrared spectra were compared with those of the corresponding L isomers. Further, the melting point diagram and the liquid–solid phase equilibrium were also determined in the coexisting systems of the active isomers and the racemic modifications. These results are shown in Table III.

Optical Resolution of *N*-*n*-Butyryl-DL-proline. The resolution of *N*-*n*-butyryl-DL-proline was carried out as follows. *N*-*n*-Butyryl-DL-proline (25.50 g) was dissolved in water (100 ml) at elevated temperature. The solution was cooled to 20 °C, seeded with *N*-*n*-butyryl-L-proline (0.20 g), and stirred at the same temperature. By refractometric and polarimetric measurements of liquid phase, it was observed that preferential crystallization of the L isomer occurred, while the D isomer remained in the solution. After 20 min, the precipitated crystals were collected by filtration, washed with a small amount of cold water, and dried to give *N*-*n*-butyryl-L-proline (2.29 g), $[\alpha]^{25}\mathrm{D}$ −97.0° (*c* 1, water), optical purity 92.0%. After the separation of the L isomer, *N*-*n*-butyryl-DL-proline (3.10 g) and a small amount of water were added to the mother liquor so that the composition of the racemic modification and water was the same as that in the initial state except that the solution contained the D isomer in excess. By seeding this supersaturated solution with *N*-*n*-butyryl-

D-proline (0.20 g), the preferential crystallization of D isomer was carried out in the same manner as described above. By repeating these procedures, L and D isomers were successively obtained. Table V shows the results obtained by the successive resolution of *N-n*-butyryl-DL-proline.

Optical Resolution of Other *N*-Acyl-DL-prolines. *N*-Acetyl-DL-proline, *N*-chloroacetyl-DL-proline, and *N*-isobutyryl-DL-proline could also be resolved in the same manner as described above. Although *N*-isobutyryl-DL-proline could not be resolved under stirring, the resolution was successful only when crystallization was induced by inoculation and was continued slowly without stirring or scratching. Conditions and results for the resolutions are summarized in Table IV.

Preparation of Optically Active Proline. *N-n*-Butyryl-L-proline obtained by the above procedure was recrystallized from water. A mixture of optically pure *N-n*-butyryl-L-proline (7.4 g) and 5 N HCl (16 ml) was refluxed for 2 h and then diluted with water (100 ml). The resulting solution was passed through a column of Amberlite IR-120 (40 ml, H^+ form). The column was washed with water and L-proline was eluted from the column with 5% NH_4OH (70 ml). The eluates were concentrated, treated with charcoal, and concentrated again to dryness. The residual crystals were dissolved in methanol (10 ml) and acetone (60 ml) was added to the solution at 5 °C. The precipitated crystals were collected, washed with acetone, and air-dried at 55 °C, giving L-proline (4.3 g), $[\alpha]^{25}D$ −85.3° (*c* 1, water). Anal. Calcd for $C_5H_9NO_2$: C, 52.16; H, 7.88; N, 12.17. Found: C, 51.49; H, 7.88; N, 12.04.

In the same procedure, D-proline was obtained from optically pure *N-n*-butyryl-D-proline, $[\alpha]^{25}D$ +85.5° (*c* 1, water). Anal. Found: C, 51.68; H, 7.95; N, 12.06.

Racemization of Optically Active *N-n*-Butyrylproline. A mixture of *N-n*-butyryl-D-proline (7.4 g) and acetic anhydride (0.38 ml) was heated in a boiling water bath for 20 min after being melted. The reaction mixture was dissolved in water (1.5 ml) and cooled at 5 °C. The precipitated crystals were collected, washed with cold water, and air-dried at 50 °C, giving *N-n*-butyryl-DL-proline (6.9 g), $[\alpha]^{25}D$ 0.0° (*c* 1, water), mp 88–89 °C. Anal. Found: N, 7.51.

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Synthesis and Metabolic Fate of Hesperetin-*3*-^{14}C

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Hesperetin-*3*-^{14}C (3′,5,7-trihydroxy-4′-methoxyflavanone-*3*-^{14}C) of specific activity 6.48 μCi/mg was prepared from acetonitrile-*2*-^{14}C by a five-step reaction sequence (8.0% overall radiochemical yield). The metabolic fate of this flavanone in vivo after oral and intraperitoneal administration to intact, bile duct ligated, and bile duct cannulated rats and in vitro with rat cecal microflora was studied. Forty percent of the radioactivity orally administered to intact rats was expired as $^{14}CO_2$, while virtually no $^{14}CO_2$ was produced upon incubation with cecal microflora. The major labeled metabolites found in both the urine of orally dosed animals and in in vitro incubations were 3-phenylpropanoic acids. It appears that bacterial enzymes were responsible for the metabolism of hesperetin-*3*-^{14}C to labeled phenylpropanoic acids, while mammalian hepatic enzymes mediated their further breakdown to benzoic acids and $^{14}CO_2$.

Flavonoids represent the single most widely occurring group of phenolic compounds found in nature (Seikel, 1964). The flavanone hesperidin (hesperetin 7-β-rutinoside), which was once designated vitamin P because of its effect on blood capillary permeability and fragility, is the predominant flavonoid in lemons and sweet oranges (*Citrus sinensis*). This material is readily converted to the aglycone (hesperetin) upon oral administration to rabbits, rats, and humans (Booth et al., 1958).

Earlier studies dealing with the metabolism of the aglycone have involved oral administration to rats (Booth et al., 1958) and in vitro treatments with rat cecal microflora (Scheline, 1968b). These studies demonstrated both the significant metabolic degradation of hesperetin and the intestinal absorption of this compound and/or its degradation products. The doses of test compound employed in these and related studies ranged from 0.15 to 1.0 g per animal.

We report here the first preparation of radiolabeled hesperetin. This labeled material provided the sensitivity

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