important to the selection of time and temperature of heat exposure for the optimum separability of the green chloroplastic proteins from the soluble white proteins.

The experiments reported in this paper have confirmed and extended the observations of others that controlled heating allows the centrifugal sedimentation of the green chloroplastic proteins while having only minimal effect on the soluble white proteins. The effects of pH, temperature, and time of exposure have been studied extensively; increasing the pH reduces the denaturation of the soluble proteins, whereas increasing either the temperature or the time of exposure has the opposite effect. By increasing the severity of the pH–temperature–time treatment, the clarity of the centrifugal supernatant can be improved markedly but only at the cost of a decreased level of soluble white protein. The results of these experiments are currently being applied in the development of a process which can separate the white from the green proteins of freshly expressed alfalfa juice on a continuous basis.

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Optical Resolution of DL-Lysine by Preferential Crystallization Procedure

Shigeki Yamada,* Masao Yamamoto, and Ichiro Chibata

To develop a practical method for the production of L-lysine, the optical resolution of synthetic DL-lysine by preferential crystallization procedure was studied. As the properties of free DL-lysine were unsuitable for this resolution method, DL-lysine was converted to various aromatic sulfonates and the suitability of each was investigated. As a result, DL-lysine-*p*-aminobenzenesulfonate was found to be resolved in a good yield by preparing a supersaturated aqueous solution of the racemic modification and seeding with crystals of the desired isomer. The mother liquor was used repeatedly for the resolution of the opposite antipode. The unused D isomer could be easily racemized by heating in an autoclave. A general method was also established to distinguish whether or not resolution of a given racemic modification was possible. However, it is not yet possible to predict what kind of sulfonates are resolvable.

L-Lysine, one of the essential amino acids, is an important substance in pharmaceutical and nutritional fields, and its market has been expanding rapidly in recent years. Today, most commercial L-lysine is produced by a fermentation method, very seldom by a synthetic method because this procedure yields racemic lysine. Also, a satisfactory resolution method suitable for industrial application has not appeared. Up until now, several fairly practical studies of the optical resolution of DL-lysine have been reported. Namely, two diastereoisomeric salts of DL-lysine with L-glutamic acid (Emmick, 1951; Rogers, 1953) and of aminocaprolactam, a synthetic intermediate of DL-lysine, with L-pyrrolidone carboxylic acid (Brenner and Rickenbacher, 1958; Nelemans *et al.*, 1963) were resolved by fractional crystallization. Also the acyl derivatives of DL-lysine, ε-benzoyl-*N*-acetyl-DL-lysine, and ε-benzoyl-DL-lysine were hydrolyzed asymmetrically by mold aminoacylase (Chibata *et al.*, 1956; Yamada *et al.*, 1956) and bacterial ε-lysine acylase (Ishikawa *et al.*, 1962), respectively. However, these methods are unsatisfactory in competition with a fermentation method. Therefore, it is very much desired to establish an advantageous resolution method for DL-lysine. In these circumstances, we have studied a practical method for the optical resolution of DL-lysine.

Although a number of methods for the optical resolution of DL-amino acids have been reported, most of them employ chemical or enzymatic procedures and reports on a preferential crystallization procedure are relatively few (Greenstein and Winitz, 1961). If successfully applied, a

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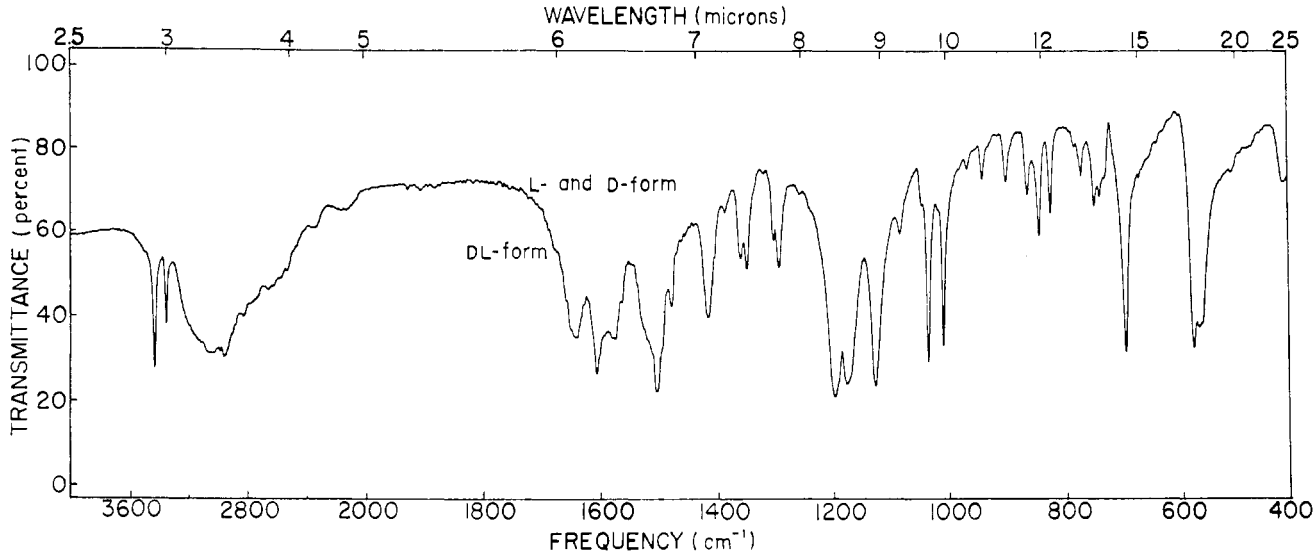


Figure 1. Infrared spectra of D-, L- and DL-Lys-*p*-ABS.

preferential crystallization procedure, which can be easily accomplished by providing seed crystals of one antipode in a supersaturated solution of the racemic modification, should be a very advantageous method for the production of optically active amino acids (Secor, 1963). However, satisfactory application of this type of simple procedure has been limited to several amino acids, such as asparagine (Piutti, 1886), histidine (Duschinsky, 1934), threonine (Velluz and Amiard, 1953), glutamic acid (Akashi, 1962; Kögl *et al.*, 1943), and aspartic acid (Haga *et al.*, 1967; Harada, 1965). The reason for this limited applicability is that most amino acids form racemic compounds instead of racemic mixtures and have no properties suitable for this resolution procedure. To accomplish optical resolution by this method, it is necessary to find conditions under which racemic modification forms a racemic mixture. However, synthetic DL-lysine itself and its ordinary salts such as mono- or dihydrochloride, sulfate, nitrate, and acetate form racemic compounds which are unsuitable for the resolution. Aromatic sulfonic acids vary greatly in their properties, and some of the salts with lysine can be expected to form racemic mixtures which can be resolved by preferential crystallization. Thus, we prepared a wide variety of aromatic sulfonates of lysine and screened the salts forming racemic mixtures. As a result, it was found that DL-lysine-*p*-aminobenzenesulfonate (DL-Lys-*p*-ABS) crystallized from water forms a racemic mixture and that the optically active isomers can be crystallized out reciprocally by seeding the supersaturated aqueous solution of the racemic modification with the respective optically active isomers. Therefore, the conditions necessary for the reciprocal resolution of the racemic modification were investigated in detail. In this report, properties of the optically active and racemic salts and methods of resolution and racemization are presented.

EXPERIMENTAL SECTION

Preparation of DL-, D-, and L-Lys-*p*-ABS. To an aqueous solution of 146.2 g (1 mol) of DL-lysine, 173.2 g (1 mol) of *p*-aminobenzenesulfonic acid was added. The mixture was heated, treated with charcoal, and filtered. The colorless filtrate was concentrated under reduced pressure and the resulting precipitate was collected. It gave 310.1 g (97.1%) of DL-Lys-*p*-ABS, mp 237–238° (dec), which was almost pure and could be used for resolution without further purification. Recrystallization from water gave colorless prisms, mp 239° (dec).

Anal. Calcd for $C_{12}H_{21}N_3O_5S$: C, 45.12; H, 6.63; N, 13.16; S, 10.04. Found: C, 45.20; H, 6.73; N, 13.09; S, 10.01.

D- and L-Lys-*p*-ABS were prepared in the same way as described above. For the D form: mp 251° (dec); $[\alpha]^{30}D$ −11.65° (c 2, NHCl). *Anal.* Found: C, 45.13; H, 6.69; N, 13.05; S, 10.10. For the L form: mp 251° (dec); $[\alpha]^{30}D$ +11.65° (c 2, NHCl). *Anal.* Found: C, 45.00; H, 6.74; N, 13.14; S, 10.06.

Infrared Spectra of D-, L-, and DL-Lys-*p*-ABS. The infrared spectra of D, L, and DL forms in KBr were identical, as shown in Figure 1.

Melting Point Diagram in Binary System of D- and L-Lys-*p*-ABS. The melting points of the mechanical mixtures of pure D and L crystals in various proportions are shown in Figure 2. The melting point diagram is symmetrical and exhibits a eutectic at the midpoint of the composition range. On the other hand, the above mechanical mixtures in various proportions were dissolved in water and then crystallized completely by drying. The melting points of the respective products were the same as those of the corresponding original mixtures.

Solubility of Lys-*p*-ABS in Water. Solubility was determined by approaching the saturation equilibrium from both undersaturation and supersaturation. Saturation equilibrium was attained by vigorous shaking in a glass-stoppered tube for 24 hr at given temperatures. The concentration of the equilibrium solution was determined by refractometry according to the standard curve previously constructed. Straight lines were obtained in the temperature–solubility curves. From the straight lines, the following equations were obtained.

$$Sr = 1.21t + 36.1$$
$$Si = 0.94t + 19.8 \text{ (from 10 to 45°)}$$

where Sr and Si are the solubility of racemic and active forms, respectively, at t° (g/100 ml of water).

Liquid–Solid Phase Equilibrium in the Coexisting Systems. The mixture of the active and the racemic forms in various proportions was dissolved at an elevated temperature and equilibrated at 25° by shaking. By refractometry and polarimetry, the equilibrium compositions of the coexisting system of the active and the racemic forms were determined, and are shown in Figure 3. The figure shows that the equilibrium points are distributed on the straight line connecting the solubility of the racemic form (point A) with that of the active form (point B).

Stability of Supersaturated Solution Containing DL-

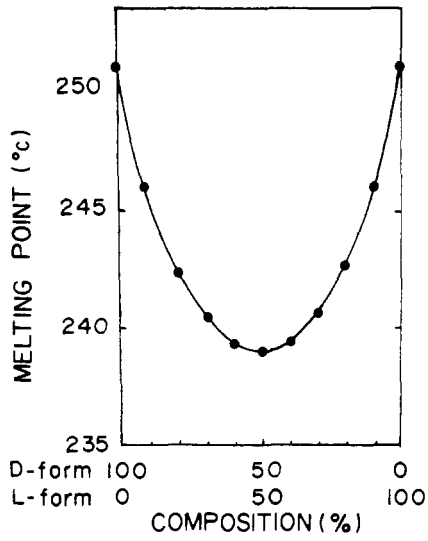


Figure 2. Melting point diagram in binary system of D- and L-Lys-*p*-ABS.

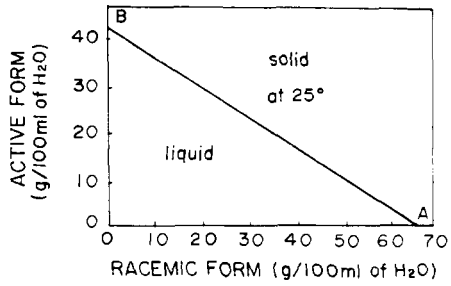


Figure 3. Liquid–solid phase equilibrium in the coexisting system of the active and the racemic forms.

and Excess D-Lys-*p*-ABS. To prepare the solution supersaturated with D isomer, 2, 4, 6, 8, or 10 g of D-Lys-*p*-ABS was added, respectively, to 100 ml of the saturated solution of DL-Lys-*p*-ABS at 25°, heated to dissolve the excess D isomer, and cooled to 25°. The behavior of crystallization from the above supersaturated solution was investigated with or without adding 0.5 g of pulverized pure D or L crystals as seed. The results are shown in Figure 4.

Resolution of DL-Lys-*p*-ABS. In a typical experiment, 38.5 g of DL-Lys-*p*-ABS and 2.5 g of L-Lys-*p*-ABS were dissolved in 50 ml of distilled water in a 100-ml Erlenmyer flask by heating. The flask was placed on a temperature-controlled water bath. The mixture was stirred slowly by a magnetic stirrer and cooled to 25°. By seeding this supersaturated solution with 0.05 g of finely pulverized L-Lys-*p*-ABS crystals, crystallization of the L isomer occurred, while the D isomer remained in the solution. During the crystallization, changes of concentration and the optical rotation were determined by refractometric and polarimetric measurements of the liquid phase. The results are shown in Figure 5. After 65 min the crystals were collected by filtration, washed with 2 ml of aqueous methanol, and dried at 50°. The crystals obtained were practically pure: yield 5.52 g; $[\alpha]^{30}_D$ +11.5° (c 2, NHCl); optical purity 98.3%; mp 249° (dec). *Anal.* Calcd for $C_{12}H_{21}N_3O_5S$: C, 45.12; H, 6.63; N, 13.16. Found: C, 45.17; H, 6.67; N, 13.05.

Reciprocal Resolution. After the separation of the L form in the above experiment, 5.77 g of solid DL-Lys-*p*-ABS (total amount of the separated crystals was 5.22 g, and the operation loss was 0.25 g) and a small amount of water were added to the filtrate so that the gross concentration was the same as that of the previous resolution. This adjustment was carried out simply by adding appropriate amounts of DL modification and water according to refractometric measurement and weight of the solution. The concentration of optically active isomer was not corrected. Thus, almost the same composition as in the previous operation was obtained, except that the predominant isomer was the opposite antipode of the previous one. Calculated compositions of the solution are shown in the second column of Table I. By seeding this supersaturated solution with 0.05 g of D isomer, the preferential crystallization of D isomer was carried out in such a manner as described previously. The process was controlled with crystallization time, 65 min in every case. Table I shows the results obtained by the 14 reciprocal resolutions.

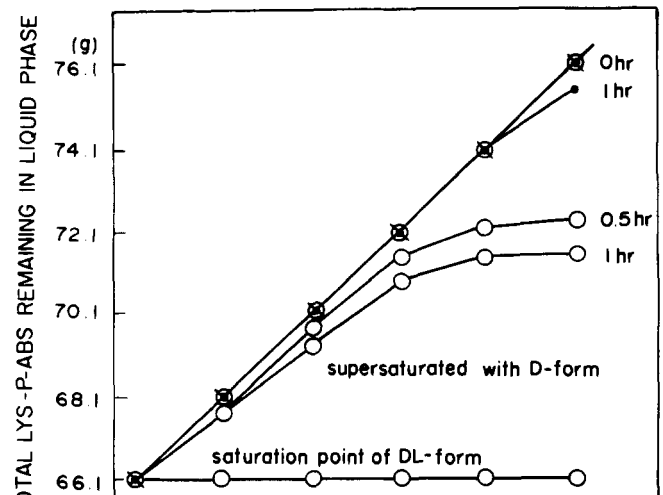


Figure 4. Stability of supersaturated solution containing DL- and D-Lys-*p*-ABS. x——x, nonseeded; ●——●, seeded with L form; O——O, seeded with D form.

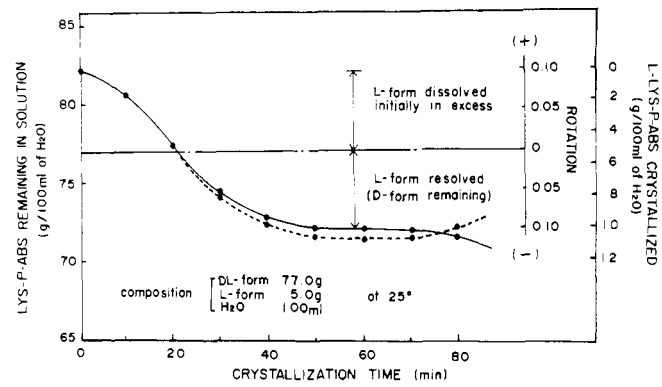


Figure 5. Time course of resolution. ——, concentration; -----, rotation.

Purification of Optically Impure Lys-*p*-ABS. Ten grams of crude L-Lys-*p*-ABS, optical purity 78.9%, was mixed with 1.6 ml of water and 30 ml of the solution saturated with DL-Lys-*p*-ABS at 25°. The mixture was then stirred vigorously for 2.5 hr at 25°. The resulting crystals were collected by filtration, washed with a small amount of 60% (v/v) aqueous methanol, and dried at 50°. By this operation, optically pure crystals of L-Lys-*p*-ABS were obtained. The yield was 7.90 g; mp 250° (dec); $[\alpha]^{30}_D$ +11.65° (c 2, NHCl). The yield was the theoretical amount. The racemic impurity was recovered as the saturated solution.

Preparation of Optically Active Lysine Hydrochlo-

Table I. Reciprocal Resolution of DL-Lys-*p*-ABS

Exp no.	Addition DL form, g	Composition		Inoculation, g	Separated crystals		Resolution rate, %
		DL form, g	Active form, g		Yield, g	Optical purity, %	
1		38.50	2.50	0.05 (L)	5.52	98.3 (L)	15.0
2	5.77	(38.13)	(2.87)	0.05 (D)	5.86	98.3 (D)	14.9
3	5.41	(38.16)	(2.84)	0.05 (L)	5.57	97.9 (L)	13.4
4	5.73	(38.44)	(2.56)	0.05 (D)	5.74	97.0 (D)	15.4
5	5.86	(38.04)	(2.96)	0.05 (L)	5.54	97.4 (L)	12.6
6	5.67	(38.61)	(2.39)	0.05 (D)	5.79	97.0 (D)	16.9
7	6.22	(37.82)	(3.18)	0.05 (L)	5.76	98.7 (L)	13.0
8	5.91	(38.54)	(2.46)	0.05 (D)	5.73	98.3 (D)	16.2
9	5.85	(37.88)	(3.12)	0.05 (L)	5.61	98.3 (L)	12.4
10	6.13	(38.66)	(2.34)	0.05 (D)	5.71	97.9 (D)	16.6
11	5.79	(37.81)	(3.19)	0.05 (L)	5.74	98.7 (L)	12.9
12	6.13	(38.57)	(2.43)	0.05 (D)	5.82	98.3 (D)	16.8
13	5.93	(37.76)	(3.24)	0.05 (L)	5.93	97.4 (L)	13.2
14	6.07	(38.51)	(2.49)	0.05 (D)	5.85	96.1 (D)	16.0
Mean	5.88	(38.23)	(2.77)	0.05	5.74	97.8	14.6

Resolution was carried out on a 50-ml scale at 25°. Crystallization time was 65 min in every case. ( ): Values calculated theoretically from analysis of separated crystals on the assumption that mechanical losses of optically active forms are negligible in process.

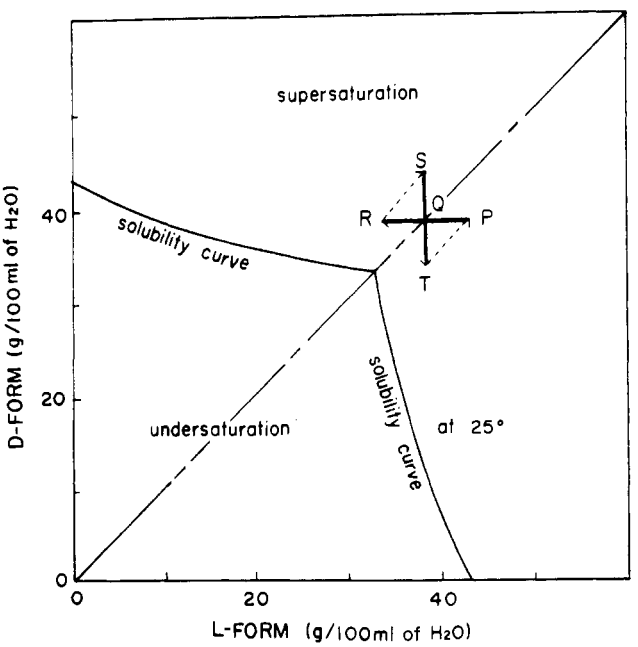


Figure 6. Illustration of alternate D and L forms resolution in phase diagram.

ride. Thirty-two grams of optically pure L-Lys-*p*-ABS was dissolved in hot water and decomposed with 25 ml of 6 *N* HCl to liberate the *p*-aminobenzenesulfonic acid. The mixture was allowed to stand in a refrigerator overnight. The resulting precipitate, which was almost pure *p*-aminobenzenesulfonic acid, was filtered and washed with cold water. The recovery was 97%. After removal of the *p*-aminobenzenesulfonic acid, water was added to the filtrate so as to make the total amount 200 ml. Then the solution was passed through a column of Amberlite IR-120 (H^+ form) to absorb the base on the resin. To remove a slight quantity of *p*-aminobenzenesulfonic acid, the column was washed with 500 ml of 0.75 *N* hydrochloric acid and then with water. From the resin, lysine was eluted with 500 ml of 2 *N* aqueous ammonia. The eluate was concentrated, adjusted to pH 4.2–4.5 with hydrochloric acid, treated with charcoal, and concentrated again. The residual crystals were suspended in 100 ml of methanol, filtered, and air dried at 97° for 24 hr to give pure L-lysine hydrochloride. The total yield was 17.6 g (95.9% of the theoretical amount), $[\alpha]^{25}_D$ +20.8° (c 2, 5 *N* HCl). *Anal.* Calcd for $C_6H_{15}O_2N_2Cl$: N, 15.34. Found: N, 15.41. In the same way, pure D-lysine hydrochloride was obtained from D-Lys-*p*-ABS: yield 17.7 g; $[\alpha]^{25}_D$ −20.8° (c 2, 5 *N* HCl). Found: N, 15.45.

Racemization of D-Lys-*p*-ABS. A mixture containing 200 g of optically pure D-Lys-*p*-ABS and 500 ml of water was heated in an autoclave at 170° for 6 hr. The reaction mixture was treated with charcoal and the colorless filtrate was evaporated under reduced pressure. The resulting precipitate was washed by suspension in 200 ml of methanol. The yield of DL-Lys-*p*-ABS was 190.8 g (95.4%), mp 237–238° (dec). The product was identical with the authentic sample prepared from DL-lysine.

RESULTS AND DISCUSSION

Generally, it is well recognized that the solid state infrared spectra of respective optical isomers are identical, but different from that of the corresponding racemic compound (Koegel *et al.*, 1957). However, in the case where racemic amino acids exist as a racemic mixture, the infrared spectrum of a racemic modification should be identical with that of the respective optical isomers. Thus, the spectra of the various sulfonates of lysine were investigated. As a result, Lys-*p*-ABS crystallized from water was found to give an identical spectrum, suggesting that the racemic modification exists as a racemic mixture. This suggestion was also supported by the melting point diagram shown in Figure 2. The solubility of the racemic modification of Lys-*p*-ABS was much higher than that of the respective antipodes. The saturated solution of the racemic modification no longer dissolved the optically active isomer. The unsaturated solution of the racemic modification dissolved the optically active isomer, and liquid compositions came to the equilibrium of the system consisting of the racemic modification and one of the optically active isomers. The above relationships of the solubility also show that the racemic modification of Lys-*p*-ABS crystallized from water is not a racemic compound but a racemic mixture. Furthermore, this salt crystallized well and the supersaturated solution was very stable. From the above experimental results, it was expected that resolution of DL-Lys-*p*-ABS could be performed by preferential crystallization procedure.

In fact, seeding a supersaturated solution of the racemic modification with crystals of one isomer (for example, L isomer) brought about preferential crystallization of the seeded L isomer, while the nonseeded D isomer remained in the solution as supersaturation. During this resolution

process, a state of supersaturation of D isomer in the solution is required to be stable in order to avoid crystallization of the racemic modification. Accordingly, assuming the mother liquor obtained by the above resolution process, a solution supersaturated with D isomer was prepared. Then the stability of this solution was investigated in both cases in the presence and the absence of seed crystals. As shown in Figure 4, the isomer existing in excess (D isomer in this example) easily crystallized only when the solution was inoculated with the same crystals as that of the excess isomer. However, no crystallization occurred during 1 hr under shaking conditions when the opposite antipode (L isomer) was inoculated or seed crystals were not added. From the experiments, the state of supersaturation of this salt was found to be stable enough to enable preferential crystallization of the desired isomer.

In the practical process, it was desirable to start resolution with supersaturated solution containing an excess of one of the isomers. For instance, the supersaturated solution containing 5 parts of the desired isomer, 77 parts of the racemic modification, and 100 parts of water was seeded with 0.1 part of the seed crystals of the same isomer present in excess under stirring at 25°. After allowing crystallization to proceed for 1 hr, about 11 parts of desired isomer crystallized from the solution. The crystals obtained by filtration were twofold greater than the excess of isomer employed initially and were almost optically pure.

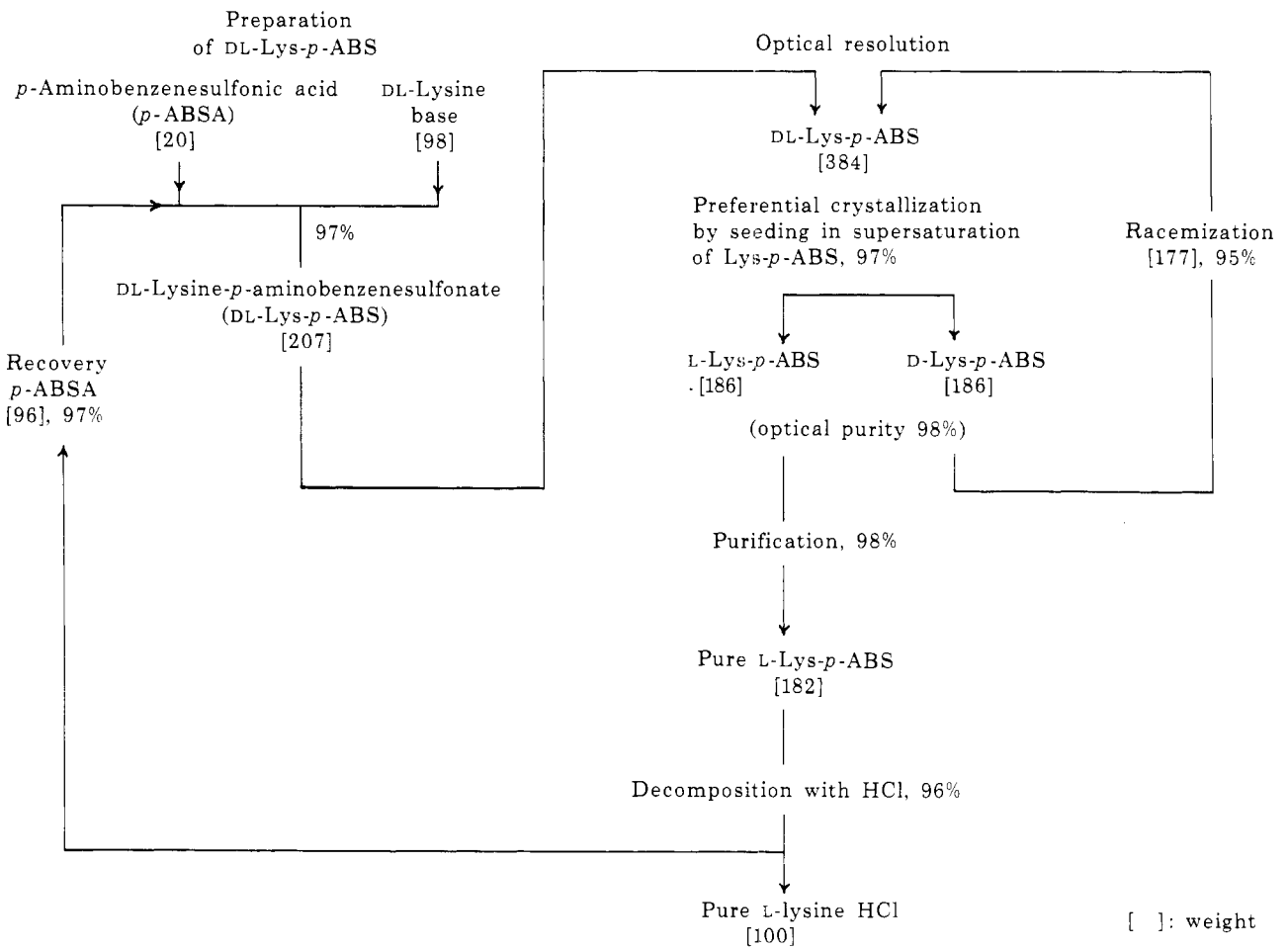
When the degree of resolution R is defined by

$$R = (WP - A - S)/B$$

W is the total weight of crystallized solids, P is the optical purity of the crystallized solids, A is the weight of the excess isomer existing initially in the original solution, S is the weight of seed crystals added to the solution, and B is the weight of the seeded isomer in the original racemic modification. R was about 15% in the above experiment. In one example, the maximum of R reached 25%. As shown in Figure 5, at 50 min after seeding, the solution came to a steady state which continued for 20 min. At 80 minutes, however, optical rotation began to drop since crystallization of the non-seeded isomer occurred.

In repeated resolutions, the same time course as above was always obtained. Therefore, 65 min could be used constantly as a crystallization time in all cases. Figure 6 illustrates by phase diagram how a resolution was carried out in the above crystallization process. Initially, the solution of composition P (which contained an excess of L isomer) was prepared at an elevated temperature and cooled to 25°. The solution was maintained at P until crystallization began. The composition of the solution moved to point Q as the L isomer existing in excess crystallized from the solution. Crystallization continued and the composition changed from point Q to point R. At point R the crystals of L isomer were filtered off. After the separation of the L isomer, the mother liquor where D isomer was dominant over L isomer was used repeatedly for the separation of D isomer by addition of the racemic modification. If the addition of the racemic modification was adjusted to the equal amount of the L isomer previously separated, the gross composition reached point S. The mixture was heated to dissolve completely and cooled to 25°

Scheme I



so that the same conditions as the previous operation were obtained, except that the solution contained D isomer in excess. As D isomer crystallized by the inoculation of D isomer, the composition changed from S to T. After the D crystals were filtered off, solid racemic modification was added to the filtrate until the gross composition reached point P. The entire cycle was repeated indefinitely and both L and D isomers were obtained reciprocally.

The results of successive resolution which was controlled with the fixed crystallization time are shown in Table I. The isomers obtained by this procedure had an optical purity of about 98% on the average. If the optical purity is not satisfactory and further purification is required, the crude products can be purified easily by recrystallization. The purification can be made without loss of the optically active isomer, according to the principle shown in Figure 3, indicating that the optically active isomer no longer dissolves in the saturated solution of the racemic modification. Namely, this purification can be performed by dissolving the mixture in a minimum amount of water required to dissolve the racemic modification in the crude crystals and allowing the pure crystals to crystallize out. However, the operation is not easy because the amount of water required to dissolve the racemic impurity is very small. Therefore, it was convenient to carry out the above operation by adding an appropriate amount of the solution saturated with the racemic modification. L-Lys-*p*-ABS thus obtained was decomposed with hydrochloric acid and converted into L-lysine hydrochloride. Insoluble *p*-aminobenzenesulfonic acid could be easily recovered quantitatively by filtration and used for resolution. On the other hand, D-Lys-*p*-ABS was completely racemized into DL modification by heating in an autoclave and used again for the resolution.

The yield of L-Lys-*p*-ABS from DL-Lys-*p*-ABS through the procedure combined resolution and racemization of Lys-*p*-ABS was 90%. The overall yield of L-lysine hydrochloride from DL-lysine base was 82% of theory. All processes and yields are summarized in Scheme I.

CONCLUSION

In the optical resolution presented here, the yield at each step is very high and the operation is simple. Also, all processes are expected to be operated automatically by sequence control system. Therefore, application of the present method for the industrial production of L-lysine is considered to be very promising if combined with a proper synthetic method for DL-lysine. In the present work we can not establish a theory to predict what kind of racemic modification forms a racemic mixture suitable for the resolution by preferential crystallization. However, it is possible to determine whether a given racemic modification is a racemic mixture or a racemic compound and to predict whether or not resolution of a given racemic modification is possible. In addition it suggests that the present simple method using aromatic sulfonates may be applied more generally for resolution of synthetic amino acids because aromatic sulfonic acids have a variety of properties and easily form salts with any kind of amino acids, so that it is very likely that some of their salts will form racemic mixtures suitable for preferential crystallization procedure. Its further application to other amino acids is under investigation.

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Possible Substitutes for Nitrite for Pigment Formation in Cured Meat Products

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Various concentrations of 24 nitrogenous ligands were examined for their ability to form ferrohemochromes with bovine myoglobin at pH 5.0 or 6.0. Methyl and hexyl nicotinate and *N,N*-diethylnicotinamide were particularly effective. Methyl and hexyl nicotinate and *N,N*-diethylnicotinamide produced stable pink pigments in cooked ground meat mixtures. These compounds were also effective, as was trigonelline, in combination with 10 or 20 ppm of nitrite in forming a stable and long-lasting pink cured meat color in such systems.

The curing of meat products generally involves the use of a mixture of sodium chloride, sugar, nitrate and/or nitrite, and often a reductant such as sodium ascorbate or sodium erythorbate. In this conventional method of curing, the nitrate and nitrite undergo reduction to nitric oxide. Nitric oxide then binds with the heme pigments myoglobin and hemoglobin to form the unstable bright red pigments nitric oxide myoglobin and nitric oxide hemoglobin. Upon heating, a relatively stable pigment, denatured globin nitric oxide ferrohemochrome, is formed.

The chemistry of the pigment formed during curing has been studied extensively and reviews are available (Fox,

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