FORMATION OF NEW CRystALLINE ENZYMES FROM CHYMOTRYPSIN

ISOLATION OF BETA AND GAMMA CHYMOTRYPSIN

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The isolation of crystalline chymotrypsinogen from fresh beef pancreas and its conversion into chymotrypsin by trypsin has been described in former publications (1).

Chymotrypsin in solution undergoes a gradual irreversible transformation into new enzymes, two of which have now been isolated in pure crystalline form. The new enzymes, called beta (β) and gamma (γ) chymotrypsins are proteins and are enzymatically indistinguishable from the original chymotrypsin but differ from it in molecular weight, crystalline form, stability in acid or alkali, solubility, etc.

The process of the irreversible transformation of chymotrypsin into the new enzymes as well as a description of the methods of their isolation in pure crystalline form and a description of certain of their properties are presented in this paper.

The procedure for preparing chymotrypsin consists essentially in adding a small amount of trypsin to a concentrated solution of pure chymotrypsinogen of pH 7.6 and allowing the solution to stand at 5°C. for 24 hours. During this time complete conversion into chymotrypsin takes place. The pH of the solution is then adjusted to 4.0 and the chymotrypsin is salted out by means of ammonium sulfate. The amorphous precipitate is redissolved in a very small amount of 0.01 normal sulfuric acid and left at 20°C. A heavy crop of rhombohedral crystals of chymotrypsin is usually obtained within 24 hours. The yield is about 50 per cent of the total chymotrypsinogen protein used.

A solution of crystalline chymotrypsin on recrystallization gives
FIG. 1. Chymotrypsin crystals. 1a, crystallized at pH 4.0. × 123. 1b, crystallized at pH 5.6. × 153.
rise either to rhombohedrons (Fig. 1a) or to long prisms (Fig. 1b), depending on the pH of the solution. In the range of pH 4.0-5.0 the solution yields rhombohedral crystals while at pH 5.2-6.0 prisms appear. The prisms, when recrystallized from a solution of pH less than 5.0 yield rhombohedrons and *vice versa*, thus proving that both forms of crystals are reversible polymorphs of one and the same substance—chymotrypsin.

![Image of needle-shaped crystals](image)

**Fig. 2.** Needle-shaped crystals consisting of a solid solution of beta, gamma, and inert protein; crystallized at pH 4.0. × 300.

The mother liquor of the first crystals of chymotrypsin still contains about 50 per cent of the original proteolytic activity in solution and when kept at room temperature at pH 4.0 a protein crystallizes in the form of fine needle-shaped crystals (Fig. 2) different from the rhombohedrons or the long prisms of chymotrypsin (2).

The rate of formation of the fine needle crystals is very slow but once started the mass of crystals continues to increase for weeks until
the solution becomes a semi-solid mass of fine crystals. The new protein crystals differ from those of chymotrypsin not only in form but also in solubility. They still possess the same type of enzymatic activity as that of chymotrypsin although there is a quantitative difference, the activity per unit weight of the new protein being about one-half that of chymotrypsin. On recrystallization at pH 4.0 the new protein always gives rise to the same fine needle-shaped crystals and all attempts to make it crystallize in the form of typical chymotrypsin rhombohedrons or prisms failed. It thus became evident that the new crystalline protein, unlike the prisms or the rhombohedrons, is not a polymorphic form of chymotrypsin.

Origin of the New Crystalline Protein.—The new crystalline protein was first obtained from the mother liquor of the chymotrypsin crystallization and the possibility existed that the protein was present in the original crude extract. It was found, however, that the material could be obtained from repeatedly recrystallized chymotrypsin which in turn had been prepared from repeatedly recrystallized chymotrypsinogen. Solubility measurements (3) have shown that chymotrypsinogen and chymotrypsin are pure proteins so that the new protein must be formed from chymotrypsin and is not present in the original extract. The method of forming the new protein from pure chymotrypsin consists in allowing a concentrated solution of recrystallized chymotrypsin to stand at pH 7.6 and 5°C. for about 3 weeks. An appreciable loss of activity takes place under these conditions without any significant loss of protein. The solution, when brought to pH 4.0 and with proper adjustment of the ammonium sulfate concentration, yields at room temperature the new protein crystals, the yield being 50-75 per cent of the total protein.

On repeated recrystallization the new protein retains the same crystalline form and also constant specific activity, which makes it appear that the new protein is a pure substance of constant composition. Measurement of the solubility of the new protein crystals in dilute ammonium sulfate solution in the presence of an excess of the solid phase shows, however, that the solubility of the crystals is not independent of the total amount of the excess crystals in suspension but continues to increase with the increase in the amount of crystals suspended. This does not accord with the phase rule criterion of a
pure substance. The plotted solubility curve (Fig. 3) showing the relation between the amount of the new protein dissolved and the total amount of protein in suspension corresponds to the theoretical solubility curve of "mixed crystals" consisting of a solid solution of two or more substances (3).

Further investigation has proved that the new protein crystals actually consist of a solid solution of several proteins, two of which have been isolated in pure crystalline form and found to be active proteolytic enzymes of the chymotrypsin type. These two crystalline enzymes named beta chymotrypsin and gamma chymotrypsin can be easily isolated from a solution of the mixed crystals in 0.4 saturated ammonium sulfate by means of fractional crystallization at various pH. At pH 5.0 or higher the solution yields on standing at 20°C. large polyhedral crystals of gamma chymotrypsin, bipyramidal in form (Fig. 4). The filtrate from the gamma crystals when brought to pH 4.2 yields fine needle crystals of beta chymotrypsin. The beta crystals generally appear in the form of leafy rosettes and, when very small, resemble the mixed crystals. The beta crystals have a tendency to form solid solutions with other proteins and it was found that the first beta crystals, although practically free of gamma protein, still contain in solid solution a great deal of inert protein which cannot be removed from the beta crystals by repeated recrystallization. It can, however, be easily removed by incubating a solution of impure beta crystals of pH 7.6 for 30 minutes at 37°C. The inert protein is then completely digested by the excess of active proteolytic

![Figure 3](image-url)

**Fig. 3.** Solubility at 10°C. of solid solution crystals in 0.4 saturated ammonium sulfate pH 4.0 in the presence of increasing quantities of solid phase.
beta protein. The latter can then be crystallized in a pure state (Fig. 5).

Methods have also been developed whereby chymotrypsin can be readily changed directly into either one of the new crystalline proteins. Thus, if a concentrated solution of chymotrypsin crystals in dilute ammonium sulfate of pH 8.0 is kept at 35°C, the chymotrypsin protein is completely changed within 90 minutes into gamma protein.

Fig. 4. Crystals of gamma chymotrypsin crystallized at pH 5.6. × 15.5.

On adjusting the pH of the solution to 5.6 large polyhedral bipyramidal gamma crystals rapidly appear. On the other hand if the change is allowed to proceed at a lower pH or lower temperature then either beta crystals or the mixed crystals of the solid solution are obtained.

On repeated recrystallization each enzyme continues to yield its own characteristic crystals. Chymotrypsin cannot be obtained again from either beta or gamma chymotrypsin. The change from
chymotrypsin to the new protein crystals is apparently an irreversible process.

The new enzymes differ from each other as well as from the original chymotrypsin not only in crystalline form but in a number of other respects, such as molecular weight, stability in acid or alkali, degree of denaturation by urea, etc. Most striking are the quantitative differences in the solubilities of the three crystalline proteins in aqueous ammonium sulfate solutions. Thus at pH 4.0 gamma is six times as soluble as beta and about eight times as soluble as chymotrypsin. A solution saturated with crystals of chymotrypsin continues to dissolve crystals of beta and vice versa, the total amount of protein dissolved being equal to the sum of the individual solubilities. The same holds true for a mixture of gamma and chymotrypsin crystals when mixed at pH 5.5. On the other hand, crystals of gamma form a solid solution with either beta or chymotrypsin crystals when mixed

Fig. 5. Crystals of beta chymotrypsin crystallized at pH 4.0. × 315.
at pH 4.0; hence the solubility of gamma at pH 4.0 is affected by the presence of either one of the other protein crystals and vice versa.

The phase rule solubility curves for the three crystalline proteins, unlike the solubility curve for the solid solution crystals, are typical of substances which contain only a small fraction of impurities.

The three enzymes, while strikingly different in many respects, are, nevertheless, chemically and enzymatically very similar. No significant differences have been found in the elementary composition nor in the free amino nitrogen and tyrosine-tryptophane content of the three proteins, and no marked quantitative differences in the rate and extent of digestion of various proteins and artificial substrates have been observed. The formation of the new enzymes from chymotrypsin is apparently due to a slight hydrolysis of the chymotrypsin molecule as evidenced by the loss of protein during the process of formation of the new enzymes as well as by the fact that their molecular weights are lower than that of the original chymotrypsin.

In the following text as well as in the figures and tables chymotrypsin is frequently designated for brevity's sake by the Greek letter alpha (α).

**Schematic Presentation of Formation of β and γ Protein from Chymotrypsin**

Chymotrypsin (rhombohedrons or prisms)

β Chymotrypsin  γ Chymotrypsin  Inert protein

Solid solution crystals (fine needles)

Fractional crystallization at pH 5.6

Filtrate to pH 4.2

Crystals of β in solid solution with inert protein

Autolysis 0.5 hour at 37°C, pH 7.6, then crystallization at pH 4.2.

β Chymotrypsin (fine needles)
EXPERIMENTAL RESULTS

I. Isolation of Beta and Gamma Chymotrypsins

1. The Formation of New Enzymes from Chymotrypsin at 5°C. and pH 7.6

Experimental Procedure.—20 gm. of semi-dry paste of crystals of chymotrypsin three times recrystallized from 0.4 saturated ammonium sulfate pH 4.0 was suspended in 48 ml. ice cold water and 12 ml. M/2 phosphate buffer pH 8.0 was then added. This brought about rapid solution of the crystals. The pH of the solution was then adjusted to 7.6 by means of a few drops of 5 N sodium hydroxide and stored at 5°C. Samples of 1 ml. were taken at various times and diluted with M/400 hydrochloric acid to proper concentrations for activity and protein nitrogen determinations. At the same time samples of 3 ml. were taken for crystallization tests. These were mixed in test tubes with 2 ml. of saturated ammonium sulfate and after adjustment of pH to 4.2 by means of several drops of N/1 sulfuric acid, were left at 20–25°C. for crystallization. The crystal suspensions were filtered after 3 days through Whatman’s No. 42 paper and the clear filtrates were analyzed for protein nitrogen; the percentage yield of crystallized protein was then calculated.

The results of the various determinations are given graphically in Fig. 6 which shows that the process of transformation of chymotrypsin at 5°C. and pH 7.6 consists of three successive stages. During the first stage, which includes approximately the first 2 or 3 days, a rapid formation of inert protein takes place as evidenced by the rapid decrease in the activity of the solution accompanied by a relatively small loss in protein. The inert protein formed interferes considerably with the crystallization of the active protein left. The solution, adjusted to the proper pH and ammonium sulfate concentration, still continues to yield crystals of normal chymotrypsin rhombohedrons, but the percentage of protein crystallized diminishes rapidly from day to day until the second stage is reached which includes the period between the third and fifth days when only a slight precipitate of spheroid granules mixed with a few rhombohedrons and fine needles appear in the crystallization samples. Finally during the third stage which extends for several weeks after the fifth day, the solution begins to yield increasing amounts of crystals again. The crystals, however, consist not of chymotrypsin rhombohedrons but entirely of fan-shaped bundles of fine needles consisting of a solid solution of beta and gamma chymotrypsins and inert protein.
The yield of the needle crystals increases rapidly from day to day for a period of about 10 days until it reaches a maximum of nearly 70 per cent of the total protein present in solution. The crystallization samples appear then as thick gels of fine crystals. On further standing autolysis takes place and the yield of crystals gradually diminishes.
The inert protein formed during the first phase of the process does not interfere with the crystallization of the new crystals since it combines with them to form a solid solution.

2. Isolation of Gamma Crystals from the "Solid Solution" Crystals

It has been mentioned that the needle crystals on recrystallization at pH 4.2 always retain the same needle form of crystals. If, however, the pH of the recrystallization mixture is adjusted to 5.2-6.0 then bipyramidal crystals of gamma chymotrypsin appear. The gamma crystals on recrystallization even at pH 4.2 yield only bipyramids and all attempts to change the gamma crystals to the original solid solution needles have so far failed.

Experimental Procedure.—10 gm. of needle solid solution crystals was dissolved in 30 ml. water and the pH was adjusted to 5.6 by means of a few drops of 5 N sodium hydroxide. 20 ml. saturated ammonium sulfate was then added and the clear solution was left at 20°C. Bipyramidal crystals gradually appeared and grew rapidly until a heavy sediment of crystals was formed within 24 hours. Crystallization was allowed to proceed for 2 days. The crystals were removed by filtration with suction on hardened paper and recrystallized several times in 0.4 saturated ammonium sulfate at pH 5.6 and finally at pH 4.2. The specific activity of the gamma crystals was about the same as that of pure chymotrypsin.

3. Isolation of Beta Crystals

The filtrate from the gamma crystals when adjusted to pH 4.2 yields fine needle crystals similar to those of the original solid solution crystals but of a lower specific activity. They were named "crude" beta crystals. The low specific activity is due to the presence of inert protein which forms a solid solution with the needle crystals. All attempts to remove the inert protein by means of fractional crystallization or repeated recrystallization were unsuccessful. It can be easily removed, however, by means of autolysis at 37°C.

Experimental Procedure.—10 gm. of three times recrystallized crude beta crystal cake was dissolved in 250 ml. water and 10 ml. 0.4 M borate buffer pH 9.0 was added. The mixture, pH 7.6, was rapidly warmed to 37°C. and left for 1 hour in a water bath at 37°C. Samples were taken at 15 minute intervals for measurements of activity and protein content. At the end of the hour the solution was cooled to 20°C. and the pH was adjusted to 4.0. Solid ammonium sulfate was added to bring the solution to 0.4 saturation. This gave rise to a slight amorphous precipitate which was filtered off with the aid of about 1 gm. Standard Super-Cel. The clear filtrate was brought with solid ammonium sulfate to 0.7 saturation. The formed precipitate was then filtered on hardened paper. The semi-dry amorphous precipitate of about 7 gm. was then dissolved in 21 ml. water and 14 ml. saturated ammonium sulfate was added. The pH was adjusted to 4.2. Crystals of fine rhomboid plates of pure beta gradually appeared. The specific activity of the pure beta crystals was about the same as that of chymotrypsin.
Fig. 7 shows that the specific activity of the solution of crude beta trypsin is gradually increased because of the digestion of the inert protein by the active beta protein.

4. Formation of Gamma Chymotrypsin from Chymotrypsin at 35°C. and pH 8.4

The formation of the new enzymes from chymotrypsin at 5°C. and pH 7.6 as described in section 1 is a slow process. The change occurs faster if the chymotrypsin is exposed to higher temperatures,
but the composition of the new materials yielded at various temperatures is not the same. It was found that when a concentrated solution of chymotrypsin is kept at 35°C. and pH 8.4 a very rapid change

![Graph](image_url)

**Fig. 8.** Formation of gamma from alpha chymotrypsin at pH 8.4 and 35°C.

occurs from chymotrypsin into gamma chymotrypsin, without formation of any beta or inert protein. The last, if formed at all, is immediately digested under these conditions.
Experimental Procedure.—A mixture was made of 16 gm. of six times recrystallized chymotrypsin crystal cake plus 24 ml. water plus 24 ml. 0.4 M borate pH 9.0. The final pH of the solution was about 8.4. The solution was left at 35°C. Samples of 0.2 ml. were taken at various times and diluted with N/400 hydrochloric acid for measurements of activity and protein content. At the same time samples of 3 ml. were taken for crystallization. These were mixed in test tubes with 2 ml. of saturated ammonium sulfate and the pH was adjusted to 5.6 by means of several drops of 1 N sulfuric acid. The mixtures were then left at 20°C. for crystallization. The crystallization mixtures were filtered after 24 hours and the protein content of the filtrates was determined; the percentage yield of crystallized protein was then calculated.

TABLE I
Formation of Gamma from Chymotrypsin at 35°C.

50 gm. alpha crystal cake + 75 ml. water + 75 ml. 0.4 M borate buffer pH 9.0; 4 hrs. at 35°C.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Original solution</td>
<td>134</td>
<td>2960</td>
<td>0.045</td>
</tr>
<tr>
<td>Original solution after 4 hrs. at 35°C</td>
<td>101</td>
<td>2260</td>
<td>0.045</td>
</tr>
<tr>
<td>Per cent</td>
<td>75</td>
<td>76</td>
<td>0.045</td>
</tr>
<tr>
<td>First crop of gamma</td>
<td>49</td>
<td>1045</td>
<td>0.047</td>
</tr>
<tr>
<td>Mother liquor</td>
<td>39</td>
<td>875</td>
<td>0.045</td>
</tr>
<tr>
<td>First recrystallization of gamma</td>
<td>45</td>
<td>880</td>
<td>0.051</td>
</tr>
<tr>
<td>Second “ “ “</td>
<td>37</td>
<td>780</td>
<td>0.048</td>
</tr>
<tr>
<td>Third “ “ “</td>
<td>32</td>
<td>670</td>
<td>0.048</td>
</tr>
<tr>
<td>Fourth “ “ “</td>
<td>29</td>
<td>560</td>
<td>0.052</td>
</tr>
</tbody>
</table>

The results are given graphically in Fig. 8. It is to be seen that crystals of chymotrypsin appeared only in the samples taken within the first 2 hours, while the later samples contained crystals of gamma protein. The rate of crystallization of each species of crystals is affected by the presence of the other materials so that there was a distinct minimum in the amount of crystalline precipitate in the sample tubes, as shown by the plotted curve for the per cent of protein crystallized. If the crystallization were allowed to proceed for a longer time the difference in the yields would gradually disappear, except for the difference between the solubility of the two species of crystals. The curves for activity and protein content of the original
chymotrypsin mixtures show that the change of chymotrypsin at 35°C. and pH 7.6 is accompanied by a simultaneous loss both of activity and protein which means that there is no accumulation of inert protein in the solution and hence the specific activity of the protein remains unchanged during the whole process. The yield and the specific activity of gamma on recrystallization are shown in Table I.

5. Final Method for Isolation of Beta and Gamma Crystals

The final procedure adopted for the isolation of the new enzymes follows.

As a starting material either chymotrypsin or the mother liquor from chymotrypsin crystallizations can be used. In the latter case the protein is first salted out in 0.7 saturated ammonium sulfate and the precipitate is then used in the following operations in the same manner as the crystal cake of chymotrypsin.

Isolation of Gamma Crystals.—Suspend 100 gm. of crystal cake of chymotrypsin in 100 ml. water. Add 50 ml. 0.5 m phosphate buffer pH 8.0 and store the clear solution for 3 weeks at about 5°C. Then add 120 ml. saturated ammonium sulfate, adjust the pH to about 5.6 by means of 5 N sulfuric acid, added drop by drop, and allow the mixture to stand at 20°C. for crystallization of gamma chymotrypsin. Filter after 3 days with suction. The filtrate (first gamma mother liquor) is stored at 5°C. Yield about 30 gm. gamma filter cake.

Recrystallization of Gamma.—Recrystallize the gamma crystals by dissolving 10 gm. in 30 ml. water and adding 20 ml. saturated ammonium sulfate. Filter after 24 hours with suction. Residue—second crystals of gamma; stored at 5°C.

Isolation of Crude Beta Crystals.—Combine filtrate with the first gamma mother liquor, adjust pH to 4.2 with 5 N sulfuric acid, and salt out the protein by adding 21 gm. solid ammonium sulfate to each 100 ml. of solution. Filter with suction. Dissolve 10 gm. of amorphous precipitate in 7.5 ml. N/100 sulfuric acid and allow solution to stand for several days at 20–25°C. until a heavy precipitate of fine needle crystals of crude beta is formed. The solution frequently turns completely into a thick fibrous gel of crystals. Filter with suction. The filtrate on standing may yield another crop of needle crystals. The total yield is about 50 gm. crude beta filter cake per 100 gm. of original chymotrypsin filter cake.

Recrystallization of Crude Beta.—Dissolve 10 gm. of crystal cake in 30 ml. of water and add 30 ml. saturated ammonium sulfate. Adjust pH to 5.6 by means of a few drops of 5 N sodium hydroxide, and after inoculation with gamma crystals allow the solution to stand for several days at 20°C. Filter off any gamma crystals formed and adjust the pH of the filtrate to 4.2. Crude beta crystals gradually appear. Filter after several days and repeat crystallization of the crude beta in the same manner.

Isolation of Pure Beta Crystals.—Dissolve 10 gm. of three times recrystallized crude beta crystal cake in 250 ml. of water, add 10 ml. 0.4 M borate buffer pH 9.0.
Heat solution to 37°C. and then let it stand at this temperature for 1 hour. Cool solution to 20°C. and adjust pH to 4.2 by means of 5 N sulfuric acid. Add with stirring 65 gm. of ammonium sulfate and if a precipitate is formed add 5 gm. Standard Super-Cel and filter with suction through 9 cm. No. 3 filter paper. Dissolve 21 gm. ammonium sulfate in each 100 ml. of the clear filtrate and filter the formed amorphous precipitate of protein with suction on hardened paper. Reject filtrate. Dissolve each gram of precipitate in 3 ml. water and add 2 ml. saturated ammonium sulfate. Adjust solution to pH 4.2 and let it stand at 20°C. An amorphous precipitate forms which gradually changes into very fine crystals. Filter after several days with suction; yield about 3 gm. of pure beta crystal cake.

The procedure for recrystallization of the "pure" beta crystals is the same as for the recrystallization of the crude beta.

II. Physical and Chemical Properties of the Various Crystalline Proteins

1. Solubility in 0.4 Saturated Ammonium Sulfate pH 4.0 at 10°C. in Presence of Increasing Amounts of the Solid Phase

Experimental Procedure.—The following operations, except crystallization, were done in a constant temperature room at 10°C. ± 0.5. The concentration of ammonium sulfate is expressed in terms of saturation at 20°C.

Crystallization.—The crystalline proteins used in the solubility tests were recrystallized several times in 0.4 saturated ammonium sulfate made up in N/10 acetate buffer of the same pH as that used in the solubility tests.

Washing of Crystals.—10–15 gm. of crystal cake was suspended in 0.4 saturated ammonium sulfate in a 100 ml. Pyrex test tube provided with a glass bead, 15 mm. in diameter. The tube completely filled with the ammonium sulfate solution was stoppered with a one-hole stopper and finally plugged with a short glass rod. Care was taken not to leave any air space in the tube. The tube was then placed horizontally in a rocking machine and allowed to rock at a slow rate so as to cause the glass bead to roll back and forth all along the tube thus keeping the suspension continuously stirred. The rocking was generally continued for 24 hours; the suspension was then filtered with suction and the clear filtrate was analyzed for activity and protein concentration. The filtered crystals were then resuspended in fresh 0.4 saturated ammonium sulfate and the whole procedure was repeated several times until the filtrates gave constant values for activity and protein concentration. These values generally became constant after 2 or 3 washings.

Solubility at 10°C. in 0.4 Saturated Ammonium Sulfate.—The crystals from the final washing were uniformly suspended in 50 ml. of 0.4 saturated ammonium sulfate. Increasing amounts of the uniform suspension from 0.1 to 15 ml. were made up to 15 ml. with 0.4 saturated ammonium sulfate and then transferred into 15 ml. test tubes, each provided with an 8 mm. Pyrex glass bead. The tubes were stoppered with one-hole rubber stoppers and then plugged with short glass
rods so as to remove all the air from the tubes. The suspensions were rocked for 24 hours and then filtered through small No. 42 filter papers. The clear filtrates, as well as the original suspensions, were analyzed for activity and protein concentration. Stirring of the suspensions for longer than 24 hours did not affect the solubility in either direction, thus proving that equilibrium was reached practically in 24 hours.

Fig. 9 shows the curves for the solubility at 10°C. of the various crystals in 0.4 saturated ammonium sulfate pH 4.0 in the presence of increasing amounts of crystalline protein added to the system. There is an enormous difference in the solubility of the gamma crystals as compared with those of beta or alpha trypsin crystals, the solubility of the last two proteins being about the same.

The solubility curves of alpha, as well as of beta and gamma crystals, consist of intersecting straight lines and correspond to the theoretical phase rule curves of substances mixed with an amount of impurity varying from 0 to 13 per cent as determined from the slopes of lines.

The solubility curve for the crude beta crystals, unlike the other curves, consists entirely of a continuous curvature similar to the curve...
shown in Fig. 3 and hence corresponds to a solubility curve typical of a solid solution.

*Mixed Solubility.*—Saturated solutions of the various crystals in 0.4 saturation ammonium sulfate pH 4.0 were made up by suspending 15 gm. of washed crystal cake in 100 ml. of solvent and shaking the suspension for 24 hours at 10°C. in the manner described before. Several portions of 15 ml. of the well mixed suspension were filtered with suction on separate papers. The filtrates were combined while each of the residues was stirred in a 15 ml. test tube with saturated solutions of various proteins or with its own saturated solution and also with 15 ml. of fresh 0.4 saturated ammonium sulfate pH 4.0. The tubes were provided with beads. The suspensions were rocked for 3 hours, then filtered with suction. The filtrates were rejected while the crystalline residues were resuspended in corresponding fresh solutions and rocked for 24 hours and then filtered. The filtrates were analyzed for activity and protein concentration while the residues were resuspended once more in corresponding fresh solutions and rocked for 48 hours longer and then filtered. The final filtrates were analyzed for activity and protein concentration as before. The results of these determinations are given in Table II which shows that a saturated solution of crystals of alpha or of beta in dilute ammonium sulfate pH 4.0 continues to dissolve crystals of the other protein until the total concentration of protein dissolved equals the sum of the solubilities of the two proteins. On the other hand, a saturated solution of gamma crystals at pH 4.0 loses protein when mixed with an excess of crystals of either alpha or beta.

### Table II

*Solubility of Alpha (Chymotrypsin), Beta, and Gamma Crystals in 0.4 Saturated Ammonium Sulfate Made Up in M/10 Acetate Buffer pH 4.0 and Saturated with the Various Crystals*

<table>
<thead>
<tr>
<th>Solid phase</th>
<th>α</th>
<th>β</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid phase-saturated solutions of</td>
<td>α</td>
<td>β</td>
<td>γ</td>
</tr>
<tr>
<td>After 24 hrs</td>
<td>8.4</td>
<td>19</td>
<td>46</td>
</tr>
<tr>
<td>After 48 hrs</td>
<td>8.4</td>
<td>21</td>
<td>51</td>
</tr>
<tr>
<td>Calculated</td>
<td>19.4</td>
<td>68</td>
<td>94.4</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>M₆ protein nitrogen per ml. filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 24 hrs</td>
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<tr>
<td>After 48 hrs</td>
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<td>Calculated</td>
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This is due to the fact that at pH 4.0 gamma protein combines with either alpha or beta to form crystals of a solid solution. The solubility of the solid solution crystals formed is apparently lower than that of the gamma crystals.

It is to be noticed that the solid solution combination between gamma and the other crystals occurs only at pH about 4.0 but not at pH above 5.5; hence, it was found possible to separate the gamma protein from its combination in solid solution with either beta or alpha chymotrypsin by fractional crystallization at pH 5.5.

**Solubility Curves of Artificial Mixtures of Crystals**

Increasing amounts of the stock suspensions of mixtures of crystals of two proteins were made up to 15 ml. with 0.4 saturated ammonium sulfate and stirred for 24-48 hours at 10°C. in the rocking machine as described before. The suspensions were then filtered and the filtrates were analyzed for protein content.

**Fig. 10.** Solubility curve of artificial mixture of crystals of alpha and beta chymotrypsin (65 per cent alpha + 35 per cent beta) in 0.4 saturated ammonium sulfate pH 4.0 at 10°C. in the presence of increasing quantities of solid phase. Slope of B C, measured = 0.31, calculated = 0.35.

Fig. 10 represents the solubility curve at pH 4.0 of a mixture of crystals of 65 parts of alpha and 35 parts of beta chymotrypsin. The curve corresponds to the theoretical curve of a mixture of two independent chemical substances, the solubility of either one of which is not affected by the presence of the solid phase of the other substance.

The same holds true for a mixture of crystals of alpha and gamma chymotrypsin in 0.4 saturated ammonium sulfate pH 5.5. The solubility curve shown in Fig. 11 corresponds to the theoretical curve of a mixture of two independent solid phases.¹

¹ The samples of the enzymes used in the “artificial mixtures” experiments contained small amounts of soluble impurities and this is shown by the fact that the solubility of the mixtures, as well as of the individual proteins, increases slightly with increase in the amount of solid phase even in the presence of a large excess of...
On the other hand, the solubility curves (Figs. 12 and 13) of the mixtures of gamma crystals with either alpha or beta chymotrypsin crystals in 0.4 saturated ammonium sulfate pH 4.0 show clearly that gamma crystals form a solid solution with either one of the other crystals. The data, however, are not sufficient to enable the quantity of the impurities to be determined so the exact values for the solubilities of the individual proteins used in these artificial mixtures experiments are unknown. Hence no comparison can be made between the solubility of the mixtures and the sums of the solubilities of the two components of each mixture.
proteins. It is to be observed that the order of the solubility of alpha and gamma chymotrypsin crystals at pH 5.5 is reversed from that at pH 4.0, the solubility of gamma crystals at pH 4.0 being about eight times as great as that of alpha chymotrypsin, while at pH 5.5 the crystals of alpha chymotrypsin are six times as soluble as the gamma crystals.

Fig. 13. Solubility curve of artificial mixture of crystals of beta and gamma chymotrypsin (35 per cent beta + 65 per cent gamma) in 0.4 saturated ammonium sulfate pH 4.0 at 10°C. The curve is of solid solution type.

Fig. 14. Cataphoresis measurements and isoelectric point determinations.
2. Cataphoresis.—Measurements were made of the rate of cataphoretic migration of collodion particles suspended in 0.01 M buffer solutions of various pH and containing 0.06 mg. of the various crystalline proteins per milliliter.

The results are given in Fig. 14 which shows that the isoelectric point is approximately identical for the three proteins.

3. Acid and Alkali Titration Curves.—Fig. 15 shows the pH titration curves for the various proteins. The data were corrected for free acid or base. The curves coincide on the acid side of the isoelectric points but diverge considerably in the range of pH 5.0–9.0.

Experimental Procedure.—The various crystalline proteins were dialyzed for 18 hours at 5°C. in running ½/200 hydrochloric acid by the method of Kunitz and Simms (4). 10 ml. samples of the dialyzed material made up in 0.1 M sodium chloride and containing a total of 3.1 gm. of protein were titrated with 0.1 M acid or alkali. The pH was measured by means of a low resistance glass electrode of Mouquin and Garman type (5) inserted in the titration vessel.

The corrections for free base in solution were negligible in the range of pH measured. The correction for free hydrochloric acid was made in the usual manner, assuming an ionic strength of $\mu = 0.1$ as due only to the salt and acid added; hence the activity coefficient of $\gamma H^+ = 0.84$ was used (6).

The isoelectric points were obtained from Fig. 14.
4. Denaturation in 0.1 N Hydrochloric Acid at 25°C.—The three proteins when made up in 0.1 N hydrochloric acid rapidly undergo denaturation which is demonstrable by the formation of protein insoluble in 1.0 M sodium chloride. Native protein is soluble in this concentration of sodium chloride.

Experimental Procedure.—10 ml. of a dialyzed solution of each material containing 1 mg. protein nitrogen per ml. was mixed with 10 ml. of 0.2 N hydrochloric acid and left at 25°C.

![Graph](image)

**Fig. 16.** Stability in 0.1 N hydrochloric acid at 25°C.

Samples of 2 ml. were taken at various times and neutralized with 2 ml. N/10 sodium hydroxide. 1.0 ml. of the neutralized solution was further diluted with N/400 hydrochloric acid to the proper concentrations for activity and protein nitrogen analysis while 2.0 ml. of the neutralized solution was added to 2 ml. 2 M sodium chloride made up in N/200 hydrochloric acid. The precipitate of denatured protein was filtered after 24 hours and the filtrate was analyzed for protein content; the amount of denatured protein was then found by difference.

The results are given in Fig. 16 which shows that alpha chymotrypsin is denatured in acid solution much faster than either beta or gamma.

5. Inactivation at pH 9.0 and 35°C.—The three enzymes when brought to pH 9.0 and kept at 35°C. undergo a gradual autolysis with a simultaneous loss of activity, but the rate of autolysis is more
rapid for beta or gamma than for alpha chymotrypsin as shown in Fig. 17.

A comparison of these results with those given in Fig. 16 shows that in acid solution alpha is less stable than beta or gamma, while at pH 9.0 the order is reversed and beta and gamma are less stable than alpha chymotrypsin.

In the range of pH 2.0-8.0 the stability of the three enzymes is about the same except for the gradual change of chymotrypsin into the new enzymes at pH above 4.0.

6. Inactivation by Urea.—The three enzymes partially lose their ability to digest proteins if exposed to the action of a concentrated urea solution. The effect of urea is almost instantaneous and is irreversible on dilution with water; the percentage loss of enzymatic activity increases with the concentration of urea used. The three enzymes, however, differ among themselves with respect to their stability in concentrated urea solutions as shown in Fig. 18, alpha being the most and gamma the least stable of the three enzymes.

7. Denaturation by Heat.—No significant difference has been found in the stability of the three enzymes when kept at 100°C. the rate of irreversible denaturation being the same for the three materials.
8. Chemical Composition, etc.—Table III contains the data for the elementary analysis as well as for some other chemical determinations. There is generally very little difference between the three enzymes in respect to most of the chemical tests. There is, however, a striking difference in the molecular weight of the three enzymes, the molecular weight of beta being 75 per cent and of gamma only 68 per cent of that of alpha.

![Graph showing inactivation by urea](image)

**Fig. 18. Inactivation by urea.** 1.0 ml. enzyme, 1.0 mg. protein nitrogen per ml. + 5 ml. of various concentrations of urea in water, 10 minutes at 20°C., then diluted 50 times with water for activity measurement in hemoglobin.

III. The Enzymatic Properties of the Various Chymotrypsin Proteins

The enzymatic behavior of the new crystalline proteins as well as of the original chymotrypsin towards various substrates is given in Table IV.

In practically all cases the rate of digestion by the three enzymes as measured by various methods at various pH is alike. Also the extent of ultimate digestion of casein or gelatin, as measured by the amount of free amino nitrogen formed, is identical for all three enzymes.

The three enzymes not only digest proteins with the same rate but
**TABLE III**

**Physicochemical Properties**

<table>
<thead>
<tr>
<th></th>
<th>α</th>
<th>β</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C</strong></td>
<td>49.80</td>
<td>50.00</td>
<td>50.06</td>
</tr>
<tr>
<td><strong>H</strong></td>
<td>7.32</td>
<td>7.38</td>
<td>7.16</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>15.83</td>
<td>15.81</td>
<td>15.69</td>
</tr>
<tr>
<td><strong>S</strong></td>
<td>0.24</td>
<td>0.27</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Ash</strong></td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Protein nitrogen as per cent total nitrogen (b)</strong></td>
<td>98</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td><strong>Amino nitrogen as per cent total nitrogen (c)</strong></td>
<td>5.0</td>
<td>5.3</td>
<td>5.5</td>
</tr>
<tr>
<td><strong>Tyrosine + tryptophane in milliequivalents per mg. total nitrogen (d)</strong></td>
<td>2.46</td>
<td>2.53</td>
<td>2.54</td>
</tr>
<tr>
<td><strong>Optical rotation (e); [α]_D per mg. nitrogen.</strong></td>
<td>-0.42</td>
<td>-0.41</td>
<td>-0.42</td>
</tr>
<tr>
<td><strong>Molecular weight (f), by osmotic pressure, average of six determinations</strong></td>
<td>40,000</td>
<td>30,000</td>
<td>27,000</td>
</tr>
<tr>
<td><strong>Ninhydrin (g)</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Biuret</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Millon</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Xanthoproteic</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(a) The elementary analyses were carried out by Dr. A. Elek in Dr. P. A. Levene's laboratory.

(b) Total and protein nitrogen in dialyzed solutions determined by micro-Kjeldahl method (7).

(c) Amino nitrogen in samples of about 2 mg. total nitrogen measured by Van Slyke's manometric method (8).

(d) 1.0 ml. containing 0.5 mg. total nitrogen + 1.0 ml. M/1 hydrochloric acid + 3.0 ml. water + 10 ml. 0.5 N sodium hydroxide + 3.0 ml. 1/3 dilution of phenol reagent of Folin and Ciocalteau (9). Color read after 10 minutes against a similar mixture containing 1 × 10⁻³ millimol tyrosine.

(e) Solution containing about 15 mg. total nitrogen per ml. (98-99 per cent protein nitrogen) was dialyzed for 24 hrs. against running M/200 hydrochloric acid at 5°C. Protein, total nitrogen, and optical rotation measured after dialysis.

(f) Osmotic pressure measurement by the method of Northrop and Kunitz (10). Inside of collodion bags: about 15 ml. enzyme solution containing about 60 mg. protein per ml. made up in 1.0 M or 0.5 M ammonium sulfate. Outside: same concentration of ammonium sulfate as inside but without protein. Triplicates were run for each salt concentration. Rocked for 48 hrs. at 5°C. Equilibrium was reached within 24 hrs. No effect of concentration of salt on the osmotic pressure was observed, proving that the Donnan effect was entirely eliminated. Protein concentration measured at the end of experiment.

(g) Ninhydrin test: 1.0 ml. enzyme containing 1.0 mg. nitrogen per ml. + 1.0 ml. 24 per cent pyridine in water + 0.2 ml.2 per cent ninhydrin (triketoxyhydrinden-hydrate) in water. Left at 25°C for 24 hrs., then made up to 25 ml. with water. No significant difference between the three materials was found in this test as well as in the other protein tests.
they also attack the substrate molecule in the same place as evidenced by the fact that when the digestion has reached its final stage by the action of any one of the enzymes it cannot be extended further by the addition of any of the other enzymes.

IV. The Mechanism of Formation of the New Enzymes

The process of formation of beta and gamma from chymotrypsin at 5°C. is accompanied by a loss of about 15 per cent of total protein and a simultaneous loss of about 40 per cent of activity; the last is due to the formation of inert protein. The transformation of chymotrypsin into gamma at 35°C. is accompanied by a loss of about 35 per cent of protein as well as by a loss of an equal portion of activity. The loss of protein as well as of activity at both temperatures proceeds at a rate approximately proportional to the rate of change of chymotrypsin into the new enzymes and the rate of loss becomes negligible after the change has been completed.

The new enzymes have lower molecular weights than the original chymotrypsin. In the case of gamma the percentage difference in the molecular weight corresponds almost quantitatively to the percentage loss in total protein during its formation from chymotrypsin at 35°C. It is thus evident that the formation of the new enzymes from chymotrypsin is essentially a hydrolytic process resulting in the cleavage of the chymotrypsin molecule. The nature of the products formed is determined by the conditions of pH and temperature. The cleavage, however, is not profound enough to affect qualitatively the enzymatic nor most of the protein properties of the molecule. The number of peptide linkages split is very small so that the observed differences either in the number of free amino groups or in the number of free carboxyl groups of the various materials are small and almost within the limits of experimental error.

The activity of beta and gamma when expressed in enzyme units per milligram protein nitrogen, or per milligram dry weight, does not differ much from that of the original chymotrypsin. The enzymatic activity thus decreases in proportion to the size of the molecule, assuming that both the molecular weight and activity measurements are certain.

The slow formation at 5°C. of beta and gamma as well as of inert
### TABLE IV

**Enzymatic Properties**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>( \alpha )</th>
<th>( \beta )</th>
<th>( \gamma )</th>
<th>Crystalline trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea hemoglobin (a) in [T.U.](^{\text{Hb}}) mg P.N.</td>
<td>0.04-0.05</td>
<td>0.035-0.045</td>
<td>0.040-0.045</td>
<td>0.17</td>
</tr>
<tr>
<td>Clotting of milk (b), rennet units/mg.</td>
<td>7-8</td>
<td>7</td>
<td>7-8</td>
<td>0</td>
</tr>
<tr>
<td>Casein in [T.U.](^{\text{Cas.}}) mg P.N. Non P.N. formation (c)</td>
<td>0.80</td>
<td>0.80</td>
<td>0.88</td>
<td>2.4</td>
</tr>
<tr>
<td>Casein in [T.U.](^{\text{Cas.}}) mg P.N. Formol titration (d)</td>
<td>0.08</td>
<td>0.087</td>
<td>0.087</td>
<td>0.18</td>
</tr>
<tr>
<td>Viscosity of gelatin pH 4.0 made up in 0.5 M ammonium sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[T.U.](^{\text{Gel. viscosity}}) mg P.N.</td>
<td>7.4</td>
<td>7.5</td>
<td>7.5</td>
<td>100</td>
</tr>
<tr>
<td>Edestin (e) [T.U.](^{\text{Ed.}}) mg P.N. ( \times 10^{-6} )</td>
<td>3.8</td>
<td>4.5</td>
<td>4.4</td>
<td>7.0</td>
</tr>
<tr>
<td>Native crystalline egg albumin (f) [T.U.](^{\text{Alb. S.}}) mg P.N.</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td>Carbobenzoxyglycyl-l-tyrosylglycine amide (g) at 35°C. Uni- molecular velocity constant per hour at 35°C. (digestion mixture containing 2.5 mg substrate and 0.025 mg enzyme protein per ml.)</td>
<td>0.38</td>
<td>0.32</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Benzoyl-l-tyrosyl-glycinamide (h). Uni- molecular velocity per hour at 5°C. (digestion mixture containing 2 mg substrate and 0.006 mg enzyme per ml.)</td>
<td>0.044</td>
<td>0.044</td>
<td>0.044</td>
<td>0</td>
</tr>
<tr>
<td>Other substrates:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sturin</td>
<td>No difference in the rate of digestion by ( \alpha, \beta, ) or ( \gamma ) was noticed.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clupein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native hemoglobin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Method of Anson and Mirsky (11). 1 ml. enzyme containing 0.01 mg protein nitrogen per ml. + 5 ml. hemoglobin. Digested 10 minutes at 35°C.

(b) Method of Kunitz (12).

(c) 10 ml. 5 per cent casein pH 7.6 + 2 ml. enzyme, 0.025 mg. protein nitrogen per ml. Digested at 35°C. Samples of 2 ml. + 2 ml. 10 per cent trichloracetic acid. Heated 10 minutes at 85°C. Filtered after 30 minutes. Micro-Kjeldahl on 2 ml. filtrate. For units see (13).
protein appears to be a simultaneous process, while at 35°C and pH 8.0 the hydrolysis is rapid and only gamma protein is formed. Exposure of pure beta protein to 35°C at pH 8.0 does not change it perceptibly into gamma protein which shows that beta is not an intermediate product in the process of transformation of chymotrypsin into gamma protein. On the other hand the solubility curve of beta crystals shows that even the highly purified material still contains from 10-15 per cent of one or more impurities which cannot be removed by recrystallization. It is thus possible that beta undergoes

(d) Procedure same as (c) except that 2 ml. samples were used for formol titration by the method of Northrop (14).

(e) 1 ml. enzyme, 0.02 mg. protein nitrogen per ml. + 5 ml. 5 per cent edestin (Hoffmann-La Roche) in 2 M sodium chloride made up in 0.1 M phosphate buffer pH 7.6. Digested 10 minutes at 35°C, then mixed with 10 ml. 5 per cent trichloracetic acid, heated 10 minutes at 85°C, filtered after 30 minutes. 5 ml. filtrate + 10 ml. 0.5 M sodium hydroxide + 3 ml. 1/3 phenol reagent. Color compared with standard tyrosine solution. [T.U.]\textsuperscript{Ed.} = millimoles tyrosine equivalents formed per 6 ml. digestion mixture per minute at 35°C.

(f) Freshly prepared crystalline egg albumin by the method of La Rosa (15). Recrystallized twice. 1 ml. enzyme 0.02 mg. protein nitrogen per ml. + 5 ml. 2 per cent egg albumin in m/10 phosphate buffer pH 7.6. Left at 35°C for 18 hrs. (+ toluene). The amount of non-protein nitrogen formed was then determined as in (c). [T.U.]\textsuperscript{Alb.} = milliequivalents non-protein nitrogen formed per minute per milligram protein nitrogen enzyme per 6 ml. digestion mixture. Evidently native egg albumin is slightly digested by the chymotrypsin but not by crystalline trypsin in agreement with the results reported by Balls and Lineweaver (16). The rate of digestion of egg albumin by the various chymotrypsins is about 1/800 of that of digestion of denatured casein.

(g) Artificial substrate specific for chymotrypsin synthesized by Drs. Bergmann and Fruton (17) who supplied us with several of their preparations. Procedure: 5 ml. substrate, 3 mg. per ml. in m/15 phosphate buffer pH 7.6 + 1 ml. enzyme, 0.025 mg. protein nitrogen per ml. at 35°C. Amino nitrogen in samples of 1 ml. measured by Van Slyke's manometric method. The velocity constant, 
\[ K = \frac{2.3 \log A_t}{A_s - A} \]  
was found to decrease slightly with time of digestion. It was extrapolated to zero time and recorded here as such.

(h) Kindly supplied by Drs. Bergmann and Fruton (17). Procedure: 25 ml. substrate, 2 mg. per ml. in m/15 phosphate pH 7.6 + 1 ml. enzyme, 0.025 mg. protein nitrogen per ml. at 5°C. Sample of 5 ml. + 1 ml. formaldehyde for formol titration with m/50 sodium hydroxide.
a further transformation either into gamma, which is very stable, or some other product.

The writer was assisted in this work by Margaret R. McDonald and Vivian Kaufman.

SUMMARY

A solution of chymotrypsin on slight hydrolysis undergoes an irreversible change into new proteins, two of which are enzymes and have been isolated in crystalline form. The new crystalline enzymes, called beta and gamma chymotrypsins, differ from the original chymotrypsin as well as from each other in many physical and chemical respects, such as molecular weight, crystalline form, solubility, and combining capacity with acid. The new enzymes still possess the same enzymatic properties as chymotrypsin. It thus appears that the irreversible change from chymotrypsin to the new enzymes does not affect the structure responsible for the enzymatic activity of the molecule.

The solubility curves of the new enzymes agree approximately with the curves for a solid phase of one component and furnish very good evidence that the preparations represent distinct substances. The various enzymes when mixed at the proper pH have a tendency to form mixed crystals of the solid solution type. Thus at pH 4.0 gamma chymotrypsin combines to form solid solution crystals with either alpha or beta chymotrypsin. Hence at this pH separation of gamma from either alpha or beta by means of fractional crystallization is impossible. At pH 5.0–6.0, however, each material crystallizes in its own characteristic form and at its own rate; thus a fractional separation of the various enzymes from each other becomes feasible.

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