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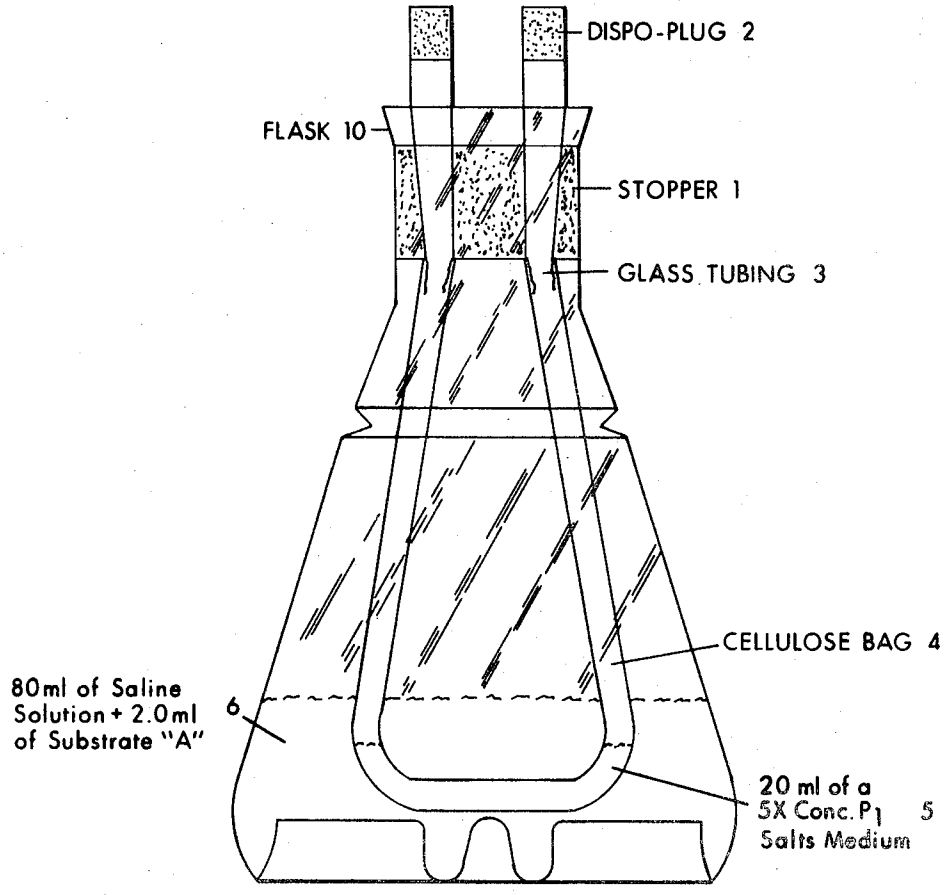


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# PRODUCTION OF AMINO ACIDS

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## ABSTRACT OF THE DISCLOSURE

Aerobic fermentation process for the production of extracellular amino acids which uses, as a primary source of carbon, a $C_1$–$C_{30}$ n-aliphatic hydrocarbon feed with an aqueous, inorganic salt growth medium, and a microorganism capable of growth on said n-aliphatic hydrocarbon feed, said process being conducted in the presence of cellulose. It is preferred that the n-aliphatic hydrocarbon be the sole source of carbon in the process, preferred n-aliphatic hydrocarbon feed being comprised primarily of $C_{11}$–$C_{30}$ n-paraffins.

Thus, the present invention is broadly concerned with a biosynthesis fermentation process for the production of high concentrations of extracellular amino acids, as well as using the residual cells for animal and human foods. More particularly, the invention is concerned with the use of $C_1$–$C_{30}$ n-aliphatic hydrocarbon feed, particularly $C_{11}$–$C_{30}$ n-paraffins as the primary source of carbon in the process. In accordance with this present invention, $C_{11}$–$C_{30}$ n-paraffins are contacted with a microorganism under fermentation conditions in the presence of cellulose to produce high yields of extracellular amino acids. This process may be carried out continuously or batchwise.

Heretofore, the microbiological production of amino acids, such as lysine, glutamic, isoleucine, valine, and the like, has reqiured the use of expensive carbohydrate substrates and precursors which had to be added to the fermentation broth. Moreover, the yields of extracellular amino acids obtained have been low and the rate of accumulation of the product very slow.



Another recent, and even more promising, technique for biologically synthesizing extracellular amino acids and food protein is by cultivating the microorganisms on petroleum substrates. This type of fermentation is usually conducted in an aqueous biosynthesis bath containing a hydrocarbon feed, an inoculant of the microorganism to be grown, an aqueous growth medium, oxygen, nitrogen and other indispensable nutrients. This technique allows the use of hydrocarbon feeds, which are widely available in the necessary quantities and are less expensive than carbohydrates. It is also known to use various biological catalysts in fermentation processes. The biosynthetic process of the present invention is applicable to the biosynthesis of all microorganisms which are capable of growth on $C_1$–$C_{30}$ n-aliphatic hydrocarbon feeds, particularly $C_{11}$–$C_{30}$ n-paraffins derived from petroleum hydrocarbon fractions.

While the present invention is applicable to a broad scope of operable bacterial microorganisms, there are a number of microorganisms which are especially suitable for hydrocarbon assimilation. These microorganisms are tabulated hereinbelow, along with their corresponding ATCC registration numbers which were secured by depositing samples with the American Type Culture Collection, 212 M Street, Northwest, Washington 7, D.C., or other designated numbers.

TABLE I

| Division | Class | Order | Family | Tribe | Genus | Species | No. ATCC |
|---|---|---|---|---|---|---|---|
| Protophyta | Schizomycetes | Pseudomonadales | Pseudomonadaceae | | Pseudomonas | Ligustri | 15522 |
| | | | | | | Pseudomallei | 15523 |
| | | | | | | Orvilla | 15524 |
| | | Eubacteriales | Micrococcaceae | | Micrococcus | Cerificans | 14987 |
| | | | | | Sarcina | Sp | QMB1518 Natick |
| | | | Brevibacteriaceae | | Brevibacterium | Insectiphilium | 15528 |
| | | | | | | Healii | 15527 |
| | | | Achromobacteracea | | Alcaligens | Sp | 15525 |
| | | | Corynebacteriaceae | | Cellumonas | Galba | 15526 |
| | | | | | Corynebacterium | Sp | 15529 |
| | | | | | | Pausometabolum | 15530 |
| | | | | | Arthrobacter | Simplex | 6946 |
| | | | | | | Sp | 19140 |
| | | Actinomycetales | Mycobacteriaceae | | Mycobacterium | Rhodchrous | |
| | | | Actinomycetaceae | | Nocardia | Sp | |
| | | | | | | Sp | |

The *Micrococcus cerificans* (14987), which was isolated and identified by Dr. R. E. Kallio et al., Journal of Bacteriology, vol. 78, No. 3, pages 441–448 (September 1959), is particularly desirable. Further identification is as follows:

## MORPHOLOGY

Cells are small, spherical, tending to be elliptical in old cultures and media high in nitrogen.

Cells from defined media average 0.5 to 1.0 microns in diameter, and from complex media cell diameters average 1.0 to 2.0 microns. Cells occur singly or in clumps. Immotile, metachromatic granules and pseuanophilic granules are not observed.

GRAM REACTION

Negative.

Colonies on defined agar are small (1 mm.), circular convex, having entire edge. Colonies on nutrient agar are larger (2 to 5 mm.), raised mucoid, generally round.

Within a species there can be many different strains comprising variations and both natural and induced mutants.

The morphology and growth reaction characteristics of other organisms listed above are given in U.S. Pat. 3,308,035 issued Mar. 7, 1967 entitled, "Process for Producing a High Protein Composition by Cultivating Microorganisms on an N-Aliphatic Hydrocarbon Feed," inventor John D. Douros, Jr.

The growth media comprise an aqueous mineral salt medium and excess oxygen. Oxygen is supplied to the cultivation substrate medium or broth in any form capable of being assimilated readily by the inoculant microorganism. Oxygen-containing compounds may also be used to supply oxygen as long as they do not adversely affect microorganism cell growth and the conversion of the oxidized hydrocarbon feed to microorganism cells. Oxygen may be supplied as an oxygen-containing gas, such as air at atmospheric or elevated pressure or oxygen-enriched air wherein the oxygen concentration may be up to 70% to 90%. In general, between about 0.1 and about 10, preferably between about 0.8 and about 2.5 volumes per minute of air are supplied to the reactor per volume of liquid in the fermentor.

Nitrogen is essential to biological growth. The source of nitrogen may be any organic or inorganic nitrogen-containing compound which is capable of releasing nitrogen in a form suitable for metabolic utilization by the growing microorganism. Suitable organic nitrogen compounds are, for example, proteins, acid-hydrolyzed proteins, enzyme-digested proteins, amino acid, yeast extract, asparagine, and urea. Suitable inorganic nitrogen compounds are ammonia, ammonium hydroxide, nitric acid or salts thereof, such as ammonium phosphate, ammonium citrate, ammonium sulfate, ammonium nitrate and ammonium acid pyrophosphate. A very convenient and satisfactory method of supplying nitrogen to the process is to employ ammonium hydroxide, ammonium phosphate or ammonium acid phosphate, which can be added as the salt per se or which can be produced in situ in the aqueous fermentation media by bubbling ammonia gas or gaseous ammonia through the broth or injecting aqueous ammonium hydroxide into the broth to which phosphoric acid was previously added, thereby forming ammonium acid phosphate.

In this way the desired pH range of about 3.0 to 8.5 is maintained and the requisite nitrogen supplied. If the microorganism comprises a yeast the preferred pH is in the range of 3.0 to 7.5 such as 4.0 to 5.0. If the microorganism comprises a bacteria the desired pH is in the range of 5.0 to 8.5, such as about 7.0. Ammonium hydroxide may be supplied to the biosynthesis bath in amounts of between about 0.08 and about 0.20, preferably between about 0.1 and about 0.15, gram of nitrogen per gram of dried cells produced. This amounts to between about 0.01 and about 1.0 wt. percent, preferably between about 0.1 and about 0.15 wt. percent, nitrogen based on the total biosynthesis bath.

In addition to the oxygen and nitrogen, it is necessary to supply requisite amounts of selected mineral nutrients in the feed medium in order to insure proper microorganism growth and maximize the assimilation of the oxidized hydrocarbon by the microorganism cells. Potassium, sodium, iron, magnesium, calcium, manganese, phosphorous, and other nutrients are included in the aqueous growth medium. These necessary materials may

The carbon source, preferably the sole carbon source, for the fermentation process is an n-aliphatic hydrocarbon feed. The n-aliphatic hydrocarbon feed contains from



potassium phosphate, potassium sulfate, potassium citrate, potassium acetate and potassium nitrate. Iron and phosphorous may be supplied in the form of their sulfates and phosphates, such as iron sulphate and iron phosphate. Usually, most of the phosphorous is supplied as ammonium phosphates. When either ammonium phosphate or ammonium acid phosphate is used, it serves as a combined source of both nitrogen and phosphorous for the microorganism cell growth.

One satisfactory composition for the fermentation media particularly for bacteria at the outset of fermentation is a follows:

| Component | Concentration (grams per liter) Can use | Usually use | Preferably use |
|---|---|---|---|
| $C_{11}$-$C_{30}$ n-aliphatic hydrocarbon | 4–120 | 5–80 | 10–50 |
| Cellulose* | 2–60 | 2. 5–40 | 5–25 |
| $K_2HPO_4$ | 0. 5–15 | 1–10 | 2–8 |
| $(NH_4)_2HPO_4$ | 5–15 | 7–13 | 8–13 |
| $NaSO_4$ | 0. 1–1. 0 | 0. 2–0. 9 | 0. 3–0. 5 |
| $FeSO_4 \cdot 7\ H_2O$ | 0. 002–0. 5 | 0. 005–0. 04 | 0. 01–0. 02 |
| $MgSO_4 \cdot 7\ H_2O$ | 0. 1–0. 7 | 0. 2–0. 6 | 0. 3–0. 3 |
| $MnSO_4 \cdot 7\ H_2O$ | 0. 002–0. 05 | 0. 005–0. 04 | 0. 01–0. 03 |
| NaCl | 0. 002–0. 05 | 0. 005–0. 04 | 0. 01–0. 08 |
| Water | Remainder to equal 100 wt. percent | | |

* Cellulose is preferably in strips such as about ⅛" to 2" wide and ½" to 6" long. Desirable strips are ¼" wide and 1" long.

Other optional mineral nutrients which may be included in trace amounts include:

| Component | Concentration (milligrams per liter) Can use | Usually use | Preferably use |
|---|---|---|---|
| $ZnSO_4 \cdot H_2O$ | 0–0. 4 | 0–0. 3 | 0–0. 2 |
| $Na_2MoO_4 \cdot H_2O$ | 0–0. 06 | 0–0. 05 | 0–0. 04 |
| $CoCl_2$ | 0–1. 2 | 0–1. 1 | 0–1. 2 |
| $H_3BO_3$ | 0–0. 08 | 0–0. 07 | 0–0. 06 |
| $CuSO_4 \cdot 5\ H_2O$ | 0–0. 3 | 0–1. 25 | 0–0. 2 |
| $CaCl_2 \cdot 6\ H_2O$ | 0–0. 14 | 0–0. 13 | 0–0. 12 |
| $NiCl_2 \cdot 6\ H_2O$ | 0–0. 01 | 0–0. 008 | 0–0. 006 |

The essential and optional nutrients may be supplied in the form of other salts or acids than those tabulated hereinabove.

A very satisfactory medium is prepared as follows:

$P_1$ Medium

| | Grams/liter of tap water |
|---|---|
| $(NH_4)_2HPO_4$ | 10 |
| $K_2HPO_4$ | 5 |
| $Na_2SO_4$ | 0.5 |

To the above is added 10 cc./liter of a salt solution A prepared as follows:

| Salt, solution A: | Grams/liter distilled water |
|---|---|
| $MgSO_4 \cdot 7H_2O$ | 40 |
| $FeSO_4 \cdot 7H_2O$ | 2 |
| $MnSO_4 \cdot 4H_2O$ | 2 |
| NaCl | 2 |

The foregoing $P_1$ medium has a pH of 7.8. A variation of the above is one in which phosphate is supplied in the form of phosphoric acid.

The temperature of the biosynthesis bath may be varied between about 20° C. and about 55° C. depending upon the specific microorganism being grown, but preferred temperatures when using bacteria are between about 25° C. and about 45° C. such as about 35° C. The pH is preferably in the range from 5.5 to 8.5 such as about 7.0. be supplied by any technique but are preferably supplied by their water-soluble salts.

Potassium may be supplied as potassium chloride, about 1 to 30 carbon atoms in the molecule preferably 11 to 30 carbon atoms. A desirable n-paraffin hydrocarbon feed contains from about 11 to 20 carbon atoms in the molecule, such as a feed of the following composition.

STRAIGHT CHAIN PARAFFIN MIXTURE
(SUBSTRATE A)

| | Weight percent |
|---|---|
| $C_{13}$ | 1.6 |
| $C_{14}$ | 4.8 |
| $C_{15}$ | 32.3 |
| $C_{16}$ | 29.8 |
| $C_{17}$ | 22.6 |
| $C_{18}$ | 8.1 |
| $C_{19}$ | 0.8 |
| | 100.0 |

Less than 0.01% aromatics.

The harvesting of the microbial cells and the amino acids accumulated in the fermentation broth can be done by suitable means. The cells may first be separated from the fermentation broth by centrifugation (e.g. closed bowl, liquid cyclones or hydroclones, evaporation (e.g. falling film, wiped film), filtration (e.g. micropore, dialysis, reverse osmosis), flocculation, settling and decantation (e.g. by adding flocculants, coagulants or filter aids or by changing pH or temperature) or any other method of separation or combination of methods. The separated, concentrated cells may then be dried by spray drying, drum drying, freeze drying, vacuum drying, tray drying, oven drying or any other drying procedure or combination of procedures to obtain a final product having extremely high protein content and no impurities detrimental to humans or animals.

The extracellular amino acids accumulated in the broth may be recovered by fractional crystallization or evaporation (e.g. falling film, wiped film), spray drying, drum drying, freeze drying, vacuum drying, tray drying, oven drying. Another general method of recovery would be the adsorption of the amino acids on ion exchange resins, followed by selective elution.

The cellulose is preferably cellulose acetate strips or cellulose bags which are prepared by treating wood pulp with acetic acid, acetic anhydride and sulfuric acid as a catalyst. The cellulose is fully acetylated (three acetate groups per glucose unit) and, at the same time, the sulfuric acid causes degradation of the cellulose polymer so that the product contains only about 200–300 glucose units per polymer chain. At this point in the process the cellulose acetate is partially hydrolyzed by the addition of water until an average of 2–2.5 acetate groups per glucose unit remain. This product is a thermoplastic.

As pointed out heretofore, unexpected, desirable results



are secured by the addition of the cellulose strips or cellulose bags to the fermentation process in that the production of extracellular amino acids is markedly increased.

The present invention may be more readily understood by reference to the drawing which illustrates one embodiment of the same. Referring specifically to the drawing, a container or flask **10** contains a two hole stopper **1**. Glass tubing **3** is inserted into the stopper **1**, to which is attached at the upper end a dispo-plug **2** and at the lower end a cellulose bag **4**. A concentrated $P_1$ salt solution **5** is positioned within the cellulose bag **4** which is positioned within the saline solution plus substrate A6.

In order to further illustrate the invention, fermentations were carried out as illustrated in the following examples.

## EXAMPLE 1

A sterile aerobic fermentation process was conducted in the apparatus illustrated in the drawing. 20 ml. of a sterile 5× concentrate of $P_1$ salts medium were added into the cellulose bag. 80 ml. of a sterile 0.85% saline solution containing 2.0 ml. of substrate A was introduced into the flask. The saline solution was inoculated with a 1% inoculum of *Micrococcus cerificans* ATCC No. 14987, which had been grown on substrate A for 24 hours, and the fermentation was conducted on a rotary mechanical shaker at 30° C. for 72 hours. The fermentation broth was periodically sampled, at which time the bacterial cells and other impurities were removed by centrifugation, and the amount of extracellular amino acids in the clear centrifugate was determined by the following assay methods. One such series of assay methods is described in the second edition of Microbiological Assay of the Vitamin B-Complex and Amino Acids (1952) by E. C. Barton Wright, Pitman Publishing Corp., New York, N.Y. Another assay method used was the Technicon Auto Analyzer which provides an automatic analytical system based upon the ion exchange chromatography system established by Spackman, Moore and Stein.

The following results were obtained.

TABLE II

[Production of extracellular amino acids using *Micrococcus cerificans* (14987)]

| | Mg./l. amino acids | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Alanine | 0 | 0 | 17 | 3,729 | 78 | 2,850 | 49 | 4,610 |
| Allo-isoleucine | 0 | 0 | ---- | 720 | P | 760 | -------- | 840 |
| Arginine | 0 | 0 | 18 | 1,520 | 87 | 780 | 60 | 1,810 |
| Aspartic acid | 0 | 0 | 21 | 4,120 | 109 | 2,540 | 68 | 4,610 |
| Cysteic acid | 0 | 0 | ---- | 260 | -------- | 210 | -------- | 260 |
| Cystine | 0 | 0 | ---- | P | -------- | P | -------- | P |
| Glutamic acid | 0 | 0 | 24 | 7,090 | 123 | 5,640 | 87 | 8,260 |
| Glycine | 0 | 0 | 29 | 3,070 | 144 | 2,030 | 78 | 3,160 |
| Histidine | 0 | 0 | ---- | 1,300 | P | 970 | P | 2,680 |
| Isoleucine | 0 | 0 | ---- | 1,370 | P | 450 | P | 1,830 |
| Leucine | 0 | 0 | 7 | 2,910 | 34 | 870 | P | 2,390 |
| Lysine | 0 | 0 | ---- | 3,650 | P | 2,660 | P | 3,970 |
| Methionine | 0 | 0 | ---- | 80 | P | P | P | P |
| Meth. sulfoxide | 0 | 0 | 5 | 440 | 25 | 440 | 10 | 920 |
| Ornithine | 0 | 0 | ---- | 5,100 | P | 3,640 | -------- | 2,120 |
| Phenylalanine | 0 | 0 | 25 | 5,440 | 124 | 4,680 | 268 | 3,790 |
| Proline | 0 | 0 | ---- | 1,240 | -------- | 950 | -------- | 1,800 |
| Serine | 0 | 0 | 19 | 4,260 | 94 | 1,850 | 80 | 3,250 |
| Threonine | 0 | 0 | 7 | 2,140 | 34 | 1,240 | P | 2,350 |
| Tyrosine | 0 | 0 | ---- | 590 | P | 100 | -------- | 920 |
| Valine | 0 | 0 | 10 | 2,350 | 50 | 1,230 | P | 2,490 |
| Total amino acids, mg./l. | 0 | 0 | 182 | 51,370 | 902 | 33,890 | 700 | 52,060 |
| Fermentation Conditions: | | | | | | | | |
| Inoculum M. cerificans (ml.) | 0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Substrate A (ml.) | 2.0 | 0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Cellulose bag | Yes | Yes | No | Yes | No | Yes | No | Yes |
| Fermentation time (days) | 3 | 3 | 1 | 1 | 2 | 2 | 3 | 3 |

NOTE.—P=Present trace amount.

## EXAMPLE 2

Additional aerobic fermentation operations were conducted using cellulose strips. A series of three sterile fermentations was carried out in 500 ml. fermentation flasks containing 100 ml. of $P_1$ medium, 2.0 ml. substrate A and 1.0 gram of cellulose strips (¼" x 1"). The medium was inoculated with a 1% inoculum of *Micrococcus cerificans* ATCC No. 14987. The fermentations were conducted on a rotary mechanical shaker at 300 r.p.m. at 30° C.

The fermentation flasks were periodically removed at 1, 2 and 3 days and the broth harvested and assayed as in Example 1. The results obtained are set forth in Table III.

TABLE III

| Amino acid | One-day fermentation mg./l. | Two-day fermentation mg./l. | Three-day fermentation mg./l. |
|---|---|---|---|
| Allo-isoleucine | 868 | 758 | 584 |
| Alanine | 2,555 | 3,610 | 2,710 |
| Arginine | 854 | 869 | 389 |
| Aspartic | 2,932 | 3,758 | 1,860 |
| S amino acids | 202 | 905 | 562 |
| Glutamic acid | 4,619 | 4,635 | 3,116 |
| Glycine | 1,914 | 4,733 | 2,783 |
| Leucine | 1,056 | 1,286 | 374 |
| Histidine | 1,321 | 1,152 | 977 |
| Isoleucine | 662 | 907 | 246 |
| Ornithine | 2,398 | 1,444 | 1,577 |
| Phenylalanine | 4,071 | 5,735 | 3,235 |
| Proline | 731 | 924 | 360 |
| Serine | 1,332 | 1,690 | 1,090 |
| Tyrosine | 243 | 523 | 124 |
| Valine | 1,345 | 1,243 | 553 |
| Total | 27,103 | 34,172 | 20,540 |

From the foregoing, it is evident that cellulose (strips or bags) substantially increase the yields of high quality amino acids.

What is claimed is:

**1.** Aerobic fermentation process for the production of extracellular amino acids which comprises incubating a fermentation broth with *Micrococcus cerificans* ATCC No. 14987, said broth comprising an aqueous inorganic salt growth medium, an oxygen-containing gas and a liquid petroleum hydrocarbon fraction as a primary source of carbon, conducting the fermentation process in the presence of cellulose acetate and under conditions adapted to promote cell growth whereby a high yield of extracellular amino acids is secured.



**2.** Process as defined by claim **1** wherein said hydrocarbon fraction comprises essentially $C_{11}$–$C_{30}$ normal paraffins.

**3.** Process as defined by claim **2** wherein said hydrocarbon fraction comprises $C_{13}$–$C_{19}$ normal paraffins and wherein the $C_{15}$–$C_{17}$ fraction is in excess of 80% by weight.

**4.** Process as defined by claim **1** wherein the cellulose is selected from the class consisting of cellulose acetate strips and cellulose acetate bags.

**5.** Process as defined by claim **1** wherein the pH is maintained in the range from 5.0 to 8.5 and wherein the temperature is maintained in the range from about 25° C. to 45° C.

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U.S. Cl. X.R.

195—100, 116, 127