fractions isolated from raw navy beans. The highest hemagglutinating activity lies in fractions 4 and 5, while fraction 3 is devoid of hemagglutinating activity but contains the highest trypsin inhibitor activity. The low trypsin inhibitor activity in all other fractions and the low hemagglutinating activity in fractions 1 and 2 may be due to incomplete separation during the isolation procedure.

Effects on growth and protein efficiency ratios obtained by feeding the various navy bean fractions to rats are presented in Table II. Rats fed the raw navy bean diet lost weight, consumed less food, and ultimately died within the experimental period of 28 days (experiment 1A). On the other hand, rats fed the autoclaved bean diet gained weight and consumed more food (experiment 1B).

All the fractions when included in the autoclaved bean diet significantly inhibited the growth of rats, as judged by protein efficiency ratio values when compared with rats fed the same fraction autoclaved (experiments 2, 3, 4, 5, and 6, Table II), the major growth-inhibiting fraction being fraction 4. Although fraction 5 contained the highest hemagglutinating activity and was included in the diet in 2.5 times greater quantity than fraction 4, it did not inhibit the growth of rats as significantly (experiment 6; $P < 0.05$) as fraction 4 (experiment 5; $P < 0.01$). It seems probable that hemagglutinating activity is not an essential factor for growth depression in rats fed raw navy beans. It remains to be seen whether the hemagglutinating activity and the growth-inhibiting activity of fraction 4 are due to the same or different factors. The work of Jaffe (*6*) and that of Honavar and coworkers (*5*) indicate that growth inhibition of rats fed black beans or kidney beans is due to their hemagglutinin content. However, it appears that the nutritional significance of hemagglutinins should be established carefully, especially in the light of very recent work of Funatsu (*4*), who separated the toxic activity from the hemagglutinating activity of ricin.

It may be that there are two types of hemagglutinins—one toxic and the other nontoxic. Fraction 5 may be identical to the nontoxic hemagglutinin isolated by Rigas and Osgood (*13*).

Experiment 4 (Table II) indicates that the navy bean trypsin inhibitor has a deleterious effect on the growth of rats. Growth depression observed from the inclusion of fraction 1 (experiment 2) and fraction 2 (experiment 3) in the diet is very difficult to rationalize. It is possible that the residual trypsin inhibitor activity present in them may account for the observed growth inhibition of rats. Recent studies of Saxena and coworkers (*14*) on raw soybean meal indicated that the major growth-inhibiting factor for chicks resides in the water-insoluble residue devoid of trypsin inhibitor activity. It may be that the growth inhibiting factor present in fraction 1 (insoluble fraction) is similar to that of soybean meal. The results of Rackis and coworkers (*12*) also indicated that there is no direct correlation between trypsin inhibitor activity of different soybean fractions and their influence on growth inhibition or pancreatic hypertrophy of rats.

In the light of the above discussion, the results presented in Table II could be explained in a different way. For example, a simple calculation of the trypsin inhibitor and hemagglutinin intake by rats fed various fractions (Table II, experiments 2A, 3A, 4A, 5A, and 6A) indicates that rats fed fraction 1 consumed the greatest while those fed fraction 4 consumed the least amount of trypsin inhibitor and hemagglutinins, and yet fraction 4 was the most growth-inhibitory. Therefore, it can be suggested that neither the hemagglutinin nor the trypsin inhibitor is toxic, but a toxic material was scattered throughout all the navy bean fractions in differing amounts. Fraction 4 appears to contain the highest and fraction 5 the least amount of that toxic material. Work is in progress to isolate and characterize a toxic factor contained in fraction 4.

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FATTY ACID RECOVERY

Recovery of Alpha-Ketoglutaric Acid from Crude Fermentation Liquors

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KOEPSELL, Stodola, and Sharpe (*5*) have found that the fermentation of glucose by certain strains of *Pseudomonas fluorescens* yields significant amounts of α-ketoglutaric acid. The object of this investigation was the development of a method for recovering the acid from such fermentation processes. The fermentation liquors contain pyruvic and α-ketoglutaric acids together with calcium carbonate and small amounts of other inorganic salts, glucose, cells, and other by-products of the fermentation. For this study fermentation liquors were provided by the Northern Utilization Research and Development Division of the Agricultural Research Service, Peoria, Ill. The particulars relative to the fermentation employed in the preparation of the crude fermentation liquors are given in Table I. The

The problem of surplus cereal grains can be partially alleviated by controlled fermentation to yield products, such as organic acids, which may be exploited industrially as foodstuffs, sequestrants, and other products. The acids must be obtained economically and profitable uses must be found. This investigation was conducted to find an economical and efficient method for recovering α-ketoglutaric acid from fermentation liquors. The method extracts the acid from an acidified broth, using cyclohexanone as the extraction solvent, separates it from the cyclohexanone by means of steam distillation, and precipitates it as its hydrated calcium salt. Yields of from 60 to 70% have been obtained from crude fermentation liquors.

Table I. Preparation of Crude Fermentation Liquors

ORGANISM, *Pseudomonas reptilivora* NRRL 6 bs. Organism was carried on slants of following composition (per cent):

Difco tryptone	0.5
Difco yeast extract	0.5
Glucose	0.1
K_2HPO_4	0.1
Difco agar	2.0
pH adjusted to 7.0	

Fresh slants were made biweekly.

INOCULUM. Transfers were made from fresh slants to 8 ml. of inoculum medium in a test tube. The tube was incubated 24 hours at 30° C. and used to inoculate a 500-ml. flask containing 100 ml. of inoculum medium. The flask was incubated for 24 hours at 30° C. on a rotary shaker, then used as inoculum for the production medium. The inoculum medium was as follows (per cent):

Glucose	5.0
Difco peptone	0.5
Difco yeast extract	0.5
$(NH_4)_2SO_4$	0.4
pH adjusted to 7.0	

PRODUCTION. The production medium had the following composition (per cent):

Glucose	9.0
$(NH_4)_2SO_4$	0.24
K_2HPO_4	0.11
$MgSO_4 \cdot 7H_2O$	0.05
Fe (ferrous ammonium sulfate or ferric chloride)	1 p.p.m.
$CaCO_3$	3.75

300 ml. of production medium were sterilized in Fernback flasks. The sterilized medium was inoculated with 3% inoculum. The flasks were incubated at 30° C. on a rotary shaker (200 r.p.m.) for 10 days. The resulting fermentation liquors were pooled, and supplied "as was" or centrifuged to remove cells.

presence of the organic acids was confirmed by paper chromatographic techniques (*5*) and the concentration of the α-ketoglutaric and pyruvic acids determined by the method of Friedemann and Haugen (*2*) as modified by Koepsell and Sharpe (*4*).

Previous to this study, two methods have been suggested for the recovery of α-ketoglutaric acid. Koepsell, Stodola, and Sharpe (*5*) used repeated extraction of an acidified and concentrated liquor, using ethyl acetate as the extraction solvent. Berger and Witt (*1*) used fractional crystallization of the calcium salt.

Experimental

The work of Koepsell, Stodola, and Sharpe (*5*) indicates that with a better extraction solvent, an extraction process for the recovery of the acid from the liquors might be both efficient and economical. A preliminary search was made in which a number of liquids were tested for their efficacy as solvents in terms of an extraction ratio, R, which is the ratio of the equilibrium concentration of α-ketoglutaric acid in the solvent phase to its concentration in the aqueous phase. These concentrations were determined by titration of samples of the aqueous phase with standard base before and after equilibration with each solvent (Table II).

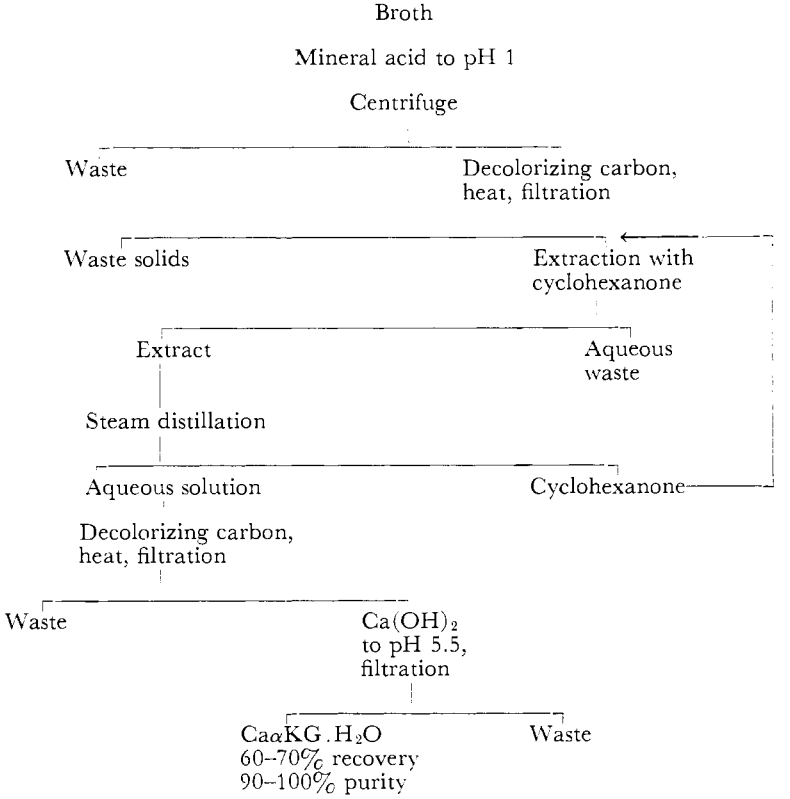
While R is not a true equilibrium constant, since it depends to some degree upon concentrations of foreign substances as well as α-ketoglutaric acid, the values obtained indicate strongly that cyclohexanone, isophorone, and possibly *tert*-amyl alcohol are the best solvents among those investigated. Further studies are summarized in Tables III and IV.

These results proved that cyclohexanone is the best solvent, although isophorone could also be used.

Process

Step 1. Acidification. Since the broth obtained from the fermentation process is a heterogeneous mixture of precipitated calcium salts, microbial cells, etc., it is necessary to free the acid and put it into solution. This is done

Flow Diagram for Isolation of α-Ketoglutaric Acid from Fermentation Broth



by adding a strong inorganic acid (sulfuric or hydrochloric acid) to a pH of 1. This forces most of the α-ketoglutaric acid into its un-ionized form and thus promotes more efficient extraction.

Step 2. Centrifugation. This step removes microbial cells and other debris which otherwise interfere with the succeeding steps. If sulfuric acid is used for the acidification, a large amount of calcium sulfate is removed in this step. To clarify the broth, very high speed centrifugation, such as with a Sharples supercentrifuge, is necessary.

Step 3. Treatment with Decolorizing Carbon. Although synthetic broths are ready for extraction after Step 2, the broths derived from the fermentation processes are colored, and show a strong tendency to emulsify with cyclohexanone. The emulsions are broken only after prolonged centrifugation. Boiling the broth with 1 gram of decolorizing carbon per 100 ml. of broth for 5 minutes removes some but not all of the color and, more important, effectively prevents the emulsification during extraction with cyclohexanone.

Table V shows that the decolorizing process does not significantly alter the α-ketoglutaric acid concentration. The slight increase in concentration may be attributed to loss of water by evaporation during boiling and filtration.

Step 4. Extraction. A further investigation of the extraction characteristics of cyclohexanone revealed that the solubility of water in cyclohexanone is 8.7% at 20° C. (*3*) while the solubility of cyclohexanone in water is 4.75% at 30° C. (*3*). Thus small but appreciable solvent losses will occur during the extraction process unless steps are taken to recover the cyclohexanone dissolved in the extracted fermentation broth.

Several measurements of the pH of aqueous solutions of α-ketoglutaric acid indicated that its first ionization constant is of the order of 6×10^{-3}. This value, along with a measured extraction ratio of 1.50 for a solution adjusted to a pH of 1, was used to estimate the variation of R with pH, and enabled one to predict that R should have a maximum value of 1.6 but would drop rapidly with an increase in pH. Experiments proved these predictions to be correct and a pH of 1.0 was chosen as the optimum for the process.

Step 5. Steam Distillation. Steam distillation provides an effective method for recovering α-ketoglutaric acid from cyclohexanone. The solvent and volatile impurities are distilled off, leaving an aqueous solution of the acid.

Known amounts of α-ketoglutaric acid were dissolved in cyclohexanone and reclaimed by steam distillation. Substantially 100% of the acid was recovered without any degradation. Similar runs with pyruvic acid yielded approximately 80% of this acid.

If the proposed process is to be economical on an industrial scale, it is necessary to re-use the recovered solvent continually. Checks were run in which the extraction characteristics of the cyclohexanone recovered by steam were compared with the pure solvent. The steam-recovered solvent which had been stripped with a 10% solution of sodium carbonate was essentially as good as the pure cyclohexanone.

Step 6. Decolorizing. It is advantageous to treat the solution recovered from the steam distillation with decolorizing carbon before the final precipitation. This removes some but not all of the yellow-brown coloration and results in a product of higher quality.

Step 7. Precipitation. Recovery of the α-ketoglutaric acid as a metallic salt through fractional crystallization provides the surest means of obtaining a good recovery of high purity. Several calcium, barium, sodium, and potassium salts were considered. Since no data on these salts were available, attempts were made to prepare the salts (*6*). The sodium and potassium salts were very difficult to prepare. The barium salt gave a poor yield, and, upon drying, appeared to be a mixture of the normal and acid salts. The calcium salt, however, was prepared easily in good yield. The product obtained after long drying at 100° C. was the monohydrated salt, as is shown in Table VI.

The solubility of calcium α-ketoglutarate monohydrate was found to be 0.36 gram per 100 ml. of water at 25° C. This was determined by equilibration of a known weight of the salt with distilled water for 5 days and determination of the weight loss of the solids. It was checked by analysis of the resulting solution for α-ketoglutaric acid.

Two methods were checked for their relative merits and for the optimal pH values to be used in the precipitation of calcium salt. In the first, solid calcium hydroxide is added slowly with vigorous stirring; in the second an excess of calcium chloride is added, after which the pH is adjusted with sodium hydroxide solution. A typical and uniform starting point for these investigations was provided by taking a large sample of

Table II. Extraction Ratios for α-Ketoglutaric Acid with Various Solvents and Water at 25° C.[a]

Solvent	C α-KG (in Solvent) / C α-KG (in Water)
Cyclohexanone	1.380
Isophorone	1.259
tert-Amyl alcohol	0.811
Benzyl alcohol	0.581
Cyclohexanol	0.546
Diethylcarbinol	0.454
Isoamyl alcohol	0.440
N-Amyl alcohol	0.398
Ethyl acetate	0.361

[a] In each case original solution had 4% of α-ketoglutaric acid and was extracted with an equal volume of solvent.

Table III. Concentration Dependence of *R* with Various Solvents

Solvent	Equilibrium Concn., μmoles/Ml. Aqueous	Solvent	R
Cyclohexanone	115.5	159.0	1.38
	59.1	79.1	1.34
	25.5	29.8	1.17
	13.2	14.4	1.10
Isophorone	119.5	150.9	1.26
	60.4	75.0	1.24
	25.7	28.4	1.10
	14.2	12.9	0.91
tert-Amyl alcohol	151.6	122.9	0.811
	78.1	60.1	0.770
	32.7	22.6	0.691
	16.4	11.1	0.677

Table IV. Extraction Ratios for Impurities with Various Solvents

	Cyclohexanone	Isophorone	*tert*-Amyl Alcohol
Pyruvic acid (1%)	0.804	0.697	0.499
Succinic acid (1%)	1.308	1.408	1.504
2-Ketogluconic acid (1%)	0.04	0	0
Glucose (0.3 g./100 ml.)	0	0	...
HCl (0.1*N*)	0.045	...	...

Table V. Effect of Decolorizing on Concentration of α-Ketoglutaric Acid

Sample	Concentration, μmoles/Ml. Before decolorizing	After decolorizing
1	229	239
2	221	228
3	305	310

Table VI. Composition of Calcium Salt of α-Ketoglutaric Acid

	% Ca	α-KG	% H_2O
Experimental	19.4[a]	72.7[b]	8.7[c]
Ca α-KG	21.7	78.3	0
Ca α-KG.H_2O	19.8	71.3	8.9
Ca (H α-KG)$_2$	12.1	87.9	0

[a] Analyzed by igniting samples to oxide and weighing; also by forming CaC_2O_4 and titrating with standard permanganate solution.
[b] Analysis by modified Friedemann and Haugen method (*2, 4*).
[c] Determined by weight loss at 100° C. under vacuum for several days.

Table VII. Results of Calcium Hydroxide Precipitations

$Ca(OH)_2$ Added, Gram	pH	Weight of Dry Precipitate, Grams	α-KG Concn. in Filtrate, μmoles/Ml.
0.45	3.7	0.19	
0.70	4.1	0.91	137
0.78	4.75	1.17	84
0.88	5.43	1.29	72
0.97[a]	6.10	1.14	106
0.90	6.65	1.21	88

[a] $Ca(OH)_2$ added and pH adjusted with concentrated HCl.

fortified natural broth and carrying it through the recovery process up to the point of the final precipitation. To test the first method six 25-ml. aliquots were taken and varying amounts of calcium hydroxide were added as shown in Table VII. The reaction proceeded slowly.

Table VIII illustrates the results of the second method, wherein calcium chloride and sodium hydroxide are added as the precipitating agent and shows that either method may be used, and neutralization need not be carried beyond a pH of 5 to 5.5. This will avoid the precipitation of calcium carbonate. To recover a higher percentage of α-ketoglutaric acid it is advisable to concentrate the filtrate and recycle it through the recovery process. The quality and purity of the salt can be improved by washing with several small portions of water before it is dried at 100° C. At this temperature the white monohydrate is obtained which is stable up to 170° C.

Table VIII. Results of Precipitation with Calcium Chloride and Sodium Hydroxide

NaOH added, Ml.	pH	Weight of Dry Precipitate, Grams	α-KG Concn. in Filtrate, μmoles/Ml.
1.97	3.60	0.73	...
2.34	4.08	1.20	97
2.66	4.80	1.26	87
2.84	5.57	1.20	90
2.86	5.86	1.28	88
2.90	6.38	1.17	96

Results

A number of small and several larger scale laboratory recovery runs have been made using the recovery process just described. The pertinent data are presented for the small scale (100- to 200-ml. initial volume of broth) runs in Table IX. In each case the starting material was a natural broth, in some cases fortified with extra α-ketoglutaric acid. All samples were acidified to a pH of 1.0 with the acid as indicated, and all precipitations are carried out by addition of calcium hydroxide to the final pH. Recovery is reported as per cent of theoretical for easy comparison.

These results represent no attempt to recover the α-ketoglutaric acid remaining in the filtrate after precipitation, and yields were presumed to be low because of the inevitable losses occurring in making filtration and separation on a small scale.

Several larger scale runs were also made; one involving a 4000-ml. sample, the other a 1300-ml. sample. Recovery in the first was 60%, and the product analyzed as 91% Ca α-KG.H_2O.

Better results were obtained in the second smaller run (1300-ml. sample) and the yield was 73%. The product was more thoroughly washed and analysis gave 100.8% of the monohydrated calcium salt. Again, no recovery from the filtrate was attempted.

Table IX. Recovery of α-Ketoglutaric Acid in Small Scale Runs

Initial α-KG Concn., μmoles/Ml.	Acid Used	Final pH	Recovery, %
209	H_2SO_4	5.0	69
222	HCl	4.5	41
226	HCl	4.9	63
239	HCl	4.5	61
239	HCl	4.5	60
240	HCl	4.7	60
305	HCl	5.0	62
322	H_2SO_4	5.2	69

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FUMIGANT RESIDUES

Residues in Milled Wheat Products Resulting from Spot Fumigation of Mill Machinery with Halogenated Liquid Fumigants

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SANITARY practice in flour mills involves a constant vigilance for infestation of any insects within the mill or mill machinery. When a problem with insects does occur, the most common practice is to control it by fumigation. The liquid halogenated fumigants are effective where the machinery is involved, being introduced directly into the machinery.

Studies have been made on residues resulting from fumigation with liquid grain fumigants as direct fumigants for grain (*1*, *5*). However, data are lacking on residues resulting from spot fumigation of mills and subsequent start-up of the milling operation. The purpose of this report is to show the safety of the use of fumigants containing ethylene dibromide, carbon tetrachloride, and ethylene dichloride in mill machinery from the standpoint of residues in products made from wheat going through the mill.