BIOSYNTHESIS OF FLAVOUR COMPONENTS IN BOURBON WHISKY

A Thesis

Presented to

The Faculty of Graduate Studies and Research

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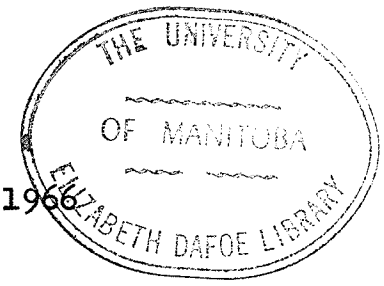
by

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LIST OF ABBREVIATIONS AND TRADE NAMES

$NADH_2$	Nicotinamide adenine dinucleotide, reduced form.
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
Tween 80	Polyoxyethylene sorbitan monooleate
PPO	2,5-Diphenyloxazole
POPOP	1:4-Bis(2-(5-phenyl)oxazolyl) benzene
Hyamine	2-Methyl-4-(1:1:3:3-tetramethylbutyl)-phenoxy-ethoxyethyl dimethyl benzyl ammonium hydroxide
(U)	Uniformly labelled
DMCS	Dichlorodimethylsilane

INTRODUCTION

Whisky is an alcoholic beverage distilled from a fermented grains mash. The main components of whisky are ethyl alcohol and water. Many other substances, however, are found in whisky in small amounts. They are the higher alcohols, organic acids, esters, aldehydes, ketones, furfural and hydrocarbons (Prescott and Dunn, 1959; Hirose, Ogawa and Kusada, 1962). These by-products, which are produced during the fermentation and partly modified during the storage of whisky, contribute largely to the flavour. The higher alcohols are quantitatively the major flavour components in whisky. The principal constituents of the higher alcohols are isoamyl alcohol, optically active amyl alcohol, isobutyl alcohol and n-propyl alcohol; of these, isoamyl alcohol is present in largest amounts. In the brewing industry, these higher alcohols have been studied to some extent, particularly the methods of their separation, determination, and biosynthesis. In the whisky industry, however, very few reports concerning the biosynthesis of higher alcohols are available.

The present study was undertaken to determine the biosynthesis of flavour alcohols during whisky fermentation with the aid of the isotopic tracer technique. Seven uniformly labelled amino acids were used in this study. The alcohols derived from each amino acid, the distribution of

radioactivity after fermentation and the effect of fermentation time on the biosynthesis of the higher alcohols were studied.

LITERATURE REVIEW

Fermentation With Leucine and the Biosynthesis of Isoamyl Alcohol

Spanyer and Thomas (1956) studied the fermentation with Saccharomyces cerevisiae of synthetic media containing dextrose and various amounts of leucine. After fermentation, fractional distillation and analysis, they found a straight line relationship between the added and the converted leucine up to a concentration of 1.23 g/l of added leucine, with 31 to 32 mole per cent of leucine converted to isoamyl alcohol. When the concentration of leucine was increased to 2.4 g/l, however, the conversion of leucine increased to 51 mole per cent.

Yamada, Yoshizawa and Sasaki (1958) found that when yeast fermented in a medium of sugar mixed with certain nitrogenous compounds other than leucine, a small quantity of amyl alcohol was always obtained. They concluded that the amyl alcohol was produced from leucine which originated from the yeast protein by autolysis.

Thoukis (1958) studied the synthesis of isoamyl alcohol by washed yeast cells of Saccaromyces cerevisiae var. ellipsoideus in 13 per cent sucrose solution by heavy inocula under non-proliferating conditions with the aid of the tracers L-leucine-C^{14}(U), L-leucine-5-C^{14}, DL-leucine-5-C^{15} and isovaleraldehyde-C^{14}. He found that 73 per cent of the radioactivity of L-leucine-C^{14} (U), 55 per cent of the

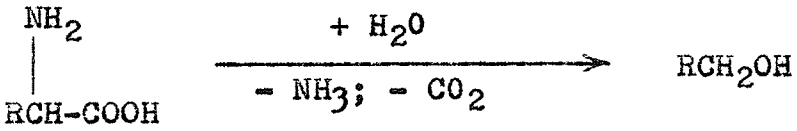
radioactivity of DL-leucine-5-C^{14} and 95 per cent of the radioactivity of isovaleraldehyde were incorporated into isoamyl alcohol. Radioactive CO_2 was found only from the uniformly labelled leucine and it accounted for 16.7 per cent of the original radioactivity of the L-leucine-C^{14} (U). Evidently, isovaleraldehyde and D-leucine could serve as precursors for the formation of isoamyl alcohol. Thoukis postulated that isoamyl alcohol could be formed from carbohydrate or from leucine with isovaleraldehyde as a possible intermediate in both pathways.

Yoshizawa et al (1961) fermented media of sucrose and various nitrogen sources with two different yeast strains. They found that a large quantity of isoamyl alcohol was formed from leucine. They used column distillation to concentrate the higher alcohols and gas chromatography for their separation. An 8-meter column with glycerol as stationary phase separated the optically active amyl alcohol and isoamyl alcohol.

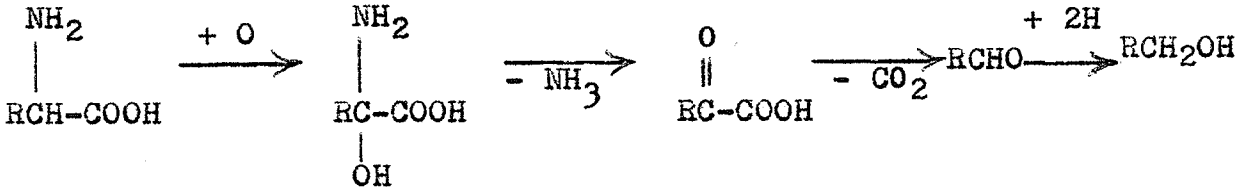
Suomulainen and Kahanpää (1963) studied the fermentation with baker's yeast of D-leucine, DL-leucine, L-leucine and L-leucine-C^{14} (U) in sucrose solutions. They found that D-leucine was neither metabolized nor converted into higher alcohols but that 65 per cent of the L-leucine was metabolized within 5 days. About 12 per cent of the original radioactivity of the L-leucine-C^{14} (U) was incorporated into isoamyl alcohol.

The CO_2 produced accounted for about 41 per cent of the radioactivity originally in the leucine.

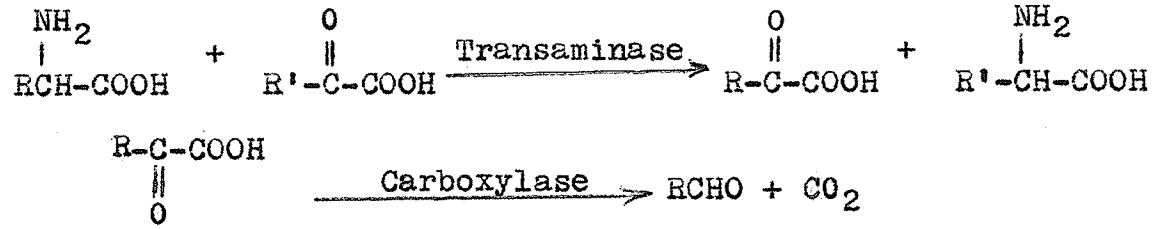
The mechanism for the biosynthesis of isoamyl alcohol and other higher alcohols has been studied since early this century. The investigations of Ehrlich (1906,1907a, 1907b, 1907c) led him to suggest that higher alcohols were produced during fermentation by deamination and decarboxylation of the amino acids according to the following reaction:



A modified scheme was proposed by Neubauer and Fromherz (1911) as follows:

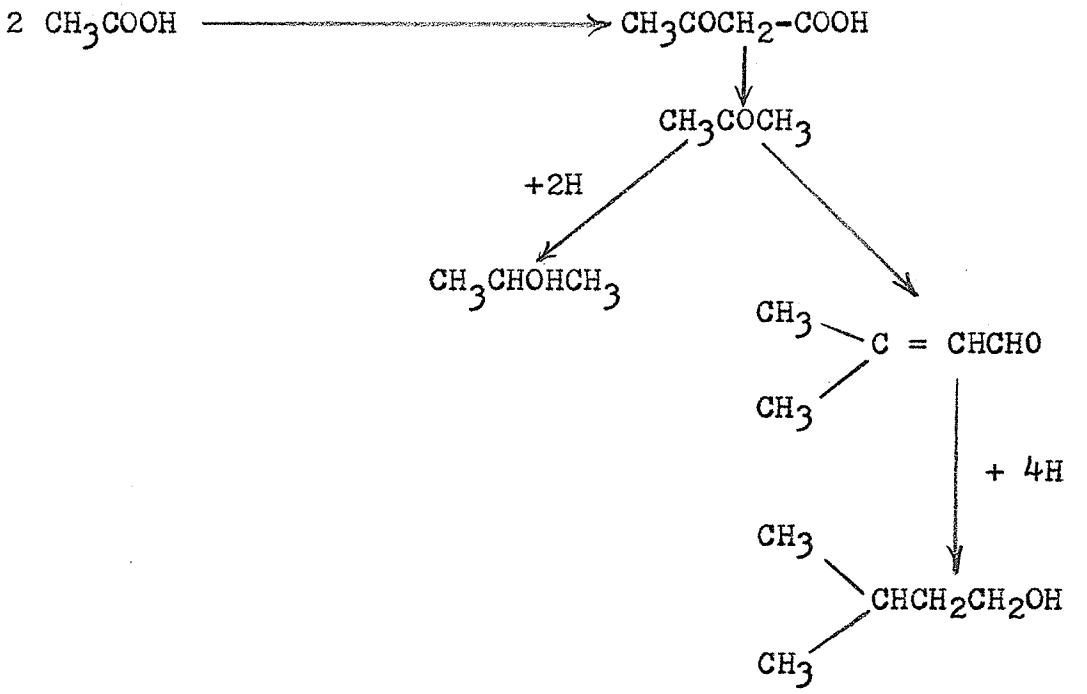


Sentheshanmuganathan and Elsden (1958) studied the fate of the amino group and the role played by glucose in the transformation of tyrosine to tyrosol. They proposed the following pathway:



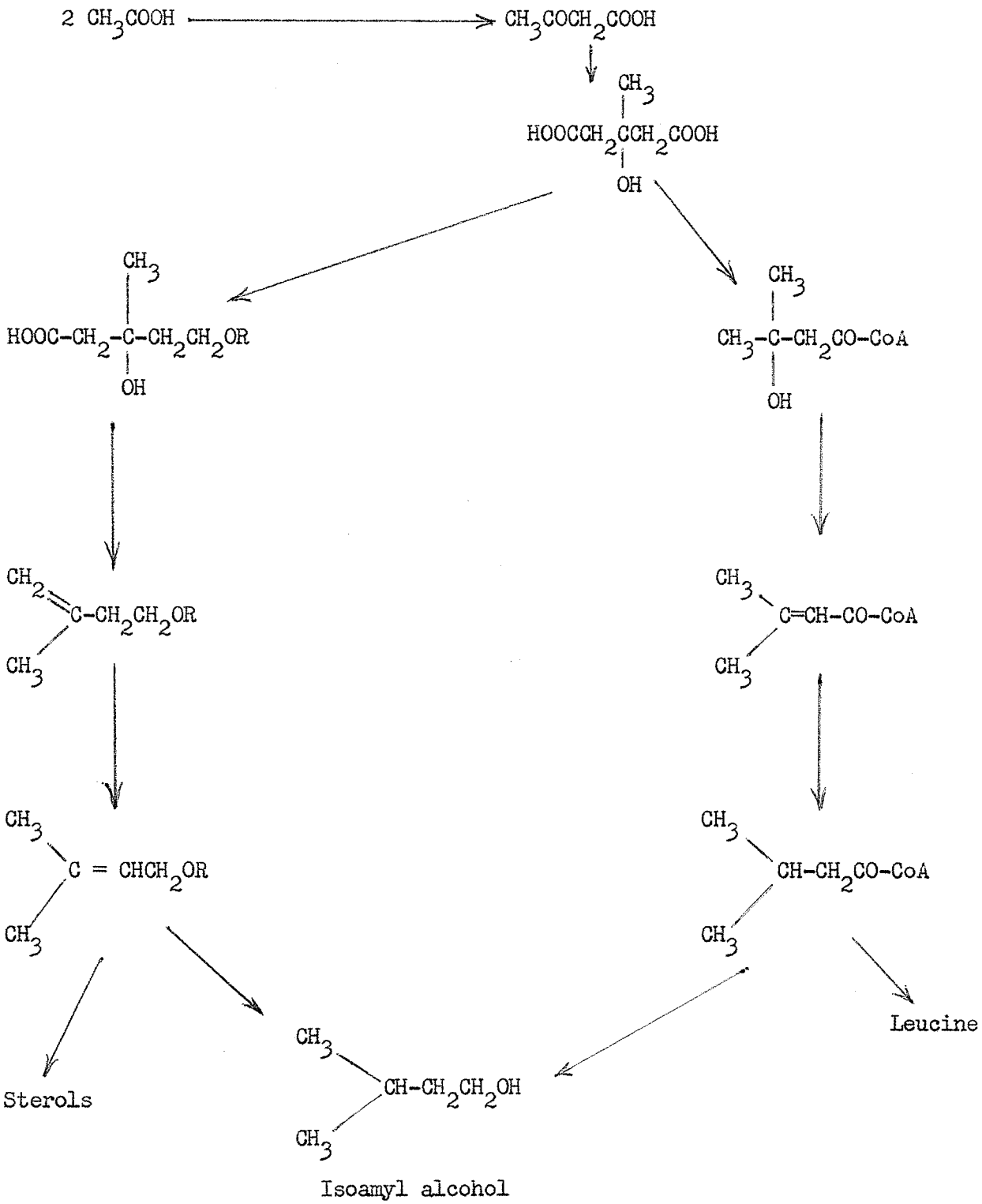
$$RCHO + NADH_2 \xrightarrow{\text{Alcohol dehydrogenase}} RCH_2OH + NAD$$

Genevois and Lafon (1957) observed that when radioactive acetate was added to a fermentation of sucrose with ammonium phosphate and asparagine as the only source of nitrogen, isoamyl alcohol with C^{14}-activity was formed. They suggested the following scheme for the synthesis of isoamyl alcohol:



Isoamyl alcohol

They also postulated that isoamyl alcohol might be formed from some of the intermediates in the biosynthesis of sterol and terpenes:



Isoamyl alcohol

Fermentation with Isoleucine and the Biosynthesis of Optically Active Amyl Alcohol

Yoshizawa et al (1961) studied the fermentation with two different yeast strains, Sake and brewer's yeast, of media of sucrose solution and isoleucine. They distilled the fermented mash, isolated the fusel oil by fractional distillation and analyzed it by gas chromatography. They found that about 50 per cent of the fusel oil was optically active amyl alcohol. The Sake yeast produced more fusel oil than the brewer's yeast.

Suomulainen and Kahanpää (1963) fermented media of sucrose and D-isoleucine, L-isoleucine or L-isoleucine-C^{14} (U) with baker's yeast. They found that the D-form was not metabolized but that 65 per cent of the L-form was metabolized within 5 days. About 15 per cent of the original activity of L-isoleucine-C^{14} was in the optically active amyl alcohol. The CO_2 accounted for about 40 per cent of the radioactivity originally in the isoleucine.

Peynand and Guimbertean (1959) found that with synthetic media up to 80 per cent of the amino acids leucine, isoleucine and valine were converted to isoamyl, active amyl, and isobutyl alcohol, respectively.

Optically active amyl alcohol formed from amino acids other than isoleucine, such as threonine and α-amino-n-butyric acid, will be reviewed later.

Fermentation with Threonine and the Biosynthesis of Related Alcohols

Yamada and Yoshizawa (1960) fermented synthetic media containing sugar, threonine and minerals with yeast Kyokai No.7. They isolated the fusel oil by fractional distillation and analyzed it by column chromatography. They report that 36.8 per cent of the threonine consumed was transformed into active amyl alcohol and that a large amount of succinic acid was produced.

In a similar experiment, Yoshizawa et al (1961) found that more than half of the higher alcohols formed was active amyl alcohol. n-Propyl alcohol was also found. When α-amino-n-butyric acid was added instead of threonine, active amyl alcohol constituted 63 per cent of the higher alcohols produced by Sake yeast and 66 per cent of the higher alcohols produced by brewer's yeast.

Yoshizawa and Yamada (1962) studied the formation of n-propyl alcohol and active amyl alcohol from threonine and α-amino-n-butyric acid by washed yeast cells. They reported that 34 per cent of the threonine assimilated and 40 per cent of the α-amino-n-butyric acid assimilated were converted to both n-propyl and active amyl alcohol. They also found that aeration decreased the formation of fusel alcohols and that the optimum pH value for their formation was 5.2.

Yoshizawa (1963) studied the formation of active amyl alcohol from α-keto-n-butyric acid using a cell-free extract of yeast. He reported that 40 per cent of the assimilated α-keto-n-butyric acid was converted to active amyl alcohol. He also found that the yield was highest at pH 5.2. Addition of NADP and NAD stimulated the synthesis while magnesium had no effect.

Yamada et al (1962) used 72 yeast strains in studies of the formation of higher alcohols from single amino acids. They found that n-propyl alcohol and active amyl alcohol both were formed from media containing threonine or α-amino-n-butyric acid. After 15 days' fermentation isoleucine was present in the media which indicated that isoleucine was an intermediate in the formation of the two alcohols.

Fermentation with Valine and the Biosynthesis of Related Alcohols

Yoshizawa et al (1961) studied the formation of higher alcohols in the fermentation of amino acids by two yeast strains. They analyzed the fusel oils by gas chromatography. They found that isobutyl alcohol constituted 87 per cent of the higher alcohol fraction formed from valine. Isobutyl alcohol was the major constituent of the higher alcohols formed also from glycine.

Yamada et al (1962) in their study of fusel oil forma-

tion, using 72 yeast strains and synthetic media with the addition of single amino acids, found that valine was formed from alanine, glycine or serine. Thus, from their earlier study (Yoshizawa et al, 1961), they concluded that isobutyl alcohol was formed from alanine, glycine or serine through the intermediate valine.

Suomalainen and Kahanpää (1963) fermented media of sucrose, D-valine, DL-valine and DL-valine-4-C^{14} with baker's yeast. They found that D-valine was not metabolized, and that 50 per cent of the L-valine in the DL-mixture was metabolized within 5 days of fermentation. Twenty-six per cent of the original radioactivity was incorporated in the isobutyl alcohol but no activity was found in the CO_2.

Yoshizawa (1964) fermented different strains of washed yeast cells and glucose solution containing acetolactic acid. He isolated the fusel oil fraction and analyzed it by gas chromatography. He found that isobutyl alcohol and α-keto-isovaleric acid were formed from acetolactic acid. He also studied the effect of aeration, vitamins and yeast strains. He found that aeration decreased the production of isobutyl alcohol and that yeasts which gave high yields of ethyl alcohol also gave high yields of isobutyl alcohol.

Fermentation with Alanine and the Biosynthesis of Related Alcohols

Yoshizawa et al (1961), in their fermentation of sugar solutions and alanine with Sake yeast and brewer's yeast, found that isobutyl alcohol constituted 55 per cent of the fusel oil when Sake yeast was used and 40 per cent when brewer's yeast was used. They concluded that isobutyl alcohol was formed from alanine. They also reported that the isoamyl alcohol peak by gas chromatography increased when alanine was added to the fermentation.

Suomalainen and Kahanpää (1963), in their fermentation of sugar solution and L-alanine with baker's yeast, found that 50 per cent of the L-alanine was metabolized and that there was some increase in the formation of isobutyl alcohol and isoamyl alcohol when alanine was added. No quantitative result was given for the formation of isoamyl alcohol or isobutyl alcohol.

Fermentation with Tyrosine

Sentheshanmuganathan and Elsden (1958) studied the formation of tyrosol from L-tyrosine with yeast. They found that the amount of tyrosol formed was proportional to the fermentation time and to the concentration of glucose in the medium up to 2.5 g/100 ml. With washed yeast cells, glucose was essential as an energy source in the formation

of tyrosol. The pathway of tyrosol formation was shown to involve transamination of tyrosine to yield p-hydroxyphenylpyruvate, decarboxylation of the pyruvate to yield p-hydroxyphenylacetaldehyde, and reduction of the aldehyde to yield tyrosol.

Sentheshanmugathan (1960) studied the transamination and decarboxylation of different amino acids including tyrosine with α-ketoglutarate by cell-free yeast extracts. He found that the amino groups of aspartic acid, leucine, n-leucine, isoleucine, valine, n-valine, methionine, tyrosine, phenylalanine and tryptophan were transferred to the α-ketoglutarate to give the corresponding α-keto-acids. He also found that the keto-acids α-ketoisocaproic acid, α-ketocaproic acid, α-ketoisovaleric acid and α-ketovaleric acid were rapidly decarboxylated by cell-free extract or purified yeast decarboxylase to give the corresponding aldehydes. Para-hydroxyphenylpyruvate, however, formed from tyrosine, was decarboxylated very slowly, even with large amounts of yeast decarboxylase. The aldehydes were reduced to the corresponding alcohols.

Fermentation with Serine

Very little work has been reported on the addition of serine to yeast fermentations. Yamada et al (1962), however, found that isobutyl alcohol was formed from serine

and suggested that valine was an intermediate.

Wang, Christensen and Cheldelin (1955) studied the biosynthesis of serine and found that it could be synthesized from glycine. Abelson and Vogel (1955), however, found that glycine and cysteine were formed from serine.

Fermentation with Other Amino Acids.

Ehrlich (1912) studied the fermentation of tryptophan and sugar with yeast. He found that tryptophol was formed.

Ingraham, Guymon and Crowell (1961) studied fermentation with a mutant strain of Saccharomyces cerevisiae. They found that n-valine gave n-butyl alcohol and n-amyl alcohol while n-leucine gave n-amyl alcohol.

Äyrapää (1963, 1965) studied the formation of phenethyl alcohol during fermentation of glucose solution containing nutrilites and buffering substances at pH 4.5 with yeast. He found that phenethyl alcohol was formed in the presence or absence of phenylalanine. The addition of phenylalanine increased the yield, but not in proportion to the amount added. He also used C^{14}-labelled phenylalanine to study the pathways of formation of phenethyl alcohol. He found that the Ehrlich pathway and the synthetic pathway were influenced by external factors in different ways. The percentage of phenylalanine transformed to phenethyl alcohol was proportional to the total nitrogen in the medium.

MATERIALS AND METHODS

Yeast Organism and Its Propagation

The yeast used in this study was *Saccharomyces cerevisiae* obtained from the Distillers' Corporation (Canada) Limited under their code number "Y-264". The yeast slant was prepared by inoculating on 5 per cent sterilized Bacto Wort Agar. The slant was incubated at 30°C for 7 to 10 days and stored at 6°C until used.

A stock solution of additives was made by dissolving 2.5 g ergosterol and 250 ml Tween 80 (Sentheshanmuganathan and Elsden, 1958) in 96 per cent ethanol with heating and making up to 1 liter volume with 96 per cent ethanol (Seagram, a).

Liquid media were prepared by diluting Malt Syrup (Standard Brands Limited, Ontario) to 18° Brix with tap water, adding 8 ml of the additives stock solution to 1 liter of this medium and sterilizing the solution in test tubes.

Yeast was inoculated from the slant into the liquid medium with a sterilized platinum needle and incubated at 30°C for 24 hours. After 24 hours' incubation, 1 ml of the culture was inoculated into another tube of liquid medium which again was incubated at 30°C for 24 hours. These daily transfers of cultures were repeated for two weeks before the culture was used for seeding a fermentation

(Seagram, b).

Corn, Rye and Malt and Their Amino Acid Analysis

Corn, rye and malt used in this study were supplied by the Distillers' Corporation. Corn, rye and malt were ground to pass through a U.S.B.S. 35 sieve, with a Wiley Mill or a Uawil-Switzerland Toronto Buhler Mill. The moisture contents of the ground materials were determined as weight difference by drying at 105°C overnight.

The amino acid contents of the ground corn, rye and malt were analysed by hydrolyzing the samples with 6N HCl under vacuum at 120°C for 16 hours, removing the HCl by vacuum evaporation at room temperature, redissolving the hydrolysate in 1/10 N HCl solution, and chromatographing this solution on an ion exchange column using a Technicon AutoAnalyzer.

Radioactive Amino Acids as Metabolic Tracers

Seven amino acids were used in this study. All were uniformly labelled with C^{14}. They were obtained from Radiochemical Center, Amersham, England through the Atomic Energy of Canada Limited. Technical data for the amino acids are shown in Table 1.

TABLE 1. Technical Data for Radioactive Amino Acids.

Amino acid	Specific Activity mc/mM	Specific Activity µc/mg	Radiochemical Purity*
L-leucine-C^{14} (U)	8.0	60.7	99%
L-isoleucine-C^{14}(U)	8.7	66.4	99%
L-threonine-C^{14}(U)	5.47	46.0	99%
L-valine-C^{14} (U)	6.9	59.0	99%
L-alanine-C^{14}(U)	20.2	227.0	98%
L-serine-C^{14}(U)	7.42	70.6	99%
L-tyrosine-C^{14}(U)	6.2	28.4	99%

* Determined by the supplier by paper chromatography.

The radioactivity of 1 µc (microcurie) is equal to 2.22×10^6 dpm (disintegrations per minute). In this report all radioactivities are expressed as dpm rather than cpm (counts per minute) in order to take into account the efficiency of the counter.

Preparation of Mash

Standard bourbon mashes composed of 65 per cent corn, 23 per cent rye and 12 per cent malt were used throughout this study. The total amount of corn, rye and malt in one mash was 43.7 g.

Tap water (280 ml) was placed in a 600 ml mash beaker and the pH adjusted to 4.0 with 2.5 N H_2SO_4. Under continual

stirring, ground malt (0.5 g) was added, the container was heated to 38°C, the ground corn (28.4 g) and rye (10.1 g) were added gradually, the pH was adjusted to 5.5 with 2.5 N H_2SO_4, the contents were heated to 100°C for 30 minutes, the container was cooled to 63°C, the remainder of the malt (4.7 g) was added, and stirring was continued for 30 minutes at 63°C. The mash was cooled to 30°C and transferred with about 15 ml sterilized water to a fermentor (a 500-ml round-bottom flask with ground glass joint). The mash thus prepared was about 250 ml in volume.

Fermentation

After the mash had been transferred to the fermentor, 10 ml of liquid yeast culture suspension and 1 - 10 μc of a radioactive amino acid in 0.2 - 2.0 ml water solution were added. The fermentor was connected to a CO_2 trapping bottle and placed in a water bath at 30°C. Fermentations were carried out at 30°C for one, two, three, five and ten days in one, two or four replicate experiments.

Carbon Dioxide Trapping

Carbon dioxide produced during fermentation was trapped in 200 ml of a 4N solution of ethanolamine in methylcellosolve contained in a gas washing bottle with a sintered glass filter on the gas dispersion tube.

Isolation of Flavour Concentrate

As soon as the fermentation time was completed, the mash was shaken for 1 - 2 min. to drive out dissolved CO_2. The fermentor flask was connected to a distillation splash head and condenser and the contents were distilled until about 99 ml had been collected in a volumetric flask. The residue from this distillation was designated Residue A. The contents of alcohol and volatile acids in the distillate were determined. The distillate was subsequently transferred to a 250-ml quick-fit flask, three boiling stones were added, and the contents were distilled through a 36-cm Vigreux column. The heating, provided by a flame, was regulated to give a slow distillation (1.5 hours total time) in order to minimize the amount of water in the final distillate. The distillation was stopped when the temperature at the top of the column reached 90°C; the inner surface of the column was then covered with water condensate. The distillate (flavour concentrate) which measured about 19 ml, was designated Fractional distillate, and the distillation residue was designated Residue B.

Determination of Fermentation Products

(a) Alcohol

The 99 to 100 ml distillate was kept at 20°C in a

water bath and made up to 100 ml with distilled water. The specific gravity of the distillate was obtained by comparing the weight of the distillate with that of the same volume of distilled water at 20°C. The alcohol content (calculated as ethyl alcohol) was found from the specific gravity from a standard conversion table (Hodgman, 1947).

(b) Volatile acids

The total distillate, after determination of specific gravity, was titrated with a standard 0.1 N NaOH solution. The amount of volatile acids in the distillate was expressed in milliequivalents (meq).

(c) Carbon dioxide

Since the trapping solution was of known volume and concentration, the amount of CO_2 produced was found by titration of the excess of ethanolamine present. A 30 per cent $BaCl_2$ solution (10 ml) and distilled water (20 ml) were added to 5 ml of the well mixed trapping solution and titrated with standard 1 N HCl solution to an end point of pH 8.3 by pH meter.

Preparation of Gas Chromatographic Columns

Two copper columns, A and B, were used.

Column A (10 feet x 1/4 inch O.D.) was packed with Chromosorb W, 30-60 mesh, which had been DMCS-treated and coated with 20 per cent of its weight of Carbowax 1540 and 0.2

per cent each of Altakerge T, Span 80 and Carbowax 400. This column gave a good separation of the alcohols with the exception of isoamyl and optically active amyl alcohol which emerged as one peak.

Column B (22 feet x 3/16 inch O.D.) was packed with Chromosorb W, 60-80 mesh, which had been DMCS-treated and coated with 6.8 per cent of triethanolamine (Sihto et al, 1962) under 29 inches of vacuum. This column gave more theoretical plates than Column A and separated isoamyl and active amyl alcohol. It was used mainly for the separation and identification of these isomers. Most peak collection work was done with Column A because of its shorter retention time.

Gas Chromatography and Peak Collection

An Aerograph Model L-90-P (Wilkens Instrument & Research Inc., California) with a thermal conductivity detector was used. The operating conditions for the two columns are shown in Table 2.

Collector tubes were made of glass tubing, 5 to 6 mm I.D., 40 to 50 cm long, slightly indented at 1-inch intervals, and bent into a U-shape. For collection of a single peak two collector tubes were connected in series to the outlet of the gas chromatograph and cooled in liquid nitrogen. Collection of the small amounts of flavour alcohols present

in the distillates gave incomplete and inconsistent recoveries. Improved recoveries of 90 to 95 per cent were usually obtained by increasing the amounts of alcohols to be chromatographed. This was effected by adding the corresponding cold alcohols (0.5 ml of a mixture of equal volumes of n-propyl alcohol, isobutyl alcohol, isoamyl alcohol and active amyl alcohol) to the radioactive alcohols contained in 2 ml of a sample of flavour concentrate for analysis. Usually 9 µl of this sample were injected into the column. When a peak had been collected, the tubes were disconnected, removed from the cooling bath, left 1 or 2 min to approach room temperature, and washed with about 1 ml ether (anhydrous, analytical grade) four times. The combined ether solutions were used for analysis of radioactivity.

TABLE 2. Operating Conditions for the Gas Chromatographic Columns

	Column A	Column B
Column temperature	110°C	90°C
Inlet temperature	175°C	170°C
Detector temperature	165°C	165°C
Outlet temperature	140°C	140°C
Helium flow rate	60 ml/min	42 ml/min
Filament current	150 mA	150 mA

Assay of Radioactivity

A Nuclear-Chicago Liquid Scintillation Counting Machine, Series 720, was used. The counting efficiency was obtained from the channel ratio - efficiency standard curve (Appendix). All radioactive samples except those collected after gas chromatography were counted in duplicate.

Four kinds of liquid scintillator counting solutions were used.

Scintillator solution A, for non-aqueous samples:

PPO	4 g
POPOP	50 mg
Toluene	1 liter

Scintillator solution B, for aqueous samples:

PPO	4 g
POPOP	100 mg
Naphthalene	50 g
Ethylene glycol monoethyl ether	167 ml
Dioxane	833 ml

Scintillator solution C, for flavour concentrate and collected peaks:

A mixture of equal volumes of solutions A and B.

Scintillator solution D, for CO_2 counting:

PPO	6 g
Toluene	667 ml
Methyl cellosolve	333 ml

Radioactivity in amino acids

An amino acid to be analysed was dissolved in a known volume of distilled water. An aliquot was pipetted into 10 ml of scintillator solution B and counted. The dpm in the amino acid was calculated.

Radioactivity in CO_2

One milliliter of the trapping solution was pipetted into 15 ml of scintillator solution D and counted for 40 minutes. The total activity in the CO_2 was calculated.

Radioactivity in the fractional distillate

One milliliter of the fractional distillate (flavour concentrate) was pipetted into 10 ml of scintillator solution C and counted. The total activity in the fractional distillate was calculated.

Radioactivity in residue B

One milliliter of residue B was pipetted into 10 ml of scintillator solution B and counted. The total activity in residue B was calculated.

Radioactivity in supernatant and yeast

Residue A was made up to 250 ml with water and blended in a Waring Blender for about 3 min to obtain a homogeneous

suspension. A 5-ml aliquot was pipetted out and mixed with 5 ml water in a centrifuge tube. The sample was centrifuged at 10,000 rpm for 40 minutes. The supernatant was decanted and made up to 10 ml with water. An aliquot (0.2 ml) was pipetted into a counting vial and mixed with 0.5 ml hyamine. The counting vial was heated to dissolve the protein particles (Herberg, 1958). Scintillator solution C (10 ml) was added and the sample was counted. The centrifuged sediment was transferred to a combustion flask with water and dried at 105°C overnight. The sample was oxidized by using the Van Slyke and Folch (1940) wet combustion method with the reagents used by Van Slyke, Plazin and Weisiger (1951) and the Stutz and Burris (1951) apparatus.. The reagents used were prepared as follows:

Wet reagent: 50 ml conc H_2SO_4, 50 ml H_3PO_4 (sp.gr. 1.7) and 1.5 g KIO_3 were heated together until all solids had dissolved.

Dry reagent: Two parts of KIO_3 and one part of $K_2Cr_2O_7$ (by weight) were ground together to a fine powder.

Dry reagent (1.5 g) was added into the flask, three boiling stones were added, 10 ml of wet reagent were added to the flask from a dropping funnel. The whole system was flushed with CO_2-free nitrogen gas. The gas stream from the combustion flask was passed through a washing column containing a solution of 4 per cent $KMnO_4$ in 2 N

H_2SO_4 and then through the CO_2-absorption device. The combustion flask was heated with a bunsen burner, at first gently, later more strongly. A greenish-brown color indicated the completion of the oxidation. The CO_2 evolved was absorbed by 10 ml of a solution of ethanolamine in methylcellosolve (2:1, by volume). The absorption device consisted of a centrifuge tube and a Vigreux column. After the combustion was completed, nitrogen gas was allowed to flush the system for 2 to 3 minutes. An aliquot (1 ml) of the CO_2 trapping solution was pipetted into 15 ml of scintillator solution D and counted. The activity in the centrifuged sediment (yeast) was calculated.

Radioactivity in gas chromatographic flavour peaks

The gas chromatographic peak in ether solution was mixed with 10 ml of scintillator solution C and counted. The total activity and the percentage incorporation in the alcohol corresponding to the chromatographic peak was calculated. The mole per cent conversion of amino acid to alcohol was calculated from the percentage incorporation taking into account the number of carbon atoms in the two components.

Flow Diagram of Fermentation and Analytical Procedures

A complete experiment for the assessment of flavour formation from a single, radioactive amino acid consisted

in fermentation, distillation, fractionation of flavour components and radioactivity counting as shown in Figure 1. The samples underlined in the figure were analysed by counting radioactivity.

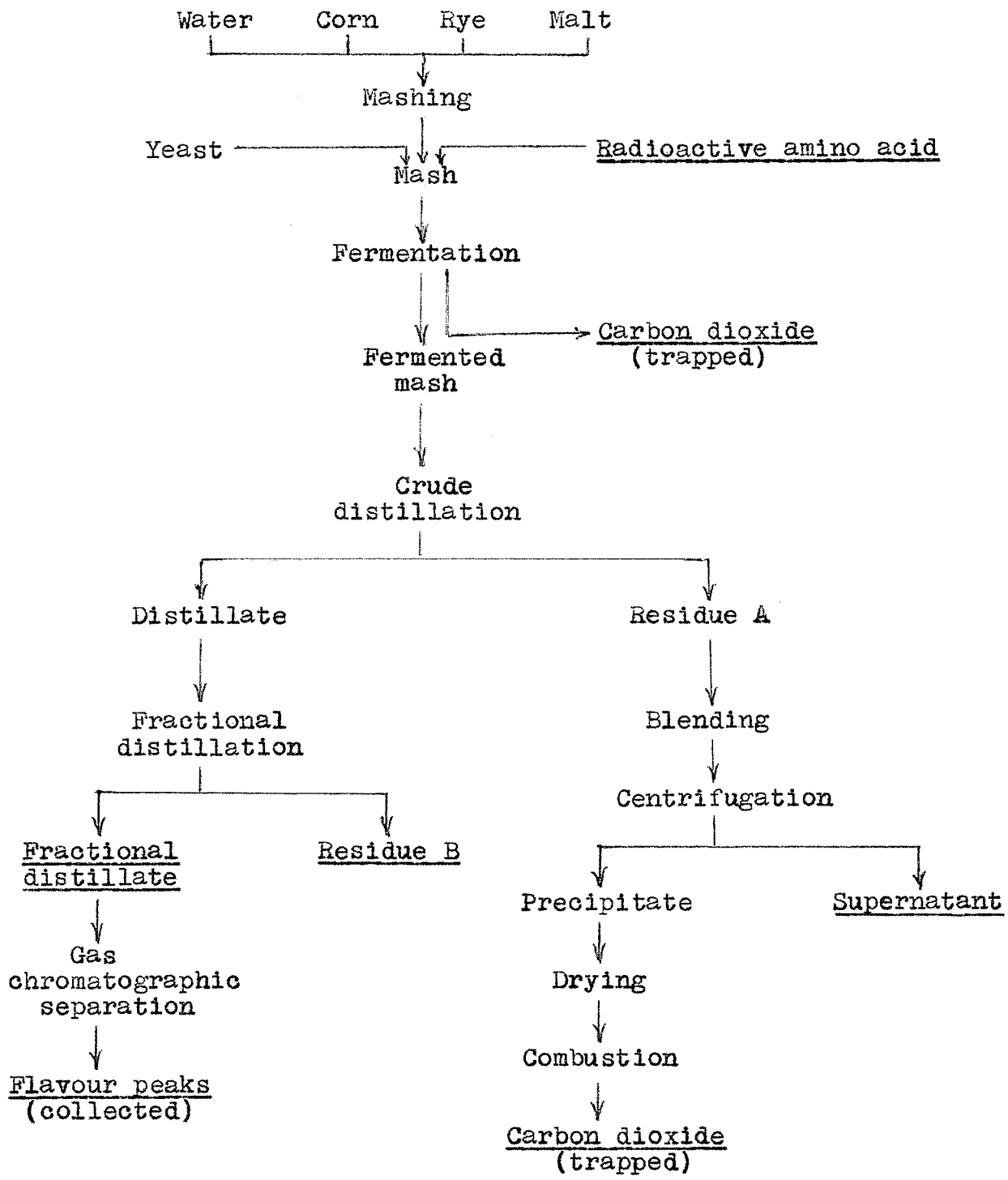


FIGURE 1. Flow Diagram of Fermentation and Analytical Procedures

RESULTS

Contents of Amino Acids and Moisture in the Raw Materials

The corn, rye and malt used for fermentation were analysed for moisture content and amino acid composition (Table 3). The amino acids are expressed as per cent by weight on dry basis for the corn, rye and malt. The amino acid contents were also calculated for a standard mash containing a mixture of 28.4 g corn, 10.1 g rye and 5.2 g malt and expressed in grams amino acid per standard mash.

Quantity of Carbon Dioxide, Alcohol, and Volatile Acid Produced in Fermentation

The quantities of CO_2, alcohol and volatile acid produced during fermentation of a standard mash for different lengths of time are summarized as the averages of 8 to 73 experiments (Table 4).

TABLE 4. Carbon Dioxide, Alcohol and Volatile Acid Produced in the Fermentation of Standard Mashes.

Fermentation time	CO_2 (g)	Alcohol (g)	Volatile acid (meq)
1 day	6.5	7.7	0.069
2 days	12.0	14.1	0.146
3 days	12.1	14.3	0.100
5 days	12.1	14.6	0.121
10 days	12.3	14.4	0.169

TABLE 3. Amino Acids and Moisture Contents in Corn, Rye and Malt.

	Corn	Rye	Malt	A standard mash
Moisture (%)	10.8	10.4	6.0	
Amino acids	(%)	(%)	(%)	(grams)
Aspartic acid	0.60	0.92	0.94	0.28
Threonine	0.32	0.39	0.40	0.14
Serine	0.45	0.51	0.44	0.18
Glutamic acid	1.90	3.22	2.35	0.89
Proline	0.96	1.46	1.25	0.44
Glycine	0.35	0.52	0.49	0.16
Alanine	0.78	0.57	0.58	0.28
Valine	0.51	0.68	0.66	0.22
Cystine	0.14	0.26	0.20	0.07
Methionine	0.14	0.17	0.14	0.06
Isoleucine	0.38	0.50	0.48	0.16
Leucine	1.16	0.81	0.82	0.41
Tyrosine	0.32	0.25	0.29	0.12
Phenylalanine	0.49	0.54	1.08	0.23
Lysine	0.26	0.46	0.50	0.13
Histidine	0.31	0.32	0.30	0.12
Arginine	0.36	0.63	0.51	0.17
Total	9.43	12.21	11.43	4.06

Gas Chromatography

Gas chromatographic analyses of a sample of fractional distillate by two different columns are shown in Figs. 2 and 3. Retention times and number of theoretical plates are listed in Table 5.

TABLE 5. Retention Times and Number of Theoretical Plates with Columns A and B.

Alcohols	Column A		Column B	
	Retention time(min)	Number of plates*	Retention time(min)	Number of plates*
Ethyl alcohol	2.8	125	11.0	482
n-Propyl alcohol	4.8	1470	16.2	1050
Isobutyl alcohol	6.2	960	18.2	915
Optically active amyl alcohol	11.4	1230	31.5	1760
Isoamyl alcohol	11.4	1230	33.5	1960

* Number of theoretical plates = $16\ (T/W)^2$, where T is the retention time in minutes and W is the peak width in minutes.

Effect of the Addition of L-leucine-C^{14}(U)

Various amounts of radioactivity, from 17×10^5 to 106×10^5 dpm, were added to standard mash batches and fermented for three days in two to four replicate experiments. A straight line relationship was found between the amount

of radioactivity added and the amount incorporated in isoamyl alcohol and CO_2 (Fig.4). About 24 per cent (23.81 per cent $\pm$ 1.38) of the original radioactivity was found in the distillate (Fig.4), most of it in the isoamyl alcohol (22.72 per cent $\pm$ 2.23). The notation ($\pm$ 1.38) used throughout this thesis refers to calculated standard deviation. About one per cent of the original activity was found in residue B. This was possibly due to incomplete distillation. When residue B was injected into the gas chromatograph, however, the only peak detected at the highest sensitivity was ethyl alcohol. About 5 per cent (5.35 per cent $\pm$ 0.18) of the original radioactivity was incorporated in the CO_2. Since under anaerobic conditions the amount of CO_2 is a good indicator for the progress of the fermentation, data on incorporation of radioactivity into CO_2 are included in Fig.4 and later related figures for reference. After 3 days of fermentation, 53 per cent of the original radioactivity had been incorporated into the yeast cells and 15 per cent remained in the supernatant.

The effect of fermentation time on the synthesis of isoamyl alcohol from L-leucine-C^{14}(U) was also studied. The results are shown in Fig.5. About 60 per cent of the final radioactivity in the isoamyl alcohol synthesized from leucine-C^{14}(U) was incorporated in the first day of fermentation, and

85 per cent after two days of fermentation. The incorporation was slowly completed during the succeeding two days.

The percentage distribution of radioactivity between different fractions after fermentation for 1 to 10 days with the same amount of L-leucine-C^{14}(U) was determined (Fig.6). In the first day about 60 per cent of the original activity was incorporated in the yeast and about 15 per cent left in the supernatant; at the end of 10 days of fermentation, only about 20 per cent remained in the yeast while about 50 per cent was found in the supernatant. The radioactivity in the yeast decreased with increasing fermentation time while the radioactivity in the supernatant increased with increasing fermentation time. The radioactivity incorporated into the CO_2 remained unchanged after 3 days of fermentation at 5.3 per cent of the original activity added. After 3 days the fermentation had apparently ceased and no more CO_2 was evolved. The incorporation of radioactivity in isoamyl alcohol, however, was only completed after five days fermentation when it reached about 26 per cent of the original activity added.

Effect of the Addition of L-isoleucine-C^{14}(U)

Various amounts of radioactivity from 20 x 10^5 to 121 x 10^5 dpm of L-isoleucine-C^{14}(U) were added to standard mash batches and fermented for three days in two to four replicate experiments. A straight line relationship was

obtained between the amount of radioactivity added and the amount of activity incorporated into optically active amyl alcohol and CO_2 (Fig.7). About 21 per cent (20.72 per cent $\pm$ 1.67) of the original radioactivity added was incorporated into the active amyl alcohol and one per cent into residue B. The radioactivity incorporated in the CO_2 was 4.8 per cent (4.84 per cent $\pm$ 0.41).

A study of the effect of fermentation time showed that 56 per cent of the final radioactivity in the active amyl alcohol was incorporated in the first day of fermentation and 85 per cent after two days of fermentation (Fig.8). The incorporation was slowly completed in the following two days.

The distribution of radioactivity between different fractions after fermentation for 1 to 10 days with the same amount of radioactivity added was determined (Fig.9). The results were similar to those for leucine. In the first day, 57 per cent of the radioactivity added was incorporated in the yeast and 14 per cent in the supernatant. The radioactivity in the yeast decreased with increasing fermentation time while the radioactivity in the supernatant increased with increasing fermentation time. At the end of 10 days of fermentation, only 21 per cent remained in the yeast while 47 per cent was found in the supernatant. The radioactivity incorporated in the CO_2 was 4.8 per cent

after 3 days of fermentation and remained unchanged up to 10 days. The fermentation was nearly completed after 3 days of fermentation.

Effect of the Addition of L-threonine-C^{14}(U)

Various amounts of radioactivity from 20 x 10^5 to 123 x 10^5 dpm of L-threonine-C^{14}(U) were added to standard mash batches and fermented for three days in single or four replicate experiments. About 20 per cent (19.46 per cent ± 0.53) of the original radioactivity was found in the distillate. Ethyl alcohol,n-propyl alcohol and optically active amyl alcohol were radioactive. A straight line relationship was found between the amount of radioactivity added and the amount incorporated in each of the three alcohols and CO_2 (Fig.10). About 8 per cent (8.08 per cent ± 0.48) of the original radioactivity added was incorporated in the ethyl alcohol, 9 per cent (8.95 per cent ± 0.51) in the optically active amyl alcohol, 2 per cent (2.08 per cent ± 0.35) in the n-propyl alcohol and 5 per cent (5.11 per cent ± 0.14) in the CO_2.

A study of the effect of fermentation time showed that about 78 per cent of the final radioactivity in the alcohols was incorporated in the first day of fermentation (Fig.11). The incorporation was nearly completed after two days of fermentation. During the first day, however,

the rate of incorporation of radioactivity for optically active amyl alcohol was lower than that for ethyl alcohol.

The distribution of radioactivity between different fractions after fermentation for 1 to 10 days with the same amount of radioactivity was determined (Fig.12). The results were similar to those for leucine and isoleucine. In the first day, 50 per cent of the original radioactivity was incorporated in the yeast and 23 per cent in the supernatant. At the end of 10 days, only 24 per cent was left in the yeast while 51 per cent was found in the supernatant. The incorporation of radioactivity into CO_2, ethyl alcohol, n-propyl alcohol, and optically active amyl alcohol was completed after two days of fermentation as shown in Figs. 11 and 12.

Effect of the Addition of L-valine-C^{14}(U)

Various amounts of radioactivity from 20 x 10^5 to 123 x 10^5 dpm of L-valine-C^{14}(U) were added to standard mash batches and fermented for three days in single or four replicate experiments. About 28 per cent (27.72 per cent $\pm$ 0.55) of the original radioactivity was found in the distillate. In this fraction, isobutyl alcohol and isoamyl alcohol were radioactive. A straight line relationship was found between the amount of radioactivity added and the amount incorporated in each of the two alcohols

and in CO_2 (Fig.13). About 14 per cent (13.53 per cent ± 0.67) of the original radioactivity was incorporated in the isobutyl alcohol, 14 per cent (13.50 per cent ± 0.56) in the isoamyl alcohol, and 10 per cent (9.40 per cent ± 0.19) in the CO_2.

Variation of the fermentation time (Fig.14) showed that about 85 per cent of the final radioactivity in the alcohols was incorporated in the first day of fermentation. The incorporation was nearly complete after the second day of fermentation except for isoamyl alcohol. In the first day, the rate of incorporation of radioactivity into isoamyl alcohol was lower than that for isobutyl alcohol.

The distribution of radioactivity between different fractions after fermentation for 1 to 10 days with the same amount of radioactivity added was determined (Fig.15). The radioactivity in CO_2 and isobutyl alcohol remained unchanged after two days of fermentation. In the first day, 14 per cent of the radioactivity was incorporated in the supernatant while at the end of 10 days of fermentation 46 per cent of the radioactivity was found in the supernatant.

Effect of the Addition of L-alanine-C^{14}(U)

Various amounts of radioactivity from 18 x 10^5 to 112 x 10^5 dpm of L-alanine-C^{14}(U) were added to standard mash batches and fermented for three days in single or four

replicate experiments. About 25 per cent (25.38 per cent ± 0.22) of the original radioactivity was found in the distillate. In this fraction, ethyl alcohol, isobutyl alcohol, isoamyl alcohol and optically active amyl alcohol were radioactive. A straight line relationship was found between the amount of radioactivity added and the amount incorporated in each of the alcohol fractions and in CO_2 (Fig.16). About 15 per cent (15.32 per cent ± 0.42) of the original radioactivity was incorporated in the ethyl alcohol, 7 per cent (6.61 per cent ± 0.47) in the amyl alcohols (largely isoamyl alcohol and a small amount of active amyl alcohol), 3 per cent (2.93 per cent ± 0.37) in the isobutyl alcohol and 29 per cent (28.77 per cent ± 0.32) in the CO_2.

A study of the effect of fermentation time on the synthesis of alcohols showed that about 85 per cent of the final radioactivity in the alcohols was incorporated in the first day of fermentation (Fig.17). The incorporation was nearly completed after the second day of fermentation.

The distribution of radioactivity between different fractions after fermentation for 1 to 10 days with the same amount of radioactivity added was determined (Fig.18). The results for alanine differed from those of the earlier amino acids in that the radioactivity incorporated in the

CO_2 was much higher, up to nearly 30 per cent of the original activity. The distribution of radioactivity between CO_2, ethyl alcohol, isobutyl alcohol and isoamyl alcohol did not change after the second day of fermentation. The distribution of radioactivity between the yeast and the supernatant changed up to the 10th day of fermentation but not as much as with leucine, isoleucine, threonine and valine.

Effect of the Addition of L-serine-C^{14}(U)

Various amounts of radioactivity from 20 x 10^5 to 175 x 10^5 dpm of L-serine-C^{14}(U) were added to standard mash batches and fermented for three days in single or four replicate experiments. About 7 per cent (6.59 per cent $\pm$ 1.82) of the original radioactivity was found in the distillate, mostly in the ethyl alcohol (6.38 per cent $\pm$ 1.88). The amount of radioactivity added and the amount incorporated in the ethyl alcohol did not give a straight line relationship (Fig.19). A nearly straight line relationship, however, was obtained between the amount of radioactivity added and that incorporated in the CO_2 (Fig.19).

Variation of the fermentation time showed that about 70 per cent of the final radioactivity in the ethyl alcohol was incorporated in the first day of fermentation (Fig.20). The incorporation was nearly complete after two

days of fermentation.

The distribution of radioactivity between different fractions after fermentation from 1 to 10 days was determined (Fig.21). In the first day of fermentation, nearly 60 per cent of the radioactivity was incorporated in the yeast cells and 30 per cent in the supernatant. At the end of 10 days of fermentation, only 36 per cent remained in the yeast while 55 per cent was found in the supernatant.

Effect of the Addition of L-tyrosine-C^{14}(U)

Various amounts of radioactivity from 22 x 10^5 to 223 x 10^5 dpm of L-tyrosine-C^{14}(U) were added to standard mash batches and fermented for three days in single experiments. A straight line relationship between the amount of radioactivity added and the amount incorporated in the CO_2 was found (Fig. 22a). Only about 3 per cent (2.55 per cent ± 0.06) of the original radioactivity was incorporated into CO_2. No component in the distillate was found radioactive. The radioactivity distribution is shown in Fig. 22b as average values for seven experiments with different amounts of radioactivity, all fermented for three days. About 58 per cent of the radioactivity was incorporated in the yeast and 33 per cent in the supernatant.

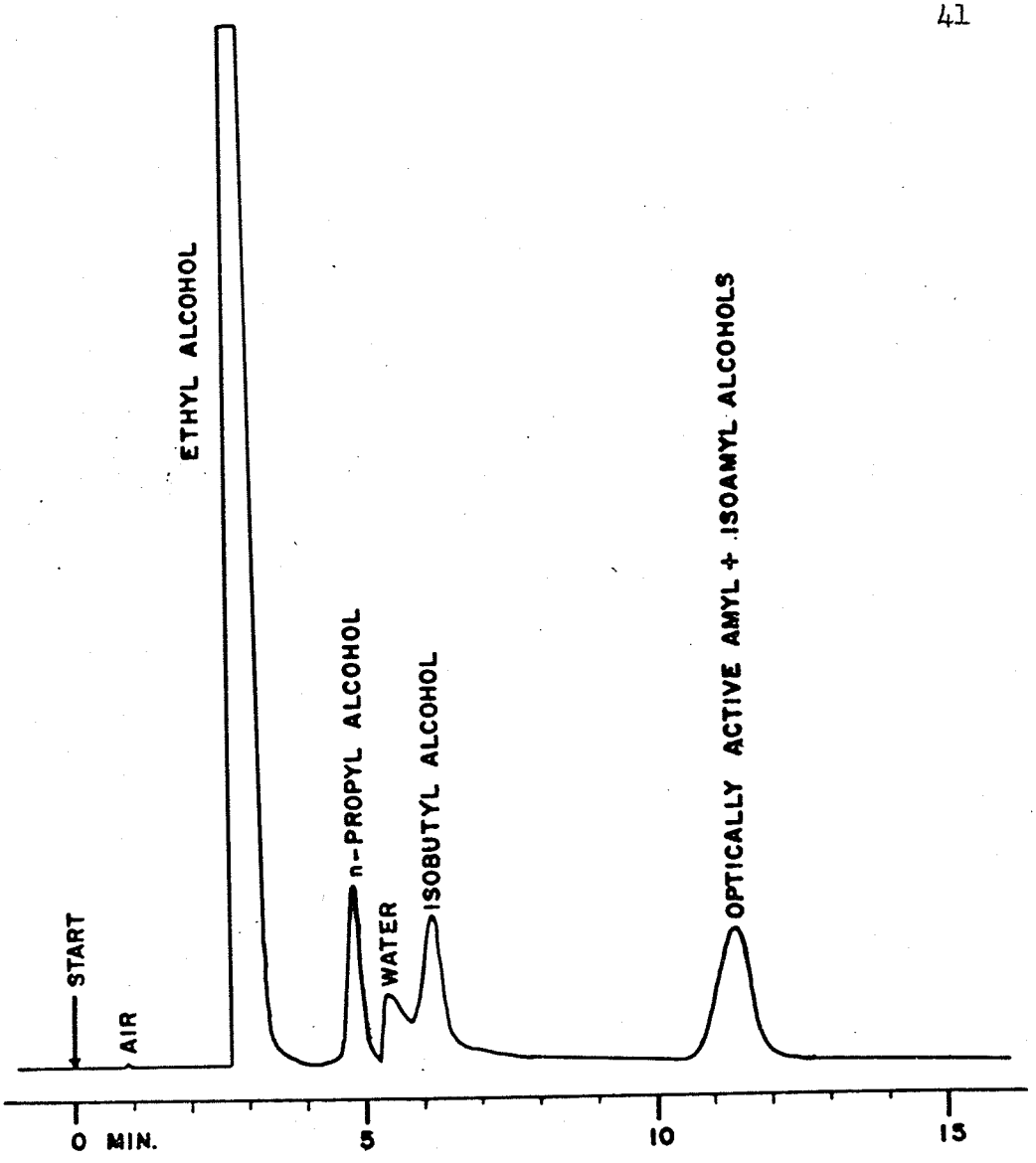


FIG. 2 GAS CHROMATOGRAM OF FRACTIONAL DISTILLATE USING COLUMN A

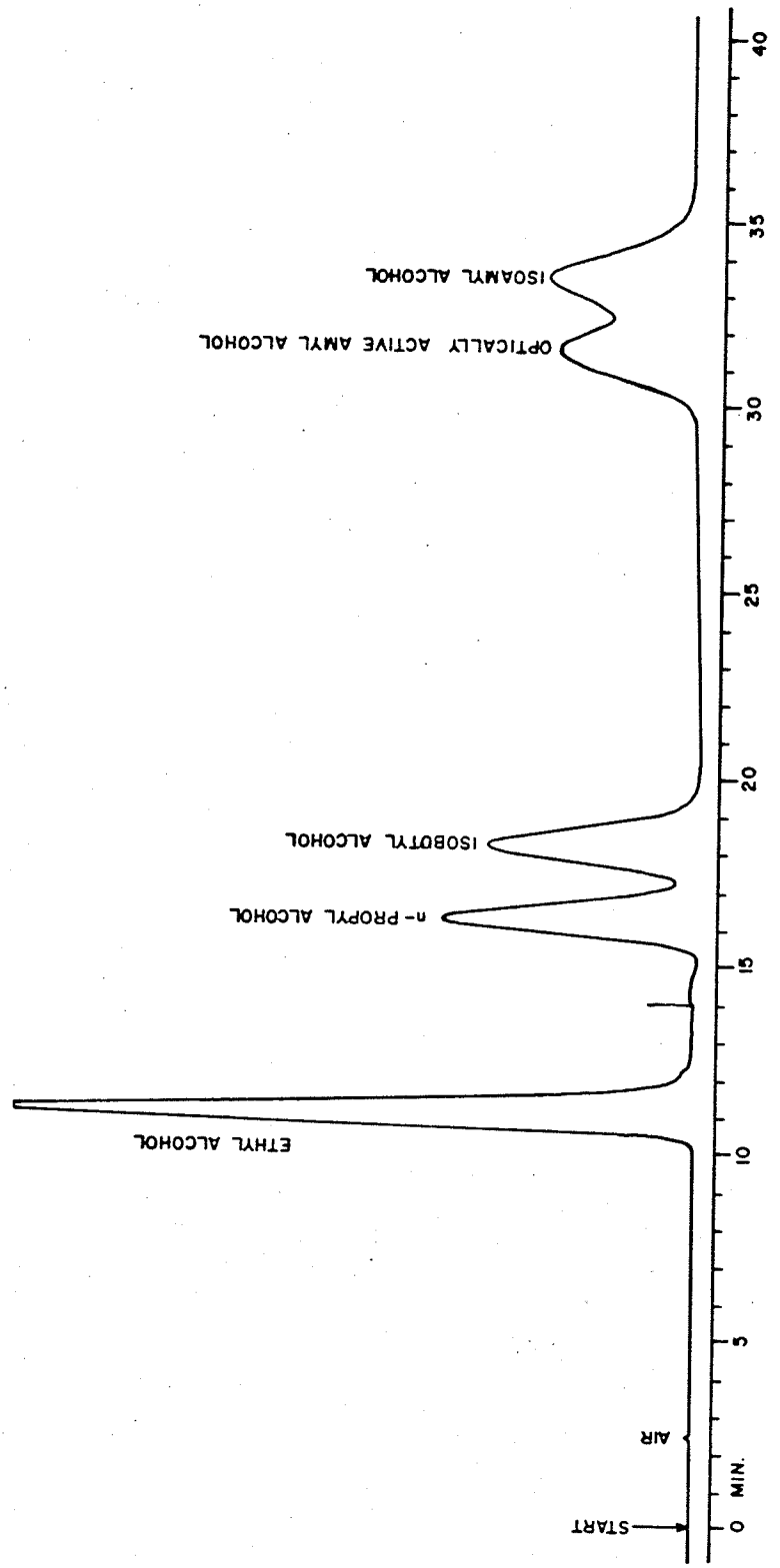


FIG. 3 GAS CHROMATOGRAM OF THE FRACTIONAL DISTILLATE USING COLUMN B

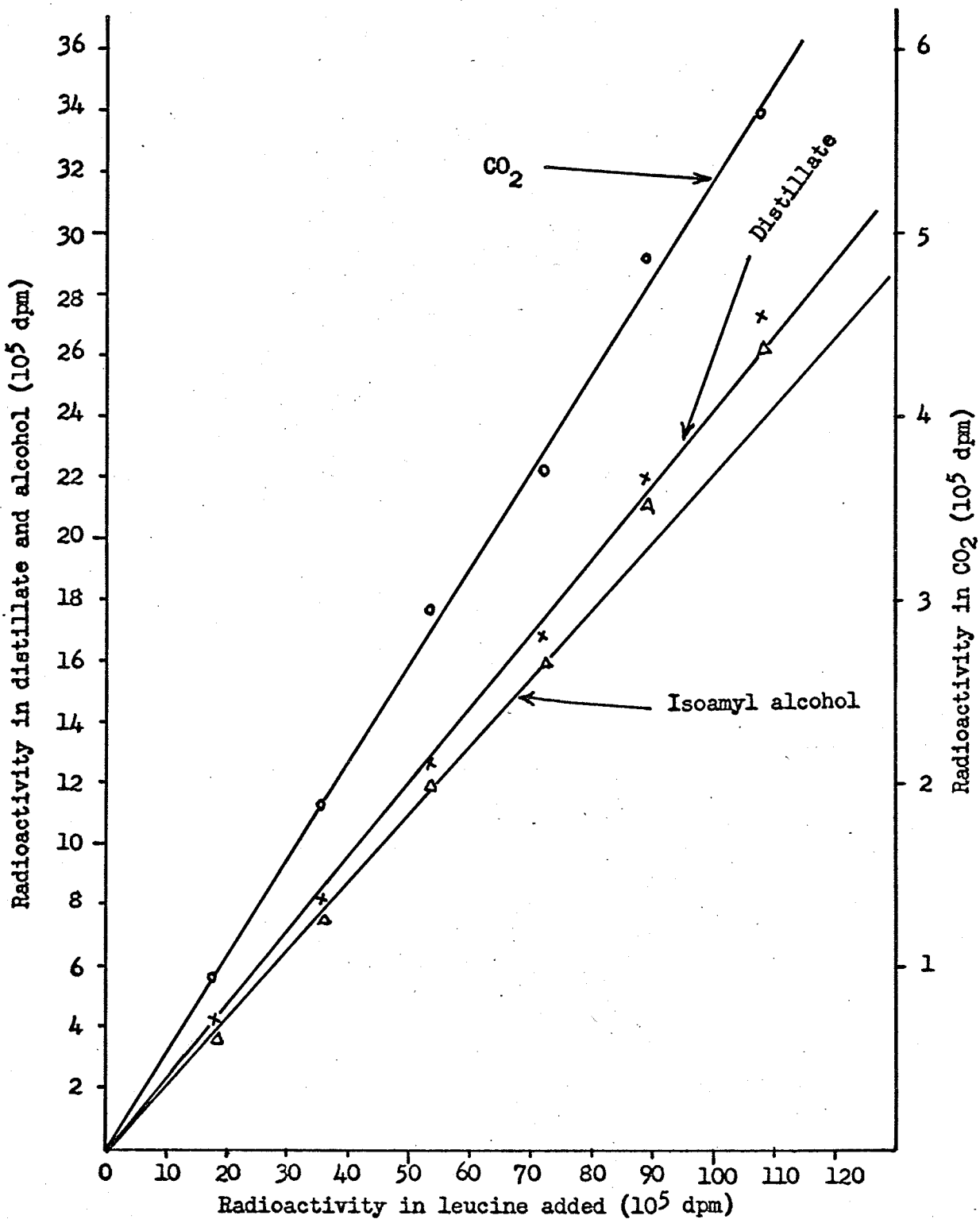
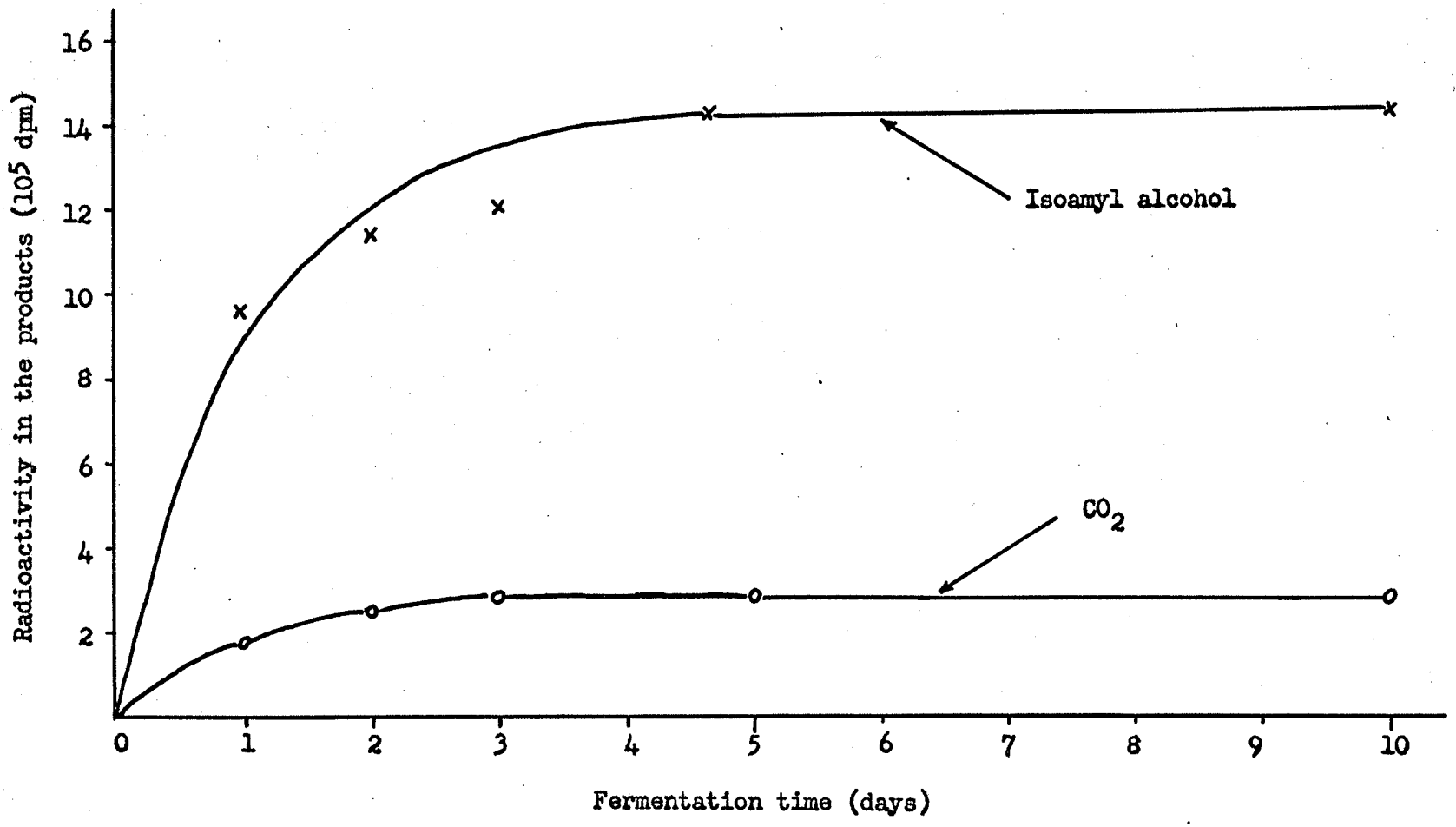


FIGURE 4. Relationship between the radioactivity in L-leucine-C^{14}(U) added and that incorporated in the products.

FIGURE 5. Effect of fermentation time on the biosynthesis of isoamyl alcohol from L-leucine-C^{14} (U) (53.3 x 10^5 dpm).



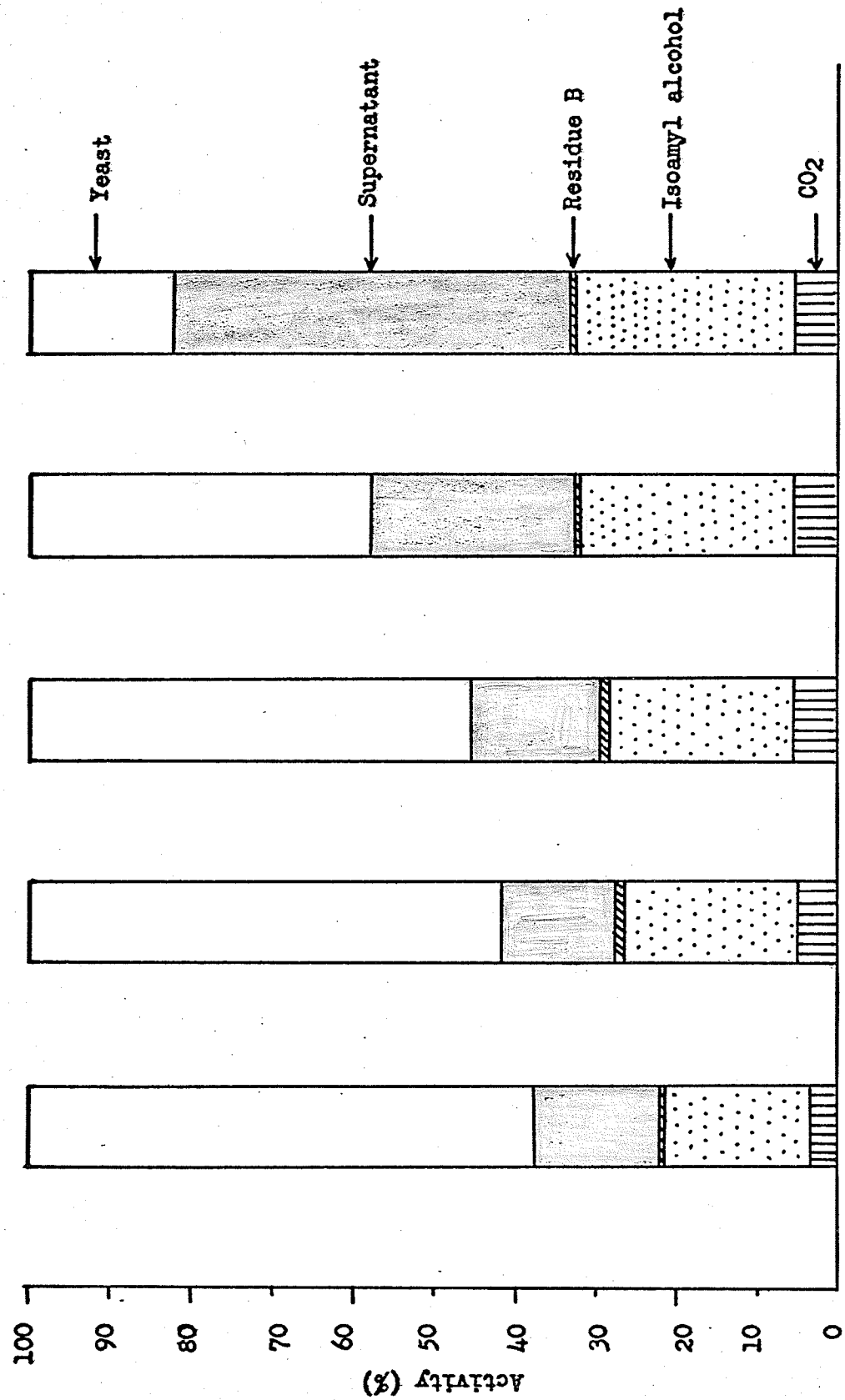


FIGURE 6. Percentage distribution of radioactivity between different fractions after fermentation with L-leucine-C^{14} (U) (53.3 x 10^5 dpm).

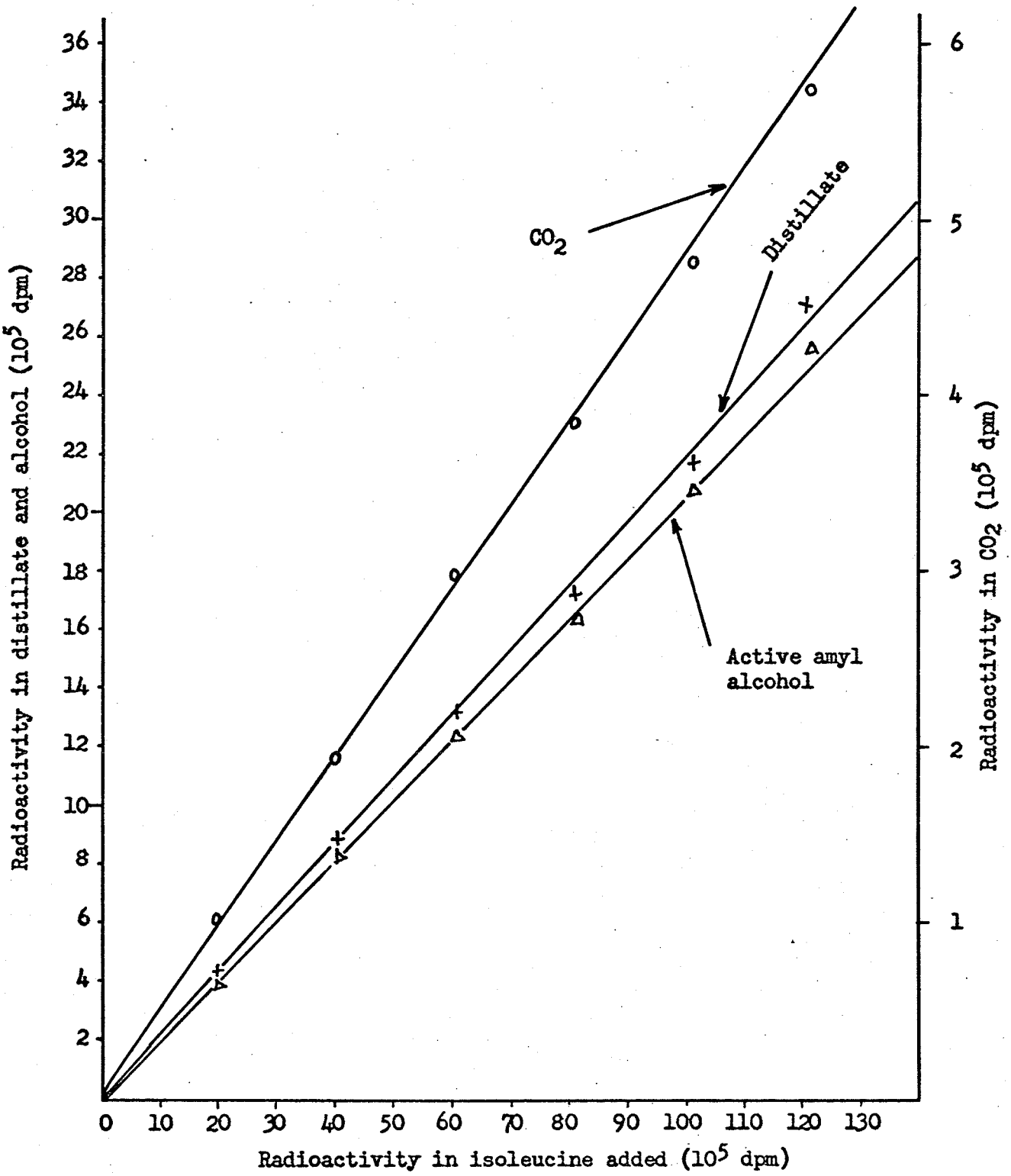


FIGURE 7. Relationship between the radioactivity in L-isoleucine-C14 (U) added and that incorporated in the products.

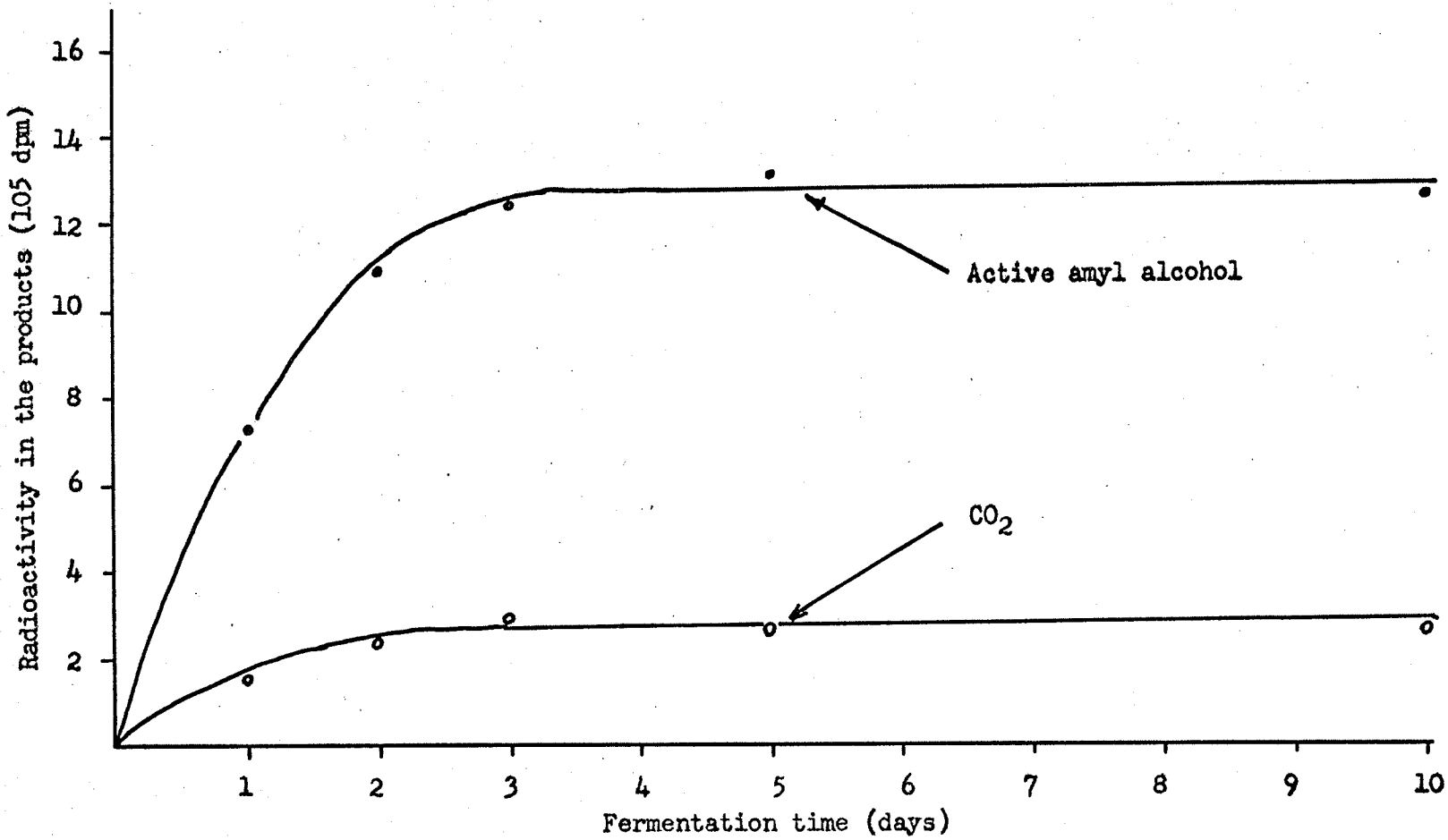


FIGURE 8. Effect of fermentation time on the biosynthesis of active amyl alcohol from L-isoleucine-C^{14} (U) (60.5 x 105 dpm).

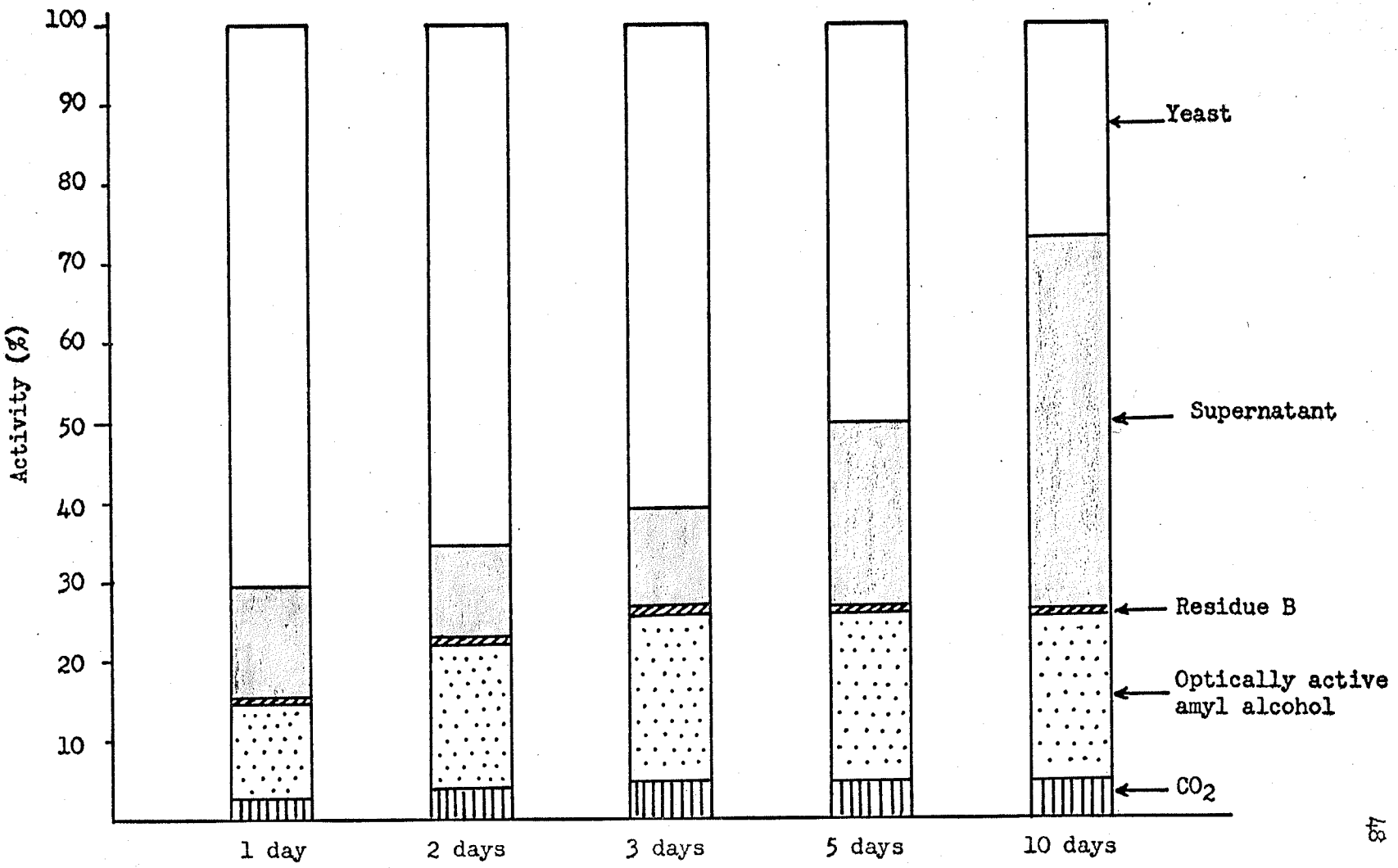


FIGURE 9. Percentage distribution of radioactivity between different fractions after fermentation with L-isoleucine-C^{14}(U) (60.5 x 10^5 dpm).

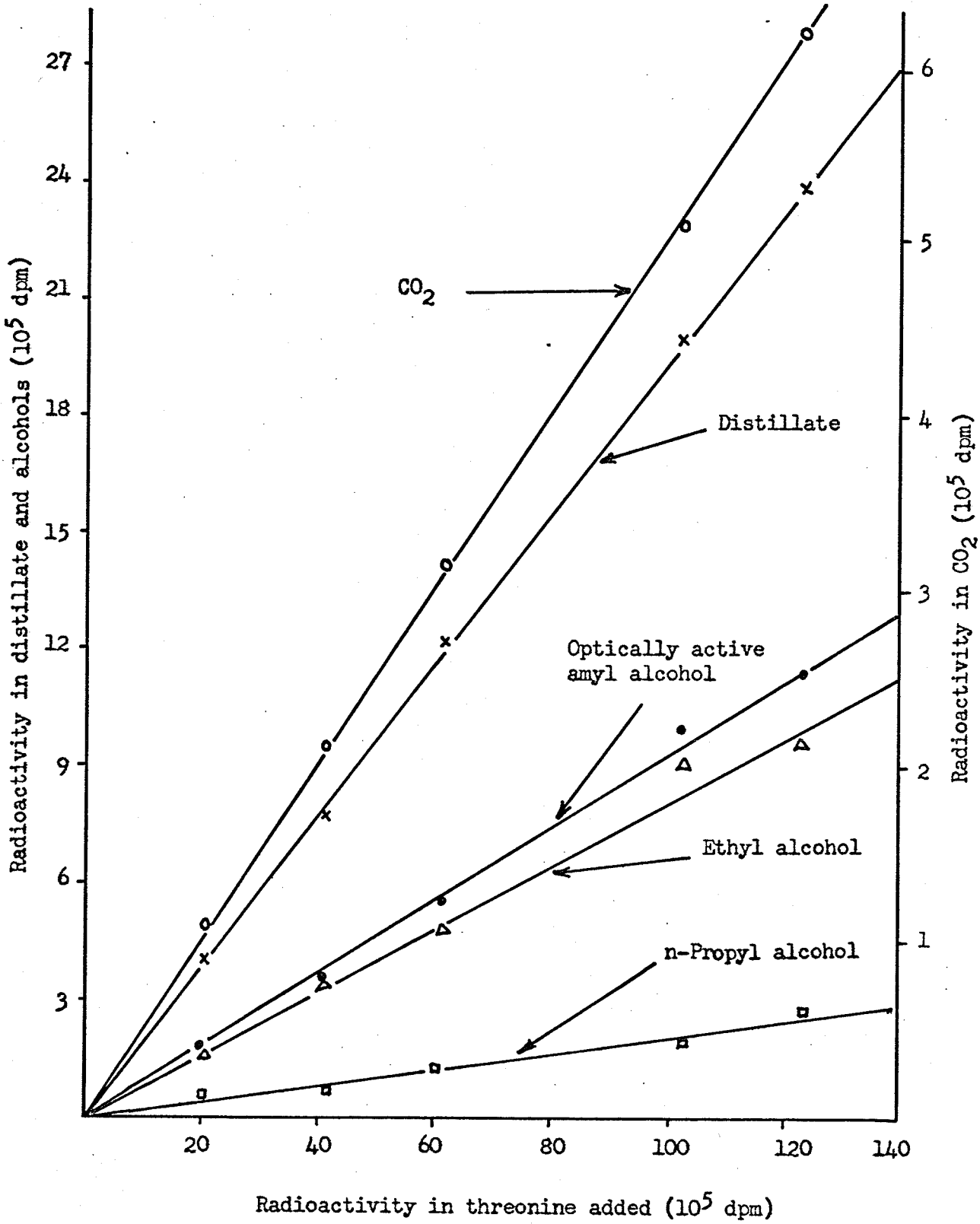


FIGURE 10. Relationship between the radioactivity in L-threonine-C^{14}(U) added and that incorporated in the products.

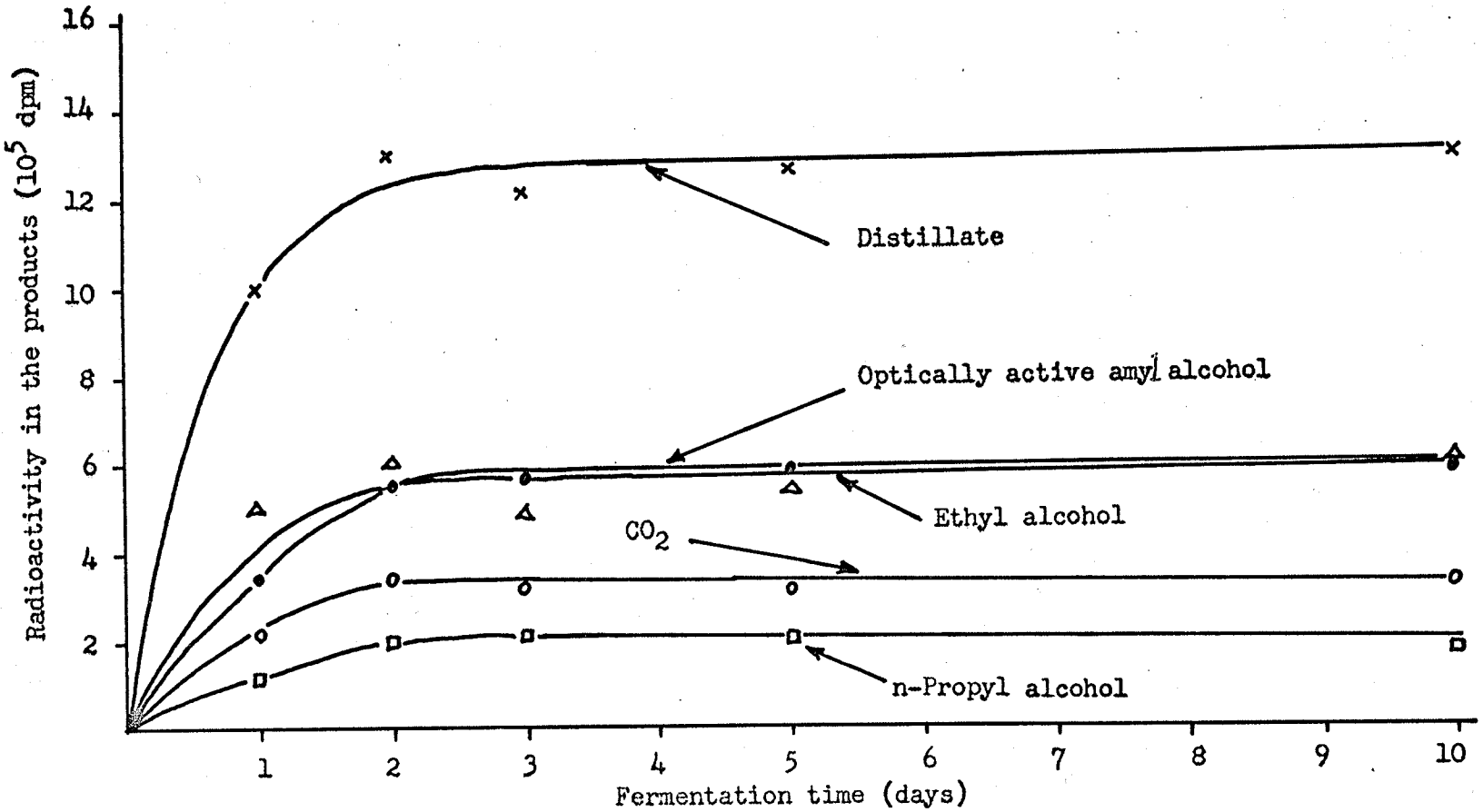


FIGURE 11. Effect of fermentation time on the biosynthesis of alcohols from L-threonine-C^{14} (U) (61.6 x 10^5 dpm).

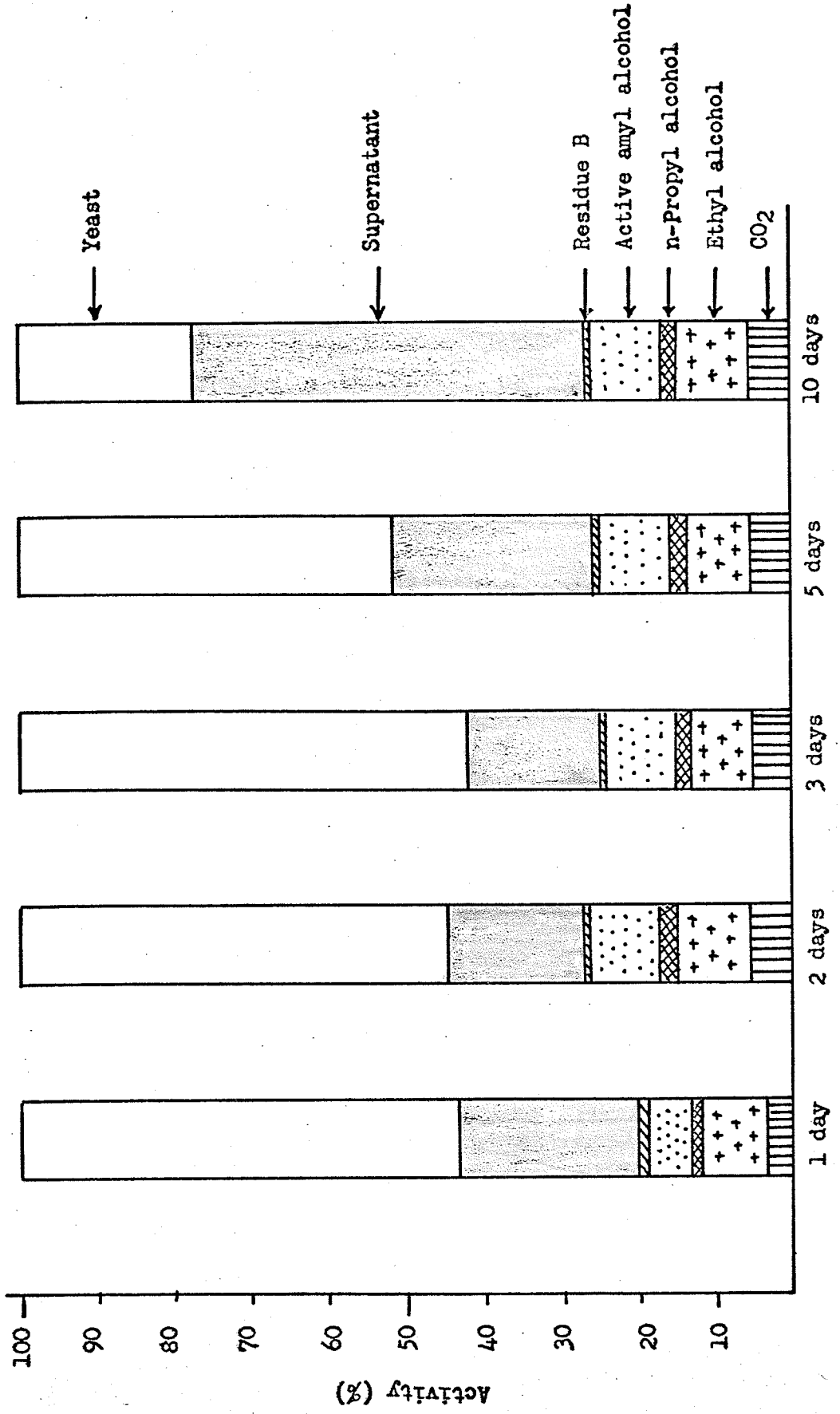


FIGURE 12. Percentage distribution of radioactivity between different fractions after fermentation with L-threonine-C^{14} (U) (61.6×10^5 dpm)

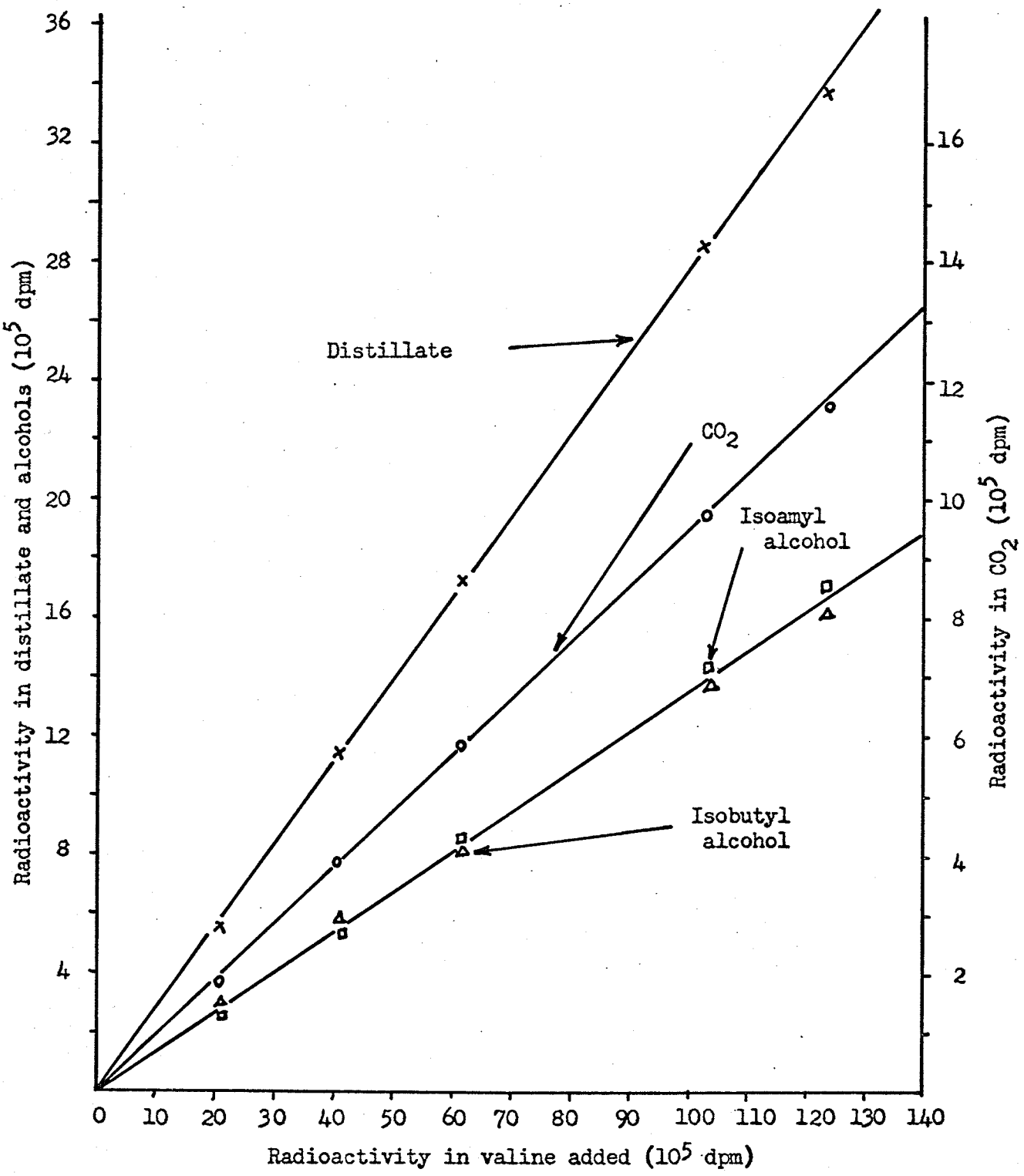


FIGURE 13. Relationship between the radioactivity in L-valine-C^{14} (U) added and that incorporated in the products.

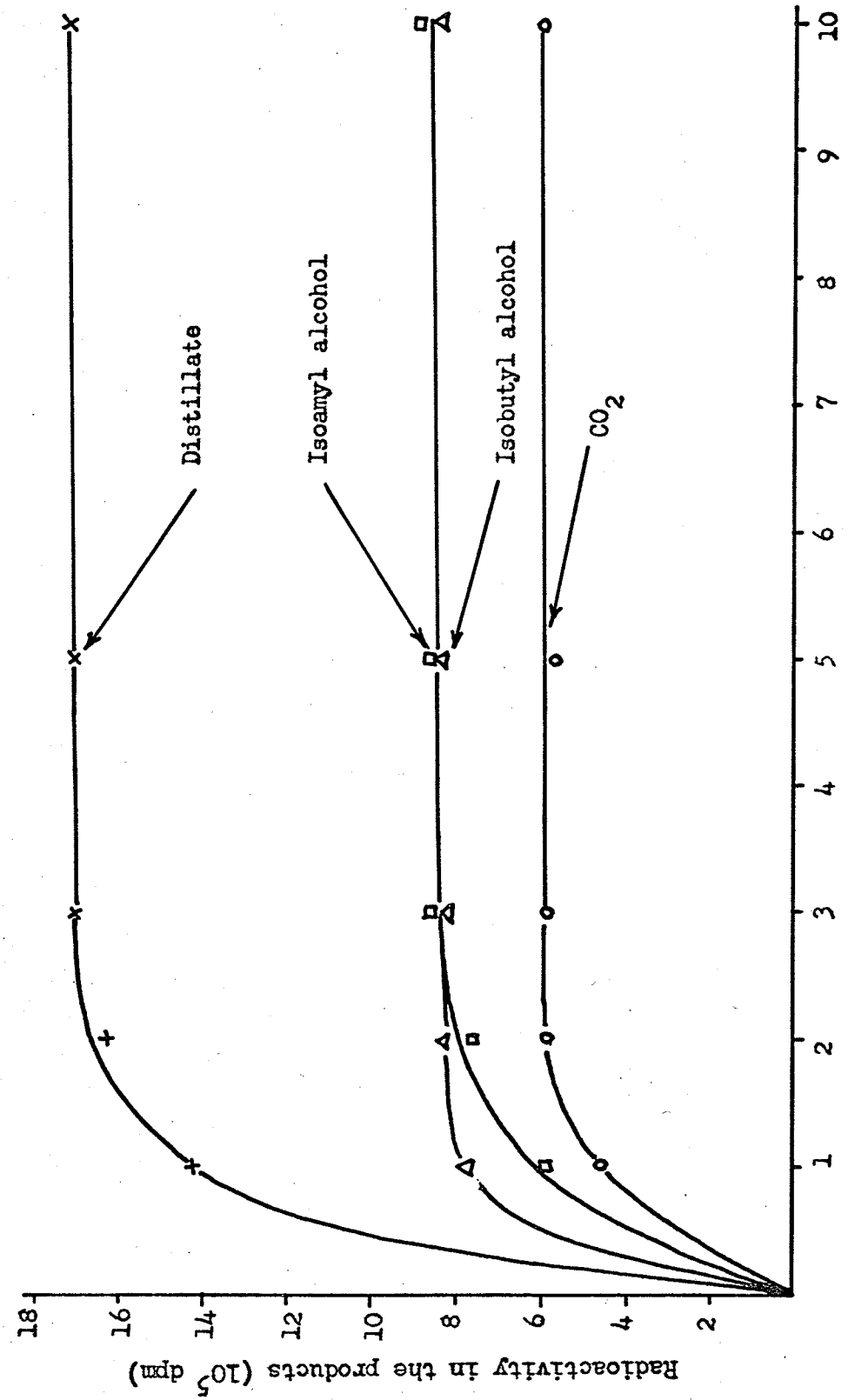


FIGURE 14. Effect of fermentation time on the biosynthesis of alcohols from L-valine-C^{14} (U) (61.6 x 10^5 dpm).

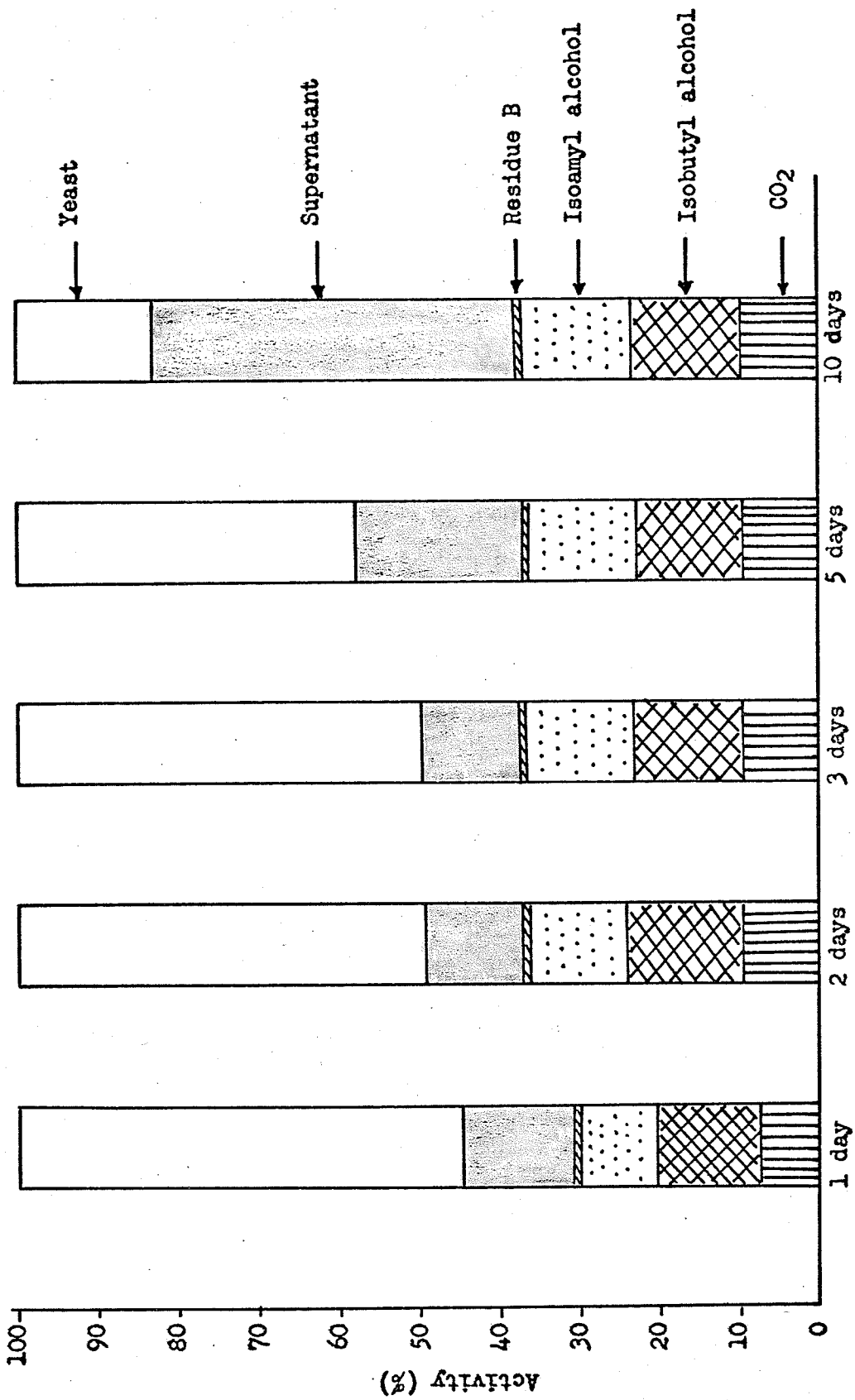


FIGURE 15. Percentage distribution of radioactivity between different fractions after fermentation with L-valine-C^{14} (U) (61.6×10^5 dpm)

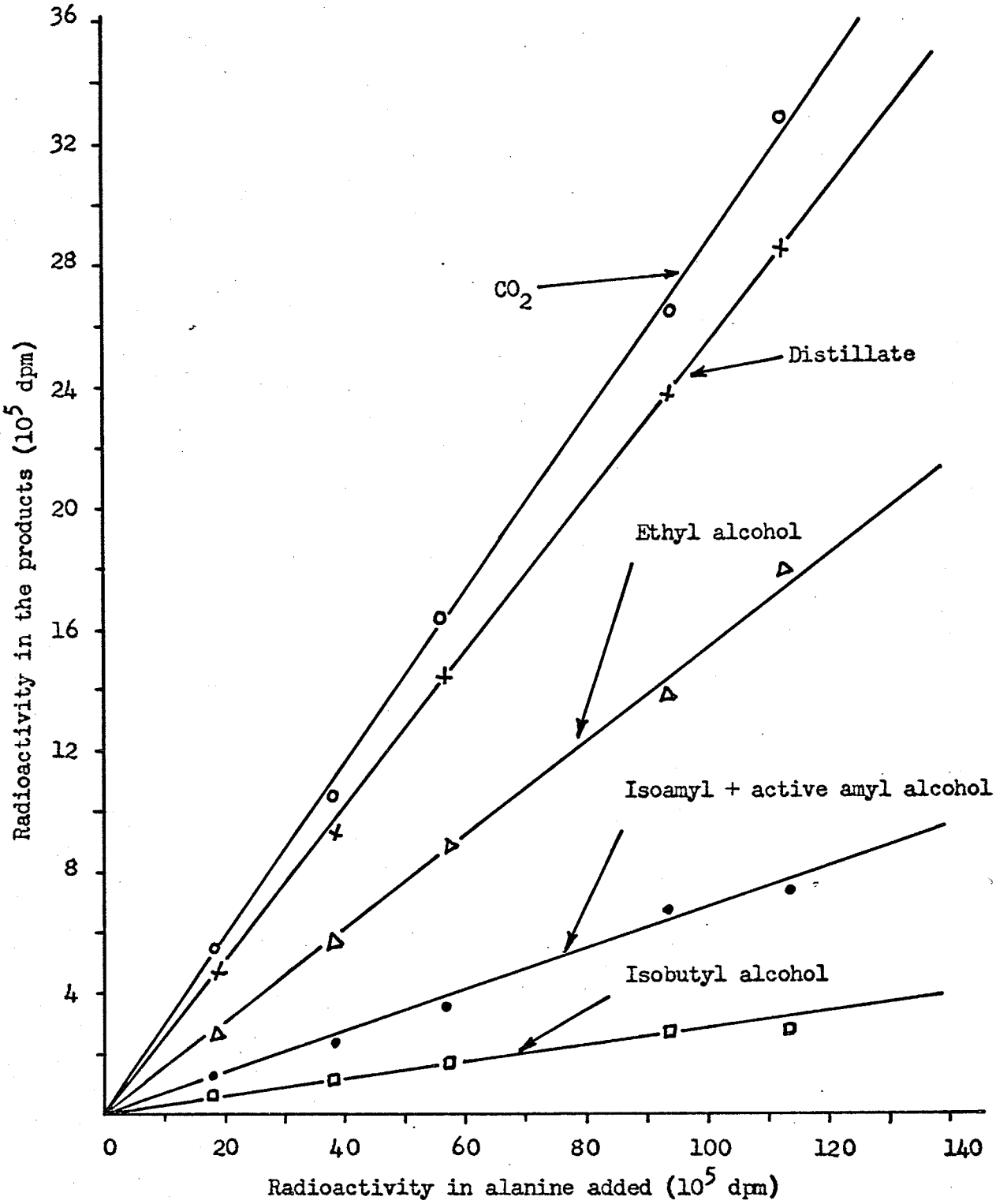


FIGURE 16. Relationship between the radioactivity in L-alanine-C^{14} (U) added and that incorporated in the products.

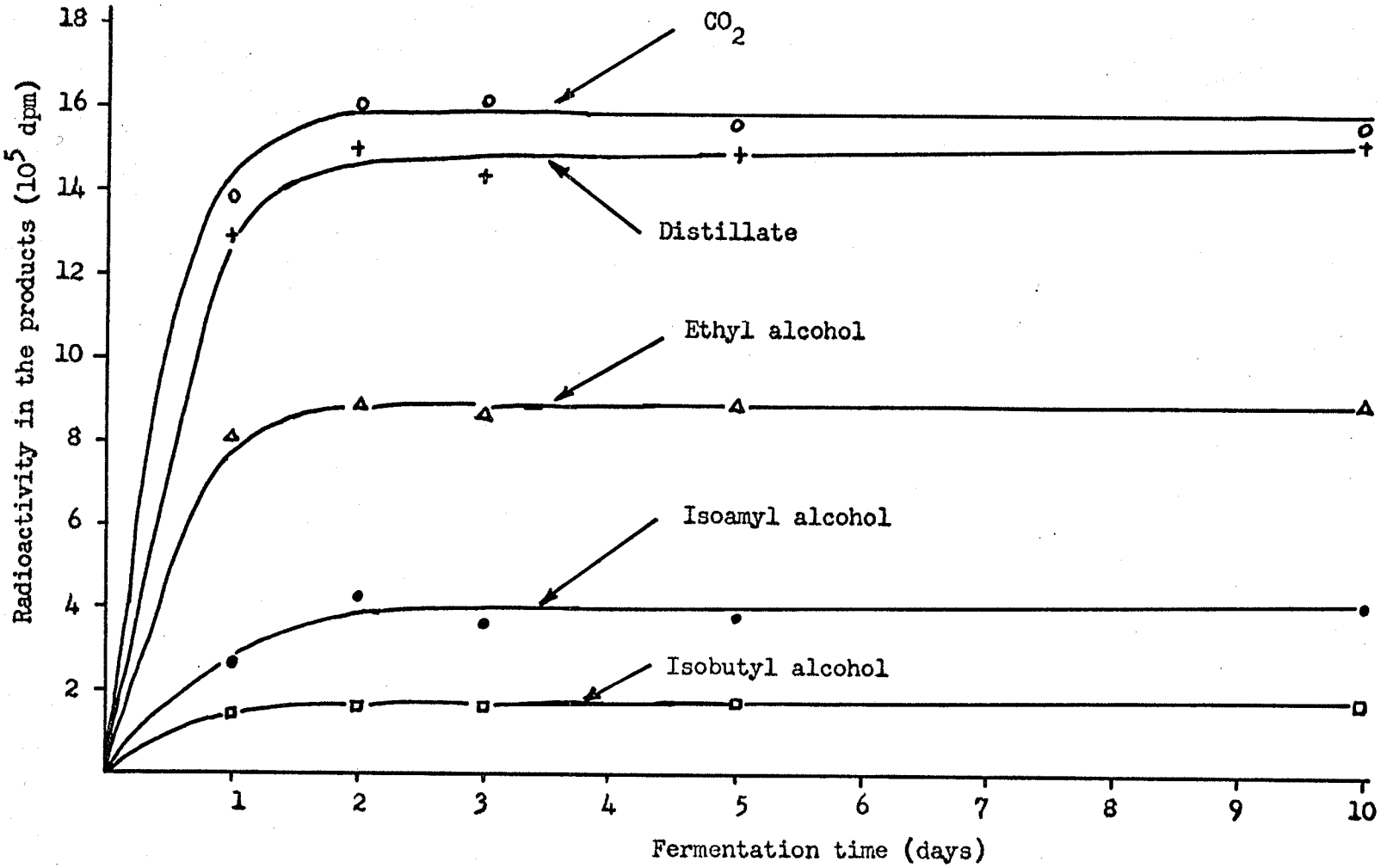


FIGURE 17. Effect of fermentation time on the biosynthesis of alcohols from L-alanine-C^{14} (U) (56.1 x 10^5 dpm)

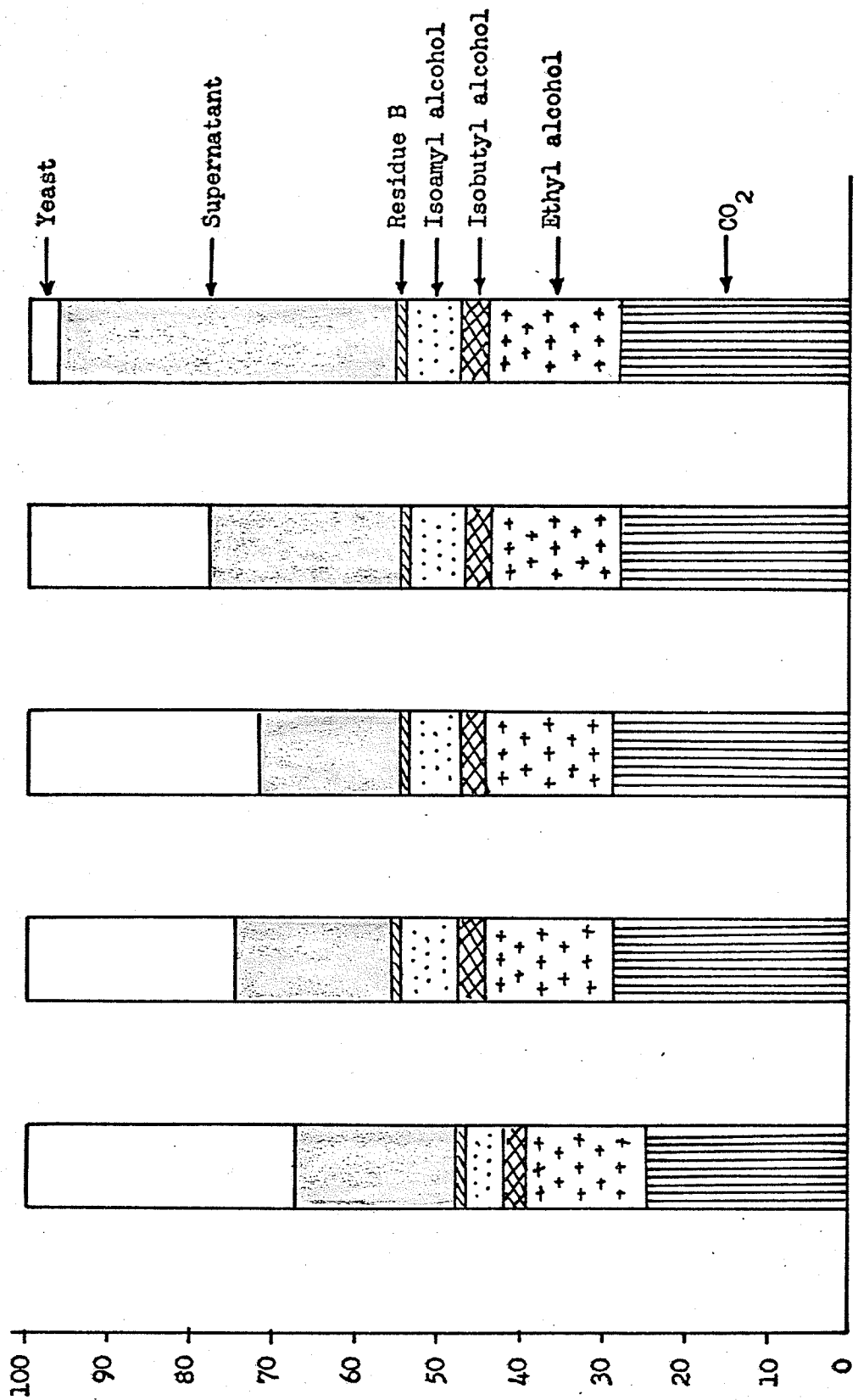


FIGURE 18. Percentage distribution of radioactivity between different fractions after fermentation with L-alanine-C^{14} (U) (56.1×10^5 dpm)

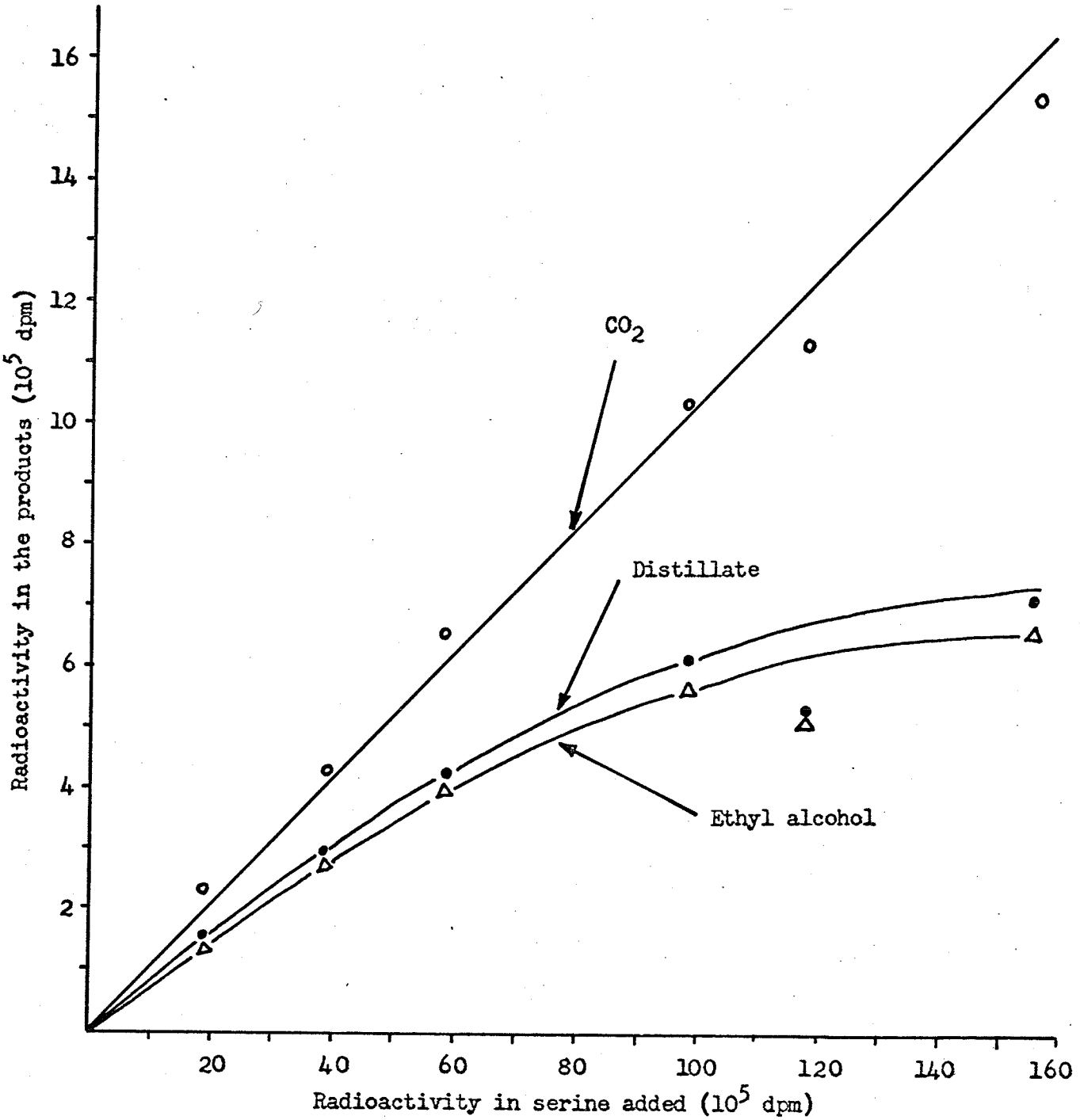


FIGURE 19. Relationship between the radioactivity in L-serine-C^{14} (U) added and that incorporated in the products.

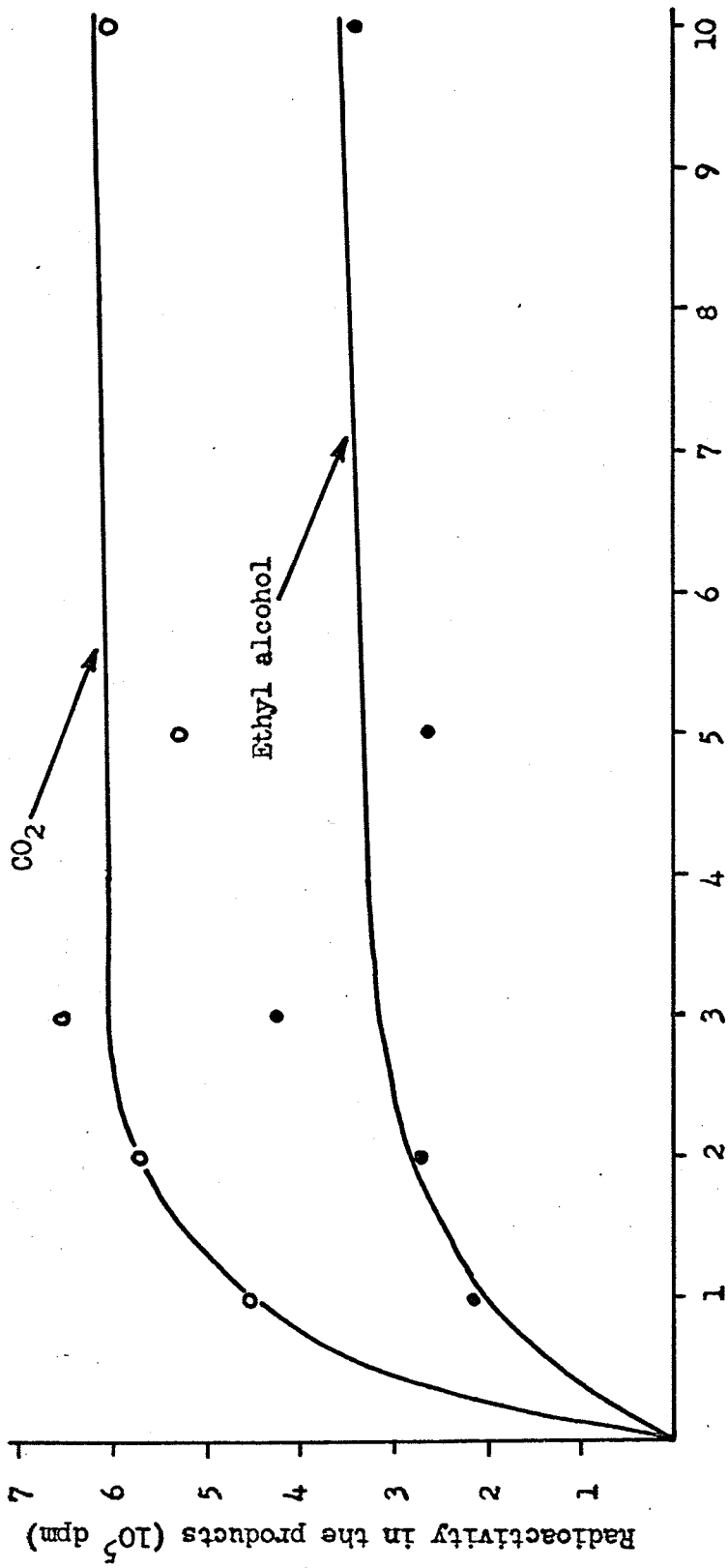


FIGURE 20. Effect of fermentation time on the biosynthesis of ethyl alcohol from L-serine-C^{14} (U) (58.8 x 105 dpm).

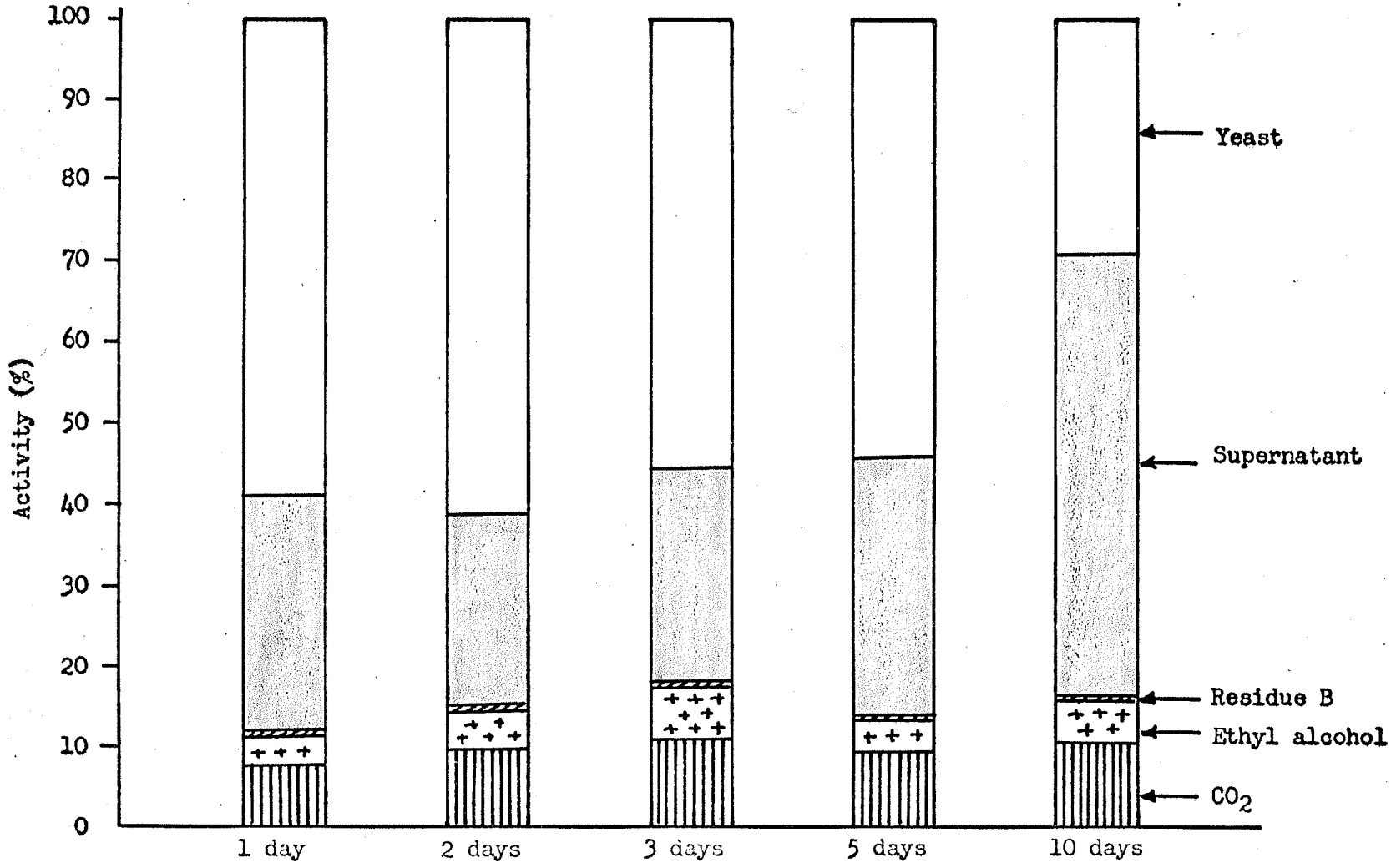
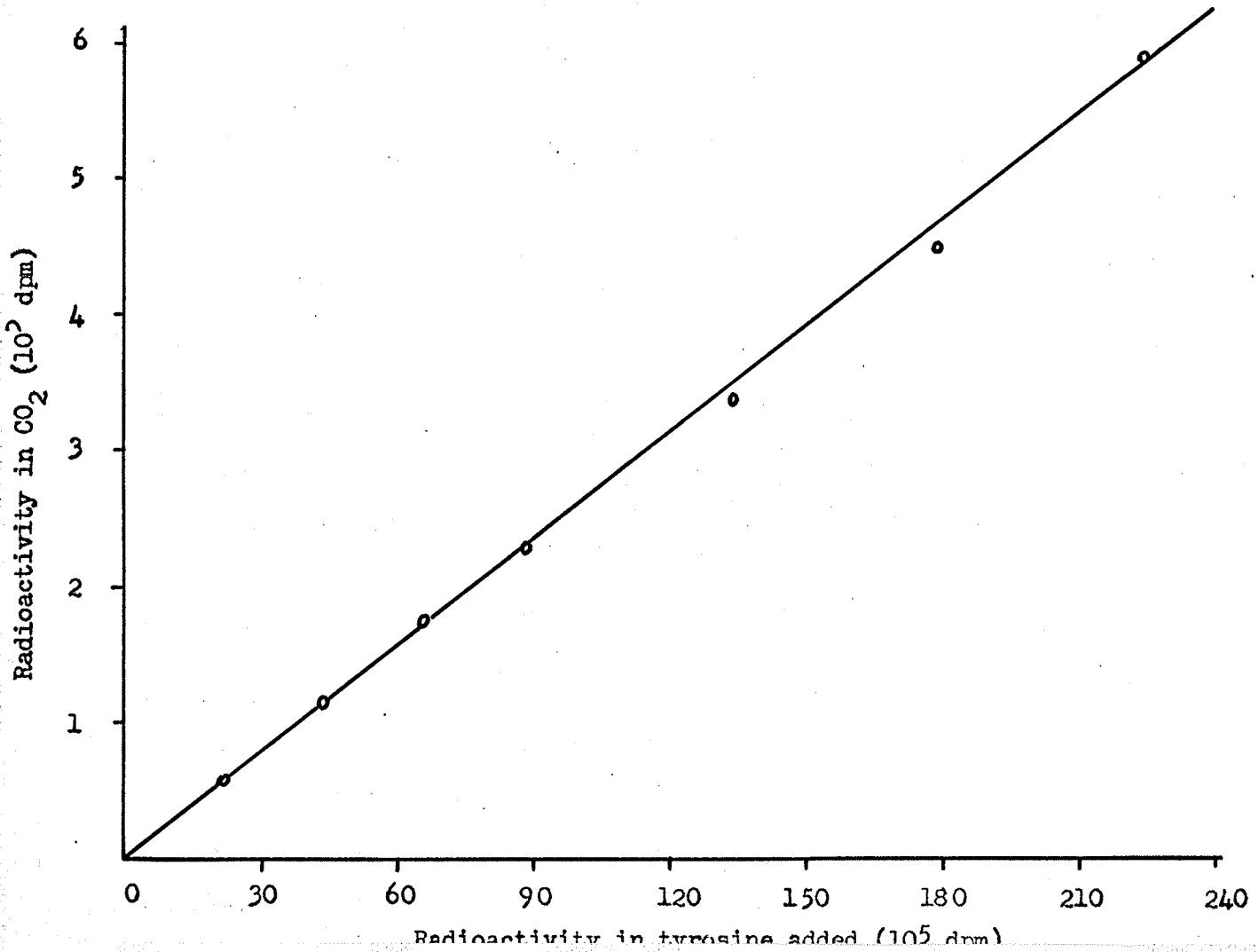


FIGURE 21. Percentage distribution of radioactivity between different fractions after fermentation with L-serine-C^{14} (U) (58.8×10^5 dpm).



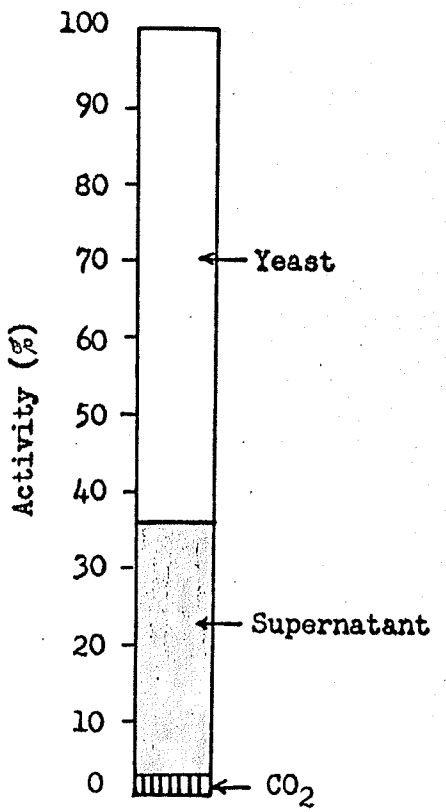


FIGURE 22b. Percentage distribution of radioactivity between different fractions after fer-

DISCUSSION

The general purpose of this work was to produce alcohol by fermentations of a standard mixture of corn, rye and malt, simulating the conditions used in the manufacture of crude whisky, and to investigate the formation of specific flavour components from amino acids present in the corn, rye and malt proteins. Radioactively labelled amino acids, the same as those present in the proteins, were added singly and in small amounts to standard fermentation mashes. After fermentation, distillation of the crude alcohol, and gas chromatographic separation of the flavour components, the individual flavour alcohols were assayed for radioactivity to determine whether they were derived from the radioactive amino acid in the mash.

The Possible Effect of Added Amino Acid Tracers on the Fermentation

All amino acids used as tracers were uniformly labelled with high specific activity as shown in Table 1. The amount used in one batch of fermentation was from 1 to 10 μc. Most of the studies were carried out with 3 μc in one batch, corresponding to approximately 0.045 mg of the amino acid (alanine, only 0.013 mg). This amount, compared with that present in the raw materials (100 to 400 mg of the different amino acids), was so minute that it should not affect the fermentation pattern.

The Progress of Fermentation

Most of the experiments showed that the fermentation and the biosynthesis were completed in three days. The majority of the flavour alcohols were formed in the first day of fermentation, the primary fermentation. Contrary to this finding, Schilfarth (1952) has proposed, from a theoretical consideration, that the bulk of the fusel oil should be formed in the secondary fermentation. In the present work, when the fermentation ceased, the yeast died and autolyzed, and the higher alcohols increased very little. In the first day of fermentation, about half of the radioactivity added was incorporated in the yeast and about 20 per cent in the supernatant. At the end of ten days of fermentation, however, only about 20 per cent of the radioactivity was left in the yeast while the radioactivity in the supernatant had increased to 50 per cent. This observation might be explained as follows: Since the bourbon whisky mash was rich in amino acids, the yeast should assimilate them by direct intact mechanism. Therefore, most of the amino acids should be incorporated in the yeast cells in the first day. When the fermentation ceased, the concentration of alcohol would have reached a maximum, the yeast would die and autolysis would occur. The materials in the cells would be broken down

enzymatically and dissolved in the water. Hence, the radioactivity would decrease in the yeast and increase in the supernatant.

The Metabolism of L-leucine-C^{14}(U)

When radioactive L-leucine was added to a standard mash and fermented, the flavour components carried the radioactive label in the isoamyl alcohol only, thus showing that L-leucine was converted to isoamyl alcohol in the fermentation.

Twenty-three per cent of the original radioactivity was incorporated in isoamyl alcohol and 5.3 per cent in CO_2 during fermentation with L-leucine-C^{14}(U). The mole per cent conversion of L-leucine-C^{14}(U) into isoamyl alcohol was 28 per cent. This result was similar to that obtained by Spanyer and Thomas (1956) who found 31 mole per cent conversion. Thoukis (1958), however, found that 73 per cent of L-leucine-C^{14}(U) was converted to isoamyl alcohol. This difference in results may be due to the different methods of fermentation. Thoukis used 13 per cent sucrose solution with a heavy inocula under non-proliferating conditions. Suomulainen and Kahanpää (1963) found that 12 per cent of the radioactivity in L-leucine-C^{14}(U) was incorporated into isoamyl alcohol. This incorporation was only about half of that obtained in the present study.

The obtained radioactivity ratio between isoamyl alcohol and CO_2 was 4.3 to 1. Thoukis obtained the same value. By observing this ratio, it was clear that the Ehrlich scheme was operating in this conversion. The theoretical ratio of radioactivity between isoamyl alcohol and CO_2 should be 5 to 1 if all the carbons in leucine were uniformly labelled and if the Ehrlich scheme were the only pathway for this conversion. The ratio obtained by Suomulainen and Kahanpää, however, was quite different. They found it to be 1 to 3.4.

The accepted mechanism for the conversion of L-leucine into isoamyl alcohol is outlined in Fig.23 (Ehrlich, 1906, 1907a, 1907b, 1907c; Neubauer and Fromherz, 1911; Sentheshanmuganathan and Elsden, 1958; Thoukis, 1958).

$$\text{Leucine} \xrightarrow[\text{deamination}]{\text{Transamination or}} \alpha\text{-Ketoisocaproate} + NH_3$$

$$\alpha\text{-Ketoisocaproate} \xrightarrow{\text{Decarboxylation}} \text{Isovaleraldehyde} + CO_2$$

$$\text{Isovaleraldehyde} + NADH_2 \xrightarrow[\text{dehydrogenase}]{\text{Alcohol}} \text{Isoamyl alcohol} + NAD$$

FIGURE 23. Pathway of Biosynthesis of Isoamyl Alcohol from Leucine

The Metabolism of L-isoleucine-C^{14}(U)

When radioactive L-isoleucine was added to a standard mash and fermented, the flavour components carried the radioactive label in the optically active amyl alcohol only, thus showing that L-isoleucine was converted to optically active amyl alcohol in the fermentation.

Twenty-one per cent of the original radioactivity was incorporated in the optically active amyl alcohol and 4.8 per cent in the CO_2 after fermentation with L-isoleucine-C^{14}(U). The mole per cent of isoleucine converted into active amyl alcohol was 25 per cent. The ratio of radioactivity between active amyl alcohol and CO_2 was 4.4 to 1, this indicating that the Ehrlich scheme was operating also in this transformation. The pathway of synthesis of active amyl alcohol is similar to that of isoamyl alcohol (Fig.23). In fermentation with sucrose and isoleucine, Suomulainen and Kahanpää (1963), found that only 15 per cent of the radioactivity of L-isoleucine-C^{14}(U) was incorporated in the optically active amyl alcohol.

The Metabolism of L-threonine-C^{14}(U)

Fermentations with L-threonine-C^{14}(U) gave radioactive ethyl alcohol, n-propyl alcohol and optically active amyl alcohol thus showing that these alcohols were formed from threonine in the fermentation. The incorporation of

radioactivity in ethyl alcohol was 8 per cent, in n-propyl alcohol 2 per cent, in active amyl alcohol 9 per cent, and in CO_2 5 per cent of the original activity added. Yamada and Yoshizawa (1960) have done extensive work in the fermentation with threonine and α-amino-n-butyric acid. They found that active amyl alcohol and n-propyl alcohol were formed and that 36.8 per cent of the threonine consumed was transformed into active amyl alcohol. Yoshizawa and Yamada (1962) found that 34 per cent of the threonine assimilated was converted into the two alcohols. In the present study, their results were qualitatively confirmed. In addition, ethyl alcohol was obtained, which has not earlier been reported.

The formation of ethyl alcohol could not be explained by the Ehrlich mechanism. The catabolism of threonine in animals, however, has been studied quite extensively. Karasek and Greenberg (1957) studied the cleavage of threonine to glycine and aldehyde. The enzyme, threonine aldolase, catalyzes this reaction; this is a sulfhydryl enzyme and is dependent on pyridoxal phosphate. The reaction is shown in the following equation:

$$CH_3\text{-}CHOH\text{-}CHNH_2\text{-}COOH \longrightarrow CH_3\text{-}CHO + CH_2NH_2\text{-}COOH$$

If this reaction occurs also in yeast, the formed acetaldehyde would easily be changed into ethyl alcohol by the

action of alcohol dehydrogenase and $NADH_2$ and would thus give a possible mechanism for the formation of ethyl alcohol in the fermentation of threonine.

The proposed mechanism of n-propyl alcohol and active amyl alcohol formation (Yoshizawa and Yamada, 1962) from threonine is given in Figure 24 together with the above suggested mechanism for the formation of ethyl alcohol.

Considering the mechanism (Fig.24), the radioactivity in the CO_2 can be calculated by the following method. In the formation of ethyl alcohol, no $C^{14}O_2$ is produced. In the formation of one mole n-propyl alcohol, one mole of $C^{14}O_2$ is produced, giving a carbon-14 ratio of 3 to 1 between the alcohol and CO_2. In the formation of one mole active amyl alcohol, one mole $C^{14}O_2$ is produced, giving a carbon-14 ratio of 3 to 1 between the alcohol and CO_2. The following radioactivities were found in the present experiments: Threonine, 100 per cent; ethyl alcohol, 8 per cent; n-propyl alcohol, 2 per cent; optically active amyl alcohol, 9 per cent; and CO_2, 5 per cent. Using the experimental values of activity for n-propyl alcohol and optically active amyl alcohol, the total activity in CO_2 was calculated as follows: $2 \times (1/3) + 9 \times (1/3) = 3.7$ per cent. The calculated activity, 3.7 per cent, was lower than the observed activity, 5 per cent. There might also be some other reaction involved causing cleavage of

threonine to CO_2.

The Metabolism of L-valine-C^{14}(U)

Fermentations with L-valine-C^{14}(U) gave radioactive isobutyl and isoamyl alcohol thus showing that these alcohols were formed from valine in the fermentation. The incorporation of radioactivity in isobutyl alcohol was 13.5 per cent, in isoamyl alcohol 13.5 per cent, and in CO_2 9.5 per cent of the original activity added. Since a uniformly labelled valine was used in this study, the mole per cent conversion of valine into isobutyl alcohol equalled 13.5 x (5/4) = 16.9 per cent. Suomalainen and Kahanpää (1963) used DL-valine-4-C^{14} as tracer and found that 26 per cent of the radioactivity added was incorporated in isobutyl alcohol, but none in isoamyl alcohol or in CO_2. Since they used valine-4-C^{14}, the mole per cent conversion of valine into isobutyl alcohol equalled the per cent incorporation, namely, 26 per cent. This value was considerably higher than that obtained in the present study. Yoshizawa et al (1961) found that only isobutyl alcohol was formed from valine. Yamada et al (1962) also reported that isobutyl alcohol was formed from valine.

The mechanism for the formation of isobutyl alcohol from valine follows the Ehrlich scheme (Yoshizawa, 1964) as shown in Figure 25. The mechanism for the formation of

isoamyl alcohol from valine (Fig.25) follows the pathway of synthesis of leucine from valine by condensation of α-ketoisovalerate with acetyl-CoA, followed by a series of reactions to form α-ketoisocaproate which undergoes decarboxylation and reduction to form isoamyl alcohol (Albert G. Moat and Fazal Ahmad, 1965).

Considering the mechanism (Fig.25), the radioactivity in the CO_2 can be calculated in the following way:

One mole valine ($5 \times C^{14}$) $\longrightarrow$ 1 mole isoamyl alcohol ($4 \times C^{14}$) + 1 mole $C^{14}O_2$

Radioactivity of CO_2 = 1/4 x radioactivity of isoamyl alcohol

One mole valine ($5 \times C^{14}$) $\longrightarrow$ 1 mole isobutyl alcohol ($4 \times C^{14}$) + 1 mole $C^{14}O_2$

Radioactivity of CO_2 = 1/4 x radioactivity of isobutyl alcohol

The percentage incorporation of radioactivity in the present experiments were as follows:

Valine, 100 per cent $\longrightarrow$
- Isobutyl alcohol, 13.5 per cent
- Isoamyl alcohol, 13.5 per cent
- CO_2, 9.5 per cent

The total radioactivity in CO_2 was calculated

$= 1/4 \times 13.5 + 1/4 \times 13.5$

$= 6.8$ per cent.

The calculated activity, 6.8 per cent, was lower than the observed activity, 9.5 per cent. There might also be some other reaction involved causing cleavage of valine to CO_2 and thus increasing the radioactivity in CO_2. In animals, valine can be catabolized into propionic acid giving 2 moles of CO_2 (Kinnorg, Takeda and Greenberg, 1955).

The Metabolism of L-alanine-C^{14}(U)

Fermentations with L-alanine-C^{14}(U) gave radioactive ethyl alcohol, isobutyl alcohol and isoamyl alcohol, thus showing that these alcohols were formed from alanine in the fermentation. A low radioactivity in the optically active amyl alcohol fraction indicated that also this alcohol was formed from alanine. This activity, however, could possibly have been caused by a contamination with isoamyl alcohol. The incorporation of activity in ethyl alcohol was 15.3 per cent, in isobutyl alcohol 3 per cent, in isoamyl alcohol (including active amyl alcohol) 6.6 per cent and in CO_2 28.8 per cent of the original activity added. Yoshizawa et al (1961) found that isobutyl alcohol was the major fusel oil component formed from alanine. Suomalainen and Kahanpää (1963) reported the formation of isobutyl and isoamyl alcohols from alanine although no quantitative data were given. The formation of ethyl alcohol from alanine apparently has not earlier been reported.

Since alanine has a three-carbon skeleton, the formation of ethyl alcohol can be explained by the Ehrlich mechanism (Fig.26). The formation of the four- and five-carbon alcohols can not be accounted for in this way. A possible mechanism is that isobutyl, isoamyl and active amyl alcohols are formed through the intermediates in the biosynthesis of valine, leucine and isoleucine, respectively, from alanine (Figs. 24, 26). Considering the mechanism (Fig.26), the radioactivity in the CO_2 can be calculated.

By deamination or transamination of alanine-C^{14}(U), pyruvate-C^{14}(U) is formed. The active acetaldehyde and acetyl-CoA used for condensation to form higher alcohols could be either radioactive or not. If they are radioactive, formed from pyruvate-C^{14}(U), the ratio of the activity between CO_2 and acetyl-CoA or active acetaldehyde will be 1 to 2. This will not effect the ratio of the activity between CO_2 and the formed isobutyl alcohol, but it will effect the ratio of activity between CO_2 and the formed isoamyl alcohol.

One mole alanine-C^{14}(U) gives 1 mole ethanol-C^{14}(U) and 1 mole $C^{14}O_2$. The ratio of radioactivity between CO_2 and ethyl alcohol will be 1 to 2.

If the active acetaldehyde is cold, one mole alanine-C^{14}(U) will give 1 mole isobutyl alcohol (2 x C^{14}) and 1 mole $C^{14}O_2$. If the active acetaldehyde is uniformly

labelled, two moles alanine-C^{14}(U) give 1 mole isobutyl alcohol (4 x C^{14}) and 2 moles $C^{14}O_2$. The ratio of the activity between CO_2 and isobutyl alcohol will be 1 to 2 in both cases.

If the active acetaldehyde or acetyl-CoA is cold, 1 mole alanine-C^{14}(U) will give 1 mole isoamyl alcohol (2 x C^{14}) and 1 mole $C^{14}O_2$. The ratio of the activity between CO_2 and isoamyl alcohol will be 1 to 2. If the active acetaldehyde and acetyl-CoA are uniformly labelled, 3 moles alanine-C^{14}(U) will give 1 mole isoamyl alcohol (5 x C^{14}) and 4 moles $C^{14}O_2$ and the ratio of the activity between CO_2 and isoamyl alcohol will be 4 to 5.

Using the experimental values of incorporated activity, the following calculations were made:

With cold active acetaldehyde and acetyl-CoA the total activity in CO_2 = 15.3 x 1/2 + 3 x 1/2 + 6.6 x 1/2 = 12.5 per cent.

With the active acetaldehyde and acetyl-CoA uniformly labelled, the total activity in CO_2 = 15.3 x 1/2 + 3 x 1/2 + 6.6 x 4/5 = 14.4 per cent.

The calculated activities, 12.5 or 14.4 per cent, were approximately only half of the experimental value, 28.8 per cent. The high experimental value for CO_2 may be explained by considering that the pyruvate first formed from alanine may not be converted only to the described alcohols (Fig.26) but also oxidized to CO_2 by means of the TCA cycle or converted to active acetaldehyde or acetyl-CoA with the liberation of CO_2.

The Metabolism of L-serine-C^{14}(U)

When radioactive L-serine was added to a standard mash and fermented, the flavour components carried radioactive label in the ethyl alcohol only, thus showing that L-serine was converted to ethyl alcohol in the fermentation.

The incorporation of radioactivity from L-serine-C^{14}(U) into ethyl alcohol was 6.4 per cent and into CO_2 10.8 per cent. After separation of the flavour alcohols by gas chromatography, the peak of isobutyl alcohol and amyl alcohol showed slight activity. This activity was not proportional to the amount of activity initially added and it was most probably caused by contamination of these alcohols with the radioactive ethyl alcohol. Yamada et al (1962) reported that isobutyl alcohol was formed from serine and they proposed a pathway for its formation with valine as an intermediate.

The Metabolism of L-tyrosine-C^{14}(U)

In fermentations with L-tyrosine-C^{14}(U), no radioactive component was detected in the distillate. Sentheshanmuganathan and Elsden (1958) reported the formation of tyrosol from tyrosine by the Ehrlich mechanism. Tyrosol, however, if obtained in the fermentation, would not appear in the distillate with the flavour components because of its

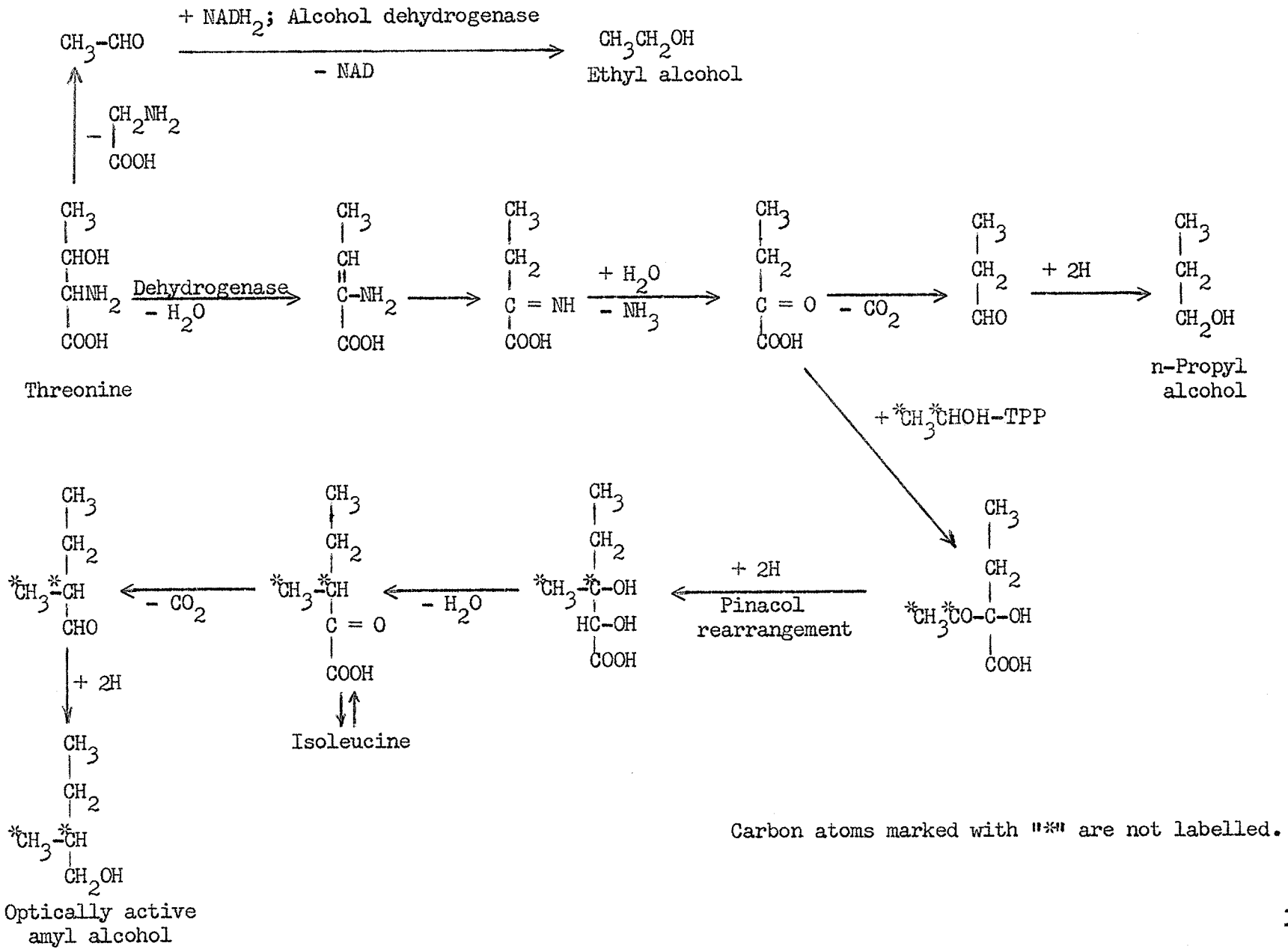
high boiling point. Tyrosine, therefore, is not considered an interesting precursor for the synthesis of flavour in bourbon whisky.

List of Alcohols Formed from the Amino Acids

The alcohols formed from amino acids by yeast fermentation in the present work and as reported by other investigators are listed in Table 6. A total of five different flavour alcohols were formed from the seven amino acids used in this study. Five of these amino acids produced amyl alcohols, which constitute the major flavour component in fusel oils.

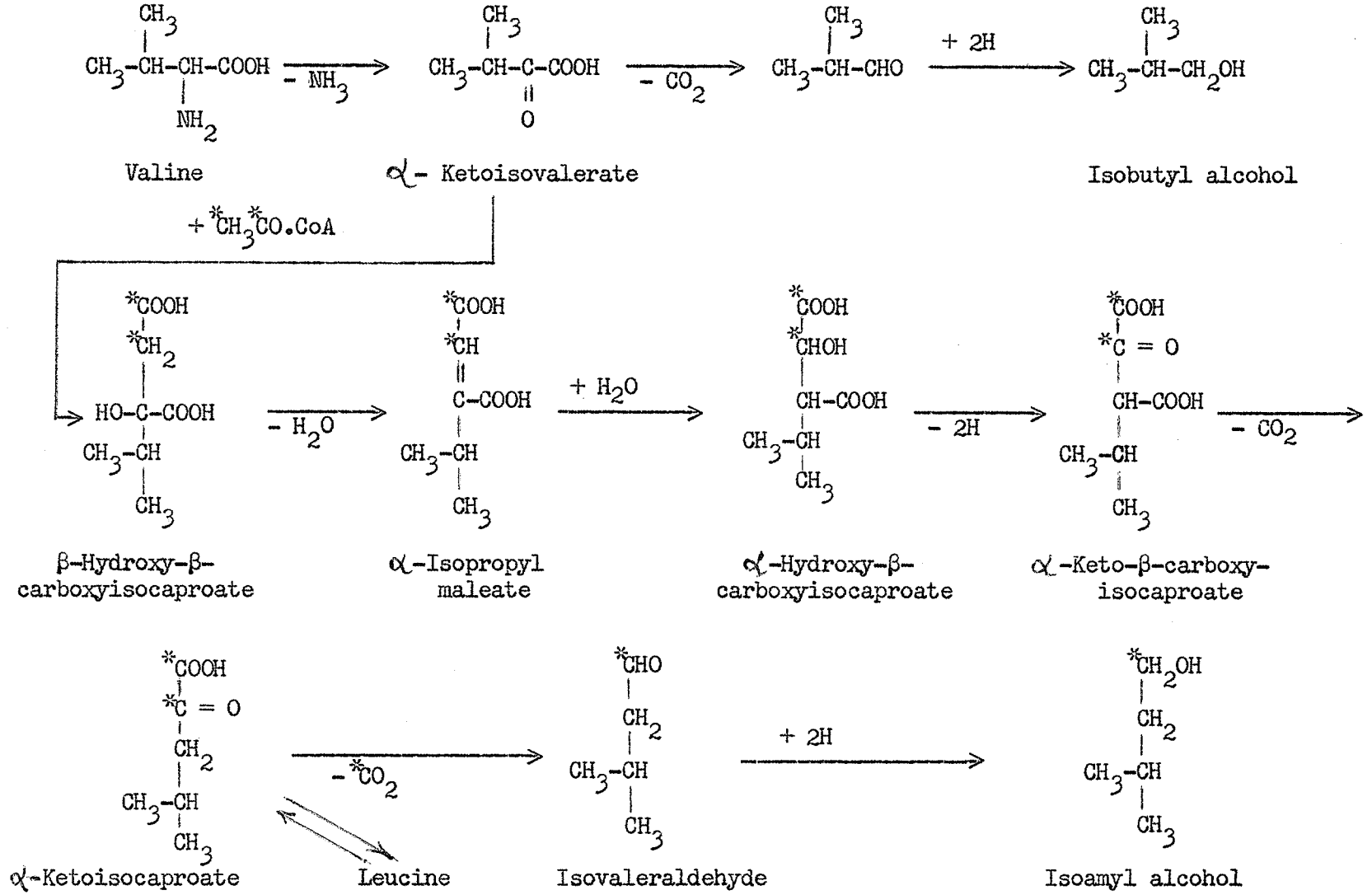
TABLE 6. Alcohols Formed From Amino Acids by Yeast Fermentation

Amino acid	Alcohols		References
	This study	Other investigators	
Leucine	Isoamyl alcohol	Isoamyl alcohol	7,11,19,26, 28,29,33, 35,38.
Isoleucine	Active amyl alcohol	Active amyl alcohol	19,28,33, 36,38
Threonine	Ethyl alcohol n-Propyl alcohol Active amyl alcohol	n-Propyl alcohol Active amyl alcohol	33,34,36, 39
Valine	Isobutyl alcohol Isoamyl alcohol	Isobutyl alcohol	19,28,33, 37,38
Alanine	Ethyl alcohol Isobutyl alcohol Isoamyl alcohol Active amyl alcohol (trace)	Isobutyl alcohol Isoamyl alcohol	28,33,38
Serine	Ethyl alcohol	Isobutyl alcohol	33
Tyrosine	None in distillate	Tyrosol	24,25
Glycine		Isobutyl alcohol	33
α-Amino-n-butyric acid		n-Propyl alcohol Active amyl alcohol	33,38,39
Norvaline		n-Butyl alcohol n-Amyl alcohol	15,33
Norleucine		n-Amyl alcohol	15,33
Tryptophan		Tryptophol	9
Phenylalanine		Phenethyl alcohol	3,4



Carbon atoms marked with "*" are not labelled.

FIGURE 24. Pathway of Biosynthesis of Alcohols from Threonine



Carbon atoms marked with an "*" are not labelled.

FIGURE 25. Pathway of Biosynthesis of Isobutyl and Isoamyl Alcohols from Valine

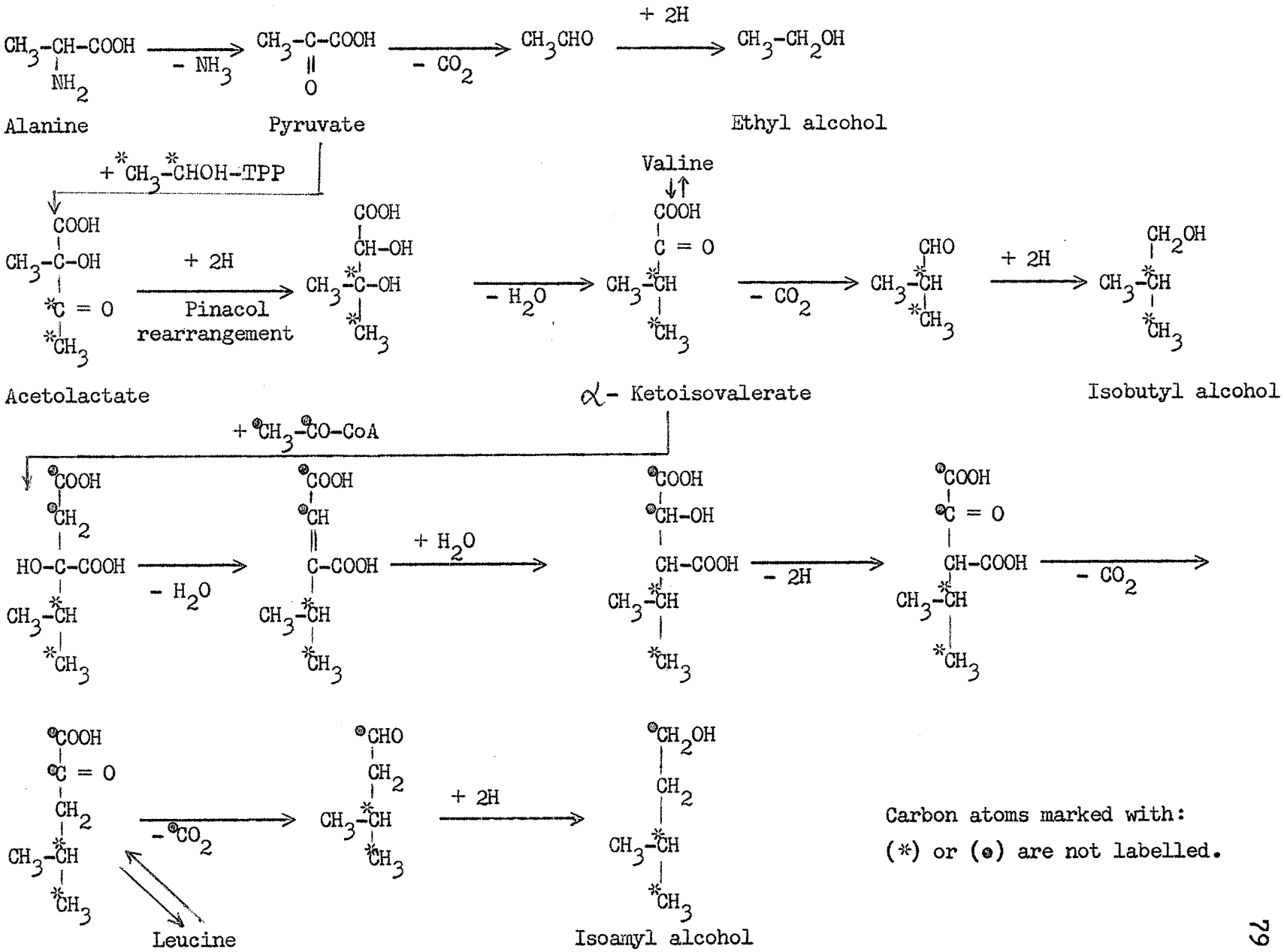


FIGURE 26. Pathway of Biosynthesis of Alcohols from Alanine

SUMMARY

The biosynthesis of the flavour alcohols in whisky was studied with the aid of radioactive tracer technique. A standard bourbon whisky mash containing corn, rye and malt was employed throughout for many small-scale fermentations to produce crude whisky. Seven carbon-14 uniformly labelled amino acids, namely, leucine, isoleucine, threonine, valine, alanine, serine, and tyrosine, were individually fermented with standard mashes and served as tracer components in this study.

The methods of mashing and fermentation were similar to those employed in bourbon whisky manufacture. The separation of flavour alcohols was achieved by gas chromatography and the eluted alcohols were collected and assayed for radioactivity. The CO_2 produced was trapped in an ethanolamine solution and also assayed for radioactivity. All radioactivity assays were performed by liquid scintillation counting.

With the exception of the experiments with serine and tyrosine, the amounts of radioactivity incorporated in the distilled alcohols were proportional to the amounts added with the amino acids. The alcohols formed from individual amino acids and the percent incorporation of radioactivity in these alcohols and in the CO_2 formed were as follows:

(a) From L-leucine-C^{14}(U): isoamyl alcohol, 22.7 per cent; CO_2, 5.3 per cent incorporation.

(b) From L-isoleucine-C^{14}(U): optically active amyl alcohol, 21 per cent; CO_2, 4.8 per cent.

(c) From L-threonine-C^{14}(U): ethyl alcohol, 8 per cent; n-propyl alcohol, 2 per cent; optically active amyl alcohol, 9 per cent; CO_2, 5 per cent.

(d) From L-valine-C^{14}(U): isobutyl alcohol, 13.5 per cent; isoamyl alcohol, 13.5 per cent; CO_2, 9.5 per cent.

(e) From L-alanine-C^{14}(U): ethyl alcohol, 15.3 per cent; isobutyl alcohol, 3 per cent; isoamyl alcohol, 6.6 per cent (including trace amounts of active amyl alcohol); CO_2, 28.8 per cent.

(f) From L-serine-C^{14}(U): ethyl alcohol, 6.4 per cent (variable); CO_2, 10.8 per cent.

(g) From L-tyrosine-C^{14}(U): no activity found in the distilled alcohols; CO_2, 2.6 per cent.

A study was made of the influence of fermentation time upon the amounts of alcohols formed. The total radioactive alcohols formed from a given amino acid reached a maximum after 2 to 5 days, designated as 100 per cent. The amounts produced before the maximum was reached are indicated below as percentage figures of the maximum. The yields of

total alcohols formed from the respective amino acids for different times of fermentation are tabulated as follows:

	1 day	2 days	3 days	5 days
L-leucine-C^{14}(U)	60%	85%	93%	100%
L-isoleucine-C^{14}(U)	56%	85%	97%	100%
L-threonine-C^{14}(U)	78%	100%		
L-valine-C^{14}(U)	85%	100%		
L-alanine-C^{14}(U)	85%	100%		
L-serine-C^{14}(U)	70%*	100%*		
L-tyrosine-C^{14}(U)	no radioactivity found in distillate			

* Results are variable

It was also found that the longer carbon chain alcohols were synthesized at a relatively slower rate than the shorter chain alcohols.

The radioactivity in the yeast decreased during the fermentation while the radioactivity in the supernatant increased.

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APPENDIX

LIQUID SCINTILLATION COUNTING CHANNEL RATIO AND COUNTING EFFICIENCY

Ratio	Efficiency	Ratio	Efficiency
0.31	79%	0.61	67%
0.32	79	0.62	66.5
0.33	79	0.63	66
0.34	79	0.64	65
0.35	79	0.65	64
0.36	79	0.66	63
0.37	79	0.67	62
0.38	79	0.68	61
0.39	79	0.69	60.5
0.40	79	0.70	60
0.41	78	0.71	59
0.42	78	0.72	58
0.43	77.5	0.73	57.5
0.44	77	0.74	57
0.45	77	0.75	56
0.46	76.5	0.76	55
0.47	76	0.77	54
0.48	75.5	0.78	53.5
0.49	75	0.79	52.5
0.50	74.5	0.80	52
0.51	74	0.81	51
0.52	73	0.82	50
0.53	72.5	0.83	49
0.54	72	0.84	48
0.55	71	0.85	47
0.56	70.5	0.86	45
0.57	70	0.87	44
0.58	69	0.88	42.5
0.59	68	0.89	41
0.60	67.5	0.90	40