amide band. The 1660 cm.$^{-1}$ band (called a σ-band) is characteristic of a solvated deuterated amide group and appears in all the copolymers. Thus in solution infrared absorption frequencies of the amide groups are observed which can be correlated with random and α-configurations of polypeptides. No quantitative measurements of the relative amounts of these forms have been attempted using these frequencies.

(3) Infrared spectra of D_2O solutions of the copolymers show no evidence of amide bands characteristic of the β-configuration. However equimolar *mixtures* of sodium poly-L-glutamate and poly-L-lysine hydrochloride gel and precipitate, concomitantly show the appearance of a β-amide I band. It may be suggested that this β-band results from the coprecipitation of the two polymers due to meeting of fairly long sequences of carboxyl and amine groups producing thereby a type of "ionic" crosslinking.

(4) Using the optical rotation data at *p*H 3, and assuming that the magnitude of b_0 represents a measure of the helix content, it may be concluded that the helix content decreases when the molar lysine content increases. From these data it appears that 30 mole per cent. L-lysine in a glutamic acid copolymer does not decrease the helix content significantly at *p*H 3, but 60 mole per cent. L-lysine decreases it to less than 25%. Estimates of helix content using optical rotatory dispersion should be regarded as preliminary[17] but are qualitatively confirmed by infrared results. Using the optical rotatory dispersion data in the manner described it appears that at approximate neutrality (*p*H 8) the helix content of these polypeptides ranges from zero in the copolymer with 30% lysine to a maximum of 30% in that with 50% lysine.

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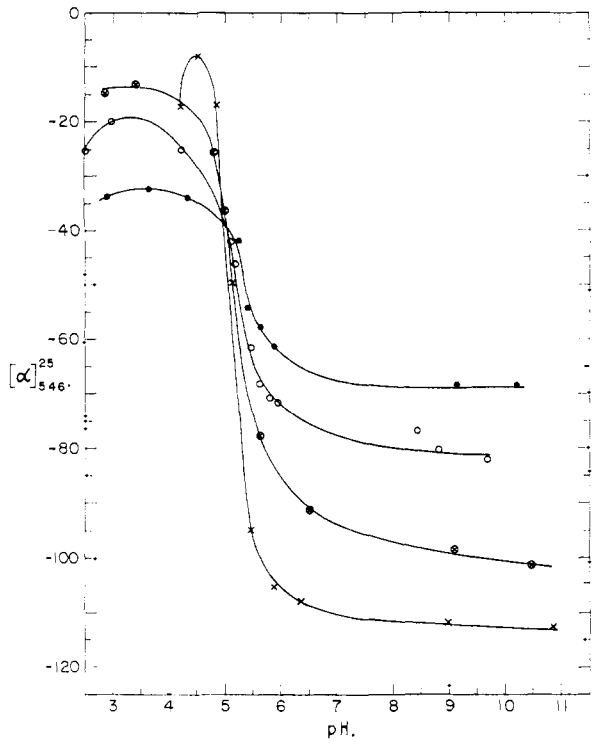

Fig. 4.—The optical rotation, $[\alpha]^{22}_{546}$, as a function of *p*H in aqueous solution: ●—●—●, 5:5 copoly-L-glutamic acid:L-lysine; O—O—O, 6:4 copoly-L-glutamic acid:L-lysine; ⊗—⊗—⊗, 7:3 copoly-L-glutamic acid:L-lysine. The poly-L-glutamic acid data, ×—×—× (ref. 4), is shown for comparison.

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Studies in Valine Biosynthesis. II. α-Acetolactate Formation in Microörganisms

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Extracts of *Saccharomyces cerevisiae* and *Escherichia coli* have been shown to be capable of converting pyruvate to α-acetolactate.

In an isotope tracer study of the biosynthesis of valine in *Torulopsis utilis*, Strassman, Thomas and Weinhouse[1] proposed a mechanism of valine biosynthesis which involves a condensation of pyruvate and acetaldehyde to yield α-acetolactate, followed by an intramolecular migration of one of the methyl groups of acetolactate from the α- to the β-carbon to yield the valine carbon skeleton. α-Acetolactate was first proposed as a biological intermediate by Krampitz,[2] who synthesized this compound and showed it to be decarboxylated to acetylmethylcarbinol (acetoin) in extracts of *Staphylococcus aureus*. Juni[3] subsequently demonstrated that α-acetolactate was an intermediate in acetoin formation in *Aerobacter aerogenes*, and found later[4] that a "pyruvic oxidase" preparation of *Escherichia coli* synthesized α-acetolactate from

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(3) E. Juni, *J. Biol. Chem.*, **195**, 715 (1952).

(4) E. Juni and G. A. Heym, *ibid.*, **218**, 365 (1956).

pyruvate. Additional evidence of α-acetolactate synthesis in *E. coli* was provided by Umbarger, *et al.*,[5] who further implicated acetolactate in valine biosynthesis by showing that a valine-requiring mutant of this organism when grown with limiting amounts of valine accumulated ten times as much acetolactate as the parent strain.

In a recent communication from this laboratory,[6] enzymatic data were reported indicating that yeast has the ability to convert acetolactate to the keto analog of valine.

However, Juni[7] found no evidence for acetolactate formation in yeast, or of its decarboxylation, and concluded that it is not an intermediate of acetoin formation in this organism. On the other hand, Dirscherl and Höfermann[8] found that fresh baker's yeast decarboxylated acetolactate and pyruvate equally rapidly.

In view of the uncertainty regarding the capability of yeast for acetolactate formation it was desirable to reinvestigate this question. The present report indicates that ordinary baker's yeast, as well as a wild strain of *E. coli* synthesize acetolactate from pyruvate.

Methods and Results

α-Acetolactic acid was prepared as the acetoxyethyl ester and was hydrolyzed to acetolactate by the method of Krampitz.[2] Sodium pyruvate-3-C^{14} was purchased from the Nuclear Instrument and Chemical Company, Chicago. It was diluted with non-isotopic sodium pyruvate to a concentration of 0.26 *M* with a specific activity of 7.7 μc./millimole. Other chemicals were commercial products.

Preparation of Yeast Extract.—Dry Fleischmann's yeast, 7.0 g., was mixed in a mortar with 50 g. of fine glass beads (Minnesota Mining and Manufacturing Company #107) and 10 ml. of 0.13 *M* potassium phosphate buffer, *p*H 5.5. The mixture was chilled to 2°, transferred with 5 ml. of buffer to an ice-salt-cooled, jacketed Waring blendor and blended a total of 2 minutes (in intermittent periods of 30 seconds in order to avoid overheating). After addition of 15 ml. of the same buffer, the beads and unbroken cells were removed by centrifugation at 4,500 r.p.m. for 15 minutes and the supernatant was decanted and recentrifuged for 30 minutes at 25,000 r.p.m. in a Spinco Model L ultracentrifuge.

Incorporation of Radioactive Pyruvic Acid into α-Acetolactic Acid.—Three Warburg vessels each containing: yeast extract, 1.0 ml.; 0.26 *M* sodium pyruvate-3-C^{14}, 1.0 ml.; 0.3 *M* sodium acetolactate, 1.0 ml.; 0.154 *M* magnesium sulfate, 0.1 ml.; and 0.01% diphosphothiamine, 0.1 ml., were incubated for 4 hours at 30° in a gas phase of 95% O_2–5% CO_2. The contents of the 3 flasks were then combined and an equal volume of absolute ethanol was added. The protein precipitate was removed by 15 minutes centrifugation at 4,500 r.p.m., the supernatant was evaporated to dryness under reduced pressure, and the residue was taken up in 10 ml. of 0.13 *M* potassium phosphate buffer, *p*H 5.5. Ten ml. of phenylhydrazine reagent (4 g. of phenylhydrazine hydrochloride and 6.4 g. of sodium acetate dissolved in water to 30 ml.) was added, and, after standing for 1 hour at room temperature, the precipitated osazone of acetylmethylcarbinol was filtered, washed extensively with hot ethanol and weighed (yield 98.8 mg. = 49%). The osazone was plated and counted, then repeatedly recrystallized from dioxane and washed with hot alcohol. The specific radioactivity of the original precipitate and of each of four successive recrystallizations were, respectively, 2530, 2560, 2580, 2525 and 2540 c.p.m. A sample of the recrystallized material was oxidized to CO_2 by dichromate[9] and the CO_2 precipitated as $BaCO_3$. It had a specific activity of 187 c.p.m. Since only 4 of the 16 carbons of this osazone represent acetolactate carbons, the specific activity of the latter can be estimated to be $4 \times 187 = 748$ c.p.m.

Juni[3] has shown that in this procedure only acetolactate reacts to form an osazone and we have confirmed this finding. In preliminary tests it was found that whereas acetaldehyde and pyruvic acid would also react with the phenylhydrazine reagent, the products were completely soluble in hot ethanol. As a further test of the specificity of this procedure, synthetic mixtures containing radioactive acetolactate (prepared synthetically from acetoacetate-2,4-C^{14}) were treated with phenylhydrazine reagent and the precipitates were filtered, washed with hot ethanol and counted. In the sample containing only 0.54 mmole of C^{14}-acetolactate the radioactivity was 436 c.p.m. In the sample containing a mixture of 0.54 mmole of C^{14}-acetolactate and 0.45 mmole of acetoin, the activity was 440 c.p.m. In a third sample, containing a mixture of 0.54 mmole of C^{14}-acetolactate, 0.17 mmole of acetoin, 0.26 mmole of pyruvate and 0.25 mmole of acetaldehyde, the activity was 434 c.p.m. Moreover, in the procedure used most of the acetoin and any acetaldehyde present are eliminated by evaporation, hence the isolated osazone of acetoin (diacetyl-(bis)-phenylhydrazone) could only have been derived from α-acetolactate. Thus we can be reasonably certain that the radioactivity incorporated into the acetolactate was not due to contamination with any of the other carbonyl compounds present in the reaction mixture.

To obtain analytical data on pyruvate disappearance, and on the appearance of acetolactate and acetoin, a pilot experiment was run at the same time with non-radioactive pyruvate, and the data thus obtained are given in Table I. Pyruvate was determined colorimetrically by the method of Friedemann and Haugen[10] and acetolactate and acetoin by Westerfeld's procedure.[11] α-Acetolactate does not produce a color in this procedure but is rapidly decarboxylated to acetoin upon acidification.[3] Hence both compounds may be determined by performing the Westerfeld test before and after treatment with dilute acid.

TABLE I

PYRUVATE DISAPPEARANCE AND ACETOLACTATE FORMATION IN EXTRACTS OF *S. cerevisiae*

Values are given in micromoles.

	Flask number 1	2	3
Pyruvate at start	260	0	260
Actolactate at start	300	300	0
Pyruvate at end	110	0	110
Acetoin at end	80	60	23
Acetolactate at end[a]	250	240	0

[a] Determined by difference between acetoin after and before acidification.

The first flask contained a mixture of pyruvate and acetolactate. After 4 hours incubation in air most of the pyruvate had disappeared, and appreciable quantities of acetolactate and acetoin were present. In flask 2, which contained acetolactate alone, most was recovered unchanged. With pyruvate alone (flask 3), only small quantities of acetoin and no acetolactate were found. Assuming that the analytical data in column 2 of Table I are representative of the experiment with labeled pyruvate, rough estimations of the radiochemical yield of acetolactate can be made, and these are shown in column 5 of Table II. About 3.7% of the pyruvate initially present, and about 6.6% of that used, was converted to acetolactate.

Accumulation of α-Acetolactic Acid.—As shown in column 4 of Table I, there was no α-acetolactic acid accumulation during incubation with pyruvate. These results are typical of many experiments in which extracts were incubated with concentrations of pyruvate between 0.05 and 0.1 *M*. However, as shown in Table III, when the concentrations of pyruvate were increased to 0.5 *M*, accumulation of α-acetolactic acid definitely occurred, though the amount varied from experiment to experiment.

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(11) W. W. Westerfeld, *ibid.*, **161**, 495 (1945).

TABLE II

RADIOCHEMICAL YIELD OF ACETOLACTATE FROM PYRUVATE-3-C^{14}

	Amount, μmoles	Spec. act., c.p.m.	Total act.,[a] c.p.m.	% of total act.
Pyruvate at start	780	38,740	90,600	100
Pyruvate at end	440	38,740	50,900	56.2
Acetolactate	900	748	3,360	3.7

[a] Total act. = spec. act. × milliatoms carbon.

TABLE III

α-ACETOLACTATE ACCUMULATION IN YEAST EXTRACT

Each flask contained 1.0 ml. of yeast extract, 0.8 ml. of 0.13 *M* potassium phosphate buffer, *p*H 7.4, 0.1 ml. of 0.15 *M* magnesium sulfate, 0.1 ml. of 0.1% diphosphothiamine and 1 ml. of 1.5 *M* sodium pyruvate. CO_2 evolution was measured manometrically. Incubation time was 2 hours at 30°, with 95% nitrogen–5% CO_2 in the gas phase. Values are given in μmoles.

Flask no.	CO_2 Evolved	Acetoin formed	Acetolactate[a] formed
1	135	47	28
2	134	53	11
3	112	52	10
4	140	50	21
5		45	28
Av.		49 ± 3	20 ± 7

[a] Determined by difference between acetoin after and before acidification.

Acetolactate Formation in *E. coli*.—It was of further interest to determine whether acetolactate is synthesized in *E. coli*. This hitherto had not been reported; however, during the progress of this work Juni and Heym[4] reported that extracts of this organism produced racemic α-acetolactic acid, and Umbarger *et al.*,[5] revealed that extracts of valine-requiring *E. coli* mutants formed α-acetolactate from pyruvate. These findings have been confirmed in the present study for a wild strain of this organism.

Preparation of Extract.—Eight liters of peptone-beef extract–yeast extract medium[3] were inoculated with *E. coli* ATCC 9637 and grown for 16 hours at 37° with rapid aeration. The cells were harvested by means of a Westphalia centrifuge and washed with 4 liters of 0.1 *M* phosphate buffer, *p*H 7.4. The cell pack, weighing 30 to 50 g., was suspended in 0.05 *M* potassium phosphate buffer, *p*H 7.0, and centrifuged at 4,000 r.p.m. for 30 minutes. After washing and centrifuging in this manner three times, cells were suspended in a volume of buffer so that the final concentration of cells was approximately 0.5 g./ml. Twenty-five ml. of the suspension was mixed with 10 g. of glass beads and subjected to sonic vibration for 40 minutes with a Raytheon 9 kc. oscillator. The beads were removed by centrifugation at 4,500 r.p.m., the supernatant was decanted, and heavy particles were centrifuged in a Spinco ultracentrifuge at 20,000 r.p.m. for 45 minutes and discarded. The extract thus prepared was either stored as such in a deep-freeze or made into an acetone powder by pouring into 20 volumes of acetone cooled to −27°. The air-dried acetone powder was then stored in a deep freeze.

TABLE IV

PYRUVATE DISAPPEARANCE AND ACETOLACTATE FORMATION IN EXTRACTS OF *E. coli*

Complete system: Tris buffer *p*H 8.3, 100 μmole; sodium pyruvate, 75 μmole; magnesium sulfate, 10μmole; ATP,[12] 3 μmole; DPN, 1.5 μmole; dialyzed extract, 0.3 ml.; water to 1 ml. Incubated in air at 30° for 90 min. Values are given in micromoles. Diphosphothiamine was not necessary for pyruvate utilization and was omitted.

Component omitted	Pyruvate utilized	Acetoin	Acetolactate
None	74	0.02	1.89
$MgSO_4$	73	.01	1.66
ATP	74	.01	1.73
DPN	26	.03	3.09

Formation of α-Acetolactate from Pyruvate.—In Table IV are given the results of a typical experiment showing acetolactate formation from pyruvate. In the complete system the recovery of pyruvate in acetolactate was 1.89 μmoles and was not lowered by omission of Mg^{++} ions, ATP or DPN^+. In fact, in the absence of DPN^+ utilization of pyruvate was diminished and the formation of acetolactate was increased.

TABLE V

ACETOLACTATE DISAPPEARANCE IN EXTRACTS OF *E. coli*

Complete system: 1.0 ml. extract; 0.1 *M* $MgSO_4$, 0.1 ml.; 0.03 *M* DPN, 0.1 ml.; 0.06 *M* ATP, 0.1 ml.; 0.05 *M* sodium α-acetolactate, 0.5 ml.; 0.05 *M* potassium phosphate buffer, *p*H 7.0 to 3.0 ml. Time 120 min., temp. 30°, gas phase 95% N_2–5% CO_2. Values are given in micromoles.

Component omitted	Acetoin found	Acetolactate found	Acetolactate used, %
None	2.73	10.5	51.2
ATP	3.56	11.7	45.6
DPN	3.98	16.2	24.6
DPN + ATP	4.31	14.6	32.0
Sonicate	4.25	21.5	0.0

The low net formation of acetolactate in these experiments is in part attributable to its further metabolism by the extract. As shown in Table V, 2 hours incubation of acetolactate in the same medium results in a loss of about 50%. A large proportion of this is accountable as acetoin, but there was also a large fraction converted to unknown products. The disappearance was decreased somewhat on omission of ATP and DPN^+.

Although the products were not identified in the present experiments, it subsequently has been found[6] that similarly prepared extracts of *S. cerevisiae* can convert α-acetolactate to α-ketoisovaleric acid. In thus demonstrating the ability of yeast to form acetolactate, these results lend support to the hypothesis advanced earlier concerning the role of this substance in valine biosynthesis and emphasize its physiological significance in the growth of microörganisms.

(12) Abbreviations used are: adenosine triphosphate, ATP; diphosphopyridine nucleotide, DPN^+; tris-hydroxymethylaminomethane, tris.

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