In the preceding publication (1) mention has been made of the syntrophism existing between the mutant strains 11A16 and 20A19 of *Escherichia coli*. These mutants, requiring isoleucine and isoleucine plus valine respectively, were able to grow together on a minimal agar plate, free of amino acids, which would not support the growth of either mutant alone. The nature of this syntrophism forms the subject of the present communication.

The culture fluids of strain 11A16 were found to be markedly inhibitory to the parent strain K-12, but this inhibition could be overcome by the addition of isoleucine. When the test for inhibition was carried out auxanographically by placing a disk of filter paper saturated with the strain 11A16 culture fluid on a minimal agar plate seeded with strain K-12, a clear zone of inhibition was found to surround the paper disk; however, inside this zone, close to the paper disk, a reversal of the inhibition could be observed. Similar experiments in which the isoleucine-and valine-requiring mutant, 20A19, was used in place of strain K-12 indicated a zone of growth of the mutant corresponding to the zone in which the inhibition of strain K-12 had been reversed in the previous experiment.

These observations indicated that compounds having biological effects similar to valine and isoleucine were accumulating in culture fluids of strain 11A16. In preliminary experiments carried out in an attempt to determine the chemical nature of these compounds and to develop pro-
cedures suitable for their isolation, it was found that both isoleucine and valine activity could be removed from culture fluids by steam distillation and by continuous extraction with ethyl ether or n-butanol under acid conditions. The active principles could be removed from the ether or n-butanol extracts by shaking with alkali. The steam distillates and ether extracts were found to be free of compounds reacting with ninhydrin. From these experiments, it appeared that the active compounds were organic acids not containing amino nitrogen.

In the isolation procedure adopted, the active components were removed from the acidified culture fluids by extraction with n-butanol, from which they were in turn extracted by dilute aqueous alkali. The alkaline extracts were concentrated, acidified, and steam-distilled. The distillate was found to contain the valine and isoleucine activity originally present in the culture fluids.

Counter-current distribution of the steam distillate between n-butanol and water resulted in a partial separation of the valine- and isoleucine-active components from one another and from the bulk of the other volatile acids. The titratable acidity and valine activity of the fractions are shown in Fig. 1.

The fractions containing the active components were subjected to paper chromatography in four solvent systems and the positions of the keto acids on the chromatograms were determined by development with semicarbazide, as described previously (2). Fractions 4 to 11 showed two
spots in all the solvent systems, corresponding in position to synthetic (1) $\alpha$-ketop-$\beta$-methyl-$n$-valeric and $\alpha$-ketoisovaleric acids, while Fraction 12 seemed to contain the latter keto acid only. The identity of the separated keto acids with the compounds responsible for the isoleucine and valine activity of the fractions was established by cutting untreated paper rectangles containing the separated keto acids from the paper chromatograms and testing them auxanographically.

Although filter paper chromatography has not as yet been accepted as rigorous proof of the identity of chemical compounds, it is felt that the failure to separate the synthetic and naturally occurring compounds in four distinct solvent systems constitutes impressive evidence that the $\alpha$-keto acids analogous to valine and isoleucine were present in culture filtrates from strain 11A16. However, in the case of the former compound, it was possible to supplement this evidence by the more traditional chemical procedures. The phenylhydrazone of the keto acid was prepared from the material in Plate 10 of the counter-current distribution experiment and found to have a melting point and ultraviolet absorption spectrum identical with those of synthetic $\alpha$-ketoisovaleric acid.

The attempt to identify the accumulating keto acid showing isoleucine activity as its phenylhydrazone was unsuccessful because insufficient material was available.$^1$ $^2$

These keto acids have been shown to fulfill the valine and isoleucine requirements of the mutant strain 20A19 (1). Thus one phase of the syntrophic effect can be explained. Strain 11A16 excreted into the agar medium the only two metabolites which strain 20A19 is unable to synthesize. It was also shown that, with excess $\alpha$-ketop-$\beta$-methyl-$n$-valeric acid, strain 20A19 was able to convert it into isoleucine and to excrete a portion of the latter into the medium, where it was readily observed by filter paper chromatography. Concomitantly, the keto acid was shown to disappear. Thus strain 20A19, after being supplied with the keto acid analogues of valine and isoleucine by strain 11A16, in turn supplied strain

$^1$ It is hardly to be expected that the phenylhydrazone of naturally occurring $\alpha$-ketop-$\beta$-methyl-$n$-valeric acid and the phenylhydrazone of the synthetic keto acid have identical melting points. The synthetic compound is a racemic mixture, while the natural compound is, in all likelihood, a single optical isomer. This factor, of course, did not interfere with the identification made by partition chromatography on filter paper with optically inactive solvents. Whether the naturally occurring keto acid is indeed optically active remains to be determined.

$^2$ It is characteristic of tube cultures (anaerobic) of strain 11A16 that $\alpha$-ketoisovaleric acid is in excess (hence, their inhibitory quality to the wild strain K-12). Shaken cultures have been shown to accumulate the keto acids also. The proportion of these keto acids in filtrates of shaken cultures apparently is such that the filtrates are not inhibitory to the wild strain. Rather, these filtrates are predominantly isoleucine-active. This problem is under investigation.
11A16 with its missing metabolite, isoleucine, the result being the syn-
trophism observed.

The demonstration of \(\alpha\)-ketoisovaleric and \(\alpha\)-keto-\(\beta\)-methyl-\(n\)-valeric acids in culture filtrates of the \textit{E. coli} mutant, strain 11A16, genetically blocked in its conversion of the latter keto acid to isoleucine, offers pre-
sumptive evidence that the keto acids are in the direct metabolic pathway
in isoleucine and valine synthesis. These findings indicate that the
mechanism, postulated by Bonner (3) for \textit{Neurospora}, has been rigorously
demonstrated as occurring in \textit{E. coli}.

**EXPERIMENTAL**

\textit{Materials}—The basal medium, organisms, amino acids, and keto acids
were the same as those described previously (1).

\textit{Culture Filtrates}—A 40 liter culture of mutant 11A16 was grown in a
50 liter round bottom flask. Growth was limited by the concentration of
\(\text{dl}\)-isoleucine added to the medium (6 \(\gamma\) per ml.). After 4 days incuba-
tion, the cells were removed by centrifugation in a Sharples supercentri-
fuge. The filtrate was made acid to Congo red, saturated with sodium
chloride,\(^3\) and extracted five times by shaking with \(n\)-butanol.

The butanol extraction removed about 90 per cent of the activity from
the culture filtrate. A further concentration was effected by extraction
with sodium hydroxide solution; this was acidified with 50 per cent \(\text{H}_2\text{SO}_4\)
and stored in the cold room. In the cold, a crystalline mass of inorganic
salts precipitated, which were removed by filtration. The acidic solution
was extracted continuously with ether for 48 hours. The ether extract
was then saturated with water and the ether removed by vacuum distil-
lation.

By these procedures, the active components were concentrated to a
volume of about 60 ml. One-half of this concentrate was made acid to
Congo red and steam-distilled. A total volume of 900 ml. was collected
in 100 ml. portions. The first 100 ml. contained by far the greater portion
of the active components.

This portion was extracted continuously with ether for 48 hours and
the ether removed \textit{in vacuo}. The acid mixture thus obtained was diluted
to 100 ml. with water saturated with \(n\)-butanol.

\textit{Preliminary Separation by Counter-Current Distribution}—The counter-
current distribution was performed by passing the water phase over \(n\)-
butanol in 250 ml. separatory funnels mounted in the shaking device
previously used in this laboratory (4, 5). Each plate consisted of a 100

\(^3\) Solvent extraction as well as steam distillation was found to be much more
efficient if a high salt concentration was employed.
ml. aqueous phase and a 100 ml. n-butanol phase. The distribution was continued until twenty-four plates were obtained.

The efficacy of the separation was judged by the distribution of acidity. The titratable acidity was determined with standard alkali, with phenolphthalein as the indicator. The titration in a two phase system (butanol-water) was difficult, but with care a fairly accurate estimation of the acidity could be obtained.

After neutralization the solutions were evaporated to dryness in vacuo and taken up in 12 ml. of distilled water. These solutions were assayed auxanographically (see Fig. 1).

**Filter Paper Chromatography**—Amino acids were chromatographed by capillary ascent (6) on Whatman No. 1 filter paper with sec-butanol plus 5 per cent formic acid, half saturated with water, as the solvent. Keto acids were chromatographed by the method previously described (2). The solvents used were n-butanol plus 5 per cent acetic acid, n-butanol plus 5 per cent propionic acid, sec-butanol plus 5 per cent acetic acid, and sec-butanol plus 5 per cent propionic acid, the solvents in each case being about 90 per cent saturated with water.

Auxanographic tests on the separated keto acids were performed by cutting a column from a fully developed, air-dried chromatogram and placing it in the form of small rectangles on agar plates seeded with the test organism. The position of the valine-active component could be detected by an inhibition zone on minimal agar plates seeded with the wild strain, *E. coli* K-12, surrounding the filter paper section containing that component. The position of the isoleucine-active component could be detected by reversal of the inhibition zone when valine was placed in the vicinity of the filter paper rectangles. Alternatively, this component could be detected on plates containing valine and seeded with strain 2OA19 (the organism requiring both isoleucine and valine or the corresponding α-keto acids). The positions determined biologically could then be compared with parallel columns cut from the same chromatogram, sprayed with the semicarbazide reagent, and examined under ultraviolet light.

**α-Ketoisovaleric Acid Phenylhydrazone** The phenylhydrazones of natural and synthetic α-ketoisovaleric acid were prepared as previously described (1). The melting point of each was 144–146°C (uncorrected), as determined by a Fisher-Johns melting point block. There was no depression upon mixing. Each phenylhydrazone exhibited molar extinctions of 15,290, 9050, and 7520 at 332, 298, and 238 mp, respectively, in ethanol, with a Beckman quartz spectrophotometer, model DU.

**Formation of Isoleucine in Strain 20A19 Cultures**—The mutant strain 20A19, requiring isoleucine or its keto acid analogue in addition to valine
or its keto acid analogue for growth, was tested for its ability to aminate \( \alpha\)-keto-\( \beta\)-methyl-\( \alpha\)-valeric acid during growth. The medium containing a limiting concentration of \( \alpha\)-valine (10 \( \gamma \) per ml.) and excess \( \alpha\)-keto-\( \beta\)-methyl-\( \alpha\)-valeric acid (50 \( \gamma \) of the sodium salt per ml.) was inoculated with strain 20A19. The filtrates from such cultures contained a factor permitting growth of strain 11A16. This factor was shown by filter paper chromatography to be isoleucine. It was also demonstrated that the \( \alpha\)-keto-\( \beta\)-methyl-\( \alpha\)-valeric acid disappeared from the medium by use of the usual chromatographic procedure. No attempts were made to demonstrate amination of \( \alpha\)-ketoisovaleric acid.

**SUMMARY**

Culture fluids of the isoleucine-deficient *Escherichia coli* mutant strain 11A16 were found to contain substances capable of supporting the growth of the isoleucine- and valine-deficient mutant strain 20A19. These substances were identified as \( \alpha\)-keto-\( \beta\)-methyl-\( \alpha\)-valeric acid and \( \alpha\)-ketoisovaleric acid, the keto analogues of isoleucine and valine respectively.

The syntrophism occurring between strains 11A16 and 20A19 on minimal agar plates may be explained by the accumulation of the keto acids by strain 11A16, supporting the growth of strain 20A19, and by a conversion of excess \( \alpha\)-keto-\( \beta\)-methyl-\( \alpha\)-valeric acid to isoleucine by strain 20A19, allowing in turn the continuous growth of strain 11A16.

**BIBLIOGRAPHY**

ISOLEUCINE AND VALINE METABOLISM OF ESCHERICHIA COLI: II. THE ACCUMULATION OF KETO ACIDS
H. Edwin Umbarger and Boris Magasanik


Access the most updated version of this article at http://www.jbc.org/content/189/1/287.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/189/1/287.citation.full.html#ref-list-1