TABLE II

ISOALLOXAZINES

	Compound	Yield, %	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	M.p., °C.
XII	$C_{16}H_{17}N_4O_7Cl$	54	46.55	46.80	4.15	4.22	235–245
XIII	$C_{16}H_{17}N_4O_7Cl$	70	46.55	46.38	4.15	4.14	276–279
XIV	$C_{16}H_{17}N_4O_7Cl \cdot H_2O$	64	44.61	44.79	4.49	4.21	289–290
XV	$C_{15}H_{15}N_4O_6Cl$	66	47.06	47.21	3.95	3.99	271–272
XVI	$C_{15}H_{15}N_4O_6Cl$	67	47.06	47.26	3.95	4.02	271–273
XVII	$C_{15}H_{15}N_4O_6Cl$	56	47.06	47.26	3.95	3.80	263–264
XVIII	$C_{15}H_{15}N_4O_6Cl$	52	47.06	47.35	3.95	3.99	282–283
XIX	$C_{17}H_{19}N_4O_7Cl$	48	47.84	47.54	4.49	4.84	255–256
XX	$C_{17}H_{19}N_4O_7Cl$	66	47.84	47.85	4.49	4.34	276–278
XXI	$C_{17}H_{20}N_4O_7$	65	52.03	52.08	5.14	5.43	285–290
XXII	$C_{17}H_{20}N_4O_8$	51	49.99	49.92	4.94	5.33	277–280

1-Chloro-4-iodo-3-nitrobenzene,[2] 4-iodo-3-nitrotoluene[6] and 4-iodo-3-nitroanisole[7] were prepared from the corresponding 4-amino compounds.

Preparation of Nitroanilines I–XI.—A synthesis of 4-chloro-2-nitro-N-(1′-D-sorbityl)-aniline is reported as representative of the method used.

Twenty grams of D-glucamine (85% pure by titration) and 50 g. of 1-chloro-4-iodo-3-nitrobenzene in 150 ml. of pyridine were heated at reflux temperature with stirring in a nitrogen atmosphere for six hours. Steam was passed into the solution until the pyridine had been removed and the resulting mixture was concentrated under reduced pressure. The residue was triturated with cold water and the solid was collected and washed with acetone giving 4.0 g. of an orange solid, m.p. 148–150°. Recrystallization from methanol gave 2.8 g. of 4-chloro-2-nitro-N-(1′-D-sorbityl)-aniline, m.p. 166–167°.

The nitroanilines are described in Table I.

Preparation of Isoalloxazines XII to XXII.—A synthesis of 6-chloro-9-(1′-D-sorbityl)-isoalloxazine is reported to illustrate the method used.

4-Chloro-2-nitro-N-(1′-D-sorbityl)-aniline (2.7 g.) in 100 ml. of 80% acetic acid was hydrogenated using 1.0 g. of palladium-on-Darco catalyst (5% palladium). After removing the catalyst the filtrate was added to a suspension of 1.5 g. of alloxan and 3.4 g. of boric acid in 110 ml. of acetic acid. After standing at room temperature for two days, the solvent was distilled under reduced pressure and two portions of ethanol were added to the residue and distilled. The residue was triturated with a minimum amount of cold water and the precipitate was collected and recrystallized from water containing about 0.5% acetic acid. A second recrystallization gave 1.8 g. of 6-chloro-9-(1′-D-orbityl)-isoalloxazine, m.p. 235–245° with softening at 210°.

The isoalloxazines are described in Table II.

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[CONTRIBUTION FROM THE DEPARTMENT OF BACTERIOLOGY AND IMMUNOLOGY, HARVARD MEDICAL SCHOOL]

Isoleucine and Valine Metabolism in *Escherichia coli*. III. A Method for the Quantitative Determination of α-Keto Acid Analogs of Isoleucine and Valine[1]

BY H. EDWIN UMBARGER AND BORIS MAGASANIK

RECEIVED MARCH 17, 1952

A method is described for the separation of mixtures of α-keto acids by paper chromatography and for the quantitative estimation of their components. The method consists of finding the positions of the keto acids by examination under ultraviolet light after treatment of the paper with semicarbazide and subsequent conversion of the semicarbazones to the corresponding 2,4-dinitrophenylhydrazones, which are extracted from the paper with alkali and determined colorimetrically. The accuracy of the method is about ±5%.

The separation and identification of the individual components in a mixture of keto acids by paper chromatography has been described in an earlier communication from this Laboratory.[2] This method proved to be of considerable value in the identification of the α-keto acid analogs of isoleucine and valine accumulating in the culture fluids of a biochemically deficient mutant of *Escherichia coli*[3] and the subsequent demonstration of their role as the immediate precursors of isoleucine and valine in a variety of microörganisms.[4]

The present paper deals with the application of this technique to the quantitative estimation of α-ketoisovaleric and α-keto-β-methyl-*n*-valeric acids in mixtures of keto acids. Preliminary experiments indicate that the same method may also be used for the estimation of pyruvic acid and α-ketoglutaric acid. Because of the great importance of α-keto acids in metabolic processes, such a method should be generally useful. Its application to the study of the transamination reactions of isoleucine and valine is described in the following paper.[5]

Experimental

Filter Paper.—Of several commonly used papers which were tested only Eaton and Dikeman (E & D) 613 was sufficiently inert to the semicarbazide treatment to permit observation of the exact location and of the extent of the keto acid spots.

(1) This work was supported in part by funds received from the Eugene Higgins Trust and by a grant from the American Cancer Society to Harvard University. This paper was presented in part before the Division of Biological Chemistry at the 119th Meeting of the American Chemical Society in Boston, Massachusetts, April 4, 1951.

(2) B. Magasanik and H. E. Umbarger, THIS JOURNAL, **72**, 2308 (1950).

(3) H. E. Umbarger and B. Magasanik, *J. Biol. Chem.*, **189**, 287 (1951).

(4) H. E. Umbarger and E. A. Adelberg, *ibid.*, **192**, 883 (1951).

(5) H. E. Umbarger and B. Magasanik, THIS JOURNAL, **74**, 4256 (1952).

Solvents.—The choice of solvents was determined by the nature of the keto acids to be separated. For the separation of α-ketoisovaleric and α-keto-β-methyl-*n*-valeric acids, a propionic acid–*s*-butanol solvent was most satisfactory. In this solvent the R_f values of these two keto acids are approximately 0.57 and 0.66, respectively, and are well separated from each other and from α-ketoglutarate with an R_f value of about 0.13. In general, it has been observed that the stronger organic acids, formic and acetic, increase the R_f values of the keto acids and, conversely, the substitution of *n*-butanol for *s*-butanol decreases the R_f values. Thus α-ketoglutaric and pyruvic acids could best be separated by using as solvent a formic and *n*-butanol mixture.

The solvent was prepared by adding 5 parts of the organic acid to 95 parts of the alcohol, in the present study, propionic acid and *s*-butanol, respectively. Distilled water was added in excess of the amount required for saturation, and the mixture allowed to "age" for 3–4 weeks. The empirical criterion for sufficient "aging" (esterification) is the ability of the water saturated solvent to maintain itself in one phase for a 16–20 hour period. The solvent components used in this Laboratory have been Eastman Kodak Co. products.

In using *s*-butanol as solvent it has occasionally been observed that certain lots would bring about almost a total destruction of the keto acids during the chromatographic operation. This has been attributed to the presence of small amounts of peroxides. Such lots of *s*-butanol may be sufficiently purified by treatment with ferrous sulfate in the usual manner. In practice, each new bottle of *s*-butanol is tested by the method of Castiglioni.[6] The test is conveniently performed as follows: Mix 4 drops of the *s*-butanol under test with 2 drops of benzaldehyde and 1 drop of concentrated sulfuric acid in a spot test plate. The appearance of a pink color indicates the presence of peroxide. It has been uniformly observed that the greater the intensity of the test the more nearly complete was the destruction of keto acids on exposure to solvent. It has been considered more convenient to reserve *s*-butanol found to be contaminated with peroxides for the chromatography of materials less labile to peroxides, such as amino acids, rather than performing a purification.

Keto Acid Reagents.—The solution used for spraying the paper contained 0.1% semicarbazide and 0.15% sodium acetate.

The 2,4-dinitrophenylhydrazine as one-quarter the concentration recommended by Friedemann and Haugen, *i.e.*, a 0.025% solution of the reagent in one-half normal hydrochloric acid. A 40% aqueous potassium hydroxide solution was used to develop the color and extract the 2,4-dinitrophenylhydrazones from the paper.

Ketoacid standards were 0.100% aqueous solutions of the sodium salts. The preparation of α-ketoisovaleric and α-keto-β-methyl-*n*-valeric acids has been described previously.[7]

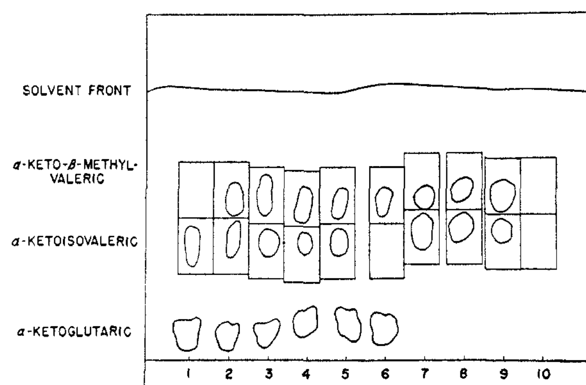

Fig. 1.—Tracing of chromatogram used for determination of α-keto acids formed during transamination: positions 1 through 6, samples; positions 1, 2 and 3, standards; position 10, blank.

(6) A. Castiglioni, *Ann. chim. applicata*, **24**, 209 (1934), Merck Index, 5th Ed., p. 669, 1940.

(7) H. E. Umbarger and J. H. Mueller, *J. Biol Chem.*, **189**, 277 (1951).

Analytical Procedure.—10–30 μl. quantities of the samples containing from 5–40 μg. of the keto acids under test were applied near the bottom of filter paper sheets with the aid of a Gilmont ultramicro buret[8] in aliquots of 2.5 μl. Between additions, the paper was dried by means of a stream of warm air from an ordinary domestic type hair dryer.

In the present studies, three sheets of filter paper were employed: one 50.0 cm. wide, the second 39.5 cm. wide, and the third 29.0 cm. A height of about 40 cm. was satisfactory. A space of 3.5 cm. was allowed between each sample or standard, with a space at each end of the paper of 4.0 cm. Thus, on the papers employed, a series of six samples and three standards (10, 20, and 30 μg.) could be applied in triplicate, the remaining three spaces serving as blanks. When it was desired to measure both α-ketoisovaleric and α-keto-β-methyl-*n*-valeric acids, both standard solutions could be applied at the same spot.

The papers were stitched with cotton thread to form cylinders. The three papers were then placed concentrically in the chromatographic chamber and the usual technique for ascending chromatography employed. After the solvent had risen about 12 inches (16–18 hours), the papers were dried with a stream of warm air. At this point, the chromatograms were examined under ultraviolet light,[9] in order to locate any non-specific areas of absorption due to artifacts in the paper or materials released by cellular debris.

The papers were then sprayed heavily with the semicarbazide reagent. It was essential that the entire paper be coated evenly with the reagent since the droplets of reagent would not diffuse from the point of contact with the paper but, rather, reacted immediately with the keto acid to form insoluble semicarbazones. Insufficient spraying resulted in a mottled appearance when viewed under ultraviolet light making the extent of the keto acid spots difficult to determine.

After spraying, the papers were dried with warm air and again examined under ultraviolet light. The semicarbazones appeared as dark shadows against the light background due to the faint fluorescence of the paper. The extent of the area covered by each semicarbazone spot was then lightly outlined by pencil.

For the determination of the amount of keto acid present, rectangular sections containing the semicarbazones under examination were removed and placed in 25 × 100 mm. test-tubes after cutting the sections into narrow strips. The size of the sections was the same for all samples and standards of any one keto acid (usually 1.25 × 2.0 inches). Filter paper blanks, also of the same size, were cut from the corresponding regions of columns containing no keto acid.

Figure 1 is a tracing of a typical chromatogram used for the separation of α-ketoglutarate, α-ketoisovaleric and α-keto-β-methyl-*n*-valeric acids present in transamination reaction mixtures. The figure shows the relative size of the rectangular sections containing standards, samples and blank which were subsequently used in an analysis for the keto acids formed from isoleucine and valine mixtures.

For the colorimetric determination, 3.00 ml. of the 2,4-dinitrophenylhydrazine solution was added to each tube, followed by 1.00 ml. of 40% KOH after 10 minutes at room temperature. The tubes were then shaken gently in a mechanical shaker for 10 minutes at room temperature to permit extraction of the 2,4-dinitrophenylhydrazones which had been formed within the paper itself. The pieces of filter paper in the test-tubes were tamped down and covered by small cotton wads. The solution could then be removed by means of a 5-ml. pipet and a rubber bulb. The cotton wad served to filter out the macerated paper yet the brief contact with the solution did not influence the determinations.

Readings of the intensity of color were made against a filter paper–reagent blank at 435 mμ using a Coleman Model 11 spectrophotometer which had been adapted for 3 ml. of solution in the standard 15 mm. square cuvettes. The intensities of color of the two remaining blanks were also determined and the readings of all three blanks averaged to obtain a correction, if necessary, for readings obtained with the standards and samples.

Figure 2 shows typical standard curves obtained in the determination of mixtures of the α-keto analogs of valine

(8) Obtained from Emil Greiner Co., New York, N. Y.

(9) "Mineralight" Model No. V-41, obtained from Ultraviolet Products Corp., Los Angeles, California.

and isoleucine. The points plotted are the averages of triplicate standards. The drop in recovery at higher concentrations with these keto acids has been observed repeatedly.[10] From such standard curves, the concentrations of keto acids in samples were computed.

Discussion

The observation that chromatograms can be sprayed with semicarbazide to reveal the exact position of the keto acids without interfering with the subsequent treatment with 2,4-dinitrophenylhydrazine was extremely pertinent to the successful development of the quantitative procedure described here. Thus it was possible to avoid the use of a guide strip[11] and to utilize the space for additional samples. The use of the guide strip presupposes parallel migration of the keto acids—a condition that one does not always obtain when the less elaborate ascending technique is employed.

The absolute recovery of the keto acids, determined by comparing the optical density given by 10 μg. of either keto acid after chromatography with that given by the same quantity of keto acid added directly to the 2,4-dinitrophenylhydrazine reagent, was found to be about 85%. The loss might be attributed to any one or more of three factors: esterification with the solvent on the paper, insufficient extraction of the 2,4-dinitrophenylhydrazones by alkali, or destruction of the keto acids by residual peroxide in the solvent. Since the absolute recovery may vary from one experiment to another, it is more desirable to determine a new standard curve each time an analysis is made.

While reasonably accurate estimates of the content of keto acids in mixtures can be obtained by using single samples, it has been found advantageous to employ triplicate determinations when more accuracy is desired. This point is illustrated in Table I, which shows the determination of α-keto-β-methyl-*n*-valeric acid in an unknown mixture when known quantities were added. The averages in this case differ by less than the ±5% which seems to be the limit of accuracy of the method.

Despite the somewhat laborious manipulation required for accurate results, the method described here would seem to be of considerable value in that preparations containing keto acids are applied to the paper in extremely small quantities without prior chemical treatment. Thus, in the series of transamination experiments reported in the following paper,[5] only 0.06–0.09-ml. portions of the 1.00-ml. reaction mixtures were sacrificed for the triplicate determinations, the remainder of the samples might conceivably have been used for other chemical or biological tests.

Further, the method would seem to be extremely versatile. Although there has been occasion in this Laboratory to determine quantitatively only mixtures of α-ketoglutaric, α-ketoisovaleric and α-keto-β-methyl-*n*-valeric acids, there is no reason to believe that by a suitable choice of solvents other combinations of keto acids could not be separated and quantitatively determined.

(10) This is in contrast to the straight line observed for α-ketoglutarate standards. The difference may well be due to the differences in solubilities of the corresponding 2,4-dinitrophenylhydrazones in alkali.

(11) E. Vischer and E. Chargaff, *J. Biol. Chem.*, **176**, 703 (1948).

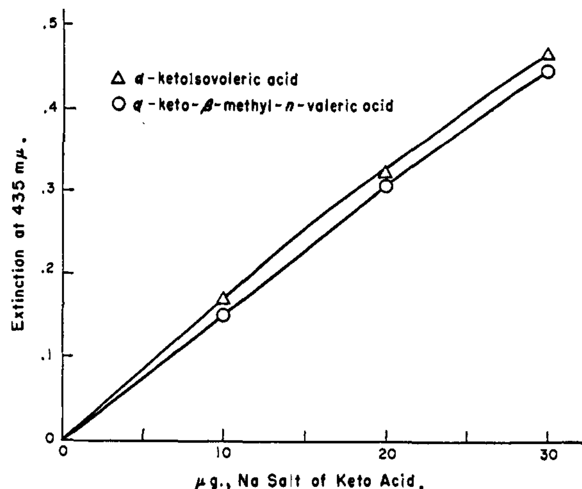

Fig. 2.—Standard curves obtained from keto acid mixtures separated chromatographically.

TABLE I

DETERMINATION OF THE KETO ACID DERIVED FROM L-ISOLEUCINE *via* TRANSAMINATION

	Keto acid content triplicate estimates[a]	Average	Error, %
Sample (20 μl.)[b]	10.3, 10.5, 10.9	10.6	..
Sample (20 μl.) + 5 μg. standard[c]	15.7, 14.7, 15.7	15.2	−3
Sample (20 μl.) +10 μg. standard	20.3, 20.3, 20.7	20.4	−1

[a] Values expressed as μg. of sodium α-keto-β-methyl-*n*-valerate as determined by reference to a standard curve. [b] Incubation mixture containing initially in one ml.: 50 μmoles of α-ketoglutarate, 4 μmoles of L-isoleucine, 10 μg. of crude pyridoxal phosphate, 5-mg. dried cells (*Escherichia coli*), 0.1 *M* phosphate buffer *p*H 8.4. Incubation 1 hour at 37°. [c] Sodium salt of *dl*-keto-β-methyl-*n*-valeric acid.

Another advantage of chromatographically separating the free keto acids rather than derivatives, such as the 2,4-dinitrophenylhydrazones,[12] is that two identical samples may be applied side by side. After development with solvent, one column may be used as a guide strip to find the positions of the various keto acids and the other used to subject any individual keto acid to a biological assay. This procedure was valuable in demonstrating the basis of the syntrophism exhibited by two genetically different isoleucine and valine requiring mutants of *E. coli.*[3]

The method is applicable, too, for the qualitative examination of reaction mixtures from transamination experiments. It is possible to demonstrate the positions of the amino acids by treating the paper with ninhydrin in the usual way after first having located the keto acids by the semicarbazide method. Thus on a single chromatogram, the essential features of transamination may be readily demonstrated.

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(12) D. Cavallini, N. Frontali and G. Toschi, *Nature*, **163**, 568 (1949).