and C_{11} dicarboxylic acid with C_4 monocarboxylic acid. To separate the monobasic from the dibasic acids, it is necessary to increase the pH of the stationary phase to 8.5 and change the butanol ratios slightly (Figure 8). The change in pH of 0.1 gives excellent resolution of both mono- and dicarboxylic acids.

It was apparent that the glycine method was equally effective in separating both mono- and dicarboxylic acids; therefore, an attempt was made to determine the range of dicarboxylic acids which could be separated effectively. The available supply of higher molecular weight dibasic acids included those from C_{11} through C_{16}.

Excellent resolution and recovery are obtained with a column at pH 9 for the C_{13} to C_{16} dicarboxylic acids (Figure 9). C_{11} and C_{12} acids are resolved on a column at pH 8.5 (Figure 8). The conflicts of C_{13} to C_{16} dibasic acids with the monobasic acid peaks were not determined because of the extremely short supply of dibasic acids. Data pertaining to recovery of dibasic acids are shown in Table II.

The author believes this method is applicable to the determination of dicarboxylic acids through C_{20} merely by increasing the pH of the stationary phase.

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Chromatography of Alpha-Keto Acid 2,4-Dinitrophenylhydrazones and Their Hydrogenation Products

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The 2,4-dinitrophenylhydrazones of a series of 35 α-keto acids (including the α-keto acid analogs of most of the common naturally occurring amino acids) have been prepared and studied in several paper chromatographic systems. The hydrazones (as prepared, or after elution from paper chromatograms) were hydrogenated under pressure in the presence of platinum oxide catalyst. Hydrogenation of the hydrazones of the α-keto acid analogs of all the amino acids studied (except cysteine) gave the corresponding amino acids, which were identified chromatographically. In several instances, more than one amino acid resulted from hydrogenation of a single keto acid hydrazone. The present results emphasize the value of combining chromatographic and hydrogenation procedures. The information presented here should serve as a useful basis for the identification of a wide variety of α-keto acids.

ALTHOUGH considerable attention has been given to the chromatographic identification of amino acids, relatively little chromatographic information has been collected concerning their α-keto analogs. Several α-keto acids have been found in nature—e.g., pyruvic, glyoxylic, oxalacetic, α-ketoglutaric, α-ketoisovaleric, α-keto-γ-methyleneglutaric—and it is probable that application of more sensitive procedures will disclose the presence of others.

A few studies on the paper chromatographic behavior of free α-keto acids have appeared (*13, 27, 31*). However, because of the instability of many α-keto acids during isolation procedures, and the fact that relatively large quantities of the free acids are usually required for identification on the chromatograms, a number of investigators (*1, 4, 20, 28, 29*) have resorted to the use of the corresponding 2,4-dinitrophenylhydrazone derivatives. Although chromatography of the 2,4-dinitrophenylhydrazones of α-keto acids is often a valuable tool for the identification and even for the quantitative determination (*3, 5, 12, 23, 26*) o-α-keto acids, several difficulties exist. For example, the chrof matographic behavior of certain 2,4-dinitrophenylhydrazones is very similar; furthermore, under some circumstances an α-keto acid 2,4-dinitrophenylhydrazone may give rise to two spots on one-dimensional paper chromatograms (and often to four spots on two-dimensional chromatograms). This is probably due to the presence of the *syn*- and *anti*-hydrazones (*10, 20, 23*). Kulonen (*11*) used a procedure for the hydrogenation of the hydrazones using aluminum amalgam, followed by paper chromatography of the resulting amino acids. Towers, Thompson, and Steward (*25*) independently developed a similar method based on catalytic hydrogenation with platinum oxide. These techniques have proved of value in the identification of several α-keto acids present in blood, urine (*11*), and certain plant tissues (*25*).

Studies in this laboratory on transamination and related problems have necessitated the use of a large number of α-keto acids, which have been prepared by synthetic organic techniques and by enzymatic oxidative deamination of the corresponding amino acid isomers (*15, 18*). In the course of these investigations the authors have had occasion to use paper chromatography for the identification of α-keto acid hydrazones, and to carry out hydrogenation of these derivatives. This paper reports paper chromatographic studies of the 2,4-dinitrophenylhydrazones of 35 α-keto acids, and of the products of hydrogenation of these compounds. This is a considerably larger series of α-keto acids than has previously been available for such study, and includes the α-keto analogs of most of the common naturally occurring amino acids. It may therefore be expected that the data presented here will be useful to those concerned with the identification of α-keto acids.

METHODS

References to the methods of preparation of the α-keto acids, and the solvents used for crystallization of the 2,4-dinitrophenyl-

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hydrazones, are given in Table I. Ascending paper chromatography was carried out on Whatman No. 4 paper, using the following solvents (*2*): (*a*) 77% ethyl alcohol; (*b*) liquefied phenol saturated with 10% aqueous sodium carbonate; (*c*) 1-propanol, ammonium hydroxide (28%), water (60:30:10); (*d*) 1-butanol, water, ethanol (50:40:10); and (*e*) methanol, benzene, 1-butanol, water (40:20:20:20).

A number of variations of these solvents were tried with no greater success than indicated in Table I. Although it cannot be maintained that the authors have found the best possible solvents, the following mixtures were found to be of relatively little value in the separation of these keto acid hydrazones: (*f*) *tert*-amyl alcohol saturated with 0.1*M* potassium phthalate buffer (pH 6.0); (*g*) lutidine, ethanol, water, diethylamine (55:20:25:1); (*h*) 1-butanol, glacial acetic acid, water (2:1:1); (*i*) phenol, 1-butanol, glacial acetic acid, water (25:12.5:50:25); (*j*) caprylic alcohol, ethanol, water, benzene (4:2:2:2); (*k*) methyl ethyl ketone, *tert*-butyl alcohol, formic acid, water (16:16:0.1:3.9); (*l*) diethyl ether, glacial acetic acid, water (50:40:10); (*m*) diethyl ether, ethyl alcohol (1:1).

The hydrazones were dissolved in ethyl acetate, ethanol, or glacial acetic acid and applied to the paper in quantities of 0.1 to 5 γ. Inspection of the chromatograms under ultraviolet light (a Hanovia Inspectolite lamp, Hanovia Chemical and Manufacturing Co., catalog No. SC-5041, with an EH-4 arc tube and filter was used) was found to be useful in detecting very small quantities (0.01 to 0.1 γ) of the hydrazones, not ordinarily visualized in white light. Where subsequent elution and hydrogenation were carried out, larger quantities of the hydrazones were applied to the paper as a series of spots.

Hydrogenation was carried out on pure samples of the 2,4-dinitrophenylhydrazones and on hydrazones eluted from specific spots on the paper chromatograms. In general, quantities of 40 to 200 γ were eluted with ethyl acetate or 10% sodium carbonate. When the latter eluting agent was used, the solution was acidified and the hydrazone extracted into ethyl acetate. The hydrazone solution was evaporated to dryness in a 12-ml. conical tube, to which 1 ml. of water and 2 mg. of platinum oxide catalyst (American Platinum Works, Newark, N. J.) were added. Several such tubes were usually placed together in a 250-ml. pressure-resistant bottle padded with cotton, and the bottle was attached to a Parr hydrogenation apparatus. After hydrogenation for 16 hours at 24° and 40 pounds per square inch, the catalyst was allowed to settle, and the clear solution was applied to paper chromatograms. Paper chromatography was carried out using the appropriate internal standards; the amino

Table I. Application of Chromatographic and Hydrogenation Procedures to α-Keto Acid 2,4-Dinitrophenylhydrazones

α-Keto Acid 2,4-Dinitrophenylhydrazone	Preparation References	Crystallization: Melting point, °C.	Crystallization: Solvent	$R_F \times 100$[a]: Solvent (*a*)	Solvent (*b*)	Solvent (*c*)	Solvent (*d*)	Solvent (*e*)	Amino Acids after Hydrogenation
Pyruvic	(*22*)	216	Water	54–63	68–74	71–78; 88–91	48–53	73–77; 79–83	Alanine
α-Ketoadipic	(*7*)	208	Water	53–58	53–60	45–51; 55–61	46–51	67–71	α-Aminoadipic acid
α-Ketobutyric	(*15*)	198	Water	72–77	61–66	84–86; 88–92	58–68	77–82	α-Aminobutyric acid
α-Ketoheptylic	(*15*)	130	Ethyl acetate, ligroin	77–86	75–88	89–94	77–86; 92–98	79–90; 98–100	α-Aminoheptylic acid
α-Keto-ε-hydroxycaproic	(*15*)	183	Water	69–73	63–69	84–87	52–59	76–79	α-Amino-ε-hydroxycaproic acid
α-Ketomalonic[b]		205	HCl	50–59	50–61	49–57	42–50	49–54; 58–63	α-Aminomalonic acid[c] glycine[c]
α-Ketophenylacetic	(*15*)	193	Water	67–75	70–76	85–91	58–68	77–82	α-Aminophenylacetic acid[c], cyclohexylglycine[c]
α-Keto-δ-guanidinovaleric	(*17*)	[d]		Streak	Streak	Streak	Streak	Streak	Arginine
α-Ketosuccinamic	(*16*)	183	Ethyl acetate	40–47	55–62	69–73	26–29; 32–36	58–66; 66–70	Asparagine[c], aspartic acid[c], alanine[c]
Oxalacetic	(*9*)	218	Water	42–51	22–34	55–72	26–40; 44–50	59–66; 74–77	Aspartic acid[c], alanine[c], β-alanine[c]
α-Keto-δ-carbamidovaleric	(*15*)	190	Water	52–61	60–72	71–76	25–28	70–74	Citrulline
β-Cyclohexylpyruvic	(*15*)	189	Water	76–83	75–84	87–91	87–94	79–87	β-Cyclohexylalanine
β-Sulfopyruvic	(*19*)	210	Alcohol	35–41	13–19	49–53	17–19; 20–23	58–65; 79–87	Cysteic acid, alanine[c]
β-Mercaptopyruvic	(*21*)	161–2	Water	64–68	60–63	79–83	42–51	74–79	Alanine
α-Keto-γ-ethiolbutyric	(*15*)	131	Water	70–76	73–82	83–90	64–73	77–82	Ethionine
α-Ketoglutaric[f]		220	Water	54–62	48–52	55–59	30–35	58–66	Glutamic acid
α-Ketoglutaramic	(*16*)	195[g]		44–53	23–30	70–75	29–32	58–63	Glutamine[c], glutamic acid[c]
Glyoxylic	(*30*)	203	Water	45–55	65–76	70–75	31–47	69–73; 76–78	Glycine
β-Imidazolylpyruvic	(*15*)	240	Ethyl acetate, ligroin	Streak	Streak	79–85	Streak	Streak	Histidine
d-α-Keto-β-methylvaleric[h]	(*14*)	176	Water	81–85	63–72	92–96	72–81	82–89	Isoleucine
α-Ketoisocaproic	(*15*)	162	Water	76–79; 81–85	63–71	93–97	79–86	84–92	Leucine
Trimethylpyruvic	(*8*)	180	Water	80–87	72–79	90–95	76–85	86–92	*tert*-Leucine
α-Keto-ε-aminocaproic	(*17*)	212	Water	71–76	75–85	79–85	67–75	91–98	Lysine[c], pipecolic acid[c]
α-Keto-γ-methiolbutyric	(*15*)	150	Water	66–79	72–79	85–88; 90–93	40–45	79–85	Methionine
α-Keto-γ-methylsulfonylbutyric	(*15*)	175	Water	52–61	65–72	74–76	30–33; 37–42	71–75	Methionine sulfone
α-Keto-δ-nitroguanidinovaleric	(*17*)	225	Glacial acetic acid	44–55	58–67	77–83	40–45	73–78	Nitroarginine[c], arginine[c]
α-Ketocaproic	(*15*)	153	Water	75–81	66–73	85–92	85–94	82–91	Norleucine
α-Ketovaleric	(*15*)	167	Water	75–81	66–72	88–93	76–85	82–87	Norvaline
α-Keto-δ-aminovaleric	(*17*)	211–2	Water	43–55	76–86	91–95	51–58	Streak	Ornithine[c], proline[c], pentahomoserine[c]
Phenylpyruvic	(*15*)	162–4; [i] 192–4		68–76; 79–83	73–83	83–89	82–93	81–91	Phenylalanine
β-Hydroxypyruvic	(*24*)	162	Ethyl acetate	56–63; 70–73	48–62	70–76; 81–84	48–53; 61–65	Streak	Serine[c], alanine[c]
α-Keto-β-hydroxybutyric	(*24*)	157–8	Ethyl acetate	63–72	55–69	73–78	52–61	77–82	Threonine[c], α-aminobutyric acid[c]
β-Indolylpyruvic	(*15*)	169	Water	64–73	71–81	80–87	82–93	79–86	Tryptophan
p-Hydroxyphenylpyruvic	(*15*)	178	Water	66–70; 72–77	58–65	84–90	71–79	79–86	Tyrosine
α-Ketoisovaleric	(*15*)	196	Water	78–85	65–70	88–92	77–82	82–90	Valine

[a] Values given in table describe ranges of spots, and represent average values of three to five determinations; composition of solvents is given under Methods.
[b] Prepared by saponification of freshly distilled ethyl oxomalonate (obtained from Cohelfred Laboratories, Chicago, Ill.).
[c] Amino acids formed in amounts of approximately same order of magnitude.
[d] Darkened at 218°, but did not melt when heated to 250°; product washed with water.
[e] Formed in trace amounts.
[f] Obtained from Nutritional Biochemicals Corp.
[g] Contained about 5% of 2,4-dinitrophenylhydrazone of α-ketoglutaric acid.
[h] *l*-isomer, *d*-isomer, and racemic form exhibited identical behavior.
[i] Crystallization from water or alcohol gives m.p. of 192–7°; crystallization from ethyl acetate and petroleum ether gives m.p. of 162–4°. The two forms are interconvertible (*6*); their chromatographic behavior was identical.

acid spots were rendered visible by dipping the dried chromatograms into a solution of 0.25% ninhydrin in acetone (*2*).

RESULTS

The solvents employed effected separation of a number of the hydrazones (Table I). Thus, separation of the members of the homologous series from glyoxylic to α-ketoheptylic acids was achieved, and the hydrazones of phenylpyruvic and *p*-hydroxyphenylpyruvic acids were distinguished from those of the aliphatic α-keto acids. However, the degree of separation of the branched chain α-keto acids from each other and from the isomeric normal α-keto acids was not great. On the other hand, hydrazones of the dicarboxylic acids in general moved more slowly than did those of the monocarboxylic acids. The dicarboxylic keto acid hydrazones were readily separated from each other and the corresponding ω-amides. The hydrazones of β-cyclohexylpyruvic and phenylpyruvic acids did not exhibit significantly different values. The hydrazones of β-hydroxypyruvic and β-hydroxy-α-ketobutyric acids gave appreciably different values from those of the corresponding unsubstituted α-keto acids. The hydrazones of the α-keto analogs of ornithine and lysine were separated from each other as were those of methionine, ethionine, and methionine sulfone. Chromatography of the hydrazones of the α-keto analogs of histidine and arginine was, in general, unsatisfactory owing to the low solubility of these derivatives.

In most instances double spots were not observed. Double spots were frequently formed when the hydrazone solutions were permitted to stand for some time prior to chromatography, and when the hydrazones were dissolved in carbonate, acidified, and then extracted with ethyl acetate. When such double spots were separately eluted and hydrogenated, both spots yielded the same amino acid. This result is consistent with the concept that these spots represent different forms of the same α-keto acid hydrazone [see (*10*, *20*, *23*)]. The occurrence of double spots obviously places a distinct limitation on the value of chromatography of the hydrazones, although where two spots were observed, they were often relatively close together.

Hydrogenation of the hydrazones resulted in the formation of the analogous α-amino acid in all but one case. Alanine was the only amino acid product of the hydrogenation of β-mercaptopyruvic acid 2,4-dinitrophenylhydrazone. Some alanine (as well as the corresponding amino acids) was also formed from the hydrazones of oxalacetic, cysteic, and α-ketosuccinamic acids. Alanine and α-aminobutyric acids were found, respectively, after hydrogenation of the hydrazones of β-hydroxypyruvic and α-keto-β-hydroxybutyric acids; neither serine nor threonine was reduced under the conditions of the hydrogenation procedure. Hydrogenation of the hydrazones of the α-keto analogs of glutamine and asparagine resulted in the formation of the corresponding dicarboxylic amino acid, indicating that some deamidation had occurred. Partial reduction of nitroarginine and of α-aminophenylacetic acid was also observed. Both lysine and pipecolic acid were formed by hydrogenation of the hydrazone of α-keto-ε-aminocaproic acid; proline and ornithine were formed from α-keto-δ-aminovaleric acid hydrazone. These α-keto acids have been found to exist in equilibrium between open-chain and cyclic forms (*17*). Hydrogenation of the hydrazone of α-ketomalonic acid gave α-aminomalonic acid and glycine; the formation of glycine is consistent with the tendency of α-aminomalonic acid to undergo decarboxylation in aqueous solution at room temperature. Under the conditions employed, hydrogenation of α-ketoglutaric acid 2,4-dinitrophenylhydrazone gave only glutamic acid; however, when very large amounts of the hydrazone were hydrogenated, a very small quantity of γ-aminobutyric acid was found. Towers and others (*25*) have reported formation of γ-aminobutyric acid and β-alanine after hydrogenation of the hydrazones of α-ketoglutaric acid and oxalacetic acids, respectively. It is to be expected that some variation may occur in the amounts of certain amino acids formed depending on the conditions of hydrogenation.

DISCUSSION

It is apparent from the R_f values given in Table I, that paper chromatography alone is of somewhat limited value for the identification of α-keto acid 2,4-dinitrophenylhydrazones, although it is very useful in excluding the presence of certain α-keto acid hydrazones. The hydrogenation procedure appears to be of considerable value in identifying the α-keto acid analogs of the known amino acids, since paper chromatography of amino acids has become a highly developed and accurate procedure (*2*). Hydrogenation of the hydrazones of the α-keto analogs of all the amino acids studied (except cysteine) gave the corresponding amino acids. Although several hydrazones gave more than one amino acid, this complication, if recognized, would not be expected to provide undue difficulty. The present results emphasize the value of combining chromatography of the hydrazones with the hydrogenation procedure. Thus, after chromatography and tentative identification of the spots, the hydrazones may be separately eluted, hydrogenated, and the resulting amino acids chromatographed. The data given in Table I suggest that these procedures provide information useful for the identification of a wide variety of α-keto acids.

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