Dehydroepiandrosterone acetate-7α(?)-H^3.[12] The solution of 103 mg. of 7α-bromo-3β-hydroxyandrost-5-en-17-one acetate was added to the suspension of 1075 mg. of 5% palladium on calcium carbonate which was prereduced. The mixture was shaken with 5 ml. hydrogen to which 2 curies of tritium were added for 1 hr. The reduction mixture was filtered, the filtrate evaporated, and the residue hydrolyzed with methanolic *N* sodium hydroxide solution at room temperature overnight. The hydrolyzed product was worked up and reacetylated. Purification of the acetate by chromatography yielded 48 mg. (57%) of crystalline dehydroepiandrosterone acetate-7α-H^3 with the benzene-ethyl acetate eluates. After recrystallization from methanol it melted at 168–171° and its infrared absorption spectrum was identical[13] with the spectrum of authentic material. The specific radioactivity of this sample was 3.5 mC per mg. On subsequent hydrolysis followed by reacetylation the specific activity remained unchanged.

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(12) Compare D. K. Fukushima, S. Lieberman and B. Praetz, *J. Am. Chem. Soc.*, **72**, 5205 (1950).

(13) The relatively low tritium concentration does not produce changes in the fingerprint region.

[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF THE UNIVERSITY OF CALIFORNIA]

Utilization of Gas Phase Chromatography for Identification of Volatile Products from Alkaline Degradation of Herqueinone

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Low molecular weight carbonyl compounds may be conveniently identified by gas chromatography of their oximes. By use of di-2-ethylhexyl phthalate as partitioning agent, there may be identified in presence of each other the aldehydes with 2–4 carbons, acetone, butanone, and 3-methyl-2-butanone. From the alkaline degradation of herqueinone, the only volatile carbonyl component detected by this method was acetaldehyde. The volatile acids from alkaline degradation of herqueinone, also examined by gas chromatography, have been found to be a mixture of formic, acetic, and isobutyric acids.

In an earlier publication[1] from this laboratory, there was reported an examination of the volatile carbonyl component obtained by heating herqueinone, the red pigment from *Penicillium herquei,* with aqueous alkali. Although the 2,4-dinitrophenylhydrazone originally precipitated from the aqueous distillate agreed rather well in properties with the derivative of acetaldehyde, it was concluded from chromatography experiments that the derivative must be either a mixture, or some other substance than the derivative of acetaldehyde. In a simultaneous publication[2] by Raistrick and co-workers, it was reported that the volatile carbonyl component is acetaldehyde, on the basis of the properties of the 2,4-dinitrophenylhydrazone. Since our earlier results based on chromatography on silicic acid and on Bentonite were probably obscured[3] by decomposition or isomerization of the hydrazone, this carbonyl component has been reexamined by use of gas phase chromatography.

Since acetaldehyde is inconveniently volatile, certain derivatives were considered for use in gas chromatography, and the oxime was found to be well adapted for gas chromatography of low molecular weight carbonyl compounds. A procedure has been developed for formation of this derivative from an aqueous solution of 5–25 mg. of the carbonyl compounds. As shown in Table I, the several compounds examined have sufficiently different retention times to permit their detection.

(1) R. E. Harman, J. Cason, F. H. Stodola, and A. L. Adkins, *J. Org. Chem.*, **20**, 1260 (1955).

(2) J. A. Galarraga, K. G. Neill, and H. Raistrick, *Biochem. J.*, **61**, 456 (1955).

(3) Work to be published in *J. Am. Chem. Soc.* on the alterations of phenylhydrazones has been carried out by Professor H. Rapoport and R. J. O'Connor in this department.

TABLE I

RETENTION TIMES OF OXIMES IN GAS PHASE CHROMATOGRAPHY

Oxime	Retention Time[a] (min.: sec.)
Acetaldehyde	3:35
Acetone	4:45
Propionaldehyde	5:10
Isobutyraldehyde	6:45
Butanone	7:55
n-Butyraldehyde	9:10
3-Methyl-2-butanone	10:55

[a] Retention time, which is given in minutes and seconds, was taken as time elapsing between injection and maximum in peak. The column was 2 meters × 8 mm. o.d., packed with 30–60 mesh Celite firebrick impregnated with 3% di-2-ethylhexyl phthalate; temperature, 88°; helium flow rate, 35 ml./min.

When the volatile neutral material from alkaline degradation of herqueinone was converted to the oxime and subjected to gas chromatography, a single peak was observed with the retention time of acetaldoxime; no other peak was observed after lapse of 55 minutes. Thus, our observations based on gas chromatography are in accord with the report of the British investigators[2] that acetaldehyde is the only steam-volatile carbonyl component

TABLE II

GAS PHASE CHROMATOGRAPHY OF ESTERS AND ACIDS

Compound	Retention Time[a] (min.:sec.)	% Yield[b]
Methyl acetate	6:10	
Methyl propionate	9:45	
Methyl isobutyrate	12:30	
Methyl trimethylacetate	14:55	
Methyl *n*-butyrate	17:30	
Methyl esters of acids from degradation[c]	6:15	1.3 ± 0.5
	12:25	28 ± 3
Butyl formate	[d]	
Butyl acetate	14:40	
Butyl isobutyrate	36:10	
Dibutyl ether	22:50	
Butyl esters of acids from degradation	[d]	1.0 ± 0.5
	15:00	3.0 ± 0.3
	24:10	
	38:20	53 ± 5
Formic acid	14:20	
Acetic acid	16:35	
Propionic acid	30:00	
Isobutyric acid	46:00	
Trimethylacetic acid	59:00	
n-Butyric acid	59:00	
Acids from degradation	14:05	2.9 ± 0.3
	16:35	2.2 ± 0.3
	46:30	42 ± 5

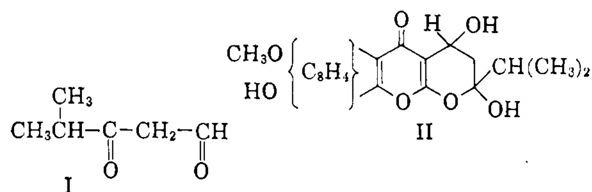
[a] Column used for the methyl esters was 4 m. × 8 mm. o.d.; partitioning agent, 3% di-2-ethylhexyl phthalate on 30–60 mesh Celite firebrick; temp., 50°, helium flow, 42 ml./min. Column used for the butyl esters was 2 m. × 9 mm. o.d.; partitioning agent, 40% high vacuum silicone grease on 30–60 mesh Chromosorb; temp., 76°; helium flow, 75 ml./min. Column used for the acids was 2 m. × 8 mm. o.d.; partitioning agent, 35% on 30–60 mesh Chromosorb of the mixture: 8 parts silicone oil DC 550, 1 part stearic acid, 1 part phosphoric acid; temp. 103°; helium flow, 115 ml./min. [b] Yields, calculated as moles of acid per mole of herqueinone, were determined by comparison of areas under the tracings from degradation products with areas under the tracings of known amounts of known compounds taken under the same conditions. The free acids and methyl esters were the product of the first degradation described in the Experimental section; the butyl esters were the product of the second degradation described. [c] Methyl formate could not be determined for it was under the large band from ether used as solvent. Methyl acetate was on the edge of this band, so the uncertainty in the measurement was relatively large, as indicated. In another chromatography at 19°, the methyl acetate band with retention time of 11:45 was resolved from ether; yield was calculated as 1.8 ± 0.2%. [d] At 76°, butyl formate was not resolved from the large band resulting from excess butyl alcohol used in esterification. At 58°, the butyl formate band appeared on the trailing edge of the butyl alcohol band, at 14:30. Comparison of area with that of a known sample of butyl formate (retention time, 14:45) indicated a yield of 1 ± 0.5% of formic acid, based on herqueinone.

recovered from alkaline degradation of herqueinone.

Failure to find 3-methyl-2-butanone, formed in acid degradation of herqueinone,[1,2] in the distillate from alkaline degradation does not unequivocally prove that this ketone is not a degradation product; however, it probably is not formed in significant amounts. When 1.0 millimole of acetaldehyde and 0.25 millimole of 3-methyl-2-butanone were added to 1*N* alkali, and the mixture was then heated to boiling and distilled, neither compound could be detected in the distillate. In contrast, however, when a solution of 1.0 millimole of the aldehyde and 0.5 millimole of the ketone was added slowly beneath the surface of the distilling alkaline solution, 86% of the 3-methyl-2-butanone was recovered.

Although it was reported previously[1] that volatile acid is liberated during alkaline degradation of herqueinone, the nature of the acid was not investigated. This volatile acid has now been examined by utilization of gas phase chromatography of the free acid, its methyl ester, and its butyl ester. As shown by the data in Table II, the principal component of this acid mixture is isobutyric acid, although small amounts of formic and acetic acids are also present. It is of interest that the yields of acetaldehyde and isobutyric acid are comparable.

It seems highly probable that the isopropyl group in the isobutyric acid from alkaline degradation of herqueinone arises from the same carbon atoms as does the isopropyl group in the 3-methyl-2-butanone obtained by acid degradation. This follows from the fact that hydrolysis of herqueinone to yield 3-methyl-2-butanone would give $C_{15}H_{12}O_7$ as the residual fragment. Hydrolytic cleavage of isobutyric acid from this fragment would leave $C_{11}H_6O_6$, a rather improbable formula. If there be only one potential isopropyl group in herqueinone, the equivalent yields of acetaldehyde and isobutyric



acid, along with the very low yields of formic and acetic acids, indicate presence in herqueinone of some structure which may give rise to structure I (or its parts) on alkaline hydrolysis. If the formyl group in structure I should be generated by a chain-cleavage step such as the reverse aldol condensation, then a different direction of chain cleavage might account for the formation of 3-methyl-2-butanone in acid degradation. Although such a partial structure as II could give rise to I (as well as small amounts of acetic acid) and is consistent with other properties of herqueinone, there is not yet available sufficient evidence to allow any firm conclusions concerning the basic ring structures present in herqueinone.

TABLE III

PHYSICAL PROPERTIES OF OXIMES

Oxime of	B.P., °C./mm. Hg Obsd.	Lit.	n_D^{25} (Obs.)	n_D (°C.) (Lit.)
Acetaldehyde	114/760	114–115/760[a]	1.4230	1.4257 (20.4)[b]
Propionaldehyde	39–42/33	77/100[c]	1.4288	1.4287 (20)[d]
n-Butyraldehyde	65–67/22	67/16.5[e]	1.4333	1.4367 (20)[f]
Isobutyraldehyde	53–54/21	139/760[a]	1.4252	1.4302 (20.5)[b]
Acetone	133/760	135/728[g]	m.p. 57–59°	m.p. 59–60°[g]
Butanone	47–48/18.5	152/763[d]	1.4367	1.4428 (20)[d]
3-Methyl-2-butanone	74.5/21	158–160/760[h]	1.4414	

[a] J. Petraczek, *Ber.*, **15**, 2784 (1882). [b] J. W. Brühl, *Z. physik. Chem.*, **16**, 214 (1895). [c] E. Bourgeois and J. Dambmann, *Ber.*, **26**, 2860 (1893). [d] C. Trapesonzjanz, *Ber.*, **26**, 1432 (1893). [e] S. Yamada, I. Chibata, and R. Tsurui, *Pharm. Bull.* (*Tokyo*), **2**, 59 (1954); *Chem. Abstr.*, **50**, 11132 (1956). [f] A. I. Vogel, W. T. Cresswell, G. H. Jeffery, and J. Leicester, *J. Chem. Soc.*, 542 (1952). [g] V. Meyer and A. Janny, *Ber.*, **15**, 1324 (1882). [h] P. Karrer, M. Gisler, E. Horlacher, F. Locher, W. Mäder, and H. Thomann, *Helv. Chim. Acta*, **5**, 478 (1922).

EXPERIMENTAL[4]

Preparation of oximes on small scale. To a solution of 10–100 mg. of the carbonyl compound in 10 ml. of water was added 3.13 g. (45 mmoles) of hydroxylamine hydrochloride. To this solution was added 2.39 g. (22.5 mmoles) of anhydrous sodium carbonate sufficiently slowly to allow control of effervescence. This mixture was extracted continuously for 1 hr. with ether which had been distilled from sulfuric acid and stored over sodium. After the extract had been dried over sodium sulfate, ether was distilled through the column until only about 1 ml. of solution remained. The column was allowed to drain for a few minutes, then the residual solution was transferred by use of a syringe to a 2-ml. volumetric flask. Ether used to wash the distillation flask was added to bring the volume to 2 ml., and this solution was used for injection in gas phase chromatography.

Oximes used to determine the retention times recorded in Table I were prepared on a larger scale and purified by distillation. Properties are found in Table III. Those oximes which were distilled at reduced pressure were found to decompose to some extent when distillation at atmospheric pressure was undertaken.

Esters used for determining retention times recorded in Table II were commercial products, except in the instances of trimethylacetic acid and isobutyric acid, in which cases commercial acids were directly esterified.

Alkaline hydrolysis of herqueinone. A solution of 744 mg. (2 mmoles) of herqueinone (isolated as previously described[1] and purified by chromatography) in 100 ml. of *N* aqueous sodium hydroxide was directly steam-distilled in a slow stream of nitrogen. During the first few seconds of distillation the odor of acetaldehyde was observed, and this was soon replaced with the odor of aldol. The distillate was collected in 4-ml. portions in ice-cooled receivers. The first 6 portions of distillate (24 ml.) gave a positive test for aldehyde with 2,4-dinitrophenylhydrazine, while the seventh portion was negative; so the first 24 ml. was treated in the manner described above for formation of oximes on a small scale. Quantitative gas phase chromatography indicated a 20% yield of acetaldehyde (based on one mole of acetaldehyde per mole of herqueinone), and no band was observed except that with the retention time of acetaldehyde.

After carbonyl components had been distilled, as described above, the alkaline aqueous solution was acidified with sulfuric acid to pH 2, and distillation was continued as water was added at about the rate it was distilled. The distillate was collected in 4 portions whose volumes were respectively 230, 60, 90, and 50 ml. Titration of these samples with 0.1*N* sodium hydroxide, using phenolphthalein as indicator, gave consumption of 10.65, 0.48, 0.38, and 0.00 ml. of alkali. Thus, total yield of acids distilled amounted to 1.15 meq. or 58% of one mole per mole of herqueinone degraded. The first 3 portions of distillate were combined, the *p*H was adjusted to 13, and the volume reduced by distillation to about 10 ml. The concentrated solution was acidified with sulfuric acid and continuously extracted with ether for 20 hr. The extract was concentrated under a column and eventually made up to a 2-ml. volume for injection in gas chromatography, following the procedure described under preparation of oximes. Chromatography was with the silicone oil–phosphoric acid–stearic acid partitioning agent, as recorded in Table II.

The solution remaining after chromatography of free acids (about 95% of the total) was allowed to react with 1.5 mmoles of diazomethane[5] for about 2 hr. The resultant solution was centrifuged to remove a small gelatinous precipitate, then made up to 5 ml. for gas chromatography as recorded for methyl esters in Table II.

In a second run, utilizing 372 mg. (1 mmole) of herqueinone, the pigment was dissolved in about 10% of the alkaline solution and added slowly to the boiling solution of the remainder of the base. To ensure recovery of acetaldehyde, ice water was circulated through the condenser. Gas chromatography indicated a yield of 50 ± 5% of acetaldehyde. The yield of acids in this run, by titration, was 59%. The solution of the acid salts obtained as described for the first run was evaporated to dryness under reduced pressure, and esters were prepared by heating the salts at 100° for 1 hr. with 1.5 ml. of *n*-butyl alcohol containing 20% by weight of concentrated sulfuric acid. The reaction mixture was dissolved in 10 ml. of ether and extracted once with water, twice with 7% sodium bicarbonate solution, and once with saturated sodium chloride solution. After the solution had been dried and concentrated to 2.6 ml. it was used for injection in gas chromatography (cf. Table II).

Distillation of 3-methyl-2-butanone and acetaldehyde from alkaline solution. (*a*) A mixture of 1 mmole of acetaldehyde and 0.25 mmole of 3-methyl-2-butanone was added to 50 ml. of cold *N* aqueous sodium hydroxide. The mixture was heated to boiling and distilled in an atmosphere of nitrogen until 10 ml. of distillate had been collected. The distillate

(4) Boiling points are uncorrected; distillations were carried out through a 65-cm. column of a simple Podbielniak design, with heated jacket and partial reflux head. Partitioning agents and dimensions of columns for gas phase chromatography are indicated in Tables I and II.

(5) The diazomethane solution, prepared according to F. Arndt, *Org. Syntheses*, **Coll. Vol. II**, 165 (1943), was not distilled but was assayed by titration.

was processed as described for small scale preparation of oximes. Gas chromatography indicated no carbonyl components in the distillate.

(b) A sample of 0.25 mmole of 3-methyl-2-butanone was distilled as described under (a). Gas chromatography indicated a recovery in the distillate of 0.2 mmole of the ketone.

(c) A solution of 1 mmole of acetaldehyde and 0.5 mmole of 3-methyl-2-butanone in 10 ml. of water was added beneath the surface of 50 ml. of a distilling N solution of aqueous alkali. Rate of addition was about equal the rate of distillation, and the procedure was as in (a) except for the gradual addition to the distilling solution. Gas chromatography indicated a recovery in the first 10 ml. of distillate of 75% of the acetaldehyde and 86% of the ketone.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER AND CO.]

(+)-2,3-Diaminosuccinic Acid

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(+)-2,3-Diaminosuccinic acid has been found in the fermentation beers of *Streptomyces rimosus*. This is the first reported isolation of a diaminosuccinic acid from a natural source.

Diaminosuccinic acid has long been known as a synthetic substance. The first preparations of *dl*- and *meso*- forms were reported as early as 1893, and many syntheses have been effected since that time.[1,2] There is no record of the isolation of this material from natural sources, though it has been reported to be a product of enzymatic synthesis from hydrazine and fumaric acid by pig liver and *E. coli* preparations.[3]

We wish to report here the isolation of an optically active form of diaminosuccinic acid from the fermentation beers of *Streptomyces rimosus*, an actinomycete better known as the producer of the antibiotic Terramycin.[4] When the acidified filtered beers were adjusted to pH 2–4, the crude acid in some cases precipitated directly as white crystals of the dihydrate, which decomposes without melting at 240–260°. The pure anhydrous compound shows $[\alpha]_D^{25}$ +28° in 5% sodium hydroxide.

The structure of the new compound follows from its elemental analysis, $C_4H_8N_2O_4$, and from its chemical and physical properties. Titration shows it to be a dibasic acid, pK_a 6.7 and 9.1. The amine nature of the two nitrogen atoms is evident from the ready formation of the dibenzoyl and di-2-naphthalenesulfonyl derivatives. The compound shows only end absorption in the ultraviolet spectrum. The infrared spectrum, (KBr) which shows strong bonded NH and OH absorption at 3–3.5μ, and broad bonded carbonyl absorption at 6.1μ, is entirely consistent with this structure. Our product is identical to synthetic *dl*-2,3-diaminosuccinic acid on two paper chromatography systems.

We were unable to detect diaminosuccinic acid in either the raw broth prior to fermentation, or in acid hydrolysates of the protein source (soy bean meal). It seems evident that diaminosuccinic acid is therefore an elaboration product of *S. rimosus*, and is not merely liberated from the vegetable protein. This conclusion is strengthened by the observation that the yields of acid (1–2 g./l.) would account for 10% of the total protein nitrogen introduced in the fermentation media.

Qualitative (paper chromatographic) examination of the beers from several strains of *S. rimosus* all showed the presence of diaminosuccinic acid. However, two strains of *S. aureofaciens* yielded no detectable quantities of diaminosuccinic acid.

EXPERIMENTAL

Isolation of (+)-2,3-diaminosuccinic acid. Streptomyces rimosus was grown for 96 hr. at 28° with aeration in a medium containing only 4% soybean meal, and 0.5% sodium nitrate. (Other media may also be used.)

Two l. of the broth were adjusted to pH 2 with HCl, filtered from mycelia, and readjusted to pH 4. After refrigerated storage (2–5°) for one week, 1.36 g. of crude (+) diaminosuccinic acid was separated by filtration. Twenty g. of the crude product isolated in a similar manner was purified by solution in 400 ml. of 5% aqueous hydrochloric acid at 80°, treatment with 2 g. "Darco G-60" charcoal, and filtration. On addition of 10N sodium hydroxide, precipitation started at pH 1, and appeared to be substantially complete at pH 4. The suspension was cooled to 5° overnight and 18 g. of white crystals were recovered by filtration. Paper chromatographic examination on a methyl ethyl ketone-acetic acid-water 3:1:1 system showed substantially one component, which gave an abnormal gray-violet ninhydrin color on spraying with detection reagent and heating the paper. A methanol-pyridine-water 8:2:1 system showed a single component with R_f between that of *meso*- and of (+)-diaminopimelic acid.

Two grams of once recrystallized diaminosuccinic acid was dissolved in 300 ml. of boiling water and cooled to 5° to yield 1.8 g. of a pure hydrated diaminosuccinic acid. Drying overnight at 20 mm. and room temperature over calcium

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(1) J. M. Farchy and J. Tafel, *Ber.*, **26**, 1980 (1893) and J. Tafel and H. Stern, *Ber.*, **38**, 1589 (1905).

(2) H. McKennis, Jr., and A. S. Yard, *J. Org. Chem.*, **23**, 980 (1958) have reported a recent study. They give an excellent series of references to chemical and biochemical work through 1957.

(3) K. P. Jacobsohn and M. Soares, *Enzymologia*, **1**, 183 (1936).

(4) Terramycin is the registered trade name of Charles Pfizer and Co. for the antibiotic oxytetracycline.