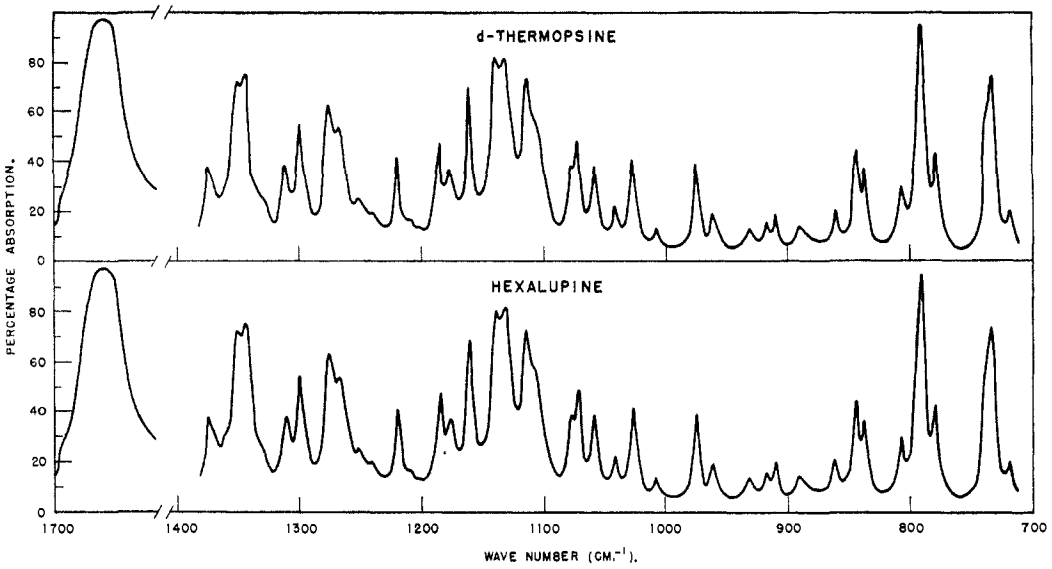

Fig. 1.

Perchlorate.—When a methanolic solution of hexalupine was carefully neutralized with 70% perchloric acid, a crystalline precipitate was obtained in colorless plates. Melting point after two recrystallizations from methanol was 284–286° (cor.) with decomposition, when the sample was inserted at 250°. No depression was obtained on a mixture with an authentic sample of *d*-thermopsine perchlorate, m.p. 286°.

Chloroplatinate.—An ethanolic solution of hexalupine was added to an aqueous solution of chloroplatinic acid, to which had been added a few drops of concentrated hydrochloric acid. The product separated rapidly in spear-like needles, which were found to be efflorescent as reported by Orechov for *l*-thermopsine chloroplatinate.[8] After recrystallization from acidified aqueous ethanol, the salt melted at 254–256° (uncor.) with decomposition (reported for *l*-thermopsine chloroplatinate, 254–256°). This melting point was not depressed by admixture with a similar salt prepared from pure *d*-thermopsine, m.p. 256–258° (uncor.).

(8) A. Orechov, S. Norkina and H. Gurewitch, *Ber.*, **66**, 625 (1933).

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC.]

Isolation and Some Chemical Properties of Grisein

BY FREDERICK A. KUEHL, JR., MARY NEALE BISHOP, LOUIS CHAIET AND KARL FOLKERS

Grisein has been isolated from the culture broth of *S. griseus* by a sequence of several steps including adsorption, elution, chromatography and countercurrent distribution. Grisein is a red amorphous powder; analytical data are in agreement with the composition $C_{40}H_{61}N_{10}O_{20}SFe$. The iron in the complex is in the ferric state and may be removed and added back to the complex. Degradation of grisein by acid hydrolysis yielded 3-methyluracil and at least two amino acids. One of the acids appears to be glutamic acid.

The production of the antibiotic grisein by a strain of *Streptomyces griseus*, obtained from Huleh peat, has been reported by Reynolds, Schatz and Waksman.[1] The antibiotic was purified[2] by adsorption from the broth on Norit A, elution with aqueous alcohol, and precipitation with methanol and acetone to give a concentrate having an activity of *ca.* 400 units/mg. and representing about 15% of the broth activity.

We have devised a process for the isolation of grisein in apparently pure form. Norit A was also used for adsorption of the antibiotic from the culture medium, but elution was found to be much more complete when aqueous pyridine was used instead of alcohol. An aqueous methanolic solution of material thus obtained gave, upon the addition of ether, precipitates having an activity of 300–500 units/mg. In order to facilitate further purification, the dried product was leached with methanol; this step doubled the activity, but gave only a 50% recovery.

Concentrates which were obtained by these preliminary steps were purified further by distribution between water and phenol–chloroform mixtures. The *p*H of the aqueous phase and the phenol content in the chloroform layer were altered to aid selective removal of impurities. The resulting product was dissolved in a little water and isopropyl alcohol was added to the solution. The precipitate, after removal, was found to have an activity of 50,000–100,000 units/mg., representing 35–40% recovery. The yields, as well as the potency of the product, were found to vary according to the potency of the starting material.

Further purification was accomplished by using the method of Martin and Synge.[3] Using silica gel saturated with *p*H 4.6 buffer and 17% phenol in chloroform (V/V), material having an activity of

(1) Reynolds, Schatz and Waksman, *Proc. Soc. Exp. Biol. and Med.*, **55**, 66 (1944).

(2) Reynolds and Waksman, *J. Bact.*, **55**, 739 (1948).

(3) Martin and Synge, *Biochem. J.*, **35**, 1358 (1941).

50,000 units/mg. could be converted to material of 160,000 units/mg. in 30% yields. The antibiotic was recovered by adding petroleum ether to the effluent and extracting with water.

The final stages of purification were accomplished by countercurrent distribution, using the system *p*H 6.7 phosphate buffer and phenol–chloroform. Distribution of material having an activity of 150,000 units/mg. through sixteen plates, collection of the "peak" fractions, and redistribution through ten plates yielded grisein having an activity of 300,000 units/mg. When this product was distributed through a ten-plate system, a distribution curve was obtained which is in close agreement with that for a pure substance.

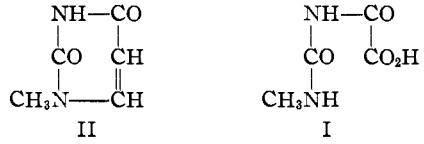
The grisein, obtained by this method, is a red amorphous powder, soluble in water and phenol. It is insoluble in all common organic solvents. It has characteristic absorption spectra with maxima at 2650 Å., $E^{1\%}_{1cm.}$ 108, and 4200 Å., $E^{1\%}_{1cm.}$ 28.9. The results of analytical and potentiometric titration data indicate that grisein is a weak acid having the composition $C_{40}H_{61}N_{10}O_{20}SFe$. Grisein forms an amorphous picrate, but no helianthate or reineckate could be prepared. It is moderately stable in aqueous acid solution, but is inactivated with methanolic hydrogen chloride. Tests with Brady reagent, Fehling solution and Tollens reagent are negative. The sugar test depending on the formation of furfural was also negative as well as the Lieberman phenol test, and the test for free C=S or =CSH groups. On iron-free grisein the test for hydroxamic acids was negative.[4]

Hydrolysis of crude grisein concentrates followed by paper strip chromatography indicated the presence of a large number of amino acids in the hydrolysate, as evidenced by ninhydrin reagent. However, on further purification, the number of spots decreased. On hydrolysis of grisein which appeared to be pure, as evidenced by distribution curves, only two ninhydrin spots were observed; one seemed to be that of glutamic acid and the other exhibited an R_F value which was not identified with that of any of the common amino acids in the preliminary strip tests. The intensities of these two spots indicate that the two amino acids are probably component parts of the grisein molecule and not trace contaminants.

The presence of iron in grisein is not surprising since Reynolds and Waksman[2] have demonstrated the necessity of iron in the growth medium for good production of this antibiotic. The iron is in the ferric state and is combined with the organic moiety of the molecule as a rather stable complex, since grisein may be extracted from aqueous solution at *p*H 2. The iron is readily removed from the complex, however, by the use of 8-hydroxyquinoline. Iron-free grisein is a colorless product exhibiting a lower antibiotic activity, 120,000 units/mg. With the removal of the iron, the absorption spectrum of the compound changes considerably; the maxima at 4200 Å. completely disappears and the peak at 2650 Å. drops to $E^{1\%}_{1cm.}$ 86. The iron may be reintroduced into the molecule with the result that the higher level of antibiotic activity is restored. The stoichiometric amount of ferric chloride must be added, however, since grisein is inactivated in the presence of excess iron. This precise combination of the desired amount of iron is best accomplished by adding excess ferric chloride, adjusting the solution to *p*H 4, and extracting the grisein from the aqueous phase with phenol–chloroform. In this manner, the excess ferric ions remain in the aqueous phase. Both grisein and iron-free grisein react with excess ferric chloride in the presence of sodium acetate and form an insoluble complex which has an activity of 200,000 units/mg.

Chromatographic purification of grisein has not been particularly satisfactory. When either alumina or magnesol was used, considerable inactivation occurred, presumably due to loss of iron. However, iron-free grisein can be chromatographed successfully on magnesol using 50% ethanol as a solvent. This chromatography is better adapted to large scale work than is countercurrent distribution, and iron-free grisein having an activity of 120,000 units/mg. may be obtained in 70% yields. The concentration of iron-free grisein in the effluent is easily followed by examining the color intensity obtained on treatment with ferric chloride.

Acid hydrolysis of grisein, followed by chloroform extraction of the hydrolysate, resulted in the isolation of a crystalline degradation product, m.p. 182–183°. This substance exhibited an absorption maximum at 2785 Å., $E^{1\%}_{1cm.}$ 524, in the ultraviolet. Analytical data established the composition $C_5H_6N_2O_2$ for the compound. Upon oxidation with potassium permanganate, the degradation product was converted to a crystalline acid, m.p. 187–188°, of the composition $C_4H_6N_2O_4$. A mixed melting point determination with ω-methyloxaluric acid (I) showed the acids to be identical. The structure of the oxidation product and the composition of the hydrolytic degradation product suggested that the latter was an N-methyluracil. Since the hydrolysis product gave a positive test with diazotized sulfanilic acid, the 3-position was indicated for the methyl group. A specimen of 3-methyluracil (II) was synthesized[5] and found to be identical with the degradation product derived from grisein.



II I

Experimental

Preparation of Crude Concentrates of Grisein.—A 650-gallon solution of culture broth possessing a microbiological potency of approximately 200 units/ml.[6] was obtained from the cultivation of a strain of *Streptomyces griseus* in the manner described by Reynolds and Waksman.[2] After this culture broth was acidified to *p*H 2.5–3.0 with phosphoric acid, it was stirred with 50 lb. of diatomaceous filter aid and filtered. The filter cake was washed with 100 gallons of water, and the combined filtrate and wash was adjusted to *p*H 7.5–8.0 with 30% sodium hydroxide solution and filtered. The filtrate was stirred with 25 lb. of activated carbon for 30 minutes and filtered. The filter cake was washed by

(4) Feigl, "Spots Tests," Third Edition, Elsevier Publishing Co., New York, N. Y., 1946, p. 353.

(5) Johnson and Heyl, *Am. Chem. J.*, **37**, 632 (1907).

(6) The microbiological assays were carried out using the cup assay procedure.

slurrying with 100 gallons of water, and collected on a filter-press. This filter cake, comprising charcoal and the adsorbed active material, was stirred for 30 minutes with a mixture of 120 gallons of water and 40 gallons of pyridine. The spent charcoal was collected in a filter-press and washed by recycling 40 gallons of 25% pyridine through the filter-press for 15 minutes. The combined eluate and washings were evaporated *in vacuo* at 40° to about 6 to 7 gallons. This concentrate was diluted with approximately 65 gallons (10 volumes) of methanol, and clarified by filtration. The active substance together with inert material was precipitated by the addition of about 10-gallon portions of the aqueous methanol solution to successive 3-gallon volumes of dry ether. The insoluble material was removed by filtration and dried in a vacuum drier. The yield from the entire batch was about 1937 g. of brown solid having a microbiological potency of approximately 320 grisein units/mg.

Thirteen and one-half kilograms of grisein concentrate (540 units/mg.) was ground in a ball mill, and then extracted with four 14-gallon portions of methanol. The methanol solution was filtered with the aid of Super-Cel. The extracted residue was suspended in acetone, collected on a filter, and dried *in vacuo*. Thirty-six pounds of crude grisein (900 units/mg.) containing Super-Cel was recovered by this method.

Purification of Crude Grisein by Partitioning between Aqueous Acid and Phenol–Chloroform.—A solution of 300 g. of grisein (750 units/mg.) in 2000 ml. of water was treated with 2.5 *N* hydrochloric acid to *p*H 1.0. This solution was stirred with a mechanical stirrer in a large separatory funnel for 5 minutes with 2000 l. of 1:1 phenol–chloroform (V/V).[7] After settling, the lower layer was discarded, and the extraction was repeated once more. The aqueous phase was then neutralized to *p*H 7 with sodium bicarbonate and extracted by stirring with two 1.5-l. portions of 3:10 phenol–chloroform (V/V). The combined 3:10 phenol–chloroform extracts were stirred with 400 ml. of water to which was added dilute hydrochloric acid in small portions until the *p*H remained at 1.0. This extraction was repeated. The combined aqueous acid solution was adjusted to *p*H 2.0 by the addition of sodium bicarbonate, and extracted with five 100-ml. portions of 1:1 phenol–chloroform (V/V). The combined 1:1 phenol–chloroform extracts were dried by filtering through filter paper and treated with a large volume of petroleum ether containing a little ether. Exhaustive extraction with water gave an extract which was washed several times with ether and concentrated *in vacuo* to a volume of 5 ml. The grisein in this aqueous concentrate was precipitated by the addition of 80 ml. of isopropyl alcohol. After washing the brown precipitate with isopropyl alcohol and drying, a grisein-containing residue of 1.33 g. (65,000 units/mg.), representing 38% of the activity, was obtained.

Purification of Grisein by Chromatographic Partitioning.[8]—A solution of 3.26 g. of grisein (45,000 units/mg.) in 45 ml. of 34% phenol–chloroform (V/V), previously saturated with *p*H 4.6–0.1 *M* citrate buffer, was diluted with chloroform to a volume of 90 ml. This solution was poured over a column of 120 g. of silica gel wet with 84 ml. of *p*H 4.6–0.1 *M* citrate buffer. The column was eluted with 17% phenol–chloroform (V/V). When the first light yellow effluent appeared, fractions were collected. The high potency material was contained in the dark red fractions, as summarized in Table I. The fractions were filtered through filter paper to remove water and inorganic material. The phenol–chloroform solution was then diluted with a large volume of petroleum ether containing a little diethyl ether,[8] and extracted exhaustively with water. The water solution was extracted with ether to remove excess phenol and then concentrated *in vacuo*.

TABLE I

CHROMATOGRAPHIC DATA

Fraction	Volume, ml.	Wt. of residue, mg.	Activity of residue
1	60		
2	33	142	155,000
3	35	105	170,000
4	35	49	150,000
5	70	102	105,000

Purification of Grisein by Countercurrent Distribution.—A grisein concentrate, 483 mg. (150,000 units/mg.), was distributed through a 15-plate system using phenol–chloroform (10 g. of crystalline phenol diluted to a volume of 100 ml. with chloroform) and 2 *M*–*p*H 6.7 phosphate buffer as the two phases. Twenty ml. of each phase was used for each distribution. The absorption at 4200 Å. was determined and the peak fractions, 10, 11, 12, and 13, were combined. The material was extracted with 1:1 phenol–chloroform (V/V). After the extract was washed with water, it was dried by filtering through filter paper. Petroleum ether was added to the extract and the material was transferred to water in the manner described previously.

This material was redistributed through a 10-plate system using 15-ml. portions of the solvents employed in the first distribution. The peak fractions, 7 and 8, were combined and worked up to give 30 mg. of grisein (270,000 units/mg.); $\lambda_{max.}$ 2650 Å., $E^{1\%}_{1cm.}$ 103; $\lambda_{max.}$ 4200 Å., $E^{1\%}_{1cm.}$ 24.

A 29-mg. portion of this material and 22 mg. of material from a similar experiment were combined, and a distribution was carried through 8 plates using 15-ml. portions each of water and phenol–chloroform (15.5 g. of crystalline phenol diluted to a volume of 100 ml. with chloroform). The peak fractions, 5 and 6, were worked up to yield 19 mg. of red powder. The sample was dried at 100° and found to have an activity of 300,000 units/mg.; $\lambda_{max.}$ 2650 Å., $E^{1\%}_{1cm.}$ 104; $\lambda_{max.}$ 4200 A., $E^{1\%}_{1cm.}$ 26.7.

One hundred and fifty mg. of purified grisein (220,000 units/mg.) was distributed through 16 plates using 2 *M*–*p*H 6.7 phosphate buffer and phenol–chloroform (10 g. crystalline phenol diluted to 100 ml. with chloroform). There was a predominate peak in the phenol–chloroform layer of plate 14, and a faint peak in the buffer phase of plate 5. When the phenol–chloroform layer was treated as previously described, 17 mg. of grisein (270,000 units/mg.; $\lambda_{max.}$ 2650 Å., $E^{1\%}_{1cm.}$ 104; $\lambda_{max.}$ 4200 Å., $E^{1\%}_{1cm.}$ 28) was obtained. The phenol–chloroform layer of plate 12, both phases of plate 13, and the water layer of plate 14 were combined and treated to yield 30.9 mg. of grisein (220,000 units/mg.; $\lambda_{max.}$ 2650 Å., $E^{1\%}_{1cm.}$ 90; $\lambda_{max.}$ 4200 Å., $E^{1\%}_{1cm.}$ 23). Plates 5 and 6 and the buffer phase of plate 7 yielded 15.8 mg. of grisein (182,000 units/mg.; $\lambda_{max.}$ 2780 Å., $E^{1\%}_{1cm.}$ 115; $\lambda_{max.}$ 4200 Å., $E^{1\%}_{1cm.}$ 24.5).

A 6.5-mg. sample of grisein (300,000 units/mg.; $\lambda_{max.}$ 2650 Å., $E^{1\%}_{1cm.}$ 108; $\lambda_{max.}$ 4200 Å., $E^{1\%}_{1cm.}$ 28.9), which was prepared by countercurrent distribution, was distributed through 10 plates, using 5-ml. portions of 2 *M*–*p*H 6.7 phosphate buffer and phenol–chloroform (10 g. of crystalline phenol diluted with chloroform to 100 ml.). The amount of grisein in each phase was determined by the absorption at 4200 Å., corrections being made for the absorption of each phase. The distribution curve was in close agreement with the theoretical curve for a pure compound.

Anal. Calcd. for $C_{40}H_{61}N_{10}O_{20}SFe$: C, 44.08; H, 5.65; N, 12.85; Fe, 5.12; mol. wt., 1090. Found: Sample A, C, 43.95; H, 5.65; N, 12.97; Fe, 5.14; eq. wt., 1034. Sample B, C, 44.10; H, 5.85.

Preparation of Iron-free Grisein.—A solution of 28 mg. of grisein in 3 ml. of water was treated with a solution of 10 mg. of 8-hydroxyquinoline in 0.5 ml. of ethanol. After standing for one hour in an ice-bath, the iron complex was removed by filtration. The filtrate was extracted six times with chloroform to remove the excess reagent, and the aqueous solution was filtered through a little Darco. The filtrate was concentrated *in vacuo* to a residue which was dissolved in acetic acid. Addition of ether caused precipitation of the grisein. The 15 mg. of material, dried at 100°, showed $\lambda_{max.}$ 2650 Å., $E^{1\%}_{1cm.}$ 86, and an activity of 120,000 units/mg.

Anal. Found: S, 3.53.

(7) Except where otherwise specified, Liquefied Phenol Merck containing *ca.* 12% water was used.

(8) On the addition of petroleum ether, occasionally a three-phase system results upon water extraction. This does not occur if a little diethyl ether is added.

Reintroduction of Iron into Iron-free Grisein.—A solution of 214 mg. of iron-free grisein (95,000 units/mg.) and 250 mg. of sodium acetate in 10 ml. of water was extracted with three 5-ml. portions of 1:3 phenol–chloroform (V/V) to remove impurities. A solution of 55 mg. of ferric chloride hexahydrate in 1 ml. of water was then added to the aqueous grisein solution. The resulting precipitate was removed by centrifugation, washed with water and acetone, and dried. The dry sample, 35 mg. (200,000 units/mg.), contained 7.6% iron.

A solution of 93 mg. of iron-free grisein ($\lambda_{max.}$ 2700 Å. $E^{1\%}_{1cm.}$ 81) in 15 ml. of water was treated with 23 mg. of ferric chloride hexahydrate in 1 ml. of water. The solution was adjusted to *p*H 4 with sodium bicarbonate and extracted with three 5-ml. portions of 1:3 phenol–chloroform (V/V). The combined phenol–chloroform extract was washed with water and dried by passing through filter paper. Addition of petroleum ether–ether and extraction with water as previously described yielded 67.2 mg. of grisein (215,000 units/mg.; $\lambda_{max.}$ 2700 Å., $E^{1\%}_{1cm.}$ 93; $\lambda_{max.}$ 4200 Å., $E^{1\%}_{1cm.}$ 23.4).

Chromatographic Purification of Iron-free Grisein.—A solution of 5.4 g. of a grisein concentrate (60,000 units/mg.) in 65 ml. of water was stirred with 2 g. of 8-hydroxyquinoline and heated. After standing for one hour at room temperature, the iron complex was centrifuged and washed with 10 ml. of water. The supernatant aqueous solution was filtered and extracted with several portions of chloroform, and then concentrated to a volume of 25 ml. This aqueous concentrate was diluted with 25 ml. of ethanol, and the solution was allowed to pass through a column consisting of a mixture of 36.5 g. of magnesol and 12 g. of polycell. Elution was carried out with 50% ethanol, and cuts were taken after the eluate produced the characteristic red color with ferric chloride solution. The results of the purification are described in Table II.

TABLE II

CHROMATOGRAPHIC DATA

Fraction	Volume (ml.)	Weight (g.)	Activity (units/mg.)	Absorption
1	9			
2	77	1.0619	120,000	$\lambda_{max.}$ 2750 Å., $E^{1\%}_{1cm.}$ 80
3	14	0.0582	110,000	

Acid Hydrolysis of Grisein.—A solution of 9.7 mg. of pure grisein in 4 ml. of 6 *N* hydrochloric acid was heated in a sealed tube at 150° for 16 hours. The insoluble material was removed by filtration and the filtrate was concentrated *in vacuo*. The wet residue was dissolved in 5 ml. of water, and the solution was continuously extracted with chloroform. The chloroform extract on concentration yielded 1.6 mg. of a crystalline residue. When recrystallized from ether–petroleum ether, the crystals melted at 178–180°. Material which was prepared similarly from the hydrolysis of less pure grisein yielded, after several crystallizations and sublimations, a degradation product of the following properties: m.p. 182–183°, $\lambda_{max.}$ 2785 Å., $E^{1\%}_{1cm.}$ 524 (in water).

Anal. Calcd. for $C_5H_6N_2O_2$: C, 47.62; H, 4.76; N, 22.23; mol. wt., 126. Found: C, 47.67; H, 4.59; N, 22.84; mol. wt., 125 (ebullioscopic in acetonitrile).

A mixture of this product with 3-methyluracil showed no melting point depression. 3-Methyluracil, m.p. 183–184°, was synthesized by the method of Johnson and Heyl.[5]

Oxidation of the Hydrolysis Product.—A solution of 10 mg. of 3-methyluracil, which was obtained from grisein, and 50 mg. of potassium permanganate in 5 ml. of water was allowed to stand at room temperature for 4.5 hours. Excess oxidant was removed with hydrogen perioxide, and the manganese dioxide was removed by centrifugation. The solution was acidified and inorganic ions were removed by allowing the solution to pass through a column of Amberlite IR-100. Evaporation of the aqueous solution *in vacuo* yielded 10 mg. of a residue which, when dissolved in ether–petroleum ether, yielded 6 mg. of a crystalline acid. Upon recrystallization, the acid melted at 187–188°. A mixture of this acid with a synthetic sample of ω-methyloxaluric acid, m.p. 186–187°, did not show a depression in the melting point.

Anal. Calcd. for $C_4H_6N_2O_4$: C, 32.87; H, 4.11; eq. wt., 146. Found: C, 32.98; H, 3.94; eq. wt., 144.

Amino Acids in Grisein.—The general procedure[9] for a one-dimensional paper chromatogram with phenol as the solvent was used for this experiment. A few milligrams of grisein (270,000 units/mg.; $\lambda_{max.}$ 2650 Å., $E^{1\%}_{1cm.}$ 104; λ_{max} 4200 Å., $E^{1\%}_{1cm.}$ 28) was hydrolyzed with 6 *N* hydrochloric acid. After the insoluble material had been removed by filtration, the filtrate was concentrated *in vacuo* to dryness. The residue was dissolved in water and a drop containing approximately 0.25 mg. of the hydrolysate was applied to the paper. After drying over a hot plate, oxidation with a drop of 1% hydrogen peroxide, neutralization with ammonia fumes, and drying, the strip was developed using phenol saturated with water as the solvent for a period of 16 hours at a temperature of 25°. After the strip was dried, it was sprayed with a 0.2% solution of ninhydrin in butanol. Two purple spots were revealed with R_F values of 0.22 and 0.34. A similar hydrolysis was made with another sample of grisein and R_F values of 0.22 and 0.34 were obtained. A mixture of cysteine and glutamic acid was developed along side of the grisein hydrolysate. Cysteine showed an R_F value of 0.12 and glutamic acid a value of 0.34. When glutamic acid was added to a grisein hydrolysate and the mixture was developed, only two spots appeared; the second one had an R_F value of 0.34 and was very intense. One of the amino acids in the hydrolysate appears to be glutamic acid.

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RAHWAY, N. J. RECEIVED AUGUST 29, 1950

(9) B. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **38**, 224 (1944).