2-methylbutanenitrile, converted by alcoholysis to ethyl 4-chloro-2-hydroxy-2-methylbutanoate.

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Experimental

2-Chloroethyl Methyl Ketone (I).—Paraformaldehyde (240 g., 8 moles) suspended by stirring in refluxing acetone (1174 ml., 16 moles) was treated with dry hydrogen chloride at such a rate as to maintain boiling of the mixture. When all paraformaldehyde had dissolved (about two hours), the mixture was stirred two hours more, cooled, treated with anhydrous calcium chloride to saturation, and left for several hours. The upper layer was separated, rapidly distilled at about 50 mm., stripped of most of its acetone by heating at atmospheric pressure, washed with water, dried and distilled *in vacuo* to yield 350 g. (41%) of I, boiling at 53° at 20 mm., 64° at 30 mm., 72° at 40 mm., 78° at 50 mm., or 123° (dec.) at 740 mm. (literature 53°[2] or 53.5°[3] at 15 mm.). The identity of I was confirmed by its chlorine content (calcd., 33.29%; found, 33.21%), its molecular refraction ($n^{23}D$ 1.4284, d^{23}_4 1.068; *MR* calcd. 25.55, *MR* found, 25.68), and its conversion to 3-methyl-1-phenylpyrazoline, m.p. 74–76° (literature 76–77°[4]), with phenylhydrazine. Slow distillation alone gave 50, or with diethylaniline[2] 65%, of methyl vinyl ketone.

Similar experiments with methyl ethyl ketone gave less than 1% 1-chloro-2-methyl-3-butanone, b.p. 58° at 20 mm. Paraldehyde, formalin and hydrogen chloride likewise produced only a very little β-chloropropionaldehyde.

I was converted by the method of Marvel and Sparberg[5] to sodium 3-oxo-1-butanesulfonate[6] in 16% yield.

Anal. Calcd. for $C_4H_7O_4SNa$: Na, 13.2. Found: Na, 13.1, 13.5.

Coupling of I with potassium thiocyanate in alcoholic solution by the method of Shriner[7] gave an uncharacterized viscous yellowish-red liquid with a foul odor, decomposing on distillation at 5 mm.

I formed the expected addition compound upon shaking with a concentrated aqueous solution of sodium bisulfite.

Anal. Calcd. for $C_4H_8O_4SClNa$: Na, 10.9. Found: Na, 10.2.

The cyanohydrin was prepared by the method of Bucherer and Grolee.[8] I (106.5 g., 1 mole) and sodium bisulfite (105 g., 1 mole) were shaken together in 200 ml. of water, and potassium cyanide (65.1 g., 1 mole) in 100 ml. of water was added in portions. The 4-chloro-2-hydroxy-2-methylbutanenitrile, which separated as a layer, was removed at once, and dried: crude yield, 46%. Vacuum distillation gave the pure nitrile, b.p. 104–106° at 13 mm., $n^{23}D$ 1.4448, d^{23}_4 1.125.

Anal. Calcd. for C_5H_8ONCl: N, 10.5; *MR*, 31.4. Found: N, 10.2; *MR*, 31.5.

The nitrile was best hydrolyzed by allowing it to stand with excess concentrated hydrochloric acid.[9] Evaporating to dryness, extracting with benzene, and removing the benzene produced a liquid that could not be crystallized and gave a fading end-point when titrated with base; it was considered to be the lactide of 4-chloro-2-hydroxy-2-methylbutanoic acid.

For alcoholysis, 60 ml. of the cyanohydrin in 100 ml. of absolute ethanol was saturated with hydrogen chloride at 0°. The mixture was refluxed for four hours, diluted with water, refluxed for one-half hour more, cooled, neutralized with sodium bicarbonate, and extracted with ether. Drying and distilling gave 25 ml. (30%) of ethyl 4-chloro-2-hydroxy-2-methylbutanoate, b.p. 105–107° at 23 mm., $n^{24}D$ 1.4385, d^{24}_4 1.106.

Anal. Calcd. for $C_7H_{13}O_3Cl$: Cl, 19.6; *MR*, 42.6. Found: Cl, 19.1; *MR*, 42.8.

Methanolysis proceeded similarly but gave two incompletely separated fractions. The one boiling at 68° at 20 mm. contained about 1% chlorine, evidently as impurity, and probably consisted chiefly of methyl 2-hydroxy-2-methyl-3-butenoate, produced by loss of hydrogen chloride from the expected ester. The latter, b.p. 98–100° at 24 mm., contained slightly less than the theoretical amount of chlorine for methyl 4-chloro-2-hydroxy-2-methylbutanoate for the same reason.

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On the Products of the Maillard Reaction[1]

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Before measurable carbon dioxide is evolved or color developed in the Maillard reaction, it is apparent that there is an interaction between simple sugars and amino acids. In the most recent contribution, Haugaard, Tumerman and Sylvestri[2] conclude that at alkaline *p*H, the initial product is a Schiff base.

In later stages, carbon dioxide, carbonyl compounds and humin-like material are observed. Wolfrom, Schuetz and Cavalieri[3] isolated hydroxymethylfurfural (HMF) from heated glucose–glycine mixtures. Gottschalk and Partridge[4] have demonstrated chromatographically two reaction products in heated mixtures of glucose and amino acids under alkaline conditions. The products were assumed to be N-glycosides. They were capable of reacting with ninhydrin and had lower R_f values in butanol–acetic acid than the original amino acid. Reaction mixtures after some fractionation were hydrolyzed with acetic acid and chromatographed, revealing a compound which co-chromatographed with authentic HMF.

In this paper, results are reported of further study of the glucose–glycine reaction. Some of the numerous reaction products have been separated by chromatography and their relation to the original reactants determined by radiographic techniques.

Experimental

Solutions described in the previous paper[5] were analyzed for reaction products, the reaction being studied at 56.5° and at 100°. The reaction at 100° was allowed to proceed for two hours, equivalent in terms of color development to 250 hours at 56.5°, whereas the reaction at 56.5° was continued for 453 hours. The mixture at the lower temperature was sampled at the beginning and end of the run. Samples (0.1 ml.) were withdrawn from the 100° mixture at 0, 2, 4, 16, 40, 64, 85 and 128 min.

Aliquots for chromatograms were prepared as follows: The 0.1-ml. sample was transferred to a 1-ml. volumetric flask and diluted to volume. Aliquots (20 μl.) of this diluted solution were applied to papers and chromatographed two-dimensionally for amino acids, sugar, non-volatile organic acids and for autoradiographs. Water-saturated phenol and water-saturated butanol–acetic acid were used for the amino acids; phenol–water and butanol–ethanol–water (52–32–15) for the sugars; butanol–formic acid and

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t-amyl alcohol–formic acid for organic acids. Ninhydrin was used to indicate amino acids, *m*-phenylenediamine for sugars and aldehydic components, and brom cresol green for organic acids, Spots in the autoradiographs were identified where possible as to class by comparison with the indicated paper chromatograms.

Autoradiographs were made from the chromatograms by placing them on 14 × 17″ (no screen) X-ray film in X-ray exposure holders. Exposures were from one to three weeks depending upon the amount of activity originally applied to the paper. After development of the films, the darkened areas were traced on the original paper to locate the zones of radioactivity. The area of the zone was subdivided into squares, approximately 2 cm. × 2 cm., matching a square hole cut in a metal shield on which the counting tube rested. The counts for the subdivisions were summated, after correction for background, to give activity for the zone. To obtain the distribution of activity on a given paper, activities for the zones were summated, and the percentage for an individual zone was thus readily computed.

The specific activities of the mixtures were determined at the beginning and end of the experiments by dry combustion of 5 to 8 mg. of reaction mixture, absorption of the CO_2 in sodium hydroxide, precipitation with barium chloride, and by counting the $BaCO_3$ on 1″ plates. Counting was performed with a TC-1 Tracerlab Autoscaler.

A portion (1 ml.) of each of the final reaction mixtures was extracted continuously for 72 hours with diethyl ether in a micro-soxhlet and the extract and residue were assayed for activity. The extract was transferred to water and chromatographed with phenol–water to determine the distribution of activity.

The remaining extract residue (0.8 ml.) was dialyzed in a collodion bag against running water until free of dialyzable material. It was then washed to remove the last traces of unreacted labeled material by addition of a solution of unlabeled glycine or glucose (depending upon which had originally been labeled) and again dialyzed until the added component had been removed. The dialysate and dialysis residue were then assayed for activity.

Some of the compounds were isolated chromatographically on the larger scale described by Flood, Hirst and Jones.[6] In two cases, dinitrophenylhydrazones (DNPH) were prepared. Absorption spectra of the isolated compounds and of the two DNPH derivatives were measured on the Beckman DU spectrophotometer. The specific activities of the compounds separated were also determined.

Results

Chromatographic results are shown in Fig. 1 and in Table I. Compounds represented by spots 1–3 are ninhydrin-reacting, while 4 to 8 react with *m*-phenylenediamine. Spot 10 is pigmented and the remaining spots grouped under 9 are located by their fluorescence.

TABLE I

PROPERTIES OF COMPOUNDS SEPARATED CHROMATOGRAPHICALLY

Spot number (Fig. 1)	Rf [a]	Rf [b]	Rf [c]	Indicator	Source of activity
1	0.40	0.15	0.29	N[d]	Gly
2	.43	.08	.20	N	Gly, Glu
3[e]	.36	.04	.18	N	Gly, Glu
4	.32	.22	.26	P[d]	Glu
5[e]	.39	.26	..	P	Glu
6	.57	.36	.29	P	Glu
7	.78	.75	.68	P	Glu
8	.90	.85	.85	P	Glu
9[f]	0.38–0.89	0.08–0.27	..	...	Glu, ? Gly
10[g]	0–0.89	0	0	...	Gly, Glu

[a,b,c] R_f values in phenol, butanol–acetic acid and butanol–ethanol, respectively. [d] N, ninhydrin; P, *m*-phenylenediamine. [e] Weak fluorescence. [f] Spots grouped under 9 in Fig. 1, broken lines; strong fluorescence. [g] Brown pigment.

(6) A. E. Flood, E. L. Hirst and J. K. N. Jones, *Nature*, **160**, 86 (1947).

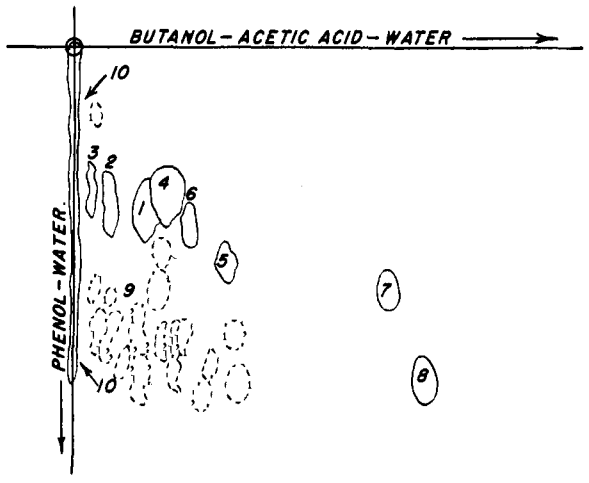

Fig. 1.—Chromatogram of the reacted Maillard reaction mixture. See text for explanation of the spot numbers.

Ninhydrin-reacting Substances. General.—The compound in spot 1 is glycine. Compound 2 can be detected after 16 min. at 100°. It accumulates with time, and as noted in Table I, is radioactive with either labeled glucose or glycine. It gives a delayed reaction with ninhydrin, appearing first as a light brown, changing slowly to the typical purple. Compound 3 behaves like 2, but appeared only at the lower temperature. R_f values are given in Table I.

Activities.—After 128 min. at 100°, spot 2 (referring here to the area marked on the paper, where compound 2 is to be found) contained 7% of the total activity from labeled glucose, 17% if from labeled glycine. At 56.6°, the respective percentages were 23 and 49. Spot 3 contained less than 2% of the total activity.

Hydrolysis.—Compound 2 after elution in water was hydrolyzed both in 5% hydrochloric acid and in 6 *N* acetic acid at 100°. Samples were withdrawn at 2, 5, 10 and 30 min. They were chromatographed two-dimensionally with phenol–water and butanol–acetic acid.

Chromatograms from the hydrochloric acid hydrolysate showed the presence of glycine and a sugar derivative detected only by radiographic means. Glycine regeneration was about 50% complete after 30 min.

Chromatograms from the acetic acid hydrolysate showed much slower liberation of the glycine. Unlike the hydrolysate with HCl, there were three compounds which reacted with *m*-phenylenediamine and which derived activity from glucose carbon. One of them co-chromatographed with HMF, *cf.* spot 8, Fig. 1.

***m*-Phenylenediamine-reacting Substances. General.**—Compound 4 is unreacted glucose. Compounds 5–8 are detected at 56.5°; all are ether-extractable; none contains glycine carboxyl carbon. Only compound 5 is found at 100° without concentration.

Activities.—Activity was detected for compounds 5 to 8 at a maximum of 3% of the total. The ether extracts derive 80 to 100% of their activity from glucose carbon and apparently contain volatile components since the total count falls markedly on standing.

Concentration.—To obtain larger quantities of compounds 5 to 8, 50 g. of glucose was heated on a steam-bath with 12.5 g. of glycine in a total volume of 125 ml. for three hours. The solution was then ether-extracted and the extract chromatographed one-way with butanol–ethanol. Compounds 5 to 8 were all present and 5 appeared to be in highest concentration. Its absorption spectrum in water shows a sharp peak at 297 mμ with a plateau at 265 mμ. It reacted in the cold to give a DNPH, absorption maximum at 410 mμ in 95% ethanol. Compounds 6 and 7 were still in low concentration and were not investigated further. Compound 8 cochromatographed with HMF in phenol–water and butanol–ethanol. Its spectrum resembles that of HMF, and the spectrum for its DNPH is superimposable on that of the HMF derivative.

Fluorescent Substances. General.—The fluorescent substances represented by broken lines in Fig. 1 show a yellowish to bluish-yellow color under a Burton ultraviolet lamp.

They do not react with the indicator sprays used. In general, R_f values are high in phenol (0.38 to 0.8) and low in butanol (below 0.3). Eleven such spots are evident at 100°, and nineteen at 56.5°.

Activities.—Activities are low but in the aggregate may represent an appreciable percentage of the total. Activity is derived from glucose, and in some cases from glycine.

Organic Acids.—Non-volatile acids were not detected by the procedures used, in chromatograms of the final reaction mixtures.

Dialysis Residues.—The dialysis residue is highly pigmented (dark brown), insoluble in water and on chromatography differs from spot 10 in that it does not move from the origin in phenol–water or in butanol mixtures. With labeled glucose, the specific activity at both temperatures approximates that of the original mixture. With labeled glycine, the specific activity at 100° was 20%, and at 56.5°, 65 to 70% that of the original.

Discussion

The reaction between glucose and glycine is complex, involving the formation of numerous compounds, as shown in Fig. 1 where the presence of more than two dozen compounds has been demonstrated. Other compounds are present in small amount, indicated by weak ninhydrin or *m*-phenylenediamine reactions, but they are not demonstrable radiographically in significant amount. They have therefore been ignored in the present discussion.

Compound 5 requires further study because of its absorption maximum in the 270–300 mμ region where furfurals show high absorption. It derives its activity solely from the glucose and its role in browning also requires evaluation.

Compound 2 has many characteristics similar to the compounds separated by Gottschalk and Partridge.[4] We hope to isolate and purify this compound, the major reaction product, except for brown pigment, and to evaluate its possible role as an intermediate in browning.

Compound 8, identified with HMF is clearly of interest because furfurals have been shown to play a part in browning of natural products.[7]

As shown by analysis of dialysis residues, more carbon is incorporated into brown pigment at the lower temperature, from glycine carboxyl carbon, than at the higher temperature. This coincides with the fact that at the lower temperature more pigment is produced per millimole of carbon dioxide from the glycine molecule.[5]

(7) E. R. Stadtman, *Advances in Food Research*, 1, 325 (1948).

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The Identity of Neomycin A,[1] Neamine[2] and the Methanolysis Product of Neomycin B and C[3]

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A comparison of the reported chemical and biological properties of these products[1–3] suggested that they might be identical and hence a fuller and more direct comparison was made, with the result that their identity has been adequately established. The free base of the methanolysis product 1 obtained from neomycins B and C[3] crystallized readily when subjected to the conditions reported for the crystallization of neamine,[2] the product isolated from the hydrolysis of neomycin with 6 *N* sulfuric acid. From the comparison of the properties of the bases and their derivatives shown in Table I there can be no doubt as to their identity. In view of the observation that both neamine and neomycin A possess antibacterial activity, of low order in the broth test but high in the agar diffusion assay,[2] and that in many of our neomycin preparations there could be demonstrated an antibacterial component with the same mobility in papergrams as neamine, it was suspected that neamine might be identical with neomycin A. A sample of the purified crystalline *p*-(*p'*-hydroxyphenylazo)-benzenesulfonate of neomycin A[4] was compared with the corresponding salt of neamine with the results also shown in Table I.

Although the identity of these products is apparent from their properties, the composition is not yet certain. The empirical formula $C_6H_{12-14}O_3N_2$ has been proposed for neamine[2] whereas the composition $C_9H_{19}O_5N_3$ has been advanced for the methanolysis product 1.[3] Since neomycin A has been found to yield on hydrolysis at 140° with 6 *N* hydrochloric acid a product with the proven composition $C_6H_{14}O_3N_2$,[5] it would therefore appear that the original product contains more than 6 carbon atoms and has either the C_9 composition or the C_{12} dimeric composition suggested.[2] Papergram studies have shown that an additional moiety formed by this further hydrolysis with strong acid is a ninhydrin positive-alkaline silver reducing material with an R_f similar to that of the diaminodesoxyhexose fragment obtained from the hydrolysis of methyl neobiosaminide.[3] Further studies of these products are being made.

Experimental

Methanolysis of Neomycin B.—A solution of 2.81 g. of neomycin B hydrochloride in 280 ml. of anhydrous methanol was refluxed for 2.5 hours with 60 ml. of 1.8 *N* methanolic hydrogen chloride. The addition of 100 ml. of anhydrous ether to the cooled solution caused the precipitation of 1.62 g. of amorphous white solid (methanolysis product 1). The mother liquor from the precipitation was concentrated to 25 ml. and diluted with 250 ml. of ether, yielding 1.00 g. of amorphous methanolysis product 2. Papergrams of these fractions show that each contains small amounts of the other but that subsequent purification results in homogeneous products.

Crystallization of Methanolysis Product 1.—A solution of 1.60 g. of the amorphous hydrochloride in 1.8 ml. of concentrated ammonium hydroxide was diluted with 146 ml. of methanol, and ammonia gas was bubbled through the solution until crystallization had begun. This solution was refrigerated for 2.5 hours and then the crystals were collected on a Buchner funnel, washed with cold absolute methanol, and dried *in vacuo*; yield 605 mg. An additional 187 mg. obtained on concentration of the mother liquor. The base was recrystallized for analysis from an ethanol–water mixture. It darkens at 230° with softening at 235° and decomposes above 280°; $[\alpha]^{24}D$ +123° (*c*, 0.7 in water).

Anal. Calcd. for $(C_6H_{14}O_3N_2)_x$: C, 44.43; H, 8.70;

(1) R. L. Peck, C. E. Hoffhine, Jr., P. Gale and K. Folkers, THIS JOURNAL, **71**, 2590 (1949).

(2) B. E. Leach and C. M. Teeters, *ibid.*, **73**, 2794 (1951).

(3) J. D. Dutcher, N. Hosansky, M. N. Donin and O. Wintersteiner, *ibid.*, **73**, 1384 (1951).

(4) The sample of neomycin A *p*-(*p'*-hydroxyphenylazo)-benzenesulfonate was obtained through the courtesy of Dr. R. L. Peck, Merck and Co., Rahway, N. J.

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