by replacing it with a methyl group (acetylglycinate). This increases ΔF by 2400 cal.

It is interesting that malonanilate ion in which the NH and carbonyl groups are interchanged has a relatively high combining constant, within 500 cal. of that of hippurate itself. This may be due to the possibility that both the NH and carbonyl groups are hydrated and the compound with the hydrated NH and carbonyl groups reversed (Fig. 1) appears structurally and configurationally quite like the original hydrated hippurate as far as the antihippurate antibodies are concerned. This is especially apparent upon examination of a three dimensional (to scale) molecular model.

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An Agent from *E. Coli* Causing Hemorrhage and Regression of an Experimental Mouse Tumor. IV. Some Nitrogenous Components of the Phospholipide Moiety[1]

BY MIYOSHI IKAWA, J. B. KOEPFLI, S. G. MUDD AND CARL NIEMANN[2]

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D-Glucosamine, ethanolamine and a hitherto unreported diamine have been found to be components of the phospholipide moiety of the mouse tumor hemorrhagic agent. The diamine, for which the name *necrosamine* is proposed, has been assigned the provisional formula $CH_3(CH_2)_{14}CH(NH_2)CH(NH_2)C_3H_7$. There is some evidence that aspartic acid may also be a component of the phospholipide moiety.

The agent which is elaborated by *E. coli* and which produces a hemorrhagic response in and causes the regression of the experimental mouse sarcoma 180 is a complex polysaccharide containing both a peptide and a phospholipide moiety.[3] It has been shown previously,[4,5] that the polysaccharide is composed of D-glucose, D-galactose and D-glucosamine, with the latter probably present as N-acetyl-D-glucosamine,[3] and that the fatty acids present in the phospholipide moiety are lauric, myristic, palmitic and D-β-hydroxymyristic acid. The phospholipide moiety[3,5] upon acid hydrolysis gave, in addition to the above ether-soluble fatty acids, a water-soluble fraction and a flocculent precipitate which was insoluble both in water and in ether.[5] It is the purpose of this communication to report on the nature of this latter substance and upon some of the other nitrogenous components of the water soluble fraction.

Treatment of the flocculent precipitate, which was obtained in *ca.* 12% yield from the phospholipide,[5] with aqueous sodium hydroxide followed by solution in ether and subsequent precipitation with methanolic hydrogen chloride gave, in good yield, a crystalline compound which appeared to be the hydrochloride of an amine. Elementary analysis of the hydrochloride, the picrate and the benzoyl derivative of this amine, and a molecular weight determination of the latter derivative, indicated that the compound in question was an acyclic saturated diamine with the molecular formula $C_{20}H_{44}N_2$. A Kuhn–Roth determination indicated the presence of at least two terminal methyl groups and thus suggested the probability of finding the amino groups in non-terminal positions. Since the diamine was observed to react with carbon disulfide to form an intramolecular dithiocarbamate salt[6] which, upon heating, lost hydrogen sulfide to form a cyclic thiourea, it was presumed that the two amino groups were in contiguous or near contiguous positions.

As the diamine under investigation appeared to be rather slowly attacked by lead tetraacetate at 25° all subsequent studies with this reagent were conducted at 60°, *cf.* Table I. Under these conditions, *i.e.*, at 60° for one hour, both ethylene glycol and 2,3-butylene glycol still consumed only one mole of the reagent per mole of substrate. However, under the same conditions, 4.4 moles of reagent was consumed per mole of diamine. It is clear from the data given in Table I that this result is not an unreasonable one for a compound of the type $-CH_2-CHNH_2-CHNH_2-CH_2-$ since lead tetraacetate, under the conditions previously speci-

TABLE I

REACTION OF LEAD TETRAACETATE WITH CERTAIN NITROGENOUS COMPOUNDS AND GLYCOLS[a]

Type	Compound	Moles $Pb(OAc)_4$ / Mole comp.
1,2-Glycols	Ethylene glycol	1.0
	2,3-Butylene glycol	1.0
Monoamines	*s*-Butylamine	0.3
	n-Decylamine	0.4
1,2-Hydroxyamines	Ethanolamine	1.5
	DL-Threonine	3.7
1,2-Diamines	Ethylenediamine	1.7
	DL-2,3-Diaminobutane	3.7
	Necrosamine	4.4
1,3-Diamines	2,4-Diaminopentane	1.2
Nitriles	Acetonitrile	0.5
	Capronitrile	0.3

[a] In glacial acetic acid for one hour at 60°.

(1) Supported from 1938 to 1943 by grants from the Argonaut Foundation and from 1948 onwards by grants from the National Cancer Institute of the U. S. Public Health Service.

(2) To whom inquiries regarding this article should be sent.

(3) M. Ikawa, J. B. Koepfli, S. G. Mudd and C. Niemann, *J. Nat. Cancer Inst.*, **13**, 157 (1952).

(4) M. Ikawa, J. B. Koepfli, S. G. Mudd and C. Niemann, THIS JOURNAL, **74**, 5219 (1952).

(5) M. Ikawa, J. B. Koepfli, S. G. Mudd and C. Niemann, *ibid.*, **75**, 1035 (1953).

(6) E. R. Buchman, A. O. Reims, T. Skei and M. J. Schlatter, *ibid.*, **64**, 2696 (1942).

fied, appears to be capable of effecting, at least in part, the dehydrogenation of imines to nitriles[7] and the further oxidation of the latter compounds presumably through attack on the α-carbon atoms.

In order to obtain further information in respect to the nature of the amino groups present in the diamine a quantitative study was made of the reaction of the diamine and amines of known structure with ninhydrin, *cf.*, Table II. The colorimetric procedure described by Moore and Stein[8] was employed and L-leucine was likewise adopted as the standard and given the molar color value of 1.00. Since the diamine gave a color value of 1.10 it was possible to conclude from this information and the data given in Table II, in respect to the behavior of primary and secondary amines of known structure, that both of the amino groups in the diamine were very probably primary amino groups. Additional evidence along these lines was obtained when it was found that the diamine gave no indication of possessing any N-alkyl groups when subjected to the usual procedure for determining such groups.[9]

TABLE II

MOLAR NINHYDRIN COLOR EQUIVALENTS OF NECROSAMINE AND RELATED COMPOUNDS[a]

Type	Compound	Molar color equivalent
Standard	L-Leucine	1.00
Pri-monoamines, nitrogen on pri-carbon atom	Methylamine	0.78
	Ethylamine	0.39
	n-Decylamine	0.41
Pri-monoamines, nitrogen on *s*-carbon atom	Cyclohexylamine	−0.02
	s-Butylamine	0.01
Pri-monoamines, nitrogen on *t*-carbon atom	2-Amino-2-methyl-1-propanol	0.02
	2-Amino-2-methyl-1,3-propanediol	0.01
	Tris-(hydroxymethyl)-aminomethane	0.01
	1-Amino-1-cyclopropanecarboxylic acid	0.09
s-Amines	Piperidine	−0.02
	Di-*n*-butylamine	0.02
Pri-diamines, nitrogen on pri-carbon atoms	Ethylenediamine	0.50
	1,4-Diaminobutane	1.18
Pri-diamines and hydroxyamines, nitrogen on *s*-carbon atoms	DL-2,3-Diaminobutane[b]	0.20
	meso-2,3-Diaminobutane[b]	0.19
	cis-1,2-Diaminocyclobutane[c]	0.42
	trans-1,2-Diaminocyclobutane[c]	1.31
	Necrosamine	1.10
	2,4-Diamino-*n*-pentane	0.68
	2-Amino-1-butanol	0.24

[a] Based upon the procedure of Moore and Stein.[8] [b] Supplied by H. J. Lucas. [c] Supplied by E. R. Buchman.

It will be seen from data given in Table II that contiguously substituted amino alcohols in which a primary amino group is bonded to a tertiary carbon atom give practically no color with ninhydrin. Thus from these and other data presented in Table II it appears that neither of the carbon atoms bearing amino groups in the diamine is tertiary. An exploratory study of the effect of configuration upon the reaction of contiguously substituted primary diamines with ninhydrin revealed that whereas *cis*-1,2-diaminocyclobutane gave approximately twice the color equivalent of the corresponding *trans* isomer there was little difference in the color equivalents of DL- and *meso*-2,3-diaminobutane.

Oxidation of the diamine with permanganate gave palmitic acid, and with lead tetraacetate, palmitaldehyde, isolated as the 2,4-dinitrophenylhydrazone. Oxidation of the diamine with periodate followed by permanganate gave, in addition to palmitic acid, a water-soluble acid fraction which, when chromatographed on paper, produced a spot corresponding to either butyric or isobutyric acid. Mixtures of these two acids could not be separated with any of the solvent systems tried.[10]

On the basis of the above observations the provisional structure $CH_3(CH_2)_{14}CHNH_2CHNH_2C_3H_7$ is proposed for the diamine isolated from the phospholipide moiety of the mouse tumor hemorrhagic agent produced by *E. coli* and since this compound has not been described previously it is suggested that it be named ***necrosamine***.

The infrared absorption spectrum of necrosamine exhibits a weakness and lack of a pronounced double peak character in the 7.25 μ region and thus suggests[11] that this compound may possess a normal carbon skeleton. Although necrosamine appears to possess two asymmetric carbon atoms no measurable optical activity was observed with solutions of either the hydrochloride or the free base. Such a result might be a consequence of an *erythro* configuration about the two asymmetric centers. However, it is clear that further study is needed to establish the structure of the terminal C_3H_7 rest and the configuration about the two asymmetric carbon atoms.

The water-soluble fraction obtained from the acid hydrolysis of the phospholipide moiety, *vide ante*, was subjected to paper chromatography and six spots, all reacting with ninhydrin and three with ammoniacal silver nitrate, were located, *cf.*, Fig. 1.

It has been noted previously[5] that the phospholipide moiety appears to contain *ca.* 12% equivalent glucosamine. The presence of D-glucosamine, in the water-soluble fraction, was established by isolation of the corresponding N-carbobenzoxy derivative and by comparative chromatography; one of the spots reacting with both ninhydrin and ammoniacal silver nitrate was identified as D-glucosamine. It is interesting that D-glucosamine, or more probably its N-acetyl derivative, is found in both the phospholipide and the carbohydrate moieties of the hemorrhagic agent. As, will be shown later, there is some evidence that at least one amino acid is also present in the phospholipide moiety it appears that in the hemorrhagic agent, as isolated, the three principal moieties may be linked

(7) R. Criegee in "Newer Methods of Preparative Organic Chemistry," Interscience Publishers, Inc., New York, N. Y., 1948.

(8) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(9) J. B. Niederl and V. Niederl, "Micromethods of Quantitative Organic Analysis," John Wiley and Sons, Inc., New York, N. Y., 1942.

(10) Alternative methods for the characterization of the lower molecular weight oxidation product are currently under investigation.

(11) R. B. Barnes, R. C. Gore, U. Liddell and V. Z. Williams, "Infrared Spectroscopy," Reinhold Publ. Corp., New York, N. Y., 1944.

in the sequence, peptide–phospholipide–polysaccharide.

Treatment of the water-soluble fraction with 2,4-dinitrofluorobenzene in the presence of excess aqueous sodium bicarbonate,[12] followed by extraction of the alkaline solution with ether and chromatographic fractionation of the ethereal extract, gave crystalline N-(2,4-dinitrophenyl)-ethanolamine. A comparative chromatogram established the identity of one of the spots which reacted with ninhydrin, but which did not reduce ammoniacal silver nitrate, with ethanolamine. Acidification of the above reaction mixture, after it had been extracted with ether, failed to give a significant amount of ether-soluble material, thus indicating that amino acids were not present in the original water-soluble acid fraction in any substantial quantity. However, paper chromatography did indicate the probable presence of aspartic acid, *cf.*, Fig. 1, and the probable absence of glutamic acid, α,α'-diaminoadipic acid, α,α'-diaminopimelic acid, serine and threonine. The spot with the greatest R_F value, and which did not reduce ammoniacal silver nitrate but which did react with ninhydrin, *cf.*, Fig. 1, was shown to be necrosamine by comparative paper chromatography.

Thus, of the original six spots, the three that reacted with ninhydrin but not with ammoniacal silver nitrate have been correlated with the presence of necrosamine, ethanolamine, and probably aspartic acid, in the water-soluble fraction. Of the remaining three spots, *i.e.*, those which reacted with both ninhydrin and ammoniacal silver nitrate, one has been definitely associated with the presence of D-glucosamine in the water-soluble fraction and the other two have not been identified. Our present knowledge of the composition of the hemorrhagic agent synthesized by *E. coli* is summarized in Table III.

TABLE III

KNOWN COMPONENTS OF THE HEMORRHAGIC AGENT

Moiety[a]	
Phospholipide	Polysaccharide
Lauric acid	D-Glucose
Myristic acid	D-Galactose
D-β-Hydroxymyristic acid	D-Glucosamine[b]
Palmitic acid	
D-Glucosamine[b]	
Ethanolamine	
Necrosamine	
Phosphoric acid	
Aspartic acid[c]	

[a] The composition of the peptide moiety has not been considered because it has been shown[3] that this fragment can be cleaved from the agent without loss in physiological activity. [b] Probably present as N-acetyl-D-glucosamine.[3] [c] The presence of this component has not been rigorously established.

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Experimental[13]

Starting Materials.—The procedure used for the isolation of the phospholipide moiety and its subsequent hydrolysis has been described previously[3,5] as has the separation, from such hydrolysates, of the water-soluble fraction and the water and ether-insoluble precipitate.

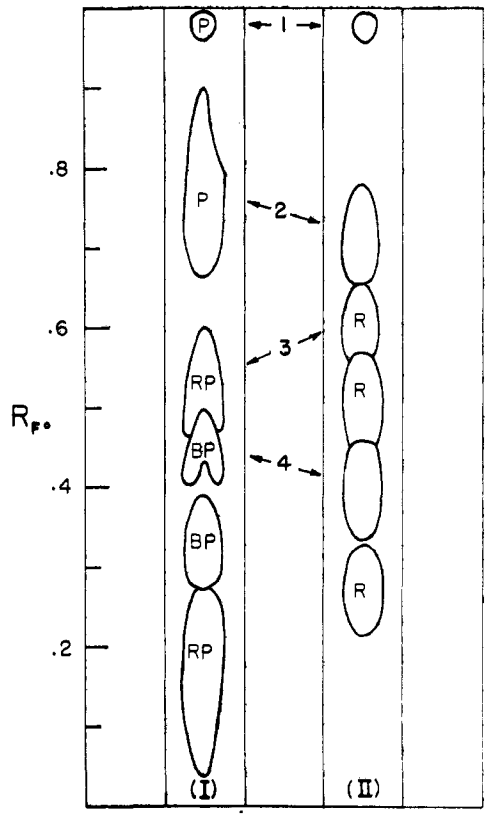

Fig. 1.—Paper chromatograms of the water-soluble fraction, I—solvent system: aqueous phenol in acetic acid atmosphere; sprayed with ninhydrin; P, purple, RP, red purple, BP, blue purple. II—solvent system: *n*-propanol–0.1 *N* aqueous ammonium hydroxide (60:40 v./v.); sprayed with ammoniacal silver nitrate; R = reduction. 1 is Necrosamine, 2 is ethanolamine, 3 is D-glucosamine, 4 is probably aspartic acid.

Isolation of Necrosamine.—The dark gray, water-insoluble, ether-insoluble precipitate was treated with an excess of *N* aqueous sodium hydroxide and the mixture extracted with ether. A slight excess of methanolic hydrogen chloride was added to the ethereal solution to give a crystalline precipitate which was recrystallized from absolute ethanol to give a product, m.p. *ca.* 275° with decomp. An attempt was made to determine the specific rotation of the hydrochloride (0.6% solution in water) and of the free base (2.5% solution in absolute ethanol) but no significant rotations were observed.

Anal. Calcd. for $C_{20}H_{44}N_2 \cdot 2HCl$ (385.5): C, 62.3; H, 12.0; N, 7.3. Found: C, 62.1; H, 12.4; N, 7.2.

The following analytical constants were also determined; neut. equiv., 194 ± 4; N-CH_3, 0.0; C-CH_3, 8.15 (*i.e.*, 2.1 groups).

The addition of an ethereal solution of picric acid to an ethereal solution of the free base gave necrosamine dipicrate, m.p. 159–161° after recrystallization from absolute ethanol.

Anal. Calcd. for $C_{32}H_{50}N_8O_{14}$ (770.8): C, 49.9; H, 6.5; N, 14.5. Found: C, 49.7; H, 6.7; N, 14.4.

Benzoylation of necrosamine, with benzoyl chloride and aqueous sodium hydroxide, gave the dibenzoyl derivative, m.p. 72.5–73.5°, after recrystallization from aqueous ethanol.

Anal. Calcd. for $C_{34}H_{52}N_2O_2$ (520.8): C, 78.4; H, 10.1; N, 5.4. Found: C, 78.2; H, 10.3; N, 5.4; mol. wt. (Rast, exaltone), 513.

Reaction of Necrosamine with Carbon Disulfide.—Necrosamine dihydrochloride, 20 mg., was added to 1 ml. of aque-

(12) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(13) All melting points are corrected.

ous *N* sodium hydroxide, the mixture extracted with ether, the ethereal extract evaporated to dryness, the residue dissolved in 2 ml. of ethanol and several drops of carbon disulfide added to this solution to give a precipitate of the intramolecular dithiocarbamate salt, which, after recrystallization from ethanol, gave 11 mg. of clusters of needles, m.p. 116–118° with decomp.

Anal. Calcd. for $C_{21}H_{44}N_2S_2$ (388.7): C, 64.9; H, 11.4. Found: C, 64.3; H, 11.0.

An ethanolic solution of the crystalline salt was heated under refluxing conditions until the odor of hydrogen sulfide was no longer present, the solution evaporated to dryness, the residue extracted with 60–70° ligroin, the extract evaporated to dryness and the residue recrystallized from aqueous ethanol to give the cyclic thiourea of necrosamine, glistening plates, m.p. 72–73°.

Anal. Calcd. for $C_{21}H_{42}N_2S_2$ (354.6): C, 71.1; H, 11.9. Found: C, 71.4; H, 11.9.

Oxidation of Necrosamine and Related Compounds with Lead Tetraacetate.—All oxidations were conducted in glacial acetic acid at 60° for one hour and the amount of reagent consumed under these conditions was determined according to Knoop, *et al.*[14] The values so obtained are summarized in Table I.

Ninhydrin Color Values of Necrosamine and Related Compounds.—The colorimetric method described by Moore and Stein[8] was employed and the intensity of the color produced by the reaction of ninhydrin with necrosamine and related compounds, under the above conditions, was determined and compared, on a molar basis, with that of L-leucine which was used as a standard and given the color value of 1.00. These data are summarized in Table II.

Oxidation of Necrosamine with Permanganate.—The free base obtained from 42 mg. of necrosamine dihydrochloride was dissolved in 15 ml. of acetone, a solution of 100 mg. of potassium permanganate in 10 ml. of acetone added, and the mixture heated under refluxing conditions for 20 minutes. The reaction mixture was diluted with 50 ml. of water, acidified with hydrochloric acid, decolorized with sodium metabisulfite, and extracted with ether. The ethereal phase was extracted with dilute aqueous sodium hydroxide and the aqueous extract acidified and again extracted with ether. This ethereal extract was evaporated to dryness, the residue extracted with 60–70° ligroin, and the residue obtained from the evaporation of the ligroin extract was recrystallized twice from aqueous ethanol to give 0.5 mg. of palmitic acid, m.p. 58–61°, which did not depress the m.p. of an authentic sample of this substance.

Oxidation of Necrosamine with Lead Tetraacetate.—This free base obtained from 38 mg. of necrosamine dihydrochloride was dissolved in 27 ml. of 0.0185 *M* lead tetraacetate in glacial acetic acid and the solution heated for one hour at 60°. Two ml. of water and 70 mg. of 2,4-dinitrophenylhydrazine were then added, the solution heated just to boiling, and sufficient water added to produce a slight turbidity. The crystals obtained upon cooling, 9.0 mg., m.p. 102–104° after recrystallization from ethanol, were again recrystallized from the same solvent to give the 2,4-dinitrophenylhydrazone of palmitaldehyde, m.p. 103–105°. This product did not depress the m.p. of an authentic specimen of the above compound but did depress the m.p. of the corresponding derivative of margaraldehyde.

Oxidation of Necrosamine with Periodate and Permanganate.—Necrosamine dihydrochloride, 11 mg., was dissolved, by warming, in a mixture of 3 ml. of water and 2 ml. of 0.14 *M* aqueous sodium periodate. Potassium permanganate, 100 mg., was then added, the solution heated under refluxing conditions for 2 minutes, 1 ml. of aqueous *N* sodium hydroxide added and the heating continued for an additional three minutes. The solution was cooled, acidified with dilute aqueous sulfuric acid, decolorized with sodium metabisulfite, extracted with ether, the ethereal phase extracted with aqueous sodium hydroxide, the aqueous phase acidified, extracted with ether, a few drops of concd. aqueous ammonium hydroxide added to the ethereal extract, and the solution evaporated to dryness. The residue was dissolved in the minimum quantity of dilute aqueous ammonium hydroxide and subjected to one dimensional paper chromatography. The chromatogram was developed with 95% ethanol containing 1% (v./v.) concd. ammonium hydroxide and the acid located by spraying with brom phenol blue as directed by Kennedy and Barker.[15] A spot corresponding to butyric or isobutyric acid was obtained. Several other solvent systems were tried, but none was able to effect a satisfactory separation of these two acids.

Paper Chromatography of the Water-soluble Fraction.—Portions of the water-soluble fraction were subjected to one dimensional paper chromatography using the ascending technique. The results obtained from these experiments are summarized in Fig. 1. The presence or absence of a particular substance was established in the usual manner, *i.e.*, by direct comparison of the chromatographic behavior of the component present in the water-soluble fraction with that of an authentic sample. Munktells chromatographic paper No. 20/150 (Sargent No. S 18860), with a weight of 150 g./sq. m., was employed throughout.

Isolation of N-Carbobenzoxy-D-glucosamine.—The partially crystalline residue obtained by evaporation of the water-soluble fraction derived from the hydrolysis of 1.96 g. of the phospholipide moiety was triturated with ethanol to give 260 mg. of a light brown crystalline solid which assayed[16] *ca.* 50% equivalent glucosamine. This solid was treated with carbobenzoxy chloride as directed by Chargaff and Bovarnick[17] to give 102 mg. of N-carbobenzoxy-D-glucosamine, m.p. 211–214°, with decomp., $[\alpha]^{25}D$ +66.2° → +80.4° in 24 hours (*c* 3.02% in pyridine). With an authentic sample, m.p. 211–214°, with decomp., $[\alpha]^{25}D$ +69.0° → +82.4° in 24 hours (*c* 3.21% in pyridine) was observed. The above rotations are somewhat higher than the value of +75.4° reported earlier.[17]

Isolation of N-(2,4-Dinitrophenyl)-ethanolamine.—The dried water-soluble fraction obtained from the hydrolysis of 721 mg. of the phospholipide moiety was treated with 2,4-dinitrofluorobenzene in the presence of excess sodium bicarbonate according to Sanger.[12] The solvent was largely removed from the ethereal extract of the aqueous bicarbonate solution and the residue was chromatographed on a 2:1 mixture of silicic acid and celite with 60–70° ligroin containing increasing amounts of acetone (each solvent containing 1% (v./v.) acetic acid) as the developing agent.[18] With 25–30% (v./v.) acetone in ligroin the lower band was eluted from the column and gave, after recrystallization from ethanol, 20 mg. of N-(2,4-dinitrophenyl)-ethanolamine, m.p. 90–91.5°.

Anal. Calcd. for $C_8H_9O_5N_3$ (227.2): C, 42.3; H, 4.0; N, 18.5. Found: C, 42.4; H, 4.1; N, 18.6.

A mixed m.p. of the above sample and an authentic specimen showed no depression and the ultraviolet absorption spectra of the two samples were found to be identical.

A second band was eluted with 33–50% acetone in ligroin and gave an amorphous yellow powder, m.p. 135–145°, after solution in ethyl acetate and precipitation with ether. This material was not identified.

The bicarbonate solution remaining after the extraction with ether, *vide ante*, was acidified and again extracted with ether. Only traces of material were present in this extract.

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