The Occurrence of ε-*N*-Methyl Lysine in Histones*

K. MURRAY

From the Department of Chemistry, Stanford University, Stanford, California

Received June 25, 1963

A hitherto unidentified component of hydrolysates of certain chromatographic fractions of histone has been isolated. From a comparison of its behavior on ion-exchange chromatography and paper chromatography with that of hydrolyzed flagellin, and from the mass spectrum of its ethyl ester, the compound has been identified as ε-*N*-methyl lysine. This amino acid has been found in histone obtained from several sources, but in all cases the quantity was very small. Radioactive ε-*N*-methyl lysine has been isolated from histone prepared from various tissues of rabbits which had been injected previously with C^{14}-(methyl)-labeled methionine. Thus methionine can donate the methyl group involved in the formation of ε-*N*-methyl lysine.

Studies of the amino acid composition of chromatographic fractions of calf thymus histone revealed the presence of a very small quantity of an unidentified substance in acid hydrolysates of some, but not all, of the histone fractions. The substance was observed in the eluate from an ion-exchange column as a shoulder on the descending side of the lysine peak (Rasmussen *et al.*, 1962) when amino acid analyses were carried out with an automatic amino acid analyszer (Spackman *et al.*, 1958). Crampton *et al.*, (1957) had also observed a small unidentified peak emerging from an ion-exchange column just after lysine in the course of their analyses of corresponding fractions of histone prepared from calf kidney, calf liver, and guinea pig testis. On the basis of a comparison of the behavior on ion-exchange chromatography of hydrolysates of the appropriate fractions of calf thymus histone with that of a hydrolysate of flagellin from *Salmonella typhimurium*, which is known to contain ε-*N*-methyl lysine (Ambler and Rees, 1959), it was tentatively suggested that the unknown substance was ε-*N*-methyl lysine (Murray and Luck, 1962). Comparisons of these hydrolysates (or components therefrom) have been extended by means of paper chromatography. The unknown substance has been isolated from a hydrolysate of calf thymus histone and the mass spectrum of its ethyl ester has been obtained. These experiments permitted the identification of the substance as ε-*N*-methyl lysine. This compound has been found in all preparations of histone from mammalian sources which have so far been examined. It was also present in wheat germ histone, but was absent from pea embryo histone, chicken erythrocyte histone, and from the basic ribosomal protein of *Escherischia coli*.

Recently, it was demonstrated that in *Escherischia coli* methionine was the methyl donor for the methylated bases found in soluble RNA (Mandel and Borek, 1961; Fleissner and Borek, 1962), and similar observations were reported on the methylation of purines in ascites cells (Biswas *et al.*, 1961). The possibility that methionine could be a methyl donor (*in vivo*) for ε-*N*-methyl lysine in histones has been investigated and this proved to be the case. While this work was in progress it was also demonstrated that methionine is a methyl donor for ε-*N*-methyl lysine in flagellin (Kerridge, 1963).

EXPERIMENTAL

Preparation of Histone Samples.—Calf thymus histone (preparation B, Rasmussen *et al.*, 1962) was prepared as previously described (Satake *et al.*, 1960). The same procedure was followed in the preparation of histone from rabbit thymus gland. Histone was also prepared from calf thymus and rabbit thymus by extraction of the washed nuclei with 0.5 N sulfuric acid, as described below for extractions from other tissues. From calf kidney, calf liver, calf spleen, lamb liver, rabbit kidney, rabbit liver, rabbit spleen, and rat liver, histone was prepared in the following manner. The tissue (100 g) was sliced, or broken if frozen, and homogenized with a 75% (v/v) solution of propan-1,2-diol in 0.01 M sodium citrate, *p*H 6.8 (400 ml) for 5 minutes at −2° in a Waring Blendor (100 v). After centrifugation at −2° (International refrigerated centrifuge) for 45 minutes at 4000 rpm, the residue was again homogenized with the propandiol-citrate solution (100 ml) and strained through a double thickness of cheesecloth to remove connective tissue before centrifugation as before. The residue was resuspended in propandiol-citrate solution (100 ml) by "flash-blending" and centrifuged for 30 minutes at 2500 rpm. These operations were carried out three times, the temperature being maintained around −2°. The residue was then suspended in 0.14 M sodium chloride solution (35 ml) and centrifuged at 0° for 20 minutes at 2000 rpm. After two further such washes with 0.14 M sodium chloride solution, the white pellet of nuclei was extracted with 0.5 N sulfuric acid (40 ml) by stirring for 15 minutes at 4°. The extract was centrifuged (Servall type SS1 centrifuge) at 2° for 30 minutes at 8000 rpm and the residue was re-extracted in a similar manner with 0.5 N sulfuric acid (40 ml), and the extract was centrifuged as before. The combined supernatants from the two acid extracts were poured into ethanol (200 ml) and the mixture was allowed to stand overnight at −5°. The clear supernatant solution was decanted and the precipitated histone sulfate was harvested by centrifugation, washed three times with ethanol, and dried *in vacuo* (yield, about 0.5 g).

Histone Fractions.—Chromatographic fractions of calf thymus histone used in analyses and starch-gel electrophoresis experiments were those already described (Rasmussen *et al.*, 1962).

Basic Protein of Escherischia coli Ribosomes.—Acetic acid–soluble protein was prepared from *E. coli* ribosomes[1] as described by Waller and Harris (1961).

Amino Acid Analyses.—Histone preparations (2–5 mg) were dissolved in constant-boiling hydrochloric acid (0.2–0.5 ml) and heated in evacuated, sealed tubes for 22 hours at 110°. The hydrolysates were evaporated to dryness *in vacuo* over phosphorus pentoxide and sodium hydroxide and the residues were dissolved in 0.2 M sodium citrate buffer, *p*H 2.2 (5 ml) immediately

* These studies were supported by a grant (C-484) from the National Institutes of Health, United States Public Health Service.

[1] Kindly donated by Dr. W. B. Wood.

prior to analysis. Amino acid analyses were carried out with a Beckman/Spinco automatic amino acid analyzer (Model 120). Analyses for basic amino acids were performed on a 50-cm column at 50° with *p*H 5.28 buffer solution in order to effect a complete separation of the unknown compound from lysine.

Paper Chromatography.—Samples were examined on Whatman No. 1 paper with the two-dimensional system described by Levy and Chung (1953). The organic phase from the mixture butan-1-ol–acetic acid–water (4:1:5) was used for the first development and the paper was dried, moistened with sodium borate buffer (0.06 M, *p*H 9.3), and again dried. The second development was effected with a mixture of *m*-cresol (25 g), phenol (25 g), and borate buffer, *p*H 9.3 (7 ml). The phenol and *m*-cresol were distilled before use. The paper was then dried and stained with ninhydrin–collidine–acetic acid reagent.

Separation of the Unknown Compound from Histone Hydrolysate.—Calf thymus histone,[2] preparation B (Rasmussen *et al.*, 1962), was hydrolyzed by refluxing in 6 N hydrochloric acid (10 g histone in 1 liter of acid) for 24 hours. The hydrolysate was concentrated to 25 ml by distillation and was decolorized by treatment with acid-washed charcoal (2 g) and was filtered. The charcoal was washed twice with water (15 ml) and the combined filtrate and washings were concentrated to about 15 ml. Portions (0.05 ml) of the concentrated hydrolysate were used for calibration of the 150-cm columns of the automatic amino acid analyzer with the *p*H 5.28 buffer solution. The remainder of the hydrolysate was then applied to these columns in portions of 0.75 ml and the eluates from the columns were collected on a fraction collector (5-minute intervals). The ninhydrin reaction was used to locate fractions containing the unknown compound; such fractions were then combined (some lysine was also present because the columns were heavily, and deliberately, overloaded) and the solution was desalted by passage through a column (30 cm × 3 cm diameter) of Dowex 50 (H^+) ion-exchange resin. This was then washed with 0.1 N hydrochloric acid and eluted with 2.5 N hydrochloric acid (Stein and Moore, 1949). Eluate fractions which (after neutralization) were ninhydrin positive were combined and evaporated to dryness *in vacuo* and the residue was dissolved in distilled water (5 ml). A small portion of this solution (5 μl) was used for paper chromatographic analysis and the remainder was acidified to *p*H 2 and again fractionated on a 150-cm column of the automatic amino acid analyzer. This time complete separation of the unknown compound from lysine was achieved and the product was desalted and recovered by the same procedure as was used in the preliminary fractionation. The final product was homogeneous when examined on the amino acid analyzer (50-cm column) and the yield was approximately 11 μmoles (about 2 mg). This calculation was based on the assumption that the leucine color equivalent of the unknown compound in the ninhydrin reaction was the same as that of lysine.

Esterification of the Unknown Compound: Mass Spectrometry.—The ethyl ester of the unknown compound (about 0.2 mg) was prepared by refluxing with dry ethanol saturated with hydrogen chloride, followed by treatment with dry ammonia in methylene chloride (Biemann *et al.*, 1961). The mass spectrum of the ester was obtained with a CEC Model 21-103C mass spectrometer having an all-glass heated inlet system (200°). The ethyl ester of lysine was prepared and examined in the same way.

[2] Kindly prepared by Dr. P. S. Rasmussen.

Experiments Concerned with Labeling (in vivo) of ε-N-Methyl Lysine in Histone.—Three 4-week-old rabbits (New Zealand white; average weight, 337 g) were injected intraperitoneally with C^{14}-(methyl)-labeled methionine (50 μc each animal). Two hours later the animals were killed. Their kidneys, livers, spleens, and thymus glands were immediately removed and weighed. Corresponding organs from the three animals were combined and histone was extracted from these tissues as described above. A portion of the histone from kidney, liver, and thymus was hydrolyzed (the yield from spleen was inadequate for this purpose) as previously described, and the hydrolysates were fractionated on a 50-cm column of Dowex 50 resin (automatic amino acid analyzer) with 0.35 M citrate buffer, *p*H 5.28 (flow rate 30 ml/hr). Eluate fractions were collected at 5-minute intervals and appropriate fractions from each of the three samples were separately freeze-dried. The freeze-dried products were dissolved in distilled water (0.5 ml) and mixed with Werbin's reagent (8 ml) (Werbin *et al.*, 1959), and their radioactivity was measured with a Packard liquid scintillation spectrometer (Series 314 E).

Starch-Gel Electrophoresis.—Starch-gels were prepared as described by Smithies (1955). The composition of the gels was 15.6 g starch per 100 ml 0.03 M sodium acetate buffer solution (4 M in urea; *p*H 4.1) and the same buffer solution (without urea) was used for a bridge electrolyte. Histone fractions were dissolved in the acetate buffer solution (without urea; 0.3–1 mg in 0.05 ml) and after electrophoresis the gels were sliced and stained with Amidoschwartz 10B (apparatus: Murray, 1962). Starch-gels of the same composition were used in preparative separations (with a continuous-elution apparatus; Murray, 1962) of histone fraction III. The histone fraction (10 mg) was dissolved in 0.03 M acetate buffer, *p*H 4.1 (0.25 ml). Appropriate eluate fractions were combined. Solutions so obtained from two experiments were passed through columns (5 cm × 1 cm diameter) of Amberlite IRC 50 resin (250 mesh) which were then washed thoroughly with water. Histone was eluted from the columns with 20% (w/v) guanidinium chloride solution and the relevant fractions were combined, dialyzed overnight, and freeze-dried. The residues (<0.5 mg) were hydrolyzed and amino acid analyses were carried out.

Results and Discussion

Identification of ε-N-Methyl Lysine.—A very small quantity of an unknown compound was observed as a peak which overlapped the descending part of the lysine peak when amino acid analyses of certain chromatographic fractions of calf thymus histone were carried out with an automatic amino acid analyzer (Rasmussen *et al.*, 1962). Complete resolution of this compound from lysine was readily effected when the analysis for basic amino acids was carried out on a 50-cm column of resin with the usual *p*H 5.28 buffer solution (Figure 1). For routine analyses of this type, a shorter column (e.g., 30 cm) would be adequate. Under the conditions of ion-exchange chromatography used, it is known that ornithine behaves identically with lysine (Spackman *et al.*, 1958) and thus, unless the unknown compound was aromatic, it was probably slightly more basic than lysine. Methylation of the α or ε amino group of lysine could well cause a change in the chromatographic behavior of lysine such as that observed. Since ε-*N*-methyl lysine was found in the flagellar protein of *Salmonella typhimurium* (Ambler and Rees, 1959), the behavior on ion-exchange chromatography of this unusual amino acid was examined.

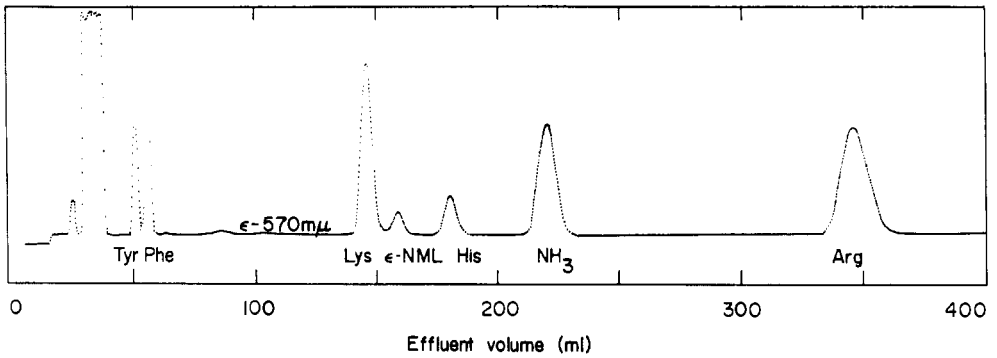

FIG. 1.—Separation of the basic amino acids on a 50-cm column (automatic amino acid analyzer) with 0.35 M citrate buffer, *p*H 5.28, at 50°. Flow rates: buffer solution, 30 ml/hr; ninhydrin solution, 15 ml/hr. The sample was a hydrolysate of calf thymus histone fraction III. ε-NML = ε-*N*-methyl lysine.

The hydrolysate of a sample of flagellin from *Salmonella typhimurium* contained a compound which in its behavior on ion-exchange chromatography with both the *p*H 5.28 buffer solution and the *p*H 4.26 buffer solution (with temperature change from 30° to 50°) was indistinguishable from the unknown component of the histone hydrolysates. When a mixture of flagellin and histone hydrolysates was applied to the column, the component eluted immediately after lysine was observed as a single sharp peak. Since ε-*N*-methyl lysine is the only unusual amino acid in flagellin, it appeared probable that the peak recorded immediately after that due to lysine was attributable to this compound, and thus that the unknown compound observed in the histone hydrolysates was α-*N*-methyl lysine (Murray and Luck, 1962). A clear separation of ε-*N*-methyl lysine from other amino acids in flagellin hydrolysate was demonstrated by two-dimensional paper chromatography (Ambler and Rees, 1959). The very low levels of the unknown compound in histone hydrolysates precluded its identification on paper chromatograms. Ion-exchange chromatographic methods were therefore used for the separation of the compound from a large-scale hydrolysate of unfractionated histone. It was necessary to carry out this separation in two stages; the first stage yielded a mixture of the unknown compound with lysine. When this mixture was examined by paper chromatography, a spot was observed which was identical with that attributed to ε-*N*-methyl lysine in a flagellin hydrolysate (Table I). This evidence supported the identification of the unknown compound as ε-*N*-methyl lysine, and also differentiated it from its isomers, α-*N*-methyl lysine and homolysine (Ambler and Rees, 1959). The second stage of the fractionation of the compound now believed to be ε-*N*-methyl lysine furnished a product which was chromatographically pure (ion-exchange chromatography).

Verification that the unknown compound was in fact ε-*N*-methyl lysine was obtained from the mass spectrum of its ethyl ester.[3] Biemann *et al.* (1961) have shown that the fragments of principal importance from ethyl esters of amino acids are the so-called "amine fragment" and "ester fragment" which arise from cleavage at the appropriate sides of the α carbon atom of the compound. The ester fragment from ε-*N*-methyl lysine ethyl ester would be observed as a peak at 102 mass units, and the amine fragment would be observed as a peak at 115 mass units. It has been shown (Biemann *et al.*, 1961) that the amine fragment of lysine ethyl ester (101 mass units) very readily loses ammonia to yield a very stable cyclised fragment of 84 mass units, which is observed as the most prominent peak of the mass spectrum. This property of the amine fragment from the lysine ester was most valuable in the utilization of mass spectrometry for the elucidation of the structure of lysopine (Biemann *et al.*, 1960). Thus in the case of ε-*N*-methyl lysine ethyl ester further breakage of the amine fragment (115 mass units) would be expected and loss of ammonia would yield a cyclized fragment of 98 mass units, or somewhat more probably due to the inductive effect of the *N*-methyl group, loss of methylamine would furnish a peak at 84 mass units; either or both of these peaks would be expected to be very prominent, and would provide further distinction of the parent compound from homolysine where the formation of such a stable cyclic fragment would be improbable since a seven-membered ring would be involved. The presence of an intense peak at 84 mass units would also distinguish α- or ε-*N*-methyl lysine from the isomeric α, β, γ, δ, and ε C-methyl isomers, each of which would furnish fragments of 115 and 98 mass units. Although the α and ε isomers of *N*-methyl lysine ethyl esters would furnish identical amine fragments, their ester fragments could permit their differentiation, since that from the α isomer would have 116 mass units whereas that from the ε isomer would have 102 mass units. However, the ester fragment in the case of lysine ethyl ester was very small, so that distinction of α and ε isomers of *N*-methyl lysine by this means alone may not be entirely satisfactory.

The mass spectrum obtained of the ethyl ester of the unknown compound is shown in Figure 2, and it is clearly in accord with the structure suggested for this compound. In addition to the molecular weight peak at 188 mass units, peaks due to the amine fragment were found at 115, 98, and 84 mass units (the last being the most prominent), as was predicted. Small peaks were found at 102 and 116 mass units; their intensities as percentage of the intensity of the peak at 84 mass units were 5.4 and 3.6, respectively. After correction for the natural abundance of heavy isotopes[3] in the immediately preceding fragments (i.e., the peaks at 101 and 115 mass units, respectively), the peak at 102 mass units was appreciably greater than the peak at 116 mass units; their corrected intensities as percentage of the intensity of the peak at 84 mass units were 5.0 and 2.2, respectively. The mass spectrum of lysine ethyl ester was also examined and again small peaks were observed at 102 and 116 mass units. The corrected relative intensities of these peaks were virtually the same as in the case of the ethyl ester of the unknown compound. As percentages of the

[3] Readers who are not familiar with applications of mass spectrometry to structural problems may wish to refer to "Mass Spectrometry. Organic Chemical Applications" by K. Biemann, McGraw-Hill Book Co., Inc., New York, 1962, and also to Biemann *et al.* (1963), particularly references 20 and 21 therein.

TABLE I

R_F VALUES OF LYSINE, ε-N-METHYL LYSINE, AND THE UNKNOWN COMPOUND, ON PAPER CHROMATOGRAMS

Developer	Flagellin Hydrolysate		Histone Hydrolysate (partially fractionated)	
	Lysine	ε-N-Methyl Lysine	Lysine	Unknown Compound
Butan-1-ol–acetic acid–water	0.12	0.14	0.11	0.13
m-Cresol–phenol–borate buffer	0.23	0.59	0.25	0.61

intensity of the peak at 84 mass units, the values were 5.3 and 1.7, respectively; a similar value for the peak at 102 mass units was calculated from the spectrum published for this compound by Biemann *et al.* (1961). It is therefore reasonable to assign the peak at 102 mass units in the mass spectrum of the ethyl ester of the unknown compound to the ester fragment of this compound. Thus, in addition to establishing the identity of the unknown component of histone as *N*-methyl lysine, the mass spectrum corroborates the chromatographic evidence that the compound is the ε isomer.

The Occurrence and Abundance of ε-N-Methyl Lysine in Histones.—The chromatographic fractions of calf thymus histone which contain ε-*N*-methyl lysine are IIaa, IIa, III, and IV (Rasmussen *et al.*, 1962) and each fraction has a very low content of ε-*N*-methyl lysine (0.5, 0.3, 0.7, and 0.8 mole % in fractions IIaa, IIa, III, and IV, respectively). It is therefore noteworthy that each fraction is found to be very heterogeneous when examined by zone electrophoresis in starch-gel (Figure 3). That each of the bands observed on the starch-gels represents a distinct protein,

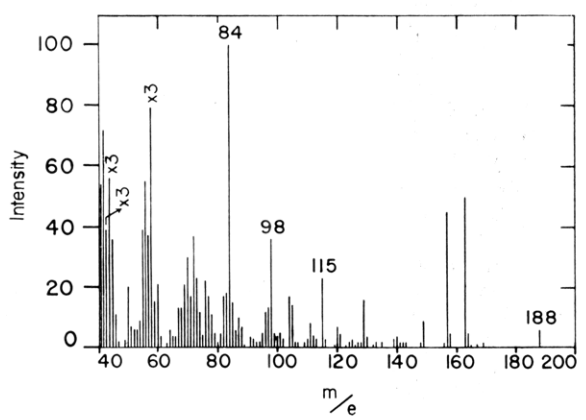

FIG. 2.—Mass spectrum of the ethyl ester of the unknown compound. Intensities are shown as percentage of the intensity of the peak at 84 mass units.

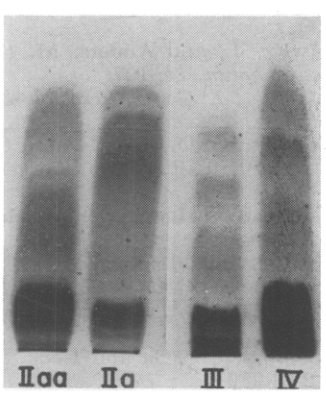

FIG. 3.—The behavior on zone electrophoresis in starch-gel of calf thymus histone fractions IIaa, IIa, III, and IV.

as opposed to aggregates of a given protein, has yet to be established by careful examination of individual bands, but it is unlikely that aggregation alone is responsible for the diversity of bands observed; this is not so in the simpler case of histone fraction IIb (Rasmussen *et al.*, 1962) and heat treatment or exposure to 4 M urea of solutions of histone fractions III and IV prior to electrophoresis had no apparent effect on the bands subsequently observed on the starch-gels. Also, preparations of histone fractions III and IV obtained by ethanol precipitation exhibited the same behavior on zone electrophoresis as did corresponding freeze-dried preparations. Attempts to separate the various components of histone fraction III by preparative starch-gel electrophoresis proved disappointing, for the resolution obtainable with the continuous elution apparatus was not satisfactory with this very complex system, and the difficulties were enhanced by the low capacity of the gel for this very complex mixture of proteins. Only the faster-moving components of histone fraction III were obtained (collectively), and here the yield was very low so that precise amino acid analyses could not be obtained. However, the data showed that the faster-moving components of histone fraction III contained a lower proportion of ε-*N*-methyl lysine than did the original histone fraction III (0.4 mole % compared with 0.7 mole % in the original fraction III). Thus histone fraction III is composite and some of its components differ in their content of ε-*N*-methyl lysine.

Histone preparations from several other sources have been examined for the presence of ε-*N*-methyl lysine. A preparation of histone from rabbit thymus was fractionated by ion-exchange chromatography on Amberlite IRC-50 with the guanidinium chloride solution eluant system and the pattern obtained was the same as that for calf thymus histone (Rasmussen *et al.*, 1962). The amino acid compositions of fractions Ia, IIb, III, and IV were very similar to those of corresponding fractions of calf thymus histone: fractions Ia and IIb contained no ε-*N*-methyl lysine, but fractions III and IV contained a small amount of this amino acid. Unfractionated preparations of histone from the following tissues were also examined: calf kidney, calf liver, calf spleen, rabbit liver, rabbit kidney, rabbit spleen, lamb liver, and rat liver. In each case, a very small quantity of ε-*N*-methyl lysine was found (not more than 0.2 mole % total recovered amino acids). A sample of wheat germ histone[4] contained 0.2 mole % ε-*N*-methyl lysine, but samples of pea embryo histone,[5] chicken erythrocyte histone,[6] and the acetic acid-soluble ribosomal protein of *Escherischia coli* did not contain ε-*N*-methyl lysine. It is very probable that the unidentified compound which Crampton *et al.* (1957) observed in their histone fraction B preparations from calf kidney, calf liver, calf thymus, and guinea pig testis was ε-*N*-methyl lysine,

[4] Kindly provided by Dr. E. Stedman.
[5] Kindly provided by Dr. J. Bonner.
[6] Kindly prepared by Dr. J. M. Neelin.

TABLE II
HISTONE YIELDS AND RADIOACTIVITY OF HYDROLYSATE FRACTIONS

	Kidney	Liver	Spleen	Thymus
Total wt. of tissue (g)	13.0	30.4	0.5	1.2
Yield of histone (mg)	71	175	1.0	10
Yield of histone (% by wt)	0.5	0.6	0.2	0.8
Histone hydrolyzed (mg)	10	22	0	3
Net counts/minute: methionine fraction	534	2436		64
Net counts/minute: lysine fraction	0	0		0
Net counts/minute: ϵ-*N*-methyl lysine fraction	22	102		15
Net counts/minute: histidine fraction	0	0		0

and that an unidentified compound observed in the hydrolysates of several β-histone fractions (E. Stedman, unpublished) also was ϵ-*N*-methyl lysine.

In a preliminary experiment to establish that the ϵ-*N*-methyl lysine found in histone hydrolysates was a true histone component, calf thymus gland was homogenized in 0.14 M sodium chloride and centrifuged. The supernatant fluid was dialyzed exhaustively against distilled water and then hydrolyzed: the hydrolysate did not contain ϵ-*N*-methyl lysine. The residue after extraction of histone from nuclei (at *p*H 0.5) was also hydrolyzed and analyzed. The small amount of ϵ-*N*-methyl lysine found (0.1 mole %) is comparable with that of the extracted histone and therefore may be attributable to the presence of unextracted histone in the residue. There was no formation of ϵ-*N*-methyl lysine when methionine and lysine were heated together under the conditions used for protein hydrolysis.

The Origin of ϵ-N-Methyl Lysine.—The possibility that methionine was a methyl donor for the ϵ-*N*-methyl lysine in histone was investigated *in vivo*. Labeled methionine (C^{14}-methyl) was injected into 4-week-old rabbits and hydrolysates of histone subsequently isolated from the kidneys, liver, and thymus glands of these animals were fractionated by ion-exchange chromatography. This experiment is summarized in Table II. Radioactive ϵ-*N*-methyl lysine was obtained from each histone hydrolysate and therefore methionine was a methyl donor. Whether ϵ-*N*-methyl lysine is incorporated as such into a polypeptide chain, or whether particular lysine residues of the completed polypeptide are methylated subsequently is a question currently under investigation, but an unequivocal answer may prove difficult to obtain. However, in *Salmonella typhimurium*, which appears to be the only other natural source of ϵ-*N*-methyl lysine,[7] there is good reason for believing that lysine residues of flagellin are methylated after incorporation into the protein (Stocker *et al.*, 1961). Studies of the hydroxylation of proline and of lysine in collagen have shown that these processes occurred under conditions where there was no protein synthesis (Manner *et al.*, 1963). Furthermore, in the case of the nucleic acids methylation occurred after polymerization of the nucleotides (Fleissner and Borek, 1962). Should methylation of lysine residues of histones occur after polypeptide synthesis, then the abundance of lysine in all histone fractions, the paucity of ϵ-*N*-methyl lysine in some fractions, and the complete absence of ϵ-*N*-methyl lysine from other fractions would suggest a very high degree of specificity for the methylation process, which would be related, presumably, to the amino acid sequence of the histone fractions involved. Very recently, a somewhat analogous specificity was reported in the case of methylation of nucleic acids (Gold *et al.*, 1963).

Although the very low level of ϵ-*N*-methyl lysine in histones makes a detailed study of the methylation process difficult, it does offer a distinct advantage for certain types of investigation because the use of radioactive methionine permits discriminative labeling of histone fractions. Two applications of this which are being pursued involve studies of tryptic peptides from appropriate histone fractions in the search for tissue or species specificity of histones, and in probing further the heterogeneity of histones.

[7] In a communication which appeared after this paper was submitted for publication, R. J. Martinez has shown that ϵ-*N*-methyl lysine may be present in the flagellae of *Spirillum serpens* (*Biochem. Biophys. Res. Commun.*, 1963, *12*, 180).

ACKNOWLEDGMENTS

The author is greatly indebted to Dr. J. Murray Luck for his interest in this work, to Dr. D. Kerridge for a gift of flagellin, to Dr. E. Stedman for unpublished information, to Drs. J. Wilson and C. Djerassi for mass spectrometry, to Dr. C. Yanofsky for making his liquid scintillation spectrometer available, and to Miss Bente B. Jensen and Mr. J. S. Applegarth for technical assistance.

ADDED IN PROOF

In a recent *in vitro* experiment, radioactive ϵ-*N*-methyl lysine was isolated from histones prepared after incubation of a homogenate of rabbit thymus glands with C^{14}-(methyl)-labeled methionine.

REFERENCES

Ambler, R. C., and Rees, M. W. (1959), *Nature 184*, 56.
Biemann, K., Lionet, C., Asselineau, J., Lederer, E., and Polonsky, J. (1960), *Biochim. Biophys. Acta 40*, 369.
Biemann, K., Seibl, J., and Gapp, F. (1961), *J. Am. Chem. Soc. 83*, 3795.
Biemann, K., Spiteller-Friedmann, M., and Spiteller, G. (1963), *J. Am. Chem. Soc. 85*, 631.
Biswas, B. B., Edmonds, M., and Abrams, R. (1961), *Biochem. Biophys. Res. Commun. 6*, 146.
Crampton, C. F., Stein, W. H., and Moore, S. (1957), *J. Biol. Chem. 225*, 363.
Fleissner, E., and Borek, E. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1199.
Gold, M., Hurwitz, J., and Anders, M. (1963), *Biochem. Biophys. Res. Commun. 11*, 107.
Kerridge, D. (1963), *J. Gen. Microbiol. 31*, Proceedings, xxiv.
Levy, A. L., and Chung, D. (1953), *Anal. Chem. 25*, 396.
Mandel, L. R., and Borek, E. (1961), *Biochem. Biophys. Res. Commun. 6*, 138.
Manner, G., Gould, B. S., Levi, A., and Schantz, A. (1963), *Fed. Proc. 22*, 229.
Murray, K. (1962), *Anal. Biochem. 3*, 415.
Murray, K., and Luck, J. M. (1962), *Fed. Proc. 21*, 410.
Rasmussen, P. S., Murray, K., and Luck, J. M. (1962), *Biochemistry 1*, 79.
Satake, K., Rasmussen, P. S., and Luck, J. M. (1960), *J. Biol. Chem. 235*, 2801.
Smithies, O. (1955), *Biochem. J. 61*, 629.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Stein, W. H., and Moore, S. (1949), *Cold Spring Harbor Symp. 14*, 179.
Stocker, B. A. D., McDonough, M. W., and Ambler, R. C. (1961), *Nature 189*, 556.
Waller, J.-P., and Harris, J. I. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 18.
Werbin, H., Chaikoff, I. L., and Imada, M. R. (1959), *Proc. Soc. Exptl. Biol. Med. 102*, 8.

Synthesis of 9-β-D-Arabinofuranosylguanine*

ELMER J. REIST AND LEON GOODMAN

From Life Sciences Research, Stanford Research Institute, Menlo Park, California

Received July 3, 1963

The synthesis of 9-β-D-arabinofuranosylguanine is described. The fusion reaction of 2,6-dichloropurine and xylofuranose tetraacetate gave, after ammonolysis and acetonide formation, crystalline 6-amino-2-chloro-9-(3′,5′-*O*-isopropylidene-β-D-xylofuranosyl)-9H-purine. Using the conventional xyloside-arabinoside conversion scheme, 6-amino-2-chloro-9-(β-D-arabinofuranosyl)-9H-purine was prepared from 6-amino-2-chloro-9-(3′,5′-*O*-isopropylidene-β-D-xylofuranosyl)-9H-purine. Deamination of 6-amino-2-chloro-9-(β-D-arabinofuranosyl)-9H-purine gave crystalline 2-chloro-6-hydroxy-9-(β-D-arabinofuranosyl)-9H-purine which was ammonolized to give the title compound.

The β-D-arabinosyl derivatives of uracil, thymine, and cytosine, naturally occurring pyrimidine nucleic acid bases, have been synthesized and have shown interesting biological activity. Chu and Fischer (1962) observed that arabinosylcytosine inhibited the conversion of cytidylic acid to 2′-deoxycytidylic acid. Evans *et al.* (1961) reported that arabinosylcytosine was a potent inhibitor of the growth of tumor cells in tissue culture and also caused striking regression of well-established tumors in mice. Pizer and Cohen (1960) observed that arabinosyluracil was not cleaved by enzymes that rupture the base-sugar bond of uridine, although it was phosphorylated to the nucleotide by the enzymes that phosphorylated deoxyuridine.

More recently, arabinofuranosyladenine has been synthesized (Lee *et al.*, 1960; Reist *et al.*, 1962) and its biological activity has been investigated. Hubert-Habert and Cohen (1962) demonstrated that arabinofuranosyladenine inhibits DNA synthesis in a purine-requiring strain of *E. coli*. In addition, arabinofuranosyladenine appeared to have affected protein synthesis as evidenced by the apparent absence of growth. Evidence was presented (Hubert-Habert and Cohen, 1962) which suggested that arabinofuranosyladenine was located primarily in the terminal nucleotide of s-RNA, a position which would be expected to have a profound influence on protein synthesis. Brink and LaPage (1963) reported that arabinofuranosyladenine inhibited the growth of a number of animal tumors and drastically lowered the incorporation of adenine-C^{14}, guanine-C^{14}, orotic acid-C^{14} and glycine-C^{14} into nucleic acid, especially DNA.

With these data in mind, it was of interest to synthesize the β-D-arabinofuranoyl derivatives of guanine and thus to complete the series of the β-D-arabinosyl derivatives of the common nucleic acid bases.

* This work was carried out under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Public Health Service, Contract No. SA-43-ph-1892. The opinions expressed in this paper are those of the authors and are not necessarily those of the Cancer Chemotherapy National Service Center. The authors are indebted to Dr. Peter Lim and staff for the paper chromatograms, optical rotations, ultraviolet spectra, and interpretation of the infrared spectra; and to Mr. O. P. Crews and his staff for the large-scale preparation of intermediates.

Guanine or a blocked derivative has not been successfully condensed directly with a sugar to give a 9-substituted nucleoside. Similarly, an acylated arabinofuranose derivative cannot be coupled directly with a purine or pyrimidine to give a β nucleoside, since the nucleoside condensation inevitably gives, as the predominant product, the anomer in which the steric relationship between the C_2′-hydroxyl and the C_1′-base is *trans* (Baker, 1957). Hence, D-arabinose in such a nucleoside condensation gives the α anomer rather than the desired β anomer (Bristow and Lythgoe, 1949). Thus indirect methods must be used, both to introduce the guanine moiety and to obtain the β linkage of 9-β-D-arabinofuranosylguanine (X).

The synthetic methods for preparing a β-arabinosylnucleoside were developed for the synthesis of arabinosyladenine (Lee *et al.*, 1960; Reist *et al.*, 1962), and were employed in the present work. A number of syntheses of guanosine have been reported (Davoll *et al.*, 1948; Davoll and Lowy, 1951; Davoll, 1958) and a modification of one of these (Davoll *et al.*, 1948) which utilized 2,6-dichloropurine (I) as the guanine precursor was used in the work under discussion. Compound I was coupled with xylofuranose tetraacetate using the elegant nucleoside fusion technique of Shimadate *et al.* (1961) to give the crude β-xylosyl derivative (II). Treatment of compound II with methanolic ammonia simultaneously deblocked the sugar portion and ammonolized the 6-chlorine to give the 2-chloro-6-aminonucleoside (III). This ammonolysis is based on the difference in reaction to nucleophiles between the 2-chlorines of 2,6-dichloropurine as noted by Montgomery and Holum (1957) and Schaeffer and Thomas (1958). Without isolation, compound III was converted with acetone and ethanesulfonic acid to crystalline 6-amino-2-chloro-9-(3′,5′-*O*-isopropylidine-β-D-xylofuranosyl)-9H-purine (IV), isolated in 35% over-all yield from compound I. Hydrogenation of compound IV over 5% palladium-on-charcoal gave crystalline 9-(3′,5′-*O*-isopropylidene-β-D-xylofuranosyl)-adenine which was identical in all respects with the authentic material (Reist *et al.*, 1962). This demonstrated unequivocally that the fusion reaction proceeded in the expected fashion to give a 9-substituted β-nucleoside.

Mesylation of compound IV gave the 2′-*O*-mesylate (V) in 95% yield. Treatment of compound V with