# Protein Methylation

Enzymatic methylation of proteins after translation may take part in control of biological activities of proteins.

Woon Ki Paik and Sangduk Kim

The structure of a protein molecule is primarily determined by the sequence of the constituent amino acids. However, the structures of certain proteins are further modified after genetic translation by alterations in the constituent amino acid residues. Such alterations come about as a result of, for example, hydroxylation, phosphorylation, acetylation, methylation, or thiolation. Hydroxylation of preformed protein occurs almost exclusively in collagen, and reviews have adequately dealt with this subject (*1*). Apart from its role in carbohydrate metabolism (*2*), phosphorylation as well as acetylation and methylation of proteins is very much studied at present partly because of the observation by Huang and Bonner that histone inhibits the DNA-dependent RNA polymerase (*3*), and that some specificity of histone as a gene regulator may be due to the fact that its side chains are often modified by acetyl or methyl groups (*4*). The phosphorylation and acetylation of histone have been reviewed (*5, 6*). During the last several years, studies on methylation of proteins have yielded many important observations. While these studies were going on, it was generally realized that protein methylation is far more complex and has more ramifications than was originally assumed. Various amino acids are methylated, their methylation involves specific enzymes, and the occurrence of methylated amino acids in nature is extremely diverse. Thus the protein methylation process might have far-reaching effects on the control of protein function in vivo. In this article we review the present status of knowledge concerning the methylation of protein and give particular emphasis to the problem of methylation of lysine, arginine, and free carboxyl groups in proteins.

Dr. Paik is senior investigator at the Fels Research Institute and associate professor in the department of biochemistry at Temple University School of Medicine. Dr. Kim is research associate at the Fels Research Institute, Philadelphia, Pennsylvania 19140.

## Natural Occurrence of the Methylated Amino Acids

Ever since ε-*N*-methyllysine ($N^{\epsilon}$-methyllysine) was found in the flagella protein of *Salmonella typhimurium* in 1959, many other methylated amino acids have been found in various natural sources and are summarized in Table 1. $N^{\epsilon}$-Methyllysine has been found in the flagella protein of *Spirillum serpens*, in histones, in myosin, in actin, and in the ribosomal protein of the water fungus *Blastocladiella emersonii* (*7–13*). Furthermore, this amino acid is also present in the free form in human brain, plasma, and urine (*14–16*). ε-*N*-Dimethyllysine ($N^{\epsilon},N^{\epsilon}$-dimethyllysine) was originally found in arginine-rich histone (*17*), and later in various muscle proteins, in flagella protein of *Salmonella typhimurium*, and in the free form in human urine (*8, 13, 16, 18, 19*). ε-*N*-Trimethyllysine ($N^{\epsilon},N^{\epsilon},N^{\epsilon}$-trimethyllysine) is present in actin (*13*), myosin (*19*), histone (*20*), cytochrome c (*21*), and human urine (*16*). Even though 1 mole of cytochrome c of wheat germ contains 2 moles of ε-*N*-trimethyllysine, cytochrome c of yeast and of *Neurospora* contains only one molecule of this amino acid (*21*). On the other hand, cytochrome c of higher animals does not seem to contain this amino acid. Our original finding of ω-*N*-methylarginine ($N^{G}$-methylarginine) in histone (*22*) was followed by its discovery in the acidic protein in the nuclei of rat liver cells (*23*) and recently in encephalitogenic basic protein from human myelin (*24*). This amino acid is also found in the free form in human urine and in brain (*16*). 3-Methylhistidine was first found in human urine; it was later shown to be present in actin, in myosin, and in histone from avian erythrocytes (*25, 26*). Finally, Liss *et al.* (*27*) and we (*28*) identified and purified an enzyme that methylates internal carboxyl groups in protein molecules with *S*-adenosyl-L-methionine as the methyl donor. Since the methylated product, an ester, is unstable to either acid or alkaline hydrolysis, the presence of this carboxymethyl dicarboxylic amino acid would be difficult to detect in nature. In addition to these various methylated amino acids mentioned above, a few rare compounds such as ε-*N*-trimethyl-5-hydroxylysine and δ-*N*-methylornithine have also been detected. However, the information on these compounds is scarce.

The fact that methylated lysine, arginine, histidine, and dicarboxylic amino acids are widely distributed might suggest a unified role of protein methylation in the modification of protein structure, such as the modification of tertiary structure or the inhibition of hydrolysis of protein by proteolytic enzymes. In addition, because proteins such as cytochrome c, myosin, actin, or encephalitogenic basic protein are highly specialized, it is also possible that the protein methylation might have a specific role for each individual protein. These possibilities are discussed below.

## Identification of the Methylated Amino Acids

$N^{\epsilon}$-Methyl-L-lysine and $N^{\epsilon},N^{\epsilon}$-dimethyl-L-lysine are commercially available. These amino acids are also easily synthesized by the method of Benoiton (*29*). $N^{\epsilon},N^{\epsilon},N^{\epsilon}$-Trimethyllysine has been synthesized by DeLange *et al.* (*21*) and by Puskás and Tyihák (*30*). $N^{G}$-Methylarginine has been obtained only in the crude state (*22*), and $N^{G},N^{G}$- and $N^{G},N'^{G}$-dimethylarginine have been synthesized by Kakimoto and Akazawa (*16*). These amino acids have been separated on the amino acid analyzer by the method originally published from our laboratory, and since then quite a few modified methods have become available (*17, 20–22*). The ion-exchange column in the automatic amino acid analyzer was eluted with 0.2*M* citrate buffer at *p*H 5.84 at a flow rate of 30 milliliters per hour. The elution was carried out at 28°C for the

first 6 hours and thereafter at 50°C. The $N^{\epsilon}$-monomethyllysine, $N^{\epsilon},N^{\epsilon}$-dimethyllysine, and $N^{\epsilon},N^{\epsilon},N^{\epsilon}$-trimethyllysine can be separated and identified by paper chromatography with an *m*-cresol–phenol system or an *n*-butanol–phenol system (*31*). $N^{\epsilon}$-Methyllysine derivatives have been reported to be present only in the arginine-rich histone fraction (*32*). However, it has also been reported that all histone fractions are methylated (*22, 33*), but this discrepancy may have been due to incomplete separation of histones.

## Methylation Occurs after the Formation of Peptide Bonds

Allfrey and his co-workers demonstrated earlier that methylation of histone in isolated calf thymus nuclei with [methyl-$^{14}$C]methionine was insensitive to puromycin (*4*). While confirming their results, we further demonstrated that, although $N^{\epsilon}$-methyl-L-lysine actively promoted the exchange reaction between adenosine triphosphate (ATP) and pyrophosphate ($PP_i$), synthetic $N^{\epsilon}$-[methyl-$^{14}$C]methyl-L-lysine was not incorporated into protein (*34*). Furthermore, *S*-adenosyl-L-methionine could replace methionine as the methyl donor (*10, 34*). Various protein methylases have been purified and were found to utilize *S*-adenosyl-L-methionine as the methyl donor (*22, 28, 35*). With the purified methylases, the methylation of the guanidino group of arginine or of the carboxyl groups of dicarboxylic amino acids are insensitive to the presence of 100 micrograms of puromycin per milliliter (*36*). These results demonstrated that the methylation of the side chain of protein occurs after the peptide bonds have been formed. Therefore, the methylation reaction constitutes one of the reactions that cause heterogeneity in protein molecules.

## Properties of Protein Methylases

As shown already in Table 1, various amino acid residues in protein molecule are methylated. Described below are three enzymes involved in protein methylation. In all cases, *S*-adenosyl-L-methionine serves as the methyl donor (see Table 2).

*Protein methylase I.* This enzyme, which we call *S*-adenosyl-L-methionine: protein-arginine methyltransferase (*37*), is localized mainly in the cytosol fraction of calf thymus and methylates the guanidino group of arginine residues in protein, particularly in added histone (*38*). Amino acid analysis of an acid hydrolyzate of enzymatically methylated protein revealed the presence of $N^{G}$-methylarginine. However, recent evidence also indicates the presence of $N^{G},N^{G}$- and $N^{G},N'^{G}$-dimethylarginine (*16, 24*). The optimum *p*H is around 7.0, with the Michaelis constant ($K_m$) being $2.1 \times 10^{-6} M$ for *S*-adenosyl-L-methionine.

*Protein methylase II.* This enzyme, which we call *S*-adenosyl-L-methionine: protein-carboxyl methyltransferase (*37*), has been extensively purified from calf thymus cytosol to homogeneity in polyacrylamide-gel electrophoresis and sedimentation anaylsis in the analytical centrifuge (*28*). The molecular weight of the enzyme is approximately 35,000. The optimum *p*H is around 6.0. The enzymatically incorporated methyl group is lost when the methylated protein undergoes either acid or

Table 1. Natural occurrence of various methylated amino acid derivatives.

| Protein | Source | Authors and year of finding | Reference |
|---|---|---|---|
| | *ε*-N-*Methyllysine* ($N^{\epsilon}$-*methyllysine*) | | |
| Flagella protein | *Salmonella typhimurium* | Ambler and Rees (1959) | (*7*) |
| | *Spirillum serpens* | Glazer *et al.* (1969) | (*8*) |
| Histone | Thymus, wheat germ | Murray (1964) | (*9*) |
| | Ascites carcinoma | Comb *et al.* (1966) | (*10*) |
| | Pea | Fambrough and Bonner (1968) | (*11*) |
| Myosin | Skeletal muscle (rabbit) | Hardy and Perry (1969) | (*12*) |
| | | Huszar and Elzinga (1969) | (*12*) |
| Actin | Amoeba | Weihing and Korn (1970) | (*13*) |
| Ribosomal protein | *Blastocladiella emersonii* | Comb *et al.* (1966) | (*10*) |
| Free amino acid | Brain (bovine) | Matsuoka *et al.* (1969) | (*14*) |
| | Plasma | Perry *et al.* (1968) | (*15*) |
| | Urine (human) | Kakimoto and Akazawa (1970) | (*16*) |
| | *ε*-N-*Dimethyllysine* ($N^{\epsilon},N^{\epsilon}$-*dimethyllysine*) | | |
| Histone | Thymus | Paik and Kim (1967) | (*17*) |
| Myosin | Soleus muscle (cat) | Kuehl and Adelstein (1970) | (*18*) |
| Actin | Amoeba | Weihing and Korn (1970) | (*13*) |
| Flagella protein | *Salmonella typhimurium* | Glazer *et al.* (1969) | (*8*) |
| Myofibrillar protein | Skeletal muscle (rabbit) | Hardy *et al.* (1970) | (*19*) |
| Free amino acid | Urine (human) | Kakimoto and Akazawa (1970) | (*16*) |
| | *ε*-N-*Trimethyllysine* ($N^{\epsilon},N^{\epsilon},N^{\epsilon}$-*trimethyllysine*) | | |
| Histone | Thymus | Hempel *et al.* (1968) | (*20*) |
| Cytochrome c | Wheat germ, *Neurospora* | DeLange *et al.* (1969) | (*21*) |
| | Yeast | DeLange *et al.* (1970) | (*21*) |
| Myosin | Skeletal muscle (rabbit) | Hardy *et al.* (1970) | (*19*) |
| Actin | Amoeba | Weihing and Korn (1970) | (*13*) |
| Free amino acid | Plant | Takemoto *et al.* (1964) | (*62*) |
| | | Larsen (1968) | (*62*) |
| | Urine (human) | Kakimoto and Akazawa (1970) | (*16*) |
| | *ε*-N-*Trimethyl*-L-*5-hydroxylysine* | | |
| Cell wall | Diatom | Nakajima and Volcani (1970) | (*63*) |
| | *ω*-N-*Methylarginine* ($N^{G}$-*methylarginine*) | | |
| Histone | Thymus | Paik and Kim (1970) | (*22*) |
| Acidic protein | Liver nuclei (rat) | Friedman *et al.* (1969) | (*23*) |
| Encephalitogenic basic protein | Myelin (human) | Baldwin and Carnegie (1971) | (*24*) |
| Free amino acid | Urine (human) | Kakimoto and Akazawa (1970) | (*16*) |
| | Brain (human) | Kakimoto and Akazawa (1970) | (*16*) |
| | *ω*-N,N-*Dimethylarginine* ($N^{G},N^{G}$-*dimethylarginine*) | | |
| Encephalitogenic basic protein | Myelin (human) | Baldwin and Carnegie (1971) | (*24*) |
| Free amino acid | Urine (human) | Kakimoto and Akazawa (1970) | (*16*) |
| | *δ*-N-*Methylornithine* | | |
| Free amino acid | Brain (bovine) | Matsuoka *et al.* (1969) | (*14*) |
| | 3-N-*Methylhistidine* | | |
| Myosin | Skeletal muscle (rabbit) | Johnson *et al.* (1967) | (*25*) |
| Actin | Skeletal muscle (rabbit) | Johnson *et al.* (1967) | (*25*) |
| | Muscle (bovine) | Asatoor and Armstrong (1967) | (*25*) |
| Histone | Erythrocyte (avian) | Gershey *et al.* (1969) | (*26*) |
| Free amino acid | Urine (human) | Tallan *et al.* (1954) | (*64*) |
| | *Carboxymethylaspartic or carboxymethylglutamic acid* | | |
| Ovalbumin* | Spleen (calf) | Liss *et al.* (1969) | (*27*) |
| Cytosol protein* | Thymus (calf) | Kim and Paik (1970) | (*28*) |

* These proteins were used as methyl acceptor for protein methylase II (*S*-adenosyl-L-methionine: protein-carboxyl methyltransferase). Carboxymethylated amino acids have not been found in natural sources, but can be detected by radiochemical methods.

Table 2. Properties of various protein methylases.

| Properties | Protein methylase (*37*) | | |
|---|---|---|---|
| | I | II | III |
| Cellular location | Cytosol and nuclei | Cytosol | Nuclei |
| Substrate protein | Histones | Gelatin, ovalbumin, pepsin | Histones |
| Amino acid residue to be methylated | Arginine | Glutamic and aspartic acid | Lysine |
| Methylated amino acid | $N^G$-Methylarginine | Carboxymethylglutamic and carboxymethylaspartic acid | $N^\epsilon$-Methylated lysines ($N^\epsilon$-, $N^\epsilon,N^\epsilon$- or $N^\epsilon,N^\epsilon,N^\epsilon$-) |
| Enzyme purification achieved | 34 | 1,700 | 2 |
| Optimum *p*H | 7.0 | 6.0 | 9.0 |
| Michaelis constant ($K_m$) for *S*-adenosyl-L-methionine | $2.1 \times 10^{-6}M$ | $1.05 \times 10^{-6}M$ | $3.0 \times 10^{-6}M$ |
| Molecular weight | | 35,000 | |
| Isoelectric point (*p*I) | | 4.85 | |

alkaline hydrolysis, and methanol may be identified (*27, 28*). Furthermore, the entire molecule of protein is not required for accepting the methyl group. Protein methylase II recognizes some feature of the amino acid sequence in the proximity of the methyl accepting site. Evidence indicated that only the internal ($\beta$ and $\gamma$) carboxyl groups but not the carboxyl terminal ($\alpha$) carboxyl groups were the sites of enzymatic methylation. The enzyme acts on gelatin, pepsin, ovalbumin, and pancreatic ribonuclease.

*Protein methylase III.* This enzyme, which we call *S*-adenosyl-L-methionine: protein-lysine methyltransferase (*37*), methylates the epsilon $NH_2$ group of lysine residues in added histone; it is found exclusively in the cell nuclei (*4, 34, 39*). Since isolated chromatin methylates its endogenous protein (*10, 40*), it is possible that this enzyme is closely related to nucleoprotein. The enzyme was slightly purified from isolated calf thymus nuclei (*35*). Contrary to the earlier finding (*39*), the partly purified enzyme does not require $Mg^{++}$. The nuclear localization of protein methylase III is not universal since ribosomes isolated from a water fungus *Blastocladiella emersonii* methylate their endogenous protein and yield $N^\epsilon$-methyllysine on hydrolysis of the methylated protein (*10*).

The question arises whether a single enzyme or several enzymes are involved in the formation of mono-, di-, or trimethylated lysine residues. Exclusive localization of the methylated lysine derivatives in protein suggests that more than one enzyme is involved in the methylation of the epsilon amino group of lysine residues; cytochrome c contains only $N^\epsilon,N^\epsilon,N^\epsilon$-trimethyllysine (*21*), whereas flagella protein from *Spirillum serpens* contains only $N^\epsilon$-monomethyllysine, but not $N^\epsilon,N^\epsilon$-dimethyl- or $N^\epsilon,N^\epsilon,N^\epsilon$-trimethyllysine (*21*), whereas flagella protein from *Spirillum serpens* contains only $N^\epsilon$-monomethyllysine, but not $N^\epsilon,N^\epsilon$-dimethyl- or $N^\epsilon,N^\epsilon,N^\epsilon$-trimethyllysine (*8*). Furthermore, a soluble preparation from Krebs 2 ascites tumor cell chromatin methylates the epsilon amino group of lysine residues of added histone. Upon acid hydrolysis $N^\epsilon,N^\epsilon,N^\epsilon$-trimethyllysine was the only methylated product detectable (*41*). The optimum *p*H of the enzymatic reaction is around 9.0, with a $K_m$ value of $3.0 \times 10^{-6}M$ for *S*-adenosyl-L-methionine.

We have developed a system by which these three methyltransferases are assayed simultaneously in the same homogenate (*42, 43*) and applied the method to the study of distribution of these enzymes in various organs of the rat. As seen in Table 3, *S*-adenosyl-L-methionine : protein-arginine methyltransferase is very high in testis, brain, and thymus, in good agreement with the findings that brain contains a large amount of $N^G$-methylarginine (*16, 24, 44*). Testis is the richest source of *S*-adenosyl-L-methionine : protein-carboxyl methyltransferase. Likewise, testis is one of the organs richest in *S*-adenosyl-L-methionine : protein-lysine methyltransferase, even though this enzyme is present in large amount in thymus. The liver is a rather poor source of methyltransferases. Furthermore, as the organ or tissue develops, or the individual becomes older, the activities of different methyltransferases change (*42*). For example, protein methylase I (*S*-adenosyl-L-methionine : protein-arginine methyltransferase) and protein methylase III (*S*-adenosyl-L-methionine : protein-lysine methyltransferase) decreased greatly in the testis during the period of growth at 4 to 8 weeks of age, when body weight increased from 100 to 200 grams. Alternatively, protein methylase II (*S*-adenosyl-L-methionine: protein-carboxyl methyltransferase) increased and then decreased during this same period. Because this age can be considered as puberty in the rat, it is possible that methyltransferase in testis might be closely related to sexual maturation.

There is a paucity of information concerning the metabolism of the methyl group that is already incorporated into protein. In order to answer some questions that arose we performed the following experiment (*45*). A synchronized culture of HeLa cells was doubly labeled for a short time with both [methyl-$^3$H]methionine and [1-$^{14}$C]methionine; both these labeled methionines become incorporated into the peptide backbone, and, in addition, the methyl-$^3$H group is also found on the side chain. Therefore, the ratio of $^3$H to $^{14}$C indicates the extent of methylation. It was found that the ratio of $^3$H to $^{14}$C in arginine-rich histone decreased continuously immediately after the addition of a large amount of unlabeled methionine, an indication of a possible demethylation mechanism. Using Chinese hamster ovary cell culture, Shepherd *et al.* (*46*) observed a rise in the amount of $N^\epsilon$-[methyl-$^{14}$C]methyllysine of arginine-rich histone fraction in S phase of growth. The peak amount was reached after termination of DNA and histone synthesis, and the radioactivity started to decline by the middle of the M phase. These results suggest that protein methylation is a dynamic process and not a terminal reaction. In fact, there is an enzyme, $\epsilon$-alkyllysinase, $\epsilon$-alkyl-L-lysine:oxygen oxidoreductase (to be assigned, E.C. 1.5.3.a), which specifically demethylates free $N^\epsilon$-methyl-L-lysine and $N^\epsilon,N^\epsilon$-dimethyl-L-lysine (*47*), but whether this enzyme demethylates the $N^\epsilon$-methyllysine residues in protein is not yet known.

## Theoretical Consideration of Protein Methylation

In order to consider the possible mechanism of methylation of protein, we must look first at the reactivity of the chemical groupings at the sites of methylation. The $pK_b$ is defined as the negative logarithm of the basic dissociation constant; thus, the stronger the base the lower the $pK_b$ value. When one of the hydrogen atoms of ammonia is substituted with a methyl group the $pK_b$ value decreases from 4.75 to 3.37 (*48*). This decrease is due to electron release by the methyl group, which increases the electron density on nitrogen and, hence, increases its affinity for a proton. The effect of a second methyl substitution is less than that of the first; the $pK_b$ of $[(CH_3)_2NH]$ is 3.22. On the other hand, when a third methyl group is introduced, the $pK_b$ value of $[(CH_3)_3N]$ becomes 4.20. The reason for this increase in the $pK_b$ value is not known. Since amines with carbon chains of varying lengths, like $[CH_3(CH_2)_n\overset{+}{N}H_3]$, have identical $pK_b$ values (*49*), the $pK_b$ value of epsilon $NH_2$ group of lysine residues in proteins may approximate 3.37, corresponding to the $pK_b$ value of $CH_3NH_2$. When the first and second methyl groups are introduced into lysine, the $pK_b$ value of the epsilon $-NH(CH_3)$ and epsilon $-N(CH_3)_2$ may be 3.22 and 4.20, respectively, Substitution with a third methyl group results in the formation of a strongly basic quaternary ammonium ion $[-N(CH_3)_3]^+$. Therefore, the basicity scale will be $-N(CH_3)_2 < -NH_2 < -NH(CH_3) < [-N(CH_3)_3]^+$ in increasing order. From this reasoning, dimethylation of the $-NH_2$ group will have an opposite effect to that of the trimethylated compound. When the interaction between two molecules is ionic, such as that between DNA and histone or between cytochrome c and cytochrome oxidase, it is quite conceivable that change in basicity may play an important role in interaction. However, since the amine may be present as the unstable hydrate $R_3NHOH$ instead of the dissociated form $[R_3N-]^+$, it is not certain how much change in basicity could be brought into the protein molecule by methyl substitution. In the case of methylation of free carboxyl groups (*27, 28*), the methylation neutralizes the charge on the carboxyl group. But in comparison, since the nonpolar methyl group increases the hydrophobicity scale of proteins (*50*), such a change in hydrophobicity might be a major consequence of methyl substitution and result in functional changes. This might be particularly noticeable when methylation occurs on a protein which binds with nonpolar substance, as in the case of enzymatic methylation of arginine residue of encephalitogenic basic protein (*24*): this basic protein binds with nonpolar lipids within myelin. Furthermore, it is also possible that the methyl substitution induces a stereochemical disturbance in the protein molecule, thereby affecting its structure and function. Which mechanism among the alternatives mentioned above predominates might depend on the kinds of amino acids and proteins involved.

Table 3. Specific activities of protein methylases I, II, and III in rat organs.

| Organs | Specific activities* | | |
|---|---|---|---|
| | I | II | III |
| Brain | 1.04 | 1.92 | 0.69 |
| Testis | 1.54 | 4.15 | 1.14 |
| Thymus | 0.96 | 1.04 | 3.21 |
| Spleen | 0.67 | 0.89 | 1.98 |
| Kidney | 0.53 | 0.40 | 0.71 |
| Heart | 0.51 | 1.17 | 0.42 |
| Pancreas | 0.48 | | |
| Liver | 0.46 | 0.29 | 1.07 |
| Muscle | | 1.52 | 0.05 |
| Lung | | 0.80 | 1.21 |

* Expressed as picomoles of *S*-adenosyl-L-[methyl-$^{14}$C]methionine used per minute per milligram of enzyme protein.

## Biological Significance

Although the biochemical significance of protein methylation remains unclear, some suggestive evidence is accumulating. Tidwell *et al.* (*32*) found, in an investigation of the time course of histone methylation during regeneration of the liver in rat, that the rate of methylation of lysine residues of histone reaches a peak at 30 hours, whereas the rate of RNA and DNA synthesis reaches a peak at around 24 hours after partial hepatectomy. Because the loosely packed euchromatin has to condense before mitosis in order to form heterochromatin, the result suggested that methylation of histone is required for this process. We have obtained a similar result with thymidine-synchronized cultures of HeLa cells (*45*). Using double labeling with [methyl-$^3$H]methionine and [1-$^{14}$C]methionine as mentioned earlier, we found that methylation occurs only in histones $F_{2a1}$ and $F_3$ (both are arginine-rich histones), and that little methylation occurs throughout the cell cycle, except during the late S phase through $G_2$ phase, at which time the methylation increased suddenly twofold. The period of peak methylation was about 3 hours after DNA synthesis reached a peak. Therefore, when histone methylation reached the peak, the synthesis of DNA was already decreasing. Furthermore, amino acid analysis of the acid hydrolyzate of the methylated histone showed that the change in the methylation occurred only on the epsilon amino group of the lysine residues, but the methylation of the guanidino group of arginine residues remained unchanged throughout the cell cycle. This change in histone methylation is highly specific, since the methylation of nonhistone residual protein was also unchanged throughout the cell cycle. These results, therefore, suggest that specific methylation of histone might be a factor that causes shutting off the DNA function before mitosis.

Precise location of $N^\epsilon$-methylated lysine residue in the glycine-rich, arginine-rich histone has been worked out independently by two groups (*51*). Desai and Foley compared the structure and the amino acid composition of histone $F_{2a1}$ fraction—also called the glycine-rich, arginine-rich (GAR) fraction—isolated from various tumor and normal tissues (*52*), and found that there were no significant differences among the histones. However, the ratio of $N^\epsilon$,$N^\epsilon$-dimethyllysine to $N^\epsilon$-monomethyllysine in the $F_{2a1}$ isolated from fast-growing tissues such as human lymphoblast, Novikoff hepatoma, fetal thymus, and bovine lymphosarcoma was only half the ratio found in calf thymus histone. This result suggests that some specificity of histone might reside in the modification of the side chains. On the other hand, the ratio of the $N^\epsilon$,$N^\epsilon$-dimethyllysine to the $N^\epsilon$-monomethyllysine did not change during hepatic regeneration (*32*). However, the rate of methyl-$^{14}$C incorporation as $N^\epsilon$,$N^\epsilon$-dimethyllysine and $N^\epsilon$-monomethyllysine changed considerably; the ratio of radioactivity in $N^\epsilon$,$N^\epsilon$-dimethyllysine to that in $N^\epsilon$-monomethyllysine decreased from 0.73 to 0.50 during hepatic regeneration.

The presence of $N^\epsilon$,$N^\epsilon$,$N^\epsilon$-trimethyllysine in cytochrome c was first reported by DeLange *et al.* (*21*). While cytochrome c of wheat germ contains 2 moles of $N^\epsilon$,$N^\epsilon$,$N^\epsilon$-trimethyllysine per

mole of cytochrome c, that of *Neurospora crassa* contains only 1 mole of the amino acid per mole of cytochrome c. Scott and Mitchell demonstrated that there are two molecular species of cytochrome c, namely $C_I$ and $C_{II}$, in the *mi*-1 (poky) mutant of *Neurospora* and that the two cytochrome c's differ in structure at residue 72 (*53*). This position is occupied in $C_{II}$ by lysine (Lys-72) and in $C_I$ by $N^{\epsilon},N^{\epsilon},N^{\epsilon}$-trimethyllysine ($Me_3$lys-72). They noted from experiments with $^{14}C$-labeled lysine that $C_{II}$ was formed first, and that this early form was converted into $C_I$ without dilution of the radioactive label. Since Lys-72 may be involved in the interaction of cytochrome c with cytochrome oxidase (*54*), methylation of $C_{II}$ may be a means for facilitating the binding of the cytochrome c to the mitochondrion.

Various $N^{\epsilon}$-methyllysine derivatives are found in various muscle proteins, as shown in Table 1; furthermore, $N^{\epsilon}$-monomethyllysine is also present in flagella proteins of *Salmonella typhimurium* and *Spirillum serpens*. The fact that various methylated amino acids are found in muscle proteins has a great potential importance, but experimental evidence suggesting causal relationship is completely lacking.

Histone as well as an acidic nuclear protein from rat liver and an encephalitogenic basic protein from human myelin contain $N^{G}$-methylarginine (*22–24*). An alkylating carcinogen, ethionine, ethylates the guanidino group of arginine residues (*23*, *33*). When encephalitogenic basic protein was incubated with *S*-adenosyl-L-methionine: protein-arginine methyltransferase in the presence of *S*-adenosyl-L-methionine, only one of 19 arginine residues in the protein was specifically methylated (*24*). This methylated arginine is at residue 107 (Arg-107) which is close to the main encephalitogenic determinant (*55*), and conceivably the methylation of this arginine residue might aid the transfer of this region of the basic protein into the nonpolar lipid environment within myelin. Furthermore, since the basic protein in aqueous solution appears to lack secondary structure, the above result indicates that *S*-adenosyl-L-methionine : protein arginine methyltransferase recognizes some feature of the amino acid sequence around Arg-107.

Kaye and Sheratzky injected immature rats with 17$\beta$-estradiol and found that *S*-adenosyl-L-methionine : protein methyltransferase activity in liver cytosol decreased to 50 percent of the control value (*56*). On the other hand, adrenalectomy did not change the activity of *S*-adenosyl-L-methionine : protein-arginine methyltransferase in the liver of rat while *S*-adenosyl-L-methionine : protein-carboxyl methyltransferase and *S*-adenosyl-L-methionine : protein-lysine methyltransferase increased approximately 70 percent (*57*). Turner and Hancock observed an increase in protein methylation activity in liver cytosol from fetal liver and hepatoma (*58*). We have also studied various methyltransferases in fast-growing hepatomas and found that only *S*-adenosyl-L-methionine : protein-arginine methyltransferase increased about 50 to 100 percent (*43*).

Methylation of the epsilon amino group of lysine residues in protein might have a physiological significance in preventing the protein from attack by intracellular proteolytic enzymes; reductive methylation of lysyl residues renders the protein resistant to the action of trypsin and decreases the number of trypsin-labile bonds (*59*). Furthermore, the rate of hydrolysis of $N^{\epsilon}$-methyl-L-lysine ethyl ester by trypsin is about 17 times slower than with L-lysine ethyl ester (*60*).

Finally, various methylated amino acids have been found free in brain, plasma, and urine (see Table 1). These amino acids were most likely derived from the hydrolysis of methylated protein in vivo, since methylation of protein occurs subsequent to the formation of peptide bonds (*4*, *34–36*). As was mentioned earlier, an enzyme $\epsilon$-alkyllysinase demethylates $N^{\epsilon}$-methyl-, and $N^{\epsilon},N^{\epsilon}$-dimethyllysine, giving rise to free L-lysine and formaldehyde (*47*). This might be the explanation for the observation that supplementation of a lysine-free diet with $N^{\epsilon}$-methyl-L-lysine supported normal growth of rats (*61*).

## Concluding Remarks

The history of protein methylation is relatively short. Since the finding of $N^{\epsilon}$-methyllysine in flagella protein of *Salmonella typhimurium*, little more than a decade has passed. We now have a much clearer understanding of the occurrence of various methylated amino acids and the enzymes involved. However, intensive biochemical efforts on elucidation of protein methylation may have just begun. Methylated amino acids are generally abundant in histones, muscle protein, cytochrome c, and brain protein. Undoubtedly, closer investigation may reveal these amino acids in other sources. In certain cases, such as methylation of histone, cytochrome c, and encephalitogenic basic protein, some suggestive evidence for function has been obtained. In contrast, not even a feeble clue is at hand for the biological significance of methylation of muscle protein. However, since methylated amino acids are found in such highly specialized and well-characterized proteins as actin, myosin, cytochrome c, encephalitogenic basic protein, and glycine-rich, arginine-rich histone, then research on protein methylation should result in a better understanding of cellular control processes.

### References and Notes


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# Toward the Reduction of Unwanted Pregnancy

An assessment of current public and private programs.

Frederick S. Jaffe

By 1965, "clearly the norm of fertility control [had] become universal in contemporary America" (*1*, p. 394). Yet between 1960 and 1965 at least 19 percent of all births were reported as unwanted by the parents at the time of conception (*2*, p. 1178).

These central and seemingly paradoxical findings of the 1965 National Fertility Study, which is the most recent national study, provide the basic framework in which current U.S. fertility control efforts can be assessed. Family planning has become a prevailing social norm, practiced in some fashion at some time by a greater proportion of Americans than is almost any other social norm. But its full potential for enabling Americans to choose freely whether and when to have children remains to be realized. The reduction of unwanted pregnancy—that is, pregnancies that are unwanted by the parents at the time of conception—provides a tangible objective for national policy, while the normative nature of family planning provides a measure of the potential interest in, and public support for, the programs required to achieve this goal.

At the outset of this analysis a key distinction should be made. The fertility control situation in the United States today is the result of the individual decisions of millions of couples seeking to realize their aspirations for themselves and their children. It is not the result of a conscious national policy. Couples who practice family planning do so because of their personal objectives, not because of family planning's potential social or demographic impact. The government's present family planning program has been designed as a means of helping individuals achieve their own goals, not as part of an official population policy. These millions of voluntary, individual decisions may well add up to a national pattern that significantly affects the future growth of the U.S. population, but they remain, both in origin and rationale, individual—not societal.

Discussions of policy issues often focus almost exclusively on the professed long-range objective, apparently on the assumption that this is the most crucial aspect of public policy. My analysis rests on a different view—one that regards public policy as comprising primarily our laws, regulations, and, most important, the allocation of our resources of funds and time. While the goals that a governmental agency or social system (or subsystem) articulates are not unimportant, a more accurate description of its policies is derived from careful analysis of its budget, which often shows disparities between its professed objectives and its actual priorites.

Throughout nearly all of our history, public policy has made it difficult for health professionals and institutions to dispense, and for couples to practice, family planning. While court decisions,

The author is director, Center for Family Planning Program Development, and vice president, Planned Parenthood-World Population, New York 10022. This article is adapted from a presentation to the U.S. Commission on Population Growth and the American Future, New Orleans, 21 January 1971.