

THE ROLE OF METHYL GROUPS
IN DIFFERENTIATION OF THE
POST-NODAL PIECES OF CHICK BLASTODERMS

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by

Michael L. Smith

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ABBREVIATIONS

A	adenosine
BrdU	5-bromodeoxyuridine
C	cytosine
cpm	counts per minute
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
G	guanosine
HCys	DL-homocysteine
hnRNA	heterogeneous nuclear ribonucleic acid
Met	L-methionine
mRNA	messenger ribonucleic acid
PNP	post-nodal piece
POPOP	1, 4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene
PPO	2, 5-diphenyloxazole
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
sRNA	soluble ribonucleic acid
T	thymine
tRNA	transfer ribonucleic acid
U	uracil, uridine

INTRODUCTION

To date, most of the experimental work concerning mechanisms for control of differentiation has been based on a number of conceptual models (see REVIEW OF LITERATURE). A common problem in these models is the need for some sort of recognition site on the nucleic acids. Since several methylated bases have been found in both RNA and DNA, the methyl group has been proposed as a potential marker for the recognition site.

Much of the evidence which has accumulated recently shows that methylation occurs to a greater extent in differentiating tissues than in adult tissues. Thus one would expect some correlation between the functions of methyl groups in genomic regulation and in initiation of differentiation. Surprisingly, very little has been reported concerning the specific missing link between nucleic acid methylation and methyl-directed differentiation.

If methylation of nucleic acids provides the missing link, why has so little work been done to demonstrate the role of methyl groups in differentiation? Perhaps one problem is that most studies of nucleic acid methylation have been conducted using tissue extracts, while studies of morphological differentiation necessarily involve intact living cells.

An experimental approach which could provide insight into this problem would be to show that methyl groups induce differentiation and, at the same time, exert at least a significant part of their influence on the nucleic acids. The addition of exogenous methyl donors to undifferentiated tissue would thus be an approach for studying methyl-induced

differentiation.

L-methionine (Met) and its derivative, S-adenosyl-L-methionine (SAM), are active biological methyl donors (Lehninger, 1975; Cantoni, 1975, 1977). Evidence indicates that Met and SAM are actively taken up by cells, from their immediate microenvironment, and incorporated into normal physiological processes. Therefore, these methyl donors could be applied to a test system of undifferentiated living tissue and observed for their ability to initiate differentiation.

The post-nodal piece of the chick embryo is an ideal model for a study of differentiation because it remains viable, yet does not differentiate, under in vitro conditions (Waddington, 1935; Butros, 1963a, 1963b).

Hensen's node, the "organizer center" of the chick embryo (Spratt, 1955), is comparable to the dorsal lip of the amphibian blastopore during early embryonic development. It is this region which influences morphogenetic movements and stimulates differentiation of the various axial "centers," such as: notochord, somites, neural centers (Spratt, 1955; Spratt and Haas, 1960a, 1960b; Shoger, 1960). When the inductive influence of Hensen's node is effectively removed by a transverse cut, the portion of the embryo posterior to this cut, the post-nodal piece, fails to differentiate into recognizable tissues (Spratt, 1952, 1955; Butros, 1967).

Numerous investigators have demonstrated that the transverse cut may be made at various distances, ranging from 0.4 to 0.8 mm, posterior to the node in order to remove all inductive and morphogenetic influence

leading to formation of axial structures (Waddington, 1935; Waheed and Mulherkar, 1967; Butros, 1960, 1962, 1965, 1967; Chauhan and Rao, 1970; Lee and Kalmus, 1975).

The initiation of differentiation in post-nodal pieces of chick embryos by the addition of methyl donating compounds would thus provide evidence for the proposed methyl-mediated control mechanisms. If the methyl groups could then be shown to become localized in the nucleus, specifically in the nucleic acids, the apparent controversy (see REVIEW OF LITERATURE) could perhaps be resolved.

In light of this information, the objectives of the present study were: (1) to investigate the potential inducing capacity of methyl groups in morphological differentiation of the undifferentiated post-nodal pieces of chick embryos, using the biological methyl donors L-methionine and S-adenosyl-L-methionine; (2) to demonstrate that the differentiation was initiated by the methyl groups rather than any other active portion of the donor molecules, using DL-homocysteine and S-adenosyl-L-homocysteine, which are the respective demethylated analogues of the methyl donors; and (3) to localize the site(s) of action of these methyl groups, using radiotracer studies.

REVIEW OF LITERATURE

Differentiation is a process by which cells become functionally and morphologically specialized entities within a living system. The cells begin with equal genetic potential but are somehow directed, during their embryonic development, into separate cell lineages. Determining how and why this specialization occurs is a central issue in developmental biology (Spratt, 1964).

When isolated embryonic tissue develops into the same differentiated tissue in vitro as it would in vivo, it is said to be "determined." Thus all the factors required for its complete development are intrinsic to the tissue. In a process called "embryonic induction" a tissue can be channeled toward a state of determination by interaction with another tissue (Tiedemann, 1966). This induction, or induced differentiation, has been demonstrated experimentally. Spemann and Mangold (1924) found that, if they explanted the dorsal lip of the blastopore of an amphibian embryo to another region of that embryo, a secondary embryo was induced. They concluded from a series of experiments that the dorsal lip of the blastopore was the "primary organizer" responsible for induction in amphibian embryos. Subsequent studies (see Hamburgh, 1971, for a review) showed that a chemical, or chemicals, contained within the tissues, could also induce differentiation. In fact, several investigators showed that the organizer tissue could be killed by a variety of physical and chemical treatments and still induce differentiation (see Hamburgh, 1971; Balinsky, 1975, for reviews). As a result of this evidence, subsequent research was directed toward identification of chemical inducing substances in tissue

extracts (Brachet, 1960). Tiedemann (1966) extracted a small nucleoprotein from 9- to 11-day old chick embryos which could induce competent amphibian gastrula ectoderm to differentiate into neural tissue. He also extracted a protein component which could induce similar gastrula ectoderm to differentiate into mesodermally-derived structures.

Still other investigators used a "conditioned medium" to test for induction (Niu and Twitty, 1953). It was proposed that, since tissue extracts contained inducing substances, the living tissues may release the same substances in vivo. To test this hypothesis, pieces of "inducer" tissue were grown in hanging drop culture for several days to "condition" the medium. The tissues were then removed and replaced with competent gastrula ectoderm. The ectoderm was shown to differentiate into mesodermally-derived structures, as a result of induction by the "conditioned" medium.

Since the tissue to be induced (ectoderm) received its stimulus from its new environment ("conditioned" medium), the next logical step was to simply maintain undifferentiated tissues in culture and use various chemicals to induce differentiation (Brachet, 1960). A number of chemicals or chemical reactive groups have been implicated as potential inducers of differentiation and morphogenesis (Berrill and Karp, 1976). Among these are: cyclic AMP (Rutter et al., 1973; McMahon, 1974), hormones (O'Malley and McGuire, 1966; Lawrence and Burden, 1973; Rutter, et al., 1973; O'Malley and Means, 1976), inorganic ions (McMahon, 1974), sulfhydryl-containing compounds (Waheed and Mulherkar, 1967; Chauhan and Rao, 1970; Lee and Kalmus, 1975), and RNA (Lee and Niu, 1973; Deshpande and Siddiqui, 1977). All of these studies are concerned with the non-

specific inductive potential of essential metabolites (McMahon, 1974), except the induction of oviduct by estrogens, which probably involves specific hormone-receptor interactions (O'Malley and McGuire, 1966).

How did these assorted metabolites effect changes so consistently? Is differentiation simply associated with normal physiological cycles as suggested by McMahon (1974), or are normal cycles related intimately to the regulation of genetic potential?

In attempts to explain these various inducing substances, several investigators have proposed models of gene regulation which would account for varied metabolic or extracellular stimuli (Britten and Davidson, 1969; Georgiev, 1969; Tsanev and Sendov, 1971; Davidson and Britten, 1973). These models are based on the assumptions that all significant developmental potential resides in the genetic code of DNA, and that the transfer of cellular information is unidirectional, i.e. from DNA to messenger RNA to protein. These assumptions are accepted as valid, although evidence has been presented which implies that DNA may not be the ultimate source of information in some animals (Deuchar, 1973).

The common themes in all of the models mentioned above are the fundamental concepts which led Jacob and Monod (1961) to hypothesize their operon model for regulation of the prokaryote genome. Although the eukaryotic cell has a much more complex organization, there are several overlapping features. First, DNA contains alternating segments of regulatory and structural regions in prokaryotes (Jacob and Monod, 1961; Davidson, 1976), which correspond to repetitive and non-repetitive nucleotide sequences in eukaryotes (Timofeeva et al., 1975; Ayres, 1977), respectively. Second, regulation involves interaction between the

structural region and a product of the regulatory region, which could be an RNA transcript (Britten and Davidson, 1969) or a translated protein (Jacob and Monod, 1961; Georgiev, 1969; Tsanev and Sendov, 1971; Davidson and Britten, 1973). Finally, the interaction involves activation of the sequences, all of which were inactive before the interaction began (Monod et al., 1963; Davidson, 1976).

At this point, the eukaryote theories of gene regulation diverge. The interaction between DNA and the surrounding blanket of basic histone proteins is unique to eukaryotes (Kornberg, 1974). These histones form an integral part of the theories mentioned above because they maintain the DNA in a repressed state until activated (see Davidson, 1976, for a review). Perhaps the histones themselves, since there are several different types (Kornberg, 1974), play an important part in regulation of the genome.

Several studies have shown that histones may, indeed, exercise significant control over morphogenesis (Kischer et al., 1966; Sherbet, 1966; Malpoix and Emelinckx, 1967; Jelinek et al., 1975). Other studies indicated that histones do not exert specific control, but rather bind in a non-specific manner to the DNA, thus preventing transcription of RNA (Sherbet, 1966). The effect appears to be much like that of actinomycin D, which inhibits DNA-dependent RNA synthesis (Sherbet, 1966; Malpoix and Emelinckx, 1967; Jelinek et al., 1975). However, unlike actinomycin D, exogenous histones produce teratogenic effects which are much more intense in older chick embryos than in younger embryos (Jelinek et al., 1975).

Because histones exert such non-specific effects on the genome

(Sherbet, 1966), and because they are so uniform across phylogenetic lines (Paul et al., 1973), histones probably do not play a major role in specific control of gene expression. Instead, their effect is probably a very generalized blanket repression. However, another group of proteins intimately associated with chromatin, the acidic non-histone chromosomal proteins, are not as uniform across phylogenetic lines, and may act as primers for specific alteration of histones so that transcription may occur (Paul et al., 1973). Apparently, the non-histones combine with either the histones or DNA to effect conformational alteration of the chromatin structure (Stein et al., 1974). This conformational change would probably involve either loosening of the binding histones or causing the DNA helix to unwind, so that DNA-dependent RNA polymerases or additional regulatory molecules could approach the DNA (Paul et al., 1973). The interactions involving non-histones could be much more specific than control by histones alone, because they are a more heterogeneous group (Stein et al., 1974; Stein et al., 1976). Also, the non-histones have been shown to include several enzymes related directly to maintenance and function of the DNA. These include DNA polymerase, polynucleotide lyase, DNase, phosphokinase, and acetyl- and methyl-transferases (Umanskii, 1975; Biswas et al., 1975). In fact, Allfrey et al., (1964) suggested that perhaps the modification of histones by these enzymatic activities (e.g., methylation) exerted sufficient control over transcription.

The question which remains is how the facts presented about chromosomal proteins can be shown to corroborate the theories of gene regulation. As mentioned above, the underlying concepts involve alternating sequences of genes, which are controlled in each cistron by transcription

products of the interspersed regulatory genes. Control of the genome is exercised by sequential and differential activation of the structural genes. The histones form an insulating blanket around the DNA until some effector molecule stimulates a specific recognition site. Presumably, the effector molecule actually belongs to a class of inducing substances, as previously described. The recognition site would be a protein group within the chromatin. Following effector entry, an active diffusible RNA segment would be transcribed which could attach to a complementary sequence (one or more of the so-called repetitive sequences) on any cistron served by that activator RNA. The attachment of activator RNA would then initiate transcription of the adjacent structural gene by allowing a polymerase or initiation factor to penetrate the histones and approach the DNA at the point of DNA-RNA complementation. The model allows for polycistronic control by either repetitive activator RNA sequences, or multiple sites for DNA-activator RNA complementation (Britten and Davidson, 1969). The functions of chromosomal proteins would be (1) to contain DNA structural gene sequences in an inactive state until they are activated, and (2) to respond to external effector molecules. The containment would probably be restricted to histones, while the differential response to effector molecules would primarily involve non-histones. In support of this approach, Wang et al. (1976) showed that non-histones from Ehrlich ascites tumor cells bind to and stimulate transcription of homologous non-repetitive DNA sequences. The non-histones also inhibit transcription of repetitive sequences, which is consistent with the Britten-Davidson model because the repetitive sequences should not be transcribed, but should serve as recognition sites

for activator attachment to DNA. A problem with this supporting evidence is that the non-histones themselves would be the activator rather than RNA. However, Davidson and Britten (1973) had recognized this potential controversy and recommended that the activator RNA could be translated into an activator protein.

At the same time that the Britten-Davidson model appeared in the literature, Georgiev (1969) proposed a similar, but independently formulated operon concept for eukaryotic gene regulation. This theory contained virtually all of the elements of the lac operon theory (Jacob and Monod, 1961), but on a scale consistent with the complexity of the eukaryotic genome. The major difference between this model and that of Britten and Davidson (1969) was that both the structural region and its associated activator complementation region would be transcribed into one (for each cistron) large heterogeneous RNA molecule. The heterogeneous RNA would be processed by ribonuclease digestion of the activator gene transcript, leaving a shorter functional RNA molecule which could be translated into enzymes or structural proteins. Presumably, the nuclease digestion would begin at the 5' end since that is the activator (repetitive sequence) region. The rationale behind the position of the activator is that, in order to allow polymerases to begin transcription, the activator would necessarily be found at the end of the structural sequence at which transcription begins (Lehninger, 1975).

The suggestion that a large RNA transcript was being produced and then enzymatically cleaved to an active form led to much experimentation (see Revel and Groner, 1978, for a review) and reevaluation of earlier concepts of messenger RNA (mRNA) production (Georgiev, 1972; Davidson

et al., 1977; Revel and Groner, 1978). This heterogeneous nuclear RNA (hnRNA) was shown to be composed of both repetitive and non-repetitive segments (see Davidson et al., 1977, for a review). The implications of such a structure being selectively degraded led to some controversy. As in his earlier report, Georgiev (1972) maintained that functional mRNA was produced by nuclease processing of the hnRNA. Davidson et al. (1977) contended that hnRNA was a regulatory transcript, not a mRNA precursor, producing either activator RNA molecules from the repetitive sequences or activator proteins from other transcripts within the hnRNA. In an extensive review, Revel and Groner (1978) support the idea of hnRNA producing mRNA via specific nuclease activity. Much of their evidence is based on the findings that mRNA segments are uniquely "capped" by methylated nucleotides.

At this point, a brief review of the literature concerning the existence of methylated bases and their significance is in order.

Transfer RNA has been found to be methylated in both prokaryotes and eukaryotes (Borek, 1963; Söll, 1971). Until recently, 5-methyl cytosine was thought to be the only modified base in the tRNA of mammals (Hall, 1971). In an extensive review, Agris and Söll (1977) have shown that a large number of methylated bases are found in tRNA's of several groups of prokaryotes and eukaryotes, including mammals. The degree of methylation appears to be directly related to the evolutionary advancement of the organisms studied (Söll, 1971). For example, E.coli phenylalanyl transfer RNA (tRNA^{phe}) contains only 2 methylated bases per molecule; yeast tRNA^{phe} contains 9 methylated nucleotides per molecule; and mammalian tRNA is methylated in at least 25% of the molecules (Hall,

1971). This suggests that tRNA methylase activity is species-specific (Tsutsui et al., 1966; Sueoka and Kano-Sueoka, 1970; Hall, 1971; Mays et al., 1973). Methylation of tRNA occurs at the poly-nucleotide level (Turkington, 1969; Sueoka and Kano-Sueoka, 1970; Hall, 1971). Söll (1971) considers methylation of tRNA a part of its maturation process.

Transfer RNA is not the only methylated nucleic acid. Hall (1971) stated that mRNA is methylated, but not extensively, because such modification could affect the message. Messenger RNA methylation has since been documented (see Rottman et al., 1976, for a review). Soluble RNA (sRNA) fractions have also been shown to be methylated (Mittelman et al., 1967; Simon et al., 1967; Leboy, 1970).

The significance of RNA methylation is not yet completely known, but much evidence has accumulated through both in vivo and in vitro studies of methylase activities, and corresponding effects in tissues (Borek, 1963; Agris and Söll, 1977; Revel and Groner, 1978). One of the most important advances in the study of methylation has been the isolation of non-methylated tRNA from the methyl-deficient auxotroph, E.coli K-12 W6 (Mandel and Borek, 1963; Baguley and Staehelin, 1968).

In a study of methyl deficient tRNA and rat liver methylases, Baguley and Staehelin (1968) showed that there were two sequences which were methylated readily by a guanine N-methyltransferase and an adenine 1-methyltransferase. The degree of methylation showed that accepting sequences for methyl groups occur at a frequency of 0.4 to 0.5 sites per tRNA molecule. This suggests that tRNA methylases may recognize specific base sequences and not whole tRNA's. Simon et al. (1967) have shown that similar activity for methylation of heterologous sRNA exists in

fetal rat and kitten brain homogenates. The enzyme activity reached a peak at birth.

In other attempts to determine functions of methylated RNAs, investigators have used various agents to stimulate or inhibit methylase activities (Leboy, 1970; Mays and Borek, 1971; Kerr, 1977; Agris and Söll, 1977). Kaye and Leboy (1968) questioned the validity of in vitro tests of methylase activity by demonstrating in both adult and newborn mouse organs, as well as mouse tumors, that the rate of RNA methylation could be increased 4- to 8-fold, and the extent of methylation could be increased 2- to 4-fold by the addition of 0.36 M ammonium acetate to the test system. The additional ions enhanced methylation of all major nucleotides, from a 2-fold increase for guanine to a 9-fold increase for cytosine. Neither the rate, extent, or pattern of methylation increased substantially more in any one tissue than in the others. In spite of the potential controversy, Leboy (1970) demonstrated significant stimulation of rat liver sRNA methylase activity by the addition of the polyamines spermine, spermidine, and putrescine. Kerr (1977) reviewed several known methylase stimulators and inhibitors. She demonstrated that a number of cations could stimulate methylase activity, including spermine, Mg^{+2} , Ca^{+2} , NH_4^+ , Li^+ , and Na^+ (Kerr, 1977). The magnesium was found to stimulate activity only slightly and, when combined with spermine, it was shown to have an antagonistic effect (Leboy, 1970; Kerr, 1977). In fact, magnesium combined with ethylenediamine tetraacetate (EDTA) exhibited marked inhibition (Leboy, 1970) of methylation.

Since stimulatory effects seemed to reflect mainly alterations of physiological conditions, no broad conclusions were reached. Inhibitor

studies, on the other hand, revealed some interesting facts. Kerr (1970) found a naturally-occurring non-dialyzable protein which inhibited RNA methylase activities in adult rat tissues, but was absent from fetal organs. Sharma et al. (1971) supported these findings and suggested that the inhibitor was either absent or suppressed in tumors and differentiating tissues, indicating that methylase activity was higher in those tissues. Nicotinamide can inhibit guanine-2-N-methyltransferase when combined with this non-dialyzable protein (Murai et al., 1972). Other inhibitors include estrogens (Sharma and Borek, 1970; Mays and Borek, 1971), acridine dyes, cytokinins, and demethylated analogues of an important biological methyl donor, S-adenosyl-L-methionine (Kerr, 1977). The effect caused by the demethylated analogue of S-adenosyl-L-methionine (SAM) appears to be competitive inhibition. Once the methyl group is freed from its donor, the donor site is available to receive another methyl group. Glycine N-methyltransferase is another competitive inhibitor, but its action is against a tRNA methylase (Kerr, 1972; Mays et al., 1973). An interesting point to note here is that S-adenosyl-L-homocysteine (demethylated SAM) competes for the methyl group of RNA methylases but not glycine N-methyltransferase (Kerr, 1972). The competitive activity of glycine N-methyltransferase increases with age (Mays et al., 1973).

The interest in RNA methylation is based on several studies which linked methylation and carcinogenesis. Alkylation of nucleic acids, in general, has been considered carcinogenic for quite some time (Datta and Datta, 1969). In fact, several known carcinogenic alkylating agents, such as methyl methanesulfonate (Whittle, 1969), dimethylsulfoxide (Ross

et al., 1972), and N-methyl nitrosourea (Rajalakshmi et al., 1978), have been shown to aberrantly methylate several nucleic acids. Craddock (1969) used methionine methyl-¹⁴C to show that nucleic acid methylation increases significantly in the presence of known carcinogens such as aflatoxin, ethionine, and dimethylnitrosamine. Crude extracts of about 30 tumors have exhibited abnormally high RNA methylase activity compared with normal tissues (Tsutsui et al., 1966; Borek, 1971). Stewart and Corrance (1969) found higher RNA methylase activity in malignant, newborn, and regenerating rat tissues than in normal tissues, suggesting some relationship between enzyme activities of cancerous and differentiating tissues. They showed that tumor extracts could even hypermethylate rat liver tRNA which had already been exposed to normal methylase activity of adult liver homogenates. Other tumor extracts which have been found to hypermethylate RNA include SV-40-induced hamster tumors (Mittelman et al., 1967), and chick liver tumors induced by Marek's disease virus (Mandel et al., 1969).

The functions of these methylated bases in RNA are not known, but evidence points toward some rather interesting conclusions. Addition of methyl groups changes hydrogen-bonding capacity and base-stacking patterns, thus providing for potential alterations of the structures of RNA species, perhaps leading to altered codon specificity (Hall, 1971). Also, chromatographic and coding properties of tRNA are changed after in vitro methylation, suggesting that methylated bases provide additional restraints or variability for codon recognition (Sueoka and Kano-Sueoka, 1970). Whether altered tRNA methylase activity is a manifestation of altered transcriptional control or a mediator of that control (Turkington,

1971) will require further investigation.

As mentioned above, methylation of nucleic acids, in general, has been implicated in carcinogenesis (Datta and Datta, 1969). In a study of Novikoff hepatomas, Sneider and Potter (1969) presented evidence linking methylation of DNA to cancer. Subsequent studies have shown that methylation induced by alkylating agents such as dimethylnitrosamine (Margison et al., 1976) and N-methyl-N-nitrosourea (Rajalakshmi et al., 1978) involve primarily the DNA of the induced tumors.

Normal tissues as well, exhibit substantial methylation, but, in animals, the methyl group is restricted to the 5-position of cytosine (Vanyushin et al., 1970; Scarano et al., 1977). 5-methylcytosine has been found in both nuclear and mitochondrial DNA (Nass, 1973). Grippo et al. (1968) reported that 60% of total 5-methylcytosine was found in monopyrimidine isostichs in sea urchin DNA. A total of 90% was found in the dinucleotide C_pG , which is a cytosine attached to the 5' end of guanosine. The highest probability for methylation of the 5-position of cytosine occurs in this dinucleotide, with less in the dinucleotide C_pT (T, thymine), and hardly any in the dinucleotides C_pC or C_pA (A, adenine) (Daskocil and Sorm, 1962). Methylation of DNA occurs at the polymer level, as in RNA, and appears to occur immediately after DNA replication (Grippo et al., 1968; Geraci et al., 1974). Recent evidence has been presented which suggests that this polymer level methylation occurs primarily in the sequence 5'-CCGG (C, cytosine; G, guanine), in which at least the second cytosine is methylated (Singer et al., 1979). In another recent report additional modified bases were found in salmon sperm DNA, including N-6-methyladenine, 2-methyladenine, and 1-methyl-

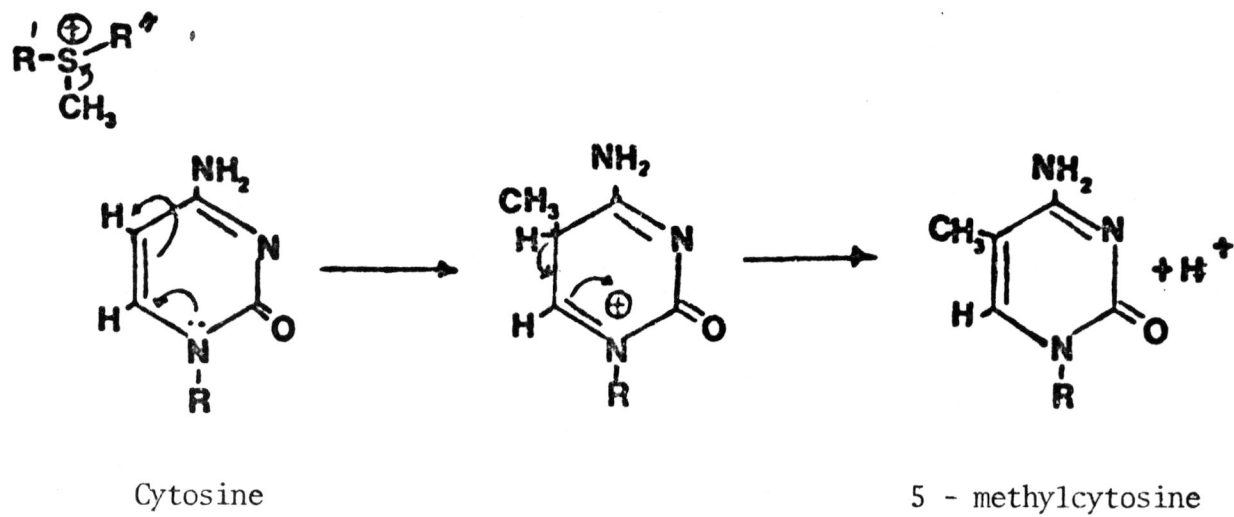
adenine (Schoen and Cooks, 1979).

The occurrence of these methylated bases in both DNA and RNA leads to the question of how the bases were methylated. Several investigators have shown that methyl groups of methionine are transferred as a unit, from the methyl donor to the acceptor molecule, rather than through the one-carbon pool and betaine (DuVigneaud *et al.*, 1956; Mandel and Borek, 1963; Tropp *et al.*, 1964; Kerr and Borek, 1973; Cantoni, 1975). This transfer involves primarily a nucleophilic substitution reaction (Figure 1) with SAM as the methyl donor (Tropp *et al.*, 1964; Borek and Srinivasan, 1965). The reaction is reported to involve some degree of coupling, so it must occur by a classical S_N2 mechanism. The transition state could be closer to the product (Gandour and Schowen, 1978) than to the anionic or cationic intermediates.

The most efficient inducer is not the known biological methyl donor, SAM, but methionine (Met). This is substantiated by Pezzoli *et al.* (1978) who reported that 70% of SAM taken up from the medium by cultured rat hepatocytes is almost immediately decarboxylated. Decarboxylated SAM is a precursor of polyamine and catecholamine biosynthesis and is not used for transfer of methyl groups (Salvatore *et al.*, 1977). On the other hand Met is adenosylated prior to use by ATP: methionine adenosyltransferase (Cantoni, 1977), thus one would expect Met to carry out more methylations than SAM in this system.

Since sulfhydryl groups have been shown to initiate differentiation (Mulherkar *et al.*, 1965; Waheed and Mulherkar, 1967; Chauhan and Rao, 1970; Lee and Kalmus, 1975), the importance of sulfur in transmethylation reactions can not be neglected. A mechanism of action for sulfur in

Figure 1.
Methylation of Cytosine



methylation reactions was proposed by Kalman (1971). He suggested a nucleophilic addition reaction between a free sulfhydryl group of the enzyme-substrate complex and a double bond of the acceptor molecule, forming a thioether intermediate. No direct evidence has been reported in favor of this mechanism, but several studies indicate that the sulfhydryl moiety is essential in SAM-mediated transmethylation reactions (Borek and Srinivasan, 1966; Kalman, 1971; Gandour and Schowen, 1978). Therefore, sulfur may function primarily as a high-energy donor/catalyst after a methyl group replaces the sulfhydryl hydrogen.

Besides their function as sulfhydryl-terminal analogues, homocysteine (HCys) and S-adenosylhomocysteine (SAH) are competitive inhibitors of methyl transfer reactions (Kerr, 1972; Salvatore *et al.*, 1977, for a review). Therefore, if methyl groups can induce differentiation, these demethylated analogues should prevent any such differentiation. Several investigators have presented evidence which supports the inhibition of SAM-mediated methylation by SAH and its analogues (Chang and Coward, 1975; Walker and Duerre, 1975; Borchardt, 1977; Michelot *et al.*, 1977; Pezzoli *et al.*, 1978). Chang and Coward (1975) showed that SAH inhibited methylation of tRNA in rat lymphocytes. Walker and Duerre (1975) reported that SAH inhibits most, if not all, transmethylation reactions involving SAM.

Once the methyl group is transferred to the nucleic acid base, it must either (1) introduce conformational alterations of the bases, (2) alter recognition and binding properties, or (3) induce base substitution. Conformational stability has been shown to be conferred on tRNA by methylation, as exhibited by significant decrease in absorbance of methylated

tRNA compared with methyl-deficient tRNA (Borek and Srinivasan, 1966). Also, the melting temperature of polyuridylic acid is much lower than that of polyribothymidylic acid (which differs from the former by addition of a 5-methyl group), indicating that the methyl groups offer more order to the molecular organization (Borek and Srinivasan, 1966).

Altered recognition has been demonstrated in both DNA and RNA (Borek and Srinivasan, 1966; Capra and Peterkovsky, 1968; Bird and Southern, 1978). Capra and Peterkovsky (1968) found that normal leucine tRNA responds equally well to poly UG (U, uracil; G, guanine) or poly UC (C, cytosine). However, methyl deficient leucine tRNA reacts preferentially with poly UC. This suggested that tRNA species which normally recognize poly UG exhibit changed codon responses when methyl-deficient. Subsequent methylation of this methyl-deficient tRNA results in changed chromatographic and coding properties. This indicates that replacement of deficient methyl groups in anticodon sequences restores proper recognition specificity (Capra and Peterkovsky, 1968). Additional evidence was reported by Bird and Southern (1978). They found that certain restriction enzymes, responsible for cleaving excess DNA after replication, selectively cleaved amplified (repeated) ribosomal DNA but did not cleave somatic (unique) ribosomal DNA. The difference was attributed to the presence of 5-methyl cytosine, rendering sites in somatic ribosomal DNA resistant to nuclease attack. The major methylated sequence is C_pG, which is the recognition sequence for all four nucleases (Bird and Southern, 1978). Revel and Groner (1978) report that 7-methylguanine is found in the 5' terminal "capping" sequence of RNA which has been cleaved from hnRNA, thus providing recognition sites for

modification nucleases.

Base replacement is thought to occur by one of two major mechanisms: enzymatic alteration (Scarano et al., 1977) or tautomeric shifts (see Jawetz et al., 1978, for a review). Holliday and Pugh (1975) and Scarano et al. (1967, 1977) have presented evidence in support of enzymatic methylation of the 5-position of cytosine, followed by enzymatic deamination, to produce thymine. This "minor thymine" is found throughout developing sea urchin embryos (Scarano et al., 1967). A possible mechanism for the methylation is shown in Figure 1. Figure 2 shows a possible mechanism for deamination of 5-methyl cytosine. The tautomeric base shift would result from altered hydrogen bonding patterns which lead to insertion of wrong bases during the next replication or transcription event. For example, 5-methyl cytosine can undergo tautomerization to bind preferentially with adenine rather than the normal purine, guanine (Jawetz et al., 1978).

Further support for the effects of methyl groups has been obtained from work with the thymine analogue, 5-bromodeoxyuridine (BrdU), which differs from thymine by substitution of a bromine atom for the 5-methyl moiety. BrdU is a known inhibitor of developmental processes (Kotzin and Baker, 1972; Levitt and Dorfman, 1972; Lee et al., 1974; Lee and Redmond, 1975) because of its ability to undergo tautomerization resulting in base transitions (Figure 3). Its effect can be alleviated by both thymine and Met, suggesting the significance of the 5-position substitution (Lee and Redmond, 1975). Evidence indicates that BrdU interferes with transcription rates and efficiency after being incorporated into DNA (Stellwagen and Tomkins, 1971; Jones and Dove, 1972; Schwartz,

Figure 2.

Hydrolytic deamination of 5 - methylcytosine

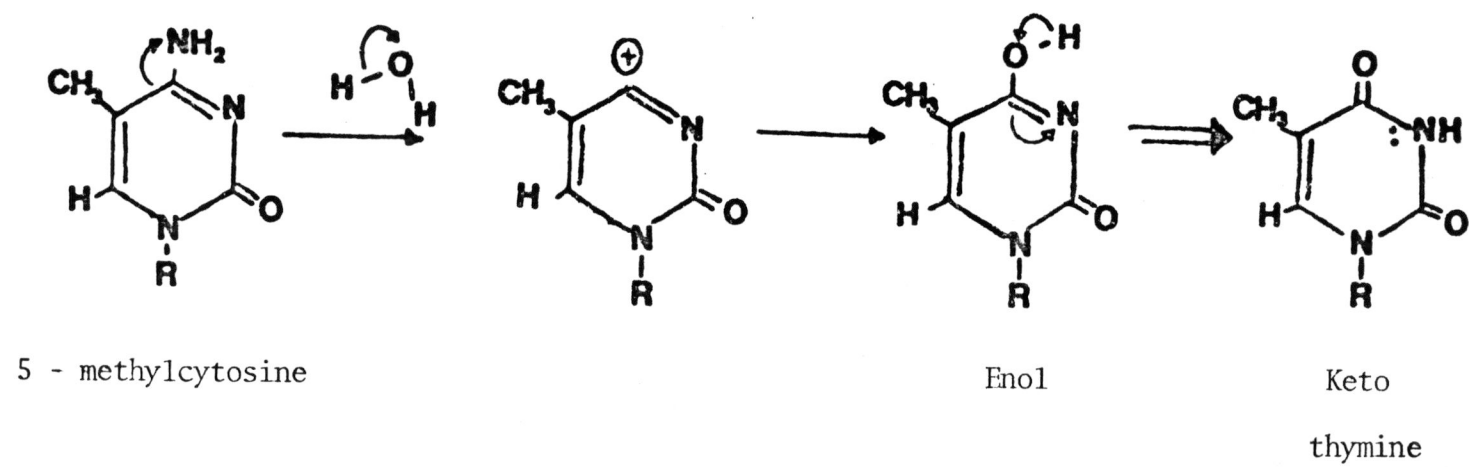
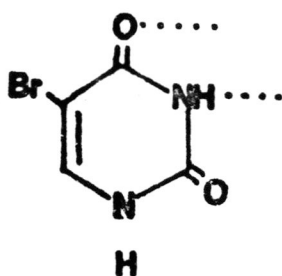
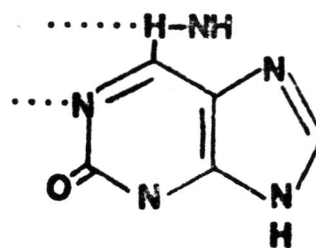


Figure 3.

BrdU-Induced Tautomeric Shift

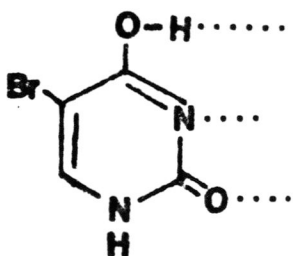


5 - bromouracil (BU)
normal keto form

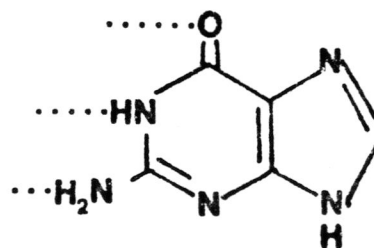


adenine (A)
normal hydrogen bonding

When incorporated into nucleic acids BU undergoes tautomerization to the enol form, which binds readily to guanine.



5 - bromouracil (BU)
enol form



guanine (G)
normal hydrogen bonding

In the next generation, a G:C pair would replace the original A:(T/BU) pair.

1977). Apparently, this effect is caused by interference with protein (histone and non-histone) binding (Schwartz, 1977) or interruption of normal strand separation prior to transcription (Jones and Dove, 1972). Lin and Riggs (1971) have reported that E.coli lac repressor binds 40 times more effectively to poly A-BrdU (A, adenine) than to poly A-T (T, thymine). In addition, the protein binds about 20 times less effectively to poly A-U (U, uracil) than to poly A-T. These results indicate that the nature of substitution at the 5-position of pyrimidines, which is exposed in the major groove of DNA, is important for repressor protein binding. Lin and Riggs (1971) suggest that, in eukaryotes, where BrdU is known to block expression of differentiated functions without greatly affecting growth and cell division, the incorporation of BrdU into operator elements of DNA may lead to tighter binding of regulatory proteins (histone or non-histone) resulting in disruption of the delicate regulatory system. In a subsequent study (Lin and Riggs, 1972), they demonstrated a 10-fold increase in lac repressor binding to BrdU-substituted operators, as compared to normal binding. The substitution at the 5-position or uracil was found to be directly related to efficiency of regulatory protein binding. In fact, the pK of the BrdU-substituted DNA-repressor complex was lower than that of normal T-substituted DNA. The melting temperature was higher with BrdU-substituted DNA. These facts indicate significantly increased stability with increasing electronegativity of the 5-position substituent (Lin and Riggs, 1972). Other investigators have reported similar binding of proteins in eukaryotes (Matthes et al., 1977).

In light of the evidence presented here, it appears that some aspects

of the models for eukaryote gene regulation discussed earlier may not be consistent with current experimental trends. For example, Davidson and Britten (1973) state that histones form a uniform blanket, surrounding the DNA, which releases the DNA upon approach of a recognized activator gene product. Evidence indicates that the histones in chromatin are not restricted to areas of repetitive or non-repetitive sequences, but are uniformly distributed throughout (Pospelov et al., 1975). Considering the relative homogeneity of histones (Kornberg, 1974; Stein et al., 1974), the responsibility for recognition of regulator (receptor) sequences would depend either on the non-histones or the activator gene products themselves. If the non-histones were responsible for recognition, they would necessarily have dual cognitive functions; first, they would recognize, and bind preferentially to, the repetitive regulator sequences; and second, they would alter chromatin conformation to allow access to DNA at the signal of the activator gene transcript. No evidence has been reported to support the dual recognition function.

The alternative approach, recognition by the activator gene products, seems somewhat more feasible. These products could introduce conformational stress into the chromatin, for example, by spatial juxtaposition alone. Presumably, the stress would lead to conformational changes in the chromatin proteins, thus exposing the DNA to the activator gene product. A problem with this approach is that, once the activator has breached the chromatin proteins, it would be confronted with numerous long segments of repetitive nucleotide sequences. Since these repetitive sequences are responsible for regulation, they must be recognized by the activator. Unless specifically modified so that each receptor/structural

gene sequence has a unique "flag", recognition would only be possible with a high number of activator gene products (one for each segment of the genome). Britten and Davidson (1969) proposed that the activator gene transcripts, i.e., RNA molecules, are responsible for differential gene activation. The only way this could occur is with a highly variable RNA population. Several reviews have presented considerable evidence in support of the large number of modifications (by methylation, primarily) of RNA species that would be required (Borek and Srinivasan, 1966; Hall, 1971; Revel and Groner, 1978).

The alternative to a highly variable activator RNA population is the presence of "flags" on the DNA itself, as mentioned above. Since 5-methyl cytosine is the only minor base in the DNA of higher animals (Vanyushin et al., 1970; Scarano et al., 1977), simply "flagging" of various regions would probably not provide sufficient variability for differential gene control. However, sequential methylation of nucleotides could result in base substitutions, either enzymatically or tautomerically, which could serve as unique and differentially expressed gene markers (Scarano, 1971; Holliday and Pugh, 1975).

If the sites to be methylated contained specific base sequences, and if methylation occurred immediately after nucleic acid synthesis, the methylated sites would be inheritable. Several investigators have shown that the dinucleotide C_pG is the site methylated most often, and with the most consistent species specificity (Grippo et al., 1968) suggest that, since DNA guanine and cytosine content is so species-specific, these bases may be able to affect mutation rates as well as control of the genome. Methylation of DNA cytosine-guanine sequences,

then, could be a highly flexible and inheritable mechanism for genetic regulation in eukaryotes.

Another point where methylation is essential is in the hnRNA maturation process. Georgiev (1969) proposed an operon concept which included one crucial feature: the selective degradation of only the repetitive sequences of the hnRNA. Davidson and Britten (1973) contended that the theory proposed by Georgiev was not supported in the literature, and that hnRNA served a regulatory function only. Since that time, a number of investigators have shown that large hnRNA sequences are partially digested by nucleases before their structural components leave the nucleus (Crick, 1979; Tsai *et al.*, 1979). The hnRNA is methylated on the 5' terminus of the structural mRNA to provide a recognition site, which prevents further nuclease digestion (see Revel and Groner, 1978, for a review).

Thus, methylation of DNA and several species of RNA provides a variety of mechanisms by which the eukaryotic genome can be controlled. Also, control of cellular and tissue differentiation is thought to depend on control of the genome. Although much evidence has accumulated concerning either control of the genome or control of differentiation, relatively little information exists which shows a direct link between methylation of nucleic acids and the process of differentiation.

MATERIALS AND METHODS

Unincubated fertile White Rock Cross hen eggs were obtained from Green Valley Hatchery in Kenly, North Carolina. The eggs were stored at 15°C until time of use (not more than 48 hours). Eggs were incubated for 18 hours at 37.5°C and 50-60% relative humidity to obtain stage 3 to 4 blastoderms (Hamburger and Hamilton, 1951). Post-nodal pieces (PNPs) were explanted according to the technique of Spratt (1947) as follows: Eggs were opened into a sterile finger bowl containing about 50 ml of sterile Ringer's solution. The blastoderms were freed from the yolk by holding them with fine-tipped forceps and making cuts about one millimeter away from the blastoderm border using iridectomy scissors. The vitelline membrane with blastoderm attached was then transferred by wide mouth pipette to a sterile petri dish containing about 20 ml of Ringer's solution. The blastoderm was dissected free of the vitelline membrane and washed with Ringer's solution to remove excess yolk granules. The area opaca was trimmed off with a sharpened sterile probe. PNPs were obtained by transecting the area pellucida 0.6 mm posterior to Hensen's node at right angle to the primitive streak (Figure 4). The isolated PNPs were then placed on culture medium ventral side up.

TISSUE CULTURE TECHNIQUE

PNPs were cultured on whole egg extract-Ringer-agar medium (Spratt and Haas, 1960a, 1960b). The whole egg extract was prepared by obtaining one fresh unincubated egg, breaking the egg into a 500 ml Erlenmeyer flask, and shaking the flask until the yolk and albumen were well mixed (5 minutes). The egg homogenate was centrifuged at 4000 rpm in the cold (4°C) for 20 minutes using sterile 50 ml Sorvall centrifuge tubes.

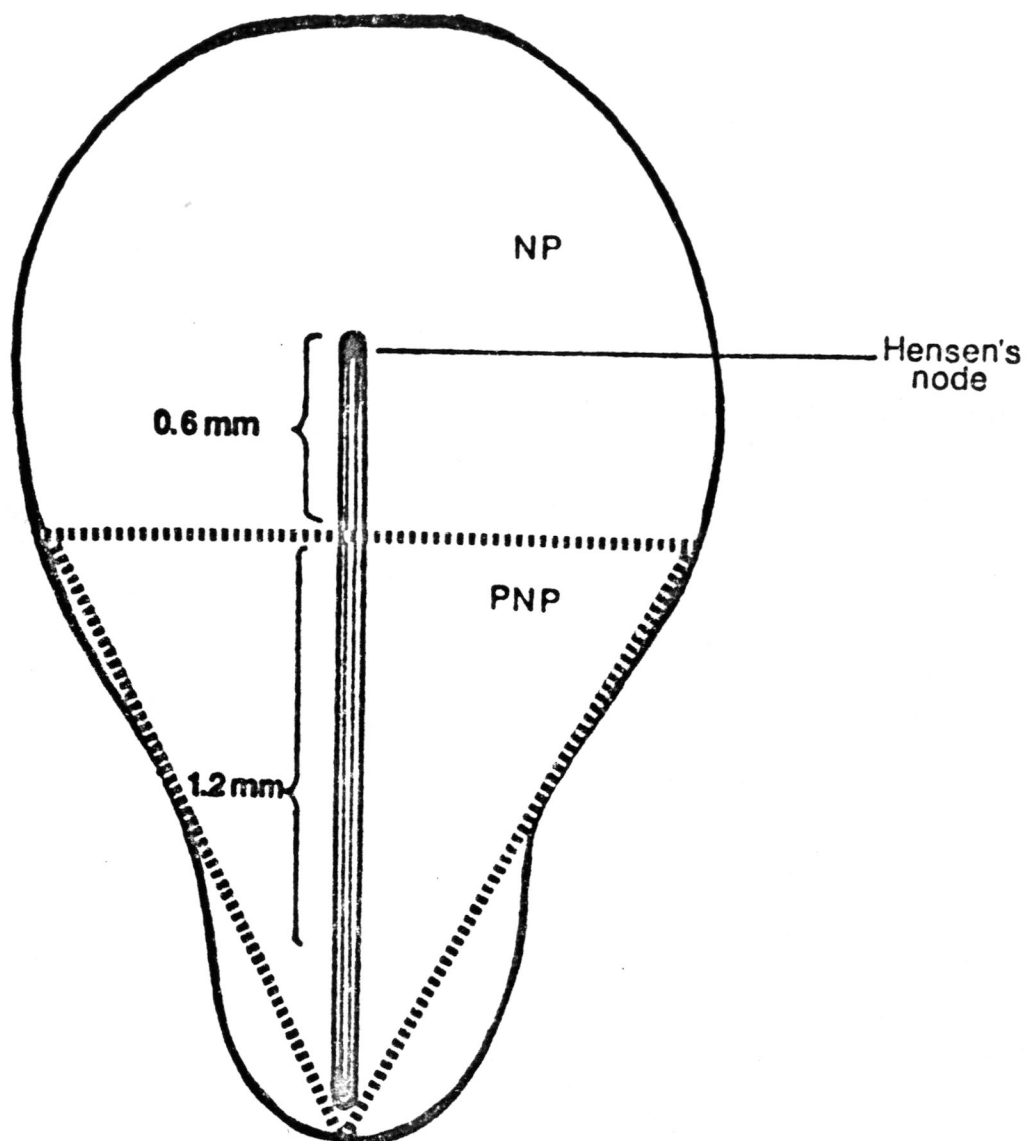


Figure 4. This is a schematic diagram of a stage 3⁺ chick blastoderm showing the approximate average length of the primitive streak and the distance posterior to Hensen's node at which transections were made. Dotted line indicates outline of post-nodal piece (PNP). NP, nodal piece.

After centrifugation, 30 ml of the supernatant were decanted and stored briefly while the agar was being prepared. Agar consisted of 0.2-0.3 gm Bacto-Agar dissolved at low boil in 30 ml of Ringer's solution. When the agar cooled to about 45°C the egg extract was added slowly. The solution was mixed thoroughly in a 250 ml Erlenmeyer flask. Aliquots of 1.5-2.0 ml of the medium were poured into sterile watch crystals supported on moist cotton rings in sterile petri dishes (Figure 5).

PNPs were treated and then incubated at 37.5°C for 2 days. Due to localized nutrient depletion and possible toxicity from waste products, PNPs were subcultured in the following manner: PNPs were removed from the medium, washed in Ringer's solution, and placed on fresh medium in culture vessels identical to those used in the initial culture period. After this transfer to fresh medium, the vessels were incubated at 37.5°C for 2 additional days. PNPs were removed from culture after 4 days and processed for morphological evaluation or quantitative analysis.

TREATMENT OF PNPs

After PNPs were placed on the initial culture medium, a drop (0.03 ml) of Ringer's solution (with or without test agent) was placed directly onto each PNP. The test agents were prepared in stock solutions of 20 µg/ml in Ringer's solution immediately before use. Dilutions of 4-18 µg/ml were prepared from the stock solutions and applied to the PNPs. Control PNPs were treated with Ringer's solution only.

METHYL DONORS - PNPs were treated with the essential amino acid L-methionine (Met) and its derivative S-adenosyl-L-methionine (SAM). Both agents are known active methyl group donors.

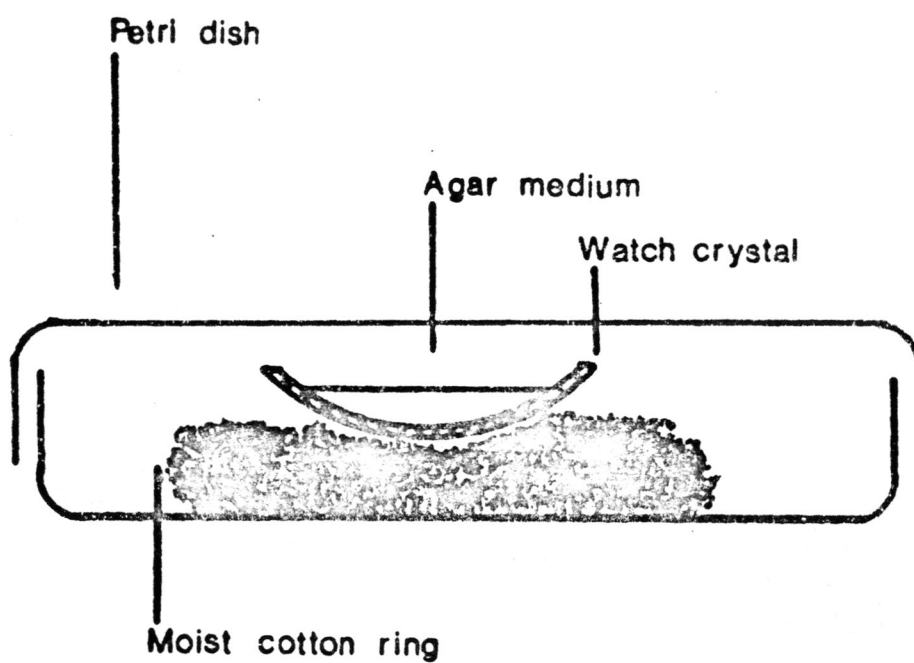


Figure 5. Schematic representation of the culture vessel used in the present study.

DEMETHYLATED ANALOGUES - PNPs were treated with DL-homocysteine (HCys) and S-adenosyl-L-homocysteine (SAH), both of which are competitive inhibitors of methyl group donation by SAM (Parsa et al., 1972; Lee and Redmond, 1975; Borchardt, 1977).

MORPHOLOGICAL STUDY OF CULTURED PNPs

LIGHT MICROSCOPY - PNPs for light microscopic observation were fixed in Bouin's fixative after four days of culture; washed in 70% ethanol to remove the picric acid crystals; dehydrated in graded ethanol series; embedded in Tissueprep (melting point, 56.5°C); sectioned at 4 μ m on a Zeiss microtome; placed on glass microslides; and stained with Delafield's hematoxylin and eosin. Sections were examined and photographed using a Zeiss STANDARD WL research microscope.

ELECTRON MICROSCOPY - PNPs for electron microscopic observation were fixed in half-strength Karnovsky's (1965) fixative (pH 7.4) according to the procedure of Sawyer (personal communication); rinsed in Ringer's solution to remove any medium; and dropped into a vial of fixative. After 30 minutes of fixation in the cold (4°C), PNPs were removed from the agar and washed in two changes of cold 0.1 M sodium cacodylate (sodium dimethyl arsenate) for 30 minutes each. PNPs were post-fixed in cold (4°C) 2% osmium tetroxide buffered with sodium cacodylate to pH 7.4 for 20 minutes, followed by washing for 10 minutes in cold 0.05 M maleic acid buffer at pH 5.2. PNPs were en bloc stained for 30 minutes with 1% uranyl acetate in 0.05 M maleic acid buffer; washed in two changes of 0.05 M maleic acid buffer; dehydrated through a graded ethanol and propylene oxide series; and embedded in Araldite 6005 with 3% plasticizer. Thin sections were cut on a Sorvall MT-2B ultramicro-

tome, contrasted with uranyl acetate and Reynolds' (1963) lead citrate, and examined on a Hitachi HS-8A electron microscope.

AUTORADIOGRAPHY

Labeled L-methionine methyl- ^3H and S-adenosyl-L-methionine methyl- ^3H were obtained from New England Nuclear, Boston, Massachusetts. Isolated PNPs were treated with $5\mu\text{Ci/ml}$ of the tritiated methyl donors during the initial culture period of 2 days. After an additional 2 days incubation period, PNPs were washed three times in Ringer's solution, then fixed in absolute ethanol:acetic acid (3:1). PNPs were dehydrated through a graded ethanol series; cleared in xylene and embedded in paraffin. Sections were cut at $4\mu\text{m}$; mounted on glass slides; deparaffinized in xylene and rehydrated through graded ethanol series.

Emulsion coating was performed in the dark with a sodium safelight (Wratten filter No. 2). Kodak NTB-2 liquid photographic emulsion was diluted with distilled water (1:1) in a water bath heated to 45°C . Slides were dipped into the emulsion back to back for 2 minutes according to the technique of Kopriwa and Leblond (1962). Slides were allowed to dry for 3 hours and placed in black plastic slide boxes containing a small amount of dessicant (Drierite). The slide boxes were then sealed with black tape and stored at 4°C for 7-10 days.

The emulsion was developed for 6 minutes in Kodak D-19 developer at 22°C , placed in a stop bath for 1 minute, and fixed for 10 minutes in Kodak acid-fixer. Sections were then stained with Delafield's hematoxylin and eosin; dehydrated through graded ethanol series; and covered with cover slips mounted in Permount. Sections were examined to determine distribution of labeled methyl groups.

EXTRACTION PROCEDURE

PNPs were prepared for liquid scintillation counting and quantitative determinations of protein, RNA, and DNA content by a modified Schmidt and Thannhauser (1945) procedure. PNPs were disrupted by trituration through a Pasteur pipet in a small volume of Ringer's solution. Acid soluble phosphates were extracted with 1.0 ml of cold (4°C) 0.5 N perchloric acid for 30 minutes. The mixture was centrifuged at 2000 rpm at 4°C for 15 minutes. The supernatant was then discarded. The pellet was washed in 0.2 N potassium acetate in absolute ethanol and centrifuged at 2000 rpm at 4°C for 15 minutes. The supernatant was discarded. Remaining lipids were extracted with 1.0 ml absolute ethanol:ether (3:1) in a water bath at 50°C for 30 minutes. The mixture was centrifuged at 2000 rpm at 4°C for 15 minutes and the ethanol:ether extraction was repeated. The lipid-free residue was then resuspended in 1.0 ml of 0.1 N potassium hydroxide for 18 hours at 37.5°C. RNA was extracted from the solution with 0.034 ml of 6N hydrochloric acid and 1.0 ml of 0.5 N perchloric acid. The mixture was centrifuged at 2000 rpm at 4°C for 15 minutes and supernatant, containing RNA, was decanted and stored at 4°C until used. The residue was resuspended in 1.0 ml of 0.5 N perchloric acid and heated at 90°C for 15 minutes. The suspension was centrifuged at 2000 rpm at 4°C for 15 minutes and supernatant, containing DNA, was decanted and stored at 4°C. The protein residue was resuspended in 2.0 ml of 1.0 N sodium hydroxide overnight at room temperature (22°C). The protein fraction was then stored at 4°C until used.

LIQUID SCINTILLATION COUNTING

PNPs were treated with tritiated Met and SAM, and cultured, as in the procedure for autoradiography. After the culture period, DNA, RNA, and protein fractions were obtained by the extraction procedure used for quantitative determinations. Protein fractions were left as precipitates from the acid extraction because sodium hydroxide causes separation of phases in the scintillation cocktail. The cocktail was prepared by dissolving 7 grams of PPO (2, 5-diphenyloxazole) and 0.3 grams of dimethyl POPOP (1, 4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene) in 1 liter of toluene. Triton X-100 was added as 1 part to 2 parts of the above solution. Plastic 20 ml scintillation vials were filled with 1 ml of test solution and 9 ml of scintillation cocktail. Vials were placed in a Beckman LS 9000 liquid scintillation counter for counts per minute (cpm) determination. Data were analyzed for relative amounts of label uptake by the three fractions (protein, DNA, RNA).

QUANTITATIVE DETERMINATIONS

DNA, RNA, and protein quantities of both control and treated PNPs were determined colorimetrically to verify viability of PNPs during the culture period. All three fractions were tested with the complementary quantitations to determine the relative degrees of purity.

PROTEIN FRACTION - The quantity of protein was determined by a reaction between the Folin-Ciocalteu reagent with the aromatic amino acids, tyrosine and tryptophan, according to a modified procedure of Lowry *et al.*, (1951). Reagent A was made up by adding together 0.5 ml of 1% copper sulfate pentahydrate, 0.5 ml of 2% potassium tartrate, and 50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide. Aliquots (0.5 ml) of

protein sample were added to 5 ml of Reagent A. After incubation at 25°C for 10 minutes, 0.5 ml of 1 N Folin-Ciocalteu reagent was added. The solution was mixed uniformly and then allowed to react at room temperature for 30 minutes in the dark. Absorbance was read at 750 nm on a Coleman-111 spectrophotometer and concentrations were extrapolated from a standard curve prepared with 0 to 300 µg/ml bovine serum albumin (Sigma Co., St. Louis, Missouri).

RNA FRACTION - The quantity of RNA was determined by measurement of pentose content by a reaction between the sugar and orcinol. The procedure was a modification of the Mejbaum (1939) orcinol reaction. Stock orcinol solution of 13.5 grams ferric ammonium sulfate and 20.0 grams recrystallized orcinol in 500 ml of distilled water was made up and stored in the cold (4°C) until used. The working orcinol reagent was prepared by combining 5 ml of orcinol stock solution, 85 ml of concentrated (12 N) hydrochloric acid and 10 ml of water. Aliquots of 1 ml of sample were added to 3 ml of the working orcinol reagent and heated in a boiling water bath for 20 minutes. The solution was allowed to cool and absorbance was read at 670 nm on a Coleman-111 spectrophotometer. Concentrations were determined by extrapolating from a standard curve prepared from 0 to 300 µg/ml of aqueous calf thymus RNA solutions (Sigma Co., St. Louis, Missouri).

DNA FRACTION - The quantity of DNA was determined by a reaction of diphenylamine with deoxyribose according to a procedure by Burton (1956). Diphenylamine stock solution was prepared by adding 1.5 grams of diphenylamine and 1.5 ml of concentrated (37 N) sulfuric acid to 100 ml of glacial acetic acid. Stock solution was stored in the cold (4°C) until

used. Diphenylamine reagent was prepared by adding 0.1 ml of acetaldehyde to 20 ml of stock. One ml of sample and 2 ml of diphenylamine reagent were mixed and incubated at 37.5°C for 18 hours. Absorbance was read at 600 nm on a Coleman-111 spectrophotometer and quantity was determined by extrapolation from a standard curve prepared from 0 to 300 µg/ml aliquots of aqueous herring sperm DNA (Sigma Co., St. Louis, Missouri).

STATISTICAL ANALYSIS OF DATA

Standard curves for protein, DNA and RNA quantitation were prepared by plotting concentration (abscissa) against absorbance (ordinate). A linear relationship was calculated by simple regression analysis utilizing the least squares method (Daniel, 1977). The equation for determining the y-intercept is as follows:

$$b = \frac{\sum x_i y_i - \frac{(\sum x_i)(\sum y_i)}{n}}{\sum x_i^2 - \frac{(\sum x_i)^2}{n}}, \text{ where } x_i \text{ and } y_i$$

represent values of concentration and absorbance, respectively; and n is the number in each sample.

Statistical validity of morphological data and quantitative analyses was determined by the use of chi-square distribution. Significant differences between two values were ascertained by the following formula:

$$\chi^2 = \sum \frac{(O - E)^2}{E}, \text{ where}$$

O is the observed value, and E is the expected value (Daniel, 1977).

RESULTS

The optimum culture period for observation of differentiation was found to be 4 days. PNPs cultured longer than 4 days (up to 10 days) exhibited no significant increase in the numbers or types of differentiated structures.

Morphological Evaluation

Controls - Examination of control whole mounts (Figure 6) revealed vesicular structures almost totally devoid of condensed cell masses, which were indicative of differentiated structures in treated PNPs. Cross sections of control PNPs (Figure 7) revealed little, if any, recognizable differentiated structure. The peripheral layer was composed of a continuous sheet of ectoderm. The underlying cells were loosely associated mesoderm, at times exhibiting acinar-like characteristics. The large cavity of the vesicular structure was lined with a thin layer of endoderm.

Treated PNPs - The highest frequencies of differentiated structures were observed when PNPs were treated with 12 $\mu\text{g}/\text{ml}$ of Met and 10 $\mu\text{g}/\text{ml}$ of SAM, except in the case of somites, in which 10 $\mu\text{g}/\text{ml}$ of Met elicited more differentiation than the higher concentrations. All micrographs in the following figures were taken from PNPs treated with 12 $\mu\text{g}/\text{ml}$ of Met, and are representative of the tissues reported for all test conditions.

Examination of whole mounts (Figure 8) revealed complicated vesicular and tubular structures, as well as localized regions of condensed tissues. Upon examination of serial sections, these condensed regions were found to contain numerous differentiated structures. A number of

these structures could not be positively identified and were not included in the data. In many cases, the tubular structures were found to be actively pulsating at the time of fixation.

Axial Structures - Although the structures were artificially stimulated to differentiate, they usually exhibited characteristic anatomical relationships. A definite correlation was seen between the frequency of occurrence of neural tissue and notochord, although notochord was observed less frequently in all cases. Lateral to the neural tissue and its underlying notochord, somites were often observed.

Neural plate tissues were found at various stages of development after the culture period, from thickened neural ectoderm (Figure 9), to deep neural grooves (Figure 10), to completely closed neural tubes (Figure 11). In some cases, multiple neural tube formation was observed (Figure 12). Neural tissue was the most frequently formed tissue (Table 1). The methyl donors exhibited significantly more induction of neural tissue than the controls. The frequency of neural tissue in PNPs treated with the methyl donors over the entire concentration range varied from a low value of 23.8% to a high value of 46.2%. These values represented statistically valid increases over the controls. On the contrary, neither of the demethylated analogues exhibited significant increases over the control values at any concentration tested.

Notochordal structures were distinct aggregates of highly vacuolated cells (Figure 13). No notochordal formations were observed in any of the controls. In general, notochordal tissue was the least frequently observed tissue, regardless of test agent used (Table 1).

Lateral to developing neural tubes, condensed regions of mesoderm were observed, which exhibited characteristic somite structure (Figure 14). These mesodermal regions displayed few extracellular spaces. At times the condensed and tightly packed cell masses mimicked the activity of overlying neural plate ectoderm. Newly formed intercellular contacts resulted in a rosette configuration. No somite structures were observed in controls. Somite development did not show a highly significant relationship to the test agents used (Table 1).

Nephric Tubules - In many instances, tubular structures composed of cuboidal to low columnar epithelium were found lateral to regions of condensed mesoderm. These structures strongly resemble developing nephric tubules (Figure 15). In general, nephric tubules were induced less frequently than neural tissue (Table 2). Values observed for PNPs treated with analogues did not exhibit significant differences from control values. However, the methyl donors exhibited significantly more induction of nephric tubules than the analogues or controls.

Cardiac Tissue - Many tubular structures were found to be actively pulsating at the time of fixation (10 to 120 beats per minute). Upon microscopic examination, several stages of developing cardiac tissue were observed, ranging from simple tubes composed of mesoderm to highly complicated arrangements of cardiac tissue, exhibiting characteristic branching patterns of muscle fibers and central vesicles containing hemopoietic elements (Figure 16). In peripheral areas, vascular sinuses were observed, which were composed of mesoderm, lined with endothelial-like cells, and contained numerous hemopoietic elements (Figure 17). The

highest frequencies of observed pulsatile structures were found in PNPs treated with 12 $\mu\text{g/ml}$ of Met and 10 $\mu\text{g/ml}$ of SAM (Table 3). Values observed for other concentrations of methyl donors did not differ significantly from the values observed for the demethylated analogues.

Pulsatile structures were also examined by electron microscopy to verify the presence of features intrinsic to cardiac muscle cells. The cells contain numerous bundles of myofibrils, characteristically branching around the nucleus (Figure 18), or ending in intercellular junctional complexes (Figure 19). Upon further examination these junctional complexes were found to be complete desmosomes, including characteristic lamination patterns and tonofibrillar arrangement. Intercellular canaliculi were frequently seen between cellular junctions (Figure 20).

Autoradiography

Labeled methyl groups were used to determine their distribution and point(s) of action within the cells. Sections of PNPs treated with labeled compounds were covered with liquid photographic emulsion. Their subsequent exposure to emitted beta particles from radioactive decay of the isotopic label resulted in production of dark grains in the emulsion directly over the labeled groups. The heaviest amount of label was found over the nuclear region (Figure 21). In some cases the heterochromatin was preferentially labelled (Figure 22).

Liquid Scintillation Counting

DNA, RNA, and protein fractions were obtained from PNPs treated with 5 $\mu\text{Ci/ml}$ of radioactively labeled test agents for determination of the relative amounts of label taken up by each fraction. The numbers

represent raw data which do not account for size or volume differences, but rather represent total counts for whole fractions (Table 4). The background count was only 48.45 counts per minute. The levels of uptake, even for the lowest value of DNA, were significantly greater than control values at the 90% confidence level.

Quantitative Determination of Viability Parameters

To verify whether or not PNPs remained viable for the duration of the culture period after treatment with test agents, DNA, RNA, and protein contents were assayed by standard colorimetric methods. The average content of DNA, RNA, and protein in control PNPs was 0.45, 19.4, and 91.0 $\mu\text{g/ml}$, respectively. In comparison, the average content of DNA, RNA, and protein in PNPs treated with the various test agents was 0.575, 21.95, and 130.45 $\mu\text{g/ml}$, respectively. These values correlate very well, thus indicating that no changes in growth and synthesis rates occurred as a result of treatment.

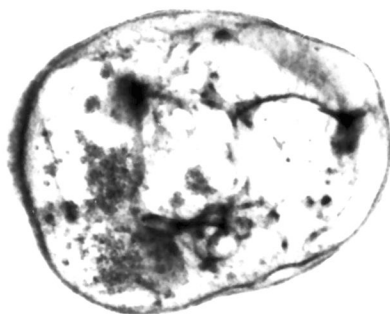


Figure 6. Whole mount of a control PNP cultured for 4 days.
x 30.

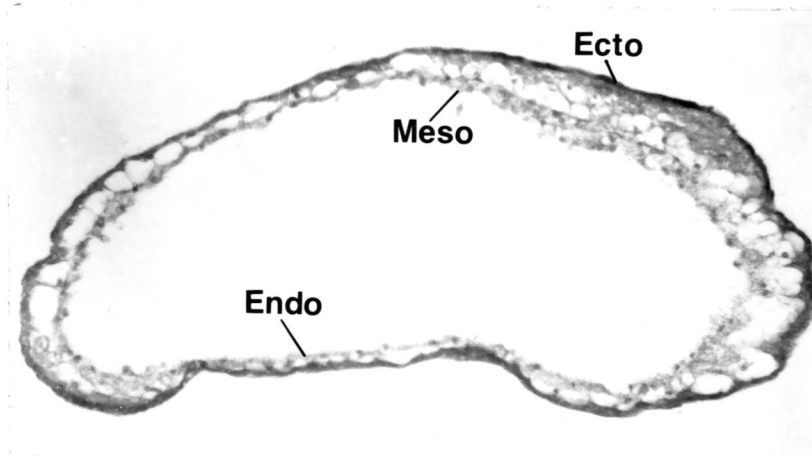


Figure 7. Cross section of a control PNP showing a large central cavity, which is devoid of any internal differentiated structure. Ecto, ectoderm; Meso, mesoderm; Endo, endoderm. x 100.

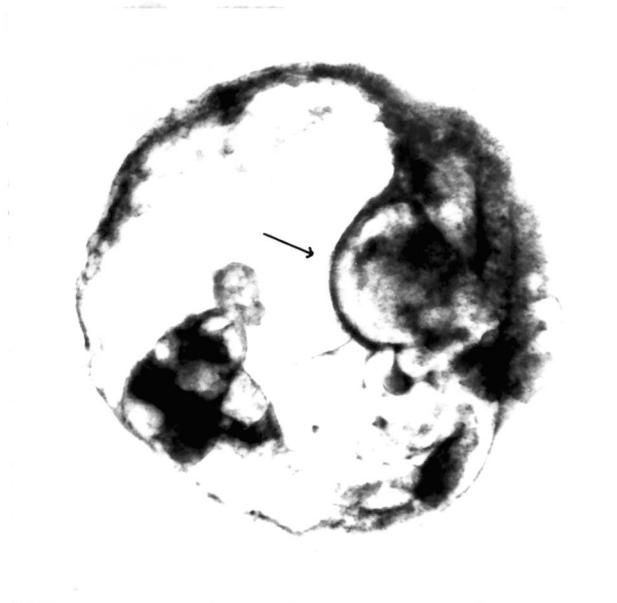


Figure 8. Whole mount of complicated PNP treated with 12 $\mu\text{g/ml}$ of L-methionine. The large vesicle (arrow) was pulsating at the time of fixation. x 30.

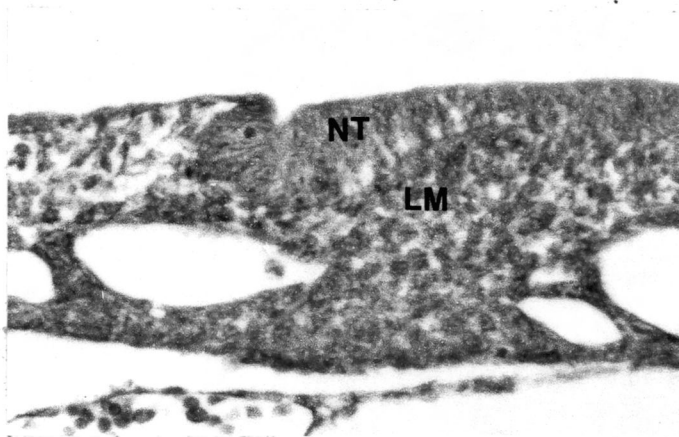


Figure 9. Cross section of PNP treated with 12 $\mu\text{g/ml}$ of L-methionine showing developing neural groove. NT, neural tissue; LM, lateral plate mesoderm. x 400.

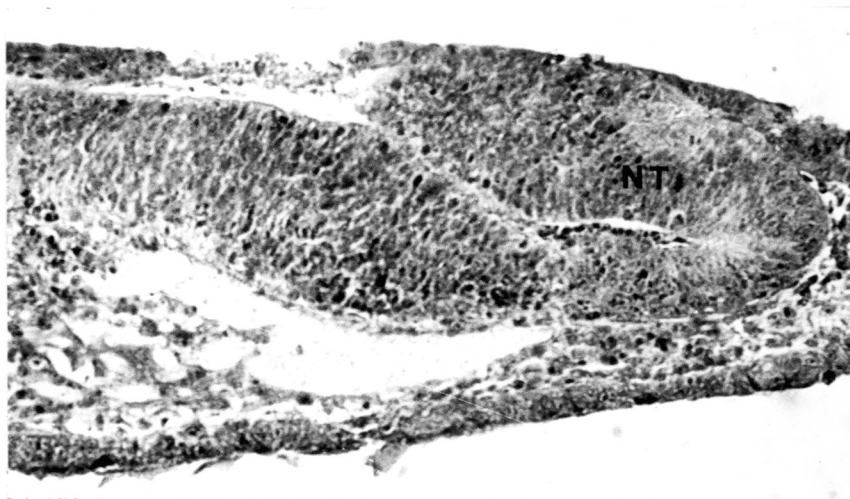


Figure 10. Cross section of a PNP showing deep neural groove.
NT, neural tissue. x 275.

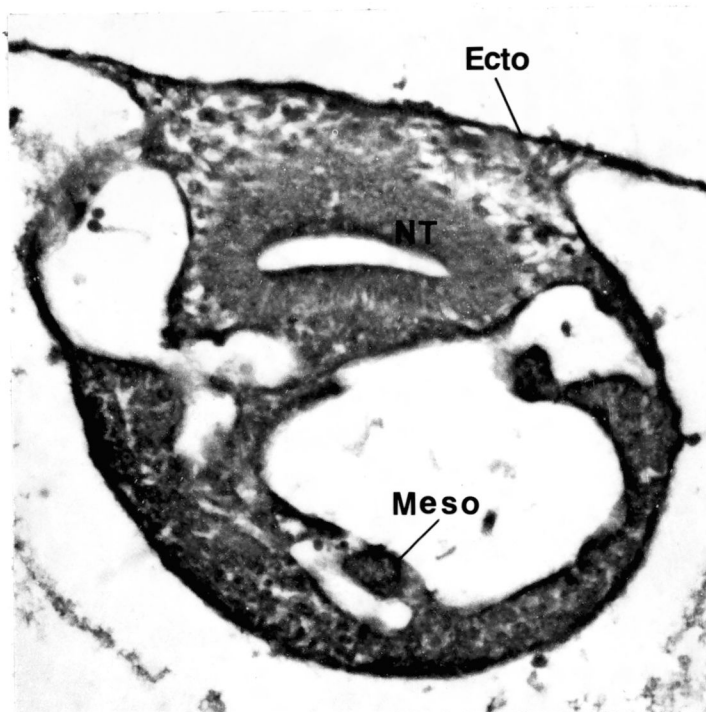


Figure 11. Cross section of a PNP showing a complete neural tube with underlying mesodermal vesicles and overlying ectoderm. NT, neural tissue; Ecto, ectoderm; Meso, mesoderm. x 640.

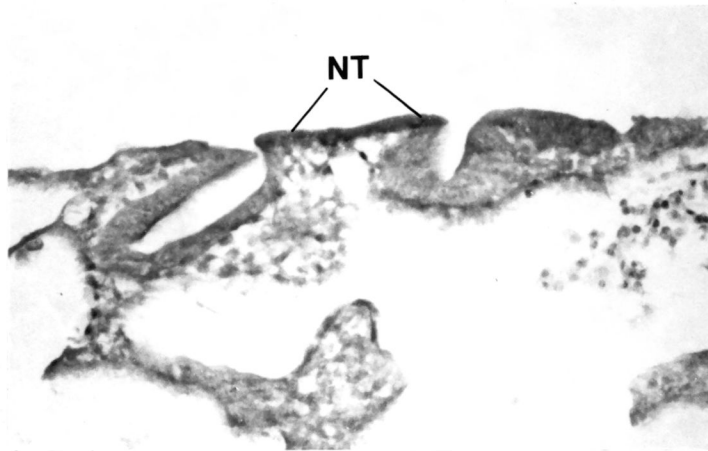


Figure 12. Cross section of a PNP showing two developing neural tubes. NT, neural tissue. x 250.

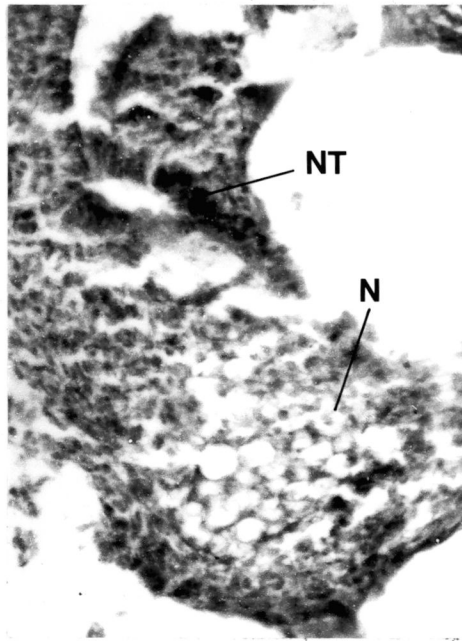


Figure 13. Cross section of a PNP showing a neural groove and its associated underlying notochord. NT, neural tissue; N, notochord. x 625.

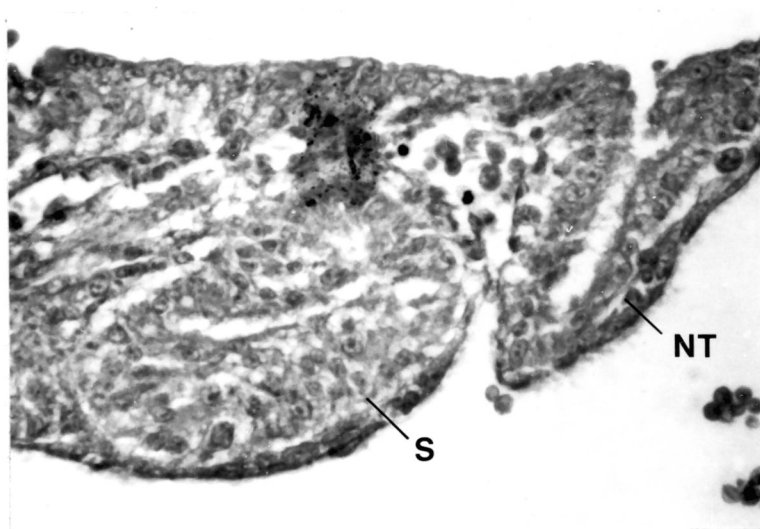


Figure 14. Cross section of a PNP showing a deep neural groove and a laterally located somite. NT, neural tissue; S, somite. x 450.



Figure 15. Cross section of a PNP showing several nephric tubules, condensed lateral plate mesoderm, and overlying thickened ectoderm. Nt, nephric tubules; LM, lateral plate mesoderm; Ecto, ectoderm. x 1250.

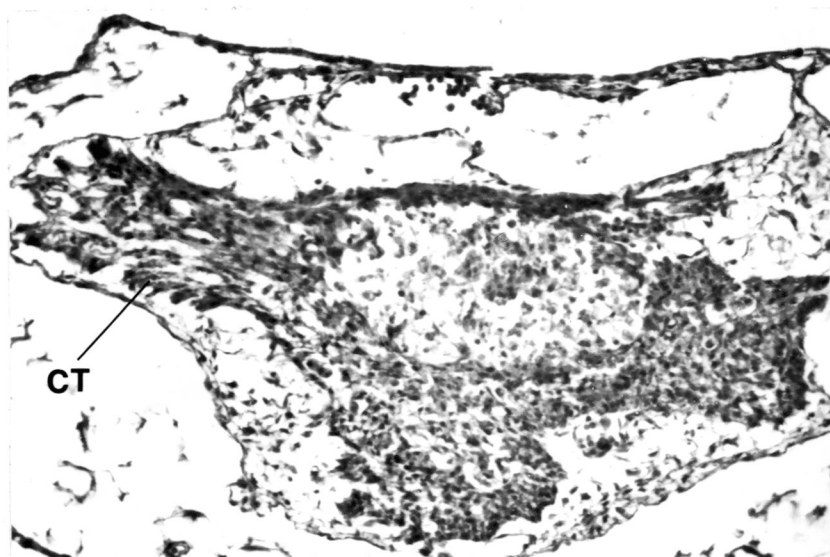


Figure 16. Cross section of a PNP showing a large area of complicated cardiac tissue and characteristic branching of the cardiac muscle fibers. CT, cardiac tissue. x 200.

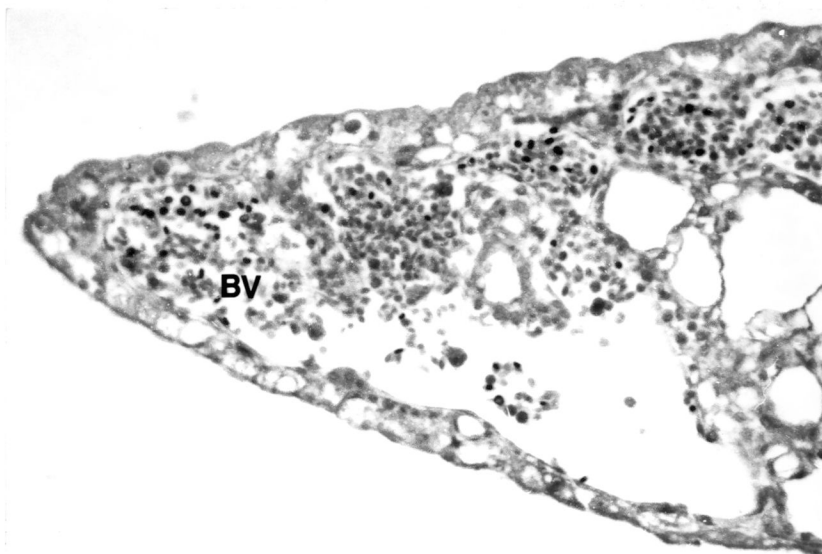


Figure 17. Cross section of a peripheral region of a PNP showing a large vascular sinus containing numerous hemopoietic elements. BV, blood vessel. x 570.

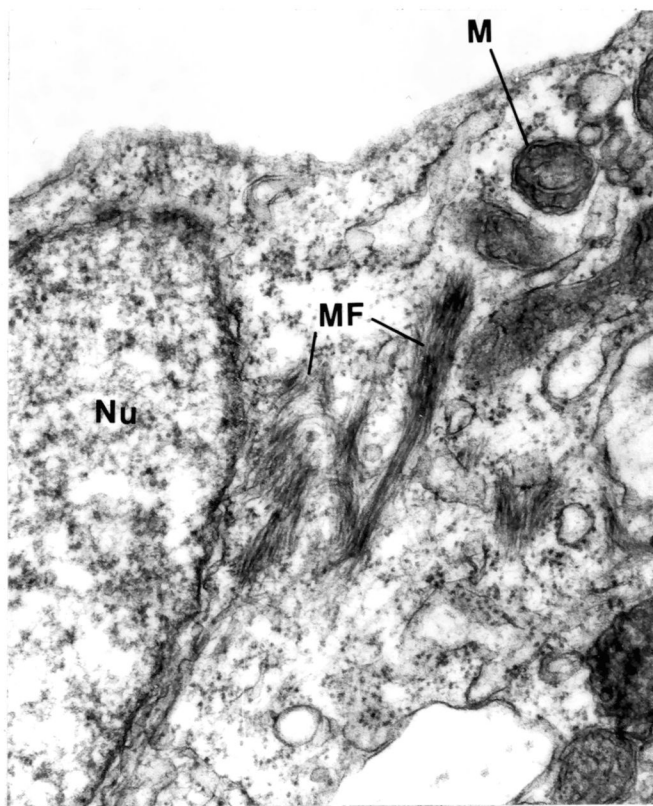


Figure 18. Electron micrograph of cardiac tissue showing characteristic branching patterns of myofibrils surrounding the nucleus. MF, myofibrils; Nu, nucleus; M, mitochondria. x 39,000.

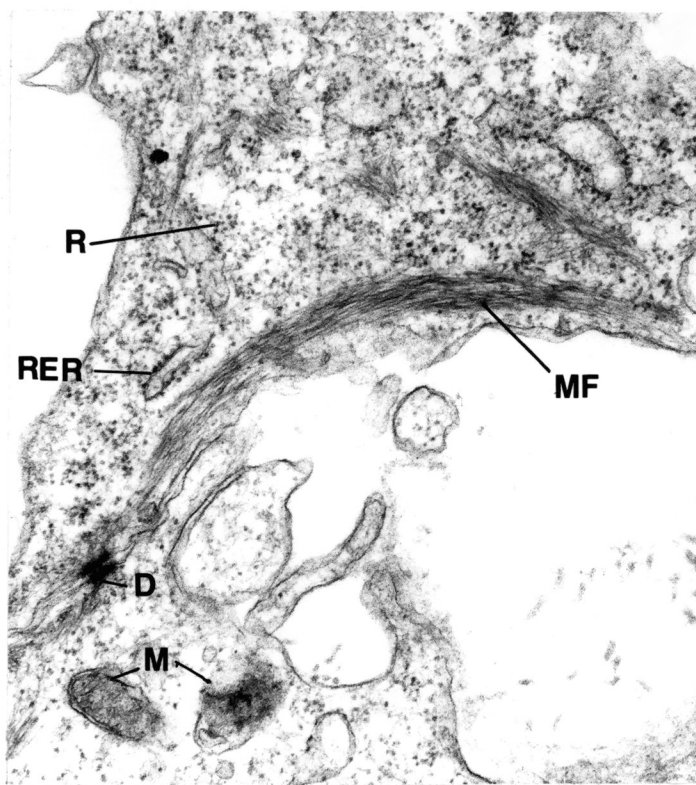


Figure 19. Electron micrograph of cardiac tissue showing large bifurcating bundle of myofibrils terminating near a junctional complex. MF, myofibrils; D, desmosome; M, mitochondria; RER, rough endoplasmic reticulum; R, ribosomes. x 39,000.

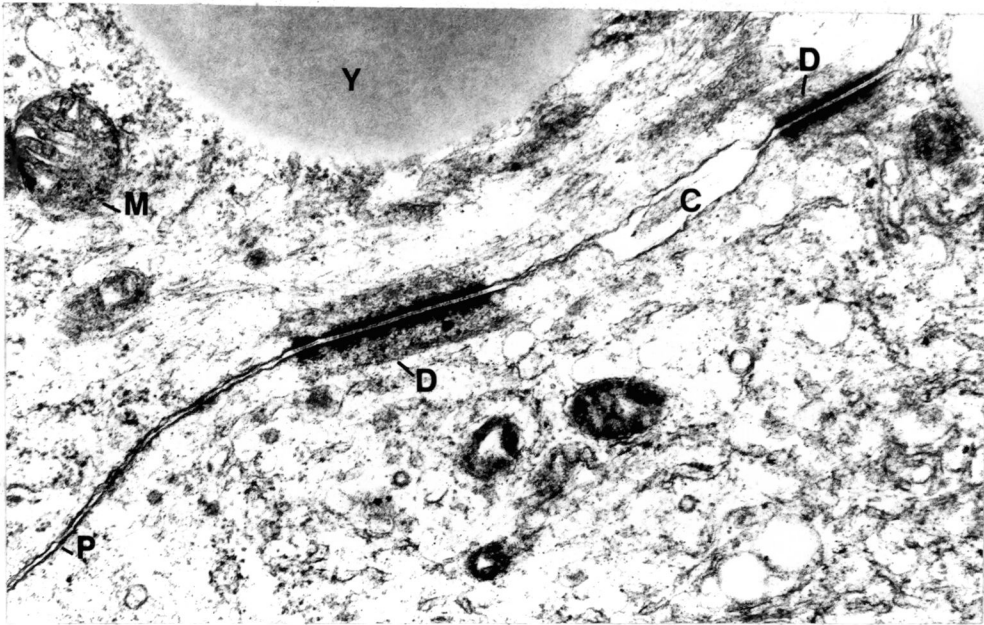


Figure 20. Electron micrograph of cardiac tissue showing specialized junctional complexes between two cells. D, desmosome; C, intercellular canaliculus; Y, yolk platelet; M, mitochondrium; P, plasmalemma. x 36,000.

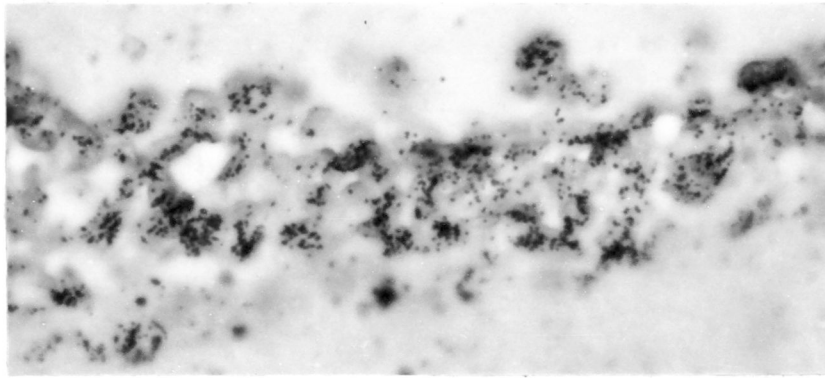


Figure 21. Autoradiograph of a cross section of a PNP treated with 5 $\mu\text{Ci/ml}$ of L-(methyl- ^3H)-methionine. Note the granules located primarily over the nuclear region.
x 420.

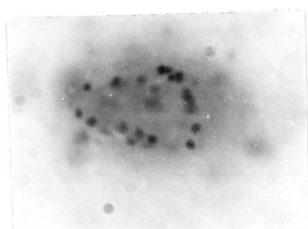


Figure 22. Autoradiograph of a cross section of a PNP treated with 5 $\mu\text{Ci/ml}$ of L-(methyl- ^3H)-methionine. Note the significant uptake of label in the peripheral areas of the nucleus, representing areas of heterochromatin. x 1550.

TABLE 1
Frequency of Axial Structures

Type of Treatment	Concentration (µg/ml)	Number of PNPs	Number of PNPs showing differentiated structures ¹		
			Neural tissue	Notochord	Somites
Control	-	56	7 (12.5)	0 (0.0)	0 (0.0)
L-methionine	8	13	4 (30.7) ²	0 (0.0)	1 (7.7) ³
	10	22	8 (36.4)	1 (4.5) ^{2,3}	4 (18.2)
	12	25	11 (44.0)	2 (8.0)	4 (16.0) ³
	14	26	10 (38.5)	0 (0.0)	2 (7.7)
S-adenosyl-L-methionine	8	21	5 (23.8) ^{2,3}	0 (0.0)	2 (9.5) ³
	10	26	12 (46.2)	3 (11.5)	5 (19.2)
	12	30	11 (36.7)	2 (6.7) ³	5 (16.7)
	14	25	7 (28.0)	0 (0.0)	1 (4.0) ^{2,3}
DL-homocysteine	8	21	1 (4.8) ²	0 (0.0)	0 (0.0)
	10	23	1 (4.3) ²	1 (4.3) ²	1 (4.3) ²
	12	26	3 (11.5) ²	0 (0.0)	1 (3.8) ²
	14	21	2 (9.5) ²	1 (4.8)	2 (9.5)
S-adenosyl-L-homocysteine	8	22	1 (4.5) ²	0 (0.0)	1 (4.5) ²
	10	26	3 (11.5) ²	0 (0.0)	3 (11.5)
	12	28	2 (7.1) ²	1 (3.6) ²	2 (7.1)
	14	21	1 (4.8) ²	0 (0.0)	2 (9.5)

1 per cent in parentheses

2 not significant compared to controls ($p < 0.05$)

3 not significant compared to analogues ($p < 0.05$)

TABLE 2
Frequency of Nephric Tubules

Type of Treatment	Concentration ($\mu\text{g/ml}$)	Number of PNPs	Number Differentiated
Control	-	56	4 (7.1)
Methionine	8	13	2 (15.4) ^{2,3}
	10	22	6 (27.3)
	12	25	9 (36.0)
	14	26	4 (15.4) ^{2,3}
S-adenosyl-L-methionine	8	21	5 (23.8)
	10	26	8 (30.8)
	12	30	7 (23.3)
	14	25	4 (16.0) ^{2,3}
Homocysteine	8	21	2 (9.5) ²
	10	23	1 (4.3) ²
	12	26	3 (11.5) ²
	14	21	1 (4.8) ²
S-adenosyl-L-homocysteine	8	22	1 (4.5) ²
	10	26	2 (7.6) ²
	12	28	2 (7.1) ²
	14	21	1 (4.8) ²

1 per cent in parentheses

2 not significant compared to controls ($p < 0.05$)

3 not significant compared to analogues ($p < 0.05$)

TABLE 3
Frequency of Cardiac Tissue

Type of Treatment	Concentration ($\mu\text{g/ml}$)	Number of PNPs	Number Differentiated
Control	-	56	2 (3.6)
L-Methionine	8	13	1 (7.7) ^{2,3}
	10	22	1 (4.5) ^{2,3}
	12	25	5 (20.0)
	14	26	1 (3.8) ^{2,3}
S-adenosyl-L-methionine	8	21	3 (14.3) ³
	10	26	5 (19.2)
	12	30	3 (10.0) ^{2,3}
	14	25	2 (8.0) ^{2,3}
DL-homocysteine	8	21	0 (0.0)
	10	23	1 (4.3) ²
	12	26	1 (3.8) ²
	14	21	0 (0.0)
S-adenosyl-L-homocysteine	8	22	0 (0.0)
	10	26	0 (0.0)
	12	28	2 (11.0) ²
	14	21	1 (6.7) ²

1 per cent in parentheses

2 not significant compared to controls ($p < 0.05$)

3 not significant compared to analogues ($p < 0.05$)

TABLE 4
Liquid Scintillation Data

Test Agent	Sample ¹ Number	Counts per minutes (CPM) ²		
		Protein	DNA	RNA
L-Methionine	1	21,695.03	332.50	1,196.31
	2	18,000.59	286.50	576.40
	3	4,127.61	354.60	1,308.58
	4	1,546.90	284.75	786.75
	5	8,400.28	239.15	897.75
	6	10,221.21	216.10	970.25
S-adenosyl-L-methionine	1	18,210.11	77.85	268.15
	2	6,381.85	95.60	341.80
	3	3,690.72	76.05	125.35
	4	27,996.39	94.90	111.80
	5	4,460.47	87.20	305.80

1 each sample represents 3 PNPs

2 all values reported are significantly higher than background (48.45 cpm) levels at values of $p < 0.05$.

DISCUSSION

Morphological differentiation in cultured post-nodal pieces (PNP) of chick blastoderms has been investigated using the biological methyl donors, L-methionine (Met) and S-adenosyl-L-methionine (SAM). Results of the present study indicated that methyl groups induce significant differentiation of both mesodermally- and ectodermally-derived structures, including neural tube, somites, nephric tubules, notochord, and cardiac tissue (which was actively pulsating at the time of fixation).

Both methyl donors contain a sulfur atom adjacent to the methyl group, which is reduced to a sulfhydryl moiety upon donation of the methyl group. Since sulfhydryl groups are known to induce differentiation (Chauhan and Rao, 1970; Lee and Kalmus, 1975), it was necessary to test the sulfhydryl-terminal demethylated analogues of Met and SAM. In both cases, the analogues (DL-homocysteine and S-adenosyl-L-homocysteine, respectively) caused significantly less differentiation than the corresponding methyl donors. It thus appears that methyl groups play a greater role in differentiation than sulfhydryl groups.

The analogues are also known for their capacity to inhibit SAM-mediated methylation reactions, both in vivo and in vitro (Chang and Coward, 1975; Borchardt, 1977). The evidence from the present study indicates that, in some cases, the analogues do, indeed, prevent differentiation or, at least, fail to induce significant differentiation compared to the controls (Table 1-3).

Once methyl groups were found to cause morphological differentiation, radiotracer studies were used to localize the methyl groups and

determine specific points of action within the cell. Evidence reported in this study indicated that the methyl groups consistently exerted much of their influence on the nucleus. In fact, results of autoradiography showed that labeled methyl groups were preferentially located in the region of the nucleus. In some cases, the exposed silver grains appeared to be associated with heterochromatin just inside the nuclear envelope but, due to the difficulty of focusing on the emulsion and cells simultaneously, the outline of the nucleus was only partially discernible.

To determine which specific fraction (DNA, RNA, or protein) of the cell was most significantly labeled, liquid scintillation counting was used. Since Met is an essential amino acid, most of the label should be incorporated into the protein fraction, with no label being incorporated into the nucleic acid fractions unless the DNA or RNA themselves were specifically methylated. Also, since a major role of SAM is to be decarboxylated and then used in polyamine biosynthesis (Pezzoli *et al.*, 1978), most of its label would be expected to remain in the protein fraction. However, the present study showed that both the DNA and RNA fractions contained significant amounts of label when compared to controls (Table 4).

Results reported here from both the morphological and radiotracer studies suggest a strong correlation between differentiation and methylation of nucleic acids.

Many investigators have reported that differential regulation of transcription is the underlying cause of differentiation in eukaryotes (Davidson, 1976). Other investigators have shown that nucleic acid methylation provides a mechanism for control of transcription (Holliday

and Pugh, 1975; Scarano et al., 1977). However, little evidence has accumulated to date which shows a direct link between nucleic acid methylation and differentiation. Before such a link can be discussed, a review of possible mechanisms of methyl group function is in order.

Once the methyl group is transferred to the nucleic acid base, its mechanism of action would involve either (1) introduction of conformational alterations of the bases, (2) alteration of recognition and binding properties, or (3) replacement of native bases.

In support of the first alternative, conformational stability has been shown to be greater in methylated polynucleotides than in similar methyl-deficient sequences (Borek and Srinivasan, 1966).

Second, numerous studies have shown that the 5-position substituent on the pyrimidine ring is responsible for histone binding and recognition. In fact, electronegative substituents, such as bromine or methyl groups, substantially increase regulator protein binding (Lin and Riggs, 1971, 1972; Matthes et al., 1977; Schwartz, 1977). Also, codon recognition properties are known to be altered by methylation (Capra and Peterkovsky, 1968).

Third, base replacement has been shown to occur by one of two methods: enzymatic alteration (Holliday and Pugh, 1975; Scarano et al., 1977) or tautomeric shifts (Jawetz et al., 1978). In either case, the result would be a transition replacement of CG pairs by AT pairs, and vice versa.

All three mechanisms would provide a functional alteration of the chromatin so that regulatory molecules could approach the nucleic acids only under certain conditions. The conformational alterations would be

necessary to expose segments of DNA to polymerases. Increased histone binding would prevent access at points along the DNA where transcription is not needed, as in the case of repetitive nucleotide sequences (Davidson and Britten, 1973). Altered codon recognition would result in mispairing and possible insertion of a different amino acid into new protein. If the different amino acid could significantly alter the secondary structure of the protein at that point, and if that point happened to be in the active site, the specificity of the enzyme could be drastically altered. Base replacement could result in similar effects.

Another function of methylation would be as a simple marker to provide a recognition site for the initiation or termination of enzyme activities associated with the polynucleotide.

The process of differentiation involves sequential and differential activation (Holliday and Pugh, 1975; Davidson, 1977) or repression (Caplan and Ordahl, 1978) of the genome, presumably by mechanisms which directly alter nucleic acid structure and functions (e.g., methylation). Control of differentiation would thus involve selective methylation of the nucleic acids, resulting in changes in transcription and translation. These changes would then lead to production of new gene products which are characteristic of specific differentiated cell types. The overlap of molecules distributed among these differentiated cell types indicates that the program of genetic expression in the development of a particular cell is a selection from the general genetic repertoire rather than completely distinct for each cell. Selective and sequential methylation provides a mechanism for this selection.

The results of this study using the methyl donors, L-methionine and S-adenosyl-L-methionine, and their demethylated analogues, DL-homocysteine and S-adenosyl-L-homocysteine, respectively, indicate that, regardless of the mechanism, methylation plays an important role in regulation of gene expression, thus substantiating the current concept of methylation as a regulator of embryonic development.

SUMMARY AND CONCLUSIONS

1. Methyl-mediated morphological differentiation has been investigated using cultured post-nodal pieces (PNPs) of chick blastoderms.
2. The biological methyl donors, L-methionine and S-adenosyl-L-methionine, elicited significantly more differentiation of recognizable structures than was observed in the controls.
3. The highest frequency of recognizable differentiated structures was observed in PNPs treated with 12 $\mu\text{g/ml}$ of L-methionine and 10 $\mu\text{g/ml}$ of S-adenosyl-L-methionine.
4. The demethylated analogues of L-methionine and S-adenosyl-L-methionine (DL-homocysteine and S-adenosyl-L-homocysteine, respectively) showed little, if any, significant differentiation of recognizable structures when compared to the controls, and significantly less than that exhibited by the methyl donors.
5. The types of differentiated structures induced by the methyl donors included the following: neural tissue, notochord, somites, nephric tubules, and cardiac tissue.
6. Results of autoradiographic studies indicated that labeled methyl groups were preferentially localized in the area of the nucleus.
7. Results of liquid scintillation counting showed that both DNA and RNA fractions were significantly labeled above control values.
8. Quantitative colorimetric determinations of DNA, RNA, and protein fractions showed that PNPs treated with any of the test agents remained viable during the entire culture period.

9. Current concepts regarding methylation, differentiation, and regulation of the eukaryotic genome were discussed in relation to the evidence reported in the present study.

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APPENDIX

INDUCED PULSATILE TISSUE IN CHICK EMBRYO EXPLANTS

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The present study was initiated to investigate the inducing ability of sulfur-containing amino acids. The sulfur atom in glutathione and cysteine is associated in the form of a sulfhydryl group. Methionine contains the sulfur atom as a sulfur-methyl moiety. To test the inducing capacity of sulfur-containing amino acids, post-nodal pieces (PNPs) of stage 3⁺ chick blastoderms were treated with 10 µg/ml of test agent and cultured for a total of 4 days on whole-egg-extract Ringer-agar medium at 37.5°C. At the end of the incubation period, PNPs were fixed and processed for microscopic and qualitative analyses. Pulsating heart tissue was used as an indicator tissue for the presence of differentiation. Glutathione induced pulsating heart tissue in 41% of treated PNPs, cysteine in 37% and methionine in 18%. These results tend to indicate that sulfur-containing amino acids play a role in the differentiation process. (Supported by Sigma Xi grant)

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FATE OF METHYL GROUPS IN THE POST-NODAL
PIECES OF EARLY CHICK EMBRYOS

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Methyl groups have been implicated as potential inducers of cellular differentiation due to their ability to induce single base changes in DNA sequences. Post-nodal pieces (PNPs), obtained from stage 3⁺ chick blastoderms, were used as a test model. L-methionine and S-adenosyl-L-methionine were tested for their inducing capacity by donation of the S-methyl group. DL-homocysteine and S-adenosyl-L-homocysteine, respectively, were used as controls. Significant morphological differentiation, indicated by recognizable axial structures, was observed as a result of treatment with methyl donors. No comparable differentiation was observed in the untreated controls or de-methylated analogue treated PNPs. Autoradiography was employed to examine the fate of methyl groups within the cell. L-methionine and S-adenosyl-L-methionine, labeled with tritium at the S-methyl group, were used for this study. Nuclear areas showed the heaviest amount of label. In addition, liquid scintillation counting was used to follow the fate of methyl groups in DNA, RNA and protein fractions extracted from PNPs by a perchloric acid procedure. DNA and RNA fractions were labeled significantly above background. The results of this study indicate that methylation of nucleic acids may result in cellular differentiation. (Supported by Sigma Xi grant)

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METHYLATION IN REGULATION OF THE EUKARYOTIC GENOME

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The occurrence and functional significance of methylated bases have been studied to some extent in nuclear DNA of eukaryotic cells. Furthermore, the possible role of DNA methylation has been correlated with the regulation of transcription leading to cell differentiation. In the chick embryo methyl groups have been reported to induce the formation of neural and cardiac tissues. Post-nodal pieces (PNPs) of stage 3 chick blastoderms were selected as ideal tissues to use for the study of cell differentiation. PNPs were cultured by the method of Spratt for 4 days. Treatment with various concentrations of active methyl donors, such as L-methionine and S-adenosyl-L-methionine, resulted in various degrees of differentiation. Localization of labeled methyl groups was accomplished using autoradiography. On the basis of the observed differentiation a mechanism is proposed to account for the location and function of the methyl groups within the nuclear material. Currently two theories for the control of the eukaryotic genome (differential gene activation and irreversible gene repression) are recognized. Results are discussed relative to these theories, with methylation suggested as a potential regulatory mechanism in cell differentiation. (Supported by Sigma Xi grant)

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THE ROLE OF METHYL GROUPS IN DIFFERENTIATION
OF CARDIAC TISSUE IN CULTURED CHICK EMBRYO EXPLANTS

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Methyl groups have been implicated in the process of differentiation. L-methionine and S-adenosyl-L-methionine, both known methyl donors, were used at various concentrations to treat post-nodal pieces (PNPs) of the chick blastoderm. DL-homocysteine and S-adenosyl-L-homocysteine, demethylated derivatives of L-methionine and S-adenosyl-L-methionine, respectively, were used as controls. PNPs were obtained by incubating fertile White Rock Cross eggs for 18 hrs and transecting isolated blastoderms at 0.6 mm posterior to Hensen's node. PNPs were cultured for 4 days on whole egg extract-Ringer agar medium and treated prior to incubation with a drop (0.03 ml) of chick Ringer's solution containing test agents. Control PNPs were similarly treated but without test agent. Differentiation was defined in functional terms as pulsatile tissue. Morphological differentiation was confirmed by light and electron microscopies. PNPs treated with methyl donors showed significant amounts of pulsatile (pre-cardiac) tissue when compared to control PNPs. These findings suggest that methyl groups are necessary for differentiation in this model. (Supported by Sigma Xi grant)

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