19*S* to 7*S* antibodies during the humoral immune response is unknown. The present results indicate that it can be blocked by 6-mercaptopurine and in such a "blocked" preparation the normal sequence of events is promptly restored by the administration of specific, reactive 7*S* antibody. How the 7*S* antibody acts in this manner is unknown and our data do not permit a choice among several possibilities. The advantage of using the 6-mercaptopurine-treated rabbit in the study of this conversion is that the early phases of this sequence are highly exaggerated and appear to be suspended in time. Thus, ample opportunities are available for attempting to modify or restore the sequence.

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Alkylation of Synthetic Polynucleotides

Abstract. *The synthetic polynucleotides may be used as models for the effects of alkylating agents on nucleic acid. Alkylation of polyadenylic acid with methyl methanesulfonate yields a polymer methylated in the one-position of adenine. This alteration in structure produces marked changes in physical properties and decreases the capacity to code for polylysine synthesis.*

There have been numerous studies of the effects of alkylating agents on nucleic acids and on their constituent bases, nucleosides, and nucleotides. Reiner and Zamenhof (*1*) and Lawley and Wallick (*2*) have isolated derivatives of guanine after the treatment of isolated deoxyribonucleic acids with dimethyl sulfate. Lawley and Brookes (*3*) isolated other derivatives of guanine, as well as derivatives of adenine, cytosine, and uracil, after similar reactions. Extensive studies of the methylation of purine nucleosides have been reported recently by Jones and Robins (*4*); their paper also reviews the occurrence of methylated bases in nucleic acids.

A systematic study of the effect of alkylating agents on synthetic polynucleotides, however, can provide much additional information on the mode of action of these compounds. Through such studies, it is possible to obtain more definite information on the nature and cause of changes in the physical properties of the polymers, as well as direct information on the biological effects of alkylation. Thus, synthetic polynucleotides that code for protein synthesis can be alkylated, and the resulting effects on their coding properties can be determined. In this paper, we report studies of this type on the methylation of polyadenylic acid.

Polyadenylic acid obtained commercially was further purified by phenol extraction, and exhaustive dialysis against 0.05*M* NaCl–0.015*M* sodium citrate solution, 0.01*M* NaCl, and glass-distilled water. Alkylations were carried out at 20°C and *p*H 7; the *p*H was maintained in the absence of buffer by an automatic titrimeter. Methyl methanesulfonate ($MeMeSO_3$) was used as alkylating agent, and the extent of alkylation was controlled by varying concentration of reagents and time of reaction (Table 1). At the termination of the reaction, cacodylate buffer (*p*H 7) was added to an ionic strength of 0.2, and the polymer was precipitated by the addition of four volumes of cold 95 percent ethanol. The precipitate was washed with ethanol, redissolved in glass-distilled water, and lyophilized.

Methylation occurred in the 1-position of adenine, as shown by the identification of 1-methyl adenine in the acid-hydrolysate. The polymer was dissolved in 1*N* HCl at a concentration of 10 mg/ml and heated in a sealed tube at 100°C for ½ hour. The hydrolysate from this treatment was chromatographed on Whatman No. 1 filter paper in the two descending solvent systems that were employed by Brookes and Lawley (*6*). Only one derivative was detected, although two faint additional spots were found in methylated polymer No. 3 after brief periods of hydrolysis. The derivative had a mobility, relative to adenine, of 1.3 in solvent A (methanol, concentrated HCl, water, 7:2:1) and of 0.7 in solvent B (*n*-butanol saturated with water-aqueous ammonia, 100:1), as compared to values of 1.3 and 0.8 found by Brookes and Lawley for 1-methyladenine.

Table 1. Methylation of polyadenylic acid.

Methylated polymer No.	Poly A (mg/ml)	$MeMeSO_3$ (mg/ml)	Reaction time (min)
1	1.33	6.2	66
2	1.22	9.7	186
3	1.10	25.3	470

Separate portions of the acid hydrolysates were chromatographed in solvent A on Whatman filter paper No. 40, and both adenine and derivative spots were eluted in cacodylate buffer (*p*H 7, ionic strength 0.2). Spectra determined in this solvent on a Beckman DU spectrophotometer against appropriate paper blanks agreed closely with the spectrum of 1-methyladenine, as presented by Brookes and Lawley. The percentage of adenine substituted in each polymer was calculated on the assumption that the molar absorptions of adenine and 1-methyladenine are equal at 260 mμ. These percentages are shown in Table 2.

The percentage of hypochromicity at 260 mμ, which reflects the degree of secondary structure in a general way, was determined by alkaline hydrolysis in the following manner. Stock solutions of polymer were prepared at room temperature in glass-distilled water at a concentration of 0.5 mg of polymer per milliliter; these were agitated gently for 4 hours and then centrifuged at 3000*g* to remove particulate matter, although no polymer gel was visible. Duplicate portions (0.11 ml) of each polymer were (i) hydrolyzed with 1 ml of 1.1*N* NaOH at 37°C for 14 hours and (ii) diluted to 5 ml in cacodylate buffer (*p*H 7.0, ionic strength 0.2) for immediate determination of absorption at 260 mμ. Base hydrolysates were neutralized, diluted in the same fashion, and com-

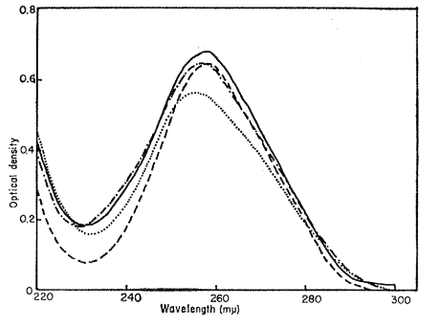

Fig. 1. Ultraviolet absorption spectra of polyA and samples of methylated polyA. The optical density scale has been adjusted to produce an optical density of 1.0 in the hydrolyzed polymer at 260 mμ. PolyA, ——; Me-polyA No. 1, ----; Me-polyA No. 2, -·-·-; Me-polyA No. 3, ···.

pared with the original (column 2 of Table 2). Exact duplication of details in this procedure is important in obtaining reproducible results.

Control experiments on acid hydrolysates showed that the base treatment introduced negligible change in the absorption of the mixture of monomers at 260 mμ, although there were other spectral changes consistent with the reported rearrangement of 1-methyladenine to 6-methylaminopurine (*6*). Accordingly, the comparison of polymer absorption with monomer absorption reflects true differences in hypochromicity among the methylated polymers. The degree of hypochromicity of the methylated polymers and of the original polyA decreased gradually when absorption was followed as a function of increasing temperature; some thermal instability was encountered on repeated heating to 90°C, however.

Complete spectrums of the original and methylated polymers determined on a Beckman DU spectrophotometer are reproduced in Fig. 1. The absorption of each polymer has been adjusted in this figure so that all of the base hydrolysates would have an optical density of 1.0 at 260 mμ on this scale. Figure 1 shows clearly the increased hypochromic effect produced by methylation and, in addition, shows a slight shift in the absorption peak to lower wave lengths with increased alkylation. Changes in spectrum between 220 and 250 mμ appear to reflect both differences in hypochromicity and absorption by the methylated bases. Thus, increased methylation may decrease the absorption in this region because of the increased hypochromicity of the polymer, or may increase the absorption because of the higher molar absorption of the methylated bases.

Sedimentation coefficients were determined in cacodylate buffer (*p*H 7, ionic strength 0.2) on a Spinco analytical ultracentrifuge equipped with ultraviolet absorption optics. The original polyA and the lightly methylated polymers all had sedimentation coefficients near 5.5, while the heavily methylated polymer (Me-polyA No. 3) had a coefficient of 8.9. The sample of polyU, the sedimentation coefficient of which is reported, was used in the melting experiments described below.

Equimolar amounts, on a nucleotide basis, of polyU and the various samples of polyA were mixed in 0.15*M* NaCl–0.015*M* sodium citrate solution, *p*H 7.1. Melting behavior (*7*) was determined in the usual way by following the optical density at 260 mμ as a function of temperature. All polyA-polyU mixtures showed a typical sudden increase in optical density at a critical temperature, the melting point. Methylation produced a substantial decrease in this temperature (column 5 of Table 2).

Ability of the polymer to code for polylysine synthesis in the *Escherichia coli* system was determined at 37°C, according to standard techniques (*8*). Results are quoted as millimicromoles of lysine incorporated per milligram of ribosomal protein at a polymer concentration of 80 μg per milliliter of reaction mixture. The marked decrease in coding ability that results from methylation is immediately apparent from these data.

It is clear, therefore, that methylation produces changes in both physical and biological properties of the synthetic polynucleotides. The increased hypochromicity and sedimentation coefficients of the alkylated polymers reflect an increase in coiling similar to that observed in alkylated DNA by Lett *et al.* (*9*). Such an effect could result from a decrease in the net charge of the molecule at *p*H 7. Brookes and Lawley (*6*) report *p*K values of 7.2 and 11.0 for 1-methyladenine, as compared to 4.2 and 9.8 for adenine.

There is a good possibility, therefore, that more of the alkylated bases are protonated at neutral *p*H and that the net charge on the polymer is thereby diminished. Protonation of unalkylated polyA is known to occur (*10*) at approximately *p*H 5 under these conditions and to produce a marked increase in helical structure, but titration studies on the alkylated polymer probably are necessary to clarify this point.

Since methylation occurs at a site participating in the hydrogen-bonding of adenine, its capacity to form complexes with opposing bases should be diminished. This effect is seen clearly in the lowering of the polyA-polyU melting temperature and probably explains the loss of coding ability of the methylated polymers. As shown by Szer and Ochoa (*11*), however, changes in secondary structure also may affect coding.

The quantitative loss of coding capacity is rather striking. Methylation of 3.6 percent of the bases reduces the incorporation of lysine to one-sixth that of the control, while methylation of 13.5 percent of the bases reduces this figure to 5 percent of control values. Similar large effects have been reported by Wahba *et al.* (*12*) for the substitution of 10 percent of bases in polyU by methyluracil or xanthine in a uracil-methyluracil or uracil-xanthine copolymer.

The significance of this work in relation to the presence of methylated

Table 2. Properties of methylated polyadenylic acids.

Polymer	Substitution (%)	Hypochromicity* (260 mμ) (%)	$S^{20,w}$	PolyA–polyU melting point (°C)	Coding capacity† (mμmole)
Control poly A	0	34.0	5.4	67.9	2.53
Me-polyA (1)	0.7	37.1	5.8	65.0	2.34
Me-polyA (2)	3.6	37.7	5.3	65.5	0.37
Me-polyA (3)	13.5	47.3	8.9	57.0	0.12
PolyU			4.5		

* (Optical density of alkaline hydrolysate minus optical density of polymer) × 100/optical density of alkaline hydrolysate. † Millimicromoles of lysine incorporated per milligram of ribosomal protein.

bases in naturally occurring nucleic acids and to the mode of action of clinical alkylating agents remains to be determined.

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Independent Action of Allele-Specific Suppressor Mutations

Abstract. *Two different allele-specific suppressor genes had an independent and nonadditive effect in restoring indoleglycerol phosphate → L-tryptophan activity in a double mutant strain lacking this activity.*

Suppressor mutations are those mutations in one gene which reverse the effects of mutations in another gene. Among the many different types of suppressor mutations are a class known as allele-specific suppressor mutations. This type of suppressor change appears to reverse the effects of only certain mutations in another gene. Examples of this type of specific suppressor effect have been reported in studies with the system described in this report, the tryptophan synthetase system of *Escherichia coli* (*1*, *2*).

The enzyme complex, tryptophan synthetase, is composed of two separable proteins subunits (*3*), designated A and B. Together, these proteins catalyze the following three reactions (*3*).

Indole + L-serine → L-tryptophan (In → Tryp) (1)

Indoleglycerol phosphate ⇄ indole + 3-phosphoglyceraldehyde (2)

Indoleglycerol phosphate + L-serine → L-tryptophan + 3-phosphoglyceraldehyde (InGP → Tryp) (3)

Many mutant strains of *E. coli* (A mutants) have been isolated which produce an altered A protein, designated A-CRM (cross-reacting material), which reacts with antibody to the normal A protein (*4*). All of the A-CRM studied to date can combine with the normal B subunit, and this complex can catalyze reaction 1, but not the other two reactions.

As reported previously, the effect of allele-specific suppressor mutations on CRM-producing A mutants and on A mutants lacking CRM was to restore small amounts of reaction 3 activity to mutant strains lacking this activity (*2*) (Table 1). The restored activity was shown to be associated with a small amount of *su*-A protein, which was similar in its properties to wild-type A protein (*2*). In the one mutant which was fully examined, the *su*-A protein was shown to differ from the corresponding CRM A protein by a single amino acid change (arginine → glycine) in its primary structure (*2*). From this observation and the extreme allele-specificity of different suppressor mutations, it appeared that each suppressor mutation had a specific and independent action in altering the primary structure of the A protein. In view of this conclusion, it was of interest to determine if two different suppressors in the same strain would have independent effects on the amount of active *su*-A protein that was formed.

A stock carrying two mutations in the A gene [CRM-producing double mutant A-11-36 (*5*)] plus the suppressors of each of these A mutations was prepared by transduction techniques for which phage Plkc was used (*6*). This stock was identified as the double mutant, double suppressor strain (designated A-11-36 su_{11}, su_{36}) on the basis of the following findings: (i) Lysates of Plkc prepared on this strain would only transduce the A-11 strain to a slow-growing *tryp*$^+$ type, identical to the A-11 su_{11} strain; (ii) the same lysates would only transduce the A-36 strain to a faster-growing *tryp*$^+$ type, identical to the A-36 su_{36} strain; (iii) the same lysates would not transduce the A-11-36 strain to any *tryp*$^+$ type.

These results confirmed that both the su_{11} and su_{36} loci were in this stock and indicated that these genes could not be co-transduced, since *tryp*$^+$ types were not found when A-11-36 was used as a recipient strain.

Two different isolates of the A-11-36 su_{11}, su_{36} strain were prepared and were grown on minimal medium and analyzed in duplicate for their A protein activities in reactions 1 and 3. The ratio of the activity of the A protein in these two reactions was considerably less in the double suppressor strain

Table 1. The enzymatic activities of the A proteins in extracts of various mutants and suppressed mutants. Abbreviations: In, indole; Tryp, tryptophan; InGP, indole-glycerol phosphate; su_{11}, su_{36}, the allele-specific suppressor genes that suppress the A-11 and A-36 mutations, respectively.

Strain	A protein activity of extracts *				InGP→Tryp/ In→Tryp activity ratio (%)
	In → Tryp		InGP → Tryp		
	Amount (units/ml)	Specific activity (units/mg)†	Amount (units/ml)	Specific activity (units/mg)†	
Wild type	75	2.5	30	1.0	40
A-36	725	22.0	<0.1	<0.003	
A-36 su_{36}	225	9.0	7.0	.28	3.2
A-11	1020	31.0	<0.1	<.003	
A-11 su_{11}	1380	55.0	12.0	.48	0.87
A-11-36	960	29.0	<0.1	<.003	
A-11-36 su_{11}, su_{36}	1640	41.0	1.60	.04	0.09

* Assayed in the presence of excess B protein. † Units per milligram of protein.