# Electron Microscopic Study of Base Sequence in Nucleic Acids. VII. Cytosine-Specific Addition of Acyl Hydrazides*

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ABSTRACT: Malonyl hydrazide reacts with cytidine 5′-monophosphate (CMP) and desoxycytidine 5′-monophosphate (dCMP) in aqueous solutions with a pH optimum of 4.2 giving addition products. Adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), uridine 5′-monophosphate (UMP), and corresponding nucleosides and deoxynucleotides do not react the same way. Acetyl hydrazide and 3,4-dicarboxybenzoylhydrazine (DCBH) enter similar reactions although the low solubility of the latter makes long reaction times necessary. 3,5-Disulfobenzoylhydrazine (DSBH) couples to CMP with a lower rate constant than do the other hydrazides studied. Polycytidylic acid (poly C) can be converted to a polymer in which 70% of the residues have been converted to the addition product with malonyl hydrazide. When ribonucleic acid (RNA) is so treated 85% of the cytosine residues can be converted to the addition product. The other bases are not altered.

The significance of these results in our electron microscopic study of nucleotide sequence are discussed.

Some time ago Beer and Moudrianakis (1962) proposed an electron microscopic procedure for the study of the sequence of bases of nucleic acids. The method requires that markers be found which are chemical groups selective in their attachment to the bases and detectable with the electron microscope. In the earlier papers we have discussed markers suitable for guanine in DNA (Moudrianakis and Beer, 1964a,b), another for labeling both guanine and uracil in RNA or guanine and thymine in DNA (Fiskin and Beer, 1965), and one suitable for thymine in DNA (Beer *et al.*, 1966; P. J. Highton, B. L. Murr, F. Shafa, and M. Beer, to be submitted).

In the present paper and in the earlier ones we have studied selective reagents which also carry anionic groups. These can be made to react with heavy cations and so rendered detectable in the electron microscope.

The present study concerns the reactions of acyl hydrazides with nucleic acids and their components. It is shown that these reagents couple more readily with CMP[1] than with the other nucleotides. Also malonyl hydrazide can be coupled with great selectivity to the cytosine base of RNA and it is possible to obtain a high degree of labeling of this base. The reactions reported here were suggested by the closely analogous cytosine-specific coupling of some semicarbazides to RNA discovered by Ukita and his collaborators (Hayatsu and Ukita, 1964; Kihugawa *et al.*, 1967).

## Materials

Acetyl hydrazide was obtained from Aldrich Chemical Co. Malonyl hydrazide was obtained by the method of Curtius and Sieber (1921). DCBH was obtained by diazomethane esterification of trimellitic anhydride in tetrahydrofuran followed by reaction with hydrazine. The ester was obtained in nearly quantitative yield (mp 103–105°). The anhydride groups were hydrolyzed by refluxing in water giving a white product (mp 145–147°). Neutralization of an aqueous solution with $NaHCO_3$ was followed by refluxing in hydrazine for 12 hr as described by Sah and Chang (1936). The resulting solution was dried over $H_2SO_4$ and the hydrazide was crystallized from water–ethanol mixture. The product formed a precipitate with salicylaldehyde and had the electrophoretic mobility of benzenedicarboxylic acid at pH 6.8.

DSBH (purchased from K & K Fine Chemicals) was converted to the trichloride by heating for 2 hr with $PCl_5$ at 145° (Bourdais and Meyer, 1961). This product was obtained in nearly quantitative yield and had a melting point of 90°. Refluxing 24 hr with excess

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[1] Abbreviations used: AMP, adenosine 5′-monophosphate; GMP, guanosine 5′-monophosphate; CMP, cytidine 5′-monophosphate; UMP, uridine 5′-monophosphate; DCBH, 3,4-dicarboxybenzoylhydrazine; DSBH, 3,5-disulfonic benzoylhydrazine.

methanol (Van Duin, 1921) and drying *in vacuo* gave the ester which on refluxing with hydrazine hydrate and hydrolysis in water gave 3,5-disulfobenzoylhydrazide.

*Anal.* Calcd: C, 24.72; H, 1.78; N, 8.12; S, 18.84. Found: C, 24.76, 24.51; H, 2.04, 2.05; N, 8.18, 8.13; S, 18.29, 18.46.

2-$^{14}$C-labeled deoxycytidine and $^{14}$C-labeled ribo- and deoxyribonucleoside 5′-monophosphates (2-$^{14}$C-labeled CMP, dCMP, UMP, dTMP, and 8-$^{14}$C-labeled GMP, dGMP, AMP, dAMP) were obtained from Schwarz Biochemicals, Inc. Poly C was obtained from Miles Chemical Co., Elkhart, Ind. $^{32}$P-labeled MS2 RNA was extracted according to the method of Fiers *et al.* (1965) from MS2 virus kindly supplied by Dr. Nathans. Pancreatic RNase was obtained from Boehringer & Soehne, Mannheim.

## Methods

*Rates of Reactions of Acyl Hydrazides with Nucleosides and Nucleotides.* Dry $^{14}$C-labeled nucleotides or nucleosides (0.4 $\mu$c) were dissolved in 45 $\mu$l of a solution of acyl hydrazide at the desired pH and concentration. For adjusting the pH acetic acid was used. The reaction mixtures so obtained were incubated at the desired temperature. For analysis 1-$\mu$l aliquots were separated by electrophoresis on Whatman 3MM paper either in 0.02 M phosphate buffer at pH 6.8 or in 0.02 M acetate buffer at pH 3.5. Sometimes the aliquots were analyzed by descending paper chromatography using the isopropyl alcohol–ammonia solvent of Hayatsu and Ukita (1964). The radioactivity was determined by scanning on a Nuclear-Chicago Actigraph scanner.

*Effect of Tempertaure on Reaction Rate of Deoxycytidine with Malonyl Hydrazide.* $^{14}$C-labeled deoxycytidine solution (1.5 $\mu$c) was dried, 18 mg of cold nucleoside was added, and the mixture was dissolved in 4 ml of 2 M solution of malonyl hydrazide at pH 4.2. The solution was divided into three portions and incubated at 37, 59, and 70°, respectively. At discrete time intervals 15-$\mu$l aliquots of solution were spotted on 3MM paper and separated by chromatography in the isopropyl alcohol–ammonia solvent, and the paper was scanned.

*Effect of the Concentration of Malonyl Hydrazide on Rate of Reaction with CMP.* $^{14}$C-labeled CMP (0.4 $\mu$c) was dried and redissolved in 45 $\mu$l of 2 and 0.2 M solutions of malonyl hydrazide at pH 4.2. The solutions were incubated at 37° and at different times 1-$\mu$l portions were separated by paper electrophoresis and scanned.

*Spectrum of the Reaction Product of CMP and Malonyl Hydrazide.* To 1 $\mu$c of a dried solution of $^{14}$C-labeled CMP were added 2 mg of cold CMP and 150 $\mu$l of 2.7 M malonyl hydrazide at pH 4.2. The solution was incubated at 37° for 96 hr. The pH was adjusted to 7.9 by addition of $Ba(OH)_2$ (Hayatsu and Ukita, 1964). Insoluble $Ba(OH)_2$ was removed by centrifugation. Two volumes of ethanol was added to precipitate the nucleotide. The deposit was washed with 55% ethanol and absolute ethanol. It was redissolved in 100 $\mu$l of $H_2O$ and electrophoresed on paper at pH 3.5. The strip was scanned, the separated ultraviolet-absorbing bands were eluted, and their spectra were taken.

*Reaction of DSBH with CMP.* To 0.4-$\mu$c portions of dried $^{14}$C-labeled CMP was added 50 $\mu$l of 1 M (maximum concentration) solution of DSBH at pH 4.0, 4.5, and 5.3. The solutions were incubated at 37°, aliquots were electrophoresed at pH 7, and the radioactivity was scanned as before.

*Reactions of Polynucleotides with Acyl Hydrazides.* POLY C PLUS MALONYL HYDRAZIDE OR ACETYL HYDRAZIDE. Poly C was dissolved in a solution of 3 M malonyl hydrazide or acetyl hydrazide. The pH was adjusted with concentrated acetic acid to 4.2 so that the final concentration of poly C was 1–2 mg/ml in a 2 M solution of acetyl hydrazide. The solution was incubated at 37°. Samples were taken out at discrete time intervals and dialyzed against $H_2O$ in the cold to remove excess of reagent. Subsequently the poly C was hydrolyzed by incubation with RNase at pH 5.8 for 8 hr at 37°. The hydrolysate was concentrated under vacuum and 10-$\mu$l aliquots were separated by paper electrophoresis in 0.04 M acetate buffer at pH 6.8 or 3.5. The resulting spots were eluted and the phosphorus content was determined by the method of Chen *et al.* (1956).

*Reaction of Malonyl Hydrazide with RNA.* To 1 ml of an aqueous solution of $^{32}$P-labeled MS2 RNA (300,000 cpm) was added 7 mg of cold yeast RNA. The RNA was precipitated with two volumes of alcohol from a solution 0.2 N in NaCl and then was redissolved in 0.9 ml of $H_2O$. The solution was made 0.2 M in malonyl hydrazide and incubated at 45° for 15 min, subsequently made 2 M in malonyl hydrazide at pH 4.2, and incubated at 37° for 90 hr.

To analyze the extent of reaction at discrete intervals samples of RNA reaction mixtures were dialyzed against 0.2 M NaCl solutions in the cold for removal of excess reagent. The RNA was then precipitated by addition of two volumes of ethanol and hydrolyzed by incubation in 0.15 N KOH at 37° for 14 hr. The hydrolysate was neutralized with $HClO_4$ at 0°, then 10 $\mu$l of hydrolysate was separated by paper electrophoresis at pH 3.5. The activity of the resulting spots was again obtained by scanning on the Actigraph scanner.

*Repeated Reaction of RNA with Fresh Reagent.* To 1.2 ml of aqueous solution of $^{32}$P-labeled MS2 RNA ($1.5 \times 10^6$ cpm ml) was added 8 mg of yeast RNA. After alcohol precipitation, the RNA was redissolved in 1 ml of 0.2 M malonyl hydrazide and incubated for 15 min at 45°. The solution was made subsequently 2 M in malonyl hydrazide at pH 4.2 and incubated at 37°. After 24 hr one-fifth of the solution was removed and analyzed for extent of reaction as described above. The remainder was dialyzed against two changes of $H_2O$ for 2 hr. The dialyzed reaction mixture was made 2 M in fresh malonyl hydrazide at pH 4.2 and incubated again. This operation of substituting reagent with fresh malonyl hydrazide was repeated two more times. At every reagent exchange a sample of the

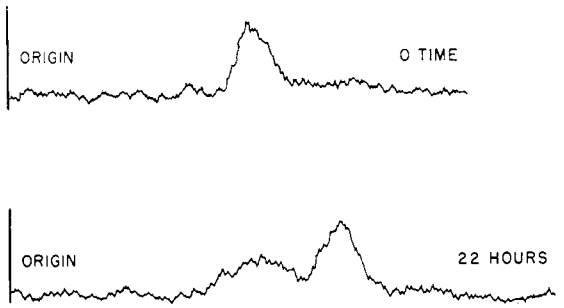


FIGURE 1: Scan of radioactivity along chromatographic chart of reaction mixture containing [2-$^{14}$C]CMP and malonyl hydrazide at pH 4.2 at zero time and 22 hr of incubation at 37°. Solvent isopropyl alcohol–ammonia–water (7:1:2). The product has a lower mobility than the unreacted nucleotide.

reaction mixture was removed and analyzed. In some experiments the reaction was carried out at an elevated temperature. Then 0.4 ml of aqueous $^{32}$P-labeled MS2 RNA ($1.5 \times 10^6$ c/mole ml) was diluted to 2 ml and made 2 M in malonyl hydrazide at pH 4.2. The solution was incubated at 55° and samples were taken at discrete intervals and analyzed for extent of reaction as already described.

## Results

*Selectivity of the Reaction of Malonyl Hydrazide with the Nucleotides.* On incubating malonyl hydrazide with some of the nucleotides a new radioactive spot appeared in the chromatographic or electrophoretic strip (see Figures 1 and 2). The activity of the spot increased with time at the expense of the unreacted material. The area under the new peak was taken as a measure of the extent of reaction. In Figure 3 the extent of reaction of malonyl hydrazide with the four ribonucleotides is given as a function of time.

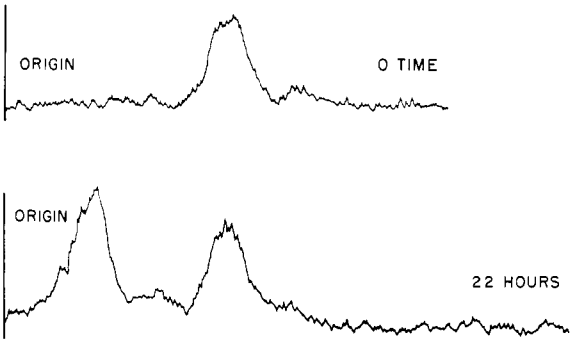


FIGURE 2: Scan of radioactivity of electrophoretic chart of same reaction mixture as in Figure 1. The buffer pH was 6.8. The greater mobility of the product than of the nucleotide suggests a greater negative charge presumably because of the added carboxyl group.

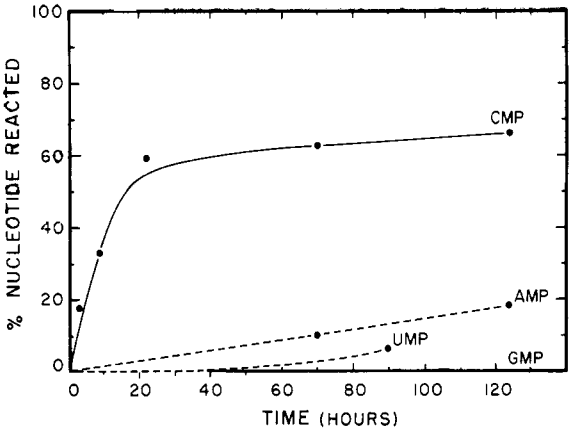


FIGURE 3: Reaction rates of ribose nucleotides with malonyl hydrazide. Reaction mixtures contained $2 \times 10^{-4}$ M $^{14}$C-labeled nucleotides, 2 M malonyl hydrazide, at pH 4.2 incubated at 37°. Aliquots were analyzed by paper electrophoresis and chromatography. Addition products are formed only with CMP (solid line). After 18 hr the reaction nearly stops. AMP, GMP, and UMP formed no addition products. After long times with UMP and AMP spots appeared which did not move in electrophoresis (broken lines).

With CMP the new product in electrophoresis moved toward the anode more rapidly than the unreacted nucleotide (Figure 2), suggesting a greater negative charge probably resulting from the coupling of the carboxyl group of malonyl hydrazide to CMP. Compared with the other nucleotides this is by far the most rapid reaction. During the first 18 hr about 60% of the CMP is converted to the product bearing a greater negative charge. On further incubation the reaction is found to be much slower.

The other nucleotides formed no product which moved more rapidly in electrophoresis. After very long times of incubation, however, in the reaction mixtures containing AMP and UMP products appeared which showed no mobility on electrophoresis at pH 6.8. Their formation is indicated in Figure 3. On the basis of mobility in descending paper chromatography using butanol–water solvent the electrophoretically immobile material obtained with AMP was shown to be largely adenosine with about 10% adenine. This hydrolysis of the nucleotide at pH 4.2 was found to occur also in the absence of the acyl hydrazide and agrees with the earlier findings of Baddiley *et al.* (1958).

On incubating the desoxynucleotides with malonyl hydrazide, dCMP gave a product which on chromatography in isopropyl alcohol–ammonia (Hayatsu and Ukita, 1964) moved more rapidly than the unreacted nucleotide and was clearly resolved from it. In a reaction time of 65 hr, 63% of the radioactivity was in this spot, as was also the case for CMP. During this time dGMP suffered a depurination of 47% and dAMP of 11%.

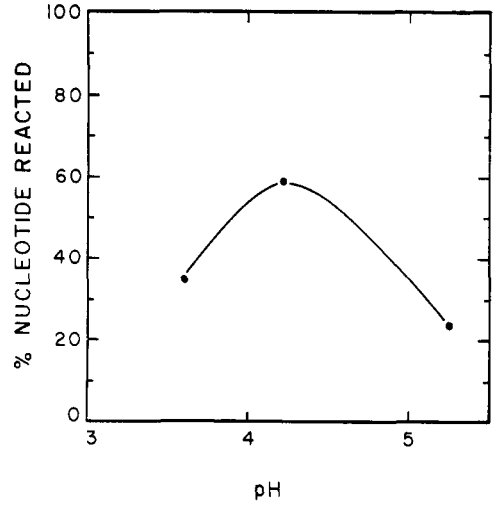


FIGURE 4: Dependence on pH of the reaction rate of CMP with malonyl hydrazide was measured by the extent of reaction after 20 hr at 37°. Content of reaction mixtures and analysis as in Figure 7. Optimal pH is 4.2.

*Effect of pH on Reaction Rate of Malonyl Hydrazide with CMP.* Figure 4 shows the dependence of the reaction rate on pH. The curve is based on the extent of reaction at 20 hr. The reaction is highly pH dependent with the optimum at about 4.2. At pH 5 and 3.5

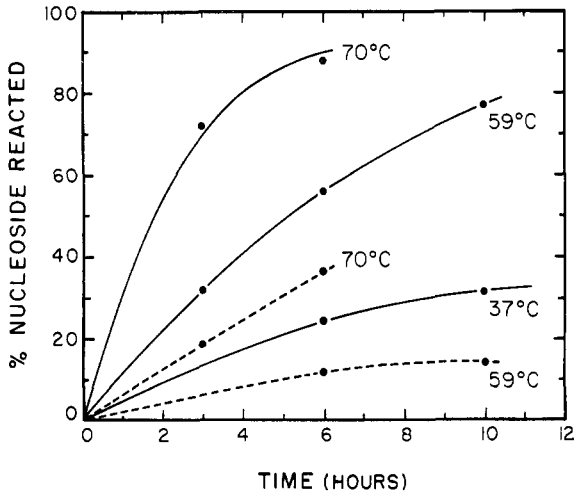


FIGURE 5: Reaction rates of CMP with malonyl hydrazide as a function of temperature. Reaction mixture contained $2 \times 10^{-4}$ M [$2^{14}$-C]CMP and 2 M malonyl hydrazide at pH 4.2. Reaction mixtures were analyzed by chromatography on Whatman 3MM paper in isopropyl alcohol–ammonia–water (7:2:1) and scanned. After incubation a new peak with lower mobility, representing the addition product, appeared (solid lines). At the higher temperature an intermediate peak appeared representing presumably a breakdown product (broken lines).

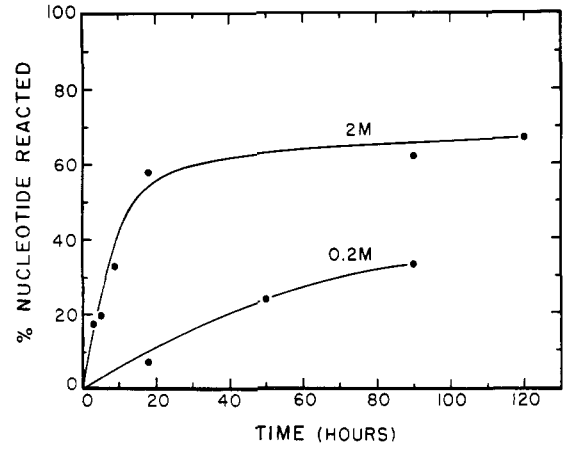


FIGURE 6: Reaction rate of CMP with malonyl hydrazide as a function of malonyl hydrazide concentration. Reaction mixtures contained $2 \times 10^{-4}$ M CMP and 2 and 0.2 M malonyl hydrazide at pH 4.2, respectively. The reaction mixtures were incubated at 37° and analyzed by paper electrophoresis at pH 7. The decrease in rate of reaction observed at the higher concentration is not observed at 0.2 M malonyl hydrazide.

the extent of reaction for the same incubation period is approximately one-half of that at pH 4.2. The optimal pH found for this reaction is identical with that found by Hayatsu and Ukita for the semicarbazide coupling reaction.

*Effect of Temperature on Reaction Rate of Malonyl Hydrazide with Desoxycytidine.* The reaction of desoxycytidine was studied at 37, 59, and 70°. The disappearance of the nucleoside is clearly faster at the higher temperatures as shown by the solid lines in Figure 5. The $Q_{10}$ of the reaction is roughly two. However, in addition to the product at higher temperatures, another material is also formed with a mobility in chromatography intermediate between that of the nucleoside and the low-temperature product found at 37°. Clearly at temperatures higher than 37° the reaction is more complicated, giving two products.

*Effect of Concentration of Malonyl Hydrazide on Rate of Reaction with CMP.* Figure 6 shows the dependence of the reaction on the concentration of malonyl hydrazide. It appears that the starting rate is approximately proportional to the concentration of the reagent over this concentration range. However, the abrupt reduction in rate observed at the higher concentration is not found at the lower concentration, indicating that the inhibition responsible for it is somehow dependent on the concentration of the reagent.

*Ultraviolet Absorption Spectrum of the Addition Product of CMP with Malonyl Hydrazide.* Figure 7 shows the ultraviolet absorption spectrum of the reaction product of CMP with malonyl hydrazide obtained by electrophoretic separation of a reaction mixture as described in Methods. To calculate the extinction coefficient the number of moles was inferred from the phos-

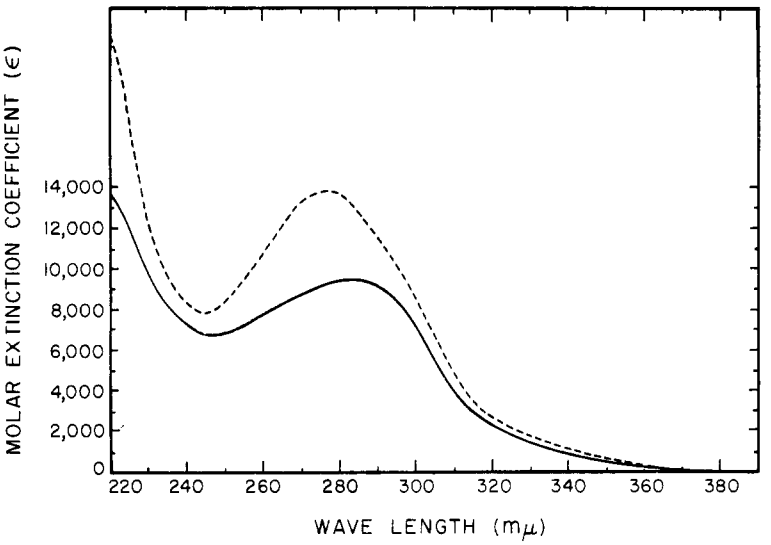


FIGURE 7: Ultraviolet absorption spectrum of the addition product of CMP with malonyl hydrazide (purified by electrophoresis) in 0.01 N HCl (pH 2). (————) addition product and (————) unreacted CMP.

phate analysis of the eluted material. The maximum, as compared with CMP, has shifted to a longer wavelength at 283 mμ; the molar extinction coefficient dropped to 9400.

*Reactions of CMP with Other Acyl Hydrazides.* CMP was found to react with acetyl hydrazide at a rate comparable to its reaction with malonyl hydrazide. After 70 hr about 60% of the nucleotide was converted to a new product. However, in electrophoresis at pH 6.8 the product did not separate from the nucleotide. A clear separation was possible by electrophoresis at pH 3.5. The product had an absorption maximum at 283 mμ ($\epsilon_{max}$ 8200).

DCBH was found to react with CMP but not with the other nucleotides. The product formed moved again more rapidly in electrophoresis at pH 6.8. However, the solubility of the reagent was low. The highest concentration which could be used was 0.12 M.

As is seen from Figure 8 the formation of product was much slower than for the more soluble malonyl hydrazide. Nevertheless, the rate constants were not appreciably different for the two reagents.

CMP was found to react with DSBH at pH 4.0. After 70 hr at 37° and a reagent concentration of 1 M, nearly 25% of the nucleotide had been converted to a product which moved faster in electrophoresis at pH 6.8 (Figure 9). At pH 4.5 the reaction was about three times slower and at pH 5.3 no product was detected in the same time.

The reaction of CMP with DSBH has a lower rate constant than with malonyl hydrazide, acetyl hydrazide, and DCBH. On the assumption that this was due to the ionized sulfonic groups we added cations to neutral-

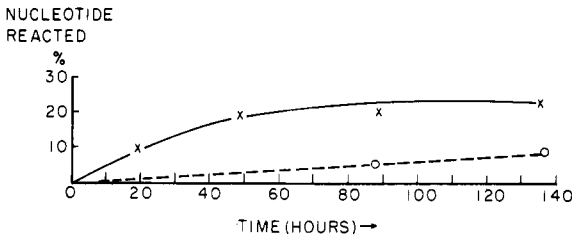


FIGURE 8: Reaction rate of CMP with DCBH. Reaction mixture contained 3 × $10^{-4}$ M 2-$^{14}$C labeled CMP in 0.12 M DCBH at pH 4.2. Reaction mixtures were incubated at 37° and analyzed by paper electrophoresis at pH 7 where a faster moving peak is observed. A breakdown product (interrupted line) which does not move in electrophoresis forms at the same rate as the addition product.

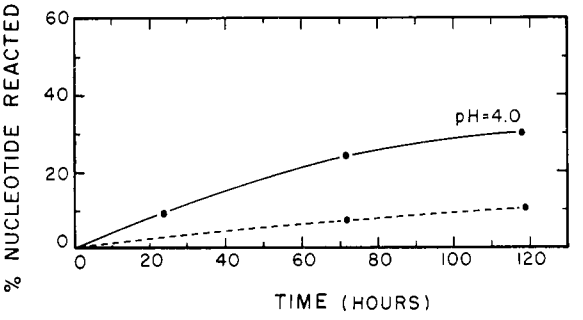


FIGURE 9: Reaction rate of CMP with DSBH. Reaction mixtures contained 2 × $10^{-4}$ M 2-$^{14}$C-labeled CMP in 1 M DSBH at pH 4. The solutions were incubated at 37° and analyzed by electrophoresis at pH 7. An addition product with higher electrophoretic mobility is formed (solid line). At the same time a breakdown product indicated by an immobile peak (broken line) is also formed.

ize them. However, in the presence of 1 M $CaCl_2$, $Mg(NO_3)_2$, or $CH_3COOTl$ the reaction occurred even more slowly.

*Reactions of Poly C with Acyl Hydrazides.* When poly C was incubated with malonyl hydrazide or acetyl hydrazide and subsequently hydrolyzed, electrophoresis again gave two spots similar to the ones obtained with CMP; using the phosphate analyses of the products obtained on electrophoresis of the hydrolysate it was found that with malonyl hydrazide in 60 hr 70% of the residues in poly C was converted to the addition products.

A sample of poly C in which 50 ± 3% of the residues had reacted with malonyl hydrazide to give the electrophoretically faster product gave on chemical analysis a N:P mass ratio of 1.56. Knowing that unreacted poly C gives N:P = 1.35 (experimentally observed 1.33) it follows that the 50 ± 3% of the reacted residues have an N:P ratio of 1.77 ± 10. The value of N:P obtained for four atoms of nitrogen per atom of phosphorus is 1.81 and so agrees with this number within experimental error. When the acetyl hydrazide was used the paper electrophoresis had to be carried out at pH 3.5 to obtain good separation of reacted from unreacted nucleotides. The results showed 78% reaction after 90 hr and 90% after 124 hr.

*Reaction of Malonyl Hydrazide with RNA.* We first wished to establish that alkaline hydrolysis does not give spurious values for the extent of reaction of the CMP moieties of the polymer. To do this a portion of poly C partly reacted with malonyl hydrazide was hydrolyzed by alkali and the remainder with RNase. On electrophoresis and analysis the two samples gave the same extent of reaction, showing that the addition product survives alkali hydrolysis. The extent of reaction with the cytidine residue in RNA was determined by separating the alkaline hydrolysate of the $^{32}P$-labeled RNA by paper electrophoresis at pH 3.5.

The scanning chart of the electrophoretic strip reveals five peaks; four of them correspond to the spots in unreacted RNA and are the four unreacted nucleotides AMP, GMP, and UMP and the unreacted portions of CMP. A fifth faster peak that is not present in the control is also found. The abundance of the unreacted CMP and that of the new peak adds up to that of CMP in the control. Also the mobility of the faster peak was the same as found for the product with CMP or the hydrolysate of altered poly C.

After 90-hr incubation at 37°, 45% of the CMP residues present in this RNA had reacted. There appeared to be also a loss of 15% in the AMP residues.

It is clear from the abrupt decrease in rate of reaction of malonyl hydrazide with CMP that after 20 hr the reagent somehow decays. In an attempt to obtain more efficient labeling of C in the RNA, therefore, in some of our experiments after a period of incubation the "aged" reagent was removed by dialysis and fresh reagent introduced into the reaction mixture as described in Methods. The results thus obtained are summarized in Table I, where we give the percentage of radioactivity found in each nucleotide after repeated incubations in fresh solutions of malonyl hydrazide. Clearly the major effect is the conversion of about 85% of the CMP residues to the faster moving product peak, $C^1MP$.

TABLE I: Abundance of Nucleotides in RNA Hydrolysate after Repeated Reaction with Malonyl Hydrazide at 37° (%).

| Nucleotide | 0 | 26 hr | 26 + 34 hr | 26 + 34 + 24 hr |
|---|---|---|---|---|
| CMP | 25 | 13 | 7 | 4 |
| AMP | 23 | 21 | 21 | 20 |
| GMP | 27 | 29 | 27 | 29 |
| UMP | 25 | 27 | 28 | 25 |
| $C^1MP$ | 0 | 10 | 17 | 22 |

TABLE II: Per Cent of Total Counts Found in Nucleotides in RNA Hydrolysate after Reaction with Malonyl Hydrazide at 55°.

| | Per Cent of Total Counts | | |
|---|---|---|---|
| Nucleotide | 0-hr Reaction | 3-hr Reaction | 6-hr Reaction |
| CMP | 25 | 9 | 5 |
| AMP | 23 | 15 | 15 |
| GMP | 27 | 31 | 32 |
| UMP | 25 | 22 | 22 |
| $C^1MP$ | 0 | 23 | 24 |

Table II gives the nucleotide analysis of RNA hydrolysates after 3 and 6 hr of reaction with malonyl hydrazide but at 55°. Again the CMP residues are largely converted to the product. In addition, however, at the elevated temperatures approximately 30% of the counts from AMP appears in the region of GMP.

## Discussion

We have shown that a number of acyl hydrazides react specifically with CMP, dCMP, and cytidine but not with the other nucleosides and nucleotides. Whenever these reagents have acid groups attached to the carboxyl group (as do malonyl hydrazide, DCBH, and DSBH) the product in electrophoresis at neutral pH moves more rapidly toward the anode than the nucleotide or nucleoside. If the reagent has no acid group attached to the carboxyl group (acetyl hydrazide) the electrophoretic mobility at neutral pH is the same for the reacted and unreacted nucleotide or nucleoside. This observation suggests that during the reaction the acid group becomes attached to the nucleoside or nucleotide. Finally, the molar ratio of P:N for the residues in poly C which had reacted with malonyl

hydrazide was 4, consistent with the coupling of hydrazide accompanied by elimination of ammonia. Such an interpretation is analogous to the results of Hayatsu and Ukita (1964) who showed the addition of certain semicarbazides to the C-6 of CMP and cytidine. The selectivity, approximate rate, pH optimum, and concentration dependence observed by them were nearly the same as found here for malonyl hydrazide and acetyl hydrazide. Thus the analogy between their reaction and the present ones appears very close. The electrophoretic sensitivity to charges on the reagent, the increased N:P ratio, as well as the similarity to the known semicarbazide addition reaction led us to the conclusion that the cytosine-specific reaction observed here is the coupling of the acyl hydrazide through its nitrogen to the C-6 of the cytosine accompanied by elimination of ammonia. The very slow reaction observed with AMP is not a coupling reaction but a hydrolytic one as is clear from the identification of the product as adenosine and the occurrence of the reaction even in the absence of acyl hydrazide.

The reactions observed with DCBH appeared similar to those with malonyl hydrazide and acetyl hydrazide. However, this reagent was much less soluble and so the conversion of CMP to the addition product proceeded much more slowly. The rate constants, however, were equal for these three reagents.

The rate constant for the reaction of DSBH is less than for the other reagents by a factor of about 2. This effect must result from the influence of the sulfonic groups which at pH 4 are ionized while the carboxyl groups of DCBH are not.

Our results show that in poly C a similar reaction occurs and at least 70% of the nucleotide residues can be reacted. This result indicates that two adjacent nucleotides can enter into reaction.

The reactions with RNA at 37° indicate that, replacing the reagent about every 24 hr, after 80 hr 85% of the CMP residues was converted to the addition product. The abundance of the other nucleotides was unchanged within experimental error. This level of marking is satisfactory for sequence analysis in that RNA so labeled when examined in the electron microscope leads to an error probability of 1 in 1000 bases after 20 micrographs are examined (Fiskin *et al.*, 1967).

When the reaction was run at 55° the CMP residue was converted more rapidly to the addition product. However at the same time some of the $^{32}P$ counts from AMP were converted to a product which had nearly the same mobility as GMP. This effect can be understood in terms of the cleavage of the adenosine phosphate bonds already shown previously. Similar scission in RNA would on hydrolysis yield adenosine and inorganic phosphate. The latter has nearly the same mobility in electrophoresis as GMP. In addition other side reactions are suggested by the excessively high value of the sum of the counts from CMP and $C^1MP$. These effects were not explored further; nevertheless, it is clear that in experiments where chain breakage is to be avoided the coupling should be performed at low temperatures.

The coupling of acyl hydrazides resembles the semicarbazides in forming addition compounds with cytidine alone among the common nucleosides. This high specificity makes them promising reagents in an electron microscopic study of base sequence.

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