The exonuclease-rich fractions from the previous step were chromatographed on hydroxylapatite (step III, Figure 13). An excellent separation of the phosphatase and exonuclease activities was obtained. The high-eluting side of the exonuclease activity peak still contained, however, some phosphatase activity and was therefore rechromatographed on hydroxyapatite (Figure 14). Interestingly enough, the exonuclease activity was eluted in a series of peaks, suggesting a heterogeneity in the enzyme molecules. The enzyme, as obtained by this method, is extremely stable at 4° in alkaline buffers. After dialysis against 0.1 M Tris-HCl (pH 9.0) in order to eliminate inhibitory phosphate, the low-eluting part of the enzyme from the rechromatography on hydroxylapatite (these fractions were used in all the work described above) showed no phosphatase activity under conditions where 2×10^{-4} phosphatase unit/exonuclease unit could have been detected.

This novel procedure has two main advantages over the method most recently developed in Laskowski's laboratory (Richards *et al.*, 1967): (a) the purification only involves chromatographic steps, avoiding cumbersome precipitations with acetone and ethanol at −17°; (b) enzyme yield is extremely high: 23% of the exonuclease activity present in the venom is obtained in an essentially phosphatase-free form after step III; an additional 35% of the activity can be obtained at an even higher level of purity after one more hydroxylapatite step.

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Serologic Specificities of Methylated Base Immune Systems*

Lawrence Levine,† Helen Van Vunakis,‡ and Robert C. Gallo

ABSTRACT: Rabbits immunized with conjugates of 1-methylguanosine–human serum albumin, N^2-dimethylguanosine–albumin, 7-methylguanosine–albumin, N^2-methylguanosine–albumin, and 5-methylcytidine–albumin produce antibodies that recognize the position of the methyl group(s) on the base. These serologic specificities were determined by gel diffusion experiments in agar containing an excess of the carrier albumin, thus eliminating the albumin antigen–antibody reaction. A minor population of the antibodies in several of the serums recognizes the normal bases. When measured by complement fixation, these latter antibodies are eliminated by dilution.

Hapten inhibition of complement fixation confirms the specificities of these immune systems.

The production of antibodies with narrow specificity toward nucleic acids is observed only in the T-even bacteriophage DNA (Murakami *et al.*, 1962; Townsend *et al.*, 1965), irradiated DNA (Levine *et al.*, 1966; Van Vunakis *et al.*, 1966), and double- and triple-stranded polyribonucleotide immune systems (Stollar, 1970). With the exception of the "conformation" specificity of antibodies to the multistrand polyribonucleotides, narrow specificities in nucleic acid immune systems result from the immunodominance of "unique" bases that either occur in nature or are produced by a chemical modification of a normal base, Thus, specificity of anti-bacteriophage DNA and the anti-irradiated DNAs has been shown to be due to the glucosylated hydroxymethylcytosine in the T-even coliphage DNAs and to the guanine photoproducts resulting from photooxidation of DNA (Van Vunakis *et al.*, 1966; Levine *et al.*, 1968) or thymine dimers resulting

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† American Cancer Society Professor of Biochemistry (Grant No. PRP-21); to whom to address correspondence.

‡ Public Health Service Research career awardee (Award No. 5-K6-AI-2372).

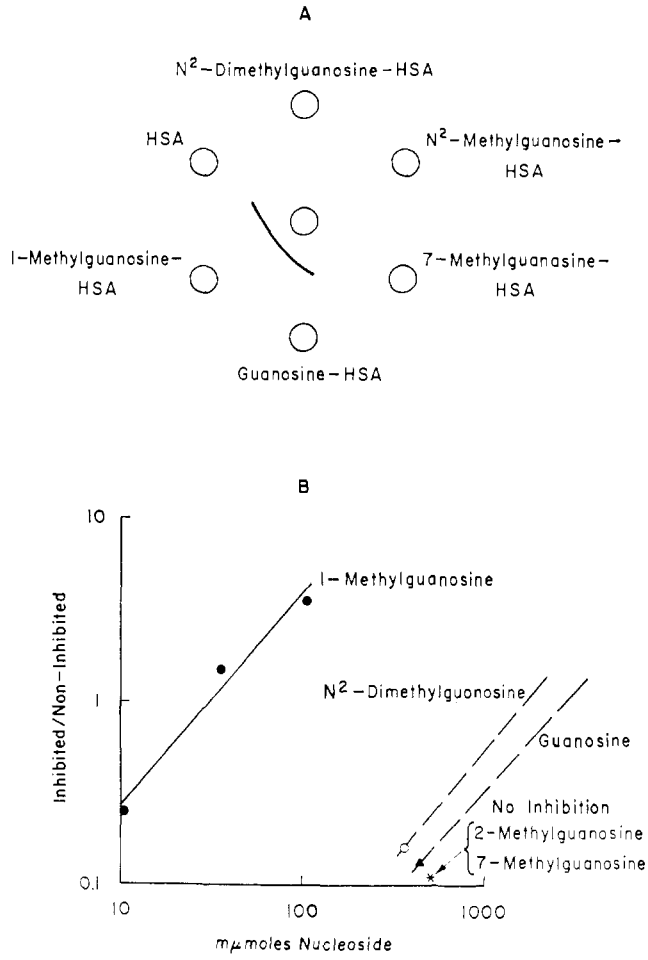

FIGURE 1. (A) Tracing of bands of precipitation seen in agar after double diffusion for 24 hr at room temperature. The agar contained human serum albumin (1 mg/ml). The antiserum (undiluted, Ra 505) is in the center. The surrounding antigen wells contained the albumin–riboside conjugates at 500 μg/ml. (B) C′-fixation inhibition of the 1-methylguanosine immune system by various ribosides. The C′-fixing system being inhibited was rabbit anti-1-methylguanosine (Ra 505-Cl, diluted 1/1200) and 1-methylguanosine–albumin (0.2 μg).

from ultraviolet irradiation of DNA (Levine *et al.*, 1966; Seaman *et al.*, 1968).

Among the naturally occurring minor bases, those that are methylated at different positions on the purine or pyrimidine nucleus should be readily identifiable by specific antibodies. Recent reports indicate that some tRNAs of some tumor tissues differ from normal tissues both qualitatively and quantitatively (Gallo and Pestka, 1970; Taylor *et al.*, 1968; Gonano and Chiarugu, 1969; Baliga *et al.*, 1969). If these differences are due to the distribution of the methylated bases between the normal and tumor tissues, as suggested by some reports (Bergquist and Matthews, 1962; Viale and Restelli, 1967; Viale *et al.*, 1967; Craddock, 1969), specific antibodies could be used to quantitate each methylated base in tissue extracts and biological fluids when measured by inhibition in a radioimmunoassay system.

We report here the production in rabbits of antibodies to 1-methylguanosine, N^2-methylguanosine, N^2-dimethylguanosine, 7-methylguanosine, and 5-methylcytidine. These antibodies are directed in part toward the methyl group as well as to the position of that methyl group on a particular base.

Materials and Methods

The methylated nucleosides used for synthesis of the immunizing antigens and for inhibition of complement (C′) fixation were purified by descending paper chromatography on Whatman No. 3MM paper in three systems: (1) isobutyric acid–H_2O–NH_4OH (99:42:4, v/v) containing 1 mM EDTA; (2) ethanol–ammonium acetate (pH 7.5) with 1 mM EDTA; and (3) isopropyl alcohol–H_2O–ammonia (850:150:13, v/v). In these systems only one ultraviolet-absorbing spot was identified. The ultraviolet-absorbing areas were cut out and the nucleosides eluted from the paper with H_2O and concentrated by lyophilization. The spectral characteristics were: 5-methylcytidine (pH 7.0) 250/260 1.04, 280/260 1.47, 290/260 1.1; 1-methylguanosine (pH 1.0) 250/260 0.84, 280/260 0.7, 290/260 0.51; 7-methylguanosine (pH 1.0) 250/260 0.85, 280/260 0.68; N^2-methylguanosine (pH 1.0) 250/260 0.79, 280/260 0.55, 290/260 0.52; N^2-dimethylguanosine (pH 1.0) 250/260 0.57, 280/260 0.55, and 290/260 0.47.

The immunizing antigens were synthesized by the procedure of Erlanger and Beiser (1964). Absorption spectra of the synthesized conjugates after exhaustive dialysis showed that in all cases successful synthesis has been achieved.

Antiserums to each nucleoside human serum albumin (hereafter simply called "albumin") conjugate were prepared in New Zealand albino rabbits (one rabbit for each immunogen) by immunization with complete Freund's adjuvant, according to the following schedule. Approximately 5 mg of each conjugate in 1.0 ml (pH 7.0) of 0.1 M phosphate buffer was emulsified with 1.0 ml of adjuvant. Animals were given the material by injection into the toepads and intramuscularly once weekly for 3 successive weeks and bled from the ear 1, 2, and 3 weeks subsequent to the final injection. Antiserums from individual bleedings were frozen and kept at −20°. The serums were examined by agar gel diffusion and by C′ fixation, according to Levine (1967).

Results

Double diffusion in agar with each of the antiserums to the nucleoside–albumin conjugates and albumin alone revealed a variable but significant amount of antibody to albumin. The antibodies to albumin could effectively be removed by absorption with albumin. Quantitative C′-fixation experiments with each antiserum, nucleoside–albumin conjugate, and albumin alone clearly demonstrated that most of the antibodies in the anti-nucleoside–albumin conjugates were directed toward the nucleosides. For example, with an antiserum to albumin–1-methylguanosine, a complete C′-fixation curve could be obtained with the homologous conjugate or with albumin at antiserum dilutions of $^1/_{1600}$ and $^1/_{100}$, respectively. At least *with respect to C′-fixing activity*, there were 16 times more antibody directed toward the nucleoside than toward the native conformation of the carrier, albumin. This difference between the C′-fixing activities of the antibodies to conjugate and the albumin carrier alone differed among the antiserums, but in no case was this difference less than sixfold. Thus, C′-fixation inhibition data could be obtained with unabsorbed antiserum.

For evaluation of the specificities of these antibodies to nucleosides by agar diffusion, albumin (1 mg/ml) was incorporated into the agar. A boundary of precipitates appeared around the antiserum well. In all cases, absorption of anti-albumin was complete. All bands with each conjugate that were observed represented nucleoside precipitin reactions.

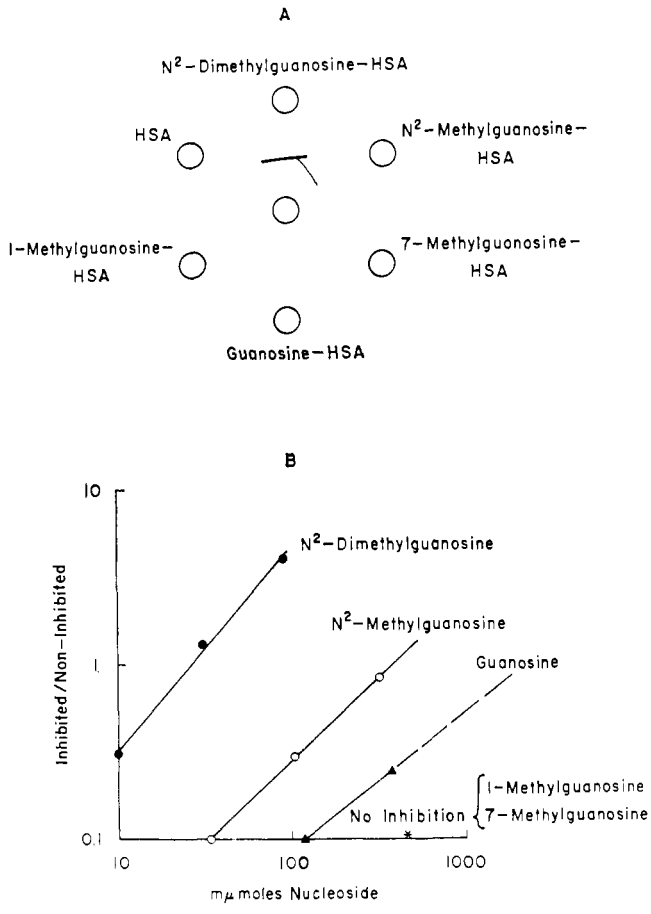

FIGURE 2: (A) Tracing of bands of precipitation seen in agar after double diffusion for 24 hr at room temperature. The agar contained human serum albumin (1 mg/ml). The antiserum (undiluted, Ra 540) is in the center. The surrounding antigen wells contained the albumin–riboside conjugates at 500 μg/ml. (B) C′-fixation inhibition by various ribosides. The C′-fixation system consisted of rabbit–anti-N^2-dimethylguanosine–albumin (Ra 540, diluted 1/1000) and N^2-dimethylguanosine–albumin (0.5 μg).

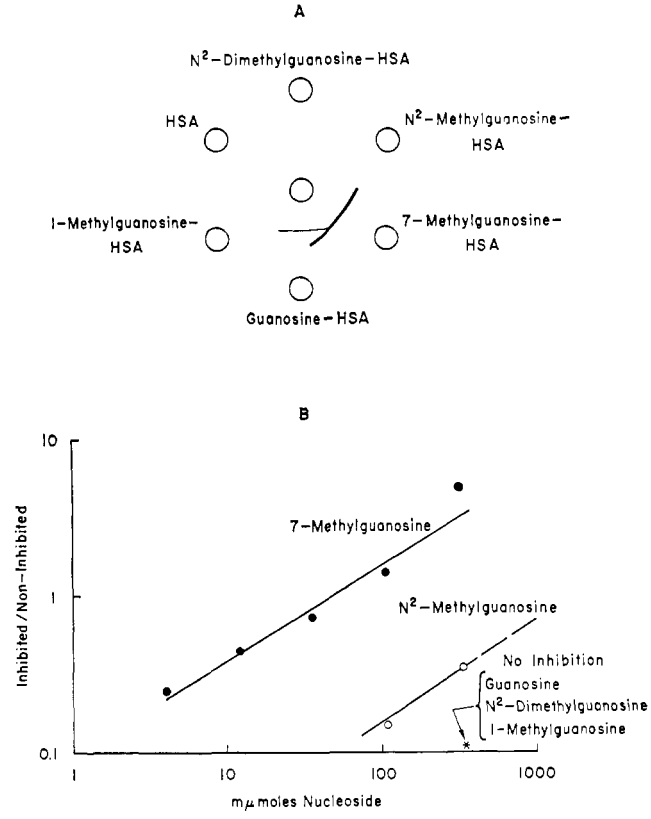

FIGURE 3: (A) Tracing of bands of precipitation seen in agar after double diffusion for 24 hr at room temperature. Albumin (1 mg/ml) was incorporated in the agar. The antiserum (undiluted, Ra 484) is in the center. The surrounding antigen wells contained the human serum albumin–riboside conjugates at 500 μg/ml. (B) C′-fixation inhibition by various ribosides. The C′-fixation system consisted of rabbit anti-7-methylguanosine–albumin (Ra 484, diluted 1/3000) and 7-methylguanosine–human serum albumin (0.2 μg).

Antibodies to 1-Methylguanosine. The conjugate, albumin–1-methylguanosine, emulsified with complete Freund's adjuvant, elicited in rabbits antibodies highly specific for 1-methylguanosine. The agar diffusion precipitin patterns and C′-fixation inhibition of the reaction between albumin–1-methylguanosine and anti-albumin–1-methylguanosine by various nucleosides are shown in Figure 1.

As measured by agar diffusion, the antibodies recognized the methyl group only on the C 1 position of the guanosine. The guanosine conjugate and the N^2-dimethyl-, N^2-methyl-, and 7-methylguanosine conjugates did not react with the anti-1-methylguanosine. This apparent specificity demonstrates the effectiveness of absorption by incorporation of albumin in the agar, since in its absence, bands of precipitation were observed with all conjugates as well as albumin, although a band of partial identity with the homologous conjugate was clearly visible.

When the antiserum was examined by inhibition of C′ fixation, this narrow specificity was confirmed. At the levels of nucleosides tested, only 1-methylguanosine was an effective inhibitor.

If the low inhibition values observed for N^2-dimethylguanosine and guanosine are significant and if the slope of the inhibition response curve remains constant, then 1-methylguanosine is at least two logs more effective. The maximum levels of nucleoside tested for inhibition were restricted due to their limited solubilities.

Antibodies to N^2-Dimethylguanosine. The data shown in Figure 2 demonstrate the narrow specificity of the N^2-dimethylguanosine immune system. However, the specificity is not as restricted as the 1-methylguanosine system. In agar diffusion experiments, only N^2-methylguanosine–albumin, among all the conjugates tested, cross reacts. This recognition of N^2-methylguanosine by anti-N^2-dimethylguanosine is also observed by C′-fixation inhibition. Although N^2-dimethylguanosine is the most effective inhibitor (50% inhibition with 50 mμmoles), N^2-methylguanosine also inhibits, but 800 mμmoles is required for 50% inhibition. From extrapolation of the curve, 5000 mμmoles of guanosine would be required for 50% inhibition. No inhibition by 1-methylguanosine or 7-methylguanosine at the 900-mμmole level was observed.

The reaction of partial identity observed in the agar diffusion experiments suggests that absorption of the antiserum to N^2-dimethylguanosine with N^2-methylguanosine–albumin will produce a serum monospecific for the N^2-dimethylguanosine residues.

Antibodies to 7-Methylguanosine. Only with the 7-methylguanosine immune system was the specificity deduced from agar diffusion and C′-fixation inhibition at variance (Figure 3). Agar diffusion experiments show cross-reactions with guanosine–albumin but not with N^2-methylguanosine–albu-

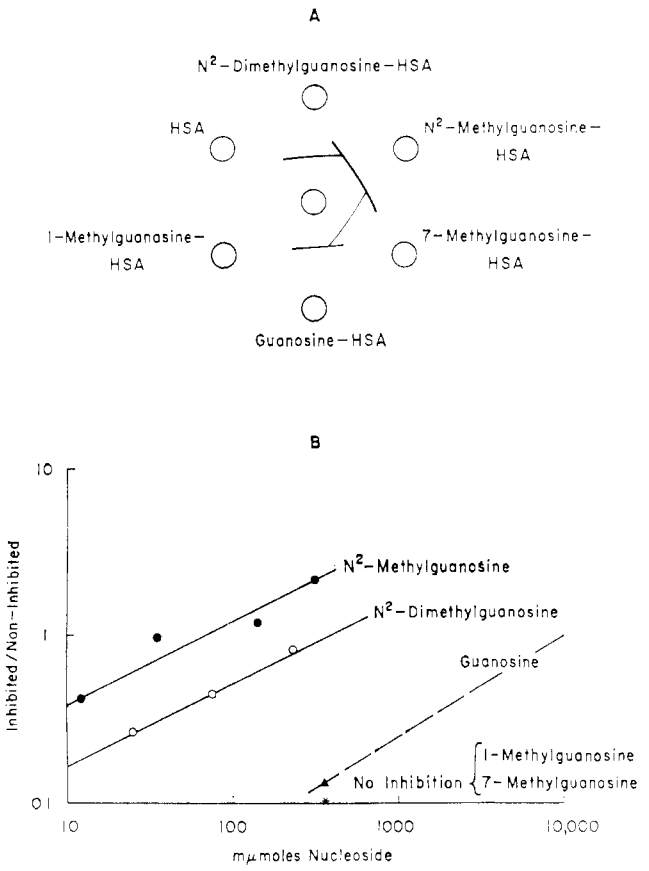

FIGURE 4: (A) Tracing of bands of precipitation seen in agar after double diffusion for 24 hr at room temperature. Albumin (1 mg/ml) was incorporated in the agar. The antiserum (undiluted, Ra 485) is in the center. The surrounding antigen wells contained the human serum albumin–riboside conjugates at 500 μg/ml. (B) C′-fixation inhibition by various ribosides. The C′-fixation system consisted of rabbit-anti-N^2-methylguanosine–human serum albumin (Ra 485, diluted 1/300) and N^2 methylguanosine–human serum albumin (1 μg).

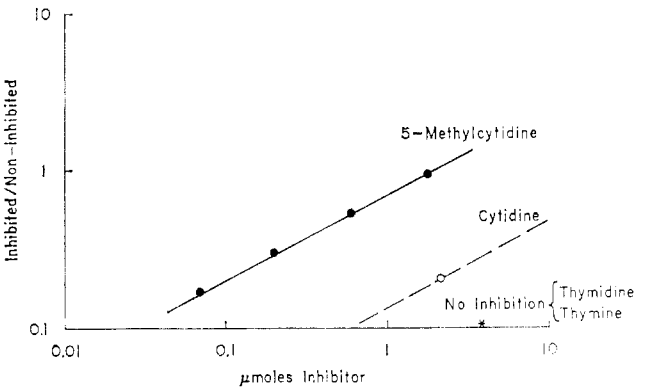

FIGURE 5: Inhibition of the 5-methylcytidine–human serum albumin C′-fixation system by 5-methylcytidine, cytidine, thymidine, and thymine. The C′-fixation system consisted of Ra 536, diluted 1/1500, and 5-methylcytidine–human serum albumin (0.5 μg).

min. In C′-fixation inhibition experiments, guanosine does not inhibit, at least at 400-mμmole levels, whereas N^2-methylguanosine shows some inhibition. Since quantitative aspects of C′-fixation may depend on the number of haptens per albumin (Reichlin *et al.*, 1965; Bethell *et al.*, 1968), C′-fixation inhibition would be a more reliable index of specificity. In any case, the specificity is again very narrow. *N*-Methylguanosine, which is the only heterologous nucleoside to inhibit, is effective only at 40 times the level of 7-methylguanosine. Again, the reaction of partial identity suggests the experimental approach for obtaining monospecificity with respect to the position of the methyl group on the guanosine, *i.e.*, absorption with the appropriate conjugate.

Antibodies to N^2-Methylguanosine. The broadest specificity among the methylated base immune systems studied was found with the N^2-methylguanosine antiserum (Figure 4). Agar diffusion experiments revealed reactions with N^2-methylguanosine–albumin, N^2-dimethylguanosine–albumin, guanosine–albumin, and 7-methylguanosine–albumin in decreasing order of precipitin intensity. Again, the various patterns of partial identities predict monospecificity after suitable absorptions. C′-fixation inhibition data, however, demonstrate that serologic specificity is narrow if methylation is not at the N^2 position. Thus, N^2-dimethylguanosine is seven times less effective an inhibitor than the homologous N^2-methylguanosine. Among 1-methylguanosine, 7-methylguanosine, and guanosine, only guanosine inhibits at the levels tested (an extrapolated value of 10,000 mμmoles would be required for 50% inhibition, a value about 50 times that required for equivalent inhibition by the homologous N^2-methylguanosine).

Antibodies to 5-Methylcytidine. Inhibition of C′ fixation between 5-methylcytidine-albumin and anti-5-methylcytidine-albumin by 5-methylcytidine, cytidine, thymidine, and thymine is illustrated in Figure 5. Only 5-methylcytidine and cytidine inhibited. The homologous 5-methylcytidine was 20 times more effective an inhibitor than cytidine. The methyl group on the 5 position of the thymidine had no effect serologically. Whereas the methylated guanosine immune systems were relatively easy to inhibit with the homologous nucleoside (50% inhibition ranging from 30 to 50 mμmoles), the methylated cytosine immune system was more difficult to inhibit with its homologous nucleoside (2000 mμmoles required for 50% inhibition). The reasons for these differences in effectiveness of inhibition are not clear at this time.

Discussion

It is clear that tumor cells (Tsutsui *et al.*, 1966; Hancock, 1968; Mandel *et al.*, 1969; McFarlane and Shaw, 1968; Mittleman *et al.*, 1967; Stewart and Corrance, 1969; Riddick and Gallo, 1970) and proliferative undifferentiated "blast"-type cells (normal or neoplastic) (Riddick and Gallo, 1970; Fujioka *et al.*, 1971; Hancock *et al.*, 1967; Rennert, 1970) contain increased levels of the group of enzymes that catalyze methylation of tRNA (tRNA methylases) compared to mature, differentiated, and nonproliferative tissues. Further, a few reports have indicated that the tRNAs themselves, in at least a few cases, may qualitatively differ between tumor cells and normal tissues (Taylor *et al.*, 1968; Gonano and Chiarugi, 1969; Baliga *et al.*, 1969; Gallo and Pestka, 1970). It has been proposed that aberrant methylation is the basis for these differences, presumably as a result of changes in tRNA methylases (Baliga *et al.*, 1969). Comparisons of the methylated bases between tumor and normal tissues have tended to substantiate these speculations (Bergquist and Matthews, 1962; Viale and Restelli, 1967; Viale *et al.*, 1967; Craddock, 1969). However, some controversy exists over this point (Randerath, 1971). Techniques used for the measurement of the methylated base content of tRNAs are usually laborious and relatively

insensitive, requiring large amounts of tissues. The use of more sensitive methods, *e.g.*, labeled methionine incorporation into tRNA, has been either impractical (requires living cells) or has been complicated by difficulties in interpretation (*e.g.*, pool size differences with the labeled methionine approach). The quantitative estimation of methylated bases by an immunoassay has the advantages of speed, sensitivity, relative ease of performance, and avoids the hazards of pool size variation, which is inherent in the labeled methionine incorporation approach.

If the tRNAs of neoplastic tissues, in fact, contain methylated bases, which differ either qualitatively or quantitatively from normal tissues, the amount of these bases and/or the ratio of one methylated base to another in biological fluids, such as blood, cerebrospinal fluid, or even urine, might be indicative of the presence and/or relative amount of the abnormal growth. Indeed, it is possible that the ratio of one base to another may even reflect the cellular origin of abnormal growth. These analyses are possible, since the turnover and catabolism of tRNA is such that purine and pyrimidine bases would be found in serum and urine. However, a more sensitive assay, capable of measuring these methylated bases in picogram quantities, will have to be developed, and attempts at this by radioassay are in progress.

In each of the five antiserums studied, antibodies that are specifically directed toward the methylated bases were present. This narrow specificity could have been predicted from the vast literature on serologic specificity. Nevertheless, the specificity for the methylated bases had to be documented.

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