Hartmann, G., Honikel, K. O., Knüsel, F., and Nüesh, J. (1967), *Biochim. Biophys. Acta 145*, 843.
Hinkle, D. C., and Chamberlin, M. J. (1970), *Cold Spring Harbor Symp. Quant. Biol. 25*, 65.
Johnston, D. E., and McClure, W. R. (1976), in RNA Polymerase, Losick, R., and Chamberlin, M. J., Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p 413.
Krakow, J. S., and von der Helm, K. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 73.
Liao, L. L., Horwitz, S. B., and Grollman, A. P. (1974), *Biochemistry 13*, 1331.
Lu, F.-J., and Anderson, S. R. (1973), *Biochemistry 12*, 3479.
Maitra, U., and Hurwitz, J. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54*, 815.
Nicholson, B. H. (1971), *Biochem. J. 123*, 117.
Pribnow, D. (1975), *J. Mol. Biol. 99*, 419.
Richardson, J. P. (1966), *Proc. Natl. Acid. Sci. U.S.A. 55*, 1616.
Riva, S., Fietta, A. M., and Sylvestri, L. G. (1972), *Biochem. Biophys. Res. Commun. 49*, 1263.
Rosen, C.-G., and Weber, G. (1969), *Biochemistry 8*, 3915.
Sadowski, P. D. (1971), *J. Biol. Chem. 246*, 209.
Schleif, R. (1969), *Nature (London) 233*, 1068.
Siddhikal, C., Erbstoeszer, J. W., and Weisblum, B. (1969), *J. Bacteriol. 99*, 151.
Weber, G., and Anderson, S. R. (1965), *Biochemistry 4*, 1942.
Weber, G., and Laurence, D. J. R. (1954), *Biochem. J. 56*, 31.
Wehrli, W., Driesel, F., Schmid, K., and Staehlin, M. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61*, 667.
Wehrli, W., and Staehelin, M. (1969), in RNA Polymerase and Transcription, Lepetit Colloquia on Biology and Medicine, Sylvestri, L., Ed., Amsterdam, North Holland Publishing Co., p 65.
Wu, C.-W., and Goldthwait, D. A. (1969), *Biochemistry 8*, 4450.
Wu, F. Y.-H., and Wu, C.-W. (1973), *Biochemistry 12*, 4343.

Lysine Transfer RNA_2 Is the Major Target for L-Ethionine in the Rat†

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ABSTRACT: Ethionine, a hepatocarcinogen, ethylates macromolecules in vivo especially tRNA of rat liver. When rats were injected with L-[*ethyl*-^{3}H]ethionine, the tRNA fraction of the liver was found to be labeled. One tRNA with the highest specific activity was purified and identified as lysine-$tRNA_2$.

It is well known that chemical carcinogens are highly reactive with some cellular macromolecules. Magee and Farber showed that tRNAs have a greater propensity for alkylation by some carcinogens than DNA (Farber and Magee, 1960; Magee and Farber, 1962). Whether the modification of tRNAs by chemical carcinogens has any relation to oncogenesis is obscure at present. However, all tumors of a wide spectrum of etiology contain a small number of isoaccepting tRNAs which are absent from the tissue of origin (Borek and Kerr, 1972). One of these tRNAs, $tRNA^{Phe}$, has been isolated from two different neoplastic tissues and each was found to contain three supernumerary modified bases (Kuchino and Borek, 1977). The interaction of the macromolecules with chemical carcinogens may be an initiating event in carcinogenesis.

Ethionine is a hepatocarcinogen in rats. Feeding of ethionine to rats results in the loss of two leucine tRNA species in the liver (Axel et al., 1967). These changes are evident much before the appearance of tumors. However, the two leucine tRNA species reappear (Yamane et al., 1976) on supplementation of the diet with copper, an inhibitor of ethionine carcinogenesis (Kamamoto et al., 1973). Ethionine interacts preferentially with liver tRNA (Farber and Magee, 1960; Farber et al., 1967; Ortwerth and Novelli, 1969), resulting predominantly in the formation of ethylated guanosines (Rosen, 1968; Pegg, 1972). However, whether the targets of the ethylation are random or specific in the population of tRNAs was obscure. We present here evidence that $tRNA^{Lys}_2$ is a specific target of alkylation by ethionine.

Materials and Methods

L-[*ethyl*-^{3}H]Ethionine (spec act. 70.1 Ci/mol) was purchased from New England Nuclear, Boston, Mass., ^{14}C- and ^{3}H-labeled L-amino acids were obtained from Amersham/Searle Corp., Arlington Heights, Ill., and RNase T_1, RNase A, RNase T_2, and bacterial alkaline phosphatase were products of Worthington Biochemical Co., St. Louis, Mo., Sigma Chemical Co., St. Louis, Mo., and Sankyo Co. Ltd., Tokyo, Japan.

Preparation of tRNA from Rat Liver after Exposure to L-Ethionine. Three female Holtzman rats (weighing 140 to 160 g) fasted overnight were injected intraperitoneally with 0.5 mCi of L-[*ethyl*-^{3}H]ethionine in 0.9% NaCl solution. After starvation for 24 h, the rats were sacrificed and the livers were rapidly removed. The tRNA was prepared by the methods described previously (Brunngraber, 1962; Zubay, 1962) and deacylated by incubation with 1.8 M Tris-HCl (pH 8.0) at 37

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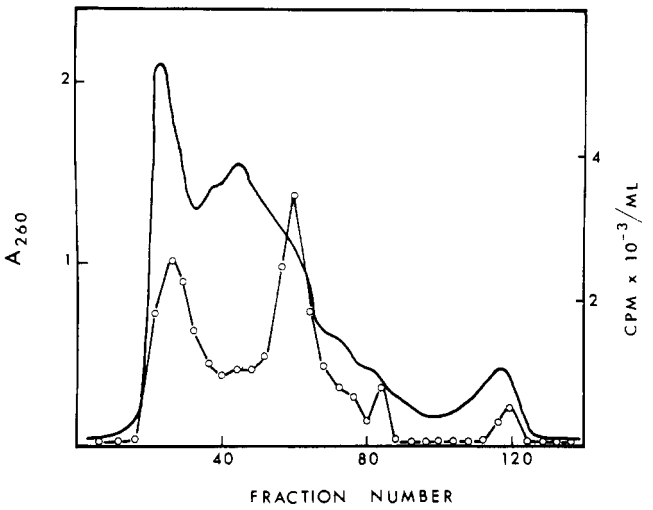

FIGURE 1: RPC-5 column chromatography of rat liver tRNA labeled with L-[*ethyl*-^{3}H]ethionine. The column (1 × 50 cm) was equilibrated with 100 mL of 0.01 M sodium acetate buffer (pH 4.7) containing 0.01 M $MgCl_2$, 0.002 M β-mercaptoethanol, and 0.4 M NaCl (buffer A). Unfractionated tRNA prepared from rat liver after 24 h of in vivo labeling was dissolved in 5 mL of buffer A and placed on a RPC-5 column. After the column was washed with 20 mL of buffer A, a linear gradient elution was carried out using 500 mL of 0.01 M sodium acetate buffer (pH 4.7), 0.01 M $MgCl_2$, 0.002 M β-mercaptoethanol, and 0.5 M NaCl in the mixing chamber, and 500 mL of 0.01 M sodium acetate buffer (pH 4.7), 0.01 M $MgCl_2$, 0.002 M β-mercaptoethanol, and 0.8 M NaCl in the reservoir. Each fraction contained 5 mL of the effluent. Aliquots (1 mL) of the effluent from a column were mixed with 10 mL of Aquasol, and the radioactivity was counted in a liquid scintillation counter. (—) Absorbance at 260 nm; (O—O) radioactivity.

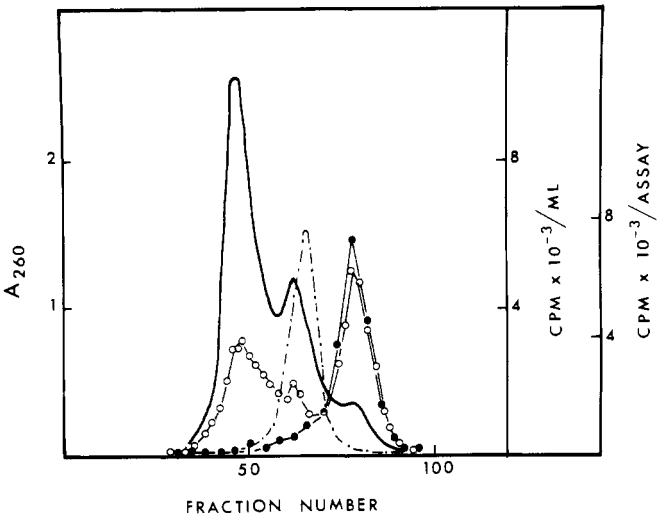

FIGURE 2: Fractionation of ^{3}H-labeled tRNA fraction with the highest specific activity on a DEAE-Sephadex A-50 column. The column (1 × 100 cm) was preequilibrated with 0.02 M Tris-HCl (pH 7.5), 0.008 $MgCl_2$, and 0.375 M NaCl. The tRNA fraction having the highest specific activity prepared from a RPC-5 column (Figure 1) was fractionated. Elution was achieved with a linear gradient obtained by placing 1 L of 0.02 M Tris-HCl (pH 7.5), 0.008 M $MgCl_2$, and 0.375 M NaCl in the mixing chamber, and 1 L of 0.02 M Tris-HCl (pH 7.5), 0.016 M $MgCl_2$, and 0.525 M NaCl in the reservoir. A volume of 5 mL of the effluent was collected per tube. (—) Absorbance at 260 nm; (O—O) radioactivity; (●—●) [^{3}H]lysine acceptor activity; (•—•) [^{3}H]histidine acceptor activity.

°C for 90 min. The tRNA was purified by gel filtration on a Sephadex G-100 column (Sharma et al., 1975).

Assay of Amino Acid Acceptor Activity of tRNA. The reaction mixture contained in a total volume of 100 μL: 10 μmol of Tris-HCl (pH 7.5), 1 μmol of $MgCl_2$, 0.2 μmol of ATP (neutralized), 0.1–1 μCi of radioactive amino acid, 10 μL of the column fraction or unit of tRNA, and a saturating amount of crude aminoacyl-tRNA synthetase (Nishimura and Weinstein, 1969). After incubation at 37 °C for 10 min, aliquots (80 μL) were applied to Whatman No. 3MM filter paper discs (diameter 23 mm). The discs were washed with cold 5% trichloroacetic acid, ethanol–ether (1:1, v/v), and finally with ether. The discs were dried and counted in a Beckman liquid scintillation counter with 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene as the scintillation fluid.

Isolation of tRNA^{Lys} from Rat Liver. Unfractionated normal rat liver tRNA was prepared from livers of female Holtzman rats and was fractionated by the DEAE[1]-Sephadex procedure (Nishimura and Weinstein, 1969). The lysine acceptor activity was separated into two peaks. The earlier eluting fractions and the late eluting fractions were pooled and were used as sources of tRNA$^{Lys}_1$ and tRNA$^{Lys}_2$, respectively. The tRNA$^{Lys}_2$ rich fraction obtained from a DEAE-Sephadex column was further purified by benzoylated DEAE-cellulose (BD-cellulose) column chromatography.

Results

tRNA isolated from livers of rats injected with 0.5 mCi of [*ethyl*-^{3}H]ethionine (70.1 Ci/mol) contained a specific radioactivity of 1050 cpm/A_{260}. To ascertain whether there are specific targets for modification by ethionine, the tRNA was subjected to a reverse-phase column chromatography (RPC-5). Figure 1 shows the distribution of radioactivity in tRNA during RPC-5 chromatography. The pattern revealed that labeling with ethionine of rat liver tRNA was not uniform. Ortwerth and Novelli (1969) have also observed preferential ethylation of a population of liver tRNA, following the administration of small amounts of radioactive ethionine.

To determine whether specific tRNAs are uniquely modified by ethionine, the tRNA fractions from 52 to 66 in Figure 1 with a specific radioactivity of 2250 cpm/A_{260} were pooled and applied to a DEAE-Sephadex A-50 column. As shown in Figure 2, about 50% of input radioactivity was eluted with lysine tRNA as the last peak. The tRNA of this region contained 16 000 cpm/A_{260} unit, which is 15-fold higher than the specific activity of unfractionated tRNA. This tRNA fraction which contained both the normal and ethylated species accepted only lysine in substantial amounts when all 20 amino acids were used to charge it (data not shown).

Rat liver tRNA^{Lys} has at least two major isoacceptors. One of them, tRNA$^{Lys}_2$, elutes at a higher NaCl concentration than tRNA^{His} on a DEAE-Sephadex A-50 column (Nishimura and Weinstein, 1969). On the other hand, tRNA$^{Lys}_1$ elutes earlier than tRNA^{His} on the same column.

To ascertain whether tRNA$^{Lys}_1$ or tRNA$^{Lys}_2$, or both, were ethylated, the tRNA was aminoacylated with [^{3}H]lysine and cochromatographed with [^{14}C]Lys-tRNA$^{Lys}_2$ purified from normal rat liver on a RPC-5 column. As shown in Figure 3, the elution position of the lysine tRNA modified by ethionine was the same as that of normal rat liver tRNA$^{Lys}_2$. The data also indicate that this lysine tRNA eluted later than tRNA^{His} from a DEAE-Sephadex A-50 column (Figure 2), which confirms the identification by the cochromatography.

The elution profiles from a DEAE-Sephadex A-25 of oligonucleotides resulting from RNase T_1 or RNase A digestion of ^{3}H-labeled tRNA together with control tRNA are

[1] Abbreviations used: BD-cellulose, benzoylated DEAE-cellulose; DEAE, diethylaminoethyl.

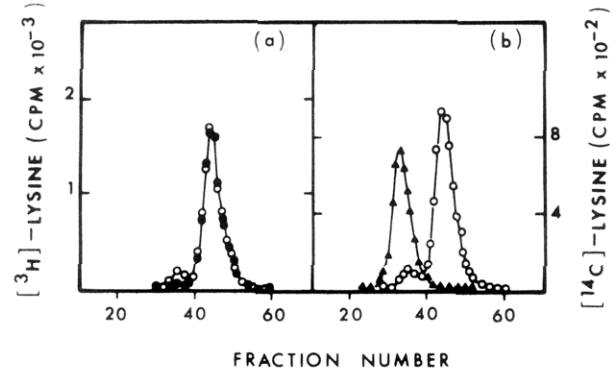

FIGURE 3: Cochromatography of purified Lys-tRNA of normal rat liver and ethionine-injected rat liver on a RPC-5 column. Normal rat liver aminoacyl-tRNA synthetase was used to aminoacylate tRNA. The conditions for chromatography were the same as described previously except a linear gradient of 0.5 M to 0.8 M NaCl was used. (a) [^{3}H]Lys-tRNA^{Lys} purified from ethionine-injected rat liver (O—O) and [^{14}C]Lys-tRNA$^{Lys}_2$ purified from normal rat liver (●—●). (b) [^{3}H]Lys-tRNA^{Lys} purified from ethionine-injected rat liver (O—O) and [^{14}C]Lys-tRNA$^{Lys}_1$ purified from normal rat liver (▲—▲).

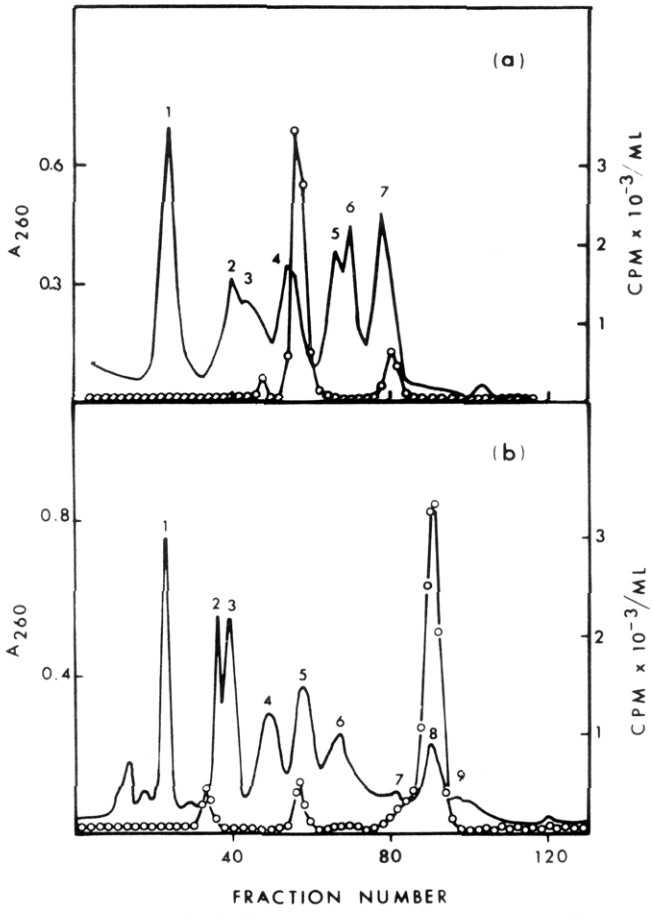

FIGURE 4: DEAE-Sephadex A-25 column chromatography of RNase digests of ^{3}H-labeled tRNA$^{Lys}_2$ purified from rat liver injected with L-[*ethyl*-^{3}H]ethionine. ^{3}H-labeled tRNA$^{Lys}_2$ containing approximately 10 000 cpm of radioactivity was mixed with 10 A_{260} units of rat liver tRNA$^{Lys}_2$, and hydrolyzed with either 10 μg of RNase T_1 or 20 μg of RNase A in 0.5 mL of 0.05 M Tris-HCl (pH 7.5) by incubation for 6 h at 37 °C. To each RNase digest was added an appropriate amount of solid urea (final concentration, 7 M), and placed on a column (0.3 × 50 cm) of DEAE-Sephadex A-25 (Seno et al., 1968). A linear gradient was achieved by placing 50 mL of 0.02 M Tris-HCl (pH 7.5), 0.14 M NaCl, and 7 M urea in the mixing chamber and 50 mL of 0.02 M Tris-HCl (pH 7.5), 0.7 M NaCl, and 7 M urea in the reservoir. Each fraction contained 0.5 mL of effluent. The radioactivity of each fraction was counted. (a) RNase A digest; (b) RNase T_1 digest; (—) absorbance at 260 nm; (O—O) radioactivity.

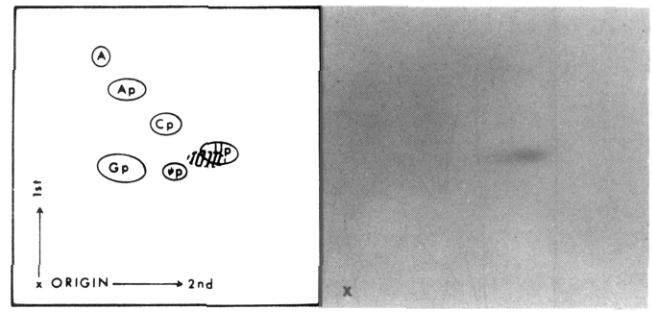

FIGURE 5: Fluorogram of a cellulose thin-layer chromatogram of the RNase T_2 digest of ^{3}H-labeled tRNA$^{Lys}_2$. Two A_{260} units of ^{3}H-labeled tRNA$^{Lys}_2$ was digested with 2 units of RNase T_2 in 50 μg of 0.05 M sodium acetate buffer (pH 4.7) by incubation for 4 h at 37 °C. The digest was subjected to two-dimensional thin-layer chromatography using the solvent system described previously (Kimura-Harada et al., 1971). Tritium-labeled nucleotides were detected on x-ray film by fluorography. (Right) Fluorogram of the hydrolysate of ^{3}H-labeled tRNA$^{Lys}_2$. (Left) The locations of nucleotides visible in UV light.

shown in Figure 4. After RNase T_1 digestion of the tRNA, about 85% of total radioactivity was eluted in the region of peak 8 (Figure 4b); with RNase A, radioactivity was detected mainly in the region of peak 4 (Figure 4a).

In addition, as can be seen in Figure 5, only one labeled base was detected on complete digestion of this lysine tRNA with RNase T_2.

Discussion

That tRNA can be a target of alkylating carcinogens has been known from the pioneering work of Magee and Farber (1962) but whether alkylation is random or specific has remained obscure. Daoud and Griffin (1976) have isolated phenylalanine tRNA–azo dye complex from rats which were fed 3′-methyl-4-dimethylaminoazobenzene. Whether the target specificity reported here is a characteristic of other alkylating carcinogens remains to be determined.

The extraordinary specificity of the ethylation of the target molecule rules out a simple chemical reaction as the mechanism of this alkylation and points, instead, to an enzymatic reaction. We may postulate that a tRNA "transethylase" which is specific for an isoaccepting species must exist in the liver. Only a small percent of the tRNA$^{Lys}_2$ was ethylated. The reason for this may be that the ethylated site is normally methylated and therefore only nascent tRNA$^{Lys}_2$ is available as a target for ethylation and methylation.

The fundamental question, whether there is a causal relationship between the alkylation of tRNAs by carcinogens and oncogenesis, is still beyond the reach of experimental probing. Indeed whether the tumor specific tRNAs found in every tumor are modified differently from those found in normal tissue counterparts has remained obscure until recently. Some investigators attempted to resolve this question by the analysis of the total tRNA populations isolated from tumor tissue. Since in every tumor there are only a few tumor specific isoaccepting tRNAs, such analyses are of doubtful significance because any changes in the structure of the few tumor specific tRNAs may be masked by the lack of changes in the preponderant species. However, we have recently analyzed pure tRNA^{Phe}, which is the most frequent isoaccepting species in tumor tissues, from two different neoplasms (Kuchino and Borek, 1977). The tumor specific tRNA^{Phe} isolated from Novikoff hepatoma and Ehrlich ascites cells both contain the same three supernumerary modified bases, one of which, 1-methylguanosine, is absent from tRNA^{Phe} of normal tissue.

What if any aberrant modifications are present in other tumor specific tRNAs is yet to be determined.

What may be the metabolic sequalae of the presence of an ethylated tRNA$^{Lys}_2$ in the population of tRNAs is obscure and its clarification will be difficult because of the numerous potencies of tRNAs and the complexity of effects which may be produced by the administration of carcinogens. For example, the tRNA isolated from livers of rats or mice given injections of large amounts of ethionine and adenine is submethylated and can act as a substrate for homologous tRNA methyltransferases (Rajalakshmi, 1973; Kerr, 1975; Wainfan et al., 1975; Wildenauer and Gross, 1974; Lu et al., 1976). It has been observed recently in our laboratory that the administration of ethionine to immature chicks produces a steroid hormone imbalance which results in a tenfold increase in serum progesterone levels (Sharma and Borek, 1977).

References

Axel, R., Weinstein, I. B., and Farber, E. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 1255–1260.

Borek, E., and Kerr, S. J. (1972), *Adv. Cancer Res. 15,* 163–190.

Brunngraber, E. F. (1962), *Biochem. Biophys. Res. Commun. 8,* 1–3.

Daoud, A. H., and Griffin, A. C. (1976), *Cancer Res. 36,* 2885–2890.

Farber, E., and Magee, P. N. (1960), *Biochem. J. 76,* 58P.

Farber, E., McConomy, J., Franzen, B., Marroquin, F., Stewart, G. A., and Magee, P. N. (1967), *Cancer Res. 27,* 1761–1772.

Kamamoto, Y., Makiura, S., Sugihara, S., Hiasa, Y., Arai, M., and Ito, N. (1973), *Cancer Res. 33,* 1129–1135.

Kerr, S. J. (1975), *Cancer Res. 35,* 2969–2973.

Kimura-Harada, F., Saneyoshi, M., and Nishimura, S. (1971), *FEBS Lett. 13,* 335–338.

Kuchino, Y., and Borek, E. (1977), *Nature (London)* (in press).

Lu, L. W., Chiang, G. H., and Randerath, K. (1976), *Nucleic Acids Res. 3,* 2243–2253.

Magee, P. N., and Farber, E. (1962), *Biochem. J. 83,* 114–124.

Nishimura, S., and Weinstein, I. B. (1969), *Biochemistry 8,* 832–842.

Ortwerth, B. J., and Novelli, G. D. (1969), *Cancer Res. 29,* 380–390.

Pegg, A. E. (1972), *Biochem. J. 128,* 59–68.

Rajalakshmi, S. (1973), *Proc. Am. Assoc. Cancer Res. 14,* 39.

Rosen, L. (1968), *Biochem. Biophys. Res. Commun. 33,* 546–550.

Seno, T., Kobayashi, M., and Nishimura, S. (1968), *Biochim. Biophys. Acta 169,* 80–94.

Sharma, O. K., and Borek, E. (1977), *Nature (London) 265,* 748–749.

Sharma, O. K., Mays, L. L., and Borek, E. (1975), *Biochemistry 14,* 509–514.

Wainfan, E., Moller, M. L., Maschio, F. A., and Balis, M. E. (1975), *Cancer Res. 35,* 2830–2835.

Wildenauer, D., and Gross, H. J. (1974), *Nucleic Acids Res. 1,* 279–288.

Yamane, Y., Sakai, K., and Kojima, S. (1976), *Gann 67,* 295–302.

Zubay, F. (1962), *J. Mol. Biol. 4,* 347–356.