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BIOLOGY DIVISION
ANNUAL PROGRESS REPORT TO
NATIONAL CANCER INSTITUTE
AEC-NCI COOPERATIVE PROGRAM ON COCARCINOGENESIS
FOR PERIOD ENDING DECEMBER 31, 1968

OAK RIDGE NATIONAL LABORATORY
operated by
UNION CARBIDE CORPORATION
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U.S. ATOMIC ENERGY COMMISSION

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BIOLOGY DIVISION

ANNUAL PROGRESS REPORT TO NATIONAL CANCER INSTITUTE

AEC-NCI COOPERATIVE PROGRAM ON COCARCINOGENESIS

for Period Ending December 31, 1968

R. F. Kimball, Director
S. F. Carson, Deputy Director

MAY 1969

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Oak Ridge, Tennessee
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for the
U. S. ATOMIC ENERGY COMMISSION

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30. Cooperative Program

AEC-NCI COCARCINOGENESIS

Biochemistry of Carcinogenesis (30.1, 30.2)

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Enzymology of Chemical Carcinogenesis (30.3–30.8)

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Mammalian Chemical Carcinogenesis (30.9–30.12)

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Chromosomal Effects of Chemicals and Radiation (30.13, 30.14)

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Biophysics of Carcinogenesis (30.15–30.27)

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Chemical Mutagenesis in Microorganisms (30.28–30.30)

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Viral Leukemogenesis (30.31, 30.32)

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Somatic Cell Genetics (30.33, 30.34)

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Inhalation Carcinogenesis (30.35–30.43)

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Biochemical Regulation (30.44–30.48)

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30.1 TRANSMISSION OF SYNTHETIC GENETIC INFORMATION BY USE OF VIRUS VECTORS

Stanfield Rogers

Efforts to build and transmit synthetic genetic information using viruses are an outgrowth of our findings that the Shope papilloma virus carries the information for the synthesis of an arginase. Infection with this virus thereby causes a reduction in the arginine blood level of certain infected animals, including rabbits, mice, rats, and man. It seemed because of this that if the information for the synthesis of a specific enzyme could be added to the virus DNA or RNA, the information could be thereby transmitted. Our first efforts using the Shope virus DNA failed, as it was not possible to add information without breaking the continuous helix, which rendered the DNA noninfective. The recent report of Goulian, Kornberg, and Sinsheimer,[1] who used ligase to tie the ends of a continuous DNA together, suggests that this now should be possible. In any event, because of this difficulty, we went to an infective linear RNA derived from the tobacco mosaic virus. By use of polynucleotide phosphorylase it has been possible to add sequences of poly-A. Tobacco plants infected with this modified RNA reveal little loss of infectivity of the RNA. Following separation of the basic peptides from extracted tobacco leaves, and the subsequent separation of polylysines from the other peptides using a Beckman PA 35 resin, and their subsequent fractionation using carboxymethyl cellulose, it has been possible to isolate and purify trimeric, tetra-, and pentalysine from plants infected with the poly-A-modified TMV-RNA. No polylysines have been detectable in extracts of normal plants, normal TMV-RNA-infected plants, or plants inoculated with a mixture of poly-A and normal TMV-RNA.[2] This model indicates that more meaningful sequences such as that for the synthesis of an enzyme can be added to a virus nucleic acid and thereby use the virus as a vector to transmit the information. Such modified viruses have the potential of curing certain of the genetic deficiency diseases and may prove useful in the therapy of cancer and certain other diseases.

As infective DNA or RNA is unlikely to produce systemic infection in mammals, efforts are under way to add synthetic protein coats such as polylysine to the modified nucleic acid to protect it from extracellular nucleases in such animals. Another possibility that is being explored is the use of subunits of normal virus protein coat in a similar way. The protective effect of TMV protein coat subunits on the Shope papilloma virus DNA is currently being tested.

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[1] M. Goulian, A. Kornberg, and R. L. Sinsheimer, *Proc. Natl. Acad. Sci. U.S.* **58**, 2321 (1967).

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30.2 REGULATION OF THE BLOOD LEVEL OF SPECIFIC METABOLITES THROUGH THE USE OF CHRONIC DIALYSIS AGAINST SPECIFIC ENZYMES

Stanfield Rogers

Repeated dialysis of the blood of patients with renal failure using artificial kidneys is well known. One of the problems of such machines is the metabolite imbalance caused by long-term dialysis. It occurred to us that such imbalance would be circumvented should the dialysis system be not allowed to act as a kidney but rather allowed to dialyze back into the blood stream. The normal kidneys of the animal or individual will then control the excretion following specific degradation of metabolites by enzymes on the exterior of the membrane. Current work in a number of laboratories is concerned with the control of metabolite blood levels through enzyme injection. Such methods are fraught with the danger of not only how much and how often to give the enzyme but because of the often fatal protein sensitivities developing upon repeated injection in time. These difficulties would be circumvented through the use of this modified form of chronic dialysis. This approach should aid in the specific therapy of cancer of various sorts as well as in the specific therapy of those with diseases such as histidinemia, this latter associated with the genetic lack of the specific enzyme histidase. A mini-Klug kidney has been kindly loaned by Dr. Converse Peirce of the Emory University School of Medicine. Our pilot studies are being done with the Shope rabbit papillomas, known to require large amounts of arginine. Arginase is the enzyme being dialyzed against.

In this system the rabbit blood arginine level may be drastically lowered with 4 to 6 hr dialysis against arginase. In rabbits repeatedly dialyzed over a period of days or dialyzed and maintained on a diet deficient in arginine, the Shope papillomas undergo a dramatic regression. As these tumors sometimes undergo a spontaneous regression, as a control the animals with greatly reduced tumor size were taken off the dialysis

and synthetic diet and full fed. The tumors then rapidly returned, indicating that the regression was caused by the therapy and not spontaneous.

30.3 USE OF VIRUSES AS CARRIERS OF ADDED GENETIC INFORMATION

Peter Pfuderer[1] Stanfield Rogers

Tobacco mosaic virus (TMV) RNA has been successfully modified by the attachment of polyadenylic acid (poly-A) moieties at the 3′ end, using ADP, polynucleotide phosphorylase, and the TMV RNA as primer in the reaction. This should lead to the production of polylysine in the infected host, either as a free polymer or attached to a viral protein.

Concomitantly, a chemical purification scheme has been developed which permits the isolation and determination of pure polylysine oligomers in the range from 1 to 30 from extracts of tobacco plants. Control runs on extracts of uninfected tobacco plants, normal TMV-RNA-infected tobacco plants, and poly-A or mixtures of poly-A and TMV-RNA-infected tobacco plants have failed to show any polylysine production in these extracts.

The poly-A-modified viral RNA, however, has been shown to produce appreciable amounts of polylysine. The polylysines produced ranged from 2 to 6 monomers in length, while the poly A added to the viral RNA was roughly 20 monomer units long. No change has been found in the protein coat of the new virus, and the polylysines seem to be produced unattached to any viral protein.

After the virus has been propagated by systemic infection for a second or third generation in the Turkish tobacco host as described above, it loses its ability to produce polylysine. Reasoning that this loss was due to a gradual takeover of wild-type unmodified virus present with the poly-A virus, an attempt was made to get a pure clone of the modified virus. The poly-A-modified RNA was purified on a sucrose density gradient, and fractions from the gradient were plaqued on a Xanthi tobacco host, which gives localized lesions typical of the mosaic infection. These plaques, which should be results of single viral RNA infections, were then reinfected and followed through several generations. To date, three of these clones have been found which produce a single polylysine polymer and seem to breed true through several generations.

Reference

[1] Enzymology section.

30.4 CHROMATOGRAPHIC COMPARISONS OF ISOACCEPTING tRNA's BETWEEN L-M CELLS IN IN VIVO CULTURE AND L-M-CELL-INDUCED TUMORS IN C_3H MICE

Wen-Kuang Yang D. H. Martin
Alfred Hellman Kiki B. Hellman[1]
G. David Novelli

As a continuation of the study of the tRNA's of L-M cells and L-M-cell-induced tumors,[2] a detailed chromatographic comparison of isoaccepting tRNA's has been carried out to determine the effect of drastic changes in the nutritional environment on the tRNA pattern in these cells. The tRNA's were isolated from washed L-M cells and from fresh solid tumor tissue. These were aminoacylated with homologous aminoacyl-tRNA synthetase preparations with the same amino acid, but carrying different labels (^3H and ^{14}C), and then co-chromatographed on the RPC-2 column.[3] The comparison has been made for 16 amino acids – serine, tyrosine, aspartate, phenylalanine, leucine, lysine, arginine, methionine, histidine, glycine, valine, alanine, isoleucine, tryptophan, proline, and threonine. Similar chromatographic patterns were observed for leucyl-, argininyl-, lysyl-, methionyl-, glycyl-, valyl-, tryptophanyl-, and prolyl-tRNA's. Significant quantitative differences in isoaccepting tRNA peaks were observed in species accepting isoleucine, alanine, threonine, and serine. Marked differences were found for tyrosyl-, phenylalanyl-, aspartyl-, and histidyl-tRNA's. A tyrosyl-tRNA migrating in front of the four tyrosyl-tRNA's which were resolved on the RPC-2 column was very prominent in L-M tumors but was completely absent in the L-M cells. Of the two phenylalanyl-tRNA's detected, the first peak was about 60% in L-M cells and below 3% in L-M tumors, whereas the second peak was about 40% in L-M cells and more than 95% in L-M tumors. The L-M cells showed two aspartyl-tRNA's, a major one and a minor one (80 and 20%); L-M tumors showed four aspartyl-tRNA's, two (8 and 2%) migrating at the same chromatographic positions as those of L-M cells and two distinct peaks (75 and 15%) migrating at different positions where no aspartyl-tRNA's from L-M cells were observed. The L-M tumors had three histidyl-tRNA peaks, of which the most prominent one in front was detected as the most minor one in the L-M cells. The alterations of chromatographic patterns of these aminoacyl-tRNA's between the L-M cells and the L-M tumors were demonstrated either by using synthetase preparations from the same source of cells and tissues or by reversing the enzyme preparations for the pre-column aminoacylation.

Further experiments showed the following facts:

1. Although tumors from the male and the female C_3H mice were found to have some differences in the growth and regression rate, no differences were detected in their tyrosyl- and aspartyl-tRNA's.

2. When the tumor tissue was trypsinized for dispersion, adapted again to in vitro culture by one passage in 19% horse serum–medium 199 and several passages in 0.5% Bacto-peptone–medium 100 as a monoloayer, and finally grown in suspension culture to obtain enough material for isolating tRNA, the chromatographic patterns of tyrosyl-, aspartyl-, and phenylalanyl-tRNA's of these in vitro grown L-M tumor cells showed those of L-M cells instead of those of L-M tumors.

3. When 5% mouse serum or 5% horse serum was included in the culture medium in the place of 0.5% Bacto-peptone for three passages, no conversion of aspartyl- and tyrosyl-tRNA's into the tumor pattern was observed.

4. Co-chromatography of tyrosyl-, aspartyl-, seryl-, and phenylalanyl-tRNA's from C_3H mouse liver with those from L-M cells or L-M tumors revealed that the isoaccepting aminoacyl-tRNA patterns of L-M tumors and the liver were similar.

5. Since tRNA's were prepared from L-M cells which were in cell suspension and from L-M tumors which were solid tissue, the possibility arose that different physical treatment of the source of material before phenol extraction might alter the chromatographic properties of isoaccepting tRNA's. This possibility was excluded by the finding that isoleucyl-, valyl- and seryl-tRNA's prepared from the cell suspensions of C_3H mouse reticulocytes were similar to those of L-M tumors in chromatographic separation.

To summarize, the results showed that the multiple isoaccepting tRNA's undergo remarkable changes in L-M cells following the drastic change from the in vitro cell culture to the in vivo environment. Whether this phenomenon results from the selection of the cell clone population or is due to adaptation of the cells at the cytoplasmic level remains to be determined.

References

[1] Present address: 17509 Princess Ann Drive, Olney, Md. 20832.

[2] W. K. Yang *et al., Biol. Div. Ann. Progr. Rept. Dec. 31, 1967,* ORNL-4240, p. 314.

[3] W. K. Yang and L. C. Waters, this report, paper 30.7.

30.5 ETHYLATION OF HEPATIC tRNA BY ETHIONINE

Lawrence Rosen Shigemi I. Simms
G. David Novelli

Ethylation by ethionine, a specific hepatocarcinogen, of liver RNA differs in two obvious ways from other unnatural agents which alkylate liver tRNA. It alkylates only tRNA to any significant extent and yields a greater variety of alkylated products. The amount of ethylation which occurs is distributed equally between the purine and pyrimidine fractions.

Ethionine is the ethyl analog of methionine, the endogenous source of methyl groups in tRNA. For this reason methylation studies with methionine were also done. Different ethylpurines and pyrimidines were synthesized and characterized to make positive identifications. The results reported below demonstrate some qualitative similarities between ethionine and methionine but also show marked differences. This demonstrates that ethionine has a different specificity and does not randomly compete with methionine for the same sites in tRNA.

In the purine fraction, only guanine is ethylated, not adenine. There are four different ethylguanines, three of which have been identified.[1] N^2-Ethyl, N^2,N^2-diethyl-, and 7-ethylguanine account for 23, 2, and 7% of the total ethylation respectively; two-thirds of the purine ethylation. An unidentified ethylpurine, probably another ethylguanine, based on electrophoretic studies, accounts for 17% of the total ethylation. These results contrast with those obtained with methionine; in the latter case both guanine and adenine are methylated.

The major ethylated purine, N^2-ethylguanine, apparently has a shorter half-life than the other ethylated purines in tRNA. This also contrasts with methylated purines, of which 7-methylguanine apparently has the shortest half-life.[2]

Approximately 80% or more of the ethylation which occurs in the pyrimidine nucleotide fraction is accounted for by 2′-*O*-ethylation of the ribose moiety. Paper chromatography demonstrates there are not less than three different 2′-*O*-ethylpyrimidine nucleotides in rat liver tRNA, and they appear to be uridylic acid derivatives, as indicated by electrophoresis. One may be assumed to be 2′-*O*-ethyluridylic acid, and the others could conceivably be 2-*O*-ethyl derivatives of minor bases related to uridylic acid such as ribothymidylic acid, pseudouridylic acid, etc. These compounds differ markedly from those obtained with the methylated pyrimidine nucleotide fraction. In the latter case,

2′-*O*-methylation accounts for 12% (maximum) of the total methylation found. The major methylated pyrimidine nucleotides are ribothymidylic and 5-methylcytidylic acids, whereas only a small amount of 5-ethylcytidylic acid has been identified.

The above results indicate that in tRNA, only guanine in the purine fraction and mainly uridylic acid (and/or its derivatives) in the pyrimidine fraction are ethylated to a major extent. For the former, only base ethylation occurs, and for the latter, mainly 2′-*O*-ethylation occurs. These findings indicate a high specificity for ethionine, different from methionine.

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[2] V. M. Craddock, S. Villa-Trevino, and P. N. Magee, *Biochem. J.* **107**, 179 (1968).

30.6 *E. COLI* tRNA: BEFORE AND AFTER BACTERIOPHAGE INFECTION

L. C. Waters G. David Novelli

When *E. coli* is infected with T2 or T4 bacteriophage, its leucine tRNA is altered. As judged by reversed-phase column chromatography the leucyl tRNA of the cells is altered quantitatively early after infection (early change[1]). Moreover, a further qualitative alteration is observed late in infection and is characterized by the appearance of one or more "new" peaks of leucine tRNA (late change[2]).

Concerning the early change, we have repeated and expanded the experiments mentioned in the previous progress report and have concluded that it results from an apparently specific inactivation of only one of at least five isoaccepting leucine tRNA's found in normal cells.[3] This inactivation does not appear to be due to a selective loss of –CCA termini nor a denaturation of the type described by Lindahl *et al.*[4] We have proposed that this early change is the result of a specific enzyme, probably a nuclease, which is phage induced. However, to date we have failed to demonstrate such an activity in cell-free extracts from phage-infected cells.

Our efforts to elucidate the origin of the "new" leucine tRNA's are continuing. In vitro labeling experiments, particularly with ^{35}S, strongly suggest that the "new" leucine tRNA's are products of *de novo* synthesis. However, these same experiments suggest that other tRNA's must also be synthesized after phage infection; that is, the specific activity of tRNA labeled after phage infection is 25% of that of a sample from normal cells labeled for the same period of time. A recent report[5] presents evidence that a considerable quantity of the nucleic acid synthesized after phage infection is host specific, although host protein synthesis is shut off immediately. In light of this report, labeling experiments as just described must be interpreted with caution. The effects of chloramphenicol (CAP) and sodium azide on the formation of the "new" tRNA's have been investigated. Chloramphenicol does not inhibit the formation of the new peaks. Sodium azide, though expected to inhibit macromolecular synthesis, did not inhibit the formation of the new peaks; however, two intermediate peaks were formed. Sodium azide did not affect the profile of tRNA from normal cells. The significance of this observation is not clear.

The control experiments using CAP have yielded some interesting results. First, in the presence of CAP, tRNA synthesis continues almost linearly with time up to at least 4 hr. This tRNA appears to have full leucine acceptor activity, a result which is in agreement with a report of Ezekiel and Valulis.[6] Second, and most interesting, is the observation that the chromatographic profile is markedly altered, similar in appearance to undermethylated tRNA's. In vitro methylation shows that the CAP tRNA is not undermethylated when compared with the usual undermethylated tRNA as obtained from a relaxed strain of *E. coli* after methionine deprivation. We are currently investigating the possibility that this CAP tRNA is defective in only one or a few of the modifications known to occur at the polynucleotide level, for example, methylation or thiolation. If so, such tRNA might prove valuable in elucidating the functional significance of such modifications.

The biological significance of the "early" and "late" change in leucine tRNA following phage infection is unknown. Studies concerning this problem are in progress.

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30.7 MICROANALYSIS OF ISOACCEPTING tRNA's BY REVERSED-PHASE COLUMN CHROMATOGRAPHY

Wen-Kuang Yang L. C. Waters

An important role for tRNA in the process of cellular regulation has been implicated by the observation of multiple isoaccepting tRNA's in the organism and by the findings that they may be altered according to the state of cellular function. This has produced a need for a generally useful method for the analysis of isoaccepting tRNA's. In this laboratory, a standard method has been devised for this purpose by employing the reversed-phase columns which were originally developed in the macromolecular separations program for the large-scale isolation of pure tRNA species. With this method and the use of precharged tRNA, sample sizes in the range of one A_{260} unit can be analyzed for the isoaccepting tRNA species of an amino acid. The main features of the method are summarized as follows:

1. tRNA is prepared from cell suspensions, fresh tissues, or frozen samples by phenol and 1 *M* NaCl extraction followed by DEAE-cellulose column chromatography.
2. Active aminoacyl-tRNA synthetases are prepared from fresh or frozen samples by subcellular fractionation, DEAE-cellulose column chromatography, and Sephadex G-100 gel filtration.
3. Conditions and criteria for satisfactory aminoacylation of the tRNA with ^{14}C- or ^{3}H-labeled amino acids are determined using the paper disk assay.
4. The labeled aminoacyl-tRNA is prepared for subsequent reversed-phase column chromatography by direct isolation from the reaction mixture on miniature DEAE-cellulose columns.
5. The samples to be analyzed are differently labeled (^{3}H or ^{14}C), mixed, and subjected to reversed-phase column chromatography. Chromatographic conditions, such as flow rate, temperature, additives (such as divalent ion, reducing agents, buffer, etc.), and NaCl gradient, are optimized to give the best recovery and resolution.
6. Columns are assayed by TCA precipitation of the individual fractions (in the presence of carrier DNA), collection on Millipore filters, and subsequent radioactivity measurements.
7. A qualitative comparison of the isoaccepting tRNA's can be made directly from the counting data. With the information obtained in feature 3, a direct quantitative comparison can be made.

This method has been applied to bacteria, fruit fly, liver and gill tissues of fish, toad liver, mouse plasma cell tumors, tissue culture L-M cells and L-M tumors, and the mouse reticulocytes. It was especially useful in the cases where the source of material for preparing tRNA was limited.

30.8 STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF tRNA PYROPHOSPHORYLASE

Audrey N. Best G. David Novelli

The purification of tRNA pyrophosphorylase has been repeated several times and extended in a large-scale process. The procedure, which has been outlined previously,[1] was modified to include a batch hydroxylapatite step after the $MnCl_2$ heat treatment. This step eliminates about 90% of inactive A_{280} material and makes possible the chromatography of 12,000 A_{280} units on the first 5 X 80 cm DEAE-cellulose column. Repeated chromatography on another DEAE-cellulose column and on a final hydroxylapatite column gives about 10 mg of purified enzyme from 3.25 kg of *Escherichia coli* B cells. The largest single loss of enzyme units occurs during or after the batch hydroxylapatite treatment, but this step appears to be necessary for large-scale work. The product of this purification compares well with smaller-scale preparations. Since no other purification steps we tested were promising, we decided to use this preparation for our enzymatic studies.

We were able to see five fairly sharp protein-staining bands by electrophoresis of the final fraction on cellulose acetate membranes in 0.02 *M* phosphate buffer, pH 6.35. Three of these bands (cut out from corresponding nonstained samples) showed incorporation of both AMP and CMP into a partially degraded tRNA preparation. It is not clear just how pure our preparation is, but we believe that this indicates one enzyme can incorporate both nucleotides. There has been no evidence of subunits during our purification procedure, as the activity always occurs in one peak.

Our final preparation did not inhibit the aminoacylation of a purified tRNA by its specific synthetase, and preliminary tests did not show aminoacyl tRNA synthetase activity. The preparation is free of acid and alkaline phosphatase activity with *p*-nitrophenyl phosphate as the substrate, but at very high concentrations it does show $CoCl_2$- and $CaCl_2$-stimulated activity at pH 6.5 toward bis(*p*-nitrophenyl) phosphate and ATP. This might indicate a contamination with the 5′-nucleotidase described by Neu,[2] and osmotic shock

treatment of the bacterial cells in the enzyme extraction step could eliminate this contamination. However, under our assay conditions for AMP (CMP) incorporation these activities are not a problem.

Our assay procedure consisted in incorporation of radioactive AMP (from ATP) into a partially purified mixed tRNA preparation from *E. coli* W (prepared by periodate oxidation of the terminal adenosine followed by amine cleavage and alkaline phosphatase treatment). This altered tRNA preparation can be repaired to about 1200 $\mu\mu$moles of AMP per A_{260} tRNApCpC. Maximum activity is noted at pH 9.5 in Bicine buffer [*N,N*-bis(2-hydroxyethyl) glycine] with 2×10^{-3} *M* ATP and about 35×10^{-6} *M* tRNApCpC (1 $A_{260} = 10^{-6}$ *M* tRNA). Under initial velocity conditions at 30°C (2-, 4-, and 6-min assay) the K_m for ATP is 1.6×10^{-4} *M*, and the K_i for CTP is 1.8×10^{-4} *M*. The K_m for the mixed tRNApCpC is 7×10^{-6} *M*. We plan to extend these studies by removing the second nucleoside from this preparation and measuring the K_m for CMP incorporation with the corresponding K_i for ATP. These experiments require rather large amounts of tRNA. It would be interesting to see if the K_m for tRNApCpC(or pC) varies with different tRNA preparations. Initial velocity rates can be increased (using optimum ATP and tRNApCpC concentrations) with increasing levels of β-mercaptoethanol up to 0.1 *M*. However, equilibration of the enzyme for at least 6 min at 0.02–0.1 *M* β-mercaptoethanol at the incubation temperature of 30° results in initial velocity values which are more linear over a fivefold increase in enzyme concentration.

We are examining several highly enriched and specific tRNA preparations which have been purified by the Chemical Technology Division, Macromolecular Separations Program, to see if the terminal nucleosides can be removed chemically without damage to the functional tRNA structure. We would like to see full charging activity by the specific synthetase after the treated tRNA has been repaired by the tRNA pyrophosphorylase. We know that $tRNA^{Phe}_{E.\ coli}$ is damaged to a certain extent by the periodate treatment. We are interested in determining under carefully controlled conditions which nucleotides can be incorporated into purified tRNA after sequential removal of the four or five terminal nucleosides from the 3′-OH terminus.

We are planning to examine the effect of enzyme and chemical treatment of purified tRNApCpC substrate in order to gain some insight into the recognition site(s) of the tRNA pyrophosphorylase for this substrate. The reverse action of the enzyme, pyrophosphorolysis of tRNA, will be studied in regard to its effectiveness with precharged tRNA.

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30.9 FURTHER OBSERVATIONS ON THE COMBINED CARCINOGENIC EFFECTS OF X RAYS AND TRIETHYLENEMELAMINE IN MICE

A. C. Upton W. D. Gude[1] J. W. Conklin

Introduction. – The radiomimetic alkylating agent triethylenemelamine (TEM) resembles x rays in certain late somatic effects on RF mice. These include shortening of the life-span, leukemogenic effects on the thymus, induction of tumors in the lung, ovary, and other organs, and the formation of lens opacities. The present study was undertaken to explore the combined effects of TEM and x rays administered concurrently, with the aim of determining whether the effects of the two agents would be additive to a degree consistent with a common mode of action.

Results and Discussion. – Ten-week-old female RF/Un mice were subjected to: (1) intraperitoneal injection of TEM, 0.25–4.0 mg/kg; (2) 25–500 r whole-body 300-kvp x irradiation (80 r/min); or (3) both TEM and x irradiation (TEM injected 1 to 5 min before irradiation). Preliminary results 30 months after treatment show that each agent given alone shortened the life-span but that the effect was greater in mice receiving both agents than in those receiving a comparable total dose of either agent alone, especially at high dose levels. Likewise, each agent alone induced thymic lymphomas and ovarian tumors, but when both agents were administered together, the incidence exceeded that induced by either agent alone, indicating that the two agents administered together exerted effects which were additive, if not synergistic, depending on the end effect in question. Since, however, a few of the mice remain alive and the lesions induced by the two agents remain to be analyzed in full, the significance of their combined effects is yet to be determined.

To explore possible mechanisms of synergistic effects of the two agents, the combined toxicity of the agents to thymus and hemopoietic cells is being analyzed with respect to effects on thymus weight and on killing of hemopoietic cells, as judged by the Till-McCulloch spleen nodule method. Preliminary results imply that x rays and TEM differ quantitatively, if not qualitatively, in their effects on blood-forming cells.

Reference

[1] Pathology and Physiology section.

30.10 ONCOGENICITY OF METHYL METHANESULFONATE (MMS) IN MALE RF MICE

N. K. Clapp R. E. Toya, Sr.[1]
A. W. Craig[2]

Introduction. – Alkylation of nucleic acids has been postulated to explain the mutagenic and carcinogenic action of certain alkylating agents; for example, dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) alkylate nucleic acids and are potent carcinogens. Conversely, methyl methanesulfonate (MMS), which methylates DNA and RNA, has not been reported to be carcinogenic hitherto.

Results and Discussion. – In a total of sixty-three 11-week-old male mice from a noninbred subline of the RF/Un strain, which received 30 mg of MMS per kilogram body weight daily in the drinking water throughout life, we found an increased incidence of lung tumors (70.2% compared with 38.9% in 162 controls) and thymic lymphomas (14.9% compared with 3.7% in controls). As measured by lung tumor induction, therefore, MMS appears to resemble DEN and DMN. DEN and DMN fail to induce leukemia, however, whereas MMS appears to resemble x rays, nitrogen mustard, TEM, and Myleran – all of which induce thymic lymphomas in RF mice.

Since alkylation occurs primarily at the 7-position of guanine in both DNA and RNA after MMS, DMN, and DEN, differences in oncogenicity between MMS, DMN, and DEN suggest that a qualitative difference may exist between the site(s) of alkylation by these agents within nucleic acids, the precise location of the alkylation along the nucleic acid chain possibly being important in the oncogenic process. Studies to determine the cocarcinogenicity of MMS with DMN and DEN are now in progress.

References

[1] Mammalian Recovery section.

[2] Paterson Laboratories, Christie Hospital, Manchester, England.

30.11 RELATIONSHIPS BETWEEN CUMULATIVE DOSE OF DIETHYLNITROSAMINE (DEN) AND INCIDENCE OF TUMORS IN RF MICE

N. K. Clapp R. E. Toya, Sr.[1]
A. W. Craig[2]

Introduction. – Diethylnitrosamine (DEN) is a potent carcinogen in a number of species of animals, inducing a high incidence of liver hepatomas, lung adenomas, and squamous cell carcinomas of the forestomach in RF mice. To determine tumor-incidence–cumulative-dose relationships, 450 eight- to ten-week-old male RF mice were divided into six different groups and given DEN in the drinking water in daily doses of 2, 3.5, 6, or 11.5 mg per kilogram body weight for treatment periods of varied lengths; cumulative doses ranged from 57 to 943 mg per kilogram body weight.

Results and Discussion. – Mean survival times for the various dose groups were markedly reduced in groups receiving high total doses and in groups where treatment periods (and drug toxicity) extended for several months. The incidence of liver tumors increased linearly with increasing cumulative dose throughout the range of doses used. In contrast, maximum incidences of lung and stomach tumors (~80 and 100% respectively) were reached at low cumulative doses, and low thresholds were suggested, while none was seen for liver oncogenesis. In three groups that received DEN at the same daily dose (6 mg/kg) but with varied treatment periods (1, 7, and 22 weeks), the higher-dose groups developed the highest incidences of tumors, despite decreased mean survival time. At the lowest dose, lung tissue would appear most sensitive, stomach intermediate in sensitivity, and liver least sensitive to DEN carcinogenesis.

References

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30.12 PRELIMINARY INFORMATION ON THE OCCURRENCE OF ETHER-LINKED LIPIDS IN TUMORS INDUCED BY DIETHYLNITROSAMINE

N. K. Clapp F. Snyder[1]

Introduction. – Previous studies have indicated that most neoplasms of animal and human origin contain increased levels of alkyl and alk-1-enyl glyceryl ether diesters and ether-linked phosphoglycerides.[2] The purpose of this study was to determine the occurrence of glyceryl ethers in chemically induced tumors of various stages of malignancy.

Results and Discussion. – Six male RF mice which were given diethylnitrosamine (DEN) in the drinking water (6 mg per kilogram body weight per day for seven weeks) developed lung adenomas, liver hepatomas, and squamous cell carcinomas of the forestomach; these were examined histologically and lipid analyses were performed. Normal nontreated tissue and nontumorous tissues from mice given DEN were analyzed similarly.

The tumors induced by DEN contained substantial quantities of ether-linked lipids, in contrast to very low or undetectable amounts in control and treated non-tumorous tissue. The ether-linked lipids in the neutral glyceride fraction of the stomach tumors appear as glyceryl ether diesters on thin-layer chromatograms. Stomach tumors which appeared more malignant histologically also contained higher amounts of glyceryl ether diesters. Lung and liver tumors were not large enough to yield adequate lipids for complete analysis. Further studies are in progress to confirm the impressions obtained in these preliminary results.

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30.13 CHEMICAL INDUCTION OF FORWARD AND REVERSE MUTATIONS IN CHINESE HAMSTER CELLS IN CULTURE

E. H. Y. Chu Patricia A. Brimer H. V. Malling

Introduction. – In previous reports,[1,2] we summarized results demonstrating the selection of biochemical mutants from Chinese hamster cells in tissue culture and the induction of forward and reverse mutations at these loci with ethyl methanesulfonate (EMS). These studies have been extended to include other chemical mutagens as well as to define the conditions of mutation expression.[3] The present experiments were designed to elucidate, if possible, the molecular mechanism of gene mutation in the hamster cells, based on reverse mutation tests with compounds whose specific mutagenic action is well established in microorganisms.[4–6]

Methods. – The chemical mutagens employed in this study were EMS, MMS (methyl methanesulfonate), MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), and ICR-170 (2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino]acridine dihydrochloride). Either spontaneously occurring or chemically induced mutant cell clones that were resistant to 8-azaguanine (azg^r) were treated with the above mutagens to induce reversions to drug sensitivity (azg^s). Prior to the reversion experiment, the possible background in the cell populations of spontaneous azg^s revertants was removed by growing cells in the presence of 30 μg/ml azaguanine. After mutagen treatment, the cells were washed and incubated for 42 hr before the selective agents were added to the medium. In this selective medium, which contained aminopterin, hypoxanthine, thymidine, and glycine, azg^s cells can grow whereas azg^r cells cannot. Both cell survival and mutation frequency were determined in terms of colony formation.

Results and Discussion. – A highly significant increase over the controls in the forward mutation frequency from azg^s to azg^r can be induced with all four mutagens. In equimolar concentrations and under similar experimental conditions, the relative effectiveness of the four compounds used is as follows: ICR-170 > MNNG > MMS > EMS.

A number of mutant clones were isolated which were shown to fully retain the resistant character after numerous cell generations in the absence of the drug. Reversion to drug sensitivity occurred either spontaneously or following mutagenic treatment (Table 30.13.1). The limited number of mutant clones tested did not permit definite conclusions to be drawn concerning the mutagenic specificity of various compounds in hamster cells. Nevertheless, several interesting facts are already evident:

1. There appear to be qualitative differences in revertibility to azg^s among the class of azg^r mutants which originated spontaneously, those induced by EMS or MMS, and those induced by ICR-170.
2. Some of the EMS- or MMS-induced mutants reverted either spontaneously or very significantly after MNNG treatment. In cases where both EMS and MNNG seemed to induce reversions, MNNG invariably was more effective than EMS.
3. Of all the 12 mutants tested which were induced by ICR-170, none was able to revert significantly to drug sensitivity.

Resistance to azaguanine in hamster cells has been shown to be due to the loss of inosinic acid–guanylic acid pyrophosphorylase activity.[1] Reversion to drug sensitivity, that is, regaining of the enzyme activity, could be due either to back mutation at the locus or to suppressor gene(s). The present data on reversion induction with specific chemical mutagens reveal the complexity and qualitative differences between azg^r mutants of different origin. The genetic alterations in the revertible mutants are likely to be single base-pair changes. Furthermore, it seems possible that the genetic alterations in azg^r mutants which can revert after treatment with EMS and MNNG are base-pair substitutions. The nonrevertible mutants can be interpreted as multiple intragenic changes or deletions.

Table 30.13.1. Distribution of Azaguanine-Resistant Mutants in Chinese Hamster Cells According to Their Spontaneous or Induced Revertibility to Drug Sensitivity

Number of Mutant Clones	Genotype	Presumptive Origin	Revertibility ($azg^r \rightarrow azg^s$)			
			Spontaneous	EMS	MNNG	ICR-170
3	azg^r	Spontaneous	+	+	+	
3			–	+	+	NT
2			+	+	–	
2			–	–	–	
2	azg^r	EMS-induced	+	+	+	
2			–	+	+	
4			–	–	+	NT
0			–	+	–	
13			–	–	–	
4	azg^r	MMS-induced	+	+	+	
3			–	+	+	NT
2			–	–	+	
0			–	+	–	
9			–	–	–	
12	azg^r	ICR-170-induced	–	–	–	–

NT = not tested.

It is clear from these data that measurement of reverse mutation frequencies cannot be used as a screening method for testing of mutagenicity. These tests have to be performed with systems where quantitative measurements of forward mutation frequencies can be obtained, such as the induction of mutations from azg^s to azg^r.

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30.14 MUTAGENICITY OF CHEMICAL CARCINOGENS IN MAMMALIAN CELLS

E. H. Y. Chu E. G. Bailiff H. V. Malling

With the availability of genetic markers and the demonstration of chemical mutagenesis in tissue culture mammalian cells, it becomes feasible to test in the same system both carcinogenicity and mutagenicity of a particular agent. We have studied the cytotoxicity and photodynamic effect[1] of several carcinogenic and non-carcinogenic polycyclic hydrocarbons. This report summarizes the preliminary results on mutation induction in Chinese hamster cells with these same series of compounds. The mutagenic change studied was from sensitivity to 8-azaguanine (wild type) to resistance.

The compounds were first dissolved in dimethylformamide and added, at appropriate concentrations, to the cell cultures. The solvent at a final concentration of 1% in the medium reduced cell survival to about 70%, but was not itself mutagenic. Cells were exposed to various polycyclic hydrocarbons for 24 hr in the dark, at the level of concentrations that would permit normal or slightly reduced survival. Our results to date clearly show that 9,10-dimethyl-1,2-benzanthracene was highly mutagenic, whereas 1,2,3,4-dibenzanthracene and 1,2,5,6-dibenzanthracene caused slight but significant increase in the forward mutation frequency. On the other hand, neither 1,2-benzpyrene nor 3,4-benzpyrene was shown to be mutagenic. These results are similar to a parallel study in *Neurospora,* with the exception that in hamster cells both 1,2,3,4- and 1,2,5,6-dibenzanthracenes exhibit mutagenic activity.

The work has been extended to include other carcinogens, such as aromatic amines. Our aim is to test the possibility that metabolic derivatives rather than the test compound per se were responsible for the final

effect. It is hoped that studies along these lines may throw some light on the somatic mutation theory of carcinogenesis.

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30.15 PROTEIN CHEMISTRY

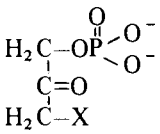
F. C. Hartman

Design and Use of Active-Site Specific Enzyme Reagents. – Chemical reagents can be endowed with a high degree of specificity for the active site of a given enzyme by incorporating into their structures those functional groups of the natural substrate required for binding to the enzyme in question. In addition to the usefulness of such reagents in basic studies concerning the structure and mechanism of action of enzymes, they are of potential value in cancer chemotherapy.[1]

In our last progress report,[2] the preparation of 1-hydroxy-3-iodo-2-propanone phosphate (HIPP) (I), a reactive derivative of dihydroxyacetone phosphate (DHAP) (II), was described. I assumed that HIPP would have an affinity for and be capable of reacting covalently with the active sites of triose phosphate isomerase (TPI), aldolase, and glycerophosphate dehydrogenase, enzymes for which DHAP is a natural substrate. During the past year, the corresponding bromo (HBPP) (III) and chloro (HCPP) (IV) compounds have been prepared, and a comprehensive investigation of their reaction with the aforementioned enzymes has been initiated.

Substantial evidence for the active-site specific modification of TPI by HIPP has been obtained and published,[3] and preliminary data indicate that HBPP reacts with TPI in a similar manner. These reagents will presumably permit structural analysis of at least a portion of the active site of TPI.

The inactivation of aldolase by HIPP is complex. Significant (50 to 70%) enzymic activity is recovered by treating the modified aldolase with cysteine or other sulfhydryl compounds. This observation, in addition to quantitative sulfhydryl determinations on the native and modified enzyme and the fact that HIPP oxidizes cysteine to cystine, suggests that the inactivation is, in part, due to the oxidation of sulfhydryl groups to disulfides. Approximately two moles of reagent per mole of aldolase are irreversibly incorporated and may account for the lack of complete restoration of activity induced by sulfhydryl compounds. HBPP and HCPP do not inactivate aldolase under the conditions used for the inactivation by HIPP.



X = I (I), OH (II), Br (III), Cl (IV)

A Heavy Atom Derivative of Rubredoxin. – The reaction of *C. past.* rubredoxin with ^{14}C-labeled α-(*p*-iodophenyl)-acetimidate has been investigated as a means of obtaining a derivative suitable for x-ray crystallographic studies.[2] Contrary to preliminary data, substitution occurs at various ϵ-amino groups, resulting in a mixture of derivatives which has resisted resolution by usual procedures. These difficulties and the fact that the elucidation by x-ray diffraction of the three-dimensional structure of rubredoxin is approaching completion in another laboratory[4] prompted me to terminate this study.

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[4] L. H. Jensen, University of Washington at Seattle.

30.16 MOLECULAR STRUCTURE OF THYMINE (*cis-syn*) PHOTODIMER

C. H. Wei J. R. Einstein

Ultraviolet irradiation of living cells damages the cellular DNA by causing bonds to form between neighboring pyrimidine residues. The thymine photodimer isolated from irradiated DNA has previously been shown to be a *cis-syn*-cyclobutane derivative. This photodimer may also be obtained by uv irradiation of frozen aqueous solutions of thymine, and was crystallized as the dipotassium and monosodium salts from alkaline alcohol-water solutions. Both forms are highly radiation sensitive, and both exhibited a slow, small change in unit-cell dimensions during data collection. Cell dimensions measured at about the middle of the data collection were, for the potassium salt, $a = 7.01$, $b = 25.97$, $c = 8.03$ A, $\beta = 99°10'$; for the sodium salt, $a = 6.56$, $b = 29.01$, $c = 6.76$ A, $\beta = 106°2'$. The space groups are Cc for the potassium salt, $P2_1/n$ for the sodium salt, with $Z = 4$ in both cases. The structure of the sodium salt ($C_{10}H_{11}O_4N_4Na \cdot H_2O$) was determined by the symbolic addition procedure and was based on 1817 independent reflections. It was shown

that x rays split the cyclobutane ring of the dimer and that the two resulting monomers fit well into the space previously occupied by the parent dimer, although individual atoms move as much as 1½ A. The structure was assumed to consist of a random distribution of dimers and monomer pairs, having related site occupancies which change during data collection. This model has been refined, with the use of a rigid-body treatment for the monomers, to a present value of 0.053 for the reliability index $R_1(F)$, based on 1014 of the strongest reflections. The atomic positions determined prior to consideration of the low-occupancy monomer sites (roughly 25% occupancy for this set of data) represented averages of the dimer and monomer positions and gave incorrect bond distances, particularly across the cyclobutane ring. However, normal bond distances were obtained for the dimer from refinement of the mixed dimer-monomer model.

The potassium salt crystals are much more mosaic than the sodium salt, and only 523 reflections were used. Although no such detailed analysis was carried out as for the sodium salt, it is evident that the dimer has roughly the same configuration in the two crystals. The following discussion refers to the sodium salt of the dimer, a view of which is shown in Fig. 30.16.1.

The cyclobutane ring is buckled, with a dihedral angle of 28°. Both pyrimidine residues are in approximately an envelope configuration, with one atom of each ring several tenths of an Angstrom unit out of the least-squares best plane of the other five atoms. The best planes, calculated for the approximately coplanar atoms of each ring, make an angle of 59° with each other. When viewed along the normal to the plane bisecting the two best planes, the two pyrimidine residues are rotated from each other by an angle of about 40°. The molecule is unusually crowded, with a distance of only 3.0 A between the two methyl carbons (normal van der Waals distance, 3.5 to 4.0 A).

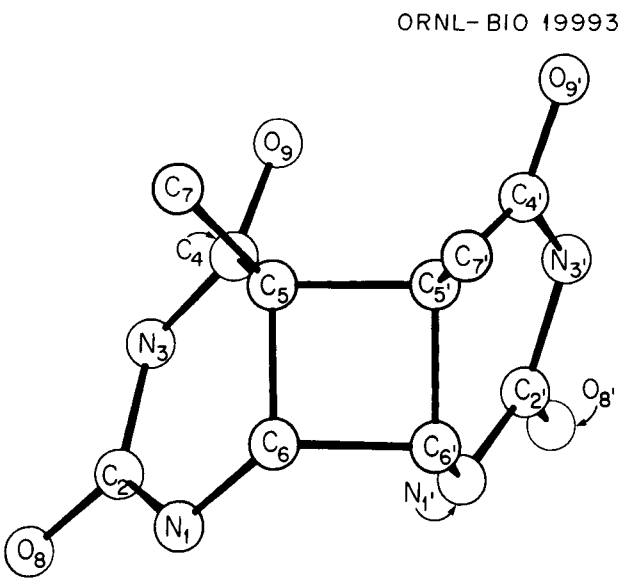

Fig. 30.16.1. The Sodium Salt of Thymine Dimer Viewed from a Direction Perpendicular to the Average Plane of the Cyclobutane Ring.

The crystal has a center of symmetry, and therefore there are two enantiomeric forms of the dimer, with right- and left-handed screw senses.

The crystal contains one water of hydration per dimer. A difference Fourier revealed all 11 hydrogen atoms. The proton replaced by the sodium ion has been removed from the N_3 atom of one pyrimidine residue; this nitrogen is coordinated to the sodium ion. The sodium is coordinated in a distorted octahedral configuration to atoms of four different dimers and to one water oxygen. Atoms of one dimer are involved in seven hydrogen bonds, four to four other dimers and three to three water oxygens. Each water is hydrogen bonded to three dimers and is coordinated to a sodium ion.

The pair of thymine monomers, which replace a dimer split by x rays, lie on nearly parallel planes spaced 3.4 A apart. Replacement of a dimer by a monomer pair is accompanied by the breaking of only two hydrogen bonds and the formation of one new hydrogen bond.

There is no free rotation about any bond in the photodimer; therefore the structure must closely approximate that of a corresponding moiety within a molecule of uv-irradiated DNA. One may attempt to explain the denaturation of DNA after uv irradiation by comparison of the dimer configuration with that of two neighboring thymine residues in the DNA helix. Several striking differences are observed. (1) In the DNA helix the distance between atoms N_1 and N_1' of neighboring thymines – the atoms bridged by sugar-phosphate-sugar – is 4.4 A, while in the dimer this distance is 2.9 A. Thus in DNA the configuration of the sugar-phosphate-sugar moiety would have to change radically on formation of the cyclobutane ring. (2) Neighboring thymine residues in DNA lie on nearly parallel planes spaced 3.4 A apart. The corresponding planes of the dimer are at an angle of 59° to each other. (3) Suppose the right-handed form of the dimer is introduced into the (right-handed) DNA helix in such a manner that the two pyrimidine planes are each tilted by about 30° to the equatorial plane of the helix, and so that the atoms

of one thymine residue lie in axial projection nearly over the corresponding atoms of one thymine residue of DNA. Then in the other pyrimidine the atoms which correspond to those forming interchain hydrogen bonds in the helix would be displaced by several Angstrom units from the required positions. Introduction of the left-handed dimer would change the screw sense of the helix. In summary, it is not difficult to rationalize the denaturation of DNA on formation of these cyclobutane dimers.

30.17 X-RAY STUDIES ON DIPEPTIDES: *N*-BROMO(AND CHLORO)ACETYL-L-PHENYLALANYL-L-PHENYLALANINE ETHYL ESTERS

J. R. Einstein C. H. Wei

In an attempt to obtain single crystals suitable for x-ray structural analysis, both bromo and chloro derivatives[1] were recrystallized from ethanol. Crystals of the bromo derivative are monoclinic with cell parameters a = 12.951 A, b = 17.617 A, c = 4.951 A, and β = 103.23°. The volume of the unit cell is 1100 A^3. The observed density of 1.39 g/cm^3 (by flotation) compares satisfactorily with the calculated value of 1.39 g/cm^3 based on two formula units of $C_{22}H_{25}N_2O_4Br$ in a unit cell. The observed systematic absence of $\{0k0\}$ for $k = 2n$ indicates probable space groups $P2_1/m$ and $P2_1$.

A set of three-dimensional counter data collected from a tiny crystal of the bromo derivative was used to compute a three-dimensional Patterson function. Atomic coordinates for the bromine atoms, obtained from an interpretation of the Patterson map, were used in calculation of Fourier syntheses, from which all nonhydrogen light atoms have been located. The refinement of the structure is in progress. The chloro derivative is shown from Weissenberg and precession photographs to be isomorphous with the bromo derivative.

Reference

[1] The samples of both derivatives were kindly furnished to us by Dr. D. G. Doherty of this Division.

30.18 AN ANALYTICAL METHOD FOR EQUATORIAL-PLANE DIFFRACTOMETRY

J. R. Einstein

A graphical and analytical method has been devised for the analysis of intensity measurements in x-ray diffraction experiments with a counter instrument utilizing equatorial-plane geometry. The method facilitates the calculation of the dependence of such measurements on the size, shape, and mosaicity of the crystal; the brilliance distribution of the source; the takeoff angle; the spectral composition of the radiation; the size of the detector aperture; the measurement technique (scan type and scan parameters, or time period of a stationary measurement); and the proximity of neighboring reflections. Crystal size and shape are, for the first time, treated in a general manner. The rest of the treatment represents a recasting of the analysis, as previously worked out by other authors, in a considerably simplified form. The method is of particular usefulness for crystals having large unit cells, for which resolution of neighboring reflections may be difficult.

30.19 ENERGY TRANSFER IN OLIGOTYROSINE COMPOUNDS – FLUORESCENCE TITRATIONS

J. A. Knopp J. W. Longworth

The initial observations on poly-L-tyrosine, where the intensity of the fluorescence of neutral tyrosyl residues was compared with the percent change of neutral to ionized tyrosyl residues, indicated that the fluorescence emission decreased much more rapidly than did the neutral tyrosyl residue concentration. These observations were used to support the concept of energy transfer in poly-L-tyrosine. This concept implied that a similar effect should be observed in oligotyrosine compounds.

We have compared the fluorescence intensity of the neutral tyrosyl residues with the percent of ionization of these residues in a series of tyrosine compounds containing one, two, three, and six residues per molecule. For the compound with one tyrosyl residue, *N*-acetyl-L-tyrosine amide, the fluorescence emission was linearly related to the percent of ionization. For all other compounds, the fluorescence emission was a nonlinear function of percent of ionization. Therefore the concept of efficient energy transfer in tyrosine compounds is confirmed.

To determine the extent of transfer, it is necessary to compare the distribution of molecular species, for example, neutral or singly ionized, with the fluorescence intensity. If the reasonable assumption is made, as we did, that the probability of a residue being ionized is equal to the fraction ionization I of the residues, then the distribution of molecules is given by the terms of the binomial series. That is, the neutral, singly ionized, doubly ionized, etc., are given by

$$(1-I)^n, \quad nI(1-I)^{n-1}, \quad \frac{n(n-1)}{2}I^2(1-I)^{n-2},$$

etc., where n is the number of residues per molecule. In the case where the ionization of one residue per molecule quenches all the other residues by energy transfer, then the fluorescence emission is only due to the neutral species, and the fluorescence intensity is proportional to $(1 - I)^n$.

We compared the experimental values with the function $(1 - I)^n$ and found that the agreement was within experimental error for all compounds except the dityrosine compounds. For the latter compounds, the differences between the experimental values and $(1 - I)^2$ were slightly greater than the experimental error. This discrepancy was interpreted as an electrostatic interaction between residues which makes the second ionization a little more difficult than expected. We conclude therefore that electronic energy transfer between neutral and ionized tyrosyl residues is 100% effective in oligotyrosine compounds up to and including hexa-L-tyrosine.

30.20 CALCULATION OF RATES OF ENERGY TRANSFER IN POLY-L-TYROSINE

J. J. ten Bosch[1] J. A. Knopp[1]

Energy transfer in poly-L-tyrosine has been demonstrated previously at both 298°K[2] and 77°K.[3,4] It appeared that ionized tyrosyl (tyrosinate) residues act as efficient energy scavengers. In order to determine whether or not tyrosyl-tyrosyl transfers preceding tyrosyl-tyrosinate ones play an important role, calculations of transfer rates were performed. Absorption and fluorescence spectra obtained previously in this laboratory[2,3] were evaluated to determine the overlap integrals corresponding to both types of transfer. Extinction coefficients and quantum yields were determined experimentally, both at 298 and 77°K. Together with information on the molecular structure found in the literature,[5] indicating a left-handed α-helical conformation, these values were applied to the theoretical formulas given by Förster's very weak coupling case of energy transfer.

It could be shown that the theory is applicable to the polytyrosine case, which means that an energy quantum is not delocalized over the polymer but that the energy may make jumps over the residue chain. The role of tyrosyl-tyrosinate transfers in the absence of tyrosyl-tyrosyl transfers could be visualized simply by calculating the probabilities that energy present in a certain residue is transferred to the tyrosinate residue. Thus a size was found for a simplified trap defined so that this probability equals 1 inside and 0 outside the trap. To determine the influence of tyrosyl-tyrosyl transfers on this picture, a complete calculation of the probability of the presence of the energy on each residue was made as a function of time and initial position of the energy. One trap was included in a 100-residue-long polymer, and the resulting set of differential equations, involving all transfer rates larger than 10^{-3} transfer per fluorescence lifetime, was solved on a CDC 1604 or IBM 360 computer. The resulting extended trap sizes and other results are shown in Table 30.20.1.

It can be seen that the calculated trap sizes are larger than the observed ones. This may be due to a polymer structure that is less ordered than the assumed structure. We believe, however, that the agreement is certainly good enough to allow the conclusion that the theory describes the actual energy transfer process.

Table 30.20.1. Trap Sizes

Temperature (°K)	When Only Direct Tyrosyl-Tyrosinate Transfers Are Considered	Both Types of Transfers	Observed
77	15.9	~27	20 ± 5[a]
298	11.3	28	25 ± 5[b]

[a]References 3 and 4.

[b]Reference 3.

References

[1] Biophysics section.

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30.21 ENERGY TRANSFER IN TYROSINE COMPOUNDS – TYROSYL-TYROSYL TRANSFERS

J. A. Knopp[1] J. W. Longworth

Calculations of the rate constants for electronic energy transfer between residues in poly-L-tyrosine demonstrated that tyrosyl-tyrosyl transfers played a role in the overall electronic energy transfer mechanism of this compound. From the magnitude of this contribution, other experiments should reveal its presence. In particular, the limiting polarization of fluorescence,

which is the polarization measured in the absence of molecular rotations, is sensitive to the difference in orientation between the absorber and emitter. Hence, if energy transfer occurs between the absorption and emission of light, this orientation must be different, and the value of polarization will be different.

We determined the limiting polarization of fluorescence resulting from different wavelengths of excitation of a series of tyrosine compounds, including poly-L-tyrosine. Compounds containing more than one tyrosyl residue per molecule exhibited a lower value of polarization than that observed for *N*-acetyl-L-tyrosine amide, which was used as the control. Therefore this is qualitative evidence for the presence of tyrosyl-tyrosyl energy transfers.

As the energy transfer rates were calculated for poly-L-tyrosine, it was possible to calculate the expected value of polarization for poly-L-tyrosine assuming a rigid left-handed α-helical conformation. It was also possible to calculate values for hexa-L-tyrosine and tri-L-tyrosine assuming the same helical conformation and a random conformation. These values are shown in Table 30.21.1 along with the appropriate experimental values. The agreement between the experimental and calculated values is not as good as might be expected. We feel that these differences between the experimental and calculated values reflect the lack of accurate information concerning the conformations of these molecules. We believe, furthermore, that the agreement is sufficient to allow the conclusion that the Förster's very weak coupling case applies to energy transfer between tyrosyl residues and that this type of transfer must be considered in developing the entire energy degradation scheme of tyrosine-containing compounds.

Table 30.21.1. Polarization of Fluorescence with 285-nm Excitation of Tyrosine Compounds Dissolved in Ethylene Glycol at 210°K

Compound	Observed Polarization	Calculated Polarization Assuming:	
		α-Helix	Random
N-Acetyl-L-tyrosine	0.380 ± 0.005		
Tri-L-tyrosine	0.198 ± 0.005		0.137
Hexa-L-tyrosine	0.126 ± 0.005	0.143	0.070
Poly-L-tyrosine	0.109 ± 0.005	0.129	0.00

Reference

[1] Biophysics section.

30.22 ENERGY TRANSFER IN OLIGOTYROSINES – LUMINESCENCE TITRATIONS

Maria Del Carmen Battista J. W. Longworth

Fluorimetric titrations of L-hexatyrosine at 300°K have demonstrated that a single ionized tyrosine residue is sufficient to completely quench the neutral residues by an intramolecular energy transfer. We wished to extend this study to low temperatures to eliminate the possibility of excited-state protolysis, and to be able to measure both singlet and triplet state behavior in the same molecule. Lowering the temperature alters the fractional ionization; so it is necessary to determine the fractional ionization I directly at the temperature of the luminescence measurements. This was accomplished by measuring the absorption spectra of the samples in quartz ESR tubes mounted in a Dewar and placed in the microfocusing attachment of the Perkin-Elmer 450 absorption spectrometer. The fluorescence of the neutral residues followed the dependence $(1 - I)^6$ at both 220 and 77°K, confirming the previous titrations at 300°K. Phosphorescence intensities were also determined, and both the neutral and ionized residues followed the expected behavior. These measurements provided definitive evidence for intramolecular electronic energy transfer at the singlet level in hexatyrosine, where a single ionized residue is sufficient to quench the other five neutral residues.

30.23 ENERGY TRANSFER AND EXCITATION SPECTRA

J. W. Longworth Maria Del Carmen Battista

Excitation spectra have been measured to show that energy absorbed by tyrosine is transferred to tryptophan in the dipeptide L-tryptophanyl-L-tyrosine. The luminescence from the dipeptide is identical with the luminescence of tryptophan. The remarks concerning transfer are general, but we will specifically consider only the tyrosine-to-tryptophan transfer. The excitation spectrum of the tryptophan emission of the dipeptide includes a contribution from the tyrosine absorption. If this transfer is not complete, that is, if there is radiationless internal conversion in tyrosine, then the excitation spectrum will not be identical to the absorption spectrum. To test for identity between the two spectra, the excitation spectrum was divided by the absorption spectrum; this quotient, when normalized, reflects the wavelength dependence of the quantum yield of emission. The quantum yield of fluorescence at 298°K of tyrosine and tryptophan was found to be constant, within 3%, from 300 to 240 nm, and with the

dipeptide L-trp-L-tyr, the quantum yield was constant to within 5%. At 280 nm, tyrosine absorbs 18% of the light absorbed by the dipeptide, and at 300 nm it has no appreciable absorption. Hence these measurements can only set an upper bound, and that is that one-third of the tyrosine absorption in the dipeptide is internally converted. Similar quantum yield studies were made with the fluorescence of the protein ribonuclease T_1, and a large dependence on the wavelength of excitation was found for the quantum yield of fluorescence of the single tryptophan of this enzyme. It was possible to account for the variation in yield by a transfer of only one-third of the energy absorbed by the nine tyrosines of the enzyme. Two-thirds of the energy absorbed by tyrosine is internally converted by radiationless transition at the singlet level.

30.24 THE EXCIPLEX NATURE OF ENDONUCLEASE FLUORESCENCE

J. W. Longworth

The endonuclease of *Staph. aureus* (E.C. 3.1.4.7) has a single tryptophan residue, and the fluorescence is dominated by emission from this residue. At 77°K the fluorescence is fine structured, and the intensity maximum is at 317 nm; but at room temperatures, the emission has no fine structure, is red shifted, and the intensity maximum lies at 335 nm. Two explanations for the effect of temperature on the character of the emission have been proposed. Mechanism 1 is that of a complex formation with a neighboring polar group by the excited state (exciplex), and mechanism 2 consists of a reorientation of the solvation shell around the excited state. To distinguish between these two mechanisms the fluorescence of the protein and model systems was measured at various temperatures in glassing solvents. Mechanism 1 is expected to lead to a common intersection point in the fluorescence since only two species are involved, whereas mechanism 2 results in a progressive shift in the intensity maximum to longer wavelengths with increasing temperature. An isoemissive point is found in the fluorescence spectra of endonuclease measured at various temperatures between 150 and 250°K, and this indicates that exciplex formation occurs at the higher temperatures. In contrast, model systems of tryptophan compounds fit the behavior expected for mechanism 2.

30.25 OPTICAL PROPERTIES OF POLYNUCLEOTIDES DENATURED BY ORGANIC SOLVENTS

J. A. Knopp[1] J. W. Longworth
R. O. Rahn[1]

A random coil conformation of polynucleotides [in particular, DNA, tRNA, $d(A\text{-}T)_n$, $d(A\text{-}C)_n$, $d(G\text{-}T)_n$, and $r(A)_n$] has been obtained by dissolving lyophilized fibers into ethylene glycol (EG) that contains 0.1 *M* NaCl. Polymers in this conformation are found to have different luminescent, circular dichroism, and thermal melting behaviors than in their duplex or single-strand stacked conformations (which exist when 5% water is present). Fluorescence at 77°K of the random coil is like that of monomers and unlike the exciplex emission from duplex polymers. Electron spin resonance and phosphorescence measurements have shown that the only triplet is that of thymine for DNA as a duplex or as a stacked single strand, but the random coil has adenine as the predominant triplet with the thymine remaining unchanged. Polyd(A-T) only phosphoresces from thymine in the random coil, whereas a mixture of polyd(A-C) and (G-T) has both an adenine and a thymine phosphorescence. This suggests that adenine and guanine can transfer energy to thymine in a random coil, but that the transfer is only short range, since thymine does not completely quench adenine in DNA.

Reference

[1] Biophysics section.

30.26 ELECTRONIC ENERGY TRANSFER IN POLYNUCLEOTIDES

J. W. Longworth R. O. Rahn[1]
J. A. Knopp[1] Maria Del Carmen Battista

The presence of intramolecular electronic energy transfers in polynucleotides has been frequently proposed and exhaustively calculated, but experimental support is rare and indirect. Electronic energy transfer is suggested by the absence of emission of a suspected donor in the presence of a potential acceptor, but to directly investigate transfer, experiments on sensitization of the acceptor or fluorescence polarization measurements must be performed. We have obtained some

qualitative results which indicate that transfers do occur. As reported in the previous abstract, the random coil of polyd(A-T) phosphoresced only from the T residues, and since the excitation spectrum is similar to the absorption, this result suggests that A transfers to T, which is consistent with the sequence of triplet energies. However, the polymer in basic solutions has emission from both A and T^-, yet the triplet energies indicate that T^- ought to transfer to A. Nevertheless the random ribopolynucleotides $r(A,U)_n$, $r(A,G)_n$ in the random coiled form at neutral and alkaline pH values always phosphoresce from the A residue alone, suggesting transfers to A – a result consistent with the known sequence of triplet levels. Likewise, the mixture of random coiled polymers polyd(G-T) and polyd(A-C) has no G emission, so that T can quench G. Hence A is an effective trap of the triplet energy in polynucleotides and can be quenched by T, as in $(A\text{-}T)_n$, but not entirely in the random coil of DNA and tRNA. Phosphorescence excitation of T is similar to the DNA absorption in a DNA duplex and is like the excitation spectrum of A in the random coil. Both observations suggest extensive intramolecular transfers. However, the excitation spectrum of the fluorescence of the random coil is different from that of the phosphorescence. This suggests that there are radiationless processes which compete with transfers at the singlet level, but since the phosphorescence excitation spectrum of one of the emitting residues resembles the absorption, then the simplest interpretation would be that the radiationless process is an intersystem crossing accompanied by triplet transfers. The polymer polyrA, in the single-strand stacked conformation, is an attractive system to apply fluorescence polarization measurements to transfer studies. Theoretical calculations by several groups indicate that within the singlet lifetime there are expected to be transfers at the singlet levels over tens of bases. However, the fluorescence polarization as a function of absorbing wavelength at 220°K in propylene glycol is identical to that found from AMP, and so there cannot be any singlet transfers. Hence, there are qualitative indications of transfers, particularly at the triplet level, and these transfers appear to depend on the polynucleotide conformation and the base sequence.

Reference

[1] Biophysics section.

30.27 LUMINESCENCE OF MIXED tRNA's FROM *ESCHERICHIA COLI*

J. W. Longworth M. P. Stulberg[1]

Native tRNA has a strong phosphorescence contribution from the thiouracil residues when excited at 330 nm. However, when excited at 260 nm, there is a completely different emission, which has no thiouracil component and has a very small quantum yield. The phosphorescence emission resembles that observed with polyrG,[2] while the fluorescence is that of an exciplex and is unlike that found from polyrG. The exciplex emission is absent in the random coiled conformation, and there is now a strong fluorescence, similar to that of the monomers. The phosphorescence is also greatly enhanced and predominantly from the A residues. There is, however, another component, which is short-lived (800 msec) and which lies to longer wavelengths than A, and has no fine structure. This component is preferentially excited by light with wavelengths longer than 290 nm. This emission resembles that previously observed with polyr(A-U), and we provisionally ascribe the emission to U. The thiouracil contribution is unaltered by forming a random coil. The emission from tRNA's at pH 12 is similar to that of the random coil. As a model for tRNA, the random copolymer $r(A,G,C,U)_n$ was investigated, and the phosphorescence was from both A and U; the exciplex fluorescence of the stacked conformation is destroyed and replaced by monomer-like emission in the random coiled conformation.

References

[1] Enzymology section.
[2] J. A. Knopp, personal communication.

30.28 RELATIONSHIP BETWEEN CARCINOGENICITY AND MUTAGENICITY OF METHYLATING COMPOUNDS

H. V. Malling F. J. de Serres

Methyl methanesulfonate (MMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) are both methylating compounds and are able to methylate the bases in the DNA. Both compounds are mutagenic in several different organisms. However, whereas MNNG is a strong

systemic carcinogen, MMS has only very limited carcinogenic activity. It is possible that the higher carcinogenic activity of MNNG than MMS is correlated with a particular type of chemical activity, and that by comparing the mutagenicity of MNNG and MMS at the molecular level, this problem can be evaluated.

Forward-Mutation Frequency. – Conidia from a genetically marked balanced two-component heterokaryon of *Neurospora crassa* were treated for 5 hr in a 0.067 *M* phosphate buffer at pH 7.0 with various concentrations of MMS. Forward mutations in the *ad-3* region induced in the heterokaryotic fraction of the conidia were obtained by the direct method. The study of the mutagenicity of MNNG in *Neurospora crassa* has been reported previously. MNNG is much more mutagenic in *Neurospora* than MMS; under similar treatment conditions with respect to pH, temperature, and shaking (4 hr treatment with 25 μM MNNG and 5 hr treatment with 20 m*M* MMS) 1.45×10^{-3} and 3.5×10^{-4} *ad-3* mutations per survivor were induced respectively.

Complementation Pattern. – The most striking difference between the mutagenesis of MNNG and MMS at the molecular level at the present stage of the analysis is in allelic complementation among the *ad-3B* mutants. *Ad-3B* mutants are either noncomplementing or have a polarized or a nonpolarized complementation pattern. Among the MNNG-induced *ad-3B* mutants, 81% had nonpolarized complementation patterns, 1% polarized complementation patterns, and 18% were noncomplementing. Among the MMS-induced *ad-3B* mutants, 39% had nonpolarized complementation patterns, 11% polarized complementation patterns, and 50% were noncomplementing.

When the spectra of complementation patterns induced by MNNG and MMS in *Neurospora* are compared with similar spectra induced by such specific mutagens as nitrous acid (NA), hydroxylamine (HA), and *O*-methylhydroxylamine (OMHA) (Table 30.28.1), tentative conclusions can be reached about the mutation mechanism of MMS and MNNG. The genetic alterations in the NA- and HA-induced mutants have been identified by tests for revertibility after treatment with specific mutagens. We found that the predominant portion of the nitrous-acid-induced base-pair transitions have GC at the mutant site, whereas most of the HA-induced mutants have AT at the mutant site. HA is an extremely specific reagent for cytosine in the DNA[1] and induces predominantly base-pair transitions from GC to AT.

It is easy to see that the spectra of the complementation patterns among mutants induced by these two mutagens reflect the genetic alteration identified at the mutant site. From what is known about the genetic code, we know that mutants induced by a GC → AT transition can be nonsense mutations, whereas mutants induced by an AT → GC transition will never give rise to a nonsense codon.

Mutants with a nonsense codon will code either for a fraction of the polypeptide chain or for no polypeptide at all. Therefore these mutants will have a polarized complementation pattern or be noncomplementing. Mutants induced by an AT → GC transition should be predominantly missense mutations, and therefore code for a complete polypeptide with only a single erroneous amino acid. The great majority of these mutants will have a nonpolarized complementation pattern, like the mutants induced by NA.

As we can see in Table 30.28.1, the spectrum of complementation patterns for a certain mutagen seems to have a diagnostic value for the determination of the genetic alteration induced by this mutagen at the molecular level. If we compare the spectrum of complementation patterns among MNNG-induced *ad-3B* mutants with the spectrum among mutants induced by NA, HA, or OMHA, which is more specific in its mutagenic action than HA, we can arrive at the tentative conclusion that mutants induced by MNNG preferentially have GC at the mutant site. The predominant mechanism of mutation induction by MNNG

Table 30.28.1. Percentage of Different Types of Complementation Patterns Among *ad-3B* Mutants Induced by Five Different Mutagens

Abbreviations:
NP = *ad-3B* mutants with nonpolarized complementation pattern
P = *ad-3B* mutants with polarized complementation pattern
NC = noncomplementing *ad-3B* mutants
MNNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
NA = nitrous acid
HA = hydroxylamine
OMHA = *O*-methylhydroxylamine
MMS = methyl methanesulfonate

Mutagen	Complementation Patterns		
	NP	P	NC
MNNG	81	1	18
NA[a]	75	7	18
HA	52	12	36
OMHA	45	12	43
MMS	37	13	50

[a]Complementation pattern among the NA-induced mutants. With a base-pair transition at the mutant site.

appears to be base-pair transition from AT to GC (this point is currently being investigated by studying the revertibility of MNNG-induced mutants after treatment with specific mutagens).

Noncomplementing mutants and mutants with polarized complementation patterns can arise from a broad spectrum of genetic alterations[2] which result in the production of polypeptide fragments or nonfunctional gene products. When the complementation spectrum of MMS-induced mutants is compared with the spectra of HA-induced and NA-induced mutants (Table 30.28.1), it resembles that of HA-induced mutants. By analogy, this suggests that most MMS-induced mutants may have AT at the mutant site, a result in good agreement with the observation that MMS reacts predominantly with guanine in the DNA. It is likely, however, that the MMS-induced mutants are composed of a wide variety of genetic alterations, similar to the EMS-induced mutants in *Neurospora.*[3]

MMS seems, therefore, to give rise to a higher frequency of mutants which specify a nonfunctional gene product than does MNNG. Compounds which induce mutations that block normal gene function completely may be more efficient at inactivation of the cell, whereas compounds which alter normal gene function are more likely to alter normal biosynthesis and metabolism. On the basis of these and similar data we have formulated the following tentative working hypothesis: that compounds which induce a high percentage of mutants in which the specific gene product has an altered function are strong carcinogens, whereas compounds which induce a high percentage of mutants in which the specific gene product is nonfunctional will only have a limited carcinogenic activity.

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30.29 GENETIC ANALYSIS OF TRIETHYLENEMELAMINE-INDUCED PURPLE MUTANTS (*ad-3*) in *NEUROSPORA CRASSA*

H. V. Malling F. J. de Serres

Triethylenemelamine (TEM) has a strong mutagenic effect in many different types of organisms (mammals, plants, microorganisms, etc.). TEM is a trifunctional alkylating agent which can react in vitro with DNA and is able to cross-link the two helixes in the DNA molecule. Of the DNA components there is some indication that TEM reacts predominantly with thymine.[1] TEM is an important antineoplastic compound but has also carcinogenic properties. It is able to induce both chromosome breaks and point mutations, but in most systems these mutational events cannot be scored simultaneously. Furthermore, TEM is one of the few chemical mutagens whose mutagenic action has been studied in the specific locus system in mice.[2]

We feel that it is important to study the mutagenic effect of TEM in a eukaryotic system, where both chromosome damage and point mutations can be detected and the genetic alterations identified at the molecular level.

In the dikaryon system of *Neurospora crassa,*[3] the following biological parameters can be studied: (1) nuclear and cytoplasmic inactivation, (2) frequency of recessive lethal mutations over the entire genome, (3) frequency of chromosomal deletions in the *ad-3* region, (4) frequency of point mutations in the two structural genes *ad-3A* and *ad-3B*, and (5) identification of the genetic alteration at the molecular level.

Conidia from a genetically balanced heterokaryon were treated for various lengths of time (20 to 170 min) with 0.01 *M* TEM dissolved in a phosphate buffer adjusted to pH 7.0. In this experiment we found the following results: (The description of the relation between mutation frequencies and the length of treatment time refers to a log-log plot. The values of the slopes are therefore equal to the power of the treatment time in this correlation.)

1. A much lower survival of the heterokaryotic fraction of the conidia compared with survival of the total population of conidia for the various times of treatment by TEM. This indicates that a part of the inactivation of the conidia by TEM is due to inactivation of the nuclei.
2. The mutation frequencies of the point mutations in *ad-3A* and *ad-3B* increase with a slope greater than 1 (1.35). The chromosome deletions ($ad\text{-}3^{IR}$) in the adenine region increase with a slope greater than 2. Since the point mutations only increased with a slope of 1.35, this indicates that the chromosome deletions in the *ad-3* region most likely are the results of two independent reactions of TEM with the chromosomes. As far as we are aware, TEM is the first chemical which has been shown to induce chromosome breaks in fungi.
3. Genetic analysis (Table 30.29.1) of the mutants by means of heterokaryon tests for complementation

Table 30.29.1. Frequency of the Different Types of Mutations Induced by TEM in the *ad-3* Region of *Neurospora crassa*

ad-3A: includes all point mutations in the *ad-3A* locus, also including the *ad-3A* mutants which have additional damages elsewhere in the genome.

ad-3B: includes all point mutations in the *ad-3B* locus, also including the *ad-3B* mutants which have additional damage elsewhere in the genome.

ad-3R+RL: includes all point mutations in either the *ad-3A* or the *ad-3B* locus which have an additional damage elsewhere in the genome.

ad-3 ^{IR}: all irreparable mutations covering either the *ad-3A* or the *ad-3B* locus or both.

Treatment (min)	Mutation Frequency per 10^6 Survivors				
	ad-3A	*ad-3B*	*ad-3R+RL*	*ad-3*^{IR}	Total Forward-Mutation Frequency (directly determined)
0	0.3	0.3	0	0	0.5
20	9.8	11.5	0.5	0.8	22.4
40	24.7	23.1	3.1	1.2	49.3
80	75.8	53.7	18.2	1.0	132.3
120	106.3	109.7	57.0	6.9	227.0
170	185.6	202.9	133.0	32.9	426.8

showed that the frequencies of *ad-3A* and *ad-3B* mutants increase with increasing length of treatment time and then with similar slopes which are both greater than 1. The frequencies of *ad-3B* mutants with a polarized complementation pattern and noncomplementing *ad-3B* mutants increased with powers of 1.67 and 1.56 of the treatment time, respectively, whereas mutants with nonpolarized complementation pattern increased in direct proportion to the length of treatment time. The frequency of the three classes of mutants within the *ad-3B* locus varies therefore with the treatment time. After 20 min treatment time the percent of nonpolarized, polarized, and noncomplementing mutants were, respectively, 65, 4, and 31; after 170 min treatment the percent of nonpolarized mutants dropped to 43, whereas the percent of polarized mutants increased to 13 and the percent of noncomplementing mutants increased to 44. From these data we can conclude that short treatments of TEM mainly give rise to point mutations and that at least more than half of these are due to base-pair substitutions, whereas mutants induced after longer treatments are derived from more drastic genetic alterations.

TEM possesses carcinogenic activities as well as antineoplastic activities. In a previous study of mutagenesis of methylating compounds with respectively potent and weak carcinogenic activity, we formulated the following working hypothesis: that compounds which induce a high percentage of mutants in which the specific gene product has an altered function are strong carcinogens, whereas compounds which induce a high percentage of mutants in which the specific gene product is nonfunctional will only have a limited carcinogenic activity.

The present data indicated therefore that long-term treatments with lower concentrations of TEM should be more carcinogenic and less antineoplastic and vice versa for short treatments with higher concentrations of TEM.

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30.30 PHOTODYNAMIC EFFECT OF POLYCYCLIC HYDROCARBONS ON *NEUROSPORA CRASSA* AND CHINESE HAMSTER CELLS

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In a previous report[1] the photodynamic effect of the two similar polycyclic hydrocarbons 1,2-benzopyrene (1,2-BP) and 3,4-benzopyrene (3,4-BP) was studied in two different systems: (1) conidia from a heterokaryon between genetically marked strains of *Neurospora* and (2) Chinese hamster cells in tissue culture. Of these two compounds, only 3,4-BP is carcinogenic and was also the only one which showed a photodynamic effect. Brookes and Lawley[2] have found a good correlation between the reactivity of a series of polycyclic hydrocarbons with DNA in the mouse skin and Iball carcinogenic index for these compounds. In the present study, we have used the same series of polycyclic hydrocarbons, namely: 1,2-BP, 3,4-BP, 9,10-dimethyl-1,2-benzanthracene (9,10-DMBA), 20-methylcholanthrene (20-MC), 1,2,5,6-dibenzanthracene (1,2,5,6-DBA), and 1,2,3,4-dibenzanthracene (1,2,3,4-DBA). None of these compounds had any toxic effect in the dark on *Neurospora* conidia. On hamster cells, only 9,10-DMBA showed a toxic effect in the dark.

Two of the three strongest carcinogens of this series of polycyclic hydrocarbons, 3,4-BP and 9,10-DMBA, showed a strong photodynamic effect in both *Neurospora* and hamster cells; the third strong carcinogen, 20-MC, did not show a pronounced photodynamic effect in either system. The compounds with weak or no carcinogenic activity (1,2-BP, 1,2,3,4-DBA, and 1,2,5,6-DBA) had no toxic effect on *Neurospora* conidia or hamster cells when the cells were posttreated with black light.

By plating *Neurospora* conidia from the heterokaryon on selective substrate, it is possible to distinguish between inactivation of the conidia due to inactivation of the nuclei or inactivation of the cytoplasm. In all cases where we found a photodynamic effect of the polycyclic hydrocarbons, the inactivation of the conidia seems to be due to inactivation of the cytoplasm.

For this series of polycyclic hydrocarbons, there seems to be a reasonably good correlation between the photodynamic effect and the carcinogenic activity of the compounds. Other compounds which are not carcinogenic, such as acridines, have shown a photodynamic effect in several biological systems. Both in *Neurospora* and Chinese hamster cells, we can study a wide spectrum of genetic effects. It seems likely that by contrasting the genetic alterations induced by a combined treatment of black light and (1) carcinogenic polycyclic hydrocarbons with (2) noncarcinogenic acridines, we would be able to predict which part of the photodynamic activity is related to the induction of cancer.

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30.31 INDUCTION OF TRANSPLANTABLE RETICULUM CELL SARCOMA IN BALB/c MICE FOLLOWING REPEATED INJECTIONS OF SHEEP ERYTHROCYTES

R. L. Tyndall J. A. Otten[1] A. C. Upton

Introduction. – Immunological studies have suggested that repeated injections of antigen might be leukemogenic. To test this possibility, thirty 12-week-old male BALB/c and eleven female AKR mice were injected intraperitoneally twice weekly with 1 ml of a 10% washed sheep red blood cell (SRBC) suspension. These injections were continued for a 12-week period in the BALB/c mice, during which time 11 of the mice died from causes unknown.

Results and Discussion. – Toward the end of treatment the surviving BALB/c mice showed signs of listlessness, ruffling of fur, and diarrhea. After cessation of treatment (12 weeks), all remaining 19 BALB/c mice showed various degrees of splenomegaly as detected by palpation. One of five animals autopsied at this time was diagnosed as leukemic on histological examination; the other four showed splenic hyperplasia. In the ensuing eight weeks, ten additional mice were autopsied, nine of which were diagnosed as leukemic and one as having splenic hyperplasia. The four remaining mice showed splenic regression shortly after cessation of treatment, but all four subsequently died from leukemia 20 to 30 weeks after SRBC treatment (Table 30.31.1).

The leukemias were classified as reticulum cell sarcomas (RCS), and all of 20 newborn BALB/c mice inoculated intraperitoneally with approximately 5×10^7 nucleated spleen cells from one of the leukemic animals developed leukemia within four weeks following injection. Malignant cells from a second donor showed similar results on transplantation. Injection of

Table 30.31.1. Effect of Repeated Inoculations of Sheep Erythrocytes in BALB/c and AKR Mice

Experiment	Strain	Treatment	Incidence of Effects				
			Morbidity[a] by End of Treatment	Splenomegaly[b] by End of Treatment	Leukemia[c]		
					Within 11 to 13 Weeks After Start of Treatment	Within 13 to 20 Weeks After Start of Treatment	Within 20 to 30 Weeks After Start of Treatment
1	BALB/c	SRBC	19/19	19/19	1/5	9/10	4/4
1	AKR	SRBC	0/12	0/12	0/12	0/12	0/12

[a]Morbidity characterized by listlessness, ruffling of fur, diarrhea; particularly pronounced following inoculation.

[b]Splenomegaly determined by abdominal palpation.

[c]Number of leukemias (majority histologically confirmed)/total number of mice necropsied.

10^5 to 10^7 x-irradiated (15,000 r) pooled malignant cells from two mice with such passaged reticulum cell sarcoma was found to transmit the disease to all of 15 recipients. The lack of spontaneous leukemia in BALB/c mice within the first year of life, observed in over 200 BALB/c mice in our animal facility as well as in other colonies, strongly indicates that the leukemias in mice following treatment with SRBC resulted from the SRBC treatment per se.

To what degree breakdown of the antigen-trapping mechanism, immunological paralysis, increased production of immunoblasts, and/or activation of latent leukemia virus(es) are involved in the induction of RCS following SRBC treatment in BALB/c mice remains to be determined. The passage of these RCS with cells irradiated with 15,000 r, however, may be indicative of a viral etiology. Also to be determined is the reproducibility of the effect of SRBC treatment of BALB/c in other mouse strains. The lack of any overt symptoms thus far in the similarly treated female AKR mice implies that the reaction may be influenced by genetic, hormonal, or other environmental factors (Table 30.31.1). Also to be explored are the effects of antigens other than SRBC and of the trauma of repeated inoculations. It is hoped that further studies presently under way will answer many of these questions.

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30.32 ALTERED PROTEIN AND ESTERASE PROFILES OF RETICULUM CELL SARCOMAS RESULTING FROM RAUSCHER LEUKEMIA VIRUS INFECTION

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Ernestine Teeter Dorothy J. Moore[1]

Introduction. – A previous report described the increased esterase isozyme activity associated with infection of both cultured and malignant spleen cells infected with the Rauscher leukemia virus.[2] The present study was undertaken to study both esterase isozyme and protein profiles of sera, spleen, and thymus tissue from leukemic BALB/c mice infected with Rauscher leukemia virus (RLV).

Results and Discussion. – Overtly leukemic and comparably aged normal BALB/c mice were bled via heart puncture, perfused with normal saline, and their tissues of interest removed and frozen at –70°C. When thawed, the tissues were homogenized and extracted in Ringer's solution, the cell debris was removed by centrifugation, and the soluble protein supernatants were electrophoresed on acrylamide gel. Esterase activity was determined using α-naphthyl butyrate as substrate and Fast Blue RR salt as the diazo coupling agent. Protein patterns were determined by staining with Aniline Blue Black.

Extracts of leukemic spleen tissue (reticulum cell sarcoma) showed a depression of prealbumin esterase and a concomitant intensification of two particular isozyme bands, when compared with normal spleen extracts. The degree of intensification in the leukemic extracts appears to be related to the degree of malignant infiltration as determined by histologic examination of sample tissue from each spleen. Protein profiles of leukemic spleen extracts revealed the acquisition of proteins with electrophoretic mobilities identical to serum albumin, transferrin, and other serum proteins, with a concomitant loss of protein bands present in normal spleen extracts. Nonmalignant thymus tissue from leukemic animals generally showed either intensification of several esterase isozymes or a generalized diminution of esterase activity. Thymus protein patterns were not apparently altered. No alterations of either esterase or protein profiles were apparent in serum from leukemic animals. Analysis of extracts from fetal or newborn spleen pools indicated a similarity between these esterase profiles and those of leukemic spleen extracts. No obvious differences, however, were observed between the protein profiles of a control uninfected (JLS V9) and those of an RLV-infected cell culture (JLS V10), neither of which showed any similarity with the sera protein pattern.

The results of these experiments indicate that the major alterations in esterase and protein profiles are associated with the malignant reticulum cell sarcoma (RCS) per se and with some as yet unexplained esterase alterations in thymus tissue. The lack of protein profile changes in the RLV-infected JLS V10 cell culture and the dissimilarity between such protein profiles and serum protein patterns indicates that the altered protein patterns in RLV-infected RCS tissue are probably a reflection of the malignant state rather than a reflection of virus production. The significance of apparent serum protein production in RCS tissue and the similarity in esterase profiles between RCS and fetal spleen tissue remain to be determined. Experiments are presently under way to determine if the analogy between fetal spleen and RCS tissue extends to other isozyme systems and to the antigenicity of the two tissues.

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30.33 SERINE AND ASPARAGINE REQUIREMENTS IN NORMAL AND LEUKEMIC HUMAN BLOOD CELLS

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Helen Vodopick[1] W. H. Lee
F. M. Faulcon[3]

It is known that some animal leukemias have a requirement for asparagine because they are sensitive to, and are repressed or even cured by, injections of asparaginase.[4] Asparaginase has also been used in the treatment of human leukemia, with the tacit assumption that there is no asparagine requirement in *normal* human bone marrow.[5] In fact there exists a paucity of data on the asparagine requirements of normal marrow and no data at all on possible requirements for the other six in vitro nonessential[6] amino acids. If asparagine has significance in this regard, then it is clear that any of the other nonessential amino acids could be equally important since asparagine is noteworthy only in that it is a member of this group.

Accordingly, we undertook the investigation of the nonessential amino acids in human granulocytic leukemia (both the acute and chronic forms) and in normal human bone marrow cells. Our experiments were mainly incorporation experiments with radioactive macromolecular precursors, experiments to determine the amount of synthesis of certain nonessential amino acids in these cells, and some experiments on the activity of enzymes involved in the biosynthesis of certain nonessential amino acids.

Figure 30.33.1 is a summary histogram of our incorporation experiments. These data are applicable to both leukemic cells and cells from normal human marrow since both cell types behave essentially the same in these experiments. The data show that while asparagine is partially required by leukemic cells it is also required by normal bone marrow. However, another nonessential amino acid, serine, is required much more severely by both cell types than is asparagine.

Table 30.33.1 compares the ability of WI-38 normal human diploid fibroblasts (a serine-independent cell type) to synthesize ^{14}C-serine from ^{14}C-glucose with this capability in leukemic cells and normal human bone marrow. WI-38 cells were highly capable of serine synthesis, while bone marrow and leukemia cells were essentially incapable of this synthesis.

We have also preliminarily examined 3-phosphoglycerate dehydrogenase activity (one of the principal enzymes in serine biosynthesis[7]) in serine-competent human cells, such as HeLa and WI-38, and in serine-dependent leukemic cells. The serine-competent cells had high activity for 3-phosphoglycerate dehydrogenase, while the leukemic cells had little or no such activity.

These results seem to indicate that a partial asparagine requirement and a more severe serine requirement are present in normal human bone marrow and in cells derived from bone marrow such as granulocytic leukemia cells.

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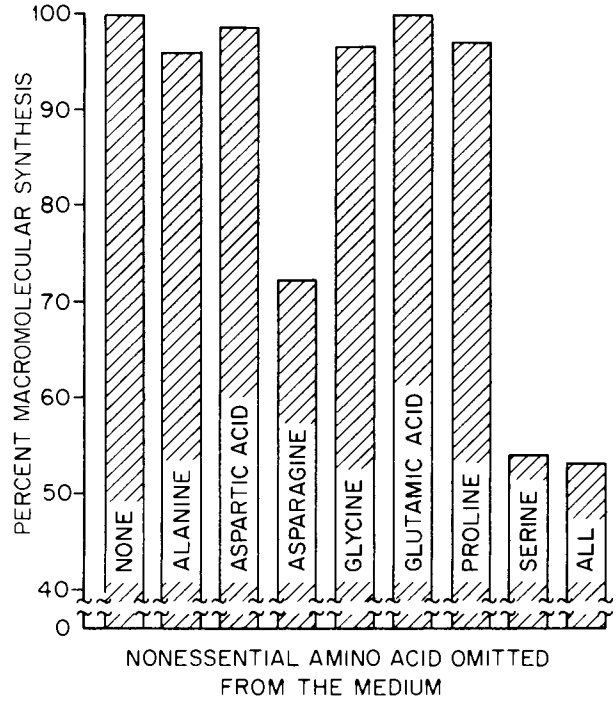

Fig. 30.33.1. The Effect on Macromolecular Synthesis of Omitting Different Nonessential Amino Acids from the Culture Media in an in Vitro Test to Assay for Nutritional Requirements in Leukemic and Normal Human Cells. Syntheses of biological macromolecules (nucleic acids and protein) were determined by incorporation of radioactive precursors. Data are average results for a number of different experiments with leukemic cells and cells of normal human bone marrow.

Table 30.33.1. Radioactivity in Total Cellular Serine After 48 hr Incubation in ^{14}C-Glucose

Cells	Counts per Minute per Micromole of Serine
WI-38 human diploid fibroblasts	317,700
Leukemic cells	2,100
Normal bone marrow cells	1,395

Thus it appears possible that a rationale may exist for leukemia therapy involving the enzyme serine dehydratase or serine antimetabolites, providing that the growth and metabolic rates of the leukemic cells are sufficiently elevated above those of the normal cells.

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30.34 A SERINE REQUIREMENT IN HUMAN LYMPHOBLASTOID ESTABLISHED CELL LINES

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We have recently presented evidence for a serine requirement in human granulocytic leukemia cells and in cells from normal human bone marrow.[2] We have also examined a number of established cell lines of human lymphoblastoid origin for their amino acid requirements. These cell lines were developed from normal subjects and from patients with various blood diseases such as infectious mononucleosis, lymphosarcoma, or chronic granulocytic leukemia.[3] All the cell lines of the lymphoblastoid type thus far examined exhibited a serine requirement. In contrast, heteroploid monolayer cells such as HeLa, diploid fibroblasts (WI-38), and a quasi-diploid squamous cell carcinoma cell line (RPMI 2650) were all serine independent.

A highly consistent and selective marker such as serine dependence can be quite useful in somatic cell genetics. Thus we are employing this marker in experiments to determine (1) the rate of spontaneous and induced reversion to serine independence and (2) whether serine dependence behaves as a dominant or recessive character upon hybridization with a serine-independent cell.

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30.35 INHALATION CARCINOGENESIS

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J. O. Brick Paul Nettesheim
R. F. Newell

The first chamber exposure phase of the role of air pollutants on the induction of pulmonary tumors in mice that have been pretreated with a radiation, viral, or combined insult is nearly complete. Eight of the groups have terminated, and the remaining six are down to small numbers of animals. The treatments and total exposure times are given in Table 30.35.1. All animals to date have remained free from pseudomonas, salmonella, internal and external parasites, and the seven murine viruses originally typed, and there has been a minimal amount of respiratory disease. The median death time of the animals in the experiment has been estimated and is presented in Table 30.35.2. The stress of living on wire or daily in the chambers is reflected in the average median death times of those with no exposure to chemicals, for example, males in pans 29 months, wire 25 months, chambers 20 months; females in pans 26 months, wire 21 months, chambers 23 months. In the males at least this can be directly correlated with the incidence of genitourinary disease, which is low in those living in pans, fourfold higher in the wire group, and sevenfold higher in the chamber group. There is an anomalous increase in median death time in six of the irradiated female groups.

The data from this study have been programmed for automatic record storage, retrieval, and analysis, and at present an examination of the various interactions between the experimental groups is in process. The age-specific incidence of lung adenomas for the first three chambers is given in Table 30.35.3. There is a consistent 100-day earlier incidence in lung adenomas in the smog-treated groups over the control and the chromic oxide group. With regard to chemical treatment the gasoline smog is also the most effective agent, as may be seen from Table 30.35.4. The total number of tumors is 15 in the washed air chamber, 16 in the chromic oxide group, and 30 in the smog-treated group. An analysis of the additional control groups with no exposure to chemicals awaits their termination.

Table 30.35.1.

A. Chamber Operations Through December 31, 1968

Chamber No.	Pretreatment	Chemical	Exposure (hr)	Completed
I	One-half irradiated	0	3133	May 1968
II	One-half irradiated	Cr_2O_3		March 1968
		52 ppm	2723	
		27 ppm	363	
III	One-half irradiated	Ozone, 1 ppm; gasoline, 30 ppm	3093	April 1968
IV	One-half irradiated – all virus	Ozone, 1 ppm; gasoline, 30 ppm	2959	May 1968
V	One-half irradiated – all virus	Cr_2O_3		June 1968
		53 ppm	2540	
		27 ppm	363	
VI	One-half irradiated – all virus	0	2813	July 1968
VII[a]	0	45 pans	0	
VIII[a]	Irradiated	45 pans	0	Dec. 1968
IX[a]	Virus	45 pans	0	
X[a]	Irradiated – all virus	45 pans	0	
XI[b]	One-half irradiated	45 pans	0	
XII[b]	One-half irradiated – all virus	45 pans	0	Nov. 1968
XIII	One-half irradiated – all virus	$CaCrO_4 \cdot H_2O$, 35 ppm	2065	
XIV	One-half irradiated	$CaCrO_4 \cdot H_2O$, 35 ppm	2171	

[a]No exposure in the chambers – kept in pans to compare with chambers I and VI controls.

[b]No exposure in chambers – kept in wire cages to compare with chambers I, VI, VII, VIII, IX, and X controls.

B. Mouse Disposition Through December 31, 1968

Total initial number of mice (all chambers to date)	7547
Sacrificed at chamber startup (pathology, etc.)	558
Total to chambers	6989
Sacrificed for 6, 12, & 18 month examinations	615
Sacrificed for electron microscopy	35
Terminal sacrifice (pathology, etc.)[c]	596
Previously found dead or sacrificed	4965
Deaths this month	122
Total deaths, all causes	6333

[c]Last 15 of each chamber category (♂ – irradiated or nonirradiated; ♀ – irradiated or nonirradiated) are sacrificed.

Table 30.35.2. Median Death Time (Months) of C57Bl/6 Mice

Chamber	Viral Pretreatment	Male		Female	
		0	100 r	0	100 r
I	0	22	20	25	25
II	0	22	20	16	18
III	0	25	22	20	25
IV	Virus	20	20	25	25
V	Virus	17	18	22	24
VI	Virus	17	20	18	25
VII	0	31		26	
VIII	0		30		26
IX	Virus	29		26	
X	Virus		28		26
XI	0	26	26	19	24
XII	Virus	25	24	20	22
XIII	Virus	(29)	21	20	19
XIV	0	(30)	22	22	20

Table 30.35.3. Lung Adenoma and Adenocarcinoma Incidence

	Time (days)						Summary
	400	500	600	700	800	900+	
Washed Air Chamber Mice							
Number with tumors	0	0	1	2	9	3	Total number of lung tumors, 15
Number of deaths	72	48	56	60	84	130	Total of animals, 450
Percentage	0	0	1.8	3.3	10.7	2.3	Total percentage, 3.3
Smog Chamber Mice							
Number with tumors	0	0	3	6	10	11	Total number of lung tumors, 30
Number of deaths	76	48	60	64	96	106	Total of animals, 450
Percentage	0	0	5.0	8.8	11.4	8.2	Total percentage, 6.7
Chromate Chamber Mice							
Number with tumors	0	1	2	2	2	9	Total number of lung tumors, 16
Number of deaths	52	48	60	68	88	134	Total number of animals, 450
Percentage	0	2.1	3.3	3.1	2.1	8.5	Total percentage, 3.6

Table 30.35.4. Lung Adenoma and Adenocarcinoma Incidence by Sex and Radiation Pretreatment

Treatment	Sex			
	Male		Female	
	Radiated	Nonradiated	Radiated	Nonradiated
Washed air chamber mice	7	3	4	1
Total number of tumors	10		5	
Smog chamber mice	10	15	4	1
Total number of tumors	25		5	
Chromic oxide chamber mice	2	8	6	0
Total number of tumors	10		6	

30.36 REQUIREMENT FOR CONTINUOUS ANTIGENIC STIMULATION IN THE DEVELOPMENT AND DIFFERENTIATION OF ANTIBODY-FORMING CELLS: THE EFFECT OF PASSIVE ANTIBODY ON THE PRIMARY AND SECONDARY IMMUNE RESPONSE

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Mary W. Francis

The levels of both antigen and specific immunoglobulin are considered important regulatory mechanisms which determine the size of antibody-producing cell compartments. Recent studies suggest that repeated antigen contact of the immunocompetent cells is required for complete differentiation. This concept of the continuous role of antigen in the immune response is consistent with the increasing body of evidence demonstrating that antigen does localize and persist in the lymphatic tissue during the primary immune response. One site in lymphatic tissue in which antigen has been demonstrated to be localized extracellularly in plasma membrane infoldings of unique reticular cells is the germinal center. Although the functional significance of this antigen deposition is still not completely resolved, experimental evidence demonstrates that there may be a causal relationship between the antigen localization and the presence and proliferation of characteristic large pyroninophilic cells of these centers. These cells, by morphologic and functional parameters, are considered immunologically competent cells. We have further suggested that during the primary immune reaction the persistence of antigen in germinal centers is

a dynamic process which is sensitive to and altered by the level of free specific antibody. In the present study we attempted to induce maximum immune progenitor cell conversion by using high antigen doses. Subsequently antigen was depleted at various intervals after priming with isologous specific antibody in order to interrupt further immune cell differentiation. It was reasoned that this condition would result in depression of the functional antibody-producing cell compartment, as measured in the intact animal, and subsequently an enhancement of the sensitized immune cell compartment, as assayed in the spleen cell transfer system. The latter is assumed to occur if an antigen is required for the continued differentiation of immunocompetent cells. These data were also correlated with a systematic study of the hyperplasia of spleen germinal centers in an attempt to better categorize the proliferating lymphoid cell compartment of these centers and to obtain further information concerning the role of the antigen localized in these centers.

Methods. – Male $BC3F_1$ mice were used in these studies. Animals were immunized with sheep erythrocyte antigen (SRBC). All injections were intraperitoneal (ip). Specific isologous antiserum was prepared by using the cell transfer method. The serum was diluted with saline to achieve 12 $\log_2$ titer units of hemagglutinin against the test antigen. The antiserum was found in a preliminary study to have a half-life of 12 days in $BC3F_1$ mice. The antiserum was also fractionated on a sucrose gradient, and essentially all activity was found in fractions previously established to contain protein less than 19S in size. Therefore the specific antibody is assumed to be primarily 7S globulin. Individual serum samples were titrated for hemagglutinin as well as hemolysin antibody. The hemolytic plaque-forming technique was used in this study. This study is a quantitative measure of cells producing hemolysin antibody.

Results and Discussion. – The effect of passive antibody on the primary response to SRBC as measured with the PFC assay was a marked decrease in the direct and indirect hemolysin-producing cells (DPFC and IPFC). However, there was a lack of correlation in the degree of antibody mediated DPFC (19S) and IPFC (7S) immune cell suppression during the primary response, the DPFC being much less depressed than the IPFC. As measured in the transfer system there was an enhanced 19S cell compartment and a depressed 7S cell compartment in one-day passively immunized mice. This was true regardless if transfers were performed at one, two, or four weeks after priming. Similarly there was an enhanced 19S cell compartment with little or no effect on the 7S sensitized cell compartment in four-day passively immunized mice. These data suggest that progeny of the antigen-stimulated progenitor cells, as a consequence of further lack of antigenic stimulation, were forced into maturation arrest.

These studies further demonstrate that isologous passive antibody suppresses germinal center growth regardless of whether the antibody is infused one, two, or four days after priming. In terms of formation of sensitized cells the marked depression of the 7S sensitized cell compartment after passive immunization in 24 hr, in contrast to the enhancement of the 19S sensitized cell compartment, corresponds to the suppressed growth of germinal centers during the primary response. Thus, if the germinal center is, as suggested, the site of proliferative expansion of immunocompetent cells, these data indicate that the center growth is related to the 7S antibody response and in the formation of "7S memory."

30.37 A COMPARATIVE STUDY OF THE IMMUNE REACTION IN GERMFREE AND CONVENTIONAL MICE

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H. E. Walburg, Jr.[1]

In recent years considerable attention has been devoted to the germinal centers of lymphatic tissue, particularly as these are structures related to the immune reaction. Results of studies performed by our group as well as others have demonstrated (1) that an antigen-stimulated proliferation of large pyroninophilic cells occurs in germinal centers and this proliferation is antigen dose dependent; (2) that the sensitized immune cell compartment develops first in lymphatic nodules at the time of germinal center hyperplasia; and (3) that during the course of an immune reaction, antigen is localized and retained extracellularly in plasma membrane infoldings of specialized reticular cells which constitute the fixed stroma of the germinal center.

A complicating factor in these immunologic studies of conventional animals is a chronic stimulation by adventitious antigens, resulting in formation of germinal centers in the spleen, Peyer's patches, and most lymph nodes. Ideally, detailed studies concerned with the growth, development, and functional significance of germinal centers require antigenically nonstimulated lymphatic tissue. The closest approximation to such ideal conditions is the germfree animal.

The purpose of the present investigation was to compare the immune capacity of intact germfree and conventional mice, in particular with regard to the

growth and development of lymphatic tissue germinal centers.

Methods. – Carworth Farm No. 1 (CF No. 1) mice were reared and maintained in the Biology Division of the Oak Ridge National Laboratory. Conventional mice used were CF No. 1, 10 to 12 weeks of age. The antigens used were sheep erythrocytes (SRBC) and ^{125}I-human gamma globulin (HGG).

At various intervals after antigen injection, animals were killed by cutting the throat, and blood was collected. One-tenth-milliliter serum aliquots and whole spleens of animals receiving ^{125}I-HGG were counted for radioactivity in a Packard Autogamma counter. Sections of spleen, liver, kidney, and mesenteric lymph node were used for autoradiography. Determinations of surface areas of the white pulp germinal centers were made by planimetry in each longitudinal spleen section. The in vivo culture system was used to follow the growth and development of the secondary antibody-forming potential.

Results. – *Primary Response to Sheep RBC.* – The latent and log phase of the hemagglutinin response was not significantly different in germfree and conventional mice. From day 6 on, the antibody level in germfree mice was consistently one to two $\log_2$ units above that in the conventional animals. Once peak titer was achieved in both groups, the hemagglutinin levels were relatively stable for the following three months.

The mean germinal center surface area increased approximately 2×10^3 μ^2 in conventional animals during the first ten days after injection of SRBC. During the same interval, the change in the germfree animals was approximately 4×10^3 μ^2. Thus there was a difference of twofold in the overall growth of germinal centers in germfree, as compared with conventional, mice. The peak size of the germinal centers in germfree animals at day 10, however, was not significantly different from that of the conventional animals at the same interval. By day 20 the mean germinal center surface area in both groups decreased to 2×10^3 μ^2, which is approximately equal to the background level in conventional mice. However, the germinal centers of germfree animals continued to decrease during the following three months.

Secondary Antibody-Forming Capacity in Germfree Mice. – One week after injection of SRBC, the spleen cells of germfree donor mice gave a six-day hemagglutinin response which was significantly above that produced by an equivalent number of spleen cells from unprimed donors, indicating that secondary antibody-forming potential was developing. Maximum level of the secondary antibody-forming capacity, however, was not reached before 30 to 60 days after priming. This peak level was maintained until five months after antigenic stimulation, at which time the experiment was terminated.

Histology and Autoradiography During the Primary Response to HGG. – One hour after injection of 1.5 mg ^{125}I-HGG in conventional mice, label was found preferentially in the marginal zones of spleen lymphatic nodules. At 6, 12, and 24 hr, the preexisting germinal centers were heavily labeled, while label decreased in other areas of the spleen (Fig. 30.37.1*A* and *B*). Between 2 and 20 days after antigen injection, the label was predominantly concentrated in germinal centers though the concentration decreased progressively with time. The overall pattern of antigen localization during the first 10 days was similar in the germfree mice as compared with the conventional mice. The major difference, however, was that although label concentrated in defined perifollicular areas of the lymphatic nodules, no active germinal centers existed (Fig. 30.37.1*C* and *D*). However, between 10 and 20 days, germinal centers formed in these regions of antigen retention. In the present study there was no deficiency in hemagglutinin-forming potential, development and growth of lymphatic tissue germinal centers, or antigen localization as well as antigen clearance in germfree mice compared with conventionally reared animals of the same strain. Rather, the data of these experiments suggest that the germfree mice may have an increased immune capacity. However, since the level of antigenic background stimulation in the conventional mouse is not controlled, the magnitude of this difference, if it is real, could be expected to vary among experiments.

Conclusion. – Based on the sequence of histologic and serologic events observed in this serial study of the primary response in germfree mice, we would suggest the following: Lymphatic tissue has two unique components, dispersed immunologically competent progenitor cells and defined areas of reticular cells, capable of localizing antigen extracellularly. After the initial antigenic stimulation, one phase of the proliferative expansion of the immunologically competent progenitor cells occurs in the region of the antigen-retaining reticular cells. This results in the accumulation of large pyroninophilic cells in this area and ultimately in the formation of a histologically well-defined germinal center. At the same time antibody becomes detectable in the serum. The serum antibody response and the growth of the germinal center simultaneously develop to peak levels. During this time the sensitized cell compartment is being established in the lymphatic nodules. Therefore the proliferation of large pyro-

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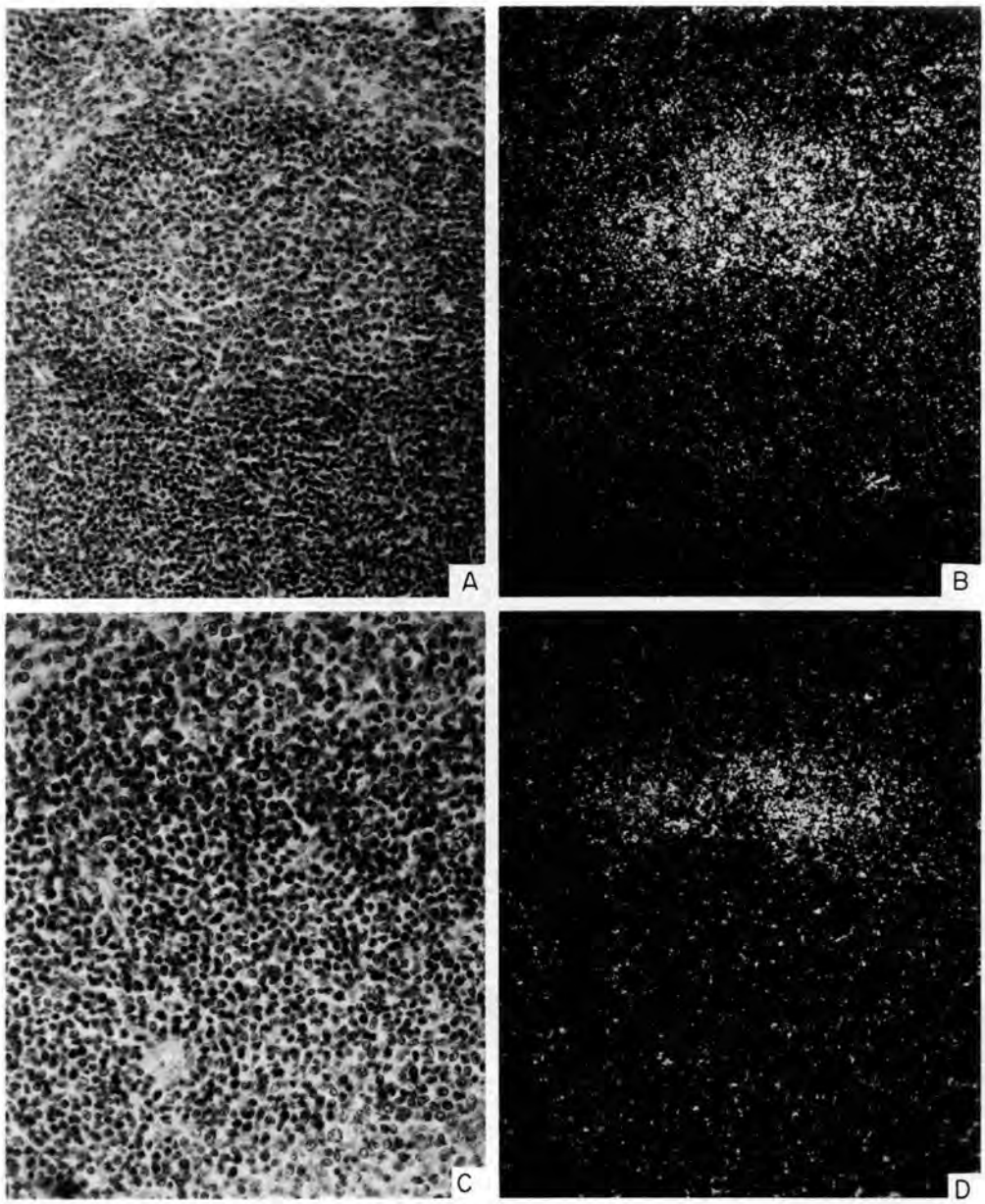

Fig. 30.37.1. ^{125}I-HGG Localization in Spleen Lymphatic Nodules During the Primary Response. *A*, active germinal center in spleen of conventional mouse 12 hr after injection; *B*, dark field photomicrograph of an autoradiogram of the identical area in the adjacent section; *C*, perifollicular region of lymphatic nodule in germfree spleen 12 hr after injection; *D*, dark-field photomicrograph of an autoradiogram of the identical area in the adjacent section.

ninophilic cells in germinal centers is probably related to both the development of antibody-producing cells as well as memory cells. Since the largest proportions of antibody-producing cells and memory cells are eventually found outside the germinal centers, the lymphoid cell population of the centers must be considered transitory. This is also supported by our previous kinetic studies of active germinal centers. The later decrease in the size of the centers probably is a manifestation of the diminishing growth rate of the antibody response as well as the memory formation. This may be a consequence of the elimination of antigen from these areas.

Reference

[1] Pathology and Physiology section.

30.38 LOCALIZATION OF ^{125}I-LABELED ANTIGEN IN GERMINAL CENTERS OF MOUSE SPLEEN: HISTOLOGIC AND ULTRASTRUCTURAL AUTORADIOGRAPHIC STUDIES OF THE SECONDARY IMMUNE REACTION

M. G. Hanna, Jr. A. K. Szakal

It has been demonstrated that during the immune reaction, antigen localizes extracellularly in plasma membrane infoldings of dendritic reticular cells located in the lymphatic tissue germinal centers. We have further demonstrated that during the primary immune reaction the persistence of antigen in germinal centers is a dynamic process which is sensitive to, and altered by, the level of specific antibody. Thus an exaggerated but synchronous response would occur when antigen is given in the presence of specific serum antibody in actively immunized animals where, although there is initial localization of antigen, the persistence in germinal centers is relatively short compared with the primary immune response.

The levels of both antigen and specific antibody are considered regulating mechanisms with regard to magnitude of the antibody-producing cell compartment. One mechanism of the selective activity of antibody would be through competition with immunologically competent cells for antigen. This selective activity would then be maximum during the secondary response and, to be optimally effective, would have to minimize or block developing and/or developed immunologically functional antigen-trapping mechanisms. Accordingly, the purpose of this investigation was to systematically study, on a histologic and ultrastructural level, ^{125}I-labeled human gamma globulin (HGG) localization in mouse spleen germinal centers during the secondary immune reaction in animals having a preexisting hemagglutinin titer.

Materials and Methods. – Male BC3F$_1$ [(C57BL ♀) × (C3H/AN ♂)F$_1$Cum] specific-pathogen-free (SPF) mice 12 to 14 weeks of age were used. Groups of mice were primed at 0 time and at three days with 1.5 mg HGG. Animals used for the serologic and histologic studies were divided into two groups, with one group receiving 1.5 mg ^{125}I-labeled HGG on day 44. Five mice were killed at 2 hr, 12 hr, and days 1, 2, 3, 4, 5, 8, and 12 after the second injection of antigen. Twenty primed animals were not injected for a secondary response, and these were killed in groups of five at days 30, 45, 52, and 62 after priming.

One-tenth-milliliter samples of serum were used for counting radioactivity, and the remainder of the serum was frozen for passive agglutinin titrations. Tissue (such as spleen, measured pieces of liver, and kidney) to be assayed for radioactivity was placed in tubes containing 1 ml Bouin's fluid. All counts were made in a Packard Autogamma counter. Tissue sections were prepared for autoradiography. Electron-microscopic autoradiography was performed on thin sections coated with Ilford L-4 nuclear research emulsion. The ultrathin sections were examined in the HUIB Hitachi electron microscope after development in Kodak Microdol X.

Histologic Autoradiography of the Secondary Response. – During the first 24 hr after the secondary injection of antigen the most dramatic histologic change was the accumulation of label in the spleen germinal centers. The amount of label in the germinal centers markedly decreased between days 2 and 3, and concentration of grains was seen in central arterioles of the lymphatic nodules. Also at this time spotty concentrations of grains were detected in the cortical lymphocyte zone of the nodules. No significant labeling could be seen in the periarterial lymphocyte mass of the nodule or in the spleen red pulp. The three-day interval corresponds to the peak retention of label counted in the spleens, and it was assumed that the majority of the grains seen at this time were associated with antigen-antibody complexes.

To better define the location of grains in the lymphatic nodules at three days after the secondary injection, studies were made of autoradiograms of 1-μ sections of Epon-embedded material. It could be seen in these autoradiograms that the grains were concentrated in capillaries of the cortical lymphocytic zone and in the central arterioles of the nodules. At days 8 to 15 the only concentrations of grains in the spleen were

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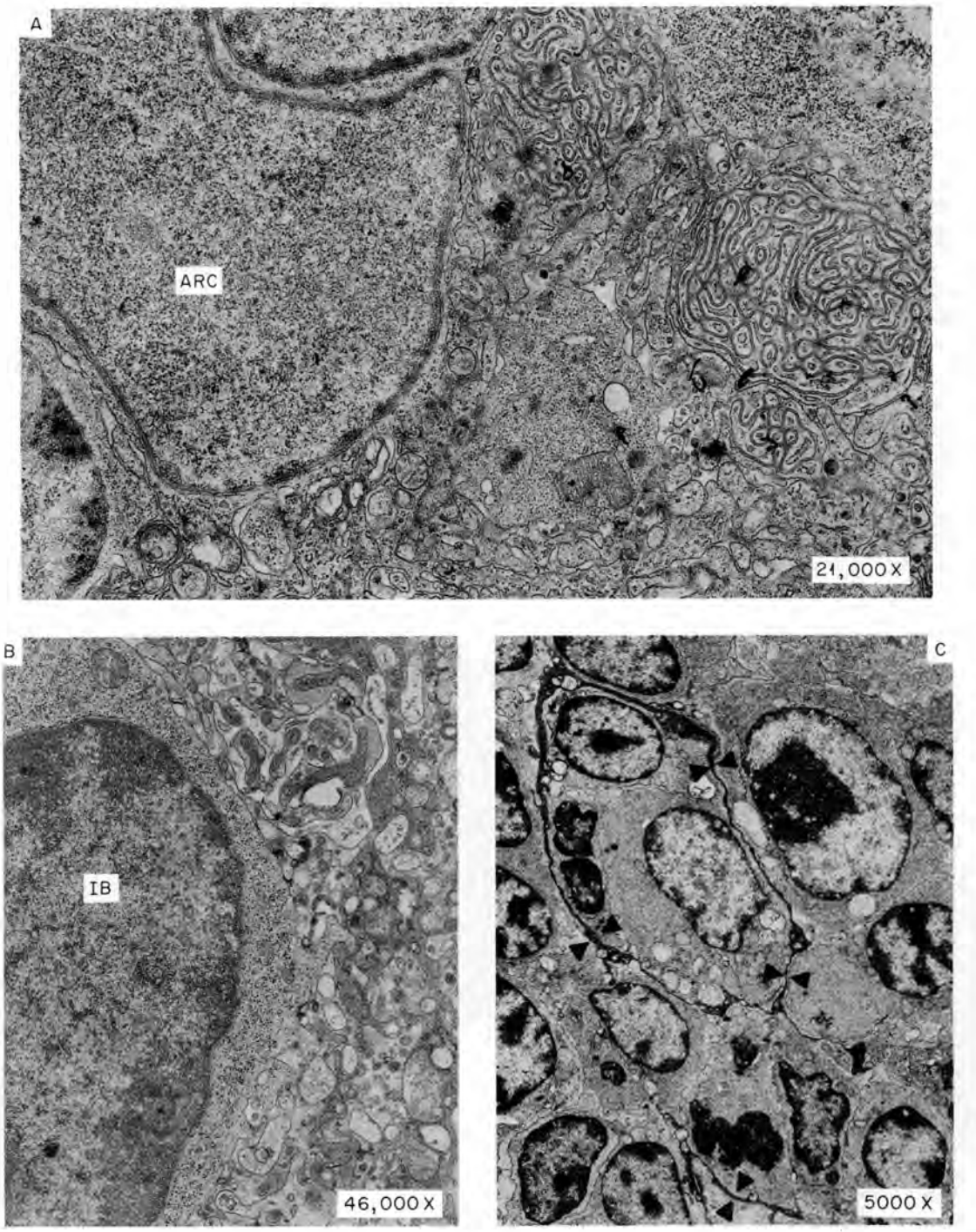

Fig. 30.38.1. *A*, **Microautoradiograms Illustrating the Distribution of Silver Grains over the Labyrinthine Plasma Membrane Infoldings of Antigen-Retaining Reticular Cell Processes (ARC) 2 hr After the Initiation of the Secondary Immune Response in Mouse Spleen Germinal Centers.** Note the uniformity of the plasma membrane infoldings. 21,000X. *B*, an electromicrograph of a portion of a germinal center at day 3, demonstrating the dilation of the membrane infoldings of the antigen-retaining cell. Note the disruption in the continuity of the plasma membrane adjacent to an immunoblast. 46,000X. *C*, electron micrographs of highly electron-dense cytoplasmic remnants of antigen-retaining reticular cells seven days after secondary injection. (▲), remnants condensed among parenchymal cells of germinal centers. 5000X. Reduced 23%.

associated with the central arterioles. The germinal centers and lymphocyte mass of the nodules were essentially cleared of detectable grains.

Ultrastructural Studies. – At 2 hr after the secondary response injection of HGG, silver grains observed in germinal centers were primarily located overy very numerous plasma membrane infoldings of antigen-retaining reticular cells (Fig. 30.38.1*A*). The extensive cytoplasmic processes containing complex and labyrinthine infoldings of plasma membranes filled the intercellular space between the large parenchymal cells (immunoblasts) of the germinal center.

By day 1 after the secondary injection of antigen there was a loss of regularity and organization of the plasma membrane infolding (Fig. 30.38.1*B*). At day 3 the disorganization of the membrane infoldings and the formation of large pools of the electron-dense material were even more pronounced. By day 7 after injection of antigen some membranous electron-dense remnants of the antigen-retaining cells were seen forming continuous networks among the free parenchymal cells of the germinal centers examined. These remnants of the processes of antigen-retaining cells appeared to be condensed among the free cells (Fig. 30.38.1*C*).

Conclusion. – The magnitude and regularity of the developed plasma membrane infoldings of antigen-retaining reticular cells in germinal centers are described. The key finding in this study was the degradation of these specifically developed plasma membrane infoldings during the early intervals of the secondary response. This response occurred in conjunction with a loss of label and an apparent loss of function of the existing antigen-trapping mechanisms in spleen germinal centers. We suggest that the cytotoxic sequelae described could be a result of complement fixation and that such a regulatory mechanism would be essential in preventing further contact of immunologically competent cells with antigen while clearing the centers for development against further antigens. A conformity of vesicles was observed in the cytoplasm of the parenchymal cells of the germinal centers and in the plasma membrane infoldings of the antigen-retaining reticular cells.

30.39 VIRUS PARTICLES IN SPLEEN GERMINAL CENTERS

M. G. Hanna, Jr. A. K. Szakal

A further aspect of the electron microscopic studies of spleen germinal centers during the immune reaction was the observation of large numbers of endogenous virus particles in the plasma membrane infoldings of antigen-retaining reticular cells. The particles measured 100 to 120 mμ in diameter, and many resembled the C-type virus particle associated with murine leukemia. Occasionally, budding of the C-type virus particles from the plasma membranes of the characteristic lymphoid cells was observed. During studies using ^{125}I-HGG, the numerous virus particles could be seen together with silver grains extracellularly in plasma membrane infoldings. This finding suggested a similarity in the mechanism of retention of the exogenous antigen and the apparently endogenous virus particles.

In light of the immunosuppressive effect of some murine leukemia viruses and their normal occurrence in spleen germinal centers, a systematic study of virus localization in germinal centers was performed. Young adult BALB/c mice received intraperitoneal injections of Rauscher virus preparation. The spleen, thymus, mesenteric lymph node, and bone marrow of these mice were studied histologically and electron microscopically at intervals between 6 hr and 20 days after injection. Numerous virus particles were observed localized extracellularly in plasma membrane infoldings of dendritic reticular cells of spleen and mesenteric lymph node germinal centers. The majority of these particles were of C type. The number of viruses in these germinal centers markedly increased between days 1 and 20 after injection. Budding of C-type particles from the parenchymal lymphoid cells of the germinal centers occurred with increasing frequency between days 1 and 20 after injection. Viruses were also observed in the thymus, bone marrow, and spleen red pulp of these experimental mice; however, the concentration of virus, even at 20 days after injection, was markedly less in these tissues compared with the concentration in germinal centers. This was also true when comparison was made between germinal centers and the associated lymphocyte mass of the lymphatic nodules.

Conclusion. – With the present state of knowledge it is impossible to determine the functional significance of virus localization in lymphatic tissue germinal centers. It is pertinent, however, that the observation was made for both endogenous as well as adventitious viruses. Also, as would be expected, the mechanism of extracellular localization (in plasma membrane infoldings of dendritic reticular cells) is identical to that which can be shown to develop for a variety of soluble and particulate antigens. A most interesting aspect of the virus localization in germinal centers is the prevalence of C-type particles and their proliferation in the lymphoid cells. In the situation of the Rauscher virus preparation in BALB/c mice, the selective retention of C-type particles might have initially been stimulated by an immune response, but ultimately resulting in the initial proliferative phase of the virus. In some way this finding must correlate to the immunosuppressive effect

of most murine leukemia viruses. Thus if the importance of antigen localization in germinal centers is to provide a unique opportunity for surface contact of antigen and lymphoid cells, then the present observations may be relevant to (1) immunologic recognition and response to viruses and (2) the opportunity of some viruses to find suitable target cells for proliferation. The latter is most relevant for murine leukemia viruses.

30.40 RADIOSENSITIVITY OF THE ANTIGEN TRAPPING MECHANISM AND ITS RELATION TO THE SUPPRESSION OF THE IMMUNE RESPONSE

Paul Nettesheim M. G. Hanna, Jr.

Our studies concerning regenerative potential after x irradiation of the cell compartment responsible for immunological memory indicated that recovery of this progenitor cell pool is dependent on the availability of uncommitted immune progenitor cells *and* persistence of antigen.[1] We therefore decided to study the effects of irradiation on the antigen-trapping and -retaining mechanism in greater detail during various stages of the immune response. The antigen chosen for this investigation was heat-aggregated ^{125}I-labeled human gamma globulin (^{125}I-HGG). We found that x ray given prior to antigenic stimulation did not completely abolish the antigen trapping in germinal centers; however, it caused an enhanced elimination of antigen from these areas. This x-ray-induced defect of the antigen retention was found to persist for several weeks (Fig. 30.40.1). The enhanced elimination of antigen could have been due to x-ray damage, either to cells responsible for trapping

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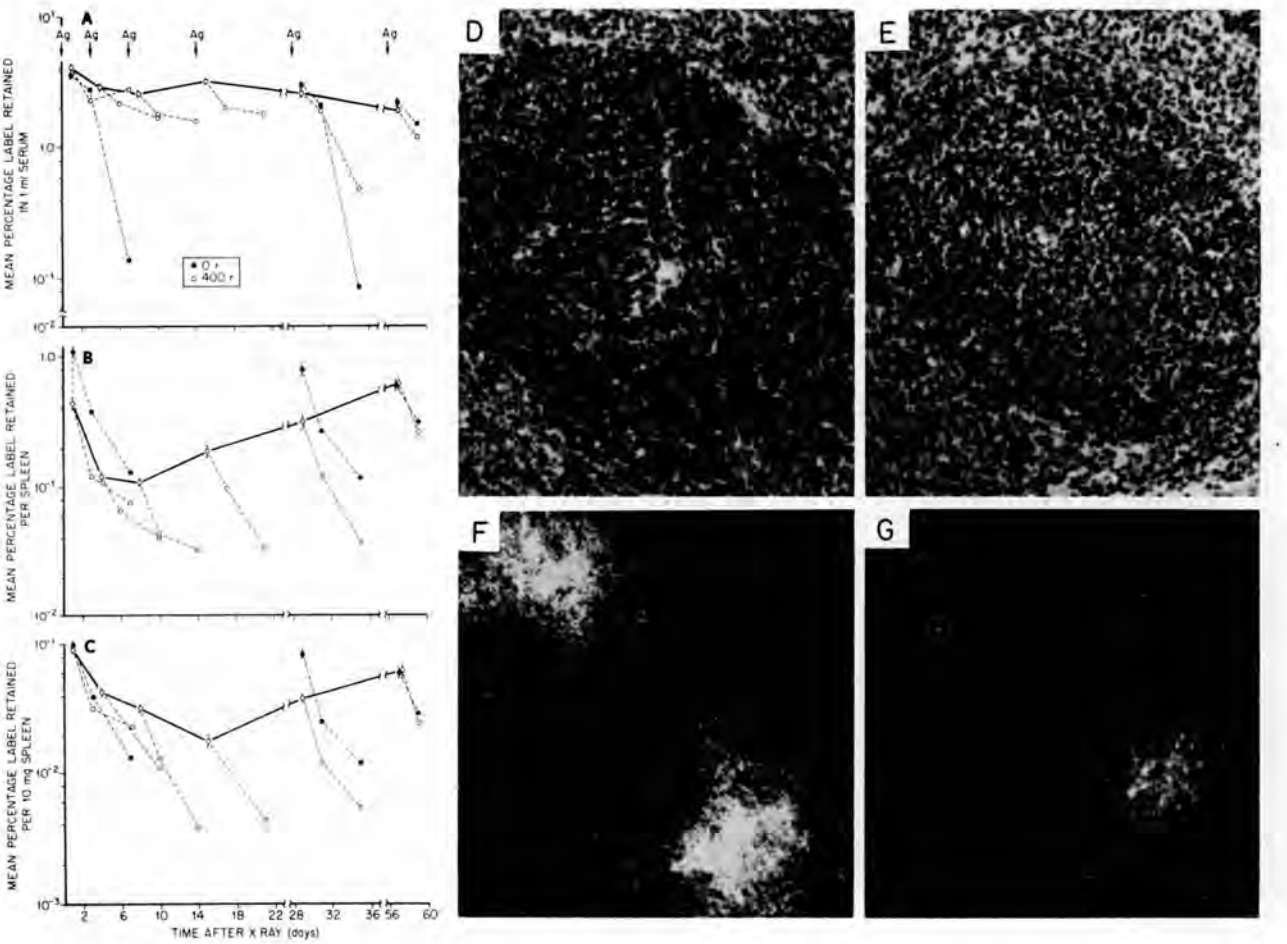

Fig. 30.40.1. Effects of X Irradiation on Retention of ^{125}I-HGG. (*A*) In serum; (*B*) in whole spleen; (*C*) per unit spleen weight: ○, irradiated mice; ●, unirradiated mice. (*D*) Spleen lymphatic nodule of unirradiated mouse; (*E*) four weeks after 600 r (H&E ~ 500X). Dark-field photomicrograph of spleen autoradiogram (~125X) one day after ^{125}I-AHGG, (*F*) of unirradiated mouse and (*G*) of mouse irradiated with 600 r four weeks previously. Enlarged 34%.

and retaining the antigen or to the immune progenitor cells, or both. In the latter case the enhanced elimination could have been a consequence of the delay of antibody production, since antibody is being implicated as a factor involved in antigen localization. To decide between these alternate possibilities, a second series of experiments was carried out in which x irradiation was given to animals with circulating 19S or 7S antibody specific for the test antigen. X ray did not affect the level of circulating antibody. It did, however, cause an enhanced elimination of the spleen in spite of the presence of specific antibody. Electron microscopic evidence suggestive of cellular damage of reticular cells implicated in antigen retention was obtained. Since these cells are known to have a low proliferative rate, this might explain the long-lasting defect in antigen-retaining capacity.

Reference

[1] P. Nettesheim and M. L. Williams, *J. Immunol.* **100**, 760 (1968).

30.41 REGENERATIVE POTENTIAL OF IMMUNOCOMPETENT CELLS INVOLVED IN THE PRIMARY AND SECONDARY ANTIBODY RESPONSE

Paul Nettesheim Mary L. Williams

The present study is a continuation of our previous attempts to characterize the various immune progenitor cell compartments stimulated in the primary and secondary antibody response. At least two distinct antigen-sensitive precursor cells are postulated in the current cytokinetic models of the antibody response. The criterion by which we hoped to distinguish between the different progenitor cell pools, if they indeed exist as separate entities, was their regenerative capacity as established during the recovery phase from x irradiation. The in vivo culture system of lymphoid cells was applied in this study, since it allows an estimation of the immune progenitor cell pool size. Figure 30.41.1 summarizes the main findings of these studies, represented by three major patterns of recovery. The primary antibody-forming potential (curve *A*) recovers very slowly from x irradiation, eventually reaching a normal level. This process does not require antigen and is, as will be discussed in more detail later, partially thymus dependent. In contrast, previous investigations revealed that the secondary antibody-forming potential does not recover when antigen is either no longer available or at too-low concentrations

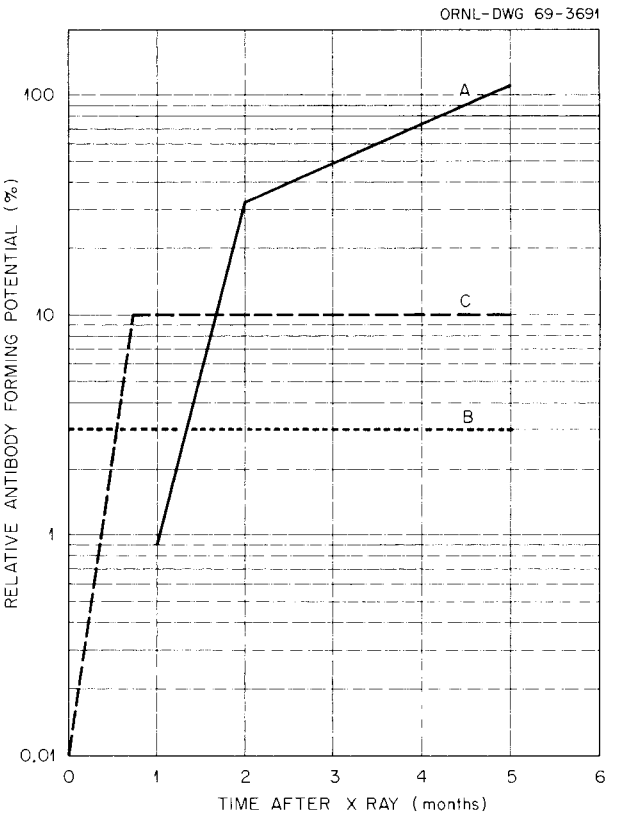

Fig. 30.41.1. Schematic Representation of the Three Major Recovery Patterns of the Immune Capacity, Observed After Sublethal X Irradiation. Primary antibody forming capacity (——). Secondary antibody forming capacity, short priming, irradiation interval (– – –). Secondary antibody forming capacity, long priming, x-irradiation interval (...).

(curve *B*), though a fast but only partial recovery is observed when antigen continues to be present for some time after the x-ray insult (curve *C*). This indicates that the cell pool which is initially triggered by antigen in the *primary* response is distinct from the cell pool predominantly responding in the secondary response. The former is part of a self-renewal system, normally maintained in a steady state, which recovers after x irradiation; the latter is a specific differentiation product of the former induced by antigenic stimulation and does not recover from injury if this stimulus is lacking. As the antigenic stimulus subsides, the production ceases. Since its self-generating capacity appears to be limited, it can be permanently reduced in size or even be abolished by insults such as x irradiation.

We then studied the effect of the thymus on the recovery of the primary immune potential in x-irradiated mice. Young adult mice were thymectomized and

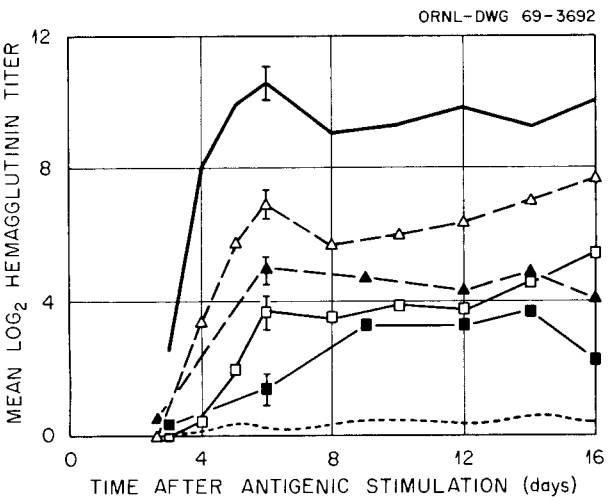

Fig. 30.41.2. Recovery of the Primary Rat Hemagglutinin Response of Normal and Thymectomized $C3_1F_1$ Mice from 600 r Whole-Body Exposure. 10^9 rat RBC were injected ip into unirradiated mice (——), and irradiated mice at the following intervals after x irradiation: 2 hr (.....); 4 weeks, normal (□–□), thymectomized (■–■); 8 weeks, normal (△–△), thymectomized (▲–▲). Microtitration method; 10 to 12 samples per point.

irradiated with sublethal x-ray exposures two weeks later. At various times thereafter an antibody response was induced by injecting 10^9 rat red blood cells. The results summarized in Fig. 30.41.2 show that recovery of immune competence is slower in thymectomized irradiated mice than in nonthymectomized irradiated controls. However, recovery from the x-ray-induced immune suppression is by no means abolished in thymectomized animals. This indicates that the immunologically competent cell or unit (since probably more than one cell type is required for initiation of the antibody response) can regenerate in the absence of the thymus, though the rate of regeneration is thymus dependent.

30.42 STUDIES ON THE MAINTENANCE OF THE LATE ANTIBODY RESPONSE

Paul Nettesheim Anna S. Hammons

Immunological memory and high levels of serum antibody are maintained for many months in animals immunized with various types of antigen. One proposed mechanism for this lengthy immunity is that antigen persists for many months in the lymphoid organs and in other organs, and continuously triggers immune progenitor cells to form new antibody-producing cells and/or memory cells to replace dying ones. Such a renewal system is particularly useful in explaining the continued antibody response, since the half-life of 7S hemagglutinating antibodies in the serum of mice has been found to be approximately four days, and that of most antibody-producing cells two to five days. X ray has been shown to interfere with antigen-induced cell proliferation and differentiation, but not with antibody production itself when given at peak of response. It was therefore used to test whether the high serum antibody levels found late in the immune response are dependent on continuous recruitment of antibody-forming cells. Mice thymectomized during adulthood were immunized with heterologous red blood cells and were irradiated four to eight weeks after immunization (i.e., at plateau of antibody response) with either 2 X 400 r (three weeks apart) or 1 X 850 r (+ 2 X 10^6 isologous bone marrow). Thymectomy was performed prior to x irradiation since it has been shown to prolong the x-ray-induced immune suppression. It was found that within the five to eight weeks following the x-ray exposure the decay rate of circulating antibody was only slightly faster in irradiated than in unirradiated control animals. In other experiments Millipore diffusion chamber cultures of spleen cells undergoing a secondary antibody response were irradiated in vitro with 1200 r on plateau of antibody response. The chamber cultures were implanted into mice and transferred every two weeks to a new set of recipients. The level of circulating antibody was measured in these recipients. It should be noted that with each chamber transfer into a new host the chamber culture (volume 0.15 ml) has to release enough antibody in order to

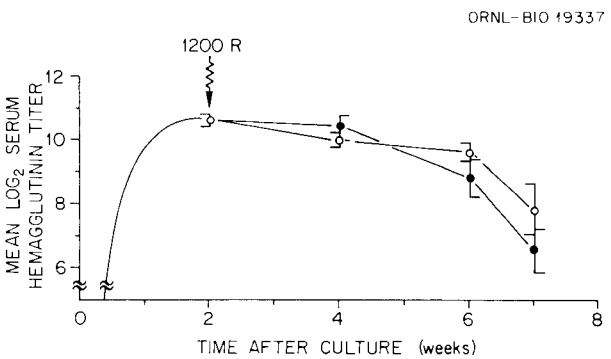

Fig. 30.42.1. Effect of X Irradiation on Serum Hemagglutinin Levels of Mice Carrying Irradiated (●) or Unirradiated (○) Diffusion Chamber Cultures. Cultures were inoculated with 25 X 10^6 primed spleen cells and 2.5 X 10^6 sheep red blood cells and irradiated with 1200 r two weeks later (see arrow). There were five to eight samples used per point. Horizontal bars indicate standard error of the mean.

raise the recipients' serum antibody from zero to levels between six and ten $\log_2$ titer units (total body fluid per 25-g mouse ~2 ml). It seems reasonable to assume that this can be accomplished only if synthesis of antibody is taking place.

The results of this experiment are summarized in Fig. 30.42.1. It can be seen that no significant differences in antibody levels were detectable between mice carrying either unirradiated or irradiated cultures. The results strongly suggest that the maintenance of the serum antibody plateau late in the immune response is dependent on a long-living population of antibody producing cells and that recruitment of such cells, if occurring at all during this phase of the response, must be very slow.

30.43 THE FATE OF 1-(4-DIMETHYLAMINOBENZYLIDENE)INDENE IN RATS

C. T. Bahner D. G. Doherty

Study of the fate of 1-(4-dimethylaminobenzylidene)indene (I) in vivo has been continued. I labeled with tritium on the aromatic ring of the benzylidene portion of the molecule was dissolved in peanut oil and administered ip to normal and tumor-bearing female rats. After two to three days the animals were killed, and tissues were taken for preparation of autoradiograms. Formalin-fixed bone marrow smears showed a high level of radioactivity. Frozen tissues were heavily radioactive, but diffuse blackening of the slides, and streaks, indicated that the radioactivity was leaking from the specimens in soluble or colloidal form. Formalin-fixed tissues embedded in paraffin and sectioned to make slides retained much less radioactivity, but it was more clearly localized. Fat cells in the abdomen were heavily labeled. Red cells in the blood vessels were consistently labeled. Liver and spleen were well but diffusely labeled. Normal liver cells retained more radioactivity than did leukemic Lymphoma 8 cells, which invaded the liver. Walker 256 tumor cells seemed more radioactive than Lymphoma 8 cells in the solid tumors, but both were well labeled. Uterus, ovary, and vagina were labeled especially in the follicular area and epithelial lining. Islet and acinar cells of the pancreas and the cortex and medulla of adrenals and thymus were radioactive. Lung and kidney tissues were moderately active. Hair follicles were consistently more radioactive than other nearby skin. In some instances there was selective labeling of one layer of the retina. Layers of cells in artery walls were labeled, but the elastic membrane in the walls was almost free from radioactivity.

30.44 TRANSLATIONAL AND TRANSCRIPTIONAL CONTROL OF TYROSINE-α-KETOGLUTARATE TRANSAMINASE SYNTHESIS IN CULTURED HEPATOMA CELLS

Kai-Lin Lee[1] F. T. Kenney

The level of tyrosine transaminase in cultured Reuber H-35 hepatoma cells is inducible by at least two and probably three discrete induction mechanisms, mediated by hydrocortisone, glucagon, and insulin respectively. Previous studies in this laboratory[2] have suggested that the transaminase mRNA in these cells may be sufficiently stable to permit discrimination between transcriptional and translational mechanisms of induction. The present experiments have revealed the following:

1. The early phases of induction by glucagon are insensitive to actinomycin D, while induction by hydrocortisone is always sensitive to this antibiotic.
2. When cells are preinduced by hydrocortisone a limitation in the response to glucagon is removed, and glucagon effects a very rapid and large induction which is completely insensitive to actinomycin.
3. Preinduction of cells with glucagon does not alter the response to subsequent addition of hydrocortisone.
4. Brief exposure of cells to hydrocortisone and subsequent removal of the hormone causes the transaminase to increase continuously for another 2 hr. Under identical experimental conditions but with pretreatment with glucagon rather than hydrocortisone, the transaminase activity immediately dropped when the glucagon was removed.
5. When hydrocortisone and glucagon were added together, the increase in transaminase was more than the additive response to each hormone alone. These results clearly indicate that the steroid hormone induces tyrosine transaminase by promoting the synthesis of transaminase mRNA, while glucagon acts at some post-transcriptional step in enzyme synthesis.

References

[1] Biochemical Regulation section.

[2] J. R. Reel and F. T. Kenney, *Proc. Natl. Acad. Sci. U.S.* **61**, 200 (1968).

30.45 HORMONAL REGULATION OF TYROSINE TRANSAMINASE IN FETAL RAT LIVER IN ORGAN CULTURE

W. D. Wicks

It now appears likely that there are three discrete mechanisms for inducing hepatic tyrosine transaminase, involving hydrocortisone, insulin, and cyclic AMP as primary inducers. Thus, when combinations of these inducers are added to explants of fetal liver in organ culture, the response of tyrosine transaminase activity is as follows: synergistic with hydrocortisone and cyclic AMP, additive with hydrocortisone and insulin, and somewhat less than additive with insulin and cyclic AMP. The synergistic response to the combination of steroid and cyclic AMP is visualized as reflecting a sequential stimulation of enzyme synthesis by the two inducers. Kinetic studies and evidence from other systems support such a possibility, and the data suggest that the steroid enhances transaminase messenger RNA formation, while cyclic AMP promotes the decoding of this messenger in some manner. The site at which insulin mediates the synthesis of the transaminase is presently unknown, but it seems clear from the combination studies and other data that it must be distinct from those affected by hydrocortisone and cyclic AMP. The lack of complete additivity in the response to insulin and cyclic AMP can be accounted for by the well-documented antagonistic effect of insulin on cyclic AMP in liver.[1] If insulin induces the transaminase by one means and independently acts to block the induction by cyclic AMP, the results would be readily explained. At present it is not clear why both insulin and cyclic AMP should induce the same enzyme, when they normally act in opposition. Epinephrine biosynthesis ultimately depends upon the supply of blood tyrosine, and it is conceivable that induction of the primary enzyme involved in tyrosine breakdown could act to reduce the availability of tyrosine and consequently lower the rate of synthesis of epinephrine. In the case of insulin this would indirectly suppress the gluconeogenic action of epinephrine on the liver. In the case of cyclic AMP, induction of the transaminase would act as feedback control since epinephrine stimulates the synthesis of this nucleotide in liver.

Experiments are now in progress aimed at delineating, at the molecular level, the different mechanisms of induction of this enzyme.

Reference

[1] L. S. Jefferson *et al.*, *J. Biol. Chem.* **243**, 1031 (1968).

30.46 ENZYMES AND FACTORS REGULATING THEIR SYNTHESIS IN HEPATOMA CELL CULTURES[1]

Kai-Lin Lee J. R. Reel F. T. Kenney

Introduction. – Most of the work on regulation of enzyme levels in normal tissues or in tumors has been done in vivo, with consequent uncertainty as to the actual factors responsible for regulating the levels of the enzymes examined. We have begun to analyze the various inducible liver enzymes in cells of the H35 hepatoma grown in tissue culture. Such an approach is essential to an understanding of hormonal regulation of enzyme synthesis, particularly in identifying with certainty which enzymes or groups of enzymes are sensitive to particular hormones. Also, it may be possible by this means to identify changes in the cultured cells which are of significance in neoplasia.

Results. – *Tryptophan Pyrrolase.* – This enzyme is inducible by glucocorticoids in vivo, in liver perfusion studies, and in the H35 hepatoma grown in vivo. In our cultured cells pyrrolase activity is not detectable, whether the cells are steroid-treated or not.

Serine Dehydrase. – This enzyme has been reported to be induced in vivo and in hepatomas grown in vivo by a variety of agents (glucocorticoids, glucagon, epinephrine, amino acid mixtures, etc.), of which we regard glucagon (cyclic AMP) as the effective agent. In our cultured cells no serine dehydrase activity could be detected, even after treatment with glucagon or hydrocortisone.

Alanine Transaminase. – This enzyme is induced in livers of glucocorticoid-treated rats, but no data have been reported to indicate that the steroid effect is direct. The enzyme level in our cultured cells is approximately the same as in the liver in vivo. Supplementation with glucocorticoids causes a two- to threefold increase in this level, but several factors suggest that this may not reflect increased synthesis of the enzyme. This point will be examined using an antiserum prepared against alanine transaminase and provided to us by Dr. H. L. Segal of SUNY, Buffalo, New York.

Glucose-6-phosphatase. – This enzyme has been reported to be induced in vivo by glucocorticoids and by glucagon and other factors which we regard as secondary to glucagon release. The phosphatase activity in our cultured cells is very low and could not be increased either by hydrocortisone or by glucagon.

Glucokinase. – This enzyme has been reported to be induced in vivo both by insulin and by glucose; as these factors are closely interrelated in vivo, there has been much controversy over the question of which is the

actual effective agent. In our cultured cells we have been unable to obtain an adequate assay for this enzyme. The assay employed involves coupling to glucose-6-phosphate dehydrogenase and measurement of TPN reduction; in the cultured cells a very active "nothing dehydrogenase" rapidly reduces TPN and obscures the glucokinase reaction.

Discussion. – From these preliminary results it is apparent that no consistent pattern has yet been established. The tyrosine transaminase in cultured hepatoma cells is present in normal amounts and responds to all of the inducing hormones previously studied in vivo, namely, hydrocortisone, glucagon, and insulin. But hydrocortisone will not induce tryptophan pyrrolase and has at best a small effect on alanine transaminase. Glucagon will induce neither serine dehydrase nor glucose-6-phosphatase. From these results the important implication is emerging that the hormones may be unable to activate genes which are thoroughly inactivated; rather, they can alter rates of transcription or of translation but only of active genetic sites. This is consistent with other aspects of hormonal regulation of enzyme synthesis.

Reference

[1] For appropriate literature citations see "Hormonal Regulation of Synthesis of Liver Enzymes," a chapter in *Mammalian Protein Metabolism,* vol. 3, ed. by H. N. Munro, Academic, New York, in press.

30.47 "SUPERINDUCTION" BY ACTINOMYCIN D: THE RESULT OF DIFFERENTIAL INHIBITION OF ENZYME SYNTHESIS AND ENZYME DEGRADATION

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The tyrosine transaminase of cell cultures of minimal deviation hepatoma cells is induced by hydrocortisone. This effect is blocked if actinomycin is added early in the course of induction, which is consistent with the concept that the steroid hormone promotes transcription. If actinomycin is withheld until the hormonal induction has reached the new steady state typical of elevated enzyme synthesis, the antibiotic causes a marked further increase in the enzyme level, an effect which has been termed "superinduction." This has now been reported in a variety of mammalian regulatory systems, and it has been attributed to the action of cytoplasmic repressors acting to limit the extent of hormonal induction.

We have measured rates of synthesis and degradation of tyrosine transaminase in both H35 and HTC cells subjected to the "superinduction" treatment. In both cell lines transaminase synthesis was inhibited by the antibiotic, falling off exponentially with an approximate half-life of 3 hr. Despite the inhibition of synthesis the enzyme level was increased. This disparity reflects a marked and rapid inhibition of degradation of the enzyme, which was quickly reduced to about 25% of the normal rate. As there is a period of 2 to 3 hr during which synthesis of the enzyme is high (though steadily dropping) and degradation is slowed, the enzyme level rises. The data of these experiments fit remarkably well the theoretical formulation

$$dE/dt = k_1 - k_2 E ,$$

where k_1 is the rate constant of synthesis and k_2 is the rate constant of degradation. This has hitherto been thought to be a rather crude approximation.

These data provide a further indication that the process of enzyme degradation is complex, as we have shown in previous in vivo experimentation. They also show clearly that "superinduction" in this experimental system is not indicative of translational repression.

30.48 HORMONAL REGULATION OF ENZYME SYNTHESIS IN CARCINOGEN-TREATED RATS

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A group of 100 pathogen-free rats were fed the hepatic carcinogen diethylnitrosamine under conditions in which the chronology of pathological changes has been documented. At intervals, groups of rats were adrenalectomized and then tested for the capacity of the liver to respond to two different types of hormonal inducers of enzyme synthesis. These were (1) the steroid hydrocortisone, which we believe acts by promoting transcription, and (2) the polypeptide glucagon, which acts at the translational level via the intermediate cyclic AMP.

With continuous carcinogen treatment the steroid-mediated induction became progressively larger, even though the animals all had massive hepatic tumors and were near death by the end of the experiment. However, the response to glucagon was progressively diminished and was virtually absent in later stages of the experiment. These results are consistent with the conclusion that the induction pathway which is sensitive to glucagon is operating without hormonal stimulation in the livers of the carcinogen-treated rats. This may indicate an effect of the carcinogen on translational processes or that the carcinogen alters cyclic AMP metabolism. These possibilities will be investigated further.