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Summary of the Workshop

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THE keynote address asked the question: Has a new era dawned for toxicology? Dr. Leon Golberg, Chemical Industry Institute of Toxicology, answered: Yes! For the time has come when molecular and cellular biologists have become conscious of the applicability of their work to toxicology, and toxicologists begin to appreciate how this basic research can contribute to an understanding of mechanisms of toxic action, to risk assessment, and to the evaluation of safety under prescribed conditions of use. Dr. Golberg attempted to provide a road map, a bird's eye view of current trends in toxicology for the benefit of nontoxicologists (taking care to avoid the topic of regulatory and political pressures). Attention was drawn to some of the more glaring gaps in present knowledge and longer term needs in toxicological research.

Analytical methods have made possible the separation and identification of trace impurities that may account for the adverse biological effects of a compound. What is being done best at present, as well as the area of most rapid and significant development, is the delineation of the metabolic fate of a compound, its pharmacokinetics, and covalent binding to—and release from—cell and tissue components. This information provides insight into species, strain, sex, and age differences in toxicity. It has often helped explain target organ specificities of toxic action. Most important of all, it has permitted progress to be made in the extrapolation of data from animal to man, and assessment of risk to man, progressing from the stage of mathematical manipulation to the rational application of data on comparative metabolism and dose-response relationships.

The voice of morphology has been muted, save for the conflicts of pathological diagnosticians. A better understanding of certain aspects of neoplasia, such as initiation, the action of promoting agents, "solid-state" carcinogenesis, and neoplastic transformation in vitro holds promise for the future. Reversibility of early changes has been looked at, and the role of nonspecific tissue damage in neoplasia, fashionably dismissed with contempt, is gradually arousing renewed interest.

Two phenomena of our age are worthy of mention. The first is the realization and documentation of the toxic hazards in natural raw and cooked food, which at long last have begun to capture scientific and public attention. The second is the increasingly frequent sequence of denunciation based on preliminary, inadequate, or flawed toxicological or epidemiological data, followed by massive efforts to repeat and substantiate these findings, often finally reaching the conclusion that the original claims were unfounded. Gradually and inevitably, a wider and more critical perspective will develop with regard to risk assessment and

* Each of the five half-day sessions of the workshop was summarized by two practicing toxicologists from the pharmaceutical industry. It was their responsibility to capture the essence of the half-day's papers and ensuing discussion. In addition, they were asked to give their own perspective on the degree of applicability of the topics covered to the field of toxicology and to identify further research needs. Thus, the parts of this summary that comment on the toxicological implications of the various papers represent a combination of the discussion of the workshop group and the particular perspective of the toxicologist rapporteurs named at the head of each session and also shown as coauthors of the SUMMARY.

the concept of acceptable risk that will reduce the burden of those currently attempting to grapple with these difficult issues.

Session I

The Cell Surface: Implications for Toxicology

1. Historical Review of Cell Membrane Architecture. DON W. FAWCETT, *Harvard*
2. Functional Components of Surface Membranes. VINCENT T. MARCHESI, *Yale*
3. Gap Junction Dynamics and Intercellular Communication. DANIEL A. GOODENOUGH, *Harvard*
4. Hormone-receptor Interactions at the Cell Membrane. MORLEY D. HOLLENBERG, *Johns Hopkins*
5. Accumulation of Acetylcholine Receptors and Acetylcholinesterase at Newly Formed Nerve-muscle Synapses. GERALD D. FISCHBACH, *Harvard*

Rapporteurs: WILLIAM F. BOUSQUET AND EMIL A. PFITZER

Introductory comments (Fawcett) provided current views of the architecture of cell membranes with reference to the familiar Davson-Danielli model as well as the more recent views of the Singer-Nicholson fluid mosaic model. Newer developments in technology, such as freeze-fracture techniques coupled with scanning electron microscopy, have contributed to the present consensus of the nature of cellular membranes by providing cleavage of the bilayer of the membrane and subsequent photography of the rough surface image of the interior of the membrane.

The cell surface is the initial contact zone for the frontier for the functional systems by which drugs exert their therapeutic and/or toxicological effects on cells. Thus, the importance of functional components of cell surface membranes was emphasized (Marchesi) with a focus on the dynamic state and mobility of the lipids in cell surfaces. The familiar phospholipid bilayer (with the ionic and polar head groups of the phospholipid molecules providing the outer hydrophilic surfaces and with the fatty acid chains being sequestered in contact with each other in the hydrophobic interior of the bilayer) is envisioned as a highly mobile structure with the individual phospholipid molecules, not only able to spin rapidly, but also able to move rapidly laterally in the plane of the membrane. In addition, individual fatty acid chains of each phospholipid may flex up and down rapidly and there may even be a slow flipping over of individual phospholipids from one surface of the membrane to the other. Despite this description of such dynamic activity, there may nevertheless be some long-range asymmetric order to the bilayer structure whose functional implications are not yet understood. In red blood cells, for example, phosphatidyl serine and phosphatidyl ethanolamine appear to predominate on the inside surface, whereas phosphatidyl choline and sphingomyelin appear predominantly on the outside surface.

Earlier models of the cell surface were depicted as a sandwich of protein monolayers on either side of the phospholipid bilayer. While retaining important structural function, the proteins of the cell surface are now envisioned as elements embedded in the phospholipid bilayer. They appear as large globules either totally implanted within the bilayer or extending in apparent random from the bilayer surface, and in some cases extending completely across the bilayer, providing contact points on both outer and inner surfaces. The proteins, like the phospholipids, are amphipathic, i.e., they are structurally asymmetric, having both hydrophilic and hydrophobic parts. Some of the proteins may provide a certain stability to the membrane, while others may provide critical receptor sites for surface contacts.

Molecules that approach the cell surface, such as sugars, peptides, or other chemicals, can combine with contact-protein-molecules to cause structural alterations of some of the protein molecules that extend into

the cell, thus having significance in modulating the transfer of information from extracellullar to intracellular loci. These contact-protein-molecules appear to have a stereochemical specificity and a unique and highly associated relationship with the amphipathic character of the phospholipids that provide the boundary around the protein. Some clusters of contact-protein-molecules also have specific functional capabilities, e.g., an interaction with external molecules can cause a configurational shift in the cluster of protein molecules that could provide an open channel for entry of molecules, such as sugars or ions, into the cell. There also appear to be proteases in the membrane that, while normally under some inhibitory system, under certain conditions can result in a self-destruction of part of the membrane without degrading the entire membrane.

The amino acid sequence of glycophorin A from cell membranes of the human erythrocyte has suggested a helical configuration of 20 uncharged amino acid residues that is just long enough to span the lipid or hydrophobic part of the phospholipid bilayer. In addition, 16 oligosaccharides (that may provide a shield or protective mechanism) were stacked on the first 50 amino acid residues on the outside of the cell. There was a cluster of acidic amino acids on the immediate outside surface and a cluster of basic amino acids in contact with the inside surface of the phospholipid bilayer. Knowledge of such protein structures and agents that react with them provide new insights into membrane transport mechanisms.

Some of the major protein fractions have been isolated from cell membranes by chemical means. One new technique has identified certain proteins involved in transport by use of the chemical agent DIDS (4,4′-diisothiocyano-2,2′-stilbene disulfonate), which inhibits transport of anions in and out of the erythrocyte. DIDS will also link covalently with peptides, so that when radiolabeled and incubated with erythrocyte membranes it will inhibit anion transport and also bind to the transport channel. Upon subsequent chemical isolation, a single protein fraction is found to be completely associated with the radioactivity, and, excitingly, this isolated protein fraction may be inserted into liposomes where it again demonstrates some degree of anion transport. This protein fraction has also been subjected to peptide analysis and was found to contain 150 amino acids (compared with 20 for glycophorin A) that might reside within the hydrophobic matrix and thus must be subject to considerable coiling within the bilayer of the membrane.

Cell-to-cell interactions (communication) may proceed by a variety of complex mechanisms, one of which is the gap junction or nexus (Goodenough). The pore diameter of these gap junctions has been estimated to be 10 to 14 Å by microinjection of fluorescent molecules. Ultrastructural studies showed initially a characteristic two-dimensional hexagonal lattice of protein subunits (connexons) in the plane of the junctional membranes. The lattice may vary with the physiological state of the cell, and experimental conditions (e.g., anoxia, low intracellular pH, or glutaraldehyde fixation) appear to influence connexon packing. Recent experiments were conducted with an apparatus that allows ultrarapid freezing of specimens without fixation or cryoprotection. Under conditions that favor low-resistance intercellular channels, the gap junction connexons had a highly fluid, disorganized appearance, whereas under conditions favoring high-resistance channels, the connexons tended to aggregate in the membrane plane to form a crystalline structure.

The cell surface is thus provided with a functional junction that can selectively allow ions and small molecules to pass from one cell to another. Conceptually, cellular communication could be interrupted via chemical insults. The cell can switch the resistance of its junctions to close off communicative pathways in response to offending stimuli. Although the switch to high resistance is most commonly observed experimentally after exposure to nonphysio-

logical chemicals, it may also be triggered by endogenous chemicals such as estrogens, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and acetylcholine.

A striking example of extended communication via the gap junction is afforded by the coupling of two myocardial cells in tissue culture through an ovarian granulosa cell when both cell types are present in coculture. Asynchronously beating myocardial cells will begin to beat in synchrony as they form gap junctions with a granulosa cell on opposite sides. This experiment elegantly demonstrates that it is not necessary to hit every cell with a stimulus in order to have multicellular response in a tissue. Due to the cell's ability to switch its junctions to high resistance, toxic lesions will normally be contained at the locus of the initial offending chemical stimulus.

Numerous gap junctions occur in the avascular lens of the vertebrate eye. Nutrient chemicals from the aqueous humor may diffuse to the inner cells via gap junctions between adjacent cell membranes. The lenticular gap junctions may be refractory to the low-to-high resistance switch, a unique condition that may eventually allow a comparative investigation of the molecular events that result in the resistance switch.

Receptor function as a specific type of cell surface contact was addressed (Hollenberg). The importance of learning more about the factors that control the mobility and clustering of hormone-receptor complexes and to characterize those membrane-localized macromolecules with which receptors can interact was emphasized.

Reliable and sensitive binding assays have led to the need to differentiate between binding at a "receptor," defined as a macromolecule that has the dual function of recognizing a ligand of interest and causing an action function that leads to a biological response, and binding at an "acceptor," defined as a macromolecule that may function solely as a recognition site for selective cellular uptake. The concept of receptors as relatively static acceptor substances has been modified by the recent observations that receptors are free to diffuse within the plane of the membrane, so that receptor-ligand complexes can potentially interact with a number of effector molecules within cell membranes. Thus, according to the "mobile receptor model" the receptor-ligand complex itself may interact with one or more specific ion channels, so as to modulate membrane transport.

Available technology for the isolation and purification of receptors for insulin and adrenergic agents allows direct application of biochemical methods for assessing the significance of ligand-receptor interactions and a more detailed study of receptor-effector coupling. The attractiveness of receptors as vehicles for studying potentially toxic substances derives from secure knowledge of their biological function in the organism, and from the relatively specific presumptions about toxicity in the whole organism that can be deduced from studies in vitro with receptors. Highly specific manifestations of toxicity involving a particular cell type could be a stimulus for the examination of that cell for hitherto unknown receptors.

The localization, properties, and factors that influence the generation and turnover of nicotinic receptors for acetylcholine in embryonic skeletal muscle cells were presented (Fischbach). Acetylcholine receptors appear early during the course of myogenesis in vitro. They are present throughout the surface membrane but small clusters of receptors or "hot spots" appear on virtually all myotubes. Myotubes can be innervated in vitro by embryonic spinal cord neurons and "hot spots" are found at each newly formed synapse. The coincidence is not due to a "seeking out" of preexisting "hot spots" by exploring motor nerves. Rather, cholinergic axons induce new clusters at sites of transmitter release. Direct nerve-muscle contact may not be required to cause clustering of receptors at synapses. Preliminary experiments indicate that receptor clustering may be mediated through release of a soluble factor that is present in cholinergic nerves. The neural

"trophic" factor is postulated to be a small molecule—possibly a peptide. The relation between receptor clustering, metabolic stability, and channel kinetics was discussed. These parameters are apparently independent of one another.

Acetylcholinesterase (AChE) appears at sites of transmitter release soon after the onset of synaptic transmission. Receptors cluster in the absence of nerve-muscle activity but AChE does not accumulate. Little if any enzyme is present at contacts formed in the presence of curare, α-bungarotoxin, or tetrodotoxin. Electrical and/or mechanical activity of the muscle fibers seems to be the crucial event.

Acetylcholine receptor clustering presents an opportunity for a direct study of agents of the neurotoxin variety and should also afford an opportunity for the mechanistic examination of agents that produce frank neurological lesions or behavioral deficits in the whole organism, which are in turn suspected of cholinergic involvement.

Group discussion regarding the possible toxicological implications of these findings elicited several points of potential relevance. These included: 1) that the progression of cataract may be related to the gap junction's inability to throw the high resistance switch; 2) that further information on structural proteins and gap junction function may provide new approaches to the manipulation of membrane transport mechanisms and to general cell behavior, such as responses to hormones and growth control; 3) the attractive possibilities for use of receptors as vehicles for the study of potentially toxic substances. This idea derives primarily from the demonstrated biological function of receptors in the organism, which allows reasonably specific presumptions about toxicity in the whole organism as deduced from in vitro studies with receptors; and 4) the potential, but as yet undefined, utility of information about phenomena such as the transmission of environmental messages, ion pumps, receptor specificity, and actions of antigenic macromolecules.

Session II

Membrane Systems of the Cytoplasm: Implications for Toxicology

1. Morphometric Analysis in the Assessment of the Response of the Liver to Drugs. ROBERT P. BOLENDER, *University of Washington*
2. Age-related Changes in Drug Disposition. DOUGLAS L. SCHMUCKER, *V.A. Hospital, San Francisco*
3. Toxicological Implications of Drug Metabolism. RICHARD M. WELCH, *Burroughs-Wellcome*

Rapporteurs: G. H. HOTTENDORF AND VIRGIL B. ROBINSON

Recent morphometric studies (Bolender) point to the potential utility of computer-simulated mathematical models in the evaluation of biological responses of cells to drugs and toxins. Such models are uniquely capable of simultaneously analyzing several complex events represented by changes in various morphometric and biochemical parameters of liver function.

Morphologically and biochemically, the liver is a remarkably heterogeneous organ, consisting of several different cell types with the hepatocyte playing the major role in biotransformation and excretion of drugs and toxins. However, the hepatocytes are also heterogeneous and a major part of the liver complexity probably results from the fact that its hepatocytic component consists of heterogeneous cells that are capable of performing and responding differently.

A three-faceted experimental approach was used in attempting to analyze these heterogeneities: 1) quantitatively relating morphological and biochemical data; 2) detecting changes both morphologically and biochemically; and 3) morphometrically sorting heterogeneous populations of cells into homogeneous subpopulations.

The quantitative morphology included the surface areas of various intrahepatocyte membranes while the biochemistry consisted of marker enzyme activity. The two parameters were combined (related) by cal-

culating enzyme density (marker enzyme activity/membrane surface area). The induction of drug metabolizing membranes in the average cell was utilized as a reference for change in this instance, while sterological sorting of the heterogeneous populations utilized quantitative morphological markers and average cell data. The response of heterogeneous hepatocytes to drugs was assessed by comparing the above parameters in treated and nontreated rats.

In presenting data on the influence of age on the response of the liver to drugs (Schmucker), it was noted that there are more than 30 million people in the United States now over 65 years of age. This fact alone suggests the need for a more intensive study of drug disposition in this particular age group. Although the maximum expected lifespan has not changed, more individuals are reaching the mean age span. Furthermore, the elderly represent the most heavily drug-treated segment of our population. Adverse drug reactions in man appear to increase with advancing age, an observation that can probably be attributed to several factors. For example, the plasma half-life of several drugs is known to increase in individuals aged 65 years and older in comparison to young adults; the half-life of diazepam and propranolol are increased four and five times, respectively.

The increased serum half-lives of drugs in aging individuals may reflect the decrease in smooth endoplasmic reticulum (SER) in hepatocytes and the reduced capacity of the liver and kidney to excrete drugs and their metabolites. In the rat, for example, hepatocyte volume and the amount of hepatic SER increase during development and maturation (1 to 16 months of age) and subsequently decrease significantly during senescence. At 30 months of age, average liver cell size is comparable to that found in 1-month-old rats, whereas these cells contain considerably less SER than the hepatocytes in young animals. On the other hand, the amount of rough endoplasmic reticulum remains fairly constant throughout the entire lifespan.

Although phenobarbital caused a faster induction of hepatic cytochrome P-450 synthesis in 1-month-old rats than in 16-month-old animals, the maximal induction level was similar in both age groups. Similar responses were found for NADPH cytochrome *c* reductase and ethylmorphine N-demethylase. However, the apparent "lag time" in the rate of induction, as measured by the concentration of cytochrome P-450 and the specific activities of the enzymes, may not represent an age-related "decline," but rather an adaptation in the synthetic activities of those livers with considerably greater amounts of SER, i.e., the mature 16-month-old rats. The "extra" SER may function in a reserve capacity and, thus, make the rapid induction of enzymes and membrane biogenesis unnecessary in mature rats in comparison to the very young *or* senescent animals.

The system primarily responsible for metabolic biotransformation of drugs in the liver is the microsomal mixed function oxidase system located in the endoplasmic reticulum of the hepatocyte (Welch). This enzyme system basically consists of two major protein fractions, cytochrome P-450(s) and NADPH-cytochrome-P-450 reductase, in a lipid environment containing phosphatidylcholine. These cytochrome(s) have been shown to be inducible with drugs and other xenobiotics and have different substrate specificities toward a variety of drugs and other xenobiotics. Although this enzyme system normally forms metabolic products with reduced pharmacological activity, many exceptions have been reported. Some products of metabolism have pronounced pharmacological activity. For instance, codeine, acetophenetidin, procaine, phenylbutazone, and a variety of other drugs are metabolized to products having significant pharmacological activity. These observations stress the need for monitoring blood levels of metabolites as well as parent drug since these products frequently lead

to structural analogs with more activity or to compounds with a somewhat different pharmacological or toxicological profile.

Carcinogens and other environmental chemicals are metabolized by the cytochrome P-450-dependent monoxygenases to alkene and arene oxides. In recent years considerable evidence has shown that the carcinogen benzo(a)pyrene is metabolized to a very potent mutagen benzo(a)pyrene 7,8-diol-9,10-epoxide and aflatoxin B is metabolized to its 2,3-oxide, which is at least 10 times more mutagenic than the original compound.

Recent studies on adverse drug reactions that involve the liver have generated considerable interest in the molecular basis of certain drug-induced toxicities. For instance, it has been proposed that the hepatotoxicity caused by isoniazid and acetaminophen results from N-hydroxylation and subsequent rearrangement to reactive electrophiles that covalently bind to macromolecules. In fact, certain barbiturates with allyl groups, like secobarbital, are oxidized to epoxides that covalently bind to liver microsomal hemoproteins and cause their rapid destruction.

The observation that covalent binding produced by such compounds as bromobenzene and its association with hepatic necrosis has also brought attention to another important detoxication system in liver cytosol that involves the sulfhydrylpeptide glutathione. Electrophilic metabolites formed in the process of oxidation are rapidly converted to inactive and easily excreted glutathione conjugates. Although the enzyme glutathione transferase is abundant in the soluble fraction of the hepatocyte, the activity of the enzyme is limited by the availability of glutathione. Combination drugs that compete for this system might be expected to produce a synergistic toxic response and diets deficient in protein may predispose one to drug-induced hepatotoxicity.

Another detoxifying enzyme, epoxide hydratase, is localized in the microsomal fraction of the hepatocyte and rapidly transforms most but not all alkene and arene epoxides to water-soluble dihydrodiols.

Group discussion of the potential of morphometric analysis suggested that such a strategic approach to the quantitative understanding of the biological responses of various cells and organs to drugs and between species awaits further refinement. The methods for morphometric measurements are novel and straightforward but necessitate the development of a large comphrehensive data base. Time and equipment requirements will probably limit the application of this strategy to a few representatives in various drug categories. Nevertheless, such information should add a valuable perspective to the evaluation of toxic responses. It was generally agreed that there is a need for more quantitation of morphological parameters and the correlation of morphological change and biochemical activity in the assessment of cellular and subcellular toxic responses to drugs. Despite the complexity of the proposed quantitative analysis, mathematical modelling and analysis through the use of computers would markedly simplify the task.

With respect to the effects of aging on the response of the liver to drugs, it was agreed that very little attention has been given to this obvious geriatric need, and research on the disposition of drugs in the aging at an early stage of clinical investigation is important. Certainly, data indicating an increase in serum half-life of some drugs and an apparent decrease in drug metabolizing capability in liver cells of the aging individual suggests that more attention be given to the determination of therapeutic regimens for this group.

With the current capability of identifying and quantitating products of metabolism, consideration must be given to the possibility of saturation of the normal physiological mechanisms in test animals when massive doses of test compounds are used. Scientifically, it is irrational to attempt extrap-

olation of human data from "solid state" toxicology studies with heroic doses that produced "hockey stick" dose response curves. Admittedly, high-dose studies in animals at huge multiples of the intended clinical dose may be useful in the assessment of potential target organ effects. However, extrapolation of these results to the safety of chronic administration of a clinical dose is unjustified. Extrapolation of toxicity data developed in animals to man should include a consideration of quantitative and qualitative interspecies differences in absorption, metabolism, distribution, and elimination. Only in this way can rational adjustments in dose differences or other expressions of comparative exposure to drugs and/or toxins be made.

Session III

The Cell Nucleus and Related Factors of Concern in Toxicological Investigations

1. Levels of Organization of the DNA in Eucaryotic Chromosomes. U. K. LAEMMLI, *Princeton*
2. Interaction of Steroid Hormones with the Nucleus. ELWOOD V. JENSEN, *University of Chicago*
3. DNA Fragmentation and DNA Repair of Mammalian Cells as an Indicator for the Complex Interaction between Carcinogens and Modulating Factors. RICHARD H. C. SAN, *British Columbia Cancer Research Center*

Rapporteurs: GREGORY S. PROBST AND ROBERT J. VAN RYZIN

Consideration of the role of histones in chromosome structure indicates that the interaction of DNA with histones constitutes one level of packing DNA into the basic nucleoprotein fiber or nucleosome (Laemmli). However, this level of organization alone cannot account for the packaging of DNA in chromosomes. A large macromolecular complex has been isolated composed of a core or scaffold of nonhistone proteins organized in the shape of the metaphase chromosome and surrounded by DNA, which exists as many loops of about 30,000 to 90,000 base pairs anchored to these scaffolding proteins. Electron micrographs supported by biochemical evidence provide for the first time convincing evidence for the fine structure of metaphase chromosomes. Thus, a scaffolding model for the metaphase chromosome structure has emerged in which a backbone of nonhistone protein is responsible for the basic structure of the chromosome and serves to organize the DNA into loops along its length. There are several orders of chromatin compaction in the structure of chromosomes. The chromosomes consist of relatively short loops radiating from a scaffold of nonhistone protein. The loops are fibers 20 to 30 nm thick that can be further uncoiled into 10-nm beaded strands consisting of nucleosomes joined by connecting segments of double-stranded DNA.

Each nucleosome consists of an octomer of two molecules each of histones H_2A, H_2B, H_3, and H_4 with the DNA coiled 1⅓ turns around each histone core. Extraction of the histones permits further uncoiling to produce the spectacular long DNA loops. As a result of these several orders of coiling there is an 8,000- to 9,000-fold contraction of DNA. The average length of DNA per chromosome is about 4.8 cm. but the average length of human metaphase chromosomes is only 6 um. Indeed, there are about 2.2 meters of DNA in the diploid chromosome complement of human cells.

Since the higher structure of the chromosome appears to be intimately dependent on both scaffold protein core and unique DNA sequences that define the loop configuration, chemically induced chromosomal aberrations may reflect the interaction of a compound with either core protein or DNA or both. Interaction of chemicals with the DNA alone may be expected to generate point mutations involving widely scattered genes, whereas chemical alteration of the core scaffold proteins may be expected to generate considerable chromosomal structural alteration, thus influencing the heritability of many genes by a single event.

The work leading to our understanding of the molecular mechanism of estrogen action was illustrated by the two-step model for estrogen-induced proliferation of rat uterus (Jensen). The first step involves entry of estrogen into the target cell without interaction with membrane receptors. Subsequent binding of estrogen to cytoplasmic receptor (estrophilin) is followed by a temperature-dependent conversion to a higher molecular weight activated complex. In the second step, the estrophilin-activated complex is translocated across the nuclear membrane where its specific interaction with the target cell chromatin acceptor modulates the synthesis of both messenger and ribosomal RNA resulting ultimately in a proliferative cellular response. Although there is evidence to suggest that the activated estrophilin complex may reflect receptor dimerization, additional experiments are required to establish the definitive nature of the activation complex as well as the molecular interaction of the activated complex with chromatin. It was speculated that activated estrophilin may resemble the progesterone receptor where it has been postulated that the activated complex is a dimeric structure, one subunit of which recognizes a protein determinant in chromatin while the other subunit interacts with the DNA determinant in chromatin.

An immunoglobulin to the nuclear form of estrophilin has recently been prepared and shown to have immunological similarity between estrophilins from several animal species. The use of this antibody may help to elucidate further the molecular mechanism of estrophilin activation, as well as the estrophilin-chromatin acceptor interaction. Due to the apparent immunological similarities of estrophilins, immediate application of estrophilin immunoglobulin may be its implementation as a diagnostic tool for the quantitative identification of estrogen-dependent breast cancer and thus be a guide to appropriate therapy. Toxicologically, the estrophilin immunoglobulin may be employed to monitor drug-induced changes in estrophilin that may arise in tissues as an early expression of estrogen-dependent neoplasia.

Measurement of DNA synthesis in vitro was discussed as a method for evaluating the role of endogenous cellular constituents in the modulation of the DNA-damaging capability of carcinogens and/or mutagens (San). While it is known that electrophilic substances generate DNA damage either by forming DNA adducts or direct DNA strand breaks and thus initiate DNA repair synthesis, little attention has been given to the examination of factors, such as antioxidants, that react with electrophiles, thus preventing the production of DNA lesions. Human fibroblast cultures provide a manipulative environment suitable for simulating the complex conditions that may exist in vivo, thus allowing a study of the interaction of carcinogens with intrinsic and extrinsic factors such as ascorbate, glutathione, hypothermia, oncogenic viruses, or genetic predisposition in the induction or inhibition of DNA repair.

Effective microsomal drug metabolism, a central modulating factor in expression of chemical-induced DNA damage, has, until recently, been obtained in fibroblast cultures only by the addition of a rat postmitochondrial fraction. While this S-9 system in vitro converts procarcinogens into ultimate carcinogens, its physiological relevance is questionable. Improved methodology in the preparation of short-term primary rat hepatocytes, known to contain the appropriate microsomal enzyme activities, has shown promise as a more physiological system for the evaluation of chemical-induced DNA damage. Initiation of hepatocyte cultures from rats pretreated to modify either metabolism, alter concentration of cell constituents such as CSH, or to examine anticarcinogens, is an attractive system to study the interaction of cellular modulating factors with suspect carcinogens. Both fibroblastic and hepatocyte culture systems for measuring DNA repair are currently being evaluated in industry and academia as a potential tool for the identifica-

tion of carcinogens as well as models for examining the mechanism of carcinogen DNA interaction. The current validation of these cell culture systems in a number of laboratories points up their potential and probable immediate usefulness to toxicology.

It was noted during discussion that the relevance of new developments in basic biological sciences to the toxicologist lies in the implementation of this new understanding both to explain the mechanism of action of toxins and to predict the toxic potential of new chemicals. The advent of new in vitro systems to examine chromosome structure, regulation of gene expression, and maintenance of genetic information provides mechanisms for gathering information about a compound prior to the initiation of whole animal studies. While these in vitro systems provide valuable mechanistic and predictive information, they only supplement and support rather than replace studies in whole animals. However, the molecular mechanism of a toxicity response, elucidated in a refined in vitro system, coupled with the overt response of an intact animal to the toxicant, provides a powerful system from which reasonable risk assessments can be made.

The achievements in scientific knowledge and refined methodologies relating to the cell nucleus have limited potential for immediate application by practicing toxicologists. Requirements for elegant electron microscopy (e.g., as described by Laemmli) are currently beyond the technical capability of most toxicology laboratories where electron microscopy is routinely limited to sectioned specimens. However, such methods may become a reality within the next decade.

Use of the estrophilin antibody in toxicology may be valuable for studying the interaction of drugs with the estrogen receptors. This avenue of research is obviously limited by the availability of the estrophilin immunoglobulin. Because of the current difficulty involved in purifying sufficient quantities of nuclear-estrophilin to use as antigen to produce the estrophilin immunoglobulin, it is unlikely that this approach to studying receptor-drug interactions would be undertaken in industry. Furthermore, this immunoglobulin may be more appropriately applied as a diagnostic tool, as mentioned earlier, or as a screening tool in the selection and development of clinical compounds directed toward regulation of estrogen action.

In contrast to chromosome structure analysis and estrophilin immunochemistry, in vitro systems for measuring chemical-induced DNA damage and repair can find immediate application in toxicology. Currently, cell culture systems for measuring DNA repair are being examined and/or validated by several toxicology laboratories. Motivated largely by proposed regulatory guidelines for mutagenicity testing, DNA repair studies are expected to be employed in conjunction with the Ames test, cytogenetic tests, and eucaryotic gene mutation tests. The overall strategy is to develop a battery of short-term in vitro tests for early evaluation of a compound prior to the initiation of long-term studies in whole animals.

Session IV

Mutagenesis: Implications for Toxicology

1. In Vitro and in Vivo Analysis of Sister Chromatid Exchange. SAMUEL A. LATT, *Harvard*
2. Application of Microbial and Mammalian Cells to the Assessment of Mutagenicity. VERNE A. RAY, *Pfizer*
3. Protection from Mutagenic Effects of Antischistosomal and Other Drugs. ERNEST BUEDING, *Johns Hopkins*
4. Intraspecies Differences in Frequency of Genes Directly Affecting Drug Disposition: The Individual Factor in Drug Response. ELLIOT S. VESELL, *Pennsylvania State University*

Rapporteurs: BERNARD BECKER AND RICHARD R. STEELMAN

Sister chromatid exchange (SCE) analyses are currently used to characterize

agents that are known to damage DNA but that are often effective at doses below those required to increase chromosome aberrations. The validity of SCE analysis as a sensitive and convenient test for mutagens-carcinogens is presently the subject of many studies (Latt). Three specific facets of SCE analysis were discussed: 1) an assessment of different techniques used for sister chromatid exchange detection; 2) an evaluation of information derived from application of SCE methodology; and 3) presentation of a hypothesis regarding the mechanism of SCE formation.

Recently developed BrdU-fluorochrome and Giemsa dye techniques have facilitated SCE research. SCEs that represent an interchange of DNA between replication products at apparently homologous loci can be detected both in cultured cells and in intact animals. The exchanges are generally detected in cytological preparations of metaphase chromosomes and presumably involve DNA breakage and reunion. Analysis of SCEs has been of practical use both for studying chromosome structure and for assessing the impact of clastogens on chromosomes. Alkylating agents are potent inducers of SCEs. Recent work has identified a large number of compounds with a wider range of SCE induction capacity, along with an increased number of compounds that apparently do not induce SCEs. The available data indicate that essentially all agents found to induce SCEs are also capable of inducing mutations and/or cancer. For the experimental toxicologist, this is the primary interest in the assay.

SCE analyses in vitro are used clinically to differentiate certain hereditary human diseases that include a predisposition to specific cancers, and also to monitor chromosome damage in cells from patients exposed to clastogenic agents during chemotherapy. For example, baseline SCE frequencies in Bloom's syndrome are greatly elevated, whereas depressed or exaggerated SCE inducibility has been observed in Fanconi's anemia and xeroderma pigmentosum, respectively.

Implicit in many of the studies described is the assumption that SCE formation bears a direct relationship to DNA repair and mutagenesis, and there is an apparent correlation between the ability of a chemical to act as a mutagen and/or carcinogen and its ability to induce SCE. However, definitive evidence of a cause and effect relationship has not been established and the precise relationship of SCE formation to DNA repair and carcinogenesis remains to be determined.

Genetic toxicology is a rapidly evolving discipline that offers promise in the selection of test compounds for chronic studies in rodents (Ray). Two toxicological end points achievable through mutagenicity studies with microbial and mammalian cells are: 1) the identification of chemicals that have a potential to interact with DNA and produce heritable effects; and 2) the identification of direct-acting carcinogens.

Point or gene mutations are detected in a variety of procaryotic or eucaryotic microbial cells in the presence of a metabolic activating system. The procedure most widely used today is the Ames Salmonella assay and it is the best validated of the microbial assays. Other assays described and compared to the Ames test include several *Escherichia coli* tests, *Aspergillus nidulans*, *Neurospora crassa*, and *Schizosaccharomyces pombe*.

Relevant comments made in describing and evaluating these microbial tests included: 1) the Ames Salmonella assay does not have a universally recognized protocol; 2) microbial assays are only a screen; 3) it is desirable to have mutagenic data available before subacute toxicity studies are started; 4) metabolic and pharmacokinetic data are needed as part of the toxicity profile; and 5) although microbial assays may be easy and inexpensive, analysis of the results can be rather complex.

Problems associated with the more frequently proposed tests were cited. These included: 1) in the Ames assay, the absence of a standardized protocol or of criteria on which scientists are in agreement as to what

constitutes a significantly positive result: 2) for *Schizosaccharomyces pombe*, the data base is presently too small to make useful correlations with other tests; and 3) for *Neurospora crassa*, that data base is more extensive than *S. pombe* but more data are needed.

Mammalian cell models are employed to detect point mutations and chromosomal aberrations. Two assays currently used are the mouse lymphoma L 5178A and the Chinese hamster ovary models. Although fewer chemicals have been assayed in these tests than in the Ames Salmonella system, sufficient evidence now exists to justify including one or the other in a battery of assays. Inclusion of these models gives perspective to the microbial assay results, broadens the detection capability of a test battery, and provides information from a mammalian test system. A tier system approach to the safety evaluation of chemicals was described along with the results of various mutagenicity assays and in vitro transformation models.

General approaches to the reduction or elimination of mutagenic effects of drugs included: 1) molecular modification; 2) coadministration of antibiotics to prevent conversion of the pharmacological agents to a mutagenic form by intestinal bacteria; and 3) use of antioxidants to prevent enzymatic conversion of nonmutagenic compounds to mutagenic metabolites (Bueding). Naturally, every approach indicated will not work with every mutagenic agent or mutagenic metabolite.

Structural analogs of hycanthone retain antischistosomal activity in mice but are not as mutagenic or toxic. A new schistosomicidal activity in mice but are not as mutagenic or toxic. A new schistosomicidal agent, 4-isothiocyano-4′-nitrodiphenylamine, was nonmutagenic either alone or in the presence of hepatic S-9 microsomal preparations in the Ames Salmonella test and in the host-mediated assay. In rodents and primates, however, mutagenic metabolites were found in the urine. When the compound was administered to germfree rats or mice, no mutagenic metabolites were found in the urine. Several antibiotics were tested to determine whether coadministration of the antibiotic and the schistosomicidal agent would prevent or reduce the mutagenic activity in the urine of normal rodents. A single dose of erythromycin, erythromycylamine, or paromomycin was highly effective in eliminating mutagenic activity without affecting the effectiveness of the drug. The antioxidants, butylated hydroxyanisole or ethoxyquin, when coadministered with the schistosomicidal agent reduced the degree of mutagenic activity in the urine of treated animals. Butylated hydroxyanisole induced certain drug metabolizing enzymes including epoxide hydratase and gluthathione-S-transferase. When antioxidants and antibiotics were used together, a synergistic reduction of mutagenic metabolites resulted.

In the discussion of interspecies differences in mutagenic frequencies, it was stressed that intraspecies differences in the alleles at some genetic loci result in differences among members of that species in structure and function of enzymes and proteins that control absorption, distribution, biotransformation, excretion, and receptor action of drugs and toxic compounds (Vesell). Such genetically controlled differences among laboratory animals of the same species, as well as among human subjects, in these enzymes and proteins indicate that not all members of a species will respond similarly after exposure to toxic chemicals. After administration of the same dose of a compound given by the same route, some members exhibit extreme toxicity; others show no toxicity whatsoever. Pharmacogenetics deals with genetically controlled differences among the members of a species in response to drugs and toxic chemicals, and more than a dozen pharmacogenetic conditions have now been described in man.

External and internal environmental factors can differentially alter the activity of certain enzymes and proteins among members of a species, thereby greatly affecting

their response after exposure to toxic compounds. Genetic mechanisms may also alter enzyme activity, as with phenobarbital- or benzypyrene-induction of hepatic mixed function oxidases.

Since individual members of a species often exhibit great variability, it is not possible to predict how a particular individual will respond to a toxic substance. Variability rather than uniformity of response should be expected.

It became apparent during discussion that many toxicologists are now using the SCE test. In a recent survey of laboratories conducted by the newly formed Genetic Toxicology Association, for example, 5 of 19 industrial laboratories had current experience with SCE techniques, and 5 others were developing the test. This fact underscores the urgent need for valid short-term predictive tests for DNA damage and/or carcinogenesis studies. It is considered likely that by standardizing the test systems, automating the staining and counting procedures, and by testing a broad series of chemical and pharmacological classes of compounds for comparative purposes routine use of this interesting technique may become a reality in 3 to 5 years.

It appears that the Ames Salmonella test in combination with other tests would detect the vast majority of direct acting carcinogens. It is difficult, however, to make definitive correlations because of the problems inherent in defining a carcinogen. Examination of published data from the saccharin, cyclamate, and nitrite experiences clearly illustrates this problem.

There is little doubt that our present assays will detect any new compound that functions as an alkylating agent. There is concern, however, that drugs that will produce specific tumors in long-term animal tests may not always be detected in mutagen assays. Thus, emphasis on testing should perhaps switch from known mutagens to commonly used agents from major chemical and pharmacological classes. In this regard, a PMA subcommittee is presently compiling data on 500 chemicals tested in one or more mutagenicity assays. It is hoped that this information will identify gaps in our knowledge, show which tests have an insufficient data base and which have the highest correlation with prolonged animal testing. Meanwhile, it is apparent that the ability of the molecular toxicologist to devise tests to detect chemical DNA disruption and repair exceeds the practicing toxicologist's ability to evaluate the validity of these tests for predicting safety or harm for people.

Session V

Lysosomes and Lysosomal Diseases in Toxicology and Concluding Lecture

1. An Overview of Lysosomes and Their Role in Physiology and Pathology. A. C. Allison, *Clinical Research Centre, Middlesex England*
2. Pre- and Postnatal Pathology, Enzyme Treatment, and Unresolved Issues in Five Lysosomal Disorders. GEORGE HUG, *University of Cincinnati*
3. Drug-induced Lysosomal Lipidosis: Biochemical Interpretations. THOMAS R. BLOHM, *Merrell Research Center*
4. Concluding Lecture: Application of Basic Concepts to Research in Toxicology. GERHARD ZBINDEN, *University of Zurich*

Rapporteurs: ROBERT DIENER AND JACK E. GRAY

Although there has been a general awareness of the role of lysosomes in cellular function for many years, the significance of these organelles in intracellular transport and detoxification of pharmacological substances, cellular products, or contaminants has not been fully appreciated. It is increasingly evident that lysosomes are not merely intracytoplasmic "garbage bags" associated with autophagocytosis. On the contrary, a variety of discrete and sophisticated functions are attributed to the many specific enzymes classified as proteases and inflammatory mediators present within lysosomes (Allison).

Experiments with macrophages and sev-

eral minerals such as asbestos, silica, and beryllium have revealed that the interaction of foreign substances with lysosomal membranes can vary remarkably. Ingested silica crystals destroy phagocytic cells within 6 hours, asbestos brings about the selective release of lysosomal enzymes, and beryllium produces extensive intracytoplasmic vacuolation and an epithelioid reaction.

During discussion it was stressed that lysosomes function at the "crossroads" of cellular metabolism and thus have central roles both in synthetic mechanisms and in degradative processes that rid the cell of unwanted organelles and substances. Although fusion of the primary lysosome with autophagic or endocytotic vacuoles can be visualized, the functional complexity of lysosomes, which contain as many as 50 different hydrolytic enzymes, is difficult to comprehend.

Increased numbers of lysosomes (some of which may be lamellated, myeloid figures) are often observed in target cells of animals given exaggerated doses of various chemical compounds. Further, the extent to which a given cell can accommodate increasing concentrations of a foreign substance, either particulate or molecular, may be expressed by the formation of giant secondary lysosomes. At the moment, however, functional and morphological correlations pertaining to the alterations that occur during these adaptive or toxic states are not well understood. The all-encompassing role of lysosomes when a foreign substance gains entrance into a cell might justify the cliché that "When things go badly for the cell, the lysosome is left holding the bag."

(We regret that the complete text of Dr. Allison's paper was not available for publication.)

Several types of naturally occurring storage disease were reviewed and the clinical features summarized (Hug). In type II glycogenosis, for example, glycogen granules accumulate within lysosomes but are not digested because of a deficiency of the enzyme glucosidase. Various treatments such as epinephrine and infusions of Niger extract have been tried but to date no satisfactory response has occurred. Other disorders presumably caused by lysosomal malfunction include Hunter's disease, Hurler's syndrome, metachromatic leukodystrophy, Tay-Sachs disease, and Chédiak-Higashi syndrome. Fibroblast cell cultures may show characteristic morphological changes.

The chemical aberrations and clinical symptomatology of human lysosomal diseases are extremely complex. Because of the complexities of the human genome, the same enzymic defect may bring about a variety of clinical accumulations in the vacuolar system of substances for the breakdown of which the missing enzyme is required.

Recognition of these conditions should be a concern for the toxicologist, since a lesion resulting from a drug-induced depletion of a specific hydrolase, for example, could conceivably resemble that caused by an inborn error of metabolism. Thus, it is important that toxicity studies be conducted with stocks of animals free of lysosomal defects, or, for specific reasons.

Presumptive biochemical steps that lead to a condition known as drug-induced lipidosis were traced (Blohm). Characteristic morphological changes in lysosomes (myeloid inclusions) are frequently found in laboratory animals given large doses of a variety of pharmaceutical agents and toxicologists are gradually becoming more aware of this. The available pieces of the puzzle were assembled and fitted together to provide a plausible biochemical explanation for the observed morphological changes. It is theorized that certain drugs that are weakly basic (pK approximately 8–10) and contain both hydrophilic and lipophilic moieties in the molecule interact with acidic phospholipids in lysosomal membranes because of the particularly low pH within the lysosome (pH 4–5), which in turn concentrates the protonated drug by as much as 1000-fold. The membranes tend to stabilize and expand and phospholipid levels are elevated.

Acid phospholipids and degradation products such as bis-(monoacyl)glyceryl phosphate tend to accumulate in the drug-stabilized membranous whorls. The determination of the degree of drug-induced phospholipidosis present in laboratory animals may have significance for the safety evaluation of a drug candidate, since severe toxicity has been associated with extreme cases of this effect. The processes by which such cellular changes are produced should be explored further and may lead to better and less expensive ways of evaluating possible adverse effects. The phenomenon has been produced in cell culture, and this would seem to offer promise as a screening technique.

During discussion it was emphasized that myeloid inclusions are the morphological hallmark of phospholipidosis. Of the several drugs that have been shown to bring about the formation of these inclusions, some induce myeloid figures more rapidly and in a greater variety of tissues. The presence of myeloid inclusions in affected cells is usually transient and unaccompanied by lesion formation, although exceptions have been observed in some affected tissues such as the retina, which apparently has a limited capacity to excrete altered lysosomes. It has been recognized, at least with some drugs, that the concentration of the drug within the target cells must exceed a rather specific level before this morphologic response becomes apparent. Furthermore, it has been shown that some cells may adapt and no longer form myeloid inclusions after the first few days of treatment.

In the final presentation, a challenge was made to investigative toxicologists to apply some of the basic concepts that had been discussed (Zbinden). Thus, appropriate targets for research must be selected and new test systems proven beyond a shadow of a doubt. Empirical toxicology tests are generally being replaced in favor of more meaningful ones.

A number of new procedures were outlined that might simplify present toxicological methods and at the same time increase their predictability. For example, the establishment of a covalent binding index could accurately categorize a new drug candidate by comparison to known strong or weak carcinogens and negative controls. Another approach is a rabbit mutagenicity test in vivo in which DNA repair synthesis is evaluated by the intratesticular injection of ^{3}H-thymidine. Since ^{3}H-thymidine is incorporated into spermatids and late spermatocytes only at a very low rate, agents that damage DNA can easily be detected by the higher amount of radioactivity present in periodically obtained ejaculates of spermatozoa. Evaluation of DNA damage, point mutations, and carcinogenicity can also be made from granuloma pouches in rats. Only after the sensitivity of a particular system is established should it be applied to new pharmaceutical or chemical candidates.

It was emphasized that pharmaceutical toxicology is a regulated science, and it behooves its practitioners to demonstrate to regulating agencies that the industry knows more about its drugs or chemicals than anyone else. Scientific disciplines must be blended to a greater extent than ever before. The superiority of any new method should be documented with convincing data so that we may: 1) develop the toxicity profile of new drugs by means of studies that are specifically appropriate; 2) temper with appropriate understanding the arbitrary imposition of toxicity studies of low yield value; and 3) learn more about the mechanisms of toxicity.