Developing Efficiency in Characterizing Drug and Substrate Reaction Schemes:

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INTRODUCTION

The medical investigator is highly tuned to the principles of reducing animal usage. The majority know that experimentation is essential to progress in the field and to the betterment of our national health and welfare. They also know that animal experimentation is costly, time-consuming, and requires diligent effort to perform correctly and appropriately. The American Physiological Society in 1913, understanding that animals and humans who were subjects for studies merited as much consideration as does the advancement of science, established their policy of preventing cruelty to animals. Over the years the explicit writings on this subject became more fully formulated, and in 1979/80 a set of formal guidelines for animal usage was sent to each member with the instructions that these guidelines be signed and posted in the laboratory. These were the guidelines on which FASEB, the Federation of American Societies of Experimental Biology, and later WHO, the World Health Organization, developed an international agreement on the principles which are listed in Table 1.

TABLE 1. WHO/CIOMS International Guiding Principles (1985)
(abbreviated wording)

1. Advancing biology requires animal studies.
2. Models and in vitro systems should be used if possible.
3. Study animals if relevant to health and biology.
4. Species appropriate and number minimal.
5. Treat animals as sentient.
6. What is painful to man is painful to animals.
7. Pain should be prevented with analgesia or anesthetic.
8. Waivers re. #7 are the prerogative of an independent review body.
9. Animals whose recovery would involve severe or chronic suffering should be killed.
10. Animal care should be best possible, veterinary approved.
11. Investigators to be qualified for animal use.

Ref: N. Howard-Jones, WHO Chronicle 39:51-56, 1985.
WHO/CIOMS = World Health Organization/Council for International Organizations of the Medical Sciences

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Code, Structure, Function, and Behavior:

This meeting concerns specific issues regarding approaches to reducing the usage of animals in research. I include the testing of the applicability of drugs to the treatment or prevention of disease without undue incidence of side effects as a research problem.

The same strategies that serve the fundamental research of discovering receptors, enzymes, proteins, and their interactions can be used in discovering toxic effects, degradation properties and so on. The context of the meeting surrounds the question of how computers may serve these ends. We are used to computers for small projects such as statistics, airplane construction, Medline searches, mathematical problems that might take days on a Cray, predicting the weather, and so on. Now we enter a new era, where the computer is beginning to serve some truly massive projects. These range in scale, in both project size and in the material under consideration, from the human genome project to the geonome project. The latter concerns our planet earth, and has been the subject of the Club of Rome and more recently the Institute of Geonomics. The topic of this conference is at an intermediate level, the Physionome Project. Let us give this a clearer context.

Large Computational Projects:

We can classify a part of the world of biological science research into a set of large scale projects about which one can conceptualize even though they may be too large to bring to realization within a few decades. These comprise the BIONOME. Following the order of complexity, and in many ways the heirarchical order of explanation, one starts with the gene and ends up with the earth, from genome to geonome. Given the genetic structure of all the species on earth, one would not have much information that helps in the day to day problems of how an individual is functioning, or how that function may be improved with pharmaceutical assistance. Nevertheless, the politics or sociology of science has deemed it appropriate to learn the structure of a least the human genome. From that we may conceptualize the next projects that are needed to bring us closer to the realities of life and function and dysfunction. The sequence, from this point of view, is first the gene, then then structure that evolves from the genes' instructions, then animal or plant function. For animals, the next stage is the function of the mind, the psyche, for the individual and then for the group. And it is the group behavior that spells the fate our favorite planet.

1. The Human Genome Project.

The genome is defined in the DNA for an individual of a species. It is therefore the ordered listing of the base pairs of the DNA in an individual human. The genotype is the structure of the genome that is the code for the structure of the organism. The phenotype is the structure of what is encoded in the genotype.

The objective of the project is to provide a prototype of the genome of a human for use in identifying the genetic basis of disease. Of the projects to follow, this is the simplest and the cheapest. It is "finite," there being only 10^9 base pairs. There are difficulties that are just being recognized: the data base structure is not yet well organized, the mechanisms for retrieval are not worked out, computer comfort and skill is low in the sequencing community, so that sequences are often sent by FAX to the database managers and then manually entered, the validation mechanisms are incomplete.

This is recognized to be a very expensive, 1 to 2 decade program. The Human Genome Project is a funded project now employing hundreds of talented scientists and technicians.

2. The Human Morphonome:

This is not yet formally defined as a project. The morphonome is a full description of the measures of the structure of an individual of the species at a particular time. This would include proteins, substrates, hormones, channels, membranes, organelles, cells, tissues, organs, and body measurements and configurations. They are the basis of life; put them together (correctly) and there is being.
Examples of components of the Human Morphonome are:

a. Quantitative descriptions of structural molecules.
b. Configuration and conformations of enzymes, transporters, channels, junctions, membranes, organelles, etc.
c. Morphogenesis and its gene regulators. Phylogeny requires a large genetic heritage, ontogeny requires only a relatively few genes.
d. The biochemical pathways, the routes for metabolic traffic.
e. Cell, tissue, and organ form.
f. Composition, material properties of cells, interstitium and tissue.
g. Anatomy, the spatial relationships between cell types, organs and organ systems.

3. The Human Physionome Project:
The physionome is a quantitative description of the physiological dynamics of the normal intact organism, from cell to sentient being. Cell-to-cell information exchange (neural, hormonal, electrical) governs biological function. Function affects phenotypic expression, and vice versa. Biological systems are regulated via multiple enzyme, humoral, and neural controllers.
Examples of components of the physionome include:

a. Molecular dynamics, energetics of conformational change, enzyme regulatory mechanisms.
b. Regulation of phenotypic expression by cell function or neighbors.
c. Biochemical kinetics-genetic models and versions for specific cell types; computational tractable reduced forms.
d. Cellular ion regulation.
e. Cell-to-cell communication may be humoral, neural, or electrical.
f. Integrated organ functions.
g. Motion and locomotion-the brains outlet.

4. The Human Psychonome:

The psychonome is the quantitative description of the psychological dynamics of the intact organism as an individual within a normal environment. Features which might be assessed as parts of the psychonome include:

a. Perception, memory (storage and recall).
b. The integrative chemistry of mood.
c. Sensory and motor integration with mood and energy levels.
d. The psyche: influences of body status.
e. The psyche: influences of physical environment.
f. The psyche: influences of psychological environment.

5.The Human Socionome:

The socionome is the description of the behaviors of groups of individuals, of the nature of interactions between social groups or societies, races, or nations. Qualitative rather than quantitative descriptions are what we have now, since this area of study has been closer to philosophy than to science. But sociology, even though measurement is difficult, is developing its measurement capabilities. The socionome includes:

a. "Natural law" (a la Rousseau)
b. The family, group, tribe, nation.
c. The common good and communal behavior.
d. Civic duty, deviations, and regulation.
e. The will of the people, bureaucracy, politics.
f. The national psyche and international behavioral issues.

6.The Geonome:

The geonome is the quantitative description of planet earth, ranging from the slow processes of plate tectonics to the more rapid processes of ozone layer depletion and weather patterns. Particular aspects include:

a. Man amongst many: interrelationships among man, animals, and plants.
b. The environment: ozone depletion, global warming, etc.
c. Preservation versus selection of species: rain forests, seas.
d. Equilibration versus the steady-state: exploitation of resources, e.g., oil usage in this pimple in time.

GOALS OF THE HUMAN PHYSIONOME PROJECT:

Building the descriptions of the dynamics of functioning, or functioning, systems upon the knowledge of the anatomy of proteins, cells, tissues and organs is in the natural sequence of scientific progression. The goals of the physionome project are huge, and currently out of sight, considering the current status of anatomic and physiologic science. Nevertheless, it is important to plan so as to give a conceptual framework for current and future efforts. The broad goals may be summarized:

1. To assemble and integrate the current knowledge of human physiology.
2. To make this knowledge available to investigators, teachers, students.
3. To foster the development of mechanisms for maximizing the information provided by any given experimental study, and in particular to apply the principles of integrative system analysis to improving understanding of physiological systems.

TOOLS FOR THE PHYSIONOME PROJECT:

1. Structured databases.
2. Computer models.
3. Computer modeling methods.
4. Resources for in numero experimentation.
5. Instructional tools for self-teaching and for classroom demonstrations.
6. Mechanisms for networked distribution.

STRATEGIES FOR THE PHYSIONOME PROJECT:

Here we begin to formulate the approaches to the project. They are hardly new, for it is the heritage of physiologists and medical scientists since the time of Claude Bernard to try to figure out how the pieces of the puzzle are fitted together. The following incomplete listing covers some aspects of what needs to be done:
1. Organize the massive information set into individually useful subsets.
2. Develop specific computer models, tested against many data sets, for each subset, e.g., in the erythrocyte, the sub- sets concerning glucose and purine handling.
3. Simplify groups of subsets into computable descriptions of the behavior of the aggregate set, e.g., volume regulation of the erythrocyte.
4. Develop generic models that can be used for many specific applications, e.g., for ionic channels, transporters, reaction sequences, blood-tissue exchange.
5. Provide reference data bases associated with each subset and sets of parameter values for each model or submodel.
6. Develop database structuring for the models that allows them to be used at a variety of heirarchical levels, while at the same time maintaining a modularity that gives recognition to the fact that most cells have the same mechanisms for transport, metabolism and ionic regulation.
7. Archive the specific models so that they can not only be retrieved by investigators, but so that they can be studied for their behavioral characteristics and used to explore the field. Here the computer serves to expand the mind and aid the intuition. Even relatively simple models go frequently far beyond one's normal intuitive capacity to predict events, which is not surprising when one considers that what we call intuition is principally a kind of logic based on a combination of experience and deduction.
8. Provide training, including computer-based self training, programs that will serve to develop a cadre of skilled teachers and investigators in the fields of human and animal health.

HOW TO MINIMIZE THE NUMBERS OF ANIMAL EXPERIMENTS

1. Have a clear, refutable hypothesis. Provoke your mind to define an alternative hypothesis, a nice way to force reexamination of the primary hypothesis, and a wonderful way to stimulate one's self to think beyond the nearest step. (Platt, 1964?)
2. Define criteria for refutation of both the hypotheses.
3. Test the new hypothesis against the accepted current working hypothesis.

a. Put working hypothesis in computable form.
b. Develop protocol for proposed in vivo experiment.
c. Run protocol "in numbers."
Identify differences between old and new hypothesis.
Express differences in experimentally measurable term,
considering sources of error.
Determine emphasis in data sampling frequency and accuracy.
Estimate relation between anticipated difference
and statistically desired number of observations.
Refine the protocol.

4. Collect some data in a few experiments.
5. Analyze each initial experiment fully before doing more:
optimize model fits to data
Seek systematic differences
Determine need for additional data
6.Estimate additional experiments and proceed.

An Example of a Research Study, attempting to minimize animal usage:
The topic: ADENOSINE, THE HYPOTHESIZED VASOREGULATOR: The basic hypothesis is that when cells are stressed so that the metabolic demands of the tissue exceed the delivery of oxygen, ATP breaks down to adenosine which is released from the cells via a facilitating transporter and diffuses to the smooth muscle cells of the terminal arterioles where is causes relaxation and vasodilation. This increases the flow, and so the process of adenosine-mediated hypoxic vasodilation is a type of closed loop control feedback. Alternative hypotheses provide for other humoral mediators of vasodilation, including potassium which is released from cardiac myocytes under the same circumstances.

The model expressing the hypothesis can be simplified to a single blood-tissue exchange unit (Figure 1, from Bassingthwaighte, Wang, and Chan, 1989) in which it is taken as given that a rise in interstitial adenosine concentration relaxes the smooth muscle. Its computer solution can be derived analytically if one ignores axial diffusion; however, a fast and highly accurate numerical solution, being 10^9 times faster than the analytic solution lends itself readily to incorporating the diffusion as well as the exchanges, and so avoids the compromise (Bassingthwaighte, Chan and Wang, 1991). The modeling follows the general stategies outlined by Bassingthwaighte and Goresky (1984).

One of the first things learned was that pulmonary endothelial uptake of adenosine in the rabbit was several times as high as that of the myocardial capillary bed. The second was that species differ. Not only did rabbit cardiac endothelial take up adenosine relatively slowly, it metabolized it down only to hypoxanthine, whereas the guinea pig heart broke it down mainly to uric acid. The rabbit endothelium lacks xanthine oxidase.

The multiple indicator dilution technique was used for the study because it provides the means for obtaining high temporal resolution data, and allows distinction between transformations occurring in endothelial cells from those in cardiomyocytes. This means that studies can be done in intact animals and humans by using

catheterization. We chose instead to use the isolated perfused heart, preserving the relationships between endothelial cells and myocytes while removing the complicating factors of erythrocyte uptake and contamination by recirculating tracer. Tracer techniques are used to allow the measurement of nonlinear transporter characteristics, while taking advantage of observing the effects of changes in non-tracer mother substance concentration on the tracer transients. The protocol for experiment is outlined in Table 2, Methods, and the diagram in Figure 2 of the process of injecting tracer into the arterial inflow , collecting outflow samples, separating injected substrate from the products of metabolism by HPLC or other chemical separation technique, to obtain a set of outflow dilution curves. These are what are analyzed using the models to obtain estimates of the parameters for transmembrane transport and for metabolic transformation. Adenosine transformed into ATP is trapped in the cells, while the breakdown products appear in the outflow, delayed only a fraction of a second by their uptake, transformation, and escape across the membrane. The metabolic sequence is shown in Figure 3; a typical set of outflow dilution curves in Figure 4.

Model solutions are fitted to the data curves in Figure 4, showing only the substrate adenosine and the two reference tracers, albumin for the vascular space and sucrose for the extracellular space and the rate of permeation through the paracellular pathway, the interendothelial clefts, linking the plasma space to the interstitium. This gives strong estimates of the permeability-surface area product for the endothelium, and reasonably good estimates, even if noisier of the subsequent transport or transformation rates. But he analysis of the outflow dilution curves of the products makes the estimates of the transformation rates quite precise; the goodness of fit of the curves is shown in Figure 5.

The distinction between events in endothelial cells and myocytes is clear, as is shown in Figure 6 (from Bassingthwaighte and Sparks (1986). Products of reactions inside the myocytes appears much later, and at lower, more diluted concentration levels, than products of intraendothelial reactions (left panel). Moreover, because less of the substrate reaches the myocyte than reaches the endothelial cell, the peak of unreacted substrate is higher in the absence of endothelial reaction.

By doing such experiments at different background concentrations of adenosine one determine the extent of competition between the tracer-labeled adenosine and the non-tracer mother substance. The apparent PS for endothelial permeation is an example, Figure 7, which shows a half maximal saturation at about 100 micromolar adenosine (Schwartz et al., 1991).

Modeling solutions obtained for this system in circumstances where the intramyocyte concentration is raised by simulating the breakdown of ATP at an abnormally high net rate, do show, in accord with the hypothesis that interstitial adenosine levels are raised, much above the levels in the effluent plasma. However, experimentally the heightened adeosine efflux is transient in circumstances where the stimulus for ATP breakdown and where the vasodilation persists beyond the transient, so that raising free interstitial adenosine is not a sufficient explanation on its own. Our

conclusion is that adenosine is only one of many regulators and that the role of the others, including potassium, should not be neglected.

WHAT WE HAVE LEARNED ABOUT CARDIAC ADENOSINE PHYSIOLOGY USING COMPUTER AIDED ANALYSIS

1. Endothelial cells dominate tracer kinetics.
2. Endothelial cells differ between heart and lung in the rabbit.
3. Species differ: guinea pig endothelial cells have xanthine oxidase, rabbit's do not.
4. Only half of tracer Ado enters ATP pool.
5. Transporters on membranes have much lower affinities than do enzymes for purine nucleoside metabolism.
6. Enzyme-to-enzyme cooperativity means they are structured into complexes.

COMPUTER MODELING OF DRUG DELIVERY

Design and run high resolution tracer dilution experiments related to membrane transport (rates and mechanisms) and rate of binding, when these may affect the rate of delivery to the effector site.

Use steady-state studies for volumes of distribution with organs (cells vs. ECF, etc.) and to compare organs with regard to charge, pH, site abundance, etc.

Assess whole body pharmacokinetics considering administration route, arrival and retention times, in blood, tissue, excretory route, and degradation. See papers by Bischoff, Lutz, Brown, in themeeting proceedings.

COMPUTER USES IN DRUG DEVELOPMENT:

1. To characterize the actions of a desired agent, choosing
   where it should act by computer modeling of the physionome.
2. Predict direct physiological effects.
3. Predict side or secondary effects.
4. Design the molecule using the morphonome.
5. Design and automate the synthesis.

ANIMAL RIGHTS AND SOCIAL RESPONSIBILITY

Greater knowledge is essential to the betterment of conditions of and for man and animals. Certain experiments can only be done in the intact organism and only in particular species. Minimizing the numbers of studies, maximizing the information per study and archiving and communicating the information is a part of the scientist's civic responsibility. For the future, and for the preservation and organization of the information that is being gathered day by day, we need to work on the Physionome

Project, integrating the knowledge by casting it in model form for making quantitative hypotheses, and storing the information in well structured databases that can be made available to other investigators.

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