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Alternatives to Animal Testing in the USP-NF : Present and Future

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CHAPTER 1 INTRODUCTION

1.1 Alternative Testing

Over the last decade, much resources, time and discussion have been spent on deriving alternatives to animal testing in all sectors of biomedical research and industry. Today, progress in biotechnology coupled with an increase in information technology has broadened the list of potential alternatives to *in vivo* testing.

As a leader in setting drug standards in the United States and several other countries, USP felt it was necessary to join the endeavor to decrease the number of animals used in testing. In 1990, the U.S. Pharmacopeial Convention passed a resolution "to replace, reduce, and refine compendial tests that require the use of animals as rapidly as science and technology permit". Consequently, the Biotechnology and Biopolymers, the Microbiology, and the Toxicity and Cell Culture Subcommittees at USP adopted an objective to seek alternative testing with emphasis on biologicals and biotechnology derived products. The following review and interpretations focus solely on these tests.

1.1.1 Evolution of the term

The concept of alternative testing was first established by Marshall Hall in the beginning of the 19th century. He developed five principles of animal experimentation aimed at minimizing pain and suffering and reducing repetitive and unnecessary experimentation. These principles became the precursors of today's (GLPs) Good Laboratory Practices and (IACUCs) Institutional Animal Care and Use Committees (1).

More recently, the topic of alternative testing came to the forefront of public conscience in 1978, when a campaign directed towards the

removal of the Draize Test from cosmetic testing was organized by animal rights advocates. The Draize test was developed in 1944, and was one of the early entrants as an *in vivo* test to evaluate ophthalmic toxicity of materials. Other tests such as the LD50 and the phenol coefficient test (related to skin damage) were developed in the 1930's. Its reliability and reproducibility and perhaps the lack of other alternative tests - be it *in vivo* or *in vitro* has brought it to widespread use in the past five decades in ocular irritancy testing in the cosmetic industry. The multimedia animal rights offensive against this test focused on Revlon, one of the leaders in the cosmetic industry and resulted in Revlon agreeing to seek alternative tests to evaluate their cosmetics. This issue ultimately led to the multisource funding of a center for Alternatives to Animal Testing at Johns Hopkins University (2).

Although animal rights advocates initiated the movement to seek alternative testing, it is the unifying concern for the welfare of animals that continues to broaden the commitment of industry and academia to seek alternative testing. The topic has become so involved that several new scientific journals have been developed which deal specifically with the topic of alternative testing. Index Medicus has also responded to the increase in publications by adding a new heading entitled "Alternatives to Animal Testing".

1.12 The three r's

Alternative methods in the broadest interpretation includes any test that refines, reduces or replaces a standard *in vivo* test (3). This concept, referred to as the three r's, is rooted in a book published in 1959 entitled "Principles of Humane Experimental Technique" by Russell and Burch (1). The three r's approach of alternative testing encompasses the entire spectrum of possibilities available. The most important point that the concept makes is that alternative testing can be accomplished by means other than replacing animal tests with *in vitro* tests. It indicates that the goal of alternative testing is to minimize the pain and suffering of research animals (reduction) by decreasing the number of animals used as well as the severity and replicates of tests required (refinement). The ideal alternative being the implementation of a validated non-animal test that is of equal scientific value as its predecessor (replacement).

1.1.3 The role of the USP Pharmacopeia

The USP founded in 1820, is a non-profit, non-government scientific organization that develops public standards for pharmaceuticals, medical devices, and diagnostics. These public standards are enforced by the Food and Drug Adminisration on the basis of the Food and Drug and Cosmetics Act which references the USP Standards. The USP public standards are designed to assure the potency, identity, purity, and quality of drugs. The safety and efficacy of drugs is the responsibility of FDA and is handled by FDA through the NDA process or the licensing process.

These USP standard requirements over the decades led to many pharmacopeial applications of animal based tests. The public interest is served by standards and the reduction of animal testing must be introduced only when advances in science and technology warrants it.

1.2 Limiting Factors

USP has an important role in recommending alternative tests. Being an independent, not-for-profit scientific organization allows USP to seek alternative tests without restrictions, conflicts of interest, or influence, thereby eliminating potential biases. However, the USP will not recommend any test or protocol which utilizes materials not available to the public. Their recognition in over 30 countries as well as the U.S. thus poses limitations on the types of tests which can be considered for compendial tests. The following is a list of considerations which USP will address when seeking alternative testing.

1.2.1 Model extrapolation

Using models to predict human response has been the basis of scientific research for more than a century. All models whether *in vitro* or *in vivo* have the objective of simulating human response. When the information derived from the model is then incorporated into a hypothesis on human response, extrapolation has occurred. The assumption in extrapolation is that the model can predict the response of the human mechanism or system of interest, so model extrapolation is limited by

limitations of the model itself. Extrapolating beyond the limitations of the model is not scientifically sound nor is it appropriate.

The relationship between the test and the human response it is simulating determines the value of extrapolated data, and the predictive ability of the test. The ultimate purpose of the test is to predict human response. Due to the intrinsic nature of models, there is no test which can totally and comprehensively predict the human response. The question is then, at what predictive value should the regulatory agency feel confident? It would be reasonable to set the minimum predictive value of the alternative test at equal to or higher than the predictive value of the official animal test. A decrease in predictive value can decrease patient safety. Therefore, it is of utmost importance that the limitations of the model's ability to simulate, and ultimately predict, human response be determined prior to the test being recommended.

1.2.2 Standardization

USP tests have been standardized in protocol and materials so that the tests perform the same across the United States and around the world. If a test requires a component which is not obtainable then the test will not be considered by USP for a referee test. Also, compendial tests may require the product under consideration be compared to a reference standard. USP establishes a reference standard, and becomes the source and supplier of the material to ensure it is standardized.

The ability of a test to be standardized is an important component to consider when seeking alternatives to animal testing. Certain types of tests lend themselves well to standardization while others do not. Generally, a non-animal test will be easier to standardize than an animal test. This is based, in part, on the fact that it is difficult or impossible to standardize an animal, the major component of an *in vivo* test. Although genetically inbred, or isogenic strains of animals are considered a means of standardizing test animals, it actually may adversely affect the test results. Inbeeding results in a loss of hybrid vigor which manifests itself in a decrease in fertility as well as immunity and overall health of the animal, which can bias the test results.

The handling, housing and care of the animal are difficult to standardize as well. The management of the animal prior to testing also directly influences the health of the animal. For example it has been shown that stress adversely affects the immune system of an animal (6). Since assay sensitivity is directly related to the response of the animal, it is evident that standardization can play an important role in animal testing. Yet, animal models are considered the "gold standard" in compendial and in quality control testing of some pharmaceuticals.

In vitro tests, on the other hand, can more easily be standardized with regard to their major components. Their equipment, reagents, media, and protocols lend themselves well to standardization, potentially eliminating the bias encountered in utilizing *in vivo* systems.

1.2.3 Validation.

Test validation is the process of comparing a particular test against a standard or definitive test. For a generalized validation scheme of compendial methods consult <1225> Validation of Compendial Methods in USP. Experimental information on the results, statistics of the test are accumulated to establish the functional parameters of the test. When seeking replacements for *in vivo* tests, validation is an extremely important, and ultimately limiting factor in the final decision on a capability.

The term validation can be viewed on two successive levels; intravalidation and intervalidation. A new test is initially validated by establishing its intrinsic parameters (intravalidation). In this phase, such characteristics as its reproducibility, accuracy, and precision are evaluated. The second level of validation occurs if the test is a replacement test for a currently validated test (intervalidation). This establishes the parameters of sensitivity and specificity of the new test as it relates to the existing test.

Sensitivity refers to the proportion of true positives that are classified as positive by the replacement test. In terms of alternative animal testing of bulk pharmaceuticals, the new test would be challenged with bulk pharmaceuticals which tested positive in the *in vivo* test. The sensitivity of the new test is determined by dividing the number of true

positives tested by the sum of true positives and false negatives identified by the new test.

Specificity refers to the proportion of true negatives which are classified as true negatives by the replacement test. Again, in terms of this scenario, the new test is challenged with bulk pharmaceuticals which tested negative in the *in vivo* test. The specificity of the new test is determined by dividing the number of true negatives tested by the sum of the true negatives and false positives identified by the new test.

A consideration in validation of a test is the time factor. Evaluating the replacement test against the existing official test is a rather long undertaking. Several years may elapse from the development of a potential alternative test to its full validation. The USP Subcommittee is faced with determining when enough data has been accumulated to recommend a new test for compendial standing. The replacement of a single compendial test with an *in vitro* test can have an extremely large impact on both animal usage and patient safety. USP's ultimate concern lies with the patient, therefore, the validation process of a potential test cannot be minimized. Consequently, USP may be in a unique position to increase the rate of validation on a test they have chosen as a potential replacement. As an alternative to waiting until the new test is validated, USP may want to consider recommending the test to be run in conjunction with the compendial test, and collate the data from these tests to determine the concordance or validity of the replacement test. The magnitude of the topic of scientific validation and regulatory agencies is reflected in the number of international workshops, programs and papers it has generated (7).

1.2.4 Correlation

The relationship between the official *in vivo* test and the alternative *in vitro* test must be thoroughly understood in order to obtain reliable data from testing. Statistically, correlation refers to the relationship between the model chosen to fit the data and the fit of the data to the model. In alternative testing, correlation refers to the relationship between the results obtained from the *in vivo* test versus the results obtained from the *in vitro* test. A"good" correlation is one in which

a high percentage of the results of the *in vitro* test are the same as the results of the *in vivo* test. An assumption is made that the official *in vivo* test already was a valid model for predicting human response. Otherwise, in view of a more correlated response, close concordance in results is of lesser importance.

An *in vivo* test utilizes the most natural test conditions available: the living animal. It also utilizes the most complex test conditions: the living animal. Consider the general results obtained when evaluating the response of an animal to a test material. Such responses as irritation, contraction, blood pressure changes, and death are utilized to evaluate the safety of a product. End point assays such as these do not explore the cellular mechanism by which these reactions occur in the animal. Yet, the majority of *in vitro* tests are evaluating a biochemical cellular response, or a segment of the response cascade. Therefore, it is important to establish a correlation between the *in vivo* and the alternative test, so that the results can be evaluated appropriately.

Determining the correlation between the *in vivo* test and a potential alternative test is an important component of ensuring that the current level of patient safety is maintained or even improved, if possible. *In vitro* tests tend to be more sensitive than *in vivo* tests. This can become problematic when an *in vivo* test is replaced by an *in vitro* compendial test. Standards of quality testing will be altered, resulting in more failures than when using the official animal test. Establishing the correlation between the *in vivo* and the *in vitro* test will determine if the *in vitro* test is more sensitive than the *in vivo* test it is replacing.

1.2.5 International Harmonization

The founders of USP worked to establish harmonization of the quality, strength, purity and potency of drugs and drug products across the United States. 170 years later, the members of USP are faced with the same task, but on a global scale. Technology advances and utilization have resulted in a world that has become a network of communication units. Today, world trade complicates the regulations placed on pharmaceutical companies, by increasing the number of countries involved with manufacture and distribution of an individual

pharmaceutical product. Multiple, and in some cases conflicting, regulations impede commerce in drugs without necessarily increasing the assurance that the product is of highest quality. By harmonizing drug standards internationally, the critical quality characteristics of drugs can be assured around the world.

A major component of establishing a drug standard - that is test and specifications - is the referee test used to evaluate it. To harmonize compendial tests, the regulations and social philosophies of the countries involved must be considered. Resolutions of the USPC in 1990 dealt with the concept of international harmonization of the major pharmacopeias; United States Pharmacopeia (USP), British Pharmacpoeia (BP), Japanese Pharmacopoeia (JP), and European Pharmacopoeia (EP). Therefore, the governing bodies of these communities and their laws need to be evaluated, in preparing future compendial test proposals by USP. The topic of alternatives to animal testing neatly ties into this challenge. When proposing an alternative test which may still involve animal use, the animal welfare laws as well as the resolutions of the governing bodies of the pharmacopeias need to be reviewed and given consideration before the test is officially recommended.

1.3 Objectives of the Review

In order to evaluate the possible reduction and refinement of animal use in the U.S. Pharmacopeia, and the potential tests available for replacement of animal compendial tests, the USP developed a project to formally collect relevant information. Although alternatives to animal testing are being sought in many areas of the U.S. Pharmacopeia, this effort focused on biological potency tests as well as general safety tests such as "depressor", "eye irritancy" and "mouse safety", for biologicals and biologically-derived products. A similar project was conducted in the Netherlands, which focused on the European perspective of this topic (8).

The objectives of this study were threefold. First, a thorough survey of current literature was necessary. This screening of literature sought to accumulate information on potential *in vitro* tests, as well as information on the trend of alternative testing. A literature review would

also provide information on the validation status of tests as well as the opinion of the scientific community on *in vitro* testing.

Second, a summary of the biological potency assays and general safety tests of the major four pharmacopeias was valuable in order to obtain an overview of their present compendial tests. Evaluating the animal requirements of European and Japanese compendial tests would provide insight into possibilities to harmonize.

Third, a summary of the pharmacopeial survey and the literature review were to be combined as a basis for recommendations, and presented to USP for review and consideration. Also, the information obtained could ultimately be incorporated into an information chapter in the U.S. Pharmacopeia.

CHAPTER 2 AVAILABLE ALTERNATIVES

2.1 Physicochemical

This group of tests analyzes biologicals by quantifying function or chemical activity *in vitro*. The following list of tests is not meant to be all inclusive, but is a representation of the most commonly used tests for quantifying and qualifying biologicals. The application to testing of particular classes of biologicals, as well as the potential limitations relating to validation, standardization, correlation and international acceptance will be discussed for each test.

2.1.1 Radioimmunoassay

The radioimmunoassay (RIA) is a commonly used quantitative analysis technique, which utilizes the immunogenic characteristics of molecules. It is a competitive binding assay which employs the antibody-antigen reaction *in vitro*, to quantitate unknown values of a molecule. Generally, molecules with a molecular weight below 1000 are non-immunogenic, thereby limiting the types of molecules which can be measured by this technique (9).

RIA technique may potentially be utilized as an alternative to animal testing for several types of molecules, since it can be validated, and it will comply with international regulations. It also avoids the

problems of correlation and extrapolation which modeling poses. Its principle limitations are based in its use for hormone potency testing, and its ability to be standardized.

To determine the potency of hormones, the USP-NF requires a bioactivity test which quantitates a biological response to a hormone. Determining the amount of hormone present alone is not sufficient for potency testing, since the presence of the hormone does not necessarily reflect its ability to produce a response. Therefore, an RIA would need to be used in conjunction with a test which measures hormone bioactivity, in order to obtain a complete potency profile.

The antibody component of this assay is one of the major limitations for standardization, since it is the ability of the antibody to bind to the molecule which determines the sensitivity and specificity of the assay. Antibody is derived from the serum of animals challenged with the molecule of interest. Antibodies produced against an antigen are considered a heterogeneous population, since they exhibit great diversity in their ability to bind to the antigen. Each species, as well as each individual animal within a species, will produce a unique population of antibody. This, coupled to the diversity found within an antibody population, reveals the difficulty in standardizing antibody in an RIA. The answer would then seem to lie in monoclonal antibodies. Since monoclonal antibodies are a homogenous population of antibodies, this would reduce the within population variance. But, the monoclonal antibody obtained from one animal will not be specific for the same epitopes as the monoclonal antibody obtained from another animal. Antibody can only be standardized if a single source were used for monoclonal antibody production. Since no single animal would be able to provide enough antibody to supply all the researchers utilizing a particular RIA, the problem of antibody standardization would remain.

2.1.2 Radioligand Receptor Assay

In vivo, the bioactivity of a hormone is determined by administering the hormone, evaluating the response of the target tissue, and converting the response to a quantitative value. *In vitro*, defining the bioactivity of a hormone is more complex. A biological response is the result of chemical changes in the cell initiated by a hormone binding to a receptor.

Utilizing the *in vivo* definition of bioactivity as the ability to produce a biological response, then *in vitro* determination of bioacivity should include a measurement of both hormone binding and metabolic change activation.

An RRA is a competitive binding assay, which utilizes receptors as the binding substance. It measures the hormone's ability to bind to a receptor, but not the activation of metabolic changes in the cell. The significance of differentiating these events is seen in the existence of antagonists which have the ability to bind to a receptor, but do not initiate a biological response. Therefore it is necessary to include other parameters in the compendial test which reflect metabolic changes within the cell. Examining the effect on guanine nucleotides, ions, and temperature, are often able to make the distinction between an antagonist and an agonist (10).

The ideal preparation for binding studies would be a homogenous population of cells with preserved biological function so that a correlation could be made between the binding of the molecule to the receptor and the cellular response (10). RRAs are usually conducted on tissue homogenates or receptors harvested from tissue. Since these types of preparations do not retain the integrity of the cell, a separate method for measuring cellular response to the hormone would need to be used in conjunction with an RRA to obtain a complete potency profile.

An RRA is an *in vitro* quantitative assay which mimics an *in vivo* mechanism, therefore its *in vitro/ in vivo* correlation should be very good. It should comply with international resolutions for alternative tests, and should lend itself well to validation. The ability of RRAs to be standardized is better than for RIAs. Receptor preparations can be stored in liquid nitrogen for up to one year, allowing standardization of the major component. A recent development in receptor binding assay application to pharmacopeial tests is the application for the biotechnology-derived human growth hormone Somatrem. The assay has been proposed in the January-February 1993 issue of Pharmacopeial Forum [Vol. 19, No. 1, p. 4518] and could also be proposed in the near future for Somatropin. The assays combines a solution of isolated specific Recombinant Human Growth Hormone Receptor with Somatrem and with USP Somatrem

Reference Standard and the quantitative detection of the complex (receptor & hormone) by HPLC.

2.1.3 High Pressure Liquid Chromatography

High Pressure Liquid Chromatography (HPLC) is a separation technique for proteins and peptides, composed of a stationary and a mobile phase. The differential migration and retention pattern of molecules through these phases characterizes the molecule. The stationary and mobile phases can be liquid or solid, and retention of the molecule can be based on size (exclusion chromatography), polarity (liquid-liquid chromatography), affinity (liquid-solid chromatography) or ion exchange (IE chromatography) of the molecule (11).

HPLC is commonly used in identity and stability testing of pharmaceuticals. It also can contribute useful information when applied to potency testing. Quantitative as well as qualitative evaluation of hormone preparations can give insight into the biological activity of the product. Naturally derived hormone products consist of a heterogenous population of hormones which may vary from source to source. Therefore, the bioactivity of the preparations may potentially vary as well. *In vitro* tests for potency must evaluate the composition of the hormone preparation as well as the activity of the hormone, in order to accurately predict bioactivity of the substance. Therefore, HPLC methods can be a useful component in an *in vitro* potency testing protocol.

There are many advantages to using HPLC as alternative tests for compendial tests. They are very sensitive, and require only small volumes of sample for evaluation. The components of an HPLC system can easily be standardized, which is necessary for any test proposed as a referee test. Also, an HPLC system is relatively not costly, and multiple samples can be run on a single column. The biological activity of the samples might or might not be preserved after HPLC testing, which could allow in specific cases further analysis to be conducted on the same sample. Establishing *in vitro/ in vivo* correlation as well as validation of the system needs to occur prior to being recommended as alternative tests for potency or general safety assays.

2.1.4 Isoelectric Focusing

Isoelectric Focusing (IEF), is a separation technique for amphoteric molecules. It is a form of electrophoresis which characterizes molecules according to their isoelectric point in a pH gradient solution. Molecules move through the electric field according to the charge on their surface. When the charged particle reaches a pH at which its net charge is zero, it has reached its isoelectric point, and will cease migrating (12). This technique can be conducted on a variety of supporting media, such as agarose, granular gel beds, and acrylamide, making it a very versatile method of analysis (13).

IEF is commonly utilized for preparative procedures, but have potential for use as analytical procedures. The molecule's tertiary structure is preserved during IEF, thus allowing characterization of molecular species in a preparation (14). This method may potentially be used to characterize the heterogenous population of hormone preparations, an important component of potency testing in *in vitro* systems. IEF can easily be standardized, and is a straightforward analysis technique. Its application and validation as an alternative test for potency evaluation needs to be researched.

2.2 Tissue Culture

In the generic sense, tissue culture includes all studies in which plant or animal cells, tissue or organs are maintained in a viable state outside the donor for periods of 24 hours or more (15). Upon initial observation, it appears as though this is not an alternative to animal testing, but merely a technique of animal testing. Closer analysis reveals that tissue culture is considered an alternative to animal testing because it reduces the pain and suffering experienced by the animals, and refines the number of animals required for the experiment. Typically, tissue for a tissue culture experiment is obtained from the sacrifice of relatively few animals, while the number of data points obtained from the study is several fold the number obtained from the individual animals. Therefore, tissue culture is an alternative method because fewer animals are used, and minimal suffering is incurred when compared to traditional *in vivo* testing. The obvious limitation here is the loss of the assay of homeostatic

mechanisms and therefore a decrease of prospects for close correlation with the *in vivo* test.

The metabolic and growth response of the tissue is solely dependent on the *in vitro* environment. Tissue is maintained *in vitro* by media, which contains the nutrients necessary for the tissue to live. Cellular requirements have not been thoroughly defined for most tissues, but it has been shown that variations in certain media components can affect the cells' metabolism (16). These changes in metabolism may result in variations of responses to pharmaceutical preparations. Therefore media must be standardized for these culture techniques to succeed as potential alternatives to animal testing.

2.2.1 Primary cell culture

This type of culture is obtained from the tissue of freshly sacrificed animals. The tissue of interest is then dissociated by chemical and mechanical manipulation into single cells or cell clusters. The cells are then plated onto a synthetic or natural matrix which allows adherence and stability for experimentation. The cells are sustained in a controlled environment and fed with nutrients necessary to supplement their metabolism. One advantage of primary tissue culture is that the tissue of interest is being utilized in the *in vitro* test thereby minimizing the amount of extrapolation. Another advantage is that a controlled environment is produced. A tissue culture plate contains multiple chambers of tissue (test units), therefore the controls and the test chambers are treated uniformly prior to experimentation. Also, individual culture plates are assumed to be homogeneous since the tissue from the animals was combined prior to dissociation. This reduces the variance between test units compared to the variance obtained when using individual animals as test units.

Primary cell cultures lend themselves well to standardization due to the controlled environment the cells are maintained in. The reagents used for culturing and making media, as well as the equipment can be readily standardized. The only component of the culturing technique which may produce some variance is the harvesting of the tissue. Since this aspect is dependent upon the expertise of the technician, some

variance may occur between technicians with respect to the tissue harvested. Precise protocols and a validation process should reduce this problem.

The *in vivo/ in vitro* correlation should be meaningful because living tissue is being utilized in the test. This is not to say that the correlation will be absolute, because of the lack of homeostatic factors. A cell reaction test such as primary cell culture may not truly depict the entire response that the model requires. In these cases, other tissue culture systems may fit the model more accurately.

2.2.2 Established cell lines

Also known as continuous cell lines, these cells have the property of unlimited division potential *in vitro* (15). Some cell lines are the result of a forced fusion of a tumor cell with a cell type which does not have the intrinsic ability to divide *in vitro*. The result is a hybrid cell which possesses the characteristics of the original cell of interest, with the additional ability for unlimited division *in vitro*. These cells are maintained in a controlled environment similar to the primary cell cultures.

The cells differ from the primary cell cultures in two aspects. First, because the cells proliferate *in vitro*, the cell cultures can be maintained for much longer periods of time. Whereas the viability of the primary cell culture is short term (days to weeks), the continuous cell line can remain viable for longer periods (months), provided there is no contamination. This may have a significant effect on the validation, since test to test variance should decrease.

Second, these cell lines can be stored in liquid nitrogen. This greatly increases the standardization potential of cell culture technique, by overcoming the technician variance during harvesting. The established cell line can be distributed from a single source, thereby certifying that the cells for testing are all from the same origin.

2.2.3 Whole organ assay

This term refers to a three-dimensional culture of undisaggregated tissue retaining some or all of the histological features of the tissue *in*

vivo (17). Certain models may require a more complex response than can be tested by a culture of cells, therefore an organ culture may be appropriate.

The organ culture requires less steps of tissue manipulation than cell culturing requires. The organ is harvested from the animal, and then placed in a controlled environment. The decrease in the complexity of the protocol would appear to be an asset for validation, yet this isn't necessarily the situation. The potential variance in this technique is shifted to the harvesting component, similar to primary cell culturing. A benefit of the whole organ culture is that the trauma of cell dissociation is avoided.

The metabolic continuity of a whole organ culture is extremely short term (hours), therefore multiple use of a single organ culture is not practical. Overall, the standardization potential of this type of culture is good, and validation should be obtainable. The *in vivo/ in vitro* correlation should be relatively high because the tissue is from the same species as the *in vivo* test. Also, a whole organ is a more complex organizational group than tissue, so the response should more closely reflect the response obtained *in vivo*. It should be noted that although the number of animals used may not be greatly reduced, the pain and suffering resulting from an *in vivo* test is removed.

2.3 Electronic

Progress in information technology is having a profound effect on scientific research. Computer technology not only increases the rate at which information is acquired, but also the amount of information that can be acquired. Utilizing available technology can potentially decrease unnecessary and repetitive testing by utilizing data that has previously been gathered.

2.3.1 Computer modeling

Simulation of an environment, a structure or a situation by a computer are all considered instances of modeling. The potential to reduce, refine, or replace animals in testing is extensive when modeling is incorporated into industrial and academic research. The number of

modeling systems is increasing rapidly. In essence, they all aim to use the power of the computer to identify and illustrate some relationship between the chemical structure and biological activity (18).

Physiological models predict biological response to a chemical or drug by collating scientific data and incorporating computer technology to produce a visual simulation of response. The limiting factor in the use of these systems is the current availability of programs for the models. The nature of models is that they represent a simplification of the real world and might not include all the relevant factors because some of the mechanisms of drug effect might not be precisely understood. The application of physiological models in compendial and in QC testing of pharmaceuticals could result in a significant reduction in the number of animals used.

Molecular modeling utilize previously acquired data to model the structure of new chemical entities. This leads to the refining of future experiments by predicting the potential activity and/or toxicity of the drug. QSAR (quantitative structure-activity relationships), is a molecular modeling method which utilizes mathematics to predict the potency of analogues in series of related compounds (19).

2.3.2 Databases

One of the most common issues that generate animosity towards the research community is the public's perception of waste. The public perceives that research is conducted independently to such a degree that experiments are being duplicated at the cost of the taxpayer and the animals. In some cases their opinion could be warranted. In industry and lately in some academic circles competition generates confidentiality, collaboration and sharing of data is excluded. Also, in the academic community, the lag time between acquisition of data and publication can lead to repetition of experiments.

Databases are resources of information which can be utilized to facilitate and coordinate testing. The term database includes any comprehensive collection of data, whether raw data, statistical data, or literature reviews. The significant aspect of databases in reference to alternative testing is their potential ability to link the research community

together. By having data available from a variety of sources, the investigator will be able to access information which may have previously been difficult to obtain. Utilizing database searches early in testing may potentially reduce animal testing by revealing that the data already exists. Databases can also function to refine animal testing by providing a thorough background search which leads to a decrease in the extent of animal testing needed.

Collaboration is the limiting factor in databases reaching their full potential. Another limitation is the standardization of data input among the various research communities. Obviously, it is not expected that the manufacturer divulge proprietary information on formulas, but merely data on raw materials. This would greatly decrease the time and fund commitment, as well as animal involvement in testing. In Britain, the Centre for Medicines Research maintains a database which incorporates information provided by over 20 pharmaceutical companies (19). In the United States, the FDA and EPA are assembling a database on chemical migration from polymeric materials to facilitate thorough and efficient review of chemical migration data (20). A database has been established by FRAME (Fund for the Replacement of Animals in Medical Experiments) which gives current information on the validation status of *in vitro* alternative tests (21).

2.4 Bioassay

Mateo Orfila in the early 19th century was the first scientist to use animals to scientifically model human response to a drug (5). He is reported to have given known quantities of drug to an animal, and then record the response of the animal, as well as grossly examine the vital organs, and analyze the tissue for the drug (5). Since that time, the animal model has served well as a means of testing drugs and bulk pharmaceuticals. As our knowledge continues to grow, so does our awareness of the limitations of animal models. Data extrapolated from animal tests is only significant if the test truly simulates the human response.

2.4.1 Mammalian

It is crucial to the significance of the data that the choice of species fits the system to be mimicked. The species or strain of the animal should resemble the system of the person being studied. For example, a potency test for follicle-stimulating hormone (FSH) should be conducted in an animal which has a similar ovulation mechanism and hormonal profile as humans. Otherwise, the data may not reflect the human mechanism. Therefore, both the nature of the study and the physiological characteristics of the animals should be considered when determining the optimal animal model.

Traditionally, mammals have served as the most commonly used models for *in vivo* testing. This is probably due to the fact that we know more about mammals than any other species. Our domestic animals mostly are mammals, providing us with food, clothing, shelter, or companionship. So, it logically follows that mammals were then chosen as tools to provide us with scientific knowledge. Over the past one hundred years of intense research with animals, our information base has grown considerably in regards to human and animal physiology. We now understand the applications and the limitations of animals, more specifically mammals, in research. Subsequently, we realized that alternative methods of animal testing cannot rule out the traditional animal model. In some testing, the model that a mammal provides cannot be substituted for an *in vitro* test. In these instances, the refinement of techniques, and the reduction in animal numbers accomplishes the goal of alternative testing.

2.4.2 Non-mammalian

Recently, the scientific community and others involved in animal testing have turned to non-mammalian animals as potential models. They offer the benefit of being subjected to less regulations than traditional laboratory animals, and in economic terms, are less expensive to maintain. The term non-mammalian is an umbrella term for all the other species in the world that do not arouse public sensibilities to the degree seen with mammals. When referencing non-mammalian species in the context of alternative testing, the term is focused to those species

which are considered - rightly or wrongly - less sentient than mammals. Two of the most commonly chosen groups are the invertebrates and the microorganisms. Invertebrates have been successfully utilized in *in vivo* and *in vitro* studies.

Microorganisms have also been used successfuly to develop *in vitro* tests for toxicity and parenteral drug testing. These tests utilize intrinsic characteristics of microorganisms, such as the luminescent activity of the Photobacterium phosphoreum, to detect toxins in a test substance (22). Other tests employing microorganisms quantitate toxic substances by measuring products of the bacteria, such as ATP released from the bacteria at cell death. Microorganism assays may be potential replacements for traditional mammalian assays in certain situations, are much less expensive, and can easily be validated and standardized. Limitation of the use of microorganisms is that classical toxicologists do not want to stray further than human or animal cells in the extrapolation of results to humans.

2.5 Combination Testing

The previous discussion of alternative options was an overview of tests which may have a part in the future of quality testing of pharmaceuticals. Each test offers a unique manner of quantitating or qualitating pharmaceuticals. Not intended to be all inclusive, these groups of tests represent the potential modes available for testing the many types of pharmaceuticals presented in the pharmacopeias of the world. These tests can be utilized alone, in combination, or as a battery of tests to obtain the necessary information to evaluate the quality, safety and efficacy of pharmaceutical products. In some instances, combining tests may lead to a more complete profile of the quality and potency of the test material.

2.5.1 Battery of in vitro tests

In vivo tests are complex, resulting in a reaction that incorporates several mechanisms. *In vitro* tests tend to be more specific, since the system is isolated from the animal. In some instances, the reaction may be the result of only a portion of the overall mechanism which was

previously examined in the *in vivo* test. Therefore, employing several *in vitro* tests can result in a more complete profile of the test material than would have been obtained from a single test. The USP currently recommends a battery of *in vitro* tests for analyzing plastics and elastomeric closures for injection, for which *in vivo* testing is indicated for materials which do not meet the requirements of the *in vitro* tests.

Application of this method of alternative testing has several benefits. First, it allows alternative testing to be implemented sooner. If an *in vitro* test which models the entire *in vivo* reaction has not been developed and/or validated yet, utilizing a battery of *in vitro* tests to evaluate the material can be a useful substitute for the interim. Second, a battery of tests may be more representative than a single test. A product may be negative in one *in vitro* test, but may be positive in another, since each test is evaluating a different mechanism. By using several *in vitro* tests, this bias can be minimized. And third, in some instances the *in vivo* test cannot be replaced by a single test, then a battery of *in vitro* tests provides the option necessary to achieve alternative testing.

2.5.2 Tier system

A two-tier approach can also consist of a combination of *in vitro* and *in vivo* tests, with the number of animals used in the *in vivo* test reduced from the original protocol. Customizing a two-tier approach to evaluate pharmaceutical materials can result in many different combinations of testing. Occasionally, the level of validation the *in vitro* tests are at should be considered so that optimal use of the tests can be achieved. For example, if an *in vitro* test has not been thoroughly validated against the official standard *in vivo* test, perhaps a two tier approach is a prospect. Here, a negative result of the first tier of *in vitro* tests is followed by a second tier of the *in vivo* test. This would reduce the exposure of animals to all those which are reactive in the first test. The *in vivo* tier serves largely as a confirmatory test. In the instances when a two-tier approach is recommended, a protocol detailing the sequence of testing should be established to minimize confusion.

The concept of a tier system has been extended by USP to a three tiers in the biological reacivity testing of plastics for containers used for

ophthalmic drugs. The aim of the three-tier system was to reduce as much as possible the use of the Eye Irritation Test by using a combination of *in vitro* and *in vivo* tests. The first tier consists in using the Biological Reactivity Tests - In Vitro <87>. If the requirements of the *in vitro* tests are met, then the second tier, consisting of two *in vivo* tests (Systemic Injection and Intracutaneous Test) is activated. If the requirements of the second-tier are met, the third-tier, the Eye Irritation Test is activated. Further refinement is being proposed by USP to delete the second and third-tier testing and require as a compendial test only the *in vitro* tests in Pharmacopeial Forum, Jan-Feb 1993, Vol. 19, No. 1, p. 455.

2.5.3 Physicochemical analytical tests with bioidentity

Biologicals such as hormones must be evaluated for their potency, which is a combination of both the quantity and the quality of the hormone. The physicochemical tests mentioned previously provided only quantitative values of the product being analyzed. Bioidentity tests qualitate the biological activity of a hormone by measuring the biological activity of the hormone. A physicochemical test used in conjunction with a Bioidentity test can provide a more complete profile of the hormone, than if either were used alone.

The biological activity of a hormone, defined as the ability of a hormone to initiate a physiological response, consists of a complex cascade of events. First the hormone must bind to a receptor. Next it must initialize the second messenger, which in turn activates the enzyme pathway to stimulate protein synthesis, and ultimately a response. It becomes apparent that a biochemical test may only evaluate one component of this cascade. For example, the RRA evaluates receptor binding, but does not determine if the hormone can initiate second messenger activity. An RIA measures a hormone's immunoactivity, which is a function of the alpha subunit of a hormone, but it is the beta portion of the hormone which determines bioactivity. In these instances, a bioidentity test might be necessary to evaluate the biological activity of the hormone.

The concept of bioidentity testing was proposed at a USP Open Conference on alternative testing, to assure the standards of potency were maintained during the changes to *in vitro* technology. A bioidentity test is small scale *in vivo* test utilized to obtain qualitative information on the preparation. The number of animals necessary for bioidentity studies is greatly reduced from the number required for established *in vivo* potency testing, since quantitation of the hormone is conducted by *in vitro* testing. Therefore, combining the analyzing characteristics of physicochemical and bioidentity tests can produce a powerful potency evaluation system for biologicals.

Chapter 3 Bioassays in the Major Pharmacopeias

3.1 Hormone Potency Assays

To achieve international harmonization while seeking alternatives to animal testing, it is first necessary to compare and contrast the present compendial tests in the major pharmacopeias. The comparative summary presented here will focus on a select number of monographs which utilize bioassays, from the USP, BP, EP, and JP.

Monographs for synthetic or naturally derived hormones require an evaluation of the potency of the drug in addition to testing of a number of quality, identity and purity characteristics. Previously, potency values were obtained by performing a biological activity test *in vivo*. Each of the following hormones present unique challenges for replacement with alternative tests, and have been chosen for their divergent representation of the dynamics of seeking alternative testing.

3.1.1 Menotropins

According to the USP, menotropins is "an extract of human post-menopausal urine containing both follicle-stimulating hormone and luteinizing hormone". The required ratio of units of FSH to LH is approximately one. The compendial test for menotropins consists of two individual tests; one to measure FSH activity, and one to measure LH activity.

Presently, the JP does not have an official monograph for menotropins. The recommended test for menotropins in the USP, BP,

and EP is similar for determining both FSH and the LH activity. The FSH activity is determined by an *in vivo* test which compares menotropins' effect on enlarging the ovaries of immature female rats to a standard preparation. The LH activity is determined by comparing menotropins' effect on increasing the mass of seminal vesicles to a standard preparation. In addition, the BP includes the option of using changes in mass of the prostate gland as a test to determine LH activity in menotropins. The required number of rats for the LH and the FSH bioassay varies among the pharmacopeias, with the BP and EP recommending a minimum of 30 rats per test, and the USP recommending a minimum of 42 rats per test. Therefore, finding an alternative test for determining the potency of menotropins would have a significant impact on animal use.

Menotropins is a naturally derived product, composed of mainly two glycoproteins. Glycoproteins exist as a heterogenous population of isohormones whose carbohydrate structures produce a variance in biological activity (23). It has been proposed that it is necessary to quantitate the isohormone content of natural hormones to obtain a complete profile of the biological activity. Two *in vitro* tests for menotropin have been discussed, but were not proposed in Pharmacopeial Forum (PF) due to their inability to address the issue of isohormones. The most appropriate *in vitro* test for LH is a cell culture test which measures the release of testosterone from mouse Leydig cells (24). The current FSH *in vitro* test measures the release of aromatase from rat granulosa cells (25) (26). Both of these tests employ significantly less animals to obtain comparable results. To address the isohormone issue, perhaps a quantitative assay which evaluates isohormone content could be used in conjunction with these two assays. A procedure has been developed to characterize isoforms of glycoproteins in an extract which combines IEF (isoelectric focusing) and IEX (ion exchange chromatography) (23). The complexity of these procedures may make them poor candidates for compendial tests. A thorough evaluation of cost, complexity, and consequence of alternative testing must occur before tests are recommended by the pharmacopeias.

3.1.2 Corticotropin

The USP definition for corticotropin is "... material containing the polypeptide hormone having the property of increasing the rate of secretion of adrenal corticosteroids, which is obtained from the anterior lobe of the pituitary of mammals used for food by man". The JP does not have an official monograph for corticotropin.

EP and consequently BP, determine the activity of corticotropin using adult hypophysectomized rats of either sex; these rats are subcutaneously injected with test or standard preparations. Three hours after injection, rats are sacrificed and their adrenal glands are removed and weighed. The adrenals are then prepared in a homogenate, and the ascorbic acid content is measured via light absorption using a colorimeter. The USP test is similar, except that the animals are required to be the same sex within a test.

One of the major concerns with the present *in vivo* test is the difficulty in maintaining an adequate supply of the reference material. The corticotropin monograph is one of the few monographs in the USP which utilize a posterior pituitary reference standard. Posterior pituitaries are becoming more difficult to obtain, therefore the proposal of a test which doesn't require posterior pituitary reference would be of significant benefit to the pharmacopeias.

A rat steroid block bioassay has been developed which appears to be equal to or superior to the USP hypophysectomized rat assay, with the additional benefit of not requiring a posterior pituitary reference. Animals are injected with dexamethazone instead of hypophysectomized, to inhibit endogenous ACTH secretion. Thus, the pituitary is kept intact, negating the need for a posterior pituitary reference. It also requires half of the animals due to the factorial design of testing, and is subsequently less costly. A harmonization concern exists because other pharmacopeias may not be willing to replace one animal assay with another, even though the goals of alternative testing are achieved through reduction of suffering and refinement of animal use.

A column perfusion system which measures corticosterone production of isolated rat adrenal cells after corticotropin stimulation has

been described in the literature (27). This system is a modified tissue culture technique which allows eluate to be collected without disruption of the cell culture. The perfusion system reacts within the range of physiological levels, but has not been validated.

An HPLC method has also been developed for measuring ACTH (28). This HPLC method does not destroy the bioactivity of ACTH, therefore it could be used as part of an *in vitro* regimen to measure corticotropin potency. As explained previously, a quantitative assay such as an HPLC would need to be used in conjunction with a qualitative assay to obtain complete potency profiles of the drug.

3.1.3 Chorionic gonadotropin

The USP definition of HCG is "a gonad-stimulating polypeptide hormone obtained from the urine of pregnant women". The EP and the BP have recommended similar tests to determine the activity of chorionic gonadotropin. Both pharmacopeias estimate the potency of HCG by comparing its ability to increase the mass of the seminal vessicles of immature rats to a standard preparation. The BP also includes the option of using the prostate gland as the target organ. Rats are injected subcutaneously with the test or standard preparation, sacrificed after a predetermined time, and the seminal vessicles are removed and weighed. The foundation for this test is based on the similar biological and immunological reactivity between LH and HCG, and the consequent reactivity of HCG with LH receptors on the seminal vessicles.

The JP describes an *in vivo* test for chorionic gonadotropin which determines its potency by comparing the effect of test preparation injection on the ovarian mass of adult female rats to the effect of standard preparation. This test is based on the biological reactivity between HCG, and LH receptors located on the ovarian follicle (29).

Presently, in the USP monograph, the activity of chorionic gonadotropin is determined by evaluating the effect of subcutaneous injection of test preparation on the mass of the uterus of young female rats, compared to the injection of standard preparation. In the Nov-Dec 1991 PF, a change in the compendial test was proposed which consisted of a bioidentity test coupled with a physicochemical test. In the

bioidentity test, the number of animals was greatly reduced, and the target tissue was changed from the uterus of female rats to the seminal vessicles of male rats. This change was proposed in conjunction with an HPLC assay, which was met with significant opposition by scientists and industry. The major concern is that a complete biological acivity profile of all of the components of the chorionic gonadotropin extract has not been identified. Measuring only chorionic gonadotropin in the extract may be misleading when determining the potency of this product. Preliminary testing using HPLC appears to be less rugged than expected at this time.

An *in vitro* system has been described in the literature to measure chorionic gonadotropin, which employs Nb2 immortalized cell lines (30). This ESTA (eluted stain assay) utilizes the reduction of a tetrazolium salt to a formazan dye by intracellular dehydrogenase as the cytochemical system in cells which respond to specific hormones (30). The stage of validation of this assay is not known, and is presented as a potential direction of alternative testing for chorionic gonadotropin which could be explored.

3.1.4 Insulin

The USP definition for insulin is "a protein, obtained from the pancreas of healthy bovine and porcine animals used for food by man, that affects the metabolism of glucose". The BP, EP, and JP all include *in vivo* assays for determining the potency of insulin. The BP offers three *in vivo* options. The first, the mouse convulsion test, is also found in the EP. Requiring no fewer than 96 mice, this test may be one of the most demanding of animal use in the pharmacopeias. The mice are randomly distributed into four groups, and injected with either the standard or test preparation. The mice are maintained at a constant temperature, and watched for 1.5 hours. The criteria for determining potency is based on the number of animals which are dead, convulsed, or lie still more than 2-3 seconds when placed on their back.

A second test is the blood glucose determination in rats. This compendial test is strictly found in the BP, and requires no fewer than 40 mice. The mice are randomly distributed into four groups, and injected with either the test or standard preparation. Thirty minutes after injection,

a blood sample is taken from the orbital sinus and the glucose concentration is determined.

The third test is the rabbit blood glucose test, and is found in the USP, BP, EP, and JP. No less than 24 rabbits are used. At 60 and 180 minutes after rabbits are injected with either test or standard preparation, a sample of blood is taken at the marginal ear vein, and the glucose concentration is determined.

In the Jan-Feb, PF 1990, the USP proposed changes in the monograph for insulin. An HPLC method was proposed to determine potency, and for determining of insulin-related substances the USP proposed a reverse-phase HPLC and a size-exclusion HPLC. The biological assay was retained as a bioidentity test, with a significant reduction in the number of rabbits used from 24 to 8. The proposal has been modified and republished in the Nov-Dec, 1992 PF, and will become a compendial test, published in a future USP-NF Supplement following review and consideration of public comments.

The EP commision has also proposed a revision of its insulin monograph. Published in the Oct 1991 Pharmeuropa, it proposed replacement of the *in vivo* assay with an HPLC method for determining insulin potency. The EP recommendation was based partially on studies published in Sept. 1990 Pharmeuropa, which favorably compared an insulin bioassay with the HPLC method (31) (32).

3.1.5 Oxytocin

The USP definition of oxytocin is "..material containing the polypeptide hormone having the property of causing contraction of uterine, vascular, and other smooth muscle, which is prepared by synthesis or obtained from the posterior lobe of the pituitary of healthy, domestic animals used for food by man". All four pharmacopias recommend the cockerel blood pressure test to determine the potency of oxytocin. A young, healthy adult cockerel is anesthetized and injected with test or standard preparation via the cannulated brachial vein or the crural vein. The blood pressure response is measured, and the potency of oxytocin is determined by statistical methods.

A second test, described in EP and BP, is the rat milk ejection test. One lactating rat is anesthetized, and the tip of the lower inguinal teat is exposed. The teat is injected with either test or standard preparation, and the milk ejection pressure response is measured.

A third test, described in the EP and the BP is an *in vitro* test measuring rat uterine contraction response to oxytocin injection. A horn of the uterus is removed from a rat, placed in a water bath to which the test or standard preparation is added. The uterine contraction response is measured, and the oxytocin potency is determined.

Since the majority, if not all, of oxytocin produced is no longer of natural origin, the USP has proposed a new compendial test which is tailored to the testing of synthetic oxytocin. Published in the July-Aug 1991 PF, it proposes the deletion of the *in vivo* chicken assay. An HPLC procedure is detailed in conjunction with a bioidentity test, the rat uterine contraction test indicated in the EP. A second iteration of that proposal will be presented in PF in 1993.

3.1.6 Somatropin

The proposed USP definition for somatropin is "a polypeptide hormone... (whose) structure corresponds to that of human growth hormone extracted from human pituitary glands. It is produced by microbial synthesis via a recombinant procedure." Presently, there is no official monograph for somatropin in any of the four major pharmacopeias. USP and EP are in the process of drafting an official monograph, having both published initial proposals in their public forum journals. USP's proposed potency test, published in the Nov-Dec 1990 PF, consists of a bioidentity and an HPLC assay. The bioidentity test is a weight variance test conducted on hypophysectomized rats. Somatropin potency is determined by administering multiple injections of the test or standard preparation over several days, and measuring the weight gain.

The bioassay for somatropin employs hypophysectomized rats, so that intrinsic levels of GH do not interfere with the externally administered somatropin. Hypophysectomizing rats is a costly, and imprecise procedure which requires the rat to undergo surgery prior to its utilization in routine testing. A novel strain of rats has developed whose basal

serum and pituitary levels of GH are 10% of normal values (33). This strain of rats are within 10% serum levels of being naturally hypophysectomized, and perhaps could be utilized in place of surgically induced hypophysectomized rats.

The EP proposed a monograph for somatropin in the Oct 1991 Pharmeuropa. While the assay for human growth factor requires a very involved bioassay due to its natural origin, the European community posed that synthetic hormones should not require bioactivity tests once product development and validation of manufacturing procedures has occurred. Therefore, the EP has proposed a series of physicochemical tests which identify the product.

There are *in vitro* assays to measure GH which may be applicable to somatropin as well. A radioreceptor assay technique has been developed by industry and is going to be proposed in an early 1993 PF to replace the proposed animal test (34). Another *in vitro* bioassay has been described in the literature which measures the activity of lactogenic hormones based on the stimulation of the growth of Nb rat lymphoma cell cultures (35). Although the test does not have the ability to discriminate between lactogenic hormones, this should not be a problem because somatropin is synthetically derived. This binding assay has the potential to be standardized, and posesses a sensitivity which exceeds that of RIAs (35). The USP is drafting a second iteration of the monograph for Somatropin, that will propose the use of an *in vitro* test, a receptor binding test, that is similar to the one already proposed for Somatrem.

3.2 Other Bioassays

Also known as biological tests, these assays are required for a variety of drugs. These safety tests utilize a significant number of animals, and are a part of the campaign for seeking alternative testing. This is not a complete list of biological tests, but a highlight of those which have received a lot of attention.

3.2.1. Depressor

The depressor test involves comparing the effect on cat blood pressure when injected with test and standard preparations. This test is

required for any product which may contain hypotensive substances in the end product, or in a component of the manufacturing process. Presently, the test is conducted using 2 healthy, adult cats. The animals are anesthetized, immobilized, and the carotid is exposed to allow continuous blood pressure monitoring. The response of the test material is compared to the response of an injection of standard histamine preparation. Due to the expense of the animals, and the fact that the animal must be sacrificed at the end of the testing, multiple products are tested on each animal to maximize its usefulness.

The depressor test for detection of histamine and histamine-like substances is required in the USP and EP. EP has discussed a new policy which would remove the histamine test to the production regulation section, thereby removing it from compendial testing. The USP believes that the histamine test is an important test for assuming patient safety when taking certain antibiotics. Therefore, they are actively seeking an *in vitro* or physicochemical test which equals the current *in vivo* cat test in sensitivity and validity. In the Mar-Apr 1991 PF, USP called upon industry to supply information on each antibiotic tested by the depressor test, to establish on a case to case basis, whether the depressor test should be required. If data can be presented which shows that there is no potential of histamine existing in the crude product, then USP may decide that the depressor test is not required for that particular product

The USP definition includes the term "histamine-like" which restricts potential replacement tests to those which have the ability to detect histamine and other common bioamines such as putricine and cadaverine. Recently, a simple HPLC method has been reported in the literature which utilizes a gradient elution system to quantitate and qualitate 9 biogenic amines simultaneously, including histamine, putricine and cadaverine (36). The potential application of this technique as an alternative to the cat depressor test is dependent upon its sensitivity and validity.

3.2.2 Pyrogen

According to the USP, the pyrogen test is designed to "limit to an acceptable level the risks of febrile reaction in the patient to the

administration by injection of the product concerned". This test is indicated in the EP, BP, JP, and USP, and is required for any parenterally administrated product. The test evaluates the difference in basal temperature of the rabbit after test and standard preparations are administered. A significant impact on animal use could result from seeking alternative testing to the pyrogen test, since it is required in over 500 monographsin the USP.

Although this test is still indicated in the pharmacopeias, it has been deleted from many monographs, and replaced by the Bacterial Endotoxin Test (BET). This *in vitro* test was added to the EP biological tests section of the methods of analysis chapter, in the 1987 Appendix. The BP included the BET in the 1989 Addendum, and the JP added it in the 1988 Supplement. In the USP, the test is applied using Limulus Amebocyte Lysate (LAL) which is obtained from aqueous extracts of the circulating amebocytes of the horseshoe crab Limulus polyphemus. An official USP test since USP XXI (1985), the BET has gained increasing attention as a substitute for the *in vivo* pyrogen test, resulting in a dramatic decrease in rabbit testing. A total of 480 monographs in USP XXII, up to 7th supplement have a BET test. Thirty two additional monographs with a pyrogen test requirement are under review and proposals for deletion and replacement by the BET method will be published in a future PF (34).

3.2.3 Eye Irritation Test

The eye irritancy test is a modification of the Draize ocular irritancy test. The test product, an extract of plastic, is placed directly in one eye of an Albino rabbit, and the other eye serves as a control. Irritancy is measured by a grading system of observations determined by USP. Neither the BP, EP, nor the JP indicate this test in any of their monographs.

The eye is a complex system, which includes epithelial cells, stroma, supporting cells, as well as a lubricating system, with its associated enzymes and electrolytes. Exposure of the ocular surface to foreign material may produce "irritation"; a very broad term, with numerous levels of effect. Therefore, it is difficult to develop *in vitro*

assays which can mimic this elaborate physiological system. A wide effort has been established by toxicologists over the past ten years to develop *in vitro* alternatives for the Draize test, because of its potential to cause pain and discomfort to the animals. The result has been the development of numerous assays, which measure irritation by evaluating cell viability, membrane integrity, cellular structure, inflammation and immunity, as well as cell recovery and repair after being challenged (37). Each tests a component of the irritancy model, and when combined, may serve as a useful *in vitro* model for ocular irritancy.

To date, none of these *in vitro* assays have been validated to a level acceptable to international regulatory agencies. Several projects have been developed around the world in order to validate some of the most promising *in vitro* tests (38) (39). The Opal Project in France and the ZEBET project in Germany are focusing on the HET-CAM test, which appears to have potential as an alternative to the rabbit test. The HET-CAM (Hens Egg Test-Chorioallantoic membrane) test is a modification of the HET test, a common embryotoxicity test. The original HET test utilized 15 day incubated hens eggs: in the European community this is considered an animal. The modified HET-CAM test utilizes 11 day incubated eggs, which by definition, are not considered animals in the European community, thus complying with international harmonization goals. It is this modification which makes the HET-CAM test potentially useful as an alternative test.

The HET-CAM test is considered an *in vitro* test on the margin of an *in vivo* test (40). Fertile, white leghorn eggs are incubated for 11 days, followed by exposure of their living vascular chorioallantoic membrane. The test or standard preparation are applied directly to the membrane, and then the CAM, blood vessels, and albumin are examined and scored. The time-dependent scores are categorized by a classification scheme analogous to the Draize categories (40). Neither of the two validation projects are complete, but preliminary data shows good correlation between the HET-CAM and the Draize test.

In the Nov-Dec 1987 PF, USP solicited comments on four *in vitro* tests as potential alternatives to the eye irritation test. Included in the article were the CAM test, the neutral red assay, the total cell protein

assay, and the rabbit corneal epithelial cells/wound healing assay. Perhaps one of these could be recommended to replace the eye irritation test.

The USP proposed the deletion of the Eye Irritancy Test in the Jan-Feb 1993 issue of PF since the use of the cytotoxicity test is an appropriate *in vitro* test for the purpose intended.

3.2.4 Mouse Safety Test

This test is categorized as a general safety test, and is indicated in the USP, and EP (BP). The safety test is a required test for medical devices and other plastic materials as well as for biologicals. In recent years its requirement in USP antibiotic monographs was deleted for almost one hundred antibiotics (34). The USP defines its application as "intended to detect in an article any unexpected, unacceptable, biological reactivity." Five healthy mice are injected intravenously with the test solution. The mice are examined 48 hours after injection for any outward symptoms which are not expected for that level of toxicity. If one or more animals is dead or showing symptoms, the test must be repeated in no less than 10 mice. For biologics, the test is conducted with both mice and guinea pigs.

In the EP (BP) the test is called the abnormal toxicity test. Its purpose is the same, but its criteria for passing is different. For their test, if one mouse dies, the test is repeated with an additional five mice. If two or more mice die, then the product fails the test. No mice must die in the repeat test for the product to pass the test.

The mouse safety test was conceived with the safety of the patient in mind. It was developed in the 1950's in response to the rapid increase in penicillin production. Since the inception of the safety test several decades ago, manufacturing regulations and QC management have evolved dramatically. Therefore, USP felt the effectiveness of the mouse safety test should be reevaluated. USP surveyed the industry [PF, July-Aug 1991, 17(4) p. 2239] on the use of the general safety test. Results of the survey indicated that of the 8,000 safety tests conducted on medical devices and other plastic materials, none had been positive. For biologicals, 34 safety tests were positive of the 16,500 conducted [Jan-

Feb 1993, PF 19 (1) p. 4654]. The Subcommittee in charge of this test will be recommending the deletion of the safety test for medical devices, but not from biologicals. The theoretical impact on animal use is staggering.

The BP Commission has decided to delete the test from 6 to 7 monographs for hormones like menotropin and corticotropin. The BP has also proposed to the EP to take the standpoint of the BP with regard to the application of the abnormal toxicity test as a routine QC test (8).

CHAPTER 4 SUMMARY AND RECOMMENDATIONS

4.1 Hormone Potency Assays

This type of assay is separated into three types of hormones. They are grouped according to similar characteristics which affect the type of alternative testing which may be utilized to measure their potency. This categorizing is done with the sole purpose of simplifying the summary and recommendations for seeking alternative testing.

4.1.1 Naturally-derived

The term naturally derived refers to those hormones which are obtained from tissue or fluid extract of animals or humans. The hormones discussed earlier which are a part of this category are menotropin, corticotropin, chorionic gonadotropin, and insulin. Naturally derived hormone products are a heterogenous supply of the hormone with respect to isoforms. It has been presented here that isoform composition is believed to produce a variance in the biological half-life of the product. Therefore, for naturally-derived hormone products, it is important to identify the isoform profile in potency testing. A technique which combines IEF and IEX has the ability to evaluate the isohormone profile of extracts.

Replacement of animal tests with *in vitro* tests for potency appears to be promising for these hormones. Insulin is close to having an official monograph which replaces the rabbit test with a physicochemical test and a small scale *in vivo* test for bioidentity.

Hormone activity is a function of the hormone's ability to stimulate a second messenger and produce a response. Cell culture systems evaluate hormone activity by measuring a cellular response to the test product. Theoretically, they may be the best overall test for evaluating hormone activity. Their limitations due to potential variability in technique could possibly be overcome with strict protocols.

Cell culture systems have been developed for most of these hormones. Culture techniques provide the closest system to *in vivo* testing, since living tissue obtained from animals or humans is used. An *in vitro* test has been developed which measures the potency of **corticotropin** by its ability to stimulate corticosterone release from cultured adrenal tissue. An alternative *in vivo* assay has been developed, the rat steroid block assay, which does not require hypophysectomizing the rats. When used in conjunction with an isoform assay, these assays may provide a more complete potency profile.

An *in vitro* cell culture test has been developed for **chorionic gonadotrophin** as well, which could be utilized for potency testing. Immortalized cell lines are stimulated with chorionic gonadotropin, and metabolic changes are evaluated.

4.1.2 Polyhormone Extracts

The **menotropin** product is naturally-derived, but is unique in that its potency is the result of two hormone activities, namely LH and FSH. Therefore, this monograph requires two separate tests to determine its potency. Two cell culture systems are at the forefront of proposed tests, but need to be validated. For LH, the mouse Leydig cell assay measures testosterone production after stimulation with LH. The granulosa cell aromatase assay is used to measure the effect of FSH on the stimulation of aromatase production. Neither of these tests have been proposed in the PF, because industry felt that the issue of isohormones was being overlooked. Since menotropins are naturally derived, the isoform characterization is a concern when evaluating its bioactivity. Perhaps if these tests can be validated, they could be proposed in conjunction with an isoform profile assay.

4.1.3 Synthetically-derived

This category consists of two types of synthetically-derived hormone products. The first, is oxytocin, which existed on the market as both naturally-derived and synthetically derived. Currently, almost all of the oxytocin on the market is synthetically derived. Synthetic hormone products are a homologous preparation of hormone, therefore, there is less consideration given to the isoform profile. Potency tests can be regimented to strictly measure activity. The USP has proposed the deletion of the chick assay potency test for **oxytocin**, and replacement by an HPLC method. Since physicochemical tests do not completely evaluate hormone activity, a rat uterine contraction test, currently indicated in the EP (BP) has been recommended for use in conjunction with the HPLC method.

The second hormone is a synthesized derivation of a natural hormone. Somatropin is produced by microbial synthesis utilizing recombinant DNA technology. Presently, there is no official monograph for **somatropin** in the major pharmacopeias, international harmonization may be achieved in the drafting of this monograph. The proposed test is a bioassay utilizing hypophysectomized rats which measures weight variance after administration of somatropin. A strain of rats has occurred whose normal intrinsic GH levels are 10% of normal rat values. Perhaps these animals could be utilized instead of hypophysectomized rats, to reduce the pain and suffering of the test animals.

An *in vitro* assay was published in the literature which utilizes Nb rat lymphoma cell growth as a measurement of lactogenic hormone potency. Although not validated, this cell culture combined with a physicochemical test has potential as an alternative to the proposed bioassay. A more recent development is the USP proposal for a receptor binding assay for Somatrem that is being also reviewed for application to Somatropin.

4.2 General Safety Tests

USP has asked industry to provide information on the production and manufacturing of their antibiotics, with regard to the potential

existence of histamine or other biogenic amines in the crude product. USP will decide on a product by product basis, whether the depressor test should be deleted from those products which show no potential for the existence of bioamines. Recently an HPLC method has been described in the literature which simultaneously measures 9 bioamines including histamine and cadaverine. This test utilizes a gradient elution system, and can be utilized on fairly crude preparations. Although not presently validated against the cat depressor test, it could become a potential alternative test for those products which require a depressor test.

USP's goal is to replace all **rabbit pyrogen tests** with the Bacterial Endotoxin Test (BET) provided that the BET can be validated for specific monographs. The USP Subcommittee has also indicated that if the USP-BET test cannot be validated it will consider the use of other LAL tests before retaining the rabbit pyrogen test. The pyrogen test has been deleted from over 400 monographs, and replaced with the BET test already. Thirty-two additional monographs are being reviewed for potential deletion of the pyrogen test.

The **eye irritation test** is controversial because it is associated in the public with the Draize test, although it is only the testing of an extract of a plastic without having to subject the rabbit-eye to a titration of the level at which irritation of the eye will occur. Numerous *in vitro* systems have been developed to replace the eye irritation test, but none have been validated to date. The HET-CAM assay is in the process of being validated against the Draize test, and appears to be a potential alternative to the eye irritation test. The USP Subcommitte has proposed the deletion of the eye irritation test since available data has shown that for extract of plastics the Biological Reactivity Tests - In Vitro are satisfactory substitutes.

The Subcommittee will be proposing the deletion of the **mouse safety test** for medical devices and other plastic products, but will maintain the requirement for biologicals. USP felt the test was becoming obsolete, based on a survey conducted by USP which solicited safety test results data from industry.

4.3 Recommendations

The establishment of alternative tests in the pharmacopeia has been a collaborative effort. The continued success of this project is dependent upon suggestions and comments of the public sector as well as upon the expertise of the relevant Subcommittees. Open communication between USP and the public is maintained through the PF, specifically the Stimuli to the Revision section. In this section, reports of authoritative groups, commentaries, scientific articles on relevant topics, and policy statements by the USP Committee of Revision are published to spark discussion on current topics facing USP and industry. In the Nov-Dec 1992 PF, an article was written by Dr. Roger Dabbah in that section, on the topic of alternative testing, which summarized the success story of USP's alternative testing project. It is hoped that the article will stimulate creative comments and suggestions of alternative tests for some of the remaining monographs. The purpose of this article is to focus on some of the remaining monographs for which alternative tests have not been officially recommended.

For hormone potency tests, *in vitro* tests have been developed to potentially replace the *in vivo* compendial tests and validation for some of these tests have been completed. Potency tests evaluate the biological activity of hormones. Traditionally this was accomplished with *in vivo* assays. Finding *in vitro* assays which can measure bioactivity has been a difficult task. A logical progression to establish an *in vitro* protocol for hormone potency is to first define hormone bioactivity, and then develop a systematic means of measuring it. Tissue cultures which can measure cellular response to hormone stimulation, or receptor site binding tests combined with HPLC methods to quantitate the hormone offer potential as a replacement protocol for *in vivo* potency testing.

The bioactivity of naturally derived and synthetically derived hormone products require different testing procedures, since each possesses unique characteristics which affect its bioactivity. Its validation of *in vitro* methods are completed, proposals of specific alternative tests for present compendial hormone potency tests will be forthcoming.

The contribution of general safety tests to establishing the safety of products for patients has recently been reevaluated. Stricter regulations

on manufacturing, and emphasis on quality control monitoring in the industry has made some of the safety testing unnecessary. The mouse safety test is an example of a test which no longer contributed to further assurance of end product safety, and so was deleted from compendial testing for antibiotics. The depressor test will also be deleted by USP if the pharmaceutical industry can provide data which proves there is no potential of bioamines being present in the product.

The eye irritation test remains an official test, but in a recent PF it was proposed to be deleted for plastics for ophthalmic drug containers. There are numerous *in vitro* tests already developed which have potential as replacement tests for the Draize test. The majority of research should focus on validating those *in vitro* tests which appear to have strong correlation to the rabbit test to replace the Draize test.

The pyrogen test has successfully been replaced by an alternative test. The BET test utilizes limulus lysate to measure the level of endotoxins in pharmaceutical products. Review of the remaining monographs containing the *in vivo* pyrogen test will continue.

CHAPTER 5 CONCLUSION

5.1 New Areas of Interest

As technology and scientific information progress, so do the potential areas which USP will be involved. Industry relies on USP to provide them with compendial tests to comply with federal regulations. For each new drug development in pharmaceutics, federal regulations and guidelines must be developed, and standards for testing must be established. Thus industry and government request USP to develop compendial tests and standards to define compliance.

5.1.1 Dermal Irritancy

The area of dermal irritancy has been proposed as an area for USP involvement. For pharmaceutical companies, topical dosage forms and dermal delivery systems have become a large market. Thus, industry and government are interested in establishing compendial tests for dermal irritancy. This area is being considered by USP, but no formal address of the topic has been published.

Limited, but significant insight into dermal irritancy testing can be obtained by examing eye irritancy testing. The eye is different in composition to the skin, but both contain a network of multiple cell types which form the respective systems. Both are testing "irritancy", a complex response of tissue to a foreign substance. Thus, *in vitro* systems such as endpoint assays, which have been successful in testing eye irritancy could perhaps be useful for testing of dermal irritancy.

The animal model most commonly used for dermal irritancy testing is the skin of the rabbit. The irritation response is based on a grading system. This test has been used by the cosmetic industry worldwide for decades to evaluate their products. *In vitro* tests have been developed to replace the rabbit test, and are currently being used by the cosmetic industry.

Several *in vitro* systems exist for evaluating dermal irritancy. Generally speaking, there are three potential models for the dermis: skin explants, cultured skin cells, and living skin equivalents. A skin explant test which utilizes the tissue from hairless mice has been described in the literature (41). Dermal irritancy is determined by measuring the release of intracellular enzymes associated with irritancy into the culture media. These type of *in vitro* tests showed potential as screening tests prior to *in vivo* testing. Perhaps, they could be proposed as a component of a two tier system, which utilizes a reduced number of rabbits for the *in vivo* testing tier.

Skin cell cultures of both human and animal origin have been developed for evaluating dermal irritancy. It has been reported that the skin permeability properties of rodents and humans are different (42). Thus, human skin cell cultures are theoretically more predictive of human response than animal skin cell cultures. The ethics of obtaining human tissue for research is controversial. Potential sources include, volunteers, patients undergoing operations, recently deceased persons and fetal tissue (43).

A cell culture derived from neonatal foreskin, and a co-culture of cells from neonatal foreskin fibroblasts grown on layers of epidermal keritinocytes have been reported in the literature (44) (45). These *in vitro* tests evaluate skin irritancy by measuring cell viability as a function of neutral red dye uptake. A correlation was shown between the dose-

response characteristics for the two tests, and human patch test scores for the surfactants tested. These cultures may potentially be useful for *in vitro* testing of dermal irritancy.

Living skin equivalent (LSE) model is one of the most advanced technologies in the generation of tissue *in vitro*. LSE is an organotypic model consisting of a collagen lattice nourished by dermal fibroblasts overlaid with a fully formed epidermis (46). This model has been modified for *in vitro* testing (LSE-T), but the methodology and data on this system have not been published.

Overall, several types of *in vitro* tests have been developed which may be useful for evaluating dermal irritancy. Incorporation of alternative testing in the field of dermal irritancy appears to be promising. A collaboration between USP and industry will be necessary to establish compendial testing if USP decided to delve into this field.

5.1.2 Immunotoxicity

Toxicity in the immune system may result either from an impairment of one or more aspects of immune function (classical immunotoxicity), or from the stimulation of tissue-damaging immune responses by drugs and chemicals (allergy) (47). Currently, the USP has no tests for evaluating the immunotoxicity of pharmaceutical products. It is recommended that USP establish compendial tests and standards for allergenicity testing.

Classical immunotoxicity testing is commonly carried out during initial phases of product development. The common animal model for this testing is the rat. The animal is injected with the test preparation, and immune system activity is monitored by measuring DTH, NK cell cytotoxicity, IL-2 production, and PGE2 production by macrophage. A more concise understanding of the immune system's response to materials has led to the focus on allergy reactions which result in tissue damage.

Presently there are two basic types of allergic reactions stimulated by chemicals and drugs; contact sensitivity and respiratory allergy. Animal models for testing contact sensitivity include the guinea pig and the mouse. Presently, no *in vitro* methods have been advanced for the identification of skin allergens (47).

There are no validated or widely applied *in vivo* or *in vitro* tests for measuring respiratory allergens (47). Metachromatic cells, located in the bronchoalveolar system, when directly exposed to inhaled allergens *in vivo*, and are believed to trigger cells during acute asthma (48). These cells have been shown to release histamine when activated (48). An *in vitro* system which measures the histamine release from cultured metachromatic cells stimulated by allergens, could possibly be used to evaluate respiratory allergy. As understanding of the biological basis for this allergy response increases, so will the potential tests for measuring the allergy.

Immunotoxicity, specifically allergy, testing poses a unique challenge to USP, due to the newness of the field. Presently, there appears to be no defined referee tests for either contact sensitivity or respiratory allergy. Therefore, USP would depend on suggestions and proposals of the public sector to form a strategy to establish standards and compendial tests for allergenicity. Since few tests are available to evaluate these immune responses, the possibility of non-animal testing being implemeted initially is reduced. As these tests are developed, USP should evaluate them for potential compendial tests.

5.2 General Conclusions

In the late 1980's, USP had decided that animal testing needed to be reevaluated, and alternative methods of testing needed to be explored. So, in 1988 an Open Conference was organized which brought together government, industry, and academic experts in relevant fields to discuss the issues involved with seeking alternative testing. the Conference provided an opportunity for US to obtain information, data, and direction for proceeding with a program to seek alternatives to animal testing in compedial tests. From this conference, appropriately entitled: USP Open Conference on Alternative Methods for Toxicity Testing - The In Vitro Option, several recommendations were made which summarized the opinions of the participants. The Conference also stimulated the 1990 USP Convention resolution to a committed search of alternative testing methods.

Replacement of *in vivo* tests with *in vitro* and physicochemical tests has many advantages. Generally, animal tests are less sensitive

than comparable *in vitro* or physicochemical tests. Also, alternatives to *in vivo* tests are usually less costly to run, and lend themselves well to multiple testing. Thirdly, animal responses may vary considerably due to genetic and handling variances, while *in vitro* and physicochemical tests tend to be more precise. Most importantly, animal welfare is a social concern, and seeking alternative tests is a morally desirable decision.

Alternative tests for biologicals and biologically-derived products have been successfully implemented or proposed for numerous monographs. Several more animal tests are being reviewed for potential replacement with *in vitro* tests. It has barely been three years since the inception of the 1990 Convention resolution, and less than 2% of the total number of monographs still contain animal tests. The impact on animal use in the pharmaceutical industry should be astounding.

USP's commitment to seeking alternative testing has been a difficult but rewarding task. The results of implementing an active program to seek alternative testing are presented in Table I.

These accomplishments have occurred by cautious analysis of information solicited from industry, academic and expert communities. USP's commitment to reducing, refining, and replacing animals in compendial tests is as exemplary as its commitment to ensuring the safety and quality of products for patients.

5.3 USP - The Future

The USP program for alternatives to animal testing is a continuous program that does not end when the USP XXIII is published in 1995. The USP Committee of Revision is totally and unambiguously commited to the resolution of the 1990 Convention to implement alternative testing "... as rapidly as science and technology permit...". This objective of the various relevant Subcommittees will be facilitated as follows:

1. Every new proposed monograph with animal testing will be examined for alternative to animal testing in a systematic fashion. Alternatives will be identified for potency, purity, identity, or quality testing and validation packages will be requested from industry.

Table 1: Summary of the USP to seek alternative testing for biologicals and biologically derived products. The 1985 USP-NF XXI is used as a baseline for comparison. The values are presented as number of monographs. (Courtesy of USP).

Progress Report
Alternatives to Animal Testing

Animal	USP XXI	USP XXII	Projected USP XXIII
Rabbit (Pyrogen)	222	37	15
Mouse	136 (14 vaccines	24	14
Rat	16	16	13
Rabbit (non-pyrogen)	15 (2 vaccines)	15[2]	6
Guinea Pig	6 (4 vaccines)	5	5
Chicken	3	3[3]	0
Monkey	2 (2 vaccines)	2	2
Cat	1	1[4]	0
Dog	1	0	0
Pigeon	1	1	1
Hamster	1 (1 vaccine)	1	1
Total	404	105[1]	67[1]
% of Monogrpahs in USP	11.2%	2.8%	1.6%
% Reduction over USP XXI		64%	77%

1 A total of 23 monographs in USP XXI, USP XXII, and USP XXIII have a CFR requirement for animal testing (vaccines).

2 Drastic reduction of rabbit in an Insulin monographs proposed in PF.

3 Deletion of animal tests proposed in PF for 3 monographs.

4 Deletion of animal test proposed in PF.

2. Worldwide developments in alternatives to animal testing will be monitored continuously to identify potential tests that could have compendial testing applications.
3. Make alternatives to animal testing one of the considerations in the worldwide harmonization of compendial testing by exchanging information with the major pharmacopeias of the world.
4. Participation in national and international symposia, meetings and congresses to promote the use of alternatives to animal testing and to report on the progress of the USP program.
5. Use a pro-active approach in the identification of alternatives to animal testing in pre-clinicals for NDA and licensing of drugs in terms of Safety and Efficacy Testing (PF, March-April 1993).

The few remaining monographs containing animal compendial tests in the 1995 USP-NF publication are projected to consist generally of vaccine monographs. Tests for vaccines are mandated by the federal government to be done on animals. To change these compendial tests would require a change in the Code of Federal Regulations. USP will be addressing this topic after alternative tests have been sought for all other compendial tests. Alternative testing for vaccines will be more complicated than previous changes have been due to the need for FDA approval of vaccine tests which do not utilize animals.

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