GENETIC VARIATION IN THE RESPONSE OF MICE TO XENOBIOTICS, *IN VITRO*

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ABSTRACT

Adverse reactions to drugs and environmental chemicals are a serious problem with up to 30% of hospital patients experiencing such problems (Venning, 1983; Ludwig and Axelsen, 1983). There is evidence that many adverse reactions arise as a result of genetically controlled sensitivity (Festing, 1987). Large genetically determined differences in response to chemicals have also been recorded in laboratory animals. However, most toxicological screening involves a single strain and fails to detect genetically determined sensitivity. Should some animals show an adverse reaction, this is usually attributed to "biological variation". As the pedigree of such animals is not normally known at the time of use there is no way of showing whether these adverse reactions were inherited (Festing, 1975,1979).

The initial aim of this project was to develop a technique for studying genetic variation in sensitivity to treatment with drugs using *in vitro* screening methods. The techniques should not require hazardous or expensive chemicals and equipment, should require a small number of animals and should be reliable and easy to perform. Several end-points were studied, and a protocol for detecting genetic differences which included four end-points and two cell types was developed.

In the second part of the project, the aim was to study genetic variation in sensitivity to Aspirin, Ethanol and Coumarin as model compounds, using the previously developed techniques in conjunction with suitable genetically-defined strains of mice.

Two cell types (macrophages and hepatocytes) were studied and several end-points were used including neutral red uptake, total protein concentration, rate of phagocytosis and LDH activity in cells and supernatant. The study involved nine strains of mice. Although statistically significant differences among inbred strains were detected, in no experiment did strain distribution pattern suggest single-locus Mendelian control. There was no evidence that response to coumarin depended on the coumarin hydroxylase (*Cyp2b*) locus nor that response to alcohol depended on the alcohol dehydrogenase locus. It is concluded that further development would be necessary to develop these methods as a way of identifying genes associated with their type of genetic variation.

GENERAL INDEX

CHAPTER I.- INTRODUCTION

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INTRODUCTION

Adverse reactions to drugs and environmental chemicals are an increasingly serious problem. Venning (1983) listed a total of 1717 fatal reports from the clinical use of various drugs in the U.K. between 1964 and 1980. Ludwig and Axelsen (1983) listed 150 drugs in clinical use which are known to have a toxic effect on the liver. Some adverse effects undoubtedly occur among people who are susceptible as a result of age, other interacting drugs, or pathological conditions. However, there is increasing evidence that many adverse reactions arise as a result of genetically controlled sensitivity (see review by Festing, 1987). Several authors have studied genetic variation in response to xenobiotics in humans: Kalow (1984), Nebert (1980), Evans (1980) and Clark (1985). Vesell and Penno (1983) have also shown that the response to many xenobiotics is strongly inherited in their studies of twins and other relatives. This genetic control is coded both by Mendelian loci and polygenic systems, and more than 20 such Mendelian loci have been identified in humans.

Large genetic differences in response to chemicals have also been recorded in laboratory animals, although fewer Mendelian loci have been isolated. For example, Mendelian loci have been described that control coumarin hydroxylase activity (Wood and Taylor, 1979), warfarin resistance (Wallace and

MacSwiney, 1976) and a cytochrome P-450-dependent enzyme which is induced by pregnenolone-16α-carbonitrile (Simmons et al., 1985). There have also been extensive studies of loci controlling P-450 enzymes, although the formal genetics of some of these has not yet been studied in detail (Festing and Eydmann, 1987a,b). However, most toxicological screening uses a single inbred strain or outbred stock and fails to detect genetic variation in sensitivity. If some animals show an adverse reaction, this is usually attributed to "biological variation". The genotype of such animals is not usually known at the time of use so there is no way of showing whether these adverse reactions are inherited (Festing, 1975, 1979, 1980, 1987). This may account for the fact that fewer Mendelian loci have been identified in laboratory animals than in humans, even though it is probably much easier to study the genetics of an adverse reaction in laboratory animals.

A lot of effort is currently being put into improving toxicological screening tests. However, while there is an increasing public demand for stricter controls of new pharmaceuticals, there is at the same time an increasing social opposition to the massive use of laboratory animals to assess the risks (Freshney, 1987; Castell and Gomez-Lechón, 1987). Thus, there is pressure to perform at least part of toxicity testing *in vitro*.

In pure research it is also desirable to reduce the numbers of animals used. In this case one advantage of *in vitro* methods is that they should provide simplified systems in which there is a greater chance of isolating single genes,

provided experiments are designed to detect genetic differences, should they occur (Festing and Eydmann, 1987a,b).

Several authors have developed simple and accurate cell culture tests for assessing toxicity *in vitro*. Typically, cells either from a continuous cell line, or from a primary culture are incubated with the compound, with the effects being quantified in a number of ways. Apart from the classical methods to assess viability (i.e. dye exclusion, fluorescent staining and enzyme leakage) a wide number of new assessment methods have been developed. These include crystal violet staining (Saotome et al., 1989), neutral red uptake (Parish and Müllbacher, 1983; Borenfreund and Puerner, 1985; Babich and Borenfreund, 1987; Fiennes et al, 1987; Borenfreund et al, 1988), nitro blue tetrazolium reduction (Segal, 1974; Rook et al., 1985), total protein determination (Shopsis and Eng, 1985), MTT uptake and conversion to formazan (Mossmann, 1983; Holt et al., 1987), methylene blue staining (Olsson et al., 1982; Oliver et al., 1989) and kenacid blue binding (Knox et al., 1986). The methods vary in their sensitivity, ease of use and the characteristic being measured. For example some, such as total protein, measure total biomass but do not distinguish between live and dead cells, while others (e.g. LDH in cells and supernatant) measure the health of the cell in terms of membrane integrity.

If *in vitro* studies are carried out on primary cell cultures from several genotypically uniform inbred strains of animals, strain differences in response would show whether the

response to the test drug was under genetic control. In *in vivo* screening the practicability of using a battery of inbred strains has been suggested in several studies (Heston, 1968; Kalter, 1965; Festing, 1975, 1979, 1980, 1987) and some simple tests have been developed for the study of genetic variation in response to xenobiotics *in vitro* (Festing and Eydmann, 1987b). In these studies, however, only one or two assessment methods were used. As pointed out by several authors (Knox et al., 1981; Castell and Gomez-Lechón, 1987; Brockman and Demarini, 1988; Saotome et al., 1989) a single *in vitro* test shows only one aspect of the action of chemicals, and no single test is able to mimic the complexities of the *in vivo* system. A battery of end-points measuring different parameters might be more appropriate for a general screening test, while individual end-points may be most useful for studying patterns of response among different strains with the aim of identifying Mendelian loci.

AIMS OF THE PROJECT

The initial aim of the project was to develop a technique for studying genetic variation in sensitivity to treatment with drugs using *in vitro* screening methods. The techniques should not require hazardous or expensive chemicals and equipment, should require a small number of animals and

should be reliable and easy to perform. Several end-points were studied, and a protocol for detecting genetic differences which included four end-points and two cell types (see materials and methods) was developed.

In the second part of the project, the aim was to study genetic variation in response to three model compounds (Aspirin, Ethanol and Coumarin), using the previously developed techniques in conjunction with suitable genetically-defined inbred strains of mice.

CHAPTER II.- INBRED STRAINS

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DEFINITION OF INBREEDING

As the use of inbred strains of mice is fundamental to these studies, it seems appropriate to review their characteristics.

Inbreeding is the mating of individuals which are related to one another by having one or more common ancestors (Festing, 1979). This may be quantified in terms of the coefficient of inbreeding F, which is the probability that the two genes at any locus are identical by descent (Falconer, 1981).

An inbred strain is one derived from a single breeding pair in the 20th or a subsequent generation of brother x sister mating or its genetic equivalent (Festing, 1975, 1979). At this time the coefficient of inbreeding, F, is at least 98.6%. However, total inbreeding is approached asymptotically, and never quite reaches 100%. In describing the characteristics of these strains, it is assumed that for all practical purposes they are fully inbred.

The recognition of the need for highly inbred mice to study the genetic factors in the development of cancer was recognized independently by several investigators, including Clarence Little, Leo Lob, Leonell Strong and Jacob Furth (Morse, 1981).

The first inbred mouse strain, DBA, was developed by

Little in 1909 and many of the most widely used mouse and rat strains were developed between 1920-1930 (Festing, 1979). At that time, L.C.Strong began the inbreeding of strains that have contributed, directly or indirectly, to the current strains A, CBA, C3H, STR, SEC and others. Simultaneously, C.C. Little began inbreeding the progenitors of strains now known as C57BL/6, C57BL/10, C57BR, C57L, and YBR, while E.C. MacDowell began the development of strains C58 and BALB/c.

Production of congenic strains was pioneered by George Snell in the 1940s as a means of studying the individual effects of the many loci involved in tissue transplantation. A number of tumours will only grow in the mouse strain in which they originated. Snell backrossed genes associated with resistance to such tumours from a "donor" strain to an inbred strain (usually C57BL/10). The resulting "congenic resistant" strains have been widely used in transplantation biology, and many congenic strains differing at non-transplantation loci have also been developed. Recombinant inbred strains are derived by systematic inbreeding from a cross of two pre-existing progenitor strains. They were devised for linkage analysis by Bailey (1971, 1981). B.A.Taylor foresaw the special advantages that recombinant strains hold for segregation analysis and gene mapping (Taylor, 1980,1989), and in 1969 commenced construction of several large sets of recombinant inbred strains with different pairs of progenitor strains.

CHARACTERISTICS OF INBRED STRAINS

Inbred strains and their derivatives have a number of properties that make them valuable in many types of research including toxicity testing, cancer research and genetic analyses. The value of such strains has been appreciated by geneticists for many years (Festing, 1975, 1979; Staats, 1981; Russell, 1985). There are several characteristics which are common to all inbred strains. These include:

Homozygosity.- As noted above, after 20 generations of brother x sister mating the coefficient of inbreeding (i.e. the probability that the two alleles at a locus are identical by descent) is at least 98.6%, so there is little heterozygosity left. This assumes that heterozygosity gives no selective advantage, which would delay the approach to complete homozygosity to some extent. However, there is always a small degree of 'residual heterozygosity' caused by the failure of the original inbreeding to reach 100% and as result of mutation.

Isogenicity.- All members of an inbred strain should trace back to a single ancestral pair. That pair will be homozygous at virtually all loci, and all progeny that they produce should be genetically identical or isogenic. In effect, an inbred strain almost represents an immortal clone of genetically identical individuals.

Long-term stability.- As a result of their homozygosity and isogenicity, there should be no genetic

segregation within an inbred strain so genetic change due to selection and/or random genetic drift should not occur. As a result, the strain should remain stable for many years.

Phenotypic uniformity.- Due to their isogenicity, inbred strains show a relatively uniform phenotype.

Individuality.- Each inbred strain has its unique genotype formed by a particular combination of alleles at each locus, and thus should have a characteristic phenotype for many different characteristics.

USE OF INBRED STRAINS IN RESEARCH AND TOXICOLOGICAL SCREENING.

Many factors can influence the susceptibility of an animal to a manufactured chemical (or drug). In toxicological screening some of these, such as route of drug administration, age weight, and health status of animals, are usually controlled at one "realistic" level. Within each species, the genotype of the individual animal may strongly influence its susceptibility to the drug. There is no general agreement on how this particular variable should be taken into account.

The principles of experimental design dictate that batches of animals used in toxicological screening should be as uniform as possible, in order to avoid experimental "noise", which reduces precision.

Most toxicological screening experiments use genetically variable outbred stocks. The use of such heterogeneous or 'noisy' experimental material reduces the precision of an experiment, leading in the long-run to an

increase in both false positive and false negative results. Some authors argue that humans are random bred, so in order to model humans realistically, or to have a better chance of finding adverse reactions, an outbred stock of animals should be used. However, a single outbred stock may not contain a wide range of phenotypes. The phenotypic variation within a single outbred stock is considerably smaller than the variation between different strains (Falconer, 1981).

An alternative strategy suggested by Heston (1968) and Kalter (1965) and explored in detail by Festing (1975, 1979, 1980) is to carry out the animal tests on a sample of 5 to 10 inbred strains of rats or mice. This should give a better chance of detecting an adverse effect and a much broader basis for extrapolating to man than the use of a single stock.

The use of several strains does not imply the use of more animals. A factorial experimental design involving several different strains can be used to increase the range of phenotypes which are tested in order to avoid the problem of strain-specific drug resistance. This factorial experiment needs to be no larger than the classical design. Fisher (1960) emphasized that factorial experiments have three main advantages over single-factor experiments: greater efficiency, greater comprehensiveness and the conclusions reached as a result of the experiment have a wider inductive basis than would be the case with a single factor experiment.

The advantages of using several inbred strains have been discussed by several authors. Festing (1979) and Haseman

and Hoel (1979) have shown that the use of a multistrain experiment need not increase the total size of the experiment. However, in the *in vitro* experiments reported here the main aim was to attempt to develop methods for studying genetic variation in response to drugs using humane methods which did not involve whole animals. For general *in vitro* screening, strain differences may not be of any practical importance as cell lines of many types and from many species are readily available.

CHAPTER III.- *IN VITRO* STUDIES

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INTRODUCTION

Procedures for estimating safety of drugs are based on exposing test animals or other organisms to a range of doses of the chemical being tested for an adequate period of time and then examining the health status of the treated organisms (Paganuzzi et al., 1981). Current legislation demands that new drugs, cosmetics, food additives, and agricultural chemicals, go through extensive toxicity testing before they are released. However, while there is an increasing public demand for stricter control of new pharmaceuticals there is also opposition to massive use of laboratory animals to evaluate the risks. Animal experiments are also expensive. There is, therefore, much pressure, both emotional and economic, to perform at least part of toxicity testing *in vitro*.

A range of models, including organ, tissue and cell cultures have been suggested as alternative methods for toxicity testing (Freshney, 1987 ; Castell and Gomez-Lechon, 1987; Knox et al., 1986). Most authors recommend a set of several different toxicity assays. As different assays measure different things, the use of a range of end-points increases the accuracy of the test.

DEFINITIONS

Tissue culture was first devised at the beginning of this century by Harrison and Carrel (Freshney, 1987) as a method for studying the behaviour of animal cells free of systemic variation that might arise in the animal both during normal homeostasis and under the stress of an experiment.

Various types of primary tissue cultures can be considered (Freshney,1987). In organ culture the whole or part of an organ is cultured and the architectural characteristic of the tissue *in vivo* is retained. In primary explant culture a fragment of tissue is placed at a glass (or plastic)/liquid interface where, following attachment, migration is promoted in the plane of the solid substrate. With cell culture the tissue is dispersed into a cell suspension.

Technical improvements made possible by the commercial supply of reliable media and sera, and by the greater control of contamination with antibiotics and clean air equipment, has made tissue culture accesible to a wide range of experiments (Freshney, 1987). A large number of permanent cell lines have been established, and these can be preserved by freezing, so that in many cases it is no longer necessary to use primary cultures. However, in these studies only primary cultures were used as the aim was to investigate the behaviour of cells of different genotypes when treated with test chemicals *in vitro*.

The number of animals needed for *in vitro* studies using primary cultures is minimal in comparison with *in vivo* studies. Depending on the cell type chosen and the assay

method, enough cells can often be obtained from each animal to test several dilutions of the test compound, possibly with many replications.

In vitro systems may also make it possible to study mechanisms of toxic action under controlled conditions (Paganuzzi et al., 1981; Castell and Gomez-Lechón, 1987). A more accurate and early observation of cytopathological effects may be observed that may lead to an easier identification of the inital cell damage.

The physiochemical environment (pH, temperature, osmotic pressure, O_2, CO_2 tension) may be controlled very precisely, and the physiological conditions may be kept relatively constant (but cannot always be defined) (Freshney, 1987).

Another advantage of *in vitro* over *in vivo* studies is that metabolic investigations using animal and human cells may help in comparing metabolic similarities and differences between man and laboratory animals, thereby providing a sound basis for the selection of species to be used in long-term testing (Paganuzzi et al., 1981). Screening tests with many variables and replicates are cheaper using tissue culture methods, and the legal, moral, and ethical questions of animal experimentation are avoided (Freshney, 1987; Festing, 1975, 1979, 1985; Knox et al., 1986; Paganuzzi et al, 1981).

A major limitation is that the measurement of toxicity *in vitro* is a purely cellular event as presently carried out (Freshney, 1987). A toxic response *in vitro* may be measured by changes in cell survival or metabolism, while the

major problem *in vivo* may involve a much more complex process associated with absorption, metabolism and excretion of the chemical. There may be no *in vitro* equivalent of some *in vivo* responses such as inflammation. Also some enzyme activities in cell cultures may be low in comparison to their activity *in vivo* (Paganuzzi et al., 1981).

Nevertheless, in many investigations the advantages of using *in vitro* methods surpass by far the disadvantages. For this reason and the fact that most toxicological screening is carried out *in vivo*, the development of *in vitro* methods that would serve for both toxicological screening and the detection of genetic variation in sensitivity was attempted.

SELECTION OF CELL TYPES

A number of cell types are capable of surviving and dividing *in vitro*. The most frequently used cell system appears to be primary cultures of fibroblastic lines of mammalian and other species, though a wide range of cell types can now be cultured. These monolayer cell cultures exhibit a morphology that is somewhat related to tissue architecture and allow cell migration, adhesion and contact regulation to be studied.

In recent years a number of papers have been published dealing with investigations on cells which are the target of the compound under investigation (Paganuzzi et al., 1981). Alveolar macrophages and other lung derived cells as well as other cells from reticuloendothelial system and blood

have been used to investigate the toxic effects of smoke components, mineral dusts, environmental contaminants and drugs. Hepatocytes have been used to investigate drugs, inorganic chemicals, solvent pesticides and other chemical action mechanisms whereas kidney cells have found application in the study of nephrotoxic heavy metals. Nervous system cells have found application in the study of neurotoxic environmental chemicals such as psychotrophic drugs. Heart and other muscle cells have been used in the study of the toxic potential of several compounds including caffein, diasepam, quinidine, propanol, butylated hydroxytoluene and butylated hydroxyanisole.

Two cell types, macrophages and hepatocytes were considered for these studies. As different toxic chemical compounds may affect different cell types, a method that included studies on, at least, two cell types should be more informative and reliable.

The use of isolated hepatocytes has become increasingly attractive in recent years as investigators have developed satisfactory cultures systems (McMahon, 1980; Paganuzzi et al, 1981; Guillouzo, 1986; Castell and Gomez-Lechón, 1987; McQueen and Williams, 1987). Isolated hepatocytes may express many of the functional activities of the intact liver, which is the primary organ involved in metabolism of xenobiotics. Therefore, hepatocytes are suitable for investigating xenobiotic metabolism, cytotoxicity and the effects of drugs on cell metabolism.

Macrophages are indispensable components of certain

defence mechanisms of the host. They are a good tool for the study of host-parasite interactions involving cellular immunity (Stuart et al, 1979; Wagner, 1980). As many adverse reactions to drugs may have an immunological component, macrophages seem an appropiate cell type to be used in toxicity studies. In several cases, macrophages from the peritoneal cavity and alveolar macrophages of mice and other species have been used for such studies (Wagner, 1980).

SELECTION OF END POINTS

One of the most widely used methods for assessing *in vitro* toxicity of chemical substances is noting their effect on cell viability (Paganuzzi et al. 1981; Freshney, 1987; Guillouzo, 1986; Tyson and Green, 1987). Viability can be evaluated by using a dye exclusion test (trypan blue, erythrosin, nigrosin or fluorescent compounds), by determining the leakage of soluble enzymes (lactic dehydrogenase (LDH) and glutamate oxalate transaminase (GOT)) or previously absorbed radioactive substances (e.g. ^{3}H.thymidine incorporation) (Paganuzzi et al., 1981).

However, according to several authors (Knox et al., 1986; Castell and Gomez-Lechón, 1987; Tyson and Green, 1987), assays of cell viability, membrane integrity or even specific metabolic effects can give misleading results when carried out on their own. Viability assays certainly represent one possible approach to a general screening protocol, but may

leave out of consideration substances that impair cell function without causing cell death (Castell and Gomez-Lechón, 1987). Such an effect, although not critical for the cell itself, is of toxicological significance, since the general homeostasis of the organism can be indirectly altered as a consequence of cell malfunction.

Some attempts have been made to develop general screening protocols that would accurately measure cell damage. Protocols including a general screening test measuring cell proliferation (Knox et al., 1986) or a battery of different assays measuring viability as well as metabolic parameters (Gomez-Lechón, 1987; Tyson and Green, 1987) have been developed.

In these studies, several assays of viability, as well as functional and metabolic measurement assays were tested to select the most accurate and reliable methods for detecting strain differences in a response (see comparison of colourimetric methods).

CHAPTER IV.- MATERIALS AND METHODS

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ANIMALS USED

The inbred strains of mice used in the experiments were chosen on the basis of their availability and genetic characteristics, as specified in each case. Some of the strains were bred on site (MRC toxicology Unit, Carshalton), and others were purchased from the National Institute for Medical Research (Mill Hill), or Harlan-Olac Ltd.(Shaws farm, Blackthorn, Bicester, Oxon). The number, sex, age, and supplier of the animals is specified for each experiment.

The origin and main characteristics of strains used in the experiments are as follows (abstracted from Festing, 1987, 1989; and Nomura et al., 1984)

A/J

Strain A is albino (coat color genes a,b,c) and was developed by Strong in 1921 from a cross of Cold Spring Harbor albino and Bagg albino. The J substrain was maintained at the Jackson Laboratory. It is well known mainly for its susceptibility to chemical induction of lung adenomas, and sensitivity to teratogens. Some macrophage defects in A/J mice have been reported. Vogel et al., (1981) found that macrophages of A/J strain were not activated by lymphokines to kill tumor cells *in vitro*. Also the proportion of macrophages found following peritoneal lavage were lower than in other strains (Festing et al., 1990).

AKR

Albino (a, B, c). Stock originated from a dealer named Detwiler in Norristown PA. Inbred by Furth as a high-leukeamia strain from 1928 to 1936, then random bred at the Rockefeller Institute for several generations. Brother x sister mating resumed by Mrs. Rhoades to F9, then C.Lynch to F21. This strain is best known for its high incidence of lymphatic Leukaemia, and for the Thy-1^a T-cell antigen, which is only present in this and a few other strains.

BALB/c

Albino (A,b,c). The strain was inbred as "Bagg albino" (hence BALB) mice by MacDowell in 1913, to Snell in 1932 (who added the /c). Now widely distributed and among the top two or three most widely used inbred strains. The strain is particularly well known for the production of plasmacytomas following intra-peritoneal injection with mineral oil. These tumours form the basis for the production of monoclonal antibodies.

BGA

Black (a). This strain was developed by backcrossing a *Gpi-1* allele from strain 129 to C57BL/6Ola by West (Edinburgh) in 1986. The *Coh*^{h} allele from strain 129, which is closely linked to *Gpi-1* has also been transferred to the C57BL/6Ola genetic background (Eydmann et al., 1991)

BXD-2

Black (a, B, D). Inbr 30+. One of a set of 24 recombinant inbred strains developed by B.A. Taylor from a cross between C57BL/6J and DBA/2J. None of the other recombinant inbred strains of this set were available for this study.

CBA/Ca

An agouti (+) strain developed by Strong in 1920 from a cross of a Bagg albino female and a DBA male. Strain CBA was selected for low mammary tumour incidence, and C3H for a high incidence. CBA is now widely distributed. Differences between some substrains are too great to be accounted for by mutation, and must be the result either of substantial residual heterozygosity, or genetic contamination. Three major branches were in existance in 1940, but these had been further subdivided by 1960. The CBA/Ca and CBA/N strains are believed to be closely related, but the latter carries the *xid* mutation (see below).

CBA/N

Agouti (+). A substrain of CBA originating from CBA/Ca (CBA/H) mice from Harwell heterozygous for the mutation *fm* (foam-cell reticulosis). A mutation *xid* (x-linked immuno deficiency) was discovered in non-*fm* stock. CBA/N mice fail to produce antibodies to certain antigens and show additional immunological disorders due to a B-lymphocyte defect (Scher, 1981).

C3H/He

Agouti (+). Strain C3H was developed by Strong in 1920 from a cross between the Bagg albino stock and DBA (see also CBA), with selection for a high incidence of mammary tumours. Now among the most widely used strains.

C57BL/6J

Black (a). This strain was developed by Little in 1921 from the mating of female 57 with male 52 from Miss Abbie Lathrop's stock. The same cross gave rise to strains C57L and C57BR. C57BL is now the most widely used of all inbred strains, though in many ways it seems to be atypical of inbred strains of laboratory mice. However, it has a good breeding performance, and has been used as the genetic background for a large number of congenic strains covering both polymorphic and mutant loci. Four major substrains A, GrFa, 6 and 10 appear to be quite similar, and any differences are consistent with what might be expected from the accumulation of new mutations and a small amount of residual heterozygosity. In contrast, the Ks substrain differs from the 6 and 10 substrains at four loci (see below). Substrains 6 and 10 were separated prior to 1937. Substrain 6 and 10 differ at the *H-9*, *Igh-2* and *Lv* loci.

C57BL/Ks

Black (a). This substrain differs from 6 and 10 substrains at the *Bgl-s*, *Bgl-t*, *cdm* and *H-2* loci, possibly as a result of genetic contamination during 1947-1948, when it

was random-bred. It resembles C57BL/6 at the *Lv* locus (at which C57BL/6 and C57BL/10 differ).

C57BL/10ScSn

Black (a). For the origin of this strain see C57BL/6.

DBA/2

Grey (a,b,d). This strain was developed by Little in 1909 from stock segregating for coat colour. It is the oldest of all inbred strains of mice. In 1929-30 crosses were made between substrains, and several new substrains established, including the widely used substrains /1 and /2. Differences between the substrains are probably too large to be accounted for by mutation, and are probably due to substantial residual heterozygosity following the crosses between substrains. The strain in well known for a high sensitivity to audiogenic seizures.

LP

White-bellied agouti with white patches (*Aw,s*). It was developed by Dunn in 1928 from a chinchilla stock from Castle and some coat colour stocks from English fanciers. To Scott, to Dickie 1947, to J 1949.

MF1

Albino (c, ?). This is an outbred stock derived from

a cross between an outbred stock designated LACA, and another stock of unknown origin.

RIII-*ro*

Albino (A,c). This strain was developed by Dobrovolskaia-Zavadskaia (Institute du Radium, Paris) in 1928. The *ro* (rough) mutation causing bent vibressae and a wet or greasy appearance to the coat occurred prior to 1952.

STS/A

Albino (c). This strain was developed by Muhlbock from mice from the Hygiene Institute Zurich in 1955.

The following F_1 hybrids were also used:

B10CF1

First generation hybrid from a cross between C57BL/10 females and BALB/c males.

CBCF1

First generation hybrids from a cross between CBA females and BALB/c males.

CBB10F1

First generation hybrids from a cross between CBA females and C57BL/10 males.

CELL CULTURE METHODS

Isolation and culture of macrophages

Peritoneal macrophages.- Peritoneal macrophages were collected from inbred mouse strains following the method described by Festing and Eydmann (1987b) with some modifications. Mice were killed by CO_2 inhalation. The skin was swabbed with 70% IMS in distilled water, cut and removed from the peritoneal wall. Three to seven ml of prewarmed (37°C) culture medium (Dulbecco's modified minimum essential medium, supplemented wiht 5% Foetal calf serum) were injected into the peritoneal cavity using a 5ml sterile plastic syringe and a sterile needle (0.6x25mm, Sabre, Berkshire). Mice were gently shaken for 1-2 minutes to increase the number of macrophages in suspension. One to four ml of the injected medium were recovered. Samples contaminated with erythrocytes were discarded.

The cell number was counted using a haemocytometer (improved Neubauer, depth 0-1mm, Gallemkamp) and each sample was diluted to $1x10^6$ cells/ml in culture medium and dispensed in 96-well microtitre tissue culture plates (Falcon, Becton Dickinson, Cowley) with an 8-channel micropipette (Titertek, Flow laboratories, Finland) at the rate of 100μl per well. 25 wells per mouse were used. After three hours of incubation at 37°C , 5% CO_2 in air in a humidified atmosphere, 100μl/well of fresh treatment-containing medium were added to the cells. Plates were placed back in the incubator until end-point assays were due. Peritoneal cells consist of a mixture of

macrophages and lymphocytes, with a small proportion of other cell types. The proportion of lymphocytes/macrophages varies between different mouse strains. The lymphocytes are non-adherent, so were washed off prior to determination of the end-point (Hunt, 1987; Festing et al., 1990).

Bone marrow macrophages.-These were obtained from femurs. The bone was removed from the leg with a sterile scalpel. Both ends were cut and cells were flushed from the femora with culture medium (DMEM + 5% FCS). Cells were counted with a haemocytometer and diluted to $1x10^6$ cells/ml in DMEM + 5% FCS. 100,000 cells/well were plated out in 96-well plates. After a period of incubation (see conduct of experiments), neutral red uptake and protein determination assays were performed as described.

Isolation and culture of Hepatocytes

Introduction

During recent years, the use of hepatocytes as a cell system for *in vitro* studies has steadily increased (McMahon, 1980; Guillouzo, 1986; McQueen and Williams , 1987). The liver is the primary organ involved in the metabolism of xenobiotics. This occurs according to various pathways which are classified into two groups: Phase I reactions which include oxidations, reductions and hydrolyses, and Phase II conjugation reactions in which the chemical is rendered more water soluble, so can more easily be excreted (Guillouzo, 1986; Castell and Gomez-Lechón, 1987). One major Phase I pathway is represented by the cytochrome P-450 dependent

mono-oxygenases located in membranes of the endoplasmic reticulum.

Isolated hepatocytes express most of the functional biochemical activities of the intact liver (phase I and phase II drug enzyme activities), and are therefore suitable for investigating xenobiotic metabolism, cytotoxicity and the effects of drugs on cellular metabolism. The rapid loss of cytochrome P-450 enzymes (from 40 to 80% of the initial activity in the first 24 hours) represent evident shortcomings. This explains why toxicological studies are usually performed on rodent hepatocyte cultures during the first two days following cell plating (Guillouzo, 1986; McQueen and Williams, 1987).

Isolated hepatocytes can be used in suspension or monolayer culture, the later having several advantages : the cells are better able to recover from alterations sustained during the isolation procedure and can be mantained for longer periods of time than suspensions (McQueen and Williams, 1987).

A major advance in the study of mature hepatocytes was the development of methods for dispersing the liver with proteolitic enzymes and harvesting intact viable cells. The introduction of collagenase as a liver-dispersing enzyme by Howard and collaborators (1967) greatly facilitated the preparation of intact cells, and when Berry and Friend (1969) introduced the use of physiological liver perfusion to make the tissue uniformly accessible to the action of collagenase and hyaluronidase, it became possible to prepare intact liver cells in high yield. Later Seglen (1973,1976) described

methods utilizing only collagenase.

Several reviews of the methods have been made since (McMahon, 1980; Guillouzo, 1986; Castell and Gomez-Lechón, 1987; McQueen and Williams, 1987; Strom et al. 1987), but preparation of hepatocytes by liver perfusion remains an art and practically every laboratory uses a general technology with individual modifications.

Procedure

The liver was perfused *in situ* following the two-step collagenase perfusion technique initially described by Berry and Friend (1969) and later improved by several authors (Seglen, 1975, McQueen and Williams, 1987). This technique was initially described for rats but it has also been described for other animal species including the mouse, rabbit, and hamster (Guillouzo, 1986; McQueen and Williams, 1987). Some modifications were introduced in order to adjust the method to the size of the test animals.

Two solutions (I and II) were run through the liver via the portal vein under physiological conditions (37°C, 5% CO_2, 95% O_2). Solution I (500 ml of Hanks' balanced solution (HBSS) without Ca^{++}, Mg^{++} and phenol red, supplemented with 8.6ml of 7.5% sodium bicarbonate) contained a chelating agent, Ethyleneglycol-bis-(b-Aminoethyl ether)N,N,N',N'-Tetraacetic acid (EGTA, 76mg/500ml) to remove Ca^{++} from tissues. Solution II (HBSS) had 25 mg of Collagenase H (Boehringer Manheim, Germany) and 100mg $CaCl_2$ (collagenase is

Ca^{++} dependent) added to enhance dispersion of the liver cells.

A diagram of the perfusion apparatus is shown in figure IV.I. Before use the apparatus was sterilised by flushing it with 500 ml distilled water, followed by 10 minutes recirculated wash with 70% industrial methylated spirits (IMS) in distilled water and a final rinse with 500 ml of sterile distilled water.

A bottle of 500ml HBSS buffer without EGTA (solution I) was placed in the water bath and the solution run through the circuit to oxygenate for 10 minutes. Before adding EGTA, 100ml of HBSS buffer were separated in a smaller bottle as solution II. EGTA was then diluted in 10ml of HBSS buffer and poured back in the 500ml bottle (solution I). Ten ml of HBSS without EGTA were used to dissolve the collagenase and $CaCl_2$, which were filtered (through a Minisart NML 0.2um filter, Sartorious, Germany) and kept in a sterile universal in a continuous flow hood close to the perfusion apparatus until the moment of use. Scissors, forceps, scalpel and blade were wiped with alcohol, two pieces (30cm) of thread cut, and a cannula (1.1x45mm, Medicut, Sherwood, Ireland) filled with heparin (Heparin sodium (mucous), Multiparin, CP Pharmaceuticals Ltd, Wrexham, U.K.) prior to the start of the perfusion.

Mice were anesthetised with Sagatal (Pentobarbitone sodium B.P., RMB Animal Health Ltd, Dagenham, U.K.) placed on a platform and wiped with 70% IMS. The skin and peritoneal wall were removed, and the portal vein cannulated (see figure

IV.I). Before starting the perfusion, the descending vena cava was cut. The cannula was connected to the circuit, trying to avoid bubbles, and solution I was run through the liver to waste. During this perfusion, the liver was excised, placed on a plastic platform and covered with a petri dish. After 10 minutes, solution I (500ml bottle) was substituted by solution II (100ml bottle). Before adding collagenase, 20ml of solution II were run through the liver to waste for 3 minutes, to wash out the remaining EGTA. The collagenase was then added and solution II recirculated by placing the liver over a funnel connected to the small bottle. After a short time the liver volume increases due to cell dispersion. When it falls back to its normal size (approximately 7-12 minutes) the perfusion is stopped. The liver was placed under a laminar flow hood. The gall bladder was removed and hepatocytes were stripped from the connective tissue stroma by cutting the liver wall with a scalpel and shaking it in William's medium E (WME) supplemented with 5% FCS. The cell suspension was passed through a sterile nylon cloth to remove cell debris and poured into a sterile universal flask. The suspension was centrifuged at 50g for 4mins., the supernatant was removed and the cell pellet resuspended in culture medium. This step was repeated twice to remove debris and non-parenchymal cells from the final suspension in order to get an homogenous suspension of parenchymal cells which constitute the main cell type in the liver.

Viability was assessed with 0.1% trypan blue dye solution. Cells with damaged cell membranes stain blue but

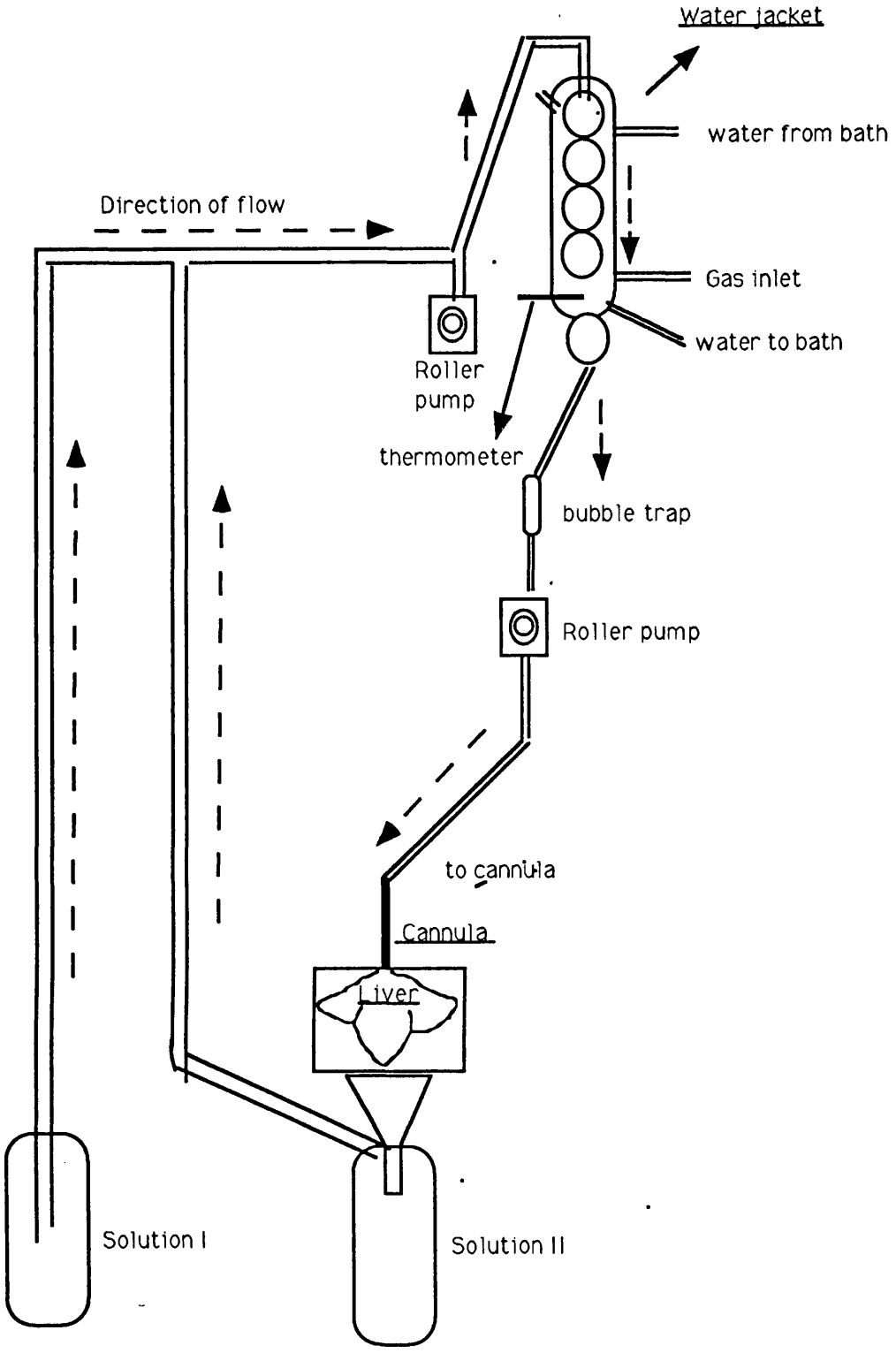

FIGURE IV.I.- Diagram of perfusion apparatus.

live cells exclude the dye and thus, appear unstained. Cell suspensions with viability lower than 60% were discarded (the normal rate of viability obtained was 60-70%). The number of cells and viability were counted using a haemocytometer and an inverted microscope.

Cell suspensions were diluted to $0.3x10^6$ in WME + 5% FCS. Three ml of cell suspension were plated out in 60mm sterile petri dishes (Falcon, Becton Dickinson, Cowley), and incubated at 37°C, 5% CO_2 in a humidified atmosphere. Hepatic parenchymal cells attach to tissue culture plates within 30 minutes, therefore short attachment times offer another means for separation, especially from endothelial cells, which require a much longer time. After 2 hours the medium was removed and replaced by fresh medium containing the test chemical. After incubation for 16-20 hours, LDH leakage, neutral red uptake and total protein determination assays were performed as described later.

TOXICITY ASSAYS

- COLOURIMETRIC METHODS

Colourimetric end-points were used for these studies for several reasons: they are generally inexpensive and easy to perform, and a large number of samples can be analysed in a relatively short time, specially if the method can be performed on 24- or 96-well plates.

Several colourimetric methods were considered. Those that required hazardous products or expensive equipment were rejected. Finally, four colourimetric methods (neutral red uptake, kenacid blue uptake, (3- (4,5-dimethylthiazol-2-il) -2,5-diphenyl tetrazolium bromide, MTT) uptake, and total protein concentration) measuring cell viability and cell function were selected and adapted to the cell systems and culture conditions. Full details are given below.

Neutral Red Uptake

Introduction

The neutral red (3-amino-m-dimethylamino-2-methyl-phenazine hydrochloride) uptake test (NR) was first described by Parish and Müllbacher (1983), and later modified and improved in several studies (Borenfreund and Puerner, 1985; Babich and Borenfreund, 1987; Borenfreund et al., 1988; Fiennes et al., 1987). It is based on the uptake of neutral red, a supravital dye, and its accumulation in the lysosomes of viable uninjured cells. This weakly cationic dye penetrates cell membranes by nonionic diffusion and binds intracellularly to anionic carboxylic and/or phosphate groups of the lysosomal matrix. Chemicals that injure the plasma or lysosomal membrane decrease the uptake and subsequent retention of the dye. Dead cells cannot retain the dye after the washing procedure.

Several advantages of using the Neutral Red uptake method can be listed. This method is easy to perform, can be assessed in 96-well plates using a microplate reader, thus allowing a large number of samples to be analysed in a short

time (Parish and Müllbacher, 1983), no radioactive materials are required (Parish and Müllbacher, 1983) and it has proved to be very sensitive in comparisons with other methods (Borenfreund et al., 1988).

Procedure

The test was performed as described by Babich and Borenfreund (1987) with some modifications. An 0.4% aqueous stock solution of the dye was stored at 4°C, and an aliquot added to complete DMEM or WMEM (used for macrophages or hepatocytes, respectively) to the final concentration required. Different procedures were followed for macrophages (cultured in 96-well plates) and hepatocytes (cultured in 60mm petri dishes):

Macrophages.- the medium containing the test chemical was removed after incubation with the cells for 3 days and replaced with 150µl/well for medium containing 50µg NR/ml. The NR-containing medium had been preincubated overnight at 37°C and was filtered prior to use to remove fine precipitates of dye crystals. The assay plate was returned to the incubator for another 3 hours to allow for the uptake of the vital dye into the lysosomes of viable cells. Thereafter, the medium was removed and plates washed 3 times with 200µl of PBS (pH= 7.2), followed by addition of 150µl of extraction solution (1:1 absolute ethanol: 0.1M citrate buffer, pH=4.2) to extract the dye from the cells. A standard set of 10 dilutions of 0.4% neutral red in extraction solution ranging from 0 to 150µg NR/ml was then placed in spare wells in each

plate. After agitation on a microtitre plate shaker, the absorbance was measured on a multiskan Titertek microplate reader at 540nm wavelength.

Hepatocytes.- After incubating the 60mm Petri dishes containing hepatocytes for 24 hours, the medium with the test substance was removed and replaced with 3 ml of medium containing 50μg NR/ml. The NR-containing medium had been incubated overnight and filtered prior to use. Plates were incubated for another 3 hours, and then washed 3 times with 4 ml of PBS (pH=7.2) followed by the addition of 3ml of extraction solution. Plates were briefly shaken and left at room temperature until a homogeneity of colour was achieved. 1.5ml of this solution were transferred with a multimicropipette to 96-well plates (10 wells/concentration were filled) and absorbance was read in a multiskan Titertek microplate reader at 540nm wavelength.

Total protein determination

Introduction

The test was performed as described by Shopsis and Eng (1985) with the modifications introduced by Festing and Eydmann (1987b). The method is based on the change of colour produced in the dye (an acidic solution of Coomassie Brilliant blue G-250) upon binding to protein (Bradford, 1976). A shift in the absorption maximum of the dye from 465nm to 595nm when binding to protein occurs, and the increase of absorption at 620nm wavelength is monitored.

Procedure

Again, different protocols were followed for macrophages and hepatocytes:

Macrophages.- After culturing cells with the test compound for five days (at 37°C, 5%CO_2 in a humidified atmosphere), the medium containing the treatment was removed by inverting the plates. 200µl/well of PBS (pH=7.2) were added and then removed by vacuum aspiration, this washing process was repeated three times. Adherent cells were lysed by addition of 100µl 0.1N NaOH. A set of standards ranging from 0 to 150 µg protein/ml (10 dilutions of BioRad Protein Standard I -bovine gamma globuline- in 0.1N NaOH) was then pipetted into spare wells. 100µl/well of staining solution (two parts of Bio-Rad reagent -solution of dye, phosphoric acid, and methanol- in three parts of distilled water) were added. Plates were left at room temperature for 20 minutes and then read in a multiskan microplate reader at 620nm wavelength.

Hepatocytes.- After 14-20 hours incubation with the test compound, the treatment-containing medium was removed (by vacuum absorption). Four ml of PBS (pH=7.2) were pipetted into each dish and removed by vacuum absorption. This washing process was repeated three times. Three ml/dish of 0.1N NaOH were added and the dish bottom was scrapped with a rubber policeman to lyse cells . Plates were shaken to get a homogeneous solution. One ml/dish was transferred to 15ml Falcon plastic tubes, and nine ml of 0.1N NaOH were added to further dilute the contents of the tube. Tubes were shaken and the dilution placed in petri dishes. One ml of each plate were

pipetted into 96-well plates (10 wells/plate). Standard dilutions were pipetted in the test plates and 100µl/well of staining solution added. After 20 minutes the plates were read in a multiskan microplate reader at 620nm.

The reason for the transfer from petri dishes to 96-well plates was the same as in the neutral red assay, namely to read large number of samples as conveniently as possible.

MTT assay

Introduction

This assay is based on the ability of mitochondrial enzymes in live but not dead cells to chemically reduce a tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-il)-2,5-diphenyl tetrazolium bromide), into a colored formazan dye which can be detected at 540nm wavelength (Mosmann, 1983; Holt et al., 1987). The MTT interacts with mitochondrial components at several points along the respiratory chain. The result of this interaction is the reduction of MTT by the succinate-tetrazolium reductase system to form a blue formazan dye which collects as crystals within the cell. The tetrazolium salt is cleaved only in active mitochondria, and so the reaction occurs only in living cells (Slater et al., 1963; Mosmann, 1983). This formazan product can be dissolved in acid-isopropanol to give a colored

solution which can be quantified spectrophotometrically. This method was studied in the first trials as a suitable assay to obtain information about both cell viability and cell function.

Procedure

This assay was performed on macrophage cultures only. The method followed is essentially that described by Mosmann (1983).

A stock solution (five mg/ml of MTT in PBS, pH= 7.2) was prepared and stored in the dark at 4°C. The stock solution was freshly prepared every week, and filter-sterilized prior to use. Plates were incubated with the test compound for five days at 37°C, 5% CO_2 in a humidified atmosphere. Thereafter, the medium with the treatment was removed (by inverting the plate) and replaced with 100μl of medium containing MTT (1:10 of stock solution in culture medium). Plates were covered with foil and returned to the incubator for another four hours. MTT-containing medium was discarded and 200μl/well of PBS (pH=7.2) pipetted into the plates and removed by inversion of the plate, this washing step was repeated three times, followed by the addition of 100μl/well of 1N HCL-isopropanol (1:24, v/v) to extract and solubilize the formazan. After agitation (10 minutes) in a microtitre plate shaker, the plates were transferred to a microplate reader and the optical density of each well was measured in a multiskan plate reader at 540nm wavelength.

Kenacid blue assay

Introduction

This method is based on a direct relationship between protein content, cell number and binding of a dye, Kenacid Blue R (Knox et al., 1986). Kenacid blue R (Kb) produces a blue precipitate on binding to protein. It was adopted for the FRAME (The fund for the replacement of animals in medical experiments) project replacing the Lowry method. The Kb assay proved to be much faster to perform and the values obtained showed less variation than was the case with the Lowry method (Knox et al., 1986). It was performed as described in that paper with small changes, and on macrophage cultures only. It was included in the first trials to test as a method suitable for measuring cell number and cell viability in a large number of samples in a short period of time with intensity of colour being assessed in 96-well plates using a microplate reader.

Procedure

A stock solution (0.4g of kenacid blue in 240ml ethanol and 630ml of distilled water) was prepared and stored at 4°C in the dark. At the end of the treatment period (five days) the medium was removed by inversion of the plate and replaced by 100µl of fixative solution (3% glutaraldehyde in PBS, pH= 7.2). After 10-20 minutes. the fixative solution was discarded and 100µl/well of stain solution (12ml glacial acetic acid in 88ml stock solution, filtered prior to use).

Plates were shaken in a microtitre plate shaker for 30 minutes. After the fixative solution was discarded, two hundred μl/well of washing solution (10:5:85 ethanol/glacial acetic acid/distilled water) were added, plates were briefly shaken and the washing solution removed by inversion of the plate. This process was repeated until there was no visible blue color in the washing solution. Thereafter, 100μl/well of desorb solution (1M potassium acetate in 70% ethanol) were added and plates were shaken for another 15 minutes. A set of standards, ranging from 0 to 40μg/ml was pipetted in the first row of the plates. The dye concentration in the desorb solution was determined spectrophotometricaly at 570nm wavelength using a multiskan Titertek plate reader.

-NON-COLOURIMETRIC METHODS

Number of cells (cell attachment)

Introduction

Cell attachment is altered by xenobiotics, and is a good measure of the effect of the treatment on the cultures though it was not possible to distinguish between live and dead cells using this method. Although it is time consuming, it was tested in the first trials as a control measurement.

Procedure

After the treatment period, cell number was measured after washing the plates with PBS (pH= 7.2) and inverting the

plates to remove the buffer and unattached cells. The washing step was repeated three times, and then the plates were placed on an inverted phase-contrast microscope and the number of cells was counted. A certain area of each well was covered with the help of a graticule. Several different fields were viewed, including those near the edge and centre of each well. This end-point was found to be unsatisfactory for several reasons. It was often difficult to distinguish between whole cells and cell fragments, it was time-consuming, and it appeared to be associated with a high measurement error. Plates were later used for total protein determination, following the procedure described above.

LDH leakage

Introduction

The measurement of cytoplasmic enzyme leakage of cell cultures exposed to toxic agents represents a sensitive method for assessing cytotoxicity and plasma membrane damage (Paganuzzi et al., 1981; Tyson and Green, 1987; Guillouzo, 1986; Castell and Gomez-lechón, 1987). Because LDH is an enzyme common to most cell types, LDH leakage can be measured and used as an indicator of cell injury in all types of cultured cells. This method was chosen for its high reliability and widespread use for testing the effect of treatments on hepatocyte cultures.

The most direct method of assessing LDH activity is

a spectophotometric measurement by monitoring the increase or decrease of absorbance of NADH at 340nm (Vassault, 1983):

$$\text{PYRUVATE} + \text{NADH} + H^{+} \xleftrightarrow{\text{LDH}} \text{L(+)-LACTATE} + NAD^{+}$$

In the pyruvate to lactate reaction, the rate of decrease is measured, whereas in the lactate to pyruvate reaction the rate of increase is measured.

In the present work, the LDH activity was measured at 30°C as the amount of pyruvate consumed, by continuously monitoring the decrease in absorbance due to oxidation of NADH at 340nm. The equilibrium is far on the side of lactate and NAD.

LDH leakage was only measured on hepatocyte cultures. It was not possible to get enough macrophages from an individual mouse to make this test a practical possibility.

Procedure

LDH activity was determined in the culture medium and cells following the procedure described by Vassault (1983) with small modifications. A stock solution of Tris-NaCl buffer (81.3mmol/l Tris, 203.2mmol/l NaCl: 4,92g Tris, 5.95g NaCl in 1L of distilled water, pH= 7.2) was prepared and stored at 4°C. Following incubation with the test compound for 16-20 hours, the culture medium was pipetted from the dishes into disposable culture tubes. Three ml 0.1% Triton X-100 detergent in PBS (pH= 7.2) were pipetted into the dishes. The dish bottom was scraped to disrupt cells and pipetted into

disposable culture tubes. Tubes were centrifuged for five minutes at 3500 g to remove cell debris. The supernatant solution was pipetted into new disposable culture tubes and placed on ice until performance of the assay, usually two hours later. If the samples were to be read the following day, tubes were placed in a sealed ice container and kept in a cold room at 4°C overnight. NADH solutions (50mg NADH/100ml Tris-NaCl buffer, pH= 7.2) were freshly prepared prior to use. Two and a half ml prewarmed (30°C) NADH solution and 200μl of sample were pipetted into disposable plastic tubes and incubated in a water bath at 30°C for 30 to 60 minutes prior to measurement. Half ml sodium pyruvate solution was pipetted into the tubes, then samples were briefly and thoroughly shaken and poured into quarz u.v. cells to be measured in a u.v. spectophotometer (Uvikon 860, Kontron instruments, Zurich). The decrease in absorbance was measured at 340nm for three minutes.

Rate of phagocytosis

Introduction

Phagocytosis is the process by which invading microorganisms are ingested by polymorphonuclear (granulocytes) and mononuclear (monocytes and macrophages) phagocytes. Phagocytosis is one of the most important host defence mechanism against bacteria (Roitt, 1988; Van Furth et al., 1979). The process of phagocytosis can be separated into several stages: opsonization of the particles by serum

factors, attachment of the opsonized particles to the cell surface, engulfment of such particles, intracellular killing of micro-organisms, and digestion of ingested particles (Van Furth, 1979; Van Oss, 1986). Thus, the measurement of phagocytosis was considered a good indicator of the metabolic activity of macrophage cultures.

Many *in vitro* assays have been developed to quantify phagocytosis. These can be divided in direct and indirect methods (Absolom, 1986; Rolland et al., 1987). Direct assessment methods include direct microscopic visualization and quantitation of the ingested particles, isotopic labeling (determination of the amount of radiolabeled particles ingested by the phagocytic cell), extraction methods (extraction, solubilization and quantitation of ingested particles by spectrophotometric means), fluorescent methods (assessment of fluorescent particles ingested by flow cytometry), and difference methods (the difference between the number of particles present in the bulk liquid pre- and post phagocytosis is measured). Indirect assessment methods include techniques for the quantitation of the events that follow the ingestion of particles by phagocytes (e.g. chemiluminescence, glycolisis, degranulation, and respiratory activity burst)

In the first trials, Chemiluminescence was tested as a measure of phagocytosis. Macrophages are known to respond by an oxidative burst to phagocytic stimuli (Trush et al., 1978; Pick and Keisari, 1981; D'Onofrio and Lohmann-Matthes, 1984; Helfand et al., 1982, Blair et al., 1988; Lavie and Gershan, 1988). This burst was found to be characterized by the

production and release of superoxide anion (O_{2-}), hydrogen peroxide (H_2O_2), and hydroxil radical (OH^-), and the emission of light (luminescence) that is strongly associated with the above mentioned oxygen radicals. This phenomenon of chemiluminescence was first described by Allen (Eastmon et al., 1980). The chemiluminescence emited was found to be amplified by luminogenic substrates such as Lucigenin and Luminol (Trush et al. 1978; Helfand et al. 1982; Müller-Peddinghaus, 1984; D'onofrio and Lohmann-Matthes; Blair et al., 1988). However, methods described in those papers using Lucigenin and Luminol were not satisfactory. The major drawback was the large number of cells needed to obtain a measurable response after activating the macrophages with opsonized zymosan (in the order of $5x10^6$ cells/sample). Similar numbers were used by Trush et al. (1978), and Easmon et al. (1980). However, Blair et al. (1988) described a method using a 96-well microtitre luminometer that substantially reduced the number of cells required for the assay. Unfortunately, the equipment (microtitre luminometer) was not available, and the measurement of chemiluminescence was discarded as being impractical. However, this assay seems to be quite rapid and reliable, and should be taken into account when looking for a method for assessing macrophage metabolism.

Methods for the measurement of H_2O_2 production were also unsuccessful. Finally, a technique for measuring the rate of phagocytosis by flow cytometry was developed. Although several methods for measuring rate of phagocytosis have been described (Kavet and Brain, 1980; Absolom, 1986), most of them

are based on tedious and time-consuming counting of the percentage of particles that have been phagocytosed by the cells. More recently, methods for the measurement of phagocytosis of fluorescent particles (latex microspheres, methacrylic copolymer nanoparticles) using the technique of flow cytometry have been described (Kavet and Brain, 1980; Stewart et al., 1986; Rolland et al., 1987). This technique is rapid, analyses a large number of cells in a short time, and determines the proportion of cells with the ingested particles and the number of particles ingested per cell (Stewart et al., 1986). Therefore, it was included in the battery of end-points for later studies as being a rapid, sensitive and reliable test of macrophage function.

Procedure

The method described by Stewart et al. (1986) was used, with some modifications. For this assay peritoneal macrophages were collected as previously described and plated out in 25-flat-bottom-well non-tissue culture plastic boxes (bacteriological plates, Sterilin) so that cells could be resuspended after incubation. $2x10^6$ cells/well were plated out and supplied with three ml of DMEM + 5% FCS. Plates were incubated for three hours and the medium was removed by suction with the help of a sterile pasteur pipette connected to a water pump (the whole procedure took place in a laminar flow hood to maintain sterile conditions) and wells were supplied with three ml of medium (DMEM + 5% FCS) containing different concentrations of treatment. Plates were left to

incubate for 16-20 hours at 37°C and 5% CO_2 in a humidified atmosphere. Before starting the phagocytosis step, five ml of culture medium containing fluorescent microspheres (diameter= 1.73 micron, SD= 0.034, Polysciences Inc., Warrington, Pennsylvania, 100 times the number of cells plated) were sonicated in a ultrasonic waterbath for 30 seconds and diluted into prewarmed (37°C) medium to a volume sufficient to add two ml of microsphere suspension to each well.

When the solution of microspheres was ready, the plates were removed from the incubator and placed in the hood, the medium was extracted by suction and two ml of microsphere containing medium were added to each well (so that $2x10^8$ microspheres approximately were added to each well). Plates were returned to the incubator for one hour, and then, washed with two ml of prewarmed (37°C) PBS supplemented with Ca^{++} to avoid cell detachment. Non-adherent cells were then gently aspirated. Finally, two ml of prewarmed (37°C) PBS + 5mM D-Glucose (filtered sterile using Minisart NML 0.2um filters, Sartorius, Germany) were added. This causes the macrophages to become detached (Hunt, 1987). After 15 minutes of incubation, when most of the plated cells are in suspension, plates were removed from the incubator, wells were flushed with the PBS + Glucose using a pasteur pipette to promote detachment. Cell suspensions were transferred to appropriately labeled five ml centrifuge tubes (Falcon, Becton Dickinson, Cowley). Plates were washed with two ml/well of PBS + 0.2% BSA and combined with cell suspensions in the appropriated tube. Tubes were centrifuged at 3500g for five minutes. After centrifugation,

the supernatant was removed and replaced with 0.3ml of PBS + 0.2% BSA. Tubes were gently shaken to resuspend the pellet. Cells were fixed by adding 0.3ml of 8% formol saline and stored at 4°C in the dark until samples were read.

The rate of phagocytosis (total number of fluorescent microspheres phagocyted per macrophage, see figure IV.II) was measured in a fluorescence actived cell sorter (FACS, Ortho Diagnostic Systems inc., 2150)

FIGURE IV.II.- Scanning electron microscopy picture of a single macrophage with beads in different stages of ingestion (picture courtesy of M.Festing)

-CELL IDENTIFICATION METHODS

Macrophage detection by fluorescent staining

Introduction

The flow cytometer may also be used to characterize sub-populations of cells. Many flow cytometer studies have involved the identification, isolation and functional characterization of sub-populations of lymphocytes carrying surface antigens detectable with fluorescein conjugated antibodies (Herzenberg and Herzenberg, 1979). This technique was used to study the percentage of macrophages contained in cell suspensions.

In their review of fluorescent staining techniques, Herzenberg and Herzenberg (1979) divided immunofluorescence staining methods in two categories: direct staining, where fluorochromes such as fluorescein and rhodamine are conjugated to the antibody which binds to the cell surface determinant, and indirect staining, where the antibody which binds to the surface determinant is not fluoresceinated and its binding is demonstrated by the binding of a second-step, fluoresceinated antibody reagent specific for determinants on the non-fluorescent first-step antibody. This latter procedure amplifies the stain because several molecules of second-step fluoresceinated antibody generally bind to one molecule of first-step reagent. This procedure was used for the detection of macrophages in mouse peritoneal cell suspensions (Festing et al., 1990).

Lymphocyte populations have been shown to be heterogeneous with respect to cell surface antigens (Lal et al., 1988). Antibodies are widely used as probes to recognize and study such specific surface molecules. Springer and collaborators (1978, 1979) prepared a cell line that secreted a monoclonal antibody (M1/70) which recognized a specific macrophage antigen (Mac-1). A method for the detection of macrophages by indirect staining using M1/70 antibodies conjugated to a second-step fluorescent conjugated antibody was developed (Ault and Springer, 1981; Festing et al., 1990). This technique was used to study the percentage of macrophages contained in cell suspensions of bone marrow and peritoneal macrophages , and after injection of inducting agents (see induction of macrophages and comparison of bone marrow and peritoneal macrophages chapters).

Procedure

The procedures described by Ault and Springer (1981) and Festing and collaborators (1990) were followed with some modifications. Bone marrow and peritoneal cell suspensions obtained as previously described were centrifuged at 1000 rpm for five minutes, the supernatant was discarded, and PBSA (PBS + 0.2% Bovine serum albumin -BSA-) was added and the pellet was resuspended. This step was repeated twice to wash the cells. Samples were resuspended in PBSA and adjusted to $1x10^7$ cells/ml One hundred μl of Normal Rabbit Serum (NRS) diluted 1:10 in PBS were added to block Fc receptors. Samples were briefly spun at 1000 rpm, the supernatant was discarded and

100μl of PBSA were added into the tubes, This step was repeated twice. The pellet was resuspended in 100μl of PBSA.

At that point, 100μl of diluted (1:10 in PBSA) anti-Mac-1-antibody (M1/70.15.1, Serotec Ltd.) were added to each sample. Tubes were left at room temperature, mixing gently occasionally to help binding. Samples were then spun down, the supernatant discarded and replaced with 200μl of fresh PBSAA (PBS + 0.2%BSA + 0.2% NaN_3). This was repeated four times. After the final centrifugation, samples were resuspended in 100μl of PBSAA. 100μl of rabbit anti-rat Ig-labelled with FITC (Serotec Ltd.) diluted 1:10 in PBSA were added to all tubes. After gentle shaking, samples were left at room temperature for 10 mins., mixing occasionally to help binding of the FITC labelled antibody to the anti-Mac-1-antibody. Samples were washed with 200μl of PBSA, gently mixed and spun down quickly, the supernatant was discarded and replaced with 100μl of PBSA. This last step was repeated four times. After the last centrifugation the pellet was resuspended in 100μl of PBSA. Samples were fixed with one ml/tube of 8% formaldehyde in 0.85% saline for use in the flow cytometer and stored at 4°C for several days (maximum 10 days).

The proportion of cells that presented fluorescence was measured by flow cytometry in a fluorescence activated cell sorter.

CHAPTER V.- CONDUCT OF EXPERIMENTS

PART I: DEVELOPMENT OF A TECHNIQUE FOR THE STUDY OF GENETIC VARIATION IN THE RESPONSE TO DRUGS

V.I.1.- INDUCTION OF MACROPHAGES USING HORSE SERUM

INTRODUCTION

The yield of cells from the peritoneal cavity may be too low to make use of some end-points such as chemiluminescence and still have sufficient cells for many replications. Even in mice and rats, this low production constitutes a drawback for the use of peritoneal macrophages, as one of the aims of *in vitro* studies is the reduction of the animals used in research. Methods for increasing the number of macrophages were examined.

The yield of macrophages can be increased by the injection of substances into the peritoneal cavity prior to harvesting the resulting peritoneal exudate. The best inducing agents are those that provoke an exudate rich in macrophages and that are readily biodegradable, leaving no trace in the cultured cells.

Stuart et al. (1979) in a review of phagocytes *in vitro*, and Hunt (1987) listed the most widely used inducing agents including horse serum, glycogen, glycerol tricaprate, proteose peptone broth, thioglycolate broth, mineral oil, casein hydrolysate and peptone water. However, the use of inducing agents has several drawbacks:

- Induced macrophages have a different chemical cyto-profile from non-induced macrophages. In particular the levels of hydrolytic enzymes are increased.

- Depending on the inducing agent used, the macrophages isolated may contain phagocytosed granules of the agent, which may interfere with the parameters under study.

- Induced exudates contain variable numbers of lymphocytes and polymorphonuclear leucocytes. This makes culture of macrophages difficult unless the exudate is harvested when macrophages are the principal cell type.

- Some inductants cause clumping of macrophages, and corresponding difficulties in counting and culture occur.

In addition to this, some agents present particular disadvantages. From the list of inducing agents given above, several were discarded for specific reasons. Macrophages induced by glycerol tricaprate show a vacuolated cytoplasm containing fat droplets, proteose peptone broth produces sticky macrophages with tendency to clump, mineral oil produces dirty exudates, persist for long time inside cells and the injected animals are often ill, and peptone water also produces sticky macrophages. From the remaining agents, casein hydrolysate, thioglycolate broth and serum, the two latter were tried. Casein was reported to take four days for the production of results, whereas the other two agents give an increase in the yield of macrophages in 48 hours (Stuart et al. 1979).

Thioglycolate injections were tried first. Many authors have reported the collection of macrophages after induction with thioglycolate, specially those working with techniques that required a large number of cells or activated macrophages, like chemiluminescence or the production of

oxygen radicals (i.e.: Müller-Peddinghaus et al., 1983; Müller-Peddinghaus, 1984; Pick and Keisari, 1980; Lavie and Gershon, 1988; D'onofrio and Lohmann-Matthes, 1984; Goldberg and Koo, 1987; Vogel et al., 1988). However, necrosis and loss of the tail was observed in several cases after thioglycolate injection, and this agent was discarded as being harmful for the animal.

Horse serum injections seemed to offer the best opportunity of increasing the production of macrophages without harming the test animal. Although the increase in macrophage yield is reported to be low (Stuart et al., 1979), it is also considered to be the least harmful of the inducing agents to the physiology of the induced macrophages. An experiment was therefore set up to study the increase in total cells and any change in the proportion of cells which were macrophages following injections with horse serum.

MATERIALS AND METHODS

Animals used

A total of 35 male RIII-*ro* mice were used for this experiment.

Horse serum dose

Half ml of prewarmed (37°C) horse serum were injected 2,3, or 4 days before macrophage extraction (see

experimental procedure). For macrophage collection, five ml of culture medium (DMEM + 5% FCS) were injected into the peritoneal cavity regardless of animal weight. Peritoneal cells were removed as previously described in the Materials and Methods chapter. The treated and control samples were pooled separately and half of the final suspensions was plated out in 96-well Titertek plates, and the other half prepared to be read by flow cytometry.

Assays

Number of cells and total protein content were determined on the 96-well plates after 1,3,5 and 7 days of culture, and the proportion of macrophages contained in the cell suspensions collected were measured by indirect staining of cell surface antigens (Mac-1, see method for identification of macrophages). All assays were performed as described in the material and methods chapter.

Experimental procedure

In the first experiment seventeen animals were used. A group of four were injected with 0.5ml of Horse Serum (37°C) four days before cell collection, four were injected three days before, and three injected two days before. Six animals were kept as controls. The number of cells obtained in each sample was counted, and then the cells were pooled and half were plated out in microtitre plates and half prepared to run through a flow cytometer for macrophage identification. This represented replicate one of the studies of percent macrophage

and total protein.

The results of this first replicate showed that there was no evidence that time before serum injection influenced the number of cells obtained, and in fact serum did not increase the number of cells (see results). In a second and third replication ten and eight mice were used, and half were injected with horse serum three days before macrophage collection. Again, half of the sample was run through a flow cytometer for the detection of macrophages in the cell suspensions, and half were plated out and incubated at 37°C, 5% CO_2 in air. Total protein and cell number were measured on the plates after 1,3,5 and 7 days of culture.

RESULTS

The number of cells obtained per sample, the proportion of macrophages contained in the cell suspensions, and the total protein and cell number measured on the plates are shown in table V.I.1.1 (page 68).

No significant difference was found among control animals and those injected with horse serum on different days using a 1-way analysis of variance ($F=1.94$, $P=0.172$ with 3 degrees of freedom for treatments and 13 DF for error).

Specific macrophage staining (see identification of macrophages in materials and methods chapter) gave similar results. Sixteen samples were obtained in three replications, but replicate samples were averaged prior to statistical

TABLE V.I.1.1.- Number of cells, percentage of cells stained by the Mac-1 technique (macrophages), total protein content expressed in means (averaged through replications) and number of cells/well.

		Samples treated with Horse Serum N= 20*	Samples untreated (control) N=15*
Number of cells		4.94×10^6	4.47×10^6
Percent of macrophages		79.1	76.8
Protein/day culture	1	45.4	45.1
	3	67.9	63.0
	5	90.1	84.7
	7	97.2	95.8
	Global Mean	75.1	72.2
No. cells/well/ day of culture	1	227	197
	3	176	134
	5	169	131
	7	157	102
	Global Mean	182	141

* = number of animals used

analysis. No significant increase in the proportion of macrophages within the cell suspensions was observed ($F=0.2$, $p>0.10$). The average proportion of cells stained in samples obtained from animals treated with Horse serum was 79.1%, and 76.8% in control samples. Although this was a small experiment, a significant difference could have been expected if horse serum was having any important effect.

Total protein determinations were performed on microtitre plates following the standard protocol after 1,3,5 and 7 days of culture. The overall means show a very small difference, an average of 75.1μg/ml of protein were measured in treated samples and 72.2μg/ml of protein in control samples. Analysis of variance showed a highly significant effect of time ($F3,14=5.5$, $P=0.01$), but no significant treatment effect or treatment x time interaction.

The number of cells counted in each well after incubation were the only data that showed some difference between treated and control samples. The average number of cells counted in samples from horse-serum treated mice was 182, and 141 in control samples. Analysis of variance showed a significant treatment effect ($F_{1,2}=7.0$, $P=0.03$), and a significant time effect ($F_{3,12}=5.3$, $P=0.02$), with the number of cells declining from an average of 212 on day one to 130 on day seven.

DISCUSSION

The results showed no significant improvement in the yield of macrophages after horse serum injections. The number of cells collected did not increase, nor did the proportion of macrophages in the cell suspension obtained after injections, or the cell protein measured after incubation. The only value that showed a slight increase after Horse serum injections was the number of cells counted in the plates after incubation. This could be due to an improvement in cell survival or an enhancement of cell attachment caused by horse serum. This difference was observed on the first day of culture. This would favour the hypothesis of enhancement in cell attachment. However, the difference seemed to increase slightly after several days of culture. The average difference in number of cells between treated and control samples was 30, 42, 38 and 50 after 1,3,5 and 7 days of culture, respectively. Thus, a slight improvement of cell survival may also occur.

However, improvement of cell attachment or cell survival was not in the scope of these studies. In addition, the likely effect of inducing agents on cell enzyme levels was considered specially inconvenient, as it was intended to measure levels of protein and enzyme release.

Other authors have found that horse serum was a useful inducing agent, so it is surprising that it had such a small effect here. It is possible that the effects are strain-specific, and that it does not work well in RIII-*ro* strain mice. This was not studied, as only a single strain was used. However, if the degree of inducement were strain

dependent, it might cause some complications in interpreting strain differences in response to toxic chemicals. On the whole, it seemed better to avoid agents which induce peritoneal macrophages.

Although other agents may show a more powerful inducing activity, they were discarded as being harmful for animals or affecting cell activity, as explained in the introduction of this chapter. In later studies no inducing agent was included in the protocol.

V.I.2.- COMPARISON OF WME AND DMEM MEDIA FOR CULTURING HEPATOCYTES

INTRODUCTION

Whereas the type of medium and culture conditions were well defined for peritoneal macrophages, less work has been done with mouse hepatocytes, though techniques for culturing rat hepatocytes are well established. Therefore, some preliminary trials were done to establish the techniques for mouse hepatocyte culture. The liver perfusion technique was adopted from the methods used for rats. The next step was the selection of a good culture medium, which in the case of hepatocytes is of special importance.

Several studies have shown that the content of cytochrome P-450 and the associated activities decline rapidly when hepatocytes were cultured in a single medium (Vind et al., 1988). Isolated hepatocytes are usually maintained in salt balanced buffer or in culture medium designed to reconstitute plasma composition (Krack et al., 1980). A variety of buffering systems have been described for the incubation of hepatocytes: a mixture of Hepes, Tes and Tricine (Seglen, 1976), Williams' medium G and E (Williams et al., 1977; Manslansky and Williams, 1982; McGowan, 1986), L-15 (Krack et al., 1980), Hams's F10 (Krack et al., 1980), Hams's F12 (Gomez-Lechón et al., 1984), Dulbecco's modified essential medium -DMEM- (Bissel, 1980; Krack et al., 1980), Waymouth's

medium (Bissel, 1980; Krack et al., 1980; Bucher, 1987; Vind et al., 1988), and Krebs-Henseleit combined with Hepes (Shaw et al., 1988). Nearly every author includes some modifications to the medium with more or less success.

The loss of cytochrome P-450 can also be temporarily prevented by supplementing the culture medium with a variety of compounds, such as hormones, and a mixture of nutrients, ligands and heme groups (Guillouzo, 1986). Serum (mainly foetal bovine serum), hormones (insulin and hydrocortison), external galactose, glucose or fructose have been widely used and their addition is described elsewhere.

Some individual compounds have been described that promote both longevity and maintenance of hepatocyte cultures. Dexamethasone (Laishes and Williams 1976; Williams et al., 1977; Guillouzo, 1986; McGowan, 1986) and recently, dimethyl sulfoxide -DMSO- (Isom, 1985; Bucher, 1987) have been observed to produce such an effect.

In this laboratory, two culture mediums, Dulbecco's minimum essential medium (DMEM) and Williams' medium E (WME) were available. Some researchers worked with WME and some with DMEM, both claiming to obtain good results with hepatocytes. While the liver perfusion technique and the LDH activity measuring method were being standardized for later use, a comparison of the two mediums, both supplemented with hormones (insulin and hydrocortisone), antibiotics (penicillin, streptomicine, and gentamycin) and L-glutamate, was undertaken to study which one gave the best results with or without supplementation with DMSO.

MATERIALS AND METHODS

Animals used

A total of seven male MF1 mice of three months of age were used for this study.

Collection of hepatocytes

The seven liver perfusions were performed on different days, following the technique described in the materials and methods chapter, with only one modification: after perfusing the liver, the cells were dispersed in Hank's balanced solution (the same buffer used for the perfusion) and divided into two equal volumes in sterile universal flasks (Sterilin, Hounslow, U.K.). After the last centrifugation, the buffer was discarded, and one of the universal flasks was filled with DMEM and the other one with WME, repeating this step in the two following centrifugations.

Experimental procedure

After centrifugating and resuspending, cells were counted and diluted to 1 x 10^6 cells/ml in their respective mediums. Three ml of cell suspension were pipetted into 60mm sterile petri dishes (Falcon, Becton Dickinson, U.K.). A total of 12 plates/replication were filled (6 plates/culture

medium). After three hours of incubation at 37°C and 5% CO_2 in air, the medium was replaced with fresh medium with or without DMSO. Three plates were filled with three ml/each of WME, three with WME supplemented with 2% (v/v) DMSO, three with DMEM and another three with DMEM + 2% DMSO. After one day of incubation at the same conditions samples of the supernatant and cells were collected to read LDH activity as described in the Materials and Methods chapter. The whole process was repeated seven times.

RESULTS

Tables V.I.2.1 and V.I.2.2 (page 76) show the LDH activity measured in cells and in the supernatant, respectively. The analysis of variance was used to assess the results and calculations were done separately for the supernatant and cells.

TABLE V.I.2.1.- Analysis of Variance for LDH in cells

Source	DF	SS	MS	F	P
Rep	6	0.316740	0.052790	8.99	0.000
Medium	1	0.008964	0.008964	1.53	0.232
DMSO	1	0.075608	0.075608	12.88	0.002
Medium*DMSO	1	0.004450	0.004450	0.76	0.395
Error	18	0.105692	0.005872		
Total	27	0.511455			

MEANS

Medium	N	LDH-cell
DMEM	14	0.36621
WME	14	0.40200

DMSO	N	LDH-cell
controls	14	0.43607
medium+DMSO	14	0.33214

TABLE V.I.2.2.- Analysis of Variance for LDH in supernatant

Source	DF	SS	MS	F	P
Rep	6	0.029120	0.004853	1.70	0.179
Medium	1	0.016321	0.016321	5.71	0.028
DMSO	1	0.000156	0.000156	0.05	0.818
Medium*DMSO	1	0.001032	0.001032	0.36	0.555
Error	18	0.051406	0.002856		
Total	27	0.098034			

MEANS

Medium	N	LDH-cell
DMEM	14	0.13286
WME	14	0.08457

DMSO	N	LDH-cell
controls	14	0.10636
medium+DMSO	14	0.11107

For the analysis of variance, the medium used (DMEM or WME), the treatment (2% DMSO or control) and replications were considered fixed effects. The design was a 2 x 2 factorial with seven replications. The results showed that DMSO had a negative effect on the cells ($F_{1,18}=12.9$, $P=0.002$). When averaged, LDH measured in cells treated with DMSO had a mean of 0.332 units whereas in controls the mean was 0.436. Also, WME gave a better result (0.402) than DMEM (0.366) but the difference was not statistically significant ($F=1.53$, $P=0.2$).

In the supernatant, a high value indicated cell damage. In this case there was a significant difference between the two media ($F_{1,18}=5.7$, $P=0.03$), with the means being 0.133 units for DMEM and 0.084 units for WME. Therefore, the use of WME appeared to result in less cell damage. However, for the supernatant, there was no significant difference between the control and the DMSO treated groups, and no significant medium x DMSO interaction effect.

DISCUSSION

It was suggested in the introduction that a variety of media could be used for hepatocyte culture. The two culture media available in this laboratory (DMEM and WME) were tried with the standard supplements (insulin, hydrocortisone, L-Glutamate, serum and antibiotics) recommended elsewhere. Among the hepatocyte culture enhancers mentioned in the

bibliography, Dexamethasone and DMSO seemed to be the most effective. However, dexamethasone had been reported to give poor results in combination with insulin (Laishes and Williams, 1976) which was being used as supplement, and it interferes with ethanol metabolism (increasing ADH activity, Crabb et al., 1989), so DMSO was prefered for the trials.

The results showed a slightly better performance of cells cultured in WME than in DMEM. WME is also a very popular culture medium among hepatotoxicologists, so it was chosen to be used in later studies with hepatocytes. On the other hand, the results obtained when the medium was supplemented with 2% (v/v) DMSO were surprising. Far from improving cell survival, a higher cell death was observed in those cultures treated with DMSO. A possible explanation was that the culture periods were too short to observe any improvement caused by DMSO, which normally appears afters several days of culture (Laishes and Williams, 1976; McMillan et al., 1991). These findings, together with the report by Isom et al. (1985) that DMSO alters the structure of proteins and nucleic acids and may directly alter gene expression led to the exclusion of DMSO, as it could jeopardize the objective of these studies.

V.I.3.- COMPARISONS OF BONE MARROW AND PERITONEAL MACROPHAGES

INTRODUCTION

Peritoneal macrophages constitute an appropriate type of cell for toxicological screening tests. However, although peritoneal macrophages gave reliable results in these studies, and their collection is quick and simple, a major drawback is the low yield of peritoneal macrophages per animal. One possible approach to this problem is the increase of macrophage production by means of inducing agents. This approach is discussed in chapter V.I.1 (Induction of macrophages using horse serum). Another possible solution is the use of alternative sources of macrophages, which is the subject of this chapter.

Macrophages can be found in the peritoneum, alveoli and in milk or colostrum (Klaus, 1987), with the peritoneum being the best source. Tissue macrophages, though often abundant, for example Kupffer cells of liver, are more difficult to obtain pure, because of the problems of dispersing cells from solid organs (Klaus, 1987). Also, it was decided to use the liver for obtaining hepatocytes rather than macrophage collection, as hepatocytes are many times more numerous than macrophages. Another alternative source of macrophages is the bone marrow. Macrophages arise from bone marrow myeloid cells by way of blood monocytes. The bone marrow contains a mixture of every blood cell type and

precursors, and has been often used as macrophage source. Although long-term cultures of bone marrow macrophages may need special culture conditions, a study was made to see if the number of macrophages present in the bone marrow was worthwhile without increasing experimental time or substantially altering the conditions already established for peritoneal macrophages.

MATERIALS AND METHODS

Animals used

Eighteen mice (nine males and nine females) of eight weeks of age from an outbred strain, MF1, were used in this experiment. Three males and three females were used in each replication.

Cell collection and culture

Peritoneal and bone marrow macrophages were collected as described in the materials and methods chapter. After cell counting and testing for erythrocytes, samples were pooled and diluted to $1x10^6$ cells/ml in culture medium (DMEM supplemented with 5% FCS and antibiotics). Half by volume of the sample was stained for Mac-1 and prepared to be run through a flow cytometer, the rest of the sample was plated out in 96-well plates (100μl/well of cell suspension, 50 wells/plate were used) following the protocols described

earlier. Plates were incubated at 37°C and 5% CO_2 in a humidified atmosphere, for the required period.

Assays

The macrophage staining technique, neutral red assay, and total protein determination were performed as described in the materials and methods chapter.

Experimental design

Each day, three males and three females were sacrified with CO_2 and bone marrow and peritoneal macrophages collected as described before. Half of the sample of bone marrow suspension and all the peritoneal suspension were used for macrophage staining and read in a flow cytometer (four samples/replication were read). The rest of the bone marrow sample was plated out (eight plates/replication were filled). After 1,3,5, and 7 days of incubation, total protein determination and neutral red assay were performed on different plates. The whole process was repeated three times.

RESULTS

An average of 54.7% of cells were stained by the Mac-1 technique in peritoneal suspensions, whereas only 32.4% of bone marrow cells were stained, showing that the proportion of macrophages was higher in peritoneal than in bone marrow suspensions. A t test (Snedecor and Cochran, 1980) of the

values obtained in the three replications gave a value of 4.0 ($P<0.001$), suggesting that the difference in proportion of stained cells in peritoneal and bone marrow samples was real.

Bone marrow cultures did not absorb neutral red dye after 1,3,5 and 7 days of incubation and the values measured in the culture medium never surpassed 1μg/ml. The protein measured in the plates was also very low and did not show the expected increase after incubation, an average of 18.4, 12.7, 14.1, and 10.6 μg of protein/ml were measured at 1,3,5 and 7 days of incubation, respectively.

DISCUSSION

Very low viabilities were detected by the neutral red assay and protein determination in cultures of bone marrow cells. Also, the number of macrophages detected by the Mac-1 technique in suspensions of bone marrow cells was very low, significantly lower than the number measured in peritoneal suspensions. Obviously, more work is needed for the development and culture of bone marrow macrophages. However, the aim of this experiment was the culture of bone marrow macrophages with the methods used for peritoneal macrophages, so that the time and expense of the general procedure were not increased. This was not possible as different nutrients and longer culture periods are needed for the development of macrophages from bone marrow cells.

Although bone marrow macrophages may be useful for specific studies, they were considered unsuitable for the projected protocol. Peritoneal macrophages are simple to culture and give reliable results, the low yield per animal being the only drawback. On the other hand, hepatocytes, the second type of cells included in the protocol, are many times more abundant than macrophages, and could to some extent reduce the number of animals needed in a screening protocol.

V.I.4.- COMPARISON OF COLOURIMETRIC METHODS

INTRODUCTION

As mentioned in the introduction (selection of cell methods) the measurement of cell death caused by test compounds is a good approach for assessing toxicity. The most widely used method for assessment of cell viability is trypan blue exclusion assessed microscopically (Paganuzi et al., 1981). However, it was rejected as an end-point for several reasons: it is subjective, depending too much on the reader's personal assessment (Holt et al., 1987), the results of the assay are affected by protein concentration and show great variability when compared with other methods (Singh and Stephens, 1986). Assays that involved the use of radiolabeled substances (e.g. ^{3}H.thymidine incorporation), although they are more objective and accurate, were also rejected because of the hazards of handling and disposal of radioactive materials. Enzyme leakage (LDH) and cell number were selected as viability assays for their later use as part of a battery of end-points.

However, as pointed out by several authors (Knox et al., 1986, Castell and Gomez-Lechón, 1987; Tyson and Green, 1987) there are a large number of chemicals that, although known to be highly toxic, would not be detected by viability assays, as these compounds impair cell function without causing cell death.

Other parameters such as mitochondrial activity, DNA synthesis, maintenance of membranes or protein content are considered more accurate (Knox et al., 1986). Several authors have described methods of measuring those parameters: crystal violet staining (Saotome et al., 1989), Neutral Red uptake (Parish and Müllbacher, 1983, Borenfreund and Puerner, 1985; Babich and Borenfreund, 1987; Fiennes et al, 1987; Borenfreund et al, 1988), Nitro Blue tetrazolium reduction (Segal, 1974; Rook et al., 1985), total protein determination (Bradford, 1976; Shopsis and Eng, 1985), MTT uptake (Mossmann, 1983; Holt et al., 1987), Methylene Blue (Olsson et al., 1982; Oliver et al., 1989), Kenacid Blue (Knox et al., 1986).

Another point to be taken into account is the time spent in performing the assay. When large numbers of individual cell cultures need to be counted, as happens in toxicology screening tests, where statistical analysis of a tissue culture experiment requires multiple replicates, a quick and reliable method is needed. Multiwell scanning spectrophotometers (ELISA readers) can measure large numbers of samples with a high degree of precision. Therefore, from the methods listed above, those that could be performed in multiwell scaning spectrophotometers and did not involve the use of hazardous products were selected. The methods studied in this experiment were: total protein determination, neutral red, kenacid blue and MTT uptake. All of them are colourimetric methods that can be quantified in multiwell readers and they measure different parameters.

The aim of this experiment was to adapt the assays

to the type of cultures used in these studies, to compare the results obtained and select those assays which were more reliable and informative.

MATERIAL AND METHODS

Animals used

Males from five mouse inbred strains (BALB/c, BXD-2, CBA/Ca, RIII-*ro*, and STS/A) and an outbred strain (MF1) of eight to twelve weeks of age were used. The strains were chosen on basis of their availability in the M.R.C. animal houses at Carshalton. A total of 90 mice (3 mice / strain/ replication) were used.

Assays

The colourimetric methods compared were Kenacid blue stain, neutral red uptake, MTT uptake and total protein determination. The assays were performed as described in the material and methods chapter. Cell number was also measured to establish the relationship between the assays and the number of cells present in the cultures.

Experimental design

Each day three mice/strain were sacrified (a total of 18/day) and peritoneal macrophages collected following the method described (see materials and methods chapter). Samples

were checked for erythrocytes and tubes from the same strain were pooled together in sterile universal flasks (Sterilin limited, Hounslow, U.K.). Cells were diluted to $1x10^6$ cells /ml adding the required amount of culture medium (DMEM). Individual wells of 96-well tissue culture microtitre plates (Falcon, Becton-Dickinson, U.K.) were inoculated with 100μl of cell suspension (12 wells/plate/strain). A total of eight plates/replication were filled.

After three hours, the plates were washed three times with PBS and 200 μl/well of medium containing six different concentrations of aspirin (0, 0.156, 0.312, 0.625, 1.25 and 2.5mM of Aspirin in DMEM supplemented with 5% FCS) were added (12 wells/concentration/plate) giving a final distribution of two wells/strain/concentration/plate. The treatment with Aspirin was included to find out the critical concentrations to be used in a later study. The location of strains and concentrations of Aspirin on each plate were changed in every replication to avoid experimental bias.

Assays were performed on the 3rd and 6th day of culture. One plate/assay/day was used. Cell number was counted on the plates used for total protein determination before performance of the assay. The protocols followed are described in the materials and methods chapter. The whole process was repeated six times.

RESULTS

All data were expressed as a proportion of the

non-aspirin control for each strain, day and replication. In the case of MTT some of the control values were extremely low, resulting in a few extremely high values which could clearly be identified as outliers. Thus, while most values were about equal to, or no more than two or three times the control values, a few were over 100 times the control values. Somewhat arbitrarily, all values over ten times the control were put in as missing values, resulting in 9/300 missing observations. In the case of protein there were three missing observations due to technical problems.

An analysis of variance (or general linear model in the two cases where there were missing values) was used to give an indication of the sensitivity of each assay method. An assay was assumed to be sensitive if it was capable of detecting the effects of strain, day or aspirin treatments with a high variance-ratio or "F" value. The analysis of variance for each of the five end-points, and the means for each strain, day and aspirin treatment are given in tables V.I.4.1 to V.I.4.6 (appendix), and a summary showing only results which achieved statistical significance is given in table V.I.4.7 (page 90).

According to this measure of sensitivity, protein gave the best results, followed by kenacid blue, with the other three methods being less sensitive.

As all results were expressed as a proportion of control values, and are therefore in the same units, a second measure of sensitivity might be the size of the experimental error variance. This is also given in table V.I.4.7. According

to this criterion, KB gave the best results, followed by cell number, protein and NRED with MTT giving the worst results.

Another point to be considered when choosing a battery of tests is whether each end-point is measuring the same basic character. This was judged by correlating each end point across all 60 observations, averaging across replications (5 strains x 2 days x 5 aspirin doses= 60 observations). The correlations are shown in table V.I.4.8 (page 90).

The results in this case are surprising. Although there were highly significant positive correlations between KB, NR and protein, the MTT results, and to a lesser extent the cell counts were negatively (and significantly) correlated with these three. The greater the KB staining, the less the MTT reduction. This was not due to a few abberant values; it was a general trend observed across all 60 treatment combinations, and can also be seen when looking at the strain means. For example, CBA/Ca cells tended to have the highest KB staining and lowest MTT reduction across all ten of the day x aspirin treatments, whereas the opposite was true with strain STS/A.

TABLE V.I.4.7.- Summary of the analysis of variance results for comparison of the colourimetric methods.

Source	DF	KB	MTT	NRED	PROT	CELL
rep	5	***	***	***	***	***
Str	5	***	***	**	***	***
day	1	***	NS	*	***	NS
asp	4	**	NS	NS	***	NS
Str*day	5	*	***	*	***	***
Str*asp	20	NS	NS	NS	NS	NS
day*asp	4	NS	NS	NS	NS	NS
Str*day*asp	20	NS	NS	NS	NS	NS
Error	295	0.014	0.791	0.567	0.118	0.063

NS p>0.05
* p<0.05
** p<0.01
*** p<0.001

TABLE V.I.4.8.- Correlations among the five end-points across 60 observations.

	KB	MTT	NRED	PROT
MTT	-0.531**			
NRED	0.369**	-0.274*		
PROT	0.699**	-0.288*	0.477**	
CELL	-0.121	0.569**	-0.089	-0.012

* p<0.05
** p<0.01

DISCUSSION

Several aspects should be considered in the selection of the colourimetric methods to be included in a general screening test, including the capability of detecting differences, the parameters measured and the experimental error obtained in the different assays.

One of the aims of this project was to study genetic variation in response to drugs using inbred strains of animals. Accordingly to this, those colourimetric methods which detected variability between strains should be preferred. Thus, protein determinations and Kenacid blue staining should be selected, as they were the most sensitive assays as shown by the analyses of variance.

However, a selection of end-points measuring different parameters has been suggested by several authors (see introduction) for general toxicology screening tests. In this way, chemicals that affect different aspects of cell function without causing cell death have more chance of being detected in a screening. Protein determination and kenacid blue staining are highly correlated so must be measuring very similar parameters, the protein content of cells. Therefore, only protein determination (easier to perform than kenacid blue staining) was included in the protocol.

The MTT assay was relatively insensitive and presented a number of technical problems, leading to many

missing values. Thus, although it appears to measure a completely different parameter from protein, it was discarded as being impractical.

Finally, protein determination and neutral red uptake were the colourimetric methods included in the protocol, which also included cell number. The counting of cells was later abandoned as being too time consuming, and the protocol was further modified with the inclusion of non colourimetric end-points (LDH leakage and rate of phagocytosis) measuring different aspects of cell viability and function.

Some comments should be made about the inclusion of non-colourimetric end-points, which increases the time and expenses of the projected protocol. The inclusion of measurement of LDH leakage and rate of phagocytosis provided useful information. No colourimetric method could be developed for obtaining similar information with the equipment and techniques available at the time of these studies. However, new colourimetric techniques are constantly published. The large volume of data obtained in a short time by colourimetric methods make them quite useful in screening tests. Time consuming assays (e.g. LDH leakage) will eventually be substituted by colourimetric techniques, and a quick and reliable battery of colourimetric methods will be devised for genetic and toxicological screening.

V.I.5.- EFFECT OF PRIOR TREATMENT WITH NEUTRAL RED ON TOTAL PROTEIN DETERMINATION

INTRODUCTION

Babich and Borenfreund (1987) combined two of the colourimetric methods included in these studies. The Neutral Red assay was followed by total protein determination on the same plates. Assuming both tests are necessary, this constituted a saving of time and test animals. However, when this procedure was tried, a reduction in the total protein in plates was observed when measured after performance of the neutral red assay. This reduction could affect the results and should be taken into account when interpreting data obtained in this way (Arranz and Festing, 1990). The following experiment was set up to study this protocol.

MATERIALS AND METHODS

Animals used

The assays were done on peritoneal macrophages collected from two mouse strains, outbred MF1 and inbred CBA/Ca. A total of 6 males (3 mice/strain) were used.

Collection and culture of cells

Peritoneal cells were collected following the method described in the materials and methods chapter. Briefly, samples were checked for erythrocytes and diluted to $1x10^6$ cells/ml in DMEM culture medium. One hundred μl of cell suspension were dispensed into 96-well Titertek plates. After three hours of incubation at 37°C, in an atmosphere of 5% CO_2 in air, the plates were washed three times in phosphate buffered saline (PBS) to remove nonadherent cells, and 200μl of DMEM containing 5% foetal bovine serum (FCS) was added to each well, and plates were returned to the incubator.

Assays

The neutral red assay (NR) and total protein determination were carried out as described in the materials and methods chapter. When neutral red and protein determination were performed on the same plate, the neutral red extraction solution was discarded by inverting the plate and total protein determination started with the three initial washes with phosphate buffer saline (PBS, pH=7.2) described in the methods section.

Experimental design

Plates containing cells from both mouse strains were cultured for 1,3,5 or 7 days. Two 'treatments' were used, the protein determination alone was done on half of the plates,

and the NR assay followed by the protein determination was done on the rest of the plates. A total of 30 wells/strain/plate were used but these were averaged before the data were analysed statistically. The experiment was repeated three times on different days using one mouse/strain/day, giving a total of 3 replicates x 4 time points x 2 strains x 2 treatments = 48 observations (each an average of 30 wells) of protein either with or without previous NR determination in a randomized complete block factorial design (Snedecor and Cochran, 1980). The data (protein) were analysed by the analysis of variance, both with and without a logarithmic transformation.

RESULTS

The mean protein estimations (on the arithmetic scale) according to the various treatments are shown in table V.I.5.1 (page 98). There were large and statistically significant differences between treatments (i.e. with or without prior NR assay; $P<0.01$), strains ($P<0.01$) and days ($P<0.01$), the total protein was 29.8μg/ml without prior NR assay of the cultures, and 14.3μg/ml with prior NR assay, a reduction of 15.5 μg or 52%.

Strain CBA/Ca averaged 13.2μg protein/ml, compared with 30.9μg/ml in MF1. This reflects a strain difference in the proportion of adherent cells among resident peritoneal cells (Festing et al., 1990), and the greater growth and/or

adherence of such cells from strain MF1 than from CBA/Ca, as noted by Festing and Eydmann (1987a,b). Averaged across both strains, the total protein increased from 16.9μg/ml on day 1 to 28μg/ml on day 7. On the arithmetic scale, there was a significant interaction effect between strain and treatment ($P<0.01$). When averaged across days the protein estimates in strain CBA/Ca decreased from 17.7 to 8.8μg/ml, a difference of 8.9μg/ml with prior NR assay. In strain MF1 the reduction was from 42.0 to 19.9μg/ml, a significantly greater difference of 22.1μg/ml. However, after a logarithmic transformation of the data, this interaction disappeared. This implies that the reduction in protein was proportional to the amount of protein in the well, and that in analysing data of this sort care should be taken to choose a suitable measurement scale.

DISCUSSION

In conclusion, the sequential use of the NR assay followed by total protein led to a reduction of about 50% in the amount of protein estimated to be present in macrophages cultured in 96-well plates. Clearly, a substantial fraction of the protein is leached or otherwise lost as a result of the NR assay. This finding does not necessarily invalidate the sequential use of these two assays. When this study was carried out, some results on genetic variation in the response to chemicals had already been obtained, and the NR assay and protein determinations had been performed on different plates.

In later studies, both assays were also performed on different plates, as a reduction in the protein values could affect comparisons to the earlier results. However, the procedure described by Babich and Borenfreund (1987) seems a good approach as it produces extra information with a low animal cost. Care should however be taken to ensure that an appropriate scale of measurement is used.

TABLE V.I.5.1.- Total protein determinations for mouse strains CBA/Ca and MF1 after 1-7 days of culture either with (NR) or without (No NR) prior NR assay.

	TOTAL PROTEIN (μg/ml)			
	CBA/Ca		MF1	
DAY	NR	No NR	NR	No NR
1	8.9	14.3	17.4	26.9
3	7.9	16.3	17.8	39.7
5	8.8	17.9	18.7	47.0
7	9.7	22.2	25.5	54.6
Mean	8.8	17.7	19.9	42.0

The pooled within-group standard deviation is 6.06, and the least significant difference (P=0.05) between any two means in the body of the table is 10.9 μg. The statistical significance of other comparisons is discussed in the text.

PART II: STUDY OF GENETIC VARIATION IN RESPONSE TO ASPIRIN, COUMARIN AND ETHANOL

V.II.1.- GENETIC VARIATION IN THE RESPONSE TO ASPIRIN

INTRODUCTION

Although Aspirin (acetylsalycilic acid) is one of the most widely used drugs, its pharmacology was not elucidated until the mid-seventies (Levy, 1979). Aspirin is rapidly hydrolized to salicylic acid in man and in rats (Levy, 1979; Wientjes and Levy, 1988). Salicylic acid is excreted or metabolized to gentisic acid and then excreted as such. Aspirin hydrolisis activity is found in most mammalian tissues (Wientjes and Levy, 1988).

Little can be found in the literature regarding the genetics that control the response to aspirin. Festing and Eydmann (1987a,b) found differences in the response to Aspirin among 21 strains of mice that suggested a multigenic control. However, some strains were particularly sensitive to Aspirin (RIII-*ro*) and two *H-2* congenic strains (BALB/c and BALB.K) also appeared to differ significantly, suggesting a possible relation between the *H-2* complex and drug response. Thus, it was thought to be worth checking the results obtained by Festing and Eydmann (1987a,b), specially those related to the *H-2* complex, using a technique that included several toxicity assays.

However, at the beginning of this study, few techniques had been developed, and in the first study

(experiment 1) only a single toxicity assay was used (total protein determination). In the second part of this study (experiment 2), the involvement of the *H-2* locus in the control of the response to aspirin was studied using a set of inbred strains (including some *H-2* congenic strains) using a technique that included three toxicity assays (neutral red uptake, total protein determination and cell number).

MATERIALS AND METHODS

- EXPERIMENT 1 -

Animals used

Males from 15 inbred/hybrid strains and one outbred stock of mice were used for this experiment: BALB/c, CBA/Ca, CBA/CaNimr, CBA/N, C57BL/Ks, C57BL/10ScSn, LP, B10CF1, CBCF1, CBB10F1, A/J, AKR, C3H/HeJ, C57BL/6J, DBA/2, and an outbred strain, MF1.

Collection of macrophages

Macrophages were collected following the technique described in the materials and methods chapter.

Assays

Total protein content was measured following the protocol described in the materials and methods chapter.

Experimental design

Each day one mouse/strain was sacrified, samples were checked for erythrocyte contamination and diluted to $1x10^6$ cells/ml in DMEM culture medium. 100µl/well of cell suspension were pipetted in 96-well plates. Fifteen wells/sample were filled. Four plates per replication and four strains per plate were used. The location of strains in the plates was changed at random in every succesive replication. After three hours of incubation at 37°C, 5% CO_2, the culture medium was removed and plates were washed three times with prewarmed (37°C) PBS. Plates were supplied with 200µl/well of treatment-containing medium (0, 1 and 5mM Aspirin in DMEM). Five wells/concentration/strain were used. Ater six days of culture the total protein content was determined on the plates following the method described in the materials and methods chapter. The whole procedure was repeated five times. A total of 80 animals (1 mouse x 16 strains x 5 replications) were used for this experiment.

-EXPERIMENT 2-

Animals used

Five inbred strains, AKR, CBA/Ca, LP, STS/A, BXD-2, three *H-2* congenic inbred strains, BALB.B, BALB/c, BALB.K and an outbred strain, MF1, were used in this experiment.

Collection of macrophages

Macrophages were collected following the technique described in the materials and methods chapter.

Assays

Total protein content, neutral red uptake and cell numbers were measured as described in the materials and methods chapter.

Experimental design

The nine strains were divided in three groups. The groups were changed randomly through replications. Every day, two mice/strain from a group were sacrified (three days were needed to obtain samples from the nine strains) and macrophages collected following the technique described in the materials and methods chapter. Samples were checked for erythrocyte contamination and diluted to $1x10^6$ cells/ml in DMEM culture medium. One hundred μl/well of cell suspension were pipetted in two 96-well plates. Fifteen wells/ strain/plate were used (a total of six plates/replication). After three hours of incubation at the standard conditions, plates were washed and supplied with 200μl/well of

aspirin-containing medium (0, 1 and 3mM Aspirin in DMEM+5%FCS). Five wells/ concentration/ strain/plate were used. After six days of culture, cell counts and total protein determinations were performed on one plate and the neutral red assay on the other plate, following the methods described in the materials and methods chapter. The whole procedure was repeated five times. A total of 90 animals (2 mice x 9 strains x 5 replications) were used for this experiment.

RESULTS

Before statistical analysis, all data were divided by their respective controls to standarise the scale. Table V.II.1.1 (page 105) summarizes the statistical analyses for both experiments.

EXPERIMENT 1.- Table V.II.1.3 (appendix) contains the protein data obtained in this first study. The analyses of variance of the protein content measured in macrophage cultures is also shown in the appendix (table V.II.1.2). The three main factors, Strains ($F=3.16$, $P=0.001$), concentration of aspirin ($F=136.16$, $P<0.001$) and replication ($F=15.11$, $P<0.001$), had large significant effects on the protein contents. The interaction Strain x Concentration of aspirin was significant ($F=4.53$, $P<0.001$). Figure V.II.1.1 (page 107) shows the

Table V.II.1.1. Summary of statistically significant effects for macrophages treated with aspirin.

Treatment	Cell type	End-point	Str (1)	Sex (2)	Str* Sex (3)	Conc (4)	Str* Conc (5)	Sex* Conc (6)
Aspirin	Macrophage	Prot(exp.1)	***			***	***	
		Prot(exp.2)	NS			NS	NS	

(1) Main effect of strain, equivalent to mean elevation of the dose-response line.

(2) Main effect of sex

(3) interaction of the above two factors

(4) Concentration of aspirin

(5) Strain x concentration interaction, equivalent to the slope of the dose-response curve for each strain.

(6) Sex x concentration interaction, equivalent to the slope of the dose-response curve for each sex.

NS = $p>0.05$
* $p<0.05$
** $p<0.01$
*** $p<0.001$

graphic representation of the results. All strains showed an increase on the protein content at the lowest concentration of aspirin (1mM), this may indicate that at low concentrations of aspirin the viability and/or cell attachment is enhanced by aspirin (cultured plates were washed before total protein content was determined, thus dead and unattached cells were theoretically removed). However, at higher concentrations of aspirin, the protein content decreased in all strains. AKR, LP, and MF1 were the most resistant strains, and C3H/HeJ, C57BL/6J and A/J the most sensitive.

EXPERIMENT 2.- Table V.II.1.5 summarises the results. The analyses of variance for protein is also shown in the appendix (table V.II.1.4). Results obtained with the neutral red assay were very low, and were not analysed as they were considered unreliable. Data on number of cells were also discarded, as at high concentrations of aspirin (5mM) dead, live and fragments of cells were not distinguishable and cell counting was unreliable.

Figure V.II.1.2 (page 108) shows the graphic representation of the protein results. Again, an increase in the protein measured was observed in all strains at low concentrations of aspirin. This effect was sustained in some strains (LP, CBA/Ca, and STS/A) at higher concentrations of aspirin (5mM), whereas in others (AKR, BXD-2, BALB/c, BALB.B, BALB.K and MF1) a decrease in protein was observed. The increase in protein at high aspirin concentrations was

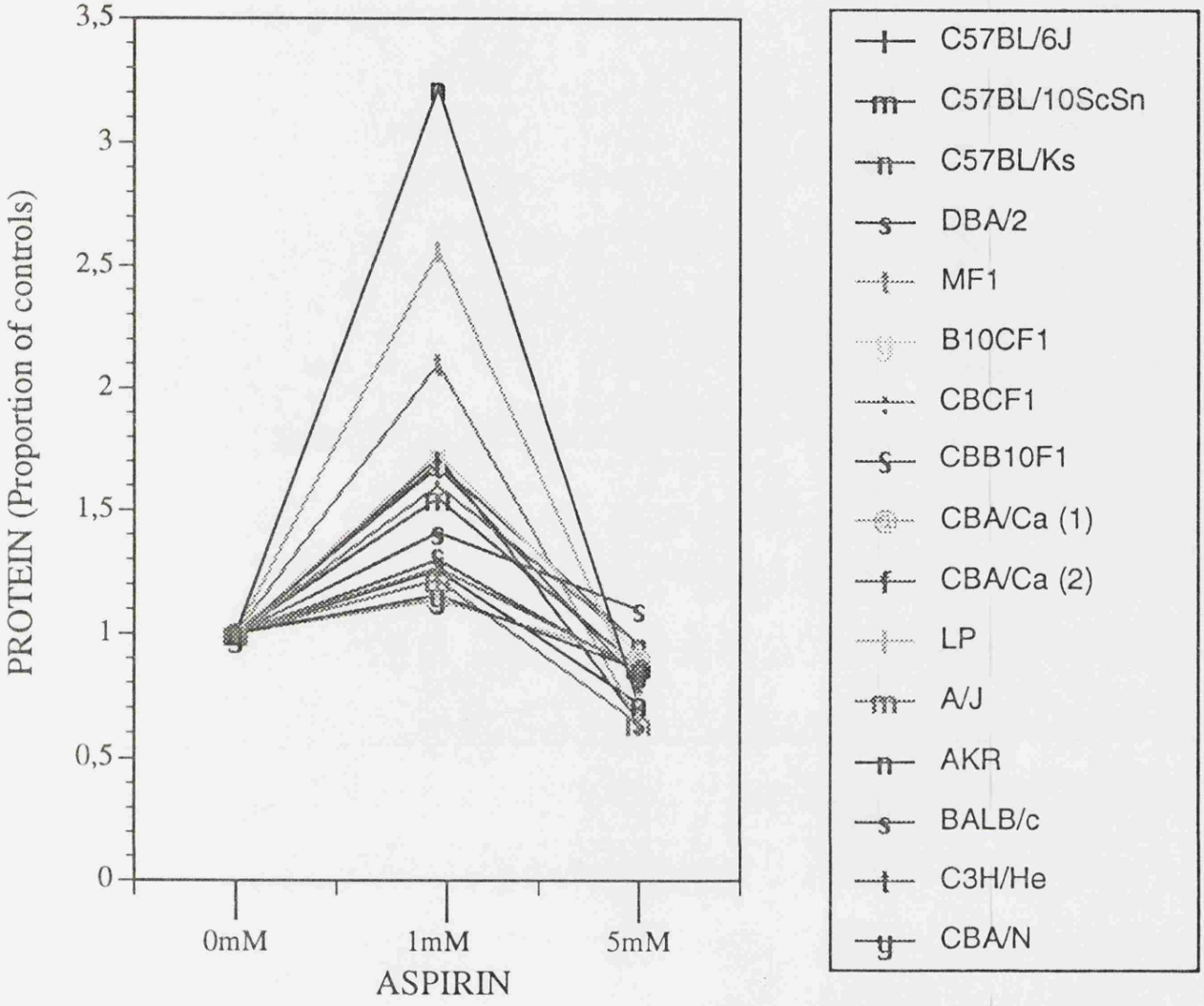

FIGURE V.II.1.1.- Protein measured in macrophage cultures treated with aspirin (experiment 1).- All strains showed an increase on the protein content at the 1mM aspirin level. However, at the 5mM level, the protein content decreased in all strains. A/J, CBA/N, and MF1 were the most resistant strains, and C3H/HeJ, C57BL/6J and LP the most sensitive.

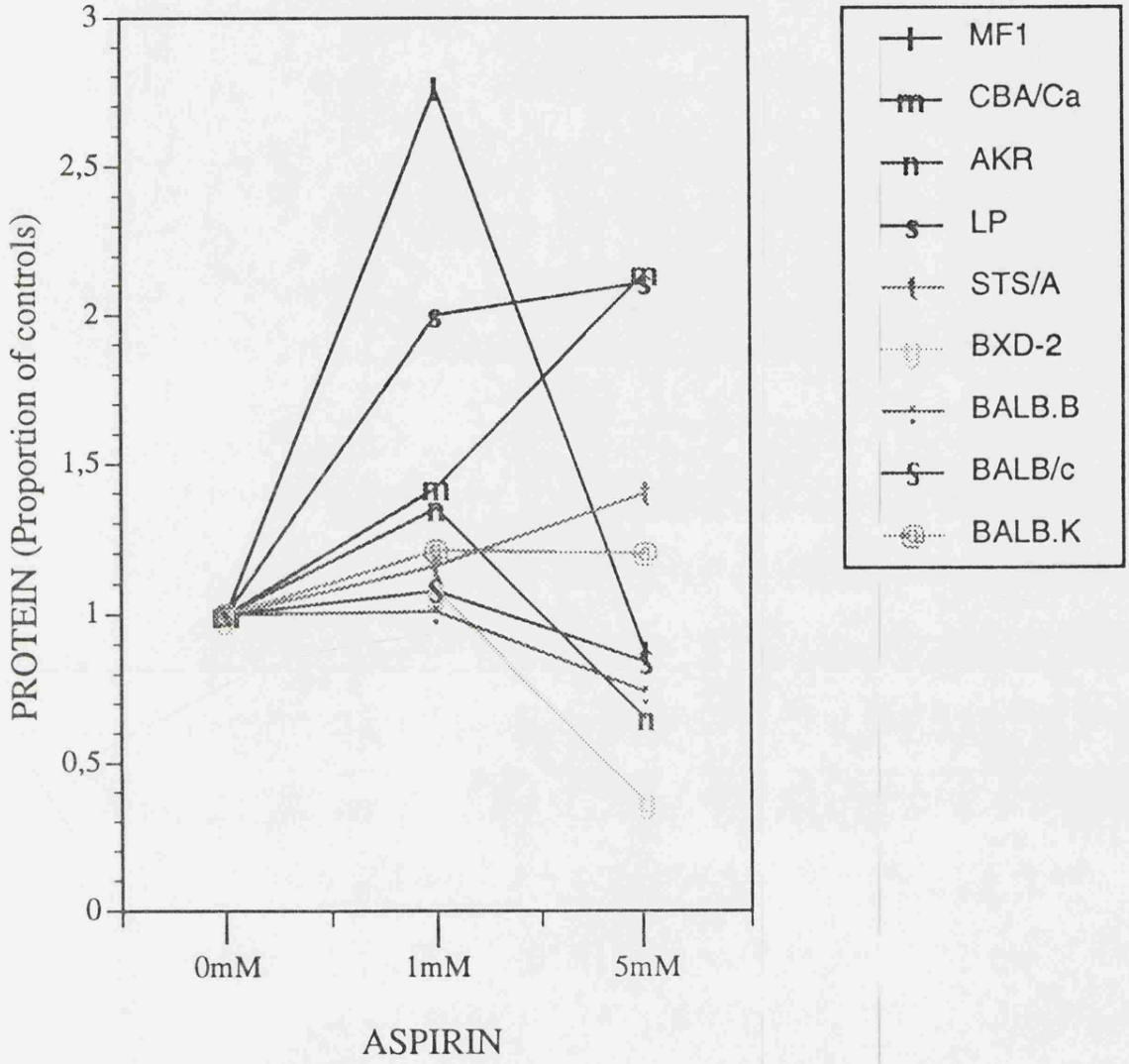

FIGURE V.II.1.2.- Protein measured in macrophage cultures treated with aspirin (-experiment 2-).- An increase in the protein content was observed in all strains at low concentrations of aspirin (1mM). This effect was sustained in some strains (LP, CBA/Ca and STS/A) at the 5mM level, whereas in others (AKR, BXD-2, BALB/c, BALB.B, BALB.K and MF1) a decrease in protein was observed. H-2 congenic strains BALB.B, BALB/c and BALB.K showed similar results.

unexpected, especially because two of the strains that showed it, LP and CBA/Ca, had not shown such an increase in the first experiment at the same concentration of aspirin. This difference may be attributed to experimental error. The analyses of variance for protein content showed significant differences among strains ($F=2.78$, $P=0.019$). However, the concentration of aspirin only showed slight effects on the results ($F=2.74$, $P=0.017$). No significant interactions were observed.

DISCUSSION

In the first part of this study (experiment 1) several inbred strains of mice were used to study genetic variation in the response to aspirin. The set of strains included some previously used in similar studies by Festing and Eydmann (1987a,b) (BALB/c, C57BL/6J, CBA/Ca, and DBA/2), two identical strains (CBA/Ca(1),bred at the M.R.C. in Carshalton, and CBA/Ca(2) or CBA/CaNimr, bred at the National Institute of Medical Research in Mill Hill) obtained from different suppliers and several others. The effect of the treatment with aspirin was measured by determining the total protein content of cell cultures. The aim of this experiment was to set up the standard culture conditions and check the reliability of the results by comparing them with those

obtained by Festing and Eydmann (1987a,b), as well as getting further information on the genetic control of the response to aspirin.

Significant strain differences were found and there was a significant interaction between strains and the concentration of aspirin. This agreed with the results obtained by Festing and Eydmann (1987a,b), although the strain means differed slightly. Similar results were obtained in strains CBA/Ca(1) and CBA/Ca(2). These results support the reliability of the procedure followed.

In a second experiment, three toxicity assays (neutral red uptake, total protein determination and cell number) were used, although the technique had not yet been developed on hepatocytes and the study was performed on macrophages only. Festing and Eydmann (1987a,b,) suggested that the *H-2* locus may be involved in the control of the response to aspirin, thus a set of *H-2* congenic strains was included to investigate this claim.

The neutral red results and cell numbers were discarded as unreliable. Significant strain differences and strain-concentration of aspirin interactions were observed with the protein determination assays.

When looking at the graphic representation of data, some unexpected results were observed. The *H-2* congenic strains BALB.B, BALB.K and BALB/c showed similar responses (see figure V.II.1.2). This fails to support the involvement of the *H-2* locus in the control of the response to aspirin treatment suggested by Festing and Eydmann (1987b). However

this point needs further study with more H-2 congenic strains. Some strains that showed resistance at the 1mM aspirin concentration were sensitive to 5mM aspirin (AKR, MF1, LP, BXD-2), and some strains that showed sensitivity to 1mM aspirin were resistant to 5mM aspirin (BALB.B, BALB.K, BALB/c, STS/A). These results should be considered with caution, as they are based on the data of only one end-point. Moreover, in later studies on the genetic variation in response to coumarin and ethanol differences were also observed between the results obtained in several assays. Thus, before drawing more conclusions based on a few data, this study was postponed until a technique to study genetic variation including several toxicity assays and cell types was developed. Moreover, it was preferred to test the technique by studying the response to a drug where there is some information of possible genetic control. Thus, the study of genetic variation in response to aspirin was put aside until a better technique to study such response had been developed and examined.

In summary, the general culture conditions were established and found to be reliable when compared to other authors' methods. However, little information was added to the knowledge of the response to aspirin. Further study was postponed until an improved technique had been developed.

V.II.2.- GENETIC VARIATION IN RESPONSE TO COUMARIN

INTRODUCTION

Coumarin is metabolized initially to hydroxycoumarin prior to phase II conjugation (Egan et al., 1990; Lake et al., 1989). Differences in liver microsomal coumarin 7-hydroxylase activity among inbred strains of mice have been reported by several authors. Wood and Conney (1974) and Wood and Taylor (1978) first discovered that basal and phenobarbital-induced coumarin 7-hydroxylase activity levels were regulated by the *Cyp2b* locus in inbred mice and mapped the locus to mouse chromosome 7. They found that basal and phenobarbital-induced rates of hepatic metabolism of coumarin to 7-hydroxycoumarin were markedly higher in DBA /2J mice than in the AKR/J, C57BL/6J, and C3H/HeJ strains. Backross and intercross mating of DBA/2J and AKR/J mice and their offspring indicated that coumarin hydroxylase activity is inherited as an autosomal trait controlled principally by a single locus.

Lush and Andrews (1978) studied coumarin in 19 strains of mice and found that they fell into 3 groups: high, intermediate and low metabolizers. These authors suggested the existence of two very closely linked genes (*Coh*-1 and *Coh*-2). In preliminary experiments they found that the gene determining the difference between the low group and the medium group is indeed linked to *Gpi*-1 on chromosome 7.

Simmons and Kasper (1983) found that coumarin hydroxylase is part of the cytochrome P450 enzyme system. The cytochrome P-450s are important microsomal and nuclear envelope enzymes which perform a critical cellular role in the oxidation of endogenous and foreign compounds. These cytochromes, though functionally related, comprise a family of enzymes that differ from one another in primary structures, substrate specifities, antigenic characteristics, and spectral properties as well as in their induction response to various xenobiotics.

Juvonen *et al*. (1985) found that pyrazole strongly induced coumarin 7-hydroxylase activity in DBA/2J mice. The group subsequently purified a coumarin-metabolizing P-450 isoenzyme from the drug-treated mice by using the high-spin spectrum of the P-450-complex as the basis for the selection of fractions from columns. This P-450 isoenzyme appeared to be the major enzyme specific to coumarin 7-hydroxylase activity in mouse liver microsomes (Juvonen et al, 1988).

A difference in the amount of P-450coh protein between the D2 and AKR mice was found to be the reason for the differences in the enzyme activity between the two mouse strains by Lang et al. (1989). Accordingly, changes at the regulatory level rather than at the structural gene would explain the genetic difference in the activity of coumarin 7-hydroxylase. Interindividual and intergroup variation in the high and low activity groups suggest that minor modifying genes may contribute to the final enzyme activity.

Negishi et al. (1989) identified type II P-$450_{15\alpha}$ as

mouse coumarin 7-hydroxylase (P-450coh). The steroid 15α-hydroxylase gene family consists of 15α-hydroxylase (type I $P\text{-}450_{15\alpha}$) and coumarin 7-hydroxylase (P-450coh or type II $P\text{-}450_{15\alpha}$). Gene *15αOH-2*, which encodes the coumarin 7-hydroxylase, might be the *Coh* locus itself.

In a review of the P450 superfamily by Nebert et al. (1989) a new nomenclature was proposed. The root symbol "*CYP*" (in capitals for human and lower case for mouse) denoting "cytochrome P450" was recommended. For a chromosomal locus, the root symbol was to be followed by an Arabic numeral designating the P450 family, and a letter designating the family. According to this recommendations, the previously named *Coh* locus was converted to the *Cyp2b* locus.

Studying coumarin-treated cells from inbred strains of known Cyp2b activity should show whether genetic variation at this locus is associated with variation in response to coumarin.

MATERIALS AND METHODS

Animals used

Eight to twelve week old mice from eight inbred strains (DBA/2, CBA/Ca, RIII-*ro*, C57BL/6J, STS/A, BXD-2, BALB/c, and BGA) and one outbred strain MF1 were used. All of them were from the MRC Toxicology Unit. These strains were

TABLE V.II.2.1.- List of strains used and the *Cyp2b* genotype described in the literature and the basal and phenobarbitone-induced activity measured by Eydmann et el. (1991, unpublished data).

STRAIN	*CYP2B* ACTIVITY (pmol/min/mg) BASAL	INDUCED	GENOTYPE	REFERENCES
DBA/2	86	262	*Cyp2b*-^{h}	Wood and Conney (1974) Lush and Andrews (1978) Juvonen et al. (1985) Negishi (1989) Lang et al. (1989) Eydmann et al. (1991)
CBA/Ca	67	101	*Cyp2b*-^{l}	Wood and Taylor (1979)
			Cyp2b-^{h}	Eydmann et al. (1991)
RIII-*ro*	33	80	unknown - presumed intermediate	
C57BL/6J	19	40	*Cyp2b*-^{l}	Wood and Conney (1974) Lush and Andrews (1978) Juvonen et al. (1985) Eydmann et al. (1991)
STS/A	29	38	unknown - presumed low	
BXD-2	82	255	*Cyp2b*-^{h}	Legraverend et al. (1984) Eydmann et al. (1991)
BALB/c	19	25	*Cyp2b*-^{l}	Eydmann et al. (1991)
BGA	---	---	*Cyp2b*-^{h}	Eydmann et al. (1991)
MF1	outbred stock			

chosen on the basis of their *Cyp2b* allele and their availability. DBA/2 and BXD-2 are *Cyp2b*$^{-h}$, C57BL/6J and BALB/c are *Cyp2b*$^{-l}$, BGA has the *Cyp2b*$^{-h}$ allele on a C57BL/Ola geneticbackground so C57BL/6J and BGA approximate a pair of congenic strains differing at the *Cyp2b* locus. Strain CBA/Ca has the *Cyp2b*$^{-h}$ genotype, but expression of the enzyme is low and it was apparently miss-classified as *Cyp2b*$^{-l}$ by Wood and Taylor in 1979 (Eydmann et al., 1991). Table V.II.2.1 (page 115) summarizes the list of strains used in this experiment and the *Cyp2b* genotype mentioned in the literature.

Coumarin dose

The coumarin-containing medium was prepared fresh every day as follows:

Coumarin (17.5 mg) was dissolved in 200µl of Ethanol, and then diluted to 5mM in 25ml of prewarmed (37° C) culture medium (DMEM + 5% FCS for macrophages, WME + 5% FCS for hepatocytes). The solution was then filtered (0.2um, Minisart NML, Sartorius, Germany). Thereafter, two different protocols were followed for macrophages and hepatocytes.

- Macrophages: Three ml of 5mM coumarin solution were diluted in 45 ml of DMEM + 5% FCS to 0.626mM, 0.312mM, 0.15mM and 0.07mM in culture medium. Once the treatment was ready, one hundred µl/well of coumarin-containing medium were added to the 100 µl/well of cell suspension contained in the 96-well plates (5 wells/concentration-including

controls/sex/strain were filled: 25 wells/mouse) and one and a half ml/well were added to the cell-containing 25-well bacteriological plates (one well/concentration/sex/strain= 5 wells/sample). Thus, the final concentrations were 0, 0.035mM, 0.07mM, 0.15mM and 0.312mM of coumarin solution.

- Hepatocytes: One and a half ml of 5mM coumarin solution were diluted in 46.5ml of prewarmed (37°C) WME + 5% FCS to give a concentration of 0.312mM. This concentration was successively diluted with WME + 5%FCS to obtain 0.15mM, 0.07mM and 0.035mM coumarin dilutions. After a initial period of culture of three hours, the medium contained was replaced by three ml of the treatment-containing medium. Three petri dishes/concentration/sex/strain were filled (15 plates/mouse) and returned to the incubator until the following day, when the end-points were performed.

Assays

Neutral red absorption assay, phagocytosis rate and protein determination were performed on macrophages, and neutral red absorption assay, LDH activity measurements and protein determinations were performed on hepatocytes.

Before starting the experiment, several trials were done to find the period of culture required to obtain the most informative results. With macrophages, the phagocytosis rate was studied after 16-20 hours of culture with the treatment, neutral red absorption on the third day of culture and protein

determinations were done on the fifth. With hepatocytes, all assays were performed after 16-20 hours of incubation with the treatment-containing medium.

Experimental design

A different protocol was followed for macrophages and hepatocytes:

- Macrophages.- Peritoneal macrophages were obtained following the standard method from the nine strains of mice listed above. Three animals/ sex/ strain/ replication were used. The nine strains were divided in groups of three, and every day a different group was used (3 animals x 2 sexes x 3 strains = 18 animals/day). The experiment was replicated three times, making a total of 162 animals used (the same 162 animals as used in the ethanol experiment). Different groups of strains were used in each replication in order to avoid bias due to "day" effects.

After collection, samples were pipetted into 96-well plates (2 plates/sex/strain = 25 wells/plate = 50 wells/sample) and into 24-well bacteriological plates (5 wells/sex/strain). After three hours of incubation, coumarin-containing medium was added.

- Hepatocytes.- On each day, one male and one female of the same strain of mice was used for liver perfusions. From a single liver enough cells could be obtained to perform the coumarin and ethanol studies. The suspension of

hepatocytes was diluted to $25x10^4$ live cells/ml in culture medium. Fifteen plates/sex/strain were filled. After 16-20 hours of incubation the end-points were studied. The process was repeated three times, and a total of 54 animals (9 strains x 2 sexes x 3 replications) were used to obtain hepatocytes for this and the ethanol experiments.

STATISTICAL ANALYSIS OF THE RESULTS OF THE STUDIES ON COUMARIN AND ETHANOL.

Each individual end-point (Phagocytosis, neutral red, protein, LDH supernatant, LDH cells) for a particular cell type (macrophage or hepatocyte) and drug (ethanol, coumarin) was analysed as a separate experiment using a general linear model analysis of variance (14 separate analyses). A split-plot design was used with the 54 main plots formed by the 9 strains x 2 sexes x 3 replications within sexes and strains. The split plots were the concentrations expressed relative to the control. In the case of the LDH supernatant end-points, the data were transformed to Y=1/X in order to normalise the data. One result of this is that the Y values decrease with increasing drug concentration, like the other end-points.

In all cases, plots of fitted versus residual values were made to ensure that a suitable scale had been used. Note that in the main plots the effect of strain and sex (averaged over all dose levels) is to be compared with the replicate within strain and sex mean square as the error term. A

significant strain or sex effect would imply that the mean value for the strain or sex averaged across all dose levels differed between strains at the relevant level of significance.

In the split plots, a significant strain x dose effect would imply that the strains did not behave uniformly with respect to the dose levels. A significant strain effect in the main plots, or a strain x dose effect in the split plots would both imply genetic variation in response to the test compound with that particular end-point.

Missing data

The statistical analysis was done using the General Linear Model program of MINITAB. Although this can tolerate some missing observations, any "nesting" must be balanced. Thus with nine strains, two sexes and three replications nested within the strains x sexes, it would not work if all data from a single individual (out of 54 mice) was missing.

As some observations were unavoidably missing, due in most cases to the difficulty of getting sufficient animals of each strain and sex, it was necessary to take account of such missing data before the analysis could be carried out.

If data from a single individual was missing, data from one of the other individuals of the same strain and sex was chosen at random to fill the gap (Replicate substitution in table V.II.2.2, page 121). Where more than one individual of a strain was missing, the analysis was carried out omitting

that strain (The strain omitted is noted in table V.II.2.2. Note that the strain was only omitted from the analysis of variance. Data on remaining individuals is still shown in the graphs). In a few cases individual observations (i.e. concentration within cell sample) were missing. This caused no problems except when the missing observation was the control (zero dose level). In this case, regression on the remaining values was used to estimate the missing value (Number of controls estimated is given in table V.II.2.2). Although this may not have been the optimum way of dealing with the missing data, it is unlikely to have caused serious bias as at most the analysis was done with 2/54 missing observations replaced in this way. A summary of the above actions is given in table V.II.2.2:

TABLE V.II.2.2.- Summary of missing observation substitutions.

Cell type	Endpoint	Drug	Replicate substituted	Strain omitted	Control estimated
Macrophage	Phag.	Ethanol	---	Strain 9	---
	Prot.	Ethanol	---	---	---
	NRed	Ethanol	---	---	---
	Phag.	Coumarin	Strain 3	Strain 1	---
	Prot.	Coumarin	Strs. 1&3	---	---
	NRed	Coumarin	---	---	---
Hepatocyte	LDH-S.	Ethanol	Strs 3&4	---	---
	LDH-C	Ethanol	Strain 3	---	1
	NRed	Ethanol	---	---	---
	Protein	Ethanol	---	Strain 3	---
	LDH-S	Coumarin	---	---	1
	LDH-C	Coumarin	---	---	1
	NRed	Coumarin	Strain 4	---	---
	Prot.	Coumarin	---	Strain 3	---

Strain 1= DBA/2; 2= BALB/c; 3= BXD-2; 4= RIII-*ro*; 9=BGA.

RESULTS

Before statistical analysis, all data were divided by their respective control values, so that all characters were expressed on a standard scale. The analyses of variance and correlations between assays for this chapter are shown in the appendix. Three main factors were considered for the analysis of variance: strains, sexes and concentration of coumarin. Results were studied separately in macrophages and hepatocytes.

Macrophages.- Tables V.II.2.10, V.II.2.11 and V.II.2.14 (appendix) and Figures V.II.2.1 to V.II.2.3 (pages 129 to 131) contain the data on neutral red uptake, protein determination and rate of phagocytosis, respectively. The analyses of variance and correlations between assays are shown in the appendix. Table V.II.2.3 (page 123) summarizes the results.

In the neutral red assay, the analysis of variance (table V.II.2.8, appendix) showed no significant main effects for strain or sex, though the concentration of aspirin had

TABLE V.II.2.3.- Summary of statistically significant effects for macrophages and hepatocytes treated with coumarin.

Treatment	Cell type	End-point	Str (1)	Sex (2)	Str* Sex (3)	Conc (4)	Str* Conc (5)	Sex* Conc (6)
Coumarin	Macrophage	NR	NS	NS	NS	***	***	NS
		Prot	NS	NS	NS	***	**	NS
		Phagocyt	NS	NS	NS	***	NS	NS
Coumarin	Hepatocyte	NR	**	NS	NS	***	**	NS
		Prot	*	*	NS	***	*	NS
		LDH-cell	*	NS	NS	***	NS	NS
		LDH-Super	*	*	*	***	***	NS

(1) Main effect of strain, equivalent to mean elevation of the dose-response line.

(2) Main effect of sex

(3) interaction of the above two factors

(4) Concentration of coumarin

(5) Strain x concentration interaction, equivalent to the slope of the dose-response curve for each strain.

(6) Sex x concentration interaction, equivalent to the slope of the dose-response curve for each sex.

NS = $p>0.05$
* $p<0.05$
** $p<0.01$
*** $p<0.001$

TABLE V.II.2.4.- Summary of strain means obtained in macrophage cultures treated with coumarin

NEUTRAL RED		PROTEIN		PHAGOCYTOSIS	
DBA/2	(0.94)	DBA/2	(1.04)	STS/A	(0.83)
C57BL/6J	(0.85)	C57BL/6J	(0.92)	BALB/c	(0.80)
STS/A	(0.84)	CBA/Ca	(0.92)	CBA/Ca	(0.79)
BALB/c	(0.77)	STS/A	(0.89)	BXD-2	(0.71)
CBA/Ca	(0.76)	BGA	(0.86)	MF1	(0.70)
BXD-2	(0.72)	BALB/c	(0.82)	BGA	(0.69)
MF1	(0.70)	BXD-2	(0.79)	RIII-*ro*	(0.67)
BGA	(0.65)	MF1	(0.77)	C57BL/6J	(0.67)
RIII-*ro*	(0.58)	RIII-*ro*	(0.76)		

TABLE V.II.2.5.- Summary of strain means obtained in hepatocyte cultures treated with coumarin

NEUT.RED		PROTEIN		LDH (CELLS)		LDH (SUP)*	
CBA/Ca	(0.57)	BXD-2	(0.74)	RIII	(0.60)	RIII	(0.52)
BL/6J	(0.51)	RIII	(0.74)	MF1	(0.53)	MF1	(0.42)
RIII	(0.50)	MF1	(0.73)	BL/6J	(0.49)	BALB/c	(0.39)
MF1	(0.43)	CBA/Ca	(0.66)	BXD-2	(0.47)	BGA	(0.35)
STS/A	(0.41)	DBA/2	(0.66)	DBA/2	(0.46)	BXD-2	(0.35)
BGA	(0.40)	BGA	(0.60)	CBA/Ca	(0.45)	CBA/Ca	(0.33)
BXD-2	(0.39)	BL/6J	(0.60)	BGA	(0.43)	STS/A	(0.30)
DBA/2	(0.38)	STS/A	(0.56)	BALB/c	(0.39)	BL/6J	(0.30)
BALB/c	(0.27)	BALB/c	(0.48)	STS/A	(0.38)	DBA/2	(0.29)

* = values multiplied by 1/x
BL/6J= C57BL/6J
RIII= RIII-*ro*

TABLE V.II.2.6.- Averaged means across assays and cell types (averages calculated after dividing the strain means by the respective assay mean) obtained in cultures treated with coumarin

MACROPHAGES		HEPATOCYTES		GLOBAL	
DBA/2	(1.22)	RIII-*ro*	(1.26)	DBA/2	(1.07)
STS/A	(1.09)	MF1	(1.11)	RIII-*ro*	(1.05)
CBA/Ca	(1.04)	CBA/Ca	(1.05)	CBA/Ca	(1.05)
C57BL/6J	(1.03)	BXD-2	(1.00)	MF1	(1.01)
BALB/c	(1.02)	C57BL/6J	(0.99)	C57BL/6J	(1.01)
BXD-2	(0.94)	BGA	(0.94)	STS/A	(0.98)
BGA	(0.93)	DBA/2	(0.92)	BXD-2	(0.97)
MF1	(0.92)	STS/A	(0.87)	BGA	(0.94)
RIII-*ro*	(0.85)	BALB/c	(0.82)	BALB/c	(0.92)

TABLE V.II.2.7.- Protein content measured by Festing and Eydmann (1987) in coumarin treated macrophages from five common strains. Data given as proportions of their respective controls.

[coumarin] strain	0.308mM	2.5mM	Mean
DBA/2	1.14	0.44	0.79
CBA/Ca	1.14	0.57	0.85
C57BL/6J	1.27	0.61	0.94
BALB/c	0.99	0.67	0.83
MF1	0.71	0.61	0.66

large significant effects (F=75.85, P<0.001). However, significant interactions were observed between strains and concentration of coumarin (F=2.98, P<0.001) which implies that the slopes of the dose-response curve varied between strains. Figure V.II.2.1 (page 129) shows the graphic representation of these results. Neutral red uptake decreased as the concentration of coumarin increased, suggesting a negative effect of coumarin on cell viability. No clear strain distribution can be discerned, although at the 0.071mM concentration three different groups can be observed: a group showing high neutral red uptake (STS/A, DBA/2, BXD-2, C57BL/6J), a group showing intermediate neutral red uptake (MF1, BALB/c) and a group of low neutral red uptake (CBA/Ca, RIII-*ro*, and BGA). However, at higher coumarin concentrations most strains presented similar decrease in neutral red uptake. Strain DBA/2 was the most resistant to coumarin, followed by C57BL/6J. RIII-*ro* was the most sensitive strain. Strain BGA also appeared to be relatively sensitive. However, there appears to be no relationship between sensitivity and the *Cyp2b* allele among the strains with a known *Cyp2b* genotype (see table V.II.2.1, page 115).

For the protein determinations, neither strain nor sex showed a significant effect on the results, though the effect of the concentration of coumarin was significant (F=76.95, P<0.001). Again, a significant interaction was observed between strains and coumarin concentration (F=2.01, P=0.007) (see table V.II.2.9, appendix). In figure V.II.2.2, a slight increase in the protein content at the 0.035mM coumarin

level was observed in most strains. However, this increase was not sustained at higher coumarin concentrations, only DBA/2 seemed to show some resistance to coumarin treatment. The graph did not present a clear distribution of strains. As with neutral red uptake, DBA/2 was the most resistant and RIII-*ro* the most sensitive strain and the *Cyp2b* genotype had no obvious influence on the results. Some of the strains used here (C57BL/6J, CBA/Ca, DBA/2, BALB/c and MF1) had already been used in a similar study of protein content in macrophages treated with coumarin by Festing and Eydmann (1987a,b). There was good agreement between the studies on strain sensitivity to coumarin by these authors and the results obtained here, especially at the 0.312mM level. DBA/2, CBA/Ca and C57BL/6J showed high mean protein content (resistance) and BALB/c and MF1 low protein (sensitivity). These results are listed in table V.II.2.7 (page 125).

No data on rate of phagocytosis could be obtained in DBA/2 mice as the strain was not available at the time when the assay was performed. In this assay, Strain and Sex had no significant effects , though the concentration of coumarin had a significant effect ($F=202.14$, $P<0.001$) and no interactions were observed (table V.II.2.12, appendix). Thus, with this assay there was no evidence of genetic variation in response to coumarin. Figure V.II.2.3 (page 131) shows that phagocytosis was not improved by coumarin treatment, and it decreased as the coumarin concentration increased in all strains. Although no clear strain distribution pattern can be observed, some clustering can be discerned at the 0.071mM

coumarin level: STS/A showed a higher rate of phagocytosis than the rest, MF1, RIII-*ro*, CBA/Ca, BXD-2 and BALB/c showed intermediate values and C57BL/6J and BGA presented low levels of phagocytosis. This distribution did not coincide with the clustering observed in the neutral red assay at the same concentration of coumarin.

Correlations between assays were calculated using the strain global means (see table V.II.2.21, appendix). The results obtained for the protein determinations and in the neutral red assay were highly correlated (0.829, $P<0.01$) whereas the correlation between the phagocytosis and neutral red results (0.503, $P>0.05$) and between phagocytosis and protein results (0.318, $P>0.05$) were low. The strain distribution pattern did not coincide between assays, probably due to the different parameters measured in each assay. It was thought that a global view of the results obtained with macrophages could provide with further information and/or help to clarify the strain distribution pattern. To summarize the results obtained with macrophages, the strain means obtained in each assay were divided by the global assay mean, and the results scored by the strains in the three assays were averaged, and a global value obtained. Similar calculations were done with the results obtained in hepatocytes. Figure V.II.2.4 (page 132) shows the graphic representation of the calculations on macrophages, in which DBA/2 showed the highest values and RIII-*ro* the lowest. Summaries of the strain means in the assays and the averaged means in macrophages are shown

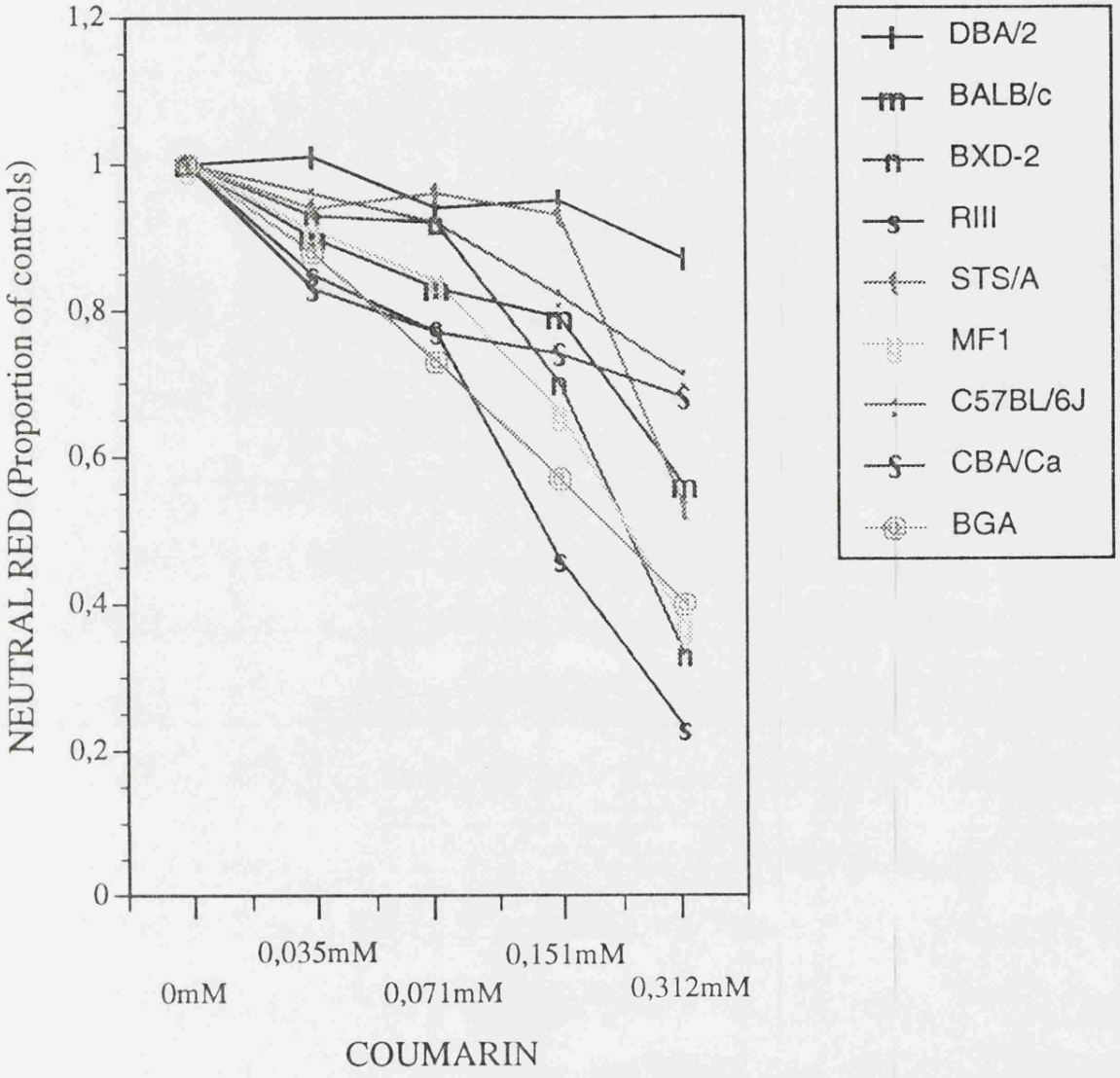

FIGURE V.II.2.1.- Neutral red uptake measured in macrophage cultures treated with coumarin.- Neutral red uptake decreased as the concentration of coumarin increased. At the 0.071mM concentration three different groups can be discerned: a group showing high uptake (STS/A, DBA/2, BXD-2 and C57BL/6J), a group showing intermediate uptake (MF1 and BALB/c) and a group of low neutral red uptake (CBA/Ca, RIII-*ro* and BGA).

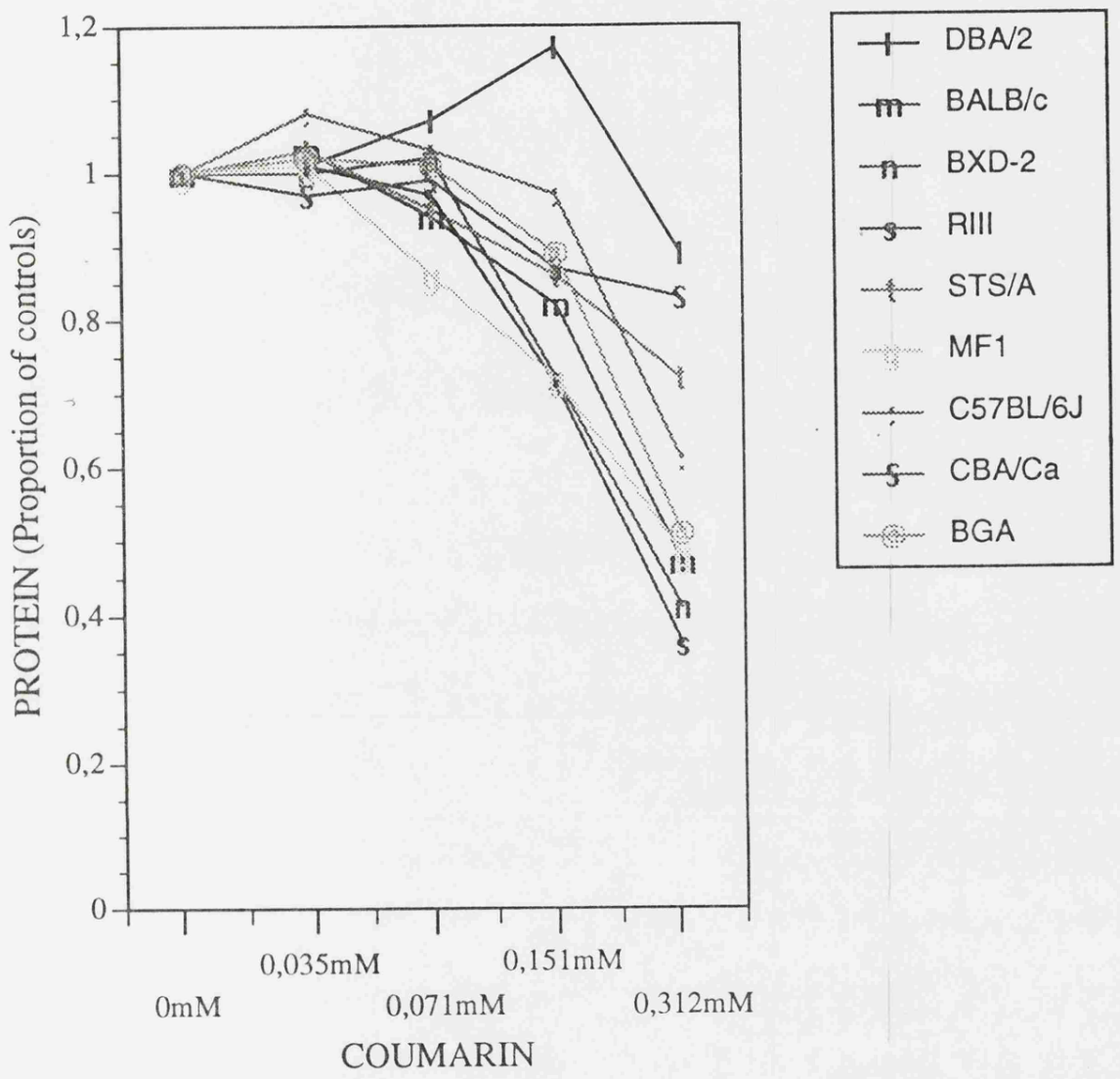

FIGURE V.II.2.2.- Protein measured in macrophage cultures treated with coumarin._The graphic representation of the results does not show a clear strain distribution pattern. As with neutral red uptake, DBA/2 was the most resistant strain and RIII-*ro* the most sensitive.

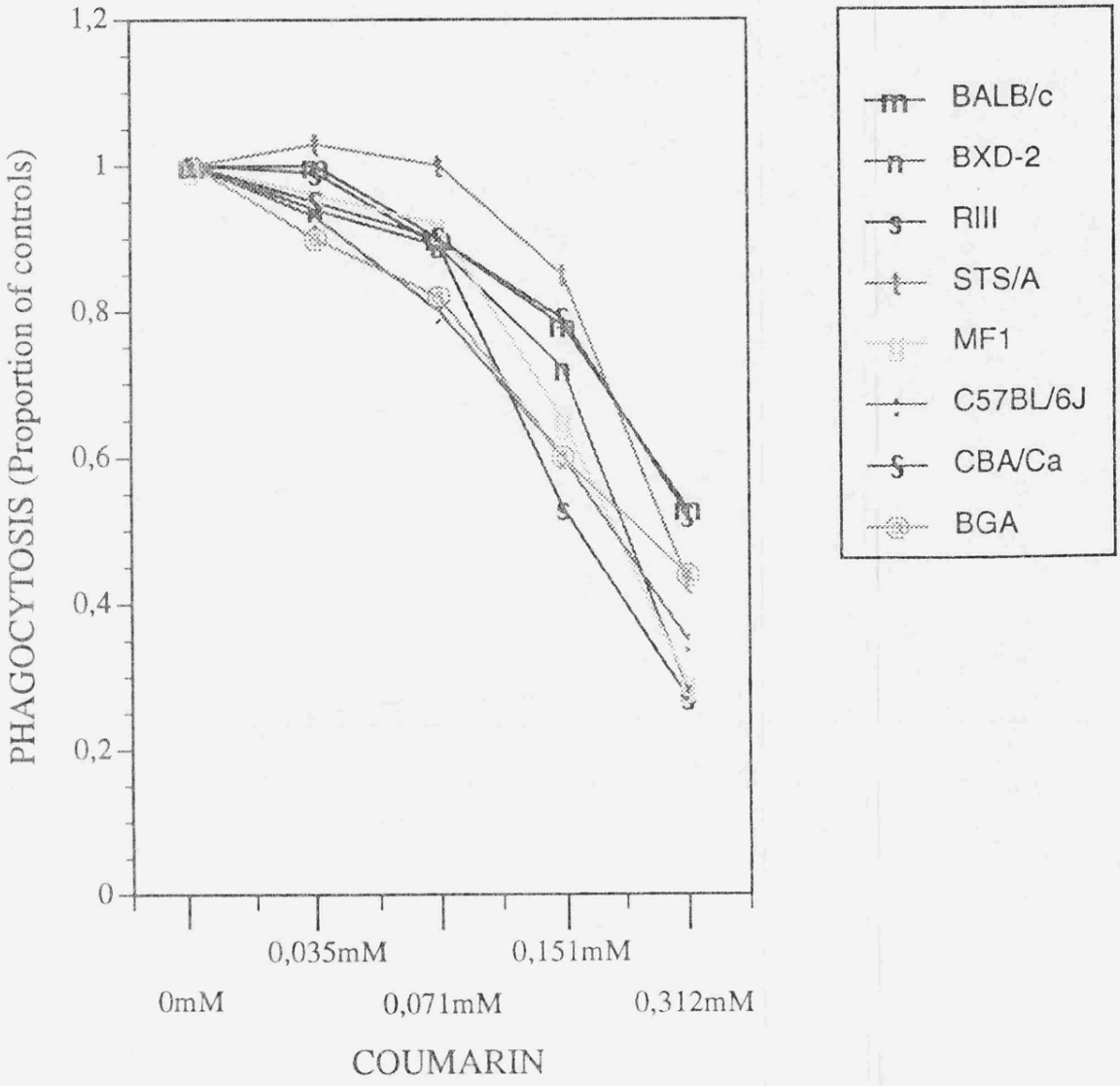

FIGURE V.II.2.3.- Rate of phagocytosis measured in macrophage cultures treated with coumarin.- No data could be obtained in DBA/2. Phagocytosis was not improved by coumarin treatment, and it decreased as the concentration of coumarin increased. Some clustering can be observed at the 0.071mM level: STS/A showed a high rate of phagocytosis, MF1, RIII-*ro*, CBA/Ca, BXD-2 and BALB/c showed intermediate values, and C57BL/6J and BGA showed a low rate of phagocytosis.

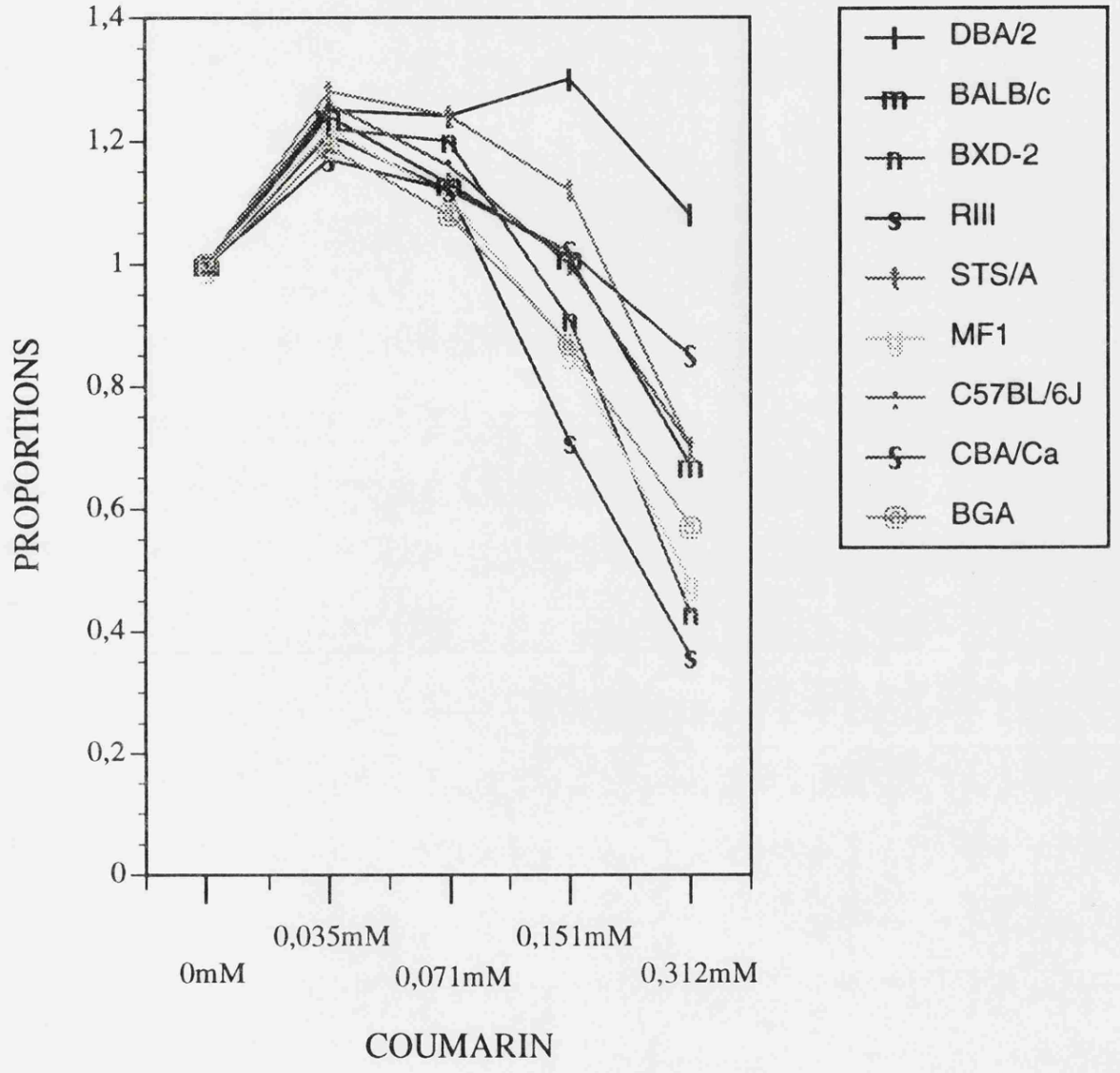

FIGURE V.II.2.4.- Averaged results (after dividing strain means by the respective assay mean) obtained in macrophage cultures treated with coumarin. No clear strain distribution pattern can be discerned. DBA/2 showed the highest values and RIII-*ro* the lowest.

in tables V.II.2.4 and V.II.2.6 (page 124), respectively. The averaged results presented DBA/2 as the most resistant strain to coumarin, STS/A, CBA/Ca, C57BL/6J, and BALB/c formed a group of intermediate values, and BXD-2, BGA, MF1 and RIII-*ro* showed low means.

Hepatocytes.- Tables V.II.2.15, V.II.2.18, V.II.2.19 and V.II.2.22 (appendix) show the neutral red uptake, total protein, and LDH activity (in cells and supernatant), respectively, measured in hepatocyte cultures treated with coumarin. These results are graphically represented in figures V.II.2.5 to V.II.2.8 (page 135 to 138).

Neutral red uptake was significantly different betwen strains ($F=3.0$, $P=0.011$) and concentrations of coumarin ($F=651$, $P<0.001$). Sex did not significantly affect the results (table V.II.2.13). There were significant interactions between strains and coumarin concentration ($F=2.27$, $P=0.002$). A marked decrease in neutral red uptake whith increasing the concentration of coumarin was observed in all strains (figure V.II.2.5). The largest strain differences were observed at the 0.071mM and 0.15mM coumarin levels, where CBA/Ca and RIII-*ro* showed the highest values, C57BL/6J, MF1, STS/A, BXD-2, DBA/2 and BGA showed intermediate values, and BALB/c presented the lowest values. This strain distribution differed from the pattern obtained with macrophages. The global results showed CBA/Ca (average protein content= 0.578) as the most resistant

strain and BALB/c (0.275) as the most sensitive, though strain differences were not very pronounced.

The Analysis of variance for protein results (table V.II.2.16, appendix) showed differences between strains ($F=2.84$, $P=0.020$) and sexes ($F=4.59$, $P=0.040$). The concentration of coumarin significantly affected the results ($F=265$, $P<0.001$). A slight interaction was observed between strains and aspirin concentration ($F=1.72$, $P=0.037$). These results are graphically shown in figure V.II.2.6 (page 136). Two groups may possibly be discerned at the 0.15mM coumarin concentration: a group of "resistant" strains including RIII-*ro* (average 0.742), BXD-2 (0.743), MF1 (0.734), CBA/Ca (0.660) and a group of "sensitive" strains including BGA (0.609), C57BL/6J (0.600), STS/A (0.563) and BALB/c (0.489). These do not correspond with the known *Cyp2b* genotypes.

The LDH activity measured in hepatocytes showed significant differences between strains ($F=2.59$, $P=0.024$) and highly significant differences between concentrations of coumarin ($F=452$, $P<0.001$). Sexes did not show significant differences ($F=0.71$, $P=0.407$). No significant interactions were observed (see table V.II.2.17, appendix). As in other assays, a steady decline in LDH activity with increasing concentrations of coumarin suggesting progressive cell death was observed in all strains (see figure V.II.2.7). RIII-*ro and MF1* mice showed the highest LDH activity in cells and STS/A and BALB/c the lowest, BXD-2, C57BL/6J, BGA and CBA/Ca showed intermediate values.

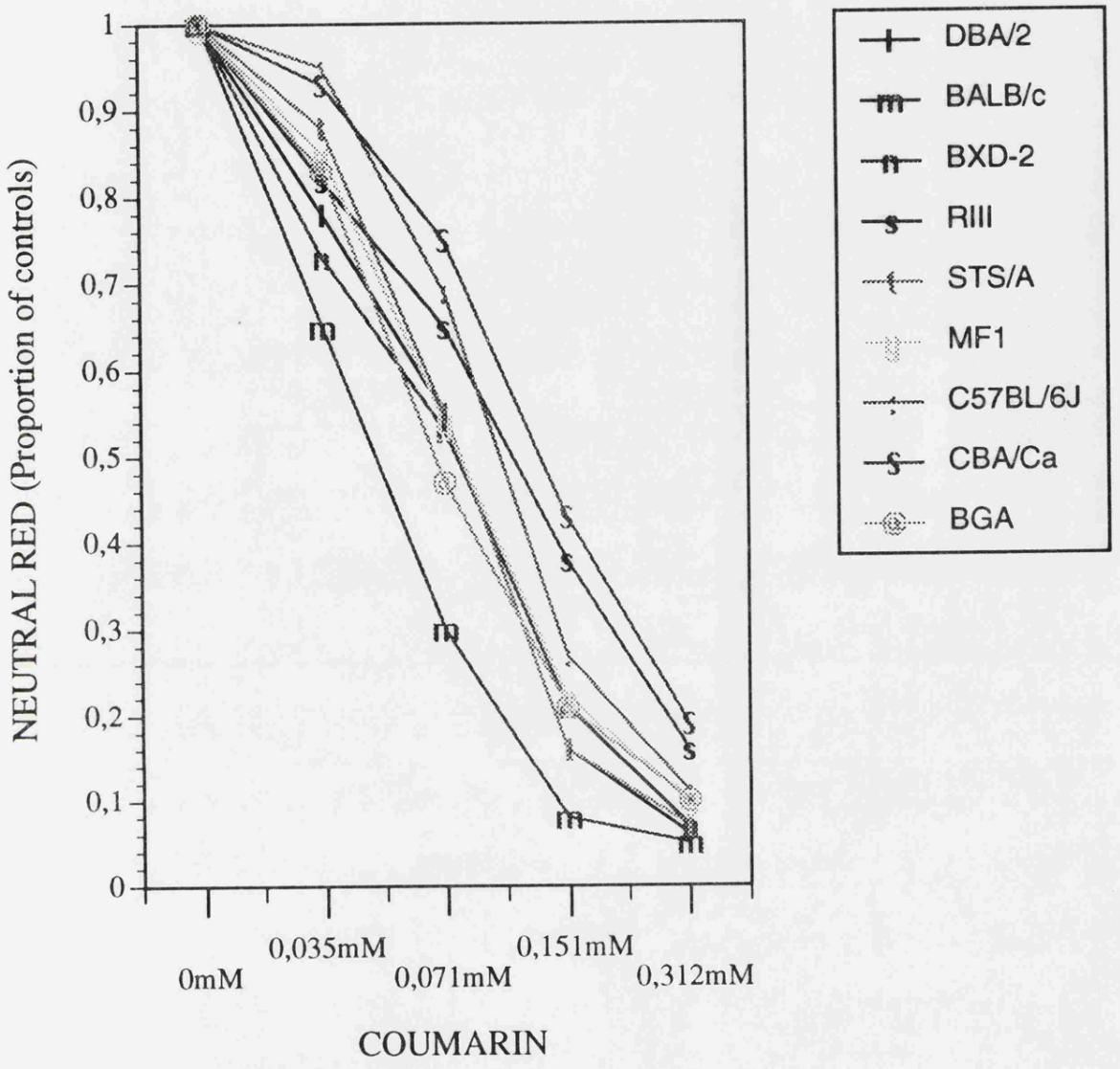

FIGURE V.II.2.5.- Neutral red uptake measured in hepatocyte cultures treated with coumarin.- A marked decrease in neutral red uptake with increasing concentrations of coumarin was observed. Some clustering of strains can be discerned at the 0.071mM and 0.151mM levels: CBA/Ca and RIII-*ro* showed the highest values, C57BL/6J, MF1, STS/A, BXD-2, DBA/2 and BGA showed intermediate values and BALB/c presented the lowest values.

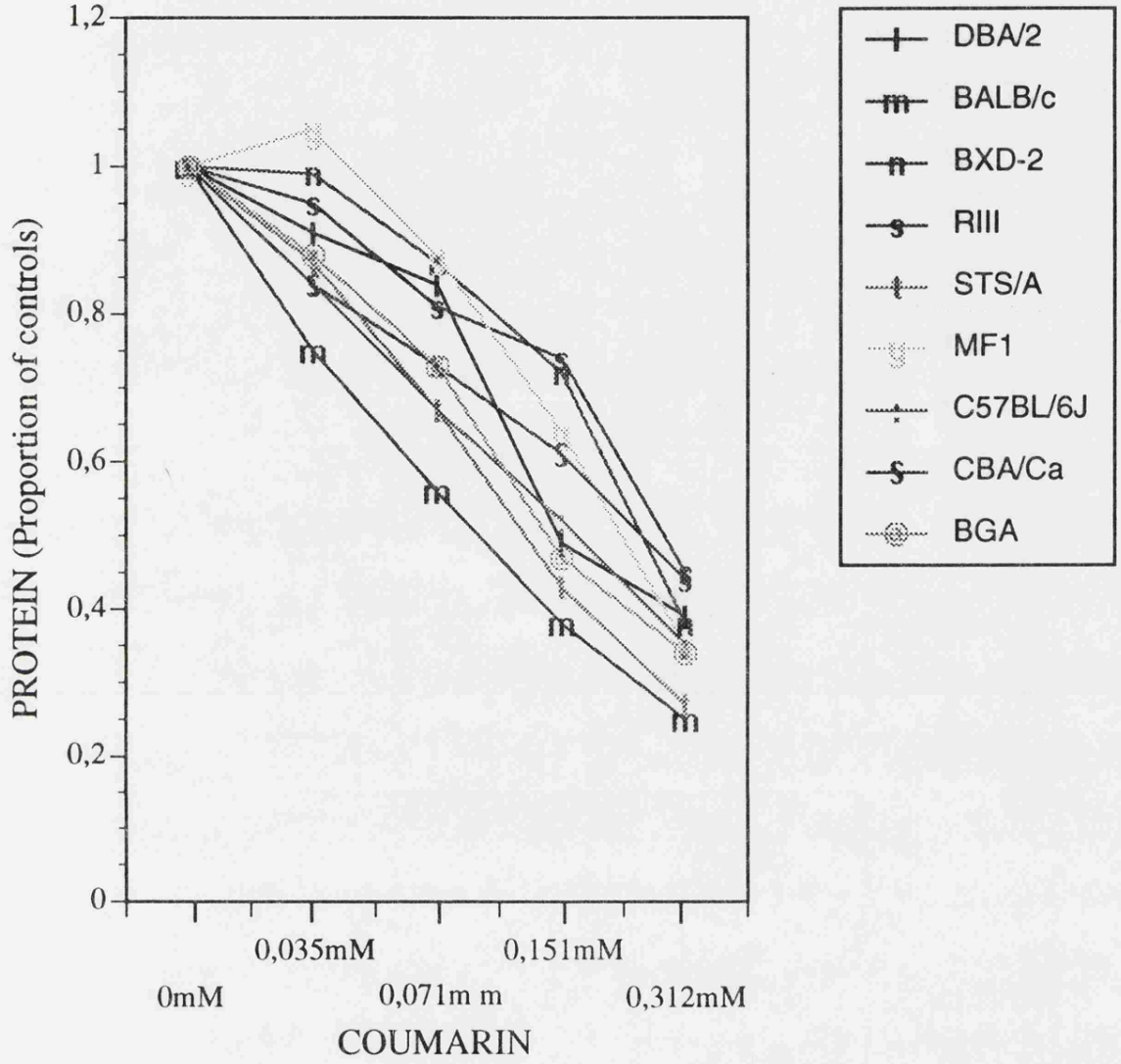

FIGURE V.II.2.6.- Protein measured in hepatocyte cultures treated with coumarin. Protein content decreased with increasing concentrations of coumarin. Two groups can be observed at the 0.151mM level: a group of "resistant" strains including RIII-*ro*, BXD-2, MF1, CBA/Ca and a group of "sensitive" strains including BGA, C57BL/6J, STS/A and BALB/c.

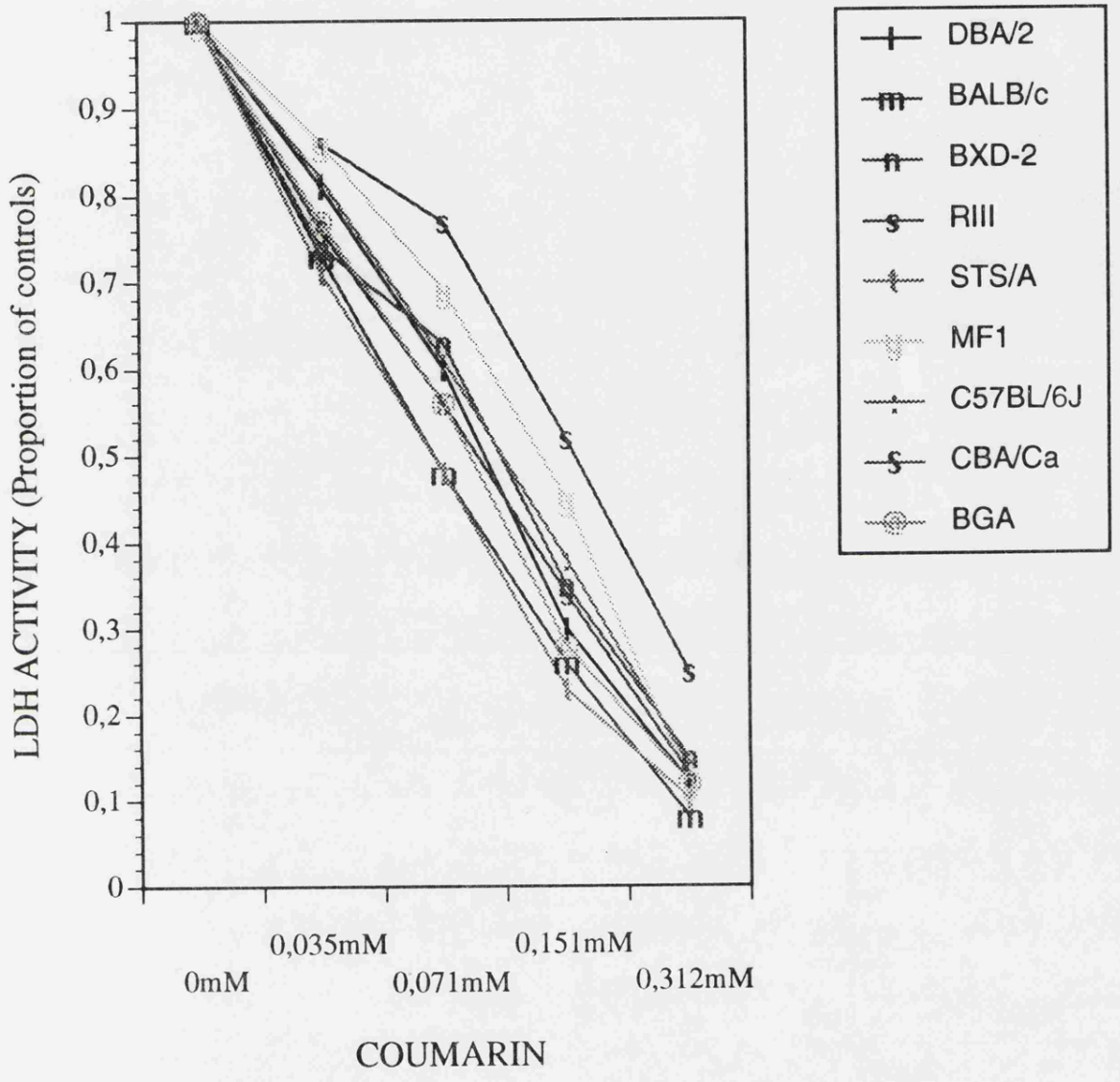

FIGURE V.II.2.7.- LDH activity measured in hepatocytes treated with coumarin. As in other assays performed in hepatocytes, a decline in LDH activity was observed with increasing concentrations of coumarin. RIII-*ro* and MF1 showed the highest values, BXD-2, C57BL/6J, BGA and CBA/Ca showed intermediate values and STS/A and BALB/c showed the lowest values.

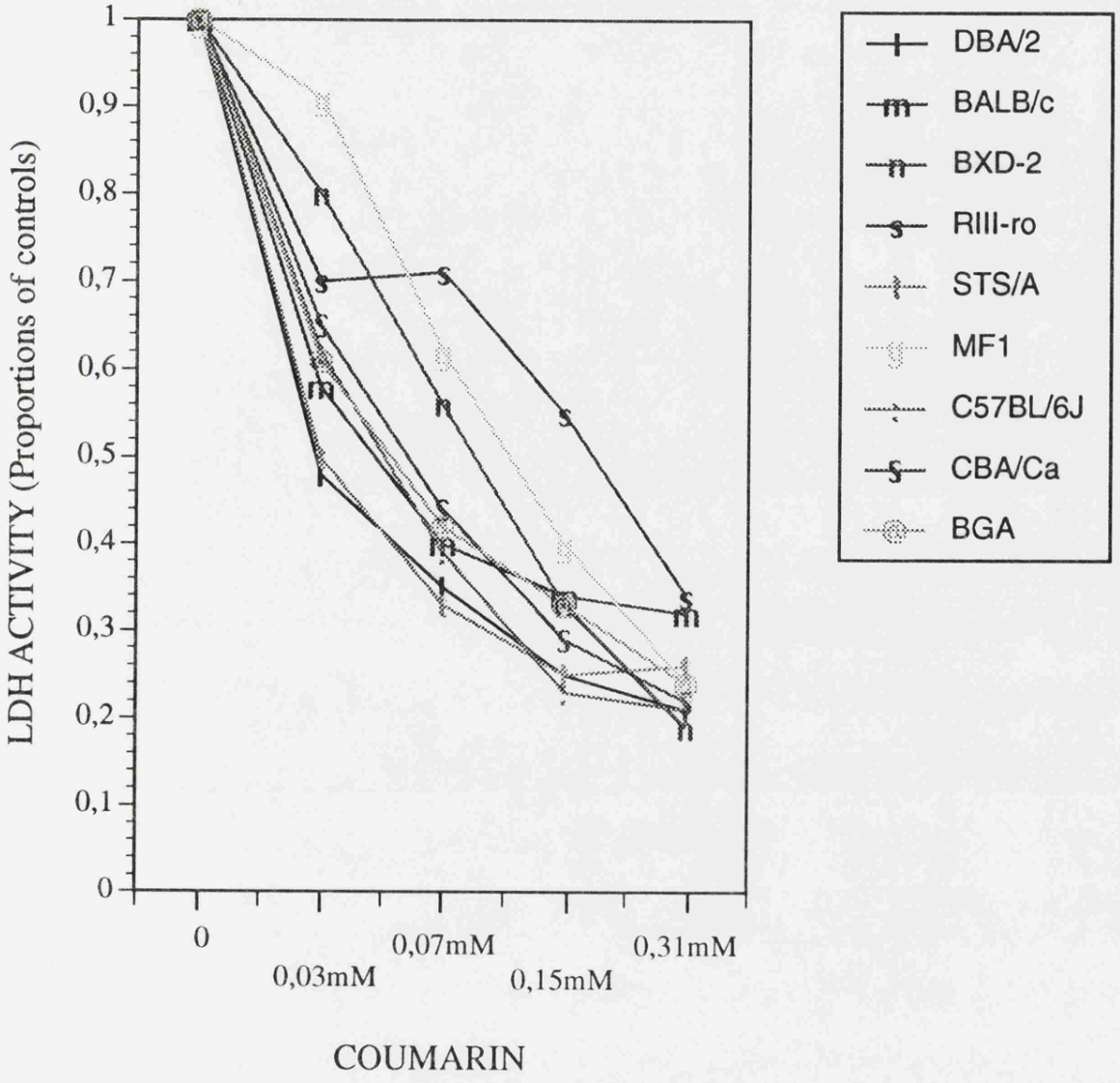

FIGURE V.II.2.8.- LDH activity measured in the supernatant of hepatocyte cultures treated with coumarin (data transformed to 1/x).- As expected, the strain distribution pattern coincided in broad terms with that obtained when measuring LDH activity in cells. RIII-*ro* and STS/A were the most resistant and most sensitive strains, respectively.

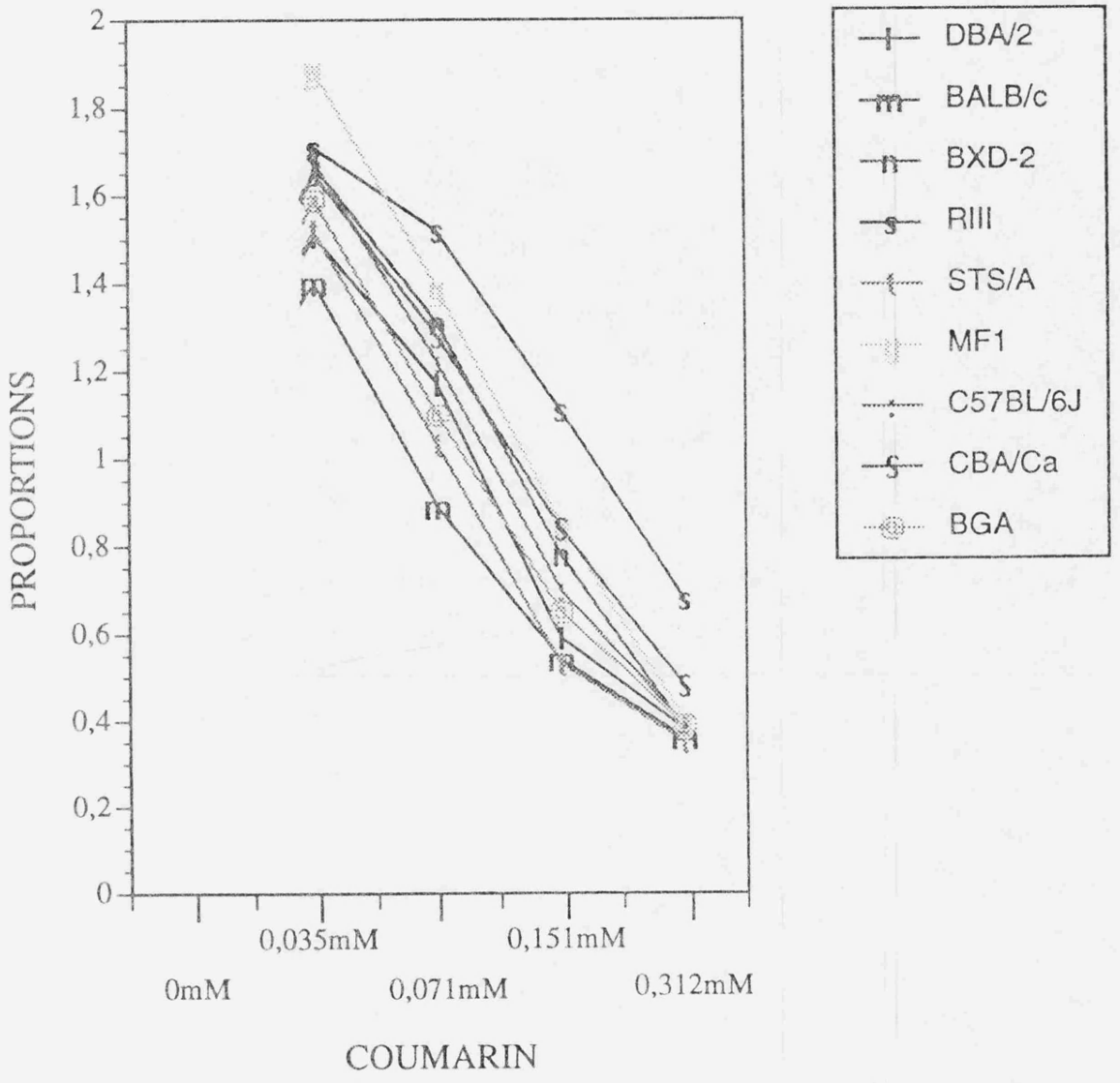

FIGURE V.II.2.9.- Averaged results (after dividing strain means by the respective assay mean) obtained in hepatocyte cultures treated with coumarin. No general pattern but an even distribution of strains can be discerned. RIII-*ro* and CBA/Ca were the most resistant strains to coumarin, followed by MF1, C57BL/6J, STS/A and BXD-2, with BGA and BALB/c being the most sensitive.

For the statistical analysis of the LDH activity measured in the supernatant of cultures (table V.II.2.20) data were transformed to 1/x in order to normalise the results. Significant differences were observed between strains ($F=3.01$, $P=0.011$) and sexes ($F=4.04$, $P=0.052$). Large differences were observed between the different coumarin concentrations ($F=168$, $P<0.001$) and there were significant interactions between strains and aspirin concentrations ($F=2.52$, $P=0.001$), and between strains and sexes ($F=2.35$, $P=0.038$). Figure V.II.2.8 (page 138) shows graphically these results. Strain RIII-*ro* seemed to be the most resistant and STS/A the most sensitive. As expected, the strain distribution pattern coincided in broad terms with the pattern obtained with the LDH activity in cells.

Correlations between LDH (cells) and protein and between LDH (supernatant) and LDH (cells) were high (0.787 and 0.703, respectively, $P<0.05$ in each case), and low between the neutral red and total protein, neutral red and LDH activity in cells and in the supernatant, and protein and LDH in the supernatant ($P>0.05$ in all cases) (see table V.II.2.21).

As in the previous case with macrophages, the low correlations between assays indicated that they supplied different information. The results obtained with the four assays were averaged (after dividing the strain means obtained in each assay by the assay global mean) in order to get a general view of the response of strain hepatocytes to coumarin (see table V.II.2.5, page 124). Figure V.II.2.9 (page 139) represents these calculations, where no particular pattern can

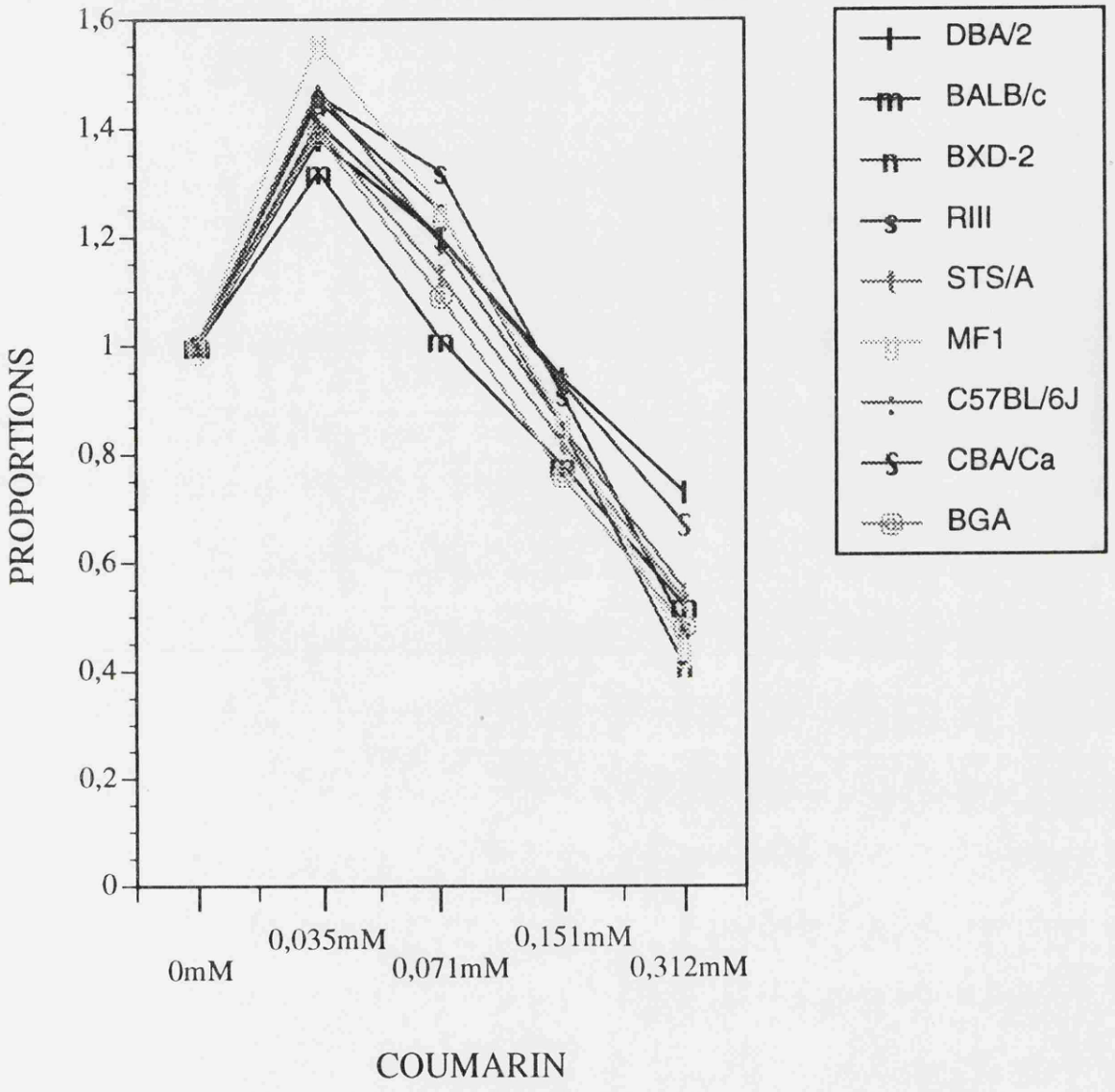

FIGURE V.II.2.10.- Averaged results obtained in macrophage and hepatocyte cultures treated with coumarin. The graphic representation of the global results shows an even strain distribution pattern. DBA/2, RIII-*ro* and CBA/Ca were the most resistant strains to coumarin, followed by MF1, C57BL/6J, STS/A and BXD-2, being BGA and BALB/c the most sensitive strains.

be discerned. RIII-*ro*, MF1 and CBA/Ca had the highest averaged values, BXD-2, C57BL/6J, BGA and DBA/2 showed intermediate values, and STS/A and BALB/c presented the lowest values.

The response of macrophages and hepatocytes from some strains differed markedly. This is commented on later in the discussion section. This was also manifest in the low correlations (using strain global means) observed between the results obtained in assays performed on macrophages and the results obtained on hepatocytes. Further calculations were made, and the macrophage and hepatocyte results were averaged to obtain a global view of the strain response to coumarin. These global results are shown in figure V.II.2.10 (page 141). Although no big strain differences could be appreciated the global results indicated that DBA/2, RIII-*ro*, and CBA/Ca were the most resistant strains to coumarin, followed by MF1, C57BL/6J, STS/A and BXD-2, being BGA and BALB/c the most sensitive.

DISCUSSION

Two main targets were aimed at in this experiment: the study of genetic variation in the response to coumarin and testing the reliability of the technique developed. Coumarin hydroxylase activity had already been studied by some authors

and found to be largely controlled by a single locus (Wood and Conney, 1974; Wood and Taylor, 1979; Wood, 1979; Negishi, 1978; Lang et al., 1989; Eydmann et al., 1991). Inbred strains of mice of known and, so far, unkown activity were used.

The differences (low correlations) observed between assays were expected. Different assays measure different parameters, and the chemical studied may affect some parameters more than others. In chapter V.1.5, where several colourimetric methods were compared, the correlations between assays had already been low, and two of the end-points used in this experiment (neutral red and protein) never gave correlation between strain means higher than 0.5. Moreover, these differences caused by the different parameters measured were one of the reasons for chosing these toxicity assays, so that more information could be gathered about the test chemical.

The toxicity assays performed on macrophages were not very informative. Differences in main effects between strains could not be detected by any of the three assays (neutral red assay, total protein and rate of phagocytosis), although some interactions between strains and concentration of coumarin in the neutral red uptake (cell viability) and protein content (cell growth) were observed. This reflected a different response among strains to the treatment, although no general pattern could be observed. It is also noticeable that a high correlation was found in the protein content obtained by Festing and Eydmann (1987a,b) and the values herein obtained in five common strains (DBA/2, CBA/Ca, C57BL/6J,

BALB/c and MF1), although the final conclusions obtained in both studies differed.

Strains DBA/2 and C57BL/6J were especially resistant to coumarin treatment as shown by the neutral red uptake and protein determination assays (see table V.II.2.4, page 119). However, the rate of phagocytosis of C57BL/6J macrophages was very low, indicating sensitivity to coumarin (no data on the rate of phagocytosis of DBA/2 macrophages were obtained). A group of strains (CBA/Ca, STS/A, BALB/c and BXD-2) showed intermediate values in the neutral red uptake and protein content, and high values in the phagocytosis assay, suggesting some resistance to coumarin. BGA generally gave low results in the three assays. In the neutral red and protein assays MF1 and RIII-*ro* were sensitive strains, whereas in the phagocytosis assay MF1 gave intermediate values and RIII-*ro* again gave low values. This disagreement between the neutral red and protein results and the phagocytosis results is reflected in the correlation matrix (table V.II.2.21), where the correlations between neutral red and protein content were high but were low between neutral red and rate of phagocytosis and between protein content and rate of phagocytosis, respectively. The results were averaged to obtain a global view of the response of macrophages of each strain to coumarin. Although no clear bimodal distribution pattern could be discerned, some differences between strains in the response to coumarin were observed. DBA/2 appeared to be clearly the most resistant strain to coumarin, followed by a group of high-intermediate resistant strains (STS/A, CBA/Ca, C57BL/6J)

and then a third group (BXD-2, BGA, MF1, RIII-*ro*) of low resistant or sensitive strains.

There was no relationship between the global response of macrophages to coumarin and the *Cyp2b* genotype. The four strains with the *Cyp2b*-^{h} genotype (DBA/2, CBA/Ca, BXD-2, and BGA) ranked 1,3,6, and 7 in resistance, respectively, whereas the two strains with the *Cyp2b*-^{l} genotype (C57BL/6J and BALB/c) ranked 4 and 5 respectively, in order of resistance. Those differences are clearly not statistically significant.

In hepatocytes, all the toxicity assays detected differences among strains. Significant interactions between strains and concentration of coumarin were also detected in neutral red uptake, protein content and LDH activity in the supernatant. In comparison with macrophages, the results obtained in hepatocytes were more informative as they showed differences which had not been detected in macrophages.

Again, no general pattern could be discerned from the figures. However, some strains showed similar results among assays. STS/A and BALB/c (except in the LDH in supernatant) always gave low values, RIII-*ro* and MF1 showed high values, BGA and CBA/Ca showed intermediate-low and intermediate-high values, respectively. The rest varied among assays. There was stronger correlation between the results obtained in hepatocytes than between the results on macrophages, especially between the protein content and the LDH activity in cells (see table V.II.2.5, page 124).

When looking at the global results obtained in hepatocytes, RIII-*ro*, MF1, and CBA/Ca appeared to be the most resistant strains, followed by BXD-2, C57BL/6J, BGA and DBA/2 forming an intermediate group, with STS/A and BALB/c the strains which showed the lowest results with hepatocytes. This strain distribution clearly differed from the pattern obtained with macrophages, though there was no evidence of any clustering of strain means. Strains that showed high values in the studies with macrophages (e.g., DBA/2 and STS/A) gave low values with hepatocytes, the most striking case being RIII-*ro*, which showed the lowest results with macrophages and the highest with hepatocytes (see table V.II.2.6, page 124). This was also manifest in the low correlations obtained between macrophage and hepatocyte results. No satisfactory explanation could be found for this, although it seems to favour the inclusion of at least two cell types in any general screening test.

When compared, no clear relations were observed between *Cyp2b* genetics and hepatocyte results. CBA/Ca, which gave high results with hepatocytes, was classified as a high activity strain by Eydmann et al. (1991). RIII-*ro* had low levels of basal 7-EOD acitivity, however it behaved as a high activity strain when induced by phenobarbitone (Eydmann et al., 1991). In this study RIII-*ro* behaved as a resistant strain. BXD-2 was classified as a high activity strain, and here it gave intermediate values. STS/A and BALB/c showed low results, indicating that they were sensitive to coumarin, and had been typed as *Cyp2b*$^{-/-}$ in the literature. However, DBA/2 and

BGA gave very low values in hepatocytes and they were classified as *Cyp2b*-^{h} strains. Also, C57BL/6J gave intermediate values, and it had been classified as a low activity strain by several authors.

From the low correlations observed between macrophage and hepatocyte results it was clear that different information was provided by the two cell types, and results were averaged to obtain a global view. The graphic representation of the averaged results (a continuos distribution pattern) did not show any evidence of a Mendelian control of the response to coumarin. Comparisons between the *Cyp2b* genotypes and the global results did not show the clear relations hoped for. DBA/2, RIII-*ro* and CBA/Ca, the most resistant strains according to the averaged results, had been described as *Cyp2b*-^{h} and *Cyp2b*-^{i} in the literature. MF1, an outbred stock subject to genetic variation, showed intermediate values. C57BL/6J, STS/A and BALB/c which gave intermediate-low global values were *Cyp2b*-^{l}. However, BGA and BXD-2 which were *Cyp2b*-^{h} and showed low global results did not fit in this relation pattern.

No single assay correlated accurately with the global results obtained after averaging the hepatocyte and macrophage data. As stated by several authors (see introduction chapter) the use of a single assay to study acute toxicity *in vitro* may produce misleading results. An example of this can be seen in the neutral red uptake measured on macrophages, where strains DBA/2 and C57BL/6J scored low and high values, suggesting sensitivity and resistance,

repectively , to coumarin. However, the overall results contradicted the neutral red data. If the purpose of a study is to assess cytotoxicity, the use of a battery of assays that increases the quantity and quality of the information obtained is favoured by these results. However, if the purpose of a study is to try to analyse genetic variation in response to a particular compound, then it would probably be best to look at each individual cell type and end point. Unfortunately, in these studies although there was clear evidence for strain differences in response of both macrophages and hepatocytes to coumarin, there was no clustering of strains as might be expected if there was control by a major gene. Nor was there any evidence that genotype at the *Cyp2b* locus had any effect of the response of either hepatocytes or macrophages to coumarin.

Although more interesting results were hoped-for, it seems that a screening protocol that detects toxicity and genetic variation in sensitivity to drugs at the same time is feasible. The technique developed here could serve as a starting point subject to improvement to increase the accuracy of the results.

V.II.3. GENETIC VARIATION IN THE RESPONSE TO ETHANOL

INTRODUCTION

The metabolism of ethanol includes a first step oxidation to acetaldehyde, which is further oxidized to acetate by aldehyde dehydrogenases in the liver (Yoshida et al., 1991; Holmes et al., 1981). More than 80% of ingested ethanol is oxidized by Alcohol dehydrogenases (Yoshida et al., 1991). In addition to this, ethanol oxidation can occur via pathways such as catalase H_2O_2 and cytochrome P-450 (Thurman and Handler, 1989).

The Alcohol dehydrogenases (ADHs) are a family of enzymes differentially expressed in mammalian tissues. They constitute a complex system composed of 3 classes of isozymes: class I, II and III (Zhang et al., 1987; Jörnvall et al., 1987; Crabb et al., 1989; Yasumani et al., 1990; Yoshida et al., 1991). Class-I ADHs are active with low concentrations of ethanol and are expressed at high levels in liver. Class-II ADHs play some role in the ethanol oxidation when the tissue ethanol levels reach high concentrations, and is primarily found in liver. Class-III ADHs oxidate long-chain alcohols and are found in all tissues examined (Yoshida et al., 1991). The genetic control of these isozymes is complex and varies between species. In inbred strains of mice a quantitative

difference in the expression of ADHs can be observed. There are some ADH-A_2 (main ethanol-active class I ADH in mouse liver) high-activity strains (C57BL/6J and YBR/K) and low-activity strains (DBA/2J and BALB/cJ). This difference is controlled by a single locus, *Adh*-1 (Zhang et al., 1987).

Aldehyde dehydrogenase (ALDH) catalize the oxidation of alcohol derivated aldehydes to their corresponding acids (Yoshida et al., 1991). ALDH activity is mainly localized in the liver. Several forms of ALDH have been observed in mammals. In mouse liver, cytosolic and mitochondrial ALDHs (AHD-$_2$ and AHD-$_5$, respectively) have been characterized.

Some studies on the genetics of the response to ethanol using *in vitro* techniques in mice have already been made. Festing and Eydmann (1987a,b) studied genetic variation in response to ethanol in macrophage cultures from inbred strains of mice. However, this study only used macrophages and a single toxicity assay (total protein determination). The aim of this project was to study the genetic variation in the response to ethanol in macrophages and hepatocytes (where ADHs seemed to be specially abundant) of mice using the selected methods to increase the information already available.

MATERIALS AND METHODS

Animals used

Eight to twelve week old mice from 8 inbred strains (DBA/2, CBA/Ca, RIII-*ro*, C57BL/6J, STS/A, BXD-2, BALB/c and BGA) and an outbred strain (MF1) were used. All of them were obtained from the M.R.C.-Toxicology Unit's stock. The same animals were used for studying genetic variation in response to coumarin.

Ethanol dose

Ethanol dilutions were prepared fresh every day. A different protocol was followed for macrophages and hepatocyte cultures.

Macrophages: 6,2 and 0% (v/v) dilutions of ethanol in prewarmed medium were prepared in a laminar flow hood. After three hours of incubation 100μl/well of ethanol-containing medium were added to the 96-well plates (already containing 100μl of cell suspension) and one and a half ml/well were added to the 25-well plates (which already contained 1.5ml/well of cell suspension) so that the final concentrations were 3,1 and 0% (v/v) of ethanol in medium.

Hepatocytes: 4,2,1 and 0% (v/v) dilutions of ethanol in prewarmed medium were prepared. After three hours of incubation, the medium was removed and three ml of ethanol-containing medium were added to the cell-containing 60mm petri dishes. Two plates/concentration were prepared.

Assays

The assessment methods used were neutral red uptake, protein determination and phagocytosis rate in macrophages, and neutral red uptake, LDH activity and protein determination in hepatocytes. Preliminary trials were done to find the period of culture required to obtain the most informative data. With hepatocytes, all assays were performed after 16-20 hours of incubation with the ethanol-containing medium. After that period of time the viability of cells would decrease quickly. With macrophages, phagocytosis rate was studied after 16-20 hours of culture with the treatment, neutral red absorption on the third day of culture and protein determinations were done on the 5th day. These periods of culture coincided with those used in the coumarin studies.

Experimental design

The experimental design used was similar to the design described in the studies of coumarin. The statistical methods used are explained in the coumarin chapter.

RESULTS

All data were divided by their respective control values before statistical analysis, so that all values were expresed on a standard scale. As in the previous chapter, results were studied separately in macrophages and hepatocytes. Table V.II.3.1 (page 154) summarizes the results of the analyses. Tables V.II.3.2, V.II.3.3 and V.II.3.4 (page 155) include summaries of the strain means obtained with macrophages, hepatocytes and the averaged strain means (after dividing the strain means by the respective assay global mean).

Macrophages.- The data on neutral red uptake, protein determinations and phagocytosis are summarized in tables V.II.3.7, V.II.3.8 and V.II.3.11 (appendix), respectively. The Analyses of variance and correlations between assays are also shown in the appendix (tables V.II.3.5, V.II.3.6 and V.II.3.9, respectively). No data on neutral red uptake and protein content in BGA and rate of phagocytosis in DBA/2 were obtained as these strains were not available at the time when the assays were performed.

TABLE V.II.3.1.- Summary of statistically significant effects for macrophages and hepatocytes treated with ethanol.

Treatment	Cell type	End-point	Str (1)	Sex (2)	Str* Sex (3)	Conc (4)	Str* Conc (5)	Sex* Conc (6)
Ethanol	Macrophage	NR	NS	NS	NS	***	**	NS
		Prot	**	NS	NS	***	NS	NS
		Phagocyt	NS	NS	NS	***	NS	NS
Ethanol	Hepatocyte	NR	NS	NS	NS	***	***	NS
		Prot	NS	NS	NS	***	**	NS
		LDH-cell	NS	NS	NS	***	***	NS
		LDH-super	NS	NS	NS	***	*	NS

(1) Main effect of strain, equivalent to mean elevation of the dose-response line.

(2) Main effect of sex

(3) interaction of the above two factors

(4) Concentration of ethanol

(5) Strain x concentration interaction, equivalent to the slope of the dose-response curve for each strain.

(6) Sex x concentration interaction, equivalent to the slope of the dose-response curve for each sex.

NS = $p>0.05$
* $p<0.05$
** $p<0.01$
*** $p<0.001$

TABLE V.II.3.2.- Summary of strain means obtained in macrophage cultures treated with ethanol.

NEUTRAL RED		PROTEIN		PHAGOCYTOSIS	
C57BL/6J	(1.02)	BXD-2	(0.99)	MF1	(0.79)
CBA/Ca	(1.00)	STS/A	(0.97)	BALB/c	(0.78)
BALB/c	(0.95)	BALB/c	(0.97)	C57BL/6J	(0.68)
MF1	(0.93)	CBA/Ca	(0.95)	CBA/Ca	(0.67)
RIII-*ro*	(0.91)	MF1	(0.92)	STS/A	(0.64)
DBA/2	(0.89)	C57BL/6J	(0.90)	BGA	(0.59)
BXD-2	(0.87)	RIII-*ro*	(0.89)	BXD-2	(0.54)
STS/A	(0.84)	DBA/2	(0.78)	RIII-*ro*	(0.45)

TABLE V.II.3.3.- Summary of strain means obtained in hepatocyte cultures treated with ethanol.

NEUT.RED		PROTEIN		LDH (CELLS)		LDH (SUP)*	
CBA/Ca	(0.55)	MF1	(0.67)	DBA/2	(0.55)	MF1	(0.35)
BL/6J	(0.48)	BXD-2	(0.66)	RIII	(0.54)	RIII	(0.32)
STS/A	(0.47)	BL/6J	(0.64)	BXD-2	(0.51)	BGA	(0.31)
DBA/2	(0.47)	DBA/2	(0.64)	MF1	(0.49)	DBA/2	(0.31)
MF1	(0.46)	RIII	(0.64)	BGA	(0.46)	BALB/c	(0.31)
RIII	(0.44)	CBA/Ca	(0.63)	BL/6J	(0.46)	BXD-2	(0.30)
BGA	(0.43)	STS/A	(0.56)	CBA/Ca	(0.46)	STS/A	(0.28)
BALB/c	(0.42)	BALB/c	(0.53)	STS/A	(0.44)	CBA/Ca	(0.27)
BXD-2	(0.38)	BGA	(0.60)	BALB/c	(0.42)	BL/6J	(0.23)

*= values multiplied by 1/x
BL/6J= C57BL/6J
RIII= RIII-*ro*

TABLE V.II.3.4.- Averaged means across assays and cell types (averages calculated after dividing strain means by the respective assay mean).

MACROPHAGES		HEPATOCYTES		GLOBAL	
BALB/c	(1.09)	MF1	(1.07)	MF1	(1.07)
MF1	(1.08)	DBA/2	(1.06)	CBA/Ca	(1.03)
CBA/Ca	(1.05)	RIII-*ro*	(1.05)	BALB/c	(1.00)
C57BL/6J	(1.04)	CBA/Ca	(1.01)	C57BL/6J	(0.99)
STS/A	(0.98)	BXD-2	(0.99)	DBA/2	(0.98)
BXD-2	(0.95)	BGA	(0.98)	BXD-2	(0.97)
BGA	(0.92)	C57BL/6J	(0.95)	RIII-*ro*	(0.96)
DBA/2	(0.90)	STS/A	(0.94)	STS/A	(0.96)
RIII-*ro*	(0.88)	BALB/c	(0.92)	BGA	(0.95)

In the neutral red assay, the main effects of strain and sex were not statistically significant (table V.II.3.5, appendix). The concentration of ethanol had large significant effects ($F=45.36$, $P<0.001$) and a significant interaction was observed between strain and concentration of ethanol ($F=3.98$, $P=0.002$). According to the neutral red results, strains C57BL/6J, MF1 and BALB/c were the most resistant, and strains DBA/2, BXD-2, and STS/A the most sensitive. Figure V.II.3.1 (page 158) shows these data. At the 1% ethanol level, some strains showed an increase in neutral red uptake (CBA/Ca, MF1 and BALB/c) indicating higher cell viability and some strains showed a decrease (DBA/2, RIII-*ro*, BXD-2, STS/A). At 3% ethanol level all strains showed a decrease in neutral red uptake except in C57BL/6J, indicating that at this concentration ethanol was toxic for the cultures.

The total protein determinations showed no significant main effects for sexes, though strains and concentration of ethanol had large significant effects ($F=3.98$, $P=0.003$ and $F=74.87$, $P<0.001$, respectively). No significant interactions were observed (table V.II.3.6). BXD-2, STS/A and BALB/c were the most resistant strains and C57BL/6J, RIII-*ro* and DBA/2 the most sensitive. Festing and Eydmann (1987a) also found significant strain differences in the protein content of cell cultures from 10 strains of mice (RIII-*ro*, MF1, CBA/Ca, CAT, BALB/c, B6CBF1, DDK, CT, C57BL/6, and DBA/2) treated with ethanol. After dividing the results obtained by those authors by their respective controls, the

resulting proportions agreed well with the results obtained here, being CBA/Ca and BALB/c the strains which showed the highest values, and RIII-*ro* and DBA/2 the strains which showed the lowest values. An increase in protein content was observed in most strains at the 1% ethanol level, indicating cell growth (see figure V.II.3.2, page 159). However, at the 3% ethanol level all strains showed a decrease in total protein.

Sexes and strains did not show significant differences in the rate of phagocytosis (table V.II.3.9). The concentration of ethanol had large significative effects ($F=169.89$, $P<0.001$). No significant interactions were observed. According to this assay, BALB/c and MF1 were the most resistant strains and BGA, RIII-*ro* and BXD-2 the most sensitive, although differences were not statistically significative. All strains showed a decrease in the rate of phagocytosis at both 1% and 3% ethanol levels (figure V.II.3.3, page 160).

No general pattern was observed among these assays and the correlations between them were low (table V.II.3.18, appendix). As in the previous study on coumarin, results were averaged across end-points (after dividing the assay strain means by the respective assay global mean) to have a global view of the treatment effects. Figure V.II.3.4 (page 161) shows graphically the averaged results. An even distribution pattern of strains was observed, being BALB/c, MF1 and CBA/Ca the most resistant strains and RIII-*ro* and BGA the most sensitive.

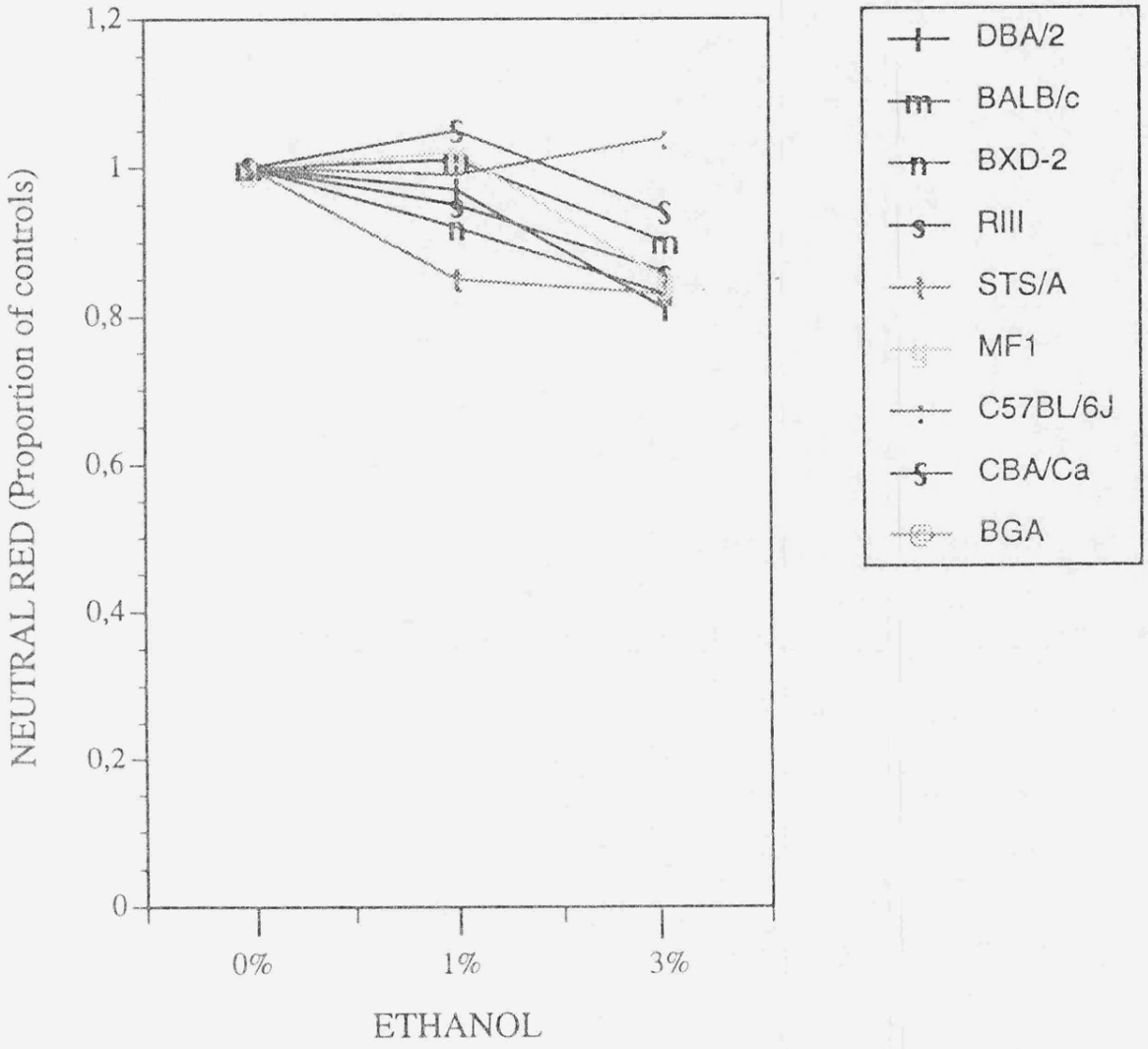

FIGURE V.II.3.1.- Neutral red uptake measured in macrophage cultures treated with ethanol. No data could be obtained in BGA mice. Some strains (CBA/Ca, MF1 and BALB/c) showed an increase in neutral red uptake at the 1% level. However, at the 3% level most strains showed a decrease. C57BL/6J, MF1 and BALB/c were the most resistant strains, and DBA/2, BXD-2 and STS/A the most sensitive strains.

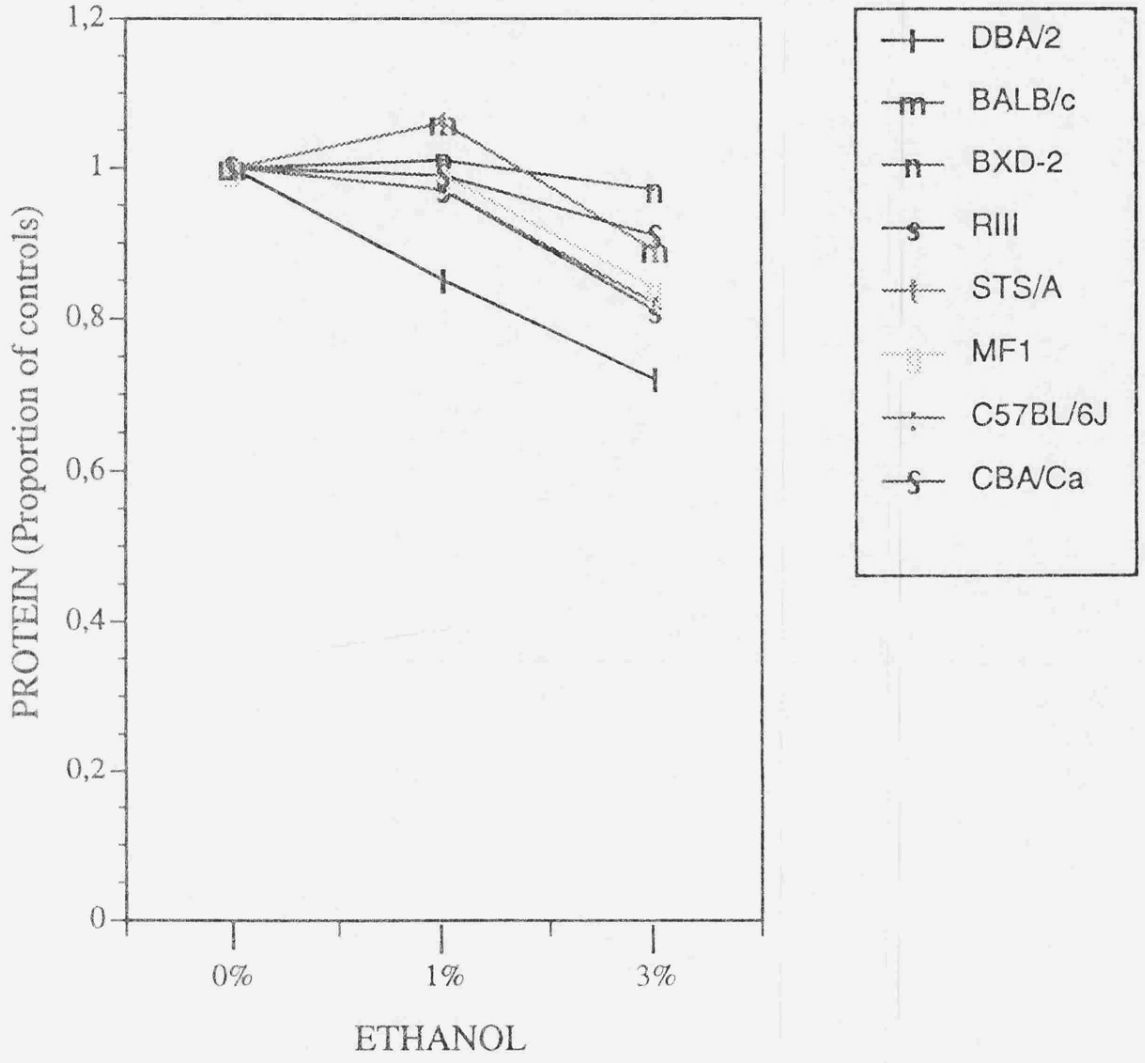

FIGURE V.II.3.2.- Protein measured in macrophage cultures treated with ethanol.- No data on BGA mice could be obtained. BXD-2, STS/A and BALB/c were the most resistant strains and C57BL/6J, RIII-*ro* and DBA/2 the most sensitive.

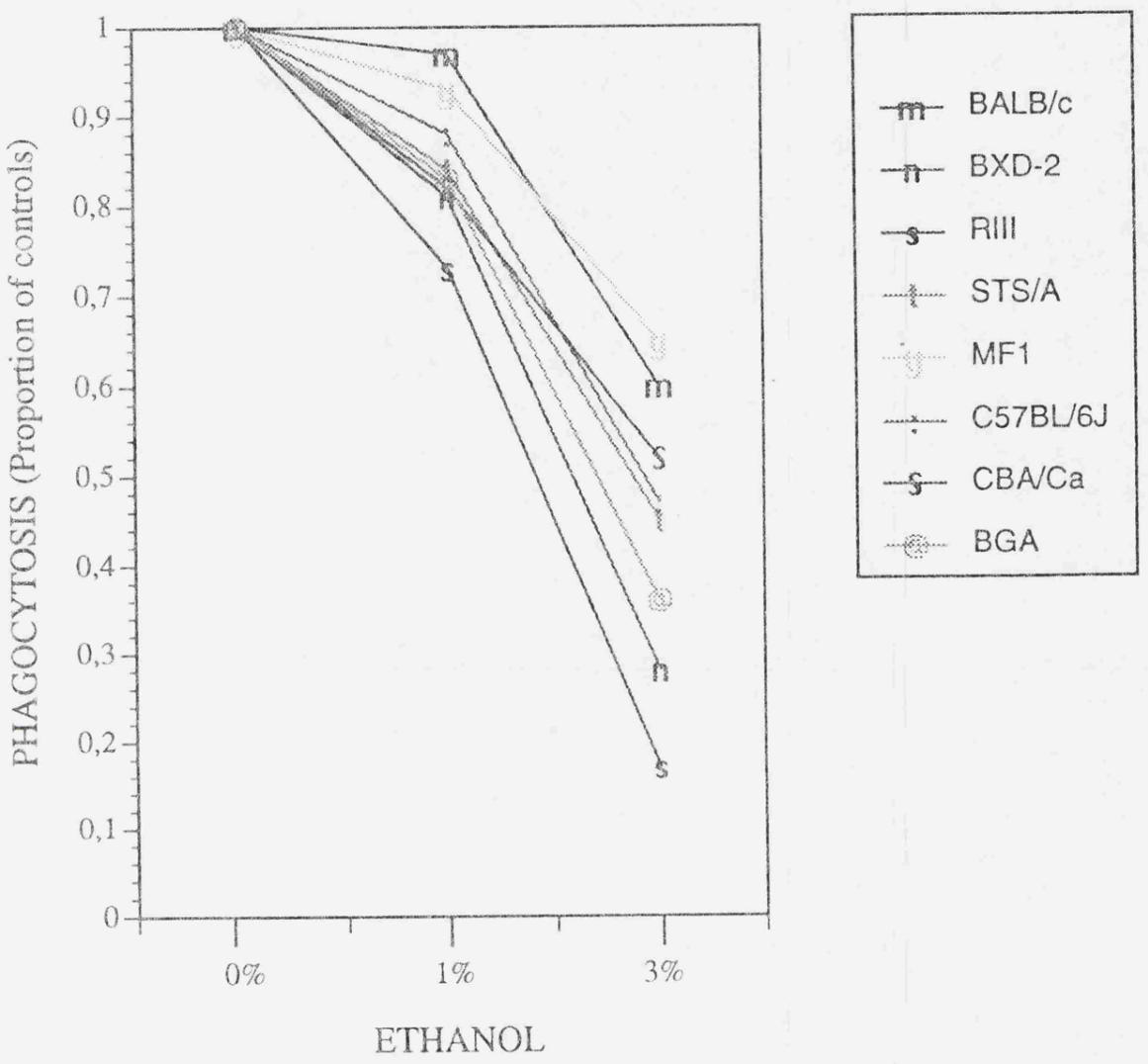

FIGURE V.II.3.3.- Rate of phagocytosis measured in hepatocyte cultures treated with ethanol. No data could be obtained in DBA/2 mice. Phagocytosis decreased with increasing concentrations of ethanol. BALB/c and MF1 were the most resistant strains and BGA, RIII-*ro* and BXD-2 the most sensitive.

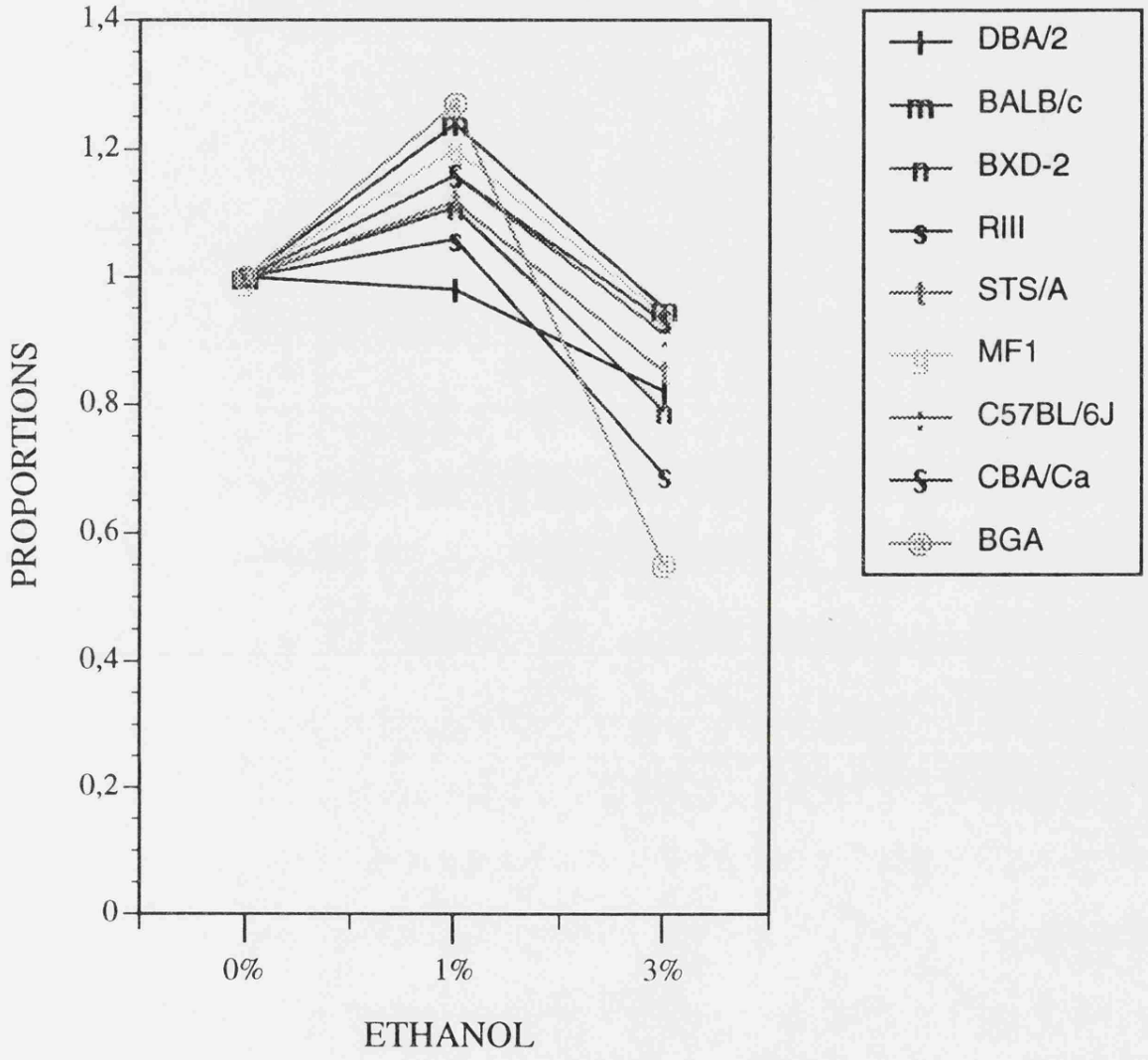

FIGURE V.II.3.4.- Averaged results (after dividing strain means by the respective assay mean) obtained in macrophage cultures treated with ethanol . An even strain distribution pattern can be observed. BALB/c, MF1 and CBA/Ca were the most resistant strains and RIII-*ro* and BGA the most sensitive.

Hepatocytes.- Data on neutral red uptake, protein determinations and LDH activity (in supernatant and cells) measured in hepatocyte cultures treated with ethanol are summarized in tables V.II.3.12, V.II.3.15, V.II.3.16 and V.II.3.19 (appendix), respectively. Graphic representations of the results are shown in figures V.II.3.5 to V.II.3.8 (pages 164 to 167). The appendix also contains the analyses of variance and correlations between assays. The results of these analyses are summarized in table V.II.3.2 (page 154).

In the neutral red assay, the analyses of variance (table V.II.3.10) showed no significant effects for strains or sexes, though the concentration of ethanol had a large significant effect ($F=873.66$, $P<0.001$). A significant interaction between the strains and the concentration of ethanol was observed ($F=2.02$, $P=0.007$). These results showed that CBA/Ca and STS/A were the most resistant strains, and BALB/c and BXD-2 the most sensitive. As can be seen in figure V.II.3.5 (page 164) the amount of neutral red uptake decreased with increasing ethanol concentrations in all strains. This differed from the results obtained with macrophages, where some increase in neutral red uptake was observed at the 1% ethanol level.

Sexes and strains showed differences in the total protein content in hepatocytes ($F=3.10$, $P=0.088$ and $F=2.10$, $P=0.072$, respectively). However, this difference was not significant at the usual 5% level (table V.II.3.13). The concentration of ethanol had large effects ($F=370.30$,

P<0.001). A significant interaction was observed between strains and concentration of ethanol, the slope of dose response curves differed (F=2.68, P<0.001). In figure V.II.3.6 it can be observed that protein content decreased in all strains, with MF1 and BXD-2 the most resistant strains and STS/A the most sensitive. Also, three different groups can be discerned in the middle of the figure. A first group formed by BXD-2 and MF1 which showed the highest results, a second group including RIII-*ro*, CBA/Ca, DBA/2, BGA and C57BL/6J which presented intermediate values, and a third cluster (BALB/c and STS/A) which showed the lowest results.

LDH activity in cells did not show significant differences among strains and among sexes (table V.II.3.17, appendix). The concentration of ethanol and the interaction strain x concentration of ethanol had large significant effects (F=671.22, P<0.001 and F=4.12, P<0.001, respectively). The results are graphically represented in figure V.II.3.8 (page 167). Two different groups can be distinguished: a group of higher values (RIII-*ro*, BXD-2, MF1 and DBA/2) and a group presenting lower values (C57BL6/6J, CBA/Ca, BGA, BALB/c and STS/A).

In the supernatant of hepatocyte cultures, the LDH acitivity measured showed no significant differences among sexes and strains (table V.II.3.14, appendix). The concentration of ethanol had a large significant effect (F=523.28, P<0.001) and there was a slight interaction between strains and concentration of ethanol (F=1.62, P=0.04). In this assay, MF1, RIII-*ro* and BGA were the most resistant strains

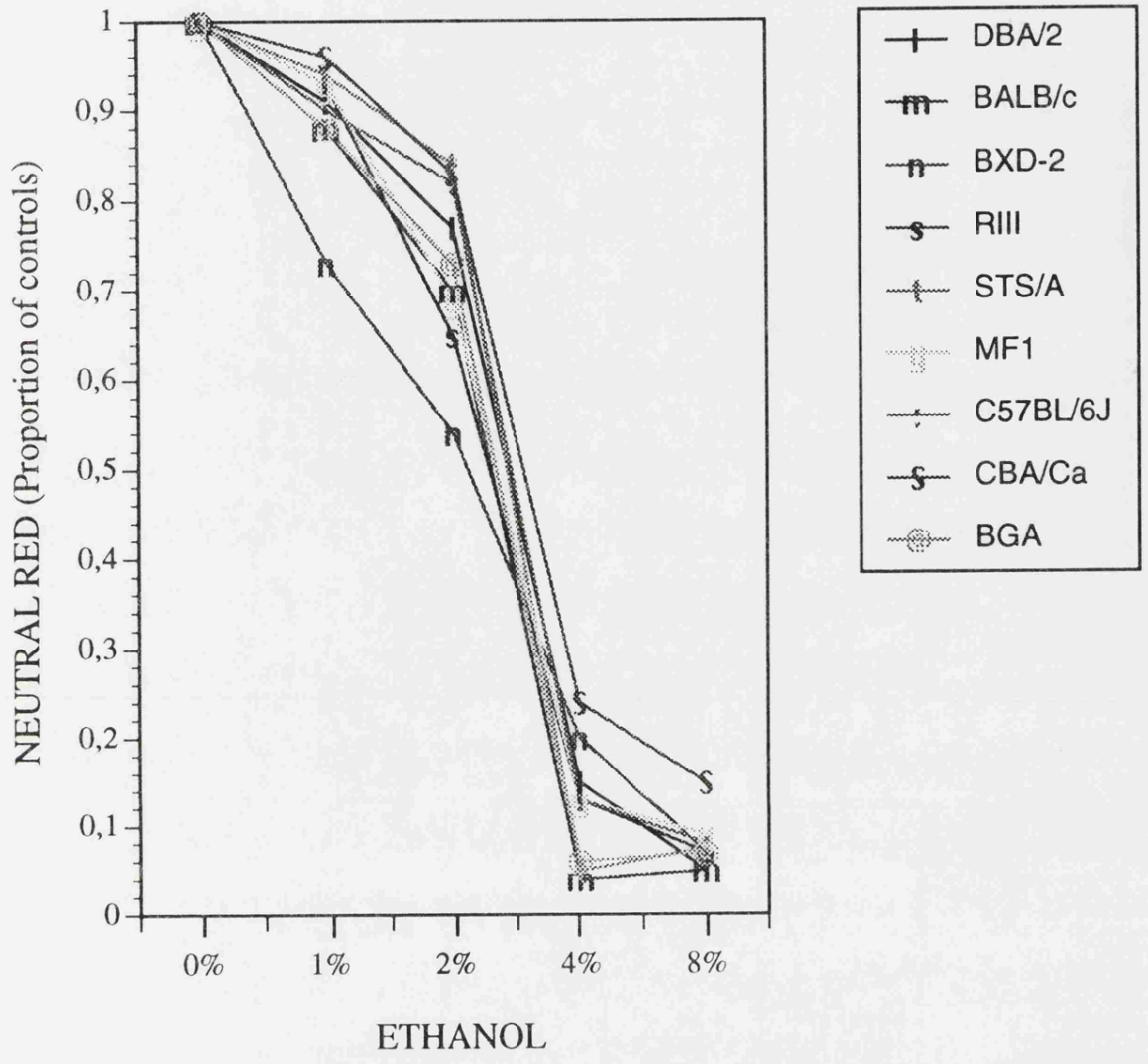

FIGURE V.II.3.5.- Neutral red uptake measured in hepatocytes treated with ethanol. A marked decrease in the neutral red uptake with increasing concentrations of ethanol was observed. Strains showed an even distribution pattern. CBA/Ca and STS/A were the most resistant strains, and BALB/c and BXD-2 the most sensitive.

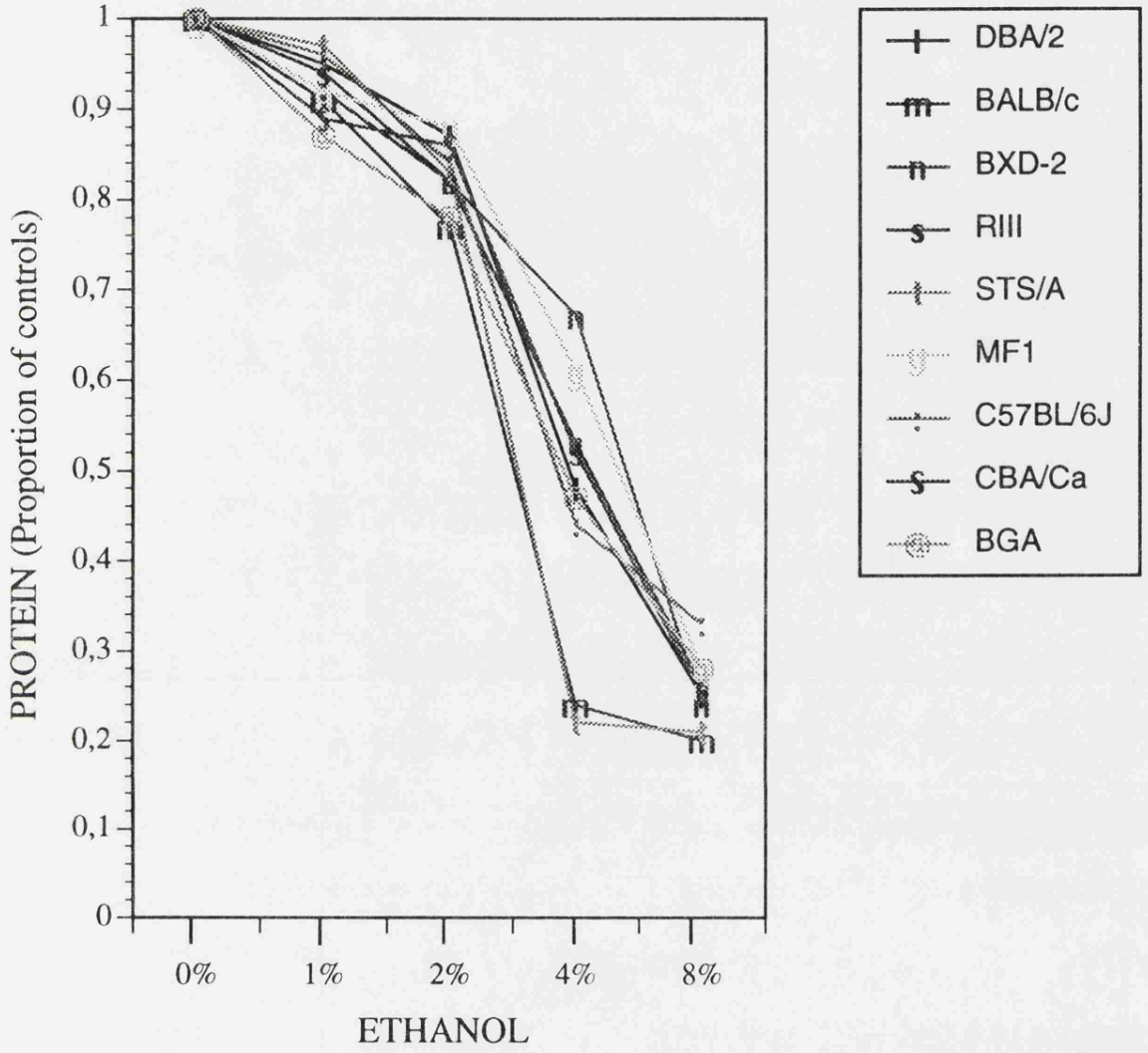

FIGURE V.II.3.6.- Protein measured in hepatocyte cultures treated with ethanol.- Protein decreased with increasing concentrations of ethanol in all strains. Three different groups can be observed at the 4% level: A first group of high protein content which includes BXD-2 and MF1, an intermediate group formed by RIII-*ro*, CBA/Ca, DBA/2, BGA and C57BL/6J, and a third group formed by BALB/c and STS/A which showed the lowest results.

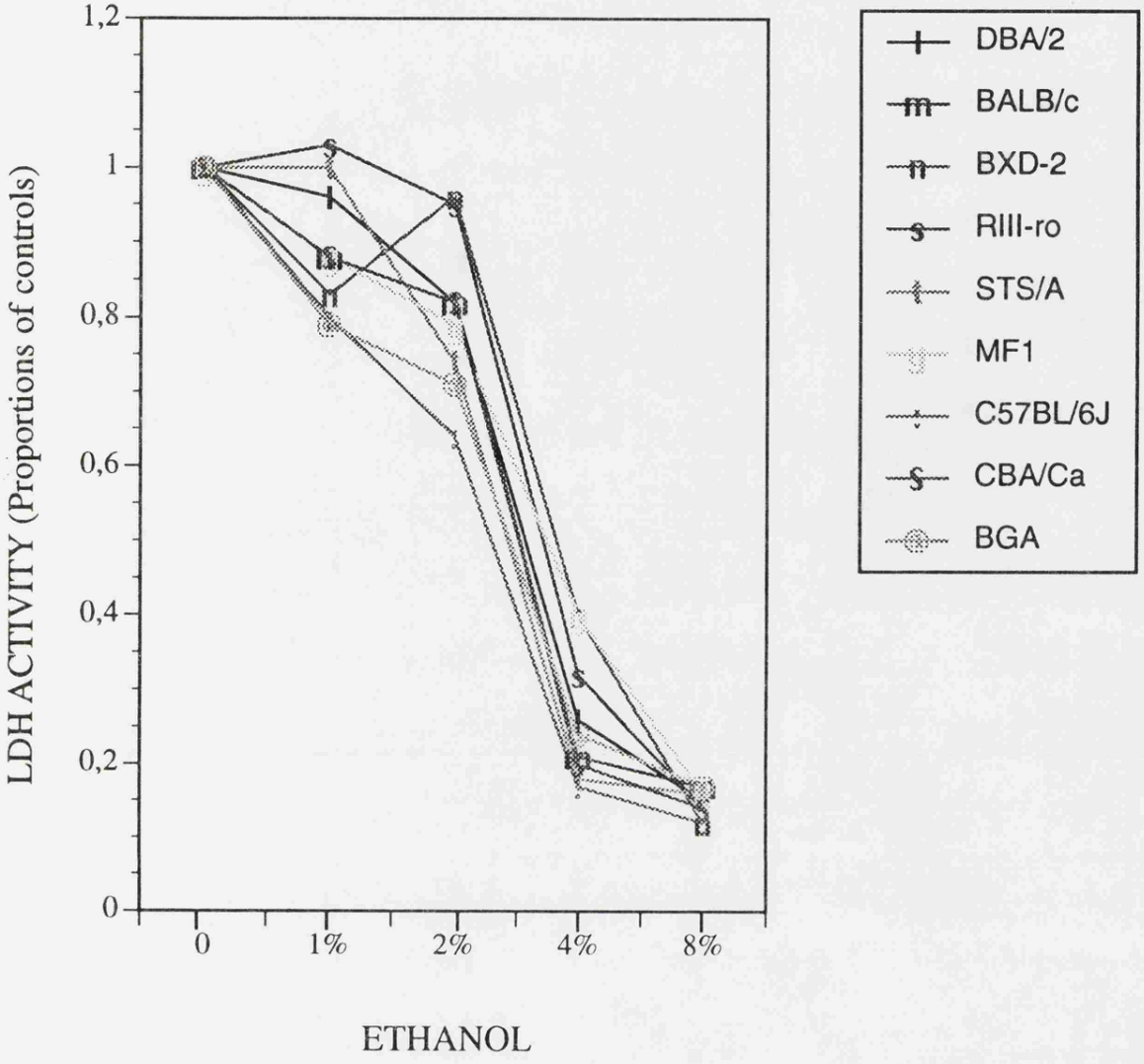

FIGURE V.II.3.7.- LDH activity measured in the supernatant of cultures treated with ethanol (data transformed to 1/x). MF1, RIII-*ro*, and BGA were the most resistant strains, and CBA/Ca and C57BL/6J the most sensitive.

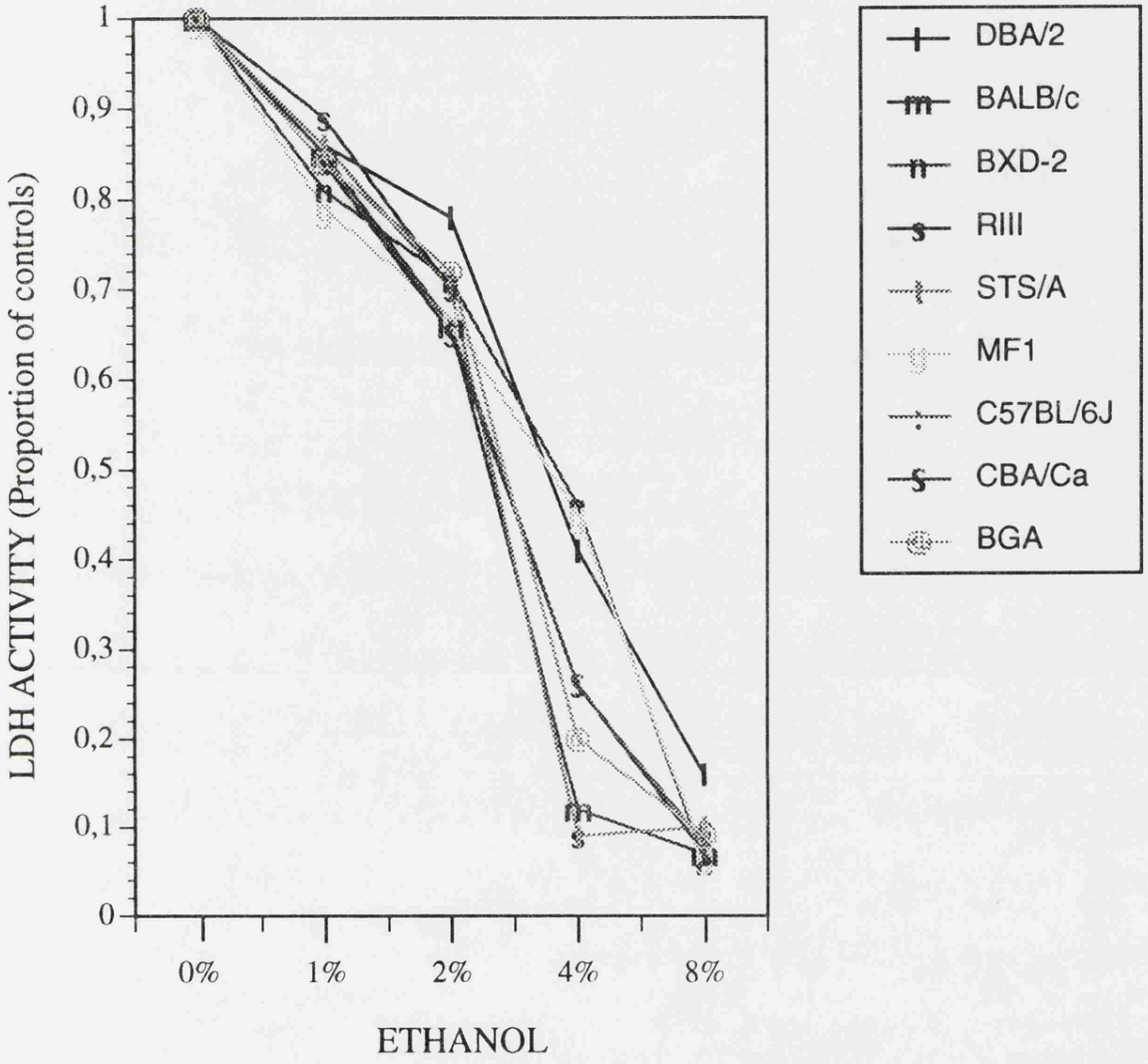

FIGURE V.II.3.8.- LDH measured in hepatocytes treated with ethanol.- Increasing concentrations of ethanol negatively affected the LDH activity measured. Two groups can be observed at the 4% level: a first group including MF1, BXD-2 and DBA/2 which showed higher values than a second group formed by C57BL/6J, CBA/Ca, BGA, BALB/c, RIII-*ro* and STS/A. However, the averaged values showed DBA/2 and RIII-*ro* as the most resistant strains and BALB/c and STS/A as the most sensitive.

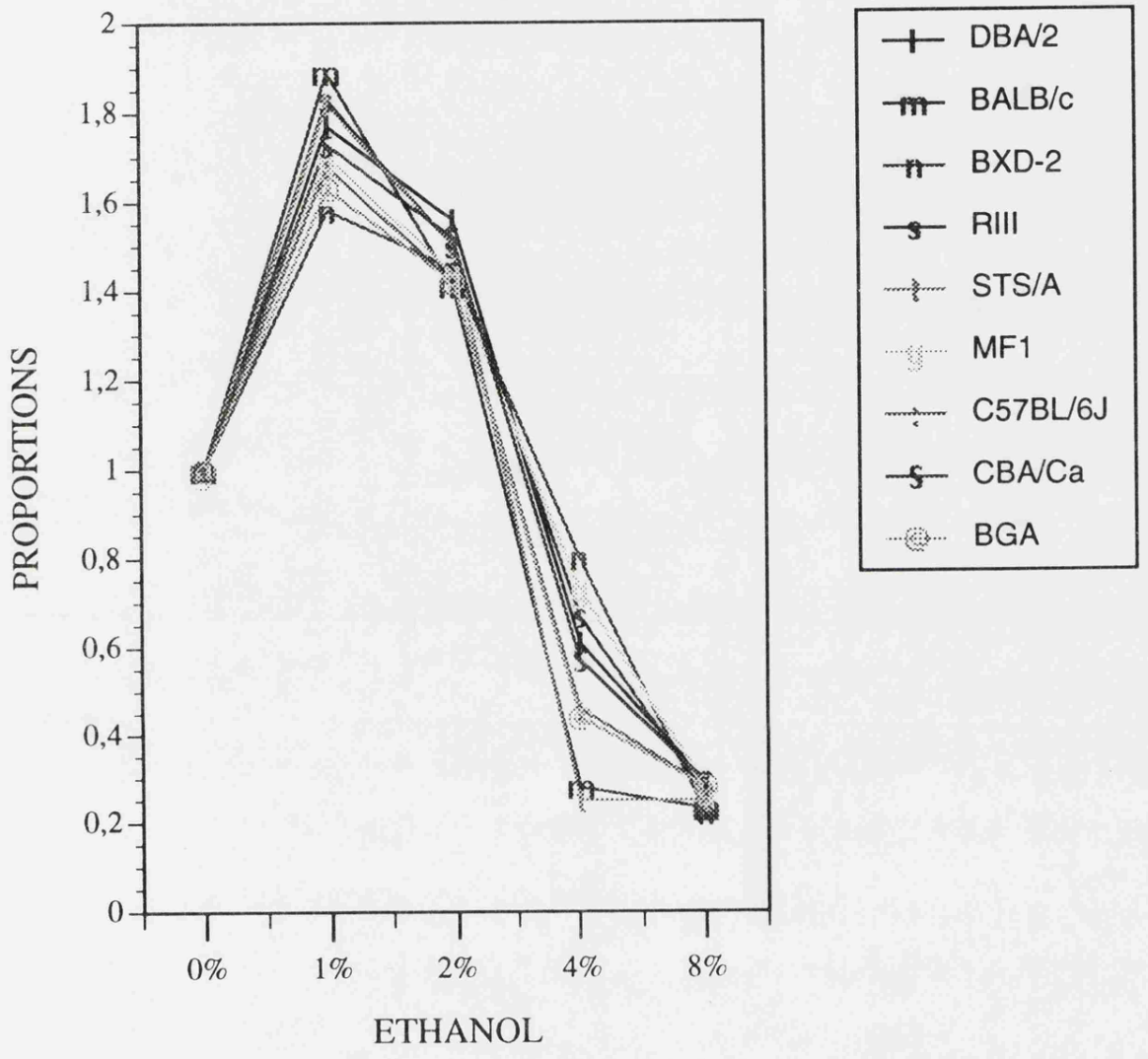

FIGURE V.II.3.9.- Averaged results (after dividing strain means by the respective assay mean) obtained in hepatocyte cultures treated with ethanol. The graph shows an even strain distribution, MF1, DBA/2 and RIII-*ro* showed the highest values, and STS/A and BALB/c the lowest.

and CBA/Ca and C57BL/6J the most sensitive (figure V.II.3.7, page 166).

There was good correlation between protein determinations and LDH activity in cells ($r=0.691$, $P<0.005$), and low between the rest ($P>0.05$) (see table V.II.3.18, appendix). As in the case of macrophages results were averaged across end-points to obtain a global view of the effect of ethanol on hepatocyte cultures. The graphic representation of these data (figure V.II.3.9) showed a continuous strain distribution pattern, with MF1, DBA/A and RIII-*ro* the strains which showed the highest values and STS/A and BALB/c the strains with the lowest values. Low correlations were also observed between the data obtained in macrophages and the data obtained in hepatocytes. This again suggested that different information was obtained and results were averaged across end-points and cell types (after dividing strain means by the respective assay mean). Tables V.II.3.3 and V.II.3.4 (page 155) summarize these results. Figure V.II.3.10 (page 170) shows the graphic representation of the global results. No great differences were observed among strains.

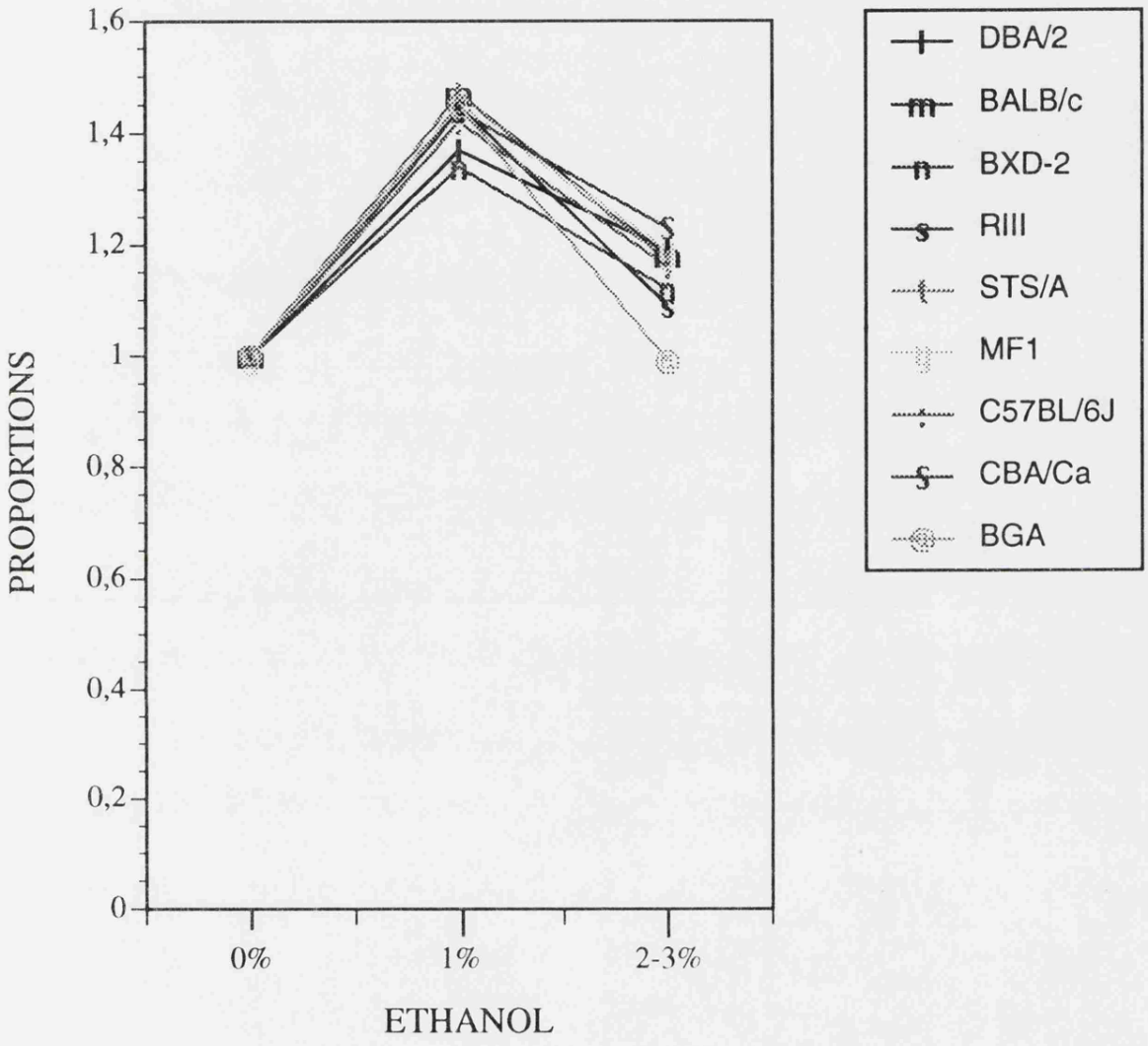

FIGURE V.II.3.10.- Averaged results obtained in macrophage and hepatocyte cultures treated with ethanol.- No clear differences were observed among strains.

DISCUSSION

In the previous chapter, the technique developed was used to study genetic variation in response to coumarin, in which a Mendelian locus was supposed to be involved. In this study, the technique was tested on the response to a drug which has been suggested to involve several loci. As explained in the introduction, the genetic control of ethanol metabolism is complex. Some previous studies on inbred strains (Festing and Eydmann, 1987a,b) on genetic variation in response to ethanol also suggested this multigenic control. However, these studies were based on the results obtained by a single toxicity assay (total protein determination), which, as seen in the coumarin chapter, may not provide as much information as would several end-points.

In macrophages, only the neutral red assay detected differences among strains in the slope of the dose-response curve to ethanol treatment, whereas the other two assays did not detect significant interactions between strains and concentration of ethanol (the strain dose response curves did not significantly differ). This could be because macrophage cultures were not very informative, as was the case in the studies on coumarin. No general strain distribution pattern can be observed in any of the figures of results obtained with

macrophages. The normal strain distribution observed in those figures seems to favour the hypothesis of a multigenic control of the response to ethanol. As in the coumarin study, the low correlations between assays suggest that several toxicity assays should be included in a general screening protocol so that no misleading conclusions are reached.

In hepatocytes, again there was no significant main effect for strains, although significant interactions between strains and concentration of ethanol were observed in all assays. These results differed from the data obtained in the coumarin study, where significant differences among strains were found in hepatocytes in all assays. Again no general pattern was observed and strains showed high or low values depending on the assay. The graphic representations of these results (figure V.II.3.9, page 168) did not show a bimodal pattern, but a normal distribution of strains. This could be taken as further evidence of a multigenic control of the response to ethanol.

The average of macrophage and hepatocyte results did not show a bimodal distribution of strains. This coincides with the normal distribution of strains found by Festing and Eydmann (1987b), showing no evidence for Mendelian control of the response to ethanol. No relation could be found between the global results and the genetics of the *Adh-A2* locus (the main class I ADH locus in mouse liver). Strains DBA/2 and BALB/c, both having low *Adh-A2* activity (Zhang et al., 1987), showed intermediate and high values, repectively, in this study, whereas C57BL/6J (Zhang et al., 1987), a high *Adh-A2*

activity strain, showed intermediate values. There were noticeable similarities between strains DBA/2 and RIII-*ro*, which gave similar values in all the assays. Further studies with recombinant strains from crosses between high and low performance strains may be worth investigating.

CHAPTER VI.- GENERAL DISCUSSION

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GENERAL DISCUSSION

One of the aims of these studies was to develop *in vitro* techniques for studying genetic variation in sensitivity to treatment with drugs.

The first part of these studies consisted of the development of such techniques. It included the selection of cell types and toxicity assays, standarization of cell cultures, and several experiments for the selection of colourimetric methods, selection of culture medium, induction of peritoneal macrophages and culture of bone marrow macrophages.

Two cell types, macrophages and hepatocytes were used. Easy and reliable methods for macrophage culture were available and standarized in this laboratory. Several established methods of liver perfusion for obtaining hepatocytes were tried and modified (change in medium composition, amount of collagenase used and time of perfusion) to achieve high yield and viability of cells. Hepatocyte culture conditions were standarized for these studies and a culture medium (WME) giving the best results from those available was selected (chapter V.I.2).

The yield of peritoneal macrophages per animal is too low for certain end-points such as chemiluminescence, so methods for increasing the number of macrophages were

examined. An attempt was made to increase macrophage production with horse serum injections, one of the least harmful inducing agents (Freshney, 1987). However, there was no significant improvement in the yield after horse serum injections. As inducing agents may affect cell activity, which may cause some complications in interpreting results, no inducing agents were included in the protocol.

Bone marrow macrophages were cultured with the same methods used for peritoneal macrophages, so that the time and expenses of the general procedure were not increased. However, different media and longer culture periods are needed for the development of macrophages from bone marrow cells. On the other hand, hepatocytes, the second type of cells included in the projected technique, are many times more abundant than macrophages, and could to some extent help to reduce the number of animals needed. However, they were not nearly so convenient as macrophages for genetics because obtaining hepatocytes is a time consuming procedure. Hepatocytes could only be obtained from about two mice/day.

Some authors have suggested that a general screening protocol for cytotoxic chemicals should include several toxicity assays (Knox et al., 1986; Castell and Gomez-Lechón, 1987; Tyson and Green, 1987). In this way, chemicals that impair some cell functions without affecting other parameters may have more chance of being detected. In the first trials with macrophages, several end-points, presumed to be measuring aspects of cell viability, number, metabolism and function were investigated following incubation with the test compound.

Other aspects considered for the selection of end-points were their reliability, ability to find differences between strains and the time consumed in performing the assay. Colourimetric methods were mostly chosen for these studies as they are inexpensive and easy to perform, and a large number of samples can be analysed in a relatively short time.

In chapter V.I.5 several colourimetric methods were selected among the large number which are available in the literature (neutral red uptake, MTT uptake, Kenacid blue stain, total protein determination). These methods were adapted to the type of cultures used and compared. As one of the aims of this project was to study genetic variation in the response to drugs, those colourimetric methods which detected variability between strains were preferred. Protein determinations and kenacid blue stain were the most sensitive assays. However, they measure the same parameter (protein content) and only total protein determination (easier to perform than the kenacid blue assay) were selected. Neutral red uptake, which measures cell viability, was also selected as being sensitive and reliable. Babich and Borenfreund (1987) combined these two methods by performing protein determination after the neutral red assay. However, a reduction in the total protein was observed when measured after performance of neutral red (Arranz and Festing, 1990). In later studies, both assays were performed on different plates. However, the procedure described by Babich and Borenfreund (1987) seems a good approach as it produces extra information with a low animal cost.

Other non-colourimetric methods were studied and adapted to the type of cultures used. LDH activity measurement is a reliable and widely used method to detect membrane integrity and was adapted to the culture conditions used. The rate of phagocytosis of fluorescent beads by macrophages which is a good measure of functional activity was also included to complete the range of techniques. Other assays, including hydrogen peroxidase production, chemiluminescence, and nitroblue tetrazolium salt (NBT) reduction were attempted using macrophages, but the methods either needed too many cells, proved to be too complex or had too low a repeatability for the large number of assays needed for genetic analyses.

Finally, the test-battery included four end-points (neutral red uptake, total protein determinations, LDH leakage and rate of phagocytosis) which measured different parameters (cell viability, protein content, membrane integrity and cell function, respectively) and two cell types (peritoneal macrophages and hepatocytes).

In the second part of these studies, the methods developed in part I were used to study the response of the cells to treatment with various test compounds (aspirin, coumarin and ethanol), and whether the response was under genetic control.

Several inbred strains of mice were used. Any difference between strains in the response to treatment with a test compound would show whether the response is under genetic control. The pattern of response may indicate if it is controlled by a single gene or a polygenic system. A bimodal

distribution in the response of the different strains would suggest (though not prove) control by a single gene. A gaussian distribution would indicate a polygenic system. Congenic strains (sets of inbred strains genotypically almost identical but differing only at a single major locus) were also used to study the effects of some specified genetic loci (e.g. *H-2* and *Cyp2b*).

At the time of studying genetic variation in response to aspirin, a technique including several end-points and cell types had not yet been completed, and this study was performed on macrophages using three toxicity assays, total protein determination, cell number and neutral red uptake (the results of which were discarded later). Although significant strain differences were observed, no evidence of a Mendelian control of the response was found. Earlier work on the response to aspirin had suggested that the major histocompatibility complex (*H-2*) could be involved in the response (Festing and Eydmann, 1987b). However, the *H-2* congenic strains BALB.B, BALB.K and BALB/c showed similar values, thus failing to support this observation.

Coumarin hydroxylase activity is largely controlled by a single locus. Several inbred strains with mice of known coumarin hydroxylase activity were used in this experiment. However, low correlations were observed between different assays, with strains showing high or low values depending on the method used for measuring the effect of the treatment. In no case was there evidence that *Cyp2b* activity had any effect on the sensitivity of the cells to coumarin. Although such a

negative results is disappointing, there is in fact no evidence that variation in *Cyp2b* activity has any effect on the toxicity of coumarin or any other substance *in vivo*. However, the global results are not in agreement with the conclusions reached by Festing and Eydmann (1987b) who tested 18 strains of known Cyp2b genotype and found that those with the *Cyp2b*-^{h} (including CBA/Ca) genotype ranked 1, 2, 3, 4 and 6th in sensitivity to 2.5mM coumarin. Further research will be needed to discover the reason for the lack of agreement between the two studies. One obvious difference is that Festing and Eydmann (1987) only found an effect of the *Cyp2b* locus at high dose levels of 2.5mM coumarin, but not at 0.308mM level, comparable to the highest level used in these investigations. However, they appeared to get a lower level of toxicity than was observed in these studies. Possibly, details of the culture procedure (like the inclusion of FCS in the collecting medium of macrophages) differed sufficiently between the two studies and may account for the observed differences.

The same technique was used to study genetic variation in response to ethanol. Previous studies on genetic variation in the response to ethanol (Festing and Eydmann, 1987a,b) gave no evidence of major genes involved in the response, and suggested multigenic control. Again low correlations between assays and cell types were obtained. No significant differences among strains were observed (except in the protein content of macrophages) although the interactions between strain and ethanol concentration were significant,

indicating that the strains behaved differently in response to ethanol. The graphic representation of the results showed a normal distribution of strains. No relation could be found between the global results and the genetics of the *Adh-A2* locus (the main *ADH* locus in mouse liver). No evidence for Mendelian control of the response to ethanol could be found.

In summary, although significant genetic control of response to ethanol could be demonstrated, there was no correlation with genotype at the *Adh-2* locus, and no evidence of clustering of strain means which might indicate the presence of a single major gene. Thus, although in vitro culture respresents a simplified system, there is no evidence that it offers major benefits in isolating genes associated with variation in response to toxic chemicals.

The results do seem to confirm that a general screening test should include several toxicity assays and cell types, as different cell types and end-points gave different estimates of over-all toxicity. However, in all cases it was the dose rate of the test compound that had the strongest effect on any given end point or cell type.

Obviously, these protocols are subject to further improvement. New and more accurate assays are constantly being developed, culture conditions improved and inbred strains are increasingly incorporated into research. Hopefully, this study may help to improve *in vitro* toxicity studies and future toxicity screening may include a investigation of the genetics of the response to the test chemical.

CHAPTER VII.- CONCLUSIONS

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CONCLUSIONS

- The results of the first part of these studies suggest that a screening protocol should include several toxicity assays. Different end-points measure different parameters, and a protocol inlcuding several assays may have more chance of detecting an adverse reaction caused by the treatment. Moreover, if the screening protocol is carried out on an appropriate set of inbred strains of animals, it could also detect genetic variation in the response to the test chemical.

- An *in vitro* toxicity screening protocol was developed including several toxicity asays (neutral red uptake, total protein determination, rate of phagocytosis and LDH activity) and two cell types (macrophages and hepatocytes) from a set of inbred strains of mice. This protocol seems to be valid for studying toxicity and genetic variation in the response to test chemicals, subject to further development and improvement.

- Genetic variation in the response to coumarin and ethanol were studied using the technique. The results

suggested a multigenic controlled response to ethanol. However, further studies with sets of congenic and recombinant strains are needed to confirm these hypotheses.

APPENDIX

LIST OF TABLES INCLUDED IN THE APPENDIX

CHAPTER V.II.3.- GENETIC VARIATION IN THE RESPONSE TO ETHANOL

V.I.4.- COMPARISON OF COLOURIMETRIC METHODS.- STATISTICAL ANALYSES AND TABLES OF MEANS.

ANALYSIS OF VARIANCE FOR EACH OF THE FIVE END-POINTS.

TABLE V.I.4.1.- Analysis of Variance for kenacid blue Stain

Source	DF	SS	MS	F	P
rep	5	8.87072	1.77415	28.83	0.000
Str	5	3.39871	0.67974	11.05	0.000
day	1	1.28283	1.28283	20.85	0.000
asp	4	0.97130	0.24282	3.95	0.004
Str*day	5	0.87711	0.17542	2.85	0.016
Str*asp	20	0.76232	0.03812	0.62	0.898
day*asp	4	0.08285	0.02071	0.34	0.853
Str*day*asp	20	0.28693	0.01435	0.23	1.000
Error	295	18.15332	0.06154		
Total	359	34.68610			

TABLE V.I.4.2.- Analysis of variance for MTT assay

Source	DF	Seq SS	Adj SS	Adj MS	F	P
rep	5	30.4532	32.7843	6.5569	8.29	0.000
Str	5	27.1406	27.4161	5.4832	6.93	0.000
day	1	1.0311	1.3373	1.3373	1.69	0.195
asp	4	0.9515	0.9155	0.2289	0.29	0.885
Str*day	5	16.9681	16.4644	3.2929	4.16	0.001
Str*asp	20	3.0268	3.2727	0.1636	0.21	1.000
day*asp	4	0.1650	0.1691	0.0423	0.05	0.995
Str*day*asp	20	2.8830	2.8830	0.1441	0.18	1.000
Error	286	226.1951	226.1951	0.7909		
Total	350	308.8145				

TABLE V.I.4.3.- Analysis of Variance for neutral red uptake

Source	DF	SS	MS	F	P
rep	5	18.0240	3.6048	6.26	0.000
Str	5	10.9187	2.1837	3.79	0.002
day	1	3.1323	3.1323	5.44	0.020
asp	4	1.4101	0.3525	0.61	0.654
Str*day	5	6.8580	1.3716	2.38	0.039
Str*asp	20	6.8646	0.3432	0.60	0.914
day*asp	4	1.1699	0.2925	0.51	0.730
Str*day*asp	20	7.5203	0.3760	0.65	0.870
Error	295	169.7652	0.5755		
Total	359	225.6631			

TABLE V.I.4.4.- Analysis of Variance for protein content

Source	DF	Seq SS	Adj SS	Adj MS	F	P
rep	5	6.4685	6.3779	1.2756	10.85	0.000
Str	5	3.2588	3.2546	0.6509	5.53	0.000
day	1	4.2612	4.3203	4.3203	36.74	0.000
asp	4	2.4305	2.4495	0.6124	5.21	0.000
Str*day	5	3.4793	3.4762	0.6952	5.91	0.000
Str*asp	20	2.3784	2.3857	0.1193	1.01	0.445
day*asp	4	0.5048	0.5126	0.1281	1.09	0.362
Str*day*asp	20	0.8518	0.8518	0.0426	0.36	0.995
Error	293	34.4568	34.4568	0.1176		
Total	357	58.0901				

TABLE V.I.4.5.- Analysis of Variance for cell number

Source	DF	SS	MS	F	P
rep	5	2.98488	0.59698	9.39	0.000
Str	5	2.11889	0.42378	6.67	0.000
day	1	0.05903	0.05903	0.93	0.336
asp	4	0.54070	0.13517	2.13	0.078
Str*day	5	1.68193	0.33639	5.29	0.000
Str*asp	20	0.58814	0.02941	0.46	0.978
day*asp	4	0.30581	0.07645	1.20	0.310
Str*day*asp	20	0.43864	0.02193	0.34	0.997
Error	295	18.75500	0.06358		
Total	359	27.47303			

TABLE V.I.4.6.- Comparison of colourimetric methods.- Results obtained on macrophage cultures treated with different Aspirin concentrations by 4 colourimetric methods (Neutral red uptake (NR), total protein determination, Kenacid blue (Kb), and MTT) and cell counting. Values are given as proportions of absolute values divided by their respective controls and were averaged through replications.

STRAIN	ASPIRIN CONCENT.	NR	PROTEIN	KB	MTT	CELL NUMBER
CBA/Ca	0.156mM	1.40	1.33	1.11	1.10	0.99
	0.312mM	1.28	1.22	1.11	1.17	0.97
	0.625mM	1.48	1.24	1.07	1.00	0.84
	1.25 mM	1.15	1.19	1.07	1.06	0.92
	2.50 mM	0.99	1.12	1.05	1.17	0.90
RIII-*ro*	0.156mM	1.00	1.16	1.16	0.98	1.01
	0.312mM	1.12	1.14	1.15	1.15	0.95
	0.612mM	1.10	1.10	1.14	1.15	0.91
	1.25 mM	0.89	0.95	0.99	1.09	0.87
	2.50 mM	0.85	0.84	0.90	1.01	0.91
MF1	0.156mM	0.73	1.45	1.35	4.57	1.12
	0.312mM	1.06	1.41	1.38	5.15	1.04
	0.625mM	1.10	1.30	1.34	9.39	1.00
	1.25 mM	1.06	1.22	1.25	5.49	0.98
	2.50 mM	1.02	1.02	1.13	11.99	0.87
BXD-2	0.156mM	1.67	1.44	1.25	0.95	1.05
	0.312mM	1.79	1.31	1.35	1.16	0.94
	0.612mM	1.50	1.33	1.28	0.96	1.02
	1.25 mM	1.29	1.06	1.24	1.02	1.02
	2.50 mM	1.14	0.95	1.13	0.89	1.02
BALB/c	0.156mM	1.18	1.17	1.07	2.08	1.17
	0.312mM	1.06	1.10	1.01	1.93	1.14
	0.612mM	1.09	1.12	1.04	1.97	1.22
	1.25 mM	1.12	1.12	1.03	1.70	1.07
	2.50 mM	1.13	1.11	1.01	1.75	1.06
STS/A	0.156mM	1.28	1.22	1.13	0.91	0.95
	0.312mM	1.36	1.26	1.26	0.95	0.96
	0.625mM	1.07	1.43	1.28	0.87	0.87
	1.25 mM	1.28	1.35	1.19	0.86	0.87
	2.50 mM	1.50	1.34	1.18	0.98	0.87

V.II.1.- GENETIC VARIATION IN THE RESPONSE TO ASPIRIN.- STATISTICAL ANALYSES AND TABLES OF MEANS

TABLE V.II.1.2.- Analysis of Variance for protein in macrophage cultures treated with aspirin -experiment 1-

Source	DF	SS	MS	F	P
replica	4	12.4534	3.1134	15.11	0.000
strain	15	10.6390	0.7093	3.44	0.000
replica*strain	60	13.4870	0.2248	1.09	0.366
aspirin	1	28.0578	28.0578	136.16	0.000
strain*aspirin	15	14.0087	0.9339	4.53	0.000
Error	64	13.1880	0.2061		
Total	159	91.8339			

F-test with denominator: replication*strain
Denominator MS = 0.22478 with 60 degrees of freedom

Numerator	DF	MS	F	P
strain	15	0.7093	3.16	0.001

TABLE V.II.1.3.- Genetic variation in the response to aspirin. Protein measured in macrophage cultures treated with aspirin (0,1 and 5mM in culture medium) -Experiment 1.

STRAIN	ASPIRIN CONCENT.	PROTEIN (μg/ml)	PROTEIN (divided by control)
C57BL/6	0 mM	23.42	
	1 mM	30.58	1.267
	5 mM	17.62	0.722
C57BL/10ScSn	0 mM	35.94	
	1 mM	55.66	1.556
	5 mM	31.10	0.879
C57BL/Ks	0 mM	41.98	
	1 mM	69.24	1.693
	5 mM	37.56	0.962
DBA/2Cbi	0 mM	62.92	
	1 mM	104.10	1.727
	5 mM	37.02	0.654
MF1	0 mM	57.22	
	1 mM	102.22	2.109
	5 mM	30.04	0.651
$B10CF_1$	0 mM	41.76	
	1 mM	67.70	1.738
	5 mM	34.82	0.922
$CBCF_1$	0 mM	31.60	
	1 mM	48.16	1.608
	5 mM	28.88	0.968
$CBB10F_1$	0 mM	41.14	
	1 mM	50.04	1.300
	5 mM	29.44	0.855
CBA/Ca (1)	0 mM	33.92	
	1 mM	37.58	1.145
	5 mM	29.84	0.905
CBA/Ca (2)	0 mM	26.80	
	1 mM	33.62	1.279
	5 mM	22.90	0.863
LP	0 mM	39.60	
	1 mM	96.10	2.564
	5 mM	29.34	0.769
A/J	0 mM	33.94	
	1 mM	38.96	1.221
	5 mM	20.26	0.646
AKR	0 mM	30.22	
	1 mM	84.10	3.222
	5 mM	21.14	0.723
BALB/c	0 mM	27.68	
	1 mM	38.02	1.416
	5 mM	29.88	1.100
C_3H/He	0 mM	50.80	
	1 mM	74.88	1.683
	5 mM	36.96	0.810
CBA/N	0 mM	34.96	
	1 mM	39.38	1.163
	5 mM	28.52	0.862

TABLE V.II.1.4.- Analysis of Variance for protein in macrophage cultures treated with aspirin -experiment 2-.

Source	DF	SS	MS	F	P
strain	8	15.2767	1.7202	3.01	0.012
replication	4	5.7534	1.4353	2.51	0.060
strain*replication	32	22.0110	0.6780	1.19	0.314
aspirin	1	1.5671	1.5680	2.74	0.107
strain*aspirin	8	8.2636	1.0330	1.81	0.111
error	33	18.8548	0.5714		
total	86	71.7266			

F-test with denominator: strain*replication
Denominator MS= 0.68784 with 32 degrees of freedom

Numerator	DF	MS	F	P
strain	8	1.910	2.78	0.019

TABLE V.II.1.5.- Genetic variation in the response to aspirin.- Protein content measured in macrophage cultures treated with Aspirin (0, 1 and 5mM in culture medium).
- Experiment 2 -

STRAIN	ASPIRIN CONCENT.	PROTEIN (μg/ml)	PROTEIN (divided by control)
MF1	0 mM	92.27	
	1 mM	172.39	2.769
	5 mM	65.98	0.879
CBA/Ca	0 mM	32.51	
	1 mM	45.54	1.426
	5 mM	64.14	2.143
AKR	0 mM	59.13	
	1 mM	83.68	1.353
	5 mM	38.60	0.659
LP	0 mM	55.15	
	1 mM	113.23	2.005
	5 mM	78.02	2.113
STS/A	0 mM	31.98	
	1 mM	37.48	1.163
	5 mM	44.39	1.401
BXD-2	0 mM	129.31	
	1 mM	117.70	1.086
	5 mM	42.70	0.379
BALB.B	0 mM	43.15	
	1 mM	43.59	1.017
	5 mM	28.87	0.749
BALB/c	0 mM	42.86	
	1 mM	46.33	1.087
	5 mM	38.54	0.842
BALB.K	0 mM	56.71	
	1 mM	67.51	1.219
	5 mM	61.31	1.200

V.II.2.- GENETIC VARIATION IN THE RESPONSE TO COUMARIN.- STATISTICAL ANALYSES AND TABLES OF MEANS

TABLE V.II.2.8.- Analysis of Variance for neutral red in macrophage cultures treated with coumarin.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	8	3.23053	3.23053	0.40382	15.19	0.000
sx	1	0.34613	0.34613	0.34613	13.02	0.000
rp(str sx)	36	7.23638	7.23638	0.20101	7.56	0.000
str*sx	8	0.19509	0.19509	0.02439	0.92	0.504
conc	3	6.04820	6.04820	2.01607	75.85	0.000
str*conc	24	1.90363	1.90363	0.07932	2.98	0.000
sx*conc	3	0.10058	0.10058	0.03353	1.26	0.290
Error	132	3.50869	3.50869	0.02658		
Total	215	22.56923				

F-test with denominator: rp(str sx)
Denominator MS = 0.20101 with 36 degrees of freedom

Numerator	DF	Seq MS	F	P
str	8	0.40382	2.01	0.073
sx	1	0.34613	1.72	0.198
str*sx	8	0.02439	0.12	0.998

TABLE V.II.2.9.- Analysis of Variance for protein in macrophage cultures treated with coumarin

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	8	0.86278	0.86278	0.10785	6.35	0.000
sx	1	0.06164	0.06164	0.06164	3.63	0.059
rp(str sx)	36	2.36724	2.36724	0.06576	3.87	0.000
str*sx	8	0.28829	0.28829	0.03604	2.12	0.038
conc	3	3.92018	3.92018	1.30673	76.95	0.000
str*conc	24	0.81894	0.81894	0.03412	2.01	0.007
sx*conc	3	0.05959	0.05959	0.01986	1.17	0.324
Error	132	2.24151	2.24151	0.01698		
Total	215	10.62015				

F-test with denominator: rp(str sx)
Denominator MS = 0.065757 with 36 degrees of freedom

Numerator	DF	Seq MS	F	P
str	8	0.10785	1.64	0.148
sx	1	0.06164	0.94	0.339
str*sx	8	0.03604	0.55	0.812

TABLE V.II.2.10.- Neutral Red absorption measured in macrophage cultures treated with coumarin. (Data given as proportion of their respective controls).

STRAIN	COUMARIN	MALES	FEMALES	MEANS
DBA/2	0.035mM	1.038	0.992	1.015
	0.071	0.943	0.955	0.949
	0.151	1.026	0.880	0.953
	0.312	0.907	0.844	0.876
	mean	0.978	0.918	0.948
BALB/c	0.035mM	0.947	0.855	0.901
	0.071	0.827	0.837	0.832
	0.151	0.824	0.759	0.791
	0.312	0.592	0.546	0.569
	mean	0.798	0.749	0.773
BXD-2	0.035mM	0.997	0.878	0.937
	0.071	0.978	0.879	0.928
	0.151	0.800	0.610	0.705
	0.312	0.388	0.287	0.337
	mean	0.791	0.663	0.727
RIII-*ro*	0.035mM	0.811	0.893	0.852
	0.071	0.765	0.790	0.778
	0.151	0.525	0.404	0.464
	0.312	0.246	0.228	0.237
	mean	0.587	0.579	0.583
STS/A	0.035mM	1.021	0.876	0.948
	0.071	1.089	0.841	0.965
	0.151	1.090	0.774	0.932
	0.312	0.460	0.609	0.535
	mean	0.915	0.775	0.845
MF1	0.035mM	0.942	0.890	0.916
	0.071	0.924	0.768	0.846
	0.151	0.657	0.672	0.665
	0.312	0.416	0.333	0.374
	mean	0.735	0.666	0.700
C57BL/6J	0.035mM	1.013	0.926	0.969
	0.071	1.011	0.847	0.929
	0.151	0.874	0.781	0.827
	0.312	0.676	0.746	0.711
	mean	0.893	0.825	0.859
CBA/Ca	0.035mM	0.831	0.843	0.837
	0.071	0.842	0.708	0.775
	0.151	0.815	0.670	0.743
	0.312	0.713	0.663	0.688
	mean	0.800	0.721	0.761
BGA	0.035mM	0.885	0.884	0.885
	0.071	0.724	0.753	0.738
	0.151	0.617	0.534	0.576
	0.312	0.414	0.397	0.406
	mean	0.660	0.642	0.642

TABLE V.II.2.11.- Protein measured in macrophage cultures treated with coumarin. (Data given as proportion of their respective controls).

STRAIN	COUMARIN	MALES	FEMALES	MEANS
DBA/2	0.035mM	1.012	1.016	1.014
	0.071	1.057	1.102	1.079
	0.151	1.006	1.349	1.177
	0.312	0.738	1.044	0.891
	mean	0.953	1.128	1.040
BALB/c	0.035mM	1.111	0.962	1.036
	0.071	0.988	0.897	0.943
	0.151	0.847	0.807	0.827
	0.312	0.494	0.461	0.478
	mean	0.860	0.782	0.821
BXD-2	0.035mM	1.069	0.940	1.004
	0.071	1.008	1.037	1.023
	0.151	0.762	0.696	0.729
	0.312	0.261	0.573	0.417
	mean	0.775	0.811	0.793
RIII-*ro*	0.035mM	1.037	0.997	1.017
	0.071	0.860	1.091	0.975
	0.151	0.590	0.832	0.711
	0.312	0.394	0.336	0.365
	mean	0.720	0.814	0.767
STS/A	0.035mM	1.024	1.039	1.031
	0.071	0.951	0.963	0.957
	0.151	0.769	0.952	0.861
	0.312	0.598	0.847	0.722
	mean	0.835	0.950	0.893
MF1	0.035mM	1.033	0.994	1.013
	0.071	0.835	0.893	0.864
	0.151	0.628	0.826	0.727
	0.312	0.481	0.497	0.489
	mean	0.744	0.803	0.773
C57BL/6J	0.035mM	1.130	1.035	1.082
	0.071	1.090	0.979	1.035
	0.151	1.134	0.817	0.976
	0.312	0.707	0.516	0.612
	mean	1.015	0.837	0.926
CBA/Ca	0.035mM	0.994	0.961	0.977
	0.071	0.893	1.095	0.995
	0.151	0.789	0.967	0.878
	0.312	0.728	0.943	0.835
	mean	0.851	0.992	0.921
BGA	0.035mM	1.001	1.046	1.023
	0.071	0.942	1.092	1.017
	0.151	0.878	0.920	0.899
	0.312	0.579	0.452	0.516
	mean	0.850	0.878	0.864

TABLE V.II.2.12.- Analysis of Variance for phagocytosis in macrophages treated with coumarin

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	7	0.41717	0.54173	0.07739	5.77	0.000
sx	1	0.00011	0.00118	0.00118	0.09	0.767
rp(str sx)	32	2.71257	2.32472	0.07265	5.42	0.000
str*sx	7	0.21964	0.17806	0.02544	1.90	0.077
conc	3	8.08205	8.13571	2.71190	202.14	0.000
str*conc	21	0.46408	0.45695	0.02176	1.62	0.057
sx*conc	3	0.05070	0.05070	0.01690	1.26	0.292
Error	109	1.46232	1.46232	0.01342		
Total	183	13.40864				

F-test with denominator: rp(str sx)
Denominator MS = 0.084768 with 32 degrees of freedom

Numerator	DF	Seq MS	F	P
str	7	0.059596	0.70	0.669
sx	1	0.000115	0.00	0.971
str*sx	7	0.031378	0.37	0.913

TABLE V.II.2.13.- Analysis of Variance for neutral red in hepatocyte cultures treated with coumarin

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	8	1.53495	1.53495	0.19187	21.45	0.000
sx	1	0.01775	0.01775	0.01775	1.98	0.161
rp(str sx)	36	2.29889	2.29889	0.06386	7.14	0.000
str*sx	8	0.25454	0.25454	0.03182	3.56	0.001
conc	3	17.47993	17.47993	5.82664	651.31	0.000
str*conc	24	0.48695	0.48695	0.02029	2.27	0.002
sx*conc	3	0.03367	0.03367	0.01122	1.25	0.293
Error	132	1.18087	1.18087	0.00895		
Total	215	23.28756				

F-test with denominator: rp(str sx)
Denominator MS = 0.063858 with 36 degrees of freedom

Numerator	DF	Seq MS	F	P
str	8	0.19187	3.00	0.011
sx	1	0.01775	0.28	0.601
str*sx	8	0.03182	0.50	0.849

TABLE V.II.2.14.- Rate of phagocytosis measured in macrophages cultured with coumarin. (Data given as proportion of their respective controls).

STRAIN	COUMARIN	MALES	FEMALES	MEANS
BALB/c	0.035mM	1.049	0.967	1.008
	0.071	0.971	0.834	0.902
	0.151	0.745	0.824	0.784
	0.312	0.509	0.565	0.537
	mean	0.818	0.798	0.808
BXD-2	0.035mM	0.935	0.953	0.944
	0.071	0.838	0.957	0.897
	0.151	0.765	0.681	0.723
	0.312	0.232	0.345	0.288
	mean	0.693	0.734	0.713
RIII-*ro*	0.035mM	0.926	1.058	0.992
	0.071	0.844	0.947	0.896
	0.151	0.595	0.475	0.535
	0.312	0.319	0.224	0.271
	mean	0.671	0.676	0.673
STS/A	0.035mM	1.031	1.042	1.036
	0.071	0.985	1.015	1.000
	0.151	0.855	0.858	0.856
	0.312	0.327	0.548	0.437
	mean	0.799	0.866	0.833
MF1	0.035mM	0.952	0.970	0.961
	0.071	0.938	0.904	0.921
	0.151	0.543	0.773	0.658
	0.312	0.219	0.370	0.294
	mean	0.663	0.754	0.709
C57BL/6J	0.035mM	0.914	0.954	0.934
	0.071	0.744	0.856	0.800
	0.151	0.644	0.557	0.600
	0.312	0.304	0.405	0.354
	mean	0.651	0.693	0.672
CBA/Ca	0.035mM	0.978	0.922	0.950
	0.071	0.940	0.860	0.900
	0.151	0.900	0.698	0.799
	0.312	0.535	0.506	0.521
	mean	0.838	0.746	0.792
BGA	0.035mM	0.891	0.917	0.904
	0.071	0.886	0.754	0.820
	0.151	0.684	0.531	0.608
	0.312	0.474	0.425	0.449
	mean	0.734	0.657	0.695

TABLE V.II.2.15.- Neutral Red absorption measured in hepatocyte cultures treated with Coumarin. (Data given as proportion of their respective controls).

STRAIN	COUMARIN	MALES	FEMALES	MEANS
DBA/2	0.035mM	0.832	0.728	0.780
	0.071	0.658	0.443	0.550
	0.151	0.198	0.128	0.163
	0.312	0.065	0.058	0.061
	mean	0.438	0.339	0.389
BALB/c	0.035mM	0.705	0.598	0.652
	0.071	0.331	0.275	0.303
	0.151	0.103	0.072	0.087
	0.312	0.076	0.043	0.059
	mean	0.304	0.247	0.275
BXD-2	0.035mM	0.694	0.784	0.739
	0.071	0.384	0.685	0.534
	0.151	0.172	0.258	0.215
	0.312	0.082	0.072	0.077
	mean	0.333	0.449	0.391
RIII-*ro*	0.035mM	0.809	0.845	0.827
	0.071	0.635	0.679	0.657
	0.151	0.421	0.351	0.386
	0.312	0.199	0.135	0.167
	mean	0.516	0.502	0.509
STS/A	0.035mM	0.911	0.851	0.881
	0.071	0.638	0.464	0.551
	0.151	0.237	0.097	0.167
	0.312	0.082	0.063	0.073
	mean	0.467	0.369	0.418
MF1	0.035mM	0.815	0.898	0.857
	0.071	0.531	0.567	0.549
	0.151	0.298	0.150	0.224
	0.312	0.114	0.092	0.103
	mean	0.440	0.427	0.433
C57BL/6J	0.035mM	0.905	1.013	0.959
	0.071	0.597	0.802	0.699
	0.151	0.298	0.259	0.278
	0.312	0.128	0.095	0.112
	mean	0.482	0.542	0.512
CBA/Ca	0.035mM	0.928	0.946	0.937
	0.071	0.750	0.756	0.753
	0.151	0.452	0.415	0.434
	0.312	0.198	0.182	0.190
	mean	0.582	0.575	0.578
BGA	0.035mM	0.846	0.828	0.837
	0.071	0.543	0.408	0.475
	0.151	0.248	0.173	0.211
	0.312	0.131	0.068	0.100
	mean	0.442	0.369	0.406

TABLE V.II.2.16.- Analysis of Variance for protein in hepatocyte cultures treated with coumarin

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	7	0.87729	0.81839	0.11691	17.36	0.000
sx	1	0.20216	0.22038	0.22038	32.73	0.000
rp(str sx)	32	1.41078	1.42689	0.04459	6.62	0.000
str*sx	7	0.22269	0.20778	0.02968	4.41	0.000
conc	3	5.37317	5.35496	1.78499	265.07	0.000
str*conc	21	0.24308	0.24304	0.01157	1.72	0.037
sx*conc	3	0.02164	0.02164	0.00721	1.07	0.364
Error	116	0.78114	0.78114	0.00673		
Total	190	9.13195				

F-test with denominator: rp(str sx)
Denominator MS = 0.044087 with 32 degrees of freedom

Numerator	DF	Seq MS	F	P
str	7	0.12533	2.84	0.020
sx	1	0.20216	4.59	0.040
str*sx	7	0.03181	0.72	0.655

TABLE V.II.2.17.- Analysis of Variance for LDH in hepatocyte cells treated with coumarin

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	8	0.92551	0.83672	0.10459	11.25	0.000
sx	1	0.03155	0.02472	0.02472	2.66	0.105
rp(str sx)	36	1.61029	1.61030	0.04473	4.81	0.000
str*sx	8	0.53662	0.44840	0.05605	6.03	0.000
conc	3	12.84796	12.62859	4.20953	452.81	0.000
str*conc	24	0.24282	0.23722	0.00988	1.06	0.395
sx*conc	3	0.06316	0.06316	0.02105	2.26	0.084
Error	127	1.18065	1.18065	0.00930		
Total	210	17.43856				

F-test with denominator: rp(str sx)
Denominator MS = 0.044730 with 36 degrees of freedom

Numerator	DF	Seq MS	F	P
str	8	0.11569	2.59	0.024
sx	1	0.03155	0.71	0.407
str*sx	8	0.06708	1.50	0.192

TABLE V.II.2.18.- Protein measured in hepatocyte cultures treated with coumarin. (Data given as proportion of their respective controls).

STRAIN	COUMARIN	MALES	FEMALES	MEANS
DBA/2	0.035mM	0.964	0.856	0.910
	0.071	0.919	0.763	0.841
	0.151	0.483	0.512	0.497
	0.312	0.455	0.327	0.391
	mean	0.705	0.614	0.660
BALB/c	0.035mM	0.831	0.668	0.750
	0.071	0.690	0.447	0.569
	0.151	0.447	0.327	0.387
	0.312	0.296	0.207	0.252
	mean	0.566	0.413	0.489
BXD-2	0.035mM	0.973	1.014	0.994
	0.071	0.858	0.882	0.870
	0.151	0.760	0.688	0.724
	0.312	0.397	0.371	0.384
	mean	0.747	0.739	0.743
RIII-*ro*	0.035mM	0.927	0.974	0.951
	0.071	0.886	0.748	0.817
	0.151	0.764	0.727	0.745
	0.312	0.455	0.457	0.456
	mean	0.758	0.727	0.742
STS/A	0.035mM	0.894	0.852	0.873
	0.071	0.795	0.556	0.675
	0.151	0.475	0.385	0.430
	0.312	0.286	0.261	0.274
	mean	0.613	0.514	0.563
MF1	0.035mM	1.006	1.097	1.051
	0.071	0.950	0.815	0.883
	0.151	0.783	0.514	0.649
	0.312	0.385	0.321	0.353
	mean	0.781	0.687	0.734
C57BL/6J	0.035mM	0.934	0.762	0.848
	0.071	0.782	0.570	0.676
	0.151	0.594	0.458	0.526
	0.312	0.366	0.333	0.350
	mean	0.669	0.531	0.600
CBA/Ca	0.035mM	0.838	0.845	0.841
	0.071	0.639	0.829	0.734
	0.151	0.528	0.709	0.618
	0.312	0.425	0.472	0.448
	mean	0.607	0.713	0.660
BGA	0.035mM	0.981	0.790	0.885
	0.071	0.772	0.689	0.731
	0.151	0.497	0.451	0.474
	0.312	0.372	0.321	0.346
	mean	0.655	0.563	0.609

TABLE V.II.2.19.- LDH activity measured in hepatocyte cells treated with coumarin. (Data given as proportion of their respective controls).

STRAIN	COUMARIN	MALES	FEMALES	MEANS
DBA/2	0.035mM	0.881	0.741	0.811
	0.071	0.618	0.583	0.600
	0.151	0.220	0.380	0.300
	0.312	0.075	0.184	0.129
	mean	0.448	0.472	0.460
BALB/c	0.035mM	0.755	0.713	0.734
	0.071	0.616	0.358	0.487
	0.151	0.340	0.184	0.262
	0.312	0.091	0.080	0.085
	mean	0.450	0.333	0.392
BXD-2	0.035mM	0.694	0.796	0.744
	0.071	0.522	0.752	0.637
	0.151	0.177	0.524	0.350
	0.312	0.066	0.244	0.155
	mean	0.365	0.578	0.472
RIII-*ro*	0.035mM	0.840	0.889	0.865
	0.071	0.783	0.771	0.777
	0.151	0.502	0.540	0.521
	0.312	0.263	0.242	0.252
	mean	0.597	0.610	0.604
STS/A	0.035mM	0.757	0.677	0.717
	0.071	0.546	0.424	0.485
	0.151	0.323	0.147	0.235
	0.312	0.125	0.086	0.105
	mean	0.438	0.333	0.386
MF1	0.035mM	0.845	0.888	0.866
	0.071	0.754	0.641	0.698
	0.151	0.528	0.379	0.453
	0.312	0.093	0.162	0.127
	mean	0.555	0.517	0.536
C57BL/6J	0.035mM	0.882	0.761	0.821
	0.071	0.714	0.519	0.617
	0.151	0.509	0.270	0.389
	0.312	0.160	0.128	0.144
	mean	0.566	0.419	0.493
CBA/Ca	0.035mM	0.702	0.827	0.764
	0.071	0.463	0.672	0.568
	0.151	0.304	0.385	0.345
	0.312	0.090	0.172	0.131
	mean	0.390	0.514	0.452
BGA	0.035mM	0.915	0.637	0.776
	0.071	0.616	0.520	0.568
	0.151	0.272	0.296	0.284
	0.312	0.110	0.131	0.120
	mean	0.478	0.396	0.437

TABLE V.II.2.20.- Analysis of Variance for LDH in supernatant of hepatocyte cultures treated with coumarin

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	8	2.42140	2.77103	0.34638	29.37	0.000
sx	1	0.40632	0.48772	0.48772	41.36	0.000
rp(str sx)	36	3.62260	3.07891	0.08553	7.25	0.000
str*sx	8	1.89263	2.08761	0.26095	22.13	0.000
conc	3	5.97978	5.95963	1.98654	168.46	0.000
str*conc	24	0.71221	0.71264	0.02969	2.52	0.000
sx*conc	3	0.01196	0.01196	0.00399	0.34	0.798
Error	128	1.50941	1.50941	0.01179		
Total	211	16.55630				

F-test with denominator: rp(str sx)
Denominator MS = 0.10063 with 36 degrees of freedom

Numerator	DF	Seq MS	F	P
str	8	0.3027	3.01	0.011
sx	1	0.4063	4.04	0.052
str*sx	8	0.2366	2.35	0.038

TABLE V.II.2.21.- Matrix of correlations of strain means between assays (coumarin chapter)

	NRHE	PROHE	LDHCE	NRMA	PROMA	PHAGO	LDHSU	GLOHE	GLOMA
PROHEP	0.44								
LDHCELL	0.49	0.78							
NRMAC	-.15	-.41	-.52						
PRMAC	0.11	-.31	-.40	0.82					
PHAGOC	-.30	-.58	-.76	0.50	0.32				
LDHSUP	0.06	0.43	0.70	-.83	-.78	-.38			
GLOHEP	0.66	0.81	0.94	-.63	-.44	-.62	0.71		
GLOMAC	-.46	-.03	-.08	0.49	0.32	0.35	-.08	-.24	
GLOBAL	0.67	0.58	0.59	0.18	0.36	-.29	0.07	0.60	0.26

PROHEP= protein measured in hepatocytes. LDHCELL= LDH measured in hepatocyte cells. NRMAC= neutral red uptake measured in macrophages. PRMAC= protein measured in macrophages. PHAGOC= phagocytosis measured in macrophages. LDHSUP= LDH activity measured in the supernatant of hepatocyte cultures. GLOHEP= averaged results obtained in hepatocytes. GLOMAC= averaged results obtained in macrophages. GLOBAL= averaged results obtained in macrophages and hepatocytes.

Statistical significance = values > 0.666 (P=0.05)
values > 0.8 (P=0.01)

TABLE V.II.2.22.- LDH activity measured in the supernatant of hepatocyte cultures treated with coumarin. (Data given as proportion of their respective controls).

STRAIN	COUMARIN	MALES	FEMALES	MEANS
DBA/2	0.035mM	2.357	1.772	2.064
	0.071	3.348	2.289	2.818
	0.151	5.057	3.002	4.030
	0.312	5.420	3.999	4.710
	mean	4.054	2.766	3.406
BALB/c	0.035mM	1.664	1.760	1.712
	0.071	2.434	2.549	2.491
	0.151	2.893	2.959	2.926
	0.312	3.248	3.008	3.128
	mean	2.560	2.569	2.564
BXD-2	0.035mM	1.531	0.975	1.253
	0.071	2.376	1.190	1.783
	0.151	4.067	2.028	3.047
	0.312	6.625	3.900	5.263
	mean	3.650	2.023	2.837
RIII-*ro*	0.035mM	1.386	1.467	1.426
	0.071	1.666	1.169	1.417
	0.151	2.319	1.327	1.823
	0.312	3.787	2.055	2.921
	mean	2.289	1.505	1.897
STS/A	0.035mM	2.118	1.895	2.007
	0.071	2.661	3.407	3.034
	0.151	3.575	4.563	4.069
	0.312	3.692	4.117	3.905
	mean	3.011	3.496	3.254
MF1	0.035mM	1.041	1.166	1.104
	0.071	1.503	1.711	1.607
	0.151	2.481	2.565	2.523
	0.312	4.296	3.879	4.088
	mean	2.330	2.330	2.330
C57BL/6J	0.035mM	1.453	1.790	1.621
	0.071	2.205	2.874	2.539
	0.151	3.589	4.999	4.294
	0.312	4.517	5.186	4.851
	mean	2.941	3.712	3.327
CBA/Ca	0.035mM	1.952	1.145	1.548
	0.071	2.791	1.728	2.260
	0.151	3.959	3.050	3.504
	0.312	5.485	3.651	4.568
	mean	3.547	2.394	2.970
BGA	0.035mM	1.582	1.689	1.636
	0.071	2.494	2.276	2.385
	0.151	3.220	2.840	3.030
	0.312	4.566	3.602	4.084
	mean	2.966	2.602	2.784

V.II.3.- GENETIC VARIATION IN THE RESPONSE TO ETHANOL.- STATISTICAL ANALYSES AND TABLES OF MEANS.

TABLE V.II.3.5.- Analysis of Variance for neutral red in macrophage cultures treated with ethanol.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	7	0.315987	0.315987	0.045141	11.27	0.000
sx	1	0.034478	0.034478	0.034478	8.61	0.006
rp(str sx)	32	1.205611	1.205611	0.037675	9.41	0.000
str*sx	7	0.105833	0.105833	0.015119	3.77	0.003
conc	1	0.181670	0.181670	0.181670	45.36	0.000
str*conc	7	0.111568	0.111568	0.015938	3.98	0.002
sx*conc	1	0.000237	0.000237	0.000237	0.06	0.809
Error	39	0.156203	0.156203	0.004005		
Total	95	2.111587				

F-test with denominator: rp(str sx)
Denominator MS = 0.037675 with 32 degrees of freedom

Numerator	DF	Seq MS	F	P
str	7	0.04514	1.20	0.332
sx	1	0.03448	0.92	0.346
str*sx	7	0.01512	0.40	0.894

TABLE V.II.3.6.- Analysis of Variance for protein in macrophage cultures treated with ethanol

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	7	0.383214	0.383214	0.054745	9.72	0.000
sx	1	0.001102	0.001102	0.001102	0.20	0.661
rp(str sx)	32	0.440207	0.440207	0.013756	2.44	0.004
str*sx	7	0.053944	0.053944	0.007706	1.37	0.246
conc	1	0.421632	0.421632	0.421632	74.87	0.000
str*conc	7	0.049467	0.049467	0.007067	1.25	0.298
sx*conc	1	0.001840	0.001840	0.001840	0.33	0.571
Error	39	0.219626	0.219626	0.005631		
Total	95	1.571033				

F-test with denominator: rp(str sx)
Denominator MS = 0.013756 with 32 degrees of freedom

Numerator	DF	Seq MS	F	P
str	7	0.054745	3.98	0.003
sx	1	0.001102	0.08	0.779
str*sx	7	0.007706	0.56	0.782

TABLE V.II.3.7.- Neutral Red measured in macrophage cultures treated with ethanol. (Data given as proportion of their respective controls).

STRAIN	ETHANOL	MALES	FEMALES	MEANS
DBA/2	1% v/v	0.911	1.030	0.971
	3%	0.777	0.852	0.815
	mean	0.844	0.941	0.893
BALB/c	1% v/v	1.019	1.008	1.014
	3%	0.869	0.938	0.903
	mean	0.944	0.973	0.959
BXD-2	1% v/v	0.828	1.017	0.922
	3%	0.784	0.878	0.831
	mean	0.806	0.947	0.877
RIII-*ro*	1% v/v	0.964	0.942	0.953
	3%	0.851	0.882	0.867
	mean	0.908	0.912	0.910
STS/A	1% v/v	0.828	0.879	0.853
	3%	0.827	0.837	0.832
	mean	0.827	0.858	0.843
MF1	1% v/v	1.015	1.030	1.023
	3%	0.793	0.907	0.850
	mean	0.904	0.968	0.936
C57BL/6J	1% v/v	1.020	0.967	0.994
	3%	1.120	0.973	1.047
	mean	1.070	0.970	1.020
CBA/Ca	1% v/v	1.037	1.078	1.058
	3%	0.930	0.962	0.946
	mean	0.983	1.020	1.002

TABLE V.II.3.8.- Protein measured in macrophage cultures treated with ethanol. (Data given as proportion of their respective controls).

STRAIN	ETHANOL	MALES	FEMALES	MEANS
DBA/2	1% v/v	0.869	0.832	0.851
	3%	0.741	0.705	0.723
	mean	0.805	0.769	0.787
BALB/c	1% v/v	1.085	1.043	1.064
	3%	0.905	0.878	0.891
	mean	0.995	0.960	0.978
BXD-2	1% v/v	0.958	1.065	1.011
	3%	0.929	1.019	0.974
	mean	0.944	1.042	0.993
RIII-*ro*	1% v/v	0.976	0.969	0.973
	3%	0.810	0.813	0.811
	mean	0.893	0.891	0.892
STS/A	1% v/v	1.081	1.049	1.065
	3%	0.920	0.867	0.893
	mean	1.000	0.958	0.979
MF1	1% v/v	1.002	0.994	0.998
	3%	0.837	0.856	0.846
	mean	0.919	0.925	0.922
C57BL/6J	1% v/v	0.996	0.960	0.978
	3%	0.804	0.840	0.822
	mean	0.900	0.900	0.900
CBA/Ca	1% v/v	0.976	1.015	0.996
	3%	0.867	0.960	0.914
	mean	0.922	0.988	0.955

TABLE V.II.3.9.- Analysis of Variance for phagocytosis in macrophages treated with ethanol

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	6	0.52655	0.54588	0.09098	4.99	0.001
sx	1	0.07876	0.06610	0.06610	3.63	0.066
rep(strxsx)	28	1.98033	1.92732	0.06883	3.78	0.000
strxsx	6	0.04172	0.02058	0.00343	0.19	0.978
conc	1	3.08275	3.09605	3.09605	169.89	0.000
strxconc	6	0.13456	0.12682	0.02114	1.16	0.351
sxxconc	1	0.06209	0.06209	0.06209	3.41	0.074
Error	33	0.60139	0.60139	0.01822		
Total	82	6.50816				

F-test with denominator: rp(str sx)
Denominator MS = 0.070726 28 degrees of freedom

Numerator	DF	Sep MS	F	P
str	6	0.087758	1.24	0.316
sx	1	0.078764	1.11	0.300
str*sx	6	0.006953	0.10	0.996

TABLE V.II.3.10.- Analysis of Variance for neutral red in hepatocyte cultures treated with ethanol

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	8	0.39178	0.39178	0.04897	4.50	0.000
sx	1	0.00341	0.00341	0.00341	0.31	0.577
rp(str sx)	36	1.51436	1.51436	0.04207	3.86	0.000
str*sx	8	0.15600	0.15600	0.01950	1.79	0.084
conc	3	28.54294	28.54294	9.51431	873.66	0.000
str*conc	24	0.52717	0.52717	0.02197	2.02	0.007
sx*conc	3	0.04056	0.04056	0.01352	1.24	0.297
Error	132	1.43750	1.43750	0.01089		
Total	215	32.61372				

F-test with denominator: rp(str sx)
Denominator MS = 0.042066 with 36 degrees of freedom

Numerator	DF	Seq MS	F	P
str	8	0.048973	1.16	0.347
sx	1	0.003412	0.08	0.777
str*sx	8	0.019500	0.46	0.873

TABLE V.II.3.11.- Rate of Phagocytosis measured in macrophages cultured with ethanol. (Data given as proportion of their respective controls).

STRAIN	ETHANOL	MALES	FEMALES	MEANS
BALB/c	1% v/v	0.927	1.012	0.970
	3%	0.523	0.676	0.600
	mean	0.725	0.844	0.785
BXD-2	1% v/v	0.799	0.828	0.813
	3%	0.234	0.333	0.284
	mean	0.516	0.581	0.548
RIII-*ro*	1% v/v	0.729	0.739	0.734
	3%	0.142	0.214	0.178
	mean	0.436	0.476	0.456
STS/A	1% v/v	0.857	0.826	0.842
	3%	0.417	0.498	0.457
	mean	0.637	0.662	0.649
MF1	1% v/v	0.992	0.875	0.934
	3%	0.567	0.751	0.659
	mean	0.780	0.813	0.796
C57BL/6J	1% v/v	0.887	0.891	0.889
	3%	0.420	0.526	0.473
	mean	0.654	0.709	0.681
CBA/Ca	1% v/v	0.812	0.841	0.827
	3%	0.477	0.581	0.529
	mean	0.645	0.711	0.678
BGA	1% v/v	0.823	0.837	0.830
	3%	0.346	0.381	0.363
	mean	0.585	0.609	0.597

TABLE V.II.3.12.- Neutral red absorption measured in hepatocyte cultures treated with ethanol. (Data given as proportion of their respective controls).

STRAIN	ETHANOL	MALES	FEMALES	MEANS
DBA/2	1% v/v	0.906	0.925	0.915
	2%	0.757	0.800	0.778
	4%	0.183	0.118	0.151
	8%	0.070	0.040	0.055
	mean	0.479	0.470	0.474
BALB/c	1% v/v	0.851	0.918	0.885
	2%	0.701	0.708	0.705
	4%	0.051	0.048	0.049
	8%	0.066	0.046	0.056
	mean	0.417	0.430	0.423
BXD-2	1% v/v	0.729	0.746	0.737
	2%	0.404	0.691	0.547
	4%	0.101	0.303	0.202
	8%	0.076	0.064	0.070
	mean	0.327	0.451	0.389
RIII-*ro*	1% v/v	0.962	0.904	0.933
	2%	0.718	0.593	0.656
	4%	0.129	0.140	0.134
	8%	0.086	0.056	0.071
	mean	0.473	0.423	0.448
STS/A	1% v/v	0.978	0.903	0.941
	2%	0.857	0.825	0.841
	4%	0.066	0.047	0.057
	8%	0.088	0.063	0.075
	mean	0.497	0.459	0.478
MF1	1% v/v	0.941	0.929	0.935
	2%	0.653	0.730	0.691
	4%	0.180	0.091	0.135
	8%	0.108	0.088	0.098
	mean	0.470	0.459	0.464
C57BL/6J	1% v/v	0.852	0.956	0.904
	2%	0.717	0.931	0.824
	4%	0.134	0.137	0.136
	8%	0.089	0.075	0.082
	mean	0.448	0.524	0.486
CBA/Ca	1% v/v	0.894	1.044	0.969
	2%	0.853	0.822	0.837
	4%	0.296	0.188	0.242
	8%	0.171	0.146	0.158
	mean	0.553	0.550	0.551
BGA	1% v/v	0.884	0.878	0.881
	2%	0.758	0.713	0.736
	4%	0.080	0.053	0.066
	8%	0.096	0.045	0.071
	mean	0.454	0.422	0.438

TABLE V.II.3.13.- Analysis of Variance for protein in hepatocyte cultures treated with ethanol

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	7	0.35748	0.35748	0.05107	5.17	0.000
sx	1	0.07542	0.07542	0.07542	7.64	0.007
rp(str sx)	32	0.77788	0.77788	0.02431	2.46	0.000
str*sx	7	0.18356	0.18356	0.02622	2.66	0.014
conc	3	10.96604	10.96604	3.65535	370.30	0.000
str*conc	21	0.55453	0.55453	0.02641	2.68	0.000
sx*conc	3	0.01107	0.01107	0.00369	0.37	0.772
Error	117	1.15495	1.15495	0.00987		
Total	191	14.08093				

F-test with denominator: rp(str sx)
Denominator MS = 0.024309 with 32 degrees of freedom

Numerator	DF	Seq MS	F	P
str	7	0.05107	2.10	0.072
sx	1	0.07542	3.10	0.088
str*sx	7	0.02622	1.08	0.399

TABLE V.II.3.14.- Analysis of Variance for LDH in supernatant of hepatocyte cultures treated with ethanol.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	8	0.67788	0.60862	0.07608	5.30	0.000
sx	1	0.06570	0.07800	0.07800	5.44	0.021
rp(str sx)	36	1.45451	1.30662	0.03630	2.53	0.000
str*sx	8	0.30057	0.28138	0.03517	2.45	0.017
conc	3	22.57972	22.52279	7.50760	523.28	0.000
str*conc	24	0.55843	0.55882	0.02328	1.62	0.045
sx*conc	3	0.00202	0.00202	0.00067	0.05	0.986
Error	129	1.85077	1.85077	0.01435		
Total	212	27.48960				

F-test with denominator: rp(str sx)
Denominator MS = 0.040403 with 36 degrees of freedom

Numerator	DF	Seq MS	F	P
str	8	0.08473	2.10	0.062
sx	1	0.06570	1.63	0.210
str*sx	8	0.03757	0.93	0.504

TABLE V.II.3.15.- Protein measured in hepatocyte cultures treated with ethanol. (Data given as proportion of their respective controls).

STRAIN	ETHANOL	MALES	FEMALES	MEANS
DBA/2	1% v/v	0.977	0.925	0.951
	2%	0.937	0.811	0.874
	4%	0.460	0.507	0.484
	8%	0.287	0.231	0.259
	mean	0.665	0.619	0.642
BALB/c	1% v/v	0.977	0.852	0.915
	2%	0.834	0.712	0.773
	4%	0.221	0.262	0.241
	8%	0.205	0.195	0.200
	mean	0.559	0.505	0.532
BXD-2	1% v/v	0.916	0.936	0.926
	2%	0.833	0.822	0.827
	4%	0.755	0.584	0.670
	8%	0.267	0.216	0.241
	mean	0.693	0.639	0.666
RIII-*ro*	1% v/v	0.954	0.935	0.944
	2%	0.906	0.743	0.824
	4%	0.580	0.483	0.531
	8%	0.269	0.263	0.266
	mean	0.677	0.606	0.642
STS/A	1% v/v	1.030	0.919	0.974
	2%	0.831	0.843	0.837
	4%	0.218	0.229	0.223
	8%	0.217	0.220	0.219
	mean	0.574	0.553	0.563
MF1	1% v/v	1.001	0.845	0.923
	2%	1.009	0.754	0.881
	4%	0.821	0.403	0.612
	8%	0.324	0.254	0.289
	mean	0.789	0.564	0.676
C57BL/6J	1% v/v	0.911	1.012	0.962
	2%	0.807	0.872	0.840
	4%	0.421	0.460	0.440
	8%	0.332	0.336	0.334
	mean	0.618	0.670	0.644
CBA/Ca	1% v/v	0.946	0.846	0.896
	2%	0.871	0.851	0.861
	4%	0.563	0.478	0.521
	8%	0.253	0.247	0.250
	mean	0.659	0.606	0.632
BGA	1% v/v	0.825	0.922	0.874
	2%	0.766	0.801	0.783
	4%	0.474	0.465	0.470
	8%	0.271	0.296	0.283
	mean	0.584	0.621	0.602

TABLE V.II.3.16.- LDH activity measured in the supernatant of hepatocyte cultures treated with ethanol. (Data given as proportion of their respective controls).

STRAIN	ETHANOL	MALES	FEMALES	MEANS
DBA/2	1% v/v	1.038	1.049	1.043
	2%	1.217	1.209	1.213
	4%	4.547	3.192	3.869
	8%	8.166	5.051	6.609
	mean	3.742	2.625	3.181
BALB/C	1% v/v	1.051	1.233	1.142
	2%	1.216	1.224	1.220
	4%	5.094	4.463	4.778
	8%	5.709	5.804	5.757
	mean	3.267	3.181	3.224
BXD-2	1% v/v	1.418	1.000	1.209
	2%	0.946	1.129	1.038
	4%	3.142	1.884	2.513
	8%	8.938	8.029	8.484
	mean	3.611	3.011	3.311
RIII-*ro*	1% v/v	1.052	0.887	0.970
	2%	1.106	0.995	1.050
	4%	4.398	1.878	3.138
	8%	9.307	4.833	7.070
	mean	3.966	2.148	3.057
STS/A	1% v/v	0.854	1.141	0.997
	2%	1.190	1.499	1.344
	4%	5.176	6.154	5.665
	8%	5.570	6.624	6.097
	mean	3.198	3.854	3.526
MF1	1% v/v	1.242	1.023	1.132
	2%	1.388	1.157	1.272
	4%	2.146	2.892	2.519
	8%	7.984	4.468	6.226
	mean	3.190	2.385	2.787
C57BL/6J	1% v/v	1.291	1.214	1.252
	2%	1.435	1.693	1.564
	4%	5.520	6.064	5.792
	8%	8.702	7.438	8.070
	mean	4.237	4.102	4.170
CBA	1% v/v	1.209	1.054	1.132
	2%	1.299	1.126	1.213
	4%	4.749	5.203	4.976
	8%	7.958	6.596	7.277
	mean	3.804	3.495	3.650
BGA	1% v/v	1.341	1.191	1.266
	2%	1.578	1.221	1.399
	4%	4.500	3.902	4.201
	8%	6.047	5.462	5.754
	mean	3.367	2.944	3.155

TABLE V.II.3.17.- Analysis of Variance for LDH in hepatocyte cells treated with ethanol

Source	DF	Seq SS	Adj SS	Adj MS	F	P
str	8	0.59179	0.46718	0.05840	6.15	0.000
sx	1	0.07815	0.04509	0.04509	4.75	0.031
rp(str sx)	36	1.39987	1.29900	0.03608	3.80	0.000
str*sx	8	0.16014	0.12232	0.01529	1.61	0.128
conc	3	19.05463	19.12757	6.37586	671.22	0.000
str*conc	24	0.93882	0.93919	0.03913	4.12	0.000
sx*conc	3	0.02026	0.02026	0.00675	0.71	0.547
Error	130	1.23486	1.23486	0.00950		
Total	213	23.47852				

F-test with denominator: rp(str sx)
Denominator MS = 0.038885 with 36 degrees of freedom

Numerator	DF	Seq MS	F	P
str	8	0.07397	1.90	0.090
sx	1	0.07815	2.01	0.165
str*sx	8	0.02002	0.51	0.837

TABLE V.II.3.18.- Matrix of correlations among strain means between assays (ethanol chapter)

	NRHEP	PRHEP	LDHCE	LDHSU	GLOHEP	NRMAC	PRMAC	PHAGO	GLOMAC
PRHEP	.079								
LDHCEL	-.16	.692							
LDHSUP	-.41	.168	.427						
GLOHEP	.194	.766	.814	.572					
NRMAC	.215	.077	-.26	-.32	-.17				
PRMAC	-.23	-.37	-.69	-.14	-.60	-.14			
PHAGOC	.474	-.08	.133	-.01	.230	-.17	-.58		
MACRO	.478	-.26	-.28	-.20	-.10	.134	-.27	.875	
GLOBAL	.463	.439	.467	.352	.742	-.05	-.67	.777	.592

PROHEP= protein measured in hepatocytes. LDHCELL= LDH measured in hepatocyte cells. NRMAC= neutral red uptake measured in macrophages. PRMAC= protein measured in macrophages. PHAGOC= phagocytosis measured in macrophages. LDHSUP= LDH activity measured in the supernatant of hepatocyte cultures. GLOHEP= averaged results obtained in hepatocytes. GLOMAC= averaged results obtained in macrophages. GLOBAL= averaged results obtained in macrophages and hepatocytes.

Statistical significance = values > 0.666 (P=0.05)
values > 0.8 (P=0.01)

TABLE V.II.3.19.- LDH activity measured in hepatocyte cells treated with ethanol. (Data given as proportion of their respective controls).

STRAIN	ETHANOL	MALES	FEMALES	MEANS
DBA/2	1% v/v	0.813	0.914	0.863
	2%	0.716	0.860	0.788
	4%	0.343	0.496	0.419
	8%	0.080	0.251	0.166
	mean	0.488	0.630	0.559
BALB/c	1% v/v	0.869	0.838	0.854
	2%	0.713	0.610	0.662
	4%	0.113	0.137	0.125
	8%	0.076	0.076	0.076
	mean	0.443	0.415	0.429
BXD-2	1% v/v	0.727	0.911	0.819
	2%	0.625	0.811	0.718
	4%	0.369	0.551	0.460
	8%	0.057	0.063	0.060
	mean	0.445	0.584	0.514
RIII-*ro*	1% v/v	0.928	0.867	0.897
	2%	0.683	0.732	0.708
	4%	0.428	0.512	0.470
	8%	0.112	0.083	0.098
	mean	0.538	0.549	0.543
STS/A	1% v/v	0.858	0.879	0.869
	2%	0.738	0.673	0.705
	4%	0.101	0.092	0.097
	8%	0.103	0.100	0.101
	mean	0.450	0.436	0.443
MF1	1% v/v	0.701	0.878	0.790
	2%	0.656	0.693	0.674
	4%	0.543	0.361	0.452
	8%	0.072	0.085	0.079
	mean	0.493	0.504	0.499
C57BL/6J	1% v/v	0.795	0.892	0.844
	2%	0.654	0.666	0.660
	4%	0.236	0.301	0.268
	8%	0.077	0.093	0.085
	mean	0.441	0.488	0.464
CBA/Ca	1% v/v	0.794	0.896	0.845
	2%	0.631	0.673	0.652
	4%	0.320	0.218	0.269
	8%	0.070	0.083	0.077
	mean	0.454	0.468	0.461
BGA	1% v/v	0.826	0.862	0.844
	2%	0.721	0.729	0.725
	4%	0.205	0.209	0.207
	8%	0.096	0.098	0.097
	mean	0.462	0.475	0.468

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