Chance factors in studies of quantitative structure-activity relationships

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limited by the number of observations and the number of variables screened. This study could not have been undertaken without which this property may indeed be a useful predictor of this toxicity. While it has been suggested that the antitumor activity of the aromatic BHG's is related to their ability to inhibit DDP in vivo, the present study shows that this property is related to the in vivo toxicity of the general class of BHG and may indeed be a useful predictor of this toxicity. However, if the aromatic BHG's are congeneric with the BQAH, then it would be expected that the antitumor selectivity is dependent on in vivo binding to an as yet undefined, alternating A-T rich site(s) in the tumor cell DNA.

Experimental Section

Equations values were determined by the chromatographic method detailed earlier for the BQAH, employing Merck cellulose F254 DC sheets as support. UV-absorbing compounds were detected by spraying with pentacyanoaquoferriate reagent, prepared as follows: Equal volumes of cold 10% aqueous solutions of sodium nitroprusside, potassium ferricyanide, and sodium hydroxide were mixed together, in that order, and the red-orange solution stood at room temperature until the color faded to a clear yellow-green (20-25 min). H2O (4 volumes) and MeCO (5 volumes) were then added, and the solution was used immediately.

Where adequate quantities of the BHG salts were available, weighed amounts were employed to prepare standard aqueous solutions of 2 mM strength. For those agents in short supply, aqueous solutions were prepared and the concentration of these was determined from the UV data quoted by Mihich et al.,

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References and Notes

(7) E. Mihich, Cancer, 20, 880 (1967).
(18) B. C. Baguley, unpublished data, this laboratory.

Chance Factors in Studies of Quantitative Structure-Activity Relationships

John G. Topliss* and Robert P. Edwards

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Multiple regression analysis is a basic statistical tool used for QSAR studies in drug design. However, there is a risk of arriving at fortuitous correlations when too many variables are screened relative to the number of available observations. In this regard, a critical distinction must be made between the number of variables screened for possible correlation and the number which actually appear in the regression equation. Using a modified Fortran stepwise multiple-regression analysis program, simulated QSAR studies employing random numbers were run for many different combinations of screened variables and observations. Under certain conditions, a substantial incidence of correlations with high r2 values were found, although the overall degree of chance correlation noted was less than that reported in a previous study. Analysis of the results has provided a basis for making judgements concerning the level of risk of encountering chance correlations for a wide range of combinations of observations and screened variables in QSAR studies using multiple-regression analysis. For illustrative purposes, some examples involving published QSAR studies have been considered and the reported correlations shown to be less significant than originally presented through the influence of unrecognized chance factors.

During the past decade, quantitative structure-activity relationships (QSAR) have been increasingly used in drug-design studies. Typically, a number of possible independent variables, usually physicochemical parameters relating to a series of compounds, are evaluated for correlation with activity values using multiple-regression analysis. The correlation equation which emerges from this analysis may contain only a small number of inde-
dependent variables out of many evaluated. The independent variables in the equation and the overall equation itself may be highly significant by standard statistical criteria. However, these criteria only relate to variables in the final equation and do not take into account how many independent variables were actually screened for possible inclusion in the equation. Clearly, the larger the number of possible independent variables considered, the greater the possibility that a correlation will occur purely by chance and this will not be reflected in the standard statistical criteria for the equation. It is therefore very important to consider not only the number of independent variables in the correlation equation itself but how many such possible variables were screened in the statistical analysis.

By way of illustration, take a case where there are ten observations and corresponding activity values. $A_1$ to $A_{10}$, and eight variables, $V_1$ to $V_8$, are considered. Multiple-regression analysis yields eq 1, where $r^2 = 0.85$, $F_{27} = 19.8$

$$A = aV_1 + bV_2 + c$$

($p < 0.005$), and $V_1$ and $V_2$ are each significant at the level $p < 0.01$. However, the statistical test for significance of the equation only relates to the variables included in the actual correlation equation and does not take account for the fact that eight variables were screened for possible correlation. The $p$ value of <0.005 for the equation shown thus overstates to some extent the probability that the relationship given by the equation is real rather than occurring by chance. The question is: how misleading is this $p$ value? The studies to be described will attempt to provide some answer to this question.

Earlier work on this problem was reported some years ago. However, these studies were too limited in scope to provide more than a demonstration that, indeed, there was a problem from chance correlations under certain conditions and to provide very general guidelines. Accordingly, in view of the interest in this problem following publication of the earlier study, plans were made to generate more extensive and detailed data.

**Method**

The basic approach was to set up a large number and wide range of simulated correlation studies using random numbers for both observations and screened variables and assess the incidence and degree of any resulting correlations.

**Computer System.** The program used was developed by modifying an existing Fortran multiple-regression analysis program (BMD02R), the essence of the modifications being to generate the data to be analyzed using a pseudo-random number generator and to accumulate statistics from each of the individual simulations and produce output reports, frequency distributions, and summary tables. The program was run on an IBM 360/158 computer performing under OS/VS2.

**Random Number Generator.** The pseudo-random numbers were generated using the IBM RANDU subroutine which employs the multiplicative congruential method and returns a vector of random numbers, each uniformly distributed in the range 0 to 1. The random numbers were scaled by a factor of 1000. Starting from a user-defined seed value (478658 was used for this study), RANDU generates a stream of pseudo-random numbers and will produce $2^{24}$ terms before repeating. By comparison of the observed against the expected frequency distribution (uniform) of numbers, RANDU is an adequate pseudo-random number generator. To better approximate pure randomness, each computer run had a different externally imposed initial seed value. In addition, only every fourth pseudo-random number was sampled from the stream.

**Computer Program Methodology.** The first step in each simulation is to generate the data matrix in the $n$ rows (one for each observation) and $n + 1$ columns (n independent variables and one dependent). The values of $r$ and $n$ are specified when the program is run. The data are then analyzed using a stepwise multiple-regression technique. At each stage of this procedure, the independent variable most highly correlated with the dependent variable is brought into the model, provided that the partial $F$-value for that variable is significant at the 10% level. Each independent variable in the regression model is then reexamined to see if it is still making a significant contribution. Any variable whose partial $F$-value is not significant at the 10% level is dropped from the model. This process continues until no more variables enter the model and none are rejected. From each simulation, the number of variables entering the regression (0 to $n$) is stored, as well as the $r^2$ statistic in the case of at least one variable entering the regression analysis. This whole process is repeated for each simulation, the number of simulations to be carried out being specified when the program is run. The results of all the simulations are then consolidated to give the following statistics: (1) the number and relative frequency for 0, 1, 2, 3, etc. variables entering the regression analysis; (2) the number and relative frequency for at least one variable entering the regression analysis; (3) for each significant correlation observed: (a) mean $r^2$ statistic, (b) median $r^2$ statistic, (c) minimum $r^2$ statistic, (d) maximum $r^2$ statistic; (4) the number and relative frequency of observed correlations by $r^2$ interval: 1.0-0.9, 0.9-0.8, 0.8-0.7, etc.

**Scope of Study.** The number of variables screened was 3-15, 20, 30, and 40, and the number of observations was 5-30, 50, 100, 200, 400, and 600. Altogether, some 300 combinations of variables and observations were examined. The number of runs for each combination ranged from 120 to 2100. It should be noted that the study was originally designed for far fewer combinations of variables and observations and runs per combination. However, due to the unexpected availability, for a short period, of a computer which was being phased out, the scope of the study was greatly expanded.

**Results**

Representative data obtained in the study are presented in Table I. The columns in the table give the number of independent variables; the number of observations; the number of runs; the frequency of a chance correlation; the average number of variables entered (for those runs for which at least one variable is entered); the maximum, minimum, and mean $r^2$ values; and the frequency of those runs where $r^2 \geq 0.5$ and 0.8. It should be noted that $p$ values for the observed correlations ranged from a minimum value of 0.10 up to 0.00001.

For the situation with ten independent variables screened and ten observations, some two-thirds of the runs gave a correlation and for these an average of 2.20 variables entered. $r^2$ ranged from 0.28 to 1.00 with a mean value of 0.64. Almost half of the total runs made gave correlations with $r^2$ values of 0.5 or higher, while about one-fourth of the total runs made gave correlations with $r^2$ values of 0.8 or higher. As the number of observations increased, there was a downward trend in $r^2$ values, so that for 15 observations only 4% of the total runs gave correlations with $r^2 \geq 0.8$ and for 30 observations no correlations with $r^2 \geq 0.8$ were found.

For 20 variables screened and only 15 observations, practically all of the runs resulted in some level of chance correlation with about 44% of the total runs resulting in a correlation with $r^2 \geq 0.8$. As the number of observations increased to 30, the number of runs yielding correlations with $r^2 \geq 0.8$ declined to about 1%. At this point, it must be noted that the degree of chance correlation found in this study was substantially less than that reported in an earlier study. Mean $r^2$ values averaged about one-half those reported in the earlier study, ranging from three-fourths down to one-fourth. The cause of this discrepancy could not be determined, since the basic records from the earlier study were not retained. However, it must be concluded that the earlier study was in error.
Table I. Data from Simulated Correlations Using Random Numbers

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<th>no.</th>
<th>no. of</th>
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Figure 1. Relationship between mean $r^2$ values and number of variables screened.

The present study was very carefully checked, and selected combinations set up and performed manually showed similar results.

From data generated in the present study, it is now possible to answer the question raised earlier concerning the $p$ value of eq 1. With ten observations and eight screened variables, the expected frequency of chance correlations comes out to be about 12%. Thus, the validity of the correlation equation is far less certain than the reported $p$ value indicates.

The results are further illustrated in Figures 1-8. The relationship between $r^2$ and the number of variables screened for varying numbers of observations is illustrated in Figure 1. It can be seen that for a constant number of observations $r^2$ increases as the number of variables screened increases. Also, for a given number of variables screened, $r^2$ increases as the number of observations decreases.

The relationship between the average number of variables (independent) entered in the correlation equation and the number of observations for different numbers of screened variables is illustrated in Figure 2. For a particular number of variables screened, the average number of variables entering the equation is relatively constant, showing a slight increase with a decrease in the number of observations over a wide range in the number of observations. However, when the number of observations approaches the number of variables screened there is a steep increase in the average number of variables entering. As might be expected, there is an increase in the number of variables entered as the number of variables screened increases.
Figure 3. Relationship between the number of observations and the probability of a chance correlation \((P_c)\) for ten screened variables with \(r^2 \geq 0.7, 0.8, \) and 0.9.

Figure 4. Relationship between the number of observations and the probability of a chance correlation for 5, 10, and 15 screened variables with \(r^2 \geq 0.8\).

In Figure 3, the relationship between the number of observations and the probability of a chance correlation with ten screened variables for various \(r^2\) values is shown. From the graph corresponding to \(r^2 \geq 0.8\), it can be seen that the probability of encountering a chance correlation at this level is about 22% for ten observations, reaching 0 for 23 observations.

The graph shown in Figure 4 gives the relationship between the number of observations and the probability of a chance correlation with \(r^2 \geq 0.8\) for 5, 10, and 15 variables. Thus, for 5 screened variables the probability of encountering a chance correlation is about 22% for 6 observations, 10% for 8 observations, 3% for 10 observations, and 1% for 12 observations.

A guideline often stated is that a certain number of observations are required in order to have much confidence in a given correlation equation. This rule of thumb is deficient on two counts: first, variables screened, rather than variables included in the equation, should be considered; second, the number of observations per variable is not a linear function of the number of variables, as it pertains to a specified level of chance correlation. In Figure 5 are plotted the number of observations per variable against the number of variables for chance correlation levels of 1% for \(r^2\) values greater than or equal to 0.5, 0.8 and 0.9. Considering the \(r^2 \geq 0.8\) curve, the number of observations per variable is 3.7 for 3 variables, dropping to 1.75 for 13 variables.

Figure 6 shows the relationship between observations and variables for a chance correlation probability of 1% or less for various \(r^2\) levels. The linear relationships shown are each statistically significant at the \(p < 0.0001\) level. This graph permits the determination of the number of observations required to screen, for example, ten variables while keeping the probability of encountering a chance correlation with \(r^2 \geq 0.8\) at the 1% level or less. From the
Figure 7. Relationship between the number of observations and the number of variables for chance correlation level $P_2 < 0.01$, with $r^2 \geq 0.7$, 0.8, and 0.9 (extrapolated).

Extrapolation of the previous graph allows one (Figure 7) to make similar estimations for larger numbers of screened variables. Thus, some 56 observations would be required to screen 40 variables while keeping the probability of encountering a chance correlation with $r^2 \geq 0.8$ at the 1% level or less.

The preceding graphs make possible the estimation of the number of observations needed to screen a specified number of variables while maintaining the probability of encountering a chance correlation of defined $r^2$ level at 1% or less. In Figure 8, the graph shows the effect on the number of required observations as the tolerated chance correlation probability increases to 5 or 10%. Again the depicted linear relationships are each statistically significant at the $p < 0.0001$ level. Thus, for 10 variables, 20 observations are needed at a chance correlation level of 1%, which reduces to 15 at the 5% level and 13-14 at the 10% level.

Some limitations must be pointed out to the approach described in this study for estimating probable chance correlation levels. In actual practice, screened variables have varying degrees of collinearity and, not infrequently, some variables are highly collinear. Generally, collinearity will be more in evidence in actual practice among a group of screened variables than among random-number simulated screened variables. To the extent that this occurs, it has the effect of reducing the number of independent variables actually operative as such. To reference a group of screened variables used in an actual problem with the random-number data, it is therefore suggested that when the correlation coefficient between two screened variables is 0.8 or higher only one of these variables should be counted in arriving at the effective number of independent variables screened. This approximate correction device should avoid serious overestimation of chance correlation effects.

A second limitation has to do with use of the stepwise multiple-regression method. This method probably misses some correlations and, as such, leads to some underestimation of the incidence of chance correlations.

Overall, the residual bias after correction for the collinearity effect tends to counterbalance the bias from use of the stepwise regression method, so the net estimation error may not be that large.

Applications to Reported Correlation Studies. In the light of the chance correlation phenomenon described, some comments will be made on a number of correlation studies reported in the literature which involved large numbers of screened variables.

The first of these reported by Peradejordi et al.1 concerned a quantum chemical approach to structure–activity relationships of tetracycline antibiotics. The correlation...
eq 2 contained seven independent variables, each sig-
ificant at the 1% level with the overall equation sign-
ificant at the 0.1% level. The $r^2$ value given was 0.986,
so that the equation accounts for almost all of the variance
in the data. There were 20 observations, and some 18
possible independent variables were screened. The ran-
dom-number simulated correlation data generated in the
current study did not include the combination of 20 ob-
servations and 18 variables. The closest reference points
were 20 observations and 20 variables, for which the in-
cidence of chance correlations with $r^2 \geq 0.9$ was about 8% 
and 17 observations and 15 variables with a 5% chance
relation incidence. The approximate risk of a chance
relation is, therefore, 5–8%. The reported correlation
equation, although it still may be valid, thus appears far
less secure than the reported $p$ value of <0.001 would
indicate. It should be noted that we are not saying that
there is a 5–8% probability that the reported equation
could arise by chance. We are saying that from 20 ob-
servations and 18 variables there is a 5–8% probability that
some correlation with $r^2 \geq 0.9$ could emerge from chance
factors alone.

Gibbons et al.\textsuperscript{5} reported on quantitative structure–
activity relationships among selected pyrimidinones and Hill
reaction inhibition. The correlation eq 3 arrived at was

$$ R^2_{p,0}= -0.46(\pm 0.15) \sigma \text{ring} + 8.92(\pm 1.85)\sigma \text{ring} + 0.046(\pm 0.008) \sigma \text{MR} - 0.15 $$

based on 17 observations, contained three independent variables, 
and had an $r^2 = 0.83$ and a $p$ value of <0.01. 
Some 20 variables were screened for possible inclusion, 
which may be equivalent to about 18 variables allowing 
for collinearity factors. This gives the combination of 17 
observations and 18 variables. The nearest reference points 
available for chance correlations are the combi-
nations of 20 observations and 20 variables and 15 observa-
tions and 15 variables, for which the correlation frequencies for $r^2 \geq 0.8$ are 17 and 28%, respectively. It 
must, therefore, be concluded that there is a substantial risk 
that the reported equation is spurious, in sharp 
contrast with the reported $p$ value of <0.01.

A study of Timmermans and van Zwieten\textsuperscript{6} on Q SAR in 
centrally acting imidazolines structurally related to clo-
andinone led to the development of eq 4. There were 27

$$ log \frac{1}{ED_{50}} = -0.00032(\pm 0.00068)(\Sigma \text{Par})^2 + 0.105(\pm 0.03) \Sigma \text{Par} - 0.695(\pm 0.17) \Delta pK_c - 5.333(\pm 1.89) \text{HOMO}(P) + 6.752(\pm 2.25) \text{EE}(P) + 2.494 $$

(4)

observations and effectively perhaps 30–35 possible in-
dependent variables screened. For 27 observations and 35 
variables using random numbers, the frequency of 
occurance of correlations with $r^2 \geq 0.9$ was approximately 45%, with approximately 11 variables entering on the
average for all significant correlations. For 27 observations and 30 variables there is about a 15% frequency with, on 
the average, seven variables entering overall. It should be 
noted that in the correlations obtained with random 
numbers the average number of variables involved exceeds 
the five in the equation of Timmermans and van Zwieten.
Therefore, the probability that the actual equation re-

ported by them is spurious is less than the 15–45% chance 
correlation incidence suggests, since $r^2$ increases as the
number of independent variables in the equation increases.
Nevertheless, it can be seen that under the conditions of the
Timmermans and van Zwieten study chance factors 
could be important and have to be addressed. Certainly, 
the stated $p$ value for the equation of <0.001 is misleading.

Discussion

An important point which needs to be considered in 
assessing the role of chance factors in correlation studies is that some 
variables screened may have a greater probability of being significant activity correlators than 
others, whereas the simulation studies treat all screened 
variables alike. In this sense, a conservative stance has 
been taken with regard to interpretation of the simulated 
correlation studies regarding the degree of chance cor-
relation risk inherent with any particular combination of 
screened variables and observations. It is clear that some 
parameters will have a much stronger physical or mech-
anic basis than others as potential activity determinants.
Thus, a $\pi$ term will be a more probably meaningful activity 
correlator than a $\pi\sigma$ cross-product term. A good approach 
in correlation studies where a large number of potential 
variables could be considered would be to initially select for 
the correlation study, where possible, a limited group of 
preferred variables. Any correlation which emerged 
would then be unlikely to be clouded by chance factors. 
A second study could then be conducted utilizing the 
complete set of variables. Variables found to be activity 
correlators in the second study but not in the first would 
be viewed as higher risk in terms of chance effects. The 
extent to which this procedure proves useful will of course 
depend on how well the total variable set can be subdivided into 
primary and secondary sets.

Having established that a particular correlation may 
have a meaningful risk associated with it from chance 
factors, the correlation should then be further examined 
to more precisely define this influence. In the case of 
correlation eq 5, which has been generated from screening

$$ Y = aX_1 + bX_2 + cX_3 + dX_4 + e $$

(5)

variables $X_1$ to $X_n$, $Y$ values can be replaced by sets (100
or more) of random numbers and the correlation analysis 
can be repeated with variables $X_1$ to $X_n$. In assessing the 
validity of eq 5, the key point would be the incidence and 
$r^2$ values of significant correlations with four independent 
variables. Such a procedure has been used by Kier and 
Hall.\textsuperscript{10,11} They also searched for correlations where all 
$X$ values were replaced by random numbers and real $Y$ 
values were retained. Since this does not preserve the 
pattern of collinearity present in the real $X$ variable set, this 
latter procedure may not provide as good an estimate of chance 
effects. It is also possible to replace selected $X$ variables 
only, with random numbers. This would be useful in 
situations where it is suspected that in a correlation 
equation certain terms, e.g., $\pi$, $\sigma$ and $\pi^2$, were real correlators 
while others were spurious. In this case, real $\pi$ and $\sigma^2$ 
numbers would be retained and the other $X$ variables 
replaced by random numbers.

In evaluating correlations for chance effects, attention 
should also be paid to whether some observations have 
been dropped from the set in order to obtain a better data 
fit. This is a fairly common occurrence and usually gives 
much higher $r^2$ values. However, sometimes the justifica-
tion given for dropping the poorly fit observations is 
questionable. The correlation equation obtained from 
utilizing all of the observations (i.e., after adding back in 
the dropped observations) should therefore be checked for
possible chance correlations, to give a better overall evaluation of the reliability of the result.

Conclusion
The results of the studies described show that chance correlations are a real phenomenon occurring when the number of variables screened for possible correlation is large compared to the number of observations. For this reason, some correlations are less significant than their standard p values indicate, as has been demonstrated by reference to some reported correlations.

The present study provides guidelines for the approximate incidence of chance correlations at specified \( r^2 \) values for various combinations of observations and screened variables. These data may be used prospectively in planning correlation studies. Thus, for a given lead structure around which further synthesis is being planned, the number of relevant independent variables to be considered may be related to the number of compounds planned for synthesis, so that an unacceptable risk of chance correlation will not be present. Specific correlations obtained under conditions where the guidelines indicate an appreciable risk of chance correlations should be individually checked as described.

Finally, it should be pointed out that there is no intention of advocating correlation studies only under conditions where there is a miniscule risk of chance correlations. However, the importance of having a true idea of the reliability of a correlation equation is obvious. It is one thing to develop and use a correlation equation which is known to be somewhat tenuous but quite another to believe that it has a solid foundation when, in fact, it has not.

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Supplementary Material Available: The complete version of Table I containing data from simulated correlations using random numbers (34 pages). Ordering information is given on any current masthead page.

References and Notes
(6) The incidence of chance correlations in the random-number simulated correlations is denoted by \( P_c \). This is to be distinguished from a \( p \) value for a specific correlation which has the standard statistical connotation.

A Preliminary Structure-Activity Study of the Mixed-Function Oxidase Inhibitor 7,8-Benzoflavone

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A series of substituted and structural analogues of 7,8-benzoflavone were examined for their ability to inhibit benzo[a]pyrene oxidation by the mixed-function oxidases found in hepatic microsomes prepared from 3-methylcholanthrene- and phenobarbital-induced rats. Of all the benzoflavones tested, only 6-amino-7,8-benzoflavone possessed significant inhibitory activity toward both classes of induced mixed-function oxidases. Parameters which were found to be necessary for maximal inhibitory activity were the maintenance of an unsubstituted or specifically substituted exocyclic phenyl group on position 2, the preservation of the pyran-4-one ring, and a 6 position which is either unsubstituted or substituted with an oxidizable moiety.

7,8-Benzoflavone (α-naphthoflavone, 7,8-BF, I), a synthetic flavanoid, is an inhibitor of microsomal mixed-function oxidase activities. These enzymes metabolize drugs, steroids, and xenobiotics, some of which are chemical carcinogens. 7,8-BF has been used for many years in mechanistic studies of the metabolic activation of carcinogens. 7,8-BF inhibits the metabolism, binding to DNA, and tumorigenesis in mouse skin of 7,12-dimethylbenz[a]anthracene and inhibits the metabolism and carcinogenicity of 3-methylcholanthrene in mouse embryo cells and mouse skin. The metabolism of benzo[a]pyrene by hepatic microsomes isolated from rats induced with 3-methylcholanthrene, 7,8-benzoflavone, and Aroclor 1254 is also inhibited by 7,8-BF. In contrast, 7,8-BF stimulates the metabolism of benzo[a]pyrene in hepatic microsomes isolated from rats induced with phenobarbital. In mouse skin, 7,8-BF inhibits benzo[a]pyrene metabolism and binding to RNA and protein, has only a marginal inhibitory effect on benzo[a]pyrene binding to DNA, and has no effect on benzo[a]pyrene-mediated tumorigenesis. Several 4'-substituted 7,8-benzoflavones have been reported to inhibit benzo[a]pyrene metabolism but none were more potent than the parent compound.

This structure-activity study is a logical and stepwise approach toward an understanding of the mechanism of action of 7,8-BF and concentrates on the examination of three areas of the 7,8-BF molecule: the exocyclic phenyl group, the 6 position of 7,8-BF, and the pyran-4-one ring.

Results and Discussion
The inhibitory activities of the flavones and related compounds were evaluated with hepatic microsomes prepared from rats induced with 3-methylcholanthrene.