Inducibility of aryl hydrocarbon hydroxylase in cultured human lymphocytes

A study of repeatability

K. A. FLETCHER, D. A. PRICE EVANS, AND M. V. CANNING

From the Department of Tropical Medicine and Department of Medicine, University of Liverpool, P.O. Box 147, Liverpool

SUMMARY Modifications to the method for estimating aryl hydrocarbon hydroxylase (AHH) activity in cultured human lymphocytes are described. Despite the improvements to the technique it was not possible to show significant 'repeatability' of values for AHH induction over a period of 2 weeks or more. Significant repeatability could be seen when a blood sample from each subject was split into duplicates. However, this level of repeatability was low when considered for quantitative genetic purposes. Possible reasons for the poor repeatability have been discussed.

Aryl hydrocarbon hydroxylase (AHH) is a member of a complex of enzymes constituting the cytochrome P-450-associated microsomal mixed-function oxidase system which is concerned with the metabolism of a wide variety of foreign hydrophobic compounds including drugs, pesticides, and carcinogens in addition to some endogenous compounds, such as steroids. The function of AHH is to convert polycyclic hydrocarbons such as benzo(a)pyrene (BP), 3-methylcholanthrene, benz(a)anthracene, and related compounds to polar metabolites which can be more easily excreted, normally following conjugation. Metabolism to these polar compounds proceeds via the formation of a reactive epoxide intermediate which can be subjected to further enzymic action or rearranges spontaneously to form a phenol. Several phenolic metabolites can be produced and it is this type of compound that is normally measured when fluorimetric assays of AHH are performed using BP as substrate.

AHH activity has been demonstrated in various tissues of animals and man (see Bast *et al.*, 1974), and is inducible in the liver and other organs of many mammalian species by both phenobarbitone and polycyclic aromatic hydrocarbons. In some inbred strains of mouse it was thought that the degree of induction of AHH was regulated by a single auto-

somal dominant gene (Nebert *et al.*, 1972), and more recent studies indicate that several alleles at two, or probably more, genetic loci are involved (Nebert *et al.*, 1975).

Investigation of the role of mixed function oxidase in the metabolism of drugs in man, and AHH in particular, was greatly facilitated by the demonstration that mitogen-stimulated human lymphocytes could be used to study the induction of AHH by polycyclic hydrocarbons (Busbee *et al.*, 1972; Whitlock *et al.*, 1972).

Controversial findings have been reported as to whether or not the degree of AHH induction is under monogenic control, and to what extent environmental and other factors influence its expression. Kellerman *et al.* (1973) reported that the distribution of AHH inducibility was trimodal as a result of a 'high' and 'low' allele operating co-dominantly at a single autosomal locus. The studies of Paigen (1976) also indicated that inducibility is controlled by a single gene. Other groups of workers (Kouri *et al.*, 1974; Coomes *et al.*, 1976; Atlas *et al.*, 1976) were unable to confirm a trimodal distribution for AHH inducibility and favoured a polygenic mode of control.

However, before a precise investigation of the mode of genetic control of AHH inducibility was undertaken it seemed important, in our opinion, to carry out a study of the 'repeatability' of the phenomenon (Falconer, 1964). We felt that only if repeatability

present to an acceptable level is it possible to say that an activity is genetically controlled to any appreciable extent. It was with this aim in mind that the experiments described in this report were performed.

Subjects and materials

Only healthy men were used in this study, ranging in age from 20 to 60 years. None of them was on any form of medication or a heavy cigarette smoker. Blood samples were always taken in the morning before 10.30.

The complete medium, used in all the experiments in this study, consisted of GIBCO MEM 158H medium supplemented with phytohaemagglutinin (Wellcome Reagents Ltd. 1 µg/ml), poke weed mitogen (GIBCO, Barker and Faines type, 10 ml; 0.005 ml/ml medium), glutamine (final concentration 2 mM), GIBCO MEM non-essential amino acids (0.01 ml solution, 100 × strength ml), preservative-free heparin (10 IU/ml), 'Crystomycin' (Glaxo Laboratories Ltd., England, 250 units penicillin and 250 µg streptomycin/ml), and 10% (w/v) sodium bicarbonate (1.0 ml/150 ml medium) which adjusted the pH of the medium to 7.4. The glutamine and sodium carbonate solutions were added through 'Swinnex' filters (Millipore (U.K.) Ltd., England).

Several batches of fetal calf sera (FCS) were investigated for their ability to induce AHH activity using leucocytes taken from three or four standard subjects who gave a range of induction ratios. A batch of FCS was chosen for all the experiments which gave relatively low control AHH levels and from two- to sixfold induction after treatment with 1.5 µM 3-MC. Though lots of the same batch of FCS were obtained from the supplier at different times, one lot was always used for a particular experiment which was normally completed in 5 to 6 weeks at the most. Aliquots of 12.5 ml FCS, sufficient for 6 cultures from one individual, were separated and stored at -70°C. This ensured that FCS was only thawed once immediately before use. Storage at -70°C ensured also that deterioration in the FCS did not occur, unlike storage at -20°C when appreciable decreases in the magnitude of induction with the passage of time were sometimes observed.

Cultures were performed in new sterile glass bottles which were used once only. Glassware used for the enzyme incubation and subsequent extraction procedures was chromic acid-treated and water-rinsed by a standard procedure. Similarly all pipettes used for the extraction process were treated with concentrated nitric acid followed by a standard water rinse. Other glassware used in the culture process was washed with Decon 90 (Decon Laboratories Ltd., Brighton, England) and again water-rinsed by a standard procedure

in a glass-washing machine (Jean Gally, S.A., Geneva, Switzerland).

Methods

In experiment 1 (see Table 1), 30 ml venous blood was taken on each occasion in sterile plastic syringes and added to 3 ml 6% dextran in 0.9% saline and 0.3 ml of a preservative-free solution of heparin (1000 IU/ml). Mixing was affected by gentle inversion of the bottle 10 times to avoid frothing of the blood and later contamination of the leucocyte-rich plasma with erythrocytes. The blood was then allowed to stand for 90 minutes in a cool place to allow the erythrocytes to sediment. The leucocyte-rich plasma was decanted as completely as possible taking care not to disturb the sediment of erythrocytes. In this manner it was found to be unnecessary to wash with hypotonic ammonia chloride solution to lyse any contaminating erythrocytes, which can lead to clumping of leucocytes and a reduction in the transformation of lymphocytes. The leucocytes were harvested by centrifugation at 100 g for 5 minutes at 20°C and, after decanting the supernatant, suspended in 12.5 ml complete medium.

In the other three experiments (2, 3, and 4: see Table 1) described in this paper the suspensions of lymphocytes used for culture were isolated by the density gradient method using a Ficoll/sodium metrizoate solution (Böyum, 1968). The sedimentation reagent (density 1.077) supplied by G. D. Searle and Co. Ltd., Bucks, England was used. Blood (30 ml) was collected in 0.3 ml preservative-free heparin (300 IU), and divided equally between two 50 ml conical centrifuge tubes. The blood was then diluted with 15 ml unsupplemented MEM medium adjusted to pH 7.4 with 10% sodium carbonate. Ficoll reagent (10 ml) previously warmed to 20 to 25°C was then carefully

Table 1 Summary of experiments

No. of experiment	Lymphocyte separation technique	Brief outline of procedure
1	Dextran	18 persons each bled on 2 different occasions; results expressed per 10 ⁶ cells
2	Ficoll	12 persons each bled on 2 different occasions; results expressed per 10 ⁶ cells
3	Ficoll	10 persons each bled on 2 different occasions; results expressed both per 10 ⁶ cells and per mg protein
4	Ficoll	(a) 10 persons each bled once and blood specimen split into duplicates; result expressed per 10 ⁶ cells (b) 6 culture tubes dealt with as follows: 3 carried through for individual biochemical assay individually; other 3 pooled and mixture divided into 3 carried forward to biochemical assay

introduced underneath the diluted blood with the aid of a lumbar puncture needle. Centrifugation was carried out at 800 g for 20 minutes at 20 to 25°C. The lymphocyte layer at the interface was carefully removed after decanting as much as possible of the upper plasma/medium layer. The lymphocytes from the pair of centrifuge tubes were pooled and washed with 15 ml unsupplemented MEM medium, recovering the cells by centrifugation at 100 g for 7 minutes. Microscopical examination of this suspension showed less than 1% contamination with granulocytes, only a small number of platelets, and 5 to 10% monocytes. After washing, the lymphocytes were then suspended in 12.5 ml complete medium. A count was then performed and the number of lymphocytes adjusted with addition of medium to approximately 1×10^6 /ml. Aliquots of 2 ml cell suspension were then added to each of 6 culture bottles which were then set up as described for the dextran-prepared cells.

Normally six cultures were set up from each individual composed of 16 ml complete medium, 2 ml fetal calf serum (GIBCO), and 2 ml leucocyte suspension containing between 2 to 6×10^6 cells.

In all experiments, the cells were then cultured for 72 hours at 37°C in an atmosphere of 4% CO₂. Three of the cultures then received 10 μ l 3 mM 3-methyl cholanthrene (3-MC, final concentration 1.5 μ M) in acetone, while the three remaining cultures, which acted as controls, received only 10 μ l acetone. After incubation for a further 20 hours, the cells were harvested by centrifugation at 340 g for 10 minutes, washed with 10 ml phosphate-buffered saline (Dulbecco 'A', pH 7.3), and finally suspended in 1 ml phosphate buffer used by Bast *et al.* (1974) for the measurement of AHH activity in blood monocytes. Small samples were taken during the harvesting and washing procedure for estimation of cell numbers and the levels of lymphocyte transformation.

Cofactors (NADH and NADPH) and bovine serum albumin were then added to each 1 ml suspension of cells (Bast *et al.*, 1974) and finally 25 μ g benzo(a)pyrene (BP) in 0.05 ml acetone was added as substrate. The same batches of cofactors, albumin, and substrate were always used throughout an experiment. The reaction was carried out at 37°C in a shaking incubator under the illumination of a single 25 W red bulb (Bast *et al.*, 1974) and was allowed to proceed for 60 minutes. The reaction was stopped by the addition of 4 ml chilled acetone/hexane (1:3, v/v). For most of the experiments the tubes were then shaken automatically for 10 minutes, though recently the contents of the tubes were vortex-mixed for 1 minute with no change in extraction efficiency. A 2 ml sample of the upper organic phase was then extracted with chilled 0.7 ml 1N NaOH by vortex-mixing for 1 minute. Between manipulations the tubes

were kept in an ice/water mixture. This is essential to avoid loss of activity and hence erroneous results are to be avoided. Almost complete loss of activity can be observed if the ambient temperature is abnormally high, for example above 30°C. This presumably reflects the instability of the hydroxylated product of BP in the organic phase, since the fluorescence of an initially cold alkali extract did not diminish when monitored continuously over a 20-minute period at 22°C.

The fluorescence of an aliquot (0.5 ml) of the lower alkali phase was measured in a Perkin-Elmer MPF recording fluorescence spectrophotometer with excitation at 396 nm. The emission spectrum was recorded from approximately 420 nm to 600 nm using a 430 nm cut-off filter. When this was done there could be no doubt that a definite peak at 522 nm was measured corresponding to the hydroxylated product of BP, and that there had been no contribution from any aqueous contamination which could occur when the hexane layer was sampled, which could be shown to give rise to fluorescence with a maximum at 460 nm which could affect the peak at 522 nm, particularly in extracts from cells with low activities of AHH.

Because of the unavailability of samples of hydroxybenzo(a)pyrene to use as a standard, the activities were referred to a standard solution of quinine sulphate (0.5 ml containing 50 ng; excitation 350 nm, emission 445 nm). Specific activities were expressed in terms of ng quinine sulphate equivalent per 10^6 cells. All values for both control and induced levels represent the mean in triplicate samples.

It was possible to confirm that 3-MC had been added to the induced cultures, and at the right concentration, by scanning the culture supernatants in the fluorimeter with excitation set at 360 nm. A sharp peak was observed with a maximum of 400 nm due to 3-MC.

Cell counts were performed on a Coulter ZB particle counter (Coulter Electronic Ltd., Harpenden, England). Total cell counts were performed with a threshold setting of 18 while transformed cells were counted at a setting of 34. Usually 70 to 80% of lymphocytes were estimated to have been enlarged and hence transformed. Microscopical examination of smears stained by the Leishman/Giemsa-Chromotrope 2R method confirmed visually that transformed cells were present.

In assays where the activity of AHH was related to the protein content of cells (experiment 3), the method of Lowry *et al.* (1951) was used for the estimation of protein. This was performed on aliquots of lymphocytes suspended in phosphate-buffered saline during the harvesting and washing procedure.

Standard statistical methods were used. The logarithmic transformations were employed for computation

because the data showed significant departure from normality.

Results

The Fig. shows the results of an experiment in which a series of duplicate dilutions of a suspension of a rat-liver homogenate were assayed by the procedure used for leucocytes. Dilutions were made so that their activities spanned the range of spectrofluorimetric readings that was usually encountered when leucocytes were assayed. This experiment was done instead of a standard curve using 3-hydroxybenz(a)-pyrene to make sure that there was a linear relation between the fluorescence measured and the hydroxylated product of BP produced under the standard conditions used in the assay. The Fig. shows that these requirements were fulfilled.

Mention had already been made of the instability of hydroxylated BP in the organic phase used for extraction at high environmental temperatures. Table 2 shows the result of an experiment in which assays of lymphocyte cultures set up from the same sample of blood were extracted with acetone/hexane mixture and then kept for 30 to 60 minutes in ice water at 2°C before extraction into alkali and reading of the fluores-

Table 2 Experiment showing effects of temperature on hydroxylated BP contents of hexane extracts of lymphocyte cultures after incubation with BP and cofactors

Lot	Time and condition of storage	ng quinine sulphate equivalents/10 ⁶ cells
1	30 min (2°C)	41.98 ± 2.93*
2	40 min (2°C)	40.91 ± 3.32
3	60 min (2°C)	39.02 ± 3.32
4	50 min (24°C)	29.85 ± 2.31

* Values of AHH specific activities are mean of 4 assays, showing standard error of mean (see text for further details)

cence. The cultures were pooled and then divided into four lots. Each lot contained four samples for assay. One lot of assays was kept at a normal room temperature (24°C) for 50 minutes before extraction and reading. The results show that though storage at 2°C of the hexane extracts beyond the time normally taken to complete the procedure did not lead to significant loss of hydroxylated BP, exposure to a normal room temperature caused a 25% loss. In our experience, as ambient temperatures rose above 24°C a disproportionate loss of hydroxylated BP occurred. Complete loss was observed on occasions when temperatures were raised above 30°C.

In experiment 1, 3 to 8 × 10⁶ leucocytes were added to each of the six cultures from an individual. The number of cells decreased to 1 to 5 × 10⁶ cells after culture. Microscopical examination showed these to be almost exclusively lymphocytes, of which about 70% were found to have been transformed into blast forms. Each of the 18 volunteers was examined on two occasions separated by at least two weeks. Though similar induction ratios were obtained in a few subjects on the two occasions, no similarity was seen in the absolute values either for control or induced cells. In view of these inconsistencies, it was decided to repeat the experiment using suspensions of lymphocytes prepared by Ficoll gradients. It was thought that the variable numbers of granulocytes and proportions of the different types of lymphocytes added to cultures might have significantly affected lymphocyte transformation and, therefore, the levels of AHH induction.

A few preliminary experiments tended to confirm these views. When cultures were performed on leucocytes isolated from the same blood sample by either dextran sedimentation or a Ficoll gradient, cells prepared by the latter method usually gave higher induction ratios and often higher specific activities for AHH in both control and induced cells.

The results of experiments carried out on a group of 12 volunteers whose blood was taken on two occasions, with an interval of at least two weeks, and processed for culture by the use of Ficoll gradients (experiment 2) showed there was little agreement between the induction ratios obtained on each occasion and virtually no similarities between the

Fig. AHH activity of a series of dilutions of a rat-liver homogenate assayed by the lymphocyte procedure.

Table 3 *Statistical evaluations of experiments*

Experiment no.	Paired <i>t</i> test* (degrees of freedom)		Ratio T/C variances due to† (degrees of freedom)			F ratio	P
	First estimation	Second estimation	Subjects	Repeats	Assays		
1	5.06 (17)	8.39 (17)	0.3144 (17)	0.1794 (18)	—	1.736	NS
2	6.05 (11)	13.99 (11)	0.2934 (11)	0.2031 (12)	—	1.445	NS
3	Cell assay	6.07 (9)	5.49 (9)	0.7375 (9)	0.1475 (10)	0.0224 (20)	Subjects/duplicates: 5.000 <0.05
	Protein assay	5.43 (9)	4.86 (9)				Duplicates/assays: 6.585 <0.01
4 (a)	6.64 (9)	4.82 (9)	0.5773 (9)	0.1007 (10)	—	5.733	<0.01

* Difference between test and control has been tested, with both quantities in its $\log_e$ form. Hence difference is equivalent to a ratio which is shown to be greater than unity (i.e. induction of AHH is demonstrated).

† Ratio test/control has been computed and $\log_e$ form used for these computations.

absolute values obtained either for controlled or induced lymphocytes. In most cases between 3 and 6 $\times 10^6$ lymphocytes were used per culture, but further experiments suggested that the optimal number of cells per culture should be 2 to 3 $\times 10^6$ lymphocytes/culture, otherwise an apparently falsely raised induction ratio was observed resulting from a reduced specific activity of AHH in the control cells when cell numbers exceeded 3 $\times 10^6$ cells/culture. Volunteers 10 to 12 were examined, adding 2 $\times 10^6$ lymphocytes/culture, and the results show that this additional step did not lead to any improvement in the repeatability of the ratios or the absolute values.

Although conditions for cell culture were maintained as reproducible as possible, it was accepted that differences in the degree of lymphocyte transformation might occur between cultures performed on the same individual on different occasions, though the rate of transformation might not alter significantly. With this possibility in mind a further group of 10 volunteers was investigated expressing the specific activity of AHH in terms of the protein content of the cells used for the assay as well as per 10^6 cells (experiment 3). All cultures were set up using 2 $\times 10^6$ lymphocytes per bottle and blood samples were, as before, taken at intervals of two weeks from each subject. Repeatability of results in the subjects was again poor and expression of specific activities of AHH in terms of cell protein showed no improvement over that using cell numbers.

A further experiment was designed to investigate whether the lack of obvious repeatability observed in the above experiments could be attributed to variations produced by the technique which are not readily controllable. A 60 ml sample of blood was taken from each of 10 volunteers, divided equally between two tubes, and treated as two separate samples (experiment 4a). The two samples were investigated on

a 'blind' basis by the technical staff performing the test. After culture and induction the triplicates of the control cultures from one of each pair of samples were pooled, mixed well, and divided into three portions again and assayed separately. The triplicates of the induced cultures were treated in an identical manner. In this way some measure of the standard error of the analytical part of the determination of AHH activity could be obtained as distinct from the cultural procedure. The results of this experiment in which 2 $\times 10^6$ lymphocytes were used per culture bottle showed good agreement between the duplicate values obtained only for blood from four volunteers. This was applied in particular to the induction ratios, whereas the absolute values of the duplicates of these individuals did not compare closely.

The intraclass correlation coefficient and its significance was computed for control activity (C), test activity (T), and the ratio T/C in all four experiments. Only in the case of T/C in experiment 4 was the value significant (0.534, $F = 3.661$, $0.025 < P < 0.05$).

When the results for the pooled triplicate and the non-pooled triplicate cultures were compared (experiment 4b), the large amount of variance between the pooled triplicate analyses of the control cells (26%) did not differ significantly ($0.05 < P < 0.1$) from the variance found between the non-pooled triplicate analyses (16%). However, the greater variance found between the triplicate analyses of the non-pooled induced cells (35%) was more significant ($0.001 < P < 0.01$) than the variance found between the triplicate analyses of the pooled induced cells (17%).

In experiments 1 and 2, the variability between subjects was not greater than among repeat estimations. In experiments 3 and 4a, the variability between subjects was greater than among repeat estimations, but the latter was still unacceptable large (Table 3).

Discussion

Several modifications to the method of assay of aryl hydrocarbon hydroxylase (Busbee *et al.*, 1972; Whitlock *et al.*, 1972; Kellerman *et al.*, 1973) were found to be necessary before accurate determinations could be made. Firstly, it was essential to keep the samples cold during the extraction procedure. Failure to do this often led to low and poor duplicate values for AHH activity. Secondly, during extraction it was important not to sample all the organic hexane phase or the alkali phase, otherwise there was a strong possibility of carry-over of contaminating substances which affected the fluorescence of the hydroxylated products of benzo(a)pyrene at 522 nm. Lastly, it was important, by performing an emission scan of the fluorescence from about 430 to 600 nm, to make sure that a well-defined peak with a maximum at 522 nm was being measured and not a shoulder on a slope, and that there was no contribution to the 522 nm peak from any other maxima, particularly that produced at 460 nm when aqueous contamination of the hexane layer had occurred.

It was useful to have proof that 3-MC had been added to the test cultures and none to the control cultures. This was provided by scanning the culture supernatants in the fluorimeter at the respective wavelengths.

It is important to emphasise these modifications because, in our experience, acceptable results were only obtained when they were carried out.

However, despite ensuring that the above modified assay was strictly adhered to, it was not possible to show significant 'repeatability' of AHH values in blood samples taken at intervals of at least two weeks. The results of the experiment performed on dextran-prepared leucocytes emphasises this point. Unlike Kouri *et al.* (1974), who did not observe induction ratios greater than 2.9, ratios between 3.0 and 6.7 were found in this experiment. In fact, ratios of this magnitude were encountered in all the experiments reported in this paper.

The following modifications of technique: Ficoll preparation, standardisation of numbers of cells, and expression of results in terms of protein content did not improve results to give significant repeatability.

With all the improvements incorporated, the 'repeatability' of T/C ratio did attain statistical significance in experiment 4. However, an intraclass correlation coefficient of 0.53 means that 47% of the variability results from methodological factors causing differences between duplicates obtained simultaneously. This 'repeatability' is too high to be of value for quantitative genetic purposes.

Additionally, the triplicate assays for both the control and induced cells showed that, despite exercis-

ing rigid control over the analytical part of the determination, large degrees of variation were obtained. These degrees of variation indicate the difficulty of eliminating unknown factors which are significantly influencing the results. A further component of variance was also added during enzyme induction in culture, even though each set of triplicate cultures was treated in an identical manner.

Kellerman *et al.* (1976) showed 'repeatability' of 0.86 for AHH ratio in 11 subjects (calculated from their Table 1), while Paigen (1976) reported that inducibility could be measured reproducibly with an average coefficient of variation of 0.13 for repeat determinations on the same individuals.

There is no suggestion in the present results of two or more distinct phenotypes and, therefore, description in terms of monogenic control is not appropriate. Taking a quantitative genetics view of the phenomenon, the estimates of repeatability (a property of populations not of individuals) in the four experiments is so high that it indicates the worthlessness of going to the labour of assessing 'heritability' in the sense used by Falconer (1964) with the present techniques.

Despite the fetal calf serum, mitogens, and other factors being rigorously controlled, the poor repeatability obtained (experiment 4) when the same sample of blood was split into duplicates indicates large variability contributed by (a) the different behaviour of the same lymphocytes in different tubes, and (b) the biochemical assay procedure.

In addition, there is the possibility that 'responsiveness' to mitogens of lymphocytes might well vary from time to time in individuals (Atlas *et al.*, 1976; Kouri *et al.*, 1974) which suggests that the use of peripheral blood monocytes for the determination of AHH inducibility might be preferable, since these cells do not require mitogen-stimulated transformation (Bast *et al.*, 1976; Lake *et al.*, 1977). The disadvantages in using these cells are the large samples of blood sometimes required to obtain sufficient monocytes and the difficulty in isolating the cells (unpublished preliminary observations). The radiometric assay for the determination of AHH activity in lymphocytes recently described by Guirgis *et al.* (1976) could also be considered. These workers reported that results using a method which measures the water soluble metabolites of benzo(a)pyrene showed improved precision over those obtained by fluorimetric assay, and that their technique gave a more reliable assessment of AHH induction. Alternatively, the assay of individual metabolites of benzo(a)pyrene, as reported by Selkirk *et al.* (1974) using high-pressure liquid chromatography may be worthy of investigation, since the pattern of these metabolites might vary between individuals, and even within individuals on different occasions.

We are grateful to the Wellcome Trust for financial support; to Mr C. D. Dewse, Nuffield Unit of Medical Genetics for help with the culture of leucocytes; and to Miss J. A. Etheridge for skilled technical assistance. We are also grateful to Dr J. L. Cunningham for his help and constructive comments and to Mr C. R. West for advice on statistical methods.

References

- Atlas, S. A., Vesell, E. S., and Nebert, D. W. (1976). Genetic control of interindividual variations in the inducibility of aryl hydrocarbon hydroxylase in cultured human lymphocytes. *Cancer Research*, **36**, 4619-4630.
- Bast, R. C., Jr., Okuda, T., Plotkin, E., Tarone, R., Rapp, H. J., and Gelboin, H. V. (1976). Development of an assay for aryl hydrocarbon (benzo(a)pyrene) hydroxylase in human peripheral blood monocytes. *Cancer Research*, **36**, 1967-1974.
- Bast, R. C., Jr., Whitlock, J. P., Jr., Miller, H., Rapp, H. J., and Gelboin, H. V. (1974). Aryl hydrocarbon (benzo(a)pyrene) hydroxylase in human peripheral blood monocytes. *Nature*, **250**, 664-665.
- Böyum, A. (1968). Separation of leucocytes from blood and bone marrow. *Scandinavian Journal of Clinical and Laboratory Investigation*, **21**, Suppl. 97.
- Busbee, D. L., Shaw, C. R., and Cantrell, E. T. (1972). Aryl hydrocarbon hydroxylase induction in human leucocytes. *Science*, **178**, 315-316.
- Coomes, M. L., Mason, W. A., Muijsen, I. E., Cantrell, E. T., Anderson, D. E., and Busbee, D. L. (1976). Aryl hydrocarbon hydroxylase and 16 α -hydroxylase in cultured human lymphocytes. *Biochemical Genetics*, **14**, 671-685.
- Falconer, D. S. (1964). *Introduction to Quantitative Genetics*, pp. 142-149. Oliver and Boyd, Edinburgh.
- Guirgis, H. A., Lynch, H. T., Mate, T., Harris, R. E., Wells, I., Caha, L., Anderson, J., Maloney, K., and Rankin, L. (1976). Aryl hydrocarbon hydroxylase activity in lymphocytes from lung cancer patients and normal controls. *Oncology*, **33**, 105-109.
- Kellerman, G., Luyten-Kellermann, M., Horning, M., and Stafford, M. (1976). Elimination of antipyrine and benzo(a)pyrene metabolism in cultured human lymphocytes. *Clinical Pharmacology and Therapeutics*, **20**, 72-80.
- Kellerman, G., Luyten-Kellermann, M., and Shaw, C. R. (1973). Genetic variation of aryl hydrocarbon hydroxylase in human lymphocytes. *American Journal of Human Genetics*, **25**, 327-331.
- Kouri, R. E., Rattie, H., III, Atlas, S. A., Niwa, A., and Nebert, D. W. (1974). Aryl hydrocarbon hydroxylase induction in human lymphocyte cultures by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Life Sciences*, **15**, 1585-1595.
- Lake, R. S., Pezzutti, M. R., Kropko, M. L., Freeman, A. E., and Igel, H. J. (1977). Measurement of benzo(a)pyrene metabolism in human monocytes. *Cancer Research*, **37**, 2530-2537.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- Nebert, D. W., Goujon, F. M., and Gielen, J. E. (1972). Aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons: a simple autosomal dominant trait in the mouse. *Nature New Biology*, **236**, 107-110.
- Nebert, D. W., Robinson, J. R., Niwa, A., Kumari, K., and Polan, A. P. (1975). Genetic expression of aryl hydrocarbon hydroxylase activity in the mouse. *Journal of Cellular Physiology*, **85**, 399-414.
- Paigen, B. (1976). Heritability of aryl hydrocarbon hydroxylase in cultured human lymphocytes. In Third International Symposium on Microsomes and Drug Oxidations. *Hoppe-Seyler's Zeitschrift für physiologische Chemie*, **357**, 1019-1067.
- Selkirk, J. K., Croy, R. G., and Gelboin, H. V. (1974). Benzo(a)pyrene metabolites: efficient and rapid separation by high pressure liquid chromatography. *Science*, **184**, 169-171.
- Whitlock, J. P., Jr., Cooker, H. L., and Gelboin, H. V. (1972). Aryl hydrocarbon (benzopyrene) hydroxylase is stimulated in human lymphocytes by mitogens and benz(a)anthracene. *Science*, **177**, 618-619.

Requests for reprints to Dr K. A. Fletcher, Department of Tropical Medicine, University of Liverpool, P.O. Box 147, Liverpool L69 3BX.