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STIMULATION OF ARYL HYDROCARBON HYDROXYLASE ACTIVITY IN CULTURED CELLS BY HUMAN AND ANIMAL SERA

A NEW *IN VITRO* APPROACH TO HUMAN DRUG METABOLISM

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Abstract—Aryl hydrocarbon hydroxylase (AHH) activity in tissue culture cells is highly sensitive to the presence of animal sera in the culture medium. Quiescent cells, grown in the absence of serum for 4–5 days appear to be growth-arrested in the G_0 phase of the cell cycle, with low AHH activity. Reintroduction of serum to these cells increases DNA synthesis, AHH activity and the accumulation of cell protein. Different sera from human and animal donors differ in their ability to stimulate these changes and activity is dependent upon the diet of donor animals. It is suggested that a system such as this may be used as a bioassay to investigate factors controlling human drug metabolism.

Aryl hydrocarbon hydroxylase (AHH) is a cytochrome P448 linked microsomal monooxygenase activity. The reactions are probably catalysed by several hemoproteins and are usually measured by the formation of water soluble products of benzo [a] pyrene, mainly 3-hydroxy benzo [a] pyrene. Numerous xenobiotics, particularly polycyclic hydrocarbons, are metabolized by this pathway [1]. The relationship between AHH activity and cancer is unclear, but it is of considerable importance to find out whether individuals differ in sensitivity to chemical carcinogens as a consequence of variations in their metabolism. Considerable inter-individual variation has been observed in AHH activity using enzyme preparations from human liver [2], full-term placenta [3], skin [4], macrophages [5] and mitogen stimulated lymphocytes [6, 7].

Lymphocytes are an accessible tissue; they are generally cultured for 72 hr in the presence of mitogens and AHH activity is measured after a further 24 hr culture with and without an inducer such as methylcholanthrene. This induction ratio is characteristic for an individual and appears to be under genetic control [8–11].

However, the induction ratio seems to be influenced by the many factors that affect lymphocyte transformation [12], such as changes in the lymphocyte membrane cholesterol composition [13, 14], and by elevated levels of α-globulin [15]. Complex seasonal changes have also been observed in AHH induction ratios [16]. In addition, one of the major problems appears to be that the mitogen stimulated lymphocyte assay for AHH activity is highly sensitive to medium composition and in particular to the batch of foetal calf serum [9]. Some batches of foetal calf serum appear to be unsatisfactory [11].

In this paper we describe a bioassay system which makes use of the sensitive response of AHH activity in a line of epithelial-like cells to various animal sera. Using this technique it is possible to investigate inter-individual differences in serum activity and to relate these to environmental factors influencing hepatic AHH activity.

MATERIALS AND METHODS

A line of cells derived from adult rat liver previously described by Montesano *et al.* [17], were grown routinely in Williams Medium E (Flow Laboratories, Irvine, Scotland) containing 10% v/v foetal calf serum (Gibco Biocult Ltd, Paisley, Scotland), 100 i.u./ml penicillin, 100 μg/ml streptomycin and 4mM L-glutamine. Cultures were maintained in a 37° humidified incubator with an air plus 3.5% CO_2 atmosphere.

Cells were prepared for the serum 'bioassay' by subculture using a 0.1% trypsin solution in Hanks balanced salt solution. Cells were seeded at a density of 5×10^5 cells/64 cm^2 Nunc petri dish in medium containing 10% v/v foetal calf serum. After attachment to the surface of the dish overnight, the growth medium was changed and cells were maintained in serum-free medium for 4–5 days. Medium was replaced each day. Under these growth conditions several rounds of cell division occur, facilitating the dilution and removal of serum factors adhering to the cell surface [18].

Rat serum was prepared as follows. Animals were exsanguinated under anaesthesia; the pooled blood being collected in a plastic universal container. The sample was left at room temperature for 2 hr before centrifuging at 3000 r.p.m. for 10 min in an MSE bench centrifuge. The serum was collected and passed through a sterile 0.22μm millipore filter before freezing.

Cultures were stimulated with prewarmed Williams Medium E containing the appropriate quantity of serum. 1,2-Benzanthracene (17.5μM) (Sigma) was included when induction studies were performed. Typically, cell monolayers were washed

three times with 0.15M NaCl and were then harvested in 4ml of 0.15M KCl using a rubber scraper. Cells were homogenized by 30 strokes of a dounce glass homogenizer and AHH activity was assayed by measuring the formation of fluorescent oxygenated metabolites of benzo [*a*] pyrene [19, 20]. Protein was measured as described by Lowry *et al.* [21].

The degree of protein accumulation observed as a result of stimulation by a serum may be defined by dividing the total protein per dish after serum stimulation by the total protein content present at the time of stimulation. This 'protein ratio' describes the proportional increase in cell protein obtained as a result of serum stimulation.

Incorporation of 6-^{3}H-thymidine (20–30 Ci/mmole, Radiochemical Centre, Amersham, Bucks) was measured over a 60 min period. Isotope (2.5μCi) was added to a 64 cm^2 petri dish containing 10ml of medium. Incorporation of label was then estimated in cell homogenates by the method of Clo *et al.* [22]. Alternatively, 0.5 μCi of isotope was added to a 7 cm^2 petri dish containing 2ml of medium. After the appropriate time interval, medium was removed using a pasteur pipette, the cell monolayer was washed three times with saline and then 1ml of cold 4%v/v perchloric acid (PCA) was carefully added to the dish. The dish was then left for 1 hr at 4° to extract PCA soluble counts before removing the PCA. The intact monolayer was then suspended in 0.5ml of 0.5M NaOH and digested at 37° for 30 min. After neutralization with HCl, samples were counted in a Tri-carb scintillation counter using Permafluor II scintillation fluid.

Efficiencies were calculated by comparing the automatic external standard (AES) ratio with that on a quench curve prepared using ^{3}H-*n*-hexadecane. These cultures were shown to be free of mycoplasma contamination by Dr D. Taylor-Robinson, MRC, Northwick Park.

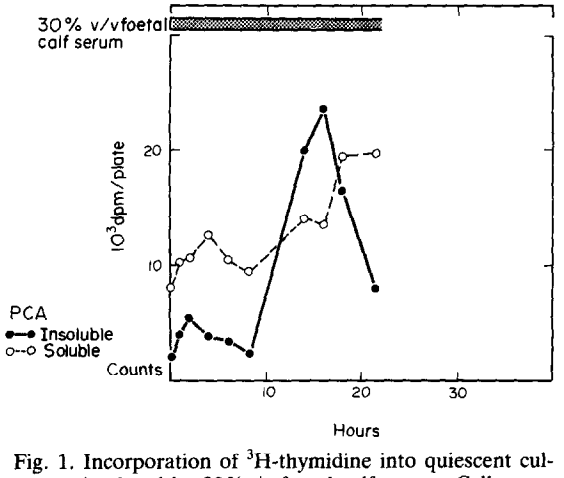

Fig. 1. Incorporation of ^{3}H-thymidine into quiescent cultures stimulated by 30%v/v foetal calf serum. Cells were grown for 4 days in the absence of serum. Cell cultures were then stimulated with fresh medium containing serum, and 0.5μCi of label was added to each 7cm^2 petri dish at the appropriate time. Cells were harvested 60 min later. Each point represents the mean of estimations made from two separate dishes.

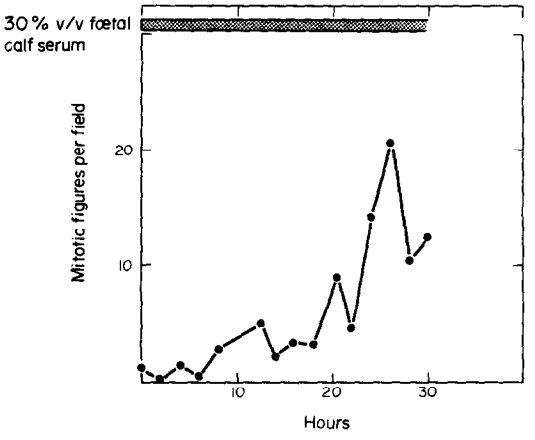

Fig. 2. The incidence of mitotic figures seen in cultures grown on glass coverslips. Cells were stimulated using fresh medium containing 30% v/v foetal calf serum, fixed in formol–saline and stained as described in Materials and Methods. The number of mitotic figures per field (400× magnification) were recorded and the arithmetic mean of 5 fields calculated.

Mitotic figures were estimated using cells grown on glass coverslips. Cells were fixed in formol–saline and stained by the Feulgen technique [23].

RESULTS

Growth in the absence of serum results in reduced AHH levels and reduced enzyme inducibility [24]. After 4–5 days in the absence of serum, a confluent monolayer of quiescent or slowly growing cells is formed. These cells exhibit reduced DNA synthesis as judged by ^{3}H-thymidine incorporation into PCA insoluble material.

Addition of fresh medium containing 30%v/v foetal calf serum is followed by a lag period of about 10 hr, after which time a wave of DNA synthesis takes place (Fig. 1). The direct estimation of mitotic figures indicates that DNA synthesis occurs some hours before mitosis (Fig. 2). It may thus be concluded that serum deprived cells treated with fresh medium containing serum emerge from the quiescent growth state in a synchronous or partially synchronous (parasynchronous) manner.

Table 1 shows the effect of stimulating serum deprived cells with medium containing 30% v/v foetal calf serum lot L/7030/D on several separate occasions. Both basal and induced AHH specific activity are stimulated whilst the total quantity of cell protein is also increased on each occasion. However, these parameters appear to be highly and separately variable from one experiment to another. Basal AHH specific activity measured 8 hr after the addition of serum ranges between 46 and 136 pmole/mg cell protein/30 min and between 43 and 58 pmole/mg cell protein/30 min at 24 hr after stimulation. Induced AHH specific activity measured 24 hr after the addition of serum varies between 325 and 512 pmole/mg cell protein/30 min.

Table 1. Effect of a single batch of foetal calf serum on AHH activity and protein accumulation*

Treatment	N	Protein μg/plate Initial	Final	Protein ratio	Final AHH specific activity −serum	+serum	Total final AHH/plate −serum	+serum	Final AHH/mg initial protein −serum	+serum
8 hr										
no inducer		4470	5450	1.22	15	80	69	421	15	95
mean ± 1.S.D.	6	±650	±1000	±0.13	±5	±32	±30	±135	±7	±29
24 hr										
no inducer		4490	6820	1.49	19	50	83	331	18	75
mean ± 1.S.D.	5	±890	±1620	±0.18	±4	±6	±18	±64	±4	±10
24 hr 17.5μM Benzanthracene		5000	6560	1.34	84	421	429	2740	86	549
mean ± 1.S.D.	5	±1240	±1450	±0.27	±29	±74	±190	±650	±38	±30

* Separate preparations of cells were treated with the same batch of foetal calf serum at different times. Cells were seeded at 5×10^5 per 64cm^2 Nunc petri dish in the presence of 10% v/v foetal calf serum. The next day, cells were transferred into fresh serum-free Williams Medium E and were cultured under these conditions for 4 days; medium was changed each day. Cells were then treated with fresh Williams Medium E containing 30% v/v foetal calf serum lot L/7030/D as described in the table, before harvesting the cells and determining protein content and AHH activity. Protein ratios were obtained by dividing final by initial protein values; specific activities for AHH are expressed as pmole/mg cell protein/30 min.

Enzyme specific activity is a poor means of expressing enzyme activity in synchronized cells [25]. Total AHH activity per plate after stimulation by serum is proportional to initial cell protein content in these cultures (Fig. 3). If it is assumed that cells contain approximately equal quantities of total protein from one cell preparation to the next, then AHH activity after serum stimulation is directly dependent upon the total number of cells present at the time of stimulation. In support of this asumption, cells grown in different media until density or contact inhibited, exhibit very similar protein contents. After 4 days in serum-free medium, this value is 0.61 mg/10^6 cells. Medium containing 10%v/v foetal calf serum gives 0.59 mg/10^6, cells whilst 10%v/v human serum gives 0.63/10^6 cells. Consequently, in these experiments AHH activity is expressed as total activity per plate divided by the total protein present at the time of stimulation.

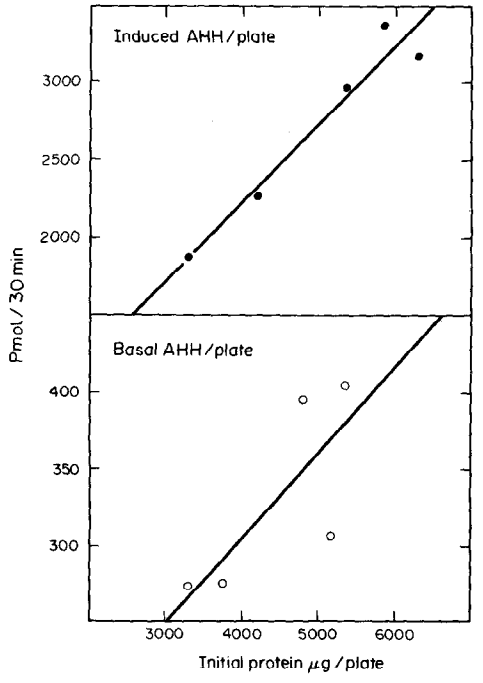

Fig. 3. The effect of initial protein density on total AHH activity per plate after serum stimulation. Cells were cultured and treated as described in Table 1. Determinations were made in duplicate 24 hr after the addition of serum. Duplicates generally differed by less than 10%. Correlation for basal and induced values are $r = 0.755$ and 0.965, respectively.

When AHH activity is expressed in this manner, 24 hr basal activity is within the range 59 to 83 pmole/mg initial protein/30 min and induced activity is between 502 and 582 pmole/mg initial protein/30 min. Basal activity measured 8 hr after stimulation still exhibits considerable variability; with a range of 65–143 pmole/mg initial protein/30 min.

Thus, basal activity exhibits much greater variability than induced activity. This may partly be a consequence of the time-course of the stimulation of basal AHH activity by serum. Although protein synthesis and accumulation continues for over 30 hr after the addition of fresh medium containing serum, AHH activity rises to a maximum by 8 hr, after which time total activity per plate slowly declines. We have also obtained evidence that cells on the G_1/S border of the cell cycle exhibit reduced enzyme synthesis (unpublished results). Consequently, AHH activity will depend upon the number of cells stimulated to enter the cycle of cell division. Clearly, determinations made at 24 hr are less sensitive to these changes, because the period spend on the G_1/S border represents only a small proportion of the total time during which enzyme synthesis takes place.

Variation in the 'protein ratio' is also a striking feature of these results, and may be attributed to a

Table 2. The effect of various sera on AHH activity in liver cells*

Treatment		No serum	Foetal calf serum 5% v/v	Foetal calf serum 30% v/v	3% casein serum 5% v/v	3% casein serum 30% v/v	20% casein serum 5% v/v	20% casein serum 30% v/v	41B serum 5% v/v	41B serum 30% v/v
8 hr no inducer	AHH	15 ±2	—	92 ±6	—	44 ±3	—	57 ±5	—	57 ±2
	Protein ratio	1.02	—	1.07	—	1.02	—	1.27	—	1.25
24 hr plus inducer	AHH	66 ±6	168 ±24	489 ±115	218 ±11	552 ±20	279 ±33	669 ±30	276 ±61	755 ±78
	Protein ratio	1.04	1.35	1.67	1.51	1.61	1.54	1.81	1.40	1.75

* Cells were treated as described in Table 1. Each value represents the mean ± standard deviation of 4 estimations taken from a single plate of cells. AHH activity is expressed as pmole/mg initial protein/30 min. Induction performed using 17.5μM Benzanthracene.

number of factors including culture conditions, the extent of cell synchronization, cell density and the type of serum used.

Having established that a characteristic stimulation of AHH activity can be obtained with a single serum, especially using induced cultures, the effects of nutrition upon the activity of pooled rat sera were investigated. Rats were fed diets containing either high or low protein contents. Serum was also obtained from rats fed the standard 41B pellet diet.

Rats fed a low protein diet have significantly less hepatic cytochrome P450 when expressed on a wet weight basis compared with rats fed a high protein diet [26], although these differences are much reduced when expressed per g of liver protein. Hepatic AHH activity appears to be more sensitive to dietary protein content and large differences are found between high and low protein groups, irrespective of how the activity is expressed.

Cells were incubated in the presence of sera

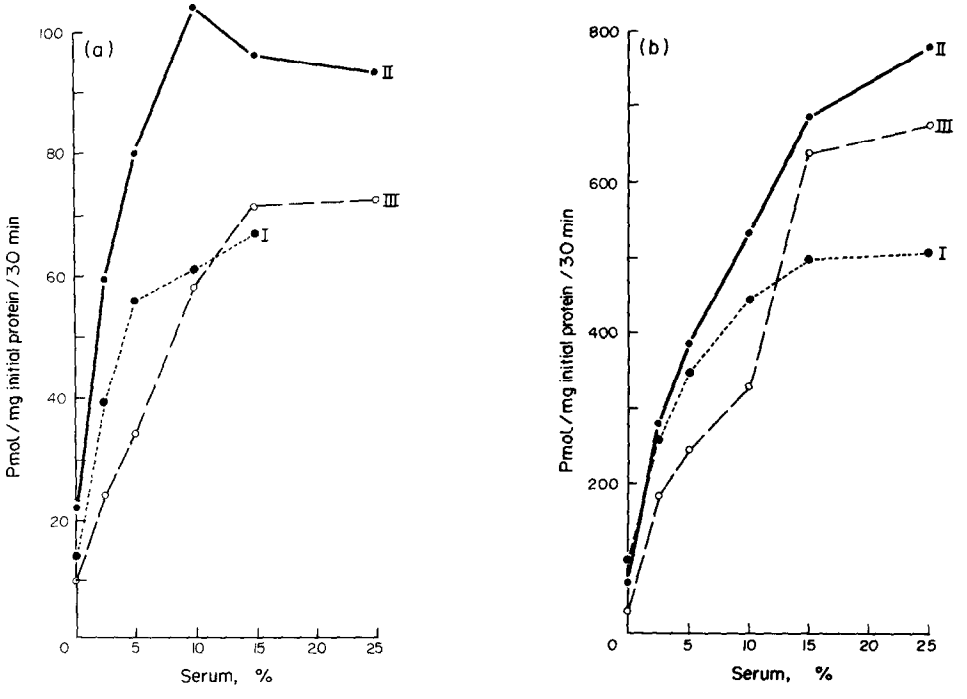

Fig. 4. Dose-response curves of AHH activity against percentage serum concentration. Panel A: Basal AHH levels measured 8 hr after stimulation with (i) 3% casein serum, (ii) 20% casein serum, (iii) 41B serum. Panel B: Induced AHH levels measured 24 hr after stimulation with (i) 3% casein serum, (ii) 20% casein serum, (iii) 41B serum. 17.5μM benzanthrazene used as inducer. AHH activity in rat liver homogenates from these donor animals was (i) 2444 ± 660 (N = 5) for rats fed the 3% casein diet, (ii) 4270 ± 517 (N = 5) for rats fed the 20% casein diet and (iii) 4704 ± 766 (N = 5) for rats fed the 41B pellet diet. Activity is expressed as nmoles/30 min/g protein.

Table 3. Kinetic constants obtained with sera from animals fed casein diets containing no fat*

Serum	Apparent V_{max} pmole/mg initial protein/30 min		Apparent K_m % v/v	
	8 hr, no inducer	24 hr, plus inducer	8 hr, no inducer	24 hr, plus inducer
3% casein	78(73–83)	584(567–601)	2.4	3.2
20% casein	111(103–120)	875(793–976)	2.1	5.4

* Lineweaver–Burk transformations of dose–response curves using regression analysis of data points shown in Fig. 4. Apparent V_{max} values are shown with the range of 1 standard deviation to either side of the mean estimate. The data has 'goodness of fit' coefficients of $r = 0.997$ and 0.998 for 3% and 20% casein serum, respectively, using induced cultures, where the difference in apparent V_{max} is significant at $P < 0.001$.

obtained from these rats. Induced AHH values were stimulated to a greater extent by sera from animals fed a high protein diet than from a low protein diet (Table 2). These effects appear to be more substantial at high serum concentrations. Basal enzyme activities measured at 8 hr after serum stimulation show a similar trend, as do measurement at 24 hr in other experiments. Protein ratios are also generally greater after treatment with sera obtained from rats fed a high protein diet.

Dose–response curves were constructed using each type of serum, prepared from a separate consignment of animals than from those used in the previous experiment. For each serum total AHH activity per plate divided by the total initial protein per plate was plotted against percentage serum concentration (Fig. 4).

Sera obtained from rats fed either 3% or 20% casein diets both give similar shaped curves with induced cultures. The 20% casein serum is more active than serum obtained from animals fed the 3% casein diet. A similar pattern can be seen with basal activities measured 8 hr after the addition of serum. Sera obtained from animals fed the 41B pellet diet, however, exhibit a dose–response curve with induced cultures of a very different shape compared to the casein diets. This serum appears to have at least one component which is only significantly active at high serum concentrations.

When these dose–response curves are transformed in the manner of Lineweaver–Burk or Hofstee, apparent K_m and V_{max} values can be obtained to represent extrapolation of data to infinite serum concentrations. Both induced and basal dose–response curves approximate to a straight line under these conditions, with the exception of that obtained using serum from rats fed the 41B pellet diet, and consequently no constants could be calculated for this serum.

Table 3 shows that only with induced cultures are the differences in AHH stimulation statistically significant. This may reflect the greater variation in results obtained with basal values. Although the exact meaning of these constants is open to question, it is clear that sera obtained from animals fed high protein diets stimulate AHH activity to a greater extent than sera obtained from animals fed low protein diets. Thus the activity of these rat sera appears to depend upon the nutritional status of the donor animal.

The demonstration of serum factors capable of stimulating drug metabolizing enzymes and the dependence of these factors upon the environment of the animals, leads to the question as to whether differences can be detected between normal human individuals in the ability of their sera to stimulate AHH activity in tissue culture cells. Blood was collected from 11 healthy volunteers and serum prepared as described in Materials and Methods.

Both basal and induced AHH activities of cells stimulated by human sera appear to vary considerably, when expressed per mg of initial protein (Fig. 5). Determinations were made only at 24 hr after the addition of 30% v/v serum. Compared to foetal calf serum (Table 1), both basal and induced values are low, whilst the corresponding protein ratios are also smaller. Basal values range between 20 and 50 pmole/mg protein/30 min, whilst induced values range between 180 and 540 pmole/mg initial protein/30 min. A strong correlation exists between basal and induced activity ($r = 0.83$, $P < 0.01$) after stimulation by human sera, indicating that both parameters may be controlled by the same set of serum factors (Fig. 5).

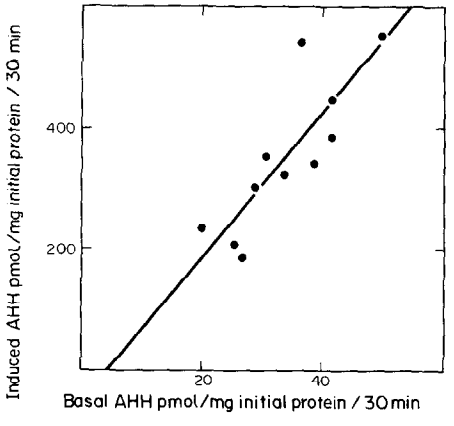

Fig. 5. Correlation of induced and basal AHH activity after stimulation of quiescent cells with different human sera. Cells were prepared as described in Table 1. All determinations were performed in triplicate 24 hr after the addition of medium containing 30%v/v human sera ± 17.5μM benzanthracene.

CONCLUSION

The experiments reported in this paper suggest that tissue culture cells which metabolize benzpyrene, and are inducible by polycyclic hydrocarbons, can be used as a relatively rapid test system to investigate factors in blood that influence human drug metabolism.

Under the conditions of culture described, AHH activity is a function of the total number of cells present and the type and concentration of serum used to stimulate these cells. The powerful effect of animal sera on AHH activity in tissue culture cells and lymphocytes has been described before [9, 11, 27, 28]. The ability of serum to stimulate AHH activity in this test system exhibits inter-individual variability and appears to be sensitive to environmental factors such as nutrition.

A strong relationship between hepatic enzyme activity and the ability of a serum to stimulate AHH activity in tissue culture cells has been demonstrated for rats. Human serum was also found to be active in this system. Despite the very small number of individuals tested, considerable inter-individual variation was observed, with a 2–3-fold range for both basal and induced activities. A similar range has been observed for the half-life of drugs such as phenacetin, acetanilide, theophylline and antipyrine [29]. It will therefore be of great interest to compare the half-lives of a number of drugs with the ability of serum from the same individuals to stimulate AHH activity in tissue culture cells.

Stimulation of these cells with serum not only stimulates AHH activity but also protein and DNA synthesis as cells resume active growth. The effect of possible fluctuations in enzyme activity during the cell cycle may be responsible for the large degree of variation seen with basal activities. Thus the value of this assay may be improved by performing experiments under conditions where less cell growth occurs, either by using higher cell densities or perhaps by using blood plasma which contains fewer growth stimulating factors. Alternatively, a more careful choice of responsive cell may considerably improve the application of these methods to the study of human drug metabolism.

Kellermann and other have studied the genetic potential for AHH synthesis of lymphocytes and other human cells. In contrast, the present assay is designed to examine the factors in the circulation, both endogenous and environmental in origin, that stimulate AHH activity in cells.

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