Genet. Res., Camb. (1978), **31**, *pp.* 177–186
With 3 text-figures
Printed in Great Britain

Genetic variation between mice in their metabolism of coumarin and its derivatives

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(*Received* 24 *November* 1977)

SUMMARY

Adult females from 19 strains of mice were injected with either coumarin or 7-ethoxycoumarin and the urinary excretion of the umbelliferone produced by the metabolism of these substances was measured. With the exception of C57L the strains fell into three classes as follows: high metabolizers (DBA/1 and DBA/2), medium metabolizers (CBA, 129/Rr, NZB and NZW) and low metabolizers (the other 12 strains). The difference in metabolizing ability between the medium group and the low group of strains was also evident when the 4-methyl derivatives of the same two substances were used. However with the 4-methyl derivatives there was no difference in metabolizing ability between the medium group and the high group. The results are interpreted as evidence that the gene *Coh* on chromosome 7 comprises two closely linked genes which determine cytochrome P-450 isozymes with different substrate specificities.

1. INTRODUCTION

Coumarin is a compound with a pleasant fragrance found in many species of plants, and was until recently used as a flavouring substance in foods (Feuer, 1974). Some mammals have a liver microsomal enzyme which can hydroxylate coumarin to form 7-hydroxycoumarin (umbelliferone, a highly fluorescent substance). Kratz & Staudinger (1967) found that different strains of domesticated rabbit (*Oryctolagus cuniculus*) have different specific activities of coumarin 7-hydroxylase in their liver microsomes. Wood & Conney (1974) found that different strains of laboratory mice (*Mus musculus*) also have different specific activities of liver microsomal coumarin 7-hydroxylase. In particular, they showed that strain DBA/2J had between three and four times the activity of strains AKR/J, C57BL/6J and C3H/HeJ. They also found that the difference between DBA/2J and AKR/J mice is determined principally by a single gene. The classification of mouse strains was extended by Lush & Arnold (1975) who found that ten more strains resembled C57BL, C3H and AKR in having a low ability to 7-hydroxylate coumarin. However three other strains, C57L, CBA/Cam and NZB, fell between the low group and DBA/2. Wood & Conney (1974) showed that mice can be assessed with respect to their ability to 7-hydroxylate coumarin *in vivo* by injecting them with coumarin and measuring the umbelliferone excreted

in their urine during the following 24 h, and this technique was also used by Lush & Arnold (1975).

Ullrich & Weber (1972) synthesized 7-ethoxycoumarin and showed that liver microsomes of mice (strain NMRI) can *O*-deethylate this substrate to produce umbelliferone. It seemed to us of interest to discover if the inter-strain variation in ability to 7-hydroxylate coumarin is also expressed as variation in the ability of the same strains to *O*-deethylate 7-ethoxycoumarin. In this paper we show that there is indeed a very close correlation between strains in their ability to metabolize these two substrates. We also show that the introduction of a methyl group at position 4 of the molecule changes the classification of the strains in such a way as to suggest that more than one isozyme may be involved in the, metabolism of the non-methylated substrates.

2. MATERIALS AND METHODS

(i) *Animals*

Nineteen strains of mice were studied. Only adult females aged between 10 and 40 weeks were used. They were housed on beechwood sawdust with paper nesting material and were protected from any potentially enzyme-inducing substances, e.g. insecticides. Water and Oxoid breeder diet were available *ad libitum*. The full designations of the strains used, and their laboratories of origin, are as follows: A2G/Lac, C57L/Lac, C3H/He/Lac, CE/Lac, DBA/1/Lac, DBA/2/Lac, F/St/Lac, ICFW/Lac, NMRI/Lac, NZB/Lac, NZW/Lac, 129/Rr/Lac (from MRC Laboratory Animals Centre, Carshalton), SM/J (Animal Breeding Research Organization, Edinburgh), BALB/c/Gr and C57BL/Gr (Department of Genetics, University College London), CBA/Cam (Department of Genetics, Cambridge University), Simpson (Kennedy Institute, London), Schneider and TO (Imperial Cancer Research Fund, London). All the above strains except Schneider and TO are inbred.

(ii) *Chemicals*

Coumarin, 4-methylumbelliferone (Koch-Light) and umbelliferone (Aldrich Chem. Co.) were of the purest grade available. 4-Methylcoumarin was synthesized for us by Mr J. Stevens (Maybridge Chem. Co.) using the method of Woodruff (1944). Professor G. Feuer also gave us an authentic sample. 7-Ethoxycoumarin was synthesized by us from umbelliferone using the method of Ullrich & Weber (1972). Professor V. Ullrich also gave us an authentic sample. 7-Ethoxy-4-methylcoumarin was synthesized using the same method, but with 4-methylumbellifer one as the starting material.

(iii) *Experimental procedure*

Each substrate was dissolved in dimethyl sulphoxide and then diluted with an equal volume of water. With 7-ethoxy-4-methylcoumarin a volume of 0·2 ml was injected i.p. into each mouse. With the other substrates the volume injected was 0·1 ml. Immediately after injection each mouse was placed in a circular stainless steel cage fitted into the top of a large polythene funnel. Drinking water

was provided, but not food. The funnel passed the excreta into a Jencon 'metabowl' which separated urine from faeces. The occasional faecal bolus which got into a urine flask did not affect the result. After 22 h in the cage the mouse was removed and the apparatus was washed through with distilled water. Each urine plus washings was further diluted with water to a suitable final volume, usually 50 or 100 ml. The ambient temperature was kept above 20 °C during the 22 h collection period.

Duplicate 2 ml samples of each diluted urine were incubated for 2 h at 37 °C with 0·2 ml of *Helix pomatia* extract (Sigma, β-glucuronidase type H-1) containing about 5500 Fishman units of β-glucuronidase activity and about 8 units of arylsulphatase activity. Each duplicate was then mixed with 3·8 ml 0·5 M glycine-NaOH buffer, pH 10·35, to give a final volume of 6·0 ml. This was divided into two 3·0 ml portions to one of which was added 0·1 ml 5% ethanol, and to the other was added 0·1 ml of 50 μM umbelliferone (or 4-methylumbelliferone) in 5% ethanol as an internal standard. The fluorescence of each solution was measured in a Baird-Atomic Fluoripoint spectrofluorimeter (λ_{ex} = 375 nm, λ_{fl} = 455 nm) and the umbelliferone, or 4-methylumbelliferone, content of the urine calculated.

Mice were typed with respect to their red cell glucose phosphate isomerase (Gpi-1) electrophoretic phenotype by the method of DeLorenzo & Ruddle (1969).

The statistical methods used were the Mann-Whitney two-tailed U test for the comparison of groups (Siegel, 1956) and the t test for the significance of a correlation.

3. RESULTS

All 19 strains were first surveyed to determine their ability to metabolize coumarin and 7-ethoxycoumarin to umbelliferone (see Fig. 1). Each mouse was injected with 3 μmole of one of the two substrates regardless of its body weight. In no strain were the mice all typed in the same experiment. The mean urinary umbelliferone excreted by the mice of each strain is given in Table 1 and plotted in Fig. 2. The results with coumarin confirm that strain DBA/2 is a good metabolizer of this substrate and show that DBA/1 is identical to it in this respect. Four strains, NZB, NZW, 129/Rr and CBA, formed a group with what we shall refer to as medium coumarin-metabolizing ability. Twelve other strains were found grouped together as relatively poor coumarin metabolizers. Strain C57L did not fit easily into either the medium or the low group and this will be commented upon later. The difference between the high group (DBA/1 and DBA/2) and the medium group is statistically significant ($P < 0{\cdot}01$), as also is the difference between the medium group and the low group ($P < 0{\cdot}01$). When 7-ethoxycoumarin was the injected substrate the above three groups were not so distinct (Fig. 2), but they are nevertheless still significantly different (high *v.* medium, $P < 0{\cdot}02$, medium *v.* low, $P < 0{\cdot}01$). There is a highly significant overall inter-strain correlation in the metabolism of the two substrates ($r = 0{\cdot}91$, $P < 0{\cdot}001$). Experiments with strains TO, DBA/1 and DBA/2 showed that the

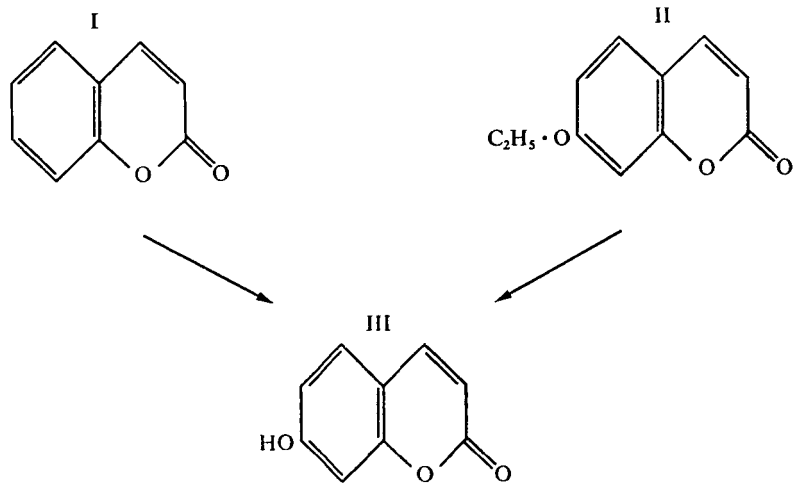

Fig. 1. The metabolism of coumarin (I) and 7-ethoxycoumarin (II) to give umbelliferone (III). The same reactions are undergone by the 4-methyl derivatives of I and II to give 4-methylumbelliferone.

Table 1. *Umbelliferone or 4-methylumbelliferone excreted by mice of each strain (mean ± S.E.M.) after injection of each of the four substrates*

Strain	n-mole umbelliferone excreted after injection of: (*a*) Coumarin (3 μmole)	(*b*) 7-Ethoxycoumarin (3 μmole)	n-mole 4-methylumbelliferone excreted after injection of: (*a*) 4-methyl coumarin (3 μmole)	(*b*) 7-Ethoxy-4-methylcoumarin (2 μmole)	Mean weight of mice injected with coumarin (g)
A2G	52 ± 6 (6)*	1340 ± 98 (6)	0 (4)	536 ± 58 (6)	21·0
BALB/c	54 ± 13 (5)	1307 ± 151 (6)	0 (2)	470 ± 43 (6)	21·7
CBA	416 ± 45 (6)	1927 ± 150 (6)	106 ± 10 (5)	483 ± 32 (6)	25·3
C57BL	77 ± 11 (6)	1309 ± 56 (6)	0 (2)	506 ± 39 (6)	24·1
C57L	304 ± 34 (7)	1464 ± 88 (6)	23 ± 5 (7)	392 ± 21 (6)	23·8
CE	126 ± 16 (4)	1481 ± 123 (5)	n.t.	n.t.	24·8
C3H/He	93 ± 8 (6)	1527 ± 106 (6)	0 (2)	509 ± 40 (6)	28·6
DBA/1	782 ± 32 (5)	2318 ± 178 (4)	168 ± 13 (6)	602 ± 41 (6)	23·0
DBA/2	735 ± 62 (7)	2329 ± 61 (4)	139 ± 5 (6)	537 ± 18 (6)	21·2
F/St	156 ± 13 (6)	1441 ± 83 (6)	0 (2)	448 ± 47 (6)	18·5
ICFW	82 ± 7 (6)	1143 ± 96 (6)	0 (2)	540 ± 44 (6)	24·6
NMRI	162 ± 22 (6)	1500 ± 100 (6)	0 (2)	434 ± 58 (6)	24·8
NZB	503 ± 35 (5)	2090 ± 141 (5)	140 ± 10 (4)	594 ± 47 (4)	28·7
NZW	419 ± 46 (4)	2282 ± 94 (4)	118 ± 12 (5)	724 ± 27 (4)	25·7
129/Rr	499 ± 79 (6)	1798 ± 71 (4)	136 ± 12 (3)	602 ± 36 (6)	23·8
Schneider	77 ± 7 (6)	1257 ± 98 (6)	0 (2)	566 ± 38 (6)	30·3
Simpson	25 ± 5 (6)	1436 ± 157 (5)	0 (2)	590 ± 15 (6)	20·1
SM	127 ± 12 (6)	1573 ± 160 (6)	0 (2)	371 ± 28 (6)	16·4
TO	81 ± 13 (8)	1288 ± 85 (6)	0 (2)	560 ± 9 (6)	27·6

n.t., Not tested. * Number of mice tested.

https://doi.org/10.1017/S0016672300017936 Published online by Cambridge University Press

percentage of each substrate metabolized and excreted was constant over a wide range of dosages (Fig. 3A).

Eighteen of the strains were surveyed for their ability to metabolize 4-methylcoumarin and 7-ethoxy-4-methylcoumarin to 4-methylumbelliferone. Each mouse was injected with either 3 μmole of 4-methylcoumarin or 2 μmole of 7-ethoxy-4-methylcoumarin regardless of its body weight. The lower dose of the second

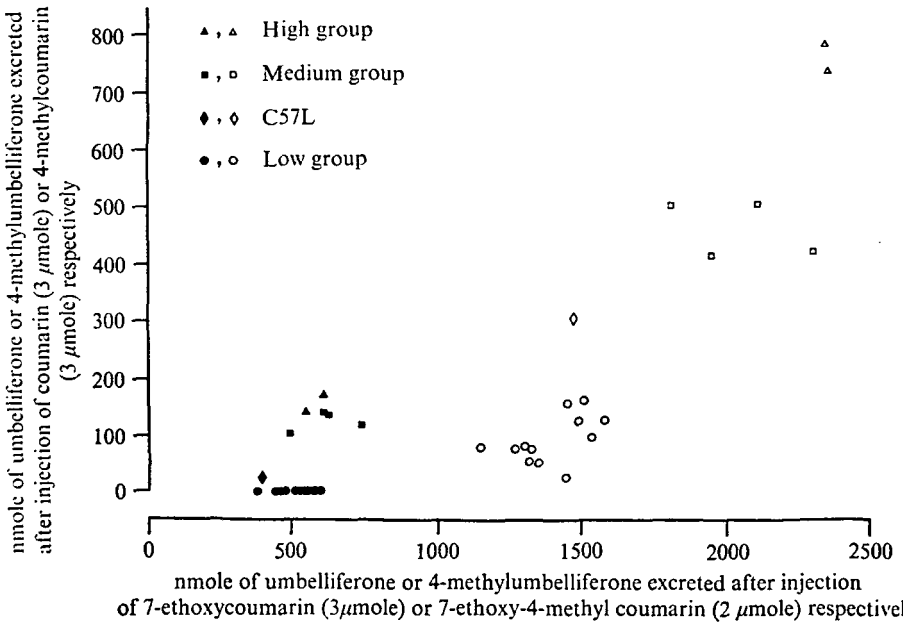

Fig. 2. Display of data in Table 1. Empty symbols refer to umbelliferone, filled symbols refer to 4-methylumbelliferone.

substrate was necessitated by its lower solubility in the solvent used. The mean urinary 4-methylumbelliferone excreted by the mice of each strain is given in Table 1 and plotted in Fig. 2. Clearly both methylated substrates were metabolized less than were their non-methylated forms. After 4-methylcoumarin injection the low group of strains excreted no detectable 4-methylumbelliferone and strain C57L excreted only a small amount. The remaining six strains formed a cluster within which there was no significant difference between the two high strains and the four medium strains. After 7-ethoxy-4-methylcoumarin injection there was again no significant difference between the two high strains and the four medium strains. However the difference between this cluster of six strains and the low group of strains was still significant ($P < 0{\cdot}05$). The results of dosage experiments with the two methylated substrates are shown in Fig. 3B and show that the percentage of 4-methylcoumarin metabolized and excreted is constant over a wide range of dosages. As mentioned above, the amount of 7-ethoxy-4-methylcoumarin which could be conveniently injected was limited to 2 μmole.

Wood & Conney (1974) showed that the high excretion of umbelliferone by

DBA/2 mice after coumarin injection is correlated with a high specific activity of liver microsomal coumarin 7-hydroxylase in that strain. This activity is principally determined by a gene, *Coh*, which is located about 6 cM distance from the gene *Gpi-1* (glucose phosphate isomerase) on chromosome 7 (A. W. Wood, 1974, personal communication; Taylor, 1976). It seemed to us that the level of umbelliferone excretion found in the strains of the medium group might be determined by a different allele of the same gene. Preliminary experiments have indicated that the gene determining the difference between the low group and the medium group is indeed linked to *Gpi-1*. Therefore on the basis of the linkage data two different alleles of the *Coh* gene could be responsible for the high and medium group of metabolizers.

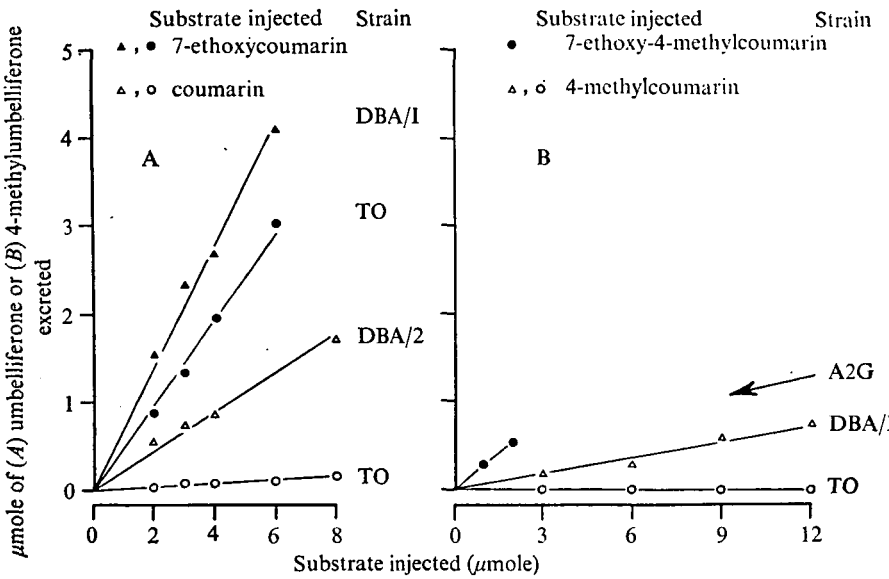

Fig. 3. The relationship between injected substrate and excreted metabolite for the four substrates used. Each symbol is the mean of between two and eight mice.

The inter-strain variation described above might have been influenced by variation in the ability of different strains to conjugate umbelliferone or 4-methylumbelliferone to form glucuronides, or possibly by variation in the ability of their kidneys to excrete the glucuronides into the urine. To check these possibilities we injected four mice from every strain except NZW and 129/Rr with 2 μmole of umbelliferone and measured how much was excreted in 22 h. The percentage excreted varied from 43% in C3H to 57% in DBA/1 and showed no correlation with the variation shown in Fig. 2. In a similar experiment with 4-methylumbelliferone each strain excreted between 80 and 95% of the dose, and again there was no correlation with the data in Fig. 2.

4. DISCUSSION

The simplest explanation of the inter-strain correlation between 7-hydroxylation of coumarin and deethylation of 7-ethoxycoumarin is that both reactions are carried out by one and the same enzyme. Creaven, Parke & Williams (1961) found that in the mouse coumarin is 7-hydroxylated by a microsomal enzyme which requires NADPH and oxygen. Wood & Conney (1974) showed that the 7-hydroxylase is inducible by phenobarbitone and is therefore probably a cytochrome P-450. However, mouse 7-ethoxycoumarin deethylase exists both as a phenobarbitone-inducible cytochrome P-450 (Ullrich & Weber, 1972) and also as a cytochrome P-448 which is inducible by 3-methylcholanthrene. A further complication is the fact that the cytochrome P-448 form is inducible only in mice which have the allele Ah^a in their genome (Nebert, Considine & Owens, 1973). There appears to be no 3-methylcholanthrene-inducible cytochrome P-448 form of coumarin 7-hydroxylase, even in mice which have the Ah^a allele (Wood & Conney, 1974). Since only a small proportion of the microsomal enzyme in an uninduced mouse is cytochrome P-448 (Lu & Levin, 1974) it seems probable that the genetic variation of 7-ethoxycoumarin deethylase described in this paper involves its cytochrome P-450 form.

The introduction of a methyl group at position 4 of coumarin or 7-ethoxycoumarin has two effects. The first effect is a reduction in the metabolism of each substrate in every strain. Indeed in the low group of strains the hydroxylation of 4-methylcoumarin is below the threshold of measurement of the very sensitive fluorimetric technique. The second effect is the elimination of the distinction between the high group of strains (DBA/1 and DBA/2) and the medium group of strains (CBA, 129/Rr, NZB and NZW). This merging of the high and medium groups which occurs when methylated substrates are used can be explained in either of the following two ways:

(1) A different allele of the gene *Coh* is present in each of the three groups of mice, as suggested by the linkage data. Each allele determines a different genetic variant, or allozyme, of the cytochrome P-450. The three allozymes have different activities towards the non-methylated substrates and so produce the three groups of metabolizing abilities in the mice. The difficulty with this hypothesis is that one must then assume that when metabolizing the *methylated* substrates the allozymes present in the medium group and the high group no longer have different activities. However, an example of a genetic difference of this kind is provided by human erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD). The normal form of G-6-PD oxidizes 2-deoxyglucose-6-phosphate at less than 4% of the rate at which it oxidizes glucose-6-phosphate, whereas with the Mediterranean variant of G-6-PD the relative rate is 25–30% (Harris, 1975).

(2) There are two forms, or isozymes, of the cytochrome P-450, each determined by a different gene. Isozyme 1 can metabolize both the methylated and the non-methylated substrates, and it is genetic variation in the activity of this

isozyme which differentiates the low group from the medium group. Isozyme 2 can metabolize only the nonmethylated substrates, and it is genetic variation of this isozyme which differentiates the high group from the medium group. Table 2 summarizes this hypothesis which in effect states that the difference in substrate specificity is due to different isozymes rather than different allozymes. An example of this kind of relationship can be seen with mammalian xanthine oxidase (EC 1.2.3.1) and aldehyde oxidase (EC 1.2.3.2) which are two very similar nonmicrosomal enzymes and are able to hydroxylate many of the same substrates. Indeed these two enzymes are so similar in every respect that they could be regarded as isozymes (Krenitsky, Neil, Elion & Hitchings, 1972). Thus although both can hydroxylate purine at position 8, only aldehyde oxidase can do this to 6-methylpurine – a situation analogous to our second hypothesis. Similarly, hypoxanthine is a good substrate for xanthine oxidase and a very poor substrate for aldehyde oxidase, but the reverse is true of 3-methylhypoxanthine (Krenitsky *et al.* 1972).

Table 2. *Summary of the hypothesis to explain the differences in metabolizing ability between the three groups of strains*

(Isozyme 1 can metabolize both the methylated and the non-methylated substrates. Isozyme 2 can metabolize only the non-methylated substrates. Each isozyme has two allozymes. Allozyme A has low activity and allozyme B has high activity. The table shows the distribution of the allozymes in the three groups of strains.)

		Classification of strains on the basis of their coumarin-metabolizing ability		
Gene	Isozyme	Low group	Medium group	High group
Coh-1	1	A	B	B
Coh-2	2	A	A	B

Wood & Conney (1974) and Wood (personal communication) classified their mice on the criterion of their ability of 7-hydroxylate coumarin. They concluded that one gene (*Coh*) determined most of the difference between the low group and DBA/2. At first sight this seems to contradict our hypothesis that two genes (*Coh-1* and *Coh-2*) differentiate these two groups (see Table 2). However this apparent contradiction disappears if, as our combined evidence indicates, both *Coh-1* and *Coh-2* are closely linked to *Gpi-1* and may therefore be very closely linked to each other. Two very closely linked genes are genetically indistinguishable from one gene unless the experiment is done on a sufficiently large scale to detect recombination. Several examples of close linkage of genes determining isozymes are now known in mammals and are understood to be the result of small tandem duplications (Lush, 1966; Eicher *et al.* 1976; Peters & Nash, 1977).

A curious but consistent feature of our data is the fact that the differences between the groups of strains are much greater when they are expressed in terms of 7-hydroxylating ability than when they are expressed in terms of deethylating

ability. For example the high group excretes a mean value of 8·2 times as much umbelliferone as the low group after coumarin injection, but only 1·7 times as much after 7-ethoxycoumarin injection. Taken by itself this might seem to be an inevitable consequence of the overall greater efficiency of 7-ethoxycoumarin metabolism; thus both the high group strains metabolized and excreted 77% of the injected 7-ethoxycoumarin in 22 h, which leaves little room for improvement. However, the same feature can be seen in the results with the methylated substrates, where only about 15% of the 7-ethoxy-4-methylcoumarin is metabolized and excreted. The presence of another isozyme of cytochrome P-450 which does not vary genetically in any of the strains tested and which can deethylate (but not hydroxylate) both the methylated and the nonmethylated substrates could produce such an effect and may be the explanation.

Strain C57L presents a problem in classification since although it falls within the low group with respect to the deethylation of both methylated and nonmethylated ethoxycoumarin it has a hydroxylating ability which is consistently too high for that group. At present we have no satisfactory explanation of the characteristics of C57L.

In rats and rabbits (Feuer, 1970; Kaighen & Williams, 1961) the 3- and 7-hydroxy metabolites are the major hydroxylated products of the microsomal metabolism of coumarin and 4-methylcoumarin. There is evidence in the rat that the 3-hydroxylase is a different enzyme from the 7-hydroxylase (Feuer, 1970). No data have yet been published on coumarin 3-hydroxylase in the mouse.

We thank Miss Jill Williams and Miss Eileen Potts for technical assistance, Dr F. Kurzer for help with the syntheses of the 7-ethoxy derivatives, and The Nuffield Foundation for financial support.

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