A Spectrofluorimetric Study of the 7-Hydroxylation of Coumarin by Liver Microsomes

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1. The fluorescence characteristics of 3- and 7-hydroxycoumarin, and 7-hydroxy- and 7-methoxy-4-methylcoumarin, have been determined. 7-Hydroxycoumarin shows excited-state ionization from pH 1 to 9. 2. A sensitive and specific fluorimetric method for the determination of 7-hydroxycoumarin (umbelliferone), and its application to liver homogenates and other tissue preparations, are described. 3. The enzymic hydroxylation of coumarin to 7-hydroxycoumarin has been studied by this method and the optimum conditions have been determined for rabbit-liver preparations. The enzymic activity was found in the microsomal fraction and required NADPH₂ and oxygen. Activity with NADH₂ was one-third of that with NADPH₂. 4. Addition of NADP was necessary for full activity of 10000g supernatant preparations of liver. Nicotinamide added during preparation preserved coenzymic activity in tissue stored at -12°. Glucose 6-phosphate had no effect on the activity of stored or fresh tissue. 5. Inhibition occurred with *p*-chloromercuribenzoate, and with the usual inhibitors of the microsomal drug-metabolizing enzymes, SKF acid, SKF 525A, and Lilly 7132, but not with 2,2'-bipyridyl. 6. Liver homogenates from rabbit, guinea pig, coypu, cat and pigeon showed activity, but preparations of rat or mouse liver, and of locust fat bodies, did not hydroxylate coumarin to umbelliferone. The enzyme system was absent from rat-liver homogenates and microsomal preparations. Moreover, rat liver also contained inhibitors of the rabbit-liver coumarin-7-hydroxylase system and of the further metabolism of umbelliferone by guinea-pig liver. Guinea-pig-liver preparations hydroxylated coumarin to umbelliferone and then converted this product into its glucuronide. 7. The coumarin-7-hydroxylase activity of female rabbit liver was two to three times that of male rabbit liver.

Kaighen & Williams (1961) have shown that coumarin is hydroxylated in the rabbit to yield in the urine all six monohydroxycoumarins. Of these 3-hydroxycoumarin was obtained in largest amount (22% of the dose), and 7-hydroxycoumarin (umbelliferone) was next in importance (12%). In the rat, however, hydroxycoumarins were not significant metabolites of coumarin in the urine, for the output of 3-hydroxycoumarin was only about 2% of the dose and that of 7-hydroxycoumarin only 0.3%. To obtain further information on this difference between rats and rabbits, it was decided to investigate the hydroxylation of coumarin *in vitro* with liver preparations. 7-Hydroxycoumarin is a highly fluorescent compound and can therefore be estimated in minute quantities. In fact, its fluorescence has been used previously in this Laboratory for the determination of β -glucuronidase (Mead, Smith & Williams, 1955) and of β -glucosidase (Robinson, 1956). Coumarin is converted into 7-hydroxycoumarin by rabbit-liver

microsomal preparations (Posner, Mitoma & Udenfriend, 1961). In the present paper a fluorimetric micro-method is described for determination of the 7-hydroxylating activity of liver preparations towards coumarin. Studies have been made mainly on rabbit liver; in a survey of other species it was found that activity is absent from rat liver. Some of the results described in the present paper have already been briefly reported (Creaven, Parke & Williams, 1962; Creaven & Williams, 1963); while the present paper was in preparation, a report on the fluorimetric method was published by Kerékjártó, Kratz & Staudinger (1964), whose data are less extensive than ours.

EXPERIMENTAL

Chemicals. The coumarin and its hydroxy derivatives and 7-hydroxy-4-methylcoumarin and its methyl ether were previously prepared and purified in this Laboratory

(Mead, Smith & Williams, 1958; Williams, 1959). Nicotinamide, the sodium salts of NADH₂, NADP and NADPH₂, disodium glucose 6-phosphate (British Drug Houses Ltd., Poole, Dorset) and glucose 6-phosphate dehydrogenase (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used as purchased. Buffers were prepared from analytical grade reagents according to Gomori (1955).

Animals. Rabbits (Chinchilla and English strains) and guinea pigs (English) were purchased from Ray Rabbitries, Lingfield, Surrey; rats (Wistar albino, and black-hooded strains) from Animal Suppliers (London) Ltd., Knebworth, Herts.; mice (I.C.I. strain) from The Scientific Animal Service, Elstree, Herts.; ferrets from Glaxo Research Farm, Ruimslip; pigeons (*Columba livia* var. *domestica*) from Regent Pet Stores, Camden Town, London; hens (Light Sussex × Rhode Island Red) from Poydon Farm, Goff's Oak, Herts.; frogs (*Rana temporaria*) from The Frog Farm, Kells, Co. Meath, Ireland; locusts (*Schistocerca gregaria*) from the Anti-Locust Research Centre, College House, Kensington, London; coypus (*Myocastor coypus*) and domestic cats were bred on the Wellcome Floor, St Mary's Hospital Medical School, London.

Preparation of enzyme solutions. Animals were killed by a blow on the back of the head, and the livers were rapidly removed, cut up and cooled on ice. Portions were quickly dried with filter paper, chopped, macerated and then homogenized for 1 min. with 3 vol. of ice-cold 1.15% KCl in an all-glass Potter-Elvehjem homogenizer driven by a Gallenkamp stirrer motor at maximum velocity (2500 rev./min., 1/75 h.p.). The homogenate was then diluted with ice-cold 1.15% KCl to 1 g. of liver/4 ml. and nicotinamide (1 m-mole/100 ml.) was added. It was then fractionated by centrifugation by standard methods (Mitoma, Posner, Reitz & Udenfriend, 1956) at 0–5° to give a '10000g supernatant' (10000g for 10 min., MSE High Speed 17 refrigerated centrifuge), a 'microsomal fraction' and a 'soluble fraction' (105000g for 1 hr., MSE Super-Speed 40 refrigerated centrifuge). Most of the experiments described below were carried out with the 10000g supernatant, which contained the microsomes plus soluble fraction. The 10000g supernatant was stored when necessary at –12°. The microsomal fraction was given three washings by resuspending in ice-cold 1.15% KCl equal to the original volume and again centrifuging at 105000g for 1 hr. The supernatants were discarded and the final microsomal pellet was resuspended in the original volume of 1.15% KCl and kept at –12°.

Conditions for incubation. In the standard procedure the above preparations (1 ml. ≡ 0.25 g. of liver and containing 10 μmoles of nicotinamide) were incubated for 15 or 30 min. with coumarin (1 μmole), 0.1 M-phosphate buffer, pH 7.4 (0.5 ml.), NADP (0.25 μmole) and disodium glucose 6-phosphate (6 μmoles) in a total volume of 3.0 ml. in 25 ml. beakers at 37° in air in a Mickle Shaking Incubator. Controls with boiled tissue, and controls in which substrate was omitted and then added after incubation, were also set up.

To determine the effect of oxygen and nitrogen on the hydroxylation of coumarin, the same amounts of substrate and cofactors as above were placed in the lower end of a Thunberg tube, and the enzyme preparation (1 ml. ≡ 0.25 g. of liver and containing 10 μmoles of nicotinamide) in the hollow stopper. The tube was evacuated for 2 min., filled with nitrogen, oxygen or air as required, and the procedure

repeated three times. The tube contents were then mixed and incubated in the Mickle incubator.

Estimation of umbelliferone. Fluorescence was determined in an Aminco-Bowman spectrophotofluorimeter, pH in a Pye Master pH-meter and light-absorption in a Unicam SP. 500 spectrophotometer.

A solution of umbelliferone (7-hydroxycoumarin) in ethanol (100 μg./ml.) was prepared and a suitable amount of this was added to appropriate volumes of water, 0.1 N-HCl, N-HCl or 0.1 N-NaOH, so that four solutions were obtained containing 5 μg. of umbelliferone/ml. of 5% ethanol. From these four solutions, there could be prepared solutions of different pH values containing 0.1 μg. of umbelliferone/ml. The pH of each solution was checked before a fluorescence determination was made. Solutions of other compounds used for fluorescence were made in a similar manner. The incubation mixtures were added to ether (5 ml.) in stoppered test tubes and shaken mechanically for 5 min. A portion (0.5 ml.) of the ether layer was diluted to 25 ml. with 0.1 M-glycine-NaOH buffer, pH 10.4, mixed, and the fluorescence intensity measured at 455 mμ with an excitation of 370 mμ. Standard solutions of umbelliferone containing 0.2–2.0 μg. were run through the same extraction and estimation procedures. The intensity of fluorescence was proportional to the concentration of umbelliferone over the range 0.2–2.0 μg./25 ml. The recovery of umbelliferone added to the above incubation mixture was 84% at 3 μg., 86% at 6 μg. and 92% at 12 μg.

Identification of umbelliferone in incubation mixtures. To the 10000g supernatant (40 ml., containing 400 μmoles of nicotinamide) from the liver of a Chinchilla doe rabbit were added coumarin (200 μmoles), NADP (10 μmoles), glucose 6-phosphate (240 μmoles) and 20 ml. of 0.1 M-phosphate buffer, pH 7.4. The solution was made up to 250 ml. with 1.15% KCl and incubated in air with shaking at 37° for 1 hr. in approximately 15 ml. portions in 100 ml. beakers. The incubation mixtures were pooled, deproteinized with aq. 30% (w/v) trichloroacetic acid (25 ml.) and centrifuged. The supernatant was continuously extracted with ether for 6 hr., and the extract was dried (Na₂SO₄) and concentrated to about 1–2 ml. Samples were chromatographed on Whatman no. 1 paper in solvents A–D (Table 1). Umbelliferone was readily detected in the incubation mixture, but neither 3-hydroxycoumarin nor its 4-, 5-, 6- and 8-isomers were detected. The limit of detection of 3-hydroxycoumarin by this method is 2 μg. In another experiment, the concentrated ether extract was chromatographed on a column (1 cm. × 25 cm.) of alumina (Spence, grade H) with light petroleum (b.p. 100–120°) containing 1% (v/v) of ether. Seven eluates (5–10 ml. each) were collected and each was concentrated to 0.1 ml. and chromatographed on paper as described above. In this case 3-hydroxycoumarin was detected in the second fraction. In a further experiment carried out similarly but with 160 ml. of homogenate, 3-hydroxycoumarin could not be detected although umbelliferone was readily found. Further, when 3-hydroxycoumarin (50 μg.) was incubated for 1 hr. with the 10000g supernatant (3 ml.) of liver homogenate plus the cofactors, no 3-hydroxycoumarin could be recovered at the end of the experiment. These results suggest that coumarin may be metabolized by liver homogenates to 3-hydroxycoumarin but that this is then further metabolized. Umbelliferone, however, is more stable in the homogenates but does tend to disappear slowly (see below).

Table 1. R_f values and colour reactions of 3- and 7-hydroxycoumarin

R_f values are for descending chromatography on Whatman no. 1 paper in solvent mixtures: A, benzene-acetic acid-water (1:1:2, by vol.), run for 4 hr.; B, butan-2-one saturated with 2N-ammonia, run for 4 hr.; C, butan-1-ol-acetic acid-water (4:1:2, by vol.), run for 8 hr.; D, propan-1-ol-ammonia (sp.gr. 0.88) (7:3, v/v), run for 8 hr. Detecting reagents were: aq. 0.01% Brentamine Fast Blue B Salt (I.C.I. Ltd.) followed by aq. saturated NaHCO_3 ; Gibbs reagent, ethanolic 0.1% 2,6-dichloroquinone-4-chloroimide, followed by aq. saturated NaHCO_3 . The excitation wavelength for fluorescence was 265 m μ .

Compound	Solvent...	R_f				Colour reactions		
		A	B	C	D	Gibbs	Brentamine	Fluorescence
3-Hydroxycoumarin		0.45	0.38	0.80	0.71	Red→green	Green	Blue
7-Hydroxycoumarin		0.20	0.65	0.80	0.69	Blue	Blue	Intense blue

RESULTS AND DISCUSSION

Fluorescence of 7-hydroxycoumarin. Fluorescence data for 3- and 7-hydroxycoumarin are given in Table 2. The hydroxyl groups in these two compounds are typical phenolic groups and it can be assumed that they have normal pK_a values of about 10. 3-Hydroxycoumarin (at 5 $\mu\text{g./ml.}$) shows a weak fluorescence at 380 m μ from pH -1 to 9 and this corresponds to the un-ionized form. From pH 9 to 12 the fluorescence shifts to 465 m μ , which corresponds to the phenolic ion. The fluorescence of this ion is very weak and at pH 10.4, the pH used for the fluorimetric determination of 7-hydroxycoumarin, it is virtually non-existent. At pH 12, the fluorescence of 3-hydroxycoumarin begins to intensify and at pH 13 it reaches a maximum (at 460 m μ), being some 18 times as intense as the 380 m μ fluorescence at low pH values. The maximum fluorescence of 3-hydroxycoumarin is 1000 times less than that of 7-hydroxycoumarin. The fluorescence at pH 13 is probably due to products of the opening of the heterocyclic ring of 3-hydroxycoumarin. Above pH 14, 3-hydroxycoumarin is non-fluorescent.

7-Hydroxycoumarin (umbelliferone) shows the phenomenon of excited-state ionization and therefore its fluorescence behaviour is completely different from that of the 3-isomer. In aqueous solution it shows maximum fluorescence as follows: $\lambda_{\text{exc.}}$ 335 m μ , λ_a 475 m μ (pH 0-1); $\lambda_{\text{exc.}}$ 335 m μ , λ_a 455 m μ (pH 1-9); $\lambda_{\text{exc.}}$ 375 m μ , λ_a 455 m μ (pH 9-14) (see Fig. 1). The fluorescence at pH 9-14 is that which would be expected from the anionic form (I) as it occurs at pH values above the expected pK_a . Below pH 9 the excitation shows the shift to shorter wavelength expected for the change to the non-ionized molecule (II), but λ_a remains that of (I). The absorbing species is thus (II) but the fluorescing species is (I), a phenomenon known as excited-state ionization (for discussion see Bridges, 1964; Williams & Bridges, 1964). At pH 1 λ_a shows a shift to 475 m μ , which is difficult to explain, since λ_a of

Table 2. Fluorescence of 3- and 7-hydroxycoumarin

Wavelengths quoted are instrumental values found in the Aminco-Bowman spectrofluorimeter. Relative intensities are based on concentration and photomultiplier readings of the instrument.

pH* of solution	Wavelength (mμ)		Relative intensity of maximum fluorescence	pH at which relative intensity was measured †
	Maximum excitation	Maximum fluorescence		
3-Hydroxycoumarin (5 μg./ml.)				
-1- 9	320	380	0.005	6
9-12	350	465	0.002	10.4
13-14	340	460	0.09	13
14		None	0.0	
7-Hydroxycoumarin (0.1 μg./ml.)				
-1 (10N-HCl)		None		
0- 1	335	475	17.5	1
2- 9	335	455	44.0	7
9-14	375	455	100.0	10†
15 (10N-KOH)		None	0.0	
Ethanol	340	385		
7-Hydroxy-4-methylcoumarin (0.1 μg./ml.)				
0	330	480	7.5‡	0
7-Methoxy-4-methylcoumarin (0.1 μg./ml.)				
0	330	385	5.2‡	0

* Range of pH over which the wavelengths given in columns 2 and 3 are found.

† 7-Hydroxycoumarin showed a constant intensity of fluorescence from pH 9.4 to 10.5 and this intensity is taken as 100.

‡ Not maximum intensities.

the non-ionized molecule (II) should be shorter than that of the anion (I). Moreover, in ethanol, umbelliferone does exhibit fluorescence (λ_a 385 m μ) at a wavelength that would be expected of (II). The fluorescing species at pH 0-1 is therefore unknown but could be a dimer hydrogen-bonded

through the free 7-hydroxyl group. Evidence in support of this was obtained through a study of the fluorescence at pH 0 of 7-methoxy-4-methylcoumarin and 7-hydroxy-4-methylcoumarin. The

latter would be expected to form a similar dimer and thus to fluoresce at the longer wavelength, but the former could not form such a dimer and would fluoresce at a shorter wavelength. These predictions were confirmed: thus 7-hydroxy-4-methylcoumarin shows λ_a 480m μ (λ_{exc} 330m μ) whereas 7-methoxy-4-methylcoumarin shows λ_a 385m μ (λ_{exc} 330m μ).

The most intense fluorescence of 7-hydroxycoumarin occurs between pH 9.4 and 10.5 (see Fig. 1). In 10N-potassium hydroxide (pH 15) and 10N-hydrochloric acid (pH -1), it is not fluorescent.

Factors affecting enzyme activity. The 7-hydroxylation of coumarin by the 10000g supernatant of rabbit-liver homogenates requires oxygen. Thus in one series of experiments the yield of umbelliferone was: in air, 0.70; in oxygen, 0.67; in nitrogen, 0.04 μ mole/g. of liver/hr. Subsequent experiments were done in air. Better yields of umbelliferone were obtained in phosphate buffers than in tris-hydrochloric acid buffers. Thus yields of umbelliferone, in μ moles/g. of liver/hr. in various buffers of pH 7.4 in a final concentration of 0.01M, were: disodium hydrogen phosphate-sodium dihydrogen phosphate, 1.04; dipotassium hydrogen phosphate-potassium dihydrogen phosphate, 1.04; tris-hydrochloric acid, 0.96; tris-maleate, 0.77; no buffer, 0.66; boiled enzyme with any buffer, 0. The amount of buffer used was 0.5ml./incubation mixture, as described above. The effect of pH on the yield of umbelliferone in phosphate and tris-hydrochloric acid buffers is shown in Fig. 2. In the former the optimum was about pH 7.3 and in the latter at pH 7.4-8.0. Kerékjártó *et al.* (1964) used tris buffer and recorded an optimum pH 8.0. If the final concentration of the sodium phosphate buffer was increased above 0.01M, the yield of umbelliferone was diminished, and at 0.05M it was halved.

Fig. 1. Fluorescence of 7-hydroxycoumarin in relation to pH. The excitation wavelength (●) and fluorescence wavelength (○) of 7-hydroxycoumarin (0.1 μ g./ml.) are shown by the upper curves. The fluorescence intensity at λ_{exc} 335m μ and λ_a 475m μ (---), λ_{exc} 335m μ and λ_a 455m μ (—) and λ_{exc} 375m μ and λ_a 455m μ (···) is shown by the lower curve. Intensities are instrumental values obtained with the Aminco-Bowman spectrophotofluorimeter with an uncalibrated photomultiplier I.P. 28 and 1/16 in. slits at positions 2, 5, 7 at maximum sensitivity.

Fig. 2. Optimum pH for enzymic hydroxylation of coumarin. Coumarin ($1.0\mu\text{mole}$) was incubated with 1.0ml. of $10000g$ supernatant preparation of rabbit-liver homogenate (containing $10\mu\text{moles}$ of nicotinamide), $6\mu\text{moles}$ of glucose 6-phosphate, $0.25\mu\text{mole}$ of NADP and 0.5ml. of 0.1M -sodium phosphate (○) or 0.05M -tris-HCl (●) buffers of different pH values in a total volume of 4.5ml. of 1.15% KCl at 37° in air for 0.5hr. Results are the mean of two experiments (s.e.m. 0.02).

Fig. 3. Effect of temperature on the enzymic hydroxylation of coumarin. Coumarin ($1.0\mu\text{mole}$) was incubated with 1.0ml. of $10000g$ supernatant preparation of rabbit-liver homogenate (containing $10\mu\text{moles}$ of nicotinamide), 0.5ml. of 0.1M -phosphate buffer, pH 7.4 , $6\mu\text{moles}$ of glucose 6-phosphate and $0.25\mu\text{mole}$ of NADP in a total volume of 3.0ml. of 1.15% KCl for 15min. in air at different temperatures. Results are the mean of two experiments (s.e.m. 0.01).

The optimum temperature was $35\text{--}37^\circ$ (see Fig. 3). The percentage of coumarin hydroxylated was maximal when the substrate concentration was $33\mu\text{M}$ but maximum yields of umbelliferone were obtained when the substrate concentration was $176\mu\text{M}$ (Fig. 4). Therefore, $1.0\mu\text{mole}$ of coumarin/incubation mixture ($330\mu\text{M}$) was used in the standard procedure. The amount of umbelliferone produced by the rabbit-liver preparation was proportional to the weight of the preparation. It

Fig. 4. Effect of substrate concentration on the enzymic hydroxylation of coumarin. The $10000g$ supernatant preparation (1.0ml.) of rabbit-liver homogenate (1ml. containing $10\mu\text{moles}$ of nicotinamide) was incubated with 0.5ml. of 0.1M -phosphate buffer, pH 7.4 , $6\mu\text{moles}$ of glucose 6-phosphate, $0.25\mu\text{mole}$ of NADP and various amounts of coumarin in a final volume of 4.5ml. of 1.15% KCl at 37° in air for 0.5hr. ●, Total yield of umbelliferone; ○, percentage of coumarin hydroxylated. Results are the mean of two experiments (s.e.m. 0.02).

was also proportional to the period of incubation up to 30min. , after which time the rate of production of umbelliferone diminished approximately linearly. Most experiments were subsequently carried out with $1.0\mu\text{mole}$ of coumarin/incubation mixture with 0.5ml. of 0.1M -disodium hydrogen phosphate-sodium dihydrogen phosphate buffer, pH 7.4 , in air at 37° for periods of 15 or 30min. with shaking.

With freshly made preparations, the addition of nicotinamide or NADP increased the yield of umbelliferone by 100% or more (Table 3), but in the presence of both cofactors the increase was no more than 20% greater than with either cofactor alone. The addition of glucose 6-phosphate to fresh tissue preparations had no significant effect on the hydroxylation with or without added nicotinamide, NADP or both (Table 3), but was added in the standard procedure to maintain uniformity. If the $10000g$ supernatant was kept for 9 days at -12° , 95% of the hydroxylating activity was lost and only 15% of the original activity could be restored by adding nicotinamide at the time of incubation. However, if nicotinamide was added before storage or NADP added before incubation some $60\text{--}70\%$ of the original activity was retained. Glucose 6-phosphate was ineffective in restoring activity lost during storage. Nicotinamide was therefore always added to homogenates during preparation, and NADP was also added always to incubation mixtures.

Table 3. *Effect of addition of nicotinamide, nicotinamide-adenine dinucleotide phosphate and glucose 6-phosphate on the enzymic hydroxylation of coumarin*

The 10000g supernatant (1ml. containing 10 μ moles of nicotinamide) from the liver of English rabbits was incubated with 1.0 μ mole of coumarin, 0.5 ml. of 0.1M-phosphate buffer, pH 7.4, and the various cofactors in a total volume of 2.9 ml. of 1.15% KCl at 37° in air for 15 min. 'Fresh supernatant' was used 2 hr. after slaughter of the animal, and 'aged supernatant' was kept for 9 days at -12° before use. Nicotinamide was added during the preparation of the 10000g supernatant; NADP and glucose 6-phosphate were added just before incubation. The yields of umbelliferone are the mean values of duplicates which differed by not more than ± 0.02 .

Additions (μ moles)			Hydroxylation (μ moles/g. of liver/hr.)		
Nicotinamide	NADP	Glucose 6-phosphate	1*	2*	3†
Fresh 10000g supernatant					
0	0	0	0.30	0.14	0.17
0	0	6	0.34	0.17	0.18
0	0.25	0	0.57	0.73	0.33
0	0.25	6	0.61	0.87	0.33
10	0	0	0.56	0.90	0.30
10	0	6	0.61	0.87	0.33
10	0.25	0	0.67	1.10	0.37
10	0.25	6	0.68	1.07	0.36
Aged 10000g supernatant					
0	0	0	0.05		
0	0	6	0.05		
0	0.25	0	0.59		
0	0.25	6	0.60		
10	0	0	0.65	(0.14)‡	
10	0	6	0.68		
10	0.25	0	0.87		
10	0.25	6	0.86		

* Adult doe.

† 4-5-week-old buck.

‡ No nicotinamide was added during preparation of the 10000g supernatant, the 10 μ moles of nicotinamide being added immediately before incubation.

Table 4. *Subcellular distribution of coumarin-hydroxylating activity and the effects of reduced nicotinamide-adenine dinucleotides*

Tissue fractions equivalent to 0.25g. of liver/ml. were prepared from rabbit-liver homogenates (Chinchilla doe), nicotinamide (10 μ moles/ml.) being added during the preparation. The fractions (1ml.) were incubated with 0.5 ml. of 0.1M-phosphate buffer, pH 7.4, 1.0 μ mole of coumarin, 6 μ moles of glucose 6-phosphate, 2 μ moles of ATP and 10 μ moles of MgCl₂, in a total volume of 3.5 ml. of 1.15% KCl, at 37° for 0.5 hr. in air. Duplicate results are shown.

Fraction	Additions (μ moles)		Hydroxylation (μ mole/g. of liver/hr.)
	NADPH ₂	NADH ₂	
10000g supernatant	0	0	0.52, 0.52
Microsomal fraction	0	0	0.02, 0.02
Soluble fraction	0	0	0.00, 0.00
Microsomal fraction + soluble fraction	0	0	0.52, 0.52
Microsomal fraction	1.0	0	0.32, 0.32
	2.0	0	0.41, 0.41
	3.0	0	0.46, 0.43
	4.0	0	0.49, 0.48
	5.0	0	0.51, 0.48
	0	1.0	0.15, 0.14
	0	5.0	0.17, 0.16

Table 5. *Effect of inhibitors on the enzymic hydroxylation of coumarin*

The 10000g supernatant (1ml. containing 10 μ moles of nicotinamide) from liver homogenates (Chinchilla rabbit) was incubated with 1.0 μ mole of coumarin, 0.5 ml. of 0.1M-phosphate buffer, pH 7.4, 0.25 μ mole of NADP and 6.0 μ moles of glucose 6-phosphate and inhibitor, in a total volume of 3.0 ml. of 1.15% KCl for 15 min. in air at 37°. The results are the mean values of two experiments.

Inhibitor	Concn. of inhibitor (mM)	Inhibition (%)
p-Chloromercuribenzoate	0.15	27
SKF acid	0.4	34
SKF 525A	0.4	30
Lilly 7132	0.4	22
2,4-Dichlorophenol	0.85	33
Ascorbic acid	1.0	22
Potassium cyanide	1.0	11
2,2'-Bipyridyl	1.4	-14
D-Glucurone	24.0	68
Sodium arsenite	3.0	0
Sodium arsenate	3.0	0
Sodium fluoride	3.0	0

drug-metabolizing enzymes, it has a requirement for oxygen and NADPH₂.

Effect of inhibitors. The hydroxylation of coumarin was inhibited by some of the established inhibitors of microsomal drug-metabolizing enzymes

Subcellular distribution of the hydroxylating system. The whole homogenate was as active as the 10000g supernatant, but no activity was found in the deposit at 10000g. The separated microsomal and soluble fractions showed no activity but the addition of the soluble to the microsomal fraction restored full activity (Table 4). However, addition to the microsomal fraction of 5 μ moles of NADPH₂/incubation mixture also restored full activity, but 5 μ moles of NADH₂ restored only one-third of the original activity. Thus the coumarin-7-hydroxylating enzyme occurs in the microsomal fraction of liver and, like many other

(Table 5), namely SKF acid (diphenyl-*n*-propyl-acetic acid), SKF 525A (β -diethylaminoethyl diphenyl-*n*-propylacetate hydrochloride) and Lilly 7132 [2-(4'-chlorophenoxy)propane-2-carboxylic acid]. *p*-Chloromercuribenzoate was also an inhibitor. This compound also inhibits the microsomal enzyme systems that hydroxylate aromatic compounds such as acetanilide (Mitoma *et al.* 1956), benzpyrene (Conney, Miller & Miller, 1957), naphthalene (Booth & Boyland, 1958) and biphenyl (P. J. Creaven, D. V. Parke & R. T. Williams, unpublished work), and the observation suggests that the coumarin-hydroxylating enzyme requires free thiol groups for its activity. 2,4-Dichlorophenol, ascorbic acid, potassium cyanide and D-glucurone were also inhibitors to some extent. No inhibition was observed with sodium arsenate, arsenite or fluoride. With 2,2'-bipyridyl, a slight stimulation of the hydroxylation was found. Inhibition by 2,2'-bipyridyl is usually taken as an indication of a requirement for ferrous iron. It has been reported that 2,2'-bipyridyl does not inhibit the microsomal hydroxylation of benzpyrene (Conney *et al.* 1957), naphthalene (Booth & Boyland, 1958) and *N*-(2-fluorenyl)acetamide (Seal & Gutman, 1959). However, 2,2'-bipyridyl does inhibit the microsomal acetanilide hydroxylase of rabbit liver and the non-microsomal phenylalanine hydroxylase of rat liver (Mitoma, 1956). It also inhibits the rabbit-liver microsomal enzyme that hydroxylates biphenyl to 4-hydroxybiphenyl (Creaven, Parke & Williams, 1962, 1964, and unpublished work), which suggests that coumarin 7-hydroxylase is different from biphenyl 4-hydroxylase.

Species variation in the hydroxylation of coumarin. The rate of conversion of coumarin into umbelliferone by the 10000g supernatant liver homogenates from various species is shown in Table 6.

(a) Rabbit. Rabbit liver is a relatively good source of the enzyme system, and female liver is about twice as active as male in both Chinchilla and English rabbits. Whether a strain difference exists is uncertain for, although the values in Table 6 suggest that female English rabbit liver is more active than Chinchilla, the males of both breeds appear to be similar.

(b) Coypu, cat and pigeon. Livers from these species hydroxylate coumarin to umbelliferone. The 10000g supernatant of coypu liver was as active as rabbit liver. However, the whole liver homogenate of the coypu was inactive, which may be due to an inhibitor in the fraction containing the nuclei and mitochondria. This type of inhibition is discussed below in the section on rat liver. Cat and pigeon livers, however, had relatively poor activity.

(c) Guinea pig. The 10000g supernatant from this species gave relatively low yields of umbelliferone; for two guinea pigs the yields were 0.05

Table 6. 7-Hydroxylation of coumarin by various species

Enzyme source: 10000g supernatant of liver homogenates. Incubation: as for Table 5, except that the period of incubation was 30 min.

Species	Hydroxylation (μ moles/g. of liver/hr.)*
Rabbit	
Chinchilla	0.99 (0.68–1.27) (5 F); 0.54 (0.34–0.67) (3 M)
English	1.41 (0.80–2.10) (3 F); 0.51 (0.44–0.65) (3 M)
Coypu	1.3, 1.3 (2 F)
Cat	0.10 (1 F); 0.34, 0.28 (2 M)
Pigeon	0.13 (1 F); 0.08, 0.16 (2 M)
Guinea pig	0.38, 0.51 (2 M)†
Rat	
Wistar albino	0 (12 rats, M and F, young and adult)
Black-hooded	0 (3 M; 3 F)
Mouse	
White albino	0 (six pooled livers)
Locust	0 (15 pooled fat bodies)

* Ranges where three or more animals were used are in parentheses; F and M, female and male.

† After acid hydrolysis of incubation mixtures; see text.

and 0.10 μ mole/g. of liver/hr. respectively. However, if the incubation mixtures were heated under reflux for 1.5 hr. with 3 ml. of 10N-hydrochloric acid the yields of umbelliferone were 0.38 and 0.51 μ mole. It appeared that the umbelliferone produced was being converted into a non-fluorescent form which was reconverted into umbelliferone by acid hydrolysis. When coumarin was incubated with rabbit preparation, as in the standard procedure, hydrolysis of the incubation mixture with acid did not increase the yield of umbelliferone.

(d) Rat. The ability to 7-hydroxylate coumarin could not be detected in any fractions of rat-liver homogenates (Wistar albino and black-hooded rats, male and female, adult, and young of 4 weeks old). This was not due to conjugation of umbelliferone, for hydrolysis of the incubation mixtures did not yield umbelliferone, and, further, umbelliferone itself was not readily metabolized by the 10000g supernatant of rat liver. The cofactors necessary for coumarin hydroxylation could be detected in rat liver, but the microsomal fraction contained no coumarin-hydroxylating enzyme. The soluble fraction from rat liver plus the microsomal fraction from rabbit liver hydroxylated coumarin, but the soluble fraction of rabbit liver plus the microsomal fraction from rat liver did not (Table 7).

Addition of rat-liver homogenates to rabbit-liver preparations showed that rat-liver homogenates contain an inhibitor of coumarin hydroxylation

Table 7. *Hydroxylation of coumarin by rat- and rabbit-liver fractions*

Microsomal fractions of rat or rabbit liver (1ml. = 250 mg. of liver and containing 10 μ moles of nicotinamide) were incubated with soluble fractions of rat or rabbit liver (1ml. = 250 mg. of liver) and coumarin (1 μ mole in 1ml. of 1.15% KCl), 0.1M-sodium phosphate buffer, pH 7.4 (0.5 ml.), nicotinamide (8 μ moles), NADP (0.25 μ mole), $MgCl_2$ (2.5 μ moles) and glucose 6-phosphate (6 μ moles) in a total volume of 4ml. for 30 min. at 37°. +, Fractions used. Incubation mixtures were set up in duplicate.

Rabbit liver*		Rat liver†		Hydroxylation (μ mole/g. of liver/hr.)
Microsomal	Soluble	Microsomal	Soluble	
+	+	—	—	0.60, 0.57
+	—	—	+	0.23, 0.26
—	+	+	—	0, 0
—	—	+	+	0, 0

* Adult female Chinchilla.

† Adult male Wistar albino.

Table 8. *Inhibition by rat liver of coumarin hydroxylation by a rabbit-liver preparation*

The 10000g supernatant of rabbit liver, with buffer and cofactors as given in Table 5, was incubated with various fractions of rat-liver homogenates (1ml. = 250 mg. of liver).

Rat-liver fraction added	Inhibition of coumarin hydroxylation* (%)		
	Expt. no....1	2	3
None (1ml. of 1.15% KCl)	0	0	0†
Whole homogenate	75	90	90
10000g supernatant	0	43	41
10000g deposit	81	90	—
18000g supernatant	—	—	43
Boiled whole homogenate	0	0	0†

* Values quoted are averages for duplicate or triplicate incubations and the variation was $\pm 2\%$.

† The umbelliferone produced was the same in both cases and in this particular experiment, for example, the umbelliferone produced was 1.26 when nothing was added and 1.27 μ moles/g. of liver/hr. when boiled whole homogenate was added.

(Table 8). The inhibitory material occurred mainly in the deposit at 10000g of rat liver, i.e. the fraction containing debris, nuclei and mitochondria. The inhibitory effect was lost when this fraction was heated in boiling water for 1min. Similar inhibitory material was found in the liver of coypu, cat and a fox, the 10000g supernatants of which can hydroxylate coumarin. Axelrod (1956) has found that inhibitors of the enzymic cleavage of aromatic ethers by rabbit liver occur in the nuclei and

Table 9. *Inhibition of umbelliferone metabolism in guinea-pig liver*

The 10000g supernatant of guinea-pig liver (1ml. containing 10 μ moles of nicotinamide), umbelliferone (42.5 μ g. in 1ml. of 1.15% KCl), 0.1M-phosphate buffer, pH 7.4 (0.5 ml.), inhibitor (0.5 ml. in 1.15% KCl), NADP (0.25 μ mole) and glucose 6-phosphate (6 μ moles) were incubated in a final volume of 3.8 ml. for 15 min. at 37°.

Inhibitor	Concn. of inhibitor (mm)	Inhibition (%)
<i>p</i> -Chloromercuribenzoate	0.1	22
Lilly 7132	1.0	16
2,2'-Bipyridyl	1.5	0
Rat liver*	1ml.	67
Rat liver* boiled	1ml.	0

* The deposit at 10000g resuspended in its original volume of 1.15% KCl (1ml. = 250 mg. of liver).

mitochondria of the liver of guinea pig, mouse, rat and dog.

(e) Mice and other species. The ability to hydroxylate coumarin was absent from mouse liver, from the liver of one ferret and from the fat body of the locust, which is able to carry out some transformations of foreign compounds (Smith, 1962). Hydroxylating activity of a low order was found in the 10000g supernatant of hen liver (two), hamster (one) and frog (six pooled livers).

Metabolism of umbelliferone in homogenates. The fate of umbelliferone in homogenates was investigated with the 10000g supernatants of guinea-pig, rabbit, rat and mouse liver. The incubations were carried out as described for coumarin except that coumarin was replaced by umbelliferone (12 μ g.). In the rabbit, rat and mouse preparations 30, 32 and 30% respectively of the umbelliferone disappeared in 30 min., but with guinea pig 70% disappeared in 5 min. and 100% in 30 min. If the preparations were heated, umbelliferone did not disappear. The process therefore appears to be enzymic. Further, all the umbelliferone that disappeared from the guinea-pig-liver incubation mixture could be recovered by refluxing the incubation mixture (2.5 ml.) with 2 ml. of 10N-hydrochloric acid for 20 min. The disappearance of umbelliferone in guinea-pig preparations was inhibited by *p*-chloromercuribenzoate, Lilly 7132 and the 10000g deposit of rat-liver homogenates (i.e. the nuclei and mitochondria), but not by 2,2'-bipyridyl (Table 9). These results suggest that umbelliferone is rapidly conjugated in the guinea-pig preparation. That umbelliferone is converted into umbelliferone glucuronide was shown by the following experiment. Umbelliferone (25 μ g. in

1ml. of 1.15% potassium chloride) and 0.1M-phosphate buffer, pH 7.4 (0.5ml.), were incubated with the 10000g supernatant of guinea-pig-liver homogenates (1ml.) at 37° for 30min. All the umbelliferone disappeared. After incubation the mixture was heated at 100°, to destroy any tissue β -glucuronidase, and centrifuged. The supernatant was then treated with 100000 Fishman units (Talalay, Fishman & Huggins, 1946) of β -glucuronidase (mollusc; L. Light and Co. Ltd., Colnbrook, Bucks.) and acetate buffer, pH 4.8, to a final concentration of 1mM, and incubated at 37° for 60min. About 60% of the original umbelliferone could be recovered by this procedure.

The enzyme system causing the disappearance of umbelliferone was present in the 10000g supernatant of guinea-pig liver. Isolated microsomes and soluble fraction were separately inactive, but when they were mixed activity was restored.

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