*Biochemical Pharmacology*, Vol. 31, No. 24, pp. 4005–4009, 1982.
Printed in Great Britain.

0006-2952/82/244005-05 $03.00/0
Pergamon Press Ltd.

# THE METABOLISM OF 7-ETHOXYCOUMARIN AND 7-HYDROXYCOUMARIN BY RAT AND HAIRLESS MOUSE SKIN STRIPS

STEPHEN J. MOLONEY,*† JOHN M. FROMSON‡ and JAMES W. BRIDGES*

*Robens Institute of Industrial and Environmental Health and Safety and Department of Biochemistry, University of Surrey, Guildford GU2 5XH, U.K.; and ‡Hoechst Pharmaceuticals Ltd., Walton Manor, Milton Keynes, U.K.

(*Received* 15 *March* 1982; *accepted* 28 *June* 1982)

**Abstract**—The metabolism of 7-ethoxycoumarin and 7-hydroxycoumarin was studied in rat and hairless mouse skin strips. These preparations supported de-ethylation, sulphation and glucuronidation reactions. The de-ethylation reaction was inducible in both species by pretreatment with either 5,6-benzoflavone or 3-methylcholanthrene. The hairless mouse strips exhibited a greater basal de-ethylase activity than rat strips, although the latter was the more responsive to inducers. Accompanying the increase in de-ethylation activity was a change in the pattern of metabolites, with a large increase in the percentage of the unconjugated metabolite. When 7-hydroxycoumarin was employed as the primary substrate the glucuronide was the major metabolite formed by strips from both species. The glucuronidation and sulphation activities were unchanged by 3-methylcholanthrene pretreatment.

Most conjugation reactions result in the elimination or detoxification of foreign compounds, although in some cases the conjugates are more toxic than their parent compounds [1]. The presence of conjugating enzymes and their interrelationships with other drug metabolizing enzymes are, therefore, important in the investigation of metabolism and toxicity of foreign compounds. Cutaneous drug metabolism has received relatively scant attention [2, 3]. The skin is continually exposed to xenobiotics yet very few reports have considered the xenobiotic metabolizing activity of the skin. The glucuronide of benzo(α)pyrene metabolites have been extracted from mouse skin homogenates after topical application of benzo(α)pyrene and 3-hydroxybenzo(α)pyrene [4]. Also, skin strips from mice and guinea-pigs have been reported to form the glucuronic acid conjugates of 2-aminophenol [5], and epithelial skin cells in culture to form the glucuronides of 4-nitrophenol and 4-aminophenol [6]. Less is known about sulphation of foreign compounds in skin. Trace amounts of what was thought to be sulphated benzo(α)pyrene metabolite have been extracted from mouse skin, but the product was not unequivocally identified [4]. However, the sulphation of the steroid dehydroepiandrosterone [7] by human skin has been reported.

This paper details an investigation of the de-ethylation of 7-ethoxycoumarin and subsequent conjugation of the product, 7-hydroxycoumarin, in skin strips, and the effect of inducers on the pattern of metabolites produced.

## MATERIALS AND METHODS

*Chemicals.* 7-Ethoxycoumarin was prepared according to the method of Ullrich and Weber [8], and was recrystallized three times from aq. methanol before use. Ketodase, a sulphatase-free β-glucuronidase preparation, was obtained from General Diagnostics (Eastleigh, U.K.). Sulphatase, type H1, was obtained from Sigma (Poole, U.K.). Leibovitz L15 medium and foetal calf serum were obtained from Gibco Biocult (Paisley, U.K.) and 4-methylumbelliferyl-β-D-glucuronide and 4-methylumbelliferyl sulphate from Koch Light (Colnbrook). 7-Hydroxycoumarin was obtained from Fluka, A.G. (Switzerland).

All other chemicals were laboratory grade or analytical grade where available.

*Animal and dosage regimens.* Male Wistar rats, University of Surrey strain, and male hairless mice were used, both at an age of 45 days at which time both species were in a growth phase of their hair cycles. All animals were allowed water and a standard laboratory diet *ad libitum*.

Intraperitoneal pretreatment regimens were for 3-methylcholanthrene, 20 mg/kg for 3 days and for 5,6-benzoflavone, 50 mg/kg for 3 days, both dissolved in corn oil. Controls received corn oil alone. Topical application of 5,6-benzoflavone was achieved using acetone as a vehicle. The hair on the mid-dorsal area of rats was clipped (Oster Clippers, Allbrooks, Rickmansworth, U.K.) before treatment. An area of approx. 24 $cm^2$ in the mid-dorsal region was used for application of each dose. One mg 5,6-benzoflavone in 0.5 ml acetone daily for 3 days was applied using a syringe and the animals were held until the acetone was dry. After topical applications the animals were housed separately. Animals were sacrificed by cervical dislocation 24 hr after the last topical or intraperitoneal administration.

*Preparation of strips and incubation conditions.* If not already clipped the hair from the backs of the rats was clipped before preparation of strips. After

† Present address: Department of Biochemistry, South Western Medical School, 5323 Harry Hines Boulevard, Dallas, Texas, U.S.A.



BP 31:24 - E

excision of the skin from the mid-dorsal region of the back, any loosely attached fat was removed from the dermal side and the skin placed into ice-cold L15 medium containing 10% v/v foetal calf serum. The strips were prepared using a block of 30 commercially available razor blades (previously degreased with ethanol and acetone) spaced 1 mm apart. The skin was placed on the blades and covered on the upper surface with a thick sheet of plastic. Pressure was gently applied to the plastic resulting in the skin being cut into strips by the blades. To ensure sharpness the blades were replaced after producing strips from three animals. The strips were returned to ice-cold medium until used for the incubation (always less than 10 min). Incubations were carried out in 10 ml open flasks containing 2 ml of a L15 medium with 10% v/v foetal calf serum at 37° in a shaking water bath. Under these conditions activities were not enhanced by incubation under an oxygen atm. and activities were linear in relation to the amount of skin for hairless mouse strips for tissue equivalent up to 60 mg dry wt/2 ml and for rat strips for tissue equivalent up to 40 mg dry wt/2 ml. Viability was shown both by the linear metabolism and by linear oxygen uptake, measured by a Gilson respirometer, over 1 hr, the longest incubation time employed.

*Assays.* The 7-ethoxycoumarin metabolites, free 7-hydroxycoumarin and its glucuronic acid and sulphate conjugates, were assayed using a sequential enzymic hydrolysis. The initial 7-ethoxycoumarin concn was 70 $\mu$M. After the appropriate incubation period at 37° in a shaking water bath the strips were removed and frozen. Two 0.75 ml aliquots of medium were taken and extracted with 6 ml hexane to remove remaining 7-ethoxycoumarin. The medium was then extracted into 4 ml ether. A 2 ml aliquot of ether extract was back extracted with 5 ml 0.2 M glycine–NaOH buffer, pH 10.6 and the fluorescence of 7-hydroxycoumarin in the aq. phase read at $\lambda$ex 370 nm and $\lambda$em 450 nm in a Perkin-Elmer MPF-4 fluorimeter. Glucuronic acid conjugates were hydrolysed with Ketodase. The 0.75 ml aliquots of L15 medium remaining after ether extraction were mixed with 0.5 ml Ketodase (a sulphatase-free glucuronidase preparation) and after adjusting the pH to 4.5 were incubated for 15 hr. The samples were then extracted with ether and the 7-hydroxycoumarin assayed. Sulphates were cleaved using arylsulphatase. To the Ketodase incubation was added 5 mg sulphatase in 0.5 ml of 0.2 M acetate buffer, pH 4.5, which was incubated for a further 15 hr. The samples were then treated as for the Ketodase incubation. Because of the low levels of sulphate conjugate observed, the possibility of interference by a carry-over of non-extracted 7-hydroxycoumarin from the Ketodase incubation was checked by comparison of this sequential method with incubation of medium, after the initial removal of free metabolite, with sulphatase and 20 mM glucaro acid 1,4 lactone (a $\beta$-glucuronidase inhibitor). Identical results were obtained. Standards of 7-hydroxycoumarin were taken through each incubation and extraction procedure. The strips that were frozen were freeze-dried and the dry wt recorded.

7-Hydroxycoumarin metabolism was assayed using a substrate concn of 70 $\mu$M. After the appropriate incubation period at 37° the strips were removed and processed as described previously. Two 0.75 ml aliquots of medium were taken and extracted three times with 6 ml ether to remove any remaining 7-hydroxycoumarin. The samples were then subjected to sequential Ketodase and sulphatase incubations as described previously for the assay of 7-ethoxycoumarin metabolites.

Possible leakage of sulphatase and $\beta$-glucuronidase activity from skin lysosomes with consequent hydrolysis of conjugates in the medium, was monitored using 4-methylumbelliferyl-$\beta$-D-glucuronide and 4-methylumbelliferyl sulphate as substrates. Either substrate was added to medium that had been preincubated with rat or hairless mouse strips for 1 hr and then the strips removed. Samples were run at both the pH of the metabolic incubations, pH 7.4, and at pH 4.5. Substrate concns used matched the amount of the relevant conjugate formed during the metabolic studies by either rat or hairless mouse strips, depending upon which type of skin the medium had been preincubated with. 4-Methylumbelliferone production was measured at hourly intervals up to 4 hr by the same assay procedure previously described for 7-hydroxycoumarin.

All activities were expressed as dry wt because accurate measurement of wet wt before the assay would demand undesirable manipulation and delay, while measurement after the assay was found to be unsatisfactory as the wet wt of the strips increased slightly with incubation time. (NB: 4 $cm^2$ rat skin or 9–10 $cm^2$ mouse skin $\simeq$ 100 mg dry wt skin $\simeq$ 1.5–2.0 mg microsomal protein.)

## RESULTS

The production of metabolites of 7-ethoxycoumarin by skin strips from untreated hairless mice is shown in Fig. 1. The glucuronide was the major metabolite with similar smaller amounts of free and sulphated 7-hydroxycoumarin being formed. When skin strips from untreated rats were used after 1 hr

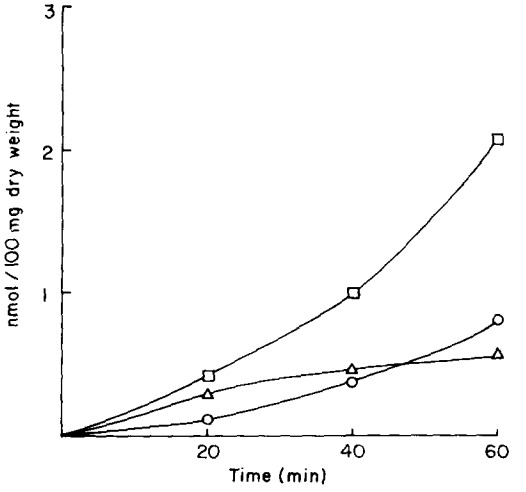


Fig. 1. Pattern of metabolism of 7-ethoxycoumarin by hairless mouse skin strips. Activities represent mean of three experiments. (△) Free 7-hydroxycoumarin; (○) sulphate; (□) glucuronide.

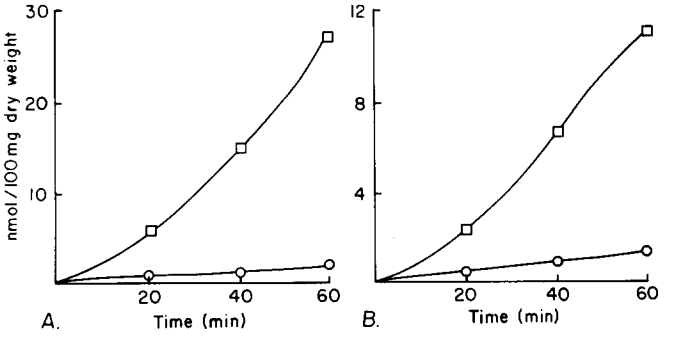


Fig. 2. Pattern of metabolism of 7-hydroxycoumarin by hairless mouse and rat skin strips. (A) Hairless mouse; (B) rat. Activities represent mean of three experiments. (○) sulphate; (□) glucuronide.

incubation with 7-ethoxycoumarin only the glucuronide was detected, the rate of formation of which was 0.11 ± 0.03 nmoles/100 mg dry wt/hr ($n = 3$). The total amount of metabolism after 1 hr incubation was 34 times greater in the hairless mouse skin strips than in the rat strips.

In contrast, when 7-hydroxycoumarin was incubated with strips from either species the patterns of metabolism were much more comparable as shown in Fig. 2. After 1 hr incubation the percentages of the total metabolites as the two conjugates were, in the rat 89% glucuronide and 11% sulphate, and in the hairless mouse 93% glucuronide and 7% sulphate. The amount of conjugates formed from 7-hydroxycoumarin by the hairless mouse skin strips was 1.7 times greater than in the rat strips.

Table 1 shows the effect of various *in vivo* pretreatments on total 7-ethoxycoumarin metabolism in skin strips. The rat exhibited the greater responsiveness to the compounds used. Intraperitoneal administration of 5,6-benzoflavone resulted in the greatest induction in the rat with topical administration of 5,6-benzoflavone and intraperitoneal administration of 3-methylcholanthrene being similar in effect. Hairless mouse skin strips showed a different pattern of induction with 5,6-benzoflavone when comparing the two routes of administration. Topical application was more effective than the intraperitoneal route. 3-Methylcholanthrene pretreatment resulted in the smallest increase.

Because of the greater responsiveness of the rat, induction dramatically decreased the difference between the abilities of the skin strips from the two species to de-ethylate 7-ethoxycoumarin. Activity in the hairless mouse strips was only two-fold greater than in rat strips after intraperitoneal 5,6-benzoflavone treatment and seven-fold greater after topical administration of the same compound.

Figure 3 shows the results of an investigation of the nature of the metabolites formed by rat skin strips after intraperitoneal administration of 5,6-benzoflavone. Major differences were observed when compared to the metabolite pattern in untreated rats, in which only the glucuronide was detectable. After induction, 7-hydroxycoumarin contributed the largest proportion of the total metabolites after 1 hr incubation (42%), while the glucuronide and sulphate accounted for 38% and 20%, respectively.

Differences in the metabolite pattern were also apparent after intraperitoneal administration of 5,6-benzoflavone to hairless mice, as shown in Fig. 4. As in the rat strips the percentage of free metabolite detected was much greater than in untreated animals. A similar pattern of metabolites was also obtained after topical pretreatment of hairless mice with 5,6-benzoflavone (data not shown). Therefore, administration of 5,6-benzoflavone leads to both qualitative and quantitative changes in the pattern of 7-ethoxycoumarin metabolites formed by rat and hairless mouse skin strips.

Sulphatase and β-glucuronidase activity was measured in medium that had been incubated with either rat or hairless mouse skin strips to examine whether the higher percentage of free metabolites found after induction could be an artifact due to an increased release of these enzymes from skin strips into the

Table 1. Induction of 7-ethoxycoumarin de-ethylase in skin strips

| Route of administration | Pretreatment | Rat (nmoles of 7-hydroxy-coumarin formed 100 mg dry wt/hr) | Percentage of control | Hairless mouse (nmoles of 7-hydroxy-coumarin formed/ 100 mg dry wt/hr) | Percentage of control |
|---|---|---|---|---|---|
| Intraperitoneal | Corn oil | 0.18 ± 0.02 | — | 2.87 ± 0.24 | — |
| | 5,6-Benzoflavone | 6.01 ± 0.41* | 3340 | 12.24 ± 3.01‡ | 430 |
| | 3-Methylcholanthrene | 4.32 ± 0.39* | 2400 | 5.72 ± 0.64† | 200 |
| Topical | Acetone | 0.13 ± 0.03 | — | 2.55 ± 0.42 | — |
| | 5,6-Benzoflavone | 3.80 ± 0.54* | 2920 | 25.10 ± 3.96* | 980 |

Activities represent the total metabolites (7-hydroxycoumarin and conjugated 7-hydroxycoumarin) and are expressed as means ± S.D. of three experiments.
*$P < 0.001$; † $< 0.005$; ‡ $< 0.01$.

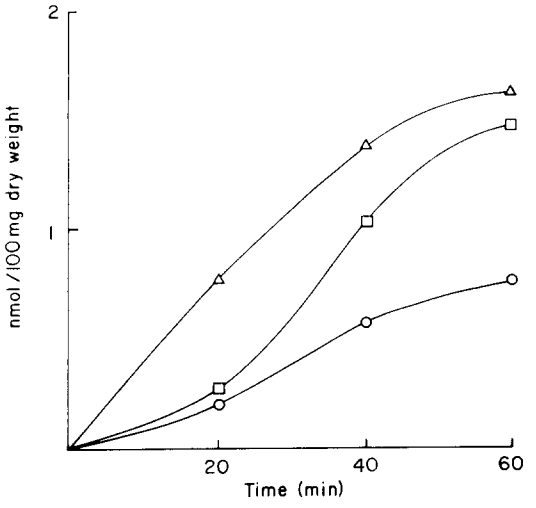


Fig. 3. Pattern of metabolism of 7-ethoxycoumarin by rat skin strips from animals induced by intraperitoneal administration of 5,6-benzoflavone. Activities represent means of three experiments. (△) Free 7-hydroxycoumarin; (○) sulphate; (□) glucuronide.

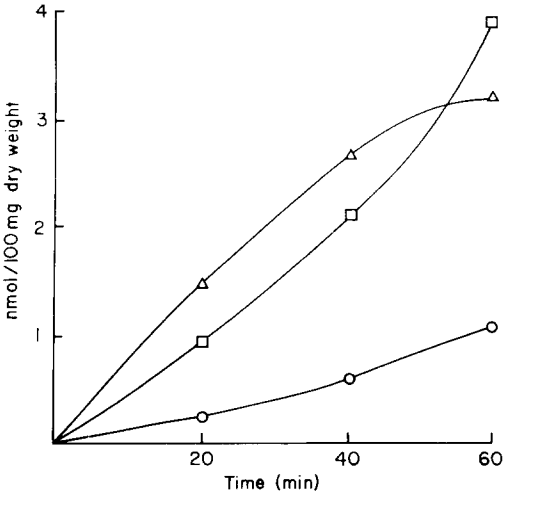


Fig. 4. Pattern of metabolism of 7-ethoxycoumarin by hairless mouse skin strips from animals induced by intraperitoneal administration of 5,6-benzoflavone. Activities represent means of three experiments. (△) Free 7-hydroxycoumarin; (○) sulphate; (□) glucuronide.

medium. No sulphatase activity could be detected. β-Glucuronidase activity was present, but it was only detectable when the medium was adjusted to pH 4.5, and longer time periods than those used in the assays were employed. No difference between control and pretreated animals could be detected.

3-Methylcholanthrene pretreatment had no detectable effect on the metabolism of 7-hydroxycoumarin with the amounts of sulphate and glucuronic acid conjugates formed being the same in pretreated and control rats and hairless mice skin strips (Table 2).

## DISCUSSION

The ability to de-ethylate 7-ethoxycoumarin was demonstrated in both rat and hairless mouse skin strips. Several differences were observed between the de-ethylating activities of the two species. These differences were in basal rate, responsiveness to inducers and effectiveness of routes of administration of inducers. These results are in good agreement with results obtained for 7-ethoxycoumarin de-ethylation in skin microsomal fractions prepared from these two species [9]. A discrepancy exists, however, between the data from hairless mouse strips and microsomes in the extent of induction expressed in these two preparations. The extent of induction over control values by topical pretreatment of 5,6-benzoflavone is approx three-fold greater in strips than that found in microsomes [9]. Furthermore, pretreatment with 3-methylcholanthrene did not change the activity in microsomes [9], but in strips resulted in a two-fold increase in activity.

An exact comparison of induction between rat skin strips and microsomes cannot be made due to the fact that control levels of 7-ethoxycoumarin de-ethylase in rat skin microsomes were non-detectable [9]. However, the extent of induction of de-ethylation reported in rat skin microsomes [9] over the level of detectability of the assay would make it appear that in the rat there is not a comparable discrepancy in induction to that seen in the hairless mouse skin strips and microsomes.

This apparent lack of induction in hairless mouse microsomes could be due to a number of reasons, such as loss of some factor(s) needed for full expression of activity or destruction of induced cytochrome

Table 2. Effect of intraperitoneal administration of 3-methylcholanthrene on the conjugation of 7-hydroxycoumarin in skin strips

| Species | Pretreatment | Glucuronide formation (nmoles/100 mg dry wt/hr) | Sulphate formation (nmoles/100 mg dry wt/hr) |
|---|---|---|---|
| Hairless mouse | Corn oil | 26.6 ± 5.8 | 0.9 ± 0.2 |
| | 3-Methylcholanthrene | 24.8 ± 2.3 | 1.2 ± 0.4 |
| Rat | Corn oil | 16.6 ± 2.9 | 1.3 ± 0.2 |
| | 3-Methylcholanthrene | 14.6 ± 2.8 | 1.1 ± 0.2 |

Results are expressed as means ± S.D. of three experiments.

P-450 during the subfractionation procedure. Further research is needed to clarify this discrepancy between the two preparations. The greater extent of induction in strips might be expected to reflect more closely the *in vivo* situation.

In both rat and hairless mouse skin strips, 7-hydroxycoumarin, whether produced by oxidative metabolism of 7-ethoxycoumarin or added directly, was metabolized to both the sulphate or glucuronic acid conjugates. The latter was the major metabolite. Both activities were unchanged by pretreatment with 3-methylcholanthrene. This is in contrast to a previous report where topical application of benzo($\alpha$)pyrene increased glucuronidation of 2-aminophenol [5] in mouse skin strips.

Following induction of 7-ethoxycoumarin metabolism in strips, unconjugated 7-hydroxycoumarin constituted a much larger percentage of the metabolites formed when compared to incubations with non-pretreated strips. This was not due to the inability of strips to conjugate 7-hydroxycoumarin, as seen from the data obtained when 7-hydroxycoumarin was used as the initial substrate. Increases in the percentage of free metabolites have also been reported during 7-ethoxycoumarin metabolism in isolated rat kidney tubules after 3-methylcholanthrene induction [10] and for 4-hydroxybiphenyl metabolism in isolated rat hepatocytes after phenobarbitone or 3-methylcholanthrene induction [11]. However, in all these cases the increase was transitory and with time the free metabolite decreased with a concomitant increase in the glucuronic acid conjugate. This has been ascribed to the free metabolite activating glucuronyltransferase [12]. In skin strips the increase was not transitory and in all cases after induction the free metabolite concn was still rising, albeit slowly, after 1 hr incubation. This may be due to the low de-ethylation activity in skin being insufficient to produce a high enough concn of metabolite to activate the glucuronyltransferase. If the large increase in unconjugated metabolite after induction in skin reflects the *in vivo* situation, it may well be of significance in the toxicity of foreign compounds to skin which is in an induced state.

The difference between the two species in the efficiency of the two routes of administration of 5,6-benzoflavone on induction, that is intraperitoneal administration giving rise to the greatest induction in rat skin and topical application giving rise to the greatest induction in hairless mouse skin, has been described and discussed for skin microsomal preparations from these animals [9] where a similar phenomenon was observed. The reason for the difference is unknown but availability of the inducer and distribution of the de-ethylating activity in the skin are thought likely to be contributing factors.

*Acknowledgement*—This work was supported by an SERC–CASE Studentship with Hoechst Pharmaceuticals Ltd.

## REFERENCES

1. G. L. Mulder, J. A. Hinson and J. R. Gillette, *Biochem. Pharmac.* **26**, 189 (1977).
2. A. Poland and E. Glover, *J. biol. Chem.* **249**, 5599 (1974).
3. R. S. Pohl, R. M. Philpot and J. R. Fouts, *Drug Metab. Dispos.* **4**, 442 (1976).
4. I. H. Stevenson and G. J. Dutton, *Biochem. J.* **77**, 19 (1960).
5. G. J. Dutton and I. H. Stevenson, *Biochim. biophys. Acta.* **58**, 633 (1962).
6. H. E. Rugstad and E. Dybing, *Eur. J. clin. Invest.* **5**, 133 (1975).
7. D. L. Berliner, J. R. Pasqualini and A. J. Gallegos, *J. invest. Derm.* **50**, 222 (1968).
8. V. Ullrich and P. Weber, *Hoppe-Seyler's Z. physiol. Chem.* **353**, 1171 (1972).
9. S. J. Moloney, J. M. Fronson and J. W. Bridges, *Biochem. Pharmac.* **31**, 4011 (1982).
10. J. W. Bridges and J. R. Fry, in *The Induction of Drug Metabolism* (eds. Estabrook and Lindenlaub), p. 343 Schallauer, Stuttgart (1978).
11. P. Wiebkin, J. R. Fry, C. Jones, R. Lowing and J. W. Bridges, *Biochem. Pharmac.* **27**, 1899 (1978).
12. J. R. Fry and J. W. Bridges, in *Progress in Drug Metabolism* (eds. J. W. Bridges and L. F. Chasseaud), Vol. 2, pp. 71–118. Wiley, Chichester (1977).