

Inheritance of Scottish-type resistance to warfarin in the Norway rat

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SUMMARY

The inheritance of resistance to the rodenticide, warfarin, in the Norway rat, *Rattus norvegicus*, derived from a wild rat population in Scotland was studied in the backcross, intercross and testcross. The resistance was found to be due to a major gene with about the same map position in Linkage Group I as the warfarin-resistance gene, *Rw*², which occurs in the wild rat population in Wales. In heterozygotes, the Scottish resistance gene, unlike the Welsh gene, is incompletely penetrant in expression, though the penetrance was found to increase markedly in response to selection. Differences between the Scottish and Welsh types of resistance suggest that the two resistance genes are allelic.

1. INTRODUCTION

Resistance to the rodenticide warfarin in wild populations of the Norway rat, *Rattus norvegicus*, has developed, apparently independently, in Scotland (Boyle, 1960), Denmark (Lund, 1964), Wales (Drummond & Bentley, 1967), Holland (Ophof & Langeveld, 1969) and the U.S.A. (Brothers, 1972; Jackson and Kaukeinen, 1972; Brookes & Bowerman, 1973). Warfarin resistance in the Welsh rat population has been shown to be due to an autosomal gene with dominant effect (Greaves & Ayres, 1967; Pool, O'Reilly, Schneiderman & Alexander, 1968), which has been mapped in Linkage Group I (Greaves & Ayres, 1969*a*). In other species a major warfarin-resistance gene, *War*, has been found in the analogous chromosome in the House mouse, *Mus musculus* (Wallace & MacSwiney, 1976), while in man a physiologically similar form of monogenic resistance to therapeutic doses of warfarin has been reported by O'Reilly (1971).

Warfarin acts by inhibiting vitamin K oxide reductase, thereby blocking the biosynthesis of blood clotting factors of the prothrombin complex. Welsh-type warfarin resistance in rats appears to involve an alteration in the reductase such that it is less sensitive to inhibition by warfarin (Bell & Caldwell, 1973), as a result of which, however, the resistant rats have an increased requirement for vitamin K (Hermodson, Suttie & Link, 1969). Studies of the vitamin K requirement of various strains of rats, in which genetical aspects were touched upon, show that Scottish-type resistant rats are intermediate between Welsh-type resistants and susceptibles in their vitamin K requirement (Greaves & Ayres, 1973; Martin, 1973).

Breeding experiments made at the University of Liverpool by D. P. Evans and P. M. Sheppard suggested that Scottish-type resistance to warfarin could be due to an autosomal gene, perhaps with incomplete dominance; owing to their limited nature, the data from these experiments, though circulated informally, were never published. However, their work provided the starting point for the study reported here, which clearly demonstrates unifactorial inheritance of Scottish-type resistance in rats.

2. METHODS

Two warfarin-resistant male *Rattus norvegicus* used to start the breeding experiment were supplied by the University of Liverpool. These animals were the offspring of a cross between warfarin-susceptible laboratory albinos and wild-type resistant rats, the latter being the offspring, born in captivity, of resistant rats caught on a farm (grid reference 766734) in Scotland close to the site from which resistance was first reported (Boyle, 1960). Warfarin-susceptible rats were drawn from the Wistar colony which had been maintained in this Laboratory, with a substantial though unspecified degree of inbreeding for approximately 20 years. All animals were maintained in conventional wire cages, with water and diet 41 B supplied in surplus.

The breeding experiment was carried out in three stages. First, resistant rats were backcrossed to the susceptible Wistar strain for four successive generations. Second, ten pairs of fourth-generation resistant rats were intercrossed. Third, the resistant offspring of the intercross were testcrossed, by backcrossing to the Wistar strain. All matings after the first backcross were monogamous. In each generation the offspring were screened for resistance at the age of 4 months by feeding them for 6 days on medium oatmeal containing 0.005% warfarin. This resistance screening treatment, which was originated by Drummond & Wilson (1968), was believed at first to be lethal to virtually all warfarin-susceptible rats, on the basis of results obtained with wild rats. However, a check made during the study showed the warfarin diet to be significantly less toxic to Wistar than to wild rats, and indicated that an estimated 6.5% of normally susceptible Wistar rats could be expected to survive the 6-day feeding period. This fact was taken into account in examining deviations from Mendelian ratios in the breeding experiment. Following the breeding experiment, a strain homozygous for the Scottish resistance gene on a Wistar background was founded.

Resistance to three anticoagulant rodenticides – warfarin, coumatetralyl and diphacinone – was compared in the homozygous strain, in an analogous strain which is homozygous for the Welsh-derived resistance gene, *Rw*², and in the Wistar strain. Coumatetralyl, like warfarin, is a derivative of 4-hydroxycoumarin, but is relatively active against warfarin-resistant rats (Greaves & Ayres, 1969*b*). Diphacinone is a highly active member of the related indane-1,3-dione series of anticoagulants. The compounds were administered subcutaneously, as a single dose in an aqueous solution of 5% powdered acacia BP, and the animals were kept under observation for three weeks, during which mortality was recorded.

Technical-grade anticoagulants were employed in all experiments.

3. RESULTS

In the screening test for resistance the consumption of food, and therefore of warfarin, was bimodally distributed (Table 1). Animals that died generally became anorectic within 2-5 days while the majority of survivors continued to eat normal amounts of the warfarin diet for the whole of the test. Thus, the dosage administered to each rat was controlled to some extent by its own tolerance. Females on average ate larger doses than males relative to their body weight, indicating, since there was no sex difference in mortality, that females had the greater tolerance for warfarin.

Table 1. Warfarin consumption (mg/kg) in relation to mortality, of rats held on a diet containing warfarin at 0.005% for 6 days: numbers of rats (*N*), mean consumption and standard error of consumption

Cross	Sex	Died			Survived		
		<i>N</i>	Mean	S.E.	<i>N</i>	Mean	S.E.
Backcross	M	47	9.8	0.4	27	15.4	1.0
	F	35	13.1	0.8	23	17.8	0.9
Intercross	M	13	10.5	0.7	19	15.1	0.5
	F	10	14.0	1.2	22	17.0	0.6
Testercross	M	125	10.2	0.2	268	15.4	0.1
	F	126	12.1	0.3	276	17.2	0.2

Table 2. Survival of offspring after 6 days on a diet containing 0.005% warfarin

(Backcrosses were the progeny of resistant rats mated to the Wistar strain in four successive backcrosses. Intercrosses were the progeny of resistant rats from the 4th backcross mated together.)

Cross	Number survived/ total	Observed survival (%)	Expected* survival (%)	$\chi^2_{(1)}$	<i>P</i>	Penetrance†
Backcrosses	50/132	37.9	53.3	12.1	< 0.001	0.69
Intercrosses	41/64	64.1	76.7	5.1	< 0.05	0.75

* Expectations based on the assumption that 6.58% of susceptible homozygotes were misclassified as resistant (see text).

† The penetrance, *p*, was calculated by solution of the following equations:

Proportion classified as resistant:	Genotype		
	<i>RR</i>	<i>RS</i>	<i>SS</i>
Backcross =	—	0.5 + (0.5 <i>p</i> × 0.0658)	
Intercross =	0.25 + 0.5 <i>p</i> + (0.25 × 0.0658)		

The survival of the progeny of the backcrosses and intercrosses is shown in Table 2. The results are given in terms of survival rather than mortality because survival is the phenotypic effect of the resistance gene under study. Families, generations, reciprocals and sexes of the progeny have been pooled because there

was no significant heterogeneity associated with any of these factors. The question to be answered by these results is whether resistance can be attributed to a single Mendelian gene with complete penetrance in the heterozygote, i.e. all heterozygotes being resistant. The Mendelian expectations (50% in the backcross and 75% in the intercross) were adjusted for the expectation mentioned earlier that 6.5% of rats homozygous for the susceptible allele were misclassified as resistant. The observed proportion of survivors is significantly less than the expected

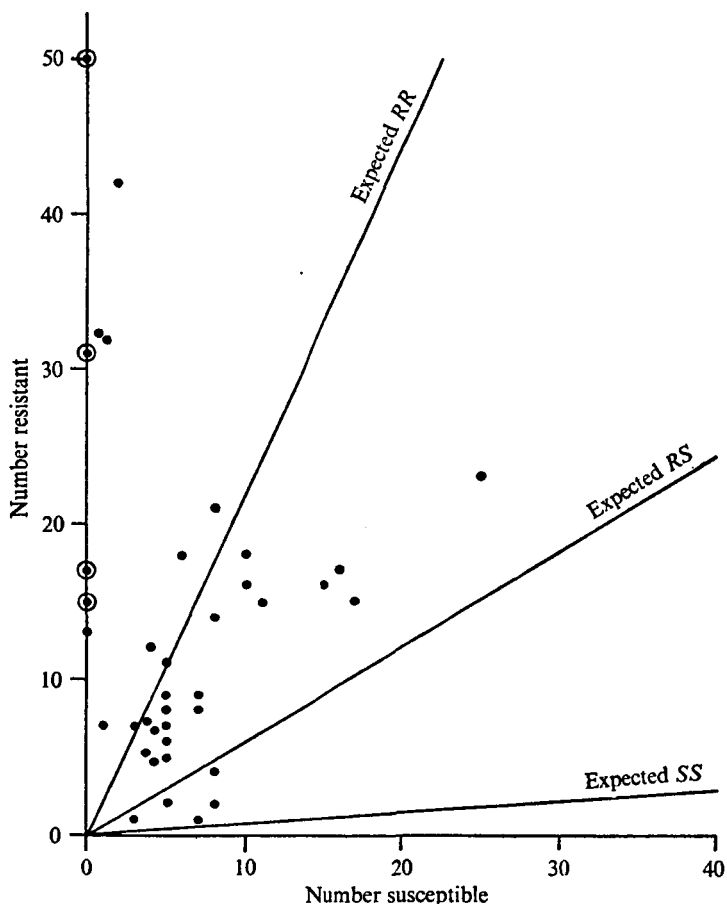


Fig. 1. Numbers of resistant and susceptible rats in 38 test-cross progenies. The resistant parents of the encircled progenies were used to found the homozygous resistant strain.

proportion in both crosses. This proves that not all heterozygotes are resistant to the dose administered in these tests. The penetrance in heterozygotes is 0.69 in the backcrosses and 0.75 in the intercrosses, assuming that all homozygotes were resistant (the calculation of penetrance is explained in Table 2). These two estimates do not differ significantly. Thus, the results of the backcrosses and intercrosses are fully consistent with the hypothesis that resistance is due to

a single gene, with all homozygotes and about 70 % of heterozygotes being resistant, and about 7 % of susceptible homozygotes being misclassified as resistant.

Forty-one out of the 64 intercross progeny were phenotypically resistant. Out of these, three failed to breed. The testcross results for the remaining 38 resistant rats are shown in Fig. 1. The expected proportions of resistant testcross progeny (100 %, 50% and 0 % respectively from *RR*, *RS* and *SS* parental genotypes) were adjusted for the expected survival of 6.58 % of susceptibles, and also for the penetrance of the resistance gene of 0.69 as previously estimated from the backcross data. The lines corresponding to the three expected ratios are indicated in Fig. 1. The χ^2 for deviation from each of the three ratios was calculated for each progeny, and the 38 progenies were assigned to the ratio of 'best fit', i.e. the ratio with the lowest χ^2 (Table 3). On this criterion the number classified *RR* (27/64) is significantly high ($\chi^2 = 9.2$; $P < 0.01$). Next, the significance of the chi squares was tested at the $P = 0.05$ level, to see which progenies could be classified unambiguously, by exclusion of two of the three possible ratios. By this criterion only 21 progenies could be classified; eight of these were significantly more resistant than would be expected from *RR*, and these are shown in a separate 'super resistant' category in Table 3. Thus, both classifications show a significant excess of resistant progenies.

Table 3. Numbers of testcross progenies assigned to each parental genotype

(The tested parents were the phenotypically resistant offspring of resistant rats from the 4th backcross, mated together.)

Criterion	<i>Super-R*</i>	<i>RR</i>	<i>RS</i>	<i>SS</i>	Total
Best fit	—	27	10	1	38
By exclusion†	8	8	4	1	21

* *Super-R* are progenies with significantly more resistants than expected from *RR* parents.

† 'By exclusion' means that in each progeny classified, the proportion of resistants is significantly different ($P < 0.05$) from that expected for every parental genotype other than the one under which it is shown.

We think that the excess of *RR* genotypes on the 'best fit' criterion and the occurrence of 'super-resistants' on the 'exclusion' criterion must mean that the penetrance of the resistance had increased. Two stages of selection for increased penetrance were brought about, firstly by intercrossing 4th generation resistant rats and, secondly, by screening their offspring. As previously mentioned, penetrance increased insignificantly from 0.69 in the backcross to 0.75 in the intercross. No overall estimate of penetrance can be made for the testcross owing to the uncertain nature of the classification of the progenies. However, it is evident from Fig. 1 that, in individual progenies, the penetrance of the resistance was virtually complete. The eight super-resistant progenies may therefore be regarded as families in which the resistance had become highly penetrant. On this interpretation the results of all the crosses are consistent with the single-gene hypothesis, with penetrance being complete in resistant homozygotes and varying

Table 4. *Mortality in the resistance screening test, in relation to coat colour, among the offspring of coloured, resistant rats*

Cross	Parents	Offspring*							
		Died				Survived			
		Albino	Intense	Dilute	Albino	Intense	Albino	Intense	Dilute
Backcross	Intense resistant x Albino susceptible (CPR/epS x epS/epS)	30 (31)	18 (11)	1 (1)	4 (3)	18 (25)	1 (1)		
Intercross 1	Intense resistant x Intense resistant (CPR/epS x CPR/epS)	9 (9)	3 (2)	1 (0)	1 (1)	7 (8)	3 (4)		
Intercross 2	Intense resistant x Albino resistant (CPR/epS x c-R/epS)	1 (1)	1 (0)	0 (0)	3 (3)	7 (8)	3 (3)		
Testcross 1	Intense resistant x Albino susceptible (CPR/epS x epS/epS)	31	4	0	8	31	8		
Testcross 2	Intense resistant x Albino susceptible (CS/cR x cS/cS)	2	2	0	6	1	0		
Testcross 3	Dilute resistant x Albino susceptible (CpR/epS x cpS/epS)	47	0	11	11	0	40		

*Survivals were adjusted (in parentheses) within each coat colour class in accordance with the penetrance estimates given in the text for the backcross and intercross, in order to combine the data for a maximum likelihood method of linkage analysis.

Table 5. *Mortality in female rats after a single subcutaneous dose of anticoagulant*

Compound	Strain	Dosage (mg/kg)										Approx. LD 50 (mg/kg)
		1.6	3.1	6.3	12.5	25	50	100	200	400	800	
Warfarin	Wistar	—	—	0/5	0/5	4/5	5/5	5/5	—	—	—	17
	Scottish	—	—	—	—	—	0/5	4/5	2/5	5/5	—	95
	Welsh	—	—	—	—	—	—	0/5	1/5	0/5	—	> 400
Coumatetralyl	Wistar	—	0/5	1/5	3/5	5/5	4/5	—	—	—	—	12
	Scottish	—	—	—	—	0/5	2/5	4/5	2/5	—	—	116
	Welsh	—	—	—	—	0/5	0/5	3/5	5/5	5/5	—	86
Diphacinone	Wistar	0/5	2/5	5/5	—	—	—	—	—	—	—	3.3
	Scottish	—	—	—	—	0/5	1/5	1/5	2/5	2/5	3/5	474
	Welsh	—	—	—	—	—	—	0/5	0/5	0/5	0/5	> 800

under selection between about 0.69 and 1.0 in heterozygotes, and with about 7% of susceptible homozygotes being misclassified under the test conditions.

Observations on the joint segregation of resistance and the Linkage Group I coat-colour characters, pink-eyed dilution (*p*) and albinism (*c*) are summarized in Table 4. The genotypes of the rats that were test-crossed were inferred from the phenotypes of their offspring. In all but Testcross 2 (Table 4) resistance entered the crosses in coupling with the dominant coat-colour characters. Pooled single-factor ratios for resistance and albinism did not differ significantly from expectation ($P > 0.5$). Linkages were estimated from the data of Table 4 by the maximum likelihood method (Carter & Falconer, 1951) and the following pooled recombination values and standard errors were obtained:

<i>c</i> - <i>R</i>	<i>c</i> - <i>p</i>	<i>p</i> - <i>R</i>
18 ± 2%	12 ± 6%	30 ± 5%

The recombination value for the *c*-*p* intercept, though on the low side, is consistent with the consolidated estimate of 18.6 ± 0.4% given by Robinson (1965), indicating that the *P*, *p* segregation, which could not be tested directly owing to epistasis by the linked gene for albinism, was essentially normal. The indicated linear arrangement of the genes is *p*-12%-*c*-18%-*R*.

The anticoagulant dose-mortality data for Wistar, Scottish and Welsh strains are given in Table 5. The ratios of the LD₅₀'s for each strain (Wistar:Scottish:Welsh) are approximately 1:5: > 20 for warfarin, 1:10:10 for coumatetralyl and 1:150: > 240 for diphacinone. Little precision can be claimed for these figures in view of the small numbers of animals used, but they indicate markedly different resistance spectra for the three strains. The Welsh strain is unique in its high resistance to warfarin and diphacinone, but similar to the Scottish strain in its response to coumatetralyl. The different resistance spectra add to the evidence that the Scottish and Welsh types of resistance are physiologically, and therefore genetically different.

4. DISCUSSION

The results show three types of evidence that Scottish-derived warfarin resistance is due to a major gene. First, the bimodal distribution of warfarin consumption by the rats (Table 1) suggests a single factor since, with multiple factors, a unimodal distribution would be expected. Secondly, survival ratios compatible with the usual unifactorial Mendelian ratios were maintained throughout the six generations of the study. The finding of partial penetrance and of modification of penetrance by the residual genotype is in contrast to the situation found with Welsh-derived warfarin resistance, which is fully penetrant in heterozygotes (Greaves & Ayres, 1967). However, control by modifiers of the expression of the major warfarin-resistance gene, *War*, in male *Mus musculus* has been described by Wallace & MacSwiney (1976).

The mode of action of the postulated penetrance modifiers is unknown. Marked strain differences in the rate of warfarin metabolism in rats have been reported by Pyörälä (1970), and individual differences have also been found (Pyörälä, 1965;

Davis, 1974), which in one case have been shown to be heritable, apparently with a polygenic basis (Pyörälä & Nevanlinna, 1968). Further, Townsend, Odam & Page (1974) found that Scottish-derived warfarin-resistant rats were slightly more resistant than Wistar rats to barbiturate treatment and that DDE selectively stimulated warfarin metabolism in the resistant strain. These studies suggest that polygenic control of warfarin metabolism could provide a basis for penetrance modification.

The third, and most convincing, evidence for a major gene is the demonstration of linkage between resistance and coat colour traits. The resistance gene has the same map position as the Welsh warfarin-resistance gene Rw^2 (Greaves & Ayres, 1969*a*). However, the Scottish gene seems to differ from the Welsh in (1) being incompletely penetrant in expression, (2) conferring a lower degree of resistance to warfarin, (3) conferring a lower susceptibility to vitamin K deficiency (Greaves & Ayres, 1973; Martin, 1973), and (4) producing a different spectrum of anticoagulant resistance. We think that the most likely explanation of the similarities and differences between the two types of resistance is that the Scottish and Welsh genes are allelic, i.e. that the Scottish resistance gene is a third allele, Rw^3 , in the series. A brief report by Dunning & Curtis (1939) indicates that a haemostatic defect resembling gross hypovitaminosis K is controlled by a recessive gene at approximately the same locus as the warfarin-resistance genes. Since the latter produce similar but less severe recessive haemostatic defects, the existence of a series of four alleles, with different effects upon warfarin toxicity, vitamin K requirement, and ultimately upon haemostasis, is readily conceivable. By extending the range of genetically based variation, such a series would increase the ability of wild rat populations to respond adaptively to selection with anticoagulant rodenticides, particularly in the presence of modifiers.

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