Genet. Res., Camb. (1977), **29**, *pp.* 215–222
With 2 text-figures
Printed in Great Britain

Unifactorial inheritance of warfarin resistance in *Rattus norvegicus* from Denmark

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(*Received* 1 *December* 1976)

SUMMARY

Wild populations of the Norway rat, *Rattus norvegicus*, in Jutland have been known to be resistant to the anticoagulant rodenticide warfarin since 1962. The inheritance of the resistance was investigated in the F_1, backcross and intercross. The results are consistent with the resistance being due to a major gene at the *Rw* locus. Resistant homozygotes, heterozygotes and susceptible homozygotes appeared to be distinguishable experimentally on the basis of differences in their susceptibility to vitamin K deficiency. The results are discussed in relation to previous studies of the inheritance of warfarin resistance in rats.

1. INTRODUCTION

Resistance to the anticoagulant rodenticide, warfarin (3-α-acetonylbenzyl-4-hydroxycoumarin) has been encountered in wild populations of *Rattus norvegicus* in several countries since 1958 (see, for example, Greaves & Ayres, 1976). In Denmark it was first discovered in 1962 on farms in the small peninsular between Velje and Horsens in eastern Jutland (Lund, 1964), and the resistant rats have since spread westwards into the interior of Jutland (Lund, 1972). It was soon established that the resistance was strongly heritable (Lund, 1967). The results of subsequent genetical studies proved, however, to be difficult to interpret as to the mode of inheritance of the resistance, and various single-gene and two-gene hypotheses were rejected (Lund, 1968, 1969).

In contrast, monogenic autosomal inheritance has been reported for cases of warfarin resistance in rats from Wales and Scotland, where the resistance seemed, at least superficially, to be similar to that found in Denmark (Greaves & Ayres, 1969, 1976). Since conditions in the Danish studies, as well as the conclusions reached differed from those in the studies of the Welsh and Scottish types of resistance, it was of interest to investigate the genetics of 'Danish-type' warfarin resistance further. For this purpose Dr M. Lund, Statens Skadedyrlaboratorium, Lyngby, Denmark, kindly sent us a wild-caught warfarin-resistant male rat from Jutland. Observations on two generations of this animal's descendants are presented here, and they show that the resistance is primarily due to a major autosomal gene.

2. METHODS

The resistant male rat received from Dr Lund was mated with five females from our warfarin-susceptible Wistar stock (which is homozygous for the linkage group I markers albinism, *c*, and pink-eyed dilution, *p*), to produce an F_1 of 49 progeny. The response to warfarin of the F_1 progeny was investigated in two experiments. Firstly, at the age of 2 months the rats were given a dose of 1·0 mg/kg of warfarin in a 10 % aqueous solution of gum acacia, by stomach tube. After 24 h a blood sample was taken directly from the ventral tail vein with a disposable plastic syringe, under light ether anaesthesia. The clotting time of the citrated blood plasma was measured by a conventional one-stage test sensitive to deficiencies of the vitamin K-dependent, prothrombin-complex blood clotting factors (the 'Two-Seven-Ten' test, Diagen Ltd., Thame, Oxfordshire, UK). Secondly, after a 1-month recovery period, the rats were placed on a diet of medium oatmeal containing 0·005 % warfarin for 6 days, and mortality was recorded over a 28-day period. This 6-day warfarin feeding treatment is lethal to the majority of warfarin-susceptible rats, and has been widely used as a standard test for resistance in *R. norvegicus* (Drummond & Wilson, 1968; Jackson *et al.* 1973; Brodie, 1976).

Next, a backcross to the susceptible strain was made by crossing an F_1 resistant male (i.e. one that had given a minimal response to the 1·0 mg/kg dose of warfarin and had subsequently survived the 6-day warfarin feeding test) with four Wistar females. This produced 89 backcross progeny which were classified as resistant or susceptible on the basis of their plasma clotting time 24 h after an oral dose of 1·0 mg/kg of warfarin, exactly as was done with the F_1 progeny.

Finally, an intercross was made by mating five pairs of resistant F_1 rats; 107 progeny were weaned of which 105 survived to be tested. The results of the tests with the F_1 and backcross progeny had suggested that the resistance was genetically similar to Welsh-type resistance. Since Welsh-type resistance is characterized by a sensitivity to vitamin K deficiency that is high in resistant homozygotes (*RR*), intermediate in heterozygotes (*RS*) and low in susceptible homozygotes (*SS*) (Hermodson, Suttie & Link, 1969), we decided to attempt to make a threefold classification (*RR*, *RS* and *SS*) of the 105 intercross progeny. This was done by placing the rats on a vitamin K-deficient diet (Greaves & Ayres, 1973) for 4 days and measuring their plasma clotting times at the beginning and after 1 and 4 days. After a recovery period of 1 month the rats were given the 6-day warfarin feeding test, in order to compare the two techniques.

Throughout the study the animals were maintained in wire-mesh cages measuring 34 × 20 × 15 cm, supported 2 cm above a metal dropping tray. All rats were supplied *ad libitum* with water and, except when on an experimental diet, with pelleted diet 41B (Oxoid Ltd, UK).

3. RESULTS

The plasma clotting times of the F_1 progeny, 24 h after the 1·0 mg/kg dose of warfarin are summarized in Fig. 1(*a*); rats with clotting times longer than 1 min are grouped together because this time indicates that the blood had negligible clotting activity. The response is bimodal which suggests, since results for males and females were similar, that the wild-caught male parent was heterozygous (*RS*) for a major autosomal gene for resistance. If rats with clotting times less than 25 sec are taken to be resistant then the proportion resistant, 20/49 (41 %), is

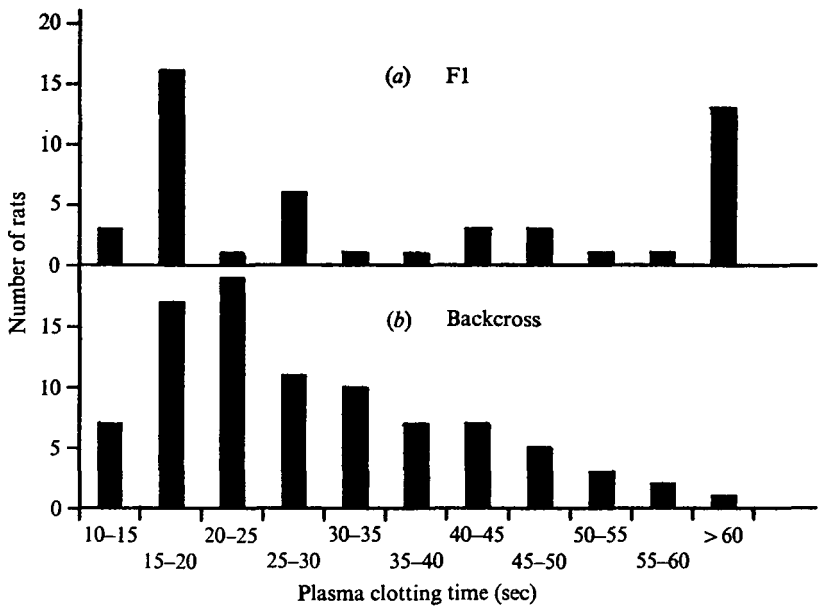

Fig. 1. The plasma clotting times of rats 24 h after an oral dose of 1·0 mg/kg of warfarin. (*a*) F_1 progeny of a wild-caught resistant male and susceptible Wistar females. (*b*) Backcross progeny of an F_1-resistant male and susceptible Wistar females.

consistent with the ratio of 0·5 expected from a backcross to the susceptible ($\chi^2 = 1{\cdot}31$; $P > 0{\cdot}2$). Twenty-three of the 49 rats (47 %) survived the 6-day warfarin feeding test, which is even closer to the expected proportion ($\chi^2 = 0{\cdot}082$; $P > 0{\cdot}7$). However, three of the rats that died had clotting times shorter than 25 sec (14, 18 and 24 sec) and seven of the rats that survived had clotting times longer than 25 sec (26, 27, 29, 41 and 43 sec). The lack of agreement between the two tests in their classification of these ten rats shows that at least one, and perhaps neither of the tests is completely dependable as an indicator of genetic resistance.

The F_1 progeny all had the same wild-type coat colour as the resistant, male parent. Since the susceptible female Wistar parents were all homozygous for albinism (*c*), pink-eyed dilution (*p*) and warfarin susceptibility (i.e. they were

cc pp SS), the wild male parent was evidently *CC PP RS*. Thus the F_1 rats used as resistant parents for the ensuing backcross and intercross were all trihybrids, *Cc Pp RS*, assuming the resistance to be due to a single major gene.

Table 1. *Segregation of warfarin resistance and coat colour traits in a trihybrid backcross* (Cc Pp RS × cc pp SS)

	Coat colour class			
	Intense (*CP*)	Dilute (*Cp*)	Albino (*c*)	Total
Resistant (*R*)	24	4	15	43
Susceptible (*S*)	10	3	33	46
Total	34	7	48	89

The plasma clotting times of the 89 backcross progeny 24 h after the 1·0 mg/kg dose of warfarin are summarized in Fig. 1(*b*). Here, the only indication of any bimodality of response is the positive skewness of the distribution, the susceptible rats presumably being included in the long upper tail. The apparently diminished response of the susceptibles to warfarin may have been due to inadvertent selection, caused by our choice of a highly resistant F_1 male to breed from. However, if the division between resistants and susceptibles is made, as before, at 25 sec, then 43/89 were resistant, again insignificantly different from the expected ratio of 0·5 ($\chi^2 = 0{\cdot}045$; $P > 0{\cdot}8$).

The 89 backcross progeny were classified by resistance and coat colour as in Table 1. The recombination fractions were calculated by the methods of Finney (1949) and Carter & Falconer (1951) to give the following estimates and standard errors. The value for the $c-p$ intercept is close to the generally accepted

$$\underbrace{p-17\pm6\,\%-c-31\pm5\,\%-R}_{34\pm7\,\%}$$

estimate of $18{\cdot}6\pm0{\cdot}4\,\%$ made by Robinson (1965). The values for the $c-R$ and $p-R$ intercepts are probably over-estimates, since it is very likely that some rats were misclassified as to their resistance or susceptibility to warfarin (Fig. 1*b*). Nevertheless, the data provide strong evidence of a major resistance gene in the same relative position in linkage group I as the *Rw* locus warfarin-resistance genes found in wild rat populations in Wales (Greaves & Ayres, 1969) and Scotland (Greaves & Ayres, 1976).

The development of vitamin K deficiency in the 105 intercross progeny is illustrated in Fig. 2. As expected, about a quarter (28/105) rapidly became severely deficient, with their clotting times increasing to more than 60 sec on the first day (Fig. 2*a*). These rats, several of which were found to have slightly elevated clotting times just before they were given the vitamin K-deficient diet, were deemed to be warfarin-resistant homozygotes (*RR*). A further 50 rats, whose clotting times rose

to more than 60 sec by the end of the 4th day, were classified as heterozygotes (*RS*). The remaining 27 rats whose clotting times remained less than 51 sec after 4 days (Fig. 2*b*) were regarded as warfarin-susceptible homozygotes (*SS*).

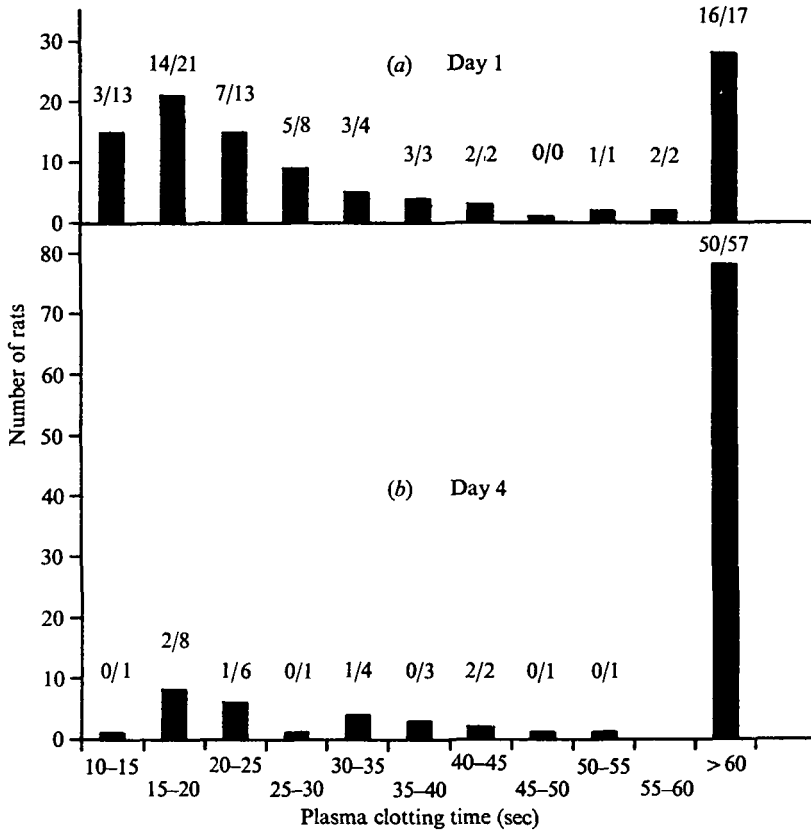

Fig. 2. The plasma clotting times of 105 rats after being held on a vitamin K deficient diet for (*a*) 1 day and (*b*) 4 days. The ratios given above each bar are the survival ratios (i.e. number survived/number tested) of the same rats when given the 6-day warfarin feeding test 1 month later. The rats were the offspring of intercrosses between F_1-resistant animals.

The classification of the intercross progeny by putative resistance genotype and coat colour is given in Table 2. The single-factor segregation for resistance agrees well with the 1:2:1 ratio expected from an intercross between heterozygotes ($\chi^2 = 0{\cdot}20$; $P > 0{\cdot}9$) and that for albinism with the expected 3:1 ratio ($\chi^2 = 1{\cdot}40$; $P > 0{\cdot}2$). The single-factor segregation for pink-eyed dilution could not be tested since the distinction between the *P* and *p* phenotypes was masked by the linked character of albinism. For the same reason no estimate could be made of recombination between pink-eyed dilution and resistance in the intercross. Recombination in the $c-p$ segment, estimated at $8 \pm 15\%$, though on the low side, is again consistent with the estimate given by Robinson (1965). The recombination value for

the $c-R$ segment is $18 \pm 4\%$, which places the resistance gene at or very close to the *Rw* locus. We regard this value as considerably more reliable than the much larger value calculated from the backcross data because the intercross progeny were classified with greater certainty.

Table 2. *Segregation of warfarin resistance and coat colour traits in a trihybrid intercross* (Cc Pp RS × Cc Pp RS)

	Coat colour class			
Putative resistance genotype	Intense (*CP*)	Dilute (*Cp*)	Albino (*c*)	Total
Homozygous resistant (*RR*)	27	1	0	28
Heterozygous (*RS*)	36	2	12	50
Homozygous susceptible (*SS*)	7	0	20	27
Total	70	3	32	105

Table 3. *Lethality of a warfarin diet to rats of different resistance genotypes*

The rats had previously been assigned among the three genotypic classes in accordance with their susceptibility to vitamin K deficiency. The data are condensed from Fig. 2.

	Putative genotype			
	RR	*RS*	*SS*	Total
Survived	16	34	6	56
Died	1	6	21	28
Total	17	40	27	84

Twenty-one of the intercross progeny, all of them *RR* or *RS*, died in the first experiment. Mortality in the remaining 84 rats in the 6-day warfarin feeding test is shown in Table 3, where it can be seen that the two criteria of resistance agreed in all except 13 cases. The warfarin diet was lethal to seven putatively resistant rats, all but one of them *RS*, suggesting that the resistance allele may be incompletely dominant in expression. However, six animals survived that had been judged to be genotypically susceptible to warfarin. The differences between the results of the two tests with these 13 rats mean again that the possibility of some misclassification cannot be ignored and therefore that linkage between albinism and resistance may possibly be closer than indicated by the calculated recombination value of 18%. A second possibility, that an allele for susceptibility to vitamin K deficiency is closely linked with the allele for warfarin resistance can be excluded on the grounds that the two traits are invariably found to occur together in rats (Greaves & Ayres, 1973) and the direct biochemical evidence that the mechanism of warfarin resistance involves an impairment of vitamin K metabolism (Bell & Caldwell, 1973). The results of all the crosses may therefore be regarded as consistent with the resistance being due to an autosomal gene at the *Rw* locus.

4. DISCUSSION

The observations presented here prove that a major warfarin-resistance gene exists in the Danish rat population. In contrast, as previously mentioned, Lund (1969) considered that the results of breeding studies with wild rats were not consistent with unifactorial inheritance of the resistance, since he found significant shortages of resistant progeny in *resistant* × *resistant* and *resistant* × *susceptible* crosses.

We believe that the apparent deficiency of resistant progeny in Lund's study can be explained in at least two ways. First, his criterion of resistance was that the rats should survive after 6 days' feeding on 0·025 % warfarin, a concentration five times as great as we used. Since even the lower concentration appeared to be lethal to some genotypically resistant animals (Table 3) it seems more than likely that the higher concentration led to significant numbers of resistant rats being classified as susceptibles. Secondly, residual genotypic variation affecting the expression of the resistance gene may have influenced Lund's results. There is some evidence for this in his observation that when two susceptible females were bred with the same resistant male they produced strikingly different proportions of resistant offspring, 0/42 from the first female and 4/9 from the second. Proportions as different as these indicate that the resistance trait can be remarkably variable in wild rat populations. Variability makes it difficult to identify resistant animals reliably (as the present results illustrate) and obscured the underlying single-factor segregation in Lund's data.

The position of the Danish allele in linkage group I and the observation that the resistance involves increased sensitivity to vitamin K deficiency mean that Danish-type resistance resembles the Welsh and Scottish types both genetically and physiologically. We may therefore suggest with considerable confidence that the Danish allele also lies at the *Rw* locus. There is some evidence that the Welsh and Scottish types of resistance are allelic (Greaves & Ayres, 1976) and it may be asked which type the Danish resistance most closely resembles. Lund (1972) has remarked that the Danish strain is similar to the Scottish and differs from the Welsh in being relatively insensitive to the synergistic effect of dietary mineral oil on warfarin toxicity. On the basis of the data presented in Table 3 the Danish resistance also seems to resemble the Scottish rather than the Welsh in being incompletely penetrant in heterozygotes (Greaves & Ayres, 1976). Against this, the Danish strain is more like the Welsh in its high susceptibility to vitamin K deficiency, as indicated both by the present results and the observation by Lund (1967) of haemorrhagic deaths among Danish resistant rats in the absence of warfarin treatment – which we have also occasionally noticed in resistant homozygotes of the Welsh, but not of the Scottish strain. Thus the evidence as to the identity of the Danish allele is fragmentary and inconclusive. More detailed studies will be needed to determine whether Danish-type resistance is identical with either of the types previously studied.

REFERENCES

BELL, R. G. & CALDWELL, P. T. (1973). Mechanism of warfarin resistance. Warfarin and the metabolism of vitamin K_1. *Biochemistry* **12**, 1759–1762.

BRODIE, J. (1976). Anticoagulant resistant rats (*Rattus norvegicus*) in Scotland. *International Pest Control* **18**, 7–10.

CARTER, T. C. & FALCONER, D. S. (1951). Stocks for detecting linkage in the mouse and the theory of their design. *Journal of Genetics* **50**, 307–323.

DRUMMOND, D. C. & WILSON, E. J. (1968). Laboratory investigations of resistance to warfarin of *Rattus norvegicus* Berk. in Montgomeryshire and Shropshire. *Annals of Applied Biology* **61**, 303–312.

FINNEY, D. J. (1949). The estimation of the frequency of recombinations. I. Matings of known phase. *Journal of Genetics* **49**, 159–176.

GREAVES, J. H. & AYRES, P. (1969). Linkages between genes for coat colour and resistance to warfarin in *Rattus norvegicus*. *Nature* **224**, 284–285.

GREAVES, J. H. & AYRES, P. (1973). Warfarin resistance and vitamin K requirement in the rat. *Laboratory Animals* **7**, 141–148.

GREAVES, J. H. & AYRES, P. B. (1976). Inheritance of Scottish-type resistance to warfarin in the Norway rat. *Genetical Research* **28**, 231–239.

HERMODSON, M. A., SUTTIE, J. W. & LINK, K. P. (1969). Warfarin metabolism and vitamin K requirement in the warfarin resistant rat. *American Journal of Physiology* **217**, 1316–1319.

JACKSON, W. B., BROOKS, J. E., BOWERMAN, A. M. & KAUKEINEN, D. (1973). Anticoagulant resistant in Norway rats in U.S. cities. *Pest Control* **41**, 56–58, 60–64, 81.

LUND, M. (1964). Resistance to warfarin in the common rat. *Nature* **203**, 778.

LUND, M. (1967). Warfarin-resistant rats in Denmark. *European and Mediterranean Plant Protection Organisation. Report of the International Conference on Rodents and Rodenticides*, Paris 1965, pp. 49–57.

LUND, M. (1968). *Government Pest Infestation Laboratory Annual Report*, 1967, p. 70.

LUND, M. (1969). Resistance to anticoagulants in Denmark. *Schriftenreihe des Vereins für Wasser-, Boden- und Lufthygiene*, no. 32, pp. 27–38.

LUND, M. (1972). Rodent resistance to the anticoagulant rodenticides, with particular reference to Denmark. *Bulletin of the World Health Organization* **47**, 611–618.

ROBINSON, R. (1965). *Genetics of the Norway rat*. Oxford: Pergamon Press.