Aust. J. Biol. Sci., 1974, **27**, 671–5

Ecological Genetics of the Wild Rabbit in Australia
I. Geographical Distribution and Biochemical Characterization of Phosphogluconate Dehydrogenase Variants

Marjorie Coggan,[A] *J. Baldwin*[B] *and B. J. Richardson*[B]

[A] Botany Department, Australian National University, P.O. Box 4, Canberra City, A.C.T. 2601.
[B] Genetics Department, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra City, A.C.T. 2601. (Reprint requests to Dr B. J. Richardson.)

Abstract

Three electrophoretically distinguishable phosphogluconate dehydrogenase (EC 1.1.1.44) isoenzyme patterns, probably resulting from the expression of two alleles at one locus, were found in natural populations of rabbits in Eastern Australia. The homopolymers and heteropolymer were isolated and characterized kinetically. The three proteins were found to be indistinguishable with respect to K_m for 6-phospho-D-gluconate and $NADP^+$ (both in the presence and absence of magnesium), energies of activation, specific activities in liver, pH profiles and thermal stabilities. The rare allele was not found near the major initial release site of the rabbit in Australia and may have arisen in Australia.

Introduction

Numerous investigations have been made of the geographical distribution of protein variation in populations with the aim of gaining some insight into mechanisms by which such variations are incorporated into natural populations (e.g. Petras 1967; Richardson and Czuppon 1969; Canham *et al.* 1970; Selander 1970; Gaines *et al.* 1971). However, few integrated studies combining (1) biochemical comparisons of the variants, (2) controlled breeding programs, (3) studies of geographical and temporal distribution of alleles, and (4) ecological investigations of the species have been attempted. This paper reports the first of a series of investigations on populations of the wild rabbit incorporating these four approaches.

The European rabbit, which was first successfully introduced into Australia in the middle of the last century (Radcliffe 1959), is now found throughout the southern half of the continent over an area of at least 3×10^6 km^2. It lives in environments ranging from arid stony deserts with an annual rainfall of 100 mm or less to subalpine valleys, wet coastal plains and subtropical grasslands. The ecology and behaviour of this agricultural pest has been studied for many years (Myers 1970).

The enzyme phosphogluconate dehydrogenase (decarboxylating) [6-phospho-D-gluconate : $NADP^+$ 2-oxidoreductase (decarboxylating); EC 1.1.1.44; hereinafter designated PGD] mediates the third step in the pentose phosphate shunt, producing half of the NADPH formed by the pathway. The enzyme has recently been purified and kinetically characterized from sheep liver (Dyson *et al.* 1973) and human red blood cells (Shih *et al.* 1968).

This paper reports the presence of a polymorphism for PGD in the wild rabbit, describes the geographical distribution of the PGD types and gives the values of a number of kinetic parameters for each partially purified isoenzyme.

Materials and Methods

Animals were collected by live trapping or shooting. Tissue samples were frozen in liquid nitrogen in the field. Heparinized blood was collected on ice and red blood cells separated by centrifugation and suspended in ethylene glycol–citrate solution (Owen 1963). All samples were stored at −12°C until prepared for electrophoretic or kinetic studies.

For electrophoretic characterization of the PGD, red blood cells were washed twice in ethylene glycol–citrate solution and ruptured with an equal volume of lysing solution (Richardson and Cox 1973). Liver, muscle and kidney samples for electrophoresis were homogenized in lysing solution, centrifuged at 3000 rev/min for 10 min in a bench centrifuge, and the supernatant was then used as a source of PGD.

Electrophoresis was carried out on both cellulose acetate and starch gels. Cellulose acetate gels (Cellogel, Chemetron, Italy) (Richardson and Cox 1973) were run for 2 h at 30 V/cm and 4°C in a tris–EDTA–borate buffer system (15 mM tris–5 mM EDTA), pH 7·5. Starch gels were run for 18 h at 2 V/cm and 4°C using a 200 mM phosphate–citrate buffer, pH 7·0. Gels were stained at room temperature in a solution containing 0·07 mM $NADP^+$, 2 mM 6-phospho-D-gluconate, 0·03 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 0·02 mM *N*-methylphenazonium methosulphate in 100 mM tris–HCl buffer, pH 8·6.

For kinetic studies of PGD, whole livers were homogenized in four volumes of ice-cold 50 mM KCl–1 mM EDTA, followed by centrifugation at 30 000 *g* for 30 min at 4°C. The fraction of the supernatant solution that precipitated in ammonium sulphate solution that was between 50 and 65% saturated was collected by centrifugation, dissolved in a small volume of 50 mM tris–HCl buffer (pH 7·5), dialysed against 500 volumes of the same buffer and stored frozen at −20°C. This preparation contained 50% of the activity present in the initial supernatant and represented a sixfold increase in specific activity. In preparations containing mixtures of PGD isoenzymes, separation was by electrophoresis on starch gels followed by elution of individual isoenzymes from the gel by homogenization and centrifugation.

Enzyme activity was followed by measuring the reduction of $NADP^+$ at 340 nm in a Gilford 2400 recording spectrophotometer. The standard assay mixture contained 50 mM tris–HCl buffer (pH 7·5), 6-phospho-D-gluconate and $NADP^+$ in a total volume of 3 ml. Assays were carried out at 37°C except where indicated. K_m values, and V_{max} values for Arrhenius plots, were determined from Lineweaver–Burk plots. Specific activities were determined from high-speed supernatants of 1 : 3 liver homogenates (1 g liver to 3 ml of 50 mM tris–HCl buffer, pH 7·4, and 1 mM EDTA). An extinction coefficient ($E\,{}^{1}_{1\,cm}{}^{\mu mol/ml}$) of $6{\cdot}22 \times 10^{-3}$ for NADPH at 340 nm was used, and protein concentrations were determined by the method of Lowry *et al.* (1951).

Results

Three electrophoretic forms of PGD were observed in the rabbit tissues examined, and animals could be classified into three groups on the basis of their PGD banding patterns (Fig. 1): animals possessing only the fastest migrating band (c), animals with only the slowest migrating band (a) and those with all three bands (a+b+c). Liver, kidney, muscle and red blood cells were examined from animals of each type to determine whether the same locus was active in each tissue of an animal. Identical patterns were found in each tissue of any given animal. The frequencies of the two PGD alleles (see Discussion) in 11 spatially separated rabbit populations in Eastern Australia are shown in Table 1 and Fig. 2.

A kinetic comparison was made of the three PDG types a, c and abc, and the b band was isolated by starch gel electrophoresis. K_m values for PGD with its substrates 6-phospho-D-gluconate and $NADP^+$, with and without $MgCl_2$, are given in Table 2, and within the limits of the method the forms cannot be considered significantly different. Substrate saturation curves were hyperbolic and the concentration of one substrate did not affect the K_m value obtained for the other. Arrhenius plots of log V_{max} as a function of the reciprocal of the absolute temperature yielded straight

parallel lines over the temperature range 10–40°C, with an energy of activation of 13·4±0·3 kcal/mol in each case.

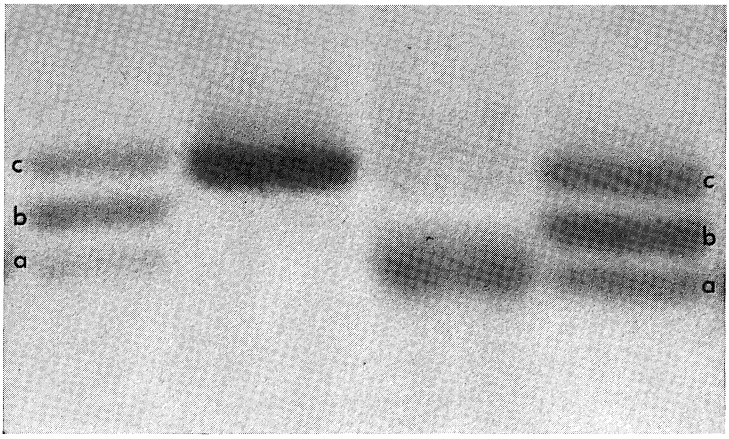

Fig. 1. Cellulose acetate gel electrophoretogram of liver homogenates from four animals, showing the three electrophoretic forms of PGD.

Table 1. A summary of the geographical data, giving collecting sites, sample sizes, the relative frequency of the rarer allele and the 95% confidence belt for this frequency

Site	*PGD*[2] frequency[A]	95% confidence belt	No. of animals	Site	*PGD*[2] frequency[A]	95% confidence belt	No. of animals
New South Wales				Victoria			
Mogo	0·00	0·00–0·06	31	Ouyen	0·13	0·07–0·22	52
Bemboka	0·00	0·00–0·08	23	Werribee	0·00	0·00–0·03	163
Cooma	0·20	0·14–0·28	69				
Snowy Plains	0·05	0·02–0·10	92	Queensland			
Braidwood	0·05	0·01–0·13	45	Injune	0·00	0·00–0·10	19
Canberra	0·05	0·03–0·08	121	Charleville	0·00	0·00–0·05	38
Urana	0·12	0·08–0·17	145				

[A] See Discussion for explanation of allele symbolism.

Table 2. Summary of comparative kinetic data for the three PGD isoenzymes

Isoenzyme	Apparent K_m values (μM): 6-Phospho-D-gluconate		NADP+		Energy of activation (kcal/mol)	Specific activity (i.u./mg protein)[A]
	No Mg^{2+}	With 10^{-2}M Mg^{2+}	No Mg^{2+}	With 10^{-2}M Mg^{2+}		
a	50	24	17	23	13·7	0·0163±0·0043 (3)
b	42	22	15	24	13·1	—
c	49	28	16	23	13·7	0·0176±0·0016 (10)
a+b+c	51	24	16	21	13·4	0·0163±0·0036 (10)

[A] Mean ± 95% confidence interval (no. of measurements in parentheses).

Specific activities of the three PGD types were determined in samples of liver (Table 2) and again showed no significant differences.

pH profiles obtained using 100 mM tris–HCl buffers in the range pH 6·0–8·1 at both V_{max} and K_m substrate levels were similar for the four PGD preparations, with

bell-shaped curves exhibiting broad maxima in the range pH 7·3–7·6. Temperature stabilities of the PGD types were indistinguishable, with 50% inactivation occurring in each case after 7 min incubation of the enzyme preparation at 60°C.

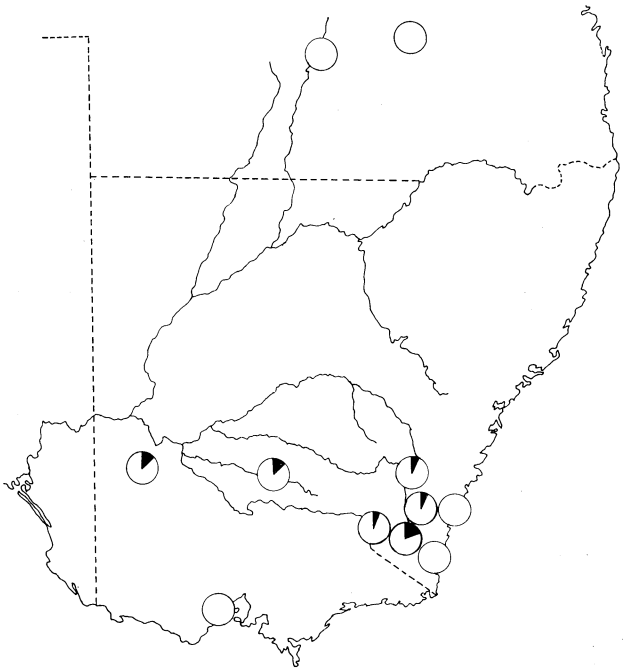

Fig. 2. Map of south-eastern Australia showing the geographical location of each sampling site and the relative frequencies of the two alleles (see Discussion) in each sample.

Discussion

The enzyme PGD is considered to be dimeric and the presence of three electrophoretically distinguishable bands of PGD activity in the rabbit can be explained in terms of two subunit types (PGD-1 and PGD-2) that associate randomly to form the three possible dimers 1–1 (band c), 2–2 (band a) and 1–2 (band b) (Fig. 1) (Shaw 1965). The distribution of PGD subunit types in the populations studied is consistent with a Hardy–Weinberg equilibrium condition of two alleles at one locus (i.e. PGD^1 and PGD^2).

Failure to detect any significant differences among the three isoenzymes raises the possibility that the two alleles are selectively indistinguishable or 'neutral' (Crow 1972). However, it is difficult to substantiate such a proposition, as selectively important differences may always exist in some unstudied property. This is a constant problem when enzymes are studied *in vitro* and removed from their normal metabolic context. In fact, selective differences between the alleles need not reside in catalytic activity *per se*, but might involve more general properties, such as surface charge distribution and its relation to other cellular components, or particular amino acid substitutions might spare an essential amino acid which is in limited supply. The frequency of the PGD^1 allele differs significantly from area to area (Table 1). The sample from Werribee is the most divergent due to apparent fixation of the common allele. This is especially interesting as the most important release of wild rabbits in Australia was near Werribee. Explanations of this data include the following possibilities: (1) that the original release did not contain the rare allele and this has either been derived from rabbits

released elsewhere in Australia or has recently arisen by mutation here, and (2) that the rare allele is at a selective disadvantage in coastal regions and has been secondarily lost in this area. The latter possibility could be supported by the fact that the rare allele is also missing from the other coastal samples studied while the former is supported by the absence of the rare allele in the peripheral samples from coastal areas and Queensland and the absence of the rare allele from four inbred strains of laboratory rabbits (McDermid, personal communication).

It is difficult to explain the distribution of the polymorphism in terms of selectively indistinguishable alleles. The relatively constant gene frequencies found over a wide central area (Fig. 2) would have to be maintained by migration (Maruyama 1972). It is then difficult to understand why the allele is missing in certain other areas, unless the rare allele is newly derived.

It seems likely that the rare allele has arisen in Australia but whether it has given rise to a transient, balanced or 'neutral' polymorphism cannot be determined at present.

Acknowledgments

The authors wish to thank Dr J. Dunsmore, Mr I. Parer, Mr W. Price, Mr P. Haycock and Mr J. Lipski, Division of Wildlife Research, CSIRO, for advice and assistance in the field, Miss R. Sheppard, Victorian Department of Crown Lands and Survey, for the material collected in Victoria, and Mrs C. Hayes, Miss P. Kell, Mr P. Bailey and Mr N. Gowen for technical assistance.

References

Canham, R. P., Birdsall, D. A., and Cameron, D. G. (1970). *Genet. Res.* **16**, 355.
Crow, J. F. (1972). *J. Hered.* **63**, 306.
Dyson, J. E. D., D'Orazio, R. E., and Hanson, W. H. (1973). *Arch. Biochem. Biophys.* **154**, 623.
Gaines, M. S., Myers, J. H., and Krebs, C. J. (1971). *Evolution* **25**, 433.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265.
Maruyama, T. (1972). *Ann. Hum. Genet.* **35**, 179.
Myers, K. (1970). Proc. Adv. Study Inst. Dynamics Numbers Population, Oosterbeek. p. 478.
Owen, R. D. (1963). In 'Methodology in Mammalian Genetics'. (Ed. W. J. Burdette.) p. 347. (Holdon Day: New York.)
Petras, M. L. (1967). *Evolution* **21**, 259.
Radcliffe, F. N. (1959). In 'Biogeography and Ecology in Australia'. (Ed. A. Keast.) p. 545. (Junk: The Hague.)
Richardson, B. J., and Cox, D. M. (1973). *Clin. Genet.* **4**, 376.
Richardson, B. J., and Czuppon, A. B. (1969). *Aust. J. Sci.* **32**, 106.
Selander, R. K. (1970). *Am. Zool.* **10**, 53.
Shaw, C. R. (1965). *Science (Wash. D. C.)* **149**, 936.
Shih, L.-Y., Justice, P., and Hsia, D. Y.-Y. (1968). *Biochem. Genet.* **1**, 359.

Manuscript received 22 April 1974