1 min after saline or hormone. Our main results, however, were obtained using a glucagon solution of 0.1 μg ml^{-1}, and 1, 2, 3 or 6 min after glucagon or 1 min after saline, liver samples were obtained by laparotomy and immediately deep frozen between 2 blocks of dry ice to be analyzed for active (A) and total phosphorylase activity[8], and cAMP[9] and glycogen content (using an anthrone method). The protein level of the 10% liver homogenate used for enzyme analysis was also determined[10]. After obtaining liver samples the animals were decapitated for collecting mixed blood for serum glucose determinations using an orthotoluidine method (test sets prepared by LACHEMA, Prague).

Results. In the screening experiment with 0.2 μg kg^{-1} glucagon no influence of fasting on the liver phosphorylase response was observed. The percentage of the total enzyme activity represented by form A in fed and fasted rats, respectively, was as follows; without hormone administration 33.6 ± 1.4 vs 23.6 ± 1.2 $p < 0.01$; 1 min after glucagon 79.1 ± 7.4 vs 80.4 ± 4.6/(means ± SEM).

In our experiment with the lower glucagon dose (table), no significant differences were found in either A or total phosphorylase activity between fed and fasted control animals. However, 0.1 μg kg^{-1} of the hormone failed to affect phosphorylase A activity in fasted animals, whereas the same dose increased it at 1, 2 and 3 min in fed rats. Substantial increases of cAMP were observed 1 min after glucagon (table), the response in the fasted group being even more pronounced than in the fed animals.

At 6 min a return of phosphorylase A vlaues to control activities and a decreased cAMP concentration was noted in fed animals. No effect of glucagon on liver glycogen content or serum glucose level of either fed or fasted animals was observed during our observation period (glucose values of control animals in mmole l^{-1}: fasted 6.6 ± 0.2, fed 8.9 ± 0.8, NS), the glycogen levels of fasted animals being extremely low as a consequence of food withdrawal (in fasted controls in mg g^{-1} 2.2 ± 0.3 in comparison with 87.5 ± 5.8 in the corresponding fed group).

Discussion. A successive lowering of rat liver phosphorylase A during fasting for several days with a decrease by about 36% after the 1st 24 h of fasting has been described[11] and a lowering of A and total enzyme activity after fasting for 24 h was reported more recently[12]. On the other hand, no effect of fasting for 19–21 h on percent phosphorylase A and total enzyme activity was found by a further group of authors[13]. In our pilot study the phosphorylase A activity of the fasted control animals was significantly lower ($p < 0.05$) than that of the fed group. The inhibitory effect of fasting was also obvious after administration of 0.1 μg kg^{-1} of glucagon. This dose was supposed to mimic portal glucagon concentrations (about 500 pg ml^{-1}) of rats subjected to the Noble-Collip drum procedure.

After the greater, probably supraphysiological glucagon dose no difference in enzyme response between fed and fasted rats was noted, therefore there seems to be an increased threshold for the glucagon effect rather than total insensitivity.

According to published reports, fasting for 18 h elevates basal and glucagon-stimulated cAMP level of isolated liver cells[14] and increases the basal cAMP level in vivo[15]. On the other hand, a decreased glucagon binding and glucagon stimulated cAMP accumulation in isolated liver cells of rats fasted for 48 h was reported[16]. In our experiments the cAMP level in the liver of control animals was uninfluenced by the food withdrawal and after glucagon it increased in fasted rats more than in fed animals. So the target of inhibition of phosphorylase by fasting should be sought somewhere on the glycogenolytic cascade between cAMP and phosphorylase. In rats fasted for 24 h a changed distribution of liver type I and type II protein kinase in the sense of predominance of type II enzyme (in contrast to the prevalence of type I in fed animals) was observed; the physiological significance of this phenomenon remains obscure[17].

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2 Š. Németh, J. Kolena, R. Kvetňanský and T. Torda, Physiol. bohemoslov. *28*, 51 (1979).
3 R.L. Noble and J.B. Collip, Q. J. exp. Physiol. *31*, 187 (1942).
4 G. Van De Werve, L. Hue and H.G. Hers, Biochem. J. *162*, 135 (1977).
5 I. Klimeš, M. Vigaš, D. Ježová, Š. Németh and J. Jurčovičová, IRCS med. Sci. *9*, 86 (1981).
6 Š. Németh, Bratisl. lek. Listy *70*, 449 (1978).
7 S. Keppens and H. DeWulf, FEBS Lett. *51*, 29 (1975).
8 W. Stalmans and H.G. Hers, Eur. J. Biochem. *54*, 341 (1975).
9 A.G. Gilman, Proc. natl Acad. Sci. USA *67*, 305 (1970).
10 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
11 H. Niemeyer, H.C. González and R. Rozzi, J. biol. Chem. *236*, 610 (1961).
12 X. Remesar and M. Alemany, Horm. Metab. Res. *12*, 19 (1980).
13 L.N. Mulmed, M.C. Gannon, D.P. Gilboe, A.W. Tan and F.Q. Nuttall, Diabetes *28*, 231 (1979).
14 J.G. Garrison and R.C. Haynes, J. biol. Chem. *248*, 5333 (1973).
15 D.E. Goldstein and R.T. Curnow, Metabolism *27*, 315 (1978).
16 M. Fouchereau-Peron, F. Rançon, P. Freychet and G. Rosselin, Endocrinology *98*, 755 (1976).
17 T.M. Chan, K.E. Steiner and J.H. Exton, J. biol. Chem. *254*, 11374 (1979).

Ketoconazole, an inhibitor of calcium transport in skeletal muscle sarcoplasmic reticulum

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Summary. Ketoconazole, an antimycotic agent, inhibits calcium binding and accumulation, and induces calcium release in sarcoplasmic reticulum. The Mg^{2+}-ATPase and the $(Ca^{2+} + Mg^{2+})$-ATPase activities are stimulated at low but inhibited at high concentrations of ketoconazole.

Ketoconazole, cis-1-acetyl-4- (4((2-(2,4-dichloro-phenyl)-2-(1H-imidazole-1-yl-methyl)-1,3-dioxalan-4-yl)methoxy)-phenyl) piperazine, an antimycotic agent, inhibits the growth of yeast by inhibiting ergosterol synthesis; this is accompanied by changes in cell membrane permeability[2]. Miconazole, an antifungal imidazole chemically related to ketoconazole, inhibits the membrane-bound plasma membrane ATPase and mitochondrial ATPase of yeast[3], and affects the exchange[4] of intracellular K^+ for extracellular H^+. It has been suggested that miconazole affects mem-

branes by competing with divalent cations for membrane binding sites[5]. Although both ketoconazole and miconazole have been shown to alter membrane permeability and function in yeast[2,5], the potential of these antimycotic imidazole compounds as inhibitors of membrane functions in other systems has not been investigated. This paper reports that ketoconazole affects sarcoplasmic reticulum from skeletal muscle by inhibiting calcium binding and uptake, by inducing calcium release and by stimulating both the Mg^{2+}-ATPase and the $(Ca^{2+}+Mg^{2+})$-ATPase activities.

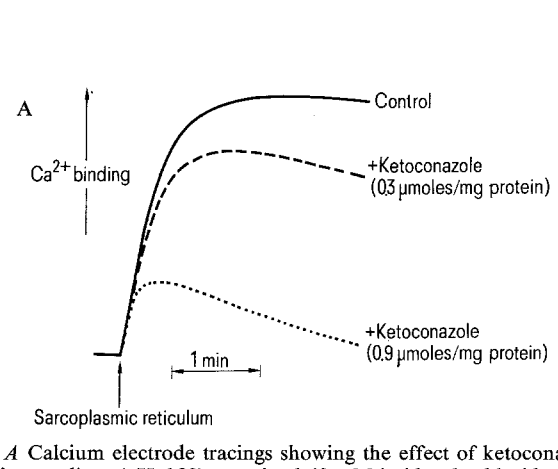
Materials and methods. ATP, bovine serum albumin, dithiothreitol (DTT) and imidazole were obtained from Sigma Chemical Corp. Ketoconazole was a gift from Dr H. Van den Bossche, Janssen Pharmaceutica. All other reagents were analytical grade. Sarcoplasmic reticulum was isolated from porcine longissimus dorsi muscle immediately post-mortem by the method of Martonosi and Feretos[6]. Calcium binding, accumulation and release were measured in a temperature-controlled vessel using a Radiometer calcium selective electrode (F2112 Ca Selectrode) connected to a Radiometer PHM 64 research pH meter and a recorder. The term 'calcium binding' is used to describe the ATP-dependent uptake of calcium in the absence of a precipitating anion, and 'calcium accumulation' to describe the uptake in the presence of the precipitating anion oxalate. Calcium binding, accumulation and release were calculated from the calcium electrode traces by back titration with calcium at the end of the experiment and also by plotting calibration curves of changes in millivolts following additions of calcium and replotting the data on a linear scale. The response of the calcium electrode was not affected by the concentrations of ketoconazole used in the experiments.

Mg^{2+}-ATPase and $(Ca^{2+}+Mg^{2+})$-ATPase activities were estimated at 37 °C by determining liberated inorganic phosphate[7]. Protein was determined according to Lowry et al.[8], using bovine serum albumin as standard.

Results and discussion. Sarcoplasmic reticulum from porcine longissimus dorsi muscle is able to bind and retain about 0.13 µmoles calcium per mg protein in the absence of any precipitating anion. In the absence of ketoconazole, all the bound calcium is retained for at least 5 min before a very slow leakage of a little calcium occurs. Figure 1A shows that ketoconazole affects both calcium binding and retention. At all ketoconazole concentrations greater than 0.15 µmoles per mg protein, there is some inhibition of calcium binding and the bound calcium is not retained by the sarcoplasmic reticulum. The figure shows that at a ketoconazole concentration of 0.3 µmoles per mg protein calcium binding is inhibited by 19%, and at 0.9 µmoles per mg protein, calcium binding is inhibited by 61%. All the bound calcium was subsequently released within 3–10 min of the commencement of calcium binding.

When added to sarcoplasmic reticulum which had previously bound exogenous calcium, ketoconazole induces a rapid release of some or all of the bound calcium. The relationship between this calcium release induced by ketoconazole and the inhibition of initial calcium binding is shown in figure 1B. The amount of calcium released depends upon the concentration of ketoconazole, with all the previously bound calcium being released by 1 µmole of ketoconazole per mg protein. At this concentration of ketoconazole initial calcium binding was only 34% of the control value.

In the presence of oxalate, calcium is accumulated by sarcoplasmic reticulum and precipitated inside the vesicles as calcium oxalate. Under these conditions large amounts of calcium can be accumulated by the sarcoplasmic reticulum, but the rate[6] is independent of the calcium concentration between 10^{-5} M and 4×10^{-4} M. Figure 2A shows that in the presence of 5 mM oxalate, ketoconazole inhibits both the total calcium accumulation and the rate of calcium accumulation. Unlike the calcium binding experiments, calcium accumulated under these conditions in the presence of ketoconazole (fig. 2A, ---- and ····) was not spontaneously released during the first 10 min following commencement of calcium accumulation, nor could rapid, complete release of accumulated calcium be induced by ketoconazole. An addition of 8.2 µmoles ketoconazole per mg protein added to the control experiment shown in figure 2A induced a very slow leakage of calcium, resulting in complete calcium release after 35 min. This is in contrast to the situation observed in the absence of oxalate, when an addition of only 1 µmole ketoconazole per mg protein caused complete release of previously bound calcium within 1 min. The effect of concentration of ketoconazole on calcium accumulation by sarcoplasmic reticulum in the



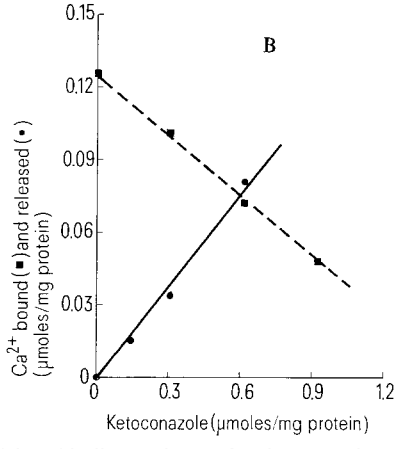


Figure 1. *A* Calcium electrode tracings showing the effect of ketoconazole on calcium binding and retention by sarcoplasmic reticulum. The reaction medium (pH 6.89) contained 40 mM imidazole chloride, 100 mM KCl, 4 mM $MgSO_4$, 1.16 mM ATP and 54 µM $CaCl_2$ in a total volume of 5.0 ml. Ketoconazole was added to the reaction medium at the appropriate concentration prior to sarcoplasmic reticulum (1.3 mg). Temperature 37 °C. *B* Effect of ketoconazole on calcium binding (■) and induction of release (●) of previously bound calcium from sarcoplasmic reticulum. For the calcium binding experiments (■), ketoconazole was added to the reaction medium before sarcoplasmic reticulum. For the induction of release (●) of previously bound calcium, ketoconazole was added to the reaction vessel 2 min after binding was complete. Calcium release was measured 1 min after ketoconazole addition.

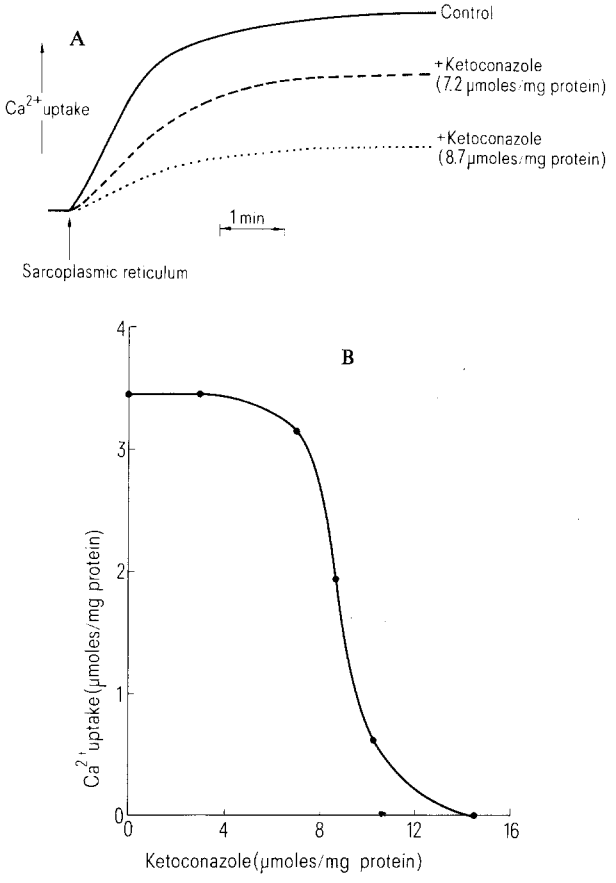


Figure 2. *A* Calcium electrode tracings showing the effect of ketoconazole on calcium accumulation. Experimental conditions as described in the legend to figure 1 except that 5 mM potassium oxalate was added to the reaction medium, and 0.08 mg sarcoplasmic reticulum protein was used instead of 1.3 mg. *B* Inhibition of sarcoplasmic reticulum calcium accumulation by ketoconazole.

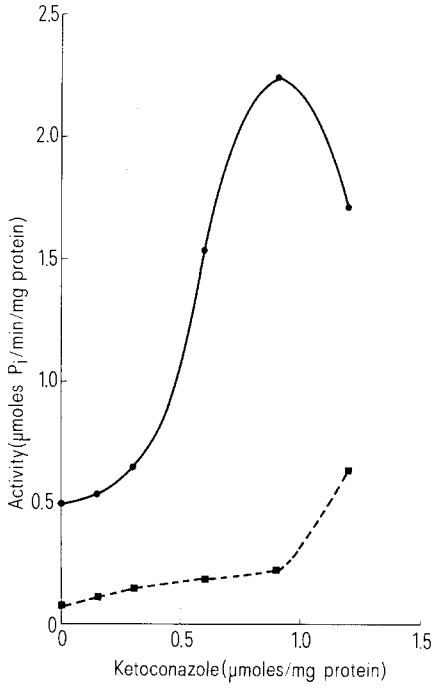


Figure 3. Effect of ketoconazole on the Mg^2-ATPase (■) and (Ca^{2+} Mg^{2+})-ATPase (●) activities of sarcoplasmic reticulum at 37 °C. The reaction medium (pH 7.2) contained 40 mM imidazole, 100 mM KCl and 4 mM $MgSO_4$. The Mg^{2+}-ATPase activity was estimated in the presence of 2 mM EDTA, and the (Ca^{2+} + Mg^{2+})-ATPase activity in 0.05 mM $CaCl_2$. Total volume, 2.0 ml. Ketoconazole was added in the appropriate concentration prior to equilibration of the reaction medium with 0.8 mg sarcoplasmic reticulum protein at 37 °C for 4 min. The reaction was started by addition of 4 mM ATP, and stopped after 1 min by addition of 2 ml ice-cold perchloric acid (4%).

presence of 5 mM oxalate is illustrated in figure 2B. Complete inhibition of calcium accumulation was obtained with 14 μmoles ketoconazole per mg protein, a concentration very much higher than that required to inhibit calcium binding (see fig. 1B). For example, 0.9 μmoles of ketoconazole per mg protein inhibits calcium binding by 61% but has no effect on calcium uptake in the presence of oxalate at this concentration.

Ketoconazole also affects the Mg^{2+}-ATPase and the (Ca^{2+} + Mg^{2+})-ATPase activities of sarcoplasmic reticulum (fig. 3). Ketoconazole stimulates the activity of both enzymes at low concentrations and inhibits at high concentrations. The activity of the Mg^{2+}-ATPase is very low initially (0.07 μmoles P_i per min per mg protein at 37 °C), and is stimulated 3-fold by 0.9 μmoles ketoconazole per mg protein (fig. 3). Further increase in the concentration of ketoconazole up to 1.2 μmoles per mg protein results in a dramatic increase in the Mg^{2+}-ATPase activity from 0.21 μmoles P_i per min per mg protein to 0.6 μmoles P_i per min per mg protein. A decrease in the Mg^{2+}-ATPase activity is observed when the concentration of ketoconazole is further increased (not shown in fig. 3). The (Ca^{2+} + Mg^{2+})-ATPase activity is progressively stimulated by ketoconazole up to 0.9 μmole ketoconazole per mg protein (fig. 3). A decrease in the (Ca^{2+} + Mg^{2+})-ATPase activity is observed between 0.9 and 1.2 μmoles ketoconazole per mg protein, a concentration at which the Mg^{2+}-ATPase activity is markedly stimulated by ketoconazole. At the same range of ketoconazole concentration, calcium binding is inhibited by 61–80% (fig. 1B).

The results suggest that ketoconazole probably affects the sarcoplasmic reticulum membranes by increasing the permeability of the sarcoplasmic reticulum to calcium. This is demonstrated by the induced release of calcium previously bound in the absence of ketoconazole, by the spontaneous release of the limited amount of calcium which binds in the presence of low concentrations of ketoconazole, and by the stimulation of ATPase activities. Ketoconazole may also compete with calcium for sarcoplasmic reticulum calcium binding sites, a possibility which is currently being investigated.

It seems most unlikely that ketoconazole has any effect on the sarcoplasmic reticulum membranes in vivo at the orally effective dose of 3.8–7.8 μg/ml in the plasma[9], since this level is far below that required in vitro to affect the functions of the sarcoplasmic reticulum. The amount of ketoconazole required to affect the function of the sarcoplasmic reticulum is at least 1000 times greater than that reported to inhibit the growth of yeast[2]. The present data show that ketoconazole can be used as an inhibitor of the calcium transport system of sarcoplasmic reticulum in in vitro studies, thus offering a useful alternative to fatty acids[10,11], chaotropic anions[12] and diethyl ether[13,14] for studies of sarcoplasmic reticulum calcium transport.

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2 H. Van den Bossche, G. Willemsens, W. Cools, F. Cornelissen, W.F. Lauwers and J. Van Cutsem, Antimicrob. Agents Chemother. *17*, 922 (1980).
3 J. Delhez, J.-P. Dufour, D. Thines and A. Goffeau, Eur. J. Biochem. *79*, 319 (1977).
4 F. Foury, M. Boutry and A. Goffeau, Archs int. Physiol. Biochim. *84*, 618 (1976).
5 K.H. Sreedhara Swamy, M. Sirsi and R. Rao, Antimicrob. Agents Chemother. *5*, 420 (1974).
6 A. Martonosi and R. Feretos, J. biol. Chem. *239*, 648 (1964).
7 R.J.L. Allen, Biochem. J. *34*, 858 (1940).
8 R.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
9 M. Borgers, M. de Brabander, H. Van den Bossche and J. Van Cutsem, Proc. 7th Congr. Human Anim. Mycol. (ISHAM), Israel, p.300. Eds E.S. Kuttin and G.L. Baum. Exerpta Medica, Amsterdam 1979.
10 W. Hasselbach and M. Makinose, Biochem. biophys. Res. Commun. *7*, 132 (1962).
11 G. Swoboda, J. Fritzche and W. Hasselbach, Eur. J. Biochem. *95*, 77 (1979).
12 R. The and W. Hasselbach, Eur. J. Biochem. *53*, 105 (1975).
13 W. Fiehn and W. Hasselbach, Eur. J. Biochem. *9*, 574 (1969).
14 G. Salama and A. Scarpa, J. biol. Chem. *255*, 6525 (1980).

Crossing-over in the female hybrids between *Drosophila simulans* and *Drosophila mauritiana*[1]

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Summary. The cross between *D. simulans* and *D. mauritiana* yields fertile hybrid females. Crossing-over occurs in such female hybrids. This system allows the transfer of pieces of chromosomes from one species into another, thus providing an experimental tool to analyze the genetic basis of evolutionary problems, as well as the regulation of gene expression during development.

Speciation is the process by which one species splits into 2 or more species. Species are defined as Mendelian populations whose members share a common gene pool, and between which gene exchange is prevented by reproductive isolating mechanisms (RIMs). Subsequently, 2 gene pools (species) evolve independently.

What are the genetic changes that underlie the development of reproductive isolation, and hence lead to the formation of new species? How many genes are involved in the establishment of the different kinds of RIMs? Do RIMs arise as a result of changes in a relatively small number of genes, or are they the by-product of accumulated changes in many loci?

It has been recognized that hybrids between different species might constitute a good system for studying evolutionary problems. Hybrids are formed between species that have diverged recently in evolution. A genetic analysis of such hybrids should help to reveal the genetic differences required to separate 2 species. Limitations arise because of the impossibility of obtaining certain hybrids due to RIMs. Furthermore, when 2 different species can be crossed and give rise to adult hybrids, the genetic analysis can usually not be taken beyond the 1st generation, either because the hybrids are sterile, or because genetic markers in the parental species are not known.

David et al.[3] reported that the cross between *D. simulans* and *D. mauritiana* yields fertile females and sterile males. Both species belong to the *melanogaster* subgroup. Several genetic markers are available in *D. simulans* and a few in *D. mauritiana*, so that it is possible to test whether crossing-over occurs in the female hybrids *simulans-mauritiana*. I have analyzed the 3 major chromosomes; the X, the 2nd and the 3rd chromosomes, for which genetic markers are available. *D. simulans* females homozygous for certain genetic markers were crossed with wild-type *D. mauritiana* males and the hybrid females of such a cross were backcrossed to *D. simulans* males homozygous for the same genetic markers as the parental females. The results are

Crossing-over between the X chromosomes, between the 2nd chromosomes and between the 3rd chromosomes in the female hybrids *simulans-mauritiana*. The numbers in parentheses represent percentages. The frequency of crossovers in female hybrids for the different chromosomal intervals were: yellow-forked *(y-f²)*: 42%; net-polychaete *(net-py)*: 49%; polychaete-plum *(py-pm)*: 31%; javelin-scarlet *(jv-st)*: 35% and scarlet-peach *(st-p)*: 41%. The flies were kept at 25 °C on standard food. Special care was taken to culture the flies in uncrowded conditions, so that competition between the different recombinant genotypes was practically eliminated. The marker mutations are described by Sturtevant[4]. *net* and *jv* are mutations homologous to the mutations described in *D. melanogaster*. *st p* homozygous flies have a phenotype clearly distinguishable from *st* or *p* alone. Some of the stocks of *D. simulans* were provided by Dr J. Puro (Turku) and the *D. mauritiana* flies were provided by Dr R.C. Woodruff (Bowling-Green).

Chromosome	Genotype of hybrid female	Genotype of *D. simulans* male	Total progeny scored	Phenotype and number of recombinants					
X	$\frac{yf^2}{++}$	$\frac{yf^2}{Y}$	1129	y 263 (23.3)			f^2 212 (18.7)		
2nd	$\frac{net\,py\,pm}{+\,+\,+}$	$\frac{net\,py\,pm}{net\,py\,pm}$	1175	net 238 (20.2)	py pm 172 (14.6)	net py 99 (8.4)	pm 104 (8.8)	net pm 72 (6.1)	py 93 (7.9)
3rd	$\frac{jv\,st\,p}{+\,+\,+}$	$\frac{jv\,st\,p}{jv\,st\,p}$	1037	jv 119 (11.4)	st p 109 (10.5)	jv st 119 (11.4)	p 162 (15.6)	jv p 73 (7)	st 70 (6.7)