The development-inducing effects of implantation of various portions of the neuroendocrine system from development- to diapause-programmed larvae of *Sarcophaga argyrostoma*

Implant	Number of larvae	Pupae developing to adult flies	
		Number	%
3 CNS+RG	27	25	92.6***
3 CNS+CC	13	5	38.5**
3 CNS	22	1	4.5 n.s.
3 RG	22	2	9.1 n.s.
Sham operated	26	1	3.8
Cold narcosis	26	1	3.8

CNS+RG, brain plus entire ring gland; CNS+CC, brain plus corpus cardiacum (i.e. with prothoracic gland and corpus allatum cut away); CNS, brain without ring gland; RG, ring gland alone. ***$p < 0.001$; **$p < 0.01$; n.s., not significant, compared with sham operated control.

hormone' is involved in programming diapause in *Sarcophaga*. On the other hand, implantation of 1, 2 or 3 CNS+RG from development-destined (short-night) larvae into diapause-committed (long-night) recipients, stimulated their development in a 'dose-dependent' fashion (fig. 2). Control implants from donors previously programmed for diapause by a series of long nights stimulated development to a much lesser degree. For example, in each pair of observations (1, 2 or 3 complexes) development was significantly higher for larvae receiving development-committed (short-night) implants (1 implant, $\chi^2 = 12.87$, $p < 0.001$; 2 implants, $\chi^2 = 10.47$, $p < 0.01$; 3 implants, $\chi^2 = 17.70$, $p < 0.001$), and 1 development-committed complex altered the diapause programme more strongly than 3 diapause-committed ones.

Our results thus show that clear differences exist between the complexes of larvae programmed for continuous development or for diapause, indicating that the endocrine potentials of such complexes are differentiated several days before diapause occurs. In this respect these observations differ from those in which endocrine differences immediately responsible for diapause or development were demonstrated[17].

To find out which portions of the neuroendocrine complex contribute to the stimulation of development, we implanted 3 CNS with corpora cardiaca (CNS+CC) from short-night donors into long-night recipients. This treatment prevented diapause in only 5 (38.5%) of the 13 insects (table). Furthermore, brains alone (CNS) or ring glands alone (RG) had no significant effect. Thus an intact PTTH-ecdysone axis is important for preventing diapause, which might suggest that prothoracotropic activity of the brain and the production of ecdysteroids were enhanced in the larvae committed for development by short nights in comparison with those committed for pupal diapause by long nights.

Our present results suggest that implanted complexes might change the hormonal balance in the recipients, in turn effecting feedback interactions[18] between the levels and timings of PTTH, ecdysteroids (and perhaps juvenile hormones)[12,19] which together regulate diapause or development in the pupa. On the other hand, implanted complexes might merely survive metamorphosis and produce their hormones, as programmed, to initiate adult development in otherwise diapausing pupae.

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2 Fraenkel, G., and Hsiao, C., J. Insect Physiol. *14* (1968) 689.
3 Saunders, D.S., J. Insect Physiol. *17* (1971) 801.
4 Denlinger, D.L., Biol. Bull. mar. biol. Lab. Woods Hole *142* (1972) 11.
5 Saunders, D.S., J. comp. Physiol. *97* (1975) 97.
6 Saunders, D.S., J. comp. Physiol. *110* (1976) 111.
7 Saunders, D.S., J. comp. Physiol. *124* (1978) 75.
8 Saunders, D.S., in: Biological Clocks in Seasonal Reproductive Cycles, p. 67. Eds B.K. Follett and D.E. Follett. John Wright, Bristol 1981.
9 Fraenkel, G., and Hsiao, C., J. Insect Physiol. *14* (1968) 707.
10 Žďárek, J., and Denlinger, D.L., J. Insect Physiol. *21* (1975) 1193.
11 Gibbs, D., J. Insect Physiol. *22* (1976) 1195.
12 Walker, G.P., and Denlinger, D.L., J. Insect Physiol. *26* (1980) 661.
13 Shaaya, E., and Karlson, P., J. Insect Physiol. *22* (1965) 65.
14 Ohtaki, T., and Takahashi, M., Jap. J. med. Sci. Biol. *25* (1972) 369.
15 Saunders, D.S., Physiol. Ent. *5* (1980) 191.
16 Denlinger, D.L., Life Sci *19* (1976) 1485.
17 Cloutier, E.J., Beck, S.D., McLeod, D.G.R., and Silhacek, D.L., Nature *195* (1962) 1222.
18 Saunders, D.S., Physiol. Ent. *6* (1981) 99.
19 Hiruma, K., Appl. Ent. Zool. *14* (1979) 76.

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Allophenic mice produced from embryos aggregated with antibody[1]

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Summary. One difficulty in the production of allophenic mice by aggregation of preimplantation embryos is that they frequently roll apart before the bonds between the blastomeres have had time to form. One solution to the problem, described here, is to pretreat one of the embryos with rabbit anti-mouse serum just prior to pushing them together. Blastocyst formation is unhampered by antibody treatment, and numerous allophenic mice have already been produced with this new procedure.

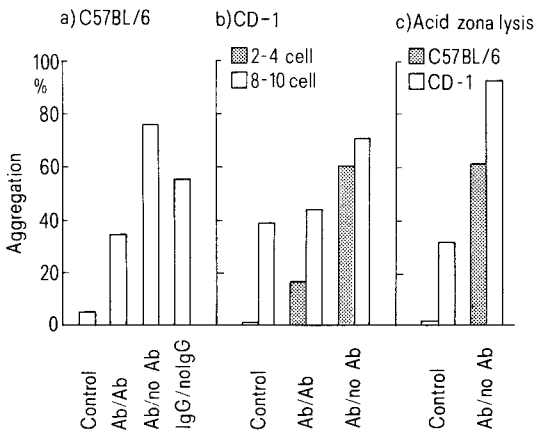
Mice composed of genotypically distinct populations of cells frequently reveal information unobtainable from studies of their pure strain counterparts. Allophenic mice represent one class of such genetic mosaics, and they are routinely produced in vitro by aggregation of preimplantation embryos from 2 strains of mice[3]. These composite embryos are then allowed to develop to term in the uteri of pseudopregnant foster mothers and thereby become fully developed mice, all of whose tissues usually contain cells from each strain[4]. The Ist part of the procedure involves

removal of the zona pellucida from appropriately staged embryos (8–10 cells) with a concentrated solution of pronase[5]. Following this, the embryos of different genotypes are placed in contact with each other, and within several minutes bonds are formed between blastomeres of the 2 embryos, thereby allowing them to develop as a unit. Prior to the formation of such bonds, the embryos are not very adherent and have a tendency to roll apart. We report here that antibodies recognizing embryonic cell surface components can be used as ligands to hold the embryos together long enough to allow the formation of their own natural bonds, and that use of such antibodies does not adversely affect the viability of treated embryos.

Methods. The mice used in these experiments were C57BL/6J and DBA/2J from the Jackson Laboratory, Bar Harbor, Maine, and random bred Swiss-ICR (CD-1) from Charles River Breeding Laboratories, Portage, Michigan.

Rabbits used for the production of antisera were New Zealand white males obtained from a local dealer. Each rabbit received multiple s.c. injections of homogenized spleen and liver (2 mice) emulsified in Freund's complete adjuvant, followed 17 days later with an i.v. injection of a cell-free homogenate of liver and spleen from 3 mice. 4 weeks later the rabbits were bled and the sera tested by hemagglutination of C57BL/6J red blood cells. Prior to their use in the embryo aggregation assays, the sera were heat inactivated at 56 °C for 1 h. The immunoglobulin (IgG) fraction was isolated as described by Levy and Sober[6].

Preimplantation embryos were flushed from the uteri of spontaneously mated females. 8–10 cell embryos were recovered the morning of the 2nd day of pregnancy (the day of vaginal plug is day 0) and 2–4 cell embryos were obtained the afternoon of day 1. Unless noted otherwise, the zona pellucida was removed by a brief treatment with 0.5% pronase[5]. Embryos were flushed and aggregated in medium composed of the following ingredients: Eagles minimum essential medium, 45 ml; heat inactivated fetal calf serum, 5 ml; 7.5% sodium bicarbonate, 0.45 ml; solution of 5000 IU/ml penicillin and 5 mg/ml streptomycin, 0.5 ml (all from Flow Laboratories); 25 mM sodium pyruvate (Cal-Biochem), 0.5 ml; DL-sodium lactate (60% syrup, Sigma), 0.1 ml. The medium was maintained continuously under an atmosphere of 5% CO_2 in air at 37 °C.



Effect of rabbit antiserum on aggregation of preimplantation embryos. Adherence was tested for untreated embryos (control); embryo pairs, both of which had been pretreated with unfractionated rabbit antiserum (Ab/Ab); embryo pairs, only one of which had been treated with antiserum (Ab/no Ab) or the IgG fraction of the antiserum (IgG/no IgG). Pronase digestion was used to remove the zona pellucida from the 8–10 cell C57BL/6J embryos in figure a and CD-1 embryos in figure b. Of the latter embryos, 2 stages were tested, 2–4 cell (crosshatched) and 8–10 cell (shaded). In figure c the effects of antiserum pretreatment was tested on agglutination on 8–10 cell embryos, from which the zonas had been removed by a brief exposure to pH 2.5 PBS, 1% polyvinylpyrrolidone (PVP-40T, Sigma). Embryos from C57BL/6J (crosshatched) and CD-1 (shaded) were tested.

The assay used to test embryo aggregation was as follows. Immediately after zona removal, pairs of embryos were placed in siliconized glass culture dishes where they were gently rolled together with a finely drawn glass rod. After they were allowed to sit undisturbed for 1 min at 37 °C, a stream of medium was gently blown over them from an egg pipette (250 µm ID). Pairs which remained bound together were recorded as successfully aggregated. This procedure was repeated 6 times for each pair. All of the results reported here for each experiment were derived from an average of 10 pairs of embryos. Antibody treatment of the embryos involved bathing them 2–3 min in a droplet of undiluted serum; unbound antibody was subsequently removed by extensive washes in medium.

Results. Sera from 2 rabbits hyperimmunized to mouse spleen and liver were harvested and, when tested against red blood cells from C57BL/6J mice, gave hemagglutination titers of 400 and 800. Results with the latter serum are given below; the former serum was also tested and gave similar results.

Results with the aggregation assay revealed a striking increase in embryo agglutination mediated by antiserum pretreatment. This was the case for 8–10 cell embryos from both C57BL/6J (fig., a) and DBA/2J (not shown).

Interestingly, the most effective procedure was to treat only 1 embryo of each pair with antiserum, although treatment of both embryos also increased their adherence in comparison with untreated controls. The response of 8–10 cell embryos from the random-bred CD-1 strain was also tested. Although the antibody treatment increased their agglutinability (fig., b), the adherence of untreated CD-1 embryos at that stage was considerably higher than that found for C57BL/6J or DBA/2J (fig., a and b). This difference suggests the involvement of genetic factors in controlling such surface interactions. CD-1 embryos from the earlier 4-cell stage were much less tacky and aggregated poorly if unaided by antibody (fig., b). When pretreated with the rabbit antiserum, such embryos become much more adherent, especially if only 1 member of each pair was antibody coated.

Besides pronase digestion, an alternate means of zona removal employed by some investigators involves treating the embryos for a brief period of time in acidified (pH 2.5) Tyrodes or phosphate buffered saline[7,8]. 8–10 cell embryos whose zonas had been removed by acid treatment were tested for aggregation with and without previous exposure to antibody. By comparison with the control pairs, aggregation was significantly enhanced when 1 member of each pair was previously exposed to antibody. This was the case for both CD-1 and C57BL/6J embryos (fig., c).

The component of the rabbit antiserum responsible for its aggregating activity is likely to be antibody. Isolated IgG from the antiserum was effective in promoting aggregation of late cleavage C57BL/6J embryos (fig., a), although it was not as active as the unfractionated antiserum. (It could be that IgM is also an active component, although this was not tested.) Since all of the adherence assays were carried out in fetal calf serum, the antiserum-enhanced agglutination observed here is unlikely to be the result of some nonspecific serum effect.

It may not always be necessary to use hyperimmune serum to promote embryo aggregation. Sera from nonimmunized rabbits were tested in the aggregation assay. Sera from 2 individual rabbits each resulted in less than 10% en-

hanced agglutinability, whereas a batch of pooled normal rabbit sera (Cappel Laboratories) increased embryo agglutinability over 60%. This was not surprising since rabbit sera are known to contain natural antibodies reactive with mouse tissues, and individual rabbits differ among themselves in the amount of such antibodies[9]. Thus, it is better to use hyperimmune serum in this procedure to ensure the presence of agglutinating antibodies.

Trials were made to determine whether antibody treatment affected the capacity of cleavage stage embryos to form blastocysts. The results indicate that exposure to antibody has no adverse effects on blastocyst formation by DBA/2J morulae. This was the case with regard to the percentage of embryos reaching blastocyst stage as well as the time at which they were formed. 39 of 43 morulae (91%) treated with antibody reached the blastocyst stage, in comparison to 36 of 39 (92%) control embryos. Moreover, we have begun to employ the rabbit antiserum to facilitate aggregation in the routine production of allophenic mice and have already produced 85 chimeric animals with the technique.

This report shows that rabbit antisera can be used to promote aggregation in the production of allophenic mice. It is effective, easy to apply, and results in no adverse effect on the embryo development. This procedure represents an alternative to that reported by Mintz et al.[10], where it was shown that the plant lectin phytohemagglutinin is also effective in mediating blastomere aggregation. Use of antibody represents an easier means of agglutinating the embryos. Application of phytohemagglutinin involves agglutinating the embryos in its presence for several minutes, followed by extensive washes to remove excess lectin from the delicately bound embryo pairs. Complete removal of unbound lectin is likely to be important, considering the well known toxic effect it has on cultured cells (for example, see Stanley et al.[11]). The procedure with antibody involves bathing the embryos in antiserum and washing them prior to aggregation, with no further need for manipulation following the formation of embryo pairs.

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2 To whom reprint requests should be addressed.

3 Mintz, B., in Methods in mammalian embryology, p. 186. Ed. J.C. Daniel. Freeman, San Francisco 1971.
4 Mintz, B., A. Rev. Genet. *8* (1974) 411.
5 Mintz, B., Science *138* (1962) 594.
6 Levy, H.B., and Sober, H.A., Proc. Soc. exp. Biol. Med. *103* (1960) 250.
7 Hall, B.V., Proc. Soc. exp. Biol. Med. *32* (1935) 747.
8 Rossant, J., and Vikj, K.M., Devl Biol. *76* (1980) 475.
9 Boyse, E.A., Hubbard, L., and Stockert, E., Transplantation *10* (1970) 446.
10 Mintz, B., Gearhart, J.D., and Guymont, A.D., Devl Biol. *31* (1973) 195.
11 Stanley, P., Caillibot, V., and Siminovitch, L., Somat. Cell Genet. *1* (1975) 3.

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New metabolites of the naturally-occurring mutagen, quercetin, the pro-mutagen, rutin and of taxifolin

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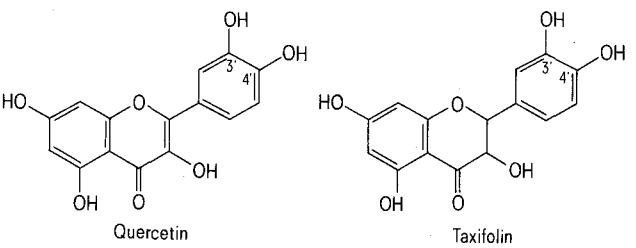
Summary. The major biliary metabolites of the mutagen, quercetin, the pro-mutagen rutin and taxifolin have been identified by EI-mass spectrometry, UV-spectroscopy and chromatographic methods as conjugates of the corresponding 3′-O-methyl ethers. The toxicological significance of these findings is discussed.

The flavonol, quercetin, which has recently been shown not only to be mutagenic in the Salmonella/microsomal assay[3,4] but also to be carcinogenic in the rat[5], is known to be widely distributed in glycosidic forms in the plant kingdom[6] and recently evidence has been presented that the quercetin glycoside, rutin, which is known to be present in edible plants, although non-mutagenic in the Salmonella/microsomal assay, is activated by incubating with a glycosidase preparation ('fecalase'), derived from the human intestine[7].

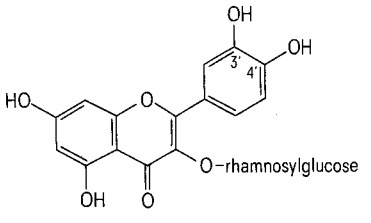
Since evidence has recently been presented that the related 3′,4′-*o*-dihydric flavanols are largely metabolized in the liver by 3′-O-methylation and that conjugates of the 3′-O-methyl ethers are subsequently excreted in large amounts in bile and urine[8–10], it appeared of interest to establish whether the mutagen, quercetin, and the 2 structurally-related compounds, rutin (quercetin-3-rhamnoglucoside) and taxifolin (dihydroquercetin) are metabolized by this route.

Experimental. Animals. Groups of 3 rats (300–350 g) of the Wistar strain were employed under conditions and on a diet previously described[11].



Quercetin

Taxifolin



Rutin