with the succinic dehydrogenase preparation in D_2O, were isolated, and their respective deuterium content was determined. The results are outlined in Table 1, experiment 2. Thus, while the malate exhibited a high deuterium content, as was anticipated, the fumarate had an exceedingly low deuterium content. Since the reaction proceeded long after the fumarase equilibrium had been reached, ample opportunity had been allowed for the fumarate to become more highly labeled if the fumarase had behaved nonstereospecifically.

Similar results have been obtained by Fisher (*5*) using crystalline fumarase. These experiments thus establish the strict stereospecific behavior of fumarase and add strength to our proposed mechanism of the anaerobic exchange reaction as catalyzed by succinic dehydrogenase.

The afore-described results with fumarase, as well as the recent studies on alcohol dehydrogenase (*6, 7*), have shown that these enzymes are endowed with the property of distinguishing between two hydrogen atoms on a methylene carbon. In order to test the possibility of a similar behavior by succinic dehydrogenase, deuteriosuccinate, obtained from an anaerobic incubation of succinate and fumarate in D_2O with a succinic dehydrogenase preparation under conditions similar to those reported in Table 1, experiment 2, was oxidized aerobically with the same enzyme, and the deuterium content of the resulting fumarate was measured. The results are given in Table 2.

The results clearly indicate that the fumarate obtained from the enzymatic oxidation of the enzymatically exchanged deuteriosuccinate retains about one-half of the deuterium, leading to the conclusion that succinic dehydrogenase is incapable of distinguishing between the hydrogens on either methylene carbon. This conclusion is based on the assumption that a single enzyme is responsible for the exchange reaction represented by Eqs. 1 and 2, and that this enzyme is also the one concerned in the aerobic oxidation of succinate. The seeming discrepancy between the deuterium content of the succinate before and after 50 percent of it had been oxidized is probably the result of an isotope effect that causes preferential oxidation of the unlabeled succinate molecules (*8*) and leads to an apparently higher deuterium content of the residual succinate and a somewhat lower than theoretical content of the resulting fumarate.

Table 2. Enzymatic oxidation of enzymatically labeled succinate.

Acids	Deuterium (atoms/molecule)
Deuteriosuccinate obtained from an anaerobic enzymatic exchange reaction	0.100
Succinate after 50 percent of above deuteriosuccinate was enzymatically oxidized	0.135
Fumarate resulting from the deuteriosuccinate oxidation	0.044

The small amount of deuterium found in the fumarate in experiment 2 of Table 1 probably arose from the initially formed deuteriosuccinate by a nonstereospecific reversal of reaction 2, and its low value probably reflects the dilution of the isotope by the initially added normal fumarate. However, in other experiments not reported here, a greater incorporation of label into fumarate was observed, the reasons for which are not clear at present. It was found, however, that this increased labeling depended on the addition of succinate, as would be expected from the scheme proposed here.

References and Notes

* Postdoctoral research fellow of the American Heart Association.

1. This paper is contribution No. 109 of the McCollum-Pratt Institute. The work was aided by a grant from the American Cancer Society, as recommended by the Committee on Growth of the National Research Council. We wish to thank H. F. Fisher for his valuable criticism of this paper.
2. E. O. Weinman, M. G. Morehouse, R. J. Winzler, *J. Biol. Chem.* **168**, 717 (1947).
3. E. C. Slater, *Biochem, J. (London)* **45**, 1 (1949).
4. D. E. Green, P. M. Kohout, S. Mii, *Biochim. et Biophys. Acta* **14**, 295 (1954).
5. H. F. Fisher, personal communication.
6. H. F. Fisher *et al., J. Biol. Chem.* **202**, 687 (1953).
7. F. A. Loewus, F. H. Westheimer, B. Vennesland, *J. Am. Chem. Soc.*, **75**, 5018 (1953).
8. M. B. Thorn, *Biochem. J. (London)* **49**, 602 (1951).

21 February 1955.

Fertilization and Normal Development of Follicular Oocytes in the Rabbit

M. C. Chang

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

The growth, maturation, and activation of rabbit follicular oocytes *in vivo* and *in vitro* (*1*) and the nuclear maturation of rabbit oocytes in culture and their fertilization in Fallopian tubes (*2*) have been studied. In the rat, the fertilization and development of follicular oocytes when transferred into the ovarian bursa of mated rats have been reported (*3*). This paper is concerned with the fertilization and normal development of follicular oocytes of rabbits recovered at various times after gonadotropic hormone injection (*4*).

The donor rabbits were intravenously injected with sheep pituitary extracts to induce ovulation, which occurs, as in the case of mating, about 10 hr later. At various intervals after injection, the ovaries were removed and suspended in serum-saline medium (equal volumes of rabbit serum and 0.9 percent NaCl) in a watch glass. Under a dissecting microscope, the follicles were broken with a needle, and the oocytes surrounded by mucous follicular cells were picked up with a pipette. In order to facilitate the manipulation of oocytes, they were treated with 0.05 percent hyaluronidase in serum-saline medium for 5 to 10 min and then were resuspended in serum-saline medium. In addition, the oocytes recovered from uninjected ani-

mals were cultured in a Carrel flask for 8 to 10 hr in the serum-saline medium. Such oocytes or those recovered after pituitary hormone injection were then transferred into the left Fallopian tube of a recipient rabbit.

The recipient rabbits were artificially inseminated and injected intravenously with sheep pituitary extracts to induce ovulation. After 6 to 7 hr, a flank incision was made under Nembutal anesthesia, and the oocytes were transferred into the left tube through the infundibulum. This time interval was chosen because spermatozoa require about 6 hr in the female tract to develop their fertilizing capacity (*5*). Since it would be difficult to distinguish between the transferred oocytes and those shed by the recipient, the left ovary was removed at the time of operation. In order to supply enough progesterone for the maintenance of pregnancy, however, a single injection of 25 mg of macrocrystalline progesterone was given subcutaneously on the fourth to sixth day after operation. The recipient rabbits were sacrificed 19 to 22 days postoperatively. Table 1 presents the observations on the number of corpora lutea in the right ovary, the number of normal fetuses, and the number of degenerations after implantation (as shown by the presence of placenta without normal fetus).

Although there is a possibility that more follicular oocytes might have been fertilized than were actually implanted, judging from the number of degenerations after implantation and the number of normal fetuses, the data definitely show that the probability of fertilization is higher than the probability of normal development subsequently. Assuming that all the fertilized ova had implanted, the probability of fertilizaton (7 to 9 percent) and that of normal development (1 to 3 percent) was low when the oocytes were recovered from uninjected animals or from animals injected with gonadotropic hormone 2 to 5 hr previously. The probability of fertilization (24 percent) and that of normal development (14 percent) increased when the oocytes were recovered from rabbits 6 to 7 hr after injection, and it increased further to 40 percent and 28 percent, respectively, 11 to 12 hr after injection. Since ovulation occurs about 10 hr after injection, it seems that the probability of fertilization was increased at about 4 or 5 hr before ovulation—that is, at the tetrad stage of the first meiosis. It is rather surprising that fertilization and development are possible in the follicular oocytes of untreated animals. It may be that these oocytes were recovered from follicles at the wave of growth, as is reported by Smelser, Walton, and Whealthem (*6*).

In the unoperated side, when the number of normal and degenerated fetuses was compared with the total number of corpora lutea present in the ovaries, 26 percent prenatal death was deduced, and of this, 15 percent (26 − 11 = 15) of ova failed either to be fertilized or to get into the tube or uterus. The prenatal death is slightly lower than that reported by Hammond (*7*).

In the transfer of fertilized rabbit ova, 54 percent normal development was observed (*8*). In the present study, the normal development is only 28 percent, even when recently shed tubal ova were transferred. Besides the possible disturbance at fertilization under the experimental conditions, it seems that unfertilized ova are less able to tolerate manipulation *in vitro* than are fertilized ova. Rabbit ova are capable of fertilization up to 6 to 8 hr after ovulation (*7, 9*) and abnormal young are obtained when aged ova are fertilized (*10*). In the present study, no abnormal fetus was observed when immature oocytes were fertilized. This

Table 1. Fertilization and normal development of follicular oocytes in the rabbit.

Stages of oocytes (hours after ovulation inducing injection)	No. of recipients	Development in the transferred side			Development in the normal side		
		Oocytes transferred	Degeneration after implantation	Normal fetuses	Corpora lutea	Degeneration after implantation	Normal fetuses
0	6	79	5 (6%)	1 (1.3%)	36	6 (17%)	27 (75%)
2–3	6	61	4 (7%)	1 (1.6%)	29	7 (24%)	20 (69%)
4–5	6	58	3 (5%)	2 (3.5%)	40	2 (5%)	35 (88%)
6–7	6	50	5 (10%)	7 (14%)	36	0	30 (83%)
8–9	6	63	3 (5%)	17 (27%)	34	2 (6%)	24 (71%)
11–12 tubal	8	50	6 (12%)	14 (28%)	36	6 (17%)	28 (78%)
0 cultured for 8–10 hr	6	88	0	0	35	4 (11%)	19 (54%)
Total					246	27 (11%)	183 (74%)

indicates the difference in nature between immature and aged ova as it affects their later development.

In a previous study of follicular oocytes of unmated or pregnant rabbits (*2*), it was found that (i) nuclear maturation as far as first polar body formation occurred when the oocytes were transferred into the Fallopian tubes, (ii) fertilization occurred at the stage of first polar body formation following transfer into the tubes of mated animals, and (iii) the proportion of fertilized ova was increased when the oocytes were cultured for 12 hr before transfer. From the results of the present study, it seems that the maturation of oocytes as a whole is probably more important for their future development than nuclear maturation, because none of the cultured oocytes developed into fetuses, although their nuclear maturation was observed after culture and their fertilization should have occurred in the Fallopian tubes. On the other hand, it may be that culture of oocytes in serum has adverse effects on the cytoplasm, even though nuclear maturation occurs.

References and Notes

1. G. Pincus and E. V. Enzmann, *J. Exptl. Med.* **62**, 665 (1935) ; *J. Morphol.* **61**, 351 (1937).
2. M. C. Chang, *J. Exptl. Zool.* **128**, No. 2 (1955).
3. R. W. Noyes, *Fertility and Sterility* **3**, 1 (1952).
4. This work was supported by grants from the Josiah Macy, Jr., Foundation and the Dickinson Memorial Fund of the Planned Parenthood Association. Thanks are due to Faith Andrews for assistance and to G. Pincus for constant interest during this study. J. Seifter of Wyeth Institute kindly supplied the hyaluronidase, and C. Gaunt of the Ciba Co. supplied the progesterone.
5. M. C. Chang, *Nature* **168**, 697 (1951).
6. G. K. Smelser, A. Walton, E. O. Wheatham, *J. Exptl. Biol.* **11**, 352 (1934).
7. J. Hammond, *J. Exptl. Biol.* **11**, 140 (1934).
8. M. C. Chang, *Nature* **161**, 978 (1948).
9. ———, *J. Exptl. Zool.* **121**, 351 (1952).
10. ———, *Federation Proc.* **11**, 24 (1952).

2 March 1955.

Tumor Induction on *Nicotiana* Species by Use of Coconut Milk and Yeast Extract

August E. Kehr

Department of Horticulture, Iowa State College, Ames

It has been known for some time that coconut milk (*1*) and more recently crown gall tumor extracts (*2*) have a growth-promoting effect on explanted tissues. This paper (*3*) deals with the induction of nongenetic plant tumors on *Nicotiana* species (which otherwise have not been observed to form tumors) by the physiological action of coconut milk and yeast extract. Tumors of different origin but of somewhat similar nature have been reported on *N. alata* (*4*).

Surface sterilized seeds of *N. glauca, N. alata,* and the tumor-forming amphidiploid, *N. glauca-langsdorffii,* were grown on 8 ml of special media (adjusted to *p*H 5.9) in 2½-in. screw-top vials. The basal tobacco medium used in all cases was that of Hildebrandt and Riker (*5*); three supplemental media were made by adding coconut milk (15 percent by volume), malt extract (5 g/lit), and yeast extract (5 g/lit) to this basal medium. Both the malt and the yeast extract were the dried commercial product, and it is possible that different lots may vary in their potency to produce tumors. Autoclaving was done at 15 lb of steam pressure for 20 min. Each treatment consisted of 20 vials, although contamination and lack of germination resulted in fewer than this in some of the final readings.

Table 1. Incidence of tumor formation in sterile culture vials on seedlings of *N. glauca* and *N. alata* on various substrates (planted 24 Oct. 1953; readings taken 1 Dec. 1953).

Medium used	*N. glauca*		*N. alata*	
	Tumors (%)	Non-tumors (%)	Tumors (%)	Non-tumors (%)
Basal (containing 0.1 μg/lit NAA)	0	100*	0	100
Basal plus 15% coconut milk	95	5	25	75
Basal plus 2½% malt extract	0	100	0	100
Basal plus 2½% yeast extract	60	40	33	66
Basal plus coconut milk and malt extract	88	12	66	33
Basal plus coconut milk and yeast extract	20	80	0	100
Basal plus malt extract and yeast extract	58	42	100	0

* Percentage of total uncontaminated vials with sprouting seedlings.

The first experiment was completed using the species *N. glauca* and *N. alata.* It can be observed from the summary in Table 1 that in no instance did tumors develop on either species when the seedlings were germinated and grown on mineral media without supplements or on media containing only malt extract. However, tumors did appear more or less consistently on all seedlings growing on media that contained coconut milk, yeast extract, and combinations containing these two materials with malt extract (Fig. 1). The tumors were white in color, except when malt extract was present in the media; in this case they appeared as a brownish mass. They varied markedly in size from those just barely detectable to those about 10 to 12 mm in diameter. The tumors appeared to arise from the stem base, but the stem layer from which the tumor cells originated is unknown. The roots did not seem to be affected. It was observed that, subsequent to the appearance of the described tumors, other tumors appeared on the larger roots of a few cultures of *N. alata.* These latter tumors appeared to be similar to those described by Tryon (*4*).

Since the basal medium in the first experiment contained 0.1 μg/lit of naphthaleneacetic acid (NAA), a