they are quinacrine-bright and AT-rich. The validity of this assumption is confirmed by the fact that centromeric heterochromatin of the 2nd pair (not quinacrine-bright) is insensitive to the effect of distamycin A. The heterochromatin of each chromosome in *Drosophila melanogaster* has a unique quantitative and qualitative identity; by in situ hybridization[7], the absence from the centromeric heterochromatin of the 2nd pair of the 1.672 g/cm^3 DNA satellite (exclusively composed of AT BP), and the presence of the 1.705 g/cm^3 and of the 1.686 g/cm^3 DNA satellites (both characterized by the presence of GC BP as well), were demonstrated. Distamycin A is clearly inactive where GC BP are predominant or strategically placed.

2. Metaphases revealing a pairing between homologous undercondensed heterochromatic sections (fig. 2, a and b), which tend to stay in close proximity even at this stage of mitosis, when the somatic pairing, characteristic of *Drosophila* chromosomes, has usually disappeared. A fusion or coalescence of heterochromatin of homologous chromosomes (particularly sex chromosomes) may be evident.

3. Metaphases with close association of undercondensed heterochromatin (fig. 2, c–f). 'Chromocenter-like' structures connecting normally condensed euchromatic arms are evident, the unaffected 2nd pair being a marker of the metaphase stage. We designate this unspecific association between heterochromatic regions as 'ectopic pairing'[8]. By means of distamycin A, which prevents the normal condensation, the physical contact between the associated heterochromatin, typical of interphase and presumably due to similarity in base sequences[9] is preserved until the metaphase.

Discussion. On the basis of the frequencies of the different patterns observed, which are an expression of the progressive degree of spiralization, a sequence in the condensation of the heterochromatin may be suggested. Ectopic pairing, homologous pairing and elongation could reflect chronological steps in the continuous spiralization process. In fact heterochromatin in the interphase may be closely and unspecifically associated; as the condensation proceeds, the pairing becomes less tight and restricted to homologous regions, gradually disappearing at the start of mitosis.

These data may be compared with similar results obtained after treatment with Hoechst 33258 of *Drosophila melanogaster* gangliar cells[10] and of *Drosophila nasuta* embryonic and gangliar cells[11]. Different degrees of decondensation have been reported for the different chromosomes of *Drosophila melanogaster*[10] (the 2nd pair being unaffected also in this case), but the sequence of the condensation process has not been indicated. In *Drosophila nasuta*[11] a difference has been reported between the 2 types of cells considered, as regards response to the agent, since the embryonic cells seem to be more sensitive. Thus they offer a better potential for analysis; however, this is limited because there is less genetical knowledge of this species.

In conclusion, the high degree of resolution in the present analysis of the condensation process may be attributed to the decondensing agent (distamycin A) and to the cell system (embryonic cells) used.

Effect of distamycin A (100 µg/ml) treatment on heterochromatin of embryonic cells of *Drosophila melanogaster*

Hours of treatment	No. of metaphases analyzed	Normal	Affected Elongation	Homologous pairing	Ectopic pairing
5	937	524 (55.92%)	277 (29.56%)	75 (8.00%)	61 (6.51%)

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Development of adenocarcinomas after transplantation of rat glandular stomachs treated in vitro with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)[1]

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Summary. Glandular stomachs of fetal and newborn Wistar rats were transplanted s.c. after treatment in vitro with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) at concentrations of 10, 5 and 1 µg/ml for 2 h. Eleven adenocarcinomas developed from 118 MNNG-treated transplants, whereas no adenocarcinomas developed from 28 untreated transplants. The incidence of adenocarcinomas in fetal glandular stomach (9/46) was significantly different ($p < 0.01$) from that in glandular stomach of newborn rats (2/67). Various types of mesenchymal tumors also developed from untreated (9/28) and MNNG-treated (20/118) transplants.

Although MNNG is a potent carcinogen and is known to induce gastric carcinomas in rats[1], there have been few studies in vitro on its carcinogenetic effect on the glandular stomach epithelium[3,4], probably owing to the difficulty of culturing epithelial cells of the glandular stomach[5,6]. We have been studying organ cultures of newborn rat glandular stomach[7]. Using the organ culture technique in combination with the transplantation method, we succeeded in transforming glandular stomach epithelium after treatment with MNNG in vitro.

Materials and methods. The glandular stomachs of fetuses (16–20 days) and newborn rats (within 72 h after birth) of

the Wistar strain (Shizuoka Experimental Animal Inc., Japan) were separated aseptically from the forestomach at the limiting ridge for use in this experiment. Explants of about 1×3 mm were made with De Wecker's iridectomy scissors under a dissecting microscope. Three explants of the mucosal surface were placed on a Millipore filter (pore size 1.2 µm) on a stainless steel grid in a 60-mm plastic organ culture dish (Falcon Plastics, USA). MNNG (Aldrich Chemical Co., Ltd, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical, USA) at concentrations of 10, 5 and 1 µg/ml in phosphate buffered saline (PBS) just before use. The concentration of DMSO never exceeded 0.1%. The explants were treated with 0.15–0.2 ml of MNNG solution, which was just enough to cover the mucosal surface, for 2 h at 37 °C under 5% CO_2 in air. After MNNG treatment, the explants on Millipore filters were washed thoroughly with PBS. Control explants were treated with 0.1% DMSO in PBS alone. The explants were then detached from the filter and finely minced with a razor. Two explants of newborn glandular stomach and three from fetuses were transplanted with a trocar into the subcutis of the back of 6-week-old male Wistar rats. The recipients were observed weekly and killed if they became moribund. The experiment was terminated in week 52. The tissues were fixed with 10% neutral formalin and stained with hematoxylin and eosin (H.E.) or alcian blue-periodic acid-Schiff reagent.

Results. A total of 146 rats received explants of the glandular stomach of MNNG-treated or untreated fetal or newborn rats. Transplantability, when expressed as the ratio of the number of rats with a cyst(s) or tumor(s) at the transplantion site to the number of rats with transplants, was very high, ranging from 78 to 96% in both MNNG-treated and untreated explants (table 1). In all, 130 of 146 (89%) transplants in recipient rats were successful.

Transplanted glandular stomachs formed various sized cysts in the subcutaneous tissue, containing sero-mucinous fluid of pH 7–8.

Microscopically, the wall of the cysts was seen to be lined with mucosal epithelium, which was classified into 2 types according to its morphological differentiation as 'specific' or 'nonspecific'. 'Specific differentiation' was the state when the cysts were lined with epithelium forming a glandular structure and pits, the types of epithelium being similar to those in the glandular stomach of mature rats (fig. 1); 'nonspecific differentiation' was the state when the cysts were lined with a few layers of undifferentiated columnar or cuboidal cells (fig. 2). Cysts were considered to be specifically differentiated when a minute area with specific differentiation was found in a large area of nonspecific differentiation.

Table 2 shows the differentiation of the mucosa of the transplanted rat glandular stomachs examined histologically. In both MNNG-treated and untreated transplants of fetal and newborn glandular stomachs, differentiation of the mucosal epithelium of the cysts was classified as 'specific' in most cases.

The tumors that developed from the transplants are listed in table 3. Adenocarcinomas of the glandular stomach developed in 11 transplants treated with MNNG; these were 9 adenocarcinomas from 46 transplants of MNNG-treated fetal glandular stomachs (19.6%) and 2 adenocarci-

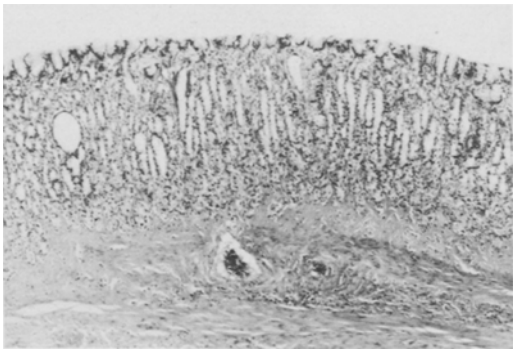

Figure 1. Mucosa of untreated fetal rat glandular stomach similar to mature gastric mucosa, which was classified as showing 'specific differentiation'. H.E. × 40.

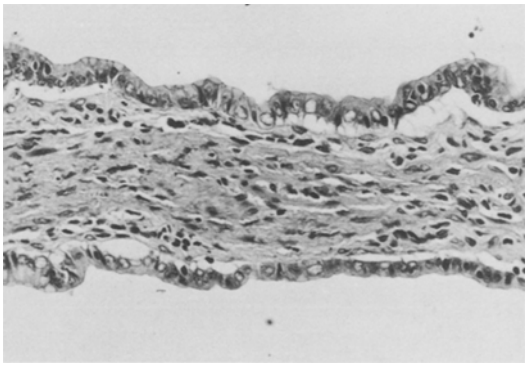

Figure 2. Mucosa of untreated newborn rat glandular stomach lined with a single layer of columnar and cuboidal epithelium, which was classified as showing 'nonspecific differentiation'. H.E. × 100.

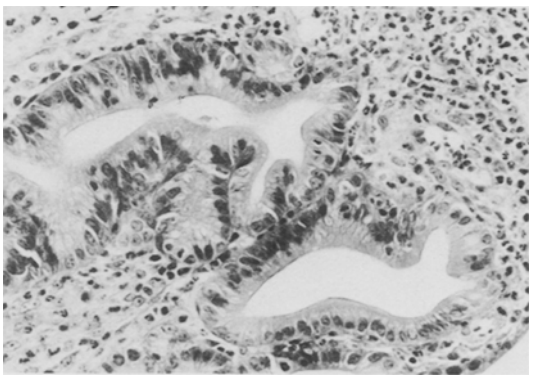

Figure 3. Tubular adenocarcinoma from transplant of fetal glandular stomach treated in vitro with 10 µg/ml of MNNG. H.E. × 100.

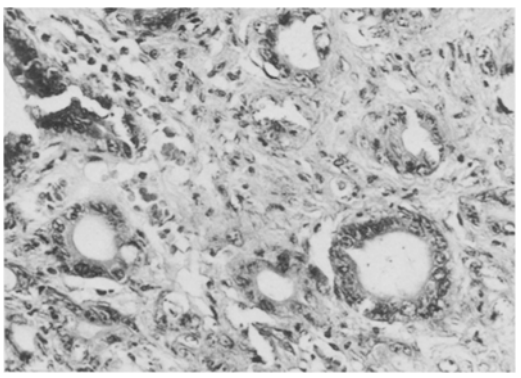

Figure 4. Tubular adenocarcinoma invading adjacent tissue from a transplant of newborn glandular stomach treated in vitro with 5 µg/ml of MNNG. H.E. × 100.

nomas from 67 transplants of MNNG-treated newborn glandular stomachs (3.0%). The incidence of adenocarcinomas in fetal glandular stomach was significantly different from that in the glandular stomach of newborn rats ($p < 0.01$ by the χ^2-test). On the contrary, no adenocarcinomas developed from 28 untreated glandular stomachs of fetal or newborn rats.

Morphologically, the tumors were identified as tubular adenocarcinomas showing strong structural atypism, such as the back-to-back phenomenon (fig. 3). The tumor cells were pleomorphic and had a round or oval hyperchromatic nucleus and basophilic cytoplasm, which was moderately PAS-positive. Mitoses were frequent. Invasion into adjacent connective tissue was often observed (fig. 4).

The other tumors which developed were 2 squamous cell carcinomas, 13 fibrosarcomas, 7 leiomyosarcomas, 5 ganglioneuroblastomas and 2 osteosarcomas. These were all found in both MNNG-treated and untreated transplants, except the squamous cell carcinomas, which were induced only in MNNG-treated transplants (table 3).

Discussion. The present experiments revealed that fetal and newborn rat glandular stomachs developed adenocarcinomas when transplanted after treatment with MNNG in vitro. The transplantability was high and the transplants survived for up to 52 weeks under the conditions used in this study. The mucosa lining the cysts was well differentiated in both MNNG-treated and untreated transplants. The cysts contained sero-mucinous fluid which showed high potential peptic activity and a different pattern of pepsinogen isozymes from that of mature rat glandular stomach, as reported in our previous paper[8].

There have been several reports on transplantation of the glandular stomach of rodents[9–12]. Of these, only that of Smith was on transplantation of carcinogen-treated glandular stomach[9]. This author transplanted fetal glandular stomach of mice together with methylcholanthrene dissolved in olive oil, and observed that the tumors developed were squamous cell carcinomas probably originating from the forestomach. This finding could be explained by data showing that the mucosa of the glandular stomach of mice is very refractory to the treatment with various carcinogens, including MNNG[13,14]. Some strains of rats are very susceptible to induction of adenocarcinomas of the glandular stomach by MNNG-treatment[2,13]. In this experiment, in vitro treatment of the glandular stomach of highly-susceptible Wistar rats with MNNG resulted in transformation of the glandular stomach mucosa and development of adenocarcinomas. Although further transplantation of the adenocarcinomas into Wistar rats was not attempted, the invasive character of the tumors suggested that they were highly malignant.

It is noteworthy that the incidence of adenocarcinomas induced by MNNG in fetal glandular stomach was dose-dependent and was significantly different from that in the glandular stomach of newborn rats ($p < 0.01$). The role of cell division in the initiation of carcinogenesis has been well established. So this difference in the incidence could be explained by the fact that the labelling index of the epithelium of the glandular stomach of rats reaches a peak at 20 days of gestation and then decreases after birth[15]. This conclusion seems to be supported by the fact that mice are sensitive to induction of lung tumors by urethane shortly after organogenesis (17 days of gestation)[16].

Many studies have been made on transplantation of tissues such as mammary gland[17], lung[18,19], ovary[20] and prostate[21] after treatment in vitro with various carcinogens. Recently, this method has been attempted with human tissues transplanted into nude mice[22,23]. Transplantation of tissues after in vitro treatment with chemicals should be considered in relation to the organ specificity of chemical carcinogens. For such studies, adult tissues are preferable. However, transplantation of the glandular stomach of adult rats has

Table 1. Transplantability of fetal and newborn rat glandular stomach

Concentration of MNNG (μg/ml)	Transplantability* Fetal glandular stomach	Newborn glandular stomach
0	11/13 (85%)	13/15 (87%)
1	15/16 (94%)	20/23 (87%)
5	14/18 (78%)	22/23 (96%)
10	16/17 (94%)	19/21 (90%)

* No. of rats with a cyst(s) or tumor(s) at transplanted site/No. of rats with transplants.

Table 2. Differentiation of transplanted rat glandular stomach

Concentration of MNNG (μg/ml)	Fetus Specific	Nonspecific	Total No. of transplants	Newborn Specific	Nonspecific	Total No. of transplants
0	13	2	15	14	1	15
1	10	5	15	13	5	18
5	19	4	23	13	5	18
10	15	4	19	15	3	18

Table 3. Development of tumors in transplanted glandular stomach

Concentration of MNNG (μg/ml)	Incidence of tumors (%)		Adeno-carcinoma		Squamous cell carcinoma		Fibro-sarcoma		Leiomyo-sarcoma		Ganglio-neuro-blastoma		Osteo-sarcoma	
	F*	NB**	F	NB	F	NB	F	NB	F	NB	F	NB	F	NB
0	6/13 (46)	3/15 (20)	0	0	0	0	3	2	1	1	1	0	1	0
1	4/16 (25)	5/23 (22)	2	1	1	0	0	2	1	1	0	1	0	0
5	7/14 (50)	6/23 (26)	2	1	0	1	1[a]	1	1	1	2	1	0	1
10	7/16 (44)	4/21 (19)	5	0	0	0	2	2	0	1[b]	0	0	0	0

* Fetal glandular stomach. ** Newborn glandular stomach. [a] plus a fibroma; [b] plus a leiomyoma.

been unsuccessful in spite of the use of various immunosuppressive agents before and after transplantation[24].
Spontaneous malignant conversion of transplanted tissues was also observed in our previous experiments[8]. It is noteworthy that no adenocarcinomas developed from untreated glandular stomach in our study, whereas there are reports that adenocarcinomas developed from untreated gastrointestinal tract of mice[11] and rats[25]. The mechanism of this phenomenon is still obscure and requires further study.

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Evidence that ADP-ribosylation is not necessary for luteinizing hormone stimulation of Leydig cell steroidogenesis[1]

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Summary. 3-Aminobenzamide, which inhibits ADP-ribosylation, did not inhibit luteinizing hormone's ability to stimulate androgen secretion by mouse testicular interstitial cells in vitro. 3-Aminobenzamide, but not its inactive analog 3-aminobenzoic acid, inhibited steroidogenesis stimulated by cholera toxin.

Luteinizing hormone (LH) stimulates steroidogenesis in Leydig cells by binding to cell surface receptors which increase the activity of adenylate cyclase via an ill defined interaction with the guanine nucleotide (G) regulatory subunit of the enzyme[2]. Cholera toxin also stimulates Leydig cell adenylate cyclase activity and steroidogenesis[3]; it does so by catalyzing the ADP-ribosylation of the G regulatory subunit of adenylate cyclase[4]. The possibility that ADP-ribosylation may be involved in the mechanism by which LH stimulates steroidogenesis is suggested by the recent observation that thyroid stimulating hormone (TSH), which is composed of an α-subunit identical to that of LH and a non-identical β-subunit, increases ADP-ribosyltransferase activity in thyroid membrane preparations[5–7]. TSH induced the ADP-ribosylation of the α-subunit of TSH and of a membrane protein tentatively identified as the G regulatory subunit of the adenylate cyclase complex[6–7].
The present study has utilized the competitive inhibitor of ADP-ribosyltransferase, 3-aminobenzamide[8], to test the hypothesis that ADP-ribosylation is involved in the mechanism by which LH stimulates Leydig cell steroidogenesis.
Materials and methods. Testicular interstitial cells were isolated from adult Swiss-Webster mice (High Oak Ranch, Goodwood, Ontario) as previously described[9]. 70,000 cells were suspended in 0.5 ml incubation medium (Medium 199 with Earle's salts and containing 1 mg/ml bovine serum albumin, 10 mM HEPES, 27 mM $NaHCO_3$, 27.5 mM glucose and 1 mM pyruvate). The incubation medium contained either 5 mM 3-aminobenzamide, 5 mM 3-aminobenzoic acid, or 2.5 µl of the solvent dimethylsulfoxide. Steroidogenesis was stimulated by the addition of LH (NIH-LH-B9) (0.1–100 ng/ml) or cholera toxin (10^{-14}–10^{-10} M).
The cells were incubated in an atmosphere of 95% O_2:5% CO_2 for 3 h in a shaking water bath at 33 °C. At the end of the incubation the cells were removed by centrifugation and the medium was stored at −70 °C. Unextracted 10–50 µl samples of thawed medium were added directly to the testosterone assay[10].

Effect of 3-aminobenzamide and its inactive analog, 3-aminobenzoic acid, on androgen production (ng/10^6 cells/3 h)

Inhibitor	Stimulus		
	None	Luteinizing hormone (0.3 ng/ml)	Cholera toxin (0.1 µM)
Control	3.8 ± 0.3	34.9 ± 2.1	29.3 ± 2.2
3-Aminobenzamide	3.6 ± 0.1	30.9 ± 1.7	11.7 ± 0.9*
3-Aminobenzoic acid	4.4 ± 0.4	35.6 ± 2.7	35.0 ± 1.9

Mean ± SEM of 5 replicates. * $p < 0.01$ vs control (Student's t-test).