Cytological Effects of Prefixation Treatment*

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ABSTRACT

The effects produced by prefixation treatments on cells in metaphase from 10-day mouse fetuses and from several embryonic stages of the frog were investigated. The technical value of some of these pretreatments is noted.

Pretreatment with isotonic solutions (both ionic and non-ionic in the case of the mouse, ionic only in the frog) generally produced a similar effect, *viz.*, chromosomal swelling with little effect on the spindle. A notable exception is provided by frog embryos preceding the neurula stage; spindle disorganization without chromosomal swelling was produced by pretreatment in isotonic modified Niu-Twitty solution, containing no divalent cations.

Pretreatment with hypotonic solutions (both ionic and non-ionic in the case of the mouse, ionic only in the frog) generally produced several major effects, *viz.*, despiralization of chromosomes, chromatid separation, and spindle disorganization. The conclusion is drawn from the mouse data that, in order to produce these effects, pretreating solutions must be of low osmotic pressure. Low ionic strength alone (*e.g.*, isotonic sucrose solutions) is not sufficient.

As differentiation of frog embryos progressed, pretreatments either of longer duration or with solutions of increasing degrees of hypotonicity were required to produce comparable intensities of the same effects.

Many of the effects on metaphases produced by hypotonic pretreatment of frog embryos were reversible by subsequent exposure to isotonic solutions.

The significance of results presented here is discussed briefly with respect to some current considerations of the macromolecular structure of chromosomes.

INTRODUCTION

Interest in techniques for the prefixation treatment of vertebrate cytological material has been increasing since the recent work of Hughes (9), Hsu (7), Hsu and Pomerat (8), and others. Many agents, often hypotonic saline solutions, have been recommended for this purpose. The resulting preparations generally have been of improved quality and have enhanced the accuracy of chromosome analyses over those made from sectioned material or from squash preparations of tissue not given such treatment. Additionally, some insight has been gained into factors which influence various mitotic phenomena. The primary technical advantage of prefixation treatment apparently has been spindle disorganization, effects on the chromosomes varying with the agent employed.

The experiments to be described were designed to compare the cytological effects obtained when the ionic composition and osmotic pressure of solutions used as media for prefixation treatment (hereinafter termed simply "pretreatment") were varied. The purpose of this investigation was twofold: to establish improved techniques for the preparation of cytological material from fetuses of the mouse, *Mus musculus*, and embryos of the frog, *Rana pipiens*, and, if possible, to derive additional information relating to the production of the observed effects.

Materials and Methods

Pretreatment and Cytological Preparation of Mouse Tissue.—Ten-day fetal mouse tissue from the ICR

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TABLE I

Solutions Used in Pretreatment of 10-Day Fetal Mouse Cells

Category	Symbol	Composition	Ionic strength	Comments
			μ	
Isotonic, ionic	MR	Mammalian Ringer solution	0.168	Richards (15)
	MMR	Modified MR	0.164	Contains no Ca^{++}: readjusted to isotonicity with NaCl
	SMR	MR:0.25 M sucrose = 1:1	0.084	
	SV	0.25 M sucrose + 0.01 M versene*	0.03	
Isotonic, non-ionic	S	0.25 M sucrose	0	Hogeboom *et al.* (6)
Hypotonic, ionic	HMR	Hypotonic MR	0.118	Total salt concentration reduced to 70 per cent of MR
	½MR	MR:water = 1:1	0.084	
	NT	Niu-Twitty solution	0.068	Niu and Twitty (14).
	MNT	Modified NT‡	0.082	Contains no Ca^{++} or Mg^{++}. King and Briggs (12)
	MNTV	MNT + 0.0005 M versene	0.082	
	½SV	0.125 M sucrose + 0.01 M versene	0.015	
	WV	0.01 M versene (aqueous)	0.03	
Hypotonic, non-ionic	½S	0.125 M sucrose	0	
	W	Water	0	
Control	CAO		—	No pretreatment: stain-fixation at room temperature (25°C.) in acetic orcein (1 per cent orcein in 60 per cent acetic acid)

* Versene is disodium salt of ethylene diamine tetraacetic acid (dihydrate), M.W. 372.35.

‡ Solution A (500 ml.), 2943 mg. NaCl, 50 mg. KCl; solution B (250 ml.), 1300 mg. Na_2HPO_4, 116 mg. KH_2PO_4; solution C (250 ml.), 200 mg. $NaHCO_3$. The solutions are sterilized by boiling and mixed after cooling. Briggs and King (3).

Swiss stock was obtained and prepared by methods essentially the same as those previously described by one of us (10). Dissection, mincing, and incubation of the fetal tissue were all done on a slide warmer at 37°C. Minces were made and pretreatments carried out in the solutions listed in Table I. Following the pretreatments specified, the minced tissue was immersed in acetic orcein (1 per cent orcein in 60 per cent acetic acid) at room temperature (approximately 25°C.). At completion of fixation-staining, slides were made by a squash method also described earlier (10).

Pretreatment and Cytological Preparation of Embryonic Frog Tissue.—Pretreatment was carried out in a manner similar to that described above for mouse tissue. Table IV summarizes the pretreatment of frog material with respect to developmental stage, regions from which cells were derived, solutions, temperatures, and exposure times.

Because of the comparative cellular fragility encountered in the early embryonic stages, it was convenient to manipulate large aggregates of cells. Pretreatment was in all cases followed by fixation-staining in acetic orcein at room temperature. After fixation-staining and before squashing, gentle tapping on the cover glass with blunt forceps resulted in better distribution of cells. Pretreatment was by immersion, without mincing, according to the conditions specified in Table IV.

Method for Making Permanent Slides.—Because of the number of different samples involved in these experiments, and the necessity for rechecking and comparing observations, it was desirable to have permanent preparations. These were secured by the following procedure, in use in these laboratories for a number of years: (*a*) freeze slide on a slab of solid CO_2 and pry off cover glass with cold razor blade (17); (*b*) immerse preparation for several hours in cold ethanol (−35°C.), dehydration taking place while the preparation remains in the frozen state (2); (*c*) mount in diaphane.

For the original squash preparations, siliconed No. 0 cover glasses and albumenized slides were used (16); for the permanent mounts, unsiliconed No. 1½, (0.16 to 0.18

mm.) cover glasses were used. Use of the combination of siliconed cover glasses and albumenized slides resulted in very little loss of material during the procedure just described. Comparison of permanent preparations with newly made temporary preparations indicated negligible differences in quality. Permanent preparations are far superior to aged temporary ones, which eventually show droplet formation and stain precipitation.

TABLE II

Pretreatment of 10-Day Fetal Mouse Cells: Effects on Metaphases

Category	Solution	Effects on chromosomes*					Spindle disorganization*				Total No. of metaphases examined
		None	Swelling	Despiralization	Chromatid separation	Dispersal	1	2	3	4	
		per cent					*per cent*				
Isotonic, ionic	MR	8	91	0	0	1	41	51	8	0	154
	MMR	4	93	0	0	3	23	38	39	0	130
	SMR	2	98	0	0	0	15	56	28	1	100
	SV	8	85	3	0	4	41	58	1	0	100
Isotonic, non-ionic	S	9	86	1	4	1	31	36	33	0	106
Hypotonic, ionic	HMR	13	70	9	1	7	18	40	42	0	106
	½MR	1	14	44	40	1	8	50	40	2	114
	NT	4	1	68	24	3	24	37	29	11	104
	MNT	1	2	62	35	0	11	27	31	30	99
	MNTV	4	3	68	24	0	45	20	23	11	119
	½SV	1	8	64	26	1	14	59	19	8	100
Hypotonic, non-ionic	½S	4	1	71	24	1	26	55	16	3	160
Control	CAO	29	71	0	0	0	71	27	2	0	154

*See text for fuller description.

Pretreatments involved incubation at 37°C. in the various solutions for 20 minutes (MMR and HMR for 60 minutes).

TABLE III

Pretreatment of 10-Day Fetal Mouse Cells with MNT: Effects of Increasing Exposure on Metaphases

Duration	Effects on chromosomes*					Spindle disorganization*				Total No. of metaphases examined
	None	Swelling	Despiralization	Chromatid separation	Dispersal	1	2	3	4	
min.	*per cent*					*per cent*				
5	8	10	57	25	0	20	40	23	17	133
10	2	2	63	33	0	30	50	14	6	161
20	1	2	62	35	0	11	27	31	30	99
30	13	1	56	31	0	26	35	27	13	105
40	1	5	50	44	0	14	38	33	15	125
50	2	6	51	39	1	25	39	30	6	164
60	3	4	49	37	6	12	36	45	7	184
Control (CAO)	29	71	0	0	0	71	27	2	0	154

* See text for fuller description.

Pretreatments involved incubation at 37°C. for the time interval specified in the first column.

Analytical Methods.—The scoring of spindle disorganization in fetal mouse metaphases was accomplished using four arbitrary categories, according to the relative degree of disorganization exhibited: (1) *none, i.e.*, appearance of spindle (Fig. 7) similar to or identical with that of spindles in control preparations (Fig. 1); (2) *slight*, some equatorial spreading of chromosomes, still too crowded for rapid and accurate counting (Fig. 2); (3) *moderate*, fair spreading of chromosomes, some overlapping, centromere orientation retained (Figs. 3, 5, and 9); (4) *extreme*, complete disruption of spindle organization with wide displacement of chromosomes (Figs. 4, 6, and 8). Each metaphase scored was placed in one of these four categories, designated class 1 to class 4, respectively.

In scoring embryonic frog metaphases, greater resolution of spindle disorganization was possible, and the following five categories were adopted. *None*, in which spindles were identical with those observed in control preparations (Figs. 10 and 14). *Slight*, in which there was some spreading of chromosomes, but much overlapping (Fig. 12). *Moderate*, showing increased spreading with some overlapping (Fig. 11). *Pronounced*, in which there was greater spreading of chromosomes with little or no overlapping, and with centromere orientation still retained (Fig. 13). *Extreme*, in which there was complete disorganization of the spindle with wide displacement of the chromosomes (Figs. 15 to 17). These categories were designated 0 through ++++, respectively, and the average degree of spindle disorganization produced by each pretreatment was noted accordingly (Table IV). A further category was necessary in classifying frog metaphases from tail tips which had been returned to isotonic solutions after hypotonic pretreatment. Such metaphases showed aggregation of chromosomes into one or more discrete groups ("aggregation," Table V); this did not occur in metaphases that received hypotonic pretreatment alone.

Effects on metaphase chromosomes were classified as follows: (1) *no effect*, chromosomes similar in appearance to those in control preparations (Figs. 1, 10, 12, and 14); (2) *swelling*, involving a variable increase in chromosomal volume and lower intensity of staining (Fig. 5); (3) *despiralization*, an increase in length of from 1.5 to 3 times the length of chromosomes in controls (Figs. 2 to 4, 7, 8, 15 to 17); (4) *chromatid separation*, marked separation of chromatids, with connection usually, but not always, retained at the centromere, often accompanied by despiralization (Figs 6. 15, to 1)7; (5) *"dispersion"* (mouse only), reduction in staining intensity, chromosome outlines not sharp, suggesting modification and/or dispersion of chromosomal constituents (Fig. 9); (6) *degeneration*

(frog only), similar to dispersion, except that chromosomes were clumped.

Each mouse metaphase was scored according to chromosome categories 1 to 5 (Tables II, III). A reference chart with photomicrographs of typical examples of each of the above effects was prepared for this purpose. In order to increase the accuracy of judging such effects, only those metaphases appearing practically intact were scored. For the same reason, only polar metaphases were accepted in cases in which there existed any evidence of the presence of a spindle. Where there existed in the same metaphase combinations of the described effects, the metaphase was entered in the category of the most pronounced effect.

In scoring the embryonic frog metaphases, the most typical effect of each pretreatment was noted, according to chromosome categories 0 to 4 (Table IV). Entries in Table IV represent the estimated degree of expression of each effect relative to the controls, using a 0 to ++++ scale. In an experiment designed to test the effect of returning stage 25 tail tips to isotonic solutions following hypotonic pretreatment, *each* metaphase was scored according to chromosome categories 1 to 4, 6, and 7, without regard to relative degree of expression (Table V).

Photomicrographic Methods.—A Spencer research microscope was used for the photomicrographs. It was equipped with a Bausch and Lomb ampliplan (high power) ocular, a Zeiss 90× 1.4 N.A. apochromatic oil immersion objective, a Spencer 1.3 N.A. achromatic condenser, and a first surface mirror. A Bausch and Lomb research lamp with ribbon filament was used with Wratten filter No. 74. Köhler illumination was employed. The negative material was Kodak contrast process orthochromatic cut film.

RESULTS

Pretreatment with Isotonic Solutions.—In the pretreatment of fetal mouse tissue, little difference was observed among the effects produced by the various isotonic solutions (Table II), regardless of their ionic strength (Table I). The major effect both of isotonic saline solutions and of 0.25 M sucrose solution was chromosomal swelling. There was present in addition a degree of apparent spindle disorganization, possibly due more to the presence of swollen chromosomes than to any direct action on the spindle.

Since the relative intensities of the effects on chromosomes are not given (Table II), it must be stated that chromosomal swelling after pretreatment in isotonic solutions is usually of a much more extreme type than that observed in the controls. The latter is considered to have resulted from endogenous reactions brought about by delay in fixation: control fetuses were usually fixed after pretreatment of experimental fetuses had been started, and were in the meanwhile held at 37°C. Such an interpretation might indicate that the swelling observed after isotonic pretreatment is not a direct result of the action of the pretreating solution, but is a consequence primarily of prolonged delay in fixation.

The chief effect of pretreating frog embryonic cells in isotonic saline solutions also is chromosomal swelling (Table IV). An exception to this is the case of frog blastula cells, which show no such effect. It should be noted that in early embryonic stages of the frog (stages 9 and 13), MNT produced spindle disorganization, while NT did not. In one experiment, the data from which are not shown in the tables, pretreatment of stage 9 cells resulted in 40 per cent of polar metaphases (total 38) having chromosomes which were countable. Pretreatment of stage 9 cells with NT resulted in only 5 per cent of polar metaphases (total 43) having chromosomes which were countable. The diameter of squashed metaphase plates was increased two- to threefold by MNT pretreatment, making possible linear measurements of the chromosomes in addition to accurate counts (4). The difference in effects produced by MNT and NT pretreatment is not apparent after gastrulation. MNT no longer produces appreciable spindle disorganization, and both MNT and NT cause swelling of chromosomes.

Pretreatment with Hypotonic Solutions.—The major effects of hypotonic solutions in the pretreatment of fetal mouse tissue were chromosomal despiralization, chromatid separation, and spindle disorganization. In many cases, these effects were quite similar among the various solutions. HMR, actually the least hypotonic of the solutions in the hypotonic ionic group, closely resembled isotonic MR in its effects, with the exception that increased spindle disorganization was observed. A similar, but more hypotonic solution, ½MR, produced chromosomal despiralization as well. The effects of NT, MNT, and ½S were similar. Neither ½MR nor ½S produced appreciable class 4 spindle disorganization.

Extremely despiralized metaphase chromosomes, such as those shown in Fig. 8, occasionally occur. Inspection of any one chromatid along its length reveals variations in diameter. Whether these variations represent a spiral or chromomeric structures could not be determined, since the diameter of such chromatids is close to the limit of resolution of the light microscope. In addition,

TABLE IV

Pretreatment of Cells of Frog Embryos: Effects on Metaphases

Temperature	Stage* and region of embryo	Solutions	Duration of exposure	Effects on chromosomes‡			Spindle disorganization‡
				Swelling	Despiralization	Chromatid separation	
°C.			*min.*				
25	9	None	—	0	0	0	0
	(animal	NT§	10	0	0	0	0
	hemisphere)	MNT§	"	0	0	0	++
		38%MNT‖	"	0	+++	+	++++
	13	None	—	+	0	0	0
	(medullary	NT	30, 60, 90, 120	++	0	0	0
	plate and	MNT	" " " "	++	0	0	+
	chorda-	38% NT‖, 38% MNT	"	0	+	+	+
	mesoderm)	" " " " " "	60	0	+	+	++
		" " " " " "	90	0	+	+	+++
		" " " " " "	120	0	++	++	+++
		W§	30	0	+	+	++
		"	60	0	++	++	+++
		"	90	0	+++	++	+++
		"	120	0	++++	+++	++++
	25	None	—	+	0	0	0
	(tail tips)	NT, MNT	30, 60, 90, 120	++	0	0	0
		38% NT, 38% MNT	" " " "	0	+	+	+
		W	"	0	+	+	+
		"	60	0	++	++	++
		"	90	0	+++	+++	+++
		"	120	0	++++	++++	++++
37	13	None	—	+	0	0	0
	(medullary	NT	5, 20	++	0	0	0
	plate and	MNT	" "	++	0	0	+
	chorda-	38% NT, 38% MNT	"	0	+	0	+
	mesoderm)	" " " " " "	20	0	+	+	++
		W	5	0	++	+	+
		"	20	0	++	+	++
	25	None	—	+	0	0	0
	(tail tips)	NT, MNT	5	+	0	0	0
		" "	15, 30	++	0	0	0
		" "	45, 60	+++	0	0	0
		38% NT, 38% MNT	5	0	+	0	+
		" " " " " "	15	0	+	+	+
		" " " " " "	30	0	++	++	++
		" " " " " "	45	0	+++	+++	++
		" " " " " "	60	0	+++	+++	+++
		W	5	0	+	0	+
		"	15	0	++	+	++
		"	30	0	+++	++	+++
		"	45	0	+++	+++	+++
		"	60	0	++++	++++	++++

* Shumway (18).

‡ Effects scored as increasing in degree (0 to ++++). See text for fuller description.

§ See Table I. Note that NT and MNT are isotonic for the frog embryo.

‖ Solution diluted with distilled water to 38 per cent of its standard concentration.

TABLE V

Pretreatment of Embryonic Frog Tail Tips (st. 25): Effects on Metaphases of Initial Pretreatment in Hypotonic Solutions, Followed by Pretreatment in Isotonic Solutions

Initial solution	Duration	Second solution	Duration	Effects on chromosomes*					Spindle disorganization*	"Aggregation"*	Total No. of metaphases examined
				None	Swelling	Despiralization	Chromatid separation	Degeneration			
	min.		*min.*	*per cent*							
None				43	54	2	2	0	0	0	136
NT	45			14	80	1	3	1	0	0	92
38% NT	45			15	3	23	59	0	++	0	201
38" "	45	NT	30	9	44	9	3	9	0	26	77
MNT	45			2	21	0	1	76	0	0	93
38% MNT	45			2	1	21	74	1	++	0	95
38" "	45	MNT	30	6	34	6	4	38	0	12	73
W	45			1	1	11	84	4	+++	C	315
"	45	NT	30	11	31	15	3	11	+	29	132
"	45	MNT	30	4	55	4	1	16	+	20	180

Pretreatments involved incubation at 37°C.

* See text for fuller description.

many of the chromosomes in Fig. 8 display symmetrical interruptions of linear continuity, not only at known sites of centromere position (arrows, *a*), but at other sites as well (arrows, *b*). These may be secondary constrictions, or perhaps potential sites of chromosome breakage of a type similar to that described by Steffensen (19) for *Tradescantia* with a calcium deficiency.

The two hypotonic solutions, 38 per cent NT and 38 per cent MNT, used for the pretreatment of frog embryonic tissues produced equivalent effects, *viz.*, chromosomal despiralization, chromatid separation, and spindle disorganization.

By virtue of some of the above mentioned effects, pretreated metaphases of frog embryos at various stages exhibited chromosomes in which the position of the centromere could be located with great accuracy. Further analysis of metaphase chromosomes such as those shown in Fig. 11 should reveal whether there exists a regular pattern of additional and still finer structural detail.

Test for the Reversibility of Effects of Hypotonic Pretreatment.—After 45 minutes' exposure at 37°C. to hypotonic solutions, tail tips from stage 25 embryos were returned to appropriate isotonic solutions. At the same time, samples of these tail tips were fixed in acetic orcein. After 30 minutes in isotonic solution, a second sample was fixed. Analysis of metaphases from these various samples gave the results summarized in Table V. These data indicate that effects on the chromosomes, *e.g.*, despiralization and chromatid separation, brought about by hypotonic pretreatment are, in general, directly reversed by subsequent exposure to the corresponding solution in isotonic strength.

An additional class of metaphase was observed in the material returned to isotonic solutions. Members of this class ("aggregation," Table V) demonstrated aggregation of chromosomes into one or more discrete groups. The latter feature distinguished these metaphases from ones which showed only the effects expected from hypotonic pretreatment. This class may in a sense represent a partial reversal of effects on the spindle of hypotonic pretreatment. Such reversal may be analogous to the reversal, to be described in the following section, of the effects of MNT plus versene on stage 8 blastula metaphases by subsequent pretreatment with NT.

Effects of Versene.—In the pretreatments of mouse tissues, addition of 0.01 M versene to 0.25 M sucrose reduced the degree of spindle disorganization, presumably a reflection of the increase in ionic strength of the solution (SV, Table I). More subtle effects of versene also were present. Among

these were many cases of apparent dispersion of chromosomes, with generally reduced sharpness in the outline of fixed and stained chromosomes. This effect, while general, was usually not pronounced enough for such metaphases to be entered in the "dispersal" category.

The addition of versene to some of the hypotonic pretreating solutions did not, at the levels of concentration used and under the conditions of the experiments on fetal mouse tissue, have pronounced influence on the morphological effects produced by the hypotonic pretreating solutions alone. Evidently, where conditions of considerably reduced ionic concentration and low osmotic pressure exist in the solution, the effects, if any, of versene are masked.

Pretreatment with MNT, to which versene in two concentrations, 0.0005 M and 0.01 M had been added, resulted in effects on both the spindle and chromosomes of stage 8 frog blastula cells. Spindle disorganization was of the type produced by MNT alone, and cannot be ascribed to the presence of versene in the medium. The major effect on the chromosomes, however, was one of apparent dispersal. Both effects were reversible by subsequent exposure of the cells to NT.

Pretreatment with Water.—Effects on fetal mouse metaphases of pretreatment at 37°C. in distilled water, even for as short a period as 5 minutes, were such as to prevent accurate analysis. Chromosomal despiralization and spindle disorganization were observed, but there was usually such marked loss of stainability that the chromosomes were barely visible under the microscope. Fig. 9 shows an example of the onset of these effects.

The effects on embryonic frog tissue of pretreatment with water were generally much less severe than those on fetal mouse tissue. The effects were, however, usually more extreme than those produced by corresponding pretreatment of the same cell types with hypotonic saline solutions (Table IV). It should be noted that the production of a given cytological effect requires the use either of increasingly prolonged exposure times or of solutions of increasing hypotonicity in pretreating tissues from increasingly advanced developmental stages. Thus, both at 25° and at 37°C., distilled water was far more efficient in the pretreatment of tail tips than were hypotonic saline solutions.

Evaluation of Methods.—The influence of certain inadequacies in the methods, particularly those employed for the investigation of fetal mouse tissue, requires mention. Variability, both in the relative intensity of spindle disorganization and in the expression of the various chromosomal effects, has resulted in some measure from uneven diffusion of pretreating solutions into particles of minced tissue. Further, while it is known that squashing has little effect on spindles of unpretreated metaphases, the fact must be kept in mind that its effects on those partially affected by pretreatment is not known. Another definite source of error is present in scoring effects of pretreatments which result in much class 4 spindle disorganization. In some cases, some metaphases are entirely lost for scoring purposes through cell breakage and complete scattering of chromosomes.

It is felt that although such methodological deficiencies are a major handicap to attempts at gathering quantitative data, they are not of sufficient magnitude to obscure the basic differences existing among effects induced by the various pretreating solutions.

DISCUSSION

It is not within the scope of this paper to review the many contributions made since 1952 to the technique of pretreatment. However, it seems appropriate to refer to some of the original work and to one recent paper on the subject.

Hughes (9) has described the effects of hypo- and hypertonic Tyrode solution on chick fibroblasts *in vitro*, studied both by observation of fixed specimens and by phase contrast time-lapse cinemicrography of living cells. Our findings in regard to effects of hypotonicity on the mitotic spindle, and to some of the types of reversibility of these effects, are in agreement with some of those described by Hughes. Hughes noted the failure of chromosomes to contract further once mitosis had been arrested by hypotonic pretreatment. However, he did not note the presence in his material of such effects on chromosomes as despiralization and chromatid separation. Hughes has indicated that dividing cells are more sensitive than intermitotic ones, and that they "may be inhibited at two main points, namely, before prophase, and before anaphase." His interpretation of the effects observed is that they may be related to different types of disturbance of the normal distribution of cations within the cell, as well as to other changes, including a shift in the normal equilibria between cell proteins and water.

The work of Hsu (7) and Hsu and Pomerat (8)

involved the prefixation treatment in hypotonic saline solutions of cells from human skin and spleen *in vitro* and cells cultured from various tissues of other mammals. Disorganization of the mitotic spindles of these cells resulted. In addition, chromosome despiralization and chromatid separation are to be seen in some of the published figures. Hsu notes overspiralization of chromosomes in some cases (Hsu, Fig. 11 A). Chromosomes identified as being in prometaphase (Hsu, Figs. 11 B to 12 D, Fig. 13) may be metaphase chromosomes despiralized by the pretreatment.

A recent technique published by Ford and Hamerton (5), developed for mouse bone marrow but said to be applicable to other tissues and species, recommends colchicine injection of the animal 1 hour previous to a 10 minute pretreatment of the tissue in hypotonic sodium citrate solution at 37°C. The authors state, "Colchicine shortens the chromosomes; hypotonic pretreatment expands the cells. Maximum dispersion of the chromosomes within the cell is thereby obtained." The excellent figures published with their paper indeed show the overspiralization of chromosomes and separation of chromatids typical of c-mitosis, but it seems questionable that maximum dispersion of chromosomes within the cell is the result simply of expansion of cells by hypotonic pretreatment. Spindle disorganization may have been caused by the action of colchicine, the ability of citrate ion to combine with divalent cations, the disruptive influence of hypotonicity, or possibly by a combination of these factors.

The pretreating solutions which in our own experiments produced spindle disorganization, despiralization of chromosomes, chromatid separation, and, in some cases, chromosomal dispersion, involved the double condition of low osmotic pressure and low ionic strength. The experiments with sucrose solutions suggest that the former may be more important than the latter. Results from pretreatment of *early* embryonic cells of the frog represent an exception, since a solution which produced marked spindle disorganization (MNT) did not fulfill the double condition, but was deficient specifically with respect to divalent cations.

The significance of many of these observations is not easily assessed in terms of recent information bearing on the macromolecular constitution of chromosomes. It has been suggested that the role of macromolecular aggregation within chromosomes may be attributed to bridges of divalent cations (13), to hydrogen bonding (1), or to a combination of ionic and covalent bonds (20). Other investigations (11) indicate that "any agent capable of influencing the nucleoprotein constituents of the cell, either by modification of the ionic environment or by the removal of some essential cellular materials, can lead to deformational changes in the chromosomes."

Similarly, the response of the spindle to some of the pretreating solutions is not easily interpreted. In view of the sensitivity of many proteins to environmental variations, it might be speculated that spindle disorganization represents changes in stage of major protein constituents of the spindle.

The precise means by which these solutions exert their various effects remain to be defined. Meanwhile, pretreatment techniques will continue to aid investigations of chromosome number and morphology in tissues which have not readily yielded to methods formerly employed.

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Explanation of Plates

Plate 211

Figs. 1 to 9 are photomicrographs from 10-day fetal mouse tissue. Metaphases in Figs. 2 to 4 and 6 to 9 resulted from hypotonic pretreatment, Fig. 5 from isotonic pretreatment. All illustrate typical examples of some of the classes indicated in Tables II and III and described in detail in the text. × 1950.

Fig. 1. Control metaphase from unpretreated fetal mouse tissue. Direct fixation-staining in acetic orcein.

Fig. 2. Despiralization of chromosomes, spindle disorganization class 2 (slight).

Fig. 3. Despiralization of chromosomes, spindle disorganization class 3 (moderate).

Fig. 4. Despiralization of chromosomes, spindle disorganization class 4 (extreme).

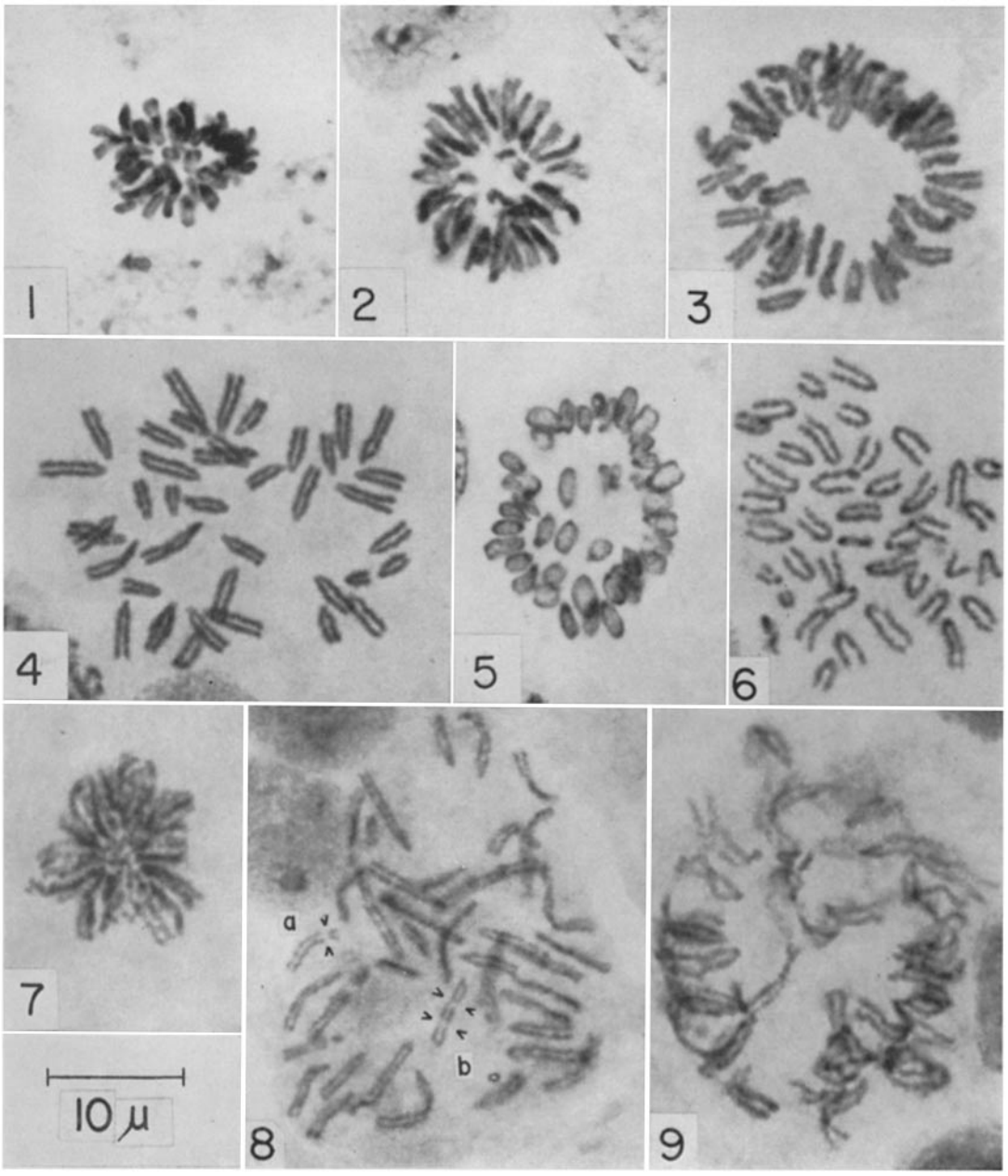
Fig. 5. Chromosomal swelling, spindle disorganization class 3.

Fig. 6. Chromatid separation, spindle disorganization class 4.

Fig. 7. Despiralization of chromosomes, spindle disorganization class 1 (none).

Fig. 8. An extreme case of chromosomal despiralization, spindle disorganization class 4. *a*, centromeric constrictions; *b*, two non-centromeric constrictions.

Fig. 9. "Dispersion" of chromosomes, spindle disorganization class 3.



(Hungerford and DiBerardino: Cytological effects of pretreatment)

PLATE 212

Figs. 10 to 17 are photomicrographs from embryonic stages of the frog. These metaphases illustrate typical examples of some of the classes indicated in Tables IV and V and described in detail in the text. Black spots are pigment granules. × 1200.

FIG. 10. Stage 9 control, direct fixation-staining in acetic orcein.

FIG. 11. Stage 9, pretreated 10 minutes in MNT at room temperature (approximately 25°C.). No effect on chromosomes, spindle disorganization class 2 (moderate).

FIG. 12. Stage 13 control, direct fixation-staining in acetic orcein. Spindle disorganization class 1 (slight).

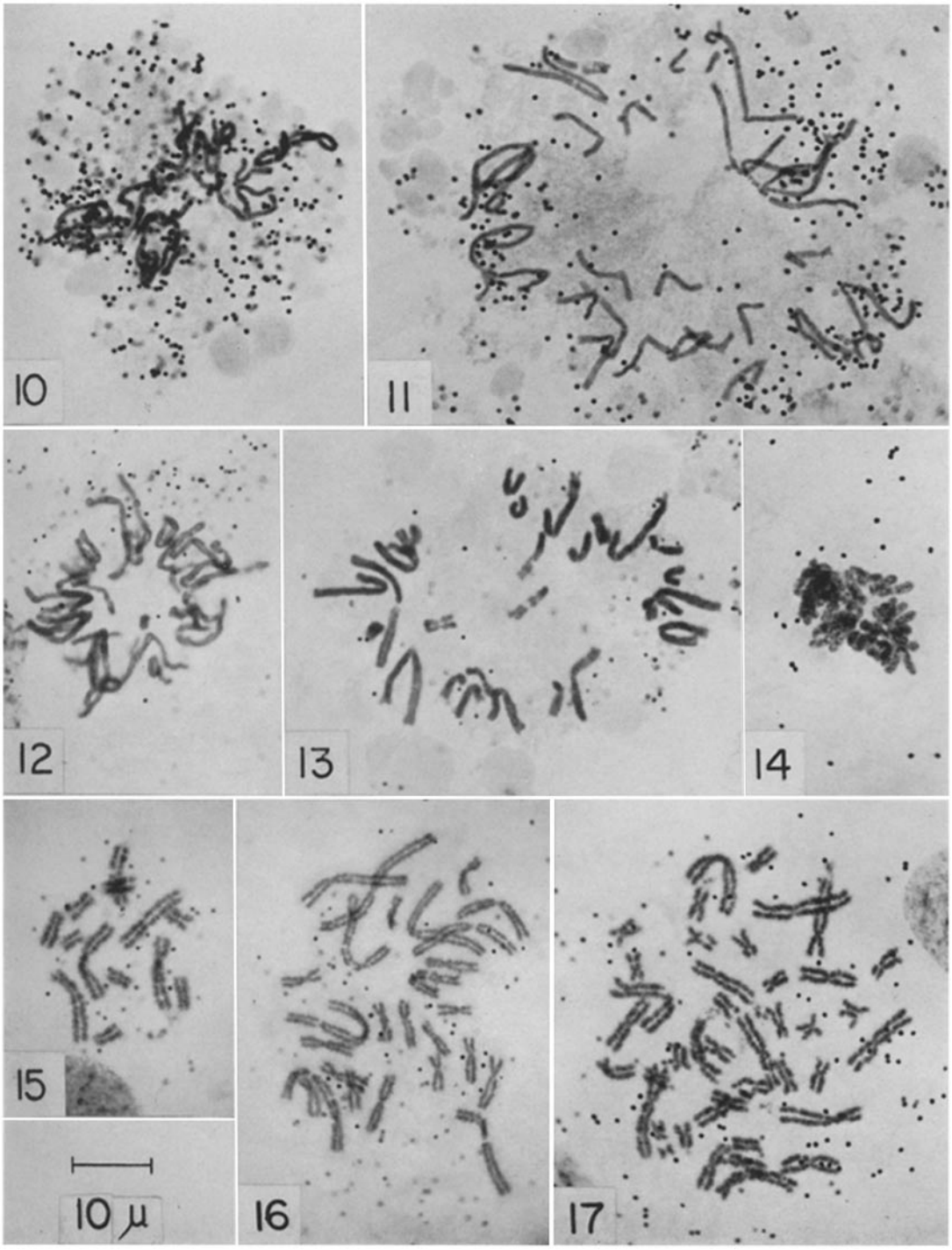
FIG. 13. Stage 13, pretreated 60 minutes in 38 per cent MNT at room temperature. No effect on chromosomes, spindle disorganization class 3 (pronounced).

FIG. 14. Stage 25 tail tip control, direct fixation-staining in acetic orcein.

FIG. 15. Stage 22 haploid tail tip, pretreated 45 minutes in 38 per cent NT at 37°C. Despiralization of chromosomes, chromatid separation, spindle disorganization class 4 (extreme).

FIG. 16. Stage 24 diploid tail tip, pretreated 120 minutes in distilled water at room temperature. Despiralization of chromosomes, chromatid separation, spindle disorganization class 4.

FIG. 17. Stage 25 triploid tail tip, pretreated 45 minutes in 38 per cent NT at 37°C. Despiralization of chromosomes, chromatid separation, spindle disorganization class 4.



(Hungerford and DiBerardino: Cytological effects of pretreatment)