[27a] 4,12 side-chain is the side-chain with the immunological specificity of O-4,12, such as the one in LPS of S form salmonella of group B.

[28] It is unlikely that the short side-chains of SR LPS result from slow synthesis of the precursors of LPS, because crude extracts of SR recombinants synthesized all the sugar nucleotides tested for as rapidly as did an extract of a 4,5,12 S-form.

[29] Similarly we can assume that the gene for O–5, which presumably controls the formation of galactose acetylase (cf. ref. 26), is not included in the gene cluster closely linked to *his*. SR recombinants which have received this gene perhaps acetylate the galactose unit in the S-specific side-chain, and show a small amount of O–5 specificity; those which have not do not show any O–5 activity (Table 2).

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*ISOLATION OF A SOLUBLE TRANSPLANTATION ANTIGEN**

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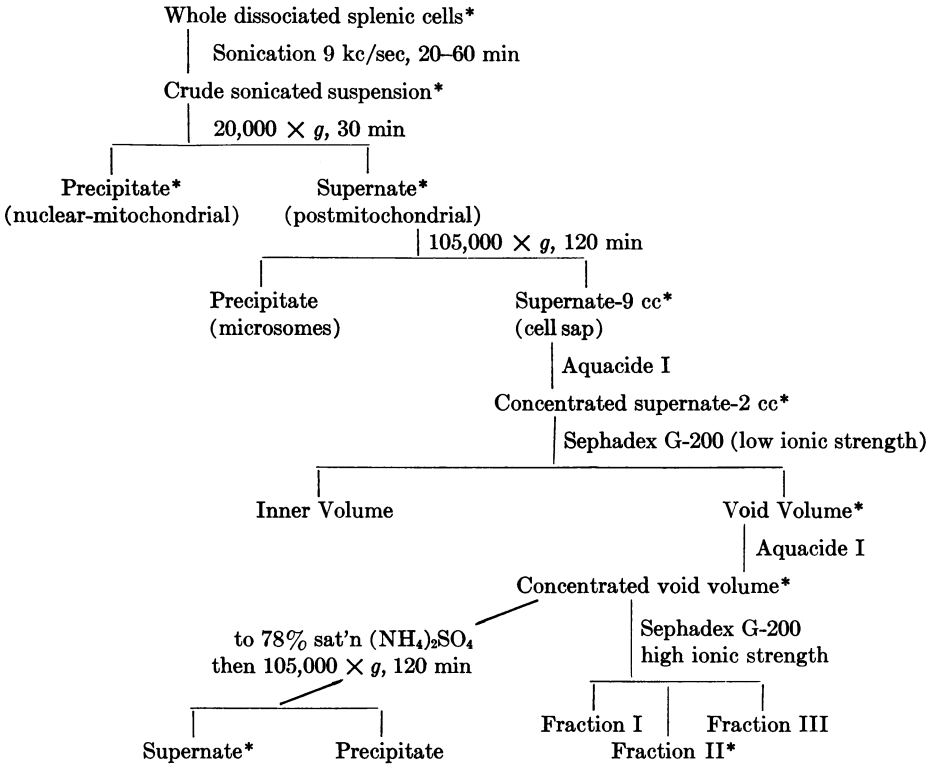
The immunological hypothesis of graft rejection is founded upon the second-set phenomenon:[1] recipient animals which have already rejected grafts reject subsequent donor grafts in accelerated fashion. According to this hypothesis, "transplantation antigens" are defined as donor-specific substances which are "released" by the graft and thereby sensitize the recipient. Operationally, the activity of tissue extracts as transplantation antigens may be demonstrated by their ability to induce more or less donor-specific sensitization such that subsequent transplants are rejected in accelerated fashion.[2]

Chemical analyses of extracts capable of inducing skin sensitization have suggested that their antigenicity depended upon, or was associated with, desoxyribonucleoproteins,[2] mucoproteins,[3] and/or lipoproteins.[4] Recently, five independent groups of investigators[4–8] have demonstrated that materials with the bulk properties of lipoproteins function as transplantation antigens in skin-sensitizing,[5] enhancing,[6] and serologic[4–8] assays. These preparations (*a*) were water-insoluble, (*b*) contained approximately equal amounts of lipide and protein and only small amounts of carbohydrate, (*c*) were inactivated by organic solvents, and (*d*) failed to move on electrophoresis at several pH's. Solubilized derivatives of these extracts[6, 8] retained serologic activity but had lost their sensitizing activity.

A soluble transplantation antigen was prepared from water-lysed tumor cells by Manson *et al.*[9] This antigen was of low potency; it could immunize against donor strain tumor or tail-skin grafts but not against body-skin grafts.

The water-soluble antigen described herein was active not only against tumor grafts but also against body-skin grafts.[10] It was isolated from normal splenic cells which were disrupted by low-frequency sonic oscillation. This antigen was histocompatibility-2 group-specific but also contained nonhistocompatibility-2 determinants.

Materials and Methods.—Materials: Inbred DBA-1/J, DBA-2/J, Balb-c/J, CBA/J, and A/J mice were obtained from the Jackson Memorial Laboratory. Six-week-old Carworth Farms White (CFW) male mice were obtained from Carworth Farms. First-set skin grafts of DBA-1/J, DBA-2/J, Balb-c/J, CBA/J, and A/J mice onto CFW recipients were completely accepted at the sixth postoperative day; on the other hand, second-set skin grafts exhibited gross and histologic stigmata of rejection by the sixth day.



Flow sheet for the preparation of the soluble antigen (* refers to active fractions)

Preparation of the antigen: Since antigens prepared by the methods of pressure decompression or homogenization yield membrane-bound insoluble antigens in the author's own and in others'[4–6, 8] hands, low-speed sonic oscillation was employed. Furthermore, since investigators employing water lysis as a primary method of cell destruction[3, 9] have not been able to extract potent soluble antigens, this procedure was abandoned, and the sonic oscillator was used to disintegrate the cells.

Whole dissociated splenic cells were prepared by pressing the organs of donor male mice through a 60-gauge stainless steel mesh into sterile Tyrodes solution (Difco). After allowing the large particles to settle for 10 min, the suspended cells were spun into a button at 1500 rpm. In order to reduce the number of red blood cells in the preparation, the button was then resuspended in 2% acetic acid in Tyrodes solution. Centrifugation at 1500 rpm yielded a precipitate of lymphoid cells. This final button of cells was resuspended in 8 ml of Tris buffer [0.05 *M* Tris (hydroxymethyl)aminomethane, 0.008 *M* magnesium chloride, 0.0025 *M* potassium chloride, 0.15 *M* sucrose adjusted to pH 7.45/25°C], and a white cell count was performed.

The cell suspension was loaded into a Raytheon model S102A 50 w/9 kc per sec magnetostriction apparatus provided with a water-cooled jacket, and was sonicated for variable lengths of time. The number of destroyed cells was determined by counting the suspension in a model B Coulter counter before and after sonic oscillation. When the initial concentration was 3–10 million cells/ml, a 10-min period of oscillation was sufficient to destroy greater than 90% of the lymphoid cells; however, when the initial concentration was up to 500 million cells/ml, a 60-min period was required.

After oscillation the suspension was centrifuged at 20,000 × *g* for 30 min yielding a "postmitochondrial" supernate and a mixed nuclear-mitochondrial precipitate. Studies on the antigenicity of these fractions have been previously reported.[11] This supernate was then centrifuged for 2 hr at 105,000 × *g* in the SW50 head of a Spinco model L ultracentrifuge. The supernate ("cell sap") of this centrifugation had a brownish opalescence, while the precipitate ("microsomes") was colorless. No additional material could be precipitated from the supernate by further centrifugation at 198,000 × *g* for 3 hr. The supernate was then placed inside dialysis tubing (#40 Visking Casing Co.) surrounded by Aquacide 1, C grade (Calbiochem), and concentrated from 9 ml to 2 ml.

The concentrated supernate was pipetted onto a 16–20 × 1.5 cm Sephadex G-200 (lot NF 3019, Pharmacia Chemicals) resin column which was equilibrated with "half Tris" (1:1 Tris buffer:distilled water), a solution of low ionic strength. The void (excluded) volume fraction and the inner (penetrating) volume fraction were collected on a Gibson automatic volumetric fraction collector. The opalescence of the cell sap fraction appeared in the inner volume, while the void volume, which contained half of the protein of the 105,000 × *g* supernate, was colorless. Both fractions were concentrated and then either analyzed or injected.

The concentrated void volume was fractionated by passage through another Sephadex G-200 resin column which this time was equilibrated with buffer at high ionic strength (full-strength Tris buffer prepared as above but with 1 *M* sodium chloride added) in order to decrease protein-protein interactions.[12] Three protein components were separated. They were individually administered to mice without concentration or dialysis. Fractionation of the concentrated void volume was also obtained by the addition of 7 ml of a saturated solution of ammonium sulfate to 2 ml of the concentrated fraction, thereby yielding a 78% saturated solution. A precipitate fraction was collected after ultracentrifugation at 105,000 × *g* for 1 hr. The supernatant fraction was concentrated with Aquacide. Both fractions were dialyzed against 250 vol of distilled water and then administered. All procedures were performed at 3°C.

Method of skin transplantation: Carworth Farms White male mice were injected subcutaneously with 0.5 ml of an antigenic preparation containing variable amounts of protein. After a specified incubation period, challenge orthotopic 6mm × 8mm skin grafts were applied according to the technique of Billingham and Medawar.[13] The animals were bandaged according to a new technique: in place of tulle gras the bed area was covered by a glass chamber sealed onto the host with Mastisol. This seal was protected by two turns of a self-adherent bandage ("Stericrepe," Beacon and Janis).[11]

On the sixth postoperative day the grafts were scored on the basis of their gross appearance, were photographed, and then were removed for histologic preparation according to the method of Medawar.[1] Microscopic analysis was performed by a modification of the method of Billingham *et al.*[14] According to this modification, grafts which appeared like first-set transplants were called "O," grafts having 25–75% epithelial destruction and evidences of an invasive-destructive reaction[15] were called "+," and grafts bearing less than 25% survival and characterized by vasculonecrotic reactions[15] were called "++."

Biochemical methods: Protein determinations were performed either by the method of Warburg and Christian[16] based upon the 260/280 mμ absorption ratio, or by a modification of the biuret method[17] using crystalline lysozyme as a standard. Linear sucrose gradients of either 5–20 or of 15–30% sucrose in Tris buffer were prepared and analyzed by the method of Martin and Ames.[18] Constant density equilibrium ultracentrifugations were performed either in 1.238 density sodium bromide (26%) for 24–30 hr or in 1.5 *M* sucrose for 15 hr.

Results.—Potency of the cell sap fraction: The 105,000 × *g* cell sap supernate was far more potent than the 105,000 × *g* "microsomal" precipitate, which only weakly and inconsistently sensitized recipients. The supernate was equally effective

TABLE 1

SENSITIZATION WITH ULTRACENTRIFUGAL FRACTIONS

Preparation	Incubation (days)	Dose (cell-equiv)*	Ratings
Tris buffer	4	—	○, ○, ○, ○, ○, ○, ○
Cell sap	4	48,000,000	+, +, +, +, +, ○
		320,000,000	+, +, +, +, ○
		850,000,000	+, +, +, ○, ○
	7	33,000,000	+, +, ○, ○, ○, ○
		55,000,000	+, +, +, +, +
		240,000,000	+, +, +, ○
		300,000,000	+, +, +, +, +, +, ○
Precipitate	4	200,000,000	+, ○, ○, ○
		500,000,000	○, ○, ○, ○
		1,500,000,000	+, ○, ○, ○, ○
	7	500,000,000	○, ○, ○, ○
		2,000,000,000	+, +, +, ○, ○, ○, ○, ○

* Spleen cell-equivalents: 1 million cell-equivalents yielded 1.26 μg of cell sap protein.

when administered four days as when administered seven days prior to challenge skin grafting (Table 1, Fig. 1).

The cell sap supernate also accelerated the rejection of solid tumor homografts. Snell *et al.*[19] demonstrated that lyophilized extracts of a DBA-2 leukemia P-1534 induced resistance against subsequent challenge with the leukemic form of the tumor. In the work presented here, this very tumor was maintained aleukemic by subcutaneous trochar passage in DBA-2/J females. Upon passage into CFW male

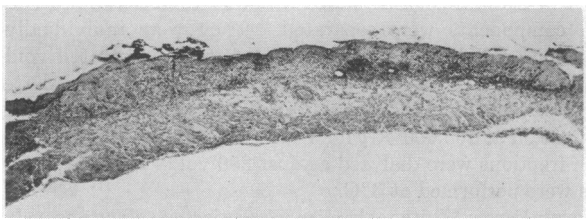


FIG. 1.—55 million cell-equivalents of DBA-1/J cell sap were administered 7 days prior to challenge skin grafting with DBA-1/J tissue. Note marked round cell infiltration, epithelial destruction, and perivenous mononuclear accumulation. This was classed as a "+" reaction.

mice the tumor was initially accepted; a 1-cm nubbin formed by the seventh day. Subsequent first-set rejection of the tumor occurred with a median survival time of 11 days. Preimmunization with DBA-2/J female splenic cells prevented the appearance of the nubbin, while preimmunization with DBA-2/J cell sap supernate accelerated its rejection (Table 2).

Specificity of the cell sap fraction: The cell sap supernatant fraction prepared from A/J (H-2a)[20] male mice was unable to immunize CFW mice against DBA-1/J (H-2q) skin transplants, but readily immunized them against A/J grafts. Likewise, DBA-1/J cell sap supernate induced sensitization against DBA-1/J skin grafts, but not against CBA/J grafts (Table 3, Fig. 2). Therefore, the ability of the cell sap fraction to accelerate graft rejection appeared to have an immunogenetic basis; it was not nonspecific.

TABLE 2

IMMUNIZATION AGAINST P-1534

Preparation	Dosage (cell-equiv)	Viability of tumor 6 days	9 days	12 days
Untreated control	—	15/15	10/15	2/15
DBA-2/J spleen cells	135,000,000	0/8		
DBA-2/J cell sap	800,000,000	11/11	4/11	0/11
Balb-c/J cell sap	825,000,000	6/6	2/6	0/6

TABLE 3

IMMUNOLOGICAL SPECIFICITY OF THE CELL SAP ANTIGEN

Antigen donor	Challenge donor	Dosage*	Immune responses
Buffer	DBA-1/J(H-2q)	—	0/15
A/J (H-2a)	DBA-1/J	900	2/9
A/J	A/J	900	7/7
DBA-1/J*	DBA-1/J	1000	5/5
	CBA/J(H-2k)		0/5
DBA-2/J (H-2d)	Balb-c/J(H-2d)	1000	6/6
DBA-2/J*	DBA-2/J	1000	4/5
	Balb-c/J		4/5
Balb-c/J*	Balb-c/J	600	6/7
	DBA-2/J		6/7
DBA-2/J*	DBA-2/J	480	4/5
	Balb-c/J		4/5
Balb-c/J*	Balb-c/J	480	3/4
	DBA-2/J		3/4

* Dosages in μg protein. † Both challenge grafts were placed on a single bed on the recipient.

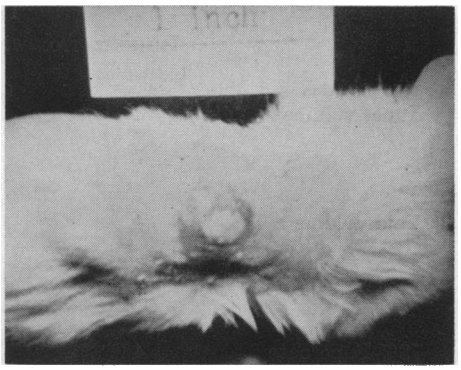


FIG. 2.—Homografts on specifically immune hosts. CFW mouse treated with one mg of DBA-1/J cell sap supernate 4 days preoperatively is carrying a DBA-1/J graft (upper left) and a CBA/J graft (lower right). Note that the DBA-1/J graft was destroyed while the CBA/J graft was unaffected.

The two mouse strains DBA-2/J and Balb-c/J belong to the same histocompatibility-2 group (H-2d).[20] The cell sap fraction of one strain (for example, Balb-c/J) sensitized CFW mice against a subsequent skin or tumor graft from the other strain (DBA-2/J), just as skin homografts from one were capable of sensitizing the recipient against subsequent challenge grafts from the other.

Nonhistocompatibility-2 (weak) antigenic differences distinguish two member strains of the same histocompatibility-2 group. DBA-2/J and Balb-c/J mouse strains differ at weak histocompatibility loci since skin grafts exchanged between them are rejected at a sluggish pace.[21] The accelerated rejection of skin grafts placed upon hosts immunized across weak histocompatibility differences demonstrates nonhistocompatibility-2 antigenic determinants. High dosages of the cell sap supernate readily induced weak determinant immunity, but lower dosages of the antigen had only negligible activity (Table 4). Therefore, the cell sap supernate appeared to possess not only histocompatibility-2 group specificity but also nonhistocompatibility-2 reactivity.

Purification of the cell sap antigen: Passage of the 105,000 × *g* supernate through a Sephadex G-200 resin column which was equilibrated with "half Tris" yielded two fractions: a void volume fraction and an inner volume fraction. While the void volume fraction had a 260 mμ/280 mμ absorption ratio of 0.8, the inner volume had an absorption ratio of 1.2–1.6. The void volume fraction readily sensitized CFW mice. On the other hand, the inner volume fraction was active only at high dosages, in spite of the fact that it contained half of the protein of its parent cell

TABLE 4

NONHISTOCOMPATIBILITY-2 REACTIVITY OF A HISTOCOMPATIBILITY-2 ANTIGEN

Antigen source	Antigen challenge	Host	Dose*	Immune responses
DBA-2/J	DBA-2/J	Balb-c/J	1000	5/7
DBA-2/J†	DBA-2/J	CFW	600	2/2
		Balb-c/J	600	2/7

* Dose in μg protein. † A single preparation injected into both recipient strains.

TABLE 5

IMMUNIZATION WITH PURIFIED FRACTIONS

Fraction	Incubation (days)	Dosage (μg protein)	Immune responses
Inner volume	4	900	+, O, O, O, O
		1600	+, O, O, O, O
	7	600	O, O, O, O
		2000	O, O
		4000	+, +, O, O, O, O
Void volume	4	180	+, O, O
		450	++, +, +, O, O
		600	++, +, +, +, +, +, +, O, O
	7	250	+, O, O
		400	+, +, O, O, O
		600	+, +, O, O, O, O
78% Supernate	4	450	++, ++, +, +, +, O, O
78% Precipitate	4	450	O, O, O
Sephadex Fx I	4	150	O, O
Sephadex Fx II	4	200	+, +, +
Sephadex Fx III	4	250	O, O, O, O

sap supernatant fraction. The antigenic power of the void volume fraction appeared to be extremely dependent upon the length of the interval between antigen administration and chellenge graft application, the incubation period. Less than half of the number of animals were immunized after a seven-day incubation period than after a four-day incubation period (Table 5).

The void volume fraction was perfectly soluble in water. After overnight dialysis against 250 vol of deionized water, ultracentrifugation at 168,000 $\times$ g for one hr failed to precipitate any material.

The void volume fraction was analyzed by ultracentrifugal, column chromatographic, and salt precipitation techniques. Equilibrium density ultracentrifugation for 24 hr in a medium of 1.238 background density (26% sodium bromide) (Fig. 3) or for 15 hr in 1.19 background density (1.5 M sucrose) precipitated the protein to the bottom third of the tube. Since ultracentrifugation in these media distributes lipoproteins in the flotsam, the void volume antigen did not have the bulk properties of a lipoprotein.

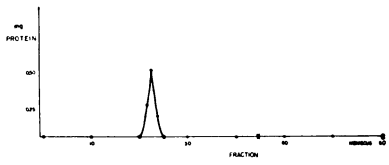


FIG. 3.—Equilibrium ultracentrifugation of the concentrated void volume fraction in 1.238 density sodium bromide. After a 24-hr run in the SW39L head at 35,000 rpm, all the protein sedimented to fractions 20–25 from the bottom of the tube.

The antigen was fractionated by adjustment to a 78 per cent saturated solution of ammonium sulfate. An inactive precipitate was recovered after 2-hr ultracentrifugation at 105,000 $\times$ g. On the other hand, at the same dosages, the overlying supernate was very potent.

Ultracentrifugation in either 5–20 per cent or in 15–30 per cent linear sucrose gradients revealed that the void volume fraction was composed of three protein components (Fig. 4). Three components were also noted upon column chromatography on Sephadex G-200 equilibrated with Tris buffer at high ionic strength (Fig. 5). Each of the components obtained by chromatography was administered individually to CFW mice, but only the second fraction possessed antigenic activity. On the basis of the rate of sedimentation of this component in sucrose gradients and on the basis of its appearance at the very front of the inner volume of Sephadex G-200, an approximate molecular weight of 200,000 was inferred.

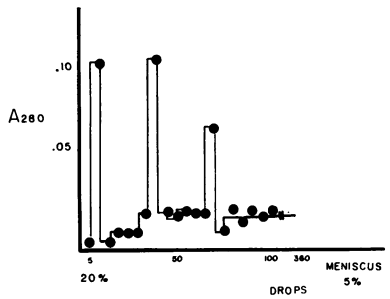


FIG. 4.—Ultracentrifugation of the concentrated void volume fraction (SW39L head, 15 hr) in a 5–20% linear sucrose gradient. Note three distinct protein peaks. The heaviest peak contained 31%, the middle peak 37%, and the lightest peak 32% of the protein.

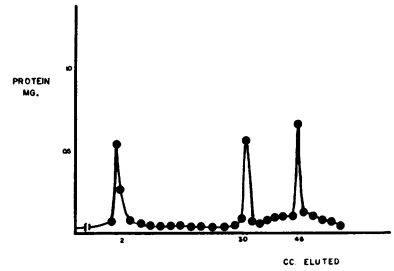


FIG. 5.—Elution pattern of the concentrated void volume fraction of Sephadex G-200 equilibrated with buffer at high ionic strength. Note three distinct peaks. The protein appeared to be almost equally distributed between the peaks: the first peak contained 33% (Fraction I), the second peak, 37% (Fraction II), and the third, 30% (Fraction III) of the total protein recovered (86% of that placed on the column).

Discussion.—Chemistry of the soluble antigen: The isolated antigen was soluble in deionized water, in dilute salt, and in 78 per cent ammonium sulfate solutions; it had an absorption maximum at 280 mμ; and it comprised 0.004 per cent of the wet weight of the whole spleens. It has not been analyzed for gross composition. However, it did not have the bulk properties of a lipoprotein, because it sedimented in media in which lipoproteins form a flotsam, namely, in a salt medium more dense than that employed by Herzenberg[4] and in a sucrose medium of the density employed by Manson.[5] Therefore, if a lipid component was present, it must have been well masked by protein. There are two explanations for the observations that both insoluble lipoprotein and soluble nonlipoprotein materials act as strong transplantation antigens. Either the soluble preparation represents a different antigen than the lipoprotein preparations, or the soluble preparation has the same reactive antigenic grouping as the lipoprotein preparations but was isolated in a different form.

Specificity of the isolated antigen: The soluble antigen possessed both histocompatibility-2 (H-2) and nonhistocompatibility-2 activity. Since mouse strains which differ at the H-2 locus also differ at other, non-H-2 loci (excepting the special case of coisogenic animals), it is necessary to adopt indirect criteria of pure H-2 activity. Gorer[22] demonstrated that hemagglutinins were only produced when a tumor graft and its host differed at the H-2 locus. On this basis some investigators have inferred that their extracts were H-2 antigens if they could induce the formation of hemagglutinins. However, there is the possibility that the parenteral administration of a non-H-2 antigen may permit it to function as an evocator of humoral antibody.

In the work described here, H-2 activity was inferred (*a*) from the cross reactions between two mouse strains which presumably only possessed H-2 groupings in common, and (*b*) from the inability of the cell sap antigen to immunize across non-H-2 differences when administered in dosages yielding strong immunity across combined H-2 and non-H-2 differences.

Non-H-2 activity was demonstrated by the capacity of high dosages of antigen to sensitize one member of an H-2 group against another member of the same group. Davies[23] has found that his lipoprotein antigenic preparation induced not only H-2 but also non-H-2 reactions. Two explanations for the presence of both activities in the same preparation may be offered. Either the preparation may contain low concentrations of contaminant non-H-2 antigen and/or the same chemical substance that carries H-2 antigenic specificity also carries non-H-2 specificities, but the latter are only active at higher dosage.

Since the active site of the transplantation antigen probably represents only a small portion of the antigenic molecule and since alterations affecting adjuvanticity are probably as destructive to activity as alterations affecting antigenicity, the chemical nature of the antigenic determinant remains unknown. The high water solubility and relative purity of the preparation presented here may facilitate the dissection of the histocompatibility determinants.

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*THE ORGANIZATION OF NUCLEI AND CHROMOSOMES IN HONEYBEE EMBRYONIC CELLS**

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Electron microscopy of thin-sectioned material has provided little insight into the macromolecular organization of nuclei and chromosomes. Recently a "whole mount" technique, originally used by Kleinschmidt[1] with bacteria and viruses, has been applied by several authors[2–4] to nucleated cells. In this technique, entire cells are spread on the air-water interface of a Langmuir trough, picked up directly on a coated grid, dehydrated, and dried by the critical point method of Anderson.[5] Such preparations, which may or may not be fixed and stained, make possible direct examination of the fine structure of entire metaphase chromosomes and of whole interphase nuclei.

Examination of a wide range of material by this method[2, 4] has shown that both nuclei and chromosomes contain a system of long, irregularly organized fibers, whose diameter according to most authors varies around 250 A; the occurrence of this approximate dimension across a range of both vertebrates and invertebrates has been impressive. Similar fibers have recently been demonstrated in thin-sectioned calf thymus nuclei,[6] and evidence is accumulating that the chromosomal fibers are nucleoprotein in nature,[4, 6] that they are the sites of messenger RNA synthesis,[6] and that they are organized as a central core surrounded by a sheath of distinctly different physical and chemical properties.[2, 4]

In the work reported here, the Kleinschmidt technique has been applied to embryonic cells of the honeybee.[7] This material is especially suitable for whole mount electron microscopy, due to the remarkably small size of the chromosomes (whose lengths and diameters are less than those of most vertebrate mitochondria) and to the correspondingly low concentration of chromatin in the interphase nuclei.

Materials and Methods.—Interphase nuclei were prepared by squashing 1–4 stage 6 ("gastrulation" stage) honeybee embryos on the tip of a glass microtool, which was then touched to the surface of a Langmuir trough containing 0.25 *M* sucrose. The spread material was picked up by touching the sucrose surface lightly with the coated side of a formvar carbon-coated grid. Grids were then washed by floating them face-down on distilled water for 5 min or less, and were mounted in a plastic grid holder under 25% ethanol. Holder and grids were dehydrated through an ethanol series (5 min each in 35, 50, 70, 95, 100, 100, 100%), and then passed through three baths of amyl acetate (10 min in each). Finally, the grids were placed in the pressure chamber of the Anderson critical point drying apparatus, in which the amyl acetate is replaced by liquid CO_2 under pressure and at reduced temperature (an ice bath); drying occurs instantly and with minimum distortion when the temperature is raised above the critical point (31.1°C).

Chromosomes were obtained by injecting late stage 2 (cleavage stage) honeybee embryos with 10^{-3} *M* colchicine (N.B. Co.), incubating the embryos at 35.5°C for one hr, and then squashing