IMMUNOGENETIC STUDIES OF THE GOLDFISH (*CARASSIUS AURATUS*)

Thesis by

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Finally, I am deeply grateful to my wife who devoted much time and energy assisting in critical experiments and in preparation of this thesis.

ABSTRACT

Transplantation experiments with goldfish scales showed that autografts became normally revascularized and persisted without any inflammation whatever. Homografts elicited an immune response, which was measured by determining median survival times and inflammatory reactions under various conditions. A plot of median survival times against temperature demonstrated that previous grafting from the same donor and higher water temperature both accelerated homograft destruction. The duration and intensity of the host's inflammatory reaction were closely associated with the rapidity of donor tissue destruction. The duration of the first inflammatory phase was dependent upon the rapidity of soft tissue destruction, while the second inflammatory phase was associated with slow digestion of the acellular scale plate. Both soft tissue and scale plate digestion showed the second-set phenomenon.

Reciprocal-homografting operations were undertaken within a pedigree in an effort to estimate the number of genetic loci concerned with scale transplant specificities. Complete cross-grafting of 23 F_1 sibs and 23 F_2 sibs, respectively, revealed no compatible combinations. Reciprocal parent-offspring homografts were also unsuccessful. It was determined that at least four independent histocompatibility loci were required to account for the mutual incompatibility observed.

Techniques were developed for bleeding and isoimmunization

of goldfish, together with methods for handling, preservation, and typing of goldfish erythrocytes. Experiments with isoimmune and rabbit antisera demonstrated the existence of numerous individual differences in the red cell antigens of goldfish.

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PART I

Scale Homotransplantation in Goldfish (*Carassius auratus*)*

1. General Introduction

During the first decade of the present century it was established that autotransplants of various normal and neoplastic tissues in adult mammals were usually permanently successful, whereas homotransplants made under the same conditions were almost always partially or completely destroyed by the host. An autotransplant is a graft involving one animal, while homotransplants are grafts between animals of the same species, but of different genetic constitutions. The numerous pioneer investigations of this early period have been reviewed in a comprehensive volume by Leo Loeb (2). In succeeding years the intensity of lymphocytic and connective tissue reactions to homografts was found to be correlated with the genetic relationship between host and donor. Loeb and his associates developed concepts of individual and organismal differentials to explain the complex manifestations of homograft incompatibility. It was supposed that

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*The material in this section, in a somewhat abridged version, has been accepted for publication (1).

the chemical specificities of gene-determined individuality differentials decided whether the substances introduced by a transplant affected the host as normal auto substances or as toxins of different potencies. The hypothetical differentials of homografts were assumed to act as antigens only secondarily.

Early in the history of transplantation studies various investigators attributed the lack of success of homografts to immune reactions of the host against the graft. In 1912 Schoene (3) found that injection of rabbits with homologous liver, spleen, or kidney immunized the animals so that a subsequent skin homograft was more rapidly destroyed, while autogenous skin was not affected. Tyzzer (4) applied the active immunity concept to the lymphocytic reaction against transplanted tumors. Marine and Manley (5) believed that the primary injury to homografts was mediated through the action of antibodies, because they found that second homotransplants of spleen were more rapidly destroyed than first ones. But proof of the primary immunological basis of homograft destruction came with the elegant, quantitative experiments of Medawar (6,7). After standardizing operative procedures and graft dosage, Medawar accurately measured the survival times of orthotopically transplanted skin grafts in rabbits. The computation of median survival times provided a statistical measure of the rate of homograft destruction. He was then able to show that skin grafts transplanted to an animal which

had already received and reacted against grafts from the same donor are the victims of an accelerated homograft destruction. This "second-set phenomenon" of actively acquired immunity has since been demonstrated with diverse tissues in various mammals and chickens.

During the last decade numerous investigations have produced evidence favoring the acquired immunity hypothesis as an explanation for the rejection of most adult tissue homografts. A recent monograph of papers (8) dealing with the relation of immunology to tissue transplantation may be cited in this connection.

Whether homografts are destroyed by serum antibodies, by contact with host cells, or by both has been uncertain. Two very recent contributions which have gone far toward elucidating the mechanism of homograft destruction will be mentioned. Billingham et al. (9), working with closely inbred lines of mice, have shown that transplantation immunity can be acquired by the introduction of lymph nodes or spleen cells from a graft-immunized animal into a tolerant animal of the same inbred strain. Such immunity could not be transferred by killed lymph nodes, massive doses of whole blood, blood leucocytes, or serum. Because the immunity acquired by the secondary hosts was conferred by cells rather than by the passive transfer of preformed antibodies, its similarity to the tuberculin reaction and to sensitization reactions of the delayed type became evident. Another experimental approach

to this problem was taken by Algire et al. (10, 11) who used diffusion chambers with porous membranes which would permit the passage of essential metabolites and thus allow survival of implanted tissues, but would prevent passage of cells of the host or of the graft. Experiments showed that homografts will survive for long periods of time in vivo within diffusion chambers, even though the host animal has been immunized. However, when cell-penetrable chambers were used which allowed host leukocytes to enter, homografts were soon destroyed. It was thus demonstrated that homografts in mice are destroyed as a result of contact with host cells, rather than by exposure to circulating non-cellular antibodies. In cell-impenetrable chambers mouse tissues survived for at least a week in nonimmunized rats but not in immunized rats, indicating that serum antibodies may be important in the destruction of heterologous grafts.

2. Homotransplantation in Cold-blooded Vertebrates

There have been few detailed studies of homotransplantation reactions in adult cold-blooded vertebrates. Most of the early investigations of the integument in Amphibia were concerned mainly with local specificity of pigmentation rather than tissue incompatibilities. Cole (12) found that homotransplants of skin in frog tadpoles one to two years old preserve their individuality only temporarily, their tissues ultimately being replaced by regenerated tissue of the

host. He considered the inflammatory reaction and disintegration of homografts as evidence that protoplasm of one individual is chemically different from that of a second individual of the same species. The definitive work of May (13) on lizards revealed that autotransplantation of skin on chameleon adults succeeded permanently, whereas all homotransplants were absorbed after they had healed in, the total absorption taking place between 60 and 90 days when the temperature was kept constant at 23.5°C. On the other hand, Collins and Adolph (14) did not observe any difference between the results of auto- and homotransplantation of skin in the adult salamander; it appeared that both remained preserved, but in both a reorganization of pigmentation took place. Later Vogel (15) investigated in detail the fate of skin transplants in adult frogs. He found that after a latent period of about one week, homografts were invariably invaded by host leucocytes. Graft tissues were then destroyed and replaced by host tissues.

Goodrich and Nichols (16) first established the existence in fish of "individuality differentials." They found that autotransplants of scales on all types of goldfish succeeded without loss of any of the tissue elements--epithelium, dermis, or fibrillary plate. But some degree of tissue incompatibility was observed in all homotransplants. This varied from that apparently effecting only the disintegration of chromatophores to that bringing about the

destruction of the whole scale and accompanied by inflammation. Later Sauter (17) and Nardi (18) working with various fishes found that homotransplants comprising scales, muscle, and ribs completely degenerated, while autotransplants succeeded. More recently Held (19) found that fin autotransplants in Platypoecilus maculatus and Xiphophorus helleri were successful when reinnervation occurred. Although 110 fin transplants were made between siblings, using mature and immature fish of both sexes in various combinations, none were successful. None of the investigators just mentioned recognized the immunological basis of homograft destruction. The chief difficulties were lack of adequate criteria for the homograft survival end point and failure to appreciate the importance of temperature controls in using poikilothermic animals.

3. Materials and Methods

The present study is concerned with the quantitative aspects of scale homograft reactions in the goldfish. Goldfish scales are highly vascularized structures with a complex histology which has been well described by Neave (20). Their location and mode of attachment greatly facilitate orthotopic transplantation and subsequent observation, and provide information about general characteristics of tissue transplantation reactions not easily obtained in other systems. Mori (21) was apparently the first to graft goldfish

scales by inserting them in empty scale pockets with a fine forceps. This simple technique has been modified in the present study so that reciprocal homografts can be made among many fish at a time, with autograft and regeneration controls. Plucking of a scale from its pocket involves the removal of (a) the epidermis covering the exposed portion of the scale plate, (b) the dermis and its contained capillaries, chromatophores etc., (c) the osteogenic and fibrogenic cells which invest the scale plate, and (d) the scale plate and guanophores (reflecting layer) lying beneath it (Figure 1). The greater part of the scale pocket is left intact. If a pocket is left empty a complete new scale is regenerated at a rate dependent on the water temperature. This is accomplished in less than a month at 28°-32°C. Throughout this paper the acellular fibrillary plate and bony layer will be called the scale plate, while the whole structure will be termed the scale according to the usage of Goodrich and Nichols (16).

Tricaine Methanesulfonate* was found to be a particularly suitable anesthetic for the prolonged operations of multiple scale grafting. Experimental fish placed in the anesthetic at a concentration of 60 mg. per liter of water become quiescent in about twenty minutes. Somewhat more anesthetic is

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*M.S.222 of the Sandoz Chemical Works, 68-72 Charlton St., New York 14, New York.

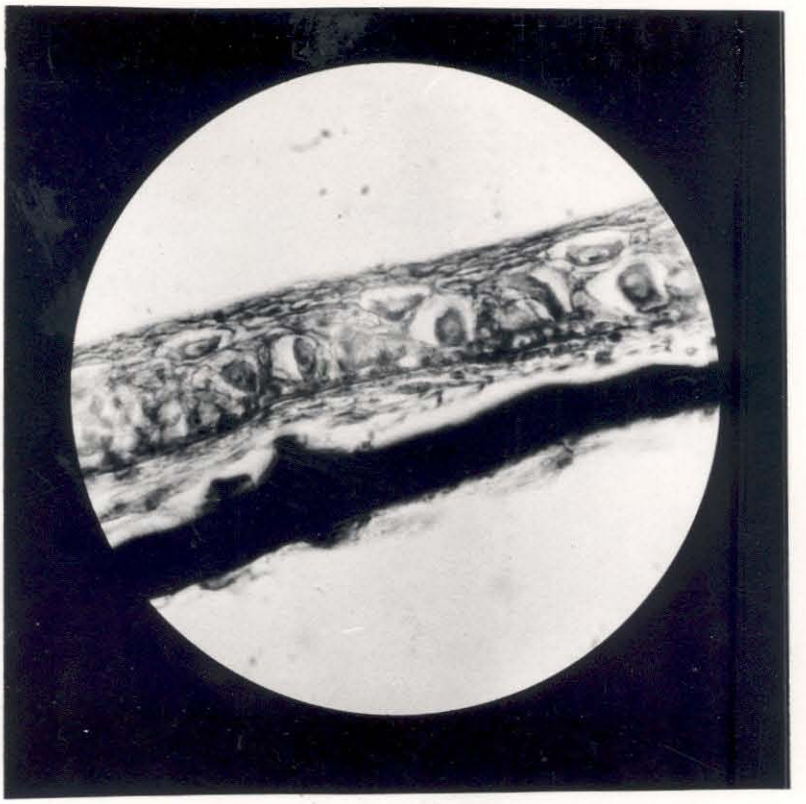

Figure 1. Normal scale in cross section. Darkly-stained scale plate underlies cellular elements. Mallory's triple stain.

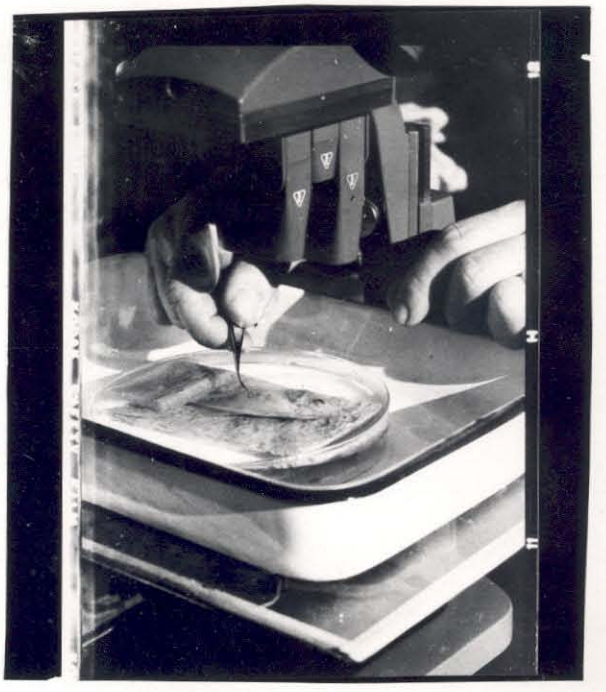

Figure 2. Technique of scale grafting showing anesthetized goldfish under microscope.

required at water temperatures about 22°C. or after fish have been repeatedly anesthetized at intervals of a few days. The fish may be kept in this solution for several hours without harmful effect, and such fish recover in a few minutes when returned to normal aquarium water. Operations are performed under a binocular dissecting microscope as shown in Figure 2. The petri dish containing anesthetic in 0.8% saline will accommodate two fish at a time lying side by side on a thin sponge. A shallow watch glass containing 0.8% saline is used to hold one scale briefly during reciprocal graft exchanges. For convenience, grafts are generally made in the row of scales just above or below the lateral line, using alternate scale positions which are numbered from the operculum (Figure 3). This procedure facilitates the operations as well as subsequent identification of each graft. The cytotoxic products of homografts elicit local inflammation in adjacent recipient tissue, but use of alternate scale positions prevents the grafts from affecting each other directly. Since scales most often do not fit closely into a foreign pocket, a fine scissors is used to trim the scale to the proper size prior to insertion. With practice, this can be done precisely and rapidly. If a scale is too small to fit properly into an homologous pocket, a larger scale from the abdomen of the same donor is used instead. The overlapping of adjacent scales holds the grafts in place. Goldfish are remarkably resistant to infection

Figure 3. Tagged fish showing scale grafts in row above lateral line. Four inflamed homografts on each side of central autograft control.

and no sterile precautions are required during or after the operation. The circuli or growth rings of the scale plate provide an easily observed record of growth and replacement. Differences between ontogenetic and regenerated scales are clear-cut; they have been described and figured by Wunder (22).

4. General Characteristics of Scale Transplantation Reactions

In each series of homografts every fish also received an autograft and the donor site was left empty as a regeneration control (Figure 3). Each fish was identified with a tag inserted through the caudal peduncle as illustrated. Histological changes in scale grafts were observed in vivo under the microscope as well as in stained sections. The reflecting layer of the scale serves as an effective mirror which facilitates detailed observation of the superficial tissues. All 379 autografts made to date have been normally revascularized and remained so without any inflammation whatever. The time required for restoration of normal circulation in autografts varies from only one day at 32°C. to 12-15 days at 10°C. At 28°C. and below, there is initially slight vasodilation and sluggish circulation in autografts as compared to adjacent normal scales. At lower temperatures this condition may persist for several days before the circulation becomes completely normal. All autograft tissue elements re-

main intact. The small area injured by the forceps is repaired, and growth of the scale continues as the fish grows (Figure 4).

In contrast, all homografts are overgrown with hyperplastic host tissue and induce capillary leakage and vasodilation in the contact zone with recipient tissue. The higher the water temperature, the more rapid and conspicuous are the manifestations of incompatibility. The cellular tissues of the 3090 homografts made thus far have invariably failed to persist. Many first-set homografts are initially revascularized as readily as autografts, but circuation is almost always sluggish, often incomplete, and persists for a few days at most before there is complete stasis. With second-set homografts there is generally no restoration of capillary circulation. In the exceptions, observed at 23°C. and 28°C., the circulation lasted for only 24 hours or less. While there is nothing to choose between auto- and homografts for the first few days at 15°C. and below, homografts made at higher temperatures induce distinctive hyperplasia within 24-48 hours. In experiments done at 28°C. and above, homografts provoke cytotoxic reactions so rapidly that there is conspicuous hemorrhage in the recipient contact zones within 24 hours after grafting. Under the latter conditions circulatory restoration is achieved in only a few first-set homografts and lasts only a day or two. Difference of sex has no apparent effect on the homograft reaction, nor differences

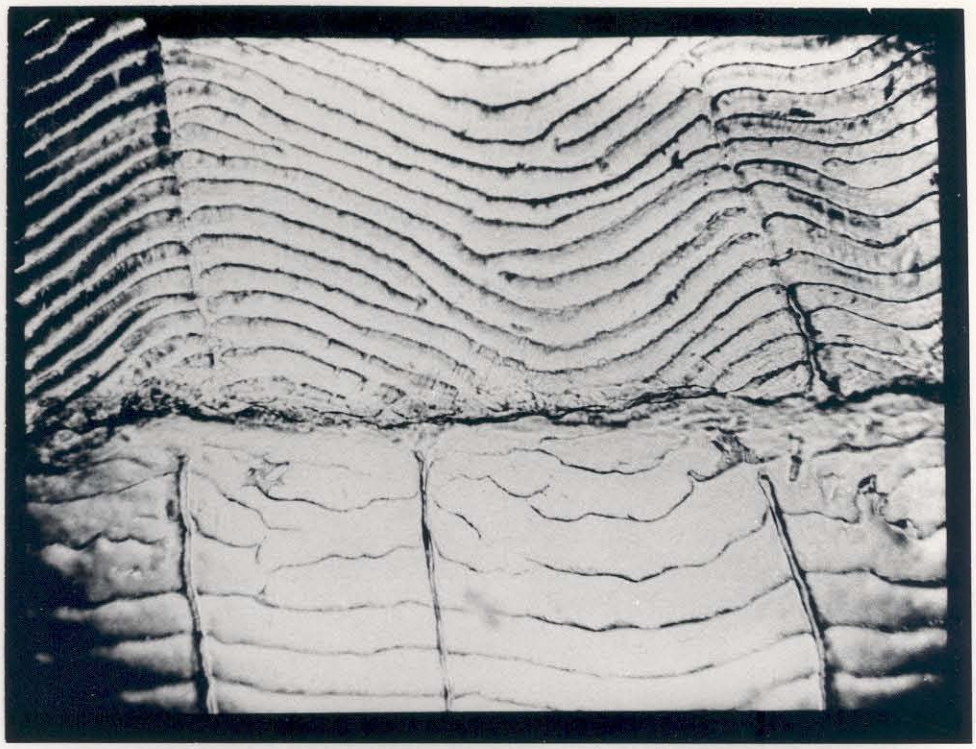

Figure 4. Autograft scale plate showing ontogenetic circuli above, and horizontal line along which graft was trimmed with new scale growth below. Alizarin red S stained in 1% aqueous KOH.

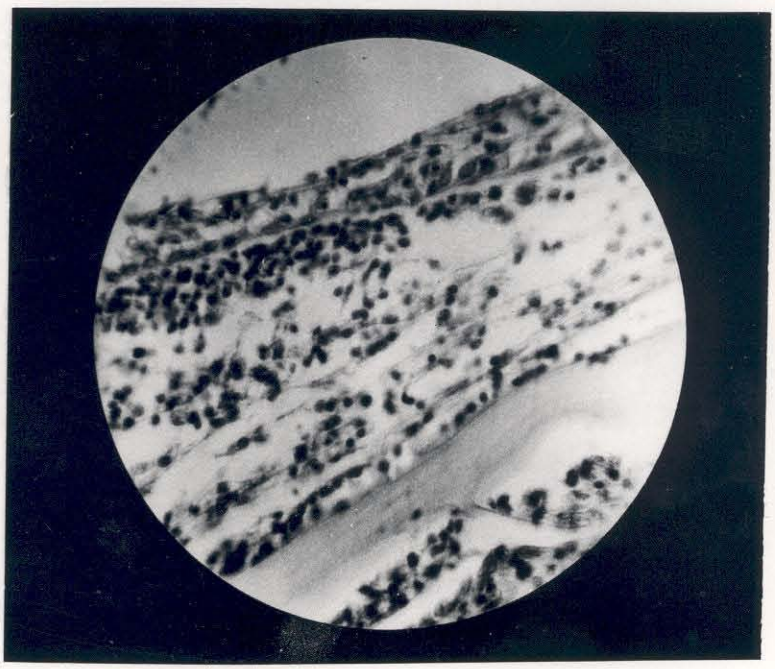

Figure 5. Scale homograft at survival end point. Note loss of cytoplasm and lymphocyte infiltration. Hematoxylin-erythrosin B stain.

of age over the interval nine months to three years.

5. Estimation of the Survival End Point

The experiments now to be described have shown that second-set homografts break down much more rapidly than first-set homografts in all recipient fish. This immune response has been measured by determining the median survival time (MST) and inflammatory reaction of scale homografts under various conditions. Determination of the end point of donor tissue breakdown has been based primarily on biological test of survival experiments, i.e., grafting of scales back to the donor at daily intervals following homotransplantation. Scales transplanted back to the donor after four or more days initially evoke inflammation and hyperplasia which persists for several days. This is attributable to the infiltration of the graft by recipient cells which are foreign to the donor. After this crisis any surviving donor tissue is revascularized and persists. It was found in all cases that some donor tissues including capillaries survived up to the time that clearing of the dense hyperplastic tissue over the homograft took place, as observed under the microscope. At the time of clearing only donor chromatophore granules and reflecting tissue remnants in process of sloughing remain on the scale plate. Fortunately the "clearing phenomenon" is quite consistent and can be applied to all phenotypes as a close approximation of the survival end point. In all the

time-mortality studies to be described, the survival end point of homografts was judged by the clearing criterion. The in vivo criteria of survival have been corroborated by stained preparations of homografts in progressive stages of breakdown. After Bouin's fixation and infiltration with dioxane and histowax, entire grafts were embedded in histowax and sectioned at 10 μ. Cross-sections representing several levels of each graft were stained with Delafield's hematoxylin and lightly counterstained with erythrosin B using the dioxane method. At the time of complete breakdown donor cells become pyknotic, then lose cytoplasm and finally fragment into chromatin droplets (Figure 5). The fate of the acellular scale plate will be considered later.

Some systematic error is probably inherent in the biological test of survival for two reasons. Destruction of donor tissue by recipient leucocytes may continue for a short time after transplantation back to the donor, and subsequent inflammation resulting from destruction of recipient cells which have infiltrated the graft may damage intact donor cells. Nevertheless, histological analysis and in vivo observation of intact tissue correlate well with results of the biological test of survival.

The rapid graphic method of Litchfield (23) has been used for the time-mortality curves. This method permits use of the data in their original form throughout. The original time-mortality data for the scale homografting experiments to

be considered are given in Table 1a, b. Calculations of MST's, standard deviations, and standard sampling errors are performed nomographically after plotting the data on logarithmic-probability paper. A logarithmic-probability plot of the results from expt. 4 is shown in Figure 6 as an illustration of the method. The solutions so obtained are as accurate as those derived by the method of probit transformations (cf. Billingham et al. (24)). Time-mortality curves (e.g. Figure 7), in which the cumulative number of grafts inflamed and destroyed is plotted against days after grafting, show that the duration and intensity of the inflammatory reaction are closely associated with the rapidity of donor tissue destruction. As determined microscopically, capillary hemorrhage in the donor-recipient contact zone always appears earlier and is more severe in second-set homografts. Inflammation is much diminished at lower temperatures and capillary hemorrhage did not appear at all in one series of three sets of homografts where tissue breakdown was followed at 8°C. However, overgrowth of recipient tissue is associated with homograft destruction at all temperatures. It is more conspicuous at higher temperatures. That homograft destruction takes place only when recipient leucocytes have direct access to graft tissues has been conclusively shown with diffusion chamber techniques by Weaver et al. (11).

Table 1a. Homograft Time-Mortality Data

	1st Set			2nd Set		
		Grafts Destroyed			Grafts Destroyed	
Expt.	Days after Grafting	No.	%	Days after Grafting	No.	%
1(A)	5	0	0	1	0	0
	7	25	78.2	2.8	3	9.38
	9	32	100	5	32	100
1(B)	5	0	0	1	0	0
	7	23	71.8	2.8	1	3.12
	9	29	90.7	5	29	90.6
	11	31	97.0	7	32	100
	14	32	100			
2(A)	4	0	0	2	0	0
	6	3	15.0	3	2	10.0
	7.2	12	60.0	4	6	30.0
	8	20	100	5	19	95.0
				6	20	100
2(B)	4	0	0	3	0	0
	6	4	20.0	4	8	40.0
	7.2	13	65.0	5	17	85.0
	8	17	85.0	6	20	100
	9.1	18	90.0			
	10.8	20	100			
3	4	0	0	1	0	0
	6	11	15.3	2.8	2	2.78
	8	63	87.5	5	66	91.5
	10	72	100	7	72	100
4	23	0	0	11	0	0
	26	2	2.33	15	9	10.2
	28	3	3.48	19	42	47.6
	34	19	22.1	24	74	84.0
	37	27	31.4	29	84	95.6
	40	42	48.8	34	88	100
	43	51	59.2			
	45	61	70.9			
	49	67	78.0			
	52	76	88.3			
	56	81	94.2			
	61	83	96.5			
	65	86	100			

Table 1b. Homograft Time-Mortality Data

	1st Set			2nd Set		
		Grafts Destroyed			Grafts Destroyed	
Expt.	Days after Grafting	No.	%	Days after Grafting	No.	%
5	13	0	0			
	17	3	3.45			
	21	16	18.4			
	25	51	58.8			
	29	63	72.4			
	34	78	89.7			
	39	87	100			
6	9	0	0	8	0	0
	13	2	2.22	11	9	10.3
	17	21	23.3	14	44	50.6
	21	42	46.6	17	73	83.9
	25	78	86.7	21	87	100
	29	90	100			
7	4	0	0	5	0	0
	6	2	2.30	7	24	27.0
	8	3	3.45	8.9	62	69.7
	10	6	6.90	11	87	97.5
	12	32	36.8	13	89	100
	14	62	71.2			
	16	85	97.8			
	18	87	100			
8	3	0	0	3	0	0
	6	4	4.50	5	38	44.7
	8	52	58.3	7	62	73.0
	10	69	77.5	10	81	95.1
	12	85	95.5	14	85	100
	14	89	100			
9	4	0	0	3	0	0
	6	30	37.5	4	27	33.7
	8	75	93.8	5	58	72.4
	10	80	100	6	76	95.0
				8	80	100
10	3.0	0	0	1.0	0	0
	4.2	40	44.4	2.2	8	8.9
	5.3	83	92.1	3.1	35	38.9
	6.2	90	100	4.1	75	83.3
				5.2	90	100

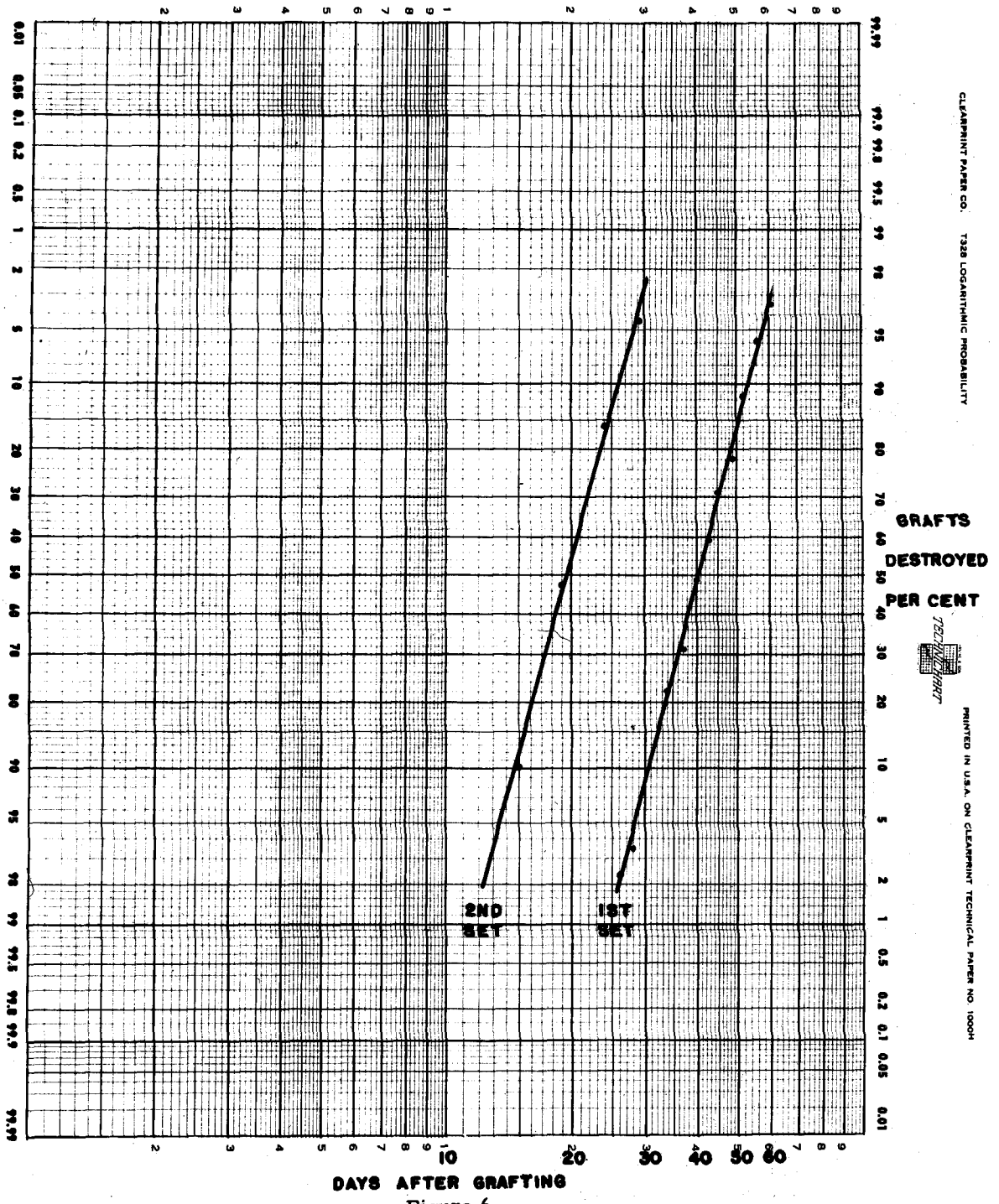
CLEARPRINT PAPER CO. T328 LOGARITHMIC PROBABILITY
GRAFTS DESTROYED PER CENT
2ND SET
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Figure 6.

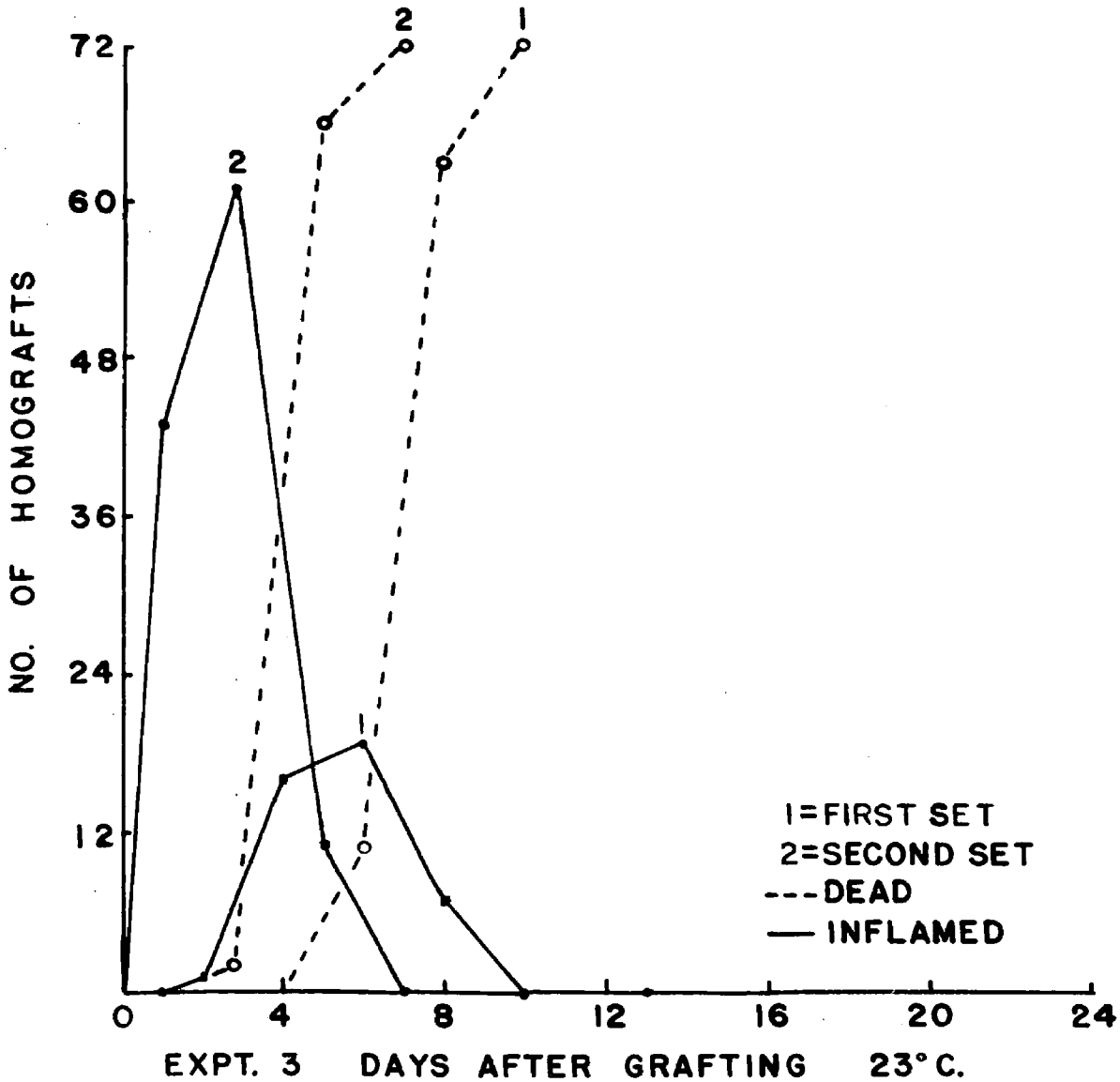
72
60
48
36
24
12
NO. OF HOMOGRAFTS
0
4
8
12
16
20
24
EXPT. 3 DAYS AFTER GRAFTING 23° C.
1=FIRST SET
2=SECOND SET
---DEAD
— INFLAMED

FIGURE 7

6. Graft Dosage Experiments

Since it was desired to quantitate homograft destruction on the basis of multiple scale grafts to each recipient, any effect of differences in graft dosage on the median survival time had to be determined. Siblings of similar size derived from the same spawning and reared together were used in order to minimize non-genetic differences. Highly inbred lines are not available. Four groups of animals were grafted as shown in Table 2, expts. 1 and 2. Each individual in expt. 1 received eight homografts, while those in expt. 2 each received half this dosage or four homografts. Half of the animals in each experiment were grafted from single donors and the remainder were grafted from four different donors. The temperature was maintained at 23°C. and graft destruction was followed by daily microscopic observations. After 26 days second-set grafts were made using the same procedure on the opposite side of the body. The results show that in the dosage range tested there is no significant dosage effect on the median survival time of first- or second-set homografts either when the same donor or different donors are involved. A comparison of time-mortality distributions (last column), i.e., of the intervals between survival end points of the first and last grafts in each set to break down, reveals a narrow distribution of survival times in first sets when all grafts are taken from single donors. When however diverse

Table 2. Scale Homograft Survival Data

Expt.		No. of Recipient Fish	No. of Grafts per Fish per Set	No. of Different Homograft Donors	Temp. °C.	Median Survival Time ± Standard Error (days) 1st Set	Median Survival Time ± Standard Error (days) 2nd Set	Interval Between 1st & 2nd Sets (days)	Time-Mortality Distribution (days) 1st Set	Time-Mortality Distribution (days) 2nd Set
1	(A)	4	8	1	23	6.6±0.2	3.5±0.2	26	4	4
	(B)	4	8	4	23	6.8±0.4	3.9±0.2	26	9	6
2	(A)	5	4	1	23	6.8±0.2	3.9±0.2	26	4	4
	(B)	5	4	4	23	7.0±0.3	4.2±0.2	26	7	3
3		8	9	7	23	6.9±0.2	3.8±0.1	25	6	5
4		10	9	9	10	40.5±1.9	19.5±0.9	72	41	23
5		10	9	9	15	24.9±1.2	---	--	25	--
6		10	9	9	16	20.5±0.9	13.9±0.5	35	18	13
7		10	9	9	19	12.6±0.3	8.0±0.2	30	13	8
8		10	9	9	21	8.3±0.3	5.4±0.4	30	11	11
9		9	9	9,8*	28	6.3±0.2	4.4±0.2	30	6	5
10		10	9	9	32	4.3±0.1	3.2±0.1	17	4	4

*One fish died after the first-set grafts were made; the graft dosage was kept the same in the second-set by placing two grafts from one donor on each recipient.

donors are used, the mortality curve extends over a longer period. This spread is attributable to the relatively prolonged survival of a few grafts. In the second sets, immunity has been developed against all graft tissues and the time-mortality curves are compressed. The survival time of individual homografts in the first set reflects the antigenicity of each graft relative to particular recipients. Variations of operative technique affecting the healing and vascular penetration of the grafts may be held chiefly responsible for the differences in survival times of grafts transplanted from the same donor to a given recipient.

7. Effect of Water Temperature on the Median Survival Time

Cushing (25) demonstrated an effect of temperature upon antibody production in fish. He found that the rate of production of agglutinins against sea urchin sperm in carp and goldfish was much faster in the fish kept at 28°C. than in fish kept at 15°C. In the present study, several comparable series of grafts were made at constant temperatures ranging from 10°C. to 32°C. The fish were of uniform gold phenotype in all experiments except #8 where shubunkins were used. Unrelated fish were selected from the large stocks of a local fish farm* to obtain a maximal response to the homografts in

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*These fish were supplied by the Altadena Water Gardens, Altadena, Calif.

each experiment. Fish were tagged when received and acclimated to the desired temperature for one week prior to grafting. Reciprocal homografts, along with autograft and regeneration controls, were made among ten fish at each temperature. Each graft was observed microscopically at intervals of one to several days depending on the rapidity of homograft destruction in each experiment. After the first-set homografts were completely destroyed, second-set homografts were made on the opposite side of the body. One or two homografts out of 90 were often lost from each set within 24 hours of an operation as a result of the activity of the fish before firm healing was achieved. The time-mortality statistics were computed on the basis of the total number of grafts retained. The results are summarized in Table 2, expts. 4 through 10. A plot of median survival times against temperature is shown in Figure 8. It is evident that the median survival times of both first- and second-set grafts are closely temperature dependent. Over the range of 10°C. to 19°C. the first-set MST's decrease linearly with an increase in temperature. Above 19°C. and particularly above 21°C., the rate of homograft destruction increases less rapidly, indicating a modification in the temperature dependence of the response. This is true also of the second-set homografts at higher temperatures. At any given temperature the median survival time of the second set is approximately one-half to two-thirds of that

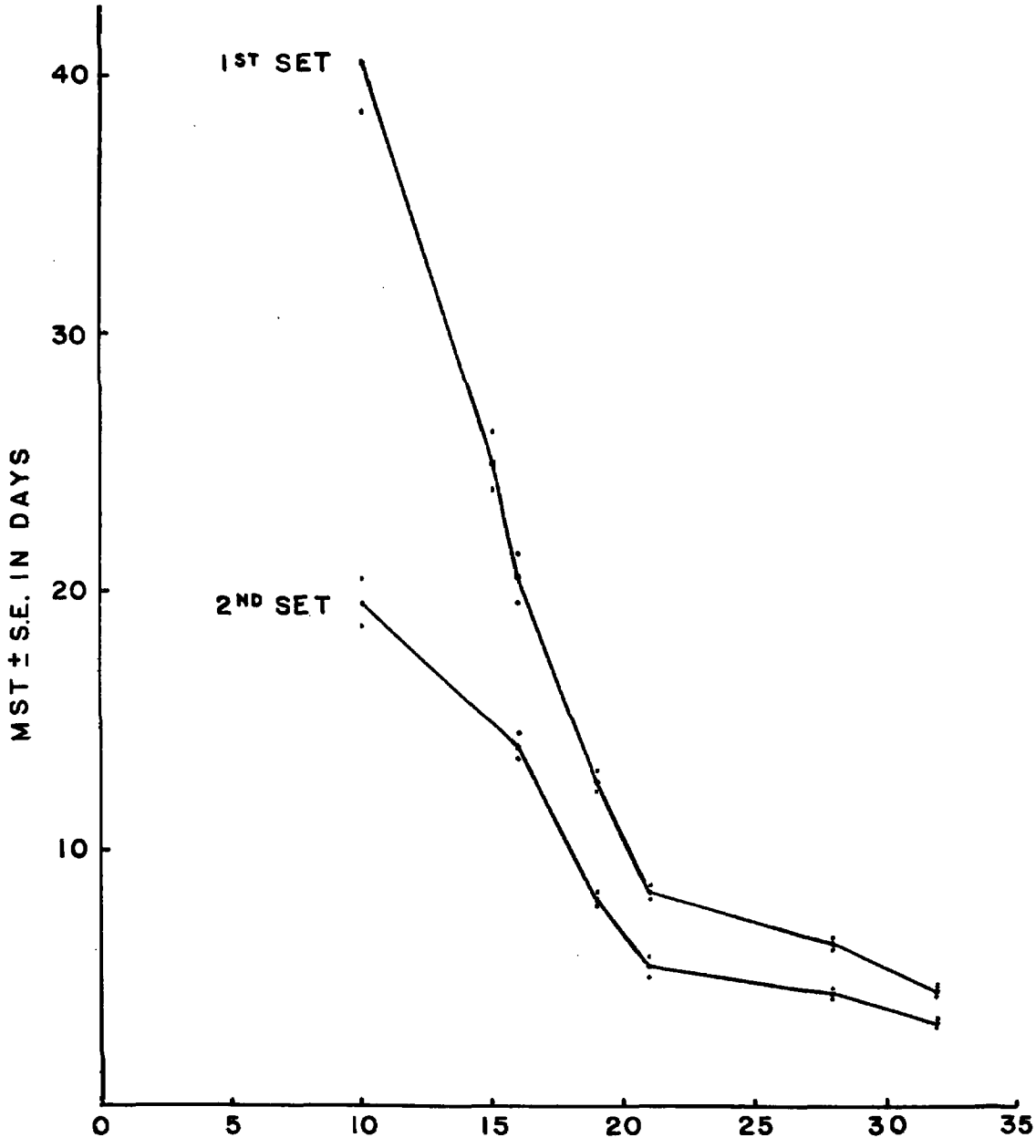
1ST SET
2ND SET
40
30
20
10
MST ± S.E. IN DAYS
0
5
10
15
20
25
30
35
WATER TEMPERATURE IN °C.

FIGURE 8

observed in the first set. It may be argued that differences in metabolic rate, affecting the rate of digestion of donor tissues by recipient leucocytes, exert the dominant influence in destruction of the second set as well as the first set. Even at 32°C., which approaches the maximum temperature at which goldfish may be maintained for prolonged periods, the rate of destruction of both sets of homografts is still higher than at 28°C. The rate at which new scales developed in the regeneration control sites also increased with the temperature, as did the rate of revascularization of autografts.

If the rate of homograft destruction in these experiments is plotted as the reciprocal of the MST and the effects of temperature are expressed in terms of Q_{10}, the following results are obtained:

Temperatures	Q_{10} of the 1st Set	Q_{10} of the 2nd Set
20°C./10°C.	3.95	3.04
25°C./15°C.	3.55	3.03
28°C./18°C.	2.34	2.13
32°C./22°C.	1.87	1.65

It is seen that the Q_{10} values decrease markedly as increasing temperatures are compared for both sets of homografts. Heilbrunn (26) points out that in general the Q_{10} of biological processes is between 2 and 3. But a process which is inhibited at low temperatures would have a high Q_{10} for the temperature

range just above these low temperatures, while there is typically a falling off in Q_{10} values at temperatures above the optimum for a given species. These considerations may be applied to the scale homograft destruction data above. Yet all the Q_{10} values are observed to fall in the range of normal, chemical reactions.

8. The Second Inflammatory Phase and Scale Plate Digestion

Thus far we have considered the aspects of homograft destruction affecting the cellular or homovital tissues of goldfish scales. In expts. 1, 2, and 3 which involved a single sibship, i.e., syngenesiotransplants in the terminology of Loeb (2), all donor scale plates were eventually reinvested with soft tissues by the recipients after digestion of the homovital tissues was completed. As shown in Figure 7, there was no renewal of inflammation around either set of homografts after the cellular tissues had broken down. That the donor scale plates did in fact persist was ascertained by periodic examination for seven months after grafting. The proximal concentric circuli observed only on ontogenetic scale plates remained intact on the homografted plates.

In the experiments where unrelated goldfish were utilized in reciprocal homografting operations, the fate of the scale plate was generally quite different. In a few instances homograft scale plates persisted and were reinvested with soft

tissues as in the sibship described above. In expt. 9 where scale plate digestion proceeded rapidly at 28°C., it was observed that no homograft plate remained completely intact. About 12% of the reconstituted scale plates of both sets represented a mosaic of donor and recipient elements as judged by the circuli (Figure 9), while in the remaining sites new scales were regenerated after complete digestion or sloughing of the homograft plates. The course of the inflammatory reaction in this experiment (Figure 10) is typical of the response to multiple homografts observed in all the experiments with unrelated fish. At each observation the severity of capillary hemorrhage (redness) in the donor-recipient contact zone was recorded for each homograft as (0), (±), (+), or (++). Although inflammation is generally more severe in second-set homografts and at the maxima in both sets, the plotted curves account only for the total number of homografts inflamed to any degree (±, +, or ++) at each observation. It should be emphasized that the autograft controls showed no inflammation at any time. One observes that the first inflammation disappears as the cellular elements of the homografts become completely digested. A second inflammatory phase is then initiated as the scale plates come into close contact with outgrowing recipient capillaries and plate digestion is soon evident. The two phases in each set overlap in the cumulative plots because of the different rates of homograft destruction. Most of the homograft plates which fail to elicit capillary hemor-

Figure 9. Mosaic donor-recipient scale plate. Partially digested donor plate above and recipient plate below. Alizarin red S stained in 1% aqueous KOH.

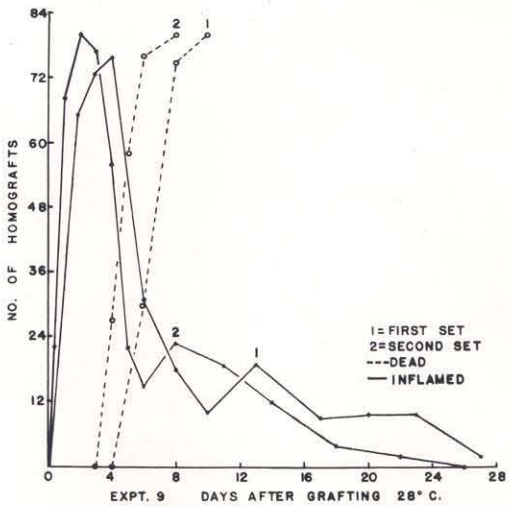

Figure 10.

rhage in the contact zone do cause peripheral hyperplasia and vasodilation during the course of plate digestion. In these instances the recipient is apparently able to remove cytotoxic substances before much local damage is done. A comparison of first- and second-set inflammatory reactions is nevertheless enlightening. While the second inflammatory phase in the first set does not reach a peak until the 13th day, the equivalent peak is reached by the second set on the 8th day. An increased overlap of these phases is also evident in second-set homografts.

Further insight into these phases was obtained by making third- and fourth-set homografts at 30 day intervals on the animals of expt. 8. The median survival times of the third and fourth sets were 5.2 ± 0.3 days and 5.2 ± 0.4 days, respectively. On this basis there was no significant increase in the rate of the reaction over that observed for the second-set homografts. However, when the inflammatory curves of the four sets are compared it is found that the second inflammatory phase begins earlier and involves more homografts in each succeeding set. The course of this phase in the second and later sets cannot be readily quantitated, because the severity of the second phase causes many scale plates to be sloughed at the outset. That the homograft scale plate by itself was capable of inducing an inflammatory reaction was demonstrated by first digesting away the soft tissues of scales in 0.1N NaOH, and then cleaning the resulting scale

plates in saline before grafting. Autografts made in this manner at 22°-24°C. were reinvested with soft tissues, while homografts elicited local hemorrhage which developed between the 10th and 15th day after grafting, and were eventually sloughed or digested. The time required for inflammation to develop was close to that observed for the second phase with intact homoscales at similar temperatures.

The mosaics are structurally a single scale plate invested with normal, vascularized tissues which show no incompatibility. But the donor portion of mosaics is always pitted or eroded, indicating that some digestion has taken place. In contrast, donor plates which are completely digested fail to fuse with newly-forming recipient scleroprotein, and continue to elicit inflammation as long as any remnant remains in contact with recipient capillaries. If the formation of mosaic scale plates was the consequence of non-antigenicity of the retained donor portion, then one would expect that the same donor-recipient combinations would give mosaics in both first and second sets. However, close correspondence is precluded by the fact that at higher temperatures many homoscale plates are sloughed at the peak of the second inflammatory phase. It was observed in expt. 9 that 10 of the 21 mosaic plates represented donor-recipient combinations common to both sets. In expt. 10 at 32°C., 22% of the scale plates became mosaics and 30 of the 40 mosaics showed donor-recipient specificity (i.e. the same donor-

recipient combinations in both sets). In expt. 9, 18 of the 21 mosaics occurred on three recipients, while in expt. 10, 25 of the 40 mosaics developed on two recipients. It is therefore certain that the formation of mosaics depends on the antigenic constitution of the recipient even though complete donor-recipient specificity is not clearly demonstrable. Donor scleroprotein in mosaics may be very slowly replaced by the recipient without visible evidence of incompatibility. All the evidence taken together indicates that at least some of the scleroproteins comprising the scale plate are antigenic, but are only slowly digested by homologous recipients. This digestion always takes place more rapidly in second-set homografts. The lack of such digestion in the syngenesiotransplants studied and the partial digestion observed in mosaics suggest that the scleroproteins in general may manifest less antigenic diversity than the homovital tissues of the scale. The experiment with treated scales involved only a few fish and it is quite possible that other kinds of treatment in larger experiments would give different results.

The possibility that damaged scale plates might be autoantigenic or digested when the collagen fibers were exposed directly to the highly vascularized dermis was also investigated. Distal sections of the scales of four fish were cut away both <u>in situ</u> and in conjunction with autografting. In all cases hyperplastic tissue appeared in the area

of the scale pocket where the scale had been cut away, but there was no inflammation. In less than three weeks at 23°C., new scleroprotein was formed in all cut-away areas to reconstitute the scales to a normal shape and size. There was therefore no indication that damaged scale plates are autoantigenic.

9. Discussion

It is rather surprising to find that autografts made at lower temperatures can survive without visible damage for one to two weeks before capillary circulation is restored. The thinness of the scale and external contact with cold water apparently facilitate respiration and metabolism in the absence of a dermal blood supply. A similar delay in revascularization is observed in first-set homografts at these temperatures. Here too there is no apparent tissue death during the early period of ischemia. The analagous resistance of mammalian skin to anoxia and general ischemia has been considered by Billingham et al. (24). The rate of breakdown of first-set scale homografts was not observed to depend on the length of time that circulation persisted. In scale transplantation, the superficial overgrowth of hyperplastic host tissue appears to provide the critical contact with all homografts. This applies especially to second-set homografts where circulatory restoration is rarely achieved at any time, whereas hyperplastic overgrowth is

more conspicuous than in the first set. From these considerations it is evident that hemal stasis cannot be used as the end point of scale homograft survival as Taylor and Lehrfeld (27) have done with rat skin grafts. Any means of estimating survival time must in the last analysis depend on biological tests of survival involving transplantation of homografts back to the original donor.

The early recognition of a first-set homograft by a recipient is a phenomenon which has not been observed in skin transplantation studies. Medawar (28) points out that the onset of the homograft reaction is preceded by a latent period during which homografts are indistinguishable from autografts transplanted in surgically equivalent ways. The existence of a latent period of several days has been taken as evidence that there is no "natural" immunity to skin homografts. With scale homografts the length of the "latent period" decreases as the temperature is increased. But at higher temperatures overgrowth of homografts by hyperplastic tissue clearly distinguishes them from autografts in only 24-48 hours. Inflammation and interference with circulatory restoration of homografts are also evident at this time. At first sight this might appear to contradict the immunological concept that homografts are destroyed as a result of prolonged contact with "immunized" recipient cells. However, recent studies of Jerne (29) with a very sensitive test system measuring specific antibody stabilization and inacti-

vation of bacteriophage T4 reveal antibody production within 48 hours after a single injection of a horse with T4 antigen. It would seem probable that vertebrates in general are capable of antibody production very soon after exposure to antigen, but that the usual in vitro test systems are not sufficiently sensitive to demonstrate this. Thus one need not invoke the presence of homotoxins or natural immunity to account for the early homograft reaction at 28°C. and above. The observations indicate that the goldfish can rapidly distinguish "self" from "not-self" and respond with an intense, local cellular reaction which mediates homograft destruction.

Although homograft survival time has been shown to be influenced by graft dosage in rabbits (Medawar, (6)) and in rats (Taylor and Lehrfeld (27)), the effect obtains only when a single set of regional lymph nodes serve the graft area, or when very minute grafts are compared to large grafts. Thus it is not surprising that two-fold differences in dosage of scale homografts made along the side of the body have slight if any effect on the median survival time. It is possible that a single scale homograft constitutes a "large dosage."

While grafts transplanted to a single recipient from genetically diverse donors survive for very different lengths of time, the time-mortality relationships were found to follow normal, sigmoid curves in cumulative plots. These curves became straight lines when plotted on logarithmic-probability

paper. This suggests that the grafts may behave almost independently, since the same number of grafts made singly to separate recipients should also give a log-normal curve. The slopes might differ significantly, however. The test for parallelism of the slopes of different time-mortality curves devised by Litchfield (23) enables one to determine whether two curves deviate significantly from parallelism. A significant deviation would indicate that the nature of the response to homografting differs in the experiments compared. Recipients from a highly inbred line would have to be used to resolve this dosage question. One might expect that multiple grafts would cooperate in bringing about their destruction to the extent that they share antigens in common. However, the scale homograft dosage experiments indicate that within the limits studied at least, the quantity of antigen digested does not modify the MST's of either the first- or second-set grafts. The fact that hyperimmunization with third- and fourth-set grafts in expt. 8 failed to further shorten the MST found in the second set is additional evidence in point.

All the cumulative time-inflammation curves show that the duration of the first inflammatory phase is closely associated with the rapidity of donor tissue destruction. When the intensity of hemorrhagic reactions around individual homografts is compared with their survival time, no relationship is evident. Moreover, certain fish were observed to

give more severe inflammatory reactions to all homografts than others in the same experiment involving reciprocal exchanges. Yet inflammation is generally more intense around second-set homografts where the median survival time is much less. It can be stated that inflammation becomes minimal in both sets as the survival end point for all homografts in an experiment is reached. The second inflammatory phase begins in the grafts first broken down while there is still some surviving tissue in the last grafts to reach the survival end point. The second phase was observed to be a consequence of relatively slow digestion of the scleroproteins comprising the scale plate. Scale plate digestion also showed the second-set phenomenon.

Goodrich and Nichols (16) state that the disintegration of chromatophores is the only constant feature of the scale homograft reaction, because some dermal tissues and surface epithelium appeared to persist in certain homografts. This is not in accord with present findings. All the cellular tissues of the 3090 homografts studied, including 1374 grafts between siblings, were destroyed by the recipients.

10. Summary

1. Goldfish scales are highly vascularized structures with a complex histology. Their location and mode of attachment greatly facilitate orthotopic transplantation and subsequent observation, and provide information about general

characteristics of tissue transplantation reactions not easily obtained in other systems. A dependable technique of scale grafting has been devised so that reciprocal homografts can be made among many fish at a time, with autograft and regeneration controls.

2. In all experiments autografts have been normally revascularized without any inflammation whatever, whereas homografts are rapidly overgrown with hyperplastic host tissue and elicit capillary leakage and vasodilation in the contact zone with recipient tissue. Second-set homografts regularly break down much more rapidly than first-set homografts. This immune response has been measured by determining the median survival time and inflammatory reactions under various conditions.

3. A plot of median survival times against temperature demonstrated that previous grafting from the same donor and higher water temperature both accelerated homograft destruction. Time-mortality curves show that the duration and intensity of the inflammatory reaction are closely associated with the rapidity of donor tissue destruction. Capillary hemorrhage in the donor-recipient contact zone always appears earlier and is more severe in second-set homografts.

4. Cumulative time-inflammation curves demonstrated that the duration of the first inflammatory phase is associated with the rapidity of soft tissue destruction. The

second inflammatory phase was observed to be a consequence of relatively slow digestion of the scleroproteins comprising the scale plate. Both soft tissue and scale plate digestion showed the second-set phenomenon.

PART II

Histocompatibility Genetics of Scale Homotransplantation

1. Introduction

The now generally accepted genetic basis of transplantation incompatibility was first formulated by Little and Tyzzer (30) in 1916 as a result of tumor transplantation experiments with closely inbred lines of mice. A few years later Little and Johnson (31) showed that the same principles applied to homotransplants of normal tissue in mice. Parents (closely inbred waltzers and albinos) failed uniformly to support implants of the splenic tissue of their hybrid progeny, whereas F_1 progeny (waltzer X albino) grew regularly the splenic tissue of either parent strain. Subsequent studies by Loeb and Wright (32) and others have confirmed this relationship between the F_1 and inbred parent strains of mammals. The extensive investigations by Snell (33) have demonstrated that susceptibility and resistance to tumor transplants are determined by multiple dominant genes, now called <u>histocompatibility genes</u>. One histocompatibility locus, the H-2 locus on the ninth chromosome in mice, has been found to account for most of the tumor transplantation specificity. Ten alleles have already been detected at this locus, which also affects the antigenic specificities of red cells. Snell has suggested

that the same genes that determine susceptibility and resistance to tumor transplants probably also determine susceptibility and resistance to normal tissue transplants, but this argument rests mainly on the transplantation relationships among inbred lines of mice and their F_1 hybrids discussed above. Recent evidence (cf. Billingham et al. (24)) indicates that the genetic requirements for successful tumor transplantation cannot be assumed to define the more exacting immunological system represented by skin homografts, because the invasive growth of tumors may override minor immunological incompatibilities, and tumors may undergo some kind of antigenic simplification in the course of repeated transplantations. Moreover, a successful tumor homograft cannot be followed very long in a given recipient, because the host soon dies. Hauschka (34) has called attention to the widespread heteroploidy in malignant tissues. Many transplantable tumors are therefore not genetically homogeneous, but represent mosaics providing opportunity for physical loss of histocompatibility genes as well as additive ploidy changes which could lead to antigenic modifications.

There have been few attempts to determine the number of genes or genetic loci responsible for the incompatibility to normal homografts which is generally observed except in closely inbred lines and identical twins. Landsteiner (35) suggested that one need not assume the existence of a huge

number of individual cell substances in one species such as Loeb (2) has maintained, since even a moderate number of antigenic characters would furnish a number of combinations (2^n for n independent factors) sufficient to explain the results of grafting incompatibility. Medawar (7) has investigated the possible number of skin antigens and skin transplantation groups in the rabbit. Pinch grafts were removed from each of 22 unrelated rabbits and transplanted reciprocally to all of the other animals. None of the grafts were permanently successful. The assumptions made with regard to the antigenic basis of the reactions were (a) that it is particulate and combinatorial in nature, and (b) that a skin homograft will fail if it contains at least one antigen that is not represented in the tissues of the recipient. The relationship of genes to antigens and allelic diversity were not considered. From the results Medawar concluded that at least seven independently combined antigens govern the grafting reactions of rabbit skin with the corollary that a rabbit may belong to one of at least 127 skin transplantation groups. Longmire et al. (36) transferred skin homografts from 71 unrelated donors to a single recipient in man and found that none survived permanently. From this they concluded that if skin transplantation groups do exist, it is unlikely that they are less than 23 in number. This estimate is certainly minimal. I. J. Good (37) points out that among the thousands of skin-homografting operations that

have been tried by surgeons just a few between unrelated individuals may have succeeded.

A general solution to the problem of how often homografts may be expected to succeed when donor and recipient are chosen at random from the population has been proposed by Good. Thus if a is a histocompatibility antigen that is present in a fraction p of the population (and accordingly absent from a fraction $1-p$), then the antigen a may be considered in four donor-recipient combinations, to the occurrence of each of which a certain probability may be attached. Only one combination defines an incompatibility situation; the probability of a successful graft (s) = $1-p+p^2$. If $p = 1/2$, $s = 3/4$, but if p is either more or less than one-half, then s is always greater than three-fourths. One may note that as p becomes smaller s approaches the value $1-p$. Good chooses to estimate the probability of a successful graft as $s \simeq (3/4)^n$, where n is the number of distinct antigens that may be present in the homograft but absent from the recipient. With n estimated at 20, the minimal value of $s \simeq 1/300$ which represents an unexpectedly high fraction in the light of clinical evidence. But n may well be more than 20, and the percentage of favorable combinations drops very rapidly as n increases.

This kind of approach to the genetic basis of homograft incompatibility is rather unrealistic because it fails to take into account the multiple allelism which is so wide-

spread in the determination of cellular antigens. The allelic diversity found at loci such as Rh (38), the B and C systems of bovine blood groups (39), and the H-2 locus in mice (40) makes it likely that numerous alleles also function in the genetic control of normal tissue compatibility. In view of the fact that there is no restriction on the number of alleles which may be present in a random sample of a population, whereas the maximal number of alleles for each locus is four with any given parents, it is apparent that relatives and especially siblings can give better estimates of the nature of genetic diversity than can unrelated individuals.

2. Experimental

Goldfish are advantageous for investigation of the genetics of histocompatibility for two reasons: (a) 100 or more progeny may be raised from a single spawning under laboratory conditions and (b) numerous scale grafts may be reciprocally exchanged and subsequently observed with much greater ease than in analogous skin grafting operations. The disadvantages are that the generation time is about one year, and that goldfish must be about nine months of age before they have attained sufficient size for convenient scale grafting.

The present experiments involve a pedigree including F_1, F_2, and backcross progenies of unrelated P_1 parents. The phenotypes of the P_1 parents differed in many genetic

traits (body color and shape, eye shape and finnage), but the F_1 parents were very similar in all respects. While it was assumed that the parents were almost certainly heterozygous for several histocompatibility alleles, reciprocal homografts were made in all parent-offspring combinations to test the possibility that offspring might accept a scale graft from one parent. The P_1 male died before the test could be made, but the backcross sibship was derived from the $P_1♀$ X $F_1♂$ cross, and the same $F_1♂$ sired the F_2 sibship. The experiment was limited by the availability of F_2 and backcross sibs which were large enough to donate scales that would be retained by the much larger P_1 and F_1 parents. Eleven fish were used in a complete cross-grafting operation - $P_1♀$, $F_1♀$ and $F_1♂$ parents, four F_2 sibs, and four backcross sibs. In this experiment and others to be described, each fish received a control autograft which was permanently successful in every case. The fate of the homografts was followed as before by <u>in vivo</u> observations under the microscope. All the homografts were destroyed in the usual manner, although the manifestations of incompatibility were somewhat less severe than those observed with unrelated fish at a similar temperature (23°C).

Much larger cross-grafting operations were undertaken with the F_1 and F_2 sibships in an effort to estimate the number of genetic loci concerned with scale transplant specificities. Previous experience with tricaine anesthetic

and grafting technique indicated that reciprocal exchanges among 20-25 sibs would approach the limit of endurance of both fish and operator. Twenty-three F_1 sibs were tagged and cross-grafted so that each fish served as a donor and recipient to all of the other sibs. All grafts were made on the left side in two rows of scales immediately above and below the lateral line with 11 homografts per row in alternate positions, giving a total of 23 X 22 = 506 homografts and 23 autografts. Because of the difficulty of handling and trimming scales of smaller specimens, the whole operation required about 15 hours to complete. The fish were first examined after four days, and nine homografts in all were found to have been lost. The combinations lost were repeated by placing new grafts in positions three rows above the lateral line. Previous experience had shown that lost homografts which were replaced by grafts to the initial pockets were frequently lost again, presumably because these pockets had healed over and would not bind a new graft firmly. The fish were examined again after 10 days and it was found that none of the repeated homografts had been lost. At this time all 506 homografts showed unmistakable evidence of donor tissue destruction and the majority of homografts had passed the survival end point.

A complete cross-grafting operation was performed in the same manner with 23 F_2 sibs. In this experiment half of the grafts were made on each of two successive days so that

only half of the fish had to be anesthetized at one time on the first day. The fish were examined on the sixth day (at 23°C.) and it was found that nine homografts had to be repeated. When the sibs were re-examined on the 12th day, the repeated homografts had been retained and all 506 homografts had reached or passed the survival end point. All autografts were intact and normally revascularized, including a few that had sustained considerable forceps injury because of their small size.

The assumptions made in the analysis of these results are (a) that each histocompatibility allele determines a particular antigenic specificity distinct from other alleles or genes at other loci and (b) that a scale homograft will fail if it contains one or more antigens different from those present in the tissues of the recipient. If one assumes that the original P_1 parents possessed maximal allelic diversity at two independent histocompatibility loci, then the F_1 progeny would fall into 16 mutually incompatible genotypes as follows:

$$P_1 = A_1A_2;B_1B_2 \times A_3A_4;B_3B_4$$

$$F_1 = \begin{array}{cccc} A_1A_3B_1B_3 & A_1A_4B_1B_3 & A_2A_3B_1B_3 & A_2A_4B_1B_3 \\ A_1A_3B_1B_4 & A_1A_4B_1B_4 & A_2A_3B_1B_4 & A_2A_4B_1B_4 \\ A_1A_3B_2B_3 & A_1A_4B_2B_3 & A_2A_3B_2B_3 & A_2A_4B_2B_3 \\ A_1A_3B_2B_4 & A_1A_4B_2B_4 & A_2A_3B_2B_4 & A_2A_4B_2B_4 \end{array}$$

Thus if 17 sibs are completely cross-grafted and mutual incompatibility obtains, then more than two loci must be involved. Such is the case in the present experiments. With three independent loci and maximal allelic diversity in parents, $4^3 = 64$ mutually incompatible genotypes are possible and equiprobable in the progeny. For n independent loci involved in a mating, it can be shown that the number of genotypes (G) possible in the progeny is: $G = g^n$, where g is the number of genotypes possible per locus, and n is the number of loci. For any particular locus with any parents, there are seven possible types of allelic combinations, each of which has different expectations with respect to homograft incompatibility in the progeny as shown in Table 3. In crosses of types 1 and 2 all of the genotypes possible in the progeny would be mutually incompatible on the basis of the locus considered. This was true of all the genotypes tested in the present experiments.

With the allelic combinations of types 1 and 2, the problem is to find the probability that a sample of N animals of G equiprobable genotypes would be mutually incompatible to homografts. The number of different samples if G.G.G... = G^N; of these, $G(G-1)(G-2)...(G-N+1)$ will all be different. Thus:

$$*p = \frac{G(G-1)(G-2)...(G-N+1)}{G^N} = \frac{G!}{(G-N)!\ G^N}$$

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*This formulation was derived by Mr. Charles Steinberg, Division of Biology, California Institute of Technology.

Table 3. Genetic Expectations with Different Types of Allelic Combinations

	Allelic Combinations Possible in Parents	No. of Genotypes Possible in Progeny	Probability That All Homografts Incompatible When N = 23
1.	$A_1A_2..X\ A_3A_4..$	$G = 4^n$, all mutually incompatible	P = 1.13% when n = 3 P =36.2 % when n = 4
2.	$A_1A_1..X\ A_2A_3..$	$G = 2^n$, all mutually incompatible	P = 1.13% when n = 6 P =12.2 % when n = 7 P =36.2 % when n = 8
3.	$A_1A_2..X\ A_2A_3..$	$G = 4^n$, some compatible combinations*	
4.	$A_1A_2..X\ A_1A_2..$	$G = 3^n$, some compatible combinations*	
5.	$A_1A_1..X\ A_1A_2..$	$G = 2^n$, some compatible combinations*	
6.	$A_1A_1..X\ A_2A_2..$	G = 1, all compatible	
7.	$A_1A_1..X\ A_1A_1..$	G = 1, all compatible	

*See text.

In the limiting case of maximal allelic diversity, it is calculated that at least four independent histocompatibility loci are required with N=23 (p=36.2%), since the probability of only one per cent is significant evidence against the assumption of three loci (cf. Table 3). If the type 2 cross obtained then seven independent loci are required to account for the results. The actual number of loci involved might of course fall between four and eight, since one would not expect every locus to possess the same type of allelic combinations in a given cross. Some compatible combinations are expected among progeny derived from crosses of types 3, 4, and 5, with the compatible fraction decreasing as n increases. The expectations with the latter types are more complex because the possible genotypes are not equipotential as compatible donors or recipients and in type 4 the genotypes in addition are not equiprobable.

Having established a least number of loci in the P_1 parents which would account for the result with the F_1 progeny, one may then estimate the probability that the F_1 parents also possessed maximal allelic diversity. Because there are only four different allelic combinations possible for each locus in the F_1 as a result of a type 1 cross, the probability is 1/4 that the F_1 parents possessed the same diversity of alleles at a given locus as the P_1 parents. For four independent loci $p=(1/4)^4 = 1/256$ that the F_1 parents possessed maximal allelic diversity--a very unlikely event. It follows

then that the F_1 parents in question showed a high probability (255/256) of sharing one or more alleles in common at one or more loci. The probability is $(3/4)^4 = 1/3$ that the F_1 parents (or any two individuals selected from the F_1) shared at least one allele in common at each of four loci. Moreover, the F_1 parents were very similar in phenotype in contrast to the P_1 parents and other F_1 sibs. Thus the expectation of finding an instance of homograft compatibility was better among the F_2 sibs than the F_1 sibs. Since no compatibility was found, one may doubt that only four loci are involved. Until compatible combinations are found in the pedigree and the individuals are subsequently bred, no upper limit for the number of histocompatibility loci may be estimated.

The assumptions made in the foregoing analysis are largely based on the one gene-one antigen relationship which has been established by investigations of the cellular antigens of mammals and birds. The apparent complexity of gene-controlled antigens and the heterogeneity of antibody populations (39,40) may well have counterparts in the field of histocompatibility. Genetic studies of normal tissue transplantation may be further complicated to the extent that linkage of H-loci obtains.

3. Summary

1. The genetic basis of normal tissue transplantation was investigated by transplanting scales within a pedigree involving F_1, F_2, and backcross progenies of unrelated P_1 parents. A complete cross-grafting operation among the $P_1♀$, $F_1♀$ and $F_1♂$ parents, four F_2 sibs and four backcross sibs revealed no successful homografts.

2. Large reciprocal-homografting operations were undertaken with the F_1 and F_2 sibships in an effort to estimate the number of genetic loci concerned with scale transplant specificities. Twenty-three F_1 sibs were cross-grafted giving a total of 23 X 22 = 506 homografts and 23 control autografts. Reciprocal exchanges were also made among 23 F_2 sibs in the same manner. Every autograft was successful, while all of the homografts were destroyed by the recipients in both sibships.

3. The number of incompatible genotypes expected in the progeny is calculated for different types of allelic combinations possible in the parents. In the limiting case of maximal allelic diversity in the parents, it was determined that at least four independent histocompatibility loci are required to account for mutual incompatibility among 23 siblings. Since the probability of finding an instance of homograft compatibility was shown to be greater among the

F_2 sibs than the F_1 sibs, it is very likely that more than four loci are involved. Until compatible combinations are found in the pedigree, no upper limit for the number of histocompatibility loci may be estimated.

PART III

Goldfish Erythrocyte Antigens and Serology

1. Introduction and Techniques

While antibody production in relation to individual differences in red cell antigens has been extensively investigated in mammals and birds (39, 41), comparatively little is known about these phenomena in the lower vertebrates. Apparently von Toth (42) was the first to investigate and detect normal hemagglutinins and hemolysins in various fish sera which would react with species-specific antigens of other fishes. However, among 11 species tested he found no normal isoantibodies for red cell antigens. All possible combinations among 25 carp (Cyprinus) were tested for normal isohemagglutinins with negative results. Later studies by various other investigators were concerned with heteroagglutinins in fish sera and species-specific antigens. The more recent investigations of normal hemagglutinins in fish sera and of species differences in red cell antigens of various fishes have been summarized by Cushing and Sprague (43, 44). Very recently Cushing and Durall (45) have detected individual differences in the red cell antigens of catfish (Ictalurus nebulosus) by means of normal isoantibodies.

The present study was aimed at detecting individual differences in the red cell antigens of goldfish and eventually

determining their mode of inheritance. No immunogenetic study of any fish using immune reagents is known to the author.

Green and Hoffman (46) have found a very broad range of isotonic values for the erythrocytes of teleosts. We have similarly found that goldfish red cells keep about equally well in 0.7% to 1.0% saline, but remain intact much longer in concentrated than in dilute suspensions, especially in homologous plasma. For washing cells and making dilutions 0.85% sodium chloride has been used as a matter of convenience. Goldfish erythrocytes have been best preserved by mixing three parts of whole blood with one part of Alsever's (47) solution. Such cells will remain intact for three weeks or more under refrigeration (5°C.). A simple technique of taking blood samples by cardiac puncture has been devised. Goldfish are wedged ventral side up between wet sponges contained in a Kimble 40100 refrigerator dish (3" X 3-3/4" X 17"). Sufficient physiological saline is placed in the dish to soak the sponges and rinse the fish prior to bleeding. Rectangular sponges of different thickness may be readily interchanged depending on the size of the fish. Since fish blood clots much more rapidly than mammalian blood, a siliconed syringe containing cold anticoagulant (isotonic citrate or Alsever's solution) is used to withdraw blood, unless of course serum is desired. A 22 G. needle on a 1.5 ml. syringe is best even with small specimens, because smaller gauges become readily clogged. The needle is usually inserted under

the third scale directly posterior to the base of the left pectoral fin and directed anterodorsally to the ventricle which lies about midway between the pectoral fins. About 0.5 ml. of blood may be safely taken from a four inch specimen, while up to 1.5 ml. may be withdrawn from a twelve inch fish. Isoimmunization may be accomplished by cardiac puncture in the same manner. The mortality rate from this procedure has been very low, even when the heart was entered repeatedly on a single occasion. The cardiac puncture technique is difficult with specimens under five inches, because the heart itself is quite small. The fact that goldfish must be two or more years old before they are large enough to provide sufficient blood for absorption analyses and preparation of reagents has proved to be a serious disadvantage in the study of individual differences in their red cell antigens.

The technique of tagging specimens and the effect of temperature on antibody production in fish have already been discussed in connection with scale homotransplantation.

2. Natural Agglutinins for Erythrocyte Antigens

Normal sera from several different goldfish were checked for natural agglutinins at 1:4 and 1:8 saline dilutions against 2% suspensions of washed red cells of 12 other goldfish in standard agglutination tests. The tests were read after 30 minutes and after 2 hours at room temperature,

with and without centrifugation. No agglutination whatever was found. Normal sera from a variety of other animals were similarly tested for natural antibodies against the red cells of eight goldfish selected at random in the hope that individual differences might be detectable in this way. This hope was not realized, because the red cells of all fish were agglutinated by a given serum to within one doubling dilution when heteroagglutinins were present. No absorption analyses were tried with normal antibodies. The results are summarized in Table 4.

Table 4. Tests for Normal Heteroagglutinins to Goldfish Erythrocytes

Serum with Heteroagglutinins	Log_2 Titer Observed	Serum Lacking Agglutinins
Rabbit	7	Guinea Pig
Horse	7	Hamster
Sheep	6	Rat
Ox	6	Carp
Chicken	5	Goldfish
Monkey	5	
Human O	4	

3. Individual Differences Detected by Isoimmune and Rabbit Antisera

Proof of the existence of individual differences in goldfish red cell antigens was first achieved by isoimmunization at room temperature. A large specimen (#3) was given

a course of nine injections via the cardiac route. Each injection consisted of 0.3 ml. of a 20% suspension of washed red cells from another fish of about the same age (four years). Injections were given on alternate days and the agglutination titer after each series of three injections was checked by trial bleedings. The blood was put into dry tubes and allowed to clot without stirring. (Breaking up the clot as it formed with a thin hardwood splint failed to increase the serum yield as it does for chicken sera, and led to considerable hemolysis.) The serum yield increased from 45% to 55% of the total blood volume with successive bleedings, presumably because the fish was becoming anemic. The titer was only 1:8 six days after the third injection, but rose to 1:512 three days after the sixth injection, and finally to 1:1024 seven days after the ninth injection. The cells of the recipient served as a negative control. The serum was stored by freezing and subsequently used with no further processing.

An absorption analysis of this high titer isoimmune serum was made using red cells from 11 goldfish of various phenotypes and presumably of different parentage. About 1 ml. of blood was taken from each fish except 7 and 25 from which only about 0.6 ml. was obtainable. The cells of each fish were washed twice in saline. A small aliquot of each sample was then removed to prepare a 2% suspension for testing and the remainder divided equally in two tubes for

absorptions. The isoimmune serum was diluted 1/8 in saline and 0.5 ml. of the diluted serum was thoroughly mixed with the packed cells of each fish for the first absorption. Each tube was centrifuged after 30 minutes at room temperature and the partially absorbed serum was transferred to the second tube for another 30 minutes to complete the absorption. The absorbed sera were then diluted 1/3 in saline for testing. Each test consisted of 0.1 ml. of diluted serum and 0.05 ml. of 2% red cells; the tests were read for agglutination after standing for 30 minutes and again after 3 hours at room temperature. Several tests which were negative at 30 minutes were weakly positive after 3 hours. The results are given in Table 5. The tests of the sera absorbed with cells of fishes 7 and 25 are not included in the table because the antibodies directed against these cells had not been completely absorbed. Fish 6 as well as the control 3 possess no antigen reacting with this serum. The positive reactions reveal the presence of at least five antibody subpopulations recognizing antigenic specificities that may be symbolized as follows:

Fish	Antigenic Factors Assigned				
1	A	B	-	-	-
3	-	-	-	-	-
6	-	-	-	-	-
7	A	B	(C	D	E)
9	A	B	C	-	-
18	A	-	-	-	-
24	A	B	-	D	-
25	A	B	-	D	-
26	A	B	-	-	-
27	A	B	-	-	-
28	A	B	-	-	E

Table 5. Absorption Analysis of Isoimmune Serum 3*

Cells	Unabsorbed Serum at 1/20	Serum absorbed by rbc of goldfish: 1	3	6	9	18	24	26	27	28	Saline Control
1	4	0	4	3	0	2	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0
7	2	2	2	2	1	2	1	1	2	2	0
9	4	2	4	4	0	4	3	3	3	3	0
18	3	0	3	3	0	0	0	0	0	0	0
24	4	1	4	4	2	3	0	2	2	2	0
25	2	2	2	3	3	1	0	2	2	3	0
26	4	0	4	4	0	2	0	0	0	0	0
27	4	0	4	4	0	3	0	0	0	0	0
28	4	3	4	4	3	4	4	3	3	0	0

*The symbols used in recording the test results are as follows:

0 - no agglutination; the button of cells formed by centrifuging resuspends evenly on gentle shaking.

1 - a few faint clumps are visible.

2 - several to many small clumps are evident.

3 - several large clumps are formed.

4 - the button breaks into one or two large clumps.

Donor fish 2 died prior to this analysis.

On the basis of this analysis fish 7 must possess at least four antigenic factors, but any two of the three factors C, D, or E in conjunction with A and B would account for the reactions observed. It is evident that there are numerous antigenic differences in the red cells of goldfish. Further tests of the above fish could not be made, because all of them succumbed to a bacterial disease.

Two additional isoimmune sera were prepared as before by nine injections of goldfish 6 and 7, respectively, with red cells of the $P_1♀$ and $P_1♂$ which were discussed in Part II of this thesis. The unabsorbed sera agglutinated the homologous cells to a titer of 1:1024. By the time the F_1 progeny were large enough to provide sufficient blood for an absorption analysis, the $P_1♂$ had died. Nevertheless, 2% suspensions of the washed red cells of 11 F_1 sibs were tested against doubling dilutions of both unabsorbed isoimmune sera. All of the F_1 cells were agglutinated within two doubling dilutions of each other by both isoimmune sera, so no differences were demonstrable among the F_1 sibs by this method. However, it is apparent that one or more antigenic specificities shared by the parents and their progeny are absent in the individuals that developed these isoimmune antibodies. These specificities represent inherited individual differences in the antigens of goldfish red cells.

Several rabbit anti-goldfish red cell sera were prepared by giving a series of injections of washed red cells into the

marginal ear veins. About 0.5 ml. of a 20% suspension of red cells was used for each injection. Ten days after the ninth injection (three injections per week) one rabbit serum showed an agglutinating titer of 1:512 and a lytic titer of 1:4096 when tested unabsorbed against the homologous cells. The lytic test consisted of 0.1 ml. of diluted antiserum (heat inactivated for complement), 0.05 ml. of 2% rbc, and 0.05 ml. of fresh guinea pig complement at 1:8. Although lysis was not complete at dilutions higher than 1:1024 for homologous cells and 1:256 for non-homologous cells, agglutination of the intact cells was observed even at 1:4096. The complement and saline controls were both negative. The higher agglutinating titer found in the presence of guinea pig complement indicates that the guinea pig serum has a "conglutinating" effect in this system.

Cross-absorption analyses were then made with the above rabbit antiserum using red cells from ten goldfish in addition to the donor. Despite great care in taking one ml. blood samples from each fish, a few samples always showed considerable hemolysis after a few hours at room temperature. In later tests hemolysis was minimized by using Alsever's solution in lieu of isotonic citrate (2% sodium citrate, 0.5% NaCl) as anticoagulant and by keeping red cells in the cold (10°C.) prior to testing. Washed cells from each fish were divided among four tubes for absorptions. To each of the first absorption tubes 0.5 ml. of antiserum at 1:8 was added.

Each of the first three absorptions was carried out at 10°C. for 15 minutes in order to minimize hemolysis. The final absorption was at room temperature for 30 minutes. After the last absorption each reagent (absorbed antiserum) was further diluted 1:4 in saline for lytic tests. Each test consisted of 0.2 ml. serum reagent and 0.1 ml. 2% rbc; after shaking, 0.1 ml. of guinea pig complement at 1:4 was added. Appropriate serum, complement, and saline controls were also run. In all four of the absorption analyses the red cells of the fish tested completely removed antibodies for each other, but left in hemolysins for the cells of the donor used in immunization. Thus only one antigen could be distinguished among the fish tested. The reciprocal removal of antibodies for red cells other than those of the donor was not a consequence or overabsorption, because whenever fewer cells (or tubes) were used in absorptions, the absorptions proved incomplete for the absorbing cells.

The extent of absorption also proved to be critical when agglutinating rather than lytic tests were made with this same rabbit antiserum. Three successive absorptions of the serum at a 1:8 saline dilution were made, followed by complete cross-tests with the cells of each of 12 goldfish. Only four of the serum reagents proved to be completely unreactive with the absorbing cells. Each of these four reagents agglutinated the cells of the same five fish, including the donor used in immunization, while the remaining seven fish

were negative to these reagents. Somewhat more blood was obtained from each fish for later tests and four absorptions were done, again with the antiserum at a 1:8 dilution. The reagents were further diluted 1:2 and 1:4 for separate tests. Antibodies directed against the individual antigen(s) previously detected were completely removed by these absorptions. The ease with which antibodies specific for individual differences are completely removed in these absorptions indicates that closely related antigens characterize the red cells of all the fish tested. This finding is analagous to the well known A_1 and A_2 specificities of human erythrocytes.

4. Attempts to Prepare Specific Antisera by Injection of Day-old Chicks

The preliminary studies just described were hampered by the small quantities of blood obtainable from fish less than nine inches long. With the standard methods detailed in the previous section, pedigree studies would be impracticable, because one would have to wait two or more years for a progeny to reach sufficient size for absorption analyses. Recent studies of Cinader and Dubert (48) suggested a possible approach to the problem. These authors found that new-born rabbits injected with human albumin (H.A.) did not form antibodies against this antigen when reinjected during adult life. Six of the rabbits injected at birth with H.A. were later injected with benzene-p-sulfonic acid-azo human

albumin and two of them produced antibodies to this antigen. These antibodies were found to be directed against the hapten and showed little or no avidity for H.A. Downe (49) found that rabbits which were given prenatal as well as a series of post-natal injections of chicken serum were still incapable of an antibody response to repeated injections of chicken serum when they were four months old (mature). Following injections of turkey serum such rabbits did produce antibodies to turkey serum which were not cross-reactive with chicken serum. Cross-reactive antibodies would normally be produced in untreated rabbits. This kind of modification of the antibody mechanism as a result of early exposure to a heterologous antigen suggested that the same approach with red cells might enable one to prepare antisera specific for individual differences in a pedigree without need for absorptions.

It was decided to try the chicken for immunization, since the immature nature of the immune mechanism with respect to homologous tissues in newly-hatched chicks had been demonstrated by Cannon and Longmire (50). About 0.5 ml. of blood was taken from each of nine goldfish (P_1♀, P_1♂, F_1♀ and F_1♂ parents, and five other F_1 sibs). The cells were washed twice and made up to 20% suspensions in saline. The red cells of each fish (donor 1) were then injected intraperitoneally into a newly-hatched chick. Three months later three of the birds were reinjected with the same

dosage of cells from a different donor (donor 2). A trial bleeding was taken just prior to the injection from the second donor and at 3-4 day intervals thereafter for a total of six bleedings. Each serum sample was titered against 2% suspensions of the cells of the first and second donors in standard agglutination tests with doubling saline dilutions of serum ranging from 1:2 to 1:8192. The results are summarized in Table 6.

In all three chicks antibodies present in the serum prior to the second injection were found to agglutinate the cells of both donors. Following the second injection of chicks 249 and 251 there was evidence of increased antibody production. However, antibodies reactive with the cells of both donors developed. The antibody activity increased more rapidly for the cells of the second donor than for the cells of the first donor. Chick 248 failed to respond to the second injection. It was clear that there had been little or no inhibition of the ability to produce antibodies against the heterologous antigens of the first donor.

In very recent experiments by Simonsen (51), chick embryos were injected with blood from adult turkeys or geese, and turkey embryos with adult chicken blood. In all three kinds of experiments there was evidence of a diminished antibody response in pretreated birds as compared with normal controls after the birds were challenged five to six weeks post-hatching by injections of blood cells from the original

Table 6. Agglutinative Titration of Chick Anti-Goldfish Red Cell Sera

Chick	First Donor (1)	Second Donor (2)	Log_2 Titer after Neonatal Injection against (1)	Log_2 Titer after Neonatal Injection against (2)	Max. Log_2 Titer after Second Donor Injection against (1)	Max. Log_2 Titer after Second Donor Injection against (2)
248	$F_1$6(P)♂	$F_1$6(P)♀	7	7	7	7
249	$P_1$6 ♀	$F_1$6-1	1	1	8	8
251	$F_1$6-1	$P_1$6 ♀	8	6	8	10

donors. But most of the tolerant birds failed to maintain the acquired tolerance when immunization was repeated or when heavier doses of antigen were employed. Quite recently, Billingham et al. (52) found that partial or incomplete tolerance may also develop when homologous red cells are injected into newly-hatched chicks. These authors showed that chickens injected with adult homologous red cells at hatching responded much more feebly to immunization from the same donors at seven weeks of age than their untreated controls. Evidently the power of a given species to produce antibodies against various types of antigens may mature at very different times.

Although the original aim of the present experiments was not realized, more extensive investigations by Owen (53) with antigenically marked human red cells have revealed that the nature of the antibody response is sometimes greatly modified as a result of immunization of neonatal recipients with heterologous erythrocytes.

5. Summary

Techniques for bleeding and isoimmunization of goldfish have been developed and methods for handling and preservation of goldfish blood cells are described. Lytic and agglutinating systems have been compared for sensitivity in the characterization of blood cell antigens. Normal goldfish sera as well as normal heteroagglutinins from various species

failed to distinguish individual red cell antigens. Experiments with isoimmune and rabbit antisera have demonstrated the existence of inherited individual differences in the red cell antigens of goldfish.

Injection of newly-hatched chicks, followed by later injection of red cells from different donors, gave little evidence of preferential production of antibodies specific for the cells of the second donor. Any tolerance that may have been acquired for the cells of the first donor was evidently too incomplete to prevent a substantial production of cross-reactive antibodies after later injection of cells from a different donor.

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TECHNIQUES FOR STUDIES OF HEMOGLOBIN SYNTHESIS IN DAPHNIA

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In recent years H. Munro Fox and colleagues (1951, 1953) have made extensive studies of the functions of hemoglobin and factors influencing hemoglobin synthesis in Daphnia. In these studies the hemoglobin concentration in the blood of living Daphnia was measured by an index method in which the color of a known dilution of laked human blood is matched with the blood colors of 10 or more individual Daphnia alined out of water on a microscope slide. The mean of the hemoglobin values, each read on a scale, gives a hemoglobin index of the population. The method is dependent upon the color matching ability of the observer and is inadequate to determine accurately the hemoglobin content of pale animals. A spectrochemical method for measurement of total hemoglobin content in experimental populations of *Daphnia magna* has been devised in this laboratory.

MATERIAL AND METHOD

A modification of the original cyanhemoglobin method for mammalian blood devised by Drabkin and Austin (1932) is utilized. Animals from a culture flask are netted and transferred to a large watch glass containing distilled water. This serves to wash the animals free of culture medium. The water is then removed with a fine pipette, and 25 adults are carefully picked up five at a time with a small, flat spatula, gently blotted with wiping tissue to remove adhering water, and transferred to a 1 ml. centrifuge tube containing 0.20 ml. of cold distilled water. After a series of cultures or aliquots of Daphnia are prepared in this way, the animals are macerated with a glass rod which fits closely into the bottom of the centrifuge tube. The tissue debris is quickly packed down again, and preparations are centrifuged for about five minutes in a clinical centrifuge. All of the pale-red supernatant is removed with a fine pipette and transferred to a 10 mm. × 75 mm. tube. Then 0.03 ml. of 0.1% $K_3Fe(CN)_6$ is added and allowed to stand for 10 minutes. Finally 0.02 ml. of 0.1% KCN is added to convert the methemoglobin to cyanhemoglobin.

The cyanide method has the advantage of inactivating the enzymes derived from the macerated animals. A very slight turbidity is present in the final preparations. This does not affect the hemoglobin determination, but it may nonetheless be cleared by the addition of one drop of concentrated ammonium hydroxide. The final preparations (0.25 ml.) are placed in 6 mm.

O.D. cuvettes which fit the No. 6-110 semi-micro adapter for use with th Coleman 6A Junior Spectrophotometer. In practice, the instrument is adjusted to 100% T with a reagent blank (0.20 ml. H_2O, 0.03 ml. 0.1% $K_3Fe(CN)_6$, 0.02 ml. 0.1% KCN).

RESULTS

Spectral-Transmittance curves of Daphnia cyanhemoglobin are shown in Figure 1. Distinct minima are obtained at 415 mu, and this is characteristic

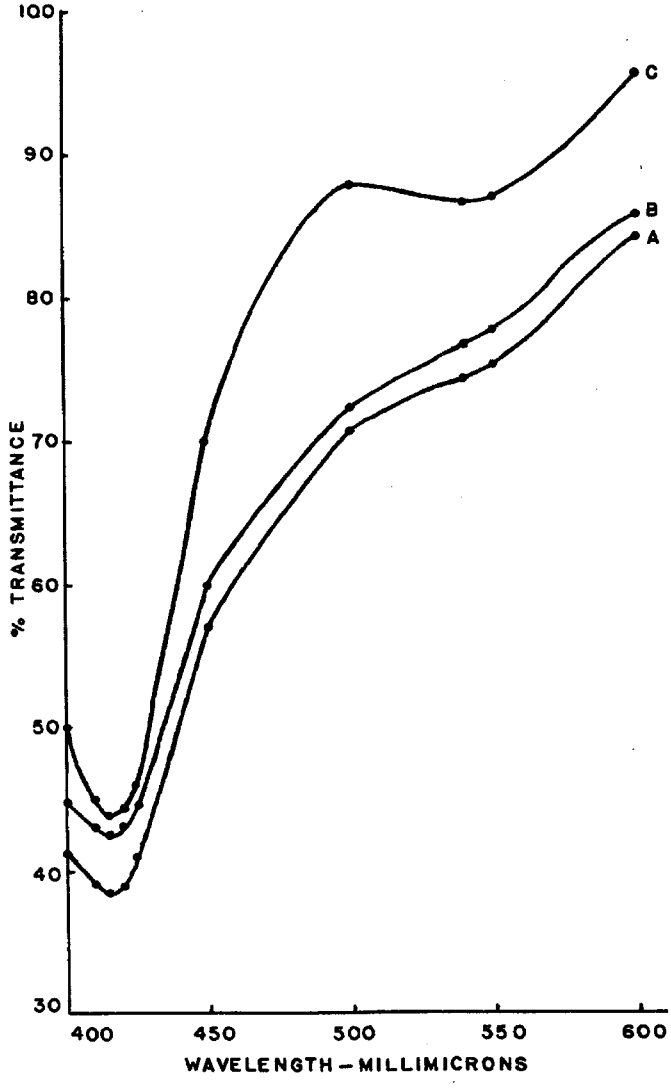

FIGURE 1. A, B. Spectral-Transmittance curves of cyanhemoglobin from two samples of 25 Daphnia each, from the same culture. C. Spectral-Transmittance curve of rabbit cyanhemoglobin standard.

of all Daphnia preparations from quite pale to dark red animals. Maximal light absorption in the 415 mu region is known to be specific for hemoglobin. The curves plotted are those of two aliquots of 25 adult Daphnia each, taken from the same culture at the same time. The Spectral-Transmittance curve of an identical volume of rabbit cyanhemoglobin of known hemoglobin concentration and with the same concentration of reagents is also shown. While the curves for the two species differ in detail, both show similar absorption maxima in the 415 mu region. The difference of 4% T between the two Daphnia samples at 415 mu has been the maximum difference observed between groups grown under identical conditions when the animals are selected for similar size and number of parthenogenetic eggs. Duplicate readings made on samples over a six hour period gave a maximal variation of only one per cent transmittance, indicating that the cyanhemoglobin preparations as well as the spectrophotometer were stable. Several Daphnia cyanhemoglobin preparations were diluted to various concentrations with water. The shape of the S-T curves over the 400–425 mu region was unaffected. Plots of per cent transmittance at 415 mu against dilution factors showed a straight line relationship in each instance, indicating that other soluble constituents were not interfering with the determination. Transmittance measurements of *D. magna* hemoglobin at this wavelength may therefore be made in conformity with the Lambert-Beer Law. Such measurements may then be converted approximately into milligrams of hemoglobin by reference to a Concentration-Transmittance graph for a rabbit cyanhemoglobin solution of known hemoglobin content measured at 415 mu, and plotted on semi-log coordinates. Thus for the reddest sample of 25 Daphnia observed in this investigation, 10% T is equivalent to 0.23 mg. Hb and for the palest sample of 25 Daphnia 67% T approximates 0.04 mg. Hb. It is apparent that at least a six-fold difference in hemoglobin content may be found in *D. magna* cultured under various conditions.

Fox et al. (1951) have shown by the hemoglobin index method that a manifold increase in the hemoglobin content of the blood in Daphnia results from oxygen deficiency in the water; pale animals become red. We have been able to verify this stimulative effect of low oxygen levels under a variety of conditions using the cyanhemoglobin method. A typical experiment will be cited.

Four 500 ml. Erlenmeyer flasks were prepared, each with 200 ml. of culture water and 40 adult Daphnia from the same stock culture. One flask was left open to the atmosphere as a control. Each of the other flasks was covered with an air tight rubber cap, sealed with paraffin wax, and the remaining air space flushed with tank nitrogen under a 2-pound pressure for one minute through two No. 22 hypodermic needles. Each flask was given air access via a No. 16, 20, and 27 needle respectively, and maintained at room temperature (21°C.-24°C.) for five days. The size of the needle determines the oxygen level in the flasks under these conditions. The hypodermic needles also maintain atmospheric pressure by allowing the carbon dioxide formed in respiration to escape. Dissolved oxygen levels were de-

termined daily by the rapid, microcolorimetric method of Loomis (1954), and were found to remain relatively constant during the period of the experiment. The results are summarized in table 1. While flasks 1 and 2 show no sig-

TABLE 1

HEMOGLOBIN CONTENT IN RELATION TO OXYGEN SATURATION

Flask	Oxygen Level[γ]	Hb Content of 25 Daphnia
1. Control	76% sat. (6.6 mg. O_2 per liter)	44.5% T (.08 mg. Hb)
2. 16 G needle	46% sat.	41.9% T (.09 mg. Hb)
3. 20 G needle	35% sat.	34.3% T (.11 mg. Hb)
4. 27 G needle	5% sat. (0.5 mg. O_2 per liter)	28.5% T (.13 mg. Hb)

[γ] Rawson's nomogram may be conveniently used for transforming oxygen values from one kind of unit to another. (Cf. Welch, 1948.)

nificant difference in terms of the experimental error (4% T), the animals in flasks 3 and 4 responded to low oxygen by a considerable increase in hemoglobin. The difference in hemoglobin content between the extremes is readily apparent to the naked eye in the living cultures. However, our preliminary experiments indicate that a week or more may be required for maximal hemoglobin synthesis by *D. magna* in response to low oxygen. The absorbance values given in Table 1 do not therefore represent the final hemoglobin contents attainable at the oxygen levels given. The oxygen level of 5 per cent saturation we have found to approach the lower limit at which *D. magna* may survive for an extended period at room temperature.

While recent investigators have been using controlled suspensions of algae such as Chlorococcum (Frank, 1952) and Chlorella (Fox and Phear, 1953) as food for Cladocera with excellent results, these have certain disadvantages in physiological studies. Such Daphnia cultures must be kept in the dark to avoid algal photosynthesis and must be stirred daily to bring the algae back into suspension. Incidental to this investigation, we have developed a semisynthetic food source for Daphnia as follows:

Distilled Water	1577 ml.
Versene Fe-3 specific*	23 ml.
Versene acid (EDTA) powder	0.1%
Ferric citrate powder	0.3%
KH_2PO_4	0.1%
Na_2HPO_4	0.1%
$Mg\ Cl_2$	0.1%
Bacto-Casitone (pH 7.1)	0.5%
Difco proteose-peptone	1.0%
Yeast extract	0.1%
Dextrose	0.5%

*The quantity used is based on Bersworth Chemical Co. Tech. Bull. No. 2 data indicating that 1 ml. Versene Fe-3 specific complexes 0.164 gms. Fe-3 at pH 8.

The ferric citrate should be dissolved in the solution of versenes in water to avoid ferric hydroxide precipitation. The other constituents may then be added in any order. The medium is autoclaved and stored in the cold. It has a pH of 7.7, a value which is quite satisfactory for *D. magna*. The versene acid is added to lower the final pH and to provide some sequestering action for magnesium and cobalt which may be limiting in hemoglobin synthesis. The use of versenes as non-metabolizable solubilizing complex-formers in culture media is described by Hutner et al. (1950). Ferric citrate is used in relatively high concentration, since the efficacy of this salt in Daphnia hemoglobin synthesis has previously been shown by Fox and Phear (1953).

The medium as given represents only a first approximation toward a satisfactory food source. In practice, 5 ml. of medium is added to each 10 liter battery jar culture every second day. Bacterial proliferation may be excessive unless there are some 200–500 animals present. Stock cultures have also been maintained in large tanks in this manner for several months. Experimental cultures have been generally set up in 500 ml. flasks with 240 ml. of water and 60 Daphnia, or one animal to 4 ml. of water. About 0.2 ml. of penicillin G sodium (Merck) at a concentration of 15 mg. per ml. may be added daily to each test culture to minimize bacteria. In this way greater quantities of media or substances being assayed for hematopoietic effects may be added without having bacteria reduce the dissolved oxygen levels. Penicillin appears to have no adverse effects on the animals.

No attempt was made to grow the Daphnia in germfree culture, although this has been done by others with a few animals for short periods (Stuart et al., 1931). If *D. magna* cannot reproduce in this medium in the complete absence of bacteria, then the protein and carbohydrate constituents may be primarily a food source for bacteria on which the Daphnia feed. In any event a single reproducible medium of this kind, embodying both organic and inorganic constituents in a buffered solution, is to be preferred over the various manure, soil, and cereal infusions generally used. Even when algal suspensions are employed, chelated metallic ions may have to be added, and one must contend with both algal and bacterial metabolism. The extent to which particulate, colloidal, and soluble constituents may be utilized or required in the diet of Cladocera is still an open question. Earlier work on Cladoceran food requirements (Stuart et al., 1931; Gellis and Clarke, 1935) is inconclusive, since the test media used were inadequate in the light of present knowledge.

Daphnia magna appears to be well suited for studies of hemoglobin synthesis. With a quantitative method of Daphnia hemoglobin determination now available, this species shows promise for assay of possible hematopoietic substances.

SUMMARY

A new spectrochemical technique for determination of hemoglobin content in pale as well as red Daphnia has been presented. By use of this method

the primary stimulus for hemoglobin synthesis of low dissolved oxygen content in the culture water has been confirmed. Spectral-Transmittance curves of Daphnia hemoglobin are figured and compared to those of rabbit hemoglobin. A method for controlling the oxygen level in culture flasks by the use of hypodermic needles and air tight rubber caps is described. A first approximation toward a semisynthetic medium for Daphnia is discussed.

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